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Research Article

Immunohistochemical Localization of AT_{1a}, AT_{1b}, and AT_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_{1a}, AT_{1b}, and AT_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_{1a} and AT_2 receptors in adrenal zona glomerulosa and medulla. AT_{1b} immunofluorescence was present in the zona glomerulosa, but not the medulla. Ultrastructural immunogold labeling for the AT_{1a} receptor in glomerulosa and medullary cells localized it to plasma membrane, endocytic vesicles, multivesicular bodies, and the nucleus. AT_{1b} and AT_2, but not AT_{1a}, immunofluorescence was observed in the anterior pituitary. Stellate cells were AT_{1b} positive while ovoid cells were AT_2 positive. In the brain, neurons were AT_{1a}, AT_{1b}, and AT_2 positive, but glia was only AT_{1b} positive. Highest levels of AT_{1a}, AT_{1b}, and AT_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_1 and AT_2 [14–19]. With the discovery of these

multiple subtypes of Ang II receptors, pharmacological studies revealed that the 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 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, 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]. It has been suggested that the medial amygdala can mediate salt appetite [30], but beyond that, the functional significance of the AT_2 in the brain and the adrenal has not been established.

The subsequent discovery that rodents express two subtypes or isoforms of the AT_1 receptor, AT_{1a} and AT_{1b}, [31–33] raises the question as to which of these two 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 AT_1 receptor-selective antagonists to bind to the AT_{1a} and AT_{1b} receptor subtypes reveal little difference in their affinities for these two subtypes [34–37].

PCR amplification of AT_{1a} and AT_{1b} mRNA in female rat adrenal, lung, vascular smooth muscle, pituitary, and brain indicated that the AT_{1a} subtype mRNA was predominant in the lung, vascular smooth muscle, and hypothalamus, while the 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 AT_1 receptor expression without altering 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 AT_{1a} and AT_{1b} mRNA expression in the adrenal indicate that the 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 AT_{1a} and AT_{1b} mRNA in rodent brain vary considerably along a continuum from a predominance of AT_{1b} expression in the female rat brain [31], to a moderate predominance of 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 AT_{1b} mRNA in the adult male rat brain [41], and to no expression of AT_{1b} mRNA in rat brain [47]. In comprehensive studies of the distribution of AT_{1a} and AT_{1b} mRNA the rat brain and pituitary [41], the AT_{1a} mRNA was found to be highly expressed in brain regions reported to mediate cardiovascular effects of Ang II, while AT_{1b} expression was very low in these regions. Conversely, AT_{1b} mRNA was very high in the anterior pituitary while AT_{1a} mRNA was low.

To determine if the distribution of AT_{1a}, AT_{1b}, and 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 AT_{1a}, AT_{1b}, and 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 AT_{1a} receptor, the peptide was PSDNMSSSAKKPASC, which corresponds to amino acids 341–355 of this 359 amino acid protein. For the AT_{1b} receptor, the peptide was SSSAKKSASFFEVE, which corresponds to amino acids 346–359 of this 359 amino acid protein. For the AT_2 receptor, the peptide was CRKSSSLREMETFVS, 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 AT_{1a}-selective antibodies, serum from rabbits immunized with the peptide corresponding to the AT_{1a} receptor subtype was affinity purified using chromatography resin cross-linked with the 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 AT_{1b} receptor peptide antigen to chromatography resin. Antibody that was not retained by the AT_{1b} resin was denoted as AT_{1a} receptor selective. Antibody that was retained by both the AT_{1a} and AT_{1b} resins was defined as nonselective for AT_{1a} or AT_{1b}-receptors. A similar strategy was used to derive AT_{1b} selective antibodies except that serum from rabbits immunized with the peptide corresponding to the AT_{1b} receptor subtype was affinity purified using chromatography resin cross-linked with the AT_{1b} peptide initially. Antibodies retained by the AT_{1b} resin were subsequently applied to the AT_{1a} resin. Antibodies retained by the AT_{1b}, but not the AT_{1a} resin, were classified as AT_{1b} selective. AT_2 receptor antibodies were affinity purified using chromatography resin cross-linked with the AT_2 receptor peptide used to generate the antibody. Antibodies retained by the AT_2 resin were eluted with high salt solution and classified as 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% NaN_3 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).

2.4. Tissue Preparation. Rats were deeply anesthetized with isoflurane or pentobarbital (65 mg/kg IP) and perfused intracardially with physiological flush solution (Tyrode's solution) containing heparin and procaine followed by histological fixative (4% paraformaldehyde with 0.05% glutaraldehyde in 0.1 M sodium phosphate, pH 7.5). Brains, pituitaries, and adrenals were removed and immersion fixed at 4°C in the same solution overnight and then stored in saline until sectioning at 50 micron thickness for immunofluorescence microscopy using a Lancer vibratome.

2.5. Immunofluorescence Histochemistry. Adrenals, pituitaries, and brains from 12 rats were used for these studies. Initially all antibodies were screened at dilutions of 1 : 100 to 1 : 10,0000 in ICC buffer (PBS with 0.25% gelatin, 2% normal goat serum 0.1% thimerosal, and 0.05% neomycin) to determine working dilutions demonstrating the highest signal and lowest background signal for each tissue. Working dilutions of angiotensin II receptor antibodies were (1 : 500) primary antibody (AT_{1a}, AT_{1b}) and 1 : 2000 AT_2 for 18–72 hours at 4°C. Control sections were incubated with primary 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 AT_{1a}, AT_{1b}, or AT_2 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% BSAc (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 AT_{1a} 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 SIs 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 AT_{1a}, AT_{1b}, and AT_2 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_2 immunoreactivity was abundantly present in both the adrenal medulla and the zona glomerulosa (Figures 2(c), 2(f), and 2(i)). The AT_2 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

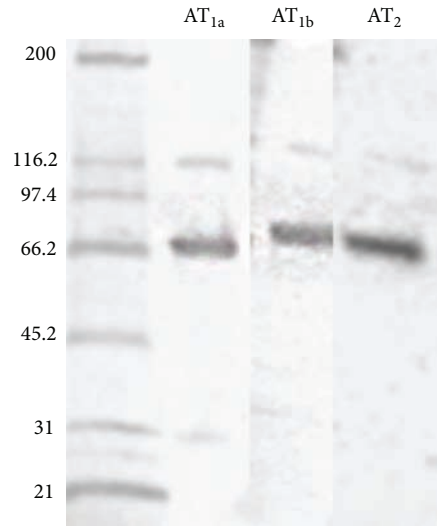


Figure 1: Western immunoblots for AT_{1a}, AT_{1b}, and AT_2 receptors of crude extracts of whole adrenals. The three receptors show major bands at ~69–75 kD and well as faint bands at about ~ 116–126 kD. AT_{1a} receptor-directed antibody (Rabbit 92578-sel), AT_{1b} receptor-directed antibody (Rabbit 92587-sel), AT_2 receptor-directed antibody (Rabbit 92595).

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_{1b} 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].

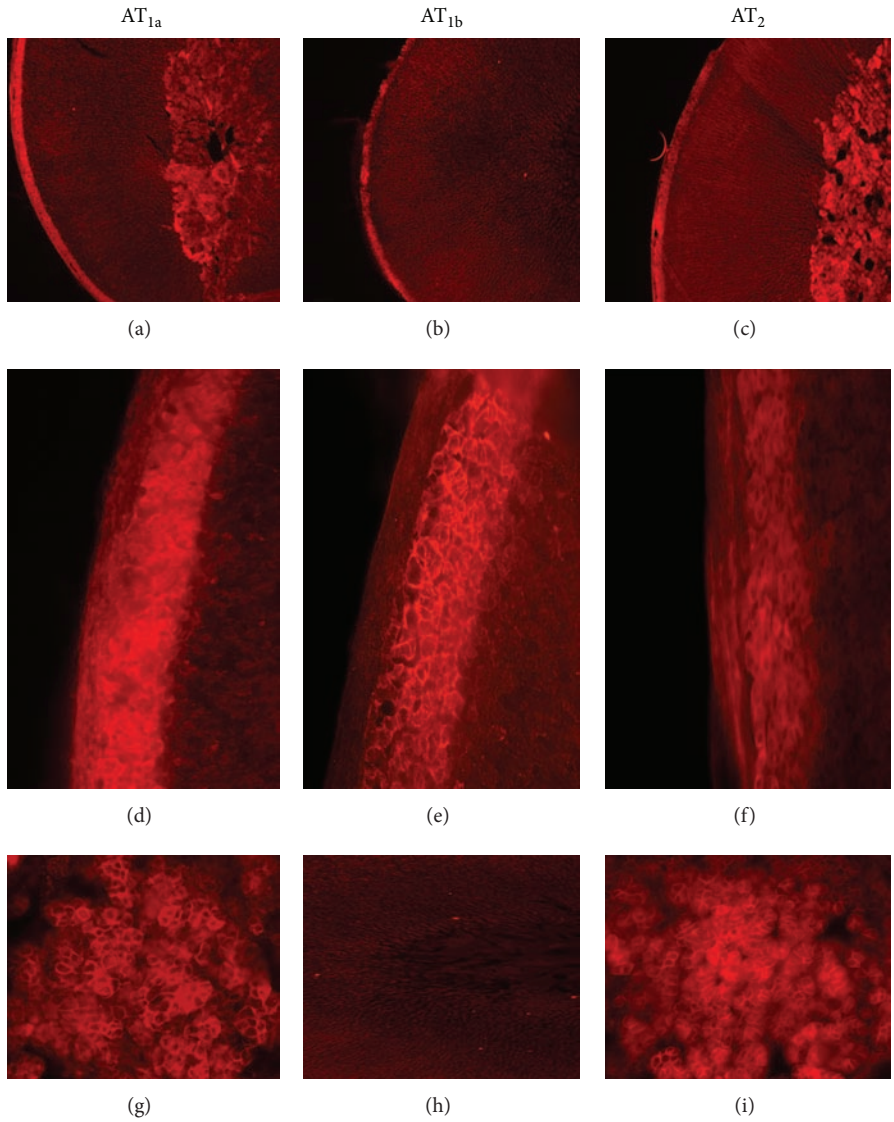
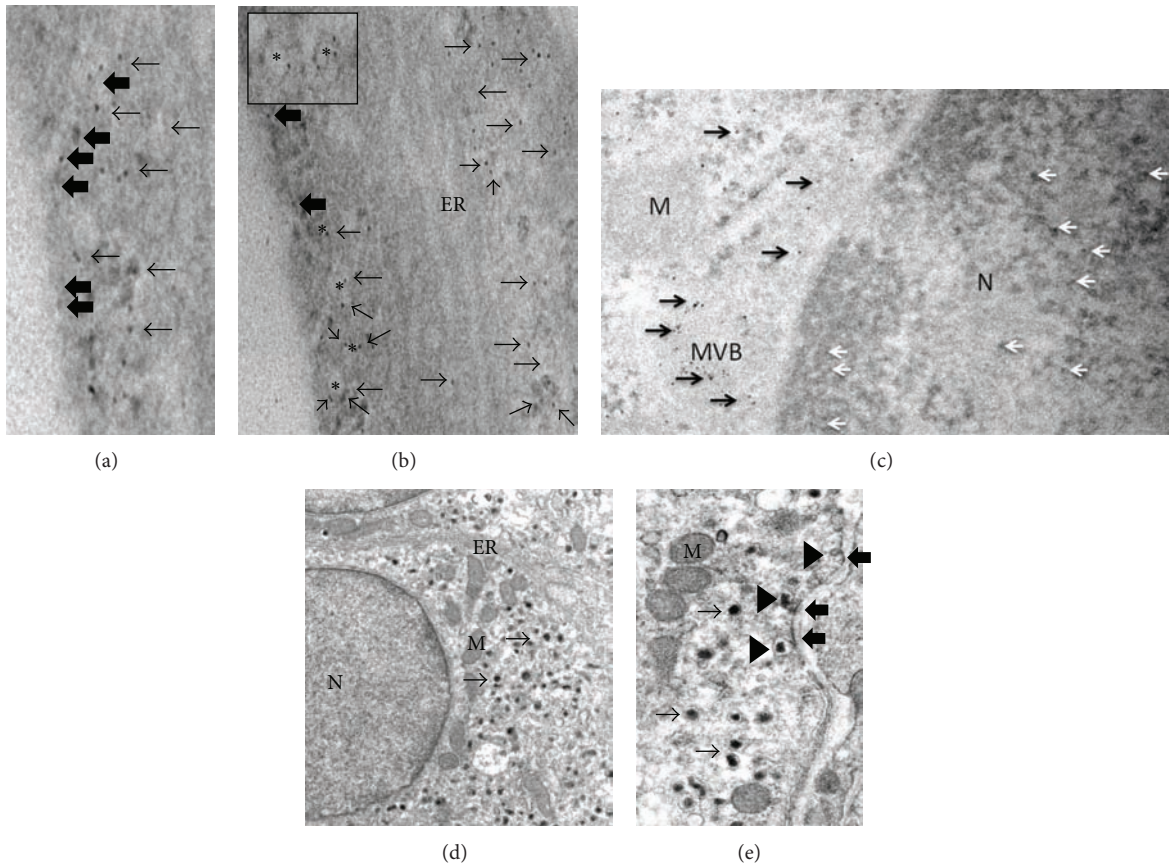


Figure 2: Immunofluorescent localization of AT_{1a}, AT_{1b}, and AT_2 receptors in rat adrenals. Survey photomicrographs show positive immunofluorescence for AT_{1a} ((a) and (d); 80x), AT_{1b} ((b) and (e)), and AT_2 ((c) and (f)) in the zona glomerulosa (160x). Positive staining for AT_{1a} ((a) and (g)) and AT_2 ((c) and (i)), but not AT_{1b} ((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 AT_{1a} and AT_{1b} 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 AT_{1a} receptor mRNA expression, but decreases AT_1 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; AT_{1a} and AT_{1b} can stimulate aldosterone release, while AT_{1a}, but not AT_{1b}, can stimulate corticosterone release in the mouse adrenal [57].

In view of the near identical pharmacological characteristics of the AT_{1a} and AT_{1b} 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 AT_{1a} receptor (accession no. P25095 http://www.ncbi.nlm.nih.gov/protein/113493 (accessed 16 March 2012) and AT_{1b} 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



(a) (b) (c) (d) (e)

FIGURE 3: Ultrastructural immunocytochemistry of the AT_{1a} receptor (using anti-AT_{1a} receptor #92578-sel in zona 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_{1a} 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_{1a} 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).

acid stretch. The closest similarities to the sequences of the AT_1 antigenic peptides in the protein database (Protein Blast) http://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastp&BLASTPROGRAMS=blastp&PAGETYPE=BlastSearch&SHOWDEFAULTS=on&LINK LOC=blasthome, accessed on February 4, 2013) were the serotonin 5 HT2b subtype with a 7 amino acid identity to the AT_{1a} peptide fragment (score = 24.0 bits) and sestrin 1 with a 7 amino acid identity to the AT_{1b} peptide fragment (score = 24.4 bits).

To generate an antibody to the AT_2 receptor, a similar strategy was applied. A C-terminal domain peptide of 15 amino acids (resembling amino acids 349 to 363) was used as the antigen. The sequence of the AT_2 receptor (accession no. P35351, http://www.ncbi.nlm.nih.gov/protein/543780 accessed on February 4, 2013) has negligible homology with either of the AT_1 receptor subtypes. The closest similarity to this peptide sequence was an immunoglobulin kappa chain (AAA41415.1) with an 8 amino acid identity to the AT_2 peptide fragment (score = 27.4 bits compared to 49.0 bits for the AT_2 receptor).

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

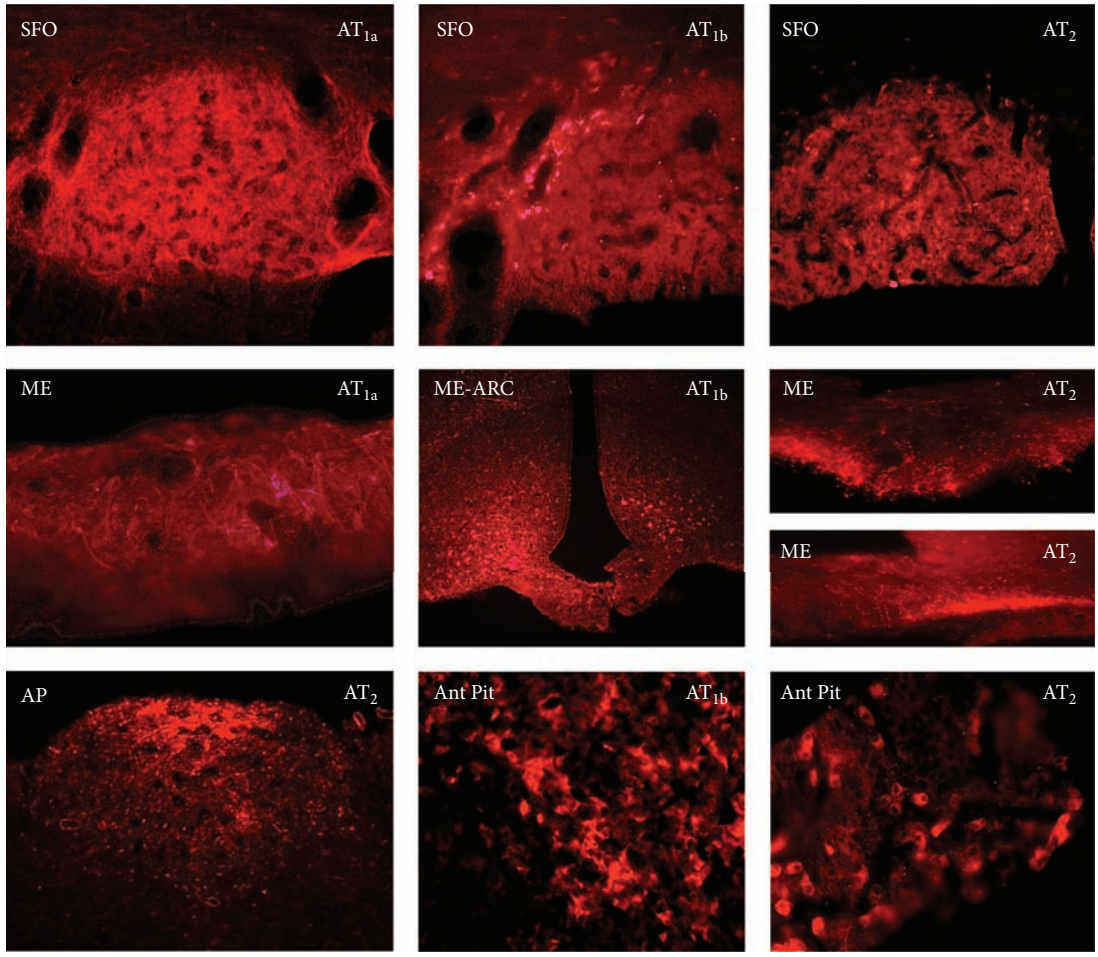


Figure 4: Circumventricular organs and pituitary AT receptor immunolocalization. Subfornical organ (top row left to right; 180x) AT_{1a}, AT_{1b}, and AT_2. Median eminence (middle row left to right AT_{1a} (160x), AT_{1b} (100x), and AT_2 (120x)). Bottom row area postrema (AP, 160x) AT_2 and anterior pituitary (pars distalis localization; 120x) of AT_{1b} (middle) and AT_2 (right). No staining for AT_{1a} was seen in the anterior pituitary.

cortex, but not in the medulla using an antibody raised against amino acids 306–359 of the human AT_1 receptor subtype. Of note, adrenals from rats kept on a low sodium diet displayed AT_1 immunoreactivity in other cortical zones (fasciculata and reticularis). The lack of adrenomedullary staining with this human antibody suggests that it may only recognize the AT_{1b} sequence in the rat. Giles et al. [67] observed AT_1 immunoreactivity and AT_1 mRNA in the zona glomerulosa of rat adrenals using an antibody directed against amino acids 350–359 of the rat AT_{1a} subtype plus a small amount of immunoreactivity in the zona fasciculata. However, there was no mention of AT_1 immunoreactivity or mRNA in the adrenal medulla.

Frei et al. [68] observed AT_1 immunoreactivity in the rat adrenal cortex and medulla using a monoclonal antibody raised against amino acids 229–246 of the human AT_1 receptor subtype. Yet, AT_2 immunoreactivity was only observed in the rat adrenal medulla using an antibody raised against amino acids 314–330 of the human AT_2 receptor subtype. On the other hand, Harada et al. [63] observed AT_2 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 AT_2 receptor and one raised against amino acids 221–363 of the human AT_2 receptor. However, they did detect a low level of AT_2 receptor-like immunoreactivity in the medulla using the latter antibody for immunohistochemical analysis. Conversely, Yiu et al. [69] reported AT_2 immunoreactivity only in the rat adrenal medulla using an antibody directed against amino acids 341–351 of the rat AT_2 subtype. Notably, they reported that this antibody failed to label brain regions known to express AT_2 receptors. Reagan et al. [70] were unable to demonstrate any AT_2 receptor immunoreactivity in the rat adrenal using a polyclonal antibody developed to recognize AT_2 receptors in N1E-115 cells.

4.3. Subcellular Localization of AT_1 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 AT_{1a}

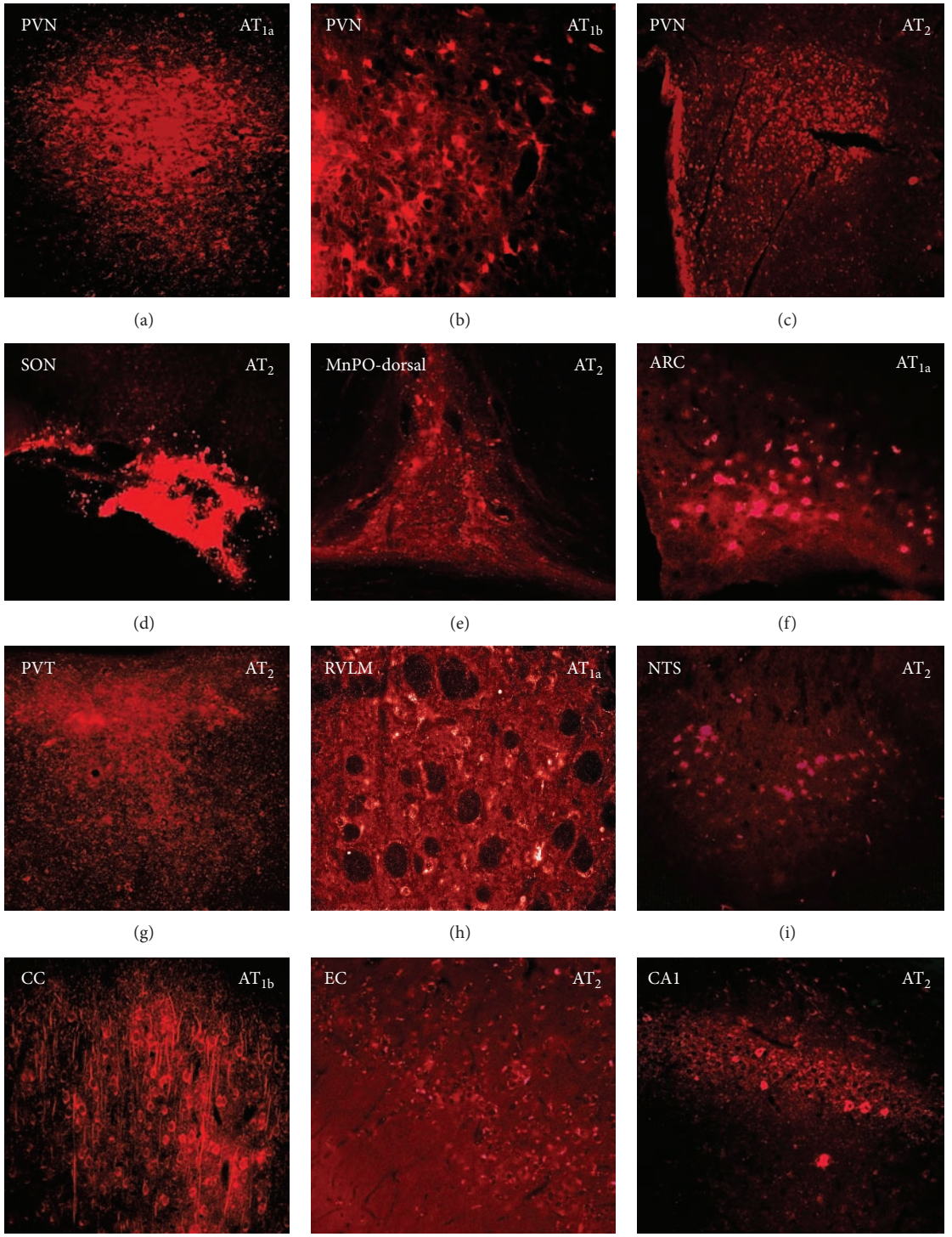


Figure 5: Immunofluorescent localization of AT receptors in various brain nuclei. Top row: hypothalamic paraventricular nucleus (PVN, 160x) AT_{1a}, AT_{1b}, and AT_2. Second row: immunofluorescence labeling (left to right) for AT_2 in supraoptic nucleus (SON; 120x) and median preoptic nucleus (MnPO-dorsal 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).

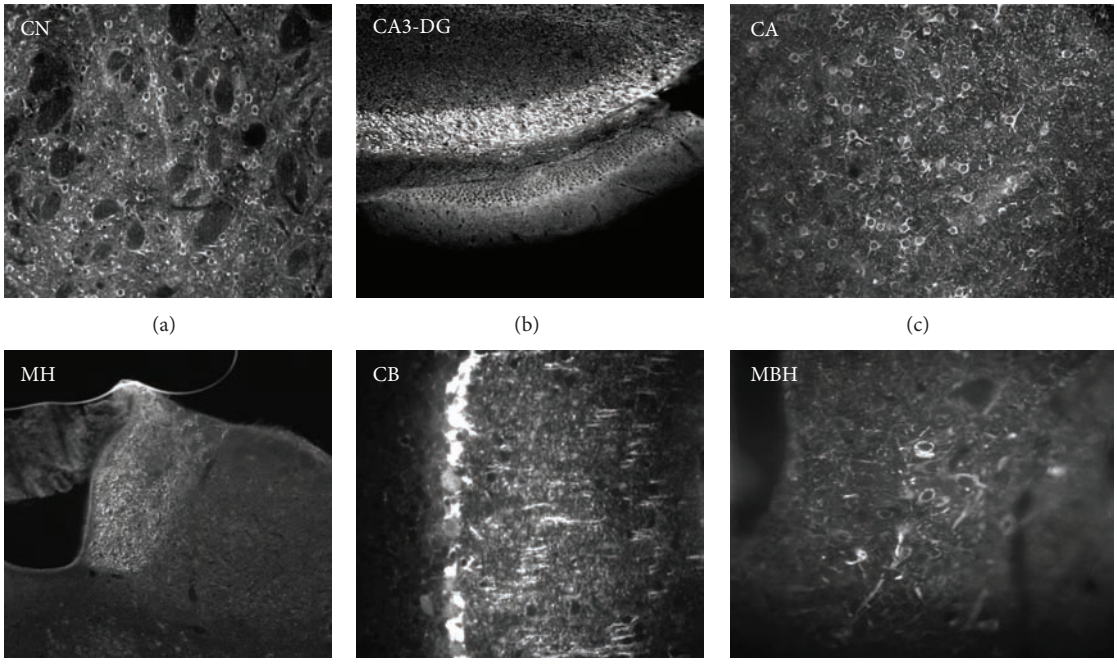


Figure 6: AT_2 receptor immunolocalization in caudate nucleus (CN, 400x), CA3-dentate gyrus of the hippocampus (CA3-DG; 80x), central nucleus of the amygdala (CA; 160x), medial habenula (MH, bottom left; 80x), AT_{1b} in cerebellar Purkinje cells (CB; bottom middle; 100x), and AT_2 in mediobasal hypothalamus (MBH; bottom right, 320x).

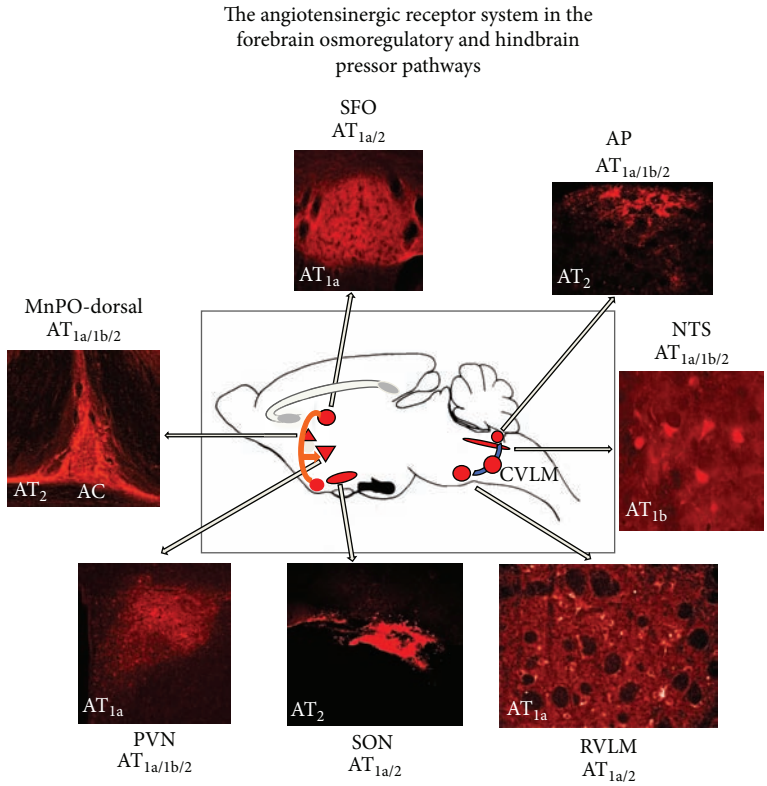


Figure 7: Diagrammatic summary of important brain nuclei in the angiotensinergic forebrain osmoregulatory pressor (orange pathway) and hindbrain pressor (blue) pathways. Note that there is more than one AT receptor in each site as given adjacent to each micrograph. A representative AT receptor for each 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 AT1-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 α-^{32}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 (KKFKK, 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_{1a} 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 organismic 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 situin 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, mediodorsal 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 AT_1 receptors [132], while microinjection of Ang II into the superior colliculus increases blood pressure via its actions at AT_2 receptors [133] consistent with radioligand binding studies indicating the presence of AT_1 or AT_2 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 AT_1 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 AT_1 receptors on vasopressinergic and oxytocinergic neurons [136]. The data reported herein is consistent with the presence of functional AT_1 receptors in the PVN and SON.

Parvocellular PVN AT_1 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 AT_{1b} mRNA in the SFO, OVLT, and cerebellum and a predominance of AT_{1b} in the hypothalamus by PCR. Conversely, Johren et al. [45] identified AT_{1a} mRNA in the SFO, OVLT, PVN, cerebral cortex and hippocampus, AT_{1b} mRNA in the cerebral cortex and hippocampus, (but not in the SFO or OVLT) and AT_2 mRNA in the medial geniculate and inferior olivary nucleus. Similarly, Lenkei et al. [41] reported a predominance of AT_{1a} mRNA expression in the SFO, OVLT, PVN, and MnPO as well as the anterior olfactory nucleus with very low AT_{1b} mRNA expression in the SFO and PVN. Lenkei et al. [137] also reported the absence of AT_{1a} and AT_{1b} 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 AT_{1a} mRNA in the pyriform cortex, basal amygdala and choroid plexus and AT_{1b} mRNA in the choroid plexus. AT_1 receptor binding has been reported in the choroid plexus [139] although at very low levels [140].

Brain AT_2 receptor mRNA shows both similarities and differences from AT_2 receptor binding in the rat brain. Noteworthy is the presence of AT_2 mRNA in the red nucleus and the absence of AT_2 mRNA in the locus coeruleus, lateral septum, and cerebellum [141]. These discrepancies have been interpreted as indicating that the red nucleus synthesizes AT_2 receptors that are only expressed on its efferent nerve terminals that project to the inferior olivary nucleus and cerebellum, while the AT_2 receptor expressing brain regions devoid of AT_2 mRNA express AT_2 receptors on the nerve terminals of its afferents from other brain regions. Lenkei et al. [142] observed AT_2 mRNA in the red nucleus. However, they also observed AT_2 mRNA in the lateral septum and locus coeruleus, as well as a much greater number of brain regions, including some traditionally AT_1 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 AT_{1a} receptor mRNA. Overall this is consistent with AT_1 receptor binding, with a few exceptions, for example, the lack of AT_1 mRNA in arcuate nucleus and median eminence, where it is postulated that the AT_1 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 AT_{1a} mRNA, but not AT_1 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 AT_1 mRNA in the RVLM and reduce it in the SFO, suggesting that enhanced activation of the RVLM by enhanced AT_1stimulation 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 AT_2 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 AT_1 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_2 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 (AB18801) and AT_2 (AB19134) 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 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_1receptors 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 immunological 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 knockout 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_1protein(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_1receptor antibodies, or (5) to discover (a) novel protein(s) that has/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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