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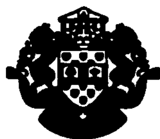

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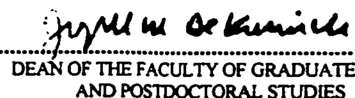

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CENTRAL EFFECTS OF PERIPHERALLEY ADMINISTERED AT_1-RECEPTOR BLOCKERS

By

Jing Zhang

Dissertation submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Master of Science in Pharmacology

Under the Supervision of Dr. Frans Leenen

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ABSTRACT

Blockade of the brain renin-angiotensin system (RAS) prevents salt-sensitive hypertension and inhibits progression of CHF. Sympathetic hyperactivity and hypertension caused by chronic treatment with ouabain or sodium-rich aCSF can be prevented by central administration of an AT_1-receptor blocker. We investigated in Wistar rats 1) the effectiveness of peripheral administration of two AT_1 - receptor blockers (losartan and embusartan) to cause central AT_1-receptor blockade, and 2) whether chronic peripheral treatment with losartan or embursartan can exert sufficient central effects to prevent the central effects of ouabain and sodium. In the first set of experiments, losartan or embursartan at 30 and 100 mg/kg were administered subcutaneously (sc) as a single dose or 1 dose daily for 6 days. The BP responses to intracerebroventricular (icv) injection of Ang II, icv infusion of Na^+-rich aCSF (0.3 M NaCl) and intravenous (iv) injection of Ang II were then measured. Losartan or embursartan at 30 and 100 mg/kg both inhibited the BP increases induced by icv Ang II and Na^+-rich aCSF. After one dose, this inhibition was more pronounced for losartan. However, after 6 days of treatment, there were no significant differences between losartan and embursartan. Losartan and embursartan blocked responses to Ang II iv similarly. In the second set of experiments, losartan or embusartan (both at 100mg/kg/day) were given sc once daily for 16 days. Ouabain or sodium-rich aCSF were given in these groups of rats by osmotic minipump for 13-14 days. The mean arterial pressure (MAP) at rest and in response to air stress, and icv injection of guanabenz (75 μg/7.5 μl, and 25 μg/2.5 μl), Ang II (30 ng/3 μl) and ouabain (0.5 μg/2 μl) at a 10

minutes interval were then measured. In control rats, chronic treatment with ouabain sc and hypertonic saline icv both increased baseline MAP by 20-25 mmHg, and enhanced 2–fold pressor responses to air stress and depressor responses to the α_2-adrenoceptor agonist guanabenz. Simultaneous treatment with losartan sc or embursartan sc fully prevented the hypertension, maintained normal responses to air stress and guanabenz, and attenuated pressor responses to acute icv injection of Ang II and ouabain. These results indicate that the results from single–dose studies may not reflect the chronic steady state. For the chronic steady state, peripheral administration of losartan as well as embursartan can cause sufficient central effects to prevent the sympathetic hyperactivity and hypertension induced by chronic peripheral ouabain and central sodium. From a therapeutic point of view, both AT_1 -receptor blockers may be similarly effective in blocking the brain RAS. However whether these findings can be extended to salt sensitive hypertension or CHF remains to be assessed.

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TABLE OF CONTENTS

ABSTRACT ii

ACKNOWLEDGEMENT iv

TABLE OF CONTENTS v

LIST OF FIGURES vii

LIST OF TABLES viii

LIST OF ABBREVIATIONS ix

General Introduction 1

1 Brain renin-angiotensin system (RAS) 1

1.1 Localization of brain RAS 1

1.2 Brain RAS and blood pressure regulation 6

2 Icv Ang II and icv hypertonic saline, central effects of a centrally administered AT_1 -receptor blocker 11

2.1 Ang II icv 11

2.2 Hypertonic saline icv 12

2.3 Ouabain and "Ouabain" icv 14

2.3.1 "Ouabain" 14

2.3.2 Ouabain 16

3 Blood-brain barrier (BBB) and AT_1 -receptor blockers 18

3.1 Blood-brain barrier 18

3.2 Peripheral administration of AT_1 -receptor blockers 24

3.2.1 CNS effects by autoradiography studies 24

3.2.2 CNS effects by functional studies 27

4 Rationale for present studies 28

Paper 1 31

Paper 2 58

Discussion **79**

1 Central effects of centrally administered AT_1-receptor blockers 79

2 Central effects of peripherally administered AT_1-receptor blockers.... 80

3 Comparing effects of losartan and embusartan administered peripherally and centrally 82

4 Effects of losartan or embusartan administered peripherally on chronic ouabain induced hypertension 83

5 Chronic icv hypertonic saline and losartan or embusartan administered peripherally 85

6 Acute vs chronic stimulation and effects of peripheral losartan or embusartan 86

7 Summary 87

8 Conclusion 89

References **90**

LIST OF FIGURES

Figure 1 Structure of losartan and embusartan 30

Figure 2 Schematic outline of study-protocol for icv and iv injections and infusions.50

Figure 3 Peak increases in MAP by icv Ang II with sc losartan or embusartan one dose or for 6 days 51

Figure 4 Peak increases in MAP by icv 0.3 M NaCl with sc losartan or embusartan one dose 51

Figure 5 Peak increases in MAP by icv 0.3 M NaCl with sc losartan or embusartan for 6 days 51

Figure 6 Peak increases in MAP by iv Ang II with sc losartan or embusartan for 6 days 51

Figure 7 Baseline MAP in rats with chronic ouabain or chronic icv 1.2 M Na+-rich aCSF treatment with and with out losartan or embusartan 78

Figure 8 Peak increases in MAP in response to air stress in rats with chronic ouabain or chronic icv 1.2 M Na^+-rich aCSF treatment with and without losartan or embusartan 78

Figure 9 Maximal decrease in MAP in response to icv injection of guanabenz in rats with chronic ouabain or chronic icv 1.2 M Na^+-rich aCSF treatment with and without losartan or embusartan 78

Figure 10 Peak increase in MAP in response to icv injection of Ang II and ouabain in rats with chronic ouabain or chronic icv 1.2 M Na^+-rich aCSF treatment with and without losartan or embusartan 78

LIST OF TABLES

Table 1: Effects of losartan and embusartan on resting MAP and HR 55

Table 2: Changes in MAP and HR after icv injection of aCSF 56

Table 3: Changes in HR to icv hypertonic saline and Ang II after six days treatment with losartan or embusartan. 57

LIST OF ABBREVIATIONS

ACE	angiotensin converting enzyme
aCSF	artificial cerebrospinal fluid
Ang II	angiotensin II
AP	area postrema
AT_1	angiotensin type 1 receptor
AT_2	angiotensin type 2 receptor
AV3V	anteroventral third ventricle
AVP	arginine vasopressin
BBB	blood-brain barrier
CHF	congestive heart failure
CVLM	central ventrolateral medulla
CNS	central nervous system
CVOs	circumventricular organs
ECF	extracellular fluid
Icv	intracerebroventricular
IR	immunoreactivity
Iv	intravenous
MAP	mitogen-activated protein
MnPO	median preoptic nucleus
NTS	nucleus of the solitary tract
OVLT	organum vasculosum laminae terminalis

PP2A	phosphatase 2A
PTPase	protein tyrosine phosphatase
PVH	paraventricular hypothalamus
PVN	paraventricular nucleus
RAS	renin angiotensin system
Sc	subcutaneous
SFO	subfornical organ
SON	supraoptic nucleus

General Introduction

1 Brain renin-angiotensin system (RAS)

Bickerton et al. (1961) first reported that circulating angiotensin II (Ang II) not only acts in peripheral tissues, but also increases the blood pressure by a direct action on the brain. Since then, much work has been done to investigate the role of Ang II in the central nervous system. Moreover, besides the classical circulatory renin angiotensin system (RAS), endogenous tissue RAS has emerged, including that in kidneys, heart and brain (Allen et al. 1999; Zhuo et al. 1998). The brain RAS, and central actions of the circulatory RAS are involved in central cardiovascular regulation and body fluid homeostasis, cyclicity of reproductive hormones, behaviour effects, and perhaps neuronal development, differentiation, learning and memory (Phillips et al. 1998; Steckelings et al. 1992; Muratani et al. 1996).

1.1 Localization of brain RAS

All components of the RAS have been identified in brain tissue. The major biosynthetic pathway of the RAS starts with the formation of Ang I from angiotensinogen by renin. Ang II is then formed from Ang I by angiotensin converting enzyme (ACE). Incubation of brain homogenates with renin generated Ang I, implying that the angiotensinogen precursor is present locally (Ganten et al. 1971). Expression of the mRNA for angiotensinogen, renin, and ACE has been demonstrated in the brain by Northern blot (Dzau et al. 1986; Whiting et al. 1991).

Renin has been identified in the brain by radioimmunoassay and immunocytochemistry (Genain et al. 1985). Its local synthesis in brain was conclusively demonstrated by the findings of renin-mRNA (Ganten et al. 1984 and Paul et al.1988), which was first reported to be localized only in neurons (Healy et al. 1984). Renin is present in high levels in nerve terminals (Paul et al. 1985) and is released from brain slices by K^+-induced depolarization or by electrical stimulation (Moffett et al. 1987). Some renin may be associated with glial cells (Inagami et al. 1980)

Angiotensinogen is the precursor peptide. Studies demonstrating an angiotensin I-generating capacity of brain extracts incubated with an excess of renin first provided the evidence for the presence of angiotensinogen in the brain (Ganten et al. 1971). Angiotensinogen, detected immunocytochemically, is predominantly located in astrocytes and ependymal cells (Deschepper et al. 1986), and angiotensinogen mRNA detected by in situ hybridization is localized mainly in astrocytes. Angiotensinogen may be produced in astrocytes and converted to Ang I by renin in the extracellular fluid; or alternatively, it may be taken up by neurons and converted intraneuronally (Bunnemann et al. 1992). However, the site of synthesis of brain angiotensins is as yet unresolved.

ACE occurs widely in the brain of various species, including humans (Chai et al.1987, 1991). The localization of ACE in the brain is associated with the endothelium of cerebral blood vessels, epithelial cells of the choroid plexus, and the plasma membranes of astrocytes in the circumventricular organs. Moderate levels of ACE occur in the neurons in the paraventricular and supraoptic hypothalamic nuclei, and in the dorsal vagal complex where its distribution coincides with that of Ang II immunoreactivity and AT_1 receptors. ACE is also found in other brain regions not

associated with the presence of Ang II immunoreactivity, including the basal ganglia, the hippocampus, the cerebellum, the inferior olivary nuclei, and the spinal trigerminal nucleus, thereby suggesting other possible actions for the enzyme in the brain (Chai et al.1995)

Ang II immunoreactive cell bodies were most prominent in magnocellular parts of the paraventricular and supraoptic nuclei, and cells were also found in parvocellular parts of the former. Other hypothalamic nuclei containing cell bodies include the suprachiasmatic nucleus, the medial preoptic area, and perifornical parts of the lateral hypothalamic area. Of considerable interest was robust staining in several of the circumventricular organs, in particular the subfornical organ, where both cells and fibers were found. In the thalamus, Ang II immunoreactive cells were found in the central medial nucleus, the nucleus reuniens, and rostral parts of the zona incerta. Two cell groups in the basal telencephalon, in the dorsal part of the bed nucleus of the stria terminalis and in the medial nucleus of the amygdala, lay at either end of an Ang II immunoreactive pathway coursing through the stria terminalis. In the midbrain, immunoreactive cells were found in the interpeduncular and peripeduncular nuclei, and one pontine cell group was detected in the most lateral part of the lateral parabrachial nucleus. The only Ang II immunoreactive cells in the medulla were in the nucleus of the solitary tract, near the margin of the area postrema. Fibers were found at all levels of the central nervous system, from the olfactory bulbs to the spinal cord, where terminal fields were observed in the substantia gelatinosa and in the intermediolateral cell column. Longitudinally oriented fibers were present throughout the periventricular fiber system

and in the medial forebrain bundle, including its caudal extension in ventrolateral parts of the brain stem (Lind et al.1985).

Ang II elicits its biological actions by binding to specific membrane-bound receptors on target cells to activate multiple, intracellular transduction pathways (Timmermans et al. 1993). Three of these, namely the AT_1, AT_2, and AT_4 receptors, are distributed in the brain as well as in peripheral tissue. In the rat, but not the human, the AT_1 receptor has two subtypes, AT_1a and AT_1b (Kakar et al. 1992). The AT_1 receptor gene (cDNA) encodes a 359-amino-acid protein with a structure typical of seven-transmembrane G protein-coupled receptors. Stimulation of AT_1 receptors by Ang II activates phospholipase C, resulting in increased intracellular calcium and inositol 1, 4, 5-triphosphate concentrations, and activates the mitogen-activated protein kinases, such as extracellular regulated kinases, by way of Src and Ras (small G proteins belonging to the Ras family of G proteins) as well as the JAK/STAT pathways (Schmitz et al. 1997). The AT_2 receptor complementary cDNA has recently been cloned. It encodes a 363 amino acid protein with unique tissue distributions and development patterns. It is 34% identical in sequence to the AT_1 receptor, sharing a seven-transmembrane domain topology (Dzau et al 1994). The signaling mechanism of AT_2 receptor action has not yet been well defined. Growth-inhibitory effects of AT_2 receptor stimulation are partly mediated by the activation of protein tyrosine phosphatase (PTPase), which results in the inactivation of AT_1 receptor- and/or growth factor-activated mitogen-activated protein (MAP) kinase. Serine/threonine phosphatase 2A (PP2A) activation, and consequent ERK inactivation through AT_2 receptor have also been reported in neuronal cells cultured from neonatal rat hypothalamus and brain stem (Horiuchi et al. 1999). Central areas involved

in cardiovascular regulation, body fluid homeostasis, and neuroendocrine function exhibit a predominance of AT_1 receptors, which bind Ang II with high affinity (Obermuller et al. 1991; Lenkei et al. 1995).

AT_1 and AT_2 receptors, and their mRNA expression, have been systematically mapped in rat brain by in vitro autoradiography (Song et al.1992), and more recently by in situ hybridization histochemistry (Lenkei et al. 1997). AT_1 receptors in the brain usually occur in areas where both ACE and Ang II immunoreactivities coexist, or where a defined action has been described for the octapeptide (Allen et al.1998). Angiotensinogen immunoreactivity is absent in CVOs implicated in cardiovascular and neuroendocrine function, such as the subfornical organ (SFO), area postrema (AP), and the organum vasculosum laminae terminalis (OVLT). However, angiotensinogen mRNA is demonstrable in the rostral part of the SFO, in the AP and in the median eminence. Ang II receptors in the CVO of rat brain have been characterized by ^{125}I autoradiography (Mendelsohn et al. 1984a, 1984b). The highest binding densities were localized in the SFO and AP, with considerable aggregation also in the OVLT and median eminence. Circulating Ang II, like other peptides, cannot cross the blood-brain barrier and localizes specifically in the CVOs (Van Houten et al. 1980). These regions represent sites at which peripherally formed Ang II may interact with the brain to regulate blood pressure. AT_1 receptors also occur at many regions inside the blood-brain barrier, such as the hypothalamic neurosecretory nuclei, and the median preoptic nucleus. In the rostral forebrain, AT_1 receptors are observed in the hypothalamic paraventricular nucleus, a region that, in conjuction with the median eminence and the anterior pituitary, is important in the regulation of pituitary function (Allen et al. 1998). In the midbrain and

hindbrain, AT_1 receptors are found mainly in regions involved in the regulation of autonomic activity and cardiovascular reflexes. These include the lateral parabrachial nucleus, a region also implicated in the regulation of fluid balance, the nucleus of the solitary tract, the dorsal motor nucleus of the vagus, the intermediate reticular nucleus, and the rostral and caudal ventrolateral medulla (Song et al.1992).

Areas implicated in the cardiovascular action of Ang II are the median preoptic area, the supraoptic, paraventricular, dorsomedial and ventromedial hypothalamic nuclei, the midbrain preriaqueductal gray, the locus ceruleus, the nucleus tractus solitarius, the dorsal motor nucleus of the vagus, and the cerebral cortex. Among these areas, the highest concentrations of angiotensinogen immunoreactivity and Ang II nerve cell body and terminal immunoreactivity were found in the paraventricular nucleus (PVN) and supraoptic nucleus (SON).

1.2 Brain RAS and blood pressure regulation

The brain renin-angiotensin system plays an important role in the regulation of blood pressure, and body fluid and electrolyte homeostasis through Ang II (Allen et al 1998). Ang II is the principle bioactive peptide of the brain RAS. A role for Ang III and IV in mediating cardiovascular actions of the brain RAS is gradually emerging (Wright et al. 1992, 1996). Ang II may have to be converted to Ang III in order to bind at the AT_1 receptor subtype, and Ang III to Ang IV in order to activate the AT_4 receptor subtype (Wright et al. 1997).

Ang II exerts diverse actions on the brain; these include modulation of drinking behaviour, salt appetite, central control of blood pressure, stimulation of pituitary hormone release, modulation of sensory function, and effects on learning and memory

(Allen et al. 1998). These actions are elicited by neurally derived Ang II acting inside the blood-brain barrier, or by systemic Ang II acting in the circumventricular organs, which have a deficient blood-brain barrier (Allen et al. 2000).

Brain Ang II may regulate blood pressure through three different mechanisms: 1) release of AVP from the hypothalamo-pituitary system, 2) inhibition of the baroreflex at the level of the NTS, and 3) increase in sympathetic nerve activity (Phillips et al. 1987). The brain structures involved in the modulation of blood pressure by Ang II are located in the forebrain (SFO, OVLT, MnPO, PVH, and SON) and in the lower brainstem (NTS, AP, and the dorsal motor nucleus of the vagus, the nucleus ambiguouss, and the ventrolateral medulla). Evidence includes:

A decrease in blood pressure observed after lesion of the AV3V region, the MnPO, and the PVH (Gutman et al. 1985).

Hypertensive effect by Ang II injected into the SFO, OVLT, MnPO, PVH, NTS, AP, and the rostral and caudal ventrolateral medulla (Lenkei et al 1997).

In addition to the angiotensinergic forebrain pathway, which connects SFO, OVLT, MnPO, PVH, and SON, other angiotensinergic neurons connect the forebrain to the brainstem. These neurons originate from PVH and project extensively into the brainstem with collateral branches in the NTS and the ventrolateral medulla. They terminate in the spinal cord at the intermediolateral cell column. The OVLT, MnPO, NTS, and AP participate in angiotensin regulation of blood pressure and contain AT_1a mRNA, AT_1 binding sites, and angiotensinergic nerve terminals, but they are devoid of AT_2 mRNA and AT_2 binding sites. Their function is to regulate blood pressure.

Central angiotensins also produce pressor responses by vasopressin release. Neuronal angiotensins (Ang II) in the CVLM may modulate AVP release by exerting a tonic facilitation on catecholaminergic neurons via AT_1 receptors. A similar effect may also occur in the PVH or SO, since neuronal Ang II has been proposed to enhance norepinephrine release from A1 and A2 nerve terminals through presynaptic AT_1 receptors.

The increase in blood pressure produced by central Ang II is also due to an inhibition of the arterial baroreflex. Phillips et al. (1998) proposed that neuronal Ang II originating from the PVH, and released in the NTS, inhibits neurotransmitter release at the level of the first synapse in the baroreflex loop through AT_1 presynaptic receptors. As proposed, the net effect would be to blunt parasympathetic activity in the dorsal motor nucleus of the vagus.

Ang II also induces an increase in blood pressure by activation of sympathetic outflow. The effect occurs via AT_1 receptors. It has been proposed that Ang II from neurons originating in the PVH and released in the NTS, the rostral ventrolateral medulla, or the intermediolateral column of the spinal cord, is responsible for the direct or indirect activation of sympathetic premotor neurons (Lenkei et al. 1997).

Some of these Ang II receptor areas are anatomically linked to the circumventricular organs, indicating a close connection between the peripheral and the central Ang II systems in the brain. There are neuroanatomical connections between SFO, OVLT and a number of forebrain structures involved in the control of blood pressure (Hartle et al. 1984). These connections are sensitive to Ang II, contain the peptide in immunoreactive terminals (Okuya et al. 1984), and contain Ang II receptors

(Plunkett et al. 1987), all of which belong to the AT_1 subtype (Tsutsumi et al. 1991). These structures are included in a continuous band connecting the SFO with the OVLT, including the median preoptic nucleus, the lamina terminalis, and a lateral connection to the PVN and the median eminence. In the PVN, Ang II immunoreactivity is concentrated in magnocellular neurons that synthesize vasopressin, and scattered cells in the parvocellular division, while most of the Ang II receptors are located in the parvocellular zone (Castren et al. 1989). Ang II can be released within the PVN (Doris et al 1988), indicating a local function for Ang II in this nucleus. Ang II occurs in nerve terminals of both the lamina interna and lamina externa of the median eminence (Ganten et al. 1977). Ang II receptors are also high in the median eminence (Mendelsohn et al. 1984b), and they are located predominantly in the lamina externa (Tsutsumi et al. 1991), where they have access to circulating Ang II. This suggests that the median eminence is another possible circumventricular site for regulation of brain and pituitary function by both peripheral and central Ang II, and another site for interaction between the central and peripheral Ang II systems. Thus, there is clear evidence to consider Ang II as one mediator in the forebrain pathways connecting the SFO and OVLT to the lamina terminalis, the PVN, and the median eminence. Since activation of circumventricular Ang II receptors by circulating Ang II results in stimulation of all structures belonging to the forebrain Ang II pathway (Ferguson 1988), this pathway could be considered the neuroanatomical connection between the peripheral and central Ang II systems.

There are many studies suggesting that the circumventricular organs are the prime targets of peripheral, circulating Ang II. The following points are made by these studies. 1) Because of their position outside the blood-brain barrier (Simpson 1981), peripheral

Ang II has easy access to the large numbers of Ang II receptors in these structures (Saavedra et al. 1989). 2) Peripheral administration of Ang II blockers prevents the central effects of peripherally administered Ang II (Phillips 1987). 3) Peripheral Ang II increases glucose utilization in circumventricular organs (Gross et al. 1985). 4) Ang II, when administered into the circumventricular organs, results in neuronal excitation and produces its central effects at doses much lower than those necessary for iv administration or for injection into the cerebral ventricular system (Sayer et al. 1984). 5) Lesions of the circumventricular organs, or severance of their central connections, diminish or abolish the effects of peripherally administered Ang II (Knowles et al. 1980, Simpson 1981). 6) Alterations in fluid homeostasis, hormonal regulation, or blood pressure result in specific, selective changes in the number of brain Ang II receptors (Nazarali et al. 1990, Saavedra et al. 1986) and in the rate of glucose utilization (Gross et al. 1985) in the circumventricular organs.

The large majority of the studies on the circumventricular organs have focused on the SFO, the OVLT, and the area postrema as principal sites for central actions of peripheral Ang II. Other circumventricular organs, such as subcommissural organ (Ghiani et al. 1988) and the median eminence (Shigematsu et al. 1986), also should be considered as target sites for peripheral Ang II, since they also contain large numbers of Ang II receptors.

Fos immunoreactivity in the rat brain after icv Ang II was compared with that induced by iv Ang II (Mckinley et al. 1995). Ang II was infused into the lateral ventricle (at 1 ng/min) or femoral vein (at 5 μg/h) of conscious rats. After 90 min, rats were killed and Fos was detected by immunohistochemistry. Both infusions caused Fos

immunoreactivity to be present in the lamina terminalis, hypothalamic supraoptic, and paraventricular nuclei, bed nucleus of the stria terminalis, and central amygdaloid nucleus. However, distribution of Fos immunoreactivity within the lamina terminalis differed with the different routes of infusion. Iv Ang II caused intense Fos immunoreactivity mainly in the SFO and OVLT. By contrast, Icv Ang II caused intense Fos immunoreactivity predominantly in the median preoptic nucleus and juxtaventricular neurons of the SFO and OVLT. These results suggest that iv Ang II induces behavioural and endocrine responses by direct actions on the SFO and OVLT, whereas icv Ang II stimulates neurons in the median preoptic nucleus as well neurons int SFO and OVLT (Mckinley et al. 1995).

2 Icv Ang II and icv hypertonic saline, central effects of a centrally administered AT_1 -receptor blocker

2.1 Ang II icv

Acute icv administration of Ang II into the third or lateral ventricles induces cardiovascular and behavioural responses, including pressor responses in a number of species besides rats (Brosnihan et al. 1979; Fink et al. 1980). Pressor responses to centrally administered Ang II may be attributed to an increase in sympathetic neuronal activity, as well as release of arginine vasopressin (AVP). In rats with peripheral blockade of AVP, icv Ang II increases renal sympathetic nerve activity and blood pressure (Huang et al. 1996). In addition to increasing sympathetic activity and AVP

release, centrally injected Ang II may induce the release of a humoral inhibitor of the Na^+, K^+-ATPase, as indicated by a decrease in 86Rb-uptake when rat arteries were incubated with plasma supernate of dogs treated with icv Ang II. The release of the humoral inhibitor was blocked by central saralasin pretreatment, consistent with the involvement of central Ang II receptors in its release (Doursout et al. 1992).

Ang II icv does not have equal access to all areas in the forebrain Ang II pathways. The OVLT contains numerous Ang II receptors stimulated by circulating Ang II (Phillips et al 1976), and ventricularly applied Ang II transported by surface tanycytes (Landas et al. 1980). Lesions in AV3V blocked the responses to both iv and icv Ang II (Buggy et al. 1978). Whether Ang II present in the cerebrospinal fluid can activate the SFO is controversial (Hoffman et al. 1976). Intraventricular saralasin reduced the binding of blood-borne [125I]angiotensin II in the subfornical organ and area postrema to a lesser extent than intravenous saralasin (Van Houten et al. 1983). In addition, the SFO receives direct Ang II-containing projections (Lind et al. 1984) and may be involved in central Ang II effects independent of stimulation by CSF-borne or blood Ang II. Thus, the OVLT is important for the effects of peripheral and cerebrospinal fluid Ang II, the median preoptic nucleus for those of brain and cerebrospinal Ang II, and the SFO for the effects of peripheral and brain Ang II.

2.2 Hypertonic saline icv

In normotensive rats, acute icv administration of hypertonic saline and ouabain cause similar sympathoexcitatory and pressor responses, which are all abolished by icv pretreatment with the AT_1 receptor blocker, losartan. Antibody Fab fragments binding brain ouabain only block responses to hypertonic saline and ouabain, but not to Ang II

(Huang et al. 1996a). This would suggest that an increase in central sodium concentration releases brain "ouabain", which exerts its sympathoexcitatory and pressor effects mostly via brain Ang II. Chronic increases in CSF sodium in normotensive rats, cause an increase in central "ouabain", sympathoexcitation, and an increase in blood pressure. Concomitant icv infusion of either Fab fragments or losartan abolished both the sympathoexcitation and increase in blood pressure supporting the role of brain "ouabain" and the brain RAS in mediating the effects of acute or chronic increases in central sodium (Huang et al. 1998).

Rohmeiss et al. (1995) reported that, after injection of the specific AT_1 receptor antagonist, losartan into the SFO, the natriuresis and blood pressure response to icv hypertonic saline was attenuated by 60%. These results demonstrated that an angiotensinergic mechanism within the SFO contributes to the pressor response to an acute icv injection of hypertonic saline and suggested that this response may be mediated via a pathway in the SFO. As an additional site, the area postrema, has been implicated in mediating pressor responses to icv hypertonic saline (Kawano et al. 1991).

After excitotoxic lesioning of the vAV3V, the pressor and tachycardiac effect of acute intracerebroventricular infusions of 0.3 mol/NaCl were significantly attenuated by ~30% in rats with systemic blockade of vasopressin mechanisms (Veerasingham et al.1997). However, vAV3V lesions fully prevented hypertension induced by chronic intracerebroventricular infusion of hypertonic saline. This difference in extent of blockade may be due to the fact that the CSF concentration of sodium obtained chronically would likely be less than the concentrations of sodium obtained by the acute injection. Small increases may act on nuclei that are sensitive to sodium, such as the

vAV3V. Higher concentrations may act on less sensitive nuclei in addition to the vAV3V (Veerasingham et al. 1999). An angiotensinergic mechanism within the SFO also contributed to the pressor response to an acute intracerebroventricular injection of hypertonic saline and Veerasingham et al. suggested that this response may be mediated via a pathway from the SFO to the paraventricular nucleus. This pathway traverses or synapses in the subcommissural MnPO, explaining why electrolytic AV3V lesions are able to abolish pressor responses to acute intracerebroventricular hypertonic saline (Rohmeiss et al. 1995). As vAV3V lesions leave the subcommissural MnPO unaffected to a large extent, it appears that the OVLT plays a greater role in mediating pressor effects of chronic intracerebroventricular infusion of hypertonic saline.

2.3 Ouabain and "Ouabain" icv

2.3.1 "Ouabain"

The term "Ouabain" is used to define brain endogenous ouabain-like compounds. The distribution of "ouabain"-containing neurons in the hypothalamus has been studied in rats and monkeys. In these studies "ouabain" was detected immunohistochemically using antibodies that recognize "ouabain" with virtually no cross-reactivity with hydrocortisone and d-aldosterone (Takahashi et al. 1988). Dense "ouabain"-immunoreactivity (IR) was detected in hypothalamic magnocellular neurons in the paraventricular nucleus (PVN) and supraoptic nucleus (SON) and its accessory nuclei. Parvocellular neurons of the PVN were also "ouabain"-positive, but the density of the immunoreactive material was significantly smaller. In the PVN and SON "ouabain"-IR was mostly observed in vasopressinergic, and to the lesser extent in oxytocinergic neurons (Yamada et al 1987).

Other ouabain positive somata and scattered fibers were found in the periventricular, perfonical, and lateral, anterior and preoptic areas (Yamada et al 1987, Yamada et al. 1992b, Ihara et al 1988). "Ouabain"-IR fibers ran from the PVN towards the SON and then together with axons from the SON through the lateral hypothalamic area to the infundibulum where they were mostly found in the inner layer of the median eminence. The same fibers were also observed adjacent to the primary capillaries of the hypophysial portal system and terminated in the posterior lobe of the pituitary gland (Yamada et al 1992a, 1992b). This suggests that magnocellular neurons of the PVN and SON are the primary source of "ouabain" released from the neurohypophysis to the circulation. Other areas where "ouabain"could be released and exert its physiologic actions are structures belonging to the lamina terminalis. Large numbers of "ouabain"-IR fibers were identified in the OVLT and its vicinity and subfornical organ (Yamada et al.1992a).

Takahashi et al. (1988) and Huang et al. (1992) showed that in conscious rats, acute icv injection of ouabain or crude hypothalamic or pituitary extracts containing ouabain-like activity cause similar increases in sympathetic activity, blood pressure and heart rate. The precise localization of effects of "ouabain" in the CNS has not yet been established. Early studies in anesthetized rats demonstrated that injection of exogenous ouabain into the lateral hypothalamus and the PVN increases blood pressure (Jones et al. 1990). To address this issue our group used either localized microinjection of Fab fragments or chemical lesions of possible target areas first the median preoptic nucleus (MnPO) and AV3V area since both these areas contain "ouabain" immunoreactive fibers. Injection of Fab fragments in the MnPO or chemical lesion of the ventral AV3V area (vAV3V; which included OVLT and the most ventral part of subcommissural part of the

MnPO), block the pressor response to icv hypertonic saline, which is known to be mediated by "ouabain" (Budzikowski et al. 1997, Veerasingham et al. 1997). Veerasingham and Leenen (Veerasingham et al. 1999) also showed that the same lesion prevents blood pressure increases during chronic infusion of sodium rich aCSF but does not affect increased responses to air jet stress.

2.3.2 Ouabain

In vitro studies suggest that nanomolar concentrations of ouabain may cause vasoconstriction in humans and rats (Blaustein 1996). Ouabain increases release, and decreases reuptake of norepinephrine from sympathetic nerve terminals, which may contribute to vasoconstriction (Tsuda et al. 1988 and Kitagawa et al. 1985). Within the adrenal gland, ouabain may increase aldosterone secretion. Chronic ouabain infusion in Wistar rats increases plasma aldosterone concentrations without affecting plasma renin activity and infusion of antibodies to ouabain decreases plasma aldosterone levels (Manunta et al. 1994) suggesting that ouabain modulates aldosterone secretion.

Chronic administration of exogenous ouabain can induce hypertension in normotensive rats. Peripheral infusion of ouabain induces hypertension in normotensive rats (Manunta et al. 1994). Huang et al. (1994) showed that in Wistar rats, icv, iv or subcutaneous administration of ouabain for 10-14 days caused dose-dependent increase in BP and HR and sympathetic outflow, associated with significantly increased "Ouabain" levels in pituitary, hypothalamus and adrenals (Huang et al. 1994). The increase in BP and HR in this model depends on the central action of ouabain since they can be prevented by chronic icv infusion of Fab fragments above mentioned (Huang et al. 1994).

Fab fragments administered into the MnPO as well as the vAV3V lesion significantly attenuated the pressor response to acute icv injection of ouabain (Budzikowski et al 1997 and Veerasingham et al. 1997).

Chronic central or peripheral administration of ouabain induces hypertension in normotensive rats (Yuan et al. 1993, Huang et al.1994). The hypertension likely results from increased sympathetic tone (Huang et al.1994), as estimated indirectly by ganglionic blockade. Chronically administered ouabain increases the content of ouabain in several brain areas (Huang et al. 1994), which are closely related to central cardiovascular regulation. This incease of ouabain in the brain appears to mediate the increase in BP, since the hypertensive effect of ouabain can be prevented by concomitant icv infusion of Fab fragments (Huang et al. 1994).

In rats the pressor/sympatho-excitatory response to acute icv ouabain can be blocked by icv pretreatment with Ang II receptor blocker saralasin or the ACE inhibitor captopril (Takahashi et al. 1984). Icv losartan markedly attenuates sympatho-excitatory and pressor responses to acute icv injection of Ang II, or ouabain (Huang et al. 1996a)). Pretreatment with icv antibody Fab fragments blocks only the effects of ouabain but not those of Ang II (Huang et al. 1996a,Yamada et al. 1994). These studies suggest that activation of brain AT_1 receptors occurs in the pathways mediating the effects of acute icv ouabain. Icv chronic losartan also prevents the central effects of chronic ouabain. Therefore, similar to acute icv ouabain (Huang et al. 1996a), a chronic increase in brain ouabain (Huang et al 1994) as a result of chronic sc. administration of ouabain appears to activate brain pathways involving AT_1 receptors, leading to sympatho-excitation and hypertension. Brain areas where brain ouabain and brain RAS may interact have not yet

been defined. In rat brain, nerve fibres of ouabain-immunopositive neurons, and Ang II receptors or other components of the brain RAS co-exist in several hypothalamic areas such as the anteroventral third ventricle, including the organum vasculosum of the lamina terminalis (Yamada et al. 1987, 1992a). In Wistar rats, the pressor response to acute icv ouabain is attenuated by losartan in the median preoptic nucleus (MnPO)(Budzikowski et al. 1997). The pathways mediating the effect of chronic ouabain and the brain RAS on baroreflex function have not been clarified either. The preoptic area (Inui et al. 1995), which is adjacent to the MnPO and is a principal location in the hypothalamus related to artereial baroreflex control, as well as brain stem ares such as the nucleus tractus solitarii (Casto et al. 1986) may participate in the relay.

3 Blood-brain barrier (BBB) and AT_1 -receptor blockers

3.1 Blood-brain barrier

The blood-brain barrier (BBB) is a vital element in the regulation of the constancy of the internal environment of the brain. The composition of the extracellular fluid of the brain is controlled within very precise limits, largely independently of the composition of the circulating blood, to provide a stable environment in which the integrative neuronal functions of the brain can optimally take place. The blood-brain barrier is formed at the level of the endothelial cells of the cerebral capillaries. These cells are characterized by having tight continuous circumferential junctions between the cells of the capillaries thus abolishing any aqueous paracellular pathways between the cells (Brightman 1992). The endothelium is thus characterized by exhibiting a high transendothelial electrical resistance in the region of 1500-2000 Ω cm^2 (Butt et al. 1990). The presence of the tight

junctions and the lack of aqueous pathways between cells greatly restrict the movement of polar solutes across the cerebral endothelium.

Some regions within the central nervous system (CNS) lack a BBB and the capillaries are fenestrated allowing the free movement of solutes between the blood and the surrounding interstitial fluid. These areas are collectively termed the circumventricular organs (CVOs) and include the choroid plexus, the median eminence, the neurohypophysis, the pineal gland, the organum vasculosum of the lamina terminalis, the subfornical organ, the subcommisural organ and the area postrema. Some of these structures, the median eminence, the neurohypophysis and pineal are neurohumoral organs specialized for the release of neuroendocrine secretion into the bloodstream. The other areas may be regarded as windows of the brain where a limited number of neurons within the immediate vicinity of the circumventricular organ have an unrestricted access to blood solutes. The access enables the brain to monitor closely the composition of the blood and to react accordingly. The ependymal cells surrounding the circumventricular organs have what appear to be tight junctions between them presumably to enclose a column of brain extracellular fluid (ECF) surrounding the CVO to prevent diffusion of interstitial solutes away from the region of the circumventricular organ (Brightman 1992). The relative surface area of the permeable fenestrated capillaries of the circumventricular organs compared to the tight BBB capillaries is 1: 5000.

The steady-state CNS concentration of a systemically administered drug will be a function of its rate of entry into the CNS less its rate of removal. The blood-brain barriers are the principle routes by which drugs enter the brain (extensively reviewed by Brightman 1992, Davson and Segal, 1996), with rates of entry dependent on the nature of

the drugs and the permeability properties of these interfaces. The main routes by which drugs are removed from the CNS are via the sink effect of CSF secretion and reabsorption (Davson and Segal, 1996) and by active efflux mechanisms at the blood-brain barrier: P-glycorprotein (Pgp) (Tatsuta et al., 1992; Schinkel et al., 1996) and the multidrug resistance-related protein (MRP) (lautier et al., 1996). The key molecular properties that appear to be important are lipid solubility, and molecular size. The more lipid-soluble a drug, the more easily it will be able to move from the polar environment of the blood into the nonpolar (lipid) environment of the endothelial cell membrane, and the greater its transfer across the blood-brain barrier (Greig, 1989).

The BBB is formed by a complex cellular system of endothelial cells, astroglia, pericytes, perivascular macrophages, and basal lamina. Compared to other tissue, brain endothelia have the most intimate cell-to-cell connections: endothelial cells adhere strongly to each other, forming structures specific to the CNS celled 'tight junctions' or zonula occludentes. They involve two opposing plasma membranes which form a membrane fusion with cytoplasmic densities on either side. These tight junctions prevent cell migration or molecule movement between endothelial cells. A continuous uniform basement membrane surrounds these brain capillaries. This basal lamina encloses contractile cells called pericytes which from an intermittent layer and probably play some roles in terms of phagocytosis activity and defense if the BBB is breached. Covering the brain capillary, astrocytic end feet build a continuous sleeve and maintain the integrity of the BBB by the synthesis and secretion of soluble growth factors essential for the endothelial cells to develop their BBB characteristics, for example the production of gamma-glutamyl transpeptidase (Schlosshauer 1993).

The anatomical locus of the BBB is the capillary wall itself. Drugs or nutrients have to reach the CNS by a transcellular route. In contrast, intercellular clefts or large fenestration's permit direct intercellular passage in other tissues. Other differences are greater number or greater volumes of mitochondria than in peripheral tissues, suggesting very active metabolism, few pinocytic vesicles and a high electrical resistance. The high resistance is characteristic of epithelia with low ionic permeabilities (Cornford et al. 1999).

The CNS has three different compartments:

- the blood compartment includes afferent and efferent arteries involved in the blood supply of molecules and brain capillaries which govern the brain tissue transfer of the molecules;
- the cerebrospinal fluid (CSF) compartment: the exchanges between CSF and brain;
- the brain tissue compartment, including both neuronal and glial cells.
- Hence, there exist, in fact, three barriers:
- the blood-CSF barrier, i.e. the choroid plexuses and pia arachnoid;
- the brain-CSF interface, i.e. ependyma
- the blood-brain barrier, i.e. the endothelial cells which represent the only one limiting transfer of molecules to the brain. Its exchange surface is 5000-fold larger than that of the blood-CSF barrier. So endothelial cells are the 'gatekeepers' of the brain. Moreover, endothelial enzymes such as monoamine oxidase A and B, catechol O-methyltransferase and GABA transaminase are involved in

neurotransmitter metabolism and constitute a metabolic barrier (Goldstein et al 1986).

The endothelium of the brain microvasculature is much tighter than that elsewhere in the body. Although brain endothelium appears to possess the same routes for transendothelial transfer as do other endothelia, the rarity of some routes leads to an extremely low overall permeability. Cells associated with brain endothelium, particularly astrocytic glial cells, appear to be involved in induction of the low permeability state. Comparison of the brain endothelium with the perineurium of peripheral nerve, part of the blood-nerve barrier, suggests that the modulation of brain endothelial permeability, seen in pathological situations, may give some physiological advantage. (Abbott et al. 1991)

Experimental observations first made by Paul Ehrlich in 1885, and by Edwin Goldman in 1901, that the CNS is not stained by intravascular water-soluble dyes, provided the first demonstration of a BBB to polar compounds. Pioneering studies of the BBB were performed in vivo using intracarotid injection single-pass techniques (Olfendorf et al. 1970). Further characterization of the BBB at the cellular level has led more recently to the development of in vitro experimental approaches. Isolated brain capillary preparations, as well as tissue culture systems using brain ECs, have proven to be a promising methodology to define the characteristics of the brain capillary endothelium at the molecular and cellular level.

Unlike peripheral endothelia, brain microvessel endothelium cells are characterized by the presence of a high transendothelia electrical resistance, intercellular tight junctions, minimal pinocytotic activity, and virtual absence of fenestration (Davson

and Segal, 1996). In vivo, the endothelial BBB actually consists of a luminal plasma membrane, the cytosol, and the abluminal membrane of the endothelial cell.

In addition to its functional barrier properties, the endothelium is capable of selective, unidirectional transcellular transfer. Many essential metabolic substances, such as glucose and some amino acids, are highly polar and have poor permeability via the membrane lipid barrier. Therefore, these substances are transported across the BBB by saturable carrier systems to meet the high metabolic demand of the brain (Crone, 1963; Davson and Segal, 1996). To achieve an effective regulation of energy/metabolic supply to the parenchyma, the BBB must be capable of responding to abluminal signals while controlling the rate of transport of metabolites and ions (i.e. K^+, Na^+, and H^+).

Most studies of BBB permeability in vivo use one of two different methodological approaches. In the first approach, a solute is injected as a bolus into the carotid artery; brain uptake or extraction is determined from a single pass of the bolus through the brain capillaries. The technique of brain uptake indexes was later introduced by Oldendorf in 1970 as an intracarotic injection single-pass method to measure cerebrovascular transport and permeability. In this method, the brain uptake of a test tracer is normalized by the use of a permeable reference tracer of known uptake (Oldendorf et al. 1970). However, because permeability is determined from a single pass through the brain, this approach has limited sensitivity and cannot be used to obtain accurate cerebrovascular permeability coefficients for poorly permeable compounds. A second methodological approach (i.e. intravenous administration, brain perfusion techniques) involves a prolonged uptake time that depends on the permeability coefficient of the solute, therefore, more sensitive than the first approach. With the

intravenous administration technique, a solute is given parentally and the plasma concentration is monitored until a specific time at which the brain content is determined. BBB permeability is calculated from the brain uptake of a solute, using a kinetic model that describes solute exchange between the plasma and CNS.

It has been difficult to quantify the amount of compound that traversed the brain endothelium alone in vivo, since numerous routes of clearance from the brain exist; thus the compound may enter the interstitium, as well as the brain parenchyma, and become sequestered intracellularly and protein bound, or become metabolized. A determination of the kinetic characteristics of transport systems has proven difficult in vivo because of the poor temporal and spatial resolution and poor access to the brain side (abluminal) of the endothelium.

3.2 Peripheral administration of AT_1 -receptor blockers

To what extent peripheral administration of AT_1 - receptor blockers blocks central effects of Ang II will depend where Ang II exerts its central effects (ie inside vs outside the BBB), and if inside the BBB whether the blocker has access to these areas. Compounds with non-polar groups are lipophilic and in general easily cross the blood-brain barrier. Compounds with polar groups are hydrophilic and have more difficulties crossing the blood-brain barrier (Grant et al. 1998).

3.2.1 CNS effects by autoradiography studies

Evidence that AT_1-receptor blockers cross the BBB comes from autoradiography. Losartan is hydrophilic and easily dissolves in water. In contrast, embursatan is lipophilic and needs to be dissolved in ethanol 20%, polyethylene 40%, and H_2O 40%.

The in vivo access of the nonpeptide angiotensin II (Ang II) antagonist, losartan 10mg/kg iv, to Ang II receptors of rat brain was investigated by vitro autoradiography in Sprague-Dawley rats with [125I]-[Sar, Ile8] Ang II as a ligand. Losartan markedly inhibited the binding to sites that contain exclusively AT_1 receptors both outside and within the blood brain barrier, such as CVO, PVN, median preoptic nucleus and NTS. The iv administration of losartan (10mg/kg, iv) resulted in a decrease of binding to the solitary tract (29±5%), the dorsal motor nucleus of the vagus (31±6%), and the paratrigeminal nucleus (35±9%). These are sites where AT_1 receptors occur predominantly. Ang II receptor binding in the circumventricular organs, the SFO, OVLT , median eminence and area postrema, were inhibited by more than 50% in rats treated with losartan. These results demonstrate that periphererally administered losartan blocks AT_1 receptor binding, not only to the circumventricular organs which are outside the blood brain barrier, but also at sites within the blood brain barrier. However, peripheral administration of losartan blocks sites outside the BBB more than these inside the BBB. These results demonstrate that losartan and/or its active metabolite readily cross the blood brain barrier in vivo and selectively inhibit binding to AT_1 receptors in specific brain nuclei (Song et al. 1991).

In another study (Zhuo et al. 1994), male Sprague-Dawley rats were administered intravenously either vehicle, or losartan at doses of 1, 3 or 10 mg/kg. The brain, kidneys and adrenals were removed at 1, 2, 8 or 24 h after administration of the antagonist. The effects of losartan on Ang II receptor binding were assessed by quantitive in vitro autoradiography. In the brain, losartan produced a dose-dependent inhibition of Ang II receptor binding to the brain structures that express exclusively, or predominantly, AT_1

receptors both outside and within the blood brain barrier. By contrast, losartan did not affect binding to the nuclei which contain exclusively, or predominatly, AT_2 receptors. As expected, AT_1 receptor binding sites that are localized outside the blood brain barrier such as those in the SFO, OVLT, median eminence and area postrema, were significantly inhibited. In the rats 60 min after losartan administration, at 1 mg/kg specific Ang II binding in these sites was inhibited by 16-45%, and binding was inhibited further by 30-67% (3mg/kg) and 47-81% (10mg/kg). In the brain nuclei that contain predominantly AT_1 receptors and are within the blood brain barrier such as the median pre-optic nucleus, bed nucleus of the stria terminalis, amygdala, fusiform nucleus, paratrigeminal nucleus, lateral parabrachial nucleus, the NTS, dorsal motor nucleus of the vagus, and spinal trigeminal nucleus, binding was also markedly inhibited by losartan in a dose-dependent manner. In these rats 60 min after losartan iv administration, at 1 mg/kg specific Ang II binding in these sites was inhibited by 4-19%, and binding was inhibited further by 9-47% (3mg/kg) and 17-67% (10mg/kg). These results show that the inhibition induced by peripheral administration of losartan was outside the BBB more than those inside the BBB (Zhuo et al. 1994).

In one more study, losartan administered sc markedly inhibited labelled Ang II binding in the liver in doses as low as 1 mg/kg (sc., 2h pretreatment), whereas, in the brain (cortex/hippocampus), 1 and 3 mg/kg (sc., 2h pretreatment) did not cause inhibition, but 10 and 30 mg/kg (sc., 2h pretreatment) caused dose-related inhibition, 30±5% and 40±5% of control values (Marshall et al. 1993). These results also suggest that peripheral administration of losartan at higher doses is able to cross the BBB and occupy central receptors.

Embusartan is a rather lipophilic AT_1 -receptor blocker. However, in whole-body autoradiographic studies in rats, after 5 mg/kg iv or 10 mg/kg oral administration of [-14C]-embusartan, relatively high radioactive concentrations were found in the liver and other peripheral tissues, but there was no penetration of the radioactivity across the blood-brain barrier (Stasch, personal communication). The absence of penetration of [-14C]-labelled compound across the BBB suggests that embusartan may have difficulties crossing the BBB and may exert less central effects than losartan.

3.2.2 CNS effects by functional studies

Evidence that AT_1 receptor blocker crosses the blood-brain barrier comes from functional studies. Functional blockade can be assessed in several ways, studies:

- To assess the effectiveness of acute vs chronic peripheral administration of an AT_1 receptor blocker to inhibit the pressor response to acute or chronic exogenous Ang II icv.
- To assess the effectiveness of acute vs chronic peripheral administration of an AT_1 receptor blocker to inhibit the pressor response to endogenous Ang II released by acute or chronic ouabain, hypertonic saline, or other stimulation.

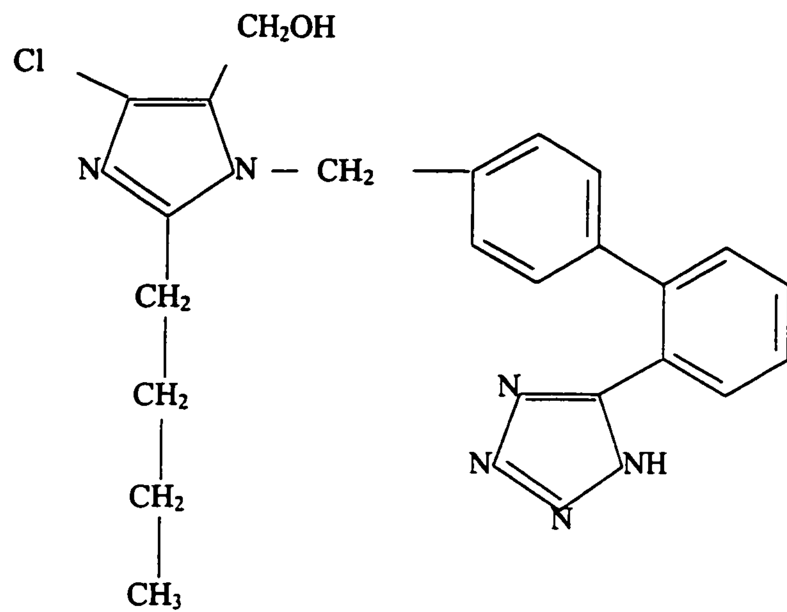
In functional studies by Culman et al. (1999), irbesartan and losartan were administered iv or p.o. at doses of 3, 10, 30 and 100 mg/kg body weight in normotensive rats. The responses to 100 ng angiotensin II injected into the lateral brain ventricle (i.c.v) were recorded for up to 3 h. The AT_1 receptor antagonists dose-dependently attenuated the pressor responses to central angiotensin AT_1 receptor stimulation of angiotension II to a similar degree (62% iv, 46% P.O.). Li et al. (1993) demonstrated that losartan 3 mg/kg iv within 5 minutes inhibited Ang II-induced increases in spontaneous firing rate of PVN

neurons. In contrast, 10 mg/kg orally did not inhibit pressor responses to icv Ang II. (Wong et al.1990), neither did 3 mg/kg/day orally for 3 days (Bui et al. 1992). Losartan 9 mg/kg injected i.p.inhibited icv Ang II induced drinking responses (Polidori et al. 1996). Similarly losartan 3 or 10 mg/kg sc reduced the increase sodium excretion and blocked the antidiuretic action induced by icv renin (Barbella et al. 1993). Low doses of losartan, particularly orally can therefore induce clear peripheral AT_1 -receptor blockade with little demonstrable central blockade, whereas at higher doses losartan appears to cross the blood-brain barrier readily and can cause marked inhibition of brain AT_1 -receptors in areas inside and outside the blood-brain barrier.

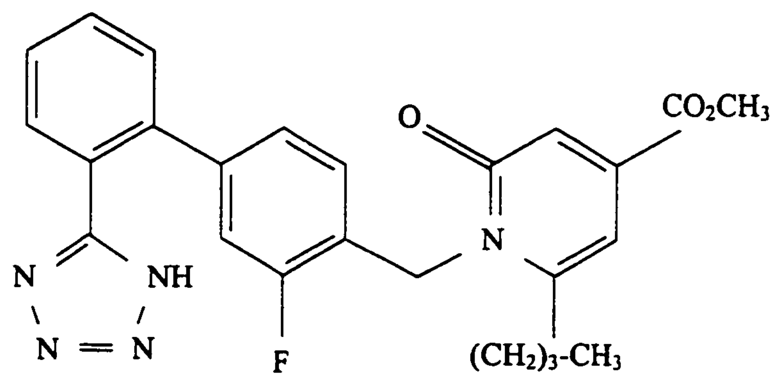
4 Rationale for present studies

Direct acute central administration of losartan blocks the increase in MAP induced by acute icv injection of Ang II, oubain and hypertonic saline (Huang et al. 1996a). Chronic central administration of losartan also blocks the hypertension and sympathoexcitation induced by chronic central administration of hypertonic saline (Huang et al. 1998b). From a therapeutic point of view, the crucial question is whether similar effects can be induced by peripheral administration of an AT_1 -receptor blocker. So far, whether peripheral administration of an AT_1-receptor blocker can prevent or reverse these central effects is still under investigation. We proposed that (1) acute and more chronic sc administration of an AT_1 -receptor blocker can prevent pressor responses induced not only by central Ang II, but also by sodium. (2) chronic sc administration of an AT_1 -receptor blocker prevents sympathetic hyperactivity and hypertension by chronic ouabain and hypertonic saline.

- to what extent peripheral administration of AT_1 - receptor blockers blocks effects of Ang II will depend on their access to central areas. Compounds with polar groups are hydrophilic substances. They are water soluble and more difficult to cross the BBB. Compounds with non-polar groups are lipophilic. They are oil soluble and in general easily cross the blood-brain barrier (Grant et al. 1998). Losartan is a rather hydrophilic AT_1 -receptor blocker, but embusartan is a rather lipophilic AT_1 -receptor blocker. Therefore, the aims of the study were:
- to determine whether acute and more chronic peripheral administration of an AT_1 -receptor blocker can prevent the pressor response induced by acute icv administration of Ang II and high sodium.
- to determine whether chronic peripheral administration of an AT_1 -receptor blocker can prevent the hypertension induced by chronic icv administration of hypertonic saline or chronic sc administration of ouabain.
- to compare the extent of central blockade induced by peripheral administration of a hydrophilic (losartan was dissolved in polar solution, ie. normal saline) versus lipophilic (embusartan was dissolved in non-polar solution, ie. ethanol 20%, polyethylene 40%, and H_2O 40%) AT_1 -receptor blocker.



Losartan



Embusartan

Figure 1 Structure of losartan and embusartan

Paper 1

PERIPHERAL ADMINISTRATION OF AT_1-RECEPTOR BLOCKERS AND PRESSOR RESPONSES TO CENTRAL ANGIOTENSIN II AND SODIUM[1]

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Abstract:

Blockade of the brain renin-angiotensin system (RAS) prevents salt-sensitive hypertension and inhibits progression of CHF. We investigated in Wistar rats the effectiveness of a lipophilic (losartan) vs a hydrophilic (embusartan) AT_1-receptor blocker to cross the blood-brain barrier and to exert central effects. Losartan or embusartan at 30 and 100 mg/kg were administered subcutaneously (sc) as a single dose or 1 dose daily for 6 days. The BP responses to intracerebroventricular (icv) injection of Ang II, icv infusion of Na^+-rich aCSF (0.3 M NaCl) and intravenous (iv) injection of Ang II were then measured. Losartan or embusartan at 30 and 100 mg/kg both inhibited the BP increases induced by icv Ang II and Na^+-rich aCSF. After one dose, this inhibition was more pronounced for losartan. However, after 6 days of treatment, there were no significant differences between losartan and embusartan. Losartan and embusartan blocked responses to Ang II iv similarly. These results indicate that results from single-dose studies may not reflect the chronic steady-state, and from a therapeutic point of view both types of AT_1-receptor blockers may be similarly effective in blocking the brain RAS.

Key Words: AT_1-receptor blockers, brain renin-angiotensin system, losartan, embusartan, angiotensin II, hypertonic saline

1. Introduction

Individual components of the renin-angiotensin system (RAS) have been found in many tissues including kidneys, heart and brain (Allen *et al.* 1999; Zhuo *et al.* 1998). Actions of Ang II in the brain, both inside and outside the blood-brain barrier, are implicated in the central regulation of blood pressure, sympathetic outflow and release of hypothalamic hormones (Muratani *et al.* 1996). In nuclei inside the blood-brain barrier, Ang II may act as a neurotransmitter. Circumventricular organs, such as the subfornical organ (SFO), the area postrema (AP) and the organum vasculosum laminae terminalis (OVLT), lack a blood-brain barrier, are rich in Ang II receptors and respond to circulating Ang II (Phillips *et al.* 1998; Zhuo *et al.* 1994). Central areas involved in cardiovascular and fluid regulation exhibit a predominance of AT_1-receptors (Tsutsumi *et al.* 1991) and central effects of Ang II on blood pressure can be blocked by central administration of AT_1-receptor blockers (Huang *et al.* 1996a).

To what extent peripheral administration of AT_1 receptor blockers also blocks these effects of Ang II will depend on their access to central areas. The penetration of drugs through the blood-brain barrier is determined by several factors, such as the degree of lipid solubility, the size of the molecule, extent of binding to plasma proteins, and possibly active transport (Davson and Segal 1996). Compounds with non-polar groups are lipophilic and in general easily cross the blood-brain barrier. Compounds with polar groups are hydrophilic and have more difficulties crossing the blood-brain barrier (Grant *et al.* 1998). Evidence that AT_1-receptor blockers cross the blood-brain barrier comes from autoradiography as well as functional studies. Single doses of losartan at 1, 3 or 10 mg/kg iv inhibited Ang II receptor binding in a dose-related manner, in brain areas containing predominately AT_1 receptors, both inside and outside the blood-brain barrier

(Zhuo *et al.* 1994). Losartan administered sc markedly inhibited labeled Ang II binding in the liver in doses as low as 1 mg/kg, whereas in the brain 1 and 3 mg/kg did not cause inhibition, but 10 and 30 mg/kg caused dose-related inhibition (Marshall *et al.* 1993). In functional studies by Culman (1998), losartan iv at doses of 3,10, 30 and 100mg/kg, dose-dependently attenuated the pressor responses to icv Ang II. Losartan 3 mg/kg iv inhibited Ang II-induced increases in spontaneous firing rate of PVN neurons within 5 minutes (Li *et al.* 1993). Losartan 9mg/kg i.p. inhibited icv Ang II induced drinking responses (Polidori *et al.* 1996). Similarly losartan 3 or 10mg/kg sc reduced the increase sodium excretion and blocked the antidiuretic action induced by icv renin (Barbella *et al.* 1993). In contrast, 10mg/kg orally did not inhibit pressor responses to icv Ang II (Wong *et al.*1990), neither did 3mg/kg/day orally for 3 days (Bui *et al.* 1992). Low doses of losartan, particularly orally can therefore induce clear peripheral AT_1-receptor blockade with little demonstrable central blockade, whereas at higher doses losartan appear to readily cross the blood-brain barrier and can cause marked inhibition of brain AT_1-receptors in areas inside and outside the blood-brain barrier.

Embusartan is a rather hydrophilic AT_1-receptor blocker. In whole-body autoradiographic studies, after 5mg/kg iv or 10mg/kg oral administration of $[^{14}C]$-embusartan, relatively high radioactivity was found in the liver and other peripheral tissues, but there was no penetration of the radioactivity across the blood-brain barrier (Stasch, personal communication). The absence of penetration of $[^{14}C]$-labelled compound across the blood-brain barrier suggests that embusartan may exert less central effects than losartan.

The brain RAS plays a pivotal role in the sympathoexcitation and hypertension caused by increases in CSF sodium (Huang *et al.* 1996a), and to high salt intake in spontaneously hypertensive rats (SHR) and Dahl salt-sensitive (Dahl S) rats (Huang *et al.* 1996b, 1998), as well as the sympathetic hyperactivity and disease progression in rats post myocardial infarction (Zhang *et al.* 1999). Central administration of an AT_1-receptor blocker inhibits these responses. From a therapeutic point of view, the crucial question is whether similar effects can be induced by peripheral administration of an AT_1-receptor blocker. The goal of the present study was therefore 2 fold: (1) to compare the extent of central blockade induced by peripheral administration of a lipophilic (losartan) versus hydrophilic (embusartan) AT_1-receptor blocker; (2) to determine whether differences in central effects persist with chronic dosing. Central blockade was assessed using the pressor responses induced by icv administration of Ang II and hypertonic saline. Icv administration of Ang II causes dose related increases in BP, likely by stimulation of neurons in the MnPO and juxta-ventricular neurons in the OVLT and SFO (Veerasingham and Leenen, 1997; McKinley *et al.* 1995). Icv administration of hypertonic saline increases sympathetic outflow and BP through AT_1-receptor stimulation (Huang et al. 1996a), mainly in nuclei of the lamina terminalis (McKinley et al. 1996) such as the MnPO and OVLT (Veerasingham and Leenen, 1999).

2. Methods

2.1. Animals and surgery

Male Wistar rats (200-250g; Charles River, Montreal, Canada) were housed at 24 °C on a 12 hrs light/dark cycle, fed regular rat chow and allowed tap water ad libitum for at least 5 days prior to entering the study. All experimental procedures were approved and carried out in accordance with the guidelines of the University of Ottawa Animal Care Committee for the use and care of laboratory animals. After 5-7 days of acclimatization, under halothane inhalation anesthesia, a guide cannula (23 gauge, stainless steel tubing) was implanted just above the left lateral cerebral ventricle and fixed on the skull of the rat. The cannula was 0.5-mm posterior and 1.4-mm lateral to the bregma, and its lower end about 0.3 mm above the ventricle (Huang *et al.* 1996a)

At least 1 week after surgery, in the early morning under halothane inhalation anesthesia, the left femoral artery and vein were cannulated with PE-10 fused to PE-50 polyethylene tubing filled with heparinized saline. After recovery from the anesthesia for 4-5 hrs, in the afternoon the intra-arterial catheter was connected to a pressure transducer for recording MAP and HR. The output signals of the transducer were amplified and fed to an IBM compatible computer with a data acquisition program. For intracerebroventricular injection, a 26 gauge stainless cannula was inserted into the guide cannula so that its tip protruded 0.8-1.0 mm into the lateral ventricle. A 20 μl volume Hamilton microsyringe was used for intracerebroventricular (icv) injections, and a microsyringe with 500 μl volume was used for icv infusion, mounted on a Sage 355 infusion pump. Unless stated otherwise, drugs used in the experiments were purchased from Sigma Chemical, St. Louis, MO.

Four separate experiments were performed to test the effects of losartan and embusartan: (1) one dose sc. at 30mg/kg (2) one dose sc. at 100mg/kg (3) 30mg/kg/day sc. as one daily injection for six days and (4) 100mg/kg/day sc. as one daily injection for six days. In each experiment, the rats were randomly divided into 3 groups: (1) control, (2) losartan, (3) embusartan. Rats in the control group were given 0.9% saline injections (1ml/kg). Injections were given in the morning about 4-5 hrs before assessments of their central effects. The doses of losartan used were based on the studies by Marshall *et al.* (1993) showing that losartan 10 and 30 mg/kg sc caused dose-related inhibition of angiotension II in the brain. Doses of embusartan were based on autoradiography studies and effectiveness studies in hypertensive rats (Stasch *et al.* 1997).

2.2. Study protocols

Following an accommodation period of 30 min, the resting mean arterial pressure (MAP) and HR were measured. Subsequently, the following assessments were performed. First, artificial cerebro-spinal fluid (aCSF) was infused icv for 10 min (3.8 □l/min). Fifteen minutes after the icv infusion of aCSF, sodium-rich aCSF (0.3 M NaCl) was infused icv for 10 min (3.8 μl/min). Fifteen minutes after the responses to 0.3 M NaCl had subsided, aCSF (3μl) and two doses of Ang II (10ng/1 μl and 30ng/3 μl for control groups, 30ng/3μl and 100ng/3μl for losartan and embusartan groups) were injected icv at a 5-min interval. After the responses to icv Ang II had disappeared and a further 10-min rest, Ang II (30ng, 100ng, or 300ng) was injected intravenously. Five minutes before each icv infusion, the AVP antagonist [β-mercapto-β, β-cyclopentamethylenepropionyl[1], O-Me-Tyr[2], Arg[8]] vasopressin (30 μg/kg) was injected intravenously to exclude peripheral effects of endogenous vasopressin. The rats were

then allowed to rest for 1 h. In the 30mg/kg one dose experiment, rats then received losartan 30 μg/3μl icv. In the 30mg/kg/day for six days experiment, rats received embusartan 30 μg/ 3μl icv. Five minutes later, AVP antagonist iv and Na^+-rich aCSF (0.3 M NaCl) icv were repeated as described above. At the end, the rats were killed by an overdose of pentobarbital iv. The accuracy of the icv cannulation was checked at autopsy with an icv injection of methylene blue.

All data are expressed as means ± S.E.M. Statistically significant differences among control, losartan and embusartan groups were determined by One-way ANOVA followed by Student-Newman-Keuls test. The level of significance was set as $p < 0.05$.

3. Results

3.1. Baseline values

In the one-dose experiments, there were no significant differences in resting MAP and HR among the groups of rats. In the six days experiments, the resting MAP was significantly decreased in rats treated with losartan and embusartan at 30mg/kg and 100mg/kg. The resting HR was significantly increased by 40-50 bpm in rats treated with losartan and embusartan at 100mg/kg (Table 1). Icv injection or infusion of aCSF caused only minor changes in BP and HR (Table 2 and Figure 3).

3.2. Responses to icv angiotensin II

Injection of Ang II icv in control rats caused clear, dose-related increases in BP. This pattern of changes in BP was similar across the four experiments (Figure 3).

One-dose treatment: Losartan significantly inhibited pressor responses to icv Ang II. This effect was dose-related with greater inhibition by 100 vs 30mg/kg sc. One dose of embusartan also inhibited pressor responses to icv Ang II. However, for each of the two doses this inhibition was less than that caused by losartan (Figure 3 top).

Treatment for six days: In contrast to single doses, treatment with losartan and embusartan for six days resulted in similar inhibition of the pressor responses to icv Ang II. The two doses caused a similar degree of blockade and a modest pressor response remained. Losartan or embusartan treatment for six days also inhibited HR responses to icv injection of Ang II (Table 3).

3.3. Responses to icv Na^+-rich aCSF

In control rats, after intravenous AVP antagonist, icv 0.3 M NaCl increased MAP within 2 min after the beginning of infusion. MAP reached plateau levels within 3-4 min

and returned to the resting level about two min after the end of infusion. This pattern of changes and peak increases (Figure 3) in BP was similar across the four experiments.

One-dose treatment (Figure 4): The increase in MAP to icv infusion of 0.3 M NaCl was significantly attenuated by losartan 30mg/kg and further by losartan 100mg/kg. However, a modest increase persisted. In contrast, the MAP response was only decreased to a minor extent by embusartan 30mg/kg or 100mg/kg. After icv injection of losartan 30 μg, the increase in MAP elicited by infusion of 0.3 M NaCl was totally blocked (Figure 4 top).

Six-day treatment (Figure 5): Chronic treatment with losartan as well as embusartan significantly attenuated the increases in MAP. The extent of this blockade did not differ for the two doses and part of the response persisted. Icv injection of embusartan at 30 μg totally blocked the MAP response to icv infusion of 0.3 M NaCl (Figure 5 top). Losartan and embusartan also inhibited HR responses to icv infusion of 0.3 M NaCl (Table 3).

3.4. Responses to iv Angiotensin II

MAP was significantly increased (Figure 6) and HR was significantly decreased (Table 2) by iv injection of Ang II 30, 100, or 300ng. Over this dose-range, ang II caused similar increase in MAP by about 40 mmHg, likely indicating maximal responses at the lowest dose. MAP and HR responses to iv Ang II were totally blocked by a single sc. injection of losartan or embusartan 100mg/kg. Similarly, MAP and HR responses to Ang II iv were totally blocked by sc. losartan or embusartan at doses of 30 and 100mg/kg/day for six days (Figure 6, Table 2).

4. Discussion

As a major new finding, the present study demonstrates that after one single sc dose, losartan inhibits the pressor responses induced by icv Ang II and Na^+-rich aCSF substantially better than embusartan, but after more chronic sc treatment, losartan and embusartan exert similar central effects. These results suggest that losartan or a metabolite crosses the blood-brain barrier easier than embusartan, and therefore may exert greater effects after acute treatment. However, during more chronic treatment, embusartan appears to gradually cross the blood-brain barrier and then exert similar central effects as losartan.

4.1. Central effects of centrally administered AT_1-receptor blockers

The pressor responses to centrally administered Ang II may be attributed to an increase in sympathetic neuronal activity as well as release of arginine vasopressin (Huang *et al.* 1996a). Combined peripheral □-adrenoceptor and V1 receptor blockade completely prevent the pressor response to central Ang II (Unger *et al.* 1981) confirming a role for both sympathetic activation and vasopressin release in the pressor response to icv Ang II.

Icv infusion of Na^+-rich aCSF increases MAP, HR, and RSNA in conscious rats. Icv pretreatment with the AT_1-receptor blocker losartan abolishes the sympathoexcitatory and pressor responses to hypertonic saline (Huang *et al.* 1996a), indicating that these responses depend on AT_1 receptor stimulation. In the present study, icv injection of losartan or embusartan totally blocked the MAP increase induced by icv infusion of 0.3 M NaCl in aCSF (Figure 4,5). These data confirm results from previous studies and demonstrate that losartan and embusartan at 30 μg icv have the same effects in the central nervous system.

4.2. Central effects of peripherally administered AT_1-receptor blockers

Losartan at single doses of 30 or 100 mg/kg sc markedly, and in a dose-related manner inhibited the MAP increases induced by icv Ang II and hypertonic saline. In contrast, embusartan at 30 mg and 100 mg/kg sc did not inhibit the BP increase induced by icv hypertonic saline, and inhibited the MAP increase induced by icv Ang II only to a minor extent. In contrast, MAP and HR responses to iv Ang II at high doses were totally blocked by both losartan and embusartan sc 100mg/kg, indicating that the sc doses of losartan and embusartan were equivalent in blocking the peripheral effects of iv Ang II. These results suggest that sc losartan crosses the blood-brain barrier more easily than sc embusartan, and therefore exerts greater central effects, at least after one dose. This finding is somewhat surprising since losartan is a hydrophilic AT_1-receptor blocker, while embusartan is a lipophilic AT_1-receptor blocker. However, losartan also generates an active metabolite, EXP 3174, which appears rapidly in plasma after systemic losartan treatment (Casjka *et al.* 1997), has a high affinity for AT_1 receptors and readily crosses the blood-brain barrier (Polidori *et al.* 1996).

In contrast to single doses, after more chronic treatment losartan as well as embusartan significantly and similarly inhibited the BP increases induced by icv angiotensin II and by icv hypertonic saline. These results suggest that during more chronic treatment, embusartan gradually crosses the blood-brain barrier and accumulates in the CNS, and then can induce the same central effects as losartan. From single-dose studies, previous studies concluded that "The degree of central angiotensin AT_1 receptor blockade following peripheral application may vary between different representatives of this class of drugs" (Culman et al. 1999) and that such a difference may have therapeutic

implications. The present study clearly demonstrates that such extrapolations from single dose studies to chronic treatment are not appropriate. An equilibrium between the blood-brain-CSF compartments will be first reached for molecules showing rapid penetration across the barriers and may take a long time for slowly penetrating substances. After a single dose losartan and embusartan indeed show clear differences in degrees of central AT_1-receptor blockade. However, within 6 days of treatment this difference has completely disappeared. Responses to both exogenous Ang II (i.e. icv Ang II) and endogenously released Ang II (i.e. by icv hypertonic saline) become blocked to a similar degree. These findings highlight the importance of chronic dosing if therapeutic relevance is a major reason for studying central effects after peripheral administration.

Losartan and embusartan administered peripherally up to 100 mg/kg only partially blocked the MAP increases induced by icv hypertonic saline, whereas losartan or embursartan at 30 μg icv fully blocked the pressor responses. The latter finding clearly indicates that the pressor response to icv hypertonic saline depends critically on central AT_1-receptor stimulation. Several factors may explain these divergent observations. Firstly, the peripheral doses may not have been large enough to provide high enough brain concentrations. This appears unlikely since during chronic treatment 30 and 100 mg/kg/day caused equivalent inhibition. Secondly, and more likely icv hypertonic saline may lead to AT_1-receptor stimulation in regions of the brain, which are accessible to icv administered losartan or embusartan but not to peripherally administered blocker.

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References

Allen, A.M., Zhuo, J., Mendelsohn, F.A., 1999. Localization of angiotensin AT_1 and AT_2 receptors. J. Am. Soc. Nephrol. 10 Suppl 11: S23-9.

Barbella, Y., Cierco, M., Israel, A., 1993. Effect of Losartan, a nonpeptide angiotensin II receptor antagonist, on drinking behavior and renal actions of centrally administered renin. Proc. Soc. Exp. Biol. Med. 202:401-6.

Bui, J.D., Kimura, B., Phillips, M.I., 1992. Losartan potassium, a nonpeptide antagonist of angiotensin II, chronically administered p.o. does not readily cross the blood-brain barrier. Eur. J. Pharmacol. 219:147-151.

Csajka, C., Buclin, T., Brunner, H.R., Biollaz, J., 1997. Pharmacokinetic-pharmacodynamic profile of angiotensin II receptor antagonists. Clin. Pharmacokinet. 32:1-29.

Culman, J., von Heyer, C., Piepenburg, B., Rascher, W., Unger, T., 1999. Effects of systemic treatment with irbesartan and losartan on central responses to angiotensin II in conscious, normotensive rats. Eur. J. Pharmacol. 367:255-65.

Davson, H., Segal, M.B., 1996. Physiology of the CSF and Blood-Brain Barrier. Chapter 4, The Blood-Brain Barrier, pgs 49-91.

Grant, G.A., Abbott, N.J., Janigro, D., 1998. Understanding the physiology of the blood-brain barrier: in vitro models. News Physiol. Sci. 13: 287-293.

Huang, B.S., Leenen, F.H.H., 1996a. Sympathoexcitatory and pressor responses to increased brain sodium and ouabain are mediated via brain ANG II. Am. J. Physiol. 270: H275-280.

Huang, B.S., Leenen, F.H.H., 1996b. Brain "ouabain" and angiotensin II in salt-sensitive hypertension in spontaneously hypertensive rats. Hypertension 28:1005-12.

Huang, B.S., Leenen, F.H.H., 1998. Both brain angiotensin II and "ouabain" contribute to sympathoexcitation and hypertension in Dahl S rats on high salt intake. Hypertension 32:1028-33.

Li, Z., Bains, J.S., Ferguson, A.V., 1993. Functional evidence that the angiotensin antagonist losartan crosses the blood-brain barrier in the rat. Brain Res. Bull. 30:33-9.

Marshall, F.H., Clark, S.A., Michel, A.D., Barnes, J.C., 1993. Binding of angiotensin antagonists to rat liver and brain membrane measured *ex vivo*. Br. J. Pharmacol.109:760-764.

McKinley, M.J., Badoer, E., Vivas, L., Oldfield, B.J., 1995. Comparison of c-*fos* expression in the lamina terminalis of conscious rats after intravenous or intracerebroventricular angiotensin. Brain Res. Bull. 37:131-7.

McKinley, M.J., McAllen, R.M., Pennington, G.L., Smardencas, A., Weisinger, R.S., Oldfield, B.J., 1996. Physiological actions of angiotensin II mediated by AT_1 and AT_2 receptors in the brain. Clin. Exp. Pharmacol. Physiol. Suppl 3:S99-104.

Muratani, H., Teruya, H., Sesoko, S., Takishita, S., Fukiyama, K., 1996. Brain angiotensin and circulatory control. Clin. Exp. Pharmacol. Physiol. 23:458-464.

Phillips, M.I., Summers, C., 1998. Angiotensin II in central nervous system physiology. Regul. Pept. 78:1-11.

Polidori, C., Ciccocioppo, R., Pompei, P., Cirillo, R., Massi, M., 1996. Functional evidence for the ability of angiotensin AT_1 receptor antagonists to cross the blood-brain barrier in rats. Eur. J. Pharmacol. 307:259-267.

Stasch, J.P., Knorr, A., Hirth-Dietrich, C., Krämer, T., Hübsch, W., Dressel, J., Fey, P., Beuck, M., Sander, E., Frobel, K., Kazda, S., 1997. Long-term blockade of the angiotensin II receptor in renin transgenic rats, salt-loaded Dahl rats, and stroke-prone spontaneously hypertensive rats. Arzneim.-Forsch/Drug Res. 47:1016-23.

Tsutsumi, K., Saavedra, J.M., 1991. Characterization and development of angiotensin II receptor subtypes (AT_1 and AT_2) in rat brain. Am. J. Physiol. 261 (1Pt 2): R209-16.

Unger, T., Rascher, W., Schuster, C., Pavlovitch, R., Schömig, A., Dietz, R., Ganten, D., 1981. Central blood pressure effects of substance P and angiotensin II: role of the sympathetic nervous system and vasopressin. Eur. J. Pharmacol. 71:33-42.

Veerasingham, S.J., Leenen, F.H.H., 1997. Excitotoxic lesions of the ventral anteroventral third ventricle and pressor responses to central sodium, ouabain and angiotensin II. Brain Res. 749:157-160.

Veerasingham, S.J., Leenen, F.H.H., 1999. Ouabain- and central sodium-induced hypertension depend on the ventral anteroventral third ventricle region. Am. J. Physiol. 276(1 Pt 2):H63-70.

Wong, P.C., Price, W.A., Reilly, T.M., Duncia, J.V., Timmermans, P.B., 1990. Antihypertensive mechanism of captopril in renal hypertensive rats: studies with a nonpeptide angiotensin II receptor antagonist and an angiotensin II monoclonal antibody. J. Pharmacol. Exp. Ther. 252:719-725.

Zhang, W., Huang, B.S., Leenen, F.H.H., 1999. Brain renin-angiotensin system and sympathetic hyperactivity in rats after myocardial infarction. Am. J. Physiol. 276: H1608-H1615.

Zhuo, J., Song, K., Abdelrahman, A., Mendelsohn, F.A.O., 1994. Blockade by intravenous Losartan of AT_1 Angiotensin II receptors in rat brain, kidney and adrenals demonstrated by *in vitro* autoradiography. Clin. Exp. Pharmacol. Physiol. 21:557-567.

Zhuo, J., Moeller, I., Jenkins, T., Chai, S.Y., Allen, A.M., Ohishi, M., Mendelsohn, F.A.O., 1998. Mapping tissue angiotensin-converting enzyme and angiotensin AT_1, AT_2 and AT_4 receptors. J. Hypertens. 16: 2027-2037.

Legends to the figures

Figure 2 Schematic outline of study-protocol for icv and iv injections and infusions

Figure 3 Line graphs showing peak increases in mean arterial pressure (MAP) by icv Ang II (10, 30, 100ng) in control groups and groups treated with losartan or embusartan, 30mg/kg (upper-left) and 100mg/kg (upper-right) one dose, or 30mg/kg/day (lower-left) and 100mg/kg/day (lower-right) for 6 days sc.

Values are mean_SEM (n=5-6/group)

*$p<0.05$ vs control group, #$p<0.05$ vs embusartan group.

Figure 4 Bar graphs showing peak increases in mean arterial pressure (MAP) by icv 0.3 M NaCl in control group and groups treated with losartan or embusartan, 30mg/kg (upper part) and 100mg/kg (lower part) one dose.

Values are mean_SEM (n=5-6/group)

*$p<0.05$ vs control group, #$p<0.05$ vs embusartan group, @$p<0.05$ vs aCSF

Figure 5 Bar graphs showing peak increases in mean arterial pressure (MAP) by icv 0.3 M NaCl in control group and groups treated with losartan and embusartan, 30mg/kg/day (upper part) and 100mg/kg/day (lower part) for 6 days sc.

Values are mean±SEM (n=5-6/group)

*$p<0.05$ vs control group, @$p<0.05$ vs aCSF

Figure 6 Bar graphs showing peak increases in mean arterial pressure (MAP) by iv Ang II (30ng, 100ng, 300ng) in control rats and rats treated with losartan or embusartan 100mg/kg one dose, or losartan or embusartan 30 or 100mg/kg/day sc. for 6 days

Values are mean_SEM (n=5-6/group)

*$p<0.05$ vs control group

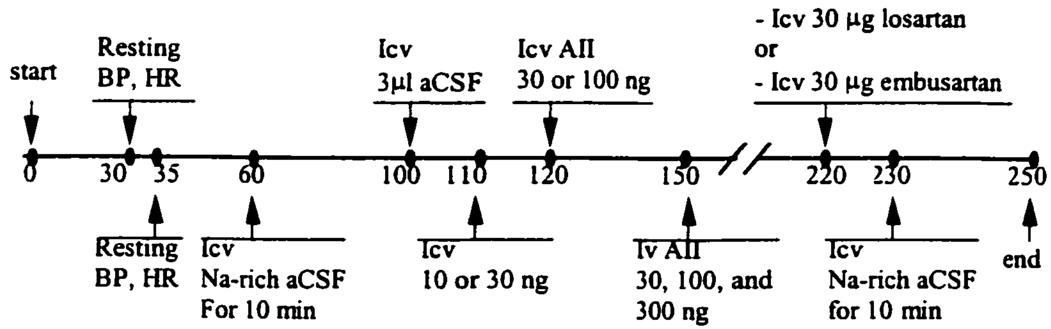

Figure 2 Schematic outline of study-protocol for icv and iv injections and infusions.

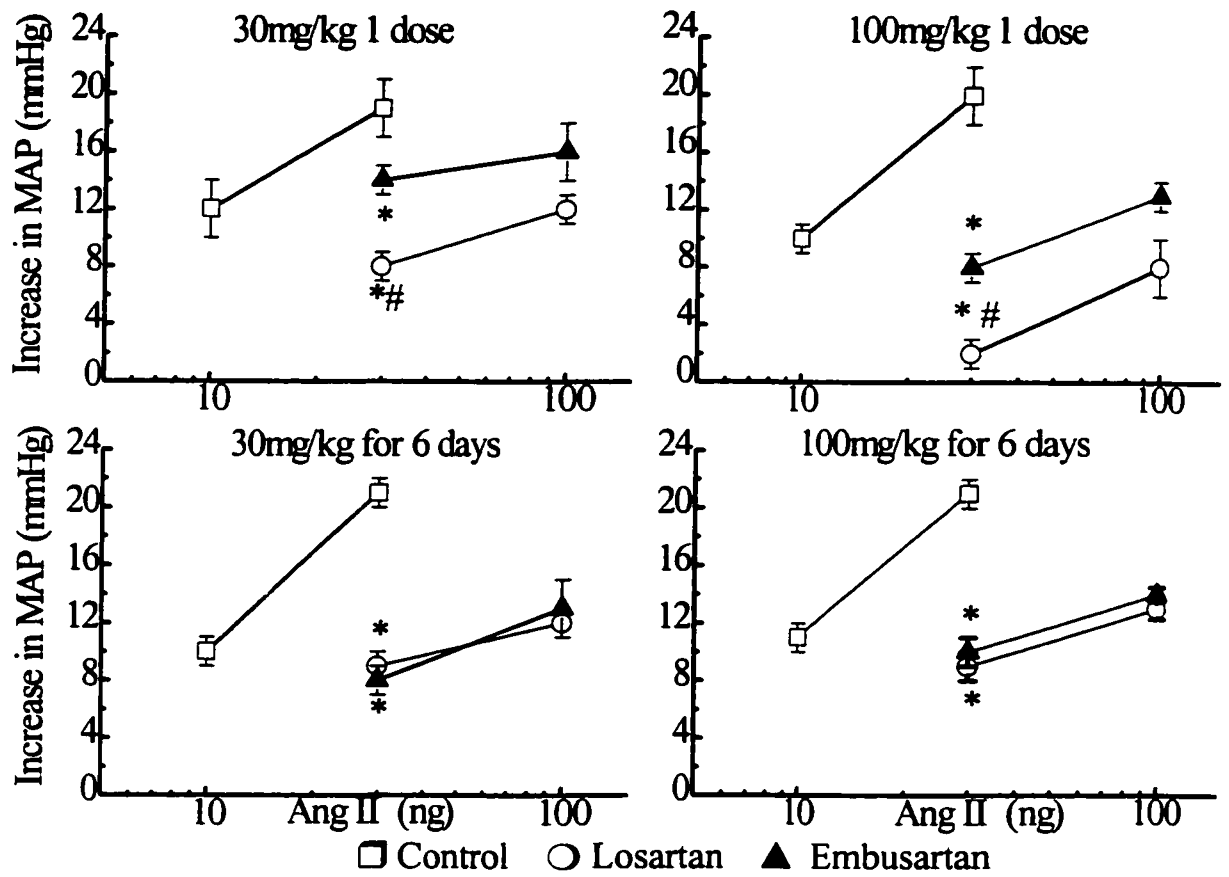

Figure 3 **Peak increases in MAP by icv Ang II with sc losartan or embusartan one dose or for 6 days**

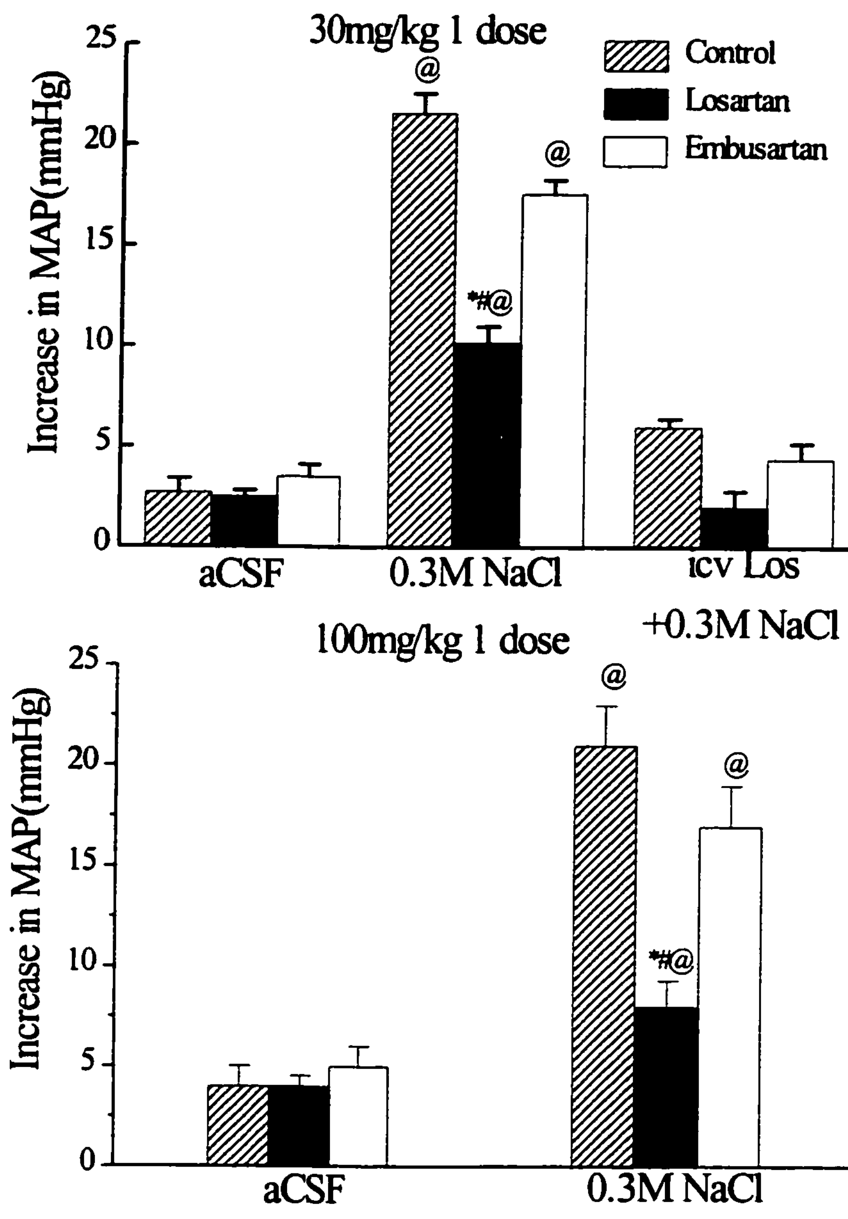

Figure 4 **Peak increases in MAP by icv 0.3 M NaCl with sc losartan or embusartan one dose**

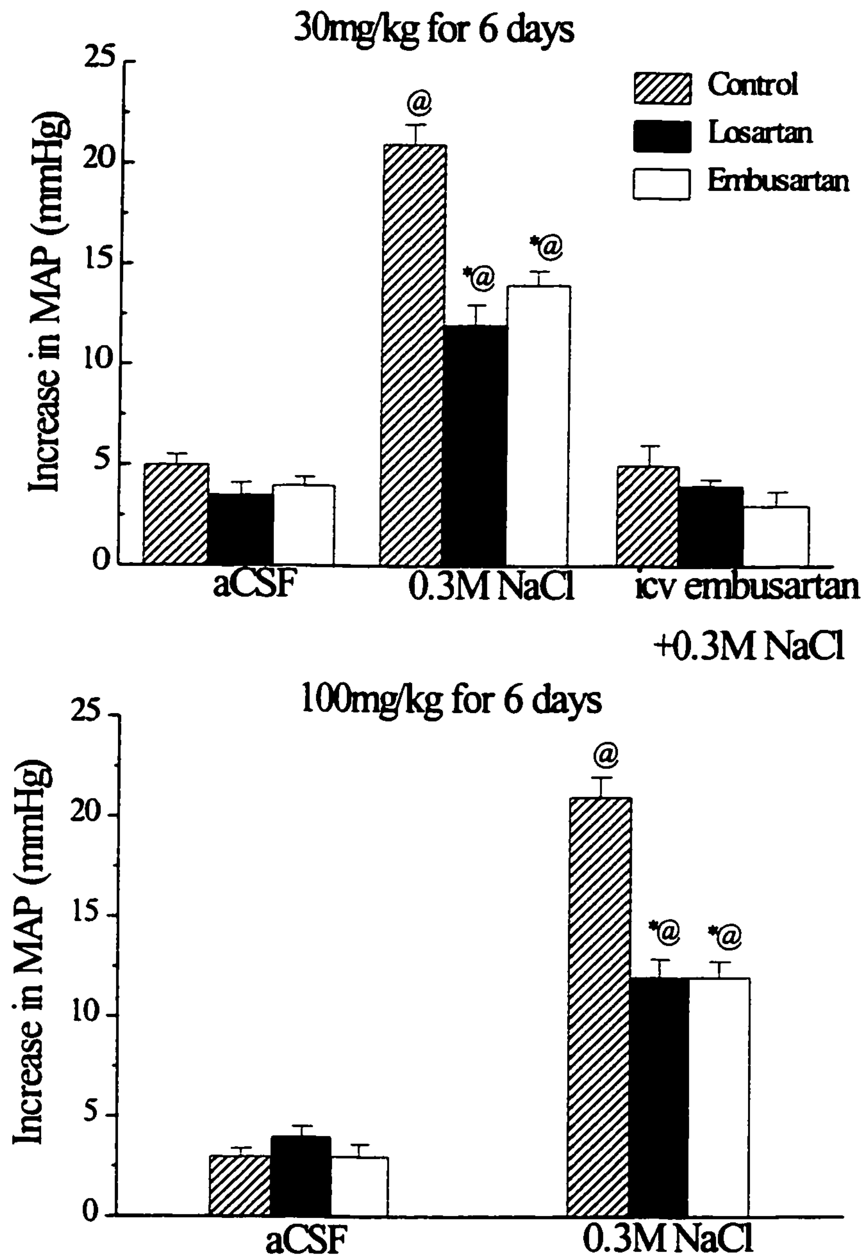

Figure 5 Peak increases in MAP by icv 0.3 M NaCl with sc losartan or embusartan for 6 days

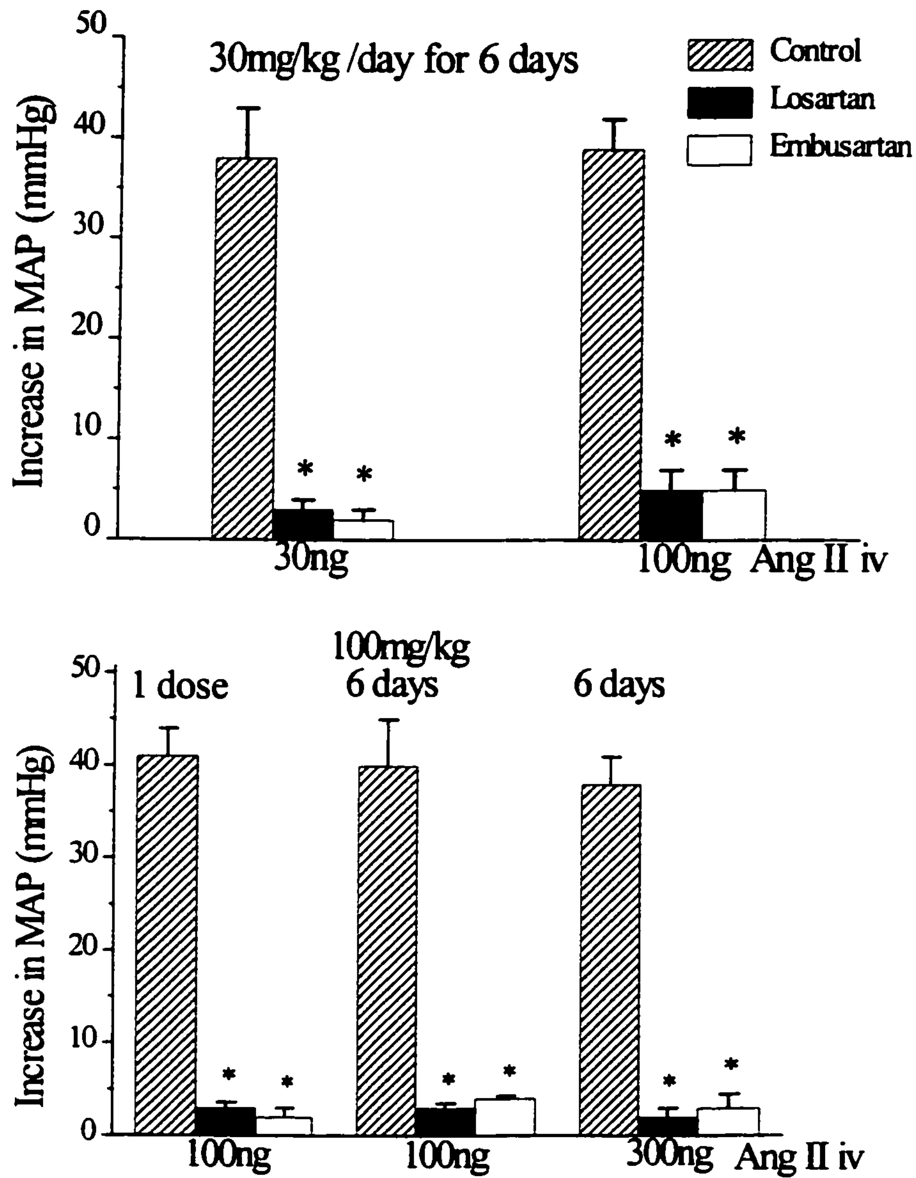

Figure 6 **Peak increases in MAP by iv Ang II with sc losartan or embusartan for 6 days**

Table 1: Effects of losartan and embusartan on resting MAP and HR

		MAP(mmHg)			HR(bpm)		
		Baseline before injections	Baseline after Na-rich aCSF icv	Baseline after Ang II icv	Baseline before injections	Baseline after Na-rich aCSF icv	Baseline after Ang II icv
One dose treatment							
Control (n=5)		105±1	106±2	106±2	420±7	398±16	420±7
Losartan	30mg/kg (n=5)	98±4	96±4	98±3	436±18	396±18	438±18
Embusartan	30mg/kg (n=6)	97±2	100±2	99±2	434±11	427±10	444±9
Control (n=5)		101±3	102±2	104±2	412±16	406±5	401±6
Losartan	100mg/kg (n=5)	102±3	106±4	109±3	385±9	394±12	426±18
Embusartan	100mg/kg (n=6)	102±2	104±3	109±2	410±7	423±6	401±8
Six day treatment							
Control (n=6)		109±3	111±4	112±4	424±6	430±14	426±10
Losartan	30mg/kg (n=9)	94±2*	99±2*	93±3*	443±14	449±18	454±16
Embusartan	30mg/kg (n=7)	92±2*	92±3*	94±3*	441±10	444±7	427±12
Control (n=6)		108±2	110±3	110±3	392±6	377±15	397±8
Losartan	100mg/kg (n=6)	84±2*	85±3*	81±3*	440±9*	454±10*	451±12*
Embusartan	100mg/kg (n=6)	83±2*	83±4*	78±2*	446±9*	454±8*	447±9*

Data are means ±SEM

* $p<0.05$, vs control.

Table 2: Changes in MAP and HR after icv injection of aCSF

One dose treatment		ΔMAP(mmHg)	ΔHR(bpm)
Control (n=5)		2±1	8±2
Losartan	30mg/kg (n=5)	2±1	2±3
Embusartan	30mg/kg (n=6)	1±1	5±2
Control (n=5)		1±1	1±3
Losartan	100mg/kg (n=5)	3±1	9±3
Embusartan	100mg/kg (n=6)	2±0	17±6
Six day treatment			
Control (n=6)		2±0	1±1
Losartan	30mg/kg (n=6)	2±0	7±2
Embusartan	30mg/kg (n=6)	2±1	2±1
Control (n=6)		2±1	7±1
Losartan	100mg/kg (n=6)	2±1	8±2
Embusartan	100mg/kg (n=6)	1±0	4±2

Data are means ±SEM

Table 3: Changes in HR to icv hypertonic saline and Ang II after six days treatment with losartan or embusartan.

30mg/kg/day for 6 days:

	Control(n=6)	Losartan(n=6)	Embusartan(n=6)
aCSF 3.8μl/min icv	20±4	16±3	16±4
0.3M NaCl 3.8μl/min icv	34±6	18±5*	17±3*
Ang II 10ng icv	22±7	_	_
Ang II 30ng icv	43±6@	17±4*	16±7*
Ang II 100ng icv	_	29±9	25±7
Ang II 30ng iv	-76±15@	10±2*	6±3*
Ang II 100ng iv	-116±20@	10±3*	4±2*

100mg/kg/day for 6 days:

	Control(n=6)	Losartan(n=6)	Embusartan(n=6)
aCSF 3.8μl/min icv	19±5	18±2	12±2
0.3M NaCl 3.8μl/min icv	24±5	18±4	19±4
Ang II 10ng icv	20±5	_	_
Ang II 30ng icv	23±6	7±3	15±6
Ang II 100ng icv	_	22±7	22±5
Ang II 100ng iv	-207±10@	3±13*	7±5*
Ang II 300ng iv	228±14@	5±1*	13±8*

Values are mean ±SEM, *p<0.05 vs control group, @p<0.05 vs baseline

Paper 2

PERIPHERAL ADMINISTRATION OF AT_1-RECEPTOR BLOCKERS PREVENTS SYMPATHETIC HYPERACTIVITY AND HYPERTENSION BY CHRONIC OUABAIN AND HYPERTONIC SALINE[1]

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Short Title: AT_1-receptor blockers and central sodium and ouabain

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ABSTRACT: Sympathetic hyperactivity and hypertension caused by chronic ouabain treatment and by central sodium can be prevented by central administration of an AT_1-receptor blocker. In the present studies, we assessed whether chronic peripheral treatment with an AT_1-receptor blocker can exert sufficient central effects to prevent these central effects of ouabain and sodium. Losartan is a lipophilic and embusartan a hydrophilic AT_1-receptor blocker. Losartan or embusartan both at (100mg/kg/day) were given sc once daily for 16 days. Ouabain (50 μg/day) was infused sc by osmotic minipump for 13-14 days. Sodium-rich aCSF (1.2M, 5 μl/hour) was infused intracerebroventricularly (icv) by osmotic minipump for 13-14 days. The resting mean arterial pressure (MAP) and the pressor responses to air stress, and icv injection of guanabenz (75 μg/7.5 μl), Ang II (30 ng/3 μl), and ouabain (0.5 μg/2 μl) were then measured. Both chronic treatment with ouabain sc and hypertonic saline icv increased baseline MAP by 20-25 mmHg, and enhanced 2-fold pressor responses to air stress and depressor responses to the α_2-adrenoceptor agonist guanabenz. Chronic treatment with losartan or embusartan at 100mg/kg fully prevented the hypertension, prevented enhanced responses to air stress and guanabenz as well as significantly attenuated pressor responses to acute icv injection of Ang II and ouabain. We conclude that peripheral administration of losartan or embusartan can cause sufficient central effects to prevent the sympathetic hyperactivity and hypertension induced by chronic peripheral ouabain and central sodium.

Key words: AT_1 receptor blocker, brain renin-angiotensin system, losartan, embusartan, angiotensin II, hypertonic saline, ouabain

INTRODUCTION

Both acute and chronic intracerebroventricular (icv) and subcutaneous (sc) infusion of ouabain elicit sympathoexcitatory and pressor effects likely via activation of central pathways involving AT_1-receptor stimulation (Huang & Leenen, 1999). Acute and chronic icv infusion of hypertonic saline causes sympathoexcitatory and pressor responses, also involving the brain RAS, since icv treatment with the AT_1 blocker losartan prevents these effects (Huang & Leenen, 1996, Huang *et al* 1998). The brain RAS also plays an important role in pathological states such as salt sensitive hypertension (Huang & Leenen, 1998) and congestive heart failure after myocardial infarction (Zhang *et al.* 1999). Chronic central administration of an AT_1-receptor blocker prevents and reverses sympathoexcitatory and/or pressor responses. From a therapeutic point of view, the crucial question is whether similar effects can be induced by peripheral administration of an AT_1-receptor blocker.

AT_1-receptor blockers cross the blood-brain barrier as demonstrated by autoradiographic as well as functional studies. Single doses of losartan at 1, 3 or 10 mg/kg iv inhibited Ang II receptor binding in a dose-related manner, in brain areas containing predominately AT_1-receptors, both inside and outside the blood-brain barrier (Zhou *et al.* 1994). Embusartan is a rather hydrophilic AT_1-receptor blocker. In whole-body autoradiographic studies, after iv 5mg/kg or oral 10mg/kg administration of [^{14}C]-embusartan, relatively high radioactivity concentrations were found in the liver, and other peripheral tissues, but there was no penetration of the radioactivity across the blood-brain barrier (Stasch, personal communication). The absence of [^{14}C]-labelled compound across the blood-brain barrier suggests that embusartan may exert less central effects than losartan.

Functional studies so far only addressed effects on acute administration of Ang II icv. Acute or chronic treatment with losartan by gavage significantly attenuates pressor responses to acute icv injection of Ang II or hypertonic saline. There is so far no evidence whether peripheral administration also can inhibit the brain RAS during chronic activation. The goal of the present study was therefore 2 fold: (1) To determine whether the hypertension induced by chronic icv administration of hypertonic saline or chronic sc administration of ouabain can be prevented by chronic peripheral administration of an AT_1-receptor blocker. (2) To determine whether the extent of central blockade differs between losartan versus embusartan. Central blockade was assessed by evaluating their effects on resting blood pressure, increases or decreases in mean arterial pressure (MAP) and HR in response to air stress, icv injection of the α_2-adrenoceptor agent guanabenz, Ang II and ouabain. Air jet stress measures activity of sympathoexcitatory pathways (Huang & Leenen, 1999) and guanabenz activity in sympathoinhibitory pathways (Koepke *et al.* 1988).

METHODS

Animals and experimental protocols:

Male Wistar rats (200-250g; Charles River, Montreal, Canada) were housed at 24°C on a 12 hrs light/dark cycle, fed regular rat chow and allowed tap water ad libitum for at least 5 days prior to entering the study. All experimental procedures were approved and carried out in accordance with the guidelines of the University of Ottawa Animal Care Committee for the use and care of laboratory animals.

Two separate experiments were performed to test the effects of chronic administration of losartan and embusartan on ouabain and hypertonic saline induced hypertension. In experiment 1, rats received a chronic subcutaneous infusion of either 0.9 % saline or ouabain 50 μg/day via osmotic pump. In experiment 2, rats received a chronic intracerebroventricular infusion of either artificial CSF (aCSF) or aCSF containing 1.2 M NaCl 5 μl/h via osmotic pump.

For experiment 1, the rats were randomly divided into 6 groups:

(1) Control (n=6): daily sc injection of 0.9 % saline 1ml/kg + sc infusion of 0.9 % saline

(2) Ouabain alone(n=6): daily sc injection of 0.9 % saline 1ml/kg + sc infusion of ouabain 50 μg/day

(3) Losartan alone (n=6): daily sc injection of losartan at 100mg/kg/day + sc infusion of 0.9 % saline

(4) Losartan + ouabain (n=6): daily sc injection of losartan at 100mg/kg/day + sc infusion of ouabain 50 μg/day

(5) Embusartan alone (n=6): daily sc injection of embusartan at 100mg/kg/day + sc infusion of 0.9 % saline

(6) Embusartan + ouabain (n=6): daily sc injection of embusartan at 100mg/kg + sc infusion of ouabain 50 μg/day

For experiment 2, the rats were also randomly divided into 6 groups:

(1) Control (n=6): daily sc injection of 0.9 % saline 1ml/kg + icv infusion of aCSF

(2) Icv hypertonic saline alone (n=6): daily sc injection of 0.9 % saline 1ml/kg + icv infusion of aCSF containing 1.2 M NaCl

(3) Losartan alone (n=6): daily sc injection of losartan at 100mg/kg/day + icv infusion of aCSF

(4) Losartan + icv hypertonic saline (n=6): daily sc injection of losartan at 100mg/kg/day + icv infusion of aCSF containing 1.2 M NaCl

(5) Embusartan alone(n=6): daily sc injection of embusartan at 100mg/kg/day + icv infusion of aCSF

(6) Embusartan + icv hypertonic saline (n=6): daily sc injection of embusartan at 100mg/kg/day + icv infusion of aCSF containing 1.2 M NaCl

Doses of losartan and embusartan were chosen based on acute and more chronic studies showing that losartan or embusartan at 30 or 100mg/kg sc markedly inhibit pressor responses to acute icv Ang II or hypertonic saline (Zhang & Leenen, unpublished data). The final assessments were performed after a 14-day infusion of ouabain or hypertonic saline. In previous studies we showed that chronic sc administration of ouabain or icv infusion of 1.2 M NaCl increases baseline MAP by 20-25 mmHg within 10-14 days (Huang *et al.* 1994, 1998).

Placement of intracerebroventricular cannula and implantation of osmotic minipump

Experiment 1. After 5-7 days of acclimatization, under halothane anesthesia, a 23-gauge guide cannula (14mm long) was fixed to the skull of the rat with acrylic cement. The lower end of the cannula was 0.5 mm above the left lateral ventricle (coordinates: 0.4 mm posterior and 1.2 mm lateral to bregma and 2.8 mm deep to dura) (Huang *et al.*1998). This cannula later served as a guide for icv injections. In 3 groups of rats, an osmotic minipump (Alzet, Model 2002) filled with ouabain dissolved in saline was implanted sc on the back. The infusion rate of ouabain was 50 μg in 12 μl per day.

Experiment 2. After 5-7 days of acclimatization, guide cannulas were implanted as in Experiment 1. In addition, a L-shaped stainless cannula was implanted into the right lateral ventricle (3.5 mm deep from dura) and fixed on the skull. By means of polyethylene tubing (PE-50 fused to PE-60), this cannula was connected to an osmotic minipump (Alzet, model 2ML2) filled with aCSF or aCSF containing 1.2M NaCl, which was located sc. on the back. The infusion rate of hypertonic saline was 5 μl per hour.

After the icv guide cannula and osmotic minipump implantation, the rats were returned to their original cage with regular food and water. They were trained to stay quietly in a small experimental cage (24 x 15 x 8 cm) in which the rat could move back and forward on three to four different occasions, each lasting 1-2 h.

Femoral artery and vein cannulation:

After 2 weeks, in the early morning, the left femoral artery and vein were cannulated with PE-10 tubing fused to PE-50 polyethylene tubing filled with heparinized saline. The catheters were tunneled subcutaneously, exteriorized at the nape and secured to the skin. Rats were given a 4-5 hr recovery period before proceeding with the experiment.

Blood Pressure and HR measurements:

The arterial catheter was connected to a transducer, and MAP and HR were recorded through an IBM-compatible computer programmed by a data acquisition program (Dataquest LabPro; Data Science International, St. Paul, MN) that allowed on line analysis of the pulsate blood pressure signal and storage of data. MAP and HR were sampled every 30 sec at a sampling rate of 500 Hz, except for air stress data in which momentary changes in MAP and HR were used. Rats were allowed an accommodation period of 30 min before resting MAP and HR were recorded.

Specific Study Protocols:

On day -2, chronic sc injection of losartan (100mg//kg/day), embursartan (100mg/kg/day), or 0.9 % saline was started. On day 0, an icv guide cannula and osmotic minipump sc were implanted. On day 13-14, in the early morning, a femoral artery and vein were cannulated. After the resting MAP and HR had been measured, standardized air stress was provided twice at a 10-min intervals by blowing the face of the rat with a jet of air (1-1.5 psi) for 30 s, from a tube located $\approx$ 3 cm in front of the rat. The average of peak changes in MAP and HR in response to the two applications of stress was used for comparisons. Icv injections were then performed using an L-shaped 30-gauge stainless-steel cannula connected to a Hamilton micro syringe via PE 10 polyethylene tubing which when inserted into the icv guide cannula protruded 1 mm into the left lateral ventricle. 3 μl of aCSF, Ang II 30ng/3μl aCSF, guanabenz (25 μg/ 2.5 μl aCSF, and 75 μg/ 7.5 μl aCSF) were injected icv at a 10 min interval, and 20 min between two guanabenz injections. Thirty minutes after the responses to guanabenz had disappeared, ouabain 0.5 μg/ 2 μl aCSF was injected icv.

At the end of the experiment, the rat was deeply anaesthetized with sodium pentobarbital, and injected icv with 5 μl of methyl blue to verify cannula placement. The brain was removed and cut through the hole of the guide cannula to assess whether the methyl blue was only in lateral ventricle.

Data Analysis:

All data are expressed as means ± S.E.M. Statistically significant differences between control, losartan and embusartan groups were determined by One-way ANOVA followed by Student-Newman-Keuls test. The level of significance was set as $p < 0.05$.

RESULTS

Baseline values:

After sc ouabain treatment for 2 weeks, resting MAP was significantly increased. Losartan or embusartan alone decreased MAP significantly. In the groups that received combined ouabain and losartan or embusartan MAP was similar to that in the groups receiving losartan or embusartan alone (Figure 7, upper panel). There were no significant differences in resting HR among the 6 groups of rats. The body weight gain over the 2 weeks of treatment was similar in all groups (data not shown).

Following icv hypertonic saline for 2 weeks, resting MAP had significantly increased. Losartan or embusartan alone decreased BP again significantly. In the groups receiving combined hypertonic saline and losartan or embusartan BP was similar to that in groups that receiving losartan or embusartan alone (Figure 7, lower panel). There were no significant differences in resting HR among the groups. The body weight gain over the 2 weeks of treatment was similar in all groups (data not shown).

Responses to Air Stress:

Ouabain induced hypertension: Air stress caused a rapid increase in MAP. Losartan and embusartan alone did not significantly affect these responses. In rats treated with ouabain, peak increases in MAP were approximately twice those in control rats. These enhanced responses did not develop when losartan or embusartan was administered sc (Figure 8, upper panel).

Hypertonic saline induced hypertension: In rats treated with icv 1.2M NaCl, peak increases in MAP were also more than twice those in control rats. These enhanced

responses did not develop when losartan or embusartan was administered sc (Figure 8, lower panel).

Responses to Intracerebroventricular Guanabenz

Ouabain induced hypertension: After icv administration of gunanabenz at either dose, MAP decreased and reached a plateau within 5 minutes. The peak responses were dose-related. In rats treated with ouabain, maximum decreases in MAP were twice those in control rats. In rats receiving ouabain concomitant with losartan or embusartan, peak decreases were similar to those in control rats (Figure 9, upper panel).

Hypertonic saline induced hypertension: In rats treated with hypertonic saline, maximum decreases in MAP were twice those in control rats. In rats receiving ouabain concomitant with losartan or embusartan, peak decreases were similar to those in control rats (Figure 9, lower panel).

Responses to Intracerebroventricular Ang II and ouabain

Ouabain induced hypertension: Icv injection of aCSF did not change BP significantly. Icv injection of Ang II or ouabain significantly increased MAP. In the control and ouabain treatment group, the increases in MAP by Ang II 30 ng icv were 18 to 20 mmHg. Sc losartan or embusartan attenuated the increases in MAP to only 6-7 mmHg. In the control and ouabain treatment group, increases in MAP by ouabain 0.5 μg icv were 13 to 15mmHg. Sc losartan or embusartan significantly attenuated these increases in MAP to 4-8 mmHg. The latter responses remained significantly larger than those induced by icv injection of aCSF (Figure 9, upper panel).

Hypertonic saline induced hypertension: In the control and ouabain treatment group, increases in MAP by Ang II 30 ng icv were 17 mmHg. Sc losartan or embusartan

attenuated the increases in MAP to 4-6 mmHg. In the control and ouabain treatment groups, increases in MAP by ouabain 0.5 μg icv were 15-17 mmHg. Sc losartan or embusartan attenuated the increases in MAP to 4-5 mmHg. However, these increases remained significantly larger than those induced by icv injection of aCSF (Figure 9, lower panel).

DISCUSSION

The present study demonstrates that in normotensive rats, sympathetic hyperactivity and increases in resting BP caused by sc infusion of ouabain or icv infusion of hypertonic saline can be fully prevented by daily sc injections of losartan or embusartan.

Consistent with other recent studies, chronic treatment with ouabain caused moderate hypertension in conscious normotensive rats (Huang *et al.*1994, Yuan *et al.* 1993 and Veerasingham & Leenen, 1999). Lesions limited to the ventral part of AV3V involving the OVLT and ventral MnPO fully prevent the hypertension induced by chronic sc administration of ouabain (Veerasingham *et al*, 1999). Peripheral mechanisms do not appear to play a significant role in the hypertension induced by sc ouabain, because central blockade of the effects of ouabain prevents hypertension in this model (Huang *et al*, 1994; Veerasingham & Leenen, 1999). In rats, icv pretreatment with the Ang II receptor blocker saralasin (Takahashi et al. 1984), or the AT_1-receptor blocker losartan (Huang *et al.* 1996) blocks sympathoexcitatory and pressor responses to acute icv ouabain. These studies suggest that activation of brain AT_1-receptors occurs in the pathways mediating the effects of acute icv ouabain. Chronic administration of ouabain leads to increased activity in sympathoexcitatory pathways, decreased activity in sympathoinhibitory pathways and the development of hypertension. All these responses can also be prevented by concomitant icv treatment with losartan (Huang *et al.* 1999).

In conscious rats acute icv hypertonic saline causes sympathoexcitatory and pressor effects which can be prevented by icv pretreatment with Fab fragments blocking brain "ouabain" or losartan (Huang & Leenen, 1996). Chronic central sodium loading

causes enhanced sympathoexcitation, impairment of baroreflexes and hypertension, which can be prevented by concomitant icv Fab fragments or losartan (Huang *et al.* 1998). These findings suggest that central pathways involving both "ouabain" and Ang II mediate the effects of chronic central sodium loading. vAV3V lesions which include the OVLT and ventral MnPO, also abolish the increase in MAP elicited by chronic infusion of hypertonic saline (Veerasingham *et al*, 1999). It appears that the OVLT and ventral MnPO play an important role in mediating pressor effects of chronic infusion of ouabain or hypertonic saline. AT_1-receptors are also present in the anteroventral third ventricle (AV3V) region, including the OVLT, MnPO and subfornical organ (SFO) (Mendelsohn *et al.* 1984), and these may be involved in the sympatho-excitatory and pressor responses to sodium or ouabain. The OVLT is one of the circumventricular organs, which is outside the blood-brain barrier. Access to the OVLT should be similar for lipophilic or hydrophilic AT_1-receptor blocker. A similar blockade can therefore be expected, assuming that the relevant AT_1-receptors are located in this area. Indeed, the present results demonstrate that chronic sc treatment with losartan or embusartan at 100mg/kg/day fully prevents the central effects of chronic ouabain and hypertonic saline. Although losartan is rather lipophilic and embusartan is rather hydrophilic, both exerted similar central effects following chronic treatment.

In a previous study, we showed that sc administration of losartan or embusartan one dose or 6 days only partially blocked the central effects of icv Ang II or hypertonic saline for 10 min (Zhang & Leenen, unpublished data). In the present study, chronic sc administration of losartan or embusartan also only partially blocked the central effects of acute icv injections of Ang II and ouabain, but fully blocked all effects of chronic

treatment with ouabain or hypertonic saline. Lesions of the ventral anteroventral third ventricle (vAV3V) also only partially inhibited pressor responses to acute icv sodium and ouabain, but fully prevented the increase in MAP elicited by chronic infusion of hypertonic saline or administration of ouabain (Veerasingham *et al.* 1997;1999). Amounts administered may contribute to this difference. Firstly, in the acute study, 0.3 M hypertonic saline was infused at 3.8μl/min. In the chronic study, 1.2 M hypertonic saline was infused at 5μl/h. The resulting CSF concentration of sodium in the acute study is likely therefore substantially higher than that in the chronic studies. Acute responses to icv hypertonic saline involve pathways from the SFO to PVN, with likely synapses in the ventral MnPO (Rohmeiss et al, 1995) and the OVLT plays a more limited role. In contrast, the OVLT appears to play a more important role in mediating pressor effects of chronic infusion of hypertonic saline (Veerasingham *et al.* 1997, 1999). Therefore, higher CSF concentrations of sodium may mainly involve pathways from the SFO to PVN, whereas lower CSF concentration sodium may only involve OVLT. Secondly, the AT_1-receptors contributing to the pressor responses to acute icv infusion of hypertonic saline may be located in areas both inside and outside the blood-brain barrier and therefore may not be fully accessible to circulating AT_1-receptor blockers. In contrast, AT_1-receptors contributing to the pressor responses to chronic icv infusion of hypertonic saline may be mainly located in the OVLT and therefore fully accessible to circulating AT_1-receptor blockers.

In conclusion, chronic infusion with ouabain sc or hypertonic saline icv both lead to increased sympathoexcitation, decreased sympathoinhibition and hypertension. These

central effects of ouabain or hypertonic saline can be fully prevented by chronic blockade of the brain RAS with losartan or with embusartan sc. A hydrophilic and lipophilic AT_1-receptor blocker were similarly effective, consistent with the concept that the relevant AT_1-receptors are located in the OVLT.

REFERENCES

Huang BS, Huang X, Harmsen E and Leenen FHH. Chronic central versus peripheral ouabain, blood pressure, and sympathetic activity in rats. Hypertension 23:1087-1090, 1994

Huang BS, Leenen FHH. Sympathoexcitatory and pressor responses to increased brain sodium and ouabain are mediated via brain ANG II. Am J Physiol 270(1 Pt 2):H275-80, 1996

Huang BS, Leenen FH. Both brain angiotensin II and "ouabain" contribute to sympathoexcitation and hypertension in Dahl S rats on high salt intake. Hypertension. 32:1028-33, 1998

Huang BS, Veerasingham SJ, and Leenen FHH. Brain "ouabain", ANG II and sympathoexcitation by chronic central sodium loading in rats. Am. J. Physiol. 274 (Heart Circ. Physiol. 43): H1269-H1276, 1998

Huang BS, Leenen FHH. Brain renin-angiotensin system and ouabain-induced sympathetic hyperactivity and hypertension in Wistar rats. Hypertension 34:107-12, 1999

Koepke JP, Jones S, DiBona GF. Sodium responsiveness of central alpha 2-adrenergic receptors in spontaneously hypertensive rats. Hypertension 11: 326-33,1988

Mendelsohn FA, Chai SY, Dunbar M. In vitro autoradiographic localization of angiotensin-converting enzyme in rat brain using 125I-labelled MK351A. J Hypertens Suppl 2(3):S41-4,1984

Rohmeiss P, Beyer C, Hocher B, Qadri F, Gretz N, Strauch M, Unger T. Osmotically induced natriuresis and blood pressure response involves angiotensin AT_1 receptors in the subfornical organ. J Hypertens 13(12 Pt 1): 399-404, 1995

Takahashi H, Matsusawa M, Suga K, Ikegaki I, Nishimura M, Yoshimura M, Ihara N, Yamada H, Sano Y. Hypothalamic digitalis-like substance is released with sodium-loading in rats. Am J Hypertens 1:146-51, 1988

Veerasingham SJ, Leenen FH. Excitotoxic lesions of the ventral anteroventral third ventricle and pressor responses to central sodium, ouabain and angiotensin II. Brain Res 21;749(1):157-60, 1997

Veerasingham SJ, Leenen FHH. Quabain and central sodium-induced hypertension depend on the ventral anteroventral third ventricle region. Am J Physiol 276(1 Pt 2): H63-70, 1999

Yuan CM, Manunta P, Hamlyn JM, Chen S, Bohen E, Yeun J, Haddy FJ, Pamnani MB. Long-term ouabain administration produces hypertension in rats. Hypertension 22:178-87, 1993

Zhang W, Huang BS, Leenen FHH. Brain renin-angiotensin system and sympathetic hyperactivity in rats after myocardial infarction. American Journal of Physiology 276: H10658-H1615, 1999

Zhuo J, Song K, Abderlrahman L, Mendelsohn FAO. Blockade by intravenous Losartan of AT_1 Angiotensin II receptors in rat brain, kidney and adrenals demonstrated by in vitro autoradiography. Clinical and Experimental Pharmacology and Physiology. 21:557-567,1994

Legends to figures

Figure 7 Bar graphs showing baseline MAP in control rats, in rats with chronic ouabain treatment with and without subcutaneous losartan or embusartan (upper panel), and in rats with chronic intracerebroventricular 1.2 M NaCl treatment with and without subcutaneous losartan or embusartan (lower panel).
Values are mean±SEM (n=6 or10 per group). *$p<0.05$ vs other groups, #$p<0.05$ vs treatment groups

Figure 8 Bar graphs showing peak increases in MAP in response to air stress in control rats, in rats with chronic ouabain treatment with and without subcutaneous losartan or embusartan (upper panel) and in rats with chronic intracerebroventricular 1.2 M NaCl treatment with or without subcutaneous losartan and embusartan (lower panel).
Values are mean±SEM (n=6 or10 per group). *$p<0.05$ vs other groups

Figure 9 Bar graphs showing maximal decreases in MAP in response to intracerebroventricular injection of guanabenz (25 and 75μg) in control rats, in rats with chronic subcutaneous ouabain with and without subcutaneous losartan or embusartan (upper panel), and in rats with chronic intracerebroventricular 1.2 M NaCl with and without subcutaneous losartan or embusartan (lower panel).
Values are mean±SEM (n=6 or 10 per group). *$p<0.05$ vs other groups at corresponding doses.

Figure 10 Bar graphs showing peak increase in MAP in response to intracerebroventricular injection of Ang II 30ng and ouabain 0.5μg in control rats and in rats with chronic ouabain treatment with and without subcutaneous losartan or embusartan (upper panel), and in rats with chronic hypertonic saline treatment with and without subcutaneous losartan or embusartan (lower panel).
Values are mean±SEM (n=6 or 10 per group).
*$p<0.05$ vs treatment groups, #$p<0.05$ vs aCSF response.

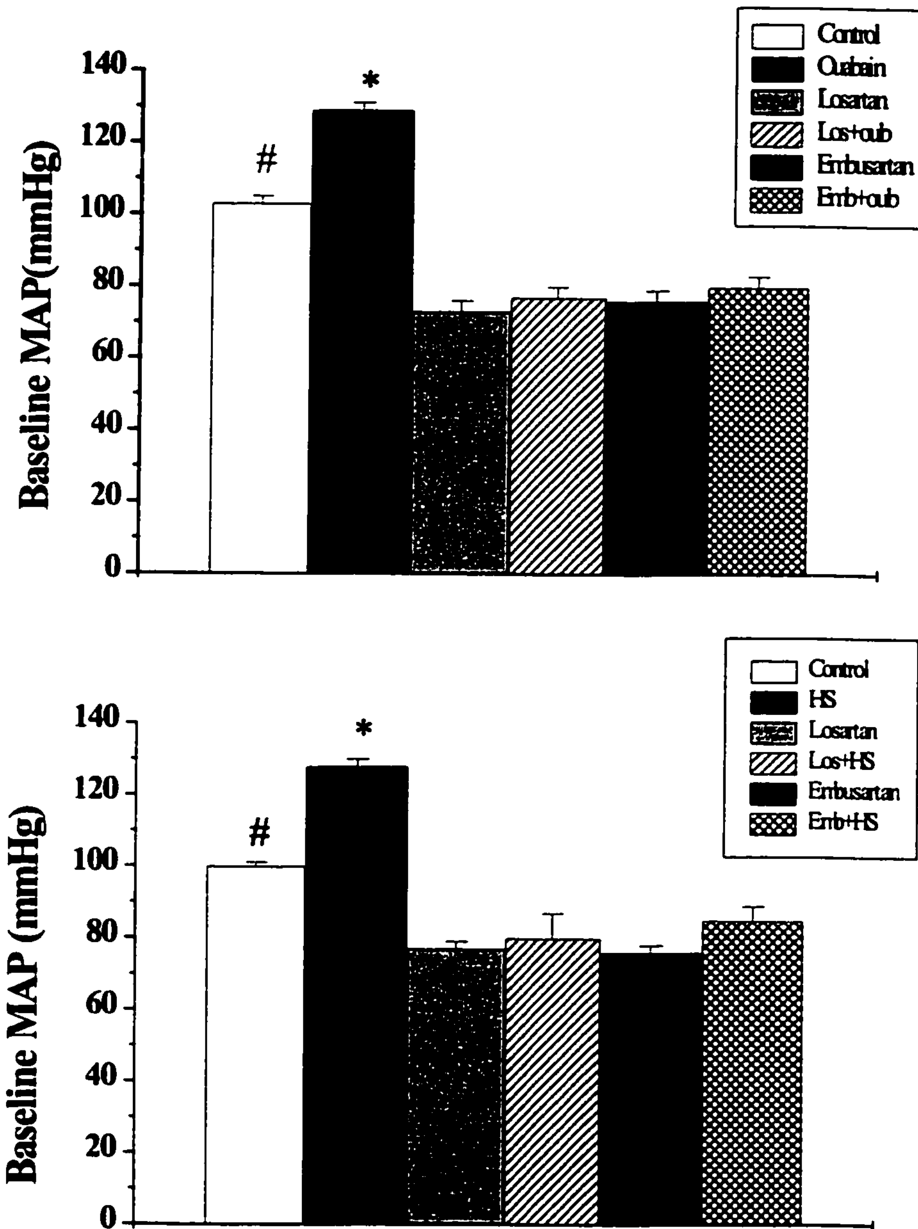

Figure 7 **Baseline MAP in rats with chronic ouabain or chronic icv 1.2 M Na+-rich aCSF treatment with and with out losartan or embusartan**

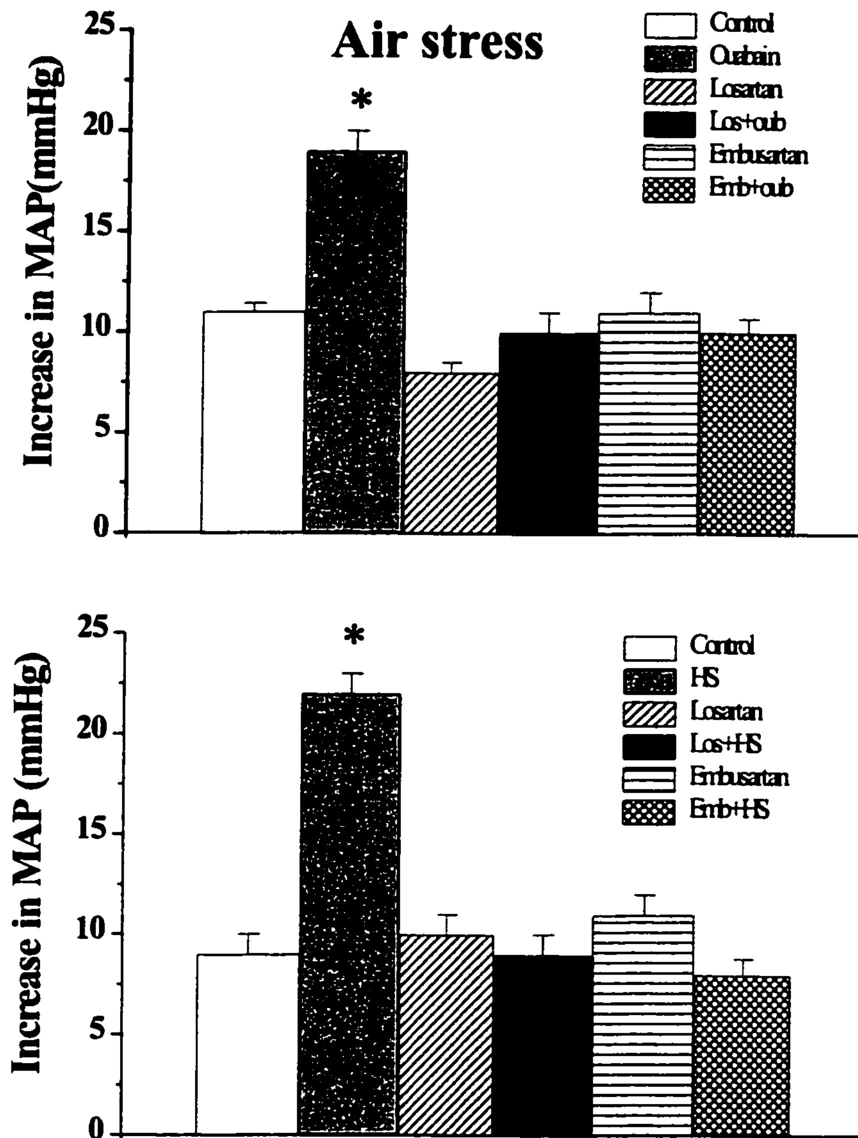

Figure 8 Peak increases in MAP in response to air stress in rats with chronic ouabain or chronic icv 1.2 M Na^{+}-rich aCSF treatment with and without losartan or embusartan

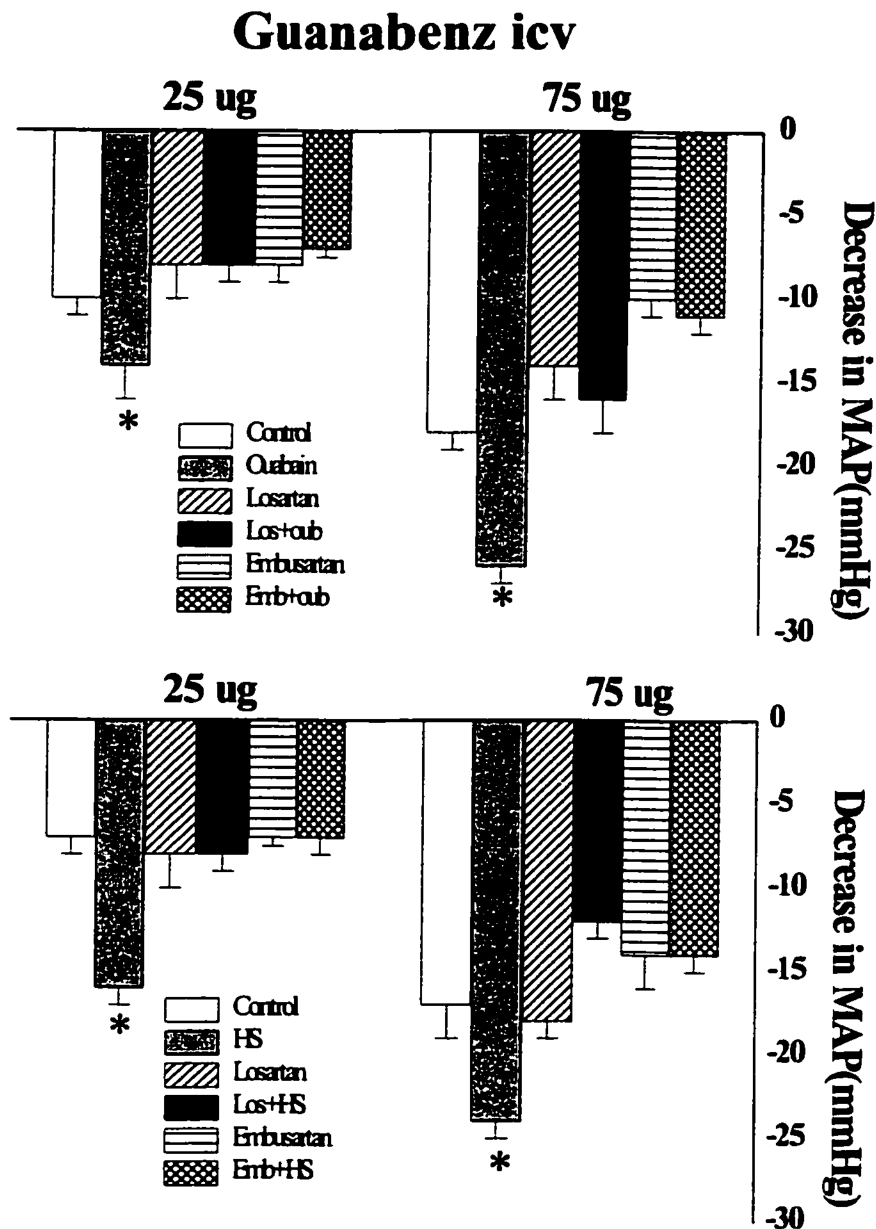

Figure 9 Maximal decrease in MAP in response to icv injection of guanabenz in rats with chronic ouabain or chronic icv 1.2 M Na^+-rich aCSF treatment with and without losartan or embusartan

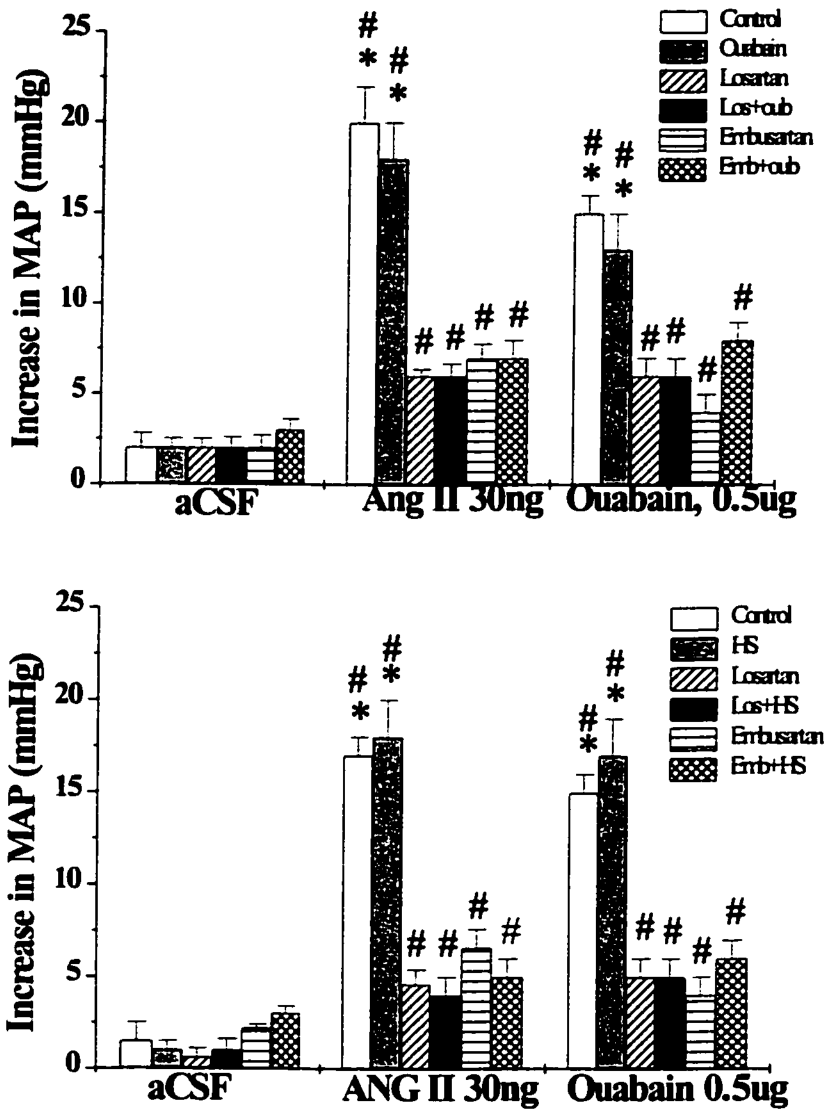

Figure 10 Peak increase in MAP in response to icv injection of Ang II and ouabain in rats with chronic ouabain or chronic icv 1.2 M Na^+-rich aCSF treatment with and without losartan or embusartan

Discussion

The present study provides several new findings regarding central effects of peripherally administered AT_1-receptor blockers. The study demonstrates that after one single sc dose, losartan inhibits the acute pressor responses induced by icv Ang II and Na+-rich aCSF substantially more than embusartan. But after 6 days sc treatment, losartan and embusartan similarly prevent the acute pressor responses induced by icv Ang II and Na+ -rich a CSF. These results suggest that losartan crosses the blood-brain barrier easier than embusartan, and therefore may exert greater effects after acute treatment. However, during 6 days treatment, embusartan appears to gradually cross the blood-brain barrier and then exerts similar central effects as losartan. The chronic study demonstrates that in normotensive rats, sympathetic hyperactivity and increases in resting BP caused by chronic sc infusion of ouabain or icv infusion of hypertonic saline can be fully prevented by daily sc injections of losartan or embusartan.

1 Central effects of centrally administered AT_1-receptor blockers

In the present study, icv injection of losartan or embusartan totally blocked the MAP increase induced by icv infusion of 0.3 M NaCl in aCSF. These data are consistent with results from previous studies (Huang et al. 1996a), and demonstrate that losartan and embusartan at 30 μg icv have the same effects in the central nervous system. The pressor responses to centrally administered Ang II may be attributed to an increase in sympathetic neuronal activity and release of arginine vasopressin (Huang et al. 1996a). Combined peripheral adrenoceptor and V1 receptor blockade completely prevent the

pressor response to central Ang II (Unger et al. 1981) confirming a role for both sympathetic activation and vasopressin release in the pressor response to icv Ang II. Icv infusion of Na^+-rich aCSF increases MAP, HR, and RSNA in conscious rats. Icv pretreatment with the AT_1-receptor blocker losartan abolishes the sympathoexcitatory and pressor responses to hypertonic saline (Huang et al. 1996a), indicating that these responses depend on central AT_1 receptor stimulation.

2 Central effects of peripherally administered AT_1-receptor blockers

Compounds with polar groups are hydrophilic substances and are easier to dissolve in polar solution. They are water soluble and more difficult to cross the BBB. Compounds with non-polar groups are lipophilic and easier to dissolve in non-polar solution. They are oil soluble and in general easily cross the blood-brain barrier (Grant et al. 1998). Losartan easily dissolves in water, whereas embusartan is a rather lipophilic AT_1 -receptor blocker (Stasch et al. 1997). Moreover, losartan generates an active metabolite EXP 3174, which appears rapidly in plasma after systemic losartan treatment (Casjka et al. 1997), has a high affinity for AT_1 receptors and readily crosses the blood-brain barrier (Polidori et al. 1996). E3174 binds to the AT_1 receptor with 10-fold greater affinity than its parent compound and it is about 15 to 20 times more potent in inhibiting Ang II-induced pressor response (Goa et al. 1996). In the present study, losartan at single doses of 30 or 100mg/kg sc markedly, and in a dose-related manner inhibited the MAP increases induced by icv Ang II and hypertonic saline. In contrast, embusartan at 30mg and 100mg/kg sc did not inhibit the BP increase induced by icv hypertonic saline,

and inhibited the MAP increase induced by icv Ang II only to a minor extent. In contrast, MAP and HR responses to iv Ang II at high doses were totally blocked by both losartan and embusartan sc 100mg/kg, indicating that the sc doses of losartan and embusartan were equivalent in blocking the peripheral effects of iv Ang II. These results suggest that sc losartan crosses the blood-brain barrier more easily than sc embusartan, and therefore exerts greater central effects, at least after one dose. These results are consistent with the characteristics of these two drugs.

In contrast to single doses, after more chronic treatment losartan as well as embusartan significantly and similarly inhibited the BP increases induced by icv angiotensin II and by icv hypertonic saline. These results suggest that during more chronic treatment, embusartan gradually crosses the blood-brain barrier and accumulates in the CNS, and then can induce the same central effects as losartan. From single dose studies, previous studies concluded that the degree of central angiotensin AT_1 receptor blockade following peripheral application may vary between different representatives of this class of drugs (Culman et al. 1999) and that such a difference may have therapeutic implications. The present study clearly demonstrates that such extrapolations from single dose studies to chronic treatment are not appropriate. An equilibrium between the blood-brain-CSF compartments will be first reached for molecules showing rapid penetration across the barriers and may take a long time for slowly penetrating substances. After a single dose losartan and embusartan indeed show clear differences in degrees of central AT_1-receptor blockade. However, within 6 days of treatment this difference has completely disappeared. Responses to both exogenous Ang II (i.e. icv Ang II) and endogenously released Ang II (i.e. by icv hypertonic saline) become blocked to a similar

degree. These findings highlight the importance of chronic dosing if therapeutic relevance is a major reason for studying central effects after peripheral administration.

3 Comparing effects of losartan and embusartan administered peripherally and centrally

In functional studies by Culman (1999), losartan iv at doses of 3, 10, 30 and 100 mg/kg, attenuated the pressor responses to icv Ang II by 7%, 21%, 24% and 63%. Li et al (1993) reported that losartan 3 mg/kg iv inhibited Ang II-induced increases in spontaneous firing rate of PVN neurons by 89% within 5 minutes. Losartan at the dose of 30 mg/kg i.p. inhibited Ang II icv induced drinking responses by 75% and 79% at 15 and 30 min after Ang II icv injection (Pilidori et al. 1996). Similarly losartan 3 or 10 mg/kg sc reduced by 16% or 33% the increase sodium excretion in response to icv renin and blocked the antidiuretic action induced by icv renin (Barbella et al. 1993).

In the present study, losartan and embusartan administered peripherally up to 100 mg/kg also only partially blocked the MAP increases induced by icv hypertonic saline and Ang II, whereas losartan or embusartan at 30 μg icv fully blocked the pressor responses. The latter finding clearly indicates that the pressor response to icv hypertonic saline depends critically on central AT_1-receptor stimulation (Huang et al. 1996). Several factors may explain these contrasting observations. Firstly, the peripheral doses may not have been large enough to provide effective brain concentrations. This appears unlikely since during chronic treatment 30 and 100 mg/kg/day caused equivalent inhibition. The bioavailability of losartan potassium is about 33%. Losartan is metabolized via hepatic carboxylation to the active metabolite E3174. The time after oral administration to

achieve peak plasma concentration is about 1 hour for losartan potassium and 3 to 4 hours for E3174 (Goa et al. 1996. Intravenous Ang II caused intense Fos immunoreactivity mainly in the SFO and OVLT. By contrast, icv Ang II caused intense Fos immunoreactivity predominantly in the median preoptic nucleus and juxtaventricular neurons of the SFO and OVLT (McKinley et al. 1995). These results suggest that iv Ang II induces endocrine responses by direct actions on the SFO and OVLT, whereas icv Ang II directly stimulates neurons in the median preoptic nucleus as well as neurons in the SFO and OVLT. Pressor response to icv hypertonic saline is mediated by an angiotensinergic mechanism in SFO (McKinley et al. 1993). It has been suggested that this response may be mediated via an angiotensinergic pathway from the SFO to the paraventricular nucleus, with likely synapses in the ventral MnPO, and the OVLT plays a more limited role (Rohmeiss et al. 1995). Maybe these brain regions are accessible to icv administered losartan or embusartan, but to a less extent to peripherally administered blocker.

4 Effects of losartan or embusartan administered peripherally on chronic ouabain induced hypertension

Huang et al (1994) suggested that in normotensive rats exogenous ouabain might act centrally to cause sympathoexcitation and thus hypertension. Electrolytic lesions limited to the ventral part of AV3V involving the OVLT and ventral MnPO fully prevent the hypertension induced by chronic sc administration of ouabain (Veerasingham et al, 1999). Peripheral mechanisms do not appear to play a significant role in the hypertension induced by sc ouabain, because central blockade of the effects of ouabain prevents

hypertension in this model (Huang et al, 1994; Veerasingham & Leenen, 1999). In rats, icv pretreatment with the Ang II receptor blocker saralasin (Takahashi et al. 1984), or the AT_1-receptor blocker losartan (Huang et al. 1996b) blocks sympathoexcitatory and pressor responses to acute icv ouabain. These studies suggest that activation of brain AT_1-receptors occur in the pathways mediating the effects of acute icv ouabain. Chronic administration of ouabain leads to increased activity in sympathoexcitatory pathways, decreased activity in sympathoinhibitory pathways and the development of hypertension. All these responses can also be prevented by concomitant icv treatment with losartan (Huang et al. 1999).

In the present study, chronic sc administration of losartan or embusartan fully blocked all effects of chronic treatment with ouabain, but only partially blocked the central effects of acute icv injection of ouabain. This difference in extent of blockade may be due to the fact that the concentration of ouabain obtained chronically would likely be less than the concentration achieved acutely, as a ~15-fold lower rate was administered chronically. Lesions of the ventral anteroventral third ventricle (vAV3V) also only partially inhibited pressor response to acute icv Na^+-rich aCSF and ouabain, but fully prevented the increase in MAP elicited by chronic administration of ouabain (Veerasingham et al. 1997, 1999). Brain areas where brain ouabain and brain RAS may interact have not yet been defined. In rat brain, nerve fibers of ouabain –immunopositive neurons, and Ang II receptors or other components of the brain RAS co-exist in several hypothalamic areas such as the anteroventral third ventricle, including the OVLT and SFO (Yamada et al. 1987, 1992). In wistar rats, the pressor response to acute icv ouabain is attenuated by losartan in MnPO (Budzikowski et al 1997). Presumaly higher

concentrations of ouabain also act on nuclei, which are different than nuclei that are most sensitive to low, more "physiologic" concentrations of ouabain.

5 Chronic icv hypertonic saline and losartan or embusartan administered peripherally

In conscious rats acute icv hypertonic saline causes sympathoexcitatory and pressor effects, which can be prevented by icv pretreatment with Fab fragments blocking brain "ouabain" or central losartan (Huang & Leenen, 1996b). Chronic sodium loading causes enhanced sympathoexcitation, impairment of baroreflexes and hypertension, which can be prevented by concomitant icv Fab fragments or losartan (Huang et al. 1998b). These findings suggest that central pathways involving both "ouabain" and Ang II mediate the effects of chronic central sodium loading. vAV3V lesions also abolish the increase in MAP elicited by chronic infusion of hypertonic saline (Veerasingham et al, 1999). It appears that the OVLT and ventral MnPO play an important role in mediating pressor effects of chronic infusion of hypertonic saline. AT_1-receptors are also present in the anteroventral third ventricle (AV3V) region, including the OVLT, MnPO and subfornical organ (SFO) (Mendelsohn et al. 1984), and these may be involved in the sympatho-excitatory and pressor responses to sodium. The OVLT is one of the circumventricular organs, which is outside the blood-brain barrier. Access to the OVLT should be similar for a lipophilic and hydrophilic AT_1-receptor blocker. A similar blockade can therefore be expected, assuming that the relevant AT_1-receptors are located in this area. Indeed, the present results demonstrate that chronic sc treatment with losartan or embusartan at 100mg/kg/day fully prevents the central effects of chronic

ouabain and hypertonic saline. These findings suggest that peripheral administration of AT_1-receptor blockers, either lipophilic or hydrophilic, at higher doses can induce the same central effects, particularly if the relevant nuclei are located outside the BBB.

6 Acute vs chronic stimulation and effects of peripheral losartan or embusartan

sc administration of losartan or embusartan one dose or for 6 days only partially blocked the central effects of icv Ang II or hypertonic saline for 10 min. Chronic sc administration of losartan or embusartan fully blocked all effects of chronic treatment with ouabain or hypertonic saline. Lesions of the ventral anteroventral third ventricle (vAV3V) also only partially inhibited pressor responses to acute icv sodium and ouabain, but fully prevented the increase in MAP elicited by chronic infusion of hypertonic saline or administration of ouabain (Veerasingham et al. 1997, 1999). Amounts administered may contribute to this difference. Firstly, in the acute study, 0.3 M hypertonic saline was infused at 3.8 µl/min. In the chronic study, 1.2 M hypertonic saline was infused at 5 µl/h. The resulting CSF concentration of sodium in the acute study is likely therefore substantially higher than that in the chronic studies. Acute responses to icv hypertonic saline involve pathways from the SFO to PVN, with likely synapses in the ventral MnPO (Rohmeiss et al, 1995) and the OVLT plays a more limited role. In contrast, the OVLT appears to play a more important role in mediating pressor effects of chronic infusion of hypertonic saline (Veerasingham et al. 1997, 1999). Thus, higher CSF concentrations of sodium may mainly involve pathways from the SFO to PVN, whereas lower CSF concentrations of sodium may only involve OVLT. Secondly, the AT_1-receptors

contributing to the pressor responses to acute icv infusion of hypertonic saline may be located in areas both inside and outside the blood-brain barrier and therefore may not be fully accessible to circulating AT_1-receptor blockers. In contrast, AT_1-receptors contributing to the pressor responses to chronic icv infusion of hypertonic saline may be mainly located in the OVLT and therefore fully accessible to circulating AT_1-receptor blockers.

7 Summary

In the one-dose experiments, there were no significant differences in resting MAP and HR among the groups of rats. In the six-day experiments, the resting MAP was significantly decreased in rats treated with losartan and embusartan at 30mg/kg and 100mg/kg. The resting HR was significantly increased by 40-50 bpm in rats treated with losartan and embusartan at 100mg/kg.

Losartan significantly inhibited pressor responses to icv Ang II. One dose of embusartan also inhibited pressor responses to icv Ang II. This effect was dose-related with greater inhibition by 100 vs 30mg/kg sc. In contrast to single doses, treatment with losartan and embusartan for six days resulted in similar inhibition of the pressor responses to icv Ang II.

The increase in MAP to icv infusion of 0.3 M NaCl was significantly attenuated by losartan 30mg/kg and further by losartan 100mg/kg. In contrast, the MAP response was only decreased to a minor extent by embusartan 30mg/kg or 100mg/kg. Six days treatment with losartan as well as embusartan significantly attenuated the increase in MAP. The extent of this blockade did not differ for the two doses and part of the response persisted.

MAP was significantly increased and HR was significantly decreased by iv injection of Ang II 30, 100, or 300ng. MAP and HR responses to iv Ang II were totally blocked by a single sc. injection of losartan or embusartan 100mg/kg. Similarly, MAP and HR responses to Ang II iv were totally blocked by sc. losartan or embusartan at doses of 30 and 100mg/kg/day for six days.

After sc ouabain treatment for 2 weeks or icv infusion of Na^+-rich aCSF for 2 weeks, resting MAP was significantly increased as compared to the control group. Both losartan and embusartan decreased baseline blood pressure and partially prevented these increases.

Air stress caused rapid increases in MAP. Losartan and embusartan alone did not significantly affect these responses. In rats treated with ouabain or icv Na+-rich aCSF, peak increases in MAP by airstress were approximately twice those in control rats. These enhanced responses did not develop when losartan or embusartan was administered sc. After icv administration of guanabenz at either dose, MAP decreases were dose-related. In rats treated with ouabain or icv Na+-rich aCSF, maximum decreases in MAP by guanabenz were twice those in control rats. In rats receiving ouabain or icv Na+-rich aCSF concomitant with losartan or embusartan, peak decreases were similar to those in control rats.

In the control, ouabain or icv Na^+-rich aCSF groups, icv injection of Ang II or ouabain significantly increased MAP. Sc losartan or embusartan only attenuated these increases in MAP.

8 Conclusion

After one single sc dose, losartan inhibits the pressor responses induced by icv Ang II and Na^+-rich aCSF substantially better than embusartan, but after six days sc treatment, losartan and embusartan exert similar central effects. These results suggest that losartan crosses the blood-brain barrier easier than embusartan, and therefore may exert greater effects after acute treatment. However, during more chronic treatment, embusartan appears to gradually cross the blood-brain barrier and then exerts similar central effects as losartan.

In normotensive rats, chronic sc administration of losartan or embusartan only partially blocked the central effects of acute icv injection of Ang II and ouabain, but fully blocked all effects of chronic treatment with ouabain or hypertonic saline. These results suggest that the AT_1-receptors contributing to the pressor responses to acute icv infusion of hypertonic saline may be located in areas both inside and outside the BBB and therefore may not be fully accessible to circulating AT_1-receptor blockers. However, AT_1-receptors contributing to the pressor responses to chronic icv infusion of hypertonic saline may be mainly located in the OVLT and therefore fully accessible to circulating AT_1-receptor blockers.

References

Abbott NJ, Revest PA. Control of brain endothelial permeability. Cerebrovasc Brain Metab Rev. 1993;(1):39-72. Review.

Allen AM, Moeller I, Jenkins TA, Zhuo J, Aldred GP, Chai SY, Mendelsohn FA. Angiotensin receptors in the nervous system. Brain Res Bull. 1998; 1;47(1):17-28. Review.

Allen AM, Zhuo J, Mendelsohn FA. Localization of angiotensin AT_1 and AT_2 receptors. J Am Soc Nephrol. 1999;10 Suppl 11:S23-9. Review.

Allen AM, Zhuo J, Mendelsohn FA. Localization and function of angiotensin AT_1 receptors. Am J Hypertens. 2000;13(1 Pt 2):31S-38S. Review.

Barbella Y, Cierco M, Israel A. Effect of Losartan, a nonpeptide angiotensin II receptor antagonist, on drinking behavior and renal actions of centrally administered renin. Proc Soc Exp Biol Med 1993, 202(4):401-6

Blaustein MP. Endogenous ouabain: role in the pathogenesis of hypertension. Kidney Int. 1996;49(6):1748-53. Review.

Bui JD, Kimura B, Phillips MI. Losartan potassium, a nonpeptide antagonist of angiotensin II, chronically administered p.o. does not readily cross the blood-brain barrier. Eur J Pharmacol. 1992;14;219(1):147-51.

Brightman M. Ultrastructure of brain endothelium. In: Bradbury, M. W. B. (ed.) Physiology and Pharmacology of the Blood-Brain Barrier. Handbook of Experimental Pharmacology 103, springer-Verlag, Berlin. 1992, pp1-22

Brosnihan KB, Berti GA, Ferrario CM. Hemodynamics of central infusion of angiotensin II in normal and sodium-depleted dogs.Am J Physiol. 1979;237(2):H139-45.

Budzikowski AS, Leenen FH. Brain 'ouabain' in the median preoptic nucleus (MnPO). Hypertension. 1997; 30:500 (Abstract).

Budzikowski AS, Leenen FH. Brain 'ouabain' in the median preoptic nucleus mediates sodium-sensitive hypertension in spontaneously hypertensive rats. Hypertension. 1997;29(2):599-605.

Buggy J, Johnson AK. Angiotensin-induced thirst: effects of third ventricle obstruction and periventricular ablation. Brain Res. 1978; 23;149(1):117-28.

Bunag RD, E. Miyajima. Baroreflex inpairment precedes hypertension during chronic cerebroventricular infusion of hypertonic sodium chloride in rats. J. Clin. Invest. 1984, 74:2065-2073

Bunnemann B, Fuxe K, Ganten D. The brain renin-angiotensin system: localization and general significance.J Cardiovasc Pharmacol. 1992;19 Suppl 6:S51-62.

Butt AM, Jones HC, Abbott NJ. Electrical resistance across the blood-brain barrier in anaesthetized rats: a developmental study. J Physiol (Lond). 1990;429:47-62.

Casto R, Phillips MI. Angiotensin II attenuates baroreflexes st muscleus tractus solitarius of rats. Am J Physiol 1986;250:R193-R198

Castren E, Saavedra JM. Angiotensin II receptors in paraventricular nucleus, subfornical organ, and pituitary gland of hypophysectomized, adrenalectomized, and vasopressin-deficient rats. Proc Natl Acad Sci U S A. 1989;86(2):725-9.

Csajka, C., Buclin, T., Brunner, H.R., Biollaz, J., Pharmacokinetic-pharmacodynamic profile of angiotensin II receptor antagonists. Clin. Pharmacokinet. 1997;32:1-29.

Chai SY, Mendelsohn FA, Paxinos G. Angiotensin converting enzyme in rat brain visualized by quantitative in vitro autoradiography.Neuroscience. 1987;20(2):615-2

Chai SY, McKinley MJ, Paxinos G, Mendelsohn FA. Angiotensin converting enzyme in the monkey (Macaca fascicularis) brain visualized by in vitro autoradiography. Neuroscience. 1991;42(2):483-95.

Chai SY, Johnston CI: Tissue distribution of angiotensin converting enzyme. In: Laragh JH, Brenner BM (editors). Hypertension: Pathophysiology, diagnosis and Management. New York: Raven Press; 1995, PP. 1683-1693

Crone C, Facilitated transfer of glucose from blood to brain tissue. J. Physiol. (Lond.) 1963; 181:103-113.

Cornford EM, Hyman S. Blood-brain barrier permeability to small and large molecules. Adv Drug Deliv Rev. 1999;5;36(2-3):145-163.

Culman J, von Heyer C, Piepenburg B, Rascher W, Unger T. Effects of systemic treatment with irbesartan and losartan on central responses to angiotensin II in conscious, normotensive rats. Eur J Pharmacol. 1999; 19;367(2-3):255-65.

Davson H and Segal MB (Eds.). The blood-brain barrier. In: Physiology of the CSF and of the Blood-Brain Barrier. New York: CRC, 1996,PP 49-91

Deschepper CF, Mellon SH, Cumin F, Baxter JD, Ganong WF Analysis by immunocytochemistry and in situ hybridization of renin and its mRNA in kidney, testis, adrenal, and pituitary of the rat.Proc Natl Acad Sci U S A. 1986;83(19):7552-6

Doris PA. Angiotensin II immunoreactivity in push-pull perfusates from the paraventricular nucleus.Peptides. 1988;9(3):481-5.

Doursout MF, Chelly JE, Liang YY, Buckley JP. The ouabain-dependent Na(+)-K+ pump and the brain renin-angiotensin system. Clin Exp Hypertens A. 1992;14(3):393-411.

Dzau VJ, Ingelfinger J, Pratt RE, Ellison KE. Identification of renin and angiotensinogen messenger RNA sequences in mouse and rat brains.Hypertension. 1986;8(6):544-8.

Dzau VJ, Mukoyama M, Pratt RE. Molecular biology of angiotensin receptors: target for drug researchJ Hypertens Suppl. 1994;12(2):S1-5. Review.

Fink GD, Bryan WJ. Forebrain control of fluid and electrolyte homeostasis and angiotensin sensitivity in rabbit.Am J Physiol. 1980;239(5):R372-6.

Ferguson AV. Systemic angiotensin acts at the subfornical organ to control the activity of paraventricular nucleus neurons with identified projections to the median eminence. Neuroendocrinology. 1988;47(6):489-97.

Fregly MJ, Rowland NE. Do peripheral and cerebroventricular injections of angiotensin II act at the same site? Studies on additivity of drinking.Brain Res Bull. 1986;16(2):249-57.

Ganten D, Minnich JL, Granger P, Hayduk K, Brecht HM, Barbeau A, Boucher R, Genest J. Angiotensin-forming enzyme in brain tissue. Science. 1971 2;173(991):64-5.

Ganten D, Fuxe K, Ganten U, Hokfelt T, Bolme P.The brain isorenin-angiotensin system: localization and biological function. Prog Brain Res. 1977;47:155-9.

Genain CP, Van Loon GR, Kotchen TA. Distribution of renin activity and angiotensinogen in rat brain. Effects of dietary sodium chloride intake on brain renin. J Clin Invest. 1985;76(5):1939-45.

Ghiani P, Uva B, Vallarino M, Mandich A, Masini MA. Angiotensin II specific receptors in subcommissural organ. Neurosci Lett 1988; 85: 212-216.

Goa KL, Wagstaff AJ. Losartan potassium: a review of its pharmacology, clinical efficacy and tolerability in the management of hypertension. Drugs. 1996; 51(5):820-45.

Goldstein GW, Betz AL. The blood-brain barrier.Sci Am. 1986;255(3):74-83.

Ganten D, Lang RE, Lehmann E, Unger T. Brain angiotensin: on the way to becoming a well-studied neuropeptide system. Biochem Pharmacol. 1984;33(22):3523-8.

Goldstein GW, Betz AL, Bowman PD, Dorovini-Zis K. In vitro studies of the blood-brain barrier using isolated brain capillaries and cultured endothelial cells. Ann N Y Acad Sci. 1986;481:202-13.

Grant GA, Abbott NJ, Janigro D. Understanding the physiology of the blood-brain barrier: in vitro models. News Physiology Science. 1998,13: 287-293

Greig, N. H. (1989). Drug delivery to the brain by blood-brain barrier circumvention and drug modification. In Neuwelt E. A. (ed.), Implications of the blood-Brain Barrier and Its Modification, vol. 1, Basic Science Studies, Plenum, New York, pp. 179-185

Gross PM, Kadekaro DW, Adrews DW, Sokoloff L, Saavedra JM, Selective metabolic stimulation of the subfornical organ and pituitary neural lobe by peripheral angiotensin II. Peptides 6 1985 [Suppl 1]:145-152.

Gutman MB, Cirello J, Morgenson GJ. The effect of paraventricular nucleus lesions on cardiovascular responses elicited by stimulation of the subfornical organ in the rat. Can J Physiol Pharmacol 1985;63:816-824

Hartle DK, Brody MJ. The angiotensin II pressor system of the rat forebrain.

Circ Res. 1984;54(4):355-66.

Healy DP, Printz MP. Distribution of immunoreactive angiotensin II, angiotensin I, angiotensinogen and renin in the central nervous system of intact and nephrectomized rats.Hypertension. 1984;6(2 Pt 2):I130-6.

Hoffman WE, Phillips MI. Regional study of cerebral ventricle sensitive sites to angiotensin II.Brain Res. 1976 9;110(2):313-30.

Horiuchi M, Akishita M, Dzau VJ. Recent progress in angiotensin II type 2 receptor research in the cardiovascular system.Hypertension. 1999;33(2):613-21. Review.

Huang BS, Harmsen E, Yu H, Leenen FH.Brain ouabain-like activity and the sympathoexcitatory and pressor effects of central sodium in rats. Circ Res. 1992;71(5):1059-66.

Huang BS, Huang X, Harmsen E, Leenen FH. Chronic central versus peripheral ouabain, blood pressure, and sympathetic activity in rats. Hypertension. 1994;23(6 Pt 2):1087-90.

Huang BS, Leenen FHH. Sympathoexcitatory and pressor responses to increased brain sodium and ouabain are mediated via brain ANG II. Am J Physiol 1996(a), 270(1 Pt 2):H275-80.

Huang, B.S., Leenen, F.H.H., Brain "ouabain" and angiotensin II in salt-sensitive hypertension in spontaneously hypertensive rats. Hypertension 1996(b), 28:1005-12.

Huang, B.S., Leenen, F.H.H., Both brain angiotensin II and "ouabain" contribute to sympathoexcitation and hypertension in Dahl S rats on high salt intake. Hypertension 1998a 32:1028-33.

Huang BS, Veerasingham SJ, and Leenen FHH. Brain "ouabain", ANG II and sympathoexcitation by chronic central sodium loading in rats. Am. J. Physiol.1998b 274 (Heart Circ. Physiol. 43): H1269-H1276.

Huang BS, Leenen FH. Brain renin-angiotensin system and ouabain-induced sympathetic hyperactivity and hypertension in Wistar rats. Hypertension. 1999;34(1):107-12.

Ihara N, Yuri K, Yamada H, Sano Y. Immunohistochemical studies on the distribution of endogenous digitalis-like substance (EDLS)-containing neurons in the rat hypothalamus, with special consideration on the possibility of their coexistence with posterior lobe hormones. Arch Histol Cytol. 1988;51(1):35-42.

Inagami T, Clemens DL, Celio MR, Brown A, Sandru L, Herschkowitz N, Hoffman LH, Kasselberg AG. Immunohistochemical localization of renin in mouse brain. Neurosci Lett. 1980 15;18(1):91-8.

Inui K, Nomura J, Murase S, Nosaka S. Fasilitation of the arterial baroreflex by the preoptic area in anesthetized rats. J Physiol 1995;488 (pt2);521-531

Jones DL, Lo S. Ouabain injected into the hypothalamus elicits pressor responses in anaesthetized rats: a mapping study. Pharmacol Biochem Behav. 1990;36(4):979-83.

Kakar SS, Riel KK, Neill JD. Differential expression of angiotensin II receptor subtype mRNAs (AT-1A and AT-1B) in the brain. Biochem Biophys Res Commun. 1992 15;185(2):688-92

Kawano Y, Barnes KL, Ferrario CM. Pressor effect of centrally administered sodium chloride: role of the ventral third ventricle region and the area postrema. J Auton Nerv Syst. 1991;35(2):153-60.

Kitagawa H, Takeda F, Kohei H. Inhibition of sympathetic nervous system by mexiletine, an antiarrhythmic agent, and its antagonism against ouabain. Arzneimittelforschung. 1985;35(1A):356-9.

Knowles WD, Phillips MI. angiotensin II responsive cells in the organ vasculosum lamina terminalis (OVLT) recorded in the hypothalamic brain slices. Brain Res 1980 197:256-259

Koepke JP, Jones S, DiBona GF. Sodium responsiveness of central alpha 2-adrenergic receptors in spontaneously hypertensive rats. Hypertension 1988;11: 326-33.

Landas S, Phillips MI, Stamler JF, Raizada MK. Visualization of specific angiotensin II binding sites in the brain by fluorescent microscopy. Science. 1980; 210(4471): 791-3.

Lautier D, Canitrot Y, Deeley RG, Cole SP. Multidrug resistance mediated by the multidrug resistance protein (MRP) gene. Biochem Pharmacol. 1996;52(7):967-77. Review.

Lenkei Z, Corvol P, Llorens-Cortes C. The angiotensin receptor subtype AT1A predominates in rat forebrain areas involved in blood pressure, body fluid homeostasis and neuroendocrine control. Brain Res Mol Brain Res. 1995;30(1):53-60.

Lenkei Z, Palkovits M, Corvol P, Llorens-Cortes C. Expression of angiotensin type-1 (AT_1) and type-2 (AT_2) receptor mRNAs in the adult rat brain: a functional neuroanatomical review. Front Neuroendocrinol. 1997;18(4):383-439.

Li Z, Bains JS, Ferguson AV. Functional evidence that the angiotensin antagonist losartan crosses the blood-brain barrier in the rat. Brain Res Bull. 1993;30(1-2):33-9.

Lind RW, Swanson LW, Ganten D. Angiotensin II immunoreactivity in the neural afferents and efferents of the subfornical organ of the rat. Brain Res. 1984;321(2):209-15.

Lin KS, Chan JY, Chan SH. Involvement of AT_2 receptors at NRVL in tonic baroreflex suppression by endogenous angiotensins. Am J Physiol. 1997;272(5 Pt 2):H2204-10.

Manunta P, Rogowski AC, Hamilton BP, Hamlyn JM. Ouabain-induced hypertension in the rat: relationships among plasma and tissue ouabain and blood pressure. J Hypertens. 1994;12(5):549-60.

Marshall FH, Clark SA, Michel AD, Barnes JC. Binding of angiotensin antagonists to rat liver and brain membranes measured ex vivo.Br J Pharmacol. 1993;109(3):760-4.

Mendelsohn FA, Chai SY, Dunbar M.In vitro autoradiographic localization of angiotensin-converting enzyme in rat brain using125I-labelled MK351A. J Hypertens Suppl. 1984(a) Dec;2(3):S41-4

McKinley, M.J., Badoer, E., Vivas, L., Oldfield, B.J., Comparison of c-*fos* expression in the lamina terminalis of conscious rats after intravenous or intracerebroventricular angiotensin. Brain Res. Bull.1993;37:131-7.

McKinley, M.J., McAllen, R.M., Pennington, G.L., Smardencas, A., Weisinger, R.S., Oldfield, B.J., Physiological actions of angiotensin II mediated by AT_1 and AT_2 receptors in the brain. Clin. Exp. Pharmacol. Physiol. Suppl 1996 3:S99-104.

Mendelsohn FA, Quirion R, Saavedra JM, Aguilera G, Catt KJ. Autoradiographic localization of angiotensin II receptors in rat brain.Proc Natl Acad Sci U S A. 1984(b);81(5):1575-9.

Moffett RB, Bumpus FM, Husain A. Cellular organization of the brain renin-angiotensin system. Life Sci. 1987;41(16):1867-79. Review.

Muratani H, Teruya H, Sesoko S, Takishita S, Fukiyama K. Brain angiotensin and circulatory control.Clin Exp Pharmacol Physiol. 1996;23(6-7):458-64. Review.

Nazarali AJ, Gutkind JS, Correa FM, Saavedra JM. Decreased angiotensin II receptors in subfornical organ of spontaneously hypertensive rats after chronic antihypertensive treatment with enalapri. Am J Hypertens 1990;3:59-61

Okuya S, Inenaga K, Kaneko T, Yamashita H Angiotensin II sensitive neurons in the supraoptic nucleus, subfornical organ and anteroventral third ventricle of rats in vitro. Brain Res. 1987;402(1):58-67.

Obermuller N, Unger T, Culman J, Gohlke P, de Gasparo M, Bottari SP. Distribution of angiotensin II receptor subtypes in rat brain nuclei. Neurosci Lett. 1991;132(1):11-5

Oldendorf WH. Measurement of brain uptake of radiolabeled substances using a tritiated water internal standard. Brain Res. 1970 Dec 1;24(2):372-6.

Paul M, Printz MP, Harms E, Unger T, Lang RE, Ganten D. Localization of renin (EC 3.4.23) and converting enzyme (EC 3.4.15.1) in nerve endings of rat brain. Brain Res. 1985 20;334(2):315-24.

Paul M, Wagner D, Metzger R, Ganten D, Lang RE, Suzuki F, Murakami K, Burbach JH, Ludwig G. Quantification of renin mRNA in various mouse tissues by a novel solution hybridization assay. J Hypertens. 1988;6(3):247-52

Phillips MI, Felix D. Specific angiotensin II receptive neurons in the cat subfornical organ.Brain Res. 1976 18;109(3):531-40.

Phillips MI. Functions of angiotensin in the central nervous system. Annu Rev Physiol. 1987;49:413-35. Review.

Phillips MI, Sumners C. Angiotensin II in central nervous system physiology.
Regul Pept. 1998 Nov 30;78(1-3):1-11. Review.

Plunkett LM, Shigematsu K, Kurihara M, Saavedra JM. Localization of angiotensin II receptors along the anteroventral third ventricle area of the rat brain. Brain Res. 1987 10;405(2):205-12.

Polidori, C., Ciccocioppo, R., Pompei, P., Cirillo, R., Massi, M., Functional evidence for the ability of angiotensin AT_1 receptor antagonists to cross the blood-brain barrier in rats. Eur. J. Pharmacol. 1996;307:259-267.

Rohmeiss P, Beyer C, Hocher B, Qadri F, Gretz N, Strauch M, Unger T. Osmotically induced natriuresis and blood pressure response involves angiotensin AT_1 receptors in the subfornical organ. J Hypertens. 1995;13(12 Pt 1):1399-404.

Saavedra JM, Correa FMA, Kurihara M, Shigematsu K. Increases number of angiotensin II receptors in the subfornical organ of spontaneously hypertensive rats. J Hypertens 1986;4: S27-S30.

Saavedra JM, Castren E, Gutkind JS, Nazarali AJ. Regulation of brain atrial natriuretic peptide and angiotensin receptors: quatitative autoradiographic studies. Int Rev Neurobiol 1989;31:257-296.

Sayer RJ, Hubbard JI, sirett NE. Rat organum vasculosum laminae terminalis in vitro: response to transmitters. Am J Physiol 1984;247:R374-R379.

Schinkel AH, Wagenaar E, Mol CA, van Deemter LP-glycoprotein in the blood-brain barrier of mice influences the brain penetration and pharmacological activity of many drugs. J Clin Invest. 1996 1;97(11):2517-24.

Schlosshauer B. The blood-brain barrier: morphology, molecules, and neurothelin. Bioessays. 1993;15(5):341-6. Review.

Schmitz U, Ishida M, Berk BC. Angiotensin II stimulates tyrosine phosphorylation of phospholipase C-gamma-associatedproteins. Characterization of a c-Src-dependent 97-kD protein in vascular smooth muscle cells. Circ Res. 1997;81(4):550-7.

Shigematsu K, Saavedra JM, Plunkett LM, Kurihara M, Correa FMA. Angiotensin II binding sites in the anteroventral-third ventrical (AV3V) area and related structures of the rat brain. Neurosci Lett 1986;67:37-41

Simpson JB. The circumventricular organs and the central actions of angiotensin. Neuroendocrinology 1981;32:248-256

Stasch, J.P., Knorr, A., Hirth-Dietrich, C., Krämer, T., Hübsch, W., Dressel, J., Fey, P., Beuck, M., Sander, E., Frobel, K., Kazda, S., Long-term blockade of the angiotensin II receptor in renin transgenic rats, salt-loaded Dahl rats, and stroke-prone spontaneously hypertensive rats. Arzneim.-Forsch/Drug Res. 47:1016-23, 1997.

Steckelings U, Lebrun C, Qadri F, Veltmar A, Unger T. Role of brain angiotensin in cardiovascular regulation. J Cardiovasc Pharmacol. 1992;19 Suppl 6:S72-9. Review.

Song KF, Zhuo JL, Mendelsohn FA. Access of peripherally administered DuP 753 to rat brain angiotensin II receptors. Br J Pharmacol. 1991;104(4):771-2.

Song K, Allen AM, Paxinos G, Mendelsohn FA. Mapping of angiotensin II receptor subtype heterogeneity in rat brain. J Comp Neurol. 1992 22;316(4):467-84.

Takahashi H, Iyoda I, Takeda K, Sasaki S, Okajima H, Yamasaki H, Yoshimura M Ijichi H. Centrally-induced vasopressore responses to sodium-potassium adenosine triphosphatase inhibitor, ouabain, may be mediated via angiotensin II in the anteroventral third ventricle in the brain. Jap Circ J 1984; 48: 1243-1250

Takahashi H, Matsuzawa M, Okabayashi H, Suga K, Ikegaki I, Yoshimura M, Ijichi H, Okamura H,Murakami S, Ibata Y. Evidence for a digitalis-like substance in the hypothalamopituitary axis in rats: implications in the central cardiovascular regulation associated with an excess intake of sodium. Jpn Circ J. 1987;51(10):1199-207.

Takahashi H, Matsusawa M, Suga K, Ikegaki I, Nishimura M, Yoshimura M, Ihara N, Yamada H, Sano Y. Hypothalamic digitalis-like substance is released with sodium-loading in rats. Am J Hypertens. 1988;1(2):146-51.

Tatsuta T, Naito M, Oh-hara T, Sugawara I, Tsuruo T. Functional involvement of P-glycoprotein in blood-brain barrier. J Biol Chem. 1992;267(28):20383-91.

Timmermans PB, Wong PC, Chiu AT, Herblin WF, Benfield P, Carini DJ, Lee RJ, Wexler RR, Saye JA, Smith RD. Angiotensin II receptors and angiotensin II receptor antagonists.Pharmacol Rev. 1993;45(2):205-51. Review.

Tsuda K, Tsuda S, Nishio I, Masuyama Y. Effects of ouabain on adrenergic neurotransmission in spontaneously hypertensive rats.Jpn Circ J. 1988;52(1):53-9.

Tsutsumi K, Saavedra JM. Characterization and development of angiotensin II receptor subtypes (AT_1 and AT_2) in rat brain. Am J Physiol. 1991;261(1 Pt 2):R209-16

Unger, T., Rascher, W., Schuster, C., Pavlovitch, R., Schömig, A., Dietz, R., Ganten, D., Central blood pressure effects of substance P and angiotensin II: role of the sympathetic nervous system and vasopressin. Eur. J. Pharmacol. 1981;71:33-42.

Van Houten M, Schiffrin EL, Mann JF, Posner BI, Boucher R. Radioautographic localization of specific binding sites for blood-borne angiotensin II in the rat brain. Brain Res. 1980 31;186(2):480-5.

Van Houten M, Mangiapane ML, Reid IA, Ganong WF. [Sar,Ala]angiotensin II in cerebrospinal fluid blocks the binding of blood-borne [125I]angiotensin II to the circumventricular organs.Neuroscience. 1983;10(4):1421-6.

Veerasingham SJ, Leenen FH. Excitotoxic lesions of the ventral anteroventral third ventricle and pressor responses to central sodium, ouabain and angiotensin II.
Brain Res. 1997 21;749(1):157-60.

Veerasingham SJ, Leenen FH. Ouabain- and central sodium-induced hypertension depend on the ventral anteroventral third ventricle region. Am J Physiol. 1999;276(1 Pt 2):H63-70.

Whiting P, Nava S, Mozley L, Eastham H, Poat J., Nucleotide Expression of angiotensin converting enzyme mRNA in rat brain. Brain Res Mol Brain Res. 1991;11(1):93-6.

Wong PC, Price WA Jr, Chiu AT, Duncia JV, Carini DJ, Wexler RR, Johnson AL, Timmermans PB. Nonpeptide angiotensin II receptor antagonists. XI. Pharmacology of EXP3174: an active metabolite of DuP 753, an orally active antihypertensive agent.
J Pharmacol Exp Ther. 1990(a);255(1):211-7

Wong, P.C., Price, W.A., Reilly, T.M., Duncia, J.V., Timmermans, P.B., Antihypertensive mechanism of captopril in renal hypertensive rats: studies with a nonpeptide angiotensin II receptor antagonist and an angiotensin II monoclonal antibody.
J. Pharmacol. Exp. Ther. 1989;250:515-22.

Wong PC, Price WA Jr, Chiu AT, Carini DJ, Duncia JV, Johnson AL, Wexler RR, Timmermans PB. Nonpeptide angiotensin II receptor antagonists. Studies with EXP9270 and DuP 753. Hypertension. 1990(b);15(6 Pt 2):823-34.

Wong PC, Hart SD, Chiu AT, Herblin WF, Carini DJ, Smith RD, Wexler RR, Timmermans PB. Pharmacology of DuP 532, a selective and noncompetitive AT_1 receptor antagonist. J Pharmacol Exp Ther. 1991 Nov;259(2):861-70.

Wright JW, Harding JW. Regulatory role of brain angiotensins in the control of physiological and behavioral responses.Brain Res Brain Res Rev. 1992;17(3):227-62. Review.

Wright JW, Bechtholt AJ, Chambers SL, Harding JW. Angiotensin III and IV activation of the brain AT_1 receptor subtype in cardiovascular function. Peptides. 1996;17(8):1365-71.

Wright JW, Harding JW. Important role for angiotensin III and IV in the brain renin-angiotensin system. Brain Res Brain Res Rev. 1997;25(1):96-124. Review.

Yamada H, Ihara N, Sano Y. Morphological evidence of endogenous digitalis-like substance (EDLS) in the rat and macaque hypothalamus,using digoxin-immunohistochemistry. Endocrinol Jpn. 1987;34(2):319-23.

Yamada H, Ihara N, Takahashi H, Yoshimura M, Sano Y. Distribution of the endogenous digitalis-like substance (EDLS)-containing neurons labeled by digoxin antibody in hypothalamus and three circumventricular organs of dog and macaque. Brain Res. 1992(a) 3;584(1-2):237-43.

Yamada H, Naruse M, Naruse K, Demura H, Takahashi H, Yoshimura M, Ochi J. Histological study on ouabain immunoreactivities in the mammalian hypothalamus. Neurosci Lett. 1992(b) 20;141(2):143-6.

Yamada K, Goto A, Nagoshi H, Hui C, Omata M. Role of brain ouabainlike compound in central nervous system-mediated natriuresis in rats. Hypertension 1994;23:1027-1031

Yuan CM, Manunta P. Hamlyn JM, Chen S, Bohen E, Yeun J, Haddy FJ, Pamnani MB. Long-term ouabain administration produces hypertension in rats. Hypertension 1993; 22:178-187

Zhang W, Huang BS, Leenen FHH. Brain renin-angiotensin system and sympathetic hyperactivity in rats after myocardial infarction. American Journal of Physiology 276: H10658-H1615, 1999

Zhuo J, Song K, Abderlrahman L, Mendelsohn FAO. Blockade by intravenous Losartan of AT_1 Angiotensin II receptors in rat brain, kidney and adrenals demonstrated by in vitro

autoradiography. Clinical and Experimental Pharmacology and Physiology. 1994; 21:557-567.

Zhuo J, Moeller I, Jenkins T, Chai SY, Allen AM, Ohishi M, Mendelsohn FA. Mapping tissue angiotensin-converting enzyme and angiotensin AT_1, AT_2 and AT_4 receptors. J Hypertens. 1998;16(12 Pt 2):2027-37. Review.