Faulk, Katelynn, The Role of Angiotensin Converting Enzyme 1within the Median Preoptic Nucleus Following Chronic Intermittent Hypoxia. Doctor of Philosophy (Biomedical Sciences), July 2016

These experiments focused on the importance of the brain renin-angiotensin system (RAS) in hypertension caused by a chronic intermittent hypoxia (CIH) model of the hypoxemia associated with sleep apnea. In the CIH model, the sustained diurnal blood pressure increase has been shown to be dependent on the transcription factor FosB and its downstream target genes such as angiotensin converting enzyme 1 (ACE1) in the median preoptoc nucleus (MnPO) in the anterior hypothalamus. These studies focused on the transcriptional regulation of MnPO ACE1 and the development of the sustained CIH hypertension.

The first project focused on ACE1 within the MnPO and its regulation by FosB during CIH. Using immunohistochemistry, ACE1 staining within the MnPO did not overlap with glial fibrillary acidic protein (GFAP), a glial cell marker. ACE1 and FosB colocalization increased within the MnPO following 7 days of CIH. A retrograde tract tracer, fluorogold, was used to determine if ACE1 positive MnPO neurons project to the paraventricular nucleus of the hypothalamus (PVN). MnPO cells containing ACE1 and FosB immunoreactivity after CIH did project to the PVN, an area known to regulate sympathetic nerve activity. Chromatin Immunoprecipitation Assay was used to authenticate an association of FosB with ACE1 within the MnPO following CIH. In the MnPO, the association of FosB with the ACE1 gene was significantly increased by CIH.

The second aim tested the functional role of MnPO ACE1 in sustained diurnal CIH hypertension. We used virally mediated expression of short hairpin RNA (shRNA) against ACE1 to significantly knockdown MnPO ACE1. Rats injected in the MnPO with shRNA against ACE1 demonstrated normal blood pressure responses to hypoxic events but the sustained blood pressure increase to CIH was significantly attenuated. ACE1 knockdown within the MnPO also decreased FosB/ΔFosB staining within the MnPO, the PVN and the rostral ventrolateral medulla (RVLM) but not in the nucleus of the solitary tract. Together, these studies suggest that MnPO ACE1 contributes to the development of sustained CIH hypertension.

The Role of Angiotensin Converting Enzyme 1 within the Median Preoptic Nucleus Following Chronic Intermittent Hypoxia

Katelynn Faulk

APPROVED:

Major Professor (Joseph Thomas Cunningham, Ph.D.)

Committee Member (Steve W. Mifflin, Ph.D.)

Committee Member (Ann M. Schreihofer, Ph.D.)

Committee Member (Rong Ma, M.D., Ph.D.)

University Member (Marianna Jung, Ph.D.)

Chair, Institute for Cardiovascular and Metabolic Diseases (Steve W. Mifflin, Ph.D.)

Dean (Meharvan Singh, Ph.D.), Graduate School of Biomedical Sciences

THE ROLE OF ANGIOTENSIN CONVERTING ENZYME 1 WITHIN THE MEDIAN PREOPTIC NUCLEUS FOLLOWING CHRONIC INTERMITTENT HYPOXIA

DISSERTATION

Presented to the Graduate Council of the

Graduate School of Biomedical Sciences

University of North Texas

Health Science Center at Fort Worth

in Partial Fulfillment of the Requirements

For the Degree of

DOCTOR OF PHILOSOPHY

By

Katelynn Faulk

Fort Worth, Texas

July 2016

ACKNOWLEDGEMENTS

Most of all, I would like to thank my mentor, Dr. J. Thomas Cunningham for his patience and guidance as I navigated through my graduate career. All that I have achieved was only possible because of his encouragement and honest reassurance. In the future, I aspire to mentor students with the same sincerity and understanding in which he mentored me.

I also acknowledge the members of my dissertation committee, Drs. Mifflin, Schreihofer, Ma, and Jung for their invaluable advice that has immensely improved this work.

I am indebted to Dr. T. Prashant Nedungadi, Mr. Joel T. Little and Mrs. Martha Bachelor for their aid and generosity in helping me conduct these experiments as well as instruction in necessary techniques.

Also, I extend my heartfelt appreciation to Brent Shell, Ashwini Saxena, Blayne Knapp, and all students in the Institute of Cardiovascular and Metabolic Diseases. Thanks as well to the colleagues, staff, and faculty at the University of North Texas Health Science Center for their support and assistance.

A special thanks to my friends and family for their love and continuous support.

TABLE OF CONTENTS

ACKNOWLEDGEMENTS ii
LIST OF TABLES v
LIST OF ILLUSTRATIONS vi
ABBREVIATIONS vii
CHAPTER I 1
DISSERTATION OVERVIEW 1
REFERENCES 8
LITERATURE REVIEW 18
OBSTRUCTIVE SLEEP APNEA AND NEURAL MECHANISMS REGULATING BLOOD PRESSURE 18
REFERENCES 26
CHAPTER II 44
THE ROLE OF ANGIOTENSIN CONVERTING ENZYME 1 WITHIN THE MEDIAN PREOPTIC NUCLEUS FOLLOWING CHRONIC INTERMITTENT HYPOXIA 44
INTRODUCTION 44
METHODS 47
RESULTS 53
DISCUSSION 65
PERSPECTIVES 68
REFERENCES 69
CHAPTER III 77
THE CRITICAL ROLE OF ANGIOTENSIN CONVERTING ENZYME 1 WITHIN THE MEDIAN PREOPTIC NUCLEUS IN THE DEVELOPMENT OF THE SUSTAINED HYPERTENSION SEEN IN CHRONIC INTERMITTENT HYPOXIA. 77
INTRODUCTION 77
METHODS 80
RESULTS 85
DISCUSSION 102
PERSPECTIVES 105

REFERENCES 106
CHAPTER IV 115
DISSERTATION PERSPECTIVE AND SIGNIFICANCE 115
CHAPTER V 119
FUTURE DIRECTIONS 119
REFERENCES 124

LIST OF TABLES

CHAPTER III-Table III-1 Baseline Measurements of MAP, HR and RR ……...……94

LIST OF ILLUSTRATIONS

CHAPTER II-Figure II-1 MAP, HR and RR of 7 Days of CIH 56

CHAPTER II-Figure II-2 ACE1 and GFAP Staining in MnPO 58

CHAPTER II-Figure II-3 ACE1 and FosB Staining and Counts inMnPO 59

CHAPTER II-Figure II-4 ACE1 Content within MnPO after CIH 60

CHAPTER II-Figure II-5 Fluorogold PVN Injection and MnPO Counts 61

CHAPTER II-Figure II-6 ACE1, Fluorogold and FosB Staining inMnPO and Counts in CIH... 62

CHAPTER II-Figure II-7 FosB Association with ACE1 Promotor Region 64

CHAPTER III – Figure III-3 Subcutaneous Ang II cFos Immuno and Cell Counts 90

CHAPTER III-Figure III-2 Effect of shACE1 on ACE1 mRNA in CIH 91

CHAPTER III-Figure III-3 ACE1 Knockdown in the MnPO and its Effect on MAP in CIH 92

CHAPTER III-Figure III-4 ACE1 Knockdown in the MnPO on HR and RR in CIH 93

CHAPTER III-Figure III-5 MnPO FosB/ΔFosB Staining 95

CHAPTER III-Figure III-6 PVN FosB/ΔFosB Staining 96

CHAPTER III-Figure III-7 Rostral NTS FosB/ΔFosB Staining 97

CHAPTER III-Figure III-8 Subpostremal NTS FosB/ΔFosB Staining 98

CHAPTER III-Figure III-9 Caudal NTS FosB/ΔFosB Staining 99

CHAPTER III-Figure III-10 Total NTS FosB/ΔFosB Counts 100

CHAPTER III-Figure III-11 RVLM FosB/ΔFosB Staining 101

CHAPTER IV-Figure IV-1 Overall Role of ACE1 in CIH 118

ABBREVIATIONS

AAV	Adeno-associated virus
ACE1	Angiotensin converting enzyme 1
ACE2	Angiotensin converting enzyme 2
ANGI	Angiotensin I
ANGII	Angiotensin II
ANG1-7	Angiotensin-(1-7)
AP-1	Activator protein - 1
AT1	Ang II receptor type 1
AT2	Ang II receptor type 2
CIH	Chronic Intermittent Hypoxia
CNS	Central nervous system
CPAP	Continuous positive airway pressure
CVLM	Caudal ventrolateral medulla
CVO	Circumventricular organ
DAB	3,3'-Diaminobenzidine
GFAP	Glial fibrillary acidic protein
GFP	Green fluorescent protein
HR	Heart Rate

ICV	Intracerebroventricular
IML	Intermediolateral tract of spinal cord
JDN	Jun dimerization protein
LCM	Laser capture microdissection
MAP	Mean Arterial Pressure
MnPO	Median preoptic nucleus
NTS	Nucleus tractus solitarius
OSA	Obstructive Sleep Apnea
OVLT	Organum vasculosum of Lamina Terminalis
PCR	Polymerase chain reaction
PVN	Paraventricular nucleus of hypothalamus
RAS	Renin-Angiotensin system
RNA	Ribonucleic Acid
RR	Respiration Rate
RVLM	Rostral ventrolateral medulla
shACE1	Short-hairpin RNA sequence against ACE1
shSCM	Short-hairpin RNA-scramble sequence
SFO	Subfornical Organ
SNA	Sympathetic Nerve Activity
SON	Supraoptic nucleus of hypothalamus
sp	Sub postremal region of the NTS
TF	Transcription Factor

CHAPTER I

DISSERTATION OVERVIEW

Chronic intermittent Hypoxia (CIH) is a model of the arterial hypoxemia seen in obstructive sleep apnea (OSA) patients[1]. CIH is used because it exhibits similar physiological aspects seen in OSA patients such as increased diurnal blood pressure, sympathetic nerve activity, renin angiotensin system (RAS) activity and an augmented chemoreflex[2-7]. OSA is linked to multiple cardiovascular consequences and therefore the pathophysiological mechanisms that lead to the sustained blood pressure seen in these patients as well as in the CIH model warrants investigation[8-11]. Since OSA patients display elevated resting SNA, a mechanism within the central nervous system may somehow become dysregulated leading the pathophysiological development of an inappropriately elevated diurnal blood pressure. A neuronal activation marker has been shown to increase within certain CNS regions following seven days of CIH[12]. This neuronal activation marker, FosB/ΔFosB, may be involved in the development of the sustained blood pressure seen in CIH. FosB/ΔFosB is also a known transcription factor and the genetic regulation it has within the CNS following CIH may be part of the pathophysiological development of the sustained blood pressure seen in CIH.

Transcription factors (TF) are ideally positioned to orchestrate changes in gene expression required to maintain cellular function in response to environmental challenges. Long

lasting neuronal regulation and plasticity that are mediated by TFs have been evident in many physiological and pathophysiological states from addiction[13] to long-term memory function[14] to circadian rythms[15, 16]. TFs are critical DNA-binding components of these pathways. The activator protein 1 (AP-1) family is a group of TFs[17]. This family of TFs include Fos, Jun, activating transcription factor (ATF), and Jun dimerization protein (JDN)[17]. One common structure for this family of TFs is the leucine zipper motif[18]. FBJ murine osteosarcoma viral oncogene homolog B (FosB) is a leucine zipper motif that is important for regulation of many families of genes[19]. Jun proteins can heterodimerize with Fos proteins to form different DNA-binding complexes with various affinities for certain target genes[20]. The dimers then may bind to AP-1 target genes with a regulatory domain sequence of TGAC/GTCA[21].

The Fos family has many members that respond in a number of ways depending on the stimuli. For example in the role of drug addiction, administration of specific drugs acutely will cause an increase in c-Fos and acute Fras in the nucleus accumbens and dorsal striatum [13, 22-24]. This acute response is fleeting in that a few hours administering the drug the acute Fos response returns to control levels[13]. However, a splice variant of FosB, ΔFosB, has been shown to not only accumulate with acute administration of drug but also with repeated administration[13]. This allows ΔFosB to act chronically due to its more stable form as compared to c-Fos and other fos proteins[13]. Additionally, ΔFosB may become even more stable after phosphorylation by casein kinase 2[25]. ΔFosB remains stable well after chronic drug administration has stopped[13]. This very property of ΔFosB makes it an attractive candidate within the central nervous system (CNS) for long-term regulation of genes expression by a number of stimuli unrelated to mechanisms that support drug addiction.

As previously mentioned, FosB/ΔFosB significantly accumulates in certain regions within the brain following CIH[12]. One region within the CNS that accumulates FosB/ΔFosB after seven days of CIH was the median preoptic nucleus (MnPO) [12]. FosB may regulate certain AP-1 target genes within the MnPO following CIH to alter blood pressure and therefore will be primary CNS region focused within this work. The MnPO resides within the lamina terminalis along the anterior wall of the third ventricle. The MnPO also receives inputs from various regions and may integrate those signals in order to affect downstream areas within the CNS to influence a variety of functions that include but are not limited to autonomic control, body fluid balance, sleep and body temperature regulation[26]. The MnPO receives inputs from two circumventricular organs (CVO), the subfornical organ (SFO) and the organum vasculosum of the lamina terminalis (OVLT) [26-30]. These two nuclei, the SFO and OVLT, do not have a functioning blood-brain-barrier and, therefore, allow signals from the circulation to be transmitted into the CNS[28]. In addition to the CVOs, areas within the hindbrain also project to the MnPO. Specifically, the NTS sends projections to the MnPO mostly from the A2 noradrengeric cell group[26, 30]. The rostral ventrolateral medulla (RVLM) also sends inputs into the MnPO from the C1 adrenergic group while the caudal ventrolateral medulla (CVLM) sends inputs from the A1 neurons[26, 30]. The MnPO, therefore, receives humoral information from the forebrain CVOs and possibly visceral afferent information from hindbrain catecholamine cells. These signals could be integrated and relay information to downstream nuclei within the brain.

The MnPO projects to many areas within the brain. In addition to receiving inputs from both the SFO and OVLT, the MnPO also sends projections to both of these nuclei[26, 31, 32]. This seems to almost be set up a regulation across these three nuclei, the MnPO, SFO and OVLT. One

study has noted intranuclear connections within the MnPO proposing a possible self-regulation of neurons within the MnPO[26]. The MnPO is also known to project to the paraventricular nucleus (PVN)[30, 33, 34], supraoptic nucleus (SON)[35, 36] and many other areas. Projections to the SON and PVN from the MnPO go to magnocellular neurons in both regions and therefore suggest that the MnPO may have a role in body fluid as well as electrolyte homeostasis[26]. The PVN also has other subregions consisting of parvocellular neurons[31-33, 36]. The MnPO projects to these cells within the PVN as well and these cells have roles in neuroendocrine function and cardiovascular regulation[33]. Through the PVN, the MnPO can regulate autonomic function since the PVN projects to the RVLM and the intermediolateral (IML) cell column of the lumbar and thoracic spinal cord [37]. The studies presented here will focus on the MnPO projections to the PVN and the role of an AP-1 target gene of FosB/ΔFosB within the MnPO has on downstream nuclei that may control blood pressure regulation.

ΔFosB within the MnPO has been shown to play a critical role in the sustained hypertension seen in CIH through dominant negative inhibition of ΔFosB[38]. After inhibiting ΔFosB within the MnPO, only the sustained blood pressure seen in CIH was attenuated[38]. Some AP-1 targets genes that were shown to be possibly regulated by ΔFosB within the MnPO in those studies included *ace1*, *ace2*, *nnos*, *enos* and a *map3k3*[38]. The current studies focused on the role of one putative AP-1 target gene, angiotensin converting enzyme 1 (ACE1), and how ACE1 within the MnPO may contribute to the sustained blood pressure seen in CIH.

ACE1 is part of the renin angiotensin system (RAS). Traditionally, RAS is a peripherally acting system that regulates a variety of functions including body fluid balance and blood pressure[39, 40]. Initially in the classical RAS, renin gets synthesized and released from

juxtaglomerular cells in the afferent arterioles of the glomeruli located in the kidney[41]. Renin gets released into the circulation under various conditions such as decreased perfusion to the kidneys triggering a change in the stretch receptors embedded in the vascular wall and increased renal sympathetic nerve activity[42, 43]. Renin circulating through the peripheral blood system cleaves angiotensinogen, which is made in the liver[44]. Angiotensinogen gets converted to angiotensin I (ANGI) by renin, which is thought to be biologically inactive[44]. This conversion to ANGI by renin is considered the first as well as rate-limiting step in the RAS system[45]. After angiotensinogen gets converted to ANGI, ACE1, primarily found in the lungs, cleaves ANGI into angiotensin II (ANGII)[46]. ANGII is an active peptide formed in this cascade that mediates vasoconstriction of blood vessels as well as increasing aldosterone secretion from the adrenal glands[39, 47, 48]. In order to explain the physiological actions of ANGII, angiotensin II receptors were discovered[49, 50]. Angiotensin II type 1 (AT1) and angiotensin II type 2 (AT2) receptors are thought to have opposite and counteracting effects[49, 50]. In addition to AT1 and AT2 receptors, other receptors related to the RAS system, such as the Mas receptor, were discovered[51, 52]. One ligand that is responsible for activating Mas receptors is angiotensin [1-7] (ANG1-7)[53]. Activation of the Mas receptor by ANG1-7 was shown to synthesize vasodilator compounds such as nitric oxide and endothelin-derived hyperpolarizing factor[54]. ANG1-7 is known to be synthesized from the conversion of ANGII to ANG1-7 by angiotensin converting enzyme 2 (ACE2), a homologue of ACE1[55]. ANG1-7 can also be synthesized with ANGII conversion to angiotensin [1-9] by ACE2 with successive conversion to ANG1-7 by ACE1[55]. The traditional RAS has been extensively explored for its effects on various physiological subjects, however, the discovery of a local RAS opened up the possibility of independently functioning a path for mechanisms of local systems within many tissue organs[56, 57].

Recently, local independent RAS components including angiotensinogen, renin, ACE1 and ANGII have been found in the brain[58-61]. More specifically, angiotensinogen was localized in astrocytes of rat brains when angiotensinogen was seen colocalized with glial fibrillary acidic protein (GFAP)[62]. Renin and ANGII was colocalized within gonadotrophs of the pituitary gland further suggesting a local RAS within the brain and the possibility of RAS influencing body fluid homeostasis, blood pressure and pituitary hormone secretion[63, 64]. Renin and angiotensinogen was also localized in other areas of the brain, including the RVLM, through the use of a double-transgenic reporter mouse model[65]. ANGII and ACE1 were found throughout the brain especially within the hypothalamus and pituitary gland [66, 67]. The expression of mRNA of AT1 receptors was observed in the hypothalamus and brainstem of normotensive Wistar Kyoto rats and even increased within those areas in spontaneously hypertensive rats[68]. These experiments further suggest a fully expressed local RAS within the brain and the possibility that a central RAS may play a critical role in body fluid regulation and blood pressure control[69]. ANGII within the brain functions as a well-known neurotransmitter[70] and ANGII within the CNS has been linked to the central control of autonomic function and increases SNA[71-77]. AT1 receptors within the SFO have also been shown to play a role in the sustained hypertension seen following CIH[78]. Administration of an AT1 receptor blocker, losartan, into the MnPO has also demonstrated attenuation of MAP in a CIH model[79]. Based on these findings it seemed plausible that the RAS within the CNS could play a role in the sustained blood pressure seen in CIH.

These studies will test the possible role of ΔFosB in controlling the expression of a putative AP-1 target gene, ACE1, following CIH and determine if this AP-1 target gene plays a role in the sustained hypertension seen in CIH. The first objective is to establish the presence of

ACE1 within the MnPO following CIH and its connection with a downstream region, the PVN. These studies will directly test the association of the transcription factor FosB with the AP-1 promoter region of MnPO ACE1 after CIH exposure. We hypothesize that MnPO neurons transcriptionally activated by CIH express ACE1 and project to the PVN. Furthermore, FosB increases its association with the ACE1 gene promoter region in the MnPO following CIH. The second objective is to functionally test the role that ACE1 has in the sustained hypertension seen in CIH. We hypothesize that ACE1downregulation within the MnPO will attenuate the sustained hypertension seen in CIH and decrease FosB staining in a downstream pathway from the MnPO that is known to regulate blood pressure.

The experiments presented here will for the first time attest the role of a putative FosB target gene, ACE1, within the MnPO for its contribution to the sustained hypertension in CIH. The mechanism of the sustained hypertension seen in CIH is an important gap to address in the existing literature and the significance of such genetic regulation within the MnPO contributing to the sustained hypertension needs elucidation to further understand the pathophysiology of obstructive sleep apnea.

REFERENCES

1. Fletcher EC, Lesske J, Behm R, Miller CC, 3rd, Stauss H and Unger T. Carotid chemoreceptors, systemic blood pressure, and chronic episodic hypoxia mimicking sleep apnea. *J Appl Physiol (1985)*. 1992;72:1978-84.
2. Fletcher EC. Invited review: Physiological consequences of intermittent hypoxia: systemic blood pressure. *J Appl Physiol (1985)*. 2001;90:1600-5.
3. Fletcher EC. Sympathetic over activity in the etiology of hypertension of obstructive sleep apnea. *Sleep*. 2003;26:15-9.
4. Narkiewicz K, van de Borne PJ, Montano N, Dyken ME, Phillips BG and Somers VK. Contribution of tonic chemoreflex activation to sympathetic activity and blood pressure in patients with obstructive sleep apnea. *Circulation*. 1998;97:943-5.
5. Prabhakar NR and Kumar GK. Mechanisms of sympathetic activation and blood pressure elevation by intermittent hypoxia. *Respir Physiol Neurobiol*. 2010;174:156-61.
6. Shell B, Faulk K and Cunningham JT. Neural Control of Blood Pressure in Chronic Intermittent Hypoxia. *Curr Hypertens Rep*. 2016;18:19.
7. Silva AQ and Schreihofer AM. Altered sympathetic reflexes and vascular reactivity in rats after exposure to chronic intermittent hypoxia. *J Physiol*. 2011;589:1463-76.
8. Morrell MJ, Finn L, Kim H, Peppard PE, Badr MS and Young T. Sleep fragmentation, awake blood pressure, and sleep-disordered breathing in a population-based study. *Am J Respir Crit Care Med*. 2000;162:2091-6.

9. Young T, Peppard P, Palta M, Hla KM, Finn L, Morgan B and Skatrud J. Population-based study of sleep-disordered breathing as a risk factor for hypertension. *Arch Intern Med*. 1997;157:1746-52.

10. Dyken ME, Somers VK, Yamada T, Ren ZY and Zimmerman MB. Investigating the relationship between stroke and obstructive sleep apnea. *Stroke*. 1996;27:401-7.

11. Shahar E, Whitney CW, Redline S, Lee ET, Newman AB, Nieto FJ, O'Connor GT, Boland LL, Schwartz JE and Samet JM. Sleep-disordered breathing and cardiovascular disease: cross-sectional results of the Sleep Heart Health Study. *Am J Respir Crit Care Med*. 2001;163:19-25.

12. Knight WD, Little JT, Carreno FR, Toney GM, Mifflin SW and Cunningham JT. Chronic intermittent hypoxia increases blood pressure and expression of FosB/DeltaFosB in central autonomic regions. *American journal of physiology Regulatory, integrative and comparative physiology*. 2011;301:R131-9.

13. Nestler EJ. Molecular basis of long-term plasticity underlying addiction. *Nat Rev Neurosci*. 2001;2:119-28.

14. Alberini CM. Transcription factors in long-term memory and synaptic plasticity. *Physiol Rev*. 2009;89:121-45.

15. Gau D, Lemberger T, von Gall C, Kretz O, Le Minh N, Gass P, Schmid W, Schibler U, Korf HW and Schutz G. Phosphorylation of CREB Ser142 regulates light-induced phase shifts of the circadian clock. *Neuron*. 2002;34:245-53.

16. Travnickova-Bendova Z, Cermakian N, Reppert SM and Sassone-Corsi P. Bimodal regulation of mPeriod promoters by CREB-dependent signaling and CLOCK/BMAL1 activity. *Proc Natl Acad Sci U S A*. 2002;99:7728-33.

17. Bohmann D, Admon A, Turner DR and Tjian R. Transcriptional regulation by the AP-1 family of enhancer-binding proteins: a nuclear target for signal transduction. *Cold Spring Harb Symp Quant Biol*. 1988;53 Pt 2:695-700.

18. Struhl K. Helix-turn-helix, zinc-finger, and leucine-zipper motifs for eukaryotic transcriptional regulatory proteins. *Trends Biochem Sci*. 1989;14:137-40.

19. Kouzarides T and Ziff E. Leucine zippers of fos, jun and GCN4 dictate dimerization specificity and thereby control DNA binding. *Nature*. 1989;340:568-71.

20. Halazonetis TD, Georgopoulos K, Greenberg ME and Leder P. c-Jun dimerizes with itself and with c-Fos, forming complexes of different DNA binding affinities. *Cell*. 1988;55:917-24.

21. Morgan JI and Curran T. Immediate-early genes: ten years on. *Trends Neurosci*. 1995;18:66-7.

22. Hope BT, Nye HE, Kelz MB, Self DW, Iadarola MJ, Nakabeppu Y, Duman RS and Nestler EJ. Induction of a long-lasting AP-1 complex composed of altered Fos-like proteins in brain by chronic cocaine and other chronic treatments. *Neuron*. 1994;13:1235-44.

23. Kelz MB and Nestler EJ. deltaFosB: a molecular switch underlying long-term neural plasticity. *Curr Opin Neurol*. 2000;13:715-20.

24. Moratalla R, Elibol B, Vallejo M and Graybiel AM. Network-level changes in expression of inducible Fos-Jun proteins in the striatum during chronic cocaine treatment and withdrawal. *Neuron*. 1996;17:147-56.

25. Ulery PG, Rudenko G and Nestler EJ. Regulation of DeltaFosB stability by phosphorylation. *J Neurosci*. 2006;26:5131-42.

26. McKinley MJ, Yao ST, Uschakov A, McAllen RM, Rundgren M and Martelli D. The median preoptic nucleus: front and centre for the regulation of body fluid, sodium, temperature, sleep and cardiovascular homeostasis. *Acta Physiol (Oxf)*. 2015;214:8-32.

27. Camacho A and Phillips MI. Horseradish peroxidase study in rat of the neural connections of the organum vasculosum of the lamina terminalis. *Neurosci Lett*. 1981;25:201-4.

28. McKinley MJ, McAllen RM, Davern P, Giles ME, Penschow J, Sunn N, Uschakov A and Oldfield BJ. The sensory circumventricular organs of the mammalian brain. *Adv Anat Embryol Cell Biol*. 2003;172:III-XII, 1-122, back cover.

29. Miselis RR, Shapiro RE and Hand PJ. Subfornical organ efferents to neural systems for control of body water. *Science*. 1979;205:1022-5.

30. Armstrong DM, Saper CB, Levey AI, Wainer BH and Terry RD. Distribution of cholinergic neurons in rat brain: demonstrated by the immunocytochemical localization of choline acetyltransferase. *J Comp Neurol*. 1983;216:53-68.

31. Johnson AK, Zardetto-Smith AM and Edwards GL. Integrative mechanisms and the maintenance of cardiovascular and body fluid homeostasis: the central processing of sensory input derived from the circumventricular organs of the lamina terminalis. *Prog Brain Res*. 1992;91:381-93.

32. Thompson RH and Swanson LW. Structural characterization of a hypothalamic visceromotor pattern generator network. *Brain Res Brain Res Rev*. 2003;41:153-202.

33. Stocker SD and Toney GM. Median preoptic neurones projecting to the hypothalamic paraventricular nucleus respond to osmotic, circulating Ang II and baroreceptor input in the rat. *J Physiol*. 2005;568:599-615.

34. Stocker SD and Toney GM. Vagal afferent input alters the discharge of osmotic and ANG II-responsive median preoptic neurons projecting to the hypothalamic paraventricular nucleus. *Brain Res*. 2007;1131:118-28.

35. Miselis RR. The efferent projections of the subfornical organ of the rat: a circumventricular organ within a neural network subserving water balance. *Brain Res*. 1981;230:1-23.

36. Gu GB and Simerly RB. Projections of the sexually dimorphic anteroventral periventricular nucleus in the female rat. *J Comp Neurol*. 1997;384:142-64.

37. Badoer E. Hypothalamic paraventricular nucleus and cardiovascular regulation. *Clin Exp Pharmacol Physiol*. 2001;28:95-9.

38. Cunningham JT, Knight WD, Mifflin SW and Nestler EJ. An Essential role for DeltaFosB in the median preoptic nucleus in the sustained hypertensive effects of chronic intermittent hypoxia. *Hypertension*. 2012;60:179-87.

39. Ferrario CM. Importance of the renin-angiotensin-aldosterone system (RAS) in the physiology and pathology of hypertension. An overview. *Drugs*. 1990;39 Suppl 2:1-8.

40. Ferrario CM. The renin-angiotensin system: importance in physiology and pathology. *J Cardiovasc Pharmacol*. 1990;15 Suppl 3:S1-5.

41. Griendling KK, Murphy TJ and Alexander RW. Molecular biology of the renin-angiotensin system. *Circulation*. 1993;87:1816-28.

42. Khayat A, Gonda S, Sen S and Smeby RR. Responses of juxtaglomerular cell suspensions to various stimuli. *Hypertension*. 1981;3:157-67.

43. Torretti J. Sympathetic control of renin release. *Annu Rev Pharmacol Toxicol*. 1982;22:167-92.

44. Gomez RA. Molecular biology of components of the renin-angiotensin system during development. *Pediatr Nephrol*. 1990;4:421-3.

45. Blundell T, Sibanda BL and Pearl L. Three-dimensional structure, specificity and catalytic mechanism of renin. *Nature*. 1983;304:273-5.

46. Heeneman S, Sluimer JC and Daemen MJ. Angiotensin-converting enzyme and vascular remodeling. *Circ Res*. 2007;101:441-54.

47. Nguyen Dinh Cat A and Touyz RM. Cell signaling of angiotensin II on vascular tone: novel mechanisms. *Curr Hypertens Rep*. 2011;13:122-8.

48. Savoia C, Burger D, Nishigaki N, Montezano A and Touyz RM. Angiotensin II and the vascular phenotype in hypertension. *Expert Rev Mol Med*. 2011;13:e11.

49. Goodfriend TL and Lin SY. Receptors for angiotensin I and II. *Circ Res*. 1970;27:163-74.

50. Lin SY and Goodfriend TL. Angiotensin receptors. *Am J Physiol*. 1970;218:1319-28.

51. Solinski HJ, Petermann F, Rothe K, Boekhoff I, Gudermann T and Breit A. Human Mas-related G protein-coupled receptors-X1 induce chemokine receptor 2 expression in rat dorsal root ganglia neurons and release of chemokine ligand 2 from the human LAD-2 mast cell line. *PLoS One*. 2013;8:e58756.

52. Jackson TR, Blair LA, Marshall J, Goedert M and Hanley MR. The mas oncogene encodes an angiotensin receptor. *Nature*. 1988;335:437-40.

53. Santos RA, Simoes e Silva AC, Maric C, Silva DM, Machado RP, de Buhr I, Heringer-Walther S, Pinheiro SV, Lopes MT, Bader M, Mendes EP, Lemos VS, Campagnole-Santos MJ, Schultheiss HP, Speth R and Walther T. Angiotensin-(1-7) is an endogenous

ligand for the G protein-coupled receptor Mas. *Proc Natl Acad Sci U S A*. 2003;100:8258-63.

54. Ferreira AJ and Santos RA. Cardiovascular actions of angiotensin-(1-7). *Braz J Med Biol Res*. 2005;38:499-507.

55. Vickers C, Hales P, Kaushik V, Dick L, Gavin J, Tang J, Godbout K, Parsons T, Baronas E, Hsieh F, Acton S, Patane M, Nichols A and Tummino P. Hydrolysis of biological peptides by human angiotensin-converting enzyme-related carboxypeptidase. *J Biol Chem*. 2002;277:14838-43.

56. Lee MA, Paul M, Bohm M and Ganten D. Effects of angiotensin-converting enzyme inhibitors on tissue renin-angiotensin systems. *Am J Cardiol*. 1992;70:12C-19C.

57. Paul M, Bachmann J and Ganten D. The tissue renin-angiotensin systems in cardiovascular disease. *Trends Cardiovasc Med*. 1992;2:94-9.

58. Morimoto S, Cassell MD, Beltz TG, Johnson AK, Davisson RL and Sigmund CD. Elevated blood pressure in transgenic mice with brain-specific expression of human angiotensinogen driven by the glial fibrillary acidic protein promoter. *Circ Res*. 2001;89:365-72.

59. Ganten D, Marquez-Julio A, Granger P, Hayduk K, Karsunky KP, Boucher R and Genest J. Renin in dog brain. *Am J Physiol*. 1971;221:1733-7.

60. Wright JW and Harding JW. The brain renin-angiotensin system: a diversity of functions and implications for CNS diseases. *Pflugers Arch*. 2013;465:133-51.

61. Paul M, Poyan Mehr A and Kreutz R. Physiology of local renin-angiotensin systems. *Physiol Rev*. 2006;86:747-803.

62. Deschepper CF, Bouhnik J and Ganong WF. Colocalization of angiotensinogen and glial fibrillary acidic protein in astrocytes in rat brain. *Brain Res*. 1986;374:195-8.

63. McKenzie JC, Naruse K and Inagami T. The renin-angiotensin system in the rat anterior pituitary: colocalization of renin and angiotensin II in gonadotrophs. *Anat Rec*. 1985;212:158-66.

64. Deschepper CF, Seidler CD, Steele MK and Ganong WF. Further studies on the localization of angiotensin-II-like immunoreactivity in the anterior pituitary gland of the male rat, comparing various antisera to pituitary hormones and their specificity. *Neuroendocrinology*. 1985;40:471-5.

65. Lavoie JL, Cassell MD, Gross KW and Sigmund CD. Adjacent expression of renin and angiotensinogen in the rostral ventrolateral medulla using a dual-reporter transgenic model. *Hypertension*. 2004;43:1116-9.

66. Brownfield MS, Reid IA, Ganten D and Ganong WF. Differential distribution of immunoreactive angiotensin and angiotensin-converting enzyme in rat brain. *Neuroscience*. 1982;7:1759-69.

67. Saavedra JM, Fernandez-Pardal J and Chevillard C. Angiotensin-converting enzyme in discrete areas of the rat forebrain and pituitary gland. *Brain Res*. 1982;245:317-25.

68. Raizada MK, Sumners C and Lu D. Angiotensin II type 1 receptor mRNA levels in the brains of normotensive and spontaneously hypertensive rats. *J Neurochem*. 1993;60:1949-52.

69. Baltatu OC, Campos LA and Bader M. Local renin-angiotensin system and the brain--a continuous quest for knowledge. *Peptides*. 2011;32:1083-6.

70. Li Z and Ferguson AV. Subfornical organ efferents to paraventricular nucleus utilize angiotensin as a neurotransmitter. *Am J Physiol*. 1993;265:R302-9.

71. Aars H and Akre S. Effect of angiotensin on sympathetic nerve activity. *Acta Physiol Scand*. 1968;74:134-41.

72. Barrett CJ, Ramchandra R, Guild SJ, Lala A, Budgett DM and Malpas SC. What sets the long-term level of renal sympathetic nerve activity: a role for angiotensin II and baroreflexes? *Circ Res*. 2003;92:1330-6.

73. DiBona GF. Central angiotensin modulation of baroreflex control of renal sympathetic nerve activity in the rat: influence of dietary sodium. *Acta Physiol Scand*. 2003;177:285-9.

74. Matsukawa T, Gotoh E, Minamisawa K, Kihara M, Ueda S, Shionoiri H and Ishii M. Effects of intravenous infusions of angiotensin II on muscle sympathetic nerve activity in humans. *Am J Physiol*. 1991;261:R690-6.

75. Yoshimoto M, Miki K, Fink GD, King A and Osborn JW. Chronic angiotensin II infusion causes differential responses in regional sympathetic nerve activity in rats. *Hypertension*. 2010;55:644-51.

76. Zucker IH and Gao L. The regulation of sympathetic nerve activity by angiotensin II involves reactive oxygen species and MAPK. *Circ Res*. 2005;97:737-9.

77. Xia H and Lazartigues E. Angiotensin-converting enzyme 2 in the brain: properties and future directions. *J Neurochem*. 2008;107:1482-94.

78. Saxena A, Little JT, Nedungadi TP and Cunningham JT. Angiotensin II type 1a receptors in subfornical organ contribute towards chronic intermittent hypoxia-associated sustained increase in mean arterial pressure. *Am J Physiol Heart Circ Physiol*. 2015;308:H435-46.

79. Knight WD, Saxena A, Shell B, Nedungadi TP, Mifflin SW and Cunningham JT. Central losartan attenuates increases in arterial pressure and expression of FosB/DeltaFosB along the autonomic axis associated with chronic intermittent hypoxia. *American journal of physiology Regulatory, integrative and comparative physiology*. 2013;305:R1051-8.

LITERATURE REVIEW

OBSTRUCTIVE SLEEP APNEA AND NEURAL MECHANISMS REGULATING BLOOD PRESSURE

One in five adults has mild obstructive sleep apnea (OSA)[1]. This translates to an average of 5-15 interruptions of normal breathing each hour or an apnea-hypopnea index of 5-15[2] . Therefore the prevalence of OSA is high. Additionally, OSA patients display an enhanced chemoreflex and elevated sympathetic nerve activity (SNA) resulting in sustained hypertension even when awake[3-8]. The elevated SNA and sustained hypertension associated with OSA have been shown to increase the risk of various detrimental cardiovascular diseases[3-7]. OSA is commonly reported in patients diagnosed with stroke[9], arrhythmias[10], heart failure and hypertension[1, 11, 12]. The common treatment for OSA is a continuous positive air pressure (CPAP) device that patients wear during sleep. CPAP compliance has continuously been an issue in that many users fail to use the device as prescribed[13]. CPAP patients also tend to not accurately portray true use time leading to unreliable monitoring of their condition although considerable efforts through the addition of smartcard or wireless data transfer have been made in an attempt to rectify these monitoring issues[13]. Nevertheless, evidence has shown that even after one year of CPAP use some physiological outcomes like muscle SNA decrease but MAP is still elevated[14]. OSA therefore is associated with many detrimental cardiovascular diseases in patients that need to be addressed[12, 15-23]. Patients with OSA and heart failure treated with CPAP failed to show a significant decrease in MAP[24]. Thus, understanding the underlying mechanisms that causes these cardiovascular outcomes in OSA patients, especially the sustained hypertension, is vital in treating this population effectively and reliably.

Chronic Intermittent Hypoxia (CIH) is model for sleep apnea in which animals are exposed to repetitive episodes of hypoxia with infusion of nitrogen at certain intervals throughout a designated sleep time[25]. CIH models the arterial hypoxemia produced during sleep apnea but not changes in thoracic pressure or hypercapnia [25]. CIH is also associated with an increase in blood pressure through elevated SNA[26-28]. The peripheral chemoreceptors are also necessary for hypertension in CIH exposed rats in that carotid body denervation blocked the blood pressure increase associated with CIH[25]. The changes in chemoreceptor sensory input may be dependent on certain mechanisms such as peroxynitrie (ONOO(-)) formation[29]. CIH has also been shown to alter rhythm generation in preBötzinger neurons that may alter inspiration activity related to autonomic control[30]. Another phenomena associated with CIH is chemoreflex augmentation of SNA that may increase SNA inappropriately in response to hypoxia[31]. Since CIH is associated with carotid body stimulation, increased SNA and blood pressure, the central nervous system (CNS) could play a role in the elevated SNA and sustained hypertension seen in CIH[32-34]. However, the specific regions and mechanism of the CNS contributing to the sustained hypertension seen in CIH remain undetermined.

Many putative mechanisms have been proposed for the elevated SNA and hypertension seen in OSA including an augmented chemoreflex[8, 35] and increased RAS activity[36]. Additionally, many regions within the CNS have been implicated in maintenance of blood pressure[37]. One area critical for the enhanced chemoreflex seen in CIH is the nucleus of the solitary tract (NTS)[38]. NTS neurons receiving inputs from the carotid body have been shown to enhance AMPA activation while decreasing NMDA responses[39]. This enhanced response to AMPA receptor stimulation would explain the enhanced chemoreflex responses seen in CIH. Glutamatergic

control via the NMDA receptor 1 and glutamate receptor 2/3 subunit has also been shown to be altered in caudal NTS neurons following CIH further suggesting chemoreflex inputs altering glutamatergic responses within the NTS following CIH stimulation[40]. NTS neurons receiving inputs from the carotid bodies have shown to decrease the function of ATP-sensitive potassium channels following CIH and therefore decreasing the outward current of potassium leading to increased excitation in those NTS neurons[41]. The increase in excitability of the NTS neurons from the decrease in ATP-sensitive potassium channels may also contribute to the enhanced response from chemoreceptor stimulation from the carotid bodies. This enhanced activation of the NTS region may then go on to activate the rostral ventrolateral medulla (RVLM) and increase SNA through both the splanchnic and renal nerves[42-45]. The increase in SNA, specifically the renal SNA, may then lead to increased activation of the systemic Renin-Angiotensin System (RAS) and therefore increase circulating ANGII[46, 47]. This increase in circulating ANGII may then go on to activate a region called the lamina terminalis within the CNS.

The lamina terminalis has been implicated in many forms of neurogenic hypertension[48]. This area contains three separate regions; the subfornical organ (SFO), median preoptic nucleus (MnPO), and organum vasculosum of the lamina terminalis (OVLT). The SFO and OVLT are circumventricular organs that lack a functional blood-brain-barrier and are sensitive to circulating peptides[49-51]. A recent study has demonstrated the vital role of angiotensin II type 1 subtype a (AT1a) receptors within the SFO in the development of the sustained hypertension seen in CIH[47]. The SFO and OVLT innervate the MnPO[52] which projects to the paraventricular nucleus (PVN)[53-55] influencing SNA and blood pressure[56-58]. The MnPO would be a significant

area to study in that it has already been shown to have a role in regulating and maintaining blood pressure[59-62]. MnPO neurons projecting to the PVN respond to various homeostatic challenges such as circulating ANGII and osmotic stimulation[54]. Electrolytic lesions of the anterior ventral region of the third ventricle, which include the MnPO, alleviate the diurnal hypertension assoicated with CIH[63]. The MnPO has abeen shown to have a role in the increase SNA and hypertension seen in spontaneously hypertensice rats[64]. The MnPO also integrates signals from other autonomic areas within the forebrain and projects to critical regions within the CNS, such as the PVN[53-55], that have been shown to directly modulate SNA and MAP. Therefore, the MnPO neurons that project to the PVN could be the site of neuronal adaption which could sustain CIH hypertension even during normoxic conditions.

Recently the transcription factor FosB in the MnPO was shown to be necessary for the maintenance of CIH hypertension during normoxia[60, 63]. FosB is known to accumulate in brain regions with chronic or intermittent stimulation[65]. Furthermore, FosB, a member of the transcription factor AP-1 family, is stably expressed following activation of the CNS[66-68] and is increased in the MnPO following CIH[60]. The transcription factor FosB has also been implicated as a mediator for adaptive changes in the CNS that contribute to drug sensitization, epilepsy and long-term potentiation (LTP)[68-72]. Thus, FosB is a good candidate to mediate changes in gene expression that support sustained hypertension associated with CIH. We have previously shown that CIH produces an increased expression of FosB within MnPO neurons [60]. Dominant-negative inhibition of ΔFosB, the more stable splice variant of FosB, in the MnPO attenuates only the sustained diurnal hypertension during normoxia and not the MAP response to a hypoxic event[63]. The inhibition of ΔFosB in the MnPO blocks CIH-induced changes in possible AP-1 target

genes, including angiotensin converting enzyme 1 (ACE1) and 2 (ACE2)[63]. Both ACE1 and ACE2 are a part of the RAS in addition to being homologues of each other[73, 74].

Chronic activation of the RAS within the systemic circulation has been shown to occur in both OSA[75, 76] and CIH[46, 77, 78]. While the systemic RAS may play a partial role in the response to hypoxia, a RAS specific to the brain may also contribute to the hypertension seen in response to CIH. It has been shown that the CNS has a unique and independent RAS[79-81]. The brain RAS has been shown to play a significant role in the homeostatic control of water-electrolyte balance and blood pressure regulation[82]. The importance of the brain RAS in the regulation blood pressure has been explored but there are still many questions to be answered[81]. Predominantly, angiotensin II (ANGII) and its receptor, angiotensin II type 1 (AT1), have been extensively investigated in the regulation of blood pressure. For example, experiments have shown that intracerebroventricular (ICV) administration of ANGII increased both heart rate and blood pressure even in chloralose-anesthetized cats[83]. Section of the spinal cord at the cervical spine of these cats also abolishes this response[83]. Blockade of AT1 receptors with losartan administered into the lateral cerebral ventricle of deoxycorticosterone acetate (DOCA)-salt hypertensive rats significantly decreased mean arterial blood pressure over time suggesting AT1 receptors play a role in the development of hypertension in a DOCA-salt treatment model[84]. Injection of valsartan, another AT1 blocker, into the RVLM of the hindbrain produced a decrease in blood pressure up to 30 mmHg in spontaneously hypertensive rats further suggesting a role of AT1 receptors in the adaptation of blood pressure[85]. In regards to the increases in blood pressure seen in CIH, central injection of losartan does significantly alleviate those increases in blood pressure[86]. The ability for even a part of the RAS to control blood pressure in a variety of

models proposes a bigger role RAS may play in many hypertensive diseases and the need to investigate other parts of the RAS within the CNS. The enzymes of the RAS, ACE1 and ACE2, within the local brain RAS are therefore reasonable candidates to investigate in the development of hypertension.

ACE1 mediates the conversion of Angiotensin I (ANGI) to ANGII, which is an established neurotransmitter of the CNS[87]. ANGII is also a known regulator of SNA[88-93]. Central administration of ANGII increases MAP and heart rate (HR) [94]. ACE1 could increase signaling in the CNS by increasing the synthesis of ANGII. ACE1 has been shown to be pro-hypertensive and contribute to harmful cardiovascular effects[95]. ACE1 inhibition within the CNS decreases adverse cardiovascular effects seen in rats subjected to myocardial infarction[96]. ACE1 also increases in the MnPO in response to water deprevation[97]. Additionally, captopril, an ACE1 blocker, given ICV reduced the pressor response of ICV ANGI administration although basal mean arterial pressure did not change[98]. However, administration of captopril chronically into the lateral ventricle mitigate the hypertension that develops in spontaneously hypertensive rats as compared to vehicle controls[99]. Although when captopril was given intravenously, spontaneous hypertension did develop in these rats[99]. This suggests ACE1 within the brain may play a role in the initial pathophysiological development of spontaneous hypertension. ACE1 within the MnPO may therefore play a role in the hypertension seen in CIH and cause significant innovation in how we conceptually look at OSA as a pathophysiological disease. However, ACE1 was not the only RAS AP-1 target seen in the dominant negative ΔFosB studies previously mentioned; ACE2 was as well[63].

ACE2 is known to form angiotensin-(1-7) (ANG1-7) from ANGII and ANG1-7 is endogenously seen within the rat brain[100]. Although ACE2 may convert ANGI to angiotensin-(1-9), ACE2 has a higher affinity for ANGII as a substrate[101]. ACE2 formation of ANG1-7 and its activation of the Mas receptor seem to counterbalance the actions of ANGII on AT1 receptors[102-105]. For example, ICV injection of ANG1-7 into female rats attenuated an aldosterone/NaCl-induced hypertension model[106]. Overexpression of ACE2 throughout the brain significantly attenuates the hypertension seen in DOCA/salt treated rats[107, 108]. The development of the hypertension seen in the DOCA/salt model may be through essentially shedding ACE2 from the cell membrane[109]. In another model of hypertension, the ANGII-induced model, ACE2 overexpression in the PVN diminishes the hypertensive outcome and therefore suggests ACE2 plays an anti-hypertensive role within the brain[110]. Overexpression of ACE2 within the RVLM attenuates glutamatergic inputs that are tonically active in spontaneously hypertensive rats[111]. As a final point, the exact role of ACE2 activity in the control of blood pressure is still controversial albeit important. One study suggested ACE2 plays a vital role in normal physiology while ACE1 plays a more critical role in pathophysiology[95, 112]. In direct contrast, central ANG1-7 was found to be important in pathophysiological conditions and its role in normal conditions minor[95, 113]. Therefore, the role ACE2 may play in the pathophysiology of sleep apnea and CIH hypertension could be of significant importance. Nevertheless, ACE1 actions in the CNS are typically described as pro-hypertensive while ACE2 is more often characterized as anti-hypertensive.

The RAS within the MnPO could be a substrate by CIH leads to sustained hypertension. Increased FosB expression in the MnPO could trigger changes in gene expression related to CIH that result in a sustained increase in MAP even in the absence of a hypoxic challenge.

Potentially, CIH would induce increased expression of FosB within the MnPO and this could in turn increase utilization of local CNS RAS. Since ACE1 is thought to be pro-hypertensive and OSA presents with a sustained increase in blood pressure, we chose to focus on the association of FosB with ACE1 in the MnPO following CIH. Therefore, increased FosB could increase ACE1 expression within the MnPO this could be a mechanism contributing to the sustained diurnal hypertension associated with CIH. This suggests that there is a possible genetic regulatory effect CIH has on MnPO neurons that could contribute to the sustained diurnal hypertension associated with CIH and would unprecedentedly show a physiological relationship between the transcription factor FosB and an AP-1 target gene, ACE1.

REFERENCES

1. Shamsuzzaman AS, Gersh BJ and Somers VK. Obstructive sleep apnea: implications for cardiac and vascular disease. *JAMA : the journal of the American Medical Association*. 2003;290:1906-14.

2. Young T, Peppard PE and Gottlieb DJ. Epidemiology of obstructive sleep apnea: a population health perspective. *American journal of respiratory and critical care medicine*. 2002;165:1217-39.

3. Carlson JT, Hedner J, Elam M, Ejnell H, Sellgren J and Wallin BG. Augmented resting sympathetic activity in awake patients with obstructive sleep apnea. *Chest*. 1993;103:1763-8.

4. Morrell MJ, Finn L, Kim H, Peppard PE, Badr MS and Young T. Sleep fragmentation, awake blood pressure, and sleep-disordered breathing in a population-based study. *American journal of respiratory and critical care medicine*. 2000;162:2091-6.

5. Smith ML, Niedermaier ON, Hardy SM, Decker MJ and Strohl KP. Role of hypoxemia in sleep apnea-induced sympathoexcitation. *Journal of the autonomic nervous system*. 1996;56:184-90.

6. Somers VK, Dyken ME, Clary MP and Abboud FM. Sympathetic neural mechanisms in obstructive sleep apnea. *The Journal of clinical investigation*. 1995;96:1897-904.

7. Young T, Peppard P, Palta M, Hla KM, Finn L, Morgan B and Skatrud J. Population-based study of sleep-disordered breathing as a risk factor for hypertension. *Archives of internal medicine*. 1997;157:1746-52.

8. Narkiewicz K, van de Borne PJ, Montano N, Dyken ME, Phillips BG and Somers VK. Contribution of tonic chemoreflex activation to sympathetic activity and blood pressure in patients with obstructive sleep apnea. *Circulation*. 1998;97:943-5.

9. Dyken ME, Somers VK, Yamada T, Ren ZY and Zimmerman MB. Investigating the relationship between stroke and obstructive sleep apnea. *Stroke; a journal of cerebral circulation*. 1996;27:401-7.

10. Hoffstein V and Mateika S. Cardiac arrhythmias, snoring, and sleep apnea. *Chest*. 1994;106:466-71.

11. Shahar E, Whitney CW, Redline S, Lee ET, Newman AB, Javier Nieto F, O'Connor GT, Boland LL, Schwartz JE and Samet JM. Sleep-disordered breathing and cardiovascular disease: cross-sectional results of the Sleep Heart Health Study. *American journal of respiratory and critical care medicine*. 2001;163:19-25.

12. Peppard PE, Young T, Palta M and Skatrud J. Prospective study of the association between sleep-disordered breathing and hypertension. *The New England journal of medicine*. 2000;342:1378-84.

13. Weaver TE. Adherence to positive airway pressure therapy. *Curr Opin Pulm Med*. 2006;12:409-13.

14. Narkiewicz K, Kato M, Phillips BG, Pesek CA, Davison DE and Somers VK. Nocturnal continuous positive airway pressure decreases daytime sympathetic traffic in obstructive sleep apnea. *Circulation*. 1999;100:2332-5.

15. Nieto FJ, Young TB, Lind BK, Shahar E, Samet JM, Redline S, D'Agostino RB, Newman AB, Lebowitz MD and Pickering TG. Association of sleep-disordered breathing, sleep apnea, and hypertension in a large community-based study. Sleep Heart Health Study. *JAMA : the journal of the American Medical Association*. 2000;283:1829-36.

16. Somers VK, White DP, Amin R, Abraham WT, Costa F, Culebras A, Daniels S, Floras JS, Hunt CE, Olson LJ, Pickering TG, Russell R, Woo M, Young T, American Heart Association Council for High Blood Pressure Research Professional Education Committee CoCC, American Heart Association Stroke C, American Heart Association Council on Cardiovascular N and American College of Cardiology F. Sleep apnea and cardiovascular disease: an American Heart Association/american College Of Cardiology Foundation Scientific Statement from the American Heart Association Council for High Blood Pressure Research Professional Education Committee, Council on Clinical Cardiology, Stroke Council, and Council On Cardiovascular Nursing. In collaboration with the National Heart, Lung, and Blood Institute National Center on Sleep Disorders Research (National Institutes of Health). *Circulation*. 2008;118:1080-111.

17. Dempsey JA, Veasey SC, Morgan BJ and O'Donnell CP. Pathophysiology of sleep apnea. *Physiological reviews*. 2010;90:47-112.

18. Garpestad E, Katayama H, Parker JA, Ringler J, Lilly J, Yasuda T, Moore RH, Strauss HW and Weiss JW. Stroke volume and cardiac output decrease at termination of obstructive apneas. *Journal of applied physiology*. 1992;73:1743-8.

19. McNicholas WT. Cardiovascular outcomes of CPAP therapy in obstructive sleep apnea syndrome. *American journal of physiology Regulatory, integrative and comparative physiology*. 2007;293:R1666-70.

20. Narkiewicz K, Wolf J, Lopez-Jimenez F and Somers VK. Obstructive sleep apnea and hypertension. *Current cardiology reports*. 2005;7:435-40.

21. Stoohs R and Guilleminault C. Cardiovascular changes associated with obstructive sleep apnea syndrome. *Journal of applied physiology*. 1992;72:583-9.

22. Wolk R, Kara T and Somers VK. Sleep-disordered breathing and cardiovascular disease. *Circulation*. 2003;108:9-12.

23. Young T and Peppard P. Sleep-disordered breathing and cardiovascular disease: epidemiologic evidence for a relationship. *Sleep*. 2000;23 Suppl 4:S122-6.

24. Mansfield DR, Gollogly NC, Kaye DM, Richardson M, Bergin P and Naughton MT. Controlled trial of continuous positive airway pressure in obstructive sleep apnea and heart failure. *American journal of respiratory and critical care medicine*. 2004;169:361-6.

25. Fletcher EC, Lesske J, Behm R, Miller CC, 3rd, Stauss H and Unger T. Carotid chemoreceptors, systemic blood pressure, and chronic episodic hypoxia mimicking sleep apnea. *J Appl Physiol (1985)*. 1992;72:1978-84.

26. Fletcher EC. Invited review: Physiological consequences of intermittent hypoxia: systemic blood pressure. *Journal of applied physiology*. 2001;90:1600-5.

27. Fletcher EC. Sympathetic over activity in the etiology of hypertension of obstructive sleep apnea. *Sleep*. 2003;26:15-9.

28. Tamisier R, Gilmartin GS, Launois SH, Pepin JL, Nespoulet H, Thomas R, Levy P and Weiss JW. A new model of chronic intermittent hypoxia in humans: effect on ventilation, sleep, and blood pressure. *Journal of applied physiology*. 2009;107:17-24.

29. Moya EA, Arias P, Varela C, Oyarce MP, Del Rio R and Iturriaga R. Intermittent Hypoxia-Induced Carotid Body Chemosensory Potentiation and Hypertension Are Critically Dependent on Peroxynitrite Formation. *Oxid Med Cell Longev*. 2016;2016:9802136.

30. Garcia AJ, 3rd, Zanella S, Dashevskiy T, Khan SA, Khuu MA, Prabhakar NR and Ramirez JM. Chronic Intermittent Hypoxia Alters Local Respiratory Circuit Function at the Level of the preBotzinger Complex. *Front Neurosci*. 2016;10:4.

31. Moraes DJ, Machado BH and Zoccal DB. Coupling of respiratory and sympathetic activities in rats submitted to chronic intermittent hypoxia. *Prog Brain Res*. 2014;212:25-38.

32. Greenberg HE, Sica AL, Scharf SM and Ruggiero DA. Expression of c-fos in the rat brainstem after chronic intermittent hypoxia. *Brain research*. 1999;816:638-45.

33. Kc P, Balan KV, Tjoe SS, Martin RJ, Lamanna JC, Haxhiu MA and Dick TE. Increased vasopressin transmission from the paraventricular nucleus to the rostral medulla augments cardiorespiratory outflow in chronic intermittent hypoxia-conditioned rats. *The Journal of physiology*. 2010;588:725-40.

34. Kc P and Dick TE. Modulation of cardiorespiratory function mediated by the paraventricular nucleus. *Respiratory physiology & neurobiology*. 2010;174:55-64.

35. Prabhakar NR and Kumar GK. Mechanisms of sympathetic activation and blood pressure elevation by intermittent hypoxia. *Respiratory physiology & neurobiology*. 2010;174:156-61.

36. Marcus NJ, Li YL, Bird CE, Schultz HD and Morgan BJ. Chronic intermittent hypoxia augments chemoreflex control of sympathetic activity: role of the angiotensin II type 1 receptor. *Respiratory physiology & neurobiology*. 2010;171:36-45.

37. Shell B, Faulk K and Cunningham JT. Neural Control of Blood Pressure in Chronic Intermittent Hypoxia. *Current hypertension reports*. 2016;18:19.

38. Kline DD. Chronic intermittent hypoxia affects integration of sensory input by neurons in the nucleus tractus solitarii. *Respiratory physiology & neurobiology*. 2010;174:29-36.

39. de Paula PM, Tolstykh G and Mifflin S. Chronic intermittent hypoxia alters NMDA and AMPA-evoked currents in NTS neurons receiving carotid body chemoreceptor inputs.

American journal of physiology Regulatory, integrative and comparative physiology. 2007;292:R2259-65.

40. Costa-Silva JH, Zoccal DB and Machado BH. Chronic intermittent hypoxia alters glutamatergic control of sympathetic and respiratory activities in the commissural NTS of rats. *American journal of physiology Regulatory, integrative and comparative physiology*. 2012;302:R785-93.

41. Zhang W, Carreno FR, Cunningham JT and Mifflin SW. Chronic sustained and intermittent hypoxia reduce function of ATP-sensitive potassium channels in nucleus of the solitary tract. *American journal of physiology Regulatory, integrative and comparative physiology*. 2008;295:R1555-62.

42. Lim K, Burke SL, Moretti JL and Head GA. Differential activation of renal sympathetic burst amplitude and frequency during hypoxia, stress and baroreflexes with chronic angiotensin treatment. *Exp Physiol*. 2015;100:1132-44.

43. Yamamoto K, Lalley P and Mifflin S. Acute intermittent optogenetic stimulation of nucleus tractus solitarius neurons induces sympathetic long-term facilitation. *American journal of physiology Regulatory, integrative and comparative physiology*. 2015;308:R266-75.

44. Dick TE, Hsieh YH, Wang N and Prabhakar N. Acute intermittent hypoxia increases both phrenic and sympathetic nerve activities in the rat. *Exp Physiol*. 2007;92:87-97.

45. Silva AQ and Schreihofer AM. Altered sympathetic reflexes and vascular reactivity in rats after exposure to chronic intermittent hypoxia. *The Journal of physiology*. 2011;589:1463-76.

46. Fletcher EC, Bao G and Li R. Renin activity and blood pressure in response to chronic episodic hypoxia. *Hypertension*. 1999;34:309-14.

47. Saxena A, Little JT, Nedungadi TP and Cunningham JT. Angiotensin II type 1a receptors in subfornical organ contribute towards chronic intermittent hypoxia-associated sustained increase in mean arterial pressure. *American journal of physiology Heart and circulatory physiology*. 2015;308:H435-46.

48. Buggy J, Fink GD, Haywood JR, Johnson AK and Brody MJ. Interruption of the maintenance phase of established hypertension by ablation of the anteroventral third ventricle (AV3V) in rats. *Clinical and experimental hypertension*. 1978;1:337-53.

49. McKinley MJ, McAllen RM, Davern P, Giles ME, Penschow J, Sunn N, Uschakov A and Oldfield BJ. The sensory circumventricular organs of the mammalian brain. *Advances in anatomy, embryology, and cell biology*. 2003;172:III-XII, 1-122, back cover.

50. Osborn JW, Fink GD, Sved AF, Toney GM and Raizada MK. Circulating angiotensin II and dietary salt: converging signals for neurogenic hypertension. *Current hypertension reports*. 2007;9:228-35.

51. Smith PM and Ferguson AV. Circulating signals as critical regulators of autonomic state--central roles for the subfornical organ. *American journal of physiology Regulatory, integrative and comparative physiology*. 2010;299:R405-15.

52. Johnson AK and Gross PM. Sensory circumventricular organs and brain homeostatic pathways. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology*. 1993;7:678-86.

53. Saper CB and Levisohn D. Afferent connections of the median preoptic nucleus in the rat: anatomical evidence for a cardiovascular integrative mechanism in the anteroventral third ventricular (AV3V) region. *Brain research*. 1983;288:21-31.

54. Stocker SD and Toney GM. Median preoptic neurones projecting to the hypothalamic paraventricular nucleus respond to osmotic, circulating Ang II and baroreceptor input in the rat. *The Journal of physiology*. 2005;568:599-615.

55. Stocker SD and Toney GM. Vagal afferent input alters the discharge of osmotic and ANG II-responsive median preoptic neurons projecting to the hypothalamic paraventricular nucleus. *Brain research*. 2007;1131:118-28.

56. Ferguson AV, Latchford KJ and Samson WK. The paraventricular nucleus of the hypothalamus - a potential target for integrative treatment of autonomic dysfunction. *Expert opinion on therapeutic targets*. 2008;12:717-27.

57. Liposits Z. Ultrastructure of hypothalamic paraventricular neurons. *Critical reviews in neurobiology*. 1993;7:89-162.

58. Stocker SD, Hunwick KJ and Toney GM. Hypothalamic paraventricular nucleus differentially supports lumbar and renal sympathetic outflow in water-deprived rats. *The Journal of physiology*. 2005;563:249-63.

59. Llewellyn T, Zheng H, Liu X, Xu B and Patel KP. Median preoptic nucleus and subfornical organ drive renal sympathetic nerve activity via a glutamatergic mechanism within the paraventricular nucleus. *American journal of physiology Regulatory, integrative and comparative physiology*. 2012;302:R424-32.

60. Knight WD, Little JT, Carreno FR, Toney GM, Mifflin SW and Cunningham JT. Chronic intermittent hypoxia increases blood pressure and expression of FosB/DeltaFosB in central autonomic regions. *American journal of physiology Regulatory, integrative and comparative physiology*. 2011;301:R131-9.

61. Ployngam T and Collister JP. Role of the median preoptic nucleus in chronic angiotensin II-induced hypertension. *Brain research*. 2008;1238:75-84.

62. Bealer SL, Metcalf CS and Heyborne R. Increased dietary sodium alters Fos expression in the lamina terminalis during intravenous angiotensin II infusion. *Experimental neurology*. 2007;204:299-306.

63. Cunningham JT, Knight WD, Mifflin SW and Nestler EJ. An Essential role for DeltaFosB in the median preoptic nucleus in the sustained hypertensive effects of chronic intermittent hypoxia. *Hypertension*. 2012;60:179-87.

64. Mourao AA, Moreira MC, Melo AB, Lopes PR, Rebelo AC, Rosa DA, Freiria-Oliveira AH, Colombari E and Pedrino GR. Does the median preoptic nucleus contribute to sympathetic hyperactivity in spontaneously hypertensive rats? *Auton Neurosci*. 2016;195:29-33.

65. Nestler EJ. Molecular basis of long-term plasticity underlying addiction. *Nature reviews Neuroscience*. 2001;2:119-28.

66. Chen J, Kelz MB, Hope BT, Nakabeppu Y and Nestler EJ. Chronic Fos-related antigens: stable variants of deltaFosB induced in brain by chronic treatments. *The Journal of neuroscience : the official journal of the Society for Neuroscience*. 1997;17:4933-41.

67. Herdegen T and Leah JD. Inducible and constitutive transcription factors in the mammalian nervous system: control of gene expression by Jun, Fos and Krox, and CREB/ATF proteins. *Brain research Brain research reviews*. 1998;28:370-490.

68. McClung CA, Ulery PG, Perrotti LI, Zachariou V, Berton O and Nestler EJ. DeltaFosB: a molecular switch for long-term adaptation in the brain. *Brain research Molecular brain research*. 2004;132:146-54.

69. McClung CA and Nestler EJ. Regulation of gene expression and cocaine reward by CREB and DeltaFosB. *Nature neuroscience*. 2003;6:1208-15.

70. Berton O, Covington HE, 3rd, Ebner K, Tsankova NM, Carle TL, Ulery P, Bhonsle A, Barrot M, Krishnan V, Singewald GM, Singewald N, Birnbaum S, Neve RL and Nestler

EJ. Induction of deltaFosB in the periaqueductal gray by stress promotes active coping responses. *Neuron*. 2007;55:289-300.

71. Winstanley CA, LaPlant Q, Theobald DE, Green TA, Bachtell RK, Perrotti LI, DiLeone RJ, Russo SJ, Garth WJ, Self DW and Nestler EJ. DeltaFosB induction in orbitofrontal cortex mediates tolerance to cocaine-induced cognitive dysfunction. *The Journal of neuroscience : the official journal of the Society for Neuroscience*. 2007;27:10497-507.

72. Howe BM, Bruno SB, Higgs KA, Stigers RL and Cunningham JT. FosB expression in the central nervous system following isotonic volume expansion in unanesthetized rats. *Experimental neurology*. 2004;187:190-8.

73. Tipnis SR, Hooper NM, Hyde R, Karran E, Christie G and Turner AJ. A human homolog of angiotensin-converting enzyme. Cloning and functional expression as a captopril-insensitive carboxypeptidase. *J Biol Chem*. 2000;275:33238-43.

74. Turner AJ and Hooper NM. The angiotensin-converting enzyme gene family: genomics and pharmacology. *Trends Pharmacol Sci*. 2002;23:177-83.

75. Moller DS, Lind P, Strunge B and Pedersen EB. Abnormal vasoactive hormones and 24-hour blood pressure in obstructive sleep apnea. *American journal of hypertension*. 2003;16:274-80.

76. Kraiczi H, Hedner J, Peker Y and Carlson J. Increased vasoconstrictor sensitivity in obstructive sleep apnea. *Journal of applied physiology*. 2000;89:493-8.

77. Fletcher EC, Orolinova N and Bader M. Blood pressure response to chronic episodic hypoxia: the renin-angiotensin system. *Journal of applied physiology*. 2002;92:627-33.

78. Foster GE, Hanly PJ, Ahmed SB, Beaudin AE, Pialoux V and Poulin MJ. Intermittent hypoxia increases arterial blood pressure in humans through a Renin-Angiotensin system-dependent mechanism. *Hypertension*. 2010;56:369-77.

79. Ganten D, Marquez-Julio A, Granger P, Hayduk K, Karsunky KP, Boucher R and Genest J. Renin in dog brain. *The American journal of physiology*. 1971;221:1733-7.

80. Wright JW and Harding JW. The brain renin-angiotensin system: a diversity of functions and implications for CNS diseases. *Pflugers Archiv : European journal of physiology*. 2012.

81. Morimoto S, Cassell MD, Beltz TG, Johnson AK, Davisson RL and Sigmund CD. Elevated blood pressure in transgenic mice with brain-specific expression of human angiotensinogen driven by the glial fibrillary acidic protein promoter. *Circulation research*. 2001;89:365-72.

82. Baltatu OC, Campos LA and Bader M. Local renin-angiotensin system and the brain--a continuous quest for knowledge. *Peptides*. 2011;32:1083-6.

83. Smookler HH, Severs WB, Kinnard WJ and Buckley JP. Centrally mediated cardiovascular effects of angiotensin II. *J Pharmacol Exp Ther*. 1966;153:485-94.

84. Park CG and Leenen FH. Effects of centrally administered losartan on deoxycorticosterone-salt hypertension rats. *J Korean Med Sci*. 2001;16:553-7.

85. Ito S, Komatsu K, Tsukamoto K, Kanmatsuse K and Sved AF. Ventrolateral medulla AT1 receptors support blood pressure in hypertensive rats. *Hypertension*. 2002;40:552-9.

86. Knight WD, Saxena A, Shell B, Nedungadi TP, Mifflin SW and Cunningham JT. Central losartan attenuates increases in arterial pressure and expression of FosB/DeltaFosB along the autonomic axis associated with chronic intermittent hypoxia. *American journal of physiology Regulatory, integrative and comparative physiology*. 2013;305:R1051-8.

87. Li Z and Ferguson AV. Subfornical organ efferents to paraventricular nucleus utilize angiotensin as a neurotransmitter. *The American journal of physiology*. 1993;265:R302-9.

88. Aars H and Akre S. Effect of angiotensin on sympathetic nerve activity. *Acta physiologica Scandinavica*. 1968;74:134-41.

89. Barrett CJ, Ramchandra R, Guild SJ, Lala A, Budgett DM and Malpas SC. What sets the long-term level of renal sympathetic nerve activity: a role for angiotensin II and baroreflexes? *Circulation research*. 2003;92:1330-6.

90. DiBona GF. Central angiotensin modulation of baroreflex control of renal sympathetic nerve activity in the rat: influence of dietary sodium. *Acta physiologica Scandinavica*. 2003;177:285-9.

91. Matsukawa T, Gotoh E, Minamisawa K, Kihara M, Ueda S, Shionoiri H and Ishii M. Effects of intravenous infusions of angiotensin II on muscle sympathetic nerve activity in humans. *The American journal of physiology*. 1991;261:R690-6.

92. Yoshimoto M, Miki K, Fink GD, King A and Osborn JW. Chronic angiotensin II infusion causes differential responses in regional sympathetic nerve activity in rats. *Hypertension*. 2010;55:644-51.

93. Zucker IH and Gao L. The regulation of sympathetic nerve activity by angiotensin II involves reactive oxygen species and MAPK. *Circulation research*. 2005;97:737-9.

94. Budzikowski AS and Leenen FH. ANG II in median preoptic nucleus and pressor responses to CSF sodium and high sodium intake in SHR. *American journal of physiology Heart and circulatory physiology*. 2001;281:H1210-6.

95. Xia H and Lazartigues E. Angiotensin-converting enzyme 2 in the brain: properties and future directions. *Journal of neurochemistry*. 2008;107:1482-94.

96. Ahmad M, White R, Tan J, Huang BS and Leenen FH. Angiotensin-converting enzyme inhibitors, inhibition of brain and peripheral angiotensin-converting enzymes, and left ventricular dysfunction in rats after myocardial infarction. *Journal of cardiovascular pharmacology*. 2008;51:565-72.

97. Bourassa EA and Speth RC. Water deprivation increases angiotensin-converting enzyme but not AT(1) receptor expression in brainstem and paraventricular nucleus of the hypothalamus of the rat. *Brain research*. 2010;1319:83-91.

98. Gaul SL, Martin GE and Sweet CS. Comparative effects of enalapril, enalaprilic acid and captopril in blocking angiotensin I-induced pressor and dipsogenic responses in spontaneously hypertensive rats. *Clin Exp Hypertens A*. 1984;6:1187-206.

99. Okuno T, Nagahama S, Lindheimer MD and Oparil S. Attenuation of the development of spontaneous hypertension in rats by chronic central administration of captopril. *Hypertension*. 1983;5:653-62.

100. Chappell MC, Brosnihan KB, Diz DI and Ferrario CM. Identification of angiotensin-(1-7) in rat brain. Evidence for differential processing of angiotensin peptides. *J Biol Chem*. 1989;264:16518-23.

101. Vickers C, Hales P, Kaushik V, Dick L, Gavin J, Tang J, Godbout K, Parsons T, Baronas E, Hsieh F, Acton S, Patane M, Nichols A and Tummino P. Hydrolysis of biological peptides by human angiotensin-converting enzyme-related carboxypeptidase. *J Biol Chem*. 2002;277:14838-43.

102. Gironacci MM, Valera MS, Yujnovsky I and Pena C. Angiotensin-(1-7) inhibitory mechanism of norepinephrine release in hypertensive rats. *Hypertension*. 2004;44:783-7.

103. Santos RA, Simoes e Silva AC, Maric C, Silva DM, Machado RP, de Buhr I, Heringer-Walther S, Pinheiro SV, Lopes MT, Bader M, Mendes EP, Lemos VS, Campagnole-Santos MJ, Schultheiss HP, Speth R and Walther T. Angiotensin-(1-7) is an endogenous ligand for the G protein-coupled receptor Mas. *Proc Natl Acad Sci U S A*. 2003;100:8258-63.

104. Gironacci MM, Cerniello FM, Longo Carbajosa NA, Goldstein J and Cerrato BD. Protective axis of the renin-angiotensin system in the brain. *Clin Sci (Lond)*. 2014;127:295-306.

105. Bader M. ACE2, angiotensin-(1-7), and Mas: the other side of the coin. *Pflugers Archiv : European journal of physiology*. 2013;465:79-85.

106. Xue B, Zhang Z, Johnson RF, Guo F, Hay M and Johnson AK. Central endogenous angiotensin-(1-7) protects against aldosterone/NaCl-induced hypertension in female rats. *American journal of physiology Heart and circulatory physiology*. 2013;305:H699-705.

107. Sriramula S, Xia H, Xu P and Lazartigues E. Brain-targeted angiotensin-converting enzyme 2 overexpression attenuates neurogenic hypertension by inhibiting cyclooxygenase-mediated inflammation. *Hypertension*. 2015;65:577-86.

108. Xia H, de Queiroz TM, Sriramula S, Feng Y, Johnson T, Mungrue IN and Lazartigues E. Brain ACE2 overexpression reduces DOCA-salt hypertension independently of endoplasmic reticulum stress. *American journal of physiology Regulatory, integrative and comparative physiology*. 2015;308:R370-8.

109. Xia H, Sriramula S, Chhabra KH and Lazartigues E. Brain angiotensin-converting enzyme type 2 shedding contributes to the development of neurogenic hypertension. *Circulation research*. 2013;113:1087-96.

110. Sriramula S, Cardinale JP, Lazartigues E and Francis J. ACE2 overexpression in the paraventricular nucleus attenuates angiotensin II-induced hypertension. *Cardiovasc Res*. 2011;92:401-8.

111. Wang YK, Shen D, Hao Q, Yu Q, Wu ZT, Deng Y, Chen YF, Yuan WJ, Hu QK, Su DF and Wang WZ. Overexpression of angiotensin-converting enzyme 2 attenuates tonically

active glutamatergic input to the rostral ventrolateral medulla in hypertensive rats. *American journal of physiology Heart and circulatory physiology*. 2014;307:H182-90.

112. Elased KM, Cunha TS, Marcondes FK and Morris M. Brain angiotensin-converting enzymes: role of angiotensin-converting enzyme 2 in processing angiotensin II in mice. *Exp Physiol*. 2008;93:665-75.

113. Dobruch J, Paczwa P, Lon S, Khosla MC and Szczepanska-Sadowska E. Hypotensive function of the brain angiotensin-(1-7) in Sprague Dawley and renin transgenic rats. *J Physiol Pharmacol*. 2003;54:371-81.

CHAPTER II

THE ROLE OF ANGIOTENSIN CONVERTING ENZYME 1 WITHIN THE MEDIAN PREOPTIC NUCLEUS FOLLOWING CHRONIC INTERMITTENT HYPOXIA

INTRODUCTION

Sleep apnea is consistently becoming a more common occurrence. According to a recent poll from The National Sleep Foundation, 26% of US individuals are at high risk for obstructive sleep apnea (OSA) [1]. In addition, sleep apnea has increased within certain demographic groups by as much as 55% over the last 20 years [2]. OSA is associated with multiple cardiovascular diseases including hypertension [3-14]. Patients with OSA have a sustained hypertension that fails to subside even in the awake hours [12]. Increased SNA that may lead to this sustained hypertension has also been observed in OSA patients [15-18]. The increase in SNA suggests that central nervous system (CNS) mechanisms may contribute to the cardiovascular sequela associated with sleep apnea.

Generally, sleep apnea results from intermittent interruptions of breathing, or apneas, that cause arterial hypoxemia, hypercapnia, and changes in thoracic pressure [5]. CIH mimics the arterial hypoxemia seen in sleep apnea patients and results in an analogous increase in SNA and sustained hypertension [19-21]. Several CNS mechanisms have been proposed that contribute to CIH hypertension such as chemoreceptor sensitization [5]. In addition, several CNS regions that

are not directly related to the chemoreceptor reflex have been investigated for their role in CIH hypertension [22, 23]. Several studies indicate that the lamina terminalis, which has been linked with several models of neurogenic hypertension [24], may contribute to CIH hypertension [25, 26]. In the lamina terminalis, the MnPO is one region that projects to the paraventricular nucleus of the hypothalamus and may influence SNA and blood pressure [27-31]. In CIH, the transcription factor FosB/ΔFosB is significantly increased in the MnPO and inhibition of ΔFosB in this region blocks the sustained component of CIH hypertension [25, 32]. These observations suggest that FosB/ΔFosB mediated changes in gene expression play an essential role specifically in the sustain hypertension associated with CIH [25]. We identified several genes that may be downstream targets of FosB/ΔFosB in the MnPO [25]. Our working hypothesis is that one or more of these CIH stimulated FosB/ΔFosB target genes within the MnPO may contribute to the sustained hypertension which occurs during nomoxia. One candidate gene that was identified is ACE1 [25]. Changes in ACE1 expression within the MnPO may be part of the mechanism by which this region contributes to increases in SNA that result in sustained hypertension associated with CIH.

Increased ACE1 expression within the MnPO may be a pro-hypertensive switch that gets chronically activated by CIH stimulation of FosB/ΔFosB and, thus, promote the inappropriate sustained blood pressure increase. ACE1 is a well-known component of RAS that converts Angiotensin I to Angiotensin II (ANGII). The CNS has been shown to have an independent RAS from the systemic RAS [33, 34]. Furthermore, ANGII has been shown to affect SNA [35-40]. We conducted experiments to test the hypothesis that MnPO neurons activated by CIH project to the PVN and that CIH increases ACE1 expression through transcriptional activation of FosB/ΔFosB

within the MnPO. In order to test this hypothesis, we utilized retrograde tract tracing to label the neurons that project from the MnPO to the PVN and further explore the ACE1 expression in those neurons following 7 days of CIH treatment. We also sought to determine the transcription factor FosB/ΔFosB association with the ACE1 gene region within the MnPO following CIH treatment with a ChIP assay.

METHODS

Animals

Adult male Sprague-Dawley rats (Charles River Laboratories, Inc., Wilmington, MA, USA) maintained on a 12:12 light/dark cycle (light period from 7am-7pm) were used. Rats were individually housed and provided with ad libitum food and water as well as being in temperature controlled rooms. All animal procedures were conducted in accordance with current National Institutes of Health guidelines and were approved by the Institutional Animal Care and Use Committee at the University of North Texas Health Science Center.

Retrograde Tracer

Rats were anesthetized with isoflurane (2-3%), and placed in a stereotaxic frame and skulls leveled to bregma and lambda after their scalps were shaved and cleaned. Each rat received a unilateral PVN injection with a retrograde tracer, Fluorogold (Fluorchrome, LLC, Denver, CO). Each injection was made using a 30 gauge injector with a 5 µL Hamilton syringe (#84851 Hamilton Reno, NV) at a volume of 200 ηl over a 5 minute period at atlas-defined coordinates of -1.80 mm (anterior/posterior), -0.4 mm (midline) and -7.6 mm (dorsal/ventral) [41]. Following the injection, the holes made in their skulls were filled with sterile gel foam and their scalps were closed with sterile absorbable suture. Rats were given at least 1 week recovery before telemetry implantation.

Radio Telemetry Transmitter Implantation

Rats were implanted with an abdominal aortic catheter attached to a CA11PA-C40 radio-telemetry transmitter using isoflurane anesthesia (2-3%), as described previously [32]. Each rat was allowed to recover for at least one week following telemetry surgery. The transmitter was secured to abdominal muscle throughout the CIH experiment. A Dataquest radio-telemetry system (Data Sciences, St. Paul, MN, USA) was used to record all physiological measurements. Mean arterial blood pressure (MAP), respiratory rate (RR) and heart rate (HR) were monitored by radio telemetry and sampled for 10 seconds every 10 minutes. Data are expressed as changes from baseline.

Chronic Intermittent Hypoxia Treatment

Baseline radio-telemetry recording occurred for 5 days prior to the start of the CIH protocol. CIH exposure was applied for 8 hours beginning at 0800 of the light phase using a 3 min hypoxia (10% O_2)/3 min normoxia (21% O_2) cycle as described previously [32]. During the remaining 16 hrs (1600-0800 h), the chambers were open to normoxic room air (21% O_2). Rats were exposed to CIH for 7 days. Controls were placed in identical chambers within the same room but were only exposed room air (21% O_2). All animals were sacrificed on the 8^{th} day following the 7 day CIH protocol. Rats were given inactin (100 mg/kg ip) and either perfused with 4% paraformaldehyde transcardially or decapitated.

Immunohistochemistry

Rats used for the immunohistochemical studies were perfused with 0.1 M phosphate buffered saline (PBS, 100-200 ml) followed by 4% paraformaldehyde (400-500 ml), as previously described [32]. Brains were then post fixed overnight and dehydrated in 30% sucrose. Each brain was cut into three sets of serial 40 μm coronal sections using a crytostat (Leica Biosystems, Buffalo Grove, IL, USA). The sections were stored in cryoprotectant at -20°C until the immunohistochemistry protocol. Sections were stained, as previously described [32, 42], for FosB (1:1000 Goat polyclonal sc-48869 Santa Cruz Biotechnology,Dallas, TX), GFAP (1:1500 Mouse monoclonal G3893, Sigma-Aldrich, St. Louis, MO) or ACE1 (1:500 Rabbit polyclonal sc-20791, Santa Cruz Biotechnology, 1:500). The FosB antibody binds to both FosB and the more stable splice variant ΔFosB and thus staining will be denoted as FosB/ΔFosB. Staining for FosB/ΔFosB was either processed with a biotinylated secondary antibody and avidin-biotin conjugated with horseradish peroxidase and diaminobenzidine (DAB) staining or stained with a fluorescently labeled secondary antibody. Tissue processed for FosB/ΔFosB DAB staining were incubated with a biotinylated horse anti-goat IgG (1:200; Vector Laboratories, Burlingame, CA), then treated with an avidin-peroxidase conjugate (Vectastain ABC Kit; Vector Laboratories) following PBS containing 0.04% 3,3′-diaminobenzidine hydrochloride and 0.04% nickel ammonium sulfate for 11 minutes. Tissue were then mounted to gel-coated slides, allowed to dry for one day and then dehydrated with serial ethanol solutions and xylene. Slides were then coverslipped with Permount mounting medium (ThermoScientific, Waltham, MA, USA). FosB/ΔFosB stained sections that were processed for indirect immunofluoresecences were incubated with a Cy2 anti-goat (1:1200; Jackson ImmunoResearch Inc., West Grove, PA) and

mounted with Vectashield HardSet mounting medium (Vector Laboratories). ACE1 staining was visualized using a Cy3 anti-rabbit (1:1200; Jackson ImmunoResearch Inc., West Grove, PA). Tissue was then imaged using an Olympus (Olympus BX41) fluorescent microscope or an inverted microscope (Olympus BX50) equipped with a spinning disk confocal unit (Olympus IX 2- DSU) and epifluorsecence. Images were collected using a Retiga-SRV camera (Q-imaging, Surrey, British Columbia, Canada). Image J was used to analyze and count labeled cells for each section. All counts were averaged across each section of nuclei.

Western Blot Analysis

The day after our 7 day CIH protocol, inactin anesthetized rats were decapitated and each brain was placed dorsal surface down in a commercially available brain matrix (Stoelting) in order to cut the brain into 1 mm coronal slabs with razor blades. Punch samples were collected from the slabs using 1 ml syringes equipped with blunt 23 gauge needles. The punches were ejected into micro-centrifuge tubes and frozen at -80°C until protein isolations and Western blot analysis were performed as previously described [43, 44]. Two to three MnPO punches from each rat were dissolved in Laemmli Buffer and run on a 12% acrylamide gradient SDS gel (Nupage Bris-Tris, Invitrogen) and transferred to a nitrocellulose membrane. Blots were washed, blocked and then incubated with primary antibodies for ACE1 (1:100 Rabbit polyclonal, SantaCruz Biotechnology) and GAPDH (1:2000 Mouse, monoclonal, EMD Millipore, Millipore Sigma, St. Louis, MO) as a control. Blots were washed again then incubated with secondary antibodies according to the primary host-species. Proteins were detected by chemiluminescent reagents (Thermo Fisher Scientific Inc., Waltham, MA, USA), imaged using G-Box (Syngene, Fredrick, MD, USA) and then analyzed for densitometry using ImageJ.

Chromatin Immunoprecipitation Analysis

Following the 7 day CIH protocol, rats were anesthetized with inactin and decapitated. Each brain was cut into 1 mm coronal slabs as described above in western blot analysis. Two MnPO punches (dorsal and ventral to the anterior commissure) from the same rat were taken from the slabs using 1 ml syringes with blunt 23 gauge needles. Punches containing the MnPO from two rats from the same treatment group were pooled and placed into the same micro-centrifuge tube. All samples were kept frozen at -80°C until ChIP assays were performed the following day. Pooled punches from two rats, four punches total, were needed in order to obtain sufficient material to execute the ChIP assay. The LowCell# ChIP Kit (kch-maglow-G16, Diagenode, USA) was utilized according to the manufacture's tissue protocol in order to analyze the association between FosB/ΔFosB and ACE1. Samples were sheared on ice using a Model 150 Sonic Dismembrator (Fisher Scientific, Pittsburg, PA, USA) and subjected to 4 rounds of 10s on 30s off at 0100% AMPS in order to achieve a base pair size between 100 – 1000 base pairs. 2 µg of a ChIP grade FosB (102) GX antibody (goat, polyclonal IgG, Santa Cruz Biotechnology) was used to detect FosB/ΔFosB bound DNA while a normal goat IgG polyclonal antibody (Abcam, Cambridge, MA) was used as a negative control. Total genomic input, FosB isolated and goat IgG isolated DNA samples were then subjected to quantitative PCR (qPCR) using iQ SYBR green Supermix (Bio-Rad Laboratories Inc., Hercules, CA) in a Bio-Rad iQTM5 iCycler system (Bio-Rad Laboratories Inc., Hercules, CA) with the following primers for ACE1 (Forward 5'-CCCGGAAATACGAAGAATTGC-3', Reverse 5'-GGCTCTCCCCACCTTGTCTC-3'). Data were normalized to input background.

Statistical Analysis

Data from immunohistochemistry studies were analyzed using one-way ANOVA with *Newman-Keuls* tests for post hoc analysis of significant main effects. Data from the radio telemetry experiments were analyzed using two-way RM ANOVA with *Student-Newman-Keuls* tests for post hoc analysis. Data from western blot and ChIP qPCR were analyzed using t-test. All tests were performed using SigmaPlot (v. 12.0, Systat Software, USA). Differences were considered statistically significant at $P < 0.05$. Data are reported as mean ± SEM.

RESULTS

MAP, HR and RR measurements following CIH. Baseline MAP, HR and RR measurements were not significantly different among the groups prior to CIH exposure. On the first day of treatment, MAP was not significantly different between the normoxic (Norm) and hypoxic (CIH) treated rats for either the dark phase or the light phase during intermittent hypoxia administration. However, MAP of the CIH treatment group significantly increased from day 2 to day 7 as compared to the Norm controls in both the dark phase and light phase ($P<0.05$; Figure 1 A & B). These results are comparable to our previous studies [32]. HR showed no significant difference between Norm and CIH treatment groups in the light phase however HR was significantly decreased in the CIH treatment group during the dark phase while CIH is not actively being administered ($P<0.05$; Figure 1 C & D). RR showed a significant increase in the CIH treatment group during the light phase as compared to Norm controls ($P<0.05$; Figure 1 E) which was expected since CIH is known to activate the chemoreflex [45]. No significant difference was found amongst the RR in the Dark period while CIH treatment was not active (Figure 1 F).

ACE1 Cell Type Localization. ACE1 staining was not colocalized with GFAP in the MnPO of rats from either the normoxic control (Figure 2 C) nor the CIH treatment group (Figure 2 F). These results suggest that in the MnPO ACE1 is not expressed by GFAP positive astrocytes (Figure 2).

Effect of CIH on ACE1 expressing cells within the MnPO. We examined FosB/ΔFosB and ACE1 staining in the MnPO (Figure 3 Top Panel) to further determine the possible activation of ACE1 expressing neurons following CIH. CIH was associated with a significant increase in the number

of FosB/ΔFosB positive neurons within the MnPO as compared to the normoxic controls ($P<0.001$; Figure 3 Bottom Panel). Interestingly, the number of FosB/ΔFosB and ACE1 positive neurons in the MnPO also significantly increased following CIH exposure ($P<0.001$; Figure 3 Bottom Panel).

Effect of CIH on ACE1 protein expression within the MnPO. ACE1 protein abundance within the MnPO significantly increased following CIH as compared to normoxic controls when normalized to GAPDH (Figure 4).

Effect of CIH on ACE1/FosB projecting to the PVN. Figure 5 (Left Panel) shows a representative fluorogold injection site the included the PVN. Brains with similar injection sites were used in the analysis ACE1 and FosB/ΔFosB localization with fluorogold. As reported above, CIH was associated with a significant increase in the numbers of FosB/ΔFosB positive cells and ACE1 positive cells within the MnPO as compared to normoxic controls ($P<0.05$; Figure 6). Fluorogold positive cells did not significantly increase within the MnPO following CIH (Figure 5 Right Panel). As shown above, MnPO cells that were positive for both FosB/ΔFosB and ACE1 significantly increased following CIH as compared to normoxic controls ($P<0.05$; Figure 6). Also CIH increased FosB/ΔFosB staining in PVN projecting cells as indicated by a significant increase in the numbers of fluorogold positive cells that were stained for FosB/ΔFosB, ($P<0.05$; Figure 6). The number of cells expressing all three (FosB/ΔFosB, ACE1 and fluorogold) labels within the MnPO significantly increased following CIH ($P<0.05$; Figure 6).

Association of FosB with ACE1. ChIP studies were used so that we could examine the possible interaction of FosB/ΔFosB with ACE1 since the gene for ACE1 contains an AP-1 regulatory site.

MnPO samples were pooled from 2 rats of identical treatment, either normoxic or CIH, in order to get sufficient material to perform ChIP. Samples were also sonicated and sheared in order to obtain an optimal size of base pairs between 100 – 1000 base pairs. qPCR was used to examine the expression of ACE1 of FosB/ΔFosB precipitated DNA following CIH or normoxia along with input genomic controls and relevant IgG negative controls. Data were normalized to input background. ACE1 association with FosB/ΔFosB was significantly increased in the MnPO following CIH as compared to the normoxic controls ($P<0.05$; Figure 7). The increase of ACE1 and FosB/ΔFosB expression following CIH suggests that ACE1 may be positively regulated by FosB/ΔFosB in the MnPO.

Figures

CHAPTER II-Figure II-1 MAP, HR and RR of 7 Days CIH

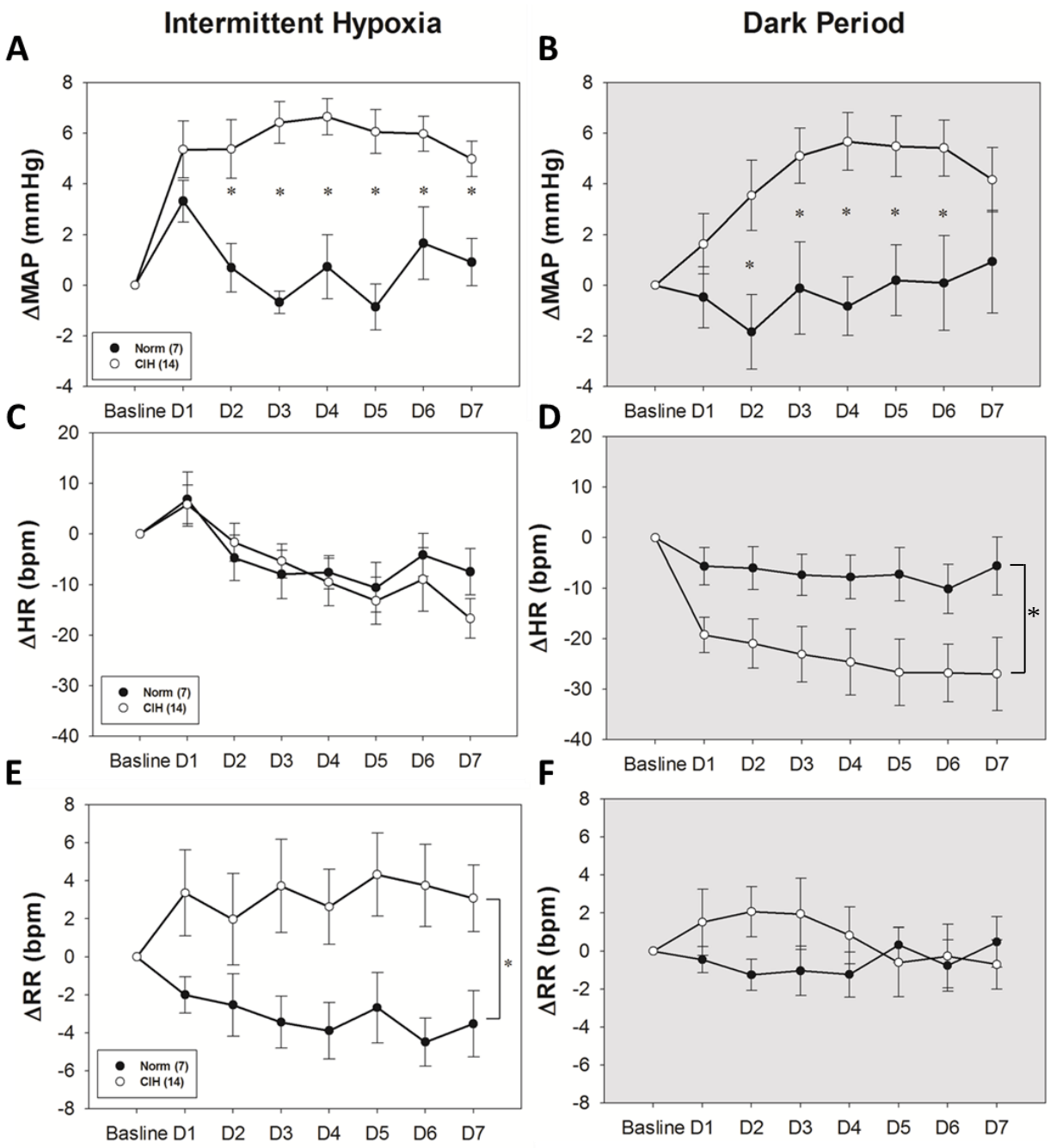


Figure 1. Effect of 7 Days (D) of CIH on MAP, HR and RR. Open circles represent chronic intermittent hypoxia (CIH) treatment while filled in circles represent normoxic controls (Norm). CIH significantly increased MAP (A & B) from D2 to D7 in both intermittent hypoxia (IH) and dark period (DK) times. HR (C & D) was significantly decreased with CIH during the DK time but not IH. RR (E & F) significantly increased with CIH during IH but not in the DK time. Data are expressed as mean±SEM and analyzed using two-way repeated measures ANOVA followed by Student-Neuman-Keuls test. * indicates $P<0.05$ in comparison to Norm.

CHAPTER II-Figure II-2 ACE1 and GFAP Staining in MnPO

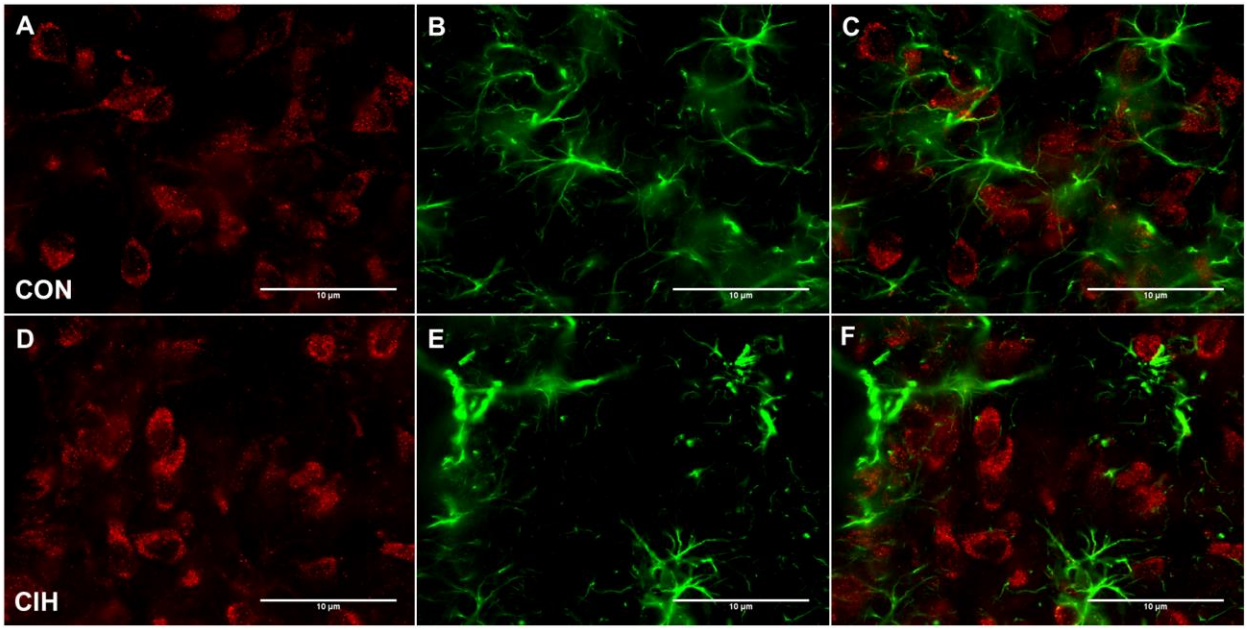


Figure 2. Immunohistochemical representation of MnPO ACE1 (A & D) and GFAP (B & E) expression. C and F represent merged ACE1 and GFAP expression. A, B and C represent normoxic controls (CON) and D, E and F represent hypoxic treated (CIH).

CHAPTER II-Figure II-3 ACE1 and FosB Staining and Counts in MnPO

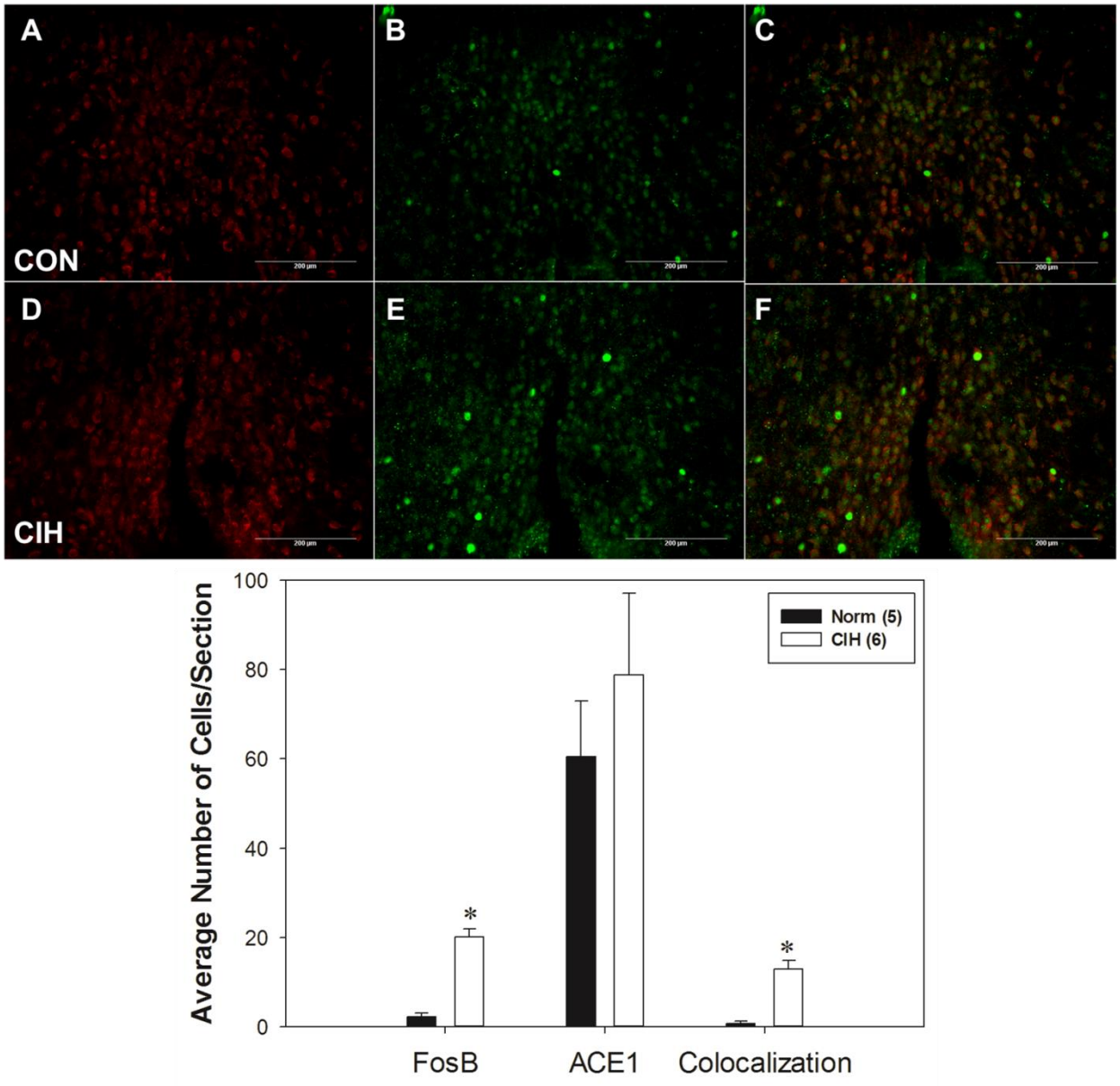


Figure 3. Top panel respresents immunohistochemistry staining of MnPO ACE1 (A & D), FosB/ΔFosB (B & E) expression. C and F represent merged ACE1 and FosB/ΔFosB expression. A, B and C represent normoxic controls (CON) and D, E and F represent hypoxic treated (CIH). Bottom panel represents average number of positive ACE1 and FosB/ΔFosB cells per section within the MnPO following 7 days of CIH treatment. Significant increases were seen in FosB/ΔFosB positive cells and cells colocalized with both ACE1 with FosB/ΔFosB in the CIH treatment group as compared to normoxic controls. No significant differences were seen within ACE1 positive cells. *indicates $P<0.001$.

CHAPTER II-Figure II-4 ACE1 content within MnPO after CIH

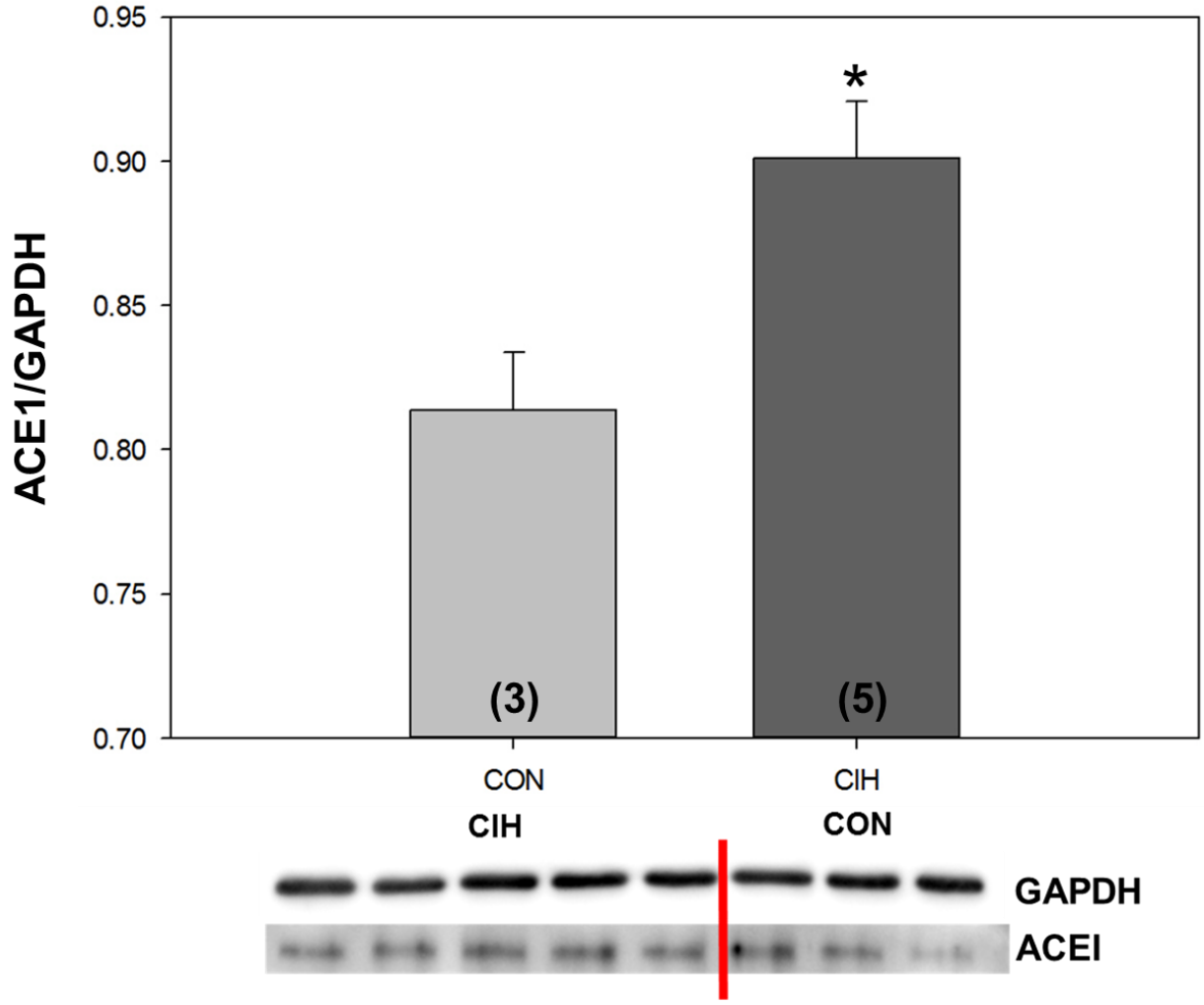


Figure 4. Western blot analysis of ACE! expression within the MnPO following normoxic (CON = 3) or hypoxic (CIH = 5) treatment. Densitometric analysis of ACE1 immunoreactivity was normalized to GAPDH. Data expressed as mean±SEM. * indicates P<0.05.

CHAPTER II-Figure II-5 Fluorogold PVN Injection and MnPO Counts

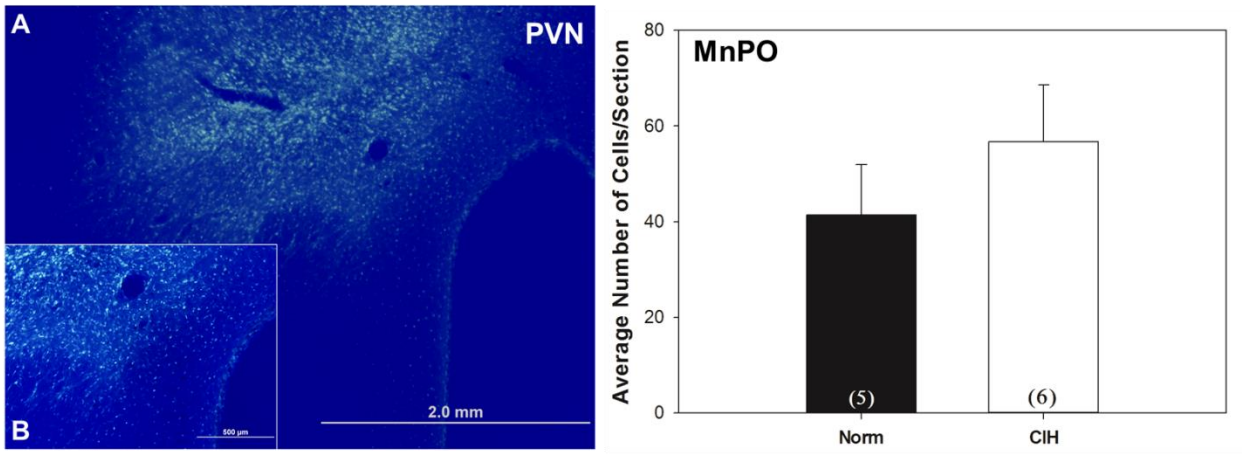
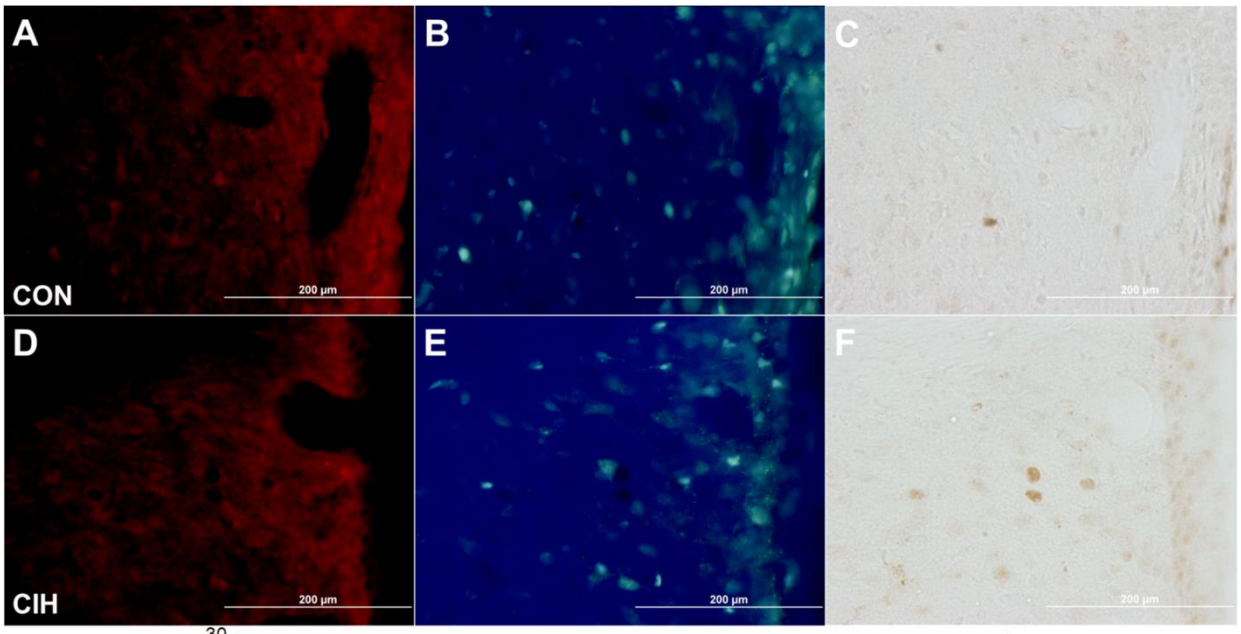


Figure 5. Left panel represents and image of a positive hit for Fluoroglod PVN unilateral injection. Right panel represents average number of fluorogold (Fgold) positive cells per section within the MnPO following CIH. No significant differences were found between normoxic controls (Norm) or hypoxic treatment (CIH; P=0.374).

CHAPTER II-Figure II-6 ACE1, Fluorogold and FosB Staining in MnPO and Counts Following CIH



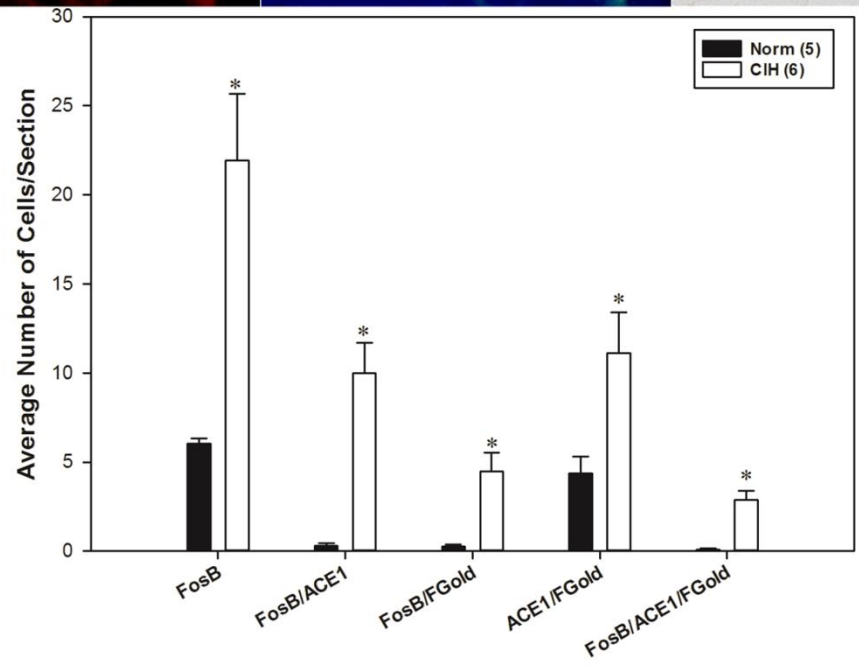


Figure 6. Top panel depicts ACE1 (A & D), Fluorogold (FGold) retrograde tracing from the PVN (B & E) and FosB/ΔFosB (C & F) expression within the MnPO. A, B and C represent normoxic treatment group (CON) while D,E and F represent CIH treatment group. Bottom panel represents average number of cells of FosB, ACE1 or Fgold expression in the MnPO following CIH. FosB/ΔFosB, ACE1, FosB/ΔFosB colocalization with Fgold, ACE1 colocalization with Fgold and colocalization of all three (ACE1, FosB/ΔFosB and Fgold) significantly increased within the MnPO following CIH as compared to normoxic controls (Norm). * indicates $P<0.05$.

CHAPTER II-Figure II-7 FosB Association with ACE1 Promotor Region

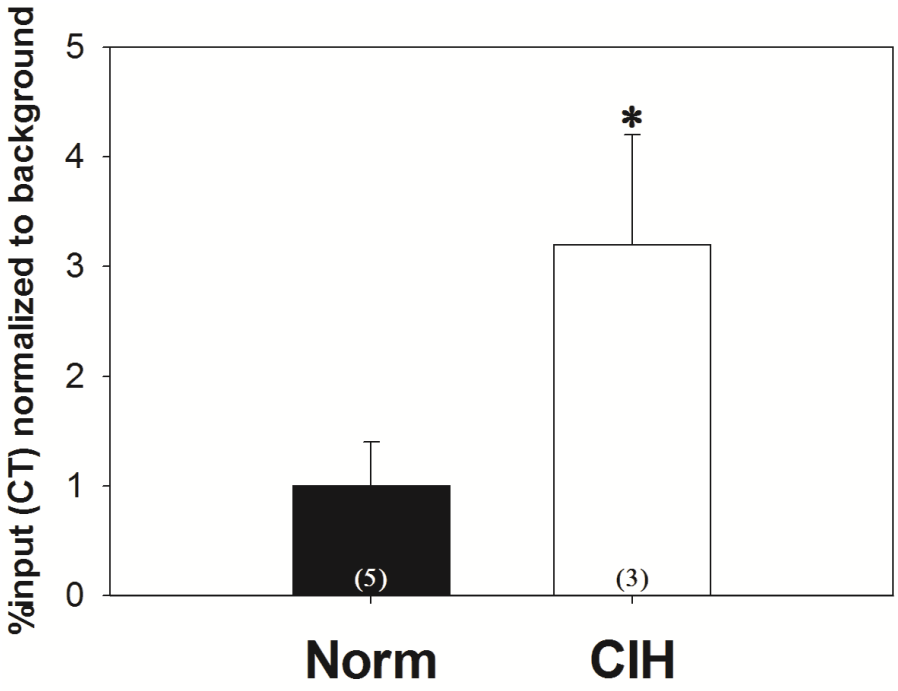


Figure 7. : FosB association with ACEI within the MnPO following 7 days of CIH. %input (CT) was normalized to background. 7 days of CIH significantly increased the association of FosB with ACEI within the MnPO as compared to normoxic controls (Norm). * indicates $P<0.05$.

DISCUSSION

Many neuronal adaptations have been implicated in animal models of CIH and our experiments focus on extending our understanding of the FosB/ΔFosB and RAS mechanisms within the CNS and how these mechanisms may contribute to the sustained diurnal MAP. ΔFosB has been shown to participate in long-term neural adaptations seen in many behaviors including drug addiction [46]. As part of the AP-1 transcription factor family, FosB/ΔFosB has been linked to sustained changes in gene expression due to its long half-life and accumulation with intermittent or repeated stimulation [46-49]. In CIH, FosB/ΔFosB significantly increases in many autonomic regions including the MnPO [25, 32]. Our previous work suggests that changes in gene expression that are mediated by FosB/ΔFosB in the MnPO play an essential role in the sustained component of CIH hypertension that occurs during normoxia [25]. The present study focused on one of these potential MnPO gene targets to better characterize its possible role in CIH hypertension. Additionally, retrograde tract tracing was also used to explore how FosB/ΔFosB activated neurons within the MnPO may influence the PVN. The main finding of this study is that CIH increases FosB/ΔFosB staining in ACE1 positive cells that project to the PVN. Furthermore, CIH significantly increases FosB/ΔFosB association with ACE1 in the MnPO suggesting that increases in ACE1 gene expression associated with CIH are due to the direct interaction of FosB/ΔFosB with ACE1. These findings are consistent with the hypothesis that FosB/ΔFosB regulation of ACE1 contributes to CIH hypertension possibly via the PVN, which may be a pathway for increasing SNA and thus sustained elevations of MAP.

ACE1 immunostaining was evident within the MnPO that did not overlap with GFAP. ACE1 expression failing to co-label with GFAP suggests ACE1 is not localized in astrocytes and may

be expressed by neurons in the MnPO. Also, ACE1 protein abundance significantly increased within the MnPO following 7 days of CIH treatment as compared to normoxic controls. These results are consistent with our earlier report that ACE1 gene expression was upregulated in the MnPO following CIH and that this effect was blocked by virally-mediated dominant negative inhibition of ΔFosB in the MnPO [25]. We have previously shown that CIH-induced FosB/ΔFosB staining in the MnPO is dependent on Angiotensin 1a receptors in the subfornical organ (SFO) [26]. Together these results indicate that activation of the peripheral RAS by CIH and the subsequent stimulation of the SFO leads to activation of the MnPO and FosB/ΔFosB-mediated upregulation of specific components of the brain RAS. This leads to the speculation that the peripheral RAS and brain RAS work together in concert to maintain CIH hypertension during normoxia. Further studies will be needed in order to fully understand the role of ACE1 within the MnPO and its involvement in the hypertension seen in CIH.

Furthermore, the MnPO has been implicated in various neural networks that regulate cardiovascular control and fluid and electrolyte balance [27, 50-52]. Activation of the MnPO appears to results in sympathoexcitation and increased blood pressure [28, 53]. The MnPO projects directly to the PVN, more specifically the parvocellular division of the PVN that in turn projects to the nucleus of the solitary tract, the rostral ventrolateral medulla, and sympathetic preganglionic neurons in the intermedial lateral column of the spinal cord [54-58]. The MnPO to PVN projection may very well regulate increases in SNA and MAP during CIH stimulation. FosB/ΔFosB positive cells significantly increased within the MnPO following CIH as compared to the normoxic controls which agree with our previous experiments [32]. The increase in FosB/ΔFosB coincides with the hypothesis that the MnPO is activated during CIH. ACE1 and FosB/ΔFosB

immunohistochemistry colocalization also significantly increased within the MnPO following CIH. This increase in FosB/ΔFosB and ACE1 colabeling may suggest CIH increases activation of MnPO neurons through the transcription factor FosB/ΔFosB and subsequently increases ACE1 which could change the strength of angiotensinergic synapses. ANGII has been shown to activate MnPO neurons that project to the PVN [28] and therefore increased FosB/ΔFosB activation of MnPO neurons containing ACE1 may project to the PVN following CIH. The results of our retrograde track tracing study from the PVN support this hypothesis.

As mentioned before, dominant-negative inhibition of ΔFosB in the MnPO blocked the increase in mRNA expression of ACE1 in CIH treated rats [25]. Also, the increase in colocalization of FosB/ΔFosB and ACE1 within the MnPO after CIH suggest a possible relationship between the two. In order to fully consider the potential for a FosB/ΔFosB and ACE1 interaction within the MnPO stimulated by CIH, ChIP experiments were conducted to determine a possible direct interaction between ACE1 and FosB/ΔFosB. Our results indicate that there was a significant increase in association between FosB/ΔFosB to the ACE1 gene region in the MnPO following 7 days of CIH. The increase in association suggests that an increase in FosB/ΔFosB activation of the MnPO targets the RAS, specifically ACE1, and that interaction may upregulate ACE1 expression. The upregulation of ACE1 within the MnPO then may go on to influence downstream regions, such as the PVN, and ultimately MAP. ACE1 was not the only gene affected by dominant-negative inhibition of ΔFosB within the MnPO and other genes may contribute to CIH hypertension in the MnPO along with ACE1. The other genes identified in this study included *map3K3, ace2*, *nos1*, and nos3 [25]. Overall, downstream regulation of ACE1 by FosB/ΔFosB within the MnPO may play an important role following CIH stimulation. However,

functional studies will be required to determine the importance of MnPO ACE1 to CIH hypertension.

PERSPECTIVES

OSA is associated with a sustained hypertension even in the absence of a hypoxia leading to increased risk for multiple cardiovascular diseases [12]. The increase in prevalence and cardiovascular health risks accompanying OSA makes it important to understand the underlying mechanisms of its associated hypertension. Previous studies have suggested the importance of the CNS as well as the brain RAS system that promotes various forms of hypertension and the sustained hypertension seen in CIH may be no different [34, 59]. This study suggests that FosB may utilize an AP-1 target gene, ACE1, in order to influence the PVN downstream of the MnPO in CIH. Investigating the role of ACE1 within the MnPO may provide a better understanding of the pathogenesis of CIH hypertension which could improve our ability to treat OSA hypertension.

REFERENCES

1. Hiestand DM, Britz P, Goldman M and Phillips B. Prevalence of symptoms and risk of sleep apnea in the US population: Results from the national sleep foundation sleep in America 2005 poll. *Chest*. 2006;130:780-6.
2. Peppard PE, Young T, Barnet JH, Palta M, Hagen EW and Hla KM. Increased prevalence of sleep-disordered breathing in adults. *Am J Epidemiol*. 2013;177:1006-14.
3. Nieto FJ, Young TB, Lind BK, Shahar E, Samet JM, Redline S, D'Agostino RB, Newman AB, Lebowitz MD and Pickering TG. Association of sleep-disordered breathing, sleep apnea, and hypertension in a large community-based study. Sleep Heart Health Study. *JAMA : the journal of the American Medical Association*. 2000;283:1829-36.
4. Somers VK, White DP, Amin R, Abraham WT, Costa F, Culebras A, Daniels S, Floras JS, Hunt CE, Olson LJ, Pickering TG, Russell R, Woo M, Young T, American Heart Association Council for High Blood Pressure Research Professional Education Committee CoCC, American Heart Association Stroke C, American Heart Association Council on Cardiovascular N and American College of Cardiology F. Sleep apnea and cardiovascular disease: an American Heart Association/american College Of Cardiology Foundation Scientific Statement from the American Heart Association Council for High Blood Pressure Research Professional Education Committee, Council on Clinical Cardiology, Stroke Council, and Council On Cardiovascular Nursing. In collaboration with the National Heart, Lung, and Blood Institute National Center on Sleep Disorders Research (National Institutes of Health). *Circulation*. 2008;118:1080-111.

5. Dempsey JA, Veasey SC, Morgan BJ and O'Donnell CP. Pathophysiology of sleep apnea. *Physiological reviews*. 2010;90:47-112.

6. Garpestad E, Katayama H, Parker JA, Ringler J, Lilly J, Yasuda T, Moore RH, Strauss HW and Weiss JW. Stroke volume and cardiac output decrease at termination of obstructive apneas. *J Appl Physiol (1985)*. 1992;73:1743-8.

7. McNicholas WT. Cardiovascular outcomes of CPAP therapy in obstructive sleep apnea syndrome. *American journal of physiology Regulatory, integrative and comparative physiology*. 2007;293:R1666-70.

8. Narkiewicz K, Wolf J, Lopez-Jimenez F and Somers VK. Obstructive sleep apnea and hypertension. *Current cardiology reports*. 2005;7:435-40.

9. Peppard PE, Young T, Palta M and Skatrud J. Prospective study of the association between sleep-disordered breathing and hypertension. *The New England journal of medicine*. 2000;342:1378-84.

10. Stoohs R and Guilleminault C. Cardiovascular changes associated with obstructive sleep apnea syndrome. *J Appl Physiol (1985)*. 1992;72:583-9.

11. Wolk R, Kara T and Somers VK. Sleep-disordered breathing and cardiovascular disease. *Circulation*. 2003;108:9-12.

12. Morrell MJ, Finn L, Kim H, Peppard PE, Badr MS and Young T. Sleep fragmentation, awake blood pressure, and sleep-disordered breathing in a population-based study. *American journal of respiratory and critical care medicine*. 2000;162:2091-6.

13. Parati G, Lombardi C and Narkiewicz K. Sleep apnea: epidemiology, pathophysiology, and relation to cardiovascular risk. *American journal of physiology Regulatory, integrative and comparative physiology*. 2007;293:R1671-83.

14. Phillips BG and Somers VK. Hypertension and obstructive sleep apnea. *Curr Hypertens Rep*. 2003;5:380-5.

15. Carlson JT, Hedner J, Elam M, Ejnell H, Sellgren J and Wallin BG. Augmented resting sympathetic activity in awake patients with obstructive sleep apnea. *Chest*. 1993;103:1763-8.

16. Smith ML, Niedermaier ON, Hardy SM, Decker MJ and Strohl KP. Role of hypoxemia in sleep apnea-induced sympathoexcitation. *Journal of the autonomic nervous system*. 1996;56:184-90.

17. Somers VK, Dyken ME, Clary MP and Abboud FM. Sympathetic neural mechanisms in obstructive sleep apnea. *The Journal of clinical investigation*. 1995;96:1897-904.

18. Young T, Peppard P, Palta M, Hla KM, Finn L, Morgan B and Skatrud J. Population-based study of sleep-disordered breathing as a risk factor for hypertension. *Archives of internal medicine*. 1997;157:1746-52.

19. Fletcher EC. Invited review: Physiological consequences of intermittent hypoxia: systemic blood pressure. *J Appl Physiol (1985)*. 2001;90:1600-5.

20. Fletcher EC. Sympathetic over activity in the etiology of hypertension of obstructive sleep apnea. *Sleep*. 2003;26:15-9.

21. Tamisier R, Gilmartin GS, Launois SH, Pepin JL, Nespoulet H, Thomas R, Levy P and Weiss JW. A new model of chronic intermittent hypoxia in humans: effect on ventilation, sleep, and blood pressure. *J Appl Physiol (1985)*. 2009;107:17-24.

22. Greenberg HE, Sica AL, Scharf SM and Ruggiero DA. Expression of c-fos in the rat brainstem after chronic intermittent hypoxia. *Brain research*. 1999;816:638-45.

23. Kc P, Balan KV, Tjoe SS, Martin RJ, Lamanna JC, Haxhiu MA and Dick TE. Increased vasopressin transmission from the paraventricular nucleus to the rostral medulla augments cardiorespiratory outflow in chronic intermittent hypoxia-conditioned rats. *The Journal of physiology*. 2010;588:725-40.

24. Buggy J, Fink GD, Haywood JR, Johnson AK and Brody MJ. Interruption of the maintenance phase of established hypertension by ablation of the anteroventral third ventricle (AV3V) in rats. *Clinical and experimental hypertension*. 1978;1:337-53.

25. Cunningham JT, Knight WD, Mifflin SW and Nestler EJ. An Essential role for DeltaFosB in the median preoptic nucleus in the sustained hypertensive effects of chronic intermittent hypoxia. *Hypertension*. 2012;60:179-87.

26. Saxena A, Little JT, Nedungadi TP and Cunningham JT. Angiotensin II type 1a receptors in subfornical organ contribute towards chronic intermittent hypoxia-associated sustained increase in mean arterial pressure. *American journal of physiology Heart and circulatory physiology*. 2015;308:H435-46.

27. Saper CB and Levisohn D. Afferent connections of the median preoptic nucleus in the rat: anatomical evidence for a cardiovascular integrative mechanism in the anteroventral third ventricular (AV3V) region. *Brain research*. 1983;288:21-31.

28. Stocker SD and Toney GM. Median preoptic neurones projecting to the hypothalamic paraventricular nucleus respond to osmotic, circulating Ang II and baroreceptor input in the rat. *The Journal of physiology*. 2005;568:599-615.

29. Stocker SD and Toney GM. Vagal afferent input alters the discharge of osmotic and ANG II-responsive median preoptic neurons projecting to the hypothalamic paraventricular nucleus. *Brain research*. 2007;1131:118-28.

30. Ferguson AV, Latchford KJ and Samson WK. The paraventricular nucleus of the hypothalamus - a potential target for integrative treatment of autonomic dysfunction. *Expert opinion on therapeutic targets*. 2008;12:717-27.

31. Stocker SD, Hunwick KJ and Toney GM. Hypothalamic paraventricular nucleus differentially supports lumbar and renal sympathetic outflow in water-deprived rats. *The Journal of physiology*. 2005;563:249-63.

32. Knight WD, Little JT, Carreno FR, Toney GM, Mifflin SW and Cunningham JT. Chronic intermittent hypoxia increases blood pressure and expression of FosB/DeltaFosB in central autonomic regions. *American journal of physiology Regulatory, integrative and comparative physiology*. 2011;301:R131-9.

33. Baltatu OC, Campos LA and Bader M. Local renin-angiotensin system and the brain--a continuous quest for knowledge. *Peptides*. 2011;32:1083-6.

34. Wright JW and Harding JW. The brain renin-angiotensin system: a diversity of functions and implications for CNS diseases. *Pflugers Arch*. 2013;465:133-51.

35. Aars H and Akre S. Effect of angiotensin on sympathetic nerve activity. *Acta physiologica Scandinavica*. 1968;74:134-41.

36. Barrett CJ, Ramchandra R, Guild SJ, Lala A, Budgett DM and Malpas SC. What sets the long-term level of renal sympathetic nerve activity: a role for angiotensin II and baroreflexes? *Circulation research*. 2003;92:1330-6.

37. DiBona GF. Central angiotensin modulation of baroreflex control of renal sympathetic nerve activity in the rat: influence of dietary sodium. *Acta physiologica Scandinavica*. 2003;177:285-9.

38. Matsukawa T, Gotoh E, Minamisawa K, Kihara M, Ueda S, Shionoiri H and Ishii M. Effects of intravenous infusions of angiotensin II on muscle sympathetic nerve activity in humans. *The American journal of physiology*. 1991;261:R690-6.

39. Yoshimoto M, Miki K, Fink GD, King A and Osborn JW. Chronic angiotensin II infusion causes differential responses in regional sympathetic nerve activity in rats. *Hypertension*. 2010;55:644-51.

40. Lim K, Burke SL, Moretti JL and Head GA. Differential activation of renal sympathetic burst amplitude and frequency during hypoxia, stress and baroreflexes with chronic angiotensin treatment. *Exp Physiol*. 2015;100:1132-44.

41. Paxinos G, Watson CR and Emson PC. AChE-stained horizontal sections of the rat brain in stereotaxic coordinates. *Journal of neuroscience methods*. 1980;3:129-49.

42. Knight WD, Saxena A, Shell B, Nedungadi TP, Mifflin SW and Cunningham JT. Central losartan attenuates increases in arterial pressure and expression of FosB/DeltaFosB along the autonomic axis associated with chronic intermittent hypoxia. *American journal of physiology Regulatory, integrative and comparative physiology*. 2013;305:R1051-8.

43. Carreno FR, Ji LL and Cunningham JT. Altered central TRPV4 expression and lipid raft association related to inappropriate vasopressin secretion in cirrhotic rats. *American journal of physiology Regulatory, integrative and comparative physiology*. 2009;296:R454-66.

44. Saxena A, Bachelor M, Park YH, Carreno FR, Nedungadi TP and Cunningham JT. Angiotensin II induces membrane trafficking of natively expressed transient receptor potential vanilloid type 4 channels in hypothalamic 4B cells. *American journal of physiology Regulatory, integrative and comparative physiology*. 2014;307:R945-55.

45. Prabhakar NR and Kumar GK. Mechanisms of sympathetic activation and blood pressure elevation by intermittent hypoxia. *Respiratory physiology & neurobiology*. 2010;174:156-61.

46. McClung CA, Ulery PG, Perrotti LI, Zachariou V, Berton O and Nestler EJ. DeltaFosB: a molecular switch for long-term adaptation in the brain. *Brain research Molecular brain research*. 2004;132:146-54.

47. Nestler EJ. Molecular basis of long-term plasticity underlying addiction. *Nature reviews Neuroscience*. 2001;2:119-28.

48. Chen J, Kelz MB, Hope BT, Nakabeppu Y and Nestler EJ. Chronic Fos-related antigens: stable variants of deltaFosB induced in brain by chronic treatments. *The Journal of neuroscience : the official journal of the Society for Neuroscience*. 1997;17:4933-41.

49. Hope BT, Nye HE, Kelz MB, Self DW, Iadarola MJ, Nakabeppu Y, Duman RS and Nestler EJ. Induction of a long-lasting AP-1 complex composed of altered Fos-like proteins in brain by chronic cocaine and other chronic treatments. *Neuron*. 1994;13:1235-1244.

50. Johnson AK, Cunningham JT and Thunhorst RL. Integrative role of the lamina terminalis in the regulation of cardiovascular and body fluid homeostasis. *Clinical and experimental pharmacology & physiology*. 1996;23:183-91.

51. McKinley MJ, Gerstberger R, Mathai ML, Oldfield BJ and Schmid H. The lamina terminalis and its role in fluid and electrolyte homeostasis. *J Clin Neurosci*. 1999;6:289-301.

52. Westerhaus MJ and Loewy AD. Sympathetic-related neurons in the preoptic region of the rat identified by viral transneuronal labeling. *J Comp Neurol*. 1999;414:361-78.

53. Llewellyn T, Zheng H, Liu X, Xu B and Patel KP. Median preoptic nucleus and subfornical organ drive renal sympathetic nerve activity via a glutamatergic mechanism within the paraventricular nucleus. *American journal of physiology Regulatory, integrative and comparative physiology*. 2012;302:R424-32.

54. Sawchenko PE and Swanson LW. The organization of forebrain afferents to the paraventricular and supraoptic nuclei of the rat. *J Comp Neurol*. 1983;218:121-44.

55. Haselton JR, Goering J and Patel KP. Parvocellular neurons of the paraventricular nucleus are involved in the reduction in renal nerve discharge during isotonic volume expansion. *J Auton Nerv Syst*. 1994;50:1-11.

56. Kannan H and Yamashita H. Connections of neurons in the region of the nucleus tractus solitarius with the hypothalamic paraventricular nucleus: their possible involvement in neural control of the cardiovascular system in rats. *Brain research*. 1985;329:205-12.

57. Patel KP and Schmid PG. Role of paraventricular nucleus (PVH) in baroreflex-mediated changes in lumbar sympathetic nerve activity and heart rate. *J Auton Nerv Syst*. 1988;22:211-9.

58. Shafton AD, Ryan A and Badoer E. Neurons in the hypothalamic paraventricular nucleus send collaterals to the spinal cord and to the rostral ventrolateral medulla in the rat. *Brain research*. 1998;801:239-43.

59. Shell B, Faulk K and Cunningham JT. Neural Control of Blood Pressure in Chronic Intermittent Hypoxia. *Current hypertension reports*. 2016;18:19.

CHAPTER III

THE CRITICAL ROLE OF ANGIOTENSIN CONVERTING ENZYME 1 WITHIN THE MEDIAN PREOPTIC NUCLEUS IN THE DEVELOPMENT OF THE SUSTAINED HYPERTENSION SEEN IN CHRONIC INTERMITTENT HYPOXIA.

INTRODUCTION

The occurrence of sleep apnea has steadily become a concern within the healthcare community. Mild obstructive sleep apnea (OSA), an apnea-hypopnea-index (AHI) of 5 or greater, may be as frequent as 28% while the prevalence of moderate OSA, an AHI of 15 or greater, may be as high as 14% [1]. Furthermore, the prevalence of sleep apnea has seen a steady increase, some as much as 55%, in many populations [2]. OSA has been well associated with hypertension and many other cardiovascular vascular diseases such as heart failure [3-14]. More specifically, OSA patients have a sustained waking hypertension seen in absence of an apnea or hypopnea event [15]. In addition to the sustained hypertension seen in OSA patients, increased sympathetic nerve activity (SNA) has also been demonstrated within this disease group [16-19]. The increase in SNA suggests a possible central nervous system (CNS) role in the sustained hypertension and adverse cardiovascular outcomes related to sleep apnea in OSA patients.

In order to model certain components of sleep apnea, chronic intermittent hypoxia (CIH) is widely used. CIH specifically models the arterial hypoxemia seen in sleep apnea patients [20]. CIH not only simulates the arterial hypoxemia from sleep apnea but the model also produces similar changes in SNA and develops sustained diurnal hypertension [21-23]. Chemoreflex augmentations as well as many other mechanisms such as changes in vasopressin transmission to the brainstem have been recognized as credible avenues for the hypertension seen in CIH [4, 24-26]. The role of the CNS in hypertension has also expanded to other areas within the CNS such as the lamina terminalis which has been implicated in many models neurogenic hypertension [27]. The lamina terminalis has similarly been associated with the hypertension seen in CIH [28, 29]. This area consists of the subfornical organ (SFO), organum vasculosum of the lamina terminalis (OVLT) and the median preoptic nucleus (MnPO). The MnPO receives inputs from the SFO and OVLT, both of which are circumventricular organs containing a leaky blood-brain-barrier [30-33]. The MnPO has projections to downstream regions, such as the paraventricular nucleus (PVN), that are known to regulate SNA as well as blood pressure [34-38]. The MnPO may be one nucleus within the CNS where homeostatic uncoupling takes place that contributes to an inappropriately sustained diurnal hypertension following CIH.

The neuronal activation of the MnPO has been linked to the sustained hypertension seen in CIH [28, 39]. Specifically the MnPO increases the expression of a chronic activation marker, FosB/ΔFosB[28, 39]. FosB/ΔFosB is a member of the AP-1 transcription factor family and expressed following CNS activation [40-42]. Inhibition of FosB/ΔFosB within the MnPO has been shown to selectively attenuate the sustained hypertension seen in CIH therefore suggesting a possible regulatory role of FosB/ΔFosB in altering downstream AP-1 genes within the MnPO [28].

Several genes were identified as possible downstream targets of FosB/ΔFosB within the MnPO following CIH and one or a combination of the genes may be responsible for the sustained CIH hypertension [28]. One putative target gene identified in the previous study is angiotensin converting enzyme 1 (ACE1)[28], a recognized part of the renin angiotensin system (RAS). The RAS has been shown to contribute to hypertension associated with CIH [43-46] and in OSA patients [47, 48]. Furthermore, the CNS is known to have a complete RAS that independently functions from the systemic RAS [49, 50]. ACE1 converts angiotensin I to angiotensin II (ANGII), a known neurotransmitter within the CNS that affects SNA [51-53]. We hypothesized that ACE1 within the MnPO plays a functional role in the sustained hypertension seen in CIH. In order to fully understand the role of ACE1 within the MnPO, viral vectors containing short hairpin RNA (shRNA) against ACE1 were used to knockdown ACE1 within the MnPO. Additionally, the downstream regions of the MnPO known for SNA regulation were assessed using the neuronal activation marker, FosB/ΔFosB. Understanding the functional role of ACE1 within the MnPO and the effects on downstream nuclei would give insight into potential mechanisms for OSA hypertension treatment.

METHODS

Animals

Adult male Sprague-Dawley rats of 6 weeks in age (Charles River Laboratories, Inc., Wilmington, MA, USA) were used for all studies. All rats were on a 12:12 light/dark cycle with a light period of 0700 to 1900 h. Rats were always individually housed and continuously provided with food and water ad libitum in temperature controlled rooms. All animal procedures were conducted according to National Institutes of Health guidelines and were approved by the Institutional Animal Care and Use Committee at the University of North Texas Health Science Center.

Virally-Mediated Gene Transfer

Rats were anesthetized with isoflurane (2-3%), their scalps shaved and cleaned, and then they were each placed in a stereotaxic apparatus. After their skulls were surgically exposed they were leveled between bregma and lambda [54]. Each rat received a microinjection targeted to the MnPO using atlas-defined coordinates of +0.9mm (midline) and -6.7mm (dorsal/ventral) with the injector placed at 8 degrees from midline [54]. After a small burr hole was made into the skull a 30 gauge injector was advanced to the aforementioned coordinates, each construct was injected into the MnPO at a volume of 500 ηl using 5 μL Hamilton syringe (#84851 Hamilton Reno, NV). After microinjection of the viral construct, a period of 5 minutes elapsed before the injector was removed from the defined coordinates in order to allow for proper absorption of the chosen AAV construct. The holes made in each skull was filled with sterile gel foam followed by closue of their scalps with sterile absorbable suture The injected viral constructs (GENEDETECT®, Auckland, NZ) were used to constitutively express an shRNA against ACE1 with green

fluorescent protein (GFP) (AAV-shACE1, titer: $1.5x10^{12}$ genomic particles/ml) or a scrambled shRNA with GFP (AAV-shSCM, titer: $1.1x10^{12}$ genomic particles/ml). One week of recovery was given before the telemetry procedure and another week of recovery prior to starting our CIH protocol at baseline approximately totaling a 3 week incubation period for the viral construct expression proceeding the first day of CIH treatment. Injection placement was verified by the GFP fluorescence using an Olympus (Olympus BX41) fluorescent microscope

Radio Telemetry Transmitter Implantation

Mean arterial blood pressure (MAP), respiratory rate (RR) and heart rate (HR) was monitored by a Dataquest radio-telemetry system (Data Sciences International, St. Paul, MN, USA) using a radio-telemetry transmitter (TA11PA-C40 DSI telemetry unit). After recovering from the MnPO microinjections for 1 week, rats were anesthetized with isoflurane (2-3%) anesthesia and implanted with an abdominal aortic catheter attached to the radio-telemetry transmitter that was secured to abdominal muscle, as described previously [39]. One week of recovery was given before the 5 day baseline measurements began followed by the 7 day CIH protocol. . All physiological measurements were monitored by this radio telemetry system and sampled for 10 seconds every 10 minutes from the first full day of baseline measurements until end of the CIH protocol. Form some analyses, data are expressed as changes from baseline.

Chronic Intermittent Hypoxia Treatment

Rats were transferred into plexiglass chambers and left at room air for 5 days of baseline measurements. For the next 7 days, CIH exposure was applied for 8 hs during the middle of the light phase of a rat using a 3 min hypoxia (10% O_2)/3 min normoxia (21% O_2) cycle as described previously [39]. During the remaining time (1600-0800), the chambers were open to room air. Normoxic controls were placed in the same room but only exposed room air (21% O_2)

throughout the CIH protocol. On the morning after the 7th day of CIH, rats were sacrificed. Rats were given inactin (100 mg/kg ip) and either perfused transcardially for immunohistochemistry or decapitated for PCR.

Laser Capture Microscopy

Rats were decapitated after anesthetization with inactin (100 mg/kg ip). Brains were immediately frozen in 2-methylbutane (Sigma-Aldrich) on dry ice and stored at -80°C until RNA extractions were performed. Fresh frozen brains were prepared for laser capture microscopy (LCM) by cutting 10 µm thick serial frozen sections at the level of the MnPO. Six sections were mounted onto PEN membrane coated slides (Catalogue# LCM0522- Arcturus Bioscience) and fixed with methanol for 30 seconds. An Arcturus Veritas Microdissection instrument (13553-00, version c), which utilizes an infrared capture laser with an ultraviolet cutting laser, was used to capture 10-15 GFP labeled MnPO neurons. As previously described, RNA was extracted and purified from each sample using an ArrayPure Nano-Scale RNA Purification Kit (Epicentre Biotechnol, Madison, WI) [55]. RNA quality was evaluated using a Nanodrop Spectophotometer (Nanodrop 2000c Spectrophotometer, ThermoScientific, Wilmington, DE) and low 260/280 sample ratios were not used because of impurity. The RNA was then amplified to aminoallyl a-RNA using the TargetAmp 2-Round Aminoallyl-aRNA Amplification Kit (epicenter Biotechnol, Madision, WI) as previously described [55-57].

Quantitative RT-PCR

Aminoallyl-aRNA from each sample was reverse-transcribed into cDNA with a Sensiscript RT Kit (Qiagen Inc., Valencia, CA) as previously described [44]. S18 and ACE1 primers weres used with the following sequences: Rsp18, forward 5'-CAGAAGGACGTGAAGGATGG-3' and reverse 5'-CAGTGGTCTTGGTGTGCTGA-3'; ACE1, forward 5'-

CCCGGAAATACGAAGAATTGC-3' and reverse 5'-GGCTCTCCCCACCTTGTCTC-3'. Rps18 was used for normalization of mRNA expression. PCR samples contained 3 µL of cDNA, 1.2 µL of each primer (forward and reverse), 3.3 µL of Rnase-free water, and 7.5 µL of iQ SYBR Green Supermix (Bio-Rad Laboratories Inc., Hercules, CA). PCR reactions were performed with the following protocol in a Bio-Rad iQTM5 iCycler system (Bio-Rad Laboratories Inc., Hercules, CA): Denaturation at 95°C for 3 minutes, 95°C for 10 seconds followed by 60°C for 1 minute (1 min 10 sec total) repeated for 50 cycles and then 65°C for 5 seconds. In each analysis, melt-curves were generated in order to identify non-specific products and primer-dimers. Data were analyzed using the $2^{-\Delta\Delta CT}$ method as reported previously from our laboratory [55-57].

Immunohistochemistry

Following the 7 day CIH protocol, rats were given inactin (100 mg/kg ip) and sacrificed. Rats were then perfused with 0.1 M phosphate buffer saline (PBS, 100-200 ml) followed by 4% PFA (400-500 ml), as previously described [28]. Brains were post fixed overnight and dehydrated in 30% sucrose. Each brain was cut in to three sets of serial 40 µm coronal sections using a crytostat. The sections were stored in cryoprotectant at -20°C until immunohistochemistry was performed. Sections were stained, as previously described [39, 46], for FosB (Goat polyclonal, Santa Cruz, 1:1000) and if staining the hindbrain tissue, dopamine β hydroxylase (DβH) (Mouse monoclonal, Millipore, 1:1000) was used. The FosB antibody from SantaCruz does not distinguish between to variants of FosB, FosB and the more stable splice variant ΔFosB so will therefore be indicated as FosB/ΔFosB staining. Tissue processed for DAB staining was incubated with a biotinylated horse anti-goat IgG (1:200; Vector Laboratories, Burlingame, CA) and treated with an avidin-peroxidase conjugate from a Vectastain ABC Kit (Vector

Laboratories). Tissue was then followed by PBS containing 0.04% 3,3′-diaminobenzidine hydrochloride and 0.04% nickel ammonium sulfate for 11 minutes. DβH staining was visualized using a Cy3 anti-mouse (1:1200; Jackson ImmunoResearch Inc., West Grove, PA). After the staining procedure, tissue was then mounted to gel-coated slides, allowed to dry for one day. The dried slides were serially dehydrated with ethanol solutions and xylene. Slides were then coverslipped with Permount mounting medium (ThermoScientific, Waltham, MA, USA) and dried for at least 48 hours before imaging. Tissue was imaged using an epifluorescent Olympus (Olympus BX41) microscope equipped with a digital camera (Olympus DP70). Image J was used to analyze and count labeled cells for each section. FosB/ΔFosB counts were averaged between sections of each brain region with the NTS subsections separately analyzed as into commissural/caudal NTS, subpostremall NTS and rostral NTS.

Statistical Analysis

Data from the immunohistochemistry were analyzed using one-way ANOVA with *Student-Newman-Keuls* tests for posthoc analysis. qRT-PCR studies were analyzed with two-way ANOVA with *Student-Newman-Keuls* tests for posthoc analysis. Baseline data from the radio telemetry recordings were analyzed either analyzed using one-way ANOVA with *Student-Newman-Keuls* tests for posthoc analysis. Data from the 7 d CIH protocol were analyzed as a change from baseline using separate two-way repeated measure ANOVA with *Student-Newman-Keuls* tests for posthoc analysis. All tests were performed using SigmaPlot (v. 12.0, Systat Software, USA). Statistically significant at was considered when $P < 0.05$. Data are reported as mean ± SEM.

RESULTS

Role of MnPO ACE1 in CIH Hypertension

Verification of ACE1 Knockdown in the MnPO. Viral constructs were used to knockdown ACE1 within the MnPO to aid in the investigation of ACE1 in the MnPO following CIH. The AAV-shSCM and AAV-shACE1 injections in the MnPO of rats treated with normoxia did not show any significant changes in ACE1 mRNA expression ($P<0.05$, Figure 2). Correspondingly, ACE1 mRNA expression significantly increased transfected MnPO cells collected from rats in the AAV-shSCM CIH treatment group as compared to the AAV-shSCM normoxic, AAV-shACE1 normoxic and the AAV-shACE1 CIH treatment groups ($P<0.05$, Figure 2). Furthermore, ACE1 mRNA expression increased within the AAV-shACE1 CIH treatment group as compared to the AAV-shSCM and AAV-shACE1 normoxic groups ($P<0.05$, Figure 2). However, the AAV-shACE1 within the CIH group showed a significant decrease of ACE1 mRNA expression as compared to the AVV-shSCM CIH group when normalized to Rsp18 ($P<0.05$, Figure 2). The AAV-shACE1 significantly blocked the increase in MnPO ACE1 message that was seen in the CIH treated rats injected with AAV-shSCM.

Effect of ACE1 Knockdown on Baseline MAP, RR and HR. 5 days prior to the first day of CIH exposure, baseline measurements were taken. There were no significant differences in baseline mean blood pressure, RR or HR measurements in both the normoxic dark phase and CIH exposure (0800-1600 h) time periods among any of the groups (Table 1).

Effect of ACE1 Knockdown on Mean Arterial Blood Pressure. During the CIH exposure (0800-1600 h), the observed changes in MAP were significantly influenced by the treatments but also

dependent on the day. MAP significantly increased in the AAV-shSCM CIH treatment group as expected when compared to the AAV-shSCM normoxic control ($P<0.05$, Figure 3). Also, the AAV-shSCM CIH treatment group significantly increased MAP from D2 through D7 as compared to the AAV-shACE1 normoxic group ($P<0.05$, Figure 3). The AAV-shSCM CIH group significantly had increased MAP on day D2 and D7 as compared to the AAV-shACE1 CIH treatment group ($P<0.05$, Figure 3). On days D1, D3, D4 and D6, the AAV-shACE1 CIH treatment group showed significantly increased MAP as compared to the AAV-shACE1 normoxic control ($P<0.05$, Figure 3). Lastly, the AAV-shACE1 CIH treatment group significantly increased MAP on IH3 and IH4 as compared to AAV-shSCM normoxic control group ($P<0.05$, Figure 3).

During the normoxic dark phase, changes in MAP were significantly affected by treatment independent of the protocol day. The changes in MAP of the AAV-shSCM CIH treatment group were significantly increased as compared to all other groups $P<0.05$, (Figure 3). There were no significant differences among the other three treatment groups: AAV-shSCM normoxic, AAV-shACE1 normoxic, and AAV-shACE1 CIH ($P<0.05$, Figure 3). These results indicate that ACE1 knockdown in the MnPO significantly blocked the sustained component of CIH hypertension that occurs during the normoxic dark phase, but does not prevent increases in blood pressure that occur during CIH exposure (0800-1600 h).

Effect of ACE1 Knockdown on Respiration Rate. During intermittent hypoxia exposure, the effects on RR were influenced by the treatments and day. There was no significant differences between the AAV-shACE1 and AAV-shSCM normoxic controls. A significant increase in RR was seen in the AAV-shSCM CIH group on days D1 and D4 as compared to the AAV-shSCM

and AAV-shACE1 normoxic control groups (P<0.05, Figure 4). Similarly, RR was significantly increased in the AAV-shACE1 CIH treatment group days D3, D4, D and D7 as compared to both AAV-shACE1 and AAV-shSCM normoxic treatment groups (P<0.05, Figure 4). Finally, on day D1, AAV-shSCM CIH was significantly increased as compared to the AAV-shACE1 CIH treatment group while on day D6 the AAV-shACE1 CIH treatment group was significantly higher than the AAV-shSCM CIH treatment group (P<0.05, Figure 4).

During the dark period with no active intermittent hypoxia exposure, RR was not different among all treatment groups (Figure 4).

Effect of ACE1 Knockdown on Heart Rate. During the intermittent hypoxia treatment period (0800-1600 h), HR showed no significant differences amongst treatment accept on the very last day of hypoxia treatment, D7. The AAV-shACE1 CIH treatment group had a significant decrease in HR as compared all other treatment groups (P<0.05, Figure 4).

While in the dark phase, AAV-shSCM and AAV-shACE1 CIH treatment groups showed significant decreases in HR as compared to both the AAV-shSCM and AAV-shACE1 normoxic control groups (P<0.05, Figure 4).

Effects of MnPO ACE1 in CIH on FosB Staining

Effect of ACE1 knockdown on FosB/ΔFosB staining in MnPO. FosB/ΔFosB staining within the MnPO was significantly increased in the AAV-shSCH CIH treatment group as compared to AAV-shSCM normoxic, AAV-shACE1 normoxic and AAV-shACE1 CIH treatment groups (P<0.05, Figure 5). In the AAV-shACE1 CIH treatment group, the numbers of FosB/ΔFosB positive cells in the MnPO were significantly increased with as compared to its AAV-shACE1

normoxic control counterpart (P<0.05, Figure 5). However, FosB/ΔFosB staining significantly decreased within the MnPO in the normoxic AAV-shACE1 group as compared to the AAV-shSCM normoxic group (P<0.05, Figure 5). These results indicated that ACE1 knockdown in the MnPO signficantly reduced FosB/ΔFosB staining in this region.

Effect of ACE1 Knockdown on FosB/ΔFosB staining in PVN. FosB/ΔFosB positive cells within the PVN was comparable between both AAV-shSCM and AAV-shACE1 normoxic controls (P>0.05, Figure 6). Both AAV-shSCM and AAV-shACE1 CIH treatment groups increased FosB/ΔFosB staining within the PVN significantly as compared to both normoxic controls of AAV-shSCM and AAV-shACE1 (P<0.05, Figure 6). Interestingly FosB/ΔFosB positive cells significantly decreased within the PVN following AAV-shACE1 microinjection into the MnPO following CIH treatment (P<0.05, Figure 6). Although CIH significantly increased FosB/ΔFosB staining in the PVN of rats injected in the MnPO with AAV-shACE1, ACE1 knockdown in the MnPO significantly attenuated this effect.

Effect of ACE1 Knockdown on FosB/ΔFosB staining in Hindbrain. In rats injected in the MNPO with AAV-shSCM and exposed to CIH, there was a significant increase in FosB/ΔFosB staining within the RVLM as compared to both normoxic treatment groups, as well as the AAV-shACE1 CIH treatment group (P<0.05, Figure 11). In contrast, ACE1 knockdown in the MnPO did not significantly influence FosB/ΔFosB staining in the NTS. The total number of FosB/ΔFosB positive cells within the entire NTS significantly increased in both CIH groups whether AAV-shSCM or AAV-shACE1 as compared to both normoxic controls (P<0.05, Figure 10). Similar increases in both CIH groups were seen in the subpostremal and caudal subdivisions of the NTS which showed a significant increase in CIH treated groups as compared to the normoxic

counterparts ($P<0.05$, Figures 8 and 9). However there was no significant difference amongst any treatment group within the rostral subdivision of the NTS (Figure 7).

Figures

CHAPTER III-Figure III-1 MnPO Microinjection of Viral Constructs

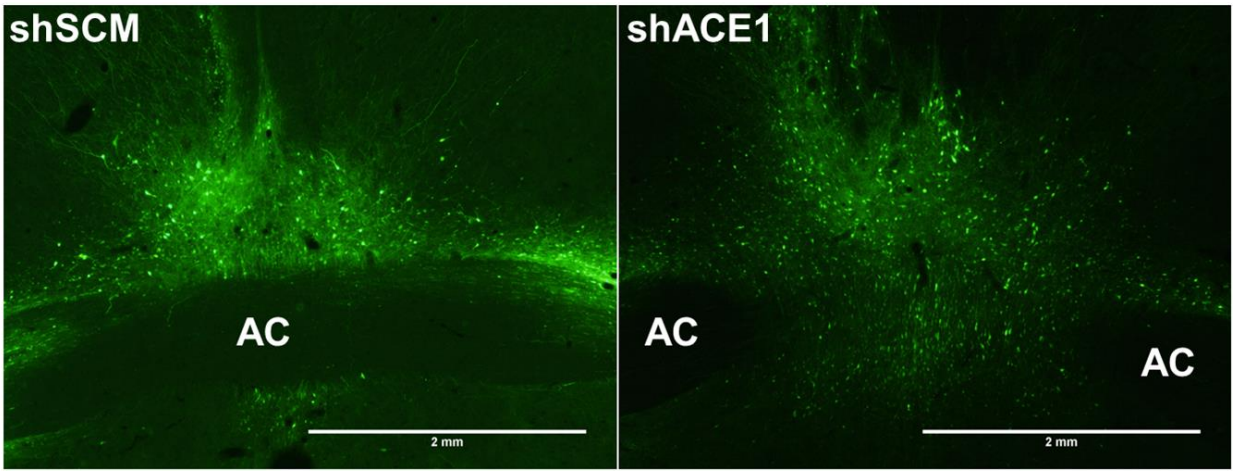


Figure 1. Representative successful viral construct microinjection hits within the MnPO. shSCM is the control scramble RNA contrast on the left while the shACE1 shRNA against ACE1 in on the right. Both are tihint he MnPO. (AC, anterior commissure).

CHAPTER III-Figure III-2 Effect of shACE1 on ACE1 mRNA in CIH

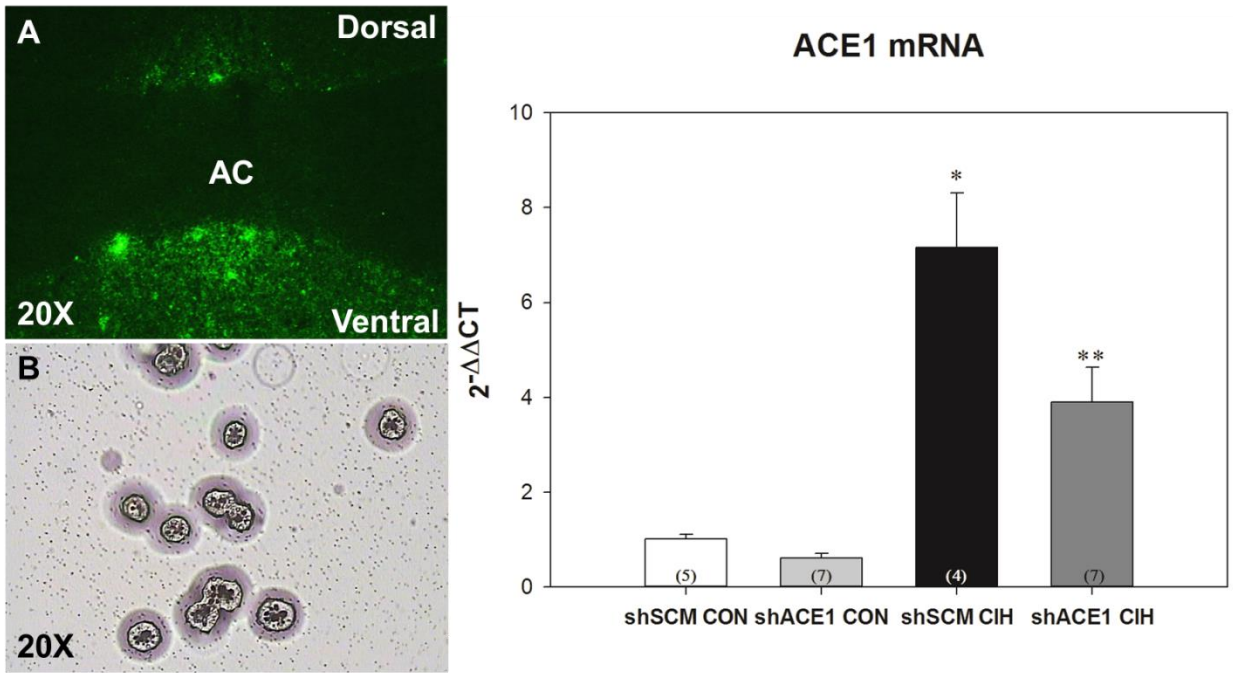


Figure 2. A & B depict fluorescent GFP reporter expressing cells within the MnPO before laser capture microdissection (LCM) and collected GFP cells on a LCM cap in brightfield respectively. AAV-shACE1 costruct successfully attenuated the 8 fold ACE1 mRNA increase following CIH within the MnPO by nearly half. (AC, anterior commissure). ($P<0.05$)

CHAPTER III-Figure III-3 Knockdown of ACE1 within the MnPO and its Effect on MAP in CIH

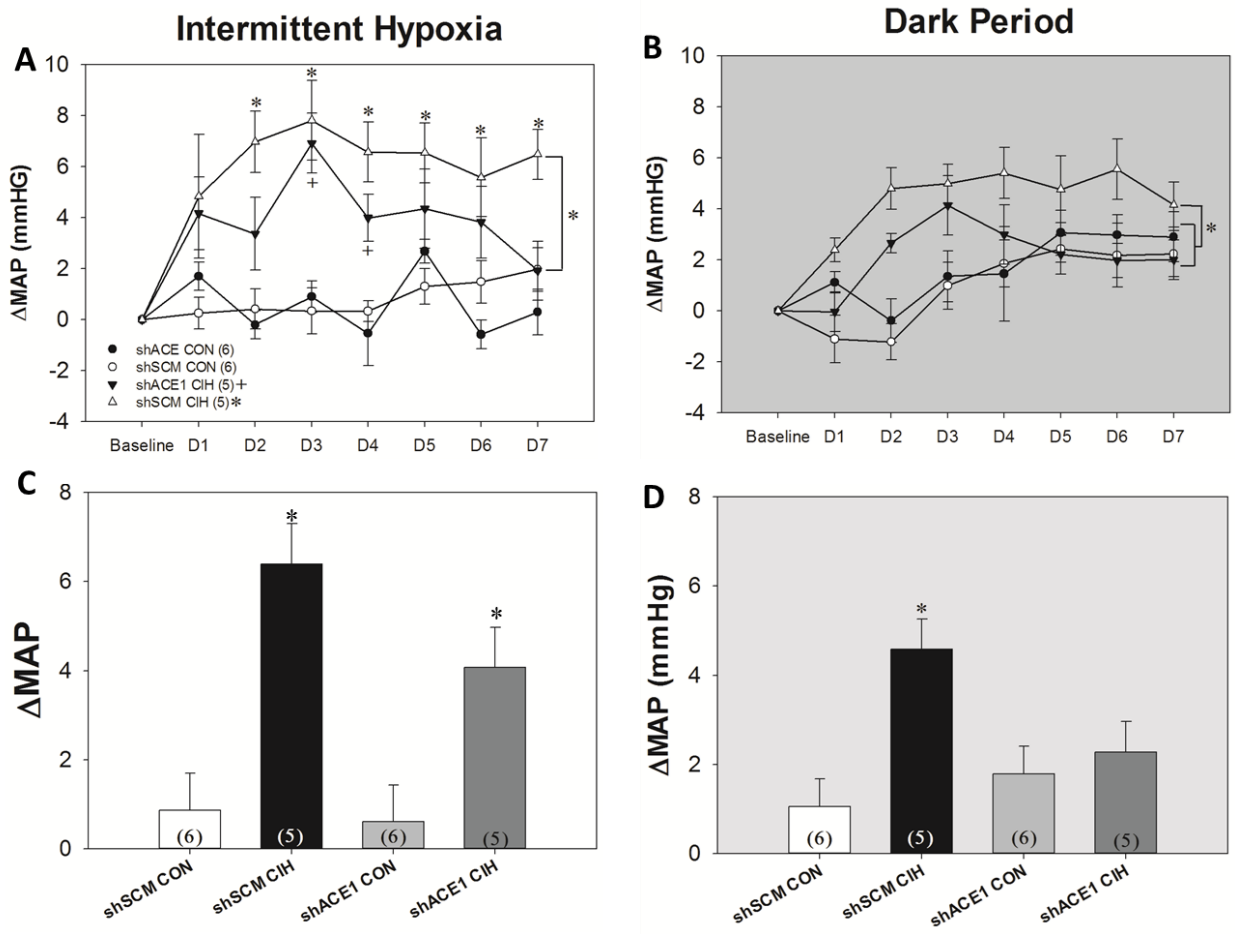


Figure 3. Knockdown of ACE1 in the MnPO successfully attenuated the sustained component of CIH hypertension (B & D). The shSCM CIH group did increase MAP during both intermittent hypoxia (IH) and dark period (DK) times as compared to controls (A & B). shSCM CIH did significantly increase as compared to normoxic controls (*P<0.05) in both IH and DK time periods (A & B). shACE1 CIH did increase MAP in the IH time period as compared to normoxic but the effect was dependent on the day of CIH treatment (+P<0.05, A & C).

CHAPTER III-Figure III-4 Knockdown of ACE1 within the MnPO and its Effect on HR and RR in CIH

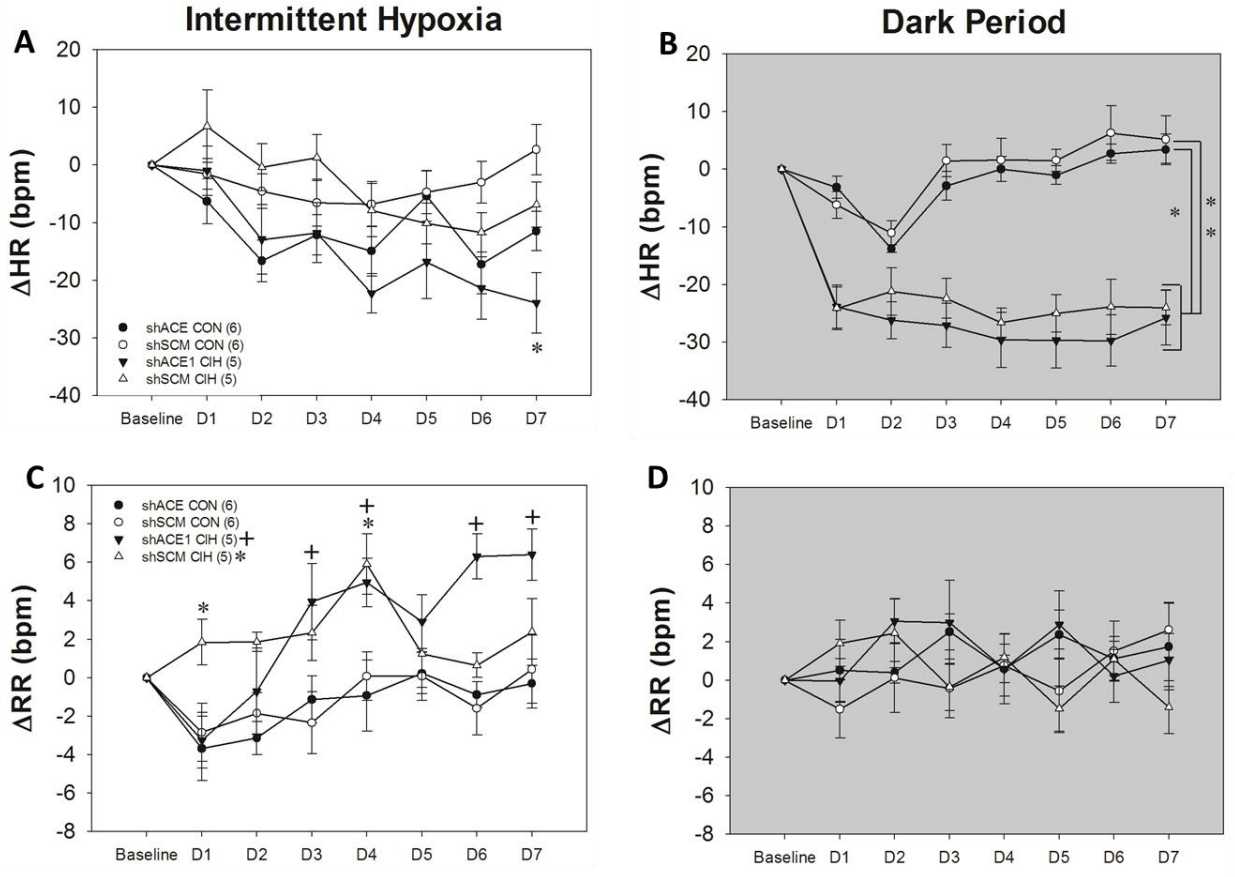


Figure 4. Knockdown of ACE1 in the MnPO did not change HR in the intermittent hypoxia (IH) time period until the very last day (D7) when the shACE1 CIH group was significantly decreased (A, *P<0.05). CIH treatment did significantly derease HR overall during the dark period (DK) time (B,*,** P<0.05). CIH treatment did increase in RR in both CIH treatment groups regardless of the viral construct used but this effect was dependent on the day of treatment (C, *, +P<0.05). RR did not significantly change amongst all groups during the DK time.

CHAPTER III-Table III-1 Baseline Measurements of MAP, HR and RR

Group	n	Time Period	MAP (mmHg)	HR (bpm)	RR (bpm)
shSCM CON	6	IH	94±3	325±9	101±2
		DK	101±4	387±7	100±2
shACE1 CON	6	IH	90±2	323±6	97±2
		DK	96±3	393±12	96±1
shSCM CIH	5	IH	93±2	322±13	96±2
		DK	98±2	383±12	98±1
shACE1 CIH	5	IH	90±2	318±6	98±2
		DK	95±2	377±6	97±2

Table 1. Baseline measurements across shSCM (Sc), shACE1 (ACE), normoxic (N) and CIH treatment groups for MAP, HR and RR. No significant differences were found amonst baseline measurements of all treatment groups.

CHAPTER III-Figure III-5 MnPO FosB/ΔFosB Staining

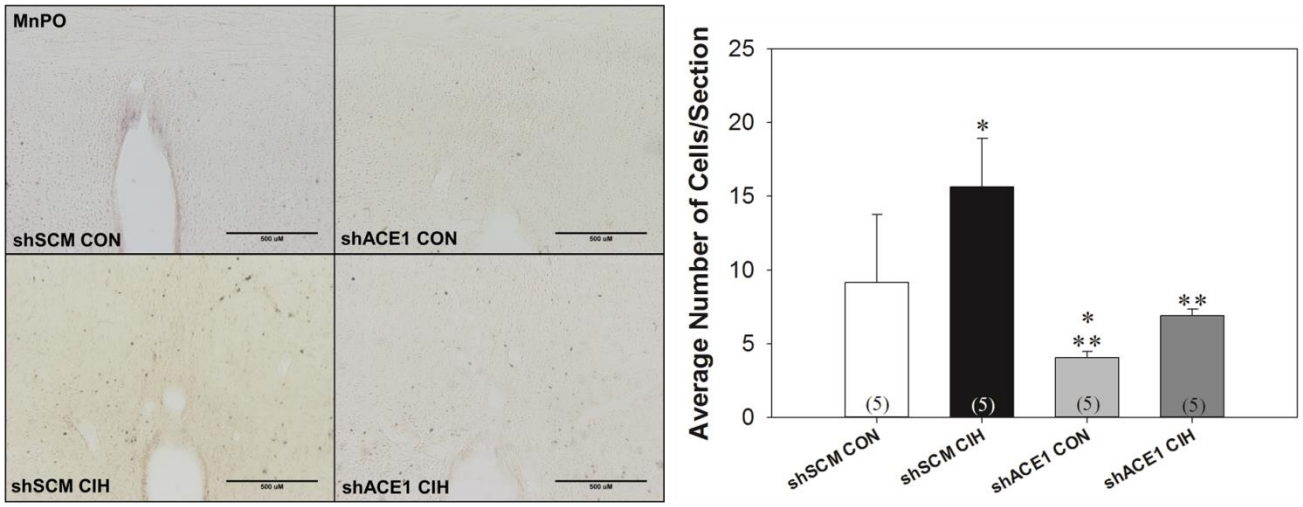


Figure 5. The left panel depicts DAB FosB/ΔFosB staining within the MnPO across all treatment groups. shSCM CIH significantly increased FosB/ΔFosB staining as compared to all other treatment groups (Right Panel, *P<0.05). ACE1 knockdown in the MnPO did significantly decrease FosB/ΔFosB staining in both normoxic (CON) and CIH treatments as compared to shSCM CIH (Right Panel, **P<0.05). shACE1 CON did decrease FosB/ΔFosB even from the shSCM CON treatment group (Right Panel, *P<0.05).

CHAPTER III-Figure III-6 PVN FosB/ΔFosB Staining

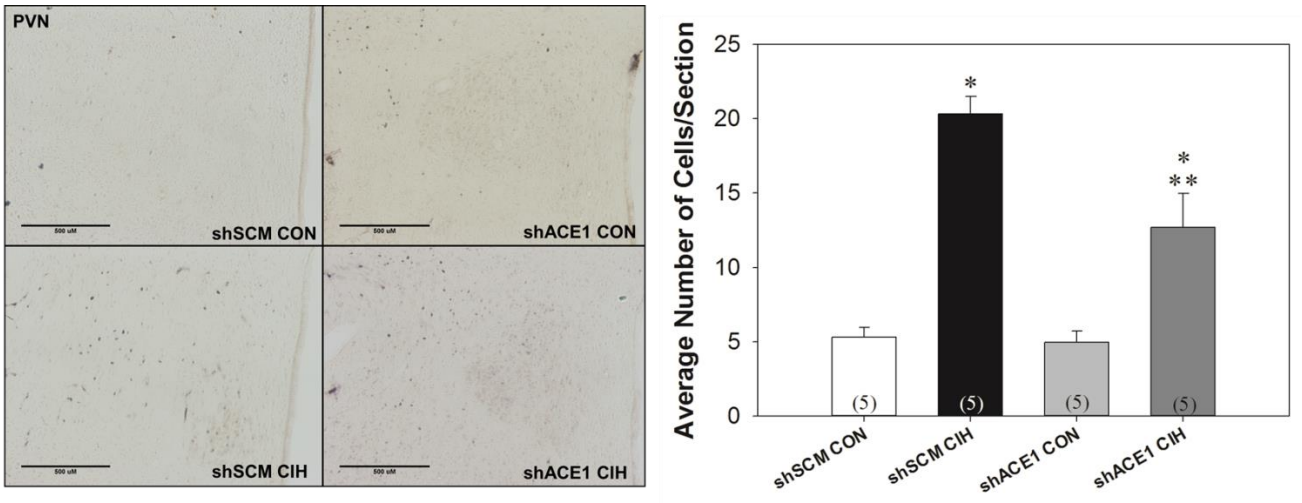


Figure 6. Left panel shows FosB/ΔFosB DAB staining in the PVN amongst all treatment groups. FosB/ΔFosB staining significantly increased in both CIH treatment groups as compared to their normoxic (CON) controls (Right Panel, *P<0.05). ACE1 knockdown within the MnPO following CIH did however decrease the FosB/ΔFosB staining in the PVN as compared to the shASCM CIH treatment group (Right Panel, **P<0.05)

CHAPTER III-Figure III-7 Rostral NTS FosB/ΔFosB Staining

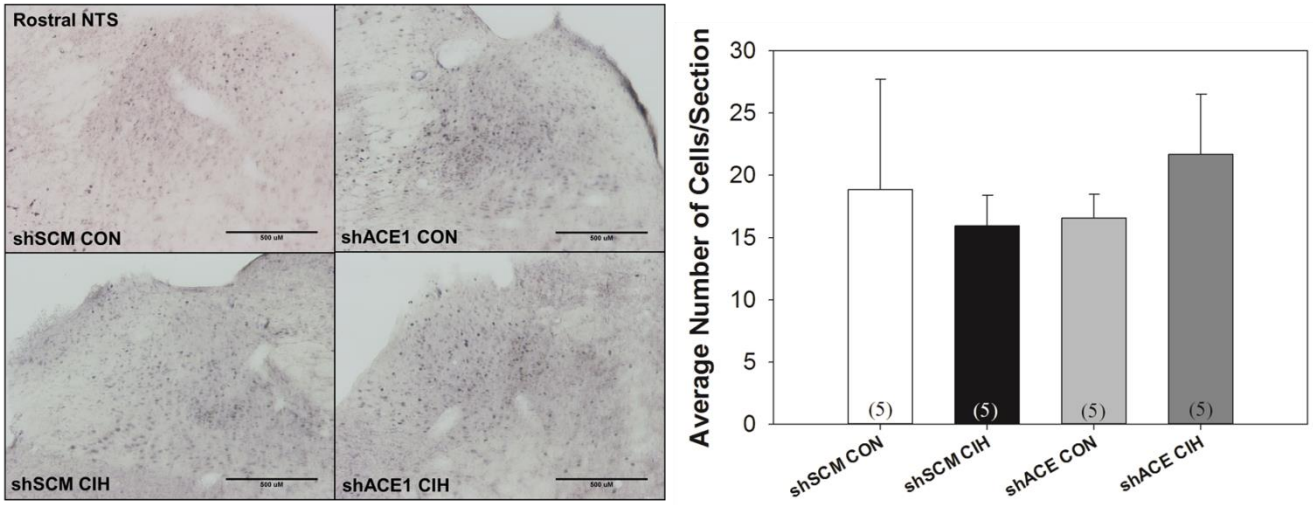


Figure 7. Left panel depicts DAB FosB/ΔFosB staining in the rostral subdivision of the NTS in all treatment groups. FosB/ΔFosB staining did not significantly change across all treatment groups (Right Panel).

CHAPTER III-Figure III-8 Subpostremal NTS FosB/ΔFosB Staining

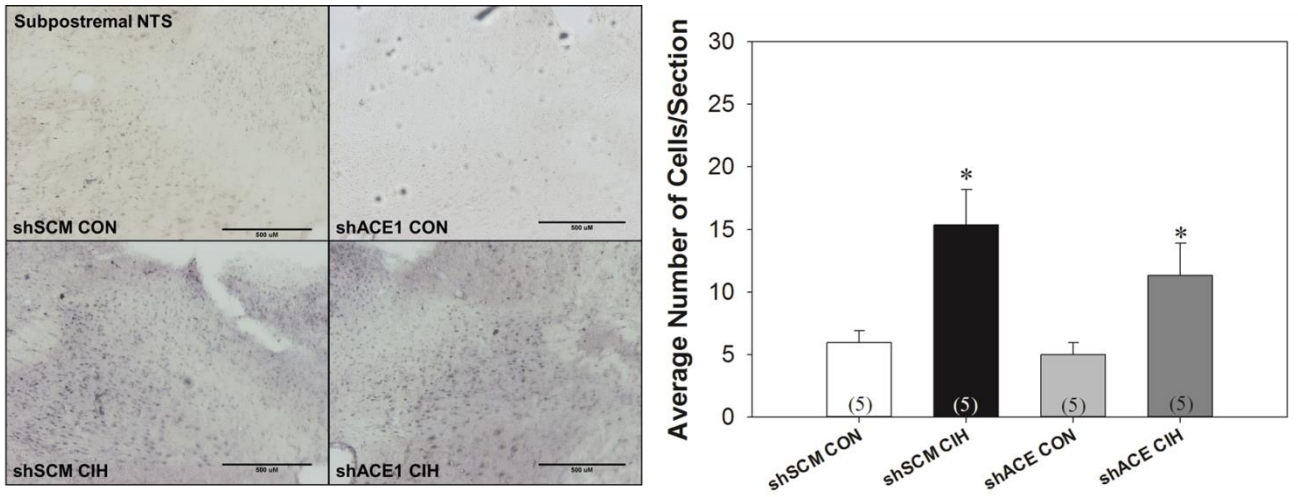


Figure 8. Left panel demonstrates FosB/ΔFosB staining in the subpostremal region of the NTS. CIH significantly increased FosB/ΔFosB staining in the subpostremal region in both shACE1 and shSCM treatments when compared to normoxic (CON) controls (Right Panel, *P<0.05).

CHAPTER III-Figure III-9 Caudal NTS FosB/ΔFosB Staining

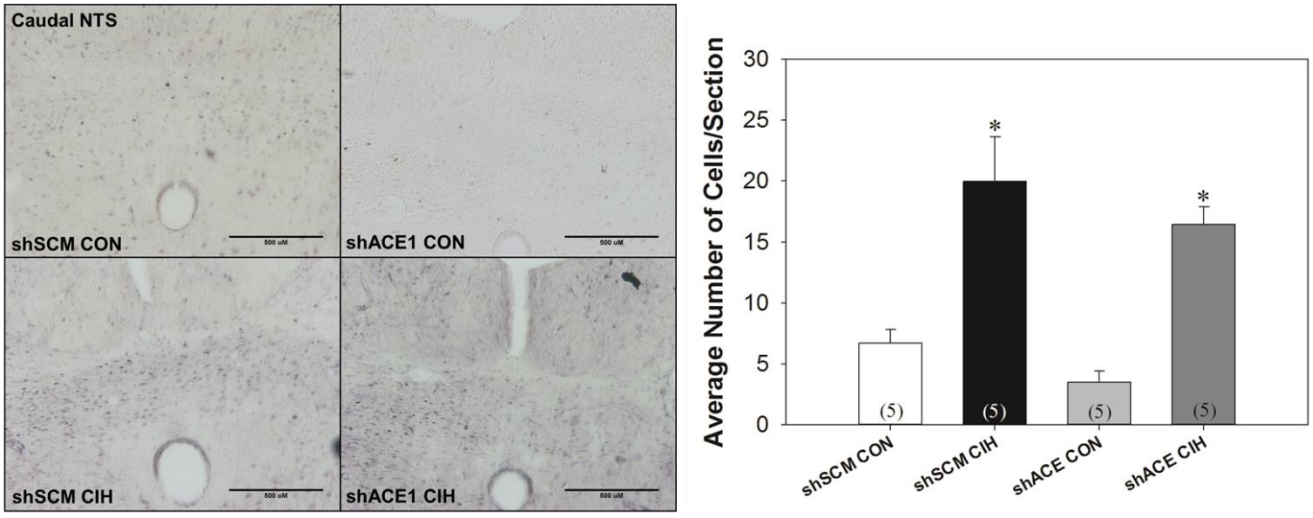


Figure 9. Left panel shows FosB/ΔFosB staining in the caudal region of the NTS. CIH significantly increased FosB/ΔFosB staining in the caudal region in both shACE1 and shSCM treatments as compared to the normoxic (CON) controls (Right Panel, *P<0.05).

CHAPTER III-Figure III-10 Total NTS FosB/ΔFosB Counts

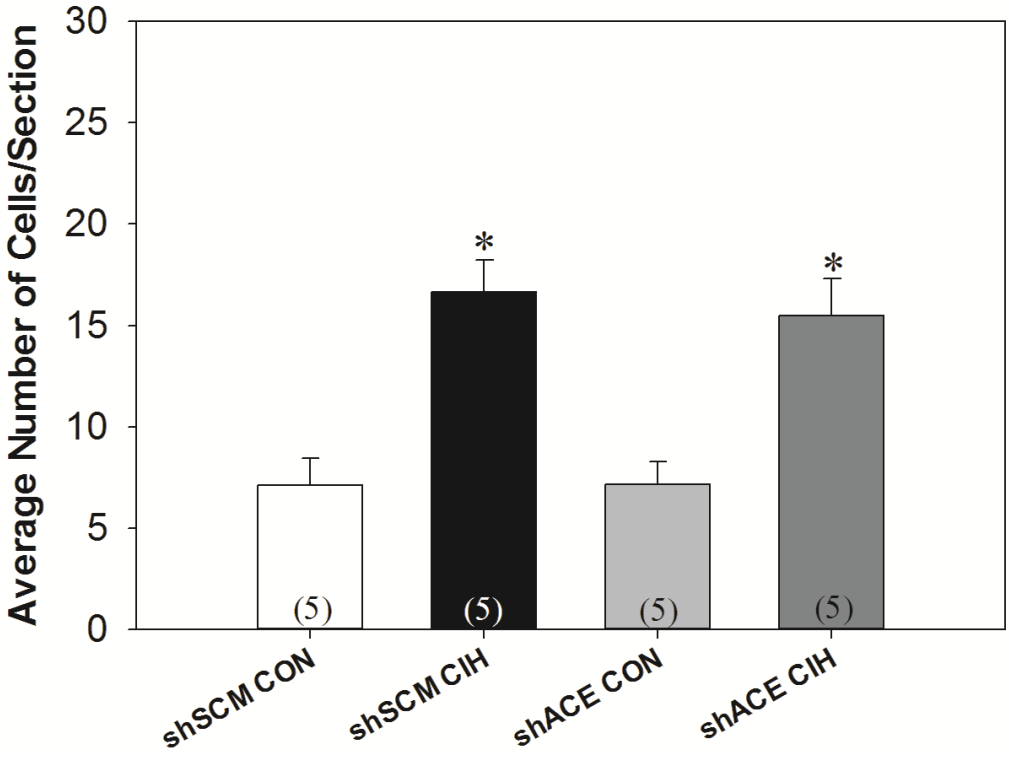


Figure 10. Graphic representation of FosB/ΔFosB scells per NTS section in the entire NTS. CIH significantly increased FosB/ΔFosB staining within the total NTS in both shACE1 and shSCM treatments as compared to the normoxic (CON) controls (*P<0.05).

CHAPTER III-Figure III-11 RVLM FosB/ΔFosB Staining

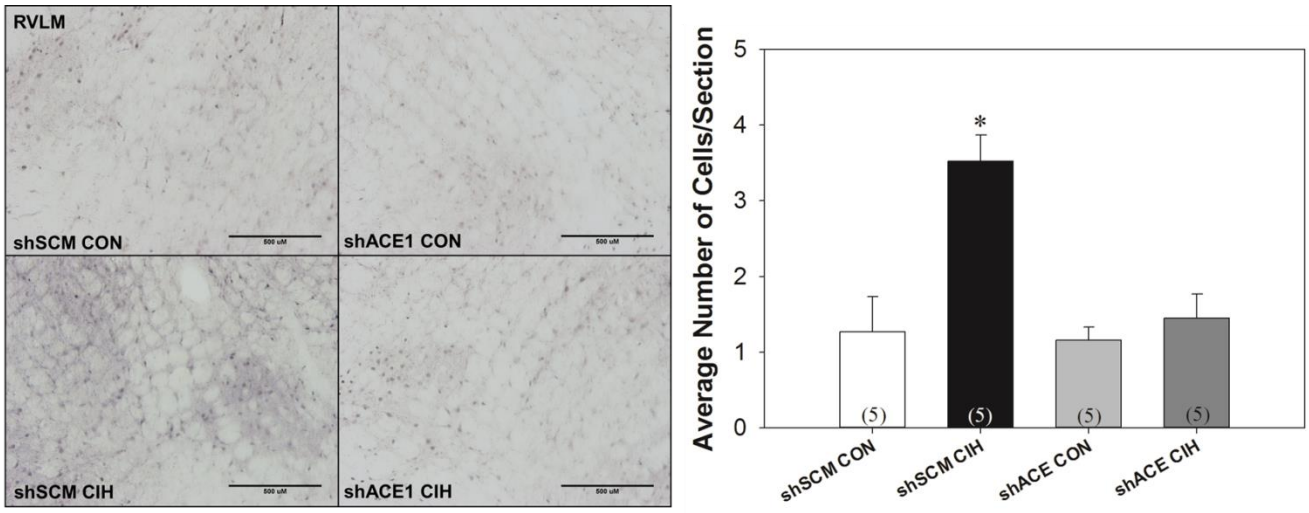


Figure 11. Left panel shows FosB/ΔFosB DAB staining in the RVLM amongst all treatment groups. FosB/ΔFosB staining significantly increased in the shSCM CIH treatment group as compared to all other groups (Right Panel, *P<0.05). ACE1 knockdown within the MnPO therefore attenuated the FosB/ΔFosB increase in staining within the PVN associated with CIH treatment.

DISCUSSION

Previously our lab demonstrated a critical role of ΔFosB within the MnPO in the sustained diurnal hypertension seen in CIH [28]. Also, several AP-1 target genes were proposed that may be regulated by ΔFosB in association with CIH. Those genes included a *map3k3, nos1, nos3, ace1* and *ace2* [28]. ACE1 may be one of the genes regulated by ΔFosB transcriptional activation within the MnPO so that blood pressure is sustained in CIH. In order to investigate the functional role ACE1 may have on CIH blood pressure, we chose to knockdown ACE1 with a shRNA delivered through an adeno-associated virus. Consistent with the results of the dominant-negative inhibition of ΔFosB studies, CIH was associated with a significant increase in ACE1 mRNA within MnPO cells of rats injected with the control vector. Injections of the AAV-shACE1 in the MnPO significantly reduced this increase but not to control levels. Preventing the increase in MnPO ACE1 message associated with CIH significantly blocked the sustained blood pressure increase normally observed during the normoxic dark phase during CIH. The blood pressure response to intermittent hypoxia (0800-1600) was not significantly or consistently altered by ACE1 knockdown in the MnPO. These results are consistent with the hypothesis that MnPO ACE1 is FosB regulated target gene that contributes to CIH hypertension. As mentioned above, our earlier studied identified several other possible gene candidates that may also play a role in the sustained hypertensive component of CIH. Further studies will need to be conducted in order to assess each candidate gene's potential role in CIH hypertension.

The FosB/ΔFosB staining was increased in the MnPO by CIH as previously shown [39, 44], However, knockdown of ACE1 within the MnPO significantly decreased this effect. ACE1 knockdown in the MnPO also significantly decreased FosB/ΔFosB staining in rats treated with

normoxia. These data suggest that ACE1 within the MnPO not only gets regulated by FosB/ΔFosB as suggested by the ΔFosB dominant negative inhibition studies [28] but that MnPO ACE1 contributes to the basal expression of FosB/ΔFosB as well. The SFO has been shown to be activated by CIH and angiotensin II type 1 subtype a (AT1a) receptors within the SFO have been implicated is the sustained CIH hypertension [39, 44]. Additionally, AT1a receptor knockdown in the SFO significantly decreased the FosB/ΔFosB staining in MnPO associated with CIH [44]. Activation of the SFO AT1a receptors may increase the activity of MnPO neurons in an AT1a receptors dependent manner that increases MnPO FosB/ΔFosB staining. Then FosB/ΔFosB may in turn regulate ACE1 expression and therefore increase production of ANGII in the MnPO. ANGII may then go to alter regions downstream from the MnPO but may also be dendritically released locally from MnPO neurons to contribute autocrine regulation of the MnPO. , Although there is to date no evidence of dendritic release of ANGII the dendritic release of other peptides such as oxytocin and vasopressin do in the supraoptic nucleus in well documented [58].,. This dendritic release of ANGII may lead to further activation of MnPO to maintain or increase FosB/ΔFosB even more. More studies are needed to understand the mechanisms by which ACE1 contributed to CIH hypertension and MnPO activity..

Previously our lab highlighted the importance of AT1a receptors within the SFO and their contribution to the sustained hypertension seen in CIH [44]. The SFO appears to be activated by the peripheral RAS that has been associated with many CIH models including our own [44, 45, 59]. The increased systemic RAS activity would be predicted to increase SFO activity through AT1a receptors which then in turn increases the activity of downstream nuclei such as the MnPO,[44]. The MnPO would be predicted to recruit the central RAS since we have shown that

ACE1 mRNA increases within the MnPO following CIH similarly to the expression seen in the ΔFosB dominant negative inhibition studies [28]. Additionally, knocking down ACE1 within the MnPO and consequently diminishing the sustained component of CIH hypertension shows that the RAS within the CNS plays a critical role in development of the hypertension seen following CIH.

A decrease in FosB/ΔFosB staining in the PVN was noted after knockdown of ACE1 within the MnPO. These results are similar to the ΔFosB dominant negative studies within the MnPO suggesting ACE1 within the MnPO influences the PVN [28]. The parvocellular neurons in the PVN are known to project to egions within the CNS that alter autonomic function such as the RVLM, the intermedial lateral column of the spinal cord and the dorsal vagal complex [60]. The majority of the FosB/ΔFosB staining seen in our study was in the parvocellular regions of the PVN and not in regions with primarily magnocellular neurons. It is possible that the increase in MnPO ACE1 associated CIH could increase ANGII signaling to the PVN. ANGII has been shown to activate the PVN influencing SNA as well as blood pressure [61-63]. The PVN projects to premotor sympathetic neurons in the RVLM and sympathetic preganglionic neurons in the IML both of which are known to affect SNA [64-66]. ACE1 knockdown within the MnPO significantly decreased FosB/ΔFosB staining in the RVLM associated with CIH. Therefore, CIH-induced FosB/ΔFosB staining in the PVN and RVLM are dependent on ACE1 in the MnPO. This suggests that these two regions are regulated by ACE1 within the MnPO as a key part of the mechanism for the sustained blood pressure component of CIH hypertension.

In contrast to the PVN and RVLM, we demonstrated that ACE1 knockdown within the MnPO did not significantly alter the FosB/ΔFosB staining in the NTS. The NTS still

significantly increased FosB/ΔFosB staining with the entire NTS and more specifically the subpostremal and caudal NTS subsections. This was similar to the ΔFosB dominant negative inhibition studies within the MnPO in that FosB/ΔFosB staining was still increased even with ΔFosB inhibition in the MnPO [28]. The lack of an effect on FosB/ΔFosB staining within the NTS by knockdown of ACE1 in the MnPO suggests that MnPO ACE1 does not contribute to this effect of CIH and that the chemoreflex mechanism that alters blood pressure is still intact. The respiration rate significantly increasing on certain days during CIH (0800-1600 h) in both CIH treated groups further suggests that this mechanism does not alter the chemoreflex augmentation seen in CIH [67].

PERSPECTIVES

The sustained hypertension associated with OSA leads to many serious cardiovascular sequela that affect mortality of these patients [7-12]. Since there is a significant prevalence of OSA and the incidence is steadily rising, the underlying mechanisms that are responsible for the sustained hypertension need to be elucidated. The CIH model sufficiently mimics the sustained hypertension and multiple mechanisms including chemoreflex augmentation and increased RAS activity have been implicated in the development of this specific form of hypertension [45]. This study provides new information about the role of ACE1 within the MnPO in the hypertension associated with CIH and the potential use of ACE blockers that cross the blood-brain-barrier in treating hypertension of mild to moderate OSA patients. Understanding the many mechanisms by which CIH influences blood pressure will only positively affect the way OSA patients with hypertension are treated within the clinic.

REFERENCES

1. Young T, Peppard PE and Gottlieb DJ. Epidemiology of obstructive sleep apnea: a population health perspective. *American journal of respiratory and critical care medicine*. 2002;165:1217-39.
2. Peppard PE, Young T, Barnet JH, Palta M, Hagen EW and Hla KM. Increased prevalence of sleep-disordered breathing in adults. *Am J Epidemiol*. 2013;177:1006-14.
3. Parati G, Lombardi C, Castagna F, Mattaliano P, Filardi PP, Agostoni P and Italian Society of Cardiology Working Group on Heart Failure m. Heart failure and sleep disorders. *Nat Rev Cardiol*. 2016;13:389-403.
4. Dempsey JA, Veasey SC, Morgan BJ and O'Donnell CP. Pathophysiology of sleep apnea. *Physiological reviews*. 2010;90:47-112.
5. Garpestad E, Katayama H, Parker JA, Ringler J, Lilly J, Yasuda T, Moore RH, Strauss HW and Weiss JW. Stroke volume and cardiac output decrease at termination of obstructive apneas. *J Appl Physiol (1985)*. 1992;73:1743-8.
6. McNicholas WT. Cardiovascular outcomes of CPAP therapy in obstructive sleep apnea syndrome. *American journal of physiology Regulatory, integrative and comparative physiology*. 2007;293:R1666-70.
7. Narkiewicz K, Wolf J, Lopez-Jimenez F and Somers VK. Obstructive sleep apnea and hypertension. *Current cardiology reports*. 2005;7:435-40.
8. Nieto FJ, Young TB, Lind BK, Shahar E, Samet JM, Redline S, D'Agostino RB, Newman AB, Lebowitz MD and Pickering TG. Association of sleep-disordered breathing, sleep apnea, and hypertension in a large community-based study. Sleep Heart

Health Study. *JAMA : the journal of the American Medical Association*. 2000;283:1829-36.

9. Parati G, Lombardi C and Narkiewicz K. Sleep apnea: epidemiology, pathophysiology, and relation to cardiovascular risk. *Am J Physiol Regul Integr Comp Physiol*. 2007;293:R1671-83.

10. Young T and Peppard P. Sleep-disordered breathing and cardiovascular disease: epidemiologic evidence for a relationship. *Sleep*. 2000;23 Suppl 4:S122-6.

11. Phillips BG and Somers VK. Hypertension and obstructive sleep apnea. *Curr Hypertens Rep*. 2003;5:380-5.

12. Somers VK, White DP, Amin R, Abraham WT, Costa F, Culebras A, Daniels S, Floras JS, Hunt CE, Olson LJ, Pickering TG, Russell R, Woo M, Young T, American Heart Association Council for High Blood Pressure Research Professional Education Committee CoCC, American Heart Association Stroke C, American Heart Association Council on Cardiovascular N and American College of Cardiology F. Sleep apnea and cardiovascular disease: an American Heart Association/american College Of Cardiology Foundation Scientific Statement from the American Heart Association Council for High Blood Pressure Research Professional Education Committee, Council on Clinical Cardiology, Stroke Council, and Council On Cardiovascular Nursing. In collaboration with the National Heart, Lung, and Blood Institute National Center on Sleep Disorders Research (National Institutes of Health). *Circulation*. 2008;118:1080-111.

13. Stoohs R and Guilleminault C. Cardiovascular changes associated with obstructive sleep apnea syndrome. *J Appl Physiol (1985)*. 1992;72:583-9.

14. Wolk R, Kara T and Somers VK. Sleep-disordered breathing and cardiovascular disease. *Circulation*. 2003;108:9-12.

15. Morrell MJ, Finn L, Kim H, Peppard PE, Badr MS and Young T. Sleep fragmentation, awake blood pressure, and sleep-disordered breathing in a population-based study. *American journal of respiratory and critical care medicine*. 2000;162:2091-6.

16. Carlson JT, Hedner J, Elam M, Ejnell H, Sellgren J and Wallin BG. Augmented resting sympathetic activity in awake patients with obstructive sleep apnea. *Chest*. 1993;103:1763-8.

17. Smith ML, Niedermaier ON, Hardy SM, Decker MJ and Strohl KP. Role of hypoxemia in sleep apnea-induced sympathoexcitation. *Journal of the autonomic nervous system*. 1996;56:184-90.

18. Somers VK, Dyken ME, Clary MP and Abboud FM. Sympathetic neural mechanisms in obstructive sleep apnea. *The Journal of clinical investigation*. 1995;96:1897-904.

19. Young T, Peppard P, Palta M, Hla KM, Finn L, Morgan B and Skatrud J. Population-based study of sleep-disordered breathing as a risk factor for hypertension. *Archives of internal medicine*. 1997;157:1746-52.

20. Fletcher EC. Invited review: Physiological consequences of intermittent hypoxia: systemic blood pressure. *J Appl Physiol (1985)*. 2001;90:1600-5.

21. Fletcher EC, Lesske J, Qian W, Miller CC, 3rd and Unger T. Repetitive, episodic hypoxia causes diurnal elevation of blood pressure in rats. *Hypertension*. 1992;19:555-61.

22. Fletcher EC. Sympathetic over activity in the etiology of hypertension of obstructive sleep apnea. *Sleep*. 2003;26:15-9.

23. Tamisier R, Gilmartin GS, Launois SH, Pepin JL, Nespoulet H, Thomas R, Levy P and Weiss JW. A new model of chronic intermittent hypoxia in humans: effect on ventilation, sleep, and blood pressure. *J Appl Physiol (1985)*. 2009;107:17-24.

24. Dempsey JA, Smith CA, Blain GM, Xie A, Gong Y and Teodorescu M. Role of central/peripheral chemoreceptors and their interdependence in the pathophysiology of sleep apnea. *Adv Exp Med Biol*. 2012;758:343-9.

25. Greenberg HE, Sica AL, Scharf SM and Ruggiero DA. Expression of c-fos in the rat brainstem after chronic intermittent hypoxia. *Brain research*. 1999;816:638-45.

26. Kc P, Balan KV, Tjoe SS, Martin RJ, Lamanna JC, Haxhiu MA and Dick TE. Increased vasopressin transmission from the paraventricular nucleus to the rostral medulla augments cardiorespiratory outflow in chronic intermittent hypoxia-conditioned rats. *The Journal of physiology*. 2010;588:725-40.

27. Buggy J, Fink GD, Haywood JR, Johnson AK and Brody MJ. Interruption of the maintenance phase of established hypertension by ablation of the anteroventral third ventricle (AV3V) in rats. *Clinical and experimental hypertension*. 1978;1:337-53.

28. Cunningham JT, Knight WD, Mifflin SW and Nestler EJ. An Essential role for DeltaFosB in the median preoptic nucleus in the sustained hypertensive effects of chronic intermittent hypoxia. *Hypertension*. 2012;60:179-87.

29. Saxena A, Bachelor M, Park YH, Carreno FR, Nedungadi TP and Cunningham JT. Angiotensin II induces membrane trafficking of natively expressed transient receptor potential vanilloid type 4 channels in hypothalamic 4B cells. *Am J Physiol Regul Integr Comp Physiol*. 2014;307:R945-55.

30. McKinley MJ, McAllen RM, Davern P, Giles ME, Penschow J, Sunn N, Uschakov A and Oldfield BJ. The sensory circumventricular organs of the mammalian brain. *Advances in anatomy, embryology, and cell biology*. 2003;172:III-XII, 1-122, back cover.

31. Osborn JW, Fink GD, Sved AF, Toney GM and Raizada MK. Circulating angiotensin II and dietary salt: converging signals for neurogenic hypertension. *Current hypertension reports*. 2007;9:228-35.

32. Smith PM and Ferguson AV. Circulating signals as critical regulators of autonomic state--central roles for the subfornical organ. *American journal of physiology Regulatory, integrative and comparative physiology*. 2010;299:R405-15.

33. Johnson AK and Gross PM. Sensory circumventricular organs and brain homeostatic pathways. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology*. 1993;7:678-86.

34. Saper CB and Levisohn D. Afferent connections of the median preoptic nucleus in the rat: anatomical evidence for a cardiovascular integrative mechanism in the anteroventral third ventricular (AV3V) region. *Brain research*. 1983;288:21-31.

35. Stocker SD and Toney GM. Median preoptic neurones projecting to the hypothalamic paraventricular nucleus respond to osmotic, circulating Ang II and baroreceptor input in the rat. *The Journal of physiology*. 2005;568:599-615.

36. Stocker SD and Toney GM. Vagal afferent input alters the discharge of osmotic and ANG II-responsive median preoptic neurons projecting to the hypothalamic paraventricular nucleus. *Brain research*. 2007;1131:118-28.

37. Ferguson AV, Latchford KJ and Samson WK. The paraventricular nucleus of the hypothalamus - a potential target for integrative treatment of autonomic dysfunction. *Expert opinion on therapeutic targets*. 2008;12:717-27.

38. Stocker SD, Hunwick KJ and Toney GM. Hypothalamic paraventricular nucleus differentially supports lumbar and renal sympathetic outflow in water-deprived rats. *The Journal of physiology*. 2005;563:249-63.

39. Knight WD, Little JT, Carreno FR, Toney GM, Mifflin SW and Cunningham JT. Chronic intermittent hypoxia increases blood pressure and expression of FosB/DeltaFosB in central autonomic regions. *American journal of physiology Regulatory, integrative and comparative physiology*. 2011;301:R131-9.

40. Chen J, Kelz MB, Hope BT, Nakabeppu Y and Nestler EJ. Chronic Fos-related antigens: stable variants of deltaFosB induced in brain by chronic treatments. *The Journal of neuroscience : the official journal of the Society for Neuroscience*. 1997;17:4933-41.

41. Herdegen T and Leah JD. Inducible and constitutive transcription factors in the mammalian nervous system: control of gene expression by Jun, Fos and Krox, and CREB/ATF proteins. *Brain research Brain research reviews*. 1998;28:370-490.

42. McClung CA, Ulery PG, Perrotti LI, Zachariou V, Berton O and Nestler EJ. DeltaFosB: a molecular switch for long-term adaptation in the brain. *Brain research Molecular brain research*. 2004;132:146-54.

43. Marcus NJ, Li YL, Bird CE, Schultz HD and Morgan BJ. Chronic intermittent hypoxia augments chemoreflex control of sympathetic activity: role of the angiotensin II type 1 receptor. *Respiratory physiology & neurobiology*. 2010;171:36-45.

44. Saxena A, Little JT, Nedungadi TP and Cunningham JT. Angiotensin II type 1a receptors in subfornical organ contribute towards chronic intermittent hypoxia-associated sustained increase in mean arterial pressure. *American journal of physiology Heart and circulatory physiology*. 2015;308:H435-46.

45. Fletcher EC, Bao G and Li R. Renin activity and blood pressure in response to chronic episodic hypoxia. *Hypertension*. 1999;34:309-14.

46. Knight WD, Saxena A, Shell B, Nedungadi TP, Mifflin SW and Cunningham JT. Central losartan attenuates increases in arterial pressure and expression of FosB/DeltaFosB along the autonomic axis associated with chronic intermittent hypoxia. *American journal of physiology Regulatory, integrative and comparative physiology*. 2013;305:R1051-8.

47. Moller DS, Lind P, Strunge B and Pedersen EB. Abnormal vasoactive hormones and 24-hour blood pressure in obstructive sleep apnea. *American journal of hypertension*. 2003;16:274-80.

48. Kraiczi H, Hedner J, Peker Y and Carlson J. Increased vasoconstrictor sensitivity in obstructive sleep apnea. *J Appl Physiol (1985)*. 2000;89:493-8.

49. Wright JW and Harding JW. The brain renin-angiotensin system: a diversity of functions and implications for CNS diseases. *Pflugers Arch*. 2013;465:133-51.

50. Ganten D, Marquez-Julio A, Granger P, Hayduk K, Karsunky KP, Boucher R and Genest J. Renin in dog brain. *The American journal of physiology*. 1971;221:1733-7.

51. Aars H and Akre S. Effect of angiotensin on sympathetic nerve activity. *Acta physiologica Scandinavica*. 1968;74:134-41.

52. Barrett CJ, Ramchandra R, Guild SJ, Lala A, Budgett DM and Malpas SC. What sets the long-term level of renal sympathetic nerve activity: a role for angiotensin II and baroreflexes? *Circulation research*. 2003;92:1330-6.

53. DiBona GF. Central angiotensin modulation of baroreflex control of renal sympathetic nerve activity in the rat: influence of dietary sodium. *Acta physiologica Scandinavica*. 2003;177:285-9.

54. Paxinos G, Watson CR and Emson PC. AChE-stained horizontal sections of the rat brain in stereotaxic coordinates. *Journal of neuroscience methods*. 1980;3:129-49.

55. Nedungadi TP and Cunningham JT. Differential regulation of TRPC4 in the vasopressin magnocellular system by water deprivation and hepatic cirrhosis in the rat. *Am J Physiol Regul Integr Comp Physiol*. 2014;306:R304-14.

56. Carreno FR, Walch JD, Dutta M, Nedungadi TP and Cunningham JT. Brain-derived neurotrophic factor-tyrosine kinase B pathway mediates NMDA receptor NR2B subunit phosphorylation in the supraoptic nuclei following progressive dehydration. *J Neuroendocrinol*. 2011;23:894-905.

57. Cunningham JT, Nedungadi TP, Walch JD, Nestler EJ and Gottlieb HB. DeltaFosB in the supraoptic nucleus contributes to hyponatremia in rats with cirrhosis. *Am J Physiol Regul Integr Comp Physiol*. 2012;303:R177-85.

58. Ludwig M and Leng G. Dendritic peptide release and peptide-dependent behaviours. *Nat Rev Neurosci*. 2006;7:126-36.

59. Yuan ZM, Chen BY, Wang PX, Li SY, Chen YL and Dong LX. [Changes of angiotensin II and its receptor during the development of chronic intermittent hypoxia-induced hypertension in rats]. *Zhonghua Jie He He Hu Xi Za Zhi*. 2004;27:577-80.

60. Swanson LW and Kuypers HG. The paraventricular nucleus of the hypothalamus: cytoarchitectonic subdivisions and organization of projections to the pituitary, dorsal vagal complex, and spinal cord as demonstrated by retrograde fluorescence double-labeling methods. *J Comp Neurol*. 1980;194:555-70.

61. Ferguson AV and Washburn DL. Angiotensin II: a peptidergic neurotransmitter in central autonomic pathways. *Prog Neurobiol*. 1998;54:169-92.

62. Latchford KJ and Ferguson AV. Angiotensin depolarizes parvocellular neurons in paraventricular nucleus through modulation of putative nonselective cationic and potassium conductances. *Am J Physiol Regul Integr Comp Physiol*. 2005;289:R52-8.

63. Lenkei Z, Corvol P and Llorens-Cortes C. The angiotensin receptor subtype AT1A predominates in rat forebrain areas involved in blood pressure, body fluid homeostasis and neuroendocrine control. *Brain Res Mol Brain Res*. 1995;30:53-60.

64. Shafton AD, Ryan A and Badoer E. Neurons in the hypothalamic paraventricular nucleus send collaterals to the spinal cord and to the rostral ventrolateral medulla in the rat. *Brain Res*. 1998;801:239-43.

65. Barman SM. Descending projections of hypothalamic neurons with sympathetic nerve-related activity. *J Neurophysiol*. 1990;64:1019-32.

66. Trzebski A and Baradziej S. Role of the rostral ventrolateral medulla in the generation of synchronized sympathetic rhythmicities in the rat. *J Auton Nerv Syst*. 1992;41:129-39.

67. Prabhakar NR, Dick TE, Nanduri J and Kumar GK. Systemic, cellular and molecular analysis of chemoreflex-mediated sympathoexcitation by chronic intermittent hypoxia. *Exp Physiol*. 2007;92:39-44.

CHAPTER IV

DISSERTATION PERSPECTIVE AND SIGNIFICANCE

Transcriptional regulation have been known to be important in many aspects of physiology. ΔFosB has been shown to play a critical role in the development of sustained hypertension seen in chronic intermittent hypoxia (CIH), especially in the median preoptic nucleus (MnPO). The work here sought to determine the role of MnPO ΔFosB in the regulation of angiotensin converting enzyme 1 (ACE1) a putative AP-1 target gene. The experiments also explored the role of ACE1 in the sustained hypertension seen during CIH.

Role of a ΔFosB AP-1 target gene, ACE1, in the MnPO with CIH

The role ACE1 within the MnPO as a FosB target gene following CIH was only moderately explored so the first objective was to determine the significance of ACE1 within the MnPO following CIH as well as determine if ACE1 MnPO cells projected to the PVN which has also been linked to CIH hypertension. We also sought to determine the relationship of FosB/ΔFosB binding to the promoter region of ACE1 after 7 days of CIH exposure. Using immunohistochemical techniques, we determined the presence of ACE1 within the MnPO. The ACE1 staining also did not overlap with a glial fibrillary acidic protein (GFAP), a known glial marker. This suggests that in the MnPO, ACE1 is found more in neurons. The cells expressing ACE1 also increased colocalization with FosB/ΔFosB after 7 days of CIH. This suggests that

more ACE1 cells within the MnPO are transcriptionally activated by FosB/ΔFosB following CIH. Using fluorogold, a retrograde tract tracer, placed into the paraventricular nucleus (PVN), we determined that the cells expressing ACE1 and that were FosB/ΔFosB positive after CIH project to the PVN. These results are consistent with our working hypothesis that ACE1 is increased in PVN-projecting MnPO neurons that are transcriptionally activated by CIH. ACE1, therefore, may get transcriptionally activated by FosB/ΔFosB following CIH and increasing PVN activity and SNA causing a sustained hypertension during CIH. Furthermore, these experiments sought to determine a more definite relationship between FosB/ΔFosB ability to bind to the ACE1 promoter region following CIH. In order to establish that possible relationship, a chromatin immunoprecipitation (ChIP) assay was used. Following 7 days of CIH, FosB/ΔFosB increased its association with the ACE1 promoter region within the MnPO. This suggests that FosB/ΔFosB does indeed bind to ACE1 within the MnPO and this association increases with CIH. These data collectively suggest ACE1 may play a role within the MnPO in the sustained hypertension seen following CIH (Figure 1A).

Role of ACE1 in the sustained Hypertension seen in CIH

The renin angiotensin system (RAS) within the central nervous system (CNS) has increasingly been recognized as a major player in the regulation of blood pressure. Based on the results described above we next tested the functional role of MnPO ACE1 in the sustained hypertension seen in CIH. We tested the role of MnPO ACE1 in CIH hypertension using viral delivery of a short hairpin RNA (shRNA) to block the increase in ACE1 mRNA within the MnPO associated with CIH. This gave us the ability to directly test the role of ACE1 within the MnPO on the blood pressure increase seen following CIH. The shRNA against ACE1, shACE1, successfully

attenuated the ACE1 mRNA increase within the MnPO following CIH by almost half. ACE1 knockdown within the MnPO selectively attenuate the sustained hypertension seen following 7 days of CIH (Figure 1B). ACE1 knockdown within the MnPO also decreased FosB/ΔFosB staining within the MnPO itself, the PVN as well as the RVLM. The decrease of FosB/ΔFosB staining in these regions suggests ACE1 may play a role in regulating those downstream nuclei and that pathway, MnPO to the PVN to the RVLM, may also be the pathway that gets amplified by increased MnPO ACE1 expression associated with CIH exposure. The ACE1 knockdown within the MnPO failed to block the increase in FosB/ΔFosB within the NTS following CIH suggesting ACE1 within the MnPO does not alter chemoreflex activation during CIH.

Perspectives

The results from both studies suggest ACE1 plays a vital role in the development of the sustained hypertension seen in CIH. The role of ACE1 in the sustained hypertension also may be through FosB/ΔFosB activation within the MnPO. The decrease in FosB/ΔFosB within the MnPO itself after ACE1 knockdown may suggest that the MnPO releases angiotensin II (ANGII) dentritically and therefore act in a paracrine or autocrine manner to affect MnPO neuron regulation. The mechanism whereby ACE1 within the MnPO regulaes downstream nuclei, such as the PVN and RVLM, remains to be determined. Further investigation needs to be done in order to determine the intracellular mechanisms whereby MnPO ACE1 alters the PVN and RVLM pathway.

Figure

CHAPTER IV-Figure IV-1 Overall Role of ACE1 in CIH

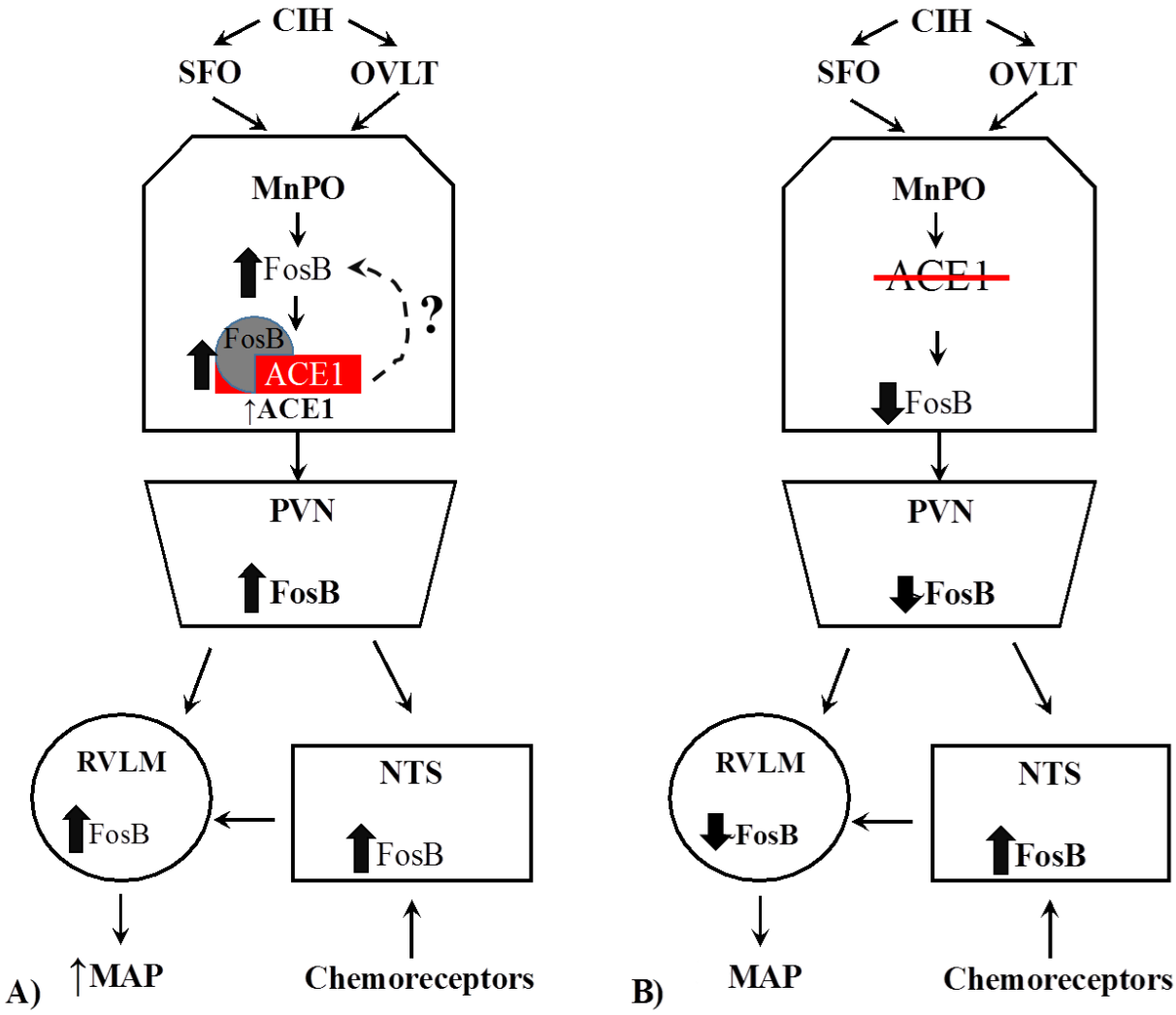


Figure 1. The role of ACE1 within the MnPO following CIH. CIH stimulates the SFO and OVLT which then may activate the MnPO. A) FosB increases within the MnPO after CIH and then FosB increases its association with the ACE1 promoter region. This increases the content of ACE1 within the MnPO. Then FosB increases in downstream regions of the MnPO, the PVN and RVLM. This pathway may be responsible for the increase in MAP seen in CIH. B) Knocking down ACE1 within the MnPO decreases FosB within the MnPO and attenuates the FosB increase in the PVN, and RVLM normally associated with CIH. This also significantly attenuated the sustained MAP seen in CIH. Knocking down ACE1 within the MnPO did not disrupt the MAP response indicative of chemoreflex pathway activation from CIH.

CHAPTER V

FUTURE DIRECTIONS

Other FosB AP-1 Target Genes in CIH

Our results demonstrate that MnPO ACE1 is a FosB regulated and contributes to the sustained component of CIH hypertension occurring during normoxia. Previously, our lab reported several possible AP-1 target genes of FosB in addition to ACE1 that may play a role in the sustained hypertension of CIH. Those additional AP-1 targets included ace2, nnos, enos and map3k3[1]. It is possible that one or more of these other gene could be directly regulated by FosB in the MnPO and work with ACE1 to maintain the increased in blood pressure produced by CIH.

MAP3K3 is a protein kinase and part of several different signaling pathways but interestingly plays a role upstream of AP-1 targets[2]. MAP3K3 also has been shown to regulate the enzymes WNK 1 and WNK4 and the lack of regulation of those WNK enzymes has been associated with a familial form of hypertension[2, 3]. MAP3K3 therefore may also play a role in the sustained hypertension seen following CIH. More experiments are needed in order to assess the role MAP3K3 may have in the development of sustained CIH. Those experiments may also utilize a similar mechanisms presented within the context in that shRNA against MAP3K3 within the MnPO could alter the blood pressure response in CIH.

Furthermore, nnos and enos are, respectively, neuronal and endothelial nitric oxide synthases which mediate the conversion of L-arginine to nitric oxide (NO)[4]. NO within the lamina terminalis has been shown to regulate blood pressure through vasopressin secretion[5, 6]. The FosB regulation of nnos or enos may also play a role in the sustained hypertension seen in CIH. NO content within the MnPO may be altered by a nitric oxide inhibitor, L-NAME[7], loaded into mini-osmotic pumps in ordered to assess the ability NO may have on blood pressure in the model of CIH.

Finally, ACE2 or angiotensin converting enzyme 2, was also shown to be a possible downstream AP-1 target of MnPO FosB[1]. ACE2 is reported to convert angiotensin II (ANGII) to angiotensin [1-7] or convert angiotensin I (ANGI) to angiotensin [1-9] (ANG1-9)[8, 9]. These end products as well as the expression of ACE2 have been shown to alter various cardiovascular functions such as hypertension and essentially ACE2 serves as a physiological break to the pro-hypertensive ACE1[8]. Investigating any one of the components, ACE2 and its end-products, may suggest a possible counter mechanism to the sustained blood pressure seen following CIH exposure. Thus, blocking the increase in MnPO ACE2 could results in a larger increase in blood pressure during CIH.

Taken together, all the possible targets of FosB, ACE2, nnos, enos and MAP3K3, along with ACE1, a suggested FosB target following CIH presented in this study, may play a role in the sustained hypertension seen in CIH in a joint mechanism and warrants further investigation. It is also possible that due to the limitations of the number of genes that could be included in the PCR array in our earlier study[1] we missed other potential MnPO FosB target genes.

RAS within the MnPO

The RAS within the brain has increasingly become a target of hypertension research[10, 11]. ACE1, as previously stated, regulates the conversion of ANGI to ANGII. The studies presented here examined the content of ACE1 within the MnPO in the sustained part of CIH hypertension; however the activity of ACE1 was not addressed. In order to advance our hypothesis, ACE1 activity within the MnPO should be measured following CIH exposure. ACE1 activity could increase in parallel with protein abundance and message expression in the MnPO and provide an additional way for blood pressure to remain sustained during CIH. Additionally, ACE2 within the MnPO could play a role in the sustained CIH hypertension. Although ACE2 experiments were previously described above in the context of FosB regulation, ACE2 may produce other various angiotensin peptides that could influence the activity of neurons in the MnPO or PVN. More specifically ACE2 could convert ANGII to ANG[1-7] within the MnPO and may activate angiotensin II type 2 receptors (AT2) or Mas receptors that affect NO signaling[12, 13]. Through these putative mechanisms, ACE2 may provide a protective mechanism against the sustained hypertension seen in CIH. Previous studies have shown that that ACE2 overexpression in the CNS protects against neurogenic hypertension[12, 13]. Also, overexpression of ACE2 within the RVLM has been shown to attenuate blood pressure in SHR rats through decreasing tonically active glutamatergic inputs to the RVLM[14]. The activation of AT2 receptors may also play a role in counteracting the sustained CIH hypertension. AT2 stimulation through compound 21, a selective AT2 receptor agonist, has been shown to attenuate deoxycorticosterone acetate (DOCA)/NaCl-induced hypertension in female rats[15]. The balance between ACE1 and ACE2 expression or activity in the CNS as well as the respective peptides they produce may be the key

integrative system that becomes unhinged following CIH exposure and a sustained hypertension therefore develops. Moreover, upstream of the ACE1/ACE2 pathway, a (pro)renin receptor (PRR) has been shown to be responsible for regulating blood pressure as well[16]. The PRR has been shown to influence similar intracellular signaling pathways as the AT1 receptor. Thus, the PPR may also play a role in hypertension development. PRR receptor key role in the development of blood pressure regulation is that PRR expression is higher compared to other RAS components in the brain[17] and PRR activation can increase RAS activity[18]. Therefore, activation of the PRR system may increase SNA. Also, overexpression of AT1 receptors in a transgenic mouse model failed to significantly change baseline blood pressure, but it did increase the cardiovascular sensitivity in these mice, implying increasing ANGII is needed for the enhancement of blood pressure[19]. Thus, PRR may play a pivotal role in the initial development of hypertension seen in CIH as compared to the vast amount of literature focusing on other RAS components[20].

Intracellular Mechanisms of ACE1 Expression within the MnPO following CIH

Although the studies presented in this body of work indicate a role of ACE1 within the MnPO in the generation of a sustained blood pressure following CIH, the intracellular mechanisms remain to be determined. In consideration of the FosB/ΔFosB staining decreasing within the MnPO with ACE1 knockdown in the MnPO itself suggest a possible role for peptides generated in the MnPO by ACE1 having a local effects on neural activity. This could be the result of the dendritic release of ANGII contributing to the stimulation of the transcription factor FosB. The dendritic release of ANGII could be similar to the mechanism of dendritic release of vasopressin within the magnocellular neurons of the PVN and the supraoptic nucleus (SON)[21, 22]. The dendritic

release of angiotensin peptide produced by ACE1 could be an autocrine or paracrine mechanism within the MnPO following a physiological challenge such as CIH.

Furthermore, the mechanism that ACE1 within the MnPO has on the PVN in respects to CIH is not known. MnPO neurons that project to the PVN have shown to decrease their neuronal activity in response to hypertonic saline in SHR rats[23]. This suggests MnPO neurons could change the activity of PVN neurons following a physiological stimulus. Additionally, ANGII is known to excite PVN neurons that project the RVLM, a known region that contributes to SNA[24]. These experiments suggest that MnPO neurons do change the activity of PVN neurons and PVN neurons that project to the RVLM get excited from ANGII application. Therefore increased ACE1 within the MnPO may increase ANGII signaling to the PVN resulting in increased RVLM activity and SNA. However, further experiments are needed to fully determine the possible mechanisms.

REFERENCES

1. Cunningham JT, Knight WD, Mifflin SW and Nestler EJ. An Essential role for DeltaFosB in the median preoptic nucleus in the sustained hypertensive effects of chronic intermittent hypoxia. *Hypertension*. 2012;60:179-87.
2. Xu LG, Li LY and Shu HB. TRAF7 potentiates MEKK3-induced AP1 and CHOP activation and induces apoptosis. *J Biol Chem*. 2004;279:17278-82.
3. Wilson FH, Disse-Nicodeme S, Choate KA, Ishikawa K, Nelson-Williams C, Desitter I, Gunel M, Milford DV, Lipkin GW, Achard JM, Feely MP, Dussol B, Berland Y, Unwin RJ, Mayan H, Simon DB, Farfel Z, Jeunemaitre X and Lifton RP. Human hypertension caused by mutations in WNK kinases. *Science*. 2001;293:1107-12.
4. Ignarro LJ. Nitric oxide. A novel signal transduction mechanism for transcellular communication. *Hypertension*. 1990;16:477-83.
5. Yamaguchi K and Hama H. A study on the mechanism by which sodium nitroprusside, a nitric oxide donor, applied to the anteroventral third ventricular region provokes facilitation of vasopressin secretion in conscious rats. *Brain Res*. 2003;968:35-43.
6. Yamaguchi K, Watanabe K and Yamaya K. Evaluation for roles of nitric oxide generated in the anteroventral third ventricular region in controlling vasopressin secretion and cardiovascular system of conscious rats. *Eur J Endocrinol*. 2000;143:523-33.
7. Rees DD, Palmer RM, Schulz R, Hodson HF and Moncada S. Characterization of three inhibitors of endothelial nitric oxide synthase in vitro and in vivo. *Br J Pharmacol*. 1990;101:746-52.

8. Xia H and Lazartigues E. Angiotensin-converting enzyme 2 in the brain: properties and future directions. *J Neurochem*. 2008;107:1482-94.

9. Donoghue M, Hsieh F, Baronas E, Godbout K, Gosselin M, Stagliano N, Donovan M, Woolf B, Robison K, Jeyaseelan R, Breitbart RE and Acton S. A novel angiotensin-converting enzyme-related carboxypeptidase (ACE2) converts angiotensin I to angiotensin 1-9. *Circ Res*. 2000;87:E1-9.

10. Takahashi H. Upregulation of the Renin-Angiotensin-aldosterone-ouabain system in the brain is the core mechanism in the genesis of all types of hypertension. *Int J Hypertens*. 2012;2012:242786.

11. Takahashi H, Yoshika M, Komiyama Y and Nishimura M. The central mechanism underlying hypertension: a review of the roles of sodium ions, epithelial sodium channels, the renin-angiotensin-aldosterone system, oxidative stress and endogenous digitalis in the brain. *Hypertens Res*. 2011;34:1147-60.

12. Feng Y, Xia H, Cai Y, Halabi CM, Becker LK, Santos RA, Speth RC, Sigmund CD and Lazartigues E. Brain-selective overexpression of human Angiotensin-converting enzyme type 2 attenuates neurogenic hypertension. *Circ Res*. 2010;106:373-82.

13. Feng Y, Xia H, Santos RA, Speth R and Lazartigues E. Angiotensin-converting enzyme 2: a new target for neurogenic hypertension. *Exp Physiol*. 2010;95:601-6.

14. Wang YK, Shen D, Hao Q, Yu Q, Wu ZT, Deng Y, Chen YF, Yuan WJ, Hu QK, Su DF and Wang WZ. Overexpression of angiotensin-converting enzyme 2 attenuates tonically active glutamatergic input to the rostral ventrolateral medulla in hypertensive rats. *Am J Physiol Heart Circ Physiol*. 2014;307:H182-90.

15. Dai SY, Zhang YP, Peng W, Shen Y and He JJ. Central Infusion of Angiotensin II Type 2 Receptor Agonist Compound 21 Attenuates DOCA/NaCl-Induced Hypertension in Female Rats. *Oxid Med Cell Longev*. 2016;2016:3981790.

16. Xu Q, Jensen DD, Peng H and Feng Y. The critical role of the central nervous system (pro)renin receptor in regulating systemic blood pressure. *Pharmacol Ther*. 2016.

17. Nguyen G, Delarue F, Burckle C, Bouzhir L, Giller T and Sraer JD. Pivotal role of the renin/prorenin receptor in angiotensin II production and cellular responses to renin. *J Clin Invest*. 2002;109:1417-27.

18. Ichihara A, Hayashi M, Kaneshiro Y, Suzuki F, Nakagawa T, Tada Y, Koura Y, Nishiyama A, Okada H, Uddin MN, Nabi AH, Ishida Y, Inagami T and Saruta T. Inhibition of diabetic nephropathy by a decoy peptide corresponding to the "handle" region for nonproteolytic activation of prorenin. *J Clin Invest*. 2004;114:1128-35.

19. Lazartigues E, Dunlay SM, Loihl AK, Sinnayah P, Lang JA, Espelund JJ, Sigmund CD and Davisson RL. Brain-selective overexpression of angiotensin (AT1) receptors causes enhanced cardiovascular sensitivity in transgenic mice. *Circ Res*. 2002;90:617-24.

20. Cuadra AE, Shan Z, Sumners C and Raizada MK. A current view of brain renin-angiotensin system: Is the (pro)renin receptor the missing link? *Pharmacol Ther*. 2010;125:27-38.

21. Ludwig M and Stern J. Multiple signalling modalities mediated by dendritic exocytosis of oxytocin and vasopressin. *Philos Trans R Soc Lond B Biol Sci*. 2015;370.

22. Du W, Stern JE and Filosa JA. Neuronal-derived nitric oxide and somatodendritically released vasopressin regulate neurovascular coupling in the rat hypothalamic supraoptic nucleus. *J Neurosci*. 2015;35:5330-41.

23. Tanaka J, Ushigome A, Matsuda M and Saito H. Responses of median preoptic neurons projecting to the hypothalamic paraventricular nucleus to osmotic stimulation in Wistar-Kyoto and spontaneously hypertensive rats. *Neurosci Lett*. 1995;191:47-50.

24. Cato MJ and Toney GM. Angiotensin II excites paraventricular nucleus neurons that innervate the rostral ventrolateral medulla: an in vitro patch-clamp study in brain slices. *J Neurophysiol*. 2005;93:403-13.