ROLE OF THE MEDIAN PREOPTIC NUCLEUS IN CHRONIC BLOOD PRESSURE REGULATION BY ANGIOTENSIN II

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The median preoptic nucleus (MnPO) receives dense reciprocal inputs from both the subfornical organ (SFO) and organum vasculosum of the lamina terminalis (OVLT), the circumventricular organs known to be important as central neural sensors of circulating angiotensin II (ANG II). This thesis proposes to establish the role of the MnPO in chronic regulation of blood pressure based on the central hypothesis that the MnPO is a crucial component of the central sympathoexcitatory circuitry necessary for chronic blood pressure control following ANG II activation of the SFO and OVLT. Throughout the studies, cardiovascular responses to either pharmacological or physiological changes of circulating ANG II activity were compared between MnPO lesioned rats and sham lesioned controls.

The first specific aim was designed to test the hypothesis that the intact MnPO is necessary for the full hypertensive response to chronic intravenous ANG II administration. In this specific aim, rats with electrolytic lesion of the MnPO displayed significantly attenuated hypertensive responses by day 7 through day 10 of ANG II infusion compared to sham lesioned rats. Therefore, we concluded that the MnPO is a crucial component of the central neuronal circuitry mediating chronic ANG II-induced hypertension.

Sub-aim 1A was designed to determine the specific role of the MnPO neurons versus fibers of passage in the attenuated hypertensive responses to ANG II observed in the MnPO lesioned rats. In line with the findings of specific aim 1, rats with ibotenic acid lesion of the MnPO demonstrated attenuated responses to the hypertensive effect of
chronic ANG II administration. However, the attenuated responses were less extensive relative to those seen in the electrolytic lesioned rats. Therefore, it was concluded that neuronal cell bodies in the MnPO are necessary for the full hypertensive response to chronic ANG II administration; however, damage of the fibers of passage partly contributes to the attenuated hypertensive responses observed in the electrolytic lesioned rats.

The second specific aim was to determine the role of the MnPO in mediating the chronic hypotensive effect of the AT$_1$ receptor antagonist, losartan. In this specific aim, rats with ibotenic acid lesion of the MnPO showed exaggerated hypotensive responses to chronic losartan administration. These findings were accompanied with a greater decline in total peripheral resistance in the MnPO lesioned rats. Therefore, we concluded that MnPO neurons do not mediate the chronic hypotensive effect of losartan and that the MnPO is not necessary for basal blood pressure control by endogenous ANG II. However, the findings suggested that the MnPO neurons likely participate in baroreflex mechanisms buffering against losartan-induced hypotension.

The last specific aim was to establish the role of the MnPO in normal blood pressure control during chronic high dietary salt intake. Although plasma sodium concentration and osmolality were raised significantly in rats with electrolytic lesion of the MnPO during high salt intake, their mean arterial pressure and heart rate were comparable with those of sham lesioned rats throughout the study. Therefore, we concluded that the MnPO is not necessary to maintain normal blood pressure during high dietary salt intake. However, MnPO lesioned rats displayed less renal sodium retention during high salt intake compared to sham lesioned rats suggesting the role of the MnPO
in the central neurohumoral control of sympathetic outflow, in particular, renal sympathetic activity, during chronic high salt intake.

In conclusion, overall, the findings in this dissertation provide important insights into the role of the MnPO in the chronic hypertension induced by ANG II. Furthermore, they provide additional evidence of the integrative role of the MnPO in chronic normal blood pressure control by circulating ANG II, plasma osmolality, and the baroreflex.
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CHAPTER I

Introduction and Review of The Literature
Introduction

Hypertension is a multi-cultural public health crisis that afflicts as many as one out of four adults of the world’s population (Kearney et al., 2005). However, despite extensive research for over several decades, the etiology and pathogenesis of many forms of hypertension remain largely unknown. Uncontrolled hypertension exposes people to serious risk of renal diseases, dementia, blindness and several cardiovascular diseases including heart failure and stroke. Therefore, effective management of hypertension is essential to decrease those risks, which requires a complete understanding of the physiology of normal blood pressure control, as well as the pathophysiology of hypertension.

Since adequate blood pressure is crucial to maintain blood perfusion to the vital organs and tissues, complex living organisms such as human beings regulate blood pressure through highly complex interconnected systems with a great degree of redundancy to maintain survival. How these complex systems function and interconnect, especially during the chronic state is not completely understood and remains an active ongoing area of research. Several organ systems, such as the renal, vascular, heart, and brain, as well as multiple neurohumoral factors are known to be critically involved in normal blood pressure regulation. Among the neurohumoral factors, angiotensin II (ANG II), a biologically highly active hormone of the renin angiotensin system (RAS), has been shown to be crucial for normal blood pressure control and is implicated in the pathogenesis of many forms of hypertension.
A well-established reverse relationship between circulating ANG II and blood volume, arterial pressure, and plasma osmolality is crucial for normal homeostatic control of blood pressure (Duggan and Ye, 1998; Hodge et al., 1966a,b; Keeton and Campbell, 1980). When plasma ANG II concentration does not decrease appropriately, such as when exogenous ANG II is administered in concert with high salt intake, hypertension is indisputably generated (Bruner et al., 1985; Osborn et al., 2003). Chronic over-activation or inappropriately high activity of RAS has been implicated in the pathogenesis of essential hypertension, a hypertension with an unknown cause, as well as many forms of secondary hypertension, including those associated with the metabolic syndrome, obesity, diabetes mellitus, heart failure, and renal diseases (Boustany et al., 2005; Grassi, 2006; Grassi et al., 1997; Hackam et al., 2007; Prasad and Quyyumi, 2004; Romero and Reckelhoff, 1999). Although ANG II exerts its hypertensive actions through several mechanisms, substantial evidence suggests that a sustained over-activation of the RAS maintains high blood pressure in part by facilitating sympathetic outflow of the brain through central neuronal mechanisms (Brooks and Osborn, 1995; Cerasola et al., 1987; Dampney et al., 2002; Dowell et al. 1996; Fink, 1997; Heesch et al., 1996; Matsukawa et al., 1991; Reid, 1992; Saxena, 1992; Tobey et al., 1983; Weekley, 1991).

The subfornical organ (SFO) and organum vasculosum of the lamina terminalis (OVLT) are two circumventricular organs (CVOs) considered to be important as primary central targets of circulating ANG II. (Buggy et al., 1978a; Haywood et al., 1983; Hendel and Collister, 2005; Mangiapane and Simpson, 1980; Marson et al., 1983; McKinley et al., 1992, 1998; Oldfield et al., 1994). The median preoptic nucleus (MnPO) is connected anatomically and functionally with the SFO and OVLT (Ciriello and Gutman, 1991;
Gutman et al., 1986; Lind et al., 1982; Miselis, 1981; Saper and Levisohn, 1983). In addition, this nucleus has been implicated in several central effects of circulating ANG II, including drinking behavior, vasopressin secretion, and acute blood pressure effects (Cunningham et al., 1991; 1992; Gardiner et al., 1985; Gutman et al., 1989; Jones, 1988; Lind and Johnson, 1982; Lind et al., 1983; Mangiapane et al., 1983; Miselis, 1982; O’Neill and Brody, 1987). This dissertation proposes that neuronal mechanisms in the MnPO are crucial for chronic blood pressure control by ANG II.

**Literature review**

**A. Renin angiotensin system and arterial pressure regulation**

1. *Formation of angiotensin II*

   The renin angiotensin system (RAS) plays a pivotal role in long-term blood pressure regulation. When arterial pressure falls below a certain threshold (65-70 mmHg in dog), renin is released from the juxtaglomerular (JG) cells of the kidney (Ehmke et al., 1987; Farhi et al., 1987; Kirchheim et al., 1985). Renin cleaves its substrate, angiotensinogen, to release the decapeptide angiotensin I, which is then cleaved by angiotensin converting enzyme, resulting in increased production of the octapeptide hormone, angiotensin II (ANG II), in the circulation.

2. *Effects of angiotensin II on arterial pressure*

   Circulating ANG II exerts its hypertensive action via multiple mechanisms, including vasoconstriction (Brown et al., 1981), enhanced sodium and water reabsorption
from the proximal renal tubules (Hall, 1986; Krieger and Cowley, 1990), increased aldososterone production from the zona glomerulosa cells of the adrenal glands (Fredlund et al., 1975; Hajnoczky et al., 1992), cardiac and vascular smooth muscle hypertrophy and remodeling (Dostal and Baker, 1992; Geisterfer et al., 1988; Griffin et al., 1991), baroreflex blunting (Campagnole-santos et al., 1988, 1992; Garner et al., 1987; Guo and abboud, 1984), and central and peripheral sympathoexcitation (Brooks and Osborn, 1995; Fink, 1997; Qiu et al., 1994; Reid, 1992; Soltis et al., 1993). Among these mechanisms, it has been well established that the sympathetic nervous excitation via central neuronal mechanism(s) in the brain is a prominent mediator of chronic blood pressure regulation by ANG II (Brooks and Osborn, 1995; Dampney et al., 2002; Osborn, 1997; Reid, 1992; Saxena, 1992).

3. Angiotensin II receptors and its signal transduction pathways

Actions of ANG II are mediated through 2 subtypes of G-protein-coupled angiotensin receptors; the angiotensin subtype I (AT$_1$) and subtype II (AT$_2$) receptors. Most of the classic effects of ANG II on arterial pressure are believed to be mediated solely by the AT$_1$ receptor, and are blocked by AT$_1$ receptor antagonists, such as losartan (Hajnoczky et al., 1992; Kaschina and Unger, 2003).

Binding of ANG II to AT$_1$ receptor initiates multiple complex intracellular signaling pathways. Acute effects of ANG II, such as vasoconstriction and aldososterone release from adrenal gland are mediated by phospholipase C (PLC)-dependent intracellular Ca$^{2+}$ release (Kaschina and Unger, 2003). Binding of ANG II to AT$_1$ receptors causes dissociation of G protein, which then activates PLC to cleave phosphatidylinositol to form inositol triphosphate (IP$_3$) and diacylglycerol (DAG). IP$_3$
induces Ca\textsuperscript{2+} release from intracellular storage sites. DAG and Ca\textsuperscript{2+} stimulate protein kinase C (PKC) leading to phosphorylation of its target proteins resulting in vascular contraction and aldosterone release. ANG II also activates phospholipase A\textsubscript{2} and phospholipase D, which have been shown to be involved in activation of DAG production, and redox-signaling pathway, respectively.

Long-term effects of ANG II, such as vascular and cardiac cell hypertrophy and proliferation, involve gene transcription and protein synthesis that are mediated by multiple protein kinase cascades, including mitogen-activated protein kinase (MAPK) and janus cytosolic protein kinase/signal transducers and activators of transcription (JAK/STAT) pathway. Activation of the JAK/STAT, and MAPK dependent pathways, as well as other protein kinase signaling pathways leads to expressions of various growth related immediate early gene, such as c-fos, c-jun, c-myc, and egr-1, which then results in increased production of multiple growth factors.

Another signaling pathway known to be important in mediating cardiovascular effects of ANG II through AT\textsubscript{1} receptors is a redox-signaling pathway. ANG II stimulates NAD(P)H oxidase, a membrane bound enzyme complex, to generate reactive oxygen species (ROS), such as superoxide. ROS inactivates nitric oxide, an endothelial derived vasodilator and smooth muscle relaxant, that has also been shown to exert inhibitory effects on central sympathetic output and blood pressure (Tseng et al., 1996). Therefore, this effect of ANG II to decrease bioavailability of nitric oxide is thought to be important in central sympathetic excitation and hypertensive effects of ANG II. In fact, it has been shown that superoxide dismutase (SOD), the enzyme that catalyzes a degradation of ROS, injected into the central lateral ventricle at the same time as ANG II can block
sympathetic excitation seen when ANG II alone is injected into the lateral ventricle (Campese et al., 2005). In addition, adenoviral-mediated delivery of cytoplasmic SOD infused intracerebroventricularly (ICV) can block hypertension induced in mice by systemic infusion of chronic sub-pressor dose of ANG II (Zimmerman et al., 2004).

B. Effects of angiotensin II on the nervous system

1. Angiotensin II influencing baroreflex function

Evidence suggests that ANG II has an effect to suppress baroreflex responses of heart rate and sympathetic activity (Campagnole-santos et al., 1992; Garner et al., 1987; Guo and Abboud, 1984). Garner et al. (1987) has shown that physiological suppression of endogenous ANG II induced by high salt intake causes an increase sensitivity of the baroreflex response of heart rate. On the other hand, an elevation of endogenous ANG II induced by low salt intake decreases sensitivity of baroreceptor-heart rate reflex. The effect of exogenous ANG II on the baroreflex sensitivity seems to be selective on the baroreflex response to an increase of arterial pressure. Guo and Abboud (1984) have shown that the baroreflex-mediated decrease in lumbar sympathetic nerve activity (LSNA) and heart rate in response to hypertension is impaired by ANG II infusion, while baroreflex mediated increased LSNA and heart rate in response to hypotension are not affected. This study is supported by reports showing that IV injection of ANG II blunts baroreflex responses of heart rate to increased blood pressure induced by phenylephrine injection but not to the decreased blood pressure induced by sodium nitropusside (Campagnole-santos et al., 1992). This attenuated effect of ANG II on reflex bradycardia has been shown to be mediated in part by ANG II actions at the central nervous system.
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(Campagnole-santos et al., 1992, 1988; Guo and Abbound, 1984; Hayashi et al., 1988; Matsumura et al., 1989). For example, Matsumura et al. (1989) has reported that injection of ANG II into the vertebral artery that supplies blood to the brain produces greater attenuation of baroreflex-induced bradycardia in response to phenylephrine infusion than intravenous injection.

2. Angiotensin II influencing sympathetic nervous activity

2.1 Sympathetic nervous system and blood pressure regulation

The sympathetic nervous system controls blood pressure mainly through adrenergic innervations in the vasculature, heart, and kidney. Sympathetic motor neurons originate from the thoracolumbar region of the spinal cord and send fibers to synapse on postganglionic neurons located within the sympathetic chain ganglia or collateral ganglia outside of the sympathetic trunk. Postganglionic neurons then project out of the ganglia to innervate various target organs and the vasculature. Adrenergic nerve terminals in the vasculature and the kidneys have been shown to be tonically active (Bencsath et al., 1982; van Brummelen et al., 1985; D’Oyley and Pang, 1990; Khan et al., 1988; Rogenes and Gottschalk, 1982). The firing rates and neurotransmitter released from the adrenergic nerve terminals can be either positively or negatively controlled by sympathetic pre-motor neurons in the rostral ventrolateral medulla (RVLM) that have efferent projections to sympathetic motor neurons in the spinal cord.

2.1.1. Sympathetic neural influences on kidney function

Strong renal sympathetic nerve stimulation acts via $\alpha$-adrenergic receptors to constrict the renal afferent and efferent arterioles, and decrease renal blood flow and glomerular filtration rates (Guyton and Hall, 2001). Low level stimulation of renal
sympathetic nerve increases sodium reabsorption in the proximal tubule and ascending loop of Henle without effecting renal blood flow and glomerular filtration rates (Dibona, 1977). In addition, activation of sympathetic nervous system via β-adrenergic receptors stimulates the release of renin from JG cells resulting in increased circulating ANG II formation. It has been shown that chronic renal adrenergic stimulation by infusing norepinephrine directly into the renal artery is able to induce hypertension that is likely mediated by sympathetic activation-induced shifting of pressure natriuresis to a higher pressure (Cowley and Lohmeier, 1979; Katholi et al., 1977). This hypertensive effect of norepinephrine infused directly into renal artery has been shown to be dependent on increased renin secretion and ANG II formation secondary to renal sympathetic activation (Reinhart et al., 1995).

2.1.2. Sympathetic neural influences on the vasculature

Norepinephrine released from sympathetic nerve terminals bind and excite α-adrenergic receptors, with less affinity to β-adrenergic receptors on the vasculature. Vasoconstrictive effects of the sympathetic nervous system are mediated by binding of norepinephrine to α-adrenergic receptors on vascular smooth muscle cells. The sympathetic nervous system contributes significantly to basal vascular tone and blood pressure as either sympathetic ganglionic or α-adrenergic receptors blockade lowers blood pressure in both experimental animals and humans (Borucki et al., 1978; D’Oyley and Pang, 1990; Zimmerman et al, 1984). Activation of the sympathetic nervous system causes both arterial and venous vasoconstriction, resulting in increased total peripheral resistance and decreased vascular capacitance, respectively. Both of these effects of ANG II lead to increased arterial pressure (Berne et al., 2004; Hottenstein and Kreulen, 1987).
2.1.3. Sympathetic neural influences on cardiac function

The sympathetic nervous system exerts both positive chronotropic and inotropic effects on the cardiac myocardium (Furnival et al., 1973). Norepinephrine released from sympathetic nerve terminals excites $\beta$-adrenergic receptors on the sino-atrial node, leading to an increase in intracellular cyclic adenosine monophosphate, resulting in an increased heart rate (Mohrman and Heller, 2003). In addition, norepinephrine interacts with $\beta$-adrenergic receptors on cardiac muscle cells, resulting in increased myocardium contractility.

2.2. Facilitating effects of circulating angiotensin II on sympathetic nervous system

Alterations of blood volume, arterial pressure, and plasma osmolality and sodium concentration cause inverse changes to circulating ANG II concentration (Duggan and Ye, 1998; Hodge et al., 1966a,b; Keeton and Campbell, 1980). Circulating ANG II has been shown to influence sympathetic activity through both peripheral and central mechanisms.

2.2.1. Peripheral effects of circulating angiotensin II on sympathetic activity

ANG II facilitates sympathetic activation at the nerve terminal through both presynaptic and postsynaptic sites (Reid, 1992). At the presynaptic nerve terminals, ANG II both enhances the release and inhibits the reuptake of noradrenaline (Khairallah et al., 1971; Majewski et al., 1984; Peach et al., 1969). At the postsynaptic site, several studies have shown an increased vasoconstrictor response to adrenergic receptor stimulation by ANG II (Dowell et al., 1996; Purdy and Weber, 1988; Reams and Bauer, 1987). Dowell et al. (1996) has demonstrated a significant potentiation of alpha-adrenergic contractile
responses of the vasculature to phenylephrine in animals that received chronic ANG II infusion for 21 days. In addition, blockade of RAS has been shown to impair tonic sympatho-adrenergic vasoconstriction in pithed (Kaufman and Vollmer, 1985) and spontaneous hypertensive rats (Moreau et al., 1993), as well as normotensive rats (Qiu et al., 1994; Soltis et al., 1993).

2.2.2. Central effects of circulating angiotensin II on sympathetic output

Substantial evidence supports the idea that variations of circulating ANG II concentration have a modulating effect on central sympathetic output (Cerasola et al., 1987; Ferrario et al., 1972; Matsukawa et al., 1991; Schmitt and Schmitt, 1968; Tobey et al., 1983; Weekley, 1991). Although a high dose of ANG II is required to produce an immediate increase of blood pressure via direct vasoconstrictive effects, low doses of ANG II can cause profound hypertension when administered chronically (in a period of days) (Ames et al., 1965; Brown et al., 1981; Dickinson et al., 1963; McCubbin et al., 1965). In fact, ANG II infusion at a low dose that produces a nearly normal plasma concentration of ANG II for 7 days causes blood pressure to increase to approximately the same level as that caused by an acute vasoconstrictive dose of ANG II (Brown et al., 1981). This finding is important since most essential hypertensive patients have plasma ANG II levels comparable or lower than those of normotensive subjects; however, their blood pressure can be markedly reduced by angiotensin converting enzyme inhibitors or AT1 receptor blockers (Brunner et al., 1979; Laragh and Brenner, 1989), suggesting an important role of RAS in maintaining high blood pressure in these essential hypertensive patients. In addition, these findings suggest the relevance of animal models of chronic
low-dose ANG II induced hypertension in the search for pathogenesis of essential hypertension.

The chronic hypertensive effect of low-dose ANG II is believed to be mediated by increased sympathetic nervous activity through central neuronal mechanisms (Csiky and Simon, 1997; Bruner and Fink, 1986; Ferrario et al., 1972; Gorbea-oppliger and Fink, 1994). This idea is supported by numerous studies. Among these are studies showing that different patterns of c-fos expression are associated with acute and chronic ANG II administration (Li et al., 1998). In fact, the rostral ventrolateral medulla (RVLM), the brain region in the medulla believed to house sympathetic premotor neurons, has shown to express c-fos only after at least 18-hr of ANG II infusion. In addition, rats infused with ANG II for 14 days demonstrated increased basal sympathetic nerve activity (Luft et al., 1989). Neonatal sympathectomy prevented increased blood pressure response to chronic infusion of ANG II in rats (Csiky and Simon, 1997). Hypertensive rats induced by a chronic sub-pressor dose of ANG II had greater depressor responses either to clonidine, a central acting sympatholytic agent (Gorbea-oppliger and Fink, 1994), or hexamethonium, a ganglionic blocker (Kline et al., 1990), than did control normotensive rats. Additionally, rats that received chronic intracerebroventricular infusion of ANG II showed greater depressor responses to sympathetic α- and β- adrenergic blockade than did normotensive rats (Bruner and Fink, 1986). In ANG II-treated rabbits, depressor responses to sympathetic ganglionic blockade with pentolinium become increasingly greater than those of saline-treated controls starting after a week of ANG II infusion (Burke et al., 2008). It should be noted although that evidence that argues against sympathetic excitatory effects of ANG II has also been presented (Hood et al., 2007;
King and Fink, 2006; Pelaez et al., 2003). For example, Hood et al. (2007) and King and Fink (2006) have shown no increased response to hexamethonium in sheep and rats chronically infused with ANG II, respectively.

C. Circumventricular organs as central sensors of circulating angiotensin II

ANG II is an octapeptide hormone that does not readily cross the blood brain barrier (BBB). Accordingly, this humoral signal gains access to the brain through specialized brain regions that lack the BBB and have a rich vascular supply called circumventricular organs (CVOs) (Fink, 1997; McKinley et al., 1998). The subfornical organ (SFO), organum vasculosum of the lamina terminalis (OVLT), and area postrema (AP) are three CVOs believed to be particularly important as the primary central sites that mediate cardiovascular effects of ANG II and plasma hyperosmolality (Ferrario et al., 1987; Fink et al., 1987; Collister and Hendel, 2003; Hendel and Collister, 2005; Oldfield et al., 1994). Among these CVOs, the SFO and OVLT are located in the lamina terminalis along the rostral border of the third ventricle. It has been shown that the SFO and OVLT are rich in AT$_1$-receptors (Bunnemann et al., 1992; Giles et al., 2001; Phillips et al., 1993), and intravenous ANG II administration induces c-fos expression in these CVOs (McKinley et al., 1992; Oldfield et al., 1994). Complete lesions of the SFO block blood pressure responses to a single acute dose of ANG II (Mangiapane and Simpson, 1980). Recently, it has been shown that an intact SFO is critical for both the chronic hypertensive response to exogenous ANG II infusion and normal blood pressure control by endogenous ANG II (Collister and Hendel, 2003; Hendel and Collister, 2005). In addition, lesions of the area called AV3V, the region surrounding the anteroventral
portion of the third cerebral ventricle, which includes the OVLT and neuronal pathways from OVLT and SFO, attenuate hypertension in a number of rat models including a renin-angiotensin dependent form of hypertension (Brody et al., 1978). Accordingly, the SFO and OVLT are important as sensors of ANG II in long-term blood pressure regulation. However, how this sensory information is processed downstream within the brain and finally influences the level of central sympathetic outflow is not fully understood.

D. Median preoptic nucleus and sympathetic excitatory effects of angiotensin II

1. Anatomical and functional connections between the MnPO and SFO and OVLT

In addition to the SFO and OVLT, parenteral administration of ANG II activates neurons in several other forebrain regions. These include the median preoptic nucleus (MnPO), magnocellular and parvocellular subdivisions of the paraventricular nucleus (PVN), supraoptic nucleus (SON), bed nucleus of stria terminalis, and suprachiasmatic nucleus (McKinley et al., 1992; Oldfield et al., 1994; Potts et al., 1999; Rowland et al., 1994). Lesion of the SFO and/or the AV3V blocks expression of c-fos in response to ANG II and hypertonic saline infusion (Hochstenbach and Ciriello, 1996; Rowland et al., 1994) in these brain regions. Consequently, these brain areas are believed to be involved in central sympathoexcitatory pathways following ANG II activation of the SFO and OVLT and therefore are thought to form downstream pathways in which signals from the SFO and OVLT are further processed. Of these brain regions, the MnPO, a nuclear group located in the lamina terminalis, right in the middle between the SFO and OVLT, has been shown to be intimately connected to the SFO and OVLT both anatomically and
functionally (Ciriello and Gutman, 1991; Gutman et al., 1986; Lind et al., 1982, 1983; Miselis, 1981; Saper and Levisohn, 1983). The MnPO receives dense reciprocal inputs from the SFO and OVLT (Miselis, 1981; Saper and Levisohn, 1983), and neurons located in this nucleus increase their metabolic activity and neuronal discharge when the SFO is either electrically stimulated or microiontophoretically applied with ANG II (Ciriello and Gutman, 1991; Tanaka et al. 1986, 1987). These excitatory responses can be blocked by applying the AT$_1$ receptor blocker, saralasin, into the MnPO (Tanaka et al, 1986). Additionally, there have been studies showing that disconnection of the SFO and MnPO disturbs drinking and pressor responses to intravenous ANG II (Miselis, 1982; Lind et al., 1983).

2. Connection between the MnPO and central and peripheral sympathetic nervous system

MnPO neurons are capable of governing the peripheral sympathetic nervous system (Stocker and Toney, 2005; Westerhaus and Loewy, 1999; Yasuda et al., 2000). A viral transneuronal labeling study has shown multi-synaptic connections between MnPO neurons and the peripheral sympathetic nervous system (Westerhaus and Loewy, 1999). Functionally, studies have shown a significant temporal correlation between spontaneous discharge of MnPO neurons and the cardiac cycle and renal sympathetic nerve activity (Stocker and Toney, 2005). In addition, electrical stimulation of the MnPO increases blood pressure and renal sympathetic nerve activity (Yasuda et al., 2000). Neuroanatomical studies have also revealed that neurons in the MnPO project to and receive inputs from other brain regions shown to contribute to the central neural pathway mediating ANG II-induced sympathoexcitation, including the parvocellular subdivision of the PVN and ventrolateral medulla (Saper and Levisohn, 1983; Sawchenko and
Swanson, 1983; Zardetto-Smith et al., 1993). ANG II microiontophoretically applied into the SFO excited MnPO neurons identified as projecting to the PVN and these responses were blocked by infusing salarasin, an ANG II type 1 receptor antagonist, into the MnPO (Tanaka et al. 1986, 1987). Intravenous ANG II activates neurons located widespread in the MnPO including neurons with axonal projections to the PVN (Stocker and Toney, 2005). In addition, lesions of the MnPO suppress ANG II induced c-fos expression in the paraventricular and supraoptic nuclei of the hypothalamus, but not the SFO, supporting the view that the MnPO is a downstream site from the SFO (Xu and Herbert, 1995). Taken together, the MnPO is therefore a probable site where neuronal signals generated by action of ANG II at the SFO and OVLT are either processed or relayed before being delivered to other downstream elements of this neuronal circuitry.

Neuroanatomical studies have shown that efferent fibers originating from the SFO and OVLT either synapse directly onto the supraoptic and paraventricular nucleus of the hypothalamus, or indirectly by first synapsing on MnPO neurons and in turn reach these hypothalamic nuclei (Miselis, 1981; Westerhaus and Loewy, 1999). Subsequently, neural fibers from the PVN project directly to both the sympathetic premotor neurons in the rostral ventrolateral medulla (RVLM) and sympathetic preganglionic neurons in the intermediolateral cell column of the spinal cord. Fig. 1 shows the proposed sympathetic excitation pathway modified from Westerhaus and Loewy (1999). Neuroanatomical connections of the PVN with the CVOs and sympathetic motor control centers in the medulla and spinal cord implicate the PVN as a key component of this neuronal circuitry. Additional evidence supporting the role of this direct pathway from SFO and OVLT to PVN in mediating the central effects of ANG II comes from several lines of observations.
SFO neurons with axonal projection to PVN increase their activity in response to intravenous ANG II infusion (Tanaka et al., 1985a,b). Direct electrical stimulation of the PVN neurons increases renal sympathetic nerve activity and blood pressure in conscious rats (Kannan et al. 1989). Furthermore, lesions of the PVN reduce blood pressure response to electrical stimulation at the SFO (Ferguson and Renuad, 1984), and disrupt drinking and blood pressure responses to ANG II injected directly into the SFO (Gutman et al. 1988). Taken together, it is well established that the direct pathways from the SFO and OVLT to PVN are crucial for the modulating effects of ANG II on central sympathetic output. However, whether the alternate pathway encompassing the MnPO has a physiological significance in central sympathetic excitation and chronic blood pressure regulation by ANG II awaits further investigation.

3. The MnPO as a potential integration site of neurohumoral signals

In addition to ANG II, numerous electrophysiological and Fos immunocytochemistry studies have shown that the MnPO neurons are also responsive to plasma hyperosmolality and/or hypernatremia induced by parenteral hypertonic saline infusion (Sly et al., 2001; Xu and Herbert, 1995). Lesions of the SFO and AV3V decrease c-fos expression in the MnPO, PVN, and SON (Hochstenbach and Ciriello, 1996). Accordingly, the MnPO is likely a downstream pathway from plasma hyperosmolality and/or hypernatremia sensed at the SFO and OVLT. Importantly, a subset of MnPO neurons is responsive to intravascular infusion of both ANG II and hypertonic saline (Stocker and Toney, 2005; McKinley et al., 1992; Oldfield et al., 1994; Potts et al., 1999; Rowland et al., 1994). Activity of ANG II and osmo-sensitive MnPO neurons are also affected by direct stimulation of peripheral baroreflex afferents, as well
as baroreflex challenges such as hemorrhage (Stocker and Toney, 2007; Tanaka et al., 1993, 2003). Studies have shown reciprocal connections between the MnPO and the nucleus of solitary tract and ventrolateral medulla, baroreflex centers in the hindbrain known to receive baroreceptor inputs from the periphery (Ciriello et al., 1985; Gieroba and Blessing, 1993; Li et al., 1992; Saper and Levesohn, 1983; Tanaka et al., 1992b). In addition, ample evidence suggests that MnPO neurons have a potential to cross communicate with both central and peripheral baroreflex arcs. (Sakamaki et al., 2004; Tanaka et al., 1992a, 1993, 2003). Taken together, these findings suggest that MnPO neurons not only function to relay sensory signals regarding circulating ANG II level and plasma osmolality from the SFO and OVLT, but processing and integrating them with signals received from other brain regions also involved in cardiovascular regulation, such as those participating in baroreceptor pathway(s). Additionally, findings that a wide variety of neurons and receptors, including glutamatergic, GABAergic, angiotensinergic, and cholinergic neurons and/or receptors coexist within the MnPO further indicate that the MnPO has a potential to produce either excitatory or inhibitory responses, depending on the type and source of signals received and processed at the same period of time (Bai and Renaud, 1998; Grob et al., 2003). This role of the MnPO as an integrative site for central autonomic responses induced by various stimuli awaits further investigation and the physiological significance of this remains to be established.
E. Functional evidence implicates the MnPO in neuronal pathway mediating central effects of ANG II

ANG II acts via central neuronal mechanisms to stimulate thirst and neurohypophyseal secretion of vasopressin and oxytocin, as well as increase blood pressure by stimulating sympathetic outflow and blunting baroreflex sensitivity (Buggy et al., 1978b; Guo and Abbound, 1984; Hsiao et al., 1977; Matsumura et al., 1989; Reid, 1992; Saxena, 1992). The MnPO has been shown to play an important role in mediating various central effects of ANG II that are believed to be initiated by the actions of ANG II at AT\textsubscript{1} receptors in the SFO and OVLT. Over several decades, a number of studies have shown that either electrolytic or excitotoxin lesions of the MnPO attenuate drinking, and vasopressin and oxytocin secretion in response to ANG II administration (Cunningham et al., 1991, 1992; Gardiner et al., 1985; Gutman et al., 1989; Jones, 1988; Lind and Johnson, 1982; Mangiapane et al., 1983). Few studies have assessed the role of MnPO in central sympathetic excitation and hypertensive effects of ANG II (Gutman et al., 1989, Jones, 1988, Fink et al., 1986, O’Neill and Brody, 1987). However, the results have been controversial. Most of these studies were conducted under an acute experimental setting in which pressure responses to single doses of ANG II were examined in MnPO lesioned rats. Lind et al. (1983) and O’Neill and Brody (1987) reported that knife cuts of the SFO and MnPO connection, as well as electrolytic ablation of both the dorsal and ventral part of the MnPO attenuated blood pressure responses to a low-dose intravenous infusion of ANG II. On the other hand, Jones (1988) and Gutman et al. (1989) showed contrary results in which lesions of the MnPO did not significantly alter the pressor responses induced by ICV and intravenous injections of ANG II. The
discrepancy of these previous reports could be explained by the difference in the extent and method of MnPO lesions, as well as dosage and duration of ANG II administration.

The role of the MnPO on the chronic hypertensive effect of ANG II was investigated in the past by Fink et al. (1986), in which the MAP response to a 5-day infusion of ANG II was shown to be not affected by MnPO lesion. However, our previous studies determining the role of SFO on blood pressure response to a chronic 10-day infusion of ANG II or the AT\textsubscript{1} antagonist, losartan, suggested that a longer period of drug infusions are required to detect effects of the lesion (Collister and Hendel, 2003; Hendel and Collister, 2005). In fact, attenuated blood pressure responses to ANG II-induced hypertension and losartan-induced hypotension in the SFO lesioned rats were not observed until day 5 and day 4 of the ANG II and losartan infusion, respectively. These attenuations of blood pressure changes occurred when it began to reach a steady state suggesting a period when blood pressure, presumably through the central sympathetic output, was fully impacted by ANG II. Therefore, a more prolonged infusion protocol of ANG II than previously performed would appear to be necessary to detect MnPO lesion effects on ANG II-induced hypertension.

**Scope and objective of thesis**

**Rational:** Substantial evidence suggests that central sympathetic output is adjusted appropriately based upon circulating ANG II concentrations. Although it is well known that peripheral ANG II accesses the brain through particular CVOs, most importantly the SFO and OVLT, the central neuronal pathway(s) that mediates sympathetic excitation
downstream from these CVOs has not been well elucidated. The MnPO receives reciprocal inputs from both the SFO and OVLT and other brain regions believed to form the sympathoexcitatory pathway following activation by ANG II at the SFO and OVLT. Although ANG II is believed to chronically control blood pressure partly by interacting with its receptor at the CVOs, whether the MnPO is necessary in mediating long-term blood pressure control is unclear.

**Central hypothesis:** This dissertation investigates the role of MnPO in chronic regulation of blood pressure by ANG II based on the central hypothesis that the MnPO is a crucial component of the central sympathetic excitation pathway necessary for blood pressure control following ANG II activation at the SFO and OVLT. The schematic diagram illustrating the central hypothesis is shown in Fig. 2.

**Specific Aim 1: What is the role of MnPO in chronic ANG II- induced hypertension?**

*The hypothesis of this study is that an intact MnPO is necessary for the full hypertensive response to chronic intravenous administration of ANG II.* The schematic diagram illustrating the hypothesis of this specific aim is shown in Fig 3. To test this hypothesis, rats were randomly assigned to either electrolytic lesion of the MnPO or sham lesioned control. In the electrolytic lesion surgery, both dorsal and ventral parts of the MnPO were electrolytically ablated. After 3 control days of 0.9% NaCl infusion, ANG II at a dose of 10 ng/kg/min was administered intravenously via femoral venous catheter for 10 days, followed by 3 recovery days of 0.9% NaCl infusion. Throughout the study protocol, mean arterial pressure (MAP) and heart rate (HR) were measured
continuously using radio-telemetry system. Daily measurements of MAP, HR, and body sodium and water balances were statistically compared between the 2 experimental groups. Based on the hypothesis, when the pathway that is necessary for mediating chronic hypertensive effects of ANG II is interrupted in rats with electrolytic lesion of the MnPO, they will demonstrate significantly attenuated hypertensive responses to chronic ANG II infusion compared to the sham lesioned rats.

**Sub-Aim 1a: Are MnPO neurons accountable for the different responses to ANG II between rats with electrolytic lesions of the MnPO and sham lesioned rats?**

To further distinguish between the relative importance of MnPO neurons from fibers of passage, excitotoxin lesion specific to cell bodies of the MnPO was conducted using ibotenic acid. *The study in sub-Aim 1a was designed to test the following hypothesis: neurons in the MnPO are (partly) responsible for the attenuated hypertensive responses to ANG II in rats with electrolytic lesion of the MnPO.* The study protocol in this sub-aim was purposely designed to be similar to that of specific aim 1 for the feasibility of comparison between the electrolytic and ibotenic acid lesioned rats, except that ibotenic acid lesion technique was performed in MnPO lesioned rats. MAP, HR, and body sodium and water balances in response to chronic ANG II infusion in rats with ibotenic acid lesion of the MnPO were compared with sham lesioned rats and electrolytic lesioned rats from the study in specific aim 1. Based on the hypothesis, rats with ibotenic acid lesion of the MnPO will show attenuated hypertensive responses to ANG II infusion. The level of attenuation in ibotenic acid lesioned rats compared to the sham lesioned rats will be similar to those of electrolytic lesioned rats if the attenuated responses seen in
electrolytic lesioned rats are solely due to damage of neuronal cell bodies of the MnPO. However, if part of this deficient response is accountable by fibers that pass through this nucleus, the attenuated response in ibotenic acid lesioned rats will be less extensive.

**Specific Aim 2: What is the role of the MnPO in chronic basal blood pressure control by endogenous ANG II?**

The hypothesis of this study was that the MnPO is a crucial component of the central neuronal pathway that mediates blood pressure control by endogenous ANG II. Fig. 4 shows a schematic diagram depicting the hypothesis of specific aim 2. In this specific aim, the AT₁ receptor antagonist, losartan, was chronically infused via osmotic minipump for 14 day to chronically block the interaction between endogenous ANG II and AT₁ receptors. To test this hypothesis, ibotenic acid lesion of the MnPO was performed and MAP, HR, cardiac output (CO), and sodium and water balances responses to chronic losartan treatment were statistically compared between rats with ibotenic acid lesion of the MnPO and sham lesioned rats. The total peripheral resistance was calculated based on MAP and CO, which provided more insight into the hemodynamic changes involved in chronic hypotension induced by losartan. Based on the hypothesis, hypotensive responses to chronic losartan treatment were expected to be attenuated in the MnPO lesioned rats compared to the sham lesioned controls.
Specific Aim 3: What is the role of MnPO in arterial pressure regulation and sodium and water homeostasis during chronic changes in dietary salt?

A proper adjustment of central sympathetic outflow is believed to be one of the homeostatic mechanisms that are important to maintain blood pressure during changes in dietary salt. Such adjustment is possibly mediated by actions of ANG II at the brain. The hypothesis of this specific aim was that the MnPO is a crucial component of neuronal circuitry that mediates appropriate adjustment of central sympathetic outflow and necessary for long-term blood pressure control during chronic high dietary salt intake. Fig. 5 shows a schematic diagram depicting the hypothesis of specific aim 3. To test this hypothesis, MAP, HR, CO, and body sodium and water balances in rats with electrolytic lesion of the MnPO were compared with those of sham lesioned rats during a 28-day protocol of normal (1.0% NaCl) and high (4.0% NaCl) salt diets. In addition, plasma sodium and osmolality were measured to determine in more detail the effects of MnPO lesion and changes in dietary salt on body fluid homeostasis. Based on the hypothesis, central sympathetic outflow of the sham lesioned rats will be correctly adjusted during chronic high salt intake based on changing ANG II levels in the circulation. Therefore, MAP responses in sham lesioned rats were expected to be relatively stable throughout the protocol. On the other hand, if the MnPO is a crucial component of the central neuronal pathway that mediates central sympathetic control by ANG II during high salt intake, MnPO lesioned rats would become salt sensitive. Their MAP would be abnormally elevated during high salt intake.
Figure 1: A schematic illustration of the proposed central pathway(s) that mediates central sympathetic excitation effect of circulating angiotensin II. The subfornical organ and organum vasculosum of the lamina terminalis sense changing levels of circulating angiotensin II and plasma osmolality and transduce these inputs into neural signals. The signals are transferred either directly to the paraventricular nucleus of the hypothalamus, or indirectly to the median preoptic nucleus and in turn reach the paraventricular nucleus. Subsequently, neural fibers from the PVN project to both the sympathetic premotor neurons in the rostral ventrolateral medulla and sympathetic preganglionic neurons in the intermediolateral cell column of the spinal cord (Westerhaus and Loewy, 1999). SFO; subfornical organ. OVLT; organum vasculosum of the lamina terminalis. MnPO; median preoptic nucleus. PVN; paraventricular nucleus. RVLM; rostral ventrolateral medulla. IML; intermediolateral cell column.
Figure 2: A schematic illustration of the central hypotheses of this thesis. The subfornical organ and organum vasculosum of the lamina terminalis sense circulating ANG II concentration, transduce the signal, and then trigger downstream pathway encompassing the median preoptic nucleus and ultimately result in an appropriate sympathetic outflow from sympathetic premotor neurons in the rostral ventrolateral medulla to the periphery. SFO; subfornical organ. OVLT; organum vasculosum of the lamina terminalis. MnPO; median preoptic nucleus. PVN; paraventricular nucleus.
Figure 3: A schematic diagram illustrating the hypothesis of specific aim 1. Chronic low dose angiotensin II infusion causes an increase of blood pressure in part by central sympathetic excitation. The median preoptic nucleus is a crucial part of this central neuronal circuitry mediating chronic hypertensive effects of angiotensin II. SFO; subfornical organ. OVLT; organum vasculosum of the lamina terminalis. MnPO; median preoptic nucleus. PVN; paraventricular nucleus.
**Figure 4:** A schematic diagram illustrating the hypothesis of specific aim 2. Chronic losartan infusion decreases blood pressure in part by central sympathetic inhibition. The median preoptic nucleus is a crucial part of this central neuronal circuitry mediating chronic hypotensive effect of losartan. SFO; subfornical organ. OVLT; organum vasculosum of the lamina terminalis. MnPO; median preoptic nucleus. PVN; paraventricular nucleus.
Figure 5: A schematic diagram illustrating the hypothesis of specific aim 3. Declination of circulating angiotensin II level is a normal homeostatic response to chronic high dietary salt intake. The median preoptic nucleus is a crucial part of the central neuronal pathway mediating declination of central sympathetic outflow secondary to decreased circulating ANG II and; therefore, necessary for maintaining normal blood pressure during high dietary salt intake. SFO; subfornical organ. OVLT; organum vasculosum of the lamina terminalis. MnPO; median preoptic nucleus. PVN; paraventricular nucleus.
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CHAPTER II

An Intact Median Preoptic Nucleus is Necessary for Chronic Angiotensin II-Induced Hypertension
An Intact Median Preoptic Nucleus is Necessary for Chronic Angiotensin II-Induced Hypertension

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Abstract

The median preoptic nucleus (MnPO) receives afferent input from the subfornical organ, a circumventricular organ that has been shown to be necessary in mediating the full chronic hypertensive response to angiotensin II (ANG II) administration. In addition, intravenous ANG II infusion has been shown to cause activation of a number of neurons in both the dorsal and ventral part of MnPO. Taken together, we hypothesized that the MnPO is necessary for the full hypertensive response observed during chronic ANG II-induced hypertension. To test this hypothesis, male Sprague Dawley rats were subjected to either sham (SHAM) or electrolytic lesion of both the dorsal and ventral part of the MnPO (MnPOx). During the same surgery, rats were instrumented with venous catheters, and radiotelemetric transducers for the intravenous administration of ANG II and the measurement of blood pressure and heart rate, respectively. Rats were then given a week recovery period. After 3 days of saline control infusion, ANG II was intravenously infused (10 ng · kg⁻¹ · Min⁻¹) in both sham and MnPOx animals for 10 consecutive days, and followed by 3 recovery days. By day 7 of Ang II infusion, MAP had increased 38 ± 3 mmHg in sham lesioned rats (n=6), but MAP of MnPOx rats (>90% MnPO ablated; n=5) had only increased 18 ± 2 mmHg. This trend continued through day 10 of ANG II treatment. These results support the hypothesis that the MnPO is necessary for the chronic hypertensive response to ANG II administration.
Introduction

The central nervous and renin-angiotensin hormonal systems interact with each other in long-term control of blood pressure (Brooks and Osborn, 1995; Dampney et al., 2002; Reid, 1992; Saxena, 1992). Much evidence supports the idea that the central nervous system monitors body fluid and adjusts sympathetic nervous output and hypothalamic-pituitary hormonal secretion based on circulating blood angiotensin II (ANG II) concentration and osmolality (Cerasola et al., 1987; Matsukawa et al., 1991; Ota et al., 1994; Schmitt and Schmitt, 1968; Scrogin et al., 1999; Tobey et al., 1983; Weekley, 1991). Such signals are directly accessible to the brain through specialized brain regions lacking the blood-brain barrier called circumventricular organs (CVOs) (Fink et al., 1987; McKinley et al., 1998). Previously, we have shown that electrolytic lesion of the subfornical organ (SFO), a CVO located on the rostral wall of the third ventricle, attenuated the hypertensive response to chronic infusion of ANG II (Hendel and Collister, 2005). Accordingly, the SFO is likely a CVO which functions as a sensor of ANG II in long-term control of blood pressure. However, how and where this sensory information is further processed downstream within the brain and finally influences activity of sympathetic premotor neurons located in the hindbrain are not fully understood.

The median preoptic nucleus, MnPO, a nuclear group located in the lamina terminalis, has been shown to connect to the SFO both anatomically and functionally (Ciriello and Gutman, 1991; Gutman et al., 1986; Lind et al., 1982; Miselis et al., 1979; Miselis, 1982; Saper and Levisohn, 1983). The MnPO receives dense afferent inputs
from the SFO (Miselis, 1981; Saper and Levisohn, 1983), and disconnection of the SFO and MnPO disturbs drinking and pressor responses to intravenous ANG II (Miselis, 1982; Lind et al., 1983). Over several decades, a large body of research has implicated the important role of the MnPO on various physiological effects of ANG II believed to be initiated by the actions of ANG II at the SFO. The role of MnPO in a dipsogenic effect of ANG II has been extensively studied, and it has been found that lesions of the MnPO reduce drinking and vasopressin secretion in response to ANG II (Cunningham et al., 1991, 1992; Gardiner and Stricker, 1985a, b; Gutman et al., 1989; Jones, 1988; Lind and Johnson, 1982; Mangiapane et al., 1983). However, few studies have been carried out to assess the role of MnPO in a central hypertensive effect of ANG II and the results have been controversial (Gutman et al., 1989; Jones, 1988; Fink et al., 1986; O’Neill and Brody, 1987).

In the present study, the role of the MnPO in chronic ANG II-induced hypertension was examined. Both the dorsal and ventral portions of the MnPO were electrolytically ablated, and the hypertensive response to a 10-day infusion of ANG II in MnPO lesioned rats was evaluated and compared with those of sham lesioned rats. Our general hypothesis has been that the MnPO is a crucial component of the central sympathoexcitatory pathway following activation of ANG II at the SFO. Specifically, in the present study, we hypothesized that the MnPO is crucial for the full hypertensive response to chronic intravenous administration of ANG II.
Experimental procedures

Adult male Sprague-Dawley rats (Charles River Laboratories, Wilmington, Mass) weighing 300-350g were used. All procedures were conducted in accordance with the National Institutes of Health guidelines and approved by the University of Minnesota Institutional Animal Care and Use committee.

Surgical procedures

Rats were randomly assigned to either a lesion of the median preoptic nucleus (MnPOx) or sham group. Pentobarbital sodium (39 mg/kg, IP) was given as preanesthetic medication, and rats were subsequently injected intramuscularly with a combination of agents (acetylpromazine, 0.2 mg/kg; butorphanol tartrate, 0.15 mg/kg; ketamine, 18.5 mg/kg) to achieve surgical anesthesia. An intramuscular injection of 4 mg tobramycin was given pre-operatively as antimicrobial prophylaxis. Anesthesized rats were then positioned in a Kopf stereotaxic apparatus.

Electrolytic lesion was targeted at both dorsal and ventral parts of the MnPO. A teflon-insulated monopolar tungsten electrode with 1.5 mm exposed at the tip was inserted midline into four predetermined coordinates along two axes: anterior-posterior (AP) relative to Bregma and dorsal-ventral (DV) relative to the surface of the sagittal sinus. The stereotaxic coordinates were selected from the rat brain atlas of Paxinos and Watson (1998). The 4 paired AP and DV coordinates (mm) used were: (-0.25, -7.4), (-0.25, -7.6), (-0.4, -6.1), (-0.4, -7.2). At each coordinate, a current of 1 Ma was passed through the electrode for 5 seconds. Sham operated rats underwent an identical
procedure as MnPOx rats, except that all DV coordinates were 2 mm less and no current was passed.

In the same surgery, all rats were implanted with radiotelemetry blood pressure transducers (model TA11PA-C40, Data Sciences International; St Paul, MN) and femoral venous catheters for 24 h-sampling of mean blood pressure (MAP) and heart rate (HR), and intravenous infusion of ANG II and saline, respectively. Skin of the inner thigh area was incised to expose the femoral artery and vein, and a femoral venous catheter was implanted. The telemetry unit consists of a fluid-filled catheter attached to the body of the transmitter/transducer. A midline abdominal incision was made in order to insert the body of the transducer into the abdomen. The telemetry catheter was passed through the abdominal wall guided by a 14-gauge stainless steel needle, inserted into the femoral artery, and advanced proximally such that the tip was located within the abdominal aorta. Both catheters were held in place by sutures. The body of the transmitter was secured to the abdominal muscle which was closed with 3/0 silk and the skin was closed with surgical staples. The intravenous catheter was advanced subcutaneously, exteriorized between the scapula through a neuron mesh button tether (Harvard Apparatus Inc, Holliston, MA), and the mesh was sutured to the interscapular muscle. The tether was connected to a spring, which was attached at the other end to a single channel hydraulic swivel above the cage, to which the femoral venous catheter was attached. Following the surgery, rats received a subcutaneous injection of 0.075 mg of butorphanol tartrate for analgesic purposes.

During the first three days after surgery, the rats received daily antimicrobial prophylactic injections of ampicillin (15 mg, IV). The rats were then started on a
continuous intravenous infusion of sterile 0.9% saline (7 ml/24 h). They were housed in individual metabolic cages in a housing facility that was maintained at a temperature of approximately 23 °C with a 12 h:12 h light-dark cycle with lights on at 7:00 AM. Rats had free access to 0.4% NaCl diet and distilled water. After the surgery, rats were allowed to recover for at least one week before the experimental protocol was started.

**Experimental protocol**

The protocol was divided into three periods: 3 days of baseline control, 10 days of intravenous ANG II infusion (10 ng · kg⁻¹ · Min⁻¹), and 3 days of recovery. ANG II was dissolved in sterile 0.9% saline and given at a rate of 7ml/24hr. During control and recovery periods, all rats received intravenous infusions of normal saline (7ml/24 hr). MAP and HR signals were sampled and recorded at 500 Hz every 1 min for 10 sec. Food intake, water intake, and urine output were measured gravimetrically daily at the same time each day (2:00 PM). Urine samples were collected and urine sodium concentration was measured using a sodium analyzer (NOVA Biomedical; Waltham, MA). Mean daily sodium intake was calculated as the sum of sodium received from the daily infusion (1 mmol/day) and the amount of dietary sodium ingested in 24 hr (0.07 mmol/g diet). Mean daily sodium excretion was calculated as the product of urine flow rate and urine sodium concentration. Mean daily water intake was calculated as the combined intake of drinking water and infusion water (7ml/24h). The daily water and sodium balance were calculated as the difference between intake and urinary excretion.
**MnPO lesion verification**

When the experimental protocol was completed, the rats were anesthetized with pentobarbital sodium (78 mg/kg), and perfused intracardially with 140 ml of heparinized saline (20 U/ml heparin in 0.9% saline), followed by 450 ml of 4% paraformaldehyde in phosphate buffer saline (PBS). The brains were then removed, post-fixed at 4°C in 4% paraformaldehyde in PBS overnight, and then transferred to 30% sucrose in PBS at 4°C for 3 days. After fixation, the brains were cut sagittally into 40-µm sections using freezing-microtome (Lipshaw Mfg.; Detroit, MI). The sections were mounted on slides, air-dried for 1 day, and then stained with cresyl violet. The location and extent of lesion was determined using a light microscope. MnPOx rats were included in the analyses if they met the following criteria: 1) more than 90% of the MnPO was damaged and 2) no or slight damage to adjacent areas of the MnPO.

**Statistical analyses**

Two-way ANOVA with repeated measures was carried out to compare each parameter between MnPOx and sham groups. A Greenhouse-Geisser adjusted P value was used to account for violations of the assumption of compound symmetry that invariably accompany this experimental design. Post-hoc multiple comparisons using Tukey-Kramer test was further conducted if the two-way ANOVA showed significant interaction between two main factors (lesion vs. day). Baseline values were derived from averages over three control days, and the differences of baselines between the two groups were determined using a Student’s t-test. A p-value of 0.05 was set as the level of
statistical significance for all statistical analyses. Values were presented as mean ± SE. All statistical procedures were performed using NCSS software (NCSS; Kaysville, UT).

**Results**

All rats included in the analyses were healthy and displayed normal behavior throughout the experimental period. Histological examination of the lesions showed that five rats from the MnPOx group adequately met the criteria to be included in the analyses. Data from these MnPOx rats were compared with those from sham lesioned rats (n = 6). Representative examples of MnPOx and sham lesion are shown in Fig. 1.

*Cardiovascular responses to ANG II infusion*

The MAP responses to ANG II in MnPOx and sham lesioned rats throughout the experimental protocol are shown in Fig. 2 (top panel). T-test analysis revealed no difference in average baseline MAP between the two groups (MnPOx, 99 ± 2 mmHg; sham, 98 ± 3 mmHg). By day 2 of ANG II infusion, MAP in both MnPOx and sham lesioned rats were significantly increased from baseline control (MnPOx, 114 ± 3 mmHg; sham, 120 ± 4 mmHg). MAP continued to increase progressively in both groups, but this response was significantly attenuated in MnPOx rats compared to sham lesioned rats by day 7 of ANG II infusion (MnPOx, 117 ± 4 mmHg; sham, 136 ± 5 mmHg). This trend continued through the remaining ANG II infusion period. Once ANG II was
discontinued, MAP of the two groups decreased gradually and returned to their baseline levels by the end of the recovery period.

Five rats had incomplete lesions of the MnPO and were not included in the final analysis. In these rats, lesions were limited to only 30 to 70% of the MnPO. By day 7 of ANG II infusion, the average MAP in these rats was 131 ± 1 mmHg. Throughout the protocol, there was no significant difference between MAP of these incomplete lesioned rats compared to sham rats.

The HR responses to ANG II are shown in Fig. 2 (bottom panel). The average baseline HR in MnPOx rats and sham lesioned rats was 444 ± 6 and 447 ± 6 beats/min, respectively. By day 3 of ANG II infusion, HR in both MnPOx and sham were significantly decreased from baseline control (MnPOx, 414.5 ± 5.6 beats/min; sham, 403.3 ± 6.5 beats/min), and this trend continued through the remaining ANG II infusion period. However, HR responses were not different between MnPOx and sham lesioned rats throughout the protocol.

**Sodium and Water balance responses**

Water balance responses are shown in Fig. 3. The average baseline water intake in MnPOx rats was 26 ± 1 ml/day, which was not different from that of the sham lesioned rats (26 ± 2 ml/day). Water intake, urine output, and water balance were not different between the two groups throughout the experimental protocol.

Sodium balance responses are shown in Fig. 4. Average baseline sodium intake was 2.9 ± 0.1 mEq/day in MnPOx rats, and 2.9 ± 0.1 mEq/day in sham lesioned rats. Average baseline sodium excretion in MnPOx and sham lesioned rats was 1.7 ± 0.3
mEq/day and 1.6 ± 0.3 mEq/day, respectively. No significant differences in sodium intake, sodium output, and sodium balance were found between the two groups throughout the protocol.

**Discussion**

The MnPO receives reciprocal inputs from the SFO and other brain regions believed to form the sympathoexcitatory pathway following activation by ANG II at the SFO (Misulis, 1981; Saper and Levisohn, 1983; Sawchenko and Swanson, 1983; Zardetto-Smith et al., 1993). In the present study, we have shown that rats with lesion of the MnPO had a significantly attenuated response to the hypertensive effects of chronic ANG II administration. In fact, we have demonstrated that when MAP reached the steady state from day 7 to 10 of the ANG II infusion, MAP in MnPOx rats had increased from baseline nearly 20 mmHg less than MAP of sham lesioned rats. This was approximately a 50% attenuation of the increased MAP seen in sham lesioned rats. These findings suggest that the MnPO is one of the crucial components of the central neural circuitry necessary for the central hypertensive response to chronic ANG II administration.

In the present study, HR was decreased significantly from baseline during day 2 to 10 of ANG II infusion in both MnPOx and sham lesioned rats. This could be explained by a baroreflex response to the elevated blood pressure induced by ANG II, although many would argue against this as the baroreceptors would reset in response to a
sustained stimulus. However, this finding is in contrast to our previous study in which no significant change of HR was observed during chronic ANG II infusion in sham lesioned rats and those with lesions of the SFO (Hendel and Collister, 2005). This difference could be explained by the higher level of increased MAP observed in this study compared to that previous study.

It has been shown previously that lesions of the AV3V region, the region surrounding the anteroventral portion of the third cerebral ventricle, attenuate hypertension in a number of rat models including a renin-angiotensin dependent form of hypertension (Brody et al., 1978). AV3V lesions include a medioventral part of the MnPO together with the anterior periventricular nuclei of the hypothalamus, and the OVLT. In the present study, lesions were limited to a more specific region, the MnPO, without damaging the other parts of the AV3V. Therefore, the attenuated response to the chronic hypertensive effect of ANG II in MnPO lesioned rats found in this study suggest that damage of the MnPO may be responsible in part for the attenuated hypertensive response to ANG II in AV3V lesioned animals as well. It also should be noted that although acute adipsia is common in AV3V lesioned animals, rats with MnPO lesions in the present study displayed normal drinking behavior throughout the study protocol, suggesting other areas of the AV3V are responsible for this behavior in the AV3V lesioned rat model.

A role of the MnPO on the central hypertensive effect of ANG II has been investigated in the past. Our findings support those that were conducted under an acute experimental setting, which found that knife cuts of the SFO and MnPO connection, and electrolytic ablation of both the dorsal and ventral part of the MnPO attenuated pressure
responses to a low-dose intravenous ANG II infusion (Lind et al., 1983; O’Neill and Brody, 1987). However, although the study by Fink et al (Fink et al., 1986) has shown that chronic MAP response to 5 days of ANG II infusion was not affected by MnPO lesion, the present findings suggest that longer duration of ANG II infusion is required to detect the lesion effect. In fact, in the present study, significant attenuation of the pressure response to ANG II was not observed until day 7 of ANG II infusion, 2 days after the end of ANG II infusion in the previous study. Therefore, the data of this current study is not inconsistent with that of the previous study, but do in fact demonstrate a role of the MnPO in the long-term hypertensive effects of ANG II.

The SFO is one of the CVOs that has been implicated as a primary central target for circulating ANG II. We have previously shown that the SFO is necessary for the full hypertensive response to chronic ANG II infusion (Hendel and Collister, 2005), and this has been shown to be mediated through superoxide production in the SFO (Zimmerman et al., 2004). Also, lesion of the SFO blocks expression of the immediate early gene, c-fos, in many brain regions that have been shown to express fos in response to intravenous infusion of hypertonic saline, and believed to be involved in central sympathoexcitatory pathways, including the MnPO, parvocellular division of the hypothalamic paraventricular nucleus, and the ventrolateral medulla (Hochstenbach and Ciriello, 1996). Part of the neural fibers that emerge from the SFO have been shown to project and synapse directly onto MnPO neurons (Miselis, 1981). Additionally, certain portions of MnPO neurons that project to hypothalamic paraventricular nucleus are activated by ANG II infusion (Stocker and Toney, 2005). One study showed that MnPO lesion diminishes c-fos expression in the paraventricular nucleus of the hypothalamus, but not in
the SFO in response to intravenous infusion of ANG II (Xu and Herbert, 1995). This supports the idea that the MnPO is a relay site from the SFO to the paraventricular nucleus. We have demonstrated in the present study the functional importance of the MnPO in the chronic hypertensive effect of ANG II. Taken together, these findings provide strong evidence to support the general hypothesis that the MnPO is part of the central sympathoexcitatory pathway downstream from the SFO and crucial for the central sympathetic response to systemic ANG II.

Neuroanatomical studies have shown that the SFO projects via two main efferent pathways (Miselis, 1981). Efferent fibers from the SFO either synapse directly onto the supraoptic and paraventricular nucleus of the hypothalamus, or relay at the MnPO before reaching these hypothalamic nuclei. Findings that some efferent fibers from the SFO bypass the MnPO may explain why MnPO lesion failed to completely block the hypertensive effects of ANG II in the present study. In addition, the hypertensive response to ANG II observed in MnPOx rats in this study may be partially explained by remaining neuronal pathways originating from other CVOs, particularly the area postrema, that has been shown to be important in the central hypertensive effect of ANG II (Fink et al., 1987). However, results from our recent study appear to diminish the role of the area postrema as a critical mediator of the chronic hypertensive effect of intravenous ANG II in this model (Nahey and Collister, 2007).

Anterograde tracing studies from the SFO (Miselis, 1981) also showed that some of the fibers that travel ventrally toward the MnPO do not actually make a synapse on this nucleus, but pass through or close by the MnPO and eventually terminate at the supraoptic and paraventricular nuclei. Thus, an argument may arise that not only neurons
in the MnPO, but also fibers of passage traveling around the periphery or passing through the MnPO were also damaged by the electrolytic ablations performed in this study. Since only MnPOx rats with lesions limited to the MnPO with no or slight damage to the adjacent area were included in the statistical analysis, we believe that neural fibers that pass by the MnPO are less likely responsible for the observed attenuated response to ANG II in this study. In fact, one rat whose lesion was missed and located in the septal diagonal band immediately anterior to the MnPO had blood pressure responses comparable to those of the sham lesioned rats (MAP increased 32 mmHg by day 7). Additionally, as a group, the partial lesioned rats average MAP was never different from the sham group. Furthermore, MnPO neurons projecting to the PVN have been shown to increase their activity in response to intravenous ANG II infusion (McKinley et al., 1992; Oldfield et al., 1994; Potts et al., 1999; Rowland et al., 1994; Stocker and Toney, 2005) supporting the idea that electrolytic ablation of the MnPO attenuates blood pressure responses to ANG II by destroying those neurons instead of fibers of passage. However, we cannot reject the possibility that fibers that directly pass through MnPO may play a part in the attenuated response observed. This distinction is critical to our overall understanding of this important pathway, and therefore to further distinguish between the relative importances of MnPO neurons from fibers of passage, current research in our lab using excitotoxin lesion specific to cell bodies of the MnPO along with retrograde tracing technique are being conducted. Nevertheless, our present findings indeed implicate the MnPO as a crucial component of central neural pathway necessary for the full hypertensive response to chronic intravenous ANG II infusion.
In conclusion, the MnPO has long been shown to mediate the central dipsogenic effect of ANG II, but relatively few studies have examined its role in chronic actions of ANG II. The present findings have expanded this knowledge by revealing a crucial role of the MnPO in the chronic hypertensive effect of ANG II. Previously, we and others have demonstrated a role of the SFO in the central hypertensive effects of ANG II. The findings that the SFO is associated with the MnPO both anatomically and functionally, together with the present observations, provide strong evidence to support the general hypothesis that the MnPO is a crucial part in the central sympathoexcitatory pathway following actions of ANG II at the SFO.

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Figure 1: Photomicrographs of 40 µm mid-sagittal sections of the median preoptic nucleus (MnPO). A. Section from a sham lesioned rat demonstrating dorsal and ventral MnPO (* and #, respectively). B. Section from a MnPOx rat demonstrating ablated dorsal and ventral MnPO. 3V = Third ventricle.
Figure 2: Average 24 h mean arterial pressure (MAP) and heart rate (HR) recorded during control period (3 days of saline), treatment (10 days of ANGII), and recovery (3 days of saline) in MnPOx and sham lesioned rats. *P < 0.05 between groups.
Figure 3: Water intake, urine output, and water balance during control, treatment, and recovery periods in MnPOx and sham lesioned rats.
Figure 4: Sodium intake, sodium excretion, and sodium balance during control, treatment, and recovery periods in MnPOx and sham lesioned rats.
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CHAPTER III

Role of the Median Preoptic Nucleus in Chronic Angiotensin II-Induced Hypertension
Role of the Median Preoptic Nucleus in Chronic Angiotensin II-Induced Hypertension

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Abstract

Several lines of evidence implicate the median preoptic nucleus (MnPO) as a downstream site of activation following binding of angiotensin II (ANG II) at the subfornical organ and organum vasculosum of the lamina terminalis. We have shown previously that electrolytic lesion of the MnPO attenuated the increased blood pressure response to chronic intravenous infusion of ANG II. However, whether MnPO neurons or fibers that pass through this region contribute to this response is not clear. In the present study, to distinguish the relative importance of MnPO neurons from fibers of passage in the hypertensive response to chronic ANG II administration, male Sprague Dawley rats were randomly assigned to either sham (Isham) or ibotenic acid lesion of the MnPO (iMnPOx). In the iMnPOx group, 200 nl of ibotenic acid in phosphate buffer saline (5 µg/µl) was injected into each of 3 predetermined coordinates targeted at the entire MnPO. After a week of recovery, rats were instrumented with venous catheters, and radiotelemetric transducers for the intravenous administration of ANG II and the measurement of mean arterial pressure (MAP) and heart rate, respectively. Rats were given another week to recover. Isham and iMnPOx animals were then infused with saline (7 ml 0.9% NaCl/day) for 3 days as a control period, followed by 10 consecutive days of intravenous ANG II infusion (10 ng·kg$^{-1}$·min$^{-1}$), and finally a recovery period similar to control. Throughout the protocol, a 0.4% NaCl diet and distilled water were provided ad libitum. By day 8 of ANG II infusion, MAP had increased 54±2 mmHg in Isham rats (n=8). The hypertensive response to ANG II was significantly attenuated in the iMnPOx rats (n=9), in which MAP had only increased 29±3 mmHg. These results support the hypothesis that
neurons of the MnPO are involved in the central neural pathway mediating the chronic hypertensive effects of ANG II.

**Introduction**

Chronic over-activation of the renin angiotensin system (RAS) has been linked to many forms of experimental and pathological hypertension (DeForrest et al., 1982; Hackam et al., 2007; Imamura, 1995; Romero and Reckelhoff, 1999). Strong evidence suggests that long-term elevation of circulating angiotensin II (ANG II) maintains high blood pressure in part by augmenting central sympathetic outflow of the brain through central neuronal mechanisms (Brooks and Osborn, 1995; Cerasola et al., 1987; Dampney et al., 2002; Dowell et al., 1996; Fink, 1997; Heesch et al., 1996; Matsukawa et al., 1991; Reid, 1992; Saxena, 1992; Tobey et al., 1983; Weekley, 1991). It has been well established that peripheral ANG II accesses the brain through circumventricular organs (CVOs), particularly the subfornical organ (SFO) and organum vasculosum of the lamina terminalis (OVLT) (McKinley et al., 1992, 1998; Oldfield et al., 1994). Lesions of these CVOs and their output tracts block or attenuate hypertension in a number of animal models, including those mediated by chronic over-activation of RAS (Brody et al., 1978; Haywood et al., 1983; Hendel and Collister, 2005; Mangiapano and Simpson, 1980; Marson et al., 1983). Therefore, the SFO and OVLT are likely initial brain sites functioning as sensors of ANG II in long-term blood pressure regulation. However, the central neuronal pathway (s) that mediates sympathetic excitation downstream from these CVOs is not completely understood.
Several lines of evidence implicate the median preoptic nucleus (MnPO), a group of neurons located on the rostral wall of the third ventricle, as a potential candidate of the downstream pathway from the SFO and OVLT. Firstly, neuroanatomical studies have revealed intense reciprocal connections between the MnPO and the SFO and OVLT, as well as other brain regions proposed to contribute to the central neuronal pathway of ANG II-induced sympathoexcitation, including the parvocellular subdivision of the hypothalamic paraventricular nucleus and ventrolateral medulla (Miselis, 1981; Saper and Levishon, 1983; Sawchenko and Swanson, 1983; Westerhaus and Loewy, 1999; Zardetto-Smith et al.; 1993). Secondly, electrophysiological and Fos immunocytochemistry studies have shown activation of neurons located widespread within the MnPO by peripherally administered ANG II (Stocker and Toney, 2005; McKinley et al., 1992; Oldfield et al., 1994; Potts et al., 1999; Rowland et al., 1994). Thirdly, a large number of lesion studies have established a crucial role of the MnPO in drinking behavior, an acute physiological effect of ANG II that is believed to be initiated by the action of ANG II at the SFO and OVLT (Cunningham et al., 1991, 1992; Gardiner and Stricker, 1985; Gutman et al., 1989; Jones, 1988; Lind and Johnson, 1982; Mangiapane et al., 1983; Tanaka and Nomura, 1993). Importantly, relatively fewer studies have examined the role of the MnPO in the chronic central actions of ANG II.

Recently, we have reported that electrolytic ablation of both the dorsal and ventral parts of the MnPO attenuates increased blood pressure responses to chronic intravenous ANG II administration (Ployngam and Collister, 2007). These findings implicate the MnPO as a crucial component of the central neuronal pathway that is necessary to mediate the full hypertensive response to chronically elevated ANG II. However, since
fibers of passage that pass through the MnPO might be unavoidably damaged by electrolytic lesion, the role of the neuronal parenchyma of the MnPO in this observed response of our previous study remains to be investigated.

Over several decades, excitotoxins, especially kainic and ibotenic acid, have been used in numerous studies to selectively cause degeneration of neuronal cell bodies in an area of interest, while leaving intact axons of passage (Kohler and Schwarcz, 1983; McLin and Steward, 2006; Nelson et al., 2007; Ota et al., 2007; Schwarcz et al., 1979; Whishaw et al., 2007). Ibotenic acid, in particular, has been used intensively and successfully to produce lesions in a number of discrete areas of the brain, such as several hypothalamic nuclei, including the MnPO (Abrahamson and Moore, 2006; Cunningham et al., 1992; Fugo et al., 2006; Konishi et al., 2007). Studies have shown that both electrolytic and excitotoxin lesion of the MnPO produce parallel results regarding deficit-drinking responses to ANG II (Cunningham et al., 1992; Mangiapane et al., 1983). However, a discrepancy of blood pressure responses to acute ANG II administration has been noted between these two lesion techniques (Jones, 1988; O’Neill and Brody, 1987). Importantly, whether this phenomenon occurs during chronic blood pressure effects of ANG II as well is not known.

The aim of the present study was to specifically examine the role of MnPO neurons in the chronic blood pressure response to ANG II. For this purpose, excitotoxin lesions specific to cell bodies of the MnPO were induced in rats using ibotenic acid, and the hypertensive response to a 10-day infusion of ANG II was evaluated and compared with those of sham lesioned rats.
Experimental procedures

Adult male Sprague Dawley rats weighing 300-350 g were used in all procedures. All procedures were approved by University of Minnesota Institutional Animal Care and Use committee in accordance with the National Institutes of Health guidelines.

Surgical procedures

Rats were randomly assigned to either ibotenic acid lesion of the MnPO (iMnPOx) or sham lesion (Isham) group. Pentobarbital sodium was given intraperitoneally (50 mg/kg) to achieve surgical anesthesia. An intramuscular injection of 4 mg Tobramycin was given for antimicrobial prophylaxis. Anesthetized rats then were positioned in a Kopf stereotaxic apparatus. Ibotenic acid lesion was targeted at the entire MnPO. An injection cannula (ID, 0.15 mm; OD, 0.30 mm; Plastic Ones, Roanoke, VG) connected to a syringe via PE50 tubing was lowered midline into three predetermined coordinates along two axes: anterior-posterior (AP) relative to Bregma and dorsal-ventral (DV) relative to the surface of the sagittal sinus. The three-paired AP and DV coordinates (mm) used were as follows: (-0.25, -7.4), (-0.4, -6.1), (-0.35, -7.2). At each coordinate, 200 nl of ibotenic acid (Sigma Aldrich, St. Louis, MO) in 1 M phosphate buffered saline (5 µg/µl) was injected over a period of 10 min. The injection cannula was left in place for approximately 10 min allowing the acid to dissolve out, before the cannula was withdrawn. Sham operation rats were performed in an identical procedure as iMnPOx rats, except that the cannula was lowered 2 mm less and no acid was injected.
At least 7 days after the lesion surgery rats underwent another surgery in which radiotelemetry blood pressure transducers (model TA11PAC40, Data Sciences International; St Paul, MN) and femoral venous catheters were placed for 24-h sampling of mean arterial pressure (MAP) and heart rate (HR), and intravenous infusion of ANG II and saline, respectively. This technique was described previously (Ployngam and Collister, 2007). The telemetry unit consists of a fluid-filled catheter attached to the body of the transmitter/transducer. An abdominal incision was performed to insert the body of the transducer into the abdomen. The telemetry catheter was passed through the abdominal wall, inserted into the left femoral artery, and advanced proximally such that the tip was situated within the abdominal aorta just below the distal renal artery. Additionally, a venous catheter was implanted into the left femoral vein. The remaining tubing was advanced subcutaneously, exteriorized between the scapulae through a spring-connected euron mesh button tether (Harvard Apparatus Inc; Holliston, MA). The spring was attached at the other end to a single channel hydraulic swivel above the cage, to which the femoral venous catheter was attached. Immediately after the surgery, rats received a subcutaneous injection of 0.075 mg of butorphanol tartrate for analgesic purposes.

During the first 3 days after surgery, the rats received daily antimicrobial prophylactic injections of ampicillin (15 mg, IV). The rats were then started on a continuous intravenous infusion of sterile 0.9% saline (7 ml/24 h). They were housed in individual metabolic cages in a housing facility that was maintained at a temperature of approximately 23 °C with a 12 h:12 h light–dark cycle with lights on at 7:00 AM. Rats
had free access to 0.4% NaCl diet and distilled water. After instrumentation, rats were allowed to recover for at least 1 week before the experimental protocol was begun.

**Experimental protocol**

The study protocol was identical to our previous report investigating the effect of electrolytic lesion of the MnPO on the chronic blood pressure response to ANG II (Ployngam and Collister, 2007). The first 3 days of baseline control were followed by 10 days of intravenous ANG II infusion (10 ng·kg⁻¹·min⁻¹), and 3 days of recovery. During control and recovery periods, all rats received intravenous infusions of normal saline (7 ml/24 h). ANG II was dissolved in sterile 0.9% saline and given at a rate of 7 ml/24 h. MAP and HR signals were sampled and recorded at 500 Hz every 1 min for 10 s. Food intake, water intake, and urine output were measured gravimetrically daily at the same time (2:00 PM). Urine samples were collected daily and urine sodium concentration was measured using a sodium analyzer (NOVA Biomedical; Waltham, MA). Mean daily sodium intake was calculated as the sum of sodium received from the daily infusion (1 mmol/day) and the amount of dietary sodium ingested in 24 h (0.07 mmol/g diet). Mean daily sodium excretion was calculated as the product of urine flow rate and urine sodium concentration. Mean daily water intake was calculated as the combined intake of drinking and infusion water (7 ml/24 h). The daily water and sodium balance were calculated as the difference between intake and urinary excretion.
**Lesion verification**

At the end of the protocol, rats were anesthetized and perfused transcardially with 4% paraformaldehyde in phosphate buffered saline. The brains were post-fixed with 4% paraformaldehyde overnight and in 30% sucrose for another 48 hr for cryoprotection. Coronal sections of the area comprising the entire MnPO according to atlas of Paxinos and Watson (1998) were cut on a sliding, freezing microtome (Lipshaw Mfg; Detroit, MI) at a thickness of 40 µm. The coronal sections were stained with Fluoro-Jade-C (Chemicon International Inc; Temecula, CA), a fluorescent, anionic stain that labels degenerating neurons. Fluoro-Jade staining was performed as described previously (McLin and Steward, 2006; Schmued and Hopkins, 2000). Briefly, sections were mounted on gelatin-coated slides, dried overnight, and the slides were then immersed in 100% ethanol for 3 min, followed by 70% ethanol for 1 min, and finally D\textsubscript{2}O for 1 min. Sections were then treated with 0.06% potassium permanganate for 15 min, while shaking gently. After rinsing with D\textsubscript{2}O, sections were immersed in 0.001% Fluoro-jade-C in 0.1% acetic acid for 30 min in the dark and then rinsed with D\textsubscript{2}O. The sections were dried overnight, immersed for 3 min in xylene substitute (Fisher Scientific, Fair Lawn, NJ), and coverslipped with the nonaqueous fluorescent mounting media DPX (Sigma-Aldrich; St. Louis, MO). Fluoro-Jade stained sections were visualized with a fluorescence microscope using a FITC-filter (Nikon Instruments Inc; New York, NY). Only MnPOx rats that had at least 90% of the MnPO area positively stained with Fluoro-jade-C, with no or few degenerate neurons observed in the adjacent area were included in the analysis.
Statistical analyses

All statistical procedures were performed using NCSS software (NCSS; Kaysville, UT). Two-way ANOVA with repeated measures was carried out to compare each parameter between iMnPOx and Isham groups. If the two-way ANOVA showed significant interaction between two main factors (lesion vs. day), post-hoc multiple comparisons using a Tukey–Kramer test was further conducted to identify days on which the two groups differed. Baseline values were derived from averages over the three control days, and the differences of baselines between the two groups were determined using a Student’s t-test. In addition, two-way ANOVA with repeated measures followed by post-hoc multiple comparisons using Tukey-Kramer test were conducted to compare MAP between iMnPOx lesioned rats from the present study and electrolytic lesioned rats (eMnPOx) from our previous study (Ployngam and Collister, 2007). A P-value of 0.05 was set as the level of statistical significance for all statistical analyses. All values were presented as mean±S.E.

Results

All rats included in the analyses were healthy and displayed normal behavior throughout the experimental protocol. Nine rats with complete lesion of the MnPO from the iMnPOx groups and 8 rats from the Isham group were included in the analyses.

Fluorojade staining revealed that ibotenic acid is highly effective in producing well-defined lesions of MnPO neural cell bodies, without affecting fibers of passage. In fact, lesions were notably discrete such that when correct positions of acid injections
were made, lesions rarely extended beyond the MnPO. As a result, most of the iMnPOx animals that were excluded from this study had missed instead of overly extensive lesions. All iMnPOx lesioned rats included in the present study had at least 90% of the MnPO neurons damaged with no or slight damage to the surrounding neural structures. In fact, areas of non-specific damage were restricted to the periventricular area surrounding the MnPO and anterior commissure. As seen in Fig 1, neither SFO nor OVLT neurons were found to be damaged following ibotenic acid lesion in any of the iMnPOx included in this study. Representative examples of iMnPOx and Isham lesion are shown in Fig 1.

**Cardiovascular response to ANG II infusion**

Fig. 2 (top panel) shows the MAP responses to ANG II in iMnPOx and Isham lesioned rats throughout the experimental protocol. No difference in 3-day baseline MAP was found between the two groups. The average baseline MAP in iMnPOx and Isham lesioned rats were 103±2 mmHg and 97±2 mm Hg, respectively. By day 2 of ANG II infusion, MAP in both iMnPOx and Isham lesioned rats were significantly increased from baseline control (iMnPOx, 119±4mmHg; Isham, 128±3mmHg). MAP increased continuously in both groups. However, this response was significantly attenuated in iMnPOx rats compared to Isham lesioned rats by day 8 of ANG II infusion (iMnPOx, 127±3 mmHg; Isham, 151±3 mm Hg). This difference continued until the finish of ANG II infusion. Once ANG II was discontinued, MAP of the two groups started to decrease gradually and returned to their baseline levels by the end of the recovery period.
The HR responses to ANG II are shown in Fig. 2 (bottom panel). HR was comparable between iMnPOx and Isham groups throughout the experimental protocol. The average baseline HR in iMnPOx rats and Isham lesioned rats was 443±7 and 424±7 beats/min, respectively. The HR was significantly decreased from baseline control by day 2 of ANG II infusion in iMnPOx (409±7 beats/min) and day 3 in Isham (390±9 beats/min) rats. HR remained lower throughout the remaining ANG II infusion period in iMnPOx lesioned rats. In contrast, it returned to baseline level by day 7 of ANG II infusion in Isham lesioned rats.

**Sodium and water balance responses**

Water balance responses are shown in Fig. 3. Baseline water intake, urine output, and water balance were comparable between the two groups. The average baseline water intake was 22±1 ml/day and 23±7 ml/day in iMnPOx and Isham lesioned rats, respectively. The average baseline urine output was 8±1 ml/day in iMnPOx and 10±2 ml/day in Isham. Water intake, urine output, and water balance were not significantly different between the two groups throughout the experimental protocol.

Sodium balance responses are shown in Fig. 4. The average baseline sodium intake was 2.7±0.1 mEq/day in iMnPOx rats, and 2.7±0.1 mEq/day in Isham lesioned rats. Average baseline sodium excretion in iMnPOx and Isham lesioned rats was 2.0±0.1 mEq/day and 2.0±0.3 mEq/day, respectively. Sodium intake, sodium output, and sodium balance were not significantly different between the two groups throughout the experimental protocol.

**MAP responses to ANG II in iMnPOx compared to electrolytic lesioned rats (eMnPOx)**
To determine the relative importance of MnPO neurons versus fibers of passage, blood pressure responses to ANG II were statistically compared between rats that underwent ibotenic acid lesion of the MnPO in the present study versus electrolytic lesion (eMnPox) from our previous study (Ployngam and Collister, 2007). For the feasibility of comparison, the present study was purposely designed such that the study protocol was analogous to that of our previous study, except that ibotenic acid lesion was performed instead of electrolytic lesion. Fig. 5 shows the results of this comparison. Average baseline MAP was similar between the two lesion groups (iMnPox, 103±2 mmHg; eMnPox, 99±3 mmHg). In rats with both lesion types, MAP increased by day 2 of ANG II infusion and continued to increase progressively until the end of ANG II treatment. Although MAP responses in electrolytic and ibotenic acid lesioned rats were both found to be attenuated by day 7 and 8 of ANG II infusion, respectively, compared to their particular sham controls, MAP responses were higher in iMnPox than in eMnPox by day 5 of ANG II infusion (iMnPox, 127±3 mmHg; eMnPox, 118±2 mmHg). This trend continued until the finish of ANG II infusion.

**Discussion**

We have previously reported that the blood pressure rise following chronic intravascular administration of ANG II was attenuated in rats with electrolytic lesion of the MnPO (Ployngam and Collister, 2007) compared to sham lesioned rats. Accordingly, neurons located in and/or fibers passing through the MnPO seem to be necessary
elements of the central neuronal pathway that mediates the hypertensive effects of circulating ANG II. Numerous axons that pass through the MnPO, but terminate elsewhere, likely originate from the SFO and OVLT, two CVOs that have been well accepted as central neural sensors of circulating ANG II. As a result, the blunted response seen in rats with electrolytic ablation of the MnPO could be explained, to a certain extent, by destruction of these axons. In the present study, ibotenic acid lesion specific to neurons of the MnPO was conducted to determine the relative role of the MnPO neural cell bodies versus fibers of passage on the chronic hypertensive effects of ANG II. The current results show that rats with lesions specific to MnPO neurons have attenuated hypertensive responses to chronic ANG II administration compared to sham lesioned rats. Therefore, the present findings suggest that MnPO neurons are a crucial component of the central neural pathway mediating the hypertensive action of chronic ANG II administration. Additionally, as one of the two main efferent pathways from the SFO and OVLT (other than the direct projection from these CVOs to the paraventricular nucleus of the hypothalamus (PVN) (Miselis, 1981)), our findings reveal that the pathway that relays to MnPO neurons is responsible for a significant portion of the hypertensive response to chronic ANG II administration.

The comparison of MAP responses between iMnPOx from the present study and eMnPOx from the previous study revealed a greater attenuation of increased MAP responses from day 5 to 10 of ANG II infusion in eMnPOx. Accordingly, fibers of passage appear to play some part in the blunted blood pressure response seen in the electrolytic lesioned rats. However, ibotenic acid lesion specific to neural cell bodies of the MnPO in the present study did cause an attenuated MAP response relative to sham
treatment. Therefore, neural cell bodies of the MnPO appear to play a necessary part in the blunted blood pressure response to ANG II seen in both ibotenic and electrolytic lesioned rats. Taken together the findings from our previous and present studies suggest that the neural circuitry necessary for the full hypertensive effect of chronic ANG II administration is dependent on MnPO neural cell bodies.

HR was decreased significantly from baseline by day 2 and 3 of ANG II infusion, respectively, in iMnPOx and Isham lesioned rats. These findings are in line with our previous study indicating a possible baroreflex response to the elevated MAP induced by ANG II (Ployngam and Collister, 2007). However, we found HR decreased equally in both iMnPOx and Isham lesioned rats during ANG II infusion, even though MAP was higher in Isham lesioned rats during the last few days of ANG II infusion. In addition, while HR in Isham normalized by day 7 of ANG II infusion, that of iMnPOx rats remained lower than their baseline control throughout the rest of ANG II infusion period. This same trend was observed in our previous study when the HR response to ANG II infusion was compared between rats with electrolytic lesion of the MnPO versus their sham controls (Ployngam and Collister, 2007). The MnPO receives reciprocal inputs from the nucleus of solitary tract and ventrolateral medulla, cardiovascular centers in the hindbrain known to receive and respond to baroreceptor inputs from the periphery (Ciriello et al., 1985; Gieroba and Blessing, 1993; Li et al., 1992; Saper and Levesohn, 1983; Tanaka et al., 1992b). In addition, compelling evidence suggests cross communication between the MnPO and both central and peripheral baroreflex centers to facilitate fine-tune control of body fluid and cardiovascular homeostasis (Sakamaki et al.; 2004; Tanaka et al., 1992a; Tanaka et al., 1993; Tanaka et al., 2003). It has been shown
previously that baroreflex-induced bradycardia is suppressed by ANG II (Campagnole-Santos et al., 1992; Garner et al., 1987; Guo and Abboud, 1984), probably in part through central neuronal mechanisms (Berecek et al., 1991; Campagnole-Santos et al., 1988; Hayashi et al., 1988; Matsumura et al., 1989). Lesions of the AV3V region, the area surrounding the anteroventral portion of the third cerebral ventricle, enhanced baroreflex sensitivity of HR to the rise of blood pressure induced by ANG II (Bealer, 1995). AV3V lesions include a medioventral part of the MnPO together with the anterior periventricular nuclei of the hypothalamus, and the OVLT. Therefore, an enhanced baroreflex-induced bradycardia in response to the elevated blood pressure induced by ANG II observed in iMnPOx rats in the present study suggests that damage of the MnPO may be responsible in part for the enhanced baroreflex response in AV3V lesioned animals of the previous study. In addition, these findings provide evidence of the possibility that central neuronal circuitry involving MnPO neurons may mediate the ANG II-induced baroreflex suppression. More conclusive evidence regarding the role of the MnPO in mediating baroreflex-modulating effects of ANG II will require future testing of baroreflex function in animals with discrete lesions of the MnPO.

Previously, few studies have performed excitotoxic lesions to establish the role of MnPO neurons in the central physiological effects of ANG II (Cunningham et al., 1991, 1992; Jones, 1988). The effect of ibotenic acid lesion of the MnPO on the dipsogenic response to ANG II has been well studied, and it has been found that it produced similar results compared to electrolytic lesions. Both lesion techniques caused deficit-drinking responses to ANG II administration (Cunningham et al., 1991, 1992; Mangiapan et al., 1983). Our studies are in agreement with these findings in that the two lesion techniques
also showed attenuated effects on the hypertensive response to chronic peripheral ANG II infusion. However, it should be noted that a differential response between electrolytic and kainic acid lesion on pressure responses to acute intracerebroventricular (ICV) injection of ANG II has been observed (Jones, 1988). While electrolytic lesion elicited a deficit blood pressure response to acute ICV ANG II injection, kainic acid lesion produced no attenuation. Nevertheless, several differences limit a direct comparison between the present study and that of Jones (1988). These include type of excitotoxin used (ibotenic acid vs. kainic acid), recovery period provided after lesion surgery (14 days in this study vs. 1 day), as well as route (IV vs. ICV) and duration of ANG II administration (10 days vs. single injection).

Chronic elevated circulating ANG II is believed to generate hypertension in part by augmenting central sympathetic outflow through neuronal circuitry in the brain (Brooks and Osborn, 1995; Fink 1997; Reid, 1992). Ample evidence implicates MnPO neurons as a crucial part of this central neural pathway mediating ANG II-induced central sympathetic excitation. MnPO neurons receive intense afferent inputs from both the SFO and OVLT, as well as other brain regions proposed to contribute to the central neural pathway of ANG II-induced sympathoexcitation, including the parvocellular subdivision of the PVN and ventrolateral medulla (Miselis, 1981; Saper and Levisohn, 1983; Sawchenko and Swanson, 1983; Westerhaus and Loewy, 1999; Zardetto-Smith et al., 1993). Numerous Fos immunoreactivity and electrophysiological studies have shown that both intravascular ANG II infusion and application of ANG II directly into the SFO increased activity of MnPO neurons (Gutman et al., 1988; McKinley et al., 1992; Oldfield et al., 1994; Potts et al, 1999; Rowland et al., 1994; Stocker and Toney, 2005;
Tanaka et al., 1986). Additionally, MnPO neurons have been shown to be capable of modulating peripheral sympathetic nervous activity (Westerhaus and Loewy; Stocker and Toney, 2005; Yasuda et al., 2000). A multi-synaptic connection of MnPO neurons to the peripheral sympathetic nervous system has been shown (Westerhaus and Loewy, 1999), as well as a significant correlation between spontaneous discharge of the MnPO neurons and the cardiac cycle and renal sympathetic nerve activity has been identified (Stocker and Toney, 2005). Lastly, electrical stimulation of the MnPO increased blood pressure and renal sympathetic nerve activity (Yasuda et al., 2000). In the present study, we have found that MnPO neurons are important for chronic hypertension induced by ANG II. Taken together, these findings suggest that MnPO neurons are part of the central sympathoexcitation circuitry activated by circulating ANG II downstream from the SFO. However, whether reduced sympathetic excitation is the mechanism behind the attenuated hypertensive response to ANG II observed in this study is not exactly known, and how MnPO neurons play a role in ANG II-induced central sympathetic excitation awaits further investigation.

A large body of research has established an important role of the MnPO on various physiological effects of ANG II that are believed to be mediated by central neural mechanisms. It has been well established that neural circuitry passing through and/or synapsing within the MnPO is necessary for drinking, vasopressin secretion, and acute blood pressure responses to ANG II (Cunningham et al., 1991; Cunningham et al., 1992; Gardiner and Stricker, 1985; Gutman et al., 1989; Jones, 1988; Lind et al., 1983; Lind and Johnson, 1982; Mangiapane et al., 1983; O’Neill and Brody, 1987). Recently, we have shown that electrolytic lesion of the MnPO attenuated increased pressure responses
to chronic intravenous ANG II infusion (Ployngam and Collister, 2007). However, it was not clear whether neural cell bodies of the MnPO or fibers of passage were responsible for the observed blunted response to ANG II. In the current study, we have added to our previous findings of the specific and important role of MnPO neurons in the chronic hypertensive effect of ANG II. In conclusion, the present study and our previous findings (Ployngam and Collister, 2007) provide strong evidence that necessitate MnPO neurons in the central neural circuitry of chronic ANG II induced hypertension.

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Figure 1: Representative photomicrographs of 40 µm coronal sections from an ibotenic lesioned rat (iMnPOx) and a sham lesioned rat (Isham). Sections of anterior part of the MnPO from iMnPOx (A) and Isham (B). Sections of dorsal (*) and ventral (#) parts of the MnPO from iMnPOx (C) and Isham (D). Sections of the SFO from iMnPOx (E) and Isham (F). Sections of the OVLT from iMnPOx (G) and Isham (H). Note degenerating neurons and synapsing fibers are stained with fluorescent stain Fluoro-jade-C in the entire MnPO in iMnPOx (panels A and C) compared to an absence of stained area in the Isham (panels B and D). No fluorescence stain was present in the SFO and OVLT in both iMnPOx and Isham (panels E-H). AC=Anterior commissure.
Figure 2: Average 24 h mean arterial pressure (MAP) and heart rate (HR) during control period, treatment, and recovery periods in ibotenic acid lesioned rats (iMnP0x) and sham lesioned rats (Sham). *P <0.05 between groups.
Figure 3: Average 24 h water intake, urine output, and water balance during control, treatment, and recovery periods in iMnPOx and Isham lesioned rats.
Figure 4: Average 24 h sodium intake, sodium excretion, and sodium balance during control, treatment, and recovery periods in iMnPOx and Isham lesioned rats.
Figure 5: Average 24 h mean arterial pressure (MAP) during control period, treatment, and recovery periods in ibotenic acid lesioned rats (iMnPOx) and electrolytic lesioned rats (eMnPOx) (between-group difference, $F = 9.63, P = 0.011$; between-day difference, $F = 45.47, P < 0.001$; group-day interaction, $F = 3.69, P = 0.022$). *$P <0.05$ between groups.
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CHAPTER IV

Role of the Median Preoptic Nucleus in the Chronic Hypotensive Effect of Losartan in Sodium-Replete Normal Rats
Role of the Median Preoptic Nucleus in the Chronic Hypotensive Effect of Losartan in Sodium-Replete Normal Rats

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Abstract

We have shown previously that the chronic hypotensive effect of the angiotensin II type I receptor blocker, losartan, is mediated in part by the subfornical organ (SFO). The median preoptic nucleus (MnPO) has been implicated as part of the neuronal pathway necessary to mediate various central effects of circulating ANG II downstream from the SFO, including acute drinking behavior as well as acute and chronic blood pressure responses. The present study was designed to test the hypothesis that the MnPO is a crucial part of the neural pathway necessary for the chronic hypotensive effect of losartan. To test this hypothesis, male Sprague-Dawley rats were subjected to either sham (SHAM) or electrolytic lesion of the MnPO (MnPOx). Rats were instrumented with radiotelemetric transducers and aortic flow probes for the continuous measurement of mean arterial pressure (MAP) and heart rate, and cardiac output (CO), respectively. Total peripheral resistance (TPR) was calculated from MAP/CO. After 3 days of baseline measurements, rats were infused intraperitoneally with losartan (10 mg/kg/d) via osmotic minipump at a rate of 5 µl/min. By day 9 of losartan treatment, MAP had decreased 34±2 mmHg in MnPOx rats (n=9), but MAP of SHAM lesioned rats (n=8) had only decreased 24±3 mmHg. These findings were accompanied by a greater decrease of TPR in MnPOx rats (MnPOx, -0.464 mmHg/ml/min; SHAM, -0.237 mmHg/ml/min), while CO remained unchanged throughout the protocol. These results do not support the hypothesis that an intact MnPO is necessary to mediate the full chronic hypotensive effect of losartan in normal rats. Instead, they appear to suggest that the MnPO may play an important role in buffering the profound hypotension induced by losartan.
Introduction

Disorders of the renin angiotensin system (RAS) and its interaction with other blood pressure control systems are thought to be involved in the pathogenesis of many forms of experimental and clinical hypertension (DeForrest et al., 1982; Hackam et al., 2007; Imamura et al., 1995; Romero and Reckelhoff, 1999). During the past several decades, RAS blockers such as angiotensin converting enzyme inhibitors (ACEi) and angiotensin II subtype 1 receptor antagonists (AT\textsubscript{1} antagonists), have become one of the first line therapies for a wide variety of hypertension. However, the mechanisms by which RAS blockers cause blood pressure to decrease, especially during a condition of normal or low RAS activity, have not been completely understood, suggesting that our knowledge of how the endogenous RAS functions and interacts with other blood pressure control systems remains incomplete.

Much evidence supports a pivotal role of the endogenous RAS in tonic sympathetic drive and basal blood pressure control during normal physiological conditions (Collister and Hendel, 2003; Collister and Osborn, 1998; Sugaya et al., 1995; Tanimoto et al., 1994). Both angiotensinogen and angiotensin II type 1a receptor deficient mice display a varying degree of chronic hypotension (Sugaya et al., 1995; Tanimoto et al., 1994). Either peripheral or central administration of the AT\textsubscript{1} antagonist, losartan, decreases renal sympathetic nerve activity in normotensive rats (Dibona et al., 1996, 1998). Additionally, we and others have shown a significant reduction of blood pressure in sodium replete normal rats after chronic blockade of RAS by AT\textsubscript{1} antagonists (Collister and Hendel, 2003; Collister and Osborn, 1998; Dibona et al., 1996; Soltis et al.,
In fact, we have shown that losartan administration for 10 days is capable of decreasing blood pressure as much as 35 mmHg in normal sodium replete rats (Collister and Hendel, 2003; Collister and Osborn, 1998).

AT$_1$ receptor antagonists probably exert their hypotensive effects by inhibiting multiple cardiovascular actions of ANG II, including renal sodium and water retention (Hall, 1986; Krieger and Cowley, 1990), vasoconstriction (Brown et al., 1981), increased aldosterone production (Fredlund et al., 1975; Hajnoczky et al., 1992), sympathoexcitation (Brooks and Osborn, 1995; Fink, 1997; Reid, 1992), and vascular hypertrophy (Griffin et al., 1991). Among these possible mechanisms, blocking of the central sympathetic excitatory effect of circulating ANG II is believed to be crucial for the chronic hypotensive effect of AT$_1$ antagonists (Dibona et al., 1996; Gorbea-oppliger and Fink, 1994). It has been well established that central effects of circulating ANG II are initiated by interaction of ANG II and AT$_1$ receptors located in circumventricular organs (CVOs) (Brody et al., 1978; Collister and Osborn, 1998; Collister and Hendel, 2003; Fink et al., 1987; Mangiapane and Simpson, 1980), which are specialized structures in the brain deficient of the blood brain barrier (BBB). Among CVOs, the area postrema (AP) and subfornical organ (SFO) appear to be important in tonic blood pressure control by endogenous ANG II, as our previous studies have shown that lesions of either of these CVOs attenuate hypotension induced by chronic intravenous losartan infusion in normal rats (Collister and Osborn, 1998; Collister and Hendel, 2003). However, the central neuronal pathway(s) essential for the hypotensive effect of this AT$_1$ antagonist, downstream from these two CVOs, has not been completely elucidated.
A large body of research has implicated the median preoptic nucleus (MnPO), a group of neurons located in the lamina terminalis behind BBB, in the neural pathway that mediates central sympathetic excitation and blood pressure effects of ANG II. The MnPO receives reciprocal inputs from the SFO, and other brain regions proposed to contribute to the central neuronal pathway of ANG II-induced sympathoexcitation, including the parvocellular subdivision of the hypothalamic paraventricular nucleus and ventrolateral medulla (Miselis, 1981; Saper and Levishon, 1983; Sawchenko and Swanson, 1983; Westerhaus and Loewy, 1999; Zardetto-Smith et al. 1993). Likewise, multisynaptic connections have been shown between the MnPO and the peripheral sympathetic nervous system (Westerhaus and Loewy, 1999). Numerous Fos immunoreactivity and electrophysiological studies have shown that both intravascular ANG II infusion and application of ANG II directly into the SFO increased activity of MnPO neurons (McKinley et al., 1992; Oldfield et al., 1994; Potts et al, 1999; Rowland et al., 1994; Stocker and Toney, 2005; Tanaka et al., 1986). An attenuation of the acute blood pressure response to intravenous ANG II has also been shown in rats with lesions of the MnPO (Lind and Johnson, 1982; O’Neill and Brody, 1987). Recently, we have demonstrated that the chronic increased blood pressure response to 10 days ANG II infusion is significantly attenuated in rats with either electrolytic or excitotoxin lesions of the MnPO (Ployngam and Collister, 2007, 2008). Taken together, it is tempting to propose that the MnPO participates in the neuronal pathway that tonically drives basal sympathetic activity and blood pressure during normal physiological conditions, and is therefore likely involved in mediating the chronic hypotensive effects of losartan.
In the present study, we hypothesized that the MnPO is a crucial part of the central neuronal pathway necessary for the chronic hypotensive effect of losartan. To test this hypothesis, ibotenic acid lesions were conducted in rats to destroy neurons in the entire MnPO. Subsequently, hemodynamic responses to chronic intraperitoneal infusion of losartan for 14 days were compared between MnPO lesioned rats and their sham lesioned controls.

**Experimental procedures**

All procedures were conducted according to the National Institutes of Health guidelines and approved by the University of Minnesota Institutional Animal Care and Use Committee. Male Sprague-Dawley rats (275-350 g; Charles River Laboratories; Wilmington, Mass) were used.

**Surgical procedures**

Rats were assigned randomly to either a lesion of the median preoptic nucleus (MnPOx) or sham lesion (SHAM) group. Pentobarbital sodium (50 mg/kg) was given intraperitoneally as an anesthetic agent. For antimicrobial prophylaxis, each rats received an intramuscular injection of 4 mg tobramycin. Anesthetized rats were then placed in a Kopf stereotaxic apparatus (Kopf Instruments; Tujunga, California).

Ibotenic acid lesion aimed at the entire MnPO was performed as previously described (Ployngam and Collister, 2008). Briefly, a dorsal midline incision was made through the skin. The Bregma and Lambda landmarks on the skull were leveled in the
same horizontal plane, and a 3 mm-hole centered on the Bregma was drilled through the skull. The two-paired AP and DV coordinates used to locate the MnPO, relative to Bregma and the surface of the sagittal sinus, respectively, were (mm): (-0.4, -6.1), (-0.35, -7.2). An injection cannula (ID, 0.15 mm; OD, 0.30 mm; Plastic Ones, Roanoke, VG) was lowered midline to each coordinate, and 200 nl of ibotenic acid (Sigma Aldrich; St. Louis, MO) in 1 M phosphate buffered saline (5 µg/µl) was injected over a period of 10 min. The injection cannula was left in place for approximately 10 min before it was withdrawn. Sham operations were identical to lesions, except that the cannula was lowered 2 mm less and no acid was infused.

The rats were allowed to recover from the lesion surgeries for at least one week. Then they were instrumented with radio-telemetric pressure transducers (model TA11PA-C40, Data Sciences International; St. Paul, MN) and aortic flow probes (model 2.5PSB, Transonic Systems; Ithaca, NY) for the purpose of continuous 24-h sampling of mean arterial pressure (MAP) and heart rate (HR), and cardiac output (CO), respectively. In this set of implantation surgeries, the rats were induced into anesthesia using pentobarbital sodium (50 mg/kg, IP). They were then intubated, and maintained with 1-2% isoflurane. Artificial ventilation was performed using a small animal respirator (Harvard Apparatus Co; Millis, MA) at a frequency of 55-60 strokes/min and a tidal volume of 2.5-3.5 ml. The pressure transducer was implanted as previously described (Ployngam and Collister, 2008). Additionally, a venous catheter was implanted into the left femoral vein and exteriorized from the skin in the midscapular region. The aortic flow probe was implanted as has been described previously by Fine et al. (2003). Briefly, the rats were positioned in dorsal recumbency. A partial median sternotomy was
performed, and the thoracic cage was retracted to clearly expose the ascending aorta. A blunt dissection was made between the ascending aorta and main pulmonary artery to provide just enough space for the flow probe to be placed around the ascending aorta. The probe cable was passed through the cranial end of the sternotomy wound and exteriorized at the same location as the venous catheter. The thorax was closed and intrathoracic negative pressure was reestablished. The flow probe cable and catheter were passed through a custom-made tether connected to a spring. Blood flow was measured by flowmeter (model TS420 perivascular flowmeter module, Transonic Systems; Ithaca, NY) via an electronic swivel (model SL6C, Kent Scientific; Torrington, CT). Immediately after surgery, the rats were given intravascular ampicillin (15 mg) and tobramycin (4 mg) for antimicrobial prophylaxis, as well as butorphanol tartrate (0.075 mg, IM) for analgesic purpose.

Upon recovery, rats were then placed individually in metabolic cages with telemetry receivers mounted behind. The housing facility was maintained at a temperature of approximately 23 °C with lights on from 7:00 AM-7:00 PM. Rats were given another dose of butorphanol tartrate (0.075 mg) on day one after the surgery. Ampicillin (15 mg, IV) and tobramycin (4 mg, IV) were given daily for another 3 days after surgery. Rats had free access to 1.0% NaCl diet and distilled water. They were allowed to recover at least 10 days after the implantation surgery before the experimental protocol was started.
**Experimental protocol**

The study protocol was started with baseline measurements for 3 days, followed by 14 days of continuous losartan infusion. At the beginning of the losartan infusion, rats were briefly anesthesized with isoflurane and equipped intraperitoneally with an osmotic minipump (model 2ML2, Alzet; Palo Alto, CA) to deliver losartan (10 mg/kg/d dissolved in 0.9% saline) at a delivery rate of 5 µl/h for 14 days. During the experimental protocol, 1.0% NaCl diet and distilled water were provided *ad lib*. Throughout the protocol, MAP, HR, CO, food intake, water intake, urine output were recorded daily in conscious rats in their home cages.

The analog signal of the CO data measured by flowmeter was transformed to digital form using an analog to digital converter (model C11V, Data Sciences International; St. Paul, MN). Throughout the protocol, MAP, HR, and CO signals were sampled and recorded at 500 Hz for 10 sec every 4 min using data acquisition and analysis software (Data Sciences International; St. Paul, MN). By assuming that mean right atrial pressure equals zero, daily total peripheral resistance (TPR) was calculated as a ratio of 24 h averaged MAP and CO. Sodium intake was calculated as a product of total food intake and sodium content in the diet (0.175 mmol/g). Urinary sodium content was measured with an ion-specific electrode (Model Nova 1, Nova Biomedical; Waltham, MA). Urinary sodium excretion was calculated as the product of urine flow rate and urinary sodium concentration.
**Plasma renin activity measurement**

Plasma samples were collected for measurement of plasma renin activity (PRA) at the second day of the control measurement period. 500 µl of whole blood was drawn from the femoral venous catheter and immediately placed into a chilled 1 ml syringe containing 1 mg EDTA in 20 µl. The EDTA blood was centrifuged at 4 °C at 1000 g for 20 min, and plasma was isolated and stored at -70 °C for later radioimmunoassay as previously described (Trostel et al., 1991).

**Lesion verification**

At the completion of the study protocol, all rats were deeply anesthetized with pentobarbital sodium and perfused via carotid artery with 4% paraformaldehyde in phosphate buffered saline. Whole brains were extracted and post-fixed with 4% paraformaldehyde overnight, then transferred to 30% sucrose for another 3 days. Coronal sections (40 µm) of the area containing the MnPO, OVLT, and SFO according to the Atlas of Paxinos and Watson (1998) were made with a freezing microtome (Lipshaw Mfg; Detroit, MI), and mounted on gelatin-coated slides. Sections were allowed to dry overnight and then stained with Fluoro-Jade-C (Chemicon International, Inc.; Temecula, CA), a fluorescent stain that labels degenerating neurons. The fluorojade staining technique was adapted from previously described techniques (McCon and Steward, 2006; Schmued and Hopkins, 2000). Briefly, brain section slides were immersed in 100% ethanol for 3 min, 70% ethanol for 1 min, and then Dh2O for 1 min. The sections were then stained with potassium permanganate for 15 min, rinsed with Dh2O for 1 min, and then immersed in 0.001% Fluoro-jade-C in 0.1% acetic acid for 30 min in the dark. After
rinsing 3 times with Dh₂O for 1 min, sections were allowed to dry overnight, and then immersed 3 times in xylene substitute (Fisher Scientific; Fair Lawn, NJ) for 2 min. Slides were then coverslipped with nonaqueous fluorescent mounting media DPX (Sigma-Aldrich; St. Louis, MO), allowed to dry for 4 hr, then visualized with an Olympus IX70 inverted fluorescence microscope using a FITC-filter (Olympus America; Melville, NY). The MnPOx rats that had at least 90% of the MnPO stained positively with Fluoro-jade-C with no or few fluorescent staining observed in the adjacent areas, including the SFO and OVLT, were included in the analysis.

**Statistical analyses**

Comparisons of each hemodynamic parameter between the MnPOx and sham groups were performed by a two-way ANOVA with repeated measures. The Tukey-Kramer multiple comparison test was used to determine on which specific days of the protocol the groups were different from one another after two-way ANOVA determined significant interaction between two main factors (lesion vs. treatment day). Baseline values were derived from the averages over three days of control measurement. The difference within groups between average baseline value and 14 days of losartan treatment was analyzed by one-way ANOVA followed by dunnett’s multiple comparison with control. PRA between the MnPOx and sham lesion groups was compared using an unpaired Student’s t-test. The level of significance was set at 0.05 for all statistical analyses. Values were presented as mean±SE. All statistical procedures were performed using statistical software (NCSS; Kaysville, UT)
Results

Fluoro-jade-C staining verified successful lesions in 9 MnPOx rats. Data from these 9 MnPOx rats were compared with that from 8 SHAM lesioned rats. Representative examples of sham and MnPOx lesions are shown in Fig. 1. In all MnPOx rats, there was minimal staining of Fluoro-jade-C in adjacent areas of the MnPO. In addition, none of the MnPOx rats included in the analyses had positive staining in the SFO and OVLT.

Plasma renin activity

PRA were measured in 4 MnPOx and 4 sham lesioned rats. At the second control day, there was no difference in PRA between MnPOx and sham lesioned rats (MnPOx, 1.39±0.16 ng ANG I • ml⁻¹ • h⁻¹; SHAM, 1.32±0.11 ng ANG I • ml⁻¹ • h⁻¹).

Cardiovascular responses to losartan infusion

Fig. 2 shows MAP and HR responses throughout the experimental protocol. There were no differences in average MAP between the two groups during the 3 days of baseline measurement (MnPOx, 101±3 mmHg; SHAM, 100±2 mmHg). By day 2 of losartan treatment, MAP in both MnPOx and sham lesioned rats was decreased significantly from baseline (MnPOx, -18.7±2 mmHg; SHAM, -13±1 mmHg). MAP decreased gradually thereafter in both groups, but a greater decrease of MAP was found in MnPOx rats by day 9 of losartan infusion (MnPOx, -34±2 mmHg; SHAM, -24±3 mmHg). This exaggerated hypotensive response in MnPOx continued until the completion of losartan treatment (Fig. 2, top panel).
Average baseline HR was 405±10 beats/min and 402±9 beats/min in MnPOx and sham lesioned rats, respectively (Fig. 2, bottom panel). HR in both MnPOx and sham lesioned rats was significantly increased from baseline on day 1-4 of losartan infusion (day 1: MnPOx, +22±4 beats/min; SHAM, +15±3 beats/min; day 4: MnPOx, +18±2 beats/min; SHAM, +10±2 beats/min), after which HR returned to baseline levels. There were no significant differences in HR between SHAM and MnPOx rats throughout the experimental protocol.

Fig. 3 shows CO and TPR responses throughout the experimental protocol. CO was obtained from 6 MnPOx and 7 sham lesioned rats. The average 3-day control measurement of CO was comparable between the two groups (MnPOx, 95±1 ml/min; SHAM, 99±5 ml/min). CO in both groups during losartan treatment remained comparable to the baselines. Although a subtle decline in CO appears to have occurred in sham lesioned rats during the first few days of losartan infusion, the changes were not found to be statistically significant. In addition, there were no significant differences in CO between SHAM and MnPOx rats throughout the experimental protocol (Fig. 3, top panel).

There were no significant differences in TPR between SHAM and MnPOx lesioned rats during the 3-day baseline measurement (MnPOx, 1.10±0.04 mmHg/ml/min; SHAM, 1.01±0.05 mmHg/ml/min) (Fig. 3, bottom panel). By day 2 of losartan infusion, although TPR in both MnPOx and sham lesioned rats were decreased significantly from baseline levels, the decrease was greater in MnPOx compared to sham lesioned rats (MnPOx, -0.219±0.002 mmHg/ml/min; SHAM, -0.069±0.001 mmHg/ml/min). Both
groups continued to show progressive decreases in TPR through the completion of losartan treatment. By day 14 of losartan treatment, TPR decreased 0.417±0.002 mmHg/ml/min in MnPOx rats, but had decreased only 0.260±0.003 mmHg/ml/min in sham lesioned rats.

**Sodium and water balance responses**

Fig. 4 shows water intake, output, and balance data. The average baseline water intake in MnPOx rats was 22±1ml/d and in sham lesioned rats was 24±1 ml/d. Water intake and urine output decreased significantly in both groups during the first 2 days of losartan treatment. However, in both groups, water balance remained comparable to the baseline control level throughout losartan infusion period. There were no significant differences of water intake, urine output, and water balance between the two groups throughout the experimental protocol.

Fig. 5 shows sodium intake, excretion, and balance data. The average baseline sodium intake was 4.2±0.2 mEq/d in MnPOx rats and 4.2±0.2 mEq/d in sham lesioned rats. Sodium intake and excretion decreased significantly from average baseline control on day 1-6 of losartan infusion in both groups. However, sodium balance in both the MnPOx and sham lesioned rats remained comparable with baseline control level throughout losartan infusion period. There were no significant differences of the sodium intake, excretion, and balance between the two groups throughout the experimental protocol.
Discussion

The present study was designed to investigate whether the MnPO participates in mediating basal blood pressure control by ANG II. In line with our previous studies (Collister and Hendel, 2003; Collister and Osborn, 1998), we have shown here a profound decrease of blood pressure during chronic losartan treatment. These findings suggest that a basal level of ANG II acting at AT$_1$ receptors is crucial to maintain basal blood pressure in normal physiological conditions. However, since losartan-induced hypotension was not found to be attenuated in MnPO lesioned rats, the current results do not support the hypothesis that the MnPO participates in the central neural circuitry that mediates the chronic hypotensive effect of losartan. Previously, we have established a crucial role of the MnPO in hypertension induced by chronic exogenous ANG II administration (Ployngam and Collister, 2007, 2008). Together, our previous and present studies seem to suggest that the MnPO does not play an important part in mediating normal blood pressure control by basal levels of circulating ANG II; however, neurons in this nucleus appear to participate in the central neural mechanisms of ANG II-induced hypertension.

Studies have shown that losartan at the dosage used in the current study (10 mg/kg/min), is capable of crossing BBB and binding to the AT$_1$ receptors located at several brain structures, including the MnPO, hypothalamic paraventricular nucleus (PVN), and rostral ventrolateral medulla (Polidori et al. 1996; Zhuo et al., 1994). Therefore, besides blocking AT$_1$ receptors at the CVOs, losartan might exert part of its
hypotensive effect by direct blockade of the intrinsic brain RAS and/or angiotensinergic pathways. MnPO neurons possess all components of the RAS. They have been found to be dense with AT$_1$ receptors (Lenkei et al., 1998), and are activated by direct application of ANG II (Bai and Renaud, 1998). Additionally, studies have shown that blockade of brain RAS by intracerebroventricular (ICV) injection of losartan reduces renal sympathetic nerve activity, improves cardiac and arterial baroreflex regulation of renal nerve activity (Dibona et al., 1996, 1998), and decreases arterial blood pressure (Yang et al., 1996). Interestingly though, it has been shown that the ICV route of AT$_1$ antagonist administration cannot reach and block the specific set of AT$_1$ receptors activated by circulating ANG II in the CVOs (Bruner and Fink, 1985). Therefore, the sympathetic nervous inhibition and hypotensive effects of ICV losartan demonstrated by Dibona et al. (1996, 1998) and Yang et al., (1996) are likely not accounted for by blocking circulating ANG II actions at the CVOs, but rather by blocking intrinsic brain RAS and/or central angiotensinergic pathways. As a result, intrinsic brain RAS and/or central angiotensinergic pathway may be involved at some degree with tonic sympathetic drive and basal blood pressure control. However, evidence obtained from this study does not appear to implicate the MnPO in neither brain intrinsic RAS nor central angiotensinergic pathways contributing to basal blood pressure control.

In line with our previous studies (Collister and Osborn, 1998; Collister and Hendel, 2003), blood pressure in both the MnPOx and sham lesioned groups decreased profoundly by day 2, and thereafter the decrease occurred gradually and reached steady state by day 7 of losartan infusion. Although daily water and sodium intakes were decreased in both the MnPOx and sham lesioned rats during the first few days (water
intake, day 1-2; sodium intake, day 1-6) of the losartan infusion, probably due to discomfort after minipump-implantation surgery, these changes are less likely to have had a significant impact on the arterial pressure, since urine output and sodium excretion were proportionally decreased, and there were no changes in water and sodium balances during losartan treatment in both the MnPOx and sham lesioned groups. Additionally, unique to this study is the measurement of CO to gain insight into the hemodynamic changes that accompany the hypotension induced by chronic losartan treatment. The novel findings of this study are that chronic losartan infusion does not much effect CO, as no significant change of CO was observed at any time throughout the study protocol in both groups. On the other hand, TPR decreased significantly by day 2 of losartan infusion, followed by a gradual decrease until the end of the study. In fact, the time course of the change observed in TPR as shown in Fig. 3 (bottom panel) was virtually identical to that seen in the arterial pressure response. Therefore, the present findings extend our previous observations that hypotension induced by chronic peripheral infusion of losartan is associated with a chronic gradual decline of TPR.

Blockade of the peripheral and central effects of ANG II by losartan could both play a part in the fall of TPR and arterial pressure observed in the present study. Peripherally, losartan may decrease TPR by impairing ANG II- potentiated sympathoadrenergic vasoconstriction, a mechanism shown to be crucial for maintaining vascular tone in pithed (Kaufman and Vollmer, 1985) and spontaneous hypertensive rats (Moreau et al., 1993), as well as normotensive rats (Qiu et al., 1994; Soltis et al., 1993). However, a large body of evidence seems to argue against an important contribution of this peripheral effect on the chronic hypotensive effect of losartan. Previously, it has been
shown that a low dose of losartan (1 mg/kg/day), which blocks the acute vasoconstrictor actions of ANG II, has no effect on arterial pressure chronically (Hornfeldt et al., 1989). In addition, losartan has been shown to completely block (within 5 minutes) the fast pressure effect of ANG II believed to be mediated solely by its vasoconstriction effect (Gorbea-oppliger et al., 1994). Our previous studies (Collister and Osborn, 1998; Collister and Hendel, 2003) and present findings have shown that the decrease in blood pressure did not reach a steady state until day 7 of chronic losartan infusion. In addition, the attenuated effect of either AP or SFO lesion on the hypotensive effect of losartan was not detected until a few days after losartan infusion had started. Therefore, due to this chronic timeline, blockade with losartan on the central sympathoexcitatory effect of ANG II likely plays a significant part in the decreased TPR observed in the present study and this central effect of losartan seems to become more important during the chronic phase of losartan-induced hypotension.

The slow inhibitory effect of losartan on central sympathetic excitation induced by ANG II could involve two delayed steps. The first delayed step is the time needed to reverse central sympathetic excitatory effects of ANG II. It has been shown that the slow pressor effect of ANG II, known to be mediated by central neuronal mechanisms, has a slow reversal time. While arterial pressure has been shown to return to baseline levels within a few minutes after stopping a brief ANG II infusion, several hours were required for arterial pressure to return to pre-ANG II level after a 7 day-ANG II infusion (Brown et al., 1981). In addition, Gorbea-Oppliger et al. (1994) has shown a longer time course for losartan to block hypertension induced by chronic ANG II infusion (hours) than that induced by an acute bolus injection of ANG II (minutes). The second delayed step could
involve the time spent for losartan to gain more access and fully block brain AT\textsubscript{1} receptors. This is believed to include time required for the formation of losartan’s active metabolize, EXP3174, which has been shown to be more potent and readily accessible to brain AT\textsubscript{1} receptors than its precursor (Polidoli et al., 1996). In addition, it appears that chronic infusion of AT\textsubscript{1} receptor antagonists is required to obtain more complete blockade of brain AT\textsubscript{1} receptors. Studies show that 14 days of infusion of the AT\textsubscript{1} receptor blocker, candesartan, results in approximately 40\% higher inhibition of brain AT\textsubscript{1} receptors than the acute dose (Nishimura et al., 2000; Song et al., 1999). In fact, Culman et al. (1999) has demonstrated that acute IV injection of losartan never completely blocks the hypertensive effect of ICV ANG II even at a dose as high as 100 mg/kg. Therefore, we believe that complete blockade of brain AT\textsubscript{1} receptors did not occur in the present study until after a few days of losartan infusion. With the two delay times added, it seems likely to explain the slowly progressive hypotensive effect that does not reach a steady state of arterial pressure until day 7 of losartan infusion.

In the present study, MnPOx rats actually showed a greater response to the hypotensive effect of losartan than sham lesioned rats. Indeed, arterial pressure in the MnPOx rats was significantly lower than that of shams by day 9 until the last day of losartan infusion. The same trend was observed in the TPR response as well. It is not clear why arterial pressure was lower in the MnPOx rats compared to sham lesioned rats toward the last several days of losartan infusion in the present study. We have previously shown differential responses to chronic losartan treatment in rats with different level of basal activity of the RAS (Collister and Nahey, 2008). As shown in the results section in the present study, this cannot be explained by a difference in the basal activity of RAS.
between the MnPOx and sham lesioned rats, as basal PRA was similar between groups. However, this finding suggests that the MnPO might be participating in central neuronal mechanisms protective against profound hypotension induced by losartan. This view is also supported by the comparable response of HR observed in the MnPOx versus sham lesioned rats, even though MAP was lower in the MnPOx rats during the last several days of losartan infusion. Studies have shown that the MnPO receives reciprocal inputs from the nucleus of the solitary tract and ventrolateral medulla, cardiovascular centers in the hindbrain known to receive and respond to baroreceptor inputs from the periphery (Ciriello et al., 1985; Gieroba and Blessing, 1993; Knuepfer et al., 1985; Li et al., 1992; Saper and Levesohn, 1983; Tanaka et al., 1992b). In addition, compelling evidence suggests cross communication between the MnPO and both central and peripheral baroreflex centers. (Sakamaki et al.; 2004; Tanaka et al., 1992a; Tanaka et al., 1993; Tanaka et al., 2003). With regard to hypotension, Fos immunocytochemistry studies have shown activation of MnPO neurons during nitroprusside-induced hypotension, but not during hypertension induced by phenylephrine infusion (Li and Dampney, 1994). Numerous reports suggest that as a reflex response to hypotension, MnPO neurons are synaptically stimulated by the A1 noradrenergic cell groups in the ventrolateral medulla which are in turn activated by afferent arterial baroreflex inputs from the periphery (Li and Dampney, 1994; Potts et al., 2000; Tanaka et al., 1992a,b; 2003). Among these are studies showing that hypovolemia (Potts et al., 2000) and hypotension (Li and Dampney, 1994) activate ventrolateral medulla neurons, which have been shown to be predominantly catecholaminergic cells. Moreover, hemorrhage induced a significant increase of noradrenaline release in the MnPO, and this response was attenuated by
previous injection of lidocaine into the A1 noradrenergic region (Tanaka et al., 2003). Electrical stimulation of the A1 noradrenergic region in the ventrolateral medulla induces excitatory responses by MnPO neurons antidromically identified as projecting to the PVN (Tanaka et al., 1992b). This excitation of MnPO neurons is blocked by the $\alpha$-adrenergic receptor antagonist, phentolamine. In addition, studies have shown that noradrenaline injected directly into the MnPO causes elevation of blood pressure and bradycardia (da Silva et al., 1995). Taken together with the present findings, we propose that the MnPO likely participates in the central baroreflex mechanisms necessary for buffering against the profound decrease of blood pressure during chronic losartan infusion. More definitive conclusions regarding this role of the MnPO will require future studies determining chronic pressure response to losartan in baroreflex deficient models.

In summary, our previous studies have established a crucial role of the MnPO in the chronic hypertensive effect of exogenous peripheral administration of ANG II. However, the present findings do not support the role of MnPO in normal blood pressure control by endogenous ANG II. Furthermore, a novel finding in the present study was that the profound hypotension induced by chronic losartan treatment was solely accounted for by a significant reduction of TPR. In addition, MAP responses to losartan were found to be exaggerated in the MnPOx compared to sham lesioned rats. These findings therefore exclude the MnPO neurons from the central neuronal mechanism(s) that mediates the chronic hypotensive effect of losartan; however, they do suggest an important role of the MnPO in baroreflex buffering of losartan-induced hypotension.
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**Figure 1**: Representative photomicrographs of 40 µm coronal sections from an ibotenic lesioned rat (MnPOx) and a sham lesioned rat (SHAM). Sections of the anterior part of the MnPO from MnPOx (A) and SHAM (B). Sections of dorsal (*) and ventral (#) parts of the MnPO from MnPOx (C) and SHAM (D). Sections of the SFO from MnPOx (E) and SHAM (F). Sections of the OVLT from MnPOx (G) and SHAM (H). Note degenerating neurons and synapsing fibers are stained with Fluoro-Jade-C in the entire MnPO in MnPOx (panels A and C) compared to an absence of stained area in the SHAM (panels B and D). No fluorescence stain was present in the SFO and OVLT in both MnPOx and SHAM (panels E–H). AC = anterior commissure.
Figure 2: Average 24-h mean arterial pressure (MAP) and heart rate (HR) during control measurement and losartan treatment in median preoptic lesioned rats (MnPOx) and sham lesioned rats (SHAM). * $P < 0.05$ between groups.
**Figure 3**: Average 24-h cardiac output (CO) and total peripheral resistance (TPR) during control measurement and losartan treatment in median preoptic lesioned rats (MnPOx) and sham lesioned rats (SHAM). * P < 0.05 between groups.
Figure 4: Average 24 h water intake, urine output, and water balance during control measurement and losartan treatment in median preoptic lesioned rats (MnPOx) and sham lesioned rats (SHAM).
Figure 5: average 24-h sodium intake, sodium excretion, and sodium balance during control measurement and losartan treatment in median preoptic lesioned rats (MnPOx) and sham lesioned rats (SHAM).
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CHAPTER V

Role of the Median Preoptic Nucleus in Arterial Pressure Regulation and Sodium and Water Homeostasis during High Dietary Salt Intake.
Role of the Median Preoptic Nucleus in Arterial Pressure Regulation and Sodium and Water Homeostasis during High Dietary Salt Intake.

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Abstract

Angiotensin II (ANG II) is potentially an important peripheral signal that modulates appropriate central sympathetic output and maintains normal arterial pressure during high salt intake. The median preoptic nucleus (MnPO) receives reciprocal inputs from the subfornical organ (SFO) and organum vasculosum of the lamina terminalis (OVLT), the circumventricular organs that have been shown to be necessary in multiple central effects of circulating ANG II involving sodium and water homeostasis. Taken together, we hypothesized that the MnPO is crucial as part of central neuronal mechanisms mediating blood pressure control by ANG II during chronic high dietary salt intake. Male Sprague Dawley rats were randomly assigned to either sham (SHAM), or electrolytic lesion of the MnPO (MnPOx). After 7-day recovery, rats were instrumented with radiotelemetric transducers and aortic flow probes for the measurement of mean arterial pressure (MAP) and heart rate (HR), and cardiac output (CO), respectively. Femoral venous catheters were also implanted to collect blood for the measurements of plasma osmolality and sodium concentration, as well as plasma renin activity. Rats were given another 10 days to recover, and then were subjected to a 28-day study protocol that included: a 7-day control period (1.0% NaCl), followed by 14 days of high salt (4.0% NaCl), and a 7-day recovery period (1.0% NaCl). Despite a slight increase of MAP observed in both the MnPOx (n=12) and sham lesioned rats (n=8) during the high salt period, there were no significant differences of MAP, HR, and CO between the two groups throughout the study protocol. These findings do not support the hypothesis that the MnPO is necessary to maintain normal blood pressure during high dietary salt intake. However, MnPOx rats showed less sodium balance than sham lesioned rats during the
first 4 days of high salt intake. Although, these results may be explained partly by plasma hyperosmolarity and hypernatremia observed in the MnPOx rats, they also shed light on the role of the MnPO in the central neuronal control of renal sodium handling during chronic high dietary salt intake.

**Introduction**

It has been well established that a decrease of sympathetic nervous activity serves as a normal physiological response to high dietary salt intake (Brooks and Osborn, 1995; Brooks et al., 2001; Luft et al., 1979). Failure of this homeostatic mechanism has been found to accompany salt-sensitive hypertension in a subset of essential hypertensive patients and several animal models of hypertension (Bouvier et al., 1987; Campese et al., 1982; Fujita et al., 1990; Huang and Wang, 2001; Jin et al., 1991). In addition, salt-sensitive hypertension can be induced in rats by clamping of sympathetic nervous activity using α-adrenergic blockade (Osborn et al., 1993). Together, these reports provide strong evidence of the link between the sympathetic nervous activity and salt intake. It remains unclear, however, how dietary salt chronically modulates sympathetic nervous activity.

High salt intake is thought to exert both inhibitory and excitatory effects on the sympathetic nervous system (Brooks et al., 2001, 2005; Toney et al., 2003). Increased plasma sodium and/or hyperosmolality secondary to high dietary salt intake are believed to induce an excitation of the sympathetic nervous system via central sodium/osmo-receptor activation (Adams et al., 2007; Chen and Toney, 2001; Toney et al., 2003). On
the other hand, sodium and water retention following high salt intake is known to suppress circulating angiotensin II (ANG II) levels resulting in an inhibition of central sympathetic nervous output. Activation of the arterial baroreflex following salt-induced volume expansion is also known to be another mechanism that participates in an inhibitory pathway of sympathetic nervous activity (Osborn and Hornfeldt, 1998). A balance of these central neural mechanisms is believed to be crucial in maintaining normal blood pressure during changes in dietary salt. However, it remains unclear how and where such balance could be achieved, leading to appropriate levels of central sympathetic outflow during high salt intake.

Several brain regions are thought to be integrative sites where peripheral ANG II, osmotic, and baroreflex signals converge and influence central sympathetic activity. Most prominent among these is the median preoptic nucleus (MnPO), a group of hypothalamic neurons located along the rostral border of the third ventricle. Firstly, neuroanatomical studies have revealed reciprocal connections between the MnPO and multiple areas known as primary central sites that receive peripheral ANG II, osmolality and baroreflex information. These include the subfornical organ (SFO) and organum vasculosum of the lamina terminalis (OVLT), two circumventricular organs shown to be important as primary central targets of circulating ANG II and osmotic substances (Miselis, 1981; Saper and Levisohn, 1983). In addition, MnPO neurons receive neuronal inputs from the nucleus of the solitary tract and ventrolateral medulla, central sites of peripheral baroreceptor afferents (Ciriello et al., 1985; Gieroba and Blessing, 1993; Li et al., 1992; Saper and Levesohn, 1983; Tanaka et al., 1992). Secondly, uni- or multisynaptic connections have been shown between the MnPO and peripheral
sympathetic nervous system (Westerhaus and Loewy, 1999), as well as several brain regions proposed to contribute to central sympathetic excitation, including the parvocellular subdivision of the hypothalamic paraventricular nucleus and ventrolateral medulla (Miselis, 1981; Saper and Levishon, 1983; Sawchenko and Swanson, 1983; Zardetto-Smith et al. 1993). Thirdly, numerous electrophysiological and Fos immunocytochemistry studies have shown that peripheral administration of both ANG II and hypertonic saline activate the MnPO neurons, importantly a subset of which responds to both of these stimuli (Stocker and Toney, 2005; McKinley et al., 1992; Oldfield et al., 1994; Potts et al., 1999; Rowland et al., 1994). Interestingly, the activity of the MnPO neurons, including those sensitive to ANG II and hyperosmolality, are also affected by direct stimulation of peripheral baroreflex afferents, as well as baroreflex challenges such as hemorrhage (Stocker and Toney, 2007; Tanaka et al., 1993, 2003). Fourthly, a large body of lesion studies has implicated the MnPO in the central neuronal pathway that mediates drinking and vasopressin secretion, central neuronal effects elicited by both peripheral ANG II and hypertonic saline (Cunningham et al., 1992; Gardiner et al., 1985; Lind and Johnson, 1982; Mangiapane et al., 1983). Finally, we have shown previously that the MnPO is necessary for the chronic hypertensive effect of ANG II, suggesting a contribution of the MnPO in the sympathoexcitatory pathway that likely mediates chronic ANG II-induced hypertension (Ployngam and Collister, 2007, 2008). Although the central neural pathway mediating sympathetic excitation following central sodium/osmo-receptor activation has not been completely elucidated, taken together with previous observations, it seems probable that the MnPO is a mutual site where parallel sympathoexcitatory pathways activated by ANG II and hyperosmolality converge and are
integrated. As a result, we proposed that the MnPO is important in the central neuronal mechanisms that control sympathetic nervous activity, and therefore blood pressure during changes in dietary salt intake.

In the present study, the role of the MnPO in chronic blood pressure control during high salt intake was investigated. We hypothesized that the MnPO is necessary for arterial pressure to be maintained in the normal range during high dietary salt intake. To test this hypothesis, rats were subjected to either MnPO lesion (MnPOx) or sham lesion operation. Subsequently, blood pressure and cardiac output responses to 14 days of 4% dietary salt were continuously monitored in MnPOx and sham lesioned rats.

**Experimental procedures**

All procedures followed the Guide for Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee of the University of Minnesota.

**Surgical procedures**

Male Sprague-Dawley rats (Charles River Laboratories; Wilmington, Mass) weighing 300-350 g were randomly assigned to either electrolytic lesion of the MnPO (MnPOx) or sham lesion (SHAM) group. Rats were anesthetized with pentobarbital sodium (50 mg/kg, IP) and then positioned in a Kopf stereotaxic apparatus (Kopf Instruments; Tujunga, California). They received tobramycin (4 mg, IM) for antimicrobial surgical prophylaxis. Electrolytic lesions of the MnPO were performed as
previously described (Ployngam and Collister, 2007). Briefly, a dorsal midline incision was made through the scalp, and Bregma and Lambda were leveled. A 3-mm hole centered on Bregma was drilled through the skull. A teflon-insulated monopolar tungsten electrode with 1.5 mm exposed at the tip was inserted midline into 4 predetermined coordinates. The coordinates used, caudal to Bregma and ventral to the surface of sagittal sinus, respectively, were (mm): (-0.25,-7.4), (-0.25,-7.6), (-0.4,-6.1), (-0.4,-7.2). At each coordinate, a 1-Ma current was passed through the electrode for 5 sec. Sham lesion surgeries were similar to electrolytic lesion, except that all ventral coordinates were 2 mm less with no current passed.

Rats were allowed to recover for 7 days. Then they were implanted with radiotelemetry blood pressure transducers (model TA11PA-C40, Data Sciences International; St. Paul, MN) and aortic flow probes (model 2.5PSB, Transonic Systems; Ithaca, NY) for continuous 24-h sampling of mean arterial pressure (MAP) and heart rate (HR), and cardiac output (CO), respectively. The rats were induced with pentobarbital sodium (50 mg/kg, IP), and anesthetically maintained with 1-2% isoflurane via endotracheal tube. Artificial ventilation was provided using a small animal respirator (Harvard Apparatus Co; Millis, MA) at a tidal volume of 2.5-3.5 ml, and frequency of 55-60 strokes/min. The pressure transducer was implanted as has been described previously (Ployngam and Collister, 2007). The venous catheter was implanted into the left femoral vein and exteriorized from the skin between the scapulae. The implantation technique for the aortic flow probe was adopted from the previous report (Fine et al, 2003). Briefly, a median sternotomy was made in rats positioned in dorsal recumbency. A blunt dissection was performed between ascending aorta and main pulmonary artery to
create a small space to place a flow probe around the ascending aorta. The probe cable was passed through the sternotomy wound and exteriorized at the interscapular region. The thoracic wound was closed and intrathoracic negative pressure was reestablished. The flow probe cable and venous catheter were passed through a mesh tether and its attached spring. Promptly after surgery, ampicillin (15 mg, IV) and tobramycin (4 mg, IV), and butorphanol tartrate (0.075 mg, IM) were given for antimicrobial prophylaxis and analgesia, respectively.

Rats were then placed individually in metabolic cages with telemetry receivers mounted behind. Probe cables were connected to a flowmeter (model T402, Transonic System; Ithaca, NY) via electronic swivels (model SL6C, Kent Scientific; Torrington, CT). The housing facility was maintained at a temperature of approximately 23 °C with 12:12 light:dark cycle (light on at 7:00 am). Ampicillin (15 mg, IV) and tobramycin (4 mg, IV) were given once daily for another 3 days after surgery. Another dose of Butorphanol tartrate (0.075 mg, IV) was given at day one after surgery. Rats were allowed to recover at least 10 days before the experimental protocol was started. During this period, 1.0 % NaCl diet and distilled water were provided *ad lib*.

**Experimental protocol**

The 28-day diet protocol was as follows: a 7 day-control period of normal salt diet (1.0% NaCl), a 14 day-period of high salt diet (4.0% NaCl), and a 7 day-recovery period of normal salt diet (1.0% NaCl). Throughout the protocol, rats were provided with *ad lib* diet and distilled water.
MAP, HR, and CO data were sampled and recorded at 500 Hz every 4 min for 10 sec using data acquisition and analysis software (Dataquest ART version 2.2, Data Sciences International; St. Paul, MN). 24 h-average TPR was calculated as a ratio of 24 h average of MAP and CO, assuming that mean right atrial pressure was equal to zero.

Daily food intake, water intake, and urine output data were collected at the same time every day (2:00 PM). Sodium intake was calculated as a product of total food intake and sodium content in the diet (1.0% NaCl diet, 0.175 mmol/g; 4.0% NaCl diet, 0.7 mmol/g). Sodium excretion was calculated as a product of urine output and urine sodium concentration. Urine sodium concentration was measured with an ion-specific electrode (Nova Biomedical; Waltham, MA).

**Measurement of plasma renin activity (PRA)**

Plasma samples for PRA measurement were collected at 10:00 pm on day 4 of the control period and day 7 of high salt period. Whole blood (500 µl) was drawn from the femoral venous catheter and promptly mixed with 1 mg EDTA (20 µl) in a chilled 1 ml syringe. 500 µl of 0.9% NaCl was infused through the intravenous catheter to replace the volume of blood removed. Whole blood was centrifuged at 4 °C at 1000 g for 20 min. Plasma was isolated and stored at -70 °C for later radioimmunoassay as previously described (Trostel et al., 1991).

**Measurement of plasma osmolality and sodium concentration**

Plasma samples were collected for measurements of plasma osmolality and sodium concentration at 10:00 pm on day 6 of the control and recovery periods, and day
12 of the high salt period. 500 µl of whole blood was drawn and placed into blood tubes containing lithium heparin. The tube was gently mixed and centrifuged at 1000 g for 20 min at 4°C. Plasma was promptly isolated and stored at -70 °C.

Plasma sodium concentration was later measured using a sodium analyzer (NOVA Biomedical; Waltham, MA). Plasma osmolality was measured with a vapor pressure osmometer (model 5500, Wescor, Inc.; Logan, UT).

**MnPO lesion verification**

At the completion of the experimental protocol, the rats were anesthetized with pentobarbital sodium (50 mg/kg), and perfused intracardially with heparinized saline (20 U/ml heparin in 0.9% NaCl solution), followed by 4% paraformaldehyde in phosphate buffered saline. Whole brains were removed, and submersed in 4% paraformaldehyde overnight at 4 °C. The brains were then transferred to 30% sucrose in PBS at 4 °C for 3 days. 40 µm sagittal sections of the brains were made using a freezing microtome (Lipshaw Mfg.; Detroit, MI). The sections were mounted on slides, allowed to dry for 1 day, and then stained with cresyl violet. Complete lesion of the MnPO was verified by light microscopy. MnPOx rats were included in the analyses if they had at least 90% of the MnPO ablated, with no or slight damage to adjacent brain regions.

**Statistical analyses**

All statistical procedures were performed with statistical software (NCSS; Kaysville, UT). Two-way ANOVA with repeated measures was conducted to compare each parameter between MnPOx and sham lesion groups. Tukey-Kramer multiple
comparison procedure was carried out to determine days at which the two groups were different from one another. Values from the 7-day control measurement were averaged to obtain baseline values. Then one-way ANOVA followed by dunnett’s multiple comparison with control was performed to determine differences within groups between baseline control and 14 days of high salt intake. Plasma sodium concentration and osmolality, as well as PRA were analyzed with two-way ANOVA followed by Tukey-Kramer multiple comparison test. Differences within groups were determined by dunnett’s multiple comparison with control. A p-value of 0.05 was set as the level of statistical significance for all statistical analyses. All values were presented as mean ± SE.

**Results**

After lesion verification, 12 MnPOx rats and 8 sham lesioned rats were included in the analyses. All of the 12 MnPOx rats had at least 90% damage of the MnPO with no or slight damage in the adjacent area. None of them had damage to the SFO and OVLT. Fig. 1 shows representative examples of sham and MnPOx lesions.

**Plasma renin activity**

PRA in 4 MnPOx and 4 sham lesioned rats is shown in Fig. 2. PRA during the control period (1.0% NaCl diet) in the MnPOx was 1.39±0.16 ng ANG I ml⁻¹ h⁻¹ and in SHAM was 1.32±0.11 ng ANG I ml⁻¹ h⁻¹. By day 7 of the high salt period (4.0% NaCl diet), PRA in the MnPO was 0.29±0.11 ANG I ml⁻¹ h⁻¹ and in SHAM was 0.52±0.11
ANG I •ml⁻¹ • h⁻¹. There were no significant differences in PRA between MnPOx and sham lesioned rats at either normal or high salt periods.

**Plasma osmolality and sodium concentration**

Plasma osmolality in 4 MnPOx and 4 sham lesioned rats is shown in Fig. 3 (top panel). Baseline plasma osmolality during control period (1.0% NaCl diet) was significantly higher in MnPOx compared to sham lesioned rats (MnPOx, 292±2 mOsm/kg H₂O; SHAM, 285±1 mOsm/kg H₂O). Plasma osmolality in MnPOx rats increased significantly from baseline by day 12 of high salt period (4.0% NaCl), while that in sham lesioned rats remained comparable to baseline value (MnPOx, 297±2 mOsm/kg H₂O; SHAM, 287±1 mOsm/kg H₂O). Plasma osmolality in MnPOx rats returned to baseline during the recovery period (1.0% NaCl diet) when diet had been changed back to normal salt.

Plasma sodium concentration in 4 MnPOx and 4 sham lesioned rats is shown in Fig. 3 (bottom panel). The baseline plasma sodium concentration during normal salt period was 147.2±0.5 mEq/l for MnPOx and 145.5±0.6 mEq/l for SHAM. Plasma sodium concentrations were not significantly different between the 2 groups at all 3 time points. However, by day 12 of the high salt period, plasma sodium concentration in MnPOx rats increased significantly from baseline (148.8±0.5 mEq/l), while this value remained unchanged in sham lesioned rats (146.3±0.9 mEq/l).
Cardiovascular responses to high dietary salt intake

Fig. 4 shows MAP and HR responses throughout the experimental protocol. The average baseline MAP of the 7-day control period (1.0% NaCl diet) in MnPOx and sham lesioned rats was 99±1 mmHg and 101±2 mmHg, respectively. The average MAP by the last day of high salt period (4.0% NaCl diet) in MnPOx and sham lesioned rats was 102±1 mmHg and 106±2 mmHg, respectively. MAP responses in MnPOx and sham lesioned rats were not significantly different throughout the study protocol (Fig. 4, top panel). However, MAP responses in both the MnPOx and sham lesioned rats were increased significantly from their average baselines during high salt period (MnPOx, (MnPOx, days 4, 5, 6, 8, 14; SHAM, 5, 8, 12, 13,14). This increase of MAP in both the MnPOx and sham lesion groups also occurred on the first day of recovery period (1.0% NaCl diet).

The average baseline HR of the 7-day control period was 397±10 beats/min and 397±23 beats/min in MnPOx and sham lesioned rats, respectively. There were no significant differences in HR between MnPOx and sham lesioned rats throughout the protocol (Fig 4, bottom panel). In addition, HR in both groups remained comparable to their baseline values during the high salt period.

Fig. 5 shows 24-h average of CO and TPR in MnPOx and sham lesioned rats throughout the study protocol. CO was obtained from 9 MnPOx and 5 sham lesioned rats. Average baseline CO was 90±4 ml/min and 99±7 ml/min in MnPOx and SHAM lesioned rats, respectively. No significant differences in CO were found between MnPOx and
sham lesioned rats throughout the study protocol (Fig. 5, top panel). CO in both groups was not significantly changed by high salt intake.

Average baseline TPR was 1.11±0.04 mmHg/ml/min and 1.00±0.10 mmHg/ml/min in MnPOx and sham lesioned rats, respectively. There were no significant differences in TPR between the two groups throughout the study (Fig. 5, bottom panel). TPR values in both groups during high salt period were not significantly different from the average baselines.

**Sodium and water balance responses to high salt**

Fig. 6 shows daily water balance data. Average baseline water intake during the 7-day control period in MnPOx and sham lesioned rats was 25.0±2.5 ml/d and 25.1±2.3 ml/d, respectively. Average baseline urine output was 9.9±1.5 ml/d and 8.6±0.8 ml/d in MnPOx and sham lesioned rats, respectively. Water intake and urine output increased significantly in both groups during the high salt period. Water intake by the last day of high salt period was 45.1±4.4 ml/d and 46.3±5.1 ml/d, respectively, in MnPOx and sham lesioned rats. However, there were no significant differences between groups in water intake, urine output, and water balance throughout the protocol.

Fig. 7 shows daily sodium balance data. Average baseline sodium intake in MnPOx and sham lesioned rats was 3.8±0.2 mEq/d and 4.4±0.3 mEq/d, respectively. Average baseline sodium excretion was 3.1±0.3 mEq/d and 3.4±0.3 mEq/d in MnPOx and sham lesioned rats, respectively. Sodium intake and excretion increased significantly in both groups when dietary salt was increased. However, while sodium intake in MnPOx rats was significantly lower than that of sham lesioned rats during the high salt period,
sodium excretion was comparable between the two groups. By the end of high salt period, sodium intake was 14.1±0.8 mEq/d and 16.8±1.1 mEq/d in MnPOx and sham lesioned rats, respectively. No significant differences in daily sodium balance were detected between the two groups throughout the study protocol.

In addition to daily water and sodium balance, cumulative values were also calculated in order to detect subtle differences in sodium and water homeostasis between the MnPOx and sham lesioned rats. Cumulative water and sodium balance data is shown in Fig. 8. No significant differences in cumulative water balance were observed between the two groups throughout the study protocol (Fig. 8, top panel). However, although cumulative sodium balance was comparable between the two groups during the 7-day control period, it was found to be higher in sham lesioned rats by day 6 of the high salt period until the end of study protocol (Fig. 8, bottom panel). Since the slope of the cumulative sodium balance plots becomes flatter during the recovery period, the significant differences between groups observed during this period probably were misleadingly influenced by cumulative differences that occurred earlier in the study protocol (Fitts, 2006). Therefore, to avoid a carried over cumulative effect and to determine more specifically which time period subtle differences of sodium balance occurred, statistical analyses using sums of sodium balance from 2 consecutive days of high salt period were also performed and compared with the average values of the control and recovery periods (Fig. 9). These analyses show that MnPOx rats had significantly lower sodium balance during the first 4 days of high salt intake, compared to sham lesioned rats.
Discussion

In the present study, we reasoned that if the integration of neurohumoral signals in the MnPO is necessary for appropriate central sympathetic output and maintenance of arterial pressure during chronic high salt intake, then blood pressure would be abnormally elevated in MnPOx compared to sham lesioned rats. Our results have shown a subtle increase of blood pressure in both the MnPOx and sham lesioned rats during high salt intake; however, no significant difference of arterial pressure was observed between the two groups throughout the study protocol. Therefore, the results of this study do not support the hypothesis that the MnPO is necessary to maintain normal blood pressure during high dietary salt intake.

Compared to those of sham lesioned rats, plasma samples of the MnPOx rats were found to be hypernatremic and hypertonic. In addition, while sham lesioned rats were able to maintain their plasma sodium concentration and osmolality within relatively stable limits when dietary salt was elevated, these parameters were raised significantly in the MnPOx rats during high salt intake. Similar findings have been reported earlier in MnPO lesioned rats that received acute hypertonic saline infusion, and were shown to have impaired ability to secrete vasopressin and oxytocin (Gardiner et al., 1985; Mangiapane et al., 1983). However, it should be noted that in the present study, blood samples were collected at a single time point during the dark period, when the rats were active and eating. Thus, it is not known whether hypertonicity was sustained chronically in the MnPOx rats. Also, any transient elevation of plasma osmolality in the sham
lesioned rats may escape detections. Nevertheless, this finding suggests that postprandial plasma hyperosmolality and hypernatremia were sustained longer in the MnPOx rats.

In the present study, a subtle but statistically significant elevation of arterial pressure was observed in both the MnPOx and sham lesioned rats during the high salt period (4.0% NaCl diet), and the first day of the recovery period (1.0% NaCl diet). This finding is in agreement with the previous study that showed arterial pressure increased in rats fed a highly concentrated sodium diet (8.0% NaCl) for a longer duration (5 wk) (Miyajima and Bunag, 1985). Hypothetically, high dietary salt is believed to chronically induce sodium and water retention, and/or increased plasma osmolality and sodium concentration. However, the mechanism(s) mediating increased arterial pressure by chronic plasma hyperosmolality and/or hypernatremia is not clear. In fact, most of the present knowledge relating to this topic is based on acute studies using rats that received hypertonic saline either via intravenous or intracarotid arterial (ICA) infusion (Chen and Toney, 2001; Toney et al., 2003; Weiss et al., 1996). Water deprivation techniques have also been used to generate a more prolonged hyperosmotic condition (Brooks et al., 2005; Scrogin et al., 1999; Stocker et al., 2004). Although there is some discrepancy between study approaches, overall, the knowledge based on these aforementioned studies suggests that plasma hyperosmolality increases blood pressure via central sympathetic excitation (Chen and Toney, 2001; Brooks et al., 2005; Scrogin et al., 1999; Toney et al., 2003; Stocker et al., 2004). Moreover, due to possible opposing effects of baroreflex buffering, intravenous hypertonic saline infusion has been shown to produce non-uniform regional sympathetic nerve responses. In fact, while blood pressure and lumbar sympathetic nerve activity are found to be elevated, renal and splanchnic sympathetic nerve activities are
reduced by intravenous hypertonic saline infusion (Weiss et al., 1996). In the present study, no significant changes of CO and TPR were observed during chronic high salt intake in both of the study groups, although there was tendency of increased CO in the sham compared to MnPOx lesioned rats. Since the increase of arterial pressure observed was subtle, and only specific vascular beds, e.g. muscular vascular beds, could be affected by the sympathetic nervous excitation, a change of TPR might not occur or be detectable. In addition, the increase of arterial pressure in sham lesioned rats could also be contributed in part by the subtle increase of CO.

Based upon the available evidence to date, sympathetic excitation by plasma hyperosmolality and ANG II likely share common central neural pathway(s). In fact, the OVLT, PVN, and RVLM, the cardiovascular brain regions known to contribute to the sympathetic excitatory circuitry of circulating ANG II, have also been shown to participate in mediating sympathetic excitation secondary to ICA hypertonic saline injection and water deprivation (Chen and Toney, 2001; Freeman and Brooks, 2007; Shi et al., 2007; Stocker et al., 2004). The MnPO has also been implicated in ANG II induced hypertension (Ployngam and Collister, 2007, 2008), and is activated by plasma hyperosmolality (Hochstenbach and Ciriello; Sly et al., 2001; Xu and Herbert, 1995). In the present study, we did not show a contribution of the MnPO in the neuronal mechanism(s) that is necessary to counteract sympathoexcitatory effects of chronic hyperosmolality. Conversely, our findings seem to be more consistent with the role of the MnPO in mediating sympathetic excitation following chronic plasma hyperosmolality. In fact, while plasma osmolality and sodium concentration were elevated in the MnPOx rats during high dietary salt intake, their arterial pressure responses remained comparable to
those of sham lesioned rats. Therefore, these findings appear to suggest a necessary role of central neuronal pathway(s) encompassing the MnPO in mediating sympathetic excitation and increased blood pressure effects of plasma hyperosmolality.

Despite no difference in arterial pressure, less positive sodium balance was observed in the MnPOx compared to sham lesioned rats during the first 4 days of high salt intake. This finding is in line with our previous study showing less positive cumulative sodium balance in SFO lesioned rats fed high salt relative to their sham lesioned controls (Hendel and Collister, 2005). In the present study, sodium intake was lower in the MnPOx rats, while sodium excretion was comparable to the sham lesioned rats. Thus, less positive sodium balance in the MnPOx rats appears to be accounted for by their inappropriately high renal sodium excretion. High plasma osmolality and sodium concentration observed in the MnPOx rats likely trigger negative feedback causing these rats to have less appetite for the high-sodium diet (Johnson, 2007). The mechanism(s) mediating enhanced renal excretion of sodium in the MnPO lesioned rats is less clear. However, although PRA decreased significantly in both MnPOx and sham lesioned rats during high salt intake, comparable PRA were observed between the two groups both during baseline and high salt periods. So the exaggerated sodium excretion seen in MnPOx rats does not seem to involve a difference in RAS activity between the two groups. In addition, this finding unlikely involves the impaired secretion of vasopressin and oxytocin that occurs in MnPOx rats (Gardiner et al., 1985; Mangiapanne et al., 1983). Although the set point of vasopressin secretion may be increased, no change of water balance seen in MnPOx rats suggests an adequate secretion of vasopressin during high salt intake. In addition, if this was the case, considering the known facilitating effect of
vasopressin on renal baroreflex sensitivity (Ferrario et al., 1987) and natriuresis effect of oxytocin (Verbalis et al., 1991), renal sodium retention would rather likely be observed in the MnPOx rats. In fact, administration of a vasopressin V1 receptor blocker has actually been shown to increase instead of decrease renal sympathetic nerve activity in water deprived rats (Scrogin et al., 2002). Other possibilities include that the renal sympathetic nerve response to high salt intake might be unsuitably low in the MnPOx rats, causing them to excrete larger amounts of sodium relative to their lower sodium intake (Dibona, 1977).

From this perspective, high dietary salt intake and plasma hyperosmolality have been shown to stimulate hepatorenal reflexes via peripheral osmoreceptors located in hepatic portal veins causing a decrease of renal sympathetic nerve activity (Morita et al., 1991, 1993; Nishida et al., 1998). Therefore, it is probable that the greater and/or more prolonged increased plasma osmolality and sodium concentration observed in the MnPOx rats during high salt intake reached the stimulation threshold of these peripheral osmoreceptors, and/or caused more intense, and prolonged stimulation. On the other hand, while activation of the osmoreceptors located peripherally suppresses renal sympathetic nerve activity, stimulation of central osmoreceptors has been shown to produce the opposite effect (Toney et al., 2003). In fact, ICA infusion of hypertonic saline produces an increase of renal sympathetic nerve activity (Chen and Toney, 2001). Therefore, the possible lower renal sympathetic nerve activity found in MnPOx rats despite higher plasma osmolality appear to support the aforementioned idea that the MnPO probably contributes to the central neural pathway mediating sympathetic excitation by central osmoreceptor excitation. This view is also supported by the acute
study performed by Yasuda et al. (2000) showing that lidocaine injected into the MnPO attenuates the increased arterial pressure and renal sympathetic nerve activity in response to intra-cerebroventricular injection of hypertonic saline. Nevertheless, the relative contribution of the central and peripheral osmoreceptor mechanisms in the sympathetic nervous response to the chronic dietary salt intake remains to be investigated, as well as the role of the MnPO in these intricate mechanisms.

In conclusion, we have shown in the present study that the arterial pressure in MnPOx rats during high salt intake was comparable to that of sham lesioned rats. These findings fail to support the hypothesis that the MnPO is necessary to maintain arterial pressure within the normal range during high dietary salt intake. However, in line with our previous study examining SFO lesioned rats, MnPOx rats of the present study displayed less renal sodium retention during high salt diet compared to sham lesioned rats. These findings could be accounted in part by the exaggerated increase of plasma osmolality and sodium concentration in response to high salt intake observed in the MnPOx rats. However, the exact role of the MnPO’s involvement in the central neural mechanism(s) mediating sympathetic excitation during chronic high dietary salt intake remains to be investigated.

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**Figure 1:** Photomicrographs of 40 µm mid-sagittal sections of the median preoptic nucleus (MnPO). (A) Section from a sham lesioned rat demonstrating dorsal (*) and ventral (#) MnPO. (B) Section from a MnPOx rats demonstrating ablated dorsal and ventral MnPO. 3V-Third ventricle.
Figure 2: Plasma renin activity at day 4 of control period (1.0% NaCl diet) and day 7 of high salt period (4.0% NaCl diet) in MnPO lesioned rats (MnPOx) and sham lesioned rats (SHAM).
Figure 3: Plasma osmolality and plasma sodium concentration at day 6 of control and recovery periods (1.0% NaCl diet), and day 12 of high salt period (4.0% NaCl diet) in median preoptic lesioned rats (MnPOx) and sham lesioned rats (SHAM). *P<0.05 between groups. #P<0.05 vs. control.
Figure 4: Average 24-h mean arterial pressure (MAP) and heart rate (HR) during control period (1.0% NaCl diet), high salt period (4.0% NaCl diet), and recovery period (1.0% NaCl diet) in median preoptic lesioned rats (MnPOx) and sham lesioned rats (SHAM).
Figure 5: Average 24-h cardiac output (CO) and total peripheral resistance (TPR) during control period (1.0% NaCl diet), high salt period (4.0% NaCl diet), and recovery period (1.0% NaCl diet) in median preoptic lesioned rats (MnPOx) and sham lesioned rats (SHAM).
Figure 6: Average 24-h water intake, urine output, and water balance during control period (1.0% NaCl diet), high salt period (4.0% NaCl diet), and recovery period (1.0% NaCl diet) in median preoptic lesioned rats (MnPOx) and sham lesioned rats (SHAM).
Figure 7: Average 24-h sodium intake, sodium excretion, and sodium balance during control period (1.0% NaCl diet), high salt period (4.0% NaCl diet), and recovery period (1.0% NaCl diet) in median preoptic lesioned rats (MnPOx) and sham lesioned rats (SHAM). *P<0.05 between groups.
Figure 8: Cumulative water and sodium balance in median preoptic lesioned rats (MnPOx) and sham lesioned rats (SHAM). *P<0.05 between groups.
Figure 9: Average 2-day sodium balance during control period (1.0% NaCl diet), high salt period (4.0% NaCl diet), and recovery period (1.0% NaCl diet) in median preoptic lesioned rats (MnPOx) and sham lesioned rats (SHAM). *P<0.05 between groups.
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CHAPTER VI

Final Discussion
**Final discussion**

The RAS plays a pivotal role in the dynamic homeostatic control of blood pressure and body water and sodium balances. In addition, chronic over-activation of the RAS participates in the pathogenesis of virtually every form of hypertension. Substantial evidence suggests that circulating ANG II modulates sympathetic activity and blood pressure to a great degree via central neuronal mechanisms in the brain (Brooks and Osborn, 1995; Cerasola et al., 1987; Dampney et al., 2002; Dowell et al. 1996; Fink, 1997; Heesch et al., 1996; Matsukawa et al., 1991; Reid, 1992; Saxena, 1992; Tobey et al., 1983; Weekley, 1991). However, such neuronal circuitry has not been completely elucidated. The objective of this dissertation was to establish the role of the MnPO in mediating chronic blood pressure regulation by circulating ANG II. By using either electrolytic or ibotenic acid lesion technique, the necessary role of the MnPO in blood pressure control by ANG II was determined chronically. Initially, the studies conducted in specific aim 1 and 2 were designed to address the participation of the MnPO on the chronic blood pressure response to increases or decreases in circulating ANG II activity, respectively, using exogenous pharmacological interventions. Subsequently, in specific aim 3, circulating ANG II activity was physiologically manipulated via an increase in dietary salt to address the contribution of the MnPO in ANG II mediated maintenance of normal blood pressure during high salt intake. Taken together, the studies performed in this dissertation add to the literature of the crucial role of central neuronal mechanisms in chronic blood pressure effects of ANG II. Most importantly, the overall findings provide
novel insights into the role of the MnPO in chronic neurohumoral regulation of blood pressure.

The study in specific aim 1 provides strong evidence of the important role of the MnPO in mediating the chronic hypertensive effect of circulating ANG II. In fact, the elevated blood pressure responses to ANG II were found to be attenuated in rats with electrolytic lesion of the MnPO relative to their sham lesioned controls by day 7 of the 10 day-ANG II infusion. The findings in this specific aim were in line with our previous study demonstrating attenuated hypertensive responses to ANG II in SFO lesioned rats (Hendel and Collister, 2005). Since the MnPO is situated behind the BBB and has been shown to be anatomically and physiologically related to the SFO where circulating ANG II interacts with AT$_1$ receptors (Ciriello and Gutman, 1991; Gutman et al, 1986, 1988; Lind and Johnson, 1982; Lind et al., 1983; Miselis, 1981, 1982; Rowland et al., 1994; Saper and Levishon, 1983; Tanaka et al., 1986, 1987; Westerhaus and Loewy, 1999; Zardetto-Smith et al. 1993), these findings suggest that the MnPO likely participates in neuronal circuitry necessary to mediate chronic hypertensive effects of ANG II downstream from the SFO. In addition, either SFO or MnPO lesion causes an attenuation of the elevated blood pressure responses specifically to the last few days of 10 days ANG II infusion, supporting the idea of the chronic nature of the central neuronal mechanisms that potentially mediate the hypertensive effect of low dose ANG II infusion. As has been reviewed in chapter 1, subtle but chronic over-activation of the RAS is likely associated with the pathogenesis of many forms of hypertension in human patients, instead of acute highly elevated RAS activity (Ames et al., 1965; Brown et al., 1981; Brunner et al., 1979; Dickinson and Lawrence, 1963; McCubbin et al., 1965).
Accordingly, the animal model of ANG II-induced hypertension used in this specific aim is highly relevant, and the findings therefore also suggest that high blood pressure in RAS-mediated hypertension seen in a number of hypertensive patients is likely maintained in part by actions of circulating ANG II through central neuronal circuitry that includes the MnPO.

The study in sub-aim 1A was performed in addition to aim 1 using ibotenic acid lesion technique in order to selectively determine the role of neuronal cell bodies in the MnPO in the chronic hypertensive effects of ANG II. In line with electrolytic lesioned rats, ibotenic acid lesioned rats demonstrated attenuated hypertensive responses by day 8 of chronic ANG II infusion. Additional comparison carried out between electrolytic and ibotenic acid lesioned rats revealed more attenuated responses in the electrolytic lesioned rats, while their sham lesioned controls were comparable to each other. These findings suggest that neuronal cell bodies located in the MnPO are important for the hypertensive responses to ANG II. However, part of the attenuated response was explained by damage of neuronal fibers that pass through the MnPO. Such fibers likely include those that project from the SFO and OVLT to the PVN and elsewhere.

During ANG II infusion, HR decreased significantly in both electrolytic and ibotenic acid lesioned rats, as well as in their sham controls, suggesting baroreflex mediated decreases in HR in response to the increased blood pressure induced by ANG II. However, while MAP increased greater in the sham lesioned rats relative to electrolytic and ibotenic acid lesioned rats, HR remained comparable between them. It has been shown previously that circulating ANG II blunts the baroreflex response of HR to hypertension (Campagnole-santos et al., 1992; Garner et al., 1987; Guo and Abboud,
1984), and this effect of ANG II is likely mediated by central neuronal mechanisms (Campagnole-santos et al., 1992, 1988; Guo and Abboud, 1984; Hayashi et al., 1988; Matsumura et al., 1989). The present findings suggest that the MnPO may play a part in the central neuronal mechanisms that mediates a suppressive effect of ANG II on the baroreflex response of HR. Furthermore, in addition to HR, it has been shown that the baroreflex response of lumbar sympathetic nerve activity to increased blood pressure is also attenuated by ANG II infusion (Guo and Abboud, 1984). Accordingly, it is likely that the suppression of the baroreflex by ANG II indirectly participates in the increases of central sympathetic outflow and blood pressure during ANG II infusion in addition to its direct excitatory effects. The lack of ANG II-induced baroreflex suppression in both the electrolytic and ibotenic acid lesioned rats may partly explain their attenuated hypertensive responses to chronic ANG II administration.

The study in specific aim 2 was conducted to assess the role of the MnPO in basal blood pressure regulation by endogenous ANG II. In line with our previous studies (Collister and Osborn, 1998; Collister and Hendel, 2003), MAP decreased profoundly and reached steady state by day 7 of losartan infusion. The same trend was also observed in TPR during losartan infusion. As has been discussed in chapter 4, although losartan may exert its hypotensive effect via blockade of ANG II acting at both centrally and peripherally located AT\textsubscript{1} receptors, the slowly developing hypotension after day 2 of losartan infusion is likely explained by gradual blockade of AT\textsubscript{1} receptors in the CVOs by losartan. This idea is supported by our previous findings that attenuated hypotensive responses to losartan were not observed until day 4 in SFO lesioned rats (Collister and Hendel, 2003) and day 8 in AP lesioned rats (Collister and Osborn, 1998), relative to
their sham controls. Additionally, since it has been reported that losartan can cross the BBB and binds to AT\textsubscript{1} receptors in the brain areas that are not reachable by circulating ANG II (Zhuo et al., 1994), the chronic hypotensive effect of losartan could possibly mediated by blockade of brain intrinsic RAS and/or central angiotensinergic pathways as well.

To gain insights into the hemodynamic responses that accompany the chronic hypotension induced by losartan, continuous CO measurement was performed in this specific aim along with continuous measurements of MAP and HR. TPR was further calculated from the product of MAP and CO. The findings show that in both the MnPO and sham lesioned rats, the decrease of MAP is explained almost solely by a decline in TPR, while CO remained unchanged from baseline throughout the losartan infusion period. These findings were supported by the observation that sodium and water balances were not affected by losartan infusion, suggesting no effect of losartan on renal handling of water and sodium excretion, although this cannot exclude the possibility that pressure diuresis/natreuresis may be shifted toward a lower steady state of arterial pressure. Additionally, as described in chapter 4 and the aforementioned paragraph, the gradual decreases of TPR, in line with MAP, is likely secondary to chronic gradual blockade of brain AT\textsubscript{1} receptors by losartan resulting in lowered central sympathetic outflow. CO measurements were conducted for the first time in this study in this rat model of chronic losartan infusion. Accordingly, the finding that losartan exerts its chronic hypotensive effect solely by causing a decrease of TPR is novel to this study.

In this specific aim, attenuated hypotensive responses to losartan were not observed in MnPO lesioned rats as had been expected based on the hypothesis.
Therefore, these findings suggest that the MnPO is not necessary for basal blood pressure control by endogenous ANG II. However, on the other hand, losartan-induced hypotension was found to be exaggerated in the MnPO lesioned rats relative to sham lesioned controls. This finding was in line with the greater decreases in TPR seen in the MnPO lesioned rats, suggesting lower sympathetic nervous activity-induced greater levels of hypotension in the MnPO lesioned rats. As described in the discussion section of the chapter 4, this exaggerated response observed in the MnPO lesioned rats likely involved a deficient baroreflex buffering of the hypotension induced by losartan infusion, indicating an important role of the MnPO in cross-talk between central sympathetic excitatory pathways and baroreflex arcs. The comparable HR responses observed between the two groups, during the time in which MAP was significantly lower in the MnPO lesioned rats further supports this idea.

The study in specific aim 3 was conducted to assess the role of the MnPO in maintaining normal blood pressure during chronic high dietary salt intake. Since salt loading causes a decrease of circulating ANG II concentration, it is believed that this negative alteration of plasma ANG II concentration sends a signal to the brain causing a decline of central sympathetic outflow to counteract the positive effects of sodium and water retention on blood pressure induced by high salt intake. MAP was found to be comparable between the MnPO and sham lesioned rats throughout the high salt period; therefore, it was concluded that the MnPO does not participate in the central neuronal mechanisms that function in maintaining normal blood pressure during high salt intake. This is in agreement with our previous studies showing that the SFO and AP as well are
not involved in this homeostatic mechanism (Collister and Osborn, 1998; Hendel and Collister, 2005).

In agreement with a previous report (Gardiner et al., 1985), MnPO lesioned rats had baseline plasma hyperosmolality and hypernatremia during normal salt intake (1.0% NaCl), suggesting a crucial role of the MnPO in neurohumoral regulation of normal body osmotic balance. This has been shown to be associated with impaired vasopressin and oxytocin secretion observed in MnPO lesioned rats (Mangiapane et al., 1983; Gardiner et al., 1985). In agreement with the latter finding, plasma osmolality and sodium concentration were increased significantly from baseline in the MnPO lesioned rats during high salt intake, while these values remained unchanged in sham lesioned rats. Although controversy exists, either hyperosmotic substances such as hypertonic sodium chloride administration or water deprivation have shown by numerous studies to have central sympathoexcitation effects secondary to elevation of plasma osmolality and/or sodium concentration (Adams et al., 2007; Brooks et al., 2005; Chen and Toney, 2001; Scrogin et al., 1999; Toney et al., 2003). In fact, the level of plasma osmolality and sodium concentration observed during high salt intake (4.0% NaCl) in the MnPO lesioned rats were greater by as much as $10\pm0.4$ mOsm/kg and $2.2\pm0.2$ mEq/l, respectively, than those of sham lesioned rats. However, no differences in MAP responses were observed between the MnPO and sham lesioned rats throughout the study. These findings shed light on a possible role of the MnPO in mediating sympathetic excitation effects of plasma hyperosmolality and/or hypernatremia. Since plasma samples were collected at only a single time point during high salt intake, whether high levels of plasma hyperosmolality and hypernatremia were sustained chronically in the MnPO
lesioned rats is not clear. Further acute and chronic studies involving animal models of plasma hyperosmolality and hypernatremia are therefore required to make a conclusive remark on this role of the MnPO.

In this specific aim, while MAP was comparable between the MnPO and sham lesioned rats, lower positive Na balance was observed in MnPO lesioned rats during the first 4 days of high dietary salt intake. These findings are in parallel with our previous study demonstrating lower positive cumulative sodium balance in SFO lesioned rats during the first 6 days of high dietary salt intake (Hendel and Collister, 2005). Therefore, findings from the previous and present studies suggest that the central neuronal pathway involving both the MnPO and SFO is important for renal handling of sodium and water excretion during high salt intake. As has been discussed in the aforementioned paragraph, these findings also suggest a role of the MnPO in mediating increased plasma hyperosmolality-induced increases in central sympathetic output, specifically to the kidneys. It has been shown that subtle stimulation of renal sympathetic nerve activity causes an increase in renal tubular reabsorption of sodium without changing renal blood flow and glomerular filtration rates (Dibona, 1977). Disruption of central neuronal pathways that mediate renal sympathetic excitatory effects of plasma hyperosmolality and/or hypernatremia in the MnPO lesioned rats may cause them to excrete inappropriately high sodium in the urine relative to their sodium intake. However, since it has been shown that stimulation of peripheral osmoreceptors oppositely causes a suppression of renal sympathetic nerve activity, the possibility that higher plasma osmolality in the MnPO lesioned rats may stimulate peripheral osmoreceptors exists as well. Future studies are needed to clarify the relative significance of central and/or
peripheral osmoreceptor stimulation during high dietary salt intake, as well as the role of
the MnPO to mediate central sympathetic responses to these stimulations.

Previous studies have shown that individual MnPO neurons are responsive to
circulating ANG II and plasma hyperosmolality, as well as baroreflex stimulation
(Johnson et al, 1996; Stocker and Toney, 2005). In addition, it has been shown that
discharge activity of ANG II and osmotic sensitive neurons in the MnPO can be
modulated by baroreflex input (Stocker and Toney, 2007). Studies performed in this
dissertation provide additional evidence of the important role of the MnPO in integrating
these neurohumoral stimuli, which then result in either net positive or negative influence
on central sympathetic activity. However, it should be noted that findings in specific aims
1-3 seem to suggest that excitatory pathways to the MnPO dominate over inhibitory
pathways, if both are activated at the same period of time. For example, firstly, specific
aim 1 has shown that the MnPO plays a positive role in the hypertension induced by
chronic ANG II infusion, potentially through central sympathetic excitation. Although
MnPO neurons likely received inhibitory input from the baroreflex arc in response to
ANG II-induced hypertension (Sakamaki et al., 2004; Stocker and Toney, 2007),
excitatory signals from the elevated circulating ANG II predominated. Secondly, in
specific aim 2, although blockade of RAS by losartan causes an inhibition of the
excitatory input from ANG II activated pathways to MnPO neurons, excitatory input
from the baroreflex arc likely predominated resulting in a net positive influence from the
MnPO on the central sympathetic outflow, and therefore blood pressure during chronic
losartan infusion. And lastly, in specific aim 3, although excitatory input to the MnPO
from circulating ANG II was diminished, excitatory input from plasma hyperosmolality
to the MnPO likely resulted in a net positive influence from the MnPO to central sympathetic outflow and blood pressure.

A number of future ideas have arisen and been noted where appropriate during the course of this dissertation. Additionally, further studies regarding the specific role of various sub-groups of MnPO neurons could be performed in the future to gain complete knowledge of the complex integrative mechanisms in the MnPO involving cardiovascular regulation. Several neuronal types and receptors have been identified in the MnPO, including angiotensinergic, noradrenergic, glutamatergic, GABAergic, orexinergic, and opioidergic neurons and/or receptors (Bai and Renaud, 1998a,b; Caeiro et al., 2006; Caeiro and Vivas, 2008; Grob et al., 2003; Marcus et al., 2001; Eyigor et al., 2001). Accordingly, in addition to cardiovascular and hydromineral homeostasis, the MnPO neurons have been shown to be involved in a number of homeostatic mechanisms, including thermoregulation, feeding behavior, stress responses, and sleep-wake pattern (Konishi et al., 2007; Kolaj et al., 2008; Kumar et al., 2008; Schwartz et al., 2008). Several studies have demonstrated distinct functions of different sub-types of MnPO neurons and/or receptors relating to cardiovascular regulation (Caeiro and Vivas, 2008; Tanaka et al., 1992a,b, 2003; Tanaka and Nomura, 1993; Schwartz et al., 2008). For instance, angiotensinergic neurons and receptors in the MnPO appear to be responsible for drinking behavior and sympathetic excitation downstream from activation of circulating ANG II at the SFO and OVLT as well as central sympathetic responses to stress (Schwartz et al., 2008; Tanaka and Nomura, 1993; Tanaka et al., 1987). Noradrenergic receptor containing neurons in the MnPO have been shown to receive and respond to neuronal inputs from baroreflex centers in the brainstem (Tanaka et al.,
Furthermore, it has been shown that opioid receptor containing neurons in the MnPO partly mediate acute pressor responses to plasma hyperosmolality (Caeiro and Vivas, 2008). Taken together this recent emerging evidence suggests complicated neuronal activities in the MnPO that remain to be elucidated in order to gain complete understanding of the role of MnPO in cardiovascular and hydromineral homeostasis.

In conclusion, this dissertation provides several novel insights into the role of the MnPO in neurohumoral control of blood pressure. Firstly, the MnPO plays an important role in mediating chronic hypertension induced by chronic low dose ANG II infusion. This finding implicates the MnPO in the central neuronal mechanisms that mediate chronic hypertension seen in RAS-dependent forms of essential hypertension as well as several forms of secondary hypertension. Secondly, the MnPO is not necessary for the chronic hypotensive effects of losartan. This finding suggests that the MnPO does not participate in the central neuronal pathway that mediates basal blood pressure control by endogenous ANG II. Thirdly, the MnPO is not important for normal blood pressure control during high dietary salt intake. Fourthly, the MnPO appears to play some role in sympathetic excitation and increased blood pressure in response to plasma hyperosmolality and/or hypernatremia. Lastly, the MnPO is likely an important integration site where 3 parallel sympathetic modulatory pathways activated by circulating ANG II, plasma hyperosmolity, or arterial baroreceptors converge. Taken together, the important cardiovascular role of the MnPO is proposed as illustrated in Fig. 6.1. Using either pharmacological or physiological manipulation of circulating ANG II activity in each 3 distinctive experiments, this dissertation establishes the crucial
integrative role of the MnPO in central neuronal mechanisms subserving chronic blood pressure homeostasis.
Figure 1: Proposed roles of the median preoptic nucleus in chronic central neural control of blood pressure homeostasis during changes in plasma angiotensin II (ANG II) concentration, plasma osmolality, and systemic blood pressure. The subfornical organ and organum vasculosum of the lamina terminalis detect changes in plasma ANG II and osmolality, transduce the signals, and then transmit them to the MnPO. The mechanism of how the A1 noradrenergic cell group becomes activated during hypotension is not clear, but likely involves hypotension-induced unloading of arterial baroreceptors causing elimination of inhibitory drive to the A1 noradrenergic cell group from the NTS or inhibitory interneurons in the cranial part of the caudal ventrolateral medulla. The A1 noradrenergic cell group then sends excitatory input to the MnPO. On the other hand, systemic hypertension causes an activation of the NTS, which then sends inhibitory input to the MnPO. Whether the downstream central sympathetic pathway is activated or suppressed by the MnPO depends on the outcome of the integration of all afferent inputs received by the MnPO. However, overall findings in this dissertation suggest that excitatory inputs to the MnPO likely predominate over inhibitory ones. SFO; subfornical organ. OVLT; organum vasculosum of the lamina terminalis. MnPO; median preoptic nucleus. PVN; paraventricular nucleus. RVLM; rostral ventrolateral medulla. NTS; nucleus tractus solitarius. ——> Excitatory input. ——> Inhibitory input. FAC; facilitation. INH; inhibition.
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