ROLE OF ANGIOTENSIN II, GLUTAMATE, NITRIC OXIDE AND AN ALDOSTERONE-OUABAIN PATHWAY IN THE PVN IN SALT - INDUCED PRESSOR RESPONSES IN RATS

by

Alexander Gabor

Department of Cellular and Molecular Medicine
Faculty of Graduate and Postdoctoral Studies
University of Ottawa

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ABSTRACT

High salt intake contributes to the development of hypertension in salt-sensitive humans and animals and the mechanistic causes are poorly understood. In Dahl salt-sensitive (S) but not salt-resistant (R) rats, high salt diet increases cerebrospinal fluid (CSF) [Na^+] and activates an aldosterone-mineralocorticoid receptor-epithelial sodium channel-endogenous ouabain (MR-ENaC-EO) neuromodulatory pathway in the brain that enhances the activity of sympatho-excitatory angiotensinergic and glutamatergic pathways, leading to an increase in sympathetic nerve activity (SNA) and blood pressure (BP). We hypothesize that high salt diet in Dahl S rats enhances Ang II release in the paraventricular nucleus (PVN), causing a decrease in local nitric oxide (NO) action and an increase in local glutamate release thereby elevating SNA, BP and heart rate (HR). The present study evaluated the effects of agonists or blockers of MR, ENaC, EO, nitric oxide synthase (NOS) or glutamate and AT_1-receptors on the BP and HR responses to acute infusions of Na^+ rich aCSF, intracerebroventricularly (icv), or in the PVN of Dahl S, R or Wistar rats or to high salt diet in Dahl S and R rats. In Wistar rats, aldosterone in the PVN enhanced the BP and HR responses to infusion of Na^+ rich aCSF in the PVN, but not in the CSF, and only the enhancement was prevented by blockers of MR, ENaC and EO in the PVN. AT_1-receptor blockers in the PVN fully blocked the enhancement by aldosterone and the responses to infusion of Na^+ rich aCSF icv, or in the PVN. Na^+ rich aCSF in the PVN caused larger increases in BP and HR in Dahl S vs. R rats and the responses to Na^+ were fully blocked by an AT_1-receptor blocker in the PVN. BP and HR responses to a NOS blocker in the PVN were the same, but L-NAME enhanced Na^+ effects more in Dahl R than S rats. High salt diet attenuated increases in BP from L-

NAME in the PVN of Dahl S but not R rats. AT_1 and glutamate receptor blockers candesartan and kynurenate in the PVN decreased BP in Dahl S but not R rats on high salt diet. At the peak BP response to candesartan, kynurenate in the PVN further decreased BP whereas candesartan did not further decrease BP at the peak BP response to kynurenate. Our findings indicate that both an acute increase in CSF [Na^+] and high salt intake in Dahl S rats increases AT_1-receptor activation and decreases NO action in the PVN thereby contributing to the pressor responses to Na^+ and presumably, to dietary salt-induced hypertension. The increased BP response to AT_1-receptor activation in the PVN of Dahl S is mediated by enhanced local glutamate receptor activation. An MR-ENaC-EO pathway in the PVN can be functionally active and further studies need to assess its role in Dahl S rats on high salt intake.

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11ß-HSD-2:	11ß-hydroxysteroid dehydrogenase type 2
2K-1C:	two-kidney, one-clip
3ß-HSD:	3ß-hydroxysteroid dehydrogenase
ACE:	angiotensin Converting enzyme
aCSF:	artificial cerebrospinal fluid
ACTH:	adrenocorticotropic hormone
AHA:	anterior hypothalamic area
AMPA:	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AP:	area postrema
AP-1:	transcription factors activator protein 1
AVP:	arginine vasopressin
BBB:	blood brain barrier
BP:	blood pressure
CHF:	chronic heart failure
CNS:	central nervous system
CP:	choroid plexus
CRH:	corticotropin releasing hormone
CSF:	cerebrospinal fluid
CVLM:	caudal ventrolateral medulla
DOCA:	deoxycorticosterone acetate
ENaC:	epithelial sodium channel
eNOS:	endothelial nitric oxide synthase
EO:	endogenous ouabain
EPSC:	excitatory postsynaptic currents
GABA:	gamma-amino butyric acid
GH:	growth hormone
GLT1:	astrocyte glutamate transporter 1
HR:	heart rate
ICV:	intracerebroventricular
IML:	intermediolateral cell column
iNOS:	inducible nitric oxide synthase
IV:	intravenous
LSNA:	lumbar sympathetic nerve activity
LT:	lamina terminalis
MAP:	mean arterial pressure
MnPO:	median preoptic nucleus
mPVN:	magnocellular subdivision of the PVN
MR:	mineralocorticoid receptor
MI:	myocardial infarction
NADPH:	nicotinamide adenine dinucleotide phosphate-oxidase
NCX:	Na^{+}/Ca^{2+} exchanger
NEDD4-2:	neural precursor cell expressed, developmentally down-regulated 4-2
NF-kappB:	nuclear factor kappa-light-chain-enhancer of activated B cells
NMDA:	N-methyl D-aspartate

nNOS:	neuronal nitric oxide synthase
NO:	nitric oxide
NOS:	nitric oxide synthase
NTS:	nucleus of the solitary tract
OVLT:	organum vasculosum of the lamina terminalis
PI:	phosphotidyl inositol
PKC:	protein kinase C
PLC:	phospholipase C
pPVN:	parvocellular subdivisions of the PVN
PVN:	paraventricular nucleus
RAS:	renin-angiotensin system
ROS:	reactive oxygen species
RVLM:	rostral ventrolateral medulla
SC:	subcutaneous
SCN:	suprachiasmatic nucleus
SD:	Sprague-Dawley
SFO:	subfornical organ
SGK1:	serum-and glucocorticoid-regulated kinase 1
SHR:	spontaneously hypertensive rat
SI:	stretch-inactivated
SK:	small conductance Ca^{2+}-activated K^{+} channel
SNA:	sympathetic nerve activity
SNS:	sympathetic nervous system
SOD:	superoxide dismutase
SON:	supraoptic nucleus
StAR:	steroidogenic acute regulatory protein
TRH:	thyroid releasing hormone
TSH:	thyroid stimulating hormone
vAV3V:	anteroventral part of the third ventricle
WKY:	Wistar-Kyoto

1. GENERAL INTRODUCTION

1.0. Overview

High dietary salt intake leads to the development of hypertension in both humans (Kawano et al. 1992) and animals (Huang et al. 2004) and contributes to vascular remodelling, cardiac hypertrophy, and stroke (Armando and Jose 2009, Weinberger 1996). In humans, salt-sensitivity of the blood pressure (BP) occurs in 40-60 % of patients with essential hypertension (Franco and Oparil 2006, Kawano et al. 1992). The extent of hypertension from high salt intake is clearly genetically determined in both humans (Miller et al. 1987, Stoll et al. 2001) and animals (Cowley et al. 2001). Despite extensive research by many groups, the genetic and mechanistic causes of salt-induced hypertension are still poorly defined. In classic studies on genetically determined salt-sensitivity, salt-sensitive hypertension has been attributed to renal factors causing an increase in sodium and water reabsorption leading to increased Na^+ and fluid retention and BP (Cowley 1992, Guyton 1991). More recent studies in a number of salt hypertension models support the role of the brain in the development of salt-induced hypertension (Brooks et al. 2005a, Huang et al. 2006a, Toney and Stocker 2010, Yemane et al. 2010). Studies by our group have made it evident that in salt-sensitive animals on high salt intake, an increase in cerebrospinal fluid (CSF) [Na^+] activates a neuromodulatory pathway involving aldosterone- "ouabain", which increases activity in angiotensinergic sympatho-excitatory pathways and thereby mediates salt-induced sympathetic hyperactivity and hypertension (Huang et al. 2006b, Leenen 2010). Numerous studies have shown that blockade of these mechanisms in the central nervous system (CNS) prevents the sympathetic hyperactivity and hypertension in eg. Dahl S rats

on high salt diet (Gomez-Sanchez et al. 1992, Huang and Leenen 1994, Huang et al. 2009, Ito et al. 2003). Where and how these mechanisms interact in the brain has not yet been resolved. All are present in the paraventricular nucleus (PVN) of the hypothalamus (Amin et al. 2005, Yamada et al. 1992b, Zhang et al. 2006), an important central site for control of sympathetic nerve activity (SNA) (Swanson and Sawchenko 1980) and the PVN is a prime candidate for mediating the sympathoexcitatory and hypertensive responses to an increase in CSF [Na^+].

In the following sections, I will provide a brief overview of the role of the brain in models of salt hypertension and outline the central neuro-anatomical and functional pathways activated by CSF [Na^+]. In addition, I will discuss signaling in the PVN in physiological conditions and in response to short-term and chronic increases in CSF [Na^+].

1.1. Salt-induced hypertension and the role of the CNS

1.1.1. Dietary salt hypertension: involvement of the kidneys and brain

Classically, mechanisms responsible for the regulation of normal BP and the development of hypertension were based on the view that long-term regulation of BP is linked to the ability of the kidneys to excrete sufficient salt to maintain normal Na^+ balance, extracellular fluid volume, and blood volume. Changes in renal function leading to a decrease in the kidney's capability to excrete Na^+ would lead to an increase in blood volume, cardiac output, and a rise in arterial pressure to maintain normal Na^+ and fluid balance (Cowley 1992, Guyton 1991). However, studies by Morgan et al. (Morgan et al. 1990) demonstrated that Dahl S rats develop similar hypertension on high salt whether a

Dahl R or S kidney was transplanted, indicating that extra-renal factors also play a critical role in salt-induced hypertension in Dahl S rats. A large body of evidence supports the role of the brain in many models of salt sensitive hypertension. First, dietary salt-induced hypertension is associated with activation of the sympathetic nervous system (SNS) in deoxycorticosterone acetate (DOCA)-salt hypertensive (O'Donaughy et al. 2006) and Dahl S rats (Huang et al. 2004), spontaneously hypertensive rats (SHR) (Qadri et al. 2003) and salt-sensitive humans (Kawano et al. 1992). Second, chemical sympathectomy, destroying sympathetic neurons in the periphery but not brain (Johnson et al. 1975, Johnson and O'Brien 1976) or chemically lesioning regions of the forebrain (Friedman et al. 1979, Goto et al. 1982) prevent the development of salt hypertension in Dahl S rats. Third, blockade of brain regions determining vasomotor tone such as the rostral ventrolateral medulla (RVLM) produces large decreases in SNA and BP in Dahl S rats on high salt, but not normal salt diet (Ito et al. 2001). However, mechanisms by which salt causes sympathetic hyperactivity and hypertension are not well understood. As discussed in the following sections, high salt intake may cause small increases in plasma [Na^+] in both salt sensitive and resistant rats (Fang et al. 2000, Habecker et al. 2003), but only salt-sensitive strains develop an increase in CSF [Na^+] (Huang et al. 2004, Nakamura and Cowley 1989) and this increase in CSF [Na^+] may activate the CNS mechanisms contributing to the salt-induced hypertension in Dahl S.

1.1.2. High salt intake, plasma and CSF [Na^+] and BP

Increased dietary salt leads to small increases in plasma [Na^+], which may (Fang et al. 2000, Habecker et al. 2003, He et al. 2005) or may not be detectable (Huang et al. 2004).

Fang et al. (2000) reported that in SHR and Wistar Kyoto (WKY) rats, 4 days of 8% high salt diet caused a similar increase in plasma [Na^+] by 3– 4 mM during both awake and sleep periods. The "small" extent of this increase is not surprising considering that a 1-2% change in plasma osmolality is enough to induce physiological and behavioural responses ie. arginine vasopressin (AVP) release and thirst that maintain systemic osmotic pressure close to a defined set-point (McKinley and Johnson 2004). Whereas high salt intake may cause detectable increases in plasma [Na^+] in both salt sensitive eg. SHR (Fang et al. 2000) and resistant rats eg. WKY, Sprague-Dawley (SD) rats (Fang et al. 2000, Habecker et al. 2003), only salt-sensitive strains develop an increase in CSF [Na^+]. In Dahl S and SHR but not Wistar or Dahl R rats, 8 % high NaCl diet caused sustained increases in CSF [Na^+] by 5- 7 mM (Huang et al. 2004). Huang et al. (2004) showed that in Dahl S and SHR on 8 % high NaCl diet, CSF [Na^+] increased between days 2- 3 and BP between days 3- 4, whereas Nakamura and Cowley (1989) showed that high salt intake increased BP by day 2 and CSF [Na^+] by day 3 in Dahl S rats. CSF [Na^+] sampling by Nakamura et al. was performed over 24-h whereas Huang et al. collected over 12-hr periods (10 AM- 10 PM). The sampling by Nakamura et al. may have missed the increases in CSF [Na^+] during nights when rats would eat their high salt diet (Huang et al. 2004). Changes in plasma [Na^+] were not detected during high salt intake in either rat strain during these studies (Huang et al. 2004, Nakamura and Cowley 1989). Intravenous (iv) infusion of ^{22}NaCl caused 5 times greater uptake of $^{22}Na^+$ in the CSF and brain parenchyma in Dahl S vs. R rats with similar BP (Simchon et al. 1999). These findings suggest that enhanced Na^+-entry across the blood brain barrier (BBB) and CSF brain barrier in Dahl S vs. R rats contribute to the increase in CSF [Na^+] on high salt diet

in Dahl S. On high salt intake, this enhanced entry in Dahl S vs. R rats becomes more pronounced (Simchon et al. 1999), suggesting that Dahl S rats have less buffering capacity compared to Dahl R. The actual mechanisms contributing to increases in CSF [Na^+] in Dahl S and SHR on high salt diet are not yet known. Production of CSF occurs mainly at the epithelium of the choroid plexus (CP) (Praetorius 2007), located mainly within the lateral and fourth brain ventricles. Enzymes and transporters on the apical (CSF-side) or basolateral surface of CP cells regulate transport of Na^+ between plasma and CSF by balancing Na^+-influx into and Na^+-efflux out of the CP cells (Amin et al. 2009, Keep et al. 1987). The Na^+/K^+/ATPase is located on the apical surface of the CP (Johansson et al. 2008, Masuzawa et al. 1984) and provides a driving force for transport of Na^+ into the CSF against a [Na^+] gradient (Amin et al. 2009, Pollay et al. 1985). Intracerebroventricular (icv) infusion of the Na^+/K^+/ATPase inhibitor ouabain decreased CSF [Na^+] by 7 mM in Wistar rats (Huang et al. 2004). The epithelial Na^+ channel (ENaC) is a voltage-insensitive Na^+ channel that allows passive transport of Na^+ ions in the direction of their concentration gradient (Palmer 1992, 1990). ENaC is expressed primarily on the apical, and to a lesser extent on the basolateral surface of the CP (Amin et al. 2009) suggesting that ENaC may contribute to Na^+ influx into CP cells both from the CSF and blood, but mainly from the CSF (Amin et al. 2009, Wang et al. 2010). Consistent with its role in transporting CSF Na^+ back into CP cells, icv infusion of the ENaC blocker benzamil increased CSF [Na^+] by 8 mM, while chronic icv infusion of Na^+ rich artificial cerebrospinal fluid (aCSF) increased expression of α and β ENaC on the apical membranes of CP cells (Wang et al. 2010), possibly to aid Na^+ transport out of the CSF. In vitro studies from our group showed that high salt diet increased intracellular

[Na^+] in the CP and attenuated the ouabain-sensitive component of Na^+-efflux in Dahl R, but not S rats. Persistent high activity of the Na^+/K^+/ATPase on high salt diet could elevate CSF [Na^+] (Amin et al. 2009). These findings suggest that aberrant regulation of Na^+ transport and Na^+/K^+/ATPase activity may be contributing to the increase in CSF [Na^+] in Dahl S rats on high salt intake. Additional studies are needed to elucidate the actual mechanisms causing a decrease in Na^+/K^+/ATPase activity in Dahl R rats on high salt.

In one study in humans, both plasma and CSF [Na^+] increased similarly by 2– 3 mM in patients with either salt-sensitive or non-salt-sensitive hypertension after 7 days on a high salt diet (16–18 g/day) vs. a low salt diet (1– 3 g/day) (Kawano et al. 1992). The authors suggested that differences in neuronal responsiveness to [Na^+] (*section 1.2.4.2.*) may explain dietary salt hypertension in humans.

1.1.3. Sympathoexcitatory and pressor responses to plasma [Na^+]

Increases in plasma [Na^+] elevate sympathetic outflow in both humans (Farquhar et al. 2006) and animals (Brooks et al. 2004, Weiss et al. 1996). In SD rats pretreated intravenously (iv) with a vasopressin antagonist or sino-aortically denervated to block the influence of inhibitory baroreceptors, iv infusion of a hypertonic 2.5 M solution of NaCl at 0.03 mL/min for 60 min increased plasma osmolality by ~15 mOsmol/L, lumbar sympathetic nerve activity (LSNA) by 30-40%, BP by 15- 20 mmHg and heart rate (HR) by 20 bpm (Stocker et al. 2008, Weiss et al. 1996). Changes in plasma [Na^+] were not described in these studies but an increase in plasma osmolality by ~15 mOsmol/L would translate to a rise in plasma [Na^+] by 7-8 mM. In humans, iv infusion of a hypertonic 0.5

M solution of NaCl for 60 min, raised plasma [Na^+] and osmolality by ~6 mM and ~10 mOsmol/L and increased muscle sympathetic nerve activity and mean arterial pressure (MAP) by ~20% and ~10 mmHg (Farquhar et al. 2005, Farquhar et al. 2006). In both rats and humans, these solutions of hypertonic saline caused parallel increases in plasma osmolality, SNA and BP that started within the first few minutes and peaked within 30-50 min after start of infusion (Farquhar et al. 2005, Farquhar et al. 2006, Stocker et al. 2008). It is not known whether these responses are mediated by increases in [Na^+] or osmolality since no studies compared the sympathetic and pressor responses to iv infusion of hypertonic solutions made from excess NaCl vs. other compounds eg. mannitol or sucrose.

Animal models with chronic increases in plasma [Na^+] showed a variety of responses in SNA and BP. In normal SD rats, high salt intake by substituting drinking water for 0.9 % NaCl for 2 weeks raised night time plasma [Na^+] by 2-3 mM and osmolality by 4-5 mosmol/L but did not change sympathetic activity, BP or HR (Adams et al. 2009, Adams et al. 2007). SD rats drinking 1 % NaCl and chronically infused subcutaneously with DOCA for 2 weeks developed an increase in plasma [Na^+] by 2-3 mM as well as an increase in sympathetic activity and BP (Brooks et al. 2005a, O'Donaughy and Brooks 2006). Intravenous infusion of an isotonic 5% dextrose solution (5DW) for 2 hours did not affect plasma Na^+ levels or LSNA and BP in normotensive SD rats (Scrogin et al. 1999). In contrast, in DOCA-salt hypertensive rats, iv infusion of 5DW for 2 hours normalized the increased plasma Na^+ and osmolality levels and decreased in parallel LSNA and BP (O'Donaughy and Brooks 2006). No studies have yet evaluated whether the increase in plasma osmolality or in plasma [Na^+] contributes to

these changes. Intracarotid infusion of a hypotonic solution, estimated to lower the osmolality of blood perfusing the forebrain but not plasma by 2 -3 % (Brooks et al. 2005b), similarly decreased BP and LSNA in DOCA-salt rats (O'Donaughy et al. 2006) but not in normal SD rats (Brooks et al. 2005b, O'Donaughy et al. 2006). Decreases in SNA and BP occurred more rapidly when infusions were performed intracarotid vs. iv, suggesting that forebrain regions (*see section 1.2.1.1*) contribute to the sympathetic and depressor responses to a decrease in plasma NaCl and osmolality in DOCA-salt rats. Lowering forebrain blood osmolality, and presumably [Na^+], did not affect SNA and BP in normal rats, suggesting that mechanisms in the brain contributing to the increase in SNA and BP in this model of chronic sympathetic hyperactivity are not active in normal conditions.

1.1.4. Sympathoexcitatory and pressor responses to CSF [Na^+]

Acute icv infusion of Na^+ rich aCSF increases SNA, BP and HR in a variety of species, including rats (Bunag and Miyajima 1984b, Huang et al. 2001b, Shah and Jandhyala 1991), mice (Chu et al. 2004, Van Huysse and Hou 2004) and sheep (May and McAllen 1997). In normal rats, icv infusion of Na^+ rich aCSF ranging from 0.2-0.45 M [Na^+] at 3.8 uL/min for 10 min raised renal sympathetic nerve activity (RSNA) by 20-50 %, BP by 8-25 mmHg and HR by 20-50 bpm (Huang et al. 2001b). Acute icv infusion of 0.3, 0.67 or 1.0 M NaCl at µL/min for 20 min raised CSF [Na^+] in the 3rd ventricle by 14, 60, and 80 mM (Kato et al. 2004). BP begins to increase within the first few minutes of icv infusion of 0.3 M Na^+ rich aCSF at 3.8 uL/ min and peak increases by ~20 mmHg occur after 5 min. RSNA and HR decrease during the first 10 min, then rise to peak increases

by 40-50 % and 30-50 bpm after 15 - 20 min (Bunag and Miyajima 1984b, Huang et al. 1992). A similar decrease in RSNA and concomitant rise in BP was observed in response to icv infusion of 0.6 M Na^+ rich aCSF at 17 uL/ min in sheep (May and McAllen 1997). Icv injection (5 uL) of 0.3 M Na^+ rich aCSF increased plasma AVP by 5 –fold (Qadri et al. 1998). AVP release in the circulation causes vasoconstriction, activating arterial baroreflex pathways and inhibition of SNA and HR (Hasser et al. 2000). Furthermore, AVP binding to V_1 receptors in neurons of the area postrema (AP), a circumventricular organ (CVO) in the brainstem able to respond to circulating levels of AVP, enhances response of neurons in the nucleus of the solitary tract (NTS) to baroreceptor afferent input thereby enhancing baroreceptor function (Bonham and Hasser 1993, Hasser et al. 1997). Indeed, chemically lesioning the AP (Peuler et al. 1990, Undesser et al. 1985) or microinjection of a V_1 receptor antagonist into the AP (Hasser and Bishop 1990) abolished the action of circulating AVP on arterial baroreflex control of the SNS. Iv injection of an AVP antagonist reversed into an increase the initial drop in RSNA and HR from an acute increase in CSF [Na^+] (Bunag and Miyajima 1984b, Huang et al. 1992) indicating that AVP release into the periphery activated sympatho-inhibitory baroreflex pathways. The initial increase in BP is likely mediated by the vasoconstrictor effects of AVP in the circulation (Huang et al. 1992), but is not affected by an AVP antagonist, suggesting that then the sympathoexcitatory effects of Na^+ rich aCSF cause the increase in BP. Icv infusion of osmotically equivalent loads of mannitol in rats (Osborne et al. 1989) or sorbitol in sheep (May and McAllen 1997) does not increase BP, indicating that pressor responses from an increase in CSF [Na^+] are mediated by changes in [Na^+] *per se* and not osmolality. Icv injection of Na^+-rich solutions made from sodium-salts

containing NaCl, Na_2SO_3, Na_2SO_4, $NaHCO_3$ or Cl^--rich solutions made from choline chloride or LiCl all increased BP (Shah and Jandhyala 1991, Wei and Wu 1979). At same concentrations (0.25 – 1M), the pressor response to NaCl solutions were 2-10 fold higher than salt solutions containing Na^+ but not Cl^- eg. $NaNO_3$, or Cl^- but not Na^+ eg. choline chloride (Shah and Jandhyala 1991), indicating that both Na^+ and Cl^- ions contribute to the BP response from acute increases in NaCl in the CSF.

Chronic icv infusion of Na^+ rich aCSF in normal SD or Wistar rats, infusing lower amounts of Na^+/min (60-170 µg Na^+/h for 1- 2 wks) compared to acute (1- 2.5 mg Na^+/h for 10-30 min) raised BP by 10 -20 mmHg (Bunag and Miyajima 1984a, Huang et al. 2006b, Huang et al. 2008a, Kawano et al. 1991b). In mice, chronic icv infusion of Na^+ rich aCSF (7 µg Na^+/h for 10 days) increased CSF [Na^+] by 5 mM and BP by ~30 mmHg (Van Huysse et al. 2011). In Wistar rats, icv infusion of Na^+ rich aCSF (0.8 M at 5 µL/h for 2 wks) raised CSF [Na^+] by 4-5 mM, increased sympathetic activity, impaired arterial baroreflex function and increased BP by 10- 20 mmHg (Huang et al. 2006b, Huang et al. 1998, Huang et al. 2008a). As measured for 2 weeks with telemetry, BP increased within the first day of Na^+ infusion, reached peak levels within day 1- 2 and remained elevated until the end of recording (Huang et al. 2008a). Chronic icv infusion (5.5 µL/h) of 1.5 M Na^+ rich aCSF for 7 days also increased CSF [Na^+] by 4 -5 mM and BP by ~15 mmHg as measured by tail-cuff plethysmography. The depressor response from iv injection of the ganglion blocker hexamethonium was larger in rats infused with 1.5 M Na^+ rich aCSF (~ -75 mmHg) vs. 0.15 M aCSF (~ -50 mmHg) on both days 1 and 7 (Kawano et al. 1991b) suggesting that an increase in sympathetic activity (*section 1.2.4.1.*) contributes to both initial and chronic phases of CSF [Na^+]- induced

hypertension. After hexamethonium, iv injection of an AVP antagonist further decreased BP by -15 mmHg on day 1 but not 7 of chronic icv infusion of Na^+ rich aCSF (Kawano et al. 1991b) suggesting that AVP release into the circulation contributes to the initial, but not chronic phase of CSF [Na^+]- induced hypertension. Plasma AVP levels were not measured on day 1 but on day 7 were not elevated (Kawano et al. 1991b), further supporting an AVP-independent mechanism in the chronic phase. Huang et al. (2008), Kawano et al. (2001) and Bunag and Miyajima (1984) reported no significant changes in HR during chronic icv infusion of Na^+ rich aCSF in rats. In contrast, in one study in mice, chronic icv infusion of Na^+ rich aCSF in mice (7 µg Na^+/h for 10 days) caused significant increases in HR by 40-70 bpm (Van Huysse et al. 2011). These findings suggest that CSF [Na^+]-induced cardiac sympathetic activation appears to be species dependent.

1.2. Central neuro-anatomical pathways and mechanisms activated by plasma and CSF [Na^+]

1.2.1. Neuro-anatomical pathways mediating responses to plasma and CSF [Na^+]

The forebrain lamina terminalis (LT) consists of the subfornical organ (SFO), the median preoptic nucleus (MnPO), and the organum vasculosum of the lamina terminalis (OVLT). These interconnected nuclei are located along the rostral wall of the 3rd ventricle (McKinley et al. 2001) (Figure 1). Dendrites of neurons in the SFO project into the ventricular lumen (Dellmann 1998) allowing them to detect changes in CSF [Na^+]. Circumventricular organs (CVOs) of the LT ie. SFO, OVLT can detect changes in plasma [Na^+] since they lack a complete BBB, allowing blood-borne osmolytes such as

Na^+ to freely cross within their local interstitium (Ferguson and Bains 1996). The MnPO shares reciprocal connections between the SFO and OVLT (Figure 1). Electrophysiological recordings from rat hypothalamic slices or explants *in vitro* have revealed that a large majority of the neurons (~ 60-100 %) in the SFO (Anderson et al. 2001, Sibbald et al. 1988), OVLT (Bourque et al. 1994, Nissen et al. 1993, Vivas et al. 1990) and MnPO (Honda et al. 1992, Honda et al. 1990b) were excited upon exposure to hypertonic solutions made from excess NaCl or mannitol, indicating that they respond to a variety of solutions that raise osmotic pressure. No studies have yet compared the extent of electrophysiological responses ie. firing frequency, membrane conductance to these hypertonic solutions on these neurons of the LT. Increases in neuronal activity in the LT can be relayed from all three nuclei to magnocellular neurons of the PVN and neighbouring supraoptic nucleus (SON) or to parvocellular neurons of the PVN (McKinley et al. 2001). Magnocellular neurons of the PVN and SON synthesize and secrete AVP and possibly endogenous ouabain (EO) into cardiovascular regulatory nuclei and the circulation (*see 1.2.3.2*) (Bourque 2008, Budzikowski et al. 1998a). Parvocellular neurons of the PVN contain autonomic neurons that project to the RVLM or sympathetic pre-ganglionic neurons of the intermediolateral cell column (IML) that excite the SNS and play a significant role in cardiovascular regulation (Figure 1-1). Electrical stimulation of the SFO, OVLT or PVN caused a sympathetically mediated increase in splanchic and renal vascular resistance and BP (Mangiapane and Brody 1986, 1987, Porter and Brody 1985). Lesioning the nuclei of the LT in normotensive rats on regular salt diet caused no changes in resting BP and HR (Adams et al. 2009, Berecek et al.

1982), suggesting that nuclei in the LT are not involved in tonic regulation of BP in normal conditions.

The PVN and SON are also sensitive to changes in $[Na^+]$ as *in vitro* perfusion with NaCl solutions ranging from 0.16-0.19 M increased firing activity in magnocellular neurons of both nuclei (Qiu et al. 2004, Voisin et al. 1999) as well as parvocellular neurons of the PVN (Chu et al. 2010). Electrophysiological studies *in vitro* showed that small increases in extracellular $[Na^+]$ by 2 -3 mM are sufficient to increase firing activity in neurons eg. the SON (Honda et al. 1990a). Compared to NaCl solutions with elevated $[Na^+]$, osmotically equivalent solutions of mannitol caused no responses in Na^+-sensitive parvocellular neurons (Chu et al. 2010) and evoked a ~3-5 fold less increase in firing activity in magnocellular neurons of the PVN and SON (Chu et al. 2010, Qiu et al. 2004). Whether an increase in CSF $[Na^+]$ can directly activate neurons in the PVN has not yet been studied. Neurons in the PVN may sense changes in CSF $[Na^+]$ through dendritic branches directly exposed to the CSF near the ependymal layer of the third ventricle (Korf et al. 1983, Qadri et al. 1998, Vigh-Teichmann and Vigh 1983).

Located at the caudal end of the 4^{th} ventricle and containing vessels that lack a BBB (Cottrell and Ferguson 2004), the AP is another CVO that can sense changes in the composition of the blood and CSF. The AP has reciprocal connections with the NTS (McKinley and Johnson 2004). These connections allow the AP to influence the sensitivity of baroreflex function (*section 1.1.4.*). Afferent inputs from arterial baroreceptors and chemoreceptors enter the NTS (Ciriello 1983), are being processed, and are sent out through excitatory projections to the caudal ventrolateral medulla (CVLM) which inhibits neurons in the RVLM involved in regulation of sympathetic

activity and BP (Natarajan and Morrison 2000). To our knowledge, no electrophysiological studies *in vitro* have yet assessed whether neurons in the AP and NTS are Na^+ or osmosensitive. However, injection (20 nL) of hypertonic solutions of NaCl (160-325 mM NaCl) throughout regions of the NTS decreased BP by 15- 45 mmHg and HR by 10-40 bpm but caused no effect in the AP (Hochstenbach and Ciriello 1994). These findings suggest that an increase in interstitial [Na^+] in the NTS decreases BP and may modulate activity of baroreceptor pathways. Although an increase in [Na^+] in the AP does not appear to directly affect BP, this CVO is involved in osmoregulation since chemically lesioning the AP caused normal rats to ingest more NaCl (Curtis et al. 1999). An increase in plasma or CSF [Na^+] may increase firing activity in neurons of the AP projecting to the lateral parabrachial nucleus which influences salt appetite via its connections to the amygdala (McKinley and Johnson 2004).

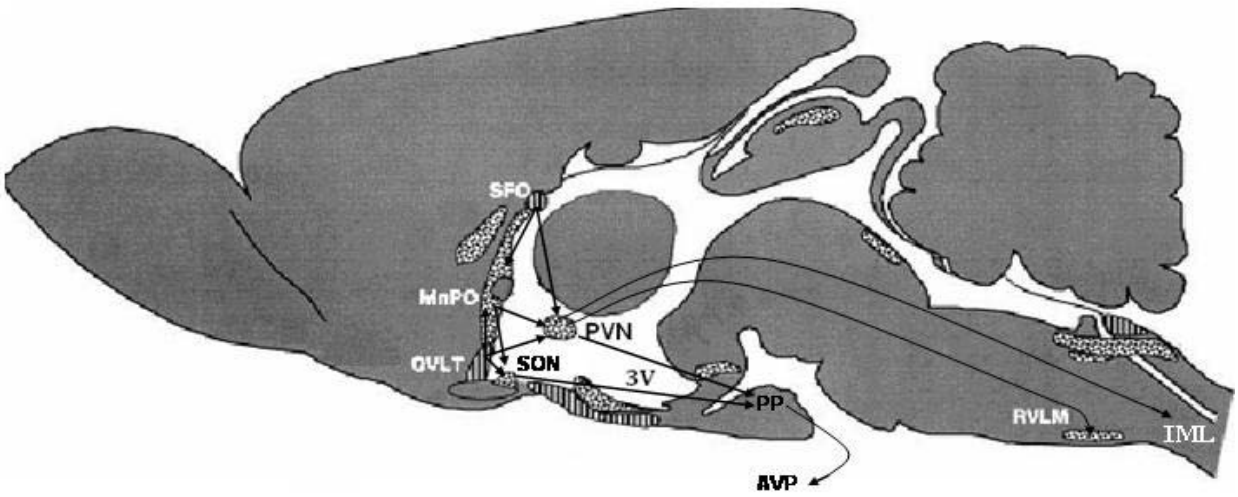

Figure 1-1: Schematic sagittal section of the rat brain indicating neural pathways involved in salt-induced hypertension. Modified from McKinley et al. (McKinley et al. 2003a). Clear area represents 3rd and 4th brain ventricles filled with cerebro-spinal fluid (CSF). SFO: subfornical organ; OVLT: organum vasculosum lamina terminalis; MnPO: median preoptic nucleus; SON: supraoptic nucleus; PVN: paraventricular nucleus; PP: posterior pituitary; RVLM: rostral ventrolateral medulla; AVP: arginine vasopressin; IML: intermediolateral cell column; 3V: 3rd ventricle.

1.2.1.1. Brain regions involved in central responses to increases in plasma [Na^+]

Intracarotid injections (150 uL) of NaCl solutions ranging from 0.4-0.8 M [Na^+] in rats caused [Na^+]-dependent increases in RSNA by 40-60%, LSNA by 60-80% and BP by 10-20 mmHg (Chen and Toney 2001, Shi et al. 2007). Sympathetic and pressor responses were also observed from hyperosmotic equivalent solutions of sucrose and mannitol, but responses to hyperosmotically equivalent NaCl solutions were ~20-30% larger (Chen and Toney 2001). Specific Na^+-sensing mechanisms may play a role in the larger responses to Na^+. Consistent with a Na^+ or osmotic driven signaling pathway from the LT to the PVN, intracarotid infusion (0.1 mL/min) of a NaCl solution with 0.7 M [Na^+], or an osmotically equivalent solution of mannitol for 10 min increased *c-fos* expression (an early marker of neuronal activation) in OVLT neurons identified to project to the PVN by retrograde labeling (Shi et al. 2008). The SFO also contributes to this [Na^+] driven signaling pathway since intracarotid injections (100 uL) of a 0.5 M solution of NaCl increased the firing activity of SFO neurons antidromically identified as projecting to the PVN or SON (Gutman et al. 1988). Severing connections between the nuclei of the LT and the PVN abolishes sympathetic and pressor responses to intracarotid injections of NaCl solutions (Antunes et al. 2006). Electrolytic lesioning of the OVLT attenuated by 30-50% the RSNA response to intracarotid injections of NaCl solutions ranging from 0.4-0.8 M [Na^+] (Shi et al. 2007). Other osmotically equivalent solutions were not utilized in this study. The PVN appears to fully mediate the sympathetic and pressor responses from an acute increase in plasma [Na^+] or osmolality since an AT_1-receptor blocker in the PVN prevented the increases in RSNA by 150-200 % and BP by 30-40 mmHg from

intracarotid injection (300 uL) of a 0.8 M hypertonic NaCl solution or an osmotically equivalent solution of mannitol (Chen and Toney 2001).

In DOCA-salt treated rats with chronically elevated plasma [Na^+], sympathetic activity and BP (*section 1.1.3.*), electrolytic lesioning of the anteroventral part of the third ventricle (vAV3V; includes the OVLT and ventral MnPO, but not SFO) (Songu-Mize et al. 1982) or PVN (Nakata et al. 1989) prevented the development of hypertension from DOCA-salt treatment, indicating that nuclei in the LT and the PVN mediate DOCA-salt induced hypertension.

In SD rats with chronically elevated plasma [Na^+] but normal levels of sympathetic activity and BP in response to drinking 0.9% NaCl for 2 weeks (*section 1.1.3*), injection of excitatory ie. glutamate, Ang II or inhibitory ie. gamma-amino butyric acid (GABA) neurotransmitters into the RVLM caused ~ 2-fold larger responses in RSNA and BP compared to rats drinking water (Adams et al. 2009, Adams et al. 2007). This effect of dietary salt was abolished by electrolytic lesions of the OVLT (Adams et al. 2009), suggesting that high salt intake in normal rats enhanced the responsiveness of sympathetic neurons in the RVLM regulating BP and this enhanced responsiveness depends on an intact OVLT. An enhanced responsiveness to excitatory inputs in the RVLM of normal rats from high salt may be balanced by an enhanced responsiveness to local inhibitory inputs eg. baroreceptor input from the CVLM via the NTS (*section 1.2.1.*), preventing a rise in SNA and BP. This enhanced responsiveness depends on an intact OVLT, but how relayed signaling from the OVLT causes enhanced responsiveness in neurons of the RVLM has not yet been studied.

1.2.1.2. Brain regions involved in central responses to increases in CSF [Na^+]

Electrolytic lesioning of the entire LT region prevented the increase in BP by ~10 mmHg from an acute icv infusion of 0.6 M Na^+ rich aCSF (1 mL/h for 20 min) in sheep (May et al. 2000), indicating that the nuclei of the LT are essential for the BP response to an acute increase in CSF [Na^+]. Larger responses in BP produced by higher [Na^+] were not investigated in this study. Consistent with the findings in sheep, covering nuclei of the LT and the 3rd ventricle region (around the PVN) with a jelly plug abolished the increase in BP by ~30 mmHg from icv infusion of a 1.5 M NaCl solution (0.2 mL over 1 min) into the lateral ventricle of dogs (Kawano et al. 1991a). Pressor responses from a brief icv infusion of 0.3 M Na^+ rich aCSF (3.8 µL/min for 10 min) in rats were fully blocked by infusion of an AT_1-receptor blocker in the MnPO (Budzikowski and Leenen 2001) and attenuated by ~50% with an AT_1-receptor blocker in the SFO (Tiruneh and Leenen, unpublished data). The SFO and OVLT share a number of reciprocal connections with the MnPO (McKinley et al. 2001). The remaining 50% of the responses to Na^+ after SFO blockade depend on the OVLT, and increased activity in both nuclei would be relayed to the MnPO. Consistent with a role for both the SFO and OVLT in the pressor responses to CSF [Na^+] in rats, excitotoxic chemical lesioning of the vAV3V region (excludes the SFO and non-ventral regions of the MnPO) attenuated by 30-50% the increases in BP by ~20 mmHg and HR by ~80 bpm from icv infusion of 0.3 M Na^+ rich aCSF (2 uL/min for 10 min) (Veerasingham and Leenen 1997).

Injection of lidocaine (a non-specific blocker of neuronal activity) in the PVN of sheep had no effect on BP alone, but prevented the increase in BP by 13 mmHg from icv infusion of 0.5 M Na^+ rich aCSF (20 µL/min for 60 min) (Frithiof et al. 2009), indicating

that an increase in neuronal activity in the PVN also mediates the pressor responses from an acute increase in CSF [Na^+]. Considering that the pressor response to CSF [Na^+] can be prevented by lesioning the entire LT or PVN and pre-sympathetic neurons in the LT influence BP via descending connections to the PVN, nuclei in the LT appear to initiate the response to CSF [Na^+], while the PVN likely integrates increased signaling from the LT. An increases in interstitial [Na^+] in the PVN may also activate local neuromodulatory pathways that enhance activity of excitatory inputs from the LT, but on their own appear not to be sufficient.

Excitotoxic chemical lesioning of the vAV3V region in Wistar rats fully blocked the increase in BP by 18 mmHg from a chronic icv infusion of 0.8 M Na^+ rich aCSF (5 µL/h for 2 weeks) (Veerasingham and Leenen 1999), indicating that nuclei in the LT also mediate the hypertension from a chronic increase in CSF [Na^+]. In Dahl S and SHR, models of chronically elevated CSF [Na^+], high salt diet increases the expression of *Fra-like* proteins (markers for long-term neuronal activation) in the nuclei of the LT and in the PVN and NTS (Budzikowski et al. 1998b, Budzikowski et al. 1997). Neurons of the LT and PVN play an important role in the development of hypertension in Dahl S rats on high salt diet since electrolytic lesioning the AV3V region (Goto et al. 1982) or the PVN (Goto et al. 1981) prevented the BP increases. Inhibition of neuronal activity in the PVN by local injection of a GABA receptor agonist decreased BP in Dahl S, but not R rats on high 8 % NaCl diet for 3 weeks (Ito et al. 2003), suggesting that enhanced neuronal activity in the PVN contributes to the maintenance of resting BP in hypertensive Dahl S rats.

1.2.2. Aldosterone-MR-ENaC-EO and the RAS in the CNS

A number of studies have established that an increase in CSF [Na^+] by icv infusion of Na^+ rich aCSF or high salt diet in Dahl S rats activates a neuromodulatory pathway involving mineralocortoicod receptors (MR), ENaC and endogenous ouabain (EO) in the CNS that enhances the activity of angiotensinergic sympathetic pathways thereby mediating the development of Na^+-induced sympathetic hyperactivity and hypertension (Gomez-Sanchez et al. 2010, Huang et al. 2006b, Leenen 2010). Considering that this EO neuromodulatory pathway increases both protein expression (Huang et al. 2011, Zhao et al. 2001) and neuronal sensitivity to tonic activity (Huang et al. 2005, Wang and Leenen 2003), it may represent an example of increased slow transmission signaling by a neuromodulator (Greengard 2001). The following sections will describe the expression, distribution and function of components of these pathways in the CNS.

1.2.2.1. Aldosterone, MR and ENaC regulation and function

Aldosterone

Aldosterone is present in cells from a wide variety of brain regions, including the cortex, hippocampus and brainstem, but is mostly highly abundant in neurons from the hypothalamus (Gomez-Sanchez et al. 1997, Huang et al. 2006b, Yongue and Roy 1987). Aldosterone in the CNS may originate from the circulation by its ability to cross the BBB (Gomez-Sanchez et al. 2005a, Yongue and Roy 1987). Although aldosterone is relatively lipophilic, it poorly penetrates the BBB due to the action of P-glycoprotein, a protein transporter located inside the BBB that pumps aldosterone back into the blood via transport through the cerebral vascular endothelium (Pardridge and Mietus 1979, Parker

et al. 2006). Most of the aldosterone in the brain of normal rats may originate from the circulation. In normal rats, high salt diet decreases both plasma and brain aldosterone content (Bayorh et al. 2005, Huang et al. 2009) and brain aldosterone becomes nearly undetectable after plasma levels are abolished by adrenalectomy (normal: ~150 pg/g; adrenalectomized: ~5 pg/g) (Gomez-Sanchez et al. 2005a, Yu et al. 2008). Accumulating evidence suggests that aldosterone can also be locally synthesized in the brain (Gomez-Sanchez et al. 2005a, Gomez-Sanchez and Gomez-Sanchez 2003). However, there is still a debate whether this locally produced aldosterone in the brain is physiologically relevant (Geerling and Loewy 2009, Gomez-Sanchez 2011, Gomez-Sanchez et al. 2005a). According to studies with adrenal tissues, in the enzymatic pathways involved in aldosterone synthesis, steroidogenic acute regulatory protein (StAR), the rate limiting enzyme, is involved in transporting cholesterol into the mitochondrial inner membrane (Stocco 2001) where it is converted to pregnenolone by P450 side chain cleavage enzyme (P450scc) (Farkash et al. 1986). Pregnenolone becomes converted into progesterone by 3ß-hydroxysteroid dehydrogenase (3ß-HSD) (Simard et al. 1993), then into deoxycorticosterone (DOC) by 21-hydroxylase in the cytosol (Shinzawa et al. 1988). In the final steps, DOC is converted into corticosterone by 11β-hydroxylase which is then converted into aldosterone by aldosterone synthase in the inner mitochondrial membrane (Vinson 2004). The mRNA for all enzymes and regulatory molecules required for the synthesis of aldosterone are expressed in the brain, including early stage enzymes such as StAR, P450scc, 3ß-HSD and late stage enzymes eg. 11β-hydroxylase, aldosterone synthase, but at much lower levels compared to the adrenal gland (Davies and MacKenzie 2003, King et al. 2002, Ye et al. 2003). The

presence of StAR mRNA was detected in whole hypothalamic tissue (Kim et al. 2004), and specifically in the MnPO and PVN of rats (Furukawa et al. 1998, Kim et al. 2003). StAR protein by immunohistochemical analysis was expressed widely in the brain, including in regions of the cortex, hypothalamus and brainstem, and its expression was present in both glia and neurons (Sierra et al. 2003). Immunohistochemical studies also showed that StAR was colocalized with P450scc in the anterior hypothalamus of mice (King et al. 2002). CYP11B2 mRNA (aldosterone synthase) was also detected in the whole hypothalamus (Gomez-Sanchez et al. 2010, Kraulis et al. 1975) and both CYP11B1 (gene for 11β-hydroxylase) and CYP11B2 mRNA were detected in the SFO, PVN and SON (Wang et al. 2010). Both CYP11B1 (MacKenzie et al. 2000, Mellon and Deschepper 1993) and CYP11B2 (MacKenzie et al. 2000, MacKenzie et al. 2002) were expressed in neurons of the hippocampus. Although these findings support that aldosterone can be locally synthesized in these hypothalamic nuclei, there is no direct evidence that such low mRNA expression levels in the brain translate into enzymatic activity that may locally synthesize aldosterone.

Little is known about the mechanisms regulating steroidogenesis and aldosterone production in the brain. Chronic icv infusion of Na^+ rich aCSF in Wistar rats (Huang et al. 2008a) and high salt diet in Dahl S rats (Huang et al. 2009) increased aldosterone and corticosterone content in the hypothalamus. An aldosterone synthase inhibitor prevented the increase in aldosterone but not corticosterone, suggesting that CSF [Na^+] activates enzymes involved in steriodogenesis in the hypothalamus (section *1.2.3.1, 1.2.3.2.*). Chronic subcutaneous (sc) infusion of Ang II for 2 weeks in Wistar rats increased Fra-like immunoreactivity in the SFO, SON and both magnocellular and parvocellular

subdivisions of the PVN (mPVN, pPVN) and raised aldosterone content in the hypothalamus (Huang et al. 2010). Icv infusion of an aldosterone synthase inhibitor prevented the increase in aldosterone and markedly attenuated the Fra expression in the mPVN and pPVN but not in the SFO and SON (Huang et al. 2010). These findings suggest that an increase in Ang II in the circulation activates neurons in the SFO and this may lead to increased production of aldosterone in the SON. Aldosterone may be locally synthesized in the SON, and increased aldosterone release may then enhance neuronal excitation in the mPVN and pPVN (*for pathways involved in this enhancement by aldosterone see 1.2.2.2.*).

Mineralocorticoid receptor

Aldosterone exerts its primary action in the CNS by binding to the MR. MR is expressed widely in the brain, mainly in the hippocampus, hypothalamus, amygdala and brainstem neurons but not glia (Gomez-Sanchez et al. 2006, Han et al. 2005). Immunohistochemical studies showed MR immunoreactivity in nuclei of the LT, PVN and SON (Amin et al. 2005, Han et al. 2005) with greater immunodensity in magnocellular neurons of the PVN and SON compared to parvocellular neurons of the PVN (Amin et al. 2005). The MR is a ligand-activated transcription factor and member of the nuclear receptor superfamily. In both the brain and periphery, MR have an equal affinity for aldosterone and corticosterone (in rodents or cortisol in humans), that is present in much higher; (~1000 fold) concentrations (Gomez-Sanchez et al. 2005a, Huang et al. 2009). In vitro assays for MR gene function indicated that gene transcriptional activity associated with MR activation is 10-fold high after binding with aldosterone vs. cortisol (Arriza et al. 1988). The enzyme 11ß-hydroxysteroid

dehydrogenase type 2 (11ß-HSD-2) converts corticosterone to an inactive metabolite and increases the likelihood of aldosterone binding to MR. Expression of 11ß-HSD-2 was found in the cerebellum, cortex, hippocampus, hypothalamus including the medial preoptic area and PVN, and in the nucleus of NTS (Moisan et al. 1990a, Moisan et al. 1990b, Zhang et al. 2006). Whether the presence of 11ß-HSD-2 alone provides aldosterone selectivity for MR in these brain regions is still under debate. The hypertension from chronic icv infusion of aldosterone can be prevented by icv infusion of corticosterone (Gomez-Sanchez et al. 1990), suggesting that corticosterone antagonizes the BP elevating effects of an increase of aldosterone in the CNS, presumably by competitively binding to the MR.

Effects of MR stimulation in the CNS appear to be site specific; its effects on salt appetite are associated with midbrain and brainstem regions (Geerling et al. 2006) whereas those involved in its sympathetic and BP effects appear to be associated with hypothalamic (Huang et al. 2011, Yu et al. 2008, Zhang et al. 2011) and brainstem regions (Kumar et al. 2006, Nakagaki et al. 2012) involved in cardiovascular regulation. Aldosterone sensitive neurons in the NTS projecting to regions of the extended amygdala are involved in regulating salt appetite (Geerling et al. 2006, Geerling and Loewy 2009). Acute icv infusion of aldosterone (300 ng/h for 2 hours in 0.145 M Na^+ aCSF) caused no change in RSNA, BP or HR but enhanced the pressor and sympathetic responses to icv infusion of aCSF containing 0.16 M Na^+. Furthermore, rats chronically infused icv with aldosterone (23 ng/h) in vehicle containing slightly elevated [Na^+] (0.15 M) had higher resting BP by 20 mmHg after 2 weeks of infusion (Wang et al. 2003a). No studies have yet determined the specific brain locations involved in mediating these effects of

aldosterone. Infusion of aldosterone into the SFO (300 ng/min for 5 min) also did not change BP or HR but enhanced the responses to Na^+ rich aCSF about 1 hour later and this effect was prevented by an MR blocker (Tiruneh and Leenen, unpublished data). Chronic icv infusion of aldosterone (23 ng/h with unstated [Na^+]) for 1 week increased Fra-like activity in PVN and BP by 20 mmHg and these effects were prevented by an MR blocker (Zhang et al. 2008). Although this study indicates that a chronic increase in aldosterone in the brain elevates Fra-like activity in the PVN, it is not known whether this effect is caused by MR stimulation directly in the PVN. A small increase in CSF [Na^+] appears to be required for both the acute and chronic effects of aldosterone.

(Intra)cellular mechanisms mediating the action of aldosterone in the CNS are not well known. Studies on kidney, heart, lung and endothelial cells indicate that they include both genomic and non-genomic mechanisms (Fuller and Young 2005, Funder 2005, Mihailidou et al. 2004). Nongenomic effects of aldosterone occur within seconds to minutes and involve activation of early signal transduction pathways leading to increased Na^+ transport independent of increases in transcription and translation (Funder 2005). These rapid effects of aldosterone can be mediated by increased activity of a number of Na^+ channels, including the Na^+/H^+ exchanger (Alzamora et al. 2000), Na^+/K^+/ATPase (Mihailidou et al. 2004) or ENaC (Zhou and Bubien 2001). Injection of aldosterone into the amygdala of rats increased saline drinking behavior within 15 min (Sakai et al. 2000), indicating that the rapid non-genomic effects of aldosterone in the amygdala cause an increase in salt appetite. Some evidence suggests that this non-genomic effect of aldosterone is mediated by a surface receptor that appears to increase GABA receptor activation in the amygdala thereby increasing Na^+ appetite (Sakai et al.

2000, Wehling et al. 1992). In the kidneys, the genomic effects of aldosterone occur with a latency period of 0.5-1 hour, are mediated through the MR and involve an increase in transcription and protein synthesis (Bhargava et al. 2001, Fuller and Young 2005). Aldosterone binds to the MR and the aldosterone-MR complex translocates into the cell nucleus upregulating serum-and glucocorticoid-regulated kinase 1 (SGK1) (Bhargava et al. 2001, McCormick et al. 2005). SGK1, in turn, inhibits neural precursor cell expressed, developmentally down-regulated 4-2 (NEDD4-2) which normally increases endocytosis and degradation of ENaC from the cell surface (Goulet et al. 1998, Hicke 1997). SGK1 also increases the activity of ENaC by phosphorylating its α-subunit (Diakov and Korbmacher 2004). Late genomic actions of aldosterone (hours to days) involve increased ENaC production by enhancing transcription of ENaC subunits through the MR mediated enhancement of transcription (Fuller and Young 2005). No studies have yet shown that aldosterone binding to MR increases ENaC expression or activity in the brain. However, expression of the ENaC regulatory genes SGK1 and NEDD4-2 has been demonstrated in hypothalamic nuclei such as the SFO, SON and PVN (Wang et al. 2010).

ENaC contributes to the regulation of Na^+ transport across a variety of epithelia, including the kidney collecting duct, lung, and distal colon (Snyder 2005). ENaC in the choroid plexus and ependyma may modulate CSF/ brain tissue [Na^+] (*see 1.1.2.*) (Amin et al. 2009, Wang and Leenen 2002, Wang et al. 2010). All three ENaC subunits (α, β, γ,) are expressed in cardiovascular regulatory regions of the brain, including the LT, PVN and SON with greater expression in magnocellular neurons of the PVN and SON compared to parvocellular neurons of the PVN (Amin et al. 2005, Teruyama et al. 2011,

Wang et al. 2010). Electrophysiological studies on magnocellular neurons of the PVN immunoreactive for vasopressin showed that bath application of the ENaC blocker benzamil reduced an inward leak current and caused hyperpolarization as well as a decrease in spontaneous firing activity (Teruyama et al. 2011), suggesting that ENaC currents contribute to resting membrane potential and may modulate neuronal activity in magnocellular neurons of the PVN. Enhanced Na^+ entry through ENaC appears to increase production/ release of neuromodulators in the hypothalamus such as EO, altering the excitability of pre-sympathetic neurons involved in BP regulation (*section 1.2.2.2.*).

1.2.2.2. Endogenous ouabain (EO)

Ouabain-like compounds have been detected in plasma and various tissues including the adrenal glands and brain (Hamlyn et al. 1991, Tymiak et al. 1993). Structural analyses indicated that one ouabain-like compound, is ouabain itself or a closely related isomer and indistinguishable from plant ouabain (Hamlyn et al. 1991, Kawamura et al. 1999, Schneider et al. 1998). Little is known about the pathways and enzymes involved in the synthesis of EO. Some evidence supports that EO is derived from the side-chain cleavage of cholesterol, with sequential metabolism of pregnenolone and progesterone intermediates (Hamlyn et al. 2003). Microarray analysis and qRTPCR studies indicated that P450scc and Δ5-3β-hydroxysteroid dehydrogenase/Δ5-Δ4- isomerase enzymes, acting in early steps of steroid biosynthesis, are upregulated in the hypothalamus of hypertensive vs. normotensive Milan rats. Gene silencing of the latter gene with siRNA decreased EO-like activity on rat adrenal medullary-derived PC-12 cells according to an $^{86}Rb^+$ uptake assay measuring Rb^+ uptake into human erythrocytes (Murrell et al. 2005).

EO production occurs in adrenal glomerulosa cells from where it is secreted into the circulation (Manunta et al. 2010, Shah et al. 1998, Sophocleous et al. 2003). Both *in vivo* data and *in vitro* studies with adrenocortical cells indicated that EO is secreted from the adrenal gland by catecholamines (Manunta et al. 2006, Manunta et al. 2010), Ang II (Shah et al. 1999) and adrenocorticotropic hormone (ACTH) (Sophocleous et al. 2003). In the brain, EO immunoreactivity was found in the PVN, SON, preoptic area and lateral area while also scattered around the CVOs (Yamada et al. 1987). Immunoreactivity was found in magnocellular neuronal somata and axons located mainly in magnocellular, and less in neighbouring parvocellular subdivisions ie. medial and dorsal (Yamada et al. 1987, Yamada et al. 1992a, Yamada et al. 1992b) as defined according to neuro-anatomical studies (Simmons and Swanson 2008, Swanson and Kuypers 1980, Swanson and Sawchenko 1980). Immunoreactive fibres originating from the PVN were found projecting towards the OVLT and the SON then subsequently together with the axons of the SON through the lateral hypothalamic area towards the inner layer of the median eminence and observed adjacent to the capillaries of the hypophysial portal vein system and terminating in the posterior pituitary (Yamada et al. 1992a, Yamada et al. 1992b). Magnocellular neurons of the PVN and SON may provide a source of EO to nuclei of the LT, parvocellular neurons of the PVN and the circulation (Budzikowski et al. 1998a). Although little is known about the mechanisms regulating EO production in the CNS, recent studies suggest that an increase in aldosterone in the brain activates an MR-ENaC neuromodulatory pathway leading to increased EO release (*section 1.2.3.1.*). Incubation with aldosterone for 24 hr *in vitro* increased EO release in the supernatant of immortalized N1 hypothalamic cells, and this effect of aldosterone was prevented by an

MR blocker (Yoshika et al. 2011). Icv infusion of an MR (Huang et al. 2006b) or ENaC blocker (Huang and Leenen 2002) prevented increases in hypothalamic EO content and sympathoexcitation from chronic icv infusion of Na^+ rich aCSF indicating that a chronic increase in CSF [Na^+] increases MR and ENaC activation, in turn, triggering the local synthesis and secretion of EO in the hypothalamus. Chronic sc infusion of Ang II increased Fra-like immunoreactivity in the SFO, SON and PVN and raised aldosterone content in the hypothalamus as well as BP. An aldosterone synthase inhibitor or MR blocker markedly attenuated Fra expression in the PVN but not in the SFO and SON and prevented the increase in aldosterone and most of the hypertension (Huang et al. 2010) (*section 1.2.2.1.*). Thus, both an increase in Ang II in the circulation and [Na^+] in the CSF may stimulate production of aldosterone in the hypothalamus leading to an increase in local EO release. One may postulate that an increase in neuronal activity in the SFO is relayed to magnocellular neurons of the SON leading to increased local production of aldosterone (*section 1.2.2.1.*). Aldosterone release in the SON or PVN would cause enhanced local EO production in magnocellular neurons and EO may exert its effects in the nuclei of the LT and/or pPVN (*further discussed in 1.2.3.*).

Ouabain binds to the α-catalytic subunit of the Na^+/K^+/ATPase thereby inhibiting the enzyme's activity (Blaustein 1993). All three catalytic subunits are expressed in the brain, α1 and α3 subunits are expressed in most neurons, whereas α2 are mainly expressed in glia (Bianco et al. 2007, McGrail et al. 1991). Alpha-2 and α3 subunits are ouabain-sensitive in most animals, whereas in rodents, but not humans, the α1 subunit is ouabain-resistant (Blanco and Mercer 1998, Lingrel 2010). Both acute icv infusion (Huang and Leenen 1996c) and chronic sc infusion of ouabain (Veerasingham and

Leenen 1999) in rats increase sympathetic activity and BP. Icv infusion of exogenous ouabain and EO from rat hypothalamic extracts cause similar sympathoexcitatory and pressor responses (Huang et al. 1992, Leenen et al. 1995). These effects are prevented by icv infusion of antibody Fab fragments (Digibind) that bind ouabain with high affinity (Curd et al. 1971, Huang et al. 1992). Pressor and HR response to icv injection of ouabain were absent in mice with a mutant, ouabain-resistant α2 Na^+/K^+/ATPase (Van Huysse et al. 2011), suggesting that the pressor responses to ouabain in the CNS of mice are mediated by ouabain binding to the α2 subunit, possibly in glial cells. Excitotoxic chemical lesioning of the AV3V region in Wistar rats blocked the increases in BP by 18 mmHg from sc infusion of ouabain for 3 weeks, indicating that nuclei in the LT mediate the hypertension from chronic sc infusion of ouabain (Veerasingham and Leenen 1999).

Mechanisms mediating the responses to EO in the CNS are not well defined. Studies with arterial and cardiac myocytes indicated that low doses of ouabain (nanomolar concentrations) bind to high ouabain binding affinity α2 (or α3 in neurons) Na^+ pump subunits (O'Brien et al. 1994, Zhang et al. 2005) comprising about ~20% of the total Na^+ pumps in most cells (Shelly et al. 2004). This elevates sub-plasma membrane [Na^+], in turn, increasing Na^+/Ca^{2+} exchanger (NCX) activity to extrude the excess Na^+, thereby raising intracellular [Ca^{2+}] (Blaustein et al. 1998, Dostanic et al. 2003, Zhang et al. 2005). Enhanced Ca^{2+} entry appears to be a mechanism by which ouabain enhances responsiveness of these cells (Arnon et al. 2000, Blaustein et al. 1998, Raina et al. 2010) and possibly neurons or glia to tonic simulation. Higher doses of ouabain (micromolar) also bind to the low ouabain affinity α1 subunit (O'Brien et al. 1994, Zhang et al. 2005), comprising the majority of the total Na^+ pumps in most cells

(Blanco and Mercer 1998, Shelly et al. 2004). Blocking this larger proportion of Na^+ pumps leads to depolarization (Arnon et al. 2000, Blaustein 1993). The acute sympathetic and pressor effects of ouabain in the CNS appear to depend on the brain renin-angiotensin system *(*RAS). Icv injection of ouabain increases SNA, BP and HR and this effect can be prevented by an AT_1-receptor blocker (Huang and Leenen 1996c, Huang et al. 1998). The BP and HR responses to icv injection of ouabain were attenuated by 55-70% in transgenic rats expressing an antisense RNA against angiotensinogen mRNA specifically in glia (angiotensinogen levels in the brain were reduced by >90%) (Huang et al. 2001a). Considering that the entire brain RAS is located in glia (*section 1.2.2.3.*) and ouabain likely exerts its effects on BP through the α2 subunit on glial cells, one may postulate that EO binding to the α2 subunit on glial cells may increase intracellular Ca^{2+} (see above). This may stimulate release of intracellular stored RAS components ie. angiotensinogen, renin or Ang II from glia (*section 1.2.2.3.*), leading to an increase in extracellular Ang II and enhanced AT_1-receptor activation in neurons. Chronic sc infusion of ouabain for 2 weeks increased Ang II content in the hypothalamus (Cheung et al. 2006). Chronic icv infusion of aldosterone increased angiotensin converting enzyme (ACE; responsible for converting Ang I to Ang II), AT_1-receptors and NADPH oxidase subunits in the PVN and SON and these effects of aldosterone are mediated by EO since they can be prevented by ouabain binding Fab fragments (Huang et al. 2011). ACE densities were significantly increased in the OVLT and tended to be increased in the SFO and MnPO of α2 Na^+/ K^+/ATPase knockout mice with ~10-35 % less hypothalamic α2 subunit protein compared to wild-type mice (Hou et al. 2009). The pressor response to icv injection of Ang I but not Ang II was enhanced in heterozygous

α2 Na^+/ K^+/ATPase knockout mice (Hou et al. 2009). These findings suggest that the chronic BP response from an increase in ouabain in the CNS is mediated by increase in ACE activity, Ang II release and AT_1-receptor activation.

1.2.2.3. Renin-angiotensin system

Angiotensin production

Angiotensins (Ang I, Ang II etc.) do not readily cross the BBB (Harding et al. 1988) and only brain regions outside the BBB such as the CVOs in the LT are directly affected by circulating angiotensins (McKinley et al. 2003a). A distinct brain RAS in cardiovascular regions inside the BBB has all precursors and enzymes required to form biologically active forms of angiotensin (Huang et al. 2011, Lavoie et al. 2004b, Morimoto et al. 2002, von Bohlen und Halbach and Albrecht 2006). In the first step of the angiotensin production, angiotensinogen is cleaved by renin to form Ang I. Renin immunoreactivity has been found in the cortex, brainstem and in hypothalamic nuclei such as the PVN and SON of rats (Fuxe et al. 1980, Hermann et al. 1987). Renin mRNA and protein levels are low in the brain and difficult to detect (Dzau et al. 1986, Iwai and Inagami 1992, Lavoie et al. 2004b). Immunohistochemical studies in transgenic mice expressing reporter genes under the control of the mouse renin promoter indicated that renin is expressed mainly in neurons, and less in glia (Hermann et al. 1987). Renin immunoreactivity was detected in neurons of the amygdala, lateral parabrachial nucleus, hippocampus, pituitary, inferior olivary nucleus, SFO, PVN, SON and RVLM (Lavoie et al. 2004a, b, von Bohlen und Halbach and Albrecht 2006). Human renin and angiotensinogen double transgenic mice expressing either intracellular or secreted renin in the brain exhibit an increase in BP

compared to their control littermates which is rapidly reduced by icv injection of losartan, indicating that renin in the brain can play a functional role in BP regulation (Lavoie et al. 2006). Renin mRNA expression in the hypothalamus of SD rats increased after 8 weeks of DOCA-salt treatment indicating that DOCA-salt treatment may influence renin expression in the hypothalamus (Nishimura et al. 1997). Imunohistochemical and situ-hybridization studies indicate that angiotensogen is expressed mainly in astrocytes but also neurons (Imboden et al. 1987, Intebi et al. 1990, Stornetta et al. 1988). Angiotensinogen has been detected in neurons and glia of the hypothalamus and brainstem such as the PVN, SON, SFO NTS (Imboden et al. 1987, McKinley et al. 2001, Thomas and Sernia 1988). Astrocytes secrete angiotensinogen into extracellular fluid where it can be converted to Ang I by renin or converted intraneuronally if taken up by neurons (Intebi et al. 1990). Similarly, Ang I can be converted to Ang II by ACE both in the extracellular fluid and intraneuronally, where it can be stored vesicles and transported to the axon terminals (Paul et al. 2006, Paul et al. 1985). Autoradiography studies detected ACE expression in the SFO, OVLT, MnPO, PVN, SON, NTS and RVLM of rats but the specific cell types were not described (Cheung et al. 2006, Dean et al. 2005, Saavedra and Chevillard 1982, Tan et al. 2005). ACE was detected in neuronal cells of rat brain tissue (Paul et al. 1985) and in vascular endothelial cells of the hypothalamus (Chai et al. 1987). In addition to renin and ACE, in the brain, Ang II can be formed directly from angiotensinogen by cathepsin G (Lippoldt et al. 1995), plasminogen activator (Gebbink 2011) and chymase (Baltatu et al. 1997).

Ang II, AT_1-receptors and angiotensinergic pathways

Ang II was localized in neurons and to a less extent in glial cells (Oldfield et al. 1989, Thomas et al. 2004) and the greatest number of Ang II containing neurons were located in nuclei such as the SFO, OVLT, MnPO, PVN, SON or RVLM (McKinley et al. 2003a, Oldfield et al. 1989, Richards et al. 1989). An extensive system of Ang II containing cell bodies, fibres and nerve terminals exists within these nuclei (Lind et al. 1985a, Lind et al. 1985b, Oldfield et al. 1989). In the PVN, Ang II immunoreactive cell bodies were found scattered in both magnocellular and parvocellular regions but more densely present in magnocellular (Lind et al. 1985a) (*see 1.3. for circuitry and functional role*). Ang II was found within synaptic vesicles in nerve terminals of the PVN (Oldfield et al. 1989, Pickel and Chan 1995) and electrical or chemical stimulation of neurons terminating in the PVN increases local Ang II release (Wright et al. 1993). Autoradiography studies reported the highest densities of AT_1 receptors in neurons of nuclei of the hypothalamus ie. OVLT, SFO, MnPO, PVN, SON (Tan et al. 2004, Wang et al. 2003b, Wang et al. 2003c) and in nuclei of the brainstem ie. NTS, RVLM and CVLM (Allen et al. 2000, Lenkei et al. 1997). In the OVLT, higher AT_1-receptor expression and responsiveness to Ang II was found in neurons within the lateral region (McKinley et al. 1998), known to project to the anterior hypothalamic area (AHA) and parvocellular region of the PVN (McKinley et al. 2003b), compared to neurons within the dorsal OVLT region projecting to magnocellular neurons of the PVN and SON (Oldfield et al. 1994). In the SFO, higher AT_1-receptor expression levels and responsiveness to Ang II assessed by Fos immunoreactivity was reported in the core, compared to the peripheral zone (McKinley et al. 1998, McKinley et al. 1992). Neurons in peripheral zone project to MnPO, SON, lateral hypothalamus and

the mPVN (Kawano and Masuko 2010, McKinley et al. 1998, Sunn et al. 2001), whereas those in the core project to the OVLT, bed nucleus of the stria terminalis and mainly the pPVN (Kawano and Masuko 2010, Swanson and Lind 1986). In the PVN, AT_1-receptor expression was found predominantly in parvocellular neurons and less in magnocellular (Oldfield et al. 2001, Thomas and Lemmer 2006). AT_1 receptors were not detected on neurons of the PVN projecting to RVLM or spinal cord (Oldfield et al. 2001) and it has not yet been elucidated where AT_1-receptor positive magnocellular neurons project (*see section 1.3.1. for indirect magnocellular and parvocellular signaling mechanisms).*

A number of studies provide evidence for an angiotensinergic sympathoexcitatory pathway between the LT-PVN-RVLM. Regions of the SFO, OVLT and MnPO known to project to parvocellular subdivisions of the PVN (McKinley et al. 1998, Sunn et al. 2001, Westerhaus and Loewy 1999) are densely filled with Ang II containing neurons (Lind et al. 1985b, Oldfield et al. 1989). Connections between the SFO and PVN appear to utilize Ang II as a neurotransmitter since stimulation of neurons in the SFO by Ang II or glutamic acid increased Ang II release in the PVN (Wright et al. 1993). Furthermore, electrical stimulation of the SFO increased the firing activity of neurons in the PVN, antidromically identified as projecting to the IML (Bains and Ferguson 1995) or the posterior pituitary (Ferguson and Washburn 1998). This excitatory response in neurons projecting to the IML was characterized by an increase in slow acting spikes, consistent with increased local Ang II release, and fast acting spikes (< 50 ms), consistent with increased release of a fast acting neurotransmitter, presumably glutamate (*section 1.3.1*) (Bains and Ferguson 1995). An AT_1-receptor blocker in the PVN blocked the slow acting, but not rapid spikes (Bains and Ferguson 1995). These findings suggest that an

increase in firing activity in neurons of the SFO is relayed to the PVN, causing increased Ang II and glutamate release onto neurons of the PVN projecting to the IML. However, these studies did not indicate the type of neuron that was recorded from eg. interneuron or spinally projecting parvocellular neuron. Furthermore, no studies have yet assessed whether this release of Ang II and glutamate in the PVN occurs from separate end terminals of angiotensinergic and glutamatergic neurons projecting from the LT, or via co-release from the same terminal. Micro-injection of Ang II in the SFO increased BP and this effect of Ang II was prevented by an AT_1-receptor blocker in the PVN (Ku et al. 1999). Llewellyn et al. (Llewellyn et al. 2012) did not confirm this, possibly attributable to the low dose of losartan injected into the PVN, which was ~10-20 fold lower than doses utilized in other studies to block the BP and HR responses to Ang II in the PVN (Chen et al. 2011). Considering that a glutamate receptor blocker in the PVN also abolished the sympathetic and pressor responses from Ang II in the SFO (Llewellyn et al. 2012), the Ang II-induced increase in neuronal activity in the SFO may be relayed to the PVN via angiotensinergic projections, activating local glutamatergic interneurons (Ferguson et al. 2008) that relay the increased neuronal activity to pre-sympathetic neurons (*further discussed in section 1.3.1.*). Studies still need to define this intricate circuitry in the PVN and investigate a possible role of both Ang II and glutamate responsive interneurons in the PVN (*see above*) in relaying increased activity to pre-sympathetic neurons. Bathing neurons of the PVN with Ang II *in vitro* excites retrogradely labeled neurons of the PVN terminating in the RVLM (Cato and Toney 2005). Disinhibiting the PVN with a GABA receptor antagonist increases RSNA and BP, and these effects are attenuated by 40-50% with an AT_1 receptor blocker in the

RVLM (Tagawa and Dampney 1999). Similarly, the increase in BP from microinjection of Ang II in the PVN can be prevented by an AT_1-receptor blocker in the RVLM (Ku et al. 1999). These findings suggest that an increase in Ang II release and decrease in GABA receptor activation in the PVN activates an angiotensinergic pathway between the PVN and RVLM, causing AT_1-receptor activation in the RVLM thereby increasing SNA and BP.

Intracellular pathways involved in AT_1-receptor activation

The intracellular effects of neuronal AT_1-receptor activation by Ang II have become more clear in recent years. AT_1-receptors are coupled to activation of phospholipase C (PLC) and the subsequent stimulation of phosphotidyl inositol (PI) hydrolysis or to the inhibition of adenylyl cylcase. PI hydrolysis effects are largely dependent on mobilization of internal calcium and/or activation of protein kinase C (PKC) activating many cellular processes (de Gasparo et al. 2000, Ferguson et al. 2001, Marrero et al. 1996). Among these, PKC phosphorylates nicotinamide adenine dinucleotide phosphate-oxidase (NADPH) oxidase (Wang et al. 2006) and its phosphorylation requires the activation of Rac1 (Zimmerman et al. 2004a), a critical G protein involved in activation of NADPH oxidase (Laufs and Liao 2000) (Figure 1-2). Ang II increased Rac1 activity in cultured neurons, an effect that was abolished by an AT_1-receptor antagonist. The increase in superoxide formation by Ang II in cultured neurons was inhibited by an adenovirus against Rac1 (Zimmerman et al. 2004a) supporting that Ang II binding to the AT_1-receptor increases NADPH oxidase via activation of Rac1. AT_1-receptor stimulation also causes a rise of intracellular Ca^{2+} (Garrido and Griendling 2009) (Figure 1-2). Ang II increases intracellular Ca^{2+} in cultured neurons which appears to be

mediated by increases in intracellular superoxide (O_2^{*-}) anion formation since they were prevented by adenoviral vectors that over-express superoxide dismutase (SOD) in the cytoplasm, or that inhibit expression of Rac1 (Zimmerman et al. 2005). Superoxide anions may also activate a p38 MAPK pathway increasing activation of transcription factors activator protein 1 (AP-1) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kappB) that modulate transcription of proteins such as K^+ channels (Soto et al. 2002, Yue et al. 2002, Zucker and Gao 2005) (Figure 1-2). NADPH oxidase catalyzes the transfer of electrons from NADPH to molecular oxygen to produce superoxide anions (Groemping et al. 2003) which directly inhibit the activity of voltage gated K^+ channels (Zucker and Gao 2005) (Figure 1-2). A decrease in activity of outward K^+ channels attenuates the outward rectifying K^+ current in response to an action potential. This effect reduces the hyperpolarization phase, during which a subsequent action potential cannot be triggered thereby enhancing action potential propagation (Wang et al. 1997, Wigmore and Lacey 2000). An increase in intracellular Ca^{2+} will also move resting membrane potential closer towards the threshold potential, promoting increased neuronal firing activity (Richards et al. 1999, Yost 1999). Acute icv infusion of Ang II increased production of superoxide anions in the SFO and PVN and elevated BP and HR. These effects were prevented by icv infusion of a selective inhibitor of NADPH oxidase (Erdos et al. 2006), indicating that icv infusion of Ang II increases production of reactive oxygen species (ROS) in SFO and PVN via an AT_1-dependent increase in local NADPH oxidase and this increase in ROS activates local pre-sympathetic neurons, leading to a rise in SNA and BP. As connections between the SFO and PVN are angiotensinergic (*see previous section*), icv infusion of Ang II may activate

AT_1-receptors in the SFO leading to an increase in AT_1-receptor activation and ROS production in the PVN, subserving the increases in SNA and BP. Chronic sc infusion of Ang II for 2 weeks increased BP by 40 mmHg and raised superoxide anion production in the SFO. These increases were abolished by injection of an adenoviral vector that over-expresses SOD into the SFO (Zimmerman et al. 2004b), suggesting that a chronic increase in circulating Ang II activates AT_1-receptors in the SFO, causing an increase in local superoxide production thereby leading to an increase in neuronal activity of pre-sympathetic neurons, sympathetic hyperactivity and hypertension.

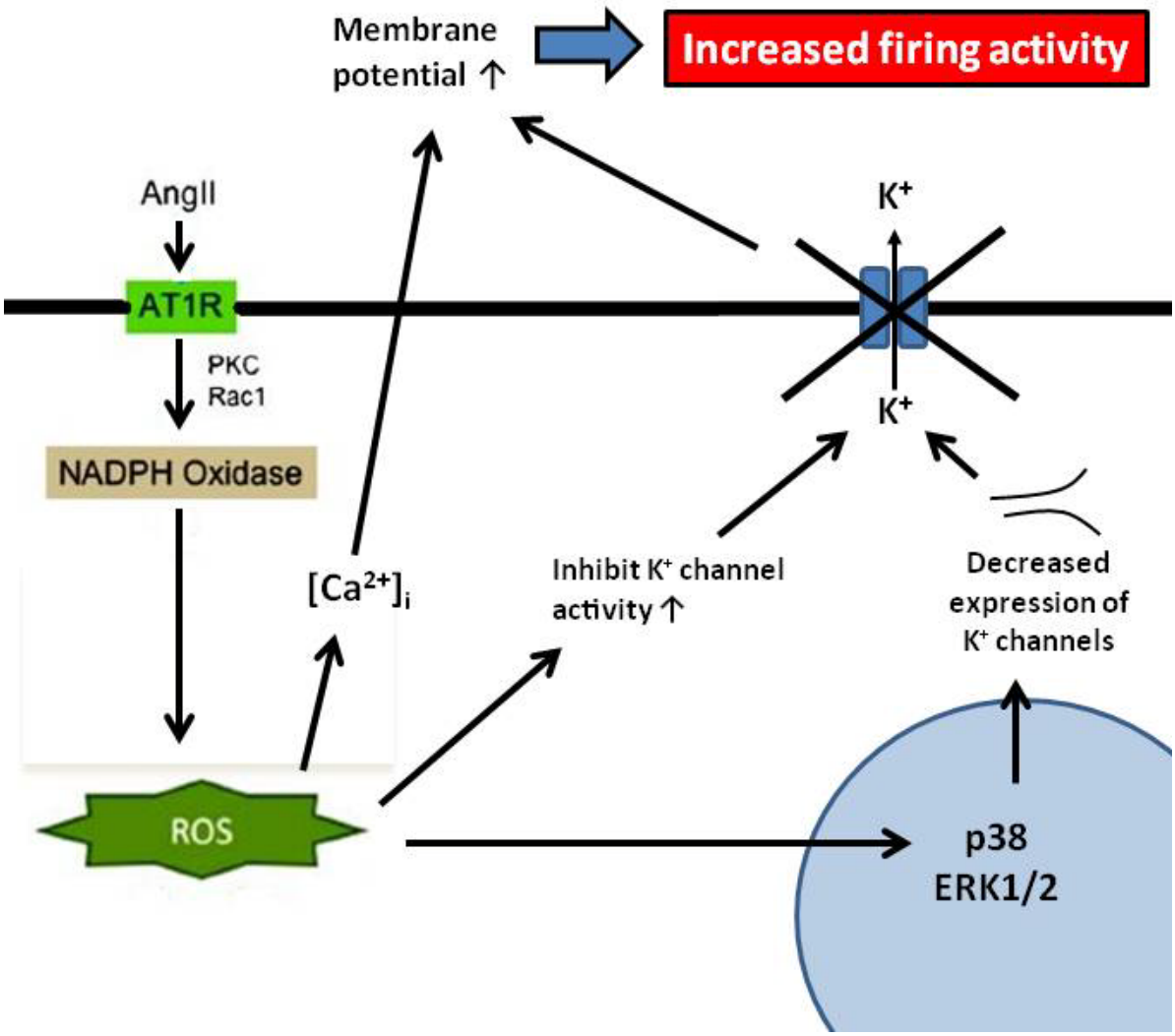

Figure 1-2: Angiotensin II signaling in the central nervous system (CNS). Ang II binding to AT_1-receptors stimulates a slow G-protein signaling pathway associated with protein kinase activation and an increase in reactive oxygen species (ROS). An increase in intracellular ROS inhibits the activity of voltage gated potassium (K^+) channels and increases intracellular calcium concentration ($[Ca^{2+}]_i$), thereby elevating membrane potential, causing increased firing activity. An increase in ROS may also activate p38 mitogen-activated protein kinase (p38MAPK) and the extracellular signal-regulated protein kinase (ERK1/2), causing activation of a number of transcription factors that decrease expression of K^+ channels, further decreasing voltage gated K^+ channel activity. *Adapted from Garrido and Griendling (2009).*

1.2.3. Functional pathways in CSF [Na^+] and dietary salt hypertension

1.2.3.1. Pathways involved in acute and chronic increases in CSF [Na^+]

Acute icv infusion of Na^+ rich aCSF (0.3-0.45 M at 3.8 µL/min for 10-20 min) increased RSNA by 30-50%, BP by 15-20 mmHg and HR by 30-50 bpm (Abrams and Osborn 2008, Huang and Leenen 1996c). These effects of Na^+ were prevented by icv infusion of benzamil (Abrams and Osborn 2008), Fab fragments or losartan (Huang and Leenen 1996c) indicating that they are mediated by ENaC activation, EO release and AT_1-receptor activation in the CNS. The pressor responses to an acute icv infusion of Na^+ rich aCSF were also prevented by Fab fragments (Budzikowski and Leenen 1997) or an AT_1-receptor blocker (Budzikowski and Leenen 2001) in the MnPO and attenuated by ~50 % by an AT_1-receptor blocker in the SFO (*section 1.2.1.2.*). Excitotoxic chemical lesioning of the OVLT and ventral part of the MnPO attenuated by 30-50 % the increases in BP from icv infusion of Na^+ rich aCSF (*section 1.2.1.2.*) supporting that all three nuclei in the LT play a role in the BP response to an acute increase in CSF [Na^+]. These studies indicate that an increase in CSF [Na^+] causes AT_1-receptor stimulation in the SFO and possibly the OVLT and increased neuronal activity from both sites is likely relayed to the MnPO. This relayed signalling then increases EO release and AT_1-receptor activation in the MnPO, but their actual sequence in the MnPO has not yet been studied. An increase in neuronal activity in the MnPO is relayed to the PVN (*section 1.2.1.*) likely causing local Ang II and glutamate release (*section 1.2.2.1*) and sympathetic hyperactivity (Figure 1-3).

Chronic icv infusion of Na^+ rich aCSF (0.8 M at 5 µL/h for 2 wks), raising CSF [Na^+] by 4-5 mM in normotensive Wistar rats increased hypothalamic aldosterone and

EO content by ~50 and ~33 %, ACE and AT_1-receptor densities in nuclei of the LT and PVN, and enhanced sympathetic activity, blunted arterial baroreflex function and increased BP by 10- 20 mmHg (Huang et al. 2006b, Huang et al. 1998). The increases in hypothalamic EO, sympathetic hyperactivity and BP were prevented by blocking MR in the CNS (Huang et al. 2006b). Aldosterone is likely the main MR agonist activating central pathways contributing to the hypertension from a chronic increase CSF [Na^+] since concomitant infusion with an aldosterone synthase inhibitor blocked the increase in hypothalamic aldosterone and prevented the chronic increase in BP in Wistar rats (Huang et al. 2008a). The aldosterone synthase inhibitor did not prevent the initial increase in BP on day 1 of chronic icv infusion of Na^+ rich aCSF but began to lower BP on day 2 of Na^+ infusion (Huang et al. 2008a). Thus, the hypertension from chronic, but perhaps not short-term increase in CSF [Na^+] in Wistar rats appears to be mediated by MR activation in the CNS likely triggered by the release of newly synthesized aldosterone in the brain. Further studies are needed to assess whether an MR blocker inhibits the initial increase in BP from chronic icv infusion of Na^+ rich aCSF in Wistar rats to exclude that release of existing aldosterone mediates the initial increase. How and where an increase in CSF [Na^+] leads to steroidogenesis has not yet been studied. Since both aldosterone and corticosteroid content in the hypothalamus were elevated during a chronic increase in CSF [Na^+] (Huang et al. 2008a), it is possible that [Na^+] increases the production of early, rate limiting and late stage enzymes involved in production of these steroids such as StAR and aldosterone synthase (*see 1.2.3.1*) (Leenen 2010). In Wistar rats with chronic icv infusion of Na^+ rich aCSF, an ENaC blocker prevented the increases in hypothalamic EO (Huang and Leenen 2002) while both an ENaC blocker and ouabain binding Fab

fragments (Huang et al. 2006b) prevented the sympathetic hyperactivity and hypertension. Fab fragments presumably prevent/reverse inhibition of the Na^+/K^+-ATPase by EO in brain areas involved in cardiovascular regulation. Fab fragments also prevented the increases in ACE and AT_1-receptor densities in cardiovascular nuclei from chronic icv infusion of Na^+ rich aCSF (Huang et al. 2006b). In addition, chronic sc infusion of ouabain increased Ang II content in the hypothalamus (Cheung et al. 2006). Icv infusion of an AT_1-receptor blocker abolished the increase in sympathetic activity and BP in Wistar rats with chronic icv infusion of Na^+ rich aCSF (Huang and Leenen 1999) or ouabain (Huang and Leenen 1996c). These findings indicate that a chronic increase in CSF [Na^+] activates a MR-ENaC-EO pathway in the CNS leading to increased AT_1-receptor activation.

Larger rates of infusion and increases in CSF [Na^+] in short-term studies (Kato et al. 2004) are required to produce similar pressor and sympathoexcitatory responses caused by lower rates of infusion and smaller increases in CSF [Na^+] in chronic studies (Huang et al. 2008a, Kawano et al. 1991b) (*section 1.1.4.*). Na^+-sensitive sympatho-excitatory pathways in the CNS appear therefore to become sensitized during a chronic increase in CSF [Na^+]. The pressor and sympathoexcitatory responses to a short-term increase in CSF [Na^+] can be enhanced by icv infusion of aldosterone and this effect of aldosterone is mediated by increased ENaC activation and EO release (Wang et al. 2003a). Chronic icv infusion of aldosterone containing slightly higher than physiological CSF [Na^+] (0.15 M Na^+) increased hypothalamic EO content (Wang et al. 2003a) and decreased nNOS in the PVN, as well as increased the NADH oxidase subunits and ACE and AT_1-receptors in the PVN, and to less extent in the SON (Huang et al. 2011) and

caused sympathetic hyperactivity and hypertension (Huang et al. 2011, Wang et al. 2003a). These effects were prevented by an ENaC blocker (Huang et al. 2011) or Fab fragments (Huang et al. 2011, Wang et al. 2003a). One may consider that an increase in CSF [Na^+] increases neuronal activity in the SFO and OVLT and this increase is relayed to magnocellular neurons of the SON and PVN leading to increased aldosterone production mainly in magnocellular neurons of the SON (*section 1.2.2.2.*). Increased aldosterone release via MR-ENaC increases EO production in magnocellular neurons of the SON and/or the PVN. EO release in nuclei of the LT or the pPVN enhances the basal activity of local Na^+-sensitive angiotensinergic sympathetic pathways. In addition, EO release in the PVN leads to an increase in NADPH oxidase subunits, ACE and AT_1-receptors densities and a decrease in nNOS expression further enhancing activity in these angiotensinergic pathways (Figure 1-3).

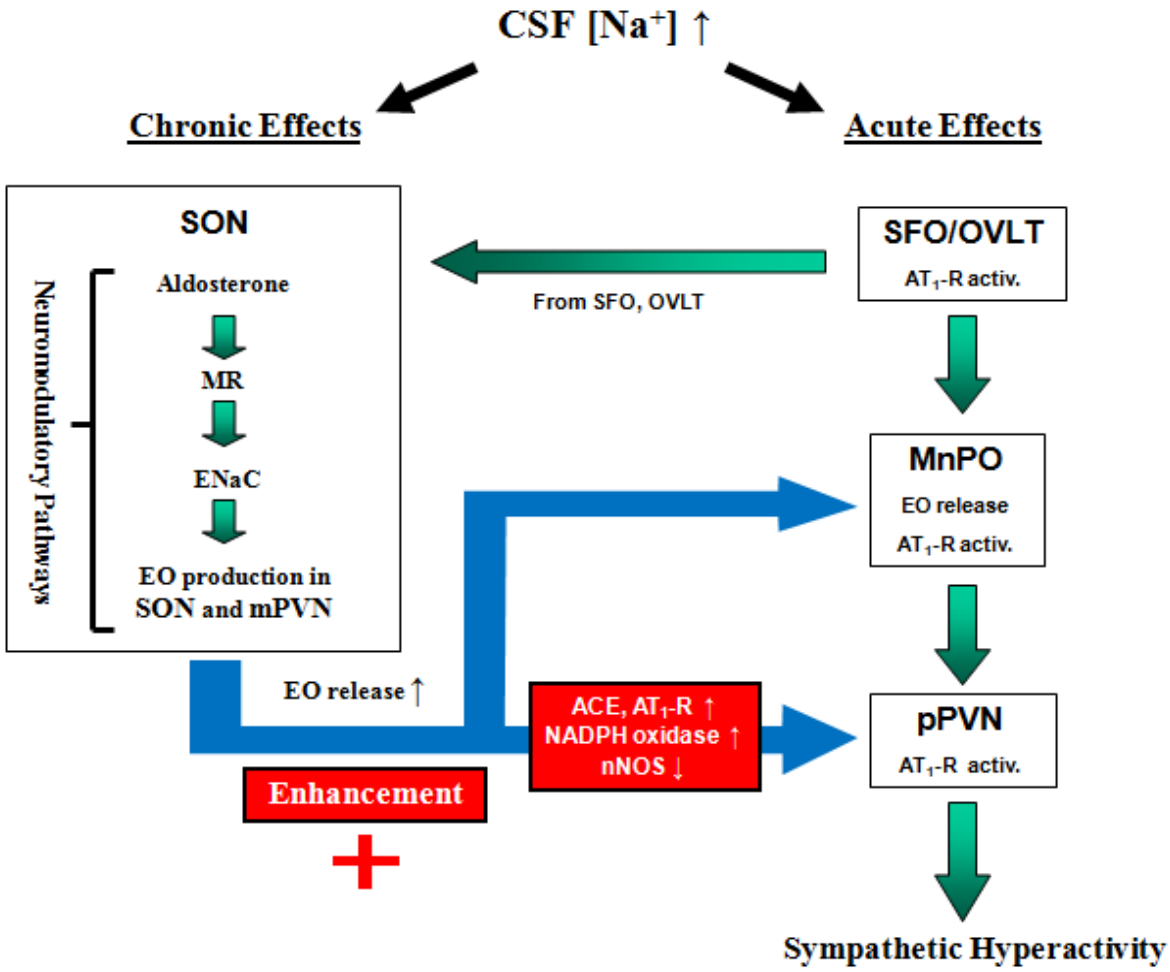

Figure 1-3: Proposed neuro-anatomical and functional pathways in the CNS activated by an acute and chronic increase in CSF [Na^+].

SFO: subfornical organ; OVLT: organum vasculosum lamina terminalis; MnPO: median preoptic nucleus; SON: supraoptic nucleus; pPVN: parvocellular paraventricular nucleus, mPVN: magnocellular paraventricular nucleus; MR: mineralocorticoid receptor; ENaC: epithelial sodium channel; EO: endogenous ouabain; ACE: angiotensin converting enzyme; AT_1-R: AT_1-receptor; NADPH: nicotinamide adenine dinucleotide phosphate-oxidase; nNOS: neuronal nitric oxide synthase.

1.2.3.2. Pathways involved in salt hypertension in Dahl S and SHR

On regular salt diet (0.3-0.6 % NaCl), BP is similar in Dahl S and R rats at a young age, whereas it tends to be elevated by 10- 20 mmHg in Dahl S rats at adult ages (>10 wks) (Huang et al. 2004, Huang et al. 2001b). In SHR on regular salt diet, BP begins to elevate around 5- 6 weeks of age, reaching between 180 and 200 mmHg during adult age (Okamoto and Aoki 1963, Pinto et al. 1998).

High salt intake (4-8 % NaCl) for 2 wks increased CSF [Na^+] by 5-7 mmol/L, BP by 15-30 mmHg and HR by 40-60 bpm in Dahl S and SHR, but not R and WKY rats (Huang et al. 2004, Nakamura and Cowley 1989). Blood pressure continued to increase with age in Dahl S rats on 8 % high NaCl diet, exceeding 185 mmHg (Pinto et al. 1998, Rapp 1982). Both acute and chronic icv infusion of Na^+ rich aCSF cause ~ 2 fold larger sympathetic and pressor responses in SHR vs. WKY rats (Huang et al. 2004, Wei and Wu 1979) and in Dahl S vs. R or Wistar rats (Huang et al. 2004, Huang et al. 2001b), indicating an increased neural responsiveness to CSF [Na^+] in Dahl S and SHR. The actual genetic variants and mechanisms contributing to the enhanced responsiveness to CSF [Na^+] in Dahl S are still unclear. Chronic icv infusion of aldosterone combined with slightly higher than physiological CSF [Na^+] increases hypothalamic EO and causes sympathetic hyperactivity and hypertension in Dahl S rats (Huang et al. 2005), to a less extent in Wistar rats (Wang et al. 2003a) and not at all in Dahl R rats (Huang et al. 2005). Considering that the RSNA, BP and HR responses to icv injection of ouabain (plant) (Huang et al. 2001b) and Ang II (Zhao et al. 2001) are similar in Dahl S and R rats, mechanisms upstream of EO's action are therefore likely responsible for the enhanced neural responses to Na^+ and aldosterone in Dahl S rats. Studies by our group in congenic

rats suggest that genetic variants on C10QTL2 contribute to this phenotype in Dahl S and/or enhance expression of genetic variants on other loci (Huang et al. 2007). The voltage-dependent calcium channel γ4 subunit, cacng4, is 1 of the genes located on C10QTL2. This channel is involved in neuronal excitation (Letts et al. 2005) and may contribute to the increased neuronal responsiveness to Na^+ in Dahl S.

High salt diet (8 % NaCl) for 2-4 weeks also increased hypothalamic content of aldosterone by ~35 %, corticosterone by ~20 % (Huang et al. 2009), EO by ~80 % (Wang and Leenen 2002), hypothalamic ACE activity by ~40 % (Zhao et al. 2001) and AT_1-receptor densities in the nuclei of the LT and PVN (Wang et al. 2003c) in Dahl S, but not R rats. In contrast to Dahl S, high salt diet (8 % NaCl) for 4 wks decreased hypothalamic content of both aldosterone and corticosterone in Dahl R rats. Contrary to the brain, high salt diet (8 % NaCl) for 2- 3 weeks lowered plasma aldosterone content in both Dahl S and R rats (Amin et al. 2011, Bayorh et al. 2005) indicating that the increased hypothalamic aldosterone from high salt intake in Dahl S is unlikely related to plasma levels since salt decreased aldosterone in the plasma. Icv infusion of an aldosterone synthase inhibitor prevented the increase in hypothalamic aldosterone and attenuated the hypertension by ~60% in Dahl S rats on high salt intake (Huang et al. 2009). Icv infusion of a steroid synthase 3β-HSD inhibitor (blocks synthesis of both aldosterone and corticosterone) or an MR blocker fully prevent the hypertension from high salt diet in Dahl S (Gomez-Sanchez et al. 2005b, Huang et al. 2009). These findings indicate that MR activation in the CNS mediates the hypertension, and aldosterone acts as the main agonist but other agonists eg. cortiscosterone may also contribute (Leenen 2010). In Dahl S and R rats, high salt diet increased EO content in the adrenal gland

(Wang and Leenen 2002) but did not appear to increase EO in the plasma (Fedorova et al. 2001, Wang and Leenen 2002). However, marinobufagenin, an endogenous Na^+/K^+/ATPase inhibitor that has greater binding affinity than EO for the α1 subunit of Na^+/K^+/ATPase (Fedorova and Bagrov 1997), was elevated in the plasma of Dahl S but not R rats at 2 or 4 weeks high salt diet (Fedorova et al. 2001, Fedorova et al. 2002). Icv infusion of an ENaC blocker prevented the increase in hypothalamic EO content (Wang and Leenen 2002), sympathetic hyperactivity and hypertension from high salt diet in Dahl S rats (Gomez-Sanchez and Gomez-Sanchez 1995, Wang and Leenen 2002). In Dahl S on high salt, Fab fragments blocked the increase in hypothalamic ACE activity (Zhao et al. 2001) while both Fab fragments (Huang et al. 2004) and an AT_1-receptor blocker (Huang and Leenen 1998) prevented the hypertension. These findings indicate that high salt diet in Dahl S rats activates the aldosterone-MR-ENaC-EO pathway in the brain leading to increased AT_1-receptor activation and sympathoexcitation and hypertension. Blockers of this pathway caused no change in resting BP in Dahl R rats on high salt or in S and R rats on regular salt diet (Huang and Leenen 1998, Huang et al. 2004, Huang et al. 2009, Wang and Leenen 2002) indicating that this pathway is not active in physiological conditions.

Compared to regular salt diet (0.3 % NaCl), high salt diet (8 % NaCl) from 5-9 weeks of age caused larger increases in hypothalamic EO (regular: +20-30%; high: +40-50%) (Leenen et al. 1993) and BP (regular: +20-30 mmHg; high: +50-60 mmHg) (Budzikowski and Leenen 2001, Leenen et al. 1993) in SHR compared to WKY rats. Acute icv injection of an MR blocker after high salt diet (8% NaCl) from 10-13 weeks of age in SHR decreased BP (from 210 to 160 mmHg) 8 hours post injection (Rahmouni et

al. 2001). In SHR, chronic icv infusion of fab fragments or losartan caused no change in BP after regular salt diet from 5-9 weeks of age whereas either blocker prevented the exacerbation of hypertension by high salt diet [resting BP at week 9 (regular Na^+: 135-145 mmHg; high Na^+: ~170 mmHg) (Huang and Leenen 1996a, b). Similarly, acute icv injection of an AT_1-receptor blocker did not affect resting BP in SHR at 20 weeks of age (DePasquale et al. 1992). These findings suggest that high salt intake exacerbates the development of hypertension in SHR and this effect of Na^+ is mediated by increased activation of the central MR-EO neuromodulatory pathway, presumably leading to increased AT_1-receptor activation in the CNS and sympathetic hyperactivity and hypertension. Since none of the blockers decreased BP in SHR on regular salt diet, this pathway in the CNS does not appear to contribute to the development of spontaneous hypertension in SHR. To our knowledge, no studies have yet elucidated the pathways contributing to the spontaneous hypertension.

Accumulating evidence reveals a dual role for brain EO in models with chronic CSF [Na^+]. Chronic icv infusion of ouabain in Wistar rats on high salt diet increased BP and HR but decreased CSF [Na^+] while fab fragments in Dahl S rats on high salt diet further increased CSF [Na^+] (Huang et al. 2004). These findings suggest that in response to increases in CSF or interstitial [Na^+] the release of EO in the CNS may act as a negative feedback to inhibit enhanced Na^+ transport via Na^+/K^+/ATPase in the CP. Although EO has been detected in the CSF of animals and humans (Borsody et al. 2006), to our knowledge, no studies have assessed its release in models of salt-induced hypertension (*section 1.1.2.*). One may consider that EO release into the CSF of Dahl S rats on high salt may be enhanced to attempt to decrease activity of the Na^+/K^+/ATPase in

the CP and blunt the increase in CSF [Na^+] (*section 1.1.2.*). In parallel, or in addition, EO binding to the Na^+/K^+/ATPase of glia in nuclei involved in cardiovascular regulation ie. SFO, OVLT or PVN may enhance the activity of pathways leading to sympathetic hyperactivity and hypertension.

A few studies have assessed where in the CNS of Dahl S and SHR the MR-ENaC-EO pathway contributes to the sympathetic hyperactivity and hypertension from high salt. Injection of fab fragments (Budzikowski and Leenen 1997) or losartan (Budzikowski and Leenen 2001) into the MnPO did not affect BP in SHR and WKY rats on regular salt diet but normalized the BP increase by 20- 25 mmHg from 5 weeks high (8 %) NaCl in SHR. These findings indicate that both EO action and AT_1-receptor activation in the MnPO contribute to the maintenance of resting BP in SHR on high but not regular salt intake. Injection of an AT_1-receptor blocker into the RVLM also did not affect BP in Dahl S and R rats on regular salt diet but normalized the BP increase by ~ 40 mmHg in Dahl S rats from 3 weeks high (8 %) NaCl diet (Ito et al. 2003) indicating that enhanced AT_1-receptor activation in the RVLM contributes to the maintenance of resting BP in hypertensive Dahl S rats on high salt intake. No studies have yet assessed whether these mechanisms in the PVN contribute to the maintenance of resting BP in Dahl S and SHR on high salt diet.

1.3. Signaling in the PVN in physiological and pathophysiological conditions

1.3.1. Overview of the PVN and signaling in physiological conditions

Overview: The PVN is a heterogeneous nucleus comprised of 3 major cell types classified according to their morphology and efferent connections (Kiss 1988, Swanson

and Kuypers 1980). Firstly, the PVN and neighbouring SON contain neurosecretory magnocellular neurons. These cells produce vasopressin, oxytocin (Swanson and Kuypers 1980) and likely EO (*see section 1.2.2.2.*). Their axon terminals are located in the capillary beds of the posterior pituitary from where they release these hormones into the circulation (Kiss 1988). Secondly, the PVN contains parvocellular neurosecretory neurons that produce a number of regulatory hormones, including corticotropin releasing hormone (CRH) (Antoni et al. 1983), thyroid releasing hormone (TRH) (Lechan and Jackson 1982) and somatostatin (Dierickx and Vandesande 1979). Their axons terminals are located in the median eminence capillary plexus, where secreted hormones become transported into the anterior pituitary through hypophyseal portal blood vessels. These hormones bind to G-protein coupled receptors on neurosecretory cells of the anterior pituitary, causing release of specific anterior pituitary hormones ie. ACTH, thyroid stimulating hormone (TSH) or growth hormone (GH) into the circulation where they seek their peripheral glandular targets (Swanson and Sawchenko 1980, Whitnall et al. 1985). Parvocellular autonomic neurons are the third cell type and are known to send projections to the IML and brainstem regions such as the NTS and RVLM (Swanson and Sawchenko 1980). Connections from these brain stem regions are reciprocal, since the PVN receives catecholaminergic inputs from the A1 cell group of the RVLM and A2 cell group of the NTS. In the hypothalamus, the PVN receives projections from the nuclei of the LT (*section 1.2.1.*), preoptic area, ventromedial and dorsomedial nuclei, anterior and lateral hypothalamic area and suprachiasmatic nucleus (SCN) (Swanson and Sawchenko 1983, 1980).

Excitatory and inhibitory neurotransmitters (glutamate and GABA) influence neuronal activity in the PVN and thereby sympathetic activity. These will be discussed in the following sections. In the PVN, the actions of the primary neurotransmitters ie. glutamate and GABA are being modulated by neuromodulators such as Ang II and nitric oxide (NO). Furthermore, the MR-ENaC-EO pathway appears to enhance the activity of these neuromodulators and primary neurotransmitters (*section 1.2.2.*).

Glutamate: Glutamate is a nonessential amino acid that primarily contributes to excitatory input in parvocellular and magnocellular neurons of the PVN (Csaki et al. 2000). Glutamate is synthesized in the cytoplasm of nerve terminals either from glucose via tricarboxylic acid cycle enzymes or from glutamine via glutaminase, and is stored in local synaptic vesicles. An action potential arriving at the nerve terminal causes release of glutamate into the synaptic cleft where it may exert its effects on postsynaptic neurons or be taken back up into the nerve terminal or into local glial cells by glutamate transporters. Glutamate taken up by glial cells in converted into glutamine which is then transported out of glial cells and into nerve terminals. This glutamine-glycine cycle maintains an adequate supply of the glutamate in the synaptic cleft (Purves 2008). Glutamatergic axons enter the PVN from other hypothalamic regions, such as the preoptic area, MnPO, SCN and AHA as well as telencephalic regions, including the lateral septum and amygdala (Csaki et al. 2000). Sympathetic and pressor responses to a glutamate analog in the MnPO or a GABA receptor blocker in the SFO or MnPO can be prevented by a glutamate receptor blocker in the PVN (Llewellyn et al. 2012) suggesting that an increase in neuronal activity in the SFO or MnPO can be relayed to the PVN via glutamatergic projections. Both glutamatergic and non-glutamatergic axons eg.

angiotensinergic (*section 1.2.2.3.*) arriving in the PVN from other brain regions may terminate onto glutamatergic interneurons within the PVN which provide an important source of synaptic integration (Ferguson et al. 2008).

Glutamate receptors can be subdivided into ionotropic and metabotropic receptors, each class containing three subclasses. Ionotropic receptors are classified as NMDA (N-Methyl-D-aspartate) and non-NMDA receptors ie. kainate and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA). Both receptor types are non-specific cation channels that are permeable to Na^+ and K^+. Glutamate binding to AMPA or kainate receptors changes their conformation and causes their ion channel to open in less than 1/1000 of a second. This allows Na^+ to flow into the cell rapidly, thereby initiating an increase in membrane potential (Platt 2007). In contrast, NMDA receptors are voltage-dependent; at resting or hyperpolarized membrane potential, a Mg^{2+} ion blocks the pore thereby preventing Na^+ to flow through the channel even if glutamate is bound. An increase in membrane potential initiated by other ion channels eg. Na^+ influx via AMPA receptors, pushes Mg^{2+} out of the pore, further increasing Na^+ conductance and strengthening the rise in membrane potential (Dingledine et al. 1999). NMDA and non-NMDA receptors are expressed on neurons in parvocellular subdivisions of the PVN (Herman et al. 2000) and immunohistochemical studies showed glutamate-immunoreactive axon terminals contacting dendrites and cell bodies of parvocellular neurons (van den Pol 1991). There are eight known glutamate metabotropic receptors divided into three groups according to their structure and function (Swanson et al. 2005). These receptors activate more slow intracellular signaling pathways ie. seconds to minutes involving secondary messengers, protein kinases and phosphatases (Greengard

2001). Consequently, these pathways may enhance or attenuate glutamatergic neurotransmission, either by modulating pre-synaptic glutamate release or by altering the activity state of post-synaptic glutamate receptors eg. NMDA (Gerber et al. 2007). For example, activation of the group I metabotropic glutamate-1 receptor (mGluR1) stimulates phospholipase C (PLC), leading to an increase in intracellular Ca^{2+} from internal stores and activation of protein kinase C (PKC), ultimately causing an increase in spontaneous or evoked glutamate release from pre-synaptic terminals (Schwartz and Alford 2000). Group I metabotropic glutamate receptors also increase the activity of post-synaptic NMDA receptors by similar intracellular pathways (Skeberdis et al. 2001). Immunohistochemical and immunocytochemical studies showed that these receptors are expressed on neuronal cell bodies and dendrites in the mPVN and the pPVN (Mateos et al. 1998, Van den Pol 1994).

Micro-injection of glutamate into the PVN increased SNA, BP and HR (Kannan et al. 1989). Specific blockers of NMDA receptors attenuated SNA and BP responses to L-glutamate in the PVN by 60-75 % and of non-NMDA receptors by 20- 30% (Li et al. 2006a), indicating that both NMDA receptors contribute to the BP responses from glutamate in the PVN. Microinjection of an mGluR1 agonist into the PVN increased LSNA, BP and HR and these responses were prevented by a NMDA receptor antagonist (Li and Pan 2010), indicating that mGluR1 activation in the PVN increases SNA, BP and HR and these effects are mediated by increased local NMDA receptor activation likely via increased glutamate release. Micro-injection of a NMDA receptor agonist in the PVN increases neuronal activity in RVLM neurons and raises BP, and these effects are attenuated by a glutamate receptor blocker in the RVLM (Yang and Coote 1998),

indicating that sympatho-excitatory glutamatergic projections from the PVN to the RVLM contribute to the pressor responses to increased glutamate release in the PVN.

GABA: GABA is the primary inhibitory neurotransmitter in the PVN and ~50 % of the synapsing nerve terminals in the PVN are immunoreactive for GABA (Decavel and Van den Pol 1990). GABA is catalyzed from glutamate by the enzyme glutamic acid decarboxylase in the cytoplasm of nerve terminals and is stored in local synaptic vesicles. Similar to glutamate, an action potential at the nerve terminal causes GABA release into the synaptic cleft where it may act on postsynaptic neurons or be taken up into the nerve terminal or local glial cells by GABA transporters. GABA may be degraded into succinate in neurons or glia by the enzymes GABA transaminase and succinic semialdehyde dehydrogenase (Purves 2008). Neurons immunoreactive for GABA are distributed throughout the PVN and homogenously among the mPVN and the pPVN (Boudaba et al. 1996). GABAergic axons entering the PVN appear to be from the anterior hypothalamic and preoptic areas (Boudaba et al. 1996, Larsen et al. 1994), the bed nucleus of the stria terminalis (Cullinan 2000, Roland and Sawchenko 1993) or the SCN (Decavel and Van den Pol 1990, Hermes and Renaud 1993). Particularly high level of GABAergic interneurons were found in the "halo zone" surrounding the nucleus and in the periventricular region (Ferguson et al. 2008, Roland and Sawchenko 1993). Immunohistochemical and retrograde labeling studies of the PVN demonstrated that GABAergic interneurons were also synaptically associated with parvocellular neurons projecting to the IML (Watkins et al. 2009).

GABA receptors are divided into two classes; $GABA_A$ and $GABA_B$ receptors. $GABA_A$ receptors are fast acting ionotropic channels that have a Cl^--ion selective pore.

GABA binding to the $GABA_A$ receptor rapidly opens the channel, causing an increase in Cl^- conductance, thereby hyperpolarizing the neuron and inhibiting action potentials. In contrast, $GABA_B$ receptors are slow acting metabotropic G-protein coupled receptors whose action is independent of Cl^- ions (Bettler et al. 2004). GABA binding to the $GABA_B$ receptors activates Gβγ subunits, thereby stimulating a slower intracellular signalling pathway that inhibits the activity of voltage gated Ca^{2+} channels, decreasing Ca^{2+} influx and release of excitatory neurotransmitters ie. glutamate from pre-synaptic terminals (Dittman and Regehr 1996, Wu and Saggau 1995). $GABA_A$ and $GABA_B$ receptors are present in the PVN. *In situ* hybridization studies detected similar levels of $GABA_A$ mRNA expression in both the mPVN and pPVN (Cullinan 2000, Wisden et al. 1992). In contrast, $GABA_B$ expression was mainly found in magnocellular subdivisions and less in parvocellular (Richards et al. 2005).

Micro-injection of a $GABA_A$ (Akine et al. 2003) or $GABA_B$ (Li and Pan 2007b) receptor agonist into the PVN decreased SNA, BP and HR. A $GABA_B$ receptor agonist decreased the frequency of glutamate mediated EPSCs in spinally projecting neurons of the PVN from a hypothalamic slice preparation (Li et al. 2008a), supporting that $GABA_B$ receptor activation on nerve terminals inhibits glutamate release onto pre-sympathetic neurons of the PVN.

Nitric Oxide: NO in the brain can act as a nontraditional neuromodulator. NO is produced by a family of three cellular nitric oxide synthases (NOS) isoforms: All three NOS isoforms; neuronal (nNOS), endothelial (eNOS) and inducible (iNOS) are found in the brain. nNOS is mostly confined to discrete neuronal populations (Anggard 1994) and is the predominant isoform responsible for the formation of NO in the PVN (Bhat et al.

1996, Gingerich and Krukoff 2005). Activation of nNOS in neurons requires a rise in intracellular Ca^{2+} concentration and can be triggered by increased Ca^{2+} influx through activation of membrane associated cation channels ie. Voltage gated Ca^{2+} channels, Na^{+}/Ca^{2+} exchanger (Zanzinger 2002) or NMDA receptors (Bredt and Snyder 1989). nNOS activation results in the formation of NO in a Ca^{2+}/calmodulin-dependent manner (Bredt and Snyder 1990, 1989) which may diffuse through the cell membrane and act on neighboring cells (Krukoff 1999, Wood and Garthwaite 1994). nNOS is expressed widely in neurons of the PVN, but far more in the magnocellular vs. parvocellular (Nylen et al. 2001, Stern 2004). Immunohistochemical studies showed that nNOS was expressed in a small proportion (6-25%) of neurons on or in close proximity to those projecting to the IML and RVLM (Li et al. 2002, Watkins et al. 2009, Weiss et al. 2001). These nNOS positive neurons were also in close proximity to GABAergic interneurons adjacent to parvocellular neurons projecting to the IML and were distinct from magnocellular neurons expressing nNOS (Watkins et al. 2009, Weiss et al. 2001). Electrophysiological studies *in vitro* indicate that NO enhances GABA release from GABAergic neurons onto pre-sympathetic neurons projecting to the IML. NO activates an intracellular guanosine monophosphate (cGMP)- protein kinase G pathway (Li et al. 2004, Li et al. 2003b) leading to decreased activation of Kv1.1 and Kv1.2 voltage gated potassium channels at the nerve terminal of these GABAergic neurons (Yang et al. 2007). Decreased activity in these channels increases neuronal activity and GABA release onto parvocellular neurons projecting to the IML or RVLM (Yang et al. 2007). An NO donor in the PVN decreases SNA, BP and HR (Zhang et al. 1997), and push-pull perfusion of the PVN with aCSF containing NO significantly elevated GABA in the perfusate (Horn et al. 1994). The

decreases in SNA and BP by the NO donor in the PVN can be inhibited by a $GABA_A$ receptor antagonist (Zhang and Patel 1998).

Ang II: The expression and distribution of Ang II and its receptors in the PVN were previously described (*section 1.2.2.3*). AT_1-receptors were not detected on pre-sympathetic neurons projecting to the RVLM or IML (*section 1.2.2.3*). Electrophysiological studies *in vitro* showed that Ang II-induced activation of AT_1-receptors on end terminals of GABAergic interneurons adjacent to pre-sympathetic neurons stimulate an intracellular $G_{i/o}$ protein signaling pathway leading to activation of NADPH oxidase (Chen and Pan 2007). The resulting increase in ROS inhibits GABA release onto pre-sympathetic neurons projecting to the RVLM (Li and Pan 2005) or IML (Li et al. 2003a). This effect of ROS is thought to involve inhibition of synaptosomal-associated protein 25 (Chen and Pan 2007, Giniatullin et al. 2006), a protein that fuses GABA storage vesicles with the plasma membrane thereby facilitating GABA release into the synaptic cleft (Giniatullin et al. 2006).

Micro-injection of Ang II in the PVN increases SNA, BP and HR. (Zhu et al. 2002). Push-pull administration of Ang II into the PVN caused an increase in local NO release. Pressor and sympathetic responses to Ang II in the PVN were enhanced by a blocker or antisense against neuronal nitric oxide synthase (nNOS), the NOS isoform primarily responsible for NO production in the PVN (Li et al. 2006b). In contrast, over-expression of nNOS significantly attenuated the pressor responses to Ang II. These findings suggest that Ang II in the PVN acutely increases local NO release, which in turn, inhibits the pressor and sympathetic responses to Ang II itself (Li et al. 2006b). Bains and Ferguson (1994) provided functional evidence for a NO mediated negative feedback

system within the PVN. The pressor responses to electrical stimulation of the SFO were enhanced by blocking NO production in the PVN by a NOS blocker. These findings support that NO may be acting in the PVN to attenuate the pressor response to local AT_1-receptor stimulation caused by stimulation of the SFO (Bains and Ferguson 1994).

Signaling and neurotransmitter interaction in physiological conditions: Injection of a glutamate (Li and Pan 2007a) or AT_1-receptor receptor blocker (Chen and Toney 2001) in the PVN of normotensive rats did not affect SNA, BP or HR whereas a $GABA_A$-receptor blocker increased local glutamate release and SNA, BP and HR (Li et al. 2006a). Pressor and sympathetic responses from a $GABA_A$-receptor blocker in the PVN were prevented by a glutamate (Chen et al. 2003, Li et al. 2006a) or AT_1-receptor blocker (Chen and Toney 2003) indicating that in physiological conditions, GABA release in the PVN tonically inhibits local glutamate release and activation of AT_1-receptors. A NOS blocker in the PVN also increased SNA, BP and HR, and these effects were prevented by a GABA receptor agonist (Zhang and Patel 1998) and a glutamate receptor blocker (Li et al. 2001) suggesting that tonic NO release in the PVN causes a tonic increase in local GABA release and supports a NO-GABA-glutamate functional pathway in the PVN. A $GABA_A$ receptor blocker increased the frequency of glutamate mediated EPSCs from retrogradely labeled PVN-RVLM projecting neurons in a hypothalamic slice preparation (Li et al. 2006a), supporting that GABA inhibits glutamate release from glutamatergic neurons onto pre-sympathetic neurons projecting to the RVLM (Figure 1-4). To our knowledge, no studies have yet assessed the mechanism(s) by which GABA release tonically inhibits AT_1-receptor activation in the PVN. Considering that AT_1-receptors were not detected on parvocellular neurons projecting to the PVN or RVLM (Oldfield et

al. 2001) and pressor responses to a $GABA_A$ receptor blocker in the PVN were fully prevented by AT_1 and glutamate receptor blockers (Chen and Toney 2001, Li et al. 2006a), glutamate release tonically inhibited by GABA in the PVN may be stimulated by tonic AT_1-receptor activation (Figure 1-4). Similar to Ang II-mediated excitation of magnocellular neurons in the PVN (Latchford and Ferguson 2004), tonic AT_1-receptor stimulation on glutamatergic neurons in the PVN may increase glutamate release onto pre-sympathetic neurons and this excitatory effect on parvocellular neurons is tonically inhibited by GABA release (Figure 1-4). However, no studies have yet assessed the extent of glutamate release by AT_1 receptor activation or the brain regions contributing to these angiotensinergic, glutamatergic or GABAergic inputs.

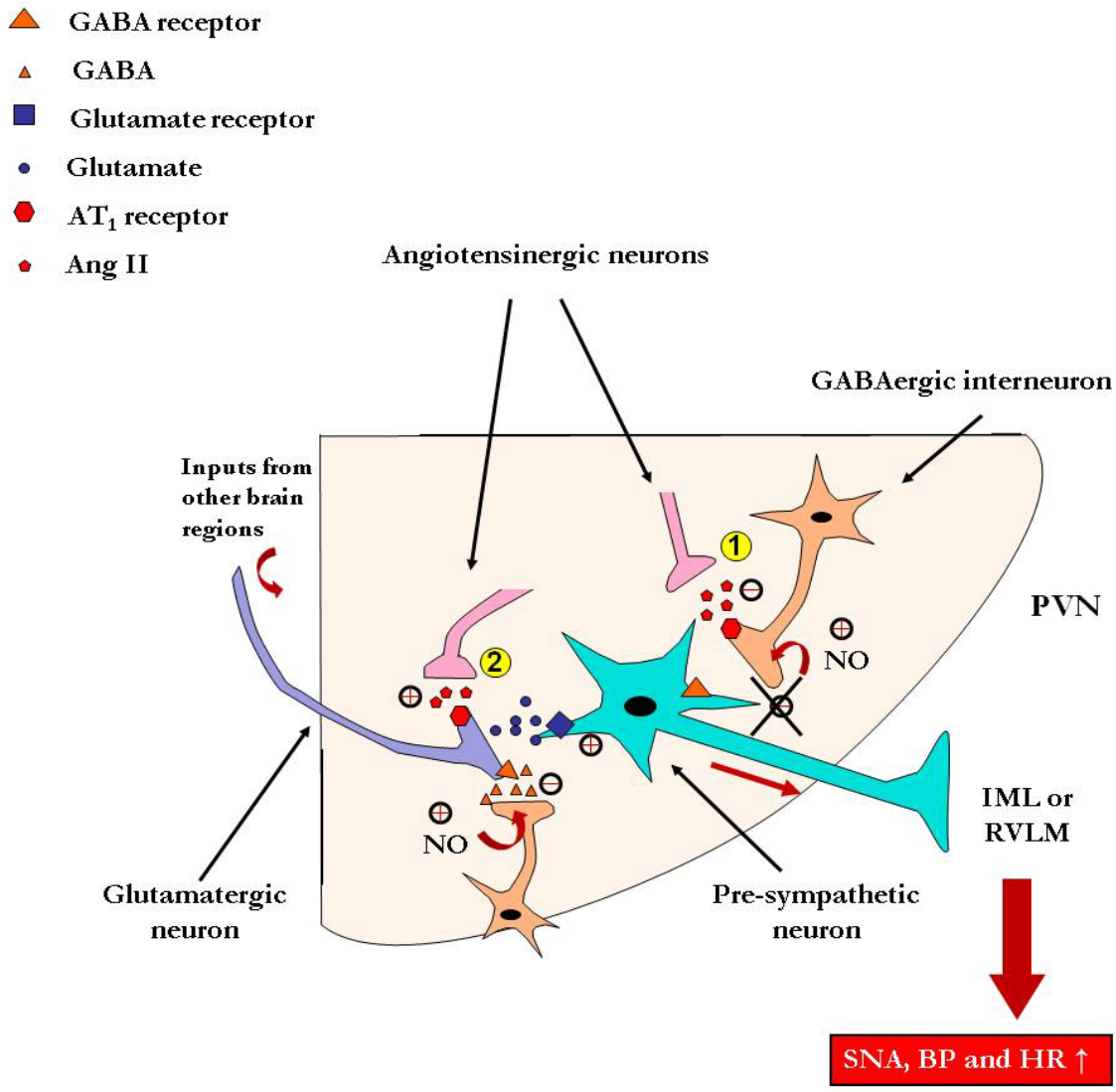

Figure 1-4: Schematic diagram of the neuronal pathways in the PVN associated with normal physiology. Glutamatergic axons from a variety of possible locations (*section 1.3.1.*) release glutamate onto pre-sympathetic neurons. Nitric oxide (NO) release in the paraventricular nucleus (PVN) tonically increases gamma-amino butyric acid (GABA) release from GABAergic interneurons thereby inhibiting the activity of adjacent glutamatergic neurons. Moreover, GABA release tonically inhibits pre-sympathetic parvocellular neurons projecting to the IML or RVLM. Ang II in the PVN activates AT_1-receptors on terminals of GABAergic interneurons (1) leading to less GABA release onto pre-sympathetic neurons. AT_1-receptor stimulation directly on glutamatergic neurons tonically inhibited by GABA (2) increases glutamate release onto pre-sympathetic neurons. IML: intermediolateral cell column; RVLM: rostral ventrolateral medulla; SNA: sympathetic nerve activity; BP: blood pressure; HR: heart rate.

1.3.2. PVN signaling in responses to an acute increase in CSF [Na^+]

Pressor responses from a short icv infusion of Na^+ rich aCSF are mediated by AT_1 receptor activation, presumably via Ang II release in the nuclei of the LT (*section 1.2.1.2, 1.2.3.1.*). An increase in neuronal activity by CSF [Na^+] in the SFO and OVLT is relayed through the MnPO to magnocellular and parvocellular neurons of the PVN (*section 1.2.1*). Direct projections exist between the SFO and OVLT to the PVN (*section 1.2.1.*) but there is no direct evidence that these projections play a role in the responses to an increase in CSF [Na^+]. Although no studies have yet defined the projections between the MnPO and PVN contributing to the responses to an increase in CSF [Na^+], they are likely angiotensinergic (*section 1.2.2.3.*) and glutamatergic (*section 1.3.1.*). An increase in Ang II release in the PVN caused by increased neuronal activity in the MnPO may increase the firing activity of pre-sympathetic neurons projecting to the IML or RVLM by 1) decreasing GABA release onto pre-sympathetic neurons (Li et al. 2003a) (*section 1.3.1.*); 2) considering that GABA receptors tonically inhibit glutamate release in normotensive rats (Li et al. 2006a), it is possible that AT_1-receptors may also inhibit GABA release from GABAergic interneurons adjacent to glutamatergic neurons. In this proposed pathway in the PVN, angiotensinergic neurons via GABAergic inhibition, enhance the activity of glutamatergic neurons capable of stimulating parvocellular neurons projecting to the RVLM or IML; and 3) increased AT_1-receptor stimulation on glutamatergic neurons in the PVN may also increase glutamate release onto pre-sympathetic neurons (*section 1.3.1.*) (Figure 1-5). An increase in glutamate release in the PVN caused by increased neuronal activity in a subset of glutamatergic neurons from the MnPO (Csaki et

al. 2000) with terminals adjacent to parvocellular neurons of the IML or RVLM may directly increase glutamate release onto pre-sympathetic neurons (Figure 1-5).

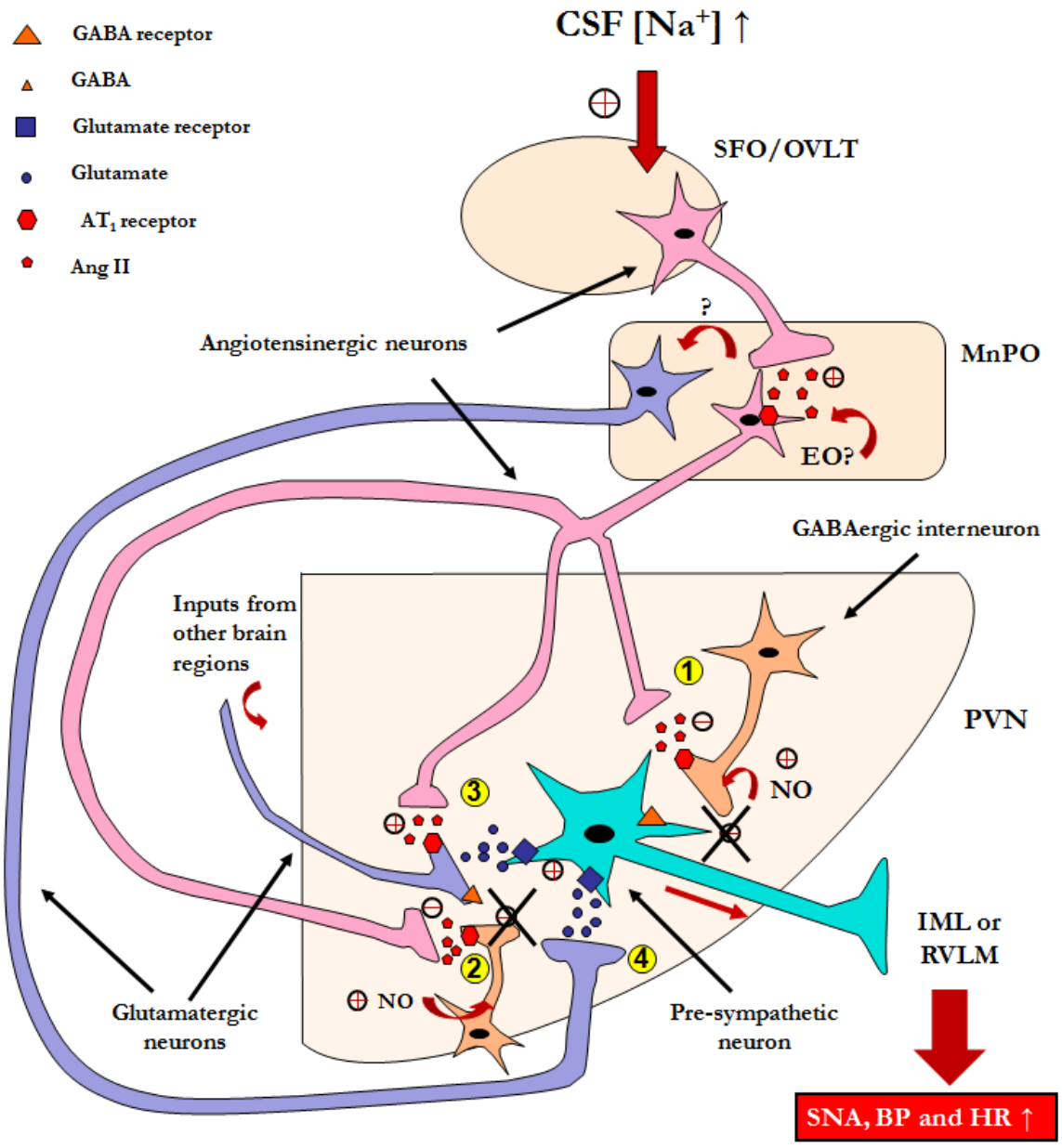

Figure 1-5: Schematic diagram of proposed neuronal pathways to/in the PVN contributing to the sympathetic and pressor responses from an acute CSF $[Na^+]$ ↑. Increases in CSF $[Na^+]$ excite angiotensinergic neurons in the subfornical organ (SFO) /organum vasculosum of the lamina terminalis (OVLT) and the increase in neuronal activity is relayed to the median preoptic nucleus (MnPO), causing increased local Ang II and endogenous ouabain (EO) release (sequence is not known, *1.2.3.1*). An increase in neuronal activity in the MnPO causes Ang II release in the paraventricular nucleus (PVN). AT_1-receptor stimulation on terminals of GABAergic interneurons causes less gamma-amino butyric acid (GABA) (1) and more glutamate release (2) onto pre-sympathetic neurons. AT_1-receptor stimulation directly on glutamatergic neurons (3) also increases glutamate release onto pre-sympathetic neurons. CSF $[Na^+]$ ↑ may also excite glutamatergic neurons in the MnPO, causing increased release of glutamate in the PVN (4). NO: nitric oxide; IML: intermediolateral cell column; RVLM: rostral ventrolateral medulla; SNA: sympathetic nerve activity; BP: blood pressure; HR: heart rate.

The PVN has been shown to play a essential role in a number of models of chronic sympathetic hyperactivity, such as SHR (Allen 2002, Li and Pan 2007a), water deprived rats (Freeman and Brooks 2007), two-kidney, one-clip (2K-1C) renal hypertensive rats (Rossi et al. 2010) and rats with chronic heart failure (CHF) (Wang et al. 2009). Inhibition of neurons in the PVN with a GABA receptor agonist decreased SNA and BP in normal rats and this effect was enhanced in water deprived (Freeman and Brooks 2007), CHF (Wang et al. 2009) and SHR (Allen 2002). In contrast, the increases in SNA, BP and HR by a GABA receptor blocker in the PVN were attenuated in rats with CHF (Wang et al. 2009), renal-wrapped hypertensive rats (Martin and Haywood 1998) and in SHR (Li and Pan 2007a). Together these findings suggest that there is a decrease in GABA release and GABA receptor activation in the PVN of these rats with chronic sympathetic hyperactivity.

Infusion of a glutamate receptor blocker in the PVN decreased SNA, BP and HR in hypertensive SHR (Li and Pan 2007a), CHF (Li et al. 2003c) and water deprived rats (Freeman and Brooks 2007) but not WKY, SD and water replete rats suggesting that increased glutamatergic input in the PVN contributes to the sympathetic hyperactivity in CHF, water deprived and SHR. mRNA and protein expression of NMDA receptor type 1 (NR_1) as well as the SNA and BP response to an NMDA receptor agonist in the PVN were increased 7 weeks post myocardial infarction (MI) in SD rats and these effects were prevented by oral administration of an AT_1-receptor blocker between 3-7 weeks post MI (Kleiber et al. 2010). *In vitro* application of Ang II on NG108-15 cells (hybrid cell line of rat neuroblastoma and mouse glioma cells expressing both AT_1 and NR_1 receptors) increased NR_1 mRNA and protein expression and this effect was prevented by an AT_1-

receptor blocker (Kleiber et al. 2010). AT_1-receptor mRNA and protein expression were also higher in CHF vs. normal rats (Zhu et al. 2004). These finding support that increased NR_1 subunit expression in the PVN contributes to the increased local glutamatergic input in CHF rats. The increased NR_1 expression in the PVN appears to be mediated by enhanced local AT_1-receptor activation, but no studies have yet assessed whether this effect of Ang II occurs in neurons co-expressing AT_1 and NR_1 receptors. An AT_1-receptor blocker in the PVN had no apparent effects in water replete rats but decreased BP in water deprived rats. The sum of the responses from an AT_1 and glutamate receptor blocker in the PVN of water deprived rats were equivalent to complete blockade with a $GABA_A$ receptor agonist (Freeman and Brooks 2007). Ang II immunoreactivity (Meyer et al. 1990) and AT_1 receptor mRNA expression (Reja et al. 2006) in the PVN were higher in hypertensive SHR compared to control WKY rats supporting that hypertensive SHR have increased angiotensinergic inputs in the PVN. These findings suggest that these models with chronic sympathetic hyperactivity are associated with an increase in excitatory angiotensinergic and glutamatergic inputs, and a decrease in inhibitory GABAergic input in the PVN.

An increase in NO production in the PVN appears to occur in several models of chronic sympathetic hyperactivity. nNOS mRNA and protein expression as well as nNOS activity in the hypothalamus and brain stem was found increased in hypertensive (14 wk old) compared to pre-hypertensive (4 wk old) SHR. No increase in hypothalamic nNOS expression was observed in equally older vs. younger WKY control rats (Plochocka-Zulinska and Krukoff 1997, Qadri et al. 2003). Oral treatment with an ACE or AT_1-receptor blocker, but not a vasodilator normalized the increased NOS activity in

the hypothalamus of hypertensive SHR (Qadri et al. 2003), indicating that the RAS and not a rise in BP mediates these increases in nNOS activity. No studies have yet assessed nNOS expression in the PVN of 2K-1C rats but NO levels in the PVN were elevated by 2-3 fold 35 days post renal clipping (Rossi et al. 2010). Injection of a dominant negative construct (DN) against nNOS in the PVN prevented the increase in local NO and further increased hypertension 35 days post renal clipping in 2K-1C rats (Rossi et al. 2010), suggesting that nNOS-mediated increases in NO in the PVN counteract the sympathetic hyperactivity and hypertension in renovascular hypertension. These studies provide evidence that enhanced nNOS expression and NO production in hypothalamic ie. PVN, and brainstem regions occur in SHR and renovascular hypertension.

Little is known about the balance of excitatory and inhibitory inputs in the PVN of Dahl S rats on high salt diet. High salt diet increased ACE and AT_1-receptor densities in the PVN of Dahl S rats (Wang et al. 2003c) suggesting that dietary salt increases angiotensinergic inputs in the PVN of Dahl S rats. *In situ* hybridization studies showed that on regular salt diet, mRNA expression levels of nNOS in the PVN were the same in Dahl S and R rats but were ~70 % larger in Dahl S and R rats compared to SD rats (Serino et al. 2001). High salt diet (8 % NaCl) for 4 wks caused a ~2 fold larger increase in mRNA levels of nNOS in the PVN of SD and Dahl S vs. Dahl R rats (Serino et al. 2001). However, no studies have yet assessed whether high salt diet causes different increases in nNOS protein abundance and NO activity in the PVN of S and R rats. On regular salt diet, there were no significant differences between nNOS activity or protein expression in the brainstem Dahl R and S rats (Tandai-Hiruma et al. 2005). High salt diet for 4 weeks increased nNOS protein expression and activity in the brainstem of Dahl S

but not R rats (Tandai-Hiruma et al. 2005). Icv injection of a nNOS inhibitor caused larger increases in RSNA, BP and HR in hypertensive Dahl S rats on 4 weeks high salt diet, than in normotensive Dahl S rats on regular salt diet (Tandai-Hiruma et al. 2005). To our knowledge, no studies have yet compared the pressor and sympathetic responses to NOS inhibitors icv, or in the PVN of Dahl S vs. R rats. Chronic icv infusion of aldosterone for 2 weeks decreased nNOS mRNA expression and protein abundance in the PVN of Wistar rats. This effect was prevented by concomitant infusion with an ENaC blocker or Fab fragments (Huang et al. 2011), indicating that a chronic increase in aldosterone in the CNS decreases nNOS expression in the PVN via increased ENaC activation and EO release. In contrast to the effects of high salt diet on nNOS expression in the PVN, one may expect that the increase in aldosterone in the CNS of Dahl S rats on high salt diet (Huang et al. 2009) (*section 1.2.3.2.*) would decrease nNOS expression in the PVN. An NO-GABA negative feedback loop associated with increased local AT_1-receptor stimulation in the PVN (*section 1.3.1*) may counter the effects of aldosterone on nNOS expression in the PVN. However, the increased NO production from this negative feedback loop is not sufficient to fully inhibit the PVN and prevent sympathetic hyperactivity and hypertension. A proposed model in the PVN associated with a chronic increase in CSF $[Na^+]$ will be further described (*section 1.4.1.).*

1.4. Rationale for the study

1.4.1. Proposed model in the PVN associated with dietary salt hypertension in Dahl S

Lesioning the PVN prevents the hypertension from high salt diet in Dahl S rats (*section 1.2.1.2.*). To our knowledge, no studies have yet assessed the mechanisms in the PVN contributing to this salt-induced hypertension. We propose that in Dahl S rats on high salt diet, increases in plasma and CSF [Na^+] activate angiotensinergic neurons in the SFO/OVLT leading to high persistent Ang II release in the PVN via relayed signaling through the MnPO (*section 1.3.2.*). This increase in angiotensinergic input to the PVN inhibits local GABAergic input and as a result enhances glutamatergic input (*section 1.3.2.*). Increases in interstitial [Na^+] in the PVN may further enhance local excitatory pathways via release of Ang II and glutamate (Jin et al. 2001, Qadri et al. 1994). This shift towards excitatory inputs increases firing activity of parvocellular neurons projecting to the IML or RVLM, causing sympathetic hyperactivity and hypertension. These ultra fast and fast reacting pathways appear chronically to be reset at a higher level by a slow neuromodulatory pathway. An increase in CSF and/or interstitial brain [Na^+] activates pathways leading to an increase in aldosterone, presumably by increased local production. No studies have yet assessed the specific nuclei of the brain, cell type(s) or mechanisms mediating this increased production of aldosterone by Na^+. A small increase in CSF [Na^+] may increase neuronal activity in the SFO and OVLT leading to increased aldosterone production in magnocellular neurons of the SON. Aldosterone released in the SON and mPVN may via MR-ENaC increase EO production in magnocellular neurons (*section 1.2.3.1.*). EO released from magnocellular nerve terminals of the SON and/or PVN to glial in the LT or the pPVN may increase Ang II release, and ACE and

AT_1-receptors in the pPVN, enhancing the activity of Na^+-sensitive angiotensinergic sympathetic pathways (*section 1.2.2.2*). An NO-GABA negative feedback loop associated with increased local AT_1-receptor stimulation in the PVN may enhance the expression of local nNOS, increasing NO production in the PVN (*section 1.3.3.*). However, enhanced EO release also increases NADPH oxidase subunits in the PVN, increasing local superoxide production, thereby diminishing the sympathoinhibitory action of tonic NO release in the PVN (*section 1.3.1.*).

In Dahl R and Wistar rats, small increases in plasma Na^+ around periods of salt intake may cause small increases in neuronal activity in CVO's of the LT ie. SFO, OVLT, and this neuronal activity is relayed to the PVN via angiotensinergic connections. Small or region specific increases in Ang II release in the PVN may activate the NO-GABA negative feedback loop, thereby inhibiting the PVN and attenuating increases in sympathetic activity and BP (Li et al. 2006b) (*section 1.3.3.*). This effect of NO would also prevent increases in firing activity in magnocellular of neurons of the SON and PVN, thereby preventing increased EO release in the PVN. Increases in neuronal activity in nuclei of the LT and PVN can also be offset by inhibitory baroreceptor input from the brainstem ie. NTS. A small increase in plasma $[Na^+]$ may activate hepatic and renal osmoreceptors projecting to the NTS (Guyenet 2006), the primary brainstem site at which baroreceptor afferents terminate (Ciriello 1983). Increased baroreceptor input to the NTS activates local GABAergic interneurons, inhibiting activity of local catecholaminergic neurons projecting to the SFO (Tanaka et al. 2002, Tanaka et al. 2001) thereby decreasing neuronal activity in the SFO. Enhanced baroreceptor input in the NTS also increases neuronal activity in neurons projecting to the PVN causing inhibition of pre-

sympathetic neurons projecting to the spinal cord, an effect that appears to depend on activation of GABAergic interneurons in the PVN (Coote 2005, Yang and Coote 2003).

1.5. Hypothesis

1) A short increase in CSF [Na^+] increases AT_1-receptor activation in the PVN thereby raising SNA, BP and HR. An increase in aldosterone in the CSF or directly in the PVN will increase MR-ENaC-EO activity in the PVN, enhancing the increased local AT_1-receptor activation from a rise in CSF [Na^+].

2) Increased AT_1-receptor activation and decreased NO action in the PVN contribute to the elevated SNA and BP in Dahl S rats on high salt diet. The BP response to the increased AT_1-receptor activation in the PVN is mediated by enhanced local glutamate receptor activation. High salt diet in Dahl S rats also activates an MR-ENaC-EO pathway in the PVN, enhancing these effects of increased local AT_1-receptor activation. None of these responses occur in Dahl R rats on high salt diet.

1.6. Objectives

1.6.1. Acute Studies

1) Assess the effects of blockers of MR, ENaC, EO and AT_1-receptors in the PVN of Wistar rats on the BP and HR responses to:
   a) Infusion of Na^+ rich aCSF alone in the PVN, or after local infusion of aldosterone.
   b) Icv infusion of Na^+ rich aCSF alone, or after infusion of aldosterone icv, or in the PVN.
2) Evaluate whether infusion of Na^+ rich aCSF in the PVN causes larger BP and HR responses in Dahl S vs. R and Wistar rats. If so, investigate whether:
   a) Blockers of MR, ENaC, EO and AT_1-receptors prevent these enhanced responses.

   Alternatively:
   b) Evaluate the effects of a NOS blocker on the responses to Na^+ rich aCSF in the PVN of Dahl S vs. R rats.

1.6.2. Chronic Studies

1) Evaluate the effects of a NOS blocker in the PVN on resting BP and HR in Dahl R and S rats on regular or high salt diet.
2) Assess the effects of acute blockade of glutamate receptors or AT_1-receptors in the PVN on resting BP and HR in Dahl S and R rats on regular or high salt intake. To assess the interaction of these mechanisms, infuse one blocker at the peak BP response to the other blocker.

1.7 Outline of approach to problem

The following manuscripts summarize our experimental approach in testing my hypotheses:

1) Manuscript #1: **Gabor A** and Leenen FH. Mechanisms in the PVN mediating local and central sodium-induced hypertension in Wistar rats. *Am J Physiol Regul Integr Comp Physiol* 296: R618-630, 2009 (Gabor and Leenen, 2009).

2) Manuscript #2: **Gabor A** and Leenen FH. Mechanisms mediating sodium-induced pressor responses in the PVN of Dahl rats. *Am J Physiol Regul Integr Comp Physiol* 301: R1338-1349, 2011 (Gabor and Leenen, 2011).

3) Manuscript # 3: **Gabor A** and Leenen FH. Cardiovascular effects of angiotensin II and glutamate in the PVN of Dahl salt-sensitive rats. *Brain Research* 1447: 28-37, 2012 (Gabor and Leenen, 2012).

Table 1-1: Drugs and treatments utilized in Manuscripts #1-3 and their primary action in the CNS.

Drug/treatment	Primary action
Aldosterone	MR agonist
Ouabain (exogenous)	Na^+/K^+/ATPase blocker
Ang II	AT_1-receptor agonist
L-NAME	NOS blocker
Eplerenone	MR blocker
Benzamil	ENaC blocker
EO Fab fragments	Bind EO
Losartan	AT_1-receptor blocker
Telmisartan	AT_1-receptor blocker
Candesartan	AT_1-receptor blocker
Kynurenate	Glutamate receptor blocker

2. MANUSCRIPT #1- MECHANISMS IN THE PVN MEDIATING LOCAL AND CENTRAL SODIUM-INDUCED HYPERTENSION IN WISTAR RATS

Alexander Gabor[a] and Frans H. H. Leenen[a]*

[a]Hypertension Unit, University of Ottawa Heart Institute,
Ottawa, Ontario, Canada.

*Thesis Supervisor

SHORT TITLE

The PVN and Central Sodium

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ABSTRACT

Sympatho-excitatory and hypertensive responses to central infusion of Na^+ rich aCSF are enhanced by aldosterone and mediated by mineralocorticoid receptors (MR) and benzamil blockable Na^+ influx leading to "ouabain" release and AT_1-receptor stimulation. The present study evaluated the functional role of these mechanisms in the paraventricular nucleus (PVN). In conscious Wistar rats, Na^+ rich aCSF was infused either directly into the PVN or intracerebroventricularly (icv) preceded by aldosterone and blockers. Infusion of Na^+ rich aCSF in the PVN caused gradual increases in BP and HR. Aldosterone and a subpressor dose of ouabain in the PVN alone did not affect BP and HR, but enhanced responses to Na^+. Eplerenone, benzamil and "ouabain"-binding fab fragments only blocked the enhancement by aldosterone, whereas losartan blocked all responses to Na^+ rich aCSF in the PVN. Increases in BP and HR by icv infusion of Na^+ rich aCSF were enhanced by aldosterone infused icv, but not in the PVN. Telmisartan in the PVN blocked again all responses. In contrast, both eplerenone and benzamil in the PVN did not change the pressor responses to icv infusion of aldosterone and Na^+ rich aCSF. These findings indicate that AT_1-receptors in the PVN mediate the responses to Na^+ rich aCSF, and their enhancement by aldosterone both locally in the PVN or in the general CSF. MR, benzamil blockable Na^+ channels or transporters and "ouabain" can be functionally active in the PVN, but in Wistar rats appear not to contribute to the pressor responses to short-term increases in CSF [Na^+].

INTRODUCTION

Intra-cerebroventricular (icv) infusion of Na^+ rich artificial cerebrospinal fluid (aCSF) activates central nervous system (CNS) pathways leading to sympathetic hyperactivity and hypertension, and these responses can be enhanced by icv infusion of aldosterone (Wang et al. 2003a). Icv infusion of fab fragments that specifically bind ouabain-like compounds ("ouabain") (Pullen et al. 2004) blocks these responses whereas icv infusion of spironolactone or benzamil inhibits increases in brain "ouabain" and hypertension in response to chronic icv infusion of Na^+ rich aCSF (Huang et al. 2006b, Wang et al. 2003a, Wang and Leenen 2003). These findings suggest that mineralocorticoid receptors (MR) and benzamil blockable Na^+ channels or transporters in the CNS mediate release of "ouabain" and the hypertension caused by a chronic increase in CSF [Na^+]. The sympatho-excitatory and hypertensive responses to icv infusion of Na^+ rich aCSF or ouabain can also be inhibited by icv losartan (Huang and Leenen 1996c). Altogether, these results suggest that in response to a chronic increase in CSF [Na^+], increased binding of an endogenous agonist (presumably aldosterone) (Huang et al. 2008a) to MR mediates activation of benzamil blockable Na^+ influx leading to "ouabain" release and AT_1-receptor stimulation. Where and how in the CNS these mechanisms interact, has not yet been studied.

Neurons in nuclei of the lamina terminalis and their efferent neural pathways to the paraventricular nucleus (PVN) are essential in coordinating the homeostatic responses to changes in CSF [Na^+] (Buggy et al. 1984, Goto et al. 1982). Located within close proximity to the ependyma of the 3rd ventricle, Na^+ sensitive neurons of the lamina terminalis may sense changes in CSF [Na^+] and the increased neuronal activity can be

relayed to the PVN. PVN neurons may also sense changes in CSF [Na^+] directly through dendritic branches exposed to the CSF near the ependymal layer of the third ventricle (Korf et al. 1983, Qadri et al. 1998, Vigh-Teichmann and Vigh 1983). Infusion of hypertonic saline in the PVN of normotensive rats causes significant increases in BP, HR and plasma arginine vasopressin (AVP) levels (Jin et al. 2001, Qadri et al. 1998). To what extent sodium sensing by the PVN contributes to the sympathoexcitatory and pressor responses to CSF [Na^+] increases has not yet been assessed, neither a possible role for a MR activated pathway in the PVN. MR and 11ß-HSD-2 activity determining selectivity of MR for aldosterone have been demonstrated in the PVN (Zhang et al. 2006). We reported expression and immunoreactivity for epithelial sodium channels (ENaC) in the PVN, mainly magnocellular neurons (Amin et al. 2005). Immunohistochemical studies also showed dense "ouabain" immunoreactivity throughout the PVN, predominantly in magnocellular neuronal somata and axons closely associated with parvocellular neurons (Yamada et al. 1992a, Yamada et al. 1992b). Angiotensinergic fibers and cell bodies, and AT_1-receptors are also present in the PVN (Lenkei et al. 1998, Lind et al. 1985b). Microinjections of angiotensin II (Ang II) or ouabain in the PVN cause increases in BP and HR (Jones and Lo 1990, Zhu et al. 2002). AT_1-receptor stimulation in the PVN may excite subpopulations of PVN neurons innervating presympathetic neurons in the rostral ventrolateral medulla (RVLM) thereby increasing sympathetic activity, BP and HR (Cato and Toney 2005).

We hypothesized firstly that the mechanisms mediating pressor responses caused by an interstitial [Na^+] increase in the PVN would be analogous to the mechanisms involved in CSF sodium-induced hypertension, specifically MR, benzamil blockable Na^+

influx and “ouabain” release leading to enhanced AT_1-receptor stimulation and secondly, that these mechanisms in the PVN contribute to the pressor responses to icv infusion of Na^+ rich aCSF. Accordingly, we assessed the role of different neuromodulators (aldosterone, ouabain) and neurotransmitters (Ang II) in the PVN in the hemodynamic responses to direct infusion into the PVN or icv infusion of Na^+ rich aCSF. Various blockers were micro-injected/infused to inhibit steps in the proposed pathway responding to an increase in $[Na^+]$.

MATERIALS AND METHODS

Animals

Male Wistar rats (250-350 g) were obtained from Charles River Breeding Laboratories, Montreal, Quebec, Canada. The rats were provided with a standard commercial rat chow and water ad libitum. All experiments were carried out according to the guidelines of the Canadian Council on Animal Care, which conform to NIH guidelines and was approved by the University of Ottawa Animal Care and Use Committee. Experiments were conducted after animals were acclimatized for 1 week.

Surgical Procedures

For all surgeries, rats were anesthetized with 2% isoflurane in oxygen. Animals were placed in a stereotaxic head frame, and the skull was leveled between bregma and lambda. For experiments involving infusion of Na^+ rich aCSF in the PVN, a section of the skull was removed, and a guide cannula (23 gauge) was positioned unilaterally 0.5 mm above the PVN according to the rat atlas of Paxinos and Watson (Paxinos and Watson 1998), 1.8 mm posterior to bregma, 0.4 mm lateral to the bregma and 7.9 mm ventral from the skull. For combined icv and PVN experiments, guide cannulas were positioned bilaterally 0.5 mm above the PVN and unilaterally above the left lateral cerebral ventricle at 0.4 mm caudal to bregma, 1.4 mm lateral to bregma and 3.5 mm ventral to dura (Paxinos and Watson 1998). Guide cannulas were secured to the skull with two jeweler's screws and acrylic cement and closed with stainless steel obturators. After 1 week recovery, the left femoral artery was cannulated with PE-50/10 polyethylene tubing filled with heparin (1,000 U/ml in 0.9% NaCl). For withdrawal of

CSF, on the day of the experiment, rats were placed in the stereotaxic head frame and a cannula was inserted into the cisterna magna, as described previously (Huang et al. 2004).

Experimental Procedures

The morning after placement of the arterial line, rats were placed in a small cage and intra-arterial catheters were connected to a pressure transducer for recordings of BP and HR via a PC equipped with software AcqKnowledge (ACQ 3.2). Systolic and diastolic pressures were determined from the raw BP signal and mean arterial pressure (MAP) was determined by adding one third of the pulse pressure to the diastolic pressure.

An "L"-shaped injection cannula (30 gauge) was lowered into the lateral ventricle or PVN through the fixed guide cannula and extended 0.5 mm past the guide. For PVN and icv infusions, injection cannulas were connected by polyethylene tubing to 10 or 500 µL Hamilton microsyringes mounted on a Harvard infusion pump (model #2400-003). Micro-injections of 100 nL were conducted for 3-5 s using a 1 uL Hamilton microsyringe. For all experiments, animals were allowed to settle and baseline levels were recorded after 20 min of stabilization and after different pretreatments before infusion of Na^+ rich aCSF. None of the pretreatments caused persistent changes in BP or HR (Table 2-1). At the end of the experiment, rats were euthanized with carbon dioxide and injected with 100 nL Evans Blue dye. Brains were removed, fixed in 2-methyl-butane at -20^{o}C, sectioned (30 µm) using a Leica cryostat and stained with neutral red. The extent of tissue damage from the cannula in the PVN was limited to the diameter of the circular injection cannula lowered into the PVN and was similar after 5 and 15 min infusion at 300 nL/min (Figure 2-1A). Micro-injection sites marked with Evans Blue were identified with a light microscope, and only data of rats whose micro-injection sites

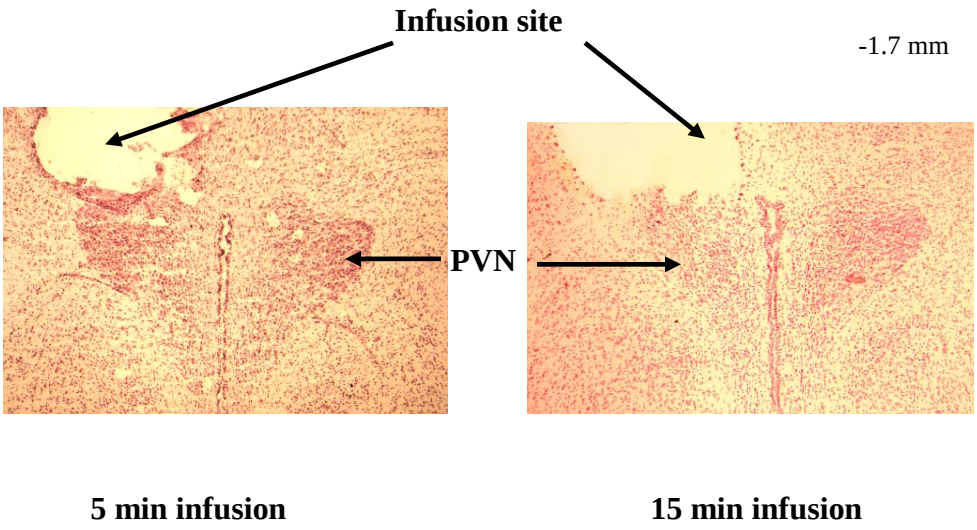
were within the lateral ventricle and the PVN were used for analysis. Correct injection sites ranged from 70-90 % for groups with unilateral infusions into the PVN and from 50-70 % for groups with combined icv and bilateral PVN infusions. To evaluate the distribution of infusions of aCSF in the PVN, Evans Blue dye (1%) dissolved in vehicle was infused at 300 nL/min (rate of Na^+ rich aCSF infusion) for 5 and 10 min. Evans Blue dye was found localized within the PVN region after 5 and 10 min infusions (Figure 2-1B).

Table 2-1: MAP and HR levels at rest and after pretreatments into the PVN or into the lateral ventricle before the start of infusion of Na^+ rich aCSF.

		First Baseline		Second Baseline After Pretreatments	
	n	MAP, mmHg	HR, beats/min	MAP, mmHg	HR, beats/min
Levels at rest and after pretreatment into the PVN					
Vehicles	15	108±2	402±3	110±2	404±4
Intravenous AVP antagonist	4	98±4	392±6	96±3	386±7
Aldosterone	15	103±2	409±4	105±2	413±4
Aldosterone + eplerenone	4	111±3	415±6	114±4	422±7
Aldosterone + benzamil	11	110±2	420±4	113±2	413±5
Aldosterone + Fab fragments	10	108±3	405±5	110±2	409±4
Losartan	11	108±2	409±4	110±2	416±5
Aldosterone + losartan	9	104±2	415±3	105±2	420±4
Subpressor dose of ouabain	5	106±2	406±5	109±3	413±6
Levels at rest and after pretreatment into the lateral ventricle					
Intracerebroventricular vehicles + vehicle in the PVN	9	102±2	398±4	104±2	404±5
Intracerebroventricular aldosterone + vehicle in the PVN	5	104±3	413±5	108±4	423±6
Intracerebroventricular aldosterone + eplerenone in the PVN	5	109±2	409±5	113±3	406±7
Intracerebroventricular aldosterone + benzamil in the PVN	5	112±2	426±7	114±3	433±6
Intracerebroventricular aldosterone + telmisartan in the PVN	5	103±3	421±5	108±4	417±5

Values are mean ± SEM

A



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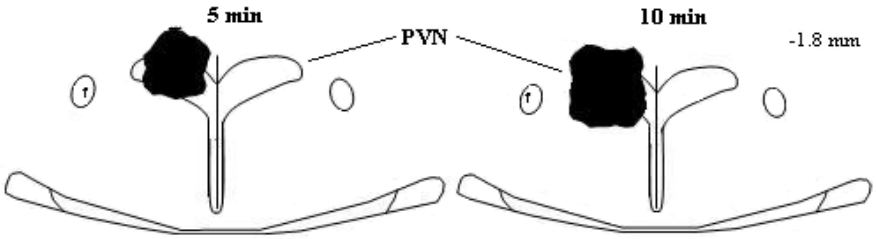


Figure 2-1: A) Representative photographs indicating the extent of damage after unilateral infusion of Na^+ rich aCSF (0.7 M Na^+) at 300 nL/min for 5 or 15 min into the PVN of 2 individual rats. B) Representative schematic representation of the distribution of Evans Blue dye after unilateral infusion of 300nL/min for 5 or 10 min in the PVN.

Experimental Protocols

Vehicles

Artificial cerebrospinal fluid (aCSF: 121 NaCl, 3.4 KCl, 1.2 $MgCl_2$, 0.6 NaH_2PO_4, 29 $NaHCO_3$, and 3.4 glucose mmol/L, pH 7.4, and osmolarity 296 mosmol/kg H_2O) was used as vehicle, except for aldosterone (ethanol and aCSF: 2:98 ratio), eplerenone (acetonitrile and aCSF: 0.1:99.9 ratio), and benzamil (propylene glycol and aCSF: 15:85 ratio). All injected compounds were purchased from Sigma or Steraloids Inc.

Experimental Protocols in the PVN

Na^+ rich aCSF in the PVN: To evaluate the responses to Na^+ rich aCSF in the PVN, Na^+ rich aCSF at 0.3 and 0.7 M Na^+ was infused into the PVN for 15 min at 300 nL/min. Na^+ rich aCSF was prepared by adjusting the [Na^+] of aCSF with additional NaCl.

Aldosterone, Eplerenone and Na^+ rich aCSF in the PVN: To evaluate whether aldosterone enhances responses to Na^+ rich aCSF in the PVN, aldosterone was infused into the PVN at 100 and 300 ng/300 nL/min for 5 min followed by 45 min recovery period to allow for activation of the early genomic effects of aldosterone (Bhargava et al. 2001, McCormick et al. 2005) and then infusion of Na^+ rich aCSF (0.3 and 0.7 M Na^+). Doses of aldosterone were based on preliminary dose response studies (data not shown). The higher dose of aldosterone was utilized for further study as limited enhancement of the responses to Na^+ rich aCSF was observed by the lower dose. To evaluate whether effects of aldosterone are mediated by MR activation, eplerenone (40 ng/300 nL/min) or its vehicle (0.1% ethanol) were infused into the PVN for 5 min followed by aldosterone infusion, 45 min recovery period and Na^+ rich aCSF (0.7 M). To our knowledge, no previous studies infused eplerenone into a brain nucleus. Based on the dose of spironolactone administered in the posterior paraventricular thalamus (Jaferi and Bhatnagar 2006) and comparing pharmacological characteristics of eplerenone and spironolactone, doses of eplerenone were generated for the PVN and assessed in preliminary dose response studies (data not shown).

To evaluate responses to aldosterone alone, this dose of aldosterone was infused into the PVN for 5 min. To assess for possible volume and osmolar effects, aldosterone (1.5 μg) or its vehicle were infused into the PVN as above followed by infusion of aCSF

(0.15 M Na^+) or of mannitol (254.3 g/L) - osmolality equivalent to 0.7 M Na^+ aCSF - at 300 nL/min for 15 min.

Aldosterone, benzamil, fab fragments and Na^+ rich aCSF in the PVN: To assess whether the effect of aldosterone in enhancing responses to Na^+ rich aCSF is mediated by benzamil blockable Na^+ channels or transporters, aldosterone was infused into the PVN at 300 ng/300 nL/min for 5 min followed by 45 min recovery period. Micro-injections of benzamil (100 ng) or vehicle were conducted prior to infusion of Na^+ rich aCSF (0.7 M Na^+) at 300 nL/min for 15 min. In an additional group of rats, benzamil (100 ng) was micro-injected prior to infusion of Na^+ rich aCSF (0.3 and 0.7 M Na^+) which also contained benzamil (50 ng/300 nL/min). The benzamil dose was based on preliminary dose ranging studies to block the enhanced response to Na^+ rich aCSF caused by aldosterone in the PVN.

To evaluate the role of endogenous "ouabain" in the response to aldosterone and Na^+ rich aCSF in the PVN, aldosterone was infused into the PVN at 300 ng/300 nL/min for 5 min followed by 45 min recovery. Ouabain-binding Fab fragments (Digibind) (Pullen et al. 2004) or its vehicle were then infused at 2.9 μg/300 nL/min for 3.5 min prior to infusion of Na^+ rich aCSF (0.3 and 0.7 M Na^+) at 300 nL/min for 15 min. The dose of fab fragments was adapted from our previous study showing blockade of the responses to icv ouabain or Na^+ rich aCSF (Huang and Leenen 1996c).

Ouabain, Ang II, and Losartan dose response studies: To evaluate the effect of ouabain and Ang II in the PVN, rats were micro-injected with vehicle or ouabain at increasing doses (20,40,80 ng) or Ang II (30,90,300 ng); doses were selected from previous studies (Jones and Lo 1990, Zhu et al. 2002). Micro-injections were performed at 30 min

intervals. To assess the dose of losartan needed to block Ang II responses, rats were first micro-injected with 23 μg losartan (Zhu et al. 2002). This caused limited blockade to 90 ng Ang II (data not shown). Subsequently, losartan was first micro-injected (20 μg), then infused (3 μg/min) for 20 min prior to Ang II which caused substantial blockade. To assess the role of AT_1-receptor activation in the response to ouabain in the PVN, losartan (20 μg) or its vehicle was first micro-injected into the PVN and then infused at 2 μg/250 nL/min for 30 min. Ouabain (80 ng) or its vehicle was then micro-injected.

Aldosterone, losartan and Na^+ rich aCSF in the PVN: To evaluate the role of AT_1-receptor activation in the responses to Na^+ rich aCSF and their enhancement by aldosterone, aldosterone or its vehicle was infused into the PVN at 300 ng/300 nL/min for 5 min. Following 45 min recovery, losartan (20 μg) or its vehicle was micro-injected into the PVN and then infused at 2 μg/250 nL/min for 30 min prior to infusion of Na^+ rich aCSF (0.3 and 0.7 M Na^+) at 300 nL/min for 15 min.

Subpressor dose of ouabain and Na^+ rich aCSF in the PVN: To evaluate whether infusion of a subpressor dose of ouabain in the PVN enhances the BP and HR responses to local infusion of Na^+ rich aCSF, ouabain or its vehicle (aCSF) was first infused into the PVN at 2 ng/300 nl/min for 5 min. This dose of ouabain was obtained from preliminary dose response studies (data not shown) and did not generate significant increases in BP and HR or cause changes in rat behavior. After 3-4 min rest, Na^+ rich aCSF (0.7 M Na^+) was infused into the PVN at 300 nL/min for 15 min. In another group of rats, the subpressor dose of ouabain was infused into the PVN prior to infusion of aCSF at 300 nL/min for 15 min.

Vasopressin release and Na^+ Rich aCSF alone or combined with aldosterone in the PVN: To evaluate the role of vasopressin in the BP responses to Na^+ rich aCSF alone, or after aldosterone, aldosterone vehicle or aldosterone (1.5 μg) were infused at 300 nL/min for 5 min followed by 40 min recovery. The arginine vasopressin antagonist [ß-mercapto-ß, ß-cyclopentamethylenepropionyl^1,*O*-Me-Tyr^2,Arg^8] vasopressin (30 μg/kg) dissolved in 0.3 mL saline (0.145 M Na^+), or its vehicle was then injected iv and after 5 min, Na^+ rich aCSF (0.7 M Na^+) was infused at 300 nL/min for 15 min into the PVN. The dose of the vasopressin antagonist was chosen from our previous study (Wang et al. 2003a).

Combined icv and PVN Experimental Protocols

Icv infusion of Na^+ rich aCSF preceded by aldosterone icv or in the PVN: To assess whether aldosterone not only icv but also in the PVN enhances the pressor responses to a CSF [Na^+] increase, aldosterone or its vehicle was infused bilaterally into the PVN at 300 ng/300 nL/min for 5 min followed by 45 min recovery period for activation of the early genomic effects of aldosterone (Bhargava et al. 2001, McCormick et al. 2005). The dose of aldosterone delivered to each side of the PVN enhanced the pressor responses to unilateral infusion of Na^+ rich aCSF in the PVN. In another group of rats, aldosterone or its vehicle was infused icv at 5 ng/3.8 ul/min for 2 hr to confirm that this treatment enhances the pressor responses to icv infusion of Na^+ rich aCSF (Wang et al. 2003a). After aldosterone was infused icv or in the PVN, icv infusion of Na^+ rich aCSF (0.3 or 0.45 M Na^+) at 3.8 uL/min for 20 min was performed according to our previous study (Wang et al. 2003a).

Icv infusion of Na^+ rich aCSF preceded by aldosterone icv and eplerenone or benzamil in the PVN: To assess the role of MR in the PVN in the pressor responses to icv infusion of Na^+ rich aCSF and their enhancement by icv infusion of aldosterone, eplerenone or its vehicle was infused bilaterally at 13.3 ng/ 300 nL/min for 15 min into the PVN. Subsequently, icv infusion of aldosterone was performed at 5 ng/3.8 ul/min for 2 hr followed by icv infusion of Na^+ rich aCSF (0.3 M Na^+) at 3.8 ul/min for 20 min. The dose of eplerenone in the PVN blocked the enhancement by aldosterone of the pressor responses to Na^+ rich aCSF in the PVN.

To assess the role of benzamil blockable Na^+ channels or transporters in the PVN in response to icv infusion of Na^+ rich aCSF after icv infusion of aldosterone, aldosterone was infused icv at 5 ng/3.8 ul/min for 2 hr. Benzamil was then infused bilaterally into the PVN at 50 ng/300 nL/min for 5 min. This dose of benzamil was within the range of doses used to inhibit the enhancement by aldosterone of the pressor responses to Na^+ rich aCSF in the PVN. Icv infusion of Na^+ rich aCSF (0.3 M Na^+) was then performed at 3.8 uL/min for 20 min.

Icv infusion of Na^+ rich aCSF preceded by aldosterone icv and telmisartan in the PVN: To assess the role of AT_1-receptors in the PVN in the pressor responses to icv infusion of Na^+ rich aCSF, the AT_1-receptor blocker telmisartan or its vehicle was infused bilaterally at 2 μg/300 nL/min for 15 min into the PVN. This dose of telmisartan blocks the pressor responses to local micro-injection of 100 ng Ang II (data not shown). Icv infusion of Na^+ rich aCSF (0.3 M Na^+) was then performed at 3.8 uL/min for 20 min. Telmisartan was used for its longer duration of action and higher binding affinity than losartan (Kakuta et al. 2005). In a subsequent group, icv infusion of aldosterone was performed at 5 ng/3.8

ul/min for 2 hr. Telmisartan was then infused bilaterally into the PVN followed by icv infusion of Na^+ rich aCSF, using the same doses as above.

CSF [Na^+] during icv infusion of Na^+ rich aCSF: To assess the effects of icv infusion of Na^+ rich aCSF on the CSF [Na^+], following cisterna magna cannulation, under mild isoflurane anesthesia, infusion of aCSF or Na^+ rich aCSF (0.3 M Na^+) at 3.8 ul/min was performed and 100–200 µl CSF samples were collected (<5 µl/s) from the cisterna magna at 20 or 60 min after start of infusion. Only one time point and infusion treatment was assessed in each rat. A Na^+ sensing electrode (Microelectrodes model #MI-425) was used to measure CSF [Na^+] in 100 ul samples. Standard curves were generated from standard solutions made from aCSF and varying amounts of NaCl.

Statistical Analysis

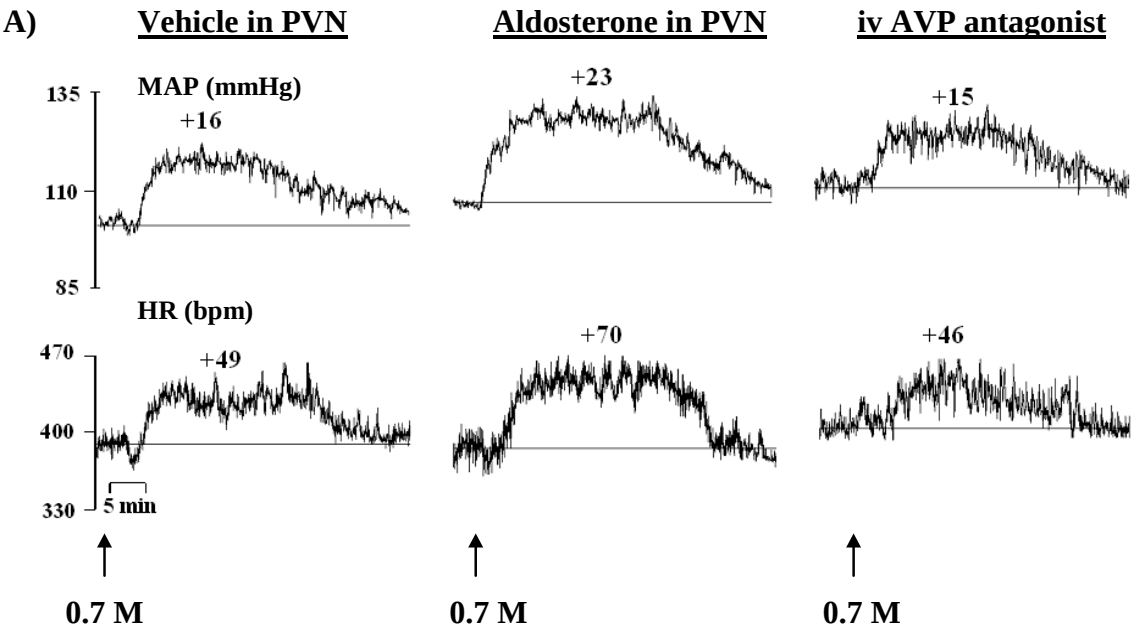
Values are expressed as means ± SE. The following statistical tests were used: 1) For comparisons among treatments, a one-way analysis of variance (ANOVA) was used followed by a Student-Newman-Keuls post hoc multiple comparison. 2) For comparisons of treatments against control, a one-way ANOVA followed by Dunnett's test was used. 3) For pairwise comparisons of the area under the curve (AUC), t-tests were used with Bonferroni adjustments for multiple comparisons. 4) For testing peak changes from baseline, paired t-tests were used. For multiple changes from baseline, a one-way ANOVA with repeated measured followed by Dunnett's test was used. The level of significance was set at $p<0.05$.

RESULTS

Studies in the PVN

Aldosterone, Eplerenone and Na^+ rich aCSF: Infusion of aCSF after either vehicle or aldosterone caused no significant changes in MAP and HR (MAP; 0±2 mmHg, HR; 0±10 bpm; and MAP; 1±1 mmHg, HR; -10±10 bpm). Mannitol (1396 mosmol/kgH_2O) after aldosterone also did not change MAP (1±2 mmHg) and HR (3±3 bpm).

Na^+ rich aCSF caused significant increases in MAP (0.3 M, 5±1 mmHg; 0.7 M, 13±1 mmHg) and HR (0.3 M, 27±5; 0.7 M, 43±6 bpm). MAP increased within 3-7 min, but HR decreased for the first few min, and then increased to levels significantly higher than resting level. Peak increases in MAP and HR occurred 11-13 min after start of Na^+ rich aCSF infusion and both had returned to baseline 35 min after end of infusion (Figure 2-2). Na^+ rich aCSF infusion at sites outside the PVN caused only minor non-significant increases in MAP (0.3 M, 2±1 mmHg; 0.7 M, 4±3 mmHg) and HR (0.3 M, 11±5; 0.7 M, 17±12 bpm).



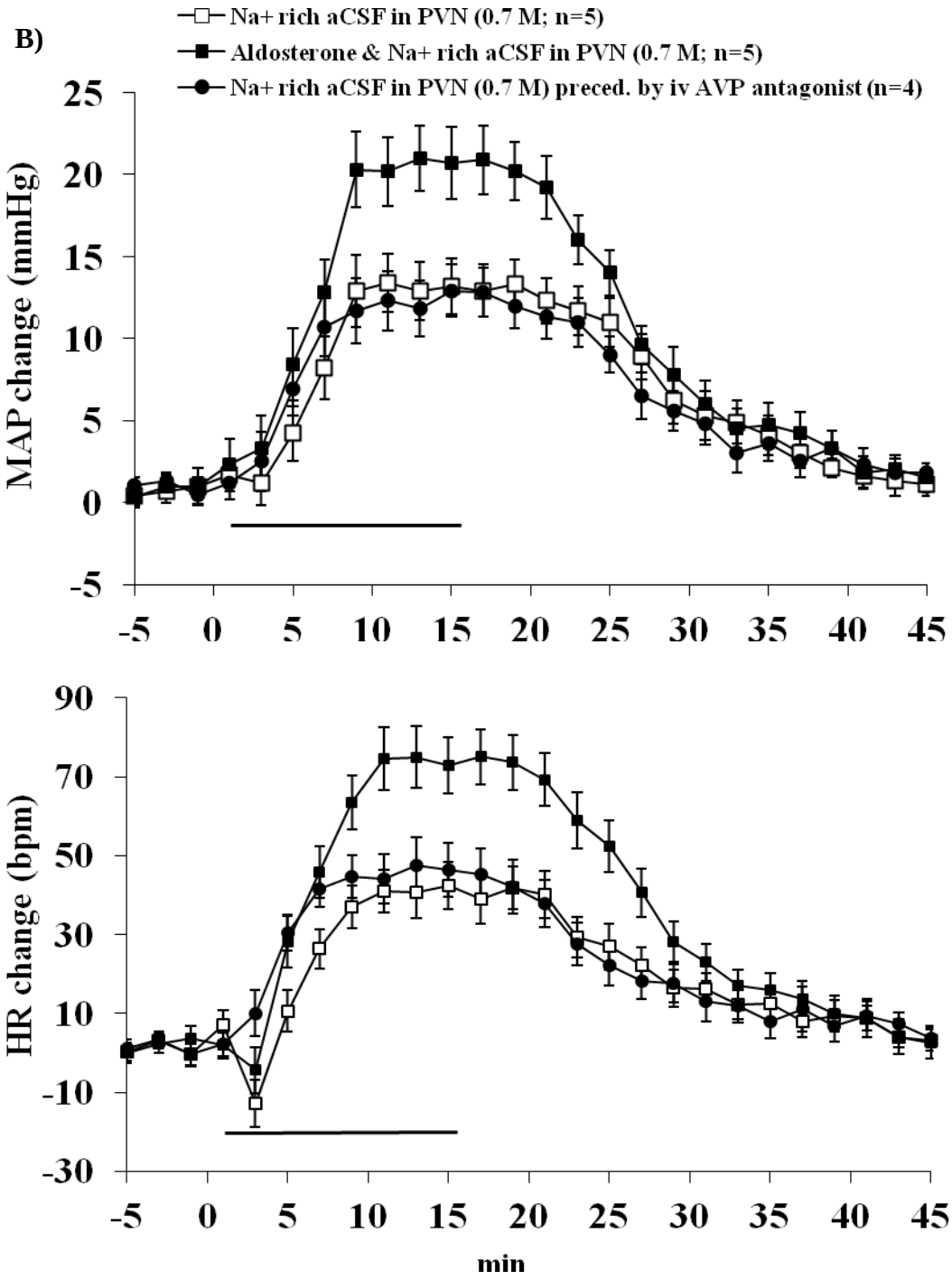


Figure 2-2: Effects of aldosterone in the PVN or of iv AVP antagonist on MAP and HR responses to infusion of Na^+ rich aCSF at 0.7 M Na^+ in the PVN. Results are presented as time tracings from one individual rat per treatment (A) or as mean ± SEM of group changes (B). By one-way ANOVA with repeated measures, Na^+ rich aCSF alone caused significant increases in MAP (7-31 min) and HR (5-31 min) and after aldosterone significant increases in MAP from 5-37 min and HR from 5-35 min. AVP antagonist did not affect pressor responses to Na^+ rich aCSF and prevented the initial decrease in HR ($p<0.05$). By t-test analysis of the AUC, aldosterone significantly enhanced the MAP and HR responses to Na^+ rich aCSF. Black line represents infusion period.

Infusion of aldosterone caused no significant changes in MAP (0±1 mmHg) and HR (10±1 bpm). After pretreatment with aldosterone, MAP and HR responses to Na^+ rich aCSF at 0.3 M Na^+ were significantly increased while there were nearly two-fold increases in both MAP and HR responses to Na^+ rich aCSF at 0.7 M Na^+ (Figure 2-2 and 2-3). The enhancement by aldosterone of the responses to Na^+ rich aCSF at 0.7 M Na^+ was blocked by eplerenone (Figure 2-3).

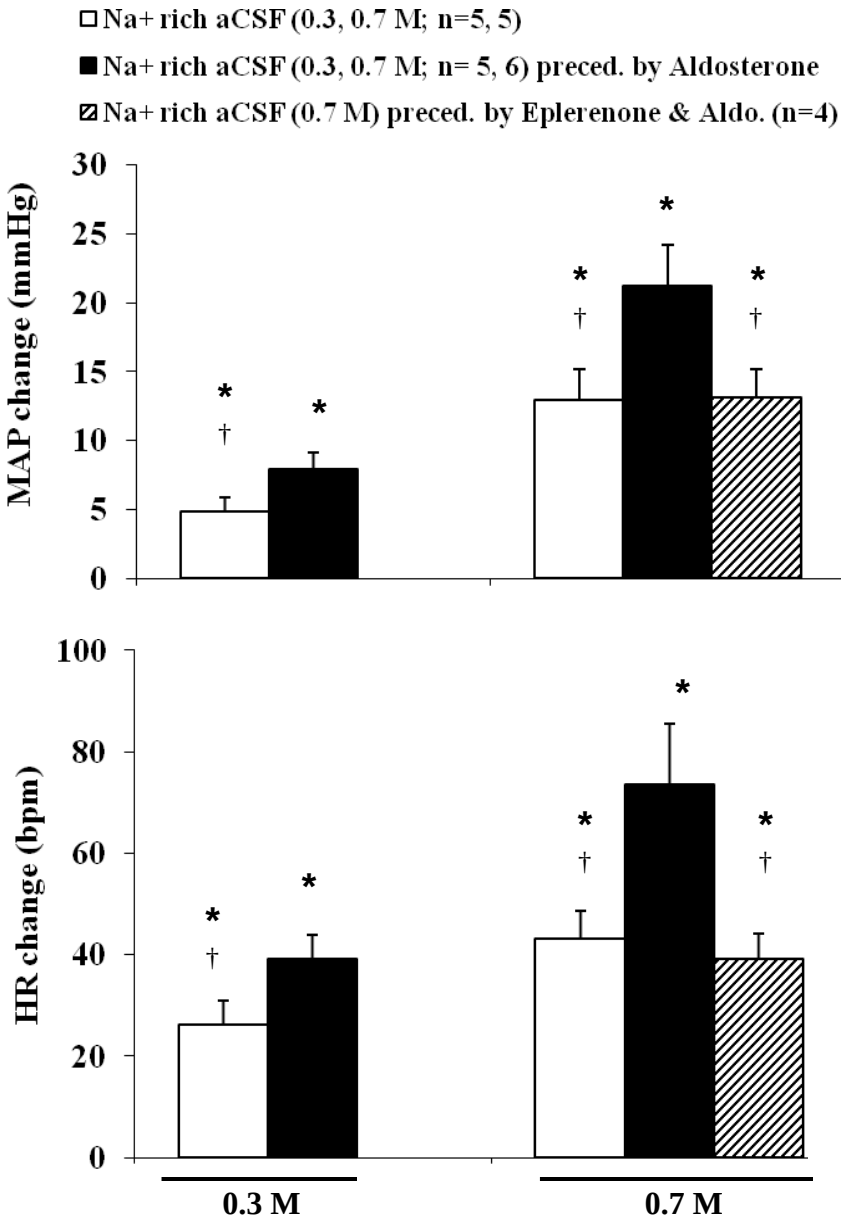
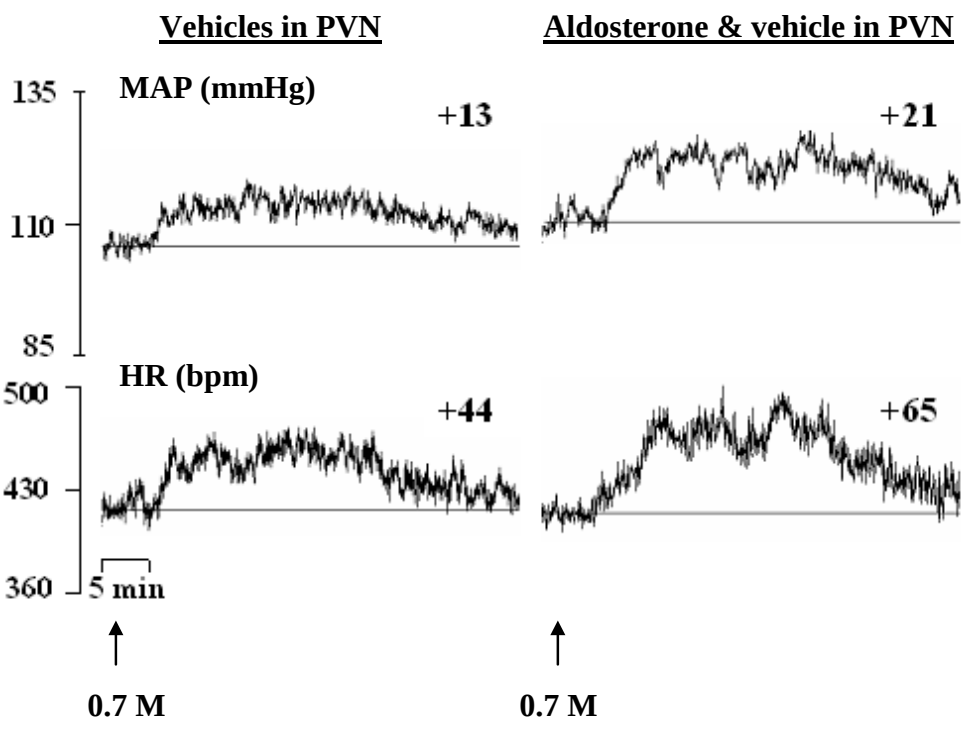
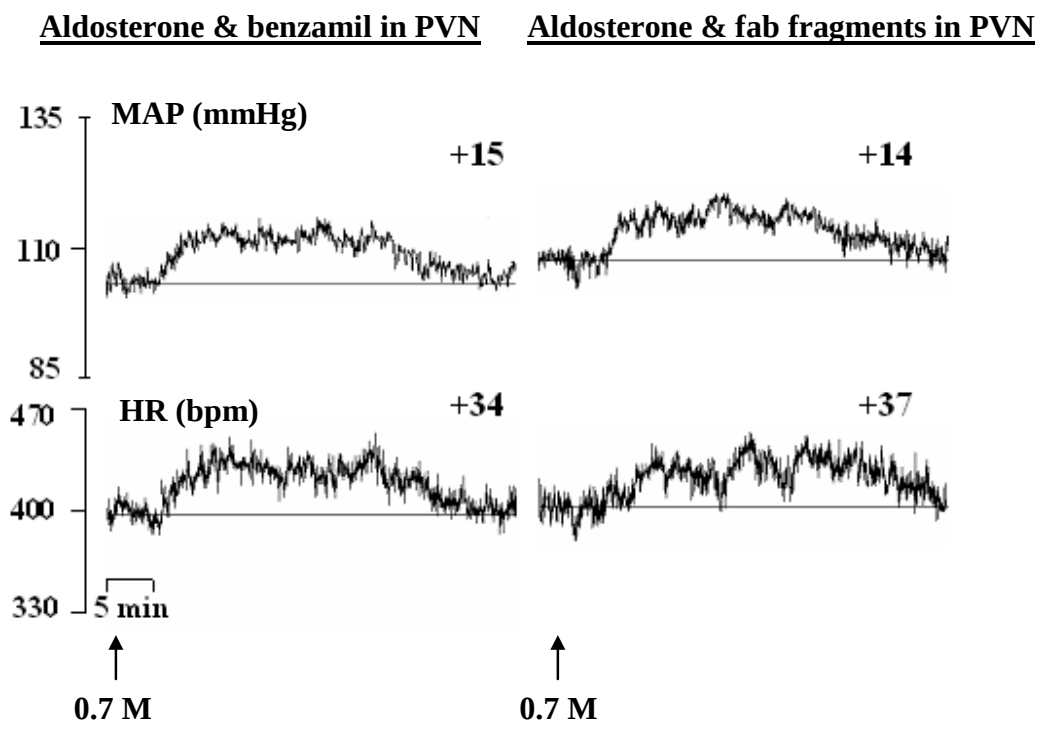


Figure 2-3: Effects of eplerenone and aldosterone in the PVN on peak MAP and HR responses to infusion of Na^+ rich aCSF (0.3 and 0.7 M Na^+) in the PVN. Values are mean ± SEM; * $p<0.05$ vs. baseline, † $p<0.05$ vs. aldosterone + Na^+ rich aCSF.

Aldosterone, benzamil, fab Fragments and Na^+ rich aCSF: Both benzamil and fab fragments fully blocked the enhancement by aldosterone of the responses to Na^+ rich aCSF (Figure 2-4). Benzamil produced similar effects when it was administered by micro-injection alone or by micro-injection followed by infusion (only the latter data shown). In groups combining aldosterone with benzamil or fab fragments, increases in MAP and HR by Na^+ rich aCSF were similar to those by Na^+ rich aCSF without pretreatment with aldosterone (Figure 2-4).

A)


Vehicles in PVN
Aldosterone & vehicle in PVN
MAP (mmHg)
135
110
85
+13
+21
HR (bpm)
500
430
360
+44
+65
5 min
0.7 M
0.7 M


Aldosterone & benzamil in PVN
Aldosterone & fab fragments in PVN
MAP (mmHg)
135
110
85
+15
+14
HR (bpm)
470
400
330
+34
+37
5 min
0.7 M
0.7 M

B)

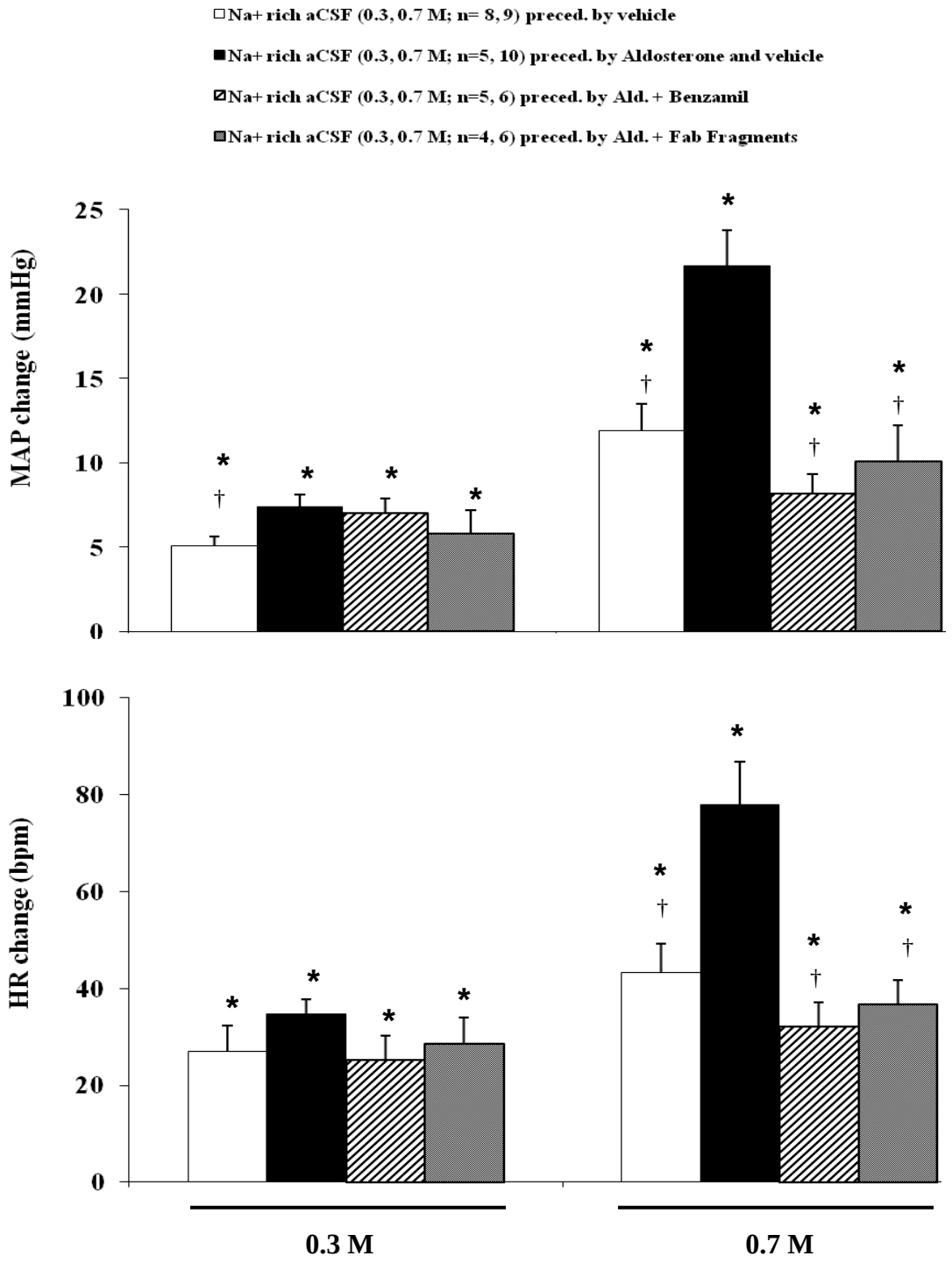


Figure 2-4: Effects of benzamil and fab fragments in the PVN on peak MAP and HR responses to infusion of Na^+ rich aCSF (0.3 and 0.7 M Na^+) in the PVN, either alone or after pretreatment with aldosterone. Results are presented as time tracings from one individual rat per treatment (A) or as mean ± SEM of group changes (B); * $p<0.05$ vs. baseline, † $p<0.05$ vs. aldosterone + vehicle.

Ouabain, Ang II, and Losartan dose response studies: Micro-injection of Ang II and ouabain in the PVN caused dose related increases in MAP and HR (Table 2-2). For Ang II, increases in MAP and HR occurred within 30 s and peak increases within 1-2 min after start of injection. For ouabain, increases in MAP and HR occurred at 2-3 min with peak increases at 6-8 min after injection. Ouabain micro-injected outside the PVN did not change MAP (3±2 mmHg) or HR (10±7 bpm).

Losartan in the PVN fully blocked the MAP and HR responses to both Ang II and ouabain (Table 2-2).

Table 2-2: Effects of losartan on peak BP and HR responses to micro-injection of Ang II (A) or ouabain (B) in the PVN.

A

	aCSF	Ang II (30 ng)	Ang II (90 ng)	Ang II (300ng)	Losartan (80 ug) + Ang II (90 ng)
	(n=5)	(n=6)	(n=6)	(n=6)	(n=4)
Δ MAP	3 ±1	14 ±1*	15 ±1*	31 ±4*	2 ±4
Δ HR	3 ±2	8 ±3	24 ±4*	44 ±3*	-2 ±5

B

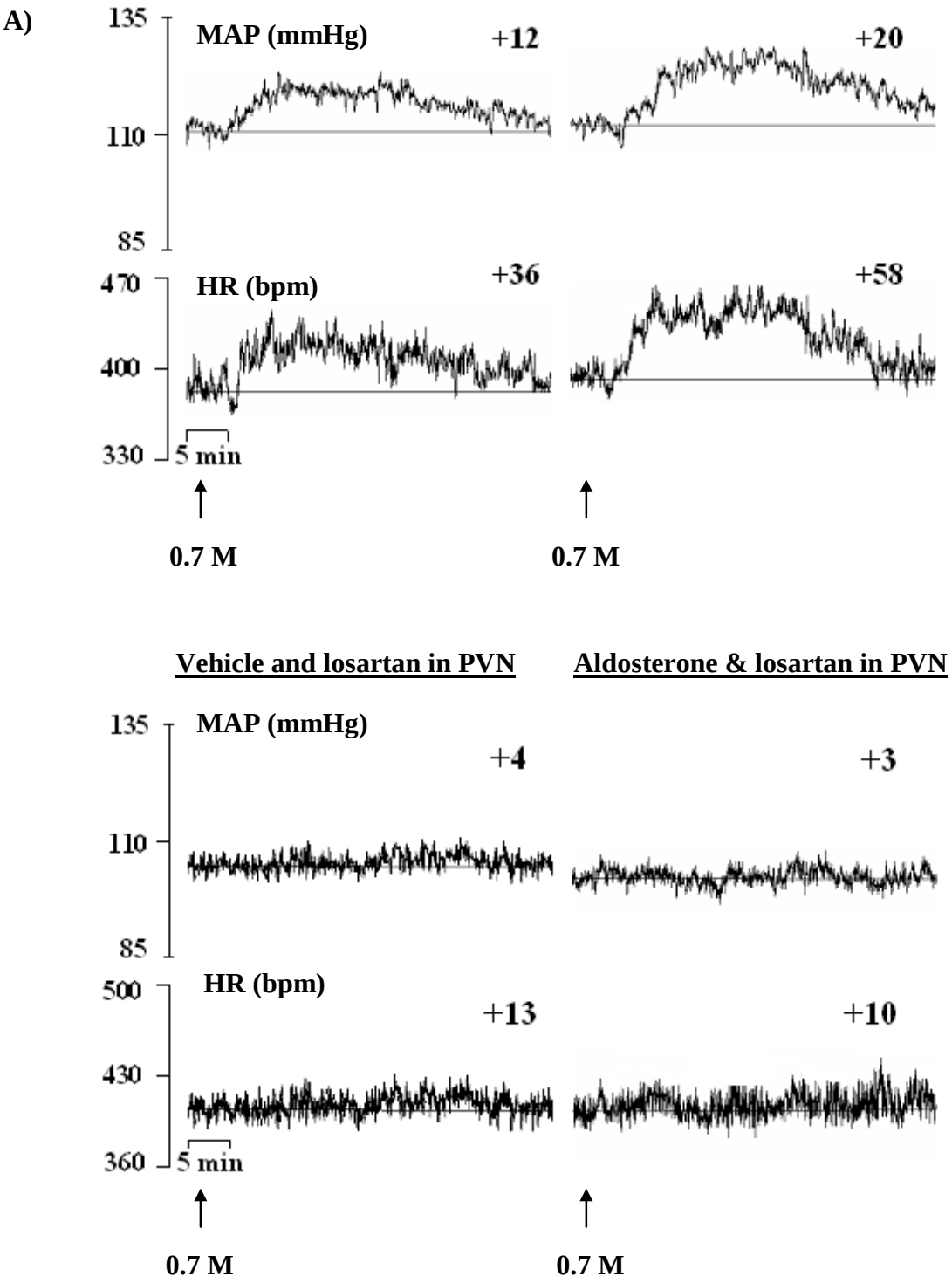
	aCSF	Ouabain (20 ng)	Ouabain (40 ng)	Ouabain (80 ng)	Losartan (80 ug) + Vehicle	Vehicle + Ouabain (80 ng)	Losartan (80 ug) + Ouabain (80 ng)
	(n=5)	(n=5)	(n=5)	(n=5)	(n=6)	(n=5)	(n=4)
Δ MAP	0 ±1	2 ±1	12 ±1*	16 ±1*	2 ±2	13±1*	1 ±1
Δ HR	0 ±0	4 ±2	18 ±4*	28 ±6*	1 ±3	62 ±11*	10 ±5

Values are mean ± SEM

* $p<0.05$ vs. aCSF

Aldosterone, losartan and Na^+ rich aCSF: After aldosterone, Na^+ rich aCSF caused again significantly larger increases in MAP and HR as compared to Na^+ rich aCSF alone

(Figure 2-5). Losartan fully prevented increases in MAP and HR to Na^+ rich aCSF either given alone or after aldosterone (Figure 2-5).



B)

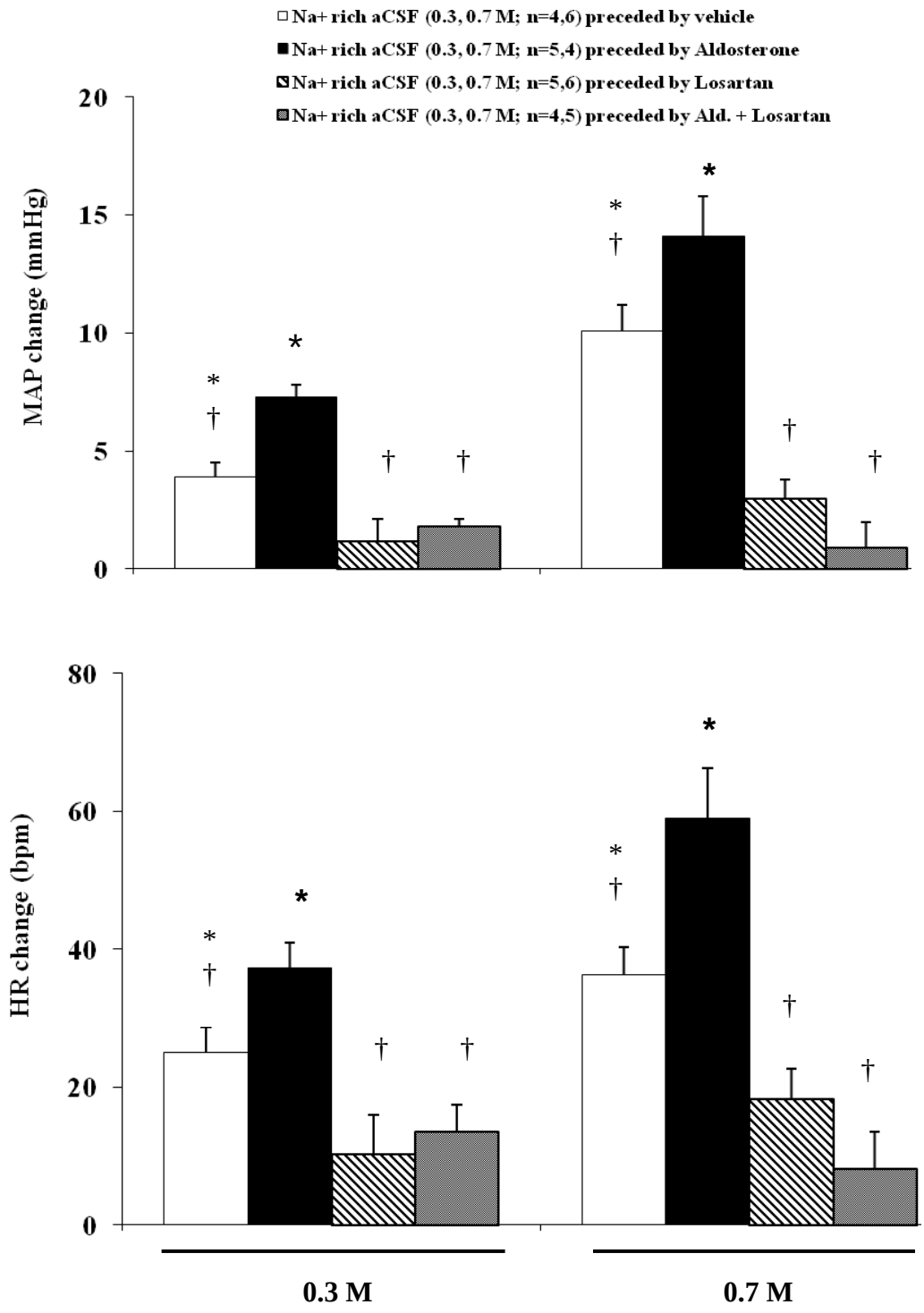
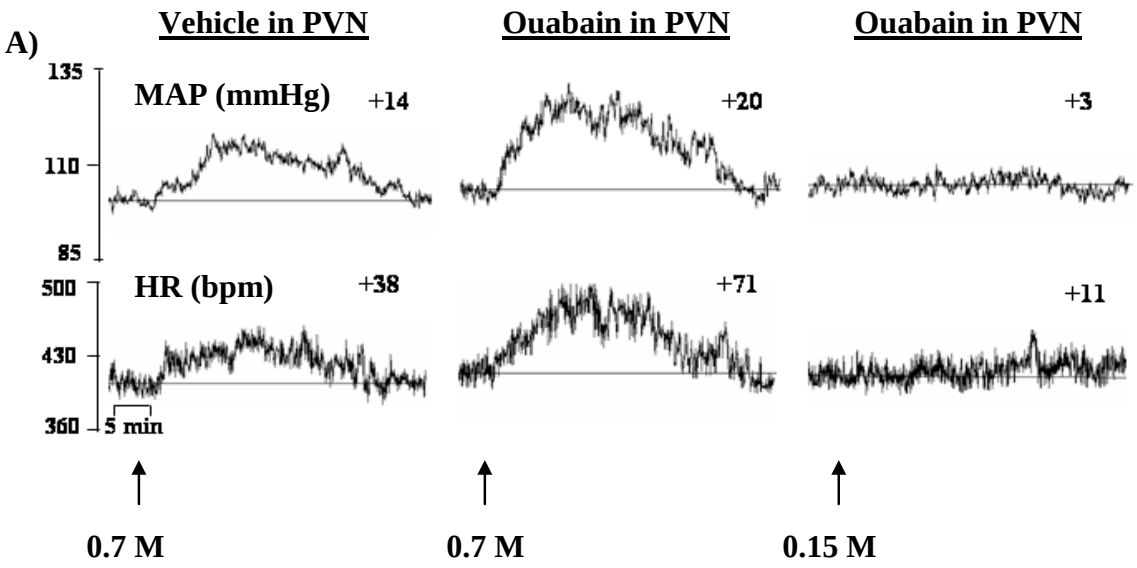


Figure 2-5: Effects of losartan in the PVN on peak MAP and HR responses to infusion of Na^+ rich aCSF (0.3 and 0.7 M Na^+) in the PVN, either alone or after aldosterone. Results are presented as time tracings from one individual rat per treatment (A) or as mean ± SEM of group changes (B); * $p<0.05$ vs. baseline, † $p<0.05$ vs. aldosterone + vehicle.

Subpressor dose of ouabain and Na^+ rich aCSF in the PVN: After infusion of a subpressor dose of ouabain in the PVN, MAP and HR responses to infusion of Na^+ rich aCSF at 0.7 M Na^+ were significantly larger compared to its effects after infusion of vehicle (Figure 2-6), whereas infusion of aCSF in the PVN caused no significant changes in MAP and HR (Figure 2-6).



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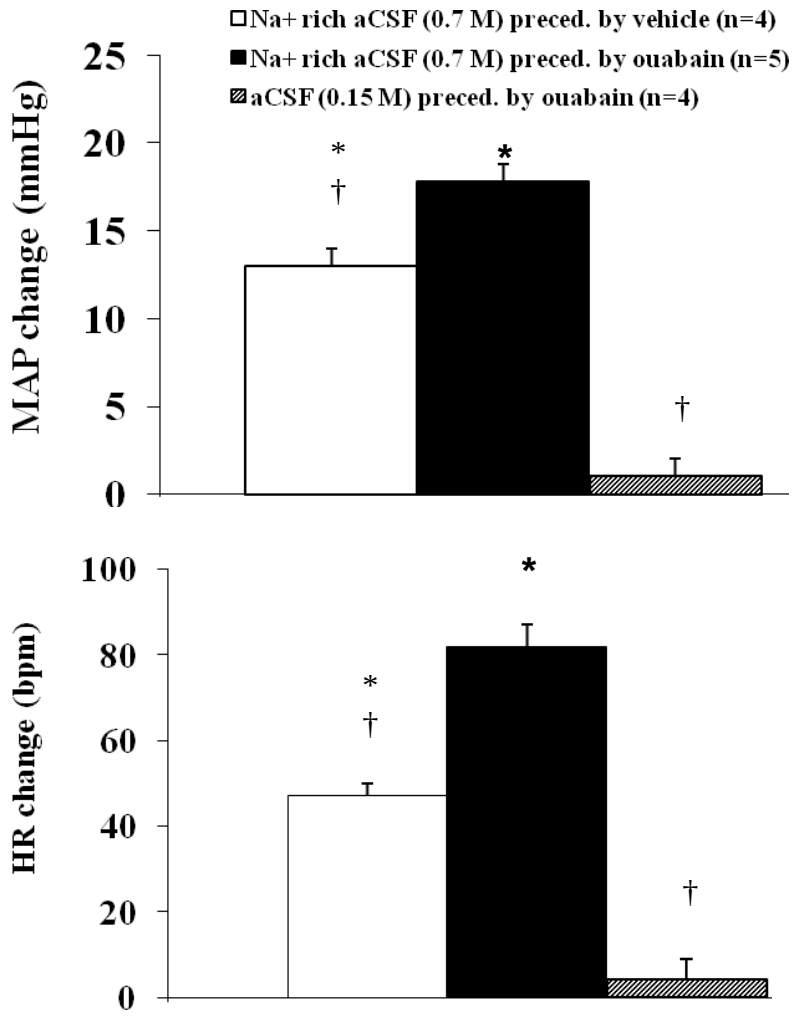


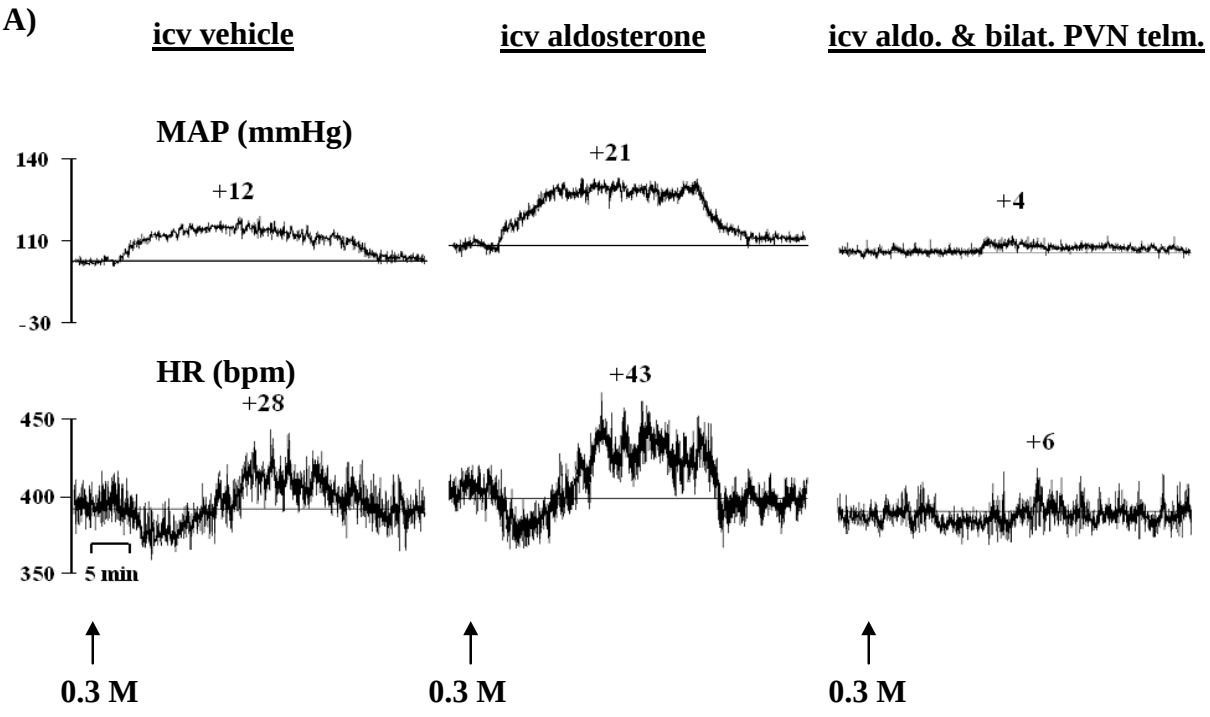
Figure 2-6: Effects of a subpressor dose of ouabain in the PVN on peak MAP and HR responses to infusion of aCSF or Na^+ rich aCSF at 0.7 M Na^+ in the PVN. Results are presented as time tracings from one individual rat per treatment (A) or as mean (± SEM) of group changes (B); * $p<0.05$ vs. baseline, † $p<0.05$ vs. ouabain + Na^+ rich aCSF.

Vasopressin release and Na^+ Rich aCSF alone or combined with aldosterone in the PVN: Infusion of Na^+ rich aCSF at 0.7 M Na^+ in the PVN caused the same gradual increases in MAP after iv pretreatment with vehicle (10±2 mmHg) or with AVP antagonist (11±2 mmHg) (Figure 2-2). Peak increases in HR were also the same (vehicle, 40±4 bpm; AVP antagonist, 43±5 bpm), but the initial decrease was no longer

observed after iv AVP antagonist (Figure 2-2). The AVP antagonist did not affect the enhancement by aldosterone of the peak MAP and HR responses to Na^+ rich aCSF at 0.7 M Na^+ (MAP, 17±2 mmHg; HR, 64±7 bpm) as compared to aldosterone and Na^+ rich aCSF alone.

Combined icv and PVN Studies

Icv infusion of Na^+ rich aCSF preceded by aldosterone icv or in the PVN: Icv infusion of aCSF caused no significant changes in MAP (3±1 mmHg) and HR (12±1 bpm). Icv infusion of Na^+ rich aCSF caused concentration dependent significant increases in MAP (0.3 M, 14±2 mmHg; 0.45 M, 23±2 mmHg) and HR (0.3 M, 29±5; 0.45 M, 48±6 bpm). MAP increases began within the first 3 min and peaked 5-7 min after start of the infusion. HR decreased for the first 6-8 min, and then increased to levels significantly higher than resting level. Both MAP and HR returned to baseline 15-20 min after end of infusion (Figure 2-7).



B)

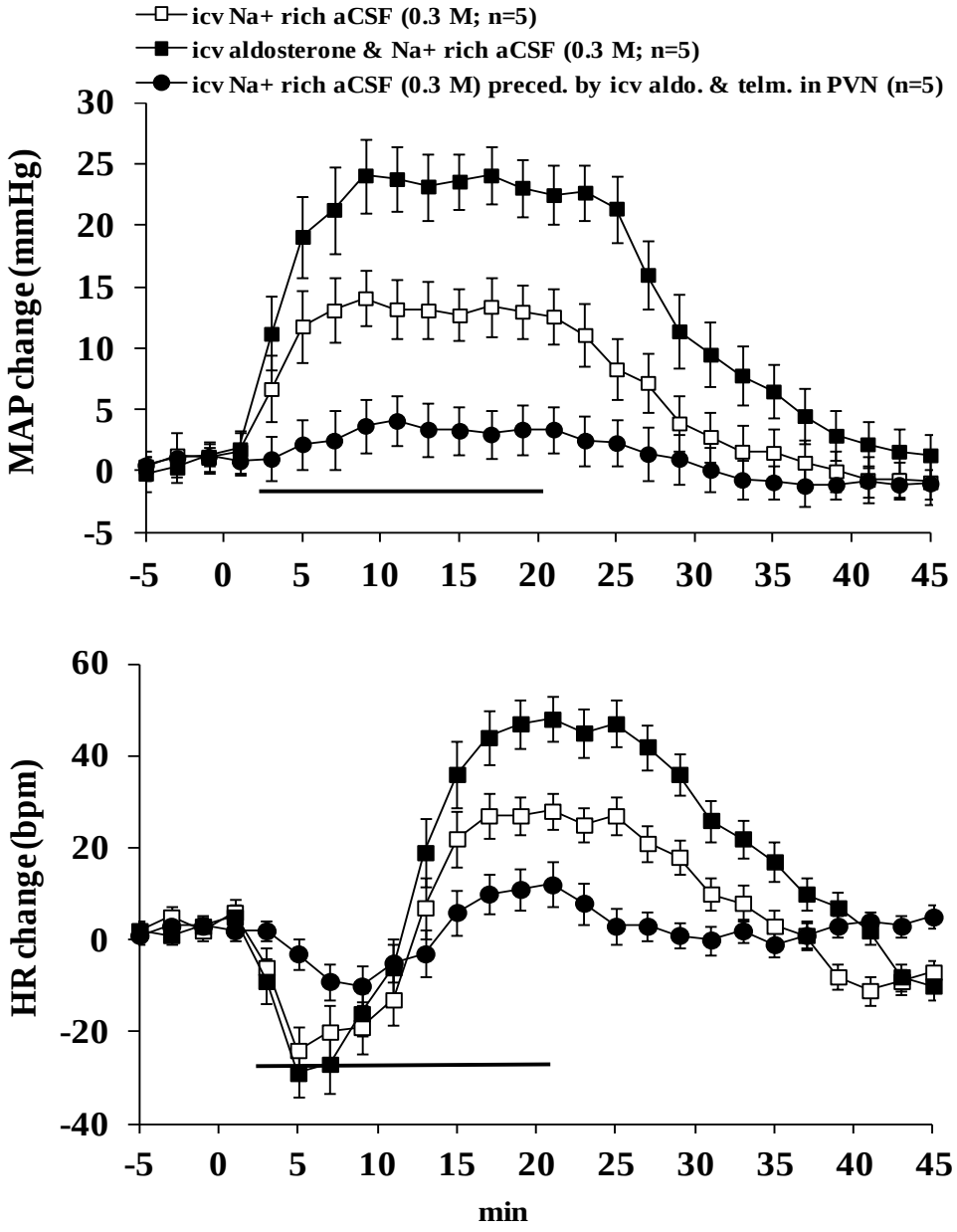


Figure 2-7: Effects of icv infusion of aldosterone alone, or preceded by bilateral infusion of telmisartan in the PVN on the peak MAP and HR responses to icv infusion of Na^+ rich aCSF at 0.3 M Na^+. Results are presented as time tracings from one individual rat per treatment (A) or as mean ± SEM of group changes (B). By one-way ANOVA with repeated measures, Na^+ rich aCSF alone caused significant increases in MAP (3-27 min) and HR (15-29 min). Aldosterone and Na^+ rich aCSF significantly increased MAP from 3-37 min and HR from 13-35 min while there were no significant increases in groups treated with telmisartan in the PVN ($p<0.05$). By t-test analysis of the AUC, aldosterone significantly enhanced MAP and HR responses to Na^+ rich aCSF. Black line represents infusion period.

After infusion of aldosterone in the PVN, peak MAP and HR responses to icv infusion of Na^+ rich aCSF ([0.3 M: MAP, 15±2 mmHg; HR, 22±4 bpm; n=6], [0.45 M: MAP, 22±3 mmHg; HR, 54±5 bpm; n=5]) were not significantly different compared to after infusion of vehicle in the PVN ([0.3 M: MAP, 14±2 mmHg; HR, 25±4 bpm; n=5], [0.45 M: MAP, 23±3 mmHg; HR, 48±6 bpm; n=5]). In contrast, the MAP and HR responses to icv infusion of Na^+ rich aCSF were significantly increased after icv infusion of aldosterone (Figure 2-8). This enhancement by aldosterone infused icv was not affected by pre-treatment with eplerenone in the PVN (Figure 2-8). Similarly, infusion of benzamil into the PVN did not change the MAP and HR responses to icv infusion of Na^+ rich aCSF after icv infusion of aldosterone (Figure 2- 8).

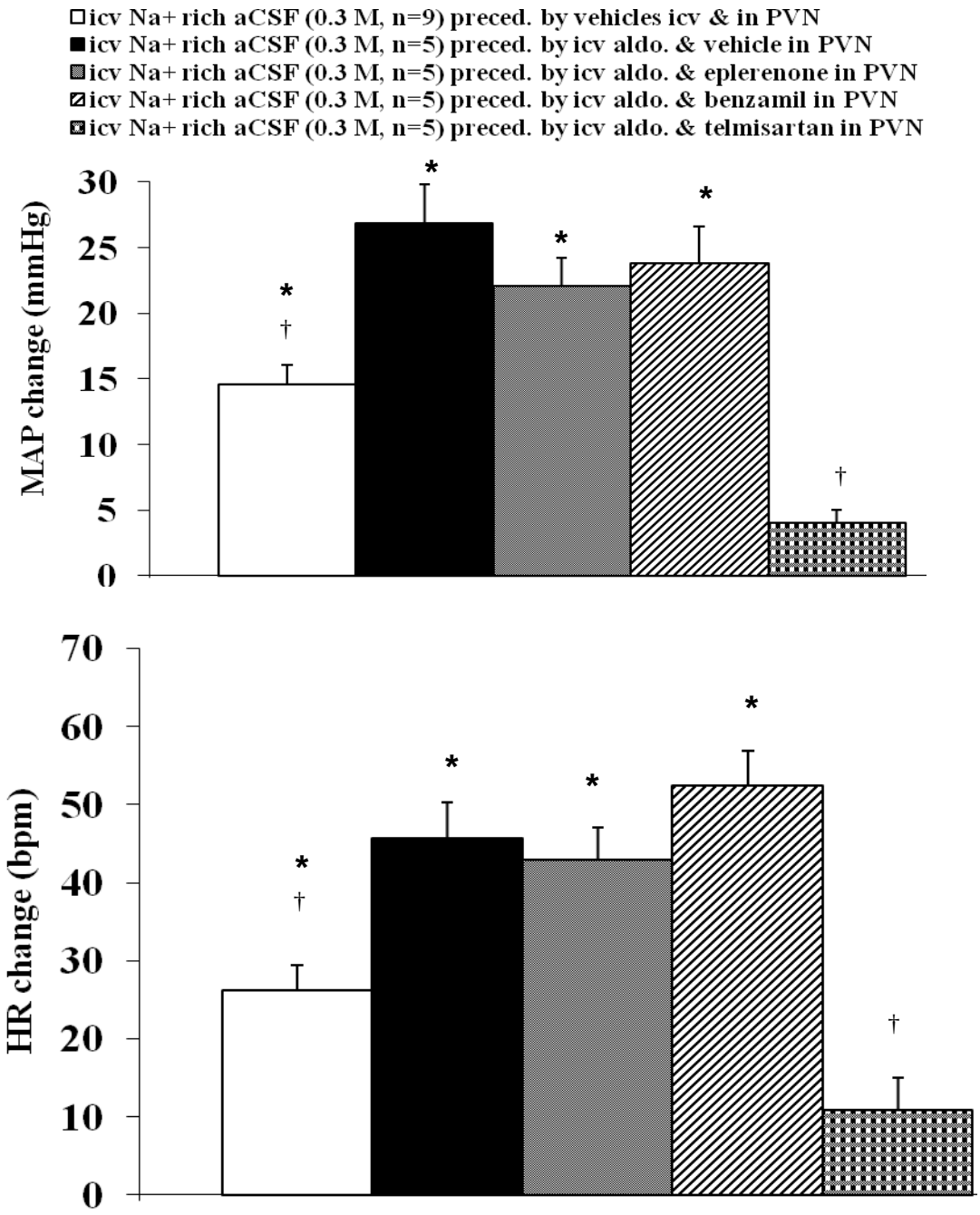


Figure 2-8: Effects of icv infusion of aldosterone and bilateral infusion of eplerenone, benzamil, telmisartan or vehicle in the PVN on peak MAP and HR responses to icv infusion of Na^+ rich aCSF (0.3 M Na^+). Values are mean ± SEM; * $p<0.05$ vs. baseline, † $p<0.05$ vs. icv aldosterone + vehicles in PVN.

Icv infusion of Na^+ rich aCSF preceded by telmisartan in the PVN: Bilateral pre-treatment of the PVN with telmisartan fully blocked BP responses to icv infusion of 0.3

M Na^+ rich aCSF: +15±1 mmHg after vehicle versus +5±1 mmHg after telmisartan as compared to +3±1 mmHg for aCSF. Telmisartan infused bilaterally in the PVN also fully prevented the BP and HR responses to icv infusion of aldosterone followed by Na^+ rich aCSF (Figure 2-8). Telmisartan infused bilaterally outside the PVN did not affect the increases in MAP and HR by icv infusion of Na^+ rich aCSF with or without aldosterone (data not shown).

Changes in CSF [Na^+] during icv infusion of Na^+ rich aCSF: Following icv infusion of aCSF (0.150 mM Na^+), CSF [Na^+] at the cisterna magna was 156±1 mM at 20 min (n=4) and 155±1 mM at 60 min (n=4). Icv infusion of Na^+ rich aCSF at 0.3 M Na^+ increased CSF [Na^+] to 169±2 mM at 20 min (n=5) and 172±2 mM at 60 min (n=4). Values for the CSF [Na^+] after 20 or 60 min of infusion did not differ significantly (p=0.33).

DISCUSSION

The present study demonstrates as new findings, that BP and HR responses to infusion of Na^+ rich aCSF in the PVN are enhanced by aldosterone and a subpressor dose of ouabain. Eplerenone, benzamil or "ouabain"-binding fab fragments prevent the enhancement by aldosterone, altogether indicating that aldosterone's effect is mediated by MR, benzamil blockable Na^+ influx and "ouabain" release. In contrast, losartan blocks all responses to aldosterone and Na^+ rich aCSF in the PVN, consistent with an essential role for AT_1-receptor stimulation in the PVN. The second finding is that BP and HR responses to icv infusion of Na^+ rich aCSF are enhanced by aldosterone infused icv, but not in the PVN and fully blocked by telmisartan in the PVN. Both eplerenone and benzamil in the PVN do not change the pressor responses to icv infusion of aldosterone and Na^+ rich aCSF. These findings suggest that AT_1-receptors in the PVN also mediate the pressor responses to CSF [Na^+] increases, but MR and benzamil blockable Na^+ influx in the PVN do not contribute to the enhancement by icv aldosterone.

Na^+ rich aCSF in the PVN

Infusion of Na^+ rich aCSF into the PVN caused concentration-related increases in BP and HR, whereas infusion of mannitol at equivalent osmolality or aCSF at same volumes generated no significant increases. These findings are similar to those reported by Jin et al. (Jin et al. 2001), and indicate that the pressor responses are primarily mediated by changes in the concentration of Na^+, and not osmolality or a volume induced stretch. The increases were fully blocked by losartan suggesting that the BP and HR responses to Na^+ rich aCSF in the PVN are mediated by local AT_1-receptor activation. Microdialysis perfusion of Na^+ rich aCSF in the PVN caused a [Na^+] dependent local Ang II release

(Qadri et al. 1994). An increase in intracellular Na^+ caused by a larger extra/intracellular gradient may increase intracellular Ca^{2+} through Na^+/Ca^{2+} channel exchangers, and thereby increase Ang II release (Doris 1988) and AT_1-receptor activation. AT_1-receptor stimulation in the PVN may excite subpopulations of PVN neurons innervating the RVLM (Cato and Toney 2005) and/or spinally projecting neurons (Li et al. 2003a), thereby increasing sympathetic activity, BP and HR (Dampney 1994). Infusion of Na^+ rich aCSF in the PVN may also activate magnocellular neurons in the PVN causing AVP release (Qadri et al. 1998). AVP may enhance sympatho-inhibitory baroreflex function (Hasser et al. 1997) and this effect likely explains the initial decrease in HR which was prevented by the AVP antagonist. However, AVP does not appear to contribute to the pressor responses since the AVP antagonist caused no changes in the increase in BP.

Aldosterone and Na^+ rich aCSF in the PVN

Infusion of aldosterone in the PVN on its own had no measurable effects on BP and HR, but enhanced the Na^+ rich aCSF induced increases in BP and HR. This enhancement of the responses to Na^+ rich aCSF was blocked by the specific MR blocker eplerenone and by benzamil, indicating that aldosterone's effects are mediated by MR activation and benzamil blockable Na^+ influx. The enhancement by aldosterone was also blocked by the antibody fab fragments, whereas similarly as aldosterone, a subpressor dose of exogenous ouabain infused into the PVN enhanced the pressor responses to local infusion of Na^+ rich aCSF. Together these findings suggest that MR stimulation via benzamil sensitive Na^+ channels or transporters on the cell surface of magnocellular neurons further increases intracellular $[Na^+]$, thereby facilitating "ouabain" release from magnocellular nerve terminals. Losartan in the PVN blocked responses to ouabain indicating that

ouabain in the PVN increases BP and HR via AT_1-receptor stimulation. Losartan also fully blocked the responses to aldosterone and Na^+ rich aCSF in the PVN, altogether indicating that the effects of endogenous "ouabain" in the PVN are also mediated via AT_1-receptor stimulation. In contrast to the rapid responses to Ang II, the pressor responses to ouabain in the PVN take several minutes to develop. A neuromodulator such as "ouabain" binds to the Na^+/K^+ATPase causing a gradual decrease in membrane potential and may thereby sensitize the neurons to tonic activity, resulting in enhanced Ang II release and/or enhanced responses to AT_1-receptor activation.

Altogether, the responses to $[Na^+]$ increases in the PVN alone or after aldosterone appear to result from two mechanisms. The first and basal mechanism involves the responses to $[Na^+]$ increases alone, where AT_1-receptor stimulation may result from a Ca^{2+} mediated mechanism. The second component involves enhancement by aldosterone of responses to $[Na^+]$ increases resulting via benzamil blockable Na^+ influx and "ouabain" release in enhanced AT_1-receptor stimulation. One may speculate that in the PVN, under basal conditions aldosterone release, and therefore activity of benzamil sensitive Na^+ channels or transporters and "ouabain" release are low, and a short infusion of Na^+ rich aCSF does not sufficiently increase this pathway to make a contribution. The present study does not address which Na^+ channels or transporters specifically mediate the benzamil sensitive Na^+ influx. However, the amount (100 ng or 0.28 nmol) infused into the PVN with a volume of 0.45 ± 0.03 mm^3 (Conn and Freeman 2000), may result in concentrations in the range of 0.5 - 2.0 mM, in which one may expect maximal inhibition of ENaC and little inhibition of other channels or transporters (Kleyman and Cragoe 1988, 1990).

Role of PVN in Responses to CSF [Na^+] increases

Consistent with our previous icv studies (Wang et al. 2003a) and its infusion in the PVN, icv infusion of aldosterone alone did not affect BP and HR, but enhanced the responses to icv infusion of Na^+ rich aCSF. In contrast, aldosterone in the PVN did not change the pressor responses to icv infusion of Na^+ rich aCSF at 0.3 or 0.45 M Na^+. The dose of aldosterone delivered to each side of the PVN was similar to the one which enhanced the pressor responses to Na^+ rich aCSF in the PVN. In addition, both eplerenone and benzamil in the PVN did not change the BP and HR responses to icv infusion of aldosterone and Na^+ rich aCSF. Doses of eplerenone and benzamil delivered to each side of the PVN were also similar to doses that blocked the enhancement by aldosterone of the pressor responses to Na^+ rich aCSF in the PVN. These findings indicate that MR and benzamil blockable Na^+ influx in the PVN are not involved in mediating the pressor responses to a short icv infusion of Na^+ rich aCSF or their enhancement by icv aldosterone. The pressor responses to icv infusion of aldosterone and Na^+ rich aCSF are mediated by benzamil blockable Na^+ channels or transporters in the brain since they can be blocked by icv infusion of benzamil (Wang et al. 2003a, Wang and Leenen 2003). We conclude from these findings that other brain regions with a more sensitive MR - benzamil sensitive Na^+ influx - "ouabain" system mediate the responses to CSF [Na^+] increases. Indeed, the pressor responses to icv infusion of Na^+ rich aCSF were completely abolished by fab fragments in the median preoptic nucleus (MnPO) demonstrating that "ouabain" in the MnPO mediates the cardiovascular responses to icv infusion of Na^+ rich aCSF (Budzikowski and Leenen 1997). Alternatively, it is possible that a short icv infusion of Na^+ rich aCSF in the lateral ventricle does not raise [Na^+] in

the PVN high enough to produce the enhancement by aldosterone observed when Na^+ rich aCSF was directly infused into the PVN. It is unlikely that differential vasopressin release accounts for the failure of aldosterone in the PVN to alter the responses to icv infusion of Na^+ rich aCSF as an AVP antagonist does not affect pressor responses to infusion of aldosterone and Na^+ rich aCSF either icv (Wang et al. 2003a) or in the PVN (present study).

Pressor responses to icv infusion of Na^+ rich aCSF can be prevented by infusion of an AT_1-receptor blocker either intracerebroventricularly (Huang and Leenen 1996c), directly into the subfornical organ (SFO) (Rohmeiss et al. 1995), MnPO (Budzikowski and Leenen 2001) or bilaterally in the PVN (present study). AT_1-receptor blockade in the PVN also fully blocks the responses to icv infusion of Na^+ rich aCSF preceded by aldosterone. Considering that the SFO and MnPO have extensive connections to the PVN (McKinley et al. 2001) and that SFO-PVN connections appear to utilize Ang II as a neurotransmitter (Li and Ferguson 1993, Wright et al. 1993), one may consider that increases in CSF [Na^+] primarily excite Na^+ sensitive nuclei in the lamina terminalis ie. SFO and MnPO and this increased neuronal activity is being relayed to the PVN via Ang II release causing local AT_1-receptor stimulation.

Limitations of the Present Study

Some limitations of the present study should be considered. Firstly, the actual local concentrations of Na^+ or aldosterone achieved in the PVN or general CSF depend on the amount infused relative to the volume of distribution and turnover of the volume. Considering that in Dahl salt sensitive (S) rats on high salt diet, CSF [Na^+] rises by 5-6 mM (Huang et al. 2004), a 13 mM increase in CSF [Na^+] after 20 min icv infusion of 0.3

M Na^+ rich aCSF in the present study does not appear to be far in excess of those achieved under pathophysiological conditions. Increases in BP and HR by Na^+ rich aCSF are fairly similar when infused either icv or directly into the PVN, but we don't know whether these similar responses are achieved by similar increases in [Na^+] at relevant sites. The pathophysiological relevance of the effects of exogenous aldosterone, infused both icv and in the PVN at possibly high doses cannot be assessed from the present study. However, the observed cardiovascular effects are clearly specific ie. MR dependent and consistent with the known actions of aldosterone. We recently reported that the sympathetic and pressor responses from chronic icv infusion of Na^+ rich aCSF in Wistar rats can be largely prevented by concomitant icv infusion with an aldosterone synthase inhibitor suggesting that endogenous aldosterone in the CNS exerts similar, if not larger effects than those observed with exogenous aldosterone (Huang et al. 2008a). Finally, "ouabain" binding fab fragments blocked the enhancement by aldosterone of the pressor responses to a local [Na^+] increase in the PVN and these responses to Na^+ were also enhanced by a subpressor dose of exogenous ouabain in the PVN. These findings are consistent with the concept that endogenous "ouabain" is an endogenous analogue of exogenous ouabain (Kawamura et al. 1999), but this obviously requires further study.

Perspectives and Significance

The present findings indicate that AT_1-receptors in the PVN are essential for mediating the pressor responses to [Na^+] increases both locally in the PVN and in the CSF. MR, benzamil sensitive Na^+ influx, possibly via ENaC, and "ouabain" in the PVN can be functionally active in response to a local increase in aldosterone and [Na^+]. These mechanisms may therefore function as a neuromodulatory pathway, enhancing the

activity of angiotensinergic sympatho-excitatory pathways. However, they may not be sensitive enough in the PVN to contribute to the pressor responses to brief increases in CSF [Na^+] and aldosterone into a lateral ventricle. Further studies are needed to address how and where the two pathways interact in response to chronic increases in CSF [Na^+] by icv infusion of Na^+ rich aCSF or by high dietary salt in genetic models of salt hypertension, such as Dahl S rats.

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3. MANUSCRIPT #2- MECHANISMS MEDIATING SODIUM-INDUCED PRESSOR RESPONSES IN THE PVN OF DAHL RATS

Alexander Gabor[a] and Frans H. H. Leenen[a]*

[a]Hypertension Unit, University of Ottawa Heart Institute, Ottawa, Ontario, Canada.

*Thesis Supervisor

SHORT TITLE

Mechanisms in BP Responses to Na^+ in PVN Dahl Rats

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ABSTRACT

Intracerebroventricular infusion of Na^+ rich artificial cerebrospinal fluid (aCSF) causes larger sympathetic and pressor responses in Dahl salt sensitive (S) versus resistant (R) or Wistar rats. Enhanced activity of the aldosterone-"ouabain" pathway or decreased nitric oxide (NO) release may contribute to this enhanced responsiveness. Where in the brain these mechanisms interact is largely unknown. The present study evaluated whether Na^+ in the PVN causes larger pressor responses in Dahl S (SS/Mcw) versus R (Dahl SS.BN13) rats and whether mineralocorticoid receptors (MR), benzamil blockable Na^+ channels, "ouabain", AT_1-receptors or NO mediate these enhanced responses. Na^+ rich aCSF in the PVN caused 30-40% larger increases in BP and HR in Dahl S versus R or Wistar rats, whereas responses to ouabain, Ang II or N_ω-Nitro-L-arginine methyl ester hydrochloride (L-NAME) in the PVN were the same. Eplerenone, benzamil or Fab fragments did not affect these responses to Na^+, whereas they were fully blocked by losartan in both Dahl S and R rats. L-NAME enhanced them more in Dahl R than S rats, thereby equalizing them in the two strains. Pressor responses to L-NAME in the PVN were attenuated by high salt diet in Dahl S, but not R rats. The results indicate that both acute and chronic increases in $[Na^+]$ in the PVN appear to inhibit NO release in the PVN of Dahl S but not R rats, thereby contributing to the enhanced pressor responses to Na^+ in Dahl S.

INTRODUCTION

High salt diet increases cerebrospinal fluid (CSF) [Na^+] (Huang et al. 2004, Nakamura and Cowley 1989) and hypothalamic aldosterone and ouabain-like compounds ("ouabain") (Huang et al. 2008c, Kawamura et al. 1999, Wang and Leenen 2002), and causes sympatho-excitation and hypertension in Dahl salt sensitive (S) but not in salt resistant (R) or Wistar rats (Huang et al. 2004, Morgan et al. 1990, Serino et al. 2001). An increase in neural responsiveness to CSF [Na^+] appears to contribute to salt sensitivity in Dahl S rats since intracerebroventricular (icv) infusion of Na^+ rich artificial CSF (aCSF) causing same increases in CSF [Na^+] as in Dahl S on high salt intake causes significantly larger increases in renal sympathetic nerve activity (RSNA), blood pressure (BP) and heart rate (HR) in Dahl S than R or Wistar rats (Huang et al. 2001b). Studies in congenic strains (Huang et al. 2007), indicate that the mechanisms contributing to the increase in CSF [Na^+] in Dahl S on high salt intake, and the enhanced central sensitivity to Na^+ appear to be different. Chronic icv infusion of aldosterone combined with slightly higher than physiological CSF [Na^+] leads to increases in hypothalamic "ouabain", sympathetic hyperactivity and hypertension in Dahl S, but not R rats (Huang et al. 2005). Considering that the RSNA, BP and HR responses to icv injection of plant ouabain are similar in Dahl S and R rats (Huang et al. 2001b, Zhao et al. 2001), mechanisms leading to enhanced release of "ouabain" may therefore contribute to the enhanced neural responses to Na^+ and aldosterone in Dahl S rats. However, the actual mechanisms contributing to the increased neuronal responsiveness to CSF [Na^+] in Dahl S rats are still unknown.

Neurons in the paraventricular nucleus (PVN) play an important role in mediating the hypertension in Dahl S rats on high salt diet since lesioning the PVN prevents the increase in BP (Goto et al. 1981). Increases in CSF [Na^+] may excite Na^+ sensitive nuclei in the lamina terminalis such as the subfornical organ (SFO) (Denton et al. 1996). This increased neuronal activity can be relayed to parvocellular neurons of the PVN projecting to the intermediolateral cell column (IML) or rostral ventrolateral medulla (RVLM), thereby increasing sympathetic activity and BP (Cato and Toney 2005, Li et al. 2003a, Li and Ferguson 1993, McKinley et al. 2001). In addition, an increase in CSF [Na^+] raises hypothalamic tissue [Na^+] (Wang et al. 2010) and may thereby enhance the firing activity of Na^+-sensitive neurons in the PVN (Gabor and Leenen 2009). In our previous study (Gabor and Leenen 2009), infusion of Na^+ rich aCSF directly into the PVN of Wistar rats caused [Na^+]-dependent increases in BP and HR and this effect was mediated by local AT_1-receptor stimulation. Aldosterone and a subpressor dose of ouabain in the PVN did not affect BP and HR, but enhanced responses to Na^+ rich aCSF. The enhancement by aldosterone could be prevented by a mineralocorticoid receptor (MR) blocker, benzamil or Fab fragments binding "ouabain". Infusion of an AT_1-receptor blocker into the PVN fully prevented the pressor responses to local infusion of ouabain or Na^+ rich aCSF combined with aldosterone, suggesting that the MR- benzamil blockable Na^+ influx- "ouabain" pathway may enhance Na^+-induced AT_1-receptor mediated responses in the PVN (Gabor and Leenen 2009).

The PVN is under tonic inhibition by nitric oxide (NO) since inhibiting NO production in the PVN by local injection of a nitric oxide synthase (NOS) blocker increases sympathetic nerve activity (SNA), BP and HR (Zhang et al. 1997). Decreases

in SNA and BP induced by an NO donor in the PVN were inhibited by a Gamma-aminobutyric acid A ($GABA_A$) receptor antagonist, while perfusion of the PVN with NO increased local GABA release, altogether suggesting that NO in the PVN tonically inhibits SNA and BP by increasing local GABA release and $GABA_A$ receptor activation (Horn et al. 1994, Zhang and Patel 1998). Electrophysiological studies provide evidence that NO enhances GABA release from GABAergic nerve terminals presynaptic to spinally projecting neurons in the PVN (Li et al. 2004, Li et al. 2002). A $GABA_A$ receptor blocker in the PVN increases local glutamate release (Li et al. 2006a), and pressor and sympathetic responses from a $GABA_A$ receptor blocker in the PVN can be prevented by a glutamate receptor blocker (Li et al. 2006a), indicating that GABA in the PVN tonically inhibits local glutamate release. Enhanced responses to neuronal stimulation in the PVN of Dahl S rats may therefore reflect enhanced activation of excitatory mechanisms and/ or less activation of inhibitory mechanisms. We hypothesized that an enhanced neuronal responsiveness to Na^+ exists in the PVN of Dahl S rats compared to its salt resistant control strain (Dahl R), and this enhanced sensitivity depends on enhanced MR- benzamil blockable Na^+ influx- "ouabain" activity. Additionally, we hypothesized that Na^+ in the PVN causes less local NO release and less inhibition of sympatho-excitatory neurons in the PVN of Dahl S versus R rats, thereby contributing to enhanced pressor responses to Na^+ in Dahl S.

The objectives of the present study were therefore: 1) to evaluate whether infusion of Na^+ rich aCSF in the PVN causes larger BP and HR responses in Dahl S versus R or Wistar rats on regular salt; 2) to investigate whether MR, benzamil blockable Na^+ channels, "ouabain", AT_1-receptors or NO contribute to these enhanced responses; and 3)

to evaluate the effects of a NOS blocker in the PVN on resting BP in Dahl S and R rats on regular or high salt diet.

MATERIALS AND METHODS

Animals and Diets

Five to 6 weeks old male Dahl S (SS/Mcw), Dahl SS.BN13 as the salt resistant control (Dahl R) and Wistar rats were obtained from Charles River Breeding Laboratories, Montreal, Quebec, Canada. Consomic Dahl SS.BN13 rats are Dahl S rats with their chromosome 13 substituted by chromosome 13 from salt resistant Brown Norway (BN) rats, and do not develop elevated BP on high salt diet (Cowley et al. 2001). The rats were provided with a standard commercial rat chow (0.3 %, 120 µmol Na^+/ gram) and water ad libitum. Experiments were conducted after animals were acclimatized for 1 week. All experiments were carried out according to the guidelines of the Canadian Council on Animal Care, which conform to National Institutes of Health (NIH) guidelines and were approved by the University of Ottawa Animal Care and Use Committee.

Surgical Procedures

For all experiments, intra-cerebral cannulations of the PVN were performed 1 week prior to infusions into the PVN. On the day of the surgery, rats were anesthetized with 2% isoflurane in oxygen. Animals were placed in a stereotaxic head frame, and the skull was leveled between bregma and lambda. A section of the skull was removed, and guide cannulas were positioned unilaterally (right side) or bilaterally 0.5 mm above the PVN according to the rat atlas of Paxinos and Watson (Paxinos and Watson 1998), 1.8 mm posterior to bregma, 0.4 mm lateral to the bregma and 7.9 mm ventral from the skull. Guide cannulas were prepared from the steel tubing of 23 gauge needles (Becton, Dickinson), cut to extend ~1 cm over the skull when positioned above the PVN. They were secured to the skull with two jeweler's screws and acrylic cement and closed with

stainless steel obturators. After 1 week recovery, the left femoral artery was cannulated with PE-50/10 polyethylene tubing filled with heparin (1,000 U/ mL in 0.9% NaCl). The left femoral vein was cannulated for intravenous (iv) injections. For pain relief, animals received subcutaneous injections of buprenorphine (0.04 mg/ kg) twice daily for 3 days following cannulations of the PVN and on the day prior to infusions in the PVN for recovery from arterial cannulations.

Experimental Procedures

On the morning after the arterial cannulation, the rats were placed in a small cage and intra-arterial catheters were connected to a pressure transducer for recordings of BP and HR via a PC equipped with software AcqKnowledge (ACQ 3.9). Systolic and diastolic pressures were determined as previously described (Gabor and Leenen 2009).

An "L"-shaped injection cannula (30 gauge) was lowered unilaterally or bilaterally into the PVN through the fixed guide cannula and extended 0.5 mm past the guide. Injection cannulas were connected by polyethylene tubing to 10 μL Hamilton microsyringes mounted on an infusion pump (model 2400-003, Harvard Apparatus). This pump was set to perform microinjections (200 nL/ 30 s) or infusions (300 nL/ min). For all experiments, animals were allowed to settle for at least 30 min prior to BP and HR recording. Baseline levels were recorded and values were determined by the average data from 2 min intervals. At the end of the experiment, rats were euthanized and 100 nL Evans Blue dye was micro-injected into the intra-cerebral infusion site. Brains were removed, frozen, sectioned using a Leica cryostat and stained with neutral red. Infusion sites were considered to be inside the PVN if the mid-point of the dye circle was inside the borders of the PVN (Figure 3-1 and 3-2-A1). Only data from rats whose micro-

injection sites were within the PVN were used for analysis. The percent of injection sites within the PVN ranged from 75 - 90 % for groups with unilateral infusions and from 60 - 70 % for groups with bilateral infusions into the PVN. For infusions into the PVN, the extent of tissue damage from the cannula was limited to the diameter of the circular injection cannula lowered into the PVN (Figure 3-2-A2). For evaluation of the distribution of infusions in the PVN, Evans Blue dye (1%) dissolved in vehicle (aCSF) was infused at 300 nL/ min for 5 and 10 min in Dahl S rats. Consistent with our previous studies in Wistar rats (Gabor and Leenen 2009), the dye was distributed largely within the PVN region after 5 min and extended beyond the PVN after 10 min (Figure 3-2-B).

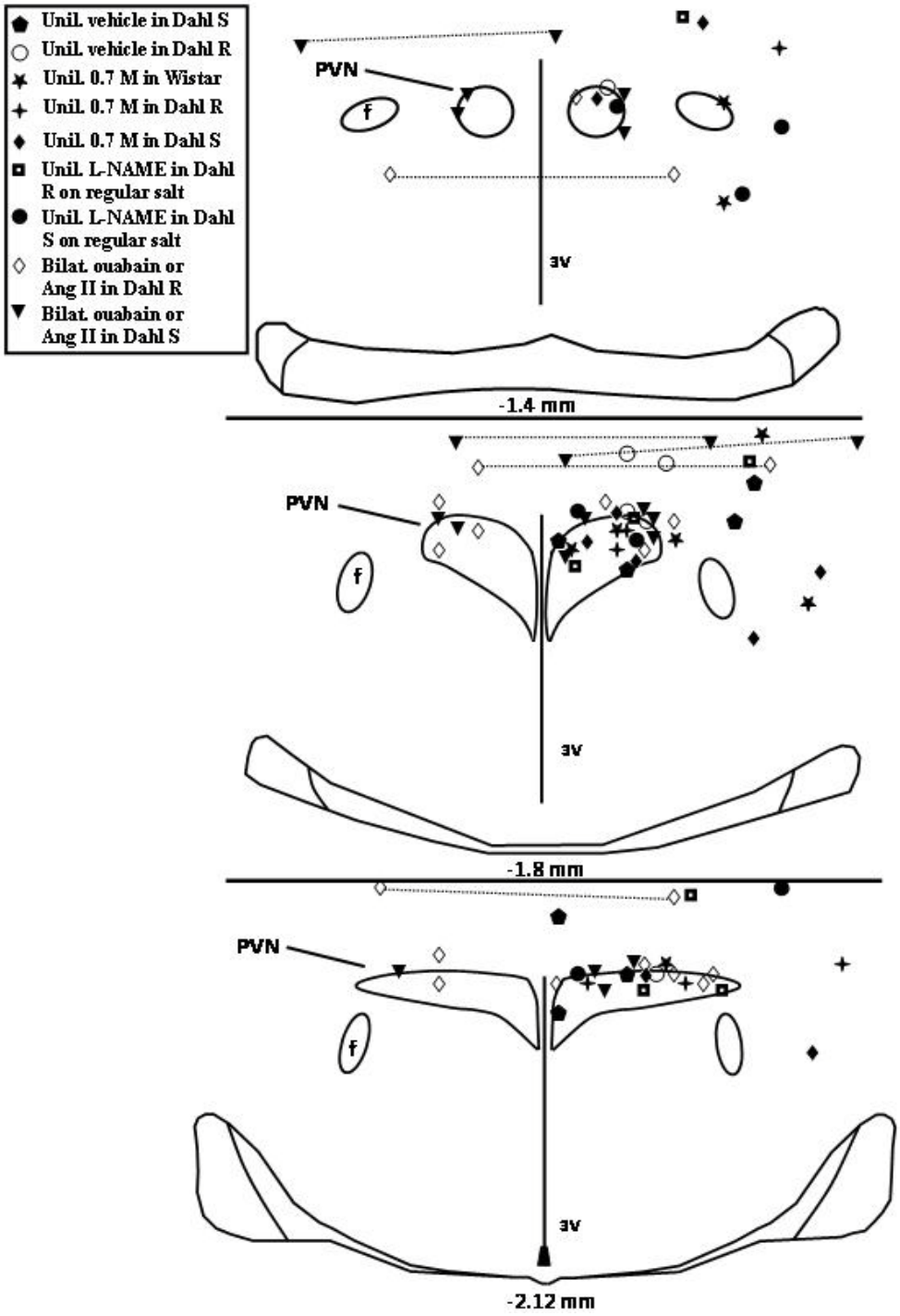

Figure 3-1: Schematic representations of infusion sites within and outside the PVN, plotted according to the location of the dye circle from 100 nL Evans Blue dye injections. Symbols represent unilateral or bilateral infusion sites from different experimental groups. Those connected by broken lines represent bilateral infusion sites outside the PVN. Remaining experimental groups not incorporated here had a similar pattern of distribution inside and outside the PVN. f indicates fornix; 3V, third ventricle.

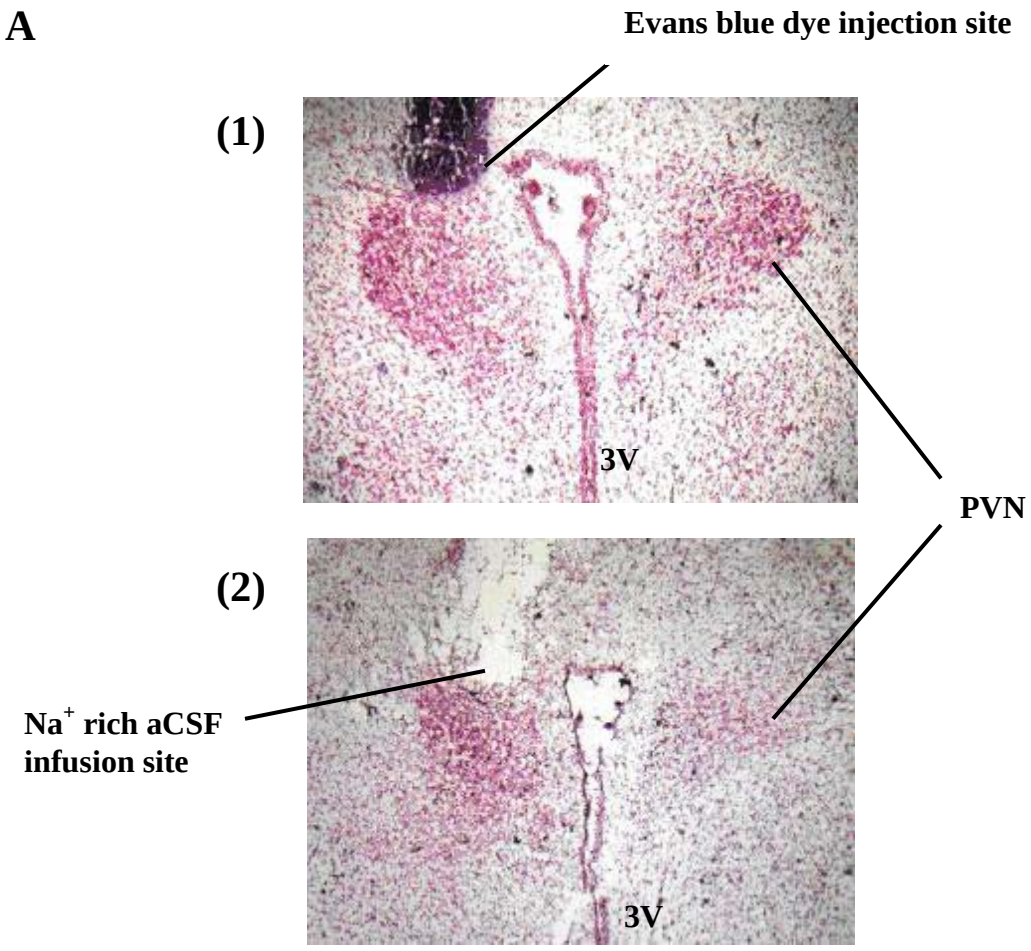

Figure 3-2: A) Representative photographs indicating of a section of the PVN utilized to verify the position of an infusion site by visualizing the spread of 100 nL Evans Blue dye (1), and extent of damage from unilateral infusion of 0.7 M Na^+ rich for 15 min into the PVN of a Dahl S rat (2). B) Schematic representation of the distribution of Evans Blue dye after unilateral infusion of 300 nL/ min for 5 or 10 min into the PVN.

Vehicles and Drugs

Artificial cerebrospinal fluid (121 NaCl, 3.4 KCl, 1.2 $MgCl_2$, 0.6 NaH_2PO_4, 29 $NaHCO_3$, and 3.4 glucose mmol/L, pH 7.4, and osmolarity 296 mosmol/kg H_2O) was used as vehicle, except for benzamil (propylene glycol and aCSF: 15:85 ratio), eplerenone (acetonitrile and aCSF: 1:99 ratio). All injected compounds were purchased from Sigma or received as gifts (see acknowledgements).

Experimental Protocols

Na^+ rich aCSF in the PVN: To evaluate whether Na^+ in the PVN causes larger pressor responses in Dahl S versus R or Wistar rats, Na^+ rich aCSF at 0.3 and 0.7 M Na^+ was infused unilaterally into the PVN for 15 min at 300 nL/min. For each rat, a 45 min resting period was given between infusion of 0.3 and 0.7 M Na^+ rich aCSF. Na^+ rich aCSF was prepared by adjusting the [Na^+] of aCSF with additional NaCl.

MR, Vasopressin and Na^+ rich aCSF in the PVN: To evaluate the role of MR or vasopressin in the enhanced pressor responses to Na^+ in the PVN of Dahl S versus R rats, unilateral infusion of eplerenone (20 ng/300 nL/min for 10 min) or vehicle was performed in Dahl S and R rats. After 10 min rest, the arginine vasopressin (AVP) antagonist [ß-mercapto-ß, ß-cyclopentamethylenepropionyl^1, *O*-Me-Tyr^2,Arg^8] vasopressin (30 μg/kg) dissolved in 0.3 mL saline (0.145 M Na^+) was injected iv and animals were given rest for 5 min prior to unilateral infusion of 0.7 M Na^+ rich aCSF at 300 nL/min for 15 min. Following 45 min rest to allow BP to return to baseline, infusion of a 10-fold higher dose of eplerenone (200 ng/300 nL/min for 10 min) or vehicle was conducted in the PVN and injection of the AVP antagonist and infusion of Na^+ rich aCSF

were repeated as previously described. Doses of eplerenone and the AVP antagonist were adapted from our previous studies (Gabor and Leenen 2009, Wang et al. 2003a).

Benzamil, Fab fragments, Losartan and Na^+ rich aCSF in the PVN: To assess whether benzamil blockable Na^+ channels, "ouabain" or AT_1-receptors mediate the enhanced pressor responses to Na^+ in the PVN of Dahl S versus R rats, unilateral infusion into the PVN of benzamil (50 ng/300 nL/min for 10 min), "ouabain"-binding Fab fragments (1 µg/300 nL/min for 10 min) or losartan (3 µg/300 nL/min for 15 min) or vehicles was performed in Dahl S and R rats. Each animal received only 1 pretreatment. Animals were given rest for 10 min prior to unilateral infusion of 0.7 M Na^+ rich aCSF at 300 nL/min for 15 min. To assess whether longer resting periods were needed to reverse binding of "ouabain" from the Na^+/K^+/ATPase (Balzan et al. 1991, Huang and Leenen 1994, Kent et al. 2004), resting periods between Fab fragments and Na^+ rich aCSF were extended to 2, 6 or 18 hours in other groups of Dahl S rats. Doses of Fab fragments, benzamil and losartan were adapted from our previous study showing blockade of the enhancement by aldosterone of the pressor responses to unilateral infusion of Na^+ rich aCSF in the PVN of Wistar rats (Gabor and Leenen 2009).

***L-NAME and Na^+ rich aCSF in the PVN*:** To evaluate whether an acute increase in $[Na^+]$ in the PVN increases local NO activity less in Dahl S versus R rats, 0.7 M Na^+ rich aCSF was infused unilaterally in the PVN at 300 nL/min for 10 min alone, or together with the non-specific NOS inhibitor N_ω-Nitro-L-arginine methyl ester hydrochloride (L-NAME) at 27 µg/300 nL/min. Forty-five min after end of these infusions, when BP had returned back to baseline, 0.7 M Na^+ rich aCSF alone, or together with L-NAME at 54 µg/300 nL/min were infused into the PVN for 10 min. Doses of L-NAME were based on

preliminary dose response experiments in Wistar rats (data not shown), and on previous studies measuring pressor responses to L-NAME in the PVN of rats (Zhang and Patel 1998).

Ouabain and Ang II in the PVN: To assess whether ouabain in the PVN causes the same pressor responses in Dahl S and R rats, vehicle was first microinjected unilaterally into the PVN. After 20 min rest, ouabain was microinjected unilaterally into the PVN at increasing doses (40, 80, 160 ng). Microinjections were performed at 30- 45 min intervals between doses of ouabain which corresponded to the period needed for BP and HR to return to baseline. To evaluate whether crosstalk between left and right sides of the PVN influence the pressor responses to ouabain in the PVN of rats, vehicle was first microinjected bilaterally into the PVN of Dahl S and R rats. After 20 min rest, ouabain (80 ng/ side) was microinjected bilaterally into the PVN of Dahl rats. To investigate whether the pressor responses to Ang II in the PVN vary in Dahl S versus R rats, Ang II (90 ng/ side) was microinjected 45 min after bilateral injection of ouabain. Doses of ouabain and Ang II in the PVN were adapted from our previous study (Gabor and Leenen 2009).

***L-NAME in the PVN of Dahl rats on regular or high salt diet*:** To evaluate whether high salt diet causes differential NO activity in the PVN of Dahl S versus R rats, Dahl S and R rats were fed either Research diets® D10001 (AIN-76A) regular (0.1 %, 44 µmol Na^+/ gram) or high (8 %, 1408 µmol Na^+/ gram) NaCl diet for 3 - 4 weeks. The regular and high NaCl diet are the same, except for the NaCl content. Unilateral cannulations of the PVN were performed 2 - 3 weeks after start of the diets, followed 1 week later by femoral artery cannulations. The morning after arterial cannulations, L-NAME (27

μg/300 nL/min) was infused unilaterally into the PVN for 10 min. Forty-five min after end of this infusion, L-NAME (54 μg/300 nL/min) was infused into the PVN for 10 min.

Statistical Analysis

Values are expressed as means ± SE. For comparison among treatments and rat strains, a two-way ANOVA was used followed by a Student-Newman-Keuls post hoc multiple comparison. For testing peak changes from baseline, paired *t*-tests were used. To test the time-course of changes from baseline, a one-way ANOVA with repeated measures followed by Dunnett's test was used. For pairwise comparisons of the area under the curve, a one or two-way ANOVA was used by a Student-Newman-Keuls post hoc multiple comparison. The level of significance was set at $P < 0.05$.

RESULTS

Resting MAP tended to be high in Dahl S versus R rats on regular salt diet (Table 3-1 and 3-2). High salt diet for 3 weeks significantly increased resting MAP by ~ 35 mmHg in Dahl S rats, but not in R rats. HR did not differ among groups (Table 3-2). There were no significant changes in baseline MAP or HR levels by the different pretreatments into the PVN prior to infusion of Na^+ rich aCSF (Table 3-1 and 3-2).

Table 3-1: Resting MAP and HR before and after pretreatments into the PVN prior to infusion of Na^+ rich aCSF. Recording of second baseline was performed 10 minutes after the end of infusion of pretreatments.

Pretreatments in the PVN	*n*	First Baseline		Second Baseline	
		MAP, mmHg	HR, beats/min	MAP, mmHg	HR, beats/min
			Dahl R		
Vehicle (1% acetonitrile in aCSF)	4	102 ± 4	395 ± 5	103 ± 4	401 ± 5
Eplerenone (2 μg)	5	105 ± 3	434 ± 7	104 ± 4	430 ± 10
Vehicle (aCSF)	3	100 ± 3	391 ± 7	103 ± 4	401 ± 6
Vehicle (15% propylene glycol in aCSF)	3	107 ± 4	418 ± 11	108 ± 5	425 ± 9
Benzamil	4	109 ± 3	439 ± 9	108 ± 3	421 ± 7
Fab fragments	5	111 ± 3	423 ± 7	115 ± 4	438 ± 6
Losartan	4	106 ± 5	416 ± 6	110 ± 5	434 ± 11
			Dahl S		
Vehicle (1% acetonitrile in aCSF)	4	111 ± 3*	427 ± 7	109 ± 4	417 ± 6
Eplerenone (2 μg)	5	108 ± 4	438 ± 8	108 ± 5	434 ± 9
Vehicle (aCSF)	3	119 ± 4*	408 ± 7	122 ± 4*	438 ± 10
Vehicle (15% propylene glycol in aCSF)	3	112 ± 3	414 ± 9	116 ± 4	420 ± 9
Benzamil	4	110 ± 5	424 ± 5	112 ± 3	438 ± 6
Fab fragments	5	117 ± 3	439 ± 11	120 ± 4	421 ± 10
Losartan	5	112 ± 2	426 ± 6	116 ± 4	441 ± 6

Values are mean ± SEM; * $p<0.05$ vs. Dahl R.

Table 3-2: Baseline MAP and HR levels prior to first or second infusion of L-NAME (270 or 540 µg), Na^+ rich aCSF (0.3 or 0.7 M) alone, or combined with L-NAME into the PVN of Dahl S and R rats on regular or high salt diet. Forty-five minutes was given after end of first infusion and recording of second baseline.

Infusions Into the PVN	*n*	First Baseline Before First Infusion		Second Baseline Before Second Infusion	
		MAP, mmHg	HR, beats/min	MAP, mmHg	HR, beats/min
			Dahl R		
0.3 M, 0.7 M Na^+-rich aCSF on regular-salt diet	4	100 ± 3	418 ± 10	102 ± 2	430 ± 12
0.7 M, 0.7 M Na^+-rich aCSF on regular-salt diet	5	104 ± 3	415 ± 9	105 ± 2	419 ± 7
0.7 M Na^+-rich aCSF + L-NAME (270 µg), 0.7 M Na^+-rich aCSF + L-NAME (540 µg) on regular-salt diet	4	110 ± 5	405 ± 8	113 ± 3	419 ± 10
L-NAME (270 µg), L-NAME (540 µg) on 3 wk regular-salt diet	4	98 ± 4	400 ± 12	100 ± 3	403 ± 11
L-NAME (270 µg), L-NAME (540 µg) on 3 wk high-salt diet	5	107 ± 5	399 ± 9	111 ± 5	386 ± 10
			Dahl S		
0.3 M, 0.7 M Na^+-rich aCSF on regular-salt diet	5	115 ± 4*	426 ± 9	117 ± 5*	432 ± 11
0.7 M, 0.7 M Na^+-rich aCSF on regular-salt diet	5	113 ± 3*	420 ± 8	112 ± 3	412 ± 10
0.7 M Na^+-rich aCSF + L-NAME (270 µg), 0.7 Na^+-rich aCSF + L-NAME (540 µg) on regular-salt diet	5	113 ± 3	415 ± 7	115 ± 2	399 ± 8
L-NAME (270 µg), L-NAME (540 µg) on 3 wk regular-salt diet	4	106 ± 2	395 ± 8	108 ± 3	399 ± 12
L-NAME (270 µg), L-NAME (540 µg) on 3 wk high-salt diet	5	143 ± 10*†	415 ± 13	138 ± 8*†	404 ± 11

Values are mean ± SEM; * $p<0.05$ vs. Dahl R, # $p<0.05$ vs. regular salt in same rat strain.

Na^+ rich aCSF in the PVN: Unilateral infusion of Na^+ rich aCSF in the PVN caused concentration dependent increases in MAP and HR in Dahl S, R and Wistar rats. In all strains, MAP increased within 3-7 min, and reached maximum by 10 min. HR decreased for the first few minutes and then increased to levels significantly higher than the resting level, reaching maximum by 12-14 min. Both MAP and HR returned to baseline within 20-30 min after end of infusion (Figure 3-3). Na^+ rich aCSF at 0.3 M Na^+ caused 15-40 % and at 0.7 M Na^+ caused 30-40 % larger peak increases in MAP and HR in Dahl S versus R or Wistar rats (Figure 3-3, 3-4). Infusion of Na^+ rich aCSF at sites outside the PVN (Figure 3-1) caused minor non significant increases in MAP and HR in Dahl S (MAP: 4±2 mmHg and HR: 8±4 bpm; n=3), Dahl R (MAP: 3±1 mmHg and HR: 9±4 bpm; n=3) and Wistar rats (MAP: 2±1 mmHg and HR: 8±3 bpm; n=4).

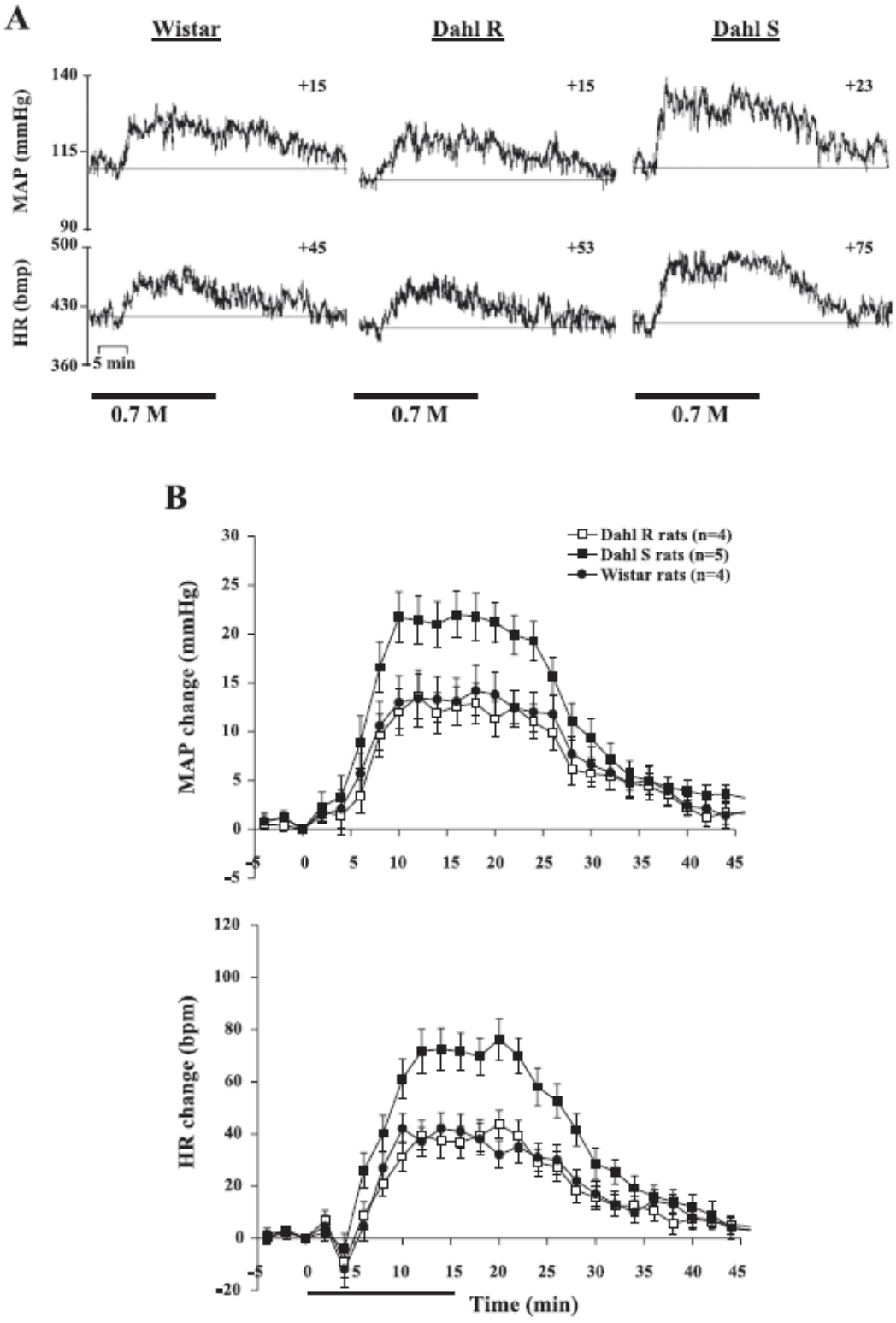

Figure 3-3: Time tracings of MAP and HR responses to unilateral infusion of 0.7 M Na^+ rich aCSF into the PVN of Dahl S, R or Wistar rats. *A*: results from 1 individual rat. *B*: group changes (mean ± SEM). By one-way ANOVA of the area under the curve, MAP and HR responses to Na^+ rich aCSF were significantly larger in Dahl S vs. R and Wistar rats (MAP: F=78.3; HR: F=30.2); [MAP or HR for Dahl S vs. R or Wistar: $P<0.001$). By one-way ANOVA with repeated measures, responses were significantly larger in Dahl S rats from 8 to 24 min for MAP and from 10 to 28 min for HR. Solid horizontal lines represent infusion period.

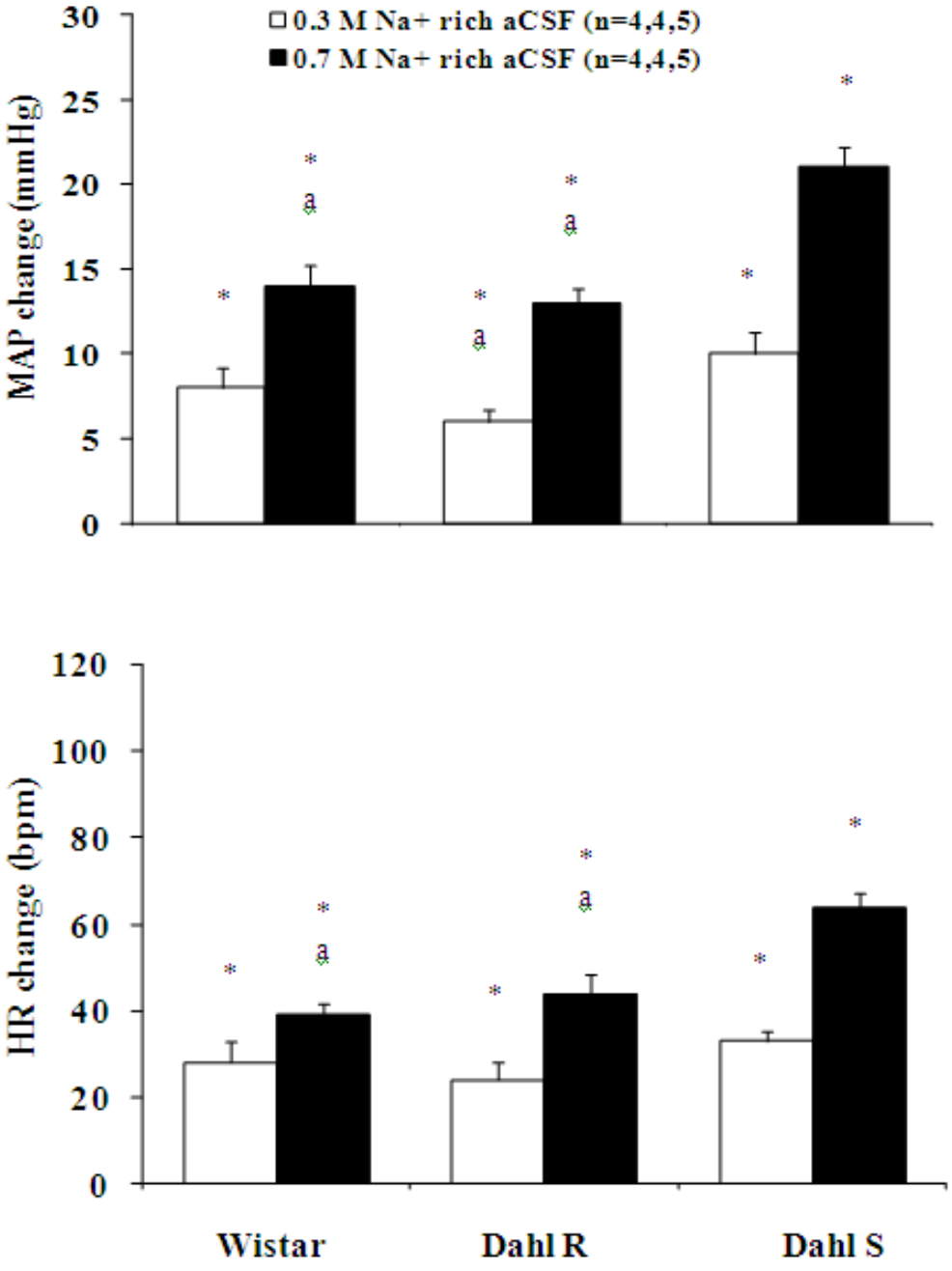

Figure 3-4: Peak MAP and HR responses to unilateral infusion of 0.3 and 0.7 M Na^+ rich aCSF into the PVN of Dahl S, R or Wistar rats. A 30 min recovery period was given between infusion of 0.3 and 0.7 M Na^+ rich aCSF. Values are mean ± SEM. By two-way ANOVA, responses in MAP and HR were significantly different between rat strains, concentrations of Na^+ rich aCSF and for the interactions between strain and Na^+ concentration (MAP: F= 37.2, 167.0, 4.6; HR: F= 21.9, 81.8, 6.2); [0.7 M in Dahl S vs. R or Wistar (MAP and HR: P<0.001); 0.3 vs. 0.7 M in Dahl R, S and Wistar (MAP and HR: P<0.001)]. * p<0.05 vs. baseline. a p<0.05 vs. Dahl S (0.3 or 0.7 M Na^+ rich aCSF).

MR, Vasopressin and Na^+ rich aCSF in the PVN: Unilateral infusion of 0.7 M Na^+ rich aCSF in the PVN caused the same peak increases in MAP and HR after local pretreatment with vehicle or eplerenone at the low dose of 200 ng in Dahl S and R rats (data not shown). Eplerenone at the high dose of 2 µg and AVP antagonist similarly did not affect the peak pressor responses to Na^+ rich aCSF in both strains (Figure 3-5). Na^+ rich aCSF no longer caused an initial decrease in HR in rats injected with AVP antagonist compared to rats treated with Na^+ rich aCSF alone per se (ie. without AVP antagonist; as in Figure 3-3). There were no differences between peak MAP and HR responses to the first and second infusion of Na^+ rich aCSF in the PVN of Dahl R and S rats [Dahl R (MAP: 14±2 versus 15±2 mmHg; HR: 57±6 versus 51±5 bpm); Dahl S (MAP: 22±2 versus 21±1 mmHg; HR: 80±9 versus 73±6 bpm)] ($P>0.05$). Unilateral infusion of vehicle for 10 min at sites outside the PVN (Figure 3-1) caused non-significant changes in MAP and HR in Dahl S (MAP: -1±2 mmHg and HR: -2±6 bpm; n=3) and Dahl R rats (MAP: 1±2 mmHg and HR: 2±9 bpm; n=2).

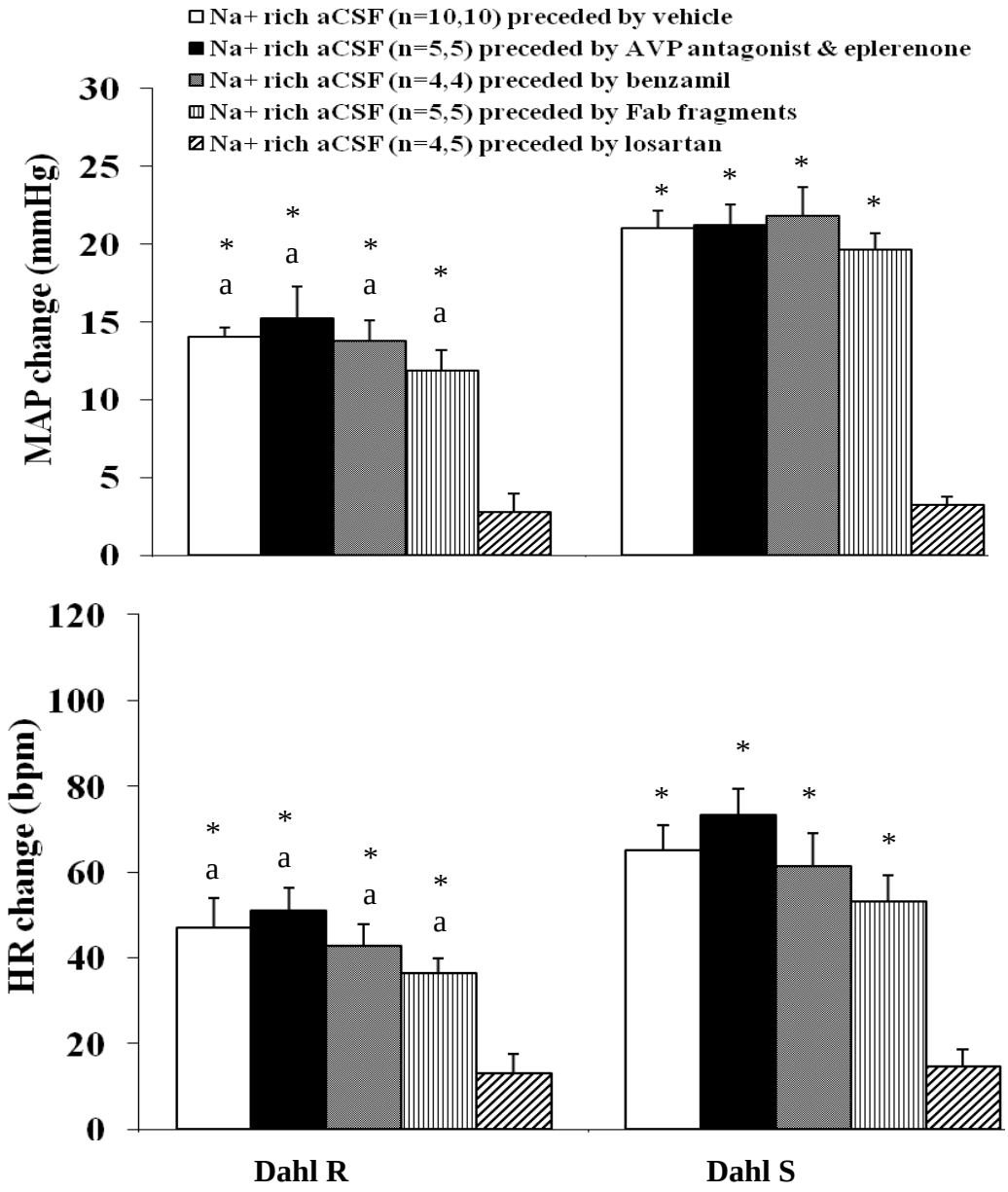

Figure 3-5: Effects of vehicles (combined data from 3), eplerenone (high dose; 2 µg), benzamil, Fab fragments or losartan in the PVN on peak MAP and HR responses to local unilateral infusion of 0.7 M Na^+ rich aCSF. Na^+ rich aCSF was infused 10 or 15 min after infusion of vehicles or blockers. For eplerenone groups, AVP antagonist was injected iv 5 min prior to infusion of Na^+ rich aCSF. Values are mean ± SEM. By two-way ANOVA of data from the eplerenone experiment, responses of MAP and HR were significantly different between rat strains (MAP: F= 46.3; HR: F= 8.7); [vehicle or eplerenone + 0.7 M in Dahl S vs. R (MAP: P<0.001; HR: P<0.03)]. By two-way ANOVA of data from benzamil, Fab fragments and losartan experiment, responses of MAP and HR were significantly different between rat strains (MAP: F= 13.5; HR: F= 42.1); [vehicles, benzamil or fab fragments + 0.7 M in Dahl S vs. R (MAP: P<0.03; HR: P<0.001)] and between blockers in the PVN (MAP: F= 20.2; HR: F= 62.7); [vehicle, benzamil or fab fragments vs. losartan in Dahl S and R (MAP and HR: P<0.008)]. By two-way ANOVA of peak increases, there were no significant differences between MAP and HR responses to Na^+ rich aCSF in the PVN preceded by the 3 different vehicles in Dahl R (MAP: 14±1, 15±2, 13±1 mmHg; HR: 54±5; 48±9; 44±10 bpm; n=4,3,3) or S rats (MAP: 19±1, 22±2, 21±1 mmHg; HR: 65±7; 59±8; 71±11 bpm; n=4,3,3). * p<0.05 vs. baseline. a p<0.05 vs. Dahl S (vehicle, eplerenone, benzamil or Fab fragments).

Benzamil, Fab fragments, Losartan, L-NAME and Na^+ rich aCSF in the PVN: Unilateral infusion of benzamil, Fab fragments or vehicles into the PVN of Dahl S and R rats did not affect peak MAP and HR responses to local infusion of 0.7 M Na^+ rich aCSF (Figure 3-5). When resting periods between Fab fragments and Na^+ rich aCSF were extended from 10 min to 2, 6 or 18 h, Fab fragments also did not affect peak MAP (2 h: 22±2 mmHg, 6 h: 20±1 mmHg and 18 h: 20±2 mmHg) and HR (2 h: 71±6 bpm, 6 h: 62±4 bpm and 18 h: 64±7 bpm) responses to Na^+ rich aCSF in Dahl S rats (n=3,5,3). In contrast, losartan fully prevented the MAP and HR responses to 0.7 M Na^+ rich aCSF in the PVN of both Dahl S and R rats (Figure 3-5).

***L-NAME and Na^+ rich aCSF in the PVN*:** The time courses of the MAP response to Na^+ rich aCSF alone, or combined with L-NAME were similar in Dahl S and R rats, whereas HR increased faster in response to L-NAME and Na^+ rich aCSF compared to Na^+ rich aCSF alone (Figure 3-6). L-NAME at both doses significantly enhanced MAP and HR responses to 0.7 M Na^+ rich aCSF in the PVN of Dahl S and R rats (Figure 3-6 and 3-7). The enhancement in MAP by L-NAME was larger in Dahl R [270 µg: +11 mmHg (+79 %)]; [540 µg: +15 mmHg (+107 %)] versus S rats [270 µg: +4 mmHg (+20%)]; [540 µg: +10 mmHg (+50%)]. Similarly, increases in HR responses were larger in Dahl R [270 µg: +60 bpm (+171%)]; [540 µg: +56 bpm (+160%)] versus S rats [270 µg: +32 bpm (+46%)]; [540 µg: +37 bpm (+53%)]. As a result, the absolute MAP and HR responses to Na^+ rich aCSF when combined with L-NAME were no longer significantly different between the two strains (Figure 3-6 and 3-7).

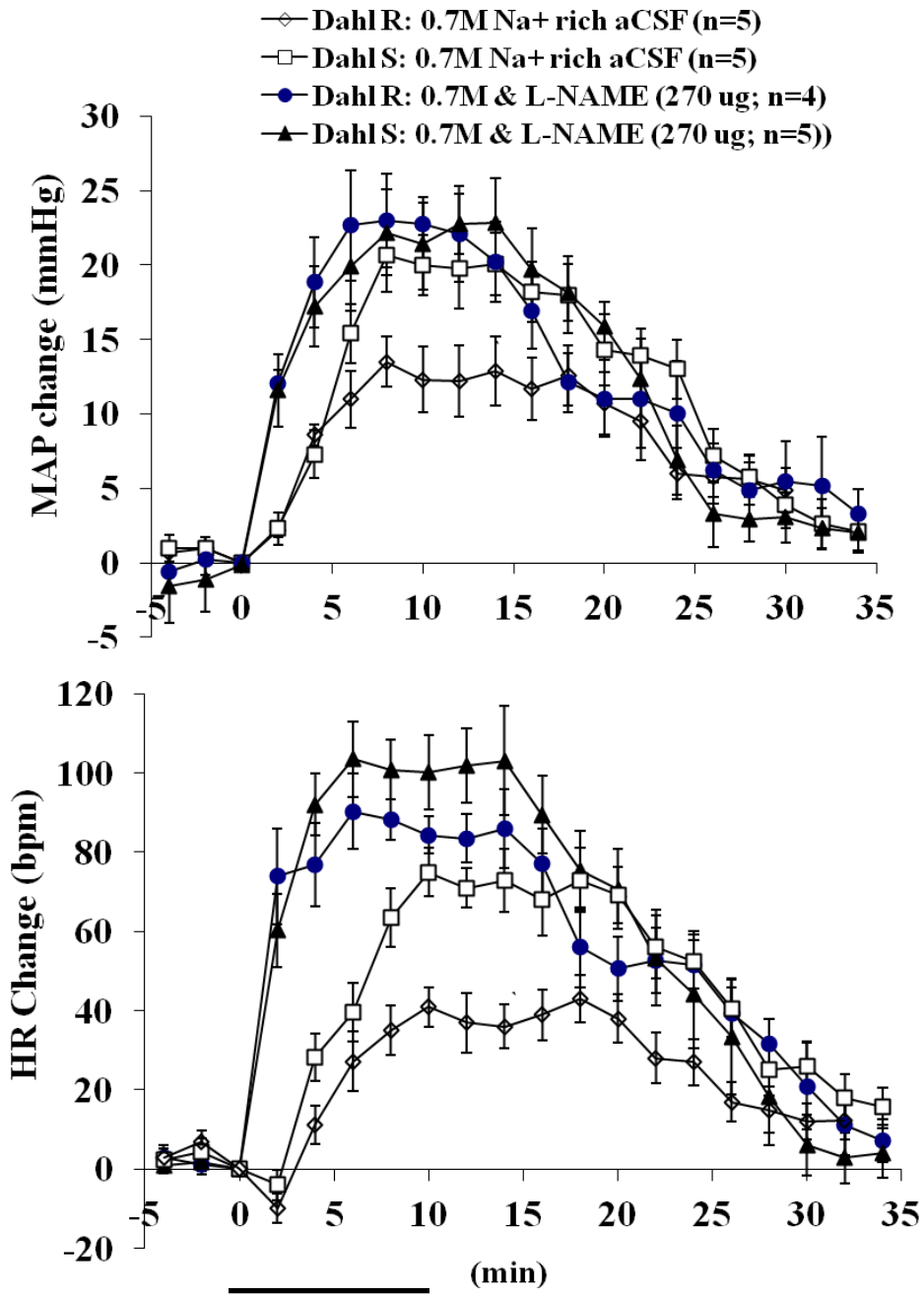

Figure 3-6: Time tracings of MAP and HR responses to unilateral infusion of 0.7 M Na^+ rich aCSF alone, or together with L-NAME (27 µg/ min) in the PVN of Dahl R and S rats on regular salt diet. Values are mean ± SEM. By two-way ANOVA of the area under the curve, responses in MAP and HR were significantly different between rat strains (MAP: *F*= 6.0; HR: *F*= 31.2); [0.7 M in Dahl S vs. R (MAP: P=0.009; HR: P<0.001)] and between treatments in the PVN (MAP: *F*= 14.4; HR: *F*= 108.7); [0.7 M vs. 0.7 M + L-NAME in Dahl R (MAP: P=0.002; HR: P<0.001)]. By one-way ANOVA with repeated measures, responses to Na^+ rich aCSF alone were significantly less in Dahl R than S rats, or from both strains infused with Na^+ rich aCSF combined with L-NAME from 6 to 18 min for MAP and from 8 to 16 min for HR. The solid horizontal lines represent the infusion period.

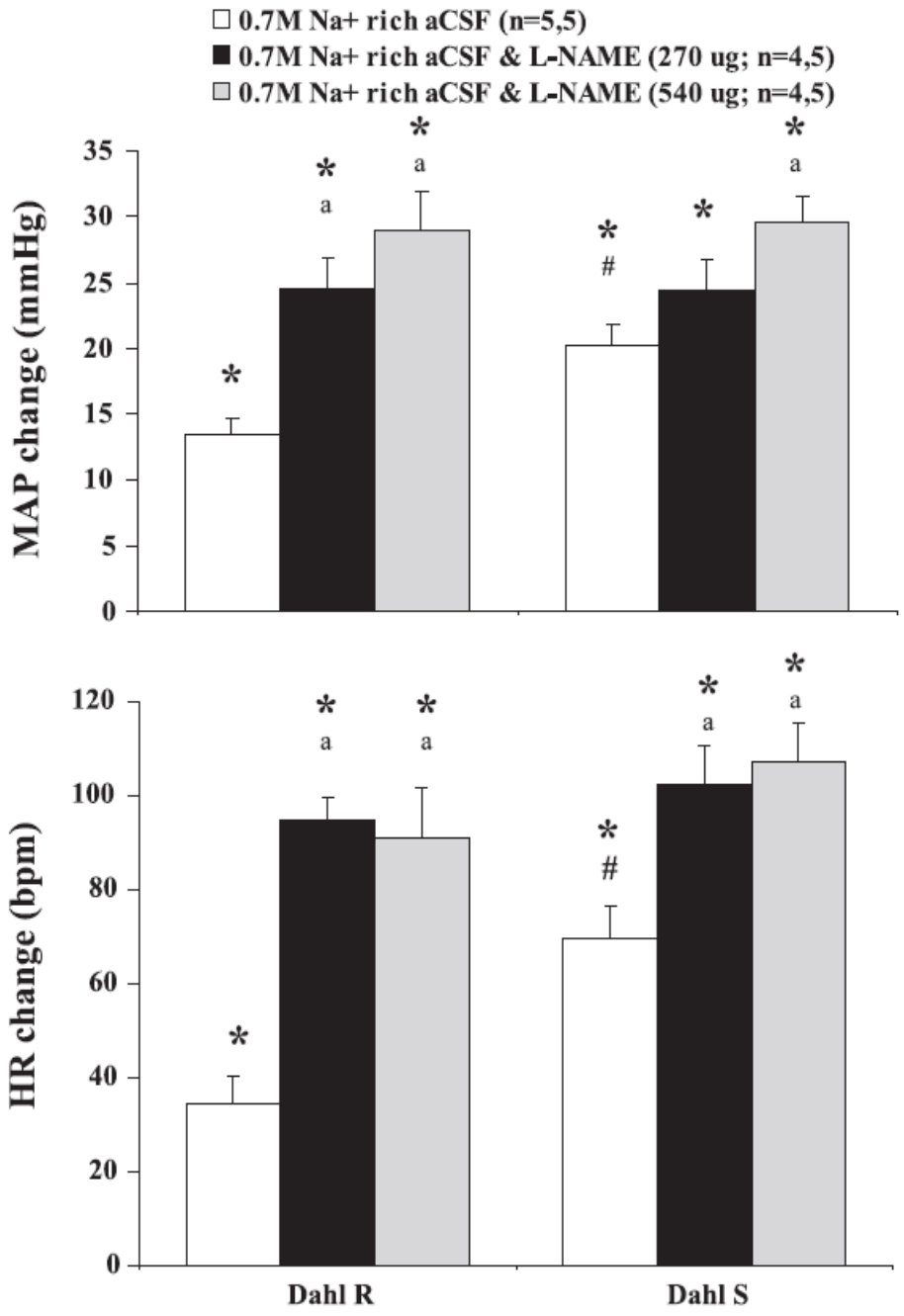

Figure 3-7: Peak MAP and HR responses to unilateral infusion of 0.7 M Na^+ rich aCSF alone, or together with L-NAME at both doses in the PVN of Dahl S and R rats. A 45 min recovery period was given between infusion of Na^+ rich aCSF together with L-NAME at 270 versus 540 µg. Values are mean ± SEM. By two-way ANOVA, responses in MAP and HR were significantly different between rat strains (MAP: F= 4.7; HR: F= 12.2); [0.7 M in Dahl S vs. R (MAP: P=0.012; HR: P=0.003)] and between treatments in the PVN (MAP: F= 22.0; HR: F= 33.7); [0.7 M vs. 0.7 M + L-NAME (270 or 540 µg) in Dahl R (MAP and HR: P<0.001); 0.7 M vs. 0.7 M + L-NAME (540 µg) in Dahl S (MAP: P=0.005; HR: P=0.001)]. * p<0.05 vs. baseline. # p<0.05 vs. Dahl R. a p<0.05 vs. 0.7M Na^+ rich aCSF alone.

Ouabain and Ang II in the PVN: Unilateral microinjection of ouabain into the PVN of Dahl S and R rats caused dose-related increases in MAP and HR (Figure 3-8). In both strains, MAP and HR decreased for the first 4-6 min and then increased to levels significantly higher than the resting level. Peak increases in MAP and HR occurred within 6-12 min and both returned to baseline within 45 min after injection of ouabain. Pressor responses to unilateral injection of ouabain (40, 80 or 160 ng) were not significantly different in Dahl S versus R rats (Figure 3-8). Bilateral injections were performed to assess whether crosstalk between left and right sides of the PVN may prevent differences between the two strains. Bilateral microinjection of ouabain (80 ng/ side) into the PVN also caused the same increases in MAP and HR in Dahl S and R rats (Figure 3-8). Compared to unilateral microinjection of ouabain, bilateral microinjection of ouabain at an equivalent dose per side (80 ng) caused nearly two-fold larger increases in MAP and HR (Figure 3-8). The time-course of changes in MAP and HR was similar for unilateral and bilateral injections of ouabain (data not shown). Bilateral injection of ouabain (80 ng/side) outside the PVN (Figure 3-1) caused small increases in MAP and HR in Dahl S (MAP: 7±2 mmHg and HR: 24±4 bpm; n=3) and Dahl R rats (MAP: 5±2 mmHg and HR: 17±5 bpm; n=3).

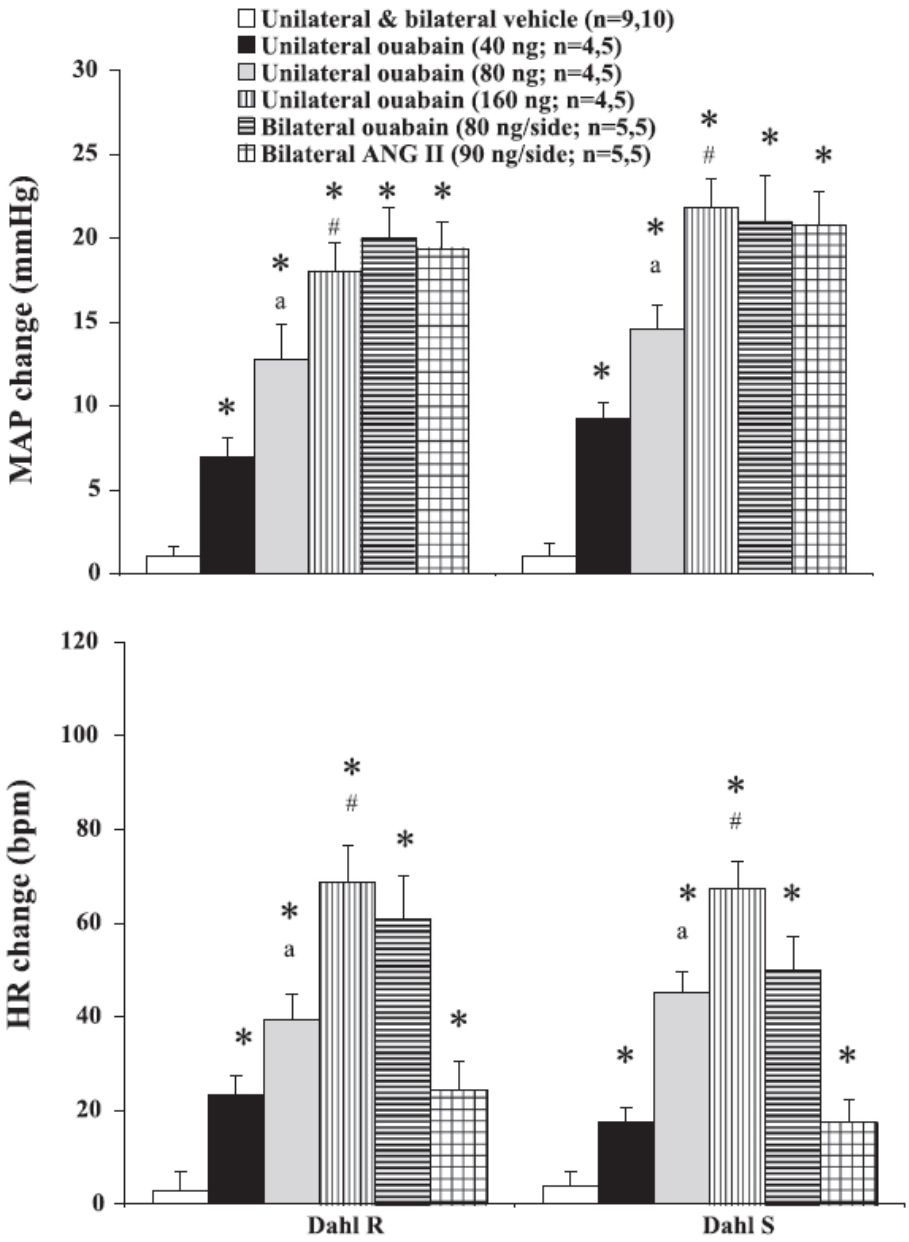

Figure 3-8: Peak MAP and HR responses to unilateral or bilateral microinjection of vehicle, ouabain (unilateral: 40, 80, 160 ng and bilateral: 80 ng/side) or Ang II (90 ng/side) into the PVN of Dahl S and R rats. Rats were treated with either unilateral or bilateral microinjections of vehicle, ouabain or Ang II. Values are mean ± SEM. By two-way ANOVA, responses in MAP and HR were significantly different between treatments in the PVN (MAP: F= 49.3; HR: F= 35.6); [Dahl S and R: vehicle vs. all other treatments (MAP and HR: P<0.03); ouabain 40 vs. 80 ng (MAP and HR: P<0.002); ouabain 80 vs. 160 ng (MAP and HR: P<0.04)]. By two-way ANOVA of the peak increases or areas under the curve (figure not shown), there were no significant differences between MAP and HR responses to ouabain or Ang II in Dahl S vs. R rats. A 20-45 min recovery period was given between injections. * p<0.05 vs. aCSF. a p<0.05 vs. ouabain (40 ng). # p<0.05 vs. ouabain (80 ng).

Bilateral microinjection of Ang II (90 ng/ side) into the PVN of Dahl S and R rats increased MAP and HR (Figure 3-8). Increases in MAP and HR occurred within 1 min and peak increases within 5 min. Both MAP and HR returned to baseline within 45 min (data not shown). Both MAP and HR responses to Ang II in the PVN were not significantly different in Dahl S versus R rats (Figure 3-8). Bilateral injection of Ang II (90 ng/side) outside the PVN (Figure 3-1) caused only minor non-significant changes in MAP and HR in Dahl S (MAP: 4±2 mmHg and HR: 7±5 bpm; n=3) and Dahl R rats (MAP: 4±1 mmHg and HR: -2±6 bpm; n=3). Bilateral injection of vehicle at sites outside the PVN also caused only minor changes in MAP and HR in Dahl S rats (MAP: 2±2 mmHg and HR: 12±8 bpm; n=3) and Dahl R (MAP: 1±2 mmHg and HR: 15±5 bpm; n=3).

***L-NAME in the PVN of Dahl rats on regular or high salt diet*:** Infusion of L-NAME (270 or 540 µg) over 10 min increased MAP and HR within 2-3 min, with maximum reached by 15 min in both strains (Figure 3-9). Both MAP and HR returned to baseline within 15-20 min after end of infusion (Figure 3-9). Infusion of L-NAME in the PVN caused the same increases in MAP and HR in Dahl S and R rats on regular salt diet and in Dahl R rats on regular and high salt diet (Figure 3-9 and 3-10). However, increases in MAP by L-NAME in Dahl S rats were significantly lower on high versus regular salt diet or compared to Dahl R rats on either diet (Figure 3-9 and 3-10). On high salt diet, increases in MAP by L-NAME in the PVN were attenuated by ~30 % in Dahl S versus R rats (Figure 3-10). In contrast, HR responses were not significantly affected by high salt diet in Dahl S. Infusion of L-NAME (540 µg) at sites outside the PVN (Figure 1) caused non-significant increases in MAP and HR in Dahl S (MAP: 3±3 mmHg and HR: 18±7

bpm; n=3) and Dahl R rats (MAP: 1±2 mmHg and HR: 7±6 bpm; n=3) on regular salt diet. MAP and HR responses to L-NAME at sites outside the PVN were not significantly different in Dahl S and R rats on high versus regular salt diet (data not shown).

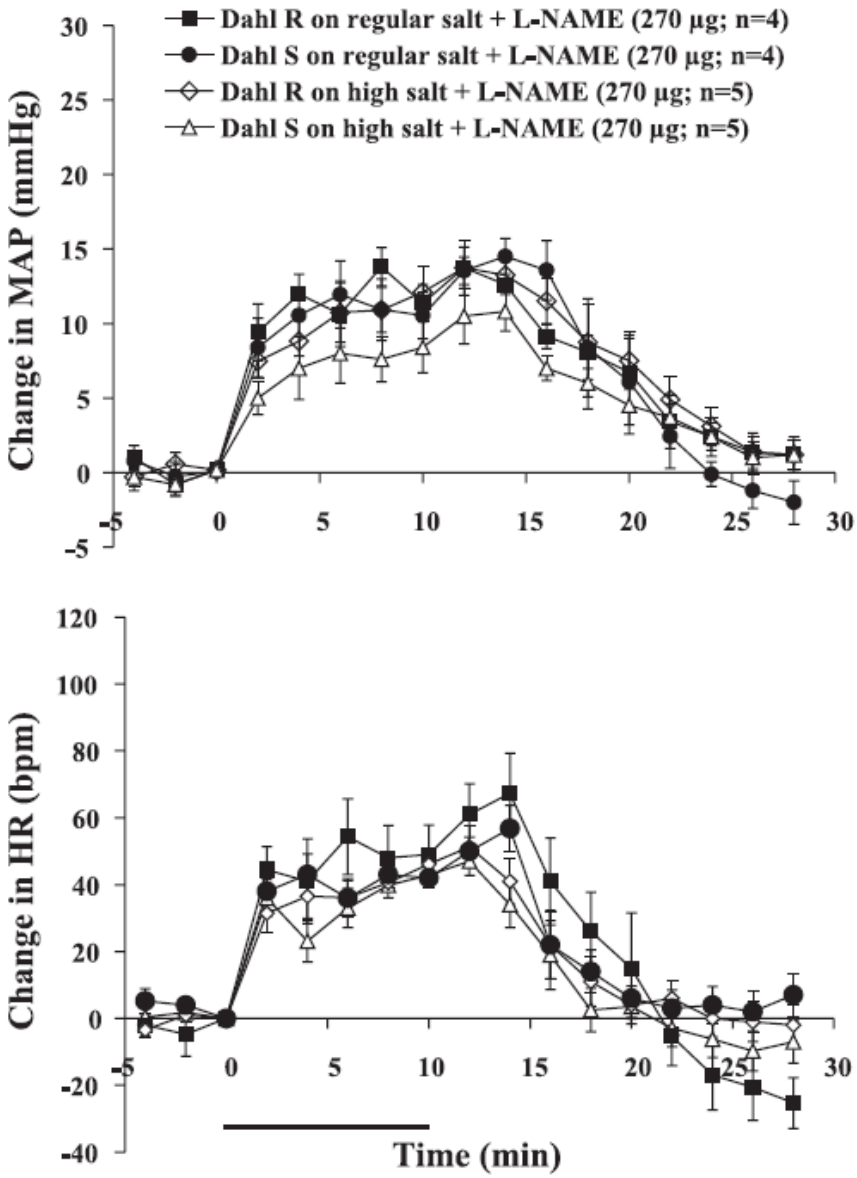

Figure 3-9: Time tracings of MAP and HR responses to unilateral infusion of L-NAME (27 µg/ min) for 10 min in the PVN of Dahl R and S rats on regular or high salt diet. Values are mean ± SEM. By two-way ANOVA of the area under the curve, responses of MAP were significantly different between rat strains and for the interactions between strain and diet (*F*= 5.0, 9.1); [high salt in Dahl S vs. R (P=0.002)]; [high vs. regular salt in Dahl S (P=0.018)]. HR responses to L-NAME were not significantly different between rat strains or diet treatments. The solid horizontal lines represent the infusion period.

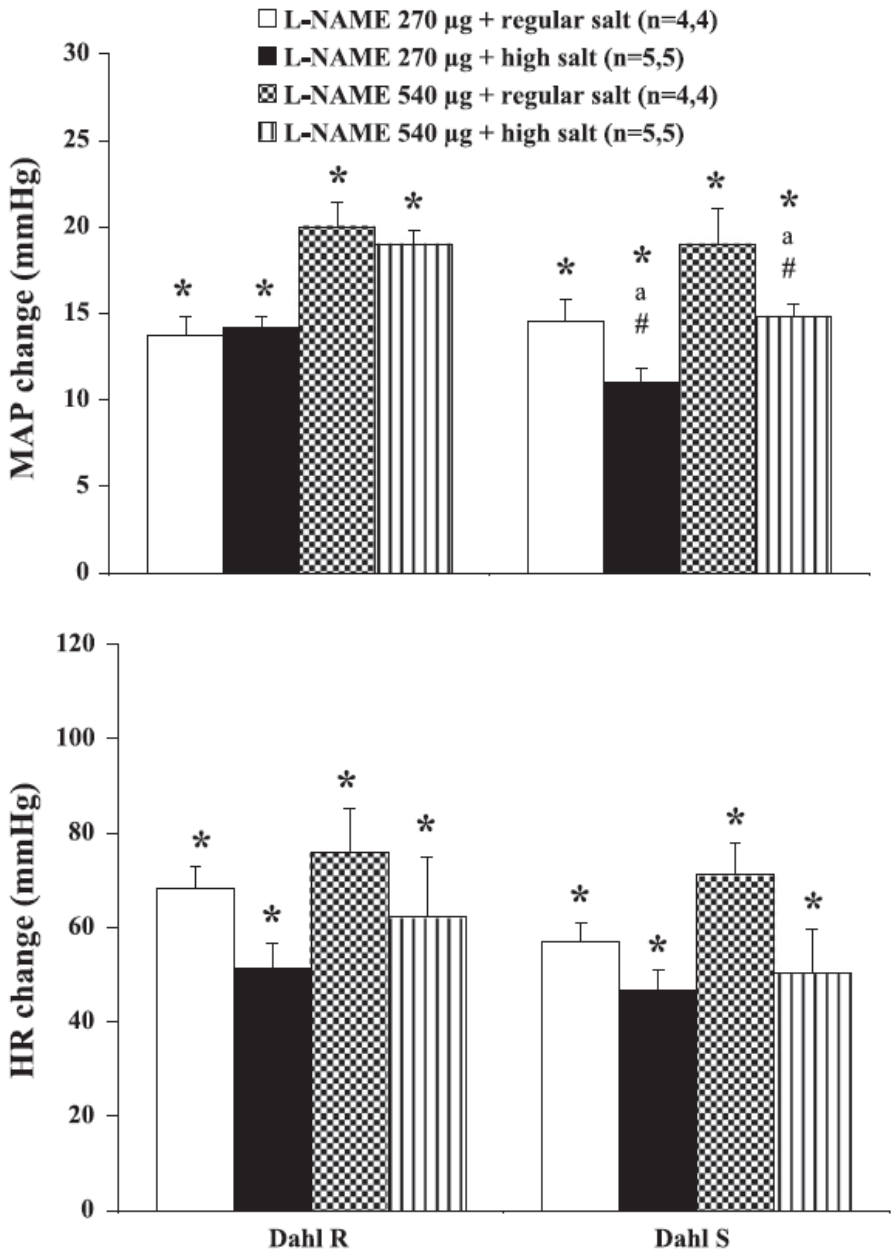

Figure 3-10: Peak MAP and HR responses to unilateral infusion of L-NAME (27 or 54 µg/ min) for 10 min in the PVN of Dahl S and R rats on regular or high salt diet. For each rat, a 45 min recovery period was given between the 2 infusions of L-NAME. Values are mean ± SEM. By two-way ANOVA, MAP responses to L-NAME (270 µg) were significant by interactions between rat strain and diet (F= 4.8); [high salt in Dahl S vs. R (P=0.018)]; [high vs. regular salt in Dahl S (P=0.016)]. MAP responses to L-NAME (540 µg) were significant by strain and diet (F= 7.1, 6.1); [high salt in Dahl S vs. R (P=0.005)]; [high vs. regular salt in Dahl S (P=0.01)]. HR responses to L-NAME at both doses were not significantly different between rat strains or diet treatments. * p<0.05 vs. baseline. a p<0.05 vs. Dahl R (540 µg). # p< 0.05 vs. L-NAME (270 or 540 µg) + regular salt.

DISCUSSION

The present study shows as new findings that infusion of Na^+ rich aCSF in the PVN causes $[Na^+]$-dependent increases in BP and HR which are significantly larger in Dahl S vs. R and Wistar rats, whereas responses to ouabain, Ang II or L-NAME in the PVN are the same. Losartan blocked all responses to Na^+ in both Dahl S and R rats, whereas L-NAME enhanced them more in Dahl R than S rats, thereby equalizing these responses in the two strains. Pressor responses to L-NAME in the PVN were attenuated by high salt diet in Dahl S, but not R rats. The results suggest that the BP and HR responses to $[Na^+]$ in the PVN of Dahl S and R rats are mediated by AT_1-receptor activation. Both acute and chronic increases in $[Na^+]$ in the PVN appear to inhibit NO release in the PVN of Dahl S, but not R rats, thereby contributing to the enhanced pressor responses to Na^+ and presumably dietary salt-induced hypertension in Dahl S.

Na^+ rich aCSF and Ang II in the PVN

High salt intake increases CSF $[Na^+]$ in Dahl S but not R or Wistar rats (Huang et al. 2004, Nakamura and Cowley 1989). To assess responsiveness to Na^+, we performed chronic icv infusion of Na^+ rich aCSF in Dahl S and R rats on regular salt to raise CSF $[Na^+]$ to levels observed in Dahl S rats on high salt diet (Huang et al. 2001b). A chronic increase in CSF $[Na^+]$ will also increase tissue $[Na^+]$ (Wang et al. 2010), and thereby activate eg. sympatho-excitatory neurons in the PVN (Gabor and Leenen 2009). In the present study, we first assessed whether this enhanced responsiveness to Na^+ exists in the PVN of Dahl S versus R and Wistar rats.

In both Wistar (Gabor and Leenen 2009) and Dahl rats, unilateral infusion of Na^+ rich aCSF in the PVN causes concentration-related increases in BP and HR. These

responses are likely mediated by changes in [Na^+] per se and not osmolality or a volume-induced stretch since infusion of mannitol at equivalent osmolality or aCSF at same volume in the PVN of Wistar rats caused no significant changes in BP or HR (Gabor and Leenen 2009, Jin et al. 2001). Similar to our previous findings in Wistar rats (Gabor and Leenen 2009), losartan blocked all responses to Na^+ rich aCSF in the Dahl R controls. This finding indicates that the pressor responses to Na^+ in the PVN of Wistar and Dahl R rats are mediated by AT_1-receptor activation. An increase in [Na^+] in the PVN increases the extra/intracellular Na^+ gradient, and intracellular Ca^{2+} through Na^+/Ca^{2+} channel exchangers (NCX). This may facilitate Ang II release (Doris 1988), resulting in AT_1-receptor activation. AT_1-receptor stimulation in the PVN may excite a subpopulation of neurons projecting to the IML (Li et al. 2003a) and/or RVLM (Cato and Toney 2005), thereby increasing sympathetic nerve activity, BP and HR (Dampney 1994). Additionally, AT_1-receptor stimulation in the PVN may activate magnocellular neurons in the PVN (Latchford and Ferguson 2004), causing AVP release (Qadri et al. 1998), thereby enhancing sympathoinhibitory baroreflex function (Hasser et al. 1997). This effect may explain why the initial decrease in HR from Na^+ rich aCSF in the PVN was prevented by an AVP antagonist, but does not appear to influence the pressor responses since the AVP antagonist did not affect the increases in BP.

Infusion of Na^+ rich aCSF into the PVN caused up to 40 % larger increases in BP and HR in Dahl S versus R or Wistar rats. Losartan also fully blocked the pressor responses to Na^+ rich aCSF in Dahl S rats indicating that the enhanced pressor responses to Na^+ in the PVN of Dahl S rats also depend on AT_1-receptor activation. Ang II in the PVN caused the same pressor responses in Dahl S and R rats, indicating that responses to

AT_1-receptor activation in the PVN per se are the same in Dahl S and R rats. The larger effect in Dahl S rats therefore likely reflects enhanced Ang II release by Na^+. Subsequent experiments investigated mechanisms potentially contributing to this difference.

MR-benzamil blockable Na^+ channel-"ouabain" pathway in the PVN

In a series of experiments, we established that the aldosterone– MR– Na^+ channel– "ouabain" pathway mediates the sympathoexcitation and hypertension in Dahl S rats on high salt or to icv infusion of Na^+ rich aCSF (see (Huang et al. 2006a, Huang et al. 2008c, Wang et al. 2003a)). Since aldosterone increases hypothalamic "ouabain" only in Dahl S rats (Huang et al. 2005) and both aldosterone and ouabain enhance responses to Na^+ (Gabor and Leenen 2009), this pathway may contribute to the enhanced responses to Na^+ in Dahl S rats. Increased release of a neuromodulator such as "ouabain" may lead to more binding to the Na^+/K^+ATPase on the surface of neurons, increasing intracellular Ca^{2+} and thereby possibly activating Ang II release (Doris 1988). Both icv (Huang et al. 1992, Huang et al. 2001b) and unilateral and bilateral micro-injection into the PVN of ouabain caused the same dose-related increases in BP and HR in Dahl S and R rats, indicating that increased responses to ouabain and presumably "ouabain" per se do not contribute to the enhanced responses to Na^+ infused either icv or in the PVN of Dahl S rats. Our findings show that eplerenone, benzamil and Fab fragments do not affect the pressor responses to Na^+ rich aCSF in the PVN of Dahl S and R rats, suggesting that MR, benzamil blockable Na^+ channels and "ouabain" are not involved in mediating the pressor responses to Na^+ in the PVN of Dahl R rats or in the enhanced pressor responses in Dahl S rats. Doses of eplerenone, benzamil and Fab fragments were similar to doses used in our previous study to block the enhancement by aldosterone of the pressor responses to

Na^+ rich aCSF in the PVN (Gabor and Leenen 2009). Fab fragments bind free "ouabain" leading to dissociation of "ouabain" from the enzyme. In Dahl S rats on high salt diet, Fab fragments injected icv began to reverse hypertension at 4 hrs (Huang and Leenen 1994), while in *in vitro* studies, addition of Fab fragments for 1 hr did not reverse "ouabain" mediated inhibition of ^{86}RB uptake (via Na^+/K^+/ATPase) by erythrocytes but did reverse the inhibition when the incubation was extended for 12 hrs (Balzan et al. 1991). After extending the time between Fab fragments and Na^+ rich aCSF to 2, 6 or 18 hours, the pressor responses to Na^+ remained enhanced, further supporting that "ouabain" is not involved in the enhanced pressor responses to Na^+ in the PVN of Dahl S rats on regular salt intake. These findings indicate that the aldosterone- "ouabain" pathway does not contribute to the enhanced responsiveness to a short-term increase in Na^+ in the PVN of Dahl S rats on regular salt diet. Activation of this pathway in the PVN may require a more chronic increase in $[Na^+]$ and may contribute to the increase in resting BP and a further increase in neuronal responsiveness to Na^+ in Dahl S rats on high salt diet. Acute infusion of blockers of this pathway in the PVN may not last long enough to cause sufficiently long inhibition of these "slow" mechanisms and chronic local infusions likely will affect /destroy neurons. As an alternative approach, injection of an adeno-associated virus (AAV) carrying a short-hairpin small-interference RNA (siRNA) against eg. MR into the PVN was recently suggested to achieve long-term inhibition (Xue et al. 2011).

nNOS in the PVN

Consistent with previous studies (Zhang et al. 1997), the NOS blocker L-NAME in the PVN increased BP and HR. L-NAME is a non-specific inhibitor of NOS, but is more specific for nNOS and eNOS vs. iNOS (Southan and Szabo 1996). BP and HR responses

to infusion of L-NAME in the PVN were inhibited by 88% in rats injected in the PVN with a dominant negative construct against nNOS (Rossi et al. 2010). These findings suggest that responses to L-NAME in the PVN are predominantly mediated by nNOS inhibition, decreasing local NO production and thereby disinhibiting the PVN. Responses to L-NAME were the same in Dahl S and R rats on regular salt diet, suggesting that on regular diet, NO release in the PVN is similar in the two strains. These functional findings are consistent with previous studies showing that mRNA expression of nNOS is the same in the PVN of Dahl S and R rats on regular diet (Serino et al. 2001).

L-NAME enhanced responses to Na^+ rich aCSF more in R than S rats and equalized their responses to Na^+. In Dahl R rats, the combined increases in BP or HR from L-NAME and Na^+ rich aCSF in the PVN were similar to the sum of those from L-NAME and Na^+ rich aCSF alone, suggesting that Na^+ did not change NOS or NO activity in the PVN of this strain. In contrast, in Dahl S, combined responses from L-NAME and Na^+ rich aCSF in the PVN were smaller than the sum of each alone. These findings suggest that an acute increase in $[Na^+]$ in the PVN inhibits local NO release in Dahl S causing larger increases in BP to Na^+. In Dahl S rats, a decrease in NO in the PVN caused by Na^+ may cause less local GABA release (Horn et al. 1994, Zhang and Patel 1998). A $GABA_A$-receptor blocker in the PVN increases SNA and BP (Li et al. 2006a, Zhang and Patel 1998), and this effect can be mostly prevented by an AT_1-receptor blocker (Chen and Toney 2003), suggesting that GABA release in the PVN tonically inhibits SNA and BP, an effect that depends on local AT_1-receptor activation. A decrease in GABA release may cause less $GABA_A$ receptor activation on angiotensinergic neurons

in the PVN, thereby disinhibiting local Ang II release. This effect of Na^+ on GABA and Ang II release in the PVN of Dahl S would enhance local AT_1-receptor activation, further increasing the activity of sympatho-excitatory neurons, thereby contributing to the enhanced pressor responses.

Na^+ in the PVN causes local Ang II release (Doris 1988), while Ang II in the PVN increases local NO release (Li et al. 2006b), enhancing local GABA release, thereby inhibiting the PVN (Horn et al. 1994). A dysfunction in this Ang II- NO- GABA negative feedback in Dahl S unlikely contributes to inhibition of NO release in the PVN by a local increase in Na^+, since pressor responses to Ang II in the PVN were the same in Dahl S and R rats.

High salt diet attenuated pressor responses to L-NAME in the PVN of Dahl S but not R rats. These findings are consistent with those from acute infusions of Na^+ and L-NAME in the PVN (as discussed above), and suggest that high salt intake inhibits NO release in the PVN of Dahl S but not R rats. Similar to acute increases in $[Na^+]$, increases in CSF $[Na^+]$ from high salt in Dahl S (Huang et al. 2004, Nakamura and Cowley 1989) may raise interstitial $[Na^+]$ in the PVN causing inhibition of local NO release, thereby disinhibiting local sympatho-excitatory neurons and contributing to the elevated SNA and BP. High salt diet does not increase CSF $[Na^+]$ in Dahl R rats (Huang et al. 2004, Nakamura and Cowley 1989). To determine whether chronic increases in CSF $[Na^+]$ cause differential NO release in the PVN of Dahl S versus R rats, BP responses to L-NAME in the PVN of Dahl S and R rats chronically icv infused with Na^+ rich aCSF need to be assessed. Since chronic icv infusion of Na^+ rich aCSF causes ~3 fold larger increases in BP in Dahl S versus R rats (Huang et al. 2001b), we expect that chronic icv

infusion of Na^+ rich aCSF also inhibits local NO release in Dahl S but not R rats, causing attenuated BP responses to L-NAME in the PVN of Dahl S. High salt diet increases mRNA expression of nNOS in the PVN of both Dahl S and R rats, but the increase is nearly two-fold larger in Dahl S (Serino et al. 2001). To our knowledge, no studies have yet assessed whether in Dahl S and R rats, high salt diet causes parallel increases in nNOS protein expression and in NO activity in the PVN. High salt diet increases AT_1-receptor densities in the PVN of Dahl S, and to a less extent in R (Wang et al. 2003c). An increase in AT_1-receptor stimulation in the PVN may enhance local release of superoxide anions (Erdos et al. 2006), scavenging NO (Krukoff 1999, Zanzinger 2002) and decreasing its effectiveness in Dahl S.

Together, our results indicate that an enhanced pressor response to Na^+ is present in the PVN of Dahl S versus R rats and is mediated by enhanced Ang II release and AT_1-receptor activation. An acute and chronic increase in Na^+ by high salt intake may inhibit NO release in the PVN of Dahl S, but not R rats, contributing to the enhanced pressor responses to Na^+, and presumably, to dietary salt-induced hypertension in Dahl S rats.

Limitations

The actual levels of Na^+ in the PVN of Dahl S rats on high salt diet have not yet been defined. It is difficult to assess whether the quantity of Na^+ infused into the PVN caused concentrations within or above the pathophysiological range. The actual interstitial $[Na^+]$ depends on the rate of infusion and spread of the infusion throughout the PVN as well as the concentration of the perfusate.

Na^+ rich aCSF infused in the PVN may spread and stimulate sympatho-excitatory neurons outside the PVN. However, the distribution of Evans Blue dye was largely

within the PVN after infusion for 5 min when BP and HR responses to Na^+ were clearly apparent and infusions in the vicinity of the PVN did not significantly increase BP or HR.

The anesthesia and (minor) surgery within 24 hours of experimental manipulation may alter central regulation of BP. To minimize possible differential effects on S versus R rats, all conditions, including the time of arterial cannulation surgeries (performed for 15-20 min in the early afternoon) and experiments performed the next morning were kept the same in both strains. A majority of studies from other laboratories performing injections into the PVN and BP recordings were conducted after acute surgery under anesthesia (Freeman and Brooks 2007, Li et al. 2006a, Ye et al. 2011, Zhang et al. 2006). These experimental approaches appear clearly less desirable. One may be concerned about manipulations of conscious rats for injections in the PVN. However, in all protocols, vehicle controls had no effect on BP or HR when infused either inside or in the vicinity of the PVN.

Perspectives and Significance

The pathophysiological relevance of Na^+ induced activation of neurons in the PVN for the hypertension associated with increases in CSF [Na^+] in Dahl S rats on high salt diet still needs to be assessed. Increases in CSF [Na^+] in Dahl S rats raise interstitial [Na^+] in the brain (Wang et al. 2010), and may thereby decrease NO activity in the PVN, and enhance its neural responsiveness to Na^+. Future studies need to assess whether a decrease in NO activity by Na^+ in the PVN of Dahl S on high salt intake enhances local AT_1-receptor activation and whether these mechanisms in the PVN contribute to the elevated SNA and BP.

ACKNOWLEDGEMENTS

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4. MANUSCRIPT #3- CARDIOVASCULAR EFFECTS OF ANGIOTENSIN II AND GLUTAMATE IN THE PVN OF DAHL SALT-SENSITIVE RATS

Alexander Gabor[a] and Frans H. H. Leenen[a]*

[a]Hypertension Unit, University of Ottawa Heart Institute,
Ottawa, Ontario, Canada.

*Thesis Supervisor

SHORT TITLE

BP effects of Ang II and Glutamate in PVN of Dahl rats

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ABSTRACT

Several models of chronic sympathetic hyperactivity are associated with an increase in excitatory angiotensinergic and glutamatergic activity, and a decrease in GABAergic activity in the PVN. The present study evaluated whether activation of glutamate and AT_1 receptors in the PVN contributes to the maintenance of resting BP in Dahl salt sensitive (S) rats on regular or high salt diet for 4 -6 weeks. Candesartan and kynurenate were infused bilaterally into the PVN and BP and heart rate (HR) were recorded. Both candesartan and kynurenate in the PVN did not change MAP and HR in normotensive Dahl salt resistant (R) and S rats on regular salt diet or in R rats on high salt diet. In hypertensive Dahl S rats on high salt diet, candesartan decreased MAP (-14±2 mmHg), and tended to increase HR (22±5 bpm). Kynurenate decreased both MAP (-22±3 mmHg) and HR (-42±7 bpm) in these rats. At the peak BP decrease by candesartan, kynurenate in the PVN further decreased BP by ~ 50 % (-14±2 mmHg), whereas candesartan did not further decrease BP at the peak BP response to kynurenate (-4±2 mmHg). These results indicate that activation of glutamate and AT_1-receptors in the PVN contributes to the maintenance of BP in hypertensive Dahl S rats, but not normotensive Dahl S and R rats. The increased BP response to AT_1-receptor activation in the PVN of hypertensive Dahl S appears to be mediated by enhanced local glutamate receptor activation, but another mechanism(s) appears to further enhance glutamate responses.

INTRODUCTION

The Dahl rat strains represent genetic models of salt-sensitive (S) versus salt-resistant (R) blood pressure (BP). Dysregulation of Na^+-homeostasis in the brain appears to play a primary role in the salt-induced hypertension in Dahl S rats (*for review, see Leenen, 2010*). In Dahl S rats, high salt diet increases cerebrospinal fluid (CSF) [Na^+] (Huang et al. 2004, Nakamura and Cowley 1989) and causes sympathetic hyperactivity and hypertension (Huang et al. 2004, Serino et al. 2001). An increase in CSF [Na^+] may excite Na^+ sensitive nuclei in the lamina terminalis (LT) such as the subfornical organ (SFO) (Anderson et al. 2001, Denton et al. 1996) or the organum vasculosum of the lamina terminalis (OVLT) (Vivas et al. 1990). This increase in neuronal activity can be relayed to parvocellular neurons of the paraventricular nucleus (PVN) projecting to the intermediolateral cell column (IML) or rostral ventrolateral medulla (RVLM), thereby increasing sympathetic activity and BP (Ito et al. 2003, Li et al. 2003a, McKinley et al. 2001). Pressor responses from a brief increase in [Na^+] in the CSF or directly in the PVN are both prevented by an AT_1-receptor blocker in the PVN (Gabor and Leenen 2009), indicating that AT_1-receptors in the PVN mediate these pressor responses to [Na^+]. A chronic increase in CSF [Na^+] by intracerebroventricular (icv) infusion of Na^+ rich aCSF or by high salt diet in Dahl S rats also increases hypothalamic tisssue aldosterone and endogenous ouabain (EO) (Huang et al. 2006b, Huang et al. 2008b), angiotensin-converting enzyme (ACE) and AT_1-receptor densities in cardiovascular nuclei such as the PVN and causes sympathetic hyperactivity and hypertension (Huang et al. 2006b, Wang et al. 2003c). Aldosterone via EO release and AT_1-receptor stimulation appears to mediate the chronic effects of [Na^+] (Huang et al. 2011, Leenen 2010). Whether AT_1-

receptor activation in the PVN contributes to the hypertension from a chronic increase in CSF [Na^+] has not yet been assessed.

In the PVN, glutamate and Ang II raise (Gabor and Leenen 2009, Kannan et al. 1989), whereas gamma-aminobutyric acid (GABA) lowers sympathetic nerve activity (SNA), BP and HR (Akine et al. 2003). Injection of a glutamate (Li and Pan 2007a) or AT_1-receptor receptor blocker (Chen and Toney 2001, Gabor and Leenen 2009) in the PVN of normotensive rats does not affect BP or sympathetic activity whereas a $GABA_A$-receptor blocker increases SNA, BP, HR and local glutamate release (Li et al. 2006a). Pressor and sympathetic responses from a $GABA_A$-receptor blocker in the PVN are prevented by a glutamate (Chen et al. 2003, Li et al. 2006a) or AT_1-receptor blocker (Chen and Toney 2003) indicating that in normal physiological conditions, GABA release in the PVN tonically inhibits local glutamate release and AT_1-receptor activation. To our knowledge, no studies have yet assessed the mechanism by which GABA release inhibits AT_1-receptor activation in the PVN. However, Ang II in the PVN decreases GABA-mediated inhibition of pre-sympathetic neurons in the PVN (Li et al. 2003a) and presumably increases local glutamate release. Similar to its effects on magnocellular neurons (Latchford and Ferguson 2004), Ang II may also increase glutamate release from glutamate interneurons to activate pre-sympathetic parvocellular neurons.

The balance of inputs in the PVN changes in several chronic models of sympathetic hyperactivity. A glutamate or AT_1-receptor blocker in the PVN decreases SNA, BP and HR in rats with chronic heart failure (CHF) (Li et al. 2003c, Zheng et al. 2009) and in spontaneously hypertensive rats (SHR) (Li and Pan 2007a). Glutamate or AT_1-receptor blockers in the PVN both decrease BP in water deprived but not replete rats

(Freeman and Brooks 2007). In contrast, decreases in SNA, BP and HR by a $GABA_A$ receptor blocker in the PVN are attenuated in rats with CHF (Wang et al. 2009), renal-wrapped hypertensive rats (Martin and Haywood 1998) and in SHR (Li and Pan 2007a). These findings suggest that conditions with chronic sympathetic hyperactivity are associated with an increase in excitatory angiotensinergic and glutamatergic inputs, and a decrease in inhibitory GABAergic input in the PVN. No studies have yet evaluated the effects of high salt diet on balance of these excitatory and inhibitory inputs in the PVN of Dahl S rats. We hypothesized that AT_1 receptor activation via increased glutamate release in the PVN also contributes to the maintenance of hypertension in Dahl S rats on high salt diet.

In the present study, we first evaluated whether increased glutamate and AT_1 receptor activation in the PVN contribute to the maintenance of elevated BP in Dahl S rats on high salt intake. We then assessed whether in the PVN of Dahl S on high salt diet the increased glutamate receptor activation is mediated by increased local AT_1-receptor activation. Accordingly, we evaluated the effects of acute blockade of glutamate receptors or AT_1-receptors in the PVN on BP and HR of Dahl S and R rats on regular or high salt intake. To assess the interaction of these mechanisms, one blocker was infused at the peak BP response to the other blocker.

MATERIALS AND METHODS

Animals and Diets

Five to 6 weeks old male Dahl S (SS/Mcw) and Dahl SS.BN13 as the salt resistant control were obtained from Charles River Breeding Laboratories, Montreal, Quebec, Canada. Consomic Dahl SS.BN13 rats are Dahl S rats with their chromosome 13 substituted by chromosome 13 from salt resistant Brown Norway rats, and do not develop elevated BP on high salt diet (Cowley et al. 2001). The rats were provided with a standard commercial rat chow (0.3 %, 120 µmol Na^+/ gram) and water ad libitum. Animals were acclimatized at our animal care facilities for 1 week and then fed either Research diets® D10001 (AIN-76A) (Cedarlane, Burlington, Ontario) regular (0.1 %, 44 µmol Na^+/ gram) or high (8 %, 1408 µmol Na^+/ gram) NaCl diet for 4 - 6 weeks. The regular and high NaCl diet are the same, except for the NaCl content. All experiments were carried out according to the guidelines of the Canadian Council on Animal Care, which conform to National Institutes of Health guidelines and were approved by the University of Ottawa Animal Care and Use Committee.

Surgical Procedures

For all experiments, intra-cerebral cannulations of the PVN were performed after 3 -5 weeks regular or high salt diet and 1 week prior to infusions into the PVN. On the day of the surgery, rats were anesthetized with 2% isoflurane in oxygen. Animals were placed in a stereotaxic head frame, and the skull was leveled between bregma and lambda. A section of the skull was removed, and guide cannulas were positioned bilaterally 0.5 mm above the PVN according to the rat atlas of Paxinos and Watson (Paxinos and Watson 1998), 1.8 mm posterior to bregma, 0.4 mm lateral to the bregma and 7.9 mm ventral

from the skull. Guide cannulas were prepared from the steel tubing of 23 gauge needles (Becton, Dickinson), cut to extend ~1 cm over the skull when positioned above the PVN. They were secured to the skull with two jeweler's screws and acrylic cement and closed with stainless steel obturators. Stainless steel plugs were inserted into the guide cannulas to prevent obstruction from foreign materials. After 1 week recovery, the left femoral artery was cannulated with PE-50/10 polyethylene tubing filled with heparin (1,000 U/ mL in 0.9% NaCl) and plugged with a stainless steel stopper. The arterial catheter was tunneled subcutaneously and externalized through a small puncture in the skin on the back of the neck. For pain relief, animals received subcutaneous injections of buprenorphine (0.04 mg/ kg) (Gabor and Leenen 2011).

Experimental Procedures

The morning after the arterial cannulation, rats were placed in a small cage, arterial plugs were removed and intra-arterial catheters were connected to a pressure transducer for recordings of BP and HR via a PC equipped with software AcqKnowledge (ACQ 3.9). Systolic and diastolic pressures (SP, DP) as well as HR were extracted from the raw BP signal by software analysis. Mean arterial pressure (MAP) was determined by adding one-third of the pulse pressure (SP - DP) to the diastolic pressure.

"L"-shaped injection cannulas (30 gauge) were lowered bilaterally into the PVN through the fixed guide cannulas and extended 0.5 mm past the guide. Injection cannulas were connected by polyethylene tubing to 10 μL Hamilton microsyringes mounted on a Harvard infusion pump (model #2400-003) to perform infusions at 300 nL/ min. Animals were then allowed to settle for at least 30 min prior to recording of baseline BP and HR levels for 10 min. For multiple infusions into the PVN, at the peak effect of the

first drug, cannulas were removed, reloaded with the second drug and relowered into the PVN within ~5 min of the end of the first infusion. At the end of the experiment, rats were euthanized in a CO_2 chamber and 150 nL Evans Blue dye was micro-injected into the intra-cerebral infusion sites. Brains were removed, frozen, sectioned using a Leica cryostat and stained with neutral red. Infusion sites were considered to be inside the PVN if the mid-point of the dye circle was inside the borders of the PVN (Figure 4-1B). Only data from rats with both micro-injection sites inside the PVN were used for analysis (Figure 4-1A). The percent of bilateral injection sites within the PVN ranged from 60 - 75 % between experimental groups. In our previous study assessing the distribution pattern of infusions in the PVN, Evan's Blue dye infusion (300 nL/min) was distributed largely within the PVN region after 5 min, and extended beyond the PVN after 10 min (Gabor and Leenen 2011).

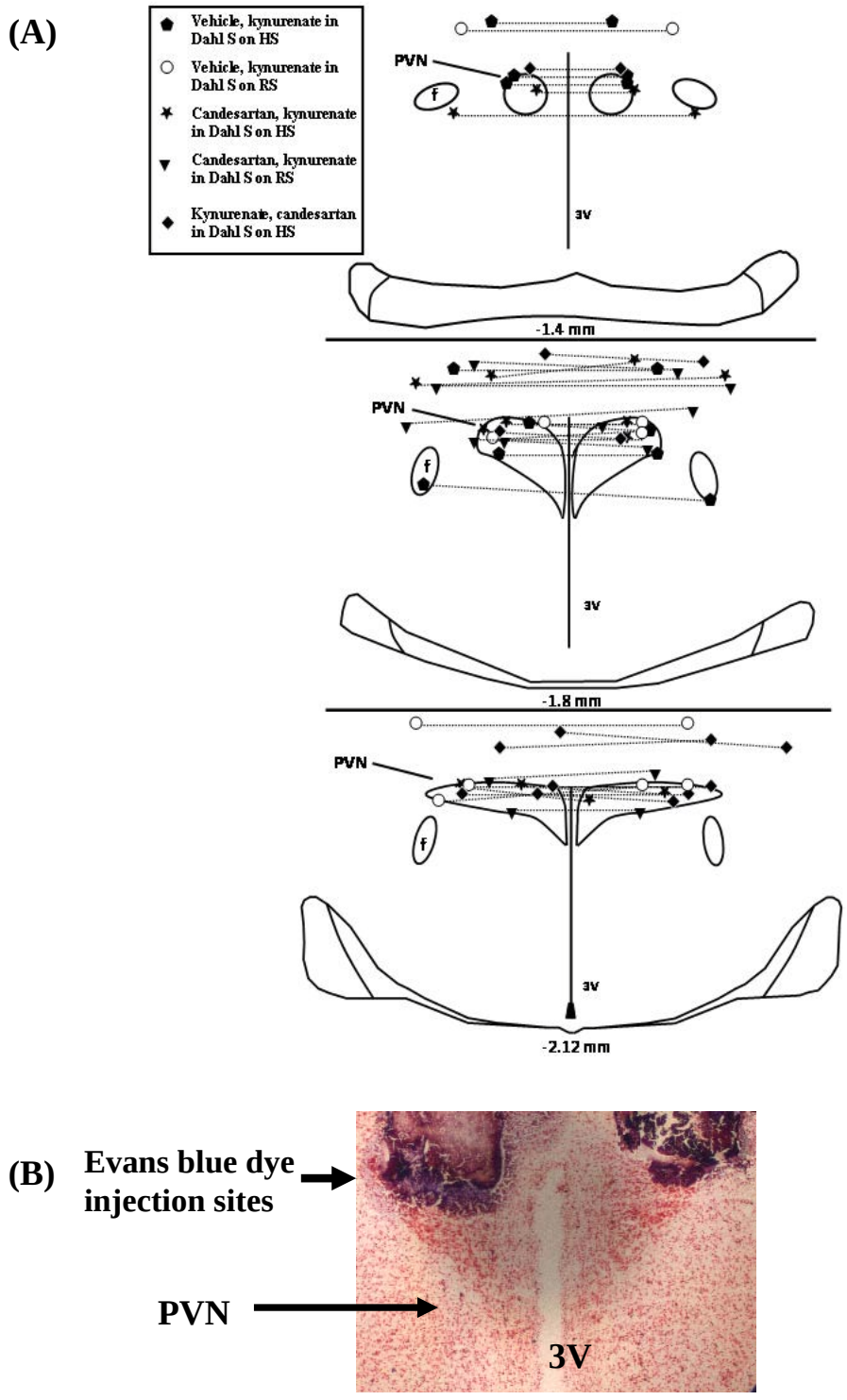

Figure 4-1: A) Schematic representation of infusion sites within and outside the PVN, plotted according to the location of dye circles from bilateral injections of 100 nL Evans Blue dye. Symbols connected by broken lines represent bilateral infusion sites from different experimental groups both inside and outside the PVN. f indicates fornix; 3V, third ventricle. B) A section of the PVN utilized to verify the position of bilateral infusion sites by visualizing the spread of 150 nL Evans Blue dye (on each side).

Artificial cerebrospinal fluid (121 NaCl, 3.4 KCl, 1.2 $MgCl_2$, 0.6 NaH_2PO_4, 29 $NaHCO_3$, and 3.4 glucose mmol/L, pH 7.4, and osmolarity 296 mosmol/kg H_2O) was used as vehicle, except for candesartan and kynurenic acid (kynurenate; dimethyl sulfoxide and aCSF: 2:98 ratio). Infusion of candesartan (0.5 µg/300 nL/min) or kynurenate (0.14 µg/300 nL/min) was performed bilaterally in the PVN (5 or 1.4 µg total on each side) for 10 min. Candesartan was chosen for its high selectivity and binding affinity for the AT_1-receptor and long half-life compared to other AT_1-receptor blockers such as losartan or valsartan (Israili 2000, Nishikawa et al. 1997). For preliminary dose response studies with Ang II, doses of candesartan were adapted from Freeman and Brooks (2007) who showed that bilateral injection of candesartan in the PVN (0.5 or 5.0 µg total on each side) lowered MAP by ~ -7 mmHg in 48 hr water deprived rats. In the present study, unilateral infusion of the higher dose in the PVN fully inhibited increases in BP and HR by 16 mmHg and 29 bpm from ANG II (90 ng) injected in the PVN (Table 4-1). The dose of kynurenate was adapted from previous studies showing that unilateral injection of 1.4 µg in the PVN prevented the increase in BP by 25 mmHg from unilateral injection of a $GABA_A$ receptor antagonist in the PVN (Chen et al. 2003) and bilateral injection of kynurenate in the PVN (0.95 µg on each side) nearly normalized BP in SHR (MAP: ~145 mmHg to ~105 mmHg) (Li and Pan 2007a). Doses of candesartan and kynurenate utilized in the present study are therefore likely sufficient to fully block AT_1 and glutamate receptors in the PVN. All injected compounds were purchased from Sigma or received as gifts (*see acknowledgements*).

Table 4-1: Effect of infusion of candesartan (0.05 or 0.5 ug/300 nL/ min for 10 min) on peak MAP and HR responses to microinjection of Ang II (90 ng/200 nL) in the PVN of Wistar rats. Each rat received unilateral injections or infusions into the PVN of vehicle, Ang II and one dose of candesartan followed by a second injection of Ang II. Five minutes was given between end of candesartan infusion and second injection of Ang II, whereas 30 min was given between all other treatments.

	Vehicle (aCSF)	Ang II (90 ng)	Candesartan (0.5 ug) + Ang II (90 ng)	Candesartan (5 ug) + Ang II (90 ng)
n	7	7	3	4
Change in MAP (mmHg)	2±1	16±2*	10±3*	1±1
Change in HR (bpm)	9±4	29±6*	31±11*	11±7

Values are mean ± SEM. * $p<0.05$ vs. vehicle

Experimental Protocols

Three experimental protocols were used in 3 different groups of Dahl rats.

Kynurenate in the PVN: To evaluate whether glutamate receptor activation in the PVN contributes to the maintenance of resting BP and HR in Dahl S rats on regular or high salt diet, bilateral infusion of vehicle (300 nL/min for 10 min per side) was performed in the PVN of Dahl S rats. Twenty minutes after vehicle infusion, kynurenate was infused into the PVN, and BP and HR were recorded until BP returned to baseline.

Candesartan followed by kynurenate in the PVN: To assess whether AT_1-receptor activation in the PVN contributes to the maintenance of resting BP and HR in Dahl S and R rats on regular or high salt diet, infusion of candesartan was performed in the PVN of Dahl S and R rats.

To evaluate whether glutamate receptor blockade in the PVN of these rats lowers BP and HR after blockade of local AT_1-receptors, kynurenate was infused into the PVN at the peak BP response to candesartan and BP and HR were recorded until BP returned to baseline.

Kynurenate followed by candesartan in the PVN: To assess whether after local kynurenate infusion, candesartan in the PVN of Dahl S on high salt diet lowers BP and HR, candesartan was infused into the PVN at the peak BP response to infusion of kynurenate.

Statistical Analysis

Values are expressed as means ± SE. For comparisons of the AUC or peak responses among treatments and rat strains, a one or two-way ANOVA was used followed by a Student-Newman-Keuls post hoc multiple comparison. For testing peak changes from baseline, paired *t*-tests were used. To test the time-course of changes from baseline, a one-way ANOVA with repeated measures followed by Dunnett's test was used. The level of significance was set at $P < 0.05$.

RESULTS

Resting MAP and HR were similar in Dahl S and R rats on regular salt diet (Table 4-2). High salt diet for 4 -6 weeks increased resting MAP by 35 - 45 mmHg in Dahl S, but not in R rats (Table 4-2). HR was significantly higher in Dahl S versus R rats on both diets (Table 4-2).

Table 4-2: Baseline MAP and HR levels prior to first or second bilateral infusion of vehicle, kynurenate (1.4 µg/ side) or candesartan (5 µg/ side) into the PVN of Dahl R and S rats on regular or high salt diet. Five to twenty-five minutes was given after end of first infusion and recording of second baseline.

		First baseline before first infusion		**Second baseline before second infusion**	
Infusions in the PVN	***n***	**MAP (mmHg)**	**HR (bpm)**	**MAP (mmHg)**	**HR (bpm)**
Vehicle, kynurenate in Dahl S on regular salt	4	109±3	442±4	110±2	442±5
Vehicle, kynurenate in Dahl S on high salt	4	153±8*	435±12	150±8*	425±11
Candesartan, kynurenate in Dahl R on reg. salt	4	106±4	408±6	104±3	409±7
Candesartan, kynurenate in Dahl R on high salt	5	112±3	421±10	110±2	425±12
Candesartan, kynurenate in Dahl S on reg. salt	4	109±2	444±7[a]	111±4	459±9[a]
Candesartan, kynurenate in Dahl S on high salt	5	149±3*[a]#	454±7[a]	135±4*[a]	466±10[a]
Kynurenate, candesartan on high salt	5	149±4#	444±16	128±6	410±15

Values are mean ± SEM; * $p<0.05$ vs. regular salt, a $p<0.05$ vs. Dahl R in same group, # $p<0.05$ vs. second infusion

Kynurenate in the PVN

Bilateral infusion of vehicle in the PVN did not change MAP or HR in Dahl S rats on regular (MAP: 1±1 mmHg; HR: 11±7 bpm) or high salt intake (Figure 4-2). Twenty minutes after vehicle infusion, kynurenate in the PVN did not change MAP (-2±2 mmHg) or HR (-16±7 bpm) (n=4) in Dahl S rats on regular salt diet. In Dahl S rats on high salt diet, kynurenate in the PVN rapidly decreased BP and HR (Figure 4-2). Both MAP and HR began to decrease within the first 5 min and peak decreases by ~ 20 mmHg and ~ 40 bpm occurred 10-15 min after start of infusion of kynurenate (Figure 4-2). Both responses started to return to baseline 15-20 min after end of kynurenate infusion. Resting MAP was lowered by kynurenate in the PVN of Dahl S rats on high salt diet from 150±8 mmHg to 128±7 mmHg, but remained partially elevated compared to Dahl S on regular diet (baseline: 109±3 mmHg; *see Table 4-1*). Bilateral infusion of vehicle or kynurenate at sites outside the PVN (Figure 4-1A) caused non-significant changes in MAP and HR in Dahl S rats on regular or high salt diet [vehicle (MAP: 1±1 mmHg; HR: 1±6 bpm, n=5); kynurenate (MAP: 1±2 mmHg; HR: -4±5 bpm), n=5].

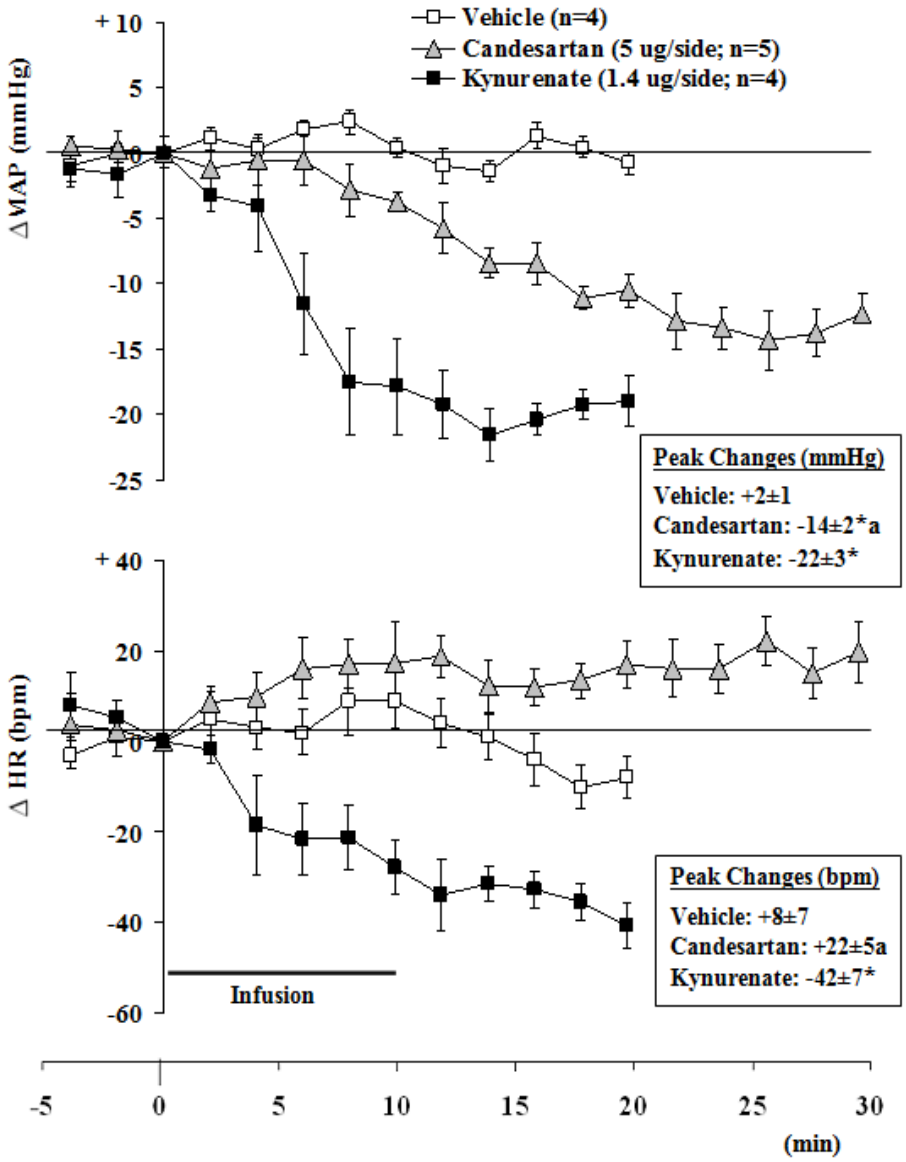

Figure 4-2: MAP and HR responses to bilateral infusion of vehicle, candesartan or kynurenate into the PVN of Dahl S rats on high salt diet. Kynurenate was infused 20 minutes after end of vehicle infusion. Values are mean ± SEM. Baseline MAP and HR are 153±8, 150±8, 149±3 mmHg and 435±12, 425±11, 454±7 bpm for vehicle, kynurenate and candesartan groups. By one-way ANOVA of the area under the curve after 20 min, MAP and HR responses were significantly different among treatments (MAP: F= 178.4; HR: F= 36.1); [vehicle vs. candesartan (MAP and HR: P<0.007); kynurenate vs. candesartan or vehicle (MAP and HR: P<0.001). By one-way ANOVA with repeated measures, MAP and HR responses to kynurenate were significantly different from vehicle from 4 to 20 min. Candesartan was significantly different from vehicle from 8 to 20 min for MAP and at 6 and between 12 to 20 min for HR. For peak response data, by one-way ANOVA, MAP and HR responses were significantly different among treatments (MAP: F= 38.4; HR: F= 39.0); [vehicle vs. kynurenate (MAP and HR: P<0.001); candesartan vs. kynurenate (MAP and HR: P<0.05); vehicle vs. candesartan (MAP: P<0.001). * p<0.05 vs. vehicle. a p<0.05 vs. kynurenate.

In Dahl R or S rats on regular salt diet, bilateral infusion of candesartan in the PVN did not change MAP (Dahl R, S: -1±1, 0±1 mmHg) or HR (Dahl R, S: -4±5, -13±3 bpm) (n=4,4). Candesartan also did not change MAP (-2±1 mmHg) or HR (8±6 bpm) in Dahl R rats on high salt diet (n=5). In Dahl S rats on high salt diet, candesartan decreased BP, but not HR (Figure 4-2). MAP began to decrease within the first 10 min and peak decreases by ~15 mmHg occurred 25-35 min after start of infusion (Figure 4-2). HR tended to increase, but this effect of candesartan was not significantly different from vehicle according to the area under the curve (AUC) (Figure 4-2). Candesartan infused outside the PVN (Figure 4-1A) did not cause significant changes in MAP or HR in Dahl S rats on regular or high salt diet [(MAP: -2±1 mmHg); (HR: -1±3 bpm), n=6].

In Dahl R or S rats on regular salt diet, kynurenate in the PVN did not change MAP (Dahl R, S: -2±1, -2±2 mmHg) or HR (Dahl R, S: -7±4, -17±6 bpm) (n=4,4) when it was infused 30 min after start of candesartan infusion. Kynurenate also did not change MAP (-2±1 mmHg) or HR (-12±5 bpm) in Dahl R rats on high salt diet (n=5). In Dahl S rats on high salt intake, at the peak BP response to candesartan, kynurenate in the PVN rapidly further decreased BP and HR (Figure 4-3 and 4-4). Peak decreases occurred ~ 10 min after start of infusion of kynurenate (Figure 4-3). Kynurenate caused significantly larger decreases in MAP, but not HR when it was infused after vehicle versus candesartan in Dahl S rats on high salt diet (-22±3 vs. -14±2 mmHg, $P<0.05$; -42±7 vs. -49±8 bpm) (Figure 4-2 and 4-4). The total decrease in MAP from candesartan followed by kynurenate in the PVN (MAP: -28±2 mmHg) was significantly larger than candesartan alone (MAP: -14±2 mmHg) ($P<0.001$), but not kynurenate after vehicle in the PVN (MAP: -22±3 mmHg) (Figure 4-2 and 4-4). The total decrease in HR from

candesartan followed by kynurenate (-47±7 bpm) was also significantly larger than candesartan alone (22±5 bpm), but not kynurenate after vehicle (-41±6 bpm). Candesartan in the PVN of Dahl S rats on high salt diet lowered resting MAP from 149±3 to 136±2 mmHg, whereas the total effect of both blockers combined nearly normalized resting MAP (from 149±3 to 122±3 mmHg).

Kynurenate followed by candesartan in the PVN

In Dahl S rats on high salt diet, at the peak BP response to kynurenate (10-15 min after start of infusion), bilateral infusion of candesartan for 10 min did not cause significant changes in MAP (-4±2 mmHg) or HR (10±6 bpm) (n=5) (Figure 4-3 and 4-4).

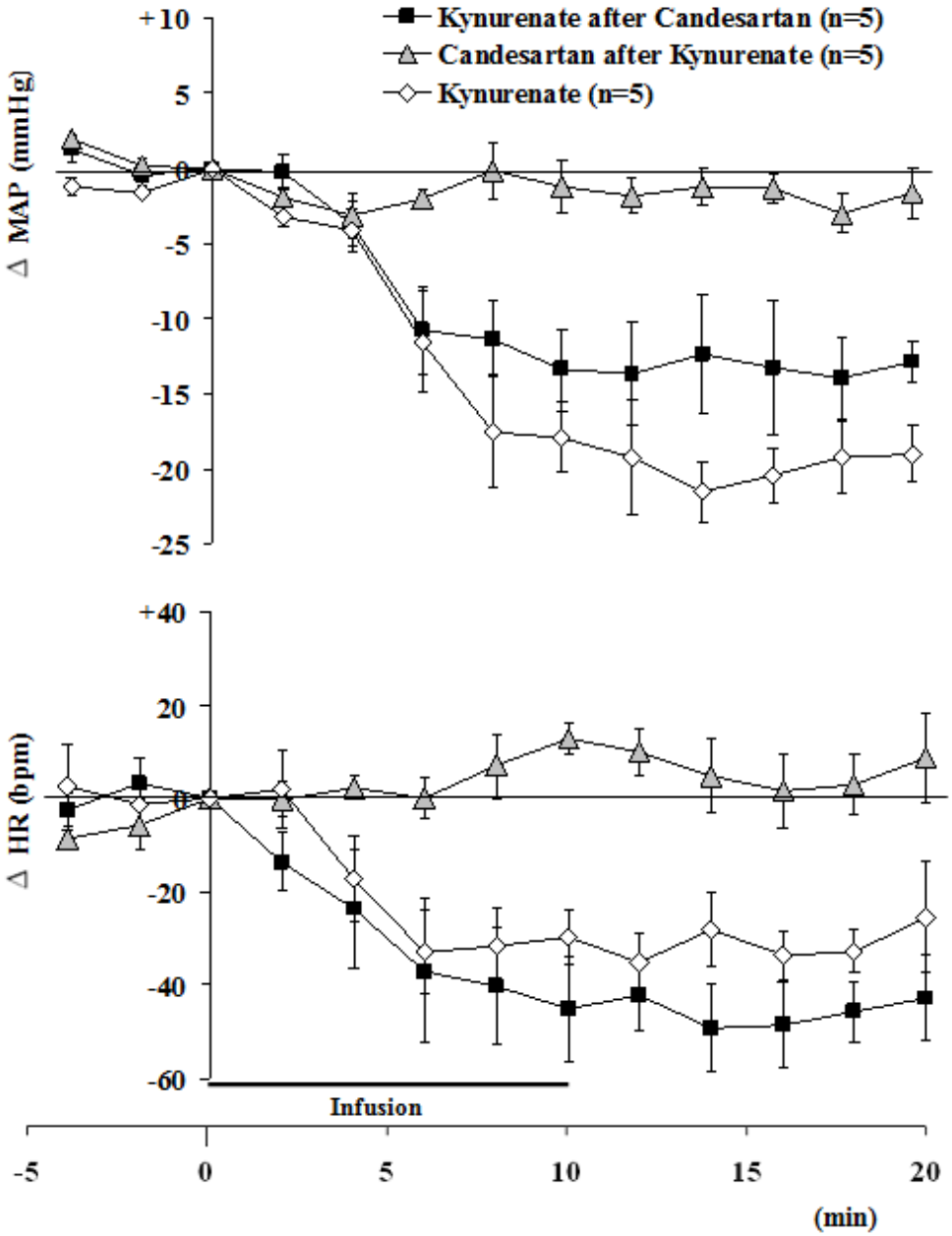

Figure 4-3: MAP and HR responses to bilateral infusion of kynurenate alone, kynurenate after candesartan and candesartan after kynurenate in the PVN of Dahl S rats on high salt diet. Kynurenate was infused 15-25 min after end of candesartan infusion, while candesartan was infused 5-10 min after end of kynurenate infusion. Baseline MAP and HR are 135±4, 128±6, 149±4 mmHg and 466±10, 410±15, 444±16 bpm for kynurenate after candesartan, candesartan after kynurenate and kynurenate groups. Values are mean ± SEM. By one-way ANOVA of the area under the curve after, MAP and HR responses were significantly different among treatments (MAP: F= 27.5; HR: F= 46.1); [kynurenate alone vs. kynurenate after candesartan or candesartan after kynurenate (MAP and HR: P<0.001); kynurenate after candesartan vs. candesartan after kynurenate (MAP P<0.05)]. By one-way ANOVA with repeated measures, decreases in MAP were significant from 6 to 20 min and for HR from 4 to 20 min.

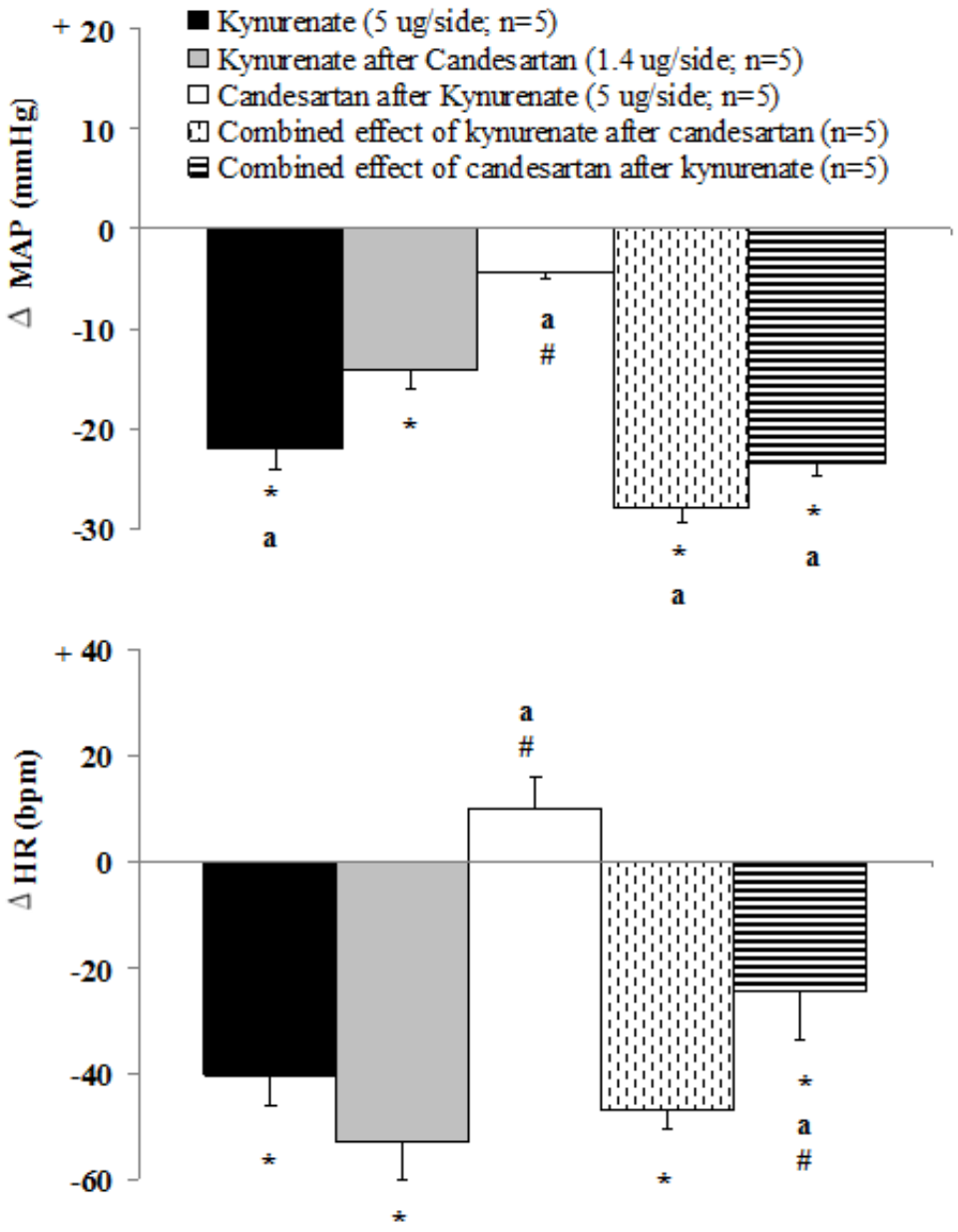

Figure 4-4: Peak MAP and HR responses to bilateral infusion of kynurenate alone, kynurenate after candesartan or candesartan after kynurenate in the PVN of Dahl S rats on regular or high salt diet and sum of the individual responses from each blocker, represented as a total effect. Kynurenate was infused 15-25 min after end of candesartan infusion, while candesartan was infused 5-10 min after end of kynurenate infusion. Values are mean ± SEM. By one-way ANOVA, responses in MAP and HR were significantly different between treatments in Dahl S rats on high salt diet (MAP: F= 32.1; HR: F= 15.3); [candesartan after kynurenate vs. all groups (MAP and HR: P<0.001); kynurenate after candesartan vs. all groups (MAP: P<0.05); combined effect of candesartan after kynurenate vs. kynurenate after candesartan or combined effect of kynurenate after candesartan (HR: P<0.04). * p<0.05 vs. baseline. a p<0.05 vs. kynurenate after candesartan. # p<0.05 vs. combined effect of kynurenate after candesartan.

DISCUSSION

The present study shows as new findings that in hypertensive Dahl S rats on high salt diet, bilateral infusion of candesartan and kynurenate in the PVN decrease BP. At the peak BP response to candesartan, kynurenate in the PVN causes a further decrease in BP, whereas candesartan does not further decrease BP at the peak BP response to kynurenate. These results indicate that activation of glutamate and AT_1-receptors in the PVN contributes to the maintenance of BP in hypertensive Dahl S rats on high salt diet. The increased BP response to AT_1-receptor activation in the PVN of hypertensive Dahl S appears to be mediated by enhanced local glutamate receptor activation, but another mechanism(s) appears to further enhance glutamate responses.

Candesartan or kynurenate in the PVN

Consistent with similar findings in normotensive Sprague Dawley (SD) (Freeman and Brooks 2007) and WKY rats (Li and Pan 2007a), bilateral infusion of candesartan or kynurenate in the PVN did not change BP or HR in Dahl R and S rats on regular salt diet. This lack of effect from candesartan or kynurenate in the PVN under normal physiological conditions may be attributed to tonic inhibition of angiotensinergic (Chen and Toney 2003) and glutamatergic neurons (Chen et al. 2003, Li et al. 2006a) in the PVN by a GABA-mediated mechanism.

Consistent with other models of chronic sympathetic hyperactivity (Freeman and Brooks 2007, Li and Pan 2007a, Li et al. 2003c), infusion of candesartan or kynurenate in the PVN lowered BP in hypertensive Dahl S rats on high salt diet. These findings indicate that increased glutamate and AT_1 receptor activation in the PVN contributes to the maintenance of resting BP in Dahl S rats on high salt diet. Glutamate receptor (Ito et

al. 2001) and AT_1-receptor (Ito et al. 2003) blockers in the RVLM decrease BP in Dahl S rats on high salt diet indicating that enhanced activation of these receptors in the RVLM also contributes to the maintenance of resting BP in hypertensive Dahl S. Kynurenate is a non-specific blocker of both N-methyl D-aspartate (NMDA) and non-NMDA channels (Bertolino et al. 1989, Birch et al. 1988). Both receptors are expressed on neurons in parvocellular subdivisions of the PVN (Herman et al. 2000) and immunohistochemical studies show glutamate-immunoreactive axon terminals contacting dendrites and cell bodies of parvocellular neurons (van den Pol 1991). Sympathetic and pressor responses to L-glutamate in the PVN are fully prevented by kynurenate in the PVN (Chen et al. 2003), while specific blockers of NMDA and non-NMDA receptors attenuate SNA and BP responses to L-glutamate by 60-75 % and 20- 30% (Li et al. 2006a), indicating that both NMDA receptors contribute to the BP responses from glutamate in the PVN. In contrast to glutamate, Ang II activates parvocellular neurons indirectly by activating AT_1-receptors on end terminals of GABAergic interneurons in the PVN, causing a decrease in GABA release onto pre-sympathetic neurons projecting to the RVLM (Li and Pan 2005) or IML (Li et al. 2003a). This effect of Ang II is dependent on downstream signaling pathways involving $G_{i/o}$ proteins and reactive oxygen species (ROS) (Chen and Pan 2007). Nitric oxide enhances GABA release from these GABAergic neurons via activation of a guanosine monophosphate (cGMP)- protein kinase G pathway (Li et al. 2003b). In Dahl S rats on high salt diet, activation of the aldosterone- EO pathway decreases NO activity in the PVN and increases local ROS production (Huang et al. 2011) via enhanced AT_1-receptor activation (Gabor and Leenen 2011, Leenen 2010) and can thereby decrease GABA release onto pre-sympathetic neurons (Chen and Pan 2007,

Li et al. 2003a). None of these changes occur in Dahl R rats on high salt, presumably because CSF [Na^+] increases in S but not R rats (Huang et al. 2004).

Decreases in BP by candesartan in the PVN of Dahl S rats on high salt began after 5 min, and peaked 25 min after start of infusion. A similar pattern of BP reduction was observed when candesartan was infused into the PVN of water deprived rats (Freeman and Brooks 2007). These effects of an AT_1-receptor blocker are likely mediated by the inhibition of slow signaling pathways associated with G-proteins (Richards et al. 1999). Decreases in BP from kynurenate were more rapid, beginning within the first few minutes and peaking within 15 min. This rapid action of kynurenate is likely caused by its inhibitory action on NMDA and non-NMDA cation channels and their associated fast signaling pathways (Lau et al. 2009).

Infusion of candesartan and kynurenate outside the PVN of Dahl S rats on high salt diet caused no significant changes in BP or HR. For rats with infusions inside the PVN, the spread of Evan's Blue dye according to histological examination was mainly inside the borders of the PVN, with no obvious leakage into the 3rd ventricle. These findings indicate that the BP response to kynurenate or candesartan in the PVN of Dahl S rats on high salt diet was mediated by blockade of glutamate or AT_1-receptors receptors inside the PVN rather than in other brain regions.

Candesartan combined with kynurenate

Combining both blockers in the PVN assesses the extent of interaction between these mechanisms in the maintenance of elevated resting BP in Dahl S rats on high salt. Glutamate release in the PVN is tonically inhibited by $GABA_A$ receptor activation (Chen et al. 2003, Li and Pan 2007a, Li et al. 2006a), while AT_1-receptor activation in the PVN

decreases local GABA release (Li et al. 2003a, Li and Pan 2005). Increased AT_1-receptor activation in the PVN of hypertensive Dahl S may inhibit GABA release and increase local glutamate release. In addition, similar to Ang II-mediated excitation of magnocellular neurons in the PVN (Latchford and Ferguson 2004), increased AT_1-receptor stimulation on a glutamate interneuron in the PVN may directly increase presynaptic glutamate release onto pre-sympathetic neurons. Candesartan caused no change in BP after it was infused at the peak BP responses to kynurenate in Dahl S on high salt indicating that the effects of increased AT_1-receptor activation in the PVN of Dahl S are fully mediated by local glutamate release. Consistent with these findings, Ang II infused in the SFO increases Ang II release in the PVN (Wright et al. 1993), SNA and BP (Ku et al. 1999) and the BP and HR responses are fully prevented by kynurenate in the PVN (Kvochina et al. 2010). Furthermore, an AT_1-receptor blocker does not change RSNA, BP and HR responses to L-glutamate in the PVN (Chen and Toney 2003), indicating that the effects from increased glutamate release in the PVN are not mediated by subsequent AT_1-receptor activation. In contrast, kynurenate after candesartan in the PVN further decreased resting MAP by -14 mmHg indicating that other mechanisms independent of AT_1-receptor stimulation in the PVN mediate part of the enhanced release of glutamate. It is possible that a subset of glutamatergic neurons in the PVN from the SFO (Bains and Ferguson 1995), anterior hypothalamic area and medial or lateral preoptic area (Csaki et al. 2000) with terminals adjacent to parvocellular neurons of the IML or RVLM are not influenced by decreased GABAergic inhibition via increased AT_1-receptor activation or direct AT_1-receptor stimulation and contribute ~ 50 % of the additional glutamate release. Alternatively, this additional AT_1-receptor independent

glutamate release could be mediated by a decrease in astrocyte glutamate transporter 1 (GLT1) activity, resulting in enhanced extracellular glutamate levels in the PVN originating from non-synaptic sources (Fleming et al. 2011).

Combining both blockers in the PVN Dahl S on high salt diet nearly normalized resting BP, but levels still remained elevated compared to those observed in rats on regular salt diet. Chronic icv infusion of an AT_1-receptor blocker fully prevents the increase in BP from 4 weeks high salt diet in Dahl S rats (Huang and Leenen 1998). Acute infusion of blockers of glutamate or AT_1-receptors in the PVN may not provide sufficient time to fully block all downstream intracellular signaling pathways contributing to the elevated SNA and BP in Dahl S on high salt intake. Injection of an adeno-associated virus (AAV) in the PVN carrying a short-hairpin small-interference RNA (siRNA) against glutamate or AT_1-receptors in the PVN can achieve long-term inhibition (Northcott et al. 2011) to assess the impact of chronic blockade specifically in the PVN.

Consistent with findings in SHR (Li and Pan 2007a) or water deprived rats (Freeman and Brooks 2007), HR decreased in response to kynurenate, whereas candesartan tended to increase HR, despite that BP responses to increased local AT_1-receptor activation in the PVN depend on local glutamate release. Fibres from parvocellular neurons of the PVN projecting through the IML innervate discrete subsets of tissues throughout the thoracic region non-uniformly at varying density levels (Swanson and Sawchenko 1980). HR responses from a $GABA_A$ receptor blocker in the PVN were abolished by an NMDA receptor blocker, but not changed by a non-NMDA receptor blocker (Li et al. 2006a) and glutamate release dependent or independent of

AT_1-receptor activation in the PVN may activate different local excitatory neurotransmitter receptors and unique sympathetic pathways affecting HR.

In conclusion, our results indicate that activation of glutamate and AT_1-receptors in the PVN contributes to the maintenance of BP in hypertensive Dahl S, but not normotensive Dahl R and S rats. Enhanced glutamate release in the PVN of hypertensive Dahl S rats appears to be caused by an increase in local AT_1-receptor activation and by another mechanism(s) that is not yet defined.

ACKNOWLEDGEMENTS

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5. GENERAL DISCUSSION

5.1. Summary of main findings

Infusion of Na^+ rich aCSF in the PVN of Wistar rats increased BP and HR. Aldosterone or a subpressor dose of ouabain in the PVN alone did not affect BP and HR, but enhanced these responses to Na^+ by 40- 60 %. Both the responses to Na^+ alone or combined with aldosterone were fully blocked by losartan, whereas only the aldosterone enhancement was blocked by eplerenone, benzamil and fab fragments. In contrast, aldosterone in the PVN did not change the BP and HR responses to icv infusion of Na^+ rich aCSF. Similarly, both eplerenone and benzamil in the PVN did not change the pressor responses to icv infusion of aldosterone and Na^+ rich aCSF. However, telmisartan in the PVN fully blocked the responses to icv infusion of Na^+ rich aCSF either alone, or after icv infusion of aldosterone.

Na^+ rich aCSF in the PVN caused 30- 40 % larger increases in BP and HR in Dahl S vs. R rats. These responses to Na^+ were not affected by eplerenone, benzamil or fab fragments, whereas they were fully blocked by losartan in both strains. BP and HR responses to L-NAME in the PVN were the same, but L-NAME enhanced Na^+ effects more in Dahl R than S rats, equalizing BP responses to Na^+ in the two strains.

High salt diet did not affect BP and HR responses to L-NAME in the PVN of Dahl R rats, but attenuated increases in BP from L-NAME in Dahl S rats. Both candesartan and kynurenate in the PVN did not change MAP and HR in normotensive Dahl R and S rats on regular salt diet or in R rats on high salt diet. In hypertensive Dahl S rats on high salt diet, candesartan and kynurenate in the PVN decreased BP. At the peak BP response to candesartan, kynurenate in the PVN further decreased BP by ~ 50

%, whereas candesartan did not further decrease BP at the peak BP response to kynurenate.

5.2. Mechanisms mediating Na^+-induced BP responses in the PVN of Wistar rats

5.2.1. Na^+ rich aCSF in the PVN

Consistent with previous studies (Jin et al. 2001), infusion of Na^+ rich aCSF in the PVN but not osmotically equivalent solutions of mannitol causes [Na^+]-dependent increases in BP and HR. Electrophysiological studies *in vitro* indicate that both solutions with elevated [Na^+] and osmolality increase firing activity in magnocellular neurons of the PVN (Qiu et al. 2004), involved in production of AVP and possibly EO (*section 1.2.1.*). In magnocellular neurons of the SON (Bourque 2008, Bourque and Oliet 1997) and PVN (Qiu et al. 2004), hyperosmotic solutions of mannitol increase the open probability of stretch-inactivated (SI) Na^+/ osmo-sensing channels thereby increasing membrane conductance and the inward flux of permeable ions ie. Na^+, Ca^{2+} (Voisin and Bourque 2002). An increase in extracellular [Na^+] caused by bath application of solutions with elevated [Na^+] increases driving force and relative Na^+ permeability through SI channels, and both an increase in membrane conductance and permeability to Na^+ leads to an increase in firing activity (Voisin and Bourque 2002). Whole cell patch-clamp recordings of parvocellular neurons of the PVN show that NaCl solutions with elevated [Na^+] cause an increase in action potential frequency via an increase in electrochemical driving force and membrane permeability to Na^+ (Chu et al. 2010). These responses were blocked by GD^{3+} (Chu et al. 2010), a non-specific blocker of a blocker of cation channels (Ciura and Bourque 2006), but the specific Na^+-transduction channel(s) contributing to

Na^+ sensing in parvocellular neurons have not yet been identified. In contrast to the action of SI channels on increases in extracellular osmolality in magnocellular neurons (Voisin and Bourque 2002, Voisin et al. 1999), hyperosmotic solutions of mannitol do not increase membrane conductance or action potential frequency in parvocellular neurons (Chu et al. 2010). These findings indicate that SI channels do not play a role in Na^+ sensing in parvocellular neurons. Thus, SI channels in magnocellular neurons and an unidentified Na^+-sensing channel(s) in parvocellular neurons transduce an increase in extracellular [Na^+] into increased firing activity.

Losartan fully blocks the BP and HR responses to Na^+ in the PVN indicating that they are mediated by AT_1-receptor activation, presumably by increased local Ang II release. Ang II is localized in both magnocellular and parvocellular neurons of the PVN (Lind et al. 1985a, Lind et al. 1985b) suggesting that both cell types may be involved in transducing extracellular [Na^+] into Ang II release in the PVN. Electrophysiological studies indicate that Ang II increases neuronal activity of magnocellular neurons of the PVN (Latchford and Ferguson 2004) and micro-injection of ANG II in the PVN dose-dependently increases AVP release into the circulation (Veltmar et al. 1992). AVP enhances sympatho-baroreflex function (Hasser et al. 1997) (*section 1.1.4.*), and the initial decrease in HR is prevented by an AVP antagonist. Gradual increases in sympathetic drive caused by increasing firing activity of spinally projecting neurons in the PVN (*discussed below*) may then override baroreflex inhibitory mechanisms and raise HR. The AVP antagonist does not affect the increase in BP from Na^+ in the PVN suggesting that AVP release does not contribute to the increase in BP. An increase in [Na^+] in the PVN may increase the activity of angiotensinergic neurons with end

terminals in parvocellular subdivisions of the PVN (van den Pol 1982). Ang II release in parvocellular subdivisions increases AT_1-receptor stimulation on end terminals of GABAergic interneurons thereby inhibiting GABA release onto spinally projecting pre-sympathetic neurons (Li et al. 2003a, Li and Pan 2005) (*section 1.3.1.*). According to our proposed model (Figure 1-4), increased AT_1-receptor stimulation may also enhance glutamate release from glutamatergic neurons adjacent to spinally projecting neurons (*section 1.3.2.*). Both of these Ang II-signaling mechanisms increase neuronal activity in pre-sympathetic neurons projecting to the IML or RVLM thereby raising SNA and BP (Dampney 1994).

An increase in neuronal activity in magnocellular neurons may increase local EO release (*section 1.2.2.2.*) and in subsequent studies, we evaluated the role of the MR-ENaC-EO pathway in the pressor responses to Na^+ rich aCSF in the PVN.

5.2.2. Aldosterone and Na^+ rich aCSF in the PVN

Consistent with infusions of aldosterone icv (Wang et al. 2003a), or in the SFO (Tiruneh and Leenen, unpublished 2012), infusion of aldosterone alone in the PVN does not affect BP and HR whereas it enhances BP and HR responses to Na^+ rich aCSF in the PVN. Eplerenone and benzamil in the PVN blocks the enhancement by aldosterone indicating that this effect of aldosterone is mediated by local MR stimulation and ENaC activation. Fab fragments also blocks this enhancement and micro-injection of a sub-pressor dose of ouabain in the PVN similarly enhances the response to Na^+. These findings indicate that aldosterone activates an MR-ENaC-EO pathway in the PVN thereby enhancing responses to a local increase in $[Na^+]$. In the PVN, MR and ENaC are expressed in magnocellular

neurons and less in parvocellular (Amin et al. 2005). It is possible that MR stimulation, likely on magnocellular neurons increases ENaC activation and raises intracellular Na^+ thereby increasing EO release at magnocellular nerve terminals (Teruyama et al. 2011). Early genomic effects of aldosterone on ENaC activation are slowly developing (> 0.5 hrs) and require an increase in transcription and translation of ENaC regulatory genes ie. SGK1 (Bhargava et al. 2001) (*section 1.2.2.1.*). The pressor and HR responses to ouabain as well as aldosterone and Na^+ rich aCSF in the PVN were fully blocked by losartan indicating that the BP effects of both endogenous and exogenous ouabain are mediated by AT_1-receptor activation. EO binding to the α2 subunit on glial cells may increase intracellular Ca^{2+} thereby stimulating the release of intracellular RAS components ie. angiotensinogen, renin (*section 1.2.2.2.*). An increase in Ang precursors in the interstitium may be taken up by angiotensinergic neurons causing enhanced Ang II production and release in response to an increase in extracellular [Na^+] (*section 4.2.1.*). Chronic effects of EO via aldosterone in the PVN involve increased expression of ACE, AT_1-receptors and NADPH oxidase subunits (Huang et al. 2011) (*section 1.2.2.2.*). Eplerenone, benzamil and Fab fragments block only the enhancement by aldosterone and not the responses to [Na^+] alone in the PVN per se. One may postulate that the activity of the MR-ENaC-EO pathway in the PVN is low and does not sufficiently contribute to the pressor responses to a local increase in [Na^+]. Alternatively, insufficient time was provided to down-regulate gene expression and turn "off" this pathway in normal conditions (*further discussed in 5.4.1.*). Next studies assessed whether these mechanisms in the PVN contribute to the BP responses to a short increase in CSF [Na^+] in Wistar rats.

5.3. Mechanisms in the PVN mediating CSF Na^+-induced pressor responses in Wistar rats

Icv infusion of Na^+ rich aCSF increases BP and HR and these responses were enhanced by infusion of aldosterone icv, but not by aldosterone in the PVN. Eplerenone and benzamil in the PVN do not change the pressor responses to icv infusion of Na^+ rich aCSF after aldosterone. In contrast, icv infusion of benzamil fully prevents the responses from icv infusion of Na^+ rich aCSF after aldosterone (Wang et al. 2003a). These findings indicate that MR-ENaC contributing to the enhanced pressor responses to an increase in CSF [Na^+] after an increase in aldosterone are not located in the PVN. Other brain regions with a more sensitive MR-ENaC-EO system mediate the enhancement by icv aldosterone of the responses to an increase in CSF [Na^+]. For example, icv infusion of Fab fragments into the MnPO prevents the BP and HR responses to icv infusion of Na^+ rich aCSF (Budzikowski and Leenen 1997) indicating that EO release in the MnPO contributes to the pressor responses to an increase in CSF [Na^+]. Aldosterone in the PVN enhances the responses to an increase in [Na^+] in the PVN but not in the CSF. It is possible that a short increase in CSF [Na^+] does not sufficiently raise [Na^+] in the PVN high enough to stimulate an enhancement by aldosterone. A chronic increase in CSF [Na^+] raises hypothalamic tissue [Na^+] (Wang et al. 2010), and whether a chronic increase in [Na^+] in the PVN enhances the activity of local neuromodulatory pathways involving aldosterone and EO still needs to be assessed.

Telmisartan in the PVN fully blocks the pressor responses to icv infusion of Na^+ rich aCSF alone, or after aldosterone. These findings indicate that AT_1-receptor activation in the PVN mediates the pressor responses to icv infusion Na^+ rich aCSF alone, and their enhancement by icv infusion of aldosterone. The pressor responses to

CSF [Na^+] can be prevented by lesioning nuclei of the LT (May et al. 2000, Veerasingham and Leenen 1997) and this brain region appears to initiate the response to CSF [Na^+] (Budzikowski and Leenen 2001) (*section 1.2.1.2.*). Considering that the nuclei of the LT have angiotensinergic projections to the PVN (*section 1.2.2.3.*), increases in CSF [Na^+] may primarily excite Na^+ sensitive nuclei in the lamina terminalis ie. SFO, OVLT and this increased neuronal activity is likely relayed to the PVN via the MnPO (*section 1.2.3.1.*) causing increased local Ang II release and AT_1-receptor stimulation. Icv infusion of aldosterone may stimulate activity of MR-ENaC-EO pathways in nuclei of the LT (*see above*) thereby enhancing activity of local angiotensinergic signaling pathways releasing Ang II in the PVN. Further studies are needed to assess how and where these MR-ENaC-EO pathways enhance the activity of angiotensinergic sympathetic pathways in responses to acute and chronic increases in CSF [Na^+].

5.4. Mechanisms mediating Na^+ induced pressor responses in the PVN of Dahl rat

5.4.1. MR-ENaC-EO and AT_1-receptors in the PVN

Consistent with our previous icv study (Huang et al. 2001b), infusion of Na^+ rich aCSF in the PVN causes larger increases in BP and HR in Dahl S vs. R or Wistar rats. Losartan prevents these responses to Na^+ in all strains. These findings indicate that an enhanced pressor response to [Na^+] is present in the PVN of Dahl S vs. R or Wistar rats and depends on AT_1-receptor activation. Micro-injection of Ang II in the PVN causes the same pressor responses in Dahl S and R rats, indicating that responses to AT_1-receptor activation per se in the PVN are the same in both rat strains. The larger effect in Dahl S rats therefore likely reflects enhanced Ang II release by Na^+. Specific Na^+-sensing

mechanisms in the PVN of Dahl R and S rats contributing to the increase in Ang II release by Na^+ have not yet been studied but are likely the same as those in Wistar rats (*section 5.2.1.*). For the mechanisms mediating the enhanced responsiveness to Na^+ in the PVN of Dahl S rats we hypothesized that the MR-ENaC-EO neuromodulatory pathway was involved. Increased release of EO in the PVN of Dahl S vs. R in response to [Na^+] would cause more Ang II production and increased local AT_1-receptor activation (*section 5.2.1.*). However, consistent with our previous icv study (Huang et al. 2001b) ouabain in the PVN causes the same pressor and HR responses in Dahl S and R rats indicating that increased responses to ouabain, and presumably EO do not contribute to the enhanced responsiveness to Na^+ in the PVN of Dahl S rats. Furthermore, eplerenone, benzamil and Fab fragments do not affect the pressor responses to Na^+ in the PVN of Dahl S and R rats suggesting that the MR-ENaC-EO pathway does not contribute to the pressor response to a short increase in [Na^+] in the PVN of Dahl R rats neither in the enhanced responses in Dahl S rats. Acute infusion of blockers of this pathway in the PVN may not provide sufficient time to down-regulate gene expression and inhibit downstream intracellular signaling pathways contributing to the enhanced responses to Na^+. However, responses to Na^+ remained the same after the time between Fab fragments and Na^+-rich aCSF was extended up to 18 h, further supporting that EO is not involved in the enhanced pressor responses to Na^+ in the PVN of Dahl S rats on the regular salt diet. Future studies need to assess whether this neuromodulatory pathway in the PVN contributes to the sympathetic hyperactivity and hypertension from a chronic increase in CSF [Na^+] such as in Dahl S rats on high salt diet. Next studies assessed the role of NOS in the enhanced responsiveness to Na^+ in the PVN of Dahl S rats.

5.4.2. NOS in the PVN

Pressor and HR responses to Na^+ rich aCSF in the PVN were larger in Dahl S vs. R rats. L-NAME enhances the responses to Na^+ more in Dahl R than S rats, thereby equalizing these responses in the two strains. These findings indicate that an acute increase in [Na^+] in the PVN increases local NO action less in Dahl S than in R rats, thereby contributing to the enhanced pressor responses in Dahl S. In the PVN, NO enhances GABA release from GABAergic neurons (Li et al. 2004, Li et al. 2003b) and GABA release tonically inhibits local glutamate release and activation of AT_1-receptors (*section 1.3.1.*). In our proposed model (Figure 1-4), a decrease in NO action by Na^+ in the PVN of Dahl S may cause less GABA release and $GABA_A$ receptor activation on glutamatergic neurons with terminals adjacent to parvocellular neurons of the IML or RVLM. This effect increases glutamate release onto pre-sympathetic neurons (Figure 1-4). Furthermore, a decrease in NO action may also attenuate GABA release from GABAergic interneurons in the PVN, causing a decrease in GABA release onto pre-sympathetic neurons (Figure 1-4). Both effects of decreased NO action by Na^+ in the PVN further increase the activity of sympathoexcitatory neurons thereby enhancing the responses to a local increase in Ang II release and AT_1-receptor activation (*section 5.2.1.*). In next studies, we assessed whether these findings extend to Dahl S and R rats on high salt diet.

5.5. Mechanisms in the PVN contributing to dietary salt hypertension in Dahl S rats

5.5.1. NOS in the PVN

L-NAME in the PVN causes the same increases in BP and HR in Dahl S and R rats on regular salt diet, indicating that on regular diet, NO action in the PVN is similar in the

two strains. Consistent with these findings, mRNA (Serino et al., 2001, *Appendix 7.1.2.*) and protein (*Appendix 7.1.2.*) expression of nNOS are the same in the PVN of Dahl S and R rats on regular diet. Considering that nNOS is the NOS isoform primarily responsible for NO production in the PVN (Rossi et al. 2010), the responses to L-NAME are likely mediated by inhibition of nNOS in the PVN, causing decreased local NO production and disinhibition of the PVN (*section 5.4.2.*).

High salt diet attenuates pressor responses to L-NAME in the PVN of Dahl S, but not R rats. These findings are consistent with those from our acute infusions of Na^+ and L-NAME in the PVN (*section 5.4.2.*) and suggest that high salt intake inhibits NO activity in the PVN of Dahl S, but not R rats. However, high salt diet increases mRNA (Serino et al., 2001, *Appendix 7.1.2.*) and protein (*Appendix 7.1.2.*) expression of nNOS in the PVN of both S and R, but decreases responses to L-NAME only in Dahl S. Higher AT_1-receptor stimulation in the PVN of S vs. R rats likely causes differences in superoxide production and NO scavenging. In Dahl R rats, small increases in plasma Na^+ around periods of salt intake (Fang et al. 2000) may cause small increases in neuronal activity in CVO's of the LT, and this neuronal activity is relayed to the PVN via angiotensinergic connections (Li and Ferguson 1993) (*section 1.2.2.3.*). Small increases in Ang II release in the PVN may activate an NO-GABA negative feedback loop (*section 1.3.3.*) thereby off-setting Ang II-induced increases in activity of pre-sympathetic neurons. In Dahl S rats, increases in neuronal activity in the SFO or OVLT from a sustained increase in CSF [Na^+] may lead to high persistent Ang II release in the PVN via relayed signaling through the MnPO (Figure 3). An NO-GABA negative feedback loop associated with increased local AT_1-receptor stimulation in the PVN may increase local

nNOS expression and NO release thereby opposing the effects of aldosterone-EO on nNOS expression (*section 1.3.3.*). However, increased AT_1-receptor stimulation in the PVN enhances local superoxide production (Erdos et al. 2006), scavenging locally produced NO (Krukoff 1999, Zanzinger 2002) more in Dahl S vs. R rats thereby reducing its effectives to inhibit the PVN in Dahl S rats (Figure 5-1). High salt (8 % NaCl) diet for 4 weeks increases AT_1-receptor binding density in the PVN by ~150 and 200 % in Dahl R and S rats (Wang et al. 2003c) supporting that dietary salt increases angiotensinergic input in both strains but more in Dahl S. As will be further discussed below, on high salt diet, an AT_1-receptor blocker in the PVN lowers BP only in Dahl S rats (*section 4.5.2.*). High salt diet increases nNOS expression and possibly NO production in Dahl R rats but does not change BP responses to L-NAME in the PVN. The scavenging effect of increased ROS in the PVN of Dahl R rats could explain why responses to L-NAME were not enhanced. Further studies are needed to evaluate whether high salt diet actually increases NO release in the PVN of Dahl R and S rats and whether increased local ROS production decreases its effectiveness in restoring local GABAergic input in Dahl S.

As increased AT_1-receptor activation could decrease NO action in the PVN, in subsequent studies we assessed whether there is increased AT_1-receptor activation in the PVN of Dahl S rats on high salt diet. We also assessed changes in glutamate receptor activation since increased AT_1-receptor activation in the PVN may enhance local glutamate release (Latchford and Ferguson 2004) (*section 1.3.1.*).

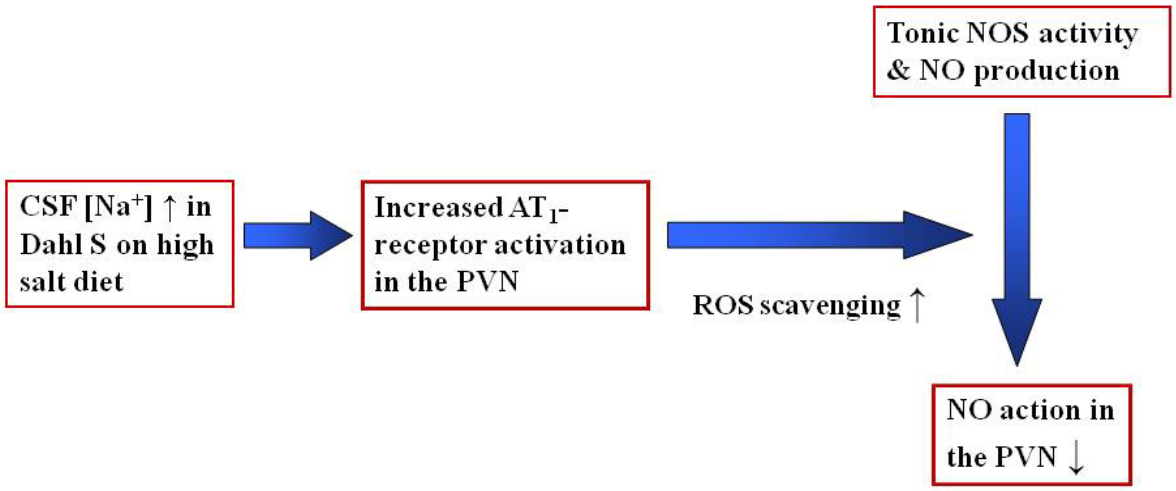

Figure 5-1: Proposed pathways contributing to decreased NO action in the PVN of Dahl S rats on high salt diet.

5.5.2. Ang II and glutamate in the PVN

Consistent with previous studies in normal rats (Freeman and Brooks 2007, Li and Pan 2007a), bilateral infusion of candesartan or kynurenate in the PVN did not change BP or HR in Dahl R and S rats on regular salt diet. These findings support that under normal conditions, GABA release in the PVN tonically inhibits glutamate release (Li et al. 2006a) and AT_1-receptors (Chen et al. 2003) (*section 1.3.1.*).

Consistent with other models with chronic sympathetic hyperactivity (Freeman and Brooks 2007, Li and Pan 2007a, Li et al. 2003c), both candesartan and kynurenate in the PVN lower BP in hypertensive Dahl S rats on high salt diet. These findings indicate that increased glutamate and AT_1 receptor activation in the PVN contributes to the maintenance of resting BP in Dahl S rats on high salt diet. An increase in CSF $[Na^+]$ in

Dahl S rats may activate neurons in the nuclei in the LT leading to increased Ang II and glutamate release in the PVN (*section 1.3.2.*). Further studies need to assess whether this increased Ang II and glutamate release in the PVN is derived from terminals of separate sets of neurons or via co-release from the terminal of the same neuron (*section 1.2.2.3.*). An increase in neuronal activity in the LT is also relayed to magnocellular neurons of the SON and PVN leading to increased aldosterone production mainly in magnocellular neurons of the SON (Huang et al. 2010). Increased aldosterone release activates MR-ENaC in magnocellular neurons of the SON and PVN causing increased EO release in the pPVN, enhancing activity of excitatory angiotensinergic sympathetic pathways (*section 1.2.3.1*; Figure 1-3). In addition, a chronic increase in CSF [Na^+] raises hypothalamic tissue [Na^+] (Wang et al. 2010) and an increase in [Na^+] in the PVN may further increase local Ang II (Qadri et al., 1994, *see above 5.2.1.*) and glutamate (Jin et al. 2001) release. According to our proposed model (*section 1.3.1*; Figure 1-4), increased AT_1-receptor activation in the PVN decreases GABA or increases glutamate release onto pre-sympathetic neurons projecting to the IML or RVLM thereby increasing their neuronal activity, SNA and BP. To determine whether the effects from increased AT_1-receptor activation in the PVN are mediated by increased local glutamate release, one blocker was infused at the peak BP response to the other blocker in Dahl S rats on high salt diet. Candesartan caused no change in BP after it was infused at the peak BP responses to kynurenate indicating that the effects of increased AT_1-receptor activation in the PVN of Dahl S are fully mediated by local glutamate release. To determine whether the increased glutamate release fully depends on AT_1-receptor activation, kynurenate was infused after candesartan in the PVN. After candesartan, kynurenate in the PVN further

decreased BP by ~50 % indicating that another mechanism(s) independent of AT_1-receptor stimulation in the PVN contributes to part of the enhanced release of glutamate. Thus, both Ang II dependent and independent pathways appear to equally contribute to elevated glutamate release in the PVN. The Ang II dependent pathway may depend on decreased GABA release from GABAergic neurons and increased glutamate release from glutamatergic neurons on pre-sympathetic neurons (*see above*). In addition, increased AT_1-receptor activation in the PVN may increase production of ROS, scavenging locally produced NO (*section 5.5.1.*) thereby decreasing GABA release in the PVN (*section 1.3.1.*). The Ang II independent pathway could be contributed by a subset of glutamatergic neurons in the PVN from the MnPO (Csaki et al. 2000) with terminals adjacent to parvocellular neurons of the IML or RVLM. These glutamatergic neurons would not be influenced by decreased GABAergic inhibition via increased AT_1-receptor activation or direct AT_1-receptor stimulation (Figure 1-5). Alternatively, chronic stimulation of group I metabotropic glutamate receptors in the PVN may enhance glutamate release or increase activity of NMDA receptors on pre-sympathetic neurons (*section 1.3.1.*), thereby contributing to the BP response to enhanced glutamate receptor activation in the PVN of Dahl S. In addition to increased excitatory and decreased inhibitory synaptic inputs in the PVN, high salt diet in Dahl S rats may alter the intrinsic membrane properties of neurons of the PVN. An increase in intracellular Ca^{2+} from a number of intracellular signalling pathways eg. AT_1-receptor signalling (*section 1.2.2.3.*), opens small conductance Ca^{2+}-activated K^+ (SK) channels, enhancing K^+ outflow through SK channels, causing hyperpolarization (Stocker 2004). This mechanism serves to dampen the excitability of pre-sympathetic neurons of the PVN (Chen and Toney 2009),

attenuating glutamatergic signalling (Li et al. 2008b). Chronic sc infusion of Ang II in SD rats on high (2 %) NaCl diet for 2 weeks diminished SK current in neurons of the PVN projecting to the RVLM from a hypothalamic slice preparation. Further studies need to assess whether a decreased SK current in pre-sympathetic neurons of the PVN contributes to the enhanced local glutamatergic input.

5.6. Conclusion

Genetically determined salt sensitivity of BP largely contributes to the development of hypertension in both humans and animals. In salt sensitive animals, high salt diet increases CSF [Na^+] and activates an aldosterone-EO neuromodulatory pathway in the brain that enhances activity of angiotensinergic sympatho-excitatory pathways and thereby mediates the salt-induced sympathetic hyperactivity and hypertension. Our findings show that the pressor and HR responses to an acute increase in CSF [Na^+] depend on AT_1-receptor activation in the PVN. An MR-ENaC-EO pathway can be functionally active in the PVN, but does not appear to contribute to these responses to CSF [Na^+]. Whether this neuromodulatory pathway in the PVN contributes to sympathetic hyperactivity and hypertension from a chronic increase in CSF [Na^+] still needs to be assessed. We also showed that an enhanced responsiveness to [Na^+] exists in the PVN of Dahl S vs. R rats. Both an acute and chronic increase in [Na^+] by high salt intake appears to less increase local NO action in Dahl S than in R rats contributing to the enhanced pressor responses to Na^+ and, presumably, to dietary salt-induced hypertension in Dahl S rats. High salt intake enhances AT_1-receptor activation in the PVN of Dahl S, but not R rats and this may lead to increased ROS production, scavenging locally produced NO more in Dahl S vs. R thereby reducing its effectiveness to stimulate

GABAergic inhibitory pathways in Dahl S. Decreased GABA release in the PVN of Dahl S rats on high salt intake may contribute to our findings that there is increased local glutamate release, but another mechanism(s) that is not yet defined appears to further enhance glutamate responses. These findings advance our knowledge of the mechanisms in the PVN contributing to salt-induced sympathetic hyperactivity and hypertension (Figure 5-2).

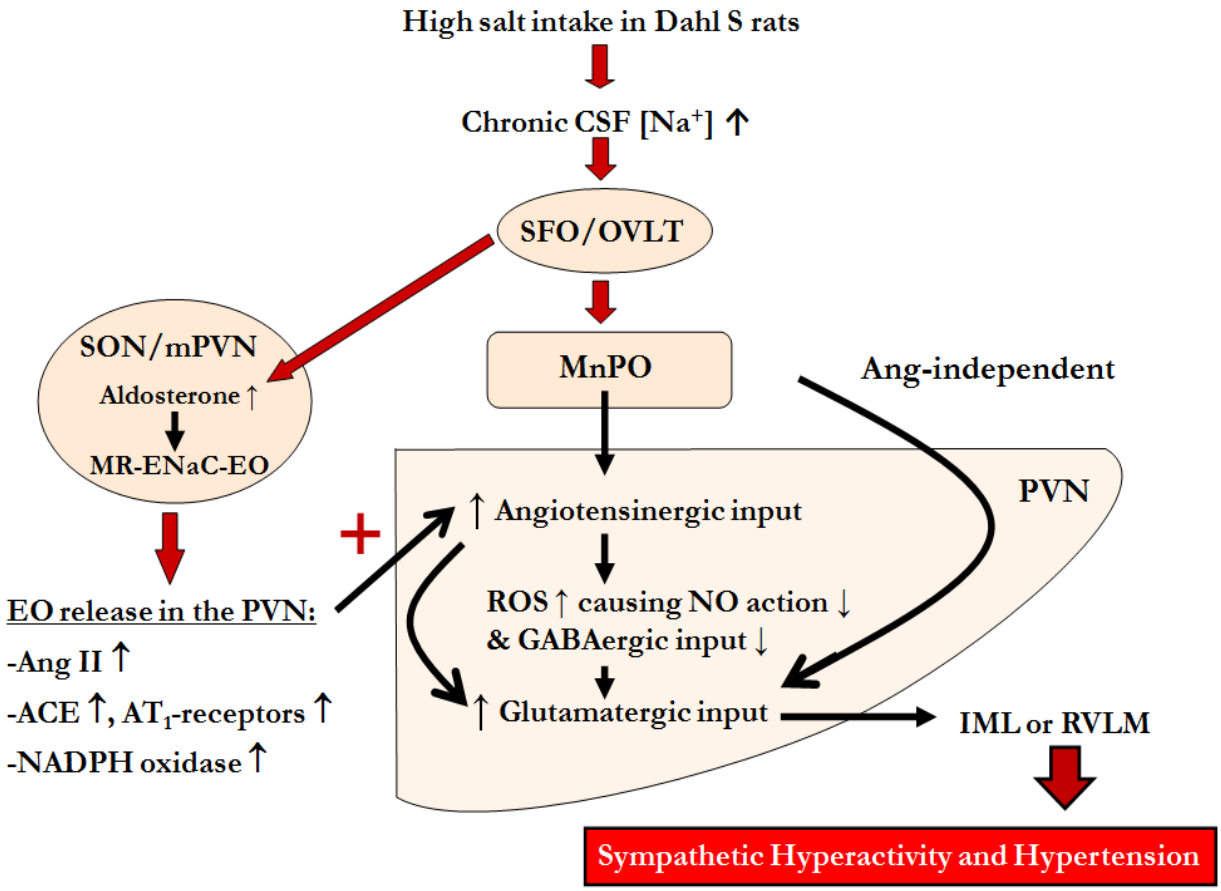

Figure 5-2: Summary of central pathways involved in a chronic increase in CSF [Na^+] from high salt diet in Dahl S rats.

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7. APPENDIX

7.1. mRNA and protein expression of nNOS in the PVN of Dahl rats

7.1.1. Brief Methods: Real Time RT-qPCR and Western Blotting

Animals and Diets

Five weeks old male Dahl S (SS/Mcw) and R (SS.BN13) rats were obtained from Charles River Breeding Laboratories, Montreal, Quebec, Canada. The rats were provided with water ad libitum and regular rat chow (0.3 %, 120 umol Na^+/ gram) for one week to become acclimatized to the laboratory rooms according to the guidelines of the University of Ottawa Animal Care Committee for the use and care of laboratory animals. Subsequently, rats were fed either Research diets® AIN-76A regular (0.1 %, 44 umol Na^+/ gram) or high (8 %, 1408 umol Na^+/ gram) NaCl diet for 3 weeks.

Real Time RT-qPCR

After 3 weeks of regular or high salt diet, Dahl S and R rats were injected with pentobarbital, perfused with phosphate buffered saline (PBS) and their brains were quickly collected and frozen at -20^{o}C with methyl-butane. Tissues were stored at -80^{o}C until further use. Brains were then sectioned on a cryostat and punches of the PVN were performed with a 0.5 mm micro-punch needle. The punches were ejected into a homogenization buffer and RNA was extracted and purifed with the Invitrogen® RNA spin column purification kit. cDNAs were synthesized by the QuaniTect® Reverse Transcription kit with integrated removal of genomic DNA contamination. The products of cDNA synthesis were amplified by real time qPCR with a primer of nNOS and Phosphoglycerate kinase 1 (PGK1) with a Roche® light cycler using fast-start DNA

Master SYBR Green. mRNA expression levels were calculated according to standard curves generated previously from runs with plasmids containing cDNA from nNOS or PGK1. Expression was normalized to PGK1 levels as an endogenous reference. Normalization was achieved by dividing the amount of nNOS cDNA by the PGK1 quantity.

Western Blotting

After 3 weeks of regular or high salt diet, Dahl S and R rats were euthanized by decapitation and their brains were removed, frozen and sectioned as previously described for qRT-PCR. Punches of the PVN were ejected into a chilled homogenization buffer containing 50 mM Tris, 150 mM NaCl, 1% sodium dodecyl sulfate, 1% sodium deoxycholic acid, 1 mM phenylmethylsulfonyl fluoride, 1 mM ethylene diamine tetracetic, 1% Triton X-100 and a protease inhibitor complex. Samples were vortexed twice for 30 s and left on ice for 1 hour. They were then frozen at -80^{o}C for 1 hour, thawed, and the quantity of protein was evaluated with a Pierce® BCA protein assay. 30 ug of protein from each sample was then loaded and run on an 8% Tris-glycine SDS-polyacrylamide gel. Protein was then transferred to a nitrocellulose membrane using a Trans-Blot cell (Biorad®) running at 0.3 A for 2 hours. Membranes were blocked with 5% milk for 30 min and cut to separate nNOS (155 kDa) and B-actin (42 kDa) protein. Membranes were incubated with an anti-nNOS mouse monoclonal antibody raised against amino acids 2-300 of nNOS of human origin (1:500, Santa Cruz) or an anti-B-actin mouse monoclonal antibody (1:10,000, Sigma). For all membranes, sheep anti-mouse with horseradish peroxidase (1:10,000, GE Healthcare) was used as a secondary antibody and incubated for 1 hour. Membranes were incubated with Western Lightning

Chemiluminescence Plus reagents (Perkin Elmer) for 1 min, and bands were visualized with a chemifluorescence digital camera. Protein expression was assessed by the density of the bands according to AlphaEase® computer software. B-actin protein expression was served as an internal control for nNOS expression. Normalization was achieved by dividing the amount of nNOS by B-actin quantity.

7.1.2. Results: mRNA and protein levels of nNOS in the PVN of Dahl rats

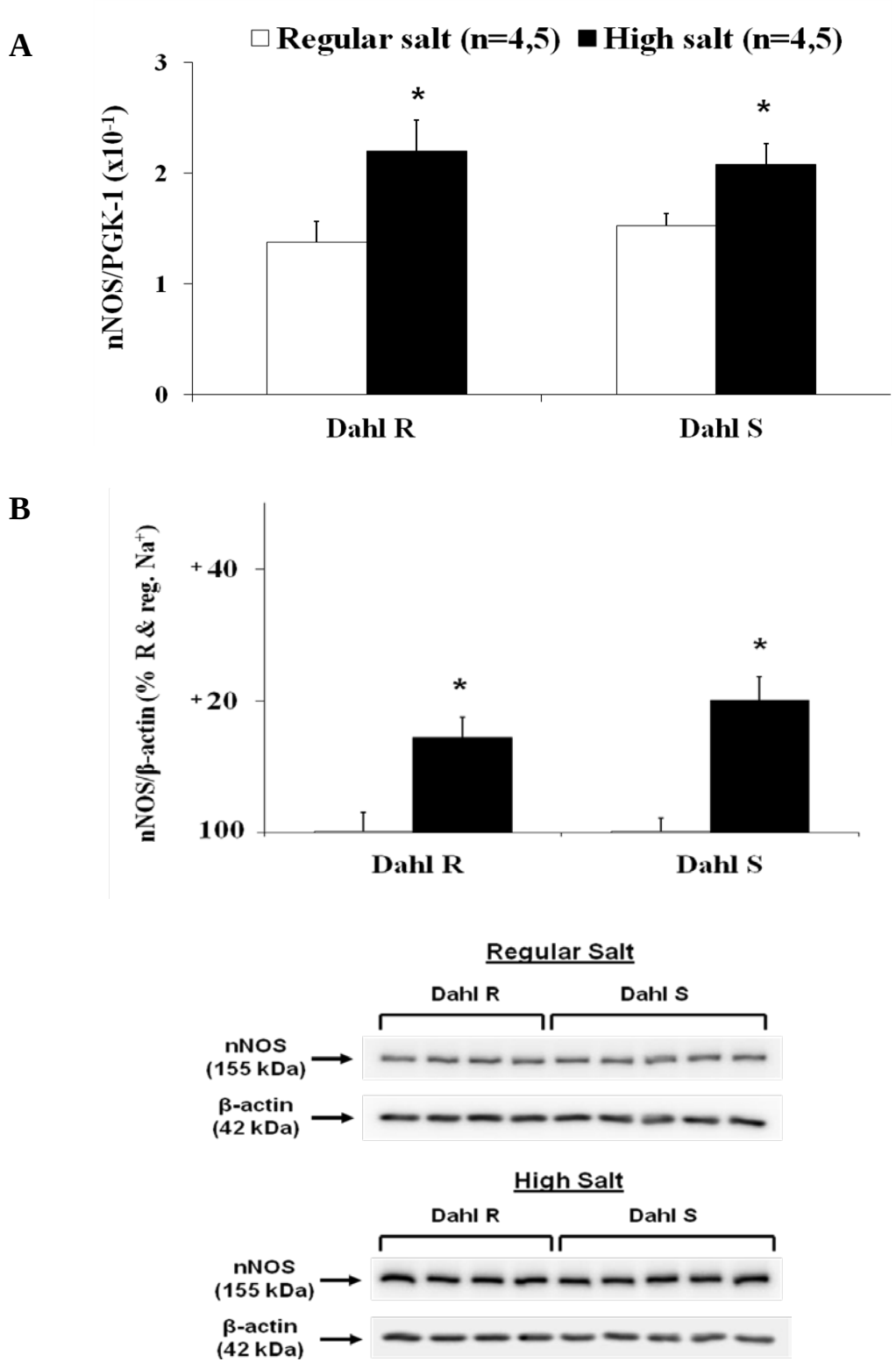

Figure 7-1: High salt diet increases nNOS mRNA (A) and protein (B) expression similarly in the PVN of Dahl S and R rats. Values are means ± SE. *p<0.05 vs. regular salt