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Renin-Angiotensin system (RAS) is a peptidergic hormonal system that is known to regulate hemodynamic and fluid balance. Clinical success of RAS inhibitors in several cardiovascular and renal diseases such as hypertension (HTN), chronic heart failure, and diabetic nephropathy underscore its involvement in their pathophysiology. Recent research efforts are not only helping us understand the mechanisms through which RAS orchestrate the pathophysiology of cardiovascular diseases but are also identifying its involvement in other pathological conditions such as mood disorders and cancer. Although, initially identified as an endocrine system in peripheral circulation, the discovery of renin in the brain ushered in the concept of 'local' or 'tissue' RAS. Now, local RAS components are increasingly identified to be present in nearly all organ systems.

The In the first project we investigated the role played by the communication between circulating Ang II and subfornical organ (SFO), a circumventricular organ lacking blood brain barrier, in chronic intermittent hypoxia (CIH) associated sustained increase in MAP even during period of normoxic breathing. Using viral mediated delivery of shRNA again Ang II type 1a receptors (AT1aR), it was found that the rats that received AT1aRshRNA in their SFO, exhibited increased MAP responses during CIH but their MAP recovered to levels of normoxic control during room air breathing. Also, disruption of this communication led to decreased FosB/ΔFosB staining in the autonomic regions of forebrain. FosB/ΔFosB staining identifies the expression of transcription factor FosB and its splice variant ΔFosB. These transcription factors are known to orchestrate transcriptional adaptations that lead to lasting neuroplastic adaptations. These data

suggest that Ang II-SFO communication is essential in neuroplastic adaptations of forebrain autonomic nuclei, which may sustain the CIH associated increase in MAP even during periods of room air breathing.

Second project was initiated to study the mechanisms through which synaptically released Ang II could affect post-synaptic neuronal sensitivity and function. It is known that bile-duct ligated rats, an experimental model of cirrhosis, exhibit impaired osmoregulation. We previously reported that bile duct ligated rats show increased presence of a non-specific cation channel (TRPV4) in the hypothalamic membrane extracts. In addition, an activated RAS is also associated with fluid-electrolyte imbalance, a hallmark feature of cirrhosis. In this project it was investigated if Ang II could translocate TRPV4 to neuronal surface *in vitro*, in a hypothalamic neuronal cell line, 4B. In 4B cells, Ang II incubation was associated with increased TRPV4 localization to the cell membrane and was also associated with increased calcium influx in response to specific TRPV4 agonist, GSK 1016790A. In addition, these effects were completely blocked in the presence of AT1R receptor antagonist (Losartan) and Src kinase inhibitor, PP2. Taken together, these data suggest that Ang II could translocate TRPV4 to neuronal membrane via Src kinase pathway. These observations could explain one of the mechanisms through which Ang II could contribute to the pathophysiological adaptations that lead to increased water retention and dilutional hyponatremia associated with chronic liver failure.

THE ROLE OF ANGIOTENSIN II

IN CENTRAL AUTONOMIC AND ENDOCRINE REGULATION

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CHAPTER I

DISSERTATION OVERVIEW

Renin-Angiotensin system (RAS) is one of most potent hormone system that plays a key role in maintain body fluid, electrolyte, and hemodynamic homeostasis [1]. Its key role in the progression of cardiovascular and endocrine pathophysiology is well recognized and supported by the mortality benefits of pharmacologic agents antagonizing different components of RAS [2]. In the last half century, which includes the discovery of renin in the brain [3], our understanding has increased in the field of 'local' RASs and their roles in several other pathophysiological conditions has becoming apparent [4].

In 1898, the study of the RAS began when Finnish physiologist Robert Tigerstedt and his mentee Per Bergman published a landmark observation regarding pressor effects of renal cortex extracts. They termed this substance renin [5]. Despite an increasing recognition of hormones of adrenal origin at that time, as introduced by Brown-Sequard [6], Tigerstedt decided to test the effect of renal cortical versus medullary extract. These findings were first reported at the International Congress of Medicine in Moscow, Russia in 1897 [7]. After returning from the conference Tigerstedt did not pursue his findings further. Hence, these serendipitous observations did not receive much recognition initially [6].

The field of RAS research was revived by a pathologist Harry Goldblatt at Case Western University, OH in 1934 [8]. His observation of renal arteriosclerosis in hypertensive patients prompted him to investigate the effects of clamping of renal arteries on blood pressure in dogs. This intervention chronically increased mean arterial pressure [8]. These experiments produced first experimental model of human hypertension and paved the way for future studies to understand the mechanism of hypertension.

It took nearly 20 years of research to identify the effector octapeptide, Angiotensin II (Ang II), that contributed to 'Goldblatt's hypertension' [9]. While the initial interest in Ang II was directed towards its role in hypertension it was not until 1950's that its role in aldosterone secretion and, hence, sodium-retaining actions on kidney were noted [10-12].

Soon, the components of 'classical' or linear cascade of RAS were identified. It begins with the synthesis of renin, a glycoprotein, in the juxtaglomerular (JG) cells of the afferent arterioles of glomeruli [13]. Under conditions of decreased renal perfusion, high sodium content in the distal tubule, and/or increased renal sympathetic nerve activity (RSNA), renin is secreted into circulation [14, 15]. While circulating, renin catalyzes the cleavage of hepatically-produced Angiotensinogen (Agen) to a decapeptide Angiotensin I (Ang I) [16]. This circulating Ang I is then cleaved by Angiotensin converting enzyme (ACE), extensively found in pulmonary endothelium, to biologically active octapeptide Angiotensin II (Ang II) [17]. With the advancement in molecular biology techniques, came pharmacologic characterization of Ang II receptors [18, 19]. While Ang II receptor type 1 and 2 (AT1R and AT2R) were initially believed

to be the yin and yang of Ang II signal transduction pathway, later identification of new biologically active splice variants of Ang I and their receptors revealed a much more complex system [20]. Nevertheless, the primary actions of the main effector peptide of RAS, Ang II, on vascular (vasoconstriction), cardiac (inotropy, chronotropy, hypertrophy, fibrosis), renal (renal tubular sodium reabsorption), and adrenal glands (aldosterone secretion) were found to be mediated by a G-protein coupled receptors (GPCR), Ang II type 1 receptors (AT1R) [21-27]. Since then, the central role of classical RAS in fluid-electrolyte and blood pressure regulation continues to remain undisputed.

While most of the research that followed involved exogenous administration of Ang II, the discovery of specific Angiotensin converting enzyme (ACE) inhibitors in 1970 revealed the tonic vasopressor effect of endogenous Ang II in healthy individuals [28, 29]. Around this time, Arthur Guyton and his colleagues, proposed that Ang II can play a role in long-term blood pressure relation by changing the set-point of renal-pressure natriuresis [30-33]. This prediction introduced a still debated concept that kidneys may play a dominant role in long-term blood pressure regulation [34].

In the 1970s, with the identification of thirst evoking effect of exogenously administered Ang II [35-39], the central effects of Ang II came to be recognized as key regulatory mechanisms. It was later identified that brain structures surrounding anterior wall of third ventricle (AV3V) or lamina terminalis were critical in integrating input from circulating Ang II and transmitting the peripheral information to the higher regulatory centers behind the blood-

brain-barrier (BBB) [40-43]. AV3V ablation studies, conducted in the laboratory of Johnson, found that central actions of Ang II not only play a role in fluid-electrolyte homeostasis [43-45], but also in long-term blood pressure regulation [46, 47]. These findings came to identify AV3V as the central site of action for circulating Ang II [48]. The three brain nuclei of AV3V were later identified as two BBB-lacking circumventricular organs (CVO), subfornical organ (SFO) and organum vasculosum of lamina terminalis (OVLT), and median preoptic nucleus (MnPO) [49]. Soon, the efferent pathways emanating from the CVO to higher homeostatic centers were identified and the role of CVO as primary central sensory organs became established [50]. Out of these two forebrain CVOs, SFO came to be recognized as a key site for the actions of circulating Ang II. SFO neurons have been shown to transcribe AT1R type a (AT1aR) mRNA, but not AT1R type b (AT1b) and AT2R mRNA [51]. Also, SFO has been demonstrated to extensively express AT1R protein [50].

While the endocrine role of circulating Ang II was increasingly recognized in central autonomic regulation [52], the discovery of renin in the brain of a dog by Ganten et al. in 1971 [3] ushered in a period of research investigating this novel aspect of RAS. This paracrine regulatory system came to be known as ‘local’ or ‘tissue’ RAS.

Although this novel concept was gathering nascent evidence in its supportSoon evidence was reported in support of [53, 54], and against [55] a local RAS. Briefly, the controversy arose based on inconsistent observation of renin mRNA in the heart tissue suggesting that the presence of renin in local tissue could be secondary to contamination with plasma and in fact

artefact in nature [55-57]. However, by the early 1990's, the presence of RAS components in brain, kidney, adrenal, heart, vasculature, reproductive organs, GIT, was fairly established. However, their functions were still elusive [54].

The brain RAS, in particular, has been extensively studied, and hence, is one of the best characterized local RAS [4]. Pertinent to the aims of this thesis, a short discussion of local RAS in the neuroendocrine regulatory region of forebrain is provided. The RAS components were identified in human and animal brain nuclei involved in autonomic and neuroendocrine function, such as hypothalamic and medullary regions [16, 58-64]. Renin protein, renin mRNA, and renin-like activity, have all been found to be present in rat brain, higher in the hypothalamus and pituitary glands, [65-68]. Angiotensinogen mRNA has been reported to be present in rat brain [69-71], and was found to be colocalized with glial marker, glial fibrillary acidic protein (GFAP) [71, 72]. Expression of Angiotensinogen mRNA and protein was observed to be higher hypothalamus and brain stem than other regions of the brain [60, 71, 73, 74]. Despite the inability of Ang II cannot cross the blood brain barrier [75-77]. However, Ang II was also found in high levels in the hypothalamus of rats [78] suggesting a biological role of locally synthesized Ang II. Later, Ang II immunoreactivity was reported in neurosecretory hypothalamus and pituitary gland was identified, and hence, the existence of intrinsic hypothalamo-neurohypophyseal local RAS was recognized [79]. The presence of Ang II receptors have been consistently reported in rat brain tissue [80-83]. AT1aR, but not AT1bR and AT2R mRNA were found to be expressed in PVN of hypothalamus [51], whereas AT1bR mRNA was noted in anterior pituitary of rats [84-86].

In the last 10 years, our understanding of functional local RAS in brain has been greatly advanced [87-92]. The genetically modified rats with a significant decrease in brain angiotensinogen exhibited a) significant reduction in basal systolic blood pressure,b) produced a high volume of low-osmolarity urine [93], and c) a reduction in plasma AVP [94]. Further support of the close association in brain RAS and central osmoregulation comes from microinjection studies. Direct infusion of hyperosmotic solutions in the PVN was found to be associated with increased Ang II immunoreactivity in PVN [95]. Microinjection of Ang II in PVN and SON of rats produced antidiuresis [96] indicating local RAS activation contributes towards neurohypophyseal secretion of AVP. Not only acute regulation, brain RAS activation also induced transcriptional events that are associated with long-term neuroplasticity in the neuroendocrine nuclei of hypothalamus after water deprivation [97-99], volume expansion [100, 101], saline-intake [102], injection of Ang I into rat SFO [103], and bile-duct ligation [104], an animal model associated with fluid-electrolyte dysregulation,. Together, these observations strongly suggest that activation of circulating RAS and/or brain RAS could play a pathogenic role in the diseases associated with fluid-electrolyte dysregulation.

Following is presented a brief review of research observation pertaining involvement of RAS in obstructive sleep apnea pathophysiology and hydromineral balance impairment observed in chronic liver failure. The research work in this dissertation is an endeavor to investigate the contribution of these two functionally overlapping regulatory systems [105], circulating and local RAS, in the pathophysiology of these diseases. In the first project, an attempt was made to identify the importance of circulating Ang II and SFO communication in chronic intermittent hypoxia associated sustained increase in blood pressure. The aim of the second project was to

investigate, in an in vitro neuronal model, if Ang II directly causes translocation of a non-specific cation-channel (TRPV4) that is increasingly implicated in osmosensory pathophysiology [106].

LITERATURE REVIEW

OBSTRUCTIVE SLEEP APNEA AND CIRCULATING RENIN ANGIOTENSIN SYSTEM

Obstructive sleep apnea (OSA) is associated with repeated episodes of nocturnal apnea of varying duration and frequency leading to oxygen desaturation and is terminated by arousal [107]. The episodes of nocturnal hypoxemia cause sympathoexcitation via chemoreflex activation [108, 109]. Over the due course of time, these intermittent hypoxic exposure leads to functional remodeling of the neural autonomic circuits [110-113] which manifests as sustained increase in the central sympathetic outflow and elevated blood pressure, even during the periods of normoxia [108, 114]. This chronic and sustained increase in sympathetic nerve activity (SNA) contributes to increased mean arterial pressure (MAP) [115-117] and hence, to cardiovascular disease [118, 119]. The pathophysiology underlying this sustained increase in sympathetic nerve activity (SNA) and mean arterial pressure (MAP) even during periods of normoxic wakefulness remains unknown.

Although positive airway pressure [continuous (CPAP) or bi-level (BiPAP)] therapies are observed to be the most effective in reducing blood pressure [120] and normalizing SNA activity in OSA patients [121, 122], however, the compliance [123], and side-effects [124] with these treatments remains a challenging issue and the decrease in blood pressure can be suboptimal [125]. Hence, it is important understand the CNS adaptations and their underlying mechanisms

that lead to chronic elevation of SNA in OSA patients. This understanding will help to develop more effective therapeutic modalities aimed to prevent cardiovascular morbidities and mortality associated with OSA [119, 126].

The autonomic centers of central nervous system (CNS) set a basal sympathetic tone or central sympathetic outflow which is modulated by peripheral chemo- and baroreflex [127]. A sustained increase in SNA is observed in OSA [128]. The increase in renal SNA (RSNA) activates the renin-Angiotensin system (RAS) [129], which is also noted to be augmented in OSA patients [130]. Ang II, a vasoactive peptide that is unable to cross the blood-brain barrier (BBB), has been implicated in increased MAP in OSA [131]. Classically, circulating angiotensin II (Ang II) is known to act on the subfornical organ (SFO) [132], [133], to promote drinking behavior [132] [134] and salt-appetite [135]. The SFO is also connected with various CNS autonomic centers [136-139] that determine resting sympathetic tone [127] but are protected behind the BBB. It is plausible that circulating Ang II is sensed by the SFO and this region transmits sensory information to the autonomic nuclei in the brain. The hypothalamic nuclei then integrate the information and reset the resting MAP set-point to a higher level.

Rat model of chronic intermittent hypoxia (CIH) has helped in our understanding of pathophysiology of OSA [113, 114, 117, 140-146]. Our animal model of chronic intermittent hypoxia (CIH), based on that introduced by Fletcher et al. [147], involves subjecting adult male Sprague-Dawley rats to episodic hypoxia by altering ambient air in specialized plexiglass chambers. We exposed the rats to alternative cycles of hypoxia (3 min, 10% O_2) followed by nomoxia (3 min, 21% O_2) for 8 hours during the light phase of 12:12 light:dark cycle. After 7

days of CIH, the rats manifested increase in MAP, not only during the light phase, but also during the dark phase of normoxic breathing, [111, 119, 130, 148, 149]. In addition, after 7 days of CIH, the rats demonstrate exaggerated hypothalamo-adrenal-adrenal (HPA) response to novel restraint stress [113], and hence, simulating HPA dysregulation noted in OSA patients [150]. Hence, we contend that our model pathophysiologically mimics neural adaptations observed in OSA and can, therefore, provide insights into the central mechanisms that contribute towards OSA associated chronic HTN [151].

The Ssubfornical organ (SFO) is a forebrain circumventricular organ (CVO) located in the anteroventral wall of third ventricle (AV3V) and is devoid of BBB [40, 152, 153]. The electrical [154, 155] and Ang II [156] mediated stimulation of SFO provokes pressor responses [156]. In addition to Ang II, the absence of BBB enables SFO to sense several other circulating peptides, such as acetylcholine (Ach) [157], amylin [158], leptin [159], atrial natriuretic peptide [160], endothelin [161], vasopressin [162], interleukin 1β [163], and estrogen [164]. This ability of SFO to sense various circulating signaling molecules enables the CNS to execute integrated regulatory physiologic responses [165].

The importance of SFO in long-term autonomic regulation of blood pressure is recognized [59, 137, 166-171]. In short, SFO is synaptically connected to the paraventricular nucleus (PVN) of hypothalamus [136, 137, 172], median preoptic (MnPO) [173, 174], lateral division of parabrachial nucleus [50, 166], nucleus of solitary tract [138], midbrain raphe [166], caudal ventrolateral medulla (CVLM) [139] and organum vasculosum of lamina terminalis (OVLT) [175]. The PVN of the hypothalamus is implicated in setting basal sympathetic tone by

directly influencing firing patterns of sympathetic premotor nuclei of RVLM and intermediolateral (IML) cell column [176, 177]. The SFO influence PVN neuronal activity by either sending direct projection [178] or by relaying signals through MnPO [179]. PVN receives predominantly GABAergic (γ-amino butyric acid) inhibitory inputs from the SFO [178] and MnPO [180, 181]. In the hypothalamus, Ang II is proposed to act presynaptic receptors and reduce this GABAergic tone on PVN neurons resulting in excitation of spinally projecting PVN neurons and increased SNA[182-184]. SFO neurons have been proposed to utilize Ang II in efferent signal transduction to the PVN [136]. Therefore, it is plausible that increased angiotensinergic outflow from SFO to the PVN resets central sympathetic outflow at a higher level, which manifests as a chronic increase in blood pressure. In support we have observed that after 7 days of CIH increase Fos-B like immunoreactivity, a transcription factor implicated in chronic neuronal activation and neural plasticity [185], in the SFO and PVN along with other lamina terminalis nuclei and brainstem autonomic nuclei. Hence, we hypothesized that knockdown of AT1aR in SFO will prevent CIH associated sustained increase in MAP.

LITERATURE REVIEW

HYDROMINERAL HOMEOSTASIS AND LOCAL RENIN ANGIOTENSIN SYSTEM

Physiologically, extracellular fluid osmolality is remarkably regulated in eukaryotic organisms with high efficiency [186]. Arginine vasopressin (AVP) is a key effector molecule through which this complex orchestration of body fluid-electrolyte homeostasis is achieved [187]. Originally discovered in 1895 as a component present in pituitary extracts [188], the antidiuretic properties of AVP were discovered in 1913 [187]. Initially, the molecular identity/ies of antidiuretic, vasoactive, and parturient molecule/s of pituitary extract hormone/s were unknown [189]. In 1951-4 Vincent du Vigneaud et al. purified this nanopeptide hormone, AVP, followed by its synthesis [187, 190, 191]. The significance of this discovery and scientific contribution was immediately recognized and Vincent du Vigneaud was awarded a Nobel Prize in Chemistry in 1955 "…for the first synthesis of a polypeptide hormone".

AVP is synthesized in magnocellular (MNC) neurons residing in the PVN and SON of hypothalamus and released from the nerve terminals in the posterior pituitary [192, 193]. Owing to these executive roles in maintaining water balance, these neurons are termed as 'command neurons' [186]. These vasopressinergic MNC neurons receive direct input from circumventricular organs (CVO), brain nuclei devoid of a blood brain barrier, located in the anterior wall of third ventricle (AV3V). The CVO neurons seem to serve as the primary osmosensory regions of the brain and regulate the firing rate of MNC and hence, alter the release

of AVP to maintain extracellular fluid (ECF) osmolarity [194]. In addition to this synaptic activation, the MNC neurons have been shown to exhibit intrinsic osmosensitivity [195-197]. Hyperosmolarity increases MNC firing rate and plasma AVP concentration, whereas hypoosmolarity decreases MNC firing rate and plasma AVP concentration [198].

In addition to these osmotic mechanisms, non-osmotic mechanisms are also known to influence the firing rate and the release of AVP from the MNC neurons [199-201]. In diverse pathological syndromes, such as hepatic cirrhosis [202], congestive heart failure [203, 204], and nephrogenic syndrome [205-207], a decrease in osmolality fails to inhibit AVP release. This feed-forward AVP release leads to water retention and contributes to the morbidity and mortality associated with these diseases [203, 204, 208-210]. In these aforementioned diseases associated with impaired osmoregulation, there is also an activated renin-Angiotensin system (RAS) [96, 174, 202, 211-215]. Our understanding of osmotic and non-osmotic mechanisms underlying the pathophysiology of these disorders is limited. In order to identify better therapeutic targets, it is important to identify the characteristics of these osmosensorsy molecules and their physiological regulation.

Nearly 25 years ago, it was reported that SON neurons depolarize in response to hypertonic solution administration [195]. Later, in a voltage-clamp experiment at resting membrane potential, it was reported that in the presence of hypertonic stimuli in rat SON neurons exhibit a depolarizing inward current [216-218]. On the other hand, hypotonic stimuli led to hyperpolarization [217, 218]. These responses were associated with increased membrane conductance caused by non-specific cation channels [196, 197, 216-218]. These observations

identified that osmotic information could translate into an alteration in the opening probability of these non-specific cation channels in specialized osmosensory neurons [186].

FThe first evidence towards the identification of these osmoregulatory non-specific cation channels came from studies in C. elegans, in which Colbert et al. demonstrated that absence of a functional Osm 9 protein led to a loss of avoidance-to-hyperosmotic-solution behavior [219]. Osm 9 shares structural homology with light-sensitive Transient receptor potential (TRP) channel found in Drosophila melanogaster [220, 221]. Blockade of hypertonicity induced responses of osmosensory neurons by ruthenium red, a non-specific TRP channel vanilloid family (TRPV) antagonist, identified TRPV channels as the putative family of mechanosensitive non-specific cation channels that could transduce osmotic information into electrical events [216, 222, 223].

In 2000, two groups independently reported TRPV type 4 (TRPV4), (initially named VR-OAC and OTRPC4) as the osmosensitive member of the TRPV family [224, 225]. Observations of impaired water balance and osmotic secretion of AVP in TRPV4-knockout ($trpv4^{-/-}$) mice suggested that TRPV4 could play a critical role in central osmoregulation and AVP release [226, 227]. In addition to TRPV4, TRPV2 [228] and an N-terminal splice variant of TRPV1 [216, 222] is also implicated in central osmosensation. Based on the ability of these different channels to heteromerize [229, 230], there have been suggestions that heteromerization of these different channel types could form a functional, stretch-inhibited osmosensory channel [231].

TRPV4 channel is one of the best-characterized TRP channels [106, 232-235]. It is a polymodally gated non-specific cation channel, which has been shown to be activated by cell-swelling [224, 225], heat, endogenous (such as arachidonic acid, anandamide, epoxyeicosatrienoic acid metabolites), and exogenous ligands (such as bisandrographolide A, citric acid, apigenin, phorbol esters, GSK 1016790A, RN-1747, and RN-1734) [106]. Newer compounds have been identified, such as HC-067047, that offer more selective inhibition of TRPV4 and aid in our understanding of this ubiquitously expressed ion channel [106, 235, 236].

Bile-duct ligated (BDL) rats simulate chronic liver failure associated with inappropriate release of AVP and dilutional hyponatremia. In BDL rats, we have reported an increased TRPV4 translocation to the membrane fraction in hypothalamic extracts [237]. In addition, we also observed an increase in plasma renin activity, an indirect marker of RAS activation. With inhibition of RAS by salt loading (2% NaCl), not only TRPV4 presence in membrane fraction was normalized but it was also associated with normalization of plasma AVP and drinking behavior [237]. We recently reported that intracerebroventricular infusion of Losartan, Ang II type 1 receptors, prevented increased drinking behavior in BDL rats [238]. The latter study indicated that an activated local RAS in the brain could play an important role in the pathophysiology of impaired osmoregulation. We hypothesized that Ang II directly translocate TRPV4 to membrane in an immortalized neuroendocrine cell line.

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CHAPTER II

ANGIOTENSIN II TYPE 1A RECEPTORS IN SUBFORNICAL ORGAN CONTRIBUTES TOWARDS CHRONIC INTERMITTENT HYPOXIA ASSOCIATED SUSTAINED INCREASE IN MEAN ARTERIAL PRESSURE

INTRODUCTION

Obstructive sleep apnea (OSA) affects about 60 million people in the US [1]. In the Wisconsin Sleep Cohort, the prevalence of OSA among a random sample of subjects aged 30 - 60 years was found to be 24% among men and 9% among women [2]. OSA is increasingly being recognized as a cause of neurogenic [3, 4] and treatment-resistant hypertension [5-9]. Despite the availability and practice of available pharmacologic and non-pharmacologic interventions, OSA continues to contribute towards increased cardiovascular [10-14, 15] and cerebrovascular [13, 16-22] morbidities and mortality. OSA is associated with a sustained increase in sympathetic nerve activity (SNA) even during periods of wakefulness and normoxia [23, 24]. In addition, long-term intermittent hypoxia is also associated with potentiated sympathetic response to acute hypoxia [25]. Together, it seems that chronic nocturnal intermittent hypoxic episodes lead to physiologic adaptations that sustains a heightened basal SNA.

An acute increase in renal sympathetic nerve activity (RSNA) stimulates release of renin from the kidneys leading to activation of renin-Angiotensin system (RAS) [26]. Angiotensin II (Ang II) is the chief effector molecule of RAS [27] and the primary receptor for the action of

Ang II is Ang II type 1 receptors (AT1R) [28, 29]. There are two subtypes of AT1R, viz. AT1R type a (AT1aR) and b (AT1bR) [30] that share 94% of sequence similarity [31]. The circulating Ang II is a vasoactive peptide and does not cross the blood-brain-barrier (BBB). For central actions, circulating Angiotensin II (Ang II) is known to act on subfornical organ (SFO) [32], a circumventricular organ (CVO) that lacks BBB and extensively express Ang II type 1 receptors (AT1R) [33] and only AT1aR mRNA [34]. Ang II actions on SFO are known to promote drinking behavior [32] [35] and salt-appetite [36]. The role of peripheral activation of RAS in the pathophysiology of CIH associated increase in mean arterial pressure (MAP) is well accepted [10]. For example, in rats exposed to CIH, peripheral administration of Losartan, a non-specific AT1R antagonist, has been shown to prevent the increase in MAP [37-39]. We have recently shown that electrolytically ablating anterior wall of third ventricle, which includes SFO, also prevents CIH associated sustained increase in MAP [40].

In this study, we hypothesized that knockdown of AT1aR in SFO will prevent sustained increase in MAP associated with CIH. To test this hypothesis, we utilized recombinant adeno-associated virus (AAV) vector, which are highly neuron specific [41], to deliver GFP-tagged small-hairpin RNA (shRNA) to silence the gene encoding for AT1aR (AT1aRshRNA) in the SFO. As a control group, a separate group was injected with shRNA with a scrambled sequence (SCMshRNA).

METHODS

Animals

All experiments 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 at Fort Worth. Adult male Sprague-Dawley rats (250-300 g body weight; Charles River) were individually housed in a temperature controlled room on a 12:12 light:dark cycle with light onset at 0700 h. Food and water was provided ad libitum. All surgeries were performed using aseptic techniques. To prevent infection and post-operative pain, each rat was treated with an antibiotic (procaine penicillin G, 30,000 U s.c.) and Buprenorphine (0.03 mg/kg s.c.) respectively. Animals were divided into four groups, 1) SCMshRNA – Normoxia (n=7), 2) AT1aRshRNA – Normoxia, (n=10), 3) SCMshRNA – CIH, (n=9), and 4) AT1aRshRNA-SFO-CIH (n=8). To validate anatomical specificity of our results, an additional group of rats received AT1aRshRNA dorsal to SFO (AT1aRshRNA-nSFO-CIH) and exposed to CIH (n=10) (Figure 1).

Microinjection of viral vectors.

The viral vectors (GENEDETECT®, Auckland, NZ) carried small hairpin RNA (shRNA) either against AT1aR or control scrambled sequence (AAV-shAT1aR or AAV-shSCM). For microinjection of viral vectors in the subfornical organ (SFO), the rats were anesthetized with isoflurane (2%) and positioned in a stereotaxic frame. After leveling the skull on the frame, midline incision was made in the skin over the skull. A small burr hole was then drilled in the skull using coordinates from the Paxinos and Watson Atlas of rat neuroanatomy. In a separate set of rats AT1aRshRNA was delivered 5mm dorsal to the anticipated SFO position to test the

anatomical validity of our results (nSFO). A stainless steel injector (3 to 4 inches) was connected to a Hamilton micro-syringe by calibrated polyethelene tubing. Through the hole, the injector was gently advanced into the brain and at the estimated location of SFO, viral vector (200 nl per rat over 15-30 seconds; 2×10^9 particles/ml) was delivered. After injection of the viral vector, the injector was left at the injection site for an additional 5 minutes. It was then gradually drawn out of the brain, the hole in the skull was filled with gel-foam, and the incision wound was surgically sutured. The rats were allowed to recover for 7 days from the surgery.

Laser-capture micro-dissection (LCM) of GFP labeled subfornical organ

Laser capture microdissection (LCM) was performed as previously described [42]. After delivery of viral vector in SFO, two separate groups of rats (SCMshRNA n=6 and AT1aRshRNA n=6) were maintained in a separate room and allowed to breath room air for 4 weeks. After 4 weeks, each rat was anesthetized with thiobutabarbital (Inactin, 100 mg/kg ip, Sigma-Aldrich, St. Louis, MO, USA) and decapitated. The brain was rapidly removed and frozen in cooled isopentane kept on dry ice. Serial frozen coronal section (10 µm thickness) containing SFO were obtained using a cryostat (Leica Biosystems, Buffalo Grove, IL, USA). The sections were mounted on Polyethylene Naphthalate (PEN) membrane coated slides (Arcturus Biosciences, Mountain View, CA, USA). Brain sections were kept frozen on dry-ice until processed for microdissection. Before microdissecting SFO, the brain sections were thawed for 30 sec on the slides and then fixed by dipping the slides in ice-cold 100% methanol for 1 min in a coplin jar. This was followed by three washes in ice-cold DEPC – PBS. The GFP expressing SFO sections were visualized and dissected using Arcturus® Veritas laser capture microdissection system (13553-00, version-c) equipped with infrared capture laser and ultraviolet cutting laser. Only one

SFO bulb exhibiting GFP expression from each animal was captured onto an Arcturus Adhesive Cap. After capturing SFO, the cap was immediately put on a 0.5 ml RNase free tube containing 30 µl of ArrayPure Nano-Scale Lysis solution and 5.0 µg of Proteinase K (Epicentre Biotechnol Inc., Madison, WI, USA). The tubes were inverted, vortexed, and stored at -80°C.

RNA extraction and amplification

Total RNA was extracted from SFO using ArrayPure Nano-Scale RNA Purification Kit (Epicentre Biotechnol Inc. Madison, WI, USA), as per manufacturer's instructions. Briefly, samples were incubated for 15 min at 65°C followed by protein precipitation (18 µl of MPC protein precipitation reagent). Samples were then vortexed and centrifuged at 10,000 g for 7 min at 4°C. Supernatant was separated and treated with 50 µl of isopropanol followed by centrifugation at 10,000 g for 5 min at 4°C to precipitate RNA. The pellet was air-dried, dissolved and incubated for 10 min in 20 µl of DNase I solution (1 µl RNase-Free DNase I and 40 µl of 1X DNase buffer) to remove contaminating DNA. Protein precipitation was carried out as previously described using MPC Protein Precipitation reagent and purified RNA was precipitated from the supernatant with isopropanol. Purified RNA pellet was air-dried and dissolved in 5 µl of RNase free water supplemented with 1 µl of ScriptGuard RNase inhibitor (Epicentre Biotechnol Inc., Madison, WI, USA). The purity and concentration of RNA was measured by a spectrophotometer (Nanodrop 2000c Spectrophotometer, ThermoScientific Wilmington, DE, USA). RNA samples were then transferred to -80°C and stored until further processing. To amplify the RNA, 1 – 2 µl per purified RNA sample was used. RNA amplification was done using TargetAmp 2-Round Aminoallyl-aRNA Amplification Kit

(Epicentre Biotechnol Inc., Madison, WI, USA), as per manufacturer's instructions and as previously reported [42].

Qantitative Real-Time PCR (qRT-PCR)

50ng of amplified Aminoallyl-aRNA from laser captured SFO was reverse transcribed to cDNA using Sensiscript RT kit (Qiagen Inc., Valencia, CA, USA), as per manufacturer's instruction. Briefly, each RT reaction was prepared by mixing 2 µl of 10X RT buffer, 2 µl of dNTP mix (final concentration 10 µM), 1 µl of RNase inhibitor (final concentration 10 U/µl), 1 µl Sensiscript reverse transcriptase, 50 ng of aRNA, and RNase-free water to yield final volume of 20 µl. The RT reactions were then incubated at 37°C for 60 min to obtain cDNA.

Each PCR reaction consisted of 2 µl of cDNA, 0.2 µl each of Forward and Reverse primers (Integrated DNA Technologies, Coralville, IA, USA), 12.5 µl of iQ SYBR Green Supermix (Bio-Rad Laboratories Inc., Hercules, CA, USA), and 10.3 µl of RNase-free water. PCR reactions were performed in a Bio-Rad iQTM5 iCycler system (Bio-Rad Laboratories Inc., Hercules, CA, USA). To normalize gene expression, 18S mRNA was also measured using qRT PCR. For each PCR experiment, no-RT and no-template controls were performed. The data was analyzed using $2^{(-\Delta\Delta Ct)}$ method, as reported previously [42].

Ang II induced drinking response and c-Fos expression experiments

A separate set of animals were tested for functional effect of AT1aR gene knockdown in SFO by measuring their water intake 2 h after subcutaneous (SC) injection of Ang II (2mg/kg body weight). All experiments were conducted between 0900 and 1000 h. Animals were tested 5 days and 1 day prior to microinjection of viral vector. The animals were injected with either

SCMshRNA (n=6) or AT1aRshRNA (n=7) in SFO as described before. After virus delivery, drinking tests were conducted every week until 4 weeks. On a typical drinking test day, animals are weighed and 30 min prior to SC Ang II injection food was removed and the water bottles were replaced with graduated drinking tubes. Baseline water intake was measured for 30 min. This was followed by SC Ang II injection and water intake was measured after 2 hours.

In addition to Ang II induced drinking response, these animals were also tested for Ang II induced c-Fos (1:2000 rabbit anti-c-Fos, Santa Cruz Biotechnologies, Santa Cruz, CA, USA) expression in neuroendocrine and autonomic nuclei of brain. On the day of sacrifice, animals were injected SC Ang II and deprived of food and water for 2 hours. After 2 hours, the animals were anesthetized with thiobutabarbital (Inactin, 100 mg/kg ip, Sigma-Aldrich, St. Louis, MO, USA) and transcardially perfused with 4% paraformaldehyde (PFA). Rats were decapitated and their brains were removed and dipped in 4% PFA for 2 hours followed by 30% Sucrose (ThermoScientific, Waltham, MA, USA) solution. After 2-3 days, serial coronal sections of frozen forebrain (40 µm thickness) were obtained using a cryostat (Leica Biosystems, Buffalo Grove, IL, USA) and stored in cryoprotectant at -20°C until processed further for immunohistochemistry (IHC).

Telemetry monitoring of blood pressure, heart rate, respiratory rate, and activity.

In animals assigned for CIH study, A Dataquest IV radio-telemetry system (Data Sciences, St. Paul, MN, USA) was used to continuously record MAP, heart rate (HR), respiratory rate (RR), and activity (Act). After 7 days of recovery from aforementioned microinjection surgery, each rat was anesthetized (isoflurane 2%) and an abdominal aortic catheter, attached to a

radiotelemetry transmitter (model TA11PA-C40), and was implanted. The transmitter was surgically secured to the abdominal muscles and the abdominal incision was closed. The rats were allowed to recover for an additional 7 days from the surgery.

Chronic Intermittent Hypoxia protocol.

For hypoxic treatment, the cages housing rats were placed in custom-built Plexiglas chambers containing O_2 monitors, as previously described [43]. In short, a typical chamber was placed over a telemetry receiver. The oxygen (O_2) level in the chamber was regulated by a set of user-controlled timers. These timers determined the opening and closing time of the valves connected to the nitrogen (100% N_2) or compressed room air (21% O_2). During this period, the chambers were closed using a replaceable air-tight lid and hypoxia was generated using a 3 min on – 3 min off cycle. Briefly, 3 min of hypoxia was generated in two steps. First, the chambers were flushed with 100% nitrogen (N_2) for 90 seconds so that the chamber O_2 level dropped from 21% to 10%. During the second stage, the nitrogen valve was closed and the chamber O_2 was allowed to remain stable at 10% for another 90 s. After which the chamber was flushed with room air for 3 min. The chamber O_2 typically recovered back to 21% in 45-60 sec. These hypoxia-normoxia cycles were repeated for 8 h during the light (nocturnal) phase, from 0800-1600 h. The effect of air-flow and the noise caused by the valves on the sleep and activity was telemetrically assessed. During 16 h of normoxia (1600h – 0800h), the lids were removed from the chambers and the animals remained exposed to room air. CIH exposure was continued for 7 days. Animals in Normoxia group were housed in the same room and allowed to breathe room

air. The experimental duration from the day of virus injection until the day of animal sacrifice was 4 weeks (Figure 2).

Data Acquisition and Analysis.

The MAP, HR, RR, and Act data was radio-telemetrically acquired using and electronically recorded by Dataquest A.R.T 2.2 (Data Sciences International, St. Paul, MN, USA). The data were sampled for 10 s every 10 m and further reduced to 1 h averages throughout the 24 h period. The telemetry transmitter offset was measured postmortem and used to adjust for any changes in MAP caused by drift in the radio signal.

Immunohistochemistry

On the morning after the last CIH exposure, rats were anesthetized using thiobutabarbital (Inactin, 100 mg/kg ip, Sigma-Aldrich, St. Louis, MO, USA) and transcardially perfused with 4% PFA. Rats were then decapitated and brains were removed and merged in 4% PFA for 2 hours and then transferred to 30% Sucrose (PBS) solution at 4°C. After 2-3 days, when brain fully sink to the bottom, forebrains were frozen and coronally sectioned (40 µm thickness) using a cryostat (Leica Biosystems, Buffalo Grove, IL, USA) and alternatively stacked in three sets. At the level of SFO, one 20 µm slice, after every three 40 µm sections, was placed directly on a gelatin coated slide and coverslipped using aqueous mounting medium (Vectashield® HardSet mounting medium, Vector Laboratories, Burlingame, CA, USA) for verification of injection site. All other stacks of 40 µm sections were stored in cryoprotectant at -20°C until further processing.

One set of forebrain sections from each rat was processed for ΔFosB (goat anti-FosB, Santa Cruz Biotechnology, Santa Cruz, CA, USA; 1:1000) expression. IHC was perfomed as previously described [43]. Sections were processed with biotinylated horse anti goat IgG (1:200; Vector Laboratories, Burlingame, CA, USA) followed by reaction with avidin-peroxidase conjugate (Vectastain ABC kit, Vector Laboratories). This was followed by reaction with 0.04% 3, 3'-diaminobenzidine hydrochloride and 0.04% nickel ammonium sulphate for 11 min. As the antibody binds with both FosB and its splice variant ΔFosB, we will refer the resultant staining as FosB/ΔFosB. Sections were then mounted on gelatin coated slides and dehydrated overnight. Next day, sections were serially dehydrated in ethanol and xylene and coverslipped using toluene based synthetic resin mounting medium (Fisher Chemical Permount^{TM} Mounting Medium, ThermoScientific, Waltham, MA, USA). After drying of the mounting medium, sections were visualized using epifluorescent microscope (Olympus BX41, Olympus, Center Valley, PA, USA) equipped with a digital camera (Olympus DP70, Olympus) and an imaging software (DP manager, v2.2.1). Images were analyzed using ImageJ (National Institutes of Health, v. 1.44p). Forebrain nuclei were identified using a rat brain atlas.

Data and Statistical analyses

Baseline recordings of MAP, HR, RR, and Act were done for 5 days prior to starting CIH. The mean was calculated for recorded variables during Light and Dark Phase. The data for 7 d of CIH are represented as the difference from the respective mean baseline value. The difference in MAP, HR, RR, and Act across groups was determined using Two-way repeated measures ANOVA. All data are expressed as mean ± SEM. Differences in mean c-Fos+ or

FosB/ΔFosB+ cell counts per section and relative mRNA expression were tested by Student's t-test. The differences were deemed significant only if the p-value was found to be less than 0.05.

RESULTS

Viral delivery of AT1aRshRNA in the SFO

Histological examination demonstrated successful delivery of viral vectors, as demonstrated by expression of GFP in the SFO. Representative photomicrographs of GFP expression in SFO are shown in Figure 3. No differences were observed in localization and intensity of GFP expression among animals that received either SCMshRNA or AT1aRshRNA. Verification of injection site was performed for all the animals.

Verification of effective knockdown of AT1aR in SFO

To verify the knockdown of AT1aR, we laser-captured GFP-expressing SFO and measured AT1aR mRNA. Representative images of GFP expressing SFO before and after capturing SFO is shown in Figures 4A and 4B. The captured SFO bulb is shown in Figure 4D. We observed that after 4 weeks of virus delivery, AT1aR mRNA was significantly lower in the rats injected with AT1aRshRNA (n=6) in comparison with those that received SCMshRNA (n=7) ($p<0.05$) (Figure 4D). We normalized AT1aR mRNA against ribosomal 18S mRNA. The GFP mRNA was not significantly different between two groups ($p>0.05$).

Effect of AT1aR knockdown in SFO on Ang II induced drinking behavior

SFO is the primary site of action for dipsogenic effects induced by peripheral administration of Ang II [32] [35]. As SFO express AT1aR mRNA [44], we tested if AT1aRshRNA delivery in the SFO results in attenuation of subcutaneously administered Ang II induced water intake. Before the injection of viral vectors, there was no difference in Ang II (2mg/kg body weight) induced water intake between the groups. After 1 week of virus injection,

however, the rats that received AT1aRshRNA (n = 7) in SFO demonstrated significantly attenuated drinking response in comparison with those that received SCMshRNA (n = 6). There was no significant within-group difference in drinking behavior over time ($p>0.05$) (Figure 5).

In addition to drinking response, circulating Ang II is known to activate central neuroendocrine [35, 45-47] and autonomic [48-51] nuclei through its actions on the SFO [52-54]. One of the markers of acute neuronal activation is transcription factor c-Fos [55, 56]. We tested if AT1aR knockdown in the SFO affect peripherally administered Ang II induced expression of c-Fos in the SFO, OVLT, MnPO, and hypothalamic paraventricular and supraoptic nuclei (PVN and SON, respectively). We found a significant reduction in c-Fos positive neurons per section in the rats injected with AT1aRshRNA in comparison with SCMshRNA ($p<0.05$). There was no significant difference in the c-Fos staining in the OVLT between two groups. Representative photomicrographs of OVLT, SFO, and MnPO are shown in Figure 6 and those of PVN and SON are shown in Figure 7. Cumulative summary data of the counts are shown in Figure 8. Among the sub-regions of PVN, significant decrease in c-Fos staining was observed in posterior magnocellular (pm), medial parvocellular (mp), and dorsal cap (dp) ($p<0.05$) (Table 1). There was a trend towards decrease in c-Fos positive neurons in ventrolateral parvocellular (vlp) and lateral parvocellular (lp) neurons, however, the difference did not achieve statistical significance.

Baseline measurements of MAP, HR, RR, and Act

Baseline recordings were done for 5 days prior to starting CIH exposures. The mean values during Light and Dark Phase were calculated and differences between groups were

measured (Table 2). There was no significant difference between baseline recordings of MAP, HR, RR, and Act between groups during light phase and dark phase ($p<0.05$). Data from each of the 7 d of CIH are termed as IH 1 through 7.

Effect of AT1aR knockdown on MAP

During Light Phase (CIH): On day 1 (IH1), a significant increase in MAP was observed in all groups that are exposed to CIH, viz. SCMshRNA-CIH, AT1aRshRNA-SFO-CIH, and AT1aRshRNA-nSFO-CIH as compared to both Normoxic groups (Two-way RM ANOVA $p<0.05$) (Figure 9A). There was no difference in MAP between SCMshRNA-CIH and AT1aRshRNA-CIH. From IH2 through IH7, SCMshRNA and AT1aRshRNA-nSFO-CIH showed significantly higher MAP than AT1aRshRNA-SFO-CIH group and both Normoxic groups (SCMshRNA-Normoxia and AT1aRshRNA-Normoxia) (Two-way RM ANOVA $p<0.05$). From IH2 through IH7, MAP in AT1aRshRNA-CIH group was not statistically different from both Normoxic groups ($p>0.05$). Groupwise comparison revealed that MAP in all three CIH groups were significantly higher than both Normoxic groups ($p<0.05$). However, among CIH groups, SCMshRNA-CIH and AT1aRshRNA-nSFO-CIH exhibited significantly higher MAP than AT1aRshRNA-CIH ($p<0.05$) (Figure 9A).

During Dark Phase (Room air breathing): On IH1, there was no difference in MAP between any groups. From IH2 through IH5, both SCMshRNA-CIH and AT1aRshRNA -nSFO-CIH were significantly higher than AT1aRshRNA-CIH and both Normoxic groups ($p<0.05$). There was no statistical difference between AT1aRshRNA-CIH and both Normoxic groups ($p>0.05$) (Figure 9B). On IH6, MAP of SCMshRNA-CIH group was significantly different from

all other groups ($p<0.05$). Again, on IH7, MAP of both SCMshRNA-CIH and AT1aRshRNA-CIH-nSFO groups were significantly higher than AT1aRshRNA-CIH and both Normoxic groups (SCMshRNA and AT1aRshRNA). Groupwise comparison revealed that MAP of SCMshRNA-CIH and AT1aRshRNA-nSFO-CIH were significantly higher from AT1aRshRNA-CIH group and both Normoxic groups (Figure 9B). There was no statistical difference in MAP of AT1aRshRNA-CIH and both Nomroxic groups. Both groups of Normoxic control animals, showed no significant difference in MAP from their respective baseline values neither during Light nor during Dark Phase during 7 d of CIH (Figures 9A and 9B).

Effect of AT1aR knockdown on HR

During light phase (CIH): Only on IH1, SCMshRNA-CIH and AT1aRshRNA-CIH were significantly higher than AT1aRshRNA-Normoxia ($p<0.05$). However, on all other days of CIH (IH2 through IH7) there was no significant differences in the HR in groups exposed to either CIH or Normoxia. Groupwise comparison did not reveal any significant difference between any of the groups ($p>0.05$) (Figure 9C).

During Dark Phase (room air breathing): Only groupwise comparison showed that HR in AT1aRshRNA-CIH and AT1aRhshRNA-CIH-nSFO were significantly lower than both Normoxic groups. Although, a statistical significance could not be achieved, the SCMshRNA-CIH group also showed a trend toward a lower HR in comparison with ATaRshRNA-Normoxia and SCMshRNA-Normoxia, ($p=0.067$ and $p=0.096$ respectively) (Figure 9D).

Effect of AT1aR knockdown on RR and Act

During Light Phase (CIH): Groupwise comparison demonstrated that RR of AT1aRshRNA-CIH and AT1aRshRNA-CIH-nSFO were significantly higher than SCM Normoxic group ($p<0.05$). SCMshRNA-CIH group also exhibited higher RR during CIH, however, the difference could not achieve statistical significance ($p=0.08$). Similarly, all three CIH exposed rats exhibited higher RR than AT1aRshRNA-Normoxia but the statistical significance could not be achieved (Figures 10A). There was no difference in RR among groups exposed to CIH ($p>0.05$). In addition, none of the groups showed any difference in Act from baseline and there were no significant differences between groups during Light Phase ($p>0.05$) (Figure 10C).

During Dark Phase (room air breathing): No significant differences were observed in RR or Act across groups during room air breathing (Figure 10B and 10D). Hence, the changes in physiological parameters during room air breathing could not be attributed to changes in sleeping pattern during light phase or due to changes in the RR or activity during dark phase.

Effect of AT1aR knockdown in SFO on CIH associated expression of FosB/ΔFosB

We have previously reported that our protocol of 7 d CIH show enhanced FosB/ΔFosB expression in OVLT, MnPO, and PVN [43]. Here we tested if AT1aRshRNA knockdown in the SFO affect CIH associated FosB/ΔFosB staining in these forebrain autonomic nuclei. We observed a significant decrease in FosB/ΔFosB expression in MnPO and parvocellular divisions (medial, dorsal, and lateral; $p<0.05$ but not ventrolateral $p=0.13$) of PVN in AT1aRshRNA-CIH rats (n=6) in comparison with SCMshRNA-CIH rats (n=6) (Figures 13). There was no

significant difference in FosB/ΔFosB expression in OVLT and magnocellular division of PVN. Representative photomicrographs of FosB/ΔFosB staining in these regions are shown in Figures 11 and 12.

Figures

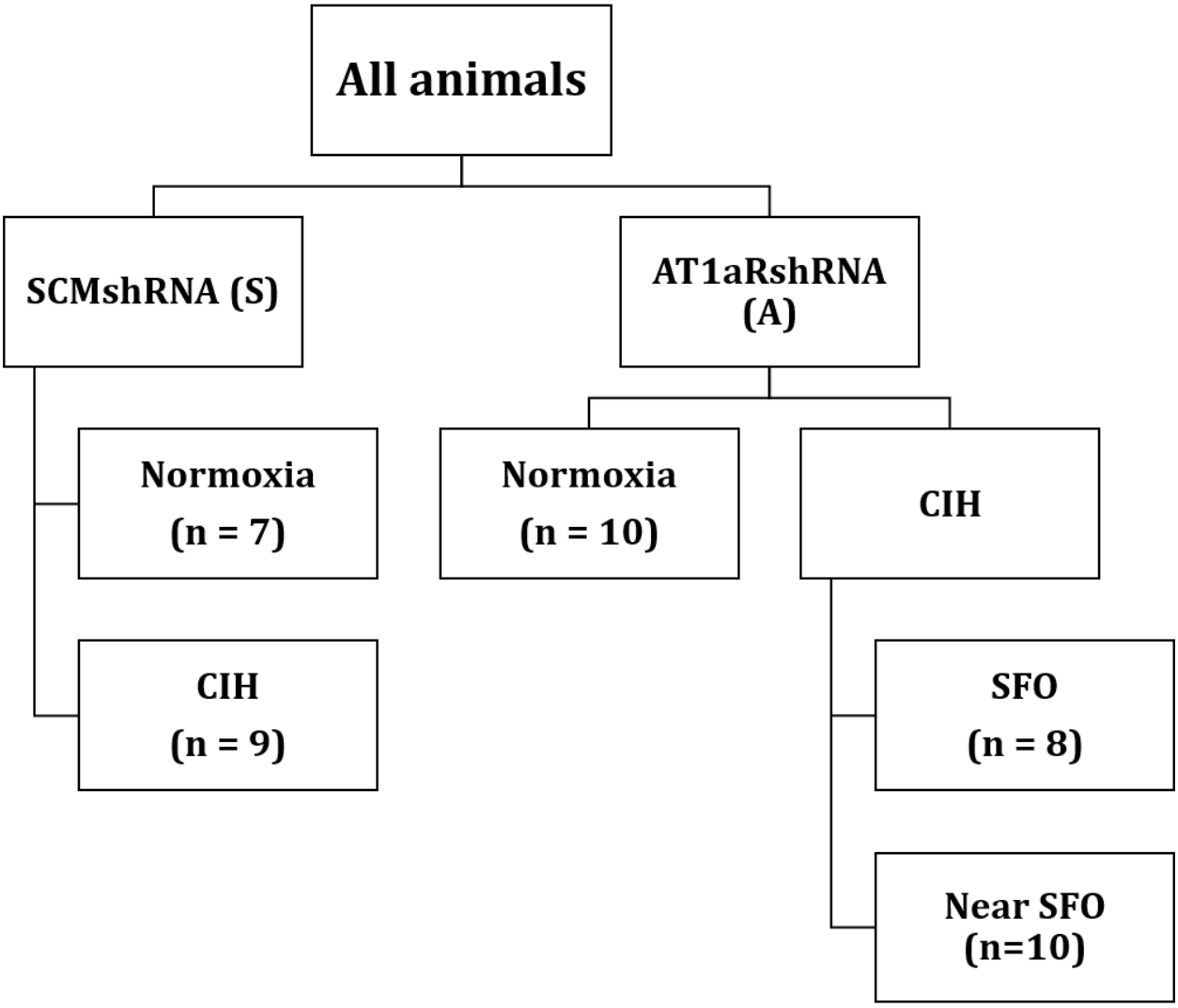

Figure 1: Schema showing animal distribution in various groups for CIH study. n = number of animals per group.

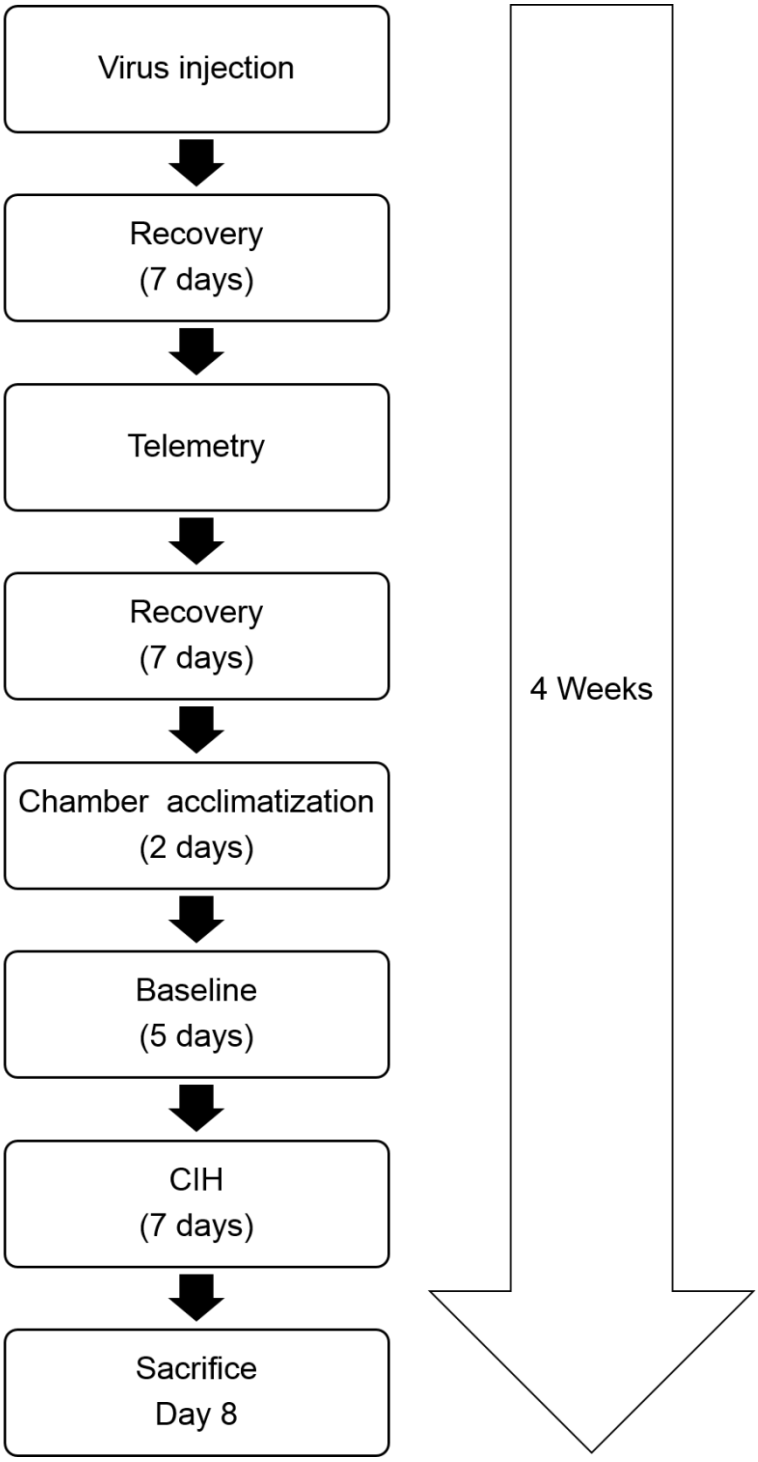

Figure 2: Schema showing experimental design and time-course of CIH study.

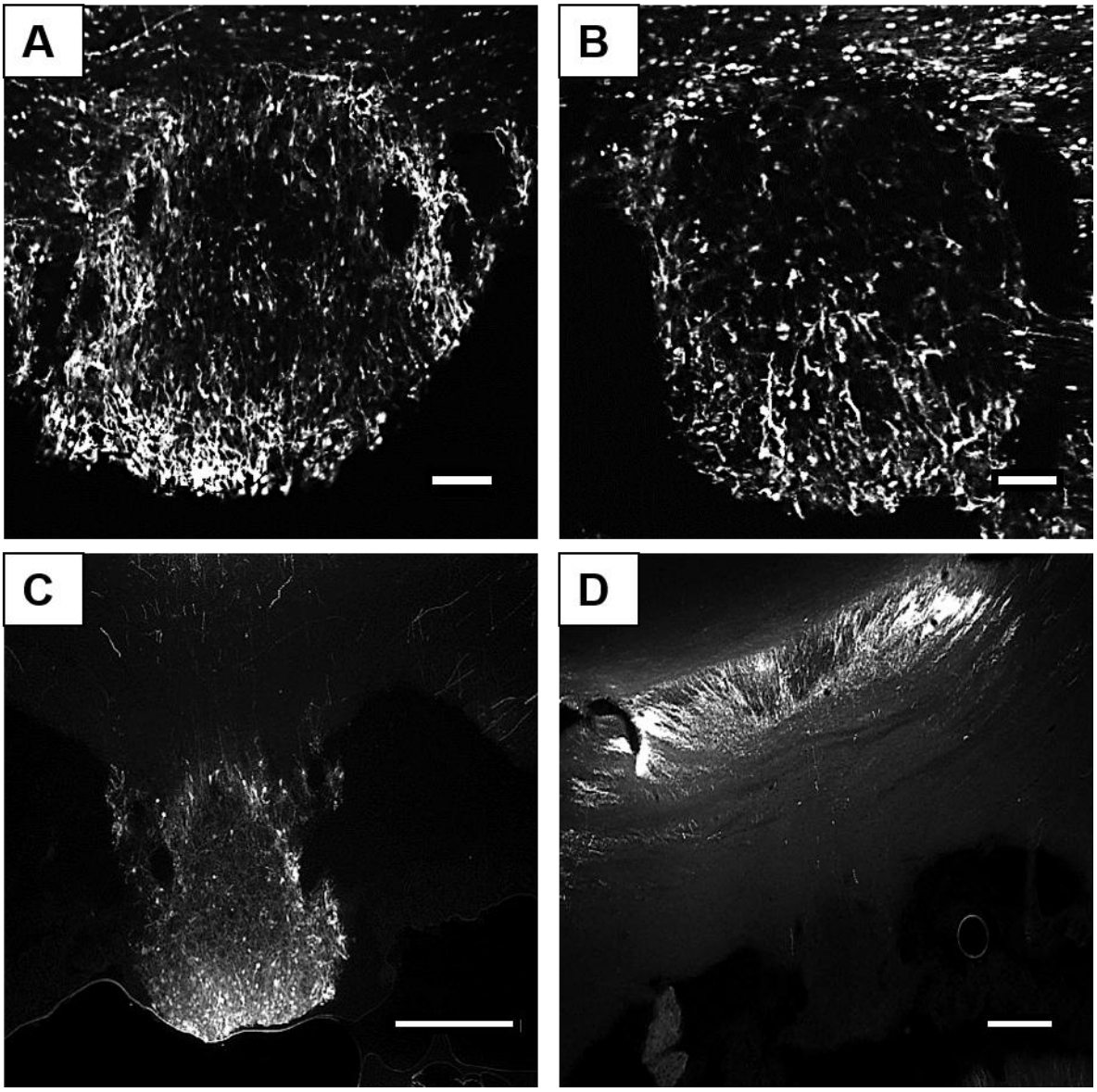

Figure 3: Equivalent transfection of the subfornical organ (SFO) neurons by AT1aRshRNA and SCMshRNA virus constructs. Higher-magnification representative fluorescent confocal photomicrographs of subfornical organ (SFO) demonstrating GFP expression in rats that received A) SCMshRNA, and B) AT1aRshRNA (Scale bar 100 μm). Lower-magnification representative fluorescent confocal photomicrograph of subfornical organ (SFO) demonstrating GFP expression in rats that received AT1aRshRNA C) in SFO and D) near SFO (nSFO) (Scale bar 500 μm).

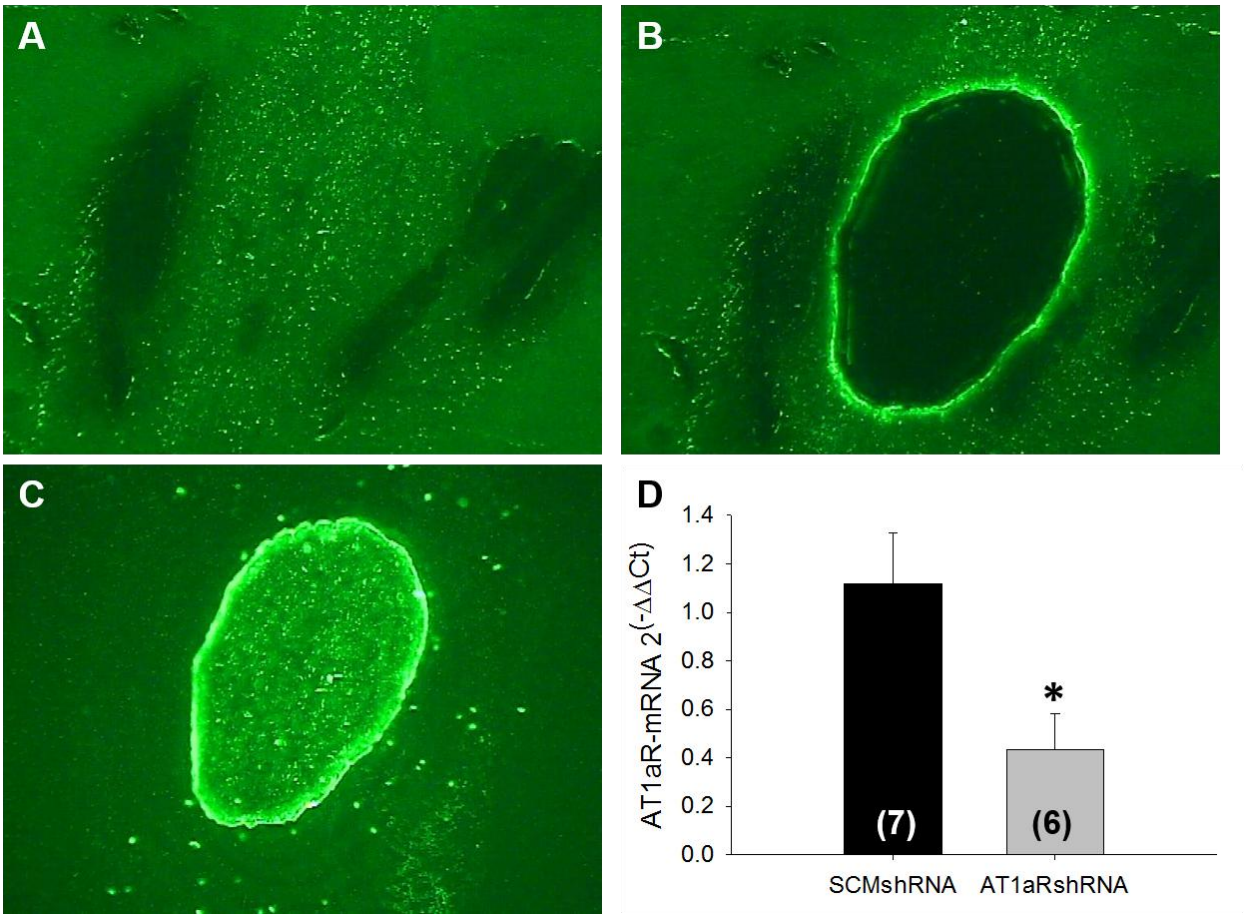

Figure 4: Laser-capture micro-dissection of SFO. Representative micrograph of a forebrain slice showing A) before and B) after capture of GFP expressing SFO and C) removed SFO. D) Bar graph showing a significant decrease in AT1aR mRNA in SFO of rats injected with AT1aRshRNA (n=6) in comparison with SCMshRNA injected rats (n=7). * indicates $p<0.05$

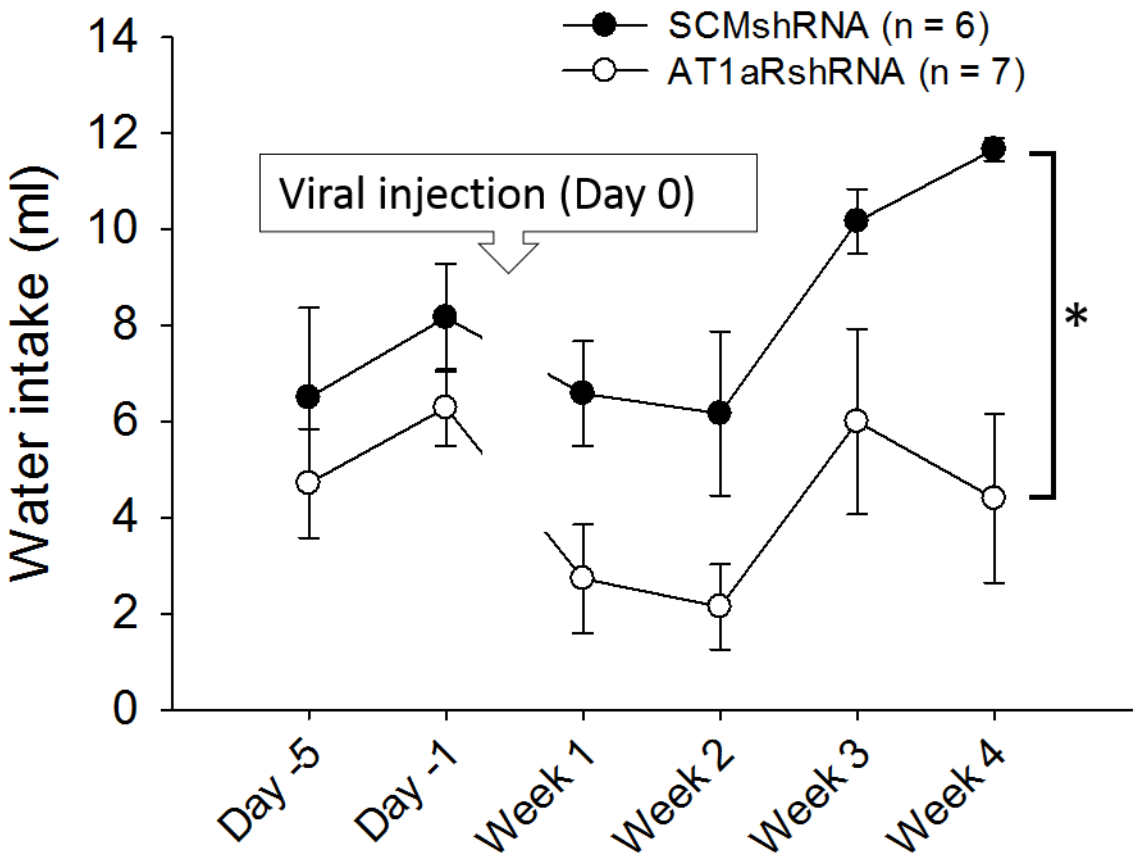

Figure 5: Decreased drinking response to SC Ang II in AT1aRshRNA group. Groupwise comparison revealed that rats that were injected with AT1aRshRNA in the SFO (n=7) exhibited significantly attenuated water intake to subcutaneously injected Ang II (2mg/kg) in comparison with SCMshRNA injected rats (n=6). * indicates $p<0.05$

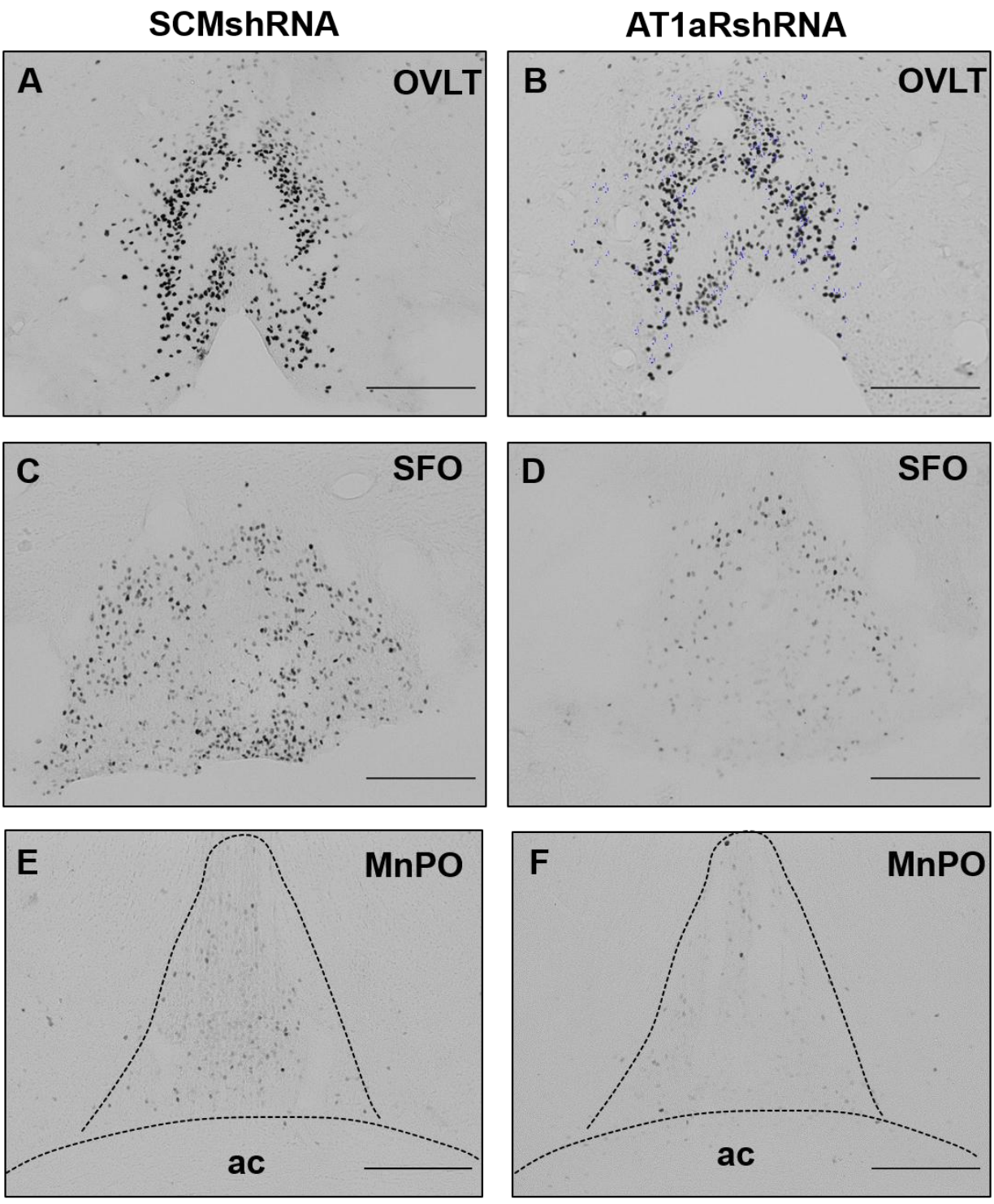

Figure 6: c-Fos expression in AV3V nuclei 2 hours after SC Ang II injection. Representative photomicrographs showing decreased c-Fos staining in A and B) OVLT; C and D) SFO; and E and F) MnPO in rats injected with AT1aRshRNA (right column) in the SFO in comparison with SCMshRNA (left column) injected rats. ac = anterior commissure. (Scale bar 200 µm)

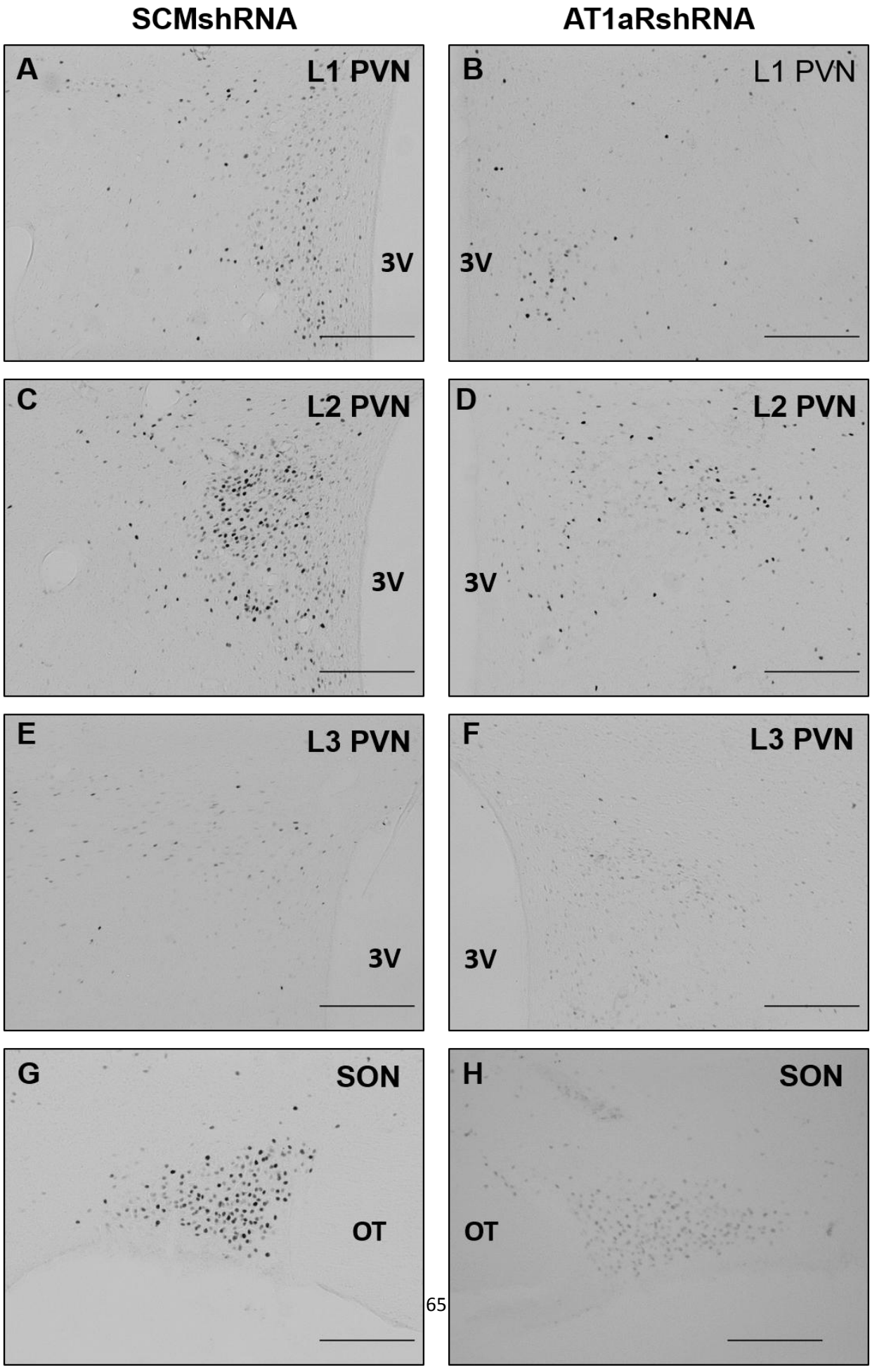
SCMshRNA
AT1aRshRNA
A
L1 PVN
3V
B
L1 PVN
3V
C
L2 PVN
3V
D
L2 PVN
3V
E
L3 PVN
3V
F
L3 PVN
3V
G
SON
OT
H
SON
OT

Figure 7: c-Fos expression in hypothalamic nuclei 2 hours after SC Ang II injection. Representative photomicrographs showing decreased c-Fos staining in A and B) anterior (L1 PVN); C and D) middle (L2 PVN); E and F) posterior (L3 PVN) paraventricular nucleus of hypothalamus; and G and H) supraoptic nucleus (SON) of hypothalamus in rats injected with AT1aRshRNA (right column) in the SFO in comparison with SCMshRNA (left column) injected rats. 3V = third ventricle, OT = optic tract. (Scale bar 200 µm)

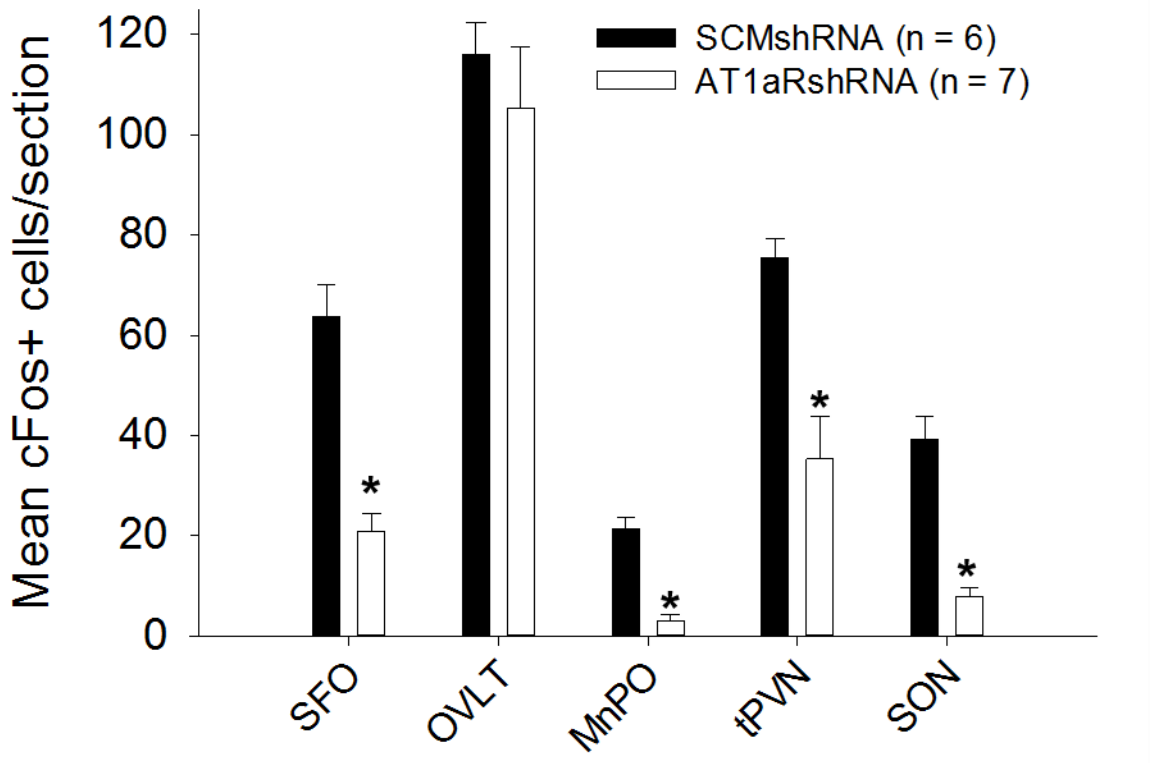

Figure 8: Mean c-Fos positive cells per section of forebrain nuclei 2 hours after SC Ang II injection. Bar graph showing average c-Fos positive cells per section in key autonomic and neuroendocrine nuclei in the forebrain of rats injected with SCHshRNA (black bars, n=6) or AT1aRshRNA (open bars, n=7) in the SFO, after 2 hours of SC Ang II injection (2mg/kg body weight). * indicates $p < 0.05$

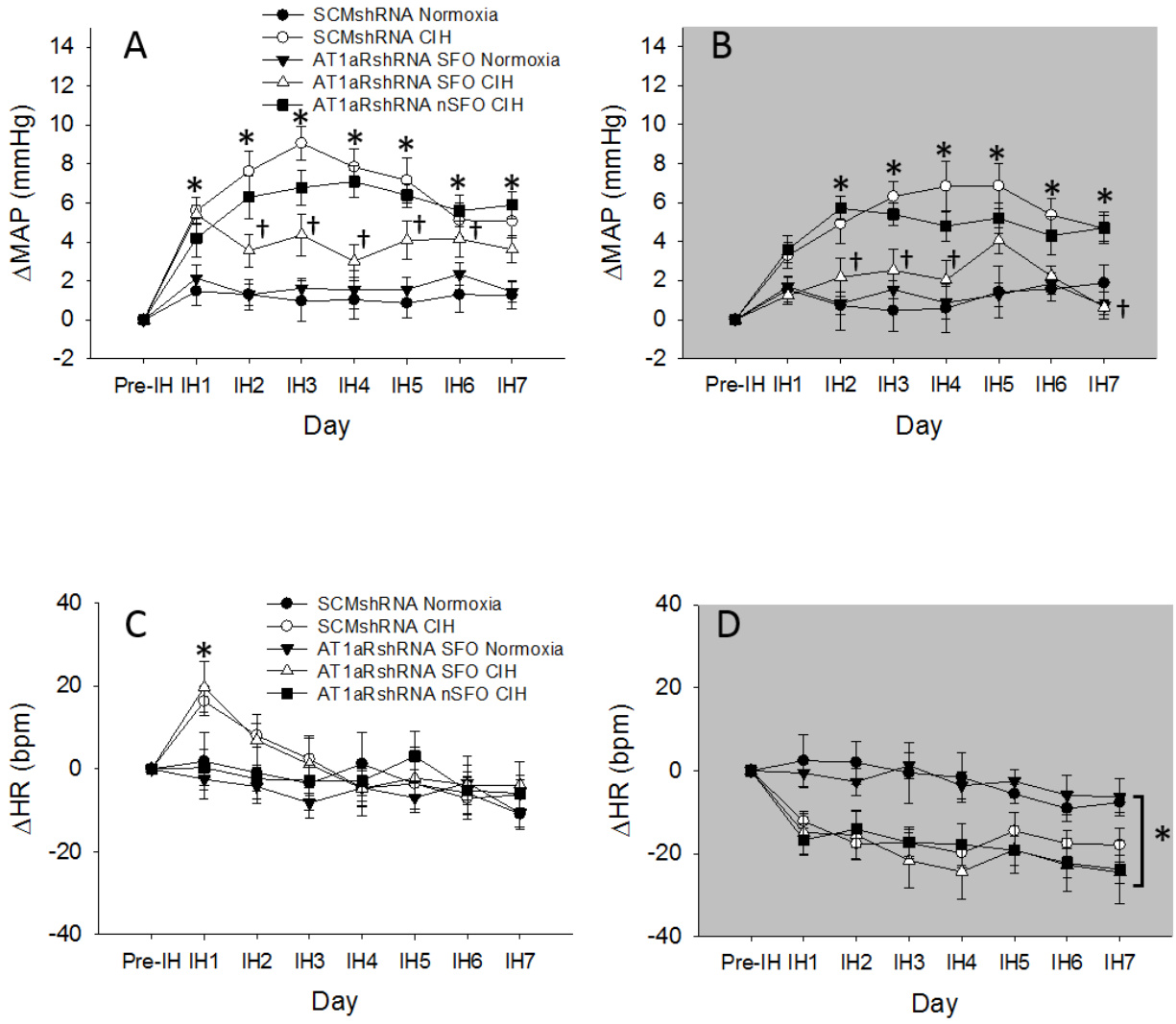

Figure 9: Effect of AT1aR knockdown in the subfornical organ (SFO) on MAP and HR: The changes from baseline (Pre-IH) are shown in MAP during A) Light Phase (CIH), and B) Dark Phase (room air breathing) and in HR during C) Light Phase (CIH), and D) Dark Phase (room air breathing). Data are expressed as mean±SEM, and analyzed using two-way repeated measures ANOVA followed by Student-Neuman-Keuls test. * indicates $p < 0.05$ in comparison with Nomoxic control; † indicates $p<0.05$ in comparison with SCMshRNA-CIH.

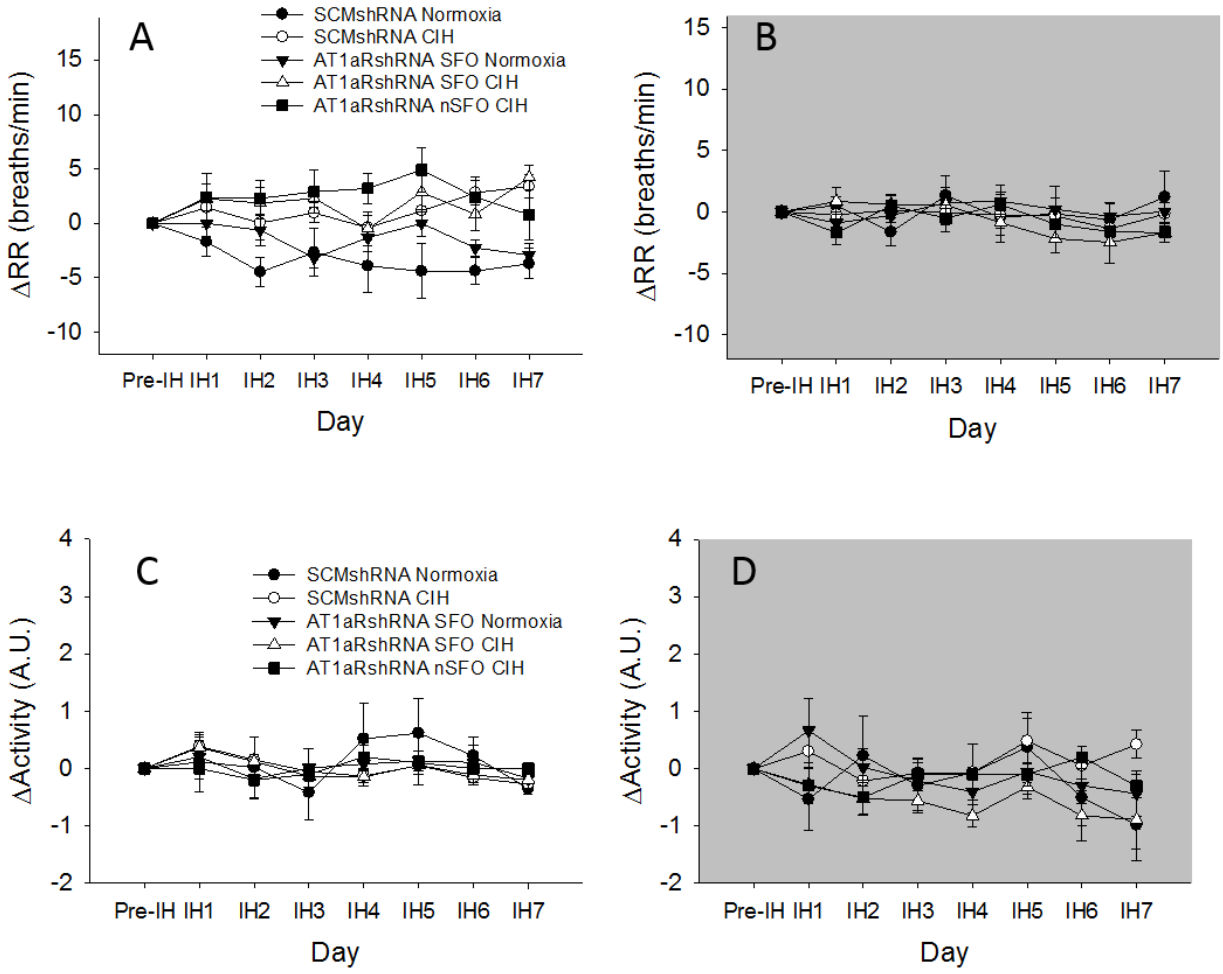

Figure 10: Effect of AT1aR knockdown in the subfornical organ (SFO) on respiratory rate (RR) and Activity. The changes from baseline (Pre-IH) are shown in HR during A) Light Phase (CIH), and B) Dark Phase (room air breathing) and in Activity during C) Light Phase (CIH), and D) Dark Phase (room air breathing). Data are expressed as mean±SEM, and analyzed using two-way repeated measures ANOVA followed by Student-Neuman-Keuls test.

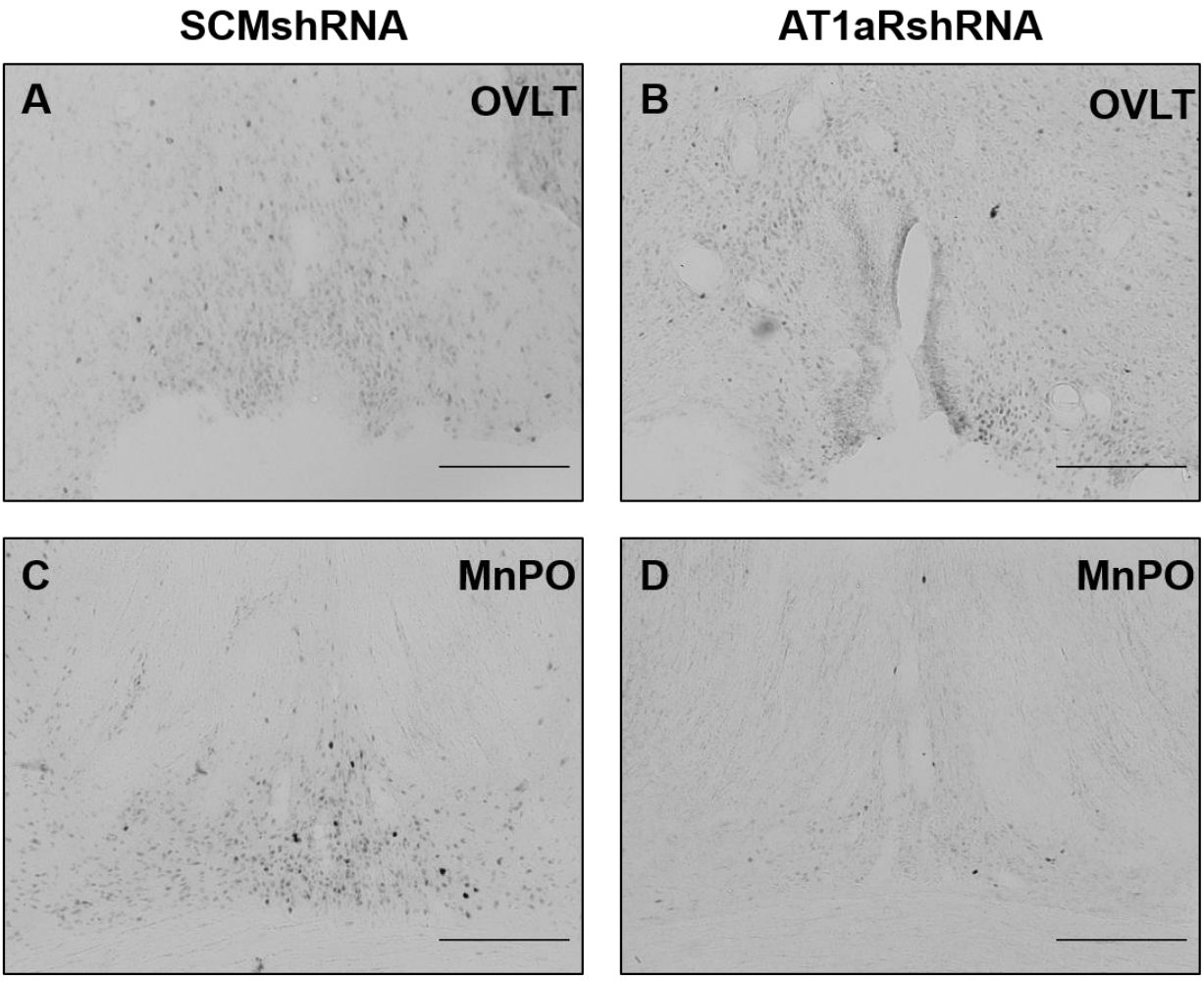

Figure 11: Effect of AT1aR knockdown in SFO on CIH associated expression of FosB/ΔFosB staining in forebrain autonomic nuclei. Representative photomicrographs showing that after 7 days of CIH exposure, rats injected with AT1aRshRNA (right column) in the SFO had decreased FosB/ΔFosB staining in A and B) OVLT; and C and D) MnPO in comparison with SCMshRNA (left column) injected rats. Scale bar is 200 µm.

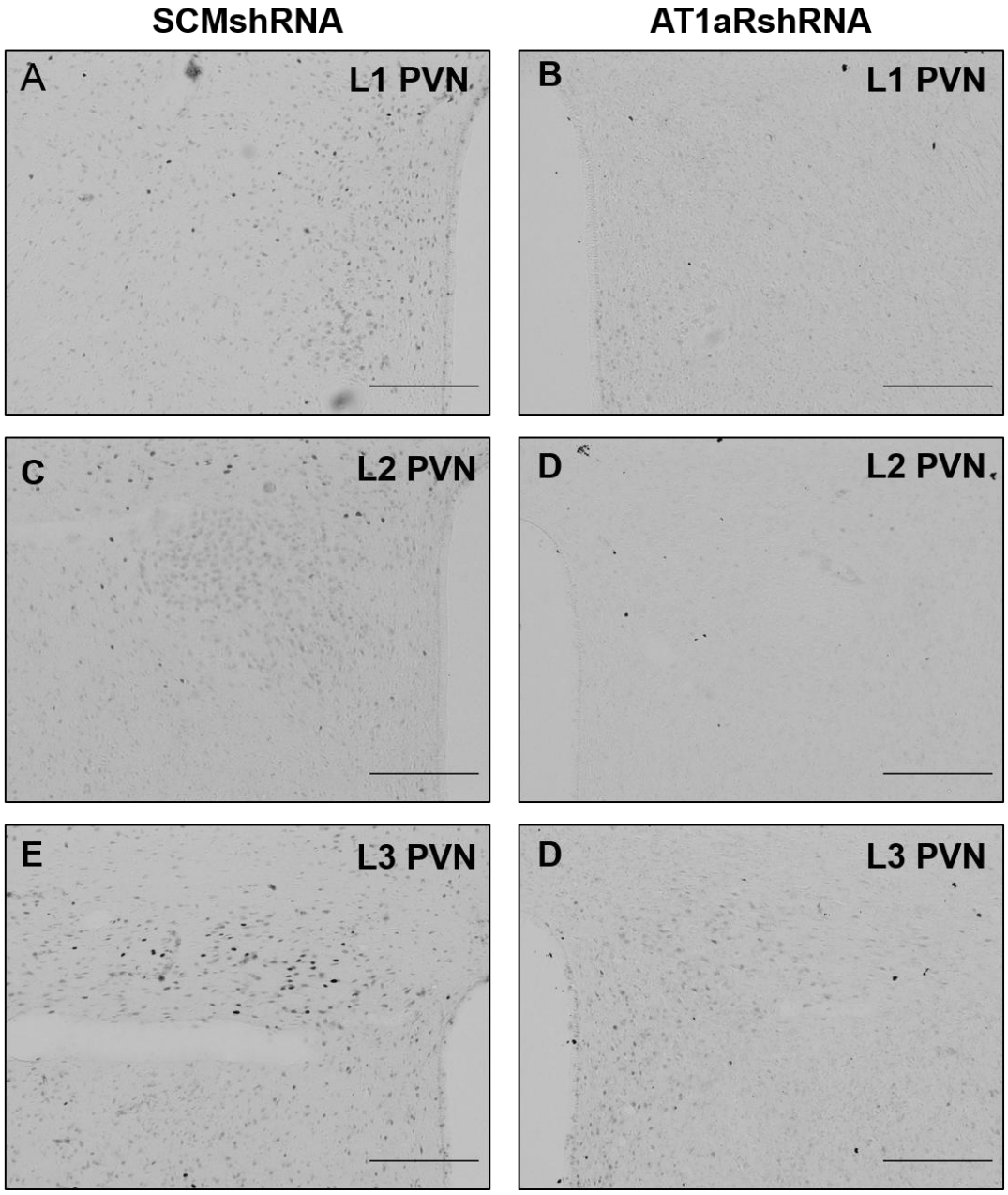

Figure 12: Effect of AT1aR knockdown in SFO on CIH associated expression of FosB/ΔFosB staining in paraventricular nucleus of hypothalamus. Representative photomicrographs showing that after 7 days of CIH exposure, rats injected with AT1aRshRNA (right column) in the SFO had decreased FosB/ΔFosB staining in A and B) anterior (L1 PVN), C and D) middle (L2 PVN), and E and F) posterior (L3 PVN) paraventricular nucleus of hypothalamus in comparison with SCMshRNA injected rats (left column). Scale bar is 200 µm.

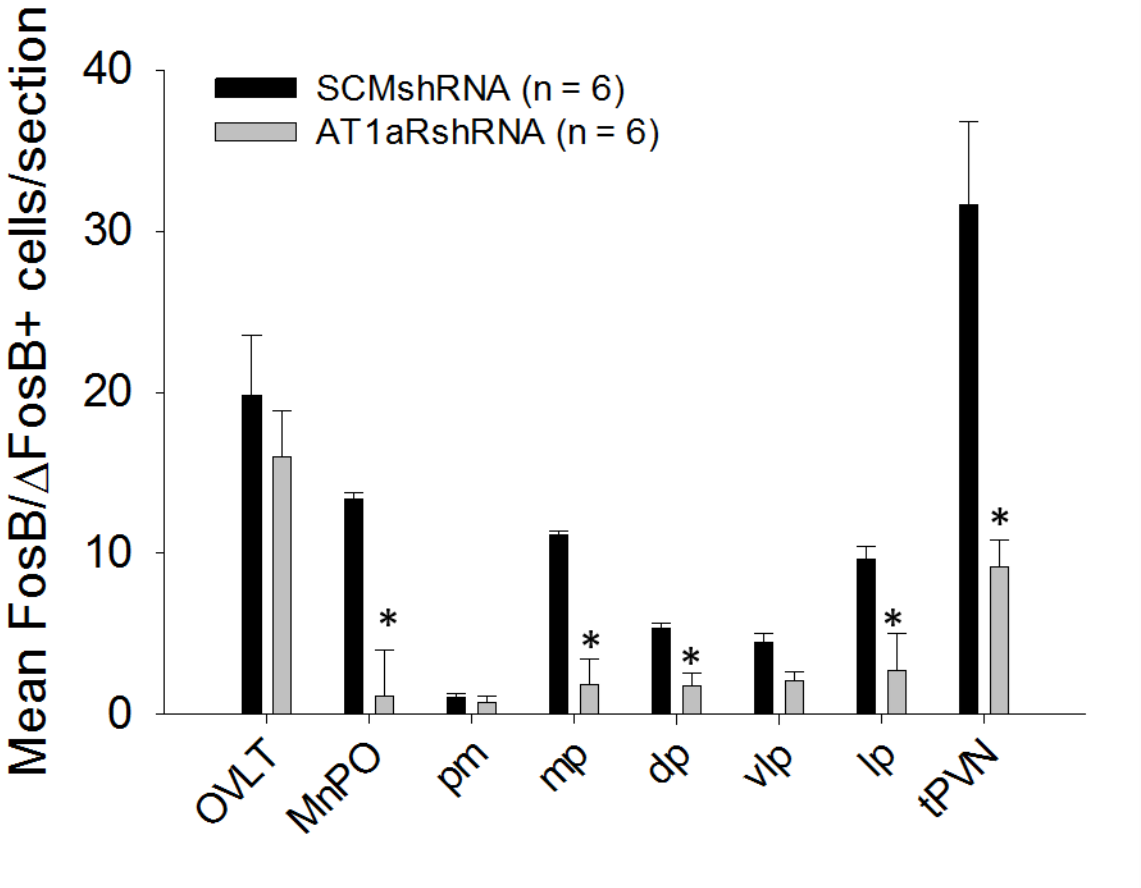

Figure 13: Mean FosB/ΔFosB positive cells per section in forebrain nuclei after 7 days of CIH. Bar graph showing a significant decrease in average FosB/ΔFosB positive cells per section in MnPO, total (tPVN) and sub-regions of PVN in the forebrain of rats injected with AT1aRshRNA (n=6) in the SFO in comparison with SCMshRNA (n=6) after exposure to 7 days of CIH. * indicates $p < 0.05$. pm = posterior magnocellular; mp = medial parvocellular, dp = dorsal cap; vlp = ventrolateral parvocellular; and lp = lateral parvocellular neurons.

PVN subdivision	SCMshRNA (n = 6)	AT1aRshRNA (n = 7)
pm	26 ± 2	8 ± 2 *
mp	16 ± 1	8 ± 2 *
dp	10 ± 1	5 ± 1 *
vlp	12 ± 1	6 ± 1
lp	12 ± 1	9 ± 2

Table 1

Mean c-Fos positive cells per section in subdivisions of paraventricular nucleus of hypothalamus of rats injected with SCMshRNA (left column) or AT1aRshRNA (right column) in the SFO and after 2 hours of subcutaneous injection of Ang II (2mg/kg body weight). Data is presented as mean ± SEM. pm = posterior magnocellular; mp = medial parvocellular, dp = dorsal cap; vlp = ventrolateral parvocellular; and lp = lateral parvocellular neurons. * indicates $p < 0.05$.

		SCMshRNA Normoxia (n = 7)	SCMshRN CIH (n = 9)	AT1aRshRNA Normoxia (n = 10)	AT1aRshRNA CIH (n = 8)
MAP (mmHg)	Light Phase	93.3 ± 2.4	95.5 ± 1.6	94.8 ± 1.7	93.1 ± 1.7
	Dark Phase	98.4 ± 2.7	99.8 ± 1.8	99.9 ± 2.1	97.9 ± 1.6
Heart Rate (beats/min)	Light Phase	343.1 ± 4.1	326.2 ± 6.0	342.5 ± 3.5	332.4 ± 4.9
	Dark Phase	398.6 ± 5.0	384.8 ± 4.4	401.7 ± 4.0	393.8 ± 4.0

Table 2

Average baseline (5 days) MAP and HR of rats injected with SCMshRNA and AT1aRshRNA during Light and Dark Phase. Data is presented as mean ± SEM. No significant difference was observed in the recorded variables.

DISCUSSION

In animal models of CIH, peripheral activation of RAS has been implicated in sustained increase in MAP [57]. In this set of experiments, we investigated if the crosstalk between circulating Ang II with the subfornical organ (SFO) neurons, a CVO lacking BBB, plays a role in sustained increase in MAP associated with CIH. The main finding of this study is that disruption of communication between circulating Ang II and SFO by knockdown of AT1aR in SFO prevents the CIH associated sustained increase in MAP. In addition, we also observed that AT1aR knockdown in the SFO is also associated with attenuated CIH induced FosB/ΔFosB staining in key autonomic regions of forebrain.

The importance of intact renal sympathetic nerves and an augmented renin-angiotensin system in CIH associated increase in MAP is well recognized [37-39, 57, 58]. Ang II is implicated to increase MAP by direct vasoconstriction [59], promoting sensation of thirst [60-63], stimulating water intake [64, 65] and salt appetite [54, 66-68], arginine vasopressin release [61, 69, 70], and regulating central sympathetic nerve discharge to the peripheral vasculature [71-77]. It is well accepted that aforementioned action of Ang II are mediated by its binding to Ang II type 1 receptors (AT1R) on SFO neurons [32, 66, 78-80]. The Ang II-SFO interaction plays an important role in central regulation of blood pressure [78, 81, 82] and electrolytic lesion of SFO has been demonstrated to block the Ang II induced hypertension [78, 79, 81]. We have reported previously that electrolytic lesion in the anterior wall of third ventricle (AV3V), which includes SFO, prevents sustained component of hypertension associated with CIH [43].

On IH1, we observed that rats that received AT1aRshRNA showed increase in MAP during hypoxia similar to SCMshRNA-CIH and AT1aRshRNA-nSFO-CIH groups. This observation indicate that AT1aR in the SFO might not play a role in acute chemoreflex mediated increase in MAP. During the Dark Phase on IH1, there was no difference in MAP of all animals, indicating recovery after first intermittent hypoxic exposure. Beginning from IH2, whereas, SCMshRNA-CIH continued to show significant elevation in MAP during light phase (CIH) and dark phase (room-air breathing) in comparison with the Normoxic control groups, the MAP in AT1aRshRNA-CIH group was not statistically higher than the Normoxic control groups. However, on groupwise comparison of the MAP during the entire 7 d period of CIH exposures, AT1aRshRNA-CIH showed significantly higher MAP than Normoxic group, indicating intact CIH mediated pressor response. Moreover, the MAP of SCMshRNA-CIH and AT1aRshRNA-nSFO-CIH groups were significantly higher than AT1aRshRNA-CIH group. It seems that AT1aR knockdown in SFO did not prevent CIH mediated increase in MAP. However, an intact Ang II-SFO communication seems to potentiate the increase in MAP after subsequent intermittent hypoxic insult and prevent recovery of baseline MAP in the absence of hypoxic challenge.

Our HR data indicate that only on IH1, the hypoxic groups exhibited significantly higher HR. There was no difference in the magnitude of tachycardic response to intermittent hypoxic challenge. Beginning IH2, the magnitude of increase in HR during CIH diminishes and fails to show any changes during CIH exposure in comparison with the Normoxic group. This trend continues for the rest of the days of CIH exposure. However, during dark phase (room air breathing), all three groups exposed to CIH continued to show significantly decreased HR in

comparison to Normoxic groups. These finding indicate that the above discussed differences in MAP between SCMshRNA-CIH and AT1aRshRNA-CIH groups cannot be attributed to changes in HR. The decrease in HR observed during dark phase could potentially indicate an effect due to hypoxic conditioning. In both humans and animals, it has been well identified that intermittent hypoxic exposures can augment vagal tone that contributes to resting bradycardia [83].

In animal models of CIH, plastic changes in the carotid chemoreceptors are observed, i.e. increased sensitivity to hypoxia [84, 85]. It has been shown that CIH mediated adaptations in the carotid chemosensory function could be driven by reactive oxygen species [86]. Recently, evidence of local RAS activation in carotid bodies of rats exposed to CIH has been provided [87]. It is hence, postulated that Ang II could contribute towards CIH associated sustained increases in MAP by resetting the hypoxic set point of chemoreflex and increase drive for sympathetic nerve activity even during normoxic breathing. However, this explanation is insufficient to account for chronic activation of the autonomic circuit in the forebrain [43]. The transcription factor FosB/ΔFosB has been shown to be responsible for long-term neuronal adaptations associated with repeated exposure to drugs of addiction [88]. We have previously demonstrated that CIH is associated with increased FosB/ΔFosB expression in the autonomic nuclei of the forebrain, including SFO, indicating their chronic activation [40]. Additionally, we have also shown that inhibiting transcriptional effect of ΔFosB in the MnPO prevents CIH associated sustained increase in MAP [40]. In our presented experiments we observed that selectively silencing AT1aR in the SFO neurons not only prevented the sustained increases in MAP after CIH but also reduced FosB/ΔFosB expression in forebrain autonomic nuclei. These

findings indicate that Ang II - SFO interaction mediated neuronal adaptations in the MnPO and PVN could be mediated by ΔFosB.

PERSPECTIVES

Obstructive sleep apnea (OSA) is a chronic disease in humans accompanied by repeated spells of apnea during sleeping. These intermittent apneic spells are associated with decrease in blood oxygen saturation [89]. This leads to chemoreflex mediated acute increase in MAP [90]. Over the time, this increase in MAP outlasts the hypoxia experienced during sleep and presents clinically as diurnal hypertension. It has been suggested that it is the hypoxia, and not hypercarbia, that drives the intermittent hypoxic stimulation mediated sympathetic nervous system activation [91]. To identify better therapeutic option, the pathophysiological mechanisms underlying OSA associated hypertension need to be investigated. Here, we report that neurohumoral communication at the level of SFO, a circumventricular organ that lacks blood brain barrier, could contribute in the sustained increase in the MAP associated with CIH.

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CHAPTER III

ANGIOTENSIN II INDUCES MEMBRANE TRANSLOCATION OF NATIVELY-EXPRESSED TRANSIENT RECEPTOR POTENTIAL VANILLOID TYPE 4 CHANNELS IN HYPOTHALAMIC CELL LINE 4B VIA SRC KINASE PATHWAY

INTRODUCTION

The Transient Receptor Potential (TRP) channel superfamily plays a central role in sensory physiology [1, 2]. The TRP subfamily vanilloid member 4 (TRPV4) is a non-selective cation channel that is calcium permeable and shows polymodal activation by diverse stimuli including, moderate heat, cell swelling, and endogenous ligands [3-5]. Functionally, it is implicated in a number of physiological systems that include endothelial function and vascular tone [6, 7], renal function [8], central osmosensation [9, 10] and regulating hydromineral homeostasis [3-5]. Mutations of TRPV4 are linked to a number of motor and sensory neuropathies and skeletal dysplasias [3, 5].

In addition, TRPV4 is expressed in CNS regions that are involved in neuroendocrine function including neurosecretory cells in the supraoptic nucleus (SON) and the paraventricular nuclei of the hypothalamus (PVN) [11, 12]. In a rodent model of cirrhosis, we have previously

reported increased TRPV4 trafficking to lipid rafts in the hypothalamic samples that contained the SON, the PVN, and the organum vasculosum of the lamina terminalis [11]. Importantly, we also found that increased TRPV4 trafficking correlated with activation of renin-angiotensin system (RAS) and was reversed after RAS inhibition [11].

Angiotensin II (Ang II) is an effector molecule of RAS [13]. Circulating Ang II is known to contribute to water and electrolyte homeostasis peripherally and centrally through its actions on blood-brain barrier deficient circumventricular organs in the forebrain and dorsal hindbrain [14, 15]. As part of the brain RAS, Ang II has also been demonstrated to act directly on the neurosecretory neurons residing in the hypothalamus [16-18] and stimulate release of AVP and corticotropin releasing factor (CRF) [19, 20]. Intracellularly, Ang II initiates multiple signal transduction pathways, including non-receptor tyrosine kinase [21-23]. The Src family of non-receptor tyrosine kinases (SFK) have been shown to regulate the function of three major TRP channel families, canonical, vanilloid, and melastatin [24-27] including TRPV4 [28].

The purpose of this study was to determine if Ang II can affect TRPV4 trafficking to the plasma membrane via SFK in hypothalamic neuroendocrine cells using the 4B cell line. Nearly a decade ago, Kasckow et al. generated the 4B cell line by immortalizing embryonic day-19 rat pup hypothalami by retrovirus-mediated transfer of the SV40 large T-antigen [29]. 4B cells exhibit neuronal phenotype, express AVP, CRF, functional CRF type-1 receptors, glucocorticoid receptors [29], and have been used to study CRF and AVP gene regulation [30-32]. To test the role of SFK in the effects of Ang II on TRPV4 translocation, we used a highly specific SFK antagonist, PP2. We conducted whole cell calcium imaging studies using the selective TRPV4

agonist, GSK 1016790A (GSK 101), and the antagonist, HC-067047, to test the functional impact of changes in TRPV4 trafficking.

METHODS

Cell culture

Rat hypothalamic 4B cells were plated at a density of $5x10^5$ in 150mm Nunc plates and grown to confluence. They were grown in DME/Ham's F12 1:1 medium supplemented with 10% newborn calf serum, 1% MEM nonessential amino acids, 1% Glutamax, 1% sodium pyruvate, and 1% pen-strep. For all experiments, cells were serum-deprived overnight.

Immunocytochemistry

Cells were plated at a density of $5x10^4$ on 18mm coverslips (Fisherbrand Microscope Cover Glass, Thermo Fisher Scientific, Waltham, MA, USA) coated with Poly-D-Lysine. Cells were serum deprived overnight before experiment day. On the day of the experiment, cells were treated with test drugs. After treatment, media was replaced with 4% paraformaldehyde in phosphate buffered saline (PFA-PBS) for 30 min to fix the cells. Cells were then washed with DPBS (Dulbecco phosphate buffered saline) three times and blocked with DPBS containing horse serum and Triton-X 100 (blocking solution) for 1 hour. The primary antibodies were mixed in the blocking solution (TRPV4 – 1:1,000, rabbit polyclonal, Sigma-Aldrich, St. Louis, MO, USA #T9075; microtubule associated protein – 2 (MAP-2) – 1:1,000, mouse monoclonal, abcam®, Cambridge, MA, USA #ab11267; glial fibrillary acidic protein (GFAP) – 1:1,000, mouse monoclonal, Sigma-Aldrich, St. Louis, MO, USA #G3893; AVP – 1:1,000, guinea pig,

Peninsula Laboratories, San Carlos, CA, USA #T5048). Coverslips were incubated with primary antibody overnight at 4°C. Next day, coverslips were incubated in fluorophore tagged secondary antibodies against respective host species (Anti rabbit Cy3 – 1:1,000, anti mouse Cy3 – 1:1,000, Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA), for 2 hours at room temperature. Then coverslips were washed three times using PBS and mounted on glass slides using mounting media (ProLong Gold reagent, Thermo Fisher Scientific Inc., Waltham, MA, USA) containing fluorescent nuclear stain, 4',6-diamidino-2-phenylindole (DAPI).

Quantitative Real Time PCR

Total cellular RNA was extracted as previously reported [33]. 4B cells were grown on 150 mm culture dish to 80% confluence. The cells were serum deprived overnight before the day of experiment. On experiment day, cells were lysed with 1ml TRIZOL reagent (Thermo Fisher Scientific, Waltham, MA, USA), according to the manufacturer's instructions. The lysate was then treated with chloroform (Thermo Fisher Scientific, Waltham, MA, USA) and centrifuged at 12,000g for 15 min. Total RNA was precipitated from the aqueous phase using isopropyl alcohol, washed with ethanol, and resuspended in RNase-free water (Qiagen Inc. Valencia, CA, USA). RNA was reverse-transcribed (RT) to cDNA with Sensiscript RT Kit reagents (Qiagen Inc. Valencia, CA, USA), as per manufacturer's instructions. Each RT reaction mixture consisted of 2 µl of 10X RT buffer, 2 µl of dNTP mix (5 mM), 2 µl of oligo-dT primer solution (10 µm), 1 µl of RNase inhibitor (10 U/µl), 1 µl of Sensiscript reverse transcriptase solution, and RNA dissolved in RNase-free water (final volume of RT reaction: 20 µl). Forward- and reverse-

primers for target genes (Table 1) were obtained from Integrated DNA Technologies (Coralville, IA, USA). PCR samples consisted of 2 µl of cDNA, 10.3 µl of RNase-free water (Qiagen Inc. Valencia, CA, USA), 0.2 µl of forward- and reverse-primer, and 12.5 µl of iQ SYBR Green Supermix (Bio Rad, Hercules, CA, USA). PCR reactions were performed (C-1000 ThermoCycler with CFX 96 Real time system; Bio Rad, Hercules, CA, USA) using following parameters: initial denaturation at 95 °C for 3 min, followed by 40 cycles of 70 sec each (40 s at 94°C; followed by 30 s at 60°C) [34]. Data were collected and analyzed using CFX manager (Bio Rad, Hercules, CA, USA). In each real-time RT-PCR analysis, no-template and no-RT controls were performed. Melting curves generated were analyzed to identify nonspecific products and primer–dimers [11, 35].

Drugs and solutions

Hank's Buffered Saline Solution (HBSS) and Dulbecco's Phosphate-Buffered Saline (DPBS) were bought from Thermo Fisher Scientific Inc. (Waltham, MA, USA). Ang II, Losartan, and GSK 1016790A were purchased from Sigma-Aldrich (St. Louis, MO, USA), and PP2, PP3, and HC 067047 were purchased from Tocris Bioscience (Bristol, UK).

Drug treatment protocol

On the day of experiment, cells were incubated in either serum-free culture medium (control) or drugs dissolved in serum-free culture medium. Cells were incubated in Ang II (100 nM) for 1 hour. To observe the effect of Losartan (Ang II receptor type 1 antagonist, 1 µM) and PP2 (selective SFK inhibitor, 10 µM), cells were incubated in either antagonist for 10 min. Then the medium was replaced with the serum-free culture medium containing Ang II (100 nM) mixed with either Losartan (1 µM) or PP2 (10µM), respectively. As a control, in a separate set of experiments, PP2 was replaced with PP3 (10µM), which is an inactive PP2 analogue,.

Western Blot

Sample preparation

Total lysate: Total protein lysate samples were prepared with modifications as described previously [36]. Briefly, after 1 hour of treatment, cells were washed twice with ice-cold DPBS (Life Technologies, Grand Island, NY, USA). Cells were then lysed in the culture dish using RIPA buffer (Thermo Fisher Scientific Inc., Waltham, MA, USA; 25mM Tris-HCl pH 7.6, 150mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS). Protease inhibitors were added to the lysis buffer (1X Halt Protease and Phosphatase inhibitor cocktail, Thermo Fisher Scientific Inc., Waltham, MA, USA). The samples were then briefly sonicated on ice and centrifuged at 12,000Xg for 5 min. The pellet was discarded and the supernatant (total lysate) was transferred to a tube and stored at -80°C until further processing.

Membrane fractions: Membrane fractions were prepared as described previously [37]. Briefly, the culture plates were rinsed twice with ice-cold DPBS. Then, 1 ml subcellular fractionation buffer (250 mM Sucrose, 20 mM HEPES, 10 mM KCl, 1.5 mM $MgCl_2$ 1 mM EDTA, 1 mM EGTA, 1 mM DTT) with protease and phosphatase inhibitors (1X Halt Protease and Phosphatase inhibitor cocktail, Thermo Fisher Scientific Inc., Waltham, MA, USA), was added to the culture dish. The cells were then scraped and lysed by passing the cells 20X through a sterile 25 gauge needle. Samples were briefly sonicated on ice and homogenate was centrifuged at 12,000g for 5 min. After discarding the pellet, the supernatant was ultracentrifuged at 100,000g (Optima MAX-XP, Beckman Coulter Inc., Indianapolis, IN, USA) for 60 min. The pellet (membrane fraction) was dissolved with brief vortex and sonication in freshly prepared RIPA buffer supplemented with protease- and phosphatase-inhibitors. This protocol of membrane fraction preparation was used to test the effects of Ang II incubation on TRPV4, either alone or in the presence of Losartan (1µM), PP2 (10µM), and PP3 (10µM).

Western blot was performed as previously described [11]. Briefly, the protein content of samples was determined by using a detergent-compatible protein assay kit (DC protein assay, Bio Rad, Hercules, CA, USA). The samples (15-20 µg of protein) were denatured by heating in 1X Laemmli buffer (62.5 mM Tris-HCl, pH 6.8, 2% (wt/vol) SDS, 160 mM DTT, 0.001% bromophenol blue, 6 M urea). Samples were then resolved in a 4-20% SDS-PAGE (Mini-PROTEAN Precast Gels, Bio Rad, Hercules, CA, USA) gradient gel in Tris-glycine buffer (25 mM Tris, 192 mM glycine, 0.1% SDS, pH 8.3, Bio Rad, Hercules, CA, USA) and transferred to a nitrocellulose membrane (50 V for 2 h). The successful transfer was determined by incubating the membrane with Ponceau S solution (Sigma-Aldrich, St. Louis, MO, USA) for 2 min. The

membrane was then washed three times with TBST (Tris buffered saline-Tween; 50 mM Tris base, 200 mM NaCl, 0.05% Tween 20), followed by treatment with 5% NFM (non-fat milk, Bio Rad, Hercules, CA, USA) buffer for 1 hour. The membrane was then incubated overnight at 4°C in primary antibody (TRPV4 – 1:1,000, rabbit polyclonal, Sigma-Aldrich, St. Louis, MO, USA #T9075; Src – 1:1,000 rabbit polyclonal, Cell Signaling Technology, Beverly, MA, USA #2123; 4G10® – 1:1,000, EMD Millipore, Billerica, MA, USA #05-1050) dissolved in 5% NFM. The next day, the membrane was rinsed three times for 10 min each with TBST and incubated in horseradish peroxidase conjugated secondary antibody against respective host species (1:2,000; Sigma-Aldrich, St. Louis, MO, USA) dissolved in 5% NFM, at room-temperature for 2 hours. The protein bands were detected using chemiluminiscence reagents (SuperSignal West Femto Chemiluminescent Substrate, Thermo Fisher Scientific Inc., Waltham, MA, USA) and images were captured using a gel imaging system (G-Box, Syngene, Fredrick, MD, USA). Densitometric analyses of the acquired images were done using ImageJ (National Institutes of Health, Bethesda, Maryland, USA). To control for the amount of protein loaded in gels, house-keeping enzyme (GAPDH, beta-actin), or lipid-raft marker (Flotillin) were probed. The membranes were incubated in stripping buffer (Restore™ PLUS Western Blot Stripping Buffer, Thermo Fisher Scientific Inc., Waltham, MA, USA) for 15 min and washed three times for 10 min each. Membranes were then blocked again with 5% NFM at room temperature for 1 hour before being incubated overnight at 4°C with primary antibodies (GAPDH – 1:2,000, mouse monoclonal, EMD Millipore, Billerica, MA, USA #MAB374; Flotillin-1 – 1:2,000, mouse monoclonal, BD Biosciences, San Jose, CA, USA #610821; THE™ beta-Actin 1:2000, mouse monoclonal, GenScript USA Inc. Piscataway, NJ, USA). The protein bands were detected as described above.

Ratiometric calcium imaging

In order to observe changes in intracellular calcium concentration, calcium-imaging was performed, as described previously [38, 39]. Briefly, 4B cells were grown on coverslips (22X22-1, Fisherbrand Microscope Cover Glass, Thermo Fisher Scientific Inc., Waltham, MA, USA) to 70-80% confluency. The cells were serum deprived overnight before the day of the experiment. The coverslips with adherent 4B cells were incubated in calcium-sensitive dye Fura-2AM (2 µM; Thermo Fisher Scientific Inc., Waltham, MA, USA) and pluronic acid (1.5 mM, F-127, Thermo Fisher Scientific Inc., Waltham, MA, USA), dissolved in serum-deprived media for 1 hour at 37^0C. The Fura-2AM media was supplemented with the respective drug solutions, as described above. For each antagonist (Losartan, PP2, and PP3), the cells were pre-treated with the antagonist drug dissolved in serum-free media for 10 min before incubation in culture media with Fura-2AM, Ang II, and respective antagonist dissolved in it, for 1 hour. The coverslips were then washed twice with HBSS (Hank's balanced buffer solution, Thermo Fisher Scientific Inc., Waltham, MA, USA). The HBSS composition was (in mM): 1.26 $CaCl_2$, 0.49 $MgCl_2$-$6H_2O$, 0.4 $MgSO_4$-$7H_2O$, 5.3 KCl, 0.44 KH_2PO_4, 137.9 NaCl, 0.33 Na_2HPO_4-$7H_2O$, 5.5 D-Glucose, and pH 7.4. After the washes, coverslip was mounted on a laminar-flow perfusion chamber (Warner Instrument Corporation, Hamden, CT, USA). The perfusion chamber was then mounted on an inverted microscope (Olympus IX81, Olympus, Melville, NY, USA) and attached to a gravity-driven flow-controlled perfusion system (Warner Instrument Corporation, Hamden, CT, USA). All calcium imaging experiments were conducted at room temperature. The cells were perfused continuously (flow rate 2ml/min) with HBSS or drug dissolved in HBSS. Cells were allowed to stabilize in the flowing perfusate for 10 min before recording baseline data. To obtain ratiometric

data, the cells were alternately illuminated with 340 nm and 380 nm wavelengths using a xenon light source (Lumen200PRO, Prior Scientific, Rockland, MD, USA). The emitted light was captured at 520 nm wavelength using a CCD camera (Hamamatsu camera controller C10600, Hamamatsu Photonics KK, Hamamatsu, Japan). Pixel-data were binned (2X2) and images were captured every 4 second. The ratiometric data was collected and analyzed using commercially available software (Slidebook 5.0, Intelligent Imaging Innovations Inc., Denver, CO, USA). Baseline data from 30-40 cells from each coverslip were collected for 3-4 min before drug administration. After achieving stable baseline data for at least 3 min, cells were perfused with specific TRPV4 agonist GSK 1016790A (GSK101) dissolved in HBSS for 1 min followed by perfusion with HBSS. Recovery data were recorded for 3 min after stopping GSK101. As previously reported [38, 39], the collected data were then normalized to baseline data averaged over 1 min before drug administration. For each cell, maximum percent change from baseline was determined and data from all the cells on each coverslip were averaged for every independent experiment and treated as one data-point. Each treatment was repeated multiple times (reported in respective data graphs) and mean data from each independent experiment was averaged and reported as group mean ± standard error of mean.

Statistics

Data are reported as group means ± standard error of the mean. Data were analyzed by One-way ANOVA followed by Student–Newman–Keuls post-hoc analysis (SigmaPlot v 5.0 Systat Software, San Jose, CA, USA). Significance was set at $p < 0.05$.

RESULTS

Characterization of 4B cells with immunocytochemistry and qRT-PCR

As reported previously [29], we confirmed the presence of AVP and CRF mRNA (Table 1) and expression of neuronal cytoskeletal protein MAP2 (microtubule-associated protein 2) (Figure 1). We also observed that these cells express male genotype based on the presence of sex-determining region Y (SRY) mRNA (Table 1) [40]. In addition, we noted that 4B cells expressed AVP but not GFAP (glial fibrillary associated protein) (Figure 1). In regards to the aims of the present study, TRPV4 mRNA expression was observed through qRT-PCR (Table 1) and protein was observed through immunocytochemical (Figure 1) and western blot analysis (Figure 2). We also identified AT1aR and AT1bR mRNA in 4B cells using qRT-PCR (Table 1).

Western Blot analyses

Consistent with earlier reports, TRPV4 antibody detected two bands with a molecular weight near 100 kD [28]. It has been shown that the higher molecular weight band is the glycosylated form of TRPV4 [28, 41]. In the membrane fractions, however, the TRPV4 antibody detected only one band. Before normalizing TRPV4 bands against GAPDH and Flotillin, we investigated the effect of different drug treatment on GAPDH expression against β-actin and found no difference in expression after drug treatments (data not shown). In total lysate, Ang II (100 nM) treatment for 1 hour did not significantly affect total TRPV4 abundance (Figure 2A).

However, in the membrane fraction, Ang II (100 nM) treatment significantly increased TRPV4 immunoreactivity ($p < 0.05$) (Figure 2B). This increase in TRPV4 abundance in membrane fraction was reversed in the presence of the AT1R antagonist, Losartan (1μM; Figure 2B).

Given the role of SFK in TRPV4 regulation, we investigated whether or not Ang II mediated TRPV4 translocation is mediated by SFK. We noted an increase in total tyrosine phosphorylation (4G10®) immunoreactivity in total lysate after treatment with Ang II (100nM) (Figure 3A). In addition, we observed an increased SFK abundance in total lysate after Ang II treatment ($p < 0.05$; Figure 3B).

Based on these results indicating that the Src Kinase pathway might be involved in Ang II incubation associated effects, we tested whether inhibition of SFK prevents the Ang II induced increase in TRPV4 translocation to plasma membrane. To ascertain this, we used a specific SFK inhibitor PP2, and its inactive analogue, PP3. We observed that after incubation in PP2 (10 μM), but not PP3 (10 μM), the increase in TRPV4 IR in membrane fraction after Ang II (100nM) treatment was reduced to control level ($p < 0.05$; Figure 2B). Hence, we concluded that Ang II induced TRPV4 translocation to lipid rafts is mediated by SFK.

Calcium imaging

To determine if increased TRPV4 trafficking to the cell membrane also translates into increased TRPV4 agonist mediated calcium entry, we performed ratiometric calcium imaging experiments using calcium-sensitive Fura-2AM dye in live 4B cells. Consistent with previous reports in HeLa cells heterologously transfected with TRPV4 [42], 4B cells demonstrated dose-dependent increases in intracellular calcium following administration of highly selective TRPV4 agonist, GSK 101 (Figure 4A). The increase in intracellular calcium produced by 100 nM GSK 101 was completely blocked by co-administration of the selective TRPV4 antagonist, HC 067047 (10 µM; Figure 4B). To verify if the increase in intracellular calcium in response to GSK 101 is due to calcium-influx, cells were incubated in $CdCl_2$ (125µM) for 10 min followed by exposure to GSK 101 mixed with the same concentration of $CdCl_2$ [43]. The GSK 101 mediated calcium transients were completely abolished in the presence of $CdCl_2$ (Figure 4B).

In order to determine if Ang II incubation potentiates TRPV4 agonist mediated calcium influx, we perfused the cells with GSK 101 (20 nM) after 1 hour incubation with 100 nM Ang II. We observed, that after incubation in Ang II, 4B cells showed a significant increase in intracellular calcium in comparison with the cells incubated with control media after exposure to the same dose of GSK101 (20 nM) (Figures 5 and 6). Similar to the results of the Western blot analyses, the enhanced calcium influx to GSK 101 that was associated with Ang II pretreatment, was abolished by co-incubations with Losartan or PP2, but not by PP3 (Figures 5 and 6). To rule out the independent effect of each antagonist on GSK 101 mediated calcium influx, separate sets of experiments were conducted by incubating the cells in each antagonist alone. We observed no

significant effects of incubation in Losartan, PP2, or PP3 alone on the responses of GSK 101 (20 nM) as compared to 4B cells incubated in control media (n = 4) (data not shown). Moreover, we found no significant effect of any of the drug treatments on the baseline 340 nm/380 nm ratio (Table 2).

Figures

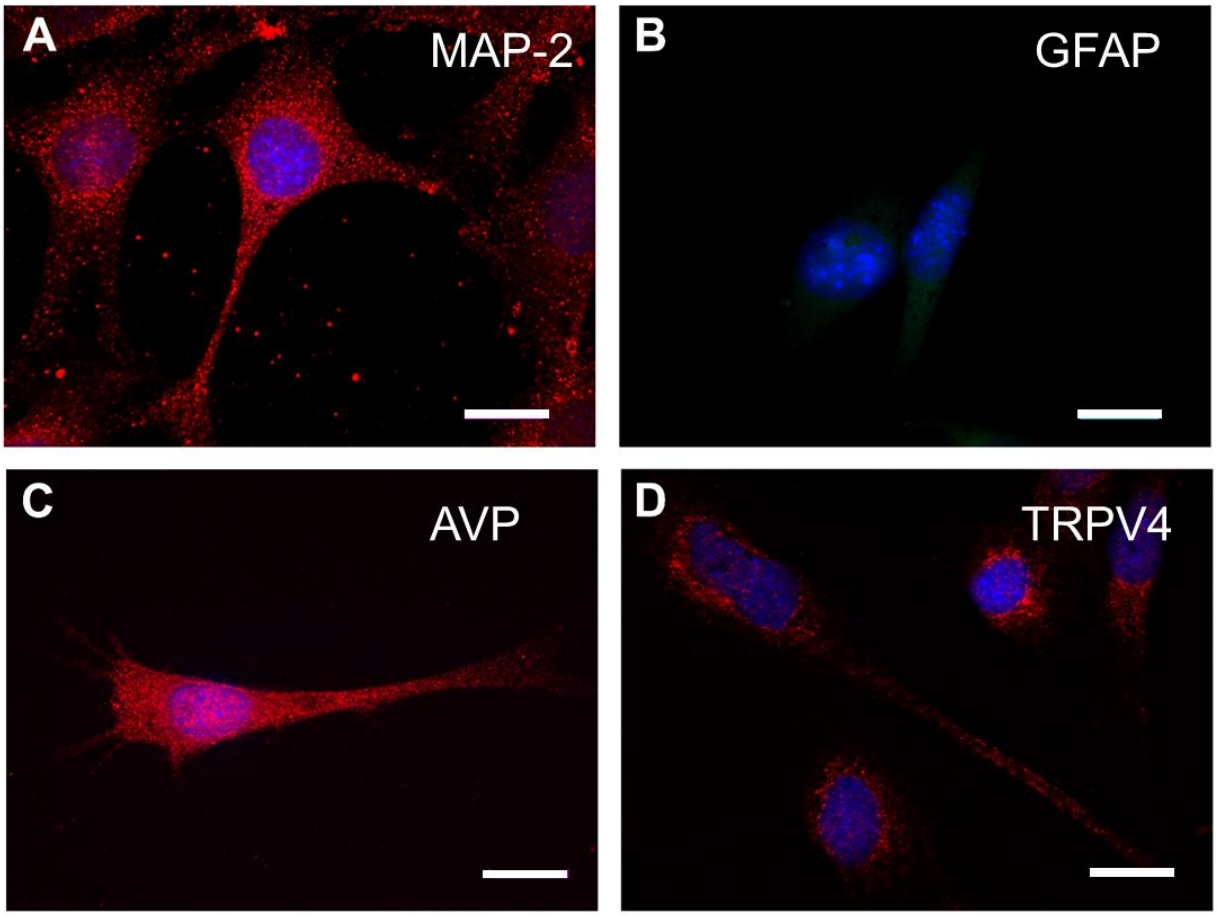
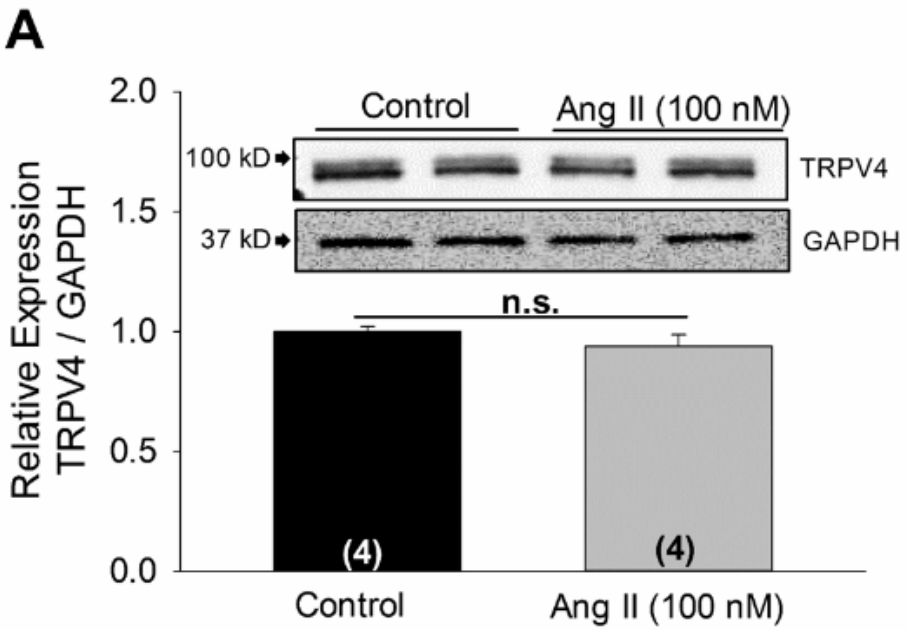


Figure 1. Immunocytochemical analyses revealed expression of A: microtubule associated protein – 2 (MAP-2) (red fluorophore), B: glial fibrillary associated protein (GFAP) (green fluorophore), C: Vasopressin (AVP) (red fluorophore), and D: Transient receptor potential channel vanilloid – type 4 (TRPV4) protein (red fluorophore). Nuclei are stained with DAPI (blue). The immunofluorescence signal for GFAP (green fluorophore) was comparable with no primary antibody control. scale bar: 10 µm


A
Control
Ang II (100 nM)
100 kD
37 kD
TRPV4
GAPDH
n.s.
Relative Expression
TRPV4 / GAPDH
2.0
1.5
1.0
0.5
0.0
(4)
(4)
Control
Ang II (100 nM)

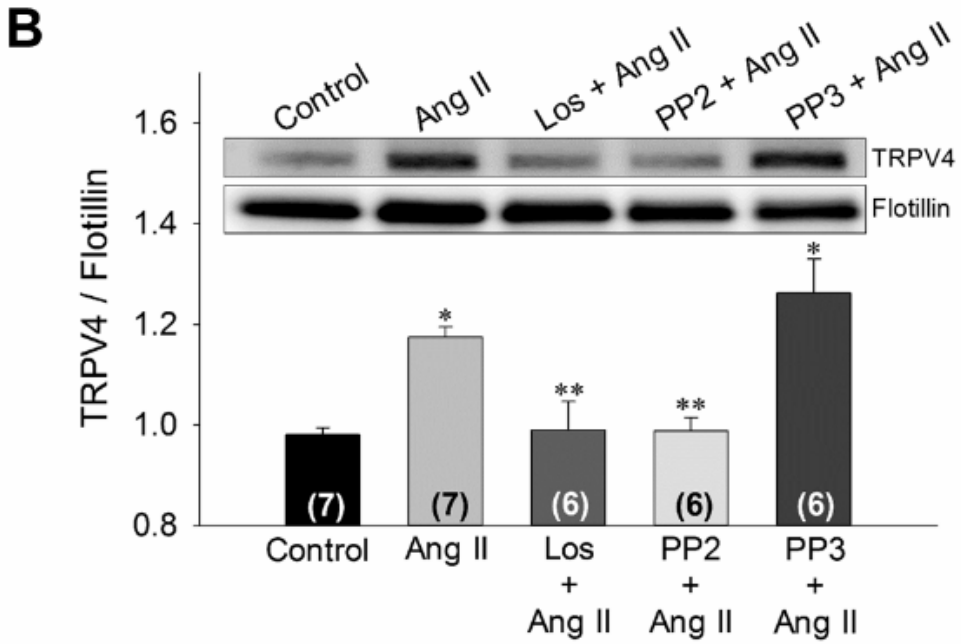

B
Control
Ang II
Los + Ang II
PP2 + Ang II
PP3 + Ang II
TRPV4
Flotillin
TRPV4 / Flotillin
1.6
1.4
1.2
1.0
0.8
*
**
**
*
(7)
(7)
(6)
(6)
(6)
Control
Ang II
Los + Ang II
PP2 + Ang II
PP3 + Ang II

Figure 2. A: Digital image of representative Western blot analysis for TRPV4 from A) total lysate of 4B cells after Ang II (100 nM) treatment for 1 hour. Densitometric analysis of TRPV4 immunoreactivity was performed and normalized with GAPDH from control cells; B: Membrane fraction of 4B cells after Ang II (100 nM) treatment without and in the presence of antagonists. Densitometric analysis of TRPV4 immunoreactivity was performed and normalized with lipid-raft marker Flotillin. Data are expressed as mean ± SEM. Number of independent experiments are provided in parenthesis. (x) = number of independent experiments; n.s. = not significat; * significantly different from Control, Los + Ang II, and PP2 + Ang II ($p < 0.05$, ANOVA followed by Student-Neuman Keuls post hoc analysis); ** significantly different from Ang II and PP3 + Ang II ($p < 0.05$, ANOVA followed by Student-Neuman Keuls post hoc analysis).

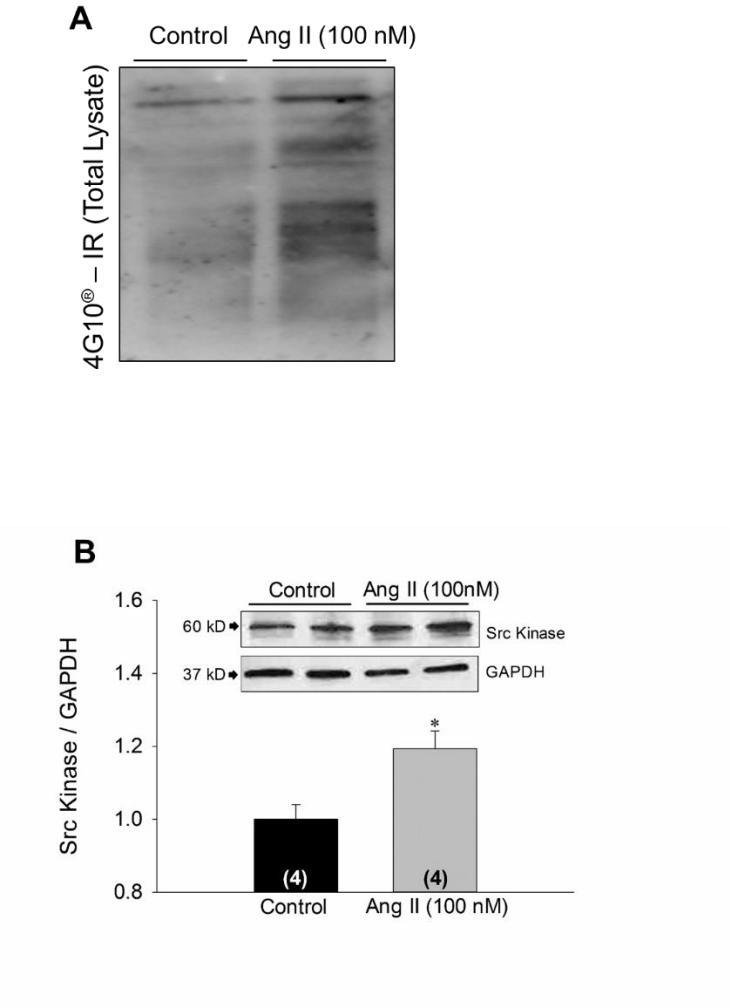


Figure 3. A) Immunoreactivity for total tyrosine phosphorylated proteins (4G10®) in total lysate (20 µg protein) after treatment with Ang II (100 nM). B) Representative western blot analysis for Src Kinase in total lysate after Ang II (100 nM) treatment. Densitometric analysis was performed after normalizing Src Kinase immunoreactivity with GAPDH. * significantly different from control ($p < 0.05$, student t-test).

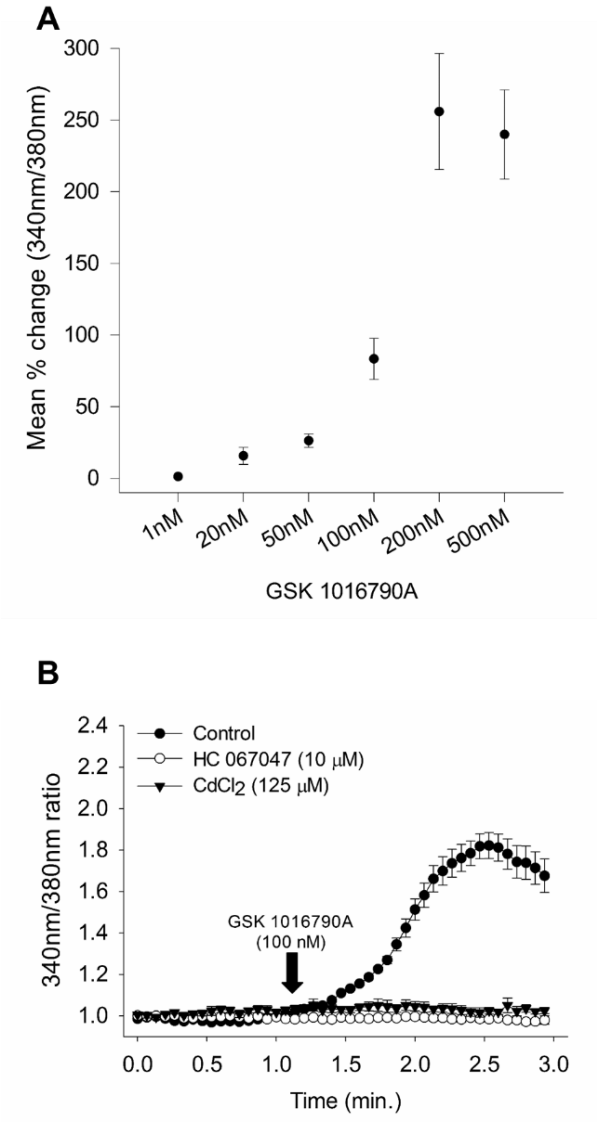


Figure 4. A: Dose dependent increase in GSK 101 mediated calcium influx in 4B cells. Mean maximum percent change in 340 nm/380 nm ratio from baseline are presented on Y-axis and the doses (nM) of GSK 101 are presented on X-axis; 1 nM (n = 4), 20 nM (n = 4), 50 nM (n = 3), 100 nM (n = 4), 200 nM (n = 4), 500 nM (n = 4). B: Mean traces of calcium response after GSK 101 (100 nM) administration in control cells and cells pretreated with TRPV4 antagonist HC 067047 and non-specific voltage gated calcium channel blocker $CdCl_2$.

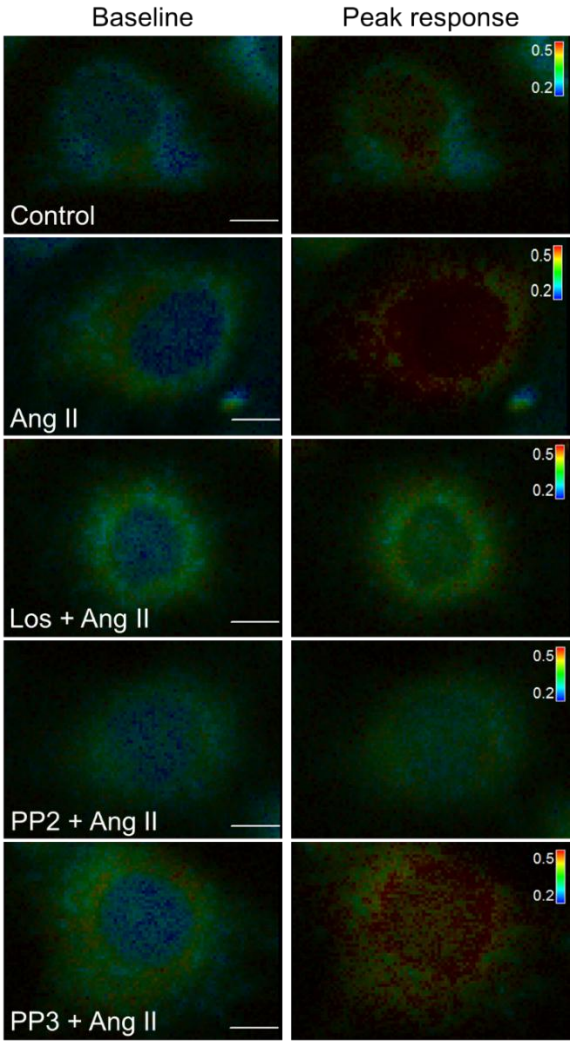
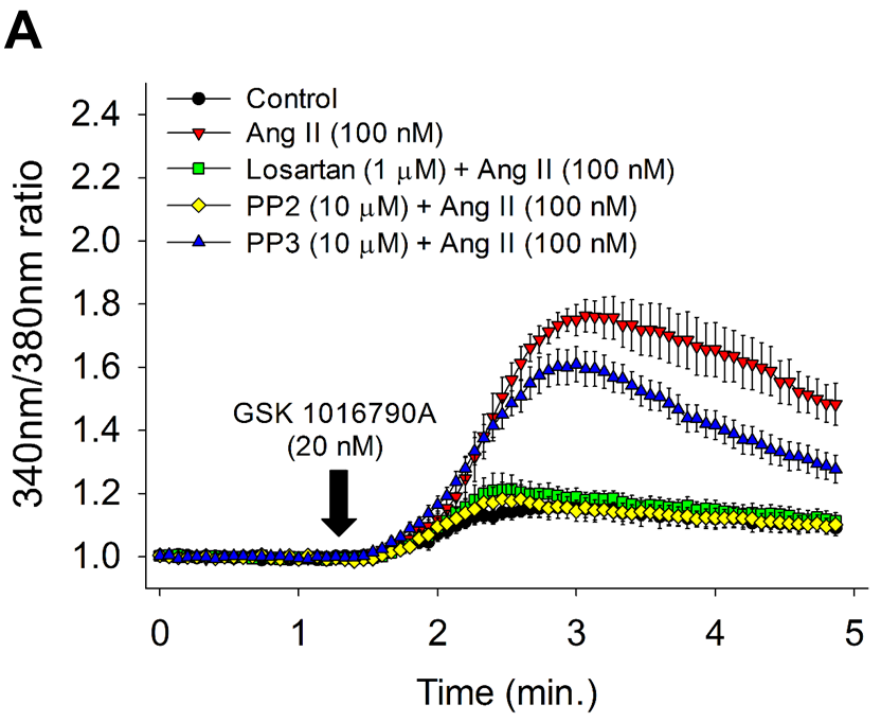


Figure 5. Representative 4B cells loaded with Fura-2AM and psuedocolored showing 340 nm/380 nm ratio. First image of respective groups shows the cell during baseline and the second image shows the cell during peak calcium response after GSK 101 (20 nM) administration. Scale bar: 10 µm.A reference bar is shown to correlate the color represented with the 340 nm/380 nm ratio value (0.2 – 0.5).


A
Control
Ang II (100 nM)
Losartan (1 μM) + Ang II (100 nM)
PP2 (10 μM) + Ang II (100 nM)
PP3 (10 μM) + Ang II (100 nM)
340nm/380nm ratio
2.4
2.2
2.0
1.8
1.6
1.4
1.2
1.0
GSK 1016790A
(20 nM)
0
1
2
3
4
5
Time (min.)

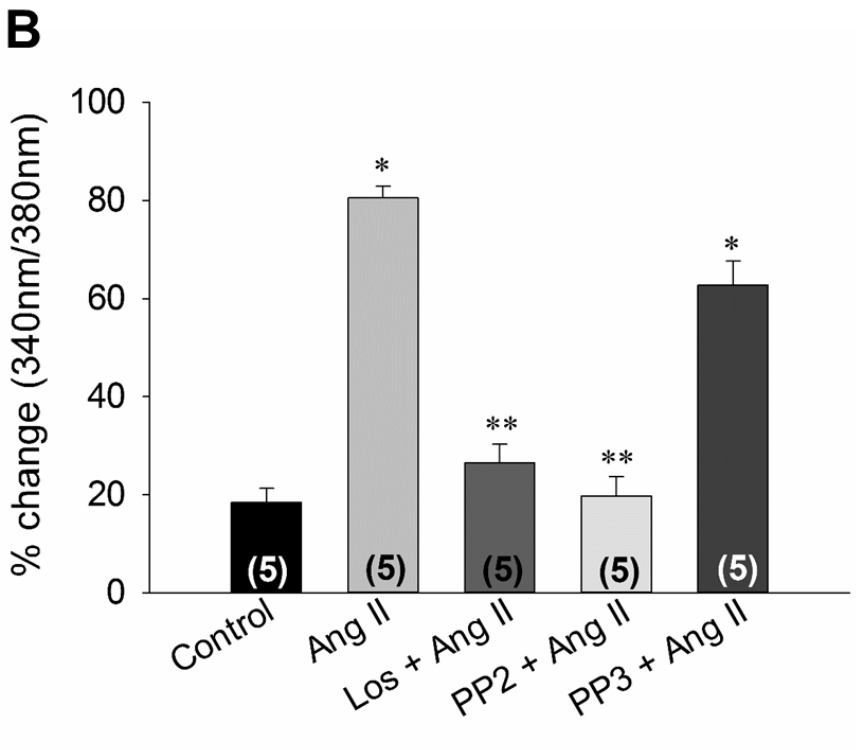

B
% change (340nm/380nm)
100
80
60
40
20
0
*
**
**
*
(5)
(5)
(5)
(5)
(5)
Control
Ang II
Los + Ang II
PP2 + Ang II
PP3 + Ang II

Figure 6. A: Mean traces of 340 nm/380 nm ratio in control cells (symbol: black circles) and after Ang II (100 nM) incubation in the absence (symbol: red inverted triangle) or presence of AT1R antagonist (Losartan 1 μM) (symbol: green square), Src Kinase inhibitor (PP2 10 μM) (symbol: yellow diamonds), and an inactive PP2 analogue (PP3 10 μM) (symbol: blue triangles). Ratio values from each cell were normalized to their respective baseline recorded over 1 min before GSK 101 exposure. Data from all cells in a coverslip at each timepoint was averaged to achieve a single mean. Experiments were repeated multiple times (number shown in parantheses). Mean GSK 101 response traces from all coverslips belonging to each treatment group were averaged to measure mean data at each timepoint. Data are presented as Mean ± SEM. B: Bar-graph showing mean maximum percent change in 340 nm/380 nm ratio after GSK 101 (20 nM) administration in 4B cells treated as described above. Maximum percent change in ratio value of each cell was measured and data from all cells in one coverslip was averaged to achieve single mean maximum change in ratio. Data from each coverslip in respective group were averaged to measure mean response in each group. (x) = Numbers of independent experiments. * significantly different from Control, Los + Ang II, and PP2 + Ang II ($p < 0.05$, ANOVA followed by Student-Neuman Keuls post hoc analysis), ** significantly different from Ang II and PP3 + Ang II ($p < 0.05$, ANOVA followed by Student-Neuman Keuls post hoc analysis).

Gene (n)	Primer sequence	Ct (mean ± SEM)
18S (4)	Forward: 5'-CAGAAGGACGTGAAGGATGG-3‘	16.624 ± 0.359
	Reverse: 5'-CAGTGGTCTTGGTGTGCTGA-3'	
SRY (2)	Forward: 5′-AAGCGCCCCATGAATGCATTTATGGT-3’	29.095 ± 1.431
	Reverse: 5′-ACACTTTAGCCCTCCGATGAGGCTGA-3’	
CRF (4)	Forward: 5'- GGAGCCGCCCATCTCTCT-3'	19.036 ± 0.063
	Reverse: 5'-TCCTGTTGCTGTGAGCTTGCT-3'	
AVPhnRNA (4)	Forward: 5'-GCCCTCACCTCTGCCTGCTA-3'	22.49 ± 0.211
	Reverse: 5'-CCTGAACGGACCACAGTGGT-3'	
TRPV4 (4)	Forward: 5'-ACAGCAACCTGGAGACTGTGCTTA-3'	23.01 ± 0.145
	Reverse: 5'-AGTCCTTGA ACTTGCGAGACAGGT-3'	
AT1aR (5)	Forward: 5'- CAAAAGGAGATGGGAGGTCA-3'	22.488 ± 0.732
	Reverse: 5'-AGCAGTTTGGCTTTGCAACT-3'	
AT1bR (3)	Forward: 5'-AGAAGAACACGCCAAGAA-3'	24.997 ± 0.738
	Reverse: 5'- TGAATGAGCACATCCAGAA-3'	

Table 1. Forward and Reverse Primers for real-time quantitative reverse transcriptase polymerase chain reaction and their respective Ct values. Numbers of independent experiments are represented in parenthesis. Data are represented as mean ± SEM. 18S: ribosomal mRNA, CRF: corticotropin releasing factor, AVPhnRNA: Vasopressin heteronuclear RNA, AT1aR and AT1bR: Ang II receptors Type 1a and 1b.

Group (n)	Baseline 340 nm/380 nm ratio (mean ± SEM)
Control (9)	0.264 ± 0.0046
Ang II (5)	0.254 ± 0.0045
Los + Ang II (5)	0.244 ± 0.0059
PP2 + Ang II (5)	0.257 ± 0.0047
PP3 + Ang II (5)	0.241 ± 0.0038
Los (4)	0.266 ± 0.0082
PP2 (4)	0.256 ± 0.0039
PP3 (4)	0.26 ± 0.0047

Table 2. Raw baseline 340 nm/380 nm ratios of 4B cells across different groups: Baseline ratio values from each cell over 1 min before GSK 101 exposure was averaged. Averaged data from all the cells on each coverslip was averaged and treated as n=1. Mean data from each coverslip from each group was then averaged and presented as mean ± SEM. The number of independent experiments for each group is shown in parenthesis. No significant differences were observed in mean baseline 340 nm/380 nm ratios after different drug treatment (One-way ANOVA).

DISCUSSION

The results of this study indicate that Ang II influences TRPV4 trafficking to the plasma membrane of neuronal 4B cells and that this effect is mediated by the SFKs. For functional validation of our molecular observations, we utilized Fura-2AM based calcium imaging experiments and used selective TRPV4 agonist GSK 101 [42, 44] to induce TRPV4 mediated calcium transients. We observed that after 1 h of Ang II incubation, GSK 101 (20 nM) administration significantly increased intracellular calcium in 4B cells in comparison with control cells. This increased calcium response was blocked by either Losartan or PP2 indicating this effect is dependent on AT1 receptors and SFKs.

Given the polymodal nature of TRPV4 [3, 45, 46], greater membrane expression of this channel could lead to greater calcium influx following osmotic, temperature or agonist stimulation. This increase in calcium entry could have important functional consequences. Nearly half a century ago, Douglas and Poisner provided evidence in support of the fundamental role played by calcium-influx in stimulus-secretion coupling for the release of AVP from the neurosecretory cells of hypothalamus [47, 48]. Intracellular calcium has also come to be recognized as a major player in the release of neuropeptides from other compartments of the cell, such as the dendrites [49].

It is well accepted that there are distinct temporal and spatial pattern in how intracellular calcium regulates release of neurotransmitter and neuropeptides. Whereas focal calcium sparks trigger presynaptic release of neurotransmitters, a diffuse increase in intracellular calcium can be

associated with neuropeptide release from dense-core vesicles [49]. Recently, it has been suggested that TRPV4 channels in vascular myoendothelial junctions mediate, spatially localized, spontaneous calcium 'sparklets' [50, 51]. It remains to be determined if TRPV4 channels generate similar spontaneous calcium sparklets in neurons and contribute to their excitability and neurotransmitter release probability. In our experiments, we did not observe localized calcium signals after GSK 101 administration. Instead, we observed a diffuse increase in intracellular calcium levels. In this initial study, the inability to resolve specific compartments of the cells is a limitation that prevents us from directly addressing this issue. It remains to be determined whether or not increased TRPV4 activity in the membrane translates into increased neurotransmitter or neuropeptide release for a given stimulus.

Recently TRPV4 has been shown to play an important role in determining the resting membrane potential and the level of spontaneous activity in magnocellular neurosecretory cells from the SON [52]. Based on these observations, it could be proposed that Ang II could increase the excitability of neuroendocrine neurons through increased membrane expression of TRPV4 which would also contribute to greater neuropeptide release by depolarizing the resting membrane potential and increasing spontaneous activity. The same study reported that TRPV1 plays a much greater role in thermosensitivity than TRPV4 under basal conditions. Therefore, it is not clear if increasing TRVP4 in the plasma membrane would alter response of neuroendocrine cells to increases in ambient temperature.

These results are consistent with our previous in vivo observations where we observed increased TRPV4 trafficking to lipid-rafts in hypothalamus in bile-duct ligated rats [11]. Further, they extend these observations by demonstrating that Ang II dependent TRPV4 trafficking is mediated by SFKs and that increased cell membrane expression of TRPV4 is associated with enhanced calcium influx. This increase in TRPV4 trafficking and function was blocked by Losartan, a AT1R antagonist, indicating that the effect of Ang II was mediated by AT1R. Our results indicate that 4B cells express both AT1aR and AT1bR. Since Losartan does not discriminate between these two AT1R subtypes it is possible that both receptor subtypes contribute to these effects. Most of the biological activity of Ang II has been shown to be mediated by AT1Rs [53, 54]. A recent study using HEK-293 and vascular smooth muscles cells reports that AT1R stimulation produced β-arrestin dependent ubiquitination of TRPV4 that decreases membrane expression of this channel [55]. These observations were supported by demonstrating reduced calcium influx to 4-α-PDD in HEK-293 cells following Ang II stimulation. There could be several possible explanations for the differences in the results between these studies including differences in cell types, heterologous expression model, and/or the doses of Ang II.

Given that 4B cells are derived from parvocellular PVN neurons, our results could be relevant to the function of the hypothalamic-pituitary-adrenal axis. Tasker and colleagues, over the past decade, have identified molecular mechanisms through which glucocorticoids inhibit the stress responsive PVN neurons of the hypothalamus. They have suggested that inhibition of medial parvocellular [56] and neurosecretory magnocellular [57, 58] PVN neurons by glucocorticoids could be executed by rapid respective inhibition and facilitation of excitatory and

inhibitory inputs to PVN neurons. They have suggested that post-synaptic Gαs-cAMP pathway mediates generation of endocannabinoids and Gβγ signaling induced generation of nitric oxide (NO) could retrogradely drive anti-excitatory and pro-inhibitory effects, respectively [58]. Recently, Boychuk et al., extended these findings to gastric pre-autonomic neurons of PVN [59]. Interestingly, they reported that in nearly half of gastric pre-autonomic PVN neurons, glucocorticoids, however, transiently increased presynaptic excitatory inputs. Their results indicate that this transient increase in excitatory input could be mediated by vanilloid family of TRP channels (TRPV1/4) [59]. Our results suggest that locally released Ang II could modulate the function of TRPV4 in this system by enhancing the excitation.

If Ang II modulation of TRPV4 trafficking alters the excitability of neuroendocrine neurons it could contribute to the pathophysiology of diseases associated with increased PVN activity. For example, it has long been realized that in congestive heart failure[60], increased sympathetic nerve activity is associated with pro-excitatory and blunted NO-mediated inhibitory adaptations in PVN neurons [61, 62]. Notably, these maladaptations are reversible after blockade of AT1R [63, 64]. These observations could suggest a putatively non-specific role played by heightened RAS activation/Ang II signaling in enhancing PVN neuronal stress reactivity. One potential post-synaptic mechanism through which Ang II could achieve this action on hypothalamic neurons is by trafficking TRPV4 to the neuronal surface. However, future investigations are needed to uncover the role of TRPV4 in complex orchestration of acute physiological response to stress and in morbidities associated with impaired HPA reactivity.

PERSPECTIVES

Physiologically, extracellular fluid osmolality is remarkably regulated in eukaryotic organisms with high efficiency. In mammals, central osmosensors regulate body fluid balance by stimulating or inhibiting the release of AVP, a neuropeptide hormone, from posterior pituitary gland. Renal action of AVP promotes water reabsorption. Physiologically, hyperosmolality increases, whereas hypoosmolality decreases plasma AVP concentration [65]. However, in diverse pathological syndromes, such as hepatic cirrhosis [66], congestive heart failure [67, 68], and nephrogenic syndrome [69-71], a decrease in osmolality fails to inhibit AVP release. This AVP release leads to water retention and contributes to the morbidity and mortality associated with these diseases [67, 68, 72-74]. The molecular mechanisms underlying inappropriate release of AVP remain to be determined. As these diseases are also associated with increased renin-angiotensin-system activation, Ang II could play a role in the pathogenesis of these syndromes. In our bile duct ligation model of cirrhosis in rodents, we reported that the inappropriate feed-forward release of AVP was associated with increased plasma renin activity [11]. Interestingly, both increased plasma AVP and RAS activation also correlated with increased TRPV4 association with lipid rafts microdomains in the plasma membrane of hypothalamic blocks that included PVN, SON, and organum vasculosum of lamina terminalis. The plasma AVP and increased TRPV4 trafficking to plasma membrane were observed to be reversed after experimental inhibition of RAS by salt loading, suggesting that Ang II could play a role in the pathogenesis of inappropriate release of AVP. The results of the current study suggest that locally released Ang II could alter the excitability of neuroendocrine neurons by increasing the membrane expression of TRPV4, which has been shown to contribute to the resting membrane

potential and spontaneous activity of magnocellular neuroendocrine cells [52]. The calcium permeability of this channel could also have function consequences for neuropeptide release from different compartments of the neuroendocrine neurons.

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CHAPTER IV

DISSERTATION PERSPECTIVE AND SIGNIFICANCE

Renin-Angiotensin system (RAS) is a dual regulatory system, with both a circulating endocrine and a local tissue paracrine system [1-3]. A major therapeutic strategy has emerged in the form of RAS inhibition for the treatment of hypertension and to decrease the risks of adverse cardiovascular outcomes [4-6]. However, the pathophysiological roles played by Ang II still remain elusive.

The main findings of this work are: 1) circulating Ang II – SFO communication appears to be essential in the sustained increase in MAP associated with CIH; 2) disruption of circulating Ang II – SFO communication is found to be associated with reduced FosB/ΔFosB staining, a transcription factor associated with lasting neuronal adaptations, in key autonomic regions of forebrain; 3) Ang II can directly induce translocation of Transient Receptor Potential channel, vanilloid family, type 4 (TRPV4), a non-specific cation channel implicated central osmosensation in neurons in vitro; 4) Ang II induced TRPV4 translocation seems to be mediated by Src kinase pathway.

Obstructive sleep apnea (OSA) is a chronic disease in humans accompanied by repeated spells of apnea during sleeping. These intermittent apneic spells are associated with decrease in blood oxygen saturation and an increase in blood carbon dioxide [7]. This leads to chemoreflex activation and concomitant increase in MAP [8]. Over the time, this increase in MAP outlasts the hypoxia experienced during sleep and present clinically as diurnal hypertension. It has been demonstrated that it is the hypoxia, and not hypercarbia, that drives the intermittent hypoxic stimulation mediated sympathetic nervous system activation [9]. To identify better therapeutic option, the pathophysiological mechanisms underlying OSA associated hypertension need to be sought and investigated.

There have been previous reports implicating activated RAS in the pathophysiology of increased MAP in animals exposed to chronic intermittent hypoxia (CIH) [10-15]. Subfornical organ (SFO), a blood brain barrier (BBB) lacking circumventricular organ in forebrain, is the primary site for central actions of circulating Ang II. It remained to be investigated if circulating Ang II interaction with the SFO neurons plays a role in neuroplastic adaptations in the autonomic nuclei of forebrain that is associated with CIH in rats [16, 17]. Ang II the main effector peptide of RAS acts through AT1R for its majority of biological activity [18-21]. Out of the two subtypes of AT1R, only AT1aR mRNA has been found in SFO [22]. Here, using virus vector mediated transfection to silence AT1aR mRNA in SFO neurons, we report that neurohumoral communication at the level of SFO could contribute in the sustained increase in the MAP associated with CIH. Also, an interruption of this circulating Ang II – SFO communication was found to be associated with a decrease in FosB/ΔFosB staining, a transcription factor implicated

in long-term neuronal adaptations. These findings identify the humoral role played circulating by Ang II in the pathophysiology of CIH associated hypertension.

In addition to circulating or classical RAS, local or tissue RAS is recognized as an important paracrine regulatory system in health and disease states [23]. In addition to blood pressure regulation, RAS has been known to play an important role in body fluid and electrolyte homeostasis [24-27]. Physiologically, extracellular fluid osmolality is remarkably regulated in eukaryotic organisms with high efficiency. In mammals, central osmosensors regulate body fluid balance by stimulating or inhibiting the release of AVP, a neuropeptide hormone, from posterior pituitary gland. Renal actions of AVP promote water reabsorption. Physiologically, hyperosmolality increases, whereas hypoosmolality decreases plasma AVP concentration [28]. However, in diverse pathological syndromes, such as hepatic cirrhosis [29], congestive heart failure [30, 31], and nephrogenic syndrome [32-34], a decrease in osmolality fails to inhibit AVP release. This AVP release leads to water retention and contributes to the morbidity and mortality associated with these diseases [30, 31, 35-37]. The molecular mechanisms underlying inappropriate release of AVP remain to be determined. As these diseases are also associated with increased renin-angiotensin-system activation, Ang II could play a role in the pathogenesis of these syndromes. Previously, in our bile duct ligation model of cirrhosis in rodents, we reported that the inappropriate feed-forward release of AVP was associated with increased plasma renin activity [38]. Interestingly, both increased plasma AVP and RAS activation also correlated with increased TRPV4 association with lipid rafts microdomains in the plasma membrane of hypothalamic blocks that included PVN, SON, and organum vasculosum of lamina terminalis.

The plasma AVP and increased TRPV4 trafficking to plasma membrane were observed to be reversed after experimental inhibition of RAS by salt loading, suggesting that Ang II could play a role in the pathogenesis of inappropriate release of AVP. The results of the current study suggest that synaptically released Ang II could alter the excitability of neuroendocrine neurons by increasing the membrane expression of TRPV4, which has been shown to contribute to the resting membrane potential and spontaneous activity of magnocellular neuroendocrine cells [39]. The calcium permeability of this channel could also have functional consequences for neuropeptide release from different compartments of the neuroendocrine neurons.

Taken together, these studies provide evidence in support of a pathophysiological role played by circulating-hormonal RAS and local RAS. First, in CIH, a model of hypertension associated with sleep apnea, circulating Ang II – SFO communication seems to drive neuroplastic changes in the key autonomic regions of forebrain and sustained increase in MAP. Second, Ang II seems to directly influence changes in TRPV4 channels, which are strongly implicated in osmosensitivity. Together these observations demonstrate that circulating and synaptically released RAS could play an important role in pathophysiology associated with hypertension and fluid-electrolyte imbalance.

Clinically, there is a significant overlap in cardiovascular (CV) disease and an impaired body-fluid balance [40-43]. Blood pressure (BP) reduction has been demonstrated to significantly lower CV risk [44-46]. Even a modest decrease in systolic blood pressure (SBP) of

2 mmHg has been reported to decrease mortality from stroke (10%) and ischemic heart disease (7%) [47]. The circulating and local RAS could synergistically drive pathogenesis and adverse event outcome of CV diseases. Hence, it is important to know if improved CV clinical outcomes associated with RAS inhibition are due to blood pressure reduction alone or to other effects such as the slowing of end-organ damage.

The Losartan Intervention For Endpoint reduction in hypertension (LIFE) trial compared composite CV endpoints [myocardial infarction (MI), stroke, or death] in patients with essential hypertension and left-ventricular hypertrophy after treatments with Losartan, an Ang II type 1 receptor blocker (ARB) or the β-blocker Atenolol [48, 49]. Although both treatments resulted comparable decrease in BP, Losartan group was associated with a 13% decrease in the relative risk (RR) of composite endpoints and a 25% reduction in the RR of fatal and non-fatal stroke [49].

In another CV outcome trial, the Heart Outcomes Prevention Evaluation (HOPE), in addition to the standard care, addition of Angiotensin converting enzyme inhibitor (ACEI), Ramipril, was compared to that of placebo in patients with one CV risk factor and evidence of vascular disease or diabetes [50-52]. The investigators noted that Ramipril treatment was associated with only 3.3/1.4 mmHg decrease in BP but the reduction in CV risk was greater than that can be attributed to BP reduction alone. They noted that ACEI was associated with reduction in RR of CV death by 26%, stroke by 32% and of MI by 20% [50-52]. These observations

provide evidences in patient population that RAS inhibition might provide better clinical outcomes than other modalities of BP reduction.

To compare different RAS inhibition strategies to prevent CV morbidity and mortality in patients with controlled BP but at high risk of vascular events, The ONgoing Telmisartan Alone and in combination with Ramipril Global End-point Trial (ONTARGET) was conducted to compare different RAS inhibition strategies to prevent CV morbidity and mortality in patients with controlled BP but at high risk of vascular events. This study showed that while combination therapy decreased SBP more than either telmisartan or ramipril alone, there were no improvements in composite endpoints (MI, stroke, hospitalization for heart failure, and death) [53].

As different drugs within same class have different pharmacokinetics, there could be differences in their beneficial effects. For example, within ARBs telmisartan has a long half-life, the highest lipophilicity, greater volume of distribution, highest affinity towards AT1R, and longest receptor dissociation half-life [54]. Given these characteristics of telmisartan, in patients with hypertension and metabolic syndrome, Yano et al. observed telmisartan reduced inflammation markers such as C-reactive protein in comparison with valsartan [55]. Also, telmisartan has been observed to slow carotid artery thickening in patients [56]. Similarly, more liposoluble ACEI, perindopril, showed normalization of Ang II/bradykinin balance, reduced

inflammation and improved endothelial function in patients with stable coronary artery disease [57].

In conclusion, RAS has evolved from its role as a blood pressure and hydromineral balance hormonal pathway to a pathogenic cascade that drives the cardiovascular disease. Recent research has advanced our understanding of this complex regulatory system and identified therapeutic candidates to achieve its efficient inhibition. However, cardiovascular disease continues to extract a huge toll on human lives in developed and developing countries alike [58, 59]. Future investigations, both basic science and clinical, are needed to advance our understanding of RAS physiology in order to develop better therapeutic options.

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CHAPTER V

FUTURE DIRECTIONS

Renin-Angiotensin system (RAS), both circulating and local, has now an established role in blood pressure and hydromineral homeostasis [1-10]. Our understanding of RAS complexity has significantly advanced in the past decade. Initially identified as a linear cascade of renin – Angiotensinogen – Angiotensin I – Angiotensin II – AT1R pathway is now known to be more complex with the identification of new enzymes, biologically-active splice variants of Angiotensinogen peptides, and their respective receptors [4, 11-14]. Moreover, these new components are not only identified as part of circulating RAS [13], but also in tissue RAS, especially brain RAS [15, 16]. Briefly, it has been identified that octapeptide Ang II could be cleaved by a membrane bound zinc-metalloprotease, ACE2, into a biologically active heptapeptide Angiotensin (1 – 7) [Ang (1-7)], which binds with strong affinity to another G-protein coupled receptor, Mas [14]. The Ang (1 – 7) – Mas receptor axis is identified to oppose Ang II – AT1R effects [17-22]. In addition, Ang II could also be metabolized by aminopeptidase A (APA) and aminopeptidase N (APN) into a heptapeptide, Ang III and a hexapeptide, Ang IV [14, 23]. While new discoveries and evidences of classical and novel RAS components and their pathophysiological role of RAS in cardiovascular disease continue to be reported, recent therapeutic research, such as vaccination against RAS components [24-26], could provide better

treatment modalities for cardiovascular diseases and pathological hydromineral balance impairments.

The research presented in this dissertation provides insights in two renin-Angiotensin system (RAS) functions, viz. 'classical' circulatory hormonal system and synaptically released Ang II. Stated below are few pertinent areas that need further investigation to advance our understanding of the role played by RAS in the pathophysiology of:

1. CIH associated sustained increase in MAP:
    a. The presented experiments demonstrate that disruption of Ang II – SFO communication results in lowering of sustained increase in mean arterial pressure (MAP) associated with chronic intermittent hypoxia (CIH). However, molecular pathways through which Ang II could alter the signaling pattern of efferent SFO neurons in CIH remains to be determined. For example, we have previously reported increased FosB/ΔFosB staining in SFO of CIH exposed rats [27]. Later, we reported that inhibiting the transcriptional effects of ΔFosB in MnPO prevents CIH associated sustained increase in MAP [28]. It remains unknown if similar inhibition of transcriptional effects of ΔFosB in SFO will show similar effects on MAP. If yes, as SFO neurons project to the MnPO [29-31], then SFO could emerge as the primary central site which initiates neural adaptations associated with pathophysiology of sleep apnea.
    b. It has been reported that circulating Ang II could increase the discharge rate of PVN-projecting SFO neurons [32]. The electrophysiological effects of CIH on firing characteristics of SFO neurons remain to be determined.

c. Neurons utilize calcium to regulate their activity, release of neurotransmitters, and transcriptional activity [33, 34]. Calcium signaling has been implicated in Ang II induced excitatory effects on SFO neurons [35, 36]. As impaired neuronal calcium dynamics are implicated in several central nervous system dysfunction [37-42], it remains to be known if CIH induces any adaptations in Ang II evoked response of SFO and if these adaptations involve alterations in calcium handling.

d. Next, as renal SNA (RSNA) is identified as an important regulator of renal RAS activation and in turn circulating Ang II could influence RSNA activity [43, 44], it is important to know if interruption of Ang II – SFO communication translates into a reduction in RSNA.

2. Liver failure associated inappropriate release of arginine vasopressin (AVP) from neurosecretory cells of hypothalamus (fluid and electrolyte imbalance):

a. The present observations indicate that Ang II can traffic putatively osmosensory TRPV4 channels to neuronal membrane. Also, increased membrane translocation is associated with an increase in TRPV4 agonist mediated calcium influx. Although, calcium influx has previously been linked with neurosecretion of AVP, known as excitation-secretion coupling [45, 46], it remains to be identified if Ang II incubation is associated with increased AVP release.

b. We have previously shown that in bile-duct ligated (BDL) rats, an animal model of chronic liver failure and inappropriate release of AVP, there is increased TRPV4 presence in the membrane fraction of hypothalamic extracts

[47]. It remains to be known if pharmacologic antagonism or genetic knockdown of TRPV4 channels in the neuroendocrine nuclei of hypothalamus (PVN and/or SON) translates into normalization of plasma AVP and drinking behavior in BDL rats.

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ABBREVIATIONS

AAV	Adeno-associated virus
Act	Activity
Ang II	Angiotensin II
AT1aR	Ang II receptor type 1 subtype a
AT1bR	Ang II receptor type 1 subtype b
AT1aRshRNA	Short-hairpin RNA against AT1aR
AVP	Arginine vasopressin
CIH	Chronic Intermittent Hypoxia
CPAP	Continuous positive airway pressure
CVLM	Caudal ventrolateral medulla
dp	dorsal parvocellular neurons of PVN
GFAP	Glial fibrillary acid protein
GFP	Green fluorescent protein
HR	Heart Rate
IML	Intermediolateral tract of spinal cord
LCM	Laser capture microdissection
lp	Lateral parvocellular neurons of PVN
MAP	Mean Arterial Pressure
MAP-2	microtubule associated protein type 2
MnPO	Median preoptic nucleus
mp	medial parvocellular neurons of PVN

NTS	Nucleus tractus solitarius
OVLT	Organum vasculosum of Lamina Terminalis
PCR	Polymerase chain reaction
pm	Posterior magnocellular neurons of PVN
PVN	Paraventricular nucleus of hypothalamus
RAS	Renin-Angiotensin system
RR	Respiratory Rate
RVLM	Rostral ventrolateral medulla
SCMshRNA	Short-hairpin RNA-scramble sequence
SFO	Subfornical Organ
SON	Supraoptic nucleus of hypothalamus
TRPV4	Transient receptor potential channel vanilloid family type 4
vlp	ventro-lateral parvocellular neurons of PVN
WB	Western Blot