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A Role for the Brain Na, K-ATPase α2 Isoform in Salt-Sensitive Hypertension : Enhanced Pressor Responses to Increased CSF Sodium and Ouabain Concentrations in Gene-Targeted Heterozygous α2 Na, K-ATPase Knockout Mice

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By

Steven F. Theriault

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For My Family

ABSTRACT

A high-salt diet raises the sodium concentration in the cerebrospinal fluid (CSF $[Na^+]$) in salt-sensitive individuals; an effect that can be mimicked by intracerebroventricular (icv) infusion of NaCl. Increasing CSF $[Na^+]$ increases the concentration of an endogenous brain ouabain-like substance(s) (OLS) that inhibits brain Na, K-ATPases, which in turn activates the brain renin-angiotensin system, augmenting sympathetic nervous system activity and blood pressure. It is unknown which of the ouabain-sensitive Na, K-ATPase α subunit isoforms (α2 or α3) in the brain mediates the pressor responses to increased CSF $[Na^+]$. We hypothesize that the α2 isoform mediates the pressor responses to elevated CSF $[Na^+]$, such that reduced expression of the α2 isoform in mice, via heterozygous gene-targeted knockout, should enhance the pressor response to icv infusion of Na^+ or ouabain, compared to wildtype litter mates. In adult (8-10 wk) mice, icv infusion of 0.225 M NaCl increased mean arterial pressure (MAP) significantly in α2 heterozygous (+/-) mice only, with greater responses in female +/- vs. male +/- mice. Western analysis of α subunit isoforms indicated that the reduction in α2 expression was greater in female +/- compared to male +/- mice, suggesting that the magnitude of the pressor responses to icv Na^+ are inversely related to α2 isoform expression. Pressor responses to icv ouabain were also exaggerated in α2+/- vs. +/+ mice, suggesting that the enhanced responses to icv Na^+ were mediated through direct downregulation of ouabain-sensitive α isoforms per se. There were no differences in α1 or α3 isoform expression between α2+/- wildtype (+/+) mice; nor were there any baseline differences in immediate upstream effectors (brain [OLS]) or putative downstream (brain angiotensin-converting

enzyme activity) effectors of cardiovascular regulation between α2 +/- and +/+ mice. These studies suggest a critical role for the α2 subunit isoform of the Na, K-ATPase in the pressor response to increased CSF [Na^+].

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LIST OF ABBREVIATIONS

+/+	wildtype
+/-	heterozygous (knockout)
ACE	Angiotensin-Converting Enzyme
ACSF	artificial cerebrospinal fluid
AngII	angiotensin II
ATP	adenosine triphosphate
AT_1R	angiotensin type 1 receptor
AVP	arginine-vasopressin
CNS	central nervous system
CSF	cerebrospinal fluid
CSF [Na^+]	Na^+ concentration in the cerebrospinal fluid
CVLM	caudal ventrolateral medulla
CVO	circumventricular organ
Dahl-R	Dahl salt-resistant rat
Dahl-S	Dahl salt-sensitive rat
ELISA	enzyme-linked immunosorbent assay
ER	endoplasmic reticulum
GABA	gamma-aminobutyric acid
GFAP	glial fibrillary acidic protein
HPLC	high performance liquid chromatography
HR	heart rate

ICV	intracerebroventricular
IV	intravenous
MAP	mean arterial pressure
MBG	marinobufagenin
MnPO	median prepoptic nucleus
NCX	Na^{+}/Ca^{++} exchanger
NKA	Na^{+}, K^{+}-ATPase
NMR	nuclear magnetic resonance
NTS	nucleus tractus solitarius
OLS	ouabain-like substance
OVLT	organum vasculosum of the lamina terminalis
PBS	phosphate-buffered saline
PBS-T	phosphate-buffered saline with 0.05% tween-20
PCR	polymerase chain reaction
PM	plasma membrane
PVDF	polyvinylidene fluoride
PVN	paraventricular nucleus
RAS	renin-angiotensin system
RSNA	renal sympathetic nervous activity
RVLM	rostral ventrolateral medulla
SBP	systolic blood pressure
SERCA	sarco/endoplasmic reticulum calcium aTPase
SFO	subfornical organ

SHR	Spontaneously hypertensive rat
SON	supraoptic nucleus
SR	sarcoplasmic reticulum
TBS-T	tris-buffered saline with 0.1% tween-20
TM	transmembrane
TSHβ	Thyroid stimulating hormone beta chain
WKY	Wistar-Kyoto rat

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Chapter 1

INTRODUCTION

1.1. The Na, K-ATPase

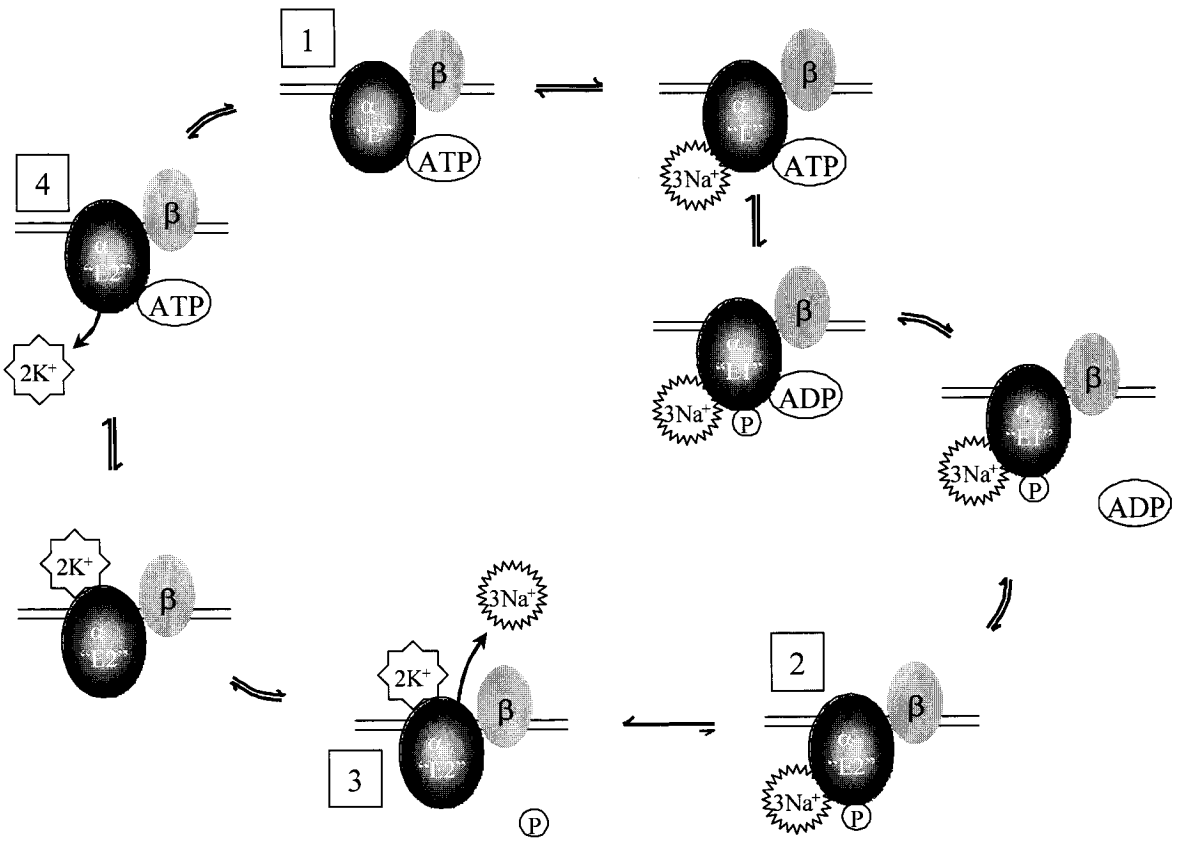
This section will provide a general overview of the Na^+, K^+-ATPase (NKA) in regards to structure, function, and its regulation. However, because of the importance of this enzyme and its variability of distribution, more detailed analysis of the role of individual subunits will be presented as they pertain to different physiological systems.

1.1.1. Function

The NKA is a membrane bound protein whose principal function is to transport 3 Na^+ ions out of the cell against its electrochemical gradient, in exchange for 2 K^+ ions into the cell. The energy for this reaction is supplied by the hydrolysis of one molecule of ATP per reaction cycle, resulting in a phosphorylated intermediate. This scheme is commonly referred to as the Albers-Post model of NKA cycling (Albers 1967; Post et al., 1972; Jorgensen, 1986; Lingrel and Kuntzweiler, 1994). Figure 1 demonstrates the ion transport cycle of the NKA. The net effect of transporting one extra positive ion out of the cell per reaction cycle is a relatively negative membrane voltage potential on the inside of the cell. Although this resting membrane potential varies from tissue to tissue, excitable tissues such as neurons and myocytes typically maintain a resting potential of approximately –70 mV.

The NKA represents the only means for sodium efflux out of the cell and this is crucial in fueling a myriad of secondary ion, water, and large molecule transport mechanisms. For instance, the buildup of Na^+ electrochemical gradients from the outside towards the interior of the cell fuels the reentry of Na^+ into the cell via transport channels such as the Na^+/Ca^{++} exchanger (facilitates Ca^{++} efflux), the Na^+/H^+ exchanger (facilitates H^+ efflux), the Na^+/glutamate cotransporter (facilitates glutamate influx), and the Na^+/glucose cotransporter (facilitates glucose influx). These are just some of the many

Figure 1. Albers-Post model of NKA cycling. The binding of ATP (1) allows for the binding of Na^+ on the intracellular surface of the α subunit. Subsequent ATP hydrolysis and α subunit phosphorylation allows for the translocation of Na^+ to the extracellular space, while promoting K^+ binding on the extracellular surface of the pump (2 to 3). The release of phosphate allows for the binding of a new molecule of ATP, which causes the translocation of K^+ into the cytoplasm (3 to 4) and the cycle continues thusly. Adapted from Jorgensen 1992, and Albers 1967.


1
β
ATP
3Na+
ADP
P
2K+
2
3
4

examples of secondary active transport mechanisms dependent upon the NKA. Another very important function in regulating Na^+ gradients is the co-regulation of water absorption by the cell, since Na^+ gradients create an osmotic gradient for water diffusion. Regulation of water absorption by cells is fundamental in maintaining cell volume (reviewed by Mobasheri et al., 2000). On a more integrative physiological level, the regulation of Na^+/water balance is fundamental in regulating total blood volume and blood pressure.

1.1.2. Structure-function relationships

The functional unit of the NKA is a heterodimer consisting of one alpha subunit and one beta subunit. Practically, this heterodimer does not exist as a single heterodimer, but tends to polymerize through α-α subunit interactions (Brotherus et al., 1983; Koster et al., 1995). The NKA is a P-type IIA ATPase (phosphorylated-intermediate type IIA) enzyme. The P-type simply means that this enzyme harnesses energy derived from the hydrolysis of the terminal (gamma) phosphate bond of ATP, and by extension, undergoes conformational changes upon binding the phosphate group (Fagan and Saier, 1994). This shift in conformation is necessary for proper enzymatic function. Type II of the P-type ATPase superfamily represents a subgroup whereby the main catalytic subunit (alpha subunit; α) is associated with a smaller subunit (beta subunit; β). Type IIA differs from type IIB, in that the main catalytic unit of a type IIA enzyme lacks a hydrophobic C-terminal domain capable of interacting with modulators such as calmodulin (Moller et al., 1996; Axelsen and Palmgren, 1998).

The α subunit (MW 100-112 kDa) is the principal catalytic subunit of the functional NKA. This subunit contains all of the cation-binding sites and ATP/ADP/PO_4 binding sites necessary for enzymatic function. The bulk of this subunit is intracellular, and both the

amino and carboxy-terminal regions are intracellular. There are 10 transmembrane helices and the extracellular loops are extremely small compared to the intracellular loops (Moller et al., 1996; Rice et al., 2001; Herbert et al., 2003). The largest intracellular loop spans between the TM4-TM5 region and contains the cation- and nucleotide-binding and phosphorylation sites responsible for ion transport. The α subunit also contains the binding sites for cardiac glycosides, which inhibit the activity of the NKA (this will be elaborated upon in section 1.1.6.2.) (reviewed by Mobasheri et al., 2000).

The α subunit is dependent upon the beta subunit, not necessarily for catalytic function per se, but rather for translocation and insertion into the plasma membrane. The β subunit is a single-membrane-spanning peptide that is glycosylated (M_r of core peptide ~35kDa and M_r of glycosylated peptide 45-55 kDa). This membrane-directing action of the β subunit is crucial for NKA activity. This is exemplified in transfection studies in Sf-9 cells using α and β subunit constructs, or studies in LLC-PK1 or kidney proximal tubule cells where b subunit expression was pre- and post-translationally modified. Changes in β subunit expression affect the formation of active holoenzyme complexes proportionally, resulting in either translocation to the cell surface, or internalization of αβ complexes, appropriately (McDonough et al., 1990; Blanco et al., 1995a; Laughery et al., 2003).

1.1.3. Identification of individual α subunit isoforms

In the late 1970's, two molecular forms of the α subunit had been identified in brain tissue of rats, termed α and α(+), which were separable by SDS-PAGE (Sweadner, 1979). The α and α(+) subunits were soon after identified in rat cardiac and adipocyte tissue (Matsuda 1984, Lytton 1985a). Studies by Matsuda et al. (1984) and Lytton (1985b) demonstrated that the α(+) isoform is more sensitive to inhibition by pyrithiamin (a thiamine

derivative) and ouabain and that insulin stimulates the activity of the α(+) isoform. These lines of evidence lent strong support to the belief that multiple isoforms of the α subunit existed. The debate was laid to rest, when in the mid 1980's, Shull and associates (1986) cloned three distinct α subunits derived from rat brain cDNA libraries, and probed for by mRNA from sheep kidney. From the work of Shull et al. (1986) "α" became known as α1, whereas "α(+)" consisted of α2 and the then-newly-discovered α3 isoform. It was also confirmed that the individual isoforms are products of unique genes, which more readily explains the variations in tissue distribution of the isoforms (Shull et al., 1986; Lingrel et al., 1990). Finally, in the early 1990's, putative mRNA transcripts of a fourth α isoform (α4) were identified in human rat testis (Shamraj and Lingrel, 1994); and later, immunoblotting techniques were used to detect the protein in testis tissue. It was determined that this polypeptide functions as a Na^+, K^+ transporter, and that this peptide binds to and can be inhibited by ouabain. Taken together, these findings prove the existence of the fourth subunit (Woo et al., 1999; Blanco et al., 1999).

1.1.4. Distribution of individual alpha subunit isoforms

1.1.4.1. Tissue distribution

Each isoform demonstrates a unique pattern of distribution. This gives the sense that an individual isoform performs a specific role(s). The mRNA distribution in major rat tissues was examined by Northern analysis (Shamraj and Lingrel 1994). Of note, it is clear that α1 is expressed ubiquitously in all tissues with little exception. With respect to the α2-4 isoforms, the analysis is somewhat misleading because the conditions used in these tissue (Northern) analyses only recognize high abundance mRNA copies and thus did not detect expression in tissues with low abundance. Nevertheless, it is apparent that in the rat, the α2

subunit is localized in skeletal muscle, heart and brain; the α3 subunit is expressed in the brain; and the α4 isoform is expressed in testis. For the most part, this tissue distribution for α2, α3 and α4 is consistent among most mammalian species albeit with some inter-species variations. For instance, although the α3 isoform is not appreciably expressed in rat cardiac tissue, it is detectable by immunoblotting techniques in human and mouse heart. Interestingly, guinea pig and sheep heart lack the α2 and α3 isoforms altogether, according to Northern blot analysis (Sweadner et al., 1994). Brain tissue in all mammalian species expresses the α2 and α3 proteins. More in-depth localization of expression patterns of each of these isoforms has revealed that the α2 subunit is located almost exclusively in glia (Cameron et a., 1994; Peng et al, 1998) while α3 is restricted to neurons (Cameron et al., 1994; Peng et al., 1997).

1.1.4.2. Subcellular distribution

At the subcellular level, the localization of isoforms is rather heterogenous among the different isoforms. In regards to α1, this isozyme, as visualized by immunofluorescence techniques shows a uniform distribution along cellular membranes, and in rat neurons, glia and vascular smooth muscle, is not limited to specific regions of the cell (Juhaszova and Blaustein, 1997a; Juhashova and Blaustein, 1997b).

Immunocytochemical techniques used to examine the cellular localization of α2 and α3 have demonstrated that in contrast to α1, α2 and α3 appear to be concentrated in domains where the outer plasma membrane comes in close contact with intracellular sarco/endoplasmic membranes (see figure 2, in section 1.1.5) (Juhaszova 1997a, Juhaszova 1997b). The α2 and α3 isoforms are often segregated to specific compartments of cells. For instance, in rat skeletal muscle cells, immunocytochemical studies have revealed that α2

is found along transverse (t-) tubular sarcolemmal membranes – important organelles involved in Ca^{++} storage (Marette et al., 1993; Hundal et al., 1994; Cougnon et al., 2002). The expression of the α3 isoform in adult neurons is polarized. Whereas dendritic and somal regions predominantly express α1, α3 is highly concentrated along axonal and synaptosomal membranes (Wetzel et al., 1999).

1.1.5. The roles of individual alpha isoforms

The previous sections have established that (1) the different alpha subunits are all products of individual genes; (2) the tissue distribution of each isoform is heterogenous and highly regulated; and that (3) the subcellular distribution of all isoforms is also differentially regulated. Therefore, although the basic ion-transporting function of the various NKA isoforms is in principle the same, the above essential differences are strongly indicative of an individual role(s) for each of these isoforms. There have been many technical challenges associated with dissecting the functions of individual isoforms by means of ATPase activity assays, ^{3}H-ouabain binding studies, etc... because many cell types contain more than one isoform with different properties, or because studies in which overexpression systems were employed suffer from the limitation that target cell lines all contain endogenous NKA.

Because of its ubiquitous distribution in cells, it has been suggested that α1 is responsible for maintaining global cellular concentrations of Na^{+} and K^{+}. Although the term "housekeeping" may be somewhat of a misnomer (as the expression and activity of α1 is regulated by a variety of factors), the term is still apt in regards to function. Its role in maintaining global Na^{+}/K^{+} ion gradients is further supported by the aforementioned observation that the distribution of α1 along cellular surfaces appears to be uniform. In certain cell types, such as epithelia of luminal structures, α1 serves a highly specialized

function. In nephron proximal tubules for instance, Na^+ passing through the lumen will be absorbed by inward Na^+ channels (Epithelial Na^+ Channel for example) and thus enter the cell. The excretion of Na^+ out of the cell into the interstitium is regulated by the activity of $\alpha 1$ along the basolateral membrane.

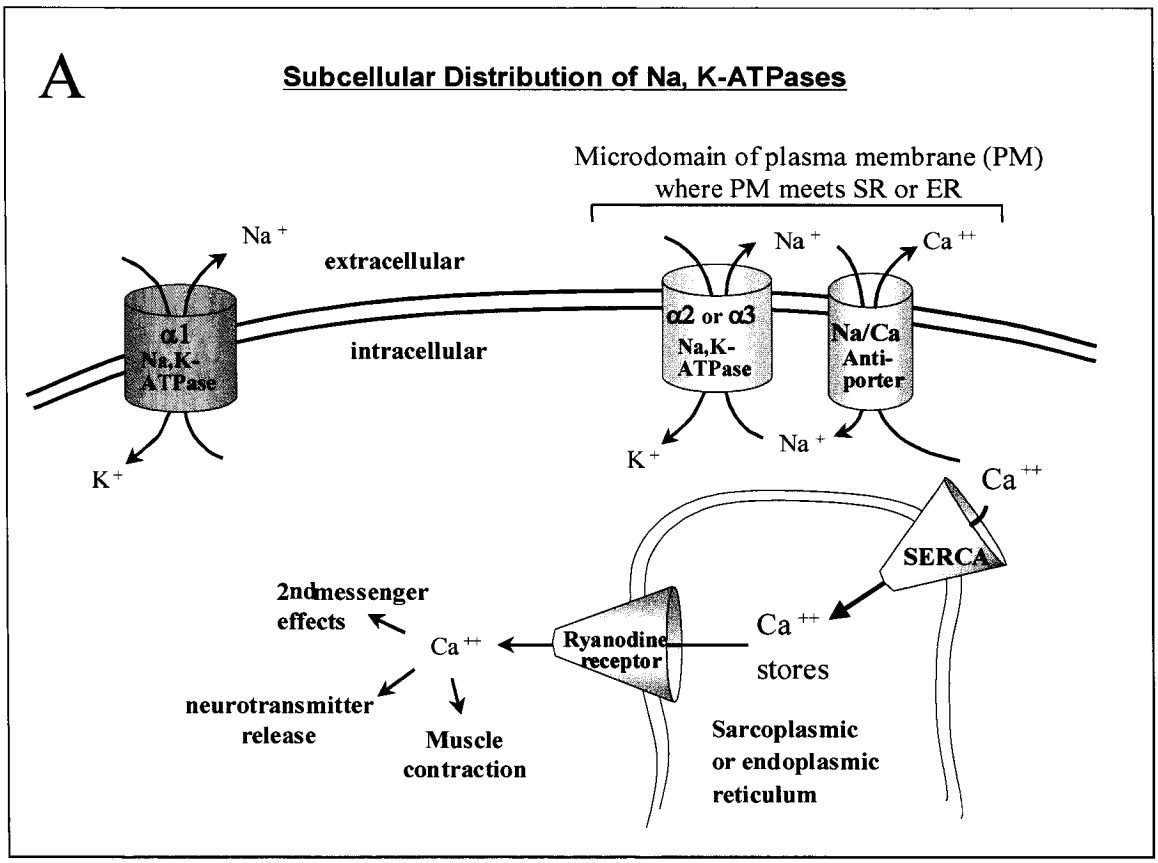
The functions of $\alpha 2$ and $\alpha 3$ are believed to be much more specific than that of $\alpha 1$. The $\alpha 3$ isoform is found predominantly in excitable neural tissue, concentrated along axonal and synaptosomal membranes. Additional studies lend significant information to the kinetic properties of $\alpha 3$. In one study by Munzer et al. (1994), HeLa cells were transfected with various α isoforms, mutated such that they were ouabain-resistant. Ouabain was added to the experimental medium to inhibit endogenous α isoforms. These studies demonstrated that the $\alpha 3$ isoform had less affinity for intracellular Na^+ than $\alpha 1$ or $\alpha 2$, as well as lower affinity for extracellular K^+ than $\alpha 1$ or $\alpha 2$. These conclusions were supported by Blanco et al. (1995a), who transfected insect sf-9 cells (from the ovary of the fall armyworm) with various baculovirus constructs expressing α and β subunit sequences. Sf-9 cells constitute an appropriate system, because they contain all of the necessary components for the assembly and expression of exogenous proteins resulting from transfection, yet they possess minimal levels of endogenous NKA isoforms (DeTomaso et al., 1993). These studies indicated that the $\alpha 3\beta 1$ NKA (the predominant combination in axonal plasma membranes) have lower cytoplasmic Na^+ and extracellular K^+ affinities than $\alpha 1\beta 1$ (Blanco et al., 1995b). In consideration of the drastic *local* ionic shifts across membranes during an action potential (i.e. local intracellular Na^+ increases, as does local extracellular K^+), it is tempting to speculate that an important function of the $\alpha 3$ isoform is to act as a large reservoir of NKA

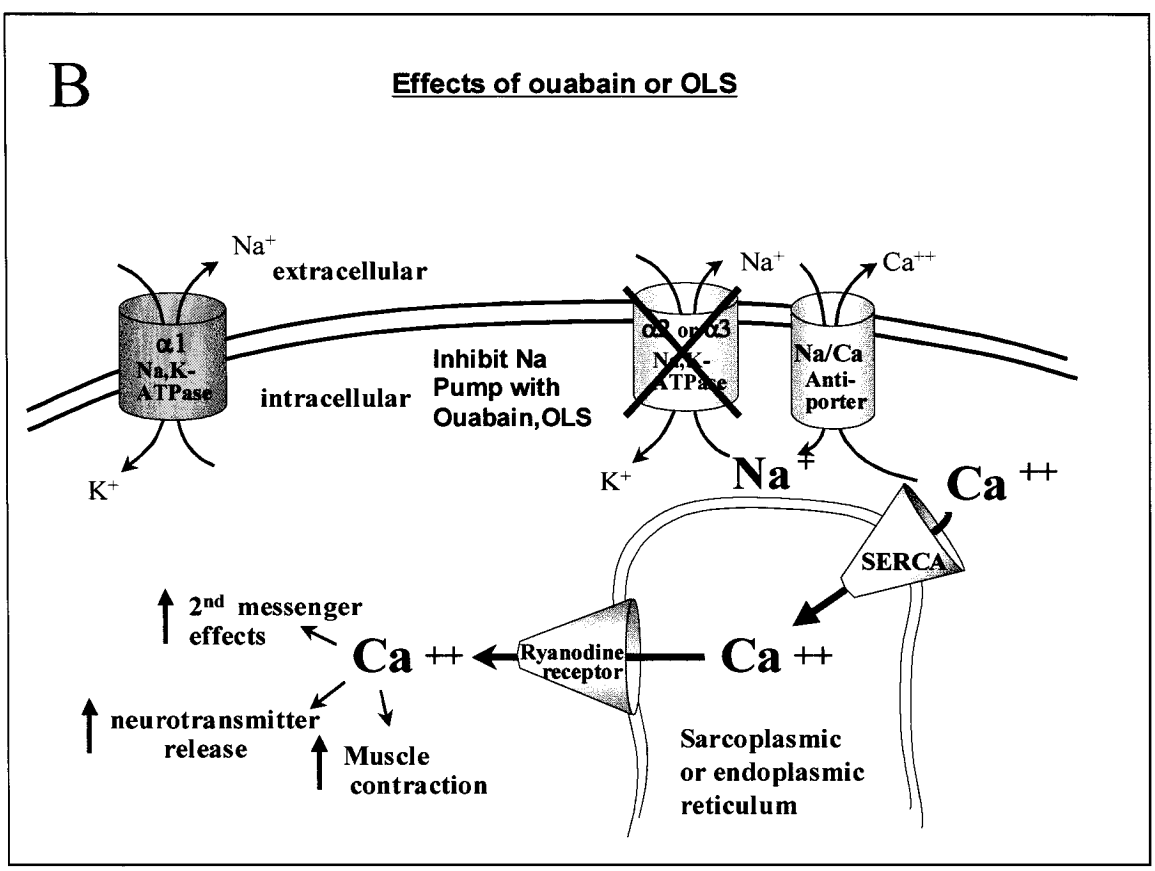
activity in excitable tissues in order to quickly restore local resting membrane potentials (Munzer et al., 1994).

The function of the α2 NKA isoform is the focus of the studies presented herein. A large body of evidence is accumulating to suggest that α2 is intricately involved in the fine-tuned regulation of Ca^{++} balance in cells. It was mentioned previously that α2 expression is limited to microdomains along the cellular plasma membrane that are in close proximity to sarco/endoplasmic membranes. These microdomains shall henceforth be referred to as plasmerosomes – a term coined by Juhaszova and Blaustein (Juhaszova and Blaustein, 1997b; Blaustein et al., 1998). The role of α2 at the plasmerosome is presented in figure 2. Plasma membrane Na^+/Ca^{++} exchangers (NCX) are also localized to these plasmerosomes, as are Sarco/Endoplasmic Reticulum Ca-ATPases (SERCA) (Blaustein et al. 2002).

The co-localization of the α2 isoform with Ca^{++} regulatory enzymes suggests a role for α2 in cellular Ca^{++} metabolism. Regulation of local Na^+ gradients at the plasmerosome, directly impacts local Ca^{++} gradients via the NCX, thus affecting Ca^{++} storage by the cell. More specifically, extracellular Na^+ gradients are maintained by the action of the NKA. The NCX uses this gradient to force Ca^{++} out of the cell, against its concentration gradient. Ca^{++} in the plasmerosome however, can also be transported across sarco/endoplasmic domains via SERCA, to be stored for later use. When an appropriate stimulus causes the release of Ca^{++} from these stores, the cell will react accordingly (e.g. muscle contraction, neurotransmitter release, gene regulation etc...). In the event that the activity of an isoform at plasmerosomes (such as α2) is inhibited, local extracellular Na^+ gradients at the plasmerosomes are diminished, in turn providing less drive for Ca^{++} efflux. In addition, elevated local $[Na^+]_I$ causes direct NCX inhibition via Na^+ binding to intracellular sites

Figure 2. The proposed effect of NKA inhibition at the plasmerosome. In (**A**), while the $\alpha 1$ NKA isoform maintains global cytoplasmic $[Na^+]$, the isoforms at the plasmerosome regulate local $[Na^+]$ between the plasma membrane and sarco/endoplasmic reticulum. This in turn regulates local Ca^{++} gradients, affecting the amount of Ca^{++} stored in the sarco/endoplasmic reticulum. In (**B**), inhibition of the NKA at the plasmerosome by ouabain or OLS results in elevated local $[Na^+]_i$. The increase in $[Na^+]_i$ inhibits secondary active transport of Ca^{++} out of the cell. As a result of increased local cytoplasmic $[Ca^{++}]$, more Ca^{++} is stored in the reticulum. Subsequent stimulation of the cell causes enhanced Ca^{++} release (relative to **A**), and exaggerated Ca^{++}-dependent cellular responses accordingly.


A
Subcellular Distribution of Na, K-ATPases
Microdomain of plasma membrane (PM) where PM meets SR or ER
Na +
extracellular
intracellular
α1 Na,K-ATPase
α2 or α3 Na,K-ATPase
Na/Ca Anti-porter
Ca ++
K +
Na +
SERCA
2nd messenger effects
Ca ++
Ryanodine receptor
Ca ++ stores
neurotransmitter release
Muscle contraction
Sarcoplasmic or endoplasmic reticulum


B
Effects of ouabain or OLS
Na+
extracellular
intracellular
α1 Na,K-ATPase
Inhibit Na Pump with Ouabain,OLS
Na/Ca Anti-porter
Ca++
K+
Na +
Ca ++
SERCA
2nd messenger effects
Ca ++
Ryanodine receptor
neurotransmitter release
Muscle contraction
Sarcoplasmic or endoplasmic reticulum

(Hilgemann et al., 1992). Examples of such inhibition may include the actions of cardiac glycosides such as ouabain (discussed in section 1.1.6.), or protein downregulation. Elevated [Ca^{++}] at the intracellular plasmerosome allows for increased transport and storage of Ca^{++} in the sarco/endoplasmic reticuli. As before, an appropriate stimulus will again cause Ca^{++} release into the cytosolic space, however, in this instance, extra Ca^{++} will be released, generating enhanced Ca^{++} effects (i.e. increased contraction, increased neurotransmitter release, etc...) (Blaustein 1993; Monteith and Blaustein 1998; Arnon et al., 2000).

1.1.6. Regulating Na, K-ATPase activity – a focus on ouabain-like substances

Even four years before the discovery of the NKA, Schatzman et al. described the inhibition of Na^+ and K^+ across plasma membranes by cardiac glycosides, and Szent-Gyorgi proposed a physiological rationale for the existence and function of possible endogenous cardiac glycosides (reviewed by Hamlyn et al., 1998). Cardiac glycosides represent a class of steroid-derived compounds that bind and inhibit α NKA subunits varyingly, depending on the type of cardiac glycoside, the type of isoform (e.g., α1,2 or 3), and the species of animal. Examining the effects of the many different family members is far too broad for the scope of this thesis. Instead, because of the well-documented involvement of endogenous "ouabain" (hereafter referred to as ouabain-like substance or OLS) in the regulation of NKA activity, blood pressure and heart rate in both acute and chronic states of sodium excess, this review will focus on the OLS specifically. It should be noted that while this review does focus on the similarities between ouabain and the OLS, for the purposes of the studies presented herein, the OLS is not explicitly defined as being identical to ouabain. Instead, OLS can be more broadly defined as any endogenous substance that is bound by Digibind®,

an antibody fragment that can bind a wide array of cardiac glycosides. Digibind® is capable of blocking blood pressure responses to Intracerebroventricular (icv) Na^+ infusion in both rats and mice (see section 1.5. for further review).

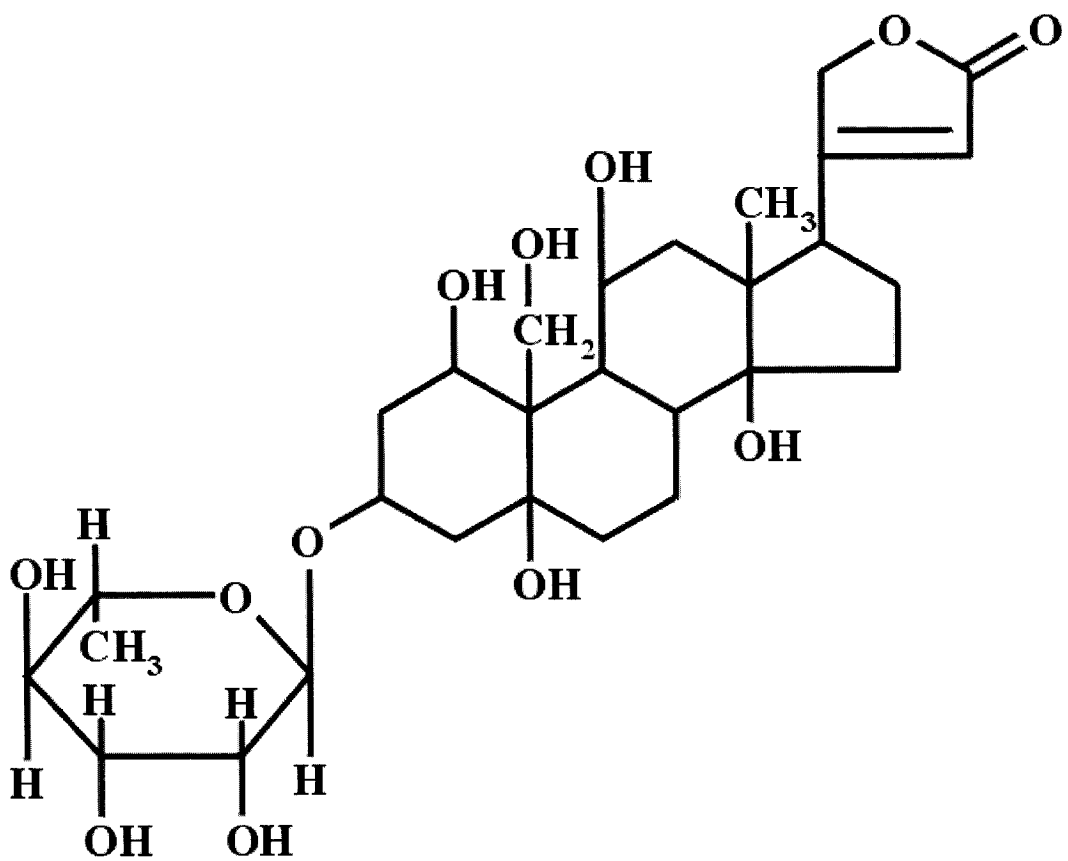
1.1.6.1. Characterization of ouabain-like substances (OLS)

Early studies by Hamlyn et al. (1991) and Mathews et al. (1991) have contributed a great deal to the characterization of the OLS in human plasma. Their analyses indicated that the OLS was indistinguishable from plant-derived ouabain, either by mass spectrometry (by native substance analysis as well as by derivitization of alcohol groups to acetyl groups), inhibiton of human erythrocyte NKA vs. exogenous ouabain, displacement of ^{3}H-ouabain, or immunoreactivity (by enzyme-linked immunosorbent assay). Later opinion was that the OLS was a stereoisomer of ouabain because studies involving napthoylation of the hydroxyl groups of the OLS and ouabain resulted in differing HPLC retention times, NMR spectra, and circular dichroic analysis (Ludens et al., 1991; Tymiak et al., 1993; Zhao et al., 1995). A more recent study by Kawamura et al. (1999) rebutted these findings however, suggesting that the differences in structural analyses under these conditions were confounded by the fact the collected OLS were stored in borosilicate glassware, and formed tetrahedral borate complexes that interfered with the napthoylation process.

1.1.6.2. Inhibition of NKA by ouabain/OLS

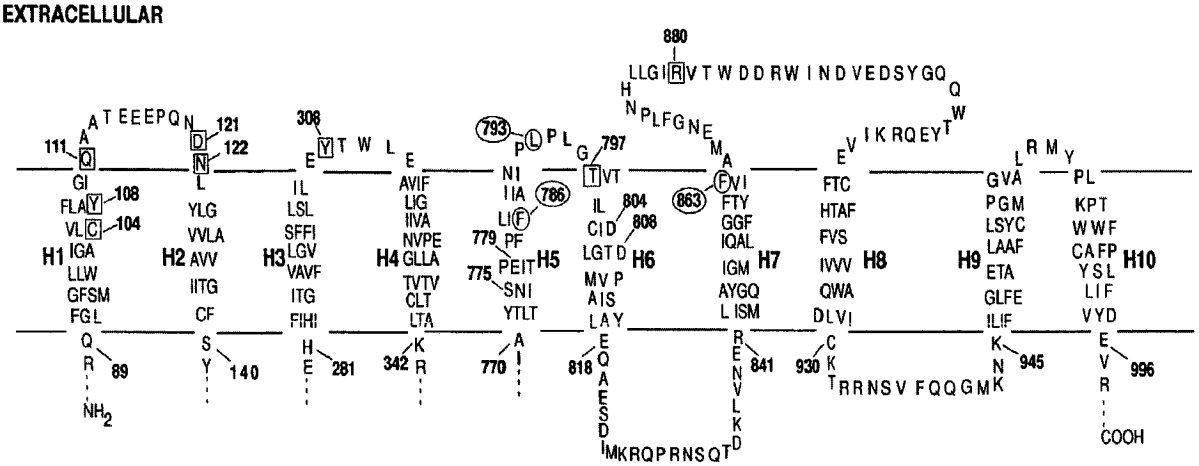
The structure of ouabain is shown in figure 3. Cardiac glycosides have three structural domains, a sugar moiety, a sterol backbone, and a lactone ring; and each contribute to the binding to NKA α subunits (From et al., 1990). However, it is still unclear how each domain contributes to NKA binding, nor is it entirely resolved where the glycosides bind to and inhibit the NKA. The α subunit of the NKA is highly conserved

Figure 3. The Structure of ouabain. There are three structural components to cardiac glycosides. In this case, ouabain is composed of a rhamnose glycoside attached to the C3 position of a sterol backbone. Ouabain is also characterized by a lactone ring at the C17 position.


O
O
OH
CH3
OH
OH
CH2
OH
OH
O
H
OH
O
CH3
H
H
H
H
OH
OH

between different isoforms and species, with the most variation present in the N-terminus; and this variation results in different sensitivities to inhibition by ouabain (or OLS). In all species, the α2 and α3 isoforms are inhibited by ouabain at low concentrations (IC_{50} of 10 – 500 nM), and are thus "ouabain-sensitive". Rodent α1 on the other hand is ~1000-fold more resistant to ouabain binding than α2 or α3 (IC_{50} of 40 - 320 μM) and is thus "ouabain-resistant" (Berrebi-Bertrand et al. 1990; O'Brien et al. 1994; and reviewed by Blanco and Mercer 1998). In contrast to rodents, the α1 isoform in most other species (such as sheep and human for example), is ouabain-sensitive, to a similar extent as the α2 and α3 isoforms in rodents. Figure 4, adapted from Palasis et al., 1996, represents a schematic diagram of important residues of the NKA in the binding of ouabain. Early mutagenesis studies determined that replacing just two residues at the borders of the TM1-TM2 extracellular hairpin loop in sheep α1 with the amino acids found in rat (i.e., Gln111Arg and Asn122Asp), was able to confer ouabain-resistance equal to that of the rat isoform (Price and Lingrel, 1988). Other mutagenesis studies showed that mutations in Asp121 of the TM1-TM2 loop, and Cys104 and Trp108 of the TM1 domain, were able to confer ouabain resistance to sheep α1, strongly implicating this region in ouabain binding, or at least affecting the affinity to ouabain (Price et al., 1989; Canessa et al., 1992; Schultheis et al., 1993). Other evidence suggests that the TM1-TM2 loop does not directly bind cardiac glycosides, but rather confers resistance by modulating dissociation rates at the true binding site located elsewhere on the α subunit. Using a monoclonal antibody directed against the sheep α1 TM1-TM2 hairpin loop does not confer ouabain resistance, but instead enhances ouabain-sensitivity (Arystarkhova et al., 1992). The expression of a chimera of the N-terminus of rat gastric H+, K+-ATPase (ouabain-insensitive) with the C-terminus of rat α1

Figure 4. Important residues in ouabain interactions with the NKA. Mutagenesis studies have shown certain residues of the NKA sequence to be important determinants of ouabain sensitivity (circled and boxed residues). Notice the localization of key residues to the extracellular surface near the H5-H6 (=TM5-TM6) hairpin, with nearby interaction from the extracellular N-terminal portion of the H7-H8 loop. Other important interactions are mediated by residues at the H1-H2 extracellular loop, particularly the membrane border residues, asn111 and asp122. This figure was taken from Palasis et al., 1996.


EXTRACELLULAR
CYTOPLASMIC
H1
H2
H3
H4
H5
H6
H7
H8
H9
H10
NH2
COOH

NKA (ouabain-insensitive), yields a highly ouabain-sensitive enzyme (Blostein et al., 1993). Furthermore, in a study of the binding of various cardiac glycosides to wildtype sheep α1 and mutated sheep α1 (mutated in the N-terminal positions outlined above), it was found that although the kinetics of cardiac glycoside binding shifted to varying degrees depending on the mutation, the ranking of binding of different cardiac glycosides did not change (O'Brien et al., 1993). These pieces of evidence suggest a true binding domain in the C-terminus of the enzyme, whose affinity to cardiac glycosides is modulated by the TM1-TM2 loop. This is supported by studies involving random mutagenesis of C-terminal domains. Mutations in Arg880, Thr797, Leu793, Phe786 and Phe863 of the sheep α1 subunit have all been implicated in conferring ouabain-resistance, and thus implicated in ouabain binding (Schultheis et al., 1993; Burns and Price, 1993; Palasis et al., 1996). Phe786 is in the intramembrane space, near the extracellular surface of the TM5-TM6 hairpin loop, while Leu793 and Thr979 are in the TM5-TM6 loop. Phe863 and Arg880 are located in the nearby N-terminal portion of the TM7-TM8 loop (Jorgensen, 1992). These residues are highly conserved in all species and in all α isoforms. The TM5-TM6 loop is crucial in binding cations (K^+) and translocating them to the cytoplasm (Arguello and Lingrel, 1995; Vilsen, 1995).

1.2. Salt-sensitive hypertension

Statistics in 2001 from the Center for Disease Control show diseases of the heart to be the number one leading cause of death among residents of the United States (Arias et al., 2003). And a major contributor to the progression of heart disease and heart failure is hypertension. A significant proportion of the North American population has high blood

pressure and the probability of developing hypertension dramatically increases with age. For instance, between 1999-2002, approximately 5% of U.S. residents under the age of 35 have hypertension (defined as having systolic blood pressure greater than 140 mmHg and diastolic blood pressure greater than 90 mmHg), approximately 30% have hypertension between ages 35-65, and ~70% of people over 65 suffer from hypertension (National Center for Health Statistics, 2004). In addition, primary essential hypertension in and of itself was the 14th leading cause of death in the United States in 2001 (Arias et al., 2003).

There is a distinct subset of hypertensive people who are salt-sensitive. By definition, salt-sensitive hypertensives demonstrate elevated blood pressure (both systolic and diastolic) upon consuming dietary salt, whereas salt-resistant people are unresponsive (or minimally responsive) to salt in their diet (de la Sierra et al., 1996; Manunta et al., 2001). Salt-sensitive hypertension is a highly polygenic condition, and all causative genetic factors remain to be elucidated. Additionally, putative causative genes are heterogeneously distributed among the human population; as such, approximately 50% of the general population of hypertensives are salt-sensitive (Weinberger et al., 1986; de la Sierra et al., 1996).

Salt-sensitive hypertension is not restricted to humans, and is in fact present in many mammalian species, including rodents. Various strains of rats, including the Dahl salt-sensitive (Dahl-S) rat and Spontaneously Hypertensive Rat (SHR), are widely used as models of salt-sensitive animals. New genetic models of mice, including the ones in this study, are becoming more and more prevalent and are breaking new ground in our understanding of salt-sensitive hypertension.

1.3. The role of the kidney in salt-sensitive hypertension

Without argument the kidney plays a fundamental role in regulating Na^+ and water balance and the development of salt-sensitive hypertension. However, while the following sections will bring to light the role the kidney plays in Na^+/water balance, they will also highlight the overriding importance of brain mechanisms crucial to the development of salt-sensitive hypertension.

1.3.1. The kidneys of Dahl-S and SHR rats

In the 1970's, Dahl et al postulated that salt-sensitivity in the Dahl-S rat (vs. the resistant Dahl-R rat) was solely dependent upon the genotype of the kidney (Dahl et al., 1974). In an elegant experiment, Dahl et al. subjected both Dahl-R and -S rats on a regular 0.3% NaCl diet to bilateral nephrectomy, followed by single renal transplants of the same or opposite genotyped kidney. This resulted in four groups: Dahl-R rat with an R or S kidney and Dahl-S rat with an R or S kidney. Uninephrectomized rats of both genotypes served as controls. On a regular sodium diet (0.3%), regardless of the genotype of the rat, recipients of Dahl S kidneys showed a 30-mmHg greater increase in systolic blood pressure (SBP) than rats of either genotype that received a Dahl R kidney. In similar experiments, Bianchi et al. (1974) transplanted kidneys from Spontaneously Hypertensive Rats (SHR) into Wistar Kyoto rats (WKY; a normotensive control strain for the SHR) and vice versa. On a regular sodium diet (1%), WKY rats with SHR kidneys had elevated SBP vs. WKY rats with WKY kidney; whereas SHR rats with WKY kidneys had reduced SBP compared to SHR rats with SHR kidneys. The kidneys of both Dahl-S and SHR rats exhibit deficient urinary excretion of Na^+ when challenged by a high-salt diet, compared to their normotensive, salt-resistant controls. However, depending on the animal model, the mechanism of Na^+ handling

deficiency varies: in an isolated kidney perfusion study, the deficiency in Na^+ excretion in the Dahl-S rat was explained by enhanced tubular reabsorption of sodium, whereas in the SHR strain it was a result of deficient glomerular filtration (Vaneckova, 2002). In both these strains of rats then, salt-sensitive animals were characterized by their inability to adequately regulate Na^+ at the level of the nephron.

1.4. Extra-renal mechanisms in salt-sensitive hypertension

The Dahl-S and SHR studies presented in the preceeding section for a regular-salt diet represent only half the story. In studies similar to the original Dahl study, the results held up on a regular-salt diet. However, when challenged with a high-salt diet (8% NaCl) after being on a regular-salt diet for 4 weeks post surgery, it was found that all transplant groups (i.e., Dahl-S rat with Dahl-R kidney, Dahl-R rat with Dahl-S kidney, or Dahl-S rat with Dahl-S kidney) demonstrated equally elevated increases in SBP by 40-60 mmHg relative to the Dahl-R rat with Dahl-R kidney group (Morgan et al., 1990). For the SHR/WKY studies, 90 days after the renal transplant surgery, rats were switched from their regular-salt to a high-salt diet (8% NaCl). Interestingly, while the SHR and WKY recipients of an SHR kidney demonstrated a further elevation in SBP by ~40 mmHg, the SHR recipients of a WKY kidney showed a further increase in SBP by ~30 mmHg, and WKY recipients of a WKY kidney also showed further elevations in SBP by 40-50 mmHg (Bianchi et al., 1974). These studies indicate that under the stress of Na^+ loading, the apparent pressor responses seem to be rather independent of renal genotype. Clearly then, there must be extra-renal mechanisms involved in the regulation of hypertensive responses to Na^+ excess.

1.5. Role of the brain in salt-sensitive hypertension

The brain is a major control center of many physiological responses, and plays a fundamental role in the mediation of hypertensive responses, in salt-sensitive hypertension. Salt-sensitive hypertension in both SHR and Dahl-S rats is mediated to a large extent by increased sympathetic nervous system activity (Takeshita et al., 1979; Oparil et al., 1988). It has also been shown recently that when SHR and Dahl S rats are switched from a regular Na^+ to a high Na^+ diet, increases in CSF sodium concentration (CSF $[Na^+]$) precede the onset of hypertension, suggesting an important role for central Na^+ in the etiology of salt-sensitive hypertension (Huang et al., 2004). Logically, it follows that an increase in CSF $[Na^+]$ by direct icv infusion would mimic the effects of a high-salt diet – a fact that has been established in both rats and mice (Huang et al., 1992; Huang and Leenen, 1992; Van Huysse and Hou, 2004). Interestingly, both icv infusion of Na^+ in Wistar rats (a "Na^+-insensitive" control strain for SHR) and a high salt diet in SHR rats yield similar elevations in CSF Na^+, and subsequent elevations in brain OLS and renin-angiotensin system (RAS) activation, renal sympathetic activity, blood pressure and heart rate (Huang et al., 1992; Huang and Leenen, 1994a; Huang and Leenen, 1994b; Huang and Leenen, 1996a; Huang and Leenen, 1996b; Huang and Leenen 1998; Huang et al., 1998). This would suggest then, that a large part of what determines one to be "salt-sensitive/resistant" is how well CSF $[Na^+]$ is maintained in response to Na^+-loading. Similar hypertensive responses can be elicited by direct icv infusion of ouabain or angiotensin II (angII). The pressor effects of a high-salt diet or icv NaCl infusion in Dahl-S and SHR rats, C57Bl/6, or Swiss Webster mice are preventable by icv administration of angiotensin II type-1 receptor (AT_1R) antagonists, or antibody fragments that bind ouabain-like substances. This suggests the hypertensive

responses to icv Na^+ are mediated by both the OLS and RAS. In addition, the responses to icv ouabain can be blocked by icv administration of AT_1R antagonists, whereas blocking the effects of ouabain has no bearing on hypertensive responses to icv angII. It can be concluded then, that elevations in brain OLS precede/cause activation of brain RAS pathways, resulting in salt-sensitive hypertension. These responses are not inhibitable by intravenous administration of AT_1R antagonists or OLS-binding antibody fragments (at the same doses that are effective icv), further implicating a CNS role in mediating salt-sensitive hypertension (Huang and Leenen, 1996a; Huang et al., 1998; Huang and Leenen, 1998; Van Huysse and Hou, 2004). In Dahl-S rats, chemical sympathectomy prevents/reverses salt-induced hypertension (Takeshita et al., 1979; Gordon et al., 1981), while in SHR rats, salt-induced hypertension is associated with elevated plasma norepinephrine levels, and the hypertension is prevented by ganglionic blockade (Winternitz and Oparil, 1982). Clearly, brain OLS and the brain renin-angiotensin system (RAS) are important effectors of sympathetic hyperactivation elicited by elevated CSF $[Na^+]$.

1.5.1. Endogenous brain OLS

1.5.1.1. Localization of brain OLS

Although the adrenal cortex is a major organ involved in the synthesis of circulating OLS (reviewed in section 1.6.2.1.), adrenalectomized SHR rats given daily aldosterone and corticosterone replacement had only minimal reductions in both circulating and brain OLS levels (Leenen et al., 1993b). This suggests that an organ(s) other than the adrenal cortex can synthesize OLS. The brain is a likely candidate. Guinea pig brain tissue extracts demonstrate an ability to inhibit ^3H-ouabain binding (Fishman, 1979), and a substance characterized as an isomer of ouabain has been isolated from bovine hypothalamic extracts

(Tymiak et al., 1993). In further support of brain-derived OLS synthesis, OLS has been isolated from isolated mouse primary astrocyte cultures (Kala et al., 2000). Immunohistochemical localization studies have highlighted the presence of ouabain in several cardiovascular regulatory areas. In the hypothalamus, OLS has been identified in the paraventricular nucleus (PVN), magnocellular nuclei and in the posterior pituitary. The immunoreactive PVN fibers run through the lateral hypothalamus to the median eminence associated with the capillaries of the hypophysial portal vein. This suggests a possible mechanism whereby OLS from the magnocellular neurons can be released into the circulation (Yamada et al., 1987; Ihara et al., 1988; Yamada et al., 1992a). In the hindbrain, OLS has been localized to the rostral ventrolateral medulla (RVLM), the ventromedial medulla, the nucleus tractus solitarius (NTS) and the dorsal motor nucleus of the vagus (Yamazato et al., 1998). As well, OLS immunoreactivity was found in circumventricular organs (CVO) such as the subfornical organ (SFO), organum vasculosum of the lamina terminalis (OVLT) and median preoptic nucleus (MnPO) (Yamada et al., 1992b). CVOs are structural nuclei that line the ventricles of the brain. They lie outside the blood-brain barrier and are highly responsive to changes in the cerebrospinal fluid milieu (for instance, elevated CSF Na^+ or ang II concentrations). Because they can serve a sensory function, they often act as relays between (patho)physiological conditions in the periphery and effector control systems in the brain.

1.5.1.2. Effects of brain OLS on cardiovascular regulation

Dahl-S rats given a high salt diet exhibit elevated pituitary, hypothalamic and pons OLS levels, in addition to elevated adrenal and plasma levels, compared to normotensive rats strains on similar diets. The increases in brain OLS precede the onset of hypertension

(Leenen et al., 1993a; Leenen et al., 1994). Independent of sodium, icv administration of ouabain causes a dose dependent elevation in mean arterial pressure (MAP), heart rate (HR) and renal sympathetic nervous system activity (RSNA) in salt-sensitive and normotensive rat strains (Huang and Leenen, 1992); and elevated MAP and HR have been demonstrated in normotensive mice (Van Huysse and Hou, 2004). Icv administration of Digibind®, an antibody fragment that binds the OLS with high affinity, prevents/reverses the pressor responses to icv Na^+ or ouabain administration (Huang et al., 1992; Leenen et al., 1994; Van Huysse and Hou, 2004). Budzikowski and Leenen (1997) have demonstrated that microinjection of Digibind® into the MnPO abolishes the pressor response to icv ouabain. In this nucleus, Digibind® had no effect on baseline blood pressure and thus it is likely that OLS here mediates pressor responses to elevated CSF Na^+, but not the maintenance of resting tone. On the otherhand, Teruya et al. (1997) have shown that microinjection of ouabain into the rostral ventrolateral medulla (RVLM) causes elevations in MAP and RSNA; and these responses can be blocked by pre-injection of Digibind®. Microinjection of Digibind® in the RVLM alone however, can significantly decrease baseline MAP and RSNA, implicating a role for OLS in the establishment of vasomotor tone in this brain area. This is logical, as pre-motor neurons in the RVLM play a crucial role in establishing and maintaining sympathetic vasomotor tone (reviewed by Dampney, 1994).

1.5.2. Brain RAS

It was not until 1961 that the brain has been shown to be responsive to the renin-angiosensin system (RAS), when in a study by Bickerton and Buckley (1961), angII-infused blood from one dog elicited hypertensive responses when infused into the brain of another dog. Since then, all components of the RAS have been located in the brain, and several of

the biological actions of angII have been characterized. The brain RAS is crucial in mediating hypertensive responses to icv Na^+ infusion, or high-salt diet in salt-sensitive individuals, if only by virtue of the fact that brain-specific antagonism of RAS components (e.g., using ACE inhibitors or AT_1R blockers) prevents the hypertensive responses to salt loading (Takahashi et al., 1984; Huang and Leenen, 1996b).

1.5.2.1. Angiotensinergic components in the brain

Most of the RAS components have been localized to areas of the brain involved in cardiovascular regulation.

Angiotensinogen, the precursor protein to angII formation has been found to be expressed in astrocytes rather ubuiquitously in the brain, but with high concentrations in the preoptic area, hypothalamus and medulla (Lynch et al., 1987; Stornetta et al., 1988; Bunnemann et al., 1992). Angiotensinogen is also an abundant component of cerebrospinal fluid (Hilgenfeldt, 1984). Renin, at the protein level, has been particularly difficult to localize in the brain. However, renin-specific activity in extracts of whole mouse brain has been demonstrated (Speck et al., 1981), and renin mRNA has been found in mouse and rat brain as determined by Northern analysis, using large quantities of total RNA (>100 μg). Recently, Morimoto et al. (2002a) generated transgenic mice in which human renin was expressed. They found that while the full-length protein was expressed in the kidney of the transgenic mice, the brain expressed a truncated form. They proposed that this enzyme could act intracellularly (as opposed to the common circulating form of renin) to convert angiotensinogen to angiotensin I. This may also explain some of the difficulties encountered in locating brain renin by immunohistochemical techniques.

Angiotensin-converting enzyme (ACE) is ubiquitously expressed in the brain, but is high in concentration in the circumventricular organs (CVO) such as the SFO, OVLT, area postrema and median eminence. ACE is also highly expressed in nuclei such as the MnPO, the NTS and dorsal motor nucleus of the vagus of the hindbrain (Saavedra and Chevillard, 1982; Chai et al., 1990; Rogerson et al., 1995). ACE has been predominantly associated with the plasma membranes of astrocytes, suggesting that angII synthesis occurs in astrocytes, or in the interstitial fluid in the vicinity of astrocytes (Pickel et al., 1986).

Angiotensin peptides including angII have been located extensively throughout the brain by immunohistochemical identification. However, neuronal cell bodies exhibiting angII immunoreactivity are limited to select brain regions including the NTS, PVN, and SFO (Lind et al., 1985; Oldfield et al., 1989).

Finally, the predominant angII receptor in the brain is the angiotensin type 1 receptor (AT_1R). AT_1Rs are found in the highest concentrations along circumventricular organs such as the SFO and area postrema, and moderately in the OVLT and median eminence. They are also highly expressed in nuclei such as the MnPO, PVN and RVLM, CVLM (caudal ventrolateral medulla), NTS and dorsal motor neuron of the vagus (Mendelsohn et al., 1984; Gehlertet al., 1986; Allen et al., 1988a; Lenkei et al., 1997).

1.5.2.2. Effects of brain RAS on cardiovascular regulation

Activation of brain RAS is critical in eliciting hypertensive responses to icv Na^+, and by extension, plays a significant role in mediating the hypertensive responses to high sodium diets in salt-sensitive animals. The sympathoexcitatory responses elicited by icv administration of Na^+ or ouabain can be prevented by prior administration of an ACE inhibitor such as captopril (thus preventing angII formation) or AT_1R blockers such as

losartan (Takahashi et al., 1984; Huang and Leenen, 1996b). Huang and Leenen (1996b) have also shown that while the pressor responses to icv ouabain could be blocked by RAS inhibitors, the pressor responses to icv angII infusion could not be attenuated by preadministration of Digibind®. This has been observated in mice as well (Van Huysse and Hou, 2004). This argues that elevated CSF Na^+ causes the release of brain OLS, in turn inhibiting ouabain-sensitive isoforms (i.e., $\alpha 2$ or $\alpha 3$) resulting in the activation of the brain RAS, leading to sympathetic hyperactivity and to enhanced pressor responses. It remains unclear though how inhibiting ouabain-sensitive isoforms results in RAS activation.

There is abundant evidence supporting the role of the brain RAS in the regulation of cardiovascular responses. Several microinjection studies have shown that injection of angII into the third ventricle, PVN, SFO, AP, NTS, RVLM all result in elevated MAP (Jensen et al., 1992; Simpson, 1981; Allen et al., 1988b; Andreatta et al., 1988). The CVOs are not shielded by the blood-brain barrier, and because they express large amounts of AT_1R, it is believed that these regions are important in mediating central effects in response to circulating angII. It appears that the circulating angII acts predominantly through the SFO and OVLT to induce thirst, vasopressin release and salt appetite (Simpson, 1981; Mangiapane et al., 1984; Fitts et al., 2000), whereas circulating angII increases blood pressure by acting on the AP (Otsuka et al., 1986).

The ventrolateral medulla represents a major area in determining sympathetic vasomotor tone. The RVLM and CVLM effectively oppose one another, by increasing sympathetic tone and blunting afferent baroreceptor signal, or decreasing sympathetic tone and enhancing baroreflex responses respectively (Dampney, 1994; Badoer et al., 1994). Microinjection of angII into the RVLM causes pressor responses, including elevated renal

sympathetic nerve activity (RSNA) (Sasaki and Dampney, 1990), and blunts the arterial baroreflex response to transient phenylephrine injection (Lin et al., 1997). In contrast, angII stimulation in the CVLM decreases MAP and HR in both SHR and WKY rats (Muratani et al., 1991). The NTS represents the first relay station for baroreflex afferents, and this nucleus contains several angiotensinergic neurons, with efferents to the RVLM and CVLM, implicating this nucleus in baroreflex control (Allen et al., 1988a).

Several neurons of the lamina terminalis including the SFO, OVLT and MnPO that express AT_1R have polysynaptic connections to the kidney via renal sympathetic nerves, implicating these regions in renal regulation of blood pressure and blood volume (Giles et al., 2001). Takagawa and Dampney (1999) demonstrated crosstalk between pressor pathways of the hypothalamic and hindbrain areas. In their study, the PVN of anaesthetized rats was activated as a consequence of disinhibition by microinjection of bicuculline – a gamma-aminobutyric acid receptor antagonist, suggesting there is tonic GABA-ergic inhibition in this nucleus. The resulting pressor responses were effectively inhibited (40-50%) by micro-injection of Losartan into the RVLM.

It is clear that central angiotensinergic mechanisms impact sympathetic drive. But what makes an animal salt-resistant (e.g., Dahl-R or WKY) or develop salt-sensitive hypertension (e.g., Dahl-S or SHR)? Much more work is needed to fully resolve this issue. When Na^+ or ouabain are infused icv in Dahl-S and Dahl-R, or SHR and WKY rats, the Dahl-S and SHR rats have significantly greater sympathoexcitatory, blood pressure and heart rate responses, and elevated baroreflex impairment vs. Dahl-R rats (Huang et al, 2001; Huang et al., 1992; Huang and Leenen, 1994b). This suggests that salt-resistant animals are less sensitive to the central effects of sodium. As previously mentioned, Huang et al. (2004)

recently reported that elevations in brain Na^+ precede the onset of hypertension in Dahl-S and SHR rats. This same study also determined that high-salt diets do not cause a significant elevation in CSF [Na^+] in normotensive strains, suggesting an impairment of brain Na^+ permeability in salt-sensitive strains (Huang et al., 2004). This is supported by previous observations that blockade of amiloride-sensitive Na^+ channels (present on the epithelial lining of the ventricles) blocked the pressor responses to icv Na^+ or ouabain in WKY rats, and of SHR rats on a high-salt diet. The effects of Na^+ channel blockade was less pronounced on SHR rats fed a regular-salt diet (Huang and Leenen, 2002).

1.5.3. Arginine-vasopressin in salt-sensitive hypertension

1.5.3.1. AVP synthesis, storage and release

Arginine-Vasopressin (AVP) is synthesized as a prohormone in paraventricular and supraoptic nuclei (SON) of the hypothalamus. The hormone is transported along the supraoptic-hypophyseal tract to synaptic terminals of magnocellular neurons located in the posterior pituitary, where it is stored and released (reviewed by Sklar and Schrier, 1983; reviewed by Kam et al., 2004). Release of AVP occurs in response to elevations in osmolarity of extracellular fluid and hemodynamic changes such as decreased blood pressure or volume. However, these factors are monitored independently, and thus, different (patho)physiological stressors can activate both regulatory pathways (for example, water deprivation can activate both regulatory pathways due to blood volume loss, as well as electrolyte concentration), whereas others will only activate one regulatory afferent (for example, salt loading activates osmotic regulatory pathways, because there are minimal hemodynamic responses) (Reviewed by Sladek, 2004). This review will briefly explore osmotic regulatory responses of AVP release.

1.5.3.2. Osmotic regulation of AVP release

The neurons that store AVP themselves are osmosensitive and will react to elevated plasma osmolarity (Mason, 1980; Oliet and Bourque, 1993). This effect sensitizes the neurons to excitatory potentials from key osmoreceptors lining structures of the anterior third ventricle, including the SFO and the OVLT. These structures lie outside the blood-brain barrier and thus are able to detect changes in plasma osmolality, as well as CSF osmolality (reviewed by Sadek, 2004). Cultures of supraoptic-hypophysial tract explants indicate that the AVP neurons do not have the capacity to release AVP in response to hyperosmotic medium alone, but can do so, if an intact OVLT is included in the explant (Sladek and Johnson, 1983). The most likely neurotransmitter relaying the osmoreceptor signal from the OVLT to the AVP neurons of the SON is glutamate (or at least a similar excitatory amino acid). Stimulation of all classes of excitatory amino acid receptor (n-methyl-D-aspartate (NMDA), non-NMDA and metabotropic (mGluR)) stimulate AVP release from supraoptic-hypophysial tract explants; whereas antagonism of NMDA and non-NMDA, but not mGluR receptors block AVP release (Sladek et al., 1998; Swenson et al., 1998; Morsette et al., 2001).

Recently, Voisin et al. (1999) have demonstrated that shifts in extracellular Na^+ concentration cause proportional changes in the mechanosensitive cation channels that are responsible for signal transduction mechanisms of osmosensitive neurons of the SON. This suggests a synergistic role for Na^+ and osmolality in mediating AVP release.

1.5.3.3. Physiological effects of AVP release

In response to hyperosmotic/hypernatremic stressors, AVP release has two predominant effects. AVP acts on cortical collecting ducts to promote water reabsorption by

promoting the translocation of intracellular aquaporin channels to the luminal membrane (reviewed by Breyer and Ando, 1994; Franchini and Cowley, 1996). This effectively increases blood volume, in an attempt to ameliorate the osmotic imbalance. AVP can also act as a direct vasoconstrictor. The AVP receptor on vascular smooth muscle cells is linked to a G protein coupled receptor that activates phospholipase C/protein kinase C and through increases in inositol triphosphate and diacylglycerol, causes the release of Ca^{++} from intracellular stores (Thibonnier et al., 1998; reviewed by Kam et al., 2004). In addition, AVP sensitizes the vasculature to the vasoconstrictor effects of norepinephrine (Noguera et al., 1997; Medina et al., 1997).

The vasoconstrictor response after salt-loading is very rapid and transient. In a study by Bunag and Miyajima (1984), 0.3 M NaCl administered icv in urethane-anaesthetized rats produced a consistent increase in mean aortic pressure by ~10 mmHg. Despite the early rise in blood pressure, they observed that initially, there was a decrease in sympathetic nervous activity. When rats were pre-treated with an AVP receptor antagonist, the early pressor responses (observed during the first 5-10 minutes after the start of NaCl infusion) were inhibited, and sympathetic activity was no longer inhibited. This study concluded that the early increase in blood pressure to icv NaCl is due to vasoconstrictor effects of AVP release, whereas the responses after 10 minutes are due to sympathetic hyperactivity.

1.5.4. A role for glia in salt-sensitive hypertension

Glia are widely recognized as important mediators of several processes in the brain. Astrocytic processes ensheath neural processes and synaptic terminals and are thus ideally positioned to affect synaptic transmission, via uptake of neurotransmitters, release of modulatory "gliotransmitters" or simply by maintaing the synaptic environment (Ventura

and Harris, 1999; Grosche et al., 1999). Glia are also capable of forming gap junctions with neurons and other glia, significantly affecting neural firing patterns (Froes et al., 1999; Alvarez-Maubecin et al., 2000). Astrocytes (and other glia) express the α2 NKA isoform, which is sensitive to ouabain. It has been established that α2 plays a major role in regulating cellular [Ca^{++}] in glia and other cells, hence it is not surprising that exposure of hippocampal preparations to ouabain (1 μM) can produce Ca^{++} transients in glia (Monteith and Blaustein, 1998). Spontaneous astrocytic Ca^{++} oscillations cause Ca^{++}-dependent release of excitatory amino acids (glutamate and aspartate); as well, the oscillations can propagate as waves to other astrocytes and in the process activate NMDA-receptor mediated inward currents in neurons along the wave path (Jeftinija et al., 1996; Parri et al., 2001).

The list of gliotransmitters released from astrocytes is extensive and includes components involved in the pressor response cascade to central Na^{+}. Many of the enzymes, or mRNAs for enzymes involved in steroid synthesis, including Cyt P450scc, 17α-hydroxylase, aldosterone synthase, and 3β-hydroxy-steroid dehydrogenase – important in the synthesis of OLS - have been localized predominantly in glia (Sanne and Krueger, 1995; Zwain and Yen, 1999). In addition, OLS is released from primary culture of mouse astrocytes, suggesting OLS in the brain is of glial origin (Kala et al., 2000). Both angiotensinogen, the precursor angiotensin peptide and brain ACE are synthesized and localized predominantly to astrocytes, suggestive of an important role for astrocytes in the mediation of brain RAS activation due to elevated CSF Na^{+}. A series of studies by Morimoto et al. provide compelling evidence for the importance of glia in RAS-mediated hypertension. In the first study, a transgenic mouse expressing human angiotensinogen under the control of a human Glial Fibrillary Acidic Protein (GFAP) promoter (thus targeted

to glia) was created. Human angiotensinogen can only be processed by human renin, and not murine renin, to form angiotensin I. Icv infusion of human renin resulted in a significant elevation in blood pressure and salt appetite; the effects of which could be blocked with icv losartan treatment (Morimoto et al., 2001). These results indicate that the glia-dependent RAS system is important in regulating blood pressure. In a subsequent study, human angiotensinogen and human renin were expressed in mouse brain under the control of either the GFAP or synapsin-1 promoter (i.e., glial vs. neural expression). While both glial renin/angiotensinogen and neural renin/angiotensinogen transgenic strains exhibited elevated blood pressure, inhibitable by icv losartan, the pressor effects in the glial transgenic mice were greater than in the neural transgenic mice (Morimoto et al., 2002b).

1.6. Peripheral (extra-renal) factors in salt-sensitive hypertension

1.6.1. Peripheral renin-angiotensin system

A comprehensive description of the role of the peripheral renin-angiotensin system (RAS) is far beyond the scope of this thesis, as several tissues (including the brain, heart, kidneys, lungs) and the general circulatory system each have their own independently regulated RAS. However, it can be generalized that the effects of angiotensin II (ang II) are hypertensive. For instance, ang II is vasoconstrictive, effectively increasing blood pressure. Several isolated arteriole perfusion studies have demonstrated a strong coupling of ang II induced elevations in $[Ca^{++}]_i$ and arteriole contraction via G-protein coupled signaling mechanisms (Edwards, 1983; Conger et al., 1993; Hansen et al., 2000). Circulating ang II promotes the secretion of OLS from zona glomerulosa cells in the adrenal cortex, via activation of AT_2 receptors (Shah et al., 1998; Shah et al., 1999). The effects of the

circulating OLS are many and will be described further below. Although there is no evidence of a direct interaction in the effects of circulating ang II and circulating OLS in augmenting vascular smooth muscle tone, it is possible that these two factors act in concert. The same ang II signaling mechanisms mediating elevations in $[Ca^{++}]_i$ also cause inhibition of NKA activity (via phosphorylation). Reductions in active NKA activity make the vasculature all the more sensitive to increases in circulating OLS, thereby further inhibiting vascular NKA activity, increasing intracellular Ca^{++} stores, and promoting enhanced vascular tone.

1.6.2. Circulating OLS

1.6.2.1. Peripheral production and secretion of OLS

Initially, OLS secretion was observed from bovine adrenocortical cells, and further work demonstrated that most of the circulating endogenous OLS originates in the zona glomerulosa cells (Hamlyn et al., 1991; Laredo et al., 1995). There is still a great deal yet to be determined concerning the endogenous synthesis of OLS, but it has been suggested that the synthesis involves side-chain cleavage of cholesterol and subsequent metabolism from pregnenolone (reviewed by Hamlyn et al., 1998). Because ang II stimulates the secretion of aldosterone and OLS, it was postulated that the biosynthesis of these two compounds follows similar routes. 11β-hydroxylase converts 11-deoxycorticosterone into corticosterone - a necessary precursor to aldosterone synthesis. Inhibition of 11β-hydroxylase in primary bovine adrenocortical cell cultures by metyrapone completely inhibited the ang II-induced secretion of OLS and aldosterone from these cells (Hamlyn et al., 2003). In a similar experiment, use of 3β-hydroxysteroid dehydrogenase reduced the conversion of pregnenolone to progesterone and blocked aldosterone synthesis, yet

augmented OLS synthesis (Hamlyn et al., 1998). These data suggest that pregnenolone is a precursor to both OLS and aldosterone synthesis, but their biosynthetic pathways diverge prior to progesterone synthesis.

1.6.2.2. Physiological role of circulating OLS

Although there is a degree of uncertainty, depending on the methodology used, it is generally accepted that the OLS concentration in mammalian serum ranges from 0.05-0.7 nM (Ferrandi et al., 1997; Harwood et al., 1997; Paci et al., 1996). In agreement with this, our group, using an enzyme-linked immunosorbent assay typically measures between 0.3-0.7 nM OLS in rat and mouse serum (Wang and Leenen, 2003; Dostanic et al., 2005).

Several studies have shown that circulating OLS increases in states of volume expansion and essential hypertension (Hamlyn et al., 1996) as well as in response to hypertonic saline in the absence of volume expansion (Yamada et al., 1997). Circulating OLS has also shown to be increased (at least in dogs) in response to elevated CSF Na^+ (Jandhyala and Ansari, 1986). The effects of circulating OLS are many. OLS is inotropic by acting at myocardial tissue to inhibit $\alpha 2$ specifically, increasing $[Ca^{++}]_i$ stores and thus promoting enhanced myocardial contraction. OLS can also inhibit $\alpha 3$ along neuromuscular junctions to promote noradrenaline release and effectively increase cardiac contraction as well (O'Brien et al, 2000; Rodriguez-Manas et al., 1992). OLS can cause increased contraction of vascular smooth muscle cells (of arteries for example) by inhibiting $\alpha 2$ and altering intracellular Ca^{++} stores (Marin et al., 1988; Weiss et al, 1993). *Ex vivo* arterial perfusion studies performed by Vassallo's group have shown that ouabain also sensitizes vascular smooth muscle to pressor substances such as phenylephrine – an artificial analog to norepinephrine (Vassallo et al., 1997; Davel et al., 2000). However, it should be noted that

in similar experiments using arteries with intact endothelium, ouabain can also act on the endothelium to promote the release of vasodilatory factors (presumably increased NO release) to counteract the vasoconstrictor effects on the smooth muscle (Rossoni et al., 1999; Rossoni et al., 2002). The above factors tend to support a role of circulating OLS in the clearance of Na^+/water from the body in order to normalize blood pressure. Increased vascular resistance and inotropy lead to elevated blood pressure and thus increased glomerular filtration. Increased renal tubular flow with decreased Na^+ reabsorption promotes Na^+ and fluid excretion.

1.7. Rationale of the current study

A high-salt diet increases CSF [Na^+] in salt-sensitive (Dahl-S and SHR on a high-salt diet), but not salt-resistant forms of hypertension. The pressor responses to icv Na^+ and high-salt diet in salt-sensitive hypertension are mediated by an endogenous OLS inhibitor of the NKA in the brain. Therefore, icv Na^+ infusion is a model for salt-sensitive hypertension. However, because no isoform-specific OLS receptor antagonists exist, it is not known if the responses to icv Na^+ in salt-sensitive hypertension are mediated by inhibition of the $\alpha1$, $\alpha2$ or $\alpha3$ NKA isoform (or a combination thereof). The fact that the $\alpha2$ and $\alpha3$ isoforms are highly ouabain-sensitive, and appear to be intimately related to the regulation of cellular Ca^{++} storage, suggests that one or both of these isoforms play a critical role in OLS-mediated responses. The only ouabain-sensitive NKA isoform that glia express is the $\alpha2$ isoform. This review has also highlighted the importance of glia, and astrocytes in particular, in the regulation of synaptic transmission, synthesis of angiotensin peptides, and blood pressure. Our hypothesis then, is that a reduction in the expression of the $\alpha2$ NKA

isoform will result in enhanced pressor responses to icv Na^+ or ouabain. To this end, we have studied the effects of icv Na^+ and ouabain in wildtype C57Bl/6 × 129/SvJ mice vs. heterozygous knockout litter mates, with reduced expression of the α2 NKA isoform. It is our expectation that the effects of OLS from icv Na^+ or exogenous icv ouabain will reduce the α2 isoform activity in astrocytes to a greater extent in the α2+/- mice vs. the +/+ mice, resulting in enhanced pressor responses.

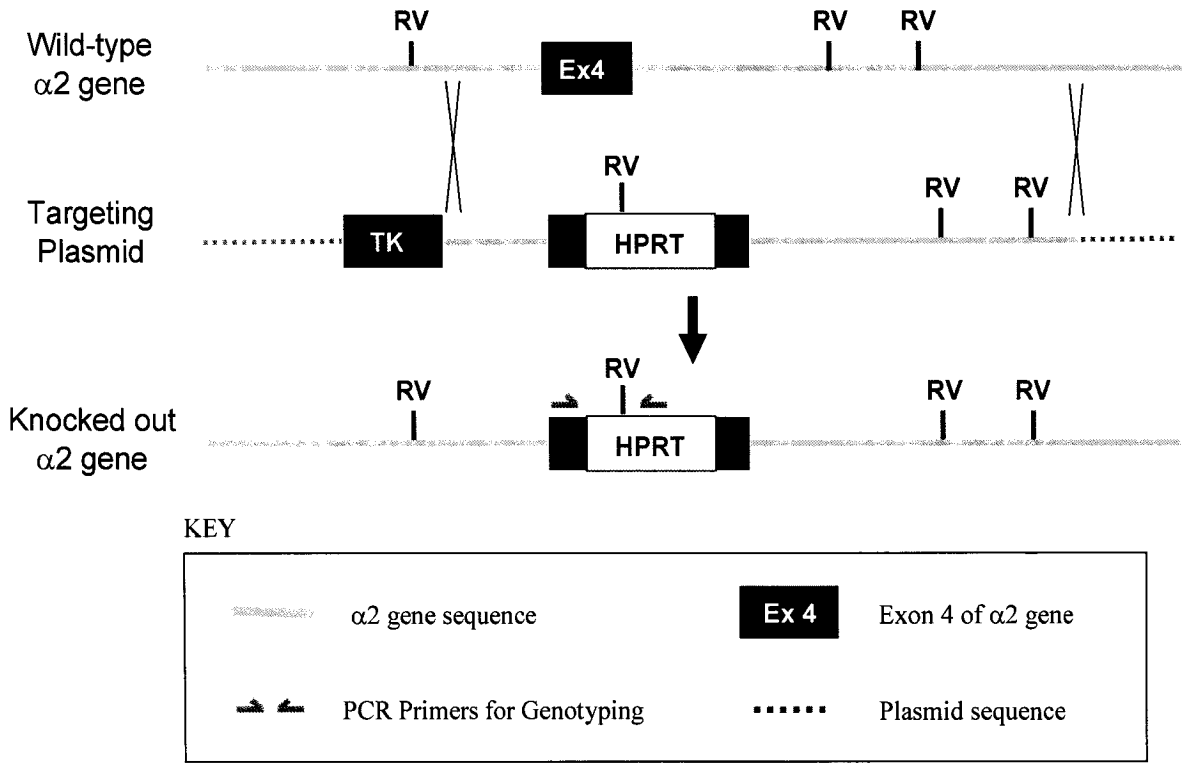
Chapter 2

METHODS AND MATERIALS

2.1. Creation of the α2 Na, K-ATPase heterozygous knockout mouse model

The heterozygous α2 knockdown (α2+/-) mice were created at the University of Cincinnati, in the laboratory of Dr. Lingrel (see James et al. (1999) for details). Figure 5 below illustrates the strategy used in creating the knockout mice. Genomic sequences of the α2 NKA were obtained by screening a 129/SvJ λ phage library with isoform-specific probes. Restriction mapping was performed, and an 8.6 kb KpnI/SmaI fragment was cloned into the pBluescript II vector (Stratagene). A hypoxanthine/guanine phosphoribosyltransferase (HPRT) expression cassette was cloned into BstBI restriction site, in exon 4 of the α2 isoform. A thymidine kinase expression cassette was also cloned into this vector, outside the α2/HPRT fragment. The targeting constructs were linearized and introduced into HPRT deficient embryonic stem cells (E14TG2a) by electroporation. The cells were grown in medium containing hypoxanthine, aminopetrin, thymidine, and gancyclovir for 9 days. Homologous recombination replaces one wildtype allele with the fragment containing the two genomic sequences flanking the HPRT cassette, providing a means for positive selection of transfected cells, while simultaneously disrupting the wildtype allele. Random recombination events were minimized throught the use of the thymidine kinase (TK) expression cassette. Random incorporation of the targeting plasmid would likely incorporate the TK cassette into the host genome. TK incorporates nucleoside analogs (such as gangyclovir) which inhibit the activity of DNA polymerase, effectively killing these cells (Mansour *et al.*, 1988). Surviving colonies were identified by polymerase chaing reaction (PCR) and Southern analysis. Correctly targeted cells were injected into blastocysts, to generate chimeric offspring. Chimeric males were crossed with C57/Black Swiss females to yield heterozygous offspring. The colony was maintained by breeding

Figure 5. Gene-targeting strategy for creating α2 NKA knockout mouse. Hypoxanthine phosphoribosyl transferase (HPRT) interrupts exon 4 of the target α2 NKA gene sequence. The inclusion of a stop codon in the HPRT cassette causes premature termination of the α2 sequence, omitting important enzyme sites such as Asp369 (the site of phosphorylation by ATP) in the translated product. A negative selection marker (Thymidine kinase (TK)) was included to safeguard against random recombination events (i.e., TK is omitted upon correct homologous recombination). RV= EcoRV restriction site. This figure was adapted and modified from James et al., 1999.


Wild-type
α2 gene
RV
Ex4
RV
RV
Targeting
Plasmid
RV
TK
HPRT
RV
RV
Knocked out
α2 gene
RV
RV
HPRT
RV
RV
KEY
α2 gene sequence
Ex 4
Exon 4 of α2 gene
PCR Primers for Genotyping
Plasmid sequence

wildtype mice with heterozygous mice, to produce only wildtype and heterozygous offspring in a 1:1 ratio.

2.2. Determination of mouse genotype by PCR

When offspring were 3 weeks old, they were weaned and tail clips (3-5 mm) were removed for DNA isolation and genotyping. DNA isolation was performed using a QIAGEN DNeasy Tissue Kit®. The tail samples were digested in a tissue lysis buffer (buffer ATL) and proteinase K, both supplied with the kit. After approximately 2 hours at 55°C with intermittent vortexing, the DNA in the samples was precipitated and poured through spin columns which adsorbs the DNA. The column was washed and the DNA eluted. PCR was then used to identify the genotypes. The validity of the PCR has been previously established by Southern analysis (James et al., 1999). The heterozygous allele is identified by two primers: one primer with the sequence 5'-CCTCTACCACGCGTCCTAG-3' hybridizes with the α2 allele and another primer with the sequence 5'-CCTACCCGCTTCCATTGCTC-3' hybridizes with the HPRT insertion cassette. The amplicon was 350 bp in length. Because the wildtype allele did not have the HPRT cassette, a wildtype mouse would not produce any PCR product. To ensure that the lack of a PCR signal was due to the lack of an HPRT-interrupted allele and not due to an error in the PCR process (for instance, not enough starting template was used to be amplified), a control set of primers was used to amplify a portion of the thyroid stimulating hormone β (THSβ) gene. The amplified product (250 bp) was obtained regardless of the genotype, provided that a sufficient amount of genomic DNA was present

2.3. Animal care

The colony was maintained at the University of Ottawa Heart Institute Animal Care and Veterinary Services vivarium. Mice were housed in group-cages in a temperature controlled environment with a 12h light/dark cycle. They were fed standard chow and given water *ad libitum*. All animal procedures and care were conducted according to procedures established by the University of Ottawa Animal Care Committee.

2.4. Cardiovascular studies

2.4.1. Surgical procedures and blood pressure measurements

8-10 week old mice (20-30g) were anesthetized using isoflurane, and a line of antithrombogenic tubing with a tapered end (MRE40, Braintree Research, Braintree, MA) filled with heparinized saline (5 U heparin/mL) was placed in the carotid artery. It was secured with sutures and exteriorized at the nape of the neck. The tubing was connected to a transducer in order to record MAP and HR. Mice were then placed in a stereotaxic frame with mouse ear-bars, nose clamp, tooth plate and anesthesia mask (David Kopf, Tujunga, CA). The head was leveled and a guide cannula was positioned with the tip just above a lateral ventricle (stereotaxic coordinates: 0.1 mm anterior, 1.0 mm lateral and 1.5 mm ventral to lambda). Jeweler's screws were then placed in the skull and the guide cannula was cemented in place with dental acrylic and plugged.

Animals were allowed to recover for 26 hours after surgery, after which time two 10-minute baseline blood pressure and heart rate measurements were recorded. Mice were then subjected to one of the protocols below.

2.4.2. Effects of icv infusion of hypertonic NaCl

Blood pressure and heart rate were measured in response to icv infusion of artificial cerebrospinal fluid (ACSF) with hypertonic salt concentrations (0.225M NaCl). The infusions were administered by a microinjection micropipette fashioned from a 27-gauge needle that protruded 1 mm beyond the tip of the guide cannula. Infusions were administered over a 60-minute period at a rate of 0.4 μL/min.

2.4.3. Effects of icv infusion of ouabain

Analogous Experiments were performed as outlined in section 2.4.2. (Effects of icv infusion of hypertonic NaCl), with ouabain instead of NaCl. A dose of 5 ng/μL ouabain in ACSF was infused at a rate of 0.4 μl/min, over the 60-minute period. These experiments were performed to assess if the effects due to icv Na^+ infusion were mediated by brain OLS per se (via NKA inhibition), or due to some unknown factor.

2.4.4. Responses to icv vs. iv ouabain infusion

The effects of ouabain mimic the effects of icv infusion of hypertonic saline in mice (Van Huysse and Hou, 2004). Responses to ouabain (5 ng/μL, 0.4 μL/min) in ACSF were measured in α2+/- and +/+ mice. Because of the adverse reactions in some of the α2+/- mice to hypertonic saline (see results), this dose of ouabain was chosen because in preliminary studies of α2+/+, minimal pressor responses were obtained (an increase of <5 mmHg in MAP). It was the hope that if this dose of ouabain caused exaggerated responses in α2+/- mice, that these responses would not be so extreme as to be fatal. To assess whether the origins of the responses were central or peripheral (i.e., outside the CNS) in nature, the same dose of ouabain was infused iv and blood pressure and heart rate were monitored accordingly.

2.5. Biochemical analysis

2.5.1. Collection of brain and serum from 8-10 week mice

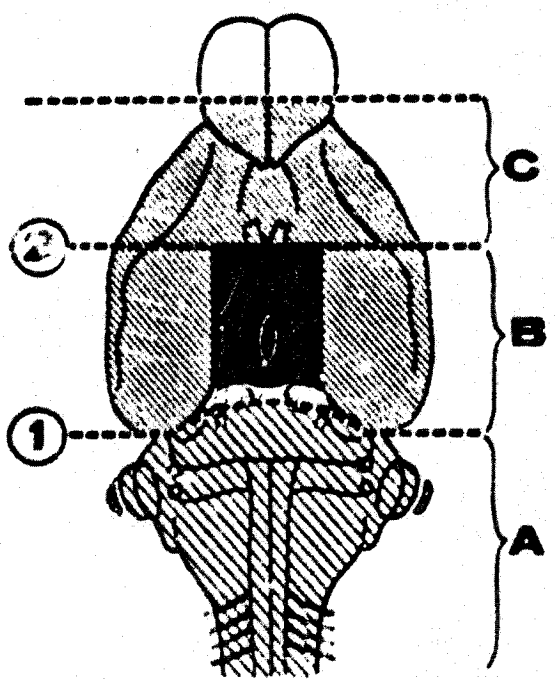
All biochemical analyses were performed on mice 8-10 weeks of age. As well, all analyses were performed on unoperated mice, that were not involved in the physiological studies. After equilibrating a CO_2 chamber (7L/min CO_2 + 3L/min O_2 for 5 minutes), mice were placed in the chamber for 3 minutes. At this point the mice were removed and were completely unresponsive to pain stimulus. A midline incision was quickly made from the peritoneal cavity to the top of the thoracic cavity. The ribcage was cut open to expose the thoracic cavity. Blood was drawn directly from the beating heart by inserting a 23G needle into the left ventricle. Approximately 0.5-1 mL of blood was collected in this fashion. The blood was stored on ice in microcentrifuge tubes for at least 30 minutes. After which time, the samples were spun at 3,000 g for 10 minutes. The serum was collected and stored at –80°C until used. Next, the mouse was decapitated and the brain excised. Brains were snap frozen in liquid nitrogen (for approximately 4 seconds) and then placed on dry ice and only after the brains were hardened were they wrapped in foil.

2.5.2. Dissection of brain – isolation of hypothalamus and pons/medulla

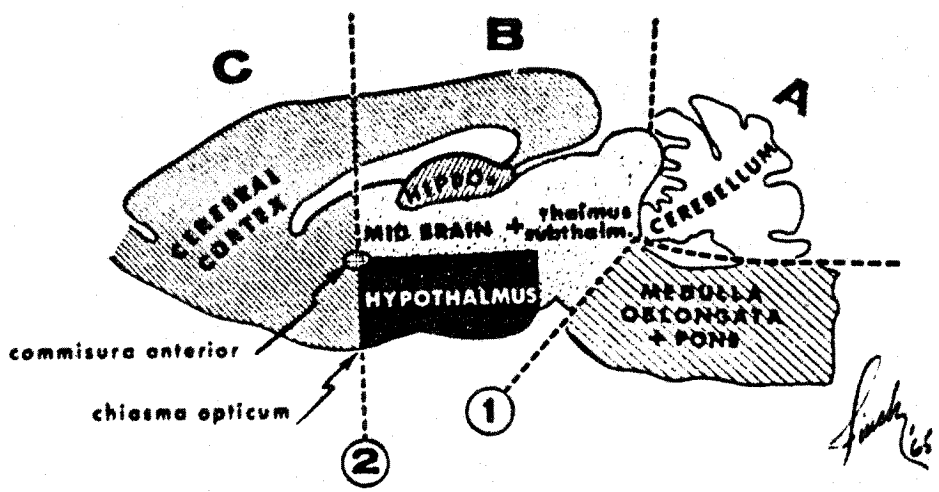
Dissection of brains was performed as outlined in figure 6 (adapted from Glowinski and Iversen, 1966). The whole brain was placed, with the ventral surface facing up, on a clean glass plate that was positioned on an open container filled with dry ice. The container itself was placed in a Styrofoam container filled with ice. The frontal cerebral cortex was removed by cutting in the coronal plane at the chiasma opticus. The hypothalamus was

Figure 6. The method of dissection for the brain. The hindbrain was cut off (panel A, cut #1) and partitioned into the cerebellum and pons/medulla oblongata (panel B, cut #1). Next, the hypothalamus (black area) was isolated by cutting off the cerebral cortex away (coronal cut at the commisura anterior – panels A and B, cut #2) and then cutting cortical tissue (sagitally) away from the median eminence (not shown in figure). Finally a transverse cut was made at the commisura anterior to segretate the hypothalamus. This figure was adapted from Glowinski and Iversen, 1966.

A


C
B
A
2
1

B


C
B
A
CEREBRAL CORTEX
HIPPOC.
MID BRAIN + thalmus nbthalm.
CEREBELLUM
HYPOTHALMUS
MEDULLA OBLONGATA + PONS
commisura anterior
chiasma opticum
1
2

isolated by cutting coronally at the commissura anterior, and then cutting cortical tissue (sagittal plane) away from the median eminence. A horizontal (transverse) section was made at the comissura anterior. The pons/medulla was isolated by first cutting away the midbrain (coronal plane) just anterior to the medulla/cerebellum. The sides of the cerebellum were cut off, which exposed the 4th ventricle. The cerebellum was cut away. Finally, the spinal cord was cut at the obex.

2.5.3. Preparation of microsomal membranes

Microsomal fractions were prepared as previously described (Chen and Liu-Shiau, 1986). Cortical brain sections were homogenized by sonication in 10 mL of sucrose-histidine buffer (SH buffer; 0.32 M sucrose, 5 mM histidine, pH 7.4) per gram of tissue in 1.5 mL microcentrifuge tubes. Samples were then centrifuged at 1000g for 10 minutes at 4°C. The supernatant was removed and transferred to a 2 mL microcentrifuge tube. The pellet was resuspended in another 10 mL of sucrose-histidine buffer per gram of (starting) tissue and recentrifuged at 1,000g for 10 minutes at 4°C. The supernatants were combined and the pellet discarded. All supernatants were centrifuged at 10,000g for 20 minutes at 4°C. The supernatants were transferred to Beckman thick wall polyallomer tubes (cat# 362333) and centrifuged at approximately 100,000g for 1 hour at 4°C in a Ti100.4 or TLA110 rotor using a Beckman Opti centrifuge. Supernatants were discarded and the final pellet was resuspended in 300 µL of sucrose-histidine buffer. Protein concentrations were determined by bicinchoninic acid assay (Pierce) in a 96-well microplate.

2.5.4. Na, K-ATPase activity assay of microsomal membranes

This assay was performed in an attempt to discern whether there was a decrease in NKA activity in microsomes from brain tissue of α2+/- and +/+ mice. This assay differentiates ouabain-sensitive activity from ouabain-resistant activity by exposing the preparations to a reaction medium without ouabain (to measure total NKA activity; i.e., α1+α2+α3), and 10^{-6} M ouabain to inhibit only the ouabain-sensitive isoforms (α2+α3). Background ATPase activity is determined by inhibiting all α isoforms with 10^{-3} M ouabain. ATPase activity is determined by measuring the hydrolysis of (^{32}P)γ-labeled ATP. The contributions of ouabain-sensitive, -resistant and background activities are derived by simple subtraction. The obvious, and major limitation to this assay is that the assay cannot resolve α2 from α3 activity, as they are both ouabain-sensitive. The assay was performed because although there is an appreciable amount of α3 in the brain, glia outnumber neurons by 10-fold, and hence the contribution of the α2 isoform to brain ouabain-sensitive NKA activity could be significant, and a decrease in activity might be measurable.

2.5.4.1. Assay setup and reaction conditions

This assay was performed as previously described, with modification (Abdelrahman et al., 1995). The microsomal fractions (as described in section 2.5.3) were prepared such that 250 μg of total protein was diluted in Sucrose-Histidine buffer to a total volume of 1000 μL and kept on ice. For each sample, three sets of tubes were prepared (total, ouabain-resistant, and background activity) each in triplicate, for a total of 9 tubes per sample. To the total and ouabain-resistant activity sets, 200 μL of 5x activity buffer was added (in mM: 500 NaCl, 100 KCl, 22.5 $MgCl_2$, 1 EDTA-Na_2, 75 Histidine, pH 7.4), while 5x background buffer was added to the background activity sets (same buffer as activity buffer, but without

KCl – i.e., without K^+, the NKA cannot cycle and is thus inhibited). 100 μL of 10x ouabain-solutions (prepared in 25 mM Histidine, pH 7.4) were added to the appropriate tubes. Thus, 10^{-2} M ouabain was added to "background" sets (i.e., final [ouabain] = 10^{-3}M), 10^{-5} M ouabain was added to "ouabain-resistant" sets (final [ouabain] = 10^{-6}M), and histidine-buffer was added to "total activity" sets (final [ouabain] = 0 M). At this point, 100 μL of sample (25 μg of total protein) was added to each tube, along with 100 μL of distilled/deionized water to make up the volume to 500 μL.

Up to this point, the samples were all maintained on ice. As quickly (and carefully) as possible, 500 μL of a radioactive labeled/unlabeled ATP solution (6 mM ATP in ddH_20, pH 7.4) was added and the tubes were placed in a gently shaking 37°C water bath for 30 minutes. The ATP solution was prepared as follows: 6 mM of a non-radioactive ATP (Sigma-Aldrich, A-2383) solution was prepared. After which, small volumes (~ 0.1-0.2 μL) of a 5'[γ-^{32}P]-ATP aqueous solution (Amersham, B0305X) were added incrementally until a 20 μL aliquot of the labeled/unlabeled solution had approximately 6000 cpm as measured in a liquid scintillation (β-radiation) counter (LKB Wallac, model 1219 Rackbeta). This method of calibration ensured that the ATP solution always has approximately 300 cpm/μL, regardless of the specific radioactivity of the labeled ATP stock. However, it should be noted that radiolabeled ATP was never used past 4 weeks from its calibration date.

After the 30-minute reaction time, the tubes were removed from the 37°C bath and placed in an ice-water bath. 500 μL of pre-prepared, chilled, acid-washed charcoal (0.5 g charcoal in every 1 mL of 1 N HCl) was added and the tubes were inverted vigourously for 30 sec. The tubes were then placed back on ice. All the tubes in the rack were vortexed simultaneously for 1 minute using a rack multi-vortex. The rack was then returned to the

ice-water bath for at least 30 minutes. By binding organic compounds, addition of the charcoal serves two purposes: (1) it effectively stops all ATPase activities in the reaction medium; and (2) it complexes with unhydrolyzed ATP, thereby separating organic (γ-^{32}P)-ATP from the inorganic γ-^{32}P produced from hydrolysis. Tubes were then centrifuged at 10,000g for 30 minutes in order to separate the charcoal and supernatant containing the hydrolyzed inorganic phosphate. Supernatant (650 μL) was then transferred to 8 mL of scintillation fluid and counted for β-radiation.

2.5.4.2. Determination of activities from radioactive counts

It was mentioned that 20 μL of the starting radioactively labeled/unlabeled ATP solution would have approximately 6000 cpm. The solution is 6 mM ATP (the concentration from the labeled ATP is negligible; i.e., typically between 0.15-0.3 nM), therefore 20 μL of solution has 120 nmol ATP. By simple equivalency, the solution is 50 cpm/nmol ATP. From this, and the counts measured from the assay, we get activity expressed as:

($CPM_{(from\ assay)}$) / (~50 CPM/nmol ATP) / (30 min) / (~25 μg protein). Or more simply, expressed as nmol ATP hydrolyzed/min/mg protein.

Finally, total ($\alpha1+\alpha2+\alpha3$) activity is determined by subtracting background CPM from 0 M ouabain activity; and ouabain-resistant ($\alpha1$) activity is calculated as activity at 10^{-6} M ouabain minus background activity. Ouabain-sensitive ($\alpha2+\alpha3$) activity then, is the difference in total and ouabain-resistant activities.

2.5.5. Western blot analysis

2.5.5.1. Gel electrophoresis

Denaturing polyacrylamide gels were prepared using Bio-Rad's Criterion III system. First, an 8% resolving gel was prepared by mixing the following components (volumes given are for 25 mL of solution): 11.5 mL ddH_20, 6.7 mL 30% acrylamide mix, 6.3 mL 1.5M Tris (pH8.8), 0.25 mL of 10% SDS, 0.25 mL of freshly prepared 10% ammonium persulfate and finally 0.015 mL N,N,N',N'-tetramethylethylenediamine (TEMED). The solution was quickly mixed and pipetted into the gel cast mold, approximately 3/4 full. Double distilled water (200 μL) was placed on top of this solution to prevent oxidation of the gel, and to minimize the formation of a meniscus. This solution set for 1 hour, after which the water was removed. A 5% stacking gel was then prepared by mixing the following components (volumes given are for 10 mL of solution): 6.8 mL ddH20, 1.7 mL of 30% acrylamide mix, 1.25 mL of 1 M Tris (pH6.8), 0.1 mLl of 10% SDS, 0.1 mL of 10% ammonium persulfate and finally 0.01 mL TEMED. This solution was also quickly mixed and pipetted into the mold until it slightly overflowed. The combs were inserted carefully as to prevent the formation of bubbles. Although this gel polymerizes rather quickly (polymerizes from 15-30 minutes), it was allowed to set for 1 hour.

At this point, microsomal membranes samples (see section 2.5.3) from each group were prepared. For α1 and α2, 2 μg of protein was to be loaded per well, and for α3, 0.2 μg of protein was to be loaded. Original samples were diluted to 2x the final concentration in 20 μl of SH buffer, then further diluted 1:2 with Laemmli buffer (Bio-Rad) containing 10% β-mercaptoethanol; the final samples therefore contained 5% β-mercaptoethanol. The samples were heated at 37°C for 15 min and immediately placed on ice afterwards. When

the gel was prepared, the combs were removed and 10 µl of each sample was loaded, except for the first lane, which consisted of 4 µl of a high-molecular weight marker (Bio-Rad). The electrophoresis tank was filled with tank buffer (0.2 M glycine, 25 mM Tris, 0.5% (w/v) sodium dodecyl sulfate, in water) and the samples were electrophoresed at 150 V for 2 hours.

2.5.5.2. Transblotting onto PVDF membrane

Near the end of the electrophoresis period, a large dish was filled with transblot buffer (0.2 M Glycine, 25 mM Tris, 20% Methanol (v/v), in water), and a transblot cassette was prepared. A fiber pad was soaked in transblot buffer and placed on the bottom of the cassette. Next, two pieces of Whatman 3MM paper, slightly larger than the gel, were also soaked in transblot buffer and placed on the fiber pad. After the electrophoresis, the gel mold was disassembled and the stacking gel was discarded. The gel was placed upside down on the 3MM sheet. The PVDF membrane was equilibrated for 3 minutes in 100% ethanol and then for at least 5 minutes in transblot buffer and then placed on the gel. Two more soaked 3MM sheets and another soaked fiber pad were placed on top. Finally the cassette was shut and placed in the transblot tank and a current of 0.3 Amps was applied for 2 hours.

2.5.5.3. Antibody probing

After the transblot, the membrane was blocked in 5% skim milk in Tris-buffered saline with 0.05% Tween-20 (TBS-T) overnight at 4°C on an orbital shaker. The following day, the membrane was washed in TBS-T for 2 x 10 minutes. All subsequent washes were also performed for 2 x 10 minutes in TBS-T. The membrane was cut in two; the upper section to be probed for the select NKA α isoform, whereas the bottom section would be

probed for the α-tubulin loading control. All primary antibodies were mouse-derived IgGs, so that a single anti-mouse secondary antibody could be used for all primary probes. The α1 isoform was probed with a6f (University of Iowa, Developmental Studies Hybridoma Bank) with a 1:1,000 dilution of the original stock. The α2 isoform was probed with McB2 (provided by Dr. Kathleen Sweadner; Pacholczyk and Sweadner, 1997) with a 1:3,000 dilution of stock. The α3 isoform was probed with MA3-915 (Upstate Biotechnologies) with a 1:30,000 dilution of the original stock. Finally, the α-tubulin was probed with B-1-3-2-5 (Sigma) at a 1:20,000 dilution of the original stock. The absolute concentrations of antibodies used were unknown. The membranes were incubated in the antibody solutions (diluted in TBS-T) for 1 hour at room temperature. After the incubation, the membranes were washed. The secondary antibody, a biotinylated goat anti-mouse antibody (Amersham) was prepared in a 1:5,000 dilution of the original stock. The membranes were incubated in the secondary antibody for 1 hour at room temperature. The membrane was then washed. A streptavidin-conjugated horseradish peroxidase solution was prepared at a 1:5,000 dilution in TBS-T and the membranes were incubated in this solution for 30 minutes at room temperature, followed again by two washes. A chemiluminescent detection reagent was employed (ECL, Amersham) to visualize the protein bands of interest. A solution of 1 part "detection reagent 1" to 1 part "detection reagent 2" to 2 parts water was prepared and the membranes were immersed in the solution for 1 minute. The pieces of the membrane were then reassembled while still damp and secured in place between clear plastic sheets in a film cassette. Films (Kodak) were exposed in a dark room for various lengths of time – typically 5 seconds, 30 seconds and 1 minute. The films were developed with an X-OMAT 270 RA Processor (Kodak).

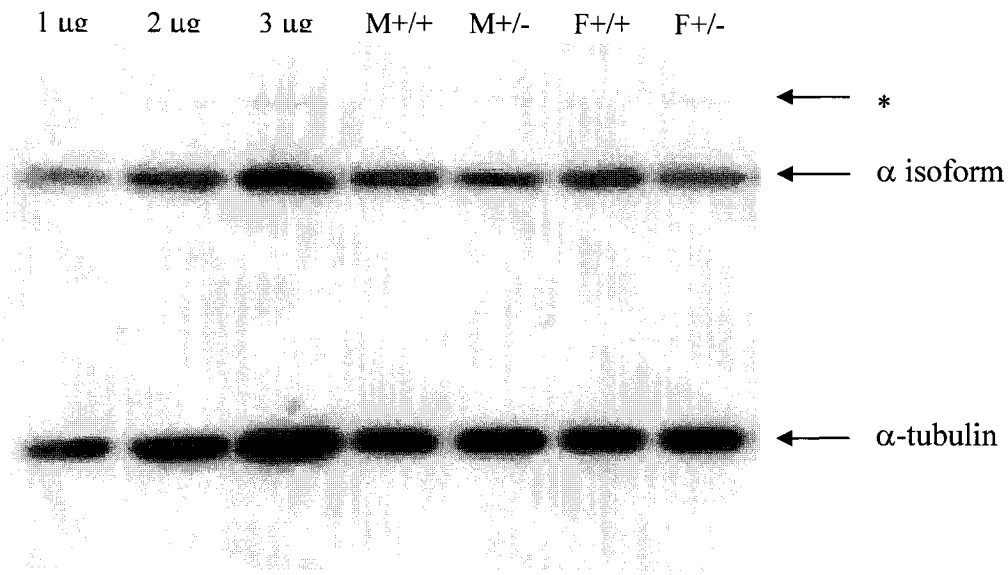
2.5.5.4. Densitometric analysis and sample quantification

The amount of α2 protein in each sample was determined semi-quantitatively by comparing the ratios of the densities of the α2 bands to their respective α-tubulin loading controls. However, in preliminary experiments it was determined that there is not a 1:1 relationship between band density on the film (as determined by Bio-Rad's Quantity One® software) and the amount of protein loaded. For instance, although there may be a linear relationship between band densities and 1, 2 and 4 μg of protein loaded, the densities would not be doubled in the 4 μg vs. 2 μg, or 2 μg vs. 1 μg bands. Therefore, in order to more accurately equate band densities with protein content, a standard curve was used on each Western blot, which consisted of a single external sample (always a male wildtype sample) prepared exactly the same way as the other samples. All other lanes were compared to this sample, and the standard itself was not included in any of the group quantifications. For α1 and α2, the standards were loaded as 1, 2, and 3 μg of total protein, whereas for the α3 sample, 0.1, 0.2, and 0.3 μg was loaded.

Figure 7 on the following page represents part of a Western blot used to measure α2 isoform content in the hypothalamus. The first three lanes represent the standard sample, whereas the following lanes show typical samples. Table 1 outlines the method of sample quantification used for Western analysis.

Figure 7. A representative section of a Western blot. This figure shows illustrates the α2 isoform (from hypothalamic microsomal preparations), depicting the use of a standard, and one sample from each group. The standard curve consists of 1,2 and 3 μg of total protein from one male wildtype sample (lanes 1-3). 2 μg of protein was loaded for each experimental sample. With regards to group labels, M and F are male and female respectively, while +/+ and +/- are wildtype and α2 heterozygous knockout mice respectively. The appropriate bands are indicated to the right of the gel. The band marked with an asterisk is the result of non-specific binding of the horseradish peroxidase.

Table 1. Method of quantifying α isoform NKA protein expression. From the gel densities of the standards, a linear regression can be obtained, relating protein amount as a function of density. For each sample, the NKA α isoform (in this case α2 NKA; "α2") and α-tubulin ("α-tub") expression can be determined relative to the standard. Once this is accomplished, the α NKA expression is compared between groups by normalizing α isoform content against α-tubulin content.



		gel densities (arbitrary units)	standard curve	amt. of protein (μg)	ratio of α2/α-tub
α2	std - 1 μg	1608	protein =		
	std - 2 μg	2743	(0.000871 x density) - 1.332		
	std - 3 μg	3905			
	M+/+	2615		1.88	**1.08**
	M+/-	2162		1.49	**0.8**
	F+/+	2530		1.81	**1.04**
	F+/-	1952		1.3	**0.77**
α-tub	std - 1 μg	2758	protein =		
	std - 2 μg	4265	(0.000479 x density) - 0.213		
	std - 3 μg	6842			
	M+/+	4087		1.74	
	M+/-	4348		1.87	
	F+/+	4091		1.74	
	F+/-	4007		1.7	

2.5.6. OLS ELISA

In order to examine whether OLS levels in the brain were different in α2 +/+ vs. +/- mice (in order to test an upstream mechanism involved in α2 NKA inhibition after salt-loading), an indirect enzyme-linked immunosorbent assay (ELISA) was developed to measure the concentration of OLS in the brain. This assay was performed as previously described (Wang *et al.*, 1998; Wang and Leenen, 2002)

2.5.6.1. Sample preparation

It should be noted that because ouabain and the OLS are photolabile, all samples were protected from light whenever possible, during sample preparation and during the assay.

Serum and brain samples were collected as described above. Between 400 and 500 μL of serum was used per sample. Serum proteins were precipitated by incubating samples with an equal volume of 0.1% trifluoroacetic acid for 3 hours, followed by centrifugation at 3,000g for 30 minutes to pellet insoluble materials. OLS in the supernatant was isolated and collected as described below.

In the brain, hypothalami, pons/medullae and frontal cortices were dissected, weighed, and sonicated in 10 ml of homogenization buffer (methanol with 2 mM ascorbic acid) per 1 g of tissue. The homogenates were centrifuged at 1,500g for 30 minutes, and the supernatants were then dried by vacuum centrifugation, overnight at room temperature. The following morning, the dried samples were reconstituted in 1 mL of 0.1% trifluoroacetic acid, and were solubilized for 3 hours. OLS was then isolated as described below.

C18 Sep-Pak Vac (Waters) columns were used to extract the OLS. All columns were assembled on a vacuum manifold. The columns were equilibrated by successively

applying 10 mL of 100% acetonitrile and 10 mL of distilled/deionized H_2O through the column, both at a rate of 5-10 mL/min. Samples were added to the pre-conditioned columns and passed through slowly at a rate of approximately 1 mL/min. The columns were washed with 10 mL of water, then with 3 mL of 2.5% acetonitrile. The OLS was then eluted from the columns by applying 3 mL of 25% acetonitrile, and passing through at a rate of ~1 mL/min. Samples were again dried down overnight by vacuum centrifugation. The following morning, samples were reconstituted in 250 µL of phosphate-buffered saline with tween-20 (PBS-T) (composition in mM: 150 NaCl, 10 NaH_2PO_4, 10 Na_2HPO_4 anhydrous; with 0.05% Tween-20, pH 7.4).

2.5.6.2. ELISA Assay Protocol

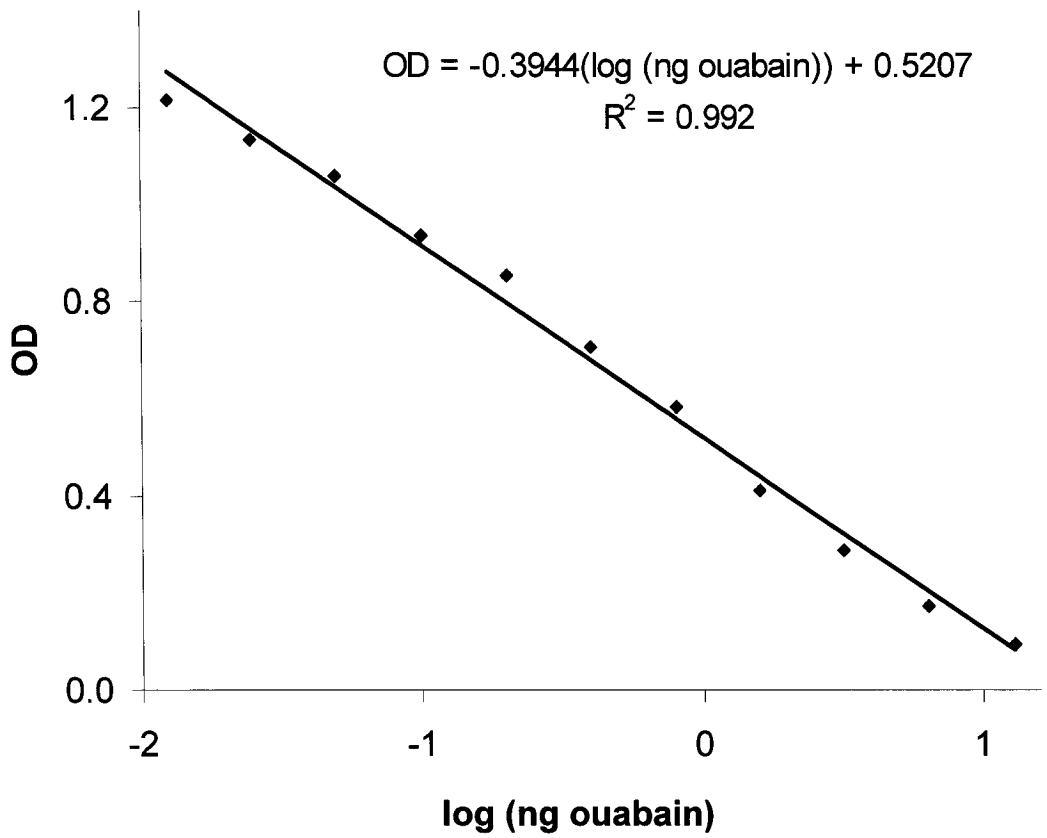
Three days before the assay, 200 µL of an ouabain-ovalbumin conjugate (Wang *et al.*, 1998) diluted to 0.6 µg/mL, was used to coat the wells of a 96-well microplate. The conjugate was diluted from its stock in a carbonate buffer (35 mM $NaHCO_3$, 15 mM Na_2CO_3, pH 9.6) and kept at 4°C. The day before the assay, the plate was washed five times with PBS-T (5 minutes per wash). All subsequent washes consisted of five washes of 5 minutes each. The plate was then blocked overnight with 5% milk poweder dissolved in PBS-T. On the day of the assay, the plate was washed and 100 µL of either sample or ouabain standard was added to the wells. The ouabain standard curve consisted of 2-fold serial dilutions ranging from 128 to 0.125 ng/mL. After the standards or samples were added, 100 µL of the primary antibody (Digibind®, sheep anti-digoxin polyclonal $F(ab')_2$ fragments from GlaxoSmithKline) at a concentration of 0.001 mg/mL, was added to each well. After incubation for 1 hour and subsequent and washing, 200 µL of horseradish peroxidase-conjugated $F(ab')_2$-specific anti-sheep IgG secondary antibody (313-036-047,

Jackson ImmunoResearch, West Grove, PA) was added to each well at a concentration of 0.001 mg/mL. After further incubation for 1 hour and washing, 200 μL of 3,3',5,5'-tetrmethylbenzidine (Sigma) was added as a colorimetric substrate. The colourimetric reaction was terminated by the addition of 50 μL of 2 M H_2SO_4; afterwhich, absorbances were measured at an emission wavelength of 450 nm, using a Bio-Rad microplate reader, model 3550-UV.

2.5.6.3. Determination of OLS Content

Figure 8 on the following page illustrates a representative standard curve obtained from ouabain standards (128 – 0.125 ng/mL) assayed in duplicate. The samples are compared to the regression equation of this curve in order to determine the amount of ouabain in the sample (in ng). Quantities are then adjusted for dilution, and the amount of starting material used.

Figure 8. Sample standard curve derived from the ELISA using ouabain as a standard. The colour development increases logarithmically from high ouabain to low ouabain, and so to obtain a linear relationshi, absorbance is compared relative to the log of the amount of ouabain in the sample. While there is no mathematical advantage to this, presenting the data in this manner allows for a visual confirmation of the integrity of the assay.


OD = -0.3944(log (ng ouabain)) + 0.5207
R2 = 0.992
1.2
0.8
0.4
0.0
OD
-2
-1
0
1
log (ng ouabain)

2.5.7. ACE Activity

In conditions of upregulated OLS in the brain (e.g., Dahl-S on a high-salt diet), a component of the RAS, angiotensin converting enzyme (ACE), is elevated (Zhao *et al.*, 2001). An increase in both ACE mRNA and protein were found in the hypothalamus and pons/medulla of Dahl-S vs. Dahl-R rats on high-salt diets. This suggests that ACE upregulation might represent a possible link between OLS inhibition of brain NKA and brain RAS activation. To determine whether the cardiovascular effects were a result of NKA inhibition, or possibly a result of altered baseline RAS in α2 heterozygous mice, basal ACE activity in hypothalamus, and pons/medulla was measured. Because ACE expression is highly associated with astrocytes, it is possible that altered glial function (as a result of decreased α2 expression) in the α2+/- mice may result in altered ACE activity, and thus altered angII production.

2.5.7.1. Sample Preparation

This assay was performed as previously described (Zhao *et al.*, 2001) with modification. Brain tissue was collected and the hypothalamus and pons/medulla were isolated as described above. Tissues were sonicated in Tris-NaCl buffer (50 mM Tris, 150 mM NaCl, pH 7.4). Samples were then centrifuged at 16,000g for 15 minutes at 4°C. The supernatant was transferred to new tubes and represents the crude homogenate used in the assay. A Bicinchoninic acid analysis for protein determination was performed on the homogenates and an aliquot of each sample was diluted to 1.6 mg/ml in Tris-NaCl buffer.

2.5.7.2. ACE Activity Assay Protocol

ACE activity is measured as the amount of histidine-leucine produced from the cleavage of hippuryl-histidine-leucine (Sigma, H-4884). The histidine-leucine produced

was conjugated with o-phthaldialdehyde (P-0657, Sigma), producing a product which can be fluorometrically quantified.

A fresh captopril solution (0.533 mM in ddH_2O) was prepared and 75 μL of it was added to two assay tubes per sample. Two other tubes were designated as captopril-. Volume substitution in captopril- tubes was done with distilled/deionized H_20. The use of captopril distinguished captopril-inhibitible proteolytic activity (i.e. ACE activity) from total proteolytic activity. Twenty-five μL of sample (40 μg of protein) was added to appropriate tubes. All captopril+ tubes were pre-incubated in a gently shaking 37°C water bath for 20 minutes. Standards tubes and captopril- tubes were kept on ice. Following the pre-incubation, captopril+ tubes were placed on ice for 2 minutes to ensure equal temperatures when the reaction is started. Also during the pre-incubation, standards tubes were setup, consisting of 0-15 μM histidine-leucine (Sigma). The histidine-leucine is first prepared as a 40 μM solution in a HEPES (50 mM) - NaCl (300 mM) solution, pH 8.3. One hundred μL of substrate, hippuryl-histidine-leucine in HEPES-NaCl, was added to all tubes, except the standards. All tubes were vortexed briefly and placed in a 37°C water bath for 1 hour. After the incubation, the tubes were removed from the water bath in the same order they were put in, and placed in an ice-water bath to slow the reaction. The reaction was then stopped by addition of 1.45 mL of 0.28 M NaOH and mixing. One hundred μL of the detection reagent, o-phthaldialdehyde (prepared as 10 mg/mL in methanol), was added to each tube. Tubes were incubated at room temperature for 10 minutes. Two hundred μL of 3 M HCl was added to each tube and samples were mixed. Tubes were placed in a cold (4°C) clinical centrifuge covered in aluminum foil (because o-phthaldialdehyde is light sensitive) and allowed to incubate for an additional 30 minutes. Samples were then centrifuged at 1500g

for 10 minutes. Each sample (325 μL) was pipetted into a 96-well opaque plate and fluorescence (excitated at 360 nm; emission wavelength measured at 480 nm) was detected by spectrofluorimetry using a POLARStar Galaxy spectrofluorimeter (BMG Labtech, GmBH).

2.5.7.3. Determination of ACE activity

The amount of Histidine-leucine produced was determined according to the standard curve (expressed as μmol histidine-leucine produced). This value was then adjusted for the reaction time and protein content, such that the final activity is expressed in μmol histidine-leucine produced/min/mg protein.

2.6. Statistical analyses

Blood pressure and heart rate responses to icv infusions of Na^+ and ouabain were analysed by 3-way ANOVA with repeated measures over time (using SAS^{TM} v.9.1, statistical analysis software; SAS Institute, Inc.), in order to take into account possible effects of gender and genotype over time (Cody and Smith, 1997). Paired t-tests were used to detect changes in blood pressure or heart rate in response to icv infusion of Na^+ or ouabain at each time point, adjusting for multiple comparisons. Specifically, for four response curve analyses per study, with 12 time points per response curve, each statistical test required an adjusted α value equal to 0.05/48 (=0.00104), to which the test had to have been less than, in order to be considered significantly different.

The biochemical data (e.g., comparisons of $\alpha 2$ protein content in female +/+ and female +/- mice) were analysed by t-tests, adjusted for multiple comparisons by Bonferonni's method. Since there were only four comparisons of interest (i.e., male+/+ vs.

male+/-, female+/+ vs. female+/-, male+/+ vs. female+/+, and male+/- vs. female+/-), the α value was adjusted so that $P < (0.05/4)$ [=0.0125] was considered statistically significant. All t-tests were done using SigmaStat (Systat Software, Inc.).

Chapter 3

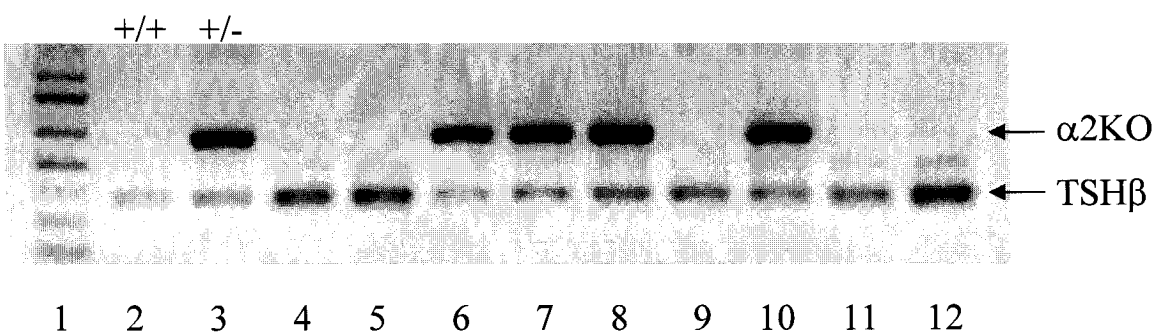
RESULTS

3.1. Genotype analysis

DNA was isolated from tail samples of 3-week old pups as described in the methods (section 2.2). PCR was then performed on the DNA in a multiplexed reaction. One primer set amplified a portion of the null allele (i.e. α2 allele interrupted by an HPRT resistance cassette), and the other primer set amplified a region of the unrelated thyroid stimulating hormone beta chain (TSHβ). The TSHβ amplicon was used as a control to ensure the presence of genomic DNA in the sample and to prevent false negatives (i.e., to ensure that the lack of an α2-HPRT PCR product in +/+ mice was not due to insufficient quantities of genomic DNA template in the sample).

In figure 9, heterozygous mice (+/-) were clearly marked by the intense α2KO band (350 bp; lanes 3, 6, 7, 8, 10) whereas the +/+ mice (lanes 2, 4, 5, 9, 11, 12) were lacking in this product. The TSHβ product (250 bp) was evident in all samples and ensured the integrity of the sample. The wildtype mice often produced a low-yield PCR product, resulting in a slight band just above the TSHβ band (evident in lanes 11, 12). This was an unidentified non-specific product from the α2KO primers; and because it was a different size than the α2KO band, it in no way interfered with the genotyping of the mice.

Figure 9. Genotype analysis of α2 +/+ and +/- by PCR. Lane 1 shows 0.5 μg of 1Kb+ lambda DNA marker. Lanes 2 and 3 are indicated as being wildtpe (+/+) and heterozygous (+/-) respectively. The null allele of the +/- mice is indicated by the presence of the α2KO band at 350 bp, whereas the wildtype mice lack this product. A part of the TSHβ gene is amplified (250 bp) in all samples in order to rule out false negatives in +/+ mice.


+/+
+/-
α2KO
TSHβ
1 2 3 4 5 6 7 8 9 10 11 12

3.2. Cardiovascular studies

Increases in CSF [Na^+], whether by icv infusion or high-salt diet in salt-sensitive animals, raises blood pressure. In rats, the early responses are mediated largely via Arginine-Vasopressin-dependent mechanisms, while the latter responses are attributable to sympathetic hyperactivity, mediated by the brain OLS and RAS. This may also be the case in mice, as the early response (0-20 min.) does not appear to be due to the OLS (Van Huysse and Hou, 2004).

3.2.1. Responses to icv infusion of ACSF-Na^+

3.2.1.1. Pressor responses to icv infusion of ACSF-Na^+

Since the OLS causes hypertension by inhibiting the NKA, the central hypothesis of this study was that a decrease in expression (via heterozygous gene knockout) of the specific isoform involved in the Na^+-induced centrally mediated pressor response to increased CSF [Na^+] would result in exaggerated increases in blood pressure in response to icv Na^+, vs. wildtype mice.

Previous studies confirm that the infusion of ACSF alone, nor ACSF with 0.150M Na^+ (approximate physiological concentration of Na^+ in the CSF) does not produce any significant pressor responses in C57Bl/6 mice - a contributing line in the current genetic model (Van Huysse, 2004).

Pressor responses to mildly hypertonic ACSF (0.225 M Na^+, at an infusion rate of 0.4 μl/min) in the α2 +/- mice and +/+ mice are shown in figure 10. When absolute MAPs are examined (figure 10A), there were significant effects of both genotype (i.e., +/+ vs. +/-) and gender in the early responses (0-20 minutes), whereas there was only a significant effect

of genotype in the late responses. However, despite the lack of a measurable i.e., statistically significant) effect of gender in the late responses, male +/- mice showed significantly elevated MAP vs. baseline from 15-35 minutes after the start of infusion, whereas female +/- mice had significantly elevated MAP vs. baseline from 10-55 minutes past the start of infusion. This would seem to indicate that the pressor responses in female +/- were more persistent than in male +/- mice. There was no significant elevation in MAP from baseline in +/+ mice.

Figure 10B shows the pressor responses to icv Na^+ as a function of differences in MAP from baseline over time. By means of this analysis, the changes in MAP from baseline were dependent on genotype in the early response, whereas the late responses were influenced by both genotype and gender. The data indicate then, that female +/- mice demonstrate an enhanced pressor response to icv Na^+ compared to female +/+ and even male +/- mice.

From table 2, it can be seen that there were no significant differences in baseline MAP between +/- and +/+ of either gender, nor between genders of either genotype. There was however a tendency for baseline blood pressures in males to be greater than in females.

Peak responses, representing maximum changes from baseline over a continuous 3-minute period, were also measured in the early (0-20 minutes after the start of infusion) and late (20-60 minutes after the start of infusion) time periods. Table 2 shows that in the early responses, both genders demonstrated greater changes in MAP in +/- vs. their respective +/+ counterparts, and to a similar magnitude (M+/- 20 ± 2 mmHg vs. M+/+ 6 ± 3; F+/- 24 ± 3 mmHg vs. F+/+ 2 ± 3). In the late responses however, only female +/- mice retained significantly greater peak changes in MAP vs. their +/+ counterpart. Although numerically

greater, the sustained peak pressor response in the female +/- group was not statistically different than the late male +/- response however (F+/- 23 ± 3 vs. M+/- 14 ± 3).

3.2.1.2. Heart rate responses to icv infusion of ACSF-Na^+

Figure 11A indicates that there was a significant effect of heart rate in both the early and late responses to icv Na^+ infusion. It is possible that the gender effects were solely due to the fact that female +/- mice had elevated (baseline) heart rates vs. female +/+ or male +/- mice, as indicated in table 2. However, as shown in figure 11B, there was a gender-dependent effect on changes in heart rate from baseline in females; and the female +/- group was the only group to demonstrate a point where the heart rate was significantly elevated vs. baseline (see figure 11A, female +/- response curve, 20 minutes after the start of infusion). There were no apparent differences in peak heart rate responses to icv Na^+ in any of the groups (table 2).

3.2.1.3. Additional responses to icv infusion of ACSF-Na^+

In addition to the +/- mice having enhanced pressor responses to icv Na^+ vs. +/+ mice, some of them also had adverse reactions to the infusions. Within 25-55 minutes from the start of Na^+ infusion, 5 female α2 +/- mice (out of 12) and 2 male +/- mice (out of 10) exhibited symptoms that have been previously observed with high-dose ouabain infusion (i.e., spasms and convulsions, unpublished observations). In addition, 3 of the female +/- mice with these symptoms died shortly after the end of the 1-hour infusion. This response was not seen in any of the wildtype mice used in these studies, nor was it seen in a previous study involving wildtype C57Bl/6 and Swiss Webster mice, in which higher doses (2.5- to 5-fold) of Na^+ were given (Van Huysse and Hou, 2004).

Figure 10. Arterial pressure response to icv Na^+ infusion. NaCl was infused icv (0.225 M NaCl in ACSF) for a 60-minute period. Absolute mean arterial pressures (MAP) are shown in (**A**), and the changes in MAP from baseline values (ΔMAP) are shown in (**B**). Group identities are shown in the legend. Data are mean ± S.E.M. Statistical key: **a** an effect of genotype, and **b** an effect of gender, separated into early (pre-20 minutes) and late (post-20 minuntes) responses to the infusion ($P<0.05$); + significant change from baseline (time=0) value ($P<0.05$).

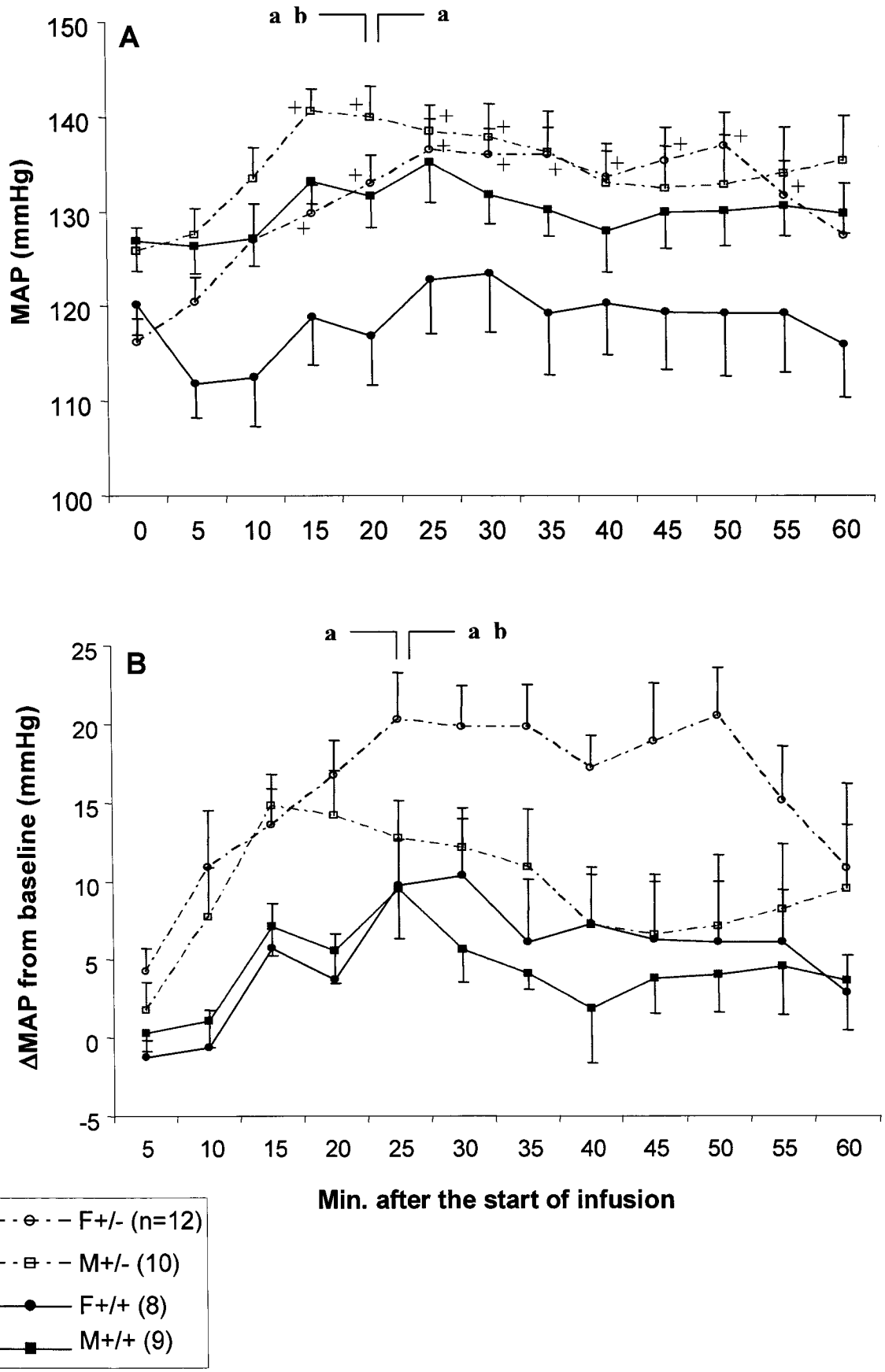

a b
a
A
150
140
130
120
110
100
MAP (mmHg)
0 5 10 15 20 25 30 35 40 45 50 55 60
a
a b
B
25
20
15
10
5
0
-5
ΔMAP from baseline (mmHg)
5 10 15 20 25 30 35 40 45 50 55 60
Min. after the start of infusion
F+/- (n=12)
M+/- (10)
F+/+ (8)
M+/+ (9)

Figure 11. Heart rate responses to icv Na^+ infusion. NaCl was infused icv (0.225 M NaCl in ACSF) for a 60-minute period. Absolute heart rates (HR) are shown in (**A**), and the changes in HR from baseline values (ΔHR) are shown in (**B**). Group identities are the same as in figure 10. Data are mean ± S.E.M. Statistical key: **a** an effect of genotype, and **b** an effect of gender, separated into early (pre-20 minutes) and late (post-20 minutes) responses to the infusion ($P<0.05$); + significant change from baseline (time=0) value ($P<0.05$).

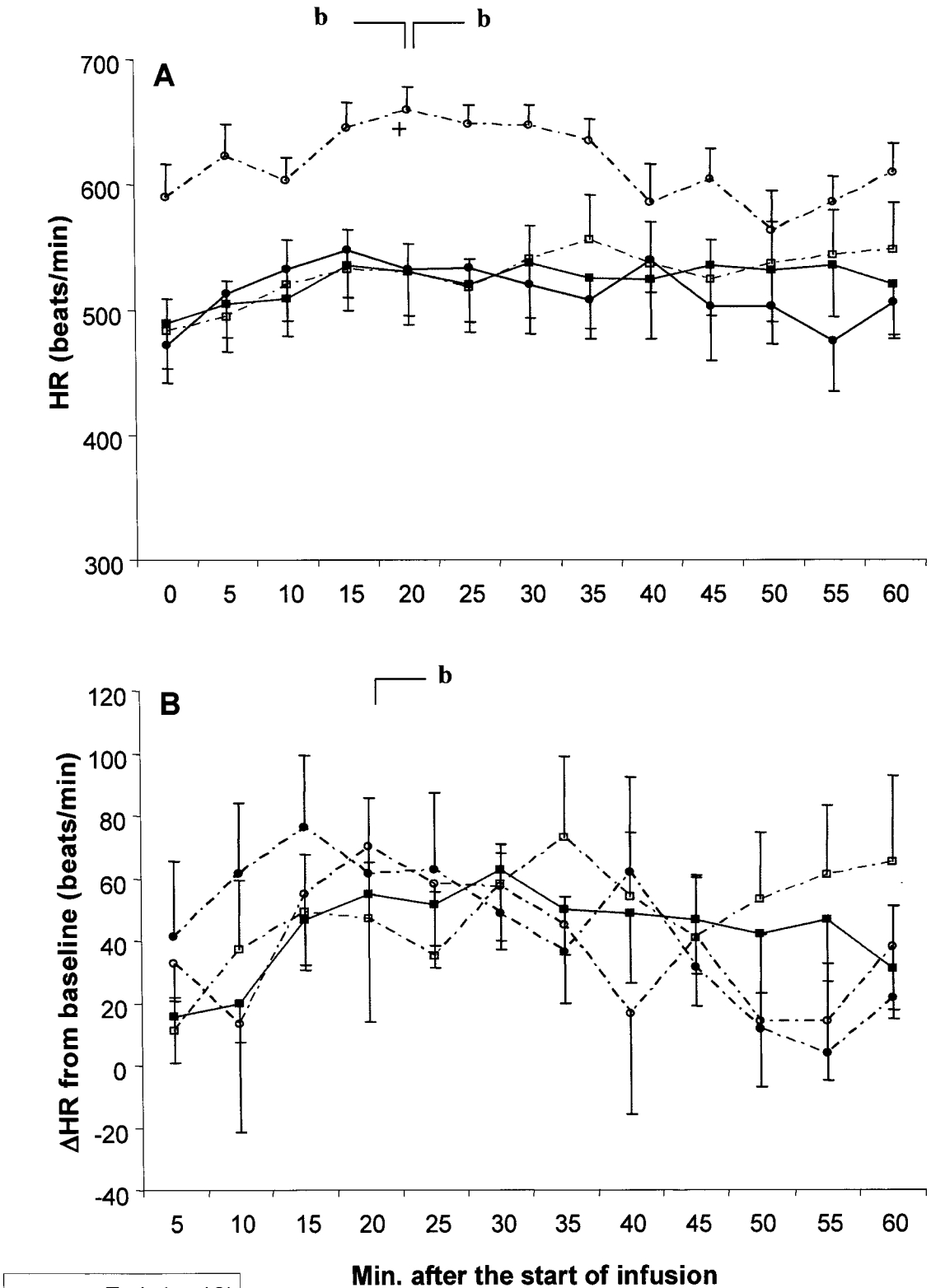

b
b
A
700
600
500
400
300
HR (beats/min)
0
5
10
15
20
25
30
35
40
45
50
55
60
b
B
120
100
80
60
40
20
0
-20
-40
ΔHR from baseline (beats/min)
5
10
15
20
25
30
35
40
45
50
55
60
Min. after the start of infusion
F+/- (n=12)
M+/- (10)
F+/+ (8)
M+/+ (9)

Table 2. Mean arterial pressure (MAP) and heart rate (HR) responses to icv infusion of 0.225 M Na^+ in ACSF (0.4 μL/min infusion rate). Baseline MAP and HR responses of male and female α2 +/+ and α2 +/- mice are shown; as well as peak changes from baseline (ΔMAP and ΔHR). Peak responses represent mean changes over a continuous 3-min. period, and are resolved into early (0-20 min.) and late (20-60 min.) responses. Data are mean ± S.E.M. * $P<0.05$ vs. +/+, same gender. # $P<0.05$ vs. male, same genotype.

	Males		Females	
	+/+ (n=9)	+/- (n=10)	+/+ (n=8)	+/- (n=12)
Baseline				
MAP	127 ± 3	126 ± 3	120 ± 3	116 ± 3
HR	489 ± 35	483 ± 26	471 ± 30	590 ± 27*#
Peak Responses from 0-20 min.				
ΔMAP	11 ± 2	20 ± 2*	2 ± 3#	24 ± 3*
ΔHR	71 ± 29	89 ± 14	66 ± 25	67 ± 20
Peak Responses from 20-60 min.				
ΔMAP	6 ± 3	14 ± 3	7 ± 3	23 ± 3*
ΔHR	38 ± 40	71 ± 20	46 ± 31	29 ± 25

3.2.2. Responses to icv infusion of ouabain in ACSF

The responses to increased CSF [Na^+] are mediated through brain OLS, since they are abolished by icv, but not iv administration of Digibind®. By extension, infusion of ouabain in the CSF mimics the hypertensive effects of icv Na^+ infusion, and high Na^+ diets in salt-sensitive individuals. In order to determine if the enhanced pressor responses in the α2 +/- mice were a result of enhanced sensitivity to NKA inhibition per se, and were not a result of altered regulation of a non-NKA protein, ouabain was infused icv and blood pressure and heart rate responses were measured. Because some of the +/- mice developed symptoms similar to acute CNS ouabain toxicity, a low dose of ouabain was infused (5 ng/μl, 0.4 μl/min) which previously has produced a response of ≤ 5 mmHg in C57Bl/6 mice (Van Huysse and Hou, 2004).

3.2.2.1. Pressor response to icv infusion of ouabain in ACSF

Figure 12 shows the effects of infusing ouabain at a dose of 5 ng/μL, at a rate of 0.4 μL/min. over a 60-minute period. Figure 12A indicates that there was a significant effect of gender on absolute MAP from 0-20 minutes after the start of infusion, possibly due to the male groups having higher baseline MAP than the female groups. After 20 minutes however, there was an effect of gender and genotype on absolute MAP. This seems intuitive, as the males retained higher blood pressures than the females throughout the study, while for either gender, the +/- groups had higher blood pressures than the +/+ groups. Despite this, only the male +/- mice showed significantly elevated MAP vs. baseline from 10-40 minutes after the start of infusion. Additionally, when arterial pressure responses were expressed as a function of difference in MAP from baseline (figure 12B), the effects of icv ouabain infusion appear to have been dependent upon genotype only.

Table 3 indicates that the peak changes in MAP from baseline to icv ouabain infusion were not different in the +/- vs. +/+ mice of either gender, nor in the same genotype between genders. This is in apparent contradiction to the previous section, but can be explained by the small sample sizes in each group (i.e., while numerically the peak changes in MAP are in agreement with the previous sections, there is insufficient power to detect statistical changes).

3.2.2.2. Heart rate response to icv infusion of ouabain in ACSF

With regards to absolute HR, at no point was the heart rate in any group significantly elevated vs. baseline (figure 13), suggesting that the (low) dose of ouabain used in this study had no effect on heart rate. Accordingly, there were no gender- or genotype-dependent heart rate responses to icv ouabain infusion. However, upon examination of the changes in heart rate from baseline (figure 13B), there appears to have been an early influence of gender on the response to icv ouabain, most likely mediated by the elevated changes in heart rate in the female +/- group. The late responses indicate that there was an effect of both gender and genotype on the heart rate. The females had greater changes in heart rate vs. baseline than the male groups, and within each gender, the +/- mice seem to have had elevated changes in heart rate from baseline compared to the +/+ mice.

Similar to the MAP data, table 3 shows that in response to icv ouabain infusion, there were no differences in peak changes of heart rate from baseline between +/- and +/+ mice of either gender, nor in similar genotypes between genders.

Figure 12. Arterial pressure responses to icv infusion of ouabain in ACSF. Ouabain was infused (5 ng/μL, 0.4 μL/min) for a 60-minute period. Absolute mean arterial pressures (MAP) are shown in (**A**), and the changes in MAP from baseline values (ΔMAP) are shown in (**B**). Group identifiers are shown in the legend. Data are mean ± S.E.M. Statistical key: **a** an effect of genotype, and **b** an effect of gender, separated into early (pre-20 minutes) and late (post-20 minutes) responses to the infusion ($P<0.05$); + significant change from baseline (time=0), $P<0.05$.

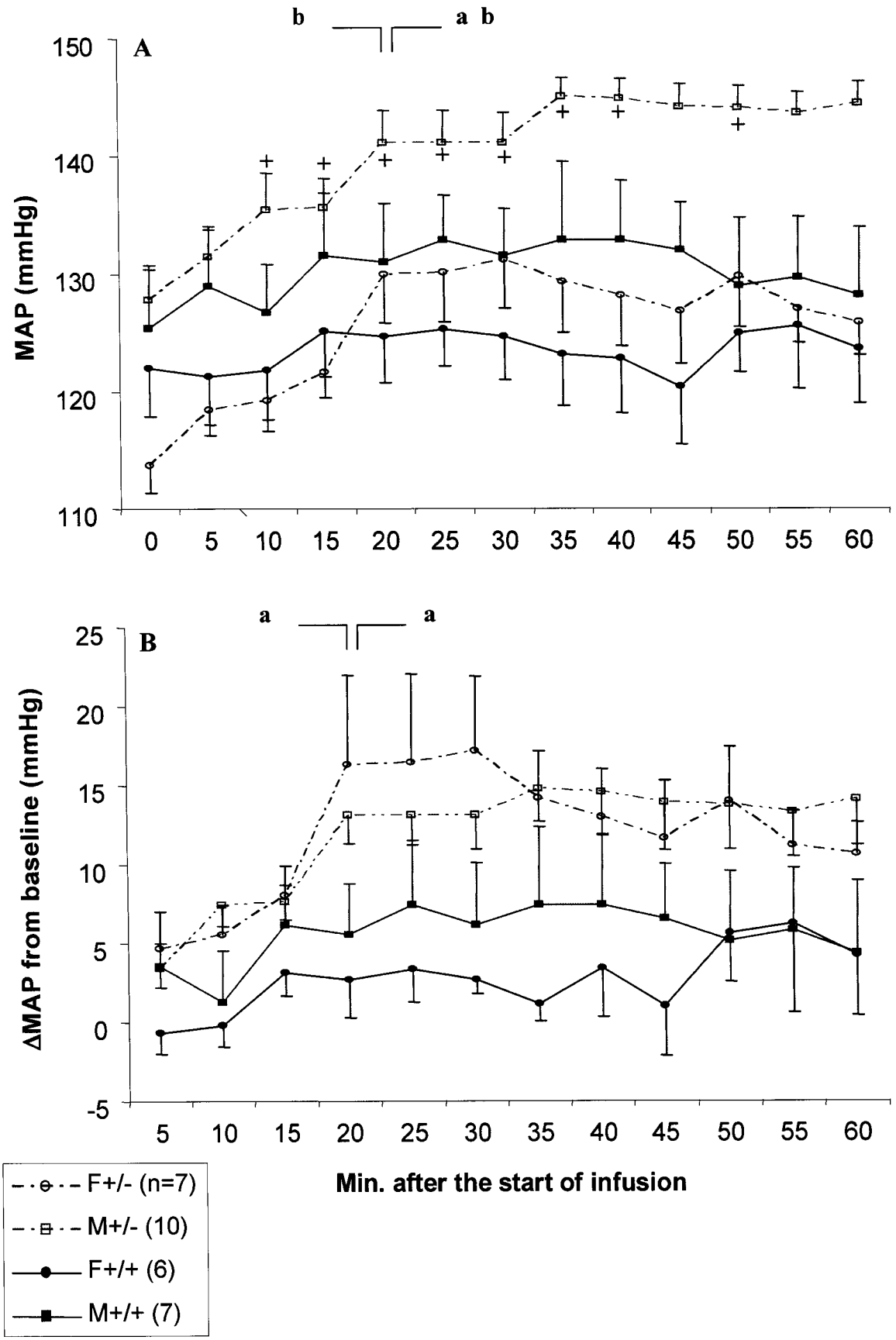

b
a b
A
150
140
130
120
110
MAP (mmHg)
0
5
10
15
20
25
30
35
40
45
50
55
60
a
a
B
25
20
15
10
5
0
-5
ΔMAP from baseline (mmHg)
5
10
15
20
25
30
35
40
45
50
55
60
Min. after the start of infusion
F+/- (n=7)
M+/- (10)
F+/+ (6)
M+/+ (7)

Figure 13. Heart rate responses to icv infusion of ouabain in aCSF. Ouabain was infused (5 ng/μL, 0.4 μL/min) for a 60-minute period. Absolute heart rates (HR) are shown in (**A**), and the changes in HR from baseline values (ΔHR) are shown in (**B**). Group identifiers are shown in the legend. Data are mean ± S.E.M. Statistical key: **a** an effect of genotype, and **b** an effect of gender, separated into early (pre-20 minutes) and late (post-20 minutes) responses to the infusion ($P<0.05$).

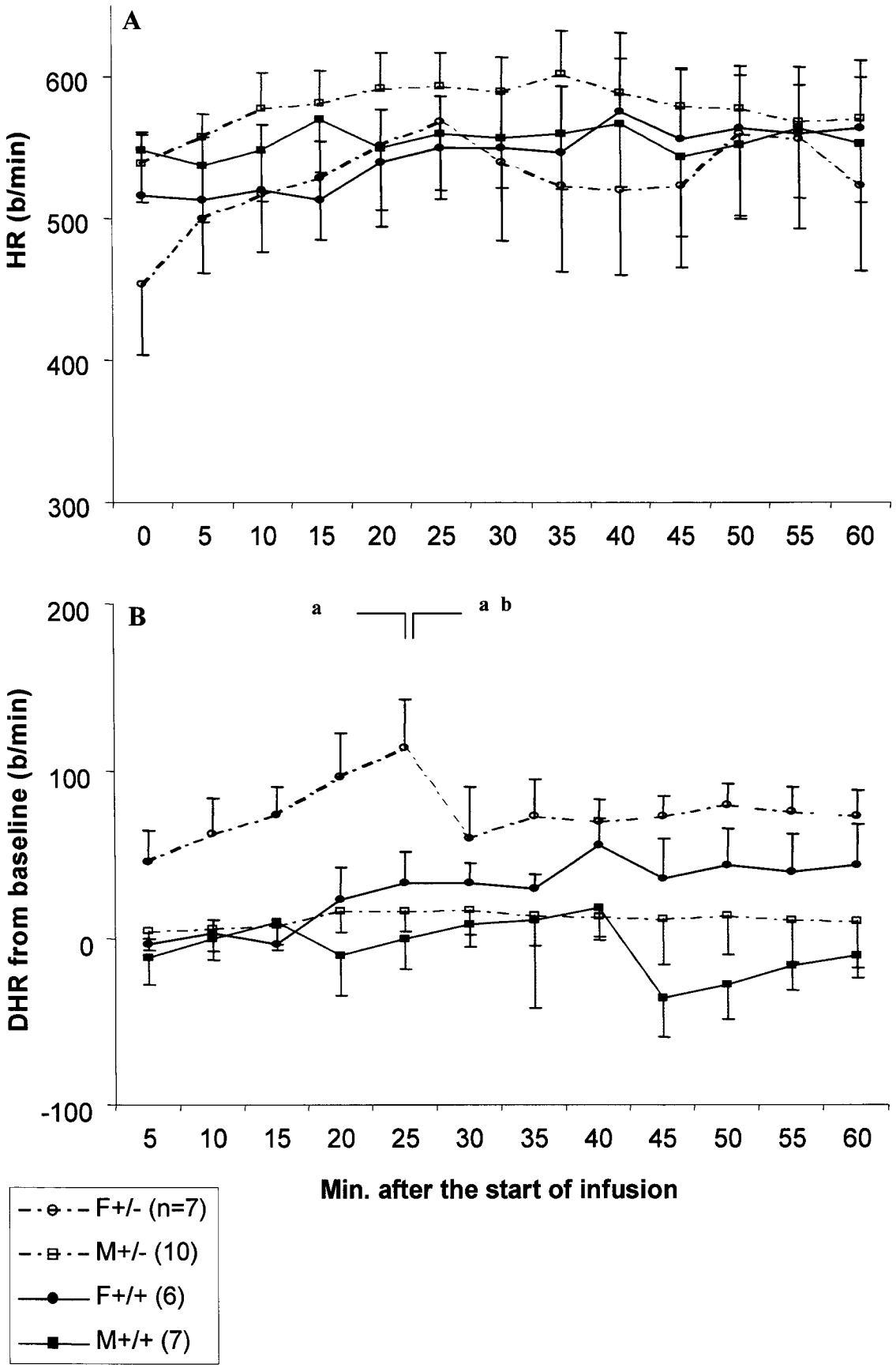

A
HR (b/min)
600
500
400
300
0 5 10 15 20 25 30 35 40 45 50 55 60
B
a
a b
DHR from baseline (b/min)
200
100
0
-100
5 10 15 20 25 30 35 40 45 50 55 60
Min. after the start of infusion
F+/- (n=7)
M+/- (10)
F+/+ (6)
M+/+ (7)

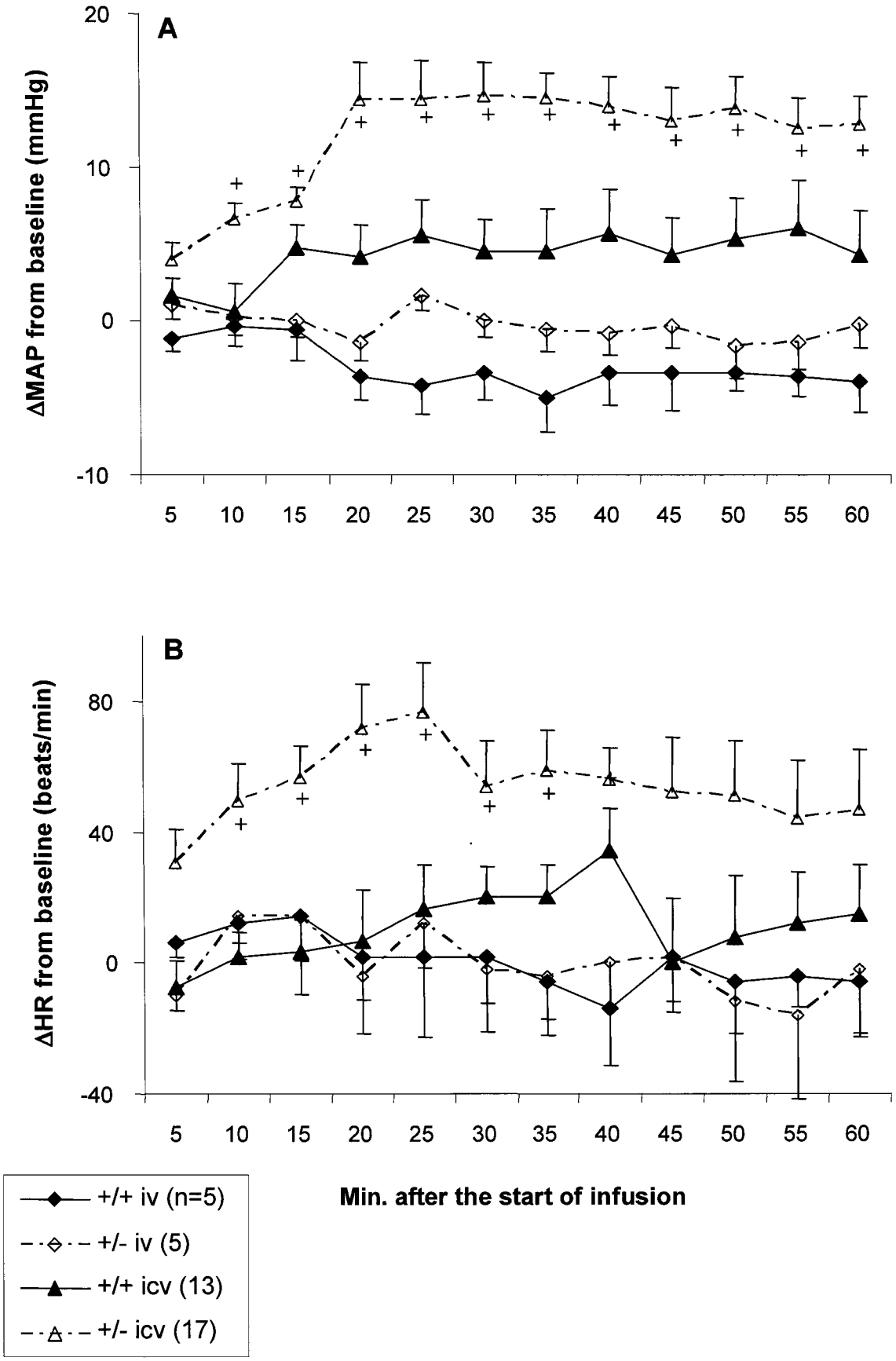
Table 3. Mean arterial pressure (MAP) and heart rate (HR) responses to icv infusion of ouabain in ACSF (5 ng/μL, 0.4 μL/min infusion rate). Baseline MAP and HR of male and female α2 +/+ and α2 +/- mice are shown; as well as peak changes from baseline (ΔMAP and ΔHR. Peak responses represent mean changes over a continuous 3-min. period, and are resolved into early (0-20 min.) and late (20-60 min.) responses. Data are mean ± S.E.M.

	Males		Females	
	+/+ (n)	**+/- (n)**	**+/+ (n)**	**+/- (n)**
Baseline				
MAP	125 ± 5 (7)	128 ± 3 (10)	122 ± 4 (6)	114 ± 6 (7)
HR	549 ± 39	539 ± 21	516 ± 44	454 ± 50
Peak Responses from 0-20 min.				
ΔMAP	8 ± 5 (4)	14 ± 1 (10)	4 ± 2 (3)	21 ± 14 (3)
ΔHR	-13 ± 33	55± 12	-7 ± 7	153 ± 74
Peak Responses from 20-60 min.				
ΔMAP	11 ± 3 (4)	17 ± 2 (10)	10 ± 8 (3)	25 ± 8 (3)
ΔHR	5 ± 17	57 ± 24	-7 ± 24	160 ± 58

3.2.3. Responses to central vs. peripheral ouabain infusion

Although the pressor responses to icv Na^+ infusion in rodents are well documented, we tested whether in this particular genetic model, the responses to central infusion of Na^+ were mediated via central mechanisms (i.e., via brain OLS). In other words, were the ouabain (or OLS) effects central in origin, or could they have been mediated by secretion of ouabain/OLS from the pituitary into the circulation, or even due to "leakage" of ouabain/OLS from the CSF into the circulatory system. This was tested by infusing ouabain iv, at the same dose as the icv studies in α2 +/+ and +/- mice. Figure 14 illustrates the blood pressure and heart rate responses to both icv and iv infusion of ouabain in α2 +/- and +/+ mice. There were clear elevations in MAP and HR from baseline in the α2 +/- ($P<0.05$) mice infused with ouabain icv, reaching a plateau at 20 minutes after the start of infusion. Although the α2 +/+ mice with icv infusions of ouabain demonstrated a consistently elevated change in MAP over time ($\leq$ 5 mmHg), this effect was not statistically significant. In contrast to the icv infusion groups, there were no changes in MAP or HR in either genotype when the same dose of ouabain was infused iv. This statement holds up even when the statistical α value used for the response curves contains no Bonferroni correction. By this it is meant that because there are effectively 48 paired t-tests needed to check every time-point (in all response curves together) vs. baseline, a test is only significant if $P < 0.05/48$ (=0.00104); and therefore, an unadjusted test would use 0.05 as an α value, and not 0.00104. These results strongly indicate that the pressor and heart rate responses to icv ouabain (and icv Na^+) were initiated through central mechanisms, and that any potential leakage into the circulatory system was non-existent, or of negligible consequence.

Figure 14. Changes in mean arterial pressure (ΔMAP) and heart rate (ΔHR) in α2+/- and +/+ mice in response to icv and iv infusion of ouabain. Mice of either genotype were infused either icv or iv with ouabain in ACSF at dose of 5 ng/mL (0.4 mL/min) over a 60-minute period. Responses to infusions are represented as changes in MAP (**A**) and HR (**B**) from baseline values (determined before the start of infusion). Group identifiers are shown in the legend. Data are mean ± S.E.M. + $P<0.05$ (adjusted for multiple comparisons) vs. baseline value.


A
ΔMAP from baseline (mmHg)
20
10
0
-10
5
10
15
20
25
30
35
40
45
50
55
60
B
ΔHR from baseline (beats/min)
80
40
0
-40
5
10
15
20
25
30
35
40
45
50
55
60
+/+ iv (n=5)
+/- iv (5)
+/+ icv (13)
+/- icv (17)
Min. after the start of infusion

3.3. Biochemical analysis

3.3.1. Na, K-ATPase activity

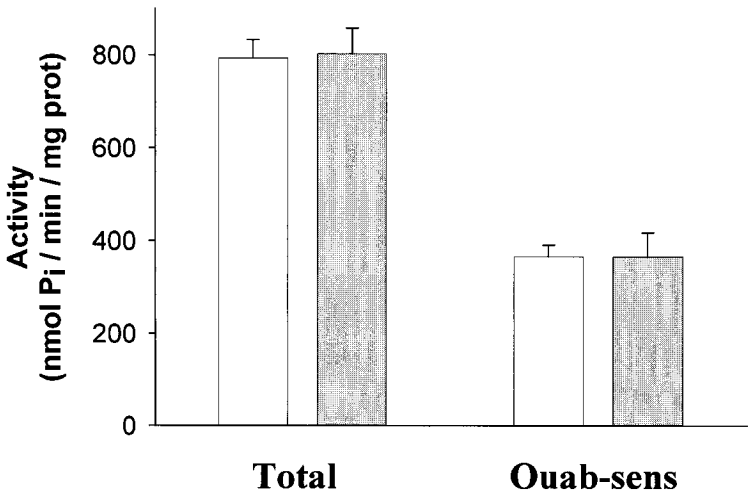
Because the α2 heterozygous mice have one null allele, these mice were expected to have decreased brain α2 activity. NKA activity from brain microsomal homogenates was measured as the amount of inorganic phosphate released (nmol P_i) per minute per mg of protein. As can be seen in figure 15, there were no discernable differences between the activity of ouabain-sensitive isoforms (α2+α3) in brain microsomal preparations.

3.3.2. Na, K-ATPase α isoform protein expression

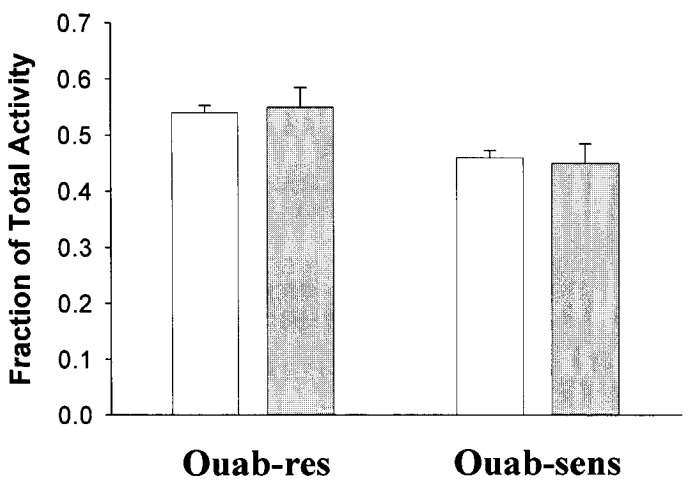
The functional effect of the heterozyous gene knockout could not be measured (i.e. there was no difference in overall (brain) ouabain-sensitive NKA activity in the α2 +/- mice vs. the +/+ mice. We chose to measure protein expression by Western blot analysis to ensure that: i) α2 protein expression was indeed decreased in the heterozygous mice; ii) the inability of the activity assay to measure differences between the genotypes was a reflection of poor resolution of the assay (e.g., discerning small amounts of α2 from large amounts of α3); and/or iii) there were no compensatory changes in α3 that could account for the lack of ouabain-sensitive NKA activity. To increase the power of the assay, two separate Western analyses were performed for each group (yielding a sample size of 9-12 per group) and the data from each Western blot were pooled. The same standard sample was used for each Western 'pair'. The NKA isoform and α-tubulin bands were standardized to their respective three point standard curves and all samples were normalized to α-tubulin.

Figure 15. Na, K-ATPase activity in whole brain microsomal preparations. Microsomes were incubated in the reaction solution containing cold ATP with trace amounts of γ-^{32}P-labeled ATP. After the ATP hydrolysis reaction was stopped, organic compounds were adsorbed onto acid-washed charcoal and centrifuged, leaving the hydrolyzed γ-^{32}P in the supernatant. An aliquot of the supernatant was added to scintillation fluid and CPM were measured with a beta-counter. CPM were adjusted to nmol P_i produced. Panel A shows total activity and ouabain-sensitive activity (α2+α3, "ouab-sens") in α2 +/- and +/+ mice. Panel B presents ouabain-resistant activity (α1, "ouab-res") and ouabain-sensitive activity as a fraction of total activity. Data are mean ± S.E.M.

A


Activity
(nmol Pi / min / mg prot)
800
600
400
200
0
Total
Ouab-sens

B

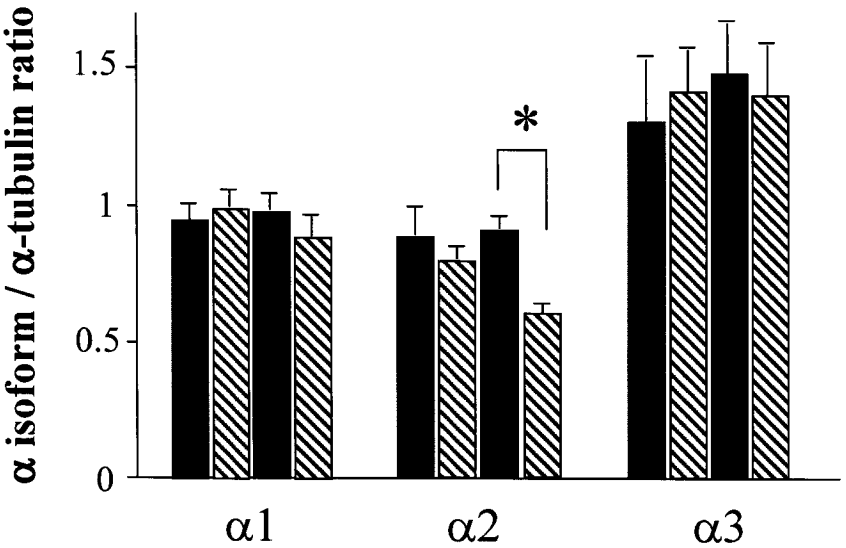

Fraction of Total Activity
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
Ouab-res
Ouab-sens

+/- (n=6)
+/+ (n=4)

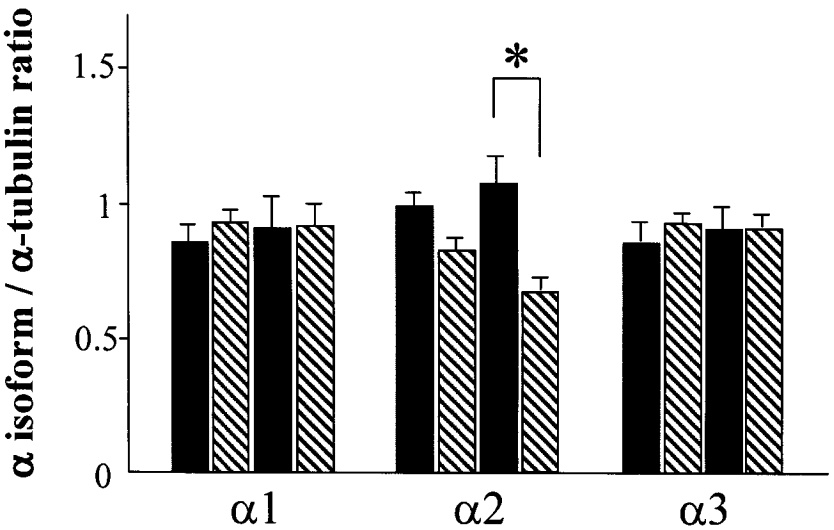
Figure 16 shows that the expression of α2 in the hypothalamus and pons/medulla (two major brain regions involved in cardiovascular regulation where the OLS is present) of α2 +/- mice was gender dependent. In the hypothalamus, α2 was reduced by 34% ($P<0.001$) in female +/- vs. +/+, while it only slightly decreased in male +/- vs. +/+ (~10%, P=NS). There were no differences between +/+ male and female. This expression pattern was similar in the pons/medulla. Female +/- had 38% less α2 protein than female +/+ mice ($P<0.001$); and although male +/- mice expressed ~16% less α2 protein than male +/+ mice, this was still not significantly different if the tests are adjusted for multiple comparisons. Neither the hypothalamus nor the pons/medulla showed any difference in the expression of the α1 or α3 isoforms between any group.

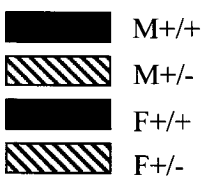
Figure 16. Expression of NKA α isoforms. Data for the hypothalamus are shown in (**A**) and the pons/medulla in (**B**). For α1 and α2, 2 μg of total protein was loaded onto an 8% acrylamide gel and later subjected to immunoblot analysis, whereas 0.2 μg of protein was loaded for α3. These samples were standardized by a three-point standard curve (1, 2 and 3 μg protein for α1/2 or 0.1, 0.2 and 0.3 μg for α3), and normalized to α-tubulin controls. Data shown were compiled from two Western blots for each isoform in each tissue. In the legend, M = male and F = female. N=9-12 per group; data are mean ± S.E.M. * $P<0.05$, vs. +/+, same gender.

A Hypothalamus



B Pons/Medulla





3.3.3. Ouabain-like substance immunoreactivity (ELISA)

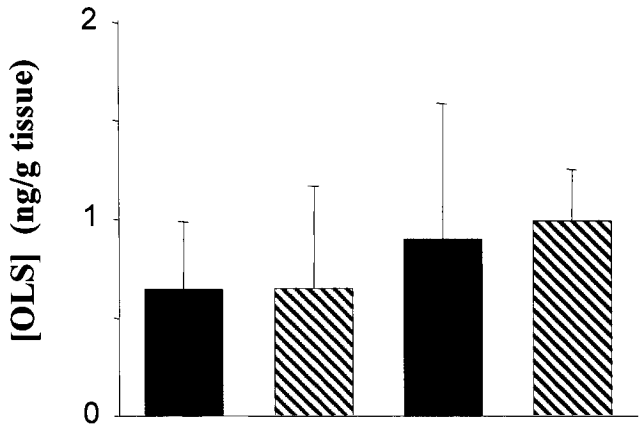
Because the centrally-mediated hypertensive responses to CSF Na^+ involve OLS (Van Huysse and Hou, 2004), we assessed whether the baseline levels of brain and serum OLS were different in +/- vs. +/+ mice. Since the circumventricular organs may respond to circulating OLS, serum levels were also examined. If [OLS] was increased in the +/- mice, this could represent a contributing factor in the enhanced response to icv Na^+ in $\alpha 2$ +/- mice. Figure 17 indicates that there were no changes in OLS levels in the hypothalamus (figure 17A), pons/medulla (figure 17B) or serum (figure 17C).

3.3.4. ACE activity

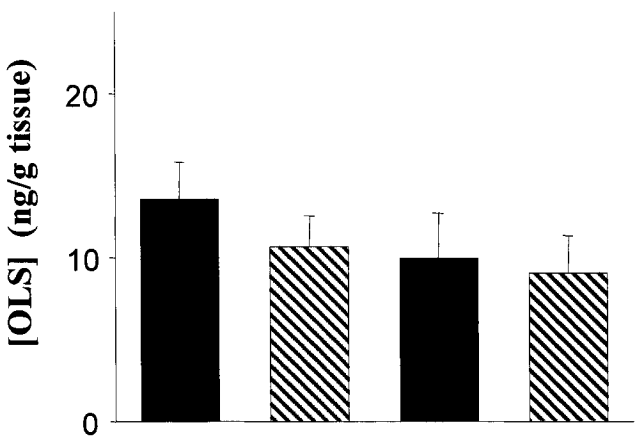
Dahl-S rats on a high salt diet show parallel increases in blood pressure, [OLS] and ACE activity in the hypothalamus and pons/medulla (Zhao et al., 2001). The renin-angiotensin system, which includes ACE, is crucial in the initiation of pressor responses to CSF-Na^+. Elevated resting levels of ACE in +/- mice vs. +/+ could represent a mechanism by which the +/- mice demonstrate enhanced pressor responses to icv Na, downstream of NKA inhibition. However, figure 18 clearly indicates that there were no differences in baseline ACE activity in hypothalamus or pons/medulla of either gender or genotype.

Figure 17. Brain and serum [OLS] as measured by ELISA in 8-10 week mice. Ouabain-like substances were isolated from brain tissue or serum and subjected to ELISA analysis as described in the methods. OLS was measured in (**A**) hypothalamus (n=5), (**B**) pons/medulla (n=5-7), and (**C**) serum (n=5-7). Group symbols (e.g., M+/+) are the same as in figure 16. Data are mean ± S.E.M.

A Hypothalamus



B Pons/medulla



C Serum

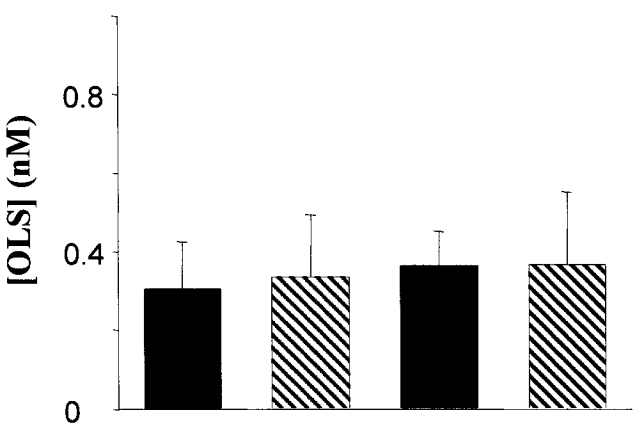
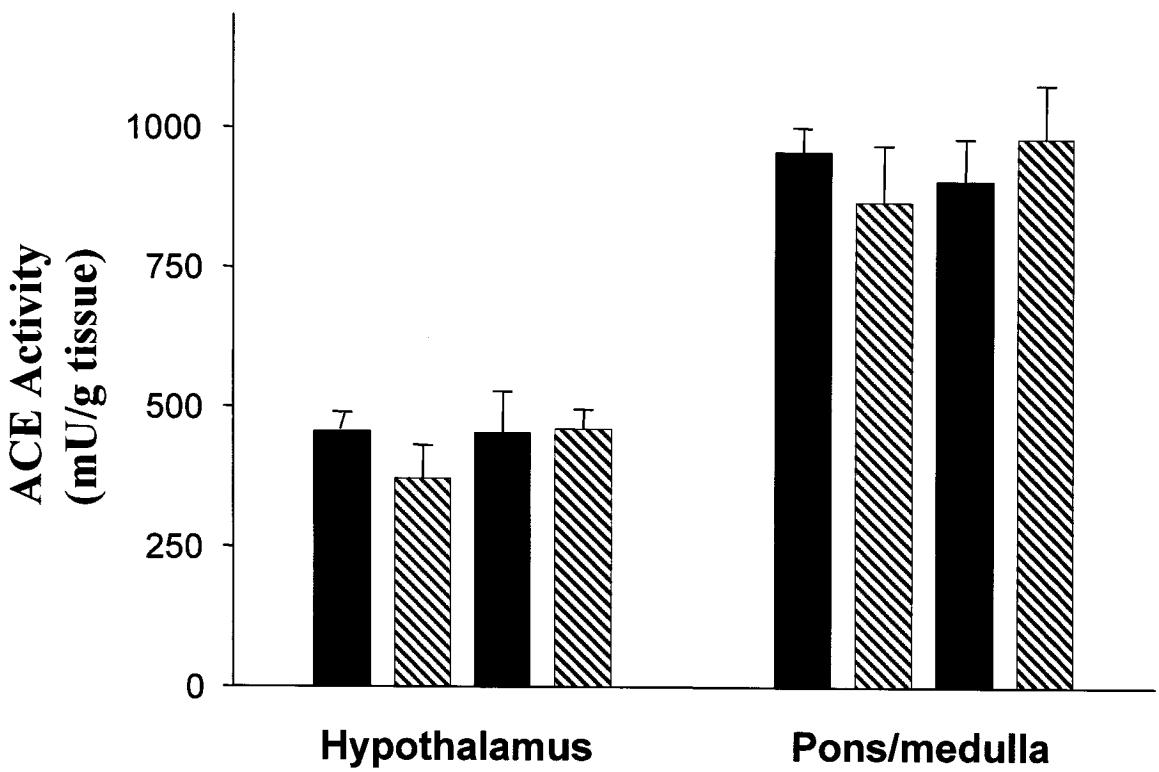
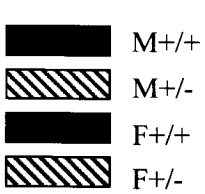


Figure 18. Baseline Brain ACE activity of 8-10 week mice. Tissues were sonicated in Tris buffer and crude homogenates were prepared as per the description in the methods section. ACE activity is the amount of captopril inhibitable proteolytic activity expressed in mU/g (nmol histidine-leucine produced/min/g protein, see methods). The legend is the same as in figure 16. For the hypothalamus n=3-4, and for the pons/medulla n=5-6. Data are mean ± S.E.M.


ACE Activity
(mU/g tissue)
1000
750
500
250
0
Hypothalamus
Pons/medulla


M+/+
M+/-
F+/+
F+/-

Chapter 4

DISCUSSION

4.1. Cardiovascular responses to icv infusion of Na^+ and ouabain

The findings of these studies suggest that the α2 isoform of the NKA is involved in the generation of centrally mediated pressor responses to increases in CSF [Na^+]. There was a consistent increase in the blood pressure response to icv Na^+ in α2 +/- vs. +/+ mice, when analyzed by either absolute values of MAP, or changes in MAP from baseline. There was also a consistent increase in the pressor response to icv infusion of ouabain in α2 +/- vs. +/+ mice. This supports the central hypothesis of this study, and because the α2 isoform is expressed predominantly in glia, these results are suggestive of a fundamental role for glia in mediating the central response to elevated CSF [Na^+].

The genotype-dependent pressor responses to icv Na^+ or ouabain occur during both the early (0-20 minutes after the start of infusion) and late, OLS-dependent responses. Because ouabain resulted in exaggerated pressor responses in α2 +/- vs. +/+ mice, this suggests a direct role for ouabain-sensitive isoforms in the mediation of salt-sensitive hypertension. In addition, only the α2 NKA isoform was reduced in expression in α2 +/- mice, implicating α2 specifically in the pathogenesis of salt-sensitive hypertension.

It was stated earlier that the sympathetically-mediated late responses are OLS-dependent. It is unexpected then, that icv infusion of ouabain would produce significant genotype-dependent effects on arterial pressure before 20 minutes after the start of infusion; which was however the case (figure 12B). If figure 12B is examined closely though, it could be argued that very early changes in MAP over time (0-15 minutes after the start of infusion of icv ouabain) are not different in the +/- mice from the +/+ mice; and that the major genotype-dependent effect seems to arise from a sharp increase in MAP response from 15-20 minutes after the start of infusion in +/- mice. Perhaps in this case then, the

ouabain-mediated increase in sympathetic tone occurs earlier (i.e., after 15 minutes) compared to icv Na^+ infusion, because the process of activating brain OLS (i.e., increasing synthesis/secretion) by hypertonic Na^+ is not required.

The heart rate responses to icv Na^+ or ouabain were not as consistent as the pressor responses. Changes in heart rate from baseline in response to icv Na^+ infusion appear to be gender-dependent – most likely a result of lesser changes in heart rate in the females. In the icv ouabain studies however, the early changes in heart rate from baseline appear to be genotype dependent – an effect likely due to the elevated response in female +/- mice. The late changes in heart rate from baseline appear to be both gender and genotype-dependent – namely, females have greater changes in heart rate from baseline than males (contrary to the icv Na^+ studies), while +/- mice have greater changes in heart rate from baseline than +/+ mice. The reason for this discrepancy is not apparent, but may involve greater impairment of baroreceptor responses in female and heterozygous mice in response to direct ouabain infusion vs. Na^+ infusion.

4.2. The central origin of the cardiovascular responses

The responses to icv Na^+ and ouabain are due to the $\alpha 2$ NKA isoform per se. The effects of icv ouabain administration would be to act on (inhibit) the NKA isoforms (particularly the ouabain-sensitive isoforms). The Western blot analyses clearly indicate that there were no changes in expression of the $\alpha 1$ or $\alpha 3$ isoforms in two major cardiovascular regulatory centers of the brain, and thus the responses to icv ouabain are due to differences in $\alpha 2$ isoform expression in the +/- mice.

These studies also argue that the pressor responses originate via central mechanisms. In these studies, pressor responses to low-dose ouabain in α2 +/- mice were only achieved when icv infusions were performed, but not when the same dose of ouabain was administered iv. These results are consistent with previous studies in which a dose of sodium, several-fold higher than the dose used in these studies (0.6 M Na^+, 0.4μl/min), administered iv has no effect on blood pressure (Van Huysse and Hou, 2004). These results support the hypothesis that elevations in CSF [Na^+] lead to pressor responses via the actions of central OLS, presumably by inhibiting ouabain-sensitive NKA α isoforms (α2 and possibly α3). Although low-dose icv ouabain infusion failed to elicit a significant response in +/+ mice, there was a consistent trend for increased blood pressure vs. baseline (~5 mmHg). This response was also consistent with previous studies showing a pressor response to icv ouabain in C57Bl/6 and Swiss Webster mice (Van Huysse and Hou, 2004).

4.3. Gender-dependent responses to icv infusion of ACSF-Na^+ / ouabain

Aside from the altered responses due to genotype (i.e. α2 +/- vs +/+), there were also important gender differences in the responses of +/- mice to icv infusion of Na^+ and ouabain. The changes in MAP from baseline due to icv Na^+ infusion were greater in the female +/- mice compared to male +/- mice (which were in turn, greater than the response in the female and male +/+ mice). The time-span in which female +/- mice had elevated arterial pressure (vs. baseline) was also longer than in the male +/- mice. In addition, female +/- mice sustained significant elevations in peak MAP vs. female +/+ mice, whereas peak changes in MAP in male +/- were only different from male +/+ mice during the early responses. Although there was not a strong effect of icv Na^+ infusion on heart rate, the female +/- group

was the only one in which heart rate was ever significantly elevated vs. baseline (figure 11A, at 20 minutes after the start of infusion).

The pressor response (changes in MAP from baseline) to icv ouabain resulted in genotype-dependent responses only (i.e., no gender differences). Additionally, only male +/- mice showed significant changes in MAP from baseline, despite female +/- changes in MAP being numerically greater. The absence of a gender-dependent effect is somewhat surprising since at nearly all time points, the difference between the changes in MAP in female +/- and female +/+ was numerically greater than the difference between male +/- and +/+ mice. This would suggest that due to the method of statistical analysis used (i.e., 3-way ANOVA with repeated measures on time for determining gender/genotype-dependent effects over time; or paired t-tests (to determine changes from baseline) with an adjusted α value set to 0.05/48 (=0.00104)), diminished the power of each test, requiring a larger sample size to adequately detect changes in the female +/- group. Similarly, table 3 indicates that the peak changes in MAP from baseline in female +/- mice was numerically greater than that of male +/- mice (which in turn were greater than male and female +/+ mice) in both the early and late responses. However, due to the small sample size present in the female +/- group, a significant difference vs. male +/- or even vs. female +/+ mice was not detected. Finally, the gender-dependent changes in heart rate from baseline from icv ouabain, were due to greater responses in the female +/- mice (figure 13B).

4.4. Mortality

Some of the α2+/- mice suffered from apparent neuro(glio?)toxic effects in response to icv Na^+ or low-dose ouabain infusion, which have been observed in mice given high-dose ouabain (e.g., spasms, convulsions; unpublished data). Three of the female +/- mice died at the end of the icv Na^+ experiments, and two female +/- mice died after the low-dose icv ouabain experiments. These responses, to these doses of Na^+ or ouabain, have not yet been documented in the +/+ mice, or in other mouse strains.

A previous study examining the brains of α2 +/- mice mice revealed no structural abnormalities/defects (Ikeda et al., 1993). Instead of resulting from an anatomical defect, the current symptoms are consistent with with susceptibility of α2 +/- mice to sodium- or ouabain-induced cellular hyperexcitability by enhancing intracellular Ca^{++} stores/release (James et al., 1999, He et al., 2001, Golovina et al., 2003). Downregulation of the α2 isoform may potentiate this excitatory effect. Indeed, Ikeda et al. (1993) have reported neural hyperactivity and impaired uptake of the excitatory neurotransmitter glutamate in α2 +/- mice. Finally, Moseley et al. (2003) have reported disruptions in normal rhythmic firing patterns in brainstem neurons controlling respiration in α2 -/- neonates. A similar homozygous knockout phenotype may be achieved in α2 +/- mice when exposure to icv ouabain or brain OLS was combined with an already reduced baseline α2 isoform expression.

The hyperresponsiveness of α2 +/- mice to elevated CSF [Na^+] or [ouabain/OLS] further implicates the α2 NKA isoform as having a critical role in mediating salt-sensitive hypertension.

4.5. Possible biochemical basis for the differences in cardiovascular responses

It has already been suggested that the effects of the brain OLS (via icv Na^+ infusion) or icv ouabain are mediated by the α2 NKA isoform. There is also an inverse relationship between the responses and α2 isoform content (as measured by Western analysis), meaning the less α2 isoform expressed in the brain, the greater the response is to icv Na^+ or ouabain. Previous biochemical studies have indicated that α2 expression in +/- mice was decreased by approximately 40% in heart (James et al., 1999), 46% in hindlimb extensor digitorum longus skeletal muscle (He et al., 2001), and ~50% in neonatal primary astrocyte culture (Golovina et al., 2002) vs. +/+ mice. The α2 isoform had not been measured in adult brain tissue in α2+/- mice prior to the present studies. Western analyses were performed in the hypothalamus and pons/medulla because these two brain regions are important in the regulation of cardiovascular function and because they contain OLS. In contrast to peripheral tissues, expression of the α2 subunit in brain tissue of adult α2+/- mice was not reduced by as much as 40-50% vs. +/+. Of interest, there were definite gender-based differences in the expression of α2 in +/- mice, but not +/+ mice; another finding not revealed in previous studies (expression was not reported at all for both genders in any tissue). In the present study, although α2 protein content in female +/- and male +/- mice was not significantly different in either the hypothalamus or pons/medulla, only the female +/- had significantly less α2 protein relative to their respective +/+ counterparts. However, it should be noted that if the α values in the inter-group t-tests were unadjusted for multiple comparisons, the results would be slightly different: In the hypothalamus, female +/- would have significantly less α2 isoform protein expression than female +/+ ($P<0.001$) and male +/- mice ($P<0.05$); In the pons/medulla, female +/- would have significantly less α2

expression than female +/+ ($P<0.001$) and male +/- mice ($P<0.05$), while male +/- would have significantly less α2 expression than male +/+ mice ($P<0.05$). The power of the statistical analysis then, when adjusted for multiple comparisons, suffers from insufficient sample size and/or large variance from sample means.

There is evidence in the literature in support of divergent roles for sex hormones in the regulation of brain NKA activity and thus the gender differences found herein. In rat brain microsomes, males had significantly enhanced ouabain-sensitive NKA activity (i.e., α2/3) vs. females, and although maximal ouabain binding (determined at high ouabain concentrations) was similar in males and females, ouabain-sensitive binding (determined at low concentrations of ouabain) was greater in males (Fraser and Sarnacki, 1989). Maximal ouabain binding represents the total NKA population (i.e., α1 + α2 + α3), whereas ouabain-sensitive binding represents the α2 + α3 NKA population. In rat-derived astrocyte culture, estrogen and progesterone treatment decreased ouabain-sensitive NKA activity, whereas testosterone treatment enhanced ouabain-sensitive NKA activity (Fraser and Swanson, 1994). *In vivo* studies have also shown that brain NKA activity varies with different stages of the estrous cycle (Rodriguez del Castillo et al., 1987). Orchidectomy in male rats decreases NKA activity, whereas testosterone replacement restores NKA activity (Guerra et al., 1987). These studies all support a role for testosterone in elevating ouabain-sensitive NKA activity (and thus possibly expression of α2), while estrogen/progesterone appears to decrease α2 activity.

There are other physiological and methodological considerations to explain the differences in α2 isoform expression in male +/- mice in the adult brain (10-16% decrease in expression vs. male +/+; P=NS), vs. previous studies showing a ~50% decrease in

expression in primary cultures of neo-natal astrocytes. Several factors are different for astrocytes regarding *in vitro* vs. *in vivo* expression of proteins. First, because the astrocyte culture is derived from neo-natal mice, these cells have not undergone ontogenic development and are thus inherently different from adult glia. Second, but much like the first point, the astrocyte cultures from α2+/- mice had never been exposed to testosterone or estrogen/progesterone, and thus there are potentially important hormonal differences in the *in vivo* and *in vitro* environments. Finally, Fink et al. (1996) and Knapp et al. (2000) have shown through *in situ* hybridization and immunohistochemical techniques, that while α2 isoform content is low in isolated oligodendrocyte cultures, it is highly abundant in oligodendrocytes of brain sections, or co-cultures of oligodendrocytes and neurons. The above studies suggest that, in neo-natally derived astrocyte culture, the α2 isoform content from α2+/- mice was a function of mRNA content (i.e., reduced by ~50% vs. α2+/+), whereas in the adult brain, α2 isoform content is highly regulated.

Finally, the data presented in Western analyses is notoriously variable, and none of the Western data presented in the above previous studies (James *et al.*, 1999; He *et al.*, 2001; Golovina *et al.*, 2002) used large groups (typically n=4-5 per group i.e., α2+/- vs. +/+); nor was a house-keeping protein used to normalize for variability in sample loading. In addition, no statistical analyses were performed. In this study, we had four groups, each of which express variable amounts (as is typical by Western analysis) of each NKA isoform, and with apparent differences in male and female +/- groups. It was therefore necessary to maximize the sensitivity of the assay, within our means. To this end, we used a larger sample size per group (n=9-12), α-tubulin analysis was included as a loading control, and finally, a standard curve was used in order to more accurately express band density (on the

film) as a function of protein content. This effectively allowed us to make statistically relevant inter-group comparisons on the expression of the α2 (and α1 and α3) isoform content.

4.6. Downstream effector to icv Na^+: the OLS.

The OLS inhibits ouabain-sensitive α isoforms (α2 in glia or α3 in neurons) and subsequently activates the local brain renin-angiotensin system (RAS). Therefore, we measured brain OLS in resting α2 +/+ and +/- animals, in order to determine if the altered pressor responses in the +/- strain could have been a result of altered baseline states of this factor.

OLS was measured in homogenates of hypothalamus, pons/medulla and serum. There were no differences in baseline OLS concentration between +/- and +/+ mice. It can therefore be concluded that the increased sensitivity of the α2+/- mice to icv Na^+ infusion was not due to elevated amounts of baseline OLS in the brain inhibiting the NKAs. Because of the reduced expression of α2 in the brain of +/- mice, an increase in resting OLS would likely lead to an elevated state of α2 inhibition, which could lead to elevated resting blood pressure and heart rate; and this was not observed. It is conceivable then, that the levels of OLS were the same in both genotypes, and the OLS inhibited the α2 NKAs in the α2+/- more than in the +/+ mice, rendering the α2 isoform-expressing brain cells of +/- mice hyperresponsive.

Serum OLS was measured, because the brain circumventricular organs (CVO) lie outside the blood-brain barrier and can respond to blood-borne substances. The CVOs represent another means by which circulating OLS could potentiate pressor responses. In

addition, circulating OLS can sensitize the vasculature and possibly even cardiac myocytes to be hyperresponsive to sympathetic stimulation, leading to increased blood pressure. Serum OLS has been shown to be elevated in states of Na^+ excess, and hypertension (Hamlyn et al., 1996; Yamada et al., 1997). In this study, there were no differences in baseline blood pressure between any of the groups, and expectedly, no differences in baseline serum [OLS].

4.7. Downstream effector to icv Na^+: ACE

When SHR are fed a high-salt diet, an increase in angiotensin-converting enzyme (ACE) activity in the midbrain has been reported (Mizuno et al., 1981); as well, Dahl-S rats fed a high-salt diet show significant increases in ACE mRNA and activity in the hypothalamus and pons/medulla (Zhao et al., 2001). In both of these settings, brain OLS is also increased (Huang and Leenen, 1992; Huang and Leenen, 1994a). Since brain ACE activity appears to correlate with the brain [OLS], inhibition of ouabain-sensitive NKA isoforms may lead to ACE activation. Likewise, a reduction in the baseline expression of one of these isoforms (as is the case in α2+/- mice) may lead to increased baseline ACE activity. Elevations in ACE activity may contribute to the increased production of brain ang II, enhancing sympathetic drive, and resulting in hypertension. We explored whether or not there were differences between baseline ACE activity in +/- and +/+ mice. Baseline ACE activity in both the hypothalamus and pons/medulla was the same in all groups. Combined with the OLS ELISA data, it is likely that the enhanced pressor responses in the +/- mice are due to lower expression of the α2 NKA isoform protein expression in these mice (vs. +/+),

and not due to baseline differences in downstream factors involved in cardiovascular responses to icv Na^+ infusion.

4.8. Limitations of the current studies

4.8.1. The genetic model

The greatest limitation in the studies presented here is the nature of the genetic model of the α2+/- mice. First, only a partial knockout can be achieved (i.e., heterozygous, +/-) mice, because homozygous knockout mice die shortly after birth from deficiencies in neurally mediated breathing mechanisms (Mosely et al., 2003). This limits the "dose-dependent" analysis of a gene-targeted knockout (i.e., +/+ vs. +/-, as opposed to +/+ vs. +/- vs. -/-). However, this point is somewhat moot as there was still an effect in the α2 heterozygous knockout mice, even with only small reductions in NKA α2 isoform expression.

The second limitation is that the knockout is systemic, as opposed to brain-specific. This limitation is of greater concern, as it makes it difficult to discern the degree of contribution from central vs. peripheral mechanisms in the blood pressure responses to icv Na^+. The expression of α2 is decreased in muscle tissue (including the heart) by approximately 40% in heterozygous mice (James et al., 1999). Although α2 expression in vascular smooth muscle has not been measured in +/- mice, it is presumably also decreased vs. +/+, and may have increased contractility. The pressor responses to icv Na^+ are mediated by elevations in sympathetic nervous activity, and part of that response involves the release of norepinephrine at the neuromuscular junctions of vascular smooth muscle cells in order to promote increased vascular resistance. It is also well documented that ouabain, acting on the vascular smooth muscle cells (presumably by inhibiting the α2

isoform) can sensitize the vasculature to the effects of norepineprine (Vassallo et al., 1997; Davel et al., 2000). Therefore, while it is clear the responses to icv Na^+ are centrally-mediated (see sections 4.2. of the discussion), the results of these studies cannot resolve whether the enhanced pressor responses in the +/- mice were due to enhanced central mechanisms superimposed upon hyperactive peripheral vasculature.

4.8.2. Determination of [OLS] by ELISA

One possible criticism of this assay is that [OLS] is measured using Digibind® - polyclonal fragment antibodies (F'[ab]$_2$) raised against digoxin. This antibody recognizes a wide spectrum of cardiac glycosides, including bufadienolides such as marinobufagenin (MBG). MBG has gained recent attention as a novel mediator of NKA activity (i.e. a specific inhibitor of the α1 NKA isoform) in Dahl-S rats on a high-salt diet (8% NaCl in diet) (Federova et al., 2002; Federova et al., 2004). In Dahl-S rats Federova et al. (2002, 2004) have demonstrated that high-salt diets result in increased circulating MBG levels. This was associated with a transition in myocardiocyte NKA α isoform expresseion towards greater expression of α1. Since Digibind® has a 20-fold higher affinity for ouabain than for MBG (Pullen, 2004), there may be an effect of MBG in our α2+/- mice (i.e., elevated circulating concentrations), that would be masked by the measurement of the ouabain-like substances. However, this is not likely the case as the studies in the Dahl-S rats were chronic, and Federova et al. (2002) describe that an initial transient OLS response induced by Na^+ loading in Dahl S rats precedes the MBG response (Federova et al., 2002), suggestive of a minimal MBG response in the acute studies presented here. Aside from all this, the use of Digibind® is able to prevent pressor responses when ouabain is infused icv, presumably by binding the ouabain. This indicates that Digibind® has a high affinity for

ouabain, making its use as a determinant of brain [OLS] relevant. In addition, to the best of our knowledge, there is no commercially available ouabain-specific antibody.

4.8.3. Determination of ACE activity

A limitation in the ACE activity assay is that active ACE fractions *in vivo* may not be well represented. The assay used in these studies measures ACE activity at the plasma membrane and any intracellularly active ACE. The classical model of ACE activation suggests that ACE is translocated to the plasma membrane which can then convert extracellular angiotensin I into angiotensin II. ACE can also be solubilized by cleavage of its intracellular C-terminal domain and released into the interstitium (or in the case of the brain, into the CSF) where it remains active. There is also evidence to support the concept that ACE is active intracellularly in neurons, where intracellular generation of ang II can lead to direct synaptic signaling (Bunneman et al., 1992). In our assay, cells were fully disrupted by sonication, and therefore active plasma membrane ACE, as well as any active intracellular ACE would be measured. However, using this method, we would not measure any active ACE that may have been solubilized and secreted into the CSF. It is possible therefore, that although we did not detect any changes in total ACE activity, there may have been differences in the ratio of intracellular ACE (whether active or inactive) and active plasma membrane ACE between groups. One possible method of resolving this issue would be to perform both total ACE activity assays, and ACE-binding studies (autoradiography) designed to measure plasma membrane [ACE] at the end of the infusion periods. This would provide information regarding the total expression of ACE (intracellular and that which is present on the plasma membrane) vs. the ACE that is only present at the plasma

membrane. The combined data would be useful for understanding the state of ACE activation due to icv Na^+ or ouabain infusion.

4.9. Conclusions and Future Directions

The current studies support a role for the α2 isoform of the NKA in mediating pressor responses to icv infusions of Na^+ and ouabain. Reduced expression of the α2 NKA isoform in brain cells (glia) of α2 +/- mice makes these mice more susceptible to the effects of inhibition by brain OLS (activated by icv Na^+ infusion), or direct ouabain infusion, resulting in enhanced sympathetically-mediated responses compared to α2+/+ mice. Clearly, the responses are central in origin, as iv administration of ouabain yields no change in arterial pressure or heart rate. It is possible however, that the effects of the centrally-mediated increase in sympathetic activity are compounded by increased vascular responsiveness in α2+/- mice (relative to +/+ mice), due to lower α2 NKA expression in vascular smooth muscle cells. Immediate future studies therefore, should focus on resolving the influence of these factors, and/or in determining to what degree sympathetic nervous system activity is elevated in α2 +/- vs. +/+ mice. Immediately after the infusion experiments are complete, plasma catecholamine levels could be monitored to assess the state of the sympathetic activity in the mouse. It is expected that the levels would be higher in α2+/- mice, suggestive of enhanced sympathetic nervous activity. In addition, immediately after experimentation, brains could be removed so that ACE activity and binding studies could be used to measure the degree of ACE activiation – a marker of brain RAS activity.

These studies examined the salt-sensitive responses in a murine model, deficient in α2 expression – a ouabain-sensitive isoform. It was also not possible to study the full effect of α2 isoform gene-deletion, because α2 homozygous knockout mice were not viable. As an alternative strategy, a newer mouse model has been developed, in which site-directed mutagenesis of the α2 NKA isoform has yielded a ouabain-resistant α2 isoform. It would be expected that similar experiments in these mice would show diminished/abolished pressor responses to icv Na^+ or ouabain in the α2-ouabain-resistant expressing mice. This alternate gene-targeted strategy has the advantage that there are no developmental/ontogenic challenges presented by the mutant isoforms, as they are regulated identically to the wildtype isoforms.

The α3 isoform of the NKA is another ouabain-sensitive isoform that, in the brain, is highly expressed in neurons. It is possible then that ouabain or OLS in the brain acts to inhibit the α3 isoform, thereby directly altering neural firing patterns or neuronal excitability/responsiveness. To explore the role α3 in salt-sensitive hypertension, parallel studies will be performed in an α3 knockout mouse (brain-specific), and in a mutated α3-ouabain-resistant mouse model, analogous to the studies of the α2 NKA isoform targeted mice. These new studies, should bring to light the role that each of the ouabain-sensitive isoforms in the brain of rodents plays in the development of salt-sensitive hypertension.

Chapter 5

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CONTRIBUTION OF COLLABORATORS

All experiments were performed under the supervision of Dr. James W. Van Huysse at the University of Ottawa Heart Institute. The experimental work outlined in chapter 2: Materials and Methods, was performed by Steven Theriault, with the exception of the creation of the α2 gene-targeted knockout mouse, animal surgery and measurement of cardiovascular responses, and ACE activity

The α2 gene-targeted heterozygous knockout mouse model was created and kindly provided to us by the laboratory of Dr. Jerry B Lingrel, at the University of Cincinnatti, Ohio.

The animal surgery, intracerebroventricular infusion experiments, and blood pressure and heart rate monitoring were performed by Dr. Xiaohong Hou. The ACE activity assays on prepared samples were performed by Stephanie Dean.

The statistical analysis of the blood pressure and heart rate responses to icv Na^+ and ouabain infusions were conducted in collaboration with Kathryn Williams (Biostatistician for the University of Ottawa Heart Institute).

AUTHORIZATIONS

Figure 5 was adapted and modified from: James, P.F., I.L. Grupp, G. Grupp, A.L. Woo, G.R. Askew, M.L. Croyle, R.A. Walsh, and J.B Lingrel. 1999. Identification of a specific role for the Na, K-ATPase alpha-2 isoform as a regulator of calcium in the heart. Molecular Cell. 3:555-63. (figure 1D of manuscript). Permission granted from Blackwell Publishing.

Figure 6 was reprinted and adapted from: Glowinski, J., and L.L. Iversen. 1966. Regional studies of catecholamines in the rat brain-I: The disposition of [^{3}H]norepinephrine, [^{3}H]dopamine and [^{3}H]dopa in various regions of the brain. J. Neurochem. 13:655-69. (Figure 1 of manuscript). Permission granted from Elsevier.

- Acquired strong communication skills by presenting scientific data at the Ontario Hypertension Society (May 2003) and Canadian Hypertension Society (Oct 2003) meetings; as well as in a graduate student seminar series
- Acquired critical review skills by attending and presenting scientific journals at a weekly journal club meeting
- Acquired critical review skills and literature review experience through writing a mock grant proposal for a an advanced biochemistry class
- Have trained laboratory technicians and B.Sc. Honours students

May 2001 – Sept. 2001 University of Ottawa Heart Institute Ottawa, ON

LABORATORY TECHNICIAN

- Primary duties included various PCR, subcloning and sequencing experiments
- Assisted in the organization and submission of operating grant proposals to the Canadian Institute of Health Research (CIHR) and Heart and Stroke Foundation of Ontario (HSFO)

Sept. 2000 – Apr. 2001 University of Ottawa Heart Institute Ottawa, ON

B.SC. STUDENT TRAINEE

- Training involved familiarization with various molecular biology techniques, including:
  - DNA/RNA isolations (from bacteria)
  - Bacterial culture and sub-cloning techniques
  - PCR
  - DNA Sequencing
  - Restriction enzyme digest analysis

Academic Awards

2003 CANADIAN HYPERTENSION SOCIETY (ONTARIO CHAPTER) MEETING
Second place in Master's level poster presentation

Publications

Dostanic I, Paul RJ, Lorenz JN, Theriault S, Van Huysse JW, Lingrel JB. The {alpha}2 isoform of Na,K-ATPase mediates ouabain-induced hypertension in mice and increased vascular contractility in vitro. Am J Physiol Heart Circ Physiol. 288:H477-85, 2005.

Under Revision: submitted to AJP: Regulatory, Integrative and Comparative Physiology: Theriault SF, Hou X, Moseley A, Dostanic I, Lingrel JB, Dean SA, Van Huysse JW. Enhanced pressor response to increased CSF sodium concentration in heterozygous {alpha}2 Na, K-ATPase knockout mice.

Volunteer Activities

2002 – 2004 Ottawa General Hospital Ottawa, ON

EMERGENCY ROOM VOLUNTEER

Have volunteered approximately 250 hours

2001 – 2004 University of Ottawa Ottawa, ON

LET'S TALK SCIENCE PROGRAM VOLUNTEER

In this program, graduate students bring science from various fields into classrooms of all grades in interesting and unique ways

Duties have included:

- Performing scientific demonstrations related to the biological sciences to students at all levels
- Coordinating scientific demonstrations with schools through teacher liaisons to the Let's Talk Science program