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Characterization of Angiotensin Ii Receptor Subtypes in the Brain

David L. Saylor
East Tennessee State University

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CHARACTERIZATION OF ANGIOTENSIN II RECEPTOR SUBTYPES IN THE BRAIN

A Dissertation

Presented to

the Faculty of the Department of Physiology

James H. Quillen College of Medicine

East Tennessee State University

In partial Fulfillment

of the Requirements for the Degree of

Doctor of Philosophy

By

David Saylor

May 1997

APPROVAL

This is to certify that the Graduate Committee of

David Saylor

met on the

4th day of December, 1996.

The committee read and examined his dissertation, supervised his defense of it in an oral examination, and decided to recommend that his study be submitted to the Graduate Council, in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Biomedical Science.

Brian P. Rowe
Chair, Graduate Committee

Barbara Turner

Robert Windingstad

Donald B Hoover

Peter J. Rim

Signed on behalf of
The Graduate Council

Interim Dean, School of Graduate Studies

ABSTRACT

CHARACTERIZATION OF ANGIOTENSIN II

RECEPTOR SUBTYPES IN THE BRAIN

by

David Saylor

The present studies explore binding, distribution, and function of angiotensin II (AII) receptors (AT_1 and AT_2) in the brain.

The discovery that sulfhydryl reducing agents masked some but not all AII receptors in the brain prompts an evaluation of commonly used binding assay buffer constituents. EDTA enhances binding (40%) at both AT_1 and AT_2 nuclei, while bacitracin did not alter binding at either receptor subtype. Phenanthroline and BSA differentially altered binding at AT_1 (220% of control) and AT_2 (118% of control) receptors. The results indicate that phenanthroline and BSA would be poor buffer constituents for studies comparing binding at AT_1 and AT_2 receptors.

AII receptors were mapped in normotensive and genetically hypertensive hamster brains and the subtype composition estimated for a number of brain nuclei and the pituitary. Binding in the hamster was similar to that previously observed in the rat brain with exceptions: 1) additional binding in the medial habenula and interpeduncular nuclei, 2) absence of binding in the inferior olive, suprachiasmatic nucleus, medial amygdala, piriform cortex, and subthalamic nucleus and 3) quantitative differences in the dorsomotor nucleus of the vagus, striatum, hippocampus and anterior pituitary. Unlike studies of the normotensive and spontaneously hypertensive rat, we found no significant differences in binding distribution, density or subtype composition when comparing normal and genetically hypertensive hamsters.

Finally, the effects of brain angiotensin II (AII) on central catecholamine utilization were determined. We found no significant differences in norepinephrine, epinephrine or dopamine utilization in rat brain homogenates following intracerebroventricular injection of AII. Although there is evidence that AII alters catecholamine utilization in some brain nuclei, these alterations appear limited (anatomically and/or quantitatively) to a relatively small portion of the brain catecholaminergic system.

The results indicate that the selection of buffer constituents is an important consideration for AII binding studies, that there are minor species differences in the distribution of AII receptors in the brain and that despite substantial functional and anatomical overlap, only a relatively small portion of the brain catecholaminergic system is modulated by angiotensin II.

DEDICATION

This dissertation is dedicated to my loving wife Teresa. Her encouragement and support have been invaluable.

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CHAPTER 1

INTRODUCTION

The Renin Angiotensin System

Nearly a century ago, Tigerstedt and Bergman (1898) obtained an extract from the renal cortex of rabbits that produced a pressor response upon intravenous injection. They named this substance 'renin' and characterized it as heat sensitive and impermeable to semi-permeable membranes, indicating that the substance was most likely (as was later confirmed) a protein. Furthermore, Tigerstedt thought the substance was probably released from the kidney into the circulation to produce its effects (this has also been confirmed).

Later, Goldblatt, a pathologist, noticed arterio- and arteriolosclerosis in hypertensive patients at autopsy. Goldblatt suspected that obstruction of blood flow to the kidney was related to hypertension. In 1934, Goldblatt *et al.* demonstrated sustained hypertension with renal ischemia using constricting clamps on the renal arteries of experimental animals. This and additional work by Goldblatt proved that this type of hypertension was produced by a humoral agent of renal origin (Goldblatt, 1947; 1948). The subsequent search for the humoral agent led to the re-discovery of renin.

Later work revealed that renin did not act alone. Page *et al.* (1940) and Braun-Menendez *et al.* (1940) independently proposed that renin might act as an enzyme and named the substrate angiotonin and hypertensin, respectively. Finally, in 1961, Page and Braun-Mendez agreed to call the new substance "angiotensin". An early assay for angiotensin in arterial blood samples demonstrated that this substance was elevated by a factor of about 20 in malignant hypertensive patients, while only about one-half of the patients with benign essential hypertension had elevated levels (Kahn *et al.*, 1952). This finding stimulated further study of renin and angiotensin. Attempts at purifying angiotensin from plasma uncovered two forms - angiotensin I (AI) and angiotensin II (AII), and another enzyme - angiotensin converting enzyme (ACE) that converted one form into the other (Skeggs *et al.*, 1954; Lentz *et al.*, 1956). These components collectively form the renin-angiotensin system (RAS) (depicted in figure 1-1) with AII being the primary affecter molecule. AII acts on

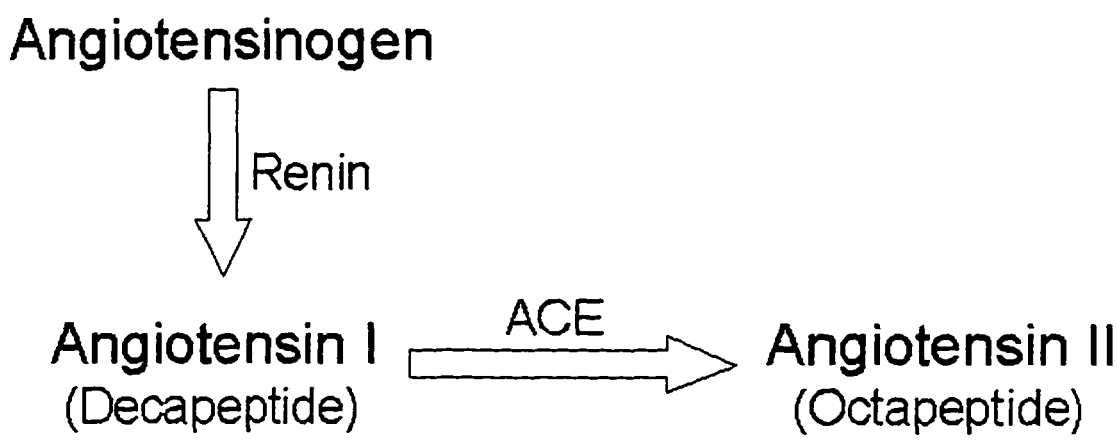

Figure 1-1. The renin-angiotensin system.

many tissues to produce a variety of effects. Most of the known actions of AII involve blood pressure regulation, sodium/fluid balance, or growth and development. Consistent with the array of actions of this peptide, a number of angiotensin receptor subtypes have been described and localized in a number of tissues. The profound effect AII has on blood pressure has made this system a primary target for antihypertensive agents, and a wide variety of drugs is currently available to block renin secretion, renin action, conversion from AI to AII, and AII receptors. Each component of this diverse and complex system will now be discussed individually.

Renin

Preprorenin (45 kD) is produced in the kidney. The human cDNA has been cloned and sequenced (Parmentier *et al.,* 1982). This precursor is processed post-translationally into the still inactive prorenin, then finally, into the active renin. The final processing of prorenin can occur intracellularly, or in the circulation following secretion (plasma prorenin levels are approximately 10^{-11} M). Renin is secreted in two forms. A two chain form is secreted by a regulated mechanism while a single chain renin (with 6 times the enzymatic activity) is constitutively secreted (Pratt *et al.,* 1983). Interestingly, circulating renin is apparently complexed to a renin-binding protein that inhibits its action (Inone *et al.,* 1990). Renin is very specific in function and substrate and does not appear to belong to any general class of proteases. However, there are some structural

similarities to the acid proteases pepsin and cathepsin D, which are also capable of producing angiotensin I (Paiva and Grandino, 1973; Hackenthal *et al.*, 1978).

The renin catalyzed conversion of angiotensinogen to AI is the physiologically regulated reaction of the RAS with plasma AII levels determined by plasma renin activity. Release of renin is regulated by a host of factors of both intra and extrarenal origin. Within the kidney, the renal arterial perfusion pressure and sodium delivery to the macula densa regulate renin release. Additional regulatory components include the renal nerves, circulating catecholamines, AII, and vasopressin. Additional stimuli, both endogenous and exogenous to the kidney, may override other factors under certain conditions. Renin release may be reflexively altered by carotid sinus arterial baroreceptors and/or by vagally mediated cardiopulmonary receptors. Additionally, psychological stresses are known to increase renin release. All of these factors are integrated by juxtaglomerular cells by a not completely understood process that probably involves a common second messenger system (Zehr *et al.*, 1980; Katz and Malvin, 1993). Products of the arachidonic acid and cyclooxygenase interaction and/or prostaglandins may be involved (Katz and Malvin, 1993).

Angiotensinogen

Angiotensinogen, a 55-60kD glycoprotein produced and constitutively secreted (Deschepper and Reudelhuber, 1990) by the liver, has been isolated and characterized. Furthermore, cDNA cloning and analysis have provided the

primary structures of rat (Ohkubo *et al.*, 1983), human (Kageyama *et al.*, 1984), and mouse (Clouston *et al.*, 1988) angiotensinogen. This tetradecapeptide differs at the N-terminus in different species (Tewksbury *et al.*, 1981), allowing polyclonal and monoclonal antibody production and direct immunoassay of the C-terminal portion (Genain *et al.*, 1984).

Angiotensinogen is present in plasma at about 1 μM and in cerebrospinal fluid (CSF) (Genain *et al.*, 1984; Ito *et al.*, 1980; Schelling *et al.*, 1983). Since the K_m of renin for angiotensinogen is around 1.25 μM, angiotensinogen is not in vast excess and alterations in plasma angiotensinogen levels can alter plasma AII levels. However, because changes in plasma angiotensinogen levels occur slowly (hours or days) this substance does not mediate acute AII responses. Angiotensinogen release is regulated by angiotensin II, glucocorticoids (especially dexamethasone), estrogens and thyroid hormones (Tewksbury, 1983; Ménard *et al.*, 1983). It is important to note that angiotensin II is a positive regulator for angiotensinogen (Li and Brasier, 1996), but negatively regulates renin (Ménard *et al.*, 1983; Hermann and Dzau, 1983). Thus, angiotensin II inhibits further angiotensin II formation and causes a replenishment of the used angiotensinogen.

Interestingly, angiotensinogen levels are increased with inflammation (Nielsen and Knudsen, 1987). AII is also elevated and may participate in the immune response during inflammation (Fernandez-Castello *et al.*, 1987).

Angiotensin Converting Enzyme

Skeggs and co-workers (1956) discovered angiotensin converting enzyme (ACE) while attempting to isolate angiotensin I. In the presence of chlorine a 'second form' of angiotensin (missing two amino acids from the C-terminal) was isolated. Independently, in the early 1960s, kininase II, which cleaves a dipeptide from bradykinin, was discovered by Yang and Erdös (1967). Yang and co-workers later demonstrated that both reactions were mediated by a single dipeptidyl decarboxypeptidase (1970). This single enzyme, therefore, inactivates a hypotensive agent and activates a pressor agent. This enzyme also cleaves des-Arg^{9}-bradykinin, neurotensin, chemotactic peptide, substance P, luteinizing hormone releasing hormone, cholecystokinin-8, [Leu^{15}]gastrin$_{11\text{-}17}$ and the opioid peptides enkephalin, heptapeptide, β-neo-endorphin, and dynorphin$_{1\text{-}8}$.

ACE exists in bound and free forms. A membrane-bound form is present in vascular beds, bound to endothelial cells. The lung, having a large vascular bed, hosts much of the ACE activity for cleaving circulating peptides including AI and bradykinin. However, membrane bound ACE is also present in arteries and veins (endothelial), microvilli, kidney proximal tubules, brush border of placenta, intestine and choroid plexus (epithelial), subfornical organ, pallidonigral dendrites and median eminence (neuroepithelial), testis, prostrate and epididymis (male genital tract). Free ACE is present in blood, urine, lung edema, amniotic fluid, cerebrospinal fluid, lymph, and seminal plasma. It is not known if the free form is

secreted or cleaved from the membrane bound C-terminal fragment (Skidgel and Erdös, 1993).

The involvement of this enzyme in the RAS is in the formation of AII from AI. The cleavage of AI has a K_m of approximately $5x10^{-5}$ M. Since AI is present in sub nanomolar concentrations, the rate of reaction is proportional to AI concentration, which is renin dependent. Therefore, this step is not physiologically regulatory. However, the metal requiring nature of this enzyme makes it a primary target for intervention. ACE inhibitors form a large and clinically important class of antihypertensive agents.

Angiotensins

Angiotensin I (AI) (figure 1-2) is formed by the action of renin on angiotensinogen. This decapeptide serves as substrate for angiotensin

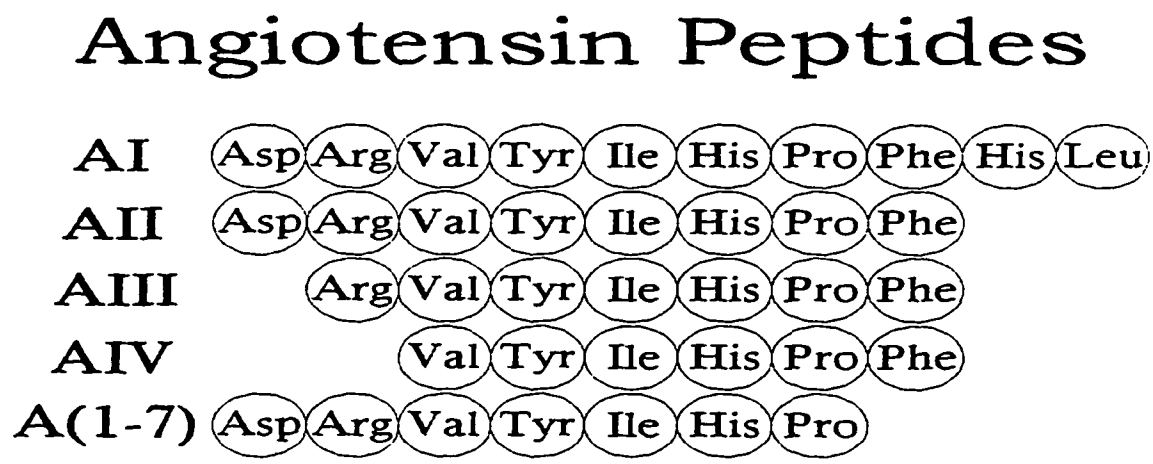

Figure 1- 2. Amino acid sequences of angiotensin peptides.

converting enzyme which removes two amino acids from the C-terminal end to produce the active octapeptide angiotensin II (AII).

AII is now known to affect multiple targets to produce a co-ordinated cardiovascular response. AII increases heart rate and contractile force, constricts blood vessel smooth muscle cells, and stimulates the pituitary to secrete vasopressin, another potent vasoconstrictor (Skeggs, Kahn, and Shumway, 1956). AII also regulates sodium and fluid balance by stimulating aldosterone release from glomerulosa cells of the adrenal cortex (Davis, 1963; Wright and Harding, 1994). AII acts in the kidney to decrease filtration by contracting glomerular mesangial cells (Fujiwara *et al.*, 1989) and smooth muscle cells in efferent arterioles (Gassee *et al.*, 1976; Gekle and Silbernagl, 1993) and excretion by increasing sodium and water re-absorption in the proximal tubule (Mitchell and Navar, 1991). AII stimulates catecholamine release in the brain (Jenkins *et al.*, 1995), and from the adrenal chromaffin cells (Kimura *et al.*, 1992). Finally, AII acts in the brain to stimulate drinking and sodium appetite as well as increases sympathetic tone and decreases parasympathetic tone (Wright and Harding, 1992). AII has also been implicated in reproduction (Morris & Paulson, 1994; Phillips *et al.*, 1995), growth and development (Huckle and Earp, 1994; Wolf, 1995), inflammation (Farber *et al.*, 1990; Weinstock *et al.*, 1987), right ventricular hypertrophy in hypertension (Waeber and Bruner, 1996), and cognition and memory (Wright and Harding, 1994).

Early attempts to antagonize AII activity were limited to the disadvantages of peptidic substitution based antagonists. Often a sarcosine substitution at position 1 was employed to increase stability. Further substitution at position 8 removes activity and may further stabilize the antagonist. More recently, non-peptidic receptor antagonists have been developed and demonstrate heterogeneity in receptor types (Chiu *et al.*, 1989; Whitebread *et al.*, 1989).

Angiotensin III (AIII) can be produced by aminopeptidase activity on AII, or from AI without AII production via aminopeptidase activity and subsequent ACE action. This peptide retains about 30% of the pressor activity of AII (Khosla *et al.*, 1974). However, AIII might play a more significant role in mediating aldosterone release (Campbell *et al.*, 1974; Mendelsohn and Kachel, 1980) and may be equipotent or more potent than AII in eliciting vasopressin release from the pituitary (Wright and Harding, 1994). Indeed, in the brain, some of the actions currently ascribed to AII may actually be mediated by AIII or other smaller peptide fragments formed locally within the tissue (Papouchado *et al.*, 1995; Unger *et al.*, 1988). In fact, AIII is equipotent or more potent than AII in activating the mas oncogene product. This receptor is structurally similar to angiotensin II receptors but is not antagonized by either selective antagonist. Furthermore this gene product is expressed in specific brain regions but is expressed in very low levels if at all at peripheral sites (Hanley, 1991).

Angiotensin IV (AIV) (amino acids 3-8 of angiotensin II) binds with high affinity to a unique receptor type but not to other angiotensin receptors.

Conversely, other angiotensins have very low affinities for the AIV receptor. AIV and AIV receptors have been localized to discrete brain regions and to a variety of other tissues (Swanson *et al.*, 1992).

Angiotensin-(1-7) (amino acids 1-7 of angiotensin II) also demonstrates activity. Schiavone *et al.* (1988) indicate that this fragment is equipotent with AII in stimulating vasopressin release. In the brain angiotensin-(1-7) mimics some but not all AII actions (Campagnole-Santos *et al.*, 1990) and appears to be more potent that AII in stimulating prostaglandin synthesis in glioma cells (Jaiswal *et al.*, 1991). However, it is unlikely that this fragment acts via AII receptors (Rowe *et al.*, 1995).

Angiotensin peptides are degraded through a variety of non-specific amino, carboxy, and endopeptidases. Additional fragmentation products are thought to be inactive.

Angiotensin Receptors

The search for non-peptidic receptor antagonists led to the discovery of angiotensin II receptor subtypes in 1989. The antagonist losartan demonstrates selectivity for the AT_1 receptor while PD123177 demonstrates AT_2 selectivity (Chiu *et al.*, 1989; Whitebread *et al.*, 1989). These receptor subtypes are also differentially sensitive to sulhydryl reducing agents. AII receptors have been localized and characterized in a variety of tissues including vascular smooth muscle, adrenal cortex, kidney, myocardium, brain, adrenal medulla, liver, uterus,

pituitary, gut, and gonads (Catt, 1993). The known actions of AII are mediated by the AT_1 receptor and are antagonized by losartan but not by PD123177.

Both AT_1 and AT_2 receptors have now been cloned and sequenced (Murphy *et al.*, 1991; Sasaki *et al.*, 1991; Nahmias and Strosberg, 1995). AT_1 receptors have been subdivided into AT_{1A} and AT_{1B} receptors based on sequence heterogeneity. However, there appears to be little if any functional difference between the AT_1 types. All three receptor types contain 7 membrane spanning regions and meet the structural requirements for G-protein linkage. AT_1 receptor are known to act through the G-proteins G_q and G_i and couple to phospholipases C, D_2, and A_2, adenylate cyclase, and calcium channels (Catt, 1993). Down regulation by AII appears to be the most significant factor regulating AT_1 expression (Lassègue *et al.*, 1994). There is some evidence that AT_2 receptors link to G_i proteins to modulate K^+ channels in primary neuronal cultures and AT_2 expression may be regulated by growth-related factors (Kang *et al.*, 1994).

Evidence suggests further heterogeneity in both AT_1 and AT_2 receptors. Initially, AT_2 receptors were thought to be not coupled to guanidine nucleotides because of observed insensitivities to GTPγS (Nahnias and Strosberg, 1995). Furthermore, some, but perhaps not all, AT_2 receptors demonstrate enhanced binding in the presence of sulfhydryl reducing agents. There is also some

evidence for a unique AT_1 receptor in renal mesangial cells but cloning and sequencing will be necessary for confirmation (Lassègue *et al.*, 1994).

Multiple Local Tissue RAS

There may actually be several independent renin angiotensin systems operating locally within certain organs. Various components of the RAS have been described in the brain, anterior pituitary, testes, ovaries, adrenal cortex, kidneys, heart, blood vessel walls, and brown and white fat (Ganong, 1994). However, many of these localizations employ very sensitive molecular methods and use polymerase chain reaction (PCR) amplification (Dostal *et al.*, 1992; Ganong, 1994). The degree of expression may not be physiologically significant and could, in some cases, represent incomplete inhibition. AII (and/or another active angiotensin fragment) probably has paracrine and/or autocrine functions in at least some of these tissues and probably acts as a neurotransmitter in the brain (Ganong, 1994). These functions may or may not interact with the hormonal RAS. However, there is some evidence that plasma renin activity alone may not account completely for plasma AII levels, suggesting a contribution by at least some local systems (Unger *et al.*, 1991).

The RAS in the Brain

The evidence for an independent local angiotensin system is most compelling in the brain. Sulfhydryl reducing agents such as dithiothreitol and mercaptoethanol have been found to differentially affect binding with respect to

angiotensin II receptor subtypes in the brain (Rowe *et al.* 1990a; Speth *et al.*, 1990). Significantly, a number of previously unreported binding sites were observed (Rowe *et al.*, 1990b) in the absence of sulfhydryl reducing agents, which were routinely included in angiotensin II binding assays in the brain prior to this discovery. Interestingly, many brain nuclei demonstrating binding are not known to be involved in cardiovascular or dipsogenic activity, suggesting that angiotensin II may have additional functionality in the brain. Additionally, most of the angiotensin II receptor populations in the brain are not accessible from the circumventricular organs, suggesting that a source of angiotensin II exists within the brain.

All of the components of the renin angiotensin system have now been localized in the brain (Moffet *et al.*, 1987; Phillips *et al.*, 1993). Angiotensinogen, angiotensin II, and angiotensin II receptors have been localized within the brain by *in situ* mRNA hybridization (Fuxe *et al.*, 1988), immunocytochemistry (Lind *et al.*, 1985), and receptor autoradiography (Gehlert *et al.*, 1986; Mendelsohn *et al.*, 1984) respectively, and demonstrate good anatomical correspondence in many brain areas (Bunnemann *et al.*, 1991).

Despite generally low levels in the brain, renin has been localized in neurons and nerve terminals and may also be present in glial cells (Fuxe *et al.*, 1980; Fischer-Ferraro *et al.*, 1971; Samani *et al.*, 1988; Yang and Neff, 1972). Most of the angiotensin converting enzyme in the brain is located in the choroid plexus and circumventricular organs with some on neuronal membranes

(Ganong, 1993). However, there are other enzymes present in the brain (like tonin and cathepsin G) that are capable of generating angiotensin II from angiotensin I or directly from angiotensinogen (Boucher *et al.,* 1974; Tonnesen *et al.* 1982).

Angiotensinogen messenger RNA (mRNA) has been localized to astrocytes, while angiotensinogen immunoreactivity has also been found in neurons. This suggests that angiotensinogen is produced only in astrocytes but is then taken up by neurons for conversion to angiotensin II. Angiotensinogen (mRNA and/or immunoreactivity), angiotensin II, and angiotensin II receptors have been localized to the subfornical organ, area postrema, median eminence, spinal trigeminal nucleus, inferior olivary nucleus, dorsal motor nucleus of the vagus. nucleus tractus solitarius, locus coeruleus, regions of the central grey, ventral nuclear complex, reuniens nucleus, mammillary nucleus, ventromedial and dorsomedial nuclei, suprachiasmatic nucleus, paraventricular and periventricular nuclei, supraoptic nucleus, medial preoptic area, median preoptic nucleus, and lateral septum.

However, angiotensinogen and angiotensin II are found in a few areas where angiotensin II receptors have not been reported. These areas include pontine central grey, the substantia nigra, the zona incerta, thalamic paraventricular nucleus, arcuate nucleus, the lateral preoptic area, medial septal nucleus, nuclei of the band of Broca, bed nucleus of the stria terminalis, and the central amygdaloid nucleus (Bunneman *et al.,* 1991).

Summary and Discussion

The kidney was linked to cardiovascular function in classical experiments by Tigerstedt and Bergman (1898). Additional work revealed a hormone system involving angiotensinogen from the liver, renin from the kidney and a converting enzyme from endothelial cells. The primary affector peptide, angiotensin II, acts on receptors present in a number of peripheral tissues and the brain to co-ordinate complex cardiovascular and fluid/salt balance responses.

Recent discoveries indicate that the renin-angiotensin system may also have paracrine/autocrine functions. Evidence is mounting for the existence of local RAS within a variety of tissues/organ systems. The most convincing evidence exists in the brain, where angiotensin II appears to act as a neurotransmitter. All of the components of the RAS have been found in the brain as well as alternative synthesis pathways capable of forming angiotensin II.

Despite the generally good co-localization of components of the renin angiotensin system within the brain, a few mismatches remain. Bunnemann *et al.* (1991) describe three major types of mismatches occurring in the brain renin angiotensin system: 1) The presence of angiotensinogen mRNA, angiotensinogen immunoreactivity, AII receptors, and the lack of angiotensin converting enzyme and/or AII in distinct areas; 2) Angiotensinogen mRNA and immunoreactivity in association with moderate to low levels of converting

enzyme, but AII and AII receptors are not detectable and 3) The lack of detectable binding sites for AII with the presence of all the other components of the RAS. The first discrepancy may be due to a relatively short half life of angiotensin II making detection of low levels difficult. With respect to the second type of discrepancy, angiotensinogen mRNA appears only in astrocytes (Bunnemann *et al.*, 1992; Stornetta *et al.*, 1988) while angiotensinogen-like immunoreactivity has also been visualized in some neurons (Intebi *et al.*, 1990; Thomas and Semia, 1988). Angiotensinogen may be produced by astrocytes and taken up by neurons at the location of the cell body, packages, transported, and converted to angiotensin II in transport or in the distant nerve terminal. Therefore, we might expect angiotensin II and angiotensin II receptors to be localized differently than angiotensinogen mRNA and immunoreactivity. Concerning the second type of discrepancy, the co-localization of all necessary components of the RAS with the exception of angiotensin II receptors, angiotensin II could be formed in (and present at) cell bodies as well as transported to nerve terminals at distant sites where AII receptors would be localized. However, the possibility remains that some angiotensin II receptors evade detection in current assays. The remaining discrepancies with respect to the absence of AII receptors where additional RAS components are present may be indicative of additional angiotensin II receptor subtypes being masked by *in vitro* conditions. Previously, the discovery of angiotensin II receptor subtypes and the subsequent unmasking of additional AII binding in the brain resolved some discrepancies in distributions

of RAS components in the brain (Rowe *et al.*, 1990b). Further evidence of additional receptor subtypes comes from recent molecular biology based experiments that examine angiotensin receptor genes for sequence and processing heterogeneity.

CHAPTER 2

SPECIFIC AIMS

The overall objective of the current research is to further characterize AT_1 and AT_2 receptors in the central nervous system. Approaches employed to meet this objective include: 1) verification of buffer medium for binding assays; 2) evaluation of central AT_1 and AT_2 receptor distributions in the hamster; and 3) characterization of the interaction between central angiotensinergic and catecholaminergic systems. Each of these experiments is described below.

Evaluation of Buffer Constituents

Early binding studies examining angiotensin II receptors employed a variety of peptidase inhibitors in the binding assay in order to preserve the labeled angiotensin II ligand. Indeed, early studies indicated that sulfhydryl reducing agents were necessary (Chen *et al.*, 1982; Printz *et al.*, 1987) to protect ^{125}I-angiotensin II. This practice presupposes that the buffer constituents do not affect the binding reaction. However, when amino and carboxy substituted angiotensin analogues (i.e. ^{125}I-[Sar^1, Ile^8]-Angiotensin II), which are less susceptible to aminopeptidase and carboxypeptidase activity, were employed in the absence of sulfhydryl reducing agents, additional angiotensin II binding was unmasked (Rowe *et al.*, 1990b). Additionally, the differential sensitivity to

sulfhydryl reducing agents corresponds to different angiotensin II receptor subtypes (Rowe *et al.*, 1990a). This precedent prompts re-evaluation of commonly used buffer constituents to determine if the procedures used provide a valid comparison of AT_1 and AT_2 binding characteristics.

Angiotensin II receptor density is measured by *in vitro* receptor autoradiography at a number of brain nuclei known to contain nearly pure populations of AT_1 or AT_2 receptors. Receptors are labeled with ^{125}I-SIAII in the presence and absence of various commonly used buffer constituents to determine if an inhibitor preferentially interferes with binding at one or the other subtype.

Receptor Localization and Subtype Characterization in the Hamster

The AT_1 and AT_2 receptor distributions are determined in normal and genetically hypertensive hamster strains. The brain angiotensin system has been implicated in the hypertension of the spontaneously hypertensive rat, which demonstrates increased levels of both angiotensin II and angiotensin II receptors (Weyhenmeyer and Phillips, 1982; Gehlert *et al.*, 1986b; Hwang *et al.*, 1986). In order to determine if this is true of other mammalian species and to attempt to correlate angiotensin II receptors in the brain with additional physiological systems, angiotensin II receptor subtypes will be examined in the brains of normotensive and spontaneously hypertensive hamsters by *in vitro* receptor autoradiography.

The Effects of Angiotensin II on Brain Catecholamine Utilization

Catecholamines have been implicated in a number of central angiotensin II responses (Fitzsimons and Setler, 1975; Reader *et al.*, 1986; Braszko and Wisniewski, 1990). Additionally, intracerebroventricular (i.c.v.) injection of norepinephrine or dopamine mimics some of the actions of i.c.v. injection of angiotensin II. Furthermore, angiotensin II (i.c.v. or microinjection via microdialysis) has been shown to alter catecholamine utilization in specific brain regions (Sumners and Phillips, 1983; Dwoskin *et al.*, 1992; Jenkins *et al.*, 1995).

Additional evidence suggests that angiotensin II may act as a neuromodulator via AT_2 receptors in the locus coeruleus (Xiong and Marshall, 1990, 1994). Specifically, angiotensin II inhibited the excitatory stimulation of glutamate in a specific manner and was sensitive to AT_2, but not AT_1 selective antagonists. Therefore, we hypothesize that angiotensin II may act, in part, by altering catecholamine utilization throughout the central catecholaminergic system.

To test this hypothesis, groups of 10 conscious, chronically canulated rats will receive i.c.v. injections of AII, artificial CSF (aCSF), AII + losartan or AII + PD123177. After decapitation, the brains will be dissected, homogenized, and assayed for catecholamine utilization by high performance liquid chromatography (HPLC).

CHAPTER 3

MATERIALS AND METHODS

In Vitro Receptor Autoradiography

Animals were anesthetized with Nembutal (80 mg/kg), perfused intracardially with chilled phosphate buffered saline, and the brains were removed quickly and stored at -20 °C. Whole brains were cryostat sectioned (20 µm, -15 °C) and thaw mounted onto sets of adjacent gelatin coated slides. In some instances, one slide from each set was thionin stained to serve as a histological reference and the other slides were returned to the freezer. Frozen slides were placed on a warming plate, pre-incubated in buffer at room temperature for 30 minutes, and transferred to buffer containing ^{125}I-[sar^{1},ile^{8}]-AII (^{125}I-SIAII) for one or two hours. The tissues were then rinsed, dried in a stream of hot air, mounted onto boards along with 20 µm thick ^{125}I standards (Microscales, Amersham, Arlington Heights, IL) and apposed to film (Kodak SB5) for 5-6 days. The film was developed with Kodak D-19 developer and ^{125}I-SIAII binding was quantified densitometrically using the MCID video based image analysis system (Imaging Research Systems, Ontario Canada). Binding was determined as fmoles ligand per gram wet weight of brain tissue and specific

binding was calculated as total ^{125}I-SIAII binding minus binding in the presence of AII (10^{-6} M).

Thionin Staining

At room temperature, slides containing freshly sectioned tissue were incubated sequentially in thionin staining solution (10 min), water (2 dips), 50% ethanol (2 dips), 70% ethanol (15 seconds), 95% ethanol (1 minute), 100% ethanol (3 minutes), and xylene (5 minutes). Finally, coverslips were mounted over the slides.

Development of Autoradiograms

Exposed film (Kodak SB5) was removed from tissue containing cassettes in the dark using a photographic safe light. Film was developed with agitation for 2 minutes in Kodak D-19 Developer, soaked (30 seconds) in a stop solution (2% acetic acid) and fixed in Kodak Rapid Fixer (5 minutes). The film was rinsed in flowing water for 20 minutes or until clear and dipped in photoflo before hanging to dry.

Densitometric Image Analysis

Radioligand binding density was quantitated using an MCID video based image analysis system. The camera, light source, and lens were adjusted as desired. The lens aperture was then adjusted to provide a green field to define the shading error adjustment. The image analysis system was then calibrated for

linear distance using a reticule. The lens aperture was then adjusted as desired and a density calibration was performed using the image of the ^{125}I standards on the autoradiogram to be quantitated.

A 0.15 x 0.15 cm^2 sampling box was used to quantitate the highest observed density (pseudocolor mode) within a given nucleus on an image corresponding to an individual brain section. For each treatment group, each brain nucleus for each animal was measured on a number of sections (the exact number depending upon the anatomical dimension of the nucleus) and an average taken. When appropriate measurements for a given nucleus were obtained from both the left and the right sides of the brain.

Analysis of Radioligand Degradation

For each animal, samples were taken from each buffer before and after 60 or 120 minute incubation of brain slices. Samples were frozen and shipped with dry ice in radioactive packaging to Dr. Robert Speth at Washington State University where they were analyzed for ^{125}I-SIAII and radiolabelled fragments by reverse phase C_{18} (microsorb) HPLC (Rainen Instr., Woburn, MA) with a radioisotope detector (Beckman Model 171, Beckman Instr., Palo Alto). Peak areas for ^{125}I labeled compounds were quantitated as height times width at half the peak height. Several peaks were resolved with 19% acetonitrile, 81% triethylamine phosphate (TEAP) at pH 3.0. The peak corresponding to ^{125}I-SIAII could not be further resolved by extending retention times by using a mobile

phase with 17% acetonitrile (pH 4.0) or by replacing TEAP with triethylamine acetate (TEAA).

Intracerebroventricular Injections

Animals were anesthetized with nembutal (70 mg/kg, intraperitoneal) and surgical anesthesia was maintained with ketamine/acepromasine (intramuscular). After trimming the top of the head, animals were placed in a stereotaxic frame. Under sterile conditions, a midline incision was made over the scalp and the skin deflected. The skull was scraped to remove membranous tissue and positioned such that the top of the skull was horizontal and flat. Stereotaxic co-ordinates were taken with a drill placed on the bregma surface feature and the drill co-ordinates were determined to be 0.9 mm posterior and 1.6 mm lateral to bregma on the left side of the animal. Holes were drilled at this site and approximately 1.5 mm anteriorly and posteriorly. Screws were placed in the anterior and posterior holes. The drill was replaced with the cannula holder and a sterile guide cannula was placed within the holder. The cannula tip was placed on bregma and the co-ordinates taken. The cannula was placed at co-ordinates 0.9 mm posterior, 1.6 mm lateral (left side), and 3.0 mm ventral to bregma. The skull was checked for dryness and any fluid on the skull surface was removed. Cranioplastic cement (liquid and powder portions) was mixed and applied, under the screw tops, covering the screws, around the cannula and into the lower threads on the cannula base. After the cement had hardened (about 20 minutes), the cannula

was released from the holder, the skin was closed around the cannula with autoclips and a dummy cannula was screwed into place inside the guide cannula.

Approximately 1 week following surgery, cannula placement was confirmed by observation of a drinking response following i.c.v. injection of angiotensin II (150 picomoles in 3 µl). A syringe (Hamilton, 10 µl) was attached via fluid filled tubing to an internal cannula designed to snap into place within the guide cannula. The internal cannula was back loaded with $5x10^{-5}$ M angiotensin II in artificial cerebrospinal fluid (aCSF) solution. The dummy cannula was replaced with the loaded internal cannula and a 3 µl injection was made. After approximately 20 seconds, the internal cannula was removed and the dummy cannula replaced. The animal was returned to the cage. The water bottle was weighed before and approximately 30 minutes following i.c.v. injection. Both time until onset of drinking and volume drank were assessed. Passing responses began drinking within 3 minutes of injection and drank >12 ml within 30 minutes of injection. Often animals failing the initial test passed on successive tests. Animals repeatedly failing drink tests (5) or considered to be in poor health were excluded from the study.

At least 24 hours following a successful drink test animals were placed into 1 of 5 experimental groups. Animals were pre-treated with a catecholamine synthesis inhibitor (∝-methyltyrosine, 200 mg/kg IP) or saline solution at 90

minutes prior to decapitation. Approximately 50 minutes prior to decapitation, internal cannula attached to syringes (Hamilton, 10 µl) and preloaded with 1 of 4 test solutions were placed within the guide cannula as described above. Bolus injections of 3 µl were delivered at 45, 30, and 15 minutes prior to decapitation and drinking responses were monitored. Decapitation was accomplished with a small animal guillotine. Immediately following decapitation, the brain was removed and dissected into the left cortex, right cortex, hindbrain and midbrain, forebrain, and cerebellum. Each brain region was weighed and homogenized in 3 ml of homogenizing solution. Following homogenization, an additional 3 ml of homogenizing solution was added. Tissue samples were then stored at -70°C until catecholamines were extracted.

Catecholamine Extraction from Brain Homogenates

Frozen tissue samples were thawed to room temperature and centrifuged for 25 minutes at 20,000xg (14000 rpm, Beckman J2-21 centrifuge with pre-cooled J20.1 rotor, set=2°C). Following centrifugation, the supernatant from each sample was collected and stored at -70°C until extraction. Catecholamines were extracted from tissue samples by differential affinity to acid washed alumina oxide (AAO). Samples (1ml) were placed into vials containing 1ml Tris buffer and 50mg AAO and the vials were vortexed and shaken for 10 minutes. The liquid was removed with a Pasteur pipette and the AAO washed twice with deionized water. With the 3rd wash, the AAO is transferred into a microfiltration apparatus

and centrifuged (Damon IEC HN-SII model, 3500rpm, 8 minutes) to dryness. Trichloroacetic acid (200 µl) was added to the AAO and vortexed gently. After 10 minutes, the trichloroacetic acid was collected by centrifugal filtration as before and stored at -70°C until HPLC quantitation.

HPLC Quantitation of Catecholamines

HPLC quantitation of catecholamines was by electrochemical detection. Mobile phase was circulated in a closed circuit from a reservoir, through a pump, injection port, guard column, analytical column, detector flow cell, and returned to the reservoir. A light helium sparge was maintained through the reservoir to keep the mobile phase degassed. The electrochemical detector applied a voltage potential of +70 mV across the mobile phase, causing reduction and oxidation of active analytes within the mobile phase. After sufficient time, the mobile phase equilibrated to the applied voltage and freshly introduced analytes oxidized and reduced above the equilibrium redox rate. Samples were introduced into the flow at the injector port. The redox current was detected, amplified, and recorded. HPLC separation of analytes produced gaussian peaks of redox activity on a current/time plot (chart recorder) with specific retention times for each analyte separated.

Catecholamine standards were used to tune mobile phase characteristics for optimum separation and to asses response factors (observed area under peak per amount of analyte in sample) for each catecholamine of interest and for

the internal standard. Similarly, standards were extracted to asses the extraction efficiency (percent of initial catecholamine present in final extract) for each catecholamine of interest and for the internal standard. Standards were extracted in buffers of varying pH levels to optimize pH of extraction.

Catecholamine extracts from rat brain tissue homogenates were injected and the peaks corresponding to NE, EPI, DA, and DHBA analyzed. NE, EPI, and DA were measured by adjusting the known initial amount of DHBA (internal standard) in 6 ml of homogenizing buffer. The internal standard amount was multiplied by the ratio of the areas under the NE, EPI and DA peaks to the area under the DHBA peak, and by the ratio of the DHBA response factor to response factors for each of the catecholamines. For example, if a chart trace indicated an area under a NE peak of 50 volt•seconds and an area for DHBA of 75 volt•seconds, with response factors of 2.53 volt•seconds/ng NE and 3.12 volt•seconds/ng DHBA and an initial amount of DHBA of 300 ng, then

$$NE = 300 \text{ ng} \bullet (50 / 75) \bullet (3.12 / 2.53) = 247 \text{ ng}.$$

The absolute amount of catecholamine was calculated as ng catecholamine per g tissue.

Materials

0.1 M Perchloric Acid ($HClO_4$): Dilute 17.1 ml of 70% (concentrated) $HClO_4$ to 2 liters.

Artificial Cerebrospinal Fluid (aCSF): Into a 100 ml volumetric flask put 1.4960g NaCl, 0.0388g KCl, 0.0382g $CaCl_2$, 0.0366g $MgCl_2$, 0.3360g $NaHCO_3$, and 0.0697g Na_2HPO_4. Dilute to the mark and stir until dissolved. Dilute this solution 1:1 when preparing agents in this vehicle.

Carrier free monoiodinated ^{125}I-[sar^1,ile^8]-angiotensin II: supplied by Dr. Robert Speth, was prepared as described previously (Speth *et al.*, 1987).

Catecholamine Standard Solutions:

DHBA (100mg/L): Dissolve 16mg DHBA•HBr into 100 ml of 0.1 M perchloric acid ($HClO_4$).

NE (75 mg/L) / EPI (25 mg/L): Dissolve 14.2 mg norepinephrine bitartrate and 4.6 mg epinephrine bitartrate into 100 ml of 0.1 M perchloric acid ($HClO_4$).

(100 ng DHBA, 75 ng NE, 25 ng EPI, 100 ng DA / ml): Combine 100 µL of each of the proceeding catecholamine standard solutions and dilute to 100 ml with 0.1 M perchloric acid ($HClO_4$).

Homogenizing Solution: Combine 14.15 g/L monochloroacetic acid, 4.5 g/L sodium hydroxide and 0.75 g/L disodium EDTA. Dissolve and adjust the pH to 3.00 - 3.05 with solid monochloroacetic acid or sodium hydroxide. Add pargyline to 350 µM (68.5 mg/L).

HPLC Mobile Phase: Dissolve 14.15 g/L monochloroacetic acid, 4.5 g/L sodium hydroxide and 0.75 g/L disodium EDTA. Adjust the pH to 3.00 to 3.05

using solid monochloroacetic acid or sodium hydroxide. Add 65 mg/L sodium octyl sulfate.

Phosphate Buffered Saline: Add 8.5 g/L sodium chloride to 10 ml/L 500 mM sodium phosphate dibasic. Adjust pH to 7.2 to 7.3.

Subbing Solution: To 500 ml of water, add 5 g gelatin. Warm to dissolve but do not exceed 60°C. To 500 ml of water, add 0.5 g chromium potassium sulfate ($CrK(SO_4)_2$:$12H_2O$). After both solutions have dissolved, mix together and filter. Add thymol to preserve and refrigerate. Warm to room temperature before using and filter after each use.

Thionin staining solution: To 600 ml boiling water, add 3.6 ml glacial acetic acid, 1.0 g thionin and 3.2 g Sodium Acetate (0.8%). When dissolved, add 400 ml deionized water and adjust the pH to 4.5 with 10 N Sodium Hydroxide.

Losartan potassium and PD123177 were supplied by Dr. Ronald Smith of DuPont (Wilmington, DE). Angiotensin peptides were obtained from Bachem (Torrance, Ca). Glacial acetic acid ($A38^{C}$-212), EDTA, ethyl alcohol ($A962^{P}$-4), sodium chloride (S671-3), sodium hydroxide (S-318), sodium phosphate dibasic (BP-331-1) and monobasic (S381), thionin stain (T-409), trichloroacetic acid (A176), and xylene ($X3^{S}$-4) were obtained from Fisher Scientific (Pittsburgh, PA).

Amastatin (A-1276), bacitracin (B-0125), bestatin (B-8385), BSA (A-7906; albumin, bovine, fraction V), catecholamine standards (dihydroxybenzylamine – DHBA, epinephrine, norepinephrine, and dopamine), dithiothreitol (DTT, D-0632), pargyline (P-8013), 1,10 phenanthroline (P-1294), phenylmethylsulfonyl fluoride

(PMSF, P-7626), sigmacote (SL-2), and trizma base (T-1503) were obtained from Sigma Chemical Co. (St. Louis, MO).

Leupeptin was obtained from Cambridge Research Biochemicals, Inc. (Wilmington, DE). Eukitt mounting medium was obtained from Aldrich chemical company (Milwaukee, WI). Sodium octyl sulfate, rapid fixer, D-19 developer, and Photo Flo were obtained from Kodak-Eastman chemical company (Rochester, NY).

Guide cannula (22 ga cut 5 mm below pedestal), internal cannula (28 ga to fit guide with 1 mm projection), dummy cannula, cannula connectors, drill bits (for 0-80 screws), and jeweler's screws (2.4 mm length, 0-80 x 3/32) were obtained from Plastics One Inc. (Roanoke, Va).

CHAPTER 4

EVALUATION OF BUFFER CONSTITUENTS FOR DIFFERENTIAL EFFECTS ON ^{125}I[SAR^1, ILE^8]-ANGIOTENSIN II BINDING AT AT_1 AND AT_2 RECEPTORS IN THE RAT BRAIN

Introduction

Two subtypes of angiotensin II (AII) receptors have been identified (Whitebread *et al.*, 1989; Chiu *et al.*, 1989a). The AT_1 receptor subtype is differentiated by high affinity ($IC_{50} = 10^{-8}$ M) for losartan (DuP753) and similar compounds, and relatively low affinity for CGP 42112A and PD123177 ($IC_{50} = 10^{-4}$ M). These compounds show the opposite pattern of selectivity for the receptor subtype designated AT_2. Receptor autoradiographic techniques have confirmed the presence of both AII receptor subtypes in discrete nuclei within the brain (Rowe *et al.*, 1990c; Gehlert *et al.*, 1990; Wamsley *et al.*, 1990; Song *et al.*, 1992; Tsutsumi and Saavedra, 1992).

Several laboratories using membrane preparations or receptor autoradiography have employed a variety of procedures to investigate AII binding with the implicit assumption that the basic methodology does not have a detrimental effect on binding to the AII receptors of interest. This has not always been the case. For example, early studies indicated that sulfhydryl reducing

agents were required to protect the radioligand from degradation (Glossman *et al.*, 1974; Bennett and Snyder, 1976) and they increased binding affinity for ^{125}I-AII in brain tissue (Chen *et al.*, 1982; Printz, 1987). Thus sulfhydryl reducing agents were widely used in binding assays for brain tissue until it was revealed that they severely impair binding specifically at AT_1 but not AT_2 receptors (Whitebread *et al.*, 1989; Chiu *et al.*, 1989b; Speth *et al.*, 1991). Accordingly, most investigators now exclude sulfhydryl reducing agents when studying AII receptors.

Most investigators are in general agreement with respect to localization and distribution of AII receptor subtypes in brain tissue and the relative affinities of various ligands at AT_1 and AT_2 sites. However, subtle differences are apparent with respect to relative binding density among brain nuclei in autoradiographic studies. Further, we have reported that the radioligand ^{125}I-[sar^1,ile^8]-AII (^{125}I-SIAII) has a four fold selectivity for brain AT_1 binding sites (Rowe *et al.*, 1992) which is not corroborated by other investigators (Chang *et al.*, 1990; Obermuller *et al.*, 1991). Prompted by the precedent set with sulfhydryl reducing agents, we became concerned that different buffer constituents, used in binding assays in different laboratories, might affect binding differentially at AT_1 and AT_2 sites. Inclusion or exclusion of such a factor in incubation cocktails might alter results obtained in different laboratories. We began by investigating the peptidase inhibitors used routinely in our autoradiographic studies, bacitracin and

EDTA, and then evaluated phenanthroline and BSA, which have been used by other investigators (Koziarz and Moore, 1989; Chang *et al.*, 1990).

Design

Evaluation of EDTA and Bacitracin

Studies in our laboratory have routinely used buffer containing 150 mM NaCl, 50 mM sodium phosphate (pH=7.1-7.2), 5 mM disodium ethylenediamine tetraacetate (EDTA), and 0.1 mM bacitracin. Sets of six adjacent sections were prepared from each of 6 rat brains. These were incubated either in the above buffer (containing EDTA and bacitracin), a buffer from which bacitracin was omitted, and a buffer from which EDTA was omitted. Total and nonspecific binding were determined for each of the three buffers by *in vitro* receptor autoradiography as described in Chapter 3 - Methods.

Evaluation of Phenanthroline and BSA

A second experiment compared our standard buffer (NaCl, $NaPO_4$, EDTA, and bacitracin) versus a buffer in which EDTA and bacitracin were replaced with 1,10 phenanthroline ($5x10^{-4}$ M) and BSA (0.2%), and one in which no peptidase inhibitors were present (EDTA and bacitracin were omitted). Sets of six adjacent sections prepared from each of 5 rat brains as described above were incubated in each of the 3 study buffers with and without an excess of unlabelled AII. Again, specific binding was assayed by *in vitro* receptor autoradiography.

Binding was also compared in buffers containing phenanthroline or BSA alone (versus phosphate-saline buffer, N=6) at the nucleus of the solitary tract (AT_1) and the inferior olivary nucleus (AT_2).

Screening of Individual Buffer Constituents

To determine the individual effects on AT_1 and AT_2 binding of additional buffer constituents, total binding (N=3) was determined in a series of 6 adjacent brain sections. Total ^{125}I-SIAII binding in control buffer (NaCl, $NaPO_4$) was compared to total binding in the presence of individual peptidase inhibitors (dithiothreitol (DTT), 5 mM; PMSF, 1 mM; amastatin, 1.25 mg/L; bestatin, 1.25 mg/L; leupeptin, 1.25 mg/L). Total binding was determined by *in vitro* receptor autoradiography for predominantly AT_1 and AT_2 nuclei.

Data Analysis and Statistics

Brain nuclei were selected and categorized by subtype predominance (>90% AT_1 or AT_2) based on a previous study (Rowe *et al.*, 1992) to facilitate comparisons of buffer effects on the two subtypes. AT_1 nuclei used include the piriform cortex (PC), suprachiasmatic nucleus (SCh), medial pre-optic nucleus (MnPO), ventral hippocampus (Vhip), paraventricular nucleus of the hypothalamus (PV), and the anterior pituitary (Pit). The AT_2 nuclei used are the medial geniculate nucleus (MG), superior colliculus (SC), subthalamic nucleus (STh), lateral septum (LS), and the mediodorsal thalamus (MD). Subtype selective competitors were not used for this study.

For each experiment, buffer effects on binding at each nucleus and recovery of intact radioligand were evaluated by a repeated measures analysis of variance and a modified Newman-Keuls test according to Winer (1971).

Results

Table 4-1A describes radioligand integrity after incubation with brain slices as a percent of intact radioligand prior to incubation in three buffers differing with respect to the presence of EDTA and bacitracin. HPLC analysis determined nearly complete recovery of intact ^{125}I-SIAII from buffer containing both bacitracin and EDTA and in buffer containing only bacitracin following incubation of rat brain slices. However, intact radioligand recovery was significantly decreased ($p<0.05$) in the absence of bacitracin. Table 4-1B compares ^{125}I-SIAII binding at rat brain nuclei in these three buffers. Figure 4-1 depicts specific binding as a percent of specific binding in buffer containing both EDTA and bacitracin at predominantly AT_1 and AT_2 nuclei. The exclusion of EDTA consistently caused a significant ($p<0.01$) reduction in specific ^{125}I-SIAII binding in all AT_1 and AT_2 predominant brain nuclei studied, with an average reduction of approximately 40%. Non-specific binding (not illustrated) was also consistently lower at all nuclei, averaging 17% less, with the omission of EDTA, but the effect was significant at only three nuclei. The exclusion of bacitracin had no effect on specific or non-specific binding at either category of brain nuclei.

TABLE 4-1. ^{125}I-[SAR^1,ILE^8]-AII RECOVERY AND SPECIFIC BINDING IN BUFFERS CONTAINING BACITRACIN AND/OR EDTA

	+Bac (-EDTA)		+EDTA +Bac		+EDTA (-Bac)
A. Radioligand Recovery	98 ± 3%	ns	98 ± 1%	*	83 ± 7%
B. Radioligand Binding					
Predominantly AT_1 Nuclei:					
Piriform Cortex	311 ± 72	**	611 ± 134	ns	667 ± 171
Suprachiasmatic n.	715 ± 59	**	1049 ± 79	ns	953 ± 63
Median Preoptic	493 ± 98	**	887 ± 111	ns	878 ± 79
Ventral Hippocampus	496 ± 67	**	700 ± 101	ns	679 ± 70
Paraventricular n.	604 ± 73	**	1140 ± 87	ns	1014 ± 87
Anterior Pituitary	2787 ± 359	**	4690 ± 484	ns	4368 ± 419
Predominantly AT_2 Nuclei:					
Mediodorsal Thalamus	226 ± 19	**	372 ± 25	ns	346 ± 27
Subthalamic n.	581 ± 42	**	887 ± 80	ns	805 ± 52
Medial Geniculate n.	318 ± 59	**	468 ± 56	ns	441 ± 65
Superior Colliculus	304 ± 28	**	577 ± 56	ns	591 ± 52
Lateral Septum	141 ± 24	**	260 ± 24	ns	286 ± 24

Binding values are reported in fmoles/gram of brain tissue ± SEM (n=6). * and ** denote significant differences ($p<0.05$ and $p<0.01$, respectively) between values in adjacent columns.

Because our recoveries exceeded 80% with reduced peptidase inhibition, we elected to utilize a minimal control buffer in the evaluation of additional buffer constituents. Table 4-2 summarizes radioligand integrity (A) and binding determinations (B) in buffers containing bacitracin and EDTA, no peptidase inhibitors, or phenanthroline and BSA. The radioligand was well preserved by the bacitracin/EDTA buffer and by the phenanthroline/BSA buffer. Intact ^{125}I-SIAII recovery was significantly reduced (but still exceeded 80%) in the buffer containing no peptidase inhibitors, consistent with the first experiment showing that deletion of bacitracin from the buffer led to increased radioligand

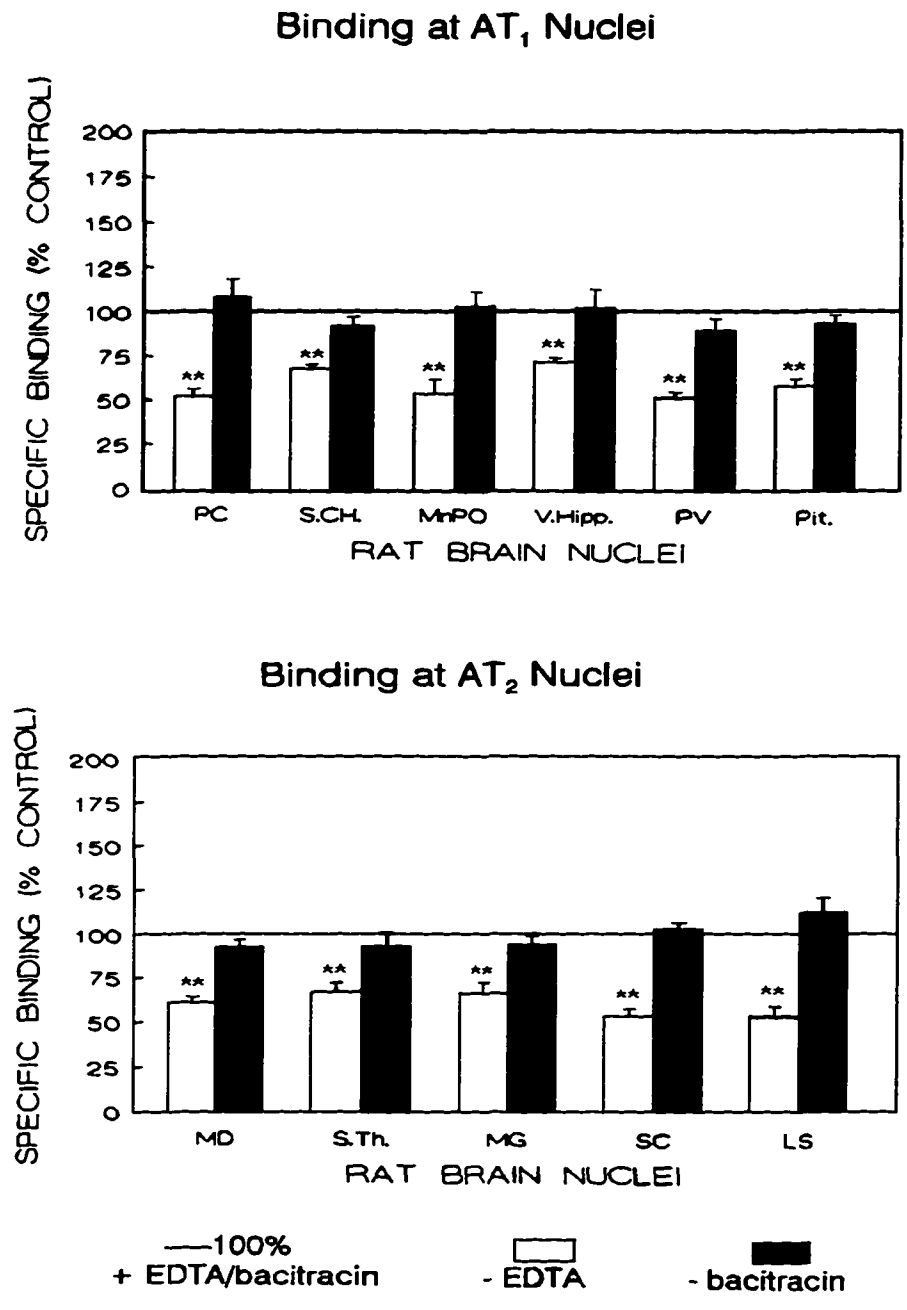

Figure 4-1. Effects of EDTA and bacitracin on specific ^{125}I-SIAII binding. The 100% line represents specific binding in the presence of bacitracin and EDTA, while white bars depict binding in the absence of EDTA and the black bars depict binding in the absence of bacitracin. Significant differences ($p<0.01$) from the control line are noted. Data are expressed as means ± SEM (N=6). Abbreviations: PC, piriform cortex; S.CH., suprachiasmatic n.; MnPO, median preoptic n.; V.Hipp., ventral hippocampus; PV, paraventricular n.; Pit., anterior pituitary; MD, mediodorsal thalamus; S.Th., subthalamic n.; MG, medial geniculate n.; SC, superior colliculus; and LS, lateral septum.

TABLE 4-2. SPECIFIC ^{125}I-[SAR^1,ILE^8]-ANGIOTENSIN II RECOVERY AND SPECIFIC BINDING IN BUFFERS CONTAINING PHENANTHROLINE/BSA, BACITRACIN/EDTA, OR NO INHIBITORS

	+Phen +BSA		+Bac +EDTA		no inhibitors
A. Radioligand Recovery	94 ± 4%	Ns	97 ± 1%	*	86 ± 4%
B. Radioligand Binding					
Predominantly AT_1 Nuclei:					
Piriform Cortex	1061 ± 225	ns	1026 ± 273	**	578 ± 112
Suprachiasmatic n.	1553 ± 334	**	1072 ± 248	*	769 ± 186
Median Preoptic	1331 ± 217	*	1024 ± 170	*	622 ± 51
Ventral Hippocampus	1055 ± 117	**	817 ± 100	**	485 ± 43
Paraventricular n.	1542 ± 191	**	1015 ± 124	**	594 ± 89
Anterior Pituitary	6285 ± 1449	**	4100 ± 780	*	2598 ± 750
Predominantly AT_2 Nuclei:					
Mediodorsal Thalamus	167 ± 8	**	289 ± 13	*	240 ± 21
Subthalamic n.	590 ± 75	**	825 ± 93	**	492 ± 48
Medial Geniculate n.	245 ± 18	**	319 ± 24	**	189 ± 20
Superior Colliculus	347 ± 48	**	458 ± 62	**	253 ± 33
Lateral Septum	152 ± 9	**	239 ± 32	**	115 ± 8

Binding values are reported in fmoles/gram of brain tissue ± SEM (n=5). * and ** denote significant differences ($p<0.05$ and $p<0.01$, respectively) between values in adjacent columns.

degradation. Figure 4-2 illustrates relative specific binding determinations in each of these buffers at predominantly AT_1 and predominantly AT_2 nuclei. In the presence of both EDTA and bacitracin, binding was increased (compared to binding in the absence of inhibitors) by an average of 39% at all brain nuclei surveyed irrespective of subtype composition. At all predominantly AT_1 nuclei (except the piriform cortex), binding was significantly higher in the phenanthroline/BSA buffer compared to both other buffers. In contrast, ^{125}I-SIAII binding at AT_2 nuclei in the phenanthroline/BSA buffer was significantly lower

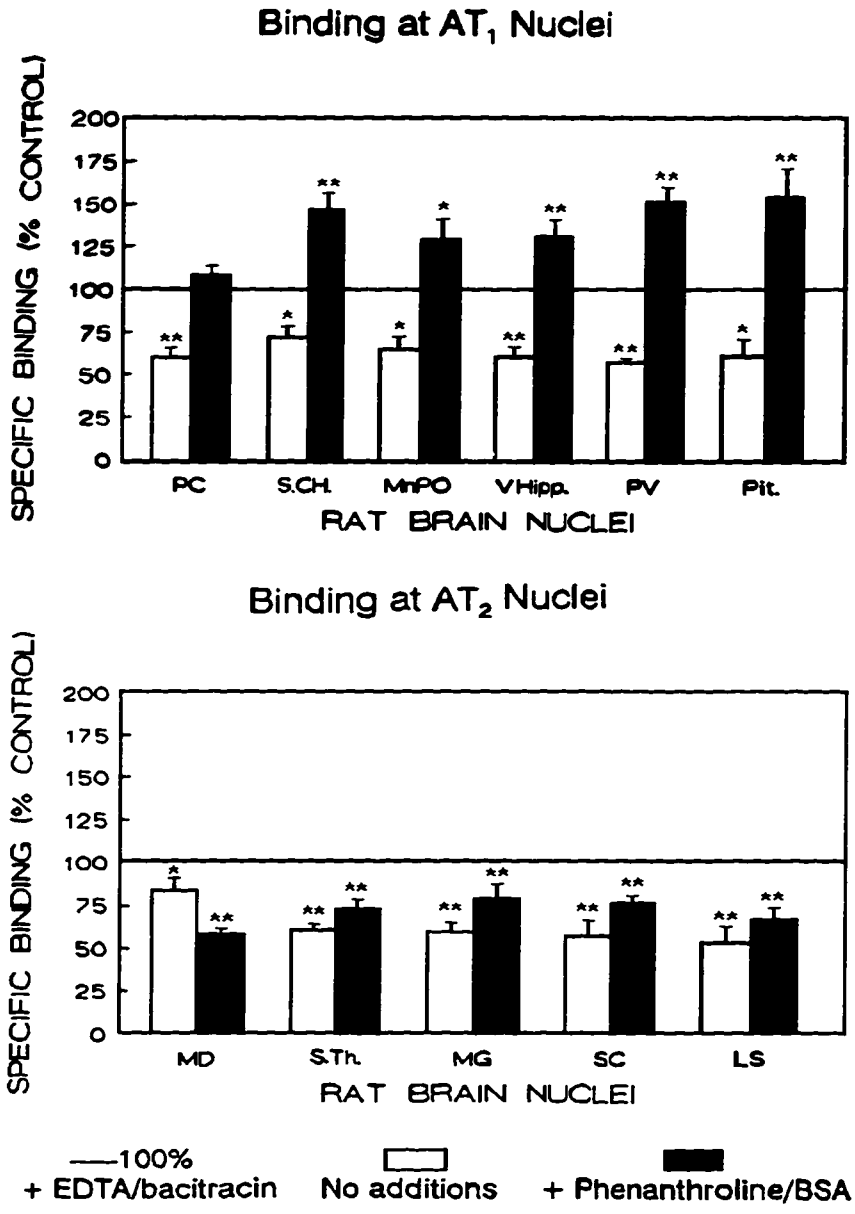

Figure 4- 2. Effects of phenanthroline and BSA on specific ^{125}I-SIAII binding. The 100% line represents specific binding in the presence of bacitracin and EDTA, while the white bars depict binding in the absence of peptidase inhibitors and the black bars depict binding with phenanthroline and BSA. Significant differences (* $p<0.5$, and ** $p<0.01$) from the 100% control line are noted. Data are expressed as means ± SEM (N=5). See brain nuclei abbreviations in the legend of Figure 4-1.

than EDTA/Bacitracin and similar to the buffer with no additions. Thus, the EDTA/bacitracin buffer affected binding consistently at AT_1 and AT_2 binding sites while the phenanthroline/BSA combination enhanced binding preferentially at AT_1 sites. Non-specific binding (not presented) was similar in all three buffers and was approximately 10-15% of total binding. Phenanthroline and BSA were evaluated individually at the nucleus of the solitary tract and the inferior olivary nucleus in each of 6 rat brains. Specific binding at the nucleus of the solitary tract (AT_1) was 782 ± 52 fmol/g in phosphate-saline buffer, 1224 ± 93 with BSA (57% increase, $p<0.01$), and 1122 ± 78 with phenanthroline (43% increase, $p<0.01$). Specific binding at the inferior olivary nucleus (AT_2) was 410 ± 30 fmol/g in phosphate-saline buffer, 356 ± 23 with BSA (NS), and 325 ± 24 with phenanthroline (21% decrease, $p<0.01$). Thus, the enhancement seen with phenanthroline and BSA at AT_1 nuclei in the previous experiment is due to individual contributions by both phenanthroline and BSA.

Additional buffer constituents were screened and the results are summarized in table 4-3. Radioligand recoveries were significantly less than 100% (after tissue incubation compared to before tissue incubation) in the presence of PMSF, amastatin, or leupeptin. Similarly, recovery in the control buffer (saline-phosphate) was 76 ± 9.1 % ($p<0.05$). Only DTT seemed to offer substantial protective effects.

TABLE 4-3. ^{125}I-[SAR^{1}, ILE^{8}]-ANGIOTENSIN II RECOVERY AND TOTAL BINDING (RELATIVE TO CONTROL) IN THE PRESENCE OF INDIVIDUAL PEPTIDASE INHIBITORS.

	DTT	PMSF	Amastatin	Bestatin	Leupeptin
A. Radioligand Recovery					
	87 ± 5.8%	69 ± 4.2%	77 ± 6.2%	76 ± 11.0%	73 ± 10.9%
Significance (vs. 100%)	Ns	P<0.01	P<0.05	ns	P<0.05
B. Total Binding (% Control)[1]					
			Predominantly AT_1 Nuclei		
PC	14 ± 0.05	102 ± 0.2	107 ± 1.6	115 ± 6.1	124 ± 10.9
Pit	12 ± 2.1	104 ± 1.2	96 ± 3.8	108 ± 5.3	99 ± 4.2
PV	24 ± 2.8	103 ± 1.1	105 ± 3.4	107 ± 9.2	106 ± 9.2
SCh	13 ± 0.8	95 ± 5.1	97 ± 3.4	110 ± 6.3	102 ± 6.7
VHipp.	14 ± 2.2	98 ± 4.1	96 ± 4.1	104 ± 2.1	111 ± 7.6
AT_1 Mean	15 ± 2.2	100 ± 1.8	100 ± 2.5	109 ± 1.7	108 ± 4.4
			Predominantly AT_2 Nuclei		
LS	131 ± 19.6	112 ± 4.4	117 ± 5.7	130 ± 17.2	150 ± 25.7
MD	124 ± 5.4	102 ± 1.7	109 ± 1.4	116 ± 4.5	119 ± 1.5
MG	111 ± 8.1	108 ± 7.5	97 ± 7.6	114 ± 10.5	119 ± 13.8
SC	139 ± 25.0	94 ± 5.6	95 ± 3.5	116 ± 3.4	132 ± 5.6
STh.	109 ± 7.0	107 ± 6.2	110 ± 7.4	117 ± 14.4	119 ± 8.3
AT_2 Mean	123 ± 5.8	105 ± 3.1	106 ± 4.2	119 ± 2.8	128 ± 6.2
Selectivity (AT_1 vs. AT_2)	P<0.05	ns	ns	ns	ns

[1] Abbreviations: piriform cortex (PC), pituitary (Pit), paraventricular n. of the hypothalamus (PV), suprachiasmatic n. (SCh), ventral hippocampus (VHipp.), lateral septum (LS), mediodorsal thalamus (MD), medial geniculate (MG), superior colliculus (SC) and subthalamic n. (STh.).

Binding values are expressed as percent of binding observed in control buffer to facilitate comparisons between nuclei. In order to ascertain the selectivity (differential effects on binding with respect to receptor subtype), the effect on binding at predominantly AT_1 nuclei is compared to that observed at predominantly AT_2 nuclei. Dithiothreitol (a sulfhydryl reducing agent) altered binding as expected, markedly inhibiting AT_1 binding while enhancing binding at AT_2 sites. This pattern produced a significant difference in relative binding at AT_1 versus AT_2 sites. Relative binding in the presence of PMSF, amastatin, bestatin and leupeptin was not significantly different with respect to receptor subtype. Therefore, these peptidase inhibitors do not differentially affect binding at AT_1 and AT_2 receptors.

Discussion

Analysis of receptor binding studies ideally presupposes that ligand and receptor metabolism do not occur. Selection of incubation constituents is often made to accomplish this goal with the expectation that these constituents will not interfere with the receptor-ligand reaction. The susceptibility of angiotensin II and its analogues to metabolism dictates the use of peptidase inhibitors in incubation cocktails. Some laboratories use radioligands such as ^{125}I-SIAII which are relatively resistant to aminopeptidase action. There is considerable variation in the selection of inhibitors for other peptidases and for other ionic buffer constituents. With regard to the criterion that constituents must not interfere with

binding, many of these procedures were established before the discovery of AII receptor subtypes and it is now apparent that this criterion was not satisfied independently for both AT_1 and AT_2 binding reactions. This important consideration was emphasized by the discovery that sulfhydryl reducing agents selectively interfere with binding at AT_1 sites (Whitebread *et al.*, 1989; Chiu *et al.*, 1989b; Speth *et al.*, 1991).

In our studies of rat brain AII receptor binding we have noted some subtle differences in data reported by various laboratories. For example, estimates of receptor subtype proportions within brain nuclei are not entirely consistent (Rowe *et al.*, 1992; Gehlert *et al.*, 1990; Song *et al.*, 1992; Tsutsumi and Saavedra, 1992). Moreover, we find that ^{125}I-SIAII shows some selectivity for brain AT_1 receptors (Rowe *et al.*, 1992) which is at variance with findings from some laboratories (Chang *et al.*, 1990; Obermuller *et al.*, 1991) but is similar to the findings of Tsutsumi and Saavedra (1992) for ^{125}I-sar^1-AII. It occurred to us that such discrepancies might result from one or more peptidase inhibitors exerting differential effects on binding at AT_1 and AT_2 receptors similar to that observed for sulfhydryl reducing agents. Table 4-4 itemizes incubation constituents used by several laboratories.

Comparison of binding at AT_1 and AT_2 sites was accomplished by sorting brain nuclei by predominant subtype (>90%) established in a previous study (Rowe *et al.*, 1992). Thus, the results were not obtained from completely

TABLE 4-4. ITEMIZED LISTING OF BUFFER CONSTITUENTS FOR SEVERAL LABORATORIES.

	Reference[1]:						
	1	2	3	4	5[2]	6[3]	7[4]
Sodium phosphate (mM)	50	50	10	10	10		
Tris (mM)						50	50
NaCl (mM)	150	150	150	120	100	125	150
$MgCl_2$ (mM)		10			6.5		
EDTA (mM)	5		5	5	5	1	5
EGTA (mM)		5					
Bacitracin (mM)		0.1		0.4	0.07	0.1	
BSA (%)		0.4	0.2	0.2	0.2	0.2	0.2
Phenanthroline (mM)					0.09		1

[1] References: 1> Rowe *et al.*, 1992; 2> Gehlert *et al.*, 1990; 3> Song *et al.*, 1992; 4> Tsutsumi and Saavedra, 1992; 5> Chang *et al.*, 1990; 6> Obermuller *et al.*, 1991; 7> Koziarz and Moore, 1989. Additional buffer constituents: [2] 0.2 mg/ml soybean trypsin inhibitor, 0.1 mM phenylmethylsulfonyl-fluoride; [3] 1.25 µg/ml each antipain, phosphoramidon, leupeptin, pepstatin A, bestatin and amastatin; [4] 1 mM phenylmethylsulfonylfluoride.

homogeneous receptor subtype populations. This approach was selected because utilization of subtype selective antagonists to mask each receptor subtype would introduce uncertainties with respect to possible differential metabolism or binding characteristics of antagonists in each experimental situation. We believe the approach is justified by the compelling internal consistency observed within the AT_1 and AT_2 categories.

We first evaluated the buffer constituents routinely used in our laboratory, EDTA and bacitracin. Addition of EDTA quantitatively increased specific binding by approximately 70% equally at both AT_1 and AT_2 binding sites. This is

interesting because radioligand degradation was unaffected by the absence or presence of EDTA, suggesting that EDTA affects binding in a manner unrelated to radioligand preservation. Three possible mechanisms might be considered: 1) EDTA chelates ions that hamper the interaction of ^{125}I-SIAII with both AII receptors, 2) EDTA interacts directly with the radioligand (and/or receptors) to enhance binding affinity, or 3) EDTA inhibits metalloproteases that degrade both AT_1 and AT_2 receptors equally. The effect of EDTA on non-specific binding is similar. If we assume this is not residual specific binding, then EDTA affects ^{125}I-SIAII but not AII receptors.

Bacitracin appears to be important for preserving radioligand, but had no effect on specific or non-specific binding. However, radioligand metabolism in the absence of bacitracin was minimal (13-17%) which probably accounts for our inability to observe differences in radioligand binding in the presence and absence of bacitracin. The presence of bacitracin may assume greater importance for the preservation of other angiotensin peptides and in binding assays where tissue/ligand ratios are higher. Parenthetically, concentrations of bacitracin greater than 0.1 mM inhibit ^{125}I-AII binding in rat brain homogenates (Speth, unpublished observation).

Some investigators use phenanthroline (0.1-1.0 mM) and BSA (0.2%) in binding assays (table 4-4). Koziarz and Moore (1989) evaluated ^{125}I-AII binding with bovine membrane preparations in the absence and presence of

1,10-phenanthroline (1 mM). Phenanthroline completely eliminated ^{125}I-AII degradation without affecting binding. Since similar results were obtained for both uterine (AT_2 predominant) and aortic (AT_1 predominant) preparations, we infer that binding was not differentially affected at AT_1 and AT_2 sites. These interesting observations prompted us to include phenanthroline in our autoradiographic system. We used a concentration of 0.5 mM phenanthroline which is lower than that used by Koziarz and Moore (1 mM) but higher than that used by Chang et al. (1990), (0.09 mM). Radioligand recovery in this study was 86.7% in the absence of inhibitors compared to 94.3% (not significantly different) in the presence of phenanthroline and BSA. The excellent radioligand recovery in the absence of inhibitors in our experiments may be attributed to the aminopeptidase resistance of the sarcosine substituted radioligand and the low tissue/radioligand ratio in these autoradiographic studies. Interestingly, however, phenanthroline and BSA had marked differential effects on binding at AT_1 nuclei as compared to AT_2 nuclei.

Addition of phenanthroline and BSA affects binding at both AT_1 and AT_2 sites in a manner that is dissimilar to EDTA. Phenanthroline chelates metal ions (Mertell and Calvin, 1956), but none that are not also chelated by EDTA. Thus, the chelating properties of phenanthroline are unlikely to account for the differential effects on binding. It is also possible that BSA contributes to the observed effects. The AT_1 receptor has disulfide bonds in its extracellular domain

(Murphy *et al.*, 1991; Sasaki *et al.*, 1991) that are crucial for binding (Whitebread *et al.*, 1989; Chiu *et al.*, 1989b). Since BSA is stabilized by 17 disulfide bonds (Budavari, 1992) it might serve as an antireductant and preserve the tertiary structure of the AT_1 receptor. We have not attempted a systematic analysis of each buffer constituent employed by multiple laboratories but we do provide evidence that procedural differences are likely to account for reported differences in binding characteristics.

This study indicates that degradation of ^{125}I-SIAII is minimal (<17%) in the complete absence of peptidase inhibitors in our autoradiographic procedures. It does not follow, however, that the observation is applicable to non-autoradiographic binding studies or to autoradiographic studies using non-sarcosine angiotensin peptides or to studies with higher tissue/incubation medium ratios. Inclusion of bacitracin or phenanthroline/BSA leads to near total recovery of radioligand. EDTA enhances binding but does so uniformly at both AT_1 and AT_2 sites. In contrast, phenanthroline and/or BSA markedly enhance binding at AT_1 sites but not at AT_2 sites. It is clear that buffer constituents affect binding at AT_1 and AT_2 sites by mechanisms that are independent of radioligand metabolism. Therefore, relative binding characteristics between AT_1 and AT_2 receptor subtypes will necessarily differ and caution must be exercised in comparing data from different laboratories.

CHAPTER 5

LOCALIZATION AND SUBTYPE CHARACTERIZATION OF ANGIOTENSIN II RECEPTORS IN THE NORMOTENSIVE AND SPONTANEOUSLY HYPERTENSIVE HAMSTER

Introduction

The presence of angiotensin II (AII) receptor subtypes has been confirmed in tissue homogenates of rat, rabbit and monkey brains (Chang and Lotti, 1991), and classification of individual brain nuclei by receptor subtype has been determined in the rat (Rowe et al., 1990c; 1991; 1992). Subtype classification of individual brain nuclei remains undetermined for other species, but binding sites have been mapped autoradiographically to individual brain nuclei in the sheep (McKinley et al., 1986), rabbit (Mendelsohn et al., 1988), dog (Speth et al., 1985), and human (McKinley et al., 1987). Some species differences are evident but binding site distributions are generally similar (Mendelsohn et al., 1990). The purpose of the first part of the present study is to define the distribution of AII receptors in the hamster brain, and further, to classify each receptor population by receptor subtype.

Genetic models of hypertension in the rat are associated with disturbances in the brain angiotensin system. For example, centrally

administered AII stimulates a larger blood pressure increase in spontaneously hypertensive rats (SHR) than in normotensive control rats (Hoffman *et al.*, 1977), and saralasin administered centrally reduces the blood pressure of SHR but not control rats (Hutchinson *et al.*, 1975; Phillips *et al.*, 1977). Further, there is a greater AII-like immunoreactivity (Weyhenmeyer and Phillips, 1982), and quantitative autoradiographic studies have shown significantly higher AII binding in SHR brains (Hwang *et al.*, 1986; Gehlert *et al.*, 1986b). We therefore compared ^{125}I-[sar^{1},ile^{8}]-AII (^{125}I-SIAII) binding in a model of genetically hypertensive hamster (Joyner *et al.*, 1988) with normotensive controls.

Design

The first experiment was designed to determine which hamster brain nuclei contain AII receptors, and further to characterize each receptor population by AII receptor subtype. Brains from 4 golden syrian hamsters (102-120 g) were prepared as described in methods (in vitro receptor autoradiography). Incubation mediums consisted of buffer with ^{125}I-SIAII (350-420 pM) alone, with losartan (10^{-5} M), with PD123177 (10^{-5} M), or with AII (10^{-6} M). Tissue sections from two animals were also incubated in the presence of both losartan and PD123177 (10^{-5} M). Receptor subtype distribution was determined by comparing specific binding of ^{125}I-SIAII in the presence of losartan or PD123177 with specific binding in the absence of competitors. The subtype selective compound losartan competes for ^{125}I-SIAII binding at the AT_1 but not at the AT_2 subtype while PD123177 competes selectively for the AT_2 binding site.

A second experiment evaluated ^{125}I-SIAII binding in a hamster model of genetic hypertension (Joyner *et al.*, 1988). Brain sections from 4 normotensive (128-133 g) and 4 hypertensive (117-144 g) hamsters were incubated with ^{125}I-SIAII (262-266 pM) as described in methods (*in vitro* receptor autoradiography). Each brain from a hypertensive hamster was processed and incubated concurrently with a brain obtained from the normotensive control strain.

Results

Sets of adjacent sections were incubated with ^{125}I-SIAII in the presence and absence of the AII subtype selective compounds losartan (AT_1 selective) and PD123177 (AT_2 selective). A typical example obtained at the level of the hypothalamus is illustrated by the autoradiograms in Figure 5-1. Losartan (10^{-5} M) competed effectively for binding sites in the hippocampus, choroid plexus, and the paraventricular nucleus of the hypothalamus (AT_1 sites), but not at the medial habenular nucleus (AT_2 site). Competition with PD123177 (10^{-5} M) produced the opposite pattern, i.e. it competed substantially for ^{125}I-SIAII binding at the medial habenular nucleus, but not at the other structures. The ^{125}I-SIAII binding was substantially displaced in all structures in sections incubated with both subtype selective compounds and produced autoradiograms very similar to those of sections incubated with AII (10^{-6} M). Similar results were obtained for various brain nuclei (autoradiograms not illustrated) which were quantitated and summarized in Table 5-1. A reciprocal competition pattern between the two

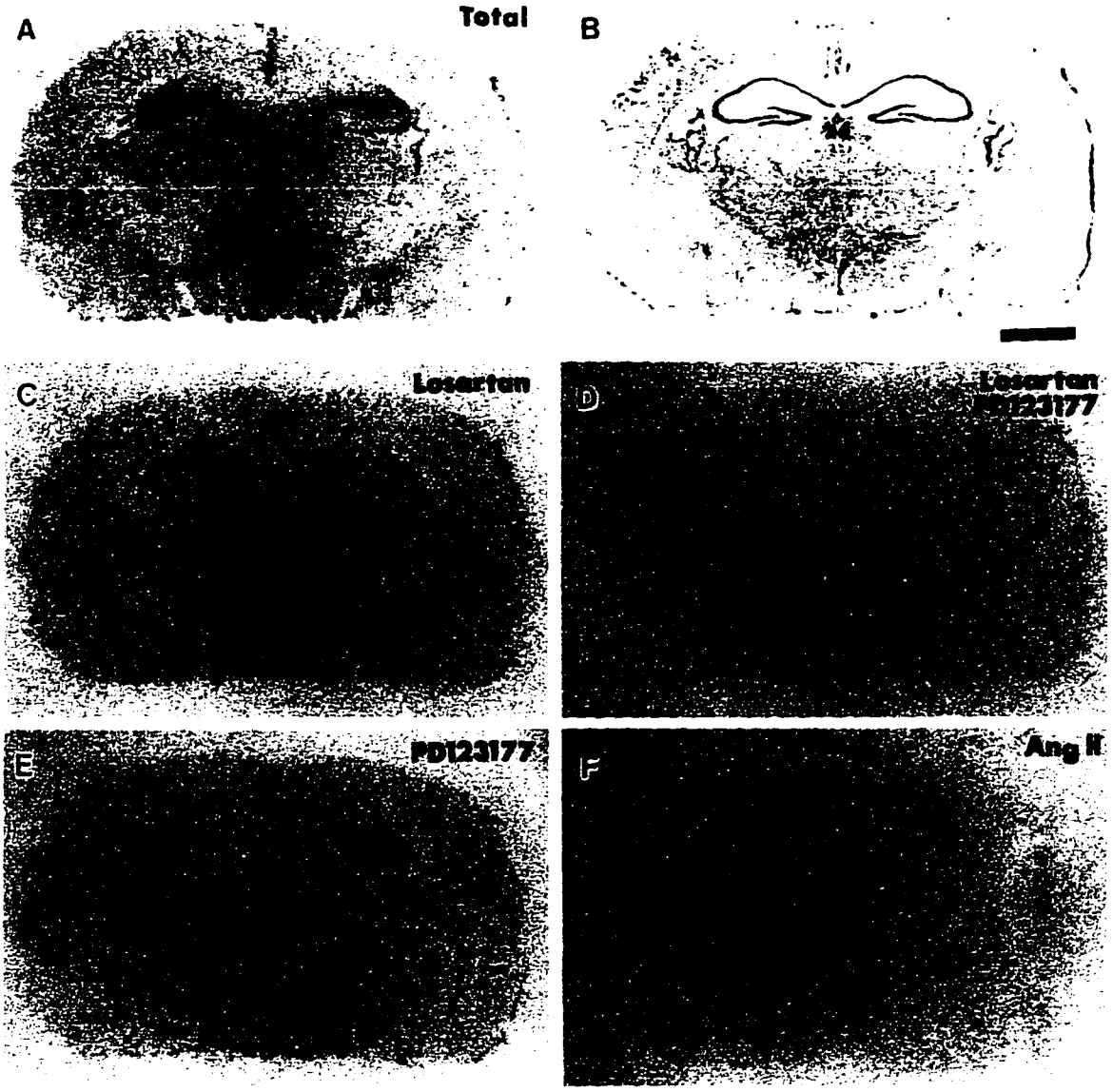

Figure 5-1. [^{125}I][Sar^{1},Ile^{8}]-angiotensin II (^{125}I-SIAII) binding with subtype selective competitors in adjacent hamster brain sections. A: total ^{125}I-SIAII binding without competitors; B: thionin stained section (Bar = 2 mm); C: sites insensitive to losartan (10^{-5} M) [medial habenula (MHb)]; D: competition with both losartan (10^{-5} M) and PD123177 (10^{-5} M); E: sites insensitive to PD123177 (10^{-5} M) [hippocampus (Hip), choroid plexus (ChP), and the [paraventricular nucleus of the hypothalamus (PV)]; F: non-specific binding not displaced by Ang II (10^{-6} M).

subtype selective compounds was evident in all brain regions. In some brain nuclei, competition by the combination of both subtype selective compounds was slightly more effective than AII. This is reflected by negative specific binding values reported in Table 5-1.

Figures 5-2 through 5-6 illustrate ^{125}I-SIAII binding without competitors for a selection of brain regions listed in Table 5-1. Each autoradiogram is accompanied by the adjacent thionin stained section to aid in the verification of structures.

Hindbrain:

Figure 5-2 illustrates ^{125}I-SIAII binding in the dorsal motor nucleus of the vagus (DMV), the nucleus of the solitary tract (NTS), and the spinal trigeminal tract. All three regions contain predominantly AT_1 receptor sites (Table 5-1). Binding to AT_1 sites is also evident in the area postrema (not illustrated) and is particularly dense in the paramedial segment of the NTS. The remaining subdivisions of the hamster NTS show modest binding compared to strikingly dense binding in the DMV. Interestingly, the DMV of the hamster was unique in that approximately one-fifth of specific ^{125}I-SIAII binding was not displaced by either losartan or PD123177 at the concentrations used. The lower panel in figure 5-2 also shows binding in the choroid plexus (ChP) of the fourth ventricle and a diffuse AT_1 binding is barely discernable in the rostral portion of the ventrolateral reticular formation. There is a notable absence of ^{125}I-SIAII binding in the inferior olive.

TABLE 5-1. [125I][SAR1,ILE8]-ANGIOTENSIN II BINDING AT HAMSTER BRAIN NUCLEI WITH AND WITHOUT SUBTYPE SELECTIVE COMPETITORS

	Specific ^{125}I-[sar^{1},ile^{8}]-Angiotensin II bound (fmoles/g tissue) [1]				Nonspecific Binding
Nucleus	Without Competition	With Losartan (10^{-5} M)	With PD123177 (10^{-5} M)	Losartan +PD123177	With AII 10^{-6} M
Pituitary	881± 69	312±73 (65%)	715±105 (19%)	67 ± 52	111 ± 11
Subfornical Organ	1064±201	353±64 (67%)	715±110 (33%)	-46 ± 59	119 ± 34
Median Preoptic n.	706± 90	159±24 (77%)	378± 94 (46%)	-13 ± 23	73 ± 11
Paraventricular n.	1263±140	197±52 (84%)	1213±121 (4%)	-118 ± 9	174 ± 18
Ventral Hippocampus	651± 79	92±26 (86%)	551± 59 (15%)	-16 ± 5	99 ± 18
Nucleus Tractus Solitarius	955± 48	362±44 (62%)	800± 78 (16%)	96 ± 11	116 ± 8
Spinal Trigeminal n.	539± 38	169±18 (67%)	389± 35 (28%)	4.5 ± 11	68 ± 6
Pedunculopontine Tg. n.	596± 72	92±8.9 (85%)	479± 47 (20%)	-19 ± 15	66 ± 12
Parabrachial n.	746± 15	231±22 (69%)	440± 22 (41%)	20 ± 8	73 ± 10
Locus Coeruleus	536± 19	365±13 (32%)	264± 60 (51%)	7 ± 6	54 ± 6
Inferior Colliculus	682± 74	494±47 (28%)	134± 18 (80%)	11 ± 3	68 ± 7
Superior Colliculus	800± 48	686±37 (14%)	105± 14 (87%)	16 ± 17	58 ± 5
Lateral Septum	532± 67	481±73 (10%)	115± 23 (78%)	26 ± 23	70 ± 7
Medial Geniculate n.	655± 78	108±10 (84%)	484± 40 (26%)	11 ± 5	69 ± 5
Dorsal Motor n. Vagus	2184±122	636±45 (71%)	2093±137 (4%)	457 ± 36	238 ± 21
Peri-aqueductal Grey	445± 36	330±22 (26%)	147± 27 (67%)	10 ± 3	56 ± 8
Interpeduncular n.	821± 37	587±96 (29%)	96± 24 (88%)	-9 ± 9	66 ± 17
Choroid Plexus	387± 42	100±15 (74%)	238± 54 (39%)	11 ± 4	61 ± 12
Medial Habenula	936± 95	894±94 (4%)	271± 17 (71%)	61 ± 36	53 ± 7

[1] Values in parentheses represent the proportion of specific binding displaced by subtype selective competition. For some nuclei, total radioligand binding in the presence of both subtype selective antagonists was less than binding in the presence of unlabelled angiotensin II, producing negative specific binding values.

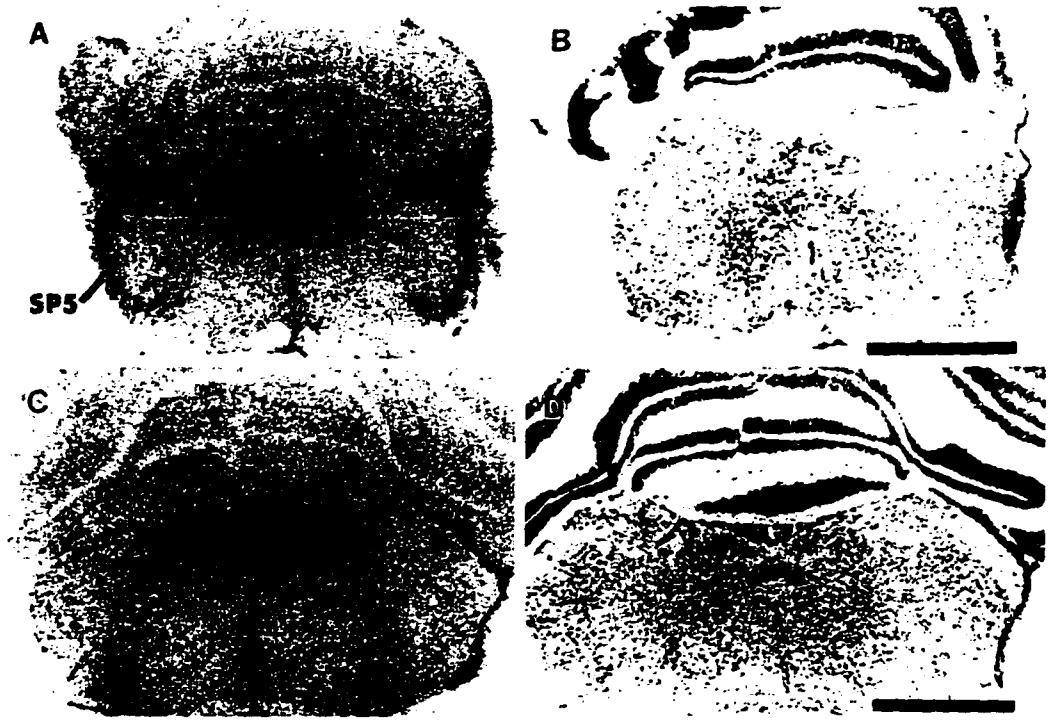

Figure 5-2. Total ^{125}I-SIAII binding (left panels) and adjacent thionin stained sections (right panels; Bar = 2 mm) at the nucleus of the solitary tract (NTS), dorsal motor nucleus of the vagus (DMV) and spinal trigeminal tract (SP5).

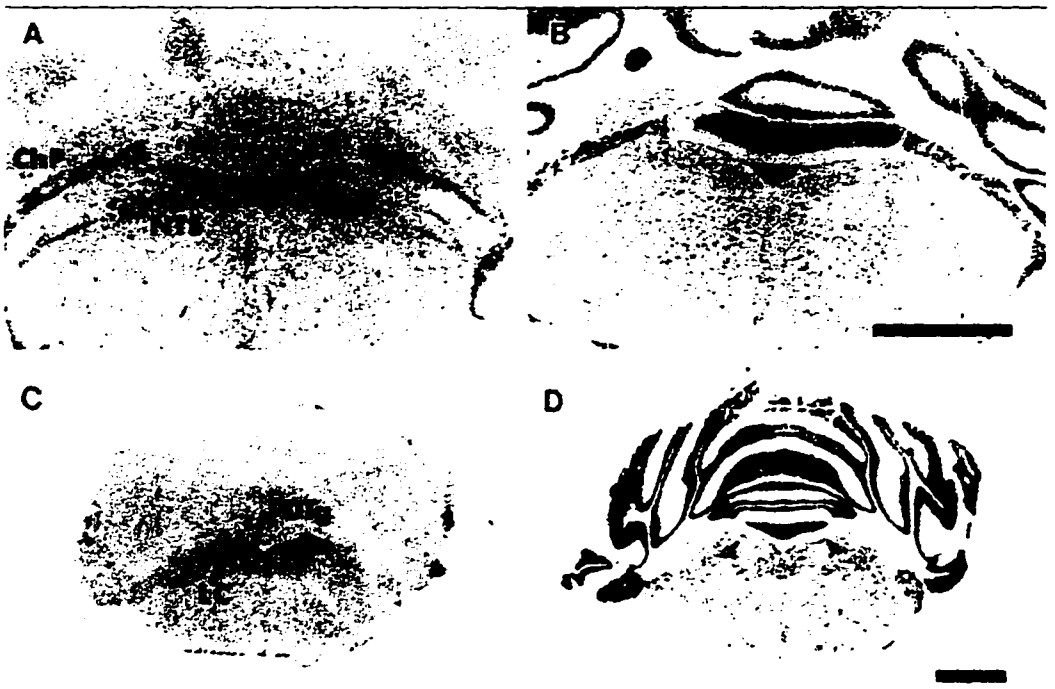

Figure 5-3. Total ^{125}I-SIAII binding (left panels) and adjacent thionin stained sections (right panels; Bar = 2 mm) at the choroid plexus (ChP), nucleus tractus solitarius (NTS) and posterodorsal tegmental nucleus (PDTg).

Figure 5-3 illustrates binding in the rostral NTS and the choroid plexus as it occurs throughout the fourth ventricle. The ^{125}I-SIAII binding in pontine structures at the level of the genu of the seventh nerve includes diffuse binding in vestibular nuclei and denser binding associated with the locus coeruleus (LC) and the posterodorsal tegmental nucleus (PDTg). Binding sites in the locus coeruleus appear to be a substantial mixture of both AT_1 and AT_2 sites (Table 5-1).

Midbrain:

The ^{125}I-SIAII binding sites on the medial border of the inferior colliculus (Figure 5-4) are predominantly AT_2. Figure 5-4 also illustrates binding sites in the parabrachial nucleus and the pedunculopontine tegmental nucleus. Binding in both nuclei is a mixture of AT_1 and AT_2 sites with AT_1 predominating.

The top panel in Figure 5-5 demonstrates binding in the superior colliculus (SC), periaqueductal grey (PAG), interpeduncular nucleus (IP), the ventral portion of the caudal hippocampus (VH), and the anterior pituitary. AT_1 binding sites are evident in the posterior segment only of the medial geniculate nucleus (MG, not illustrated). Diffuse, predominately AT_2 binding is evident in the periaqueductal grey (PAG). Binding is conspicuously absent in the subthalamic nucleus of the hamster.

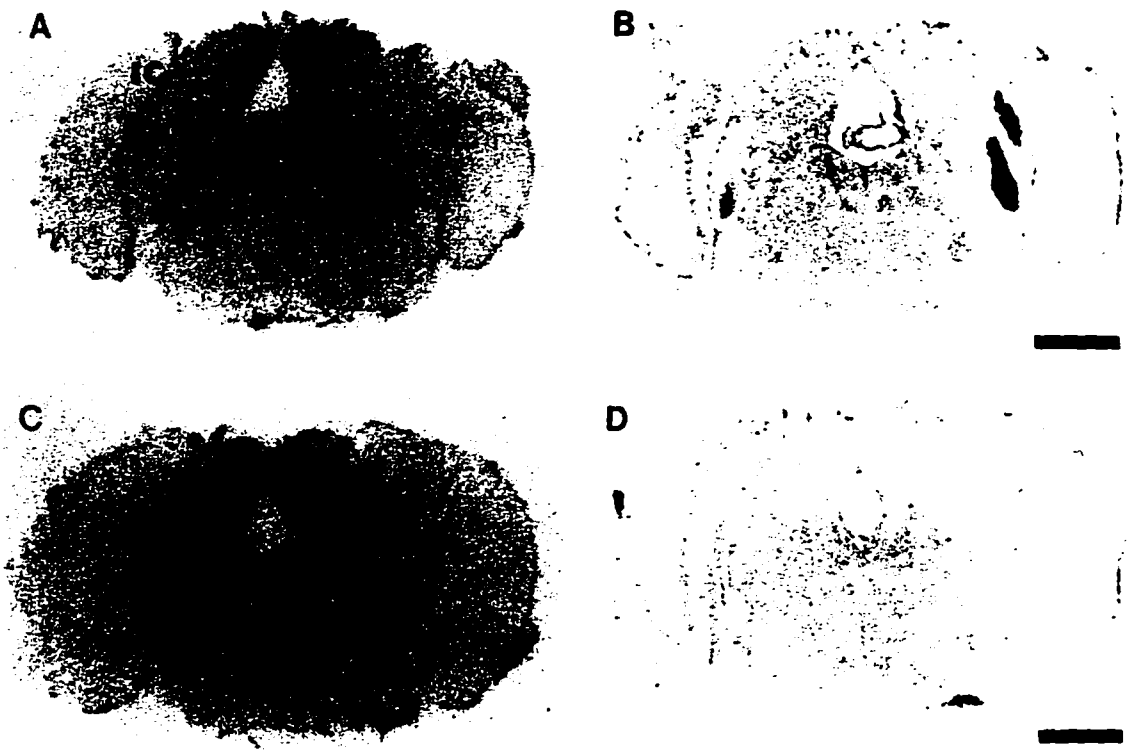

Figure 5-4. Total ^{125}I-SIAII binding (left panels) and adjacent thionin stained sections (right panels; Bar = 2 mm) at the inferior colliculus (IC), parabrachial nucleus (PB) and pedunculopontine tegmental nucleus (PPTg).

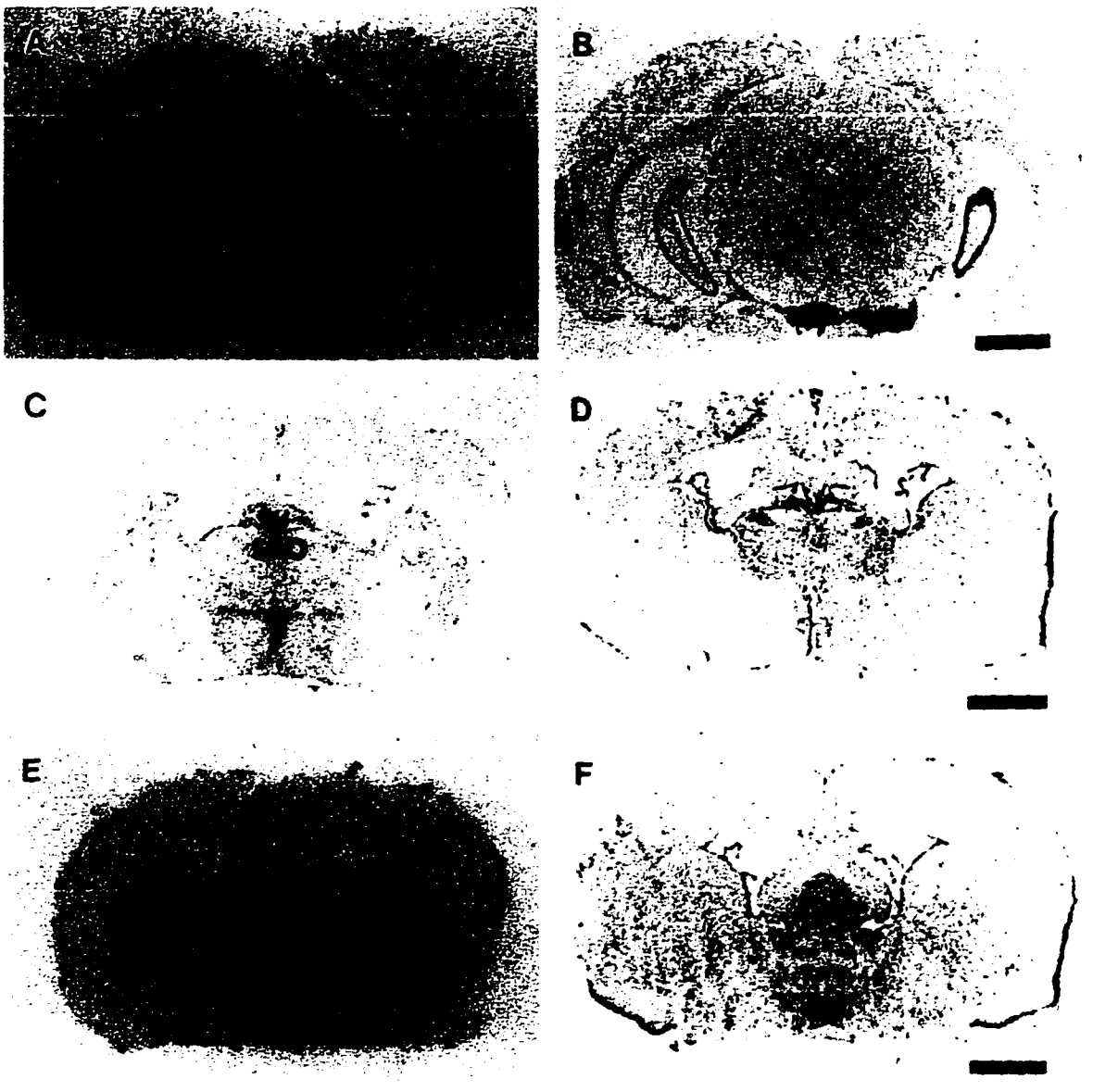

Figure 5-5. Total ^{125}I-SIAII binding (left panels) and adjacent thionin stained sections (right panels; Bar = 2 mm) at the superior colliculus (SC), periaqueductal grey (PAG), ventral hippocampus (VH), interpeduncular nucleus (IP), pituitary (Pit), subfornical organ (SFO) and median preoptic nucleus (MnPO).

Forebrain

Figure 5-1 shows ^{125}I-SIAII binding in the hippocampus, medial habenular nucleus (MHb) and paraventricular nucleus (PV). The dense binding in the MHb is predominately AT_2, while the other structures represent mostly AT_1 binding sites as described above. Binding is notably absent at the suprachiasmatic nucleus, medial amygdala and lateral olfactory tract. Figures 5-5 and 5-6 illustrate ^{125}I-SIAII binding in the subfornical organ (SFO), median preoptic nucleus (MnPO), organum vasculosum of the lamina terminalis (OVLT) and lateral septum (LS). Binding was also observed in the supraoptic nucleus (not illustrated). Binding is absent in the piriform cortex while the striatum has diffuse but conspicuous binding (Figure 5-6).

Hypertensive Hamsters

Table 5-2 describes specific ^{125}I-SIAII binding in the brain nuclei of 4 hypertensive hamsters (systolic, 140-151 mm Hg) and 4 normotensive controls (systolic, 80-87 mm Hg). There were no significant differences in ^{125}I-SIAII binding for any of the nuclei studied.

Discussion

The distribution of AII receptors in the hamster brain is similar to that reported in the rat and other species. The similarities are particularly prominent in

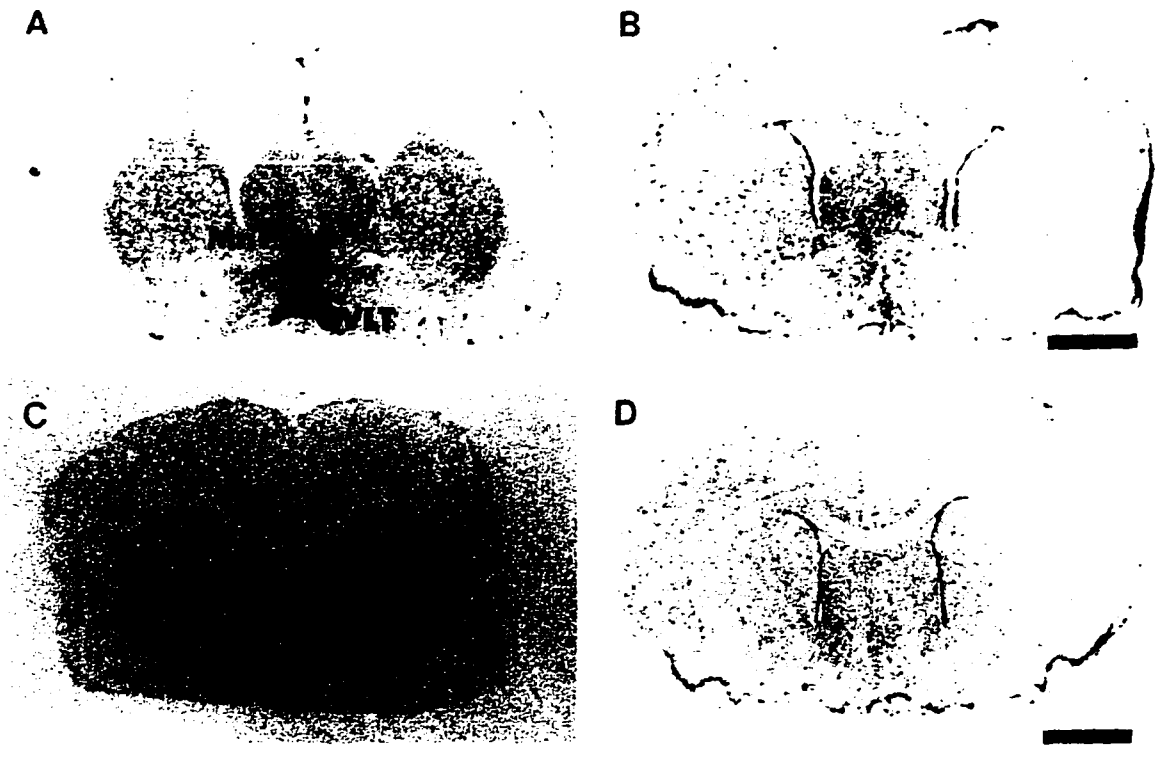

Figure 5-6. Total ^{125}I-SIAII binding (left panels) and adjacent thionin stained sections (right panels; Bar = 2 mm) at the median preoptic nucleus (MnPO), organum vasculosum of the lamina terminalis (OVLT) and lateral septum (LS).

nuclei associated with cardiovascular or dipsogenic functions. For example, the SFO, OVLT, and MnPO have been linked to dipsogenic actions of AII, and the SFO, OVLT, AP, SC, PAG, and NTS have been implicated in the mediation of pressor responses to AII (Speth *et al.*, 1988). All of these brain nuclei contain high densities of AII receptors in both hamster and rat. Other nuclei involved in cardiovascular function where dense ^{125}I-SIAII binding is observed in both species include the PV, MnPO, and DMV. Recent studies have demonstrated

TABLE 5-2. SPECIFIC ^{125}I-[SAR^{1}, ILE^{8}]-ANGIOTENSIN II BINDING AT BRAIN NUCLEI OF NORMOTENSIVE AND HYPERTENSIVE HAMSTER STRAINS.

Nucleus	Normotensive Hamsters	Hypertensive Hamsters
Pituitary	479 ± 98	603 ± 32
Subfornical Organ	860 ± 223	905 ± 116
Median Preoptic n.	362 ± 71	285 ± 20
Paraventricular n.	764 ± 40	819 ± 70
Ventral Hippocampus	353 ± 26	371 ± 34
Nucleus Tractus Solitarius	559 ± 53	559 ± 53
Spinal Trigeminal n.	261 ± 19	263 ± 19
Pedunculopontine Tegmental n.	304 ± 32	249 ± 44
Parabrachial n.	466 ± 31	501 ± 48
Locus Coeruleus	227 ± 18	217 ± 30
Inferior Colliculus	271 ± 12	264 ± 36
Superior Colliculus	323 ± 36	347 ± 55
Lateral Septum	198 ± 29	210 ± 27
Medial Geniculate n.	213 ± 3	277 ± 35
Dorsal Motor n. of the Vagus	1429 ± 234	1454 ± 158
Peri-aqueductal Grey	159 ± 20	145 ± 19
Interpeduncular n.	453 ± 35	528 ± 78
Choroid Plexus	226 ± 41	166 ± 32
Medial Habenula	503 ± 58	407 ± 53
Organum Vasculosum of the Lamina Terminalis	791 ± 112	728 ± 120

blood pressure responses to AII administered locally at the ventrolateral medulla (Muratani *et al.*, 1991) of rats. Discernable but weak ^{125}I-SIAII binding is localized in this brain region of both the hamster and the rat.

At several nuclei, ^{125}I-SIAII binding was notably different between species. For example, AII receptors have been localized at the inferior olive, suprachiasmatic nucleus, subthalamic nucleus, medial amygdala, piriform cortex and various thalamic structures of the rat, but ^{125}I-SIAII binding is conspicuously absent at these regions of the hamster brain. With the exception of the

subthalamic nucleus and inferior olive of the human brain (Allen *et al.,* 1991), there are no reports of binding in these regions in other species either. There is particularly dense ^{125}I-SIAII binding in the anterior pituitary of the rat, but relatively weak binding in the hamster. Binding in the hamster hippocampus is more extensive than that observed in the rat, and binding in the striatum of the hamster is more conspicuous than the rat. Binding in the DMV of the hamster is particularly dense. Intense binding observed in the interpeduncular nucleus and the medial habenula of the hamster was particularly interesting. AII binding is minimal or absent at these nuclei in the rat brain, and unreported in other species with the exception of the interpeduncular nucleus of the rabbit (Mendelsohn *et al.,* 1988) and human (Allen *et al.,* 1991).

The medial habenula and interpeduncular nuclei communicate via the habenulopeduncular tract (Herrick, 1965; Contestabile and Villani, 1983). This system has connections to the olfactory and limbic system via several nuclei which also contain AII receptors including: septum (Herkenham and Nauta, 1977), suprachiasmatic nucleus (of the rat) (Sofroniew and Weindl, 1978), locus coeruleus and dorsal tegmental region (Jones and Moore, 1977), central gray (Marchand *et al.,* 1980), and the ventral tegmental nucleus (Contestabile and Flumerfelt, 1981). The habenula may be important in linking olfactory sensory information with motor areas in the brainstem, or in mediating certain emotional behaviors (Crosby *et al.,* 1962; Herrick, 1965; Woodburne, 1967). Lesions of the medial and lateral habenular nuclei result in the impairment of the ability of rats to

find food via smell (Rausch and Long, 1971) and furthermore, lesions of the anterior olfactory nucleus are related to degeneration in the dorsomedial thalamus and habenula in the opossum (Ferrer, 1969). Perhaps AII influences salt or water seeking behaviors via this system and species variations are related to differing physiological adaptations to respective natural environments.

The apparent absence of AII receptors at the subthalamic nucleus, inferior olive, piriform cortex, medial amygdala, and suprachiasmatic nucleus in the hamster, but not in the rat is interesting. These structures process a variety of sensory information (Paxinos, 1985) and it is difficult to understand how these two species might function differently in this regard. However, the role of the suprachiasmatic nucleus in processing photoperiod information to influence circadian and reproductive cycles (Card and Moore, 1984; Raisman, 1975) is likely to be different between the species.

Data for losartan and PD123177 competition at hamster brain nuclei resembled those observed in rat brain nuclei (Rowe *et al.*, 1992). The percentage of binding displaced by losartan and PD123177 at each nucleus (Table 1) approximates the fraction of AT_1 and AT_2 receptors respectively. This is based upon assumptions that: 1) the concentration of each competitor is considerably higher than the IC_{50} for its higher affinity site and lower than the IC_{50} for its low affinity site, 2) AT_1 and AT_2 are the only high affinity sites for ^{125}I-SIAII, and binding kinetics for the radioligand are identical at both sites. These data are not available for the hamster, but ^{125}I-SIAII shows some selectivity for AT_1 receptors

in the rat brain. However, since the combination of both competitors competed effectively for all ^{125}I-SIAII binding in the hamster brain, and the sums of percentage competition for each competitor approximate 100% for most nuclei, we believe the values for percent inhibition for each competitor in Table 1 closely reflect the fractional subtype distribution. It is noteworthy that all but two nuclei show similar patterns of subtype predominance in both the hamster and the rat. The two exceptions are the medial geniculate nucleus, predominantly AT_1 in the hamster and predominantly AT_2 in the rat, and the periaqueductal grey that contains mostly AT_2 sites in the hamster and AT_1 in the rat. Strikingly, there seems to be a fraction (approximately 21%) of specific ^{125}I-SIAII binding sites uniquely within the dorsal motor nucleus of the vagus that is not displaced by competition with both losartan and PD123177. Further work will be required to determine if this is a third receptor subtype, but the existence of binding sites insensitive to both losartan and PD123177 has also been reported in *Xenopus laevis* oocytes (Ji *et al.*, 1990).

Angiotensin II binding is evident in several hamster brain nuclei associated with cardiovascular function and several studies indicate disturbances of the brain angiotensin system in the spontaneously hypertensive rat. Specifically, AII receptor binding is elevated at the subfornical organ, nucleus of the solitary tract, dorsal motor nucleus of the vagus, locus coeruleus, supraoptic nucleus, and the organum vasculosum of the lamina terminalis of the SHR (Gehlert *et al.*, 1986b; Hwang *et al.*, 1986) and we investigated the possibility of similar anomalies in a

genetic model of hypertensive hamster (Joyner *et al.*, 1988). We found no significant ^{125}I-SIAII binding differences in the hypertensive model as compared to the normotensive control strain at any of the 20 nuclei studied. Thus, the brain AII system does not appear to be implicated in this hamster model of genetic hypertension.

In summary, ^{125}I-SIAII binding in the hamster brain was localized in generally the same nuclei as reported for other species, and the receptor subtype distribution was similar to that in the rat brain. In particular, a predominance of AT_1 receptors was observed in nuclei associated with AII mediated cardiovascular and dipsogenic functions in other species. Thus, AII mediated central control of cardiovascular function might be operationally similar in all mammalian species. In some hamster brain nuclei the presence or absence of ^{125}I-SIAII binding is at variance with observations in other species. This occurs at nuclei where a role for AII has not been investigated and analysis of species mismatches might provide clues with regard to possible AII functions at these nuclei.

CHAPTER 6

CHARACTERIZATION OF THE EFFECTS OF CENTRALLY ADMINISTERED ANGIOTENSIN II ON BRAIN CATECHOLAMINE UTILIZATION

Introduction

Angiotensin and catecholamine systems are known to interact in a number of ways. Peripherally, angiotensin II stimulates the release of norepinephrine from adrenal chromaffin cells (Marley and Bunn, 1988.), postganglionic neurons (Li *et al.*, 1988), sympathetic nerve terminals in the vasculature (Wong *et al.*, 1992), and brown adipose tissue (Cassis and Dwoskin, 1991). Peripheral interaction between angiotensin and catecholamine systems reflects functional commonality between the two systems. Both have potent and profound cardiovascular actions.

Additionally, angiotensin and catecholamine systems exist within the brain. Anatomically, a number of brain nuclei appear to belong to both systems. Angiotensin immunoreactivity and angiotensin II receptors have been co-localized with catecholamine rich cell bodies and/or nerve terminals in the locus coeruleus, nucleus of the solitary tract, paraventricular nucleus of the hypothalamus (PV), median preoptic nucleus, intermediolateral cell column and in the caudal and rostral ventrolateral medulla (Jenkins *et al.*, 1995). Again, the

commonality of these sites relates to cardiovascular related functions. Peripheral angiotensin II is known to act at the circumventricular organs to activate pathways involving the PV, supraoptic nucleus (SON), nucleus of the solitary tract, and eventually, the rostral and caudal ventrolateral medulla, to influence autonomic output to the peripheral cardiovascular system (Phillips, 1987; McKinley *et al.,* 1990).

Central angiotensin II also acts on receptors in the PV and SON to stimulate release of the potent vasoconstrictor, arginine vasopressin (AVP) into the peripheral circulation. Additionally, angiotensin II stimulates drinking through complex pathways involving the subfornical organ, the anterior wall of the third ventricle, median preoptic nucleus, and the organum vasculosum of the lamina terminalis (Johnson and Edwards, 1990; Mangiapane *et al.,* 1983). Catecholamines have been implicated in both of these responses (Harland *et al.,* 1989; reviewed in Jenkins *et al.,* 1995). Additionally, AII increases norepinephrine utilization in the anteroventral third ventricle of spontaneously hypertensive rats (Tsukashima *et al.,* 1996). Sumners and Phillips (1983) demonstrated changes in norepinephrine and dopamine utilization in the hypothalamus, striatum and subfornical organ following intracerebroventricular injection of angiotensin II. Apparently, angiotensin II produces these responses, at least in part, by modulating catecholamine utilization in these brain regions.

More recently, central angiotensin II has been implicated in the processing of motor, visual, auditory, olfactory, pain, and thermal information as well as in

cognition and learning (Barnes *et al.*, 1992; reviewed in Wright and Harding, 1994). Three central catecholamine systems have been implicated in learning and memory: 1) an α-adrenergic system with neurons in the locus coeruleus projecting through the median forebrain bundle into the limbic forebrain (including amygdala), hypothalamus and neocortex; 2) a dopaminergic system with neurons around the interpeduncular nucleus projecting to the substantia nigra and through the median forebrain bundle to the hypothalamus and prosencephalon, and projections from the substantia nigra to the striatum; and 3) a β-adrenergic inhibitory system in the basolateral amygdala (Gorelick *et al.*, 1975).

Angiotensin II is known to stimulate synthesis and release of catecholamines in the striatum and hypothalamus. Approximately 45% of all noradrenergic neurons in the CNS originate at the locus coeruleus (Swanson, 1976), an area known to contain angiotensin II receptors on noradrenergic neurons (Rowe *et al.*, 1990d). Angiotensin II receptors also exist on dopamine containing cell bodies in the substantia nigra and on their nerve terminals in the striatum (Jenkins *et al.*, 1995). Angiotensin II receptors also occur in additional brain nuclei associated with the three catecholaminergic systems described above (Wright and Harding, 1994). Furthermore, dopamine antagonists block the cognitive effects of angiotensin II (Hyttel and Christensen, 1983). It seems likely

that angiotensin II affects memory and learning, and may tend to act in general, though modulation of catecholaminergic systems.

Interestingly, there is some evidence that catecholaminergic modulation may be mediated, in part, through AT_2 receptors (Xiong and Marshall, 1994). Confirmation of this finding would dramatically increase our understanding of the physiological role of AT_2 receptors in the brain.

We hypothesize that angiotensin mediates many of its actions through catecholaminergic systems and that a major portion of the central catecholamine utilization may be influenced by central angiotensin activity. Furthermore, the AT_2 receptor may mediate a portion of this interaction.

Design and Methods

Preliminary experiments were performed to evaluate brain dissection and intracerebroventricular injection methodologies and dosage and timing for α-methyltyrosine pretreatment. The α-methyltyrosine pretreatment protocol was chosen based on the effects observed in the preliminary experiments. The desired effect was a 50% reduction in catecholamine levels. The rationale for this dosage is that catecholamine levels of 50% of normal is optimal for observing either increases (resulting in further depletion) or decreases (resulting in less depletion) in catecholamine utilization. All animals were screened with a drink test as described in chapter 3. Rats were pretreated with the catecholamine synthesis inhibitor α-methyltyrosine (200 mg/kg, IP) or saline (IP). Animals

TABLE 6-1. CONTROL AND TREATMENT GROUPS FOR CHARACTERIZATION OF CENTRAL ANGIOTENSIN II EFFECTS ON BRAIN CATECHOLAMINE UTILIZATION.

Pretreatment (IP):	Saline	α-Methyltyrosine			
i.c.v. treatment:	aCSF	aCSF	AII	AII + Losartan	AII + PD123177
N:	9	10	10	10	10

treated with α-methyltyrosine were divided into four groups in a balanced design (table 6-1). Forty-five minutes following pretreatment, animals received a series of three intracerebroventricular injections as described in chapter 3 (figure 6-1). Intracerebroventricular injections were given at fifteen minute intervals and animals were decapitated fifteen minutes after the final injection. Brains were immediately dissected on ice into left and right cortices, hindbrain, forebrain and cerebellum. Brain regions were immediately homogenized and stored at -60°C. Catecholamines (norepinephrine, epinephrine and dopamine) were extracted and quantitated as described in chapter 3.

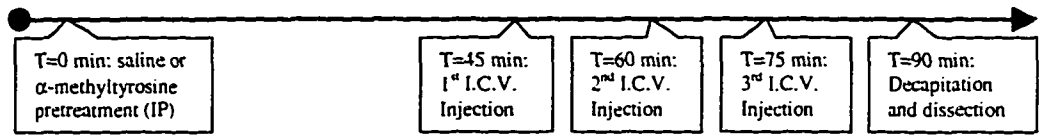

Figure 6-1. Time line for pretreatment and intracerebroventricular injections.

The effect of α-methyltyrosine was determined by a Student's t-test comparing saline pre-treated aCSF animals with α-methyltyrosine pre-treated aCSF animals. Differences between α-methyltyrosine treatment groups were assessed by one-way analysis of variance. Confidence levels of 0.05 and 0.01 were used in determining significance levels in all tests.

Results

Animals surgically prepared for intracerebroventricular injections were screened for correct cannula placement using a test administration of angiotensin II (150 picomoles in 3 µl). Animals passing this drinking (table 6-2) test were randomly assigned to five experimental groups according to table 6-1. Catecholamine utilization was determined for norepinephrine, epinephrine and

TABLE 6-2. DRINKING (ML) INDUCED BY INTRACEREBROVENTRICULAR INJECTION OF 150 PICOMOLES OF ANGIOTENSIN II IN 3 µL OF ARTIFICIAL CSF, ARRANGED ACCORDING TO SUBSEQUENT EXPERIMENTAL GROUPING.

saline pretreatment	α-methyltyrosine pretreatment			
aCSF	aCSF	AII	AII + Losartan	AII + PD123177
16.5	15.9	16.6	11.2	11.2
15.2	10.4	14.9	15.6	17.5
18.7	15.8	13.3	19.0	15.0
14.9	16.4	15.1	15.7	16.0
17.1	13.9	17.7	17.4	14.0
12.8	15.4	13.5	14.1	15.2
17.3	16.2	13.8	18.9	12.4
14.3	12.3	15.8	18.5	13.5
12.6	13.9	11.7	14.0	13.7
	15.3	13.8	13.2	16.8

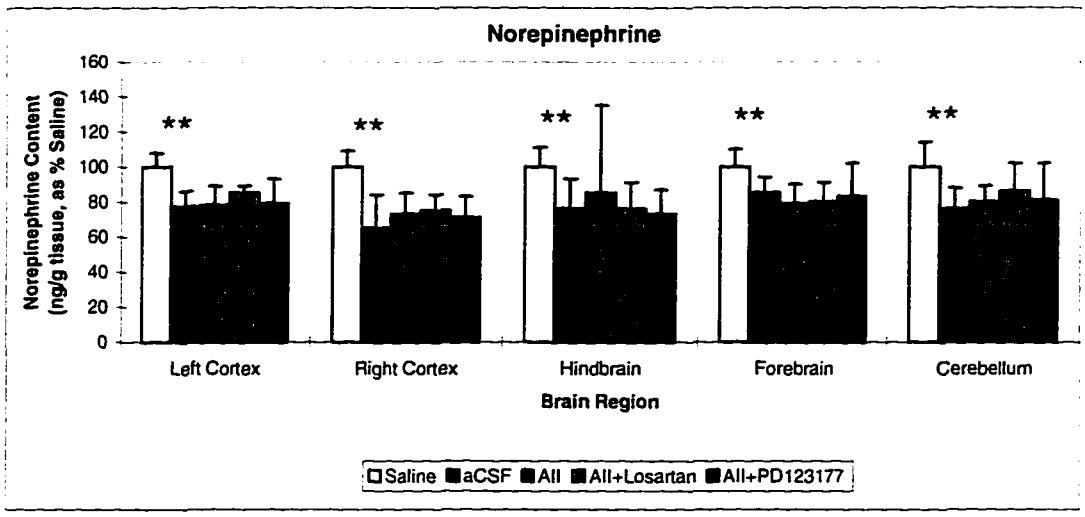

Figure 6-2. Relative norepinephrine content in animals pretreated with saline (IP) and receiving i.c.v. injections of aCSF (saline) or α-methyltyrosine (IP) and i.c.v. injections of either aCSF, angiotensin II, angiotensin II + losartan, or angiotensin II + PD123177. Values for the saline group are 231 (left cortex), 227 (right cortex), 415 (hindbrain), 465 (forebrain), and 138 (cerebellum) ng norepinephrine per gram tissue. Error bars indicate standard deviations. ** denotes significant differences ($p<0.01$) between saline and α-methyltyrosine pre-treated animals receiving aCSF by i.c.v. injection.

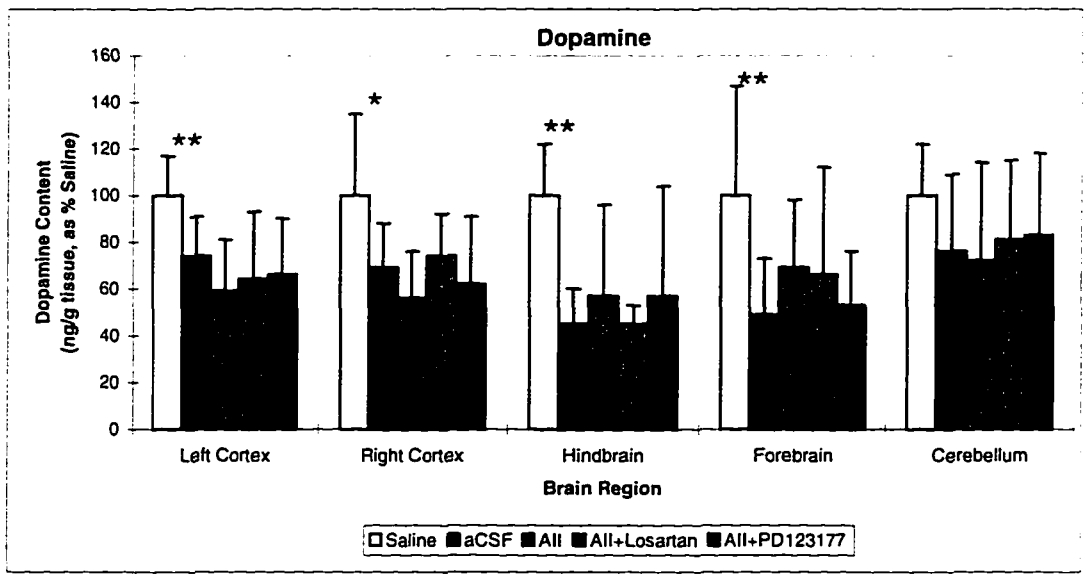

Figure 6-3. Relative dopamine content in animals pretreated with saline (IP) and receiving i.c.v. injections of aCSF (saline) or α-methyltyrosine (IP) and i.c.v. injections of either aCSF, angiotensin II, angiotensin II + losartan, or angiotensin II + PD123177. Values for the saline group are 2044 (left cortex), 1679 (right cortex), 81 (hindbrain), 936 (forebrain), and 58 (cerebellum) ng dopamine per gram tissue. Error bars indicate standard deviations. * and ** denote significant differences ($p<0.05$ and $p<0.01$, respectively) between saline and α-methyltyrosine pre-treated animals receiving aCSF by i.c.v. injection.

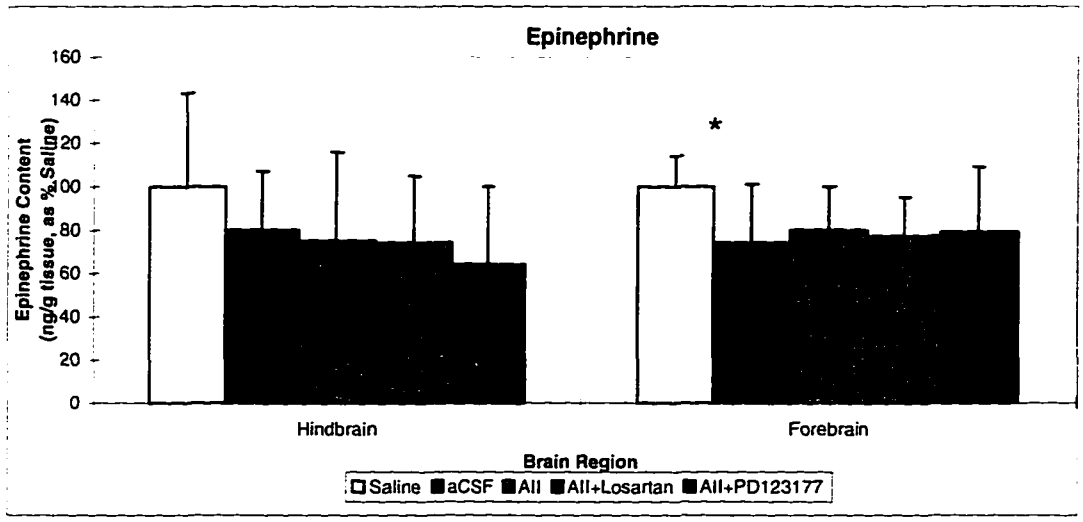

Figure 6-4. Relative epinephrine content in animals pretreated with saline (IP) and receiving i.c.v. injections of aCSF (saline) or α-methyltyrosine (IP) and i.c.v. injections of either aCSF, angiotensin II, angiotensin II + losartan, or angiotensin II + PD123177. Values for the saline group are 5.9 (hindbrain) and 5.5 (forebrain) ng epinephrine per gram tissue. Error bars indicate standard deviations. * denotes significant differences ($p<0.05$) between saline and α-methyltyrosine pre-treated animals receiving aCSF by i.c.v. injection.

dopamine in the presence and absence of the catecholamine synthesis inhibitor α-methyltyrosine (figures 6-2 - 6-4). Catecholamine levels are adjusted for wet tissue weight and expressed as a percentage of saline pretreated levels to facilitate comparisons. All brain regions demonstrated a highly significant ($p<0.01$) decrease (24 ± 3%) in norepinephrine levels in animals pretreated with α-methyltyrosine (figure 6-2). Similarly, dopamine levels (figure 6-3) in α-methyltyrosine animals were 63 ± 6% of saline pretreated animals (significant in four of the five brain regions). Epinephrine levels were detectable in only the hindbrain and forebrain (figure 6-4) and demonstrated a significant

α-methyltyrosine effect in only the forebrain. Unexpectedly, there were no significant differences observed between α-methyltyrosine treated animals receiving intracerebroventricular injections of aCSF, AII, or AII in addition to either subtype selective antagonist. This was true for all three catecholamines observed in all five brain regions.

Discussion

Our methods were sufficiently sensitive to detect 15% changes in catecholamine content following treatment with α-methyltyrosine. The finding of no angiotensin II effects implies that despite the extensive anatomical and functional overlap between the central angiotensinergic and catecholaminergic systems, the portion of the catecholaminergic system that is influenced by intracerebroventricularly administered AII represents a small fraction of the total central catecholamine content.

This unexpected finding is difficult to explain considering reports based on alternative experimental designs employed by other laboratories. Sumners and Phillips (1983) demonstrated significant alterations in catecholamine utilization in the hypothalamus and striatum micropunches following intracerebroventricular angiotensin II administration. These finding have been corroborated by Stadler *et al.* (1992) and Mendelsohn *et al.* (1993). Furthermore, the catecholaminergic actions of angiotensin II appear to be more pronounced in spontaneously hypertensive rats (Tsukashima *et al.*, 1996). Recent reports indicate that

angiotensin II regulates tyrosine hydroxylase gene expression (Yu *et al.*, 1996) and norepinephrine transport system (Lu *et al.*, 1996) in neuronal cultures of both normotensive and spontaneously hypertensive rats.

There is some evidence that angiotensin II modulates the responsiveness of catecholamine containing neurons to other stimuli. Xiong and Marshall (1990; 1994) have demonstrated that AII modulates the response of locus coeruleus neurons to glutamate excitation via an AT_2 receptor mediated pathway. Similarly, angiotensin II potentiates potassium induced norepinephrine (Qadri *et al.*, 1991) and dopamine (Badoer *et al.*, 1990) release in the hypothalamus. Furthermore, in the periphery, angiotensin II facilitated adrenal catecholamine release in response to splanchnic nerve stimulation in hemorrhaged dogs (Kimura *et al.*, 1992). However, angiotensin II probably activates catecholaminergic neurons in a direct manner through a pathway that is synergistic to those of alternative stimuli.

Nevertheless, even a less direct role for angiotensin II in regulating catecholamine utilization would fail to completely explain our results. However, additional angiotensin II activity and/or secondary effects of i.c.v. injection may have interfered with our measurements. We occasionally observed slight motor effects and some catatonia following i.c.v. injections, with subsequent detrimental effects on drinking responses for AII treatment groups. These effects occurred without regard to treatment group and the few affected animals did not

demonstrate aberrant characteristics with regard to unaffected drinking responses. Furthermore, the AII treatment group demonstrated no correlation between catecholamine response and volume drank (NE correlation depicted in figure 6-5) nor time until onset of drinking (not shown). Therefore, the inclusion or exclusion of these animals does not significantly alter our findings. However, these observances indicate the potential existence of similar effects that may be less observable and more frequent, but that may be equally or more detrimental with respect to catecholamine responses. These effects probably involve pathways and brain areas adjacent to the target injection site that are inconsistently affected depending on the exact cannula placement.

Additionally, we cannot rule out the possibility that angiotensin II acts directly on catecholaminergic neurons in only a few very discrete anatomical

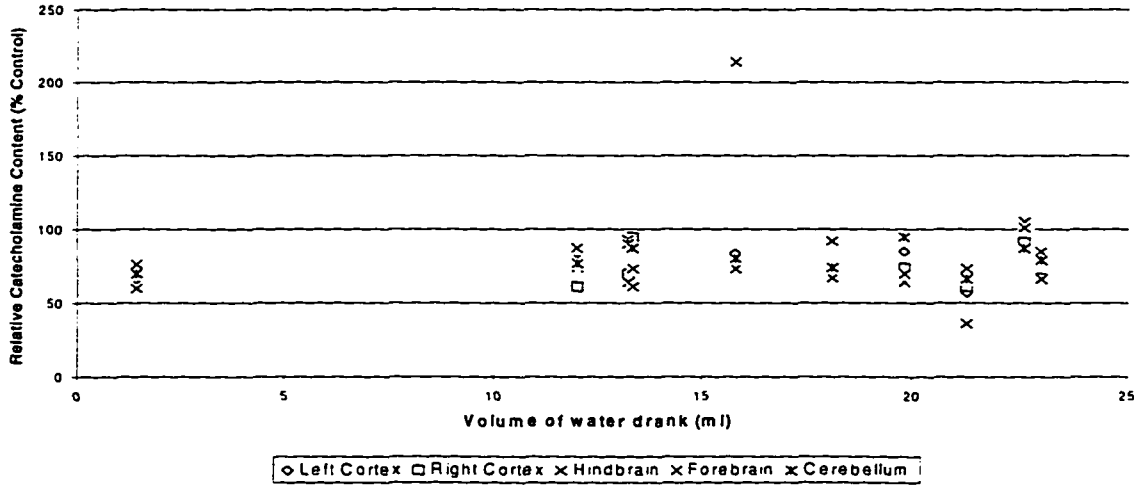

Figure 6-5. Correlation of norepinephrine and drinking responses following intracerebroventricular administration of angiotensin II.

locations. In our preparation, such responses may be masked by the background changes in catecholamine content throughout the remainder of the brain. It is also possible that angiotensin II alters catecholamine utilization differently in different brain nuclei. The interaction of AT_1 and AT_2 receptors may also differ in different brain areas.

The experimental design was chosen to obtain a summation (within brain regions) of potentially small individual catecholamine responses for AT_1 and AT_2 receptors. Our results indicate that the nature of the interaction between the central angiotensin II and catecholaminergic systems makes this approach inappropriate. Because we failed to detect a significant angiotensin II effect, the use of subtype selective antagonists failed to provide additional information.

CHAPTER 7

SUMMARY, CONCLUSIONS, AND PERSPECTIVES

Nearly a century has been invested in studying the renin-angiotensin system. Many rewards are already apparent. Inhibition of the peripheral renin-angiotensin system has proven very effective in the management of some forms of hypertension. The discovery of angiotensin II receptor subtypes and the availability of selective antagonists will likely provide these benefits with little or no side effects.

The discovery of a separate angiotensin system in the brain may lead to additional clinical advances. In addition to cardiovascular regulation, the central angiotensin system has been implicated in the processing of many types of sensory information, and in memory and cognition. And we continue to discover additional functions of this system. These findings suggest that this system may offer insight into certain dementia including Alzheimer's disease.

The diverse activity of the angiotensinergic system is possible because of the diversity of angiotensin receptor subtypes and active peptides. Angiotensin-(1-7) and angiotensin IV appear to act through a unique receptor subtypes (Ambühl *et al.*, 1994; Swanson *et al.*, 1992) and there are two types of angiotensin II receptors (AT_1 and AT_2). Further characterization of these receptors and isolation of the subtypes will allow the many actions of this

system to be studied independently. The utilization of modern tools of molecular biology are already providing insight into these receptor populations (Nahmias and Strosberg, 1995) and angiotensin peptides (Lynch and Peach, 1991).

Our research demonstrates the significance of *in vitro* conditions and underscores the need to validate *in vitro* procedures and to be aware of assumptions implicit in these procedures. We have validated the binding assay employed in our laboratory and demonstrated subtype selective effects for some commonly used buffer constituents. We also ascertained the degree of degradation in the absence and presence of individual peptidase inhibitors for our substituted radioligand. We then utilized this system in two approaches to characterize further the functional role of central angiotensin II.

We compared the anatomical distributions of normal hamsters, genetically hypertensive hamsters and the previously determined distribution in the rat. Identification of sites differing in angiotensin II receptor content and subtype composition (inferior olive, suprachiasmatic nucleus, medial amygdala, piriform cortex, subthalamic nucleus, anterior pituitary, hippocampus, striatum, dorsomotor nucleus of the vagus, medial habenula, and the interpeduncular nucleus) may direct more focused investigations. These differences may correlate to physiological differences between the two species.

Unlike genetically hypertensive and normotensive rats, hamsters did not demonstrate elevated levels of angiotensin II receptors in the brains of hypertensive animals. This finding illustrates the complexity of hypertension

pathology and indicates that the hamster model may (in some cases) provide an alternative model to the spontaneously hypertensive rat. The differences between these models probably relate to different mechanisms of hypertension.

We also investigated the interaction between angiotensin and catecholamine systems within the brain. Our intent was to demonstrate extensive general interaction between the two systems and confirm a role for the AT_2 receptor subtype in this interaction. Our findings suggest that interactions between these systems are more complex and less homogenous than we anticipated. Additional work is necessary to determine the nature of these interactions.

Even though we were unable to answer all of our questions, we have uncovered some of the complexity of the central angiotensin system. Now, we must 'zoom in' and isolate specific characteristics for more direct examination. The species differences in receptor binding include many brain nuclei at which the function of angiotensin II is not presently known. Additional work may associate these differences with known physiological differences between the animal models. Much work needs to be done to examine the potential neuromodulatory role for angiotensin II in catecholaminergic systems. The exact role each of these systems and their interaction in memory formation and cognition remains to be determined. Perseverance and continuing advances in technology will eventually resolve these challenges.

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VITA

David Lee Saylor

Education:

Bachelor of Science
Major: Chemistry; Minor: Biology
Awarded: May 1990
College of Arts and Sciences
East Tennessee State University

Doctorate of Philosophy in Biomedical Science
Concentration: Physiology
To Be Awarded: May 1997
Quillen College of Medicine
East Tennessee State University

Special Skills:

In Vitro Receptor Autoradiography, Quantitative computer assisted densitometric image analysis, stereotaxic rat brain microinjection, catecholamine extraction and HPLC analysis from tissue homogenates, Diverse knowledge of computer hardware / software / programming.

Awards and Achievements:

Dean's List – East Tennessee State University, Spring Semester, 1990.
Department of the Army Certificate of Training – NBC Defense.
Certificate of Achievement – 22nd Support Command (TAA), U.S. Army Central Command, Department of the Army.
Certificate of Achievement – 20th Engineer Brigade (Combat) (Airborne Corps), Saudi Arabia, Operation Desert Shield/Storm.
Army Service Ribbon, National Defense Service Medal, Army Lapel Button, Marksmanship Badge M-16, Southwest Asia Service Medal w/ Bronze Service Star – 1, Kuwait Liberation Medal, SW-Asia-SM-BSS-2.
Awarded 1993 Graduate Student Physiology Award for maintaining highest exam average of graduate students in the Medical Physiology course.
Honorable Discharge, United States Army Reserve, Effective: 30 Aug 94.

Professional Experience:

1985 – 1988: Computer Laboratory Student Worker
Dr. Dan Fetters
King College
Bristol, Tennessee

1987 – 1988: Organic Chemistry Laboratory Instructor
Mr. Weaver
King College
Bristol, Tennessee

1990 – 1991: Research Technician I
Dr. Brian Rowe
Quillen College of Medicine
East Tennessee State University
Johnson City, Tennessee

1992 – 1995: Graduate Assistantship
Department of Physiology
Quillen College of Medicine
East Tennessee State University
Johnson City, Tennessee

1993 – 1995: Tutor for Medical Physiology Course
Quillen College of Medicine
East Tennessee State University
Johnson City, Tennessee

1997 – Present: Post-Doctoral Fellow
Dr. Danquing Wu
Department of Pharmacology and Physiology
University of Rochester Medical Center
Rochester, New York.

Publications:

Articles:

Rowe, B.P., **Saylor**, D.L. and Speth, R.C. (1990). Novel angiotensin II binding sites in the mesopontine area of the rat brain. *Brain Res.* 534: 129-34.

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