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**Salt-Induced Hypertension:
Central Regulation by
Ouabain-Like Compounds and Angiotensin II**

By

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**Submitted to the School of Graduate Studies
in Partial Fulfillment of the Requirements
for the Degree of
Doctor of Philosophy**

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AUTHORIZATION

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CONTRIBUTION OF COLLABORATORS

All studies were conducted under the supervision of Frans H.H. Leenen at the University of Ottawa Heart Institute. All experimental work presented in Chapters 2, 3 and 4 was carried out by Shereeni J. Veerasingham. The Manuscripts presented in these Chapters were written by S. J. Veerasingham.

Appendix: Experiments presented in Table 1, 2 and 3 were performed by Bing S. Huang (University of Ottawa Heart Institute). The experiments presented in Table 4 and Figure 1 were carried out by Faranak V. Ansari in collaboration with S. J. Veerasingham who performed all the surgery and blood pressure measurements, analyzed the data and wrote the manuscript.

ABSTRACT

We hypothesized that the ventral anteroventral third ventricle region (vAV3V), which mainly comprises the organum vasculosum laminae terminalis (OVLT) and extends ~100 μm into the median preoptic nucleus, mediates salt-induced sympathoexcitation and hypertension via AT_1 receptor activation. We utilized ibotenic acid lesions of the vAV3V to determine a role of neurons in this area in pressor responses to intracerebroventricular (icv) administration of hypertonic saline (0.3M NaCl, 2 $\mu\text{l}/\text{min}$ for 10 min), ouabain (0.3 and 0.6 μg) and angiotensin II (Ang II; 10 and 30 ng), and in two rat models of hypertension in which mechanisms of salt-sensitive hypertension are mimicked: chronic icv hypertonic saline (0.8 M NaCl, 5 $\mu\text{l}/\text{h}$ icv for 2 weeks) to increase CSF $[\text{Na}^+]$ and chronic subcutaneous (sc) ouabain (50 $\mu\text{g}/\text{d}$ sc for 3 weeks) to mimic increases in central ouabain-like compound content. ^{125}I -Sar¹Ile⁸-Ang II binding to AT_1 receptors was determined by *in vitro* receptor autoradiography to determine if changes in AT_1 receptor density accompany the development of salt-induced hypertension in Dahl salt-sensitive (S) rats. In conscious vAV3V lesioned Wistar rats with systemic vasopressin receptor blockade, pressor and tachycardic responses to 0.3 M NaCl and ouabain, were significantly attenuated by 26-32 % whereas responses to Ang II were unaffected. Resting mean arterial pressure (MAP) in sham-operated rats that received chronic hypertonic saline infusions or ouabain treatment was significantly higher (17-19 %) than in aCSF or placebo treated controls. vAV3V lesions abolished the chronic hypertonic saline or ouabain induced increases in MAP and the enhanced depressor responses to ganglionic blockade with hexamethonium. AT_1 binding in the OVLT, the supraoptic nucleus and the paraventricular nucleus did not differ between Dahl S and R rats on regular salt diet, and was decreased similarly on high salt diet.

Within the subfornical organ AT_1 receptor binding did not differ between Dahl S and R rats on regular salt diet and decreased in both strains on the high salt diet, but decreased more in hypertensive Dahl S versus the normotensive Dahl R rats (50 % versus 23 %). In conclusion, the vAV3V area appears to play a crucial role in mediating salt-induced hypertension, possibly via sympathetic activation, but autoradiography did not provide evidence for differential AT_1 stimulation in the vAV3V.

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LIST OF ABBREVIATIONS

ACE	angiotensin converting enzyme
aCSF	artificial cerebrospinal fluid
ACTH	adrenocorticotrophic hormone
Ang I	angiotensin I
Ang II, ANG II	angiotensin II
Ang III	angiotensin III
Ang IV	angiotensin IV
AP	area postrema
AVP	arginine vasopressin
AV3V	anteroventral third ventricle
CVO	circumventricular organs
Dahl R	Dahl salt-resistant
Dahl S	Dahl salt-sensitive
DMH	dorsomedial hypothalamus
DVC	dorsal vagal complex
FLI	Fos-like immunoreactivity
FRA	Fos Related Antigen
HHIF	hypothalamic hypophysary inhibitory factor
HNa-aCSF	aCSF containing 0.3 M NaCl
HPLC	high performance liquid chromatography
HR	heart rate

HS	hypertonic saline
icv	intracerebroventricular
LC	locus coeruleus
MAP	mean arterial pressure
MnPO	median preoptic nucleus
Na ⁺ K ⁺ -ATPase	sodium-potassium-activated adenosine triphosphatase
NMDA	<i>N</i> -methyl-D-aspartate
NMR	nuclear magnetic resonance
NTS	nucleus tractus solitarius
OLC, "ouabain"	endogenous ouabain-like compounds
OVLT	organum vasculosum laminae terminalis
PBS	phosphate buffered saline
PVN	paraventricular nucleus
RAS	renin-angiotensin system
RVLM	rostral ventrolateral medulla
sc	subcutaneous
SCh	suprachiasmatic nuclei
SFO	subfornical organ
SON	supraoptic nucleus
vAV3V	ventral part of the anteroventral third ventricle
VLM	ventrolateral medulla
6-OHDA	6-hydroxydopamine

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Chapter 1

General Introduction

Preface

Essential hypertension afflicts approximately 15 % of the population, and poses an increased risk to cardiovascular disease such as stroke, myocardial infarction, and end stage renal disease resulting in significant morbidity and mortality. Hypertension is a polygenic disease resulting from the interaction of a number of genetic and environmental factors (Hamet et al.1997). One major environmental determinant is dietary salt consumption. Interpopulation studies have demonstrated a positive correlation between dietary sodium intake and blood pressure. The prevalence of hypertension is virtually non-existent, and blood pressure does not increase with age in populations in which dietary sodium intake is less than 600 mg/d (Simchon et al.1991). Conversely, in populations with a high sodium intake the incidence of hypertension is high (Simchon et al.1989). Dosage requirements of antihypertensive drugs other than calcium antagonists are reduced by salt restriction (Kurtz and Morris, 1983; Whitescarver et al.1986) underscoring the importance of salt in the maintenance of hypertension. Furthermore, in animal models that are genetically predisposed to hypertension, high salt intake exaggerates its development (Takeshita et al.1979; Oparil et al.1988).

Despite a wealth of information resulting from decades of studies, the mechanisms involved in mediating the increase in blood pressure resulting from a high dietary salt intake are incompletely understood. A complex interaction between neuroendocrine factors and the kidney may underlie the propensity for salt retention and development of salt-induced hypertension. An increasing number of studies have demonstrated a critical role for the central nervous system in salt-induced hypertension. High salt intake

suppresses sympathetic nervous system activity in normotensive animals whereas it stimulates sympathetic activity in salt-sensitive animal models of hypertension and human subjects (Chen et al.1988; Kotchen et al.1991). The sympathetic nervous system, in addition to influencing vasomotor tone, participates in the long-term regulation of blood pressure by increasing vascular muscle membrane permeability to sodium, causing trophic effects on vascular smooth muscle leading to increased vascular resistance and responses to vasoconstrictor stimuli; and renal sympathetic nerve activity promotes anti-natriuresis and renin secretion (Mark, 1996). Studies in our laboratory have demonstrated that central inhibition of the sodium-potassium-activated adenosine triphosphatase (Na^+K^+ -ATPase) by endogenous ouabain-like compounds (OLC), as well as the central actions of the brain renin-angiotensin system (RAS) increases sympathetic activity in rat models of salt-induced hypertension (Huang and Leenen, 1996a; Huang and Leenen, 1998). This literature review will provide an overview of the Na^+K^+ -ATPase, its endogenous ligand inhibitor, OLC, and the brain RAS, followed by a critical review of the present understanding of their role in central mechanisms that mediate salt-induced hypertension in an animal model of essential hypertension, the Dahl salt-sensitive rat.

1.1 The Na^+K^+ -ATPase and its Endogenous Ligand Inhibitor, OLC

1.1.1 Structure and Function of the Na^+K^+ -ATPase

The Na^+K^+ -ATPase (EC 3.6.1.37) is a membrane associated protein complex expressed in nearly all eukaryotic cells (reviewed by Rose and Valdes Jr., 1994). It consists of two dimers, each composed of noncovalently associated α (112 kDa) and β

(55 kDa) subunits. The α -subunit is the catalytic subunit and contains all ligand binding sites including the cardiac glycoside binding site. It is proposed to have 7-8 transmembrane domains whereas the β -subunit has a single hydrophobic transmembrane domain and is highly glycosylated on its extracellular surface. The β -subunit is thought to orient and stabilize the α -subunit in the membrane, but also appears to be essential for the function of the Na^+K^+ -ATPase. A smaller γ subunit (10 kDa) is also present and may function to increase the affinity of the Na^+K^+ -ATPase for ATP.

The Na^+K^+ -ATPase utilizes the energy released from the intracellular hydrolysis of ATP to couple the export of three Na^+ ions out of the cell to the import of two K^+ ions into the cell thus setting up an electrochemical gradient across the cell membrane. A number of transport mechanisms are coupled to the transport of Na^+ back into the cell along its concentration gradient including the co-transport of glucose, amino acids, chloride and phosphate ions and the counter-transport of protons and calcium. The activity of the Na^+K^+ -ATPase is thus essential for the maintenance of the resting membrane potential and cellular electrolyte homeostasis.

The four known isoforms of the α -subunit (α_1 , α_2 , α_3 and α_4) as well as the four known β -subunit isoforms (β_1 , β_2 , β_3 and the β isoform of the H^+K^+ -ATPase) are encoded by different genes and are differentially expressed in various organs. In rodents, the α_1 isoform is 1000-fold more resistant to ouabain than other isoforms. This ouabain resistance of the rodent α_1 is not observed in other species. The α_1 isoform is ubiquitously expressed whereas the other isoforms, α_2 , α_3 , and α_4 are distributed in a tissue-specific manner e.g. the α_3 isoform is primarily associated with the central nervous system.

1.1.2 *Endogenous Ouabain-Like Compounds (OLC)*

A highly conserved binding domain for cardiac glycosides on the α - subunit of the Na^+K^+ -ATPase suggests the presence of endogenous ligand inhibitors that modulate its activity. In 1953, Szent-Gyorgyi speculated on the presence of a digitalis-like substance: "We need not be astonished ... if we find digitalis-like substances in any cell ... The digitalis cardiac glycosides, if I may say so, are no drugs at all: they are substitutes for a missing screw in our machinery, which had a cardinal role in one of the most basic physiological regulations...". Gruber et al. (1980) provided the first experimental evidence to support this hypothesis. They demonstrated the presence of a substance with digoxin-like immunoreactivity in plasma of dogs following acute volume-expansion with saline (Gruber et al.1980). This was followed by reports by Schreiber et al. (1981) that a digoxin-like substance was detectable in rats with pressure-induced cardiac overload.

1.1.2a *Chemical Characterization of OLC*

Characterization of OLC has been hindered by the extreme paucity of material available from tissues and non-specific interferences in enzyme assays. Candidates for the endogenous digitalis-like factor have included a number of steroid compounds that share a cyclopentanoperhydrophenanthrene nucleus with a lactone ring on position C 17, including progesterone derivatives and dehydroepiandosterone sulphate, as well as non-steroidal compounds, namely ascorbic acid, peptides, non-esterified fatty acids and lysophospholipids (reviewed by Kolbel and Schreiber, 1996). Hamlyn and co-workers (1991) characterized an inhibitor isolated from human plasma using high performance liquid chromatography (HPLC) or an affinity-based chromatography as indistinguishable

from ouabain by multiple functional criteria including competition with ^3H -ouabain for binding to $\text{Na}^+\text{K}^+\text{ATPase}$, and inhibition of ^{86}Rb uptake of human erythrocytes and isolated $\text{Na}^+\text{K}^+\text{-ATPase}$. Further analysis of the inhibitor using mass spectrometry suggested that it was a stereo-isomer of ouabain (Mathews et al.1991). Endogenous $\text{Na}^+\text{K}^+\text{-ATPase}$ inhibitors have also been isolated from bovine adrenals (Schneider et al.1998), and bovine hypothalamus as well as guinea pig brain (Tymiak et al.1993; Fishman, 1979). Ion spray spectrometry of the compound purified from bovine hypothalamus indicated a molecular weight equivalent to ouabain. However, naphthoylation of the hydroxyl groups revealed differing retention times following HPLC for the naphthoylated derivatives of the endogenous compound and of ouabain, and differing nuclear magnetic resonance (NMR) profiles suggesting that the endogenous compound is an isomer of ouabain in which the location or configuration of one or more hydroxyl groups is/are altered (Hamlyn et al.1996). However, a more recent study demonstrated that the NMR spectra was due to the unexpected formation of a tetrahedral borate complex of the endogenous compound in the borosilicate glassware used during past structural analysis and attributed the retention time of naphthoylated derivatives following HPLC to glycerol contamination (Kawamura et al.1999). This study concluded that the endogenous compound was ouabain, but may exist *in vivo* in complex with inorganic elements. Schneider et al. (1998) characterized the compound isolated from bovine adrenals as ouabain by ultraviolet and mass spectroscopy and NMR.

Endogenous $\text{Na}^+\text{K}^+\text{-ATPase}$ inhibitors that are distinct from OLC have also been detected. A non-peptide, non-lipid inhibitor termed hypothalamic hypophysary inhibitory

factor (HHIF) was isolated from bovine hypothalamus and pituitary (Illescas et al.1990). This compound inhibited the $\text{Na}^+\text{K}^+\text{ATPase}$ in a dose related manner and competed with ouabain for binding to the enzyme, but was not immuno-reactive to antibodies raised against digoxin or ouabain (Sancho et al.1993; Huang et al.1997). A dihydro-digitalis-like factor was detected in bovine adrenal cortex and human serum (Qazzaz et al.1996a) and a substance chromatographically identical to authentic digoxin was characterized from human pleural fluids and urine (De Angelis et al.1997; Weinberg et al.1997). Another inhibitor, isolated from peritoneal dialysate of volume-expanded renal failure patients, like ouabain inhibits the $\text{Na}^+\text{K}^+\text{ATPase}$ via the cardiac glycoside binding site and is immuno-reactive to fab fragments of an anti-digoxin immunoglobulin G which binds digoxin, ouabain and other related cardenolides with high affinity, but differs in α -isoform sensitivity (Tao et al.1996a; Tao et al.1996b). In contrast to ouabain, this substance is a potent inhibitor of the rodent α_1 isoform and has a greater inhibitory effect on the α_2 compared to the α_3 isoform (Tao et al.1996b). Some studies have characterized endogenous bufodienolide-like $\text{Na}^+\text{K}^+\text{ATPase}$ inhibitors. Hilton and co-workers (Hilton et al.1996; Wright et al.1997) isolated an inhibitor identified by mass spectrometry as $3\beta,14\alpha,20:21$ -bufenolide in the plasma of a volume expanded patient and in human placenta. A marinobufagenin-like inhibitor has also been detected in human plasma and urine (Bagrov et al.1996). In addition, Samuelov and Lichtstein (1997) have characterized an inhibitor from bovine cataractous lens as 19-norbufalin. Sich et al. (1996) reported the presence of three distinct inhibitors in plasma that exhibit proscillaridin A immunoreactivity. One of these compounds co-eluted with authentic ouabain and another

with proscillaridin A following HPLC.

In the literature the terms endogenous digitalis-like substance/factor, endogenous "ouabain", ouabain-like factor and OLC have been used interchangeably to indicate endogenous Na^+K^+ -ATPase inhibitors with an ouabain-like steroid structure (Figure 1) that act via the cardiac glycoside binding site. From the evidence presented it appears that several endogenous Na^+K^+ -ATPase inhibitors exist. As it is likely that more than one inhibitor is assessed by commonly used methods, and one of these inhibitors is ouabain, we use the term OLC.

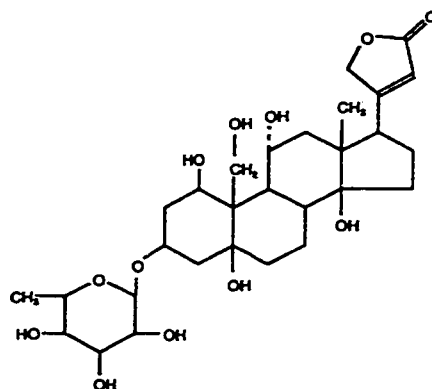


Figure 1. Structure of Ouabain

1.1.2b Source(s) and Distribution of OLC

Several investigators have independently confirmed the presence of OLC in human plasma (Paci et al.1996; Worgall et al.1996; Balzan et al.1997; Ferrandi et al.1997; Harwood et al.1997). Reported normal ranges for OLC varies widely between laboratories from 20-60 pmol/l assayed by radioimmunoassay reported by Naruse et al. (1994) to 0.29-0.44 nmol/l using radioimmunoassay or 0.6-1.0 nmol/l assayed by

enzyme-linked immunosorbent assay reported by Hamlyn and Manunta (1992). Some studies, however, failed to detect OLC (Doris et al.1994; Gomez-Sanchez et al.1994; Lewis et al.1994). Differences in detection of OLC and concentrations obtained appear to be due to differences in the specificity of the antibody used or the sensitivity of the assay employed. Antolovic et al. (1998) identified a cardiac glycoside specific binding protein of 26 kDa in bovine plasma which may be involved in transport of OLC to receptor sites supporting the presence of endogenous cardiac glycosides in mammals.

Adrenal venous OLC levels are approximately 6-fold greater than systemic arterial levels in dogs (Boulanger et al.1993) suggesting that the adrenals may secrete OLC. High levels of OLC have been detected in bovine adrenal cortex (Qazzaz and Valdes, 1996b). Ludens et al. (1992) reported that plasma OLC levels fall progressively after total adrenalectomy but not after adrenal medullectomy in rats that did not receive steroid substitution. Ludens et al. (1992) using rat adrenal cortex, and Laredo et al. (1994) utilizing bovine adrenocortical cells in culture have demonstrated OLC secretion from adrenocortical cells. In addition, intact perfused adrenal glands retain the ability to secrete OLC (Hinson et al.1998). Taken together, these studies suggest that the adrenal cortex is a source of circulating OLC. However, OLC concentrations following bilateral adrenalectomy in patients with Cushings disease, presumably on steroidal substitution, were similar to that of healthy subjects (Naruse et al.1994). Furthermore, in adrenalectomized spontaneously hypertensive rats that received daily substitution of aldosterone and corticosterone, only minor decreases in circulating and brain OLC were observed, and OLC increased in response to high salt intake (Leenen et al.1993a). This

suggests that an organ(s) other than the adrenal cortex contributes to production of OLC.

The brain appears to synthesize OLC. High levels of OLC have been detected in bovine and guinea pig brain, especially in the hypothalamus and the pituitary (Fishman, 1979; Tymiak et al.1993) and hypothalamic extracts have a 10,000-fold greater ability to inhibit the Na^+K^+ -ATPase compared to extracts from plasma (Alaghband-Zadeh et al.1983). In *in vitro* cultures, rat fetal hypothalamic, but not cortical, cells secrete a Na^+K^+ ATPase inhibitor (Morgan et al.1985). OLC is also released by primary cultures of mouse astrocytic cells (Kala et al. 2000). A series of studies have provided morphological evidence for the presence of OLC in the brains of rats, dogs and macaques utilizing immunohistochemistry using digoxin- or ouabain-specific antibodies (Yamada et al.1987; Ihara et al.1988; Yamada et al.1992a; Yamada et al.1992b). Distribution of immunoreactive neurons was similar across species, and for both antibodies. Ouabain and digoxin immunoreactivity was detected in areas involved in cardiovascular and osmoregulation. Immunoreactive neurons were localized in the paraventricular nucleus (PVN), supraoptic nucleus (SON), magnocellular accessory nuclei of the hypothalamus, and the posterior pituitary (Yamada et al.1987; Ihara et al.1988; Yamada et al.1992a; Yamada et al.1992b). Immunoreactive fibres from the PVN course through the lateral hypothalamic area to the infundibulum where they terminate in the inner layer of the median eminence (Yamada et al.1987; Yamada et al.1992a; Yamada et al.1992b). These nerve fibres were associated with primary capillaries of hypophysial portal veins (Yamada et al.1992a). Digoxin-immunoreactivity in the PVN and SON was co-localized with vasopressin and to a lesser extent with oxytocin (Ihara et al.1988). It is tempting to speculate that like

vasopressin and oxytocin, OLC may be produced in magnocellular neurons and released into the circulation from the posterior pituitary. Dense immunoreactivity was also observed in the organum vasculosum laminae terminalis (OVLT) and the SFO (Yamada et al.1992a). Immuno-positive fibres projecting to the OVLT were detected, while few were detected near the SFO. Some other areas contained a few immuno-positive somata and fibres, namely, the median preoptic nucleus (MnPO), periventricular, perifornical, anterior and lateral hypothalamic, and preoptic areas (Ihara et al.1988; Yamada et al.1992b). In more recent studies, ouabain-immunoreactive neurons have been detected in the medulla: rostral ventrolateral medulla (RVLM), ventromedial medulla, nucleus ambiguus, caudal raphe, nucleus tractus solitarius (NTS) and dorsal motor nucleus of the vagus (Yamazato et al.1998). A similar pattern of staining was obtained using fab fragments of a digoxin-specific antibody which also binds ouabain (Yamazato et al.1998). These studies suggest that areas within the hypothalamus and medulla may serve as sites of action of OLC.

Ouabain, digoxin and related steroids are composed of a steroid nucleus with one to four sugar moieties. The sugar moiety of ouabain, rhamnose, is found only in plants suggesting that rhamnose for the synthesis of OLC is most likely absorbed from the gastrointestinal tract. Steroids are synthesized from cholesterol mostly in the adrenals, but also in other tissues including the brain, where they are synthesized either de novo from cholesterol, or by the metabolism of circulating precursors (reviewed by Baulieu, 1991). Precursors of steroids such as pregnenolone and dehydroepiandrosterone are present in the brain at higher concentrations than in plasma and persist despite castration and

adrenalectomy (Corpechot et al.1981; Corpechot et al.1983). Enzymes, or the mRNA for the enzymes, involved in steroidogenesis, including the cytochrome P450 enzymes scc, 17 α -hydroxylase, aldosterone synthase, and 3- β -hydroxy-steroid dehydrogenase, have been localized mainly to glial cell types but in some cases are also found in neurons and are differentially distributed in the brain supporting the concept of neurosteroid biosynthesis in the brain (Sanne and Krueger, 1995; Gomez-Sanchez et al.1997; Zwain and Yen, 1999; Brown et al.2000). Despite the fact that the enzymes involved in the synthesis of OLC in mammals are unknown, it seems likely that the steroid nucleus for OLC could be synthesized in the brain as well as in the adrenals.

It appears that both the adrenal cortex and the posterior pituitary contribute to circulating OLC and their relative roles require further investigation. High levels of OLC have also been detected in the heart, kidneys, mammary gland and placenta but it is not known if these organs contribute to circulating levels of OLC. The evidence presented suggests that in addition to being an endocrine product, OLC may also act in an autocrine or paracrine fashion on ouabain-sensitive sites in the brain in a similar fashion to that seen with angiotensins in the circulation and in intrinsic tissue renin-angiotensin systems.

1.1.2c Physiological Role of OLC

Stimuli for the secretion of OLC are poorly understood. It appears to be secreted in response to changes in Na⁺ concentrations, and/or extracellular volume expansion. Several studies have demonstrated that volume expanded states and essential hypertension are associated with an increase in circulating levels of OLC (reviewed by Hamlyn et al.1996; Goto and Yamada, 1998). Yamada et al. (1997) demonstrated an

increase in plasma OLC in response to a hypertonic sodium load in the absence of volume expansion in rats indicating that volume expansion may not be the primary stimulus for OLC secretion. Increases in CSF Na^+ also result in increases in plasma OLC (Jandhyala and Ansari, 1986; Takahashi et al.1988), and appear to cause secretion of OLC within the brain (Huang et al.1992; Huang and Leenen, 1996b). In rats, a transient increase in CSF Na^+ concentration is associated with a decrease in hypothalamic OLC content (Takahashi et al.1988), whereas a chronic increase in CSF Na^+ concentration increases hypothalamic OLC content (Huang et al.1998) suggesting that a chronically elevated CSF Na^+ concentration increases OLC production and/or accumulation within the hypothalamus.

Ang II, via AT_2 receptors stimulates the secretion of OLC from bovine adrenal zona glomerulosa cells (Shah et al.1999). Hinson et al. (1998) reported that adrenocorticotrophic hormone (ACTH) stimulated OLC release in 3 of 5 perfusions from intact isolated rat adrenal preparations. Administration of synacthen, a synthetic ATCH, did not affect plasma OLC concentrations in humans (Butt et al.1998). It is therefore likely that Ang II, and possibly ACTH, regulate OLC secretion from the adrenals.

OLC binds the cardiac glycoside binding domain on the α -subunit of the Na^+K^+ -ATPase and inhibits its activity. It is likely that the effects of OLC in various tissues would relate to the relative expression of the ouabain-sensitive α isoforms ie the α_3 and α_2 isoforms. Modest inhibition of the Na^+K^+ -ATPase by OLC affects many Na^+ gradient dependent transport processes, including neurotransmitter uptake and storage, cytosolic pH and slowing the extrusion of Ca^{2+} via the $\text{Na}^+\text{-Ca}^{2+}$ exchanger (reviewed by Blaustein 1993). However, in resting cells cytosolic Ca^{2+} concentrations remain relatively stable

whereas in activated cells Ca^{2+} redistribution is amplified by the release of stored Ca^{2+} resulting in an increase in cytosolic Ca^{2+} concentration and accounts for most of the effects of Na^+K^+ -ATPase inhibition.

OLC is present in plasma of humans at a concentration ranging from 25 - 750 pM (Goto and Yamada, 1998) which appears low given the vast receptor pool. However, the actions of OLC are likely to be related to its concentration in the vicinity of physiologically relevant receptors and may depend on tissue and cellular distribution of ouabain-sensitive isoforms of the α -subunit ie the α_3 and α_2 isoforms in rodents and all isoforms in non-rodents. Juhaszova and Blaustein (1997a; 1997b) demonstrated a ubiquitous distribution for the α_1 isoform in plasma membrane and a more confined, reticular distribution of the α_2 and α_3 isoforms that paralleled the underlying endoplasmic or sarcoplasmic reticulum and colocalized with the Na^+ - Ca^{2+} exchanger in rat astrocytes and arterial myocytes. The ouabain-sensitive α isoforms may therefore regulate Na^+ , and indirectly Ca^{2+} , in a restricted cytosolic space overlying the reticulum thereby modulating reticular Ca^{2+} release and subsequent Ca^{2+} signalling.

In vitro studies suggest that nanomolar concentrations of ouabain may cause vasoconstriction in humans and rats by influencing vascular responsiveness to vasoconstrictors (Woolfson et al.1990; Woolfson and Poston, 1991; Weiss et al.1993; Songu-Mize et al.1994). Within the adrenal gland, ouabain may increase aldosterone secretion (Szalay, 1993). Chronic ouabain infusion in rats increases plasma aldosterone concentrations without affecting plasma renin activity (Manunta et al.1994) suggesting that ouabain modulates aldosterone secretion. Ouabain causes natriuresis via action on the

kidneys (Blaustein, 1993). On the myocardium, ouabain acts to increase the force of contraction (Blaustein, 1993).

The brain is very susceptible to the effects of OLC as it expresses the most ouabain-sensitive isoform of the α -subunit of the Na^+K^+ -ATPase, α_3 . Takahashi et al. (1984) and we (Huang et al. 1992) demonstrated that intracerebroventricular (icv) injections of ouabain, or hypothalamic extracts containing OLC, cause similar increases in renal sympathetic nerve activity, blood pressure and heart rate. The pressor response to icv injections of ouabain was prevented by prior injection of fab fragments that bind ouabain into the MnPO (Budzikowski and Leenen, 1997) indicating that the MnPO mediates the pressor response to exogenous ouabain. However, in untreated rats, microinjection of the fab fragments did not affect resting blood pressure indicating that OLC within this nucleus does not contribute appreciably to maintaining resting blood pressure. Jones and Lo (1990) mapped ouabain-sensitive sites in the hypothalamus of anaesthetized rats using low dose (20ng) injections of ouabain. Injections in the PVN elicited the largest pressor response. Other areas that exhibited a consistent pressor response were the anterior hypothalamic area and the posterior hypothalamus. Studies using northern analysis and *in situ* hybridization indicate that mRNA for α_3 and β_1 isoforms of the Na^+K^+ -ATPase are expressed at higher levels than mRNA for α_1 and α_2 isoforms in the hypothalamus (Schneider et al. 1988; Antonelli et al. 1995). High levels of hybridization signal for α_3 were found in ventromedial hypothalamus, supraoptic nucleus, paraventricular nucleus and the anterior hypothalamic area (Antonelli et al. 1995). The distribution of high affinity ^3H -ouabain binding sites varies in different regions of the brain, with widespread

distribution in the hypothalamus (Antonelli et al.1995). Within the PVN ^3H -ouabain binding density was lower than in other areas of the hypothalamus (Antonelli et al.1995). As this nucleus is very sensitive to local microinjections of ouabain (Jones and Lo, 1990), has high levels of α_3 and β_1 mRNA (Antonelli et al.1995), but has relatively low binding density for ^3H -ouabain, it is possible that there is a greater rate of turnover of the enzyme in this nucleus compared to other areas in the hypothalamus. Alternatively, the lower binding density for ^3H -ouabain may reflect increased occupancy of the binding site by endogenous OLC which may not have dissociated prior to the assay. OLC within the RVLM appears to contribute to the maintenance of sympathetic outflow as blockade of the effects of OLC within the RVLM by microinjection of fab fragments that bind digoxin and ouabain, or of an anti-ouabain antibody, decreased baseline blood pressure and renal sympathetic nerve activity and prevented pressor and sympathoexcitatory responses to subsequent injections of ouabain (Teruya et al.1997). The presence of putative OLC-producing neurons in areas of the brain known to participate in the regulation of sympathetic outflow (see section 1.1.2) supports a role for OLC in the central regulation of blood pressure. Furthermore, an ouabain-like inhibitor extracted from rat cerebral cortex (Endobain E) increases norepinephrine release from hypothalamic tissue in a concentration-dependent manner suggesting that the actions of this inhibitor may result in increases in noradrenergic activity (Vatta et al.1999). Taken together, experimental evidence suggests that OLC may act at various central sites to increase sympathetic outflow thereby regulating blood pressure, but pathways involved in this process have not yet been determined.

1.2 The Brain Renin-Angiotensin System (RAS)

The sympathoexcitatory and pressor responses to icv administration of ouabain can be prevented by icv pretreatment with captopril, an angiotensin converting enzyme inhibitor, or the angiotensin receptor antagonists, saralasin and losartan (Takahashi et al.1984; Huang and Leenen, 1996b). These findings indicate that OLC-induced increases in sympathetic activity and pressor responses are mediated by activation of the brain RAS.

In 1961, Bickerton and Buckley first reported that circulating Angiotensin II (Ang II) is able to act on the central nervous system to increase blood pressure (Bickerton and Buckley, 1961). In addition to being regarded as a circulating hormone, Ang II is now regarded as an effector peptide of intrinsic tissue RASs including that in the brain. More recent evidence suggests that Angiotensin III (Ang III, Ang II 2-8) may be a major effector peptide of the brain RAS and a role for Angiotensin IV (Ang IV, Ang II 3-8) is emerging (reviewed by Wright and Harding, 1997). The brain RAS and central actions of the circulatory RAS are involved in central cardiovascular regulation, body fluid homeostasis including regulation of thirst and sodium appetite, cyclicity of reproductive hormones and sexual behavior, cerebral blood flow, neuronal development and differentiation, and learning and memory (reviewed by Phillips, 1987; Wright and Harding, 1997; Steckelings et al.1992; Muratani et al.1996).

1.2.1 Distribution of Components of the RAS in Brain Tissue

All components of the RAS have been identified in brain tissue including angiotensinogen, the precursor for angiotensins, the protease renin that cleaves

angiotensinogen to the decapeptide angiotensin I (Ang I), angiotensin converting enzyme (ACE), which converts Ang I to the octapeptide Ang II, as well as the aminopeptidases A and N, which are the main enzymes that cause N-terminal cleavage of Ang II to Ang III, and Ang III to Ang IV respectively. Expression of the mRNA for angiotensinogen, renin and ACE has been demonstrated in the brain of several species, including rats consistent with the concept of a local brain RAS (Dzau et al.1986; Whiting et al.1991). Incubation of brain homogenates with renin generates Ang I implying that the precursor angiotensinogen is present locally (Ganten et al.1971). Bunnemann et al. (1992) localized angiotensinogen mRNA predominantly in astrocytes. Angiotensinogen was localized immunohistochemically mainly in astrocytes and ependymal cells (Deschepper et al.1986), but was also detected in neurons (Imboden et al.1987). The presence of angiotensinogen has also been demonstrated in the CSF (Moffett, 1987). Following initial reports on central renin activity (Ganten et al.1971; Fischer-Ferraro et al.1971) the presence of brain renin that is independent of circulating renin, distinct from other proteases, active *in vivo* and inhibited by renin specific antibodies was confirmed (Hirose et al.1978; Speck et al.1981). Renin activity was widely distributed with particularly high concentrations in the pituitary, the choroid plexus, the hypothalamus, the cerebellum, and the amygdaloid nucleus (Genain et al.1985). Brain ACE is similar to peripheral ACE with respect to optimum pH, chloride dependency, and inhibition by various inhibitors (Printz et al.1982) but isozymes with different molecular weights have been demonstrated in the brain (Strittmatter et al.1985; Hooper and Turner, 1987). Brain ACE is ubiquitous in distribution but is highly expressed in the choroid plexus and circumventricular organs

(CVO), where it is primarily associated with plasma membranes of astrocytes. Like ACE from peripheral sources, brain ACE is nonspecific in action i.e. in addition to converting Ang I to Ang II, ACE of central origin degrades kinins and neuropeptides such as opioid peptides and substance P. Aminopeptidase A appears to be heterogeneously distributed in the brain with the highest concentrations of aminopeptidase A activity detected in the pituitary and CVO, and lower concentrations in the median eminence, the arcuate nucleus, the SON and PVN and the choroid plexus (Zini et al.1997). Aminopeptidase N is diffusely distributed through out the gray matter of the brain (Barnes et al.1988).

The site of synthesis of angiotensins in brain tissue is as yet unresolved. Bunnemann et al. (1992) suggested that angiotensinogen may be produced in astrocytes and converted to Ang I by renin in the extracellular fluid or alternatively may be taken up by neurons and converted intraneuronally. Renin and ACE activity have been colocalized in synaptosomes supporting the concept of intraneuronal synthesis of Ang I and Ang II (Paul et al.1985). However, renin has also been detected in oligodendrocytes and ACE has been detected extracellularly (Inagami et al.1980; Phillips, 1987). After conversion of Ang I to Ang II by ACE, Ang II is further acted on by aminopeptidase A to form the heptapeptide Ang III which is converted to the hexapeptide Ang IV by aminopeptidase N. Ang II has been thought of as the first biologically active molecule in this cascade. However, an increasing number of studies indicate that conversion of Ang II to Ang III is required for part of the actions of Ang II (Zini et al.1996; Song et al.1997). An alternate pathway exists whereby Ang I is converted to the nonapeptide des-Asp¹-Ang I which is acted on by ACE to form Ang III. Rat hypothalamic homogenates almost exclusively degrade Ang

I to des-Asp¹-Ang I rather than Ang II (Sim and Qiu, 1994). The presence of these angiotensin peptides in the brain despite the blood brain barrier and their persistence in nephrectomized rats (Ganten et al.1983) indicates that the peripheral RAS does not contribute to these peptides. Angiotensin peptides have also been isolated from neuronal cell cultures further strengthening the concept of an intrinsic brain RAS (Raizada et al.1982). Details of studies demonstrating the presence of RAS peptides and enzymes in the brain have been extensively reviewed elsewhere (Phillips et al.1979; Saavedra, 1992).

Four main angiotensin receptor subtypes have been described (reviewed by Wright and Harding, 1995; Unger et al.1996). Three of these, namely the AT₁, AT₂ and AT₄ receptors, are distributed in the brain as well as in peripheral tissue. The distribution of these receptors within the brain is comparable across mammalian species. Two AT₁ receptor subtypes have been identified in rodents, AT_{1A} and AT_{1B}, (Murphy et al.1991; Elton et al.1992; Sandberg et al.1992; Sasamura et al.1992) and their genes localized to chromosome 17 and 2 respectively. These AT₁ subtypes are 96 % identical with respect to amino acid sequences encoded by the coding region but are markedly different in non-coding regions suggesting different regulatory mechanisms. In humans only a single gene for the AT₁ receptor (on chromosome 3) has been identified. The AT₁ receptor subtypes share comparable binding characteristics, binding Ang II with higher affinity than Ang III, but are differentially distributed. Central areas involved in cardiovascular regulation, body fluid homeostasis and neuroendocrine function exhibit a predominance of AT₁ receptors (Obermuller et al.1991; Tsutsumi and Saavedra, 1991). These areas include the SFO, OVLT, PVN, area postrema (AP) and NTS which exhibit high densities and the

MnPO which exhibits moderate amounts of the receptor, and in rats are of the AT_{1A} subtype (Lenkei et al.1995). AT_{1B} receptors are distributed mainly in the anterior pituitary, but its mRNA is also expressed in the OVLT and SFO (Kakar et al.1992; Lenkei et al.1995). Other areas that express moderate levels of AT₁ receptors include the amygdala, piriform cortex and lateral olfactory tract. In adult rat brain, AT₂ receptors, which bind Ang III with higher affinity than Ang II, are mainly distributed in regions participating in sensory and limbic functions including the medial geniculate, hypoglossal nucleus, inferior olivary nucleus, the basal ganglia, thalamus (Song et al.1992; Lenkei et al.1996). The AT₂ receptor is also moderately expressed in areas that participate in cardiovascular regulation such as the locus coeruleus and medial amygdala (Song et al.1992; Lenkei et al.1996). Lesser densities of both AT₁ and AT₂ receptors are seen in the cerebellum, cingulate gyrus and septum. The AT₄ receptor binds Ang IV with high affinity, but has a very low affinity for ligands that bind to AT₁ or AT₂ receptors. Structures with high densities of AT₄ receptors include the cerebral and piriform cortices, the hippocampus, superior colliculus, basal ganglia, thalamus, nucleus accumbens, medial habenula, lateral geniculate, periaqueductal gray, ventral tegmental area, cerebellum and inferior olivary nucleus (Roberts et al.1995). Several of these structures regulate sensory and motor integration, cognitive processes and memory supporting a role for Ang IV in these processes.

Central areas with a cardiovascular regulatory role in which all components of the RAS are present include the median preoptic area, the PVN, SON, dorsomedial and ventromedial hypothalamic nuclei, the midbrain periaqueductal gray, the locus ceruleus,

the nucleus tractus solitarius, the dorsal motor nucleus of the vagus and in very low concentrations, the cerebral cortex (Bunnemann et al.1992). Among these areas, the highest concentrations of angiotensinogen immunoreactivity and Ang II nerve cell body and terminal immunoreactivity were found in the PVN and the SON (Healy and Printz, 1984). Angiotensinogen immunoreactivity is absent in CVO implicated in cardiovascular and neuroendocrine function such as the SFO, AP and the OVLT (Bunnemann et al.1992). However, angiotensinogen mRNA is demonstrable in the rostral part of the SFO, the AP and in the median eminence (Bunnemann et al.1993) and high binding densities for Ang II receptors are found in the SFO and AP, with moderate densities also found in the OVLT and median eminence. (Mendelsohn et al.1984; Gehlert et al.1986). CVO express a deficient blood brain barrier and have a high capillary density, fenestrated capillaries, large perivascular spaces and specialized ependymal cells (tanycytes) enabling them to detect circulating substances and transducing the information to neural messages (Gross and Weindl, 1987). Circulating angiotensins, like other peptides, cannot cross the blood brain barrier and not surprisingly bind specifically to CVO (Van Houten et al.1980). This may explain very low amounts of demonstrable angiotensinogen in CVO as those that express very high Ang II binding sites, SFO and OVLT, have been implicated in the action of blood-borne and CSF-borne Ang II.

Steckelings et al. (1992) summarized the major localization of Ang II neuronal perikarya, receptors and fibres (Figure 2).

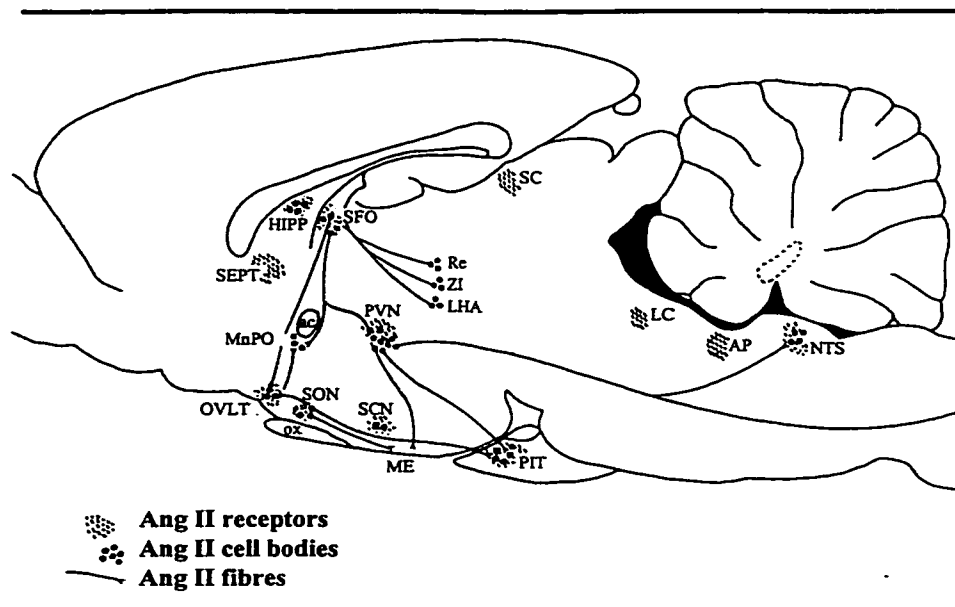


Figure 2. A schematic representation of a mid-sagittal section through a rat brain showing Ang II pathways. ac, anterior commissure; AP, area postrema; HIPP, hippocampus; LC, locus coeruleus; LHA, lateral hypothalamic area; Me, median eminence; MnPO, median preoptic nucleus; NTS, nucleus tractus solitarius; OVLT, organum vasculosum laminae terminalis; ox, optic chiasm; PIT, pituitary; PVN, paraventricular nucleus; Re, reuniens thalamic nucleus; SC, superior colliculus; SCN, suprachiasmatic nucleus; SEPT, septum; SFO, subfornical organ; SON, supraoptic nucleus; ZI, zona incerta. (Adapted from Steckelings et al. 1992)

To demonstrate angiotensinergic pathways, anatomical tract tracing in combination with immunohistochemical staining for Ang II has been used to identify immunoreactive somata and axonal projections to sites where Ang II is presumably utilized as a neurotransmitter. Lind et al. (1984) demonstrated two major angiotensinergic efferent pathways from the SFO, one projecting to the MnPO and the other to the PVN. The SFO also has an afferent projection arising in perifornical parts of the lateral hypothalamic area (Lind et al. 1984). In addition, Jhamandas et al. (1989) demonstrated that 46 % of SFO neurons retrogradely labelled from the SON were also labelled for angiotensin. Fewer cells demonstrating both the retrograde tracer and immunoreactivity for angiotensin were

demonstrable in the MnPO and OVLT. The presence of these pathways is supported by electrophysiological and microinjection studies (Akaishi et al.1981; Jhamandas et al.1989; Wright et al.1993). Lind et al. (1985) suggested that angiotensinergic projections may include those from the PVN to presympathetic neurons in the intermediolateral cell column (IML) of the spinal cord and to the posterior pituitary. Ang II immunoreactive fibres have also been identified in the pons within the lateral parabrachial nucleus and area postrema, and within the medulla in the NTS and IML, but the origin of these fibres was not identified.

1.2.2 Role of the Brain RAS in Cardiovascular Regulation

The brain RAS, via its effector peptides, mediates osmo- and cardiovascular regulation by acting at various areas within the brain to enhance sympathetic outflow, blunt baroreceptor reflex gain and stimulate release of vasopressin and ACTH (reviewed by Phillips, 1987; Unger et al.1988; Ferguson and Washburn, 1998). These centrally mediated effects may be achieved by either the actions of angiotensins synthesized by the brain, or the actions of circulating Ang II on CVO. In this review we focus mainly on the actions of angiotensins synthesized by the brain. In transgenic rats deficient in brain angiotensinogen, mean arterial pressure tended to be lower than control rats with a functional brain RAS (Huang et al.2000a) suggesting a role for the brain RAS in cardiovascular control. Extracellular recordings have demonstrated an excitatory effect of exogenous Ang II in a number of brain areas involved in cardiovascular regulation including the SFO (Li and Ferguson, 1993), MnPO (Tanaka and Nomura, 1993), OVLT (Knowles and Phillips, 1980), PVN (Li and Ferguson, 1993), lateral hypothalamus

(Tanaka et al.1986), AP (Carpenter et al.1988; Papas et al.1990), NTS (Barnes et al.1990; Hegarty et al.1996), and dorsal motor nucleus of the vagus (Barnes et al.1990). In some cases an inhibitory effect has also been observed but may be attributable to indirect actions of Ang II on inhibitory interneurons. In order to investigate the role of Ang II in central cardiovascular regulation microinjections of exogenous Ang II into various brain areas have been widely used to mimic the effects of endogenously released Ang II. However, the variability in responses to microinjections emphasizes the limitations of this approach when used in isolation to provide an understanding of functional roles of Ang II in specific areas.

The brain RAS appears to modulate tonic sympathetic drive generated by vasomotor neurons of the ventrolateral medulla (VLM). Microinjection of Ang II into the VLM of rats elicits cardiovascular changes that parallel, but are of smaller magnitude than those produced by microinjection of the excitatory amino acid, glutamic acid. Ang II injected into the caudal VLM produces depressor and bradycardic effects that are opposite to the pressor and tachycardic effects elicited by injection into the RVLM (Muratani et al.1991). Ang II responsive sites in the RVLM were localized to the subretrofacial nucleus which contains neurons that project to sympathetic preganglionic neurons of the intermediolateral cell column (Ross et al.1984; Dampney et al.1987). Chan et al. (1991) have shown that 30 % of RVLM neurons with spinal projections are excited by iontophoretic application of Ang II. Sasaki and Dampney demonstrated that microinjection of Ang II into the RVLM increases renal sympathetic nerve activity (Sasaki and Dampney, 1990). Microinjections of either Ang II or Ang III into the RVLM

cause comparable pressor responses in rabbits (Sasaki et al.1993). Combined treatment with bestatin which inhibits conversion of Ang III to Ang IV, and either Ang II or Ang III prolonged the responses suggesting that extending the half-life of Ang III was crucial for the prolonged response. In cats and rabbits, topical application or microinjection of Ang II antagonists into the VLM produced blood pressure and heart rate changes opposite to those evoked by Ang II (Andreatta et al.1988; Sasaki and Dampney, 1990). Bilateral injection of an Ang II antagonist into the RVLM in normotensive rats reduced blood pressure to virtually spinal levels (Ito and Sved, 1996) lending credence to the concept for a role of the brain RAS in modulation of tonic sympathetic drive generated by vasomotor neurons of the VLM.

Ang II action in the VLM also appears to be involved in modulation of phasic cardiovascular function. In anesthetized rats, microinjection of Ang II into the RVLM suppressed the arterial baroreflex response and this effect could be prevented by co-administration of either the nonpeptide AT₁ or AT₂ receptor antagonist, losartan or PD123319 respectively (Lin et al.1997). In the same study, microinjection of PD 123319 elicited an enhancement of baroreflex responses whereas losartan had no significant effect indicating a role for AT₂ receptors in the effect of endogenous Ang II in the RVLM. In anesthetized rabbits, saralasin, a peptide Ang II receptor antagonist, facilitated sympathetic baroreflex function and decreased resting renal sympathetic nerve activity when microinfused into the RVLM and inhibited baroreflex response when microinfused into the caudal VLM (Saigusa et al.1996). The presence of Ang II receptors associated with vagal efferents within the NTS, the first relay station of the baroreflex pathway is

also consistent with a modulatory role of the brain RAS in baroreflex function (Allen et al.1988). Microinjection of Ang II into the NTS of anaesthetized rats resulted in depressor responses at low doses (1 ng) or a pressor response at higher doses (10 ng) accompanied by bradycardia (Rettig et al.1986; Fow et al.1994). Bilateral microinjection of the Ang II antagonist (Sar¹,Thr⁸) Ang II, into the NTS enhanced baroreflex mediated bradycardia (Compagnole-Santos et al.1988). In conscious rats microinjection of saralasin had a similar effect (Michelini and Bonagamba, 1990). Both Ang II and Ang III, administered icv, decreased baroreflex sensitivity for reflex bradycardia (Lin et al.1988; Campagnole-Santos et al.1992). Overall these studies suggest that Ang II, and perhaps Ang III, decreases the gain of the baroreflex. While pressor responses to Ang II in the NTS appear to be mediated by sympathetic activation, the attenuation of reflex bradycardia has been attributed to a decrease in parasympathetic activity.

Circulating Ang II binds to receptors on CVO and modulates central cardiovascular control. The SFO, OVLT and the AP appear to be the main CVO that mediate this effect (Fink et al.1980; McKinley et al.1995; Potts et al.1999). These CVO are very sensitive to Ang II with microinjection of 0.1 ng or less of Ang II into the SFO, the third ventricle close to the OVLT or the area postrema (AP) resulting in pressor responses (Simpson and Routtenberg, 1973; Phillips, 1978; Lowes et al.1993). Lesion studies have indicated that the SFO is critical in mediating the pressor responses to circulating Ang II (Mangiapanne and Simpson, 1980) whereas modulation of the baroreflex control of heart rate by circulating Ang II has been attributed to its action on the AP (Matsukawa and Reid, 1990).

1.2.3 Responses to Intracerebroventricular Administration of Angiotensins

Although Ang II is present in the CSF and its secretion is regulated during the estrous cycle (Ghazi et al.1994) and is reportedly elevated in spontaneously hypertensive rats (Hermann et al.1984), its relevance in cardiovascular regulation is not known.

Administration of Ang II icv has been used to provide insight into the overall central response to exogenous Ang II and mechanisms involved in mediating these responses.

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 including rats (Severs and Daniels-Severs, 1973; Brosnihan et al.1979; Fink and Bryan, 1980; Fitzsimons, 1980). Pressor responses to centrally administered Ang II may be attributed to an increase in sympathetic neuronal activity as well as a release of vasopressin. Peripheral sympathectomy results in a prolonged latency for the pressor response to icv Ang II implying the presence of a fast component in the pressor response induced by sympathetic neural activation (Falcon et al.1978). Inhibition of vasopressin's actions by peripheral V_1 receptor blockade, vasopressin antibodies or hypophysectomy attenuates the pressor response to icv Ang II (Haack and Moehring, 1978; Unger et al.1981). Furthermore, in rats with diabetes insipidus that lack vasopressin, pressor responses to central Ang II are depressed (Hutchinson et al.1976). The action of Ang II on vasopressin release appears to depend on the prior conversion of Ang II to Ang III as icv administration of 3-amino-4-thiol-butyl sulfonate (EC33), an aminopeptidase A inhibitor, inhibited Ang II-induced vasopressin release in a dose dependent manner (Zini et al.1996). Combined peripheral α -adrenoceptor and V_1 receptor blockade completely

prevents 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.

Ang III, administered icv, increases blood pressure to a similar extent as icv Ang II whereas Ang IV causes a smaller pressor effect (Wright et al.1985; Wright et al.1996). Prior icv administration of an antiserum with anticatalytic activity against aminopeptidase A reduced Ang II-induced pressor responses by 73 %, but had no effect on the actions of Ang III (Song et al.1997). This study suggest that conversion of Ang II to Ang III may be required to mediate a major part of the pressor actions of Ang II. Central pretreatment with the AT₁ receptor antagonist, losartan, blocked pressor responses to Ang II, and analogues of Ang II, Ang III and Ang IV (Huang and Leenen, 1996b; Wright et al.1996) indicating that the AT₁ receptor mediates pressor actions of angiotensin peptides. Blockade of central AT₂ receptors using PD123177 potentiated Ang II-induced drinking and vasopressin release (Hohle et al.1995) suggesting that AT₂ receptors exert a tonic inhibitory control on some of the stimulatory actions of Ang II on AT₁ receptors. Pretreatment with divalinal (HED1291), a specific AT₄ receptor antagonist, did not affect pressor responses induced by analogues of the angiotensin peptides (Wright et al.1996) indicating that the AT₄ receptor does not mediate the pressor responses.

In addition to increasing sympathetic activity and vasopressin 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 (Buckley et al.1986; Doursout et al.1991). The release of this humoral inhibitor was blocked by central saralasin consistent with the

involvement of central Ang II receptors in its release (Doursout et al.1991).

Pressor responses to icv Ang II are prevented by electrolytic lesions of the area surrounding the ventral part of the third ventricular wall (Hartle et al.1982; Fink et al.1983). Two of the nuclei within this region, the OVLT and the MnPO, are activated in response to icv Ang II implicating these areas in mediation of the pressor effects of CSF Ang II (McKinley et al.1995).

1.2.4 Regulation of the Brain RAS by High Salt Diet

Components of the brain RAS are regulated by osmotic stimuli including salt intake. In normotensive Wistar rats, high salt diet increases renin mRNA in hypothalamic homogenates following 10 days of the diet and remained elevated following 8 weeks of high salt intake despite suppression in renal renin mRNA (Nishimura et al.1997). Mizuno et al.(1981) reported a significant increase in ACE activity in the midbrain following a high salt diet in spontaneously hypertensive rats. Increases in brain ACE mRNA and activity are associated with the development of hypertension in Dahl salt-sensitive (Dahl S) rats, a model of salt-induced hypertension (Zhao et al.2000, see section 1.3.1). These studies indicate that a high salt intake upregulates components of the brain RAS.

The regulation of receptors would be expected to play a decisive role in modifying the activity of the brain RAS. AT₁ receptors, the main Ang II receptor subtype expressed in areas involved in body fluid homeostasis, belong to a G protein-coupled receptor family and mediates phosphoinositide hydrolysis whereas the AT₂ receptor mediates changes in cyclic GMP levels (Sumners and Raizada, 1993). The receptors are regulated at various levels including regulation of gene expression, regulation by its ligands ie homologous

downregulation and rapid onset desensitization (tachyphylaxis), and by regulation of signal transduction mechanisms. The 5'-noncoding region of the rat AT_{1A} gene contains a TATA box, three glucocorticoid responsive elements, AP1 and AP2 binding sites and a cAMP recognition element suggesting that its expression can be regulated via these sites (Harris and Inagami, 1995). Ang II stimulates AT₁ receptor internalization of cloned AT₁ receptors transiently expressed in COS-7 cells but this effect is not elicited by phorbol esters or other agents that mimic the process of Ang II signaling (Harris and Inagami, 1995). However, in primary cultures of neuronal cells, phorbol esters and forskolin cause a time- and dose-dependent increase in AT₁ receptor mRNA indicating that protein kinase C and protein kinase A may regulate AT₁ receptor gene expression in neurons (Lu et al. 1994). Furthermore, as forskolin failed to influence AT₁ receptor mRNA or Ang II receptors in astroglial cultures, it appears that neuronal AT₁ receptor expression and regulation is distinct from that in astroglial cells. Few studies have addressed the in vivo regulation of brain AT₁ receptors by high salt diet. In Dahl S rats, high dietary salt intake increases AT₁ receptor mRNA levels in brain homogenates (Strehlow et al. 1999). However, it is not known in which nuclei the mRNA for the receptor increased and whether the AT₁ receptor protein expression was increased as well.

1.3 Salt-Induced Hypertension in Dahl S Rats

Lewis K. Dahl selectively bred Sprague Dawley rats for sensitivity or resistance to the hypertensive effect of high salt diet. In Dahl S rats, a high dietary salt intake exacerbates the development of hypertension whereas their salt-resistant controls, Dahl salt-resistant

(Dahl R) rats, remain normotensive. When fed a regular salt diet containing less than 1 % NaCl, Dahl S rats take longer to become (mildly) hypertensive and by 9 weeks of age are often reported to have blood pressures 15-20 mmHg higher than age matched Dahl R rats.

1.3.1 Peripheral Mechanisms in Dahl S Hypertension

In Dahl S rats, an 8 % NaCl diet causes a rapid onset of hypertension, initially caused by blood volume expansion and elevated cardiac output, and later maintained by an increase in total peripheral resistance as cardiac output rapidly returns to normal despite persisting hypervolemia (Simchon et al.1989; Simchon et al.1991).

A number of mechanisms that contribute to the development and maintenance of hypertension in this model have been identified. Dahl et al. (1974; Dahl and Heine, 1975) demonstrated that a kidney from a Dahl S rat transplanted into a bilaterally nephrectomized Dahl R rat caused the development of hypertension in the Dahl R rat. Conversely, a Dahl R kidney transplanted into a Dahl S rat with either bilateral or unilateral nephrectomy attenuated the development of hypertension implicating a renal functional defect in the pathogenesis of Dahl S hypertension. The pressure-natriuresis curve of Dahl S kidneys is shifted towards the pressure axis (Tobian et al.1978) resulting in salt and water retention when Dahl rats are fed a high salt diet. Most studies have indicated that Dahl S rats retain sodium on a high salt diet, probably due to a renal functional defect and an inappropriate renal vasoconstriction (Roman, 1986; Roman and Kaldunski, 1991; Tsunooka and Morita, 1997; Simchon et al.1999). Using an isolated perfused kidney preparation, Tobian et al. (1979) provided evidence for a humoral agent in plasma of Dahl S rats that causes sodium retention. In addition, a number of

biochemical derangements that might impair kidney function in Dahl S hypertension have been identified including a decreased production of cyclic GMP (Simchon et al.1996), kallikrein (Sustarsic et al.1981), prostaglandin E₂ (Limas et al.1981) or dopamine (Sakamoto et al.1994). Furthermore, administration of L-arginine, the substrate for nitric oxide production, improves salt excretion, returns the renal pressure-natriuresis curve to normal and prevents salt-induced hypertension in Dahl S rats indicating that an inadequate production of nitric oxide impairs kidney function in Dahl S hypertension (Chen and Sanders, 1991; Chen et al.1993).

Adrenalectomy prevents the development of Dahl S hypertension (Iwai et al.1969). However, this study did not clarify whether steroidal substitution was used. As adrenalectomy without adequate steroidal substitution prevents nearly all forms of hypertension, the role of adrenal function in the development of salt-induced hypertension in this model requires further clarification. Adrenals of Dahl S rats exhibit increased 18-hydroxylation and lower 11- β -hydroxylation of deoxycorticosterone than Dahl R rats, but high salt diet had no effect on this pattern (Rapp and Dahl, 1971a; Rapp and Dahl, 1971b). Alleles at the genetic locus responsible for the steroid pattern, *Hyp-1*, cosegregate with the increase in blood pressure with high salt diet in F₂ populations of crosses of Dahl S and R rats, and appear to be responsible for about a 16 mmHg increase in blood pressure in Dahl S rats on high salt diet (Rapp and Dahl, 1972).

Defects in vascular function have also been implicated in Dahl S hypertension. Inhibition of endogenous microvascular nitric oxide synthesis causes arteriolar constriction in Dahl S rats on regular salt diet but had no effect on vascular tone in Dahl S

rats on high salt diet (Boegehold, 1992) indicating that hypertensive Dahl S rats have suppressed basal arteriolar synthesis/release of nitric oxide, a major determinant of blood vessel tone. This impaired production/release of nitric oxide may contribute to the increase in vascular resistance and therefore, maintenance of hypertension in these rats. Plasma OLC increased more rapidly in Dahl S compared to Dahl R rats on high salt diet being significantly higher following 1 week of high salt diet (Leenen et al.1994) suggesting that plasma OLC may contribute to the development of hypertension via inhibition of the Na^+K^+ -ATPase in vasculature leading to a subsequent increase in cytosolic Ca^{2+} causing vasoconstriction. However, following 3 weeks of high salt diet plasma OLC was comparable in Dahl S and R rats (Leenen et al.1994) indicating that it is unlikely that plasma OLC contributes to the maintenance of hypertension in this model. Ouabain-sensitive Na^+K^+ -ATPase activity was increased in the aorta of hypertensive Dahl S rats compared to Dahl R rats (Overbeck et al.1981). This appears contrary to predicted effects of OLC on vasculature. However, as the aorta is not representative of resistance vessels it may not reflect effects of circulating OLC. Alternatively, it is possible that OLC may have dissociated during the assay and the increased Na^+K^+ -ATPase activity reflects an upregulation of the enzyme.

1.3.2 Neural Mechanisms in Dahl S Hypertension

Neural mechanisms appear to be critical for the development of Dahl S hypertension as chemical sympathectomy (Friedman et al.1979; Takeshita et al.1979), ganglionic blockade (Gordon et al.1981) and lesions of the area surrounding the anteroventral third ventricle wall (Goto et al.1982) and the PVN (Azar et al.1981; Goto et al.1981b;

Ernsberger et al.1985; discussed in section 1.6) prevent or attenuate the development hypertension in this model.

The mechanisms by which high salt intake initiates central mechanisms that cause hypertension are unresolved. One possibility is through a differential change in CSF sodium concentration induced by high salt diet in Dahl S versus Dahl R rats. Intravenous administration of $^{22}\text{NaCl}$ resulted in a significantly greater increase in CSF and brain ^{22}Na in Dahl S rats than in Dahl R rats suggesting a greater permeability of the blood-brain barrier to sodium in Dahl S rats (Simchon et al.1999). A high salt diet also caused a further increase in CSF ^{22}Na compared to regular salt diet in Dahl S rats but not in Dahl R rats (Simchon et al.1999). Nakamura and Cowley Jr. (1989) demonstrated that 3 days after the initiation of a high salt (4 % NaCl) intake, CSF sodium concentration increased ~ 4 mM/l and remained elevated for the duration of the diet in Dahl S compared to concentrations in Dahl R rats. As the increase in blood pressure in Dahl S rats on the high salt diet began before the increase in CSF sodium concentration, the increase in CSF sodium concentration may not be the stimulus for the initial rise in blood pressure but could contribute to subsequent increases in blood pressure. However, in this study initial changes in CSF sodium concentrations may have been underestimated as CSF samples were collected over a 24 h period between 8 and 11 am. Because rats are nocturnal animals, this may not accurately reflect increases following dietary salt intake at night. In addition, in Dahl S rats, pressor and renal sympathetic nerve activity responses to icv sodium were twice as large as that in Dahl R rats even on a regular salt diet (Goto et al.1981a; Huang and Leenen, 2000b), indicating an increased sensitivity of pathways

contributing to responses to an increase in CSF sodium in Dahl S rats. Therefore, it appears that salt-induced hypertension in Dahl S rats may result from a larger increase in CSF sodium concentration in Dahl S versus R rats on high salt diet compounded by the greater sensitivity of pressor mechanisms to an increase in CSF sodium concentrations in Dahl S rats.

The increase in blood pressure caused by a high salt diet in Dahl S rats is associated with decreases in sympathoinhibition and increases in sympathoexcitation resulting in an increase in sympathetic neuronal activity (Huang and Leenen, 1994). In these studies sympathetic activity was estimated by assessment of renal sympathetic nerve activity and cardiovascular responses to air jet stress and to icv injection of guanabenz, an α_2 -adrenoceptor agonist. On high salt diet, Dahl S rats exhibit an enhanced response to guanabenz possibly reflecting an upregulation and/or decreased α_2 -receptor occupancy in the anterior hypothalamus as a result of decreased norepinephrine release to sympathoinhibitory neurons (Koepke et al.1988; Wyss et al.1988).

1.3.2.1 Brain OLC and the Brain RAS Mediate Dahl S Hypertension

A number of functional changes in central mechanisms have been reported in Dahl S hypertension including differences in regulation of central nitric oxide, OLC and the RAS. In this section we focus on the role of brain OLC and the RAS. Other mechanisms were considered outside the scope of this review.

In Dahl S rats centrally released OLC plays a critical role in the development or exacerbation of hypertension when these rats are fed a high salt diet. Dahl S rats on high dietary salt intake exhibit increases in hypothalamic, pituitary and pons, as well as adrenal

OLC content compared to their normotensive controls on similar diet that precede the development of hypertension (Leenen et al.1993b; Leenen et al.1994). Concomitant chronic icv administration of fab fragments that bind ouabain and related steroids with high affinity (Butler et al.1977; Leenen et al.1995) prevented the salt-induced sympathoexcitation and increase in blood pressure (Huang and Leenen, 1994; Huang and Leenen, 1995). Administration of the fab fragments iv at doses similar to that were effective in preventing salt-induced hypertension in these rats when used icv, were ineffective in preventing salt-induced increases in blood pressure indicating that the antihypertensive effect of the fab fragments administered icv at the dose used were central. Although there is a small increase in brain OLC content in normotensive rats on a high salt diet, there is no attendant increase in sympathetic activity or blood pressure and icv administration of the fab fragments do not change blood pressure. It is possible that the OLC concentrations in Dahl R rats on high salt diet do not reach the threshold required for an increase in blood pressure.

The brain RAS also appears to play a crucial role in salt induced hypertension in Dahl S rats. Chronic central infusion of CV-11974, a non-peptide AT₁ receptor antagonist, prevented the development of hypertension in Dahl-Iwai salt-sensitive rats on high salt diet (Teruya et al.1995). Our group demonstrated that chronic blockade of central AT₁ receptors by icv losartan in Dahl S rats on high salt diet prevented exacerbation of hypertension and normalized the salt-induced enhancement of mean arterial pressure and renal sympathetic nerve activity responses to icv guanabenz and to air jet stress (Huang and Leenen, 1998). These studies suggest that AT₁ receptor activation contributes to the

increase in sympathetic activity and exacerbation of hypertension in Dahl S and Dahl-Iwai salt-sensitive rats on a high salt diet. As the activity of the circulatory RAS is decreased or remains unchanged on a high salt diet in Dahl S rats (Bouhnik et al. 1992; Zhao et al. 2000) it is unlikely that central AT₁ receptor activation is due to circulating Ang II in Dahl S hypertension. Pressor responses to icv Ang II were twice as large in Dahl S rats compared to Dahl R rats on regular salt diet suggesting an increased sensitivity to Ang II (Goto et al. 1981a). Biochemical assessment of activity of the brain RAS in this model is limited. Strehlow et al. (1999) reported an increase in AT₁ receptor mRNA in brain homogenates of hypertensive Dahl S rats following 6 weeks of high salt diet. A recent study by our group assessed changes in ACE mRNA and ACE activity within dissected brain regions during the development of hypertension in Dahl S rats (Zhao et al. 2000). ACE mRNA, assessed by quantitative reverse transcriptase-polymerase chain reaction, and ACE activity in hypothalamic and pons homogenates increased in Dahl S rats on regular salt diet compared to their normotensive controls at 9 weeks of age. When fed a high salt diet from 4-9 weeks of age ACE mRNA increased further, approximately 3-fold in the hypothalamus and about 2-fold in the pons, in Dahl S but not Dahl R rats. We also demonstrated an increase in ACE activity in the hypothalamus and pons in Dahl S rats on high salt diet compared with Dahl S rats on regular salt diet and Dahl R rats on either diet. Concomitant icv administration of fab fragments that bind OLC prevented the salt-induced increase in blood pressure as well attenuated the increases in ACE mRNA and activity in the hypothalamus and pons in Dahl S rats on high salt diet to levels in Dahl S rats on regular salt. This suggests that the

increase in ACE that accompanies salt-induced hypertension in Dahl S rats is OLC dependent.

1.4 Pressor Responses to Centrally Administered NaCl

As discussed in section 1.3.2, high sodium intake may cause larger increases in CSF sodium in salt-sensitive versus resistant rats. In addition, salt-sensitive rats may have a greater response to similar increases in CSF sodium. As an increase in CSF sodium concentration may initiate or contribute to the development of salt-induced hypertension (discussed in section 1.3.2), icv administration of hypertonic saline has been used to investigate central mechanisms that may mediate salt-induced hypertension. In normotensive rats, icv injection of hypertonic saline causes vasopressin release and sympathoexcitation in the presence of a vasopressin antagonist, leading to a pressor response; a drinking response and natriuresis (Rohmeiss et al.1995b; Huang and Leenen, 1996b). Ang II, administered icv, elicits very similar responses (see section 1.2.2). The sympathoexcitatory and pressor responses to central hypertonic saline injections as well as central injections of Ang II are inhibited by prior icv administration of an AT₁ receptor blocker, losartan (Rohmeiss et al.1995b; Huang and Leenen, 1996b). This suggests that the responses to both icv hypertonic saline and Ang II are mediated by AT₁ receptors. Blockade of central AT₂ receptors results in exaggerated vasopressin release and dipsogenic responses to icv Ang II and hypertonic NaCl suggesting that AT₂ receptors exert a tonic inhibitory effect on AT₁ receptor mediated osmoregulation (Hohle et al.1995; Hohle et al.1996).

Ouabain administered icv causes similar sympathoexcitatory and pressor responses as that seen with icv hypertonic saline or Ang II and these responses can also be prevented by icv pretreatment with captopril, an angiotensin converting enzyme inhibitor, or the Ang II receptor antagonists, saralasin and losartan (Takahashi et al.1984; Huang and Leenen, 1996b). On the other hand, fab fragments that bind ouabain and related steroids, block responses to hypertonic saline and ouabain but not responses to Ang II (Huang and Leenen, 1996b). This would suggest that an increase in central sodium concentration releases brain OLC which exerts its sympathoexcitatory and pressor effects mostly via brain Ang II.

Chronic icv infusion of hypertonic saline in normotensive Wistar rats cause an increase in brain OLC, sympathoexcitation and an increase in blood pressure mimicking the effects of high dietary sodium in salt-sensitive rats (Huang et al.1998, Appendix Table 1). As sympathoexcitatory and pressor responses are elicited by icv hypertonic saline but not by other hypertonic solutions or ammonium chloride, it is likely that the responses to hypertonic saline are attributable to central sodium ion concentration rather than hyperosmolality or chloride ion concentration (Bunag and Miyajima, 1984). Concomitant icv infusion of either fab fragments that bind ouabain, or losartan, abolished the sympathoexcitation and increase in blood pressure supporting the role of both brain OLC and the brain RAS in mediating the effects of acute or chronic increases in central sodium (Huang et al.1998). Thus if changes in central sodium concentrations do indeed occur on high sodium intake, these cause a similar pattern of central changes as caused by high sodium intake in Dahl S rats and may therefore play a major role.

1.5 Ouabain-Induced Hypertension

Chronic ouabain administration, either intravenously, subcutaneously or icv, causes moderate hypertension in conscious rats (Yuan et al.1993; Huang et al.1994; Manunta et al.1994; Wang et al.1997; Veerasingham et al.2000a). Some studies have failed to demonstrate a hypertensive effect with chronic ouabain treatment (Yasujima et al.1986; Sekihara et al.1992; Li et al.1995; Wang et al.1999). These studies either used lower doses (Li et al.1995; Wang et al.1999), once weekly doses (Sekihara et al.1992), or shorter treatment periods (Yasujima et al.1986) as blood pressure does not increase significantly during the first week of peripheral ouabain administration (Manunta et al.1994). The mechanisms responsible for ouabain-induced hypertension are not yet resolved. Chronic ouabain treatment is associated with an increase in total peripheral resistance (Yuan et al.1993) and the authors therefore suggested that inhibition of Na⁺K⁺-ATPase activity in vascular smooth muscle, resulting in vasoconstriction, may contribute to ouabain-induced hypertension. However, hypertension caused by chronic ouabain administration is reversed by ganglionic blockade (Huang et al.1994) and therefore more likely to be due to an increase in sympathetic outflow resulting in increased vascular tone. The sympathoexcitatory response to air-jet stress and sympathoinhibitory response to icv guanabenz are enhanced in ouabain-treated rats (Huang and Leenen, 1999). The enhanced response to guanabenz following ouabain treatment is consistent with a decrease in activity in sympathoinhibitory pathways, resulting in an enhanced response to an exogenous α_2 -adrenoceptor agonist (Koepke et al.1988). Ouabain, a polar compound, does not readily cross the blood brain barrier but accumulates in the hypothalamus, pons

and pituitary following chronic systemic administration (Huang et al.1994; Manunta et al.1994). Ouabain-induced hypertension is prevented by concurrent icv infusion of fab fragments that bind ouabain (Huang et al.1994). It is therefore likely that central mechanisms play a significant role in the development of ouabain-induced hypertension. The delay in the development of hypertension in response to peripheral ouabain administration may be because several days are required for ouabain to accumulate in the brain in amounts necessary to cause a significant inhibition of central Na^+K^+ -ATPase. In addition, ouabain, like other cardiac glycosides, may augment arterial baroreflex function which would temporarily inhibit increases in blood pressure (Ferrari et al.1981). In normotensive rats treated with ouabain and high salt diet, chronic sinoaortic denervation results in increases in blood pressure within 5 days to the same extent as 12 days of ouabain and high salt treatment in rats with intact arterial baroreflex mechanisms (Huang et al.1999) indicating that the baroreflex opposes the pressor response to combined ouabain and high salt treatment.

We recently demonstrated that ouabain-induced sympathoexcitation and hypertension in rats are also prevented by concomitant icv administration of losartan (Huang and Leenen, 1999). Losartan also significantly improved the sensitivity of the arterial baroreflex control of renal sympathetic nerve activity and heart rate preventing the ouabain-induced tendency toward desensitization of arterial baroreflex. This study indicates that AT_1 receptor activation occurs in the pathways mediating the effects of chronic ouabain administration, similar to that seen with acute icv injection of ouabain (section 1.4). Other neurotransmitters acting within these pathways may also play a role

in mediating ouabain-induced hypertension. In anaesthetized normotensive rats, the sympathoexcitatory and pressor actions of ouabain within the RVLM appear to be partly mediated by acetylcholine as prior microinjection of gallamine, a M2 muscarinic antagonist, inhibited subsequent ouabain-induced responses (Teruya et al.1997). Local perfusion of ouabain into the nucleus accumbens causes extracellular release of dopamine and γ -aminobutyric acid. (Tanganelli et al.1994). Whether similar mechanisms contribute to the hypertensive effect of chronic ouabain administration is yet to be determined.

1.6 Brain Regions Involved in Regulation of Salt-Induced Hypertension

The central pathways that mediate sympathoexcitation and hypertension induced by either central sodium administration or a high salt diet are not well understood. Expression of inducible transcription factors, proteins encoded by immediate early genes, are involved in regulation of target gene transcription and has been widely used to identify neuronal activation in multi-synaptic pathways. The most widely used is the expression of members of the *fos* and *jun* gene families. Hypertonic saline, administered icv, induced the expression of c-Fos and c-Jun within the OVLT, MnPO, SFO, PVN and SON (Moellenhoff et al.1998). Central pretreatment with losartan prevented the expression of c-Fos and c-Jun in these areas (Moellenhoff et al.1998). Central administration of Ang II causes similar sympathoexcitatory and pressor responses, as well as similar patterns of immediate early gene expression to that seen with icv hypertonic saline (McKinley et al.1995; Huang and Leenen, 1996b). These studies suggest that an angiotensinergic pathway(s) mediates the response to central sodium administration.

Whether the same pathway(s) is involved in mediating the hypertensive effect of a chronic elevation of CSF Na⁺ concentration is not known.

During chronic exposure to the inducing stimulus Fos down-regulates (Winston et al.1990; Rosen et al.1994) making it unsuitable for detection of chronic neuronal activation. Protein products of other members of the *c-fos* gene family, Fos B, Fos Related Antigen (Fra) 1 and Fra 2, exhibit prolonged induction times and persistent expression during chronic exposure to the stimulus (Morgan and Curran, 1989; Sharp et al.1991). Detection of these proteins, Fos-like immunoreactivity (FLI), has been utilized to identify neuronal populations, including cardiovascular regulatory areas (Budzikowski et al.1996; Budzikowski et al.1998; Vahid-Ansari and Leenen, 1998), activated in response to a chronic stimulus. We assessed FLI during the development of salt-induced hypertension in Dahl S rats (Budzikowski et al.1998). A differential increase in neuronal FLI was observed in the MnPO, PVN and SON in hypertensive Dahl S rats compared to Dahl R rats on high salt diet suggesting an involvement of these areas in mediating salt-induced hypertension.

Central neuronal activation mechanisms in ouabain-induced hypertension appear to be distinct from that in the Dahl S rats with significant increases in FLI only within the SON and LC, despite the fact that development of hypertension on high dietary salt intake in Dahl S rats is dependent on an increase in endogenous brain ouabain-like compounds (Huang and Leenen, 1994; Huang and Leenen, 1995). Within the SON, ouabain-induced FLI mostly within the ventral half of the nucleus suggesting a possible involvement of vasopressinergic neurons (Appendix, Figure 1). Dense immunoreactivity to endogenous

OLC has been demonstrated in magnocellular neurons of the SON and was colocalized with vasopressin, and to a lesser extent with oxytocin (Yamada et al.1987; Ihara et al.1988; Yamada et al.1992b). It is therefore tempting to speculate that the increase in FLI expression in ouabain-induced hypertension in the SON relates to an accumulation of exogenous ouabain in this nucleus. The SON also receives noradrenergic input from the LC which may be involved in the baroreflex regulation of vasopressin release (Banks and Harris, 1984). An increase in FLI expression within the LC in ouabain treated rats may therefore relate to a compensatory mechanism that attempts to oppose the increase in blood pressure. Differences in FLI expression during the development of ouabain-induced hypertension and Dahl S hypertension may be accounted for by the fact that in addition to neurons that express FLI in response to OLC, high salt diet in Dahl S rats may activate a number of neuronal populations involved either in osmo-regulation or leading up to the production of OLC.

Pressor responsiveness to electrical stimulation of the ventromedial hypothalamus is enhanced in Dahl S rats compared to Dahl R rats (Bunag et al.1983) suggesting that this region, or fibres that pass through this region, contribute to differences in blood pressure regulation between these strains. Hypertension in Dahl S rats on a high salt diet is attenuated by electrolytic lesions of the PVN (Goto et al.1981b; Ernsberger et al.1985), whereas electrolytic or radio-frequency lesions of the PVN, suprachiasmatic nuclei (SCh) and the intervening periventricular area (Azar et al.1981; Ernsberger et al.1985) prevented the development of hypertension. indicating that the PVN mediates salt-induced hypertension. Lesions of only the SCh enhanced the increase in blood pressure in

Dahl S rats fed a high salt diet (Goto et al.1981b).

1.6.1 The Anteroventral Third Ventricle (AV3V) Region

Another area that has been implicated in mediating salt-induced hypertension is the anteroventral third ventricle (AV3V) region. The AV3V integrates cardiovascular regulation and fluid and electrolyte homeostasis (Brody, 1988; McKinley et al.1996). Neural pathways arising from the AV3V have been shown to influence blood pressure and participate in modulation of the baroreceptor reflex. In addition, the integrity of this region is essential for the development of several forms of experimental hypertension (Brody et al.1978) including salt-induced hypertension in Dahl S rats (Goto et al.1982).

The AV3V was originally designated in the rat brain on the basis of a pattern of physiological changes and deficits produced when this area was destroyed or isolated from the rest of the CNS. As its name implies, the AV3V forms the anterior wall of the ventral part of the third ventricle (Figure 3). The region extends from the optic chiasm dorsally to the anterior commissure. The area comprises the OVLT dorso-ventrally, just dorsal to the optic chiasm, and more dorsally the ventral MnPO up to the level where it is partially interrupted by the anterior commissure. More caudally, it includes the anteroventral periventricular nucleus and the anterior part of periventricular preoptic nucleus.

The AV3V has direct afferent and efferent neural connections with several cerebral regions involved in cardiovascular and body fluid regulation as well as internal interconnections between its component nuclei.

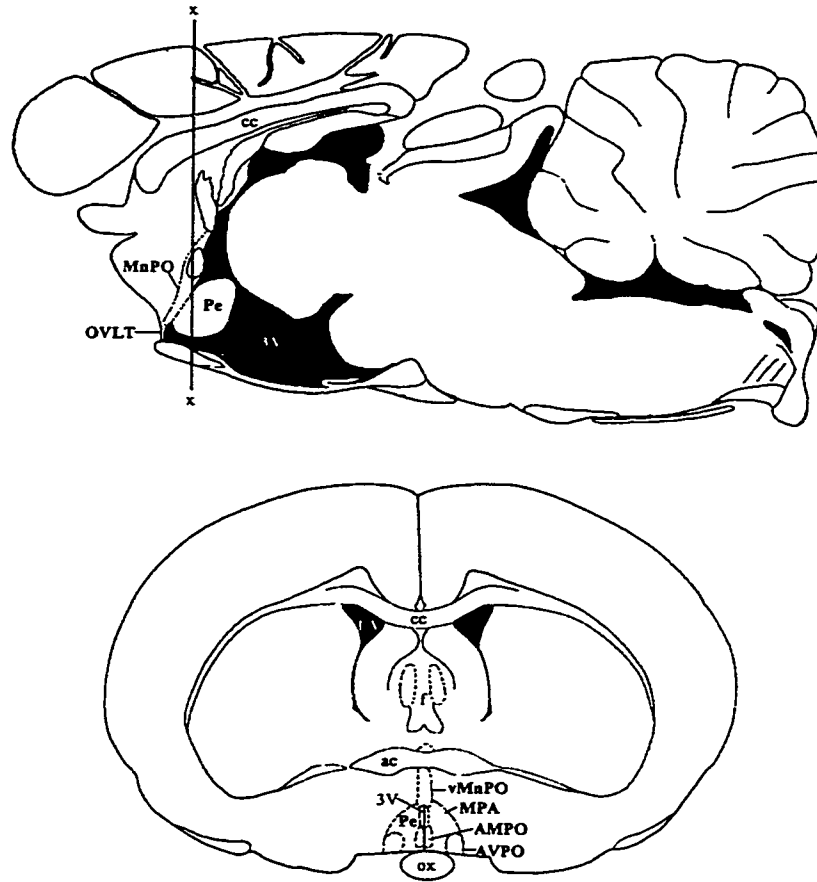


Figure 3. A schematic representation of a mid-sagittal (upper diagram) and a coronal section (at plane x-x, lower diagram) through the AV3V region of the rat brain. Nuclei of the AV3V region are highlighted in bold font. ac, anterior commissure; AMPO, anterior medial preoptic nucleus; AVPO, anteroventral preoptic nucleus; cc, corpus callosum; f, fornix; LV, lateral venticle; vMnPO, median preoptic nucleus, ventral; MPA, medial preoptic area; OVLT, organum vasculosum laminae terminalis; ox, optic chiasm; Pe, periventricular nucleus; 3V, third ventricle.

In addition, the AV3V contains fibres of passage that course through the region en route to other target nuclei, most notably a pathway from the SFO to the PVN. The afferent and efferent connectivity of the main nuclei of the AV3V, the OVLT and MnPO, to other cardiovascular regulatory areas are summarized in Table 1.

Table 1. Neural Connections of the OVLT and MnPO

	OVLT	MnPO
Lamina terminalis		
Subfornical organ	A, E	A, E
Median preoptic n.	A, E	
OVLT		A, E
Preoptic region		
Anteroventral periventricular preoptic n.		A
Lateral preoptic area	A	A
Median preoptic area	A	E
Preoptic periventricular n.		A
Hypothalamus		
Anterior hypothalamic area	A	
Arcuate n.	A	A
Dorsal perifornical area		E
Lateral hypothalamus	A	
Paraventricular n.	A, E	A, E
Supraoptic n.	E	E
Suprachiasmatic n.		E
Ventromedial hypothalamic n.	A	
Midbrain		
A5 and A7 noradrenergic group		E
Dorsal raphe (B7,B8)		E
Locus coeruleus (A6)	A	A, E
Central grey	A	A
Lateral parabrachial n.		A, E
Raphe magnus (B3)		E
Raphe pontis (B5)		A, E
Hindbrain		
Ventrolateral medulla		A
Nucleus Tractus Solitarius		A

Summary of reported neural connections of the organum vasculosum laminae terminalis (OVLT) and median preoptic nucleus (MnPO) . A indicates an afferent input to, and E indicates efferents from the OVLT or MnPO to the corresponding site in the left column. n, nucleus. (Adapted from McKinley et al. 1999)

The MnPO has reciprocal connections with the OVLT and SFO. In addition, these two CVO are reciprocally connected. All nuclei of the lamina terminalis ie the OVLT, MnPO

and SFO provide direct innervation of the SON and the magnocellular part of the PVN providing the major forebrain input to these vasopressin secreting neurons. The SFO and the OVLT also send inputs via a synaptic relay in the MnPO to magnocellular neurons of the SON and PVN.

An interaction of OLC and Ang II in this region is supported by histological findings. Immunoreactivity to ouabain in nerve terminals is observed in the AV3V including the OVLT (Yamada et al.1992a). In these areas AT_1 receptors and other components of the brain RAS are also densely distributed (Plunkett et al.1987; Bunnemann et al.1992). Studies that utilized electrolytic lesions of the AV3V showed that this region mediates the pressor effects of icv injections of hypertonic saline, Ang II and ouabain (Hartle et al.1982; Buggy et al.1984; Takahashi et al.1984). AV3V lesions also attenuated volume-expansion dependent release of a Na^+K^+ -ATPase inhibitor (Songu-Mize et al.1987).

1.7 Rationale and Overview of the Study

Several nuclei/areas have been identified to be involved in the sympathoexcitatory and hypertensive responses to central sodium and high salt intake in Dahl S rat. However, at present it is still quite unclear what the actual stimulus is and what the actual pathways are leading to sympathoexcitation.

In the brain, both OLC and AT_1 receptor stimulation appear to contribute to the increase in sympathetic outflow and development of Dahl S hypertension as evidenced by the data presented. Our group has hypothesized that a high dietary salt intake in salt-sensitive rats alters central control of blood pressure by transiently or intermittently

increasing CSF sodium concentration causing an increase in brain OLC and subsequent AT_1 receptor stimulation leading to an increase in sympathetic activity and hypertension. To gain further insight into mechanisms and brain regions involved in salt-induced hypertension we have utilized two rat models that mimic particular mechanisms. We simulated the increase in CSF Na^+ concentration in Dahl S rats on high salt diet using chronic icv administration of hypertonic NaCl (Huang et al.1998). A chronic elevation in CSF Na^+ concentration of ~ 6 mmol/l causes an increase in brain OLC content, and an increase in sympathetic activity and blood pressure, similar to that seen in Dahl S hypertension (Huang et al.1998). In addition, we have used chronic subcutaneous ouabain administration to mimic the increase in brain OLC seen in salt-induced hypertension in Dahl S rats. In Dahl S hypertension central AT_1 receptor blockade prevents the development of sympathoexcitation and hypertension indicating that the brain RAS plays a critical role (Teruya et al.1995; Huang and Leenen, 1998). Central AT_1 receptor blockade also prevents the increase in sympathetic activity and hypertension caused by a chronic elevation of CSF Na^+ concentration (Huang et al.1998, Appendix Table 3) and subcutaneous ouabain administration (Huang and Leenen, 1999). However, particular brain areas involved are not known as these studies utilized icv administration of the AT_1 receptor antagonist.

The AV3V may be expected to be involved in cardiovascular control based on its neuronal connections to numerous cardiovascular areas of the brain. Studies using electrolytic ablation of the AV3V have indicated that this region mediates Dahl S hypertension as well as pressor responses to acute icv administration of hypertonic saline

and ouabain (Goto et al.1982; Buggy et al.1984; Takahashi et al.1984). However, electrolytic ablation of the AV3V destroys cell bodies as well as fibres of passage. There are fibres of passage that traverse the AV3V, notably a pathway from the SFO to the OVLT (Oldfield and McKinley, 1995). Interruption of these connections could account for some of the effects of an electrolytic lesion. Furthermore, the AV3V area is a large region including a number of component nuclei (see section 1.6.1). Therefore the results from these studies using electrolytic lesions are difficult to interpret. As the AV3V contains high densities of AT₁ receptors, especially within its most ventral nucleus, the OVLT (Shigematsu et al. 1986; Plunkett et al. 1987; Lenkei et al. 1995), it is possible that an angiotensinergic mechanism within this area mediates salt-induced hypertension. We **hypothesized that the area limited to the ventral part of the AV3V region (vAV3V), which includes the OVLT and the part of the MnPO that overlies the dorsal cap of the OVLT, mediates salt-induced sympathoexcitation and hypertension via AT₁ receptor stimulation.**

We determined whether the vAV3V mediates salt-induced hypertension by utilizing an excitatory amino acid, ibotenic acid, to selectively destroy neurons within the vAV3V. The extent of lesions are most frequently determined by the detection of gliosis following the lesion. However, discrete lesions are often difficult to discern and gliosis typically extends outside the limits of a lesioned area. We therefore used the absence of immunoreactivity to Neu-N, a neuron specific nuclear antigen, as previously described by Morassutti et al. (1994) to determine the extent of the lesion. We also attempted to identify areas in which AT₁ receptor activation mediates salt-induced hypertension by

assessing changes in Ang II receptor densities during the development of Dahl S hypertension.

The objectives of the research presented were:

1. to determine whether the vAV3V ie the OVLT and overlying MnPO, mediates pressor responses to icv injections of hypertonic saline, ouabain and Ang II.
2. to determine if the vAV3V mediates hypertension induced by a chronic icv hypertonic NaCl administration and chronic sc ouabain.
3. to assess whether the development of salt-induced hypertension in Dahl S rats is accompanied by differential changes in brain AT₁ receptor densities in the vAV3V area compared to their salt-resistant controls, Dahl R rats.

Chapter 2

Excitotoxic lesions of the ventral anteroventral third ventricle and pressor responses to central sodium, ouabain and angiotensin II

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**Short Communication
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ABSTRACT

To clarify the role of neurones in the anteroventral third ventricle (AV3V) area in cardiovascular responses to CSF sodium, ouabain and angiotensin II (ANG II), we employed excitotoxic lesions of the ventral AV3V (vAV3V). In conscious lesioned Wistar rats with systemic vasopressin blockade, pressor and tachycardiac responses to intracerebroventricular (i.c.v.) artificial CSF containing 0.3 M NaCl or ouabain were significantly attenuated by 26-32% whereas responses to ANG II were not affected. Thus, in rats with systemic blockade of vasopressin mechanisms, the vAV3V region partially mediates acute pressor responses to i.c.v. sodium and ouabain but not to ANG II.

Keywords: Anteroventral third ventricle; Excitotoxic lesion; Ouabain; Cerebrospinal fluid sodium; Angiotensin II

Both brain endogenous ouabain-like compound(s) ("ouabain") and brain angiotensin II (ANG II) are implicated in the pressor responses to an increase in brain sodium concentration. Intracerebroventricular (i.c.v.) administration of hypertonic saline (HS) and ouabain cause similar sympathoexcitatory and pressor responses which are attenuated or abolished by pretreatment with Fab fragments which bind ouabain and related steroids [4]. This suggests that these responses to centrally administered HS are mediated by brain "ouabain". Icv ANG II also mimics responses to i.c.v. hypertonic saline and AT₁ receptor blockade attenuates the pressor response to HS [5] consistent with a role for AT₁

receptor stimulation. I.c.v. losartan blunts responses to i.c.v. hypertonic saline, ouabain and ANG II whereas Fab fragments only block responses to hypertonic saline and ouabain [5]. This would suggest that an increase in central sodium concentration increases brain "ouabain", exerting its sympathoexcitatory and pressor effects mostly via brain ANG II.

Tissue surrounding the most anteroventral part of the third ventricle (AV3V) integrates fluid and electrolyte homeostasis and cardiovascular regulation [6,7]. Ventricular obstruction studies demonstrate that HS or ANG II administered i.c.v. need to contact the anterior third ventricle to cause pressor effects [1,3,8]. In addition, electrolytic ablation of the AV3V abolishes pressor responses to both icv HS and ANG II [1,2] and attenuates pressor responses to i.c.v. ouabain [15]. In vitro single unit recordings demonstrate that the median preoptic nucleus (MnPO), a component of the AV3V region, is sensitive to HS and ANG II [16]. The AV3V contains ANG II receptors and the organum vasculosum of the lamina terminalis (OVLT), the most ventral component of the AV3V, contains nerve terminals densely immunoreactive to ouabain [9,12,18]. This region may therefore be involved in mediating the pressor effects of an increase in CSF sodium concentration via brain "ouabain" and ANG II.

In the present study excitotoxic lesioning was used to clarify the role of the ventral AV3V (vAV3V) region in pressor responses to i.c.v. sodium, ANG II and ouabain. Chemical lesions leave fibres of passage intact in contrast to studies using electrolytic ablation. As pressor responses to osmotic stimuli and ANG II involve arginine vasopressin (AVP) release and sympathetic neural activation, systemic AVP blockade was used to estimate the sympathetic component of the pressor responses.

Male Wistar rats (150-200 g, Charles River, Montreal, Canada) were lesioned in the vAV3V region (an angle of 6° to the midline, 0.1 mm posterior, 0.8 mm lateral to bregma and 8.2 mm deep to the dura) with 185 nl of ibotenic acid (10 µg/µl, Research Biochemicals International, Natick, MA, USA) in artificial CSF (aCSF) pressure ejected from a micropipette (tip OD 50 µm) under pentobarbital anaesthesia [14]. Sham-lesioned animals received only vehicle. A 23-gauge stainless steel guide cannula was fixed to the skull such that its lower end was ~ 0.3 mm above the lateral ventricle [4]. AV3V lesions result in adipsia. Lesioned rats were therefore postsurgically allowed access to sucrose solutions (10%, 5% and 2% sucrose in water for 2 days each) followed by regular water. Fluid intake and body weight were monitored.

Eight to 10 days after initial surgery, rats were reanesthetized by halothane/oxygen inhalation and instrumented with femoral arterial and venous catheters for recording mean arterial pressure (MAP) and heart rate (HR) and systemic injections respectively [4]. A 31-gauge tubing inserted into the guide cannula such that it protruded 0.8-1.0 mm into the ventricle was used for i.c.v. injections. All drugs were purchased from Sigma Chemical Co., St Louis, MO, USA.

After a recovery period of 3-4 h, resting MAP and HR were assessed in unrestrained rats. aCSF and aCSF containing 0.3 M NaCl (HNa-aCSF) were then infused i.c.v. (2 µl/min for 10 min) 15 min apart. After 25 min, two doses of ANG II (10 ng and 30 ng/1-3 µl aCSF) were injected i.c.v. at a 5 min interval. After a 35 min recovery two doses of ouabain (0.3 µg and 0.6 µg ouabain/1-2 µl aCSF) were injected i.c.v. at a 5 min interval. To assess the effect of injection volume, 3 µl aCSF was injected i.c.v. All treatments

were preceded 5 min by an i.v. injection of AVP antagonist (d(CH₂)₅Tyr(Me)AVP 30 µg/kg) [5] to exclude the systemic effects of endogenous vasopressin.

Rats were then deeply anaesthetized with sodium pentobarbital, injected i.c.v. with 2 µl of 25% India ink in saline to verify guide cannula placement and perfused transcardially with 50 ml of 10 mM phosphate buffered saline (PBS) followed by 150 ml 4% paraformaldehyde in 100 mM PBS containing 0.4% picric acid. The brain was removed, post-fixed for 90 min and cryoprotected in 10% sucrose in PBS for 1 wk. Serial 16 µm sections were cut on a cryostat and lesions were verified by visualizing an absence of immunoreactivity to A60, an antibody that recognizes a neurone specific nuclear antigen, NeuN [11]. All antibodies were diluted in 10 mM PBS (pH 7.4) containing 0.3% Triton X-100 and incubations were at 35 °C. A 80 min primary incubation with A60 (1:200) was followed by PBS washes and incubation in a sheep anti-mouse biotinylated Ig (Amersham, Oakville, Ont., 1:100) for 60 mins. After further PBS washes sections were incubated with streptavidin horse radish peroxidase complex (Amersham, Oakville, Ont., 1:200) for 30 mins. Immunoreactive cells were visualized using diaminobenzidine as a chromogen.

All data are expressed as means ± S.E.M. Statistically significant differences between resting MAP and HR and responses to i.c.v. treatments were determined by paired *t*-tests. Two-way ANOVA followed by Student-Newman-Keuls test was used for all other comparisons. The level of significance was set as $P < 0.05$.

Histological verification of lesions confirmed consistent lesioning of tissue limited to the vAV3V region, i.e. the OVLT and encroaching on the most ventral part of the

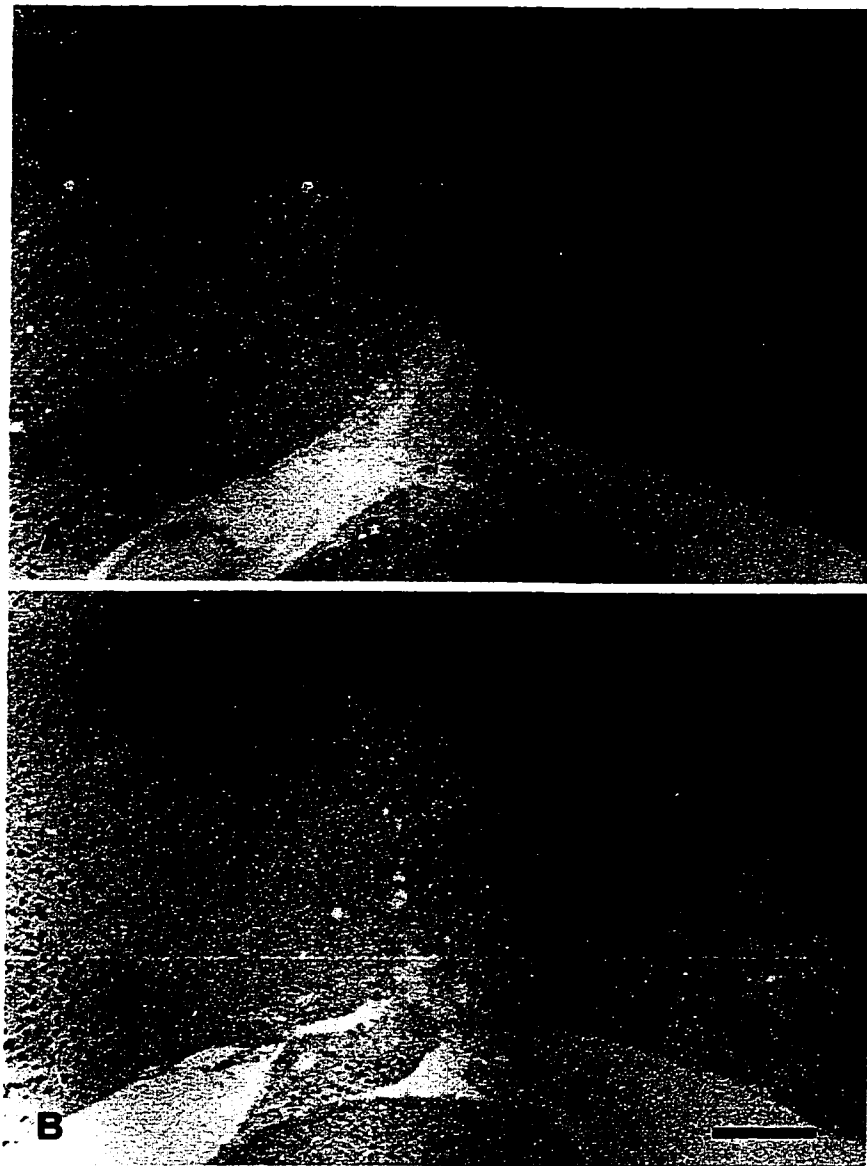


Fig. 1. Photomicrographs of NeuN immunoreactivity in the AV3V region of a typical sham-lesioned (A) and lesioned (B) rat. Arrowhead points to lesioned area. Scale bar indicates 250 μ m in A and B.

subcommissural median preoptic nucleus (MnPO) in 8 rats. Three rats with unilateral lesions were excluded from the analysis. Sham-lesioned rats ($n = 7$) showed no/minimal damage to the AV3V. Fig. 1 shows NeuN immunoreactivity in the AV3V of typical lesioned and sham-lesioned rats. Absence of damage to other periventricular areas and correct i.c.v. guide cannula placement was confirmed in these rats.

Fluid intake was similar in the sham-lesioned and lesioned groups on the day prior to the experiment (157 ± 16 and 153 ± 11 ml/kg/24 h respectively). Body weight gain was similar in both groups (62 ± 6 and 61 ± 15 g in sham- and vAV3V-lesioned rats respectively).

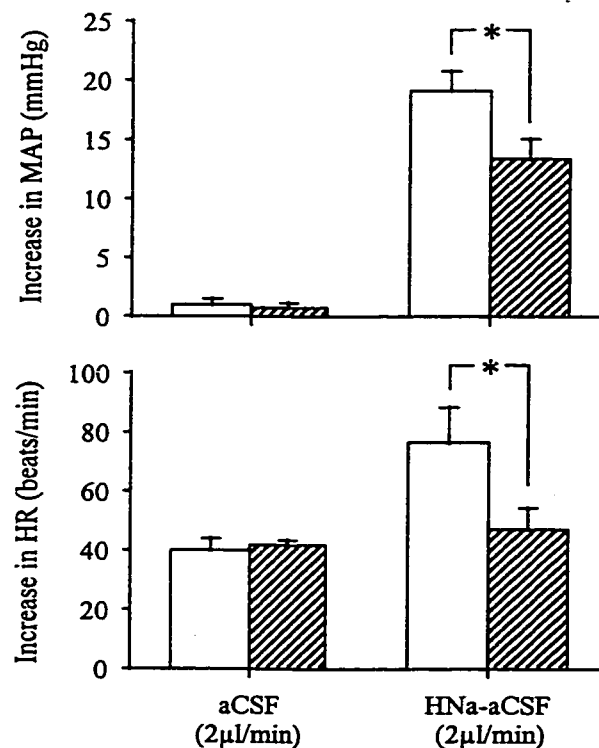


Fig. 2. Peak increases in MAP (upper panel) and HR (lower panel) in response to i.c.v. aCSF and HNa-aCSF infusion in sham-lesioned (open bars, $n = 7$) and vAV3V-lesioned (hatched bars, $n = 8$) rats. * $P < 0.05$.

Sham-lesioned and AV3V-lesioned rats did not differ significantly in resting MAP (86 ± 6 and 93 ± 3 mm Hg respectively) or HR (410 ± 7 and 423 ± 13 beats/min respectively). Intravenous injection of AVP antagonist caused transient non-significant decreases in MAP and HR. I.c.v. infusion of aCSF caused non-significant increases in MAP and HR in both groups. HNa-aCSF significantly increased both MAP and HR over resting values in the sham-lesioned group. Ventral AV3V lesions significantly attenuated MAP and abolished HR responses to i.c.v. HNa-aCSF (Fig. 2). ANG II and ouabain injected i.c.v. in sham-lesioned rats elicited significant dose-dependent increases in MAP accompanied by significant acceleration in HR.

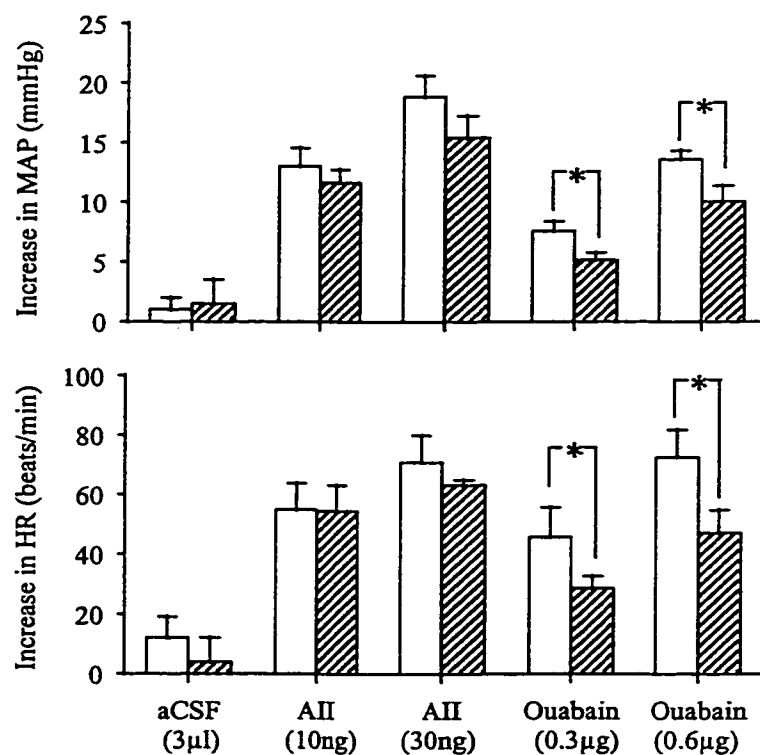


Fig. 3. Peak increases in MAP (upper panel) and HR (lower panel) in response to i.c.v. aCSF, ANG II and ouabain injections in sham-lesioned (open bars, $n = 7$) and vAV3V-lesioned (hatched bars, $n = 8$) rats. * $P < 0.05$.

Control injections of aCSF caused minor non-significant increases in both MAP and HR (Fig. 3). Lesions of the vAV3V significantly attenuated MAP and HR responses to ouabain by 26-32% but did not significantly affect responses to ANG II (Fig. 3).

We have previously shown that brain "ouabain" and ANG II are both involved in the pressor effects of increased central sodium and that brain "ouabain" acts upstream to ANG II [5]. The present study demonstrates that in rats with systemic AVP blockade, neurones in the vAV3V region partially mediate the pressor and tachycardiac effects of i.c.v. sodium and ouabain, whereas responses to i.c.v. ANG II appear not to be mediated by this area.

Our results confirm and expand on those of a study by Takahashi et al. which demonstrated an attenuation of the pressor effect of i.c.v. ouabain following electrolytic AV3V lesions [15].

Our findings differ from those of earlier studies which demonstrate a more complete blockade of pressor responses to an increase in i.c.v. sodium or ANG II with AV3V ablation [1,2]. These studies used electrolytic lesions that firstly, lesioned a larger area involving the whole subcommissural MnPO and secondly, lesioned fibres of passage besides neuronal perikarya. Rohmeiss et al. demonstrated that the pressor response to i.c.v. hypertonic saline is mediated by an angiotensinergic mechanism in the subfornical organ (SFO) [13]. It has been suggested that this response may be mediated via a pathway from the SFO to the paraventricular nucleus [13]. As this pathway traverses the AV3V it would be destroyed by electrolytic lesions of the AV3V but not by excitotoxic lesions, explaining the discrepancy in degree of inhibition of pressor responses to i.c.v.

sodium between our study and those of earlier studies. Centrally administered ANG II causes cellular activation in the MnPO and juxta ventricular neurones of the SFO and OVLT [10]. Xu and Herbert demonstrated suppression of cellular activation to i.c.v. ANG II following electrolytic lesion of the subcommissural MnPO, despite an intact OVLT in central sites which receive efferents from the AV3V region [17]. This suggests that the MnPO mediates responses to i.c.v. ANG II and that an additional pathway involving the SFO is present. Since our lesions largely spared the subcommissural MnPO and would not have destroyed fibres of passage from the SFO that course through the MnPO, it is not surprising that we did not see attenuations to pressor responses to icv ANG II. Finally, we cannot exclude that the discrepancy between previous studies and the present one may relate to the absence of an AVP mediated pressor response to ANG II in the present study.

These results demonstrate that in rats with systemic AVP blockade a discrete area of the vAV3V region is involved in mediating part of the pressor responses to i.c.v. sodium and ouabain but not to ANG II.

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Chapter 3

Ouabain and Central Sodium Induced Hypertension Depend on the Ventral Anteroventral Third Ventricle Region

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ABSTRACT

To examine the role of the ventral anteroventral third ventricle (vAV3V) in the hypertension induced by chronic subcutaneous (sc) ouabain and intracerebroventricular (icv) hypertonic saline, neurons in this area were destroyed by microinjection of an excitotoxin, ibotenic acid. Sham or lesioned Wistar rats were administered ouabain (50 µg/d) or placebo for three weeks from sc implanted controlled release pellets, or artificial CSF or CSF containing 0.8 mol/L NaCl (5 µl/h) infused icv for 2 weeks. At the end of the experiment, mean arterial pressure (MAP) and heart rate (HR) at rest and in response to ganglionic blockade by intravenous hexamethonium (30 mg/kg) were assessed. In rats infused with hypertonic saline responses to air jet stress were also assessed. Baseline MAP in sham operated rats receiving icv hypertonic saline or sc ouabain was significantly higher than in control rats (115±1 vs 97±3 and 121±3 vs 103±3 mmHg respectively). Ventral AV3V lesions abolished the increase in MAP elicited by chronic infusion of hypertonic saline or administration of ouabain. Sham rats treated with hypertonic saline or ouabain exhibited significantly enhanced decreases in MAP to hexamethonium but lesioned rats did not. Rats infused with hypertonic saline demonstrated enhanced responses to air jet stress which was similar in sham operated and lesioned rats. These results demonstrate that neurons in the vAV3V are essential for the hypertension induced by icv hypertonic saline and sc ouabain, possibly by increasing sympathetic tone. Cardiovascular responses to air jet stress appear not to be mediated by the vAV3V.

Key words: hypertonic saline, ouabain, ventral anteroventral third ventricle, excitotoxic lesion, air jet stress, ganglionic blockade

The presence of endogenous ligand inhibitors of Na^+, K^+ -ATPase activity in humans and other mammals has been well established. An inhibitor characterized as ouabain or a stereo-isomer of ouabain [7,24] has been described in mammalian plasma and may be secreted by the adrenal cortex [22]. Endogenous Na^+, K^+ -ATPase inhibitors have also been isolated from mammalian brain [9] and may be produced in the hypothalamus [1,6,27]. Tymiak *et al* [35] have identified one of these inhibitors as an isomer of ouabain.

Chronic administration of exogenous ouabain for 10 days or more, either peripherally or centrally, induces hypertension in normotensive rats [11,23,39]. The mechanisms responsible for the ouabain induced hypertension are still unresolved. A central mechanism is supported by the fact that intracerebroventricular (icv) administration of Fab fragments of antibodies which bind ouabain and related steroids with high affinity [2] prevent the hypertension induced by subcutaneous (sc) ouabain and that ganglionic blockade reverses the hypertension [11].

In normotensive rats, icv administration of hypothalamic extracts containing endogenous brain ouabain-like compounds, ouabain or hypertonic saline cause similar sympathoexcitatory and pressor responses [10]. These responses are abolished by icv pretreatment with anti-ouabain Fab fragments [10]. This suggests that these responses to acute icv administration of hypertonic saline are mediated by a brain ouabain-like compound. Chronic icv infusion of hypertonic saline increases brain ouabain-like compound concentrations accompanied by a sustained increase in blood pressure, sympathoexcitation and enhanced pressor responses to air jet stress in normotensive rats [13,25]. Concurrent infusion icv of anti-ouabain Fab fragments prevents the hypertension

induced by chronic icv infusion of hypertonic saline indicating that a brain ouabain-like compound mediates this chronic pressor effect [13].

One central region that may be involved in mediating the pressor effects of chronic hypertonic saline and ouabain is the tissue surrounding the most anteroventral part of the third ventricle (AV3V). This region integrates fluid and electrolyte homeostasis and autonomic regulation [15,16]. Ventricular obstruction studies demonstrate that hypertonic saline administered icv needs to contact the anterior third ventricle wall to cause pressor effects [4]. In addition, electrolytic ablation of the AV3V abolishes pressor responses to icv hypertonic saline [4] and attenuates pressor responses to icv ouabain [33]. We demonstrated that neurons in the ventral AV3V (vAV3V) partly mediate pressor and tachycardic responses to acute icv administration of hypertonic saline and ouabain in rats with systemic vasopressin blockade [36]. The organum vasculosum laminae terminalis (OVLT), the most ventral component of the AV3V, contains nerve terminals densely immunoreactive to ouabain [38]. Endogenous ouabain-like compounds in the vAV3V may therefore be involved in mediating the pressor effects of an increase in CSF sodium concentration.

In this study we utilized microinjections of the excitotoxin - ibotenic acid, to destroy neurons in the vAV3V to determine whether the hypertension induced by chronic icv administration of hypertonic saline and chronic sc administration of ouabain is dependent on neurons in this area. Ouabain was administered sc as long term icv administration may cause neurotoxic effects. To assess the involvement of autonomic mechanisms we evaluated the effects of ganglionic blockade. Cardiovascular responses to air jet stress were assessed in rats treated with icv hypertonic saline to ascertain involvement of the

vAV3V in the enhancement of these responses seen in this model [13].

METHODS

Male Wistar rats (Charles River, Montreal, Canada) weighing 150-200 g were housed in a climatized room at 24 °C on a 12 h light/dark cycle, fed regular rat chow and allowed tap water *ad libitum* for at least 3 days prior to entering the study. Body weight was monitored weekly. All experimental procedures were approved and carried out in accordance with the guidelines of the University of Ottawa Animal Care Committee for the care and use of laboratory animals. All chemicals were purchased from BDH Inc., Toronto, Ontario except where otherwise noted.

Experimental Protocols

Experiment 1: Controlled time-release ouabain pellets (50 µg/d) or placebo pellets were implanted subcutaneously 1 day following either vAV3V or sham lesion. Lesioned rats were provided access to graded sucrose solutions postsurgically (10%, 5% and 2% sucrose in water for 2 days each) followed by regular water to ensure fluid intake comparable to that in sham lesioned rats [36]. Rats were fed high sodium diet (1370 µmol sodium/g, Harlan Sprague-Dawley, Inc., Madison, WI) for the last 10 days of the experimental period to accelerate increases in blood pressure [11]. At 20-21 days, blood pressure and heart rate at rest and in response to an intravenous injection of the ganglionic blocker, hexamethonium (30 mg/kg), were assessed.

Experiment 2: Sham or vAV3V lesioned rats received chronic icv infusions of either artificial cerebrospinal fluid (aCSF) or aCSF containing 0.8 mol/L NaCl via osmotic

minipumps. Lesioned rats were provided sucrose solutions as in the previous experiment. After 14 days of infusion, blood pressure and heart rate were assessed at rest followed by responses to a standardized air jet stress of a 1.5 PSI air stream directed on the face of the rat for 20 seconds. After a 10 minute recovery period in which MAP and HR returned to resting levels, the air jet stress was repeated. After a further 10 minutes, responses to hexamethonium were recorded as described in the previous experiment.

Surgical Procedures

Excitotoxic Lesions: Ventral AV3V/sham lesions and placement of icv guide canulae were carried out under sodium pentobarbital anaesthesia (50 mg/kg, ip). The rat was placed in a stereotaxic frame (David Kopf Instruments, Tujunga, Calif.) such that the top of the skull was level between bregma and lambda. Body temperature of the rat was maintained by a homeothermic blanket during surgery. A small hole was drilled to expose the brain overlying the co-ordinates for vAV3V ablation and icv guide canula placement. A glass micropipette (tip size 50 μm O.D.) filled with a solution of ibotenic acid [14] (10 $\mu\text{g}/\mu\text{l}$, Research Biochemicals International, Natick, Ma.) in aCSF (composition in mmol/L: NaCl 133.3, KCl 3.4, CaCl_2 1.3, MgCl_2 1.2, NaH_2PO_4 0.6, NaHCO_3 32.0, glucose 3.4; pH 7.4), or aCSF only, was placed in the vAV3V region (coordinates with respect to bregma: an angle of 6° to the sagittal plane, 0.1 mm posterior, 0.8 mm lateral and 8.2 mm deep to the dura). Animals in the lesioned group received 185 nl of ibotenic acid solution pressure ejected from the micropipette over a period of 5 minutes using a pressure ejection system (Picospritzer General Valve Corp., Fairfield, N.J.). Sham-lesioned animals received identical treatment but only vehicle was injected. The

micropipette was left *in situ* for 10 minutes to prevent backflow.

Intracerebroventricular canula placement and osmotic minipump implantation: Icv canulae were placed following vAV3V or sham lesion. A 23 gauge right-angled stainless steel canula was placed into the lateral ventricle (coordinates with respect to bregma: 0.5 mm posterior, 1.4 mm lateral and 3.9 mm deep to the dura) and fixed to the skull with small screws and acrylic cement (HCG Hygenic Corporation, St. Catharines, Ont., Canada). The icv cannula was connected by polyethylene tubing (PE 50/60 combination) to an osmotic minipump (model 2ML2, Alza Corp., Palo Alto, California) which was implanted subcutaneously on the back of the rat. Osmotic minipumps were filled with 2.3 ml of either aCSF or aCSF containing 0.8 mol/L NaCl and delivered infusate at a mean pumping rate of 5.0 μ l/h. Penicillin G (30 000 I.U., Longsil, Victoriaville, PQ., Canada) was injected subcutaneously after surgery for prophylaxis.

Pellet implantation: The day after excitotoxic/sham lesioning rats were reanaesthetized by halothane/oxygen inhalation. A small incision was made on the back of the rat and 2 controlled time-release ouabain/placebo pellets (Innovative Research, Sarasota, FL) were implanted subcutaneously. Each pellet (0.5 mg) releases a constant amount of ouabain (25 μ g/d) or vehicle over a 21 day period.

Arterial and venous cannulation: On the morning of the experiment, rats were reanaesthetized by halothane/oxygen inhalation and instrumented with right femoral artery and vein catheters (polyethylene catheter, PE10/50 combination) filled with heparinized saline (1000 I.U./ml heparin). The catheters were tunnelled subcutaneously and exteriorized at the nape. Blood pressure measurements were taken after a 4 hour

recovery period.

Blood Pressure and Heart Rate Measurements

The arterial catheter was connected to a pressure transducer for recording MAP and HR. The bridge output signal of the transducer was amplified (Transbridge TBM4, World Precision Instruments, Sarasota, FL) and fed to an IBM compatible computer programmed by a data acquisition program (Dataquest LabPro, Data Science International, St. Paul, MN) which allowed online analysis of the pulsatile blood pressure signal (sampling rate 500 Hz) and storage of data. MAP and HR measurements represent averages of 10 second periods except for air stress data in which momentary changes in MAP and HR were used. Rats were allowed an accommodation period of 30 minutes prior to recording resting MAP and HR.

Intracerebroventricular Canula Placement and Lesion Verification

At the end of the experiment, the rats were deeply anaesthetized with sodium pentobarbital (65 mg/kg, ip) and injected icv with 2 μ l of 20% India ink in saline to verify guide canula placement. Patency and connection of the catheter to the icv canula and osmotic minipump was also checked. The rats were then perfused transcardially with 100 ml of 10 mmol/L phosphate buffered saline (PBS) followed by 150 ml 4% paraformaldehyde in 100 mmol/L PBS containing 0.4% picric acid. The brain was removed, post-fixed for 90 minutes and cryoprotected in 10% sucrose in PBS for 1 week. Serial 16 μ m sections were cut on a cryostat and lesions were verified by visualizing an absence of immunoreactivity to A60, an antibody that recognizes a neuron specific nuclear antigen, NeuN [26]. All antibodies were diluted in 10 mmol/L PBS (pH 7.4)

containing 0.3% Triton X100 and incubations were carried out in a humidified chamber at 35 °C. Sections were washed between incubations in 10 mmol/L PBS three times. A 80 minute primary incubation with A60 (1:200) was followed by PBS washes and incubation in a sheep anti-mouse biotinylated Ig (Amersham, Oakville, Ontario, 1:100) for 60 minutes. After further PBS washes sections were incubated with streptavidin horse radish peroxidase complex (Amersham, Oakville, Ontario, 1:200) for 30 minutes. Immunoreactive cells were visualized using diaminobenzidine as a chromogen. The extent of the lesions was visualized as an absence or marked decrease of immunoreactivity to NeuN and mapped onto projection drawings of the rat brain from individual animals using a stereotaxic atlas as a guide [28]. In a separate group of sham and lesioned rats (n=4 per group) the degree and extent of the lesion was assessed by counting immunoreactive neurons in specific AV3V nuclei using an image analysis system equipped with MetaMorph software (Universal Imaging Corporation, West Chester, PA) calibrated for the objective used (10x). Following image acquisition and contrast enhancement, thresholding was performed to exclude weakly stained nuclei and criteria were set to exclude nuclear fragments (< 8 μm). Immunoreactive neurons were counted within a 100 μm^2 area in each nucleus in two representative sections per rat and averages from the two counts were used in statistical analysis. Slides were coded so that experimental groups were not known at the time of quantification.

Statistical Analysis

A total of seven rats from the four lesioned groups were excluded prospectively from the analysis due to unilateral vAV3V ablation. In the second experiment a further seven

rats were excluded due to incorrect icv cannula placement (n=2), or blocked/disconnected catheters from minipumps (n=5). Final numbers of rats per group were as follows: sham operated groups receiving placebo or aCSF (n=7 per group), sham operated rats receiving ouabain or 0.8M NaCl (n=7 and 8 respectively), lesioned rats receiving placebo or aCSF (n=7 and 6 respectively) and lesioned rats receiving ouabain or 0.8M NaCl (n=8 per group). Values are presented as means $\pm$ S.E.M. Changes in MAP or HR in response to either hexamethonium or air jet stress from resting levels within groups were determined by paired t-tests. Comparisons of NeuN positive neurons between sham and lesioned groups was determined by unpaired t-tests and all other group comparisons were determined by two-way ANOVA followed by Student-Newman-Keuls test. The level of significance was set at $p < 0.05$.

RESULTS

Extent of Lesions

Histological verification of lesions confirmed consistent lesioning of tissue limited to the vAV3V region. Specifically, there was a marked decrease in NeuN immunoreactivity in lesioned versus sham operated rats within the OVLT (9 ± 1 vs 32 ± 2 neurons/100 μm^2 , $p < 0.05$; arrowheads in Fig. 1B) and the most ventral part (approximately 100 μm) of the subcommissural median preoptic nucleus (MnPO, 6 ± 1 vs 14 ± 2 neurons/100 μm^2 , $p < 0.05$; arrowheads in Fig. 1D). NeuN immunoreactivity was not significantly different between sham and lesioned rats in the remaining MnPO (42 ± 3 vs 40 ± 2 neurons/100 μm^2), the periventricular hypothalamic nucleus (8 ± 1 vs 10 ± 1 neurons/100 μm^2), anterior

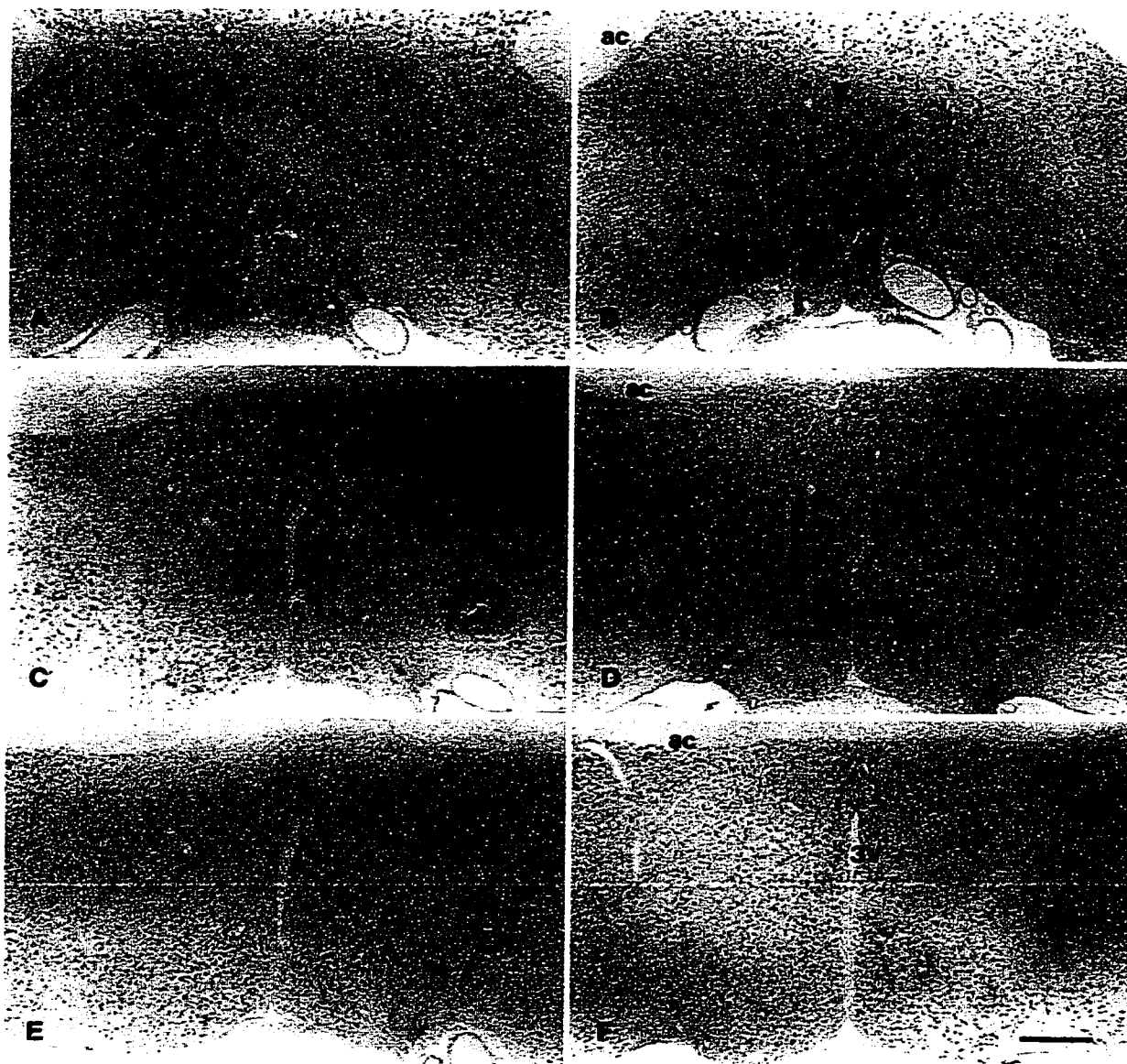


Fig. 1. Photomicrographs of neuron-specific nuclear antigen (NeuN) immunoreactivity of a typical sham-operated (*A*, *C*, and *E*) and lesioned (*B*, *D*, and *F*) rat through the anteroventral part of the third ventricle (AV3V) region (coordinates with reference to bregma: *A* and *B*, -0.10 mm; *C* and *D*, -0.25 mm; and *E* and *F*, -0.35 mm). arrowheads point to lesioned area. Scale bar indicates 500 μ m. 3V, third ventricle; ac, anterior commissure.

medial preoptic nucleus (14 ± 2 vs 17 ± 2 neurons/ $100 \mu\text{m}^2$) and anteroventral preoptic nucleus (18 ± 2 vs 16 ± 1 neurons/ $100 \mu\text{m}^2$). Fig. 1 shows Neu-N immunoreactivity in sections taken through the AV3V region of typical lesioned and sham operated rats. Final body weights were not significantly different between sham and vAV3V lesioned rats in either experiment (data not shown).

Effect of vAV3V Lesion on Responses to Chronic Subcutaneous Ouabain

Administration

Resting MAP in sham operated rats receiving chronic ouabain was significantly higher compared to those on placebo treatment. Ventral AV3V lesions abolished the increase in MAP elicited by chronic ouabain treatment (Fig. 2). Resting HR did not differ significantly between groups.

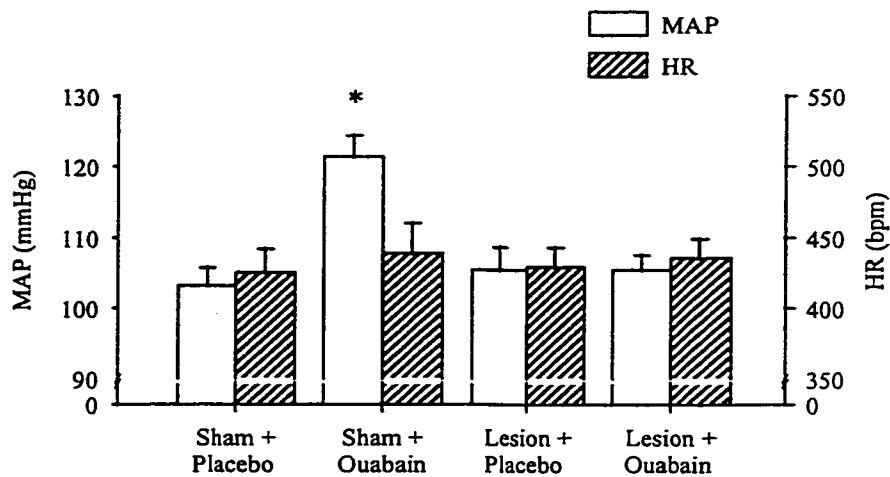


Fig. 2. Baseline mean arterial pressure (MAP) and heart rate (HR) in sham- and vAV3V-lesioned rats treated for 3 wk with ouabain ($50 \mu\text{g/day sc}$) or placebo. bpm, Beats/min. Values are presented as means $\pm$ SE; $n = 7-8$ rats. * $P < 0.05$ vs. other groups.

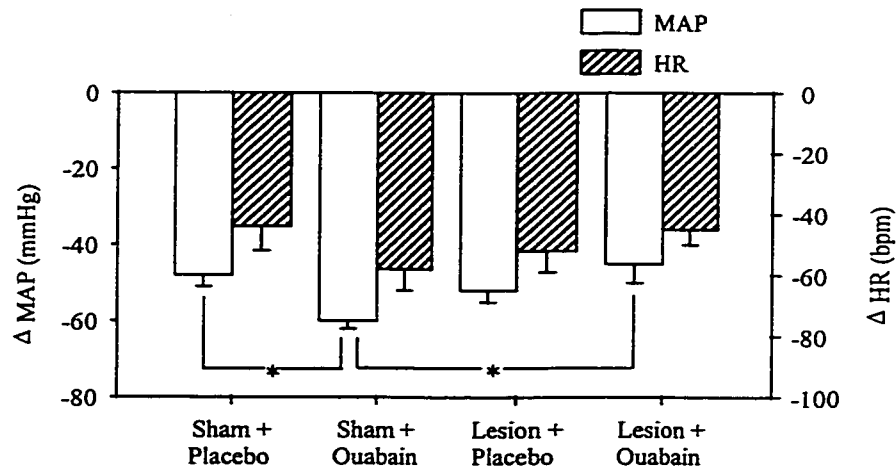


Fig. 3. Peak decreases in MAP and HR in response to hexamethonium (30 mg/kg iv) in sham- and vAV3V-lesioned rats treated for 3 wk with ouabain (50 µg/day sc) or placebo. Values are presented as means ± SE; $n = 7-8$ rats. * $P < 0.05$ as indicated.

The extent of decrease in MAP caused by intravenous hexamethonium was significantly enhanced in sham operated rats treated with ouabain but not in lesioned rats treated with ouabain (Fig. 3) resulting in similar MAP in all groups after hexamethonium (sham + placebo: 60 ± 5 ; sham + ouabain: 60 ± 3 ; lesion + placebo: 54 ± 3 and lesion + ouabain: 59 ± 4 mmHg; n.s.). Decreases in HR were not significantly different between groups.

Effect of vAV3V Lesion on Responses to Chronic Intracerebroventricular Hypertonic Saline Administration

Resting MAP in sham operated rats receiving chronic icv hypertonic saline infusion was significantly higher than those receiving icv aCSF. Lesions of the vAV3V prevented the increase in MAP elicited by chronic icv hypertonic saline infusion (Fig. 4). Resting HR was not significantly different between groups.

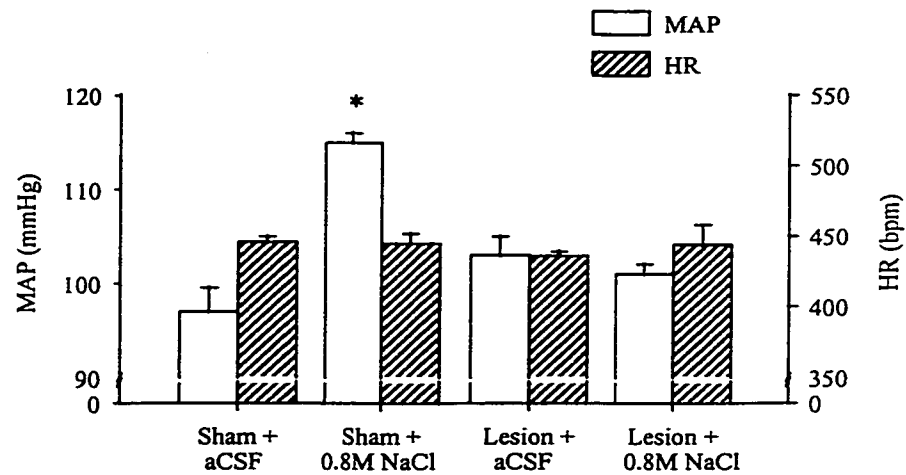


Fig. 4. Baseline MAP and HR in sham- and vAV3V-lesioned rats treated with artificial cerebrospinal fluid (aCSF) or aCSF containing 0.8 mol/l NaCl (5 μ l/h icv) for 2 wk. Values are presented as means $\pm$ SE; n = 6-8 rats. * P < 0.05 vs. other groups.

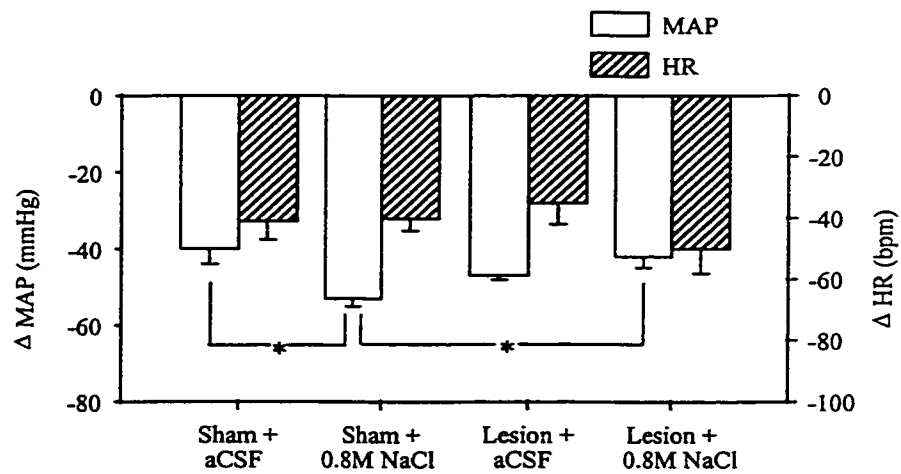


Fig. 5. Peak decreases in MAP and HR in response to hexamethonium (30 mg/kg iv) in sham- and vAV3V-lesioned rats treated with aCSF or aCSF containing 0.8 mol/l NaCl (5 μ l/h icv) for 2 wk. Values are presented as means $\pm$ SE; n = 6-8 rats. * P < 0.05 as indicated.

The extent of decrease in MAP caused by intravenous hexamethonium was significantly enhanced in sham operated rats receiving icv hypertonic saline but was not

enhanced in lesioned rats treated similarly (Fig. 5). After hexamethonium treatment MAP was not significantly different between groups (sham + aCSF: 56 ± 4 ; sham + 0.8M NaCl: 59 ± 3 ; lesion + aCSF: 61 ± 2 and lesion + 0.8M NaCl: 59 ± 3 mmHg). Decreases in HR were not significantly different between groups.

Sham operated rats infused with hypertonic saline exhibited significantly enhanced MAP and HR responses to air jet stress compared to either sham or lesioned rats treated with aCSF infusion (Table 1). Lesioned rats receiving icv hypertonic saline exhibited similarly enhanced responses to air jet stress.

Table 1: Peak increases in MAP and HR in response to air jet stress in sham- and vAV3V-lesioned rats treated with aCSF or NaCl for 2 wk.

Group	Treatment	MAP, mmHg	HR, beats/min
Sham-lesioned	aCSF	6 ± 2	20 ± 2
	NaCl (0.8 M)	$13 \pm 1^*$	$39 \pm 2^*$
vAV3V-lesioned	aCSF	9 ± 1	22 ± 1
	NaCl (0.8 M)	$14 \pm 2^*$	$38 \pm 2^*$

Values are presented as means $\pm$ SE; n = 6 - 8 rats. MAP, mean arterial pressure; HR, heart rate; aCSF, artificial cerebrospinal fluid; vAV3V, ventral anteroventral third ventricle. NaCl was infused intracerebroventricularly at a pumping rate of 5 μ l/h. * $P < 0.05$ vs. aCSF groups.

DISCUSSION

The present study demonstrates that the vAV3V region is essential for the hypertension induced by chronic administration of hypertonic saline icv or of ouabain sc. Since excitotoxic lesions spare axons passing through and nerve terminals within the area of lesion [30], it is likely that neuronal somata in the vAV3V mediate this effect. In

addition, vAV3V lesions prevent enhanced depressor responses to ganglionic blockade but not enhanced cardiovascular responses to air jet stress.

Consistent with other recent studies, chronic treatment with ouabain caused moderate hypertension in conscious rats [11,23,39]. Takahashi *et al* reported an attenuation of the pressor effect of acute icv administration of ouabain following electrolytic AV3V lesions involving the whole subcommissural MnPO and the anterior hypothalamic periventricular area [33]. Lesions limited to the vAV3V also attenuate the pressor and tachycardic responses to acute icv administration of ouabain in rats with systemic vasopressin blockade [36]. In the present study, vAV3V lesions fully prevented hypertension induced by chronic subcutaneous administration of ouabain likely because a comparatively smaller dose was administered which presumably would act only on nuclei most sensitive to ouabain. A larger (acute) dose would be able to act on less sensitive nuclei as well as the vAV3V. The finding that the enhanced depressor responses to ganglionic blockade elicited by chronic administration of ouabain were prevented by vAV3V lesions suggests that this region mediates sympathetic activation in ouabain induced hypertension. Following ganglionic blockade, blood pressure was similar in all groups consistent with a role for sympathetic activation in ouabain induced hypertension. Administration of a vasopressin receptor antagonist after ganglionic blockade only caused a further 5 mmHg decrease in MAP in rats treated chronically with ouabain [11], suggesting that release of vasopressin contributes only in a minor way to the pressor effect of chronic treatment with ouabain. Ouabain was administered s.c. and therefore could also act peripherally to cause hypertension e.g. by inhibiting the $\text{Na}^+,\text{K}^+,\text{ATPase}$ in vascular smooth muscle

resulting in vasoconstriction [31]. However, it is not likely that peripheral mechanisms play a significant role in the hypertension induced by sc ouabain as central blockade of the effects of ouabain prevents hypertension in this model [11].

We previously reported that treatment with ouabain for two weeks at a dose similar to that used in the present study caused hypertension accompanied by tachycardia [11]. In the present study, rats were treated with ouabain for three weeks and did not exhibit tachycardia. This is consistent with the findings of Manunta *et al* [23], who did not find an accompanying tachycardia following five weeks of treatment with ouabain. It is likely that in the more chronic phase of treatment control systems adapt causing the tachycardia to diminish.

Consistent with an earlier study [5] icv infusion of 0.8 mol/L NaCl caused mild hypertension in conscious rats. As sympathoexcitatory and pressor responses are elicited by icv hypertonic saline but not by other hypertonic solutions or ammonium chloride, it is likely that the responses to hypertonic saline are attributable to sodium ions rather than hyperosmolality or chloride ions [5]. Infusion of 0.8 mol/L NaCl at 5.0 μ l/h for 14 days increases CSF sodium ion concentration by ~ 6 mmol/L [13].

Our results complement earlier studies that demonstrated a complete blockade of pressor responses to an acute icv administration of hypertonic saline by electrolytic AV3V ablation [3,4]. Ventral AV3V lesions attenuated the pressor and tachycardic effect of acute icv infusions of 0.3 mol/L NaCl by ~ 30 % in rats with systemic blockade of vasopressin mechanisms [36]. In the present study vAV3V lesions fully prevented hypertension induced by chronic icv infusion of hypertonic saline. This difference in

extent of blockade may be due to the fact that the concentration of sodium obtained chronically would likely be less than the concentration achieved acutely as an approximately 10 fold lower rate was administered chronically. Presumably smaller concentrations of sodium acted only on the nuclei most sensitive to sodium. Higher concentrations may act on less sensitive nuclei in addition to the vAV3V. Rohmeiss *et al* demonstrated that an angiotensinergic mechanism within the subfornical organ (SFO) mediates the pressor response to an *acute* icv injection of hypertonic saline, and suggested that this response may be mediated via a pathway from the SFO to the paraventricular nucleus [29]. This pathway traverses or synapses in the subcommisural MnPO explaining why electrolytic AV3V lesions are able to abolish pressor responses to acute icv hypertonic saline. As vAV3V lesions leave the subcommisural MnPO unaffected to a large extent, it appears that the OVLT plays a greater role in mediating pressor effects of *chronic* icv infusion of hypertonic saline. In dogs, an additional site, the area postrema has been implicated in mediating pressor responses to icv hypertonic saline [17].

Deficits in vasopressin release mechanisms have been demonstrated with electrolytic AV3V lesions [20,37]. It is not known if excitotoxic lesions of the vAV3V also cause a deficit in vasopressin release. As blockade of vasopressin mechanisms was not used in the present study, it is possible that release of vasopressin may have contributed to the hypertension induced by hypertonic saline. Vasopressin contributes to the pressor effect of hypertonic saline in the early phase (day 1) of chronic icv infusion. In the chronic phase (day 7) elevated plasma catecholamine levels and augmented depressor responses to ganglionic blockade suggest an increase in sympathetic activation [18]. In the present

study vAV3V lesions prevented the enhanced depressor response to ganglionic blockade elicited by chronic icv administration of hypertonic saline. In addition, despite mild hypertension in sham operated rats receiving hypertonic saline, resting MAP was similar in all groups following ganglionic blockade. It is therefore likely that sympathetic activation mediated by the vAV3V persisted in sham operated rats and accounts for the greater part of the pressor effect of chronic icv administration of hypertonic saline.

In rats receiving chronic icv infusion of hypertonic saline, vAV3V lesions prevent enhanced depressor responses to ganglionic blockade but not enhanced cardiovascular responses to air jet stress. Therefore, although the vAV3V appears to mediate the increase in baseline sympathetic tone due to icv administration of hypertonic saline, it does not mediate the enhanced cardiovascular responses to air jet stress exhibited by this model. This is consistent with studies in salt-sensitive models of hypertension. Hatton *et al* reported that electrolytic ablation of the AV3V in borderline hypertensive rats fed high salt diet did not affect cardiovascular responses to air stress [8]. In spontaneously hypertensive rats on high salt diet enhanced cardiovascular responses to air jet stress can be prevented by icv administration of Fab fragments but not by blockade of ouabain-like compounds in the MnPO [12,34]. Although various brain areas have been proposed to mediate the cardiovascular responses to air jet stress [19,21,32], it appears that neurons releasing ouabain-like compounds in area(s) other than the AV3V are involved in mediating the responses to sodium loading.

In conclusion, this study demonstrates that the vAV3V is essential for hypertension that is induced by chronic icv hypertonic saline and sc ouabain possibly via sympathetic

activation. As both these models of hypertension exhibit an elevation in central ouabain and can be prevented by blockade of central ouabain [11,13], it is tempting to speculate that the vAV3V mediates the pressor response to icv hypertonic saline via a release of ouabain-like compounds in the vAV3V. In this regard neurons in the vAV3V may act as the primary sodium sensor or may act as a "relay" in mediating these effects and the primary sodium sensor may be located in the SFO or elsewhere.

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Chapter 4

Brain Angiotensin II Receptor Density in Salt-induced Hypertension in Dahl Rats

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ABSTRACT

Salt-induced hypertension in Dahl salt-sensitive (S) rats can be prevented by intracerebroventricular administration of an angiotensin II type 1 (AT₁) receptor antagonist. The objective of this study was to assess whether changes in brain AT₁ receptor density accompany the development of salt-induced hypertension in Dahl rats. Dahl S and salt-resistant (R) rats were fed either regular (101 µmol Na/g) or high (1370 µmol Na/g) salt diet from 4 to 6 weeks of age. At the end of the dietary period, ¹²⁵I-sar¹ile⁸-angiotensin II binding to AT₁ and AT₂ receptors by quantitative in vitro receptor autoradiography as well as resting mean arterial pressure (MAP) was assessed. The resting MAP in Dahl S rats on high salt diet was significantly higher than in other groups. AT₁ receptor binding did not differ between strains on regular salt diet. High salt diet decreased AT₁ receptor binding by 25-30 % in both strains in the organum vasculosum laminae terminalis (OVLT), the suprachiasmatic nucleus (SCh) and the paraventricular nucleus (PVN). Within the subfornical organ (SFO) AT₁ receptor binding did not differ between Dahl S and R rats on regular salt diet (346±10 vs. 306±14 fmol/mg resp., NS, n=4) and decreased in both strains on the high salt diet, but decreased more in Dahl S versus Dahl R rats (175±15 vs. 235±8 fmol/mg resp., p<0.05, n=4). AT₂ receptor binding did not differ between strains or diet in any of the regions quantified. AT₁ receptors within the SFO, OVLT, PVN and SCh nuclei may be involved in osmo-regulatory mechanisms. In Dahl S rats, the development of salt-induced hypertension is associated with an enhanced down-regulation of AT₁ receptors in the SFO. One may speculate that this decrease in receptor binding *in vitro* may reflect an increase in angiotensin II receptor

binding *in vivo* followed by internalization of the receptor complex.

Keywords: AT₁ receptors, subfornical organ, organum vasculosum laminae terminalis, suprachiasmatic nucleus, paraventricular nucleus, Dahl rats

The brain Renin Angiotensin System (RAS) is involved in sodium homeostasis and central cardiovascular regulation. Components of the brain RAS are distributed in central regions that mediate these functions including the septum, the preoptic region, hypothalamic nuclei, the midbrain, and nuclei of the medulla oblongata ¹. These areas express a predominance of AT₁ receptors ¹⁻³ which bind angiotensin II (Ang II) and angiotensin III (Ang III) with high affinity. AT₁ receptor stimulation appears to play a critical role in the development of salt-induced hypertension in the Dahl salt-sensitive (Dahl S) rat, a genetic model of salt-sensitive hypertension. Chronic central infusion of CV-11974, a non-peptide AT₁ receptor antagonist, prevented the development of hypertension in Dahl-Iwai salt-sensitive rats on a high salt diet ⁴. In addition, our group demonstrated that chronic blockade of central AT₁ receptors, by intracerebroventricular infusion of losartan, prevents sympathoexcitation and exacerbation of hypertension in Dahl S rats on high salt diet ⁵.

Lesion studies indicate that a difference in function of certain hypothalamic areas/nuclei may mediate salt induced hypertension in Dahl S rats. Lesions of the anteroventral third ventricle region ⁶ or bilateral lesions of the paraventricular nucleus (PVN) ^{7,8} attenuated salt-induced hypertension in this model. Lesions of the anteromedial

hypothalamus, which included the PVN, suprachiasmatic nuclei and intervening periventricular tissue, prevented the development of hypertension in Dahl S rats on high salt diet ^{8,9}. As these regions contain components of the brain RAS ^{1,10}, they may mediate salt induced hypertension via an increase in its activity, that is, synthesis and release of its effector peptides. In addition to the production or release of angiotensins, the availability of receptors that mediate their effects would be expected to determine the efficacy of the brain RAS.

Biochemical assessment of activity of the brain RAS in this model is limited. In hypertensive Dahl S rats, AT₁ receptor mRNA increased 3-fold in brain homogenates compared to Dahl R rats following 6 weeks of high salt diet ¹¹. A high salt diet caused a marked increase in angiotensin converting enzyme (ACE) mRNA and activity measured in hypothalamic and pons homogenates in Dahl S rats compared to Dahl R rats ¹².

Although these studies suggest an enhanced activity of the brain RAS during the development of hypertension in this model, an assessment of particular brain nuclei/areas in which the brain RAS is involved in Dahl S hypertension is unavailable. The objective of the present study was therefore to identify and assess changes in Ang II receptor density induced by high dietary salt intake within brain areas involved in sodium homeostasis and cardiovascular regulation in Dahl rats.

METHODS

Male Dahl R and S rats (Harlan Sprague Dawley, Madison, WI) age 3-4 weeks, were housed in a temperature-controlled environment at 24 °C on a 12 h light/dark cycle, fed

regular rat chow and allowed tap water *ad libitum* for at least 3 days prior to entering the study. All experimental procedures were carried out in accordance with the guidelines of the Medical Research Council of Canada outlined in the Guide for the Care and Use of Experimental animals. All chemicals were purchased from Sigma (Oakville, Ontario, Canada) except where otherwise noted.

Dietary Treatments

Separate groups of rats were used for blood pressure measurements and determination of Ang II receptor binding. At 4 weeks of age, rats were randomly assigned to receive either a regular salt (101 $\mu\text{mol Na/g}$) or a high salt (1370 $\mu\text{mol Na/g}$, Teklad, Madison, WI) diet for 2 weeks. Following 2 weeks of a high salt diet Dahl S rats are in the early developmental phase of hypertension with attendant increases in hypothalamic and pons ACE activity ¹².

Blood Pressure and Heart Rate Measurement

At the end of the dietary period, rats were anaesthetized by halothane/oxygen inhalation and a polyethylene catheter was inserted into the left carotid artery, tunneled subcutaneously and exteriorized at the nape. Following an overnight recovery period, resting mean arterial pressure (MAP) and heart rate (HR), were recorded using a data acquisition program (Dataquest LabPro, Data Science International, St. Paul, MN) which allowed online analysis of the pulsatile blood pressure signal from the arterial catheter (averages of 10 s periods, sampling rate 500 Hz). Rats were allowed an accommodation period of 30 minutes prior to recording resting MAP and HR.

Quantitative In Vitro Ang II Receptor Autoradiography

Procedures for quantitative assessment of Ang II binding by *in vitro* receptor autoradiography were performed according to a standard protocol ¹³. Briefly, rats were decapitated and the brains removed rapidly, frozen in 2-methylbutane at -40 °C and stored at -20 °C. All tissues were processed within a 1 week period. Serial 20 µm thick sections were cut on a cryostat, thaw-mounted onto Superfrost Plus® microscope slides (VWR, West Chester, PA) and dehydrated overnight in a desiccator at 4 °C. Sections were preincubated in a buffer (10 mM sodium phosphate, 120 mM NaCl, 5 mM di-sodium EDTA, pH 7.4) containing 0.2 % bovine serum albumin for 15 min at 20 °C. The absence of peptidase or protease inhibitors in the preincubation buffer promotes the dissociation and degradation of endogenous Ang II. Sections were then incubated in the same buffer containing 0.2 % bovine serum albumin, 0.5 mg/ml bacitracin, and ¹²⁵I-sar¹Ile⁸-Ang II (140 pmol/l, Washington State University Peptide Radioiodination Service Centre, Pullman, WA). AT₁ and AT₂ receptor binding was determined by including either PD 123319 (10⁻⁵ M), an AT₂ receptor antagonist, or losartan (10⁻⁵ M, Merck Research Laboratories, Rahway, NJ), an AT₁ receptor antagonist, to exclude AT₂ and AT₁ receptor binding respectively. Non-specific binding was determined in the presence of 1 µM Ang II. Following four successive 1 min washes in ice-cold buffer to remove non-specifically bound ligand, sections were air-dried and then exposed to Biomax MR2 film (VWR, Mississauga, Ontario) for 24-48 h alongside methylacrylate ¹²⁵I standards (Washington State University Peptide Radioiodination Service Centre, Pullman, WA). Film was processed using a Kodak X-OMAT 270 RA automatic processor (Rochester, New York,

NY) and relative optical density within outlined brain areas was quantified by computerized densitometry following calibration to relative optical densities of the ^{125}I standards using a computer assisted image analysis system, AIS/C (Imaging Research Inc., St. Catherines, Ontario, Canada) and expressed as fmol/mg tissue. Specific binding density was determined by subtraction of non-specific binding from total binding for each receptor subtype. Animals were coded such that experimental groups were not known during densitometry.

Statistical Analysis

Data are presented as means $\pm$ SE. All comparisons between groups were determined by a two-way analysis of variance followed by the Student-Newman-Keuls test where applicable. The level of significance was set at $P < 0.05$.

RESULTS

Resting Hemodynamics

Resting MAP was similar in Dahl R and S rats fed a regular salt diet for 2 weeks.

Following 2 weeks of diet, MAP in Dahl S rats on high salt diet was significantly higher compared to Dahl S rats on regular salt diet or Dahl R rats on either high or regular salt diet (123 ± 2 vs. 105 ± 3 , 101 ± 3 or 103 ± 3 mmHg resp.; $p < 0.05$; $n = 6$ per group).

Resting HR did not differ significantly between groups (data not shown). Body weight gain following regular salt diet for 2 weeks was similar in Dahl R and Dahl S rats (Δ : 115 ± 6 and 125 ± 10 g; NS) and was inhibited to a similar extent in both the Dahl R and Dahl S groups (16-18 %) on high salt diet.

Ang II Receptor Density

At 6 weeks of age, ^{125}I -sar¹ile⁸-angiotensin II binding to AT₁ receptors was detected in high densities in the organum vasculosum laminae terminalis (OVLT), subfornical organ (SFO), suprachiasmatic nucleus (SCh) and PVN, and in moderate densities in the median preoptic nucleus, area postrema (AP) and the dorsal vagal complex (DVC), ie the nucleus tractus solitarius (NTS) and the motor nucleus of the vagus. Binding density was quantified in the DVC as distinction between the NTS and the motor nucleus of the vagus on autoradiograms was difficult. In most cardiovascular or osmo-regulatory areas AT₁ receptor binding did not differ significantly between the two strains on regular salt intake at 6 weeks of age (Table 1). In rats fed the high salt diet from 4 to 6 weeks of age, AT₁ receptor binding decreased by 25-30 % in both strains in the OVLT, the SCh and the PVN. Figure 1 shows typical images of AT₁ receptor binding densities in sections taken through the SFO of Dahl R and S rats following 2 weeks of dietary treatment. AT₁ receptor binding was similar in the SFO of Dahl R and Dahl S rats on regular salt diet, and decreased in both strains following 2 weeks of high salt diet. This decrease was significantly greater in Dahl S versus R rats on high salt diet (Figure 2). AT₁ receptor binding within the vMnPO, the AP and the DVC was not affected by high salt intake in either strain (Table 1). In the piriform cortex, an area not involved in cardiovascular regulation or body fluid homeostasis, AT₁ receptor binding did not differ between groups on either diet (Table 1).

AT₂ receptor binding in the locus ceruleus (LC) was comparable between Dahl S and

R rats at 6 weeks of age on regular salt diet (125 ± 36 and 151 ± 11 fmol/mg resp., NS) and did not change significantly on high salt diet from 4-6 weeks of age in either Dahl S or Dahl R rats (153 ± 6 and 164 ± 19 fmol/mg resp. NS). AT_2 receptor binding in the inferior olivary nucleus, an area not involved in cardiovascular regulation or body fluid homeostasis, did not differ between groups (data not shown). Non-specific binding ranged from 10 to 15 fmol/mg in brain areas assessed.

Table 1. Effect of high salt diet on AT_1 receptor binding density in Dahl rats.

Diet/Strain	AT_1 Receptor Binding Density (fmol/mg)						
	OVLT	vMnPO	SCh	PVN	DVC	AP	Pir
Regular Salt							
Dahl R	280±12	182±14	280±14	268±13	153±27	158±16	118±16
Dahl S	304±10	198±17	268±13	244±13	148±15	164±31	126±17
High Salt							
Dahl R	211±12*	166±18	183±23*	194±14*	156±10	150±10	101±20
Dahl S	226±19*	168±18	199±15*	185±18*	155±14	146±26	108±18

AT_1 receptor binding density within cardiovascular regulatory areas in Dahl salt-resistant (Dahl R) and Dahl salt-sensitive (Dahl S) rats fed regular salt (101 μ mol Na/g) or high salt (1370 μ mol Na/g) diet from 4 to 6 weeks of age. Values are means $\pm$ SE, n = 4 per group, * p < 0.05 vs regular salt groups for same dietary period. OVLT, organum vasculosum laminae terminalis; vMnPO, median preoptic nucleus, ventral; SCh, suprachiasmatic nucleus; PVN, paraventricular nucleus; DVC, dorsal vagal complex; AP, area postrema; Pir, piriform cortex.

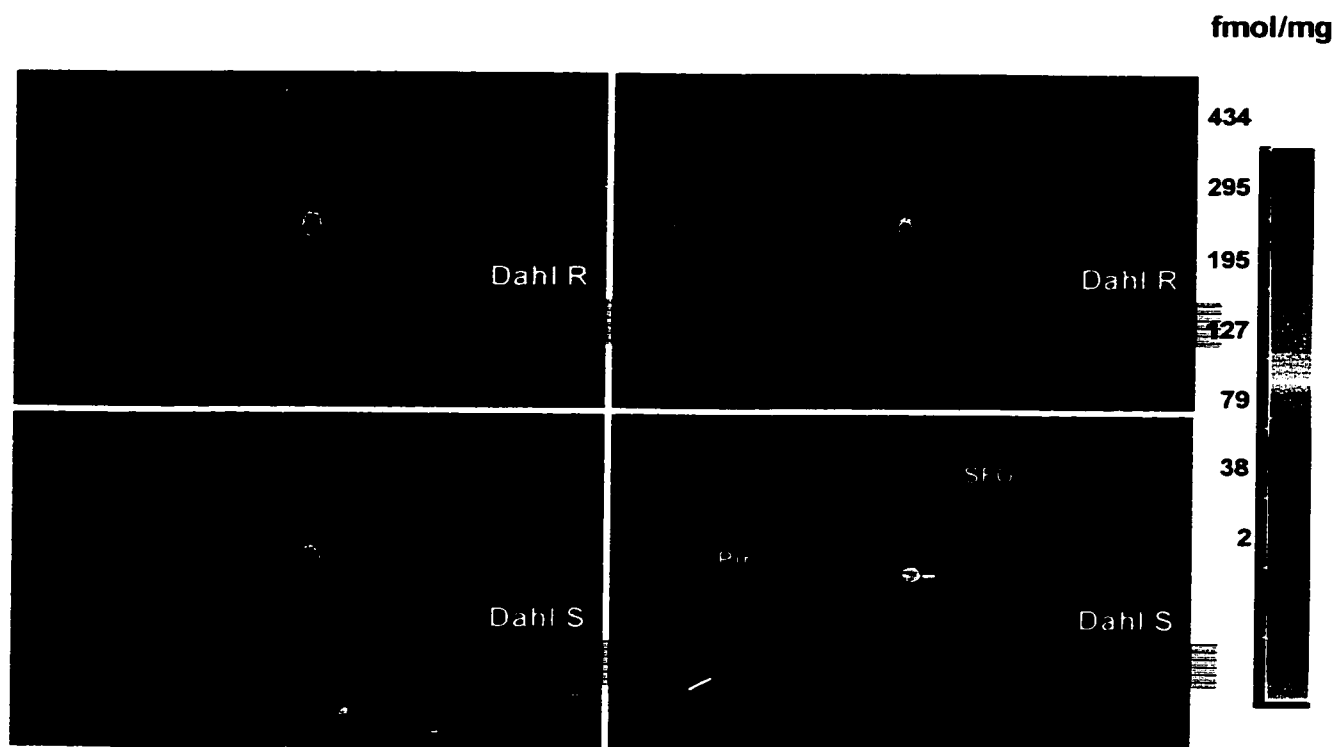


Figure 1. Computer generated images of AT_1 receptor binding densities in the subforminal organ (SFO) of Dahl salt-resistant (Dahl R) and Dahl salt-sensitive (Dahl S) rats on regular salt ($101 \mu\text{mol/g Na}$) or high salt ($1370 \mu\text{mol/g Na}$) diet from 4 to 6 weeks of age. AT_1 binding was similar in Dahl R and Dahl S rats on regular salt diet and decreased in both strains on high salt diet, with an enhanced decrease in Dahl S rats on high salt diet. Pir, piriform cortex.

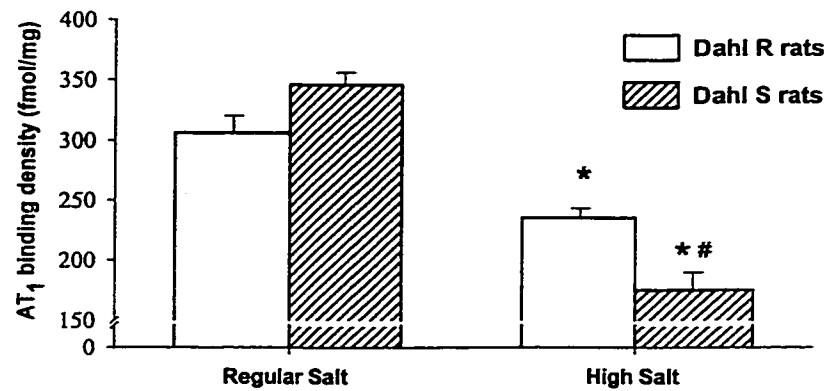


Figure 2. AT_1 receptor binding density within the SFO in Dahl salt-resistant (Dahl R) and Dahl salt-sensitive (Dahl S) rats fed regular (101 $\mu\text{mol Na/g}$) or high (1370 $\mu\text{mol Na/g}$) diet from 4 to 6 weeks of age. Values are presented as means $\pm$ SE; $n = 4$ per group. * $P < 0.05$ vs. same strain on regular salt diet, # $P < 0.05$ vs. Dahl R rats on same diet.

DISCUSSION

The present study demonstrates that a high dietary salt intake from 4 to 6 weeks of age causes a decrease in AT_1 receptor binding in both Dahl R as well as Dahl S rats in the OVLT, the Sch and the PVN. The high salt diet-induced increase in MAP in Dahl S rats was accompanied by a more pronounced decrease in AT_1 binding within the SFO in Dahl S versus Dahl R rats. AT_2 receptor binding did not differ between strains at 6 weeks of age on either regular or high salt diet.

The circulatory RAS, as well as intrinsic tissue RAS including that in the brain, appear to be regulated in distinct and organ specific manner in response to dietary salt

^{11,14,15}. In rats the angiotensin receptors in the brain are predominantly of the AT_{1A} subtype and are expressed in high densities in the SFO, OVLT, PVN, area postrema (AP) and NTS, and in moderate amounts in the MnPO ¹. In the OVLT and SFO, AT_{1B} receptor mRNA is also expressed ^{1,16}. The regulation of AT₁ receptors in the brain, which predominate in areas involved in sodium homeostasis and cardiovascular regulation, is expected to be one of the critical components in regulating the activity of the brain RAS in response to salt. In normotensive rats, a high salt diet for 3 weeks caused an upregulation of AT_{1A} receptor mRNA by 35 %, but no significant change in AT_{1B} mRNA in decorticated brain homogenates ¹⁷. In normotensive rats that drank 1 % NaCl solution for 4 weeks, Ang II receptor binding in homogenates of the hypothalamus, thalamus, striatum, midbrain and cortex was decreased by 7-31% ¹⁸. In another study, Ang II receptor binding to whole brain homogenates from normotensive Wistar rats fed a high salt diet for 2 to 3 weeks showed minor changes ($-6.3 \pm 4.4\%$) compared to binding in homogenates from rats on regular salt diet ¹⁹. In this study the use of whole brain homogenates may have masked differences in binding densities in specific brain regions/nuclei between rats on regular and high salt diet. It therefore appears that in normotensive rats AT₁ receptor mRNA increases and Ang II binding decreases in the brain in response to a high salt diet.

Strehlow et al. ¹¹ reported a 3-fold increase in AT₁ receptor mRNA in whole brain homogenates following 6 weeks of high salt diet in hypertensive Dahl S rats, but not in their normotensive controls, Dahl R rats, indicating a difference in AT₁ receptor regulation in response to dietary salt between Dahl S and R rats. In the present study, we

demonstrate a decrease in ^{125}I -Sar¹Ile⁸-Ang II binding to AT₁ receptors in the OVLT, Sch, PVN and the SFO in both Dahl R and S rats following 2 weeks of a high salt diet. The fact that brain AT₁ receptor mRNA increases in Dahl S rats on high salt diet whereas AT₁ receptor density decreases in response to high salt diet may have several explanations. Quantification of AT₁ receptor mRNA reflects message for production of AT₁ receptors and would include AT₁ receptors at the cell membrane as well as internalized receptors. ^{125}I -Sar¹Ile⁸-Ang II binding to AT₁ receptors reflects binding at the cell membrane and therefore only the number of AT₁ receptors in the cell membrane. It is unlikely that this decrease in AT₁ receptor binding reflects increased receptor occupancy as ligands would have dissociated or degraded during the initial incubation in the absence of peptidase or protease inhibitors. An increase in AT₁ mRNA combined with a decrease in membrane receptor density could therefore represent an increase in receptor turnover. AT₁ receptors in the brain appear to be regulated in response to its ligand as chronic intracerebroventricular infusions of Ang II in normotensive rats result in an increase in AT₁ receptor mRNA and protein in whole brain homogenates ²⁰. We speculate that a decrease in receptor density *in vitro* in the present study may reflect a marked increase in AT₁ receptor occupancy *in vivo* followed by receptor internalization and then an increase in mRNA for the receptor. In Dahl R rats on high salt diet AT₁ receptor mRNA did not increase ¹¹ but AT₁ receptor binding did decrease in several nuclei. These findings may point to an actual down-regulation of the receptor, or may indicate that an increase in AT₁ receptor occupancy *in vivo* and subsequent internalization of the receptor-agonist complex resulted in decreased membrane receptor density. The increase in AT₁ receptor

mRNA seen in other normotensive strains and salt-sensitive Dahl S rats is absent in Dahl R rats. It is therefore possible that Dahl R rats have altered regulatory mechanisms for AT₁ receptor production in response to high salt diet and production of AT₁ receptor receptors does not increase. Whether this contributes to the salt-resistance of this strain, cannot be assessed from the present findings. Finally, as our binding study was performed following 2 weeks of high salt diet and Strehlow et al.¹¹ assessed AT₁ receptor mRNA after 6 weeks of high salt diet, one cannot exclude that AT₁ receptors in Dahl S rats are differentially regulated depending on the time period of the high salt diet.

AT₁ receptor stimulation is critical for salt-induced hypertension as central blockade of AT₁ receptors prevents the exacerbation of hypertension in Dahl S rats on a high salt diet^{4,5}. AT₁ receptor activation may result either from the action of angiotensins synthesized by the brain, or the actions of circulating Ang II on circumventricular organs which lack a blood-brain barrier. In this study, we observed that salt-induced hypertension in Dahl S rats is associated with a more pronounced decrease in AT₁ receptors in the SFO of Dahl S rats compared to Dahl R rats. The SFO, a circumventricular organ, almost exclusively expresses AT₁ receptors and mediates pressor and osmoregulatory effects of circulating Ang II^{21,22}. Bouhnik et al.²³ reported similar plasma Ang II concentrations in young Dahl S and R rats on regular and high salt diet for 4 weeks. In the substrain of Dahl S rats we used, at 6 weeks of age on regular salt diet or after a high salt diet from 4 to 6 weeks of age, plasma Ang II concentrations are lower than in Dahl R rats²⁴. Despite lower plasma Ang II concentrations in Dahl S compared to Dahl R rats, it appears that Ang II concentrations are not regulated in

response to high salt diet for this duration of time in either strain ²⁴. It is therefore unlikely that the decrease in AT₁ receptor density observed in the SFO on high salt diet in either Dahl R or Dahl S rats in the present study relates to circulating Ang II. However, one cannot exclude that an increase in affinity of AT₁ receptors in the SFO may have contributed to increased *in vivo* binding and consequent homologous down-regulation. Ang II may also have been derived from circulating Ang I, cleaved by the high concentrations of ACE found in the SFO. This explanation seems unlikely as Dahl S rats on either regular or high salt diet also have markedly lower plasma Ang I concentrations than Dahl R rats on similar diets ²⁴. Alternatively, Ang II may have been released from synaptic input to the SFO. Afferents to the SFO include input from cardiovascular regulatory areas such as the medial septal nucleus, OVLT, MnPO, anterior hypothalamic area, PVN and the LC ²¹. The enhanced decrease in AT₁ receptor binding in Dahl S versus Dahl R rats may reflect increased AT₁ receptor stimulation of the SFO. Electrical stimulation of the SFO appears to result in pressor responses due to post-synaptic actions of Ang II and appear to be mediated via a pathway to the PVN ²⁵. It is therefore possible that enhanced AT₁ receptor stimulation in the SFO contributes to the development of hypertension. Alternatively, the enhanced decrease in binding may be a compensatory mechanism to counteract the increase in blood pressure.

The SFO influences sodium homeostasis and blood pressure regulation via its connections to a number of nuclei, particularly via angiotensinergic connections to the MnPO, OVLT and PVN ^{21,26-28}. On the basis of lesion experiments, the suprachiasmatic nucleus is proposed to contribute to regulation of feeding and drinking ²⁹. As decreased

¹²⁵I-Sar¹Ile⁸-Ang II binding to AT₁ receptors in the SFO, OVLT, PVN and SCh was observed in both rat strains on high salt diet, it is likely that in these areas decreases in receptor density reflect elevated locally released Ang II which may mediate osmoregulatory mechanisms.

Activation of AT₂ receptors appears to exert a tonic inhibitory control on some of the stimulatory actions of Ang II on AT₁ receptors³⁰ but it is not known which nuclei/areas mediate this effect. Consistent with previous reports^{3,31} we did not detect any binding to AT₂ receptors in areas known to participate in osmoregulation such as the SFO, MnPO, OVLT and PVN. The LC is the major source of the brain's noradrenergic system, expresses AT₂ receptors and has proposed roles in sleep, memory and vigilance³²⁻³⁴. Chemical stimulation of noradrenergic cell bodies in the LC decreases blood pressure, renal sympathetic activity and heart rate^{35,36}. Efferents from the LC to the supraoptic nucleus have been implicated in the baroreflex regulation of vasopressin release³⁷, indicating that the LC also plays a role in central regulation of blood pressure. However, in the present study AT₂ receptor binding within the LC was similar between strains on either regular or high salt diet. AT₂ receptors do not undergo internalization of the receptor-agonist complex³⁸ and therefore an increased *in vivo* receptor stimulation may not result in a decrease in membrane receptor density. Therefore, this does not exclude that AT₂ receptor stimulation in this nucleus is involved in pathways contributing to differences in resting blood pressure or responsiveness to stressors.

In conclusion, salt-induced hypertension in Dahl S rats is accompanied by an enhanced decrease in AT₁ receptor density within the SFO. Further studies will be

required to determine whether this reflects an angiotensinergic mechanism within the SFO which contributes to the development of salt-induced hypertension, or a compensatory mechanism to counteract the increase in blood pressure. Salt-induced decreases in AT₁ receptor binding in the OVLT, the SCh and the PVN probably reflect osmoregulatory mechanisms as the extent of changes in receptor binding was similar in Dahl R and Dahl S rats on high salt diet.

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Chapter 5

General Discussion

5.1 Summary

The research presented in this thesis investigates the role of the vAV3V, ie the OVLT and the overlying part of the subcommissural MnPO, in salt-induced hypertension. Previous studies (Nakamura and Cowley Jr, 1989; Simchon et al.1999) have suggested that a differential increase in CSF sodium concentrations induced by high dietary salt may activate central pathways that mediate sympathoexcitation and hypertension. In earlier studies, we have demonstrated that hypertension induced by a chronic increase in CSF sodium concentration, as well as, salt-induced hypertension in Dahl S rats, are mediated by the effects of OLC and AT_1 receptor stimulation (Teruya et al.1995; Huang and Leenen, 1997; Huang et al.1998). The study presented in Chapter 2 demonstrated that the pressor and tachycardic effects of short-term icv infusions of hypertonic saline and icv injections of ouabain were partially mediated by the vAV3V in rats with systemic vasopressin receptor blockade (Veerasingham and Leenen, 1997). The next experiments were designed to investigate the role of the vAV3V in the development of sympathoexcitation and hypertension in two rat models of hypertension in which mechanisms of salt-sensitive hypertension are mimicked, ie an increase in central sodium and OLC concentrations. We demonstrated that the vAV3V region mediates hypertension induced by chronic icv administration of hypertonic saline or chronic subcutaneous ouabain administration, possibly via sympathetic activation (Veerasingham and Leenen, 1999, Chapter 3). The vAV3V region, in particular the OVLT, has high densities of AT_1 receptors. In the next experiment, we sought to determine if changes in AT_1 receptor density within the vAV3V accompany the development of salt-induced hypertension in

Dahl S rats. Salt-induced hypertension in Dahl S rats was accompanied by an enhanced AT_1 receptor down-regulation within the SFO but not in the OVLT, the major nucleus of the vAV3V (Veerasingham and Leenen, 2000b, Chapter 4). Salt-induced decreases in AT_1 receptor binding in the OVLT, the SCh and the PVN are likely to reflect an angiotensinergic mechanism involved in osmoregulation and regulation of feeding as the extent of changes in receptor binding was similar in Dahl R and Dahl S rats on high salt diet (Veerasingham and Leenen, 2000b, Chapter 4). The vAV3V area therefore appears to play a crucial role in mediating hypertension in models of salt-induced hypertension, possibly via sympathetic activation mediated by a non-angiotensinergic mechanism.

5.2 The Advantages of Excitotoxic Lesions

In the experiments presented in Chapters 2 and 3, the vAV3V was lesioned by microinjections of an excitotoxin, ibotenic acid. Ibotenic acid-induced neurotoxicity is mediated via *N*-methyl-D-aspartate (NMDA) type glutamate receptors on neuronal perikarya (Zinkland et al.1992). Cell death is due to an increase in intracellular Ca^{2+} concentration resulting from Ca^{2+} entry via the NMDA receptor-channel complex (Choi, 1987; Mayer and Westbrook, 1987). Ibotenic acid-induced lesions spare nerve axons and terminals within the lesioned area where there are few or no receptors for it (Schwarcz et al. 1979) and therefore provides a more selective lesion compared to mechanical, electrolytic or radio-frequency techniques which would damage fibres of passage in addition to neuronal soma within the lesioned area. Furthermore, microinjections of an excitotoxin produce a more discrete lesion than mechanical, electrolytic or radio-

frequency techniques as the extent of the lesion can be well contained by controlling the volume of injection. In contrast to other excitatory amino acids, ibotenic acid does not cause lesions remote to its target (Schwarcz et al. 1979; Guldin and Markowitsch, 1981; Kohler and Schwarcz, 1981). Ibotenic acid provides a more restricted lesion than kainic acid, another frequently used excitotoxin (Schwarcz et al. 1979; Guldin and Markowitsch, 1981). It also ablates tissue resistant to kainic acid (Kohler and Schwarcz, 1981) and does not produce epileptiform activity seen in rats after kainic acid use (Guldin and Markowitsch, 1981; Jellestad and Grahnstedt, 1985). It has been suggested that the precision of ibotenic acid lesions is due to rapid decarboxylation of the excitotoxin to muscimol, a non-toxic GABA agonist (Kohler et al. 1979; Schwarcz et al. 1979). In our studies neuronal degeneration along the track made by the injection micropipette was avoided by leaving the micropipette in place for 10 minutes following the injection of ibotenic acid, as previously described (Jarrard, 1989). Lesions were verified by visualizing the absence of immunoreactivity to NeuN, a neuron specific nuclear antigen as previously reported (Morassutti et al. 1994). This method delineates the lesioned area by an absence of neuronal nuclei which provides greater precision than visualization of gliosis which typically extends into the area surrounding the lesioned area.

The studies presented in Chapters 2 and 3 therefore likely indicate that neuronal somata within the vAV3V contribute to the pressor and tachycardic responses to acute icv hypertonic saline and ouabain administration, and are essential for (icv) hypertonic saline- and (sc) ouabain-induced hypertension.

5.3 The vAV3V and Pressor Effects of icv Hypertonic Saline

Hypertonic saline administered as icv injections or short-term infusions increases arterial pressure in various species including rats (Andersson et al. 1972; Chiu and Sawyer, 1974; Bunag and Miyajima, 1984; Abe et al. 1989; Wells et al. 1990). Consistent with these studies, short-term (10 min) icv infusions of hypertonic saline resulted in ~18 mmHg increase in MAP in the present study. The pressor response to icv NaCl appears to be due to the changes in CSF Na⁺ and Cl⁻ concentrations rather than to a net change in CSF osmolality as icv injections of hypertonic solutions of sucrose, glycerol or sorbitol either did not change blood pressure, or caused a much smaller pressor response (May and McAllen, 1997; Bunag and Miyajima, 1984; Shah and Jandhyala, 1991). The pressor response to icv hypertonic saline appears to be mediated by an increase in sympathetic outflow as well as release of vasopressin (Thrasher et al. 1980; Bunag and Miyajima, 1984; Wells et al. 1990; Shah and Jandhyala, 1991). Chiu and Sawyer (1974) reported that guanethidine treatment inhibiting release of endogenous norepinephrine from post-ganglionic adrenergic nerve endings suppressed pressor responses to icv hypertonic saline in cats suggesting that an increase in sympathetic outflow mediated the response. However, results obtained from recordings of multifibre sympathetic nerve preparations during icv infusions of hypertonic saline have varied from increases in baroreceptor-denervated cats (Schad and Seller, 1975) or dogs (Takishita and Ferrario, 1983) to decreases in baroreceptor-denervated cats (Tobey et al. 1983), as well as carotid-sinus denervated dogs (Takishita and Ferrario, 1983) and intact sheep (May and McAllen, 1997). The reasons for the discrepancies in the results of these studies are not clear and

may relate to different experimental conditions. In rats, icv hypertonic NaCl causes an initial decrease in heart rate and sympathetic nerve activity (in the first 5 min of infusion), followed by an increase in both heart rate and sympathetic nerve activity to greater than preinfusion levels (Bunag and Miyajima, 1984). In rats that were pretreated with a vasopressin antagonist, the early pressor response to icv hypertonic saline infusion was inhibited, and sympathetic nerve activity increased from the onset of infusion (Bunag and Miyajima, 1984). It is therefore likely that the early pressor response to icv hypertonic NaCl results from vasopressin release and the arterial baroreceptor reflex response to the increase in blood pressure causes the initial decreases in heart rate and sympathetic nerve activity. Infusions of ammonium chloride increased blood pressure but did not affect sympathetic nerve activity (Bunag and Miyajima, 1984). Furthermore, pressor responses to choline chloride were attenuated by iv administration of a vasopressin antagonist but not by ganglionic blockade indicating that vasopressin release, rather than an increase in sympathetic activity, mediates the increase in blood pressure in response to Cl⁻ (Shah and Jandhyala, 1991).

5.3.1 Short-Term Infusions

Lesions of the vAV3V attenuated the pressor and tachycardic responses to short-term (10 min) icv hypertonic saline infusions by ~30 %. Our findings differ from those of an earlier study in which AV3V lesions prevented pressor responses to acute icv injection of hypertonic saline (Buggy et al. 1984). Electrolytic AV3V lesions disrupt vasopressin secretion (Sladek and Johnson, 1983; Gruber et al. 1986; Wilkin et al. 1986; Morris et al. 1994). It is not known whether excitotoxic lesions of the vAV3V also cause deficits in

vasopressin release. However, we assessed responses in rats that received systemic injections of a vasopressin receptor antagonist thereby excluding a vasopressin mediated pressor response. On the other hand, electrolytic lesions firstly lesion a larger area involving the whole subcommissural MnPO and the anterior hypothalamic periventricular area and secondly, lesion fibres of passage as well as neuronal somata. Destruction of fibres of passage during electrolytic AV3V lesions may account for some of the effects of these lesions, such as the initial increase in resting blood pressure (Bealer and Van Huysse, 1989). Rohmeiss et al. (1995a) demonstrated that microinjection of losartan into the SFO attenuated the pressor response to icv hypertonic saline indicating that the response to a significant extent is mediated by an angiotensinergic mechanism in the SFO. It has been suggested that this response may be mediated via a pathway from the SFO to the paraventricular nucleus (Rohmeiss et al. 1995a). As this pathway synapses in, or traverses the AV3V it would be destroyed by electrolytic lesions of the AV3V but not by excitotoxic lesions limited to the ventral part of the region (vAV3V lesions) possibly explaining the discrepancy in the degree of inhibition of pressor responses to icv hypertonic saline between our study and those of earlier studies (Buggy et al. 1984). The major nucleus within the vAV3V, the OVLT, has putative roles as an osmoreceptor (McKinley et al. 1996). In particular, neurons within the dorsal cap of the OVLT project to the SON, the major site of production of vasopressin in the rat, and are activated in response to osmotic stimuli (Oldfield et al. 1991). Neuronal activation of the OVLT has also been demonstrated in response to icv administration of hypertonic saline (Solano-Flores et al. 1993). Therefore, in addition to the SFO, the OVLT appears to also

contribute to responses to icv hypertonic saline.

5.3.2 *Chronic Infusions*

Lesions of the vAV3V prevented the development of hypertension in rats that received chronic icv administration of hypertonic saline. The greater degree of effectiveness of vAV3V lesions in preventing the pressor response of chronic icv infusions compared to short-term infusions may have several explanations. Firstly, chronic infusions (0.8 M NaCl, 5 μ l/h) would likely have resulted in lower CSF sodium concentrations compared to 10 min infusions (0.3M NaCl, 2 μ l/min) as a ~10-fold lower dose was administered chronically. Presumably, pressor responses to CSF sodium concentrations achieved with chronic infusions are mediated via the vAV3V whereas higher concentrations achieved with short-term infusions may have acted on nuclei less sensitive to sodium in addition to the vAV3V. Furthermore, as a vasopressin antagonist was not administered to rats in this experiment, it is possible that hypertonic saline-induced vasopressin release may have contributed to the development of hypertension. Kawano et al. (1991) reported that vasopressin mediates the pressor effect of icv hypertonic saline infusion during the early phase (day 1), whereas during the chronic phase of infusion (day 7), elevated catecholamines and augmented depressor responses to ganglionic blockade suggest an increase in sympathetic activation. Electrical stimulation of the anterior hypothalamus elicited smaller decreases in blood pressure and sympathetic nerve activity in rats that received chronic icv infusions of hypertonic saline compared to rats with control infusions of aCSF (Miyajima and Bunag, 1984). Depressor responses to iv injections of histamine was similar in both groups indicating a similar cardiovascular

responsiveness in both groups. It therefore seems likely that decreased anterior hypothalamic inhibition of vascular sympathetic tone contributes to chronic icv hypertonic saline-induced hypertension. In the present study vAV3V lesions prevented the enhanced depressor response to ganglionic blockade elicited by chronic icv administration of hypertonic saline. In addition, despite mild hypertension in sham operated rats receiving hypertonic saline, resting MAP was similar in all groups following ganglionic blockade. It is therefore likely that sympathetic activation mediated by the vAV3V persisted in sham operated rats and accounts for the greater part of the hypertension caused by chronic icv administration of hypertonic saline.

5.3 The vAV3V and Ouabain-Induced Pressor Responses

Consistent with previous studies (Takahashi et al.1984; Huang and Leenen, 1996b) we have reported a pressor response to icv injections of ouabain. Excitotoxic lesions of the vAV3V attenuated pressor and HR responses to icv injections of ouabain by ~30 %. These findings are consistent with an earlier report by Takahashi et al. (1984) that electrolytic AV3V lesions attenuated the pressor responses of icv ouabain injections. In this study the lesions attenuated the pressor response to a greater extent (~60 %). This could be due to either the larger region lesioned with an AV3V lesion, lesion of fibres of passage through the AV3V that could possibly mediate the responses, or the larger dose of ouabain used in the study (1 µg vs. 0.6 µg used in the our study) affecting more brain regions.

In rats that received chronic sc ouabain treatment, vAV3V lesions prevented the

development of ouabain-induced hypertension. It is likely that vAV3V lesions caused a greater degree of blockade of the pressor response to ouabain in rats that received chronic sc ouabain treatment compared to rats that received icv injections of ouabain because the chronic sc treatment probably resulted in lower central concentrations of ouabain which presumably would act only on nuclei most sensitive to it whereas a larger dose (acute icv injections) would be able to act on alternate less sensitive nuclei as well as the vAV3V. An increase in vascular tone in ouabain-induced hypertension could be due to an increase in sympathetic outflow as hypertension caused by chronic ouabain administration, is reversed by ganglionic blockade (Huang et al. 1994). Following chronic sc administration, ouabain accumulates in the hypothalamus, pons and pituitary (Huang et al. 1994). As central blockade of the effects of ouabain using chronic icv administration of antibody fab fragments that bind ouabain prevents sc ouabain-induced hypertension, it is likely that a central action of ouabain is mostly responsible for the development of hypertension in this model (Huang et al. 1994). In the present study, the enhanced depressor responses to ganglionic blockade elicited by chronic sc administration of ouabain were prevented by vAV3V lesions suggesting that this region mediates autonomic mechanisms, possibly sympathetic activation, in ouabain-induced hypertension. Following ganglionic blockade, blood pressure was similar in all groups consistent with a role for sympathetic activation in ouabain-induced hypertension. The development of ouabain-induced hypertension is accompanied by an increase in FLI, a marker of neuronal activation, within the SON, mostly within the ventral half of the nucleus, suggesting a possible involvement of vasopressinergic neurons (Veerasingham et

al.2000a, Appendix Fig. 1). However, administration of a vasopressin receptor antagonist after ganglionic blockade only caused a further 5 mmHg decrease in MAP in rats treated chronically with ouabain (Huang et al. 1994), suggesting that release of vasopressin contributes only in a minor way to mechanisms responsible for ouabain-induced hypertension. In this experiment ouabain was administered sc and could therefore also act peripherally to cause hypertension eg by inhibiting the Na^+K^+ -ATPase in vascular smooth muscle resulting in vasoconstriction (Yuan et al. 1993). However, as central blockade of the effects of ouabain completely prevents sc ouabain-induced hypertension a central mechanism is favoured.

5.4 Cardiovascular Responses to Air Jet Stress Are Not Mediated by the vAV3V

Consistent with our previous report (Huang et al.1998, Appendix: Table 1), cardiovascular responses to a form of environmental stress, air jet stress, were enhanced in rats that received chronic icv infusions of hypertonic saline. Various brain areas have been proposed to mediate the cardiovascular responses to air jet stress. DiMicco's group has demonstrated that disinhibition of neurons within the dorsomedial hypothalamus (DMH) or posterior hypothalamus using GABA A receptor antagonists evokes physiological changes similar to that seen in acute experimental stress (Wilbert et al. 1988; DiMicco et al. 1996). Conversely, microinjection of a GABA A agonist, muscimol, into the DMH abolishes air stress-induced increases in arterial pressure and heart rate (Stotz-Potter et al. 1996), whereas in the posterior hypothalamus it abolishes the increase in heart rate but not arterial pressure (Lisa et al. 1989). Blockade of excitatory amino acid

receptors within the DMH also attenuates stress-induced cardiovascular changes (DiMicco et al. 1996). These studies indicate that within the DMH, a balance of activation of inhibitory GABA A receptors and excitatory amino acid receptors mediates stress-induced cardiovascular responses. Air jet stress for 90 minutes increases AT_{1A} receptor mRNA in the PVN and AT_2 receptor mRNA in the locus coeruleus (LC, Dumont et al. 1999). However, it is not known whether the stress-induced regulation of Ang II receptors in these areas is involved in mediating cardiovascular responses.

In rats that received chronic infusions of hypertonic saline, lesions of the vAV3V did not prevent enhanced cardiovascular responses to air jet stress. As these lesions prevented enhanced depressor responses to ganglionic blockade, it appears that the vAV3V mediates the increase in basal sympathetic tone due to icv administration of hypertonic saline, but not the enhanced cardiovascular responses to air jet stress exhibited by this model. This is consistent with studies in salt-sensitive models of hypertension. Hatton et al. reported that electrolytic ablation of the AV3V in borderline hypertensive rats fed high salt diet did not affect cardiovascular responses to air stress (Hatton et al. 1991). In rats that received chronic icv administration of hypertonic saline, or models of salt-sensitive hypertension such as spontaneously hypertensive rats or Dahl rats on high salt diet, enhanced cardiovascular responses to air jet stress can be prevented by icv administration of antibody fab fragments that bind OLC, or icv treatment with losartan, indicating that the central action of OLC and central AT_1 receptor stimulation is required for these stress-induced responses (Huang et al. 1998; Huang and Leenen, 1998; Huang and Leenen, 1996a). In spontaneously hypertensive rats on high salt diet, administration of these fab

fragments in the MnPO did not affect cardiovascular responses to air jet stress (Budzikowski and Leenen, 1997). It is therefore likely that OLC and Ang II act in area(s) other than the AV3V to mediate the enhanced cardiovascular responses to air jet stress.

5.5 Pressor Responses to icv Ang II are Not Mediated by the vAV3V

Ang II, administered icv, results in an increase in sympathetic nerve activity and blood pressure, similar to responses to icv injections of hypertonic saline (Ferrario, 1983; Huang and Leenen, 1996b). In our study, pressor responses to icv Ang II (10 or 30 ng) were not affected by lesions of the vAV3V area. Earlier studies have demonstrated a blockade of pressor responses to icv Ang II following electrolytic ablation, or chemical sympathectomy using 6-hydroxydopamine (6-OHDA), of the AV3V region (Buggy et al. 1984; Bellin et al. 1987). Combined treatment of the AV3V with 6-OHDA and desmethyl-imipramine, a drug that blocks the uptake of 6-OHDA into adrenergic but not dopaminergic nerve terminals therefore sparing adrenergic neurons, did not affect pressor responses to Ang II (Bellin et al. 1988). This study suggests a role for adrenergic neurons within the AV3V region in mediating pressor responses to Ang II. Centrally administered Ang II results in an increase in Fos expression in the MnPO and juxta-ventricular neurons of the SFO and OVLT indicating cellular activation in response to central Ang II in these areas (McKinley et al. 1995). Xu and Herbert demonstrated suppression of cellular activation to icv Ang II following electrolytic lesion of the subcommissural MnPO despite an intact OVLT in central sites which receive efferents from the AV3V region (Herbert and Xu, 1995). This suggests that the MnPO, or fibres of passage through the

MnPO, mediates responses to icv Ang II. Since our lesions did not affect the subcommissural MnPO to a large extent and would not have destroyed fibres of passage from the SFO that traverse the MnPO, it is not surprising that we did not see attenuations of pressor responses to icv Ang II. In contrast, electrolytic lesions of the AV3V region would have ablated both the subcommissural MnPO as well as fibers of passage through it, thus explaining why these lesions prevent icv Ang II-induced pressor responses.

5.6 Salt-Induced Hypertension in Dahl S Rats is Associated With Enhanced AT₁ Receptor Down-Regulation in the SFO, But Not the vAV3V

Central AT₁ receptor blockade prevents salt-induced sympathoexcitation and hypertension in Dahl S rats (Teruya et al. 1995; Huang and Leenen, 1997) indicating a critical role for AT₁ receptor activation in mediating salt-induced hypertension. Pressor responses to icv Ang II are enhanced in Dahl S, but not Dahl R rats on high salt diet (Goto et al. 1981a). In addition, sympathoexcitatory and pressor responses to icv administration of Ang I are also enhanced in Dahl S, but not Dahl R rats on high salt diet (Zhao et al. 2000a) indicating an increased central responsiveness to either Ang II or Ang III. However, particular brain nuclei/areas involved are not known as these studies utilized icv administration of AT₁ receptor antagonists, Ang I or Ang II. Our group has demonstrated that the development of salt-induced hypertension in Dahl S rats is accompanied by increases in ACE mRNA and ACE activity in hypothalamic and pons homogenates (Zhao et al. 2000a). However, despite similar Ang I concentrations in hypothalamic homogenates between strains on either regular or high salt diet,

hypothalamic Ang II concentrations of Dahl S rats on regular salt diet were lower than in Dahl R rats on a similar diet and were not regulated by high salt diet in either strain (Zhao et al. 2000a). Similar Ang II concentrations in Dahl S and R rats on high salt diet despite a marked increase in ACE activity in the Dahl S rats may be explained by an increased metabolism to Ang III or increased cellular internalization following binding to receptors. Alternatively, increases in Ang II within particular hypothalamic nuclei may not have been detected because homogenates of the whole hypothalamus were used for the assay.

Lesion studies have implicated the AV3V (Goto et al. 1982), the PVN (Goto et al. 1981b; Ernsberger et al. 1985) and the anteromedial hypothalamus (Azar et al. 1981; Ernsberger et al. 1985) which includes the PVN, SCh and intervening periventricular tissue, in mediating salt-induced hypertension in this model. As these regions contain components of the brain RAS (Healy and Printz, 1984; Lenkei et al. 1995), they may mediate salt-induced hypertension via an increase in its activity, that is, synthesis and release of its effector peptides. In addition, the regulation of AT₁ receptors in these areas may be critical in modifying the activity of the brain RAS in response to salt. In our study, high salt diet decreased ¹²⁵I-Sar¹Ile⁸-Ang II binding to AT₁ receptors by 25-30 % in both Dahl R and Dahl S rats in the OVLT, the SCh and the PVN. These nuclei contribute to osmoregulation and the regulation of feeding and drinking. Magnocellular neurons of the PVN synthesize vasopressin, oxytocin and transport these peptides to the neural lobe where they are released into the circulation in response to a various stimuli including perturbations in sodium homeostasis and blood pressure (Renaud and Bourque, 1991). Magnocellular neurons of the PVN may also possibly be the site of OLC production

which may be released in response to osmotic stimuli in a similar fashion to vasopressin and oxytocin (see section 1.1.2b). However, the majority of AT₁ binding sites are distributed in the parvocellular PVN which projects mainly to the external layer of the median eminence and to autonomic preganglionic and related nuclei. The OVLT has reciprocal connections to the PVN and also projects to the SON (see Table 1, section 1.6.1) and these pathways are implicated in vasopressin release in response to hypertonicity (McKinley et al. 1996). On the basis of lesion experiments, the SCh is proposed to contribute to regulation of feeding and drinking (Van den Pol and Powley, 1979). As AT₁ receptor binding was decreased similarly in both strains in the OVLT, SCh and PVN, it is likely that the decrease in AT₁ receptor binding reflects an angiotensinergic mechanism involved in osmoregulation in response to high salt diet in these rats. As AT₁ receptor binding was not significantly different between strains on high salt diet in the OVLT, the major nucleus of the vAV3V, or the overlying MnPO, it appears that angiotensinergic mechanisms in this area are similar in both strains and therefore are not likely to contribute to salt-induced hypertension in Dahl S rats.

The SFO is known to influence sodium homeostasis and blood pressure regulation via its connections to a number of nuclei, particularly via angiotensinergic connections to the MnPO, OVLT, PVN and SON (Johnson et al. 1992; Ferguson, 1992). Salt-induced hypertension in Dahl S rats following 2 weeks of a high salt diet was associated with a more pronounced decrease of AT₁ receptor density in the SFO compared to Dahl R rats. The SFO, a circumventricular organ, is known to mediate pressor and osmoregulatory effects of circulating Ang II (Simpson, J.B. and A. Routtenberg, 1973; Mangiavane and

Simpson, 1980) and predominantly expresses AT₁ receptors (Lenkei et al. 1997).

However, it is not likely that the decrease in AT₁ receptor density observed in the SFO on high salt diet in either Dahl R or Dahl S rats relates to circulating Ang II as plasma Ang II concentrations are reportedly similar, or lower than in Dahl R rats, and are not altered in either strain by a high salt diet (Zhao et al. 2000b; Bouhnik et al. 1992). However, one cannot exclude that an increase in affinity of AT₁ receptors in the SFO may have contributed to increased *in vivo* binding and consequent homologous down-regulation. Ang II may also have been derived from circulating Ang I, cleaved by the high concentrations of ACE found in the SFO. This explanation seems unlikely as Dahl S rats on either regular or high salt diet also have markedly lower plasma Ang I concentrations than Dahl R rats on similar diets (Zhao et al. 2000b). Alternatively, Ang II may have been released from synaptic input to the SFO. Afferents to the SFO include input from cardiovascular regulatory areas such as the medial septal nucleus, OVLT, MnPO, anterior hypothalamic area, PVN and the LC (McKinley et al. 1999). The enhanced decrease in AT₁ receptor density in Dahl S versus Dahl R rats may reflect increased AT₁ receptor stimulation of the SFO. Electrical stimulation of the SFO appears to result in pressor responses due to post-synaptic actions of Ang II and appear to be mediated via a pathway to the PVN (Ferguson and Washburn, 1998). It is therefore possible that enhanced AT₁ receptor stimulation in the SFO contributes to the development of hypertension. Alternatively, the enhanced decrease in receptor density may be a compensatory mechanism to counteract the increase in blood pressure.

The salt-induced decreases in AT₁ receptor density we observed within the SFO,

OVLT, SCh and PVN in both Dahl R and Dahl S rats extend the findings of a previous report in which a decrease in Ang II receptor binding (without subtype distinction) in homogenates of the hypothalamus, thalamus, striatum, midbrain and cortex was observed in normotensive Wistar Kyoto rats that drank 1 % NaCl for 4 weeks (Mizuno and Fukuchi, 1981). In another study, high salt diet for 2 to 3 weeks only induced minor changes (-6.3 ± 4.4 %) in Ang II receptor binding to whole brain homogenates from normotensive Wistar rats (Mann et al. 1980). The use of whole brain homogenates in this study may have masked differences in binding densities in specific brain regions/nuclei between rats on regular and high salt diet. A high salt diet for 3 weeks also results in an upregulation of AT_{1A} receptor mRNA in decorticated brain homogenates in normotensive rats (Sandberg et al. 1994). Similarly, in Dahl S rats, a high salt diet for 6 weeks increases AT₁ receptor mRNA in whole brain homogenates three times as much as in Dahl S rats on regular salt diet (Strehlow et al. 1999). It is not clear why brain AT₁ receptor mRNA increases in Dahl S and normotensive rats on high salt diet whereas AT₁ receptor binding decreases in response to high salt intake. An increase in AT₁ mRNA combined with a decrease in receptor binding could represent an increase in receptor turnover as AT₁ receptor mRNA reflects message for production of AT₁ receptors and would include AT₁ receptors at the cell membrane as well as internalized receptors whereas AT₁ receptor binding reflects receptor density at the cell membrane only. As ligands would have dissociated or degraded during the initial incubation in the absence of peptidase or protease inhibitors, it is unlikely that the decrease in AT₁ receptor binding reflects increased receptor occupancy in our study. AT₁ receptors in the brain appear to be

regulated in response to its ligand as chronic icv infusions of Ang II in normotensive rats result in an increase in AT₁ receptor mRNA and protein in whole brain homogenates (Porter, 1999). We speculate that a decrease in receptor density *in vitro* in the present study may reflect a marked increase in AT₁ receptor occupancy *in vivo* followed by receptor internalization and a subsequent increase in the receptor mRNA. As receptor density assessments were performed following 2 weeks of high salt diet in our study and Strehlow et al. (1999) assessed AT₁ receptor mRNA after 6 weeks of high salt diet, one cannot exclude that AT₁ receptors in Dahl S rats are differentially regulated depending on the time period of the high salt diet.

Strehlow et al. (1999) reported that in Dahl R rats, 6 weeks of high salt diet does not significantly change AT₁ receptor mRNA. In the present study, AT₁ receptor density decreased in several nuclei in Dahl R rats on high salt diet. These findings may point to an actual down-regulation of the receptor, or that an increase in AT₁ receptor occupancy *in vivo* and subsequent internalization of the receptor-ligand complex resulted in decreased *in vitro* membrane receptor density. The increase in AT₁ receptor mRNA seen in other normotensive strains and salt-sensitive Dahl S rats on a high salt intake is absent in Dahl R rats indicating that these rats exhibit altered regulatory mechanisms for AT₁ receptor production in response to high salt intake compared to Dahl S rats or normotensive rats. It is possible that AT₁ receptor turnover is not increased in Dahl R rats in response to high salt diet. Whether this contributes to the salt-resistance of this strain, cannot be assessed from the present study.

Activation of AT₂ receptors appears to exert a tonic inhibitory control on some of the

stimulatory actions of Ang II on AT₁ receptors (Hohle et al.1995) but it is not known which nuclei/areas mediate this effect. Consistent with previous reports (Tsutsumi and Saveedra, 1991; Sanvito et al.1997) we did not detect any binding to AT₂ receptors in areas known to participate in osmoregulation such as the SFO, MnPO, OVLT and PVN. We detected moderate AT₂ receptor densities in the LC, the major source of the brain's noradrenergic system. The LC has proposed roles in sleep, memory and vigilance (Hobson et al.1975; Aston-Jones, 1985; Sara and Devaues, 1989). LC may also play a role in central regulation of blood pressure as chemical stimulation of noradrenergic cell bodies in the LC decreases blood pressure, renal sympathetic activity and HR (Miyawaki et al. 1991; Sved and Fleston, 1987) and efferents from the LC to the supraoptic nucleus have been implicated in the baroreflex regulation of vasopressin release (Banks and Harris, 1984). In the present study AT₂ receptor binding within the LC was similar between strains on either regular or high salt diet. AT₂ receptors do not undergo internalization of the receptor-agonist complex (Hein et al. 1997). Furthermore, Csikos et al. (1998) have indicated that an endogenous ligand for the AT₂ receptor prevents degradation of the receptor. Therefore, an increased *in vivo* receptor stimulation may not result in a decrease in membrane receptor density. The absence of differences in AT₂ receptor densities does not, therefore, exclude that AT₂ receptor stimulation in this nucleus is involved in pathways contributing to differences in resting blood pressure or responsiveness to stressors.

5.7 Conclusions

The major findings presented in this thesis corroborate previous reports that the

AV3V region is critical for the pressor and tachycardic responses to hypertonic saline and ouabain and extends these studies by narrowing down the area that mediates these responses to the vAV3V. The vAV3V area also plays a crucial role in mediating hypertension in models that mimic mechanisms of salt-sensitive hypertension, such as an increase in CSF sodium concentration and an increase in central OLC content. It appears that enhanced sympathetic activation in these models of hypertension may be mediated via the vAV3V. Previous studies have indicated that the integrity of the AV3V region is essential for the development of salt-induced hypertension in Dahl S rats (Goto et al. 1982) and that salt-induced sympathoexcitation and hypertension require AT₁ receptor stimulation (Teruya et al. 1995; Huang and Leenen, 1997). We demonstrate that salt-induced hypertension in Dahl S rats is accompanied by an enhanced decrease in AT₁ receptor density within the SFO but not in the OVLT, the major nucleus of the vAV3V. Further investigation will be required to determine whether this reflects an angiotensinergic mechanism within the SFO which contributes to the development of salt-induced hypertension, or a compensatory mechanism to counteract the increase in blood pressure. Salt-induced decreases in AT₁ receptor density in the OVLT, the Sch and the PVN probably reflect osmoregulatory mechanisms as the extent of changes in receptor density was similar in Dahl R and Dahl S rats on high salt diet. In conclusion, the vAV3V area therefore appears to play a crucial role in mediating hypertension in models of salt-induced hypertension, possibly via sympathetic activation mediated by a non-angiotensinergic mechanism.

5.8 Addendum

The following clarification was added following the oral defence:

Chapter 2, page 59: The three rats excluded from the analysis should be described as having an off-midline lesion rather than a unilateral lesion.

Chapter 3, Figure 1, D and E: NeuN immunoreactivity was not significantly different between sham and lesioned rats in the periventricular hypothalamic nucleus, anterior medial preoptic nucleus and anteroventral preoptic nucleus.

Chapter 6

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Chapter 7

Appendix

Table 1, 2 and 3 are from: Huang, B.S., Veerasingham, S.J., and Leenen, F.H.H. Brain "ouabain", ANG II, and sympathoexcitation by chronic central sodium loading in rats. *Am J. Physiol.* 274: H1269-H1276, 1998.

Table 1. Brain "ouabain" content and MAP and HR at rest and in response to air stress in rats treated with aCSF or aCSF containing 0.8 M or 1.2 M NaCl for 14 days

	aCSF	aCSF + 0.8 M NaCl	aCSF + 1.2 M NaCl
<i>n</i>	8	9	9
Brain "ouabain", µg/g tissue			
Hypothalamus	2.5 ± 0.1	3.6 ± 0.1*	4.1 ± 0.1 [#]
Pituitary	42.8 ± 1.5	64.7 ± 1.3*	78.1 ± 2.1 [#]
Pons	1.8 ± 0.1	2.7 ± 0.1*	3.0 ± 0.1*
Baseline hemodynamics			
MAP, mmHg	91 ± 2	113 ± 3*	117 ± 2*
HR, beats/min	401 ± 11	431 ± 11	439 ± 21
Responses to air stress			
Increase in MAP			
Absolute, mmHg	7 ± 1	14 ± 1*	15 ± 1*
Percent, % baseline	8 ± 1	12 ± 1*	13 ± 1*
Increase in HR			
Absolute, beats/min	21 ± 2	41 ± 5*	53 ± 5*
Percent, % baseline	5 ± 1	10 ± 2*	12 ± 2*

Values are means ± SE; n = no. of rats studied. MAP, mean arterial pressure; HR, heart rate; aCSF, artificial cerebrospinal fluid; "ouabain", brain ouabain-like activity. * *P* < 0.05 vs. aCSF. [#] *P* < 0.05 vs. other groups.

Table 2. Plasma and CSF sodium and potassium concentrations and resting MAP and HR in rats infused intracerebroventricularly with aCSF or aCSF containing 0.8 M or 1.2 M NaCl for 14 days

	aCSF	aCSF + 0.8 M NaCl	aCSF + 1.2 M NaCl
<i>n</i>	5	5	5
Plasma			
Na ⁺ , mmol/l	143 ± 3	137 ± 3	136 ± 4
K ⁺ , mmol/l	3.5 ± 0.1	3.4 ± 0.1	3.6 ± 0.2
CSF			
Na ⁺ , mmol/l	146 ± 2	152 ± 2*	160 ± 3*
K ⁺ , mmol/l	2.9 ± 0.1	2.9 ± 0.1	2.8 ± 0.1
Baseline hemodynamics			
MAP, mmHg	93 ± 3	112 ± 3*	118 ± 2*
HR, beats/min	394 ± 12	421 ± 11	435 ± 14*

Values are means ± SE; n = no. of rats studied. * *P* < 0.05 vs. aCSF; # *P* < 0.05 vs. other groups.

Table 3. Resting MAP, HR, CVP, and body weight in rats after treatment with intracerebroventricular aCSF plus γ-globulins, or 0.8 M NaCl plus γ-globulins, Fab fragments, or losartan for 14 days

	aCSF	0.8 M NaCl in aCSF		
	γ-Glob, <i>n</i> = 7	γ-Glob, <i>n</i> = 7	Fab frag, <i>n</i> = 7	losartan, <i>n</i> = 8
MAP, mmHg	93 ± 2	121 ± 2*	98 ± 3	96 ± 6
HR, beats/min	412 ± 11	442 ± 8*	422 ± 6	417 ± 13
CVP, mmHg	2.8 ± 0.5	2.5 ± 0.3	2.7 ± 0.6	2.5 ± 0.5
Body wt gain,g	91 ± 5	81 ± 6	83 ± 5	82 ± 4

Values are means ± SE; n = no. of rats studied. CVP, central venous pressure; γ-Glob, γ-globulins; Fab frag, Fab fragments. * *P* < 0.05 vs. other groups. # *P* < 0.05 vs. aCSF with γ-globulins.

Table 4, blood pressures and Figure 1 are from: Veerasingham, S.J., Vahid-Ansari, F., and Leenen, F.H.H. Neuronal Fos-like immunoreactivity in ouabain-induced hypertension. *Brain Res.* In press.

Table 4. Number of FLI-positive neurons/section in ouabain-induced hypertension

	Number of Immunoreactive Neurons/Section							
	vMnPO	PVNm	PVNp	AHA	SON	CG	LC	rNTS
7 d infusion								
Saline	6 ± 1	4 ± 1	10 ± 2	1 ± 1	0 ± 0	10 ± 1	0 ± 0	8 ± 1
Ouabain	5 ± 1	5 ± 2	12 ± 2	2 ± 1	1 ± 1	10 ± 1	0 ± 0	7 ± 1
14 d infusion								
Saline	9 ± 1	12 ± 2	6 ± 3	4 ± 1	2 ± 2	18 ± 4	0 ± 0	8 ± 1
Ouabain	7 ± 2	15 ± 3	10 ± 1	6 ± 1	9 ± 2*	13 ± 1	4 ± 2*	8 ± 1

Peak increases in the number of FLI positive neurons per section within cardiovascular regulatory areas in rats treated with sc saline or ouabain (50 µg/d) infusions for 7 or 14 d. Values are presented as means ± S.E.M.; n = 6; * p < 0.01 vs saline treated group for the same infusion period. vMnPO, ventral median preoptic nucleus; PVNm, paraventricular nucleus, magnocellular division; PVNp, paraventricular nucleus, parvocellular division; AHA, anterior hypothalamic area; SON, supraoptic nucleus; CG, central grey; LC, locus ceruleus; rNTS, rostral part of the nucleus tractus solitarius.

Blood pressures: Resting MAP in ouabain-treated rats was significantly higher than in control rats at 14 but not at 7 days (125 ± 4 vs 101 ± 6, p < 0.05 and 102 ± 4 vs 98 ± 6 NS respectively). Resting HR did not differ significantly between groups at either time point.

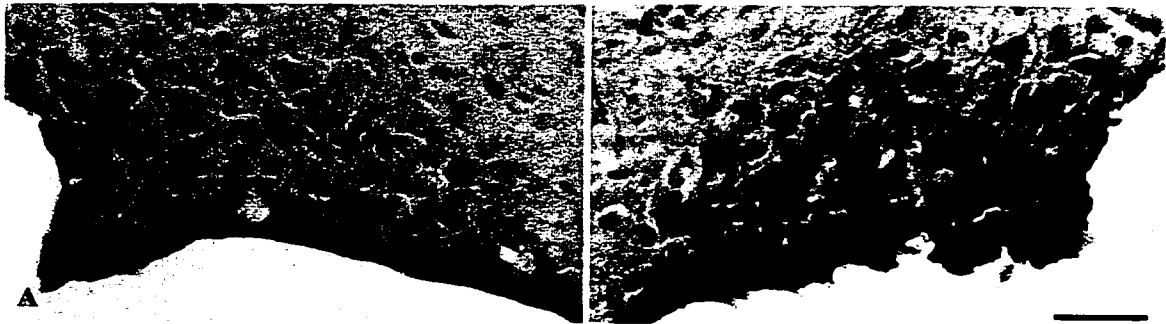


Figure 1. FLI in the SON in ouabain-induced hypertension. FLI was visualized using glucose/diaminobenzidine intensified with nickel in 30 μm thick coronal sections of the SON in rats that received 14-day sc infusions of either saline (A) or ouabain (B; 50 $\mu\text{g}/\text{day}$). B demonstrates a marked increase in FLI within the SON of the ouabain-treated rat. Scale bar: 100 μm in A and B.