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The brain Renin Angiotensin System (RAS) is quickly becoming recognized as a critical mediator of blood pressure and body fluid homeostasis. In the forebrain, the median preoptic nucleus (MnPO) responds to Angiotensin II (Ang II) stimulation by increasing thirst and blood pressure. Understanding how this nuclei regulates blood pressure and body fluid homeostasis in response to Ang II has the potential to open new therapeutic avenues for treatment of hypertension. In the studies following series of studies I investigated the role of MnPO Angiotensin Type Ia receptors in the sustained hypertension induced by Chronic Intermittent Hypoxia (CIH) and thirst regulation.

The first project focuses on the role of the MnPO in the sustained hypertension of CIH. Sleep apnea leads to hypertension that persists throughout the waking period. The neural mechanisms that underlie this pathophysiological increase in blood pressure are not well known. CIH is a model of the hypoxemia experienced by sleep apnea sufferers. This model produces a sustained increase in blood pressure, and numerous studies have indicated that the central renin-angiotensin system is involved in this prolonged hypertension. The MnPO receives inputs from the subfornical organ (SFO) and the organum vasculosum lamina terminalis, both circumventricular organs (CVOs) outside the blood brain barrier allowing for it to receive input from nuclei exposed to peripherally circulating hormones. Downstream the MnPO projects to brain regions that control sympathetic activity, such as the paraventricular nucleus (PVN), making it a prime target for integrating peripheral signals with central sympathetic drive. Previous studies have shown that administration of Losartan, and AT1 receptor blocker, directly into the ventricles of

the brain prevents the sustained component of CIH induced hypertension and decreases FosB in the MnPO. Inhibition of the transcription factor FosB in the median preoptic nucleus via a dominant negative construct prevents the sustained hypertension from CIH. CIH also increases Angiotensin Type 1a receptor mRNA in the MnPO via the FosB transcription factor. We hypothesize that knockdown of AT1a receptors locally in the MnPO will prevent the sustained hypertension of CIH. Utilizing a short hairpin RNA we knocked down the Angiotensin Type 1a receptor in the MnPO to determine its contribution to 7 days of CIH hypertension. AT1a receptor knockdown in the median preoptic nucleus blocked the sustained component of hypertension from CIH as compared to scramble injected animals. Immunohistochemistry revealed significantly less FosB positive cells in the MnPO of animals injected with the knockdown vector compared to animals given the control vector prior to 7 days of CIH. The rostral ventrolateral medulla, a key regulator of sympathetic outflow, also expressed significantly less FosB with the AT1a knockdown in the MnPO. Our results indicate that AT1a receptors in the MnPO are regulated by FosB and contribute to CIH hypertension.

Thirst is a critical function the MnPO regulates and the second project aimed to determine if Ang II is necessary for thirst generation at the MnPO. Ang II is known to produce a dipsogenic response when administered peripherally, or centrally. Peripheral Ang II acting as a hormone activates CVOs, brain regions lacking a blood brain barrier, such as the SFO. These circumventricular organs synapse upon the MnPO which leads to activation of higher cortical centers and thirst. Intracerebroventricular (ICV) administration of Ang II produces thirst without interacting with CVOs by directly activating the MnPO, and it is therefore hypothesized that Ang II is the neurotransmitter released at the CVO-MnPO junction. We sought to test this hypothesis by knocking down AT1a receptors in the MnPO using a short hairpin RNA complimentary for the

receptor subtype. Knockdown of AT1a in the MnPO significantly reduced drinking in animals administered ICV Ang II. This reduction in water consumption was mirrored by a reduction in cFos, a transcription factor and marker of neuronal activity, in the MnPO, SON, and PVN. Thirst generated by subcutaneous administration of Ang II was unaffected by the knockdown of AT1a in the MnPO. cFos counts in the SFO, OVLT, MnPO, PVN, and SON also were not significantly influenced. These data indicates that AT1a receptors in the MnPO are not necessary for water intake stimulated by peripheral Ang II. While experimental administration of Ang II directly to the ventricles is sufficient to induce drinking it may not play a prominent role in stimulating drinking under physiological conditions.

The Role of Angiotensin II in Central Control of Blood Pressure and Body Fluid Homeostasis

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THE ROLE OF ANGIOTENSIN II
IN CENTRAL CONTROL OF BLOOD PRESSURE AND BODY FLUID HOMEOSTASIS

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ABBREVIATIONS

AAV	Adeno-associated virus
ACE	Angiotensin converting enzyme
aCSF	Artificial cerebrospinal fluid
ACT	Activity
Ang II	Angiotensin II
AT1aR	Ang II receptor type 1 subtype a
AT1bR	Ang II receptor type 1 subtype b
AT2	Ang II receptor type 2
AT1ashRNA	Short-hairpin RNA against AT1aR
AVP	Arginine vasopressin
CIH	Chronic Intermittent Hypoxia
CNS	Central nervous system
CPAP	Continuous positive airway pressure
CVLM	Caudal ventrolateral medulla
CVO	Circumventricular organ
DAB	3,3'-Diaminobenzidine
DMNX	Dorsal motor nucleus of the vagus
dp	dorsal parvocellular neurons of PVN
GFP	Green fluorescent protein
HR	Heart Rate
ICV	Intracerebroventricular

IML	Intermediolateral tract of spinal cord
LCM	Laser capture microdissection
lp	Lateral parvocellular neurons of PVN
MAP	Mean Arterial Pressure
MnPO	Median preoptic nucleus
mp	medial parvocellular neurons of PVN
NA	Nucleus ambiguus
NTS	Nucleus tractus solitarius
OVL	Organum vasculosum of Lamina Terminalis
OXT	Oxytocin
pc	Pre-commisural region of the NTS
PCR	Polymerase chain reaction
pm	Posterior magnocellular neurons of PVN
PSNA	Parasympathetic Nerve Activity
PVN	Paraventricular nucleus of hypothalamus
RAS	Renin-Angiotensin system
RNA	Ribonucleic Acid
RR	Respiratory Rate
RVLM	Rostral ventrolateral medulla
SCRshRNA	Short-hairpin RNA-scramble sequence
SA	Sleep Apnea
SFO	Subfornical Organ
SNA	Sympathetic Nerve Activity

SON	Supraoptic nucleus of hypothalamus
sp	Sub postremal region of the NTS
vlp	ventro-lateral parvocellular neurons of PVN

CHAPTER I

DISSERTATION OVERVIEW

The Renin Angiotensin System (RAS) has long been known as a powerful peripherally acting hormone system that is critical for maintenance of blood pressure and body fluid homeostasis[1, 2]. The traditional RAS cascade begins with the release of the enzyme renin from the renin secreting cells (juxtaglomerular cells) of the kidney. Renin cleaves the large peptide angiotensinogen which is produced in the liver and results in the production of angiotensin I. Angiotensin I is cleaved a second time typically in the lungs by angiotensin converting enzyme (ACE1) producing Angiotensin II (Ang II). Ang II is traditionally thought to be the effector molecule of the system increasing blood pressure through vasoconstriction and stimulating thirst through its actions on the brain. All of this still holds true, but the RAS is substantially more complex with the discovery of multiple splice variants of the angiotensin molecule each with their own receptors, tissue specific RAS systems, and an increasingly convoluted role in disease[3-5].

Discovery of the Ang II occurred as a result of trying to identify why renal ischemia via obstructing renal arteries produced systemic hypertension[6, 7]. The finding that local blood flow impediments generated a systemic pathological condition lead to the search for a humoral factor

that was produced in response to the ischemia. Previously, ground renal cortex extract had been used to experimentally increase blood pressure, but the effector molecule had never been extracted[8]. After extraction and isolation of numerous candidates Ang was found to be the effector peptide responsible for vasoconstriction.

Ang II, as the names suggests, produces constriction (or “tension) in the vasculature (“angio), increasing systemic vascular resistance, and subsequently, increased arterial blood pressure[9-11]. In addition, in the zona glomerulosa of the adrenal gland, Ang II stimulates release of the mineralocorticoid aldosterone. Together Ang II and aldosterone increases renal tubule sodium reabsorption resulting in water retention as well[12], further enhancing Ang II’s blood pressure effect on blood pressure. Peripheral facing regions of the brain, the CVOs, also receive input from Ang II functioning as a hormone[13-16]. In response to Ang II binding there is increased sympathetic nerve activity, release of arginine vasopressin (AVP), and thirst generation[17-19]. All together these actions serve to increase blood pressure either through increased sympathetic drive or water retention and acquisition through thirst generation.

More recent work has identified tissue specific RAS in organs such as the kidney, heart, and intestines[5], which serve to modulate the actions of their specific organ. Pertinent to the work of this dissertation the brain has a separate RAS that does not function as a hormone, but rather as a neurotransmitter[19, 20]. Ang II functions as a peptide neurotransmitter, and much like the peripheral hormone RAS it exerts its effects through binding to GPCRs such as the angiotensin type 1 receptor (AT1)[20, 21]. All of the components of the RAS have been found through the brain including renin protein, RNA, and renin enzymatic activity cleaving angiotensinogen to Ang II[22, 23]. The precursor peptide, angiotensinogen, is also present throughout the

hypothalamus[24, 25]. Ang II is also present in the hypothalamus and in the hindbrain, and plays a role in central control of body fluid regulation and autonomic control[22, 26-29]. The entire RAS cascade in the brain functions in the same way as it does in the periphery. Rats bred to be deficient in brain angiotensinogen exhibit diminished drinking responses to peripherally administered renin, and are also hypotensive[30]. After crossing these angiotensinogen deficient rats with rats that overexpress Ang II and therefore develop hypertension, the Ang II overexpression/Angiotensinogen null rodents do not develop as severe hypertension[30]. Predictably, intracerebroventricular infusion of Ang II results in an increase in systemic arterial pressure[31-33].

The primary focus of the brain RAS in the context of this work is in the MnPO. This nucleus lies on the anterior wall of the third ventricle and is located inside the blood brain barrier. It is reciprocally innervated by two circumventricular nuclei, the subfornical organ (SFO), and the organum vasculosum of the lamina terminalis (OVLT), allowing input from regions outside the blood brain barrier[34, 35]. In the hypothalamus, the MnPO projects to the PVN[36, 37], SON[38], and the arcuate nucleus[34, 39]. The midbrain has reciprocal connections from the locus ceruleus, raphe pontis, and lateral parabrachial[34, 36]. Even more caudal, the hindbrain inputs to the MnPO arise from the nucleus tractus solitarius (NTS) mostly from the A2 region, and the RVLM[34].

The AT1 receptor is abundant in the MnPO and the primary subtype is the AT1a receptor[27, 40, 41]. Neurons in the MnPO respond to Ang II stimulation in two ways, 1) depolarization by reduced potassium efflux or 2) greater hyperpolarization via potentiation of GABA through

augmented chloride influx[40, 42]. AT₂ receptors have also been detected in the MnPO, but their function is not well explored[43, 44].

The MnPO plays a role in four major functions: thermoregulation, sleep, salt and volume regulation, and cardiovascular control; the latter two functions are pertinent to this dissertation.

The MnPO is a regulator of cardiovascular control through its autonomic control, and more specifically regulation over sympathetic nerve activity. This action makes the MnPO a target for control of hypertension[45-47]. The final function of the MnPO is in salt and volume regulation through its effects on vasopressin release and fluid intake. Some neurons in the MnPO are directly salt sensitive, but the whole nucleus receives inputs from CVOs that are in direct contact with the blood[41, 48, 49]. This allows the MnPO to control blood volume as well as plasma and CNS sodium concentrations.

In the context of the work presented, we are interested in the effects of Ang II on this MnPO.

The first area of focus is on the ability of the MnPO to drive hypertension through Ang II receptor stimulation throughout Chronic Intermittent Hypoxia (CIH). The second project focuses on the ability of Ang II to generate thirst through stimulation of the AT_{1a} receptors on the MnPO. The work presented seeks to understand the dual role Ang II plays in control over these overlapping but separate functions of the MnPO.

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PUBLISHED REVIEW PART 1

NEURAL CONTROL OF BLOOD PRESSURE IN CHRONIC INTERMITTENT HYPOXIA

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Abstract

Sleep Apnea (SA) is increasing in prevalence and is commonly comorbid with hypertension. Chronic Intermittent Hypoxia is used to model the arterial hypoxemia seen in SA, and through this paradigm the mechanisms that underlie SA induced hypertension are becoming clear. Cyclic hypoxic exposure during sleep chronically stimulates the carotid chemoreflexes, inducing sensory long term facilitation, and drives sympathetic outflow from the hindbrain. The elevated sympathetic tone drives hypertension and renal sympathetic activity to the kidneys resulting in increased plasma renin activity and eventually Angiotensin II (Ang II) peripherally. Upon waking, when respiration is normalized, the sympathetic activity does not diminish. This is partially because of adaptations leading to overactivation of hindbrain regions controlling sympathetic outflow such as the Nucleus Tractus Solitarius (NTS), and Rostral Ventrolateral Medulla (RVLM). The sustained sympathetic activity is also due to enhanced synaptic signaling from the forebrain through the Paraventricular Nucleus (PVN). During the waking hours, when the chemoreceptors are not exposed to hypoxia, the forebrain circumventricular organs (CVOs) are stimulated by peripherally circulating Ang II from the elevated plasma renin activity. The CVOs and Median Preoptic Nucleus chronically activate the PVN due to the Ang II signaling. All together this leads to elevated nocturnal mean arterial pressure (MAP) as a response to hypoxemia, as well as inappropriately elevated diurnal MAP in response to the maladaptations.

Sleep Apnea

Sleep apnea (SA) commonly refers to a group of disorders that are characterized by periodic interruption of respiration, or hypoventilatory breathing during sleep. Sufferers experience airway obstruction, hypoxia, hypercapnia, and sleep fragmentation from arousal during periods of insufficient oxygenation. The Wisconsin Sleep Cohort Study indicates that the number of individuals with sleep apnea has been steadily increasing. Depending on the sub-population examined, the prevalence of moderate to severe SA has increased between 14 to 55% since 1994[1]. The Wisconsin Sleep Study also identified a variety of cardiovascular sequelae associated with sleep apnea including hypertension[2]. The hypertension associated with sleep apnea is sustained during the diurnal cycle and is accompanied by chronically elevated sympathetic nerve activity (SNA). Activation of the chemoreflex in sleep apnea leads to increased SNA which drives increased blood pressure[3]. Elevated SNA has been observed in SA patients in numerous studies, and even short bouts of voluntary apnea result in increased SNA[4-6].

A better understanding of the central nervous system (CNS) mechanisms that lead to chronic SNA and diurnal hypertension could be critical to understanding the pathophysiology of SA. While a number of reviews have comprehensively discussed the pathophysiology of sleep apnea [7], this work will focus on such CNS events related to hypertension in intermittent hypoxia which models the hypoxemia associated with SA.

Chronic Intermittent Hypoxia

Fletcher conceived the idea that exposing rodents to cyclical hypoxia would appropriately mimic the hypoxemia experienced by SA patients[8, 9]. Animals in this model increase their blood pressure in proportion to elevations reported in SA sufferers[10-12]. This hypertension is accompanied by increased muscle SNA during the hypoxic exposure, and during normoxia in SA patients [3, 13]. The chronic intermittent hypoxia (CIH) model produces the same pathophysiological increase in blood pressure seen with SA by only mirroring the hypoxic exposures. In response to CIH, animal models exhibit elevated renal[14, 15] and splanchnic SNA[16, 17] along with increased phrenic nerve activity[16]. CIH also leads to enhanced sympathetic responses to chemoreceptor stimulation [18, 19]. Therefore, CIH alone is sufficient to produce the hypertension without any need for the obstruction or sleep fragmentation present in SA.

Many labs have adapted this protocol in order to understand the mechanisms that produce CIH hypertension and to gain insight into the pathophysiology of SA. Patients with SA exhibit different durations and severity of hypoxemia and so there are a variety of CIH models for SA. Eventually this variation in condition will allow for comparison between cycle time and severity of hypoxia in the disease process, but until enough studies are completed the lack of consensus makes it difficult to determine what the defining characteristics of the disease process are and what is specific to the idiosyncratic CIH protocols. A more standardized CIH protocol may be beneficial in the short term to tease out the core features of the disease process.

Hypertension Initiation During CIH

The carotid bodies that lie in the bifurcation of the internal and external carotid arteries sense blood oxygenation. This tissue does not sense oxygen content directly, but rather sufficient pressure of oxygenation[20]. Adequate pressure of oxygen in the arteries results in carbon monoxide (CO) production by heme oxygenase-2 in the glomus cells, and CO suppresses the production of dihydrogen sulfide (H₂S) through a PKG mediated pathway[21]. This is reversed in hypoxia and H₂S increases carotid body firing by inhibiting potassium channels and increasing calcium influx[22]

Carotid body stimulation during the hypoxia in CIH results in increased afferent signals via the glossopharyngeal nerve to the Nucleus Tractus Solitarius (NTS). This signaling is essential for sympathetic activation in CIH and numerous studies have verified that carotid body denervation prevents CIH hypertension [8, 23]. The acute increase in SNA from CIH is a productive response to diminished oxygenation and helps to maintain adequate perfusion and oxygenation to essential organs such as the heart and the brain.

Patients with SA exhibit higher baseline muscle sympathetic nerve activity (MSNA) and blood pressure during periods of normal oxygenation indicating that some part of the adaptive response remains after the end of the hypoxic challenge[13]. These same subjects have significantly lower blood pressure and MSNA in response to a 15 minute inhalation of 100% oxygen, but not room air[13]. The abnormally high MSNA during normal oxygenation indicates that the carotid chemoreceptors may be inappropriately active during normal oxygenation. Chronic activation of carotid chemoreceptors is present in cat and rodent CIH models as well[24-26]. Abnormal chemoreceptor activation may be due to sensory long term facilitation (sLTF) in the glomus cell

of the carotid body[24]. Glomus cells are the oxygen sensing cells in the carotid bodies and induce firing of the glossopharyngeal afferents in response to inadequate oxygenation. The carotid bodies also possess a renin independent pathway capable of producing Ang II[27, 28]. Locally administered Ang II briefly increases carotid afferent firing when applied continuously, and results in sLTF when applied in discrete intervals[29]. This same phenomenon is produced by application of Serotonin (5-HT) as well[30, 31]. Both 5-HT and Ang II function by activating NADPH Oxidase 2 (NOX 2) which produces reactive oxygen species (ROS). The presence of ROS is essential for sLTF to occur[30]. Repetitive stimulation of the glomus cells via Ang II or 5-HT during CIH causes sLTF and results in increased basal and chemoreflex stimulation which may help to maintain the elevated MAP seen in SA patients.

After a week of CIH, the chronic chemoreflex stimulation from hypoxemia leads to a rightward resetting of the arterial pressure and renal SNA (rSNA) baroreflex curve. This resetting occurs without any changes in sensitivity so the baroreflex is not inhibited after a 7 day exposure to CIH, but there is greater discharge at each pressure point[32]. In a separate study, animals exhibited exaggerated chemoreflex responses to acute hypoxic exposures with elevated sympathetic discharge after 2 weeks of CIH[17]. This elevated discharge was not coupled with an enhanced pressor response though. IV application of phenylephrine, an α -adrenergic agonist, revealed that the CIH animals had an attenuated vasoconstrictor response to phenylephrine. These same anaesthetized CIH animals had a greater decrease in MAP relative their normoxic counterparts after ganglionic blockade with mecamylamine providing evidence that the increased arterial pressure after CIH is not completely governed by sympathetic vasoconstriction[17]. At this same two week time point, another study found that there was not an increase in total

peripheral resistance, but there was an increase in cardiac output in CIH treated animals providing support for the hypothesis that CIH induced hypertension not solely a result of vasoconstriction[33]. With CIH protocols of 30 days or longer, there is impaired baroreflex control of heart rate[34, 35]. Overall, the increased sympathetic outflow at the early stages of CIH may desensitize adrenergic receptors in the vasculature and hypertension may be driven by increased cardiac output and baroreflex impairment at later time points.

Maintaining Hypertension – Hindbrain

Increased chemoreceptor activation stimulates the NTS which leads to activation of the rostral ventrolateral medulla (RVLM) and increased renal[14, 15], splanchnic, and phrenic SNA [16, 17]. rSNA leads to increased plasma renin activity (PRA), and through the Renin Angiotensin System (RAS), elevated circulating Ang II peripherally[36].

Glutamate is the major neurotransmitter released by chemoreceptor afferents[37], and CIH has complex effects on glutamate neurotransmission in the NTS. For example, 10 days of CIH increases the excitability of second order NTS neurons due to enhanced spontaneous neurotransmitter release, but this effect is offset by a decrease in amplitude of evoked excitatory postsynaptic currents (EPSCs) [38]. Consistent with this observation, it has recently been reported that the number of active synapses is reduced in the NTS after CIH [39]. Moreover, CIH may differentially affect the sensitivity of second order NTS neurons to glutamate with AMPA mediated responses being enhanced while NMDA responses are decreased [40]. These observations suggest that, while chemoreceptor sensitivity may be increased by CIH, compensatory mechanisms at the level of the NTS may reduce the overall impact of this effect on autonomic control.

In addition to chemoreceptor stimulation, hypoxia activates K_{ATP} currents in neurons and numerous other cell types causing hyperpolarization that may protect vulnerable tissue during adverse events that result in ATP depletion. After 7 days of CIH, acute hypoxic exposure to a NTS slice preparation results in less outward potassium current, and therefore less protection and more excitability in NTS neurons receiving carotid body inputs[41]. The K_{ATP} channels normally dampen excitability in the neurons, but CIH diminishes this potassium current in the NTS allowing for increased activation. This lack of potassium efflux could contribute to a more robust sympathetic response to hypoxemia.

Enhanced chemoreceptor activation during CIH also augments signaling in the pre-sympathetic hindbrain nuclei. Catecholamine producing Tyrosine Hydroxylase (TH) positive A2 neurons located in the NTS are one subtype which receives direct inputs from the chemoreceptors [42, 43]. The more caudal aspects of the NTS are responsible for modulation of sympathetic outflow in part through their connections with the RVLM. Knockdown of TH, and therefore catecholamine production in A2 neurons, reduces the sustained component of CIH hypertension [44]. Δ FosB staining in the Paraventricular Nucleus (PVN) of the hypothalamus after CIH was significantly reduced in the rats that received TH knockdown in the NTS. In these studies, knockdown of TH was achieved via an adenoviral vector (AAV) specific for neurons which demonstrates the importance of neural inputs in the NTS rather than glia for the progression of hypertension from CIH. This is reinforced by another study that acutely administered fluorocitrate into the NTS of CIH animals to inhibit glia and found no change in baseline, or post CIH sympathetic activity from the animals[45]. A more chronic inhibition of glial cells may unmask a role for the tripartite synapse in regulation of CIH sympathetic control via purinergic

signaling though. Glial release of ATP onto NTS neurons that project to the RVLM enhances synaptic transmission via activation of P2X receptors[46]. Downstream from the NTS, purinergic inputs to the RVLM are augmented after a 10 day CIH protocol, and this effect is most likely mediated by an increase in P2X3 and P2X4 receptors[47].

CIH also influences hindbrain circuits that couple respiratory and sympathetic outflow. After 10 bouts of brief hypoxic exposure rats showed a marked increase in phrenic and splanchnic sympathetic activity[16]. Recording both of these variables simultaneously revealed that splanchnic and phrenic activity was coupled and that splanchnic discharge primarily occurred during the expiratory phase of respiration. A longer 10 day CIH exposure also resulted in increased abdominal sympathetic activity that coupled with the expiration phase of respiration[48]. This respiratory coupling may be due in part to some C1 RVLM neurons that show increased excitability from respiratory neurons that are most likely expiratory[49].

While all of the studies presented so far have focused on increasing sympathetic outflow there is also evidence for diminished parasympathetic control over heart rate. A 4 week model of CIH that includes hypercapnia along with hypoxia (CIHH) resulted in increased inhibitory neurotransmission to cardiac vagal neurons in the Nucleus Ambiguus and Dorsal Motor Nucleus of the Vagus [50]. This was coupled with a reduction in excitatory glutamatergic neurotransmission and indicates that parasympathetic outflow is reduced following CIH. The diminished parasympathetic outflow blunts the acute bradycardic response to CIHH, but does not result in a sustained increase in heart rate, and overall may contribute to poor baroreflex sensitivity in longer term CIH.

Maintaining Hypertension – PVN

The PVN is an important regulatory region for blood pressure control due to its parvocellular regions that influence SNA as well as the hypothalamic-pituitary-adrenal axis. In addition, its magnocellular region is in direct control of vasopressin which maintains body fluid regulation. The PVN receives inputs related to the chemoreflex [42, 51], and from forebrain regions that sense circulating Ang II such as the Subfornical Organ (SFO) as well as integrating centers such as the Median Preoptic Nucleus (MnPO) [52, 53]. This pivotal location makes it an attractive target for intervention in the SA disease process. After 7 days of CIH the transcription factor Δ FosB, a marker for neuronal activity, is significantly increased in the medial parvocellular, dorsal parvocellular, and lateral parvocellular subregions of the PVN [11]. Reducing the activity of the PVN acutely in an anaesthetized preparation via microinjection of the GABA agonist muscimol produced greater decreases in MAP and lumbar SNA in 7 day CIH exposed animals than in controls[54]. In agreement with the acute data, chronic bilateral infusion of muscimol over 14 days of CIH prevented the sustained hypertensive component from developing [55]. Together these observations indicate that the PVN contributes to CIH hypertension.

Numerous studies have demonstrated a role for Ang II acting as a peptide neurotransmitter in hypertension[56-58]. CIH induced neurogenic hypertension is also partially driven by Ang II. Chronic intracerebroventricular (ICV) infusions of Losartan, an Angiotensin Type 1 (AT1) receptor blocker significantly attenuated the sustained component of CIH hypertension that occurs during the normoxic dark phase[59]. In addition, ICV losartan blocked increases in Δ FosB staining in the PVN as well as the RVLM, NTS, and MnPO [59]. Similarly, bilateral infusion of losartan directly in the PVN prevented an absolute increase in MAP after 14 days of

CIH [55]. This beneficial effect on CIH hypertension was also present when AT₂ or Ang 1-7 receptors were blocked, indicating that multiple different Angiotensin peptide variants and receptors are contributing to the increase in firing from the PVN.

AT₁ stimulation is partially responsible for the increase MAP in CIH so understanding regulation of AT₁ receptor expression may be a critical to understanding the pathogenesis of CIH hypertension. Neuronal Nitric Oxide Synthase (nNOS), which synthesizes the gaseous neurotransmitter nitric oxide, represses AT₁ receptor expression in hypothalamic cell culture via a PKG mediated mechanism [60]. A significant decrease in neurons expressing nNOS RNA and histological staining for nNOS was observed in the ventral parvocellular PVN of rats after 35 days CIH [61]. This study did not find a difference in the magnocellular region, but also did not measure nNOS activity. In a separate study, a mouse model of 35 days of CIH resulted in a decrease of NO production in the dendrites of PVN neurons[62]. Decreased NO production was preceded by the internalization of NR1, an NMDA receptor subunit in neurons with nNOS expressing dendrites on day 14 of CIH. This internalization reduces NMDA mediated inward currents and may play a role in the decrease of nNOS expression and therefore NO production. The authors propose that the neurons examined in this study are potentially magnocellular since nNOS in the PVN is scarcely expressed in parvocellular preautonomic or GABAergic PVN neurons [63]. Conversely, nNOS in the PVN has been identified in the magnocellular neurosecretory neurons in numerous studies [64-66]. It has been shown that the dendrites from magnocellular neurons may be in close proximity to PVN pre-autonomic neurons and that their release of vasopressin can regulate the activity of PVN pre-autonomic neurons and sympathetic outflow [67]. It could be speculated that changes in NO release from magnocellular dendrites

may influence AT1 receptor expression in PVN parvocellular neurons. It remains to be determined if this type of interaction between these two PVN cell types contributes to CIH hypertension.

Plasma vasopressin (AVP) is not increased after 35 days of CIH, and plasma osmolality is not perturbed after 7 days of CIH [11, 62]. However, recent studies indicate that AVP in PVN parvocellular neurons may contribute to increased sympathetic drive. Among the parvocellular neurons in the PVN that express vasopressin there is a subset that project caudally to the RVLM [68-70]. After 8 days of CIH, the number of RVLM neurons positive for the AVP receptor V1A significantly increases. Functionally, when the PVN is disinhibited by the GABA antagonist bicuculine, there is increased sympathetic drive from the RLVM which can be stopped with a V1A blocker. Animals that were exposed to 8 days of CIH required significantly more pharmacological V1A blocker to normalize blood pressure. These results suggest that a vasopressin projection from the PVN to the RVLM may regulate sympathetic outflow during CIH and that CIH is associated with an increase in the excitatory drive from this pathway.

In addition to increasing sympathetic outflow, the PVN contributes to diminishing parasympathetic discharge as well. A subpopulation of parvocellular neurons in the PVN project to the cardiac vagal neurons (CVN), and after exposure to 4 weeks of CIHH these parasympathetic control centers exhibit reduced excitatory glutamatergic input after photo-stimulation of the PVN[71]. The CVNs neurons also showed a reduction in EPSC frequency and amplitude after photo-stimulation stimulation of PVN. This supports the increased inhibitory input to the CVNs and demonstrates a role for the PVN in diminished parasympathetic tone after SA.

Maintaining Hypertension – Forebrain

Both sleep apnea and CIH increase PRA and RAS [36, 72]. Increased peripherally circulating Ang II can bind to CVOs in the forebrain and contribute to the pathophysiological increase in sympathetic tone. After 7 days of CIH, there is increased Δ FosB staining in the SFO, Organum Vasculosum Lamina Terminalis (OVLT), and MnPO[11]. These observations are consistent with the hypothesis that circulating Ang II is activating the circumventricular organs, SFO and OVLT, which in turn stimulate the MnPO to drive sympathetic tone through its connections with the PVN. Electrolytic lesion of the AV3V region that contains MnPO, OVLT, and SFO afferents prevents the sustained increase in MAP during waking, but does not affect the MAP increase during intermittent hypoxia[73].

Supporting the idea that peripheral Ang II plays a role in CIH induced hypertension, knockdown of AT1a receptors in the SFO via an AAV prevents the sustained component of hypertension from CIH[36]. In addition to the functional effect, the knockdown decreased the expression of Δ FosB in the downstream regions of the MnPO and PVN of rats. This suggests that SFO stimulation is necessary to drive the increase MAP during the waking hours and Δ FosB expression in both the MnPO and the PVN.

Both the MnPO and SFO have projections to the PVN, and both increase Δ FosB in response to CIH. The redundant connections to the PVN from these regions to the PVN have not been explored in CIH and the contribution of each nuclei separately is not well defined. ICV administration of Losartan decreased Δ FosB expression in the MnPO along with numerous other autonomic nuclei. Δ FosB is an AP-1 transcription factor that signals for increased neuronal activity and transcriptionally modulates the expression of numerous genes. A dominant negative

inhibition of Δ FosB in the MnPO via a virus expressing Δ JunD also prevented sustained hypertension from CIH[73]. This decrease in MAP along with a reduction in the number of Δ FosB positive cells in the PVN, RVLM, and NTS demonstrates an essential role for the MnPO in CIH induced hypertension. Furthermore, several genes were identified as regulated by Δ FosB in the MnPO such as Angiotensin Converting Enzyme (ACE), Angiotensin Converting Enzyme 2, Angiotensin Type 1a Receptor (unpublished observation), and nNOS. Future experiments will examine how these Δ FosB regulated genes contribute to CIH hypertension. It could be that, as described above for the NTS, some of these changes in gene expression contribute to the development of CIH hypertension (ACE & AT1a) while others represent homeostatic changes (ACE2 & nNOS) that reduce the magnitude of CIH hypertension.

Summary

Utilizing CIH as a model of the hypoxemia associated with SA has allowed for unprecedented exploration into the early mechanisms that could underlie SA induced hypertension. Initially, hypertension may arise from repeated chemoreflex stimulation of the sympathetic nervous system (Figure 1). This point is supported by the observations that removal of the carotid body prevents or eliminates the hypertension. This repeated exposure to hypoxemia acutely leads to increased blood pressure and through rSNA increases PRA. At the same time the glomus cells of the carotid body could be contributing to the generation of sLTF due to increased ROS and Ang II. The combination of increased peripheral Ang II and sensitized chemoreception by both may be required to sustain elevated blood pressure and increased SNA during the normoxic waking hours. During the waking hours the hindbrain continues to be overactive and this leads to increased SNA. Augmented signaling from the carotid bodies during normal oxygenation along

with changes in excitability and synaptic sensitivity in the NTS could lead to increased activation of the RVLM, and through A2 projections of the NTS, the PVN (Figure 1).

Peripheral Ang II may play an additional role in supporting sustained elevations in blood pressure and SNA associated with CIH. Circulating Ang II stimulates the SFO, and possibly the OVLT, which in turn stimulates the MnPO leading to increased expression of Δ FosB (Figure 1). Δ FosB mediated changes in gene expression in the MnPO are necessary for the sustained component of CIH hypertension that occurs during normoxia. Ang II signaling is likely an important component of these central pathways as ICV Losartan administration prevents the sustained component CIH hypertension. Furthermore, three of the candidate Δ FosB regulated genes in the MnPO are part of the brain RAS such as ACE and the AT1a receptor. Enhanced angiotensin signaling at the level of the MnPO could create a feed forward loop that contributes to sympathetic drive through the PVN, where information from the lamina terminalis could be integrated with chemoreceptor information from the hindbrain (Figure 1). The evidence described above indicates that experimental manipulation effecting either the lamina terminalis, the PVN, or the hindbrain are sufficient to abrogate CIH hypertension suggesting that the entire network may be required for the sustained component of CIH hypertension that is similar to diurnal hypertension in SA patients.

Perspectives

All of the pathophysiological processes described above could lead to potential avenues for the future treatment of hypertension associated with SA. Alternative treatment options are needed for patients who do not experience any therapeutic effect on blood pressure from CPAP. Even amongst individuals who adhere to CPAP for 4 hours per night, at least 25% do not show a

therapeutic benefit reducing hypertension[74, 75]. This leaves a significant number of individuals at risk for cardiovascular sequelae despite some relief from the hypoxemia. A novel analysis of circulating miRNA in plasma has identified potential biomarkers that may help to identify these non-responders so that they can be medicated appropriately along with CPAP[76].

A lack of response to CPAP makes intuitive sense in light of the processes examined. Treating the hypoxemia with CPAP after a prolonged disease period only alleviates the pathophysiological mechanisms that occur acutely during the apneic period. The forebrain and hindbrain mechanisms altered by long term exposure to hypoxia may continue to drive the high blood pressure until neural adaptations have been reversed. Angiotensin receptor blockers (ARB) are a prime candidate to treat hypertension associated with SA through the brain. Losartan[77, 78] and Candesartan[79] are among the ARBs that have functional evidence that they cross the blood brain barrier. Despite this evidence, there has been no detection of the active form of these ARBs in the brain and, in animal models, central administration of losartan produces a more robust and consistent decrease in blood pressure compared to peripheral administration[59, 80]. A more blood brain barrier permeable ARB could be an important addition to the therapeutic tools available for SA induced hypertension. A number of the mechanisms that have been identified in animal models of CIH appear to be sufficient to establish and sustain hypertension, but SA is increasingly associated with treatment resistant hypertension[81]. For most SA patients, single modality therapies may be sufficient for treatment, but long term sufferers with resistant hypertension may benefit from targeting multiple mechanisms to significantly reduce MAP.

Unfortunately, around 80% of those who are afflicted with sleep apnea remain undiagnosed[82]. An essential step for the pathophysiology outlined here to be effectively treated, and treated early, is to diagnose SA. Utilizing a similar circulating biomarkers approach in the blood there could be a cheaper, simpler way to identify those suffering from sleep apnea. A circulating biomarker approach would be a useful tool in identifying individuals who are sleep apneic during their routine blood draws and examinations rather than through self or partner identification. This would not only provide information on who should undergo polysomnography, but would allow for some degree of intervention in underserved populations that may not be able to afford a sleep study. It is possible that these biomarkers could be related to the mechanisms that sustain hypertension during normoxia.

Overall, CIH has proven to be an extremely useful model to dissect the neurological pathogenesis of sleep apnea. Further studies utilizing this design will provide insight into the onset and maintenance of the disease. This will allow for treatments that are not palliative, but actively ameliorating the hypertension.

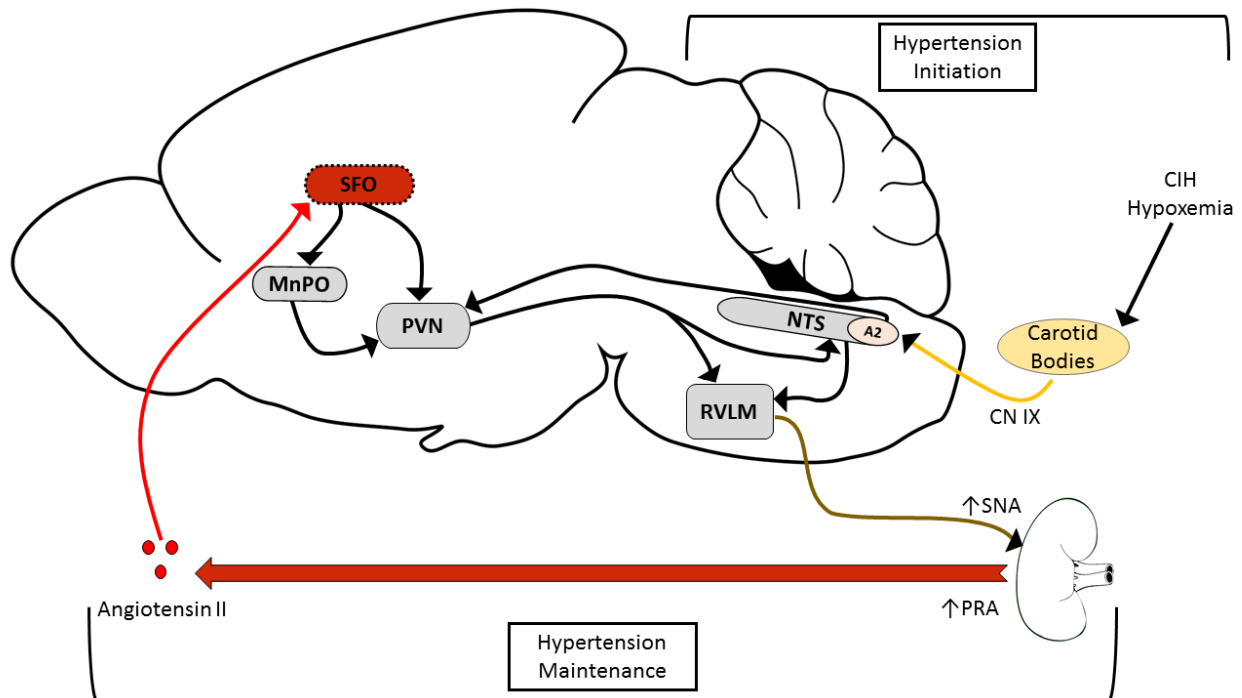


Figure 1: Hypertension Initiation -The hypoxemia from CIH or Sleep Apnea drives the activation of the carotid bodies. This in turn activates the NTS and the RVLM to increase sympathetic tone. In addition, the sympathetic outflow to the kidneys increases plasma renin activity (PRA) and results in increased circulating Angiotensin II (Ang II) during both the hypoxic period and later during the normoxic period. **Hypertension Maintenance** – Circulating Ang II activates SFO which synapses and activates MnPO. Both of these nuclei stimulate the PVN. The NTS also has afferent connections to PVN that could converge with excitation from the SFO and MnPO. Together with enhanced activity from the NTS due to altered chemoreceptor function, these forebrain mechanisms could contribute to the maintenance of the hypertension during the normoxic periods of CIH.

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LITERATURE REVIEW PART 2

ANGIOTENSIN II AND BODY FLUID HOMEOSTASIS

Body fluid homeostasis is essential for organismal survival and is therefore tightly regulated. Control of body fluids directly affects blood pressure and osmolality which are essential to maintain homeostasis. In order to exert control over renal losses in fluid there is a complex set of autonomic and endocrine responses that modify renal function, as well as behavior. Retention of salt and water can be exerted by release of Aldosterone and AVP respectively. Ang II exerts some control in release of both of these hormones preventing loss of fluid and electrolytes and preserving blood volume and osmolality.

Even with a variety of retention mechanisms there is still a necessary loss of body fluid through respiration, perspiration, and excretion. In order to restore body fluid homeostasis, thirst generation and subsequent fluid consumption is necessary. This is mediated by networks similar to those that control cardiovascular autonomic and endocrine function. Again, Ang II plays a key role in driving pathways to retain water, but also in stimulating thirst to drive drinking behavior and restore body water balance.

Thirst can be generated through a decrease in blood pressure as well. Maintenance of blood pressure can independently signal for increased fluid consumption. Hypovolemia, a true loss in blood volume and therefore a decrease in cardiac output is a potent stimulator of Ang II. Diminished pressure at cardiopulmonary and carotid baroreceptors drives sympathetic nerve activity to release Ang II. Ang II production occurs in order to further increase sympathetic tone, vasoconstriction, and thirst[1, 2]. This also occurs when there is only an apparent loss of blood volume through reductions in venous return. Dogs instrumented with an occlusive cuff on the vena cava to reduce venous return increased fluid consumption in response to the apparent, but not actual, hypovolemia[3, 4]. This response occurs due to an unloading of the sinoaortic or cardiopulmonary baroreceptors and thirst can be reliably reproduced by denervation of these receptors[5]. The converse is also true as loading the baroreceptors through inflating a balloon in the pulmonary artery where it meets the right atrium reduces thirst and water intake[6]. These manipulations indicate that while fluid conservation mechanisms and thirst can be elicited in response to depletion, they can also be initiated in apparent depletion, or as a means to maintain blood pressure.

Aside from the effects of the baroreceptors on thirst and drinking behavior, Ang II also induces thirst with hypovolemia. Fitzsimons and colleagues identified that nephrectomy would diminish thirst in response to hypovolemia[7]. Further analysis of kidney extracts revealed that the dipsogenic agents were renin[7] and Ang II[8]. These early experiments relied on supraphysiological doses of Ang II to generate thirst which generated controversy as to its role in normal function[9]. Lower and lower doses were used until a 200pmol/ml infusion was found to act as the threshold for thirst[10-13]. Normal circulating Ang II concentrations are below

60pg/mL[14-16]. More recent studies have shown that mice lacking the angiotensinogen gene do not drink in response to hypovolemia further indicating a physiological role for Ang II induced thirst[2]. These studies identified the peripheral factors that were capable of generating thirst in response to hypovolemia, but thirst could be generated through changes in osmolality as well.

Perturbations to body fluid homeostasis through changes in osmolality also influence thirst and water retention. Verney's seminal study indicated that intracarotid administration of NaCl or dextrose results in water retention, whereas urea does not[17]. This effect occurs for salt and dextrose because they act as an effective osmole, whereas the urea does not. Verney termed cells that would most likely shrink in response to these effective osmoles as "osmoreceptors" even though their specific identity was unknown[17]. Thirst is a centrally driven mechanism so identifying brain regions that could eliminate dipsogenic responses provides insight to the identity of the osmoreceptors.

The anterior regions of the third ventricle, later identified as the lamina terminalis, including the highly vascularized SFO was hypothesized to play a role in body fluid homeostasis[18]. Administration of low doses of Ang II the SFO resulted in a reliable dipsogenic response[19]. Lesions of the SFO individually[20, 21] and surrounding nuclei, the MnPO, and OVLT generate profound adipsia[22, 23]. Animals that undergo total lamina terminalis lesions must be presented with sweetened water to prevent wasting[5]. All of this evidence indicated that the lamina terminals played a crucial role in thirst, and suggested that the lamina terminals may contain the osmoreceptors Verney coined, but still did not verify their location. Ablation of the OVLT increases the hypertonicity required to elicit drinking, and reduces the quantity of fluid consumed[24]. *In vitro* studies indicate that after dissociation of OVLT neurons exposure to

hypertonic solutions causes the neurons to depolarize[25-27]. The OVLT is not the only osmosensory unit of the lamina terminalis though as the SFO too has been shown to respond to osmotic challenges[28]. The MnPO responds to changes in CNS sodium, and there are a subset of neurons which hyperpolarize in response to hyponatriuric artificial cerebrospinal fluid (aCSF)[29].

There is substantial evidence to indicate that entirety of the lamina terminalis may play some role in osmoreception and thirst generation in response to Ang II. The role of each individual nuclei and their specific interactions with Ang II in order to modulate thirst is not well known. Research presented here helps to elaborate on the specific role of AT1 receptors in generating thirst at the MnPO in response to Ang II administration and systemic hypertonicity.

OVERALL HYPOTHESIS AND SPECIFIC AIMS

Ang II is capable of generating thirst at the MnPO as well as exerting control over autonomic regulation through modulation of sympathetic outflow[30]. Given the dual proposed role of Ang II in the MnPO we sought to explore the physiological consequences of its absence.

Overall Hypothesis: Ang II Receptors in the Median Preoptic Nucleus (MnPO) are necessary for both autonomic regulation and dipsogenic responses.

Specific Aim I: To test the role of MnPO AT1a receptors in CIH hypertension.

In order to test this hypothesis, we first examined the role AT1 receptors play in autonomic control of hypertension in CIH. Increased sympathetic outflow is a hallmark of CIH and various forebrain regions, including the MnPO, have been implicated in the pathogenesis[31-33]. Utilizing a shRNA sequence complementary for AT1a receptors we will determine the role AT1a receptors play in management of blood pressure in response to CIH

Specific Hypothesis I: Knockdown of AT1a Receptors in the MnPO will block the sustained hypertension associated with CIH.

Dominant negative inhibition of FosB in the MnPO prevents the sustained component of hypertension, and Fos activity has been previously linked to AT1 receptor upregulation[33-35]. Given this information we expect knockdown of AT1a receptors to prevent FosB expression in the MnPO and prevent the sustained component of hypertension from CIH

Specific Aim II: MnPO AT1a receptors are necessary for Ang II induced thirst.

AT1 receptors in the MnPO also have been implicated in the MnPO's ability to regulate thirst. Lesion of the MnPO reduces thirst in response to subcutaneous and ICV Ang II[36, 37]. We will test the specific role of MnPO AT1a receptors by selectively eliminating them via shRNA against AT1a.

Specific Hypothesis II: Knockdown of AT1a Receptors in the MnPO will diminish drinking responses to centrally and peripherally administered Ang II.

ICV Ang II produces thirst, and lesions of the MnPO, either electrolytically or through ibotenic acid, both reduce this drinking response[36, 37]. We expect knockdown of AT1a to produce a similar effect. Administration of Ang II peripherally through subcutaneous administration does not directly act on the MnPO, but does stimulate the CVOs to drive the MnPO to stimulate drinking. This CVO-MnPO pathway is presumed to utilize Ang II as the primary neurotransmitter and we seek to establish this through our AT1a specific knockdown.

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CHAPTER II

**A VITAL ROLE FOR THE MEDIAN PREOPTIC AT_{1a} RECEPTOR IN THE SUSTAINED
HYPERTENSION OF CHRONIC INTERMITTENT HYPOXIA**

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ABSTRACT

Sleep Apnea leads to hypertension that persists throughout the diurnal period. The neural mechanisms that underlie this pathophysiological increase in blood pressure are not well known. Chronic Intermittent Hypoxia (CIH) is a model of the hypoxemia from sleep apnea. This model produces a sustained increase in blood pressure, and the central renin-angiotensin system has been implicated in this sustained hypertension. The median preoptic nucleus receives inputs from circumventricular organs outside the blood brain barrier and projects to regions that control sympathetic activity making it a prime target for integrating peripheral signals with central sympathetic drive. Inhibition of FosB in the median preoptic nucleus prevents the sustained CIH hypertension. CIH also increases Angiotensin Type 1a receptor in the median preoptic nucleus via FosB. Utilizing a short hairpin RNA we knocked down the Angiotensin Type 1a receptor in the median preoptic nucleus to determine its contribution to CIH hypertension. AT1a receptor knockdown in the median preoptic nucleus blocked the sustained component of hypertension from CIH as compared to scramble injected animals. Immunohistochemistry revealed significantly less FosB positive cells in the median preoptic nucleus of animals injected with the knockdown vector compared to animals given the control vector after 7 days of CIH. The rostral ventrolateral medulla, a key regulator of sympathetic outflow, also expressed significantly less FosB with the AT1a knockdown in the median preoptic nucleus. Our results indicate that AT1a receptors in the MnPO are regulated by FosB and contribute to CIH hypertension.

INTRODUCTION

Sleep apnea (SA) is characterized by repeated disturbances to normal respiration leading to a number of physiological changes in part due to the intermittent hypoxemia[1]. SA is associated with elevated sympathetic outflow and mean arterial pressure (MAP) which persist beyond the hypoxic sleeping hours[1-3]. Long term chronic sympathetic activity and elevated MAP produce a variety of cardiovascular sequelae such as hypertension[4]. In order to clinically address the chronic sympathoexcitation a deeper understanding of the pathophysiological mechanisms is necessary. Chronic intermittent hypoxia (CIH) is an experimental model of the episodic hypoxemia experienced by SA patients[5]. This model effectively produces the increased SNA and MAP seen in SA patients after only a few days of exposure[1, 6]. A number of mechanisms that contribute to the sympathoexcitation in SA have been proposed, including changes in chemoreceptor function[6] and respiratory-sympathetic coupling[7] leading to chronic activation of sympathetic control centers.

Peripheral RAS has long been implicated in the pathophysiology of SA, but recent studies in animal models have demonstrated an additional distinct neural mechanism that is essential for the sustained component of hypertension[8-10]. This mechanism involves the lamina terminalis which demonstrates increased FosB/ Δ FosB staining following 7 days of CIH along with other brain regions involved in central autonomic regulation such as the paraventricular nucleus (PVN) of the hypothalamus, the nucleus tractus solitarius (NTS), and rostral ventrolateral medulla (RVLM) of the hindbrain[11]. The FosB staining in the lamina terminalis appears to be related to activation of the Renin-Angiotensin system (RAS) that occurs during CIH.

Circulating Angiotensin II (Ang II), a major effector peptide hormone, binds to the subfornical organ (SFO), and organum vasculosum lamina terminalis (OVLT) of the lamina terminalis. These two nuclei are highly vascularized and lack a blood brain barrier allowing peripherally circulating Ang II to access neurons in these regions. Both of these nuclei synapse upon the median preoptic nucleus (MnPO) which is situated between the SFO and OVLT along the anterior wall of the third ventricle but lies within the blood brain barrier[12]. All three of these areas contain angiotensin receptors[13]. Early studies by Fletcher's laboratory demonstrated a role for the RAS in CIH hypertension [14, 15]. More recent studies on CIH have linked the RAS to modulation of peripheral chemoreceptors[16] and activation of the lamina terminalis[8-10]. Chronic intracerebroventricular (ICV) infusions of the angiotensin receptor antagonist losartan block both the sustained component of CIH hypertension that occurs during the normoxia and FosB staining in the lamina terminalis[8]. Similar effects are produced by virally mediated knock down of AT1a receptors in the SFO [9]. Dominant negative inhibition of MnPO FosB via overexpression of JunD results in a reduction in the sustained hypertension of CIH[10]. Previous studies in other animal models suggest that the AP-1 transcription factor family, which includes FosB, may regulate the expression of AT1a receptors in the CNS providing a mechanism in which FosB inhibition results in lower MAP after CIH[17, 18].

The ability of FosB to potentially regulate MnPO RAS components in CIH induced hypertension lead us to investigate a role of FosB and AT1a signaling in this region. We hypothesized that AT1a signaling in the MnPO plays a vital role in the sustained component of CIH hypertension.

METHODS

Animal Care

Experiments were performed according to the National Institute of Health guide for the care and use of laboratory animals and the University of North Texas Health Science Center Institutional Animal Care and Use Committee. These experiments used 6 week old (250-300g) adult male Sprague-Dawley rats (Charles River Laboratory, Wilmington, MA). Animals were individually housed in 8"x9" cages in temperature controlled rooms with a 12 hour light dark cycle with the light phase lasting from 7am-7pm. Food and water were available ad libitum and animals were housed in 8" by 9" cages. Surgeries were performed using aseptic technique, and post-operative infection prevented by subcutaneous administration of procaine penicillin G (30,000 U). A non-steroidal anti-inflammatory drug, carprofen (Rimadyl, 2mg po. VWR, Radnor PA), was given before and after surgery for pain management.

Viral Vectors

Viral vectors used in these experiments were purchased from GENEDETECT (Auckland, New Zealand). The adeno-associated viruses (rAAV1/2) either contained a small hairpin RNA sequence to match the AT1a receptor (AAV-AT1ashRNA) or a scrambled (AAV-SCRshRNA) sequence. Viruses were used undiluted at a titer of 1.1×10^{12} genomic particles/ml. Both viruses expressed green fluorescent protein to verify accuracy of the injection location.

Microinjections

Rats were anaesthetized with 2% isoflurane and their scalps were shaved and disinfected with alcohol and iodine. Each rat was then placed in a Kopf stereotaxic head frame (David Kopf

Instruments, Tujung, CA). To ensure accurate injections skulls were leveled along two cranial suture landmarks, bregma and lambda. The injector was angled 8° from medial to lateral and the injection coordinates used for the MnPO were 0.9 mm lateral, and 6.7 mm ventral from bregma [19] . After drilling a burr hole at the site of injection, a 30 gauge steel injector was lowered to the MnPO and 200-300nl of AAV-AT1ashRNA delivered. The injector was connected to a Hamilton 5µL syringe (#84851 Hamilton Reno, NV) by calibrated polyethylene tubing that was used to determine the injection volume. The injector remained inserted for 5 minutes to allow for absorption then the injector was slowly backed out of the skull. Gel foam was packed in to the opening in the cranium. Absorbable antibiotic suture was used to close the incision site and minimize post-surgical infection. Rats were allowed 7 days recovery before telemetry instrumentation.

Telemetry

All rats were implanted with the Data Sciences International (DSI St. Paul, Minnesota) TA11PA-C40 telemetry unit. This telemetry was used in conjunction with the Dataquest A.R.T.™ 4 acquisition system. Prior to implantation rats were anaesthetized with 2% isoflurane and their abdomens were shaved, and then cleaned and sterilized with 100% ethanol and iodine. A midline incision was made from the xyphoid process to the start of the pelvis. The abdominal aorta was located, isolated, tied off with silk, and after a puncture with a 23-gauge needle the tip of the telemetry was inserted. Vetbond (3M, St. Paul, MN) and woven cellulose gauze was used to adhere the telemetry unit to the abdominal aorta. The battery of the telemetry unit was secured to the abdominal muscle via prolene suture, and the incision site closed with absorbable vicryl antibiotic suture. Animals were allowed 7 days to recover after telemetry implantation. During

the experiments, heart rate (HR), respiration rate (RR), activity (ACT), and mean arterial pressure (MAP) are all measured for 10 seconds every 10 minutes via the radio telemetry system and transmitted to Dataquest.

Chronic Intermittent Hypoxia

Animals were transferred to CIH chambers a week after telemetry instrumentations and 2 weeks after virus microinjections. Animals were housed in a 8"x 9" cage that was placed inside of custom plexi-glass chambers. Rats were housed in these chambers for a 5 day baseline period prior to the start of the 7 day CIH protocol. CIH occurred in 6 minute cycles; 3 minutes of 21% oxygen room air pumped in, 90 seconds of nitrogen pumped in to lower the chamber O₂ to 10% oxygen, and then 90 seconds of maintenance at 10% oxygen[9, 11]. This cycle repeated for 10 times per hour, 8 hours a day (8am-4pm to match sleeping hours of the rats) for 80 total cycles[20] each day for 7 days. Animals were exposed to room air for the remainder of the 24 hour period (4pm-8am).

FosB inhibition

Two weeks prior to baseline rats were anesthetized with isoflurane (2%) and injected in the MnPO with an AAV vector containing a dominant negative construct against FosB, Δ JunD and GFP or a control vector containing only GFP. After a two week recovery period the rats were exposed CIH for 7 days. On the morning of the 8th day, the rats were anesthetized with inactin (100 mg/kg ip) and sacrificed[10]. After extraction 23 gauge coronal punches containing the MnPO were harvested from each brain. The MnPO was identified histologically by the presence of the anterior commissure. RNA was extracted from the samples using the PARIS kit (Ambion,

ThermoFisher Scientific, Grand Island, NY) according to the manufacturers' instructions and used for qRT-PCR analysis.

Laser Capture Microdissection and qRT-PCR

On the morning of the 8th day after CIH animals were anesthetized with inactin (100 mg/kg ip), sacrificed, and their brains removed. Each brain was immediately snap frozen in ice-cold isopentane. Frozen brains were sectioned at 10µm on a Leica CM 1950 cryostat (Leica Biosystems, Buffalo Grove, IL, USA) onto Polyethylene Naphthalate membrane coated slides (Arcturus Biosciences, Mountain View, CA, USA). A laser capture microdissection system (Arcturus® Veritas, 13553-00, version-c) was used to identify and laser capture MnPOs that were GFP positive. Captured regions were placed on Arcturus CapSure Macro LCM Caps (LCM0211) and RNA was purified using the epicenter ArrayPure™ Nano-scale RNA Purification Kit according to manufacturer's instructions[21]. The purified RNA underwent amplification using the epicenter TargetAmp™ 2-Round AminoallylaRNA Amplification Kit 1.0. and purified using the RNeasy® MinElute® Cleanup Kit (Madison, WI). Quality and purity of the RNA was verified spectrophotometrically on a Nanodrop 2000c (ThermoScientific Wilmington, DE, USA). RNA was considered pure when the 260nm/280nm wavelength absorbance ratio was above the standard RNA purity of 1.8 according to Nanodrop instructions. 40ng of the resulting purified RNA was used for reverse transcriptase (rt) PCR via the Sensiscript® Reverse Transcription Kit as per our previous publications[9, 21-24].

The cDNA that was produced from reverse transcription was used for quantitative PCR.

Individual 15µL qPCR reactions consisting of 1.8µL of cDNA, 1.2µL of a primer mix, 7.5µL of iQ SYBR Green Supermix, and 4.5µL RNase free water were performed in a 96 well plate. A

Bio-Rad iQTM5 iCycler 191 system thermocycler (Bio-Rad Laboratories Inc., Hercules, CA, USA) was used for perform the qPCR. Each reaction was performed in duplicate and reflectance units (RFU) data from the iCycler was analyzed according to the $2^{-\Delta\Delta C_t}$ relative reference method in accordance with our previous publications[21-24]. Primers used in conjunction with syber green are listed below.

Primers

Δ FosB

Forward 5'-AGGCAGAGCTGGAGTCGGAGAT-3'

Reverse 5'-GCCGAGGACTTGAAGTTCACCTCG-3'

S18

Forward 5'-CAGAAGGACGTGAAGGATGG-3'

Reverse 5'-CAGTGGTCTTGGTGTGCTGA-3'

AT1a

Forward 5'-ACTCACAGCAACCCTCCAAG-3'

Reverse 5'-ATCACCACCAAGCTGTTTCC-3'

AT1b

Forward 5'-AGAAGAACACGCCAAGAA-3'

Reverse 5'-TGAATGAGCACATCCAGAA-3'

Immunohistochemistry

Rats from each treatment group were prepared for immunohistochemistry and their brains were processed for FosB staining as previously described[20]. After 7 days of CIH or normoxia, rats were anesthetized with inactin (100 mg/kg ip) and perfused with PBS followed by 4% paraformaldehyde in PBS. The brains were removed from their vaults and placed in 4%

paraformaldehyde overnight and transferred to 30% sucrose solution for dehydration the next morning. After dehydration, hindbrains and forebrains were separately sectioned at 40 microns using a Leica cryostat. Three sets of serial sections were placed in cryoprotectant[25] and stored at -20°C. One set of sections was bleached with 30% peroxide, and stained for FosB/ Δ FosB using a goat primary antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA; 1:1000). Sections were incubated in the primary antibody for 3 days at 4°C[20]. This primary antibody was reacted with a biotinylated anti-goat secondary, and finally visualized using 3,3'-diaminobenzidine hydrochloride to allow for FosB expression counts. Hindbrain sections were co-labeled with a mouse DBH antibody (Millipore, Billerica, MA; 1:1,000), and aCY3-labeled anti-mouse secondary antibody in order to identify the catecholaminergic NTS, CVLM, and RVLM neurons. Stained sections were imaged on an epifluorescent microscope (Olympus BX41, Olympus, Center Valley, PA, USA) with a digital camera (Olympus DP70) and FosB was counted utilizing NIH Image J software (National Institutes of Health, v1.49). Counts were averaged between sections of each nuclei and sub-sections of the NTS and PVN.

Statistical Analysis

Based upon pilot and previous data the number of animals required for the studies has been calculated using power analysis for a $p < 0.05$ and an $\alpha \geq 0.80$. Baseline data for telemetry recordings that were obtained during the 3-5 days prior to the start of CIH were analyzed for between group differences using two way repeated measure ANOVA for treatment and condition. These baseline measurements were averaged for each rat and the averages were used to calculate Δ MAP, Δ HR, Δ RR, and Δ ACT measurements from baseline. The delta data were analyzed by separate two way mixed effects ANOVA. Post hoc analysis on between group differences was

performed using the Student Newman-Kuel's (SNK) test. Within group differences for absolute values for each condition were analyzed using separate repeated measures ANOVA followed by a Holm-Sidak post hoc test to compare against average absolute baseline conditions. Data from the mRNA quantification generated from qPCR were analyzed with a one way ANOVA for condition. FosB data were analyzed for between group effects using separate one way ANOVA followed by SNK post-hoc tests for condition. All analyses were performed on the SigmaPlot statistical software package (SigmaPlot v.12, Systat Software, Inc., San Jose, CA). Statistical significance was set at $P < 0.05$. All data are reported as a mean \pm one SEM. All data was tested for equal variance and normality to insure parametric statistics were appropriate.

RESULTS

Regulation of MnPO AT1a by CIH

In order to determine the role of AT1a receptors in the MnPO in CIH hypertension, we first tested the effects of FosB inhibition on changes in MnPO AT1a message associated with CIH. Seven days of CIH was associated with a significant increase of AT1aR message in the MnPO and this increase was blocked by the inhibition of FosB with the dominant negative Δ JunD (Figure 1-E).

Next, we investigated whether this CIH-induced increase in AT1a message could be blocked using an AAV vector approach. AAV-shAT1a injections in the MnPO prevented the increase in AT1aR message in this region that is normally associated with CIH. CIH was associated with a significant increase in AT1a message in rats that were injected in the MnPO with the control scramble vector (Figure 1-F). The AT1aR message in the MnPO of rats injected with AAV-SCR and exposed to CIH were significantly higher compared to all of the other treatment groups (Figure 1). AT1a message measured from the MnPO of rats injected with AAV-shAT1a and exposed to CIH were not different from the normoxic control groups ($P > .05$). CIH did not significantly affect AT1b message in the MnPO. AT1b message from the two CIH exposed groups were not significantly different from the rats exposed to normoxia and injected with AAV-SCR. AT1b was significantly elevated in normoxic AAV-shAT1a animals, but not in any other treatment group ($P < .05$) (Figure 1-F).

Role of MnPO AT1a Receptors in CIH Hypertension

Injections of AAV-shAT1a did not influence basal MAP, HR (Table 1), RR, or ACT as compared to AAV-shSCR injected rats ($P>.05$). CIH significantly increased MAP from baseline in rats injected with AAV-shSCR and rats injected with AAV-shAT1a that did not include the MnPO (“Miss”) (Table 1). These increases were observed from 0800 to 1600 during CIH exposure (“light” phase) and in the normoxic dark phase on each day of the 7 day protocol. During CIH exposure from 0800 to 1600 h, the delta changes in MAP were not different among the three CIH treated groups ($P>.05$, Figure 2-A). Rats injected with AAV-shSCR and the rats injected with AAV-shAT1a outside of the MnPO demonstrated changes in MAP that were significantly increased as compared to the two normoxic groups (Figure 2-A). The rats injected in the MnPO with AAV-shAT1a did demonstrate significant increases in MAP although not on every day of CIH (Figure 2-A). The rats exposed to normoxia did not show changes in MAP from baseline during the 7 day protocol regardless of which construct was injected in the MnPO as well as no overall difference between light and dark phase blood pressure ($P>.05$, Figure 2-A).

In rats with confirmed injections of AAV-shAT1a in the MnPO, CIH did not significantly increase blood pressure from baseline during the normoxic dark phase (Fig 2-B). During the normoxic dark phase, rats injected with AAV-shSCR or AAV-shAT1a outside of the MnPO and exposed to CIH demonstrated changes in the MAP that were significantly greater than rats injected in the MnPO with AAV-shAT1a treated with CIH and rats from the two groups maintained in normoxia (Figure 2-B). The sustained changes in MAP produced by CIH in rats injected in the MnPO with AAV-shAT1a were significantly decreased as compared by a 2 Way

RM ANOVA to the two other CIH treated groups and not different from the normoxic groups (Figure 2-B).

A significant difference was found overall between dark phase Δ HR in normoxic AAV-shAT1a and hypoxic AAV-shAT1a miss animals (Fig 2-D). There was also a significant difference overall in RR during CIH ($P < .05$, Figure 3-A). No significant differences were found overall between any of the groups for CIH Δ HR (Figure 2), Normoxic Δ RR, or Δ ACT ($P > .05$, Figure 3). No significant difference was found in activity among the groups ($P > .05$).

Effects of MnPO AT1a Knockdown on CIH induced FosB Staining.

FosB/ Δ FosB staining in OVLT and SFO was not significantly different between the two groups exposed to CIH independent of which viral vector that they received, and the rats injected with the AAV-AT1a were not significantly increased as compared to the two normoxic groups (Figure 4-B). In the MnPO, CIH AAV-shSCR animals had significantly more cells expressing FosB/ Δ FosB than the CIH knockdown group, or the normoxic groups (Figure 4-B). AAV-shAT1a injected and AAV-SCR injected rats exposed to CIH had greater FosB/ Δ FosB staining in the PVN than either normoxic group (Figure 5-B). Similar patterns were observed in the PVN subregions (Figure 5-B).

The CVLM revealed no significant difference in FosB expression between any of the groups. In the RVLM, AAV-shSCR CIH animals expressed significantly more FosB/ Δ FosB positive cells than CIH AAV-shAT1a or normoxic animals ($P < .05$). Overall, CIH significantly increased FosB staining in the NTS of rats injected with either AAV-AT1a or AAV-SCR and there was no significant difference between the two CIH treated groups (Figure 6-C). While similar results were observed in the commissural and pre-commissural NTS, AAV-AT1a injections in the MnPO were

associated with a significant reduction in FosB staining in the subpostremal NTS as compared to CIH treated rats that had been injected with AAV-SCR in MnPO ($P < .05$, Figure 6-C)

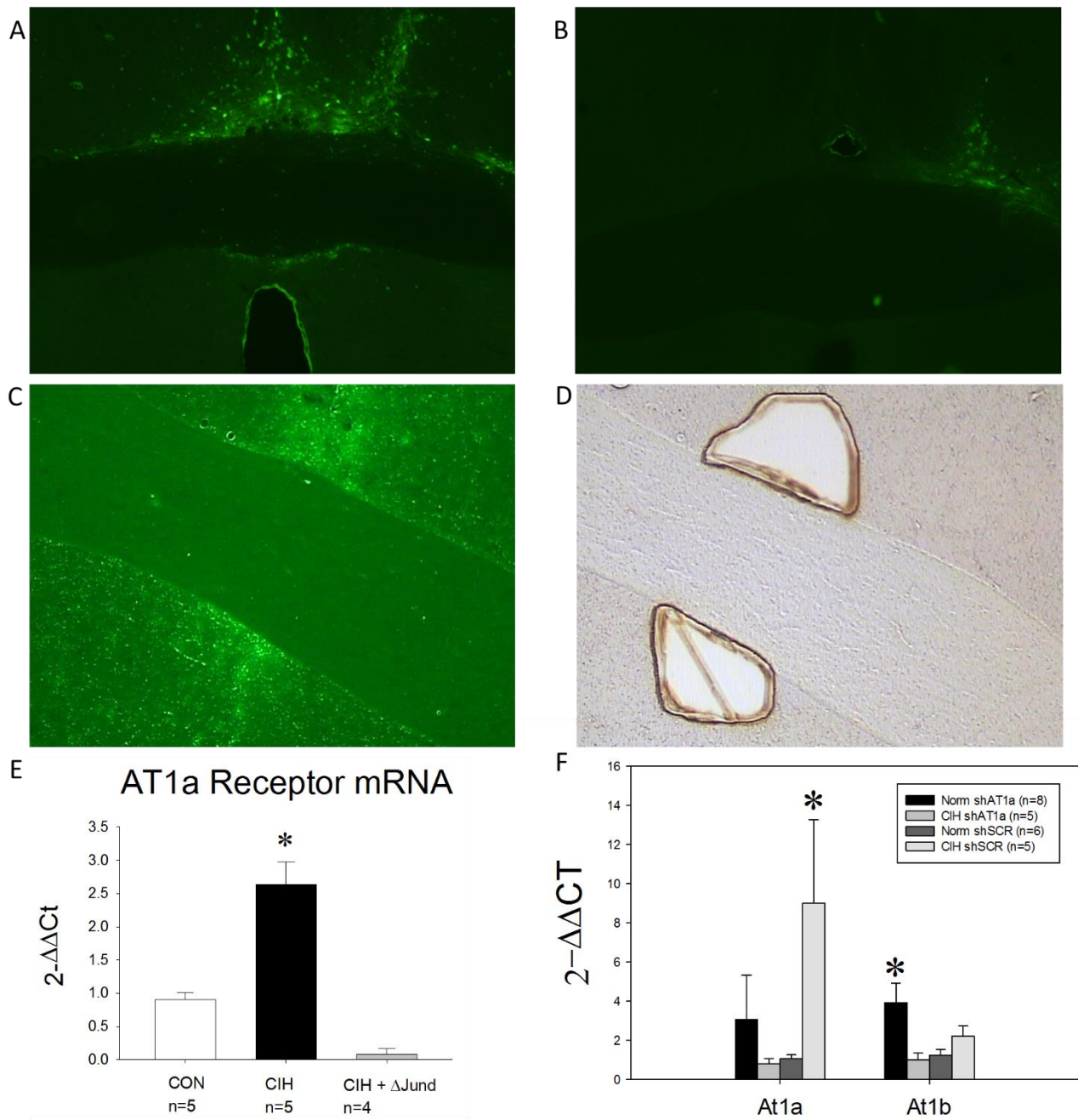


Figure 1. A & B. Representative images of a successful MnPO hit and miss respectively. C & D. Laser capture microdissection before with fluorescence (C) and after capture in brightfield (D).

E. CIH increases AT1a Receptors through FosB. 7 Days of CIH increases the expression of AT1a Receptor mRNA from punch sections of the MnPO. Presence of the Δ JunD Dominant Negative construct prevents the elevation in AT1a Receptor message compared with the controls and CIH animals (*P<.05). F. shAT1a prevents the increase of AT1a expression after 7 days of CIH. qRT-PCR from Laser captured MnPOs demonstrate the ability of shAT1a to prevent an increase in AT1a receptor message after 7 days of CIH as compared to CIH SCR animals (*P<.05). Expression of AT1a in CIH shAT1a was comparable to normoxic counterparts (P>.05). AT1b expression was significantly higher in normoxic shAT1a injected animals (*P<.05).

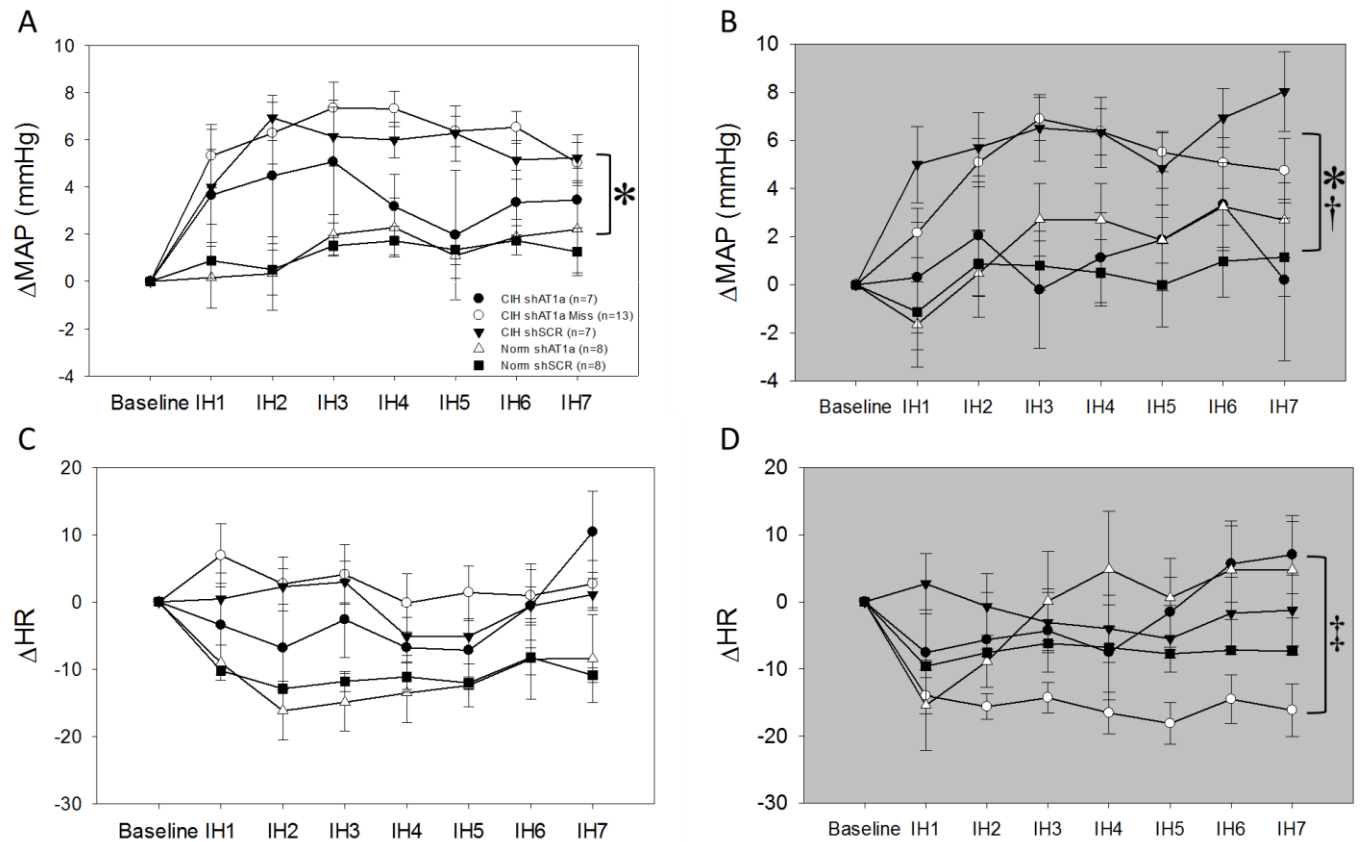


Figure 2. Knockdown of AT1a receptors in the MnPO prevents the sustained component of hypertension from CIH. A. CIH SCR and CIH shAT1a miss animals had significantly elevated blood pressure during CIH as compared to normoxic controls ($*P<.05$), but their pressure was not significantly different from AT1a knockdown animals ($P>.05$). B. During the normoxic dark phase the hypertension from CIH was prevented as the CIH scramble and virus miss groups were significantly different from the normoxic animals ($*P<.05$) and AT1a Injected CIH animals ($\dagger P<.05$). C & D. There was no difference in heart rate amongst any groups during CIH, but there was a significant different between CIH AT1a knockdown animals and their normoxic counterparts during the normoxic dark phase ($\ddagger P>.05$).

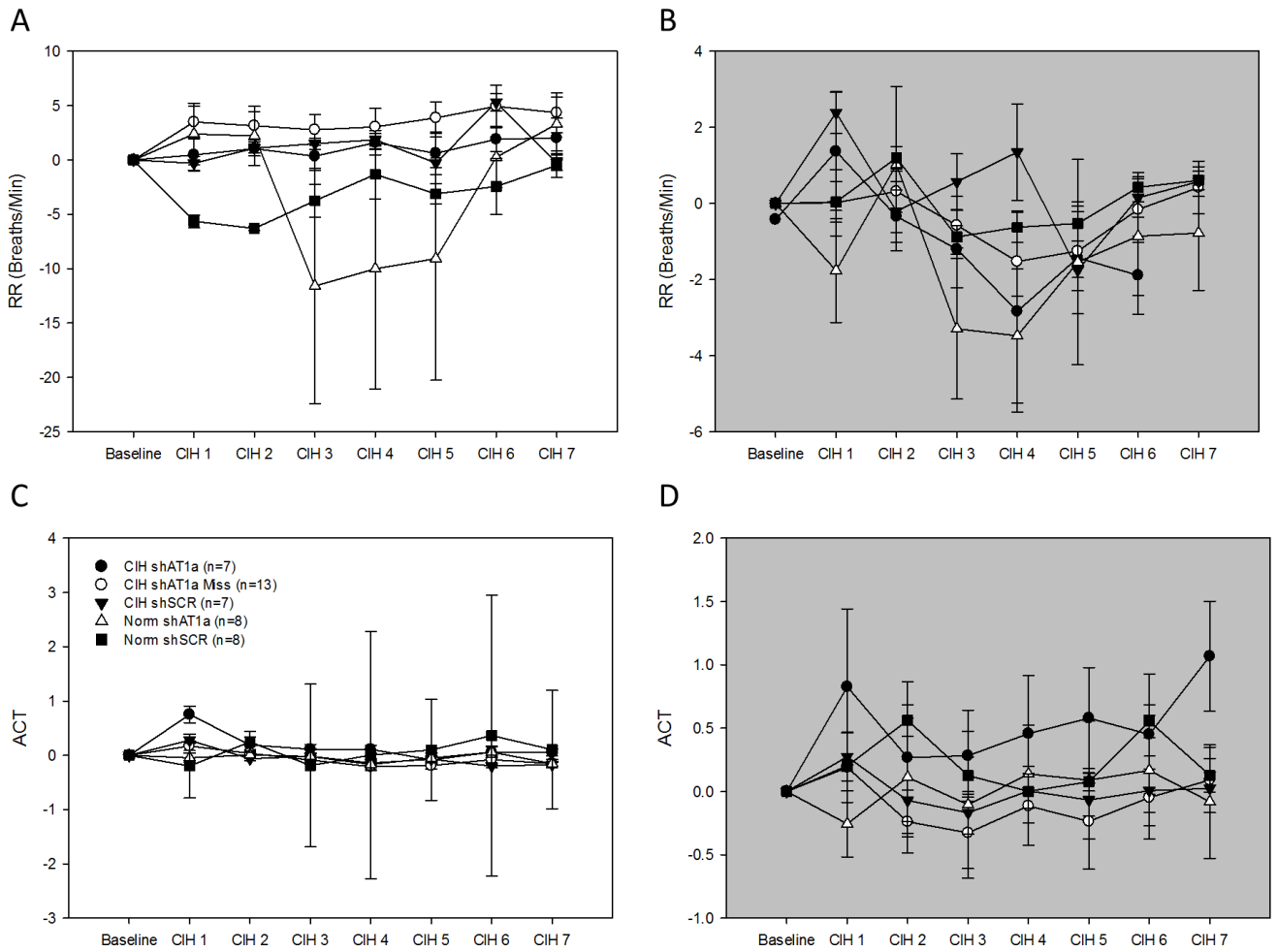


Figure 3. Respiration and Activity. Figures A and B represent the respiration rate during CIH (A) and normoxia (B). There was a significant difference overall in RR during CIH ($P < .05$). Panels C and D represent the CIH and normoxic activity of the animals respectively. There was no difference observed in activity.

	shSCR Norm n=8	shSCR CIH n=7	shAT1a KD Norm n=8	shAT1a KD CIH Hit n=7	shAT1a KD CIH Miss n=13
MAP (mmHg)					
Light Baseline	90.8±2.3	91.7±2.3	92.5±2.3	96.9±2.6	91.7±1.8
Light CIH	92.1±2.3	97.3±2.4 **	94.1±2.3	100.8±2.4	98.6±1.8 **
Dark Baseline	99.1±2.4	96±2.4	100.4±2.3	102.1±2.7	97.4±1.9
Dark CIH	99.7±2.4	101.4±2.6 *	102±2.4	103.3±2.6	102.7±1.9**
Heart Rate					
Light Baseline	313.8±8.3	328.5±8.5	326.1±8.3	344.1±9.5	323.6±6.6
Light CIH	302.3±8.3	327.9±8.9	314.2±8.3	344.3±8.9	325±6.5
Dark Baseline	386.7±7.8	391.5±8	386.2±7.8	391.7±9	382.1±6.2
Dark CIH	379±8.3	378.2±8.9	384.3±8.3	386.3±8.9	366.5±6.5

Table 1. Average Blood pressure and heart rate throughout the baseline and CIH periods separated by light and dark periods. No significant differences exist between the baselines of any groups. Within the shSCR CIH animals there was a significant difference between the baseline average and the 7 days of CIH both during the light hypoxic periods (*. $P < .05$), and during the dark normoxic period (** $P < .001$). The same trend was true of the shAT1a KD CIH Miss animals that had significantly higher pressure during the 7 days of CIH both during the light hypoxic and dark normoxic periods (** $P < .001$)

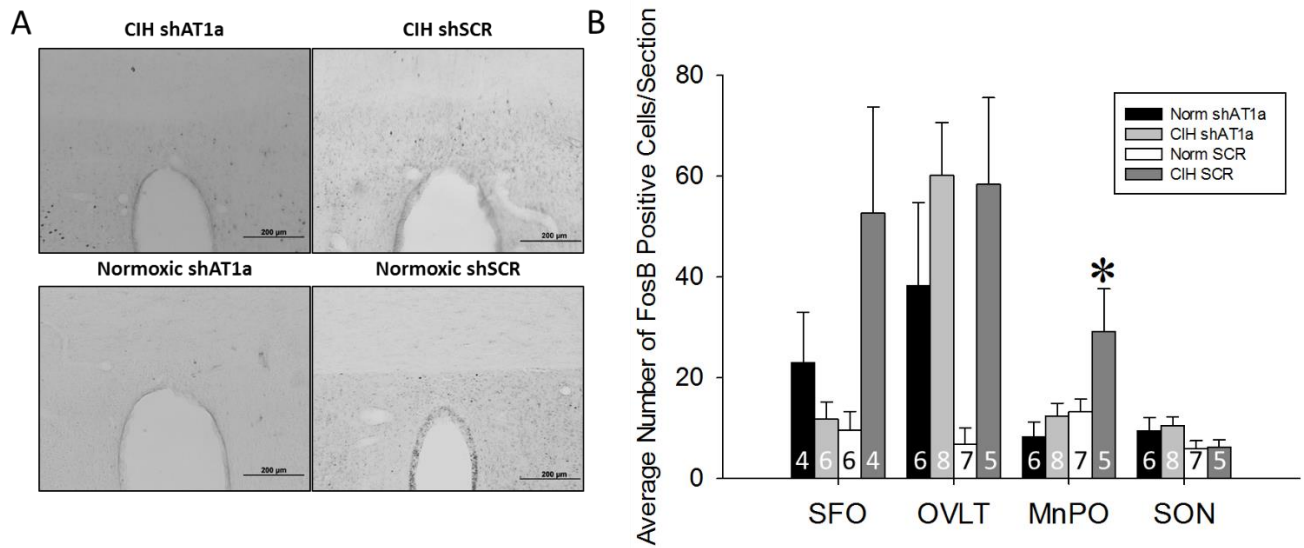


Figure 4. A. Representative examples of DAB visualized FosB expression in MnPOs. B. CIH SCR animals expressed significantly more FosB in the MnPO than normoxic controls or CIH shAT1a animals (* $P < 0.05$). Knockdown of AT1a receptors successfully prevented increased FosB expression in the MnPO. CIH shAT1a animals were comparable to normoxic controls ($P > 0.05$). No significant differences exist for the SFO, OVLT, or SON.

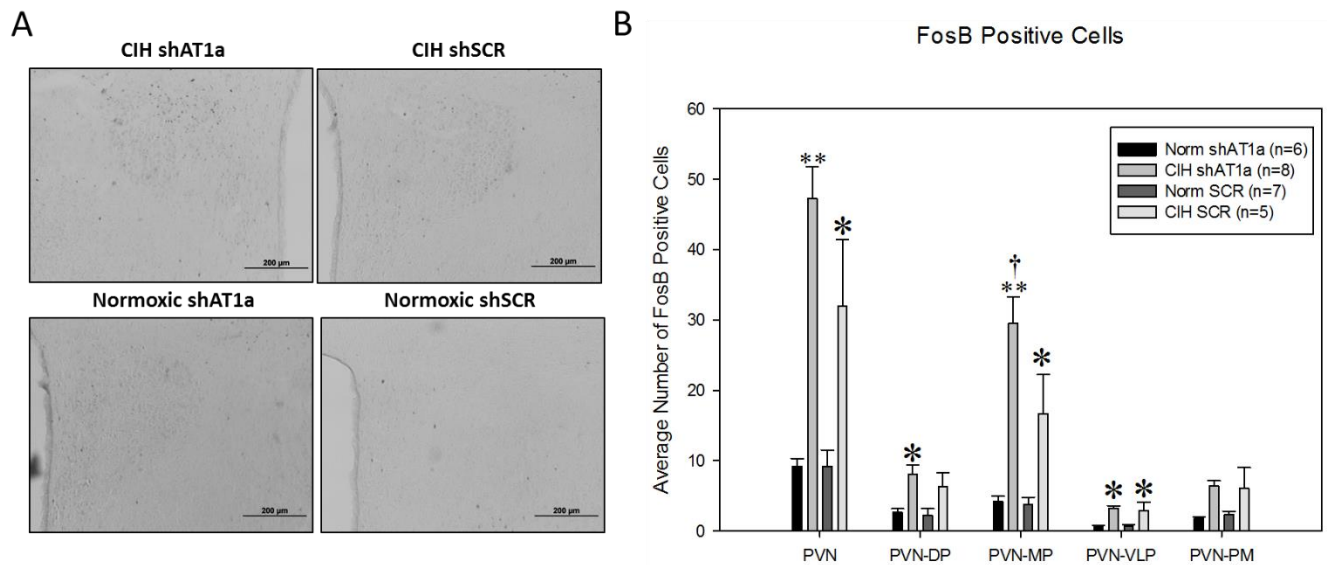


Figure 5. A. Representative examples of DAB visualized PVN sections. B. Both scramble (SCR) (* $P < .05$) and virus injected CIH animals (CIH shAT1a) (** $P < .001$) had significantly increased FosB expression their PVNs as compared to normoxic controls both overall and in the median parvocellular region (mp). CIH shAT1a injected animals also exhibited higher FosB than their scramble normoxic counterparts in the median parvocellular (PVN-MP) PVN ($\dagger P < .05$). FosB was elevated in CIH animals in the ventrolateral parvocellular region as well (* $P < .05$ vs normoxic animals), and the CIH shAT1a animals had elevated FosB in the dorsal parvocellular region (* $P < .05$ vs normoxic animals). This suggests that the prevention of hypertension by MnPO AT1a knockdown may not have been mediated through the PVN.

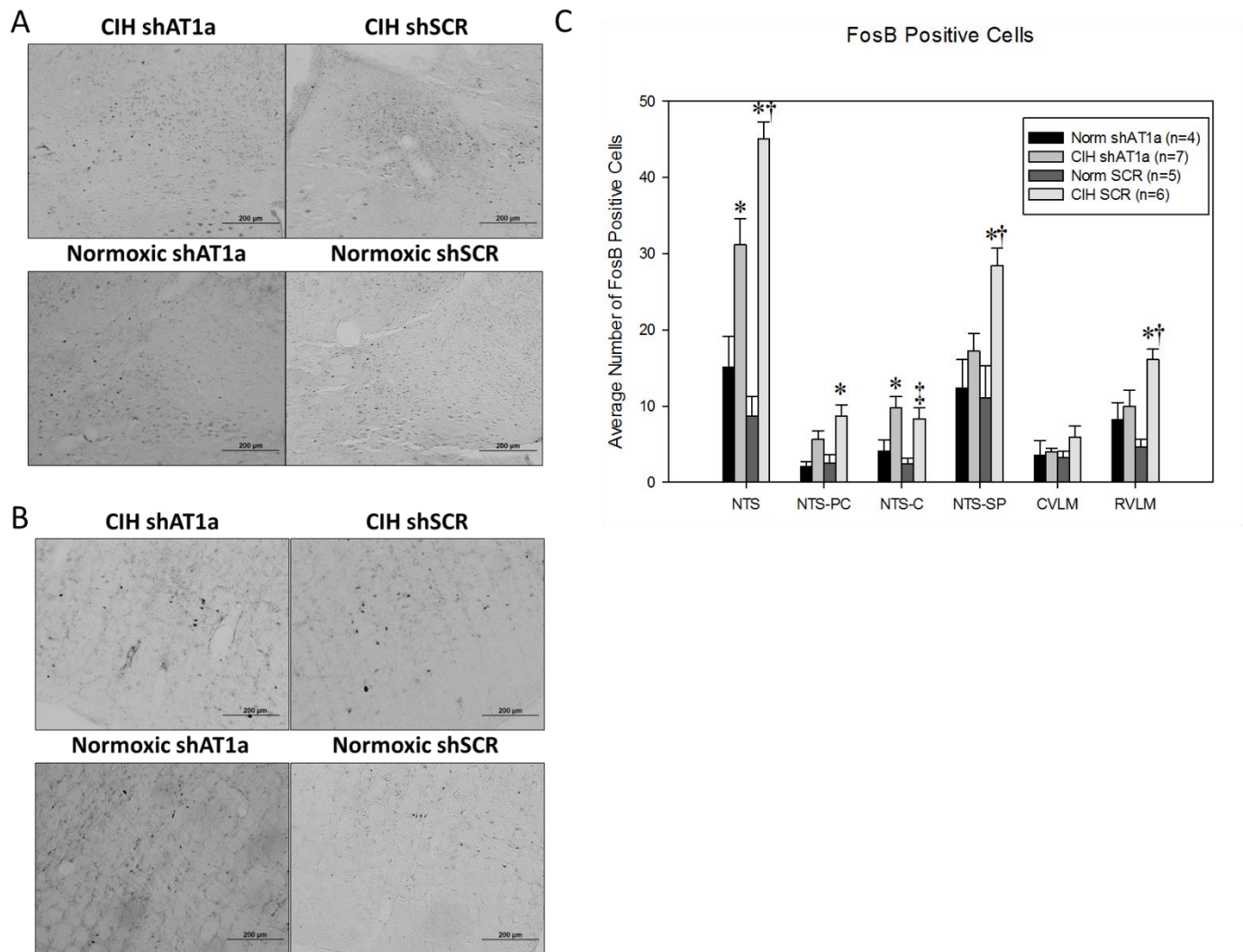


Figure 6. A. Representative examples of DAB visualized Subpostremal (SP) Nucleus Tractus Solitarius (NTS). B. Representative images of the rostral ventrolateral medulla (RVLM). C.

There was an overall difference in FosB expression in the Nucleus Tractus Solitarius ($P < .001$), and specific differences between Scramble CIH (CIH SCR) animals compared to normoxic animals ($*P < .05$) and shAT1a CIH animals ($\dagger P < .05$). Subsection analysis in the pre-commissural region found a significant difference between Scramble CIH animals and both normoxic groups ($*P < .05$). shAT1a CIH animals had significantly more FosB staining than either normoxic group

and AAV-SCR CIH animals expressed more FosB than their normoxic scramble counterparts. In the subpostremal NTS (NTS-SP) SCR CIH animals expressed more FosB positive cells than either the normoxic control groups (* $P < .05$) or the shAT1a CIH animals ($\dagger P < .05$) (Panel A). CIH SCR animals also exhibited significantly more FosB in the RVLM as compared to normoxic controls or CIH shAt1a animals (* $P < .05$) ($\dagger P < .05$).

DISCUSSION

AT1a receptor message in the MnPO was significantly increased following 7 days of CIH. This same 7 day CIH protocol has previously been shown to increase MnPO staining for the transcription factor FosB[20]. We have previously shown that dominant negative inhibition of FosB in the MnPO prevented the sustained increase in blood pressure associated with CIH that is observed during normoxia without affecting the blood pressure increases that occur during the intermittent hypoxia[10]. In the current study, we also used shRNA knockdown of AT1a receptors in the MnPO to test AT1a's role in CIH hypertension. The results show that the shRNA injection in the MnPO blocked the increase in AT1a receptor message associated with CIH without affecting message for the AT1b receptor. Functionally, AT1a receptor knockdown in the MnPO affected CIH hypertension in the same manner as FosB inhibition. The sustained component of the hypertension that occurs during normoxia was blocked by AT1a receptor knockdown in the MnPO (Figure 2-B) while the increase in blood pressure that was recorded during the intermittent hypoxia exposures from 0800-1600 was not different from rats exposed to CIH but injected with a control vector or shRNA injections that missed the MnPO (Figure 2-A).

Unexpectedly, normoxic animals loaded with the virus expressed significantly more AT1b receptor message. There was no difference in cardiovascular variables between normoxic shAT1a animals and their scramble controls indicating the increase in AT1b expression had no functional effect. This is not unexpected as the two receptors share over 90% amino acid sequence homology[26, 27]. Expression of AT1b in a model system of chinese hamster ovaries revealed that both receptor subtypes stimulate the same intracellular pathway[28]. The differences between the AT1 receptors is likely limited to location of expression and not

pharmacology or function. The lack of the sustained component of hypertension is most likely from the reduction in AT1a expression after CIH.

Overall, the results of the present study suggest that the AT1a receptor is a downstream target of FosB in the MnPO that contributes to CIH hypertension. This is consistent with previous studies which indicated that AP-1 transcription factors can increase AT1 receptor expression [17, 18]. In our initial study that used dominant negative inhibition of FosB [10], our PCR array analysis identified other components of the RAS such as ACE1 and ACE2 as possible downstream targets of FosB but did not include the AT1a receptor.

Although the effects of AT1a receptor knockdown and dominant negative inhibition of FosB in the MnPO produced similar effects on CIH hypertension, there were differences in how these manipulations influenced CIH-induced FosB staining. The PVN exhibited a marked increase in FosB expression in response to CIH in both the knockdown animals and their scramble counterparts (Figure 4-B). The increase in FosB expression has previously been seen in numerous CIH studies[8-10], but the AT1a receptor knockdown failed to prevent this increase in FosB expression despite blocking the sustained component of the CIH hypertension. In previous studies of CIH hypertension from our group, CNS treatments that have diminished CIH hypertension have also significantly reduced FosB staining in parvocellular PVN[8-10, 29]. Chronic infusion of AT1 antagonists into the PVN are also capable of preventing hypertension from CIH[30]. The PVN has been shown to directly contribute to increase sympathetic nerve activity associated with CIH[31]. Given the role of the PVN in CIH hypertension, it is possible that this FosB staining may indicate activation of a subset of PVN neurons not responsible for sympathetic activity. CIH has been shown to sensitize the HPA axis[32] and this aspect of PVN

function was not assessed in the present study so increased FosB expression in the PVN may not be related to sympathetic outflow. Alternatively, the differences could be related to other functions of the AT1a receptor in this region. For example, sodium sensitive MnPO neurons require AT1 receptors for their inhibitory response to GABA and removing AT1a receptors may remove this brake allowing for increased stimulation of neurons in the PVN. Recent work has shown that chronic activation of a subset of oxytocin producing PVN neurons projecting to the dorsal motor nucleus of the vagus are capable of preventing the sustained component of hypertension from CIH with hypercapnia[33].

Overall, there was a significant increase in NTS FosB staining between the rats exposed to CIH and those who were not (Figure 5-C). Subsection analysis revealed that in the subpostremal NTS the numbers of FosB positive neurons associated with CIH was significantly lower in rats injected with AT1a shRNA as compared with rats injected with scrambled shRNA. This more rostral segment of the NTS is responsible for sympathetic respiratory control. Following CIH, numerous labs have found respiratory coupled sympathetic outflow is increased at the end of expiration[34-36]. This increase in expiratory sympathetic drive has been postulated to be partially responsible for the sustained hypertension. The ability of the MnPO AT1a knockdown to prevent an increase in subpostremal NTS FosB may indicate some role in this reported change in sympathetic respiratory coupling.

The RVLM is a major source of sympathetic premotor neurons that contribute to blood pressure regulation. AT1a knockdown in the MnPO prevented the increase in FosB staining in the RVLM that was seen in CIH treated rats injected with the control vector. This result is consistent with the effects of AT1a knockdown in the MnPO on CIH hypertension and suggests that decreased

SNA contributed to the reduction in blood pressure potentially through changes in vascular tone and cardiac output.

We previously demonstrated that ICV administration of losartan is more effective than subcutaneous losartan at preventing CIH hypertension[8]. However, this broad administration does not provide information on the contribution of individual nuclei. Our current study sought to identify the contribution of the MnPO to the central blockade of Ang II using a virally mediated approach that was specific to the MnPO. Knockdown of AT1a in the MnPO prevented the sustained component of hypertension from CIH most likely by blocking inputs from the SFO and therefore preventing the MnPO from stimulating downstream nuclei that control SNA.

In response to AT1a knockdown there was a decrease in FosB expression locally in the MnPO which indicates AT1a's role in stimulating FosB expression. AT1 receptors are known to increase in expression or sensitivity in response to stimulation, and Fos proteins play a key role in this response, at least neuronally[37, 38]. By eliminating the AT1a receptor there is a reduction in both the activation of NADPH oxidase, and activation of CREB which may work independently, or in concert to drive cFos then FosB to transcriptionally activate AT1 receptor production[18, 39]. Taken together, dominant negative inhibition of FosB prevents the increased AT1a expression and it seems that AT1a stimulation of the MnPO increases FosB transcription factor which in turn drives AT1a expression. A breakdown at either the angiotensin signaling or the transcriptional activation is sufficient to prevent the blood pressure increase from CIH.

We previously have demonstrated an overall effect of AT1 inhibition in CIH hypertension via ICV losartan administration[8], and followed up by demonstrating the role of MnPO FosB in CIH hypertension. The current study highlights the critical role that AT1a receptors in the MnPO

play in the sustained hypertension from CIH. This finding helps to solidify the role of central RAS in the pathogenesis of hypertension from CIH, and provides future avenues of treatment that may be more efficacious and less palliative. The RVLM is a major source of sympathetic premotor neurons that contribute to blood pressure regulation. AT1a knockdown in the MnPO prevented the increase in FosB staining in the RVLM that was seen in CIH treated rats injected with the control vector. This result is consistent the effects of AT1a knockdown in the MnPO on CIH hypertension and suggests that decreased SNA contributed to the reduction in blood pressure through changes in vascular tone and cardiac output.

PERSPECTIVES

The pathophysiology of SA is poorly understood in part due to the fact that sufferers cannot self-identify as sleep apneic. While the disease progresses silently, the sequelae become indistinguishable from the inciting pathophysiology and determining what initiated hypertension as opposed to what maintains it becomes difficult. The 7 day model of CIH allows for study of the initiating factors in the pathogenesis of neurogenic hypertension. RAS activation both peripherally and centrally has been shown to be necessary for the development of CIH hypertension[9, 10, 40-42]. Our study demonstrated the role MnPO AT1a receptors play in the onset of neurogenic hypertension from CIH. Treatment with AT1 receptor blockers that can cross the blood brain barrier may provide relief from hypertension as well as impede the progression of the neuropathology in the forebrain. There are numerous other pathophysiological process occurring simultaneously though such as sLTF in the chemoreceptors and weakened K_{ATP} responses to hypoxia [43, 44]. In order to reliably treat hypertension from advanced sleep apnea, it may be necessary to target multiple mechanisms. More studies are needed to determine

if a multi-tiered approach is necessary to target dysfunction in the forebrain separate from the chemoreceptors and hindbrain.

LIMITATIONS

The current study was unable to directly measure AT1 receptor protein expression due to a lack of effective antibodies[45]. In addition, there were no direct measurements of Ang II binding so it is impossible to quantify the efficacy of the AT1a receptor knockdown relative to the blood pressure response of the animals. The lack of sustained hypertension in AT1a knockdown animals while speculated to be through reduced sympathetic outflow may be mediated by other mechanisms since direct measurements of sympathetic nerve activity were not performed.

qPCR measurements of AT1a and AT1b were performed, but other receptors that bind Ang II such as AT2 may play a role in the prevention of the sustained hypertension from CIH. A more rigorous analysis may reveal Ang II is binding to alternative receptors in the absence of AT1a.

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CHAPTER III

MEDIAN PREOPTIC AT1A RECEPTORS NOT NECESSARY FOR THIRST GENERATION FROM SUBCUTANEOUS ANGIOTENSIN II

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ABSTRACT

Angiotensin II is known to produce a dipsogenic response when administered peripherally, or centrally. Peripheral Ang II acting as a hormone activates circumventricular organs (CVOs), brain regions lacking a blood brain barrier, such as the subfornical organ. These circumventricular organs synapse upon the median preoptic (MnPO) nucleus which leads to activation of higher cortical centers and thirst. Intracerebroventricular (ICV) administration of Ang II produces thirst without interacting with CVOs by directly activating the MnPO and it is therefore presumed that Ang II is the neurotransmitter release at the CVO-MnPO junction. We sought to test this hypothesis by knocking down AT1a receptors in the MnPO using a shRNA complimentary for the receptor subtype. Knockdown of AT1a in the MnPO significantly reduced drinking in animals administered ICV Ang II ($P < .05$). This was mirrored by a reduction in cFos, a marker of neuronal activity, in the MnPO, SON, and PVN ($P < .05$). Thirst generated by subcutaneous administration of Ang II was unaffected by the knockdown of AT1a in the MnPO ($P > .05$), and cFos staining amongst the SFO, OVLT, MnPO, PVN, or SON also was not significantly influenced ($P > .05$). This data indicates that AT1aR receptors in the MnPO are not necessary for water intake stimulated by peripheral ANG II. While experimental administration of Ang II directly to the ventricles is sufficient to induce drinking it may not play a prominent role in physiological drinking.

INTRODUCTION

Peripheral Angiotensin II (Ang II) is known to generate thirst[1, 2], but there is a paucity of information available as to the specific mechanisms underpinning how this peripheral hormone drives a central dipsogenic response. Ang II binds to circumventricular organs (CVOs), nuclei outside the blood brain barrier that receive inputs from the blood through fenestrated capillaries, such as the subfornical organ (SFO) and organum vasculosum lamina terminalis (OVLT). Both of these CVOs express angiotensin type 1 (AT1) receptors[3], and increase their firing in response to Ang II stimulation[4]. The SFO and OVLT both have efferent projections to the median preoptic nucleus (MnPO), and stimulate MnPO activity[5], presumably utilizing Ang II as a peptide neurotransmitter but that has not been determined. The MnPO then stimulates the magnocellular region of the paraventricular nucleus (PVN) of the hypothalamus and the supraoptic nucleus (SON) to release vasopressin in order to retain water while simultaneously activating the parvocellular regions of the PVN to drive higher cognitive centers to stimulate thirst [6].

The actions of Ang II at the CVOs are well explored. Early studies indicated that peripheral Ang II drives thirst[1, 2], and this occurs at a dipsogenic threshold concentration of Ang II at 200pg/mL[7]. Acute administration of Ang II to the SFO in particular demonstrated the role of this sensory CVO in drinking behaviour[8, 9]. Lesions of the SFO predictably diminish the drinking response to peripheral Ang II[10], but not the response to intracerebroventricular (ICV) administration directly to the lateral ventricle[10, 11]. Specific knockdown of AT1a receptors via shRNA in the SFO also diminish drinking response to peripherally administered Ang II[12]. Some of the neurons responding to peripheral Ang II may express transcription factor ETV-1 as

optogenetic stimulation of these neurons elicits drinking[13]. Overall, Ang II alone has been demonstrated to be capable of generating thirst through its interactions with the SFO.

While the SFO is essential to drinking responses to peripheral Ang II it is not the only nuclei that modulates thirst in response to elevations in plasma Ang II. Inside of the blood brain barrier lesions of the MnPO either electrolytically[14] or via ibotenic acid[15, 16] also reduce drinking in response to Ang II. These lesion studies provide evidence for the SFO to MnPO pathway for peripheral Ang II to generate thirst. ICV administration of Ang II can generate thirst even when the SFO is lesioned so long as the MnPO is intact[5, 17]. ICV Ang II does not interact with the SFO since it lies outside the blood brain barrier so thirst can be driven by Ang II directly stimulating the MnPO[11, 18]. Stimulation of the SFO via Ang II increases firing rate of the MnPO[4, 5]. Together, these results seem to indicate that peripheral Ang II stimulates the SFO, which in turn stimulates the MnPO potentially utilizing Ang II to promote thirst.

Direct bath application of Ang II to slices containing the MnPO reveals two distinct subpopulations of neurons. One subpopulation gradually depolarizes in response to Ang II via reduced efflux of K^{+} [19]. The second, sodium sensitive, population of neurons exhibit no immediate effect from Ang II, but when stimulated with GABA the neurons exhibit an enhanced hyperpolarization[20]. One or both of these populations may participate in the thirst signaling when Ang II is applied to the MnPO.

Hypertonic saline also produces a robust drinking response, but this is not thought to be through an Ang II mediated mechanism at the level of the MnPO. Administration of ibotenic acid to lesion cells via excitotoxicity while under ketamine anaesthesia to blockade NMDA receptors selectively preserves the Ang II drinking response[15, 16]. This drinking response, presumably

separate from Angiotensin, acts through a similar downstream neural network through the PVN and higher cortical centers.

Our current study sought to determine if Ang II functions as both a hormone and a neurotransmitter in examining drinking responses to peripheral Ang II. We knocked down AT1a receptors by injecting viral vectors with shRNA within the MnPO and assessed drinking responses to either subcutaneous hypertonic saline, subcutaneous Ang II, or ICV Ang II. We hypothesized that knockdown of AT1a receptors in the MnPO would reduce thirst to both subcutaneous and ICV Ang II, but not to subcutaneous hypertonic saline.

METHODS

Animal Use and Care

All experiments outlined below were performed according to the National Institute of Health guide for the care and use of laboratory animals and approved by the University of North Texas Health Science Center Institutional Animal Care and Use Committee. All studies used 6 week old (250-300g) adult male Sprague-Dawley rats (Charles River Laboratory, Wilmington, MA). All rats were individually housed in temperature controlled rooms with a 12 hour light dark cycle. The light phase lasted from 7am-7pm. Animals were given feed and water *ad libitum*, and housed in 8"x9" cages. All surgeries were performed using aseptic technique, and subcutaneous procaine penicillin G (30,000 U) was used as prophylactic post-operative infection treatment. Carprofen (Rimadyl, 2mg po), a non-steroidal anti-inflammatory drug, was given before and after surgery for pain management.

Viral Vectors

These studies used commercially available recombinant adeno-associated viral (rAAV1/2) vectors from GENEDIRECT (Auckland, New Zealand). The AAVs contained a small hairpin RNA constructs that were either a complementary sequence for the AT1a receptor (AAV-T1ashRNA) or a scrambled (AAV-SCRshRNA) sequence. Both viruses also contained constructs for green fluorescent protein (GFP) as a reporter. and were used undiluted at a titer of 1.1×10^{12} genomic particles/ml.

Microinjections and Intracerebroventricular Cannula Implantation

Animals were induced and anaesthetized with 2% isoflurane and their scalps were shaved and disinfected with alcohol and iodine. The rats were placed into a stereotaxic frame (David Kopf Instruments, Tujung, CA). After securing them into the frame, a midline incision exposed the skull to reveal bregma and lambda which were used to level the skull. All microinjections for the MnPO were at the following stereotaxic coordinates: 0.1 mm posterior, 0.9 mm lateral, and 6.7 mm ventral from bregma at 8° from vertical [21]. Each rats received a 200-300 nl injection of either AAV-AT1a or AAV-SCR. For rats receiving a chronic intracerebroventricular (ICV) cannula, a craniotomy was performed at -1.0 mm posterior; +1.5 mm lateral to bregma. A cannula (Plastics One, Roanoke VA), which extended 4.5 mm below the pedestal, was implanted and secured via Loctite (Henkel, Westlake Ohio) and dental cement (CO-ORAL-ITE Dental Mfg Co, Diamond Springs CA) adhering the cannula to three screws mounted in the skull. The cannula was capped with an obturator to obstruct the opening and prevent infection. After cannula implantation animals were given an analgesic and allowed 14 days of recovery before drinking tests.

Drinking Test

Separate groups of rats were tested for drinking responses stimulated by peripherally or centrally administered Ang II. All tests were conducted during the light phase between 1100-1500 hours. For each test, animals were food and water deprived for one hour in advance in order to ensure they were not sated prior to the test. After the hour of depravation, water was returned for a 30

minute pre-test. Water bottles were weighed for consumption in the pre-test, and then animals received subcutaneous or ICV injections and water intake was measured over the next 2 hours. Bottles were weighed at the end of the two hour drinking test and food was returned. Separate groups of rats were administered 2 mg/kg subcutaneous Ang II, or 2 ng/uL Ang II in aCSF on days 14 and 18. All animals were given 3% NaCl (1 ml/100 g/bw) on day 16 for the drinking test. Rats tested for drinking responses to subcutaneous ANG II were also tested with subcutaneous Ang II 7 days and 3 days prior to stereotaxic surgery in order to select for sufficient fluid consumption of 2mL. ICV animals drink a reliable amount in response to ICV Ang II and did not undergo a pre-test[16, 22, 23]. All animals were sacrificed on day 21 after a mock drinking test in which Ang II was administered, but water was never returned in order to stimulate cFos expression and determine neural regions activated by the Ang II.

Immunohistochemistry

Animals were anesthetized with inactin (100 mg/kg ip) and sacrificed on day 21 via transcardiac perfusion with 4% paraformaldehyde (PFA). Brains were divided between hindbrain and forebrain then postfixed for 24 hours in PFA. Following postfixation, the brains were dehydrated in 30% sucrose until they equalized with the sucrose gradient. Brains were serial sectioned at 40 μ m using a Leica CM 1950 cryostat. Separate sets of serial sections were stored in cryoprotectant at -20°C. One set of the serial sections was utilized for cFos immunohistochemistry. Sections were incubated with 30% hydrogen peroxide, then with a primary goat anti-rat antibody against cFos (1:2000, Santa Cruz Biotechnology, Santa Cruz, CA, USA). After 3 days of incubation at 4°

C the primary antibody was conjugated with a biotinylated anti-goat secondary anti-body (1:200, Vector Laboratories, Burlingame, CA) and visualized using 3,3'-diaminobenzidine hydrochloride. In order for PVN subsection analysis, the tissue was co-labeled for vasopressin using a primary antibody raised in Guinea Pig (1:1000, Peninsula Laboratories, San Carlos, CA) and visualized with anti-guinea pig secondary antibody conjugated with CY3 (1:200, Jackson ImmunoResearch, West Grove, PA). Stained tissue were placed on gelatin coated slides and coverslipped using permount. Regions of interest were imaged using an epifluorescent-equipped microscope (Olympus BX41, Olympus, Center Valley, PA, USA) with a digital camera (Olympus DP70). cFos positive cell counting was performed utilizing NIH Image J software (National Institutes of Health, v1.49). Counts were averaged between sections of each nuclei and sub-sections of the PVN for statistical analysis via a one way ANOVA[24].

Statistical Analysis

All statistical analysis was performed using the SigmaPlot statistical software package (SigmaPlot v.12, Systat Software, Inc., San Jose, CA). Based upon pilot and previous data the number of animals required for the studies has been calculated using power analysis for a $p < 0.05$ and an $\alpha \geq 0.80$. Osmolality, hematocrit, and immunohistochemistry cell counts were analyzed using a one way ANOVA to compare between experimental group conditions. Drinking test consumption, and consumption by weight was analyzed using a two way repeated measures ANOVA.

RESULTS

Hematocrit, Osmolality, and Subcutaneous Hyperosmolar Solution

No differences were observed for osmolality (Figure 1-A) or hematocrit (Figure 1-B) across all groups and conditions ($P > .05$). All experimental groups were not found to be different across hemodynamic variables upon sacrifice (Figure 1). No differences were observed among the groups for drinking responses when challenged with 3% NaCl ($P > .05$, Figure 2).

Drinking Tests

Peripheral ANG II: Prior to stereotaxic surgery, there were no differences among the groups for drinking responses to 2 mg/kg subcutaneous Ang II for the two tests ($P > 0.05$; Figure 3).

Injections of AAV-AT1a in the MnPO did not influence the drinking responses to subcutaneous ANG II on either post test days 14 or 18 ($P > 0.05$, Figure 3). There was a significant decrease overall between pre-test day 2 and day 14 ($P < .05$, Figure 3), and a significant increase between pre days 1 and 2 ($P < .05$, Figure 3) indicating a potentiation of Ang II response from pre day 1 that diminished in the 14 days post surgery. Consumption by weight also exhibited a significant difference between Pre Day 2 and Day 18 ($P < .05$, Figure 3-A), and Pre Day 1 and Day 14 ($P < .05$, Figure 3-A) indicating decreased response to Ang II 14 days after microinjection. Overall, this indicates that MnPO AT1a receptors are not necessary for drinking in response to subcutaneous Ang II.

There were differences in water intake that were not related to the AAV treatments between the second pre-surgical and first post-surgical drinking tests ($P < .05$). The amount of water consumed during the second pre-surgical drinking test was significantly greater than water intake recorded during the first post-surgical drinking test. These trends remained when data was normalized by weight as there was no difference between any groups on individual days ($P > .05$), but a difference between consumption of all groups between Pre day 2 and post day 14 ($P < .05$).

Central ANG II: Ang II administered into the lateral ventricle also produced a marked increase in water intake in the rats injected in the MnPO with scrambled shRNA and rats with injections of AT1a shRNA outside of the MnPO (“Miss”) on day 18 ($P < .05$; Figure 3). The rats with AAV-AT1a injections in the MnPO drank significantly less than the other groups during post-surgical day 18 drinking tests ($P < .05$, Figure 3). These results were the same after normalizing water intake by body weight ($P < .05$, Figure 3). In contrast to the results of the test with subcutaneous injected ANG II, AT1a receptors in the MnPO do contribute to drinking responses produced by ICV ANG II.

The rats injected with AAV-shRNA outside of the MnPO and AAV-SCR both drank significantly more water in absolute consumption during the second post-surgical drinking test (day 18) as compared to the first (day 14) ($P < .05$). This potentiation effect was not observed in the rats with AAV-AT1a injection that included the MnPO ($P > 0.05$, Figure 3)

Immunohistochemistry

Peripheral Ang II: Analysis of cFos staining in the subcutaneous injection groups revealed robust cFos expression in the SFO, OVLT, MnPO, SON, and PVN (Figure 4). There were no

statistically significant differences for cFos staining in the listed nuclei amongst the three different treatment groups ($P > .05$, Figure 5). This indicates that AT1a knockdown in the MnPO failed to prevent cFos expression in response to subcutaneous Ang II in any of the regions that were analyzed. Similar results were observed amongst the subsection analysis in the PVN ($P > .05$).

Central Ang II: AT1a knockdown in MnPO significantly reduced cFos staining in the MnPO associated with ICV ANG II as compared to rats injected in the MnPO with AAV-SCR or AAV-AT1a injections that missed MnPO ($P < .05$, Figure 6). The AT1a receptor knockdown animals also exhibited less cFos expression in the SON as compared to miss animals. There were no differences in Fos staining amongst the groups in the PVN overall, but subsection analysis showed that AAV-AT1a injection in the MnPO significantly decreased cFos in median parvocellular, and posterior magnocellular subsections of the PVN ($P < .05$). Among the ICV injected animals there was not reduced cFos expression in the SFO and OVLT since they are outside the blood brain barrier.

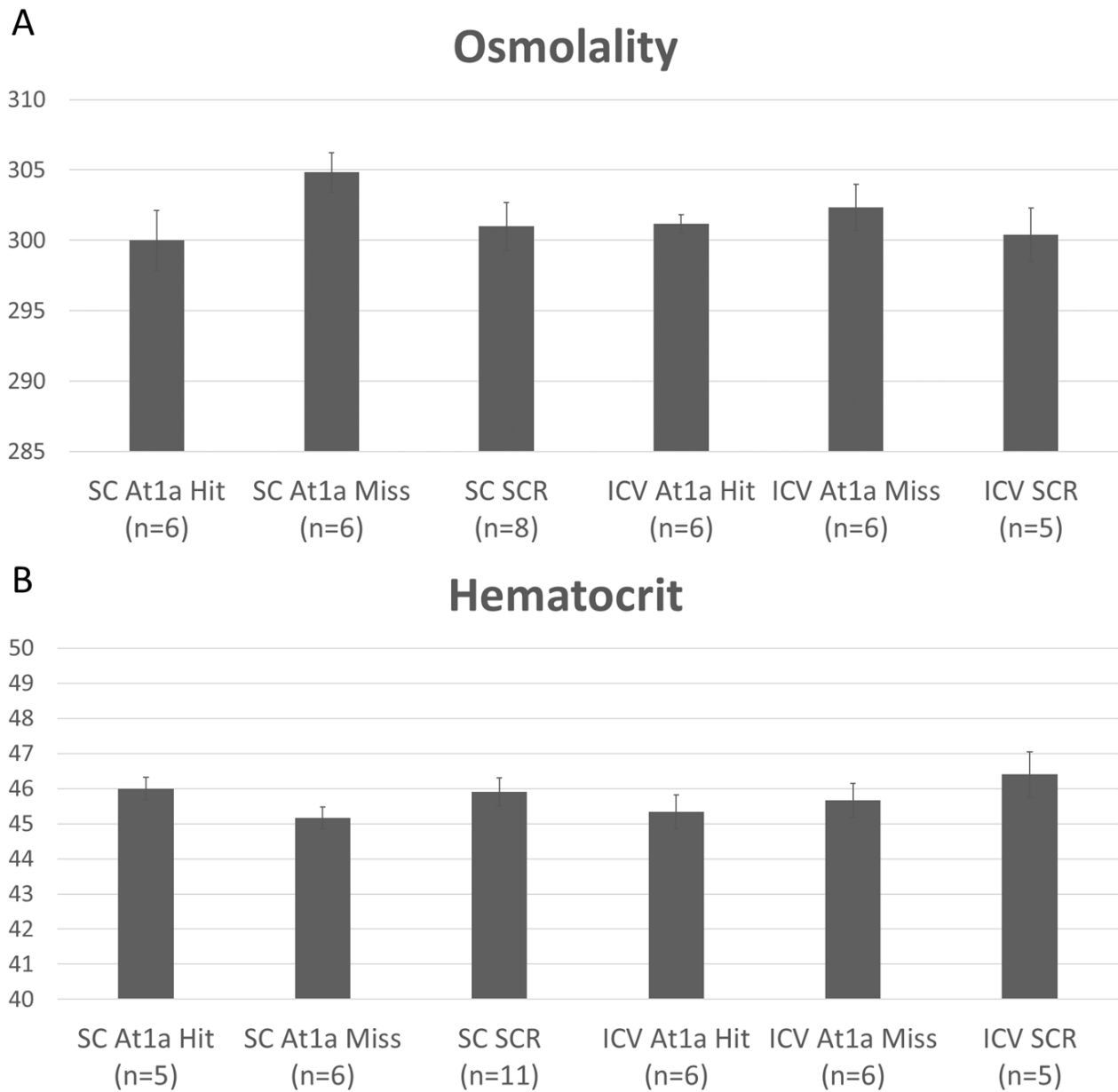


Figure 1. No difference in osmolality or hematocrit. A. No significant difference between any experimental groups in osmolality. B. No significant difference in hematocrit between experimental groups.

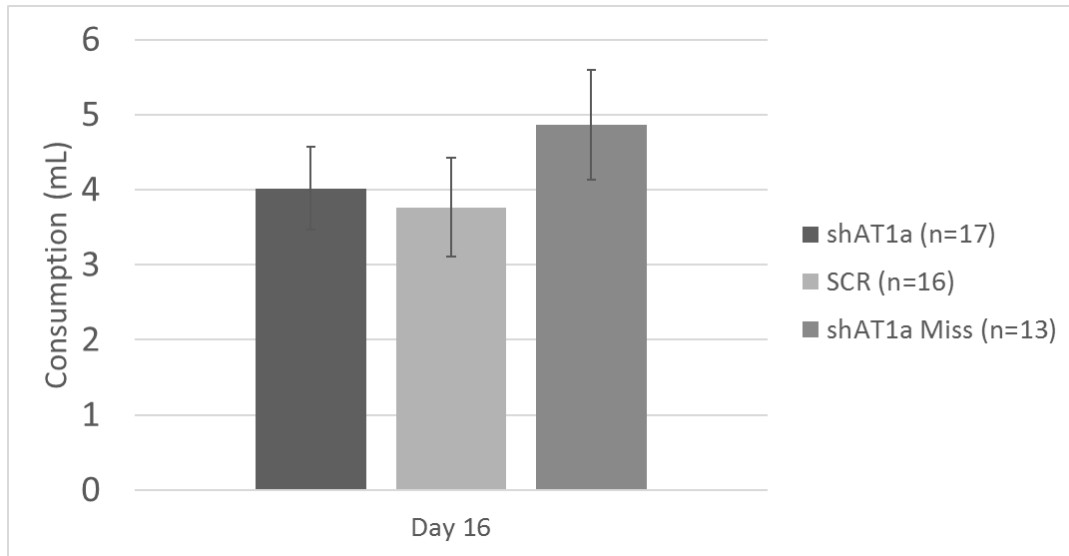


Figure 2. Subcutaneous injection of hyperosmolar solution induces thirst equally amongst all experimental groups ($P > .05$). Knockdown of AT1a receptors has not impact on drinking in response to hyperosmolar solution

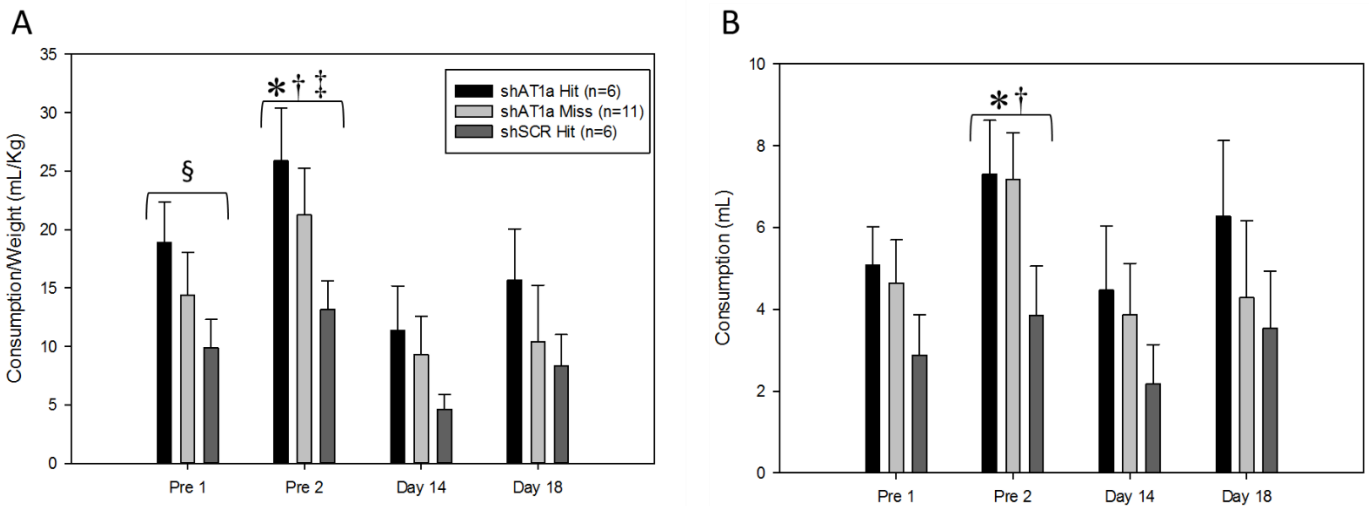


Figure 3. Knockdown of AT1a Receptors in the MnPO do not affect drinking response to subcutaneous Ang II administration. A. No significant differences between experimental groups on pre or post test days. A significance difference overall in consumption between Pre Day 2 and Post Day 14 in consumption and consumption/weight (* $P < .05$) as well as between Pre Day 1 and Pre Day 2 († $P < .05$) indicating the potentiation of Ang II on Pre Day 2. In terms of consumption by weight there was also a significant difference between Pre Day 2 and Day 18 (‡ $P < .05$), and Pre Day 1 and Day 14 (§ $P < .05$) indicating decreased response to Ang II 14 days after microinjection.

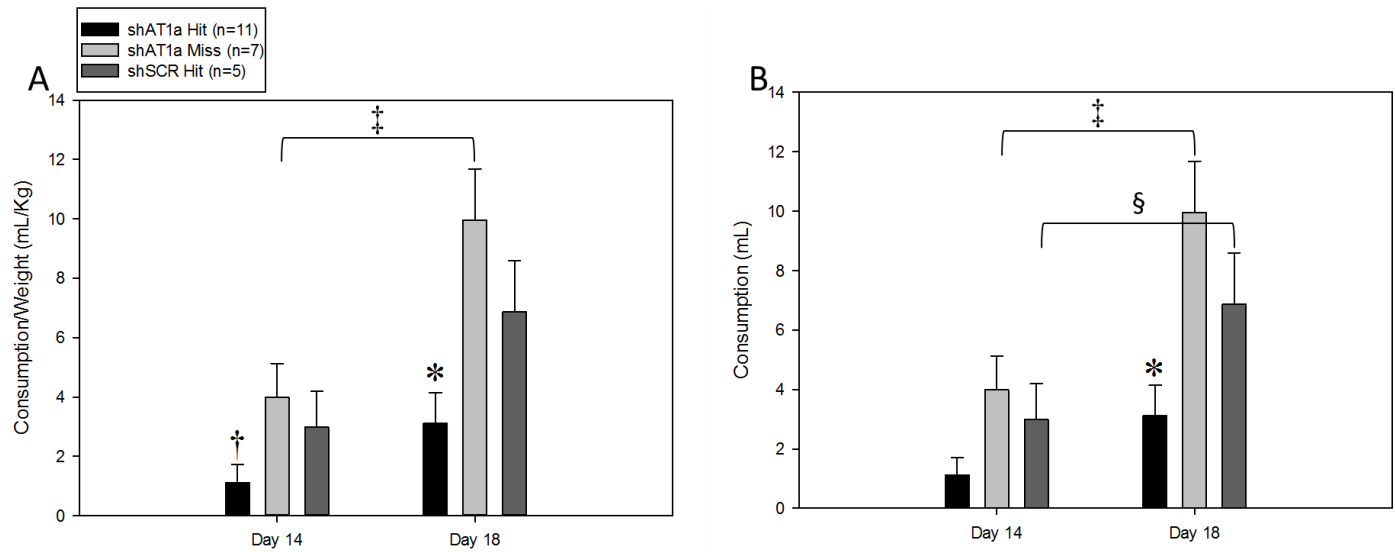
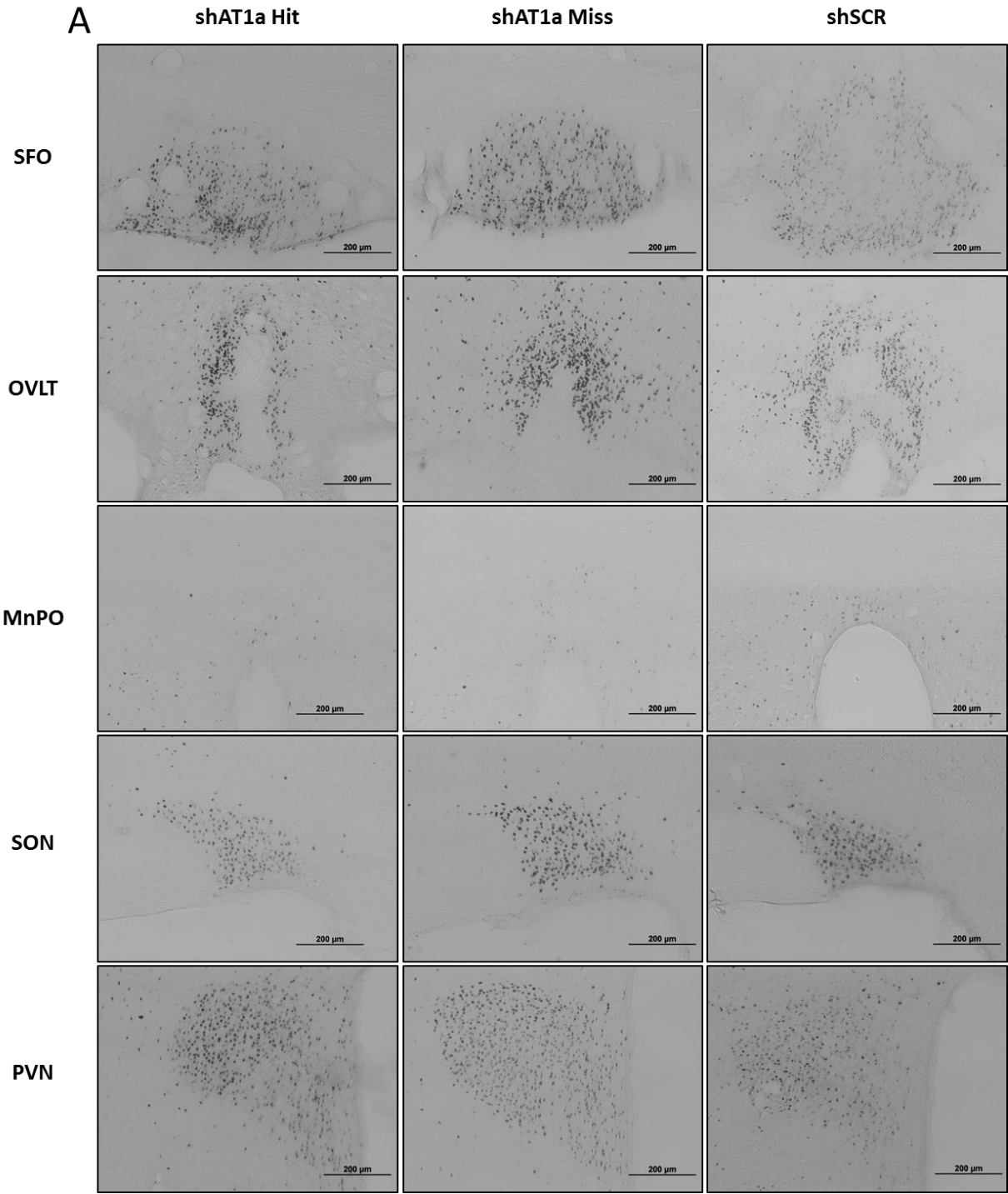


Figure 4. Knockdown of AT1a Reduces Drinking in Response to ICV Ang II Administration.

Significantly less consumption in the shAT1a group as compared to the shAT1a miss and Scramble controls in total consumption and consumption/weight on day 18 (* $P < .05$). There was also a significant difference in consumption/weight between the shAT1a Hit and Miss groups on Day 14 († $P < .05$). There was a significant increase in drinking in the shAT1a miss group between Day 14 and Day 18 in both consumption and consumption/weight (‡ $P < .05$), and a significance difference between scramble absolute consumption between days 14 and 18 (§ $P < .05$).



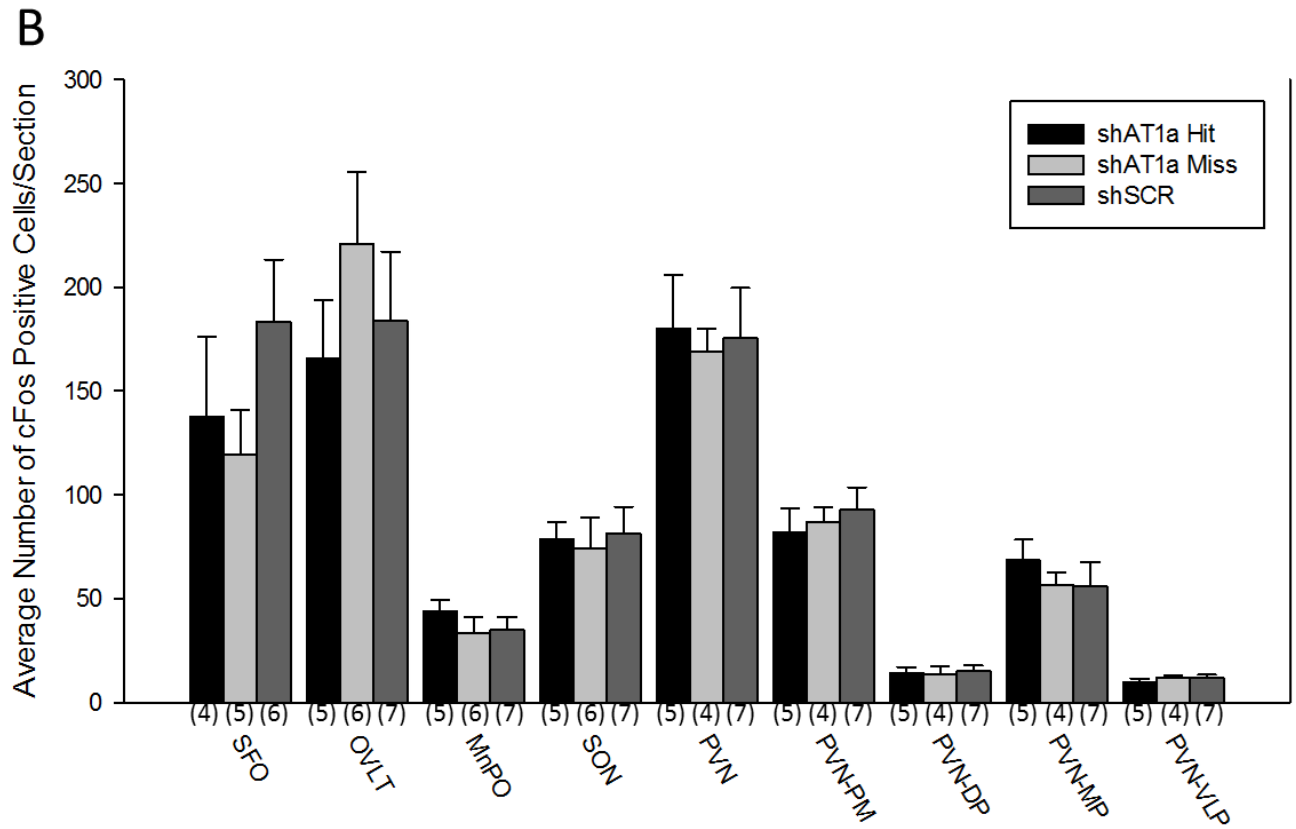
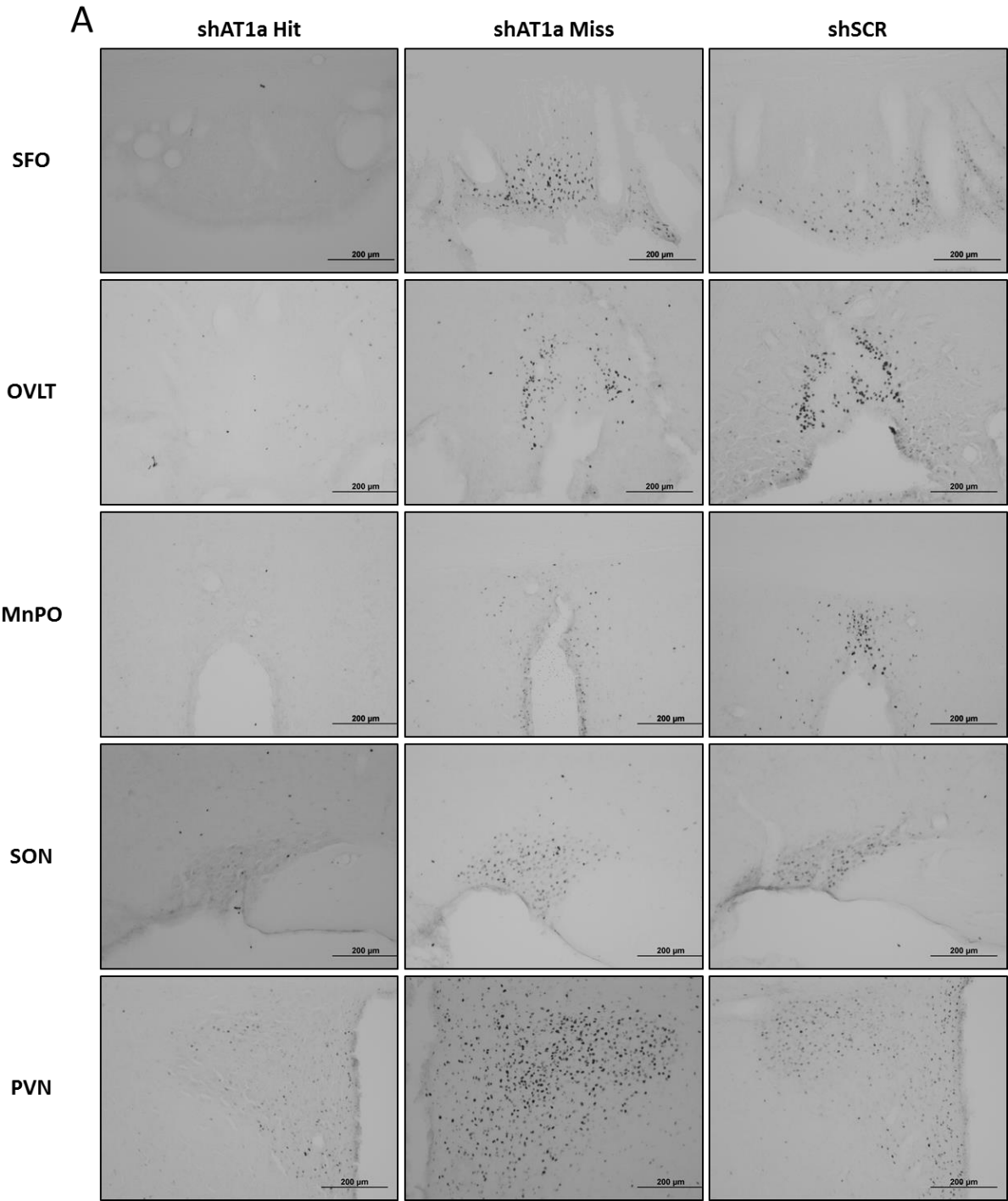


Figure 5. A. Representative DAB staining for cFos in the SFO, OVLT, MnPO, SON, and PVN after subcutaneous Ang II. B MnPO AT1a Knockdown does not affect cFos expression in response to subcutaneous Ang II administration. No significant differences found between groups in any nuclei or subnuclei ($P > .05$).



B

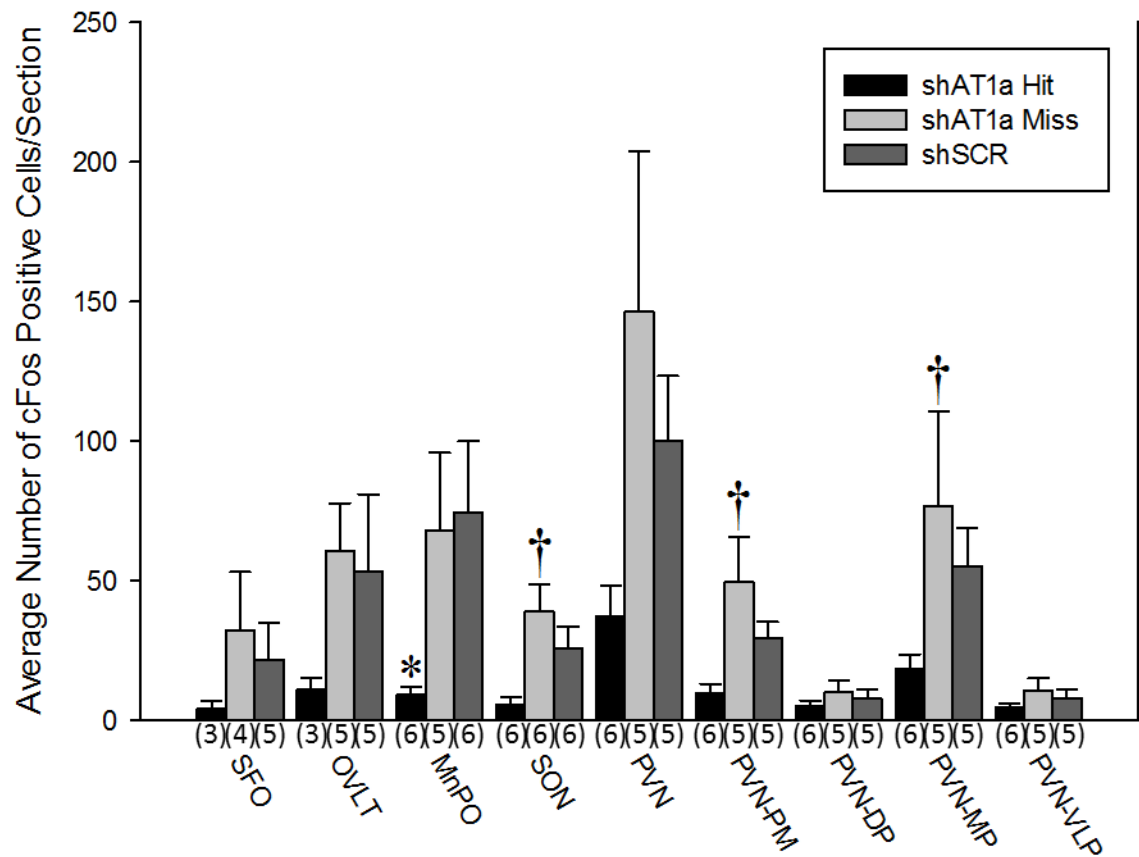


Figure 6. A. Representative DAB staining throughout nuclei associated with thirst after ICV Ang II. B. MnPO knockdown of AT1a receptors reduces cFos expression in response to ICV Ang II administration. shAT1a hit animals exhibited significantly less cFos expression compared to shAT1a miss and scramble animals in the MnPO (* $P < .05$). shAT1a miss animals had significantly higher expression than shAT1a hit animals in the SON, magnocellular PVN, and median parvocellular PVN († $P < .05$).

DISCUSSION

The results demonstrate that AT1a receptors in the MnPO are necessary for water intake stimulated by central ANG II but not peripheral ANG II. These findings were not anticipated since it has been proposed that water intake stimulated by peripheral ANG II recruits a central RAS system in which ANG II acts as a neurotransmitter at the level of the MnPO[25, 26]. The results of the Fos staining experiments are consistent with the behavioral results. AT1a receptor knockdown in the MnPO significantly decreased the numbers of Fos positive neurons in the MnPO and SON following ICV ANG II but had no effect on Fos staining associated with subcutaneous ANG II treatment. Together these results challenge the long standing model of ANG II in the physiological regulation of drinking behavior.

One possible explanation for these results may be provided by in vitro electrophysiology studies of the SFO-MnPO pathway. These studies have shown that stimulation of the SFO produces both fast inhibitory and excitatory postsynaptic effects that are mediated by GABA and glutamate respectively [27, 28]. Stimulation of the SFO by circulating ANG II could, therefore, cause the release of multiple neurotransmitters that influence the activity of MnPO neurons. In this context, the elimination of ANG II signaling in MnPO by knocking down AT1a receptors may not be sufficient to block thirst associated with peripherally administered ANG II. Centrally injected ANG II would bypass the SFO and act directly in the MnPO to generate drinking behavior. ANG II has been shown to have direct depolarizing effects on some MnPO neurons [19] which could be necessary for central ANG II to produce drinking behavior. We have previously shown that AT1a receptor knockdown in the SFO or MnPO each blocked the

sustained component of CIH hypertension. The results of these previous studies, along with our current data suggest that different populations of MnPO neurons are involved in autonomic regulation versus fluid intake and that the autonomic regulatory circuit is the one that uses ANG II as a hormone and a neurotransmitter.

Role of MnPO AT1a Receptors in Hyperosmotic Thirst

The MnPO regulates body fluid homeostasis through its inputs from CVOs[17, 29], as well as its direct ability to sense sodium[20]. Lesions of the MnPO diminish drinking in response to hypertonic saline providing a role for the MnPO in regulating blood tonicity[14-16]. Utilizing ibotenic acid lesions coupled with NMDA blockade provided evidence that there were distinct subpopulations of neurons, or at least distinct receptors, that controlled drinking responses to Ang II as opposed to hypertonic saline[16]. Our data supports this as knockdown of the AT1a receptors in the MnPO had no effect on thirst generated through peripheral hypertonic saline administration, but did significantly reduce drinking in response to ICV Ang II. The MnPO is capable of reducing acute drinking in response to hypertonic saline, but does not mediate this response through AT1 receptors.

Role of MnPO AT1a Receptors in Ang II Thirst

Ang II has long been known to play a role in thirst whether administered peripherally[1, 2, 7] or centrally[30, 31]. ICV injection of 2ng of Ang II generated a considerable drinking response in both scramble injected and AAV-shAT1a miss animals and this response was significantly greater on the second drinking test day (Figure 4). The heightened response on the second day is attributable to the ability of Ang II to potentiate AT1 signaling through transcriptional activation

and increased receptor production[32]. AAV-shAT1a injection into the MnPO significantly attenuated the drinking response to ICV Ang II (Figure 4). Knockdown of AT1a in the MnPO also prevented a significant increase in drinking between drinking tests on Day 14 and 18. These results align with prior publications that ICV Ang II, acting as a peptide neurotransmitter in the MnPO, stimulates thirst, and therefore our knockdown of the receptor prevents the dipsogenic response. In addition to reducing water consumption, AAV-shAT1a animals exhibited a significant decrease in cFos expression in their MnPO as well as their SON, median parvocellular, and magnocellular PVN. Decreased Fos expression indicates that Ang II may be responsible for the induction of this transcription factor, and that in response to ICV Ang II there is both a central drive for thirst as well as vasopressin release.

Subcutaneous Ang II administration produced a markedly different response as compared to ICV Ang II administration. As expected, 2mg/kg SC Ang II resulted in an appreciable drinking response in the scramble and AAV-shAT1a miss animals, but unexpectedly this drinking response was not diminished by AAV-shAT1a's presence in the MnPO. The knockdown animals exhibited a drinking response equal in magnitude to the controls, and also exhibited a potentiation of the drinking response on the second test day (day 18). This result is unexpected given both the ability of ICV Ang II to stimulate drinking through the MnPO, and lesions of the MnPO to reduce drinking from subcutaneous Ang II[14, 15]. These results indicate that Ang II at the MnPO is sufficient to induce drinking, but does not appear to be necessary.

Immunohistochemistry supported this conclusion as there was no significant difference between FosB expression in the CVOs, MnPO, PVN, or SON in any of the experimental groups (Figure 4).

Numerous studies have demonstrated the ability of the MnPO to produce a dipsogenic response through AT1 receptor stimulation[6, 15, 16, 33, 34] and this seemingly provides evidence that these receptors are a crucial component to peripheral Ang II's ability to acutely regulate body fluid homeostasis[5, 17, 18]. Utilizing a shRNA mediated knockdown of AT1a our data indicates that Ang II signaling at the MnPO is not necessary. Ang II stimulates the SFO and OVLT, which in turn increase the firing rate of the MnPO neurons[4, 5], but this is not predominantly mediated through Ang II. Glutamatergic inputs may be sufficient to drive the acute dipsogenic response to Ang II though NMDA receptors[16]. Ang II is a certainly a key element in generating thirst, but data from this study demonstrates that at the MnPO, other neurotransmitters may play a more dominant role.

PERSPECTIVES

Physiological regulation of thirst plays a central role in an organisms ability to regulate blood volume, blood pressure, osmolality, and fluid compartment distribution. Control over thirst is pivotal to maintaining homeostasis. The lamina terminalis has long been known to increase thirst in response to angiotensinergic inputs, but the specific mechanisms that transduced and communicated the signal was unknown. The current work adds to a growing body of literature that supports peripheral Ang II for thirst generation through the SFO. The novel aspect of this study arises from thirst generation without AT1 receptors in the MnPO. The ability to generate thirst through the MnPO without Ang II indicates that there are other neurotransmitters that may play a dominant role in the thirst signal propagation. Further studies will be necessary to elucidate the specific neurotransmitter(s) responsible for this signal.

LIMITATIONS

This study utilized supraphysiological doses on Ang II, and while this was capable of generating thirst a physiological dose of ICV Ang II may not have the same effect. In addition, without identifying the specific neurotransmitter responsible for thirst generation at the SFO-MnPO junction it is impossible to tell if Ang II has an effect under pathophysiological conditions. There was no control group for animals without Ang II administration, and while no animal consumed an appreciable amount of fluid during the pre-test period there may have been an effect of handling and injection alone.

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CHAPTER IV

DISSERTATION PERSPECTIVES AND SIGNIFICANCE

The RAS plays a major role in regulation of blood pressure and body fluid homeostasis. It performs this role by acting peripherally as a hormone, and centrally as a peptide neurotransmitter. The work presented here explored both of these roles in the context of hypertension generated from Chronic Intermittent Hypoxia (CIH), and the ability of Ang II to generate thirst in the MnPO. The major results from these projects are outlined below.

Role of AT1a in CIH Induced Hypertension

CIH, a model of the hypoxemia experienced by sleep apnea sufferers, produces a sustained increase in blood pressure in response to repeated bouts of hypoxia. The first project aimed at elucidating forebrain mechanisms that contribute to this sustained hypertension. The major findings of this first study are that shRNA against AT1a receptors successfully prevents the increase in AT1a mRNA induced by CIH. AT1a receptor message normally increases in response to 7 days of CIH. Inhibition of FosB in the MnPO via a dominant negative construct prevents the increase in AT1a receptor message, suggesting the FosB transcription factor may be

upstream of AT1a receptor expression. Knockdown of AT1a receptor message in the MnPO via shRNA prevented the sustained component of hypertension. After knockdown of AT1a in the MnPO, the MnPO, NTS, and RVLM expressed significantly less FosB, indicating a decrease in neuronal activity in these regions. The PVN exhibited the characteristic increase in FosB staining after 7 days of CIH suggesting it was unaffected by the MnPO knockdown, or a different subset of neurons were active due to the lack of AT1a receptors in the MnPO (Figure 1).

Role of MnPO AT1a Receptors in Thirst

The second project focused on the role of Ang II to induce thirst through the MnPO. In response to AT1a knockdown in the MnPO there was no difference in thirst generated through peripheral Ang II administration. Immunohistochemistry revealed there was high cFos expression throughout the SFO, OVLT, MnPO, PVN, and SON with no difference between animals with or without AT1a receptor knockdown. The opposite was true of animals given the AT1a knockdown virus and then exposed to ICV Ang II, as these animals drank significantly less than their control counterparts (i.e., with intact AT1a receptors). This reduction in water consumption was mirrored by a reduction in cFos in the MnPO as compared to scramble controls and AT1a miss animals (Figure 2).

Perspectives

These results taken together demonstrates the complex role that Ang II plays in the ability of the MnPO to regulate blood pressure and body fluid homeostasis. It is interesting to note that while the MnPO is essential for Ang II based hypertension, it was not required to elicit thirst indicating that these may be pharmacologically distinct pathways. A subset of MnPO neurons that respond

to Ang II are also salt sensitive, and this may indicate an ability for salt the to exacerbate the hypertension generated from CIH, or create a condition in which peripheral Ang II is capable of generating thirst. Clinically this may indicate that sufferers of sleep apnea may benefit from intervention with a more blood brain barrier permeable angiotensin receptor blocker as this would act directly on the MnPO AT1 receptors. This drug regiment would be unlikely to effect their baseline fluid homeostasis.

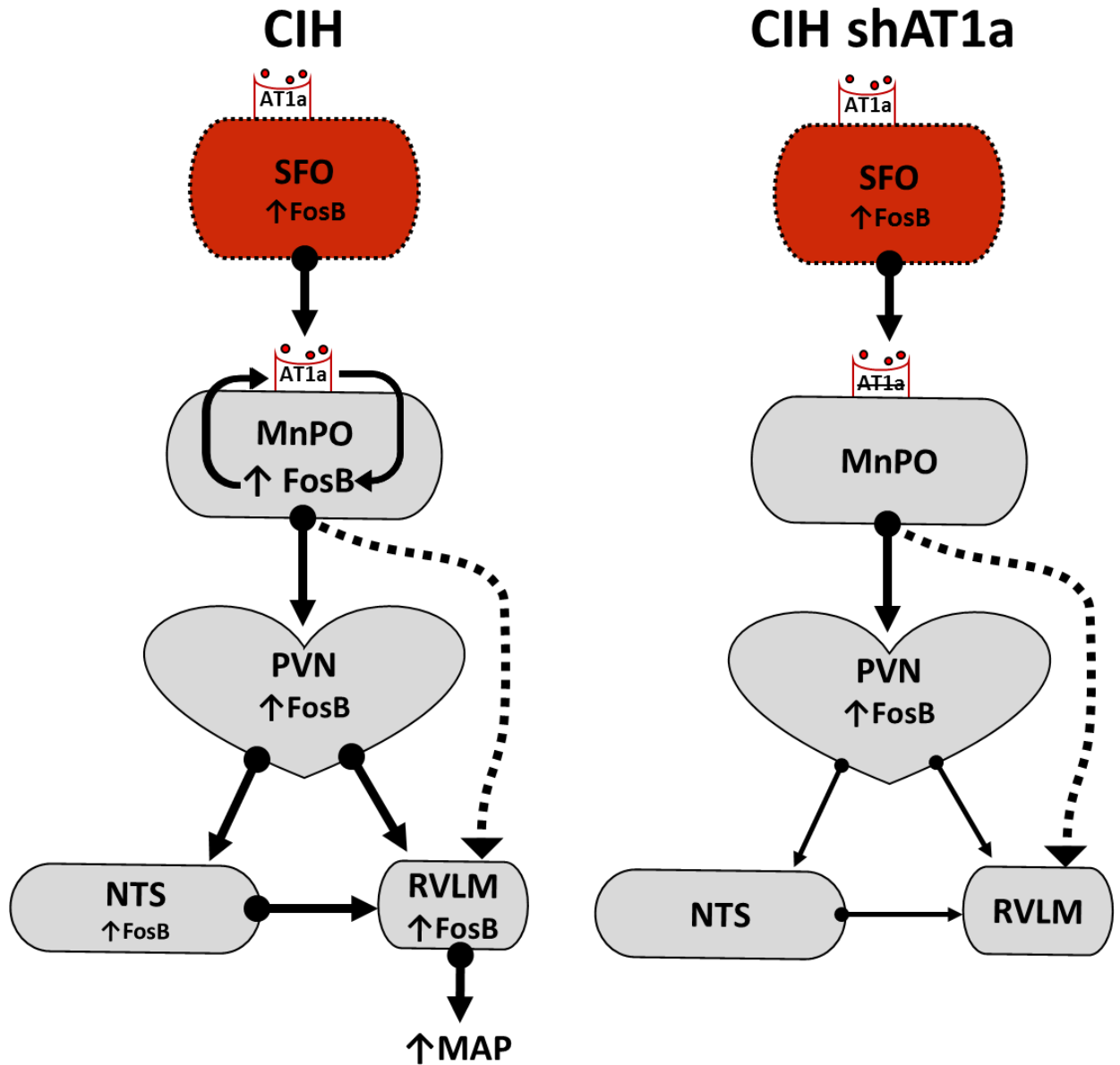


Figure 1. Left Panel: Effects of MnPO AT1a Knockdown during CIH. Normally circulating Ang II (red circles) binds to the SFO, stimulating the circumventricular organ and causing synaptic release of Ang II on to the MnPO. The MnPO in response to this stimulation increases FosB expression which will increase AT1a expression allowing for more input from the SFO.

Downstream the MnPO stimulates the PVN to drive the sustained component of hypertension through the NTS and RVLM by increasing SNA. This increased SNA can then drive high blood pressure during the waking hours. Right Panel: The knockout of AT1a receptors in the MnPO prevents the stimulation of MnPO AT1a receptors, the increase in FosB, and the activation of the RVLM, which suppresses SNA and prevents an increase in MAP. The PVN remains activated, but the specific neurons that are stimulated are not yet known. There may be projections that arise from the MnPO that could directly affect sympathetic outflow at the RVLM, but they are not yet fully characterized.

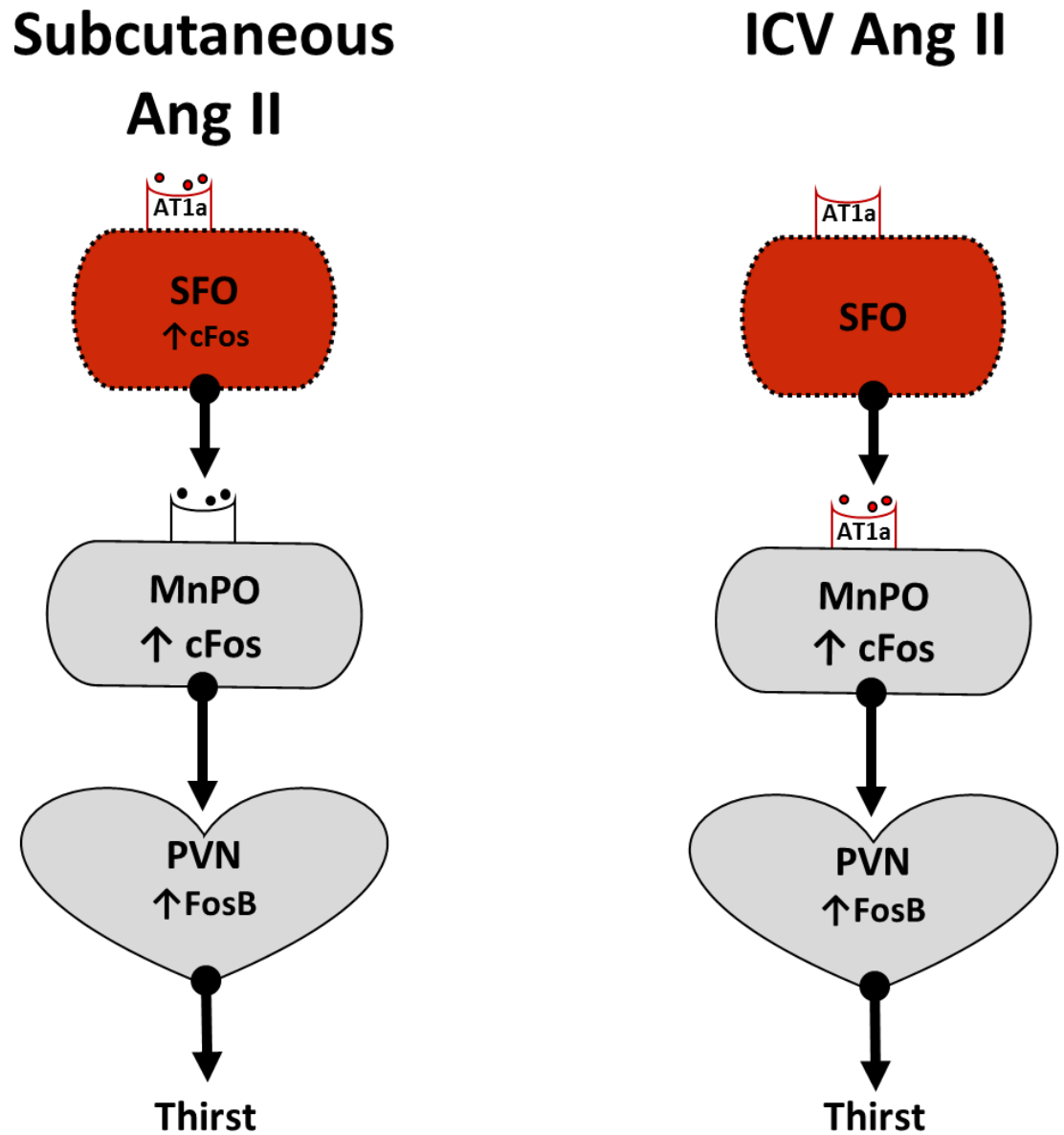


Figure 2. Role of MnPO Ang II in thirst generation. Left Panel: Ang II administered subcutaneously stimulates the MnPO driving cFos expression. Ang II is not necessary (black circles and black receptor are unidentified) at the SFO-MnPO junction in order to drive cFos

expression or stimulate the PVN downstream. The MnPO then stimulates the PVN and thirst is generated. ICV Ang II administration does not interact with the SFO since it is a circumventricular organ. The ICV Ang II directly stimulates the MnPO initiating cFOS and resulting in activation of the PVN and thirst.

CHAPTER V

FUTURE DIRECTIONS

Long-term effects of MnPO and PVN AT1a Receptors on Blood Pressure

Longer term durations beyond 7 days of CIH do not increase the magnitude of elevated blood pressure increase[1-3], but there are potentially other changes that occur with prolonged CIH. Hypertension can increase blood brain barrier permeability in regions such as the PVN and RVLM[4]. Direct application of Ang II receptor blockers to the PVN has been shown to prevent the hypertension induced by CIH[5]. Taken together these findings could indicate a shift in the SA disease process where the MnPO AT1a receptors play a key role early in the pathophysiology, but as the blood brain barrier becomes permeable around the PVN, direct Ang II interactions could drive the disease process during the later periods of pathophysiology. More studies are needed to understand what happens to the blood brain barrier in chronic hypertension and how the sleep apnea disease process progresses beyond the initiating 7 days (approximately 1 year in humans).

Direct Measurement of Sympathetic Nerve Activity after MnPO AT1a Knockdown

The results from the presented studies suggest that knockdown of AT1a receptors in the MnPO reduce sympathetic nerve activity, but this should be verified through direct measurement. There is increasing evidence that CIH both increases SNA and decreases PSNA and both could potentially be affected through AT1a knockdown[1, 6, 7].

Increased PVN FosB after MnPO AT1a Knockdown

Investigation into the increase in FosB positive neurons described in this study would help to clarify how MnPO AT1a knockdown exerts its beneficial effects on post CIH blood pressure. Recent studies have indicated that the PVN contains neurons projecting to the dorsal motor nucleus of the vagus (DMNX) and nucleus ambiguus, both of which control parasympathetic outflow[1, 2, 6-8]. A subset of the PVN neurons releases oxytocin (OXT) and this increases parasympathetic activity, and prevents the sustained component of hypertension after 21 days of CIH[7]. Given the increase in FosB positive neurons in the PVN retrograde track tracing from the DMNX coupled with FosB immunohistochemistry could demonstrate that some of the FosB positive neurons are those pre-parasympathetic neurons.

Potential Role of MnPO AT2 Receptors

The MnPO possesses AT2 receptors in addition to AT1a and 1b receptors[9]. All three of these receptor subtypes bind Ang II. After binding AT1a and 1b receptors work through similar transduction pathways within the cell[10], but AT2 activates a different pathway and tends to have the opposite effects of AT1[11]. It is possible that through knockdown of AT1a receptors AT2 activation was increased. Selective blockade of AT2 via PD 123319 during normoxic and

CIH conditions could determine the contribution of this receptor to the MnPO's control of autonomic regulation during baseline and CIH conditions.

Role of Salt in MnPO Thirst Generation

The MnPO possesses two distinct neuron phenotypes in terms of their response to Ang II. The first responds to Ang II by a slow depolarization that results from decreased potassium efflux[12, 13]. The second group of neurons responds by potentiating the effects of GABA through increased chloride influx[13]. This second group is sodium sensitive and hyperpolarizes in response to hyponatriuric artificial cerebrospinal fluid (aCSF). Salt and Ang II may interact to dictate thirst. High salt hyperosmotic aCSF coupled with Ang II may increase thirst more than simply hyperosmotic aCSF alone.

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