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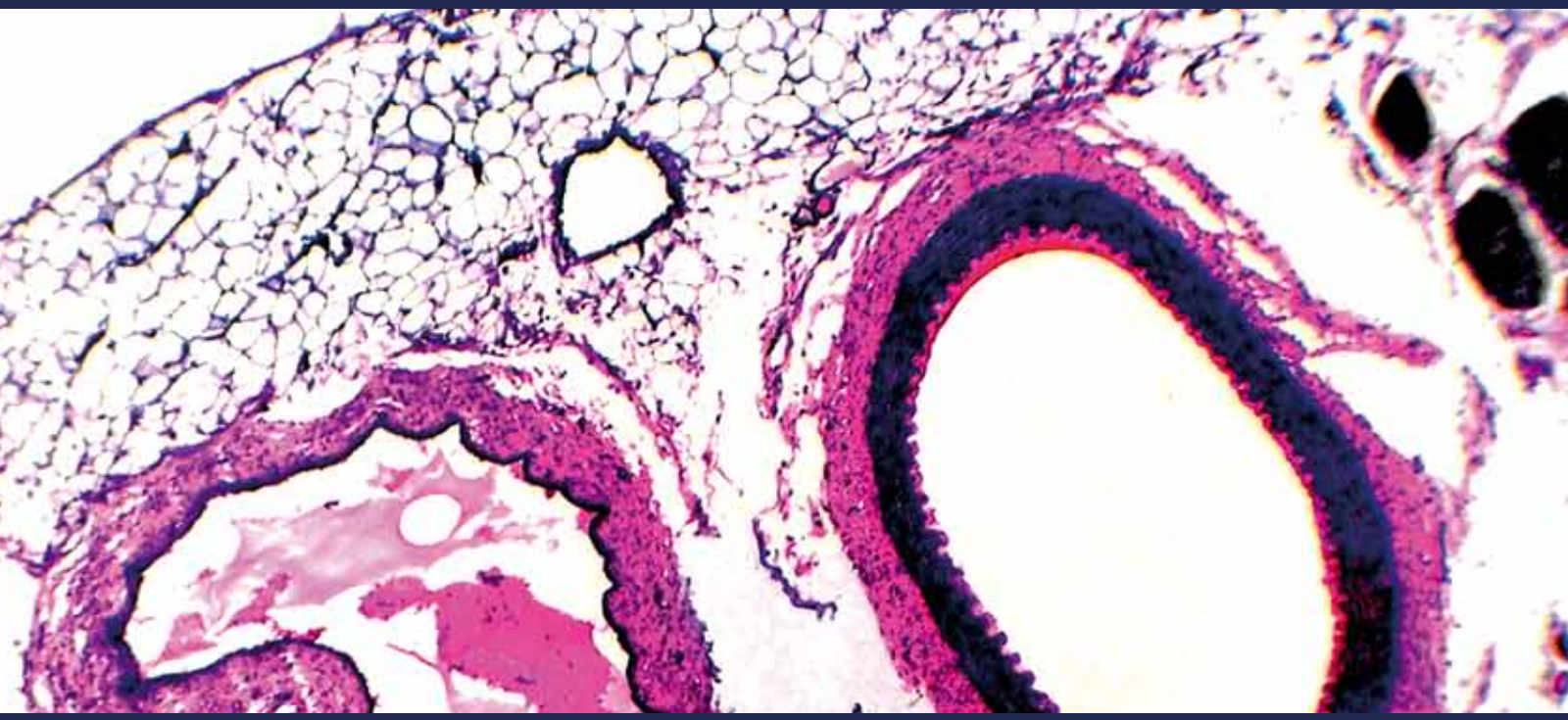
# Brain RAS: Hypertension and Beyond

Guest Editors: Marc de Gasparo, Robert C. Speth, Ovidiu C. Baltatu, and Patrick Vanderheyden



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# Editorial Board

# Contents

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*Editorial*

# Brain RAS: Hypertension and Beyond

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Cardiac output and vascular resistance are the cornerstones of blood pressure regulation, which is achieved through neural, humoral, and local tissue factors.

The sympathetic nervous system (SNS) and the renin-angiotensin system (RAS) play a major role in the control of blood pressure. Angiotensins act as endocrine, paracrine, and autocrine regulators. The peripheral physiological and pathophysiological actions of the RAS are well established. The existence and functional relevance of the RAS in the brain is increasingly recognized as a major regulator of the cardiovascular (CV) system and a significant drug target for antihypertensive and other CV therapeutics. All the constituents of the RAS occur in the brain and participate in the regulation of blood pressure through sympathetic activation and vasopressin release. In addition, an interconnection between neurotransmitters and the brain RAS affects behavior and neurological diseases, for example, Parkinson's, and Alzheimer's diseases. Moreover, the clinical efficacy of renin and ACE inhibitors and angiotensin receptor blockers (ARBs) and the presence of their targets in the brain illustrate the synergistic interaction between brain and peripheral RAS.

This special issue illustrates some aspects of the brain RAS pathway and function including its effect on the circadian rhythm of blood pressure.

The RAS has been described in the brain. Using subtype specific antibodies, C. Premer et al. observed selective expression of AT1a, AT1b, and AT2 receptor subtypes in neurons and glia in a large number of brain regions including the subfornical organ, median eminence, area postrema, paraventricular, and solitary tract nucleus of the rat brain as well as in the pituitary and adrenal.

Ang II formation in the pineal gland and glial cells appears to depend on alternative pathways including chymase (L. A. Campos et al.). One possibility might be that the prorenin receptor (PRR) binds prorenin or renin from circulation to form Ang I and chymase to form Ang II. The brain PRR appears to initiate the brain angiotensin peptide formation (W. Li et al.). Indeed, PRR is expressed ubiquitously in the brain with the highest expression levels in the pituitary and frontal lobe. Recent findings indicate that PRR has RAS independent roles associated with the vacuolar proton-ATPase and the Wnt signaling pathways (W. Li et al.). PRR in the brain could play a pivotal role in neural regulation of blood pressure and body fluid homeostasis.

In addition, AT4/IRAP and Mas receptors are also present in the brain. Aminopeptidases (and other angiotensins degrading enzymes, e.g., ACE2 and endopeptidase), which form fragments such as Ang III, Ang IV, Ang 2–10, Ang 1–9, and Ang 1–7, are also the topic of several reports (A. B. Segarra et al.; M. A. Clark et al.).

Formation of Ang III in the brain may promote hypertension while Ang IV, which inhibits vasopressinase activity and may have a therapeutic value for cognitive function in the brain.

There is still a debate regarding the relative importance of Ang II and Ang III in the brain. Using astrocytes in culture and an inhibitor of aminopeptidase A to prevent conversion of Ang II to Ang III, M. A. Clark et al. demonstrate that both

Ang II and Ang III induce phosphorylation of MAPK and JNK and stimulate astrocyte growth equipotently.

Ang IV binds to the AT4 receptor. While the $AT_4$ receptor has been convincingly shown to be the insulin-regulated aminopeptidase IRAP, (also known as vasopressinase and cysteine aminopeptidase), others have suggested that the physiological action of Ang IV may also be mediated through the tyrosine kinase c-Met receptor. Regardless of this controversy, binding of Ang IV causes inhibition of the catalytic activity of the peptidase activity of the IRAP receptor and therefore increases AVP and oxytocin, glucose uptake, and cognitive processes. Intracerebroventricular injection of Ang IV improves memory and learning in the rat. The potential of IRAP inhibitors able to cross the blood brain barrier is discussed by H. Andersson and M. Hallberg.

Clearly, the brain RAS regulates sympathetic activity and norepinephrine (NE) release (K. Tsuda), and hyperactivity of the SNS is clearly involved in the cardiovascular pathology. Ang II through the AT1 receptor and MAPK stimulation affects noradrenergic nerve terminals in the paraventricular nucleus of the hypothalamus (PVN), inhibiting $K^+$ channel and stimulating $Ca^{++}$ channels causing NE release. Also, brain aldosterone-mineralocorticoid receptor- (MR-) ouabain pathway might have a pivotal role in Ang II-induced neuronal activation and pressor responses (K. Tsuda). In contrast, Ang 1–7, a metabolite of both Ang I and Ang II, reduces NE release through BK and NO stimulation (M. Nautiyal et al.).

Regulation of the baroreflex is central to CV regulation, and cardiac autonomic imbalance (decreased cardiovagal and increased sympathetic tone) causes baroreflex dysfunction. Ang II acting through the AT1 receptor and Ang 1–7 acting through the mas-receptor counterbalance each other in the brain through AMPK and mitochondrial NADPH oxidase stimulation and superoxide production (M. Nautiyal et al.; B. P. Campagnaro et al.). Ang II also stimulates dopamine $\beta$-hydroxylase in the striatum regulating the baroreflex (Tsuda). It also stimulates central cholinergic neuronal activity. The frontal cortex and CV system are reciprocally and asymmetrically organized: the frontal cortical activity appears to control CV function. Indeed, whereas there is an increased parasympathetic nervous system (PNS) activity in the left prefrontal cortex, one observes a decreased SNS activity in the right one (A. B. Segarra et al.). This may suggest that stimulation of the frontal cortical activity could lower blood pressure.

Several contributions focus on the AT2 receptor, the functions of which still remain poorly understood. Interestingly, the hypothalamus expresses both AT1 and AT2 receptors that could counterbalance each other's effects (C. Premer et al.). Further, the AT2 receptor is involved in neuronal disorders and appears to play a major role in the regulation of cognitive function through a balance between phosphorylation and kinase activity versus phosphatase activity (M. O. Guimond and N. Gallo-Payet). AT2 receptors also stimulate neurite outgrowth, migration and excitability and reduce oxidative stress. Moreover, binding of Ang II to the AT2 receptor stimulates bradykinin and nitric oxide production and improves cerebral blood flow preventing ischemic damage and dementia (M. Mogi et al.). In addition, the AT2 receptor appears to play a role in metabolic syndrome as it regulates appetite and increases glucose uptake. Finally, it is proposed that the AT2 receptor behaves like a gate keeper of cellular and tissue homeostasis (M. O. Guimond and N. Gallo-Payet).

Some signaling pathways involved in the AT2 receptor function are described. A family of AT2 receptor interacting proteins (ATIP) through the C-terminal domain of the receptor affect neurite outgrowth and neuronal differentiation (S. Rodrigues-Ferreira et al.). Such interaction of ATIP with the nicotinic acetylcholine receptor may also affect cognitive function (M. Mogi et al.). In fact, the evidence in favor of a neuroprotective action of AT2 receptor stimulation is compelling (M. Mogi et al.; M. O. Guimond and N. Gallo-Payet).

Interestingly, the RAS in the brain and in the periphery is differently regulated by sodium (H. Takahashi). Whereas a sodium load suppresses the peripheral RAS and reduces sodium reabsorption from the renal tubules, it activates the brain RAS to retain more sodium. The mechanism involves the brain endothelial $Na^+$ channel, activation of brain aldosterone, and endogenous digoxin-like substances, causing stimulation of the sympathetic system and increased blood pressure (H. Takahashi).

Circadian rhythms in neural and endocrine systems regulated by the molecular clock in the suprachiasmatic nucleus affect cardiovascular and metabolic disorders. Blood pressure variations during the day and night derive from the change in the dominant sympathetic tone. L. A. Campos et al. demonstrate here that Ang II acts upon AT1 receptors in the pineal gland to stimulate tryptophan hydroxylase activity and melatonin formation. This decreases sympathetic activity and increases parasympathetic activity. This reduces oxygen-free radicals and increases nitric oxide availability causing a decrease in blood pressure. Such behavior is typical of the physiological night tone reduction of blood pressure (dipper hypertensive patient), whereas the nondipper hypertensive shows an impaired melatonin formation during the night with an inverse circadian blood pressure profile. Pinealectomy reverses the condition, increasing sympathetic activity and the adrenocorticotropin axis. The brain RAS appears therefore to be a major regulator of circadian variation of blood pressure through its effect on light cycle shifts. Medical treatment coordinated with biological rhythms (chronotherapy) combined with inhibition of the brain RAS may be a means of individualizing treatment of hypertension related to circadian rhythms. Interestingly, increased melatonin release also improves insulin sensitivity. Supporting this finding, decreased melatonin is observed in type 2 diabetes and a melatonin gene receptor mutation was associated with increased risk of type 2 diabetes. Melatonin, a nutritional supplement, may therefore be a good therapeutic complement for diabetic patients. Although pineal Ang II stimulates melatonin (L. A. Campos et al.), a beneficial effect of this approach on type II diabetes remains to be demonstrated.

S. Lattanzi et al. review the effectiveness of ARBs given in acute stroke. In a meta-analysis of three trials, they conclude that there is no place for blood pressure lowering treatment

in the acute phase of stroke. However, there may be a U-shaped relationship between BP and outcome: too low blood pressure may result in brain hypoperfusion and thus in a worse outcome. Two ongoing large studies in acute ischemic and haemorrhagic stroke (ENOS and INTERACT) will help to select the best approach of blood pressure management.

In summary, an abundant body of evidence indicates an important regulatory role for the brain RAS in cardiovascular homeostasis and disease beyond blood pressure regulation. Moreover, accumulating evidence reveals brain RAS roles in brain-specific functions and diseases such as cognitive dysfunction, dementia, Alzheimer's and Parkinson's diseases. Further research is needed to better understand detailed mechanisms of brain RAS in these and other diseases to possibly develop new diagnostic strategies.

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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 $\mu$g/mL of antigenic peptide at the working dilution). Also antibodies were immunoprecipitated from their working dilutions by incubation with 100 $\mu$L $AT_{1a}$, $AT_{1b}$, 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

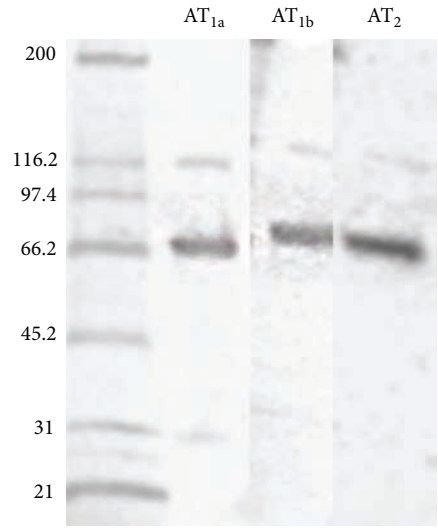



Figure 1: Western immunoblots for $AT_{1a}$, $AT_{1b}$, and $AT_2$ receptors of crude extracts of whole adrenals. The three receptors show major bands at ~69–75 kD and well as faint bands at about ~ 116–126 kD. $AT_{1a}$ receptor-directed antibody (Rabbit 92578-sel), $AT_{1b}$ receptor-directed antibody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

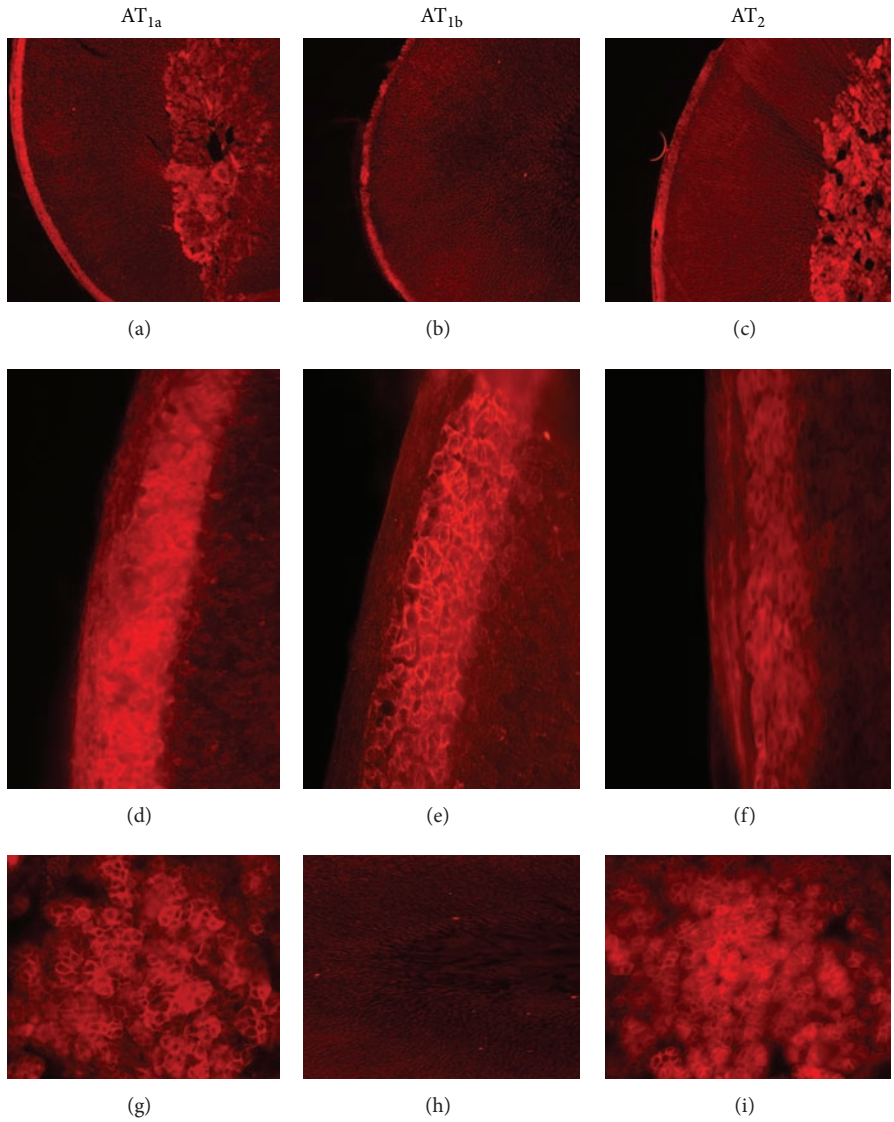
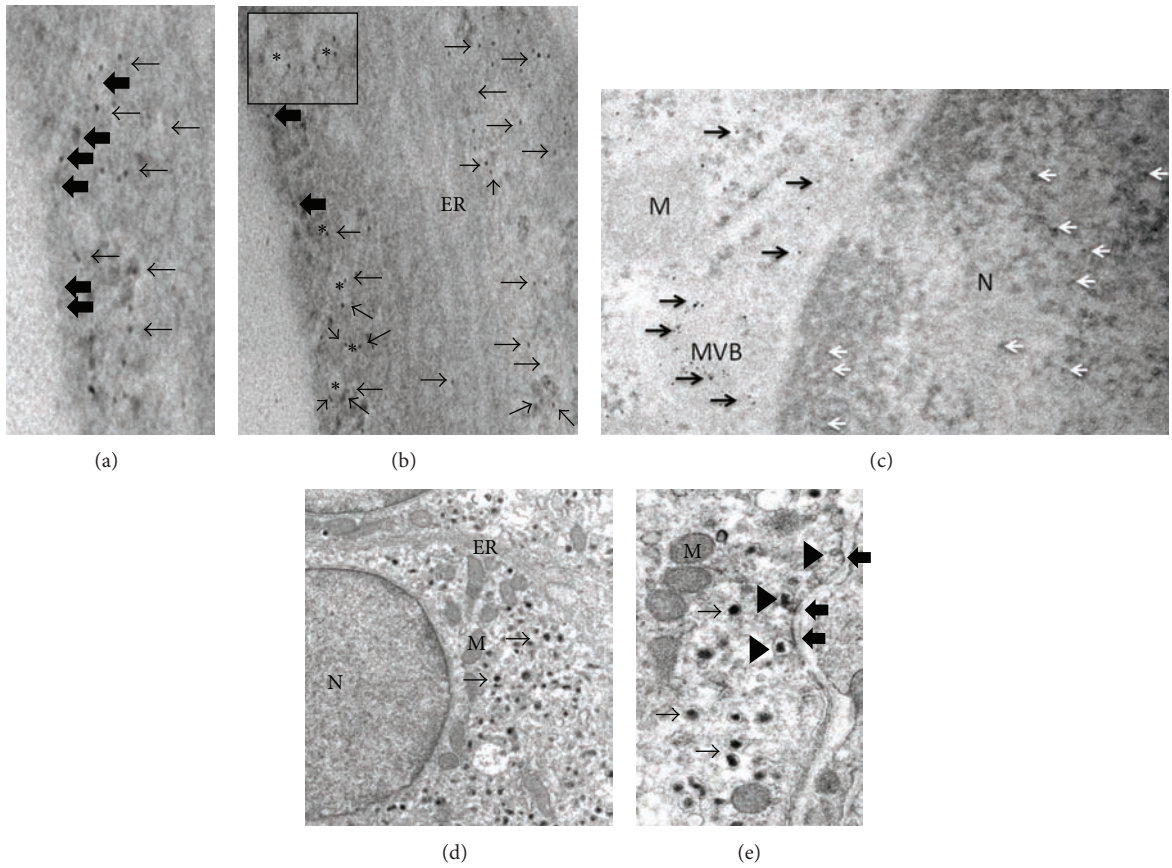



Figure 2: Immunofluorescent localization of $AT_{1a}$, $AT_{1b}$, and $AT_2$ receptors in rat adrenals. Survey photomicrographs show positive immunofluorescence for $AT_{1a}$ ((a) and (d); 80x), $AT_{1b}$ ((b) and (e)), and $AT_2$ ((c) and (f)) in the zona glomerulosa (160x). Positive staining for $AT_{1a}$ ((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

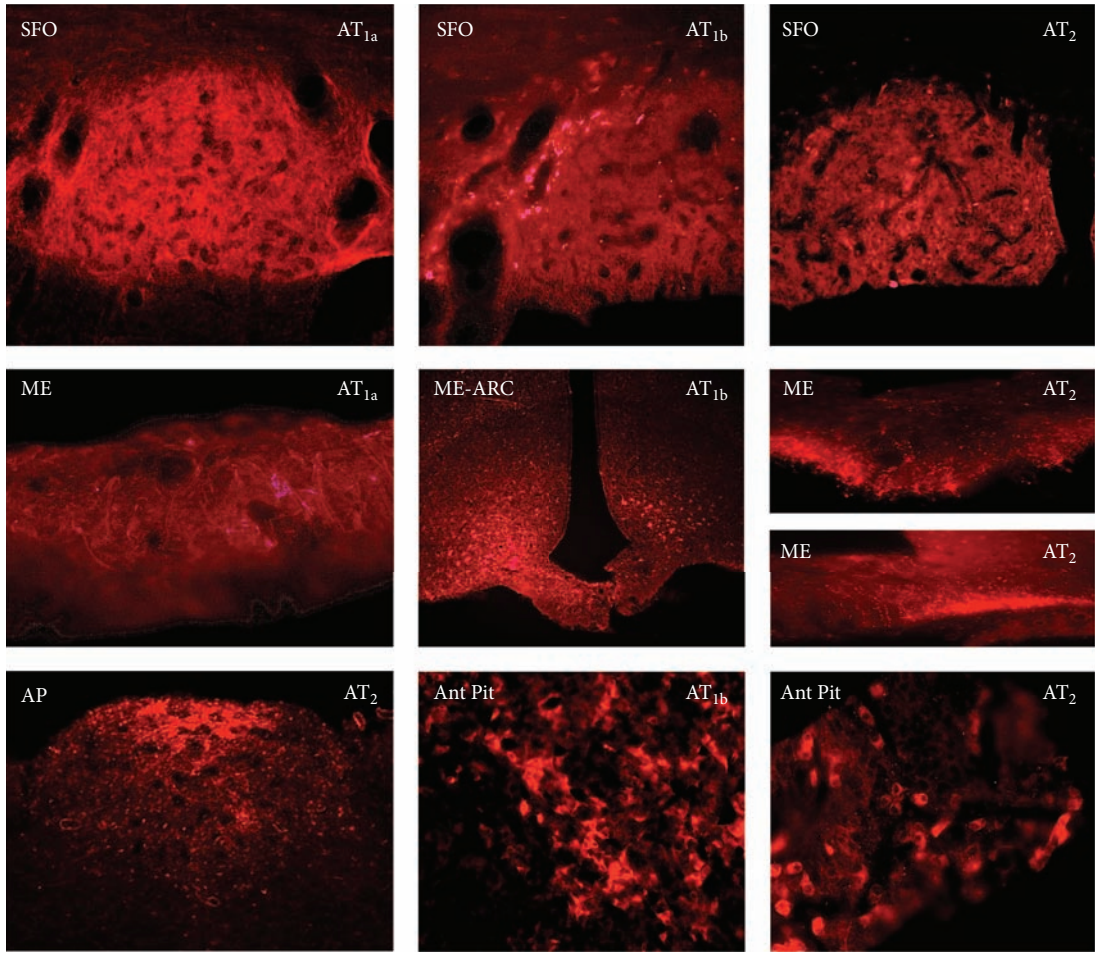



Figure 4: Circumventricular organs and pituitary AT receptor immunolocalization. Subfornical organ (top row left to right; 180x) $AT_{1a}$, $AT_{1b}$, and $AT_2$. Median eminence (middle row left to right $AT_{1a}$ (160x), $AT_{1b}$ (100x), and $AT_2$ (120x)). Bottom row area postrema (AP, 160x) $AT_2$ 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 $AT_{1a}$

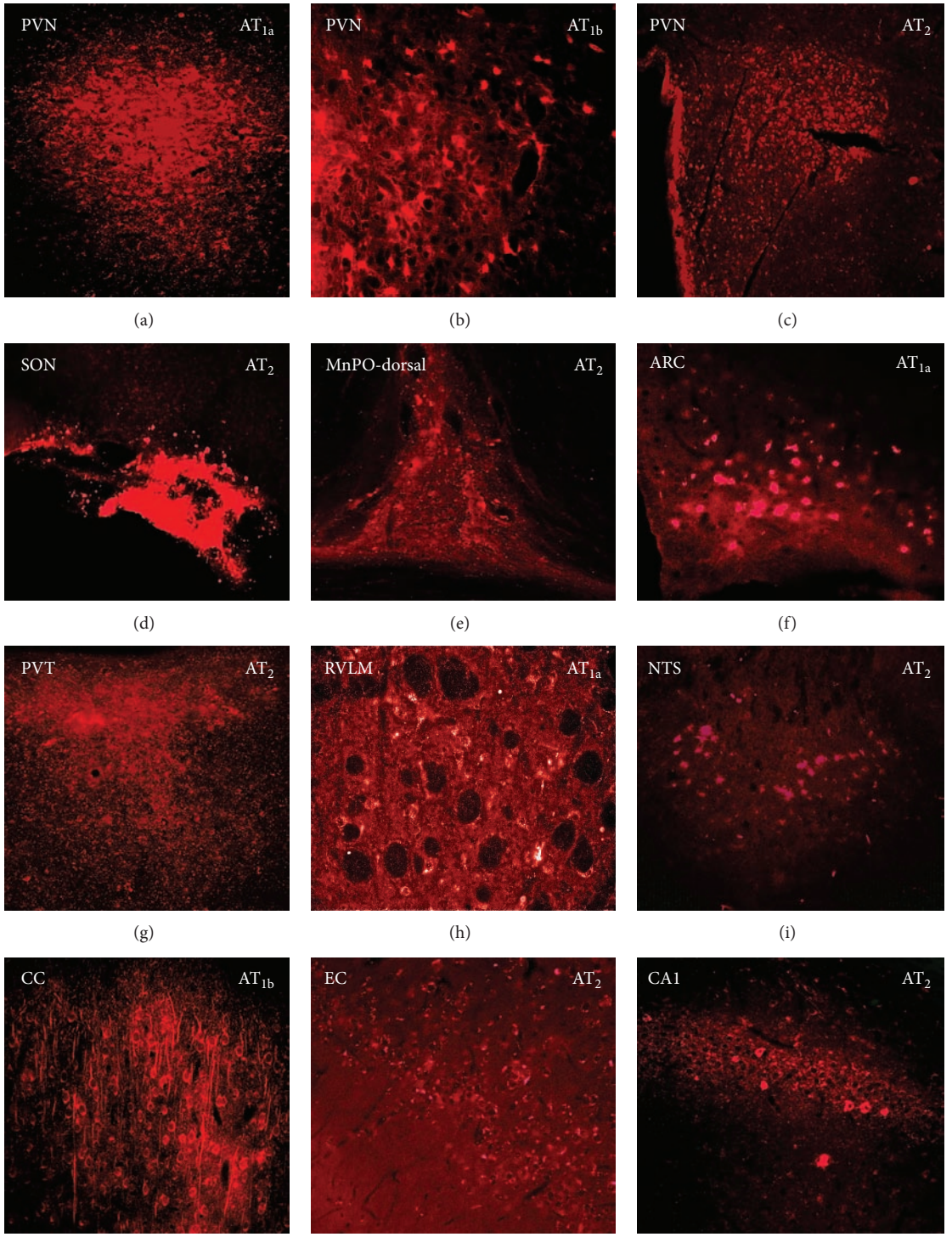



Figure 5: Immunofluorescent localization of AT receptors in various brain nuclei. Top row: hypothalamic paraventricular nucleus (PVN, 160x) $AT_{1a}$, $AT_{1b}$, and $AT_2$. Second row: immunofluorescence labeling (left to right) for $AT_2$ in supraoptic nucleus (SON; 120x) and median preoptic nucleus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

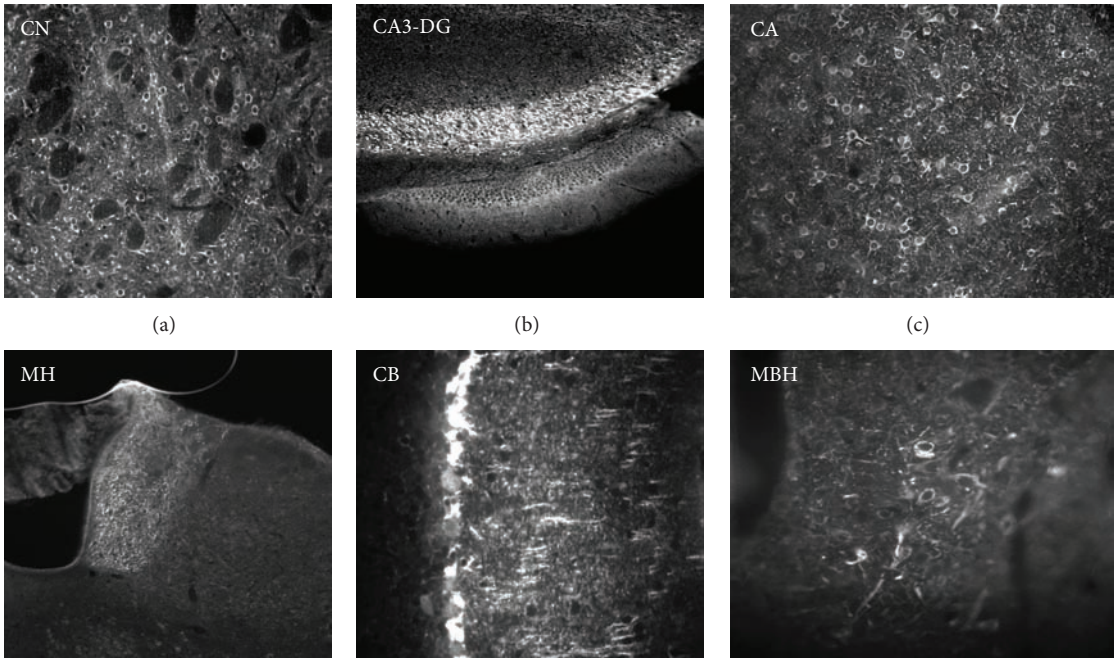



Figure 6: $AT_2$ receptor immunolocalization in caudate nucleus (CN, 400x), CA3-dentate gyrus of the hippocampus (CA3-DG; 80x), central nucleus of the amygdala (CA; 160x), medial habenula (MH, bottom left; 80x), $AT_{1b}$ in cerebellar Purkinje cells (CB; bottom middle; 100x), and $AT_2$ in mediobasal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

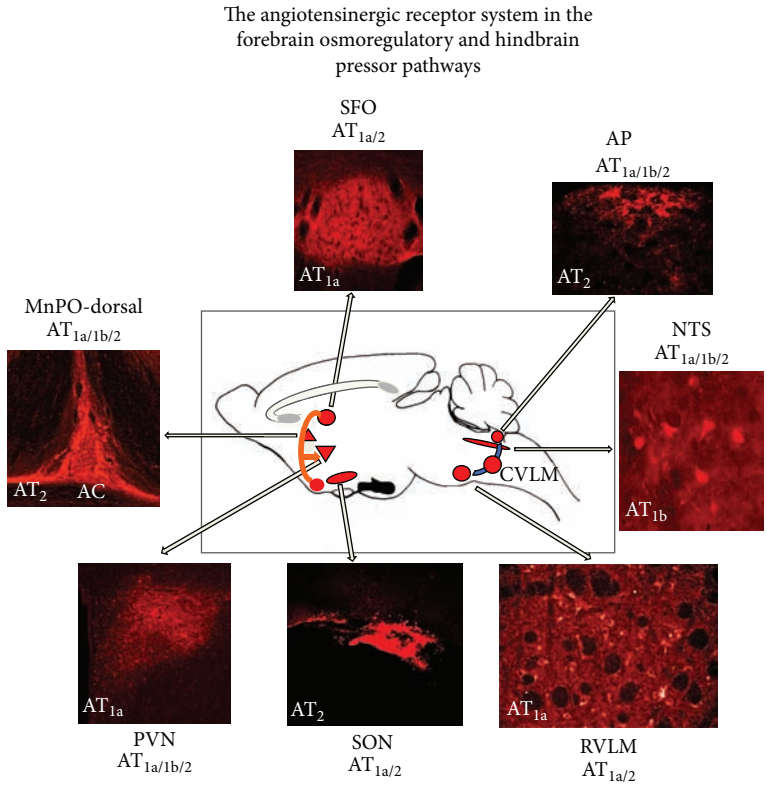



Figure 7: Diagrammatic summary of important brain nuclei in the angiotensinergic forebrain osmoregulatory pressor (orange pathway) and hindbrain pressor (blue) pathways. Note that there is more than one AT receptor in each site as given adjacent to each micrograph. A representative AT receptor for each 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 $\alpha$-$^{32}$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 $AT_1$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 $AT_2$ 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. $AT_1$receptors are phosphorylated by G protein receptor kinase GRK2 (formerly known as $\beta$ adrenergic receptor kinase, BARK1) leading to $\beta$-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-$AT_1$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 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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## References

[1] J. H. Laragh, M. Angers, W. G. Kelly, and S. Lieberman, "Hypotensive agents and pressor substances. The effect of epinephrine, norepinephrine, angiotensin II, and others on the secretory rate of aldosterone in man," *The Journal of the American Medical Association*, vol. 174, pp. 234–240, 1960.

[2] J. O. Davis, P. M. Hartroft, E. O. Titus, C. C. J. Carpenter, C. R. Ayers, and H. E. Spiegel, "The role of the renin-angiotensin system in the control of aldosterone secretion," *The Journal of Clinical Investigation*, vol. 41, no. 2, pp. 378–389, 1962.

[3] W. Feldberg and G. P. Lewis, "The action of peptides on the adrenal medulla. Release of adrenaline by bradykinin and angiotensin," *The Journal of physiology*, vol. 171, pp. 98–108, 1964.

[4] D. Ganten, J. L. Minnich, P. Granger et al., "Angiotensin-forming enzyme in brain tissue," *Science*, vol. 173, no. 3991, pp. 64–65, 1971.

[5] R. L. Stornetta, C. L. Hawelu-Johnson, P. G. Guyenet, and K. R. Lynch, "Astrocytes synthesize angiotensinogen in brain," *Science*, vol. 242, no. 4884, pp. 1444–1446, 1988.

[6] R. W. Lind, L. W. Swanson, and D. Ganten, "Organization of angiotensin II immunoreactive cells and fibers in the rat central nervous system. An immunohistochemical study," *Neuroendocrinology*, vol. 40, no. 1, pp. 2–24, 1985.

[7] D. A. Booth, "Mechanism of action of norepinephrine in eliciting an eating response on injection into the rat hypothalamus," *Journal of Pharmacology and Experimental Therapeutics*, vol. 160, no. 2, pp. 336–348, 1968.

[8] I. A. Reid and D. J. Ramsay, "The effects of intracerebroventricular administration of renin on drinking and blood pressure," *Endocrinology*, vol. 97, no. 3, pp. 536–542, 1975.

[9] W. B. Severs and A. E. Daniels-Severs, "Effects of angiotensin on the central nervous system," *Pharmacological Reviews*, vol. 25, no. 3, pp. 415–449, 1973.

[10] M. I. Phillips and C. Sumners, "Angiotensin II in central nervous system physiology," *Regulatory Peptides*, vol. 78, no. 1–3, pp. 1–11, 1998.

[11] J. W. Wright and J. W. Harding, "The brain renin-angiotensin system: a diversity of functions and implications for CNS diseases," *Pflügers Archiv*, vol. 465, pp. 133–151, 2013.

[12] W. F. Ganong, "Blood, pituitary, and brain renin-angiotensin systems and regulation of secretion of anterior pituitary gland," *Frontiers in Neuroendocrinology*, vol. 14, no. 3, pp. 233–249, 1993.

[13] E. Vila-Porcile and P. Corvol, "Angiotensinogen, prorenin, and renin are co-localized in the secretory granules of all glandular cells of the rat anterior pituitary: an immunoultrastructural study," *Journal of Histochemistry and Cytochemistry*, vol. 46, no. 3, pp. 301–311, 1998.

[14] A. T. Chiu, W. F. Herblin, D. E. McCall et al., "Identification of angiotensin II receptor subtypes," *Biochemical and Biophysical Research Communications*, vol. 165, no. 1, pp. 196–203, 1989.

[15] S. Whitebread, M. Mele, B. Kamber, and M. De Gasparo, "Preliminary biochemical characterization of two angiotensin II receptor subtypes," *Biochemical and Biophysical Research Communications*, vol. 163, no. 1, pp. 284–291, 1989.

[16] T. J. Murphy, R. W. Alexander, K. K. Griendling, M. S. Runge, and K. E. Bernstein, "Isolation of a cDNA encoding the vascular type-1 angiotensin II receptor," *Nature*, vol. 351, no. 6323, pp. 233–236, 1991.

[17] K. Sasaki, Y. Yamano, S. Bardhan et al., "Cloning and expression of a complementary DNA encoding a bovine adrenal angiotensin II type-1 receptor," *Nature*, vol. 351, no. 6323, pp. 230–233, 1991.

[18] Y. Kambayashi, S. Bardhan, K. Takahashi et al., "Molecular cloning of a novel angiotensin II receptor isoform involved in phosphotyrosine phosphatase inhibition," *Journal of Biological Chemistry*, vol. 268, no. 33, pp. 24543–24546, 1993.

[19] M. Mukoyama, M. Nakajima, M. Horiuchi, H. Sasamura, R. E. Pratt, and V. J. Dzau, "Expression cloning of type 2 angiotensin II receptor reveals a unique class of seven-transmembrane receptors," *Journal of Biological Chemistry*, vol. 268, no. 33, pp. 24539–24542, 1993.

[20] S. P. Tofovic, A. S. Pong, and E. K. Jackson, "Effects of angiotensin subtype 1 and subtype 2 receptor antagonists in normotensive versus hypertensive rats," *Hypertension*, vol. 18, no. 6, pp. 774–782, 1991.

[21] T. Hano, M. Mizukoshi, A. Baba, N. Nakamura, and I. Nishio, "Angiotensin II subtype 1 receptor modulates epinephrine release from isolated rat adrenal gland," *Blood Pressure, Supplement*, vol. 3, Supplement 5, pp. 105–108, 1994.

[22] R. F. Kirby, R. L. Thunhorst, and A. K. Johnson, "Effects of a non-peptide angiotensin receptor antagonist on drinking and blood pressure responses to centrally administered angiotensins in the rat," *Brain Research*, vol. 576, no. 2, pp. 348–350, 1992.

[23] D. C. Hogarty, E. A. Speakman, V. Puig, and M. I. Phillips, "The role of angiotensin, $AT_1$ and $AT_2$ receptors in the pressor, drinking and vasopressin responses to central angiotensin," *Brain Research*, vol. 586, no. 2, pp. 289–294, 1992.

[24] E. Lazartigues, P. Sinnayah, G. Augoyard, C. Gharib, A. K. Johnson, and R. L. Davisson, "Enhanced water and salt intake in transgenic mice with brain-restricted overexpression of angiotensin ($AT_1$) receptors," *American Journal of Physiology*, vol. 295, no. 5, pp. R1539–R1545, 2008.

[25] D. S. A. Colombari, J. V. Menani, and A. K. Johnson, "Forebrain angiotensin type 1 receptors and parabrachial serotonin in the control of NaCl and water intake," *American Journal of Physiology*, vol. 271, no. 6, pp. R1470–R1476, 1996.

[26] L. A. A. Camargo, W. A. Saad, S. Simões, T. A. B. Santos, and W. Abrão Saad, "Interaction between paraventricular nucleus and septal area in the control of physiological responses induced by angiotensin II," *Brazilian Journal of Medical and Biological Research*, vol. 35, no. 9, pp. 1017–1023, 2002.

[27] B. P. Rowe, K. L. Grove, D. L. Saylor, and R. C. Speth, "Angiotensin II receptor subtypes in the rat brain," *European Journal of Pharmacology*, vol. 186, no. 2-3, pp. 339–342, 1990.

[28] K. Tsutsumi and J. M. Saavedra, "Quantitative autoradiography reveals different angiotensin II receptor subtypes in selected rat brain nuclei," *Journal of Neurochemistry*, vol. 56, no. 1, pp. 348–351, 1991.

[29] K. Song, A. M. Allen, G. Paxinos, and F. A. O. Mendelsohn, "Angiotensin II receptor subtypes in rat brain," *Clinical and Experimental Pharmacology and Physiology*, vol. 18, no. 2, pp. 93–96, 1991.

[30] M. N. Nitabach, J. Schulkin, and A. N. Epstein, "The medial amygdala is part of a mineralocorticoid-sensitive circuit controlling NaCl intake in the rat," *Behavioural Brain Research*, vol. 35, no. 2, pp. 127–134, 1989.

[31] S. S. Kakar, K. K. Riel, and J. D. Neill, "Differential expression of angiotensin II receptor subtype mRNAs (AT-1A and AT-1B) in the brain," *Biochemical and Biophysical Research Communications*, vol. 185, no. 2, pp. 688–692, 1992.

[32] N. Iwai and T. Inagami, "Identification of two subtypes in the rat type I angiotensin II receptor," *FEBS Letters*, vol. 298, no. 2-3, pp. 257–260, 1992.

[33] T. S. Elton, C. C. Stephan, G. R. Taylor et al., "Isolation of two distinct type I angiotensin II receptor genes," *Biochemical and Biophysical Research Communications*, vol. 184, no. 2, pp. 1067–1073, 1992.

[34] A. T. Chiu, J. Dunscomb, J. Kosierowski et al., "The ligand binding signatures of the rat $AT_{1a}$, $AT_{1b}$ and the human $AT_1$ receptors are essentially identical," *Biochemical and Biophysical Research Communications*, vol. 197, no. 2, pp. 440–449, 1993.

[35] K. Sandberg, H. Ji, A. J. L. Clark, H. Shapira, and K. J. Catt, "Cloning and expression of a novel angiotensin II receptor subtype," *Journal of Biological Chemistry*, vol. 267, no. 14, pp. 9455–9458, 1992.

[36] Y. Tian, A. J. Baukal, K. Sandberg, K. E. Bernstein, T. Balla, and K. J. Catt, "Properties of $AT_{1a}$ and $AT_{1b}$ angiotensin receptors expressed in adrenocortical Y-1 cells," *American Journal of Physiology*, vol. 270, no. 5, pp. E831–E839, 1996.

[37] R. C. Speth, "Sarcosine1,glycine8 angiotensin II is an AT 1 angiotensin II receptor subtype selective antagonist," *Regulatory Peptides*, vol. 115, no. 3, pp. 203–209, 2003.

[38] S. S. Kakar, J. C. Sellers, D. C. Devor, L. C. Musgrove, and J. D. Neill, "Angiotensin II type-1 receptor subtype cDNAs: differential tissue expression and hormonal regulation," *Biochemical and Biophysical Research Communications*, vol. 183, no. 3, pp. 1090–1096, 1992.

[39] O. Jöhren, C. Golsch, A. Dendorfer, F. Qadri, W. Häuser, and P. Dominiak, "Differential expression of $AT_1$ receptors in the pituitary and adrenal gland of SHR and WKY," *Hypertension*, vol. 41, no. 4, pp. 984–990, 2003.

[40] J. M. Burson, G. Aguilera, K. W. Gross, and C. D. Sigmund, "Differential expression of angiotensin receptor 1A and 1B in mouse," *American Journal of Physiology*, vol. 267, no. 2, pp. E260–E267, 1994.

[41] Z. Lenkei, P. Corvol, and C. Llorens-Cortes, "The angiotensin receptor subtype $AT_{1a}$ predominates in rat forebrain areas involved in blood pressure, body fluid homeostasis and neuroendocrine control," *Molecular Brain Research*, vol. 30, no. 1, pp. 53–60, 1995.

[42] Y. Chen and M. Morris, "Differentiation of brain angiotensin type 1a and 1b receptor mRNAs a specific effect of dehydration," *Hypertension*, vol. 37, no. 2, pp. 692–697, 2001.

[43] K. Krishnamurthi, J. G. Verbalis, W. Zheng, Z. Wu, L. B. Clerch, and K. Sandberg, "Estrogen regulates angiotensin $AT_1$ receptor expression via cytosolic proteins that bind to the 5' leader sequence of the receptor mRNA," *Endocrinology*, vol. 140, no. 11, pp. 5435–5438, 1999.

[44] J. M. Gasc, S. Shanmugam, M. Sibony, and P. Corvol, "Tissue-specific expression of type 1 angiotensin II receptor subtypes: an in situ hybridization study," *Hypertension*, vol. 24, no. 5, pp. 531–537, 1994.

[45] O. Johren, T. Inagami, and J. M. Saavedra, "$AT_{1a}$, $AT_{1b}$, and $AT_2$ angiotensin II receptor subtype gene expression in rat brain," *NeuroReport*, vol. 6, no. 18, pp. 2549–2552, 1995.

[46] M. Jezova, I. Armando, C. Bregonzio et al., "Angiotensin II $AT_1$ and $AT_2$ receptors contribute to maintain basal adrenomedullary norepinephrine synthesis and tyrosine hydroxylase transcription," *Endocrinology*, vol. 144, no. 5, pp. 2092–2101, 2003.

[47] Z. Lenkei, M. Palkovits, P. Corvol, and C. Llorens-Cortès, "Expression of angiotensin type-1 ($AT_1$) and type-2 ($AT_2$) receptor mRNAs in the adult rat brain: a functional neuroanatomical review," *Frontiers in Neuroendocrinology*, vol. 18, no. 4, pp. 383–439, 1997.

[48] T. P. Hopp and K. R. Woods, "Prediction of protein antigenic determinants from amino acid sequences," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 78, no. 6, pp. 3824–3828, 1981.

[49] Y. Kitami, T. Okura, K. Marumoto, R. Wakamiya, and K. Hiwada, "Differential gene expression and regulation of type-1 angiotensin II receptor subtypes in the rat," *Biochemical and Biophysical Research Communications*, vol. 188, no. 1, pp. 446–452, 1992.

[50] A. T. Chiu, J. H. Dunscomb, D. E. McCall, P. Benfield, W. Baubonis, and B. Sauer, "Characterization of angiotensin $AT_{1a}$ receptor isoform by its ligand binding signature," *Regulatory Peptides*, vol. 44, no. 2, pp. 141–147, 1993.

[51] H. Jo, E. K. Yang, W. J. Lee, K. Y. Park, H. J. Kim, and J. S. Park, "Gene expression of central and peripheral renin-angiotensin system components upon dietary sodium intake in rats," *Regulatory Peptides*, vol. 67, no. 2, pp. 115–121, 1996.

[52] C. Llorens-Cortes, B. Greenberg, H. Huang, and P. Corvol, "Tissular expression and regulation of type 1 angiotensin II receptor subtypes by quantitative reverse transcriptase-polymerase chain reaction analysis," *Hypertension*, vol. 24, no. 5, pp. 538–548, 1994.

[53] J. Qiu, S. H. Nelson, R. C. Speth, and D. H. Wang, "Regulation of adrenal angiotensin receptor subtypes: a possible mechanism for sympathectomy-induced adrenal hypertrophy," *Journal of Hypertension*, vol. 17, no. 7, pp. 933–940, 1999.

[54] N. Iwai, T. Inagami, N. Ohmichi, Y. Nakamura, Y. Saeki, and M. Kinoshita, "Differential regulation of rat $AT_{1a}$ and $AT_{1b}$ receptor mRNA," *Biochemical and Biophysical Research Communications*, vol. 188, no. 1, pp. 298–303, 1992.

[55] D. H. Wang, Y. Du, H. Zhao, J. P. Granger, R. C. Speth, and D. J. Dipette, "Regulation of Angiotensin type 1 receptor and its gene expression: role in renal growth," *Journal of the American Society of Nephrology*, vol. 8, no. 2, pp. 193–198, 1997.

[56] M. M. Martin, E. J. Lee, J. A. Buckenberger, T. D. Schmittgen, and T. S. Elton, "MicroRNA-155 regulates human angiotensin II type 1 receptor expression in fibroblast," *Journal of Biological Chemistry*, vol. 281, no. 27, pp. 18277–18284, 2006.

[57] M. Naruse, A. Tanabe, T. Sugaya et al., "Deferential roles of angiotensin receptor subtypes in adrenocortical function in mice," *Life Sciences*, vol. 63, no. 18, pp. 1593–1598, 1998.

[58] K. Song, J. Zhuo, A. M. Allen, G. Paxinos, and F. A. O. Mendelsohn, "Angiotensin II receptor subtypes in rat brain and peripheral tissues," *Cardiology*, vol. 79, Supplement 1, pp. 45–54, 1991.

[59] A. Israel, L. M. Plunkett, and J. M. Saavedra, "Quantitative autoradiographic characterization of receptors for angiotensin II and other neuropeptides in individual brain nuclei and peripheral tissues from single rats," *Cellular and Molecular Neurobiology*, vol. 5, no. 3, pp. 211–222, 1985.

[60] D. P. Healy, A. R. Maciejewski, and M. P. Printz, "Autoradiographic localization of [125I]-angiotensin II binding sites in the rat adrenal gland," *Endocrinology*, vol. 116, no. 3, pp. 1221–1223, 1985.

[61] K. G. Birukov, S. Lehoux, A. A. Birukova, R. Merval, V. A. Tkachuk, and A. Tedgui, "Increased pressure induces sustained protein kinase C-independent herbimycin A-sensitive activation of extracellular signal-related kinase 1/2 in the rabbit aorta in organ culture," *Circulation Research*, vol. 81, no. 6, pp. 895–903, 1997.

[62] R. Wakamiya, K. Kohara, and K. Hiwada, "Gene expression of the type-1 angiotensin II receptor in rat adrenal gland," *Blood Pressure*, vol. 3, Supplement 5, pp. 109–112, 1994.

[63] K. Harada, H. Matsuoka, N. Fujimoto et al., "Localization of type-2 angiotensin II receptor in adrenal gland," *Journal of Histochemistry and Cytochemistry*, vol. 58, no. 7, pp. 585–593, 2010.

[64] B. Peters, S. Clausmeyer, P. Teubner et al., "Changes of $AT_2$ receptor levels in the rat adrenal cortex and medulla induced by bilateral nephrectomy and its modulation by circulating ANG II," *Journal of Histochemistry and Cytochemistry*, vol. 49, no. 5, pp. 649–656, 2001.

[65] W. G. Paxton, M. Runge, C. Horaist, C. Cohen, R. W. Alexander, and K. E. Bernstein, "Immunohistochemical localization of rat angiotensin II $AT_1$ receptor," *American Journal of Physiology*, vol. 264, no. 6, pp. F989–F995, 1993.

[66] J. G. Lehoux, I. M. Bird, N. Briere, D. Martel, and L. Ducharme, "Influence of dietary sodium restriction on angiotensin II receptors in rat adrenals," *Endocrinology*, vol. 138, no. 12, pp. 5238–5247, 1997.

[67] M. E. Giles, R. T. Fernley, Y. Nakamura et al., "Characterization of a specific antibody to the rat angiotensin II $AT_1$ receptor," *Journal of Histochemistry and Cytochemistry*, vol. 47, no. 4, pp. 507–515, 1999.

[68] N. Frei, J. Weissenberger, A. G. Beck-Sickinger, M. Höfliger, J. Weis, and H. Imboden, "Immunocytochemical localization of angiotensin II receptor subtypes and angiotensin II with monoclonal antibodies in the rat adrenal gland," *Regulatory Peptides*, vol. 101, no. 1–3, pp. 149–155, 2001.

[69] A. K. L. Yiu, P. F. Wong, S. Y. Yeung, S. M. Lam, S. K. S. Luk, and W. T. Cheung, "Immunohistochemical localization of type-II ($AT_2$) angiotensin receptors with a polyclonal antibody against a peptide from the C-terminal tail," *Regulatory Peptides*, vol. 70, no. 1, pp. 15–21, 1997.

[70] L. P. Reagan, R. R. Sakai, and S. J. Fluharty, "Immunological analysis of angiotensin $AT_2$ receptors in peripheral tissues of neonatal and adult rats," *Regulatory Peptides*, vol. 65, no. 2, pp. 159–164, 1996.

[71] S. S. G. Ferguson, "Evolving concepts in G protein-coupled receptor endocytosis: the role in receptor desensitization and signaling," *Pharmacological Reviews*, vol. 53, no. 1, pp. 1–24, 2001.

[72] L. Hunyady, A. J. Baukal, Z. Gáborik et al., "Differential PI 3-kinase dependence of early and late phases of recycling of the internalized $AT_1$ angiotensin receptor," *Journal of Cell Biology*, vol. 157, no. 7, pp. 1211–1222, 2002.

[73] R. Kumar, V. P. Singh, and K. M. Baker, "The intracellular renin-angiotensin system: a new paradigm," *Trends in Endocrinology and Metabolism*, vol. 18, no. 5, pp. 208–214, 2007.

[74] B. Boivin, G. Vaniotis, B. G. Allen, and T. E. Hébert, "G protein-coupled receptors in and on the cell nucleus: a new signaling paradigm?" *Journal of Receptors and Signal Transduction*, vol. 28, no. 1-2, pp. 15–28, 2008.

[75] A. L. Robertson and P. A. Khairallah, "Angiotensin II: rapid localization in nuclei of smooth and cardiac muscle," *Science*, vol. 172, no. 3988, pp. 1138–1139, 1971.

[76] R. Re and M. Parab, "Effect of angiotensin II on RNA synthesis by isolated nuclei," *Life Sciences*, vol. 34, no. 7, pp. 647–651, 1984.

[77] A. T. Chiu, D. E. McCall, T. T. Nguyen et al., "Discrimination of angiotensin II receptor subtypes by dithiothreitol," *European Journal of Pharmacology*, vol. 170, no. 1-2, pp. 117–118, 1989.

[78] P. Eggena, J. H. Zhu, K. Clegg, and J. D. Barrett, "Nuclear angiotensin receptors induce transcription of renin and angiotensinogen mRNA," *Hypertension*, vol. 22, no. 4, pp. 496–501, 1993.

[79] P. Eggena, J. H. Zhu, S. Sereevinyayut et al., "Hepatic angiotensin II nuclear receptors and transcription of growth-related factors," *Journal of Hypertension*, vol. 14, no. 8, pp. 961–968, 1996.

[80] R. N. Re, D. L. Vizard, J. Brown, and S. E. Bryan, "Angiotensin II receptors in chromatin fragments generated by micrococcal nuclease," *Biochemical and Biophysical Research Communications*, vol. 119, no. 1, pp. 220–227, 1984.

[81] G. W. Booz, K. M. Conrad, A. L. Hess, H. A. Singer, and K. M. Baker, "Angiotensin-II-binding sites on hepatocyte nuclei," *Endocrinology*, vol. 130, no. 6, pp. 3641–3649, 1992.

[82] S. S. Tang, H. Rogg, R. Schumacher, and V. J. Dzau, "Characterization of nuclear angiotensin-II-binding sites in rat liver and comparison with plasma membrane receptors," *Endocrinology*, vol. 131, no. 1, pp. 374–380, 1992.

[83] H. Licea, M. R. Walters, and L. G. Navar, "Renal nuclear angiotensin II receptors in normal and hypertensive rats," *Acta physiologica Hungarica*, vol. 89, no. 4, pp. 427–438, 2002.

[84] A. Tadevosyan, A. Maguy, L. R. Villeneuve et al., "Nuclear-delimited angiotensin receptor-mediated signaling regulates cardiomyocyte gene expression," *Journal of Biological Chemistry*, vol. 285, no. 29, pp. 22338–22349, 2010.

[85] R. Chen, Y. V. Mukhin, M. N. Garnovskaya et al., "A functional angiotensin II receptor-GFP fusion protein: evidence for agonist-dependent nuclear translocation," *American Journal of Physiology*, vol. 279, no. 3, pp. F440–F448, 2000.

[86] T. A. Morinelli, J. R. Raymond, A. Baldys et al., "Identification of a putative nuclear localization sequence within ANG II $AT_{1a}$ receptor associated with nuclear activation," *American Journal of Physiology*, vol. 292, no. 4, pp. C1398–C1408, 2007.

[87] D. Lu, H. Yang, G. Shaw, and M. K. Raizada, "Angiotensin II-induced nuclear targeting of the angiotensin type 1 ($AT_1$) receptor in brain neurons," *Endocrinology*, vol. 139, pp. 365–375, 1998.

[88] D. K. Lee, A. J. Lança, R. Cheng et al., "Agonist-independent nuclear localization of the apelin, angiotensin AT 1, and bradykinin B2 receptors," *Journal of Biological Chemistry*, vol. 279, no. 9, pp. 7901–7908, 2004.

[89] T. L. Goodfriend and M. J. Peach, "Angiotensin III: (Des aspartic acid) angiotensin II. Evidence and speculation for its role as an important agonist in the renin angiotensin system," *Circulation Research*, vol. 36, no. 6, Supplement 1, pp. 38–48, 1975.

[90] S. Shanmugam, Z. G. Lenkei, J. M. R. Gasc, P. L. Corvol, and C. M. Llorens-Cortes, "Ontogeny of angiotensin II type 2 ($AT_2$) receptor mRNA in the rat," *Kidney International*, vol. 47, no. 4, pp. 1095–1100, 1995.

[91] A. Israel, F. M. A. Correa, M. Niwa, and J. M. Saavedra, "Quantitative measurement of angiotensin II (A II) receptors in discrete regions of rat brain, pituitary and adrenal gland by autoradiography," *Clinical and Experimental Hypertension A*, vol. 6, no. 10-11, pp. 1761–1764, 1984.

[92] R. C. Speth, J. K. Wamsley, and D. R. Gehlert, "Angiotensin II receptor localization in the canine CNS," *Brain Research*, vol. 326, no. 1, pp. 137–143, 1985.

[93] D. R. Gehlert, R. C. Speth, and J. K. Wamsley, "Distribution of [125I]angiotensin II binding sites in the rat brain: a quantitative autoradiographic study," *Neuroscience*, vol. 18, no. 4, pp. 837–856, 1986.

[94] K. Tsutsumi and J. M. Saavedra, "Angiotensin-II receptor subtypes in median eminence and basal forebrain areas involved in regulation of pituitary function," *Endocrinology*, vol. 129, no. 6, pp. 3001–3008, 1991.

[95] J. M. Saavedra, "Brain and pituitary angiotensin," *Endocrine Reviews*, vol. 13, no. 2, pp. 329–380, 1992.

[96] M. Pawlikowski, "Immunohistochemical detection of angiotensin receptors $AT_1$ and $AT_2$ in normal rat pituitary gland, estrogen-induced rat pituitary tumor and human pituitary adenomas," *Folia Histochemica et Cytobiologica*, vol. 44, no. 3, pp. 173–177, 2006.

[97] S. G. Shelat, L. P. Reagan, J. L. King, S. J. Fluharty, and L. M. Flanagan-Cato, "Analysis of angiotensin type 2 receptors in vasopressinergic neurons and pituitary in the rat," *Regulatory Peptides*, vol. 73, no. 2, pp. 103–112, 1998.

[98] Z. Lenkei, A. M. Nuyt, D. Grouselle, P. Corvol, and C. Llorens-Cortès, "Identification of endocrine cell populations expressing the $AT_{1b}$ subtype of angiotensin II receptors in the anterior pituitary," *Endocrinology*, vol. 140, no. 1, pp. 472–477, 1999.

[99] G. L. Sanvitto, O. Jöhren, W. Häuser, and J. M. Saavedra, "Water deprivation upregulates ANG II $AT_1$ binding and mRNA in rat subfornical organ and anterior pituitary," *American Journal of Physiology*, vol. 273, no. 1, pp. E156–E163, 1997.

[100] C. Moreau, R. Rasolojanahary, A. J. Zamora, A. Enjalbert, C. Kordon, and C. Llorens-Cortes, "Expression of angiotensin II receptor subtypes $AT_{1a}$ and $AT_{1b}$ in enriched fractions of dispersed rat pituitary cells," *Neuroendocrinology*, vol. 66, no. 6, pp. 416–425, 1997.

[101] J. P. Bonjour and R. L. Malvin, "Stimulation of ADH release by the renin-angiotensin system," *The American journal of physiology*, vol. 218, no. 6, pp. 1555–1559, 1970.

[102] V. L. Brooks, L. C. Keil, and I. A. Reid, "Role of the renin-angiotensin system in the control of vasopressin secretion in conscious dogs," *Circulation Research*, vol. 58, no. 6, pp. 829–838, 1986.

[103] B. G. Zimmerman, "Effect of acute sympathectomy on responses to angiotensin and norepinephrine," *Circulation research*, vol. 11, pp. 780–787, 1962.

[104] B. G. Zimmerman, "Adrenergic facilitation by angiotensin: does it serve a physiological function?" *Clinical Science*, vol. 60, no. 4, pp. 343–348, 1981.

[105] G. Aguilera, C. L. Hyde, and K. J. Catt, "Angiotensin II receptors and prolactin release in pituitary lactotrophs," *Endocrinology*, vol. 111, no. 4, pp. 1045–1050, 1982.

[106] M. K. Steele, S. M. McCann, and A. Negro-Vilar, "Modulation by dopamine and estradiol of the central effects of angiotensin II on anterior pituitary hormone release," *Endocrinology*, vol. 111, no. 3, pp. 722–729, 1982.

[107] M. T. Bluet-Pajot, J. Epelbaum, D. Gourdji, C. Hammond, and C. Kordon, "Hypothalamic and hypophyseal regulation of growth hormone secretion," *Cellular and Molecular Neurobiology*, vol. 18, no. 1, pp. 101–123, 1998.

[108] E. Spinedi, L. Herrera, and A. Chisari, "Angiotensin II (AII) and adrenocorticotropin release: modulation by estradiol of the AII biological activity and binding characteristics in anterior pituitary dispersed cells," *Endocrinology*, vol. 123, no. 1, pp. 641–646, 1988.

[109] A. Seltzer, J. E. B. Pinto, P. N. Viglione et al., "Estrogens regulate angiotensin-converting enzyme and angiotensin receptors in female rat anterior pituitary," *Neuroendocrinology*, vol. 55, no. 4, pp. 460–467, 1992.

[110] O. Johren, G. L. Sanvitto, G. Egidy, and J. M. Saavedra, "Angiotensin II $AT_{1a}$ receptor mRNA expression is induced by estrogen- progesterone in dopaminergic neurons of the female rat arcuate nucleus," *Journal of Neuroscience*, vol. 17, no. 21, pp. 8283–8292, 1997.

[111] T. Inagami, S. Eguchi, K. Numaguchi et al., "Cross-talk between angiotensin II receptors and the tyrosine kinases and phosphatases," *Journal of the American Society of Nephrology*, vol. 10, Supplement 11, no. 1, pp. S57–S61, 1999.

[112] C. H. Gelband, M. Zhu, D. Lu et al., "Functional interactions between neuronal $AT_1$ and $AT_2$ receptors," *Endocrinology*, vol. 138, no. 5, pp. 2195–2198, 1997.

[113] H. Y. Sohn, U. Raff, A. Hoffmann et al., "Differential role of angiotensin II receptor subtypes on endothelial superoxide formation," *British Journal of Pharmacology*, vol. 131, no. 4, pp. 667–672, 2000.

[114] C. Sumners, W. Tang, W. Paulding, and M. K. Raizada, "Peptide receptors in astroglia: focus on angiotensin II and atrial natriuretic peptide," *GLIA*, vol. 11, no. 2, pp. 110–116, 1994.

[115] J. M. Saavedra, "Emerging features of brain angiotensin receptors," *Regulatory Peptides*, vol. 85, no. 1, pp. 31–45, 1999.

[116] L. Füchtbauer, M. Groth-Rasmussen, T. H. Holm et al., "Angiotensin II Type 1 receptor ($AT_1$) signaling in astrocytes regulates synaptic degeneration-induced leukocyte entry to the central nervous system," *Brain, Behavior, and Immunity*, vol. 25, no. 5, pp. 897–904, 2011.

[117] L. E. Downie, K. Vessey, A. Miller et al., "Neuronal and glial cell expression of angiotensin II type 1 ($AT_1$) and type 2 ($AT_2$) receptors in the rat retina," *Neuroscience*, vol. 161, no. 1, pp. 195–213, 2009.

[118] J. Benicky, E. Sánchez-Lemus, M. Honda et al., "Angiotensin II $AT_1$ receptor blockade ameliorates brain inflammation," *Neuropsychopharmacology*, vol. 36, no. 4, pp. 857–870, 2011.

[119] P. Shi, M. K. Raizada, and C. Sumners, "Brain cytokines as neuromodulators in cardiovascular control," *Clinical and Experimental Pharmacology and Physiology*, vol. 37, no. 2, pp. e52–e57, 2010.

[120] P. Shi, C. Diez-Freire, J. Y. Jun et al., "Brain microglial cytokines in neurogenic hypertension," *Hypertension*, vol. 56, no. 2, pp. 297–303, 2010.

[121] R. K. Bickerton and J. P. Buckley, "Evidence for a central mechanism in angiotensin induced hypertension," *Proceedings of the Society for Experimental Biology and Medicine*, vol. 106, pp. 834–836, 1961.

[122] H. Glossmann, A. J. Baukal, and K. J. Catt, "Properties of angiotensin II receptors in the bovine and rat adrenal cortex," *Journal of Biological Chemistry*, vol. 249, no. 3, pp. 825–834, 1974.

[123] J. P. Bennett Jr. and S. H. Snyder, "Angiotensin II binding to mammalian brain membranes," *Journal of Biological Chemistry*, vol. 251, no. 23, pp. 7423–7430, 1976.

[124] M. Van Houten, E. L. Schiffrin, and J. F. E. Mann, "Radioautographic localization of specific binding sites for blood-borne angiotensin II in the rat brain," *Brain Research*, vol. 186, no. 2, pp. 480–485, 1980.

[125] F. A. O. Mendelsohn, R. Quirion, J. M. Saavedra, G. Aguilera, and K. J. Catt, "Autoradiographic localization of angiotensin II receptors in rat brain," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 81, no. 5, pp. 1575–1579, 1984.

[126] B. P. Rowe, D. L. Saylor, and R. C. Speth, "Analysis of angiotensin II receptor subtypes in individual rat brain nuclei," *Neuroendocrinology*, vol. 55, no. 5, pp. 563–573, 1992.

[127] A. N. Epstein, J. T. Fitzsimons, and B. J. Rolls, "Drinking induced by injection of angiotensin into the rain of the rat," *Journal of Physiology*, vol. 210, no. 2, pp. 457–474, 1970.

[128] A. K. Johnson and A. N. Epstein, "The cerebral ventricles as the avenue for the dipsogenic action of intracranial angiotensin," *Brain Research*, vol. 86, no. 3, pp. 399–418, 1975.

[129] A. V. Ferguson, D. L. S. Washburn, and K. J. Latchford, "Hormonal and neurotransmitter roles for angiotensin in the regulation of central autonomic function," *Proceedings of the Society for Experimental Biology and Medicine*, vol. 226, no. 2, pp. 85–96, 2001.

[130] M. A. Fontes, O. Baltatu, S. M. Caligiorne et al., "Angiotensin peptides acting at rostral ventrolateral medulla contribute to hypertension of TGR(mREN2)27 rats," *Physiol Genomics*, vol. 2, no. 3, pp. 137–142, 2000.

[131] R. Casto and M. I. Phillips, "Cardiovascular actions of microinjections of angiotensin II in the brain stem of rats," *American Journal of Physiology*, vol. 246, no. 5, Part 2, pp. R811–R816, 1984.

[132] M. D'Amico, F. C. Di, L. Berrino, and F. Rossi, "$AT_1$ receptors mediate pressor responses induced by angiotensin II in the periaqueductal gray area of rats," *Life Sciences*, vol. 61, no. 1, pp. PL17–PL20, 1997.

[133] M. D'Amico, F. C. Di, F. Rossi, and T. D. Warner, "Role of $AT_2$ receptors in the cardiovascular events following microinjection of angiotensin II into the superior colliculus of anaesthetised rats," *Naunyn-Schmiedeberg's Archives of Pharmacology*, vol. 357, no. 2, pp. 121–125, 1998.

[134] M. J. McKinley, E. Badoer, L. Vivas, and B. J. Oldfield, "Comparison of c-fos expression in the lamina terminalis of conscious rats after intravenous or intracerebroventricular angiotensin," *Brain Research Bulletin*, vol. 37, no. 2, pp. 131–137, 1995.

[135] T. Nagatomo, K. Inenaga, and H. Yamashita, "Transient outward current in adult rat supraoptic neurones with slice patch-clamp technique: inhibition by angiotensin II," *Journal of Physiology*, vol. 485, no. 1, pp. 87–96, 1995.

[136] C. W. Bourque, D. L. Voisin, and Y. Chakfe, "Stretch-inactivated cation channels: cellular targets for modulation of osmosensitivity in supraoptic neurons," *Progress in Brain Research*, vol. 139, pp. 85–94, 2002.

[137] Z. Lenkei, P. Corvol, and C. Llorens-Cortes, "Comparative expression of vasopressin and angiotensin type-1 receptor mRNA in rat hypothalamic nuclei: a double in situ hybridization study," *Molecular Brain Research*, vol. 34, no. 1, pp. 135–142, 1995.

[138] O. Jöhren and J. M. Saavedra, "Expression of $AT_{1a}$ and $AT_{1b}$ angiotensin II receptor messenger RNA in forebrain of 2-wk-old rats," *American Journal of Physiology*, vol. 271, no. 1, pp. E104–E112, 1996.

[139] D. R. Gehlert, S. L. Gackenheimer, and D. A. Schober, "Autoradiographic localization of subtypes of angiotensin II antagonist binding in the rat brain," *Neuroscience*, vol. 44, no. 2, pp. 501–514, 1991.

[140] K. Tsutsumi and J. M. Saavedra, "Characterization and development of angiotensin II receptor subtypes ($AT_1$ and $AT_2$) in rat brain," *American Journal of Physiology*, vol. 261, no. 1, pp. R209–R216, 1991.

[141] O. Jöhren, T. Inagami, and J. M. Saavedra, "Localization of $AT_2$ angiotensin II receptor gene expression in rat brain by in situ hybridization histochemistry," *Molecular Brain Research*, vol. 37, no. 1-2, pp. 192–200, 1996.

[142] Z. Lenkei, M. Palkovits, P. Corvol, and C. Llorens-Cortes, "Distribution of angiotensin II type-2 receptor ($AT_2$) mRNA expression in the adult rat brain," *Journal of Comparative Neurology*, vol. 373, pp. 322–339, 1996.

[143] F. C. Nunes and V. A. Braga, "Chronic angiotensin II infusion modulates angiotensin II type I receptor expression in the subfornical organ and the rostral ventrolateral medulla in hypertensive rats," *Journal of the Renin-Angiotensin-Aldosterone System*, vol. 12, pp. 440–445, 2011.

[144] L. P. Reagan, M. Theveniau, X. D. Yang et al., "Development of polyclonal antibodies against angiotensin type 2 receptors," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 90, pp. 7956–7960, 1993.

[145] M. I. Phillips, L. Shen, E. M. Richards, and M. K. Raizada, "Immunohistochemical mapping of angiotensin $AT_1$ receptors in the brain," *Regulatory Peptides*, vol. 44, no. 2, pp. 95–107, 1993.

[146] S. G. Wei, Y. Yu, Z. H. Zhang, R. M. Weiss, and R. B. Felder, "Mitogen-activated protein kinases mediate upregulation of hypothalamic angiotensin II type 1 receptors in heart failure rats," *Hypertension*, vol. 52, no. 4, pp. 679–686, 2008.

[147] A. Shekhar, P. L. Johnson, T. J. Sajdyk et al., "Angiotensin-II is a putative neurotransmitter in lactate-induced panic-like responses in rats with disruption of GABAergic inhibition in the dorsomedial hypothalamus," *Journal of Neuroscience*, vol. 26, no. 36, pp. 9205–9215, 2006.

[148] E. Moellenhoff, A. Blume, J. Culman et al., "Effect of repetitive icv injections of ANG II on c-Fos and $AT_1$-receptor expression in the rat brain," *American Journal of Physiology*, vol. 280, no. 4, pp. R1095–R1104, 2001.

[149] N. E. Rowland, B. H. Li, M. J. Fregly, and G. C. Smith, "Fos induced in brain of spontaneously hypertensive rats by angiotensin II and co-localization with AT-1 receptors," *Brain Research*, vol. 675, no. 1-2, pp. 127–134, 1995.

[150] Y. M. Kang, Y. Ma, C. Elks, J. P. Zheng, Z. M. Yang, and J. Francis, "Cross-talk between cytokines and renin-angiotensin in hypothalamic paraventricular nucleus in heart failure: role of nuclear factor-$\kappa$B," *Cardiovascular Research*, vol. 79, no. 4, pp. 671–678, 2008.

[151] Y. M. Kang, Y. Ma, J. P. Zheng et al., "Brain nuclear factor-kappa B activation contributes to neurohumoral excitation in

angiotensin II-induced hypertension," *Cardiovascular Research*, vol. 82, no. 3, pp. 503–512, 2009.

[152] L. P. Reagan, L. M. Flanagan-Cato, D. K. Yee, L. Y. Ma, R. R. Sakai, and S. J. Fluharty, "Immunohistochemical mapping of angiotensin type 2 ($AT_2$) receptors in rat brain," *Brain Research*, vol. 662, no. 1-2, pp. 45–59, 1994.

[153] J. Huang, Y. Hara, J. Anrather, R. C. Speth, C. Iadecola, and V. M. Pickel, "Angiotensin II subtype 1A ($AT_{1a}$) receptors in the rat sensory vagal complex: subcellular localization and association with endogenous angiotensin," *Neuroscience*, vol. 122, no. 1, pp. 21–36, 2003.

[154] G. Wang, J. Anrather, J. Huang, R. C. Speth, V. M. Pickel, and C. Iadecola, "NADPH oxidase contributes to angiotensin II signaling in the nucleus tractus solitarius," *Journal of Neuroscience*, vol. 24, no. 24, pp. 5516–5524, 2004.

[155] M. J. Glass, J. Huang, R. C. Speth, C. Iadecola, and V. M. Pickel, "Angiotensin II AT-1A receptor immunolabeling in rat medial nucleus tractus solitarius neurons: subcellular targeting and relationships with catecholamines," *Neuroscience*, vol. 130, no. 3, pp. 713–723, 2005.

[156] G. Wang, T. A. Milner, R. C. Speth et al., "Sex differences in angiotensin signaling in bulbospinal neurons in the rat rostral ventrolateral medulla," *American Journal of Physiology*, vol. 295, no. 4, pp. R1149–R1157, 2008.

[157] J. P. Pierce, J. Kievits, B. Graustein, R. C. Speth, C. Iadecola, and T. A. Milner, "Sex differences in the subcellular distribution of angiotensin type 1 receptors and NADPH oxidase subunits in the dendrites of C1 neurons in the rat rostral ventrolateral medulla," *Neuroscience*, vol. 163, no. 1, pp. 329–338, 2009.

[158] C. G. Coleman, J. Anrather, C. Iadecola, and V. M. Pickel, "Angiotensin II type 2 receptors have a major somatodendritic distribution in vasopressin-containing neurons in the mouse hypothalamic paraventricular nucleus," *Neuroscience*, vol. 163, no. 1, pp. 129–142, 2009.

[159] G. Wang, C. G. Coleman, M. J. Glass et al., "Angiotensin II type 2 receptor-coupled nitric oxide production modulates free radical availability and voltage-gated Ca2+ currents in NTS neurons," *American Journal of Physiology*, vol. 302, pp. R1076–R1083, 2012.

[160] L. Gao and I. H. Zucker, "$AT_2$ receptor signaling and sympathetic regulation," *Current Opinion in Pharmacology*, vol. 11, no. 2, pp. 124–130, 2011.

[161] I. H. Zucker, H. D. Schultz, K. P. Patel, W. Wang, and L. Gao, "Regulation of central angiotensin type 1 receptors and sympathetic outflow in heart failure," *American Journal of Physiology*, vol. 297, no. 5, pp. H1557–H1566, 2009.

[162] L. Gao, W. Wang, Y. L. Li et al., "Sympathoexcitation by central ANG II: roles for $AT_1$ receptor upregulation and NAD(P)H oxidase in RVLM," *American Journal of Physiology*, vol. 288, no. 5, pp. H2271–H2279, 2005.

[163] L. Gao, W. Wang, W. Wang, H. Li, C. Sumners, and I. H. Zucker, "Effects of angiotensin type 2 receptor overexpression in the rostral ventrolateral medulla on blood pressure and urine excretion in normal rats," *Hypertension*, vol. 51, no. 2, pp. 521–527, 2008.

[164] D. C. Merrill, M. W. Thompson, C. L. Carney et al., "Chronic hypertension and altered baroreflex responses in transgenic mice containing the human renin and human angiotensinogen genes," *Journal of Clinical Investigation*, vol. 97, no. 4, pp. 1047–1055, 1996.

[165] H. Xia, Y. Feng, T. D. Obr, P. J. Hickman, and E. Lazartigues, "Angiotensin II type 1 receptor-mediated reduction of angiotensin-converting enzyme 2 activity in the brain impairs baroreflex function in hypertensive mice," *Hypertension*, vol. 53, no. 2, pp. 210–216, 2009.

[166] A. I. Rodriguez-Perez, R. Valenzuela, B. Villar-Cheda, M. J. Guerra, J. L. Lanciego, and J. L. Labandeira-Garcia, "Estrogen and angiotensin interaction in the substantia nigra. Relevance to postmenopausal Parkinson's disease," *Experimental Neurology*, vol. 224, no. 2, pp. 517–526, 2010.

[167] J. Rodriguez-Pallares, P. Rey, J. A. Parga, A. Muñoz, M. J. Guerra, and J. L. Labandeira-Garcia, "Brain angiotensin enhances dopaminergic cell death via microglial activation and NADPH-derived ROS," *Neurobiology of Disease*, vol. 31, no. 1, pp. 58–73, 2008.

[168] B. Joglar, J. Rodriguez-Pallares, A. I. Rodriguez-Perez, P. Rey, M. J. Guerra, and J. L. Labandeira-Garcia, "The inflammatory response in the MPTP model of Parkinson's disease is mediated by brain angiotensin: relevance to progression of the disease," *Journal of Neurochemistry*, vol. 109, no. 2, pp. 656–669, 2009.

[169] P. Garrido-Gil, R. Valenzuela, B. Villar-Cheda, J. L. Lanciego, and J. L. Labandeira-Garcia, "Expression of angiotensinogen and receptors for angiotensin and prorenin in the monkey and human substantia nigra: an intracellular renin-angiotensin system in the nigra," *Brain Structure and Function*, vol. 218, no. 2, pp. 373–388, 2012.

[170] R. C. Speth, W. T. Barry, M. S. Smith, and K. L. Grove, "A comparison of brain angiotensin II receptors during lactation and diestrus of the estrous cycle in the rat," *American Journal of Physiology*, vol. 277, no. 3, pp. R904–R909, 1999.

[171] B. Zelezna, E. M. Richards, W. Tang, D. Lu, C. Sumners, and M. K. Raizada, "Characterization of a polyclonal anti-peptide antibody to the angiotensin II type-1 ($AT_1$) receptor," *Biochemical and Biophysical Research Communications*, vol. 183, no. 2, pp. 781–788, 1992.

[172] D. F. Guo, I. Chenier, V. Tardif, S. N. Orlov, and T. Inagami, "Type 1 angiotensin II receptor-associated protein ARAP1 binds and recycles the receptor to the plasma membrane," *Biochemical and Biophysical Research Communications*, vol. 310, no. 4, pp. 1254–1265, 2003.

[173] B. Deslauriers, C. Ponce, C. Lombard, R. Larguier, J. C. Bonnafous, and J. Marie, "N-glycosylation requirements for the $AT_{1a}$ angiotensin II receptor delivery to the plasma membrane," *Biochemical Journal*, vol. 339, Part 2, pp. 397–405, 1999.

[174] S. AbdAlla, H. Lother, and U. Quitterer, "$AT_1$-receptor heterodimers show enhanced G-protein activation and altered receptor sequestration," *Nature*, vol. 407, no. 6800, pp. 94–98, 2000.

[175] J. L. Cook, R. N. Re, D. L. DeHaro, J. M. Abadie, M. Peters, and J. Alam, "The trafficking protein GABARAP binds to and enhances plasma membrane expression and function of the angiotensin II type 1 receptor," *Circulation Research*, vol. 102, no. 12, pp. 1539–1547, 2008.

[176] M. J. McKinley, E. Badoer, and B. J. Oldfield, "Intravenous angiotensin II induces Fos-immunoreactivity in circumventricular organs of the lamina terminalis," *Brain Research*, vol. 594, no. 2, pp. 295–300, 1992.

[177] H. A. Rockman, K. R. Chien, D. J. U. Choi et al., "Expression of a $\beta$-adrenergic receptor kinase 1 inhibitor prevents the development of myocardial failure in gene-targeted mice," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 95, no. 12, pp. 7000–7005, 1998.

[178] J. L. Cook, A. Singh, D. DeHaro, J. Alam, and R. N. Re, "Expression of a naturally occurring angiotensin $AT_1$ receptor

cleavage fragment elicits caspase-activation and apoptosis," *American Journal of Physiology*, vol. 301, pp. C1175–C1185, 2011.

[179] J. Benicky, R. Hafko, E. Sanchez-Lemus, G. Aguilera, and J. M. Saavedra, "Six commercially available angiotensin II $AT_1$ receptor antibodies are non-specific," *Cellular and Molecular Neurobiology*, vol. 32, pp. 1353–1365, 2012.

[180] J. M. Adams, J. J. McCarthy, and S. D. Stocker, "Excess dietary salt alters angiotensinergic regulation of neurons in the rostral ventrolateral medulla," *Hypertension*, vol. 52, no. 5, pp. 932–937, 2008.

[181] M. Herrera, M. A. Sparks, A. R. Alfonso-Pecchio, and L. M. T. M. Coffman, "Lack of specificity of commercial antibodies leads to misidentification of Angiotensin type 1 receptor protein," *Hypertension*, vol. 61, pp. 253–258, 2013.

[182] M. C. Michel, T. Wieland, and G. Tsujimoto, "How reliable are G-protein-coupled receptor antibodies?" *Naunyn-Schmiedeberg's Archives of Pharmacology*, vol. 379, no. 4, pp. 385–388, 2009.

[183] B. Xue, T. G. Beltz, Y. Yu et al., "Central interactions of aldosterone and angiotensin II in aldosterone- and angiotensin II-induced hypertension," *American Journal of Physiology*, vol. 300, no. 2, pp. H555–H564, 2011.

[184] M. Ito, M. I. Oliverio, P. J. Mannon et al., "Regulation of blood pressure by the type 1A angiotensin II receptor gene," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 92, no. 8, pp. 3521–3525, 1995.

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*Research Article*

# Distinct Molecular Effects of Angiotensin II and Angiotensin III in Rat Astrocytes

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It is postulated that central effects of angiotensin (Ang) II may be indirect due to rapid conversion to Ang III by aminopeptidase A (APA). Previously, we showed that Ang II and Ang III induced mitogen-activated protein (MAP) kinases ERK1/2 and stress-activated protein kinase/Jun-terminal kinases (SAPK/JNK) phosphorylation in cultured rat astrocytes. Most importantly, both peptides were equipotent in causing phosphorylation of these MAP kinases. In these studies, we used brainstem and cerebellum astrocytes to determine whether Ang II's phosphorylation of these MAP kinases is due to the conversion of the peptide to Ang III. We pretreated astrocytes with 10 $\mu$M amastatin A or 100 $\mu$M glutamate phosphonate, selective APA inhibitors, prior to stimulating with either Ang II or Ang III. Both peptides were equipotent in stimulating ERK1/2 and SAPK/JNK phosphorylation. The APA inhibitors failed to prevent Ang II- and Ang III-mediated phosphorylation of the MAP kinases. Further, pretreatment of astrocytes with the APA inhibitors did not affect Ang II- or Ang III-induced astrocyte growth. These findings suggest that both peptides directly induce phosphorylation of these MAP kinases as well as induce astrocyte growth. These studies establish both peptides as biologically active with similar intracellular and physiological effects.

## 1. Introduction

Mitogen-activated protein (MAP) kinases constitute a superfamily of serine/threonine protein kinases involved in the regulation of a number of intracellular pathways associated with cellular growth, apoptosis, cellular differentiation, transformation of cells, and vascular contraction [1–4]. We have shown that angiotensin (Ang) II via activation of $AT_1$ receptors increases the expression of MAP kinases in primary cultures of rat astrocytes [5–7]. ERK1/2 MAP kinases were shown to mediate Ang II-induced astrocyte growth and Ang II-induced c-Fos and c-Myc expression [5, 6, 8]. We have also established that Ang II induces the phosphorylation of stress-activated protein kinase/Jun-terminal kinase (SAPK/JNK) MAP kinases leading to cellular proliferation in cultured rat astrocytes, an effect that was also mediated by the $AT_1$ Ang receptors [7]. Our findings suggest that Ang II signals through these two different MAP kinase pathways in astrocytes.

More recently, we showed that Ang III also induces the phosphorylation of ERK1/2 and SAPK/JNK MAP kinases in these cells [9, 10]. Moreover, Ang III was equipotent to Ang II in causing these MAP kinases phosphorylation and occurred via interaction with the Ang $AT_1$ receptor. Ang III also induced astrocyte growth, however, not to a similar extent as Ang II [9].

Similar to our intracellular findings, *in vivo* studies have established that both peptides have similar physiologically relevant effects. For example, intracerebroventricular (ICV) injection of Ang II or Ang III caused a similar dose-dependent increase in blood pressure. Since Ang II is quickly cleaved by aminopeptidase A (APA) into Ang III, the true effector was unknown [11, 12]. In spontaneously hypertensive rats (SHR), injection of both peptides caused a prolonged blood pressure response compared to controls. However, pretreatment with bestatin, an aminopeptidase B (APB) inhibitor, potentiated and prolonged the elevated blood pressure response to Ang III in SHR [13]. Since bestatin

inhibits the breakdown of Ang III, these findings suggest that Ang III was a key player in the blood pressure response elicited by the activation of the renin angiotensin system.

Findings from several studies have implicated Ang III as the active peptide in the central nervous system. Reaux et al. [14] showed that preventing Ang II conversion to Ang III using EC33 (a selective APA inhibitor) blocked the pressor response of exogenous Ang II in rats, suggesting that the conversion of Ang II to Ang III is required to increase blood pressure in these animals. Moreover, ICV injection of EC33 caused a dose-dependent decrease in blood pressure, while ICV injection of PC18 (an APB inhibitor) increased blood pressure, an effect that was prevented by pretreatment with the $AT_1$ receptor antagonist, losartan [14]. These findings and ours suggest that Ang III is a major effector peptide of the brain renin angiotensin system, and this peptide is involved in central control of blood pressure. In addition, these findings and others implicate Ang III, not Ang II, as the active peptide of the central renin angiotensin system leading to the putative "Ang III hypothesis." Therefore, in this study, we used specific APA inhibitors to establish whether Ang II-mediated phosphorylation of ERK1/2 and SAPK/JNK MAP kinases in astrocytes was a result of Ang II conversion to Ang III. This is highly possible since APA is expressed in astrocytes [15] and neurons [16, 17]. As shown by others, inhibition of this enzyme has attenuated certain effects of Ang II. These studies were conducted in brainstem and cerebellum astrocytes to allow correlation of the current results with our previous findings [9, 10]. Moreover, astrocytes are the main source of brain angiotensinogen [18], the Ang II and Ang III precursors, and thus are ideal brain-derived cells to study the signaling pathways of these peptides.

## 2. Materials and Methods

*2.1. Materials.* Tissue culture supplies such as Dulbecco's Modified Eagles Medium (DMEM)/F12 (1 : 1), fetal bovine serum (FBS), antibiotic solution, and trypsin/EDTA were purchased from VWR (Grand Island, NY, USA). Ang II and Ang III were obtained from Bachem (Torrance, CA, USA). The APA inhibitor glutamate phosphonate (4-amino-4-phosphonobutyric acid, GluP) was generously supplied by Dr. Robert Speth (Nova Southeastern University, FL, USA), while amastatin (AMA) was purchased from Calbiochem (La Jolla, CA, USA). $^3$H-Thymidine (2000 Ci/mmole) was purchased from MP Biomedicals (Solon, OH, USA). The phosphospecific ERK1/2 antibody, the phosphospecific SAPK/JNK antibody (Tyr751), the ERK1/2 antibody, and the SAPK/JNK antibody were purchased from Cell Signaling Technology (Beverly, MA, USA). Protein measurement supplies, gel electrophoresis, and Western blotting supplies including BCA protein reagents, acrylamide, ECL chemiluminescent reagents, and nitrocellulose membrane were purchased from either GE Health Care (Piscataway, NJ, USA) or Biorad Laboratories (Hercules, CA, USA) or from Pierce Biotechnology (Rockford, IL, USA). All other chemicals were purchased from either VWR international (Suwannee, GA, USA) or Sigma (St. Louis, MO, USA).

*2.2. Preparation of Astrocytes.* Timed, pregnant Sprague-Dawley rats were obtained from Charles River Laboratories (Wilmington, MA, USA) and maintained in the ALAAC-accredited animal facility of Nova Southeastern University. Primary cultures of astrocytes were prepared from the brainstem and cerebellum of 2-3-day-old neonatal pups by physical dissociation as previously described [6, 8, 19]. Cells were maintained in DMEM/F12 with 10% FBS, 100 $\mu$g/mL penicillin, and 100 units/mL streptomycin at 37°C in a humidified $CO_2$ incubator (5% $CO_2$ and 95% air). Cultures were fed every 3 to 4 days until confluent. Confluent monolayers were placed in DMEM/F12 containing 10 mM HEPES, pH 7.5, 10% FBS, and antibiotics and shaken overnight to remove oligodendrocytes. Astrocytes were detached with trypsin/EDTA (0.05% trypsin, 0.53 mM EDTA), replated at a ratio of 1 to 10, and grown to about 85% confluence prior to use. Isolated cells showed a positive immunoreactivity with an antibody against glial fibrillary acidic protein and negative immunoreactivity with markers for neurons, fibroblasts, or oligodendrocytes.

*2.3. Cell Treatments.* Cultured brainstem and cerebellum astrocytes growing on 100 mm culture dishes were made quiescent by a 48-hour treatment with serum-free media. Astrocytes were pretreated with either 100 $\mu$M GluP or 10 $\mu$M AMA for 15 minutes. Following the pretreatments, the cells were subsequently stimulated with 100 nM Ang II or 100 nM Ang III for 10 minutes. For comparative purposes, cells were also incubated with the inhibitors alone. Basal and stimulated levels of the MAP kinases were determined in the presence of DMSO in experiments involving AMA. The concentrations of the GluP and AMA were not cytotoxic based on measurements of the mitochondrial uptake of the tetrazolium dye, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), to its insoluble formazan form (data not shown). Cell lysates from all experiments were prepared by washing the astrocyte monolayers with phosphate-buffered saline containing 0.01 mM $NaVO_4$ to prevent the dephosphorylation of activated phosphorylated proteins. Cells were solubilized in supplemented lysis buffer (100 mM NaCl, 50 mM NaF, 5 mM EDTA, 1% Triton X-100, 50 mM Tris-HCl, 0.01 mM $NaVO_4$, 0.1 mM PMSF, 0.6 $\mu$M leupeptin, and pH 7.4) for 30 minutes on ice. The supernatants were clarified by centrifugation (12000 ×g for 10 minutes, 4°C), and the protein concentrations were subsequently measured using the BCA method.

*2.4. Western Blot Analysis.* Western blotting protocols to determine ERK1/2 phosphorylation and JNK phosphorylation were previously described [7, 10]. Essentially, solubilized proteins were separated in 10% polyacrylamide gels and transferred to nitrocellulose membranes. Nonspecific binding to the membranes was prevented by treating the membranes with 5% Blotto and probing with either the specific activated phosphorylated form of the ERK1/2 antibody or the activated phosphorylated form of the SAPK/JNK antibody. After incubating with the primary antibodies, the membranes were probed with goat anti-rabbit antibody coupled to horseradish peroxidase, the immunoreactive bands

visualized using ECL reagents, and the data quantified by densitometry. To quantify protein loading, membranes were also probed with antibodies for the ERK1/2 protein and SAPK/JNK protein. The immunoreactive bands were visualized using ECL reagents and quantified by densitometry.

*2.5. Measurement of DNA Synthesis.* Subconfluent monolayers of cells growing in 24-well culture dishes were made quiescent by 48-hours treatment with serum-free media. Individual wells were then pretreated for 15 minutes with 100 $\mu$M GluP or 10 $\mu$M AMA. Subsequently, the cells were treated with either 100 nM Ang II or 100 nM Ang III for 48 hours. For comparative purposes, some wells did not receive any peptides or were treated with DMSO (the vehicle for AMA) or were only treated with the inhibitors alone. $^3$H-Thymidine (0.25 Ci/mL culture medium) was added during the last 24 hours of treatment. Newly synthesized DNA was precipitated with 5% TCA, dissolved in 0.25 N NaOH, and quantified by liquid scintillation spectrometry as previously described [20].

*2.6. Statistical Analysis.* All data are expressed as the mean ± SEM of 6 or more experiments, as indicated. $t$-tests or repeated measures of one-way analysis of variance (ANOVA) with Dunnett's posttest were used to compare treatment groups with groups treated with no chemicals or those treated with the inhibitors, using PRISM (GraphPad). The criterion for statistical significance was set at $P < 0.05$.

## 3. Results

*3.1. Effect of APA Inhibitors on ERK1/2 MAP Kinase Phosphorylation by the Peptides.* In previous studies, we showed that Ang II and Ang III interact with Ang $AT_1$ receptors to significantly increase ERK1/2 MAP kinase phosphorylation to a similar extent [10]. To determine whether the effects observed with Ang II were due to its conversion to Ang III, cerebellar and brainstem astrocytes were pretreated for 15 minutes with 10 $\mu$M AMA or 100 $\mu$M GluP. The cells were subsequently stimulated with either 100 nM Ang II or 100 nM Ang III. As shown in Figure 1, both Ang II and Ang III significantly and equipotently induced ERK1/2 MAP kinase phosphorylation in brainstem astrocytes. The APA inhibitors AMA (Figure 1(a)) and GluP (Figure 1(b)) were ineffective in preventing Ang II- and Ang III-induced ERK1/2 phosphorylation. Similarly, in cerebellar astrocytes, both peptides induced ERK1/2 MAP kinase phosphorylation, effects that were not affected by pretreatment with the APA inhibitors (Figures 1(c) and 1(d)). These findings suggest that the two peptides directly induced phosphorylation of this MAP kinase pathway.

*3.2. Effect of APA Inhibitors on SAPK/JNK MAP Kinase Phosphorylation by the Peptides.* As shown previously [9] and in Figure 2, Ang II and Ang III via interactions with the Ang $AT_1$ receptor induced SAPK/JNK MAP kinase phosphorylation similarly in brainstem astrocytes. Pretreatment with the APA inhibitors AMA (Figure 2(a)) and GluP (Figure 2(b)) failed to prevent Ang II-mediated and Ang III-mediated SAPK/JNK MAP kinase phosphorylation. In cerebellar astrocytes, the two peptides were equipotent in inducing SAPK/JNK MAP kinase phosphorylation (Figures 2(c) and 2(d)). Both APA inhibitors were unable to prevent Ang II-induced and Ang III-induced SAPK/JNK MAP kinase phosphorylation in cerebellar astrocytes as well (Figures 2(c) and 2(d)). Interestingly, brainstem astrocytes were more sensitive to the effects of both peptides, exhibiting higher levels of SAPK/JNK phosphorylation as compared to cerebellum astrocytes. In addition, our findings also suggest that both peptides have direct effects to induce SAPK/JNK MAP kinase in brainstem and cerebellum astrocytes.

*3.3. Effects of APA Inhibitors on Ang Peptide Astrocyte DNA Synthesis.* Both Ang II and Ang III have been shown to induce cellular proliferation of astrocytes [10]. To determine whether inhibition of APA affected the mitotic effects of Ang II and Ang III, brainstem and cerebellar astrocytes were pretreated with the APA inhibitors (10 $\mu$M AMA and 100 $\mu$M GluP) followed by stimulation with the peptides. As shown in Figure 3, both Ang II and Ang III induced cellular proliferation of brainstem and cerebellum astrocytes. However, Ang II was more potent at inducing astrocyte proliferation in both brainstem and cerebellum astrocytes, a finding that we observed previously [9, 10]. The APA inhibitors AMA and GluP (Figures 3(a) and 3(b)) were ineffective at preventing the proliferative effects of Ang II and Ang III, suggesting that both peptides had direct effects on cell proliferation.

## 4. Discussion

We and others have established that the angiotensin peptides Ang II and Ang III have important cellular effects in the central nervous system. However, in the intact brain, controversy still surrounds the synthesis, degradation, and physiological effects of Ang II, the primary peptide produced by the renin angiotensin system. One such controversy is known as the "Ang III Hypothesis." The premise of this theory is that the physiologically relevant peptide in the brain that binds to and activates the $AT_1$ receptor is Ang III, not Ang II. This putative "Ang III Hypothesis" suggests that Ang II is converted to Ang III in order to activate Ang $AT_1$ receptors [14, 21–25]. Studies have shown that centrally produced Ang II is rapidly degraded by several enzymes (primarily APA), leading to the accumulation of Ang III [26]. It is the degradation of Ang II to Ang III by APA that is the most documented breakdown pathway for Ang II and is the focus of most studies. Since the metabolism of Ang II is relatively rapid, this has helped to fuel the Ang III Hypothesis.

There are a few studies that support the putative Ang III hypothesis. Most of these studies have focused on using APA inhibitors such as AMA to block Ang II conversion to Ang III, then measuring effects of the initial Ang II treatment on blood pressures of normotensive and hypertensive animals [11–14]. A few studies have also focused on the Ang receptor(s) that Ang III interacts with to cause effects on

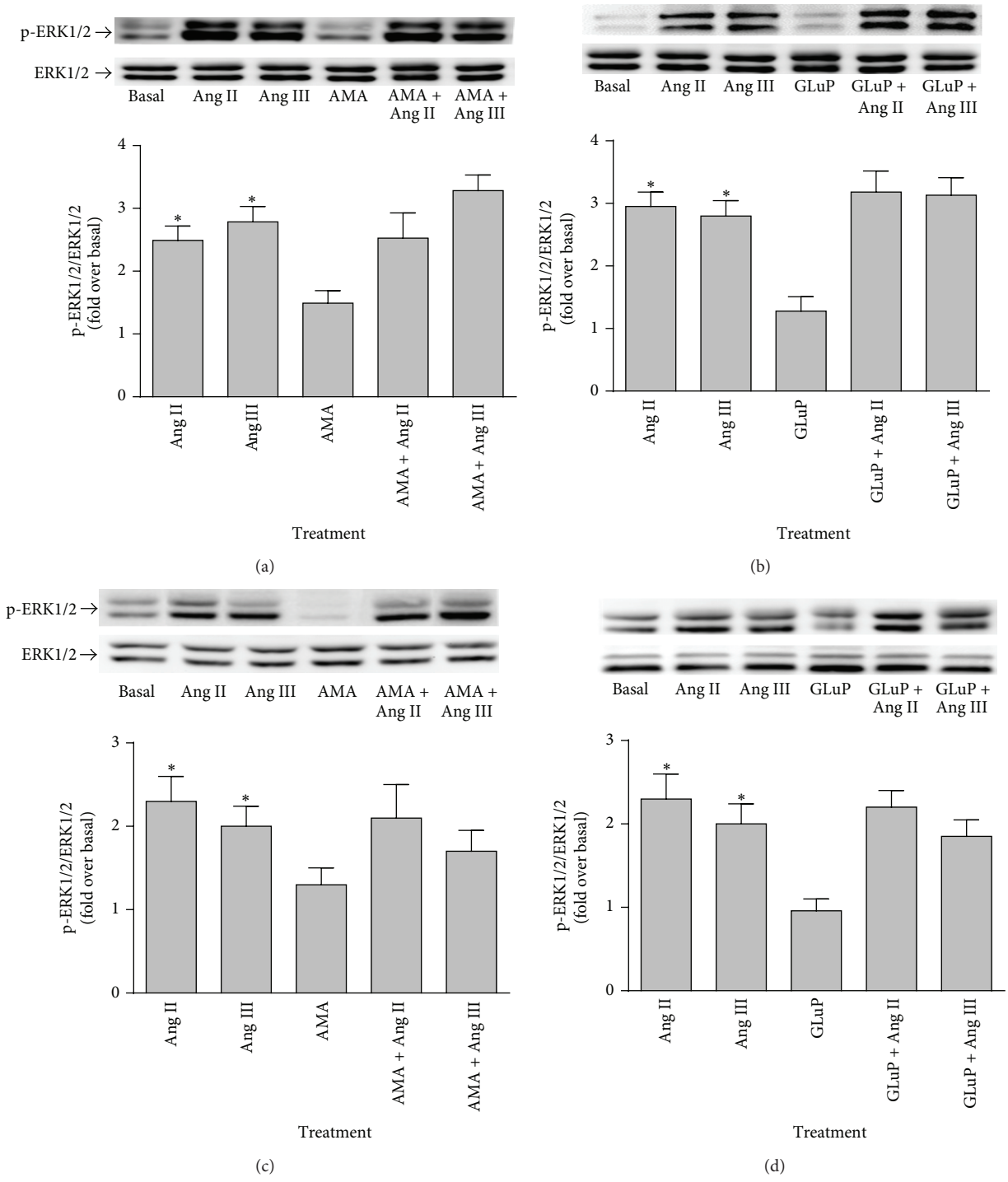


Figure 1: Effect of AMA and GluP on Ang II- and Ang III-induced ERK1/2 protein phosphorylation. Quiescent monolayers of brainstem ((a) and (b)) or cerebellum ((c) and (d)) astrocytes were pretreated with 10 $\mu$M AMA or 100 $\mu$M GluP for 15 minutes. The cells were subsequently stimulated with 100 nM Ang II or 100 nM Ang III for 10 minutes. Phosphorylated-ERK1/2 immunoreactive protein levels were measured by Western blot analysis using an antibody specific for the phosphorylated form of ERK1/2. Protein loading was quantified using the ERK1/2 protein antibody. The data were analyzed by densitometry, and the amount of phosphorylation was calculated as the fold increase over basal in the presence of vehicle. Each value represents the mean ± SEM of preparations of brainstem and cerebellum astrocytes from 6 or more litters of neonatal rat pups. * denotes that $P < 0.05$ as compared to basal levels for ERK1/2 expression in astrocytes prepared from the brainstem and the cerebellum.

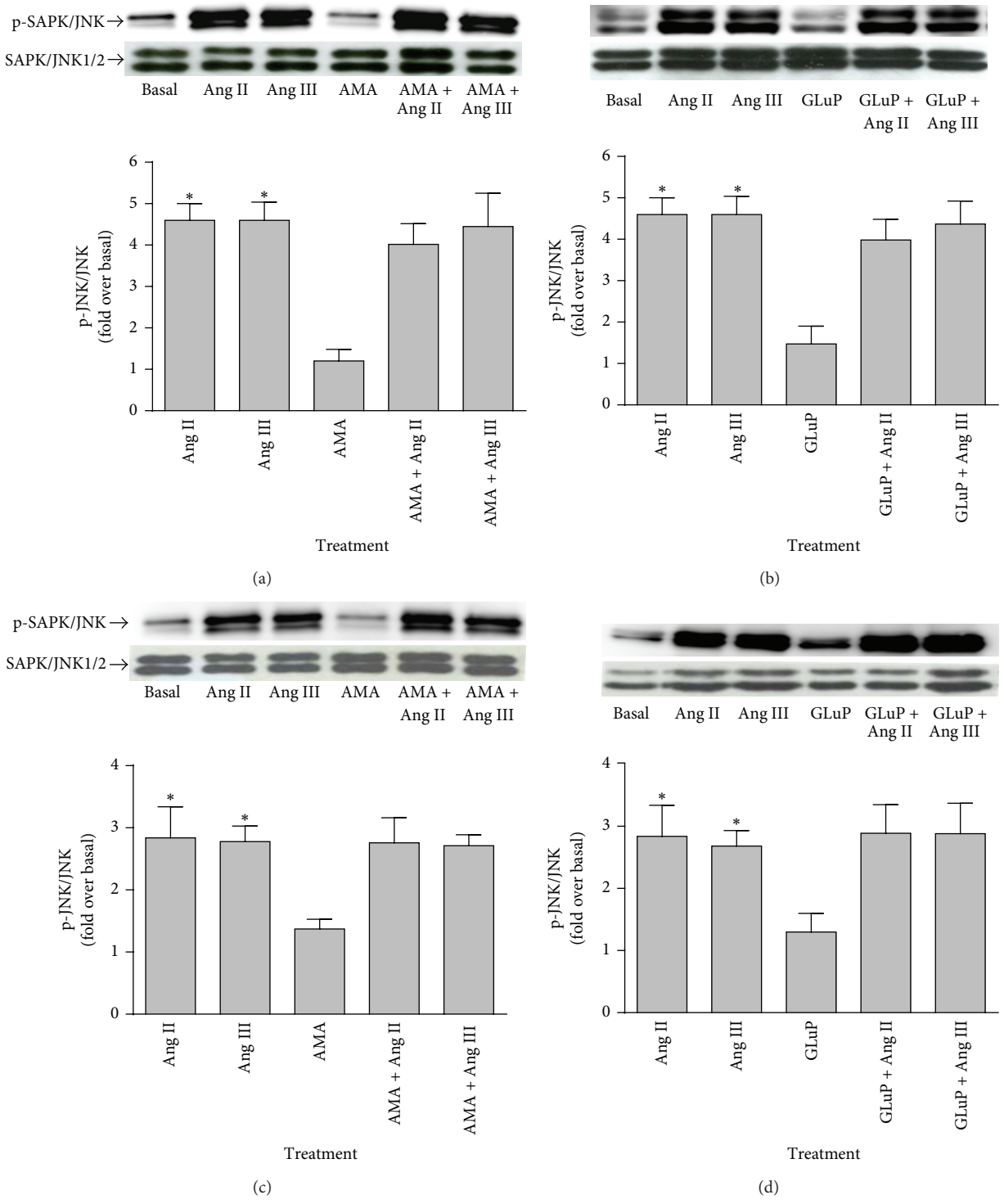


Figure 2: Effect of AMA and GluP on Ang II- and Ang III-induced SAPK/JNK protein phosphorylation. Quiescent monolayers of brainstem ((a) and (b)) and cerebellum ((c) and (d)) astrocytes were pretreated with 10 $\mu$M AMA or 100 $\mu$M GluP for 15 minutes. The cells were subsequently stimulated with 100 nM Ang II or 100 nM Ang III for 10 minutes. Phosphorylated-SAPK/JNK immunoreactive protein levels were measured by Western blot analysis using an antibody specific for the phosphorylated form of SAPK/JNK. Protein loading was quantified using the SAPK/JNK protein antibody. The data were analyzed by densitometry, and the amount of phosphorylation was calculated as the fold increase over basal in the presence of vehicle. Each value represents the mean ± SEM of preparations of brainstem and cerebellum astrocytes from 6 or more litters of neonatal rat pups. * denotes that $P < 0.05$ as compared to basal levels for SAPK/JNK expression in astrocytes prepared from the brainstem and the cerebellum.

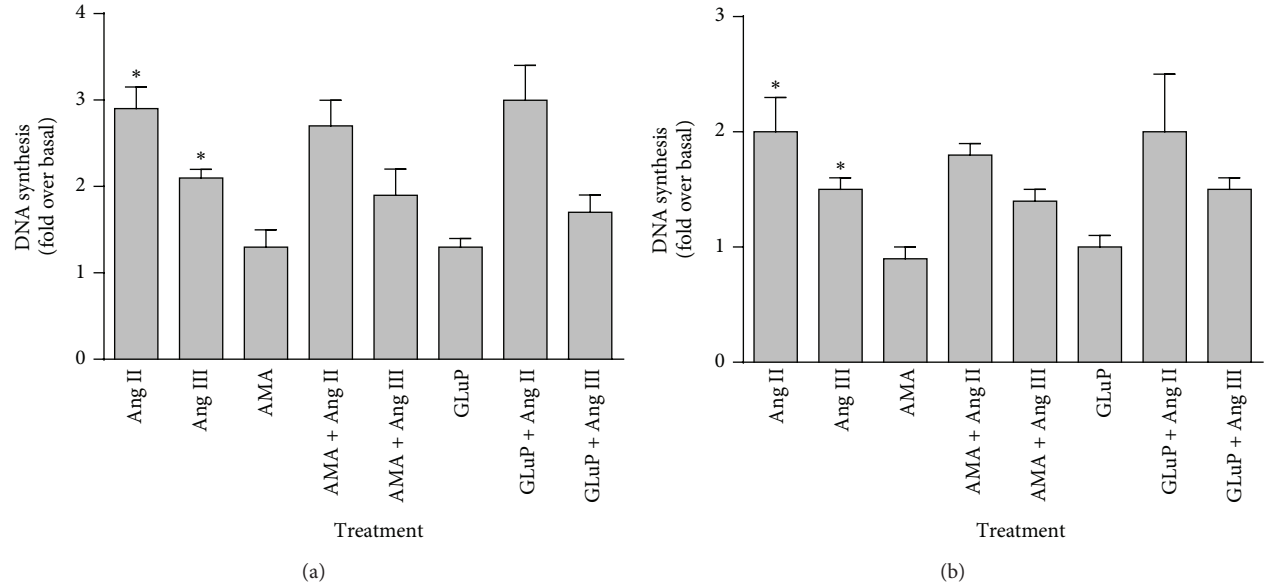


Figure 3: Effect of AMA and GluP on Ang II- and Ang III-induced DNA synthesis. Quiescent monolayers of brainstem (a) and cerebellum (b) astrocytes were incubated for 48 hours with 100 nM Ang II or Ang III in the presence and absence of 10 $\mu$M AMA or 100 $\mu$M GluP. During the last 24 hours of treatment, $^3$H-Thymidine was added and DNA synthesis measured as described. Cells were also treated with the inhibitors alone. Each value represents the mean ± SEM of preparations of brainstem and cerebellum astrocytes from 6 or more litters of neonatal rat pups. *denotes that $P < 0.05$ as compared to basal levels of DNA synthesis.

blood pressure. An *in vitro* study by Harding et al. [22] showed that Ang III was more potent than Ang II at eliciting responses from paraventricular nucleus receptors isolated from normal rats and SHR. The Ang II response was blocked by the addition of AMA, the selective APA inhibitor, and was ineffective at preventing the Ang III response [22].

There are a number of studies that refute the theory that Ang II conversion to Ang III is necessary for $AT_1$ receptor binding. Kokje et al. [27] showed that aminopeptidase-resistant Ang II analogs, when ICV-adminiistered, caused pressor and dipsogenic activities similar to or greater than Ang II. In addition, there were no differences in Ang II, its aminopeptidase-resistant analogs, and Ang III initial peak pressor responses, findings that do not support a requisite formation of Ang III to elicit a response. It has also been established that $^{125}$I-Ang II can bind to brain $AT_1$ receptors, an effect that does not require conversion to $^{125}$I-Ang III [28].

In the current studies, we sought to determine whether some of the effects that we have previously observed with Ang II are modulated in the presence of the APA inhibitors, AMA and GluP. AMA was selected since it is an APA inhibitor that is used by many investigators to study Ang II metabolism [26]. GluP was selected since it has recently received much attention as a direct APA inhibitor with a better selectivity for APA [28]. This enzyme was selected as a target for inhibition since it is the major enzyme involved in Ang II conversion to Ang III [26, 29].

In these studies, we showed that Ang III and Ang II had similar effects to induce ERK1/2 and SAPK/JNK MAP kinases phosphorylation, effects that we have previously observed [9, 10]. Blocking the ability of APA to convert Ang II to Ang III did not significantly affect the ability of Ang II to induce phosphorylation of these MAP kinases. In addition, both Ang II and Ang III induced astrocyte proliferation although Ang II was more potent, an effect we have observed previously. Further, blocking the activity of APA failed to prevent the proliferative effects of both peptides. Overall, our findings suggest that Ang II has direct effects to stimulate MAP kinases and to induce astrocyte growth since preventing its conversion to Ang III did not affect the ability of the peptide to induce MAP kinase phosphorylation or its ability to cause astrocyte proliferation. These findings fail to support the Ang III hypothesis and suggest that in astrocytes, Ang II has agonistic and mitogenic properties of its own. Most importantly, these studies also support a role for Ang III as a central peptide.

Ang III agonistic properties were observed a number of years ago [30]; however, the relevance of this peptide as a key player in the renin angiotensin system is not yet appreciated. In astrocytes, our studies support mitogenic properties of Ang III since it directly activates ERK1/2 and SAPK/JNK MAP kinases and causes astrocyte growth [9, 10]. Most importantly, Ang III-induced astrocyte proliferation occurred via the MAP kinase pathways suggesting an important role for these pathways in Ang III effects [9, 10]. It is notable that in most instances, Ang III agonistic effects in the brain are similar to Ang II. Both peptides have been shown to induce similar central-mediated increases in blood pressure or mean arterial pressure [30, 31] and equivalent central effects on thirst and sodium appetite [32]. Centrally administered Ang III was as potent as Ang II in causing pressor and renal effects in rats on normal and high sodium

diets [33]. Ang II and Ang III induced the central expression of the transcription factors c-Fos, c-Jun, and Krox-24 with the same efficacy [34]. Peripherally, Ang III was equipotent to Ang II with respect to blood pressure increases, aldosterone secretion, and renal functions [35]. On the other hand, we have shown that the ability of Ang III to cause proliferation of astrocytes was differentiated from Ang II, exhibiting a similar effect as Ang II at lower doses. At the higher doses of the peptides, astrocytes were more responsive to Ang II [10]. Thus, our studies and others have shown that Ang II and Ang III may have equivalent effects on several physiological processes but differential effects on others. Overall, our findings suggest a relevant physiological role of both Ang II and Ang III in the body, in particular in the brain.

It has also been suggested that the APA inhibitors may be acting as agonist/antagonist of the $AT_1$ receptor [26]. Thus, in the presence of the inhibitors, the $AT_1$ receptor responses would be modulated. However, in our hands, pretreating with the APA inhibitors had no effect on both Ang II and Ang III to induce the MAP kinases phosphorylation, and they did not affect the proliferative effects of both peptides. Since both Ang II and Ang III responses are mediated by interaction with the $AT_1$ receptor, these inhibitors are apparently not acting in an agonist/antagonist capacity in our system. The specificity and the efficacy of the APA inhibitors used in this study may be questionable and/or controversial. However, our findings are credible since most of the studies that are used to support the Ang III Hypothesis use an APA inhibitor (mostly AMA) to prevent Ang II conversion to Ang III [14, 22], suggesting that the results of our current study are directly comparable to others.

Even though a central renin angiotensin system is widely accepted, there are still few controversies that surround its existence, a major one being the Ang III Hypothesis. Our findings suggest that in brainstem and cerebellum astrocytes, both Ang II and Ang III are direct potent central mitogens and that their activities are unaffected by APA inhibitors. Thus, *in vitro*, these findings suggest that both peptides are important modulators of the actions of the brain renin angiotensni system. The relevance of these findings must be corroborated *in vivo* since cell cultures cannot give an overall picture of all actions of these peptides in the brain. It is important that we establish the major peptides that govern the actions of the central renin angiotensin system due to the significance of this system in many physiological and disease processes. Thus, our findings are essential in establishing the relevance of both Ang II and Ang III as direct acting endogenous central peptides.

## Conflict of Interests

The authors declare that they have no conflict of interests.

## Acknowledgments

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## References

[1] T. Force and J. V. Bonventre, "Growth factors and mitogen-activated protein kinases," *Hypertension*, vol. 31, no. 1, pp. 152–161, 1998.

[2] S. Mii, R. A. Khalil, K. G. Morgan, J. A. Ware, and K. C. Kent, "Mitogen-activated protein kinase and proliferation of human vascular smooth muscle cells," *American Journal of Physiology*, vol. 270, no. 1, pp. H142–H150, 1996.

[3] R. M. Touyz and E. L. Schiffrin, "Signal transduction in hypertension: part I," *Current Opinion in Nephrology and Hypertension*, vol. 2, no. 1, pp. 5–16, 1993.

[4] R. M. Touyz, M. El Mabrouk, G. He, X. H. Wu, and E. L. Schiffrin, "Mitogen-activated protein/extracellular signal-regulated kinase inhibition attenuates angiotensin II-mediated signaling and contraction in spontaneously hypertensive rat vascular smooth muscle cells," *Circulation Research*, vol. 84, no. 5, pp. 505–515, 1999.

[5] M. Clark, "Angiotensin II activates mitogen-activated protein kinases and stimulates growth in rat medullary astrocytes," *The FASEB Journal*, Article ID A1169, 2001.

[6] M. A. Clark and N. Gonzalez, "Angiotensin II stimulates rat astrocyte mitogen-activated protein kinase activity and growth through EGF and PDGF receptor transactivation," *Regulatory Peptides*, vol. 144, no. 1–3, pp. 115–122, 2007.

[7] M. A. Clark, G. Guillaume, and H. C. Pierre-Louis, "Angiotensin II induces proliferation of cultured rat astrocytes through c-Jun N-terminal kinase," *Brain Research Bulletin*, vol. 75, no. 1, pp. 101–106, 2008.

[8] M. A. Clark and N. Gonzalez, "Src and Pyk2 mediate angiotensin II effects in cultured rat astrocytes," *Regulatory Peptides*, vol. 143, no. 1–3, pp. 47–55, 2007.

[9] M. A. Clark, C. Nguyen, and H. Tran, "Angiotensin III induces c-Jun N-terminal kinase leading to proliferation of rat astrocytes," *Neurochemical Research*, vol. 37, no. 7, pp. 1475–1481, 2012.

[10] M. A. Clark, H. Tran, and C. Nguyen, "Angiotensin III stimulates ERK1/2 mitogen-activated protein kinases and astrocyte growth in cultured rat astrocytes," *Neuropeptides*, vol. 45, no. 5, pp. 329–335, 2011.

[11] R. H. Abhold, M. J. Sullivan, J. W. Wright, and J. W. Harding, "Binding, degradation and pressor activity of angiotensins II and III after aminopeptidase inhibition with amastatin and bestatin," *Journal of Pharmacology and Experimental Therapeutics*, vol. 242, no. 3, pp. 957–962, 1987.

[12] S. Zini, M. C. Fournie-Zaluski, E. Chauvel, B. P. Roques, P. Corvol, and C. Llorens-Cortes, "Identification of metabolic pathways of brain angiotensin II and III using specific aminopeptidase inhibitors: predominant role of angiotensin III in the control of vasopressin release," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 93, no. 21, pp. 11968–11973, 1996.

[13] J. W. Wright, M. J. Sullivan, and J. W. Harding, "Dysfunction of central angiotensinergic aminopeptidase activity in spontaneously hypertensive rats," *Neuroscience Letters*, vol. 61, no. 3, pp. 351–356, 1985.

[14] A. Reaux, M. C. Fournie-Zaluski, C. David et al., "Aminopeptidase A inhibitors as potential central antihypertensive agents," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 96, no. 23, pp. 13415–13420, 1999.

[15] M. J. Ramírez-Expósito, J. M. Martínez-Martos, M. D. Mayas et al., "Oleate, linoleate and cholesterol differently modify aspartyl- and glutamyl-aminopeptidase activities in primary cultures of rat astrocytes," *Comparative Biochemistry and Physiology C*, vol. 128, no. 1, pp. 113–118, 2001.

[16] B. Stragier, D. De Bundel, S. Sarre et al., "Involvement of insulin-regulated aminopeptidase in the effects of the renin-angiotensin fragment angiotensin IV: a review," *Heart Failure Reviews*, vol. 13, no. 3, pp. 321–337, 2008.

[17] B. Stragier, S. Sarre, P. Vanderheyden et al., "Metabolism of angiotensin II is required for its *in vivo* effect on dopamine release in the striatum of the rat," *Journal of Neurochemistry*, vol. 90, no. 5, pp. 1251–1257, 2004.

[18] R. L. Stornetta, C. L. Hawelu-Johnson, P. G. Guyenet, and K. R. Lynch, "Astrocytes synthesize angiotensinogen in brain," *Science*, vol. 242, no. 4884, pp. 1444–1446, 1988.

[19] E. A. Tallant and J. T. Higson, "Angiotensin II activates distinct signal transduction pathways in astrocytes isolated from neonatal rat brain," *Glia*, vol. 19, no. 4, pp. 333–342, 1997.

[20] E. J. Freeman, G. M. Chisolm, C. M. Ferrario, and E. A. Tallant, "Angiotensin-(1–7) inhibits vascular smooth muscle cell growth," *Hypertension*, vol. 28, no. 1, pp. 104–108, 1996.

[21] M. A. Devynck, M. G. Pernollet, and P. G. Matthews, "Specific receptors for des-Asp1-angiotensin II ("angiotensin III") in rat adrenals," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 74, no. 9, pp. 4029–4032, 1977.

[22] J. W. Harding, D. Felix, M. J. Sullivan et al., "The pivotal role at angiotensin III in the brain angiotensin system," *Proceedings of the Western Pharmacology Society*, vol. 30, pp. 11–15, 1987.

[23] A. Reaux, M. C. Fournie-Zaluski, and C. Llorens-Cortes, "Angiotensin III: a central regulator of vasopressin release and blood pressure," *Trends in Endocrinology and Metabolism*, vol. 12, no. 4, pp. 157–162, 2001.

[24] J. W. Wright, E. Tamura-Myers, W. L. Wilson et al., "Conversion of brain angiotensin II to angiotensin III is critical for pressor response in rats," *American Journal of Physiology*, vol. 284, no. 3, pp. R725–R733, 2003.

[25] J. W. Harding and D. Felix, "The effects of the aminopeptidase inhibitors amastatin and bestatin on angiotensin-evoked neuronal activity in rat brain," *Brain Research*, vol. 424, no. 2, pp. 299–304, 1987.

[26] R. C. Speth and V. T. Karamyan, "The significance of brain aminopeptidases in the regulation of the actions of angiotensin peptides in the brain," *Heart Failure Reviews*, vol. 13, no. 3, pp. 299–309, 2008.

[27] R. J. Kokje, W. L. Wilson, T. E. Brown, V. T. Karamyan, J. W. Wright, and R. C. Speth, "Central pressor actions of aminopeptidase-resistant angiotensin II analogs: challenging the angiotensin III hypothesis," *Hypertension*, vol. 49, no. 6, pp. 1328–1335, 2007.

[28] V. T. Karamyan, R. Gadepalli, J. M. Rimoldi, and R. C. Speth, "Brain AT1 angiotensin receptor subtype binding: importance of peptidase inhibition for identification of angiotensin II as its endogenous ligand," *Journal of Pharmacology and Experimental Therapeutics*, vol. 331, no. 1, pp. 170–177, 2009.

[29] J. W. Wright, S. Mizutani, and J. W. Harding, "Focus on brain angiotensin III and aminopeptidase A in the control of hypertension," *International Journal of Hypertension*, vol. 2012, Article ID 124758, 12 pages, 2012.

[30] J. R. Blair-West, K. D. Carey, D. A. Denton, L. J. Madden, R. S. Weisinger, and R. E. Shade, "Possible contribution of brain angiotensin III to ingestive behaviors in baboons," *American Journal of Physiology*, vol. 281, no. 5, pp. R1633–R1636, 2001.

[31] C. J. Tseng, L. L. Chou, L. P. Ger, and C. S. Tung, "Cardiovascular effects of angiotensin III in brainstem nuclei of normotensive and hypertensive rats," *Journal of Pharmacology and Experimental Therapeutics*, vol. 268, no. 2, pp. 558–564, 1994.

[32] W. L. Wilson, B. P. Roques, C. Llorens-Cortes, R. C. Speth, J. W. Harding, and J. W. Wright, "Roles of brain angiotensins II and III in thirst and sodium appetite," *Brain Research*, vol. 1060, no. 1-2, pp. 108–117, 2005.

[33] C. Y. Chen and W. C. Huang, "Pressor and renal effects of intracerebroventricularly administered angiotensins II and III in rats," *Kidney and Blood Pressure Research*, vol. 23, no. 2, pp. 95–105, 2000.

[34] A. Blume, C. Undeutsch, Y. Zhao, E. Kaschina, J. Culman, and T. Unger, "ANG III induces expression of inducible transcription factors of AP-1 and Krox families in rat brain," *American Journal of Physiology*, vol. 289, no. 3, pp. R845–R850, 2005.

[35] I. Gammelgaard, S. Wamberg, and P. Bie, "Systemic effects of angiotensin III in conscious dogs during acute double blockade of the renin-angiotensin-aldosterone-system," *Acta Physiologica*, vol. 188, no. 2, pp. 129–138, 2006.

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*Research Article*

# The Brain-Heart Connection: Frontal Cortex and Left Ventricle Angiotensinase Activities in Control and Captopril-Treated Hypertensive Rats—A Bilateral Study

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The model of *neurovisceral integration* suggests that the frontal cortex (FC) and the cardiovascular function are reciprocally and asymmetrically connected. We analyzed several angiotensinase activities in the heart left ventricle (VT) of control and captopril-treated SHR, and we search for a relationship between these activities and those determined in the left and right FC. Captopril was administered in drinking water for 4 weeks. Samples from the left VT and from the left and right FC were obtained. Soluble and membrane-bound enzymatic activities were measured fluorometrically using arylamides as substrates. The weight of heart significantly decreased after treatment with captopril, mainly, due to the reduction of the left VT weight. In the VT, no differences for soluble activities were observed between control and treated SHR. In contrast, a generalized significant reduction was observed for membrane-bound activities. The most significant correlations between FC and VT were observed in the right FC of the captopril-treated group. The other correlations, right FC versus VT and left FC versus VT in controls and left FC versus VT in the captopril group, were few and low. These results confirm that the connection between FC and cardiovascular system is asymmetrically organized.

## 1. Introduction

Frontal cortex (FC) and cardiovascular functions are reciprocally connected, as part of the model of *neurovisceral integration* [1]. This connection is asymmetric [2] and a neurochemical substrate may underlie this lateralization [3]. Compared with vehicle-treated spontaneously hypertensive rats (SHRs), we recently reported an inverted bilateral behavior of angiotensinase activities between left/right FC and plasma after captopril treatment. The asymmetries between left and right FC markedly increased compared to the control group. We suggested that these results might reflect a systematized lateralized neuroendocrine response between brain and cardiovascular functions involving the autonomic nervous system [4]. There are evidences suggesting that the hyperactivity of the sympathetic nervous system is involved in the cardiac pathologies related to neurological accidents such as cerebral infarction or head traumas, contributing to their high mortality rates [5]. Similarly, it has been also proposed that the autonomic imbalance in which the sympathetic nervous system predominates over the parasympathetic may be the pathway that connects impaired cognitive processes involving frontal cortex functions and altered heart functions [6, 7]. In addition, it was reported that unilateral prefrontal cortex lesions can alter emotional and cardiovascular autonomic responses, depending on which hemisphere was injured: there was a predominant parasympathetic activation by the left prefrontal cortex but a sympathetic inhibition by the right prefrontal cortex [8]. Aspartyl- (AspAP), glutamyl- (GluAP), alanyl- (AlaAP), and cystinyl-aminopeptidase (CysAP) are aminopeptidases (AP) involved in the metabolism of angiotensin peptides [9]. Based on these evidences of neuroendocrine correlations between brain and cardiovascular function and on our previous report showing

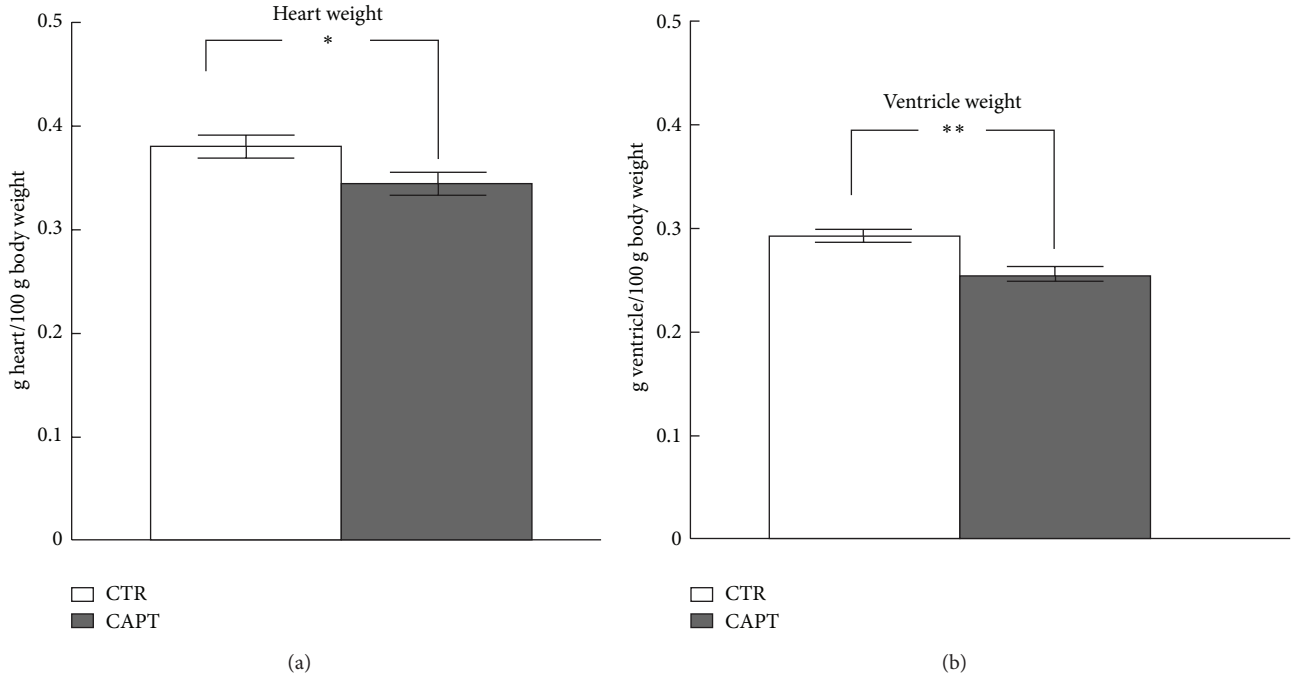


Figure 1: Total heart and left ventricle weight (g) in control (CTR) and captopril- (CAPT-) treated animals, as measured at the end of the treatment period (four weeks). The values represent the mean ± SEM of 10 animals in each group. $^{*}P < 0.05$; $^{**}P < 0.01$.

an asymmetrical effect of captopril between FC and plasma [4], it is therefore essential to analyze those angiotensinase activities in the heart ventricle (VT) of control and captopril-treated hypertensive rats and to search for a possible relationship between these activities in VT and the same determined in the left and right FC.

## 2. Material and Methods

All of the experimental procedures involving animals were performed in accordance with the European Communities Council Directive 86/609/EEC and were approved by the Bioethics Committee of the University of Jaén. Twenty adult male SHRs were divided into control ($n = 10$) and captopril-treated ($n = 10$) groups. Captopril (100 mg/kg p.o.) was administered daily in drinking water (0.5 mL/100 mg body weight) for 4 weeks. The systolic blood pressure (SBP) was monitored by the plethysmographic method throughout the experimental period. At the end of the treatment period, after recording the SBP, each rat was perfused with saline under equithesin anesthesia, and the left and right FC and samples from the left VT were obtained as previously described [4, 10]. Briefly, the brain samples were dissected according to the stereotaxic atlas of Paxinos and Watson [11]. For each group, the left and right frontal lobes 11.20 mm anterior to the interaural line were collected separately [12]. In addition, the heart was removed and weighed and the left ventricle was immediately dissected and weighed and a left ventricular sample was obtained. Soluble (SOL) and membrane-bound (MB) Aspartyl- (AspAP), glutamyl- (GluAP), alanyl- (AlaAP), and cystinyl-aminopeptidase (CysAP) activities were measured fluorometrically using acrylamides as substrates, as previously described [4]. Student's $t$-test was used to compare the data from control and captopril-treated SHRs, and the paired Student's $t$-test was used for left FC versus right FC comparisons [4]. The Pearson correlation coefficient of the left or right frontal cortex and plasma AP activities was computed using SPSS13.0 and STATA 90. $P$ values below 0.05 were considered significant.

## 3. Results

The results of the present research are reported in Figures 1, 2, and 3 and in Table 1. The SBP of captopril-treated SHRs was 47 mm Hg (or 30%) lower than that of control rats ($P < 0.001$) [4]. The weight of total heart decreased significantly after captopril treatment ($P < 0.05$) mainly due to a reduction in the left ventricle weight ($P < 0.01$) (Figure 1). In a previous study [4], we observed that the asymmetries for MB activities markedly increased in frontal cortex after captopril treatment compared to the control group, whereas the bilateral pattern (left versus right differences) of SOL activities did not substantially change. There was a left predominance for GluAP but a right one for AlaAP and CysAP [4].

In the present study, no differences for SOL GluAP, AlaAP, and CysAP activities were observed between the control and treated SHRs in the ventricle (Figure 2). No detectable activity was measured for SOL AspAP. However, a generalized significant reduction ($P < 0.05$) was observed for all MB AP activities ($P < 0.05$) after captopril except for AspAP that

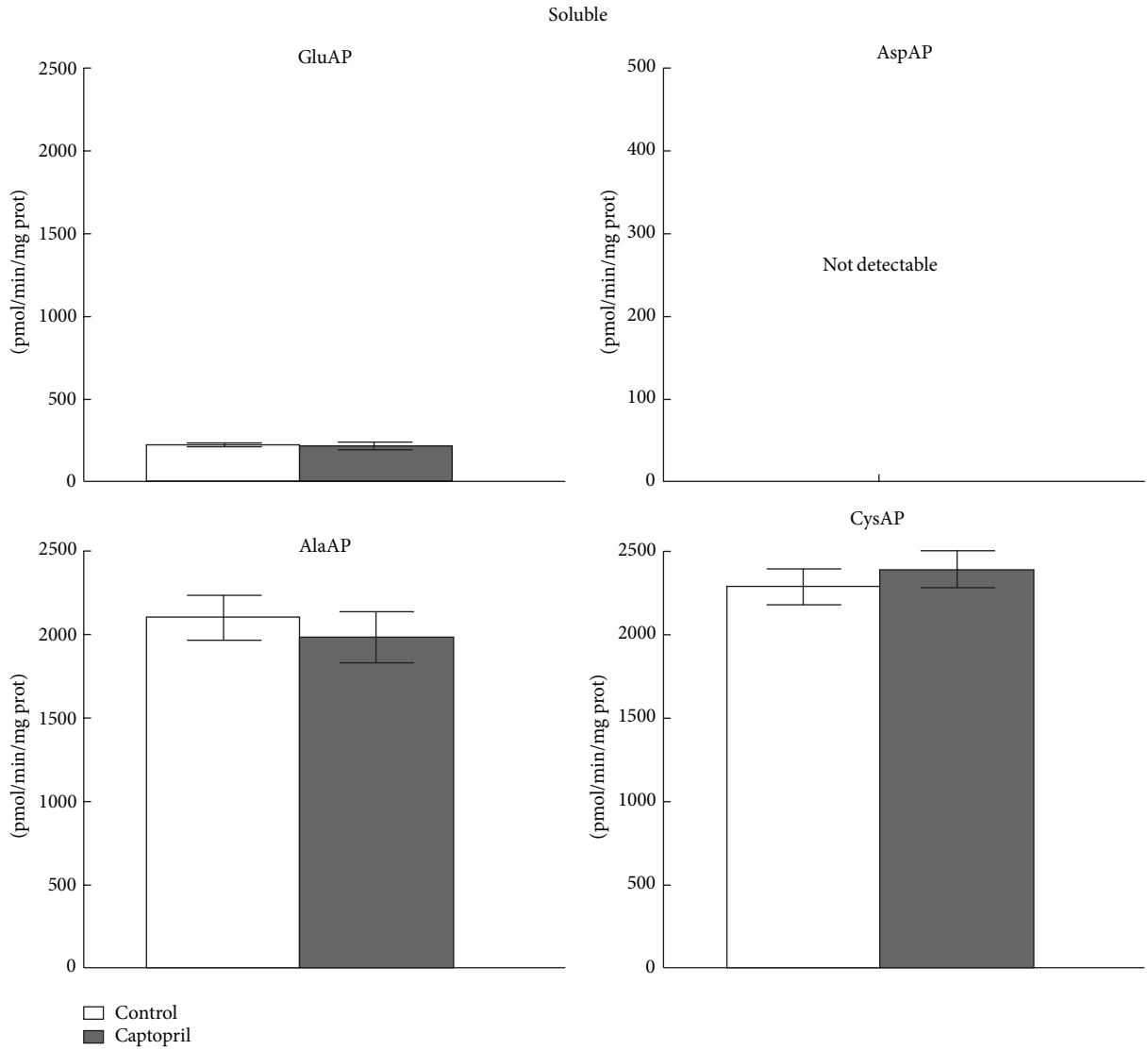


Figure 2: Soluble GluAP, AlaAP, and CysAP activities on the left ventricle of control ($n$ = 10) and captopril-treated ($n$ = 10) spontaneously hypertensive rats. The values represent the mean ± SEM of specific GluAP, AlaAP, and CysAP activities expressed as picomoles of glutamyl-, alanyl- or cystinyl-$\beta$-naphthylamide hydrolyzed per min per mg of protein.

did not reach statistical significance versus the control group (Figure 3).

There are correlations between FC and VT: the majority and most significant correlations were observed with the right FC of the captopril-treated group. Interestingly, the correlations involving MB activities of the right FC were negative for GluAP (left FC predominant) and positive for AlaAP and CysAP (right FC predominant). The other correlations, right FC versus VT and left FC versus VT in controls and left FC versus VT in the captopril group, were few and low. Surprisingly, the opposite was previously observed between FC and plasma [4]. The plasma AP activities correlated significantly with those in the right FC in the control rats, whereas they correlated with activities in the left FC in the captopril-treated group.

## 4. Discussion

Our results demonstrated that captopril modified angiotensinase activities in heart and in brain, as previously reported [4], and that there is a significant correlation in the levels of these enzymatic activities between both organs.

Several components of a local cardiac RAS can result of an uptake from the circulation but a functional intracrine RAS in the heart also exists [13]. Moreover, ganglionic neurons in human heart express angiotensinogen and are able to generate Ang II [14]. As a whole, the local heart RAS might be involved in cellular hypertrophy and cardiac arrhythmias as well as in the regulation of heart cell volume through the action of Ang II on the $AT_1$ receptor [15]. In addition, the presence of Ang IV in the left ventricle was previously

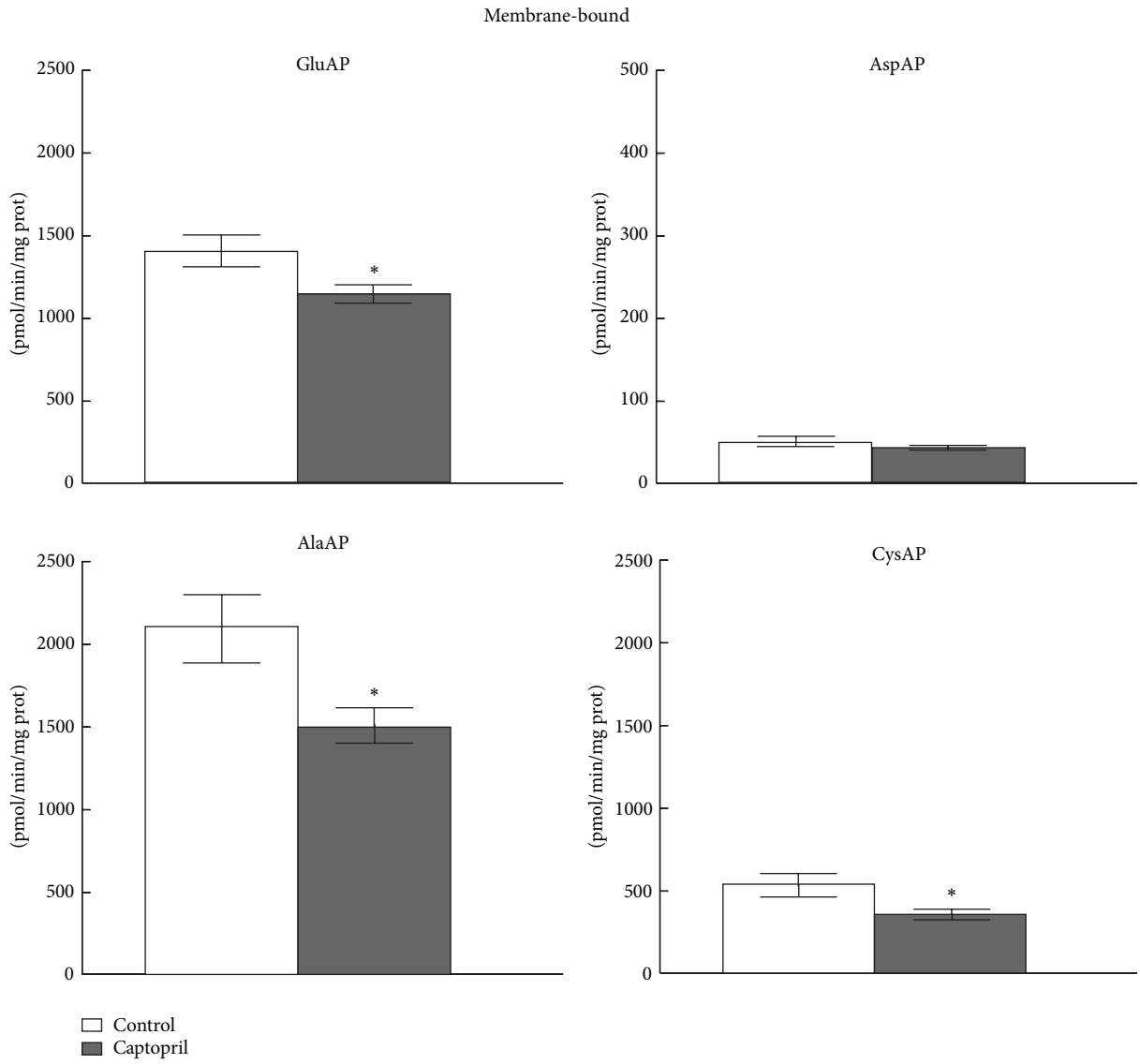


Figure 3: Membrane-bound GluAP, AspAP, AlaAP, and CysAP activities in the left ventricle of control ($n = 10$) and captopril-treated ($n = 10$) spontaneously hypertensive rats. The values represent the mean ± SEM of specific GluAP, AspAP, AlaAP, and CysAP activities expressed as picomoles of glutamyl-, aspartyl-, alanyl-, or cystinyl-$\beta$-naphthylamide hydrolyzed per min per mg of protein. $^{*}P < 0.05$.

described [16]. It was also observed that there was 10-fold more $AT_4$ receptor than $AT_1$ receptor in rabbit myocardium where they might exert opposite effects to those of Ang II through the binding of Ang IV [16].

The present results of lower MB aminopeptidase activities in ventricle may indicate a local reduction in the metabolism of angiotensin peptides after treatment with captopril. More specifically, the results suggest a lower metabolism/higher availability of Ang II (metabolized to Ang III by GluAP), Ang III (metabolized to Ang IV by AlaAP), and Ang IV (also metabolized by AlaAP) in captopril-treated animals. On the other hand, Ang IV binds specifically to the $AT_4$ receptor, which was proposed to be identical to the insulin-regulated aminopeptidase (IRAP) [17]. Indeed, the high affinity-binding site for Ang IV is absent in IRAP-KO mice [18], and the brains of these mice are protected against ischemic damage as Ang IV does in control animals [19]. Cystein aminopeptidase (CysAP), also called oxytocinase or vasopressinase (EC 3.4.11.3), is considered the human variant of IRAP [20]. These enzymes can therefore be considered identical. However, the identity of the $AT_4$ receptor remains in dispute, and its identification with IRAP is still controversial. Indeed, Ang IV has a very rapid effect on signaling molecules [21] at pico/nanomolar concentration, whereas the effect of Ang IV on enzyme inhibition such as on accumulation of endogenous IRAP substrates is slow. Also, the concentration of Ang IV producing a biological effect is below that needed to inhibit IRAP [22]. It was therefore proposed that the physiological action of Ang IV was mediated through the tyrosine-kinase cMet receptor whose function overlap with those of Ang IV/$AT_4$, that is, memory facilitation, cerebroprotection, seizure, neurite

Table 1: Frontal cortex versus ventricle.

| Left frontal cortex | | | Right frontal cortex | | |
|---|---|---|---|---|---|
| FC versus VT | $r$ | $P$ | FC versus VT | $r$ | $P$ |
| | | | Control | | |
| SOL GluAP versus MB CysAP | −0.733 | 0.03 | | | |
| MB GluAP versus SOL AlaAP | +0.703 | 0.05 | No correlations | | |
| MB GluAP versus MB GluAP | +0.693 | 0.05 | | | |
| | | | Captopril | | |
| MB GluAP versus MB CysAP | +0.766 | 0.02 | SOL CysAP versus SOL AlaAP | +0.700 | 0.05 |
| | | | SOL GluAP versus SOL AlaAP | +0.722 | 0.04 |
| | | | SOL AlaAP versus MB CysAP | −0.749 | 0.03 |
| | | | SOL GluAP versus MB CysAP | −0.781 | 0.02 |
| | | | MB CysAP versus MB AlaAP | +0.692 | 0.05 |
| | | | MB AlaAP versus MB AspAP | +0.805 | 0.02 |
| | | | MB GluAP versus MB AspAP | −0.870 | 0.01 |
| | | | MB CysAP versus MB CysAP | +0.695 | 0.05 |
| | | | MB AlaAP versus MB GluAP | +0.742 | 0.03 |
| | | | MB CysAP versus MB GluAP | +0.707 | 0.04 |

Correlations between the left or right frontal cortex (FC) soluble (SOL) or membrane-bound (MB) AP activities versus SOL or MB left ventricle (VT) AP activities in the control and captopril-treated animals. Pearson's correlation coefficients ($r$) and $P$ values are indicated and specify the significance of the differences between these correlations.

outgrowth, cerebral blood flow, depression, and Parkinson's and Alzheimer's diseases [22].

Independently of the discussion on the real nature of the Ang IV binding site, it was proposed that the binding of Ang IV to its receptor results in the inhibition of the receptor's metabolic activity, reducing the catabolism of its substrates and consequently increasing their availability and extending their action [20]. Ang IV could therefore regulate glucose uptake modulating CysAP activity: CysAP is indeed colocalized with the glucose transporter GLUT4. In the presence of insulin, CysAP and GLUT4 are expressed in the plasma membrane, where GLUT4 induces glucose uptake [20]. Therefore, a decrease in CysAP activity as observed here would imply high levels of Ang IV and increased glucose uptake, which would also improve heart function [23].

These results are in agreement with previous reports indicating that the left ventricular expression of the glucose transporter GLUT4 is regulated by insulin at the transcriptional level [24] and that captopril increased myocardial oxygen and glucose uptake [25]. Therefore, considering these possibilities, the general reduction in the metabolism of Ang peptides in ventricle under captopril treatment might lead to a situation in which Ang peptides counteract each other resulting in an improvement of cardiovascular function.

As previously stated, brain asymmetry is not a static but rather a dynamic phenomenon in which both environmental and endogenous factors act as modulators [3]. In addition, this asymmetry extends to the neurovisceral integration through the asymmetrical functioning of the autonomic nervous system [4, 26–28]. For example, unilateral brain manipulations may not only produce alterations in brain bilaterality [27] but also have significant asymmetrical peripheral consequences in plasma and heart function [26, 28]. In addition, drug treatments may also influence brain bilaterality acting directly and/or indirectly through mechanisms involving the neurovisceral integration model and the asymmetrical functioning of the autonomic nervous system [4]. This hypothesis may agree with the data observed by Chi et al. in 2003 [29] demonstrating that "several genes critical in the establishment of left-right asymmetry were expressed preferentially in venous endothelial cells, suggesting coordination between vascular differentiation and body plan development." The authors highlighted the importance of the similarity of the migration paths of blood vessels and nerves during development, which reflects their mutual functional interaction [29].

It is particularly interesting that the present results are in contrast with those previously observed in plasma [4] where, in the control rats, the plasma AP activities correlated significantly with those in the right FC, whereas they correlated with those in the left FC in the captopril-treated group. These results strongly support that such responses were due to an asymmetry in the organization of the autonomic nervous system's innervations of the blood vessels and heart [4, 26, 28]. Remarkably, the peripheral autonomic innervation not only uses the classic autonomic neurotransmitters acetyl-choline or norepinephrine but also other neuropeptides including angiotensin II that colocalize with them [30]. Thus, neuronal Ang II acts as a neuropeptide and synaptic cotransmitter in the periphery of the sympathetic nervous system [30]. In addition, the present results agree with the notion that essentially the right cortical activity regulates cardiovascular function [2]. Captopril influences heart [31] and brain [32] function and is a clear choice for the treatment of heart failure [33]. In addition, heart function [1] and heart failure [5] affect brain function, and, vice versa, left or right stroke differently influences the heart function [28]. Indeed, the stimulation of the left insular cortex in epileptic patients

before temporal lobectomy causes bradycardia and depressor responses, whereas the opposite happens after stimulation of the right cortex indicating a left parasympathetic and a right orthosympathetic dominance, respectively [34]. Cardiac autonomic derangements and arrhythmias have been mainly described in patients suffering right-sided stroke with insular involvement [35]. A reduced respiratory heart rate variability, a reflex mainly under parasympathetic control, was associated with increased mortality after right-sided stroke, suggesting that the risk of sudden death may be correlated with lateralization and the location of the brain infarct after stroke [36]. These and other evidences have led some authors to recommend a prolonged and intensive cardiovascular monitoring in patients manifesting right-sided stroke [37].

There is therefore a clear reciprocal interaction between heart and brain in physiological and pathological conditions [1, 5] that may be modulated by captopril as suggested by the present data. The brain RAS participates in the development of cardiac hypertrophy and fibrosis through the modulation of the autonomic nervous system. Furthermore, the inhibition of the sympathetic hyperactivity after myocardial infarction through suppression of the brain RAS appears to have a beneficial effect [38]. The blood-brain barrier permeability of captopril is negligible, but it has a marked effect on cerebral blood flow autoregulation when injected intravenously [39]. In addition, it causes a sympathetic inhibition in SHRs after chronic administration suggesting that ACE inhibition may be protective for cerebral metabolism against ischemic insult [40]. Therefore, our present results suggest that captopril may centrally modulate the function of the autonomic nervous system leading to a higher connectivity between the right FC and the left ventricle. This could be particularly beneficial after a right-sided stroke.

The asymmetrical organization of the nervous system is the result of evolutionary adaptation [3, 41]. This is a dynamic phenomenon in which both environmental and endogenous physiological or pathologic factors act as modulators [3]. It has been hypothesized that the brain is intrinsically asymmetric. Virtually all brain functions are organized, more or less, in an asymmetrical fashion. It was speculated that imbalances in established brain asymmetries (toward symmetry or toward increasing asymmetry) might lead to neuropathological deviations in the functions of the nervous system, including the peripheral consequences due to changes in the modulation of the autonomic nervous system [3, 42, 43]. A deeper knowledge of how deviations in physiological lateralizations may lead to neuropathological consequences and how some exogenous factors may influence in physiological laterality, is important in order to be able to search for therapeutic tools that balance those deviations.

Captopril asymmetrically modifies angiotensinase activities in frontal cortex and in ventricle and, consequently, affects the functions they are involved in. However, these effects are not independent but according to our results, mutually interdependent. Therefore, but when appropriately characterized and systematized, the present results open new therapeutic consequences in cardiovascular treatment (presumably not only limited to specific drugs) that should be taken into account in the design of protocols for the evaluation of cardiovascular treatments. The present study provides further molecular support for the involvement of the RAS in the brain frontal cortex-heart connection and adds, for the first time, biochemical evidence on the lateralized functioning of that connection. If this connection is asymmetric and the consequences of unilateral brain insults differ depending on the injured side, the necessity of characterizing appropriately the neurovisceral integration of the RAS is relevant and deserves further preclinical work.

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## References

[1] J. F. Thayer and R. D. Lane, "Claude Bernard and the heart-brain connection: further elaboration of a model of neurovisceral integration," *Neuroscience and Biobehavioral Reviews*, vol. 33, no. 2, pp. 81–88, 2009.

[2] P. S. Foster and D. W. Harrison, "Magnitude of cerebral asymmetry at rest: covariation with baseline cardiovascular activity," *Brain and Cognition*, vol. 61, no. 3, pp. 286–297, 2006.

[3] M. Ramírez, I. Prieto, F. Vives, M. de Gasparo, and F. Alba, "Neuropeptides, neuropeptidases and brain asymmetry," *Current Protein and Peptide Science*, vol. 5, no. 6, pp. 497–506, 2004.

[4] A. B. Segarra, I. Prieto, I. Banegas et al., "Asymmetrical effect of captopril on the angiotensinase activity in frontal cortex and plasma of the spontaneously hypertensive rats: expanding the model of neuroendocrine integration," *Behavioural Brain Research*, vol. 230, no. 2, pp. 423–427, 2012.

[5] M. A. Samuels, "The brain-heart connection," *Circulation*, vol. 116, no. 1, pp. 77–84, 2007.

[6] J. F. Thayer and J. F. Brosschot, "Psychosomatics and psychopathology: looking up and down from the brain," *Psychoneuroendocrinology*, vol. 30, no. 10, pp. 1050–1058, 2005.

[7] J. F. Thayer and R. D. Lane, "The role of vagal function in the risk for cardiovascular disease and mortality," *Biological Psychology*, vol. 74, no. 2, pp. 224–242, 2007.

[8] M. J. Hilz, O. Devinsky, H. Szczepanska, J. C. Borod, H. Marthol, and M. Tutaj, "Right ventromedial prefrontal lesions result in paradoxical cardiovascular activation with emotional stimuli," *Brain*, vol. 129, no. 12, pp. 3343–3355, 2006.

[9] M. Ramírez, I. Prieto, F. Alba, F. Vives, I. Banegas, and M. de Gasparo, "Role of central and peripheral aminopeptidase activities in the control of blood pressure: a working hypothesis," *Heart Failure Reviews*, vol. 13, no. 3, pp. 339–353, 2008.

[10] I. Prieto, F. Hermoso, M. De Gasparo et al., "Aminopeptidase activity in renovascular hypertension," *Medical Science Monitor*, vol. 9, no. 1, pp. BR31–BR36, 2003.

[11] G. Paxinos and C. Watson, *The Rat Brain in Stereotaxic Coordinates*, Academic Press, London, UK, 4th edition, 1998.

[12] A. B. Segarra, J. I. Ruiz-Sanz, M. B. Ruiz-Larrea et al., "The profile of fatty acids in frontal cortex of rats depends on the type of fat used in the diet and correlates with neuropeptidase activities," *Hormone and Metabolic Research*, vol. 43, no. 2, pp. 86–91, 2011.

[13] W. C. De Mello and E. D. Frohlich, "On the local cardiac renin angiotensin system. Basic and clinical implications," *Peptides*, vol. 32, no. 8, pp. 1774–1779, 2011.

[14] J. Patil, S. Stucki, J. Nussberger et al., "Angiotensinergic and noradrenergic neurons in the rat and human heart," *Regulatory Peptides*, vol. 167, no. 1, pp. 31–41, 2011.

[15] G. O. A. Naik, G. W. Moe, and P. W. Armstrong, "Specific and non-specific measurements of tissue angiotensin II cascade members," *Journal of Pharmaceutical and Biomedical Analysis*, vol. 24, no. 5-6, pp. 947–955, 2001.

[16] B. K. Slinker, Y. Wu, A. J. Brennan, K. B. Campbell, and J. W. Harding, "Angiotensin IV has mixed effects on left ventricle systolic function and speeds relaxation," *Cardiovascular Research*, vol. 42, no. 3, pp. 660–669, 1999.

[17] A. L. Albiston, S. G. McDowall, D. Matsacos et al., "Evidence that the angiotensin IV (AT(4)) receptor is the enzyme insulin-regulated aminopeptidase," *Journal of Biological Chemistry*, vol. 276, no. 52, pp. 48623–48626, 2001.

[18] A. L. Albiston, R. N. Fernando, H. R. Yeatman et al., "Gene knockout of insulin-regulated aminopeptidase: loss of the specific binding site for angiotensin IV and age-related deficit in spatial memory," *Neurobiology of Learning and Memory*, vol. 93, no. 1, pp. 19–30, 2010.

[19] V. Pham, A. L. Albiston, C. E. Downes et al., "Insulin-regulated aminopeptidase deficiency provides protection against ischemic stroke in mice," *Journal of Neurotrauma*, vol. 29, no. 6, pp. 1243–1248, 2012.

[20] B. Stragier, D. De Bundel, S. Sarre et al., "Involvement of insulin-regulated aminopeptidase in the effects of the renin-angiotensin fragment angiotensin IV: a review," *Heart Failure Reviews*, vol. 13, no. 3, pp. 321–337, 2008.

[21] Y. D. Li, E. R. Block, and J. M. Patel, "Activation of multiple signaling modules is critical in angiotensin IV-induced lung endothelial cell proliferation," *American Journal of Physiology*, vol. 283, no. 4, pp. L707–L716, 2002.

[22] J. W. Wright and J. W. Harding, "Brain renin-angiotensin-A new look at an old system," *Progress in Neurobiology*, vol. 95, no. 1, pp. 49–67, 2011.

[23] M. Ramírez, I. Banegas, A. B. Segarra et al., "Bilateral distribution of oxytocinase activity in the medial prefrontal cortex of spontaneously hypertensive rats with experimental hemiparkinsonism," in *Mechanisms in Parkinson's Disease—Models and Treatments*, J. Dushanova, Ed., InTech, 2012.

[24] S. Petersen, M. Bähr, and J. Eckel, "Insulin-dependent regulation of Glut4 gene expression in ventricular cardiomyocytes: evidence for a direct effect on Glut4 transcription," *Biochemical and Biophysical Research Communications*, vol. 213, no. 2, pp. 533–540, 1995.

[25] F. Randsbaek, H. H. Kimose, T. Bjerre, U. Møldrup, H. E. Bøtker, and T. T. Nielsen, "Captopril-induced glutamate release at the start of reperfusion after cold cardioplegic storage of pig hearts," *Journal of Thoracic and Cardiovascular Surgery*, vol. 119, no. 5, pp. 1030–1038, 2000.

[26] I. Banegas, I. Prieto, F. Vives et al., "Asymmetrical response of aminopeptidase A and nitric oxide in plasma of normotensive and hypertensive rats with experimental hemiparkinsonism," *Neuropharmacology*, vol. 56, no. 3, pp. 573–579, 2009.

[27] I. Banegas, I. Prieto, F. Vives et al., "Lateralized response of oxytocinase activity in the medial prefrontal cortex of a unilateral rat model of Parkinson's disease," *Behavioural Brain Research*, vol. 213, no. 2, pp. 328–331, 2010.

[28] I. Banegas, I. Prieto, A. B. Segarra et al., "Blood pressure increased dramatically in hypertensive rats after left hemisphere lesions with 6-hydroxydopamine," *Neuroscience Letters*, vol. 500, no. 2, pp. 148–150, 2011.

[29] J. T. Chi, H. Y. Chang, G. Haraldsen et al., "Endothelial cell diversity revealed by global expression profiling," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 100, no. 19, pp. 10623–10628, 2003.

[30] J. Bohlender and H. Imboden, "Angiotensinergic neurotransmission in the peripheral autonomic nervous system," *Frontiers in Bioscience*, vol. 17, no. 7, pp. 2419–2432, 2011.

[31] A. L. M. Swislocki, T. L. Kinney Lapier, D. T. Khuu, K. Y. Fann, M. Tait, and K. J. Rodnick, "Metabolic, hemodynamic, and cardiac effects of captopril in young, spontaneously hypertensive rats," *American Journal of Hypertension*, vol. 12, no. 6, pp. 581–589, 1999.

[32] J. J. Braszko, W. Karwowska-Polecka, D. Halicka, and P. R. Gard, "Captopril and enalapril improve cognition and depressed mood in hypertensive patients," *Journal of Basic and Clinical Physiology and Pharmacology*, vol. 14, no. 4, pp. 323–343, 2003.

[33] C. Demers, A. Mody, K. K. Teo, and R. S. McKelvie, "ACE inhibitors in heart failure: what more do we need to know?" *American Journal of Cardiovascular Drugs*, vol. 5, no. 6, pp. 351–359, 2005.

[34] S. M. Oppenheimer, A. Gelb, J. P. Girvin, and V. C. Hachinski, "Cardiovascular effects of human insular cortex stimulation," *Neurology*, vol. 42, no. 9, pp. 1727–1732, 1992.

[35] F. Colivicchi, A. Bassi, M. Santini, and C. Caltagirone, "Cardiac autonomic derangement and arrhythmias in right-sided stroke with insular involvement," *Stroke*, vol. 35, no. 9, pp. 2094–2098, 2004.

[36] H. K. Naver, C. Blomstrand, and B. Gunnar Wallin, "Reduced heart rate variability after right-sided stroke," *Stroke*, vol. 27, no. 2, pp. 247–251, 1996.

[37] R. T. F. Cheung and V. Hachinski, "Cardiac effects of stroke," *Current Treatment Options in Cardiovascular Medicine*, vol. 6, no. 3, pp. 199–207, 2004.

[38] L. A. Campos, M. Bader, and O. C. Baltatu, "Brain Renin-Angiotensin system in hypertension, cardiac hypertrophy, and heart failure," *Frontiers in Physiology*, vol. 2, article 115, 2011.

[39] D. I. Barry, O. B. Paulson, and J. O. Jarden, "Effects of captopril on cerebral blood flow in normotensive and hypertensive rats," *American Journal of Medicine*, vol. 76, no. 5, pp. 79–85, 1984.

[40] K. H. Berecek, K. A. Kirk, S. Nagahama, and S. Oparil, "Sympathetic function in spontaneously hypertensive rats after chronic administration of captopril," *American Journal of Physiology*, vol. 252, no. 4, part 2, pp. H796–H806, 1987.

[41] G. Vallortigara, "The evolutionary psychology of left and right: costs and benefits of lateralization," *Developmental Psychobiology*, vol. 48, no. 6, pp. 418–427, 2006.

[42] A. Samara and G. T. Tsangaris, "Brain asymmetry: both sides of the store," *Expert Review of Proteomics*, vol. 8, no. 6, pp. 693–703, 2011.

[43] M. E. Rentería, "Cerebral asymmetry: a quantitative, multifactorial, and plastic brain phenotype," *Twin Research and Human Genetics*, vol. 15, no. 3, pp. 401–413, 2012.

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*Review Article*

# Elevated Blood Pressure in the Acute Phase of Stroke and the Role of Angiotensin Receptor Blockers

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Raised blood pressure (BP) is common after stroke but its causes, effects, and management still remain uncertain. We performed a systematic review of randomized controlled trials that investigated the effects of the angiotensin receptor blockers (ARBs) administered in the acute phase (≤72 hours) of stroke on death and dependency. Trials were identified from searching three electronic databases (Medline, Cochrane Library and Web of Science Database). Three trials involving 3728 patients were included. Significant difference in BP values between treatment and placebo was found in two studies. No effect of the treatment was seen on dependency, death and vascular events at one, three or six months; the cumulative mortality and the number of vascular events at 12 months differed significantly in favour of treatment in one small trial which stopped prematurely. Evidence raises doubts over the hypothesis of a specific effect of ARBs on short- and medium-term outcomes of stroke. It is not possible to rule out that different drugs might have different effects. Further trials are desirable to clarify whether current findings are generalizable or there are subgroups of patients or different approaches to BP management for which a treatment benefit can be obtained.

## 1. Introduction

Raised blood pressure (BP) is common after acute stroke, whether of ischaemic or haemorrhagic type. It exists in more than three quarters of patients, of which about half have a history of hypertension [1], and it declines spontaneously in two-thirds of cases returning to prestroke levels over the first week. Its decrease usually occurs 4–10 days after stroke, but in a significant percentage of patients it falls by about 25–30% just within the first 24 hours; particularly when they are moved to a quiet room, they are allowed to rest and their bladder is empty [2].

Mechanisms and effects of elevated BP in this clinical setting have not been well understood. It might be attributable to either one more of the following conditions: preexisting, inadequately treated or undiagnosed hypertension, stress of hospitalization, raised intracranial pressure, haematoma expansion, damage to autonomic centers and abnormal baroceptor sensitivity, neuroendocrine response with activation of sympathetic nervous system, renin-angiotensin axis and/or glucocorticoid system, and myocardial changes [3–6].

Most of the studies, although not all, have found that high BP in the acute phase of stroke, whether measured as casual or 24 hours ambulatory readings, is associated with a poor outcome [7–9] and an increased risk of death and dependency [10–14]; a U-shaped relationship between BP values and outcome has been described in different studies [15–17]. Recent evidence suggests that not only BP but even its derived indices and other haemodynamic measures as mid blood pressure, mean arterial pressure, BP variability, heart rate, pulse pressure, and rate-pressure product are related to functional outcome [18–20]. The association is thought to be related to the early stroke recurrence and the development of cerebral edema and greater serious haemorrhagic transformation in ischaemic stroke [21, 22] and to the haematoma expansion in primary intracerebral haemorrhage [23].

While observational studies show that high BP is independently associated with a poor outcome, suggesting that it should be lowered, pathophysiology argues that lowering BP will reduce cerebral blood flow when cerebral autoregulatory mechanisms are impaired. Additionally, in acute ischaemic

stroke the infarcted brain tissue may be surrounded by a "penumbra" zone of underperfused but viable tissue where cerebral blood flow extremely depends on the systemic BP and collaterals until the occluded artery is recanalized. Lowering BP carries the risk of jeopardizing the perfusion of this area leading to an increase of brain infarction or perihematoma ischaemia. Spontaneous thrombolysis may also occur, and the ischaemic area may become hyperaemic; at this stage a very high BP might cause propagation of infarct-related brain oedema or haemorrhagic transformation of the infarct. Unfortunately there is no sure clinical correlate of spontaneous thrombolysis, and in routine clinical care it is not possible to judge when it is better to leave a very high BP untreated or when it is necessary to intervene.

In summary, there is still debate in whether, when and how high BP should be lowered (epidemiological evidence) or not (pathophysiological concerns). Different antihypertensive drug classes might have differential effects [24] considering both their action in lowering BP and specific organ effects: for example $\beta$-blockers might be detrimental [25], and the use of calcium channel blockers was associated with a worsening of outcome in some studies, especially those testing intravenous formulation [26], perhaps because of a reduction in cerebral perfusion.

Our purpose is to investigate through a research of the recent literature the effect of the angiotensin receptor blockers (ARBs) administered in the acute phase of stroke on death and dependency.

## 2. Methods

*2.1. Identification and Inclusion of Trials.* We used Medline (1985 to December 2011; any language) to identify randomized controlled trials of ARBs in patients within 72 hours of stroke. We selected trials of more than 100 patients which assessed the effect on death or dependency and recorded coronary heart disease events or stroke (irrespective of whether BP lowering was considered the mechanism of action). Search terms were "blood pressure lowering", "blood pressure reduction", "antihypertensive", or "hypertension" or "receptor, angiotensin/antagonists and inhibitors" or the name of all ARBs listed in the British National Formulary as keywords or text words. Limits were Medline publication type "clinical trial" or "controlled clinical trial" or "randomized controlled trial", or "meta-analysis". We also searched the Cochrane Collaboration and Web of Science databases and the citations in trials and meta-analysis. Randomized trials were included irrespective of participants' age, disease status, BP before treatment, and the use of other drugs. Confounded trials (in which ≥2 active treatment were compared in the absence of a control arm) were excluded.

*2.2. Blood Pressure and Outcome.* The authors extracted data independently from identified publications with respect to patients' number, sex, age, previous medical history, stroke subtype, time from stroke to enrollment, baseline BP, difference in BP between treatment and control groups, follow up period, grade of dependency, and death. Outcome events included stroke (all, fatal, and nonfatal), myocardial infarction (all), total vascular events (combined stroke, MI, and vascular death), and mortality (all-cause, vascular).

## 3. Results

*3.1. Trials.* Three randomized placebo-controlled multicenter studies fulfilled the inclusion criteria (Table 1), each of which had been published [27–29]. The combined sample size was 3728 with almost two-thirds of the data coming from one study (SCAST). In two studies (ACCESS and PRoFESS) all patients recruited had ischaemic stroke, while in the SCAST trial 85.4% of patients had ischaemic and 13.5% haemorrhagic stroke; mean time from stroke to enrollment varied from 17.6 to 57.6 hours, and recruitment was limited to patients with high BP. Main characteristics of each trial are summarized in Table 2.

In the ACCESS study, protocol considered a 7-day placebo controlled phase: treatment was started with 4 mg candesartan cilexetil daily or placebo on day 1; on day 2, dosage was increased to 8 or 16 mg candesartan cilexitil or placebo if BP exceeded 160 mmHg systolic or 100 mmHg diastolic; target was a 10–15% BP reduction within 24 hours. On day 7, a 24-hour BP profile was obtained in all patients: in those in the candesartan cilexitil group who showed a hypertensive profile (mean daytime BP > 135/85 mmHg) dosage was increased or an additional antihypertensive drug was added; in placebo arm, candesartan cilexitil was started in patients with hypertensive profile to lower BP to <140/90 mmHg (office BP) or < 135/85 mmHg (mean daytime BP), while those with a normotensive profile did not receive antihypertensive medication. Follow-up examinations were performed after 3, 6, and 12 months.

The PRoFESS study compared the effect of telmisartan (80 mg daily) versus placebo and combined aspirin (25 mg twice daily) and extended release dipyridamole (200 mg daily) versus clopidogrel (75 mg daily) in a 2 × 2 factorial design in patients with recent ischaemic stroke followed up for a mean duration of 30 months. The subgroup analysis reviewed included only patients randomized within 72 hours of stroke onset, evaluated at 1 and 3 months.

In the SCAST trial, patients with diagnosis of stroke (ischaemic or haemorrhagic) presenting within 30 hours of symptom onset were allocated in a 1 : 1 ratio to treatment with candesartan or placebo. There was a fixed-dose escalation scheme: 4 mg on day 1, 8 mg on day 2 and 16 mg on days 3–7. Subsequent evaluations took place on day 7 and at 1 and 6 months; to avoid important differences in treatment during followup, candesartan was the advised antihypertensive agent and was provided free of charge.

In each trial there were no significant differences regarding the use of concomitant medication on hospital admission or during follow-up between the active treatment and placebo groups.

*3.2. Baseline Findings.* In the ACCESS trial mean BP on hospital admission was 198/103 and on study onset 189/99; baseline severity of stroke was not directly reported but patients had a mean Barthel Index (BI) of 62; no data are

Table 1: Included trials.

| Study | Year | Study design | Antihypertensive agent |
|---|---|---|---|
| Acute Candesartan Cilexetil Therapy in Stroke Survivors (ACCESS) [27] | 2003 | Prospective, double-blind, placebo-controlled, randomized, multicenter phase II study. | Candesartan cilexitil |
| Prevention Regimen for Effectively Avoiding Second Strokes (PRoFESS) [28]* | 2008 | Prospective, double-blind, placebo-controlled, randomized, multicenter phase III study. | Telmisartan |
| The angiotensin-receptor blocker candesartan for treatment of acute stroke (SCAST) [29] | 2011 | Prospective, double-blind, placebo-controlled, randomized, multicenter phase III study. | Candesartan cilexitil |

*In the PRoFESS trial a subgroup analysis has been considered.

Table 2: Main characteristics of the included trials.

| Study | ACCESS [27] | PRoFESS [28]* | SCATS [29] |
|---|---|---|---|
| Main inclusion criteria | Motor deficit, cerebral CT scan excluding intracranial hemorrhage, onset of symptoms within 72 hours, necessity to treat hypertension according to current recommendations#. | Ischemic stroke within 72 hours from onset of symptoms, age older than 55 years or age 50–54 years if 2 additional vascular risk factors present, seated systolic BP 121 to 180 mmHg, seated diastolic BP ≥110 mmHg, neurological and clinical stability. | Patients aged 18 years or older with a clinical diagnosis of stroke (ischaemic or haemorrhagic), presenting within 30 hours of symptom onset and with systolic BP higher than 140 mmHg. |
| Relevant exclusion criteria | Age ≥85 years, occlusion or ≥70% stenosis of the internal carotid artery, malignant hypertension, manifest cardiac failure (NYHA class III and IV), high-grade aortic or mitral stenosis, unstable angina pectoris, contraindications against candesartan cilexetil. | Dysphagia preventing oral medication, mRS >3 at time of randomization, severe known renal insufficiency or renal artery stenosis or coronary artery disease or recent MI, hyperkalemia, uncorrected volume or sodium depletion, schedule for carotid endarterectomy, currently using or needing ARB | SSS consciousness score ≤2, premorbid mRS ≥4, clear indication for or contraindications to or current treatment with an ARB. |
| Treatment design | Candesartan cilexetil (4–16 mg daily according to BP levels)+. | Telmisartan (80 mg daily). | Candesartan cilexetil (4 mg on day 1, 8 mg on day 2 and 16 mg on days 3–7)†. |
| Follow-up evaluation | On day 7, at 3, 6, and 12 months. | On day 7, at 1, and 3 months. | On day 7, at 1, and 6 months. |
| Primary endpoints | Case fatality and disability (measured as BI) at 3 months. | Combined death or dependency (measured as mRS) at 30 days. | Composite endpoint of vascular death, nonfatal MI or nonfatal stroke and functional status (measured as mRS) at 6 months. |
| Secondary endpoints | Overall mortality and cerebrovascular and cardiovascular events at 12 months. | Overall mortality and cerebrovascular and cardiovascular events at 7, 30, and 90 days. | Stroke progression‡; neurological status at 7 days (measured as SSS); overall mortality, cerebrovascular and cardiovascular events, functional outcome (measured as BI) at 6 months. |

*In the PRoFESS trial a subgroup analysis has been considered.

#This was assumed when the mean of at least 2 blood pressure measurements was ≥200 mmHg systolic and/or ≥110 mmHg diastolic 6 to 24 hours after admission or ≥180 mmHg systolic and/or ≥105 mmHg diastolic 24 to 36 hours after admission.

+Candesartan cilexetil 4 mg daily on day 1; on day 2, dosage was increased to 8 or 16 mg if blood pressure exceeded 160 mmHg systolic or 100 mmHg diastolic. At the end of the placebo-controlled 7-days phase, in candesartan cilexetil-treated patients the dosage was increased or an additional antihypertensive drug was added only in the case of a hypertensive profile (mean daytime blood pressure ≥135/85 mm Hg). In placebo-treated patients candesartan cilexetil was started only in presence of a hypertensive profile.

†Dose adjustments were made if systolic blood pressure was lower than 120 mmHg or when clinically indicated.

‡Stroke progression was defined as a neurological deterioration of 2 or more points on the SSS occurring within the first 72 h of stroke onset and believed to be caused by the index stroke, after exclusion of recurrent stroke or systemic reasons for deterioration.

ARB: angiotensin receptor blocker; BI: barthel index; BP: blood pressure; MI: myocardial infarction; mRS: modified ranking scale; NYHA: New York Heart Association; SSS: Scandinavian stroke scale.

available about etiopathogenesis of strokes. In the PRoFESS subgroup analysis mean baseline BP was 147/84 mmHg and mean National Institutes of Health stroke scale (NIHSS) at admission was 3. According to TOAST classification, strokes were due to small artery occlusion in 59.5%, large-artery atherosclerosis in 20.9%, cardioembolism in 1.4%, and to undetermined or other determined etiologies in the remaining cases. In the SCAST study mean baseline BP was 171/90 mmHg and patients enrolled had a mean Scandinavian stroke scale (SSS) score of 41, equivalent to a NIHSS of 8 [30]. Strokes have been described following topographic rather than etiological criteria: according to Oxfordshire Community Stroke Project (OCSP) classification, strokes were distributed as total anterior syndrome in 8%, partial anterior syndrome in 48.7%, posterior syndrome in 14%, lacunar syndrome in 28.9%, unknown in <1%. Main baseline findings of the included studies are illustrated in Table 3.

*3.3. Blood Pressure and Outcomes.* In the ACCESS population no significant difference in BP was evident between the groups during the placebo-controlled phase and in the subsequent followup; at 7 days mean BP was 160/87 mmHg, at 3-months 150/85 mmHg and at 12 months 147/83 mmHg. Only two patients in the placebo arm had a normotensive profile on day 7 and did not receive the antihypertensive drug. In the PRoFESS subgroup analysis, telmisartan lowered significantly systolic BP (SBP) by 6 to 7 mmHg and diastolic BP (DBP) by 2 to 4 mmHg over the whole study course: on day 7, BP was135.3 (17.8)/78.4 (10.8) mmHg in treatment arm and 141.4 (17.0)/81.6 (11.0) mmHg in placebo group, with a difference of 6.1/3.2 mmHg ($P < 0.0001$); on day 30, mean BP was 135.7/79.6 mmHg versus 142.6/83.1 mmHg (treatment versus placebo) with a difference of 6.9/3.6 mmHg ($P < 0.0001$); on day 90, mean SBP was 134.5 (19.9) mmHg (treatment) versus 140.3 (19.0) (placebo) with a difference of 5.8 mmHg ($P < 0.0001$) and mean DBP was 79.2 (11.1) mmHg versus 81.5 (11.2) mmHg (treatment versus placebo) with a mean difference of 2.4 mmHg ($P = 0.0002$).

In the SCAST trial BP fell in both groups during treatment but was significantly lower in patients allocated candesartan than in those on placebo ($P \leq 0.001$ for days 2–7); on day 7, mean BP was 147/82 mmHg (SD 23/14) in the candesartan group and 152/84 mmHg (SD 22/14) in the placebo group. The mean difference in SBP on day 7 was 5 mmHg (95% CI 3–7; $P < 0.0001$) and the mean difference in DBP was 2 mmHg (1–3; $P = 0.001$). During the 6-month followup, mean BP values were similar in the two groups, and at 6 months the mean BP was 143/81 mmHg in both groups.

In the ACCESS the BI revealed no significant difference on study onset and after 3 months (candesartan cilexetil versus placebo, day 0: 60.0 ± 30.2 versus 64.1 ± 27.5; 3 months: 87.0 ± 22.9 versus 88.9 ± 19.9). The cumulative 12-month mortality (candesartan cilexetil versus placebo: 2.9% versus 7.2%; $P = 0.07$) and the number of vascular events (candesartan cilexetil versus placebo: 9.8% versus 18.7%; $P = 0.026$) differed significantly in favor of the candesartan cilexetil group; the odds ratio was 0.475 (95% CI, 0.252 to 0.895). The clinical benefit was independent of BP values and mainly attributable to a lower incidence of myocardial ischaemic events. Drug tolerance and number or type of undesirable effects did not differ significantly between the groups.

In the PRoFESS subgroup analysis combined death or dependency (mRS at 30 days, with adjustment for baseline covariates) did not differ whether analyzed as an ordinal outcome (ordered mRS categories: 0, 1, 2, 3, 4–6 to maintain proportionality) (OR, 1.03; 95% CI, 0.84–1.26; $P = 0.81$) or with dichotomization of the data at the median (mRS 0-1 versus 2–6; OR, 1.00; 95% CI, 0.77–1.29). There was no significant difference between the treatment groups for the distribution of ordinal stroke events (fatal, dependent (mRS 2–5), independent (mRS 0,1), TIA, none), for the time to recurrence ($P = 0.40$) and, similarly, for other events (i.e., death, stroke recurrence, MI, and combined vascular events) at 7, 30, or 90 days. Serious adverse events were similar between telmisartan and control.

In the SCAST trial the analysis of the first co-primary effect variable, the cumulative risk of the composite endpoint of vascular death, stroke or myocardial infarction, showed no significant difference between candesartan and placebo (unadjusted analysis HR 1.09, 95% CI 0.84–1.41; $P = 0.53$; adjusted analysis HR 1.09, 0.84–1.41; $P = 0.52$; per-protocol analysis HR 1.11, 0.85–1.46; $P = 0.46$). Regarding the second co-primary effect variable, the functional outcome at 6 months, no significant difference was seen across the mRS categories (unadjusted ordinal regression analysis, OR 1.13, 95% CI 0.97–1.32; $P = 0.12$); similar results have been obtained in both the fixed dichotomy (mRS 3–6 versus 0–2) analysis (unfavourable outcomes in 35% of patients on candesartan and in 33% of patients allocated on placebo; OR 1.12, 0.90–1.41, $P = 0.32$; RR 1.06, 0.93–1.19, $P = 0.39$) and the sliding dichotomy analysis using the SSS scores at baseline (unfavourable outcomes in 56% of patients on candesartan and 52% of patients on placebo; OR 1.16, 95% CI 0.97–1.38, $P = 0.11$; RR 1.07, 95% CI 0.99–1.16, $P = 0.11$). For all the secondary outcomes assessed there were no significant differences between treatment and placebo: for all events (death from any cause, vascular death, ischaemic stroke, haemorrhagic stroke, all strokes, myocardial infarction, stroke progression, symptomatic hypotension, renal failure, and symptomatic venous thromboembolism: RR 1.47, 95% CI 1.01–2.13; $P = 0.04$), for SSS score at 7 days ($P = 0.13$) and for BI at 6 months (0.47). There were no significant differences between the groups for the adverse events reported by the investigators.

Significant differences in BP values between treatment and placebo were therefore found in the PRoFESS and SCAST trials, but in only the first it remained significant during the follow-up; conversely, in the ACCESS study a significant difference was never found.

In all trials no effect of the active treatment was seen on dependency, death and vascular events at one, three or six months; in the ACCESS study a decrease in cumulative mortality and number of vascular, mainly myocardial, events emerged in candesartan group at 12 months.

Table 3: Patients characteristics at enrollment.

| Study | ACCESS [27] treatment/placebo | PRoFESS [28]* treatment/placebo | SCATS [29] treatment/placebo |
|---|---|---|---|
| Number of patients | 173/166 | 647/713 | 1017/1012 |
| Age | 68.3 (9.3)/67.8 (9.4) | 66.8 (8.8)/67.1 (9.2) | 70.8 (11.2)/71.0 (11.0) |
| Male (%) | 86 (49.7)/86 (51.8) | 420 (64.9)/464 (65.1) | 612 (60.2)/564 (55.7) |
| Clinical history | | | |
| Previous stroke/TIA | NA | 160 (24.7)/184 (25.8) | 252 (24.8)/204 (20.2) |
| Atrial fibrillation | NA | 10 (1.6)/14 (2.0) | 190 (18.7)/186 (18.4) |
| Hypertension | NA | 453 (70.0)/503 (70.6) | 676 (66.5)/670 (66.2) |
| Diabetes mellitus | 67 (38.7)/58 (35.0) | 176 (27.2)/198 (27.8) | 163 (16.0)/157 (15.5) |
| Hyperlipidemia | 74 (42.8)/75 (45.2) | 264 (40.8)/283 (39.7) | NA |
| Ischemic heart disease | 38 (22)/32 (19.3) | 95 (14.7)/104 (14.6) | NA |
| Time from stroke (hours) | 29.9/29.7 | 57.6 (16.8)/57.6 (16.8) | 17.6 (8.1)/17.9 (8.1) |
| Blood pressure (mmHg) | | | |
| Systolic | 188 (20.9)/190 (19.7) | 146 (16.2)/147 (16.3) | 171.2 (19.0)/171.6 (19.2) |
| Diastolic | 99 (14.9)/99 (13.0) | 84 (10.1)/84 (10.2) | 90.3 (13.9)/90.6 (14.2) |
| Clinical severity | 60.0 (30.2)/64.1 (27.5) at BI | 2.9 (2.8)/3.1 (2.9) at NIHSS | 40.6 (12.3)/40.5 (12.6) at SSS |

*In the PRoFESS trial a subgroup analysis has been considered.
Data are *n* (%) or mean (SD). NA: not assessed. BI: Barthel Index; mRS: modified ranking scale; NIHSS: National Institutes of Health Stroke Scale; SSS: Scandinavian stroke scale.

## 4. Discussion

While the primary prevention of stroke through the treatment of hypertension is well established and evidence from randomized controlled trials suggests the use of antihypertensive agents for the secondary prevention of vascular events in patients with previous stroke or transient ischaemic attack, the management of BP immediately after stroke has been an enigmatic controversy for more than two decades, exemplified by a debate published back in 1985 [31], and still remains uncertain. In experimental studies on rats, candesartan has been shown to be neuroprotective with both reduction of neurovascular damage, demonstrated by decreased infarct size, hemoglobin content and oedema in the ischaemic brain hemisphere, and improvement of neurological outcome. These favorable effects were evident with an early post ischemic stroke drug administration, at doses that did not affect or moderately lowered BP [32, 33], suggesting a multimodal protective effect, partly due to BP lowering and partly due to pleiotropic, vascular and neuronal, actions. Homeostatic defense processes against postischaemic brain damage, or almost a part of them, are pressure dependent; presumably there are feedbacks and fine interrelationships among BP, the processes limiting the infarct size and the mechanisms involved in the recovery of ischemic tissue. On the other side, competitive inhibition of the binding of angiotensin (AT) II to $AT_1$ receptors allows an unopposed activation of the $AT_2$ receptors which has been hypothesized to be protective in focal cerebral ischaemia being responsible of several events as recruitment of cerebral collaterals, normalization of cerebrovascular autoregulation, enhancement of neuronal resistance to anoxia, inhibition of inducible nitric oxide synthetase, reduction of oxidative damage, prevention of apoptosis, promotion of angiogenesis and attenuation of inflammation, endothelial disfunction and prothrombosis [34, 35].

Translating these experimental findings to human stroke patients is quite difficult. The clinician who faces a patient in the acute phase of stroke with elevated BP has these main alternatives: he may choose to continue or not a preexisting antihypertensive drug or to introduce or not a new one. In the Continue Or Stop post-Stroke Antihypertensives Collaborative study [36], continuation compared with cessation of preexisting antihypertensive drugs for a two weeks period after acute stroke was not associated with a substantial reduction in two-week death or dependency, cardiovascular event rate or mortality at six months. Besides, clinical trials evaluating the effects of different BP lowering drugs administered in the acute phase of stroke have given conflicting results in respect of functional outcome, and the evidence to guide the practicing clinician is still rudimentary. Sharp reduction in arterial BP has been sought to determine a worse short and long-term prognosis [37, 38], while a moderate and caution reduction might be safe and even improve long term mortality and reduce recurrent vascular events [39, 40]. Not only the degree of BP reduction but even the nature of the pharmacological agent itself, considering its specific mechanism of action and in the perspective of possible drug-class related benefits, has been and still remain matter of debate.

The purpose of this systematic review is to investigate the role of a specific class drug, the ARBs, early administered after stroke. None of the included studies demonstrated a significant benefit of active treatment on functional outcome and stroke recurrence at short and medium term, but some evaluations should be taken in account. Firstly, the mean BP at enrollment was much higher in the ACCESS study (189/99 mmHg) in respect to both the PRoFESS and SCAST

trials (147/84 mmHg and 171/90 mmHg, resp.). Secondly, relevant differences existed in stroke severity: patients enrolled in the PRoFESS had very mild stroke in respect to those participating to the SCAST and above all to the ACCESS who had a significantly greater impairment. Thirdly, the effect of treatment on BP: candesartan did not alter BP in the ACCESS while in the PRoFESS, despite the fact that BP at baseline was reasonably well controlled, telmisartan further reduced it; also in the SCAST trial BP fell in both treatment and placebo but was significantly lower in patients allocated candesartan although difference disappeared during the followup. Finally, the achievement of endpoints: neither functional outcome nor number of vascular events have been positively affected by treatment but in the ACCESS study, although the primary outcome (disability at 3 months) was neutral, treatment with candesartan was associated with a significant reduction in secondary outcome including the 12-month mortality and vascular events. Taking into account the U-shaped relationship between BP and outcome in acute stroke, the main advantage deriving from lowering BP treatment would be expected in the ACCESS trial which included patients with severely elevated BP. Even in this study, however, 3-month outcome has not been influenced by treatment and the reduction in the number of vascular events observed in favor of the candesartan cilexitil group was mainly due to a lower incidence of myocardial ischaemic events but not of recurrent cerebral ischaemic events which did not significantly contribute to the difference in cardiovascular morbidity and mortality. Much more surprising is the fact that in this study BP has not been affected by treatment, raising the question around the existence of a drug specific effect of the $AT_1$ receptor blockade, beyond the hemodynamic activity, able to modulate vascular remodeling and to affect cardiovascular survival with a benefit that did not arise immediately but appeared to increase over the time. Analogous data have been acquired from various cardiac intervention studies suggesting the hypothesis that early neurohumoral inhibition has similar beneficial effects in both cerebral and myocardial ischaemia, although the underlying mechanism is not resolved. These findings, however, have not been confirmed in the PRoFESS and SCAST trials, and such discrepancies may simply reflect a false positive finding of a small trial which was stopped prematurely on the basis of an interim analysis, requiring more investigations.

Taking their similarities and differences, the studies we have considered raise doubts over the hypothesis of a specific effect of angiotensin receptors blockade in acute stroke; moreover, conclusions are fully compatible with those of a recent meta-analysis [29] and a regularly update Cochrane survey [41] of randomized controlled trials of BP lowering drugs in acute stroke. According to currently available evidence there is no clear evidence of benefit for routine BP lowering treatment in the acute phase of stroke; many pitfalls, however, still remain to explain. Firstly, BP management in this clinical setting may have to be tailored with respect to the underlying etiology, and other parameters than baseline BP may need to taken into consideration [42, 43]. Patients with atherosclerotic or lacunar strokes are often affected by chronic hypertension with subsequent arterial stiffness and shift of the cerebral blood flow autoregulatory curve to the right. Consequently, they may tolerate elevated BP in acute stroke more efficiently, and BP values considered "normal" for the general population may be inadequate to perfuse the ischaemic brain. These patients usually present also diffuse atherosclerotic lesions in cerebral vessels which compromise the patency of collateral circulation: high BP may be needed to enhance perfusion of the ischaemic penumbra zone. On the contrary, in the cardioembolic strokes patients may only have atrial fibrillation in absence of arterial hypertension or significant atherosclerotic stenosis and may need only moderately elevated BP to promote perfusion through a patent collateral circulation; moreover, cardioembolic strokes tend to be of larger size with a higher risk of edema and hemorrhagic transformation in case of raised levels of BP. Unfortunately, the trials considered neither reported nor stratified outcome data according to the aetiopathogenesis of strokes. Secondly, the interrelationships between baseline BP, stroke severity, and administration of antihypertensive agents have not been well understood but an interaction could not be excluded; in the PRoFESS study the failure to show beneficial effect of telmisartan may reflect that patients had only mild hypertension and mild stroke. Thirdly, favourable effect of treatment may show a significant time interaction; a post hoc analysis of the main PRoFESS study indicated that recurrence was lower with telmisartan after the first six months of treatment [44], and in the ACCESS benefit on overall mortality and vascular events did not arise immediately but instead appeared to increase during follow up. The included studies presented followup ranging from 3 to 12 months and have not been designed to investigate long term effects of treatment.

Although a benefit of early treatment has not emerged, it is noteworthy that a substantial safety has resulted without difference in number or type of undesirable effects in treatment and placebo groups; since chronic lowering BP reduces strokes recurrence [45, 46] it may be safe to start such treatment even acutely in selected subgroups. Many other issues like the timing of starting the treatment, the degree, and rapidity of BP reduction, also taking account of the initial level, and the formulation, route of administration, and doses of different pharmacological agents should be further addressed; it is not possible to rule out that different drugs might have different effects.

Our analyses are not ideal in some respects. Firstly, trial-level data rather than individual patients' data were assessed since the latter were not available to us; analyses based on individual patients' data are generally superior and allow subgroup analyses to be performed. Secondly, the inhomogeneity of the patients' population and of the design of the study methods regarding cut offs for BP, stroke severity, time of assessment of the outcome variables; for the PRoFESS study, we have considered a subgroup of the patients entered into the main large secondary prevention trial, such that patients' characteristics reflect the inclusion criteria for a study of vascular prophylaxis rather than acute intervention. Thirdly, we could not assess the effect of lowering BP in patients with different subtypes of strokes since trials did not report these data separately.

## 5. Conclusion and Future Research

The currently available studies did not identify any clear indication that treatment with the ARBs is beneficial in patients with acute stroke and raised BP on functional outcome and stroke recurrence at short and medium term. Many issues, however, should be still considered; two large studies involving more than 2500 patients with acute ischaemic (ENOS) and haemorrhagic stroke (ENOS, INTERACT 2) [47, 48] are ongoing, and it is favorable these and future trials will help to clarify many unresolved questions and whether there are subgroups of patients or different approaches to BP management for which a treatment benefit can be obtained.

## Conflict of Interest

All authors have no conflict of interests.

## References


[1] P. Bath, J. Chalmers, W. Powers et al., "International Society of Hypertension (ISH): statement on the management of blood pressure in acute stroke," *Journal of Hypertension*, vol. 21, no. 4, pp. 665–672, 2003.

[2] M. Britton, A. Carlsson, and U. de Faire, "Blood pressure course in patients with acute stroke and matched controls," *Stroke*, vol. 17, no. 5, pp. 861–864, 1986.

[3] H. Fodstad, P. J. Kelly, and M. Buchfelder, "History of the Cushing reflex," *Neurosurgery*, vol. 59, no. 5, pp. 1132–1137, 2006.

[4] K. Ohwaki, E. Yano, H. Nagashima, M. Hirata, T. Nakagomi, and A. Tamura, "Blood pressure management in acute intracerebral hemorrhage: relationship between elevated blood pressure and hematoma enlargement," *Stroke*, vol. 35, no. 6, pp. 1364–1367, 2004.

[5] T. G. Robinson, M. James, J. Youde, R. Panerai, and J. Potter, "Cardiac baroreceptor sensitivity is impaired after acute stroke," *Stroke*, vol. 28, no. 9, pp. 1671–1676, 1997.

[6] G. Orlandi, S. Fannucchi, G. Strata et al., "Transient autonomic nervous system dysfunction during hyperacute stroke," *Acta Neurologica Scandinavica*, vol. 102, no. 5, pp. 317–321, 2000.

[7] B. Carlberg, K. Asplund, and E. Hagg, "The prognostic value of admission blood pressure in patients with acute stroke," *Stroke*, vol. 24, no. 9, pp. 1372–1375, 1993.

[8] B. K. Dandapani, S. Suzuki, R. E. Kelley, Y. Reyes-Iglesias, and R. C. Duncan, "Relation between blood pressure and outcome in intracerebral hemorrhage," *Stroke*, vol. 26, no. 1, pp. 21–24, 1995.

[9] T. Robinson, A. Waddington, S. Ward-Close, N. Taub, and J. Potter, "The predictive role of 24-hour compared to casual blood pressure levels on outcome following acute stroke," *Cerebrovascular Diseases*, vol. 7, no. 5, pp. 264–272, 1997.

[10] B. Carlberg, K. Asplund, and E. Hagg, "The prognostic value of admission blood pressure in patients with acute stroke," *Stroke*, vol. 24, no. 9, pp. 1372–1375, 1993.

[11] M. Willmot, J. Leonardi-Bee, and P. M. W. Bath, "High blood pressure in acute stroke and subsequent outcome: a systematic review," *Hypertension*, vol. 43, no. 1, pp. 18–24, 2004.

[12] V. Tikhonoff, H. Zhang, T. Richart, and J. A. Staessen, "Blood pressure as a prognostic factor after acute stroke," *The Lancet Neurology*, vol. 8, no. 10, pp. 938–948, 2009.

[13] M. R. Keezer, A. Y. X. Yu, B. Zhu, C. Wolfson, and R. Côté, "Blood pressure and antihypertensive therapy as predictors of early outcome in acute ischemic stroke," *Cerebrovascular Diseases*, vol. 25, no. 3, pp. 202–208, 2008.

[14] L. G. Stead, R. M. Gilmore, W. W. Decker, A. L. Weaver, and R. D. Brown, "Initial emergency department blood pressure as predictor of survival after acute ischemic stroke," *Neurology*, vol. 65, no. 8, pp. 1179–1183, 2005.

[15] J. Castillo, R. Leira, M. M. García, J. Serena, M. Blanco, and A. Dávalos, "Blood pressure decrease during the acute phase of ischemic stroke is associated with brain injury and poor stroke outcome," *Stroke*, vol. 35, no. 2, pp. 520–527, 2004.

[16] J. Leonardi-Bee, P. M. W. Bath, S. J. Phillips, and P. A. G. Sandercock, "Blood pressure and clinical outcomes in the International Stroke Trial," *Stroke*, vol. 33, no. 5, pp. 1315–1320, 2002.

[17] N. Ahmed and G. Wahlgren, "High initial blood pressure after acute stroke is associated with poor functional outcome," *Journal of Internal Medicine*, vol. 249, no. 5, pp. 467–473, 2001.

[18] N. Sprigg, L. J. Gray, P. M. Bath et al., "Relationship between outcome and baseline blood pressure and other haemodynamic measures in acute ischaemic stroke: data from the TAIST trial," *Journal of Hypertension*, vol. 24, no. 7, pp. 1413–1417, 2006.

[19] M. Yong, H. C. Diener, M. Kaste, and J. Mau, "Characteristics of blood pressure profiles as predictors of long-term outcome after acute ischemic stroke," *Stroke*, vol. 36, no. 12, pp. 2619–2625, 2005.

[20] S. L. Dawson, B. N. Manktelow, T. G. Robinson, R. B. Panerai, and J. F. Potter, "Which parameters of beat-to-beat blood pressure and variability best predict early outcome after acute ischemic stroke?" *Stroke*, vol. 31, no. 2, pp. 463–468, 2000.

[21] H. P. Adams, G. del Zoppo, M. J. Alberts et al., "Guidelines for the early management of adults with ischemic stroke: a guideline from the American heart association/American stroke association stroke council, clinical cardiology council, cardiovascular radiology and intervention council, and the atherosclerotic peripheral vascular disease and quality of care outcomes in research interdisciplinary working groups: the American Academy of Neurology affirms the value of this guideline as an educational tool for neurologists," *Stroke*, vol. 38, no. 5, pp. 1655–1711, 2007.

[22] J. Leonardi-Bee, P. M. W. Bath, S. J. Phillips, and P. A. G. Sandercock, "Blood pressure and clinical outcomes in the International Stroke Trial," *Stroke*, vol. 33, no. 5, pp. 1315–1320, 2002.

[23] B. K. Dandapani, S. Suzuki, R. E. Kelley, Y. Reyes-Iglesias, and R. C. Duncan, "Relation between blood pressure and outcome in intracerebral hemorrhage," *Stroke*, vol. 26, no. 1, pp. 21–24, 1995.

[24] G. M. Sare, L. J. Gray, and P. M. W. Bath, "Effect of antihypertensive agents on cerebral blood flow and flow velocity in acute ischaemic stroke: systematic review of controlled studies," *Journal of Hypertension*, vol. 26, no. 6, pp. 1058–1064, 2008.

[25] Blood Pressure in Acute Stroke Collaboration, "Vasoactive drugs for acute stroke," *Cochrane Database of Systematic Reviews*, no. 4, Article ID CD002839, 2000.

[26] N. Ahmed, P. Näsman, and N. G. Wahlgren, "Effect of intravenous nimodipine on blood pressure and outcome after acute stroke," *Stroke*, vol. 31, no. 6, pp. 1250–1255, 2000.

[27] J. Schrader, S. Lüders, A. Kulschewski et al., "The ACCESS study: evaluation of acute candesartan cilexetil therapy in stroke survivors," *Stroke*, vol. 34, no. 7, pp. 1699–1703, 2003.

[28] P. M. W. Bath, R. H. Martin, Y. Palesch et al., "Effect of telmisartan on functional outcome, recurrence, and blood pressure in patients with acute mild ischemic stroke: a PRoFESS subgroup analysis," *Stroke*, vol. 40, no. 11, pp. 3541–3546, 2009.

[29] E. C. Sandset, P. M. W. Bath, G. Boysen et al., "The angiotensin-receptor blocker candesartan for treatment of acute stroke (SCAST): a randomised, placebo-controlled, double-blind trial," *The Lancet*, vol. 377, no. 9767, pp. 741–750, 2011.

[30] L. J. Gray, M. Ali, P. D. Lyden, and P. M. W. Bath, "Interconversion of the National Institutes of Health Stroke Scale and Scandinavian Stroke Scale in Acute Stroke," *Journal of Stroke and Cerebrovascular Diseases*, vol. 18, no. 6, pp. 466–468, 2009.

[31] V. Hachinski, "Hypertension in acute ischemic strokes," *Archives of Neurology*, vol. 42, no. 10, p. 1002, 1985.

[32] S. C. Fagan, A. Kozak, W. D. Hill et al., "Hypertension after experimental cerebral ischemia: candesartan provides neurovascular protection," *Journal of Hypertension*, vol. 24, no. 3, pp. 535–539, 2006.

[33] J. Brdon, S. Kaiser, F. Hagemann, Y. Zhao, J. Culman, and P. Gohlke, "Comparison between early and delayed systemic treatment with candesartan of rats after ischaemic stroke," *Journal of Hypertension*, vol. 25, no. 1, pp. 187–196, 2007.

[34] Y. Nishimura, T. Ito, and J. M. Saavedra, "Angiotensin II AT1 blockade normalizes cerebrovascular autoregulation and reduces cerebral ischemia in spontaneously hypertensive rats," *Stroke*, vol. 31, no. 10, pp. 2478–2486, 2000.

[35] M. Iwai, H. W. Liu, R. Chen et al., "Possible inhibition of focal cerebral ischemia by angiotensin II type 2 receptor stimulation," *Circulation*, vol. 110, no. 7, pp. 843–848, 2004.

[36] T. G. Robinson, J. F. Potter, G. A. Ford et al., "Effects of antihypertensive treatment after acute stroke in the Continue Or Stop post-Stroke Antihypertensives Collaborative Study (COSSACS): a prospective, randomised, open, blinded-endpoint trial," *The Lancet Neurology*, vol. 9, no. 8, pp. 767–775, 2010.

[37] J. Castillo, R. Leira, M. M. García, J. Serena, M. Blanco, and A. Dávalos, "Blood pressure decrease during the acute phase of ischemic stroke is associated with brain injury and poor stroke outcome," *Stroke*, vol. 35, no. 2, pp. 520–527, 2004.

[38] N. Ahmed, P. Näsman, and N. G. Wahlgren, "Effect of intravenous nimodipine on blood pressure and outcome after acute stroke," *Stroke*, vol. 31, no. 6, pp. 1250–1255, 2000.

[39] J. F. Potter, T. G. Robinson, G. A. Ford et al., "Controlling hypertension and hypotension immediately post-stroke (CHHIPS): a randomised, placebo-controlled, double-blind pilot trial," *The Lancet Neurology*, vol. 8, no. 1, pp. 48–56, 2009.

[40] K. Fujii, B. L. Weno, G. L. Baumbach, and D. D. Heistad, "Effect of antihypertensive treatment on focal cerebral infarction," *Hypertension*, vol. 19, no. 6, pp. 713–716, 1992.

[41] Blood Pressure in Acute Stroke Collaboration (BASC), "Interventions for deliberately altering blood pressure in acute stroke (Cochrane Review)," in *The Cochrane Library*, Issue 1, John Wiley & Sons, Chichester, UK, 2004.

[42] G. Ntaios, D. Lambrou, and P. Michel, "Blood pressure changes in acute ischemic stroke and outcome with respect to stroke etiology," *Neurology*, vol. 79, pp. 1440–1448, 2012.

[43] G. Ntaios, D. Lambrou, and P. Michel, "Blood pressure change and outcome in acute ischemic stroke: the impact of baseline values, previous hypertensive disease and previous antihypertensive treatment," *Journal of Hypertension*, vol. 29, no. 8, pp. 1583–1589, 2011.

[44] S. Yusuf, H. C. Diener, R. L. Sacco et al., "Telmisartan to prevent recurrent stroke and cardiovascular events," *The New England Journal of Medicine*, vol. 359, no. 12, pp. 1225–1237, 2008.

[45] PATS Collaborating Group, "Post-stroke antihypertensive treatment study. A preliminary result," *Chinese Medical Journal*, vol. 108, pp. 710–717, 1995.

[46] S. MacMahon, B. Neal, C. Tzourio et al., "Randomised trial of a perindopril-based blood-pressure-lowering regimen among 6105 individuals with previous stroke or transient ischaemic attack," *The Lancet*, vol. 358, no. 9287, pp. 1033–1041, 2001.

[47] D. Thomas, P. M. Bath, K. Lees et al., "Glyceryl trinitrate versus control, and continuing vs. stopping temporarily prior antihypertensive therapy, in acute stroke: rationale and design of the Efficacy of Nitric Oxide in Stroke (ENOS) trial (ISRCTN99414122)," *International Journal of Stroke*, vol. 1, no. 4, pp. 245–249, 2006.

[48] C. Delcourt, Y. Huang, J. Wang et al., "The second (main) phase of an open, randomised, multicentre study to investigate the effectiveness of an intensive blood pressure reduction in acute cerebral haemorrhage trial (INTERACT2)," *International Journal of Stroke*, vol. 5, no. 2, pp. 110–116, 2010.

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*Review Article*

# AT2 Receptor-Interacting Proteins ATIPs in the Brain

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A complete renin-angiotensin system (RAS) is locally expressed in the brain and fulfills important functions. Angiotensin II, the major biologically active peptide of the RAS, acts via binding to two main receptor subtypes designated AT1 and AT2. The present paper focuses on AT2 receptors, which have been reported to have neuroprotective effects on stroke, degenerative diseases, and cognitive functions. Our group has identified a family of AT2 receptor interacting proteins (ATIPs) comprising three major members (ATIP1, ATIP3, and ATIP4) with different intracellular localization. Of interest, all ATIP members are expressed in brain tissues and carry a conserved domain able to interact with the AT2 receptor intracellular tail, suggesting a role in AT2-mediated brain functions. We summarize here current knowledge on the ATIP family of proteins, and we present new experimental evidence showing interaction defects between ATIP1 and two mutant forms of the AT2 receptor identified in cases of mental retardation. These studies point to a functional role of the AT2/ATIP1 axis in cognition.

## 1. Introduction

The renin-angiotensin system (RAS), a major regulator of blood pressure and cardiovascular functions, is now fully recognized as playing important roles in the brain [1–6]. Among the active peptides generated by the RAS, angiotensin II (AngII) stands as the best characterized. This octapeptide binds to two receptor subtypes, namely, AT1 and AT2, that belong to the superfamily of seven transmembrane domains receptors. In most tissues and cell lines, AT1 appears as a driving receptor that mediates most effects of AngII by activating classical heterotrimeric G proteins and intracellular signaling cascades [6, 7]. In contrast, AT2 is generally considered as an AT1-counteracting receptor that involves nonclassical signaling pathways and does not necessarily require exposure to AngII [6, 8]. Over the past few years, several AT2 receptor interacting partners have been identified [8–12], among which SHP-1 [8, 9], PLZF [13], and ATIP1/ATBP50 [12, 14, 15] that regulate AT2 receptor trafficking, internalization and/or activation. In this paper, we will focus on the family of ATIP proteins and their potential roles in AT2-mediated brain functions.

## 2. AT2 Receptor in the Brain

In contrast to the ubiquitous AT1 receptor, the AT2 subtype is predominantly expressed during embryonic development and is restricted to few sites in the adult [8, 17, 18]. In the central nervous system, AT2 expression is high during fetal life and remains elevated in the adult in specific areas involved in cognition, behavior, and locomotion [1, 18–20]. AT2 is mainly expressed in neurons and mediates neuronal differentiation [21–27], survival [28–30] and regeneration [31–33] through the regulation of protein kinases and phosphatases

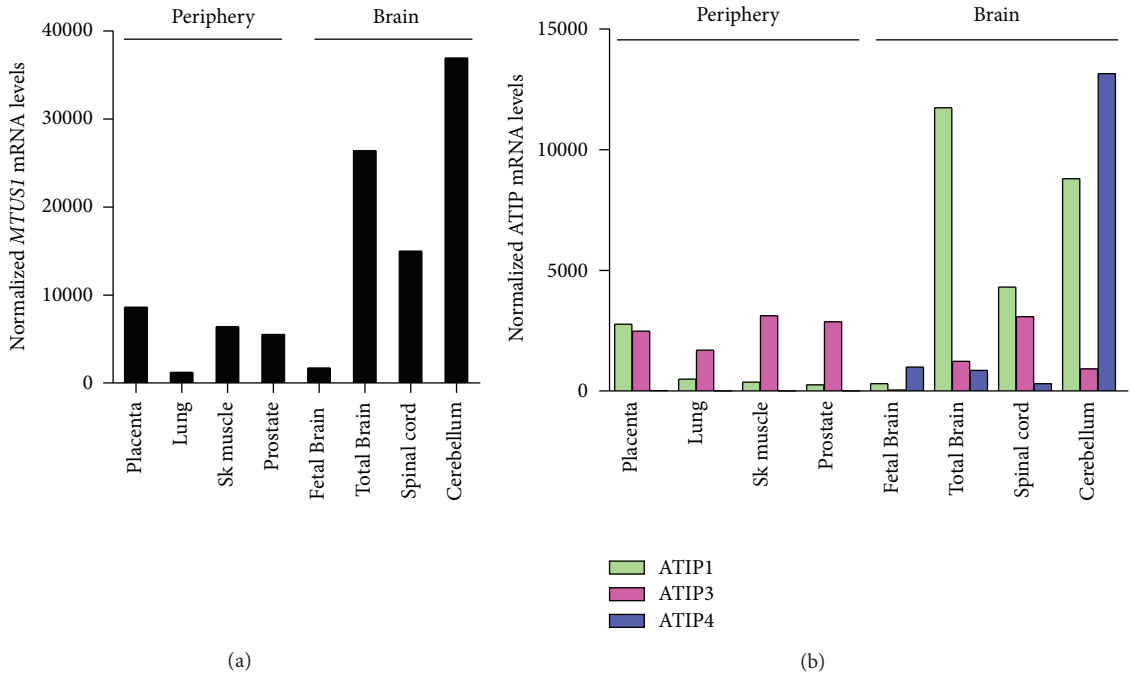


Figure 1: Distribution profiles of ATIP transcripts in normal human tissues. (a) Real-time PCR using probes common to all ATIP transcripts (3′ exons). (b) Real-time PCR using probes specific for each ATIP transcript (5′ exons). Results presented are from Di Benedetto et al., 2006 [16], and are normalized relative to the levels of human acidic ribosomal phosphoprotein P0 (RPLP0). Sk. muscle: skeletal muscle.

[8, 34–38], and the reorganization of the cytoskeleton [39, 40]. Functional *in vivo* studies tend to indicate protective effects of the AT2 receptor against stroke, Alzheimer disease, and cognitive impairment [26, 33, 41–45]. Involvement of AT2 receptors in cognition has also been suggested by the identification of mutations in the corresponding AGTR2 gene in several cases of mental retardation [46–48]. However these results remain a matter of debate [49–52], and functional alterations of AT2 receptors in mental retardation have still to be demonstrated. Further analyses of AT2 signaling pathways and AT2 interacting partners in the brain [53] following or not receptor activation with compound 21 (M024), a new selective AT2 ligand [54–56], should bring further insights on the effects of AT2 receptors in normal and pathological situations.

## 3. A Family of AT2 Receptor-Interacting Proteins (ATIP)

A family of AT2 receptor-interacting proteins (ATIPs) has been identified by a two-hybrid system cloning strategy using as a bait the 52 carboxy-terminal residues of the AT2 receptor [14, 15]. Three major human ATIP members (ATIP1, ATIP3, and ATIP4) are encoded by alternative exon splicing [16] and from alternative promoters [57] present on a single gene designated *MTUS1*. This gene contains 17 coding exons encompassing more than 112 kilobases and localizes at chromosomal position 8p22 [16]. All three ATIP transcripts use the same 3′ exons of the gene, and therefore encoded proteins are identical in their carboxy-terminal (395 amino acids) portion, which carries the AT2 receptor-interacting domain [12, 14, 16]. Thus, each ATIP member is in principle able to interact with AT2, although to date, only ATIP1 has been formally demonstrated to bind AT2 in living cells [14, 15, 34]. Expression of all three mRNA species has been detected in nonpathological human tissues by real-time PCR analysis using probes specific for each splice isoform [16] (Figure 1). ATIP1 and ATIP3 are ubiquitous whereas ATIP4 expression is restricted to the central nervous system. All ATIP transcripts were found expressed in every brain area examined [16], ATIP1 being predominant in all brain regions except cerebellum and fetal brain in which ATIP4 represents the major ATIP species.

## 4. The ATIP1/AT2 Axis in Neuronal Differentiation

ATIP1 (also designated MTSG1 and ATBP50 in the mouse) is the first characterized member of the ATIP protein family [14, 15, 58]. ATIP1 is a cytosolic protein that inhibits cell proliferation, receptor tyrosine kinase signaling, and ERK phosphorylation and contributes to the trafficking of the AT2 receptor from the Golgi to the cell membrane.

Real-time PCR analysis of ATIP transcripts in human tissues has revealed that ATIP1 is ubiquitous and the most abundant ATIP mRNA species expressed in the brain [16] (Figure 1). However, only few studies have investigated the effects of ATIP1 in brain functions. In rat fetal neurons, ATIP1 is constitutively associated with the AT2 receptor at the cell membrane and is part of a multimeric complex

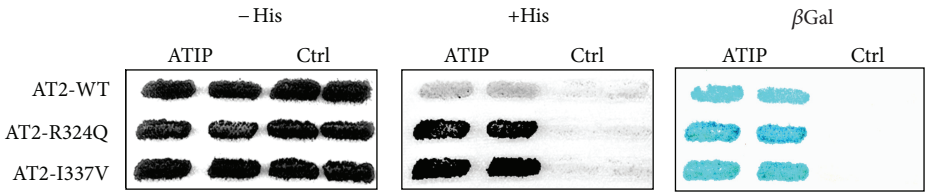


Figure 2: Interaction between ATIP1 and wild-type or mutated AT2 receptors. The C-terminus domain of ATIP1 (ATIP) interacts with the C-terminal region of human AT2 receptor, either wild-type (WT) or mutated (R324Q, I337V). The yeast reporter strain HF7 expressing the pairs of indicated hybrid proteins was analyzed for histidine auxotrophy and $\beta$-galactosidase expression as described [14]. Transformants were plated on medium with histidine (left), without histidine (His, middle), or replica-plated on Whatman filters and tested for $\beta$-galactosidase activity ($\beta$Gal, right). Growth in the absence of histidine and blue color in the $\beta$-galactosidase assay indicate interaction between hybrid proteins (WT: wild-type sequence; Ctrl; empty vector).

comprising the AT2 receptor and the SHP-1 tyrosine phosphatase [34]. Upon AT2 receptor activation by AngII, ATIP1 and SHP-1 remain associated but detach from the AT2 receptor and translocate from the cell membrane to the nucleus. In the nucleus, the ATIP1/SHP-1 complex activates the transcription of the methyl methanesulfonate sensitive 2 (MMS2) gene, thereby contributing to AT2-mediated neuronal differentiation [34]. These data suggest that detachment of ATIP1 from the AT2 receptor, rather than its association, may trigger activation of AT2 signaling pathways. Accordingly, dissociation of ATIP1/AT2 complexes following AngII stimulation has also been reported in transfected Chinese Hamster Ovary (CHO) cells [14].

## 5. ATIP1/AT2 Alterations in Mental Retardation

ATIP1 interacts with the C-terminal intracellular portion of the AT2 receptor [14, 15]. Interestingly, two nonconservative amino acid substitutions (R324Q and I337V) in the carboxy-terminal sequence of the human AT2 receptor have been identified in cases of mental retardation [46], prompting us to investigate whether these alterations may impact on the ability of the AT2 receptor to recruit ATIP1. We addressed this question using the two-hybrid system in yeast. The last 52 amino acids of the human AT2 receptor, either wild-type or mutated (R324Q or I337V), were PCR-amplified and subcloned into the pGBT9 vector in frame with the Gal4-DNA binding domain. The AT2-interacting domain of ATIP1 was subcloned into the VP16 vector. Interactions were assayed as previously described in the HF7 yeast strain which contains Histidine and beta-galactosidase reporter genes [14]. Interaction between the C-terminal domain of ATIP1 and the C-terminal region of the human AT2 receptor was confirmed (Figure 2). To our surprise, the interaction of ATIP1 with each of the mutated forms of the AT2 receptor was stronger compared to the interaction with wild-type AT2, suggesting that AT2 mutants may exhibit higher affinity for ATIP1. These data raise the interesting possibility that mutated AT2 receptors may retain ATIP1 at the cell membrane upon AngII stimulation. Further experiments are required to explore this hypothesis. We speculate that AT2 mutations (R324Q and I337V) identified in mental retardation may impair the intracellular activity of the receptor by preventing the release of ATIP1. These results suggest for the first time that dysfunctions in the AT2/ATIP1 axis may be involved in mental retardation. They point to a role for ATIP1 in brain functions and relaunch the debate on the functional involvement of AT2 receptors in mental retardation.

## 6. Microtubule-Associated ATIP3

As mentioned before, ATIP3 is identical to ATIP1 in the carboxy-terminal region carrying the AT2-interacting domain; however whether this isoform indeed interacts with the AT2 receptor in living cells remains to be determined. QPCR analyses revealed that ATIP3 transcripts are expressed in all human tissues including in the central nervous system [16]. However, ATIP3 functions in the brain have not yet been investigated. Of interest, ATIP3 closely associates with microtubules (Figure 3) [12, 59, 60], suggesting possible roles of this protein in diverse biological functions associated with cytoskeleton remodeling. Indeed, ATIP3 localizes to the mitotic spindle during cell division and acts as a potent antimitotic protein that inhibits cancer cell proliferation *in vitro* and tumor growth *in vivo*, in line with tumor suppressor effects of ATIP3 reported in breast cancer [59].

In the brain, microtubules play essential roles by regulating neuronal differentiation, neurite outgrowth, and cell migration [61, 62]. Alterations of microtubule-associated proteins such as tau are strongly associated with the occurrence of neurodegenerative pathologies, including Alzheimer disease [63, 64], in which AT2 receptors have also been implicated [43, 44, 65]. Whether microtubule-associated effects of ATIP3 may also contribute to the regulation of brain functions, in response or not to AT2 receptor stimulation, is a question that deserves further studies.

## 7. Brain-Specific Expression of ATIP4

The cDNA cloning and functional characterization of the ATIP4 isoform have not been undertaken to date. ATIP4 presents two interesting features that make it a good candidate for mediating AT2 functions in the brain. First, expression of the ATIP4 mRNA is restricted to the brain and remains undetectable in peripheral tissues [16] (Figure 1). Of note, ATIP4 mRNA levels are highest in the fetal brain and

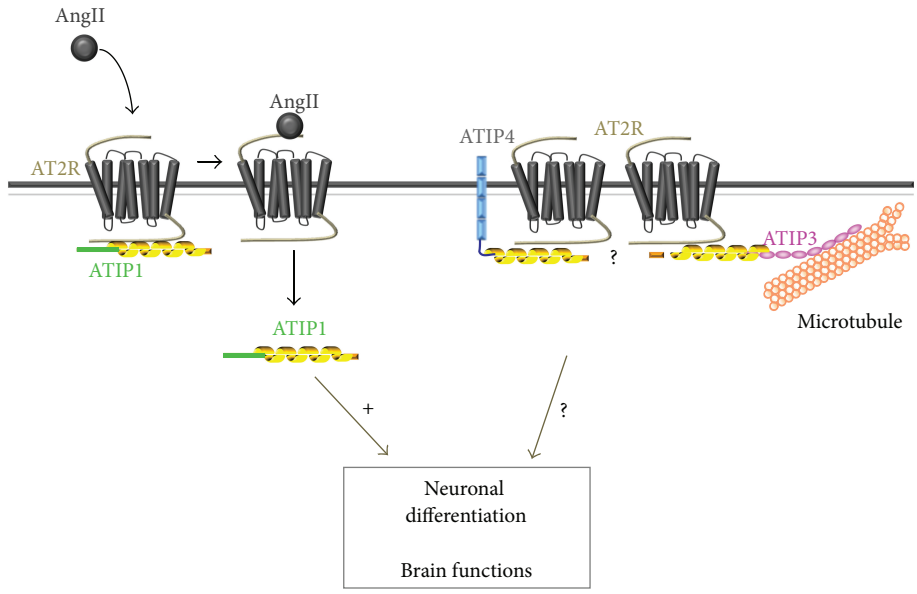


Figure 3: Schematic representation of ATIPs localization and interaction with AT2 receptors. ATIP1 is constitutively associated with the AT2 receptor at the cell membrane in rat fetal neurons and dissociates from the receptor upon AngII stimulation [34]. Putative interactions of ATIP3 and ATIP4 with AT2 receptors through their respective carboxy-terminal regions are represented.

in the cerebellum, which are two regions of abundant AT2 receptor expression in human brain [20]. Second, the amino acid sequence of ATIP4 contains a stretch of 24 hydrophobic residues flanked by charged residues, which is the hallmark of intrinsic membrane-spanning domains. Based on these *in silico* observations, it is tempting to speculate that ATIP4 might be structurally organized as a transmembrane protein with a short (36 residues) N-terminal extracellular domain and an intracellular region (456 residues) able to interact with the AT2 receptor (Figure 3). Future studies should be designed to investigate whether ATIP4 and AT2 are indeed colocalized at the plasma membrane in neuronal cells, and whether they functionally interact to regulate important brain functions. Prominent ATIP4 expression in the cerebellum compared to other regions of the brain (Figure 1) may suggest involvement of this ATIP isoform in functions related to locomotion, behavior, and/or cognition.

## 8. Concluding Remarks

Since the discovery of a new family of AT2 receptor interacting proteins in 2004, the question of whether these polypeptides may play a role in normal and/or pathological brain functions has not been addressed. Notably, all ATIP members are abundantly expressed in the brain and share the same C-terminal domain able to interact with the AT2 receptor, suggesting that each ATIP member may contribute to brain AT2 receptor functions. A functional AT2/ATIP1 axis has been previously reported to be involved in rat fetal neuron differentiation. We present here evidence that AT2/ATIP1 interactions are altered by *AGTR2* mutations identified in cases of mental retardation. These data relaunch the debate on the implication of AT2 receptors in mental retardation and point to *MTUS1* as an attractive target gene in human brain pathologies.

## Conflict of Interests

The authors have no conflict of interests to declare.

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## References

[1] C. Llorens-Cortes and F. A. Mendelsohn, "Organisation and functional role of the brain angiotensin system," *Journal of the Renin-Angiotensin-Aldosterone System*, vol. 3, supplement 1, pp. S39–S48, 2002.

[2] M. I. Phillips and E. M. De Oliveira, "Brain renin angiotensin in disease," *Journal of Molecular Medicine*, vol. 86, no. 6, pp. 715–722, 2008.

[3] A. E. Cuadra, Z. Shan, C. Sumners, and M. K. Raizada, "A current view of brain renin-angiotensin system: is the (pro)renin receptor the missing link?" *Pharmacology and Therapeutics*, vol. 125, no. 1, pp. 27–38, 2010.

[4] A. G. Dupont and S. Brouwers, "Brain angiotensin peptides regulate sympathetic tone and blood pressure," *Journal of Hypertension*, vol. 28, no. 8, pp. 1599–1610, 2010.

[5] J. W. Wright and J. W. Harding, "Brain renin-angiotensin-A new look at an old system," *Progress in Neurobiology*, vol. 95, no. 1, pp. 49–67, 2011.

[6] M. Horiuchi, J. Iwanami, and M. Mogi, "Regulation of angiotensin II receptors beyond the classical pathway," *Clinical Science*, vol. 123, no. 4, pp. 193–203, 2012.

[7] S. Higuchi, H. Ohtsu, H. Suzuki, H. Shirai, G. D. Frank, and S. Eguchi, "Angiotensin II signal transduction through the AT1 receptor: novel insights into mechanisms and pathophysiology," *Clinical Science*, vol. 112, no. 7-8, pp. 417–428, 2007.

[8] S. Nouet and C. Nahmias, "Signal transduction from the angiotensin II AT2 receptor," *Trends in Endocrinology and Metabolism*, vol. 11, no. 1, pp. 1–6, 2000.

[9] M. Mogi, M. Iwai, and M. Horiuchi, "Emerging concepts of regulation of angiotensin II receptors: new players and targets for traditional receptors," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 27, no. 12, pp. 2532–2539, 2007.

[10] E. R. Porrello, L. M. Delbridge, and W. G. Thomas, "The angiotensin II type 2 (AT2) receptor: an enigmatic seven transmembrane receptor," *Frontiers in Bioscience*, vol. 14, no. 3, pp. 958–972, 2009.

[11] M. Mogi, M. Iwai, and M. Horiuchi, "New insights into the regulation of angiotensin receptors," *Current Opinion in Nephrology and Hypertension*, vol. 18, no. 2, pp. 138–143, 2009.

[12] S. Rodrigues-Ferreira and C. Nahmias, "An ATIPical family of angiotensin II AT2 receptor-interacting proteins," *Trends in Endocrinology and Metabolism*, vol. 21, no. 11, pp. 684–690, 2010.

[13] T. Senbonmatsu, T. Saito, E. J. Landon et al., "A novel angiotensin II type 2 receptor signaling pathway: possible role in cardiac hypertrophy," *EMBO Journal*, vol. 22, no. 24, pp. 6471–6482, 2003.

[14] S. Nouet, N. Amzallag, J. M. Li et al., "Trans-inactivation of receptor tyrosine kinases by novel angiotensin II AT2 receptor-interacting protein, ATIP," *Journal of Biological Chemistry*, vol. 279, no. 28, pp. 28989–28997, 2004.

[15] C. J. Wruck, H. Funke-Kaiser, T. Pufe et al., "Regulation of transport of the angiotensin AT2 receptor by a novel membrane-associated Golgi protein," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 25, no. 1, pp. 57–64, 2005.

[16] M. Di Benedetto, I. Bièche, F. Deshayes et al., "Structural organization and expression of human MTUS1, a candidate 8p22 tumor suppressor gene encoding a family of angiotensin II AT2 receptor-interacting proteins, ATIP," *Gene*, vol. 380, no. 2, pp. 127–136, 2006.

[17] S. Shanmugam and K. Sandberg, "Ontogeny of angiotensin II receptors," *Cell Biology International*, vol. 20, no. 3, pp. 169–176, 1996.

[18] A. M. Nuyt, Z. Lenkei, M. Palkovits, P. Corvol, and C. Llorens-Cortes, "Ontogeny of angiotensin II type 2 receptor mRNA expression in fetal and neonatal rat brain," *Journal of Comparative Neurology*, vol. 407, no. 2, pp. 193–206, 1999.

[19] Z. Lenkei, M. Palkovits, P. Corvol, and C. Llorens-Cortès, "Expression of angiotensin type-1 (AT1) and type-2 (AT2) receptor mRNAs in the adult rat brain: a functional neuroanatomical review," *Frontiers in Neuroendocrinology*, vol. 18, no. 4, pp. 383–439, 1997.

[20] A. M. Allen, D. P. MacGregor, M. J. McKinley, and F. A. Mendelsohn, "Angiotensin II receptors in the human brain," *Regulatory Peptides*, vol. 79, no. 1, pp. 1–7, 1999.

[21] S. Meffert, M. Stoll, U. M. Steckelings, S. P. Bottari, and T. Unger, "The angiotensin II AT2 receptor inhibits proliferation and promotes differentiation in PC12W cells," *Molecular and Cellular Endocrinology*, vol. 122, no. 1, pp. 59–67, 1996.

[22] L. Laflamme, M. De Gasparo, J. M. Gallo, M. D. Payet, and N. Gallo-Payet, "Angiotensin II induction of neurite outgrowth by AT2 receptors in NG108-15 cells. Effect counteracted by the AT1 receptors," *Journal of Biological Chemistry*, vol. 271, no. 37, pp. 22729–22735, 1996.

[23] F. Côté, T. H. Do, L. Laflamme, J. M. Gallo, and N. Gallo-Payet, "Activation of the AT2 receptor of angiotensin II induces neurite outgrowth and cell migration in microexplant cultures of the cerebellum," *Journal of Biological Chemistry*, vol. 274, no. 44, pp. 31686–31692, 1999.

[24] L. Gendron, M. D. Payet, and N. Gallo-Payet, "The angiotensin type 2 receptor of angiotensin II and neuronal differentiation: from observations to mechanisms," *Journal of Molecular Endocrinology*, vol. 31, no. 3, pp. 359–372, 2003.

[25] M. O. Guimond, C. Wallinder, M. Alterman, A. Hallberg, and N. Gallo-Payet, "Comparative functional properties of two structurally similar selective nonpeptide drug-like ligands for the angiotensin II type-2 (AT(2)) receptor. Effects on neurite outgrowth in NG108-15 cells," *European Journal of Pharmacology*, vol. 699, no. 1–3, pp. 160–171, 2012.

[26] B. Maul, O. von Bohlen und Halbach, A. Becker et al., "Impaired spatial memory and altered dendritic spine morphology in angiotensin II type 2 receptor-deficient mice," *Journal of Molecular Medicine*, vol. 86, no. 5, pp. 563–571, 2008.

[27] U. M. Steckelings, F. Rompe, E. Kaschina et al., "The past, present and future of angiotensin II type 2 receptor stimulation," *Journal of the Renin-Angiotensin-Aldosterone System*, vol. 11, no. 1, pp. 67–73, 2010.

[28] J. Li, J. Culman, H. Hörtnagl et al., "Angiotensin AT2 receptor protects against cerebral ischemia-induced neuronal injury," *The FASEB Journal*, vol. 19, no. 6, pp. 617–619, 2005.

[29] N. Hobara, M. Goda, N. Yoshida et al., "Angiotensin II type 2 receptors facilitate reinnervation of phenol-lesioned vascular calcitonin gene-related peptide-containing nerves in rat mesenteric arteries," *Neuroscience*, vol. 150, no. 3, pp. 730–741, 2007.

[30] M. Horiuchi, M. Mogi, and M. Iwai, "The angiotensin II type 2 receptor in the brain," *Journal of the Renin-Angiotensin-Aldosterone System*, vol. 11, no. 1, pp. 1–6, 2010.

[31] R. Lucius, S. Gallinat, P. Rosenstiel, T. Herdegen, J. Sievers, and T. Unger, "The angiotensin II type 2 (AT2) receptor promotes axonal regeneration in the optic nerve of adult rats," *Journal of Experimental Medicine*, vol. 188, no. 4, pp. 661–670, 1998.

[32] K. Reinecke, R. Lucius, A. Reinecke, U. Rickert, T. Herdegen, and T. Unger, "Angiotensin II accelerates functional recovery in the rat sciatic nerve in vivo: role of the AT2 receptor and the transcription factor NF-kappaB.," *The FASEB Journal*, vol. 17, no. 14, pp. 2094–2096, 2003.

[33] P. Namsolleck, F. Boato, K. Schwengel et al., "AT2-receptor stimulation enhances axonal plasticity after spinal cord injury by upregulating BDNF expression," *Neurobiology of Disease*. In press.

[34] J. M. Li, M. Mogi, K. Tsukuda et al., "Angiotensin II-induced neural differentiation via angiotensin II type 2 (AT2) receptor-MMS2 cascade involving interaction between AT2 receptor-interacting protein and Src homology 2 domain-containing protein-tyrosine phosphatase 1," *Molecular Endocrinology*, vol. 21, no. 2, pp. 499–511, 2007.

[35] B. Plouffe, M. O. Guimond, H. Beaudry, and N. Gallo-Payet, "Role of tyrosine kinase receptors in angiotensin II AT2 receptor signaling: involvement in neurite outgrowth and in p42/p44mapk activation in NG108-15 cells," *Endocrinology*, vol. 147, no. 10, pp. 4646–4654, 2006.

[36] M. O. Guimond, C. Roberge, and N. Gallo-Payet, "Fyn is involved in angiotensin II type 2 receptor-induced neurite outgrowth, but not in p42/p44mapk in NG108-15 cells," *Molecular and Cellular Neuroscience*, vol. 45, no. 3, pp. 201–212, 2010.

[37] K. Bedecs, N. Elbaz, M. Sutren et al., "Angiotensin II type 2 receptors mediate inhibition of mitogen-activated protein kinase cascade and functional activation of SHP-1 tyrosine phosphatase," *Biochemical Journal*, vol. 325, no. 2, pp. 449–454, 1997.

[38] N. Elbaz, K. Bedecs, M. Masson, M. Sutren, A. D. Strosberg, and C. Nahmias, "Functional trans-inactivation of insulin receptor kinase by growth-inhibitory angiotensin II AT2 receptor," *Molecular Endocrinology*, vol. 14, no. 6, pp. 795–804, 2000.

[39] U. Stroth, S. Meffert, S. Gallinat, and T. Unger, "Angiotensin II and NGF differentially influence microtubule proteins in PC12W cells: role of the AT2 receptor," *Molecular Brain Research*, vol. 53, no. 1-2, pp. 187–195, 1998.

[40] P. Kilian, S. Campbell, L. Bilodeau et al., "Angiotensin II type 2 receptor stimulation increases the rate of NG108-15 cell migration via actin depolymerization," *Endocrinology*, vol. 149, no. 6, pp. 2923–2933, 2008.

[41] C. A. McCarthy, A. Vinh, J. K. Callaway, and R. E. Widdop, "Angiotensin AT2 receptor stimulation causes neuroprotection in a conscious rat model of stroke," *Stroke*, vol. 40, no. 4, pp. 1482–1489, 2009.

[42] P. Gelosa, A. Pignieri, L. Fändriks et al., "Stimulation of AT2 receptor exerts beneficial effects in stroke-prone rats: focus on renal damage," *Journal of Hypertension*, vol. 27, no. 12, pp. 2444–2451, 2009.

[43] N. Gallo-Payet, M. O. Guimond, L. Bilodeau, C. Wallinder, M. Alterman, and A. Hallberg, "Angiotensin II, a neuropeptide at the frontier between endocrinology and neuroscience: is there a link between the angiotensin II Type 2 receptor and Alzheimer's disease?" *Frontiers in Endocrinology*, vol. 2, p. 17, 2011.

[44] F. Jing, M. Mogi, A. Sakata et al., "Direct stimulation of angiotensin II type 2 receptor enhances spatial memory," *Journal of Cerebral Blood Flow & Metabolism*, vol. 32, no. 2, pp. 248–255, 2012.

[45] U. M. Steckelings, L. Paulis, P. Namsolleck, and T. Unger, "AT2 receptor agonists: hypertension and beyond," *Current Opinion in Nephrology & Hypertension*, vol. 21, no. 2, pp. 142–146, 2012.

[46] V. S. Vervoort, M. A. Beachem, P. S. Edwards et al., "AGTR2 mutations in X-linked mental retardation," *Science*, vol. 296, no. 5577, pp. 2401–2403, 2002.

[47] T. Ylisaukko-oja, K. Rehnström, R. Vanhala, C. Tengström, J. Lähdetie, and I. Järvelä, "Identification of two AGTR2 mutations in male patients with non-syndromic mental retardation," *Human Genetics*, vol. 114, no. 2, pp. 211–213, 2004.

[48] E. Takeshita, E. Nakagawa, K. Nakatani, M. Sasaki, and Y. Goto, "Novel AGTR2 missense mutation in a Japanese boy with severe mental retardation, pervasive developmental disorder, and epilepsy," *Brain and Development*, vol. 34, no. 9, pp. 776–779, 2012.

[49] T. Bienvenu, K. Poirier, H. Van Esch et al., "Rare polymorphic variants of the AGTR2 gene in boys with non-specific mental retardation," *Journal of Medical Genetics*, vol. 40, no. 5, pp. 357–359, 2003.

[50] J. Erdmann, S. Dähmlow, M. Guse et al., "The assertion that a G21V mutation in AGTR2 causes mental retardation is not supported by other studies (multiple letters)," *Human Genetics*, vol. 114, no. 4, pp. 396–397, 2004.

[51] D. Huang, W. Sun, and C. M. Strom, "Sequence variations in AGTR2 are unlikely to be associated with x-linked mental retardation," *American Journal of Medical Genetics*, vol. 139, no. 3, pp. 243–244, 2005.

[52] V. S. Vervoort, G. Guzauskas, J. Archie, C. E. Schwartz, R. E. Stevenson, and A. K. Srivastava, "AGTR2 in brain development and function," *American Journal of Medical Genetics*, vol. 140, no. 5, pp. 419–420, 2006.

[53] K. Seidel, S. Kirsch, K. Lucht et al., "The promyelocytic leukemia zinc finger (PLZF) protein exerts neuroprotective effects in neuronal cells and is dysregulated in experimental stroke," *Brain Pathology*, vol. 21, no. 1, pp. 31–43, 2011.

[54] Y. Wan, C. Wallinder, B. Plouffe et al., "Design, synthesis, and biological evaluation, of the first selective nonpeptide AT2 receptor agonist," *Journal of Medicinal Chemistry*, vol. 47, no. 24, pp. 5995–6008, 2004.

[55] J. Georgsson, C. Sköld, M. Botros et al., "Synthesis of a new class of druglike angiotensin II C-terminal mimics with affinity for the AT2 receptor," *Journal of Medicinal Chemistry*, vol. 50, no. 7, pp. 1711–1715, 2007.

[56] U. M. Steckelings, M. Larhed, A. Hallberg et al., "Non-peptide AT2-receptor agonists," *Current Opinion in Pharmacology*, vol. 11, no. 2, pp. 187–192, 2011.

[57] J. Yu, X. Liu, H. Ye, and X. Zhou, "Genomic characterization of the human mitochondrial tumor suppressor gene 1 (MTUS1): 5' cloning and preliminary analysis of the multiple gene promoters," *BMC Research Notes*, vol. 2, p. 109, 2009.

[58] S. Seibold, C. Rudroff, M. Weber, J. Galle, C. Wanner, and M. Marx, "Identification of a new tumor suppressor gene located at chromosome 8p21.3-22.," *The FASEB Journal*, vol. 17, no. 9, pp. 1180–1182, 2003.

[59] S. Rodrigues-Ferreira, A. Di Tommaso, A. Dimitrov et al., "8p22 MTUS1 gene product ATIP3 is a novel anti-mitotic protein underexpressed in invasive breast carcinoma of poor prognosis," *PLoS ONE*, vol. 4, no. 10, Article ID e7239, 2009.

[60] A. Molina, S. Rodrigues-Ferreira, A. Di Tommaso, and C. Nahmias, "[ATIP, a novel superfamily of microtubule-associated proteins].," *Médecine Sciences*, vol. 27, no. 3, pp. 244–246, 2011.

[61] C. C. Hoogenraad and F. Bradke, "Control of neuronal polarity and plasticity-a renaissance for microtubules?" *Trends in Cell Biology*, vol. 19, no. 12, pp. 669–676, 2009.

[62] F. E. Poulain and A. Sobel, "The microtubule network and neuronal morphogenesis: dynamic and coordinated orchestration through multiple players," *Molecular and Cellular Neuroscience*, vol. 43, no. 1, pp. 15–32, 2010.

[63] K. Iqbal, F. Liu, C. X. Gong, and I. Grundke-Iqbal, "Tau in Alzheimer disease and related tauopathies," *Current Alzheimer Research*, vol. 7, no. 8, pp. 656–664, 2010.

[64] L. M. Ittner and J. Götz, "Amyloid-$\beta$ and tau-a toxic pas de deux in Alzheimer's disease," *Nature Reviews Neuroscience*, vol. 12, no. 2, pp. 65–72, 2011.

[65] S. AbdAlla, H. Lother, A. El Missiry et al., "Angiotensin II AT2 receptor oligomers mediate G-protein dysfunction in an animal model of Alzheimer disease," *Journal of Biological Chemistry*, vol. 284, no. 10, pp. 6554–6565, 2009.

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*Review Article*

# The Brain Renin-Angiotensin System and Mitochondrial Function: Influence on Blood Pressure and Baroreflex in Transgenic Rat Strains

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Mitochondrial dysfunction is implicated in many cardiovascular diseases, including hypertension, and may be associated with an overactive renin-angiotensin system (RAS). Angiotensin (Ang) II, a potent vasoconstrictor hormone of the RAS, also impairs baroreflex and mitochondrial function. Most deleterious cardiovascular actions of Ang II are thought to be mediated by NADPH-oxidase- (NOX-) derived reactive oxygen species (ROS) that may also stimulate mitochondrial oxidant release and alter redox-sensitive signaling pathways in the brain. Within the RAS, the actions of Ang II are counterbalanced by Ang-(1–7), a vasodilatory peptide known to mitigate against increased oxidant stress. A balance between Ang II and Ang-(1–7) within the brain dorsal medulla contributes to maintenance of normal blood pressure and proper functioning of the arterial baroreceptor reflex for control of heart rate. We propose that Ang-(1–7) may negatively regulate the redox signaling pathways activated by Ang II to maintain normal blood pressure, baroreflex, and mitochondrial function through attenuating ROS (NOX-generated and/or mitochondrial).

## 1. Introduction

The renin-angiotensin system (RAS), and in particular angiotensin (Ang) II, is implicated in the impairment of arterial baroreflex function and reduction of heart rate variability (HRV) commonly associated with hypertension [1–4]. However, more recent studies suggest that a part of the deficit in sensitivity of the baroreflex function (BRS) in hypertension results from a reduction in Ang-(1–7), an alternative product of the RAS, rather than a frank increase in Ang II [5, 6]. Ang II blockade attenuates oxidant production and improves mitochondrial function in peripheral tissues in various experimental models of hypertension [7–10]. The contributions of Ang-(1–7) to the beneficial effects of Ang II blockers are increasingly recognized [11–16], but few studies have directly addressed the role of Ang-(1–7) in mitochondrial function. In this paper, we summarize (1) the role of Ang II in reactive oxygen species (ROS) generation and (2) the implication of ROS and redox-signaling on blood pressure, baroreflex, and mitochondrial function, with a particular focus on potential mechanisms for the counterbalancing role of Ang-(1–7) (Figure 1). Furthermore, we highlight the recent studies in transgenic rats with altered brain RAS (summarized in Figure 2) as a tool to study changes in brain ROS and signaling pathways in response to Ang peptides [Ang II and Ang-(1–7)] and their effect on BRS and mitochondrial function. The transgenic (mRen2)27 rat strain which overexpresses the murine Ren2 gene is hypertensive and has impaired BRS for control of heart rate (HR) with high levels of Ang II relative to Ang-(1–7) in the brain medullary tissue compared to the normotensive Sprague-Dawley (SD) rats [17, 18]. In contrast, transgenic rats with low glial angiotensinogen (ASrAOGEN) have lower mean arterial pressure (MAP) and HR suggesting decreased

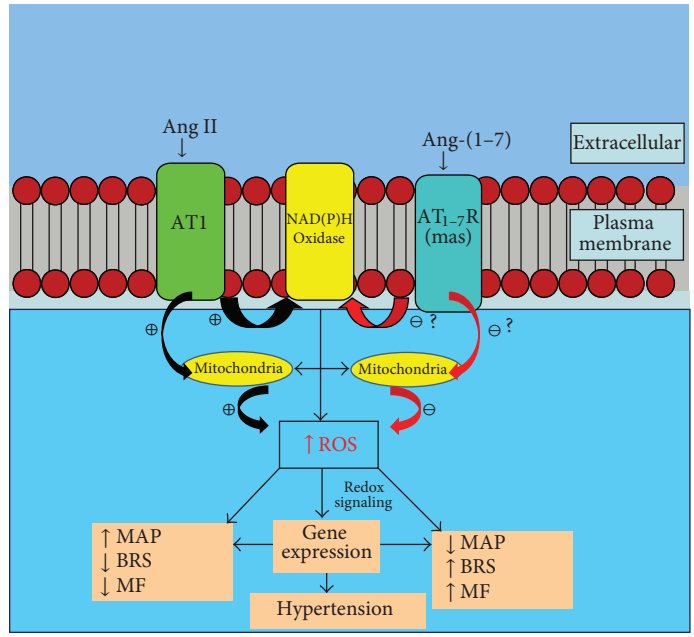



Figure 1: Proposed model: Ang-(1–7) through indirect (NADPH oxidase-mediated ROS) and/or direct interactions with mitochondria can attenuate ROS in dorsal medulla resulting in reduced blood pressure and enhanced baroreflex and mitochondrial function. ROS: reactive oxygen species; MAP: mean arterial pressure; BRS: baroreflex sensitivity; MF: Mitochondrial function; ?: not known.

| (mRen2)27 | Sprague-dawley | ASrAOGEN |
|---|---|---|
| Overexpress murine mRen2 gene | Parent control strain | Overexpress antisense oligonucleotide to AOGEN |
| Hypertensive | Normotensive | Hypotensive |
| High Ang II/Ang-(1–7) in medullary tissue | | Low Ang II/Ang-(1–7) in medullary tissue |
| Low BRS compared to control SD | | High BRS compared to control SD |

Figure 2: Major characteristics of (mRen2)27, Sprague-Dawley (SD), and ASrAOGEN rat strains with respect to hemodynamic and baroreflex function. AOGEN: angiotensinogen, BRS: baroreflex sensitivity, RAS: renin-angiotensin system.

sympathetic nerve activity and enhanced BRS for control of HR relative to SD rats [19, 20]. While we would expect that both Ang II and Ang-(1–7) would be reduced in the glial cells, nonglial sources (neuronal and/or circulating) of angiotensinogen and Ang peptides appear to be intact in ASrAOGEN rats [21]. Tissue levels of Ang II relative to Ang-(1–7) in the medulla have not been reported; however, blockade of Ang II actions by an AT1 receptor antagonist revealed that there was no Ang II tone attenuating the BRS in anesthesized ASrAOGEN rats and we conclude that glia-derived Ang II is responsible for this action. In contrast, since blockade of endogenous Ang-(1–7) attenuates BRS in both younger and older anesthesized ASrAOGEN rats, a nonglial source of Ang-(1–7) likely contributes to the preservation of BRS in these animals [5, 22]. Thus, there appears to be low Ang II but maintenance of Ang-(1–7) tone contributing to the enhanced BRS seen in the medulla of these animals.

## 2. Angiotensin Peptides and ROS Generation in the Brain

Overactivation of the RAS in pathological conditions, such as hypertension, results in excessive ROS production through the prooxidant actions of Ang II [24, 25]. The contribution of cytoplasmic NADPH-oxidase-(NOX)-generated ROS by Ang II in neurogenic hypertension is well established [26–28]. Ang II also stimulates mitochondrial ROS; both as a result of cytoplasmic NOX-derived ROS or direct effects on mitochondria [29–32]. Scavenging mitochondrial ROS, through agents such as Mito-TEMPO that preferentially targets the mitochondria, prevents Ang II-induced hypertension in mice [31, 33]. Antioxidant therapies targeting mitochondria are suggested to disrupt the mitochondrial ROS-dependent stimulation of cytoplasmic NOX activity, thereby providing beneficial effects in hypertension [34].

However, recent studies show that the NOX isoforms are also present within mitochondria [35–37] and the contribution of mitochondrial NOX to overall mitochondrial ROS in hypertension is unknown. Molecular interventions to target specific NOX isoforms within the mitochondria or other cellular organelle are required to address this issue.

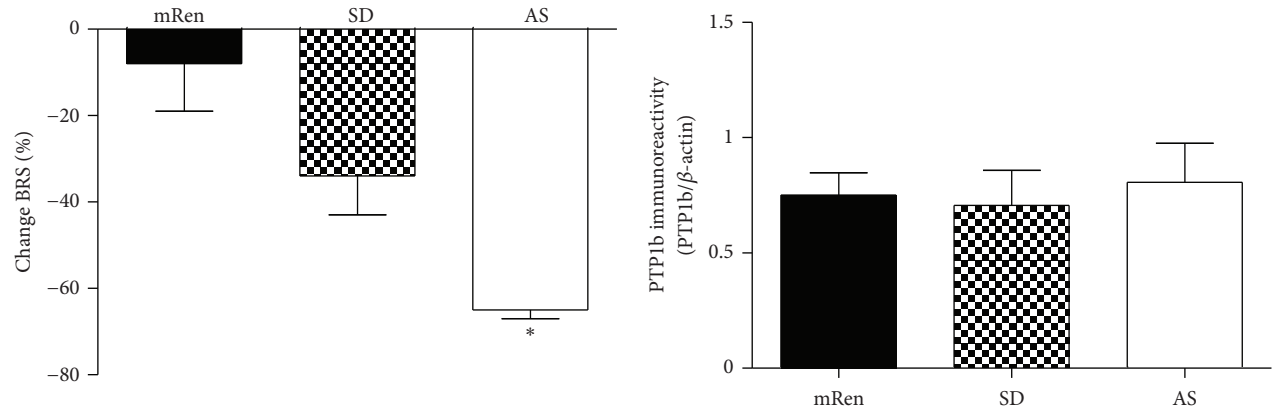
Ang-(1–7) has emerged as a major counter-regulatory peptide to Ang II actions and may serve to inhibit Ang II-stimulated ROS production through inhibiting NOX and/or increased ROS scavenging by augmenting antioxidant enzymes such as catalase [38, 39]. Indeed we find that higher Ang II actions relative to Ang-(1–7) in the brain dorsal medulla of hypertensive (mRen2)27 rats are associated with increased cytoplasmic NOX activity and ROS in isolated brain dorsal medullary mitochondria compared with the hypotensive ASrAOGEN [with higher Ang-(1–7) actions relative to Ang II] or the normotensive SD rats [40]. The levels of ROS were similar in the ASrAOGEN rats compared to SD rats under basal conditions suggesting that Ang-(1–7) may serve to inhibit NOX and/or activate antioxidant enzymes in response to Ang II stimulation. Although this concept is supported by several studies [38, 41], it has not been investigated directly in the brain to our knowledge. Thus, in this respect, it would be interesting to test whether blockade of endogenous Ang-(1–7) in ASrAOGEN rats will result in increased NOX activity/ROS levels in response to Ang II infusion/microinjection in the brain.

## 3. ROS and Redox-Signaling in the Brain: Influences on Blood Pressure, Baroreflex, and Mitochondrial Function

Excessive ROS in brain contributes to increased sympathetic outflow [42, 43] and impairs mitochondrial [8, 31, 44, 45] and BRS function [46–48]. Our recent studies find that Ang-(1–7) via chronic ICV infusion improved vagal function independent of any blood pressure lowering effect in transgenic hypertensive (mRen2)27 rats [49]. This effect was in contrast to the response to $AT_1$ receptor antagonist, candesartan, which normalized blood pressure but did not significantly improve the vagal indices of BRS or HRV. We have yet to determine the effect of these treatments on mitochondrial ROS, but neither treatment altered cytoplasmic NOX activity. Central infusion of the ROS scavenger tempol did not lower blood pressure or influence indices of baroreflex function, but significantly reduced cytoplasmic NOX activity, suggesting independence from ROS-related mechanisms for blood pressure lowering and autonomic nervous system balance in the hypertensive (mRen2)27 strain. However, we do not know whether tempol efficiently targets mitochondrial ROS or the extent that alterations in mitochondrial ROS would influence blood pressure and/or BRS in the transgenic rats. Dikalova and colleagues have reported blood pressure lowering effects of Mito-TEMPO in both Ang II-induced and DOCA salt hypertension in mice while a similar dose of tempol alone did not lower blood pressure in this study [33]. Furthermore, mitochondria are in close structural proximity to the endoplasmic reticulum (ER), and ER stress is implicated in mitochondrial dysfunction [50]. Indeed, the recent study by Young and colleagues link Ang II-induced hypertension to ER and oxidative stress in the brain [51]. These results provide a compelling case to investigate the effects of mitochondrial ROS, independent of cytoplasmic NOX.

AngII/$AT_1$ receptor/NOX-derived ROS are implicated in the activation of the MAP Kinases (MAPK) p38 and ERK1/2 that contribute to an impaired BRS and the pressor effects of Ang II in the RVLM [52–54]. A role for $AT_1$ receptors and MAPKs in activation of mitochondrial apoptotic pathways in neural regulation of blood pressure and BRS is also apparent [54]. However, hypertensive (mRen2)27 rats which show an increased NOX activity in the brain dorsal medulla but not activated p38, ERK1/2, or JNK-1 in comparison to SD rats suggesting a lack of association of MAPK signaling pathways with high blood pressure or oxidative stress [40]. In contrast, (mRen2)27 rats have an upregulated phosphoinositol 3 kinase (PI3 K) pathway that contributes to the elevated MAP and impaired BRS [55]. Hypotensive ASrAOGEN rats with normal NOX activity exhibit reduced levels of phosphorylated ERK1/2 and JNK-1 but not p38 in the brain dorsal medulla [40]. These animals have significantly higher expression of MAPK phosphatase-1 [MKP-1, a negative regulator of MAPK signaling [40]] supporting the concept that Ang-(1–7) increases regulatory phosphatases that may buffer against acute Ang II-stimulated signaling. Indeed, ASrAOGEN rats show greater impairments in the BRS for control of HR following acute solitary tract nucleus inhibition of protein tyrosine phosphatase 1b (PTP1b), a negative regulator of the PI3 K pathway, suggestive of increased expression and/or activity of this phosphatase within the dorsal medulla (Figure 3(a)). However, protein expression of total PTP1b (phosphorylated and nonphosphorylated forms) is similar in the dorsal medulla among the three rat strains under baseline conditions (Figure 3(b)). Therefore, given the functional differences observed following inhibition of PTP1b activity, quantification of the phosphorylated active form of PTP1b is necessary to confirm increased PTP1b activity in the ASrAOGEN rats. Differences in ROS [higher in (mRen2)27 versus SD or AsrAOGEN] or the upstream-regulatory kinases/phosphatases can modulate the phosphatase activity by changes in phosphorylation status at a number of different sites, despite lack of changes in the total protein [56]. While an upregulation of phosphatase expression and activity within the dorsal medulla may contribute to the enhanced resting BRS in the ASrAOGEN animals relative to the normal baroreflex function in SD rats [23], the lack of endogenous PTP1b tone in transgenic (mRen2)27 rats (Figure 3(a)) could result in increased PI3 K activity that contributes to an impaired BRS and increased MAP in these animals [55].

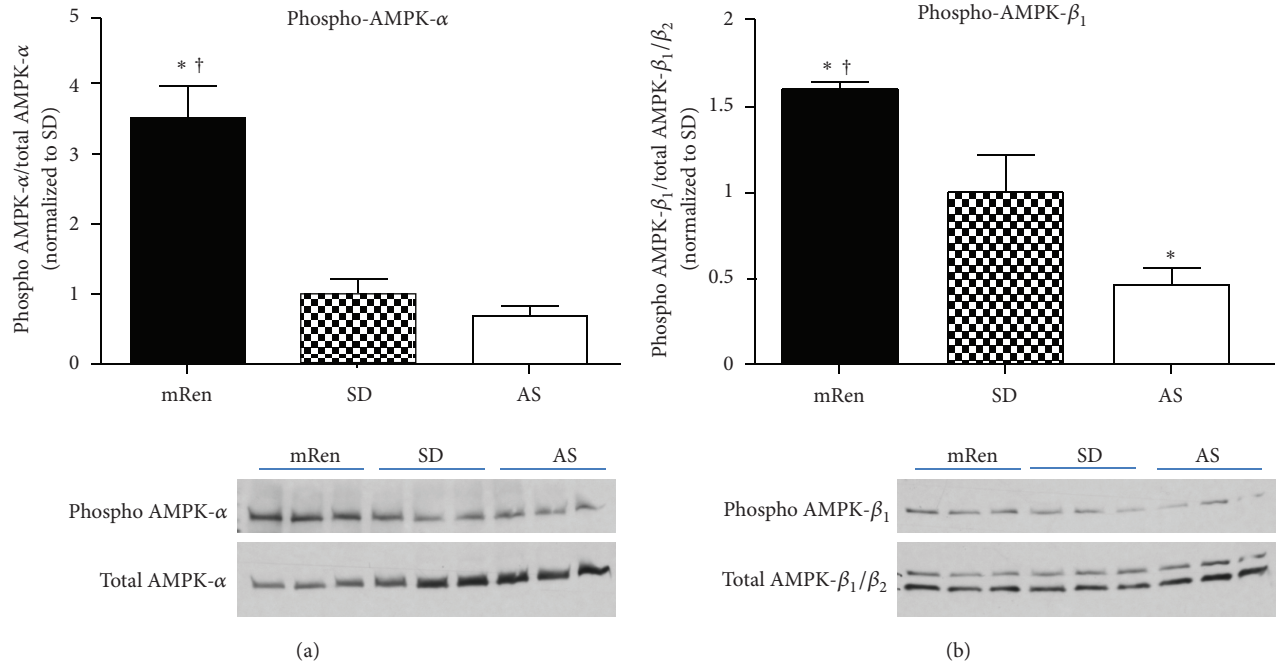
An interesting paradox to the beneficial role of these regulatory phosphates is that both MKP-1 and PTP1b have negative effects on metabolic function [57, 58]. In this regard, global knockdown of these phosphatases improves insulin-sensitivity and prevents diet-induced obesity [59, 60]. MKP-1 is suggested to impair mitochondrial biogenesis in skeletal muscle in response to a high-fat diet through negative regulation of the p38 MAPKs [61]. However, ASrAOGEN rats that




(a) Change in BRS in response to PTP1b inhibition in the NTS

(b) PTP1b expression in brain dorsal medulla

Figure 3: ASrAOGEN (AS) rats show significantly greater reduction in the evoked BRS for control of HR following inhibition of PTP1b (a), despite similar levels of the PTP1b protein in dorsal medulla of the three strains (b). Mean ± SEM ($n$ = 4–6 per group for solitary tract nucleus (NTS) microinjections, $n$ = 6 for Western blotting); $^{*}P < 0.05$ versus (mRen2)27 rats. Data replotted from [23] for the Sprague-Dawley (SD) rats and original data presented for ASrAOGEN and (mRen2)27 [mRen] rats. Western blotting carried out as published for SD rats [23] and original data presented for ASrAOGEN and (mRen2)27 rats. Note the PTP1b antibody recognizes both phosphorylated and nonphosphorylated forms.




(a)

(b)

Figure 4: Hypertensive (mRen2)27 rats show significantly increased phosphorylated AMP-Kinase (AMPK) in the brain dorsal medulla. AMPK-$\alpha$ (a) and $\beta_1$ (b) activities were measured by Western blot hybridization using phospho-specific antibodies (Cell Signaling) in brain dorsal medulla tissues from (mRen2)27 [mRen], Sprague-Dawley (SD), and ASrAOGEN (AS) rats. Top: Densitometry analyses of phoshorylated protein levels normalized to total AMPK-$\alpha$ and $\beta_1/\beta_2$; bottom: representative Western blots. Data are mean ± SEM ($n$ = 3–6 per group); $^{*}P < 0.05$ versus SD; $^{\dagger}P < 0.05$ versus AS rats.

have increased activity of these phosphatases at least in dorsal medulla are resistant to diet-induced obesity and spared age-related decline in cardiovascular and metabolic functions [62, 63]. These animals have increased life-span and their phenotype mimics animals with long-term RAS blockade where improved mitochondrial function is reported [7, 62–64]. Whether the brain-specific actions of these phosphatases contribute to the beneficial metabolic effects in ASrAOGEN rats is of interest and currently unknown. Thus, further studies dissecting the role of these brain signaling pathways

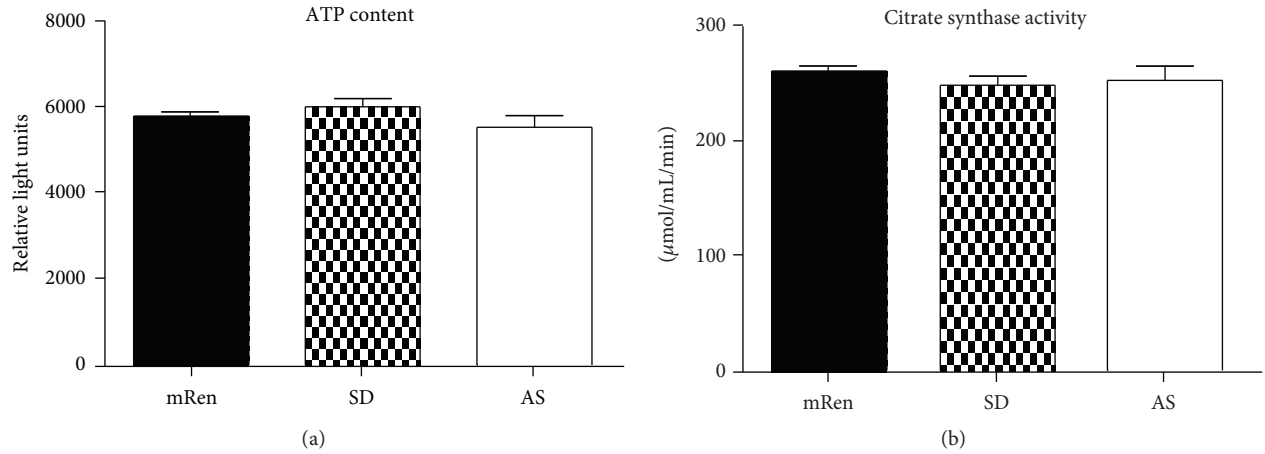



Figure 5: Brain dorsal medullary ATP levels (a) and mitochondrial content as measured using citrate synthase (b) was not different among hypertensive (mRen2)27 [mRen], Sprague-Dawley (SD) or hypotensive ASrAOGEN (AS) rats. ATP levels were determined using a chemiluminescent assay (Promega) and citrate synthase activity was measured using the assay kit (Sigma) in tissue homogenates. Mean ± SEM ($n$ = 6 per group).

in regulating MAP, BRS, mitochondrial, and metabolic functions are warranted.

Altered mitochondrial oxidant and/or energy levels are associated with a stimulated AMP-activated protein kinase (AMPK) pathway that is activated in response to depleted cellular energy levels, to restore mitochondrial biogenesis and ATP levels [65, 66]. AMPK was significantly activated (phosphorylated AMPK-$\alpha$ and $\beta_1$ subunits, Figures 4(a) and 4(b), resp.,) in the dorsal medulla of transgenic (mRen2)27 rats that exhibit increased cytoplasmic NOX activity and ROS levels in the brain dorsal medullary mitochondria relative to SD rats [40]. While we expected lower ATP levels (Figure 5(a)) and the mitochondrial content/number (assessed indirectly using the marker of mitochondrial health or activity, citrate synthase enzyme activity, Figure 5(b)) in the (mRen2)27 rats, these markers were not different in the dorsal medulla of the three strains. Therefore, activation of AMPK in the (mRen2)27 rats may represent a compensatory response to restore normal ATP and mitochondrial activity in the hypertensive strain in the face of increased ROS. Additional studies are necessary to address whether (1) blockade of AMPK activation lowers mitochondrial content and depletes ATP levels and (2) targeting mitochondrial ROS improves MAP, BRS, and mitochondrial function in the hypertensive (mRen2)27 rats.

## 4. Conclusions and Perspectives

Mitochondria-derived ROS which often accompanies impaired autonomic function is an emerging therapeutic target in hypertension [31, 33, 34, 45–47]. Increased cellular ROS may manifest as impaired BRS for the control of HR and reduced HRV (decreased parasympathetic outflow or vagal tone); and these indices of autonomic imbalance are associated with increased overall mortality, independent of blood pressure. Therefore, determining the key cellular mechanisms underlying the beneficial actions of Ang-(1–7) (such as altered kinase-phosphatase signaling) in influencing baroreflex function may help elucidate new therapeutic targets for reducing cardiometabolic pathologies. While Ang-(1–7) has been investigated for its role in attenuation of ROS, studies specifically addressing the mitochondria are lacking and few investigators are studying the interactions in brain. Thus, targeting improved vagal and mitochondrial function in addition to MAP may provide better target organ protection than lowering blood pressure alone, leading to reductions in all cause mortality.

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## References

[1] D. B. Averill and D. I. Diz, "Angiotensin peptides and baroreflex control of sympathetic outflow: pathways and mechanisms of the medulla oblongata," *Brain Research Bulletin*, vol. 51, no. 2, pp. 119–128, 2000.

[2] M. J. Campagnole-Santos, S. B. Heringer, E. N. Batista, M. C. Khosla, and R. A. S. Santos, "Differential baroreceptor reflex modulation by centrally infused angiotensin peptides," *American Journal of Physiology*, vol. 263, no. 1, part 2, pp. R89–R94, 1992.

[3] D. I. Diz, J. A. Jessup, B. M. Westwood et al., "Angiotensin peptides as neurotransmitters/neuromodulators in the dorsomedial medulla," *Clinical and Experimental Pharmacology and Physiology*, vol. 29, no. 5-6, pp. 473–482, 2002.

[4] D. I. Diz, A. C. Arnold, M. Nautiyal, K. Isa, H. A. Shaltout, and E. A. Tallant, "Angiotensin peptides and central autonomic regulation," *Current Opinion in Pharmacology*, vol. 11, no. 2, pp. 131–137, 2011.

[5] A. C. Arnold, A. Sakima, D. Ganten, C. M. Ferrario, and D. I. Diz, "Modulation of reflex function by endogenous angiotensins in older transgenic rats with low glial angiotensinogen," *Hypertension*, vol. 51, no. 5, pp. 1326–1331, 2008.

[6] A. Sakima, D. B. Averill, P. E. Gallagher et al., "Impaired heart rate baroreflex in older rats: Role of endogenous angiotensin-(1–7) at the nucleus tractus solitarii," *Hypertension*, vol. 46, no. 2, pp. 333–340, 2005.

[7] E. M. de Cavanagh, B. Piotrkowski, N. Basso et al., "Enalapril and losartan attenuate mitochondrial dysfunction in aged rats," *The FASEB Journal*, vol. 17, no. 9, pp. 1096–1098, 2003.

[8] E. M. V. de Cavanagh, J. E. Toblli, L. Ferder et al., "Angiotensin II blockade improves mitochondrial function in spontaneouslyhypertensive rats," *Cellular and Molecular Biology*, vol. 51, no. 6, pp. 573–578, 2005.

[9] E. M. V. de Cavanagh, J. E. Toblli, L. Ferder, B. Piotrkowski, I. Stella, and F. Inserra, "Renal mitochondrial dysfunction in spontaneously hypertensive rats is attenuated by losartan but not by amlodipine," *American Journal of Physiology*, vol. 290, no. 6, pp. R1616–R1625, 2006.

[10] E. M. V. de Cavanagh, L. Ferder, J. E. Toblli et al., "Renal mitochondrial impairment is attenuated by AT1 blockade in experimental type I diabetes," *American Journal of Physiology*, vol. 294, no. 1, pp. H456–H465, 2008.

[11] I. F. Benter, M. H. M. Yousif, F. M. Al-Saleh, R. Raghupathy, M. C. Chappell, and D. I. Diz, "Angiotensin-(1–7) blockade attenuates captopril- or hydralazine-induced cardiovascular protection in spontaneously hypertensive rats treated with NG-nitro-l-arginine methyl ester," *Journal of Cardiovascular Pharmacology*, vol. 57, no. 5, pp. 559–567, 2011.

[12] H. A. Shaltout, J. C. Rose, M. C. Chappell, and D. I. Diz, "Angiotensin-(1–7) deficiency and baroreflex impairment precede the antenatal Betamethasone exposure-induced elevation in blood pressure," *Hypertension*, vol. 59, no. 2, pp. 453–458, 2012.

[13] S. Sriramula, J. P. Cardinale, E. Lazartigues, and J. Francis, "ACE2 overexpression in the paraventricular nucleus attenuates angiotensin II-induced hypertension," *Cardiovascular Research*, vol. 92, no. 3, pp. 401–408, 2011.

[14] T. M. Gwathmey, K. D. Pendergrass, S. D. Reid, J. C. Rose, D. I. Diz, and M. C. Chappell, "Angiotensin-(1–7)-angiotensin-converting enzyme 2 attenuates reactive oxygen species formation to angiotensin ii within the cell nucleus," *Hypertension*, vol. 55, no. 1, pp. 166–171, 2010.

[15] M. C. Chappell, "Emerging evidence for a functional angiotensin-converting enzyme 2-angiotensin-(1–7)-Mas receptor axis: more than regulation of blood pressure?" *Hypertension*, vol. 50, no. 4, pp. 596–599, 2007.

[16] R. A. S. Santos, A. J. Ferreira, and A. C. Simões e Silva, "Recent advances in the angiotensin-converting enzyme 2-angiotensin(1–7)-Mas axis," *Experimental Physiology*, vol. 93, no. 5, pp. 519–527, 2008.

[17] J. J. Mullins, J. Peters, and D. Ganten, "Fulminant hypertension in transgenic rats harbouring the mouse Ren-2 gene," *Nature*, vol. 344, no. 6266, pp. 541–544, 1990.

[18] J. J. Mullins and D. Ganten, "Transgenic animals: new approaches to hypertension research," *Journal of Hypertension*, vol. 8, no. 7, pp. S35–S37, 1990.

[19] M. Schinke, M. Bohm, G. Bricca, A. Lippoldt, M. Bader, and D. Ganten, "Antisense RNA expression modulates angiotensinogen synthesis in cell culture and in the brain of transgenic rats," *Hypertension*, vol. 26, no. 3, pp. 547–546, 1995.

[20] M. Schinke, O. Baltatu, M. Böhm et al., "Blood pressure reduction and diabetes insipidus in transgenic rats deficient in brain angiotensinogen," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 96, no. 7, pp. 3975–3980, 1999.

[21] A. C. Arnold, A. Sakima, S. O. Kasper, S. Vinsant, M. A. Garcia-Espinosa, and D. I. Diz, "The brain renin-angiotensin system and cardiovascular responses to stress: insights from transgenic rats with low brain angiotensinogen," *Journal of Applied Physiology*, vol. 113, no. 12, pp. 1929–1936, 2012.

[22] A. Sakima, D. B. Averill, S. O. Kasper et al., "Baroreceptor reflex regulation in anesthetized transgenic rats with low glia-derived angiotensinogen," *American Journal of Physiology*, vol. 292, no. 3, pp. H1412–H1419, 2007.

[23] A. C. Arnold, M. Nautiyal, and D. I. Diz, "Protein phosphatase 1b in the solitary tract nucleus is necessary for normal baroreflex function," *Journal of Cardiovascular Pharmacology*, vol. 59, no. 5, pp. 472–478, 2012.

[24] K. K. Griendling and M. Ushio-Fukai, "Reactive oxygen species as mediators of angiotensin II signaling," *Regulatory Peptides*, vol. 91, no. 1–3, pp. 21–27, 2000.

[25] S. R. Datla and K. K. Griendling, "Reactive oxygen species, NADPH oxidases, and hypertension," *Hypertension*, vol. 56, no. 3, pp. 325–330, 2010.

[26] M. C. Zimmerman, E. Lazartigues, J. A. Lang et al., "Superoxide mediates the actions of angiotensin II in the central nervous system," *Circulation Research*, vol. 91, no. 11, pp. 1038–1045, 2002.

[27] M. C. Zimmerman, R. P. Dunlay, E. Lazartigues et al., "Requirement for Rac1-dependent NADPH oxidase in the cardiovascular and dipsogenic actions of angiotensin II in the brain," *Circulation Research*, vol. 95, no. 5, pp. 532–539, 2004.

[28] G. Wang, J. Anrather, J. Huang, R. C. Speth, V. M. Pickel, and C. Iadecola, "NADPH oxidase contributes to angiotensin II signaling in the nucleus tractus solitarius," *Journal of Neuroscience*, vol. 24, no. 24, pp. 5516–5524, 2004.

[29] E. M. V. de Cavanagh, F. Inserra, M. Ferder, and L. Ferder, "From mitochondria to disease: role of the renin-angiotensin system," *American Journal of Nephrology*, vol. 27, no. 6, pp. 545–553, 2007.

[30] S. I. Dikalov, W. Li, A. K. Doughan, R. R. Blanco, and A. M. Zafari, "Mitochondrial reactive oxygen species and calcium uptake regulate activation of phagocytic NADPH oxidase," *American Journal of Physiology*, vol. 302, no. 10, pp. R1134–R1142, 2012.

[31] A. K. Doughan, D. G. Harrison, and S. I. Dikalov, "Molecular mechanisms of angiotensin II-mediated mitochondrial dysfunction: linking mitochondrial oxidative damage and vascular endothelial dysfunction," *Circulation Research*, vol. 102, no. 4, pp. 488–496, 2008.

[32] S. Kimura, G. X. Zhang, A. Nishiyama et al., "Mitochondria-derived reactive oxygen species and vascular MAP kinases: comparison of angiotensin II and diazoxide," *Hypertension*, vol. 45, no. 3, pp. 438–444, 2005.

[33] A. E. Dikalova, A. T. Bikineyeva, K. Budzyn et al., "Therapeutic targeting of mitochondrial superoxide in hypertension," *Circulation Research*, vol. 107, no. 1, pp. 106–116, 2010.

[34] S. I. Dikalov and R. R. Nazarewicz, "Angiotensin II-induced production of mitochondrial reactive oxygen species: potential mechanisms and relevance for cardiovascular disease," *Antioxidants and Redox Signaling*. In press.

[35] K. Block, Y. Gorin, and H. E. Abboud, "Subcellular localization of Nox4 and regulation in diabetes," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 106, no. 34, pp. 14385–14390, 2009.

[36] S. M. Kim, Y. G. Kim, K. H. Jeong et al., "Angiotensin II-induced mitochondrial Nox4 is a major endogenous source of oxidative stress in kidney tubular cells," *PLoS ONE*, vol. 7, no. 7, Article ID e39739, 2012.

[37] J. Kuroda, T. Ago, S. Matsushima, P. Zhai, M. D. Schneider, and J. Sadoshima, "NADPH oxidase 4 (Nox4) is a major source of oxidative stress in the failing heart," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 107, no. 35, pp. 15565–15570, 2010.

[38] I. F. Benter, M. H. M. Yousif, G. S. Dhaunsi, J. Kaur, M. C. Chappell, and D. I. Diz, "Angiotensin-(1–7) prevents activation of NADPH oxidase and renal vascular dysfunction in diabetic hypertensive rats," *American Journal of Nephrology*, vol. 28, no. 1, pp. 25–33, 2007.

[39] G. S. Dhaunsi, M. H. M. Yousif, S. Akhtar, M. C. Chappell, D. I. Diz, and I. F. Benter, "Angiotensin-(1–7) prevents diabetes-induced attenuation in PPAR-$\gamma$ and catalase activities," *European Journal of Pharmacology*, vol. 638, no. 1–3, pp. 108–114, 2010.

[40] M. Nautiyal, P. V. Katakam, D. W. Busija et al., "Differences in oxidative stress status and expression of MKP-1 in dorsal medulla of transgenic rats with altered brain renin-angiotensin system," *American Journal of Physiology*, vol. 303, no. 8, pp. R799–R806, 2012.

[41] M. H. Yousif, G. S. Dhaunsi, B. M. Makki, B. A. Qabazard, S. Akhtar, and I. F. Benter, "Characterization of Angiotensin-(1–7) effects on the cardiovascular system in an experimental model of type-1 diabetes," *Pharmacological Research*, vol. 66, no. 3, pp. 269–275, 2012.

[42] Y. Hirooka, "Role of reactive oxygen species in brainstem in neural mechanisms of hypertension," *Autonomic Neuroscience: Basic and Clinical*, vol. 142, no. 1-2, pp. 20–24, 2008.

[43] J. R. Peterson, R. V. Sharma, and R. L. Davisson, "Reactive oxygen species in the neuropathogenesis of hypertension," *Current Hypertension Reports*, vol. 8, no. 3, pp. 232–241, 2006.

[44] S. H. H. Chan, K. L. H. Wu, A. Y. W. Chang, M. H. Tai, and J. Y. H. Chan, "Oxidative impairment of mitochondrial electron transport chain complexes in rostral ventrolateral medulla contributes to neurogenic hypertension," *Hypertension*, vol. 53, no. 2, pp. 217–227, 2009.

[45] M. Nozoe, Y. Hirooka, Y. Koga et al., "Mitochondria-derived reactive oxygen species mediate sympathoexcitation induced by angiotensin II in the rostral ventrolateral medulla," *Journal of Hypertension*, vol. 26, no. 11, pp. 2176–2184, 2008.

[46] J. Y. Jun, J. Zubcevic, Y. Qi, and M. K. Raizada, "Scavenging of mitochondrial ros in the brain prevents neurogenic hypertension," in *Proceedings of the 25th International Symposium on Cerebral Blood Flow, Metabolism and Function*, 2011.

[47] K. Ogawa, Y. Hirooka, K. Shinohara, T. Kishi, and K. Sunagawa, "Inhibition of oxidative stress in rostral ventrolateral medulla improves impaired baroreflex sensitivity in stroke-prone spontaneously hypertensive rats," *International Heart Journal*, vol. 53, no. 3, pp. 193–198, 2012.

[48] V. A. Braga, E. Colombari, and M. G. Jovita, "Angiotensin II-derived reactive oxygen species underpinning the processing of the cardiovascular reflexes in the medulla oblongata," *Neuroscience Bulletin*, vol. 27, no. 4, pp. 269–274, 2011.

[49] M. Nautiyal, H. A. Shaltout, D. C. de Lima, N. K. do, M. C. Chappell, and D. I. Diz, "Central angiotensin-(1–7) improves vagal function independent of blood pressure in hypertensive (mRen2)27 rats," *Hypertension*, vol. 60, no. 5, pp. 1257–1265, 2012.

[50] J. D. Malhotra and R. J. Kaufman, "ER stress and its functional link to mitochondria: role in cell survival and death," *Cold Spring Harbor Perspectives in Biology*, vol. 3, no. 9, Article ID a004424, 2011.

[51] C. N. Young, X. Cao, M. R. Guruju et al., "ER stress in the brain subfornical organ mediates angiotensin-dependent hypertension.," *Journal of Clinical Investigation*, vol. 122, no. 11, pp. 3960–3964, 2012.

[52] S. H. H. Chan, K. S. Hsu, C. C. Huang, L. L. Wang, C. C. Ou, and J. Y. H. Chan, "NADPH oxidase-derived superoxide anion mediates angiotensin II-induced pressor effect via activation of p38 mitogen-activated protein kinase in the rostral ventrolateral medulla," *Circulation Research*, vol. 97, no. 8, pp. 772–780, 2005.

[53] S. H. H. Chan, L. L. Wang, H. L. Tseng, and J. Y. H. Chan, "Upregulation of AT1 receptor gene on activation of protein kinase C$\beta$/nicotinamide adenine dinucleotide diphosphate oxidase/ERK1/2/c-fos signaling cascade mediates long-term pressor effect of angiotensin II in rostral ventrolateral medulla," *Journal of Hypertension*, vol. 25, no. 9, pp. 1845–1861, 2007.

[54] T. Kishi, Y. Hirooka, S. Konno, K. Ogawa, and K. Sunagawa, "Angiotensin II type 1 receptor-activated caspase-3 through ras/mitogen-activated protein kinase/extracellular signal-regulated kinase in the rostral ventrolateral medulla is involved in sympathoexcitation in stroke-prone spontaneously hypertensive rats," *Hypertension*, vol. 55, no. 2, pp. 291–297, 2010.

[55] E. M. Logan, A. A. Aileru, H. A. Shaltout, D. B. Averill, and D. I. Diz, "The functional role of PI3K in maintenance of blood pressure and baroreflex suppression in (mRen2)27 and mRen2.Lewis rat," *Journal of Cardiovascular Pharmacology*, vol. 58, no. 4, pp. 367–373, 2011.

[56] M. Soulsby and A. M. Bennett, "Physiological signaling specificity by protein tyrosine phosphatases," *Physiology*, vol. 24, no. 5, pp. 281–289, 2009.

[57] R. J. R. Flach and A. M. Bennett, "Mitogen-activated protein kinase phosphatase-1—a potential therapeutic target in metabolic disease," *Expert Opinion on Therapeutic Targets*, vol. 14, no. 12, pp. 1323–1332, 2010.

[58] R. C. Tsou and K. K. Bence, "The genetics of PTPN1 and obesity: insights from mouse models of tissue-specific PTP1B deficiency," *Journal of Obesity*, vol. 2012, Article ID 926857, 8 pages, 2012.

[59] M. Elchebly, P. Payette, E. Michaliszyn et al., "Increased insulin sensitivity and obesity resistance in mice lacking the protein tyrosine phosphatase-1B gene," *Science*, vol. 283, no. 5407, pp. 1544–1548, 1999.

[60] J. J. Wu, R. J. Roth, E. J. Anderson et al., "Mice lacking MAP kinase phosphatase-1 have enhanced MAP kinase activity and resistance to diet-induced obesity," *Cell Metabolism*, vol. 4, no. 1, pp. 61–73, 2006.

[61] R. J. Roth, A. M. Le, L. Zhang et al., "MAPK phosphatase-1 facilitates the loss of oxidative myofibers associated with obesity in mice," *Journal of Clinical Investigation*, vol. 119, no. 12, pp. 3817–3829, 2009.

[62] S. O. Kasper, C. S. Carter, C. M. Ferrario et al., "Growth, metabolism, and blood pressure disturbances during aging in

transgenic rats with altered brain renin-angiotensin systems," *Physiological Genomics*, vol. 23, no. 3, pp. 311–317, 2005.

[63] S. O. Kasper, C. M. Ferrario, D. Ganten, and D. I. Diz, "Rats with low brain angiotensinogen do not exhibit insulin resistance during early aging," *Endocrine*, vol. 30, no. 2, pp. 167–174, 2006.

[64] D. I. Diz, S. O. Kasper, A. Sakima, and C. M. Ferrario, "Aging and the brain renin-angiotensin system: insights from studies in transgenic rats," *Cleveland Clinic Journal of Medicine*, vol. 74, supplement 1, pp. S95–S98, 2007.

[65] R. Bergeron, J. M. Ren, K. S. Cadman et al., "Chronic activation of AMP kinase results in NRF-1 activation and mitochondrial biogenesis," *American Journal of Physiology*, vol. 281, no. 6, pp. E1340–E1346, 2001.

[66] L. H. Young, J. Li, S. J. Baron, and R. R. Russell, "AMP-activated protein kinase: a key stress signaling pathway in the heart," *Trends in Cardiovascular Medicine*, vol. 15, no. 3, pp. 110–118, 2005.

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*Review Article*

# The Angiotensin-Melatonin Axis

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Accumulating evidence indicates that various biological and neuroendocrine circadian rhythms may be disrupted in cardiovascular and metabolic disorders. These circadian alterations may contribute to the progression of disease. Our studies direct to an important role of angiotensin II and melatonin in the modulation of circadian rhythms. The brain renin-angiotensin system (RAS) may modulate melatonin synthesis, a hormone with well-established roles in regulating circadian rhythms. Angiotensin production in the central nervous system may not only influence hypertension but also appears to affect the circadian rhythm of blood pressure. Drugs acting on RAS have been proven effective in the treatment of cardiovascular and metabolic disorders including hypertension and diabetes mellitus (DM). On the other hand, since melatonin is capable of ameliorating metabolic abnormalities in DM and insulin resistance, the beneficial effects of RAS blockade could be improved through combined RAS blocker and melatonin therapy. Contemporary research is evidencing the existence of specific clock genes forming central and peripheral clocks governing circadian rhythms. Further research on the interaction between these two neurohormones and the clock genes governing circadian clocks may progress our understanding on the pathophysiology of disease with possible impact on chronotherapeutic strategies.

## 1. Introduction

The renin-angiotensin system (RAS) is considered as a major endocrine regulator of cardiovascular homeostasis. The RAS acts in endocrine, paracrine, and autocrine manner in several organs and systems exercising various organ-specific actions with effects on the cardiovascular system [1]. Several lines of evidence from integrative physiology and functional genomics to molecular and genetic levels indicate that the RAS, circulating (endocrine) or tissue (paracrine and autocrine), is one of the major drivers of hypertension and cardiovascular diseases [1–3]. This knowledge led to the successful development of drugs to block the RAS system (angiotensin converting enzyme inhibitors, angiotensin receptor blockers, and renin inhibitors) that proved efficacious in the treatment of hypertension and other cardiovascular diseases [4].

Melatonin is produced by the pineal gland predominantly during night and it is considered as a major hormone regulating the circadian rhythmicity of several biological systems [5]. Research on melatonin functions revealed that this is not only a regulator of the biological circadian clock [5] but also it has a variety of biological functions [6]. Melatonin appears to be involved in various diseases, such as sleep disorders, dementia, mood disorders, cancer, and diabetes [7].

Both angiotensin and melatonin are synthesized in the brain. Angiotensin produced locally in central nervous system in nuclei involved in cardiovascular and fluid-electrolyte homeostasis interacts with other systems, such as sympathetic, vasopressinergic ones [1, 8]. Moreover, there is a local pineal RAS that modulates the synthesis of melatonin, which represents the main hormonal output of the pineal gland [9, 10]. The RAS is classically involved in cardiovascular and metabolic pathophysiology while melatonin deals with

circadian rhythms. In this paper, we aimed at evidencing interference at several levels between the RAS and melatonin to modulate cardiovascular and metabolic pathophysiology (Table 1).

## 2. Angiotensin-Melatonin Axis

The postulation of a local RAS in the brain has led to discovery of brain-specific roles of angiotensin II (Ang II). A local production of active angiotensins has been documented in several brain nuclei and regions. Of these, the two neuroendocrine glands situated in the brain possess high levels of Ang II-forming activities [9]. This led us to postulate the presence of a local RAS in the pineal gland. Angiotensinogen, the precursor of the RAS, has been identified in pineal glial cells while the receptors type AT1b are localized in pinealocytes [10]. As part of the enzymatic cascade producing Ang II, we identified angiotensin converting enzyme (ACE) and chymase but not renin, indicating the existence of nonrenin pathways [9, 10]. By utilizing both pharmacological and transgenic strategies, we could demonstrate that locally produced Ang II in the pineal can modulate the melatonin synthesis [11]. Melatonin represents the main hormonal output of the pineal gland and it is considered as an important modulator of circadian rhythms. Our studies indicate that Ang II acts on the pinealocyte AT1 receptors to influence the synthesis and activity of tryptophan hydroxylase (TPH), the rate-limiting enzyme of melatonin synthesis. The demonstration of a functional pineal RAS interfering with melatonin synthesis indicates that this may affect melatonin roles, such as in modulation of circadian rhythms (Figure 1).

The circadian system comprised of a group of specialized genes is a key integrator of metabolism and behavior that synchronizes physiological processes. Circadian oscillators are present not only in the suprachiasmatic nucleus (SCN) which is considered to be the master clock but also in peripheral tissues, including cardiovascular organs [12]. Several animal studies are identifying roles for clock genes in cardiovascular and metabolic physiology and pathophysiology [13]. Genetic manipulation of clock genes in transgenic mouse models has uncovered new functions of internal clocks in pathogenesis of cardiovascular diseases [14]. Since our and other studies point to an important role of the RAS in the modulation of circadian rhythms of blood pressure [15, 16], an interaction with the genes governing circadian clock of cardiovascular tissues might be conceivable. An interaction between the RAS and the circadian system has been suggested to contribute to the development of inverted BP profile in transgenic rats harboring the mouse Ren-2 renin gene, TGR(mREN)27 [17, 18].

The interaction between angiotensin and melatonin both centrally and peripherally in hypertension and cardiovascular diseases has to be studied. At pineal level, the challenge is to understand in a hypertensive patient with elevated levels of angiotensin how melatonin release is affected: is there a feedback loop of high levels of circulating angiotensin impacting on either potentiating more melatonin release to counteract hypertension, or if there is a lack of effect, that is, resistance to central angiotensin, that resets the pineal gland axis and reduces melatonin output, which then may further potentiate hypertension. Further studies are necessary to decipher and dissect possible interactions between the RAS, melatonin, and the clock genes governing circadian clock.

Table 1: Opposing roles of angiotensin II and melatonin in cardiovascular and metabolic pathophysiology.

| Angiotensin | Melatonin |
|---|---|
| Blood pressure: direct effects | |
| Increase, vasoconstriction | Decrease, vasodilation |
| Blood pressure: circadian rhythm | |
| Nondipper/riser hypertension | Decreased levels in nondipper hypertension, chronobiotic |
| Central clock: suprachiasmatic nucleus | |
| Precursor and receptors present | Receptors present |
| Sympathetic nervous system | |
| Stimulation | Sympatholytic |
| Oxidative stress | |
| Increase | Decrease |
| Inflammation | |
| Increase | Decrease |
| Central clock: suprachiasmatic nucleus | |
| Precursor and receptors present | Receptors present |
| Insulin | |
| Insulin resistance | Increase in insulin sensitivity |

## 3. Antagonistic Effects of Angiotensin and Melatonin in Cardiovascular and Metabolic Systems

*3.1. Cardiovascular System.* A cardiovascular role of melatonin has been suggested already 40 years ago by the description of a pinealectomy-induced experimental hypertension model [19–21]. Melatonin receptors are present in the vasculature and mediate vascular constriction and vasodilation through MT1 and MT2 receptors, respectively [22]. Melatonin administration generally induces a decrease in blood pressure [22]. One possible mechanism contributing to the melatonin hypotensive effects is through its sympatholytic properties [23]. On the other hand, a marked reduction of circulating melatonin has been observed in cardiovascular diseases [24]. These findings suggest antagonistic activities of angiotensin and melatonin in the cardiovascular system (Table 1). The mechanisms by which melatonin is antagonizing Ang II actions in cardiovascular and metabolic diseases are comprising its antihypertensive, antioxidant, and anti-inflammatory functions [24]. Melatonin has direct free radical scavenging and indirect antioxidant activity. Through these marked antioxidant properties, melatonin has cardioprotective effects, in particular in myocardial damage after ischemia-reperfusion [25].

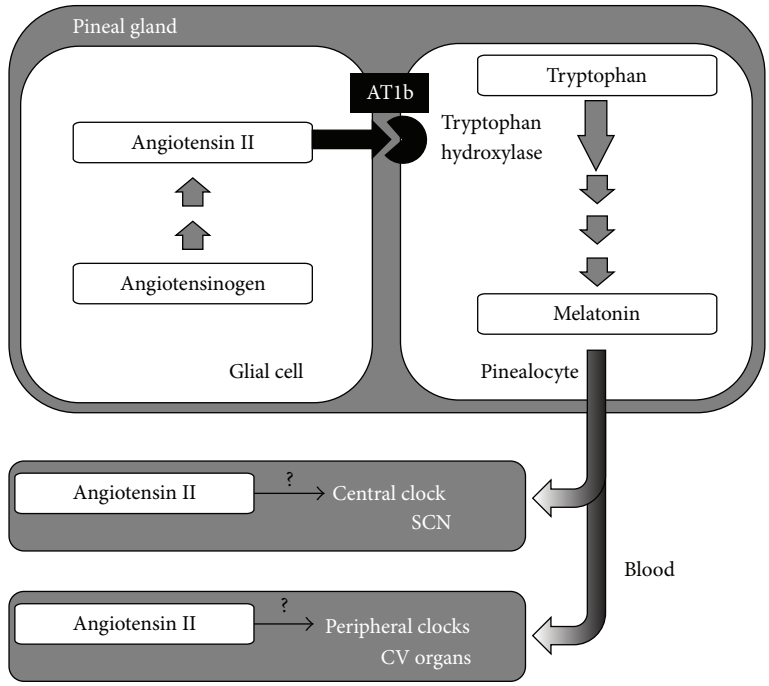


Figure 1: Pineal renin-angiotensin system (RAS) interacts with melatonin synthesis. Angiotensin II, produced from angiotensinogen produced by glial cells, acts on AT1b receptors present on pinealocytes to stimulate tryptophan hydroxylase, which is the rate-limiting enzyme in melatonin synthesis. Both angiotensin and melatonin may interact to regulate rhythmicity either centrally in the suprachiasmatic nucleus (SCN) or peripherally in clocks present in several cardiovascular organs.

*3.2. Circadian Rhythms of the Cardiovascular System.* Multiple clinical studies have implicated blood pressure (BP) and heart rate (HR) variability in the diagnosis and prognosis of arterial hypertension and cardiovascular diseases. In healthy individuals, there is a circadian variation of BP with a nocturnal fall of 10%–20% during the sleep period [26]. In hypertensive patients, this circadian rhythm may disappear or even become inverted. Therefore, according to the BP circadian alterations, patients have been classified as "dippers" when the mean nighttime BP is ≥10% lower than the mean daytime BP, as "nondippers" when the reduction is <10% or as "risers" when it is higher [27]. Nondippers and risers are at an increased risk for target organ damage and cardiovascular events [28, 29]. Moreover, a circadian pattern becomes quite obvious in the occurrence of acute cardiovascular diseases, such as ischemia, infarction, stroke, and sudden death, and new chronotherapeutic approaches in antihypertensive therapy are trying to exploit the knowledge of circadian rhythms in order to reduce these events [29]. Therefore, a better understanding of the molecular biology and pathophysiology of nondipper hypertension will lead to a better understanding of the disease and possibly lead to new diagnostic tools or therapeutic strategies. Further studies investigating the molecular mechanisms of the circadian regulation of the cardiovascular system should hopefully reveal new diagnostic tools or treatment algorithms for disease.

Our group was the first to do demonstrate that chronic Ang II infusion may induce a shift in the circadian BP rhythm (Figure 2) [16]. Ang II infused subcutaneously at doses of up to 250 ng/kg per minute that does not produce direct vasoconstriction is described as "slow pressor" or "subpressor" and can induce a gradual increase of BP. Chronic infusion (days to weeks) of subpressor Ang II subcutaneously induces nondipper hypertension similarly with the renovascular and other forms of human hypertension where the circadian variation of blood pressure is altered [16, 30, 31]. Alterations in the circadian BP rhythm are not synchronized with alterations of heart rate or locomotor activity, contributing to the concept that the circadian variability in blood pressure and heart rate are differentially regulated [15, 16]. We further hypothesized that the brain RAS might be involved in the Ang II-induced BP circadian shift. To test this hypothesis, we studied a transgenic rat that has reduced angiotensinogen levels in the brain through expression of an antisense RNA against angiotensinogen, induced by means of the astrocyte-specific glial fibrillary acidic protein promoter [32]. Ang II infusion in the TGR(ASrAOGEN) transgenic rats did not induce a BP circadian shift, indicating that peripheral RAS interacts with the brain RAS to induce not only hypertension [2] but also a BP circadian shift [15, 16]. We employed the TGR(ASrAOGEN) to investigate if the brain RAS is involved in circadian rhythm reentrainment to light phase shifts. The BP and HR acrophases (peak time of curve fitting) in TGR(ASrAOGEN) rats readjusted to light shifts significantly slower than in control (Sprague-Dawley) rats [15, 16]. However, the acrophases of locomotor activity changed similarly in both strains. These data suggest that

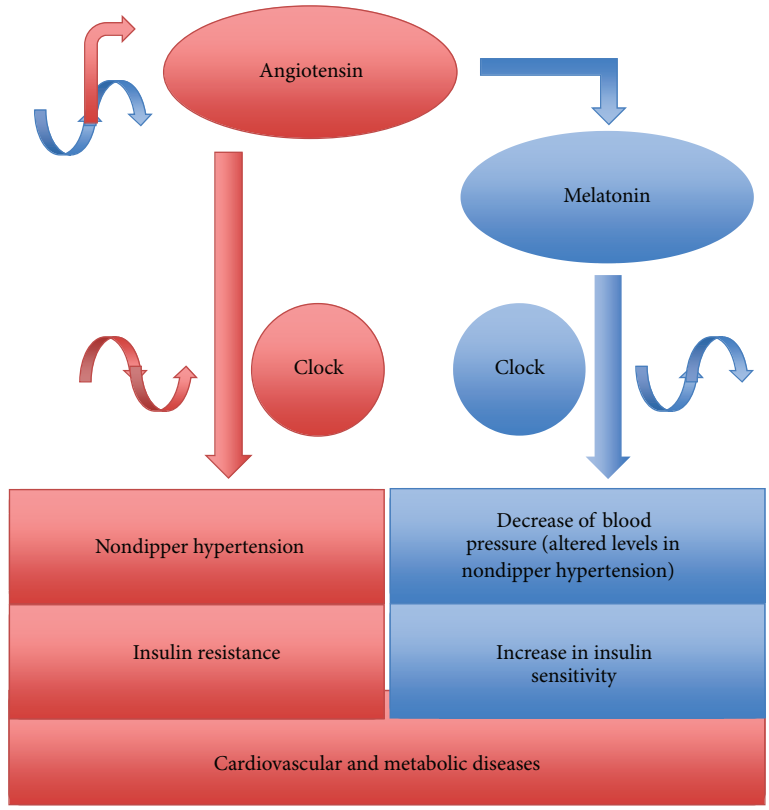


Figure 2: Angiotensin versus melatonin in cardiovascular and metabolic diseases. An increase in angiotensin induces nondipper/riser hypertension, which is characterized by a decrease in melatonin. Angiotensin and melatonin have opposing effects on insulin sensitivity.

treatment with RAS blockers with high penetrability of the blood-brain barrier (such as candesartan and valsartan) could slow the resynchronization of cardiovascular system in jet-lag conditions of travelers adapting to a new time zone.

Research on the circadian actions of Ang II has preoccupied several research groups. Among these, the group of Lemmer has provided several lines of evidence on the importance and the mechanisms regulating cardiovascular circadian rhythms [29]. Lemmer et al. provided significant insights in the pathophysiology of a transgenic rat model, TGR(mREN2)27 [17, 18]. The TGR(mREN2)27 is a well-characterized model of malignant hypertension due to an overactive RAS as it harbors the mouse salivary gland renin gene (mREN2). The TGR(mREN2)27 not only become hypertensive and develop target-organ damage but also exhibit an inverted circadian rhythm of BP, which makes them a valuable model to further study the molecular biology of circadian rhythms. However, detailed mechanisms responsible for the Ang II-induced nondipper hypertension are still not well understood. The circadian rhythms that are a characteristic of most of the physiological parameters are governed by biological clocks. Ablation of the SCN of the hypothalamus that serves as the main zeitgeber for such circadian rhythms eliminates BP, HR, and locomotor activity [33, 34]. Ang II might interact directly or through melatonin to influence the 24-h rhythmic expression of clock genes in SCN (Figure 1). Evidence on a mutual relationship between melatonin and circadian oscillators has recently been reviewed by Hardeland [35]. Also, an impaired nocturnal melatonin secretion has been detected in nondipper hypertensive patients (Figure 2) [36]. Besides melatonin, the brain RAS might influence the central circadian clock present in the hypothalamic SCN either directly or through vasopressin that is another hormone with demonstrated roles in circadian rhythms [8, 11, 37].

Ang II and melatonin might interact with circadian oscillators present not only in the SCN but also in peripheral tissues, including cardiovascular organs (Figure 1) [12]. Several animal studies are identifying roles for clock genes in cardiovascular physiology [13]. Genetic manipulation of clock genes in transgenic mouse models has uncovered new functions of internal clocks in pathogenesis of cardiovascular diseases [14]. Recent evidence is suggesting that a disruption of central or peripheral clocks may contribute to the progression of cardiovascular diseases [38]. Researching for further insights on the roles of biological clocks in cardiovascular organs shall provide acumens into the relevance of the circadian rhythms in cardiovascular pathology.

*3.3. Metabolic Syndrome, Insulin Resistance, and Diabetes.* Several lines of evidence including successful therapies with drugs acting on RAS are demonstrating roles of RAS in diabetes mellitus and metabolic syndrome. Putnam et al. recently reviewed the accumulating evidence describing the RAS as a "target of and contributor to dyslipidemias, altered glucose homeostasis, and hypertension of the metabolic

syndrome" [39]. Angiotensin II causes insulin resistance through activation of the AT1R and increased production of mineralocorticoids [40]. However, the underlying mechanisms of Ang II leading to insulin resistance remain to be fully elucidated. Melatonin on the other hand induces an increased insulin sensitivity [35]. Evidence for a link between melatonin and insulin came from pinealectomized animals that develop diabetogenic syndrome characterized by insulin resistance and a 50% reduction of GLUT4 in adipose and muscular tissue [41]. Moreover, it was demonstrated that the absence of melatonin in pinealectomized animals impairs the temporal organization of several metabolic functions associated to the carbohydrate metabolism, such as daily insulin secretion, adaptation to starvation, and exercise [42–44]. This dramatic picture can be partially or totally restored by melatonin reposition or restricted feeding [45, 46]. Melatonin acting through $MT_1$ membrane receptors is able to induce insulin receptor phosphorylation at the same time that mobilizes several intracellular transduction steps in common to insulin signaling [47]. Melatonin is able to restore insulin sensitivity and regulates food ingestion and body weight and abdominal adiposity in old rats [48].

Not only melatonin regulates insulin but also insulin can act on *in vitro* pineal glands potentiating the noradrenergic-induced melatonin synthesis, regulating the activity of the enzymes tryptophan hydroxylase and N-acetyltransferase through after-transcriptional mechanisms [49].

The first reports on diabetes and melatonin production showed that diabetic rats and mice, chemically-induced by alloxan (ALX) or streptozotocin (STZ), presented a decrease in melatonin synthesis and plasma levels [50, 51], although Champney et al. [52] reported no alterations in the level of melatonin in diabetic rats. Melatonin was also observed to suppress the onset of type 1 diabetes in nonobese mice, while pinealectomy had the opposite effect [53]. In type 2 diabetic patients and diabetic Goto Kakizaki rats a decreased serum melatonin level was observed [54]. In STZ-induced diabetic animals, melatonin was shown to decrease serum lipid oxidation [55] and protein glycosylation [56], as well as regulate the activity of antioxidant enzymes, improving the protection against the oxidative damage caused by diabetes [57–59]. Despite that, melatonin was not able to normalize hyperglycemia and/or body weight in these animals [55, 60, 61] and lower levels of the indolamine were observed in peripheral tissues like pancreas, kidney, spleen, and duodenum [62].

## 4. Conclusions and Perspectives

Accumulating evidence suggests that not only angiotensin interferes with melatonin synthesis and release but also both hormones interact at several levels having opposing effects in cardiovascular and metabolic pathophysiology (Table 1). Furthermore, evidence is indicating that not only melatonin but also angiotensin may interfere with circadian rhythms. The intimal regulatory mechanisms of interference between the two systems both centrally and peripherally, at synthesis and action levels, in homeostatic and disease conditions await further investigation. On how peripheral RAS interacts with the pineal RAS in hypertension and cardiovascular disease and if the sympathetic nervous system or other systems are involved in this interaction are still open questions.

Contemporary progress in chronobiology directs to an important role of clock-associated genes in the progression of cardiovascular and metabolic diseases. Since angiotensin appears to be involved in the modulation of circadian rhythms of blood pressure, an interaction with the clock genes seems likely. Therefore, we believe that further research on the molecular biology of circadian alterations involving interactions between angiotensin, melatonin, and clock genes may have an impact on cardiovascular and metabolic pathophysiology leading to new chronotherapeutic strategies.

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## References

[1] M. Bader, J. Peters, O. Baltatu, D. N. Müller, F. C. Luft, and D. Ganten, "Tissue renin-angiotensin systems: new insights from experimental animal models in hypertension research," *Journal of Molecular Medicine*, vol. 79, no. 2, pp. 76–102, 2001.

[2] L. A. Campos, M. Bader, and O. C. Baltatu, "Brain renin-angiotensin system in hypertension, cardiac hypertrophy, and heart failure," *Frontiers in Physiology*, vol. 2, artile 115, 2011.

[3] O. C. Baltatu, L. A. Campos, and M. Bader, "Local renin-angiotensin system and the brain: a continuous quest for knowledge," *Peptides*, vol. 32, no. 5, pp. 1083–1086, 2011.

[4] T. G. von Lueder and H. Krum, "RAAS inhibitors and cardiovascular protection in large scale trials," *Cardiovascular Drugs and Therapy*, 2012. In press.

[5] P. Pevet and E. Challet, "Melatonin: both master clock output and internal time-giver in the circadian clocks network," *Journal of Physiology Paris*, vol. 105, no. 4-6, pp. 170–182, 2011.

[6] R. Hardeland, "Neurobiology, pathophysiology, and treatment of melatonin deficiency and dysfunction," *The Scientific World Journal*, vol. 2012, Article ID 640389, 18 pages, 2012.

[7] A. Carpentieri, G. Díaz de Barboza, V. Areco, and N. Tolosa de Talamoni, "New perspectives in melatonin uses," *Pharmacological Research*, vol. 65, no. 4, pp. 437–444, 2012.

[8] L. A. Campos, A. S. Couto, R. Iliescu et al., "Differential regulation of central vasopressin receptors in transgenic rats with low brain angiotensinogen," *Regulatory Peptides*, vol. 119, no. 3, pp. 177–182, 2004.

[9] O. Baltatu, H. Nishimura, S. Hoffmann et al., "High levels of human chymase expression in the pineal and pituitary glands," *Brain Research*, vol. 752, no. 1-2, pp. 269–278, 1997.

[10] O. Baltatu, A. Lippoldt, A. Hansson, D. Ganten, and M. Bader, "Local renin-angiotensin system in the pineal gland," *Molecular Brain Research*, vol. 54, no. 2, pp. 237–242, 1998.

[11] O. Baltatu, S. C. Afeche, S. H. José dos Santos et al., "Locally synthesized angiotensin modulates pineal melatonin generation," *Journal of Neurochemistry*, vol. 80, no. 2, pp. 328–334, 2002.

[12] D. F. Reilly, E. J. Westgate, and G. A. FitzGerald, "Peripheral circadian clocks in the vasculature," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 27, no. 8, pp. 1694–1705, 2007.

[13] H. Duez and B. Staels, "Nuclear receptors linking circadian rhythms and cardiometabolic control," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 30, no. 8, pp. 1529–1534, 2010.

[14] N. Takeda and K. Maemura, "Circadian clock and vascular disease," *Hypertension Research*, vol. 33, no. 7, pp. 645–651, 2010.

[15] L. A. Campos, R. Plehm, J. Cipolla-Neto, M. Bader, and O. C. Baltatu, "Altered circadian rhythm reentrainment to light phase shifts in rats with low levels of brain angiotensinogen," *The American Journal of Physiology*, vol. 290, no. 4, pp. R1122–R1127, 2006.

[16] O. Baltatu, B. J. Janssen, G. Bricca et al., "Alterations in blood pressure and heart rate variability in transgenic rats with low brain angiotensinogen," *Hypertension*, vol. 37, no. 2, pp. 408–413, 2001.

[17] I. Herichová, B. Mravec, K. Stebelová et al., "Rhythmic clock gene expression in heart, kidney and some brain nuclei involved in blood pressure control in hypertensive TGR(mREN-2)27 rats," *Molecular and Cellular Biochemistry*, vol. 296, no. 1-2, pp. 25–34, 2007.

[18] B. Lemmer, K. Witte, H. Enzminger, S. Schiffer, and S. Hauptfleisch, "Transgenic TGR(mREN2)27 rats as a model for disturbed circadian organization at the level of the brain, the heart, and the kidneys," *Chronobiology International*, vol. 20, no. 4, pp. 711–738, 2003.

[19] S. W. Holmes and D. Sugden, "The effect of melatonin on pinealectomy induced hypertension in the rat," *British Journal of Pharmacology*, vol. 56, no. 3, pp. 360–361, 1976.

[20] H. Karppanen, S. Lahovaara, P. Mannisto, and H. Vapaatalo, "Plasma renin activity and in vitro synthesis of aldosterone by the adrenal glands of rats with spontaneous, renal, or pinealectomy induced hypertension," *Acta Physiologica Scandinavica*, vol. 94, no. 2, pp. 184–188, 1975.

[21] P. J. Meneuvonen and H. Karppanen, "Effects of hydrochlorothiazide, furosemide and ethacrynic acid on pinealectomy-induced hypertension in rats," *Annales Medicinae Experimentalis et Biologiae Fenniae*, vol. 49, no. 3, pp. 120–124, 1971.

[22] R. M. Slominski, R. J. Reiter, N. Schlabritz-Loutsevitch, R. S. Ostrom, and A. T. Slominski, "Melatonin membrane receptors in peripheral tissues: distribution and functions," *Molecular and Cellular Endocrinology*, vol. 351, no. 2, pp. 152–166, 2012.

[23] S. Tengattini, R. J. Reiter, D. X. Tan, M. P. Terron, L. F. Rodella, and R. Rezzani, "Cardiovascular diseases: protective effects of melatonin," *Journal of Pineal Research*, vol. 44, no. 1, pp. 16–25, 2008.

[24] A. Dominguez-Rodriguez, "Melatonin in cardiovascular disease," *Expert Opinion on Investigational Drugs*, vol. 21, no. 11, pp. 1593–1596, 2012.

[25] A. Dominguez-Rodriguez, P. Abreu-Gonzalez, and P. Avanzas, "The role of melatonin in acute myocardial infarction," *Frontiers in Bioscience*, vol. 17, no. 7, pp. 2433–2441, 2011.

[26] F. Fabbian, M. H. Smolensky, R. Tiseo et al., "Dipper and non-dipper blood pressure 24-Hour patterns: circadian rhythm-dependent physiologic and pathophysiologic mechanisms," *Chronobiology International*, 2012. In press.

[27] "Guidelines for the clinical use of 24 hour ambulatory blood pressure monitoring (ABPM) (JCS 2010)-Digest version," *Circulation Journal*, vol. 76, no. 2, pp. 508–519, 2012.

[28] R. C. Hermida, D. E. Ayala, A. Mojon, and J. R. Fernandez, "Blunted sleep-time relative blood pressure decline increases cardiovascular risk independent of blood pressure level-the "normotensive non-dipper" paradox," *Chronobiology International*, 2012. In press.

[29] B. Lemmer, "The importance of circadian rhythms on drug response in hypertension and coronary heart disease-from mice and man," *Pharmacology and Therapeutics*, vol. 111, no. 3, pp. 629–651, 2006.

[30] G. Simon, G. Abraham, and G. Cserep, "Pressor and subpressor angiotensin II administration. Two experimental models of hypertension," *The American Journal of Hypertension*, vol. 8, no. 6, pp. 645–650, 1995.

[31] O. Baltatu, J. A. Silva Jr., D. Ganten, and M. Bader, "The brain renin-angiotensin system modulates angiotensin II-induced hypertension and cardiac hypertrophy," *Hypertension*, vol. 35, no. 1, pp. 409–412, 2000.

[32] M. Schinke, O. Baltatu, M. Böhm et al., "Blood pressure reduction and diabetes insipidus in transgenic rats deficient in brain angiotensinogen," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 96, no. 7, pp. 3975–3980, 1999.

[33] B. J. A. Janssen, C. M. Tyssen, H. Duindam, and W. J. Rietveld, "Suprachiasmatic lesions eliminate 24-h blood pressure variability in rats," *Physiology and Behavior*, vol. 55, no. 2, pp. 307–311, 1994.

[34] K. Witte, A. Schnecko, R. M. Buijs et al., "Effects of SCN lesions on circadian blood pressure rhythm in normotensive and transgenic hypertensive rats," *Chronobiology International*, vol. 15, no. 2, pp. 135–145, 1998.

[35] R. Hardeland, "Melatonin in aging and disease-multiple consequences of reduced secretion, options and limits of treatment," *Aging and Disease*, vol. 3, no. 2, pp. 149–225, 2012.

[36] M. Jonas, D. Garfinkel, N. Zisapel, M. Laudon, and E. Grossman, "Impaired nocturnal melatonin secretion in non-dipper hypertensive patients," *Blood Pressure*, vol. 12, no. 1, pp. 19–24, 2003.

[37] L. A. Campos, S. C. Afeche, R. Plehm et al., "Altered circadian rhythm reentrainment and pineal indoles in rats with low brain angiotensinogen," *Journal of Hypertension*, vol. 20, p. 1086, 2002.

[38] N. Takeda and K. Maemura, "Circadian clock and cardiovascular disease," *Journal of Cardiology*, vol. 57, no. 3, pp. 249–256, 2011.

[39] K. Putnam, R. Shoemaker, F. Yiannikouris, and L. A. Cassis, "The renin-angiotensin system: a target of and contributor to dyslipidemias, altered glucose homeostasis, and hypertension of the metabolic syndrome," *The American Journal of Physiology*, vol. 302, no. 6, pp. H1219–H1230, 2012.

[40] G. Lastra-Lastra, J. R. Sowers, K. Restrepo-Erazo, C. Manrique-Acevedo, and G. Lastra-González, "Role of aldosterone and angiotensin II in insulin resistance: an update," *Clinical Endocrinology*, vol. 71, no. 1, pp. 1–6, 2009.

[41] F. B. Lima, U. F. Machado, I. Bartol et al., "Pinealectomy causes glucose intolerance and decreases adipose cell responsiveness to insulin in rats," *The American Journal of Physiology*, vol. 275, no. 6, pp. E934–E941, 1998.

[42] C. N. Borges-Silva, M. I. C. Alonso-Vale, S. M. Franzói-De-Moraes et al., "Pinealectomy impairs adipose tissue adaptability to exercise in rats," *Journal of Pineal Research*, vol. 38, no. 4, pp. 278–283, 2005.

[43] M. I. Cardoso Alonso-Vale, G. F. Anhê, C. N. Borges-Silva et al., "Pinealectomy alters adipose tissue adaptability to fasting in rats," *Metabolism*, vol. 53, no. 4, pp. 500–506, 2004.

[44] M. C. Picinato, E. P. Haber, A. R. Carpinelli, and J. Cipolla-Neto, "Daily rhythm of glucose-induced insulin secretion by isolated islets from intact and pinealectomized rat," *Journal of Pineal Research*, vol. 33, no. 3, pp. 172–177, 2002.

[45] M. M. Zanquetta, P. M. Seraphim, D. H. Sumida, J. Cipolla-Neto, and U. F. Machado, "Calorie restriction reduces pinealectomy-induced insulin resistance by improving GLUT4 gene expression and its translocation to the plasma membrane," *Journal of Pineal Research*, vol. 35, no. 3, pp. 141–148, 2003.

[46] F. B. Lima, D. H. Matsushita, N. S. Hell, M. S. Dolnikoff, M. M. Okamoto, and J. Cipolla Neto, "The regulation of insulin action in isolated adipocytes. Role of the periodicity of food intake, time of day and melatonin," *Brazilian Journal of Medical and Biological Research*, vol. 27, no. 4, pp. 995–1000, 1994.

[47] G. F. Anhê, L. C. Caperuto, M. Pereira-Da-Silva et al., "In vivo activation of insulin receptor tyrosine kinase by melatonin in the rat hypothalamus," *Journal of Neurochemistry*, vol. 90, no. 3, pp. 559–566, 2004.

[48] D. D. Rasmussen, B. M. Boldt, C. W. Wilkinson, S. M. Yellon, and A. M. Matsumoto, "Daily melatonin administration at middle age suppresses male rat visceral fat, plasma leptin, and plasma insulin to youthful levels," *Endocrinology*, vol. 140, no. 2, pp. 1009–1012, 1999.

[49] R. A. P. Garcia, S. C. Afeche, J. H. Scialfa et al., "Insulin modulates norepinephrine-mediated melatonin synthesis in cultured rat pineal gland," *Life Sciences*, vol. 82, no. 1-2, pp. 108–114, 2008.

[50] S. F. Pang, F. Tang, and P. L. Tang, "Alloxan-induced diabetes and the pineal gland: differential effects on the levels of pineal N-acetylserotonin, pineal melatonin, and serum melatonin," *Journal of Pineal Research*, vol. 2, no. 1, pp. 79–85, 1985.

[51] T. H. Champney, G. C. Brainard, B. A. Richardson, and R. J. Reiter, "Experimentally-induced diabetes reduces nocturnal pineal melatonin content in the Syrian hamster," *Comparative Biochemistry and Physiology A*, vol. 76, no. 1, pp. 199–201, 1983.

[52] T. H. Champney, A. P. Holtorf, C. M. Craft, and R. J. Reiter, "Hormonal modulation of pineal melatonin synthesis in rats and Syrian hamsters: effects of streptozotocin-induced diabetes and insulin injections," *Comparative Biochemistry and Physiology*, vol. 83, no. 2, pp. 391–395, 1986.

[53] A. Conti and G. J. Maestroni, "Role of the pineal gland and melatonin in the development of autoimmune diabetes in non-obese diabetic mice," *Journal of Pineal Research*, vol. 20, no. 3, pp. 164–172, 1996.

[54] E. Peschke, T. Frese, E. Chankiewitz et al., "Diabetic Goto Kakizaki rats as well as type 2 diabetic patients show a decreased diurnal serum melatonin level and an increased pancreatic melatonin-receptor status," *Journal of Pineal Research*, vol. 40, no. 2, pp. 135–143, 2006.

[55] A. Armagan, E. Uz, H. R. Yilmaz, S. Soyupek, T. Oksay, and N. Ozcelik, "Effects of melatonin on lipid peroxidation and antioxidant enzymes in streptozotocin-induced diabetic rat testis," *Asian Journal of Andrology*, vol. 8, no. 5, pp. 595–600, 2006.

[56] P. L. Montilla, J. F. Vargas, I. F. Túnez et al., "Oxidative stress in diabetic rats induced by streptozotocin: protective effects of melatonin," *Journal of Pineal Research*, vol. 25, no. 2, pp. 94–100, 1998.

[57] A. Guven, O. Yavuz, M. Cam et al., "Effects of melatonin on streptozotocin-induced diabetic liver injury in rats," *Acta Histochemica*, vol. 108, no. 2, pp. 85–93, 2006.

[58] K. Winiarska, T. Fraczyk, D. Malinska, J. Drozak, and J. Bryla, "Melatonin attenuates diabetes-induced oxidative stress in rabbits," *Journal of Pineal Research*, vol. 40, no. 2, pp. 168–176, 2006.

[59] M. Kanter, H. Uysal, T. Karaca, and H. O. Sagmanligil, "Depression of glucose levels and partial restoration of pancreatic $\beta$-cell damage by melatonin in streptozotocin-induced diabetic rats," *Archives of Toxicology*, vol. 80, no. 6, pp. 362–369, 2006.

[60] E. J. Sudnikovich, Y. Z. Maksimchik, S. V. Zabrodskaya et al., "Melatonin attenuates metabolic disorders due to streptozotocin-induced diabetes in rats," *European Journal of Pharmacology*, vol. 569, no. 3, pp. 180–187, 2007.

[61] H. Vural, T. Sabuncu, S. Oktay Arslan, and N. Aksoy, "Melatonin inhibits lipid peroxidation and stimulates the antioxidant status of diabetic rats," *Journal of Pineal Research*, vol. 31, no. 3, pp. 193–198, 2001.

[62] K. Stebelová, I. Herichová, and M. Zeman, "Diabetes induces changes in melatonin concentrations in peripheral tissues of rat," *Neuroendocrinology Letters*, vol. 28, no. 2, pp. 159–165, 2007.

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*Review Article*

# RAS in Pregnancy and Preeclampsia and Eclampsia

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Preeclampsia is a common disease of pregnancy characterized by the presence of hypertension and commitment of many organs, including the brain, secondary to generalized endothelial dysfunction. Its etiology is not known precisely, but it involved several factors, highlighting the renin angiotensin system (RAS), which would have an important role in the origin of multisystem involvement. This paper reviews the evidence supporting the involvement of RAS in triggering the disease, in addition to the components of this system that would be involved and how it eventually produces brain engagement.

## 1. Introduction

Preeclampsia is a major complication of pregnancy and corresponds to a major cause of both maternal and fetal morbidity and mortality [1–3]. It is a condition that produces a compromise of many organs, including the brain causing seizures, a condition known as eclampsia [4, 5]. The pathophysiology is not well understood, but it involves different factors, such as genetic, immunological, and inflammatory [6, 7]. In recent years there is a series of studies linking the renin angiotensin system (RAS) with preeclampsia [8–10], in the sense that the alteration of this system would be involved in the pathogenesis of this disease, as this could trigger the different characteristics in this pathology, including brain involvement.

## 2. RAS in Normal Pregnancy

RAS is a system that functions as an important regulator of blood pressure, electrolyte balance, and fluid homeostasis [11]. This system comprises the inactive peptide angiotensinogen, which is converted to angiotensin I and then the active peptide angiotensin II (Ang II) through the action of renin and angiotensin-converting enzyme (ACE) [12]. Ang II exerts its action primarily through the AT1 receptor, located widely in different tissues, including the syncytiotrophoblast [10].

During pregnancy usually occurs overexpression of many components of the RAS, both in the blood and tissues. There is an increase in plasma renin mainly by extrarenal production [13]. There is also a higher-level production of angiotensinogen liver secondary to increased circulating estrogens. ACE is the only component that has been shown to decrease during normal pregnancy, but equally there is a higher plasma concentration of Ang II [8, 13].

There is an upregulation of RAS components during normal pregnancy, but there is also a decrease in sensitivity to Ang II, whereby these women are resistant to the pressor effect of this molecule, requiring twice Ang II by intravenous infusion compared with nonpregnant women to achieve a similarly vasomotor response [14].

It is thought that this might be related to the monomer structure of AT1 during uncomplicated pregnancies, unlike the heterodimeric structure observed in terms of sensitivity to Ang II [15]. In addition, estrogens produce a shift in the formation of angiotensin peptides, reducing the formation of Ang II and increasing the production of Ang-(1–7), which has a vasodilator role [16].

Furthermore, in addition to a systemic RAS, RAS also exists in uteroplacental territory [13]. This unit consists

of a placental portion, corresponding to fetal tissue, and a decidual, which is of maternal origin, and in both all components of the RAS are secreted. Therefore, there are 2 RAS systems: placental and decidual. The latter could be related to the pregnancy-associated vascular remodeling of the spiral arteries [17].

## 3. Pathophysiology of Preeclampsia: Role of RAS

Preeclampsia corresponds to a multisystem disorder characterized by increased peripheral vascular resistance, increased platelet aggregation, and systemic endothelial dysfunction [18]. Corresponds to a multifactorial disease, involving genetic and environmental components, a defective extravillous trophoblast invasion, an impaired immune tolerance between maternal, fetal and placental tissues and maternal inflammatory disorders [19, 20]. Clinically it is characterized by the presence of hypertension and proteinuria from the 2nd half of pregnancy, and the only effective treatment is the termination of pregnancy [21].

From a physiological point of view, preeclampsia is defined as a disease of two stages [22]. The first is the placental stage that occurs during the first 20 weeks of gestation. In this, the phenomena of remodeling of the vascular walls of the spiral arteries do not develop properly, resulting in abnormal placentation, thus prompting ischemic placenta [23]. The second stage occurs during the second half of pregnancy and is known as the systemic stage. This is the clinical stage of preeclampsia, in which there is an exaggerated maternal systemic inflammatory response and endothelial dysfunction as a central element [24–26]. Between these two stages are some mediators, which are understood as molecules released by the placenta and are capable of transmitting this placental damage and translate into a systemic involvement. Mediators most studied are oxidative stress, microfragments of syncytiotrophoblast (STBM), and antiangiogenic proteins [27].

There is a considerable amount of evidence supporting the role of angiogenic factors in triggering preeclampsia, and these are the tyrosine-like soluble factor (sFlt-1) and soluble endoglin (s-Eng) [28, 29]. These molecules bind to angiogenic proteins such as VEGF and prevent them from joining their membrane receptors on endothelial cells, leading to endothelial dysfunction [30]. It was observed that these factors are elevated about 6–8 weeks before the start of the clinical picture of preeclampsia, and their plasma concentrations are related to the severity of the disease [31, 32]. In animal models it has been found that inoculation of these can produce hypertension, proteinuria, and hepatic involvement, symptoms characteristic of preeclampsia [33, 34]. It is also observed that hypoxia causes increased secretion of these factors [35].

In patients with preeclampsia, dysregulation has been observed in the RAS compared to healthy pregnancies. The levels of renin, Ang I, and Ang II are lower than in uncomplicated pregnancies [36]. Despite this decrease in the expression of RAS components, in patients suffering from preeclampsia increased sensitivity to Ang II exists, showing an exaggerated pressor response to Ang II.

## 4. AT1 Receptors Autoantibodies in Preeclampsia

In recent years there is a wealth of evidence supporting the AT1 autoantibodies (AT1-AA) in the pathogenesis of preeclampsia. These correspond to IgG autoantibodies that bind to a seven-amino acid sequence present on the second extracellular loop of the AT1 receptor [37, 38]. They are present in the plasma of patients with preeclampsia and are able to increase the beating rate of the cultured cardiomyocytes [39]. Many research papers show that these autoantibodies are elevated in patients with preeclampsia, but not in uncomplicated pregnancies.

*In vitro* and *in vivo* studies have determined its role in triggering preeclampsia. These antibodies bind to the AT1 receptors of different cell groups, triggering its pathological action [40]. It has been observed that in human trophoblast cells AT1-AA substances induce the generation of reactive oxygen species (ROS) intracellularly through NDPH oxidase activation [41]. In addition, these same cells stimulate the release of PAI-1, resulting in a decreased trophoblast invasiveness [42, 43], generating a defect of placentation. This increase in PAI-1 is also observed in mesangial cells, which can produce a decrease in extracellular matrix degradation and increased subendothelial fibrin deposition thereby determining renal damage leading to proteinuria and a decreased glomerular filtration rate [44]. It has also been observed that AT1-AA binds to endothelial and vascular cells, causing endothelial damage and vasoconstriction [45, 46].

All these actions could explain endothelial dysfunction, increased peripheral vascular resistance, and impaired coagulation system observed in preeclampsia.

In animal models it has been observed that the inoculation of AT1-AA from patients with preeclampsia is capable of reproducing the characteristics of the disease [47, 48]. Reports in rats showed that surgically induced placental ischemia may cause increased levels of AT1-AA and trigger hypertension and proteinuria [49–51]. It was also observed that these autoantibodies stimulate the release of sFlt-1 and s-eng by the placenta, key proteins in triggering endothelial dysfunction [52, 53].

In the same way pregnant human studies show that placental perfusion abnormality, evaluated with Doppler ultrasound of the uterine arteries, is associated with increased plasma concentrations of AT1-AA before the onset of the disease and that plasmatic levels of these autoantibodies correlate with the severity [54]. The concentration of these autoantibodies is higher in cases of severe preeclampsia, also having a linear correlation with proteinuria and hypertension. This is further confirmed by the fact that in milder cases, as moderate preeclampsia and gestational hypertension, levels of AT1-AA are higher than in normotensive pregnancies, but lower than in severe preeclampsia. Therefore, AT1-AA would be a key element in the pathogenesis of preeclampsia and modulate the secretion

of important factors responsible for the pathophysiology [55].

## 5. Eclampsia: Loss of Autoregulation of Cerebral Blood Flow and Possible Role of RAS

One of the most serious complications of preeclampsia is eclampsia, which corresponds to the presence of seizures in the context of a patient with hypertension and proteinuria [5, 56]. However, currently the eclampsia is being seen as a manifestation of a much larger entity than pregnancy, known as posterior reversible encephalopathy (PRES), which is produced by other conditions such as hypertensive encephalopathy or use of immunosuppressive drugs. In these cases there is a characteristic increase in blood pressure and/or alteration of endothelial permeability [57–60].

The PRES is characterized by the presence of well-defined signs and symptoms associated with the presence of specific neuroimaging. Among the symptoms are headache, nausea, vomiting, visual disturbances, and seizures [61, 62]. Diagnosis is by observation of symmetrical hyperintense lesions and bilateral parietooccipital level on MRI, suggestive of vasogenic edema [63].

The exact pathophysiology of PRES is not known with certainty, but is thought to be due to alterations of the vasculature and cerebral perfusion [64]. With the increase in blood pressure, brain responds with its vasculature vasoconstriction, which determines an increase of cerebral perfusion pressure (CPP). As this CPP remains persistently high, it will produce a pressure transmission to the distal small cerebral vessels, causing endothelial damage and muscle dysfunction of cerebral vascular territory [65, 66]. This phenomenon is known as barotrauma, corresponding to forced dilation of arterioles and distal opening of endothelial tight junctions, determining a disruption of the blood-brain barrier (BBB), which leads to increased permeability of this, resulting in a vasogenic edema [67].

However, not all cases of PRES relate to alterations in arterial pressure. One study shows that 16% of cases of eclampsia occur in normotensive patients, and only 13% of cases were associated with severe hypertension [68, 69]. Therefore, the loss of autoregulation of cerebral perfusion secondary to hypertension does not explain all cases of PRES, so that alteration of endothelial permeability by disruption of the BBB ought to play an important role [67]. A recent study shows that preeclampsia altered BBB permeability independently of blood pressure. In this report it is determined that plasma from patients with preeclampsia significantly increases the permeability of the BBB, compared to plasma from patients with normal pregnancies [70]. These findings support the concept of the pathophysiology of PRES that is determined by hemodynamic factors and factors altering endothelial function.

Various reports have identified the involvement of RAS in the blood-brain barrier disruption in other medical conditions [71]. Therefore, the presence of AT1-AA in patients with preeclampsia could play a key role in triggering PRES. It is shown that these autoantibodies produced an increase in peripheral vascular resistance and hypertension, which leads to a systemic endothelial dysfunction via ROS secretion and antiangiogenic proteins [37, 40], so directly involved in the conditions that can trigger PRES.

## 6. Management of Preeclampsia and Eclampsia Prevention

Currently, the only effective treatment for preeclampsia is the termination of pregnancy, which in very early pregnancies may not be the best alternative, as this results in increased perinatal morbidity and mortality secondary to prematurity [72]. Therefore, it is important to seek management strategies from the physiological point of view, and in that sense, the management of RAS alteration appears to be a logical choice, given the strong evidence about an excessive AT1 receptor activation during preeclampsia.

Regarding eclampsia, the drug of choice for prevention and management is magnesium sulfate. This drug reduces the risk of seizures in patients with severe preeclampsia [73]. Its mechanism of action is not entirely clear, but it has been shown to be capable of reducing the CPP [74]. However, it should be administered intravenously and usually for 48 hrs, in patient hospitalized and only for short periods of time.

Many studies determined that the addition of losartan (AT1 receptors blocker) or neutralizing antibodies of the AT1-AA (7-amino acid peptide epitope or 7-aa) blocks the effect of AT1-AA, further confirming that the action of these antibodies is through activation of AT1 receptors (z). Animal studies with surgically induced placental ischemia demonstrate that the addition of these compounds significantly reduces arterial pressure [49], but this effect was not seen with ACE inhibitors [75]. In another report, AT1-AA purified from pregnant women was injected in mice. They triggered hypertension and proteinuria, were significantly reduced or abolished when administered losartan or 7-aa [47]. Adoptive transfer studies in pregnant mice have demonstrated that release of antiangiogenic factors and proinflammatory cytokines resulting from autoantibody-mediated receptor activation is blocked by the addition of 7-aa or AT1 receptor antagonist [10, 37].

The main problem with AT1 receptors blockers is that they increase the risk of fetal malformations, such as oligohydramnios, pulmonary hypoplasia, transient renal failure, preterm delivery, and Potter syndrome [76–78], so its use during pregnancy should be avoided. However, AT1 receptors blockers can be used postpartum, and considering that currently about 30% of eclampsia occur in postpartum [56, 79] and that AT1-AA levels can remain high for a long time [80], AT1 receptors blockers become an interesting strategy for the management of hypertension during the postpartum period and to decrease the incidence of eclampsia, as well as controlling blood pressure; they block the action of endothelial AT1-AA, which is still present in the postpartum period.

Neutralizing antibodies 7-aa have the advantage of not inhibiting the AT1 receptor; they only block the AT1-AA, without changing completely the action of Ang II.

Therefore, it seems a useful strategy for the management of preeclampsia. However, we must await the development of further studies to determine its safety during pregnancy.

## 7. Discussion

During preeclampsia there is an alteration of the RAS. The presence of AT1-AA determines triggering a series of actions in tissues and organs that would result in an increase in peripheral vascular resistance, altered coagulation, renal impairment, and systemic endothelial dysfunction. These alterations can generate the commitment of many organs, including the brain, producing a PRES.

Blocking the action of these autoantibodies using losartan or 7-aa substantially decreases the damage caused by AT1-AA, creating an opportunity for the management and prevention of complications of preeclampsia.

## References


[1] B. Sibai, G. Dekker, and M. Kupferminc, "Pre-eclampsia," *The Lancet*, vol. 365, no. 9461, pp. 785–799, 2005.

[2] L. Duley, "Maternal mortality associated with hypertensive disorders of pregnancy in Africa, Asia, Latin America and the Caribbean," *British Journal of Obstetrics and Gynaecology*, vol. 99, no. 7, pp. 547–553, 1992.

[3] L. Duley, "The global impact of pre-eclampsia and eclampsia," *Seminars in Perinatology*, vol. 33, no. 3, pp. 130–137, 2009.

[4] E. R. Norwitz, C. D. Hsu, and J. T. Repke, "Acute complications of preeclampsia," *Clinical Obstetrics and Gynecology*, vol. 45, no. 2, pp. 308–329, 2002.

[5] G. G. Zeeman, "Neurologic complications of pre-eclampsia," *Seminars in Perinatology*, vol. 33, no. 3, pp. 166–172, 2009.

[6] J. M. Roberts and D. W. Cooper, "Pathogenesis and genetics of pre-eclampsia," *The Lancet*, vol. 357, no. 9249, pp. 53–56, 2001.

[7] J. L. James, G. S. Whitley, and J. E. Cartwright, "Pre-eclampsia: fitting together the placental, immune and cardiovascular pieces," *Journal of Pathology*, vol. 221, no. 4, pp. 363–378, 2010.

[8] D. M. Shah, "Role of the renin-angiotensin system in the pathogenesis of preeclampsia," *American Journal of Physiology*, vol. 288, no. 4, pp. F614–F625, 2005.

[9] R. A. Irani and Y. Xia, "The functional role of the renin-angiotensin system in pregnancy and preeclampsia," *Placenta*, vol. 29, no. 9, pp. 763–771, 2008.

[10] R. A. Irani and Y. Xia, "Renin angiotensin signaling in normal pregnancy and preeclampsia," *Seminars in Nephrology*, vol. 31, no. 1, pp. 47–58, 2011.

[11] D. M. Shah, "The role of RAS in the pathogenesis of preeclampsia," *Current Hypertension Reports*, vol. 8, no. 2, pp. 144–152, 2005.

[12] W. R. Welches, K. B. Brosnihan, and C. M. Ferrario, "A comparison of the properties and enzymatic activities of three angiotensin processing enzymes: angiotensin converting enzyme, prolyl endopeptidase and neutral endopeptidase 24.11," *Life Sciences*, vol. 52, no. 18, pp. 1461–1480, 1993.

[13] L. Anton and K. B. Brosnihan, "Systemic and uteroplacental renin-angiotensin system in normal and pre-eclamptic pregnancies," *Therapeutic Advances in Cardiovascular Disease*, vol. 2, no. 5, pp. 349–362, 2008.

[14] R. Abdul-Karim and N. S. Assali, "Pressor response to angiotensin in pregnant and non-pregnant women," *American Journal of Obstetrics and Gynecology*, vol. 82, pp. 246–251, 1961.

[15] S. AbdAlla, H. Lother, A. El Massiery, and U. Quitterer, "Increased AT1 receptor heterodimers in preeclampsia mediate enhanced angiotensin II responsiveness," *Nature Medicine*, vol. 7, no. 9, pp. 1003–1009, 2001.

[16] K. B. Brosnihan, L. A. A. Neves, L. Anton, J. Joyner, G. Valdes, and D. C. Merrill, "Enhanced expression of Ang-(1–7) during pregnancy," *Brazilian Journal of Medical and Biological Research*, vol. 37, no. 8, pp. 1255–1262, 2004.

[17] T. Morgan, C. Craven, J. M. Lalouel, and K. Ward, "Angiotensinogen Thr235 variant is associated with abnormal physiologic change of the uterine spiral arteries in first-trimester decidua," *American Journal of Obstetrics and Gynecology*, vol. 180, no. 1 I, pp. 95–102, 1999.

[18] L. Poston, "Endothelial dysfunction in pre-eclampsia," *Pharmacological Reports*, vol. 58, pp. 69–74, 2006.

[19] C. W. G. Redman and I. L. Sargent, "Placental stress and pre-eclampsia: a revised view," *Placenta*, vol. 30, pp. S38–S42, 2009.

[20] P. Kaufmann, S. Black, and B. Huppertz, "Endovascular trophoblast invasion: implications for the pathogenesis of intrauterine growth retardation and preeclampsia," *Biology of Reproduction*, vol. 69, no. 1, pp. 1–7, 2003.

[21] ACOG Committee on Obstetric Practice, "ACOG practice bulletin. Diagnosis and management of preeclampsia and eclampsia. Number 33, January 2002. American college of obstetricians and gynecologists," *International Journal of Gynecology and Obstetrics*, vol. 77, no. 1, pp. 67–75, 2002.

[22] C. W. Redman and I. L. Sargent, "Latest advances in understanding preeclampsia," *Science*, vol. 308, no. 5728, pp. 1592–1594, 2005.

[23] V. Chaddha, S. Viero, B. Huppertz, and J. Kingdom, "Developmental biology of the placenta and the origins of placental insufficiency," *Seminars in Fetal and Neonatal Medicine*, vol. 9, no. 5, pp. 357–369, 2004.

[24] C. W. G. Redman and I. L. Sargent, "Pre-eclampsia, the placenta and the maternal systemic inflammatory response—a review," *Placenta*, vol. 24, pp. S21–S27, 2003.

[25] F. Bernardi, F. Guolo, T. Bortolin, F. Petronilho, and F. Dal-Pizzol, "Oxidative stress and inflammatory markers in normal pregnancy and preeclampsia," *Journal of Obstetrics and Gynaecology Research*, vol. 34, no. 6, pp. 948–951, 2008.

[26] J. P. Granger, B. T. Alexander, M. T. Llinas, W. A. Bennett, and R. A. Khalil, "Pathophysiology of preeclampsia: linking placental ischemia/hypoxia with microvascular dysfunction," *Microcirculation*, vol. 9, no. 3, pp. 147–160, 2002.

[27] R. J. Levine and S. A. Karumanchi, "Circulating angiogenic factors in preeclampsia," *Clinical Obstetrics and Gynecology*, vol. 48, no. 2, pp. 372–386, 2005.

[28] S. Maynard, F. H. Epstein, and S. A. Karumanchi, "Preeclampsia and angiogenic imbalance," *Annual Review of Medicine*, vol. 59, pp. 61–78, 2008.

[29] A. Wang, S. Rana, and S. A. Karumanchi, "Preeclampsia: the role of angiogenic factors in its pathogenesis," *Physiology*, vol. 24, no. 3, pp. 147–158, 2009.

[30] R. L. Kendall and K. A. Thomas, "Inhibition of vascular endothelial cell growth factor activity by an endogenously encoded soluble receptor," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 90, no. 22, pp. 10705–10709, 1993.

[31] R. J. Levine, S. E. Maynard, C. Qian et al., "Circulating angiogenic factors and the risk of preeclampsia," *The New England Journal of Medicine*, vol. 350, no. 7, pp. 672–683, 2004.

[32] R. J. Levine, C. Lam, C. Qian et al., "Soluble endoglin and other circulating antiangiogenic factors in preeclampsia," *The New England Journal of Medicine*, vol. 355, no. 10, pp. 992–1005, 2006.

[33] S. E. Maynard, J. Y. Min, J. Merchan et al., "Excess placental soluble fms-like tyrosine kinase 1 (sFlt1) may contribute to endothelial dysfunction hypertension, and proteinuria in preeclampsia," *Journal of Clinical Investigation*, vol. 111, no. 5, pp. 649–658, 2003.

[34] S. Venkatesha, M. Toporsian, C. Lam et al., "Soluble endoglin contributes to the pathogenesis of preeclampsia," *Nature Medicine*, vol. 12, no. 6, pp. 642–649, 2006.

[35] A. Makris, C. Thornton, J. Thompson et al., "Uteroplacental ischemia results in proteinuric hypertension and elevated sFLT-1," *Kidney International*, vol. 71, no. 10, pp. 977–984, 2007.

[36] F. Herse, A. C. Staff, L. Hering, D. N. Müller, F. C. Luft, and R. Dechend, "AT1-receptor autoantibodies and uteroplacental RAS in pregnancy and pre-eclampsia," *Journal of Molecular Medicine*, vol. 86, no. 6, pp. 697–703, 2008.

[37] Y. Xia, C. Z. Cissy, S. M. Ramin, and R. E. Kellems, "Angiotensin receptors, autoimmunity, and preeclampsia," *Journal of Immunology*, vol. 179, no. 6, pp. 3391–3395, 2007.

[38] Y. Xia and R. Kellems, "Receptor-activating autoantibodiesand disease: preeclampsia and bevond," *Expert Review of Clinical Immunology*, vol. 7, no. 5, pp. 659–674, 2011.

[39] G. Wallukat, V. Homuth, T. Fischer et al., "Patients with preeclampsia develop agonistic autoantibodies against the angiotensin AT1 receptor," *Journal of Clinical Investigation*, vol. 103, no. 7, pp. 945–952, 1999.

[40] B. LaMarca, K. Wallace, and J. Granger, "Role of angiotensin II type I receptor agonistic autoantibodies (AT1-AA) in preeclampsia," *Current Opinion in Pharmacology*, vol. 11, no. 2, pp. 175–179, 2011.

[41] R. Dechend, C. Viedt, D. N. Müller et al., "AT1 receptor agonistic antibodies from preeclamptic patients stimulate NADPH oxidase," *Circulation*, vol. 107, no. 12, pp. 1632–1639, 2003.

[42] Y. Xia, H. Y. Wen, and R. E. Kellems, "Angiotensin II inhibits human trophoblast invasion through AT1 receptor activation," *The Journal of Biological Chemistry*, vol. 277, no. 27, pp. 24601–24608, 2002.

[43] Y. Xia, H. Wen, S. Bobst, M. C. Day, and R. E. Kellems, "Maternal autoantibodies from preeclamptic patients activate angiotensin receptors on human trophoblast cells," *Journal of the Society for Gynecologic Investigation*, vol. 10, no. 2, pp. 82–93, 2003.

[44] S. M. Bobst, M. C. Day, L. C. Gilstrap, Y. Xia, and R. E. Kellems, "Maternal autoantibodies from preeclamptic patients activate angiotensin receptors on human mesangial cells and induce interleukin-6 and plasminogen activator inhibitor-1 secretion," *American Journal of Hypertension*, vol. 18, no. 3, pp. 330–336, 2005.

[45] R. Dechend, V. Homuth, G. Wallukat et al., "AT1 receptor agonistic antibodies from preeclamptic patients cause vascular cells to express tissue factor," *Circulation*, vol. 101, no. 20, pp. 2382–2387, 2000.

[46] X. Yang, F. Wang, H. Chang et al., "Autoantibody against AT1 receptor from preeclamptic patients induces vasoconstriction through angiotensin receptor activation," *Journal of Hypertension*, vol. 26, no. 8, pp. 1629–1635, 2008.

[47] C. C. Zhou, Y. Zhang, R. A. Irani et al., "Angiotensin receptor agonistic autoantibodies induce pre-eclampsia in pregnant mice," *Nature Medicine*, vol. 14, pp. 855–862, 2008.

[48] B. LaMarca, M. Parrish, L. F. Ray et al., "Hypertension in response to autoantibodies to the angiotensin II type i receptor (AT1-AA) in pregnant rats: role of endothelin-1," *Hypertension*, vol. 54, no. 4, pp. 905–909, 2009.

[49] B. LaMarca, G. Wallukat, M. Llinas, F. Herse, R. Dechend, and J. P. Granger, "Autoantibodies to the angiotensin type I receptor in response to placental ischemia and tumor necrosis factor $\alpha$ in pregnant rats," *Hypertension*, vol. 52, no. 6, pp. 1168–1172, 2008.

[50] J. P. Granger, B. B. LaMarca, K. Cockrell et al., "Reduced uterine perfusion pressure (RUPP) model for studying cardiovascular-renal dysfunction in response to placental ischemia," *Methods in Molecular Medicine*, vol. 122, pp. 383–392, 2006.

[51] B. B. LaMarca, W. A. Bennett, B. Alexander et al., "Hypertension produced by reductions in uterine perfusion in the pregnant rat: role of tumor necrosis factor-$\alpha$," *Hypertension*, vol. 46, no. 4, pp. 1022–1025, 2005.

[52] C. C. Zhou, S. Ahmad, T. Mi et al., "Autoantibody from women with preeclampsia induces soluble Fms-like tyrosine kinase-1 production via angiotensin type 1 receptor and calcineurin/nuclear factor of activated T-cells signaling," *Hypertension*, vol. 51, no. 4, pp. 1010–1019, 2008.

[53] C. C. Zhou, R. A. Irani, Y. Zhang et al., "Angiotensin receptor agonistic autoantibody-mediated tumor necrosis factor-$\alpha$ induction contributes to increased soluble endoglin production in preeclampsia," *Circulation*, vol. 121, no. 3, pp. 436–444, 2010.

[54] T. Walther, G. Wallukat, A. Jank et al., "Angiotensin II type 1 receptor agonistic antibodies reflect fundamental alterations in the uteroplacental vasculature," *Hypertension*, vol. 46, no. 6, pp. 1275–1279, 2005.

[55] A. H. Siddiqui, R. A. Irani, S. C. Blackwell, S. M. Ramin, R. E. Kellems, and Y. Xia, "Angiotensin receptor agonistic autoantibody is highly prevalent in preeclampsia: correlation with disease severity," *Hypertension*, vol. 55, no. 2, pp. 386–393, 2010.

[56] B. M. Sibai, "Diagnosis, prevention, and management of eclampsia," *Obstetrics and Gynecology*, vol. 105, no. 2, pp. 402–410, 2005.

[57] J. Hinchey, C. Chaves, B. Appignani et al., "A reversible posterior leukoencephalopathy syndrome," *The New England Journal of Medicine*, vol. 334, no. 8, pp. 494–500, 1996.

[58] J. Tollemar, O. Ringden, B. G. Ericzon et al., "Cyclosporine-associated central nervous system toxicity," *The New England Journal of Medicine*, vol. 318, no. 12, pp. 788–789, 1988.

[59] J. P. Sloane, K. Y. Lwin, M. E. Gore et al., "Disturbannce of blood-brain barrier after bone-marrow transplantation," *The Lancet*, vol. 2, no. 8449, pp. 280–281, 1985.

[60] M. Verbeke, J. van de Voorde, L. de Ridder, and N. Lameire, "Functional analysis of vascular dysfunction in cyclosporin treated rats," *Cardiovascular Research*, vol. 28, no. 8, pp. 1152–1156, 1994.

[61] V. H. Lee, E. F. M. Wijdicks, E. M. Manno, and A. A. Rabinstein, "Clinical spectrum of reversible posterior leukoencephalopathy syndrome," *Archives of Neurology*, vol. 65, no. 2, pp. 205–210, 2008.

[62] W. S. Bartynski, "Posterior reversible encephalopathy syndrome, part 1: fundamental imaging and clinical features," *American Journal of Neuroradiology*, vol. 29, no. 6, pp. 1036–1042, 2008.

[63] W. S. Bartynski and J. F. Boardman, "Distinct imaging patterns and lesion distribution in posterior reversible encephalopathy

syndrome," *American Journal of Neuroradiology*, vol. 28, no. 7, pp. 1320–1327, 2007.

[64] E. Oehm, M. Reinhard, C. Keck, T. Els, J. Spreer, and A. Hetzel, "Impaired dynamic cerebral autoregulation in eclampsia," *Ultrasound in Obstetrics and Gynecology*, vol. 22, no. 4, pp. 395–398, 2003.

[65] E. Oehm, A. Hetzel, T. Els et al., "Cerebral hemodynamics and autoregulation in reversible posterior leukoencephalopathy syndrome caused by pre-/eclampsia," *Cerebrovascular Diseases*, vol. 22, no. 2-3, pp. 204–208, 2006.

[66] M. A. Belfort, M. W. Varner, D. S. Dizon-Townson, C. Grunewald, and H. Nisell, "Cerebral perfusion pressure, and not cerebral blood flow, may be the critical determinant of intracranial injury in preeclampsia: a new hypothesis," *American Journal of Obstetrics and Gynecology*, vol. 187, no. 3, pp. 626–634, 2002.

[67] M. J. Cipolla, "Cerebrovascular function in pregnancy and eclampsia," *Hypertension*, vol. 50, no. 1, pp. 14–24, 2007.

[68] F. Mattar and B. M. Sabai, "Eclampsia. VIII. Risk factors for maternal mortality," *American Journal of Obstetrics and Gynecology*, vol. 182, no. 2, pp. 307–312, 2000.

[69] B. M. Sibai, "Eclampsia. VI. Maternal-perinatal outcome in 254 ocnsecutive cases," *American Journal of Obstetrics and Gynecology*, vol. 163, no. 3, pp. 1049–1055, 1990.

[70] O. Amburgey, A. C. Chapman, V. May, I. M. Bernstein, and M. J. Cipolla, "Plasma from preeclamptic women increases blood-brain barrier permeability: role of vascular endothelial growth factor signaling," *Hypertension*, vol. 56, no. 5, pp. 1003–1008, 2010.

[71] N. Pelisch, N. Hosomi, M. Ueno et al., "Blockade of AT1 receptors protects the blood-brain barrier and improves cognition in dahl salt-sensitive hypertensive rats," *American Journal of Hypertension*, vol. 24, no. 3, pp. 362–368, 2011.

[72] B. M. Sibai, "Diagnosis and management of gestational hypertension and preeclampsia," *Obstetrics and Gynecology*, vol. 102, no. 1, pp. 181–192, 2003.

[73] D. Altman, G. Carroli, L. Duley et al., "Do women with pre-eclampsia, and their babies, benefit from magnesium sulphate? The Magpie trial: a randomised placebo-controlled trial," *The Lancet*, vol. 359, no. 9321, pp. 1877–1890, 2002.

[74] M. Belfort, J. Allred, and G. Dildy, "Magnesium sulfate decreases cerebral perfusion pressure in preeclampsia," *Hypertension in Pregnancy*, vol. 27, no. 4, pp. 315–327, 2008.

[75] B. T. Alexander, K. Cockrell, F. D. Cline, M. T. Llinas, M. Sedeek, and J. P. Granger, "Effect of angiotensin II synthesis blockade on the hypertensive response to chronic reductions in uterine perfusion pressure in pregnant rats," *Hypertension*, vol. 38, no. 3, pp. 742–745, 2001.

[76] N. Roger, I. Popovic, P. Madelenat, and D. Mahieu-Caputo, "Fetal toxicity of angiotensin-II-receptor inhibitors. Case report," *Gynecologie Obstetrique Fertilite*, vol. 35, no. 6, pp. 556–560, 2007.

[77] M. A. Bos-Thompson, D. Hillarire-Buys, F. Muller et al., "Fetal toxic effects of angiotensin II receptor antagonists: case report and follow-up after birth," *The Annals of Pharmacotherapy*, vol. 39, no. 1, pp. 157–161, 2005.

[78] K. Kato, M. Okuda, H. Ishikawa, T. Takahashi, and F. Hirahara, "Oligohydramnios and pulmonary hypoplasia: a case in which involvement of an angiotensin II receptor antagonist was suspected," *Journal of Obstetrics and Gynaecology Research*, vol. 34, no. 2, pp. 242–246, 2008.

[79] C. A. Hubel, G. Wallukat, M. Wolf et al., "Agonistic angiotensin II type 1 receptor autoantibodies in postpartum women with a history of preeclampsia," *Hypertension*, vol. 49, no. 3, pp. 612–617, 2007.

[80] M. C. Chames, J. C. Livingston, T. S. Invster et al., "Late postpartum eclampsia: a preventable disease?" *American Journal of Obstetrics and Gynecology*, vol. 186, no. 6, pp. 1174–1177, 2002.

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*Review Article*

# The Angiotensin II Type 2 Receptor in Brain Functions: An Update

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Angiotensin II (Ang II) is the main active product of the renin-angiotensin system (RAS), mediating its action via two major receptors, namely, the Ang II type 1 ($AT_1$) receptor and the type 2 ($AT_2$) receptor. Recent results also implicate several other members of the renin-angiotensin system in various aspects of brain functions. The first aim of this paper is to summarize the current state of knowledge regarding the properties and signaling of the $AT_2$ receptor, its expression in the brain, and its well-established effects. Secondly, we will highlight the potential role of the $AT_2$ receptor in cognitive function, neurological disorders and in the regulation of appetite and the possible link with development of metabolic disorders. The potential utility of novel nonpeptide selective $AT_2$ receptor ligands in clarifying potential roles of this receptor in physiology will also be discussed. If confirmed, these new pharmacological tools should help to improve impaired cognitive performance, not only through its action on brain microcirculation and inflammation, but also through more specific effects on neurons. However, the overall physiological relevance of the $AT_2$ receptor in the brain must also consider the Ang IV/$AT_4$ receptor.

## 1. Introduction

A major advance in the field of the renin-angiotensin system (RAS) was the discovery of a complete RAS in the brain, independent from the peripheral system, by Jacques Genest's laboratory in Montreal in 1971 [1] (for reviews see [2–4]). Further studies by Mendelsohn et al. [5, 6] and Unger et al. [7] corroborated these observations, using biochemical, pharmacological, and autoradiographic approaches. It was found that brain levels of angiotensin II (Ang II) are higher than its circulating levels, suggesting independence of the two systems (review in [3, 4]). The various components of RAS (angiotensin-converting enzyme (ACE), Ang II and Ang II receptors) are all found in the adult brain in areas involved in the regulation of fluid and electrolyte balance, in the regulation of arterial pressure and vasopressin release, and regulation of the autonomic system [8, 9]. They are also present in structures involved in cognition, behavior, and locomotion. In particular, over the past 10 years, several advances have been made regarding the role of Ang II in various brain functions, including cerebroprotection, stress, depression, and memory consolidation [3, 4, 10–12]. Moreover, many evidences highlight the potential function of Ang II in the etiology of certain neurodegenerative diseases, including Alzheimer's and Parkinson's disease, seizures, and the development of metabolic syndrome and diabetes (review in [4, 13, 14]).

In the classical view, synthesis of Ang II begins with the conversion of angiotensinogen into Ang I by the enzyme renin. Ang I is then converted by ACE into Ang II (a.a. 1–8), which is then metabolized to Ang III (a.a. 2–8) and Ang IV (a.a. 3–8). Ang II and Ang IV can be further converted into Ang (1–7) and Ang (3–7) (Figure 1). Ang II binds to two main receptors, namely, the angiotensin type 1 ($AT_1$) and type 2 ($AT_2$) receptors, both belonging to the G-protein coupled receptor (GPCR) family [4, 12, 13]. Aside from this common link, these two receptors otherwise carry very little similarities. Indeed, their actions are generally opposite and while the $AT_1$ receptor is expressed abundantly in several tissues, expression of $AT_2$ receptor is limited to specific tissues and brain areas, where its concentration is generally low compared to the $AT_1$ receptor. Most of the known effects of Ang II are due to activation of the $AT_1$ receptor, including vasoconstriction, cellular growth, and proliferation. On the

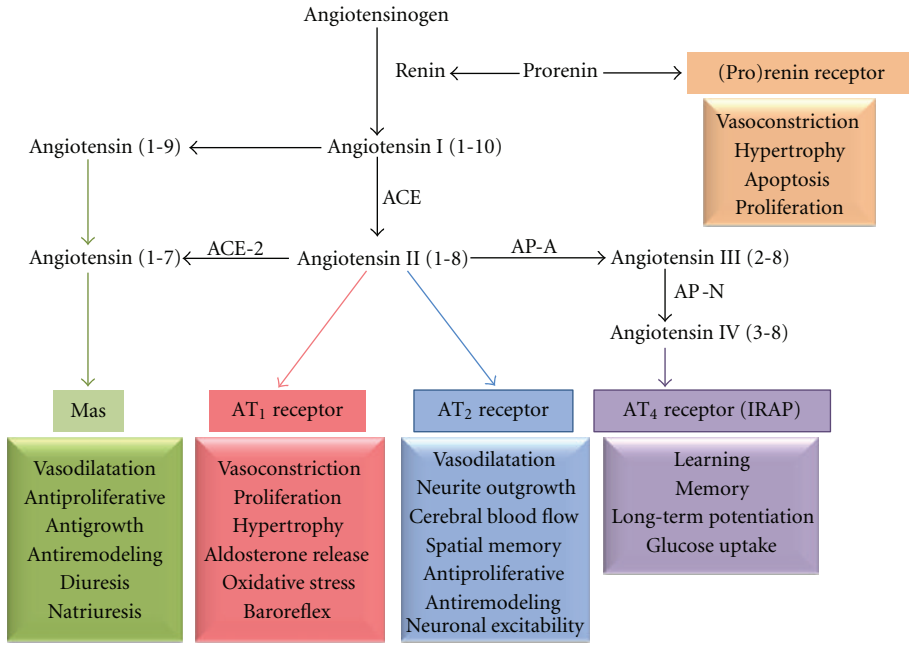


Figure 1: Summary of the brain renin angiotensin system (RAS). The figure summarizes the conversion of angiotensinogen to angiotensins I and II through fragments. The biologically active forms include angiotensins II, III, IV, and (1–7). The main enzymatic pathways are mediated by renin and angiotensin converting enzyme ACE or ACE2, AP-N and AP-A. The major brain effects of angiotensins are mediated by $AT_1$, $AT_2$, $AT_4$, prorenin, and Mas receptors. The functions associated with each receptor are indicated. ACE: angiotensin converting enzyme; AP-A: aminopeptidase A; AP-N: aminopeptidase N, adapted from Phillips and Oliveira, 2008 [3] and from Wright and Harding, 2012 [4].

other hand, it is generally assumed that the $AT_2$ receptor counteracts the action of the $AT_1$ receptor, promoting vasodilatation, apoptosis, and antigrowth effects. In addition to the classical $AT_1$ and $AT_2$ receptors, more recent studies have identified other receptors for RAS components, such as the (pro)renin receptor, the Ang (1–7) Mas receptor, and the Ang IV receptor ($AT_4$ receptor, called IRAP for insulin-regulated aminopeptidase), all of which are expressed in the brain (for reviews, see [4, 12, 13]) (Figure 1). In particular, several studies have shown that Ang IV-$AT_4$ receptor/IRAP have important functions in the brain, related to cognition and memory. In addition, in many situations, Ang IV acts as an inhibitor of $AT_1$ receptor actions [15–17].

The present paper is focused on the known and suggested roles of the $AT_2$ receptor in brain functions related to neuronal activities and cognitive disorders as well as the potential link between metabolic syndrome and cognitive functions. The role of the $AT_2$ receptor in the central regulation of blood pressure, thirst and related pathologies, such as hypertension, stroke, or ischemic damage, will not be discussed here; these latter topics are covered in recent reviews (see [11, 18–20]). For further detail, readers are also invited to consult several excellent reviews describing recent up-to-date advances pertaining to the various active Ang II-derived ligands and their receptors [4, 12, 13] However, considering that there are some similarities between the $AT_2$ receptor and the Ang IV/$AT_4$ receptor, comparisons between the two will be included, when appropriate.

## 2. The Type 2 Receptor—A Nonclassical GPCR

Three important periods highlight the history of the $AT_2$ receptor: (i) its discovery in the 1980s, (ii) its cloning in 1991–1993, and (iii) the generation of transgenic mice in 1995. However, because Ang II has a similar affinity for both of its receptors, it has been difficult to discriminate between the latter using nonselective ligands such as native Ang II or peptide analogs such as Saralasin or SarIle. At the end of 1980s, tools enabling to distinguish these Ang II receptors became available. The first tools included the nonpeptide antagonists losartan (previously known as DUP753) and PD123,177, and peptide ligands such as CGP42112A and p-aminophenylalanine (review in [2]). Seminal autoradiographic studies [5, 6], followed by biochemical studies by the groups of Marc de Gasparo, Peter Timmermans, and Robert Speth revealed the presence of two binding sites for Ang II, which differed in their expression pattern and biochemical properties [21–25]. However, the conclusive evidence for the existence of two receptors came from their cloning in the early 1990s [26, 27]. Despite the fact that both receptors belong to the large 7-transmembrane domain family of GPCR, $AT_1$ and $AT_2$ share only ~34% amino acid sequence identity. Moreover, while the $AT_2$ receptor displays most of the structural features of a GPCR, it is usually considered as an atypical member of this family. Indeed, although some of its signaling pathways involve a $G_i$-dependent mechanism, most of the known effects of the $AT_2$

receptor are independent from G-protein coupling mechanisms (for reviews, see [14, 20, 28–31]). Moreover it fails to induce all of the classical signaling pathways such as cAMP, production of inositol triphosphate, or intracellular calcium release (for reviews, see [28, 29]).

*2.1. Selective Ligands of the $AT_2$ Receptor.* In fact, $AT_2$ receptor functions are still unclear both in many physiological and pathophysiological situations, mainly because research on this receptor has long been hampered by at least three challenges, namely, (i) the low and unusual expression of this receptor, (ii) its nonclassical signaling, and (iii) the absence of appropriate selective ligands. While several nonpeptide ligands with a large spectrum of selectivity for the $AT_1$ receptor are readily available, only a few have been developed for the $AT_2$ receptor. Until recently, physiological and pharmacological assessments of the $AT_2$ receptors were obtained using either the antagonist PD123,319 (a modification of the initial PD123,177) or the more common $AT_2$ receptor agonist CGP42112A, although two other peptides have also shown agonistic properties on $AT_2$ receptors, namely, the agonist *p*-aminophenylalanine [22] and novokin [32, 33]. However, the fact that CGP42112A is only a partial agonist, combined with its short half-life, has hampered its further use in *in vivo* studies (review in [20, 34, 35]). Thus, many hypotheses regarding $AT_2$ receptor functions in physiological situations have emerged from indirect observations, either using transgenic animals, or blockade of the $AT_1$ receptor. In 2004, Wan and collaborators synthesized the first selective nonpeptide $AT_2$ receptor agonist, called compound 21 (C21) [36], now renamed M024 [37]. Since then, an increasing number of studies have used C21/M024 for both *in vitro* and *in vivo* studies (see further below) to better investigate the selective role of the $AT_2$ receptor (for review, see [20, 34]).

## 3. Overview of the Signaling Pathways of the $AT_2$ Receptor in the Brain

Signaling pathways associated with the $AT_2$ receptor primarily involve a balance between phosphatase and kinase activity. The final outcomes vary according to whether the cell is undifferentiated or differentiated and whether angiotensin $AT_1$ receptors are also expressed or not. Although there is still much controversy surrounding the effects of the $AT_2$ receptor in peripheral systems, its role and mechanisms of action in the brain have gained much greater consensus [14, 31, 38].

*3.1. Signaling Pathways of the $AT_2$ Receptor Leading to Neurite Outgrowth.* In neuronal models, studies on functionality and signaling have usually been conducted simultaneously. Most of the data, including our own findings using NG108-15 cells, indicate that the effects of the $AT_2$ receptor on neurite outgrowth involve four main complementary signaling cascades (Figure 2). The first cascade entails a decrease in $p21^{RAS}$ [39] and protein kinase C$\alpha$ (PKC$\alpha$) activities [40], both of which are involved in the switch from proliferation to differentiation. Simultaneously, a second cascade, initiated by Rap1/B-Raf, induces a delayed and sustained phosphorylation of $p42/p44^{mapk}$ [39, 41]. In both NG108-15 [39] and PC12W [42] cells, this sustained activation is essential for inducing neurite outgrowth. The initial activation of Rap1 by the $AT_2$ receptor is not direct, but rather mediated by phosphorylation of the tropomyosin-related kinase receptor A (TrkA) [43], through the intervention of a Src family kinase member [44]. The third cascade comprises nitric oxide (NO) and cGMP. In NG108-15 cells, we [45] and others [46] have shown that external application of NO is sufficient to induce neurite outgrowth and elongation. In these cells, neuronal NO synthase (nNOS) activation and cGMP production induced by the $AT_2$ receptor is dependent on the $G\alpha_i$ protein. However, cGMP is not involved in Ang II-induced activation of $p42/p44^{mapk}$ [45]. More recently, Li et al. [47] have shown that after $AT_2$ receptor stimulation, the $AT_2$ receptor interacting protein, ATIP [48], interacts with the tyrosine phosphatase SHP-1. The complex then translocates into the nucleus where it transactivates the ubiquitin-conjugating enzyme variants called methyl methanesulfonate sensitive 2 (MMS2), resulting in neural differentiation and protection (reviewed in [31, 49]). Finally, interaction of $AT_2$ with certain receptor tyrosine kinases may also induce neurite outgrowth. For example, in a model of fructose-induced insulin-resistant rats, authors also demonstrated that neurite outgrowth of dorsal root ganglia (DRG) neurons induced by the $AT_2$ receptor was facilitated by activation of the phosphatidylinositol 3-kinase (PI3K)/Akt pathway, suggesting the existence of a crosstalk between the $AT_2$ receptor and the insulin receptor [50]. In addition, several phosphatases, such as PP2A [51–54] and Src homology region 2 domain-containing phosphatase-1 (SHP-1), are clearly associated with the mechanism of action of the $AT_2$ receptor. Finally, certain pathways associated with $AT_2$ receptor activation may be through interaction with protein partners such as the promyelocytic zinc finger (PLZF) protein [55, 56] or the peroxisome proliferator-activated receptor gamma (PPAR$\gamma$) [57] (for review, see [29, 31, 58–60]).

*3.2. Ang-II-Independent Effects of the $AT_2$ Receptor.* It should be noted that while the main effects of $AT_1$ and $AT_2$ receptors are dependent of Ang II binding, some evidences also suggest that they may have certain ligand-independent effects (review in [29, 31]). For example, $AT_1$ receptors can be activated by mechanical stress, independently from Ang II binding and/or stimulation of $p42/p44^{mapk}$ [61, 62]. Similarly, $AT_2$ receptor overexpression in CHO cells, R3T3 fibroblasts, and vascular smooth muscle cells enhances apoptosis signaling simply by its overexpression [63]. Another study also observed that the $AT_2$ receptor, when expressed as a constitutive homooligomer, leads to G-protein dysfunction and symptoms of neurodegeneration without Ang II stimulation [64]. Although the mechanism underpinning this effect still requires further investigation, it appears that the C-terminal portion of the $AT_2$ receptor is essential, since expression of a mutant $AT_2$ receptor truncated in its C-terminal region is unable to form oligomers. Aside from its effect on cell survival, Ang II-independent effects of the $AT_2$ receptor also include modulation of gene expression, at least in human coronary artery endothelial cells, where $AT_2$ receptor overexpression modulates more genes than

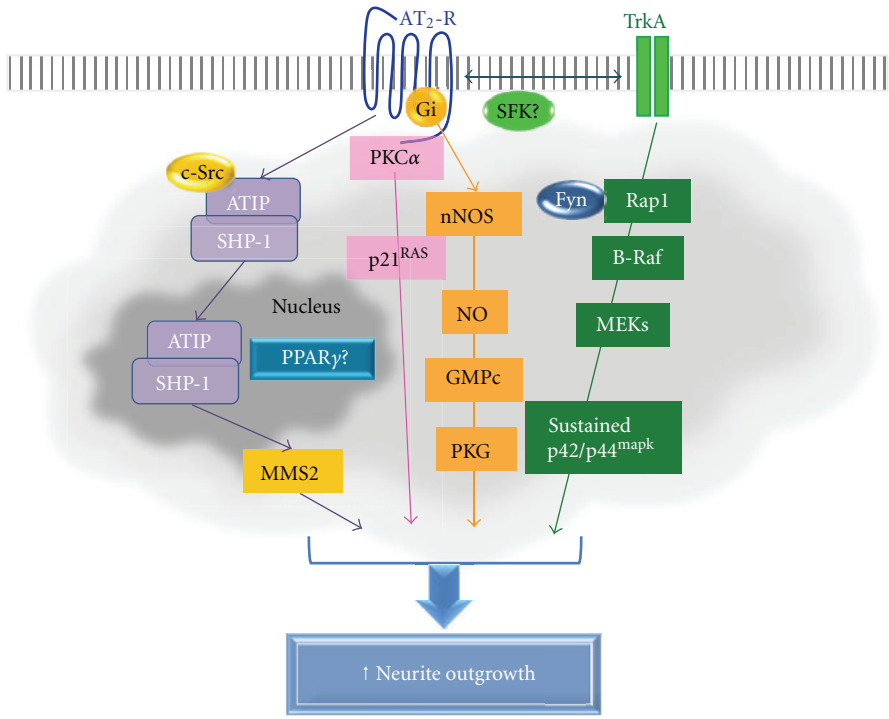


Figure 2: Overview of the main signaling pathways implicated in the action of $AT_2$ receptor leading to neurite outgrowth.

CGP42112A stimulation [65]. Although these $AT_2$-regulated genes are associated with many cellular functions, including cell migration, protein processing, intracellular signaling, and DNA repair, it is still unknown whether Ang II-independent effects of the $AT_2$ receptor are associated with protective effects in neuronal function. Moreover, an Ang II-independent effect of the $AT_2$ receptor was observed in a model overexpressing the receptor, and thus its relevance in physiological situations is still unknown. Clearly, many questions still remain to be elucidated in order to fully understand how the $AT_2$ receptor exerts its effects on brain functions. However, new recent insights in $AT_2$ receptor signaling have been achieved which could partly explain some of the observed discrepancies (see Section 8).

*3.3. Signaling Pathways Associated with Ang IV and the $AT_4$ Receptor.* Interestingly, despite the high similarity between central biological effects associated with $AT_2$ and $AT_4$ receptor stimulation (memory processing, long-term potentiation facilitation, protective function in cognitive loss) (see Section 5), the signaling pathways induced by these two receptors are clearly different. In contrast with the $AT_2$ receptor, the $AT_4$ receptor is a single transmembrane receptor, initially known for its aminopeptidase activity, leading to peptide processing of oxytocin, vasopressin, Ang III, met-enkephalin, somatostatin, and other neuropeptides (review in [4, 12]). Ang IV has also been shown to increase endothelial NO synthase activity via mobilization of intracellular calcium [66, 67] and to stimulate the PI3 K/PKB (protein kinase B) pathway [68] in endothelial cells. A recent study also showed that Ang IV treatment in diet-induced hyperglycemic mice increased the interaction between IRAP and PI3 K, leading to activation of Akt and the glucose transporter type-4 (GLUT4) translocation. This pathway was also associated with an improvement in glucose tolerance [69], suggesting that Ang IV may be implicated in insulin signaling and development of diabetes. Finally, in some studies, Ang IV was also found to elicit rapid activation of other selected kinases including ERK1/2, p38 kinase, focal adhesion kinase, and paxillin (review in [70]). Nevertheless, it appears that these signaling effects of Ang IV are model dependent, and none of these pathways have been observed in neuronal cells. Therefore, although renewed interest in Ang IV has recently emerged with the recognition that the Ang IV/IRAP/$AT_4$ receptor plays an important role in cognition and pain [4, 12, 71], it is still unknown to date whether signaling pathways associated with Ang IV stimulation are implicated in these effects.

## 4. Expression and Roles of the $AT_2$ Receptor in the Brain

All of the various RAS components in the brain, and in particular the $AT_2$ receptor, are highly expressed during fetal life, suggesting that they could play key roles during development. On the other hand, in the adult, $AT_2$ receptor expression in the brain is limited to certain specific areas (review in [72–75]). For instance, the $AT_2$ receptor has been found at high levels in the medulla oblongata (control of autonomous functions), septum and amygdala (associated

with anxiety-like behavior), thalamus (sensory perception), and superior colliculus (control of eye movements in response to visual information) as well as in the subthalamic nucleus and cerebellum (areas associated with learning of motor functions). It is also expressed with the $AT_1$ receptor in areas involved with cardiovascular functions, learning and behavior (cingulate cortex, molecular layer of the cerebellar cortex, superior colliculus, paraventricular nuclei hippocampus) (for extensive mapping, see [72, 73]). More recently, expression of the $AT_2$ receptor was also detected in the substantia nigra pars compacta, the area involved in dopaminergic signals and associated with Parkinson's disease [76], and in the hippocampus [64, 77]. At the cellular level, the $AT_2$ receptor is expressed in neurons, but not in astrocytes [28, 72, 78]. Evidence also suggests that the $AT_2$ receptor is expressed in the vasculature wall, where it acts on cerebral blood flow (review in [31, 49]). In addition, the presence of a non-$AT_1$/non-$AT_2$ receptor in the CNS has been suggested, which displays high affinity for Ang I, II, and III [79].

Interestingly, the Ang IV receptor IRAP is also observed in structures classically associated with cognitive processes and sensory and motor functions (including hippocampus, thalamic nuclei, caudate putamen, cerebellum, neocortex, lateral geniculate body, inferior olivary nucleus, superior colliculus, ventral tegmental area, and brain stem) (review in [12, 80, 81]). In contrast, the Ang (1–7) Mas receptor is expressed in brain areas involved in central cardiovascular regulation (in particular, the nucleus of the solitary tract (NTS), rostral ventrolateral medulla (RVLM), caudal ventrolateral medulla (CVLM), inferior olive, parvo- and magnocellular portions of the paraventricular nucleus (PVN), supraoptic nucleus, and lateral preoptic area) (review in [82]). In some instances, these areas also contain $AT_1$ and $AT_2$ receptors.

One of the first roles of the $AT_2$ receptor to be identified was the modulation of neuronal excitability (review in [28, 83]). In cells of neuronal origin, activation of the $AT_2$ receptor decreases activity of T-type calcium channels [84, 85] while stimulating a delayed-rectifier $K^+$ current ($I_K$) and a transient $K^+$ current ($I_A$) [86]. Using primary cultures of cortical neurons, studies by Grammatopoulos et al. [87] have shown that II neuroprotection against chemical hypoxia was mediated by activation of a delayed rectifier $K^+$ channel, an effect exemplified by simultaneous blockade of the $AT_1$ receptor. Moreover, in preparations of locus coeruleus brain slices [88], angiotensin II, through the $AT_2$ receptor, was found to depress glutamate depolarization and excitatory postsynaptic potentials. In the superior colliculus, both $AT_1$ and $AT_2$ receptors are involved in sensory visuomotor integration. Lastly, Ang II, through $AT_2$ receptor stimulation with CGP42112A, has also been shown to induce a strong suppressive effect on visual neuronal activity. Together, these results indicate that $AT_2$ receptor modulation of potassium and calcium channels activity may impact neuronal functions.

The second and best recognized effect of the $AT_2$ receptor is stimulation of neurite outgrowth in various cell types from neuronal origin (NG108-15 and PC12W cells) as well as in primary cultures of neurons from retinal explants [89], in neurospheres from mouse fetal brain [90], cerebellar neuronal cells [91], and the cortex [47] (review in [3, 10, 14, 34]). This $AT_2$-induced neurite elongation is characterized by an increase in mature neural cell markers, such as $\beta$III-tubulin and MAP2 [47, 92]. It is also associated with a rise in neuronal migration [54, 91] and neuronal survival following ischemia-induced neuronal injury [93]. These effects may be important not only for developmental differentiation, but also after injury-induced regeneration. Indeed, a beneficial effect of the $AT_2$ receptor in nerve regeneration has been observed following both optic [89] and sciatic [94] nerve crush or in perivascular nerves implicated in vasodilation [95]. This implication of the $AT_2$ receptor in neuronal regeneration has even led to the suggestion that Ang II, via the $AT_2$ receptor, could act as a neurotrophic factor. In summary, the $AT_2$ receptor, by its function in the modulation of neuronal excitability, neurite elongation, migration, and nerve regeneration, may be an important factor in the regulation of central nervous system activity and cognitive function either following nerve injury or during the development of neurodegenerative disease (Figure 3).

In addition to neuronal differentiation, which is of paramount importance in nerve regeneration, the $AT_2$ receptor also stimulates differentiation of hematopoietic cells, a key process during regeneration and reconstruction. Indeed, ischemic damage is characterized by infiltration of a number of hematopoietic cells such as leukocytes, platelets, macrophages, and leukocytes [96]. In particular, the $AT_2$ receptor has the capacity to induce differentiation of human monocytes into dendritic cells [97]. Supporting a protective effect of the $AT_2$ receptor is the observation that ischemic damages were found to be greater in mice with hematopoietic cells deleted in $AT_2$ receptor expression [98]. These findings suggest that expression and activation of the $AT_2$ receptor in hematopoietic cells may be part of its beneficial effect following brain injury, although the mechanism involved remains to be investigated (review in [99]).

Some of the initial hypotheses regarding the potential role of the $AT_2$ receptor *in vivo* were confirmed in 1995 when two independent groups developed $AT_2$ knockout mice by targeted gene deletion [100, 101]. Surprisingly, despite the fact that the $AT_2$ receptor is highly expressed during fetal development in many tissues, including skin, kidney, brain, and heart, it appears that mice lacking the $AT_2$ receptor do not exhibit any major anatomical defects. However, these mice exhibit markedly reduced exploratory behavior as well as altered thirst reaction, lower body temperature, slightly elevated mean arterial pressure, and a stronger vasoconstrictive response to Ang II [100–102]. Moreover, they exacerbate stronger symptoms when exposed to pathological situations. For example, these mice display an accelerated pathological response when exposed to cardiovascular disease induction. They also exhibit larger cerebral infarct size following medial cerebral artery occlusion (MCAO) [93], stronger cognitive deficits following ischemia [103], and faster progression of atherosclerosis [104]. The various actions of the $AT_2$ receptor currently documented in the brain are summarized in Figure 3.

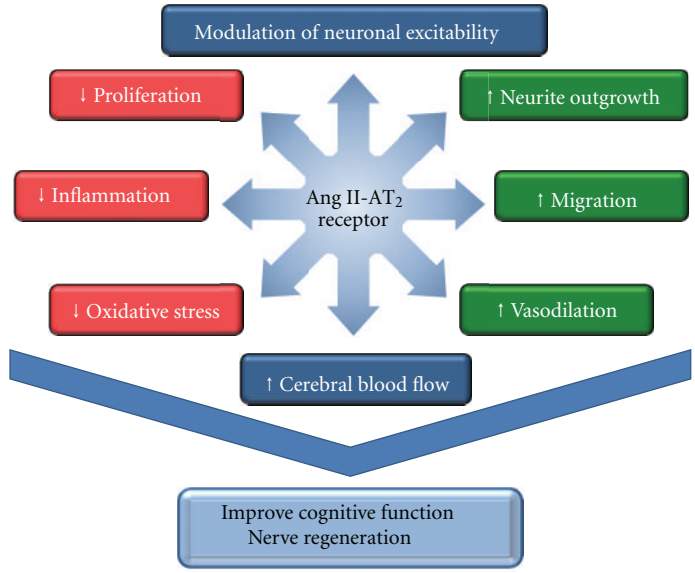


Figure 3: Summary of the known cellular effects of the $AT_2$ receptor. Stimulation of the $AT_2$ receptor of angiotensin II results in the activation of several intracellular cascades, resulting in a decrease in proliferation and in growth-promoting effects, as well as a decrease in inflammatory cytokines and in reactive oxygen species, hence decreasing oxidative stress. On the other hand, such activation increases neuronal excitability, by acting on $K^+$ and $Ca^{2+}$ channel activity, and increases neurite outgrowth and migration of neurons, favoring neuronal plasticity. Stimulation of the $AT_2$ receptor is also known for its effect on vasodilation, thus increasing cerebral blood flow, and to increase glucose uptake, which improves insulin sensitivity. These cellular events in turn decrease or increase important physiological functions, which in general have reached a significant degree of consensus. Together, the $AT_2$ receptor may appear as a gatekeeper of cellular and tissue homeostasis. Indeed, most current studies suggest that $AT_2$ receptor activation may have a protective role in various pathological situations.

## 5. Role of the $AT_2$ Receptor in Cognitive Function and Neurological Disorders

The first evidence for the implication of the $AT_2$ receptor in cognition resulted from studies in knockout mice. Indeed, $AT_2$-deficient mice suffer from perturbations in exploratory behavior and locomotor activity [100, 101], as well as displaying an anxiety-like behavior [105]. Moreover, in the adult, inhibition of the $AT_2$ receptor with the $AT_2$ receptor antagonist PD123,319 has been reported to abolish the Ang II-induced acquisition of conditioned avoidance responses [106]. These results strongly support that, in addition to a role during development, the $AT_2$ receptor may be involved in cognitive processes in the adult. Furthermore, although the $AT_2$ receptor is expressed at low levels in many areas of the nervous system, it may be reexpressed in certain pathological conditions such as optic [89] or sciatic [94] nerve transection, stroke [107], and certain neurodegenerative diseases such as Alzheimer's disease [108]. For example, Ge and Barnes [108] found that $AT_2$ receptor expression is diminished in Parkinson's disease (caudate nucleus and cerebellum) but enhanced in Huntington's disease (caudate nucleus). In Alzheimer's disease, the temporal cortex of the adult brain exhibits an increased expression while the hippocampus displays a decreased expression of the $AT_2$ receptor. In most of these situations, the $AT_2$ receptor is described as having beneficial effects in improving neuroprotection by acting not only on neurons, but also on blood circulation. Indeed, recent studies have clearly shown a protective role of the $AT_2$ receptor following brain ischemia and demonstrated that expression and activation of the $AT_2$ receptor may decrease brain damage and restore cognitive loss following middle cerebral artery occlusion (for review, see [11, 14, 49]). Altogether, these data suggest that the $AT_2$ receptor may play an important role in maintaining functions of the human brain.

*5.1. AGTR2 Mutations in Intellectual Disability.* Intellectual disability, previously described as mental retardation [109], affects approximately 1–3% of the population, of which a large number are associated with mutations on chromosome X. Among these, certain mutations on *AGTR2* coding for the angiotensin $AT_2$ receptor have been identified. Mutations in the *AGTR2* gene correlate with the development of human X-linked intellectual disability [110]. Indeed, 9 patients with X-linked intellectual disability were shown to have mutations in the *AGTR2* gene associated with decreased expression of the $AT_2$ receptor, including a complete loss of expression in a woman with an IQ of 44. Clinical features of these mutations ranged from moderate to severe intellectual disability, seizure, and manifestations of autism, thus supporting the hypothesis that the $AT_2$ receptor is required for brain development and for the maintenance of neuronal connections involved in learning and memory. This hypothesis was further corroborated by two other studies reporting mutations of *AGTR2* in patients suffering from intellectual

disability, seizures, restlessness, hyperactivity, and disrupted speech development [111, 112]. However, other studies failed to link any *AGTR2* mutations with intellectual disability, observing no difference in mutation incidence between the latter and control groups [113–115]. Differences among control cohort selection, number of patients per group, and ethnical variations between different studies could explain the discrepancies in these findings. Hence, it remains unclear whether mutations in *AGTR2* are associated or not with intellectual disability.

*5.2. $AT_2$ Receptor in Alzheimer's Disease (AD).* Amyloid-$\beta$ (A$\beta$) deposition in senile plaques and the presence of neurofibrillary tangles are the main pathological hallmarks of AD. However, other structural and functional alterations, including inflammation, increased oxidative stress and vascular damage/ischemia, are also associated with AD and other neurodegenerative diseases. These alterations may contribute to neuronal and synaptic dysfunction and loss, as well as the ensuing cognitive deficits and dementia of this disorder [116–121] (for recent review, see [11, 14, 49]). Clinical studies have documented that treatment with antihypertensive drugs is associated with an improvement in cognitive function (review in [122–124]). More recent studies have shown that treatment with angiotensin II receptor blockers (ARBs) is associated with a decrease in AD and dementia progression with a greater efficacy compared to ACE inhibitors [125, 126]. In particular, Tsukada et al. [127] have shown that cognitive deficit induced by A$\beta$ (1–42) in mice was improved by pretreatment with a low dose of telmisartan partly because of peroxisome proliferator-activated receptor-gamma (PPAR$\gamma$) activation. Corroborating the hypothesis that ARBs could be beneficial in reducing the onset of AD, Kume et al. [128] recently observed that AD hypertensive patients treated with telmisartan presented no decrease in cognitive functions test scores, but an increased cerebral blood flow, suggesting that treatment with this ARB could reduce AD progression. Altogether, studies conducted using various models of cognitive disorders have reported improved memory and cognitive processes and/or attenuation of A$\beta$1–42 oligomerization following treatment with ARBs, particularly valsartan [129], losartan [130], telmisartan [128, 131], and olmesartan [132] (now called metabosartans for ARBs with a PPAR$\gamma$ agonistic effect) (review in [11, 14, 49, 133]). More recently, a study using direct stimulation of the $AT_2$ receptor with the selective agonist C21/M024 demonstrated similar effects in an AD mouse model [134]. In this latter study, Jing et al. observed that intracerebroventricular injections of A$\beta$ (1–40) in mice induced a poorer performance in the Morris water maze and that this effect was reversed by coadministration of C21/M024, indicating that direct stimulation of $AT_2$ receptors improves spatial memory functions. Stroke is also one of the most important causes of cognitive impairment and dementia (review in [11, 49]). There is increasing evidence suggesting that activation of the $AT_2$ receptor could improve cerebral blood flow and microcirculation as well as decrease inflammation [93, 103, 135, 136], both being associated with improvement in cognitive function following cerebral ischemia [90, 93, 103, 137] (for reviews, see [3, 10, 14, 49]). As reported by Iwai et al. [103] and summarized by Horiuchi and Mogi [49] and Mogi and Horiuchi [11], superoxide anion production was found to be more markedly enhanced in $AT_2$ receptor-deficient mice compared to wild-type animals in a model of MCAO. These observations suggest that $AT_2$ receptor stimulation has a protective effect on ischemic brain lesions, at least partly through modulation of cerebral blood flow and superoxide production. Moreover, beneficial effects of ARBs on these parameters were less evident in $AT_2$ receptor-deficient mice [103]. Similar approaches were also used in other studies suggesting a beneficial effect of the $AT_2$ receptor on cognitive functions [90, 135, 136].

However, not only blood circulation but also neuronal functions can be improved by activation of $AT_2$ receptors. Indeed, activation of $AT_2$ receptors in neurons is also associated with a decrease in apoptosis signaling. Grammatopoulos et al. demonstrated in cultures of primary cortical neurons that angiotensin decreased sodium azide-induced apoptosis through $AT_2$ receptor activation [138] by reducing caspase-3 activation [139]. Finally, we and others have shown that the $AT_2$ receptor can activate the tyrosine kinase Fyn [44] and the phosphatase PP2A [51, 52, 54], both of which are key regulators of the phosphorylation of the microtubule associated protein tau. Hyperphosphorylation of tau is of paramount importance in the development of Alzheimer's disease by forming neurofibrillary tangles and leading to microtubule depolymerization. Thus, $AT_2$ receptor activation may participate to the control of equilibrium between tau phosphorylation and dephosphorylation.

Another key observation is the effect of estrogen receptors on $AT_2$ receptor functions. In 2008, Chakrabarty et al. [140] demonstrated that estrogen, through its E2 receptor, induced neurite elongation of dorsal root ganglion and that this effect was dependent on $AT_2$ receptor activation. Moreover, it has been observed that ischemic damage in $AT_2$-deficient mice was greater in females than in males, while no significant sex-different change was observed in $AT_2$-expressing mice [135]. These results suggest that the existence of some level of crosstalk between $AT_2$ receptor and estrogen and that $AT_2$ receptor could be necessary for E2 receptors to elicit its full effect on neuronal physiology. Thus, while stimulation of the $AT_2$ receptor and/or inhibition of the $AT_1$ receptor could lead to potential therapeutic avenues in neurodegenerative disease, the interaction between $AT_2$ receptor and estrogen should also be considered. Indeed, there are some differences in response to RAS stimulation according to gender (review in [141]). Nonetheless, the clear demonstration of an $AT_2$ receptor effect in Alzheimer's disease remains to be firmly demonstrated, mainly because in most studies to date the presence of $AT_1$ receptors and $AT_2$ receptors in the hippocampus has not been studied. Indeed, to our knowledge, only two studies have documented the presence of $AT_2$ receptors in the hippocampus, one in a model of Alzheimer's disease [64], the other in a model of epilepsy [77]. On the other hand, presence of the $AT_2$ receptor was not detected in the hippocampus in various studies from the Llorens-Cortes group [72, 73]. However, these studies were performed with healthy and middle-aged animals.

It should be mentioned that, in addition to the $AT_2$ receptor, the Ang IV/$AT_4$ receptor may also have a protective effect on cognitive function. Indeed, Braszko's group was the first to report that intracerebroventricular injections of Ang II and Ang IV were equivalent in facilitating exploratory behavior in rats tested in an open field and improved recall of passive avoidance conditioning in the acquisition of active avoidance conditioning [142, 143], results that were confirmed by others in subsequent studies (review in [71]). This strongly suggests an important function of Ang IV and its receptor in learning and memory processes and could represent a new therapeutic target in the treatment of memory loss associated with dementia (review in [71]). One mechanism proposed to explain this beneficial effect of Ang IV on cognitive function is the colocalization of IRAP with the glucose transporter GLUT4. In hippocampal pyramidal neurons, IRAP and GLUT4 are localized in secretory vesicles responsive to insulin (review in [70, 71, 80]), suggesting that Ang IV, by binding to IRAP, may increase GLUT4 membrane expression thus facilitating glucose uptake, as observed in adipocytes [144] (review in [145]).

*5.3. $AT_2$ Receptor and Parkinson's Disease.* In addition to AD and stroke, some evidences also suggest that central RAS could be implicated in the development of Parkinson's disease. Parkinson's is the second most common neurodegenerative disorder and is characterized by the progressive cell death of midbrain dopaminergic neurons in the substantia nigra and the presence of protein inclusions leading to formation of Lewy bodies (review in [146]). Although the mechanisms leading to Parkinson's disease are still unclear, it appears that mitochondrial dysfunction, oxidative stress, and inflammation are key factors to its progression [147]. Recently, ARBs have been shown to reduce lipid peroxidation and protein oxidation while protecting dopaminergic neurons in the substantia nigra in a rat model of Parkinson's disease [76, 148]. However, whether such action is due solely to a blockade of the $AT_1$ receptor or also from activation of the $AT_2$ receptor is not yet clearly established (review in [147]). Activation of the $AT_2$ receptor is able to stimulate differentiation of mesencephalic precursor cells into dopaminergic neurons, suggesting that stimulation of the $AT_2$ receptor could be useful in increasing the production of dopaminergic neurons in Parkinson's disease [149]. Moreover, Grammatopoulos et al. observed a protection against rotenone-induced oxidative stress and associated cell death in dopaminergic neurons following Ang II stimulation. This protective effect was prevented by the presence of the $AT_2$ receptor antagonist PD123,319, but was increased in the presence of the $AT_1$ receptor antagonist losartan [150]. More recently, it has been observed that during the aging process in rats, $AT_2$ receptor expression is decreased in dopaminergic neurons, as opposed to an increase in $AT_1$ receptor expression. This was associated with an enhancement of prooxidative and proinflammatory markers in the substantia nigra, leading to an increase in dopaminergic neuronal death [151]. Although these findings do not indicate a role of $AT_2$ receptor in the development of Parkinson's disease, they strongly suggest that such modifications in Ang II receptors during the natural aging process may increase the risk of Parkinson's disease.

## 6. Role of the $AT_2$ Receptor in the Regulation of Appetite

Obesity, which is characterized by excess body fat accumulation [152], is associated with an increased risk of diabetes, hypertension, and dyslipidemia. It is also one of the major components of the metabolic syndrome. Studies have demonstrated $AT_2$ receptor expression in tissues associated with glucose metabolism, including pancreatic [153–155] and adipose tissues [13, 30]. For example, expression of the $AT_2$ receptor in the pancreas has been shown to be important for fetal pancreatic development [156] while in the adult, it is associated with protection against pancreatic fibrosis [157] and a decrease in pancreatic tumor growth [158, 159]. The following is a summary of what is currently known regarding the potential function of the $AT_2$ receptor in the regulation of appetite, glucose metabolism, and its potential role in metabolic syndrome.

There are some evidences suggesting that Ang II could be implicated in food intake: for example, Ang II suppresses food intake after central infusion [32, 160, 161] while blockade of the $AT_1$ receptor by telmisartan is associated with a decrease in body weight [162]. Furthermore, both $AT_1$ and $AT_2$ receptors are expressed in the hypothalamus, which is implicated in the central regulation of food intake. In 2008, Ohinata et al. [32] observed that the decrease in food intake induced by centrally administrated Ang II was inhibited by PD123,319, and absent in $AT_2$-KO mice, suggesting that this effect was mediated by the $AT_2$ receptor. A similar effect was observed using novokin, a potent analog of ovokinin with $AT_2$ receptor agonistic properties [163]. Although the mechanism underlying this $AT_2$ receptor-associated decrease in food intake remains unclear, it may be linked to its capacity to modulate T-type calcium channels, since a recent study demonstrated that inhibition of these channels inhibits weight gain in mice fed with a high-fat diet [164]. However, this hypothesis remains to be explored. Studies conducted to date with the selective $AT_2$ agonist C21/M024 do not describe such differences between C21/M024-treated animals compared to the control group [165, 166], suggesting that the duration of C21/M024 treatment may have been too short to induce any modification in body weight. Thus, in this latter instance, even if the $AT_2$ receptor was observed to decrease food intake, it was probably not sufficient to induce a decrease in body weight, at least following short periods of stimulation (<8 weeks). Moreover, it has also been observed that inhibition of ACE by captopril also reduced body weight of mice fed with a high fat diet [167]. This decrease in body weight, however, was not associated with a decrease in food intake, suggesting that other mechanisms were regulated by Ang II. Since Ang II is no longer available in captopril-treated mice, these results suggest that other members of the RAS, independent of Ang II, could be implicated in the regulation of food intake and body weight. These observations should therefore be considered when interpreting results obtained with ARBs.

## 7. Link between Metabolic Syndrome and Alzheimer's Disease: Is There a Place for the $AT_2$ Receptor?

A number of excellent reviews have recently been published regarding the potential link between insulin resistance, metabolic syndrome, and neurodegenerative disorders (both AD and vascular dementia) [168–177], including the involvement of RAS in this process [90, 133, 178]. Development of AD is closely associated with a reduction in cerebral glucose utilization, even in the early stages of the disease. In fact, cerebral metabolism in the AD brain decreases prior to the onset of cognitive decline, suggesting that energy failure could represent one of the earliest hallmarks of AD. Induction of insulin resistance in AD animal models aggravate both amyloid and tau accumulation [179, 180], leading several investigators to refer to Alzheimer's disease as type 3 diabetes (review in [168]). One aspect of this relationship is the loss of insulin signaling in the insulin-resistant brain. In addition to the many peripheral complications associated with dysfunction in insulin sensitivity, it appears that brain insulin signaling plays crucial central functions in the regulation of energy balance (food intake, body weight) as well as in learning and memory (review in [171]). Moreover, inflammation, increase in oxidative stress, and mitochondrial dysfunctions are key features of type 2 diabetes (T2D) that are also shared in Alzheimer's disease.

In T2D patients, results of a major clinical study (Study on Cognition and Prognosis in the Elderly, SCOPE) [181] and a clinical double-blind study [182] have revealed that ARBs have a further therapeutic effect on impaired cognitive function beyond their antihypertensive effects compared with other antihypertensive drugs. Similarly, Tsukuda et al. [131, 183] have demonstrated that candesartan improves impaired cognitive function induced by T2D, with multiple beneficial effects. Two of these effects may be through PPAR$\gamma$ or through $AT_2$ receptor activation. Therefore, improvement of metabolic syndrome may also be beneficial in decreasing associated cognitive decline. This would contribute to better insulin signaling in the brain and, therefore, a slowing of cognitive decline associated with brain insulin resistance.

## 8. New Insights in AT2 Receptor Knowledge and Perspectives: What Remains to Be Done?

As pointed out recently [18, 20], there is still an ongoing debate as to the putative role of the $AT_2$ receptor in physiology, and whether this role is deleterious or beneficial. Summarized below are some of the new advances in $AT_2$ receptor signaling that could have important insights in $AT_2$ receptor-associated brain functions.

*8.1. Homo- and Heterodimerization.* Although GPCRs have traditionally been thought to act as monomers (review in [184]), it is now well accepted that many GPCRs can form dimers which could affect both their trafficking and function. In this context, homodimerization of $AT_1$ and $AT_2$ receptors, as well as $AT_1$/$AT_2$ heterodimer formations, has been reported. For example, the $AT_2$ receptor is known to undergo homodimerization, a property which enhances apoptosis [185]. In addition, AbdAlla et al. [186] reported that the $AT_2$ receptor undergoes heterodimerization with the $AT_1$ receptor in transfected PC12 cells, in fetal fibroblasts, and in myometrial biopsies. In an animal model of Alzheimer's disease, the same group demonstrated that A$\beta$ induces the formation of cross-linked $AT_2$ receptor oligomers [64, 187]. Notably, oligomers of $AT_2$ receptors have also been observed in prefrontal cortex specimens of Alzheimer's disease patients, while being completely absent in specimens of nondemented control individuals, thus lending further support for a role of the $AT_2$ receptor in cognitive function. Heterodimerization between the $AT_2$ receptor and bradykinin has also been described in PC12W cells [188]. It is already known that bradykinin mediates $AT_2$ receptor-induced NO production [189–191]. This interaction between the two receptors has been shown to enhance phosphorylation of various kinases, including $p42/p44^{mapk}$ and $p38^{mapk}$. Therefore, it would appear that homo- and heterodimerization of the $AT_2$ receptor may have a role in its regulation. However, these initial observations still require confirmation before being accepted as important regulatory aspects of Ang II receptor signaling and functions (review in [29, 31]). The use of recently developed methodologies such as FRET/BRET technology has confirmed efficient heterodimerization of the $AT_1$ receptor with the bradykinin receptor B2 [192], indicating the potential clinical significance of GPCR oligomerization [29, 31]. Moreover, recent studies have identified intracellular crosstalk pathways between the $AT_1$ receptor and the $AT_2$ receptor at the gene expression level. Indeed, $AT_1$ receptor activation enhances $AT_2$ receptor mRNA degradation, while $AT_2$ receptor activation increases its own mRNA transcription [193].

*8.2. PPAR$\gamma$: Could It Be the Missing Link?* There is existing confusion regarding the mechanism of action of specific ARBs, since some also have partial PPAR$\gamma$ agonistic activity (such as telmisartan, irbesartan, and candesartan). There is some evidence suggesting that this PPAR$\gamma$ activation following blockade of the $AT_1$ receptor could be part of its anti-inflammatory and antioxidative effects, leading to neuroprotection against ischemia and A$\beta$ accumulation [127, 194, 195]. Indeed, neuroprotective effects of PPAR$\gamma$ agonists, such as pioglitazone, have been observed during neural cell differentiation and death, and in inflammatory and neurodegenerative conditions, including amyotrophic lateral sclerosis, Alzheimer's disease and Parkinson's disease models, as well as stroke [196, 197]. PPAR$\gamma$ is a transcriptional factor regulating the expression of multiple genes, thereby promoting the differentiation and development of various tissues, specifically adipose tissue, brain, placenta, and skin (review in [197]). In addition, certain studies have indicated that $AT_2$ receptor stimulation increases PPAR$\gamma$ expression and transcriptional activity, at least in PC2W cells [57] and neurons [131]. The final targets of these pathways are gene expression and phosphorylation of various microtubule-associated proteins, which modulate microtubule stability/dynamics responsible for neurite elongation. This observation is noteworthy, especially with regard to

the implication of PPAR$\gamma$ in NGF-induced neurite outgrowth [198] which clearly suggests a possible crosstalk between the $AT_2$ receptor and NGF pathways. This hypothesis is furthermore reinforced by the observation that inhibition of the NGF receptor TrkA significantly decreases $AT_2$ receptor-induced neurite outgrowth [43]. Moreover, Iwai et al., using atherosclerotic ApoE-KO mice with an $AT_2$ receptor deficiency (AT2R/ApoE double knockout mice), observed that the lack of $AT_2$ receptor expression decreased the expression of PPAR$\gamma$ in adipocytes [199]. These observations strongly suggest a link between the $AT_2$ receptor and PPAR$\gamma$ functions. Considering that similar neuroprotective effects were also associated with the $AT_2$ receptor, it may be hypothesized that activation of PPAR$\gamma$ could be shared both by ARBs and $AT_2$ receptor stimulation.

## 9. Could the $AT_2$ Receptor Be an Attractive Therapeutic Target?

One of the biggest challenges in studying the $AT_2$ receptor is applying observations stemming from the use of cell lines to *in vivo* models. Indeed, studies using cell lines expressing the $AT_2$ receptor either endogenously or via transfection have provided paramount information regarding its intracellular mechanisms of action. However, associating these mechanisms with biological functions has proven to be much more difficult. As indicated previously, synthesis and characterization of the selective $AT_2$ receptor selective agonist C21/M024 in 2004 or of the recently developed antagonist [200] has provided long-awaited tools to bypass the difficulty of using traditional $AT_2$ receptor ligands such as CGP42112A or PD123,319. Since then, many studies have allowed significant advances in the understanding of $AT_2$ receptor functions. Nonetheless, one would have thought that this new $AT_2$ receptor ligand would have resolved certain controversies surrounding this enigmatic receptor. However, eight years after the first characterization of this compound, the clear demonstration of an $AT_2$ receptor effect in the brain remains to be unequivocally established. One major input since C21/M024 was first described is that selective stimulation of the $AT_2$ receptor does not decrease blood pressure [134, 165, 166, 201–206]. These results are quite surprising, considering previous reports emanating from the indirect *in vivo* manipulation studies which associated $AT_2$ receptor activation with vasodilation and a decrease in mean arterial pressure. However, blockade of the $AT_1$ receptor with ARBs not only allows stimulation of the $AT_2$ receptor, the only Ang II receptor available in this condition, but also increases the bioavailability of Ang II for ACE2 and aminopeptidase to produce Ang IV and Ang (1–7), both of which exert vasodilatory effects (Figure 1). Nevertheless, beyond its blood-pressure lowering effects, *in vivo* studies using C21/M024 have described a protective role of the $AT_2$ receptor in vascular remodeling [166, 207], in poststroke cardiac [208] and renal function [165, 205] as well as in cognitive functions [134]. Furthermore, observation of $AT_2$ receptors in tissues associated with glucose metabolism, such as the pancreas and adipose tissue, suggests that the $AT_2$ receptor could also be beneficial in metabolic syndrome and associated cognitive loss. However, the selectivity of C21/M024 for the $AT_2$ receptor has also been recently challenged and differs according to the dosage used and/or route of administration and, importantly, according to experimental conditions. In particular, a recent observation by Verdonk et al. [209], whereby C21/M024 induced vasorelaxation of preconstricted iliac arteries from SHR Wistar rats and C57BL/6 mice as well as in $AT_2$-deficient mice, has rekindled the debate on the relevance of the $AT_2$ receptor and the selectivity of $AT_2$ receptor ligands in physiological functions. Moreover, this effect of C21/M024 in arteries was only partially blocked by PD123,319. These latest findings raise the possibility that C21/M024 could exhibit certain $AT_2$ receptor-independent effects. Therefore, the question still remains: is the $AT_2$ receptor a potential therapeutic target and could it replace or increase beneficial effects associated with ARBs?

## 10. Conclusion

As described in the aforementioned sections, $AT_2$ receptor activation may act at several stages in the cascade of alterations leading to cognitive impairment and neuronal dysfunction. An increasing number of studies suggest that the protective effects of ARBs on brain damage and cognition may result not only from the inhibition of $AT_1$ receptor effects, but also from the beneficial effect due to unopposed activation of the $AT_2$ receptor. In addition, the relationship between impaired energy metabolism/obesity/insulin resistance and the increased risk of dementia emphasizes the view that the mechanisms of action of the $AT_2$ receptor may have a beneficial protective effect. However, the physiological relevance of the $AT_2$ receptor in the brain will need to be compared with at least two other components of RAS, namely, the ACE2/Ang-(1–7)/Mas complex and Ang IV/$AT_4$ receptor/IRAP in the brain (Figure 1).

Lifestyle-related disorders, such as hypertension, T2D, and obesity, are also implicated as risk factors for dementia. In this regard, two recent publications have established that direct $AT_2$ receptor stimulation with C21/M024 improves insulin sensitivity in a rat model of diet-induced insulin resistance [210] and in type 2 diabetic mice [211]. Thus, any treatment aimed at improving insulin resistance or cognitive functions is likely to slow down symptoms and improve quality of life associated with these age-related disorders. Furthermore, the recent development of selective $AT_2$ receptor agonists should facilitate efforts to elucidate distinct roles of the $AT_2$ receptor in brain physiology, supporting or disproving the hypothesis that the $AT_2$ receptor helps improve a number of brain impairments related to neuronal plasticity and morphology, microcirculation and inflammation (Figure 3), all of which are altered in certain neurological disorders.

There is clearly still much work to be accomplished to fully understand the role of the $AT_2$ receptor in normal versuspathological conditions and to determine whether $AT_2$ receptor agonists could represent an attractive therapeutic target. In this aspect, compounds such as C21/M024 as well as other recently synthesized highly selective nonpeptide $AT_2$ receptor ligands (all leading to neurite outgrowth in NG108-15 cells) and their effectiveness to induce $AT_2$

receptor-dependent effects need to be further explored [212–216].

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## References

[1] D. Ganten, A. Marquez-Julio, P. Granger et al., "Renin in dog brain," *The American Journal of Physiology*, vol. 221, no. 6, pp. 1733–1737, 1971.

[2] M. de Gasparo, K. J. Catt, T. Inagami, J. W. Wright, and T. Unger, "International union of pharmacology. XXIII. The angiotensin II receptors," *Pharmacological Reviews*, vol. 52, no. 3, pp. 415–472, 2000.

[3] M. I. Phillips and E. M. de Oliveira, "Brain renin angiotensin in disease," *Journal of Molecular Medicine*, vol. 86, no. 6, pp. 715–722, 2008.

[4] J. W. Wright and J. W. Harding, "The brain renin-angiotensin system: a diversity of functions and implications for CNS diseases," *Pflügers Archiv*. In press.

[5] F. A. Mendelsohn, R. Quirion, J. M. Saavedra, G. Aguilera, and K. J. Catt, "Autoradiographic localization of angiotensin II receptors in rat brain," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 81, no. 5, pp. 1575–1579, 1984.

[6] F. A. O. Mendelsohn, A. M. Allen, J. Clevers, D. A. Denton, E. Tarjan, and M. J. McKinley, "Localization of angiotensin II receptor binding in rabbit brain by in vitro autoradiography," *Journal of Comparative Neurology*, vol. 270, no. 3, pp. 372–384, 1988.

[7] T. Unger, E. Badoer, D. Ganten, R. E. Lang, and R. Rettig, "Brain angiotensin: pathways and pharmacology," *Circulation*, vol. 77, no. 6, pp. 40–54, 1988.

[8] W. B. Severs and A. E. Daniels-Severs, "Effects of angiotensin on the central nervous system," *Pharmacological Reviews*, vol. 25, no. 3, pp. 415–449, 1973.

[9] M. I. Phillips, "Functions of angiotensin in the central nervous system," *Annual Review of Physiology*, vol. 49, pp. 413–435, 1987.

[10] M. Horiuchi, M. Mogi, and M. Iwai, "The angiotensin II type 2 receptor in the brain," *Journal of the Renin-Angiotensin-Aldosterone System*, vol. 11, no. 1, pp. 1–6, 2010.

[11] M. Mogi and M. Horiuchi, "Effect of angiotensin II type 2 receptor on stroke, cognitive impairment and neurodegenerative diseases," *Geriatrics & Gerontology International*. In press.

[12] J. W. Wright and J. W. Harding, "Brain renin-angiotensin—a new look at an old system," *Progress in Neurobiology*, vol. 95, no. 1, pp. 49–67, 2011.

[13] A. D. de Kloet, E. G. Krause, and S. C. Woods, "The renin angiotensin system and the metabolic syndrome," *Physiology and Behavior*, vol. 100, no. 5, pp. 525–534, 2010.

[14] N. Gallo-Payet, M. O. Guimond, L. Bilodeau, C. Wallinder, M. Alterman, and A. Hallberg, "Angiotensin II, a neuropeptide at the frontier between endocrinology and neuroscience: is there a link between the angiotensin II type 2 receptor ($AT_2R$) and Alzheimer's disease?" *Frontiers in Endocrinology*, vol. 2, article 17, pp. 1–10, 2011.

[15] R. Yang, I. Smolders, D. De Bundel et al., "Brain and peripheral angiotensin II type 1 receptors mediate renal vasoconstrictor and blood pressure responses to angiotensin IV in the rat," *Journal of Hypertension*, vol. 26, no. 5, pp. 998–1007, 2008.

[16] J. W. Wright, A. V. Miller-Wing, M. J. Shaffer et al., "Angiotensin II(3-8) (ANG IV) hippocampal binding: potential role in the facilitation of memory," *Brain Research Bulletin*, vol. 32, no. 5, pp. 497–502, 1993.

[17] J. W. Wright, A. J. Bechtholt, S. L. Chambers, and J. W. Harding, "Angiotensin III and IV activation of the brain $AT_1$ receptor subtype in cardiovascular function," *Peptides*, vol. 17, no. 8, pp. 1365–1371, 1996.

[18] U. M. Steckelings, L. Paulis, P. Namsolleck, and T. Unger, "$AT_2$ receptor agonists: hypertension and beyond," *Current Opinion in Nephrology and Hypertension*, vol. 21, pp. 142–146, 2012.

[19] U. M. Steckelings, F. Rompe, E. Kaschina, and T. Unger, "The evolving story of the RAAS in hypertension, diabetes and CV disease—moving from macrovascular to microvascular targets," *Fundamental and Clinical Pharmacology*, vol. 23, no. 6, pp. 693–703, 2009.

[20] K. Verdonk, A. H. Danser, and J. H. van Esch, "Angiotensin II type 2 receptor agonists: where should they be applied?" *Expert Opinion on Investigational Drugs*, vol. 21, no. 4, pp. 501–513, 2012.

[21] S. Whitebread, M. Mele, B. Kamber, and M. De Gasparo, "Preliminary biochemical characterization of two angiotensin II receptor subtypes," *Biochemical and Biophysical Research Communications*, vol. 163, no. 1, pp. 284–291, 1989.

[22] R. C. Speth and K. H. Kim, "Discrimination of two angiotensin II receptor subtypes with a selective agonist analogue of angiotensin II, p-aminophenylalanine6 angiotensin II," *Biochemical and Biophysical Research Communications*, vol. 169, no. 3, pp. 997–1006, 1990.

[23] A. T. Chiu, W. F. Herblin, D. E. McCall et al., "Identification of angiotensin II receptor subtypes," *Biochemical and Biophysical Research Communications*, vol. 165, no. 1, pp. 196–203, 1989.

[24] A. T. Chiu, D. E. McCall, T. T. Nguyen et al., "Discrimination of angiotensin II receptor subtypes by dithiothreitol," *European Journal of Pharmacology*, vol. 170, no. 1-2, pp. 117–118, 1989.

[25] R. C. Speth, B. P. Rowe, K. L. Grove, M. R. Carter, and D. Saylor, "Sulfhydryl reducing agents distinguish two subtypes of angiotensin II receptors in the rat brain," *Brain Research*, vol. 548, no. 1-2, pp. 1–8, 1991.

[26] M. Nakajima, M. Mukoyama, R. E. Pratt, M. Horiuchi, and V. J. Dzau, "Cloning of cDNA and analysis of the gene for mouse angiotensin II type 2 receptor," *Biochemical and Biophysical Research Communications*, vol. 197, no. 2, pp. 393–399, 1993.

[27] Y. Kambayashi, S. Bardhan, K. Takahashi et al., "Molecular cloning of a novel angiotensin II receptor isoform involved in phosphotyrosine phosphatase inhibition," *Journal of Biological Chemistry*, vol. 268, no. 33, pp. 24543–24546, 1993.

[28] L. Gendron, M. D. Payet, and N. Gallo-Payet, "The angiotensin type 2 receptor of angiotensin II and neuronal differentiation: from observations to mechanisms," *Journal of Molecular Endocrinology*, vol. 31, no. 3, pp. 359–372, 2003.

[29] E. R. Porrello, L. M. D. Delbridge, and W. G. Thomas, "The angiotensin II type 2 ($AT_2$) receptor: an enigmatic seven transmembrane receptor," *Frontiers in Bioscience*, vol. 14, no. 3, pp. 958–972, 2009.

[30] N. Gallo-Payet, M. Shum, J. P. Baillargeon et al., "$AT_2$ receptor agonists: exploiting the beneficial arm of Ang II signaling," *Current Hypertension Reviews*, vol. 8, pp. 47–59, 2012.

[31] M. Horiuchi, J. Iwanami, and M. Mogi, "Regulation of angiotensin II receptors beyond the classical pathway," *Clinical Science*, vol. 123, no. 4, pp. 193–203, 2012.

[32] K. Ohinata, Y. Fujiwara, S. Fukumoto, M. Iwai, M. Horiuchi, and M. Yoshikawa, "Angiotensin II and III suppress food intake via angiotensin $AT_2$ receptor and prostaglandin EP4 receptor in mice," *FEBS Letters*, vol. 582, no. 5, pp. 773–777, 2008.

[33] Y. Yamada, D. Yamauchi, H. Usui et al., "Hypotensive activity of novokinin, a potent analogue of ovokinin(2–7), is mediated by angiotensin $AT_2$ receptor and prostaglandin IP receptor," *Peptides*, vol. 29, no. 3, pp. 412–418, 2008.

[34] U. M. Steckelings, F. Rompe, E. Kaschina et al., "The past, present and future of angiotensin II type 2 receptor stimulation," *Journal of the Renin-Angiotensin-Aldosterone System*, vol. 11, no. 1, pp. 67–73, 2010.

[35] T. Unger and B. Dahlöf, "Compound 21, the first orally active, selective agonist of the angiotensin type 2 receptor ($AT_2$): implications for $AT_2$ receptor research and therapeutic potential," *Journal of the Renin-Angiotensin-Aldosterone System*, vol. 11, no. 1, pp. 75–77, 2010.

[36] Y. Wan, C. Wallinder, B. Plouffe et al., "Design, synthesis, and biological evaluation, of the first selective nonpeptide $AT_2$ receptor agonist," *Journal of Medicinal Chemistry*, vol. 47, no. 24, pp. 5995–6008, 2004.

[37] J. Georgsson, C. Sköld, M. Botros et al., "Synthesis of a new class of druglike angiotensin II C-terminal mimics with affinity for the $AT_2$ receptor," *Journal of Medicinal Chemistry*, vol. 50, no. 7, pp. 1711–1715, 2007.

[38] U. M. Steckelings, E. Kaschina, and T. Unger, "The $AT_2$ receptor—a matter of love and hate," *Peptides*, vol. 26, no. 8, pp. 1401–1409, 2005.

[39] L. Gendron, L. Laflamme, N. Rivard, C. Asselin, M. D. Payet, and N. Gallo-Payet, "Signals from the $AT_2$ (angiotensin type 2) receptor of angiotensin II inhibit $p21^{RAS}$ and activate MAPK (mitogen-activated protein kinase) to induce morphological neuronal differentiation in NG108–15 cells," *Molecular Endocrinology*, vol. 13, no. 9, pp. 1615–1626, 1999.

[40] H. Beaudry, L. Gendron, M. O. Guimond, M. D. Payet, and N. Gallo-Payet, "Involvement of protein kinase C$\alpha$ (PKC$\alpha$) in the early action of Angiotensin II type 2 ($AT_2$) effects on neurite outgrowth in NG108–15 cells: $AT_2$-receptor inhibits PKC$\alpha$ and $p21^{RAS}$ activity," *Endocrinology*, vol. 147, no. 9, pp. 4263–4272, 2006.

[41] L. Gendron, J. F. Oligny, M. D. Payet, and N. Gallo-Payet, "Cyclic AMP-independent involvement of Rap1/B-Raf in the angiotensin II $AT_2$ receptor signaling pathway in NG108–15 cells," *Journal of Biological Chemistry*, vol. 278, no. 6, pp. 3606–3614, 2003.

[42] U. Stroth, A. Blume, K. Mielke, and T. Unger, "Angiotensin $AT_2$ receptor stimulates ERK1 and ERK2 in quiescent but inhibits ERK in NGF-stimulated PC12W cells," *Molecular Brain Research*, vol. 78, no. 1-2, pp. 175–180, 2000.

[43] B. Plouffe, M. O. Guimond, H. Beaudry, and N. Gallo-Payet, "Role of tyrosine kinase receptors in angiotensin II $AT_2$ receptor signaling: involvement in neurite outgrowth and in $p42/p44^{mapk}$ activation in NG108–15 cells," *Endocrinology*, vol. 147, no. 10, pp. 4646–4654, 2006.

[44] M. O. Guimond, C. Roberge, and N. Gallo-Payet, "Fyn is involved in angiotensin II type 2 receptor-induced neurite outgrowth, but not in $p42/p44^{mapk}$ in NG108–15 cells," *Molecular and Cellular Neuroscience*, vol. 45, no. 3, pp. 201–212, 2010.

[45] L. Gendron, F. Côté, M. D. Payet, and N. Gallo-Payet, "Nitric oxide and cyclic GMP are involved in angiotensin II $AT_2$ receptor effects on neurite outgrowth in NG108–15 cells," *Neuroendocrinology*, vol. 75, no. 1, pp. 70–81, 2002.

[46] D. Müller, K. J. Greenland, R. C. Speth, and R. Middendorff, "Neuronal differentiation of NG108–15 cells has impact on nitric oxide- and membrane (natriuretic peptide receptor-A) cyclic GMP-generating proteins," *Molecular and Cellular Endocrinology*, vol. 320, no. 1-2, pp. 118–127, 2010.

[47] J. M. Li, M. Mogi, K. Tsukuda et al., "Angiotensin II-induced neural differentiation via angiotensin II type 2 ($AT_2$) receptor-MMS2 cascade involving interaction between $AT_2$ receptor-interacting protein and Src homology 2 domain-containing protein-tyrosine phosphatase 1," *Molecular Endocrinology*, vol. 21, no. 2, pp. 499–511, 2007.

[48] S. Nouet, N. Amzallag, J. M. Li et al., "Trans-inactivation of receptor tyrosine kinases by novel angiotensin II $AT_2$ receptor-interacting protein, ATIP," *Journal of Biological Chemistry*, vol. 279, no. 28, pp. 28989–28997, 2004.

[49] M. Horiuchi and M. Mogi, "Role of angiotensin II receptor subtype activation in cognitive function and ischaemic brain damage," *British Journal of Pharmacology*, vol. 163, no. 6, pp. 1122–1130, 2011.

[50] N. Hashikawa-Hobara, N. Hashikawa, Y. Inoue et al., "Candesartan cilexetil improves angiotensin II type 2 receptor-mediated neurite outgrowth via the PI3K-Akt pathway in fructose-induced insulin-resistant rats," *Diabetes*, vol. 61, no. 4, pp. 925–932, 2012.

[51] X. C. Huang, E. M. Richards, and C. Sumners, "Angiotensin II type 2 receptor-mediated stimulation of protein phosphatase 2A in rat hypothalamic/brainstem neuronal cocultures," *Journal of Neurochemistry*, vol. 65, no. 5, pp. 2131–2137, 1995.

[52] X. C. Huang, E. M. Richards, and C. Sumners, "Mitogen-activated protein kinases in rat brain neuronal cultures are activated by angiotensin II type 1 receptors and inhibited by angiotensin II type 2 receptors," *Journal of Biological Chemistry*, vol. 271, no. 26, pp. 15635–15641, 1996.

[53] R. Caballero, R. Gómez, I. Moreno et al., "Interaction of angiotensin II with the angiotensin type 2 receptor inhibits the cardiac transient outward potassium current," *Cardiovascular Research*, vol. 62, no. 1, pp. 86–95, 2004.

[54] P. Kilian, S. Campbell, L. Bilodeau et al., "Angiotensin II type 2 receptor stimulation increases the rate of NG108–15 cell migration via actin depolymerization," *Endocrinology*, vol. 149, no. 6, pp. 2923–2933, 2008.

[55] K. Seidel, S. Kirsch, K. Lucht et al., "The promyelocytic leukemia zinc finger (PLZF) protein exerts neuroprotective effects in neuronal cells and is dysregulated in experimental stroke," *Brain Pathology*, vol. 21, no. 1, pp. 31–43, 2011.

[56] T. Senbonmatsu, T. Saito, E. J. Landon et al., "A novel angiotensin II type 2 receptor signaling pathway: possible role in cardiac hypertrophy," *EMBO Journal*, vol. 22, no. 24, pp. 6471–6482, 2003.

[57] Y. Zhao, A. Foryst-Ludwig, D. Bruemmer et al., "Angiotensin II induces peroxisome proliferator-activated receptor gamma in PC12W cells via angiotensin type 2 receptor activation," *Journal of Neurochemistry*, vol. 94, no. 5, pp. 1395–1401, 2005.

[58] M. Mogi, M. Iwai, and M. Horiuchi, "Emerging concepts of regulation of angiotensin II receptors: new players and targets for traditional receptors," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 27, no. 12, pp. 2532–2539, 2007.

[59] H. Funke-Kaiser, J. Reinemund, U. M. Steckelings, and T. Unger, "Adapter proteins and promoter regulation of the angiotensin $AT_2$ receptor—implications for cardiac pathophysiology," *Journal of the Renin-Angiotensin-Aldosterone System*, vol. 11, no. 1, pp. 7–17, 2010.

[60] S. Rodrigues-Ferreira and C. Nahmias, "An ATIPical family of angiotensin II $AT_2$ receptor-interacting proteins," *Trends in Endocrinology and Metabolism*, vol. 21, no. 11, pp. 684–690, 2010.

[61] Y. Zou, H. Akazawa, Y. Qin et al., "Mechanical stress activates angiotensin II type 1 receptor without the involvement of angiotensin II," *Nature Cell Biology*, vol. 6, no. 6, pp. 499–506, 2004.

[62] N. Yasuda, H. Akazawa, Y. Qin, Y. Zou, and I. Komuro, "A novel mechanism of mechanical stress-induced angiotensin II type 1-receptor activation without the involvement of angiotensin II," *Naunyn-Schmiedeberg's Archives of Pharmacology*, vol. 377, no. 4–6, pp. 393–399, 2008.

[63] S. I. Miura and S. S. Karnik, "Ligand-independent signals from angiotensin II type 2 receptor induce apoptosis," *The EMBO Journal*, vol. 19, no. 15, pp. 4026–4035, 2000.

[64] S. AbdAlla, H. Lother, A. El Missiry et al., "Angiotensin II $AT_2$ receptor oligomers mediate G-protein dysfunction in an animal model of Alzheimer disease," *Journal of Biological Chemistry*, vol. 284, no. 10, pp. 6554–6565, 2009.

[65] B. L. Falcón, S. J. Veerasingham, C. Sumners, and M. K. Raizada, "Angiotensin II type 2 receptor-mediated gene expression profiling in human coronary artery endothelial cells," *Hypertension*, vol. 45, no. 4, pp. 692–697, 2005.

[66] J. M. Patel, Y. D. Li, J. Zhang, C. H. Gelband, M. K. Raizada, and E. R. Block, "Increased expression of calreticulin is linked to ANG IV-mediated activation of lung endothelial NOS," *American Journal of Physiology*, vol. 277, no. 4, pp. L794–L801, 1999.

[67] J. M. Patel, J. R. Martens, Y. D. Li, C. H. Gelband, M. K. Raizada, and E. R. Block, "Angiotensin IV receptor-mediated activation of lung endothelial NOS is associated with vasorelaxation," *American Journal of Physiology*, vol. 275, no. 6, pp. L1061–L1068, 1998.

[68] Y. D. Li, E. R. Block, and J. M. Patel, "Activation of multiple signaling modules is critical in angiotensin IV-induced lung endothelial cell proliferation," *American Journal of Physiology*, vol. 283, no. 4, pp. L707–L716, 2002.

[69] Y. C. Wong, M. K. Sim, and K. O. Lee, "Des-aspartate-angiotensin-I and angiotensin IV improve glucose tolerance and insulin signalling in diet-induced hyperglycaemic mice," *Biochemical Pharmacology*, vol. 82, no. 9, pp. 1198–1208, 2011.

[70] D. De Bundel, I. Smolders, P. Vanderheyden, and Y. Michotte, "Ang II and Ang IV: unraveling the mechanism of action on synaptic plasticity, memory, and epilepsy," *CNS Neuroscience and Therapeutics*, vol. 14, no. 4, pp. 315–339, 2008.

[71] J. W. Wright and J. W. Harding, "The angiotensin $AT_4$ receptor subtype as a target for the treatment of memory dysfunction associated with Alzheimer's disease," *Journal of the Renin-Angiotensin-Aldosterone System*, vol. 9, no. 4, pp. 226–237, 2008.

[72] Z. Lenkei, M. Palkovits, P. Corvol, and C. Llorens-Cortes, "Distribution of angiotensin II type-2 receptor ($AT_2$) mRNA expression in the adult rat brain," *The Journal of Comparative Neurology*, vol. 373, no. 3, pp. 322–339, 1996.

[73] Z. Lenkei, M. Palkovits, P. Corvol, and C. Llorens-Cortès, "Expression of angiotensin type-1 ($AT_1$) and type-2 ($AT_2$) receptor mRNAs in the adult rat brain: a functional neuroanatomical review," *Frontiers in Neuroendocrinology*, vol. 18, no. 4, pp. 383–439, 1997.

[74] K. Song, A. M. Allen, G. Paxinos, and F. A. O. Mendelsohn, "Angiotensin II receptor subtypes in rat brain," *Clinical and Experimental Pharmacology and Physiology*, vol. 18, no. 2, pp. 93–96, 1991.

[75] K. Song, A. M. Allen, G. Paxinos, and F. A. O. Mendelsohn, "Mapping of angiotensin II receptor subtype heterogeneity in rat brain," *Journal of Comparative Neurology*, vol. 316, no. 4, pp. 467–484, 1992.

[76] T. N. Grammatopoulos, S. M. Jones, F. A. Ahmadi et al., "Angiotensin type I receptor antagonist losartan, reduces MPTP-induced degeneration of dopaminergic neurons in substantia nigra," *Molecular Neurodegeneration*, vol. 2, article no. 1, 2007.

[77] G. A. Argañaraz, A. C. Konno, S. R. Perosa et al., "The renin-angiotensin system is upregulated in the cortex and hippocampus of patients with temporal lobe epilepsy related to mesial temporal sclerosis," *Epilepsia*, vol. 49, no. 8, pp. 1348–1357, 2008.

[78] S. P. Bottari, N. Obermuller, Y. Bogdal, K. R. Zahs, and C. F. Deschepper, "Characterization and distribution of angiotensin II binding sites in fetal and neonatal astrocytes from different rat brain regions," *Brain Research*, vol. 585, no. 1-2, pp. 372–376, 1992.

[79] V. T. Karamyan and R. C. Speth, "Identification of a novel non-$AT_1$, non-$AT_2$ angiotensin binding site in the rat brain," *Brain Research*, vol. 1143, no. 1, pp. 83–91, 2007.

[80] S. Y. Chai, R. Fernando, G. Peck et al., "The angiotensin IV/$AT_4$ receptor," *Cellular and Molecular Life Sciences*, vol. 61, no. 21, pp. 2728–2737, 2004.

[81] P. M. L. Vanderheyden, "From angiotensin IV binding site to $AT_4$ receptor," *Molecular and Cellular Endocrinology*, vol. 302, no. 2, pp. 159–166, 2009.

[82] R. A. S. Santos, F. Frézard, and A. J. Ferreira, "Angiotensin-(1-7): blood, heart and blood vessels," *Current Medicinal Chemistry: Cardiovascular and Hematological Agents*, vol. 3, no. 4, pp. 383–391, 2005.

[83] L. Gao and I. H. Zucker, "$AT_2$ receptor signaling and sympathetic regulation," *Current Opinion in Pharmacology*, vol. 11, no. 2, pp. 124–130, 2011.

[84] B. Buisson, S. P. Bottari, M. De Gasparo, N. Gallo-Payet, and M. D. Payet, "The angiotensin $AT_2$ receptor modulates T-type calcium current in non-differentiated NG108–15 cells," *FEBS Letters*, vol. 309, no. 2, pp. 161–164, 1992.

[85] B. Buisson, L. Laflamme, S. P. Bottari, M. De Gasparo, N. Gallo-Payet, and M. D. Payet, "A G protein is involved in the angiotensin $AT_2$ receptor inhibition of the T-type calcium current in non-differentiated NG108–15 cells," *Journal of Biological Chemistry*, vol. 270, no. 4, pp. 1670–1674, 1995.

[86] J. Kang, P. Posner, and C. Sumners, "Angiotensin II type 2 receptor stimulation of neuronal $K^+$ currents involves an inhibitory GTP binding protein," *American Journal of Physiology*, vol. 267, no. 5, pp. C1389–C1397, 1994.

[87] T. N. Grammatopoulos, V. Johnson, S. A. Moore, R. Andres, and J. A. Weyhenmeyer, "Angiotensin type 2 receptor neuroprotection against chemical hypoxia is dependent on the delayed rectifier $K^+$ channel, $Na^+/Ca^{2+}$ exchanger and

$Na^+/K^+$ ATPase in primary cortical cultures," *Neuroscience Research*, vol. 50, no. 3, pp. 299–306, 2004.

[88] H. Xiong, "Angiotensin II depresses glutamate depolarizations and excitatory postsynaptic potentials in locus coeruleus through angiotensin II subtype 2 receptors," *Neuroscience*, vol. 62, no. 1, pp. 163–175, 1994.

[89] R. Lucius, S. Gallinat, P. Rosenstiel, T. Herdegen, J. Sievers, and T. Unger, "The angiotensin II type 2 ($AT_2$) receptor promotes axonal regeneration in the optic nerve of adult rats," *Journal of Experimental Medicine*, vol. 188, no. 4, pp. 661–670, 1998.

[90] M. Mogi, J. M. Li, J. Iwanami et al., "Angiotensin II type-2 receptor stimulation prevents neural damage by transcriptional activation of methyl methanesulfonate sensitive 2," *Hypertension*, vol. 48, no. 1, pp. 141–148, 2006.

[91] F. Côté, T. H. Do, L. Laflamme, J. M. Gallo, and N. Gallo-Payet, "Activation of the $AT_2$ receptor of angiotensin II induces neurite outgrowth and cell migration in microexplant cultures of the cerebellum," *Journal of Biological Chemistry*, vol. 274, no. 44, pp. 31686–31692, 1999.

[92] L. Laflamme, M. De Gasparo, J. M. Gallo, M. D. Payet, and N. Gallo-Payet, "Angiotensin II induction of neurite outgrowth by $AT_2$ receptors in NG108–15 cells. Effect counteracted by the $AT_1$ receptors," *Journal of Biological Chemistry*, vol. 271, no. 37, pp. 22729–22735, 1996.

[93] J. Li, J. Culman, H. Hörtnagl et al., "Angiotensin $AT_2$ receptor protects against cerebral ischemia-induced neuronal injury," *FASEB Journal*, vol. 19, no. 6, pp. 617–619, 2005.

[94] S. Gallinat, M. Yu, A. Dorst, T. Unger, and T. Herdegen, "Sciatic nerve transection evokes lasting up-regulation of angiotensin $AT_2$ and $AT_1$ receptor mRNA in adult rat dorsal root ganglia and sciatic nerves," *Molecular Brain Research*, vol. 57, no. 1, pp. 111–122, 1998.

[95] N. Hobara, M. Goda, N. Yoshida et al., "Angiotensin II type 2 receptors facilitate reinnervation of phenol-lesioned vascular calcitonin gene-related peptide-containing nerves in rat mesenteric arteries," *Neuroscience*, vol. 150, no. 3, pp. 730–741, 2007.

[96] A. Okamura, H. Rakugi, M. Ohishi et al., "Upregulation of renin-angiotensin system during differentiation of monocytes to macrophages," *Journal of Hypertension*, vol. 17, no. 4, pp. 537–545, 1999.

[97] K. A. Nahmod, M. E. Vermeulen, S. Raiden et al., "Control of dendritic cell differentiation by angiotensin II," *The FASEB Journal*, vol. 17, no. 3, pp. 491–493, 2003.

[98] J. Iwanami, M. Mogi, K. Tsukuda et al., "Effect of angiotensin II type 2 receptor deletion in hematopoietic cells on brain ischemia-reperfusion injury," *Hypertension*, vol. 58, no. 3, pp. 404–409, 2011.

[99] C. V. Borlongan, L. E. Glover, N. Tajiri, Y. Kaneko, and T. B. Freeman, "The great migration of bone marrow-derived stem cells toward the ischemic brain: therapeutic implications for stroke and other neurological disorders," *Progress in Neurobiology*, vol. 95, no. 2, pp. 213–228, 2011.

[100] L. Rein, G. S. Barsh, R. E. Pratt, V. J. Dzau, and B. K. Kobilka, "Behavioural and cardiovascular effects of disrupting the angiotensin II type-2 receptor gene in mice," *Nature*, vol. 377, no. 6551, pp. 744–747, 1995.

[101] T. Ichiki, P. A. Labosky, C. Shiota et al., "Effects on blood pressure exploratory behaviour of mice lacking angiotensin II type 2 receptor," *Nature*, vol. 377, no. 6551, pp. 748–750, 1995.

[102] H. M. Siragy, T. Inagami, T. Ichiki, and R. M. Carey, "Sustained hypersensitivity to angiotensin II and its mechanism in mice lacking the subtype-2 ($AT_2$) angiotensin receptor," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 96, no. 11, pp. 6506–6510, 1999.

[103] M. Iwai, H. W. Liu, R. Chen et al., "Possible inhibition of focal cerebral ischemia by angiotensin II type 2 receptor stimulation," *Circulation*, vol. 110, no. 7, pp. 843–848, 2004.

[104] M. Iwai, R. Chen, Z. Li et al., "Deletion of angiotensin II type 2 receptor exaggerated atherosclerosis in apolipoprotein E-null mice," *Circulation*, vol. 112, no. 11, pp. 1636–1643, 2005.

[105] S. Okuyama, T. Sakagawa, S. Chaki, Y. Imagawa, T. Ichiki, and T. Inagami, "Anxiety-like behavior in mice lacking the angiotensin II type-2 receptor," *Brain Research*, vol. 821, no. 1, pp. 150–159, 1999.

[106] J. J. Braszko, "$AT_2$ but not $AT_1$ receptor antagonism abolishes angiotensin II increase of the acquisition of conditioned avoidance responses in rats," *Behavioural Brain Research*, vol. 131, no. 1-2, pp. 79–86, 2002.

[107] I. Makino, K. Shibata, Y. Ohgami, M. Fujiwara, and T. Furukawa, "Transient upregulation of the $AT_2$ receptor mRNA level after global ischemia in the rat brain," *Neuropeptides*, vol. 30, no. 6, pp. 596–601, 1996.

[108] J. Ge and N. M. Barnes, "Alterations in angiotensin $AT_1$ and $AT_2$ receptor subtype levels in brain regions from patients with neurodegenerative disorders," *European Journal of Pharmacology*, vol. 297, no. 3, pp. 299–306, 1996.

[109] R. L. Schalock, R. A. Luckasson, K. A. Shogren et al., "The renaming of mental retardation: understanding the change to the term intellectual disability," *Intellectual and Developmental Disabilities*, vol. 45, no. 2, pp. 116–124, 2007.

[110] V. S. Vervoort, M. A. Beachem, P. S. Edwards et al., "AGTR2 mutations in X-linked mental retardation," *Science*, vol. 296, no. 5577, pp. 2401–2403, 2002.

[111] E. Takeshita, E. Nakagawa, K. Nakatani, M. Sasaki, and Y. I. Goto, "Novel AGTR2 missense mutation in a Japanese boy with severe mental retardation, pervasive developmental disorder, and epilepsy," *Brain & Development*, vol. 34, pp. 776–779, 2012.

[112] T. Ylisaukko-oja, K. Rehnström, R. Vanhala, C. Tengström, J. Lähdetie, and I. Järvelä, "Identification of two AGTR2 mutations in male patients with non-syndromic mental retardation," *Human Genetics*, vol. 114, no. 2, pp. 211–213, 2004.

[113] T. Bienvenu, K. Poirier, H. Van Esch et al., "Rare polymorphic variants of the AGTR2 gene in boys with non-specific mental retardation," *Journal of Medical Genetics*, vol. 40, no. 5, pp. 357–359, 2003.

[114] J. Erdmann, S. Dähmlow, M. Guse et al., "The assertion that a G21V mutation in AGTR2 causes mental retardation is not supported by other studies," *Human Genetics*, vol. 114, no. 4, pp. 396–397, 2004.

[115] D. Huang, W. Sun, and C. M. Strom, "Sequence variations in AGTR2 are unlikely to be associated with x-linked mental retardation," *American Journal of Medical Genetics*, vol. 139, no. 3, pp. 243–244, 2005.

[116] V. Boissonneault, M. Filali, M. Lessard, J. Relton, G. Wong, and S. Rivest, "Powerful beneficial effects of macrophage colony-stimulating factor on $\beta$-amyloid deposition and cognitive impairment in Alzheimers disease," *Brain*, vol. 132, no. 4, pp. 1078–1092, 2009.

[117] C. Iadecola, "Neurovascular regulation in the normal brain and in Alzheimer's disease," *Nature Reviews Neuroscience*, vol. 5, no. 5, pp. 347–360, 2004.

[118] F. M. LaFerla, K. N. Green, and S. Oddo, "Intracellular amyloid-$\beta$ in Alzheimer's disease," *Nature Reviews Neuroscience*, vol. 8, no. 7, pp. 499–509, 2007.

[119] L. Mucke, "Neuroscience: Alzheimer's disease," *Nature*, vol. 461, no. 7266, pp. 895–897, 2009.

[120] P. T. Nelson, H. Braak, and W. R. Markesbery, "Neuropathology and cognitive impairment in alzheimer disease: a complex but coherent relationship," *Journal of Neuropathology and Experimental Neurology*, vol. 68, no. 1, pp. 1–14, 2009.

[121] B. V. Zlokovic, "Neurovascular mechanisms of Alzheimer's neurodegeneration," *Trends in Neurosciences*, vol. 28, no. 4, pp. 202–208, 2005.

[122] P. R. Gard and J. M. Rusted, "Angiotensin and Alzheimer's disease: therapeutic prospects," *Expert Review of Neurotherapeutics*, vol. 4, no. 1, pp. 87–96, 2004.

[123] J. Stegbauer and T. M. Coffman, "New insights into angiotensin receptor actions: from blood pressure to aging," *Current Opinion in Nephrology and Hypertension*, vol. 20, no. 1, pp. 84–88, 2011.

[124] K. Shah, S. U. Qureshi, M. Johnson, N. Parikh, P. E. Schulz, and M. E. Kunik, "Does use of antihypertensive drugs affect the incidence or progression of dementia? A systematic review," *American Journal Geriatric Pharmacotherapy*, vol. 7, no. 5, pp. 250–261, 2009.

[125] N. M. Davies, P. G. Kehoe, Y. Ben-Shlomo, and R. M. Martin, "Associations of anti-hypertensive treatments with Alzheimer's disease, vascular dementia, and other dementias," *Journal of Alzheimer's Disease*, vol. 26, no. 4, pp. 699–708, 2011.

[126] N. C. Li, A. Lee, R. A. Whitmer et al., "Use of angiotensin receptor blockers and risk of dementia in a predominantly male population: prospective cohort analysis," *BMJ*, vol. 340, Article ID b5465, 2010.

[127] K. Tsukuda, M. Mogi, J. Iwanami et al., "Cognitive deficit in amyloid-$\beta$-injected mice was improved by pretreatment with a low dose of telmisartan partly because of peroxisome proliferator-activated receptor-$\gamma$ activation," *Hypertension*, vol. 54, no. 4, pp. 782–787, 2009.

[128] K. Kume, H. Hanyu, H. Sakurai, Y. Takada, T. Onuma, and T. Iwamoto, "Effects of telmisartan on cognition and regional cerebral blood flow in hypertensive patients with Alzheimer's disease," *Geriatrics & Gerontology International*, vol. 12, no. 2, pp. 207–214, 2012.

[129] J. Wang, L. Ho, L. Chen et al., "Valsartan lowers brain $\beta$-amyloid protein levels and improves spatial learning in a mouse model of Alzheimer disease," *Journal of Clinical Investigation*, vol. 117, no. 11, pp. 3393–3402, 2007.

[130] L. Danielyan, R. Klein, L. R. Hanson et al., "Protective effects of intranasal losartan in the APP/PS1 transgenic mouse model of Alzheimer disease," *Rejuvenation Research*, vol. 13, no. 2-3, pp. 195–201, 2010.

[131] M. Mogi, J. M. Li, K. Tsukuda et al., "Telmisartan prevented cognitive decline partly due to PPAR-$\gamma$ activation," *Biochemical and Biophysical Research Communications*, vol. 375, no. 3, pp. 446–449, 2008.

[132] S. Takeda, N. Sato, D. Takeuchi et al., "Angiotensin receptor blocker prevented $\beta$-amyloid-induced cognitive impairment associated with recovery of neurovascular coupling," *Hypertension*, vol. 54, no. 6, pp. 1345–1352, 2009.

[133] M. Mogi and M. Horiuchi, "Effects of angiotensin II receptor blockers on dementia," *Hypertension Research*, vol. 32, no. 9, pp. 738–740, 2009.

[134] F. Jing, M. Mogi, A. Sakata et al., "Direct stimulation of angiotensin II type 2 receptor enhances spatial memory," *Journal of Cerebral Blood Flow and Metabolism*, vol. 32, no. 2, pp. 248–255, 2012.

[135] A. Sakata, M. Mogi, J. Iwanami et al., "Sex-different effect of angiotensin II type 2 receptor on ischemic brain injury and cognitive function," *Brain Research*, vol. 1300, pp. 14–23, 2009.

[136] J. Zhou, J. Pavel, M. Macova et al., "$AT_1$ receptor blockade regulates the local angiotensin II system in cerebral microvessels from spontaneously hypertensive rats," *Stroke*, vol. 37, no. 5, pp. 1271–1276, 2006.

[137] C. A. McCarthy, A. Vinh, J. K. Callaway, and R. E. Widdop, "Angiotensin $AT_2$ receptor stimulation causes neuroprotection in a conscious rat model of stroke," *Stroke*, vol. 40, no. 4, pp. 1482–1489, 2009.

[138] T. Grammatopoulos, K. Morris, P. Ferguson, and J. Weyhenmeyer, "Angiotensin protects cortical neurons from hypoxic-induced apoptosis via the angiotensin type 2 receptor," *Molecular Brain Research*, vol. 99, no. 2, pp. 114–124, 2002.

[139] T. N. Grammatopoulos, K. Morris, C. Bachar, S. Moore, R. Andres, and J. A. Weyhenmeyer, "Angiotensin II attenuates chemical hypoxia-induced caspase-3 activation in primary cortical neuronal cultures," *Brain Research Bulletin*, vol. 62, no. 4, pp. 297–303, 2004.

[140] A. Chakrabarty, A. Blacklock, S. Svojanovsky, and P. G. Smith, "Estrogen elicits dorsal root ganglion axon sprouting via a renin-angiotensin system," *Endocrinology*, vol. 149, no. 7, pp. 3452–3460, 2008.

[141] J. C. Sullivan, "Sex and the renin-angiotensin system: inequality between the sexes in response to RAS stimulation and inhibition," *American Journal of Physiology*, vol. 294, no. 4, pp. R1220–R1226, 2008.

[142] J. J. Braszko, G. Kupryszewski, B. Witczuk, and K. Wisniewski, "Angiotensin II-(3-8)-hexapeptide affects motor activity, performance of passive avoidance and a conditioned avoidance response in rats," *Neuroscience*, vol. 27, no. 3, pp. 777–783, 1988.

[143] J. J. Braszko, J. Wlasienko, W. Koziolkiewicz, A. Janecka, and K. Wisniewski, "The 3-7 fragment of angiotensin II is probably responsible for its psychoactive properties," *Brain Research*, vol. 542, no. 1, pp. 49–54, 1991.

[144] S. B. Waters, M. D'Auria, S. S. Martin, C. Nguyen, L. M. Kozma, and K. L. Luskey, "The amino terminus of insulin-responsive aminopeptidase causes Glut4 translocation in 3T3-L1 adipocytes," *Journal of Biological Chemistry*, vol. 272, no. 37, pp. 23323–23327, 1997.

[145] A. L. Albiston, G. R. Peck, H. R. Yeatman, R. Fernando, S. Ye, and S. Y. Chai, "Therapeutic targeting of insulin-regulated aminopeptidase: heads and tails?" *Pharmacology and Therapeutics*, vol. 116, no. 3, pp. 417–427, 2007.

[146] K. Beyer, "Mechanistic aspects of Parkinson's disease: $\alpha$-synuclein and the biomembrane," *Cell Biochemistry and Biophysics*, vol. 47, no. 2, pp. 285–299, 2007.

[147] B. Mertens, P. Vanderheyden, Y. Michotte, and S. Sarre, "The role of the central renin-angiotensin system in Parkinson's disease," *Journal of the Renin-Angiotensin-Aldosterone System*, vol. 11, no. 1, pp. 49–56, 2010.

[148] P. Rey, A. Lopez-Real, S. Sanchez-Iglesias, A. Muñoz, R. Soto-Otero, and J. L. Labandeira-Garcia, "Angiotensin type-1-receptor antagonists reduce 6-hydroxydopamine toxicity for dopaminergic neurons," *Neurobiology of Aging*, vol. 28, no. 4, pp. 555–567, 2007.

[149] J. Rodriguez-Pallares, C. R. Quiroz, J. A. Parga, M. J. Guerra, and J. L. Labandeira-Garcia, "Angiotensin II increases differentiation of dopaminergic neurons from mesencephalic precursors via angiotensin type 2 receptors," *European Journal of Neuroscience*, vol. 20, no. 6, pp. 1489–1498, 2004.

[150] T. N. Grammatopoulos, F. Ahmadi, S. M. Jones, M. W. Fariss, J. A. Weyhenmeyer, and W. M. Zawada, "Angiotensin II protects cultured midbrain dopaminergic neurons against rotenone-induced cell death," *Brain Research*, vol. 1045, no. 1-2, pp. 64–71, 2005.

[151] B. Villar-Cheda, R. Valenzuela, A. I. Rodriguez-Perez, M. J. Guerra, and J. L. Labandeira-Garcia, "Aging-related changes in the nigral angiotensin system enhances proinflammatory and pro-oxidative markers and 6-OHDA-induced dopaminergic degeneration," *Neurobiology of Aging*, vol. 33, no. 1, pp. e201–e211, 2012.

[152] D. W. Haslam and W. P. T. James, "Obesity," *Lancet*, vol. 366, no. 9492, pp. 1197–1209, 2005.

[153] P. S. Leung, H. C. Chan, L. X. M. Fu, and P. Y. D. Wong, "Localization of angiotensin II receptor subtypes $AT_1$ and $AT_2$ in the pancreas of rodents," *Journal of Endocrinology*, vol. 153, no. 2, pp. 269–274, 1997.

[154] P. S. Leung, W. P. Chan, T. P. Wong, and C. Sernia, "Expression and localization of the renin-angiotensin system in the rat pancreas," *Journal of Endocrinology*, vol. 160, no. 1, pp. 13–19, 1999.

[155] S. W. Tsang, C. H. K. Cheng, and P. S. Leung, "The role of the pancreatic renin-angiotensin system in acinar digestive enzyme secretion and in acute pancreatitis," *Regulatory Peptides*, vol. 119, no. 3, pp. 213–219, 2004.

[156] K. K. Leung, J. Liang, M. T. Ma, and P. S. Leung, "Angiotensin II type 2 receptor is critical for the development of human fetal pancreatic progenitor cells into islet-like cell clusters and their potential for transplantation," *Stem Cells*, vol. 30, no. 3, pp. 525–536, 2012.

[157] B. Ulmasov, Z. Xu, L. H. Tetri, T. Inagami, and B. A. Neuschwander-Tetri, "Protective role of angiotensin II type 2 receptor signaling in a mouse model of pancreatic fibrosis," *American Journal of Physiology*, vol. 296, no. 2, pp. G284–G294, 2009.

[158] C. Doi, N. Egashira, A. Kawabata et al., "Angiotensin II type 2 receptor signaling significantly attenuates growth of murine pancreatic carcinoma grafts in syngeneic mice," *BMC Cancer*, vol. 10, article no. 67, 2010.

[159] K. Y. Lam and P. S. Leung, "Regulation and expression of a renin-angiotensin system in human pancreas and pancreatic endocrine tumours," *European Journal of Endocrinology*, vol. 146, no. 4, pp. 567–572, 2002.

[160] J. P. Porter and K. R. Potratz, "Effect of intracerebroventricular angiotensin II on body weight and food intake in adult rats," *American Journal of Physiology*, vol. 287, no. 2, pp. R422–R428, 2004.

[161] J. P. Porter, J. M. Anderson, R. J. Robison, and A. C. Phillips, "Effect of central angiotensin II on body weight gain in young rats," *Brain Research*, vol. 959, no. 1, pp. 20–28, 2003.

[162] G. Aubert, M. Burnier, A. Dulloo et al., "Neuroendocrine characterization and anorexigenic effects of telmisartan in diet- and glitazone-induced weight gain," *Metabolism*, vol. 59, no. 1, pp. 25–32, 2010.

[163] K. Ohinata, Y. Fujiwata, F. Shingo, I. Masaru, H. Masatsugu, and M. Yoshikawa, "Orally administered novokinin, an angiotensin $AT_2$ receptor agonist, suppresses food intake via prostaglandin $E_2$-dependent mechanism in mice," *Peptides*, vol. 30, no. 6, pp. 1105–1108, 2009.

[164] V. N. Uebele, A. L. Gotter, C. E. Nuss et al., "Antagonism of T-type calcium channels inhibits high-fat diet-induced weight gain in mice," *Journal of Clinical Investigation*, vol. 119, no. 6, pp. 1659–1667, 2009.

[165] P. Gelosa, A. Pignieri, L. Fä et al., "Stimulation of $AT_2$ receptor exerts beneficial effects in stroke-prone rats: focus on renal damage," *Journal of Hypertension*, vol. 27, no. 12, pp. 2444–2451, 2009.

[166] A. Rehman, A. Leibowitz, N. Yamamoto, Y. Rautureau, P. Paradis, and E. L. Schiffrin, "Angiotensin type 2 receptor agonist compound 21 reduces vascular injury and myocardial fibrosis in stroke-prone spontaneously hypertensive rats," *Hypertension*, vol. 59, no. 2, pp. 291–299, 2012.

[167] R. S. Weisinger, T. K. Stanley, D. P. Begg, H. S. Weisinger, K. J. Spark, and M. Jois, "Angiotensin converting enzyme inhibition lowers body weight and improves glucose tolerance in C57BL/6J mice maintained on a high fat diet," *Physiology and Behavior*, vol. 98, no. 1-2, pp. 192–197, 2009.

[168] B. Kim and E. L. Feldman, "Insulin resistance in the nervous system," *Trends in Endocrinology and Metabolism*, vol. 23, no. 3, pp. 133–141, 2012.

[169] S. M. de la Monte, "Insulin resistance and Alzheimer's disease," *BMB Reports*, vol. 42, no. 8, pp. 475–481, 2009.

[170] S. M. de la Monte, "Contributions of brain insulin resistance and deficiency in amyloid-related neurodegeneration in Alzheimer's disease," *Drugs*, vol. 72, no. 1, pp. 49–66, 2012.

[171] D. Bosco, A. Fava, M. Plastino, T. Montalcini, and A. Pujia, "Possible implications of insulin resistance and glucose metabolism in Alzheimer's disease pathogenesis," *Journal of Cellular and Molecular Medicine*, vol. 15, no. 9, pp. 1807–1821, 2011.

[172] G. J. Biessels and L. J. Kappelle, "Increased risk of Alzheimer's disease in type II diabetes: insulin resistance of the brain or insulin-induced amyloid pathology?" *Biochemical Society Transactions*, vol. 33, no. 5, pp. 1041–1044, 2005.

[173] S. Craft, "Insulin resistance syndrome and Alzheimer disease: pathophysiologic mechanisms and therapeutic implications," *Alzheimer Disease and Associated Disorders*, vol. 20, no. 4, pp. 298–301, 2006.

[174] S. Craft, "The role of metabolic disorders in Alzheimer disease and vascular dementia: two roads converged," *Archives of Neurology*, vol. 66, no. 3, pp. 300–305, 2009.

[175] R. A. Whitmer, E. P. Gunderson, E. Barrett-Connor, C. P. Quesenberry Jr., and K. Yaffe, "Obesity in middle age and future risk of dementia: a 27 year longitudinal population based study," *British Medical Journal*, vol. 330, no. 7504, pp. 1360–1362, 2005.

[176] K. Yaffe, T. Blackwell, A. M. Kanaya, N. Davidowitz, E. Barrett-Connor, and K. Krueger, "Diabetes, impaired fasting glucose, and development of cognitive impairment in older women," *Neurology*, vol. 63, no. 4, pp. 658–663, 2004.

[177] K. Yaffe, A. Kanaya, K. Lindquist et al., "The metabolic syndrome, inflammation, and risk of cognitive decline," *Journal of the American Medical Association*, vol. 292, no. 18, pp. 2237–2242, 2004.

[178] C. Qiu, B. Winblad, and L. Fratiglioni, "The age-dependent relation of blood pressure to cognitive function and dementia," *Lancet Neurology*, vol. 4, no. 8, pp. 487–499, 2005.

[179] Y. D. Ke, F. Delerue, A. Gladbach, J. Götz, and L. M. Ittner, "Experimental diabetes mellitus exacerbates Tau pathology in a transgenic mouse model of Alzheimer's disease," *PLoS One*, vol. 4, no. 11, Article ID e7917, 2009.

[180] M. Kohjima, Y. Sun, and L. Chan, "Increased food intake leads to obesity and insulin resistance in the Tg2576 Alzheimer's disease mouse model," *Endocrinology*, vol. 151, no. 4, pp. 1532–1540, 2010.

[181] H. Lithell, L. Hansson, I. Skoog et al., "The study on cognition and prognosis in the elderly (SCOPE): principal results of a randomized double-blind intervention trial," *Journal of Hypertension*, vol. 21, no. 5, pp. 875–886, 2003.

[182] M. A. Tedesco, G. Ratti, S. Mennella et al., "Comparison of losartan and hydrochlorothiazide on cognitive function and quality of life in hypertensive patients," *American Journal of Hypertension*, vol. 12, no. 11 I, pp. 1130–1134, 1999.

[183] K. Tsukuda, M. Mogi, J. M. Li et al., "Amelioration of cognitive impairment in the type-2 diabetic mouse by the angiotensin II type-1 receptor blocker candesartan," *Hypertension*, vol. 50, no. 6, pp. 1099–1105, 2007.

[184] S. C. Prinster, C. Hague, and R. A. Hall, "Heterodimerization of G protein-coupled receptors: specificity and functional significance," *Pharmacological Reviews*, vol. 57, no. 3, pp. 289–298, 2005.

[185] S. I. Miura, S. S. Karrik, and K. Saku, "Constitutively active homo-oligomeric angiotensin II type 2 receptor induces cell signaling independent of receptor conformation and ligand stimulation," *Journal of Biological Chemistry*, vol. 280, no. 18, pp. 18237–18244, 2005.

[186] S. AbdAlla, H. Lother, A. M. Abdel-tawab, and U. Quitterer, "The angiotensin II $AT_2$ receptor is an $AT_1$ receptor antagonist," *Journal of Biological Chemistry*, vol. 276, no. 43, pp. 39721–39726, 2001.

[187] S. AbdAlla, H. Lother, A. El Missiry et al., "Dominant negative $AT_2$ receptor oligomers induce G-protein arrest and symptoms of neurodegeneration," *Journal of Biological Chemistry*, vol. 284, no. 10, pp. 6566–6574, 2009.

[188] P. M. Abadir, A. Periasamy, R. M. Carey, and H. M. Siragy, "Angiotensin II type 2 receptor-bradykinin $B_2$ receptor functional heterodimerization," *Hypertension*, vol. 48, no. 2, pp. 316–322, 2006.

[189] H. M. Siragy and R. M. Carey, "The subtype-2 ($AT_2$) angiotensin receptor regulates renal cyclic guanosine 3′, 5′-monophosphate and $AT_1$ receptor-mediated prostaglandin $E_2$ production in conscious rats," *Journal of Clinical Investigation*, vol. 97, no. 8, pp. 1978–1982, 1996.

[190] P. Gohlke, C. Pees, and T. Unger, "$AT_2$ receptor stimulation increases aortic cyclic GMP in SHRSP by a kinin-dependent mechanism," *Hypertension*, vol. 31, no. 1, pp. 349–355, 1998.

[191] C. D. Searles and D. G. Harrison, "The interaction of nitric oxide, bradykinin, and the angiotensin II type 2 receptor: lessons learned from transgenic mice," *Journal of Clinical Investigation*, vol. 104, no. 8, pp. 1013–1014, 1999.

[192] U. Quitterer, A. Pohl, A. Langer, S. Koller, and S. Abdalla, "A cleavable signal peptide enhances cell surface delivery and heterodimerization of Cerulean-tagged angiotensin II $AT_1$ and bradykinin B2 receptor," *Biochemical and Biophysical Research Communications*, vol. 409, no. 3, pp. 544–549, 2011.

[193] K. Shibata, I. Makino, H. Shibaguchi, M. Niwa, T. Katsuragi, and T. Furukawa, "Up-regulation of angiotensin type 2 receptor mRNA by angiotensin II in rat cortical cells," *Biochemical and Biophysical Research Communications*, vol. 239, no. 2, pp. 633–637, 1997.

[194] J. Iwanami, M. Mogi, K. Tsukuda et al., "Low dose of telmisartan prevents ischemic brain damage with peroxisome proliferator-activated receptor-$\gamma$ activation in diabetic mice," *Journal of Hypertension*, vol. 28, no. 8, pp. 1730–1737, 2010.

[195] K. Washida, M. Ihara, K. Nishio et al., "Nonhypotensive dose of telmisartan attenuates cognitive impairment partially due to peroxisome proliferator-activated receptor-$\gamma$ activation in mice with chronic cerebral hypoperfusion," *Stroke*, vol. 41, no. 8, pp. 1798–1806, 2010.

[196] R. Clasen, M. Schupp, A. Foryst-Ludwig et al., "PPAR$\gamma$-activating angiotensin type-1 receptor blockers induce adiponectin," *Hypertension*, vol. 46, no. 1, pp. 137–143, 2005.

[197] W. Gillespie, N. Tyagi, and S. C. Tyagi, "Role of PPAR$\gamma$, a nuclear hormone receptor in neuroprotection," *Indian Journal of Biochemistry and Biophysics*, vol. 48, no. 2, pp. 73–81, 2011.

[198] K. M. Fuenzalida, M. C. Aguilera, D. G. Piderit et al., "Peroxisome proliferator-activated receptor $\gamma$ is a novel target of the nerve growth factor signaling pathway in PC12 cells," *Journal of Biological Chemistry*, vol. 280, no. 10, pp. 9604–9609, 2005.

[199] M. Iwai, Y. Tomono, S. Inaba et al., "$AT_2$ receptor deficiency attenuates adipocyte differentiation and decreases adipocyte number in atherosclerotic mice," *American Journal of Hypertension*, vol. 22, no. 7, pp. 784–791, 2009.

[200] A. M. Murugaiah, X. Wu, C. Wallinder et al., "From the first selective non-peptide $AT_2$ receptor agonist to structurally related antagonists," *Journal of Medicinal Chemistry*, vol. 55, no. 5, pp. 2265–2278, 2012.

[201] Q. Ali and T. Hussain, "$AT_2$ receptor non-peptide agonist C21 promotes natriuresis in obese Zucker rats," *Hypertension Research*, vol. 35, pp. 654–660, 2012.

[202] S. Bosnyak, I. K. Welungoda, A. Hallberg, M. Alterman, R. E. Widdop, and E. S. Jones, "Stimulation of angiotensin $AT_2$ receptors by the non-peptide agonist, Compound 21, evokes vasodepressor effects in conscious spontaneously hypertensive rats," *British Journal of Pharmacology*, vol. 159, no. 3, pp. 709–716, 2010.

[203] J. Gao, H. Zhang, K. D. Le, J. Chao, and L. Gao, "Activation of central angiotensin type 2 receptors suppresses norepinephrine excretion and blood pressure in conscious rats," *American Journal of Hypertension*, vol. 24, no. 6, pp. 724–730, 2011.

[204] A. B. Jehle, Y. Xu, J. M. Dimaria et al., "A nonpeptide angiotensin II type 2 receptor agonist does not attenuate post-myocardial infarction left ventricular remodeling in mice," *Journal of Cardiovascular Pharmacology*, vol. 59, no. 4, pp. 363–368, 2012.

[205] L. C. Matavelli, J. Huang, and H. M. Siragy, "Angiotensin $AT_2$ receptor stimulation inhibits early renal inflammation in renovascular hypertension," *Hypertension*, vol. 57, no. 2, pp. 308–313, 2011.

[206] L. Paulis, S. T. Becker, K. Lucht et al., "Direct angiotensin II type 2 receptor stimulation in Nomega-nitro-L-arginine-methyl ester-induced hypertension: the effect on pulse wave velocity and aortic remodeling," *Hypertension*, vol. 59, no. 2, pp. 485–492, 2012.

[207] L. Paulis and T. Unger, "Novel therapeutic targets for hypertension," *Nature Reviews Cardiology*, vol. 7, no. 8, pp. 431–441, 2010.

[208] E. Kaschina, A. Grzesiak, J. Li et al., "Angiotensin II type 2 receptor stimulation: a novel option of therapeutic interference with the renin-angiotensin system in myocardial infarction?" *Circulation*, vol. 118, no. 24, pp. 2523–2532, 2008.

[209] K. Verdonk, M. Durik, N. Abd-Alla et al., "Compound 21 induces vasorelaxation via an endothelium- and angiotensin II type 2 receptor-independent mechanism," *Hypertension*, vol. 60, pp. 722–729, 2012.

[210] M. Shum, S. Pinard, M. O. Guimond et al., "Angiotensin II Type 2 receptor promotes adipocyte differentiation and restores adipocyte size in high fat/high fructose diet-induced insulin resistance in rats," *American Journal of Physiology*. In press.

[211] K. Ohshima, M. Mogi, F. Jing et al., "Direct angiotensin II type 2 receptor stimulation ameliorates insulin resistance

in type 2 diabetes mice with PPARgamma activation," *PLoS One*, vol. 7, no. 11, Article ID e48387, 2012.

[212] A. K. Mahalingam, Y. Wan, A. M. S. Murugaiah et al., "Selective angiotensin II $AT_2$ receptor agonists with reduced CYP 450 inhibition," *Bioorganic and Medicinal Chemistry*, vol. 18, no. 12, pp. 4570–4590, 2010.

[213] A. M. S. Murugaiah, C. Wallinder, A. K. Mahalingam et al., "Selective angiotensin II $AT_2$ receptor agonists devoid of the imidazole ring system," *Bioorganic and Medicinal Chemistry*, vol. 15, no. 22, pp. 7166–7183, 2007.

[214] U. Rosenström, C. Sköld, B. Plouffe et al., "New selective $AT_2$ receptor ligands encompassing a $\gamma$-turn mimetic replacing the amino acid residues 4-5 of angiotensin II act as agonists," *Journal of Medicinal Chemistry*, vol. 48, no. 12, pp. 4009–4024, 2005.

[215] C. Wallinder, M. Botros, U. Rosenström et al., "Selective angiotensin II $AT_2$ receptor agonists: benzamide structure-activity relationships," *Bioorganic and Medicinal Chemistry*, vol. 16, no. 14, pp. 6841–6849, 2008.

[216] X. Wu, Y. Wan, A. K. Mahalingam et al., "Selective angiotensin II $AT_2$ receptor agonists: arylbenzylimidazole structure-activity relationships," *Journal of Medicinal Chemistry*, vol. 49, no. 24, pp. 7160–7168, 2006.

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*Review Article*

# The Prorenin and (Pro)renin Receptor: New Players in the Brain Renin-Angiotensin System?

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It is well known that the brain renin-angiotensin (RAS) system plays an essential role in the development of hypertension, mainly through the modulation of autonomic activities and vasopressin release. However, how the brain synthesizes angiotensin (Ang) II has been a debate for decades, largely due to the low renin activity. This paper first describes the expression of the vasoconstrictive arm of RAS components in the brain as well as their physiological and pathophysiological significance. It then focus on the (pro)renin receptor (PRR), a newly discovered component of the RAS which has a high level in the brain. We review the role of prorenin and PRR in peripheral organs and emphasize the involvement of brain PRR in the pathogenesis of hypertension. Some future perspectives in PRR research are heighted with respect to novel therapeutic target for the treatment of hypertension and other cardiovascular diseases.

## 1. Introduction

The renin-angiotensin system (RAS) plays an important role in the physiological and pathophysiological regulation of blood pressure (BP) and cardiovascular function. Renin, the rate-limiting enzyme of the RAS, was discovered more than a hundred years ago by Tigerstedt [1]. Angiotensinogen (AGT) is cleaved to angiotensin (Ang) I by renin, which is released from the juxtaglomerular apparatus. Ang I is then further converted into the octapeptide, Ang II, by the angiotensin-converting enzyme (ACE). Ang II is the main effector peptide in this system. Via binding to Ang II type 1 receptor (AT1R), Ang II stimulates vasoconstriction and secretion of the steroid hormone, aldosterone, which mediates sodium reabsorption and water retention [2]. In addition to the classical view of endocrine RAS, local RAS has been identified in various tissues, which interacts with the endocrine RAS [3–6].

The first evidence for the existence of brain RAS was demonstrated by Brickerton and Buckley [7] who showed that administration of Ang II into the brain caused an increase in BP. It is well accepted now that RAS is intrinsic to the brain and plays an integral role in the pathophysiological regulation of cardiovascular function [8, 9]. The activities of brain RAS are achieved by influencing the autonomic nervous system, the baroreflex sensitivity, vasopressin (AVP) release, and the thirst and salt appetite [10–13]. Brain Ang II also acts as a neuropeptide via AT1R to increase the excitability of neurons in the cardiovascular regulatory centers of the hypothalamus and brainstem [14, 15]. Chronic central infusion of Ang II elicits an increase in arterial pressure and enhances the sensitivity of cardiac sympathetic afferent reflex via AT1R [16]. Human renin and AGT double transgenic mice exhibit Ang II-dependent hypertension, which can be attenuated by AVP antagonist suggesting an interaction between the brain RAS and AVP in hypertension [17, 18].

Despite a multitude of evidence supporting the importance of Ang II action in the brain, conjecture remains about how Ang II forms locally in the brain because brain renin activity is undetectable using currently available methods [19, 20]. Prorenin has been recognized as a precursor of renin, but with very low angiotensinogenase activity [21]. Interestingly, we recently reported the existence of prorenin protein in brain tissues and the brain prorenin levels were ten-fold higher compared with renin levels [22].

The (pro)renin receptor (PRR) is a newly discovered component of the RAS which is able to promote Ang II formation via binding to renin or prorenin [23]. This timely discovery may shed new light on a possible pathway for Ang II formation in the brain regions behind the blood-brain barrier. Our paper focuses on the influence of brain RAS on the regulation of cardiovascular function with a specific emphasis on recent evidence concerning the role of brain PRR in the regulation of BP and cardiovascular homeostasis.

## 2. The Brain RAS

Most brain areas are separated from the circulation by the blood-brain barrier which is impermeable to Ang II [24]. Therefore, Ang II needs to be generated locally or transported via transcytosis mechanism [25] to interact with its receptors located on neurons and astrocytes of the central nervous system (CNS). Genetic and pharmacological studies have demonstrated the intrinsic existence of brain RAS components [8, 9]. Furthermore, the pathophysiological significance of a brain RAS is supported by observations of increased RAS activities in the brain cardiovascular regulatory areas of hypertensive animal models [26, 27].

## 3. Renin

Renin is the rate-limiting enzyme for the formation of Ang I. Renin-like enzymatic activity within the brain was first reported by Ganten et al. [28]. However, the isolation of detectable active renin from the brain has remained unsuccessful for decades after its discovery [29]. Although the brain contains a large amount of enzymes, such as cathepsins [30] and tonin [31], which can also generate Ang peptides from AGT, the relevance of these alternative enzymatic pathways for Ang II generation in the brain remains elusive [32]. The most direct evidence supporting renin gene expression in the brain has come from Dr. Sigmund's group [33] who utilized an enhanced green fluorescence protein (eGFP) gene as a reporter for renin expression. They found renin promoter activity in the neurons of the cerebellum and hippocampus and in the cardiovascular regulatory regions such as the rostral ventrolateral medulla (RVLM), the subfornical organ (SFO), the paraventricular nucleus (PVN), and the supraoptic nucleus (SON). This group also discovered two different forms of renin in the brain, the intracellular (icRenin) and secret (sRenin) renin, derived from two different renin transcripts [34]. The relevance of icRenin is still unclear. This renin is a truncated form of prorenin lacking the first third of the prosegment, which remains intracellularly, whereas the sRenin secrets to the extracellular space. Due to the lack of machinery to cleave prorenin to renin in the brain [35], the sRenin in the brain is possibly prorenin. Although true renin activity exists at a finite level in the brain, trypsin or acid treated brain extracts showed a marked increase in renin activity. In addition, the inactive form of renin has been successfully isolated from the brain which could be activated by trypsin and inhibited by antirenin antibody *in vitro* [36]. This inactive form of renin may be the main source of renin-like activity in the brain. Pharmacological studies have provided strong support for the renin signal in the brain. For example, despite the difficulty of directly measuring renin activity in brain tissue, intracerebroventricular (ICV) infusion of the renin inhibitor, aliskiren, prevented sympathetic hyperactivity and hypertension as well as desensitization of arterial baroreflex function in Dahl salt-sensitive rats on a high salt diet [37]. These *in vivo* studies provide support for the existence of renin or renin-like activity in the brain and its role in hypertension.

## 4. Angiotensinogen and ACE

In the CNS, the main source of AGT synthesis is from the astroglia [38]. However, AGT expression has also been found in pure neuron cultures [39]. The AGT protein and its transcripts have been reported in the cardiovascular regulatory regions of the brain by different groups [40–42]. Transgenic rats expressing an antisense RNA against AGT mRNA specifically in the brain exhibited lower BP, polyuria, and reduction in plasma AVP [43] indicating that AGT is synthesized in brain and plays an essential role in BP regulation.

The ACE is a zinc metalloprotease which hydrolyzes the carboxyl terminal dipeptide His-Leu of Ang I to form Ang II. High densities of ACE were visualized in the choroid plexus, SFO, the caudate putamen, and the substantial nigra by autoradiography [44]. The ACE activity is present in the renin-containing synaptosomes of the neurons suggesting that intraneuronal synthesis of Ang I and Ang II is possible in synaptosomes [45]. The ICV delivery of the human ACE gene increased sympathetic activity, BP, and heart rate which were accompanied by increased Ang II and AVP production. These effects were abolished by ICV administration of an ACE inhibitor suggesting the importance of brain ACE in Ang II formation [26].

## 5. Angiotensin II and Angiotensin II Type I Receptor

The actions of Ang II are mediated predominantly by a seven transmembrane domain Gq-protein coupled receptor designated as AT1R. Effects of the AT1R are mediated by multiple intracellular signaling pathways, starting with G-protein and phospholipase activation, followed by an increase in intracellular inositol trisphosphate and calcium, which result in vasoconstriction, cell proliferation, and fibrosis [46, 47]. The AT1R is localized with high densities in the anterior pituitary, area postrema, lateral geniculate body, inferior olivary nucleus, median eminence, nucleus of the solitary tract (NTS), the anterior ventral third ventricle region, PVN, SON, and SFO [48]. The AT1R expression levels are tightly regulated by cardiovascular status and Ang II levels. Higher levels of AT1R were found in the hypothalamus of spontaneously hypertensive rat (SHR) and the SFO, PVN, and NTS of animals with chronic heart failure [27, 49]. Others have shown that chronic Ang II infusion

upregulates AT1R mRNA and protein levels in the PVN and SFO via activation of intracellular mitogen-activated protein kinase (MAPK) signaling pathways [50]. Constitutive AT1R overexpression in the astroglia of RVLM resulted in a chronic increase in BP indicating that increased AT1 receptor activity is a primary determinant of efferent drive from RVLM [51]. Although Ang II is a small octapeptide, it is unable to permeate the blood-brain barrier and the blood-cerebrospinal fluid barrier [24, 52]. However, Ang II immunoreactivity has been found in the cell bodies of magnocellular and parvocellular neurons in the PVN and the magnocellular neurons in the SON [53] and Ang II-stained fibers have been found at all levels of the CNS, from the olfactory bulbs to the spinal cord. Furthermore, brain Ang II content was significantly increased in bilaterally nephrectomized rats despite diminishment of plasma Ang II to a very low level [54]. The body of evidence suggests that brain Ang II is synthesized locally and can be regulated independently of peripheral Ang II.

## 6. Prorenin

Prorenin, the precursor of renin, is cleaved to its active form by the removal of the 43 amino acid prosegment [21]. Whereas the renal juxtaglomerular cells constitute the most important source of circulating renin [55], a number of extrarenal tissues including the adrenal glands, ovary, testis, placenta, and retina produce prorenin [56]. This also explains the finding that the plasma prorenin level is 10 fold higher than that of renin [57]. However, prorenin has very low enzymatic activity in the plasma. Only two percent of prorenin exists as the "open form", in which the prosegment of prorenin undergoes a conformational change and exposes the enzyme's active site [58]. This process is also called nonproteolytic activation of prorenin. The role of non-proteolytic activation of prorenin remains debatable due to the controversial phenotype in prorenin transgenic animal models. Liver-specific transgenic rats with a 400-fold increase in circulating prorenin exhibited severe renal lesions and hypertrophic cardiomyocyte with normal BP [59]. In contrast, transgenic animals with inducible or constitutive overexpression of prorenin, despite expressing 13–179 folds higher circulating prorenin, did not display cardiac or kidney damage. However, they did develop moderate Ang II dependent hypertension, since the BP was reduced by an ACE inhibitor [60, 61]. The reason for the presence of different phenotypes in the prorenin transgenic animal model remains unclear but could be due to the difference in the levels of plasma prorenin or the species. Although Mercure et al. [61] showed that Ang II is responsible for the increase in BP in the prorenin transgenic mice; the high plasma prorenin levels present in his study might not exist in physiological or even pathophysiological states [62]. The formation of Ang II in Mercure's model may be due to the activity of the two percent "open form" prorenin in the plasma [63]. However, in all three models, the increase in prorenin levels is limited to the circulation and liver. Thus, the role of local or tissue prorenin in cardiovascular regulation remains inconclusive.

## 7. (Pro)renin Receptor

In the year 2002, a new component of RAS was identified in human mesangial cells and named the (pro)renin receptor (PRR) because PRR binds both renin and prorenin [23]. The PRR gene is identical to ATPase 6 accessory protein2 (ATP6AP2) and is located on the X chromosome. PRR gene encodes a 350-amino-acidand ubiquitously expresses a single transmembrane protein with a large N-terminal extracellular domain which binds both renin and prorenin with affinities in the nanomolar range [64, 65]. Immunofluorescence observed by confocal microscope demonstrated that PRR was located on the cell surface, as well as intracellular compartments especially on the perinuclear space [22, 23, 66]. *In vitro* binding studies have shown that prorenin binds to PRR with a 3-4 fold higher affinity than renin, indicating that prorenin is a preferred ligand for PRR compared with renin [64, 65]. The binding of renin to PRR increases enzymatic activity 5-fold higher than the nonreceptor-bound renin [23]. The binding of prorenin to PRR induces a conformational change; the prosegment is removed from the catalytic cleft and the active site is accessible to AGT leading to full nonproteolytic activation of prorenin [23]. Interestingly, this phenomenon is reversible and prorenin eluted from the receptor reverts to its inactive form. The binding of renin to PRR is independent of the active site and receptor-bound renin or prorenin is not internalized or degraded [64, 67]. Thus, the discovery of the PRR has shed light on an alternate pathway for nonproteolytic activation of prorenin. Although the formation and role of intracellular Ang II has been previously reported [68, 69], there is a lack of attention to whether the intracellular PRR and prorenin contributes to the intracellular Ang II formation. It is likely that in tissues lacking a mechanism to cleave prorenin to renin, activation of prorenin via binding to PRR may contribute to intracellular Ang II formation. Several *in vitro* studies have been completed to assess the ability of prorenin to form Ang II via binding to the PRR. At a concentration of nanomolar range, which is much higher than the circulation level in physiological conditions, prorenin binds to PRR and exhibits enzymatic activity similarly to that of renin [64]. Furthermore, a recent *in vitro* study showed that the PRR activation by prorenin to generate Ang II requires about 800 fold higher prorenin concentration above normal plasma levels; the Ang II-independent activation requires an even higher prorenin concentration [70]. Combining these observations, prorenin may act mainly in the tissues either extracellularly or intracellularly, where its concentration may be high enough to activate Ang II-dependent or independent signals [70].

Transgenic rats with human PRR expression in vascular smooth muscle cells have been shown to exhibit hypertension and increased plasma aldosterone at six months, suggesting a pathological role of PRR in raising BP [71]. This study speculated that the rise in BP may have been caused by increased plasma aldosterone levels in these rats, but whether the increase in aldosterone depends on Ang II was not directly tested by the authors. In another transgenic model, ubiquitous expression of human PRR in rats resulted

in proteinuria, glomerulosclerosis, MAPK activation, and cyclooxygenase-2 upregulation. These rats exhibited normal BP and renal RAS activity suggesting an Ang II independent nephropathy [72]. A previous *in vitro* study has shown that rat prorenin/renin was able to activate human PRR [65]. The phenotype of these transgenic rats could be due to the activation of human PRR transgenes by endogenous rat prorenin/renin. The reason for the differences in the phenotypes of these two models is not clear, but could be attributed to tissue targeting strategy differences. However, these studies clearly suggest that the pathological changes seen in these models might be due to the non-RAS dependent PRR rather than RAS dependent effects. In addition, binding of renin or prorenin to the PRR directly triggered intracellular MAPK signaling cascades in several cell types, which up-regulated the expression of profibrotic genes such as plasminogen activator inhibitor type (PAI)-1, collagens, and fibronectin [73–75]. Despite Ang II type 1 and 2 receptor blockade, renin or prorenin was still able to induce a long-lasting ERK 1/2 phosphorylation by binding to PRR, which was not blocked by aliskiren. Since aliskiren is a renin inhibitor which blocks the breakdown of angiotensinogen by renin, but does not affect the binding of renin to PRR and the PRR mediated signaling, it is clear that PRR mediates RAS independent signaling pathways [73].

Global PRR knockout is lethal in mice indicating an essential role of PRR in embryonic development [76]. Recently, several tissue specific PRR knockout mouse models have been generated; [77–79] these *in vivo* studies provide additional insight into functions of PRR which are independent of RAS activation. PRR knockout in cardiomyocytes of mice led to heart failure and death within four weeks after birth accompanied by deacidification of the intracellular vesicles [77]. This outcome was also reported in the mice with a PRR deletion in the podocytes [78, 79]. In both cases, the defect appears to be associated with an inability to acidify intracellular compartments and a dysfunction of vATPase, suggesting a PRR effect unrelated to RAS *in vivo*. Moreover, PRR functions as a physiological adaptor between vATPase and the Wnt receptors [80]. Wnt via binding to its receptor, low-density lipoprotein receptor-related protein 6 (LRP6), induces receptor aggregation and phosphorylation of LRP6, resulting in the stabilization of $\beta$-catenin. The Wnt/$\beta$-catenin signaling is fundamental for a normal patterned embryo. In the adult, however, Wnt signaling is involved in cell proliferation and tissue homeostasis and has been implicated in certain pathologies, such as cancer and diabetes. By employing a genome-wide small inhibitory RNA (siRNA) screen, Cruciat et al. [80] identified PRR as part of the Wnt receptor complex, acting as a specific adaptor between LRP6 and vATPase where PRR and vATPase mediated Wnt signaling during anteroposterior patterning of Xenopus early CNS development.

## 8. The PRR in the CNS

A mutation of the PRR gene, resulting in frame deletion of exon 4 is associated with X-linked mental retardation and epilepsy pointing to an important role of PRR in the CNS [81]. PRR mRNA is widely expressed in various regions of the human brain with the highest expression levels in the pituitary and frontal lobe [82]. Immunostaining showed that PRR is colocalized with oxytocin and AVP in the magnocellular neurons of the PVN and SON indicating that PRR may be related to the central control of water-electrolyte homeostasis and BP [82]. Similarly, a wide distribution of PRR mRNA was found in key regions of the mouse brain involved in the regulation of BP and body fluid homeostasis [83]. Furthermore, PRR protein is expressed throughout the brain in cardiovascular regions including the SFO, PVN, nucleus of raphe pallidus, NTS, and RVLM as well as in noncardiovascular regulatory regions [22]. Immunofluorescence staining for PRR, neuron-specific nuclear protein (NeuN, neuron marker), and glial fibrillary acidic protein (GFAP, astroglia marker) revealed that PRR is expressed in astroglia, but with prominent expression in the neurons (Figure 1). Neuronal cells from the hypothalamus and brainstem of normotensive rat brains express PRR with levels 3-fold higher than that seen in astroglial cells from the same brain areas [66]. Moreover, PRR mRNA and protein levels were increased in brain cardiovascular regulatory regions in hypertensive animals [22, 84]. Knockdown of PRR in the SON was associated with attenuation of hypertension and a decrease in plasma AVP in SHR [84]. We recently reported that PRR knockdown in the brain attenuated Ang II-dependent hypertension in human renin and AGT double transgenic mice [22]. This effect was associated with a decrease in AVP levels, sympathetic tone, and improvement of baroreflex sensitivity indicating a role of PRR in the autonomic regulation of hypertension. However, whether PRR regulates BP and cardiovascular homeostasis via Ang II dependent or independent pathways remains inconclusive.

Shan et al. [84] demonstrated that coincubation of human prorenin and AGT in isolated neurons evoked a dose- and time-dependent increase in Ang I and II formation indicating the ability of prorenin to generate angiotensin peptides in neurons possibly by binding to PRR. To address whether PRR mediates Ang II formation in the CNS, our laboratory recently measured Ang II levels in human renin and AGT double transgenic mice following brain-targeted PRR knockdown using PRR short hairpin RNA; Ang II levels were significantly decreased after PRR knockdown in the hypothalamus (Figure 2) and the levels were associated with a decrease of PRR levels in this region, as reported previously [22]. These data show that in hypertension, PRR may be involved in Ang II formation in the CNS. We also recently reported that the prorenin protein exists in mouse brain tissue, and its expression level is 10-fold higher than that of renin. In light of the facts that (1) there is abundant PRR expression in the brain, (2) prorenin exists in the brain despite an extremely low renin activity, and (3) manipulation of PRR modulates the Ang II levels; we propose that the binding of prorenin to PRR may initiate the rate-limiting step for angiotensin peptides formation in the CNS. The limitation of the knock down-based hypothesis is that PRR also acts as an accessory protein for vATPase, which is important for vesicular acidification, an important

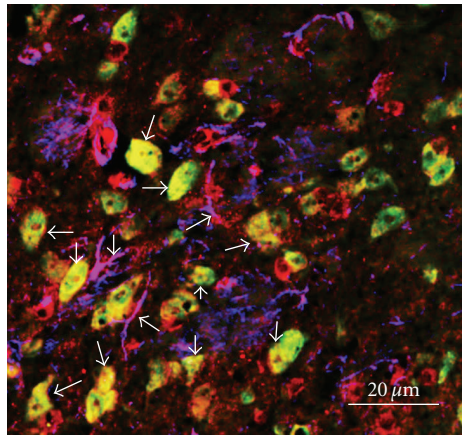


Figure 1: (Pro)renin receptor expression in the C57Bl/6J mouse brain. Triple immunofluorescence staining for PRR (red), neuron-specific nuclear protein (green, neuron marker), and glial fibrillary acidic protein (blue, astroglia marker) revealed that PRR is expressed in both neurons and astroglia with prominent expression in the neurons. Arrows indicate the colocalization of PRR with neurons or astroglia.

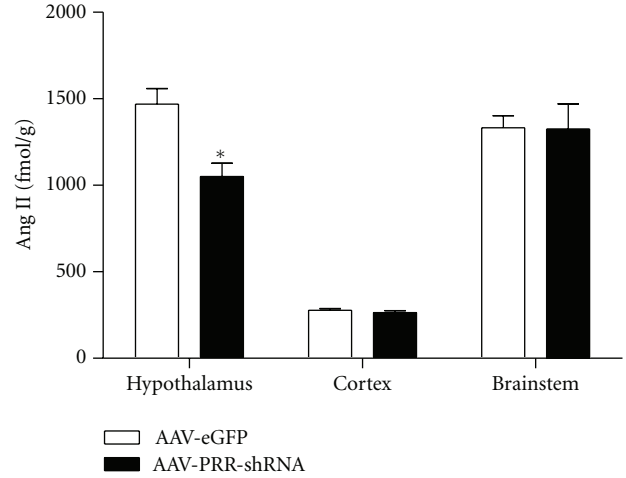


Figure 2: ICV delivery of (pro)renin receptor shRNA reduces brain Ang II level in the hypothalamus. The human renin and AGT double transgenic mice were ICV injected with AAV-PRR short hairpin RNA (AAV-PRR-shRNA, $3.5 \times 10^{11}$ Vg/100nl) or control virus. Two weeks after virus injection, brain tissues were harvested for analysis of Ang II levels in the hypothalamus, cortex, and brainstem. ($n = 6$/group). $^*P < 0.05$ versus AAV-enhanced green fluorescent protein (AAV-eGFP) treatment.

function in neuronal cells. Although the role of vATPase in Ang II formation has not been reported, it is difficult to distinguish the effects of PRR silencing on RAS-related prorenin inactivation and vATPase dysfunction. So far, the role of RAS independent signaling pathways of PRR in the CNS remains unknown.

## 9. Conclusion and Perspective

Emerging evidence supports the argument that brain RAS signals contribute to the development of hypertension. Interruption of these signals is beneficial to the control of BP in hypertension. All components of the RAS exist in the brain, but the renin level is extremely low. Prorenin and PRR may be the missing piece of the puzzle of the brain RAS since prorenin is conferred a nonproteolytic activation when binding to the PRR. Although further study is needed to test this hypothesis, evidence which support this case include that: (1) prorenin is the dominant form of total renin (prorenin and renin) in the brain, (2) PRR is highly expressed in the CNS, (3) brain PRR expression levels are increased in several hypertensive animal models, (4) knockdown of the PRR in the hypothalamus is associated with reduction in Ang II formation in this region during hypertension, and (5) reduced PRR expression level is associated with reduced BP in Ang II-dependent hypertension. Conversely, the RAS-unrelated PRR function via vATPase is critical to acidification of the intracellular compartments and might be important in the modulation of autonomic function since vATPase is responsible for neurotransmitter transportation and storage in the neuron vesicles [85]. We conclude that PRR might be an essential component of the tissue RAS, at least in the CNS. On the other hand, the RAS-unrelated PRR function in the CNS needs further investigation. Thus, targeting PRR can be an innovative, new strategy for the treatment of hypertension. Understanding the physiological and pathophysiological role of PRR in hypertension models would move this possibility forward. Because the PRR plays an essential role in embryonic development, generation of PRR antagonist or inducible tissue-specific PRR knockout animal models would be ideal tools to study the role of PRR in adult diseases models.

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## References

[1] R. Tigerstedt, P. N. Bergman, and Kreislauf, "Niere und Kreislauf," *Archives of Physiology*, pp. 223–271, 1898.

[2] S. J. Cleland and J. L. Reid, "The renin-angiotensin system and the heart: a historical review," *Heart*, vol. 76, no. 3, pp. 7–12, 1996.

[3] M. Bader, J. Peters, O. Baltatu, D. N. Müller, F. C. Luft, and D. Ganten, "Tissue renin-angiotensin systems: new insights from experimental animal models in hypertension research," *Journal of Molecular Medicine*, vol. 79, no. 2, pp. 76–102, 2001.

[4] O. Baltatu, J. A. Silva Jr., D. Ganten, and M. Bader, "The brain renin-angiotensin system modulates angiotensin II-induced

hypertension and cardiac hypertrophy," *Hypertension*, vol. 35, no. 1, pp. 409–412, 2000.

[5] L. J. Dell'Italia, Q. C. Meng, E. Balcells et al., "Compartmentalization of angiotensin II generation in the dog heart: evidence for independent mechanisms in intravascular and interstitial spaces," *Journal of Clinical Investigation*, vol. 100, no. 2, pp. 253–258, 1997.

[6] L. G. Navar, L. M. Harrison-Bernard, A. Nishiyama, and H. Kobori, "Regulation of intrarenal angiotensin II in hypertension," *Hypertension*, vol. 39, no. 2, pp. 316–322, 2002.

[7] R. K. Bickerton and J. P. Buckley, "Evidence for a central mechanism in angiotensin-induced hypertension," *Proceedings of the Society for Experimental Biology and Medicine*, pp. 836–843, 1961.

[8] R. A. L. Dampney, "Functional organization of central pathways regulating the cardiovascular system," *Physiological Reviews*, vol. 74, no. 2, pp. 323–364, 1994.

[9] S. Morimoto, M. D. Cassell, T. G. Beltz, A. K. Johnson, R. L. Davisson, and C. D. Sigmund, "Elevated blood pressure in transgenic mice with brain-specific expression of human angiotensinogen driven by the glial fibrillary acidic protein promoter," *Circulation Research*, vol. 89, no. 4, pp. 365–372, 2001.

[10] G. Aguilera and A. Kiss, "Regulation of the hypothalmic-pituitary-adrenal axis and vasopressin secretion: role of angiotensin II," *Advances in Experimental Medicine and Biology*, vol. 396, pp. 105–112, 1996.

[11] G. D. Fink, "Long-term sympatho-excitatory effect of angiotensin II: a mechanism of spontaneous and renovascular hypertension," *Clinical and Experimental Pharmacology and Physiology*, vol. 24, no. 1, pp. 91–95, 1997.

[12] D. B. Averill and D. I. Diz, "Angiotensin peptides and baroreflex control of sympathetic outflow: pathways and mechanisms of the medulla oblongata," *Brain Research Bulletin*, vol. 51, no. 2, pp. 119–128, 2000.

[13] J. T. Fitzsimons, "Angiotensin, thirst, and sodium appetite," *Physiological Reviews*, vol. 78, no. 3, pp. 583–686, 1998.

[14] K. L. Barnes, D. M. DeWeese, and M. C. Andresen, "Angiotensin potentiates excitatory sensory synaptic transmission to medial solitary tract nucleus neurons," *American Journal of Physiology*, vol. 284, no. 5, pp. R1340–R1353, 2003.

[15] D. P. Li, S. R. Chen, and H. L. Pan, "Angiotensin II stimulates spinally projecting paraventricular neurons through presynaptic disinhibition," *Journal of Neuroscience*, vol. 23, no. 12, pp. 5041–5049, 2003.

[16] R. Ma, H. D. Schultz, and W. Wang, "Chronic central infusion of ANG II potentiates cardiac sympathetic afferent reflex in dogs," *American Journal of Physiology*, vol. 277, no. 1, pp. H15–H22, 1999.

[17] S. Morimoto, M. D. Cassell, T. G. Beltz, A. K. Johnson, R. L. Davisson, and C. D. Sigmund, "Elevated blood pressure in transgenic mice with brain-specific expression of human angiotensinogen driven by the glial fibrillary acidic protein promoter," *Circulation Research*, vol. 89, no. 4, pp. 365–372, 2001.

[18] S. Morimoto, M. D. Cassell, and C. D. Sigmund, "Glia- and neuron-specific expression of the renin-angiotensin system in brain alters blood pressure, water intake, and salt preference," *Journal of Biological Chemistry*, vol. 277, no. 36, pp. 33235–33241, 2002.

[19] E. Hackenthal, R. Hackenthal, and U. Hilgenfeldt, "Isorenin, pseudorenin, cathepsin D and renin. A comparative enzymatic study of angiotensin-forming enzymes," *Biochimica et Biophysica Acta*, vol. 522, no. 2, pp. 574–588, 1978.

[20] A. Lippoldt, K. Fuxe, and C. Luft, "A view of renin in the brain," *Journal of Molecular Medicine*, vol. 79, no. 2, pp. 71–73, 2001.

[21] C. Mercure, G. Thibault, S. Lussier-Cacan, J. Davignon, E. L. Schiffrin, and T. L. Reudelhuber, "Molecular analysis of human prorenin prosegment variants in vitro and in vivo," *Journal of Biological Chemistry*, vol. 270, no. 27, pp. 16355–16359, 1995.

[22] W. Li, H. Peng, T. Cao et al., "Brain-targeted, (pro)renin receptor knockdown attenuates angiotensin II-dependent hypertension," *Hypertension*, vol. 59, pp. 1188–1194, 2012.

[23] G. Nguyen, F. Delarue, C. Burcklé, L. Bouzhir, T. Giller, and J. D. Sraer, "Pivotal role of the renin/prorenin receptor in angiotensin II production and cellular responses to renin," *Journal of Clinical Investigation*, vol. 109, no. 11, pp. 1417–1427, 2002.

[24] P. Schelling, J. S. Hutchinson, and U. Ganten, "Impermeability of the blood cerebrospinal fluid barrier for angiotensin II in rats," *Clinical Science and Molecular Medicine*, vol. 51, no. 3, pp. 399–402, 1976.

[25] J. M. Rose and K. L. Audus, "Receptor-mediated angiotensin II transcytosis by brain microvessel endothelial cells," *Peptides*, vol. 19, no. 6, pp. 1023–1030, 1998.

[26] S. Nakamura, A. Moriguchi, R. Morishita et al., "Activation of the brain angiotensin system by in vivo human angiotensin-converting enzyme gene transfer in rats," *Hypertension*, vol. 34, no. 2, pp. 302–308, 1999.

[27] N. L. Han and M. K. Sim, "Hypothalamic angiotensin receptor subtypes in normotensive and hypertensive rats," *American Journal of Physiology*, vol. 275, no. 2, pp. H703–H709, 1998.

[28] D. Ganten, J. L. Minnich, P. Granger et al., "Angiotensin-forming enzyme in brain tissue," *Science*, vol. 173, no. 3991, pp. 64–65, 1971.

[29] M. Bader and D. Ganten, "It's renin in the brain: transgenic animals elucidate the brain renin-angiotensin system," *Circulation Research*, vol. 90, no. 1, pp. 8–10, 2002.

[30] L. B. Klickstein, C. E. Kaempfer, and B. U. Wintroub, "The granulocyte-angiotensin system. Angiotensin I-converting activity of cathepsin G," *Journal of Biological Chemistry*, vol. 257, no. 24, pp. 15042–15046, 1982.

[31] E. S. L. Lomez, R. C. Araujo, M. Bader, J. B. Pesquero, and J. L. Pesquero, "Tonin and kallikrein in the brain of transgenic rat line expressing human tissue kallikrein," *Hypertension*, vol. 39, no. 2, pp. 229–232, 2002.

[32] C. C. Cardoso, N. Alenina, A. J. Ferreira et al., "Increased blood pressure and water intake in transgenic mice expressing rat tonin in the brain," *Biological Chemistry*, vol. 391, no. 4, pp. 435–441, 2010.

[33] J. L. Lavoie, M. D. Cassell, K. W. Gross, and C. D. Sigmund, "Localization of renin expressing cells in the brain, by use of a REN-eGFP transgenic model," *Physiological Genomics*, vol. 16, pp. 240–246, 2004.

[34] D. Xu, G. R. Borges, J. L. Grobe, C. J. Pelham, B. Yang, and C. D. Sigmund, "Preservation of intracellular renin expression is insufficient to compensate for genetic loss of secreted renin," *Hypertension*, vol. 54, no. 6, pp. 1240–1247, 2009.

[35] W. A. Hsueh, E. J. Carlson, D. O'Connor, and S. Warren, "Renin requires a structural alteration prior to activation by renal kallikrein," *Journal of Clinical Endocrinology and Metabolism*, vol. 51, no. 9, pp. 942–944, 1980.

[36] S. Hirose, M. Naruse, M. Ohtsuki, and T. Inagami, "Totally inactive renin zymogen and different forms of active renin in

hog brain tissues," *Journal of Biological Chemistry*, vol. 256, no. 11, pp. 5572–5576, 1981.

[37] B. S. Huang, R. A. White, L. Bi, and F. H. Leenen, "Central infusion of aliskiren prevents sympathetic hyperactivity and hypertension in Dahl salt-sensitive rats on high salt intake," *American Journal of Physiology*, vol. 302, pp. R825–R832, 2012.

[38] A. D. Intebi, M. S. Flaxman, W. F. Ganong, and C. F. Deschepper, "Angiotensinogen production by rat astroglial cells in vitro and in vivo," *Neuroscience*, vol. 34, no. 3, pp. 545–554, 1990.

[39] K. Hermann, M. I. Phillips, U. Hilgenfeldt, and M. K. Raizada, "Biosynthesis of angiotensinogen and angiotensins by brain cells in primary culture," *Journal of Neurochemistry*, vol. 51, no. 2, pp. 398–405, 1988.

[40] M. Aronsson, K. Almasan, K. Fuxe et al., "Evidence for the existence of angiotensinogen mRNA in magnocellular paraventricular hypothalamic neurons," *Acta Physiologica Scandinavica*, vol. 132, no. 4, pp. 585–586, 1988.

[41] M. Palkovits, E. Mezey, M. Fodor et al., "Neurotransmitters and neuropeptides in the baroreceptor reflex arc: connections between the nucleus of the solitary tract and the ventrolateral medulla oblongata in the rat," *Clinical and Experimental Hypertension*, vol. 17, no. 1-2, pp. 101–113, 1995.

[42] M. Tham, M. K. Sim, and F. R. Tang, "Location of renin-angiotensin system components in the hypoglossal nucleus of the rat," *Regulatory Peptides*, vol. 101, no. 1 3, pp. 51–57, 2001.

[43] M. Schinke, O. Baltatu, M. Böhm et al., "Blood pressure reduction and diabetes insipidus in transgenic rats deficient in brain angiotensinogen," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 96, no. 7, pp. 3975–3980, 1999.

[44] S. M. Strittmatter, M. M. Lo, J. A. Javitch, and S. H. Snyder, "Autoradiographic visualization of angiotensin-converting enzyme in rat brain with [3H]captopril: localization to a striatonigral pathway," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 81, no. 5, pp. 1599–1603, 1984.

[45] M. Paul, M. P. Printz, and E. Harms, "Localization of renin (EC 3.4.23) and converting enzyme (EC 3.4.15.1) in nerve endings of rat brain," *Brain Research*, vol. 334, no. 2, pp. 315–324, 1985.

[46] C. Berry, R. Touyz, A. F. Dominiczak, R. C. Webb, and D. G. Johns, "Angiotensin receptors: signaling, vascular pathophysiology, and interactions with ceramide," *American Journal of Physiology*, vol. 281, no. 6, pp. H2337–H2365, 2001.

[47] E. Kaschina and T. Unger, "Angiotensin AT1/AT2 receptors: regulation, signalling and function," *Blood Pressure*, vol. 12, no. 2, pp. 70–88, 2003.

[48] J. W. Wright and J. W. Harding, "Brain renin-angiotensin—a new look at an old system," *Progress in Neurobiology*, vol. 95, no. 1, pp. 49–67, 2011.

[49] R. Yoshimura, T. Sato, T. Kawada et al., "Increased brain angiotensin receptor in rats with chronic high-output heart failure," *Journal of Cardiac Failure*, vol. 6, no. 1, pp. 66–72, 2000.

[50] S. G. Wei, Y. Yu, Z. H. Zhang, and R. B. Felder, "Angiotensin II upregulates hypothalamic AT1 receptor expression in rats via the mitogen-activated protein kinase pathway," *American Journal of Physiology*, vol. 296, no. 5, pp. H1425–H1433, 2009.

[51] A. M. Allen, J. K. Dosanjh, M. Erac, S. Dassanayake, R. D. Hannan, and W. G. Thomas, "Expression of constitutively active angiotensin receptors in the rostral ventrolateral medulla increases blood pressure," *Hypertension*, vol. 47, no. 6, pp. 1054–1061, 2006.

[52] L. Volicer and C. G. Loew, "Penetration of angiotensin II into the brain," *Neuropharmacology*, vol. 10, no. 5, pp. 631–634, 1971.

[53] R. W. Lind, L. W. Swanson, and D. Ganten, "Organization of angiotensin II immunoreactive cells and fibers in the rat central nervous system. An immunohistochemical study," *Neuroendocrinology*, vol. 40, no. 1, pp. 2–24, 1985.

[54] M. R. Trolliet and M. I. Phillips, "The effect of chronic bilateral nephrectomy on plasma and brain angiotensin," *Journal of Hypertension*, vol. 10, no. 1, pp. 29–36, 1992.

[55] P. B. Persson, "Renin: origin, secretion and synthesis," *Journal of Physiology*, vol. 552, no. 3, pp. 667–671, 2003.

[56] D. J. Campbell, "Critical review of prorenin and (pro)renin receptor research," *Hypertension*, vol. 51, no. 5, pp. 1259–1264, 2008.

[57] J. Nussberger, M. de Gasparo, L. Juillerat et al., "Rapid measurement of total and active renin: plasma concentrations during acute and sustained converting enzyme inhibition with CGS 14824A," *Clinical and Experimental Hypertension A*, vol. 9, no. 8-9, pp. 1353–1366, 1987.

[58] F. Suzuki, M. Hayakawa, T. Nakagawa et al., "Human prorenin has "gate and handle" regions for its non-proteolytic activation," *Journal of Biological Chemistry*, vol. 278, no. 25, pp. 22217–22222, 2003.

[59] M. Véniant, J. Ménard, P. Bruneval, S. Morley, M. F. Gonzales, and J. Mullins, "Vascular damage without hypertension in transgenic rats expressing prorenin exclusively in the liver," *Journal of Clinical Investigation*, vol. 98, no. 9, pp. 1966–1970, 1996.

[60] B. Peters, O. Grisk, B. Becher et al., "Dose-dependent titration of prorenin and blood pressure in Cyp1a1ren-2 transgenic rats: absence of prorenin-induced glomerulosclerosis," *Journal of Hypertension*, vol. 26, no. 1, pp. 102–109, 2008.

[61] C. Mercure, G. Prescott, M. J. Lacombe, D. W. Silversides, and T. L. Reudelhuber, "Chronic increases in circulating prorenin are not associated with renal or cardiac pathologies," *Hypertension*, vol. 53, no. 6, pp. 1062–1069, 2009.

[62] J. Deinum, B. Rønn, E. Mathiesen, F. H. M. Derkx, W. C. J. Hop, and M. A. D. H. Schalekamp, "Increase in serum prorenin precedes onset of microalbuminuria in patients with insulin-dependent diabetes mellitus," *Diabetologia*, vol. 42, no. 8, pp. 1006–1010, 1999.

[63] W. W. Batenburg and A. H. Danser, "(Pro)renin and its receptors: pathophysiological implications," *Clinical Science*, vol. 123, pp. 121–133, 2012.

[64] A. H. M. N. Nabi, A. Kageshima, M. Uddin, T. Nakagawa, E. Y. Park, and F. Suzuki, "Binding properties of rat prorenin and renin to the recombinant rat renin/prorenin receptor prepared by a baculovirus expression system," *International Journal of Molecular Medicine*, vol. 18, no. 3, pp. 483–488, 2006.

[65] W. W. Batenburg, M. Krop, I. M. Garrelds et al., "Prorenin is the endogenous agonist of the (pro)renin receptor. Binding kinetics of renin and prorenin in rat vascular smooth muscle cells overexpressing the human (pro)renin receptor," *Journal of Hypertension*, vol. 25, no. 12, pp. 2441–2453, 2007.

[66] Z. Shan, A. E. Cuadra, C. Sumners, and M. K. Raizada, "Characterization of a functional (pro)renin receptor in rat brain neurons," *Experimental Physiology*, vol. 93, no. 5, pp. 701–708, 2008.

[67] G. Nguyen, F. Delarue, J. Berrou, E. Rondeau, and J. D. Sraer, "Specific receptor binding of renin on human mesangial cells in culture increases plasminogen activator inhibitor-1 antigen," *Kidney International*, vol. 50, no. 6, pp. 1897–1903, 1996.

[68] R. Singh, D. Choubey, J. Chen, and D. J. Leehey, "Inhibition of intracellular Angiotensin II formation blocks high glucose effect on mesangial matrix," *Regulatory Peptides*, vol. 158, no. 1–3, pp. 103–109, 2009.

[69] R. Singh, "Jak2-independent activation of stat3 by intracellular angiotensin II in human mesangial cells," *Journal of Signal Transduction*, vol. 2011, Article ID 257862, 10 pages, 2011.

[70] W. W. Batenburg, X. Lu, F. Leijten, U. Maschke, D. N. Muller, and A. H. Danser, "Renin- and prorenin-induced effects in rat vascular smooth muscle cells overexpressing the human (pro)renin receptor: does (pro)renin-(pro)renin receptor interaction actually occur?" *Hypertension*, vol. 58, pp. 1111–1119, 2011.

[71] C. A. Burcklé, A. H. J. Danser, D. N. Müller et al., "Elevated blood pressure and heart rate in human renin receptor transgenic rats," *Hypertension*, vol. 47, no. 3, pp. 552–556, 2006.

[72] Y. Kaneshiro, A. Ichihara, M. Sakoda et al., "Slowly progressive, angiotensin II-independent glomerulosclerosis in human (pro)renin receptor-transgenic rats," *Journal of the American Society of Nephrology*, vol. 18, no. 6, pp. 1789–1795, 2007.

[73] S. Feldt, W. W. Batenburg, I. Mazak et al., "Prorenin and renin-induced extracellular signal-regulated kinase 1/2 activation in monocytes is not blocked by aliskiren or the handle-region peptide," *Hypertension*, vol. 51, no. 3, pp. 682–688, 2008.

[74] M. Sakoda, A. Ichihara, Y. Kaneshiro et al., "(Pro)renin receptor-mediated activation of mitogen-activated protein kinases in human vascular smooth muscle cells," *Hypertension Research*, vol. 30, no. 11, pp. 1139–1146, 2007.

[75] Y. Huang, S. Wongamorntham, J. Kasting et al., "Renin increases mesangial cell transforming growth factor-$\beta$1 and matrix proteins through receptor-mediated, angiotensin II-independent mechanisms," *Kidney International*, vol. 69, no. 1, pp. 105–113, 2006.

[76] G. Sihn, A. Rousselle, L. Vilianovitch, C. Burckle, and M. Bader, "Physiology of the (pro)renin receptor: wnt of change," *Kidney International*, vol. 78, no. 3, pp. 246–256, 2010.

[77] K. Kinouchi, A. Ichihara, M. Sano et al., "The (Pro)renin receptor/ATP6AP2 is essential for vacuolar H +-ATPase assembly in murine cardiomyocytes," *Circulation Research*, vol. 107, no. 1, pp. 30–34, 2010.

[78] F. Riediger, I. Quack, F. Qadri et al., "Prorenin receptor is essential for podocyte autophagy and survival," *Journal of the American Society of Nephrology*, vol. 22, pp. 2193–2202, 2011.

[79] Y. Oshima, K. Kinouchi, A. Ichihara et al., "Prorenin receptor is essential for normal podocyte structure and function," *Journal of the American Society of Nephrology*, vol. 22, no. 12, pp. 2203–2212, 2011.

[80] C. M. Cruciat, B. Ohkawara, S. P. Acebron et al., "Requirement of prorenin receptor and vacuolar H+-ATPase-mediated acidification for Wnt signaling," *Science*, vol. 327, no. 5964, pp. 459–463, 2010.

[81] J. Ramser, F. E. Abidi, C. A. Burckle et al., "A unique exonic splice enhancer mutation in a family with X-linked mental retardation and epilepsy points to a novel role of the renin receptor," *Human Molecular Genetics*, vol. 14, no. 8, pp. 1019–1027, 2005.

[82] K. Takahashi, K. Hiraishi, T. Hirose et al., "Expression of (pro)renin receptor in the human brain and pituitary, and co-localisation with arginine vasopressin and oxytocin in the hypothalamus," *Journal of Neuroendocrinology*, vol. 22, no. 5, pp. 453–459, 2010.

[83] A. Contrepas, J. Walker, A. Koulakoff et al., "A role of the (pro)renin receptor in neuronal cell differentiation," *American Journal of Physiology*, vol. 297, no. 2, pp. R250–R257, 2009.

[84] Z. Shan, P. Shi, A. E. Cuadra et al., "Involvement of the brain (pro)renin receptor in cardiovascular homeostasis," *Circulation Research*, vol. 107, no. 7, pp. 934–938, 2010.

[85] Y. Moriyama, M. Maeda, and M. Futai, "The role of V-ATPase in neuronal and endocrine systems," *Journal of Experimental Biology*, vol. 172, pp. 171–178, 1992.

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*Review Article*

# Upregulation of the Renin-Angiotensin-Aldosterone-Ouabain System in the Brain Is the Core Mechanism in the Genesis of All Types of Hypertension

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Basic research using animal models points to a causal role of the central nervous system in essential hypertension; however, since clinical research is technically difficult to perform, this connection has not been confirmed in humans. Recently, renal nerve ablation in humans proved to continuously decrease blood pressure in resistant hypertension. Furthermore, when electrical stimulation was continuously applied to the carotid baroreceptor nerve of human adults, their blood pressure lowered. These findings promoted the concept that the central nervous system may actually be involved in the pathogenesis of essential hypertension, which is closely associated with excess sodium intake. We have demonstrated that endogenous digitalis plays a key role in hypertension associated with excess sodium intake via sympathetic activation in rats. Increased sodium concentration inside the brain activates epithelial sodium channels and the renin-angiotensin-aldosterone system in the brain. Aldosterone releases ouabain from neurons in the paraventricular nucleus in the hypothalamus. Angiotensin II and aldosterone of peripheral origin reach the brain to augment sympathetic outflow. Collectively essential hypertension associated with excess sodium intake and obesity, renovascular hypertension, and primary aldosteronism and pseudoaldosteronism all seem to have a common cause originating from the central nervous system.

## 1. Introduction

Blood pressure (BP) is a physiological phenomenon like body temperature, respiration, and pulse rate; its dysregulation most often results in hypertension. BP regulation is operated via sympathetic and parasympathetic nerves, as well as the release of pituitary hormones. However, antihypertensive agents such as diuretics, beta blockers, alpha blockers, calcium channel blockers (CCBs), mineralocorticoid receptor blockers (MRBs), and antagonists for the renin-angiotensin system (RAS) effectively lower the BP, and their sites action lie in the peripheral tissues. These antihypertensive treatments seem to have little or no connection with the central nervous system (CNS) regulation of BP; thus, if a central mechanism does exist, its actual regulatory role is believed to be minimal, and, therefore, any such central mechanism of hypertension has not been investigated extensively. However, evidence from animal models has demonstrated the role of sympathetic nervous system activity (SNSA) in hypertension [1–3]. For example, renal denervation consistently lowers the BP in a variety of animal models of hypertension [4–6]. In line with this, renal denervation by catheter-based radiofrequency ablation technology has been shown to effectively lower BP in patients with resistant hypertension at least for 2 years [7]. As Guyton's model [8] predicted that the renal function to excrete sodium is the infinite determinant of hypertension, denervation natriuresis may be the mechanism of action of renal denervation, demonstrating the crucial role of peripheral SNSA. Furthermore, interventional activation of the carotid baroreflex via continuous electrical stimulation with an implantable device has been shown to effectively lower BP by decreasing SNSA in patients with resistant hypertension [9]. This evidence indicates that the central setting level of BP regulation is elevated at the higher level

in hypertensive patients. These 2 important observations led us to consider the role of SNSA in the genesis of hypertension in humans.

In addition to these findings, other evidence to confirm the role of CNS in the genesis of hypertension has been revealed: a small dose of angiotensin II (Ang II) continuously administered subcutaneously (SC) gradually elevates BP, which can be abolished by intracerebroventricular (ICV) pretreatment with either an aldosterone synthase inhibitor or an MRB in rats [10]. Similar findings have been demonstrated with aldosterone: SC administration of aldosterone with 1% NaCl saline, as drinking water in rats gradually elevates BP, which is blunted by ICV pretreatment with either an Ang II AT-1 receptor blocker (ARB) or MRB [11]. These observations indicate that secondary hypertension such as primary aldosteronism, pseudoaldosteronism, and renovascular hypertension should be classified as centrally induced hypertension. Furthermore, central abolition of BP increases with ARB or MRB treatment, indicating that the major classes of antihypertensive agents may primarily be acting at a central site to lower BP. In fact, reflex tachycardia is absent when treated with the abovementioned antihypertensive agents, indicating that these agents lower BP by acting at a central site to set the BP to a lower level. In this paper, these are the particular points of discussion.

## 2. Arterial Hypertension and SNSA

The role of the autonomic nervous system activity in the pathogenesis of essential hypertension has been extensively studied [12–14]. Measurement of urinary excretion of norepinephrine has shown increased SNSA in animal models of hypertension, such as spontaneously hypertensive rats [15], Dahl salt sensitive rats [16], and models of deoxycorticosterone acetate (DOCA)-salt hypertension [17], Goldblatt renovascular hypertension [18], and renal mass-reduced hypertension [19]. Furthermore, there are numerous reports showing elevated urinary excretion of catecholamines in humans [20]; however, the difference between normal controls and hypertensive subjects is not high enough to establish the role of SNSA in hypertension.

On the other hand, SNSA is notably elevated in young subjects with labile hypertension who are believed to be in an early phase of essential hypertension [21]. SNSA is clearly increased among obese subjects with metabolic syndrome who are prone to be hypertensive and is decreased with a significant reduction in body weight [22, 23]. Augmented SNSA may be due to increased leptin, which is secreted from fat cells and stimulates SNSA via actions on the arcuate nucleus in the hypothalamus, and suppresses appetite [24]. When rats are fed a high-fat diet, they become resistant to appetite suppression but not resistant to sympathetic activation leading to hypertension [25]. However, SNSA is not much augmented in those hypertensive animals and humans, rather it is nearly normal due to suppression by the BP elevation caused by the initial increase in SNSA; that is, the SNSA converges to a near-normal level after a certain level of increase in the BP caused by an initial increase in SNSA. The subtle elevation of SNSA will be maintaining the elevated BP.

## 3. Sodium, SNSA, and Hypertension

Our ancestors living inland during the Stone Age would have consumed natural foods like wild animals do, and sodium intake would have been suitable at the minimum for survival. Epidemiological surveys of Yanomamo Indians in Amazon, Brazil, who, until recently, had been living like the people in the Stone Age did, revealed that their urinary excretion of sodium was only 0.9 mmol/day (equivalent to 0.53 g of sodium chloride) [26]. This is roughly 1/20th of the average sodium intake in modern societies, and this small amount of sodium was proven to be sufficient to live. In fact, all vertebrates, except human beings and domestic animals, consume only natural food without adding sodium salt. The average BP of the Yanomamo Indians was 96.0/60.6 mmHg, and the level did not elevate depending on age, which is in contrast to the observation in modern populations. The average life span of people who lived in the Stone Age is thought to be approximately 30 years, which is too low to cause atherosclerotic vascular complications, even if hypertension existed. Rather, higher BP would be better to supply enough blood to the principal organs and skeletal muscles. Renovascular hypertension may be a good example: when renal blood supply is impaired due to narrowing of renal arteries, the RAS is activated to raise the BP to maintain sufficient blood supply to the kidney. Thus, hypertension might have been a benefit for people in the Stone Age who were often attacked by enemies and wild animals; people who had a prompt elevation in BP could survive and leave offspring. To maintain a high BP, large amount of sodium would be needed, and thus, salt-sensitive subjects would be selected to survive. Those whose bodies promptly elevated the BP upon exposure to stress could succeed in genealogical history. Therefore, their offspring are the people living now, and we are salt-sensitive and prone to hypertension. Mechanisms to retain as much sodium as possible were required, the most powerful of which is the renin-angiotensin-aldosterone system (RAAS) [27]. In subjects who consume low amounts of sodium, the RAAS is working at full strength, as is the case for patients with Bartter syndrome. In addition, increased sympathetic outflow accelerates sodium reabsorption from the renal tubules via renal nerves [28]. Similarly, insulin acts to retain sodium available at renal tubules; this action is exaggerated in the early stage of diabetes mellitus and metabolic syndrome associated with obesity. The precise mechanism of how insulin retains sodium at the renal tubules is now well known.

Together, strong sodium-retaining mechanisms were a desirable trait and selected in later generations. Therefore, hypertension easily develops after excess intake of sodium. This trait was selected to correct our life style with a lower intake of sodium; as a result, the incidence of stroke is markedly decreased in Japan [29]. Understanding the mechanism of induction of hypertension after excess intake of sodium will help to devise efficient measures to control

the BP even after excessive sodium intake. A recent approach to elucidate these mechanisms revealed that the brain RAAS and endogenous digitalis are essentially involved in the induction of hypertension.

## 4. Endogenous Digitalis underlies the Connection of Sodium and Hypertension

Continuous administration of mineralocorticoids causes sodium accumulation, which results in natriuresis at a certain point, described as an escape phenomenon [30]. Factors associated with natriuresis include the glomerular filtration rate of the kidney, aldosterone, and other factors. The others are referred to as third factors [31]. Since it is well known that endogenous digitalis, an inhibitor for $Na^+/K^+$-ATPase, circulates and that the activity of tissue $Na^+/K^+$-ATPase decreases during sodium loading [32], endogenous digitalis is the most probable candidate for a third factor. Digoxin has been used for treating patients with congestive heart failure or tachyarrhythmias. Because it may cause intoxication, serum digoxin-like immunoreactivity (DLI) levels have been monitored, and significant levels of DLI have been detected in subjects not consuming digitalis glycosides [33]; this suggests the presence of endogenous digoxin. In fact, digoxin has been detected in human plasma by liquid chromatography and mass spectrometry [34]. Further, the turnover ratio of DLI in the hypothalamus and its plasma concentration increase with increasing sodium loading [35]. This suggests that digoxin may be produced in the hypothalamus and released into circulation. In fact, increased DLI concentrations were detected in the plasma of DOCA-salt hypertensive rats [36]. In humans, urinary excretion of DLI was found to be correlated with urinary excretions of sodium or BP during a medical checkup [33]. However, the DLI concentrations were not high enough to completely explain the pressor mechanism.

Ouabain, another water-soluble cardenolide, is known to be of endogenous origin [37]. Administration of low-dose ouabain causes sustained elevation of BP [38]. The Milan hypertensive rat model is known to have a mutation coding adducin, which augments the renal $Na^+/K^+$-ATPase to increase sodium accumulation and hypertension [39]. In this model, plasma ouabain levels are increased. PST2238, or rostafuroxin, is an analogue of digitoxygenin, and it antagonizes the action of ouabain and decreases BP in Milan hypertensive rats [40]. Since hypertension induced by low-dose ouabain is abolished by rostafloxin [41], ouabain must be directly involved in causing hypertension. Like Milan hypertensive rats, humans with adducin polymorphisms have elevated circulating ouabain levels, and rostafuroxin was found to be effective in lowering their BP [42]. However, results of a large-scale clinical study did not conclusively prove this [43].

Immunohistochemical approach using anti-ouabain or anti-digoxin antibody revealed that neurons in the paraventricular nucleus (PVN) and supraoptic nucleus (SON) showed ouabain- or digoxin-like immunoreactivity (OLI or DLI) [44–46]. Both OLI and DLI are detected not only in neuronal cell bodies but also in their dendrites and varicosities, which resembles the distribution of neuroendocrine hormones. The nerve fibers densely extend to the median eminence, subfornical organ (SFO), and organum vasculosum of the laminae terminalis (OVLT). Sodium loading increased the turnover ratio of DLI in the hypothalamus [35], and destruction of microtubules by ICV injection of colchicine increased DLI in the hypothalamus [35]. Therefore, DLI is clearly produced in the hypothalamus, PVN, and SON, which are stimulated by sodium loading. Although we did not explore similar experiments concerning the OLI, similar results are expected.

Since a low dose of ouabain injected into the lateral ventricle or the hypothalamus of rats increases the BP along with increasing the peripheral SNSA [47, 48], it seems likely that there are receptors for ouabain and endogenous ouabain acts as a neurotransmitter to increase SNSA.

Immortalized N1 cells are thought to be of PVN origin because these cells release vasopressin and oxytocin when stimulated [49]. Experiments on N1 cell line cultured in a serum-free condition showed that ouabain was released from these cells in a time-dependent manner; therefore, ouabain must be produced in the hypothalamus.

## 5. Brain RAAS and Sodium Metabolism (Figure 1)

In a clinical setting, BP in essential hypertension can be easily controlled with excellent antihypertensive agents such as diuretics, CCBs, angiotensin I converting enzyme inhibitors (ACEIs), ARBs, and MRBs. Sites where these agents act are crucial for BP regulation. In particular, ACEIs and ARBs are very effective in controlling BP and preventing complications, which indicates that RAAS is essential to the pathogenesis of hypertension. These drugs are effective in high-renin patients but still serve to lower BP even in those with low plasma renin activity (PRA) [50]. The RAAS has not been considered to be involved in sodium-sensitive hypertension because PRA is suppressed with sodium loading. On the other hand, in addition to the renal-renin and adrenal aldosterone system, RAAS was found to exist in other tissues such as the salivary gland and brain [51]. When sodium was loaded, the classical RAAS was suppressed, but the brain RAAS was activated [52]. The classical RAAS regulates sodium balance via a negative feedback, but the brain RAAS forms a positive feedback cycle to retain more sodium via increased renal SNSA. In the CNS, sodium loading upregulates the messenger RNA for renin, ACE, and angiotensin II AT-1 receptor [53]. Therefore, ICV administration of Ang II augmented pressor responses in sodium-loaded rats. Furthermore, aldosterone is present in the brain, and its level increases with sodium loading, leading to increased SNSA [54]. The hypotensive effects of MRB are greater in the low-renin essential hypertensive patients than in the normal renin hypertensives [55], which may be explained by the activated RAAS in the brain. Since Ang II administered into the CNS increases the BP along with increasing the SNSA [56], augmented RAAS in the brain

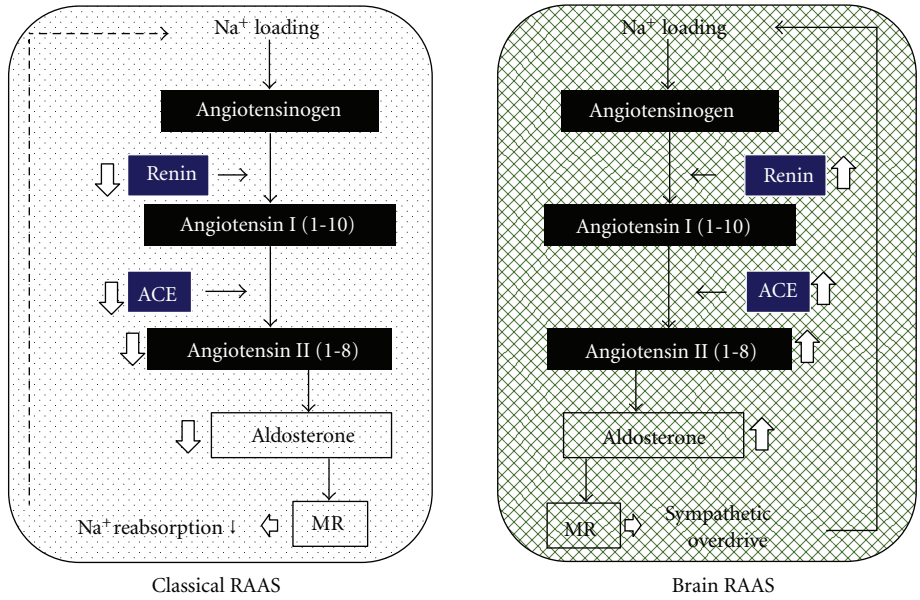


Figure 1: The classical RAAS (renal-renin and adrenal aldosterone system) regulates sodium balance via a negative feedback, but the brain RAAS forms a positive feedback cycle to retain more sodium via increased renal SNSA.

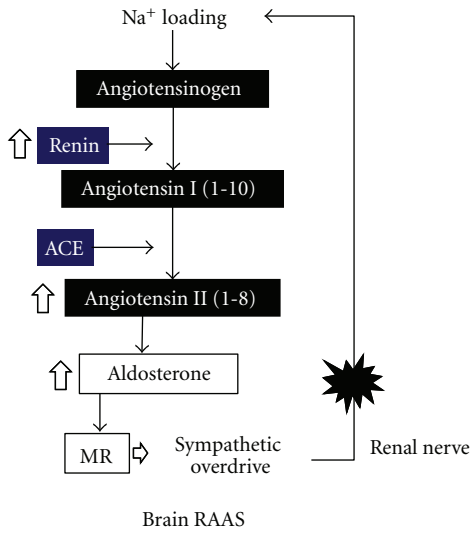


Figure 2: The most important component of the positive feedback cycle may be the renal nerve because renal nerve ablation lowers blood pressure even in humans, and inhibitors of RAAS lower blood pressure even in low-renin essential hypertensives.

induced by sodium loading may be a probable cause of essential hypertension. ICV injections of hypertonic saline cause elevation of both BP and plasma DLI, which can be blocked by ICV pretreatment with ARB [57]. Therefore, increased brain RAAS by sodium has been suggested to be involved in hypertensive actions of sodium. Thereby, abdominal SNSA, an upstream event of renal nerve stimulation, is markedly increased, as confirmed by the decreased renal blood flow observed using radioactive microspheres that indicate activated renal SNSA [58]. Increased renal SNSA decreases renal excretion of sodium by decreasing renal blood flow and increasing renal tubular sodium reabsorption [59]. Thus, increased intracranial sodium concentration forms a positive circuit to retain sodium in the body (Figure 2).

## 6. Epithelial Sodium Channels May Be a Sensor for Sodium Concentration

The mechanism underlying SNSA activation by sodium intake cannot be explained by osmotic stimuli, since osmotic stimulation by urea does not increase the BP [60]. Epithelial sodium channels (ENaC) were thought to act as sensors for sodium in the brain because these channels sense sodium on the tongue [61]. This was proven because the ENaC-specific inhibitor, benzamil, abolished the pressor responses to ICV injections of hypertonic saline in a dose-dependent manner [61]. Wang et al. [62] explored the connection of ENaC with brain ouabain. They found that ICV administration of hypertonic NaCl with a small dose of aldosterone caused pressor responses accompanied by increased SNSA. This response could be abolished by ICV pretreatment with benzamil. Further, ICV pretreatment with Digibind, an inhibitor of ouabain and digoxin, blocked the pressor effects. These findings indicate that ENaC is a sensor of sodium in the brain, and its downstream effects consist of aldosterone and ouabain/digoxin release, causing an elevation in BP.

## 7. Relationship between RAAS and Digitalis in the Brain (Table 1)

As mentioned above, ICV pretreatment with ARB can inhibit the pressor responses and DLI release into circulation caused by ICV injections of hypertonic saline in rats. Sodium

Table 1: Intracranial pretreatments with one of these agents blunt pressor responses caused by centrally administered sodium, sodium-induced hypertension in Dahl salt-sensitive rats, hypertension caused by subcutaneously injected angiotensin II, or aldosterone.

| |
|---|
| (i) AT-1 receptor blocker |
| (ii) Mineralocorticoid receptor blocker |
| (iii) Aldosterone synthase inhibitor |
| (iv) Epithelial sodium channel (ENaC) blocker |
| (v) Antidigitalis blocking antibody |
| (vi) Anti-oxidative agent |

loading upregulates RAS in the brain. ICV pretreatment with spironolactone, an MRB, blocks both BP rises and increased OLI in the hypothalamus and pituitary caused by ICV administration of hypertonic NaCl [63], which indicates that sodium loading increases aldosterone in the brain [54]. In fact, aldosterone-like immunoreactivity in the hypothalamus is increased in rats after sodium loading. Since ICV pretreatment with spironolactone decreases the intrahypothalamic content of OLI, ouabain may be located downstream of aldosterone release. We recently found that aldosterone caused a dose-dependent release of OLI from cultured N1 cells of PVN origin [49].

## 8. The Central Effects of Ang II or Aldosterone of Peripheral Origin

It has long been known that pressor responses occur due to increased peripheral SNSA after Ang II is administered via vertebral artery [64]. ICV administration of Ang II leads to similar pressor responses [56]. Therefore, Ang II directly acts on the CNS. Because pressor responses can be blocked by ICV pretreatment with ARB, the effect is mediated via AT-1 receptors of Ang II [65]. For example, this response is attenuated in subfornical organ-lesioned rats, which means that one of the regions where Ang II acts is the subfornical organ [65]. SC injection of low-dose Ang II for several days leads to gradual increase in BP in approximately 3 days [10]. ICV pretreatment with an inhibitor of aldosterone synthase abolished the pressor response: it reduced the BP elevation induced by a rather high SC dose of Ang II by approximately 80%. Similar blocking effects can be obtained by eplerenone, an MRB, as well as Digibind. A similar finding has been reported by other researchers [11] who showed that RU28318, an MRB, abolishes the pressor responses caused by SC injection of Ang II. These findings are in absolute contrast to the classical interpretation that Ang II causes hypertension by constricting the arterial beds and increasing cardiac contraction. That MRBs are effective in blocking the central actions of Ang II indicates that Ang II produces aldosterone in the brain. Specifically, the RAAS may be playing a role in the brain similar to its role in the peripheral system [66].

SC injections of aldosterone in addition to 1% saline as drinking water gradually increased the BP by about 30 mmHg; this is similar to the results for Ang II administration [11]. Water intake is concomitantly increased as BP rises. Thereby, ICV administration of irbesartan, an ARB, RU28318, or spironolactone almost completely blocked the pressor responses and water intake. The pressor response can also be suppressed by ICV pretreatments with either apocynin (an NADPH oxidase inhibitor) or tempol (a reactive oxygen species scavenger); this indicates that the response is mediated by oxidative stress caused by aldosterone in the brain. However, water intake was not suppressed by suppression of oxidative stress, suggesting that the pressor mechanism is independent from the 1% saline intake response.

Because ICV pretreatment with MRB abolishes pressor responses to systemic administration of aldosterone, it follows that aldosterone of adrenocortical origin can act at a central site to increase the BP. Like the interpretation for Ang II, conclusion of this unique evidence is entirely different from the classical interpretation that aldosterone causes hypertension by acting at the renal tubule to increase sodium reabsorption. The novel findings that both Ang II and aldosterone primarily act in the CNS to cause hypertension are epoch-making discoveries that radically challenge the classical interpretation (Figure 3).

## 9. Supposed Common Central Mechanism of a Variety of Models of Hypertension

A hypertensive state means that the set point of the baroreceptor reflex has shifted to a higher level, and the sensitivity is decreased. Thus, when Ang II production is blocked with ACEI, the set point is lowered to the normal level and the sensitivity recovers [67]. It follows, therefore, that Ang II is critically involved in the pathogenesis of hypertension. The lowering of set point is not secondary to normalizing the BP since the set point is still at the lower BP level after treatment with ACEI, even when the BP has been elevated to the hypertensive level with intravenous infusions of phenylephrine. These findings indicate that inhibition of RAAS completely normalizes BP regulation. The previously mentioned evidence indicates that the involved RAAS is not only of peripheral origin, but also of central origin. However, since the vasomotor center exists in the CNS but not in the periphery, Ang II or AT-1 receptors in the brain will be primarily involved in changing the set point of the baroreceptor reflex mechanism.

The epidemiological and experimental evidence previously discussed suggests that a human being who lived in an environment with minimum intake of sodium during long periods of Stone Age acquired a rigid mechanism to retain sodium; this mechanism primarily consists of RAAS and SNSA. Meanwhile, a high BP would be a desirable feature for living in low-salt conditions because people with a high BP could be aroused more rapidly and fight more vigorously than those with lower BP. Atherosclerosis was not a problem, as these people did not live long. However, significant changes in the sodium environment occurred during a short period, and BP fluctuation required

Table 2: Sodium retention can be achieved by a variety of causes.

| |
|---|
| (i) Excess intake of sodium; essential hypertension |
| (ii) Impaired renal excretion |
| (a) Renal insufficiency and renal failure |
| (b) Insulin resistance accompanied by obesity and/or the early stage of type II diabetes mellitus |
| (c) Increased aldosterone production; primary aldosteronism, idiopathic hyperaldosteronism, renovascular hypertension, pheochromocytoma |
| (d) Other mineralocorticoid excess; 17**a**-hydroxylase deficiency, 11**b**-hydroxylase deficiency, apparent mineralocorticoid excess syndrome and deoxycorticosterone-producing tumor |
| (e) Exaggerated renal sodium reabsorption; Liddle syndrome |

Table 3: A list of secondary hypertension, supposedly caused by direct central actions of angiotensin II or mineralocorticoid.

| |
|---|
| Angiotensin II |
| Renovascular hypertension, aortic coarctation, renin-producing tumor, and pheochromocytoma |
| Mineralocorticoids |
| Aldosterone; primary aldosteronism, idiopathic hyperaldosteronism, renovascular hypertension, and pheochromocytoma |
| Other mineralocorticoids; 17**a**-hydroxylase deficiency, 11**b**-hydroxylase deficiency, apparent mineralocorticoid excess syndrome, and deoxycorticosterone-producing tumor |

correction; thus, the renal-renin and adrenal aldosterone systems were equipped to form negative feedback. On the other hand, brain RAAS may have been forming a positive feedback system to retain sodium and maintain the BP at a high level. Specifically, when sodium was loaded, opposing mechanisms may be operational at the periphery and the CNS. Therefore, human beings having a predisposition to hypertension can easily elevate the BP with increased sodium environment, which would result in sodium-sensitive essential hypertension. Similar mechanisms might be playing a role in renal hypertension, obesity-related hypertension, and senile hypertension with reduced renal function. In renovascular hypertension, increased production of Ang II and aldosterone might be directly acting at the central site to cause hypertension. Similarly, in primary aldosteronism and pseudoaldosteronism, MR in the CNS might be mainly involved in the genesis of hypertension. Circumventricular organs surrounding the third ventricle such as SFO, OVLT, and area postrema are outside of the blood-brain barrier, are anatomically different from other brain tissues, and show dense distribution of AT-1 receptors. These areas are thought to sense information from systemic circulation. For instance, when the SFO was electrically destroyed, hypertension caused by chronic SC infusion of Ang II was suppressed [65], indicating that Ang II acts at the SFO. Further, hypotensive effects of losartan, an ARB, are reduced, which means that ARB is actually acting at the SFO to reduce BP. Therefore, Ang II and aldosterone produced in the blood may be acting at these central sites to elicit sympathetic hyperactivity (Tables 2 and 3).

Thus, the central mechanism might be involved in every type of hypertension.

## 10. Relationship between the Central Vasopressor Mechanism and Antihypertensive Agents (Figure 4)

As mentioned previously, the central cascade causing hypertension may consist of $Na^+$, ENaC, RAAS, digitalis, oxidative stress, and SNSA. Therefore, agents acting at one of these components will be excellent antihypertensive agents. In fact, many antihypertensive agents have been identified during the long history of screening for treatment of hypertension, and the currently available antihypertensive agents seem to be acting at any one of the components of this cascade. For example, the role of diuretics is easy to understand, because they reduce sodium loading. Although ENaC inhibitors such as amiloride and triamterene are known to reduce BP [68], these are not used as first-line antihypertensive agents primarily because of their low receptor selectivity. Presently, blockers of RAAS such as ACEIs, ARBs, direct renin inhibitors (DRIs), and MRBs are the most frequently used agents. These may be acting at the core of the mechanism causing hypertension, and therefore, are the most reliable agents. Canrenone and rostafloxin (PST2238) are digitalis antagonists, which also reduce BP [69, 70]. However, there remain some concerns particularly in their potency and receptor selectivity. Antioxidants may be effective antihypertensive agents, since tempol is known to reduce BP in hypertensive rats [71]. Centrally acting sympatholytic agents such as $\alpha_2$-adrenoceptor agonists or imidazoline receptor agonists are well known to reduce the BP [72], but adverse effects such as drowsiness and dry mouth limit their usage. $\alpha_1$-Adrenoceptor antagonists are also used for the treatment of hypertension [73] and are

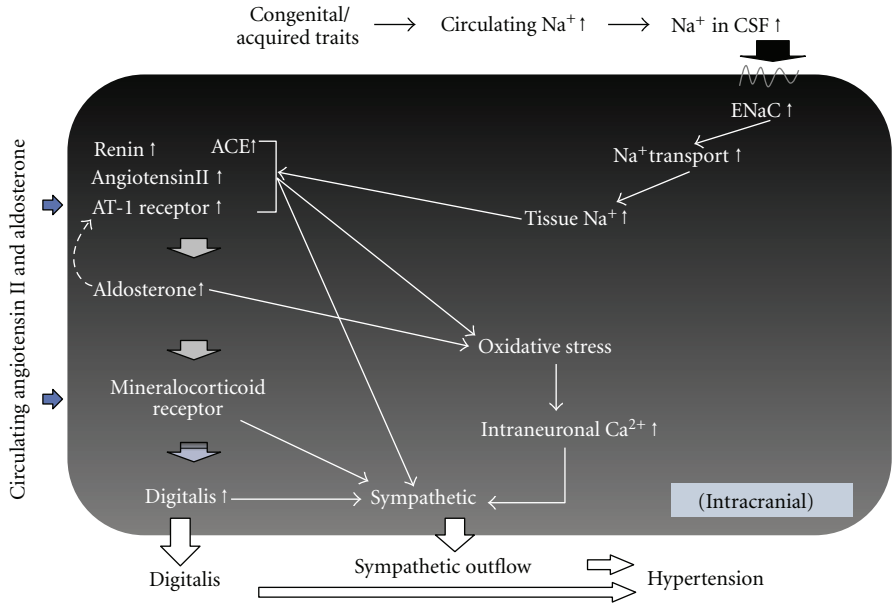


Figure 3: Supposed common central mechanism of a variety of models of hypertension. The dotted line indicates the possible actions of aldosterone on the RAS activation in the brain.

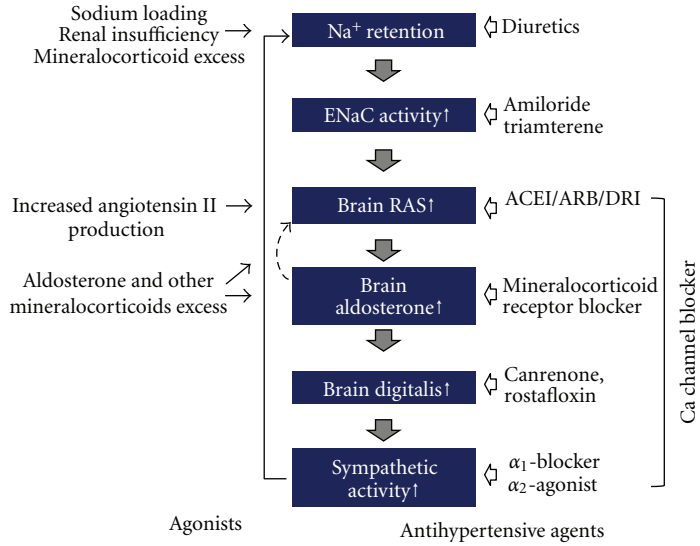


Figure 4: A supposed cascade of genesis of hypertension and acting sites of antihypertensive agents. In fact, when we treated hypertensive patients with these agents, reflex tachycardia is missing. This means that these antihypertensive agents are resetting the blood pressure regulatory center to a lower level besides their original actions such as vasodilation and diuresis.

known to act at the CNS level to decrease SNSA in rats [74]. CCBs are widely used in the clinical setting and are known to cause sympathetic inhibition at the CNS in rats [75]. Although short-acting and potent CCBs cause sympathetic activation by the baroreceptor reflex, gradual decreases in BP with slow-acting agents do not cause reflex tachycardia [76]. The fact that reflex tachycardia is not caused by the agents listed before indicates that at least the first and second lines of antihypertensive agents are actually acting at a CNS site to regulate BP and shifting the set point to a lower level.

In general, the blood-brain barrier blocks the entry of agents into the brain tissue. Therefore, concentrations of systemically administered agents, except the lipid-soluble agents, in the brain are very low. Therefore, it is believed that antihypertensive agents, except for $\alpha_2$-adrenoceptor agonists, do not affect the brain function. However, as mentioned earlier, these agents act at the circumventricular organs where the barrier is lacking.

Currently available antihypertensive agents have been screened for many years and can reduce the BP comfortably without stimulating the heart and finally improve our

prognosis. This may be because these agents are acting at the core mechanism of hypertension. In other words, the common site of actions of these agents is the cause of hypertension. Thus, it may be desirable to target development of novel agents with selective action at the central mechanism regulating BP in future studies.

## References


[1] K. R. Borkowski and P. Quinn, "Adrenaline and the development of spontaneous hypertension in rats," *Journal of Autonomic Pharmacology*, vol. 5, no. 2, pp. 89–100, 1985.

[2] M. Bouvier and J. De Champlain, "Increased apparent norepinephrine release rate in anesthetized DOCA-salt hypertensive rats," *Clinical and Experimental Hypertension A*, vol. 7, no. 11, pp. 1629–1645, 1985.

[3] A. Cabassi, S. Vinci, M. Calzolari, G. Bruschi, and A. Borghetti, "Regional sympathetic activity in pre-hypertensive phase of spontaneously hypertensive rats," *Life Sciences*, vol. 62, no. 12, pp. 1111–1118, 1998.

[4] H. Takahashi, I. Iyoda, H. Yamasaki et al., "Retardation of the development of hypertension in DOCA-salt rats by renal denervation," *Japanese Circulation Journal*, vol. 48, no. 6, pp. 567–574, 1984.

[5] R. L. Kline, P. M. Kelton, and P. F. Mercer, "Effect of renal denervation on the development of hypertension in spontaneously hypertensive rats," *Canadian Journal of Physiology and Pharmacology*, vol. 56, no. 5, pp. 818–822, 1978.

[6] V. M. Campese and E. Kogosov, "Renal afferent denervation prevents hypertension in rats with chronic renal failure," *Hypertension*, vol. 25, no. 4, part 2, pp. 878–882, 1995.

[7] H. Krum, M. Schlaich, R. Whitbourn et al., "Catheter-based renal sympathetic denervation for resistant hypertension: a multicentre safety and proof-of-principle cohort study," *The Lancet*, vol. 373, no. 9671, pp. 1275–1281, 2009.

[8] A. C. Guyton, "Long-term arterial pressure control: an analysis from animal experiments and computer and graphic models," *American Journal of Physiology*, vol. 259, no. 5, part 2, pp. R865–R877, 1990.

[9] J. D. Bisognano, G. Bakris, M. K. Nadim et al., "Baroreflex activation therapy lowers blood pressure in patients with resistant hypertension: results from the double-blind, randomized, placebo-controlled rheos pivotal trial," *Journal of the American College of Cardiology*, vol. 58, no. 7, pp. 765–773, 2011.

[10] B. S. Huang, S. Ahmadi, M. Ahmad, R. A. White, and F. H. H. Leenen, "Central neuronal activation and pressor responses induced by circulating ANG II: role of the brain aldosterone-"ouabain" pathway," *American Journal of Physiology*, vol. 299, no. 2, pp. H422–H430, 2010.

[11] B. Xue, T. G. Beltz, Y. Yu et al., "Central interactions of aldosterone and angiotensin II in aldosterone- and angiotensin II-induced hypertension," *American Journal of Physiology*, vol. 300, no. 2, pp. H555–H564, 2011.

[12] S. E. Kjeldsen, B. Flaaten, I. Eide, A. Helgeland, and P. Leren, "Increased peripheral release of noradrenaline and uptake of adrenaline in essential hypertension?" *Clinical Science*, vol. 61, supplement 7, pp. 215s–217s, 1981.

[13] D. Levitan, S. G. Massry, M. Romoff, and V. M. Campese, "Plasma catecholamines and autonomic nervous system function in patients with early renal insufficiency and hypertension: effect of clonidine," *Nephron*, vol. 36, no. 1, pp. 24–29, 1984.

[14] R. Franco-Morselli, J. L. Elghozi, E. Joly, S. Di Giuilio, and P. Meyer, "Increased plasma adrenaline concentrations in benign essential hypertension," *British Medical Journal*, vol. 2, no. 6097, pp. 1251–1254, 1977.

[15] H. Grobecker, M. F. Roizen, V. Weise, J. M. Saavedra, and I. J. Kopin, "Sympathoadrenal medullary activity in young, spontaneously hypertensive rats," *Nature*, vol. 258, no. 5532, pp. 267–268, 1975.

[16] M. J. Kenney, D. A. Morgan, and A. L. Mark, "Sympathetic nerve responses to sustained stimulation of somatic afferents in Dahl rats," *Journal of Hypertension*, vol. 9, no. 10, pp. 963–968, 1991.

[17] M. Bouvier and J. de Champlain, "Increased basal and reactive plasma norepinephrine and epinephrine levels in awake DOCA-salt hypertensive rats," *Journal of the Autonomic Nervous System*, vol. 15, no. 2, pp. 191–195, 1986.

[18] R. E. Katholi, S. R. Winternitz, and S. Oparil, "Decrease in peripheral sympathetic nervous system activity following renal denervation or unclipping in the one-kidney one-clip Goldblatt hypertensive rat," *The Journal of Clinical Investigation*, vol. 69, no. 1, pp. 55–62, 1982.

[19] W. J. Stekiel, S. J. Contney, and J. H. Lombard, "Sympathetic neural control of vascular muscle in reduced renal mass hypertension," *Hypertension*, vol. 17, no. 6, part 2, pp. 1185–1191, 1991.

[20] D. S. Goldstein, "Plasma catecholamines and essential hypertension. An analytical review," *Hypertension*, vol. 5, no. 1, pp. 86–99, 1983.

[21] A. Penesova, Z. Radikova, E. Cizmarova et al., "The role of norepinephrine and insulin resistance in an early stage of hypertension," *Annals of the New York Academy of Sciences*, vol. 1148, pp. 490–494, 2008.

[22] M. D. Esler, N. Eikelis, E. Lambert, and N. Straznicky, "Neural mechanisms and management of obesity-related hypertension," *Current Cardiology Reports*, vol. 10, no. 6, pp. 456–463, 2008.

[23] G. Grassi, G. Seravalle, F. Quarti-Trevano et al., "Excessive sympathetic activation in heart failure with obesity and metabolic syndrome: characteristics and mechanisms," *Hypertension*, vol. 49, no. 3, pp. 535–541, 2007.

[24] M. M. Smith and C. T. Minson, "Obesity and adipokines: effects on sympathetic overactivity," *Journal of Physiology*, vol. 590, part 8, pp. 1787–1801, 2012.

[25] K. Rahmouni, D. A. Morgan, G. M. Morgan, A. L. Mark, and W. G. Haynes, "Role of selective leptin resistance in diet-induced obesity hypertension," *Diabetes*, vol. 54, no. 7, pp. 2012–2018, 2005.

[26] J. J. Mancilha-Carvalho, R. de Oliveira, and R. J. Esposito, "Blood pressure and electrolyte excretion in the Yanomamo Indians, an isolated population," *Journal of Human Hypertension*, vol. 3, no. 5, pp. 309–314, 1989.

[27] A. D. M. Riquier-Brison, P. K. K. Leong, K. Pihakaski-Maunsbach, and A. A. McDonough, "Angiotensin II stimulates trafficking of NHE3, NaPi2, and associated proteins into the proximal tubule microvilli," *American Journal of Physiology*, vol. 298, no. 1, pp. F177–F186, 2010.

[28] G. F. DiBona, "Neurogenic regulation of renal tubular sodium readsorption," *American Journal of Physiology*, vol. 233, no. 2, pp. F73–F81, 1977.

[29] M. Kubo, Y. Kiyohara, I. Kato et al., "Trends in the incidence, mortality, and survival rate of cardiovascular disease in a Japanese community: the Hisayama study," *Stroke*, vol. 34, no. 10, pp. 2349–2354, 2003.

[30] J. Möhring, B. Möhring, and S. Just, "Description of the DOCA escape phenomenon in the rat," *Naunyn Schmiedeberg's Archives of Pharmacology*, vol. 266, no. 4, pp. 406–407, 1970.

[31] F. Krück and H. J. Kramer, "Third factor and edema formation," *Contributions to Nephrology*, vol. 13, pp. 12–20, 1978.

[32] P. A. Doris, "Endogenous inhibitors of the Na,K pump," *Mineral and Electrolyte Metabolism*, vol. 22, no. 5-6, pp. 303–310, 1996.

[33] H. Takahashi, M. Matsusawa, H. Okabayashi et al., "Endogenous digitalislike substance in an adult population in Japan," *American Journal of Hypertension*, vol. 1, no. 3, part 3, pp. 168s–172s, 1988.

[34] Y. Komiyama, N. Nishimura, X. H. Dong et al., "Liquid chromatography mass spectrometric analysis of ouabainlike factor in biological fluid," *Hypertension Research*, vol. 23, supplement, pp. S21–S27, 2000.

[35] H. Takahashi, M. Matsusawa, K. Suga et al., "Hypothalamic digitalis-like substance is released with sodium-loading in rats," *American Journal of Hypertension*, vol. 1, no. 2, pp. 146–151, 1988.

[36] I. Kojima, S. Yoshihara, and E. Ogata, "Involvement of endogenous digitalis-like substance in genesis of deoxycorticosterone-salt hypertension," *Life Sciences*, vol. 30, no. 21, pp. 1775–1781, 1982.

[37] J. M. Hamlyn, M. P. Blaustein, S. Bova et al., "Identification and characterization of a ouabain-like compound from human plasma," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 88, no. 14, pp. 6259–6263, 1991.

[38] C. M. Yuan, P. Manunta, J. M. Hamlyn et al., "Long-term ouabain administration produces hypertension in rats," *Hypertension*, vol. 22, no. 2, pp. 178–187, 1993.

[39] G. Tripodi, A. Piscone, G. Borsani et al., "Molecular cloning of an adducin-like protein: evidence of a polymorphism in the normotensive and hypertensive rats of the milan strain," *Biochemical and Biophysical Research Communications*, vol. 177, no. 3, pp. 939–947, 1991.

[40] P. Manunta, M. Ferrandi, E. Messaggio, and P. Ferrari, "A new antihypertensive agent that antagonizes the prohypertensive effect of endogenous Ouabain and Adducin," *Cardiovascular and Hematological Agents in Medicinal Chemistry*, vol. 4, no. 1, pp. 61–66, 2006.

[41] P. Ferrari, L. Torielli, M. Ferrandi et al., "PST2238: a new antihypertensive compound that antagonizes the long-term pressor effect of ouabain," *Journal of Pharmacology and Experimental Therapeutics*, vol. 285, no. 1, pp. 83–94, 1998.

[42] G. Bianchi, M. G. Tripodi, G. Casari et al., "$\alpha$-Adducin may control blood pressure both in rats and humans," *Clinical and Experimental Pharmacology and Physiology*, vol. 22, no. 1, pp. S7–S9, 1995.

[43] J. A. Staessen, L. Thijs, K. Stolarz-Skrzypek et al., "Main results of the ouabain and adducin for Specific Intervention on Sodium in Hypertension Trial (OASIS-HT): a randomized placebo-controlled phase-2 dose-finding study of rostafuroxin," *Trials*, vol. 12, pp. 13–27, 2011.

[44] H. Yamada, M. Naruse, K. Naruse et al., "Histological study on ouabain immunoreactivities in the mammalian hypothalamus," *Neuroscience Letters*, vol. 141, no. 2, pp. 143–146, 1992.

[45] H. Takahashi, M. Matsuzawa, H. Okabayashi et al., "Evidence for a digitalis-like substance in the hypothalamopituitary axis in rats: implications in the central cardiovascular regulation associated with an excess intake of sodium," *Japanese Circulation Journal*, vol. 51, no. 10, pp. 1199–1207, 1987.

[46] H. Yamada, N. Ihara, H. Takahashi, M. Yoshimura, and Y. Sano, "Distribution of the endogenous digitalis-like substance (EDLS)-containing neurons labeled by digoxin antibody in hypothalamus and three circumventricular organs of dog and macaque," *Brain Research*, vol. 584, no. 1-2, pp. 237–243, 1992.

[47] H. Takahashi, I. Iyoda, K. Takeda et al., "Centrally-induced vasopressor responses to sodium-potassium adenosine triphosphatase inhibitor, ouabain, may be mediated via angiotensin II in the anteroventral third ventricle in the brain," *Japanese Circulation Journal*, vol. 48, no. 11, pp. 1243–1250, 1984.

[48] I. Iyoda, H. Takahashi, L. C. Lee et al., "Cardiovascular and sympathetic responses to ouabain injected into the hypothalamus in rats," *Cardiovascular Research*, vol. 20, no. 4, pp. 294–298, 1986.

[49] M. Yoshika, Y. Komiyama, and H. Takahashi, "An ouabain-like factor is secreted from immortalized hypothalamic cells in an aldosterone-dependent manner," *Neurochemistry International*, vol. 59, no. 2, pp. 104–108, 2011.

[50] J. Minami, T. Ishimitsu, and H. Matsuoka, "Is there overlap in blood-pressure response to the blockers of the renin-angiotensin system between lower and higher renin subjects?" *American Journal of Hypertension*, vol. 21, no. 2, pp. 130–131, 2008.

[51] S. Hirose, T. Ohsawa, T. Inagami, and K. Murakami, "Brain renin from bovine anterior pituitary: isolation and properties," *The Journal of Biological Chemistry*, vol. 257, no. 11, pp. 6316–6321, 1982.

[52] M. Tada, A. Fukamizu, M. S. Seo, S. Takahashi, and K. Murakami, "Renin expression in the kidney and brain is reciprocally controlled by captopril," *Biochemical and Biophysical Research Communications*, vol. 159, no. 3, pp. 1065–1071, 1989.

[53] M. Nishimura, A. Nanbu, K. Ohtsuka et al., "Sodium intake regulates renin gene expression differently in the hypothalamus and kidney of rats," *Journal of Hypertension*, vol. 15, no. 5, pp. 509–516, 1997.

[54] B. S. Huang, W. J. Cheung, H. Wang, J. Tan, R. A. White, and F. H. H. Leenen, "Activation of brain renin-angiotensin-aldosterone system by central sodium in Wistar rats," *American Journal of Physiology*, vol. 291, no. 3, pp. H1109–H1117, 2006.

[55] M. H. Weinberger, W. B. White, L. M. Ruilope et al., "Effects of eplerenone versus losartan in patients with low-renin hypertension," *American Heart Journal*, vol. 150, no. 3, pp. 426–433, 2005.

[56] J. F. E. Mann, W. Rascher, A. Schomig et al., "Contribution of the sympathetic nervous system to the centrally-induced pressor action of angiotensin II in rats," *Clinical and Experimental Pharmacology and Physiology*, vol. 9, no. 2, pp. 193–201, 1982.

[57] H. Takahasi, M. Matsusawa, I. Ikegaki et al., "Brain renin-angiotensin system and the hypothalamic, digitalis-like Na+, K+-ATPase inhibitor in rats," *Clinical and Experimental Hypertension A*, vol. 10, no. 6, pp. 1285–1287, 1988.

[58] M. Sakamoto, M. Nishimura, and H. Takahashi, "Brain atrial natriuretic peptide family abolishes cardiovascular haemodynamic alterations caused by hypertonic saline in rats," *Clinical and Experimental Pharmacology and Physiology*, vol. 26, no. 9, pp. 684–690, 1999.

[59] G. F. DiBona, "Neural regulation of renal tubular sodium reabsorption and renin secretion," *Federation Proceedings*, vol. 44, no. 13, pp. 2816–2822, 1985.

[60] B. S. Huang, B. N. Van Vliet, and F. H. H. Leenen, "Increases in CSF [Na+] precede the increases in blood pressure in Dahl

S rats and SHR on a high-salt diet," *American Journal of Physiology*, vol. 287, no. 3, pp. H1160–H1166, 2004.

[61] M. Nishimura, K. Ohtsuka, A. Nanbu, H. Takahashi, and M. Yoshimura, "Benzamil blockade of brain $Na^+$ channels averts $Na^+$-induced hypertension in rats," *American Journal of Physiology*, vol. 274, no. 3, part 2, pp. R635–R644, 1998.

[62] H. Wang, B. S. Huang, and F. H. H. Leenen, "Brain sodium channels and ouabainlike compounds mediate central aldosterone-induced hypertension," *American Journal of Physiology*, vol. 285, no. 6, pp. H2516–H2523, 2003.

[63] B. S. Huang, W. J. Cheung, H. Wang, J. Tan, R. A. White, and F. H. H. Leenen, "Activation of brain renin-angiotensin-aldosterone system by central sodium in Wistar rats," *American Journal of Physiology*, vol. 291, no. 3, pp. H1109–H1117, 2006.

[64] K. Fukiyama, J. W. McCubbin, and I. H. Page, "Chronic hypertension elicited by infusion of angiotensin into vertebral arteries of unanaesthetized dogs," *Clinical Science*, vol. 40, no. 3, pp. 283–291, 1971.

[65] J. P. Collister and M. D. Hendel, "Chronic effects of angiotensin II and AT1 receptor antagonists in subfornical organ-lesioned rats," *Clinical and Experimental Pharmacology and Physiology*, vol. 32, no. 5-6, pp. 462–466, 2005.

[66] Z. H. Zhang, Y. Yu, Y. M. Kang, S. G. Wei, and R. B. Felder, "Aldosterone acts centrally to increase brain renin-angiotensin system activity and oxidative stress in normal rats," *American Journal of Physiology*, vol. 294, no. 2, pp. H1067–H1074, 2008.

[67] C. M. Heesch, M. E. Crandall, and J. A. Turbek, "Converting enzyme inhibitors cause pressure-independent resetting of baroreflex control of sympathetic outflow," *American Journal of Physiology*, vol. 270, no. 4, part 2, pp. R728–R737, 1996.

[68] B. S. Heran, J. M. Chen, J. J. Wang, and J. M. Wright, "Blood pressure lowering efficacy of potassium-sparing diuretics (that block the epithelial sodium channel) for primary hypertension," *Cochrane Database of Systematic Reviews*, no. 1, Article ID CD008167, 2010.

[69] A. M. Grandi, D. Imperiale, R. Santillo et al., "Aldosterone antagonist improves diastolic function in essential hypertension," *Hypertension*, vol. 40, no. 5, pp. 647–652, 2002.

[70] P. Ferrari, "Rostafuroxin: an ouabain-inhibitor counteracting specific forms of hypertension," *Biochimica et Biophysica Acta*, vol. 1802, no. 12, pp. 1254–1258, 2010.

[71] K. W. Mathis, M. Venegas-Pont, C. W. Masterson, N. J. Stewart, K. L. Wasson, and M. J. Ryan, "Oxidative stress promotes hypertension and albuminuria during the autoimmune disease systemic lupus erythematosus," *Hypertension*, vol. 59, no. 3, pp. 673–679, 2012.

[72] J. Brown, G. Branche, M. Streets, A. King, V. Dowdy, and J. Batts, "Centrally acting alpha-2 agonists, peripherally acting adrenergic-blocking drugs, and direct vasodilators in the treatment of mild and moderate essential hypertension," *Clinical Cardiology*, vol. 12, supplement 4, pp. IV78–IV81, 1989.

[73] N. Chapman, C. Y. Chen, T. Fujita et al., "Time to re-appraise the role of alpha-1 adrenoceptor antagonists in the management of hypertension?" *Journal of Hypertension*, vol. 28, no. 9, pp. 1796–1803, 2010.

[74] H. Takahashi, H. Okabayashi, K. Suga, M. Matsuzawa, I. Ikegaki, and M. Yoshimura, "Sympatholytic effects of the intravenously injected alpha1-adrenergic blocker, bunazosin, in anaesthetized rats," *Journal of Hypertension*, vol. 5, no. 6, pp. 677–682, 1987.

[75] I. Iyoda, H. Takahashi, K. Takeda et al., "Centrally-induced vasodepressor responses to diltiazem, a calcium channel blocker, in rats," *Journal of Hypertension*, vol. 3, no. 6, pp. 639–644, 1985.

[76] B. S. Huang, P. P. Murzenok, and F. H. H. Leenen, "Sympathoinhibitory and depressor responses to long-term infusion of nifedipine in spontaneously hypertensive rats on high-salt diet," *Journal of Cardiovascular Pharmacology*, vol. 36, no. 6, pp. 704–710, 2000.

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*Review Article*

# Roles of Brain Angiotensin II in Cognitive Function and Dementia

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The brain renin-angiotensin system (RAS) has been highlighted as having a pathological role in stroke, dementia, and neurodegenerative disease. Particularly, in dementia, epidemiological studies indicate a preventive effect of RAS blockade on cognitive impairment in Alzheimer disease (AD). Moreover, basic experiments suggest a role of brain angiotensin II in neural injury, neuroinflammation, and cognitive function and that RAS blockade attenuates cognitive impairment in rodent dementia models of AD. Therefore, RAS regulation is expected to have therapeutic potential for AD. Here, we discuss the role of angiotensin II in cognitive impairment and AD. Angiotensin II binds to the type 2 receptor ($AT_2$) and works mainly by binding with the type 1 receptor ($AT_1$). $AT_2$ receptor signaling plays a role in protection against multiple-organ damage. A direct $AT_2$ receptor agonist is now available and is expected to reduce inflammation and oxidative stress and enhance cell differentiation. We and other groups reported that $AT_2$ receptor activation enhances neuronal differentiation and neurite outgrowth in the brain. Here, we also review the effect of the $AT_2$ receptor on cognitive function. RAS modulation may be a new therapeutic option for dementia including AD in the future.

## 1. Introduction

The renin-angiotensin system (RAS) in the brain is well known to be involved in systemic blood pressure control, including the regulation of cerebral blood flow [1]. Angiotensin II, a major player in RAS mainly via the angiotensin type 1 ($AT_1$) receptor, plays an important role in the pathophysiology of tissue dysfunction [2, 3]; therefore, RAS blockade by $AT_1$ receptor blockers (ARBs) and angiotensin converting enzyme inhibitors (ACEIs), which are widely used as antihypertensive drugs, is expected to prevent multiple-organ damage. Cognitive impairment and dementia are common serious health problems that impair quality of life in the elderly. Previous reports indicate the possibility that treatment with antihypertensive agents prevents the impairment of quality of life including cognitive performance [4, 5]. Possible beneficial effects of RAS blockade on cognitive function are also being highlighted in the clinical field [6, 7]. An epidemiological study by Li et al. recently showed that male subjects treated with ARBs exhibited a significant reduction in the incidence and progression of Alzheimer disease (AD) and dementia compared with those treated with ACEIs and other cardiovascular drugs [8]. Moreover, Davies et al. also reported that patients diagnosed with dementia had fewer prescriptions for ARBs and ACEIs. Interestingly, the inverse associations with AD were stronger for ARBs compared with ACEIs [9]. In contrast, Ohrui et al. demonstrated that long-term use of ACEIs may have a protective role against the development of AD, probably through their direct effects on RAS in the brain [10]. In a subanalysis of the Study on Cognition and Prognosis in the Elderly (SCOPE) trial, hypertensives treated with an ARB, candesartan, showed less decline of specific areas of cognitive function such as attention and episodic memory [11]. However, almost all large clinical intervention trials have shown no significant difference in the incidence of dementia between treatment with ARBs or ACEIs and the placebo group. The Ongoing telmisartan alone and in combination with ramipril global endpoint trial (ONTARGET) and the parallel telmisartan randomized assessment study in ACE intolerant subjects with cardiovascular disease (TRANSCEND) trial showed no clear effects on cognitive

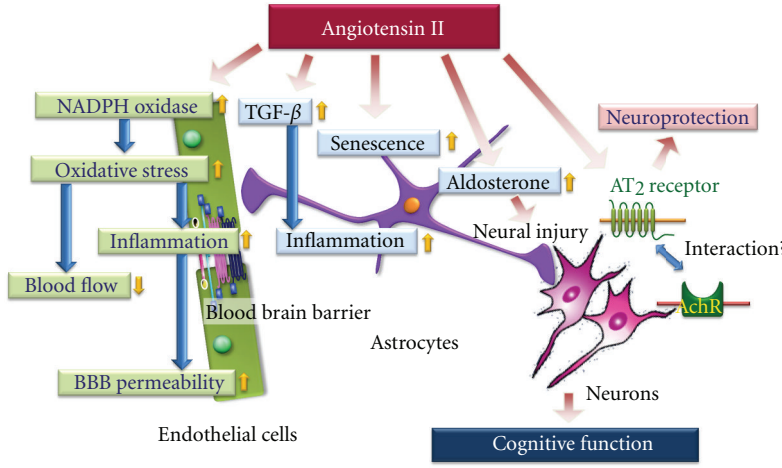


Figure 1: Possible effect of angiotensin II on neurovascular unit. $AT_2$: angiotensin II type 2 receptor, AchR: acetylcholine receptor, BBB: blood brain barrier, and TGF-$\beta$: transforming growth factor $\beta$.

outcomes [12]. The reason why RAS blockade failed to prevent dementia may be the short-term observation for the long-term preclinical disease stage of dementia; however, the detailed explanation is not clear. Another reason is the selection of hypertensive patients, who have high cardiovascular disease morbidity, in these trials. A large number of these patients are likely to go on to develop dementia, most likely with strong vascular underpinning. In these trials, vascular dementia and AD are not well distinguished because most studies focused on dementia as subanalysis. As described in the review by Kehoe and Passmore, RAS has multifunctional involvement not only in vascular dementia but also in AD [13]. Therefore, in such specific groups with cardiovascular risk, the distinction of dementia subtype is very important in comparing the incidence of dementia.

The effect of angiotensin II on cognition has been examined in basic studies. Although the blood-brain barrier is impermeable for all RAS components, the local brain RAS has possible physiological and pharmacological functions in the neuronal system [14]. Gard reviewed the contradictory role of angiotensin II in memory and learning in animal studies [15]. Angiotensin II enhances memory and learning in rodents [16, 17], but other studies suggest that angiotensin II decreases cognition [18]. To assess the paradoxical effect of angiotensin II on cognitive function, we therefore performed cognitive tests in mice with continuous activation of angiotensin II, using transgenic mice carrying both the human renin and angiotensinogen genes (hRN/hANG-Tg) [19]. Interestingly, the avoidance rate in hRN/hANG-Tg mice did not increase from 14 weeks of age; however, that from 8 to 13 weeks of age tended to be higher than that in wild-type mice. These findings suggest that the acute or subacute effect of angiotensin II may enhance cognitive function, but chronic treatment with angiotensin II may exhaust neural function and result in cognitive impairment. Angiotensin II induces cerebrovascular remodeling, promotes vascular inflammation and oxidative stress, and results in impairment of regulation of cerebral blood flow (CBF) [20, 21]. Moreover, endothelial function in cerebral vessels was impaired in a genetic model of angiotensin-II-dependent hypertension [22, 23]. On the other hand, Lanz et al. showed that angiotensin II induced sustained central nervous system (CNS) inflammation via transforming growth factor-(TGF-)$\beta$ in an experimental autoimmune encephalomyelitis (EAE) mouse model [24]. Furthermore, angiotensin II induced astrocyte senescence, which is involved in age-associated neurodegenerative disease via superoxide production [25]. In contrast, a centrally active ACE inhibitor, perindopril, was reported to prevent cognitive impairment in chronic central hypoperfusion rats [26] and Alzheimer disease model mice [27]. These reports indicate that continuous angiotensin II stimulation impairs cognitive function via stimulation of the $AT_1$ receptor with "environmental degradation of neurons" such as a decrease in CBF and an increase in oxidative stress, CNS inflammation, and cellular senescence in the brain. Such multiple stimuli by angiotensin II induce cognitive impairment following neuronal degeneration.

## 2. Effects of Angiotensin II on Amyloid $\beta$ Metabolism and Cholinergic System

There are two major proposed pathomechanisms of AD; the amyloid cascade hypothesis and the cholinergic hypothesis. Amyloid $\beta$ (A$\beta$) is a 39–42 amino acid peptide, produced by cleavage of amyloid precursor protein (APP) [28]. A$\beta$ (1–42) causes the neurodegenerative abnormalities that lead to clinical AD [29]. Although the effect of angiotensin converting enzyme on A$\beta$ metabolism is one of the hot topics in the relation between RAS and AD [30], it seems that angiotensin II does not directly affect A$\beta$ secretion or secretase activity via activation of the $AT_1$ receptor [31]. On the other hand, blockade of RAS may affect A$\beta$ metabolism. For example, an ARB, valsartan, was able to attenuate oligomerization of amyloid $\beta$ peptides into high molecular weight oligomeric peptides [32]. Moreover,

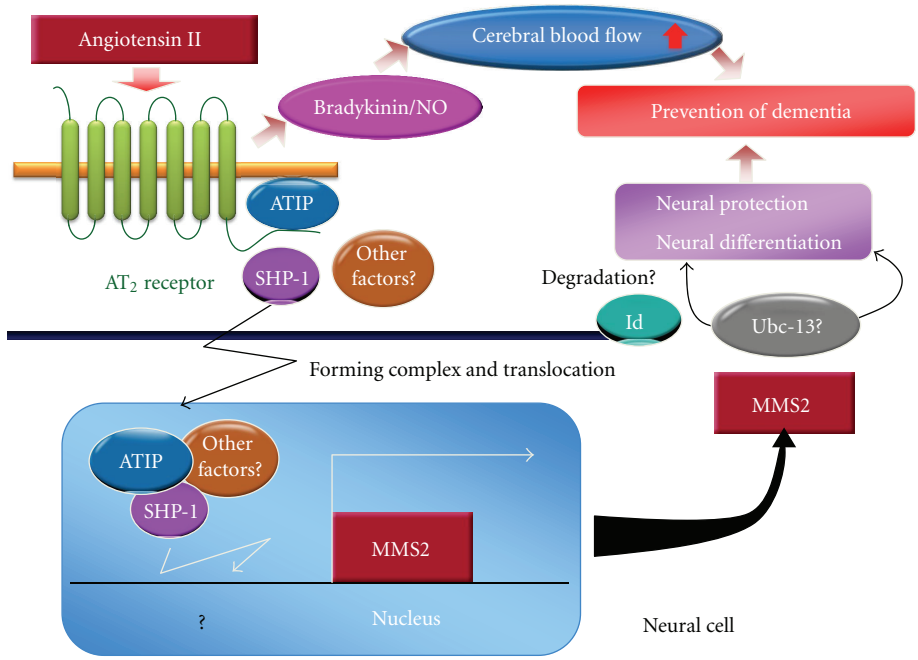


Figure 2: Effect of angiotensin II type 2 receptor signaling on cognitive function. $AT_2$: angiotensin II type 2 receptor, ATIP: $AT_2$ receptor-interacting protein, Id1: inhibitor of DNA binding protein 1, MMS2: methyl methanesulfonate-sensitive 2, NO: nitric oxide, SHP-1: Src homology 2 domain-containing protein-tyrosine phosphatase 1, and Ubc-13: ubiquitin conjugating enzyme 13.

treatment with valsartan also disrupted the development of amyloid $\beta$-mediated cognitive impairment in Tg2576 mice, a model of Alzheimer disease; however, it is reported that this beneficial effect is not observed with treatment with other ARBs. We previously reported that A$\beta$ (1–40) concentration in the brain of ddY mice that underwent intracerebroventricular injection of A$\beta$ (1–40) was significantly decreased by treatment with an ARB, telmisartan [33]. Moreover, Danielyan et al. reported that intranasal administration of losartan exerts direct neuroprotective effects via its A$\beta$-reducing and anti-inflammatory effects in the central nervous system [34]. These results indicate that treatment with ARBs may have a beneficial effect on A$\beta$-induced brain injury through unknown mechanisms on A$\beta$ metabolism by angiotensin II inhibition. On the other hand, brain-penetrating ACEIs such as perindopril prevent cognitive impairment in mice with intracerebroventricular A$\beta$ (1–40) injection via attenuation of oxidative stress and hippocampal astrocyte activation [35]. ACE activity is increased in the hippocampus of these AD mice and suppressed by perindopril treatment. Although there is concern that ACEIs may enhance brain A$\beta$ (1–42) deposition from basic research [36] because ACE converts A$\beta$ (1–42), which plays a causative role in the development of Alzheimer disease, to A$\beta$ (1–40) [37], recent pilot clinical trials showed that ramipril inhibits cerebrospinal fluid (CSF) ACE activity, but did not influence CSF A$\beta$ (1–42) and cognition [38]. The effects of other RAS components involving angiotensin-II-generating enzymes on cognition have also been discussed. A$\beta$ clearance is induced by many kinds of degrading enzyme such as neprilysin (NEP), insulin-degrading enzyme, and endothelin-converting enzyme. Angiotensin II is also generated by degradation of angiotensinogen and angiotensin I by tonins, cathepsins, and chymases as well as ACE. Gene polymorphism in cathepsin G, one of the angiotensin generating enzymes, showed no significant association with AD [39]. In our knowledge, no report has examined the relation between tonin, chymase, and dementia; however, inhibition of angiotensin generating enzymes may also inhibit A$\beta$ degradation. Therefore, it is difficult to assess the effect on A$\beta$ metabolism of drugs that inhibit angiotensin II based on degrading angiotensinogen. Further investigation is necessary to understand the relation among angiotensin II, ACE, other degrading enzymes, and A$\beta$ metabolism.

In the cholinergic hypothesis, AD is also characterized by a loss of neurons, especially those expressing nicotinic acetylcholine receptors (nAChR) [40, 41]. To improve the cognitive deficit in AD, one promising drug target currently under investigation is the neuronal nicotinic alpha7 acetylcholine receptor ($\alpha$7nAChR) [42, 43]. Although there are few reports about the correlation between $\alpha$7nAChR and angiotensin II, Marrero's group has demonstrated that angiotensin II blocks nicotine-mediated neuroprotection against A$\beta$ (1–42) via activation of the tyrosine phosphatase, SHP-1 [44]. They also showed that angiotensin II inhibits $\alpha$7nAChR-induced activation of the JAK2-PI-3 K cascade in PC12 cells through $AT_2$ receptor-induced SHP-1 activation [45]. However, $AT_2$ receptor-induced SHP-1 activation also induces cerebellar development and neural differentiation [46, 47]. Moreover, A$\beta$ triggered $AT_2$ receptor oligomerization in

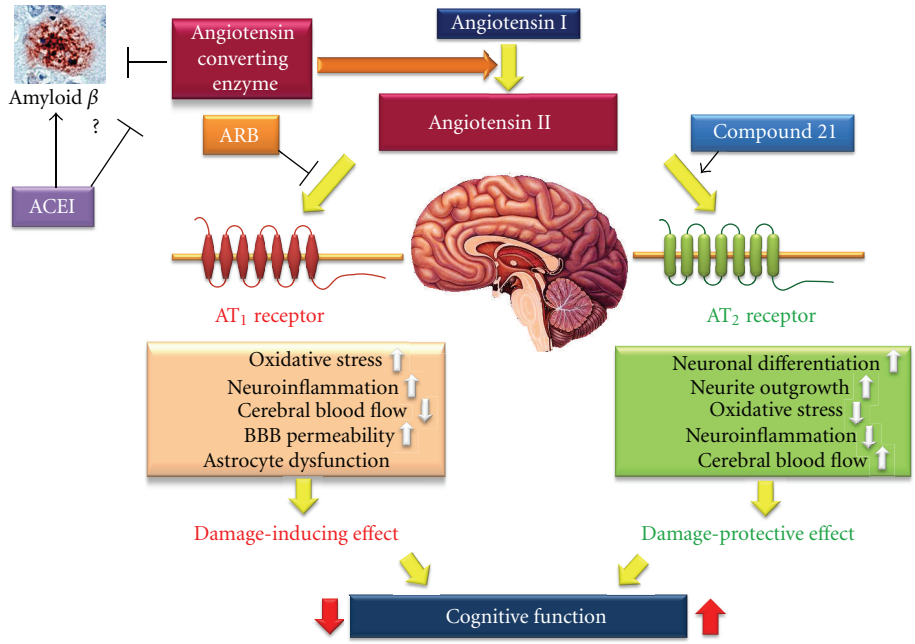


FIGURE 3: Effect of angiotensin II on cognitive function. ACE: angiotensin converting enzyme inhibitor, $AT_1$: angiotensin II type 1 receptor, $AT_2$: angiotensin II type 2 receptor, and ARB: angiotensin II type 1 receptor blocker.

the hippocampus [48] and impaired coupling of the muscarinic acetylcholine receptor (mAChR) to heterotrimeric GTP-binding proteins (G$\alpha$ q/11) [49]. Therefore, the $AT_2$ receptor may interact with the cholinergic system; however, the actual effect of angiotensin II mediated by AChRs is still an enigma (Figure 1).

## 3. Effects of Angiotensin II on Neurovascular Unit

Nonneuronal cells such as vascular cells and glia (astrocytes, microglia and oligodendroglia) comprise the "neurovascular unit" and could play important roles in disease pathogenesis [50]. Especially, CBF functions in concert as a part of the neurovascular unit to maintain homeostasis of the cerebral microenvironment [51]. Iadecola and colleagues demonstrated that angiotensin II increases the production of reactive oxygen species (ROS) in cerebral microvessels via gp91phox (nox-2), a subunit of NADPH oxidase [20, 51]. Moreover, recently they also demonstrated that slow infusion of the pressor angiotensin II induces attenuation of the increase in CBF induced by neural activity (whisker stimulation) and by endothelium-dependent vasodilators, without elevation of mean arterial pressure (MAP) [52]. Such an effect of angiotensin II reduces blood supply and contributes to increased susceptibility to dementia. Interestingly, this angiotensin-induced cerebrovascular dysregulation was attenuated in female compared with male mice [53]. This sexual dimorphism of the cerebral blood-vessel response to angiotensin II may be implicated in the sex difference in cognitive impairment reported in epidemiological studies [54]. On the other hand, Takeda et al. demonstrated that the ARB olmesartan ameliorates amyloid $\beta$-induced impairment of functional hyperemia evoked by whisker stimulation via a decrease in oxidative stress in brain microvessels [55]. Recently, Zhang et al. reported that angiotensin II increases cerebral microvasculature inflammation via induction of oxidative stress and leads to immune-endothelial interaction, resulting in enhancement of BBB permeability [56]. Therefore, angiotensin-II-induced oxidative stress may have a key role in dysfunction of the neurovascular unit (Figure 1).

On the other hand, several reports indicate the effect of angiotensin II on astrocytes to be neuroinflammation, neuronal damage and astrocyte senescence. For example, Lanz et al. clearly demonstrated that angiotensin II acts as a paracrine mediator, sustaining inflammation in the CNS via TGF-$\beta$ upregulation in astrocytes [24]. We also reported that aldosterone secretion induced by angiotensin II in astrocytes enhances neuronal damage due to angiotensin II [57]. Moreover, Liu et al. showed that angiotensin II induces astrocyte senescence via superoxide production [25]. These findings of astrocyte dysfunction induced by angiotensin II also explain the crucial role of angiotensin II in dysfunction of the neurovascular unit (Figure 1).

## 4. Effect of $AT_2$ Receptors on Cognition and Dementia

The major actions of angiotensin II are mediated by the $AT_1$ receptor, whereas the role of a second receptor subtype known as the angiotensin II type 2 ($AT_2$) receptor is suggested to be protecting of the brain [58]. In the brain, $AT_2$ receptors are expressed not only in the vascular wall but also in areas related to learning and control of motor

activity [59, 60]. Mice with deletion of the $AT_2$ receptor were reported to exhibit worse cognitive function compared with wild-type mice [60]. Reinecke et al. demonstrated the possibility that stimulation of the $AT_2$ receptor may promote cell differentiation and regeneration in neuronal tissue [61] and that $AT_2$ receptor stimulation supported neuronal survival and neurite outgrowth in response to ischemia-induced neuronal injury [62]. We also demonstrated that $AT_2$ receptor signaling enhanced neural differentiation and the repair of damaged DNA through induction of a neural differentiating factor, methyl methanesulfonate-sensitive 2 (MMS2), which is one of the ubiquitin conjugating enzyme variants [47]. Moreover, Gallo-Payet et al. reported that angiotensin II induces neural differentiation and neurite outgrowth via mitogen-activated protein kinase [63] or nitric oxide [64] through $AT_2$ receptor activation, and is involved in cerebellar development [65]. Therefore, direct $AT_2$ receptor stimulation is expected to have a beneficial effect on cognitive function. We examined the possibility that direct stimulation of the $AT_2$ receptor by a newly generated direct $AT_2$ receptor agonist, Compound 21 (C21), would enhance cognitive function [66]. Daily intraperitoneal injection of C21 for 2 weeks significantly enhanced spatial learning evaluated by the Morris water maze test in C57BL6 mice, but this effect was not observed in $AT_2$ receptor-deficient mice. C21 treatment increased cerebral blood flow assessed by laser speckle flowmetry and hippocampal field-excitatory postsynaptic potential. Moreover, treatment with C21 prevented cognitive decline in an Alzheimer disease mouse model with intracerebroventricular injection of amyloid $\beta$ (1–40). $AT_2$ receptor activation is reported to stimulate the release of NO/cGMP and may mediate vascular relaxation and blood flow indirectly through modulation of bradykinin release [67]. In our model, C21-induced cognitive enhancement was attenuated by coadministration of icatibant, a bradykinin $B_2$ receptor antagonist. Therefore, direct activation of the $AT_2$ receptor improves spatial learning via an increase in microcirculation, partly through modulation of bradykinin. The preventive effect of $AT_2$ receptor signaling on dementia is summarized in Figure 2. Clinical use of C21 is expected to be a new therapeutic option in patients with dementia.

## 5. Conclusion

Continuous stimulation with angiotensin II may damage neurons via multiple cascades through $AT_1$ receptor stimulation. On the other hand, stimulation of the $AT_2$ receptor is expected to prevent neural damage and cognitive impairment (Figure 3). However, it is difficult to perform clinical intervention studies to confirm the results of animal studies because of the long-term progression of cognitive impairment. Moreover, in clinical practice, it is not possible to exclude the antihypertensive effect of RAS blockade on cognition in patients with hypertension. However, RAS modulation may be a new therapeutic option for dementia including AD in the future. Therefore, the hypothesis that RAS regulation affects future cognitive function should be confirmed with carefully designed clinical studies.

## Abbreviations

| | |
|---|---|
| AD: | Alzheimer disease |
| ARB: | Angiotensin II type 1 ($AT_1$) receptor blocker |
| $AT_1$ receptor: | Angiotensin II type 1 receptor |
| $AT_2$ receptor: | Angiotensin II type 2 receptor |
| ACE: | Angiotensin converting enzyme |
| CBF: | Cerebral surface blood flow |
| hRN/hANG-Tg: | Human renin and angiotensinogen genes |
| RAS: | Renin-angiotensin system |
| si: | Small interfering |
| MMS2: | Methyl methanesulfonate-sensitive 2. |

## Conflict of Interests

The authors declare no conflict of interests.

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## References

[1] M. de Gasparo, K. J. Catt, T. Inagami, J. W. Wright, and T. Unger, "International union of pharmacology. XXIII. The angiotensin II receptors," *Pharmacological Reviews*, vol. 52, no. 3, pp. 415–472, 2000.

[2] R. E. Schmieder, K. F. Hilgers, M. P. Schlaich, and B. M. Schmidt, "Renin-angiotensin system and cardiovascular risk," *The Lancet*, vol. 369, no. 9568, pp. 1208–1219, 2007.

[3] J. Iwanami, M. Mogi, M. Iwai, and M. Horiuchi, "Inhibition of the renin-angiotensin system and target organ protection," *Hypertension Research*, vol. 32, no. 4, pp. 229–237, 2009.

[4] R. Fogari and A. Zoppi, "Effect of antihypertensive agents on quality of life in the elderly," *Drugs and Aging*, vol. 21, no. 6, pp. 377–393, 2004.

[5] A. Fletcher, "Quality of life in the management of hypertension," *Clinical and Experimental Hypertension*, vol. 21, no. 5-6, pp. 961–972, 1999.

[6] J. W. Wright and J. W. Harding, "The brain RAS and Alzheimer's disease," *Experimental Neurology*, vol. 223, no. 2, pp. 326–333, 2010.

[7] M. Mogi and M. Horiuchi, "Effects of angiotensin II receptor blockers on dementia," *Hypertension Research*, vol. 32, no. 9, pp. 738–740, 2009.

[8] N. C. Li, A. Lee, R. A. Whitmer et al., "Use of angiotensin receptor blockers and risk of dementia in a predominantly male population: prospective cohort analysis," *British Medical Journal*, vol. 340, p. b5465, 2010.

[9] N. M. Davies, P. G. Kehoe, Y. Ben-Shlomo, and R. M. Martin, "Associations of anti-hypertensive treatments with Alzheimer's disease, vascular dementia, and other dementias," *Journal of Alzheimer's Disease*, vol. 26, no. 4, pp. 699–708, 2011.

[10] T. Ohrui, T. Matsui, M. Yamaya et al., "Angiotensin-converting enzyme inhibitors and incidence of Alzheimer's disease in Japan [7]," *Journal of the American Geriatrics Society*, vol. 52, no. 4, pp. 649–650, 2004.

[11] B. K. Saxby, F. Harrington, K. A. Wesnes, I. G. McKeith, and G. A. Ford, "Candesartan and cognitive decline in older patients with hypertension: a substudy of the SCOPE trial," *Neurology*, vol. 70, no. 19, pp. 1858–1866, 2008.

[12] C. Anderson, K. Teo, P. Gao et al., "Renin-angiotensin system blockade and cognitive function in patients at high risk of cardiovascular disease: analysis of data from the ONTARGET and TRANSCEND studies," *The Lancet Neurology*, vol. 10, no. 1, pp. 43–53, 2011.

[13] P. G. Kehoe and P. A. Passmore, "The renin-angiotensin system and antihypertensive drugs in Alzheimer's disease: current standing of the angiotensin hypothesis?" *Journal of Alzheimer's Disease*, vol. 30, supplement 2, pp. S251–S268, 2012.

[14] J. W. Wright and J. W. Harding, "Brain renin-angiotensin-A new look at an old system," *Progress in Neurobiology*, vol. 95, no. 1, pp. 49–67, 2011.

[15] P. R. Gard, "The role of angiotensin II in cognition and behaviour," *European Journal of Pharmacology*, vol. 438, no. 1-2, pp. 1–14, 2002.

[16] V. Georgiev and D. Yonkov, "Participation of angiotensin II in learning and memory. I. Interaction of angiotensin II with saralasin," *Methods and Findings in Experimental and Clinical Pharmacology*, vol. 7, no. 8, pp. 415–418, 1985.

[17] A. Kułakowska, W. Karwowska, K. Wiśniewski, and J. J. Braszko, "Losartan influences behavioural effects of angiotensin II in rats," *Pharmacological Research*, vol. 34, no. 3-4, pp. 109–115, 1996.

[18] V. Raghavendra, K. Chopra, and S. K. Kulkarni, "Involvement of cholinergic system in losartan-induced facilitation of spatial and short-term working memory," *Neuropeptides*, vol. 32, no. 5, pp. 417–421, 1998.

[19] S. Inaba, M. Iwai, M. Furuno et al., "Continuous activation of renin-angiotensin system impairs cognitive function in renin/angiotensinogen transgenic mice," *Hypertension*, vol. 53, no. 2, pp. 356–362, 2009.

[20] K. Kazama, J. Anrather, P. Zhou et al., "Angiotensin II impairs neurovascular coupling in neocortex through NADPH oxidase-derived radicals," *Circulation Research*, vol. 95, no. 10, pp. 1019–1026, 2004.

[21] Y. Wei, A. T. Whaley-Connell, K. Chen et al., "NADPH oxidase contributes to vascular inflammation, insulin resistance, and remodeling in the transgenic (mRen2) rat," *Hypertension*, vol. 50, no. 2, pp. 384–391, 2007.

[22] S. P. Didion, C. D. Sigmund, and F. M. Faraci, "Impaired endothelial function in transgenic mice expressing both human renin and human angiotensinogen," *Stroke*, vol. 31, no. 3, pp. 760–765, 2000.

[23] F. M. Faraci, K. G. Lamping, M. L. Modrick, M. J. Ryan, C. D. Sigmund, and S. P. Didion, "Cerebral vascular effects of angiotensin II: new insights from genetic models," *Journal of Cerebral Blood Flow and Metabolism*, vol. 26, no. 4, pp. 449–455, 2006.

[24] T. V. Lanz, Z. Ding, P. P. Ho et al., "Angiotensin II sustains brain inflammation in mice via TGF-$\beta$," *Journal of Clinical Investigation*, vol. 120, no. 8, pp. 2782–2794, 2010.

[25] G. Liu, N. Hosomi, H. Hitomi et al., "Angiotensin II induces human astrocyte senescence through reactive oxygen species production," *Hypertension Research*, vol. 34, no. 4, pp. 479–483, 2011.

[26] K. Yamada, T. Horita, M. Takayama et al., "Effect of a centrally active angiotensin converting enzyme inhibitor, perindopril, on cognitive performance in chronic cerebral hypo-perfusion rats," *Brain Research*, vol. 1421, no. 1, pp. 10–120, 2011.

[27] K. Yamada, S. Uchida, S. Takahashi et al., "Effect of a centrally active angiotensin-converting enzyme inhibitor, perindopril, on cognitive performance in a mouse model of Alzheimer's disease," *Brain Research C*, vol. 1352, pp. 176–186, 2010.

[28] W. E. Van Nostrand, J. Davis-Salinas, and S. M. Saporito-Irwin, "Amyloid $\beta$-protein induces the cerebrovascular cellular pathology of Alzheimer's disease and related disorders," *Annals of the New York Academy of Sciences*, vol. 777, pp. 297–302, 1996.

[29] J. Hardy and D. J. Selkoe, "The amyloid hypothesis of Alzheimer's disease: progress and problems on the road to therapeutics," *Science*, vol. 297, no. 5580, pp. 353–356, 2002.

[30] K. Zou and M. Michikawa, "Angiotensin-converting enzyme as a potential target for treatment of Alzheimer's disease: inhibition or activation?" *Reviews in the Neurosciences*, vol. 19, no. 4-5, pp. 203–212, 2008.

[31] B. R. Wang, J. Q. Shi, Y. D. Zhang, D. L. Zhu, and J. P. Shi, "Angiotensin II does not directly affect A$\beta$ secretion or $\beta$-/$\gamma$-secretase activity via activation of angiotensin II type 1 receptor," *Neuroscience Letters*, vol. 500, no. 2, pp. 103–107, 2011.

[32] J. Wang, L. Ho, L. Chen et al., "Valsartan lowers brain $\beta$-amyloid protein levels and improves spatial learning in a mouse model of Alzheimer disease," *Journal of Clinical Investigation*, vol. 117, no. 11, pp. 3393–3402, 2007.

[33] K. Tsukuda, M. Mogi, J. Iwanami et al., "Cognitive deficit in amyloid-$\beta$-injected mice was improved by pretreatment with a low dose of telmisartan partly because of peroxisome proliferator-activated receptor-$\gamma$ activation," *Hypertension*, vol. 54, no. 4, pp. 782–787, 2009.

[34] L. Danielyan, R. Klein, L. R. Hanson et al., "Protective effects of intranasal losartan in the APP/PS1 transgenic mouse model of Alzheimer disease," *Rejuvenation Research*, vol. 13, no. 2-3, pp. 195–201, 2010.

[35] Y. F. Dong, K. Kataoka, Y. Tokutomi et al., "Perindopril, a centrally active angiotensin-converting enzyme inhibitor, prevents cognitive impairment in mouse models of Alzheimer's disease," *FASEB Journal*, vol. 25, no. 9, pp. 2911–2920, 2011.

[36] M. L. Hemming and D. J. Selkoe, "Amyloid $\beta$-protein is degraded by cellular angiotensin-converting enzyme (ACE) and elevated by an ACE inhibitor," *Journal of Biological Chemistry*, vol. 280, no. 45, pp. 37644–37650, 2005.

[37] K. Zou, T. Maeda, A. Watanabe et al., "A$\beta$42-to-A$\beta$40- and angiotensin-converting activities in different domains of angiotensin-converting enzyme," *Journal of Biological Chemistry*, vol. 284, no. 46, pp. 31914–31920, 2009.

[38] W. Wharton, J. H. Stein, C. Korcarz et al., "The effects of ramipril in individuals at risk for Alzheimer's disease: results of a pilot clinical trial," *Journal of Alzheimer's Disease*, vol. 32, no. 1, pp. 147–156, 2012.

[39] T. J. Bhojak, S. T. DeKosky, M. Ganguli, and M. I. Kamboh, "Genetic polymorphism in the cathepsin G gene and the risk of Alzheimer's disease," *Neuroscience Letters*, vol. 309, no. 2, pp. 138–140, 2001.

[40] A. Kadir, O. Almkvist, A. Wall, B. Långström, and A. Nordberg, "PET imaging of cortical 11C-nicotine binding correlates with the cognitive function of attention in Alzheimer's disease," *Psychopharmacology*, vol. 188, no. 4, pp. 509–520, 2006.

[41] S. D. Buckingham, A. K. Jones, L. A. Brown, and D. B. Sattelle, "Nicotinic acetylcholine receptor signalling: roles in alzheimer's disease and amyloid neuroprotection," *Pharmacological Reviews*, vol. 61, no. 1, pp. 39–61, 2009.

[42] H. Y. Wang, D. H. S. Lee, M. R. D'Andrea, P. A. Peterson, R. P. Shank, and A. B. Reitz, "$\beta$-Amyloid1-42 binds to $\alpha$7 nicotinic acetylcholine receptor with high affinity. Implications for Alzheimer's disease pathology," *Journal of Biological Chemistry*, vol. 275, no. 8, pp. 5626–5632, 2000.

[43] H. R. Parri, C. M. Hernandez, and K. T. Dineley, "Research update: alpha7 nicotinic acetylcholine receptor mechanisms in Alzheimer's disease," *Biochemical Pharmacology*, vol. 82, no. 8, pp. 931–942, 2011.

[44] S. Shaw, M. Bencherif, and M. B. Marrero, "Angiotensin II blocks nicotine-mediated neuroprotection against $\beta$-amyloid (1–42) via activation of the tyrosine phosphatase SHP-1," *Journal of Neuroscience*, vol. 23, no. 35, pp. 11224–11228, 2003.

[45] M. B. Marrero and M. Bencherif, "Convergence of alpha 7 nicotinic acetylcholine receptor-activated pathways for anti-apoptosis and anti-inflammation: central role for JAK2 activation of STAT3 and NF-$\kappa$B," *Brain Research C*, vol. 1256, pp. 1–7, 2009.

[46] L. R. Seguin, R. S. Villarreal, and G. M. Ciuffo, "AT2 receptors recruit c-Src, SHP-1 and FAK upon activation by ang II in PND15 rat hindbrain," *Neurochemistry International*, vol. 60, no. 2, pp. 199–207, 2012.

[47] J. M. Li, M. Mogi, K. Tsukuda et al., "Angiotensin II-induced neural differentiation via angiotensin II type 2 (AT2) receptor-MMS2 cascade involving interaction between AT 2 receptor-interacting protein and Src homology 2 domain-containing protein-tyrosine phosphatase 1," *Molecular Endocrinology*, vol. 21, no. 2, pp. 499–511, 2007.

[48] S. AbdAlla, H. Lother, A. El Missiry et al., "Angiotensin II AT2 receptor oligomers mediate G-protein dysfunction in an animal model of Alzheimer disease," *Journal of Biological Chemistry*, vol. 284, no. 10, pp. 6554–6565, 2009.

[49] P. J. Tienari, B. De Strooper, E. Ikonen et al., "The $\beta$-amyloid domain is essential for axonal sorting of amyloid precursor protein," *EMBO Journal*, vol. 15, no. 19, pp. 5218–5229, 1996.

[50] C. Iadecola, "The overlap between neurodegenerative and vascular factors in the pathogenesis of dementia," *Acta Neuropathologica*, vol. 120, no. 3, pp. 287–296, 2010.

[51] H. Girouard, L. Park, J. Anrather, P. Zhou, and C. Iadecola, "Angiotensin II attenuates endothelium-dependent responses in the cerebral microcirculation through nox-2-derived radicals," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 26, no. 4, pp. 826–832, 2006.

[52] C. Capone, G. Faraco, L. Park, X. Cao, R. L. Davisson, and C. Iadecola, "The cerebrovascular dysfunction induced by slow pressor doses of angiotensin II precedes the development of hypertension," *American Journal of Physiology-Heart and Circulatory Physiology*, vol. 300, no. 1, pp. H397–H407, 2011.

[53] H. Girouard, A. Lessard, C. Capone, T. A. Milner, and C. Iadecola, "The neurovascular dysfunction induced by angiotensin II in the mouse neocortex is sexually dimorphic," *American Journal of Physiology-Heart and Circulatory Physiology*, vol. 294, no. 1, pp. H156–H163, 2008.

[54] G. A. Laughlin, L. K. McEvoy, D. von Muhlen et al., "Sex differences in the association of Framingham cardiac risk score with cognitive decline in community-dwelling elders without clinical heart disease," *Psychosomatic Medicine*, vol. 73, no. 8, pp. 683–689, 2011.

[55] S. Takeda, N. Sato, D. Takeuchi et al., "Angiotensin receptor blocker prevented $\beta$-amyloid-induced cognitive impairment associated with recovery of neurovascular coupling," *Hypertension*, vol. 54, no. 6, pp. 1345–1352, 2009.

[56] M. Zhang, Y. Mao, S. H. Ramirez, R. F. Tuma, and T. Chabrashvili, "Angiotensin II induced cerebral microvascular inflammation and increased blood-brain barrier permeability via oxidative stress," *Neuroscience*, vol. 171, no. 3, pp. 852–858, 2010.

[57] L. J. Min, M. Mogi, J. Iwanami et al., "Angiotensin II and aldosterone-induced neuronal damage in neurons through an astrocyte-dependent mechanism," *Hypertension Research*, vol. 34, no. 6, pp. 773–778, 2011.

[58] M. Horiuchi, M. Mogi, and M. Iwai, "The angiotensin II type 2 receptor in the brain," *Journal of the Renin-Angiotensin-Aldosterone System*, vol. 11, no. 1, pp. 1–6, 2010.

[59] M. Iwai, H. W. Liu, R. Chen et al., "Possible inhibition of focal cerebral ischemia by angiotensin II type 2 receptor stimulation," *Circulation*, vol. 110, no. 7, pp. 843–848, 2004.

[60] M. Mogi, J. M. Li, J. Iwanami et al., "Angiotensin II type-2 receptor stimulation prevents neural damage by transcriptional activation of methyl methanesulfonate sensitive 2," *Hypertension*, vol. 48, no. 1, pp. 141–148, 2006.

[61] K. Reinecke, R. Lucius, A. Reinecke, U. Rickert, T. Herdegen, and T. Unger, "Angiotensin II accelerates functional recovery in the rat sciatic nerve in vivo: role of the AT2 receptor and the transcription factor NF-kappaB," *FASEB Journal*, vol. 17, no. 14, pp. 2094–2096, 2003.

[62] J. Li, J. Culman, H. Hörtnagl et al., "Angiotensin AT2 receptor protects against cerebral ischemia-induced neuronal injury," *FASEB Journal*, vol. 19, no. 6, pp. 617–619, 2005.

[63] L. Gendron, L. Laflamme, N. Rivard, C. Asselin, M. D. Payet, and N. Gallo-Payet, "Signals from the AT2 (angiotensin type 2) receptor of angiotensin II inhibit p21(ras) and activate MAPK (mitogen-activated protein kinase) to induce morphological neuronal differentiation in NG108-15 cells," *Molecular Endocrinology*, vol. 13, no. 9, pp. 1615–1626, 1999.

[64] F. Cote, L. Laflamme, M. D. Payet, and N. Gallo-Payet, "Nitric oxide, a new second messenger involved in the action of angiotensin II on neuronal differentiation of NG108-15 cells," *Endocrine Research*, vol. 24, no. 3-4, pp. 403–407, 1998.

[65] F. Côté, T. H. Do, L. Laflamme, J. M. Gallo, and N. Gallo-Payet, "Activation of the AT2 receptor of angiotensin II induces neurite outgrowth and cell migration in microexplant cultures of the cerebellum," *Journal of Biological Chemistry*, vol. 274, no. 44, pp. 31686–31692, 1999.

[66] F. Jing, M. Mogi, A. Sakata et al., "Direct stimulation of angiotensin II type 2 receptor enhances spatial memory," *Journal of Cerebral Blood Flow and Metabolism*, vol. 32, no. 2, pp. 248–255, 2011.

[67] O. Jöhren, A. Dendorfer, and P. Dominiak, "Cardiovascular and renal function of angiotensin II type-2 receptors," *Cardiovascular Research*, vol. 62, no. 3, pp. 460–467, 2004.

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*Review Article*

# Discovery of Inhibitors of Insulin-Regulated Aminopeptidase as Cognitive Enhancers

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The hexapeptide angiotensin IV (Ang IV) is a metabolite of angiotensin II (Ang II) and plays a central role in the brain. It was reported more than two decades ago that intracerebroventricular injection of Ang IV improved memory and learning in the rat. Several hypotheses have been put forward to explain the positive effects of Ang IV and related analogues on cognition. It has been proposed that the insulin-regulated aminopeptidase (IRAP) is the main target of Ang IV. This paper discusses progress in the discovery of inhibitors of IRAP as potential enhancers of cognitive functions. Very potent inhibitors of the protease have been synthesised, but pharmacokinetic issues (including problems associated with crossing the blood-brain barrier) remain to be solved. The paper also briefly presents an overview of the status in the discovery of inhibitors of ACE and renin, and of AT1R antagonists and AT2R agonists, in order to enable other discovery processes within the RAS system to be compared. The paper focuses on the relationship between binding affinities/inhibition capacity and the structures of the ligands that interact with the target proteins.

## 1. Introduction

Neuropeptides participate in the transmission or modulation of signals in the central nervous system (CNS) [1]. Hence, these peptides are engaged in neurological functions that include those related to cognition and memory, mood, the experience of pain, stress, reaction to reward, control of the intake of food, and neuroendocrinological regulation. The physiological action of neuropeptides is terminated by proteolytic degradation, and this is most often mediated by extracellular proteases anchored in the cell membrane. In this respect, neuropeptides differ from classic transmitters. Limited hydrolysis of neuroactive peptides may lead to the fragments being formed with either similar or very different biological activities [2]. The conversion of angiotensin II (Ang II) to angiotensin IV (Ang IV) is a good example of the latter. This type of biotransformation results from the action of more or less specific endoproteases. Several proteases that are capable of releasing bioactive fragments from their substrates have been identified in various CNS tissues [3, 4].

We discuss in this paper the renin-angiotensin system (RAS) and describe briefly how the two proteases, the angiotensin converting enzyme (ACE) and renin, have served and continue to serve as drug targets. We discuss briefly the two major receptors of the parent peptide angiotensin II, AT1R and AT2R, and we describe related antagonists and agonists to these receptors. Finally, we direct our focus to the hexapeptide Ang IV, which plays a central role in the brain. It has been suggested that the insulin-regulated aminopeptidase (IRAP) is the major target for Ang IV in the brain, and we therefore discuss in more detail recent progress in the discovery of inhibitors of IRAP. This paper concentrates on the molecular structures of the ligands that interact with the target proteins.

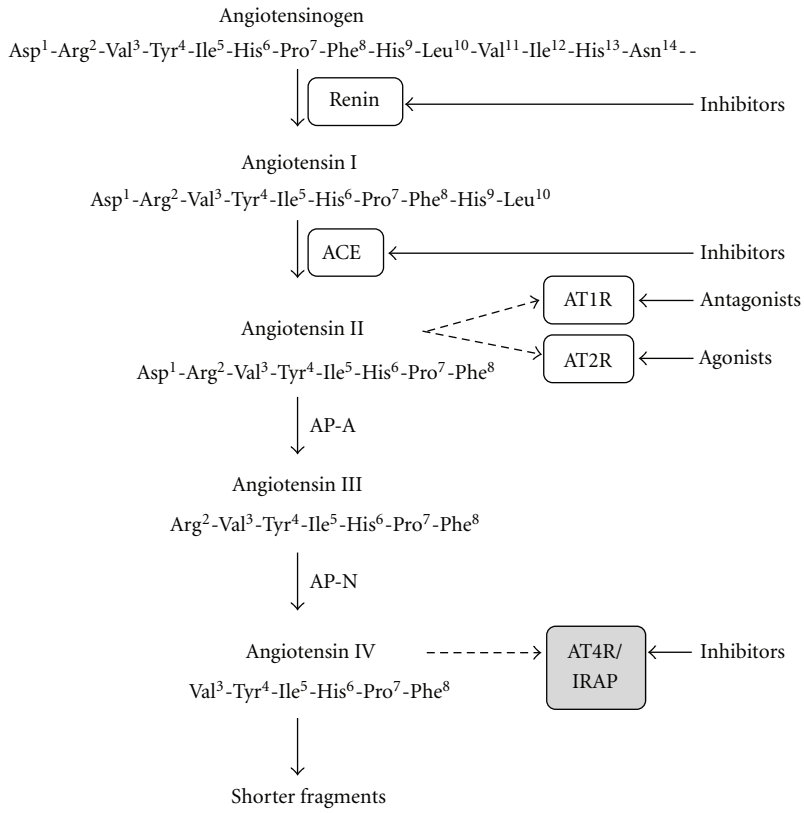



Figure 1: A part of the renin-angiotensin system (RAS), including selected degradation products and drug targets.

## 2. Proteolytic Processing

Angiotensin II (Ang II) is formed from angiotensin I (Ang I), which is an essentially inactive peptide derived from circulating and tissue angiotensinogen (Figure 1). The aspartyl protease renin liberates Ang I from angiotensinogen. The proteolytic cleavage of angiotensin I to produce Ang II is mediated mainly by the metalloproteinase ACE, an established target for drug therapy. Enzymatic cleavage by chymase, carboxypeptidase, catepsin G or tonin are alternative routes by which Ang II can be produced [5]. As in the cases of the tachykinins and the opioid peptides, metabolism of Ang II results in the formation of several fragments with biological activities that differ from those of the parent peptides. Proteolytic cleavage by glutamyl aminopeptidase A (AP-A) and membrane alanyl aminopeptidase N (AP-N), for example, results in the sequential removal of single amino acid residues from the N-terminal end of the peptide, to form Ang III (Ang II(2–8)) and Ang IV (Ang II(3–8)), respectively [6]. These peptides are important neuropeptide fragments in the CNS [7–10]. Ang IV plays a particularly important role, and its mechanism of action is distinct [11–14]. It is noteworthy that Ang IV can be formed by the action of aminopeptidases on Ang I before it is converted to Ang II [15]. A previously unknown human Ang II-related peptide, denoted Ang A, has recently been discovered [16]. This peptide, ($Ala^1$)-Ang II, is not a product of proteolysis but is derived from decarboxylation of the aspartic acid residue of Ang II [16]. It acts as a full agonist with properties that are similar to those of Ang II [17].

Chymotrypsin and dipeptidyl carboxypeptidase can further process Ang IV and the fragment Ang (3–7) to form inactive fragments and amino acid residues [18–23]. Ang (3–7) is formed from Ang IV by carboxypeptidase P (Carb-P) and propyl oligopeptidase (PO) cleavage. Chymotrypsin can hydrolyse bonds to Val, Tyr, and Ile, and this is an important property to consider when designing metabolically stable Ang IV analogues and Ang IV peptide mimetics. Furthermore, Ang II can be converted to the bioactive Ang (1–7) by the proteolytic removal of the C-terminal phenylalanine by Carb-P [12], the action of the monopeptidase ACE2 [24], and by ACE cleavage of the Phe-His from Ang (1–9) [25].

Figure 1 shows selected degradation products and five major drug targets. There are several other potential targets for drugs in the RAS, such as the Ang (1–7)/Mas receptor [26, 27] and the aminopeptidase A (AP-A), but these will not be discussed here (for a recent paper, see [28]). Neither will antagonists to AT2R as potential future drugs be discussed. This review will focus on the discovery of IRAP inhibitors.

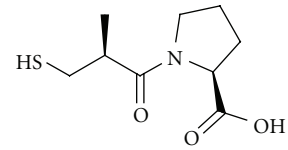
## 3. Inhibitors of Angiotensin Converting Enzyme and Renin

Angiotensin II (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe) is an important modulator of cardiovascular function and exerts a pronounced hypertensive effect. Forty years ago, it was

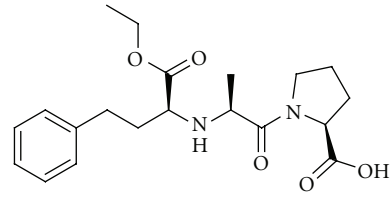
discovered that minor modifications of the amino acid residue sequence of Ang II, such as the replacement of the phenylalanine residue at the C-terminal by a residue with an aliphatic side chain, created peptides that block the action of Ang II. Two such peptides, saralasin ((Sar[1], Ala[8])-Ang II) and sarile ((Sar[1], Ile[8])-Ang II), in which the N-terminal sarcosine residue enhances the effect, were evaluated in clinical trials [29, 30]. However, due to the peptidic character neither saralasin, approved by FDA for limited applications, nor the more potent sarile found long term use in clinic [31–33]. They did, however, become important research tools and clinical observations from treatment with these two peptides confirmed that the RAS is a very relevant target for drug intervention. Hence, the two major proteases, renin and ACE, that are responsible for the degradation of the precursor protein angiotensinogen to the effector peptide Ang II, became attractive drug targets (Figure 1).

ACE inhibitors were subsequently disclosed [34, 35]. The design of the first ACE inhibitor took advantage of the similarity of ACE to the metalloproteinase carboxypeptidase A, which is inhibited by 2-benzyl succinic acid. It was also known that isolated peptides from extracts of venom from the Brazilian pit viper *Bothrops jararaca* have an antihypertensive effect, inhibiting the conversion of Ang I to Ang II. These two insights allowed the elegant and very rapid discovery of the zinc-binding thiol compound captopril, which entered the market in 1978. The nanopeptide (Pyr-Trp-Pro-Arg-Pro-Gln-Ile-Pro-Pro), known as "teprotide," had the highest *in vivo* potency among the venom peptides. It contains a proline residue at the C-terminal that is retained in captopril (Figure 2) [36]. Attempts to improve metabolic stability and to minimize the principal side effects of captopril, rashes and loss of taste, that it was hypothesized that the thiol group was responsible for, led to the once daily prodrug enalapril (Figure 2) that entered the market in 1985 [37, 38]. In enalapril, the thiol group had been replaced by a zinc-coordinating carboxyl group. The high-resolution X-ray crystal structure of enalapril (after liberation of the free carboxyl group from the prodrug) is now available [39] and shows that the N-terminal carboxyl group interacts with the catalytic zinc ion at the active site of ACE. The 3D structure of the membrane-bound ACE, however, was not known when the first ACE inhibitors were designed. Subsequently there was a large number of ACE inhibitors introduced, for example, ramipril, quinapril, perindopril, lisinopril, and benazepril, that today are extensively used in clinic [40].

Attempts to inhibit the other major protease involved in the proteolytic processing, the aspartyl protease renin, continued for decades, but it was not until 2007 that the first such inhibitor, aliskiren (Figure 3), reached the market [41–43]. The development of renin inhibitors was often hampered by the peptidic character of the potential inhibitors being studied, leading as it did to limited metabolic stability, poor absorption, and, as a consequence of this, low oral bioavailability. It was, furthermore, difficult to predict effects in humans from results obtained in animal models. The compounds were transition-state analogues, in which the peptide bond to be cleaved had been substituted by a group that mimicked the transition state. The design of the

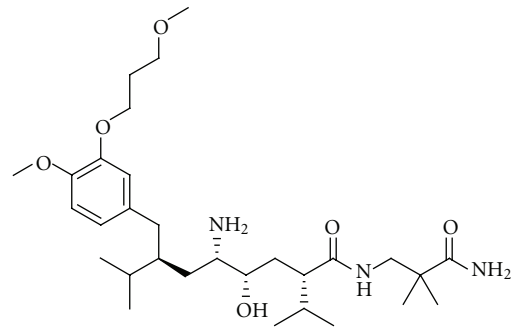



Captopril




Enalapril

Figure 2: Captopril and enalapril, inhibitors of the angiotensin converting enzyme (ACE).




Aliskiren

Figure 3: The structure of the renin inhibitor aliskiren.

transition-state analogue aliskiren was aided by modelling and the availability of X-ray structures [44]. Experience gained during the renin inhibitor programmes benefited greatly programmes to discover HIV protease inhibitors, which were, in fact, on the market ten years before aliskiren. Significant progress has recently been made in identifying new potent non-peptide "direct renin inhibitors", but no such inhibitors have progressed to late stage development.

## 4. Angiotensin II AT1 Receptor Antagonists and AT2 Receptor Agonists

The first angiotensin receptor blocker (ARB) to be used in the clinic, the peptide saralasin, was not orally active and its duration of action was very short. Clinical results obtained with saralasin, however, demonstrated clearly that the Ang II receptor was a suitable target for drugs. The first non-peptide ARB, losartan (Figure 4), was introduced onto the market in 1995. Losartan is characterized by the tetrazole moiety, which serves as a carboxylate bioisostere, at the biphenyl unit and by the readily oxidized hydroxymethyl group. Losartan

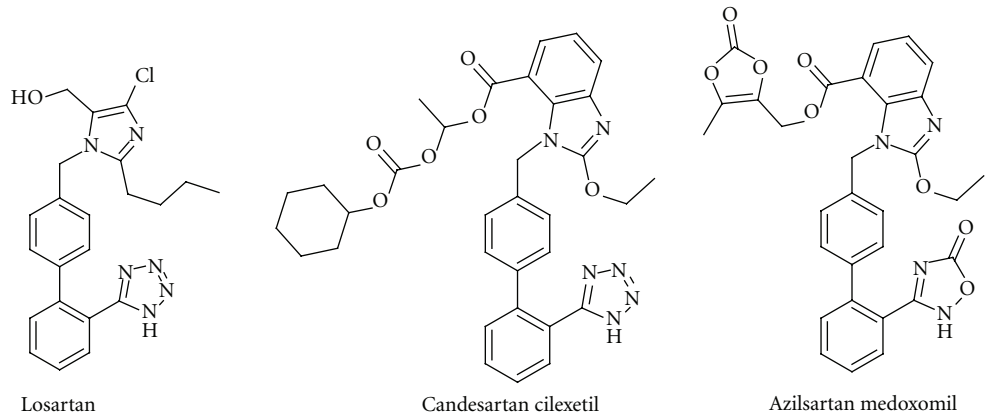
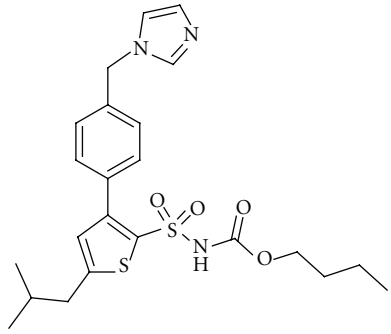



Figure 4: Three examples of Ang II AT1 receptor blockers (ARBs).

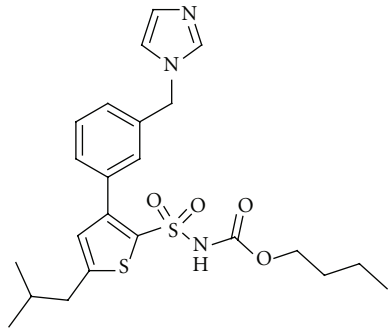
is converted *in vivo* by oxidation of the hydroxymethyl group to the more potent carboxylic acid metabolite. For a review on the discovery of angiotensin receptor blockers, see [45]. Several other AT1R antagonists are now in clinical use, for example, eprosartan, olmesartan, telmisartan, valsartan, and candesartan. The latter is administered as a prodrug, candesartan cilexetil (Figure 4). This liberates candesartan, which binds strongly to the AT1 receptor [46, 47]. It is believed that an optimal clinical effect of these drugs, known collectively as "sartans," requires high levels of target occupancy [48, 49]. It should be mentioned in this context that an increasing number of examples in the literature suggest that the *in vivo* duration of drug action of AT1R blockers, for example, depends not only on macroscopic pharmacokinetic properties such as plasma half-life and the time needed to equilibrate between the plasma and the effect compartments, but also on long-lasting target binding and rebinding [50–52]. The prodrug azilsartan medoxomil (Figure 4), which has a less common carboxylic acid bioisostere attached to the biphenyl scaffold, was approved in 2011 and is the latest member of the ARB group [53–55]. It is well established that AT1R antagonists are at least as effective as ACE inhibitors, $\beta$-blockers, and calcium channel antagonists in reducing cardiovascular morbidity and mortality [56, 57]. Furthermore, it is reported that losartan [58] and candesartan [59–61] have positive effects on cognition in elderly patients. Interestingly, TRV120023 representing a $\beta$-arrestin-biased AT1R ligand has cardioprotective and functional properties *in vivo* which are distinct from losartan. It has been suggest that this novel class of drugs that is G protein independent but $\beta$-arrestin selective may provide an advantage over conventional ARBs by supporting cardiac function and reducing cellular injury during acute cardiac injury [62, 63]. Anyhow, efforts to develop new and better chemical entities that block the AT1 receptor are limited. There is strong competition in this research field, which may partly explain this decision, but efforts to develop single compounds that can block both the AT1R and the receptor of the very potent vasoconstrictor endothelin A are in progress [64]. Furthermore, dual antagonism of AT1R and neutral endopeptidase inhibition has recently attracted interest. Clinical results from the dual inhibitor LCZ696, now in phases II-III, are promising [65].

Recently, the AT2 receptor has emerged as a new target for drug therapy [66–68]. The AT2 receptor is abundant in fetal tissues but in adults this G protein-coupled receptor remains abundant only in certain tissues such as vascular endothelium [69] and brain areas [70, 71]. The AT2 receptor is present in higher density in distinct regions of the brain and it has been suggested that it is involved in growth development and exploratory behaviour [72, 73]. It is expressed in the locus coeruleus, ventral and dorsal parts of lateral septum, the superior colliculus, and subthalamic nucleus, in the nuclei of many cells of the thalamus, and in nuclei in cells of the inferior olive. Both the AT1 and AT2 receptors are expressed in the cingulate cortex, the molecular layer of the cerebellar cortex, the superior colliculus and paraventricular nuclei [70, 74–76]. AT2R RNA and the receptor protein have recently been identified in the substantia nigra pars compacta [77] and in the hippocampus [78, 79]. Thus, the receptor is present in the adult in areas associated with control and learning of motor activity, sensory areas, and selected structures of the limbic system [80]. It has been suggested that modulation of AT2R signalling can improve cognitive performance in persons with Alzheimer's disease (AD) not only through the action of an AT2R agonist on blood flow/brain microcirculation but also through its more specific effects on neurons [81]. Activation of the AT2 receptor affects neuronal cell differentiation and nerve regeneration [82–84]. Interestingly, several peripheral effects that are mediated through the AT2 receptor oppose other effects that are mediated through the AT1 receptor, suggesting that a similar balance may exist in the CNS [85, 86]. It is worth noting that the AT2 receptor is re-expressed in some disease conditions such as heart failure, renal failure, myocardial infarction, hypertension, and some brain disorders [71, 87–92]. The AT2R mediates vasodilatory, antiproliferative and anti-inflammatory effects [93].

Several potent drug-like and selective AT2R agonists have been disclosed [94–96]. The first of these receptor-selective agonists, M024 or "compound 21" (M024/C21, Figure 5),



M024/C21



M132/C38

Figure 5: The structures of the selective AT2 receptor agonist M024/C21 and the structurally similar M132/C38, which acts as an AT2 receptor antagonist.

was first reported in 2004, and has been extensively studied since then [94]. It possesses a sulfonyl carbamate entity as a metabolically stable carboxylic acid isostere and exhibits a striking structural similarity to several AT1R antagonists. Compound M024/C21, developed through a series of modifications to the non-selective AT1R agonist L-162,313 [97], stimulates neurite outgrowth in neuronal cells (which express only AT2R) [94] through the sustained activation of p42/p44 mapk. It decreases dopamine synthesis in the rat striatum, and some results suggest that the AT1 and AT2 receptors in the striatum exert opposite effects on dopamine synthesis, rather than dopamine release [98]. The compound enhances cognitive functions in mice [81, 99]. It improves myocardial function independently of blood pressure after myocardial infarction in normotensive Wistar rats [100] and has a pronounced anti-inflammatory effect [101]. M024/C21 gives improved vascular stiffness and lower collagen concentrations in the aorta and myocardium of stroke-prone spontaneously hypertensive rats [102]. It was recently suggested that the combination of M024/C21 with antihypertensive treatment might lead to vasculoprotective effects even beyond the blood-pressure-reducing effect [66, 103].

It is expected that bioavailability in the brain of the drug-like M024/C21 will be low [104]. This is expected to be the case also for M132/C38 (Figure 5), a selective AT2R antagonist with a very similar structure as M024/C21. Nevertheless, this AT2R agonist/antagonist pair, in which the two compounds possess similar pharmacokinetics, should be an important and useful tool in studying the RAS. Furthermore, these molecules may serve as a starting point in medicinal chemistry programmes aimed at discovering molecules that are active as AT2R agonists in the brain after oral administration. No selective AT2R agonists have still entered phase I clinical trial. On the contrary, EMA401, a lipophilic structural analogue of the commonly used research tool, the AT2 receptor antagonist PD123319 is in clinical trials. This antagonist is developed for neuropathic pain [105].

## 5. Inhibitors of Insulin-Regulated Aminopeptidase

In 1988, Braszko et al. reported that intracerebroventricular (i.c.v.) injection of the Ang II metabolite Ang IV (Val-Tyr-Ile-His-Pro-Phe, Figure 6) improved memory and learning in the rat [106]. They showed that Ang IV affects motor activity, the performance of passive avoidance, and a conditioned avoidance response. Various animal models were subsequently investigated, and results were obtained for Barnes maze, swim mazes, and radial arm mazes [8, 14, 107–110]. Not only Ang IV but also related analogues were studied, such as the endogenous LVV-hemorphin-7 (Leu-Val-Val-Tyr-Pro-Trp-Thr-Glu-Arg-Phe), which has structural similarities to Ang IV at the N-terminal part of the peptide, in that it has a tyrosine residue attached to lipophilic amino acid residues [111]. LVV-hemorphin-7 is a powerful promoter of memory retention and retrieval in rats [107]. The observation that Ang IV improves processes related to memory and learning has attracted considerable interest in recent years. Excellent reviews have been published describing the role of Ang IV in the brain [112–114]. Efficient new chemical entities for the treatment of the cognitive decline associated with Alzheimer's disease, brain trauma, and cerebral ischemia are needed since the clinical studies of the cholinesterase inhibitors and NMDA antagonists used today have been mostly disappointing [115–118]. Thus, new improved enhancers of cognitive functions are desired and the receptor(s) involved in the beneficial effects of Ang IV has emerged as a relevant new target for drug intervention.

A specific binding site for Ang IV was identified in 1992, and was later named the AT4 receptor [119–121]. Harding et al. found high densities of binding sites in areas of the brain associated with cognitive, sensory and motor functions, including the hippocampus [120].

*5.1. Ligands with Affinity to the AT4 Receptor.* Shortly after the discovery of the binding sites, systematic structure-activity relationship studies (SAR) were commenced by Wright and Harding and it became clear that the Val-Tyr-Ile tripeptide motif in the N-terminal part of Ang IV was of critical importance for binding affinity [122]. This conclusion relied on classic glycine and d-amino acid scans and various other alterations of the amino acid residues of

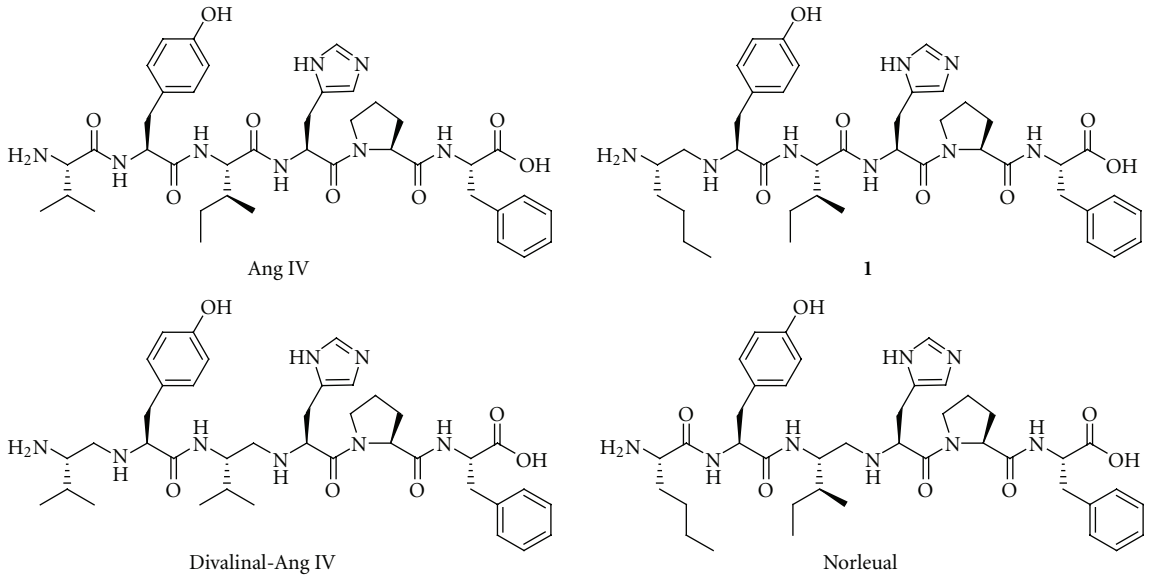


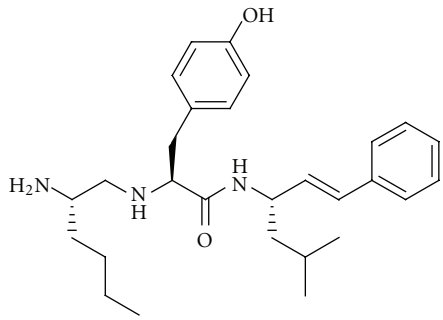

Figure 6: Ang IV and three hexapeptide analogues that incorporate one or two reduced peptide bonds ($\Psi[CH_2NH]$) as peptide bond bioisostere.

Ang IV [123, 124]. Hydrophobic residues at position one, and norleucine in particular, rendered very high binding affinities, while substitution of the N-terminal amine by acetylation or methylation lead to low affinity ligands. Thus, a residue with straight aliphatic side-chain combined with a primary amine function was found to be preferred in the N-terminal. The C-terminal part, on the other hand, could be altered without affecting the binding affinity dramatically. However, truncations with the exception of removal of the C-terminal phenylalanine residue were found to be unproductive [122].

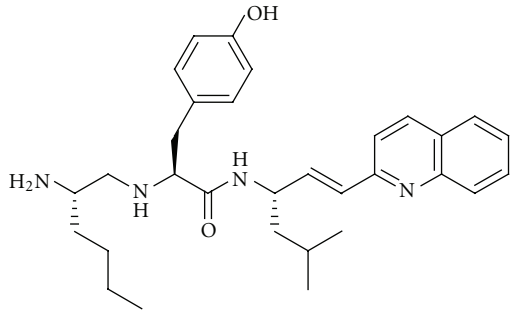
The hexapeptide Ang IV and its peptide analogues are prone to undergo proteolytic cleavage but a significant improvement of the metabolic stability could be achieved by reduction of the peptide bond between $Val^1$ and $Tyr^2$ of Ang IV with most of the affinity retained. Analogue **1** (Figure 6) constitute another such example, encompassing a reduced peptide bond ($\Psi[CH_2NH]$) between residues one and two and thus two amine sites that can be protonated [123]. Divalinal-Ang IV and norleual (Figure 6) are two other pseudopeptides comprising reduced peptide bonds that were studied in some detail and served as important research tools [125, 126].

Kobori et al. at Taisho Pharmaceuticals filed patent applications in the late 1990s that disclosed a series of compounds that bind strongly to guinea pig hippocampus membranes [127, 128]. They had deduced this from competitive experiments with radiolabeled [$^{125}$I]Ang IV. The compounds with the highest binding affinity are characterized by a straight fourcarbon chain at position one and a reduced amide bond ($\Psi[CH_2NH]$) between residues one and two. These high-affinity compounds have also a styrene moiety that replaces His-Pro-Phe in Ang IV, (compound **2** and quinoline **3** in Figure 7) and they have IC50 values lower than 1 nM. Ligand **4** in Figure 7 has three basic amino groups and was produced by reducing the amide bond between residues two and three in compound **2**. This compound has a binding affinity for hippocampus membranes that is 40 times lower than that of compound **2**. A ligand essentially devoid of affinity was obtained by maintaining the peptide bond between residues one and two intact and reducing the bond ($\Psi[CH_2NH]$) between residues two and three. Furthermore, reduction of the *trans* double bond at the C-terminal leads to considerably lower affinities. The potent ligands synthesized by Kobori et al. have to the best of our knowledge not been evaluated as IRAP inhibitors.

*5.2. Inhibitors of Insulin-Regulated Aminopeptidase (IRAP).* A receptor for Ang IV was purified from bovine adrenal membranes in 2001. This AT4 receptor was identified as the insulin-regulated aminopeptidase (IRAP) [129]. The IRAP/$AT_4$ receptor has attracted considerable interest in recent years as a potential target for pharmaceuticals aimed for the treatment of cognitive disorders [112, 130–133]. IRAP is a single-spanning transmembrane zinc-metallopeptidase that belongs to the M1 family of aminopeptidases. IRAP has been identified as cystinyl aminopeptidase (CAP, EC 3.4.11.3), placental leucine aminopeptidase (P-LAP, soluble human homologue), oxytocinase, gp160, or vp165 [134–137]. The insulin-regulated aminopeptidase has been cloned and characterized in adipocytes in vesicles that contain the insulin-regulated glucose transporter GLUT4 [138]. IRAP contains three domains [136, 139–141]: an intracellular region that is involved in intracellular localization and redistribution, a hydrophobic transmembrane segment, and an extracellular region that contains the catalytic site. The M1 family shares a consensus His-Glu-Xaa-Xaa-His-$(Xaa)_{18}$-Glu zinc-binding motif that is essential for enzymatic activity, and a Gly-Xaa-Met-Glu-Asn (Xaa = Ala in IRAP)




**2**

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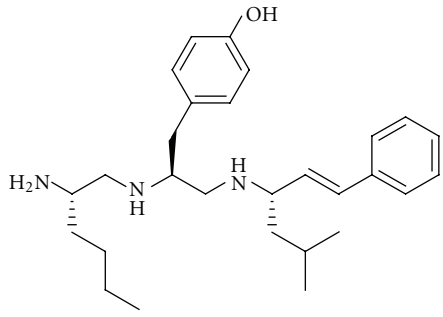
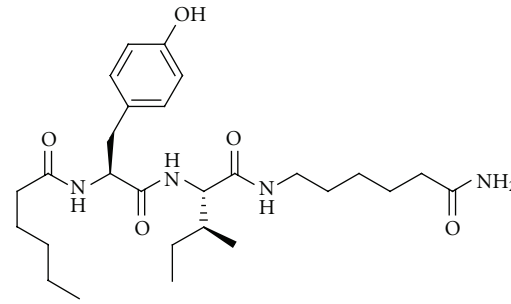



**4**

Figure 7: Highaffinity Ang IV receptor binding analogues that incorporate one or two reduced peptide bonds ($\Psi[CH_2NH]$) and a styrene moiety replacing the C-terminal tripeptide His-Pro-Phe of Ang IV.




PNB-0408

Figure 8: The structure of the compound PNB-0408, which crosses the blood-brain barrier.

exopeptidase motif. The zinc ion is coordinated to the two His residues, the second Glu residue, and a water molecule, which is anticipated to be activated by the other Glu residue during the hydrolytic step [142–144]. Mutational analyses have shown that the Gly-Ala-Met-Glu-Asn motif is important in the recognition of the N-terminal of both substrates and competitive inhibitors [139, 145, 146]. An important and characteristic property of the aminopeptidase is its ability to cleave the N-terminal amino acid residue from several bioactive peptides *in vitro*, including Met-enkephalin and Leu-enkephalin, dynorphin A, neurokinin A, cholecystokinin-8, somatostatin, oxytocin, and vasopressin [135, 147, 148].

Several hypotheses have been presented to explain the ability of Ang IV and its analogues to enhance cognitive functions [25, 149, 150]. One hypothesis concerns substrates such as vasopressin, somatostatin, and cholecystokinin, whose half-lives are prolonged when IRAP is inhibited by Ang IV, LVV-hemorphin-7 or analogous derivatives [151, 152]. These substrates improve parameters associated with cognition [153], and vasopressin and oxytocin are considered to be the main substrates of IRAP [148, 154]. It was recently shown that somatostatin has an impact on memory processing through its action on the somatostatin receptor subtype 3 [155]. A second hypothesis proposes that IRAP acts as a classical receptor that transfers information across the cell membrane after receptor binding, while a third is that the Ang IV analogues prolong the localisation of IRAP and GLUT4 at the cell surface, and thereby modulate the uptake of glucose into neurons and other cells [112, 150, 156, 157]. Furthermore, it has been proposed that the metallopeptidases in the same family as IRAP, such as aminopeptidase N (AP-N, EC 3.4.11.2), are targets [158]. Both Ang IV and LVV-hemorphin-7 inhibit AP-N activity [159]. Alternative macromolecular targets for Ang IV have been proposed, such as c-Met, a tyrosine kinase receptor that binds hepatocyte growth factor (HGF) and that is associated with memory and learning consolidation [160, 161]. Norleual (Figure 6) inhibits HGF-mediated effects at picomolar concentrations and blocks [$^{125}$I]HGF binding to c-Met.

The receptor or receptors that are involved in the positive identity identities of the effects of Ang IV and its analogues is still not clear, but both IRAP and c-Met are probably involved [114]. The availability of IRAP/Ang IV receptor ligands that are able to penetrate into the brain is important in order to obtain mechanistic insights. Wright and Harding have synthesised PNB-0408 (N-hexanoyl-Tyr-Ile-N′-(5-carbamoylpentyl)amide, Figure 8) and have shown that it crosses the blood-brain barrier and enhances cognitive activity [133, 162, 163]. This compound should be a very useful research tool. The activity profiles of this modified tripeptide and related analogues have now been established in several models of dementia [133].

Wright and Harding, and their group in USA, have been pioneers in identifying compounds that bind strongly to the

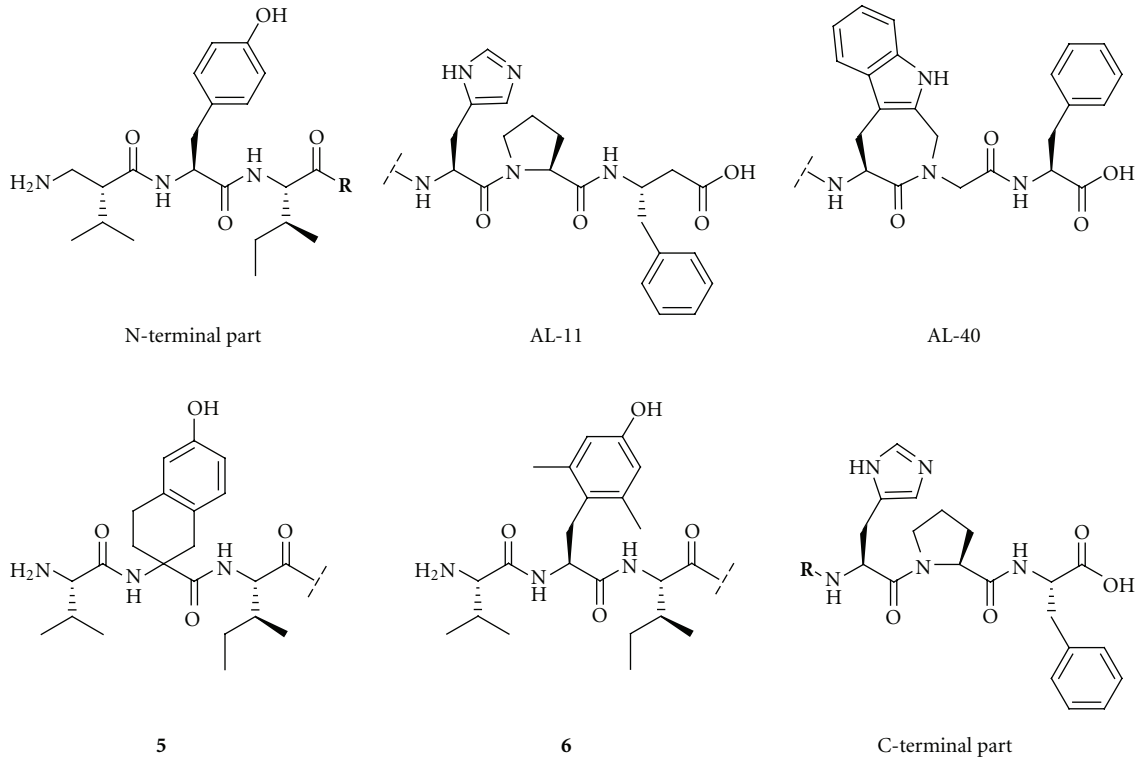



Figure 9: Examples of recently identified IRAP inhibitors.

Ang IV receptor, as are also Kobori et al. in Japan. Several other research groups have devoted considerable efforts in recent years to developing small molecules that interact with the Ang IV receptor, for example, in Belgium, Sweden, and (in particular) in Australia. These latter three groups have focussed on making efficient inhibitors of IRAP, and they have used slightly different and complementary approaches. The major objectives have been to identify powerful, selective inhibitors that are metabolically stable, and that resist, in particular, degradation by IRAP itself and related peptidases. To make inhibitors with high bioavailability in brain after oral administration is a tremendous challenge.

Many of the ligands previously identified as high-affinity binders were found to inhibit IRAP [111, 164]. Metal chelators (such as EDTA and phenanthroline) had previously been routinely used in experiments that measured binding affinity. It now became important to determine the ability of ligands to inhibit the hydrolysis of synthetic substrates in the absence of metal chelators. Ligands had different potencies and frequently different rank orders in the IRAP assay in the absence of chelators. It was suggested that the differences were a result of the absence of zinc in the active site when metal chelators were present [159, 164–166]. Thus, it became obvious that chelators must be omitted to obtain physiologically relevant results [167]. Furthermore, the results illustrated also the importance of synthesising inhibitors that are metabolically stable and are not substrates of IRAP.

Lukaszuk et al. in Belgium have recently performed a $\beta$-homoamino acid scan of Ang IV [168]. Replacement of Val$^1$ by (R)-$\beta^2$hVal and replacement of Phe$^6$ by $\beta^3$hPhe led to a metabolically stable and potent IRAP inhibitor, AL-11 (Figure 9). This has a high selectivity for IRAP over AP-N and the AT1 receptor. It has been reported that Ang IV affects blood pressure by a process that is mediated by the AT1 receptor [169]. The His$^4$ and Pro$^5$ residues were subsequently replaced by other, conformationally constrained, residues [170]. Incorporating (R)-$\beta^2$hVal$^1$ and Aia$^4$-Gly$^5$ gave a compound that is highly selective and stable and has a high inhibitory effect (AL-40, Figure 9) [168, 170]. Ang IV itself is only a weak inhibitor of the catalytic activity of IRAP, as has been shown by experiments with the metabolically stable tritiated Ang IV analogue [$^3$H]AL-11, in combination with the selective AP-N inhibitor 7B which is a phosphinic transition-state analogue [171] and in the absence of metal chelators. Adding metal chelators creates the apoform of IRAP [167, 172], and it is important to note that the active form and apoforms of IRAP react differently [173]. Hence, much of the previous results refer to binding to the apoenzyme rather than to the catalytically active enzyme [122–124, 174]. Ascher et al. have recently discussed the regulation of the peptidase activity of IRAP in detail [175].

The Belgian group also performed an extensive study that examined the roles of Tyr$^2$, Pro$^5$, and Phe$^6$ in Ang IV by introducing conformational constraints at the different amino acid residues. The study confirmed that conformational constraints are important in obtaining selectivity. Replacing Tyr$^2$ by any one of several conformationally constrained residues impairs the activity of the peptide, while modifications at the C-terminal are more acceptable.

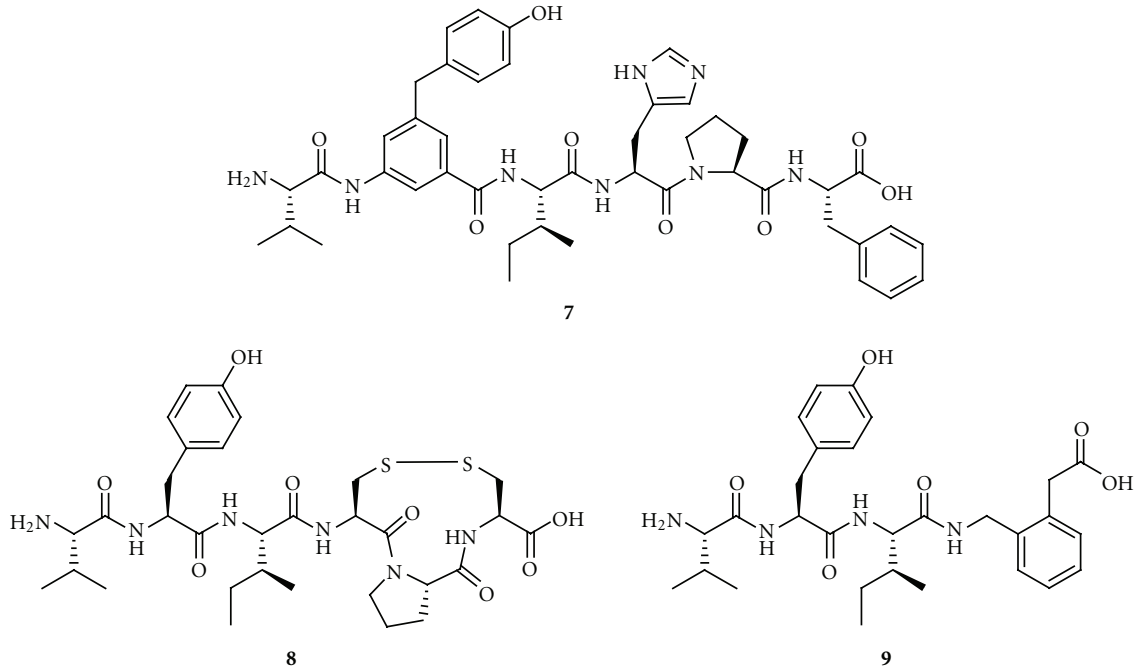



Figure 10: The structures of the Ang IV peptidomimetics **7**–**9**.

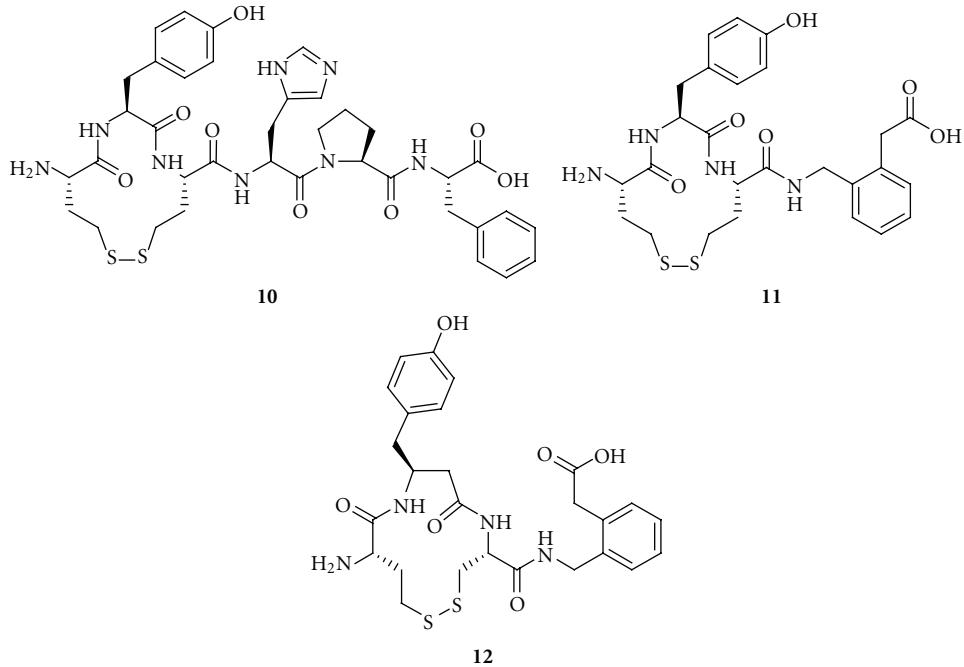
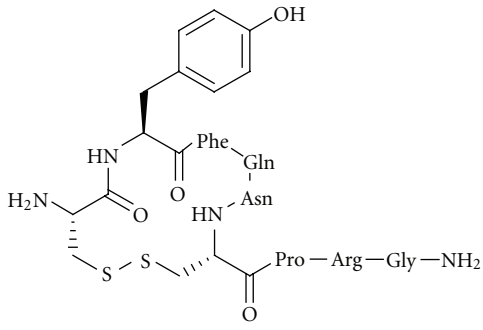


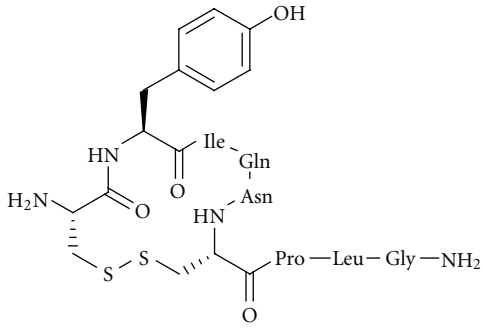

Figure 11: The structures of three IRAP inhibitors from a series of disulfide-cyclized Ang IV peptidomimetics. The ability to inhibit IRAP increases from **10** to **12**. Both **11** and **12** have 2000-fold selectivity for IRAP over AP-N.

Analogues **5** and **6** (Figure 9), for example, inhibit IRAP to a very low degree. This suggests that the orientation of the $Tyr^2$ side chain is critical for activity [176].

Our approach in Sweden to obtain active inhibitors of IRAP has been based on an ambition to determine various bioactive conformations by introducing local steric constraints. We have also worked to create various secondary structures by side chain cyclizations. Incorporation of a 4-hydroxydiphenylmethane scaffold as a substitute for $Tyr^2$ as part of the attempts to create steric constraint, as in Compound **7** (Figure 10), is deleterious for activity. This result again suggested that position two is susceptible

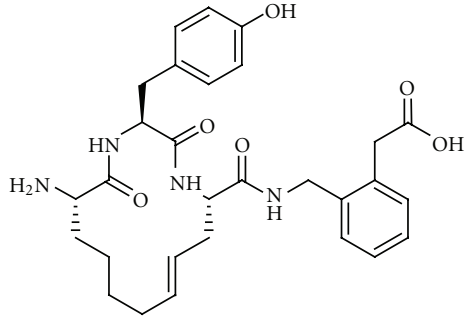



Vasopressin

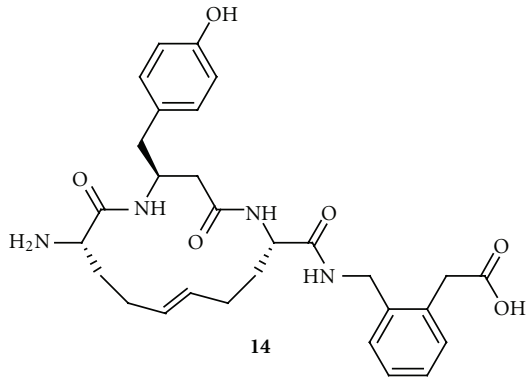



Oxytocin

Figure 12: The macrocyclic disulfides vasopressin and oxytocin are substrates of IRAP.




**13**




**14**

Figure 13: Macrocyclic Ang IV peptidomimetics that strongly inhibit IRAP.

to structural manipulation [177]. A series of macrocyclizations was performed in order to obtain improved inhibitors and to better understand how Ang IV binds to IRAP [174, 178–180]. Cyclization of [$Cys^4$, $Cys^6$]Ang IV to form an 11-membered ring resulted in a compound (**8**, $K_i$ = 26 nM, Figure 10) that is more potent than Ang IV ($K_i$ = 62 nM) as an IRAP inhibitor. Previous conformational analyses of Ang II suggested that such a macrocyclic system would tend to adopt an inverse $\gamma$-turn [181, 182], and the replacement of His-Pro-Phe by a 2-(aminomethyl)phenylacetic acid moiety designed to mimic an inverse $\gamma$-turn resulted in compound **9** (Figure 10). This compound has a $K_i$ value of 44 nM and a much simpler structure than previous candidates [171].

Cyclization of [$Cys^1$, $Cys^3$]Ang IV gives an inactive inhibitor with an 11-membered ring system, while a compound with fair inhibitory potency and a 13-membered macrocycle is obtained using [$Hcy^1$, $Hcy^3$]Ang IV (**10**, $K_i$ = 303 nM, Figure 11). The hybrid of **9** and **10** is a potent inhibitor (**11**, $K_i$ = 23 nM, Figure 11), and, importantly, selective for IRAP over AP-N. Thus, it appears that that Ang IV adopts a $\gamma$-turn at the C-terminal when binding to IRAP, while an open, less well-defined turn conformation is present at the N-terminal. Further structural optimisation produced compound **12** (Figure 11), which has a $\beta^3$hTyr residue in a 13-membered macrocycle. Its $K_i$ value is 3.3 nM. This compound is 20 times more potent than Ang IV and exhibit a 2000-fold selectivity for IRAP over AP-N [180]. Removal of the carboxyl group at the C-terminal gives less efficient inhibitors of IRAP. Hence, macrocyclizations by oxidative disulfide formations can provide very potent IRAP inhibitors.

Macrocyclizations, obtained by applying the metathesis reaction, are very attractive in efforts to make potent compounds with high oral bioavailability [183, 184]. This observation and the knowledge that compounds such as vasopressin and oxytocin (Figure 12) are macrocyclic in the N-terminal part and substrates of IRAP [148] prompted the synthesis of carba analogues. It was believed that these would be efficient IRAP inhibitors that were more metabolically stable.

A large series of macrocyclic compounds were prepared, of which the compounds labelled **13** and **14** (Figure 13) have the lowest $K_i$ values, 4.1 nM and 1.8 nM, respectively [179]. These carba analogues are also the most metabolically stable analogues that were synthesized in the programme. In the absence of chelators, the binding affinity of the 14-membered macrocyclic compounds to IRAP is 10 times higher than that of Ang IV. N-Methylation of the peptide bond between residues one and two reduces the activity, suggesting that the amide nitrogen and the N-terminal primary amine nitrogen are coordinated to the zinc atom in the active site of the protease [179]. Incorporation of a methylene group adjacent to the N-terminal amino group, as in AL-11 and AL-40 (Figure 9), and replacement of the C-terminal carboxyl group with bioisosteres seem to be the obvious next steps in efforts to improve the inhibitors. The amide bond between residues one and two, which is present in all of the macrocyclic inhibitors that have been reported, is the major target for IRAP. Thus, exchanging this bond

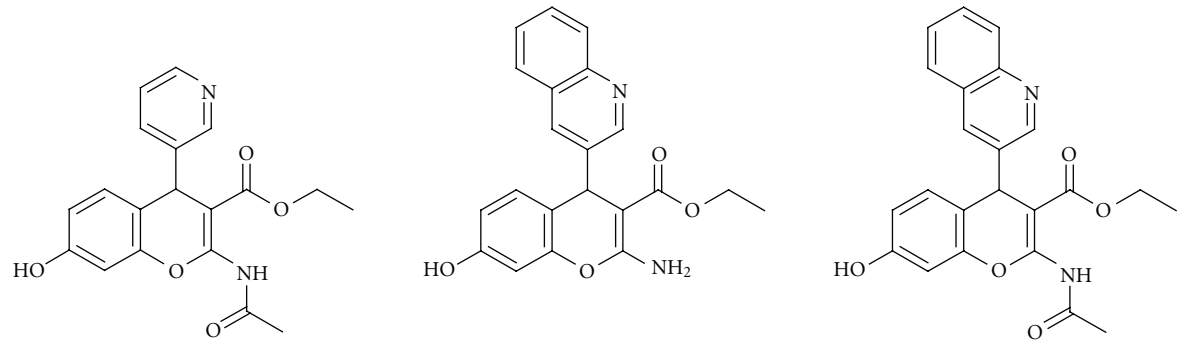



Figure 14: Recently identified potent non-peptidic IRAP inhibitors with a benzopyran scaffold.

for a proteolytically inert $CH_2NH$ fragment may improve the properties. Such a $CH_2NH$ fragment should provide also a strong coordination to zinc.

The Australian group discovered a series of IRAP inhibitors based on a benzopyran system as scaffold. These drug-like compounds should be more metabolically stable than the peptidomimetics previously discussed [113]. While compounds **13** and **14** were produced through various modifications of pre-existing compounds, primarily macrocyclizations of Ang IV itself, inhibitors based on benzopyrans originate from a structurebased design process and virtual screening.

The 3D structure of IRAP is not known. Thunnissen et al. therefore used the structurally related human leukotriene $A_4$ hydrolase [185], which also belongs to the M1 aminopeptidase family, in an *in silico* screening aimed at identifying potential inhibitors [186]. The process involved the *in silico* screening of 1.5 million commercially available compounds against a model structure homologous to IRAP, identification of hits, biological evaluation of hits, and optimisation of structures. Several potent drug-like benzopyrans were identified as IRAP inhibitors. Among those, the racemic pyridine derivative HFI-419, and the quinoline derivatives HFI-435 and HFI-437 (Figure 14), are selective for IRAP, with $K_i$ values of 420, 360, and 20 nM, respectively. The compounds bind with low affinity to structurally related enzymes, such as AP-N and leukotriene $A_4$ hydrolase itself, despite the latter having been used in the homology modelling [186]. Computational docking suggests that the *S*-isomer is the preferred binding mode in all examples, and two alternate binding conformations for these structurally analogous inhibitors have been proposed [187].

The quinoline compounds HFI-435 and HFI-437 cannot adopt the same binding mode as the pyridinyl compound HFI-419 for steric reasons, but HFI-435 and HFI-437 seem to adopt binding modes that allow a stronger interaction with the zinc ion through coordination with the nitrogen atom in the quinoline heterocycle. It has been predicted that the quinoline compounds are more active than the pyridinyl compounds, partly due to a more favourable coordination to the zinc ion in IRAP. In addition, computational docking experiments have suggested that $Phe^{544}$ of IRAP provides an important hydrophobic packing point at one side of the active site [187]. No comparative modelling has been carried out, but it is tempting to suggest that the N-terminal of Ang IV, the macrocyclic compound **13** and the quinoline HIF-437 bind to IRAP as shown in Figure 15. The amide nitrogen (or the amide oxygen) and/or the N-terminal nitrogen atom may interact with the zinc atom.

Albiston et al. demonstrated that *i.c.v.* administration of HFI-419 enhances memory in two memory paradigms. The performance of rats in a task involving spontaneous alternation of spatial working memory after administration of compound HFI-419 [186] was similar to the performance after administration of Ang IV and LVV-hemorphin-7 [14]. Results from *in vivo* experiments with this drug-like class of compounds strongly support the strategy of using IRAP as a target for cognitive enhancers. The benzopyran class of IRAP inhibitors provides promising leads for further development.

## 6. Conclusions

Experimental data from various animal models demonstrate that inhibitors of IRAP facilitate memory. The explanations to the beneficial effects on a biochemical level are not clear and many alternative hypotheses have been proposed. Further studies to elucidate the mechanism of action are needed. In this context, metabolically stable IRAP inhibitors, able to cross the blood-brain barrier and with fair bioavailability in brain should serve as important research tools. Such inhibitors could also provide new chemical entities and enhancers of cognition for future treatments of the memory loss associated with ageing and AD. All data with IRAP inhibitors available today were achieved after i.c.v. administration and new inhibitors with oral bioavailability are highly desirable. There are in brief two different approaches addressing the discovery of such inhibitors. One starts from Ang IV itself. After subsequent truncations and macrocyclizations, stabilizing favourable conformations, potent inhibitors could be obtained. However, these are still peptidic in character. The other approach originates from a virtual screening and the utility of a homology model

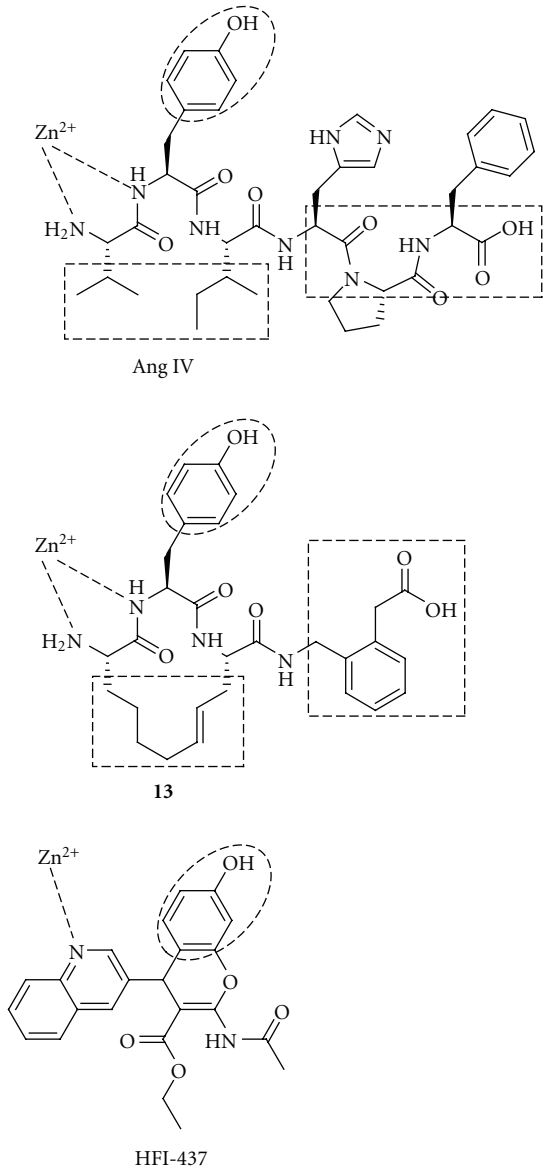



Figure 15: Ang IV, a peptidomimetic (**13**) and a non-peptidic (HFI-437) IRAP inhibitor. The rectangles and circles show proposed recognition elements important for interaction with IRAP.

of IRAP. A series of drug-like small inhibitors with high potential for further optimization were identified by this process. The major challenge remaining for these inhibitors is to enable them to cross the blood-brain barrier.

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## References

[1] A. J. Kastin, J. E. Zadina, R. D. Olson, and W. A. Banks, "The history of neuropeptide research: version 5.a," *Annals of the New York Academy of Sciences*, vol. 780, pp. 1–18, 1996.

[2] M. Hallberg and F. Nyberg, "Neuropeptide conversion to bioactive fragments—an important pathway in neuromodulation," *Current Protein and Peptide Science*, vol. 4, no. 1, pp. 31–44, 2003.

[3] S. Y. Chai, R. Fernando, S. Ye, G. R. Peck, and A. L. Albiston, "Insulin-regulated aminopeptidase, in Proteases in biology and disease," in *Aminopeptidases in Biology and Disease*, N. M. Hooper and U. Lendeckel, Eds., pp. 61–81, Kluwer Academic/Plenum Publishers, New York, NY, USA, 2004.

[4] M. Hallberg, P. Le Greves, and F. Nyberg, "Neuropeptide processing," in *Proteases in Biology and Disease, Proteases in the Brain*, U. Lendeckel and N. M. Hooper, Eds., pp. 203–234, Springer Science, New York, NY, USA, 2005.

[5] C. I. Johnston and J. Risvanis, "Preclinical pharmacology of angiotensin II receptor antagonists: update and outstanding issues," *American Journal of Hypertension*, vol. 10, no. 12, pp. 306S–310S, 1997.

[6] F. Fyhrquist and O. Saijonmaa, "Renin-angiotensin system revisited," *Journal of Internal Medicine*, vol. 264, no. 3, pp. 224–236, 2008.

[7] J. R. Blair-West, K. D. Carey, D. A. Denton, L. J. Madden, R. S. Weisinger, and R. E. Shade, "Possible contribution of brain angiotensin III to ingestive behaviors in baboons," *American Journal of Physiology*, vol. 281, no. 5, pp. R1633–R1636, 2001.

[8] J. W. Wright, A. V. Miller-Wing, M. J. Shaffer et al., "Angiotensin II(3-8) (ANG IV) hippocampal binding: potential role in the facilitation of memory," *Brain Research Bulletin*, vol. 32, no. 5, pp. 497–502, 1993.

[9] J. Lee, S. Y. Chai, F. A. O. Mendelsohn, M. J. Morris, and A. M. Allen, "Potentiation of cholinergic transmission in the rat hippocampus by angiotensin IV and LVV-hemorphin-7," *Neuropharmacology*, vol. 40, no. 4, pp. 618–623, 2001.

[10] M. Cesari, G. P. Rossi, and A. C. Pessina, "Biological properties of the angiotensin peptides other than angiotensin II: implications for hypertension and cardiovascular diseases," *Journal of Hypertension*, vol. 20, no. 5, pp. 793–799, 2002.

[11] K. L. Hall, S. Venkateswaran, J. M. Hanesworth, M. E. Schelling, and J. W. Harding, "Characterization of a functional angiotensin IV receptor on coronary microvascular endothelial cells," *Regulatory Peptides*, vol. 58, no. 3, pp. 107–115, 1995.

[12] J. W. Wright and J. W. Harding, "Important roles for angiotensin III and IV in the brain renin-angiotensin system," *Brain Research Reviews*, vol. 25, no. 1, pp. 96–124, 1997.

[13] E. S. Pederson, R. Krishnan, J. W. Harding, and J. W. Wright, "A role for the angiotensin AT4 receptor subtype in overcoming scopolamine-induced spatial memory deficits," *Regulatory Peptides*, vol. 102, no. 2-3, pp. 147–156, 2001.

[14] D. De Bundel, I. Smolders, R. Yang, A. L. Albiston, Y. Michotte, and S. Y. Chai, "Angiotensin IV and LVV-haemorphin 7 enhance spatial working memory in rats: effects on hippocampal glucose levels and blood flow," *Neurobiology of Learning and Memory*, vol. 92, no. 1, pp. 19–26, 2009.

[15] R. Ardaillou and D. Chansel, "Synthesis and effects of active fragments of angiotensin II," *Kidney International*, vol. 52, no. 6, pp. 1458–1468, 1997.

[16] V. Jankowski, R. Vanholder, M. Van Der Giet et al., "Mass-spectrometric identification of a novel angiotensin peptide in human plasma," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 27, no. 2, pp. 297–302, 2007.

[17] R. Yang, I. Smolders, P. Vanderheyden et al., "Pressor and renal hemodynamic effects of the novel angiotensin

a peptide are angiotensin II type 1A receptor dependent," *Hypertension*, vol. 57, no. 5, pp. 956–964, 2011.

[18] I. Banegas, I. Prieto, F. Vives et al., "Brain aminopeptidases and hypertension," *Journal of the Renin-Angiotensin-Aldosterone System*, vol. 7, no. 3, pp. 129–134, 2006.

[19] T. L. Reudelhuber, "The renin-angiotensin system: peptides and enzymes beyond angiotensin II," *Current Opinion in Nephrology and Hypertension*, vol. 14, no. 2, pp. 155–159, 2005.

[20] J. M. Saavedra, "Brain and pituitary angiotensin," *Endocrine Reviews*, vol. 13, no. 2, pp. 329–380, 1992.

[21] R. C. Speth, T. E. Brown, R. D. Barnes, and J. W. Wright, "Brain angiotensinergic activity: the state of our current knowledge," *Proceedings of the Western Pharmacology Society*, vol. 46, pp. 11–15, 2003.

[22] T. Unger, E. Badoer, D. Ganten, R. E. Lang, and R. Rettig, "Brain angiotensin: pathways and pharmacology," *Circulation*, vol. 77, no. 6, pp. I-40–I-54, 1988.

[23] C. I. Johnston, "Biochemistry and pharmacology of the renin-angiotensin system," *Drugs*, vol. 39, supplement 1, pp. 21–31, 1990.

[24] C. M. Ferrario and M. C. Chappell, "Novel angiotensin peptides," *Cellular and Molecular Life Sciences*, vol. 61, no. 21, pp. 2720–2727, 2004.

[25] G. Vauquelin, Y. Michotte, I. Smolders et al., "Cellular targets for angiotensin II fragments: pharmacological and molecular evidence," *Journal of the Renin-Angiotensin-Aldosterone System*, vol. 3, no. 4, pp. 195–204, 2002.

[26] S. V. B. Pinheiro, A. C. Simões e Silva, W. O. Sampaio et al., "Nonpeptide AVE 0991 is an angiotensin-(1-7) receptor mas agonist in the mouse kidney," *Hypertension*, vol. 44, no. 4, pp. 490–496, 2004.

[27] A. J. Ferreira, T. M. Murca, R. A. Fraga-Silva, C. H. Castro, M. K. Raizada, and R. A. Santos, "New cardiovascular and pulmonary therapeutic strategies based on the Angiotensin-converting enzyme 2/angiotensin-(1-7)/mas receptor axis," *International Journal of Hypertension*, vol. 2012, Article ID 147825, 2012.

[28] J. W. Wright, S. Mizutani, and J. W. Harding, "Focus on brain angiotensin III and aminopeptidase A in the control of hypertension," *International Journal of Hypertension*, vol. 2012, Article ID 124758, 2012.

[29] M. C. Khosla, R. A. Leese, W. L. Maloy, A. T. Ferreira, R. R. Smeby, and F. M. Bumpus, "Synthesis of some analogs of angiotensin II as specific antagonists of the parent hormone," *Journal of Medicinal Chemistry*, vol. 15, no. 8, pp. 792–795, 1972.

[30] E. Haber, "The role of renin in normal and pathological cardiovascular homeostasis," *Circulation*, vol. 54, no. 6, pp. 849–861, 1976.

[31] H. R. Brunner, H. Gavras, J. H. Laragh, and R. Keenan, "Angiotensin II blockade in man by SAR ALA angiotensin II for understanding and treatment of high blood pressure," *Lancet*, vol. 2, no. 7837, pp. 1045–1048, 1973.

[32] D. H. P. Streeten, G. H. Anderson Jr., and T. G. Dalakos, "Angiotensin blockade: its clinical significance," *The American Journal of Medicine*, vol. 60, no. 6, pp. 817–824, 1976.

[33] T. Ogihara, T. Yamamoto, and Y. Kumahara, "Clinical applications of synthetic angiotensin II analogue," *Japanese Circulation Journal*, vol. 38, no. 11, pp. 997–1003, 1974.

[34] M. A. Ondetti, B. Rubin, and D. W. Cushman, "Design of specific inhibitors of angiotensin converting enzyme: new class of orally active antihypertensive agents," *Science*, vol. 196, no. 4288, pp. 441–444, 1977.

[35] M. A. Ondetti and D. W. Cushman, "Inhibition of the renin-angiotensin system. A new approach to the therapy of hypertension," *Journal of Medicinal Chemistry*, vol. 24, no. 4, pp. 355–361, 1981.

[36] M. A. Ondetti and D. W. Cushman, "Inhibitors of angiotensin converting enzyme," in *Biochemical Regulation of Blood Pressure*, R. L. Soffer, Ed., pp. 165–204, Wiley, New York, NY, USA, 1981.

[37] P. A. Todd and R. C. Heel, "Enalapril. A review of its pharmacodynamic and pharmacokinetic properties, and therapeutic use in hypertension and congestive heart failure," *Drugs*, vol. 31, no. 3, pp. 198–248, 1986.

[38] P. A. Todd and K. L. Goa, "Enalapril. A reappraisal of its pharmacology and therapeutic use in hypertension," *Drugs*, vol. 43, no. 3, pp. 346–381, 1992.

[39] R. Natesh, S. L. U. Schwager, H. R. Evans, E. D. Sturrock, and K. R. Acharya, "Structural details on the binding of antihypertensive drugs captopril and enalaprilat to human testicular angiotensin I-converting enzyme," *Biochemistry*, vol. 43, no. 27, pp. 8718–8724, 2004.

[40] J. H. Bauer, "Angiotensin converting enzyme inhibitors," *American Journal of Hypertension*, vol. 3, no. 4, pp. 331–337, 1990.

[41] M. Azizi, R. Webb, J. Nussberger, and N. K. Hollenberg, "Renin inhibition with aliskiren: where are we now, and where are we going?" *Journal of Hypertension*, vol. 24, no. 2, pp. 243–256, 2006.

[42] M. M. Ibrahim, "RAS inhibition in hypertension," *Journal of Human Hypertension*, vol. 20, no. 2, pp. 101–108, 2006.

[43] C. Werner, M. Baumhäkel, K. K. Teo et al., "RAS blockade with ARB and ACE inhibitors: current perspective on rationale and patient selection," *Clinical Research in Cardiology*, vol. 97, no. 7, pp. 418–431, 2008.

[44] J. Maibaum, S. Stutz, R. Göschke et al., "Structural modification of the P2′ position of 2,7-dialkyl-substituted 5(S)-amino-4(S)-hydroxy-8-phenyl-octanecarboxamides: the discovery of aliskiren, a potent nonpeptide human renin inhibitor active after once daily dosing in marmosets," *Journal of Medicinal Chemistry*, vol. 50, no. 20, pp. 4832–4844, 2007.

[45] R. R. Wexler, W. J. Greenlee, J. D. Irvin et al., "Nonpeptide angiotensin II receptor antagonists: the next generation in antihypertensive therapy," *Journal of Medicinal Chemistry*, vol. 39, no. 3, pp. 625–656, 1996.

[46] C. A. Stoukides, H. J. McVoy, and A. F. Kaul, "Candesartan cilexetil: an angiotensin II receptor blocker," *Annals of Pharmacotherapy*, vol. 33, no. 12, pp. 1287–1298, 1999.

[47] C. H. Gleiter, C. Jägle, U. Gresser, and K. Mörike, "Candesartan," *Cardiovascular Drug Reviews*, vol. 22, no. 4, pp. 263–284, 2004.

[48] S. E. Kjeldsen, J. Stlhammar, P. Hasvold, J. Bodegard, U. Olsson, and D. Russell, "Effects of losartan vs candesartan in reducing cardiovascular events in the primary treatment of hypertension," *Journal of Human Hypertension*, vol. 24, no. 4, pp. 263–273, 2010.

[49] M. Eklind-Cervenka, L. Benson, U. Dahlström, M. Edner, M. Rosenqvist, and L. H. Lund, "Association of candesartan vs losartan with all-cause mortality in patients with heart failure," *Journal of the American Medical Association*, vol. 305, no. 2, pp. 175–182, 2011.

[50] G. Vauquelin, F. Fierens, and I. V. Liefde, "Long-lasting angiotensin type 1 receptor binding and protection by candesartan: comparison with other biphenyl-tetrazole sartans," *Journal of Hypertension*, vol. 24, no. 1, pp. S23–S30, 2006.

[51] R. A. Copeland, "The dynamics of drug-target interactions: drug-target residence time and its impact on efficacy and safety," *Expert Opinion on Drug Discovery*, vol. 5, no. 4, pp. 305–310, 2010.

[52] G. Vauquelin and S. J. Charlton, "Long-lasting target binding and rebinding as mechanisms to prolong in vivo drug action," *British Journal of Pharmacology*, vol. 161, no. 3, pp. 488–508, 2010.

[53] W. B. White, M. A. Weber, D. Sica et al., "Effects of the angiotensin receptor blocker azilsartan medoxomil versus olmesartan and valsartan on ambulatory and clinic blood pressure in patients with stages 1 and 2 hypertension," *Hypertension*, vol. 57, no. 3, pp. 413–420, 2011.

[54] G. L. Bakris, D. Sica, M. Weber et al., "The comparative effects of azilsartan medoxomil and olmesartan on ambulatory and clinic blood pressure," *Journal of Clinical Hypertension*, vol. 13, no. 2, pp. 81–88, 2011.

[55] P. Naik, P. Murumkar, R. Giridhar, and M. R. Yadav, "Angiotensin II receptor type 1 (AT1) selective nonpeptidic antagonists—a perspective," *Bioorganic and Medicinal Chemistry*, vol. 18, no. 24, pp. 8418–8456, 2010.

[56] B. Dahlöf, R. B. Devereux, S. E. Kjeldsen et al., "Cardiovascular morbidity and mortality in the Losartan Intervention for Endpoint reduction in hypertension study (LIFE): a randomised trial against atenolol," *Lancet*, vol. 359, no. 9311, pp. 995–1003, 2002.

[57] S. Julius, S. E. Kjeldsen, M. Weber et al., "Outcomes in hypertensive patients at high cardiovascular risk treated with regimens based on valsartan or amlodipine: the VALUE randomised trial," *Lancet*, vol. 363, no. 9426, pp. 2022–2031, 2004.

[58] R. Fogari, A. Mugellini, A. Zoppi et al., "Influence of losartan and atenolol on memory function in very elderly hypertensive patients," *Journal of Human Hypertension*, vol. 17, no. 11, pp. 781–785, 2003.

[59] L. Hansson, H. Lithell, I. Skoog et al., "Study on COgnition and Prognosis in the Elderly (SCOPE)," *Blood Pressure*, vol. 8, no. 3, pp. 177–183, 1999.

[60] P. Trenkwalder, "The Study on COgnition and Prognosis in the Elderly (SCOPE)—recent analyses," *Journal of Hypertension*, vol. 24, no. 1, pp. S107–S114, 2006.

[61] A. Zanchetti and D. Elmfeldt, "Findings and implications of the Study on COgnition and Prognosis in the Elderly (SCOPE)—a review," *Blood Pressure*, vol. 15, no. 2, pp. 71–79, 2006.

[62] K. S. Kim, D. Abraham, B. Williams, J. D. Violin, L. Mao, and H. A. Rockman, "Beta-Arrestin-biased AT1R stimulation promotes cell survival during acute cardiac injury," *American Journal of Physiology*, vol. 303, pp. H1001–H1010, 2012.

[63] G. Boerrigter, D. G. Soergel, J. D. Violin, M. W. Lark, and J. C. Burnett Jr., "TRV120027, a novel beta-arrestin biased ligand at the angiotensin II type I receptor, unloads the heart and maintains renal function when added to furosemide in experimental heart failure," *Circulation: Heart Failure*, vol. 5, pp. 627–634, 2012.

[64] N. S. Kirkby, P. W. F. Hadoke, A. J. Bagnall, and D. J. Webb, "The endothelin system as a therapeutic target in cardiovascular disease: great expectations or bleak house?" *British Journal of Pharmacology*, vol. 153, no. 6, pp. 1105–1119, 2008.

[65] L. M. Ruilope, A. Dukat, M. Böhm, Y. Lacourcière, J. Gong, and M. P. Lefkowitz, "Blood-pressure reduction with LCZ696, a novel dual-acting inhibitor of the angiotensin II receptor and neprilysin: a randomised, double-blind, placebo-controlled, active comparator study," *The Lancet*, vol. 375, no. 9722, pp. 1255–1266, 2010.

[66] L. Paulis, U. M. Steckelings, and T. Unger, "Key advances in antihypertensive treatment," *Nature Reviews Cardiology*, vol. 9, pp. 276–285, 2011.

[67] L. Paulis and T. Unger, "Novel therapeutic targets for hypertension," *Nature Reviews Cardiology*, vol. 7, no. 8, pp. 431–441, 2010.

[68] N. Gallo-Payet, M. Shum, J. P. Baillargeon et al., "AT2 receptor agonists: exploiting the beneficial arm of ang II signaling," *Current Hypertension Reviews*, vol. 8, pp. 47–59, 2012.

[69] H. Yamada, M. Akishita, M. Ito et al., "AT2 receptor and vascular smooth muscle cell differentiation in vascular development," *Hypertension*, vol. 33, no. 6, pp. 1414–1419, 1999.

[70] Z. Lenkei, M. Palkovits, P. Corvol, and C. Llorens-Cortès, "Expression of angiotensin type-1 (AT1) and type-2 (AT2) receptor mRNAs in the adult rat brain: a functional neuroanatomical review," *Frontiers in Neuroendocrinology*, vol. 18, no. 4, pp. 383–439, 1997.

[71] M. Horiuchi, M. Mogi, and M. Iwai, "The angiotensin II type 2 receptor in the brain," *Journal of the Renin-Angiotensin-Aldosterone System*, vol. 11, no. 1, pp. 1–6, 2010.

[72] T. Ichiki, P. A. Labosky, C. Shiota et al., "Effects on blood pressure exploratory behaviour of mice lacking angiotensin II type 2 receptor," *Nature*, vol. 377, no. 6551, pp. 748–750, 1995.

[73] C. Tebbs, M. K. Pratten, and F. B. Pipkin, "Angiotensin II is a growth factor in the peri-implantation rat embryo," *Journal of Anatomy*, vol. 195, no. 1, pp. 75–86, 1999.

[74] M. A. Millan, D. M. Jacobowitz, G. Aguilera, and K. J. Catt, "Differential distribution of AT1 and AT2 angiotensin II receptor subtypes in the rat brain during development," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 88, no. 24, pp. 11440–11444, 1991.

[75] K. Tsutsumi and J. M. Saavedra, "Characterization and development of angiotensin II receptor subtypes (AT1 and AT2) in rat brain," *American Journal of Physiology*, vol. 261, no. 1, pp. R209–R216, 1991.

[76] Z. Lenkei, M. Palkovits, P. Corvol, and C. Llorens-Cortes, "Distribution of angiotensin II type-2 receptor (AT2) mRNA expression in the adult rat brain," *The Journal of Comparative Neurology*, vol. 373, pp. 322–339, 1996.

[77] T. N. Grammatopoulos, S. M. Jones, F. A. Ahmadi et al., "Angiotensin type I receptor antagonist losartan, reduces MPTP-induced degeneration of dopaminergic neurons in substantia nigra," *Molecular Neurodegeneration*, vol. 2, no. 1, article 1, 2007.

[78] G. A. Argañaraz, A. C. Konno, S. R. Perosa et al., "The renin-angiotensin system is upregulated in the cortex and hippocampus of patients with temporal lobe epilepsy related to mesial temporal sclerosis," *Epilepsia*, vol. 49, no. 8, pp. 1348–1357, 2008.

[79] S. Abdalla, H. Lother, A. El Missiry et al., "Angiotensin II AT2 receptor oligomers mediate G-protein dysfunction in an animal model of Alzheimer disease," *Journal of Biological Chemistry*, vol. 284, no. 10, pp. 6554–6565, 2009.

[80] N. Gallo-Payet, M. O. Guimond, L. Bilodeau, C. Wallinder, M. Alterman, and A. Hallberg, "Angiotensin II, a neuropeptide at the frontier between endocrinology and neuroscience: is there a link between the angiotensin II type 2 receptor and Alzheimer's disease?" *Front Endocrinology*, vol. 2, article 17, 2011.

[81] F. Jing, M. Mogi, A. Sakata et al., "Direct stimulation of angiotensin II type 2 receptor enhances spatial memory," *Journal of Cerebral Blood Flow & Metabolism*, vol. 32, pp. 248–255, 2012.

[82] S. Meffert, M. Stoll, U. M. Steckelings, S. P. Bottari, and T. Unger, "The angiotensin II AT2 receptor inhibits proliferation and promotes differentiation in PC12W cells," *Molecular and Cellular Endocrinology*, vol. 122, no. 1, pp. 59–67, 1996.

[83] L. Laflamme, M. De Gasparo, J. M. Gallo, M. D. Payet, and N. Gallo-Payet, "Angiotensin II induction of neurite outgrowth by AT2 receptors in NG108-15 cells. Effect counteracted by the AT1 receptors," *Journal of Biological Chemistry*, vol. 271, no. 37, pp. 22729–22735, 1996.

[84] R. Lucius, S. Gallinat, P. Rosenstiel, T. Herdegen, J. Sievers, and T. Unger, "The angiotensin II type 2 (AT2) receptor promotes axonal regeneration in the optic nerve of adult rats," *Journal of Experimental Medicine*, vol. 188, no. 4, pp. 661–670, 1998.

[85] M. De Gasparo, K. J. Catt, T. Inagami, J. W. Wright, and T. Unger, "International union of pharmacology. XXIII. The angiotensin II receptors," *Pharmacological Reviews*, vol. 52, no. 3, pp. 415–472, 2000.

[86] R. M. Carey, Z. Q. Wang, and H. M. Siragy, "Role of the angiotensin type 2 receptor in the regulation of blood pressure and renal function," *Hypertension*, vol. 35, no. 1, pp. 155–163, 2000.

[87] U. M. Steckelings, F. Rompe, E. Kaschina et al., "The past, present and future of angiotensin II type 2 receptor stimulation," *Journal of the Renin-Angiotensin-Aldosterone System*, vol. 11, no. 1, pp. 67–73, 2010.

[88] R. M. Carey, "Angiotensin type-2 receptors and cardiovascular function: are angiotensin type-2 receptors protective?" *Current Opinion in Cardiology*, vol. 20, no. 4, pp. 264–269, 2005.

[89] C. Savoia, R. M. Touyz, M. Volpe, and E. L. Schiffrin, "Angiotensin type 2 receptor in resistance arteries of type 2 diabetic hypertensive patients," *Hypertension*, vol. 49, no. 2, pp. 341–346, 2007.

[90] H. M. Siragy, "The angiotensin II type 2 receptor and the kidney," *Journal of the Renin-Angiotensin-Aldosterone System*, vol. 11, no. 1, pp. 33–36, 2010.

[91] T. Unger, "Targeting cardiovascular protection: the concept of dual renin-angiotensin system control," *MedGenMed Medscape General Medicine*, vol. 10, article S4, 2008.

[92] C. A. Lemarié and E. L. Schiffrin, "The angiotensin II type 2 receptor in cardiovascular disease," *Journal of the Renin-Angiotensin-Aldosterone System*, vol. 11, no. 1, pp. 19–31, 2010.

[93] U. M. Steckelings, E. Kaschina, and T. Unger, "The AT2 receptor—a matter of love and hate," *Peptides*, vol. 26, no. 8, pp. 1401–1409, 2005.

[94] Y. Wan, C. Wallinder, B. Plouffe et al., "Design, synthesis, and biological evaluation, of the first selective nonpeptide AT2 receptor agonist," *Journal of Medicinal Chemistry*, vol. 47, no. 24, pp. 5995–6008, 2004.

[95] X. Wu, Y. Wan, A. K. Mahalingam et al., "Selective angiotensin II AT2 receptor agonists: arylbenzylimidazole structure-activity relationships," *Journal of Medicinal Chemistry*, vol. 49, no. 24, pp. 7160–7168, 2006.

[96] A. M. S. Murugaiah, C. Wallinder, A. K. Mahalingam et al., "Selective angiotensin II AT2 receptor agonists devoid of the imidazole ring system," *Bioorganic and Medicinal Chemistry*, vol. 15, no. 22, pp. 7166–7183, 2007.

[97] S. D. Kivlighn, W. R. Huckle, G. J. Zingaro et al., "Discovery of L-162,313: a nonpeptide that mimics the biological actions of angiotensin II," *American Journal of Physiology*, vol. 268, no. 3, pp. R820–R823, 1995.

[98] B. Mertens, P. Vanderheyden, Y. Michotte, and S. Sarre, "Direct angiotensin II type 2 receptor stimulation decreases dopamine synthesis in the rat striatum," *Neuropharmacology*, vol. 58, no. 7, pp. 1038–1044, 2010.

[99] M. Mogi and M. Horiuchi, "Effect of angiotensin II type 2 receptor on stroke, cognitive impairment and neurodegenerative diseases," *Geriatric Gerontology*. In press.

[100] E. Kaschina, A. Grzesiak, J. Li et al., "Angiotensin II type 2 receptor stimulation: a novel option of therapeutic interference with the renin-angiotensin system in myocardial infarction?" *Circulation*, vol. 118, no. 24, pp. 2523–2532, 2008.

[101] F. Rompe, M. Artuc, A. Hallberg et al., "Direct angiotensin II type 2 receptor stimulation acts anti-inflammatory through epoxyeicosatrienoic acid and inhibition of nuclear factor κb," *Hypertension*, vol. 55, no. 4, pp. 924–931, 2010.

[102] A. Rehman, A. Leibowitz, N. Yamamoto et al., "Angiotensin type 2 receptor agonist compound 21 reduces vascular injury and myocardial fibrosis in stroke-prone spontaneously hypertensive rats," *Hypertension*, vol. 59, pp. 291–299, 2012.

[103] S. Foulquier, U. M. Steckelings, and T. Unger, "Impact of the AT(2) receptor agonist C21 on blood pressure and beyond," *Current Hypertension Reports*, vol. 14, no. 5, pp. 403–409, 2012.

[104] N. Shraim, B. Mertens, R. Clinckers, S. Sarre, Y. Michotte, and A. Van Eeckhaut, "Microbore liquid chromatography with UV detection to study the in vivo passage of compound 21, a non-peptidergic AT2 receptor agonist, to the striatum in rats," *Journal of Neuroscience Methods*, vol. 202, pp. 137–142, 2011.

[105] "Spinifex EMA401 meets primary endpoint in phase 2 trail," in *Pharmaceutical Business Review*, Progressive Media Group, London, UK, 2012.

[106] J. J. Braszko, G. Kupryszewski, B. Witczuk, and K. Wisniewski, "Angiotensin II-(3-8)-hexapeptide affects motor activity, performance of passive avoidance and a conditioned avoidance response in rats," *Neuroscience*, vol. 27, no. 3, pp. 777–783, 1988.

[107] J. Lee, A. L. Albiston, A. M. Allen et al., "Effect of I.C.V. injection of AT4 receptor ligands, NLE 1-angiotensin IV and LVV-hemorphin 7, on spatial learning in rats," *Neuroscience*, vol. 124, no. 2, pp. 341–349, 2004.

[108] J. W. Wright, J. A. Clemens, J. A. Panetta et al., "Effects of LY231617 and angiotensin IV on ischemia-induced deficits in circular water maze and passive avoidance performance in rats," *Brain Research*, vol. 717, no. 1-2, pp. 1–11, 1996.

[109] J. W. Wright, L. Stubley, E. S. Pederson, E. A. Kramár, J. M. Hanesworth, and J. W. Harding, "Contributions of the brain angiotensin IV-AT4 receptor subtype system to spatial learning," *Journal of Neuroscience*, vol. 19, no. 10, pp. 3952–3961, 1999.

[110] J. J. Braszko, P. Wielgat, and A. Walesiuk, "Effect of D3 dopamine receptors blockade on the cognitive effects of angiotensin IV in rats," *Neuropeptides*, vol. 42, no. 3, pp. 301–309, 2008.

[111] J. Lee, T. Mustafa, S. G. McDowall et al., "Structure-activity study of LVV-hemorphin-7: angiotensin AT4 receptor ligand and inhibitor of insulin-regulated aminopeptidase," *Journal of Pharmacology and Experimental Therapeutics*, vol. 305, no. 1, pp. 205–211, 2003.

[112] S. Y. Chai, H. R. Yeatman, M. W. Parker et al., "Development of cognitive enhancers based on inhibition of insulin-regulated aminopeptidase," *BMC Neuroscience*, vol. 9, no. 2, article S14, 2008.

[113] A. L. Albiston, S. Diwakarla, R. N. Fernando et al., "Identification and development of specific inhibitors for insulin-regulated aminopeptidase as a new class of cognitive enhancers," *British Journal of Pharmacology*, vol. 164, no. 1, pp. 37–47, 2011.

[114] J. W. Wright and J. W. Harding, "Brain renin-angiotensin-A new look at an old system," *Progress in Neurobiology*, vol. 95, no. 1, pp. 49–67, 2011.

[115] J. Birks, "Cholinesterase inhibitors for Alzheimer's disease," *Cochrane Database of Systematic Reviews*, no. 1, Article ID CD005593, 2006.

[116] K. M. Cosman, L. L. Boyle, and A. P. Porsteinsson, "Memantine in the treatment of mild-to-moderate Alzheimer's disease," *Expert Opinion on Pharmacotherapy*, vol. 8, no. 2, pp. 203–214, 2007.

[117] P. M. Doraiswamy and G. L. Xiong, "Pharmacological strategies for the prevention of Alzheimer's disease," *Expert Opinion on Pharmacotherapy*, vol. 7, no. 1, pp. 1–10, 2006.

[118] P. Raina, P. Santaguida, A. Ismaila et al., "Effectiveness of cholinesterase inhibitors and memantine for treating dementia: evidence review for a clinical practice guideline," *Annals of Internal Medicine*, vol. 148, no. 5, pp. 379–397, 2008.

[119] G. N. Swanson, J. M. Hanesworth, M. F. Sardinia et al., "Discovery of a distinct binding site for angiotensin II (3-8), a putative angiotensin IV receptor," *Regulatory Peptides*, vol. 40, no. 3, pp. 409–419, 1992.

[120] J. W. Harding, V. I. Cook, A. V. Miller-Wing et al., "Identification of an AII (3-8) [AIV] binding site in guinea pig hippocampus," *Brain Research*, vol. 583, no. 1-2, pp. 340–343, 1992.

[121] M. De Gasparo, A. Husain, W. Alexander et al., "Proposed update of angiotensin receptor nomenclature," *Hypertension*, vol. 25, no. 5, pp. 924–927, 1995.

[122] M. F. Sardinia, J. M. Hanesworth, L. T. Krebs, and J. W. Harding, "AT4 receptor binding characteristics: D-Amino acid- and glycine-substituted peptides," *Peptides*, vol. 14, no. 5, pp. 949–954, 1993.

[123] M. F. Sardinia, "AT4 receptor Structure-Binding relationship: N-terminal-modified angiotensin IV analogues," *Peptides*, vol. 15, no. 8, pp. 1399–1406, 1994.

[124] R. Krishnan, J. M. Hanesworth, J. W. Wright, and J. W. Harding, "Structure-binding studies of the adrenal AT4 receptor: analysis of position two- and three-modified angiotensin IV analogs," *Peptides*, vol. 20, no. 8, pp. 915–920, 1999.

[125] L. T. Krebs, E. A. Kramár, J. M. Hanesworth et al., "Characterization of the binding properties and physiological action of divalinal-angiotensin IV, a putative AT4 receptor antagonist," *Regulatory Peptides*, vol. 67, no. 2, pp. 123–130, 1996.

[126] E. A. Kramár, D. L. Armstrong, S. Ikeda, M. J. Wayner, J. W. Harding, and J. W. Wright, "The effects of angiotensin IV analogs on long-term potentiation within the CA1 region of the hippocampus in vitro," *Brain Research*, vol. 897, no. 1-2, pp. 114–121, 2001.

[127] T. Kobori, K. Goda, K. Sugimoto, T. Ota, and K. Tomisawa, "Preparation of peptide derivatives as angiotensin IV receptor agonists," 1997, WO 97/03093 A1.

[128] T. Kobori, K. Goda, K. Sugimoto, T. Ota, and K. Tomisawa, "Preparation of amino acid derivatives as angiotensin IV receptor agonists," 1998, WO 98/05624 A1.

[129] A. L. Albiston, S. G. McDowall, D. Matsacos et al., "Evidence that the angiotensin IV (AT4) receptor is the enzyme insulin-regulated aminopeptidase," *Journal of Biological Chemistry*, vol. 276, no. 52, pp. 48623–48626, 2001.

[130] M. S. Wolfe, "Therapeutic strategies for Alzheimer's disease," *Nature Reviews Drug Discovery*, vol. 1, no. 11, pp. 859–866, 2002.

[131] M. Hallberg, "Targeting the insulin-regulated aminopeptidase/AT4 receptor for cognitive disorders," *Drug News and Perspectives*, vol. 22, no. 3, pp. 133–139, 2009.

[132] P. R. Gard, "Cognitive-enhancing effects of angiotensin IV," *BMC Neuroscience*, vol. 9, no. 2, article S15, 2008.

[133] J. W. Wright and J. W. Harding, "The angiotensin AT4 receptor subtype as a target for the treatment of memory dysfunction associated with Alzheimer's disease," *Journal of the Renin-Angiotensin-Aldosterone System*, vol. 9, no. 4, pp. 226–237, 2008.

[134] D. De Bundel, I. Smolders, P. Vanderheyden, and Y. Michotte, "Ang II and Ang IV: unraveling the mechanism of action on synaptic plasticity, memory, and epilepsy," *CNS Neuroscience and Therapeutics*, vol. 14, no. 4, pp. 315–339, 2008.

[135] M. Tsujimoto, S. Mizutani, H. Adachi, M. Kimura, H. Nakazato, and Y. Tomoda, "Identification of human placental leucine aminopeptidase as oxytocinase," *Archives of Biochemistry and Biophysics*, vol. 292, no. 2, pp. 388–392, 1992.

[136] T. Rogi, M. Tsujimoto, H. Nakazato, S. Mizutani, and Y. Tomoda, "Human placental leucine aminopeptidase/oxytocinase: a new member of type II membrane-spanning zinc metallopeptidase family," *Journal of Biological Chemistry*, vol. 271, no. 1, pp. 56–61, 1996.

[137] T. E. Rasmussen, S. Pedraza-Diaz, R. Hardre, P. G. Laustsen, A. G. Carrion, and T. Kristensen, "Structure of the human oxytocinase/insulin-regulated aminopeptidase gene and localization to chromosome 5q21," *European Journal of Biochemistry*, vol. 267, no. 8, pp. 2297–2306, 2000.

[138] S. R. Keller, H. M. Scott, C. C. Mastick, R. Aebersold, and G. E. Lienhard, "Cloning and characterization of a novel insulin-regulated membrane aminopeptidase from Glut4 vesicles," *Journal of Biological Chemistry*, vol. 270, no. 40, pp. 23612–23618, 1995.

[139] P. G. Laustsen, S. Vang, and T. Kristensen, "Mutational analysis of the active site of human insulin-regulated aminopeptidase," *European Journal of Biochemistry*, vol. 268, no. 1, pp. 98–104, 2001.

[140] P. G. Laustsen, T. E. Rasmussen, K. Petersen et al., "The complete amino acid sequence of human placental oxytocinase," *Biochimica et Biophysica Acta*, vol. 1352, no. 1, pp. 1–7, 1997.

[141] A. L. Albiston, G. R. Peck, H. R. Yeatman, R. Fernando, S. Ye, and S. Y. Chai, "Therapeutic targeting of insulin-regulated aminopeptidase: heads and tails?" *Pharmacology and Therapeutics*, vol. 116, no. 3, pp. 417–427, 2007.

[142] K. Ito, Y. Nakajima, Y. Onohara et al., "Crystal structure of aminopeptidase N (Proteobacteria alanyl aminopeptidase) from Escherichia coli and conformational change of methionine 260 involved in substrate recognition," *Journal of Biological Chemistry*, vol. 281, no. 44, pp. 33664–33676, 2006.

[143] F. Tholander, A. Muroya, B. P. Roques, M. C. Fournié-Zaluski, M. M. G. M. Thunnissen, and J. Z. Haeggström, "Structure-based dissection of the active site chemistry of leukotriene A4 hydrolase: implications for M1 aminopeptidases and inhibitor design," *Chemistry and Biology*, vol. 15, no. 9, pp. 920–929, 2008.

[144] N. Luciani, C. Marie-Claire, E. Ruffet, A. Beaumont, B. P. Roques, and M. C. Fournié-Zaluski, "Characterization of

Glu350 as a critical residue involved in the N-terminal amine binding site of aminopeptidase N (EC 3.4.11.2): insights into its mechanism of action," *Biochemistry*, vol. 37, no. 2, pp. 686–692, 1998.

[145] S. Ye, S. Y. Chai, R. A. Lew, and A. L. Albiston, "Insulin-regulated aminopeptidase: analysis of peptide substrate and inhibitor binding to the catalytic domain," *Biological Chemistry*, vol. 388, no. 4, pp. 399–403, 2007.

[146] S. Ye, S. Y. Chai, R. A. Lew et al., "Identification of modulating residues defining the catalytic cleft of insulin-regulated aminopeptidase," *Biochemistry and Cell Biology*, vol. 86, no. 3, pp. 251–261, 2008.

[147] H. Matsumoto, T. Rogi, K. Yamashiro et al., "Characterization of a recombinant soluble form of human placental leucine aminopeptidase/oxytocinase expressed in Chinese hamster ovary cells," *European Journal of Biochemistry*, vol. 267, no. 1, pp. 46–52, 2000.

[148] M. G. Wallis, M. F. Lankford, and S. R. Keller, "Vasopressin is a physiological substrate for the insulin-regulated aminopeptidase IRAP," *American Journal of Physiology*, vol. 293, no. 4, pp. E1092–E1102, 2007.

[149] A. L. Albiston, T. Mustafa, S. G. McDowall, F. A. O. Mendelsohn, J. Lee, and S. Y. Chai, "AT4 receptor is insulin-regulated membrane aminopeptidase: potential mechanisms of memory enhancement," *Trends in Endocrinology and Metabolism*, vol. 14, no. 2, pp. 72–77, 2003.

[150] S. Y. Chai, R. Fernando, G. Peck et al., "The angiotensin IV/AT4 receptor," *Cellular and Molecular Life Sciences*, vol. 61, no. 21, pp. 2728–2737, 2004.

[151] G. L. Kovacs and D. De Wied, "Peptidergic modulation of learning and memory processes," *Pharmacological Reviews*, vol. 46, no. 3, pp. 269–291, 1994.

[152] B. Alescio-Lautier, V. Paban, and B. Soumireu-Mourat, "Neuromodulation of memory in the hippocampus by vasopressin," *European Journal of Pharmacology*, vol. 405, no. 1–3, pp. 63–72, 2000.

[153] M. A. Gulpinar and B. C. Yegen, "The physiology of learning and memory: role of peptides and stress," *Current Protein & Peptide Science*, vol. 5, pp. 457–473, 2004.

[154] A. L. Albiston, R. N. Fernando, H. R. Yeatman et al., "Gene knockout of insulin-regulated aminopeptidase: loss of the specific binding site for angiotensin IV and age-related deficit in spatial memory," *Neurobiology of Learning and Memory*, vol. 93, no. 1, pp. 19–30, 2010.

[155] E. B. Einstein, C. A. Patterson, B. J. Hon et al., "Somatostatin signaling in neuronal cilia is critical for object recognition memory," *Journal of Neuroscience*, vol. 30, no. 12, pp. 4306–4314, 2010.

[156] R. N. Fernando, A. L. Albiston, and S. Y. Chai, "The insulin-regulated aminopeptidase IRAP is colocalised with GLUT4 in the mouse hippocampus—potential role in modulation of glucose uptake in neurones?" *European Journal of Neuroscience*, vol. 28, no. 3, pp. 588–598, 2008.

[157] P. M. L. Vanderheyden, "From angiotensin IV binding site to AT4 receptor," *Molecular and Cellular Endocrinology*, vol. 302, no. 2, pp. 159–166, 2009.

[158] I. Garreau, D. Chansel, S. Vandermeersch, I. Fruitier, J. M. Piot, and R. Ardaillou, "Hemorphins inhibit angiotensin IV binding and interact with aminopeptidase N," *Peptides*, vol. 19, no. 8, pp. 1339–1348, 1998.

[159] H. Demaegdt, P. J. Lenaerts, J. Swales et al., "Angiotensin AT4 receptor ligand interaction with cystinyl aminopeptidase and aminopeptidase N: [125I]Angiotensin IV only binds to the cystinyl aminopeptidase apo-enzyme," *European Journal of Pharmacology*, vol. 546, no. 1–3, pp. 19–27, 2006.

[160] J. W. Wright, B. J. Yamamoto, and J. W. Harding, "Angiotensin receptor subtype mediated physiologies and behaviors: new discoveries and clinical targets," *Progress in Neurobiology*, vol. 84, no. 2, pp. 157–181, 2008.

[161] B. J. Yamamoto, P. D. Elias, J. A. Masino et al., "The angiotensin IV analog Nle-Tyr-Leu-$\psi$-(CH2-NH 2)3-4-His-Pro-Phe (Norleual) can act as a hepatocyte growth factor/c-met inhibitor," *Journal of Pharmacology and Experimental Therapeutics*, vol. 333, no. 1, pp. 161–173, 2010.

[162] J. W. Wright and J. W. Harding, "The brain angiotensin IV/AT4 receptor system as a new target for the treatment of Alzheimer's disease," *Drug Development Research*, vol. 70, no. 7, pp. 472–480, 2009.

[163] A. McCoy, *Pharmacokinetic characterization of angiotensin IV analogs with therapeutic potential for cancer and dementia [thesis from Department of Veterinary and Comparative Anatomy, Pharmacology, and Physiology]*, Washington State University, Washington, DC, USA, 2010.

[164] R. A. Lew, T. Mustafa, S. Ye, S. G. McDowall, S. Y. Chai, and A. L. Albiston, "Angiotensin AT4 ligands are potent, competitive inhibitors of insulin regulated aminopeptidase (IRAP)," *Journal of Neurochemistry*, vol. 86, no. 2, pp. 344–350, 2003.

[165] H. Demaegdt, H. Laeremans, J. P. De Backer et al., "Synergistic modulation of cystinyl aminopeptidase by divalent cation chelators," *Biochemical Pharmacology*, vol. 68, no. 5, pp. 893–900, 2004.

[166] H. Laeremans, H. Demaegdt, J. P. De Backer et al., "Metal ion modulation of cystinyl aminopeptidase," *Biochemical Journal*, vol. 390, no. 1, pp. 351–357, 2005.

[167] H. Demaegdt, A. Lukaszuk, E. De Buyser et al., "Selective labeling of IRAP by the tritiated AT4 receptor ligand [3H]Angiotensin IV and its stable analog [3H]AL-11," *Molecular and Cellular Endocrinology*, vol. 311, no. 1-2, pp. 77–86, 2009.

[168] A. Lukaszuk, H. Demaegdt, E. Szemenyei et al., "$\beta$-homo-amino acid scan of angiotensin IV," *Journal of Medicinal Chemistry*, vol. 51, no. 7, pp. 2291–2296, 2008.

[169] R. Yang, I. Smolders, D. De Bundel et al., "Brain and peripheral angiotensin II type 1 receptors mediate renal vasoconstrictor and blood pressure responses to angiotensin IV in the rat," *Journal of Hypertension*, vol. 26, no. 5, pp. 998–1007, 2008.

[170] A. Lukaszuk, H. Demaegdt, D. Feytens, P. Vanderheyden, G. Vauquelin, and D. Tourwé, "The replacement of His(4) in angiotensin IV by conformationally constrained residues provides highly potent and selective analogues," *Journal of Medicinal Chemistry*, vol. 52, no. 18, pp. 5612–5618, 2009.

[171] H. Chen, B. P. Roques, and M. C. Fournié-Zaluski, "Design of the first highly potent and selective aminopeptidase N (EC 3.4.11.2) inhibitor," *Bioorganic and Medicinal Chemistry Letters*, vol. 9, no. 11, pp. 1511–1516, 1999.

[172] H. Demaegdt, J. P. De Backer, A. Lukaszuk et al., "Angiotensin IV displays only low affinity for native insulin-regulated aminopeptidase (IRAP)," *Fundamental and Clinical Pharmacology*, vol. 26, pp. 194–197, 2012.

[173] H. Demaegdt, L. Smitz, J. P. De Backer et al., "Translocation of the insulin-regulated aminopeptidase to the cell surface: detection by radioligand binding," *British Journal of Pharmacology*, vol. 154, no. 4, pp. 872–881, 2008.

[174] A. Axén, H. Andersson, G. Lindeberg et al., "Small potent ligands to the insulin-regulated aminopeptidase (IRAP)/AT4

receptor," *Journal of Peptide Science*, vol. 13, no. 7, pp. 434–444, 2007.

[175] D. B. Ascher, B. A. Cromer, C. J. Morton et al., "Regulation of insulin-regulated membrane aminopeptidase activity by its C-terminal domain," *Biochemistry*, vol. 50, no. 13, pp. 2611–2622, 2011.

[176] A. Lukaszuk, H. Demaegdt, I. van den Eynde, P. Vanderheyden, G. Vauquelin, and D. Tourwé, "Conformational constraints in angiotensin IV to probe the role of Tyr2, Pro5 and Phe6," *Journal of Peptide Science*, vol. 17, no. 8, pp. 545–553, 2011.

[177] H. Andersson, H. Demaegdt, G. Vauquelin, G. Lindeberg, A. Karlén, and M. Hallberg, "Ligands to the (IRAP)/AT4 receptor encompassing a 4-hydroxydiphenylmethane scaffold replacing Tyr2," *Bioorganic and Medicinal Chemistry*, vol. 16, no. 14, pp. 6924–6935, 2008.

[178] A. Axén, G. Lindeberg, H. Demaegdt, G. Vauquelin, A. Karlén, and M. Hallberg, "Cyclic insulin-regulated aminopeptidase (IRAP)/AT4 receptor ligands," *Journal of Peptide Science*, vol. 12, no. 11, pp. 705–713, 2006.

[179] H. Andersson, H. Demaegdt, A. Johnsson et al., "Potent macrocyclic inhibitors of insulin-regulated aminopeptidase (IRAP) by olefin ring-closing metathesis," *Journal of Medicinal Chemistry*, vol. 54, no. 11, pp. 3779–3792, 2011.

[180] H. Andersson, H. Demaegdt, G. Vauquelin et al., "Disulfide cyclized tripeptide analogues of angiotensin IV as potent and selective inhibitors of insulin-regulated aminopeptidase (IRAP)," *Journal of Medicinal Chemistry*, vol. 53, no. 22, pp. 8059–8071, 2010.

[181] B. Schmidt, S. Lindman, W. Tong et al., "Design, synthesis, and biological activities of four angiotensin II receptor ligands with $\gamma$-turn mimetics replacing amino acid residues 3–5," *Journal of Medicinal Chemistry*, vol. 40, no. 6, pp. 903–919, 1997.

[182] S. Lindman, G. Lindeberg, A. Gogoll, F. Nyberg, A. Karlén, and A. Hallberg, "Synthesis, receptor binding affinities and conformational properties of cyclic methylenedithioether analogues of angiotensin II," *Bioorganic and Medicinal Chemistry*, vol. 9, no. 3, pp. 763–772, 2001.

[183] E. Marsault and M. L. Peterson, "Macrocycles are great cycles: applications, opportunities, and challenges of synthetic macrocycles in drug discovery," *Journal of Medicinal Chemistry*, vol. 54, no. 7, pp. 1961–2004, 2011.

[184] E. M. Driggers, S. P. Hale, J. Lee, and N. K. Terrett, "The exploration of macrocycles for drug discovery—An underexploited structural class," *Nature Reviews Drug Discovery*, vol. 7, no. 7, pp. 608–624, 2008.

[185] M. M. G. M. Thunnissen, P. Nordlund, and J. Z. Haeggström, "Crystal structure of human leukotriene A4 hydrolase, a bifunctional enzyme in inflammation," *Nature Structural Biology*, vol. 8, no. 2, pp. 131–135, 2001.

[186] A. L. Albiston, C. J. Morton, L. N. Hooi et al., "Identification and characterization of a new cognitive enhancer based on inhibition of insulin-regulated aminopeptidase," *FASEB Journal*, vol. 22, no. 12, pp. 4209–4217, 2008.

[187] A. L. Albiston, V. Pham, S. Ye et al., "Phenylalanine-544 plays a key role in substrate and inhibitor binding by providing a hydrophobic packing point at the active site of insulin-regulated aminopeptidase," *Molecular Pharmacology*, vol. 78, no. 4, pp. 600–607, 2010.

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*Review Article*

# Renin-Angiotensin System and Sympathetic Neurotransmitter Release in the Central Nervous System of Hypertension

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Many Studies suggest that changes in sympathetic nerve activity in the central nervous system might have a crucial role in blood pressure control. The present paper discusses evidence in support of the concept that the brain renin-angiotensin system (RAS) might be linked to sympathetic nerve activity in hypertension. The amount of neurotransmitter release from sympathetic nerve endings can be regulated by presynaptic receptors located on nerve terminals. It has been proposed that alterations in sympathetic nervous activity in the central nervous system of hypertension might be partially due to abnormalities in presynaptic modulation of neurotransmitter release. Recent evidence indicates that all components of the RAS have been identified in the brain. It has been proposed that the brain RAS may actively participate in the modulation of neurotransmitter release and influence the central sympathetic outflow to the periphery. This paper summarizes the results of studies to evaluate the possible relationship between the brain RAS and sympathetic neurotransmitter release in the central nervous system of hypertension.

## 1. Introduction

There is increasing evidence to suggest that sympathetic nervous activity in both central and peripheral nervous systems may play a major role in the regulation of blood pressure, and that hypertension is accompanied characteristically by increased sympathetic nervous activity in both humans and animal models [1, 2]. Pharmacologic studies have demonstrated that depletion of central and peripheral catecholamine stores could prevent or attenuate the development of hypertension [3, 4]. The concept on the release of sympathetic neurotransmitters from nerve endings has been considerably refined by the demonstration of multiple presynaptic receptors, which were shown to either facilitate or inhibit their release [5, 6]. The renin-angiotensin system (RAS) including angiotensin receptors is widely distributed in the brain [7–9]. It is proposed that the RAS facilitates the sympathetic nervous system and that angiotensin-converting enzyme (ACE) inhibition and angiotensin receptor blockade are antiadrenergic [10, 11]. However, the interaction between the brain RAS and sympathetic nervous system are not fully understood. In the present paper, we discuss the relationship between the brain RAS and sympathetic neurotransmitter release and further evaluate the role of RAS in the regulation of sympathetic nerve activity in the central nervous system of hypertension.

## 2. Amount of Norepinephrine Release in the Central Nervous System of Hypertension

Augmented norepinephrine (NE) release and catecholamine synthesis as well as tyrosine hydroxylase gene expression have been reported at central sites related to blood pressure regulation in adult spontaneously hypertensive rats (SHR) [12, 13]. In a study presented earlier, Qualy and Westfall [14] observed that stimulation-evoked NE release from paraventricular hypothalamic nucleus in hypertensive rats was significantly increased in comparison with normotensive rats. We have been demonstrating that the stimulation-evoked NE release from hypothalamic slices was significantly greater in SHR than in normotensive Wistar-Kyoto (WKY) rats, suggesting that the release of NE from the hypothalamus may contribute to the elevated sympathetic nerve

activity in hypertension [15–22]. It was also demonstrated that, by using electrophysiological method, higher discharge rates were detected in neurons of the rostral ventrolateral medulla (RVLM) of neonatal SHR [23]. On the other hand, the stimulation-evoked NE release in the slices of whole medulla oblongata was not significantly different between SHR and WKY rats [24–29]. Recently, Teschemacher et al. [30] demonstrated that although overall electrophysiological characteristics of C1 and A2 catecholaminergic neurons in the brain were compatible between SHR and WKY rats, the angiotensin II- (Ang II-) induced $Ca^{2+}$-mobilization was reduced in A2 neurons of SHR. Because A2 neurons are a part of an antihypertensive circuit, the reduced sensitivity of the A2 cells to Ang II might further compromise their homeostatic role in SHR. There might be regional differences in the amount of NE release in the central nervous system of hypertensive models.

In human hypertensive subjects, Esler et al. [31] studied the brain NE release and its relation to peripheral sympathetic nervous activity by using transmitter washout method. They showed that overall NE overflow into the internal jugular vein was significantly increased in subjects with essential hypertension compared with normotensive subjects. The finding indicated that central sympathetic tone might be activated in essential hypertension, although precise mechanisms regulating central sympathetic neurotransmitter release in human hypertension is not fully understood.

## 3. Role of Renin-Angiotensin System in the Regulation of Norepinephrine Release in the Central Nervous System

*3.1. Angiotensin II.* It was shown that angiotensin I (Ang I) and Ang II injected to the central nervous system significantly elevated blood pressure [32]. Davern and Head [33] showed that the chronic subcutaneous infusion of Ang II caused rapid and marked neuronal activation in circumventricular organs, such as subfornical organ, the nucleus of the solitary tract, paraventricular nucleus, and supraoptic nucleus. In an in vitro study presented earlier, Garcia-Sevilla et al. [34] demonstrated that Ang II facilitated in a concentration-dependent manner the potassium-evoked NE release in the rabbit hypothalamus, which was antagonized by saralasin. The result indicated that the increase in NE release might be mediated through presynaptic angiotensin facilitatory receptors on noradrenergic nerve terminals. It was also shown that Ang II increased the potassium-evoked NE release from slices of rat parietal cortex, and that the effect was blocked by saralasin, but not by the Ca channel blocker, nimodipine [35]. Moreover, the facilitative action of Ang II on NE release might be pronounced in the hypothalamus of SHR compared with normotensive rats [36]. In a microdialysis study, Qadri et al. [37] showed that intracerebroventricular administration of 100 ng of Ang II increased blood pressure and NE release in anterior hypothalamus of conscious rats, which was antagonized by the Ang II receptor blocker. By using the similar method, Stadler et al. [38] also reported that Ang II led to significant dose-dependent increases of NE release in the paraventricular nucleus.

Several lines of evidence demonstrate that function and signaling of the angiotensin type 1 ($AT_1$) receptors are quite different from the angiotensin type 2 ($AT_2$) sites and that these receptors may exert opposite effects on blood pressure regulation [39]. Gelband et al. [40] demonstrated that neuronal $AT_1$ receptors might have a pivotal role in NE neuromodulation, and that evoked NE neuromodulation might involve $AT_1$ receptor-mediated, losartan-dependent, rapid NE release, inhibition of potassium-channels and stimulation of $Ca^{2+}$-channels. Furthermore, they proposed that $AT_1$ receptor-mediated enhanced neuromodulation might involve the Ras-Raf-MAP kinase cascade and lead to an increase in NE transporter, tyrosine hydroxylase, and dopamine $\beta$-hydroxylase mRNA transcription. On the other hand, neuronal $AT_2$ receptors might signal via a Gi-protein and be coupled to activation of PP2A and PLA2, and stimulation of potassium-channels. Nap et al. [41] showed that prejunctional $AT_1$ receptors might belong to the $AT_{1B}$ receptor subtype because $AT_{1B}$ receptor inhibition by high concentrations of PD 123319 could suppress the Ang II-augmented noradrenergic transmission. Gironacci et al. [36] demonstrated that Ang-(1-7), which is synthesized by angiotensin converting enzyme 2 (ACE2), significantly decreased the potassium-induced NE release in the hypothalamus of SHR by stimulating the $AT_2$ receptors. In addition, they showed that the inhibitory effect of Ang-(1-7) on NE release was blocked by the nitric oxide (NO) synthase inhibitor and the bradykinin (BK) $B_2$ receptor antagonist. The finding indicated that Ang-(1-7) reduced NE release from the hypothalamus of SHR via the $AT_2$ receptors, acting through a BK/NO-mediated mechanism. Recent findings have also revealed that the Mas oncogene may act as a receptor for Ang-(1-7) [42–44]. It is strongly suggested that activation of the ACE2-Ang-(1-7)-Mas axis might act as a counterregulatory system against the ACE-Ang II-$AT_1$ receptor axis [42–44].

Bourassa et al. [45] demonstrated that $AT_1$ receptor binding in both RVLM and caudal ventrolateral medulla as well as dorsomedial medulla was increased in SHR compared with normotensive rats. Conversely, expression of the novel, non-$AT_1$, non-$AT_2$, Ang II and III binding site, which was recently discovered, might be decreased in the RVLM and dorsomedial medulla of SHR [45]. They proposed that increased $AT_1$ receptor binding in the RVLM might contribute to the hypertension of SHR, whereas reduced radioligand binding to the novel, non-$AT_1$, non-$AT_2$, angiotensin binding site in the RVLM of SHR might indicate a role for this binding site to reduce blood pressure via its interactions with Ang II and III.

In this context, the facilitative effect of Ang II on NE release might be an important factor in the excitation of sympathetic tone in the central nervous system, although further studies should be performed to assess more thoroughly the precise roles of the different types of Ang II receptors in the regulation of central sympathetic nerve activity in hypertension.

*3.2. Angiotensin-Converting Enzyme Inhibitors.* All components of the RAS have been identified in the brain, and the brain RAS might actively participate not only in blood pressure elevation, but also in target organ damages [7–9, 46]. It is proposed that the inhibition of brain ACE activity may be associated with blood pressure reduction induced by ACE inhibitors (ACEIs). Captopril, a widely accepted ACEI, may block the conversion of Ang I to Ang II and has been used as an effective antihypertensive agent in both human and experimental hypertension [47, 48]. Several studies have provided evidence that the distribution of the target sites for ACEIs might be widespread [49, 50]. It was demonstrated that captopril administered centrally significantly lowered blood pressure in intact conscious SHR [51]. Intracerebroventricular administration of captopril significantly suppressed the pressor responses to Ang I given by the same route in SHR [52]. Baum et al. [53] also observed the attenuation of pressor responses to intracerebroventricular Ang I by ACEI in conscious rats. It was shown that oral administration of captopril caused the inhibition of brain ACE activities [50, 54, 55]. It was demonstrated that the ACE activities in various tissues 1 hour after oral administration of several ACEIs and that captopril significantly reduced the ACE activities in aorta, heart, kidney, lung, and brain. Berecek et al. [56] showed that chronic intracerebroventricular injection of captopril attenuated the development of hypertension in young SHR in association with a depression in whole animal reactivity to vasoactive agents and an increased baroreflex sensitivity. It was also observed that central administration of captopril produced a significant depression in vascular reactivity to vasoconstrictor agents in the isolated perfused kidney of SHR in vivo [57]. The findings propose the hypothesis that central action of captopril might be, at least in part, related to vascular relaxation.

The different structures of ACEIs may influence their tissue distribution and routes of elimination. Cushman et al. [55] examined the effects of various ACEIs on brain ACE activity in SHR. They showed that not only captopril, but also zofenopril produced a modest, short-lasting inhibition of ACE activity in the SHR brain. On the other hand, fosinopril, lisinopril, and SQ 29,852 had delayed but long-lasting inhibitory actions, and ramipril and enalapril showed no effects. More studies are necessary to determine the distribution, binding activity to ACE, and metabolism of each ACEI in the brain.

In an in vitro study presented previously, we showed that captopril significantly inhibited the stimulation-evoked NE release in slices of rat hypothalamus and medulla oblongata [58], as well as in peripheral tissues, such as rat mesenteric arteries [59]. It might be possible that the inhibition of NE release by captopril might be partially due to a reduction in Ang II formation in the central nervous system. The modulation of central NE release by captopril might reduce the sympathetic outflow to the periphery, which could partially explain the hypotensive effects of the ACEI. Recently, Bolterman et al. [60] showed that captopril selectively lowered Ang II, oxidative stress, and endothelin in SHR. On the other hand, it was demonstrated that captopril had an asymmetrical effect on the angiotensinase activity in frontal cortex and plasma of SHR [61]. It might be possible that multiple neuroendocrine actions of ACEIs in the brain could, at least in part, contribute to their blood pressure-lowering efficacy in both human and experimental hypertension.

*3.3. Angiotensin Receptor Blockers.* It was shown that Ang II administered intracerebroventricularly at a dose that induces drinking behavior in rats significantly increased potassium-stimulated release of NE in the hypothalamus [62]. It can be suggested that Ang II is important primarily in pathological states and that NE plays a substantial role in the brain Ang II-induced drinking response. Furthermore, losartan, an angiotensin receptor blocker (ARB), significantly inhibited the potassium-stimulated NE release in the hypothalamus, acting via the AT1 receptor subtype [62]. Averill et al. [63] showed that losartan attenuated the pressor and sympathetic overactivity induced by Ang II and L-glutamate (an excitatory amino acid) in RVLM of SHR. Huang et al. [64] showed that central infusion of an $AT_1$ receptor blocker prevented sympathetic hyperactivity and hypertension in Dahl salt-sensitive hypertensive rats on high salt diet.

Previous studies have reported only a limited ability of systemic ARB to cross the blood brain barrier [65–69]. Gohlke et al. [65] reported that orally applied $AT_1$ receptor blocker candesartan suppressed the central responses of Ang II in a dose- and time-dependent manner in conscious rats, indicating that the $AT_1$ receptor blocker might effectively inhibit the centrally mediated action of Ang II upon peripheral application. It was also shown that peripherally administered candesartan markedly decreased $AT_1$ binding areas outside (subfornical organ and area postrema) and inside (paraventricular nucleus of the hypothalamus and nucleus of the solitary tract) the blood-brain barrier [66, 67]. Unger [68] proposed that candesartan might be the most effective ARB in crossing the blood-brain barrier. On the other hand, Pelisch et al. [69] demonstrated that CV-11974, the active form of candesartan, was undetectable in brain tissue after oral candesartan treatment, suggesting that candesartan may not cross the intact blood-brain barrier in its active form, or may reach the brain at undetectable levels. They proposed the possibility that undetectable levels of ARB in the brain tissue would be enough to modulate the brain RAS. Further studies are required to determine the relationship between the molecular characteristics of ARBs and their properties to cross the blood-brain barrier [69].

In human hypertensive subjects, Esler [70] proposed that the ability of ARBs to antagonize neural presynaptic angiotensin $AT_1$ receptors appears to differ markedly between the individual agents in this drug class. Recently, Krum et al. [71] examined whether ARBs inhibited central sympathetic outflow in human subjects. Using the whole body NE spillover method in humans with essential hypertension, they demonstrated that eprosartan and losartan did not materially inhibit central sympathetic outflow or act presynaptically to reduce NE release at existing rates of nerve firing. They concluded that sympathetic nervous inhibition might not be a major component of the blood

pressure-lowering action of ARBs in subjects with essential hypertension.

On the other hand, de Champlain et al. [72] showed that the hypotensive action of valsartan may be mediated in part by an inhibition of the sympathetic baroreflex in patients with essential hypertension. It was also demonstrated that valsartan not only decreased blood pressure, but also shifted the baroreflex set point in hypertensive subjects [73]. Additional studies are necessary to determine the potential effects of ARBs on sympathetic nerve activity in the central nervous system of both human and experimental hypertension.

*3.4. Direct Renin Inhibitors.* Recent years have seen the development of the nonpeptide, orally long-term effective direct renin inhibitor (DRI), aliskiren, which may block the initial stages of the RAS and exert a sustained antihypertensive action in hypertensive subjects [74–76].

With regard to the influences of aliskiren on central sympathetic nervous system, Huang et al. [64] examined whether central infusion of aliskiren prevented sympathetic hyperactivity and hypertension in Dahl salt-sensitive hypertensive rats on high salt diet. Intracerebroventricular infusion of aliskiren markedly inhibited the increase in Ang II levels in the cerebrospinal fluid and in blood pressure caused by intracerebroventricular infusion of rat renin. In Dahl-salt sensitive rats, high salt intake increased resting blood pressure, enhanced pressor and sympathoexcitatory responses to air stress, and desensitized arterial baroreflex function. All of these effects were significantly prevented by intracerebroventricular infusion of aliskiren. These results indicated that intracerebroventricular infusions of aliskiren were effective in preventing salt-induced sympathetic hyperactivity and hypertension in Dahl-salt sensitive rats. Because the ARB also exerted similar effects [64], the result strongly confirms the idea that renin in the brain plays an important role in the salt-induced hypertension.

In a clinical study, it was shown that aliskiren significantly reduced sympathetic hyperactivity and blood pressure in patients with chronic kidney disease (CKD) [77]. However, Fogari et al. [78] reported that the increase in plasma NE evoked by Ca channel blocker, amlodipine, was not reduced by aliskiren addition in hypertensive subjects. It is strongly suggested that DRI might directly inhibit sympathetic neurotransmitter release, and that the hypotensive action of DRI might largely depend on its inhibitory effect on the sympathetic nerve activity in the central nervous system.

*3.5. (Pro)renin Receptor.* Recent evidence indicates that the (pro)renin receptor (PRR), which is a newly discovered member of the brain RAS, might contribute to the pathogenesis of hypertension [79–82]. Li et al. [83] demonstrated that PRR protein was highly expressed in neurons and upregulated in the subfornical organ and the periventricular nucleus in Ang II-dependent hypertensive mice. In addition, they found that PRR knockdown in the brain significantly decreased blood pressure in renin-angiotensinogen transgenic hypertensive mice, which was associated with a decrease in sympathetic tone and improvement of spontaneous baroreflex sensitivity. Furthermore, PRR knockdown was associated with downregulation of the $AT_1$ receptors. It is proposed that PRR blockade in the central nervous system might represent a novel approach for the treatment of neurogenic hypertension.

*3.6. Mineralcorticoid Receptor.* It has been shown that aldosterone as well as Ang II might act within the central nervous system and cause the sympathetic hyperactivity to increase blood pressure [84–87]. Huang et al. [84] proposed that brain aldosterone-mineralcorticoid receptor (MR)-ouabain pathway might have a pivotal role in Ang II-induced neuronal activation and pressor responses. The sympathoexcitatory effect of aldosterone was blocked by intracerebroventricular of MR antagonist [86]. It was also shown that central infusion of aldosteone synthase inhibitor prevented sympathetic hyperactivity and hypertension by central sodium loading [88]. In a clinical study, Raheja et al. [89] showed that MR blockade by spironolactone prevented chlorthalidone-induced sympathetic activation in hypertensive subjects. Blockade of MR receptors and inhibition of aldosterone synthesis in the central nervous system might lead to a reduction in systemic blood pressure, although it remains unclear whether MR receptor blockers might exert greater antihypertensive effects than other RAS inhibitors.

*3.7. Oxidative Stress.* The involvement of oxidative stress is implicated in the pathogenesis of hypertension. It was demonstrated that superoxide anions in the RVLM, which might generate hydroxyl radicals, were increased in SHR-SP (stroke prone), suggesting that increased oxidative stress would lead to enhancing the central sympathetic outflow [90, 91]. Recent evidence indicates the possible link between Ang II and NADPH oxidase-derived oxidative stress in the central nervous system [92, 93]. Mertens et al. [93] proposed that $AT_1$ receptors might activate the NADPH oxidase complex which could be the most important source of reactive oxygen species (ROS) and suggested that the produced superoxide anion would be converted into $H_2O_2$ by superoxide dismutase or combine with NO to generate peroxynitrite, thereby decreasing NO-bioavailability and promoting lipid and protein oxidation. Additional studies are necessary in order to further unravel the implications of oxidative stress in the Ang II-induced neurotoxicity in the brain.

## 4. Renin Angiotensin System and Other Neurotransmitter Release in the Central Nervous System

*4.1. Dopamine Release.* There is increasing evidence to suggest that dopaminergic nerve activity in the central nervous system may play a crucial role in the regulation of blood pressure [94–102]. Recent evidence has suggested a functional interaction between brain angiotensin mechanisms and dopamine neurons, although the influences of the

RAS on central dopaminergic activities in hypertension are controversial [94–102].

It has been demonstrated that ACE is localized throughout the brain and, importantly, is found in neurons of striatum and nigrostriatal tract [48]. In addition, there has been increasing evidence in favor of the involvement of nigrostriatal dopaminergic systems in the pathogenesis of hypertension. Linthorst et al. [95] reported that substantia nigra lesions caused a profound attenuation of the development of hypertension in SHR. Brown et al. [103] showed that the Ang II-induced dopamine release was completely blocked by the $AT_1$ receptor antagonist, losartan, but not by the $AT_2$ receptor antagonist, PD 123177, suggesting that Ang II acting via the $AT_1$ receptor subtype might facilitate the release of dopamine, in the rat striatum. By using the microdialysis method, Mendelsohn et al. [104] showed that administration of Ang II into the rat striatum caused an increase in the release of dihydroxyphenylacetic acid (DOPAC), a dopamine metabolite, and proposed that dopamine release was under a tonic facilitative influence of Ang II.

On the other hand, it has been proposed that the nigrostriatal dopaminergic system may mediate baroreflex sensitivity in rats because both striatal dopamine release and baroreflex responses produced by phenylephrine and carotid occlusion were attenuated by lesions of the nigrostriatal dopaminergic pathway [105]. It has also been reported that centrally administered Ang II increased exploratory behavior in rats and that this effect was antagonized by the dopamine receptor antagonist sulpiride. The finding indicated that Ang II potentiated central dopaminergic effects and dopamine-mediated behavioral responses [106]. Banks et al. [107] demonstrated that the ACE inhibitor captopril inhibited apomorphine-induced oral stereotypy in rats. The observation suggests that a decrease in Ang II formation caused by the ACE inhibitor could block nigrostriatal output. In an in vitro study, we examined the effects of captopril on the release of dopamine, and further determined a possible role of RAS in the regulation of dopaminergic neurotransmission in the central nervous system [108]. We showed that captopril inhibited the release of dopamine in the rat striatum in a dose-dependent manner [108]. Although the mechanisms underlying the neurosuppressive effects of captopril remain to be elucidated, the finding suggests that the inhibition of dopaminergic neurotransmission may be related to the central action of the ACEI.

Jenkins et al. [109] observed that $AT_1$ receptors have been identified on dopamine containing cells in the substantia nigra and striatum of human brain using receptor autoradiography. Mertens et al. [110] showed that $AT_1$ and $AT_2$ receptors might exert an opposite effect on the modulation of DA synthesis in the striatum. Speth and Karamyan [111] demonstrated that the novel, non-$AT_1$, non-$AT_2$ binding site for angiotensin peptides as a mediator of nontraditional actions of Ang II, could have a role in the stimulation of dopamine release from the striatum.

Several studies have been made to elucidate the possible link between the brain RAS and the dopaminergic cell death in Parkinson's disease [93, 112, 113]. One hypothesis is that $AT_1$ receptors might play a pivotal role in Parkinson's disease. As mentioned above, the stimulation of $AT_1$ receptors might lead to the activation of the NADPH oxidase complex and the generation of ROS [92, 93]. Mertens et al. [93] proposed that $AT_2$ receptor agonists alone or in combination with $AT_1$ receptor blockers might effectively improve the pathological conditions in Parkinson's disease. It was also shown that chronic treatment of ACEI increased striatal dopamine content in the MPTP-mouse [112]. On the other hand, it was demonstrated that administration of PRR blocker significantly decreased 6-hydroxydopamine-induced dopamine cell death in the cultures, suggesting that the potential neuroprotective strategies for dopamine neurons in Parkinson's disease should address not only Ang II but also PRR signaling [113].

In this context, it can be speculated that the striatal dopaminergic system may actively participate in the control of blood pressure and behavioral responses, although further studies should be performed to assess the precise role of the RAS in the regulation of central dopaminergic nerve activity and its modulation by the RAS inhibitors.

*4.2. Acetylcholine Release.* Previous studies have shown that the central cholinergic system may also actively participate in blood pressure control [114–123]. Buccafusco and Spector [114] demonstrated that cholinergic stimulation of the central nervous system by direct receptor agonists produced pressor responses involving activation of central muscarinic receptor sites. It was shown that pressor responses induced by intracerebroventricular injection of Ang II were significantly blocked by hemicholinium-3 (an inhibitor of Ach synthesis), which may indicate a possible interaction between the cholinergic nervous system and the RAS in the brain [115]. Vargas and Brezenoff [116] also reported that the decrease in Ach content of the hypothalamus, striatum, and brainstem caused by hemicholinium-3 was associated with a reduction in systemic blood pressure in SHR. In an in vitro study, we have shown that captopril significantly inhibited the stimulation-evoked Ach release in rat striatum [108]. The finding may propose the hypothesis that the inhibition of central cholinergic activity could contribute, at least in part, to the hypotensive mechanisms of captopril.

Recently, the possible link between the brain RAS and the pathophysiology of Alzheimer's disease has been documented. Tota et al. [124, 125] observed that perindopril and candesartan significantly ameliorated the scopolamine-induced impairment in memory, cerebral blood flow, and cholinergic function in mice. In addition, they suggested that activation of $AT_1$ receptors might be involved in the scopolamine-induced amnesia and that $AT_2$ receptors could contribute to the beneficial effects of the RAS inhibitors. The findings might propose the idea that inhibition of the brain RAS in hypertensive subjects would be neuroprotective against Alzheimer's disease, although further studies are necessary to assess more thoroughly the relationships between the RAS and central cholinergic nerve activity and their roles in the regulation of blood pressure and neurological functions.

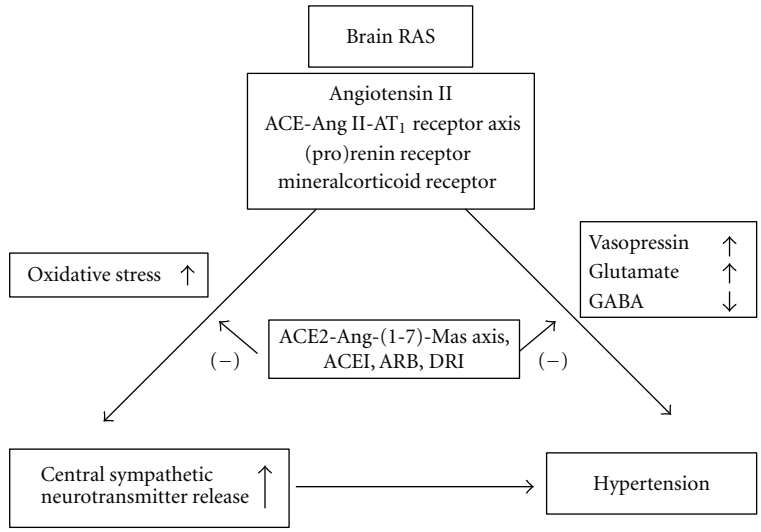


Figure 1: Schematic demonstration of the possible relationship between the brain RAS and sympathetic neurotransmitter release in hypertension. RAS: renin-angiotensin system, ACE: angiotensin converting enzyme, ACEI: angiotensin converting enzyme inhibitor, ARB: angiotensin receptor blocker, DRI: direct renin inhibitor, GABA: $\gamma$-aminobutyric acid, (−): inhibition.

*4.3. Vasopressin Release.* Brain RAS also modulates the cardiovascular and fluid-electrolyte homeostasis by interacting hypothalamic-pituitary axis and vasopressin release [126, 127]. It was shown that the angiotensinogen-deficient rats had lower plasma levels of vasopressin and an altered central vasopressinergic system [128, 129]. It was also demonstrated that PRR receptor knockdown significantly reduced $AT_1$ receptors and vasopressin levels in the renin-angiotensinogen double-transgenic hypertensive mice [83], suggesting that the brain RAS might have a pivotal role in the regulatory mechanisms of vasopressin neurotransmission.

*4.4. Glutamate/GABA ($\gamma$-Aminobutyric Acid) Release.* The role of the brain RAS on glutamate/GABA neurotransmission has also been described [130–132]. It was shown that bilateral microdialysis of the $AT_1$ receptor blocker, ZD7155, into the RVLM significantly decreased glutamate and increased GABA levels [130]. In contrast, administration of $AT_2$ receptor blocker, PD 123319, increased glutamate and decreased GABA levels within the RVLM [131]. Fujita et al. [132] demonstrated that administration of candesartan suppressed ischemia-induced increases in the extracellular glutamate with a concomitant reduction in the production of ROS in the retinal ischemia-reperfusion injury model of the rat, indicating that candesartan might protect neurons by decreasing extracellular glutamate after reperfusion and by attenuating oxidative stress via a modulation of the $AT_1$ receptor signaling. These findings suggested that the RAS might have a crucial role in the regulation of cardiovascular and neurological functions by modulating glutamate/GABA release in the brain.

## 5. Conclusion

All components of the RAS have been identified in the central nervous system, and the brain RAS may regulate blood pressure by modulating sympathetic nerve activity. It has been proposed that the RAS may have a stimulatory influence on the sympathetic nervous system. The brain RAS may augment presynaptic facilitation of sympathetic neurotransmitter release and enhance the central sympathetic outflow. In the present paper we discussed the relationship between the brain RAS and sympathetic neurotransmitter release in hypertension (Figure 1). Ang II strongly potentiates sympathetic neurotransmitter release in the central nervous system. In contrast, the inhibitors of the RAS, such as ACEIs, ARBs, and DRIs might suppress sympathetic hyperactivity in the brain. The release of vasopressin, glutamate, and GABA could also be altered by the RAS inhibition. Although the clinical significance of the modulation of central sympathetic neurotransmitter release by the RAS inhibitors is not fully understood, the current findings may be consistent with the idea that the neurosuppressive effect could partially contribute to their hypotensive action in hypertension.

Clearly, more studies are required to further evaluate the precise role of the brain RAS in the control of sympathetic nerve activity, blood pressure, and neurological functions. In addition, better knowledge of the cellular mechanisms in the brain RAS could provide useful information concerning the development of a more specific and more physiological approach to hypertensive research.

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## References


[1] S. Julius, "Autonomic nervous system dysregulation in human hypertension," *American Journal of Cardiology*, vol. 67, no. 10, pp. 3B–7B, 1991.

[2] S. Kasparov and A. G. Teschemacher, "Altered central catecholaminergic transmission and cardiovascular disease," *Experimental Physiology*, vol. 93, no. 6, pp. 725–740, 2008.

[3] G. Haeusler, L. Finch, and H. Thoenen, "Central adrenergic neurones and the initiation and development of experimental hypertension," *Experientia*, vol. 28, no. 10, pp. 1200–1203, 1972.

[4] H. Vapaatalo, R. Hackman, P. Anttila, V. Vainionpää, and P. J. Neuvonen, "Effects of 6-hydroxydopamine on spontaneously hypertensive rats," *Naunyn-Schmiedeberg's Archives of Pharmacology*, vol. 284, no. 1, pp. 1–13, 1974.

[5] S. Z. Langer, "Presynaptic autoreceptors regulating transmitter release," *Neurochemistry International*, vol. 52, no. 1, pp. 26–30, 2008.

[6] K. Tsuda and Y. Masuyama, "Presynaptic regulation of neurotransmitter release in hypertension," *Clinical and Experimental Pharmacology and Physiology*, vol. 18, no. 7, pp. 455–467, 1991.

[7] D. Ganten and G. Speck, "The brain renin-angiotensin system. A model for the synthesis of peptides in the brain," *Biochemical Pharmacology*, vol. 27, no. 20, pp. 2379–2389, 1978.

[8] M. Bader, J. Peters, O. Baltatu, D. N. Müller, F. C. Luft, and D. Ganten, "Tissue renin-angiotensin systems: new insights from experimental animal models in hypertension research," *Journal of Molecular Medicine*, vol. 79, no. 2, pp. 76–102, 2001.

[9] O. C. Baltatu, L. A. Campos, and M. Bader, "Local renin-angiotensin system and the brain—a continuous quest for knowledge," *Peptides*, vol. 32, no. 5, pp. 1083–1086, 2011.

[10] D. I. Diz, A. C. Arnold, M. Nautiyal, K. Isa, H. A. Shaltout, and E. A. Tallant, "Angiotensin peptides and central autonomic regulation," *Current Opinion in Pharmacology*, vol. 11, no. 2, pp. 131–137, 2011.

[11] J. C. Balt, M. J. Mathy, M. Pfaffendorf, and P. A. Van Zwieten, "Sympatho-inhibitory properties of various $AT_1$ receptor antagonists," *Journal of Hypertension*, vol. 20, no. 5, pp. S3–S11, 2002.

[12] K. Pacak, G. Yadid, G. Jakab, J. W. M. Lenders, I. J. Kopin, and D. S. Goldstein, "In vivo hypothalamic release and synthesis of catecholamines in spontaneously hypertensive rats," *Hypertension*, vol. 22, no. 4, pp. 467–478, 1993.

[13] V. Reja, A. K. Goodchild, J. K. Phillips, and P. M. Pilowsky, "Tyrosine hydroxylase gene expression in ventrolateral medulla oblongata of WKY and SHR: a quantitative real-time polymerase chain reaction study," *Autonomic Neuroscience: Basic and Clinical*, vol. 98, no. 1-2, pp. 79–84, 2002.

[14] J. M. Qualy and T. C. Westfall, "Release of norepinephrine from the paraventricular hypothalamic nucleus of hypertensive rats," *American Journal of Physiology—Heart and Circulatory Physiology*, vol. 254, no. 5, part 2, pp. H993–H1003, 1988.

[15] K. Tsuda, H. Yokoo, and M. Goldstein, "Neuropeptide Y and galanin in norepinephrine release in hypothalamic slices," *Hypertension*, vol. 14, no. 1, pp. 81–86, 1989.

[16] K. Tsuda, S. Tsuda, M. Goldstein, and Y. Masuyama, "Effects of calcitonin gene-related peptide on [$^3$H]norepinephrine release in medulla oblongata of spontaneously hypertensive rats," *European Journal of Pharmacology*, vol. 191, no. 1, pp. 101–105, 1990.

[17] K. Tsuda, S. Tsuda, M. Goldstein, and Y. Masuyama, "Effects of neuropeptide Y on norepinephrine release in hypothalamic slices of spontaneously hypertensive rats," *European Journal of Pharmacology*, vol. 182, no. 1, pp. 175–179, 1990.

[18] K. Tsuda, S. Tsuda, M. Goldstein, and Y. Masuyama, "Effects of Bay K 8644, a $Ca^{2+}$ channel agonist, on [$^3$H]norepinephrine release in hypothalamus of spontaneously hypertensive rats," *European Journal of Pharmacology*, vol. 194, no. 1, pp. 111–114, 1991.

[19] K. Tsuda, S. Tsuda, I. Nishio, Y. Masuyama, and M. Goldstein, "Calcitonin gene-related peptide in noradrenergic transmission in rat hypothalamus," *Hypertension*, vol. 19, no. 6, pp. 639–642, 1992.

[20] K. Tsuda, S. Tsuda, M. Goldstein, I. Nishio, and Y. Masuyama, "Modulation of noradrenergic transmission by neuropeptide Y and presynaptic $\alpha$2 adrenergic receptors in the hypothalamus of spontaneously hypertensive rats," *Japanese Heart Journal*, vol. 33, no. 2, pp. 229–238, 1992.

[21] K. Tsuda, S. Tsuda, I. Nishio, Y. Masuyama, and M. Goldstein, "Synergistic effects of BAY K 8644 and bradykinin on norepinephrine release in the hypothalamus of spontaneously hypertensive rats," *Clinical and Experimental Pharmacology and Physiology*, vol. 22, supplement 1, pp. S54–S57, 1995.

[22] K. Tsuda, S. Tsuda, I. Nishio, and Y. Masuyama, "Role of dihydropyridine-sensitive calcium channels in the regulation of norepinephrine release in hypertension," *Journal of Cardiovascular Pharmacology*, vol. 38, no. 1, pp. S27–S31, 2001.

[23] T. Matsuura, H. Kumagai, A. Kawai et al., "Rostral ventrolateral medulla neurons of neonatal Wistar-Kyoto and spontaneously hypertensive rats," *Hypertension*, vol. 40, no. 4, pp. 560–565, 2002.

[24] K. Tsuda, M. Goldstein, and Y. Masuyama, "Neuropeptide Y and galanin enhance the inhibitory effects of clonidine on norepinephrine release from medulla oblongata of rats," *American Journal of Hypertension*, vol. 3, no. 10, pp. 800–802, 1990.

[25] K. Tsuda, S. Tsuda, Y. Masuyama, and M. Goldstein, "Norepinephrine release and neuropeptide Y in medulla oblongata of spontaneously hypertensive rats," *Hypertension*, vol. 15, no. 6, pp. 784–790, 1990.

[26] K. Tsuda, S. Tsuda, I. Nishio, Y. Masuyama, and M. Goldstein, "Modulation of norepinephrine release by galanin in rat medulla oblongata," *Hypertension*, vol. 20, no. 3, pp. 361–366, 1992.

[27] K. Tsuda, S. Tsuda, I. Nishio, Y. Masuyama, and M. Goldstein, "Glutamatergic regulation of [$^3$H]-noradrenaline release in the medulla oblongata of normotensive and spontaneously hypertensive rats," *Journal of Hypertension*, vol. 12, no. 5, pp. 517–522, 1994.

[28] K. Tsuda, S. Tsuda, I. Nishio et al., "Effects of $\beta$-endorphin on norepinephrine release in hypertension," *Journal of Cardiovascular Pharmacology*, vol. 36, no. 6, pp. S65–S67, 2000.

[29] K. Tsuda, S. Tsuda, and I. Nishio, "Role of $\alpha$2-adrenergic receptors and cyclic adenosine monophosphate-dependent protein kinase in the regulation of norepinephrine release in the central nervous system of spontaneously hypertensive rats," *Journal of Cardiovascular Pharmacology*, vol. 42, supplement 1, pp. S81–S85, 2003.

[30] A. G. Teschemacher, S. Wang, M. K. Raizada, J. F. R. Paton, and S. Kasparov, "Area-specific differences in transmitter

release in central catecholaminergic neurons of spontaneously hypertensive rats," *Hypertension*, vol. 52, no. 2, pp. 351–358, 2008.

[31] M. D. Esler, G. W. Lambert, C. Ferrier et al., "Central nervous system noradrenergic control of sympathetic outflow in normotensive and hypertensive humans," *Clinical and Experimental Hypertension*, vol. 17, no. 1-2, pp. 409–423, 1995.

[32] G. D. Fink, J. R. Haywood, W. J. Bryan, W. Packwood, and M. J. Brody, "Central site for pressor action of blood-borne angiotensin in rat," *The American Journal of Physiology*, vol. 239, no. 3, pp. R358–R361, 1980.

[33] P. J. Davern and G. A. Head, "Fos-related antigen immunoreactivity after acute and chronic angiotensin II-induced hypertension in the rabbit brain," *Hypertension*, vol. 49, no. 5, pp. 1170–1177, 2007.

[34] J. A. Garcia-Sevilla, M. L. Dubocovich, and S. Z. Langer, "Angiotensin II facilitates the potassium-evoked release of $^3$H-noradrenaline from the rabbit hypothalamus," *European Journal of Pharmacology*, vol. 56, no. 1-2, pp. 173–176, 1979.

[35] Y. Huang, J. Rogers, and G. Henderson, "Effects of angiotensin II on [$^3$H]noradrenaline release and phosphatidylinositol hydrolysis in the parietal cortex and locus coeruleus of the rat," *Journal of Neurochemistry*, vol. 49, no. 5, pp. 1541–1549, 1987.

[36] M. M. Gironacci, M. S. Valera, I. Yujnovsky, and C. Peña, "Angiotensin-(1–7) inhibitory mechanism of norepinephrine release in hypertensive rats," *Hypertension*, vol. 44, no. 5, pp. 783–787, 2004.

[37] F. Qadri, E. Badoer, T. Stadler, and T. Unger, "Angiotensin II-induced noradrenaline release from anterior hypothalamus in conscious rats: a brain microdialysis study," *Brain Research*, vol. 563, no. 1-2, pp. 137–141, 1991.

[38] T. Stadler, A. Veltmar, F. Qadri, and T. Unger, "Angiotensin II evokes noradrenaline release from the paraventricular nucleus in conscious rats," *Brain Research*, vol. 569, no. 1, pp. 117–122, 1992.

[39] U. M. Steckelings, L. Paulis, P. Namsolleck, and T. Unger, "$AT_2$ receptor agonists: hypertension and beyond," *Current Opinion in Nephrology and Hypertension*, vol. 21, no. 2, pp. 142–146, 2012.

[40] C. H. Gelband, C. Sumners, D. Lu, and M. K. Raizada, "Angiotensin receptors and norepinephrine neuromodulation: implications of functional coupling," *Regulatory Peptides*, vol. 73, no. 3, pp. 141–147, 1998.

[41] A. Nap, J. C. Balt, M. J. Mathy, M. Pfaffendorf, and P. A. Van Zwieten, "Different $AT_1$ receptor subtypes at pre- and postjunctional sites: $AT_{1A}$ versus $AT_{1B}$ receptors," *Journal of Cardiovascular Pharmacology*, vol. 43, no. 1, pp. 14–20, 2004.

[42] M. Iwai and M. Horiuchi, "Devil and angel in the renin-angiotensin system: ACE-angiotensin II-$AT_1$ receptor axis vs. ACE2-angiotensin-(1–7)-Mas receptor axis," *Hypertension Research*, vol. 32, no. 7, pp. 533–536, 2009.

[43] M. Iwai, H. Nakaoka, I. Senba, H. Kanno, T. Moritani, and M. Horiuchi, "Possible involvement of angiotensin-converting enzyme 2 and Mas activation in inhibitory effects of angiotensin II type 1 receptor blockade on vascular remodeling," *Hypertension*, vol. 60, no. 1, pp. 137–144, 2012.

[44] A. J. Ferreira, M. Bader, and R. A. Santos, "Therapeutic targeting of the angiotensin-converting enzyme2/Angiotensin-(1-7)/Mas cascade in the renin-angiotensin system: a potent review," *Expert Opinion on Therapeutic Patients*, vol. 22, no. 5, pp. 567–574, 2012.

[45] E. A. Bourassa, X. Fang, X. Li, A. F. Sved, and R. C. Speth, "$AT_1$ angiotensin II receptor and novel non-$AT_1$, non-$AT_2$ angiotensin II/III binding site in brainstem cardiovascular regulatory centers of the spontaneously hypertensive rat," *Brain Research*, vol. 1359, pp. 98–106, 2010.

[46] O. Baltatu, J. A. Silva, D. Ganten, and M. Bader, "The brain renin-angiotensin system modulates angiotensin II-induced hypertension and cardiac hypertrophy," *Hypertension*, vol. 35, no. 1, part 2, pp. 409–412, 2000.

[47] G. L. Plosker and D. McTavish, "Captopril: a review of its pharmacology and therapeutic efficacy after myocardial infarction and in ischaemic heart disease," *Drugs & Aging*, vol. 7, no. 3, pp. 226–253, 1995.

[48] S. M. Strittmatter, M. M. Lo, J. A. Javitch, and S. H. Snyder, "Autoradiographic visualization of angiotensin-converting enzyme in rat brain with [$^3$H]captopril: localization to a striatonigral pathway," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 81, no. 5, pp. 1599–1603, 1984.

[49] T. Unger, B. Schull, W. Rascher, R. E. Lang, and D. Ganten, "Selective activation of the converting enzyme inhibitor MK 421 and comparison of its active diacid form with captopril in different tissues of the rat," *Biochemical Pharmacology*, vol. 31, no. 19, pp. 3063–3070, 1982.

[50] M. L. Cohen and K. D. Kurz, "Angiotensin converting enzyme inhibition in tissues from spontaneously hypertensive rats after treatment with captopril or MK-421," *Journal of Pharmacology and Experimental Therapeutics*, vol. 220, no. 1, pp. 63–69, 1982.

[51] J. S. Hutchinson and F. A. O. Mendelsohn, "Hypotensive effects of captopril administered centrally in intact conscious spontaneously hypertensive rats and peripherally in anephric anaesthetized spontaneously hypertensive rats," *Clinical and Experimental Pharmacology and Physiology*, vol. 7, no. 5, pp. 555–558, 1980.

[52] T. Unger, I. Kaufman-Buehler, B. Schoelkens, and D. Ganten, "Brain converting enzyme inhibition: a possible mechanism for the antihypertensive action of captopril in spontaneously hypertensive rats," *European Journal of Pharmacology*, vol. 70, no. 4, pp. 467–478, 1981.

[53] T. Baum, F. T. Becker, and E. J. Sybertz, "Attenuation of pressor responses to intracerebroventricular angiotensin I by angiotensin converting enzyme inhibitors and their effects on systemic blood pressure in conscious rats," *Life Sciences*, vol. 32, no. 12, pp. 1297–1303, 1983.

[54] M. L. Cohen and K. Kurz, "Captopril and MK-421: stability on storage, distribution to the central nervous system, and onset of activity," *Federation Proceedings*, vol. 42, no. 2, pp. 171–175, 1983.

[55] D. W. Cushman, F. L. Wang, W. C. Fung, C. M. Harvey, and J. M. DeForrest, "Differentiation of angiotensin-converting enzyme (ACE) inhibitors by their selective inhibition of ACE in physiologically important target organs," *American Journal of Hypertension*, vol. 2, no. 4, pp. 294–306, 1989.

[56] K. H. Berecek, T. Okuno, S. Nagahama, and S. Oparil, "Altered vascular reactivity and baroreflex sensitivity induced by chronic central administration of captopril in the spontaneously hypertensive rat," *Hypertension*, vol. 5, no. 5, pp. 689–700, 1983.

[57] K. H. Berecek and D. N. Shier, "Alterations in renal vascular reactivity induced by chronic central administration of captopril in the spontaneously hypertensive rat," *Clinical and Experimental Hypertension A*, vol. 8, no. 7, pp. 1081–1106, 1986.

[58] K. Tsuda, S. Tsuda, I. Nishio, Y. Masuyama, and M. Goldstein, "Effects of captopril on [$^3$H]-norepinephrine release in rat central nervous system," *Clinical and Experimental Pharmacology and Physiology*, vol. 22, no. 9, pp. 610–613, 1995.

[59] K. Tsuda, H. Shima, M. Kuchii, I. Nishio, and Y. Masuyama, "Effects of captopril on neurosecretion and vascular responsiveness in hypertension," *Clinical and Experimental Hypertension A*, vol. 9, no. 2-3, pp. 375–379, 1987.

[60] R. J. Bolterman, M. C. Manriquez, M. C. Ortiz Ruiz, L. A. Juncos, and J. C. Romero, "Effects of captopril on the renin angiotensin system, oxidative stress, and endothelin in normal and hypertensive rats," *Hypertension*, vol. 46, no. 4, pp. 943–947, 2005.

[61] A. B. Segarra, I. Prieto, I. Banegas et al., "Asymmetrical effect of captopril on the angiotensinase activity in frontal cortex and plasma of the spontaneously hypertensive rats: expanding the model of neuroendocrine integration," *Behavioural Brain Research*, vol. 230, no. 2, pp. 423–427, 2012.

[62] S. Stancheva, L. Alova, and M. Stefanova, "Effect of peptide and nonpeptide antagonists of angiotensin II receptors on noradrenaline release in hypothalamus of rats with angiotensin II-induced increase of water intake," *Pharmacological Reports*, vol. 61, no. 6, pp. 1206–1210, 2009.

[63] D. B. Averill, T. Tsuchihashi, M. C. Khosla, and C. M. Ferrario, "Losartan, nonpeptide angiotensin II-type 1 ($AT_1$) receptor antagonist, attenuates pressor and sympathoexcitatory responses evoked by angiotensin II and L-glutamate in rostral ventrolateral medulla," *Brain Research*, vol. 665, no. 2, pp. 245–252, 1994.

[64] B. S. Huang, R. A. White, L. Bi, and F. H. Leenen, "Central infusion of aliskiren prevents sympathetic hyperactivity and hypertension in Dahl salt-sensitive rats on high salt intake," *American Journal of Physiology*, vol. 302, no. 7, pp. R825–R832, 2012.

[65] P. Gohlke, S. Von Kügelgen, T. Jürgensen et al., "Effects of orally applied candesartan cilexetil on central responses to angiotensin II in conscious rats," *Journal of Hypertension*, vol. 20, no. 5, pp. 909–918, 2002.

[66] Y. Nishimura, T. Ito, K. Hoe, and J. M. Saavedra, "Chronic peripheral administration of the angiotensin II $AT_1$ receptor antagonist candesartan blocks brain $AT_1$ receptors," *Brain Research*, vol. 871, no. 1, pp. 29–38, 2000.

[67] Y. Nishimura, T. Ito, and J. M. Saavedra, "Angiotensin II $AT_1$ blockade normalizes cerebrovascular autoregulation and reduces cerebral ischemia in spontaneously hypertensive rats," *Stroke*, vol. 31, no. 10, pp. 2478–2486, 2000.

[68] T. Unger, "Inhibiting angiotensin receptors in the brain: possible therapeutic implications," *Current Medical Research and Opinion*, vol. 19, no. 5, pp. 449–451, 2003.

[69] N. Pelisch, N. Hosomi, M. Ueno et al., "Systemic candesartan reduces brain angiotensin II via downregulation of brain renin-angiotensin system," *Hypertension Research*, vol. 33, no. 2, pp. 161–164, 2010.

[70] M. Esler, "Differentiation in the effects of the angiotensin II receptor blocker class on autonomic function," *Journal of Hypertension*, vol. 20, no. 5, pp. S13–S19, 2002.

[71] H. Krum, E. Lambert, E. Windebank, D. J. Campbell, and M. Esler, "Effect of angiotensin II receptor blockade on autonomic nervous system function in patients with essential hypertension," *American Journal of Physiology*, vol. 290, no. 4, pp. H1706–H1712, 2006.

[72] J. de Champlain, M. Karas, L. Assouline et al., "Effects of valsartan or amlodipine alone or in combination on plasma catecholamine levels at rest and during standing in hypertensive patients," *Journal of Clinical Hypertension*, vol. 9, no. 3, pp. 168–178, 2007.

[73] J. Struck, P. Muck, D. Trübger et al., "Effects of selective angiotensin II receptor blockade on sympathetic nerve activity in primary hypertensive subjects," *Journal of Hypertension*, vol. 20, no. 6, pp. 1143–1149, 2002.

[74] F. Angeli, G. Reboldi, G. Mazzotta, C. Poltronieri, and P. Verdecchia, "Safety and efficacy of aliskiren in the treatment of hypertension: a systematic overview," *Expert Opinion on Drug Safety*, vol. 11, no. 4, pp. 659–670, 2012.

[75] R. Dusing, P. Brunel, I. Baek, and F. Baschiera, "Sustained decrease in blood pressure following missed doses of aliskiren or telmisartan: the ASSERTIVE double-blind, randomized study," *Journal of Hypertension*, vol. 30, no. 5, pp. 1029–1040, 2012.

[76] A. Virdis, L. Ghiadoni, A. A. Qasem et al., "Effect of aliskiren treatment on endothelium-dependent vasodilation and aortic stiffness in essential hypertensive patients," *European Heart Journal*, vol. 33, no. 12, pp. 1530–1538, 2012.

[77] L. Siddiqi, P. L. Oey, and P. J. Blankestijn, "Aliskiren reduces sympathetic nerve activity and blood pressure in chronic kidney disease patients," *Nephrology Dialysis Transplantation*, vol. 26, no. 9, pp. 2930–2934, 2011.

[78] R. Fogari, A. Zoppi, A. Mugellini et al., "Effect of aliskiren addition to amlodipine on ankle edema in hypertensive patients: a three-way crossover study," *Expert Opinion on Pharmacotherapy*, vol. 12, no. 9, pp. 1351–1358, 2011.

[79] G. Nguyen, F. Delarue, C. Burcklé, L. Bouzhir, T. Giller, and J. D. Sraer, "Pivotal role of the renin/prorenin receptor in angiotensin II production and cellular responses to renin," *Journal of Clinical Investigation*, vol. 109, no. 11, pp. 1417–1427, 2002.

[80] G. Nguyen, "Renin, (pro)renin and receptor: an update," *Clinical Science*, vol. 120, no. 5, pp. 169–178, 2011.

[81] C. A. Burcklé, A. H. J. Danser, D. N. Müller et al., "Elevated blood pressure and heart rate in human renin receptor transgenic rats," *Hypertension*, vol. 47, no. 3, pp. 552–556, 2006.

[82] Z. Shan, P. Shi, A. E. Cuadra et al., "Involvement of the brain (pro)renin receptor in cardiovascular homeostasis," *Circulation Research*, vol. 107, no. 7, pp. 934–938, 2010.

[83] W. Li, H. Peng, T. Cao, R. Sato et al., "Brain-targeted (pro)renin receptor knockdown attenuates angiotensin II-dependent," *Hypertension*, vol. 59, no. 6, pp. 1188–1194, 2012.

[84] B. S. Huang, S. Ahmadi, M. Ahmad, R. A. White, and F. H. H. Leenen, "Central neuronal activation and pressor responses induced by circulating ANG II: role of the brain aldosterone-"ouabain" pathway," *American Journal of Physiology—Heart and Circulatory Physiology*, vol. 299, no. 2, pp. H422–H430, 2010.

[85] B. Xue, T. G. Beltz, Y. Yu et al., "Central interactions of aldosterone and angiotensin II in aldosterone- and angiotensin II-induced hypertension," *American Journal of Physiology—Heart and Circulatory Physiology*, vol. 300, no. 2, pp. H555–H564, 2011.

[86] Z. H. Zhang, Y. Yu, Y. M. Kang, S. G. Wei, and R. B. Felder, "Aldosterone acts centrally to increase brain renin-angiotensin system activity and oxidative stress in normal rats," *American Journal of Physiology—Heart and Circulatory Physiology*, vol. 294, no. 2, pp. H1067–H1074, 2008.

[87] F. H. H. Leenen, M. RuzickaW, and J. S. Floras, "Central sympathetic inhibition by mineralcorticoid receptor but not

angiotensin II type 1 receptor blockade. Are prescribed doses too low?" *Hypertension*, vol. 60, no. 2, pp. 278–280, 2012.

[88] B. S. Huang, R. A. White, M. Ahmad, A. Y. Jeng, and F. H. H. Leenen, "Central infusion of aldosterone synthase inhibitor prevents sympathetic hyperactivity and hypertension by central $Na^+$ in Wistar rats," *American Journal of Physiology—Regulatory Integrative and Comparative Physiology*, vol. 295, no. 1, pp. R166–R172, 2008.

[89] P. Raheja, A. Price, Z. Wang et al., "Spironolactone prevents chlorthalidone-induced sympathetic activation and insulin resistance in hypertensive patients," *Hypertension*, vol. 60, no. 2, pp. 319–325, 2012.

[90] T. Kishi, Y. Hirooka, Y. Kimura, K. Ito, H. Shimokawa, and A. Takeshita, "Increased reactive oxygen species in rostral ventrolateral medulla contribute to neural mechanisms of hypertension in stroke-prone spontaneously hypertensive rats," *Circulation*, vol. 109, no. 19, pp. 2357–2362, 2004.

[91] Y. Hirooka, "Oxidative stress in the cardiovascular center has a pivotal role in the sympathetic activation in hypertension," *Hypertension Research*, vol. 34, no. 4, pp. 407–412, 2011.

[92] F. Liu, J. Havens, Q. Yu et al., "The link between angiotensin II-mediated anxiety and mood disorders with NADPH oxidase-induced oxidative stress," *International Journal of Physiology, Pathophysiology and Pharmacology*, vol. 4, no. 1, pp. 28–35, 2012.

[93] B. Mertens, P. Vanderheyden, Y. Michotte, and S. Sarre, "The role of the central renin-angiotensin system in Parkinson's disease," *Journal of the Renin-Angiotensin-Aldosterone System*, vol. 11, no. 1, pp. 49–56, 2010.

[94] A. C. E. Linthorst, H. De Lang, W. De Jong, and D. H. G. Versteeg, "Effect of the dopamine $D_2$ receptor agonist quinpirole on the in vivo release of dopamine in the caudate nucleus of hypertensive rats," *European Journal of Pharmacology*, vol. 201, no. 2-3, pp. 125–133, 1991.

[95] A. C. E. Linthorst, P. L. M. Van Giersbergen, M. Gras, D. H. G. Versteeg, and W. De Jong, "The nigrostriatal dopamine system: role in the development of hypertension in spontaneously hypertensive rats," *Brain Research*, vol. 639, no. 2, pp. 261–268, 1994.

[96] K. Tsuda, S. Tsuda, Y. Masuyama, and M. Goldstein, "Alterations in catecholamine release in the central nervous system of spontaneously hypertensive rats," *Japanese Heart Journal*, vol. 32, no. 5, pp. 701–709, 1991.

[97] K. Tsuda, S. Tsuda, I. Nishio, Y. Masuyama, and M. Goldstein, "Facilitatory effects of diltiazem on dopamine release in the central nervous system. Focus on interactions with $D_2$ autoreceptors and guanosine triphosphate binding proteins," *American Journal of Hypertension*, vol. 5, no. 9, pp. 642–647, 1992.

[98] K. Tsuda, S. Tsuda, M. Goldstein, and Y. Masuyama, "Effects of verapamil and diltiazem on dopamine release in the central nervous system of spontaneously hypertensive rats," *Clinical and Experimental Pharmacology and Physiology*, vol. 20, no. 10, pp. 641–645, 1993.

[99] K. Tsuda, S. Tsuda, I. Nishio, Y. Masuyama, and M. Goldstein, "Modulation of [$^3$H]dopamine release by neuropeptide Y in rat striatal slices," *European Journal of Pharmacology*, vol. 321, no. 1, pp. 5–11, 1997.

[100] K. Tsuda, S. Tsuda, I. Nishio, Y. Masuyama, and M. Goldstein, "Effects of galanin on dopamine release in the central nervous system of normotensive and spontaneously hypertensive rats," *American Journal of Hypertension*, vol. 11, no. 12, pp. 1475–1479, 1998.

[101] E. Badoer, H. Wurth, D. Turck et al., "Selective local action of angiotensin II on dopaminergic neurons in the rat hypothalamus in vivo," *Naunyn-Schmiedeberg's Archives of Pharmacology*, vol. 340, no. 1, pp. 31–35, 1989.

[102] T. A. Jenkins, S. Y. Chai, and F. A. O. Mendelsohn, "Effect of angiotensin II on striatal dopamine release in the spontaneous hypertensive rat," *Clinical and Experimental Hypertension*, vol. 19, no. 5-6, pp. 645–658, 1997.

[103] D. C. Brown, L. J. Steward, J. Ge, and N. M. Barnes, "Ability of angiotensin II to modulate striatal dopamine release via the $AT_1$ receptor in vitro and in vivo," *British Journal of Pharmacology*, vol. 118, no. 2, pp. 414–420, 1996.

[104] F. A. O. Mendelsohn, T. A. Jenkins, and S. F. Berkovic, "Effects of angiotensin II on dopamine and serotonin turnover in the striatum of conscious rats," *Brain Research*, vol. 613, no. 2, pp. 221–229, 1993.

[105] S. F. Lu, H. J. Young, and M. T. Lin, "Nigrostriatal dopamine system mediates baroreflex sensitivity in rats," *Neuroscience Letters*, vol. 190, no. 1, pp. 17–20, 1995.

[106] V. Georgiev, L. Gyorgy, D. Getova, and V. Markovska, "Some central effects of angiotensin II. Interactions with dopaminergic transmission," *Acta Physiologica et Pharmacologica Bulgarica*, vol. 11, no. 4, pp. 19–26, 1985.

[107] R. J. A. Banks, L. Mozley, and C. T. Dourish, "The angiotensin converting enzyme inhibitors captopril and enalapril inhibit apomorphine-induced oral stereotypy in the rat," *Neuroscience*, vol. 58, no. 4, pp. 799–805, 1994.

[108] K. Tsuda, S. Tsuda, I. Nishio, Y. Masuyama, and M. Goldstein, "Captopril inhibits both dopaminergic and cholinergic neurotransmission in the central nervous system," *Clinical and Experimental Pharmacology and Physiology*, vol. 25, no. 11, pp. 904–907, 1998.

[109] T. A. Jenkins, A. M. Allen, S. Y. Chai, and F. A. O. Mendelsohn, "Interactions of angiotensin II with central catecholamines," *Clinical and Experimental Hypertension*, vol. 17, no. 1-2, pp. 267–280, 1995.

[110] B. Mertens, P. Vanderheyden, Y. Michotte, and S. Sarre, "Direct angiotensin II type 2 receptor stimulation decreases dopamine synthesis in the rat striatum," *Neuropharmacology*, vol. 58, no. 7, pp. 1038–1044, 2010.

[111] R. C. Speth and V. T. Karamyan, "Brain angiotensin receptors and binding proteins," *Naunyn-Schmiedeberg's Archives of Pharmacology*, vol. 377, no. 4–6, pp. 283–293, 2008.

[112] T. A. Jenkins, J. Y. F. Wong, D. W. Howells, F. A. O. Mendelsohn, and S. Y. Chai, "Effect of chronic angiotensin-converting enzyme inhibition on striatal dopamine content in the MPTP-treated mouse," *Journal of Neurochemistry*, vol. 73, no. 1, pp. 214–219, 1999.

[113] R. Valenzuela, P. Barroso-Chinea, B. Villar-Cheda et al., "Location of prorenin receptors in primate substantia nigra: effects on dopaminergic cell death," *Journal of Neuropathology and Experimental Neurology*, vol. 69, no. 11, pp. 1130–1142, 2010.

[114] J. J. Buccafusco and S. Spector, "Role of central cholinergic neurons in experimental hypertension," *Journal of Cardiovascular Pharmacology*, vol. 2, no. 4, pp. 347–355, 1980.

[115] J. J. Buccafusco and M. Serra, "Role of cholinergic neurons in the cardiovascular responses evoked by central injection of bradykinin or angiotensin II in conscious rats," *European Journal of Pharmacology*, vol. 113, no. 1, pp. 43–51, 1985.

[116] H. M. Vargas and H. E. Brezenoff, "Suprression of hypertension during chronic reduction of brain acetylcholine in spontaneously hypertensive rats," *Journal of Hypertension*, vol. 6, no. 9, pp. 739–745, 1988.

[117] K. Tsuda, S. Tsuda, Y. Masuyama, and M. Goldstein, "Effects of verapamil on [$^3$H]acetylcholine release in the striatum of spontaneously hypertensive rats," *European Journal of Pharmacology*, vol. 216, no. 2, pp. 319–322, 1992.

[118] K. Tsuda, S. Tsuda, I. Nishio, Y. Masuyama, and M. Goldstein, "Effects of nicardipine on the release of acetylcholine in the rat central nervous system," *Japanese Circulation Journal*, vol. 57, no. 10, pp. 993–999, 1993.

[119] K. Tsuda, S. Tsuda, Y. Masuyama, and M. Goldstein, "Effects of diltiazem on [$^3$H]-acetylcholine release in rat central nervous system," *Clinical and Experimental Pharmacology and Physiology*, vol. 21, no. 7, pp. 533–537, 1994.

[120] K. Tsuda, S. Tsuda, M. Goldstein, and Y. Masuyama, "Inhibitory effects of verapamil on [$^3$H]-acetylcholine release in the central nervous system of Sprague-Dawley rats," *Clinical and Experimental Pharmacology and Physiology*, vol. 21, no. 7, pp. 527–532, 1994.

[121] K. Tsuda, S. Tsuda, M. Goldstein, I. Nishio, and Y. Masuyama, "Glutamatergic regulation of [$^3$H]acetylcholine release in striatal slices of normotensive and spontaneously hypertensive rats," *Neurochemistry International*, vol. 29, no. 3, pp. 231–237, 1996.

[122] K. Tsuda, S. Tsuda, I. Nishio, and Y. Masuyama, "The release of acetylcholine and its dopaminergic regulation in the central nervous system of spontaneously hypertensive rats," *Journal of Cardiovascular Pharmacology*, vol. 34, supplement 4, pp. S53–S57, 1999.

[123] K. Tsuda, S. Tsuda, and I. Nishio, "Role of protein kinase C in the regulation of acetylcholine release in the central nervous system of spontaneously hypertensive rats," *Journal of Cardiovascular Pharmacology*, vol. 41, supplement 1, pp. S57–S60, 2003.

[124] S. Tota, C. Nath, A. K. Najmi, R. Shukla, and K. Hanif, "Inhibition of central angiotensin converting enzyme ameliorates scopolamine induced memory impairment in mice: role of cholinergic neurotransmission, cerebral blood flow and brain energy metabolism," *Behavioural Brain Research*, vol. 232, no. 1, pp. 66–76, 2012.

[125] S. Tota, K. Hanif, P. K. Kamat, A. K. Najmi, and C. Nath, "Role of central angiotensin receptors in scopolamine-induced impairment in memory, cerebral blood flow, and cholinergic function," *Psychopharmacology (Berlin)*, vol. 222, no. 2, pp. 185–202, 2012.

[126] L. A. Campos, M. Bader, and O. C. Baltatu, "Brain renin-angiotensin system in hypertension, cardiac hypertrophy, and heart failure," *Frontiers in Physiology*, vol. 2, article 115, 2011.

[127] O. Baltatu, L. A. Campos, and M. Bader, "Genetic targeting of the brain renin-angiotensin system in transgenic rats: impact on stress-induced renin release," *Acta Physiologica Scandinavica*, vol. 181, no. 4, pp. 579–584, 2004.

[128] M. Schinke, O. Baltatu, M. Böhm et al., "Blood pressure reduction and diabetes insipidus in transgenic rats deficient in brain angiotensinogen," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 96, no. 7, pp. 3975–3980, 1999.

[129] L. A. Campos, A. S. Couto, R. Iliescu et al., "Differential regulation of central vasopressin receptors in transgenic rats with low brain angiotensinogen," *Regulatory Peptides*, vol. 119, no. 3, pp. 177–182, 2004.

[130] D. Patel, M. Böhlke, S. Phattanarudee, S. Kabadi, T. J. Maher, and A. Ally, "Cardiovascular responses and neurotransmitter changes during blockade of angiotensin II receptors within the ventrolateral medulla," *Neuroscience Research*, vol. 60, no. 3, pp. 340–348, 2008.

[131] A. Tedesco and A. Ally, "Angiotensin II type-2 ($AT_2$) receptor antagonism alters cardiovascular responses to static exercise and simultaneously changes glutamate/GABA levels within the ventrolateral medulla," *Neuroscience Research*, vol. 64, no. 4, pp. 372–379, 2009.

[132] T. Fujita, K. Hirooka, T. Nakamura et al., "Neuroprotective effects of angiotensin II type 1 receptor ($AT_1$-R) blocker via modulating $AT_1$-R signaling and decreased extracellular glutamate levels," *Investigative Ophthalmology and Visual Science*, vol. 53, no. 7, pp. 4099–4110, 2012.

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*Review Article*

# Cardiac-Autonomic Imbalance and Baroreflex Dysfunction in the Renovascular Angiotensin-Dependent Hypertensive Mouse

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Mouse models provide powerful tools for studying the mechanisms underlying the dysfunction of the autonomic reflex control of cardiovascular function and those involved in cardiovascular diseases. The established murine model of two-kidney, one-clip (2K1C) angiotensin II-dependent hypertension represents a useful tool for studying the neural control of cardiovascular function. In this paper, we discuss the main contributions from our laboratory and others regarding cardiac-autonomic imbalance and baroreflex dysfunction. We show recent data from the angiotensin-dependent hypertensive mouse demonstrating DNA damage and oxidative stress using the comet assay and flow cytometry, respectively. Finally, we highlight the relationships between angiotensin and peripheral and central nervous system areas of cardiovascular control and oxidative stress in the 2K1C hypertensive mouse.

## 1. Introduction

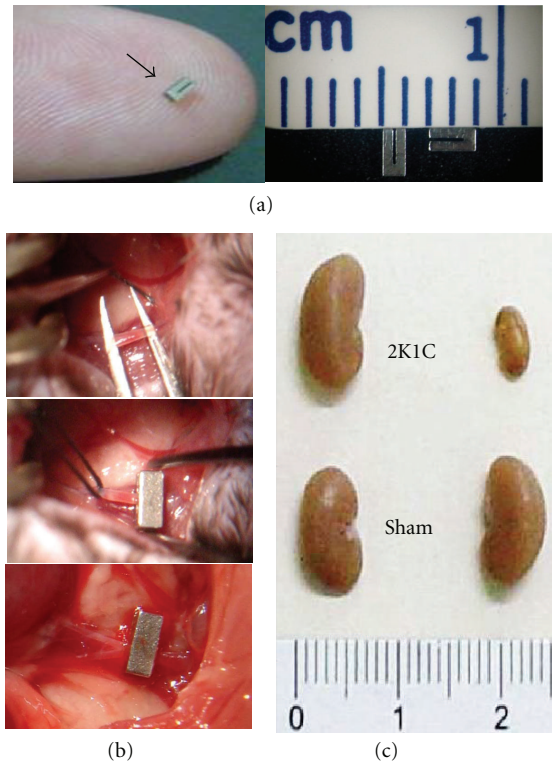
The sympathetic nervous system has an excitatory action on the heart and blood vessels, whereas the parasympathetic cardiovagal innervation has an inhibitory action on the heart [1]. Cardiac output and vascular resistance are the main determinants of arterial blood pressure (BP), which is maintained with minimal oscillations by baroreceptors located at the carotid sinus and aortic arch that transmit their signals to integrative medullary areas [1, 2]. Thus, the balanced activity of the efferent autonomic nervous system and arterial baroreceptors is essential for the control of the cardiovascular system to achieve optimal blood flow to the organs of the body.

As recently reviewed [3], conditions of exaggerated and sustained sympathetic activity, reduced parasympathetic activity, and baroreflex dysfunction are important cardiovascular risks. Over the past decades, our laboratory has shown that these pathological conditions are present as a result of the hypertension induced by the activation of the renin-angiotensin system (RAS) in the rat [4, 5], which are also observed in the RAS-dependent hypertensive mouse [6, 7].

In this paper, we will highlight the characteristics of the murine model of RAS-dependent hypertension, provide new insights into the role played by oxidative oxygen species (ROS) in the integrative brain areas, and discuss which findings are expected to be revealed next.

## 2. Induction of 2K1C Hypertension in the Mouse

For decades, the rat has been used to study the relationship between RAS and the autonomic nervous system. However, genetic discoveries and advances in molecular biotechnologies have provided the opportunity to develop many mouse models for human diseases. Although a major disadvantage of this animal is the small size, advances in surgical techniques have overcome this limitation, allowing




(a) (b) (c)

Figure 1: The procedure used to induce angiotensin-dependent hypertension in the mouse. A solid, stainless steel clip with an opening width of 0.12 mm (a) is placed around the left renal artery to cause stenosis (b), which results in the atrophy of the clipped kidney and hypertrophy of the contralateral, nonclipped kidney (c) and hypertension.

Table 1: Cardiovascular parameters in 2K1C mice compared to Sham mice, two weeks after clipping.

| Parameter | Sham | 2K1C | Reference no. |
|---|---|---|---|
| Mean arterial pressure (mmHg) | ~100–115 | ~120–135 | [13, 14] |
| Heart rate (bpm) | ~500–570 | ~650 | [14] |
| Cardiac weight/body weight index (mg/g) wet (dry) | ~4.1 (~1.0) | ~4.5 (~1.3) | [13, 14] |
| Nonclipped kidney weight (mg) | 140–160 | 170–210 | [14, 15] |
| Clipped kidney weight (mg) | 150–160 | 70–90 | [14, 15] |

for studies of cerebral [8, 9], cardiac [10], vascular [11], and renal [12] functions.

In our laboratory, we used the procedure established by Wiesel et al. [13] to develop a murine model of two-kidney, one-clip (2K1C) hypertension [6, 7, 11, 14]. To minimize variability, a solid stainless steel clip with an opening width of 0.12 mm is placed around the left renal artery (Figure 1) to constrict it and to chronically reduce the perfusion of the left kidney while leaving the other kidney untouched. A mouse body weight of 23 g and clip lumen size of 0.12 mm allows for the induction of hypertension without causing renal infarction [13]. As illustrated in Figure 1, two weeks after clipping, 2K1C mice show atrophy of the clipped (left) kidney and hypertrophy of the contralateral, nonclipped (right) kidney.

Two weeks after renal artery clipping, 2K1C mice already exhibit arterial hypertension (Table 1) with similar levels observed at four weeks [13]. Similar to the 2K1C hypertensive rat that develops cardiac hypertrophy [4, 5], our laboratory has shown a similar phenotype in the 2K1C hypertensive mouse [6, 14]. The development of cardiac hypertrophy is thought to be the result of increased angiotensin II levels through the stimulation of protein and DNA synthesis in cardiac cells [16].

## 3. Systemic and Central Renin-Angiotensin Systems

An advantage to using the C57BL/6 mouse for the induction of RAS-dependent hypertension is that it is a prototype of strains with a single renin gene [17], that is, this mouse does not behave differently from the rat in the 2K1C model of renovascular hypertension. As shown in Tables 1 and 2, the high BP in this model is due to a rapid increase in plasma renin levels (~3-fold) in response to a reduction in the perfusion pressure in the stenotic kidney, which secretes renin from juxtaglomerular cells. This is followed

Table 2: Average values of plasma renin, angiotensin I, II and 1-7 in 2K1C mice compared to Sham mice, two weeks after clipping.

| Parameter | Sham | 2K1C | Reference no. |
|---|---|---|---|
| Renin (ng Ang I/mL/hr)* | ~1000 | ~3000 | [13] |
| Angiotensin I (pmol/mL) | ~80 | ~160 | [14] |
| Angiotensin II (pmol/mL) | ~30 | ~140 | [14] |
| Angiotensin 1-7 (pmol/mL) | ~90 | ~180 | [14] |

* Measured with a microassay based on angiotensin I trapping by antibody.

by a subsequent increase in plasma angiotensin I, which is further converted to the vasoactive angiotensin II (~4.5-fold). Pressure diuresis and hypertrophy of the contralateral kidney (Figure 1, Table 1) prevents hypervolemia [13, 14]. As recently demonstrated by our laboratory, 2K1C mice also show augmented levels of angiotensin 1-7 (Table 2), which is an angiotensin I metabolite formed by a pathway that is independent of angiotensin-converting enzyme (ACE) [18]. Interestingly, knocking-out the angiotensin 1-7 receptor Mas exacerbates the course of 2K1C hypertension in mice [19]. The observed increase in the level of this peptide in the 2K1C mouse seems to serve as an important endogenous, physiological counterbalancing mechanism that partially attenuates the hypertensinogenic actions of activated RAS [18].

In some brain areas, including the rostral ventrolateral medulla (RVLM), hypothalamic paraventricular nucleus (PVN), and subfornical organ (SFO), a local RAS has been identified to act as a critical mediator of chronic hypertension in the 2K1C mouse model [15, 20, 21]. The SFO is a circumventricular region that has a fenestrated vasculature that could permit the entry of increased circulating levels of angiotensin II in addition to residual locally produced angiotensin, leading to the stimulation of the local production of angiotensin II in other brain areas protected by the blood-brain barrier [20].

## 4. Imbalance of the Cardiac Autonomic Nervous System

An imbalance of the autonomic nervous system, as often occurs in conjunction with several cardiovascular diseases, affects BP and HR variability [22, 23], which may be associated with targeted organ damage and an increased risk of morbidity and mortality [3]. Central areas that are involved in the autonomic control of the cardiovascular system include the rostral ventrolateral and ventromedial medulla (RVLM and RVMM), the caudal ventrolateral medulla (CVLM), PVN, and SFO [24–26]. The signals that are generated in the sinoaortic baroreceptor endings are transmitted through the afferents of cranial nerves XI and X to the nucleus tractus solitarius (NTS), followed by the CVLM, and are processed in the RVLM. The RVLM also integrates inputs from the SFO and PVN, providing a major input to the preganglionic neurons of the sympathetic nervous system [15, 20, 27]. Thus, through the integrative processing of central areas, the autonomic sympathetic and parasympathetic nervous systems provide control to the cardiovascular system and the optimal perfusion of organs in accordance with their metabolic needs.

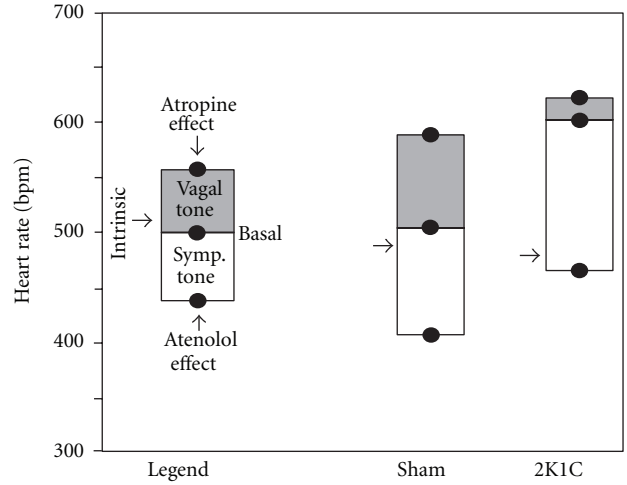



Figure 2: Typical imbalance of cardiac autonomic tones in the angiotensin-dependent hypertensive mouse. The cardiovagal tone is represented by the tachycardia observed following the administration of the muscarinic blocker atropine, and the cardiac sympathetic tone is represented by the bradycardia observed after administering the $\beta$-adrenergic blocker atenolol. The heart rate after the double blockade indicates the intrinsic heart rate.

The parasympathetic cardiovagal and sympathetic tones in the mouse have traditionally been assessed through pharmacological methods involving a $\beta_1$-blocker (atenolol), a muscarinic, cholinergic receptor blocker (atropine methyl nitrate) or a double blockade of those receptors [28, 29]. The increase in HR after administering atropine reflects the cardiovagal tone present under baseline resting conditions, and the decrease in HR after atenolol administration reflects cardiac sympathetic tone (Figure 2); a double blockade enables the determination of the intrinsic HR. In the wild-type mouse under resting conditions, a balance between the sympathetic and parasympathetic activities has been reported [7], with a predominance of the sympathetic tone over the cardiovagal tone under special conditions [30].

As shown in Figure 2, the autonomic control of HR in 2K1C hypertensive mice is characterized by an increased cardiac sympathetic tone, whereas the parasympathetic cardiovagal tone is decreased when compared to sham mice [7]. This condition in humans and animal models of cardiovascular diseases represents a major risk factor for cardiovascular mortality [3]. Angiotensin II mediates the increased activity of the sympathetic nerve to the heart in experimental models of RAS-dependent hypertension [32]. In rats, it has been suggested that an infusion of angiotensin II contributes to tachycardia by increasing the intrinsic HR [33]. However, this does not appear to be the case in the 2K1C mouse model, which shows tachycardia without marked changes in this hemodynamic parameter [6]. Considering that the neuronal nitric oxide synthase-(nNOS-) deficient mouse exhibits tachycardia primarily due to abnormal cardiac autonomic control [34] and that

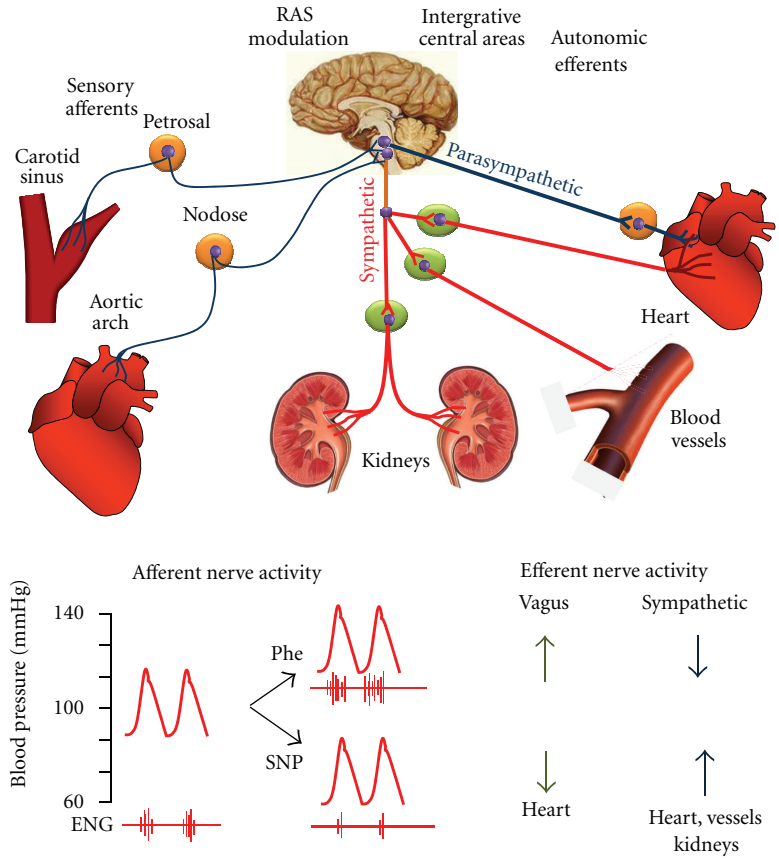



Figure 3: Neuroreflex control of circulation. The top panel illustrates the main neural components of the baroreflex arch. The bottom panel shows a schematic illustration of evaluation of the baroreflex function using the vasoactive agent phenylephrine (Phe) and sodium nitroprusside (SNP) in the murine model of renovascular hypertension. ENG, electroneurogram. The scheme is based on previous publications [3, 31].

endothelial nitric oxide synthase (eNOS) gene therapy restores the basal HR in 2K1C mice [7], it is possible that nitric oxide (NO) plays a role in the autonomic control of HR in this model of RAS-dependent hypertension.

## 5. Baroreflex Dysfunction

Among the neural systems that control cardiovascular function, the baroreflex is a neural mechanism that acts moment-to-moment to maintain BP with minimal fluctuations [1]. With each arterial systole, mechanosensitive nerve endings located at the carotid sinuses and the aortic arch generate bursts of action potentials that are transmitted to the NTS in the medulla oblongata. Here, the signals are integrated and result in the maintenance of a balanced parasympathetic outflow to the heart and a sympathetic outflow to the heart, vessels and kidneys (Figure 3, top panel). As illustrated in Figure 3 (bottom panel), an immediate rise in BP evokes a reflexive increase in cardiovagal inhibitory activity and a decrease in cardiac and vascular sympathetic excitatory activity, resulting in an immediate correction of BP. Conversely, in response to a rapid decrease in BP, cardiovagal activity is diminished and cardiac and vascular sympathetic activity increase to return the BP to normal values.

In our laboratory, the sensitivity of the baroreflex has been traditionally assessed through pharmacological approaches in conscious animals. An acute, phenylephrine- (Phe-) induced increase in BP leads to an increase in the number of action potentials generated at each discharge and, consequently, to a reflexive increase in parasympathetic and a decrease in sympathetic nerve activities. The opposite is observed during an acute, sodium nitroprusside- (SNP-) induced decrease in BP. Peak values of mean arterial pressure (MAP) and HR in response to Phe and SNP injections are fitted to a sigmoidal logistic equation, which is used to determine the gain (first derivative of the curve) and the maximum reflex tachycardia (upper plateau) and reflex bradycardia (lower plateau) [5, 35]. Considering the small size of the mouse, it is more appropriate to evaluate the baroreflex function in conscious mice by injecting a single dose or by slowly infusing Phe and SNP to avoid volume overloading.

A disruption in the balance between parasympathetic and sympathetic tones, as discussed above, can lead to an impairment in baroreflex sensitivity, as has been demonstrated by our laboratory in different models of hypertension [5, 36–38]. Figure 4 shows representative sigmoidal barocurves of a 2K1C mouse compared to a sham animal. The 2K1C mouse curve is shifted to the right of

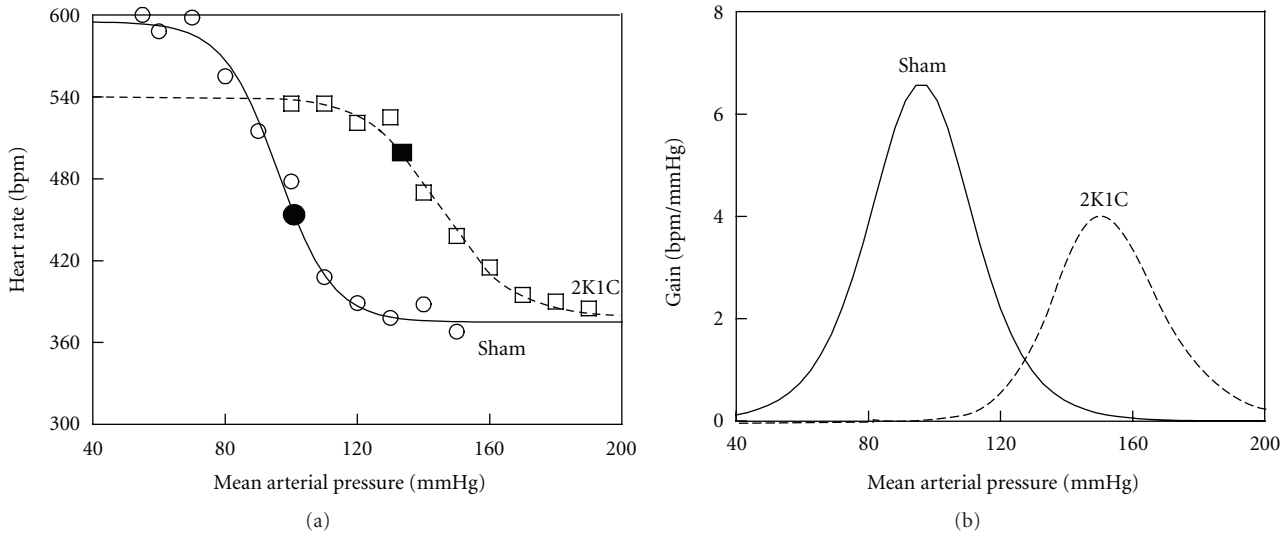



Figure 4: Plots showing typical reflex heart rate changes as a function of drug-induced changes in arterial pressure using logistic, sigmoidal-fitting barocurve analysis (a) and baroreflex gains calculated from the first derivative of the sigmoid function (b) comparing 2K1C with sham mice. The small circles and squares indicate individual changes in heart rate in response to every 10 mmHg of drug-induced changes in arterial pressure.

the sham mouse, closely following the high levels of MAP at the midpoint of the curve. The lower slope of the fitting curve indicates impaired baroreflex sensitivity in the 2K1C mouse. We exclude the possibility that the decreased baroreflex sensitivity in 2K1C mice could be due to a limited chronotropic reserve to respond to increases in HR, as the upper plateau of the barocurve of 2K1C mice was below of that observed for sham mice. Based on observations from our laboratory and others, a reasonable explanation for this finding is that, apart from its pressure effect, adventitial angiotensin II and its $AT_1$ receptors at the aortic arch (and probably at the carotid sinus) act by decreasing the sensitivity of aortic afferents during physiological changes in BP, thus contributing to the impairment of the baroreflex function in cardiovascular diseases [31, 39, 40]. Interestingly, in the rat, central endogenous angiotensin 1-7 has been shown to counterbalance the angiotensin II-induced baroreflex dysfunction [41]. Moreover, a lack of the angiotensin 1-7 Mas receptor-induced baroreflex dysfunction in mice [42]; however, this has not yet been investigated in the renovascular 2K1C mouse model.

## 6. DNA Damage and Oxidative Stress

There is mounting evidence that increased oxidative stress contributes to increased cardiac and vascular sympathetic tone and decreased baroreflex sensitivity in cardiovascular diseases, including hypertension, as recently reviewed elsewhere [3, 21]. Because ROS play a crucial role in RAS signaling [21, 43, 44], a key mechanism by which angiotensin II influences the heart and vessel function could be through its ability to activate ROS production [20, 45]. ROS have been shown to mediate the actions of angiotensin II at the ganglionic [46] and central nervous system levels, resulting in excessive sympathetic drive to the heart [45, 47, 48]. In our laboratory, we currently use the comet assay associated with dihydroethidium (DHE) staining to evaluate oxidative stress in different cells and tissues of the 2K1C hypertensive mouse.

The intracellular oxidation of DHE to the fluorescent dye ethidium has been previously used as an indicator of superoxide generation [49]. DHE is freely permeable to cell membranes and can be directly oxidized to ethidium bromide in cell cytoplasm by the superoxide anion [50, 51]. Ethidium bromide becomes trapped in the nucleus by intercalating within DNA, leading to an increase in ethidium fluorescence in the cell nucleus. DHE itself fluoresces blue in the cell cytoplasm, while the oxidized form ethidium fluoresces red following DNA intercalation. Blood cells can be used to assess ROS generation by superoxide detection with DHE.

The most important, biologically active oxidant in the cardiovascular system, superoxide is a highly reactive and short-lived radical responsible for ROS generation. In addition, it can interact with nearby molecules such as DNA, and thus play a key role in inducing DNA oxidative damage [52, 53]. The comet assay is recognized as a versatile and sensitive method for quantifying and analyzing DNA fragmentation in individual cells and can be used to assess DNA exhibiting oxidative damage. The basic principle of the comet assay is the migration of DNA in an agarose matrix under electrophoretic conditions. As a result of this migration, the cells look like comets under microscopic visualization, with a head (intact DNA) and a tail containing DNA fragments. Individual blood cells are embedded in low-melting-point agarose and spread on a common microscope slide. Membranes, soluble cell constituents, and histones are removed by lysing with detergent and high-salt solution. Following the lysis procedure, the slides are placed in an electrophoresis chamber filled with an alkaline

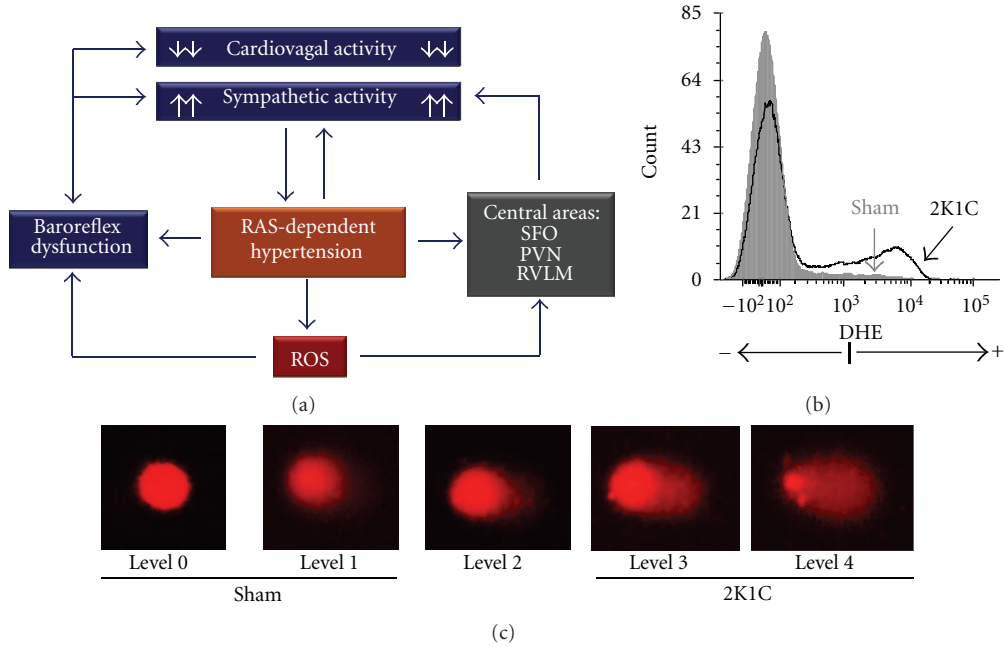



Figure 5: Relationship between RAS activation, ROS production, and baroreflex dysfunction in the 2K1C mouse. (a) Effects of renal clipping-induced high plasma levels of angiotensin II on peripheral and central neural areas controlling cardiovascular function mediated by reactive oxygen species (ROS); (b) typical flow cytometric analysis with the dihydroethidium assay (DHE) showing elevated production of superoxide in 2K1C mice; (c) comet assay illustrating the detection of greater levels of DNA damage (comet-tail fragmentation) in the 2K1C mice.

buffer (pH > 13) for DNA unwinding. Then, the DNA undergoes electrophoresis, allowing for the migration of DNA fragments out of the nucleus in an electrical field towards the anode. Staining is usually performed with a DNA-specific fluorescent dye such as ethidium bromide and observed using a fluorescence microscope. The result of this migration is a bright fluorescent head and tail that gives the appearance of a comet. The relative content of DNA in the tail indicates the amount of DNA damage.

As illustrated in Figure 5, the incidence of genomic fragmentation is visually scored into five levels according to the comet-tail size. RAS-dependent hypertensive mice predominantly present comets with elevated DNA damage levels (3 and 4) in whole blood cells. In addition, flow cytometric analysis of blood cells shows an augmentation of DHE staining in these animals, which indicates that 2K1C hypertension increases superoxide generation, in turn, leading to DNA fragmentation in whole blood cells. Ongoing studies are focused on the effects of RAS-induced hypertension in the cells of different tissues.

## 7. The Relationship between RAS, ROS, and the Autonomic Control of Cardiovascular Function

Our finding of increased DNA damage and ROS production in the 2K1C mouse is in agreement with other studies that found an accumulation of superoxide at the ganglionic level [46] and in different brain integrative areas such as the PVN in this murine model of RAS-induced hypertension [15, 20, 45]. It is thought that the involvement of the PVN in 2K1C hypertension occurs through the activation of RVLM-projecting parvocellular neurons in this region, leading to increased sympathoexcitation [21, 45]. Based on the above data, a plausible mechanism involved in baroreflex dysfunction and the imbalance of the parasympathetic (diminished) and sympathetic (increased) tones appears to be an excessive generation of ROS in the circulatory system and in both the peripheral and central components of the baroreflex. As recently reviewed, ROS is an insidious and ubiquitous promoter of sympathoexcitation and baroreflex dysfunction that can accelerate or worsen cardiovascular disease processes and cardiovascular risks [3, 21].

## 8. New Insights in Therapeutic Approaches to Improve Cardiovascular Function in 2K1C Mice

Although therapies have been aimed at nullifying the undesirable effects of angiotensin II, some investigators have focused on demonstrating the importance of the counter-balanced effects of angiotensin 1-7 [18], which is increased in the 2K1C hypertensive mouse [14]. For example, it has been shown that enalapril treatment increases the sensitivity of the baroreflex in the rat, and that this effect was reversed by an i.c.v. infusion of the selective angiotensin 1-7 antagonist, D-Ala7-Ang-1-7 (A-779) [41]. Similar results were observed when A-799 was injected into the CVLM in 2K1C hypertensive rats [54]. Recently, others have shown that the knockout of the angiotensin 1-7 Mas receptor in mice exacerbates

the course of 2K1C hypertension [19]. Considering the identification of the angiotensin-converting enzyme homologue ACE2 as an angiotensin peptide-processing enzyme and of Mas as a receptor for angiotensin 1-7, this axis is a putative target for the development of new cardiovascular drugs [18]. Furthermore, there has been a lack of studies evaluating the effects of peripheral and central manipulation of angiotensin 1-7 on cardiac autonomic tones and the baroreflex function in the 2K1C mouse model.

As recently reviewed [21], there is growing evidence that acute or chronic antioxidant treatment decreases BP and sympathetic activity and improves the baroreflex control of HR in 2K1C rats. Furthermore, tempol or vitamin C administered systemically or into the RVLM or PVN diminishes BP and sympathetic activity [21], highlighting the pivotal role played by central integrative areas controlling cardiovascular function in RAS-dependent renovascular hypertension.

Some gene therapies have also been tested in studies of 2K1C hypertensive mice, but up until now, no favorable results have been observed. For example, Gava et al. [7] used gene therapy in 2K1C mice and observed that it prevented the development of hypertension but not baroreflex dysfunction. Burmeister et al. [15] tested the hypothesis that excessive superoxide anion production in the PVN contributes to the development and maintenance of renovascular hypertension by delivering an adenovirus encoding superoxide dismutase (AdCuZnSOD) to the PVN. They observed that this prevents the elevation in superoxide anions and abolishes renovascular hypertension. However, this approach has not yet been used to evaluate effects on baroreflex dysfunction in the 2K1C mouse.

## 9. Conclusions and Perspectives

In the past few years, the mouse model of 2K1C hypertension has greatly contributed to the understanding of the relationships between RAS and neural control of cardiovascular function. In addition to the actions of systemic angiotensin II, it has been demonstrated that a local RAS in RVLM, PVN and SFO brain areas act as a critical mediator of chronic hypertension in this experimental model. The 2K1C hypertensive mouse exhibits a cardiac autonomic imbalance characterized by an increased sympathetic tone and a decreased vagal tone, beyond impaired baroreflex sensitivity. In addition to the demonstrations that ROS play a crucial role in RAS signaling at the ganglionic and central nervous system levels, there are growing evidences that DNA damage and increased oxidative stress contribute to the increased cardiac and vascular sympathetic tone and decreased baroreflex sensitivity in the renovascular hypertension. It is well known that angiotensin II increases superoxide production through the activation of NADPH oxidase. Gene therapies by delivering an adenovirus encoding eNOS or enzymes that prevent the elevation of superoxide anions have shown to improve the cardiac autonomic control of HR and baroreflex sensitivity and to prevent renovascular hypertension in the murine model. Although therapies have been aimed at nullifying the undesirable effects of angiotensin II, a putative target for the development of new cardiovascular drugs is the angiotensin 1-7 which induces the release of NO and diminishes NADPH oxidase activation, counteracting the effects of angiotensin II. Therefore, future studies should address potential strategies to decrease oxidative stress and to prevent or restore the cardiac autonomic balance and the baroreflex function in the mouse model of renovascular hypertension.

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## References

[1] E. C. Vasquez, S. S. Meyrelles, H. Mauad, and A. M. Cabral, "Neural reflex regulation of arterial pressure in pathophysiological conditions: interplay among the baroreflex, the cardiopulmonary reflexes and the chemoreflex," *Brazilian Journal of Medical and Biological Research*, vol. 30, no. 4, pp. 521–532, 1997.

[2] M. W. Chapleau, Z. Li, S. S. Meyrelles, X. Ma, and F. M. Abboud, "Mechanisms determining sensitivity of baroreceptor afferents in health and disease," *Annals of the New York Academy of Sciences*, vol. 940, pp. 1–19, 2001.

[3] F. M. Abboud, "The Walter B. Cannon Memorial Award Lecture, 2009. Physiology in perspective: the wisdom of the body. In search of autonomic balance: the good, the bad, and the ugly," *American Journal of Physiology*, vol. 298, no. 6, pp. R1449–R1467, 2010.

[4] A. M. Cabral, A. Antonio, M. R. Moyses, and E. C. Vasquez, "Left ventricular hypertrophy differences between male and female renovascular hypertensive rats," *Brazilian Journal of Medical and Biological Research*, vol. 21, no. 3, pp. 633–635, 1988.

[5] M. R. Moyses, A. M. Cabral, D. Marcal, and E. C. Vasquez, "Sigmoidal curve-fitting of baroreceptor sensitivity in renovascular 2K1C hypertensive rats," *Brazilian Journal of Medical and Biological Research*, vol. 27, no. 6, pp. 1419–1424, 1994.

[6] V. A. Peotta, A. L. Gava, E. C. Vasquez, and S. S. Meyrelles, "Evaluation of baroreflex control of heart rate in renovascular hypertensive mice," *Canadian Journal of Physiology and Pharmacology*, vol. 85, no. 8, pp. 761–766, 2007.

[7] A. L. Gava, V. A. Peotta, A. M. Cabral, E. C. Vasquez, and S. S. Meyrelles, "Overexpression of eNOS prevents the development of renovascular hypertension in mice," *Canadian Journal of Physiology and Pharmacology*, vol. 86, no. 7, pp. 458–464, 2008.

[8] E. C. Vasquez, R. F. Johnson, T. G. Beltz, R. E. Haskell, B. L. Davidson, and A. K. Johnson, "Replication-deficient adenovirus vector transfer of gfp reporter gene into supraoptic nucleus and subfornical organ neurons," *Experimental Neurology*, vol. 154, no. 2, pp. 353–365, 1998.

[9] E. C. Vasquez, T. G. Beltz, S. Meyrelles, and A. K. Johnson, "Adenovirus-mediated gene delivery to hypothalamic magnocellular neurons in mice," *Hypertension*, vol. 34, no. 4, pp. 756–761, 1999.

[10] T. M. C. Pereira, B. V. Nogueira, L. C. F. Lima et al., "Cardiac and vascular changes in elderly atherosclerotic mice: the

influence of gender," *Lipids in Health and Disease*, vol. 9, article 87, 2010.

[11] B. V. Nogueira, V. A. Peotta, S. S. Meyrelles, and E. C. Vasquez, "Evaluation of aortic remodeling in apolipoprotein E-deficient mice and renovascular hypertensive mice," *Archives of Medical Research*, vol. 38, no. 8, pp. 816–821, 2007.

[12] C. M. Balarini, M. Z. T. Oliveira, T. M. C. Pereira et al., "Hypercholesterolemia promotes early renal dysfunction in apolipoprotein E-deficient mice," *Lipids in Health and Disease*, vol. 10, article 220, 2011.

[13] P. Wiesel, L. Mazzolai, J. Nussberger, and T. Pedrazzini, "Two-kidney, one clip and one-kidney, one clip hypertension in mice," *Hypertension*, vol. 29, no. 4, pp. 1025–1030, 1997.

[14] B. V. Nogueira, Z. Palomino, M. L. Porto et al., "Granulocyte colony stimulating factor prevents kidney infarction and attenuates renovascular hypertension," *Cellular Physiology and Biochemistry*, vol. 29, no. 1-2, pp. 143–152, 2012.

[15] M. A. Burmeister, C. N. Young, V. A. Braga, S. D. Butler, R. V. Sharma, and R. L. Davisson, "In vivo bioluminescence imaging reveals redox-regulated activator protein-1 activation in paraventricular nucleus of mice with renovascular hypertension," *Hypertension*, vol. 57, no. 2, pp. 289–297, 2011.

[16] J. I. Sadoshima and S. Izumo, "Molecular characterization of angiotensin II-induced hypertrophy of cardiac myocytes and hyperplasia of cardiac fibroblasts: critical role of the AT1 receptor subtype," *Circulation Research*, vol. 73, no. 3, pp. 413–423, 1993.

[17] L. J. Field and K. W. Gross, "*Ren-1* and *Ren-2* loci are expressed in mouse kidney," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 82, no. 18, pp. 6196–6200, 1985.

[18] R. A. Santos and A. J. Ferreira, "Angiotensin-(1–7) and the renin-angiotensin system," *Current Opinion in Nephrology and Hypertension*, vol. 16, no. 2, pp. 122–128, 2007.

[19] D. Rakuan, M. Bürgelová, I. Vanková et al., "Knockout of angiotensin 1–7 receptor mas worsens the course of two-kidney, one-clip goldblatt hypertension: roles of nitric oxide deficiency and enhanced vascular responsiveness to angiotensin II," *Kidney and Blood Pressure Research*, vol. 33, no. 6, pp. 476–488, 2010.

[20] V. A. Braga, I. A. Medeiros, T. P. Ribeiro, M. S. França-Silva, M. S. Botelho-Ono, and D. D. Guimarães, "Angiotensin-II-induced reactive oxygen species along the SFO-PVN-RVLM pathway: implications in neurogenic hypertension," *Brazilian Journal of Medical and Biological Research*, vol. 44, no. 9, pp. 871–876, 2011.

[21] R. R. Campos, E. B. Oliveira-Sales, E. E. Nishi, M. A. Boim, M. S. Dolnikoff, and C. T. Bergamaschi, "The role of oxidative stress in renovascular hypertension," *Clinical and Experimental Pharmacology and Physiology*, vol. 38, no. 2, pp. 144–152, 2011.

[22] R. Agarwal, "Regulation of circadian blood pressure: from mice to astronauts," *Current Opinion in Nephrology and Hypertension*, vol. 19, no. 1, pp. 51–58, 2010.

[23] B. J. A. Janssen and J. F. M. Smits, "Autonomic control of blood pressure in mice: basic physiology and effects of genetic modification," *American Journal of Physiology*, vol. 282, no. 6, pp. R1545–R1564, 2002.

[24] M. J. Brody, K. J. Varner, E. C. Vasquez, and S. J. Lewis, "Central nervous system and the pathogenesis of hypertension: sites and mechanisms," *Hypertension*, vol. 18, no. 5, supplement, pp. I-7–I-12, 1991.

[25] K. J. Varner, D. S. Rutherford, E. C. Vasquez, and M. J. Brody, "Identification of cardiovascular neurons in the rostral ventromedial medulla in anesthetized rats," *Hypertension*, vol. 19, no. 2, supplement, pp. II193–II197, 1992.

[26] K. J. Varner, E. C. Vasquez, and M. J. Brody, "Lesions in rostral ventromedial or rostral ventrolateral medulla block neurogenic hypertension," *Hypertension*, vol. 24, no. 1, pp. 91–96, 1994.

[27] E. C. Vasquez, S. J. Lewis, K. J. Varner, and M. J. Brody, "Chronic lesion of rostral ventrolateral medulla in spontaneously hypertensive rats," *Hypertension*, vol. 19, no. 2, supplement, pp. II154–II158, 1992.

[28] M. W. Chapleau and R. Sabharwal, "Methods of assessing vagus nerve activity and reflexes," *Heart Failure Reviews*, vol. 16, no. 2, pp. 109–127, 2011.

[29] C. N. Young and R. L. Davisson, "In vivo assessment of neurocardiovascular regulation in the mouse: principles, progress, and prospects," *American Journal of Physiology*, vol. 301, no. 3, pp. H654–H662, 2011.

[30] S. J. Swoap, C. Li, J. Wess, A. D. Parsons, T. D. Williams, and J. M. Overton, "Vagal tone dominates autonomic control of mouse heart rate at thermoneutrality," *American Journal of Physiology*, vol. 294, no. 4, pp. H1581–H1588, 2008.

[31] E. C. Vasquez, V. A. Peotta, and S. S. Meyrelles, "Cardiovascular autonomic imbalance and baroreflex dysfunction in the apolipoprotein E-deficient mouse," *Cellular Physiology and Biochemistry*, vol. 29, no. 5-6, pp. 635–646, 2012.

[32] A. M. Cabral and E. C. Vasquez, "Time course of cardiac sympathetic and vagal tone changes in renovascular hypertensive rats," *American Journal of Hypertension*, vol. 4, no. 10 I, pp. 815–819, 1991.

[33] S. L. Bealer, "Systemic angiotensin II alters intrinsic heart rate through central mechanisms," *Brain Research Bulletin*, vol. 58, no. 1, pp. 61–65, 2002.

[34] P. Jumrussirikul, J. Dinerman, T. M. Dawson et al., "Interaction between neuronal nitric oxide synthase and inhibitory G protein activity in heart rate regulation in conscious mice," *Journal of Clinical Investigation*, vol. 102, no. 7, pp. 1279–1285, 1998.

[35] S. S. Meyrelles, H. Mauad, S. C. B. Mathias, A. M. Cabral, and E. C. Vasquez, "Effects of myocardial hypertrophy on neural reflexes controlling cardiovascular function," *Journal of the Autonomic Nervous System*, vol. 73, no. 2-3, pp. 135–142, 1998.

[36] L. C. Schenberg, C. A. L. Brandao, and E. C. Vasquez, "Role of periaqueductal gray matter in hypertension in spontaneously hypertensive rats," *Hypertension*, vol. 26, no. 6, pp. 1125–1128, 1995.

[37] K. N. Sampaio, H. Mauad, V. C. Biancardi et al., "Cardiovascular changes following acute and chronic chemical lesions of the dorsal periaqueductal gray in conscious rats," *Journal of the Autonomic Nervous System*, vol. 76, no. 2-3, pp. 99–107, 1999.

[38] V. A. Peotta, E. C. Vasquez, and S. S. Meyrelles, "Cardiovascular neural reflexes in L-NAME-induced hypertension in mice," *Hypertension*, vol. 38, no. 3, pp. 555–559, 2001.

[39] C. M. Dos Santos, E. D. Moreira, E. M. Krieger, and L. C. Michelini, "Chronic AT1 receptor blockade alters aortic nerve activity in hypertension," *Hypertension*, vol. 31, no. 4, pp. 973–977, 1998.

[40] K. Arakawa and H. Urata, "Hypothesis regarding the pathophysiological role of alternative pathways of angiotensin II formation in atherosclerosis," *Hypertension*, vol. 36, no. 4, pp. 638–641, 2000.

[41] R. R. Britto, R. A. S. Santos, C. R. Fagundes-Moura, M. C. Khosla, and M. J. Campagnole-Santos, "Role of angiotensin-(1–7) in the modulation of the baroreflex in renovascular

hypertensive rats," *Hypertension*, vol. 30, no. 3, pp. 549–556, 1997.

[42] M. M. De Moura, R. A. S. Dos Santos, M. J. Campagnole-Santos et al., "Altered cardiovascular reflexes responses in conscious Angiotensin-(1–7) receptor Mas-knockout mice," *Peptides*, vol. 31, no. 10, pp. 1934–1939, 2010.

[43] Y. C. Chan and P. S. Leung, "The renin-angiotensin system and reactive oxygen species: implications in pancreatitis," *Antioxidants and Redox Signaling*, vol. 15, no. 10, pp. 2743–2755, 2011.

[44] A. C. Montezano and R. M. Touyz, "Oxidative stress, Noxs, and hypertension: experimental evidence and clinical controversies," *Annals of Medicine*, vol. 44, supplement 1, pp. S2–S16, 2012.

[45] E. B. Oliveira-Sales, E. E. Nishi, B. A. Carillo et al., "Oxidative stress in the sympathetic premotor neurons contributes to sympathetic activation in renovascular hypertension," *American Journal of Hypertension*, vol. 22, no. 5, pp. 484–492, 2009.

[46] X. Ma, H. J. Zhang, C. A. Whiteis, K. C. Kregel, F. M. Abboud, and M. W. Chapleau, "Oxidative stress in sympathetic ganglia: a possible mechanism of increased sympathetic nerve activity and impaired baroreflex sensitivity in atherosclerosis," *Hypertension*, vol. 44, p. 523, 2004.

[47] M. C. Zimmerman, E. Lazartigues, R. V. Sharma, and R. L. Davisson, "Hypertension caused by angiotensin II infusion involves increased superoxide production in the central nervous system," *Circulation Research*, vol. 95, no. 2, pp. 210–216, 2004.

[48] L. Gao, W. Wang, Y. L. Li et al., "Sympathoexcitation by central ANG II: roles for AT1 receptor upregulation and NAD(P)H oxidase in RVLM," *American Journal of Physiology*, vol. 288, no. 5, pp. H2271–H2279, 2005.

[49] T. L. Vanden Hoek, C. Li, Z. Shao, P. T. Schumacker, and L. B. Becker, "Significant levels of oxidants are generated by isolated cardiomyocytes during ischemia prior to reperfusion," *Journal of Molecular and Cellular Cardiology*, vol. 29, no. 9, pp. 2571–2583, 1997.

[50] G. Rothe and G. Valet, "Flow cytometric analysis of respiratory burst activity in phagocytes with hydroethidine and 2',7'-dichlorofluorescin," *Journal of Leukocyte Biology*, vol. 47, no. 5, pp. 440–448, 1990.

[51] H. D. Guthrie and G. R. Welch, "Determination of intracellular reactive oxygen species and high mitochondrial membrane potential in Percoll-treated viable boar sperm using fluorescence-activated flow cytometry," *Journal of Animal Science*, vol. 84, no. 8, pp. 2089–2100, 2006.

[52] J. Cadet, C. D'Ham, T. Douki, J. P. Pouget, J. L. Ravanat, and S. Sauvaigo, "Facts and artifacts in the measurement of oxidative base damage to DNA," *Free Radical Research*, vol. 29, no. 6, pp. 541–550, 1998.

[53] U. Schmid, H. Stopper, F. Schweda, N. Queisser, and N. Schupp, "Angiotensin II induces DNA damage in the kidney," *Cancer Research*, vol. 68, no. 22, pp. 9239–9246, 2008.

[54] L. M. Cangussu, U. G. M. de Castro, R. D. P. Machado et al., "Angiotensin-(1–7) antagonist, A-779, microinjection into the caudal ventrolateral medulla of renovascular hypertensive rats restores baroreflex bradycardia," *Peptides*, vol. 30, no. 10, pp. 1921–1927, 2009.