Research Article

Immunohistochemical Localization of AT\textsubscript{1a}, AT\textsubscript{1b}, and AT\textsubscript{2} Angiotensin II Receptor Subtypes in the Rat Adrenal, Pituitary, and Brain with a Perspective Commentary

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Angiotensin II increases blood pressure and stimulates thirst and sodium appetite in the brain. It also stimulates secretion of aldosterone from the adrenal zona glomerulosa and epinephrine from the adrenal medulla. The rat has 3 subtypes of angiotensin II receptors: AT\textsubscript{1a}, AT\textsubscript{1b}, and AT\textsubscript{2}. mRNAs for all three subtypes occur in the adrenal and brain. To immunohistochemically differentiate these receptor subtypes, rabbits were immunized with C-terminal fragments of these subtypes to generate receptor subtype-specific antibodies. Immunofluorescence revealed AT\textsubscript{1a} and AT\textsubscript{2} receptors in adrenal zona glomerulosa and medulla. AT\textsubscript{1b} immunofluorescence was present in the zona glomerulosa, but not the medulla. Ultrastructural immunogold labeling for the AT\textsubscript{1a} receptor in glomerulosa and medullary cells localized it to plasma membrane, endocytic vesicles, multivesicular bodies, and the nucleus. AT\textsubscript{1b} and AT\textsubscript{2}, but not AT\textsubscript{1a}, immunofluorescence was observed in the anterior pituitary. Stellate cells were AT\textsubscript{1b} positive while ovoid cells were AT\textsubscript{2} positive. In the brain, neurons were AT\textsubscript{1a}, AT\textsubscript{1b}, and AT\textsubscript{2} positive, but glia was only AT\textsubscript{1b} positive. Highest levels of AT\textsubscript{1a}, AT\textsubscript{1b}, and AT\textsubscript{2} receptor immunofluorescence were in the subfornical organ, median eminence, area postrema, paraventricular nucleus, and solitary tract nucleus. These studies complement those employing different techniques to characterize Ang II receptors.

1. Introduction

The ability of angiotensins II (Ang II) and III (Ang III) to stimulate aldosterone [1, 2] and epinephrine [3] release from the adrenal gland is well established. The central nervous system and adenohypophyseal effects of these peptides are also well documented and numerous. While the effects of Ang II on the adrenal are thought to arise primarily from blood-borne Ang II, it is clear that there is a local brain angiotensinergic system as illustrated by biochemical, immunohistochemical, behavioral, physiological, and receptor binding studies [4–8] and reviews [9–11]. The anterior pituitary also appears to be subject to both blood-borne and local angiotensinergic systems, as well as receiving indirect regulatory signals from brain angiotensinergic activity [12, 13].

In mammals, there are two primary Ang II receptor subtypes, AT\textsubscript{1} and AT\textsubscript{2} [14–19]. With the discovery of these
multiple subtypes of Ang II receptors, pharmacological studies revealed that the \( \text{AT}_1 \) subtype mediated both aldosterone [20] and epinephrine [21] release as well as pressor [22, 23], dipsogenic [22–24], and sodium appetite [24–26] responses to Ang II. The localization of \( \text{AT}_1 \) receptors in the rat brain regions mediating pressor and dipsogenic actions of Ang II, such as the subfornical organ (SFO), median preoptic nucleus (MnPO), organum vasculosum of the lamina terminalis (OVLT) paraventricular nucleus of the hypothalamus (PVN), nucleus of the solitary tract (NTS), and area postrema [27–29] is consistent with this role. In contrast, \( \text{AT}_2 \) receptors tend to be distributed in sensory, motor, and emotional regions of the brain, for example, superior colliculus, medial geniculate nucleus, locus coeruleus, lateral septum, medial amygdala, subthalamic nucleus, and inferior olivary nucleus [27–29].

The subsequent discovery that rodents express two subtypes or isoforms of the \( \text{AT}_1 \) receptor, \( \text{AT}_{1a} \) and \( \text{AT}_{1b} \), [31–33] raises the question as to which of these subtypes may be mediating adrenal hormone release and the physiological effects of Ang II in the brain and pituitary. Pharmacological studies of the ability of angiotensins and \( \text{AT}_1 \) receptor-selective antagonists to bind to the \( \text{AT}_{1a} \) and \( \text{AT}_{1b} \) receptor subtypes reveal little difference in their affinities for these two subtypes [34–37].

PCR amplification of \( \text{AT}_{1a} \) and \( \text{AT}_{1b} \) mRNA in female rat adrenal, lung, vascular smooth muscle, pituitary, and brain indicated that the \( \text{AT}_{1a} \) subtype mRNA was predominant in the lung, vascular smooth muscle, and hypothalamus, while the \( \text{AT}_{1b} \) subtype was predominant in the adrenal, pituitary, subfornical organ, and organum vasculosum of the lamina terminalis [31, 38]. Both PCR amplification [31, 35, 38–40] and in situ hybridization [39, 41, 42] have been used to compare the expression of mRNA for these two subtypes in the adrenal and brain. However, the expression of mRNA does not always correspond with the expression of the protein it encodes. For example, estrogen treatment can reduce \( \text{AT}_1 \) receptor expression without altering \( \text{AT}_1 \) mRNA expression presumably via posttranscriptional inhibition of mRNA translation [43]. Moreover, in neuronal tissues, the receptors may be expressed on axonal terminals distant from their perikaryal mRNA.

Studies of \( \text{AT}_{1a} \) and \( \text{AT}_{1b} \) mRNA expression in the adrenal indicate that the \( \text{AT}_{1b} \) subtype mRNA is predominant in the rat adrenal [35, 38, 39, 44], but that it is absent in the adrenal medulla [44–46]. Studies of \( \text{AT}_{1a} \) and \( \text{AT}_{1b} \) mRNA in rodent brain vary considerably along a continuum from a predominance of \( \text{AT}_{1b} \) expression in the female rat brain [31], to a moderate predominance of \( \text{AT}_{1a} \) in the male mouse brain [40, 42], a differential distribution of the mRNAs in a two-week-old male rat brain [45], to very low expression of \( \text{AT}_{1b} \) mRNA in the adult male rat brain [41], and to no expression of \( \text{AT}_{1b} \) mRNA in rat brain [47]. In comprehensive studies of the distribution of \( \text{AT}_{1a} \) and \( \text{AT}_{1b} \) mRNA the rat brain and pituitary [41], the \( \text{AT}_{1a} \) mRNA was found to be highly expressed in brain regions reported to mediate cardiovascular effects of Ang II, while \( \text{AT}_{1b} \) expression was very low in these regions. Conversely, \( \text{AT}_{1b} \) mRNA was very high in the anterior pituitary while \( \text{AT}_{1a} \) mRNA was low.

To determine if the distribution of \( \text{AT}_{1a} \), \( \text{AT}_{1b} \), and \( \text{AT}_2 \) receptor subtype protein in the rat adrenal, pituitary, and brain corresponds to the distribution of the mRNAs for these subtypes, this study uses fluorescence immunohistochemistry with antibodies directed at unique peptide fragments of each of these three subtypes to localize these receptors.

2. Materials and Methods

2.1. Antibody Preparation. Antipeptide antibodies were generated against fragments of rat \( \text{AT}_{1a} \), \( \text{AT}_{1b} \), and \( \text{AT}_2 \) receptors. Peptides candidates were selected by computer analysis of full length receptors retrieved from the NCBI protein database (http://www.ncbi.nlm.nih.gov/protein) and by Hopp-Woods analysis [48] for optimal antigenicity. Peptides corresponding to receptor fragments near the carboxy terminal tail of the receptor subtypes where there is a 2 amino acid difference were synthesized by solid phase peptide synthesis. For the \( \text{AT}_{1a} \) receptor, the peptide was PSDNMSSSALKPASC, which corresponds to amino acids 341–355 of this 359 amino acid protein. For the \( \text{AT}_{1b} \) receptor, the peptide was SSASKSASFFFEVE, which corresponds to amino acids 346–359 of this 359 amino acid protein. For the \( \text{AT}_2 \) receptor, the peptide was CRKSSLREMETFYS, which corresponds to amino acids 349–363 of this 363 amino acid protein (except that it contained a glutamic acid in position 358 versus an aspartic acid). The peptides were compared with the protein database (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to establish the uniqueness of the peptide sequences from other known proteins.

Peptides were conjugated to keyhole limpet hemocyanin (KLH) and injected into rabbits at approximately monthly intervals for 6 months. Serum was obtained from the rabbits and affinity purified. To obtain \( \text{AT}_{1a} \)-selective antibodies, serum from rabbits immunized with the peptide corresponding to the \( \text{AT}_{1a} \) receptor subtype was affinity purified using chromatography resin cross-linked with the \( \text{AT}_{1a} \) peptide. Antibodies retained by this resin were eluted with a high salt solution and the eluate was then applied to an affinity column made by cross-linking the \( \text{AT}_{1b} \) receptor peptide antigen to chromatography resin. Antibody that was not retained by the \( \text{AT}_{1b} \) resin was denoted as \( \text{AT}_{1a} \) receptor selective. Antibody that was retained by both the \( \text{AT}_{1a} \) and \( \text{AT}_{1b} \) resins was defined as nonselective for \( \text{AT}_{1a} \) or \( \text{AT}_{1b} \)-receptors. A similar strategy was used to derive \( \text{AT}_{1b} \) selective antibodies except that serum from rabbits immunized with the peptide corresponding to the \( \text{AT}_{1b} \) receptor subtype was affinity purified using chromatography resin cross-linked with the \( \text{AT}_{1b} \) peptide initially. Antibodies retained by the \( \text{AT}_{1b} \) resin were subsequently applied to the \( \text{AT}_{1a} \) resin. Antibodies retained by the \( \text{AT}_{1b} \) but not the \( \text{AT}_{1a} \) resin, were classified as \( \text{AT}_{1b} \) selective. \( \text{AT}_2 \) receptor antibodies were affinity purified using chromatography resin cross-linked with the \( \text{AT}_2 \) receptor peptide used to generate the antibody. Antibodies retained by the \( \text{AT}_2 \) resin were eluted with high salt solution and classified as \( \text{AT}_2 \) selective.
2.2. Animals. Adult male Sprague-Dawley rats (225–300 g body weight; Harlan, Sprague Dawley) were kept in an AAALAC approved vivarium (12:12 Light:Dark). Standard lab chow and water were available ad lib. Animals were kept in the vivarium for at least two weeks prior to use and were housed two per cage. All procedures were approved by the University of Wisconsin, School of Veterinary Medicine Animal Care Committee.

2.3. Western Immunoblotting. Fresh or frozen, whole or dissected rat adrenals (n = 4) were employed. A 2 mm slab was cut from the center of the adrenal and the medulla was removed by punch. The cortex was dissected away from the medulla. Tissues were homogenized in one complete mini protease inhibitor tablet (Roche, Indianapolis, IN) dissolved in 7 mL of RIPA buffer (Millipore, Billerica, MD). Lysates were sonicated for 5 minutes and cleared of debris by centrifugation at 15000 rpm for 20 minutes. Samples were normalized so as to amount of protein present via BCA assay (Thermo Scientific, Rockford, IL).

Samples were dissolved 1:1 in loading buffer with beta mercaptoethanol and boiled at 95°C for 4 minutes before loading. Proteins were separated via SDS-PAGE and transferred to PDVF membrane (Bio-Rad, Hercules, CA). Transfer conditions were wet (1 hour at 100 volts). Membranes were incubated for one hour in tris buffered saline containing 0.05% tween-20 (TBST), 5% powdered milk, and 1% bovine serum albumin. Blots were incubated in primary antibodies overnight at 4°C. Primary antibodies (Table 1) were diluted in TBST with 0.2% Na3 as a preservative. Blots were incubated in secondary antibody for 45 minutes. Secondary antibody goat anti-rabbit HRP (KPL, Gaithersburg, MD) was diluted 1:100,000 in 20 mL TBST with 2 uL streptavidin HRP (Sigma Aldrich, St Louis, MO). Developing solutions used in this study were LumiGLO immunoblotting reagent (KPL) and Supersignal West Pico Substrate (Thermo Scientific).

Samples were run on SDS-PAGE gels, transferred to PDVF membrane, blocked in 10% milk in TBST (1 hour), and incubated with primary antibodies at working dilution overnight at 4°C. The primary antibody dilution for AT1a receptors was 1:500 and for AT2 receptors, respectively, antibodies incubated with an excess of the antigenic peptides (20 μg/mL of antigenic peptide at the working dilution). Also antibodies were immunoprecipitated from their working dilutions by incubation with 100 μL AT1a, AT1b, or AT2 affinity gels and then the supernatant was used in place of the antibody solution. Sections were then incubated with Cy3-labeled goat anti-rabbit IgG and then mounted onto poly-L-lysine slides. Slides were viewed and analyzed utilizing a Nikon Eclipse E600 epifluorescence microscope with UV illumination, and a digital camera (Spot RT, Diagnostic Products).

2.6. Immunoelectron Microscopy. Adrenals from 7 rats were used for ultrastructural immunocytochemistry (N = 4 rats for immunogold detection and n = 3 rats for peroxidase. For both methods, rats were perfused as described above and postfixed for 24 hours in 4% paraformaldehyde with 0.1% glutaraldehyde, washed in PBS and vibratome sectioned at 50 micron thickness. The sections were incubated in 0.1% sodium borohydride 15 minutes, permeabilized in 0.05% triton for one hour, and blocked in either 0.5% BSA (Aurion, Arnhem, Gelderland, The Netherlands) for one hour for immunogold detection or ICC buffer for immunoperoxidase detection prior to overnight exposure to primary antibody. The primary antibody dilution for AT1a receptors was 1:500 for both immunogold and immunoperoxidase.

For the immunogold method antibody-labeled receptor was detected using ultrasmall gold (Aurion, 0.8 nanometer average size) diluted 1:100 in phosphate buffer and incubated overnight. Tissues were then postfixed in 2.5% glutaraldehyde for 30 minutes. The immunological signal was silver intensified by incubation in R-Gent SE-EM (Aurion) for one hour. For immunoperoxidase detection antibody-bound receptor was incubated with peroxidase labeled goat anti-rabbit IgG-Fab (1:250 overnight in the refrigerator). Peroxidase signal was visualized by incubation in diaminobenzidine (30 mg %) and hydrogen peroxide (0.01%) for 10 minutes in 0.1 M Tris HCL, pH 7.5. Then both immunogold and immunoperoxidase sections were rinsed in 0.1 M sodium phosphate buffer, fixed with osmium, dehydrated through an alcohol series to propylene oxide, and flat embedded in EMBED 812 resin (Electron Microscopy Sciences, Hatfield, PA).

Ultrathin sections were cut and adsorbed to grids coated with Formvar film (Electron Microscopy Sciences), and contrasted with uranyl acetate and lead citrate. All samples were examined and photographed with a Philips CM 120 STEM electron microscope and a Megaview 3 Sls digital camera (Olympus, Munster, Westphalia, Germany) in combination with the software program iTEM (Olympus) at the University of Wisconsin Madison Electron Microscope Facility.

3. Results

Western blotting of protein extracts of the adrenal with the 3 antibodies revealed primary ~69, ~75, and ~71 kD bands for the AT1a, AT1b, and AT2 receptors, respectively, with secondary bands of ~116, ~126, and ~119, respectively (Figure 1). This suggests that the solubilized receptor was
Table 1: Tabulated summary of comparative regional and cellular distribution of Ang II receptor-immunoreactivity in rat brain and pituitary.

| Region                                | AT_{1a} | AT_{1b} | AT_{2} |
|---------------------------------------|---------|---------|--------|
| Neocortex                             |         |         |        |
| Lamina I-II                           | ND      | 0"/5'  | 1"/1'  |
| Lamina III-IV                         | ND      | 4"/5'  | 0"/4'  |
| Lamina V-VI                           | ND      | 4"/4'  | 3"/3'  |
| Basal (Anterior) Forebrain            |         |         |        |
| Entorhinal cortex                     | 2"/0'  | 0"/0'  | 5"/1'  |
| Hippocampus                           |         |         |        |
| CA1                                   | 1"/0'  | ND     | 4"/5'  |
| CA3                                   | 1"/0'  | ND     | 5"/5'  |
| Dentate Gyrus                         | ND      | ND     | 2"/2'  |
| Central Amygdala                      | 1"/0'  | ND     | 4"/3'  |
| Caudate nucleus                       | 3"/1'  | 1"/0'  | 5"/3'  |
| Thalamus                              |         |         |        |
| Medial Dorsal Thalamus                | 0"/0'  | 0"/0'  | 5"/2'  |
| Periventricular nucleus of the thalamus | 0"/0' | 0"/0'  | 3"/5'  |
| Medial Habenula                       | 1"/0'  | 0"/0'  | 5"/3'  |
| Lateral Habenula                      | 0"/0'  | 0"/0'  | 0"/0'  |
| Septal Area                           |         |         |        |
| Dorsal Median preoptic nucleus        | 2"/3'  | 3"/4'  | 4"/5'  |
| Medial Septum                         | 0"/0'  | 0"/0'  | 0"/2'  |
| Lateral Septum                        | 2"/1'  | 1"/1'  | 2"/0'  |
| Hypothalamus                          |         |         |        |
| Anterior Hypothalamic Area            | 2"/3'  | 1"/1'  | 3"/4'  |
| Lateral Hypothalamic Area             | 3"/2'  | 4"/5'  | 3"/0'  |
| Paraventricular nucleus               | 4"/0'  | 5"/5'  | 5"/3'  |
| Periventricular area                  | 3"/0'  | 2"/5   | 5"/0'  |
| Suprachiasmatic nucleus               | 1"/0'  | 0"/0'  | 2"/5   |
| Arcuate nucleus                       | 5"/4'  | 5"/5'  | 4"/0'  |
| Circumventricular Organs/Pituitary    |         |         |        |
| Median Eminence                       | 0"/2'  | 0"/3'  | 0"/4'  |
| Subfornical Organ                     | 4"/5'  | 0"/2'  | 3"/5'  |
| Area Postrema                         | 3"/0'  | 4"/4'  | 3"/4'  |
| Posterior Pituitary (pars nervosa)    | 0"/3'  | 0"/2'  | ND     |
| Anterior Pituitary (pars distalis)    | 0"/0'  | 5"/0'  | 5"/0'  |
| Stellate cells                        | 0"/0'  | 5"/0'  | 1"/0'  |
| Ovoid cells                           | 0"/0'  | 1"/0'  | 4"/0'  |
| Cerebellum                            |         |         |        |
| Purkinje Cells                        | 0"/0'  | 5"/5'  | ND     |
| Hindbrain                             |         |         |        |
| RVLM                                  | 4"/2'  | 2"/2'  | ND     |
| NTS                                   | 3"/4'  | 5"/2'  | 3"/4'  |

Key: * refers to neuronal cell bodies; † refers to fibers. Scored on a scale from 0 to 5. ND is not determined. No AT_{1a} immunoreactivity was observed in glia. AT_{1b} and AT_{2} immunoreactivity were observed in glia.

glycosylated since the theoretical molecular weights of the deglycosylated receptors are 40759 Daltons for the AT_{1a}, 40781 Daltons for the AT_{1b}, and 41200 Daltons for the AT_{2} receptor. The secondary bands most likely represent dimerized receptors or receptor-protein complexes. Immunofluorescent staining of the adrenal with the 3 antibodies gave differing discrete staining patterns in the adrenal. Using a working dilution of 1:500 AT_{1a}, immunoreactivity was seen in both the adrenal medulla and the zona glomerulosa (Figures 2(a), 2(d), and 2(g)). The staining
was primarily cytoplasmic in both regions, although in the medulla, localization to the cell membrane is apparent in some cells (Figure 2(g)). AT\(_{1b}\) immunoreactivity was present in abundance in the zona glomerulosa of the adrenal (Figures 2(b) and 2(e)). The immunofluorescence was primarily localized to the cell membrane (Figure 2(e)). Weak AT\(_{1b}\) immunoreactivity was also present in the zona reticulata (Figure 2(e)). AT\(_{1b}\) immunostaining was nearly nonexistent in the medulla (Figure 2(h)).

AT\(_{1a}\) immunoreactivity was abundantly present in both the adrenal medulla and the zona glomerulosa (Figures 2(c), 2(f), and 2(i)). The AT\(_{1a}\) immunofluorescence was also primarily cytoplasmic although a plasma membrane localization was seen in many medullary cells (Figure 2(i)). No immunofluorescent signal was seen in any sections incubated with the antigenic peptide preadsorbed antibodies (not shown).

Immunoelectron microscopic analysis of the subcellular localization of AT\(_{1a}\) receptors in the zona glomerulosa and medulla is shown in Figure 3. Both cell membrane and cytoplasmic labeling for AT\(_{1a}\) receptors was seen in these cells. AT\(_{1a}\) receptor immunogold labeling of endocytic vesicles and mature multivesicular vesicular bodies was seen in glomerulosa cells (Figures 3(b) and 3(c)) and immunoperoxidase labeling of cell membrane and newly forming endocytic vesicles was seen in medullary cells (Figure 3(e)). Intranuclear AT\(_{1a}\) receptor immunogold staining was observed in cells of the zona glomerulosa. However, AT\(_{1a}\) receptor-immunogold staining was not evident in mitochondria or endoplasmic reticulum of either glomerulosa or medullary cells.

AT\(_{1b}\) immunoreactivity was observed in the pars distalis of the anterior pituitary. It was primarily localized to stellate cells, but significant numbers of ovoid cells were also immunopositive. By contrast, AT\(_{1a}\) immunoreactivity was not observed in the pituitary (Figure 4). AT\(_{2}\) receptor immunoreactivity also was observed in the pars distalis of the anterior pituitary, primarily in ovoid cells. AT\(_{1a}\) and AT\(_{1b}\) receptor immunoreactivity was observed on nerve fibers in the posterior pituitary (Table 1). No Ang II receptor immunoreactivity was observed in the intermediate lobe of the pituitary.

In sections from the brain, neurons were immunopositive for all three receptors, but glial cells showing astrocytic (and microglial, Figure 4 center panel) characteristics were immunopositive only for AT\(_{1b}\). Immunoreactivity for all three angiotensin receptor subtypes was present in abundance in brain regions reported to have high angiotensin receptor density by ligand binding studies and other immunohistochemistry studies (Figures 4–7, Table 1). These regions include the SFO, median eminence, PVN, NTS, and area postrema (Figures 4 and 5, Table 1). In all five of these locations, we demonstrated the presence of all three receptors, although their distribution within each region was not identical (Table 1). Of note, AT\(_{1b}\) receptor immunoreactivity was present in the magnocellular division of the PVN while AT\(_{2}\) receptor immunoreactivity was present in the supraoptic nucleus (SON) (Figure 5). AT\(_{2}\) receptors were more widely distributed than AT\(_{1a}\) and AT\(_{1b}\) receptors in the brain, and their immunoreactivity was found in every region in which AT\(_{1}\) receptor immunoreactivity was observed (Table 1). AT\(_{2}\) receptor immunoreactivity was found exclusively in the amygdala, piriform cortex, thalamus, and medial epithalamus (Figures 5 and 6, Table 1).

Angiotensin II receptor immunoreactivity also was found in rat brain regions generally reported to have low expression of Ang II receptors. These include neurons in the cerebral cortex (AT\(_{1a}\) and AT\(_{2}\)), hippocampus (AT\(_{1a}\) and AT\(_{2}\)), caudate nucleus (AT\(_{1a}\), AT\(_{1b}\) and AT\(_{2}\)), and SON (AT\(_{2}\)) (Figure 5).

4. Discussion

4.1. Antibody Development Strategy. The results of these studies unequivocally demonstrate a differential distribution of AT\(_{1a}\), AT\(_{1b}\), and AT\(_{2}\) receptor immunostaining. This was accomplished by precise epitope targeting within the C-terminus of each receptor, selective antipeptide affinity chromatographic purification methods, Western blotting, and tissue specificity studies in adrenal and pituitary where the distribution of these AT receptor-expressing cells has been established by in situ hybridization and receptor binding studies.

The initial identification of the two subtypes of AT\(_{1}\) Ang II receptors in rodents demonstrated the presence of mRNA for both the AT\(_{1a}\) and AT\(_{1b}\) subtype in the rat adrenals [32, 38, 49]. The AT\(_{1b}\) was identified as the predominant AT\(_{1}\) receptor subtype in the rat adrenal based on mRNA expression [38, 49]. While these initial observations have been confirmed in the rat adrenal [50, 51], the AT\(_{1a}\) is considered to be the predominant AT\(_{1}\) receptor subtype in all other rat tissues except the anterior pituitary based on mRNA expression [38, 52].
Figure 2: Immunofluorescent localization of AT₁a, AT₁b, and AT₂ receptors in rat adrenals. Survey photomicrographs show positive immunofluorescence for AT₁a ((a) and (d); 80x), AT₁b ((b) and (e)), and AT₂ ((c) and (f)) in the zona glomerulosa (160x). Positive staining for AT₁a ((a) and (g)) and AT₂ ((c) and (i)), but not AT₁b ((b) and (h)), is present in the adrenal medulla. The antibodies used were those used in Figure 1.

It is important to be able to discriminate AT₁a and AT₁b receptor protein expression, because their mRNAs are differentially regulated [31, 39, 49, 52–54]. Furthermore, it is important to determine if the changes in mRNA expression translate into changes in expression of these receptor subtypes, because mRNA expression does not always correlate with protein expression. For example, in the kidney losartan increases AT₁a receptor mRNA expression, but decreases AT₁ receptor binding [55]. The existence of miRNAs for angiotensin receptors, for example, miR-155 [56] further erodes the value of mRNA levels as indicators of angiotensin receptor protein expression. Functionality of the subtypes may also differ; AT₁a and AT₁b can stimulate aldosterone release, while AT₁a, but not AT₁b, can stimulate corticosterone release in the mouse adrenal [57].

In view of the near identical pharmacological characteristics of the AT₁a and AT₁b receptor subtypes [34–36], the only way to discriminate these two proteins is to exploit immunological differences arising from differences in their amino acid sequences. While the AT₁a receptor (accession no. P25095 http://www.ncbi.nlm.nih.gov/protein/113493 (accessed 16 March 2012) and AT₁b receptor (accession no. NP 112271) http://www.ncbi.nlm.nih.gov/protein/82524858NP112271 (accessed 16 March 2012) subtypes are encoded by separate genes, they are ∼95% identical and are both made up of 359 amino acids [33, 38]. Thus there are only a few regions of these receptors where they differ substantially in amino acid sequence. One of these regions, near the carboxy terminus of the receptor proteins (amino acids 352 to 355), has 2 different amino acids in this 4 amino
Figure 3: Ultrastructural immunocytochemistry of the AT$_1$ receptor (using anti-AT$_1$ receptor #92578-selinzona glomerulosa (Figures 3(a) through 3(c); 48,000x) and adrenal medulla (Figures 3(d) and 3(e)). Immunogold ultrastructural analysis of zona glomerulosa shows AT$_1$ receptor (showing localization at the cell membrane (bold arrows; Figures 3(a) and 3(b)), in the cytoplasm (line arrows; Figures 3(a) through 3(c)), and on the surface of endocytic vesicles (insert Figure 3(b)). Immunogold particles were also seen in a multivesicular body (MVB) and in the nucleus (Figure 3(c)). Immunoperoxidase staining of adrenal medullary cells reveals a large number of AT$_1$ positive vesicles (line arrows; Figure 3(d); 20,000x and Figure 3(e); 48,000x), patches of membrane receptors (block arrows; Figure 3(e)), and apparent omega body fusion with the cell membrane (arrow heads; Figure 3(e)). Note the lack of localization in the mitochondria (M) and endoplasmic reticulum (ER).

4.2. Adrenal AT Receptor Subtype Localization. The presence of AT$_{1a}$, AT$_{1b}$, and AT$_2$ angiotensin receptor subtype immunoreactivity in the rat adrenal was clearly demonstrated in this study. AT$_{1a}$ and AT$_2$ receptor subtype immunoreactivities were found in both the zona glomerulosa and medulla, which is consistent with receptor binding studies [37, 58–61] and mRNA studies [44, 61–64]. AT$_{1b}$ receptor was not observed in the adrenal medulla, but was present in the zona glomerulosa. This is consistent with in situ hybridization studies of the distribution of AT$_{1b}$ mRNA in the adrenal [39, 44, 46, 54].

Other studies of the localization of Ang II receptor subtype immunoreactivity in the adrenal have given mixed and controversial results. Paxton et al. [65] observed AT$_1$ receptor immunoreactivity in the zona glomerulosa of the rat adrenal with an antibody prepared against amino acids 15–24 of the rat AT$_{1a}$ and AT$_{1b}$ receptor. However, they did not observe any AT$_1$ receptor immunoreactivity in the adrenal medulla. Similarly, Lehoux et al. [66] observed AT$_1$ immunoreactivity in the zona glomerulosa of the rat adrenal.
cortex, but not in the medulla using an antibody raised against amino acids 306–359 of the human AT₁ receptor subtype. Of note, adrenals from rats kept on a low sodium diet displayed AT₁ immunoreactivity in other cortical zones (fasciculata and reticularis). The lack of adrenomedullary staining with this human antibody suggests that it may only recognize the AT₁b sequence in the rat. Giles et al. [67] observed AT₁ immunoreactivity and AT₁ mRNA in the zona glomerulosa of rat adrenals using an antibody directed against amino acids 350–359 of the rat AT₁ subtype plus a small amount of immunoreactivity in the zona fasciculata. However, there was no mention of AT₁ immunoreactivity or mRNA in the adrenal medulla.

Frei et al. [68] observed AT₁ immunoreactivity in the rat adrenal cortex and medulla using a monoclonal antibody raised against amino acids 229–246 of the human AT₁ receptor subtype. Yet, AT₂ immunoreactivity was only observed in the rat adrenal medulla using an antibody raised against amino acids 314–330 of the human AT₂ receptor subtype. On the other hand, Harada et al. [63] observed AT₂ receptor immunoreactivity in immunoblots of the rat adrenal cortex, but not in the medulla using two different antibodies—one raised against amino acids 21–35 of the rat AT₂ receptor and one raised against amino acids 221–363 of the human AT₂ receptor. However, they did detect a low level of AT₂ receptor-like immunoreactivity in the medulla using the latter antibody for immunohistochemical analysis. Conversely, Yiu et al. [69] reported AT₂ immunoreactivity only in the rat adrenal medulla using an antibody directed against amino acids 341–351 of the rat AT₂ subtype. Notably, they reported that this antibody failed to label brain regions known to express AT₂ receptors. Reagan et al. [70] were unable to demonstrate any AT₂ receptor immunoreactivity in the rat adrenal using a polyclonal antibody developed to recognize AT₂ receptors in NIE-115 cells.

4.3. Subcellular Localization of AT₁ Receptors. Localization of immunofluorescence for all three angiotensin receptor subtypes to the cell membrane as well as the cytoplasm in the adrenal is consistent with the behavior of other G protein coupled receptors that are functionally expressed on cell membranes but undergo receptor-mediated internalization [71]. The electron microscopic localization of AT₁ₐ
Figure 5: Immunofluorescent localization of AT receptors in various brain nuclei. Top row: hypothalamic paraventricular nucleus (PVN, 160x) AT$_{1a}$, AT$_{1b}$, and AT$_2$. Second row: immunofluorescence labeling (left to right) for AT$_2$ in supraoptic nucleus (SON; 120x) and median preoptic nucleus (MnPO-dorsal part: 80x), and AT$_{1a}$ localization labeling of arcuate nucleus (ARC; 160x). Third row, (left to right): AT$_2$ in the periventricular nucleus of the thalamus (PVT; 160x), AT$_{1a}$ receptor in the rostral ventrolateral medulla (RVLM, 100x), and AT$_2$ receptor in nucleus of the solitary tract (NTS; 160x). Bottom row (left to right): AT$_{1a}$ in frontal parietal cortex (160x), AT$_2$ in entorhinal cortex (80x), and AT$_2$ in hippocampus CA1 (120x).
The angiotensinergic receptor system in the forebrain osmoregulatory and hindbrain pressor pathways

Figure 6: AT$_2$ receptor immunolocalization in caudate nucleus (CN, 400x), CA3-dentate gyrus of the hippocampus (CA3-DG; 80x), central nucleus of the amygdala (CA; 160x), medial habenula (MH, bottom left; 80x), AT$_{1b}$ in cerebellar Purkinje cells (CB; bottom middle; 100x), and AT$_2$ in mediobasal hypothalamus (MBH; bottom right, 320x).

Figure 7: Diagrammatic summary of important brain nuclei in the angiotensinergic forebrain osmoregulatory pressor (orange pathway) and hindbrain pressor (blue) pathways. Note that there is more than one AT receptor in each site as given adjacent to each micrograph. A representative AT receptor for each site is shown within the figure.
immunoreactivity to putative developing endosomes still in contact with the cell membrane (Figure 3(e)) is consistent with receptor mediated endocytosis as the mechanism of angiotensin receptor internalization [72]. In addition, there is now a considerable body of evidence supporting the existence of an intracellular RAS which signals via AT\(_1\) receptors [73].

Noteworthy in our study is the nuclear localization of adrenal AT\(_{1a}\) receptors. The ability of G protein coupled receptors to localize and signal directly to the cell nucleus is firmly established [74] and likely includes angiotensin receptors. Beginning with the electron microscopic studies localizing \(^3\)H-Ang II to myocardial cell nuclei [75], it has been suspected that Ang II receptors are present in cell nuclei. The existence of nuclear Ang II receptors was subsequently documented in isolated hepatic nuclei by Re and Parab [76] who showed that Ang II increased RNA polymerase II activity, increasing RNA synthesis. Notably, they used 4 mM dithiothreitol an inhibitor of Ang II binding to AT\(_1\) receptors [77], suggesting that the Ang II effect might be mediated by AT\(_2\) receptors. Eggena et al. [78] showed that AT\(_1\) receptor subtype binding was present in rat hepatic cell nuclei and that Ang II could specifically induce transcription of mRNA for renin and angiotensinogen in isolated rat liver nuclei. Moreover, hepatic nuclear AT\(_1\) receptor binding and functionality could be dynamically regulated by adrenalectomy and nephrectomy [79]. Re et al. [80] and Eggena et al. [79] reported that nuclear Ang II receptor binding was associated with nuclear chromatin. Of note, Re et al. [80] observed \(^{125}\)I-Ang II binding to nuclear chromatin in the presence of 5 mM dithiothreitol, again suggesting that \(^{125}\)I-Ang II may be binding to AT\(_2\) receptors [15, 77]. The relative abundance of AT\(_{1a}\) binding within the nucleus, but not the nuclear membrane of the glomerulosa cells in this study, is consistent with localization to nuclear chromatin. AT\(_1\) receptor binding sites have also been identified in rat hepatocyte nuclear membranes by Booz et al. [81] and Tang et al. [82]. Interestingly, Tang et al. [82] determined that the majority of the AT\(_1\)-like binding of Ang II in hepatocyte nuclei was bound to a soluble intranuclear protein. Licea et al. [83] demonstrated nuclear Ang II receptor binding in nuclei of rat renal cortex. Tadevosyan et al. [84] showed that Ang II could stimulate \(^\alpha,\beta\)P-UTP incorporation into RNA and increase NF-kappaB mRNA expression in isolated rat heart cardiomyocyte nuclei suggesting a nuclear site of action of Ang II.

Additional evidence supporting a nuclear localization of angiotensin receptors includes studies using an AT\(_1\) receptor-GFP fusion construct which translocates to the nucleus in Chinese hamster ovary cells [85] and human embryonic kidney (HEK-293) cells [86], as well as immunohistochemical studies showing colocalization of AT\(_1\) and AT\(_2\) immunoreactivity with the nuclear membrane markers nucleoporin-62 and histone-3 [84]. Moreover, the AT\(_1\) receptor contains a nuclear localization signal motif (KKF KKK, 307-11) in its intracellular carboxy terminal tail [87], which promotes its translocation to the cell nucleus. Mutation of one amino acid in this motif (K307Q) in an AT\(_{1b}\) r-GFP receptor construct prevents it from localizing to the nucleus of HEK293 cells [86]. Of note, both agonist induced [87] and agonist independent [71, 88] nuclear localization of AT\(_1\) receptors has been reported.

While there are no published reports of adrenal nuclear angiotensin receptor binding or function, Eggena et al. [78] reported preliminary data suggesting that Ang II could stimulate RNA transcription in isolated adrenal nuclei. In addition, Goodfriend and Peach [89] suggested that Ang III can act intracellularly in the zona glomerulosa to promote aldosterone production.

4.4. Pituitary AT Receptor Subtype Localization. Both AT\(_{1b}\) and AT\(_2\) receptor immunoreactivities were present in high amounts in the anterior pituitary. As noted previously mRNA for AT\(_{1b}\) receptors is abundant in the anterior pituitary, while AT\(_{1a}\) mRNA is much less abundant and AT\(_2\) mRNA is not observed in the anterior pituitary [47, 90]. Autoradiography and radioligand binding studies have demonstrated a high density of Ang II receptors in the anterior pituitary [37, 91–93]. This binding displays AT\(_1\) receptor characteristics, and little or no AT\(_2\) receptor binding has been observed [27, 94]. AT\(_{1b}\) expression was highest in stellate cells, while AT\(_2\) expression was highest in ovoid cells. Both AT\(_{1a}\) and AT\(_{1b}\) immunoreactivity was present on nerve fibers in the posterior pituitary. The ability of Ang II to affect the release of pituitary hormones is well known [95]. There are no reports of Ang II receptor binding in the posterior pituitary of the rat, although there is one report of AT\(_1\) receptor-immunoreactivity in nerve fibers and cell bodies in the posterior pituitary [96] and one report of AT\(_2\) receptor-immunoreactivity in the posterior pituitary as well as in the vasopressinergic magnocellular division of the PVN and the SON [97]. mRNA studies indicate a predominance of the AT\(_{1b}\) subtype in the anterior pituitary of the rat [38, 98–100], with little or no AT\(_{1a}\) and AT\(_2\) mRNA.

Many of the pituitary hormone-releasing effects of Ang II occur in the hypothalamus and those effects are discussed below. However, some of the pituitary hormone releasing of Ang II occur directly in the pituitary. Systemically administered Ang II stimulates vasopressin release from the posterior pituitary of the dog [101, 102]; however, this may not generalize to the rat. AT\(_{1a}\) and AT\(_{1b}\) receptors on nerve fibers in the rat posterior pituitary [96] could mediate these effects of Ang II, reminiscent of the mechanism whereby Ang II acts on sympathetic nerve terminals to stimulate norepinephrine release [103, 104].

Radioligand binding studies have revealed high levels of Ang II receptor binding in a lactotroph enriched pituitary preparation [105]. mRNA studies indicate that AT\(_{1b}\) receptors appear most often on lactotrophs, being present on more than 50% of all lactotrophs [98]. The appearance of AT\(_{1b}\) immunoreactivity in ovoid cells is consistent with these receptors being present on lactotrophs. It has been reported that AT\(_{1b}\) mRNA is present in a somatotroph cell line [100]. Somatotrophs are also ovoid in shape and blood-borne Ang II can inhibit growth hormone release [106], although it has also been reported that Ang II synthesized by and released from lactotrophs can stimulate the release of growth hormone from
somatotrophs, [107] suggesting that somatotrophs may have excitatory AT$_1$ receptors and inhibitory AT$_2$ receptors.

ACTH release from dissociated corticotrophs in the anterior pituitary is also stimulated by Ang II in vitro [108]. The stimulation decreases with supraphysiological estradiol exposure in vivo and correlates positively with reductions in Ang II receptor binding caused by in vivo supraphysiological estradiol exposure [108]. Autoradiographic studies of AT$_1$ receptor binding in the anterior pituitary indicate that AT$_1$ receptor binding varies with the estrous cycle and that exogenous estrogen decreases anterior pituitary AT$_1$ receptor binding in ovariectomized rats [109]. mRNA for AT$_1b$ receptors in the anterior pituitary is also suppressed by estrogen treatment [38, 110]. The appearance of high levels of AT$_1b$ immunoreactivity in stellate cells in this study is consistent with these receptors being present on corticotrophs.

There is one report of AT$_2$ receptor immunoreactivity in pituitary adenoma blood vessels in humans [96], leading to the hypothesis that AT$_2$ receptors in could participate in tumor-induced angiogenesis.

4.5. Brain AT Receptor Subtype Localization. These studies describe a widespread distribution of AT$_{1a}$, AT$_{1b}$, and AT$_2$ receptor immunoreactivity throughout the rat brain. The receptors were expressed abundantly in a number of brain regions that constitute the cardiovascular regulatory circuits of the brain, as well as the noncardiovascular regulatory regions of the brain. There was considerable variation in the degree of expression of the receptors in different regions reminiscent of the profound differences in radioligand binding for Ang II receptors, particularly among the AT$_1$ receptors. AT$_2$ receptors displayed an unanticipated widespread distribution throughout the rat brain, which contrasts with their limited distribution as indicated by radioligand binding studies. While AT$_1$ receptors are considered to play the predominant role of mediating the actions of Ang II in the brain, AT$_2$ receptors are increasingly recognized as having an important role as physiological antagonists of AT$_1$ receptor effects. The codistribution of AT$_1$ and AT$_2$ receptors in several brain regions as well as the adrenal is consistent with the concept of colocalization of these two subtypes in the same cells as counter regulators to each other at the cellular level as well as on an organism level [111–113].

The selective expression of AT$_{1b}$ receptors on astrocytes suggests that there is a cell-specific expression of Ang II receptor subtypes in the brain. Functional AT$_1$ receptors are present in primary cultures of astroglia from rat brain [114], but questions have been raised as to whether this expression could reflect an altered phenotype of cultured cells not seen in situ in a living brain [115]. In contrast, Füchtbauer et al. [116] observed AT$_1$ immunoreactivity (Santa Cruz, sc-579, amino acids 306–359) in astrocytes of the outer molecular layer of the dentate gyrus of the mouse brain, but did not see AT$_1$ immunoreactivity in the microglia. Of note, retinal astrocytes also express AT$_1$ receptor immunoreactivity (Alomone, #AAR-011 amino acids 4–18) while amacrine cells in the rat retina display AT$_2$ immunoreactivity (Alomone, no. AAR12, amino acids 21–35) [117]. These reports and our observations suggest that glia do express AT$_1$ receptors and that they are of the AT$_{1b}$ subtype. Since astrocytes are the primary source of angiotensinogen in the brain, the AT$_{1b}$ receptor may play a role in regulating angiotensinogen in the brain.

The expression of AT$_{1b}$ receptor immunoreactivity on cells with the morphological characteristics of microglia suggests that this receptor subtype mediates the proinflammatory effects of Ang II. AT$_1$ receptor antagonism blocks the activation of microglia in an animal model of brain inflammation [118]. Proinflammatory cytokine participation in the pressor actions of Ang II in the brain is reversible by AT$_1$ antagonists [119, 120], suggesting that microglial AT$_1$ receptors may play a role in blood pressure regulation as well as inflammation.

The concept of the presence of Ang II receptors in the brain was firmly established by the cross-perfusion studies of Bickerton and Buckley [121] showing that blood-borne Ang II had sympathoexcitatory effects mediated by the brain. Since that time, a multitude of methodological approaches have been used to map the distribution of Ang II receptors in the brain. Early radioligand binding studies of brain Ang II receptors [122, 123] indicated that Ang II receptors were located in regions within the blood-brain barrier, for example, cerebellum, hypothalamus, thalamus, septum, and midbrain, as well as outside the blood brain barrier. The first receptor autoradiographic study of brain Ang II receptors for blood-borne Ang II clearly demonstrated their presence in 4 circumventricular organs (CVOs): the SFO, OVLT, median eminence, and area postrema [124]. In vitro receptor autoradiographic studies of the rat brain confirmed the localization of Ang II receptors in these CVOs and revealed a widespread distribution of discrete populations of Ang II receptors in a large number of brain nuclei [93, 125]. Subsequent receptor autoradiographic studies using Ang II receptor subtype specific competing ligands indicated that both AT$_1$ and AT$_2$ receptors were present in the brain and were differentially distributed [27, 58]. Regions containing high densities of AT$_1$ receptor binding include regions associated with dipsogenesis and cardiovascular regulation, for example, SFO, OVLT, MnPO, PVN, NTS, dorsal motor nucleus of the vagus, area postrema, rostral ventrolateral medulla (RVLM), as well as noncardiovascular regulatory regions, for example, pyriform cortex, subiculum, and spinal trigeminal nucleus. Generally, regions containing high densities of AT$_2$ receptor binding are unrelated to blood pressure regulation and dipsogenesis, for example, mediadorsal thalamus, inferior olivary nucleus, medial geniculate, and subthalamic nucleus. While many regions have a strong predominance of one or the other subtype, several brain regions show both AT$_1$ and AT$_2$ receptor binding, for example, parabrachial nuclei, pedunculopontine tegmental nucleus, locus coeruleus, and superior colliculus [126].

Localized injection of exogenous Ang II has been used to map the distribution of brain Ang II receptors. Early studies directed at determining sites of action of Ang II assessed its behavioral and physiological effects. Subsequent studies using iontophoretic or pressure injection of Ang II via micropipettes have focused on its cellular effects. Early mapping of Ang II receptors mediating its dipsogenic
effects indicated a widespread distribution in the forebrain [127]. However, a subsequent study [128] revealed that all the active sites were targeted with a cannula that traversed the anterior cerebral ventricles, and that only when Ang II leaked into the ventricles that a dipsogenic response occurred. Microinjection of Ang II into the SFO and PVN is excitatory to these neurons [129]. Microinjection of Ang II into the RVLM [130], area postrema, and NTS [131] increases blood pressure. Microinjection of Ang II into the periaqueductal gray increases blood pressure via its actions at AT1 receptors [132], while microinjection of Ang II into the superior colliculus increases blood pressure via its actions at AT2 receptors [133] consistent with radioligand binding studies indicating the presence of AT1 or AT2 receptors in these regions [27]. Lastly, the distribution of angiotensin responsive neurons has been determined using induction of fos expression as a functional marker [134].

A major controversy involves the presence or absence of Ang II receptors on vasopressinergic and oxytocinergic neurons in the SON and the magnocellular division of the PVN. Stimulation of vasopressin and oxytocin release from the posterior pituitary results from stimulation of the magnocellular neurons in the PVN and SON. In this study, all 3 Ang II receptor subtypes were highly expressed in the magnocellular divisions of the PVN. Radioligand binding studies of Ang II receptors reveal high expression of AT1 receptors in the parvocellular region of the PVN and low expression of Ang II receptors in the magnocellular division of the PVN and SON (as described in the previous section). Similarly, mRNA studies (succeeding section) have failed to demonstrate measurable Ang II receptor synthesizing capacity in these regions. However, electrophysiological studies suggest that neurons in these regions are responsive to Ang II. Nagatomo et al. [135] showed that Ang II inhibited potassium currents in SON neurons using patch clamping in brain slices. Ang II has a direct excitatory effect in the SON, which is consistent with the presence of AT1 receptors on vasopressinergic and oxytocinergic neurons [136]. The data reported herein is consistent with the presence of functional AT1 receptors in the PVN and SON.

Parvocellular PVN AT1 receptors revealed by radioligand binding and mRNA assays are well placed to stimulate CRH neurons in the PVN to release corticotrophin releasing hormone (CRH) from their nerve terminals in the median eminence into the hypothalamo-hypophyseal portal vessels to act upon corticotrophs in the anterior pituitary. In this study, all 3 Ang II receptor subtypes were highly expressed in the parvocellular division of the PVN. The use of in situ hybridization or PCR for localization of mRNA to determine sites of synthesis of proteins has been widely used to localize Ang II receptor subtypes in the brain. Kakar et al. [31] reported a predominance of AT1b mRNA in the SON and MnPO as well as the anterior olfactory nucleus with very low AT1b mRNA expression in the SFO and PVN. Lenkei et al. [137] also reported the absence of AT1a and AT1b mRNA in the vasopressin positive neurons and GFAP positive astroglia in the SON and PVN. In the two-week-old rat brains, Jöhren and Saavedra [138] also observed AT1a mRNA in the pyriform cortex, basal amygdala and choroid plexus and AT1b mRNA in the choroid plexus. AT1 receptor binding has been reported in the choroid plexus [139] although at very low levels [140].

Brain AT2 receptor mRNA shows both similarities and differences from AT1 receptor binding in the rat brain. Noteworthy is the presence of AT2 mRNA in the red nucleus and the absence of AT2 mRNA in the locus coeruleus, lateral septum, and cerebellum [141]. These discrepancies have been interpreted as indicating that the red nucleus synthesizes AT2 receptors that are only expressed on its efferent nerve terminals that project to the inferior olivary nucleus and cerebellum, while the AT2 receptor expressing brain regions devoid of AT2 mRNA express AT2 receptors on the nerve terminals of its afferents from other brain regions. Lenkei et al. [142] observed AT2 mRNA in the red nucleus. However, they also observed AT2 mRNA in the lateral septum and locus coeruleus, as well as a much greater number of brain regions, including some traditionally AT1 predominant regions such as the NTS and spinal trigeminal nucleus. Lenkei et al. [47] also did a comprehensive in situ hybridization analysis of the rat brain AT1a receptor mRNA. Overall this is consistent with AT1a receptor binding, with a few exceptions, for example, the lack of AT1a mRNA in arcuate nucleus and median eminence, where it is postulated that the AT1 receptors occur on nerve terminals of hypothalamic neurons that synthesize dopamine or releasing hormones and release them into the hypothalamo-hypophyseal portal system to act upon endocrine cells of the anterior pituitary. There are also some brain regions that express AT1b mRNA, but not AT1a receptor binding, such as hippocampus CA1 and CA2 and some thalamic and brainstem nuclei [47]. An area of considerable cardiovascular regulatory significance is the RVLM. Chronic Ang II infusion was shown to up-regulate AT1 mRNA in the RVLM and reduce it in the SFO, suggesting that enhanced activation of the RVLM by enhanced AT1 stimulation increases sympathetic nervous system activity [143].

There are a large number of studies that have used immunohistochemistry and Western blotting to identify and localize Ang II receptor subtypes in the central nervous system. The receptor antigens are generally peptide fragments from different domains of the receptor protein, although one antibody [144] was generated from a purified AT1 receptor protein. Some antibodies target an extracellular domain near the amino terminal for example, Santa Cruz Biotechnology, SC-1173 (amino acids 15–24), the transmembrane spanning regions of the receptor, intra- and extracellular domains between the transmembrane spanning domains, the third intracellular loop (amino acids 225–237) of the AT1 receptor (Chemicon), and the intracellular carboxy terminal domain. Several of these studies have used antibodies directed against
the same carboxy terminal regions of the AT$_{1a}$ (Abcam, AB18801), the AT$_{1a}$ or the AT$_{1b}$ (Advanced Targeting Systems, AB-N25AP, AB-N26AP, or AB-N27AP), and the AT$_2$ receptors (Abcam, AB19134; Advanced Targeting Systems, AB-N28AP) that were used for generation of these antibodies.

Localization of AT$_1$ receptor immunoreactivity in the brain was first done by Phillips et al., [145] using the 225–237 antibody directed to the third intracellular loop of the AT$_1$ receptor. They showed extensive distribution of AT$_1$ immunoreactivity in areas identified by receptor autoradiography to have Ang II receptors. Cardiovascular regulatory regions that were AT$_1$ immunopositive included the PVN, OVLT, SFO, area postrema, NTS, RVLM, and nucleus ambiguous. AT$_1$ immunopositive neurons were also present in the SON, and magnocellular division of the PVN, medial septal nucleus, LC, superior and inferior olivary nuclei, hypoglossal nucleus, ventral horn of the spinal cord and other regions not generally viewed as AT$_1$ receptor targets of Ang II. Conversely, some areas reported to express Ang II receptor binding sites, for example, pyriform cortex, suprachiasmatic nucleus did not show AT$_1$ immunoreactivity. They suggested that Ang II via AT$_1$ receptors may have an expanded role in the CNS beyond that considered at that time.

Other studies also report the presence of AT$_1$ receptors in the SON and/or the magnocellular division of the PVN using either an amino terminal peptide fragment directed antibody, AB18801 and AB-N27AP [146, 147] and the antibody directed against the 225–237 fragment of the AT$_1$ receptor [148, 149]. Of note, the number of cells in the magnocellular division of the PVN expressing AT$_1$ receptor using AB18801 was dramatically increased in rats with induced heart failure [146]. Two other studies observed an increase in total PVN AT$_1$ receptor immunoreactivity (Abcam unspecified). In the first study, PVN AT$_1$ immunoreactivity was increased in a rat model of heart failure [150]. In the second study, PVN AT$_1$ immunoreactivity was increased with chronic intravenous Ang II infusion that was only partially reversed by ICV losartan infusion [151].

Using an antibody against purified AT$_2$ receptor protein, Reagan et al. [152] immunohistochemically localized AT$_2$ receptor immunoreactivity in the rat brain. Regions reported to have AT$_2$ receptor binding and/or mRNA that were immunopositive included the locus coeruleus and several thalamic nuclei. Other regions reported to be AT$_2$ expressing included the amygdala and the Purkinje cell layer of the cerebellum. In addition, AT$_2$ immunoreactivity was present in the magnocellular division of the PVN and SON which further confirms observations in our study. However, as noted above, this antibody did not label the adrenal [70].

A series of studies have used the AT$_{1a}$ carboxy-terminal fragment-directed antibody to identify AT$_1$ receptor immunoreactivity in the area postrema, NTS and RVLM at the electron microscopic level. AT$_{1a}$ immunoreactivity was present in neuronal cell bodies, dendrites, axon terminals, perivascular glial processes of astrocytes, fibroblasts, and vascular endothelial cells in the area postrema and dorsomedial NTS [153]. This AT$_{1a}$ immunoreactivity colocalized with the gp91$^{phox}$ subunit of NADPH oxidase in neuronal cell bodies, dendrites, and putative vagal afferents in the medial NTS [154]. Dendritic processes of the medial NTS containing AT$_{1a}$ immunoreactivity also were positive for tyrosine hydroxylase (TH) or adjacent to TH containing axons [155]. In the TH positive neurons of the RVLM, AT$_1$ receptor expression was greater in female rats than in male rats [156], and this increase was associated with a higher estrogen state (proestrus versus diestrus) and increased plasma membrane expression of AT$_1$ immunoreactivity [157]. This same group has used the AT$_2$ fragment directed antibody (AB19134) to identify AT$_2$ receptor immunoreactivity in the PVN and NTS at the electron microscopic level [158, 159]. These studies have co-localized AT$_1$ immunoreactivity with neuronal nitric oxide synthase (nNOS) in neuronal cell bodies and dendrites in the medial NTS [159], and with vasopressin in neuronal cell bodies and dendrites in the PVN [158]. This latter observation contrasts with the studies of Lenkei et al. [142], who did not find AT$_2$ receptor mRNA in the PVN.

Extensive studies of AT$_1$ and AT$_2$ immunoreactivity in the RVLM and NTS in animal models of heart failure have been carried out by Gao, Zucker and colleagues using AT$_1$ and AT$_2$ antibodies, primarily SC-1173 and SC-9040 [160, 161]. AT$_1$ receptors in the RVLM and NTS showed increased AT$_1$ immunoreactivity, while AT$_2$ receptors showed decreased immunoreactivity. Infusion of Ang II into the brain of rabbits to simulate a heart failure model increased AT$_1$ receptor immunoreactivity in the RVLM [162]. Interestingly, viral transfection of AT$_2$ receptors into the RVLM, which was documented with increased AT$_2$ immunoreactivity, suppressed sympathetic activity in normal rats [163]. In a mouse model of hypertension, the RA mouse [164], immunoreactivity for AT$_1$ (SC-1173) in the NTS and RVLM, was not shown to be up regulated [165].

AT$_1$ (ABI8801) and AT$_2$ (AB9134) immunoreactivity in the substantia nigra (SN) colocalized with TH in neurons, GFAP in astrocytes and OX-6 and OX-42 in activated microglia [166–168]. Using different carboxy terminal directed AT$_1$ and AT$_2$ antibodies for Western blotting, it was shown that estrogen treatment of ovariectomized rats, which was protective against 6-hydroxydopamine induced neurotoxicity in the SN, decreased AT$_1$ and increased AT$_2$ expression in the SN [166]. Of note, no change in AT$_1$ receptor mRNA was observed [166]. These researchers also observed AT$_1$ and AT$_2$ immunoreactivity (Santa Cruz, SC-579 and SC-9040) in dopaminergic neurons, astrocytes and microglia in both monkey and human SN [169].

The dorsomedial hypothalamus (DMH), a brain region that exhibits high AT$_1$ receptor density [170], also displays AT$_1$ immunoreactivity using the AB-N27AP [147]. This brain region is associated with the cardiovascular manifestations of panic disorder and direct administration of an AT$_1$ receptor antagonist into the DMH blocks this component of the panic disorder in an animal model of panic disorder [147].

Giles et al., [67] using the 350–359 carboxy terminal peptide directed AT$_{1a}$ antibody, observed strong AT$_1$ receptor immunoreactivity in numerous brain regions including the SFO, OVLT, MnPO, the parvocellular division of the PVN,
several other hypothalamic nuclei, and the NTS, corresponding well with radioligand binding and mRNA studies of the distribution of brain AT$_1$ receptors.

4.6. Perspective on the Use of Antibodies for the Study of Angiotensin Receptors. The ambiguity associated with studies of angiotensin receptors using different methods, whether by radioligand binding, receptor autoradiography, mRNA, local application of Ang II, electrophysiology, fos induction, or by immunoreactivity, necessitates considerable stringency in the analysis and interpretation of the data. Strengths of the immunohistochemical studies reported herein are as follows: (1) there is no known peptide sequence that closely mimics those used to generate these antibodies, (2) the antibodies were affinity purified to eliminate antibodies that did not recognize the antigenic peptide, (3) antibody binding is blocked by incubation with an excess of the antigenic peptide (preadsorption control), (4) Western blots indicate that the primary bands of labeled protein have molecular weights within the range of those previously observed for glycosylated, dimerized or chaperone protein linked angiotensin receptors [68, 171–175], and (5) the anatomical pattern of immunoreactivity correlates with radioligand binding for AT$_1$ receptors [37, 59], agonist-induced c-fos expression [176], and the distribution of mRNA encoding the protein [44].

Weaknesses of this and other immunohistochemical approaches are as follows: (1) one cannot rule out the possibility that another protein could present an epitope similar to that recognized by these antibodies leading to a false positive, (2) there are posttranslational modifications of the receptor proteins that may mask the antigenic sites that they recognize, for example, phosphorylation of serine residues in Ang II receptors by a variety of protein kinases. The C-terminal domains chosen for generation of these antibodies contain several serines which when phosphorylated may mask the epitopes for the antibodies. AT$_1$ receptors are phosphorylated by G protein receptor kinase GRK2 (formerly known as β adrenergic receptor kinase, BARK1) leading to β-arrestin binding to the intracellular domain of the AT$_1$ receptors which may also mask the epitopes [177]. An additional post-translational modification is proteolytic cleavage of the receptor into smaller fragments following internalization. Cook et al. [178] demonstrated formation of a 54 amino acid carboxy terminal fragment of the rat AT$_{1a}$ receptor that translocated to the nucleus and induced apoptosis in a variety of cell types. Thus it is possible that the immunoreactivity observed herein is not that of the full length receptor. (3) Receptors undergo protein-protein interactions such as receptor dimerization or interactions with chaperone proteins which have the potential to mask the antigenic site on the receptor; (4) inability to document the loss of immunological reactivity in an animal in which the receptor protein has been eliminated, for example, receptor knockouts. A recent publication [179] using Western blotting and immunofluorescence has challenged the specificity of 6 commercially available AT$_1$ receptor antibodies, including one previously questioned by Adams et al., [180] based upon the presence of immunoreactive material in mice in which the AT$_{1a}$ receptor is disrupted. The specificity of 3 AT$_1$ receptor antibodies, Alomone Labs #AAR-011, Santa Cruz sc-1173, and Abcam 18801, has also been challenged based upon expression of immunoreactivity in AT$_{1a}$ and AT$_{1b}$ knockout mice [181]. A generalized challenge to the ability of antibodies to selectively recognize G protein-coupled receptors (GPCR) based on apparent nonspecificity of 49 GPCR antibodies to 19 different GPCRs (the AT$_1$ and AT$_2$ receptors were not among the 19 GPCRs) has called into question the validity of immunolological identification of GPCRs [182]. However, Xue et al., [183] using the same antibody as Adams et al., [180] demonstrated knockdown of AT$_1$ receptor immunoreactivity in the PVN. Of note, the AT$_{1a}$ gene disruption [184] does not eliminate the carboxy terminal coding domain of the receptor that includes the peptide sequences used to generate several of those antibodies. If this portion of the receptor is still expressed it could explain the residual presence of AT$_{1a}$ immunoreactive material in these knockout mice. However, the amino terminal sequence used to generate SC-1173 (amino acids 15–24) is in the deleted part; thus, it remains questionable whether the siRNA knockdown in the rat brain or the knockout of the mouse AT$_{1a}$ receptor gives the correct information regarding the specificity of this and other AT$_1$ receptor antibodies.

One approach to resolve this question is to determine the identity of the protein in the band that the AT$_1$ receptor antibodies recognize in both wild-type and AT$_1$ receptor knockout mice. This has the potential to either (1) validate the immunological identification of AT$_1$ receptor protein thereby calling into question the efficacy of the AT$_1$ receptor knock-out technology, (2) to discover a heretofore unknown subtype of the AT$_1$ receptor with an mRNA sequence that somehow evaded recognition by homology cloning approaches, (3) to identify (a) non-AT$_1$ protein(s) that colocalize(s) with AT$_1$ receptors and display (a) sufficiently similar epitope(s) as to be recognized by a variety of AT$_1$ receptor antibodies, (4) to discover (a) proteins with no relationship to AT$_1$ receptors that coincidentally express the same epitope(s) as the AT$_1$ receptor antibodies, or (5) to discover (a) novel protein(s) that have not yet been identified.

Until such questions are definitively answered, immunohistochemical studies, despite their known and potential limitations, can complement other types of analyses, which are also subject to a variety of differing limitations.

In conclusion, antibodies that can differentiate the 3 different angiotensin II receptor subtypes in the rat were used to immunohistochemically label angiotensin II receptor subtype-like immunoreactivity in the rat adrenal, pituitary, and brain. The pattern of staining corroborates mRNA, radioligand binding, and functional studies of adrenal and anterior pituitary angiotensin receptors. This indicates that AT$_{1a}$ and AT$_2$ receptor subtypes occur in the zona glomerulosa and medulla of normal rats, the AT$_{1b}$ subtype occurs only in the zona glomerulosa of normal rats while the AT$_{1b}$ is the subtype predominantly expressed in the anterior pituitary. The localization of Ang II receptor immunoreactivity in the brain is in large part consistent with radioligand binding, mRNA, Ang II-induced fos expression, and functional studies; however, differences between these immunoreactivity
observations and observations obtained from some other techniques are yet to be resolved.

**Disclosure**

R. C. Speth has licensed these antibodies for commercial sale to Advanced Targeting Systems, Inc., San Diego, CA, USA (92121). The immunochemical studies conducted by M. Brownfield did not benefit ImmunoStar (i.e., they do not offer these antibodies).

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