THE ROLE OF THE RENIN-ANGIOTENSIN SYSTEM IN THE CENTRAL NERVOUS SYSTEM

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THE ROLE OF THE RENIN-ANGIOTENSIN SYSTEM IN THE CENTRAL NERVOUS SYSTEM

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Table of Contents

04 ***Editorial: The Role of the Renin-Angiotensin System in the Central Nervous System***
Natalia P. Rocha, Ana Cristina Simões e Silva and Antonio Lucio Teixeira

07 ***Interactions of the Brain Renin-Angiotensin-System (RAS) and Inflammation in the Sensitization of Hypertension***
Baojian Xue, Yuping Zhang and Alan Kim Johnson

18 ***Pathobiological Interactions of Local Bone Marrow Renin-Angiotensin System and Central Nervous System in Systemic Arterial Hypertension***
Rafiye Ciftciler and Ibrahim Celalettin Haznedaroglu

26 ***Brain Renin–Angiotensin System at the Intersect of Physical and Cognitive Frailty***
Caglar Cosardereliogl u, Lolita S. Nidadavolu, Claudene J. George, Esther S. Oh, David A. Bennett, Jeremy D. Walston and Peter M. Abadir

55 ***Peripheral Levels of Renin-Angiotensin System Components are Associated With Cognitive Performance in Huntington's Disease***
Natalia P. Rocha, Courtney Cleary, Gabriela D. Colpo, Erin Furr Stimming and Antonio L. Teixeira

62 ***Epilepsy Seizures in Spontaneously Hypertensive Rats After Acoustic Stimulation: Role of Renin–Angiotensin System***
Christiane Becari, Giorgia Lemes Pereira, José A. C. Oliveira, Katarzyna Polonis, Norberto Garcia-Cairasco, Claudio M. Costa-Neto and Marilia G. A. G. Pereira

70 ***Decreased Plasma Levels of Angiotensin-Converting Enzyme Among Patients With Bipolar Disorder***
Marsal Sanches, Gabriela D. Colpo, Valeria A. Cuellar, Taya Bockmann, Deevakar Rogith, Jair C. Soares and Antonio L. Teixeira

76 ***Transcriptomic Analysis of Mouse Brain After Traumatic Brain Injury Reveals That the Angiotensin Receptor Blocker Candesartan Acts Through Novel Pathways***
Peter J. Attilio, Dustin M. Snapper, Milan Rusnak, Akira Isaac, Anthony R. Soltis, Matthew D. Wilkerson, Clifton L. Dalgard and Aviva J. Symes

96 ***Circulating Angiotensin-(1–7) Is Reduced in Alzheimer's Disease Patients and Correlates With White Matter Abnormalities: Results From a Pilot Study***
Victor Teatini Ribeiro, Thiago Macedo e Cordeiro, Roberta da Silva Filha, Lucas Giandoni Perez, Paulo Caramelli, Antônio Lúcio Teixeira, Leonardo Cruz de Souza and Ana Cristina Simões e Silva

106 ***Angiotensin-(1-7) Central Mechanisms After ICV Infusion in Hypertensive Transgenic (mRen2)27 Rats***
Lucas M. Kangussu, Marcella Nunes Melo-Braga, Bruna Soares de Souza Lima, Robson A. S. Santos, Hélida Monteiro de Andrade and Maria José Campagnole-Santos

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Editorial: The Role of the Renin-Angiotensin System in the Central Nervous System

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Editorial on the Research Topic

The Role of the Renin-Angiotensin System in the Central Nervous System

The Renin-Angiotensin System (RAS) has been known for decades as a system involved in blood pressure control through the regulation of volemia and systemic vascular resistance. More importantly, angiotensin-converting enzyme (ACE) inhibitors and angiotensin (Ang) II receptor antagonists have considerably contributed to decreasing morbidity and mortality due to hypertension (Basso and Terragno, 2001), bringing the RAS to the front light of cardiovascular medicine.

The classical vasopressor effects of the RAS involve a series of physiological pathways initiated by renin, an enzyme secreted by the kidneys. Renin hydrolyzes angiotensinogen to produce Ang I which, in turn, is cleaved by the ACE into Ang II. The main effects of the RAS are thought to be exerted by Ang II through the angiotensin type 1 (AT_1) receptor. ACE, Ang II, and AT_1 receptor constitute the main players of the classical arm of RAS, whose effects include vasoconstriction, aldosterone secretion, and fluid retention.

Our knowledge about the RAS has grown exponentially over the years as a multitude of other enzymes, peptides, and receptors have been identified. In this context, it is worth highlighting Ang-(1–7), a heptapeptide produced mainly by the cleavage of Ang II by an enzyme homolog to ACE, the ACE2 (Donoghue et al., 2000; Tipnis et al., 2000). Most physiological effects of Ang-(1–7) through its receptor "Mas" antagonize the classical RAS actions. Therefore, the RAS arm composed of ACE2/Ang-(1–7)/Mas receptor is regarded as a counter-regulatory axis of the classical arm of the RAS (Santos et al., 2008).

Our understanding of the physiological roles of the RAS has also evolved based on the findings of RAS components in "unlikely" places—for example, renin (a kidney enzyme) in the brain. As a result, the concept of local RAS was introduced (Paul et al., 2006), and herein we highlight the brain RAS. At first, the physiological effects linked to the brain RAS were associated with cardiovascular and body fluid homeostasis, such as the control of drinking behavior, salt appetite, vasopressin release, baroreflex, and sympathetic/parasympathetic activity. Later, it became clear that RAS actions in the central nervous system (CNS) are not limited to blood pressure and water-electrolyte balance regulation. Actually, the RAS is involved in several brain functions, including cognition, motor, and emotional/behavioral modulation (Rocha et al., 2018a). In this regard, the RAS has been implicated in the pathophysiology or related to the clinical phenotype observed in different neuropsychiatric conditions, such as Alzheimer' disease (AD) (Rocha et al., 2018b),

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Parkinson's disease (Rocha et al., 2016), schizophrenia (Mohite et al., 2018), and mood disorders (Mohite et al., 2020).

The manuscripts published in the current Research Topic discuss the role played by the RAS in the CNS and in CNS-related diseases. Regarding the traditional role attributed to the RAS (i.e., blood pressure control), Xue et al. revisited the evidence on how the RAS and immune mechanisms in the CNS synergistically induce a hypertensive response sensitization contributing to the pathogenesis of systemic hypertension. For example, stressors such as low-dose of Ang II and high-fat diet can induce the upregulation of both RAS and inflammatory elements, which will act on brain nuclei involved in blood pressure regulation through systemic-brain communication (Xue et al.). A second review discussed the interactions between peripheral- and CNS- RAS in the pathophysiology of systemic hypertension. The authors emphasized the relevance of the bidirectional crosstalk between the bone marrow RAS and the CNS RAS in atherosclerosis and hypertension. They also presented potential pharmacological approaches for treating cardiovascular diseases by activating the ACE2/Ang-(1–7)/Mas receptor pathway (Ciftciler and Haznedaroglu). Focusing on the CNS-related mechanisms underlying the antihypertensive effects mediated by Ang-(1–7), the original study performed by Kangussu et al. demonstrated that chronic intracerebroventricular infusion of Ang-(1–7) modulates both inflammatory and RAS-related responses in the hypothalamus. More specifically, i.c.v. administration of Ang-(1–7) resulted in changes in inflammatory (TNF decrease and IL-10 increase) and RAS-related components (reduced AT_1 receptor expression and ACE activity) in the hypothalamus, besides reducing cardiac hypertrophy in hypertensive transgenic (mRen2)27 rats (Kangussu et al.).

Hypertension is a common comorbidity and also a risk factor for neuropsychiatric diseases. Accordingly, the RAS may be regarded as a common pathophysiological pathway shared by these conditions. For example, hypertension is commonly observed in individuals with epilepsy, while lower blood pressure has been associated with reduced frequency and severity of seizures in patients with epilepsy. Becari et al. investigated whether the RAS is involved in seizures evoked by chronic acoustic stimulation in spontaneously hypertensive rats (SHRs). The chronic treatment with RAS-acting agents, such as ACE inhibitors and AT_1 receptor antagonists, reduced blood pressure in SHR but did not influence seizures. The authors concluded that the RAS might not play a major role in the mechanisms underlying audiogenic seizures in SHR (Becari et al.).

In addition to the role played by the RAS in CNS control of blood pressure and epilepsy, recent studies have investigated the involvement of the RAS in neurodegenerative diseases. The rationale behind these studies relies on the connection between RAS and different mechanisms implicated in neurodegeneration, including oxidative stress, neuroinflammation, endothelial dysfunction, microglial polarization, and alterations in neurotransmitter secretion, as reviewed by Cosarderelioglu et al. An exploratory study assessed whether peripheral levels of RAS components are associated with structural brain measures in patients with AD. Patients with AD presented reduced plasma levels of Ang-(1–7) in comparison with age-matched controls. Among patients with AD, lower levels of Ang-(1–7) were associated with decreased white matter hypointensities volumes. These findings corroborate the hypothesis that the RAS counter-regulatory/neuroprotective arm is downregulated in AD [here evidenced by lower levels of Ang-(1–7)], and possibly associated with cerebrovascular lesions in AD (Ribeiro et al.). Peripheral levels of RAS components were also investigated in relation to clinical outcomes (specifically cognitive performance) in patients with Huntington's disease (HD). In the study performed by Rocha et al., higher levels of ACE2 were associated with better scores in the Verbal Fluency Test, while higher levels of Ang II correlated with worse scores on the Mini-Mental State Examination in patients with HD. These results corroborate the proposed hypothesis of an imbalance between the classical (ACE/Ang II/AT_1 receptor) and the counter-regulatory/neuroprotective [ACE2/Ang-(1–7)/Mas receptor] arms of the RAS, being the former associated with negative clinical outcomes and the latter with positive effects in HD (Rocha et al.). Given the aforementioned results, it is tempting to hypothesize that the ACE/Ang II/AT_1 receptor axis inhibition and/or ACE2/Ang-(1–7)/Mas receptor axis activation would result in neuroprotective effects and/or positive clinical outcomes. With this perspective in mind, the treatment with candesartan (an AT_1 antagonist with documented ability to cross the blood-brain barrier) was tested as neuroprotective in a preclinical model of traumatic brain injury and showed to improve functional recovery and reduce neuropathology. Attilio et al. investigated, on this Research Topic, through RNA sequence analysis the mechanisms by which candesartan treatment influences the response to injury. Candesartan induced changes in genes involved in angiogenesis, inflammatory response, and extracellular matrix regulation (Attilio et al.). This study confirmed that AT_1 receptor antagonists are neuroprotective possibly due to a combination of effects, including anti-inflammatory properties. It is worth mentioning that the use of AT_1 receptor antagonists not only blocks the Ang II/AT_1 receptor signaling but also upregulates the Ang-(1–7)/Mas receptor pathway, thus the neuroprotective effects of such drugs are possibly due to the ACE2/Ang-(1–7)/Mas receptor axis activation as well (Rocha et al., 2018a).

Lastly, the role of the RAS in the CNS was investigated in the context of psychiatric disorders. Bipolar disorder (BD) is one of the most severe mental illnesses, with high rates of medical comorbidities and functional impairment. The etiopathogenesis of BD is yet to be elucidated and among the several pathways that might be involved, Sanches et al. hypothesized that dysfunctions in the RAS might play a role in the pathophysiology of this complex disorder. They reported no differences between patients with BD and age-matched controls regarding the plasma levels of ACE, ACE2, Ang-(1–7), and Ang II. However, non-euthymic patients with BD (i.e. patients experiencing mood episodes) had significantly lower ACE levels compared to controls. This study reinforces previous findings that RAS-related changes are associated with clinical outcomes in neuropsychiatric conditions (Sanches et al.). Given the medical and societal burden associated with psychiatric disorders and the associated therapeutic challenges, investigating the role of RAS in this

context might open new venues for understanding the biology of these disorders and, ultimately, novel pharmacological strategies.

In conclusion, this Research Topic highlights the emerging role of RAS in the CNS under physiological and pathophysiological conditions. Despite several gaps and controversies in the field, the investigation of RAS mechanisms in the CNS seems a promising endeavor. Future studies may shed light on pathophysiological processes underlying neuropsychiatric diseases and novel therapeutic targets for these devastating conditions.

AUTHOR CONTRIBUTIONS

NR wrote the first draft of the manuscript. ACSS and AT wrote sections of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

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REVIEW
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Interactions of the Brain Renin-Angiotensin-System (RAS) and Inflammation in the Sensitization of Hypertension

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Mounting evidence indicates that the renin-angiotensin (RAS) and immune systems interact with one another in the central nervous system (CNS) and that they are importantly involved in the pathogenesis of hypertension. Components comprising the classic RAS were first identified in the periphery, and subsequently, similar factors were found to be generated *de novo* in many different organs including the brain. There is humoral-neural coupling between the systemic and brain RASs, which is important for controlling sympathetic tone and the release of endocrine factors that collectively determine blood pressure (BP). Similar to the interactions between the systemic and brain RASs is the communication between the peripheral and brain immune systems. Systemic inflammation activates the brain's immune response. Importantly, the RAS and inflammatory factors act synergistically in brain regions involved in the regulation of BP. This review presents evidence of how such interactions between the brain RAS and central immune mechanisms contribute to the pathogenesis of hypertension. Emphasis focuses on the role of these interactions to induce neuroplastic changes in a central neural network resulting in hypertensive response sensitization (HTRS). Neuroplasticity and HTRS can be induced by challenges (stressors) presented earlier in life such as a low-dose of angiotensin II or high fat diet (HFD) feeding in adults. Similarly, the offspring of mothers with gestational hypertension or of mothers ingesting a HFD during pregnancy are reprogrammed and manifest HTRS when exposed to new stressors as adults. Consideration of the actions and interactions of the brain RAS and inflammatory mediators in the context of the induction and expression of HTRS will provide insights into the etiology of high BP that may lead to new strategies for the prevention and treatment of hypertension.

Keywords: RAS, inflammation, CNS, hypertension, perinatal programming

INTRODUCTION

Hypertension is a major risk factor for the development of cardiovascular diseases that are responsible for high morbidity and mortality. Approximately one-third of adults in the United States have hypertension, and only half of hypertensive patients show a satisfactory response to treatment (Go et al., 2014; Fryar et al., 2017). This may be because many of the existing antihypertensive therapies target peripheral mechanisms and are not the most effective for treating those forms of hypertension that are driven from the central nervous system (CNS) (Go et al., 2014). A better understanding of CNS mechanisms underlying the pathogenesis of hypertension could lead to the discovery of novel strategies for preventing and treating high blood pressure (BP).

CNS mechanisms driving sympathetic nervous system (SNS) overactivity play an important role in the development and progression of many forms of experimental hypertension (Haspula and Clark, 2018). The brain regions involved in sympathetic regulation include several nuclei located in the brainstem and hypothalamus such as rostral ventrolateral medulla (RVLM), hypothalamic paraventricular nucleus (PVN) and subfornical organ (SFO) (Guyenet, 2006; Dampney, 2016). Accumulating evidence indicates that renin-angiotensin system (RAS) components and inflammatory mediators act on brain nuclei within a central neural network to increase SNS drive that in turn, generates hypertension (Winklewski et al., 2015; Huber et al., 2017). This review presents evidence on how the actions and interactions between components of the brain RAS and CNS immune factors contribute to the pathogenesis of hypertension.

About ten years ago, we discovered that the CNS can be reprogrammed by mild non-pressor challenges that induce a neurological state that is expressed as a sensitized hypertensive response when a challenge (stressor) is presented once again. Thicausal mechanisms in prenatals hypertensive response sensitization (HTRS) is the result of the induction of CNS neuroplasticity in the brain neural pathways controlling the tone of the SNS (Johnson et al., 2015; Johnson and Xue, 2018). Our findings implicate both the brain RAS and CNS inflammatory mechanisms in the neuroplasticity that is responsible for HTRS. This review will focus on the mechanisms of the induction of HTRS produced by the presentation of physiological and dietary challenges presented earlier in the life span in both adults and during the prenatal period.

Besides a prohypertensive axis of the RAS, an antihypertensive axis of the RAS has also been identified and studied extensively regarding its protective role in the development of hypertension (Xu et al., 2011; Medina and Arnold, 2019; Sumners et al., 2020). This review also will discuss briefly recent evidence describing the protective effects of the RAS antihypertensive axis on HTRS.

The RAS and Proinflammatory Cytokines (PICs) in the CNS

The hypothalamus is a key brain region contributing to the regulation of the cardiovascular system, fluid balance, and energy homeostasis. Maintenance of these functions requires the integration of information from neural and humoral signals initially derived from the systemic (aka the circulating or classic) RAS and the systemic immune system. The SFO and organum vasculosum (OVLT) lie along the lamina terminalis (LT) and the area postrema (AP), located in the caudal medulla, function as sensory circumventricular organs (SCVOs) (Johnson and Gross, 1993). By virtue of lacking a blood-brain barrier, SCVOs can monitor blood-borne humoral factors, including angiotensin (ANG) II, leptin, extracellular osmolality and sodium (Mimee et al., 2013). Information reflecting the levels of blood-borne factors sensed by these structures is then projected through efferent pathways either directly or indirectly to hypothalamic regions including the paraventricular hypothalamic nucleus (PVN), ventromedial hypothalamus (VMH) and the arcuate nucleus (ARC) (Guyenet, 2006). In the hindbrain, there are important cardiovascular nuclei in addition to the AP. These include the solitary tract nucleus (NTS) and RVLM, which have bidirectional connectivity with those hypothalamic nuclei controlling sympathetic activity and BP (Guyenet, 2006; Dampney, 2016).

The RAS plays a fundamental role in the regulation of BP and hydromineral homeostasis. ANG II generated by angiotensin converting enzyme (ACE) 1 acts on ANG type 1 receptors (AT1-R) to produce its major physiological and pathophysiological effects, including direct acting vasoconstriction, baroreflex modulation, sympathetic activation, aldosterone release, triggering oxidative stress, and inflammation (Huber et al., 2017; Miller and Arnold, 2019). Components of the RAS (renin, angiotensinogen, ACE1 and AT1-R) associated with the synthetic cascade generating ANG II comprise a prohypertensive axis of the RAS. There also is a RAS anti-hypertensive axis in which ACE2 cleaves ANG II to form ANG-(1-7) that binds the Mas G protein-coupled receptor (Mas-R) to counter-regulate the effects produced by ANG II/AT1-R interaction. ANG II acting on angiotensin type 2 receptors (AT2-R) also exerts actions that oppose those of ANG II/AT1-R (Xu et al., 2011; Santos et al., 2018; Miller and Arnold, 2019).

The metabolic cascade of the systemic RAS (aka as the circulating or systemic RAS), involves renal renin release to act on renin substrate to generate ANG I and then via ACE1 action, ANG II. Following the elucidation of the components of the classic RAS, comparable elements were identified in many body organs including the brain. The so-called brain RAS is associated with neurons, astrocytes and microglia functions in a paracrine, autocrine, and even intracrine signaling role (Huber et al., 2017; Jackson et al., 2018; Nakagawa et al., 2020). Many CNS nuclei (i.e., the LT, PVN, RVLM, NTS and AP) implicated in cardiovascular control and fluid balance are rich in AT1-R-positive cells and are often innervated by axons containing some components of the RAS (Sumners et al., 2020).

Recent studies demonstrate that either AT2-R or ACE2/ANG-(1-7)/Mas-R are present either within or adjacent to cardiovascular nuclei in the brainstem and hypothalamus that express the AT1-R (Gao and Zucker, 2011; Gao et al., 2012; Sumners et al., 2015, 2020; Medina and Arnold, 2019). Functionally, in contrast to the prohypertensive effects of brain AT1-R activation to increase sympathetic activity and BP, either

overexpression or selective activation of central AT2-R decreases BP and plasma norepinephrine levels at least partially by reducing sympathetic outflow (Gao et al., 2008, 2011). Similarly, central infusion of ANG-(1-7) or overexpression of ACE2 has been shown to counteract most typical actions of ANG II or aldosterone and to reduce neurogenic hypertension (Feng et al., 2008, 2010) through decreasing the expression of AT1-R, ACE1 and PICs, and increasing AT2-R and Mas-R expression and nitric oxide production in the PVN (Sriramula et al., 2011).

Recent studies demonstrate that ACE2 is expressed on GABAergic neurons that in turn synapse on excitatory neurons within the hypothalamus (Mukerjee et al., 2019; Xu et al., 2019). ADAM17, an enzyme that catalyzes the cleavage of membrane anchored ACE2, is colocalized with AT1-R. Activation of ADAM17 in glutamatergic neurons leads to a selective increase of sympathetic activity, ultimately contributing to dysautonomia and neurogenic hypertension produced by ANG II or aldosterone (Mukerjee et al., 2019; Xu et al., 2019). These results highlight the importance of both the prohypertensive and antihypertensive axes of the RAS in BP regulation by the CNS.

Systemic treatment with exogenous pyrogen (eg., lipopolysaccharide; LPS) or PIC administration induces enhanced expression of PICs in the brain. Because PICs are large lipophobic polypeptides, they are unlikely to pass the blood-brain barrier. Therefore, as in the case of classic brain RAS communication, the mode of body-brain signaling is worthy of consideration. Current evidence indicates that there are five putative means of informing the brain of systemic inflammation and elevated peripheral PICs. First, sensory circumventricular organs, most notably the OVLT have been implicated in the mediation of fever. There is evidence to indicate that the pyrogenic effect of interleukin (IL)-6 is produced by action on the OVLT to release prostaglandin E_2, which in turn serves as a humoral mediator to communicate with surrounding tissue in the mode of a volume transmitter (Blatteis et al., 1983; Stitt, 1985; Blatteis, 1990; Harre et al., 2002). Second, there are active transport mechanisms for tumor necrosis factor-α (TNF-α). IL-β and IL-6 from blood into the brain (Banks et al., 1995). Third, brain perivascular cells, which have characteristics of macrophages, are responsive to low systemic doses of LPS and IL-1β. They also release prostaglandin E_2 to act in a paracrine manner to affect surrounding neural activity (Ericsson et al., 1994; Schiltz and Sawchenko, 2002). Fourth, there is unequivocal evidence showing that PICs, through their binding to receptors on brain endothelial cells, activate a humoral-neural signaling pathway and evoke fever by eliciting prostaglandin E2 synthesis in these cells (Blomqvist and Engblom, 2018). Fifth, visceral afferents arising from the periphery are activated by intraperitoneal administration of LPS or a PIC. Subdiaphragmatic vagotomy abolishes neural activation in the CNS and the central expression of PICs produced by intraperitoneal administration of proinflammatory agents (Maier et al., 1998; Goehler et al., 2000; Dantzer, 2018).

The brain has its own innate immune system, which involves microglia as the resident immune cell. Recent studies indicate that microglia and astrocytes in the brainstem and hypothalamus participate in orchestrating many cardiovascular and metabolic functions (Guyenet, 2006; Dampney, 2016). Glial cell activation involves mobilization of inflammatory signaling pathways involving dynamic changes in the expression and activity of several mediators, such as toll-like receptor 4 (TLR4) and IκB kinase-β/NF-κB, and to release a variety of PICs including TNF-α, IL-6 and IL-1β (De Git and Adan, 2015). Microglial activation and increased PICs in the periphery and CNS have been implicated in the pathophysiology of a variety of cardiometabolic diseases including heart failure, hypertension, and diabetes mellitus (Marina et al., 2016). Recent studies demonstrate that the high BP of the spontaneously hypertensive rat (SHR) and ANG II-induced hypertension are accompanied by microglial activation within the PVN along with elevated PICs (Shi et al., 2010; Shen et al., 2015). The increased levels of blood-borne and central PICs in cardiovascular disease states lead to the neurohumoral activation including sympathoexcitation and upregulation of central RAS activity (Wei et al., 2015, 2018). Blockade of brain microglia or targeted depletion of activated microglia within the PVN attenuates ANG II-induced hypertension, decreases PVN cytokines and reduces cardiac hypertrophy (Shi et al., 2010; Shen et al., 2015). These results support a role for microglial activation and the release of PICs in the pathogenesis of hypertension.

Interactions of the Brain RAS and PICs in the Pathogenesis of Renin-Angiotensin-Elicited Hypertension

Increased local production of ANG II is one of the main mechanisms responsible for the development and progression of hypertension. Apart from the control of SNS activity and extracellular volume regulation, ANG II also acts as a stimulator of PICs and promotes activation of the brain innate immune system (Shi et al., 2010; Sriramula et al., 2011; Shen et al., 2015). Conversely, both systemic PICs and brain PICs reciprocally interact with the RAS (Winklewski et al., 2015). As previously noted, the body-brain communication of these two systems includes both afferent neural and the humoral pathways over which circulating ANG II and PICs activate the central neural network controlling cardiovascular function (Wei et al., 2013, 2015, 2018). Because either the RAS or inflammatory mediators can contribute individually to the pathogenesis of hypertension, the interaction between RAS components and PICs are likely to have important synergistic effects (Yu et al., 2013; Xue et al., 2016a).

Toll-like receptors are pathogen receptors, which are a major component of the innate immune system (Akira, 2003; Hedayat et al., 2011). A growing body of findings demonstrate that TLR4s modulate inflammatory responses implicated in the development of hypertension. As a prototypic TLR4 ligand, LPS administered acutely activates microglia and increases PICs in the brain and this response is attenuated by blockade of AT1-R (Benicky et al., 2009, 2011). Chronic LPS infusion results in a sustained increase in BP that is accompanied by microglial activation, increased PIC expression and elevated reactive oxygen species (ROS) production in the RVLM (Wu et al., 2012). Francis and colleagues

reported that ANG II upregulates TLR4 expression in the PVN. Chronic blockade of brain TLR4 significantly attenuated ANG II-induced hypertension and cardiac hypertrophy. Blockade of TLR4 receptors resulted in reduced ROS generation, myocardial inflammation, NF-κB activity, expression of TNF-α and IL-1β, and altered prohypertensive and antihypertensive RAS components. The blockade resulted in tilting the balance in favor of the antihypertensive response (Dange et al., 2014). SHR have more TLR4s in the PVN, and blockade of these receptors in the PVN prevented the increase in BP and attenuated cardiac hypertrophy by downregulation of inflammatory components and upregulation of anti-inflammatory mediators in the PVN (Dange et al., 2015). These results indicate that in different forms of hypertension, TLR4 is a mediator for microglial activation that further engages the RAS and promotes PIC and ROS production.

NF-κB is a key regulator of PIC expression and the inflammatory response observed in hypertension. In ANG II-induced hypertension, peripheral ANG II infusion increases PICs, ROS, and prohypertensive RAS components (ACE1 and AT1-R) and decreases the antihypertensive components (ACE2 and Mas-R) within the PVN. Bilateral PVN NF-κB blockade decreases the prohypertensive RAS axis and increases the protective antihypertensive RAS axis through an ROS-mediated mechanism. The result is an attenuation of the ANG II-induced hypertensive response (Kang et al., 2009; Cardinale et al., 2012). Furthermore, central pharmacological blockade or genetic knockout of TNF-α has been shown to prevent cardiac hypertrophy and to lower BP in animals with hypertension induced by ANG II through mechanisms similar to that of central blockade of NF-κB (Sriramula et al., 2008, 2013).

Endoplasmic reticulum (ER) stress and autophagy are also implicated in the development of hypertension. In ANG II-induced hypertensive mice there is upregulation of many members of the ER stress pathway in the SFO. Central inhibition of ER stress prevented ANG II-induced hypertension (Young et al., 2012). Likewise, SHRs exhibited greater ER stress and autophagic dysfunction in the RVLM that precedes the development of the hypertensive phenotype, which is dependent on oxidative stress (Chao et al., 2013). Activation of AT1-R increases ER stress and ROS production in the SFO that further activates NF-κB during the development of ANG II-induced hypertension (Young et al., 2015). Collectively, these data suggest that ER stress and autophagy, as well as their interactions with the RAS and PICs in the brain, represent novel cellular and molecular mechanisms underlying the development of neurogenic hypertension.

Recent studies demonstrate that blood-borne PICs induce a pressor response and sympathetic activation through acting on the SFO to increase RAS activity and inflammation. Blockade of AT1-R or ACE or knockdown of AT1-R in the SFO attenuates those PIC-elicited responses (Wei et al., 2013, 2015, 2018; Yu et al., 2018). Tumor necrosis factor-alpha converting enzyme (TACE) generates a soluble form of TNF-α. TACE is upregulated in the SFO and PVN by central infusions of ANG II or IL-1β. The SFO knockdown of the TNF-α receptor 1 or central blockade of TACE ameliorates sympathetic excitation and impaired cardiac hemodynamics in heart failure (Yu et al., 2017, 2019; Korim et al., 2019). These results suggest that the SFO-mediated sympathoexcitatory response to PICs depends on the ambient level of activity of the central RAS and PICs.

Collectively, the studies just described indicate that crosstalk between the RAS and TLR4/NF-κB/TNF-α as well as ER stress and autophagy within the brain tips the balance of the RAS in favor of the prohypertensive axis while decreasing the antihypertensive axis thereby enhancing cardiovascular inflammation. This process results in triggering positive feedback from periphery to brain to drive a progressive increase in sympathetic tone and the pathogenesis of hypertension.

Brain Inflammation and RAS Activation in Obesity-Induced Hypertension

Obesity is present in epidemic proportions and is a major risk factor for type II diabetes mellitus and hypertension. Either obesity *per se* or consuming a high fat diet (HFD) produces a chronic low-grade inflammatory state characterized by activation of microglia, astrocytes, and increased expression of PICs in the CNS. The mediators of the brain innate immune system, including TLR4, IκB kinase-β/NF-κB (IKKβ/NF-κB), ER stress and autophagy, are involved in obesity-related inflammatory signaling (De Git and Adan, 2015). For example, chronic HFD feeding resulted in increased expression and activity of TLR4 in the hypothalamus (Ropelle et al., 2010; Wang et al., 2012). Eating a HFD for just one day is sufficient to increase hypothalamic IKKβ/NF-κB signaling (Thaler et al., 2012). IKKβ/NF-κB signaling then acts both upstream and downstream from ER stress and autophagy that contributes to the development of leptin resistance and obesity (Zhang et al., 2008; Meng and Cai, 2011). In contrast, brain knockout or central pharmacological inhibition of TLR4 or IKKβ/NF-κB prevents hypothalamic inflammation, improves leptin sensitivity and protects against HFD-induced obesity (Zhang et al., 2008; Kleinridders et al., 2009; Milanski et al., 2009; Meng and Cai, 2011). These results indicate that increased PICs induced by brain innate immune system mediators contribute to the elevated SNS activity and BP in obesity (Hall et al., 2015).

Growing evidence indicates that obesity is associated with overactivation of the RAS in humans and animals (Kalupahana and Moustaid-Moussa, 2012). In addition to its well documented role in BP control, the RAS is involved also in energy homeostasis. Both the classic RAS and adipose tissue RAS play roles in promoting energy storage, whereas in contrast, the brain RAS increases energy expenditure (Grobe et al., 2010; De Kloet et al., 2013). The difference between the peripheral and central RAS effects suggests the presence of a negative feedback mechanism that is responsible for maintaining energy balance (Prior et al., 2010). However, a consequence of the presence of a putative peripheral-central negative feedback pathway for the maintenance of energy balance is that it activates the RAS and PICs to drive increased SNS activity and BP. This may be the crux of the mechanisms underlying the development of obesity-related hypertension. This notion is supported by recent studies showing that although animals with diet-induced obesity (DIO) can restore body weight, insulin levels and leptin sensitivity

to normal after being switched to a normal fat diet, BP, renal sympathetic nerve activity (RSNA) and the activity of central RAS and PICs remain high (Prior et al., 2010; Maric et al., 2014). Genetic disruption of AT1-R in leptin-expressing or agouti-related peptide (AGRP)-expressing cells in the ARC abolishes the thermogenic increase in sympathetic nerve activity in response to leptin, HFD, and deoxycorticosterone acetate (DOCA)-salt (Claflin et al., 2017; Deng and Grobe, 2019). In rabbits, three weeks of HFD feeding led to increased BP, heart rate (HR) and RSNA. These are responses mediated by neurogenic mechanisms (Armitage et al., 2012; Lim et al., 2013). Therefore, obesity itself may induce hypothalamic inflammation and sensitization of the brain to circulating sympathoexcitatory factors such as those from the RAS, and these initiate increased BP.

In obese humans and in animal models of DIO, increased SNS activity and BP have also been demonstrated to be associated with increased leptin and activation of a leptin/melanocortin 4 receptor (MC4R) pathway (Hall et al., 2002; Tchernof and Despres, 2013). The CNS structures that mediate leptin's action include the ARC and PVN and extrahypothalamic regions including the NTS and SFO. Microinjection of leptin into the ARC or PVN increased sympathetic activity (Harlan et al., 2011; Shi et al., 2015), whereas, leptin receptor deletion in the ARC or SFO attenuated the increase in sympathetic activity evoked by leptin (Harlan et al., 2011; Young et al., 2013). In either leptin deficient or leptin receptor deficient mice, the lack of leptin signaling led to attenuated microglial activation and reduced expression of several inflammatory mediators. Since leptin can be considered as a PIC (Lord, 2006), it may be that it is in this role that it produces microglial activation (Gao et al., 2014). De Kloet et al. reported that HFD and obesity activate microglia and astrocytes with a subsequent increase in PICs in the SFO and PVN. This effect was ANG II dependent as some of the responses were reversed by deletion of PVN AT1-R (De Kloet et al., 2014). Other studies have shown that with central inhibition of ACE1, blockade of AT1-Rs or global knockout of AT1-R, there is an abolition of the increase in sympathetic nerve activity to leptin. This suggests that leptin interacts with the brain RAS to regulate sympathetic activity and BP (Hilzendeger et al., 2012; Xue et al., 2016b). Meanwhile, it has been shown that obesity-associated activation of the hypothalamic NF-κB/IKK-β pathway also mediates leptin functions in the CNS (Purkayastha and Cai, 2013). These findings suggest that the interactions and synergism between leptin, the RAS and PICs may be responsible for the obesity-induced increase in sympathetic activity that generates hypertension in DIO.

Brain RAS Activation and Inflammation in Hypertensive Response Sensitization (HTRS) in Adult Animals

There are many examples of changes in behavioral and physiological responses resulting from prior experience. The impact of exposure to certain stimuli or physiological states can last a lifetime. When a response entails an increase in magnitude because of an antecedent condition, it is considered as an example of sensitization (Kandel et al., 2014). Sensitization involving the CNS has been studied in many conditions but until recently has received no attention in investigation of the pathogenesis of hypertension (Johnson et al., 2015; Johnson and Xue, 2018). In order to study sensitization of the hypertensive response and the role of the CNS in the sensitization process, we developed an Induction-Delay-Expression (IND-DEL-EXP) experimental model. During IND, rats are exposed to various types of challenges that do not produce any sustained increase in BP when administered by themselves. Rats then are allowed a rest period (i.e., DEL) to allow for demonstration of the permanence of the induced state and metabolism of any hormones or drugs that might have been administered. After the DEL, a sensitized hypertensive response to a slow-pressor dose of ANG II was observed in these rats when challenged during a period of EXP. Brains can also be collected at the end of DEL for regional (the LT and PVN) analysis for gene expression of RAS components, PICs and indicators of microglial activation (Johnson et al., 2015; Johnson and Xue, 2018).

We found that peripheral or central pretreatment with non-pressor doses of ANG II, aldosterone, or TNF-α sensitized the hypertensive response to a slow-pressor dose of ANG II. At the end of DEL the mild challenges used for IND upregulated mRNA expression of several components of the RAS and PICs and of microglial marker in the brain, whereas central inhibition of AT1-R, mineralocorticoid receptors, and inflammation reversed the changes in gene expression and also prevented the sensitization of ANG II-elicited hypertension seen during EXP. Importantly, the results showed that either central blockade of the RAS or central blockade of inflammation during IND attenuated both the upregulated expression of RAS components and PICs regardless of the mild challenges used for IND (Xue et al., 2012a,b, 2016a,b). Such findings suggest that a previous encounter with even a mild stressor can chronically upregulate the brain RAS and induce neuroinflammatory cellular and molecular mediators in brain pathways that control sympathetic tone. The result is an increase in neural excitability that is expressed as HTRS when challenged once again with an old or new stressor. The capacity of the RAS components or PICs to mutually up-regulate their expression may be indicative of actions within central sensitization-related positive feed-forward systems which can accelerate the onset and rate of development of hypertension.

In studies of dietary sensitization, 3 weeks of HFD feeding increased white adipose tissue mass, plasma leptin levels and mRNA expression of leptin and leptin receptor in both the LT and PVN (Xue et al., 2016a). Central administration of leptin mimicked HFD sensitization of ANG II-induced hypertension while central leptin antagonist prevented the sensitization (Xue et al., 2016b). Central inhibition of AT1-R, TNF-α synthesis or microglial activation significantly attenuated either HFD- or leptin-induced upregulation of RAS activity, inflammation in the LT and PVN, and HFD or leptin sensitization of ANG II-induced hypertension. These observations indicate that a HFD can predispose rats to display HTRS through leptin-mediated upregulation of RAS components and PICs in the LT and PVN. This leptin-dependent increase in RAS activity and PICs within the CNS may offer a causal link between obesity and hypertension (Xue et al., 2016a,b).

Despite the prohypertensive axis of the RAS playing a predominant role in the sensitization of hypertension, the antihypertensive axis of the RAS has a protective effect against the sensitization process. This is based on its counter-regulatory effects on hypertension. In our studies, low doses of ANG II or aldosterone infusion during IND not only enhances ANG II-induced increases in the expression of renin, AT1-R, and ACE mRNA, but also upregulates mRNA expression of ACE2 and AT2-R in forebrain nuclei (Xue et al., 2012a,b). Furthermore, central ANG-(1-7) normalized the increased mRNA expression of AT1-R and ACE produced by ANG II given during IND while the mRNA expression of ACE2 and Mas-R remained high and abolished the ANG II-induced sensitization in male rats (Xue et al., 2014). The results indicate that the antihypertensive axis of the RAS plays an important counter-regulatory role in protecting against sensitization of ANG II-induced hypertension. The enhanced central ACE2 and AT2-R expression may reflect the activation of inhibitory mechanisms that attempt to buffer against the pressor actions of ANG II and act to keep BP in check (Xue et al., 2012a,b, 2014).

Taken together, our studies provide insight into the actions of the RAS and of PICs in the hypothalamus to mediate cardiovascular sensitizing effects of mild physiological or metabolic challenges, even though these factors do not induce any apparent immediate abnormalities in the cardiovascular function. The demonstrations of sensitization also indicate that challenges to homeostasis can act to reprogram the neural network controlling BP to generate an enhanced pressor response when a hypertensive stimulus is either sustained or encountered at a point later in life (Johnson et al., 2015; Johnson and Xue, 2018) (**Figure 1**).

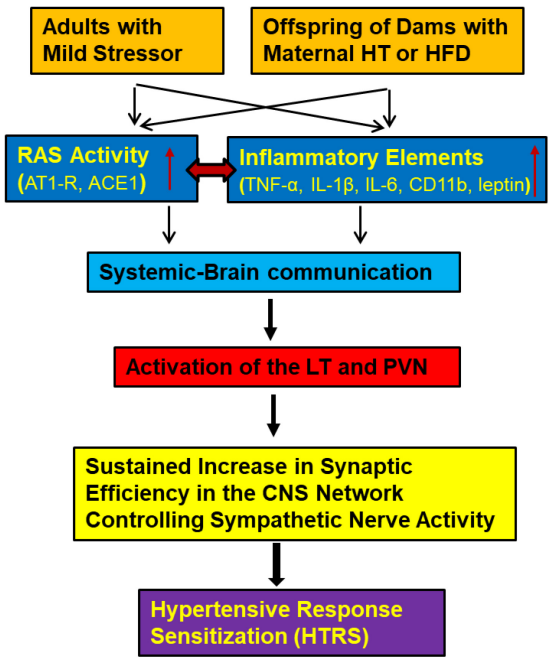

FIGURE 1 | Central cascade of factors mediating hypertensive response sensitization (HTRS). In adult animals challenged with mild physiological or psychological stress or in offspring from dams with maternal hypertension (HT) or high fat diet (HFD) feeding, both the renin-angiotensin system (RAS) and inflammatory elements will be upregulated and act on brain nuclei involved in blood pressure regulation through systemic-brain communication. These central RAS and inflammatory mechanisms will synergistically induce a HTRS. (ACE1, angiotensin converting enzyme 1; AT1-R, angiotensin II type 1 receptor; TNF-α, tumor necrosis factor-α; IL-1β, interleukin-1β; IL-6, interleukin-6; CD11b, cluster of differentiation molecule 11b; LT, the lamina terminalis; PVN, the paraventricular nucleus of hypothalamus).

Brain RAS Activation and Inflammation and Maternal Hypertension- or HFD-Induced Sensitization of Hypertension in Offspring

There is a strong association between maternal health during pregnancy and cardiovascular disease in adult offspring. Many kinds of prenatal insult such as hypertension, obesity or HFD, protein restriction, uteroplacental perfusion insufficiency or glucocorticoid treatment during pregnancy have been demonstrated to have consequences on health of the offspring. Such prenatal challenges alter responses to environmental stressors and predispose human and experimental animals to cardiovascular and metabolic disorders (Reynolds et al., 2013; Alexander et al., 2015).

Increased RSNA and overactivity of the RAS and PICs have been implicated as causal mechanisms in prenatal programming of hypertension (Wu and Berecek, 1993; Lopes et al., 2008; Mizuno et al., 2013, 2014; Washburn et al., 2015; Deng et al., 2016). Either renal denervation (RD) or chronic blockade of the RAS and inflammation reduces elevated BP through restoration of renal and arterial function in offspring of dams after different types of insult to the mother during pregnancy (Wu and Berecek, 1993; Langley-Evans and Jackson, 1995; Intapad et al., 2013; Mansuri et al., 2015; Deng et al., 2016). Recent studies also report that expression of the AT1-R in the SFO and the OVLT was increased in the offspring of protein-restricted dam. Intracerebroventricular injections of an ACE1 inhibitor or an AT1-R antagonist significantly reduced BP in these offspring (Pladys et al., 2004).

The prevalence of obesity in women of childbearing age and during pregnancy has steadily increased over the past two decades (Flegal et al., 2012). Prior et al. demonstrated that the offspring of rabbits fed a HFD had markedly elevated RSNA and pressor responses to central leptin, enhanced sympathetic responses to ghrelin and an exaggerated sympathetic response to acute air-jet stress when compared with offspring from mothers fed a normal fat control diet. This suggests that the higher levels of BP and RSNA may be attributable to changes in central pathways regulating SNS activity (Prior et al., 2014). Also, maternal HFD was associated with hypothalamic inflammation, impaired baroreflex sensitivity and reduced HR variability, implicating abnormalities in autonomic control in the offspring (Samuelsson et al., 2010; Deng et al., 2016; Cesar and Pisani, 2017).

Maternal separation during early life, so called early life stress, sensitizes rats to ANG II-induced hypertension in adult life. Early

life stress offspring displayed renal dysfunction, elevated renal and systemic sympathetic activity, enhanced ANG II-mediated vasoconstriction and vascular inflammation and reduced endothelial nitric oxide buffering capacity (Loria et al., 2010, 2011, 2013a,b).

Our studies investigating the effects of gestational maternal challenges on the offspring when they reach adulthood have focused on the role of reprogramming central mechanisms involving the RAS and PICs on SNA. This was done by inducing sensitization of the offspring during the prenatal period by subjecting pregnant dams to ANG II-elicited hypertension or to HFD feeding (Xue et al., 2017; Zhang et al., 2018; Wang et al., 2020). In the offspring of hypertensive dams, we found that the adult male offspring showed upregulated expression of both RAS components and PICs in the LT and PVN and displayed HTRS to a slow-pressor dose of ANG II when compared with the offspring of normotensive dams. Either renal denervation or captopril treatment administered between weaning and adulthood blocked HTRS and reversed the changes in RAS and PIC mRNA expression of the hypertensive dam offspring (Xue et al., 2017). Likewise, in adult offspring of HFD fed dams, upregulated expression of RAS components, NADPH oxidase and PICs were evident in the LT and PVN. These offspring exhibited normal BP at 10 weeks of age, but had blunted cardiac baroreflex function and elevated autonomic sensitivity to central challenges with ANG II or TNF-α. The offspring also showed a significantly greater hypertensive response to a slow-pressor dose of ANG II that was accompanied by an enhanced upregulation of mRNA expression of RAS components, NADPH oxidase and PICs in the LT and PVN (Zhang et al., 2018). These studies further demonstrate that systemic blockade of either the RAS or inflammation in offspring abolished upregulated brain RAS and PICs and sensitization of the hypertensive response to ANG II produced by the pregnant mothers eating a HFD (Wang et al., 2020). These results indicate that maternal HFD feeding during either pregnancy or lactation is sufficient for early life programming of the central HTRS to predispose the next generation to a greater risk of hypertension. The studies also provide a potential therapeutic option to reduce the impact of maternal obesity or HFD on adverse outcomes, especially hypertension, in the offspring.

Sex-specific effects are widely observed in perinatal developmental programming studies and female offspring are protected as compared to male offspring (Dasinger and Alexander, 2016; Colafella and Denton, 2018). We also have found that there are sex differences in sensitization of ANG II hypertension in adult animals and in the offspring of maternal hypertensive mothers, where female animals are protected against the sensitization of hypertension (Xue et al., 2014, 2018).

Taken together, these experiments demonstrate that reprogramming of the mechanisms controlling BP during a sensitive prenatal period produces long-lasting phenotypic changes in the CNS and increased responsiveness to a prohypertensive challenge (**Figure 1**).

SUMMARY AND CONCLUSION

In this review, we have described the roles of the RAS and inflammation in the CNS in the development of hypertension. These two systems, either independently or synergistically, activate brain nuclei involved in BP regulation to increase SNS drive that in turn, generates renin-angiotensin-elicited or obesity-related hypertension. We further highlighted the interactions of the RAS and inflammation in the HTRS.

The predisposition of the offspring of mothers exposed to various insults during pregnancy to display HTRS appears to be very similar to sensitization of this response produced by prior challenges that were found in adult animals. The permanence of HTRS is associated with CNS reprogramming instantiated as changes in expression of RAS components and of PICs. The studies on the new models of sensitization provide many possibilities to elucidate central mechanisms underlying the long-term maintenance of sensitization and the etiology of hypertension.

Because maternal hypertension and HFD/obesity are common and rapidly increasing, it is likely that future generations will be at increased risk for cardiovascular diseases including hypertension. Because only half of hypertensive patients show a satisfactory response to conventional treatment (Go et al., 2014; Fryar et al., 2017), it is critical to identify new therapeutic strategies that effectively prevent hypertension or that are useful in treating the disorder. This is especially true for the kinds of high BP that are driven from the CNS. These insights may ultimately reduce the clinical, social and economic burden produced by hypertension.

AUTHOR CONTRIBUTIONS

BX conceived, drafted, revised, and approved the manuscript. YZ revised and approved the manuscript. AJ conceived, edited, revised, and approved the manuscript. All authors contributed to the article and approved the submitted version.

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Pathobiological Interactions of Local Bone Marrow Renin-Angiotensin System and Central Nervous System in Systemic Arterial Hypertension

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Circulating renin-angiotensin system (RAS) and local paracrin-autocrin-intracrin tissue-based RAS participate in numerous pathobiological events. Pro-inflammatory, pro-fibrotic, and pro-thrombotic consequences associated with local RAS activation have been detected at cellular and molecular level. Regenerative progenitor cell therapy in response to RAS modulating pharmacotherapy has emerged as an adjunct in the context of endothelial cell injury and regeneration to improve regeneration of the vascular endothelium. Local hematopoietic bone marrow (BM) RAS symbolizes the place of cross-interaction between vascular biology and cellular events from embryogenesis to definitive hematopoiesis underlying vascular atherosclerosis. The BM microenvironment also contains Mas receptors, which control the proliferative role of Ang 1-7 on hematopoietic stem cells. Ang 1-7 is produced from Ang-II or Ang-I with the help of ACE2. Various tissues and organs also have an effect on the RAS system. The leukocytes contain and synthesize immunoreactive angiotensinogen species capable of producing angiotensin in the basal state or after incubation with renin. The significance of RAS employment in atherosclerosis and hypertension was indicated by novel bidirectional Central Nervous System (CNS) RAS–BM RAS communications. Myeloid cells generated within the context of hematopoietic BM RAS are considered as the initiators and decision shapers in atherosclerosis. Macrophages in the atherosclerotic lesions contain angiotensin peptides by which RAS blockers inhibit monocyte activation and adherence. Furthermore, vascular biology in relation to inflammation and neoplasia is also affected by local tissue RAS. The purpose of this article is to outline interactions of circulating and local angiotensin systems, especially local bone marrow RAS, in the vascular pathobiological microenvironment of CNS.

Keywords: renin-angiotensin system, bone marrow, atherosclerosis, hypertension, central nervous system

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INTRODUCTION

As previously known, renin-angiotensin system (RAS) was described as an endocrine system that regulates blood pressure and body electrolyte balance (1). Recently RAS, which is considered as an "ubiquitous" system with various effects on tissue physiology and homeostasis, has been shown to be locally expressed in different tissues (2). With the detection of novel RAS components, the emerging tissue RAS concept has expanded the physiological and clinical

location of the RAS (2). Circulating local RAS and local paracrin-autocrin-intracrin tissue-based RAS participate in numerous pathobiological events. Pro-inflammatory, pro-fibrotic, and pro-thrombotic consequences associated with local RAS activation have been detected at cellular and molecular level (2). For these reasons, it is important to know RAS components, tissue-specific expressions of RAS and how they can change under pathological circumstances (2). There is a RAS located in the bone marrow (BM) microenvironment and within the hematopoietic stem cells (HSC). The concept of local BM RAS, which is active in primitive and definitive hematopoiesis, had been proposed by Haznedaroglu and coworkers about three decades ago (3, 4).

RAS molecules, particularly ACE, ACE2, AGT, AGTR1, AGTR2, AKR1C4, AKR1D1, ANPEP, ATP6AP2, CMA1, CPA3, CTSA, CTSD, CTSG, CYP11A1, CYP11B1, CYP11B2, CYP17A1, CYP21A2, DPP3, EGFR, ENPEP, GPER, HSD11B1, HSD11B2, IGF2R, KLK1, LNPEP, MAS1, MME, NR3C1, NR3C2, PREP, REN, RNPEP, THOP1 are available in the BM (5). These molecules affect the entire stem cell-oriented hematopoiesis. Local BM RAS seems to be the major station of cross-talk between hematopoietic stem/progenitor cells and their afferent functions within the heart, kidney and Central Nervous System (CNS) as a (patho)biological trigger for vascular atherosclerosis (6). There is also a local CNS RAS. The importance of RAS and BM-RAS activity in atherosclerosis and hypertension was indicated by novel bidirectional CNS RAS–BM RAS communications (7–9). The purpose of this article is to outline interactions of circulating and local angiotensin systems, especially local bone marrow RAS, in the vascular pathobiological microenvironment of CNS.

LOCAL BONE MARROW RENIN ANGIOTENSIN SYSTEM: DEFINITION AND A BRIEF HISTORY

Major RAS molecules, including renin, angiotensinogen, angiotensin receptors and ACE, are all found in the BM microenvironment (10). Haznedaroglu et al. first proposed the idea of a local hematopoietic RAS in BM in 1996. The study supposed that there is a locally active RAS in the BM that affects the production, proliferation growth, and differentiation of hematopoietic cells (3). Later, evidence of a local RAS in the BM increased day by day. Locally active BM RAS influences important pathways in physiological and pathological blood cell production by autocrine, paracrine and intracrine routes (11, 12). The development of hematopoietic niche, erythropoiesis, myelopoiesis, thrombopoiesis and other cellular linage is controlled by local BM RAS peptides (13–16). Additionally, many important pathobiological events such as cellular proliferative events, mobilization, angiogenesis, fibrosis, and apoptosis in the cytokine network are affected by RAS molecules (11, 12, 17, 18). Local RAS in the BM stromal niche controls important hematopoietic functions (10, 12–15, 19, 20). The BM stromal microenvironment contains important peptides that are components of the RAS (11, 12).

BM stromal microenvironment includes AT1R and AT2R (angiotensin type 1 and type 2 receptors, respectively) and inhibitory tetrapeptide AcSDKP (N-acetyl-Ser-Asp-Lys-Pro) (11, 12). The major RAS effector agent angiotensin II (Ang II) performs its impacts on the hematopoietic system by activating the AT1Rs and AT2Rs, along with the BM microenvironment (11, 12). As a result of ACE's (CD143) disrupting the inhibitory tetrapetide, AcSDKP, priming of stem cells into S-phase is triggered (16, 21). Additionally, Ang-II stimulates the AT1/AT2 receptors, so it has stimulating or inhibitory effects on erythropoietin, thrombopoietin and other hematopoietic cytokines in normal hematopoiesis and myeloproliferative diseases (16, 22, 23). Multiple clinical studies have been made to evaluate the role of local BM RAS in several diseases (18, 24–30).

Phase I/II clinical trials of a pharmaceutical agent of peptide angiotensin 1-7 (Ang-1-7) have been directed to assess the role of local BM RAS in different diseases (18, 24, 25). Other participating components of Ang II and RAS such as Ang IV [Ang-(3-8)] and Ang-(1-7) play a regulatory role on the cardiovascular system (26). Moreover, ACE2 and Mas receptor are very important components of the option line of RAS and are expressed in Hematopoietic stem/progenitor cells (HSPCs) (27). Activation of the ACE2/Ang-(1-7)/MasR axis stimulates the functions of HSPCs related to vascular repair and repulses dysfunctions caused by chronic pathological situations (27). On the other hand, white blood cells locally produce angiotensin peptides (28–30). Gomez et al. showed that circulating rat leukocytes express the angiotensinogen gene. It showed that leukocytes contain and synthesize immunoreactive angiotensinogen species that can produce angiotensin in the basal state or after incubation with renin. As a system that produces angiotensin, leukocytes can be important in modulating inflammatory responses, tissue damage, and cardiovascular pathology such as hypertension (28).

The supposition that local autocrine BM RAS may be effective in neoplastic hematopoiesis supports the prominent functions of local RAS in primitive embryonic hematopoiesis (31–33). Likewise the considerable functions of local RAS, which are involved in primitive embryonic hematopoiesis, also reinforce the supposition that local autocrine BM RAS may have an effective role in neoplastic hematopoiesis (19). Critical RAS modulating agents such as renin, ACE, angiotensinogen, and AngII have been previously described in leukemic malignant cells (34–36). Recently, Yamashita et al. showed that angiotensin-(1-12) generation is revealed in the BM of rats. Chymase-mediated Ang II production in BM was importantly higher than ACE-mediated and 280-fold higher than that in the heart (37). CD68 positive myeloid lineage cells, especially myeloid progenitors, have higher chymase expression than CD68 negative lymphoid lineage cells in BM (37).

LOCAL BONE MARROW RENIN ANGIOTENSIN SYSTEM AND ATHEROSCLEROSIS

Local BM RAS has significant effects on hematopoietic systems, particularly on myeloid, and erythroid cells (3, 12, 19). Local BM RAS is involved in the regulation of important peptides

that control hematopoiesis. With the help of ACE, Ang I transformed into Ang II, while bioactive SP, Ac-SDKP and Ag 1-7 were inactivated by ACE. Also, during this process, substance P (SP) is secreted from nerve endings. BM stromal and hematopoietic cells secrete RAS peptides by the AT1 and NK1 receptors that coordinate the effect of Ang II and SP, respectively as depicted in **Figure 1**. Additionally, it has been proven that important receptors of Ang 1-7 and MAS are available in the BM stroma (38). The BM microenvironment contains Mas receptors, which control the proliferative role of Ang 1-7 on HSCs. Ang 1-7 is created from Ang-II or Ang-I with the help of ACE2. Hematopoietic recovery after myelosuppression increases with Angiotensin (1-7)(5, 17). Besides all these, RAS plays a role in the pathogenesis of various diseases (42).

RAS is an extremely complex system consisting of a series of enzymes, peptides and receptors known to play a role in the formation of hypertension and atherosclerosis (43). It has been shown that most of the enzymes and peptides described initially as a hormonal system of RAS can be made locally in various organs, including blood vessels (43). RAS has a critical function in the management of blood flow, fluid volume, blood pressure and electrolyte balance (6). The overworking of RAS participates in the pathogenesis of various clinical situations, such as the beginning and progression of atherosclerosis (6). Ang II is the peptide known as the main last effector molecule of the classical RAS line, consisting of ACE, Ang II and angiotensin receptor type I (AT1R) (43). Ang II is produced in the brain, kidney and blood vessels. Ang II triggers hypertension through transcription activation, reactive oxygen species (ROS) production, inflammation and numerous alternative cellular events (44, 45). Ang II takes part in all stages of the pathogenesis, up to early lesion formation, growth, progression and plaque rupture, and as a result causes atherosclerosis (44, 45). Oxidative stress and inflammation make up the majority of the mechanism of action of Ang II on atherosclerosis (46). Inflammatory cells in atherosclerotic lesions are generally thought to originate from BM (15). Angiotensin II improves erythroid differentiation in the BM by interacting with the AT1R (15). Fukuda et al. analyzed a few BM chimeric mice whose BM cells were positive or negative for AT1R (47). They demonstrated that AT1aR in BM cells attend in the pathogenesis of atherosclerosis (47). Another study showed that AT1a receptor was expressed by human BM $CD34^{+}$, $CD38^{-}$ cells, and lymphocytes (48). Ang II has been reported to stimulate the differentiation of human CD34 + hematopoietic progenitors from cord blood (48).

Besides all these, atherosclerosis due to hypercholesterolemia is affected by the pharmacological antagonism of AT1 receptors or the reduction of the AT1A receptor (49). Hypercholesterolemia induces the production of angiotensin peptides for the effect of AT1A receptor deficiency on atherogenesis (49). There is also a close relation between cardiac RAS and the hematopoietic BM RAS (3, 12). Myocardial tissue healing through HSC plasticity shows the interaction between local cardiac RAS and hematopoietic RAS (23).

ATHEROSCLEROSIS AND CENTRAL NERVOUS SYSTEM

Atherosclerosis is often assessed as a chronic inflammatory disease, because inflammation has a significant function in all stages of atherosclerosis (50, 51). Studies have shown that atherosclerotic disease is often the cause of the onset of ischemic cerebro-vascular events (52–54). The atherogenic process is attended by flow-mediated inflammatory alterations in endothelial cells (EC) (55). In early-stage atherosclerosis, endothelial damage, abnormal lipid metabolism and hemodynamic damage are the causes of the disease (55). In late-stage atherosclerosis, lots of macrophages and inflammatory cytokines leak into the vascular wall, excrete matrix metalloproteinases (MMPs) and result in plaque rupture, bleeding and thrombosis (56). The harmonious effect of all proinflammatory signals on the plaque increases inflammation and also prevents the regeneration of structural elements that support the mechanical stability of the inflamed tissue (57).

Atherosclerosis is a chronic inflammatory syndrome that affects the unity and activity of major blood vessels which supplies the brain. As a result, chronic inflammation in the vessels impairs cerebral blood flow and neurovascular communication, which is important for cerebrovascular function (58). Chronic atherosclerosis has an effect on the brain throughout the life of people (59–62). Atherosclerosis leads to impaired vascular integrity, causing cognitive decline, stroke and vascular dementia (58). Atherosclerosis is most effective on large and medium-sized arteries, including internal carotid and vertebral arteries. Atherosclerosis and associated cardiovascular diseases can cause a wide variety of vascular diseases and lesions in the brain. These lesions caused by atherosclerosis occur as a result of arterial stiffness and inflammation (63). All these events take place with a complex mechanism.

There is an association between the autonomic nervous system (ANS) and BM cells. This relationship between BM stromal cells, HSCs and nerve terminals has been defined as the 'neuro-reticular complex' (64, 65). A distinctive feature of early hypertension is endothelial dysfunction (8). BM-derived endothelial progenitor cells (EPC) participate in the healing of damaged endothelium. Studies have shown that EPC numbers and functions are lower in patients with hypertension and cardiovascular disease (66–69).

CENTRAL NERVOUS SYSTEM AND HYPERTENSION

All components of RAS are found in the brain (70, 71). The first finding that Ang (1-7) could be produced in areas of the CNS was obtained from studies of the hydrolysis of [125I]-Ang I in brain homogenates (72). It has been shown as immunostaining for Ang (1-7) in the paraventricular, supraoptic and suprachiasmatic nuclei of the hypothalamus, in the stria terminalis bed nucleus, substantia innominata, median exclusivity and neurohypophysis (73), (74). Other studies have shown the effect of the immune

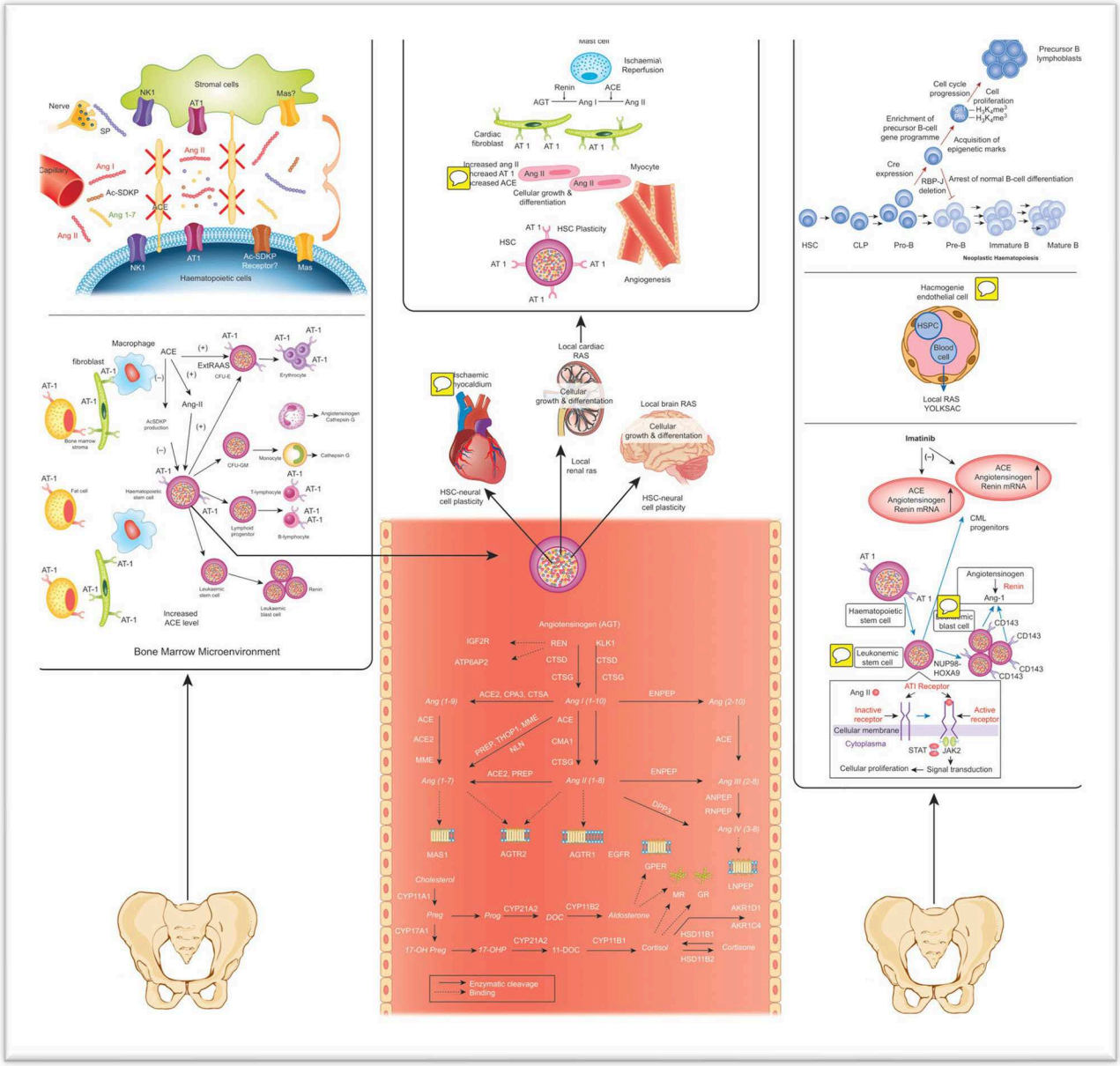

FIGURE 1 | The effect of local RAS on normal and leukemic hematopoiesis, as well as plasticity and systemic circulation, are shown (19). Ang I, Ang II, Ac-SDKP, and Ang 1-7 are secreted in different tissues and transferred to the BM through circulation. SP is secreted from the nerve end protruding into the BM. ACE converts Ang II from Ang I and reduces bioactive SP, Ac-SDKP and Ang 1-7 (38). Mas, with the Ang 1-7 receptor, is detected in the BM. Peptides in RAS can stimulate hematopoietic cells (39). Furthermore, local BM RAS has a function in the development, formation, proliferation, and differentiation of hematopoietic progenitors. Angiotensin peptides and steroid hormones were symbolized in gray. Lymphoid cells cannot differentiate throughout the normal B-cell pathway (40). Therefore, the leader B-cell gene undergoes epigenetic initiation and enrichment of a precursor B-cell gene programme. As a result, cell cycle progression and cell proliferation are observed in mutant cells. This leads to the enlargement of a lymphoblast population and the development of leading B cell leukemia (5, 19, 23, 38, 40, 41). HSPC, Hematopoietic stem/progenitor cells; ACE, Angiotensin converting enzyme; CML, Chronic myeloid leukemia; Ang, Angiotensinogen; CFU-GM/E, Colonyforming units-Granulocyte-Macrophage/erythroid; JAS/STAT, Janus kinase/Signal transduction and transcription; BC, Blood cell; Ang, Angiotensin; Preg, Pregnanolone; Prog, Progesterone; DOC, deoxycortisol; 17-OHP, 17-OH Progesterone; ACE, angiotensin I converting enzyme; ACE2, angiotensin I converting enzyme type 2; AGTR1, angiotensin II type 1 receptor; AGTR2, angiotensin II type 2 receptor; AKRIC4, aldo-ketoreductase family 1 member C4; AKRID1, aldo-ketoreductase family 1 member D1; ANPEP, alanyl-aminopeptidase; ATP6AP2, prorenin/renin receptor; CMA1, chymase 1; CPA3, carboxypeptidase A3; CTSA, cathepsin A; CTSD, cathepsin D; CTSG, cathepsin G; CYP11A1, cytochrome P450 family 11 subfamily A polypeptide 1; CYP11B1, cortisolsynthase; CYP11B2, aldosteronesynthase; CYP17A1, cytochrome P450 family 17 subfamily A polypeptide 1; CYP21A2, cytochrome P450 enzyme family 21 subfamily A polypeptide 2; DPP3, dipeptidyl-peptidase 3; ENPEP, glutamylaminopeptidase (aminopeptidase A); GR, glucocorticoidreceptor; HSD11B1, hydroxysteroid (11-beta) dehydrogenase 1; HSD11B2, hydroxysteroid (11-beta) dehydrogenase 2; IGF2R, insulin-like growth factor 2 receptor; KLK1, tissuekallikrein; LNPEP, leucyl/cystinylaminopeptidase; MAS1, MAS1 proto-oncogene; MME, membranemetallo-endopeptidase; MR, mineralocorticoidreceptor; NLN, neurolysin (metallopeptidase M3 family); PREP, prolylendopeptidase; REN, renin; RNPEP, arginylaminopeptidase (aminopeptidase B); THOP1, thimetoligopeptidase 1. Images of IGF2R36, ATP6AP237, MR38, GR39, G-protein coupled receptors (AGTR1, AGTR2, GPER, and MAS1) 40 and LNPEP41 (19).

system and neuroimmune pathways in hypertensive patients (9, 75).

Hypertension is a very significant risk factor for cardiovascular diseases. It remains a global public health problem (75). Various effects such as salt sensitivity and high systemic RAS activity are included in the pathophysiology of hypertension (76, 77). Moreover, studies have shown that disruptions in activity within the cardiovascular CNS fields as a result of increased sympathetic and reduced parasympathetic impulse to the peripheral organs cause end organ injury, vascular/endothelial dysfunction, and hormonal instability (78, 79). Recently, significant advances have been made in the treatment of hypertension using ACE inhibitors or AT1 receptor blockers, diuretics, α-adrenoreceptor antagonists, RAS inhibitors and calcium channel blockers (75). Molecular and neuronal changes at the brainstem and hypothalamus level have been shown to contribute to neurogenic hypertension. Studies have shown that sympathetic activation not only starts hypertension but also protects it (75). The first evidence of the significance of the autonomic nervous system for BM cell homeostasis was obtained from studies demonstrating the regulation of BM cell activity (80). It was showed that the release of BM HSPCs is rhythmically regulated in a circadian manner, for which sympathetic drive is essential (80–82).

LOCAL BONE MARROW RENIN ANGIOTENSIN SYSTEM AND CENTRAL NERVOUS SYSTEM WITHIN THE CONTEXT OF ESSENTIAL HYPERTENSION

BM has an essential function in hematopoiesis regulation (19). BM HSPCs are in communication with cells of secondary lymphoid organs, such as the spleen, which control HSPC differentiation and maturation (83, 84). The relationship of BM and HSPCs with hypertension has recently attracted interest. It is thought to be a bidirectional brain-BM communication hypothesis, and this hypothesis is based on various evidences (7, 9).

Ang II related HT lead to a recommendation that activation of microglia in the pre-sympathetic cardio-regulatory brain fields may precede both the activation of the sympathetic drive and the increase in blood pressure (9, 75). Prohypertensive signals such as increased Ang II cause neurovascular-glial inflammation in the cardio-regulator areas of the brain (7). Dysfunctional ANS output is characterized by increment in the sympathetic and a reduction in the parasympathetic impulse to the periphery, including BM (7). This process induces an increment in peripheral Ang II (7). Thus, it results in a permanent increment in the inflammatory cells and a decrease in the endothelial progenitor cells (7). Increased inflammatory cells contribute to vascular and tissue damage, while reduced endothelial progenitor cells contribute to repair of this harm, leading to cardiorenal pathology (7). Extravasation of inflammatory cells into pre-sympathetic brain areas and increased somatic afferent input from BM to the brain contribute to neurovascular-glial inflammation (7). So, it directs the dysfunctional ANS output to the environment. These pathways are circumstances that cause cardiovascular and kidney pathophysiology and resistant hypertension (7). On the other hand, in Ang II-induced hypertension, BM-induced AT1R receptors limit mononuclear cell aggregation in the kidney. Thus, it reduces the chronic hypertensive response, possibly through the arrangement of vasoactive cytokines (85). Dysfunctional ANS output is characterized by a sympathetic increase and a decrease in parasympathetic impulses and is involved in the pathophysiology of hypertension (7, 9).

One study demonstrated that a dysfunctional BM ANS is correlated with imbalanced EPCs and inflammatory cells in hypertension (80). They demonstrated that in their study presympathetic neuronal activation in a spontaneously hypertensive rat was related with an accelerated retrograde transfer of the gren fluorescent protein–labeled pseudorabies virus from the BM in mangasese-enhanced MRI (80). Pro-hypertensive markers, such as increased brain and systemic RAS, cause neurovascular-glial inflammation in the brain cardio-regulator sites (27). All this evidence supports the bidirectional brain-BM interaction hypothesis for cardiovascular system homeostasis and hypertension. The effect of somatic afferent input from the BM to the CNS continues to be investigated (27).

BM has an important role in neurogenic hypertension. Memory T cells are present in the BM. T-cell activation has a significant function in hypertension (9). The BM is the primary source of EPC, which play a significant function in endothelial repair in arterial or renal injury (86, 87). Chronic elevation in BM norepinephrine may disrupt the role of EPCs and this may be important in the terms of hypertension. These interactions strengthen the connection between the CNS and BM (9). Cross-communication between ANS and BM vasculature may be an important mechanism of the pathophysiology of hypertension. NE has a vasoconstrictor role in the BM and plays a significant function in controlling blood flow (9, 88). Prohypertensive signals such as Ang II cause neuro-vascular-glial inflammation in the cardio-regulator sites of the brain (7–9). Dysfunctional ANS output is represented by an increase in sympathetic and a reduction in parasympathetic impulses to the environment, including BM. As a result of these events, there is a permanent increase in inflammatory cells and a reduce in EPCs. As a result, these increased inflammatory cells cause vascular and tissue damage, while the reduction of endothelial progenitor cells causes a reduction in repair of this damage, leading to cardio-renal pathologies (7, 9). The combination of increased somatic afferent input from BM to the brain through the extravasation of inflammatory cells into pre-sympathetic brain areas and activation of TRPV1 channels contributes to neuro-vascular-glial inflammation (89–91). All these processes sustain the emerging cardiovascular and kidney pathophysiology and resistant hypertension (7).

NEW PHARMACOLOGICAL APPROACHES FOR RAS

There are lots of described important therapeutic usages for Angiotensin (1-7) and analogs on treating cardiovascular diseases

and atherosclerosis. Some studies showed new pharmacological approaches. One of them investigated a BM-specific adrenergic beta 1 and beta 2 knock out mouse chimera (AdrB1.B2 KO) to research how sympathetic impulse to the bone influences transcripts and miRNAs in the hypothalamic paraventricular nucleus (92). The results showed that there are molecular axes involved in neural-immune interactions that can serve as targets of therapeutic treatment for a dysfunctional ANS (92). Ahmari et al. produced a mouse chimera in which the BM was irradiated and exactly reconstituted with BM from beta 1 and 2 adrenergic receptor KO mice (93). The study showed that genetic ablation of beta 1 and 2 adrenergic receptors in the BM directs an impressive modification in the BM immune system mediators. This results in decreased circulation levels of a subtype of T cells, neutrophils and macrophages (93). To research Ang-(1-7)-dependent Mas receptor function, Yang et al. used apoE-KO and apoE/Mas-KO mice with Ang-(1-7) or saline for 6 weeks (94). To check whether Ang-(1-7) regulates atherosclerosis through a NO-dependent pathway, apoE-KO mice were used with the NO synthase inhibitor in the presence or lack of Ang-(1-7) (94). Ang-(1-7) has been shown to have protective vascular effects through Mas receptor activation (94).

FUTURE PROSPECTS AND HYPOTHESES

In light of all these data, it is thought that the autonomous control of the BM has an important function in hypertension and there is bidirectional communication between the brain and the BM in this procedure (7). It is thought that there will be important developments that will provide new therapeutic targets in the therapy of hypertension with new studies in this area. It is obvious that there will be innovations at genetic level in the treatment of hypertension with the clarification of this process. Angiotensin peptides on HSCs can be edited with clustered regularly interspaced short palindromic repeats (CRISPR). Cellular therapy and gene edition can contribute to the treatment of essential hypertension. It can change the biology of HSCs that will go to the CNS in an anti-atherogenic and anti-hypertensive direction by genomic edition with CRISPR, by down-regulating AT1 receptors on the HSCs and upregulating MAS receptors. Another therapeutic target will be active microglia, which can affect the activity of neurons in the cardio-regulatory regions of the brain, which can control BM and effect the role of BM HSPCs (7). In addition to the known effects of RAS in many areas, future research and clinical studies are required to explain the different roles of local tissue RAS, including local BM RAS, and to use them as therapeutic targets.

AUTHOR CONTRIBUTIONS

RC was responsible for the writing of the article. IH served as scientific adviser, drafted the article, and revised it critically for important intellectual content. All authors contributed to the article and approved the submitted version.

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REVIEW
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Brain Renin–Angiotensin System at the Intersect of Physical and Cognitive Frailty

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The renin–angiotensin system (RAS) was initially considered to be part of the endocrine system regulating water and electrolyte balance, systemic vascular resistance, blood pressure, and cardiovascular homeostasis. It was later discovered that intracrine and local forms of RAS exist in the brain apart from the endocrine RAS. This brain-specific RAS plays essential roles in brain homeostasis by acting mainly through four angiotensin receptor subtypes; AT_1R, AT_2R, MasR, and AT_4R. These receptors have opposing effects; AT_1R promotes vasoconstriction, proliferation, inflammation, and oxidative stress while AT_2R and MasR counteract the effects of AT_1R. AT_4R is critical for dopamine and acetylcholine release and mediates learning and memory consolidation. Consequently, aging-associated dysregulation of the angiotensin receptor subtypes may lead to adverse clinical outcomes such as Alzheimer's disease and frailty via excessive oxidative stress, neuroinflammation, endothelial dysfunction, microglial polarization, and alterations in neurotransmitter secretion. In this article, we review the brain RAS from this standpoint. After discussing the functions of individual brain RAS components and their intracellular and intracranial locations, we focus on the relationships among brain RAS, aging, frailty, and specific neurodegenerative diseases, such as Alzheimer's disease, Parkinson's disease, and vascular cognitive impairment, through oxidative stress, neuroinflammation, and vascular dysfunction. Finally, we discuss the effects of RAS-modulating drugs on the brain RAS and their use in novel treatment approaches.

Keywords: renin–angiotensin system, RAS, brain, neurodegenerative diseases, neuroinflammation, oxidative stress, physical and cognitive frailty, aging

One of the biggest challenges of healthcare in the 21st century is the care of older adults suffering from cognitive impairment and frailty because of their high medical, economic, psychological, and social burden. Frailty, among the most common geriatric syndromes, is characterized by failure of homeostatic mechanisms, diminished physical function, and reduced age-related physiologic reserve leading to decreased ability to cope with stressors and increased vulnerability to adverse outcomes (Fried et al., 2001; Ma and Chan, 2020). There is growing evidence linking frailty and cognitive impairment (Boyle et al., 2010; Subra et al., 2012; Robertson et al., 2013; Ma et al., 2019; Wallace et al., 2019). Although studies in

epidemiology and pathology have shown strong associations between frailty and cognitive impairment, the association's biological basis still remains elusive. One study found shared pathologies associated with both (Buchman et al., 2014). There are likely additional shared mechanisms linking the two, including inflammation, oxidative stress, mitochondrial damage, and cellular regeneration failure. The identification of this biological link can lead to new preventive and therapeutic interventions for both conditions. In this regard, the following section introduces the renin–angiotensin system, a critical hormonal system that can provide further insight into the biological links between frailty and cognition.

INTRODUCTION: AN OVERVIEW OF THE CLASSICAL AND LOCAL RENIN–ANGIOTENSIN SYSTEM

The renin–angiotensin system (RAS) was first identified as part of the endocrine system, which regulates water and electrolyte balance, systemic vascular resistance, aldosterone release, and cardiovascular homeostasis. After the discovery of renin (Tigerstedt and Bergman, 1898), it took 40 years to isolate the other component of the RAS, angiotensin, by two groups simultaneously (Braun-Menendez et al., 1940; Page and Helmer, 1940). The classical RAS is activated by the release of renin from juxtaglomerular cells of renal afferent arterioles into the circulation. As the first and rate-limiting step, renin converts the precursor molecule angiotensinogen to angiotensin I (Ang I), which is then transformed to angiotensin II (Ang II) by angiotensin-converting enzyme (ACE), localized mostly in the endothelial cells of the lungs. Within this system, Ang II is a primary bioactive product, leading to antagonistic effects including vasoconstriction and vasodilation by, respectively, binding to angiotensin II type 1 receptor (AT_1R) and angiotensin II type 2 receptor (AT_2R) (Griendling et al., 1993; Unger et al., 1996; Vajapey et al., 2014).

Beyond the classical endocrine (circulating) RAS, additional research identified the autocrine (cell to the same cell type) and paracrine (cell to different cell type) effects of RAS. Further, it has been shown that RAS can be locally synthesized and act in many tissues including endothelial cells, adrenal and pituitary glands, testis, ovary, kidney, heart, and eye (Dzau, 1988; Paul et al., 2006; Vajapey et al., 2014). This form of RAS was termed local or tissue RAS. The broad clinical relevance of local RAS is shown in **Figure 1**. Among the local RASs, brain RAS (b-RAS), discovered by Ganten et al. (1971) has a particular importance, as systemic RAS components cannot access most brain regions because of the blood–brain barrier (BBB) (Wright and Harding, 2013). Besides these tissue-level RAS, subcellular functional units of RAS in organelles such as mitochondria and nuclei were revealed by different research groups (Abadir et al., 2011, 2012; Gwathmey et al., 2012).

Further research on b-RAS has shown that it has complicated effects on the central nervous system beyond its well-known roles, including sodium retention, vascular, and blood pressure control. There is growing evidence of b-RAS' impact on oxidative stress, endothelial dysfunction, microglial polarization, neuroinflammation, brain homeostasis, alterations in neurotransmitter secretion, cognition, and even aging and frailty (Rodriguez-Pallares et al., 2008; Abadir, 2011; De Silva and Faraci, 2013; Wright and Harding, 2013; Labandeira-Garcia et al., 2017; Forrester et al., 2018). The use of classical RAS-acting drugs like angiotensin receptor blockers (ARB) or ACE inhibitors (ACEI) for the regulation of b-RAS has long been under investigation.

After reviewing the structure and function of b-RAS, we consider its roles in aging and neurodegenerative diseases with respect to oxidative stress, neuroinflammation, and vascular dysfunction. Finally, we summarize the effects of angiotensin system modulators on b-RAS and their therapeutic potential.

STRUCTURE OF THE BRAIN RENIN–ANGIOTENSIN SYSTEM

There are two main RASs in the brain: circulating and local (Saavedra, 2005; Grobe et al., 2008). Circulating RAS exerts its effect via the circumventricular organs, which are regions of the brain that lack BBB and project to nuclei in the hypothalamus and medulla (Lenkei et al., 1997; Gao and Zucker, 2011). In contrast, b-RAS, which is the independent local RAS of the brain, can synthesize all components of the circulatory RAS (Harding et al., 1988; Wright and Harding, 2013). Despite a few recent studies with conflicting results (van Thiel et al., 2017), it is generally accepted that there is *de novo* production of RAS components as well as active RAS genes and their promoter regions in the brain (Fuxe et al., 1980; Hermann et al., 1987; Harding et al., 1988; Keisuke et al., 2017). In a double-transgenic mouse, it was demonstrated that there are specific renin-expressing cells near angiotensinogen-expressing cells, specifically within the rostral ventrolateral medulla (RVLM) (Lavoie et al., 2004a,b).

Angiotensin Ligands and Peptidases Within the Brain

Angiotensinogen is mainly produced and secreted from astrocytes within the brain to be cleaved into various neuroactive angiotensin peptides (Stornetta et al., 1988; Milsted et al., 1990). Conversion of angiotensinogen to the decapeptide Ang I is catalyzed by renin. Then, the zinc metalloprotease ACE hydrolyzes the carboxy-terminal dipeptide of Ang I to form the octapeptide Ang II (Wright and Harding, 2013). Cathepsin and chymase can also hydrolyze Ang I (Unger and Li, 2004; Mogi et al., 2012). Glutamyl aminopeptidase A (AP-A) cleaves the aspartate residue at the N-terminal of Ang II to form the heptapeptide angiotensin III (Ang III), which is then converted to the hexapeptide angiotensin IV (Ang IV) by alanyl aminopeptidase N (AP-N) cleaving arginine at the N-terminal. Ang IV can be further converted

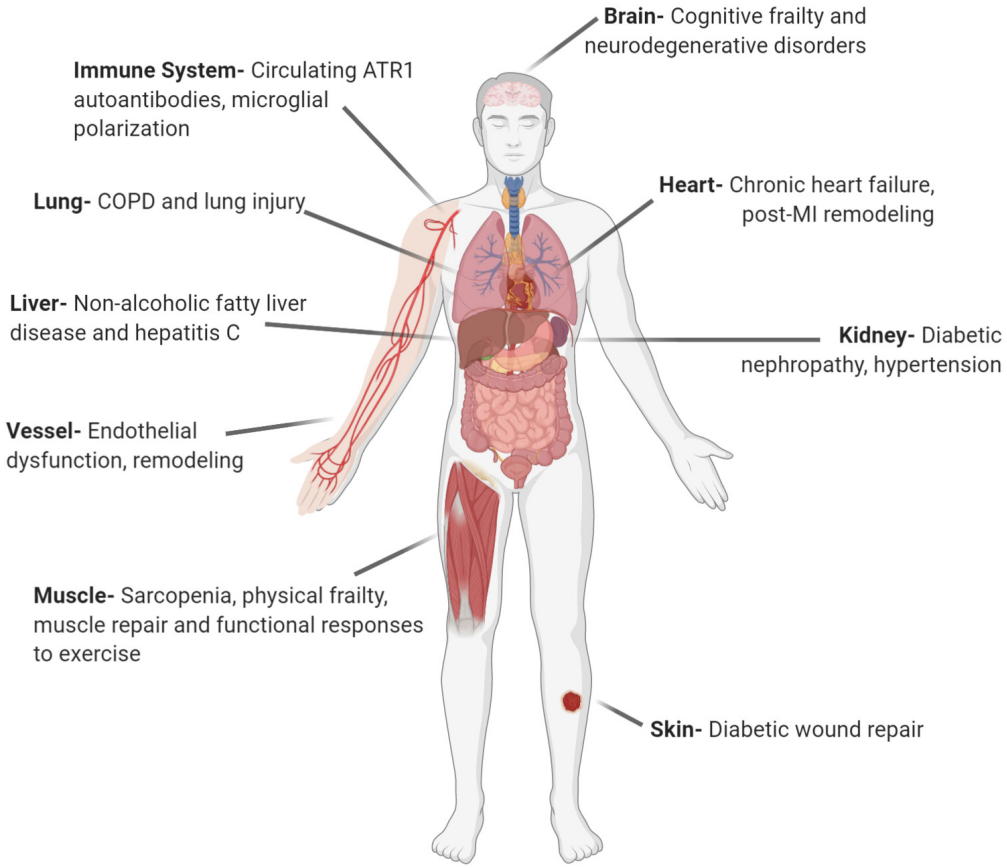

FIGURE 1 | Local angiotensin systems have broad clinical relevance. AT_1R, angiotensin receptor type 1; COPD, chronic obstructive pulmonary disease; MI, myocardial infarction (Created with BioRender.com).

to Ang (3–7) by carboxypeptidase P and prolyl oligopeptidase. Alternatively, Ang II can be converted to Ang (1–7) by carboxypeptidase P and ACE2, which is an isoform of ACE (Wright and Harding, 2013). ACE2 can also convert Ang I to Ang (1–9). Ang (1–7) can be converted from Ang (1–9) by ACE or from Ang I by neutral endopeptidase (Jiang et al., 2013). A recently discovered component of RAS is alamandine, which is formed by the decarboxylation of Ang (1–7) (Lautner et al., 2013). Alternatively, alamandine can be generated by ACE2 cleaving angiotensin A, which is obtained by decarboxylation of Ang II (Lautner et al., 2013). Ang II, Ang (1–7), and Ang IV are the main neuroactive angiotensin peptides that trigger signal transduction as they bind to their cognate receptors. The entire pathway is illustrated in **Figure 2**.

In classical RAS, renin is formed by the cleavage of prorenin. However, renin activation that generally occurs in secretory granules of renal juxtaglomerular cells is not as common in extrarenal tissues such as the brain (Hirose et al., 1981). Renin concentration is low within neurons and astrocytes (Grobe et al., 2008; Bodiga and Bodiga, 2013). However, prorenin receptor (PRR), which activates prorenin without prosegment removal, is highly expressed in neurons and some microglial cells in several cardiovascular brain regions such as the subfornical organ (SFO), paraventricular nucleus (PVN), the nucleus of the solitary tract (NTS), and RVLM as well as non-cardiovascular brain regions such as brain cortex and basal ganglia (Nguyen et al., 2002; Valenzuela et al., 2010; Li et al., 2012a,b; Garrido-Gil et al., 2013b, 2017; Xu et al., 2016). Overactivation of this system can lead to cognitive impairment by the activation of the Ang II/AT_1R axis (Wright and Harding, 2011; Bodiga and Bodiga, 2013). Besides increasing the catalytic activity of prorenin and renin to hydrolyze angiotensinogen into angiotensin, PRR has Ang II-independent effects (Nguyen and Contrepas, 2008; Shan et al., 2008). In this context, PRR can initiate its own signaling pathway and induce its pro-oxidative effects (Labandeira-Garcia et al., 2017). Although it was shown that there is a relationship between PRR and neural development (Trepiccione et al., 2016), RAS-independent functions of PRR within the brain are not yet clear (Nakagawa et al., 2020).

Furthermore, another brain-specific renin isoform called renin-b was also discovered (Lee-Kirsch et al., 1999; Sinn and Sigmund, 2000). While renin-b was initially believed to be effective in the intracellular generation

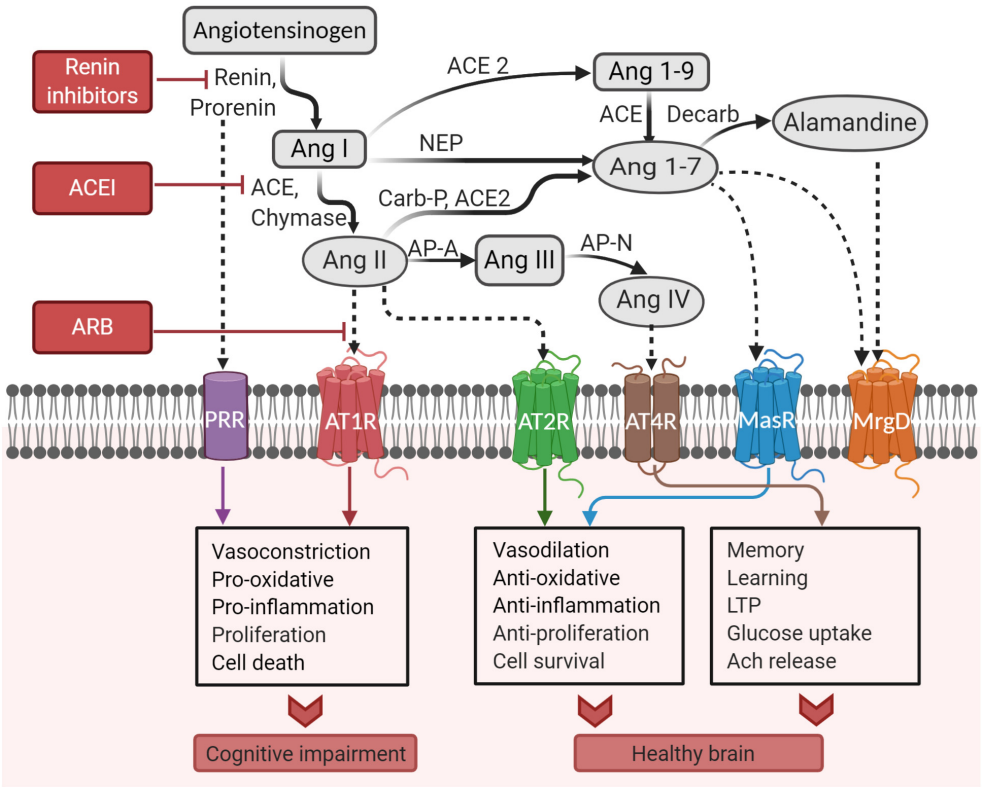

FIGURE 2 | Pathways of the brain renin–angiotensin system. ACE, angiotensin-converting enzyme; ACEI, angiotensin-converting enzyme inhibitor; Ang, angiotensin; AP-A, aminopeptidase A; AP-N, aminopeptidase N; ARB, angiotensin receptor blocker; AT_1R, angiotensin II type I receptor; AT_2R, angiotensin II type 2 receptor; AT_4R, angiotensin IV receptor; Carb-P, carboxypeptidase-P; decarb, decarboxylase; LTP, long-term potentiation; MasR, Mas receptor; MrgD, Mas-related-G protein-coupled receptor; NEP, neutral endopeptidase; PRR, prorenin receptor (Created with BioRender.com).

of Ang II, it is now thought to be more involved in intracellular regulation of b-RAS (Grobe et al., 2008; Keisuke et al., 2017).

Angiotensin Receptors and Their Locations Within the Brain

Ang II acts through two receptor subtypes: AT_1R and AT_2R, both of which are members of the G-protein-coupled receptor (GPCR) family (Miura et al., 2013). Ang III also binds to these receptors although forms higher-affinity bonds with AT_2R and lower-affinity bonds with AT_1R compared to Ang II (Wright and Harding, 2011). Human AT_1R contains 359 amino acids and has a molecular mass of 41 kDa, while AT_2R, which is 34% identical to AT_1R, consists of 363 amino acids and also has a mass of 41 kDa (de Gasparo et al., 2000; Zhang et al., 2017). Binding of Ang II to AT_1R, accompanied by changes in its transmembrane 3–6 conformation, induces cell signaling (Inoue et al., 1997; Ghanouni et al., 2001; Miura et al., 2013). AT_1Rs and AT_2Rs were identified mostly in the cortex, hippocampus, and basal ganglia in humans and many animals (Lenkei et al., 1996; Rodriguez-Pallares et al., 2008; Joglar et al., 2009; Valenzuela et al., 2010; Garrido-Gil et al., 2013b, 2017; Jackson et al., 2018). There are two types of AT_1Rs: AT1A and AT1B. The AT1A receptor is mainly expressed in brain regions contributing to homeostatic regulation of blood pressure and electrolyte balance (Jöhren et al., 1995; MacGregor et al., 1995; Lenkei et al., 1997), whereas the AT1B receptor is seen in structures involved in memory and higher brain functions such as the cerebral cortex and hippocampus (Jöhren et al., 1995). AT_2R is present in brain regions involved in learning and memory, particularly the hippocampus, cingulate cortex, lateral septum, and locus coeruleus, but also the superior colliculus, thalamic and subthalamic nuclei, and inferior olive (Millan et al., 1991; Lenkei et al., 1996). AT_1Rs and AT_2Rs have been identified mostly in neurons, astrocytes, oligodendrocytes, and microglia of those regions (Tsutsumi and Saavedra, 1991; Fogarty and Matute, 2001; Rodriguez-Pallares et al., 2008; Joglar et al., 2009; Garrido-Gil et al., 2013b, 2017). However, a few studies could not identify AT_1Rs in microglia (Lenkei et al., 1997; Benicky et al., 2009) likely because of their methodology and the fact that AT_1Rs are undetectable in microglia's non-activated state and then upregulated only upon activation (Lanz et al., 2010; Rodriguez-Perez et al., 2015; Sun et al., 2015; Labandeira-Garcia et al., 2017). A more recent investigation of cellular locations of AT_1R and AT_2R under normal and hypertensive conditions showed that AT_1Rs and AT_2Rs are localized to neurons rather than astrocytes and microglia in PVN, NTS, arcuate nucleus, dorsomedial hypothalamus, area postrema, median preoptic

nucleus, SFO, and organum vasculosum of the lamina terminalis (Sumners et al., 2020).

Another receptor is angiotensin receptor 4 (AT_4R), to which Ang IV binds. However, in high concentrations of Ang IV, it can bind to AT_1R (Jackson et al., 2018). Unlike other angiotensin receptors, AT_4R is identical to insulin-regulated aminopeptidase (IRAP), which is a type 2 transmembrane protein of the gluzincin aminopeptidase family, including homologous aminopeptidases AP-A and AP-N (Lee et al., 2003; Farag et al., 2017; Wright and Harding, 2019). AT_4Rs are more abundant in sensory and cognitive regions of the brain in contrast to AT_1Rs that are abundant in cardiovascular and osmoregulatory areas (Wyse et al., 1995). The fact that AT_4R and cholinergic receptors are closely localized suggests that AT_4Rs have a potential role in learning and memory (Wilson et al., 2009). AT_4Rs are present in many components of the central nervous system, particularly the caudate, putamen, cerebellum, anterior pituitary globus pallidus, neocortex, CA1–CA3 pyramidal layers of hippocampus, nucleus basalis of Meynert, lateral geniculate body, ventral lateral thalamic nucleus, motor neurons of the brain stem, and ventral horn of the spinal cord (Wright and Harding, 2011). At the cellular level, it is well known that AT_4R is located on neurons (Chai et al., 2000; Albiston et al., 2001). However, there are conflicting results about their presence on astrocytes (Greenland et al., 1996; Jackson et al., 2018; O'Connor and Clark, 2018). In a recent study, the presence of IRAP/AT_4R was shown in both pinealocytes and astrocytes in the rat pineal gland (Abrahão et al., 2019).

Ang (1–7) binds to Mas receptors (MasRs) with the highest affinity, but it can also bind to Mas-related-G protein-coupled receptors (MrgDs) and AT_2Rs with low affinity (Elased et al., 2008; Bernstein et al., 2014; Tetzner et al., 2016). MasRs are GPCRs with high concentrations in the brain structures associated with memory and cognition such as the hippocampus, but also the piriform cortex involved in smell (Freund et al., 2012). Although they were seen in both neuronal and glial cells, the MasR immunoreactivity was higher in neurons than in glia (Costa-Besada et al., 2018). Recently discovered MrgDs are also GPCRs, and their primary ligand is alamandine (Lautner et al., 2013). The discovery of MrgDs has added another level of complexity into RAS, and further studies are needed for a complete understanding of their roles within the brain.

Hetero-dimerizations have been observed between GPCRs of the RAS. While AT_1R and bradykinin B2 receptor hetero-dimerization enhances G-protein activation (Quitterer et al., 2011; Miura et al., 2013), AT_2R/AT_1R and MasR/AT_1R heterodimers directly antagonize and inactivate AT_1Rs, leading to a decrease in AT_1R signaling cascade (AbdAlla et al., 2001). Also, AT_2Rs and MasRs may form heterodimers as they have similar signaling mechanisms, and are functionally dependent on each other (Leonhardt et al., 2017). In this regard, it was shown that MasR protein expression and mRNA levels in the substantia nigra (SN) of AT_2R knockout (KO) mice decreased, rendering Ang (1–7) ineffective in astrocytes, whereas MasR expression did not change in AT_1R KO mice (Villela et al., 2015; Leonhardt et al., 2017). However, notably, AT_2R expression and ACE2 and Ang 1–7 levels decreased in the absence of the Ang II/AT_1R axis (Villar-Cheda et al., 2010).

Angiotensin receptors can also be located intracellularly. Specifically, they have been found in the nucleus, mitochondria, neurosecretory vesicles, and the plasma membrane (Sadoshima et al., 1993; Vila-Porcile and Corvol, 1998; Sherrod et al., 2005; Peters, 2008; Abadir et al., 2011). Intracellular RAS can be regulated independently from the systemic circulation (Abadir et al., 2012). Whether circulating (classical), local, or subcellular (intracrine) RAS, the main system processes are the same (Kumar R. et al., 2012). Ang II and enzymes such as renin and ACE can be localized to cytoplasm and nuclei; ACE also has been found on the endoplasmic reticulum (Shen et al., 2008; Abadir et al., 2012).

FUNCTIONS OF RENIN–ANGIOTENSIN SYSTEM RECEPTORS

Here, we review the general functions of RAS receptors and discuss their cell type-specific functions within the brain.

Prelude to the Renin–Angiotensin System Signaling and Functions

AT_1R and AT_2R stimulation generally lead to opposing actions. While AT_1R mediates vasoconstriction, cellular proliferation, cell growth, and production of superoxide, AT_2R mediates vasodilatation and has both anti-oxidant and anti-inflammatory properties (Abadir, 2011; Abadir et al., 2011).

Although Ang II can bind to both AT_1R and AT_2R, ACE upregulation prompts AT_1R signaling (Jackson et al., 2018). After G-protein coupling stimulation of AT_1R with Ang II, second messenger signaling, which consists of inositol trisphosphate, diacylglycerol, and arachidonic acid, initiates activation of downstream effectors, such as phospholipases C, A, and D. Response of these signals can differ across tissues. Protein kinase C, Akt, intracellular protein kinases (such as receptor and non-receptor tyrosine kinases), and serine/threonine kinases [such as mitogen-activated protein kinase (MAPK) family kinases] are activated by the AT_1R signaling cascade (Forrester et al., 2018). Overactivation of AT_1R cascade may result in hypertrophy, vascular remodeling, and hyperplasia (Suzuki et al., 2005).

The Ang II/AT_2Rs axis signals through three major transduction pathways that seem to oppose the actions of AT_1R. AT_2R activates several protein phosphatases and nitric oxide (NO)/cyclic GMP system and stimulates phospholipase A2, with subsequent release of arachidonic acid (Abadir, 2011). AT_2R also inhibits cell growth and proliferation by inhibiting autophosphorylation of insulin and epidermal growth factor receptors (Steckelings et al., 2010). Furthermore, AT_1R blockade increases angiotensinogen and AT_2R stimulation by inhibition of negative feedback (Carey et al., 2001). The AT_1R blockade also increases renal NO, which is blunted by concomitant AT_2R blockade (Siragy et al., 2000), suggesting AT_2R's role in increasing NO production via direct stimulation of NO synthase (NOS) or indirectly through bradykinin-dependent mechanisms (Abadir et al., 2003).

Receptor Functions According to Cell Type

Neurons

Neurons have all types of angiotensin receptors on their cell membrane; also, they have an intracellular angiotensin system that works separately. It has been shown that neurons have intracrine RAS, including AT_1Rs, AT_2Rs, and MasRs in their mitochondria and nuclei (Valenzuela et al., 2016; Villar-Cheda et al., 2017; Costa-Besada et al., 2018). Although astrocytes synthesize most of the angiotensinogen within the brain, neurons can also synthesize angiotensinogen. Angiotensinogen immunoreactivity is found in the entire brain, predominantly in areas responsible for water and electrolyte balance such as the SFO, PVN, NTS, and RVLM (Grobe et al., 2008). This wide distribution of angiotensinogen in the brain shows that b-RAS is not limited to cardiovascular regulatory functions (Bodiga and Bodiga, 2013). As neurons contain RAS enzymes (e.g., intracellular renin, prorenin, etc.), angiotensinogen can be converted to active angiotensin peptides intracellularly (Lee-Kirsch et al., 1999; Abadir et al., 2012; Jackson et al., 2018).

The Ang II/AT_1R axis produces reactive oxygen species (ROS) and causes oxidative stress by NADPH oxidase in neurons, as in other tissues. Conversely, the Ang II/AT_2R axis and Ang (1–7)/MasR axis produces NO and inhibits superoxide increment (Costa-Besada et al., 2018). In neurons, while the Ang II/AT_1R axis and the (Pro)renin/PRR axis have pro-oxidative and pro-inflammatory properties, the Ang II/AT_2R axis and the Ang (1–7)/MasR axis act as the protective arm of the RAS (Costa-Besada et al., 2018). Mitochondrial AT_2Rs are more common than AT_1Rs in dopaminergic neurons (Valenzuela et al., 2016). Notably, even though MasR is less concentrated in mitochondria than the rest of the cell, both ACE2 and Ang (1–7) are more abundant in mitochondria (Costa-Besada et al., 2018). This study supports a recent finding suggesting that MrgDRs and AT_2Rs are additional Ang (1–7) receptors (Tetzner et al., 2016).

In neurons, Ang II, Ang III, and AT_2Rs are colocalized in the mitochondrial inner membrane (Abadir et al., 2011). This colocalization is important since mitochondrial NOS, a possible mitochondrial respiration regulatory enzyme, is also known to exist in the mitochondrial inner membrane (Ghafourifar and Richter, 1997). Also, it was shown that the AT_2R agonist CGP421140 activates NO production in a dose-dependent manner, and that AT_2R blocker PD-123319 mitigates it (Abadir et al., 2011).

As with mitochondrial RAS, nuclear RAS is essential for the regulation of oxidative stress. It maintains the balance between detrimental and protective pathways of RAS through transcription and trafficking of additional receptor types (Zawada et al., 2015; Villar-Cheda et al., 2017; Costa-Besada et al., 2018). When nuclear AT_1Rs are activated, both oxidative and anti-oxidative mechanisms are initiated. An increase in mRNA levels of PRR, angiotensinogen, and renin are seen, leading to intracellular synthesis of more Ang II and Ang (1–7). Concomitantly, protective AT_2R levels increase. AT_2Rs are then delivered to different organelles such as mitochondria and cell membranes. While Ang (1–7) shows its protective effects, increased Ang (1–7) levels suppresses AT_2R expression (Villar-Cheda et al., 2017; Costa-Besada et al., 2018). These compensatory mechanisms can be dysfunctional in aging and cognitive disorders (Labandeira-Garcia et al., 2017).

Overactivation of the Ang II/AT_1R axis has many detrimental effects on the brain, such as hypertension, neuroinflammation, increased oxidative stress, BBB disruption, and neurotoxicity. Neuronal AT_1R activation *in situ* or brain slices may increase the firing rate of neurons in specific brain regions, such as the SFO, PVN, and RVLM (Knowles and Phillips, 1980; Sumners et al., 2002). This effect can be reversed by losartan, an ARB (Sumners et al., 2002). The Ang II/AT_1R axis enhances sympathetic neurotransmitter release in the central nervous system. In particular, the release of vasopressin, dopamine, and norepinephrine is facilitated (Stadler et al., 1992; Medelsohn et al., 1993; Tsuda, 2012). Also, studies have investigated the effects of the Ang II/AT_1R axis b-RAS on inhibitory GABA and excitatory glutamate transmitters. It was shown that Ang II/AT_1R axis decreases GABA and enhances glutamate release (Tedesco and Ally, 2009; Fujita et al., 2012; Tsuda, 2012). Notably, there is evidence that activation of AT_2R and MasR functions in opposition to AT_1R's neurosecretory effect (Tsuda, 2012; de Kloet et al., 2016).

In contrast to the effects of Ang II/AT_1R signaling, Ang II induces neuroprotective mechanisms, NO production, neurite outgrowth, and brain development through AT_2R activation, thus improving cognitive function (Farag et al., 2017). In this regard, impaired AT_2R signaling results in AT_1R-mediated oxidative stress and neuroinflammation, which may lead to impaired cognition. For example, within the hippocampus, decreased AT_2R activation was shown to cause dendritic spine abnormalities and spatial memory deficits (Maul et al., 2008). As another neuroprotective mechanism, AT_2R activation improved neuronal survival and neurological deficits via increased VEGF production after ischemic injury in rodents (Mateos et al., 2016). AT_2R activation also enhances the repair of damaged DNA and differentiation in the nervous system through induction of methyl methanesulfonate sensitive 2, a ubiquitin-conjugating enzyme variant (Mogi and Horiuchi, 2013).

Another neuroprotective axis is Ang (1–7)/MasR, which limits the pressor, angiogenic, and proliferative actions of Ang II (Ho and Nation, 2018). This axis has both anti-oxidant and anti-inflammatory properties (Jiang et al., 2013). It has essential roles in learning, memory, neuroprotection, and cell survival (Wright et al., 2013; Farag et al., 2017). Besides, the Ang (1–7)/MasR axis is well known for promoting the production of arachidonic acid and activation of endothelial and neuronal NO synthase, which are crucial for object recognition memory and long-term potentiation (LTP) in the hippocampus and amygdala (Hellner et al., 2005; Yang et al., 2010). Indeed, deficient object recognition memory was observed in MasR KO mice (Lazaroni et al., 2012). This axis also plays a neuroprotective role against ischemic stroke by significantly increasing the density of brain capillaries, improving regional cerebral blood flow (CBF), and decreasing infarct volume and neurological deficits (Jiang et al., 2013). This role was further confirmed by a study in which the brain angiogenic effects of Ang (1–7) were attenuated with MasR

antagonist A-779 (Jiang et al., 2014). A recently discovered effect of Ang (1–7)/MasR axis is an increase of glucose utilization and decrease of insulin resistance (IR) (Williams et al., 2016; Loloi et al., 2018; Wright and Harding, 2019). Consistent with this effect, chronic administration of Ang-(1–7) improved glucose tolerance in fructose-fed rats (Guimaraes et al., 2014).

As discussed above, AT_4Rs are mostly located on neurons, specifically in the sensory and cognitive regions. One of the first studies presenting behavioral evidence for the cognitive effects of AT_4Rs showed improvement in the learning and memory functions of rats with scopolamine-induced deficits in the circular water maze task by using an Ang IV analog intracerebroventricularly (Pederson et al., 1998). Previous work suggested that actions of Ang IV are partially mediated by IRAP which codistributes with the insulin-regulated glucose transporter (GLUT 4) (Albiston et al., 2001, 2003). In this context, AT_4Rs show some of their effects by inhibiting the catalytic activity of IRAP (Lew et al., 2003; Singh and Karnik, 2016; Abrahão et al., 2019). With this inhibition, Ang IV extends the half-life of several pro-cognitive endogenous peptides such as vasopressin, oxytocin, somatostatin, and endothelial NOS, which have been shown to enhance memory consolidation and retrieval. A parallel AT_4R/IRAP mediated pathway that enhances memory is through neuronal glucose uptake via regulation of GLUT4 vesicular trafficking (Chai et al., 2004; Fernando et al., 2008; Wright and Harding, 2011; Farag et al., 2017). Moreover, AT_4R activation induces a non-*N*-methyl-D-aspartate (non-NMDA)-dependent form of LTP via increases in intracellular calcium influx (Davis et al., 2006). Regarding the cognitive effects of Ang IV, IRAP inhibitors are shown to improve memory (De Bundel et al., 2009; Albiston et al., 2011; Mountford et al., 2014). Another suggested neuroprotective pathway of Ang IV/AT_4R is through hepatic growth factor and type 1 tyrosine kinase receptor (c-Met) signaling. As a mediator of the HGF/c-Met pathway, Ang IV stimulates c-Met (Ma et al., 2003; Wright and Harding, 2011), which then attenuates neurodegenerative changes by facilitating the non-NMDA–dependent LTP pathway and increasing dendritic arborization in the hippocampus (Akimoto et al., 2004; Shimamura et al., 2006; Tyndall and Walikonis, 2007). Additionally, the HGF/c-Met system shows cerebroprotective effects by facilitating CBF. All of these functions of the HGF/c-Met system overlap with those of the AngIV/AT4 system (Wright and Harding, 2015). Another manifestation of the learning and memory effects of Ang IV is through regulation of neurotransmitter secretion. In this regard, it has been hypothesized that Ang IV modulates serotonin, dopamine, and acetylcholine release (Lee et al., 2001; Fernando et al., 2008; Gard, 2008). This hypothesis has been supported by studies showing close localization of AT_4Rs and D2 receptors, correlation of IRAP with cholinergic cell bodies and terminals, and limitation of AngIV-induced cognitive facilitation via blockade of the D2 and D4 dopamine receptors (Chai et al., 2000; Braszko, 2006, 2009; Wright and Harding, 2011).

Astrocytes

Astrocytes, microglial cells, and oligodendrocytes are the principal glial cells of the brain. Astrocytes are the most common type of glial cells. Astrocytes have essential roles, such as supporting brain tissue, regulating the chemical content of extracellular space, restricting the spread of neurotransmitter molecules toward unwanted regions by encircling synaptic junctions, and removing many neurotransmitters from the synaptic cleft (Bear et al., 2016; Almad and Maragakis, 2018). These functions make them essential not only for support but also for LTP, thus memory.

Astrocytes are the main angiotensinogen source of the brain. In particular, they have AT_1R, AT_2R, and MasR on their cell membranes, mitochondria, and nuclei (Fogarty and Matute, 2001; Garrido-Gil et al., 2013b; Costa-Besada et al., 2018). Although not entirely clear, few studies demonstrate the existence of AT_4Rs on the astrocytes (Greenland et al., 1996; Holownia and Braszko, 2007). As with neurons, over-activation of AT_1Rs on astrocytes contribute to oxidative stress, inflammation, cognitive impairment, and cell death while Ang II and Ang (1–7) have protective roles. However, AT_1Rs on astrocytes differ from those on neurons in that astrocytic AT_1R activation can affect the permeability of BBB, although it is commonly accepted that overactivation of Ang II/AT_1R axis increases the permeability of BBB (Biancardi and Stern, 2016; Guo et al., 2019). A study, however, reported counterintuitive results such that astrocyte-derived Ang II restricts BBB permeability with tight junction stabilization, thus diminishing peripheral immune cell entry to the brain (Wosik et al., 2007; Füchtbauer et al., 2010).

Microglia

Microglial cells are macrophages that mediate immune and inflammatory responses of the brain (Bear et al., 2016). They can be in two states: resting or activated. In normal conditions, microglia remain in the resting state due to immunosuppressive proteins secreted by neurons. Contrary to their name, resting-state microglia cells actively scan their surrounding environment to detect any abnormalities in brain homeostasis. When they are activated, they can polarize into two distinct substates: proinflammatory/classically activated (M1) and anti-inflammatory/alternatively activated (M2) substates (Labandeira-Garcia et al., 2017). The M1 substate exacerbates neuronal death by secreting pro-inflammatory mediators and free radicals. In contrast, the M2 substrate, being immunoregulatory microglia, promotes brain repair/regeneration, produces growth factors and anti-inflammatory cytokines protecting neurons, and reduces inflammation (Labandeira-Garcia et al., 2017). An inadequate transition from the proinflammatory M1 to immunoregulatory M2 substrate in the presence of any brain lesions may result in the prolonged release of inflammatory cytokines and ROS, which is followed by increased neuroinflammation and neurodegeneration (Kigerl et al., 2009; Heneka et al., 2015; Tang and Le, 2016). It is known that RAS has roles in this immunoregulatory response as it does in the peripheral immune system (Labandeira-Garcia et al., 2017). In particular, RAS affects microglia via AT_1Rs, AT_2Rs, and MasRs on mitochondria, nuclei, and cell membranes (Garrido-Gil et al., 2013b; Regenhardt et al., 2013; Costa-Besada et al., 2018). Under non-pathologic conditions, the presence of AT_1R and AT_2R

are undetectable, unlike the MasR, which can be observed in healthy microglia (Regenhardt et al., 2013; Labandeira-Garcia et al., 2017; Costa-Besada et al., 2018). However, as M1 microglia exerts its pro-inflammatory response, AT_1Rs and AT_2Rs are upregulated. In this way, activation of nuclear AT_1R upregulates itself and leads to a shift toward an M1 phenotype. AT_1R-mediated activation of the M1 pro-inflammatory response was suggested to be the mechanism that exacerbates cell death and inflammation, ultimately leading to impaired cognition (Bernstein et al., 2014). Similar to what is observed with AT_1R, activation of nuclear AT_2R upregulates itself and leads to a shift toward an M2 phenotype. This shift further leads to the production of anti-inflammatory cytokines such as IL-10 and IL-4 and the upregulation of phagocytic receptors that help synaptic clearance (Rodriguez-Pallares et al., 2008; Regenhardt et al., 2013; Biancardi et al., 2015; Fouda et al., 2017). MasR and AT_2R, both of which have antioxidant and anti-inflammatory properties, also enhance the production of brain-derived neurotrophic factor, known to promote cell survival and synaptic plasticity (Bernstein et al., 2014) and improve cognition (Liu et al., 2016). While AT_2R expression is usually upregulated alongside AT_1R as a compensatory mechanism, this relationship was shown to be blunted in aging (Labandeira-Garcia et al., 2017).

Oligodendrocytes

Oligodendrocytes are axon-myelinating cells. To our knowledge, they have AT_1Rs and AT_2Rs on their cell membrane, which is known to have opposing effects. In particular, for oligodendrocytes, AT_1Rs lead to demyelination, while AT_2Rs promote re-myelination to enhance synaptic transmission and improve neuronal communication (Valero-Esquitino et al., 2015; Jackson et al., 2018).

THE LINK BETWEEN RAS, OXIDATIVE STRESS, NEUROINFLAMMATION, AND VASCULAR DYSFUNCTION

As reviewed in the previous section, functions of RAS within the brain are not limited to hypertension. Dysregulation of these functions can have detrimental effects on the brain. In this regard, a role for b-RAS has been identified in many different neuropsychiatric disorders, including anxiety, depressive disorder, and alcoholism within the brain (Labandeira-Garcia et al., 2017). Above all, the b-RAS may also lead to chronic neurodegenerative diseases by playing a pivotal role in oxidative stress and neuroinflammation (Barnham et al., 2004). In the following subsections, we will describe the mechanisms through which RAS contributes to oxidative stress and neuroinflammation.

Oxidative Stress

Although ROS have essential roles in metabolism, cell signaling, and the proper formation of learning and memory processes under physiological conditions (Chandel et al., 2000; Massaad and Klann, 2011; Chandel, 2014), in excessive amounts, they can lead to oxidative stress (Li et al., 2013). ROS are produced as a primary product of NADPH-oxidase (NOX) and secondary products of many other enzymatic processes such as those including xanthine oxidase, cyclooxygenases, uncoupled NOS, and the mitochondrial electron transport chain (De Silva and Faraci, 2013). Among them, membrane NOX complexes and mitochondria are the two main sources of ROS (Babior, 2004). Notably, there is a NOX-derived ROS-mediated cross-talk between them, which can further enhance the production of ROS by mitochondria (Cai, 2005). Furthermore, accumulated amounts of ROS can impair mitochondrial integrity, decrease ATP production, and lead to more mitochondria-derived ROS. It is known that mitochondrial-derived ROS contribute to cellular dysfunction by reaching the cytoplasm, demonstrating additional detrimental consequences.

Reactive oxygen species-associated oxidative stress leads to structural and functional modifications of proteins via oxidation. These modifications can increase the hydrophobicity of some proteins, thus leading to protein aggregation. To restrain cellular toxicity as a consequence of protein aggregation, effective removal of oxidized and damaged proteins is essential. This removal can be performed by either proteasome-mediated protein degradation or the autophagic pathway. However, ROS can also impair the proteasome system, thus leading to reduced protein degradation and accumulation of abnormal proteins such as synuclein, tau, or huntingtin in neurodegenerative diseases (Dröge, 2002; Turrens, 2003). ROS-related inhibition of the proteasome-based protein degradation upregulates autophagy. Although autophagy in response to mild oxidative stress is neuroprotective, its excessive or chronic upregulation promotes cellular death (Dasuri et al., 2013). With these pathways, ROS production and the destructive effects of oxidative stress can be further exacerbated (Gao et al., 2014). Notably, both proteasome-mediated degradation and autophagy demonstrate age-associated dysfunction and the effects of ROS on these processes contributes to the development of age-related neurodegenerative diseases (Grune et al., 2004; Dasuri et al., 2013).

There are seven isoforms of NOX. Among them, NOX1, NOX2, and NOX4 have been identified in the brain (Miller et al., 2007). Overstimulation of the Ang II/AT_1R axis can activate these brain NOX complexes and generate excessive amounts of ROS (Chan et al., 2005). In this regard, it has been demonstrated that ROS is elevated in the cerebrum with acute and chronic administration of Ang II via activation of NOX2 (Girouard et al., 2006; Chrissobolis et al., 2012). As extracellular Ang II binds to membrane AT_1R, it activates NOX2, increases intracellular Ca^{2+} levels, and generates intracellular oxidative stress (Wang, 2004). In addition, Ang II is known to be capable of destroying endothelium-dependent vasodilation in the cerebral circulation. However, interestingly, Chrissobolis et al. (2012) showed that these deleterious effects of Ang II did not occur in NOX2 deficient mice treated with Ang II. Oxidative stress also mediates Ang II-induced inward remodeling and hypertrophy in cerebral arterioles that blunt cerebral perfusion in hypertension. Consistent with previous findings, this inward remodeling was also not observed in NOX2 deficient mice (Chan and Baumbach, 2013). Activation of nuclear and mitochondrial AT_1Rs produce NOX4-derived ROS in neurons by coupling

to phosphoinositol-3 kinase and protein kinase C activation (Hongpaisan et al., 2004; Kimura et al., 2005; Abadir et al., 2012; Valenzuela et al., 2016; Villar-Cheda et al., 2017). Importantly, activation of nuclear AT_1Rs regulates gene expression and triggers several mechanisms that protect cells against oxidative stress (Villar-Cheda et al., 2017). In particular, nuclear RAS increases expression of AT_2R mRNA and angiotensinogen and activates Ang II/AT_2R and Ang (1–7)/MasR axes to counteract AT_1R effects in neurons (Villar-Cheda et al., 2010, 2017; Costa-Besada et al., 2018).

Finally, local Ang II levels impair insulin signaling and contribute to IR by impacting insulin-stimulated increases in insulin receptor substrate1-associated phosphoinositol-3 kinase activity (Folli et al., 1999). In addition to Ang II, hyperglycemia is also known to induce oxidative stress. Therefore, the combined effect of Ang II and hyperglycemia may exacerbate oxidative stress damage in diabetic tissue (Chen et al., 2007; Fukumoto et al., 2008). Separately, abnormalities in brain insulin signaling pathways are associated with cognitive impairment and AD pathology (Arvanitakis et al., 2020).

Besides increasing ROS generation, the RAS can alter the mitochondrial redox balance by diminishing the activity of scavenging enzymes. Many scavengers, including superoxide dismutase (SOD), catalase, and glutathione, have an essential role in reducing oxidative stress in the brain. There are three forms of SOD: cytosolic copper-zinc SOD (CuZnSOD; SOD1), mitochondrial manganese SOD (MnSOD; SOD2), and extracellular CuZnSOD (SOD3). SOD1 and SOD2 are especially crucial for age-related brain disorders (Gao et al., 2014). In transgenic mouse models of Alzheimer's disease (AD) pathology, the deletion of one allele of SOD2 increased amyloid plaque formation while the deletion of SOD1 increased β-amyloid oligomerization, cognitive impairment, and neuronal dysfunction (Esposito et al., 2006; Murakami et al., 2011). Furthermore, overexpression of SOD was shown to be related to decreased susceptibility to β-amyloid-induced neurotoxicity and ischemic brain injury (Celsi et al., 2004; Chen et al., 2011), yet it has been shown that the activity of antioxidant molecules glutathione, SOD, and catalase decrease with activation of the RAS (Bechara et al., 2005; Rodriguez-Iturbe et al., 2007; Xiong et al., 2010). Consistent with this observation, NO bioavailability and SOD activity increased, and endothelial function improved in humans after treatment with the AT_1R blocker losartan (Hornig et al., 2001).

ROS production in the brain is high due to its high oxygen consumption, and the oxidative metabolism of neurotransmitters, making the brain extremely vulnerable to additional free radical attacks (Sian et al., 1994; Kumar H. et al., 2012). Therefore, regulation of RAS is particularly essential as it may lead to impaired neurotransmitter release. Within the basal ganglia, cell death and dysfunction increased with AT_1R upregulation in dopaminergic neurons (Garrido-Gil et al., 2013a; Zawada et al., 2015; Ou et al., 2016). By contrast, in the same type of cells, Ang (1–7)/MasR and Ang II/AT_2R axes reduced ROS production and mitochondrial respiration (Costa-Besada et al., 2018). Further, it has been shown that upregulated ACE expression reduced acetylcholine release from cholinergic neurons (Barnes et al., 1992; Tota et al., 2012). This RAS-mediated upregulation of oxidative stress leads to the release of pro-inflammatory response exacerbating cell death (Garrido-Gil et al., 2013a; Ou et al., 2016).

Neuroinflammation

Within the brain, microglial cells are the most significant contributor to NOX-derived ROS (Gao et al., 2012). Among NOX isoforms, NOX2 is the primary source of extracellular ROS in microglia (Gao et al., 2014). NOX-derived ROS also affect intracellular signaling pathways related to microglial activation, proliferation, and release of pro-inflammatory signals (Shacter, 2000; Qin et al., 2004; Shulaev and Oliver, 2006; Gao et al., 2014). Although activation of microglial cells is necessary for removing dead cells and debris, exacerbation of this inflammatory cascade can harm the surrounding neurons and cause the progression of neurodegeneration (Vowinckel et al., 1997).

As discussed in Section "Receptor Functions According to Cell Type," the Ang II/AT_1R axis plays a role in the transition of microglia between its activated substates proinflammatory M1 and anti-inflammatory M2. In particular, AT_1R-induced NOX activation regulates the shift between M1 and M2, with upregulation of the AT_1R/NOX axis promoting pro-inflammatory and suppressing anti-inflammatory substates. With this signal cascade, microglia further exacerbate ROS production (Choi et al., 2012). This mechanism was also supported by a study showing that AT_1R antagonists reduce M1 microglia activation and promote M2 microglia polarization (Saavedra, 2017). In microglial cells, AT_1R-induced NOX activation is mediated by protein kinase C (Joglar et al., 2009). The initial NOX-derived superoxides are amplified by activation of nuclear factor-kβ (NF-kβ) and the RhoA/Rho kinase pathway, which further increased NOX activation, hence yielding ROS production (Borrajo et al., 2014; Rodriguez-Perez et al., 2015). With a feed-forward mechanism, Rho-kinase activation upregulates AT_1R expression via NF-kβ in microglial cells. Activation of the microglial RhoA/Rho kinase pathway is a significant modulator of the actin cytoskeleton and mediates microglial polarization and neurodegeneration (Labandeira-Garcia et al., 2015, 2017). Moreover, Ang II/AT_1R axis can increase toll-like receptor 4, which is known to mediate microglial activation and neuroinflammation (Biancardi et al., 2015; Rietdijk et al., 2016). It was shown that Ang II and prorenin promotes the production of ROS and pro-inflammatory cytokines [e.g., interleukin-1 beta (IL-1β) and IL-6] while reducing anti-inflammatory IL-10 levels (Winklewski et al., 2015). As an example, a recent study showed that Ang II administration leads to a pro-inflammatory response in the hippocampus with an increase in hippocampal CD68-positive cells (Takane et al., 2017).

In astrocytes, the Ang II/AT_1R axis mediates the production of IL-6 and ROS via NF-kβ/ROS pathway (Gowrisankar and Clark, 2016). Also, Ang III can induce signal transducer and activator of transcription 3, which is a crucial signal transduction protein that mediates cell differentiation, proliferation, apoptosis, and inflammation in astrocytes (Costantino and Barlocco, 2008; Kandalam et al., 2015).

There are also inflammation-regulating mechanisms induced through RAS receptors. Neuronal AT_2R signaling can lead to a downregulated signal transducer and activator of transcription 1 and 3 phosphorylation that can suppress microglia activation (Jackson et al., 2018). AT_4R was shown to mediate anti-inflammatory effects in a mouse model of AD (Royea et al., 2017). Thus, there is a delicate balance between Ang II/AT_1R/NOX pro-oxidative and pro-inflammatory axes and Ang II/AT_2R – Ang (1–7)/MasR antioxidant and anti-inflammatory axis, which determine RAS effects within the brain.

The precise reason for sex differences (premenopausal women vs. men) in the prevalence of cerebrovascular diseases is not yet fully understood. However, an association of Ang II-estrogen was suggested as a possible mechanism due to Ang II's sexually dimorphic effects on the cerebral vasculature (De Silva and Faraci, 2013). Consistent with this suggestion, it was shown that activation of microglial estrogen receptor B suppresses Ang II-induced increase in levels of several significant mediators (e.g., IL-1β and rho kinase) of the microglial inflammatory response (Rodriguez-Perez et al., 2015).

Vascular Dysfunction

Renin–angiotensin system can affect CBF through several mechanisms. The first such mechanism acts through Ang II-induced hypertension, which is characterized by many forms of alterations in the cerebral vasculature, including pathological remodeling of arteries, vasoconstriction, diminished cerebrovascular autoregulation, cerebrovascular inflammation, and decreased vascular compliance (Faraci and Heistad, 1990). A consequence of this RAS/hypertension-based mechanism is the development of an impaired response to reduced cerebral perfusion, which may render the brain vulnerable to ischemia and cellular injuries (Saavedra, 2017).

Renin–angiotensin system can also cause vascular dysfunction independent of hypertension. Based on its pro-oxidative and pro-inflammatory properties, it can cause vasoconstriction, endothelial damage, BBB breakdown, and disruption of neurovascular coupling (Ahmed et al., 2019). Impairments of neurovascular coupling, defining the alterations in CBF according to local neural activity, was shown to be associated with Ang II independent of hypertension based on evidence in phenylephrine-induced hypertensive mice showing no impairment in their neurovascular coupling (Kazama et al., 2003; Capone et al., 2011). Similarly, the detrimental effects of the Ang II/AT_1R axis on BBB permeability have not been seen in a deoxycorticosterone acetate-induced hypertension state (Rodrigues and Granger, 2012). Ang II can induce vasoconstriction via smooth muscle- and endothelium-dependent mechanisms. First of all, smooth muscle contraction generally occurs via AT_1R-mediated activation of phospholipase C, resulting in inositol 1,4,5-triphosphate and diacylglycerol production. These products mediate two distinct pathways, which commonly result in smooth muscle contraction via activation of several protein kinases, such as myosin light chain kinase and Rho-kinase (Hilgers and Webb, 2005). Ang II-induced constriction of basilar arteries was destroyed by Rho-kinase inhibitor Y-27632 (Faraci et al., 2006). Another path to vasoconstriction is endothelium-dependent constriction via cyclooxygenase- and/or superoxide-dependent mechanisms mediated by AT_1R in cerebral endothelium (De Silva et al., 2009). Finally, the Ang II/AT_1R axis causes an alteration in endothelium-dependent vasodilation by decreasing the bioavailability of NO (Intengan and Schiffrin, 2001).

AGE- AND DISEASE-RELATED CHANGES IN THE RAS

The pathophysiologies reviewed in the last section generally develop in the elderly. In this section, we discuss their connection with aging and how they lead to frailty and neurodegenerative diseases.

RAS, Aging, and Frailty

Aging and RAS are interrelated. Age-associated unbalanced activation of RAS influences the aging phenotype. RAS component levels changing with age accelerate cellular senescence and age-related tissue/organ dysfunction, thus leading to chronic age-related diseases through various mechanisms including oxidative stress, inflammation, apoptosis, and vascular changes. Several studies investigated the relationship between RAS, longevity, and neurodegenerative diseases. The relationship between RAS and longevity was first shown in mice by demonstrating that disruption of the AT_1R promotes longevity via attenuation of oxidative stress and pro-survival gene induction (Benigni et al., 2009). Benigni et al. (2013) also showed the association between prolonged life span and reduced AT_1R protein in humans with AT_1R gene variation. Furthermore, it has been shown in multiple studies that Ang II mediates premature senescence (Kunieda et al., 2006; Tsai et al., 2016).

Changes in RAS components with age are differently regulated in circulating and local RAS (Abadir, 2011). While circulating RAS components have lower levels in older animals, perhaps, due to age-related increase in blood pressure (Diz, 2008), AT_1R levels in local RAS are found to be upregulated (Kobori et al., 2007; Abadir, 2011; Abadir et al., 2011; Rodriguez-Perez et al., 2019). Previous work investigating changes in AT_1R and AT_2R level with age showed that mitochondrial and nuclear AT_1R levels increased significantly with age (Gwathmey et al., 2010; Abadir et al., 2011) as opposed to decreased AT_2R expression (Millan et al., 1991). Furthermore, the maximal expression of AT_2R was found in developing fetal tissues, followed by an immediate reduction after birth and maintenance of relatively lower levels in adulthood (Carey and Siragy, 2003). This was also confirmed by increased AT_1R/AT_2R expression ratios in aged animals (Garcia-Garrote et al., 2019). This was attributed to age-associated changes in the distribution of Ang II receptors, with nuclear AT_1Rs becoming the most common (85%) in older ages as opposed to nuclear AT_2Rs being the most common (80%) at young ages (Gwathmey et al., 2010). These distributional alterations were also shown in mitochondria of aged mice (Abadir et al., 2011). However, the same study showed that *in vivo* administration of AT_1R blocker losartan for 20 weeks increased the number of mitochondrial AT_2Rs and improved bioenergetics

of the aging mitochondria without affecting the expression of mitochondrial AT_1Rs (Abadir et al., 2011).

The b-RAS distribution also shows age-related differences. In a recent study, a progressive decrease in the expression of AT_2Rs with aging was shown in the SN (Rodriguez-Perez et al., 2019). Notably, in the same study, several changes in external appearance, spontaneous motor behavior, RAS components, and pro-oxidative and pro-inflammatory markers were found in young AT_2R KO mice as being similar to those observed in aged wild type mice. Regarding RAS changes, elevated Ang II and AT_1R levels and decreased mRNA/protein expression of other protective RAS receptors were observed both in aged wild type and AT_2R-KO mice (Rodriguez-Perez et al., 2019). Another study using AT_2R-KO mice found increased AT_1R expression in the brain ventricular–subventricular zone accompanied by a marked decrease in proliferation and generation of neuroblasts. This decrease was inhibited by treatment with the AT_1R antagonist candesartan. Further, increased proliferation and generation of neuroblasts were shown in wild type mice with the administration of AT_2R agonist C21 (Garcia-Garrote et al., 2019). Similarly, it was shown that AT_1R blockade can enhance hippocampal neurogenesis in hypertensive state (Bhat et al., 2018; Drews et al., 2019). Interestingly, neurogenesis was enhanced in hippocampal dentate gyrus of adult rodents exposed to short-term heat as a result of activation of AT_1R due to increase in Ang II (Koyama et al., 2018). This finding was in contrast to the aforementioned literature and might be because of the transient increase of Ang II with short-term application of the heat. However, further research is needed for a more definitive explanation. In another study about the importance of RAS on neurogenesis showed that permanent depletion of ACE2 impaired the effect of running on neurogenesis in the adult hippocampus (Klempin et al., 2018). Finally, similar observations were made for MasRs (Gwathmey et al., 2010) such that the protective Ang1–7/Mas axis expression decreased in aged rats similar to AT_2R (Villar-Cheda et al., 2010; Costa-Besada et al., 2018).

Mitochondrial dysfunction has a pivotal role in cell aging. The distributional changes in RAS receptor levels in mitochondria with aging in favor of AT_1Rs elevates mitochondrial ROS levels. This can diminish mitochondrial integrity and function, leading to decreased ATP generation and further production of ROS and peroxynitrite, a cytotoxic anion that inhibits mitochondrial electron transport (Sastre et al., 2000; Abadir et al., 2012). ROS have been proposed as the leading molecular factor in the aging process (Harman, 1956). One cellular aging mechanism is one in which increased mitochondrial ROS levels lead to oxidation of mitochondrial protein/lipid and DNA mutations whose accumulations are linked to senescence and apoptosis (Abadir et al., 2012; Vajapey et al., 2014). Aggregation of modified proteins due to enhanced RAS activity could be the mechanism of accelerated premature aging in AD (Cooper et al., 2018).

Consistent with the detrimental effects of AT_1Rs on mitochondria, it was shown that deletion of AT_1Rs resulted in a marked prolongation of life span in mice by upregulating multiple mitochondrial and prosurvival genes, nicotinamide phosphoribosyltransferase (Nampt) and sirtuin 3 (SIRT3) in the kidney (Benigni et al., 2009). Similarly, it was shown within the brain that overactivation of the Ang II/AT_1R/NOX axis leads to increased cell vulnerability to oxidative stress by decreasing levels of SIRT3, which would typically suppress pro-oxidative RAS axis with a feed-forward mechanism (Diaz-Ruiz et al., 2020). The same study also showed that these effects could be counteracted by treatment with AT1 antagonists in aged rats (Diaz-Ruiz et al., 2020). On the other hand, SIRT1 and Ang II/AT_1R axis, both of which have roles in neuroinflammation, oxidative stress, and aging-related cell degeneration, also regulate each other, and this regulation is impaired in aged animals (Diaz-Ruiz et al., 2015). Further, lower SIRT1 in brain is associated with more brain tau pathology (Julien et al., 2009). In addition, glial-derived Ang II appears to participate in age-related impairments of autonomic function such that glial angiotensinogen deficiency prevents these impairments (Diz, 2008). The intracellular Ang II/AT_1R/NOX axis generates superoxide anions that promote the uncoupling of endothelial NOS, which in turn reduces NO availability and mitochondrial NOS activity with aging (Valdez et al., 2004). Finally, the Ang II/AT_1R axis accelerates cellular senescence by inducing telomere shortening and cell cycle arrest, which can be reversed by losartan (Feng et al., 2011; Conti et al., 2012). Similar to Ang II, agonistic angiotensin II type 1 receptor autoantibodies can activate the AT_1R (Herse and LaMarca, 2013), which is consistent with the finding that higher levels of autoantibodies are associated with higher levels of inflammatory cytokines, weaker grip strength, slower walking speed, and increased risk for frailty (Abadir et al., 2017).

The compensatory mechanism of the b-RAS, which decreases oxidative stress and neuroinflammation, becomes less effective with age due to reduced AT_2R with age (Villar-Cheda et al., 2010, 2012; Lu et al., 2015). In aged animals, this decrease in AT_2R expression was shown to lead to a further increase in the pro-oxidative, pro-inflammatory effects and neuron vulnerability induced by activation of upregulated AT_1Rs (Labandeira-Garcia et al., 2017). As an example of neuronal vulnerability, neurotoxins lead to more dopaminergic neuron loss in aged compared to younger animals (McCormack et al., 2004).

The imbalance developing with age between pro-inflammatory and protective arms of RAS leads to inflammaging, which refers to aging-related pro-inflammatory changes observed in several tissues (Abadir, 2011; Labandeira-Garcia et al., 2017). This is associated with the pathogenesis of common and disabling diseases of older age, functional decline, frailty, and increased mortality (Abadir, 2011). Consistent with this, increased NOX activity due to RAS was seen in age-related diseases such as diabetes and atherosclerosis (Griendling et al., 2000; Mehta and Griendling, 2007). Aging and neuroinflammation in brain tissue together lead to exacerbated responses to lesions and neurodegeneration, hence being major risk factors for neurodegenerative diseases such as AD and Parkinson's disease (PD) (Collier et al., 2007; Labandeira-Garcia et al., 2017).

Lifelong accumulation of neuropathologies occurs in neurodegenerative diseases prior to clinical diagnosis (Braak and Braak, 1991; Bennett et al., 2012). Although neuropathologies are responsible, in large part, for the onset of dementia, they only account for a fraction of cognitive decline (Boyle et al., 2013, 2018, 2019). In this regard, frailty may be a critical determinant

of the cognitive impairment in both AD and stroke (Bennett et al., 2012; Taylor-Rowan et al., 2019). A strong interaction between frailty and pathologies was shown in AD such that high frailty renders subjects more vulnerable to pathologies with both more frequent development of the disease and worse cognitive decline. By contrast, people with low frailty are more resilient to neuropathologies (Wallace et al., 2019).

Frailty has also been shown to be an independent factor determining both the prevalence of stroke and post-stroke mortality rates (Palmer et al., 2019; Evans et al., 2020). The last decade has witnessed a considerable increase in research on frailty. Frailty is strongly influenced by multiple factors, including inflammation, oxidant state, vascular regulations, and mitochondria dysfunctions, and apoptosis. RAS plays a broad and essential role in the regulation of these factors, and it is closely associated with both physical and cognitive frailty phenotypes. Given the availability of RAS-acting drugs and the canonical role of RAS in both frailty and pathological mechanisms of age-related neurodegenerative diseases, which is discussed in the subsequent sections, RAS deserves further research as a potential therapeutic agent in age-related diseases.

Alzheimer's Disease

Alzheimer's disease is a complicated neurodegenerative disease characterized by progressive neuronal losses and cognitive impairment. Based on the brain autopsies of 83 patients with and without dementia, Braak and Braak (1991) described the pathologic characteristics of the disease as degeneration of specific nerve cells, presence of neuritic plaques, and neurofibrillary tangles (NFT). The most prominent pathological changes were found to be the extracellular amyloid plaques and intraneuronal NFT accumulations, which had started to take place years before the appearance of clinical symptoms (Braak and Braak, 1991; Mcgeer and Mcgeer, 1995). Although it is possible that these accumulations are not causal in AD pathogenesis, they characterize AD as a unique neurodegenerative disease among different diseases that can lead to dementia. However, classical diagnostic approach focused solely on clinical syndromes of AD regardless of these unique AD neuropathologies. This has resulted in misdiagnosis and limited our understanding of AD on a biological basis. Thus, recently, a purely biological definition of AD was introduced based on *in vivo* identification and postmortem examination of distinctive AD neuropathologies (Jack et al., 2018). This new research framework recommends referring to the clinical symptoms of AD without neuropathologic verification as Alzheimer's clinical syndrome instead of what has been called 'probable or possible AD' according to the traditional approach (Jack et al., 2018). Various RAS-related mechanisms have been suggested as contributors to its pathogeneses. The b-RAS is involved with processes of learning and memory, neuronal differentiation, and nerve regeneration via several mechanisms, including oxidative stress, neuroinflammation, and vasculopathy, as discussed in the previous sections. The relation of AD and other pathologies to cognitive decline follow complex patterns (Boyle et al., 2017; Wilson et al., 2019). A few years before death the rate of cognitive decline increases markedly due to terminal decline (Wilson et al., 2012). Several predisposing factors, such as genetics, age, and possibly environmental toxins, contribute to the development of initial lesions like senile plaques and NFTs. These products lead to an inflammatory response, microglial activation, and cytokine release, which then accelerates neuronal dysfunction and cognitive decline (Mcgeer and Mcgeer, 1995). In this regard, inflammation was thought to contribute to the progressive neuropathology of AD (McGeer and McGeer, 2013). As discussed in Section "Receptor Functions According to Cell Type," the Ang II/AT_1R axis of the b-RAS is an important contributor to neuroinflammation by enhancing microglial activation and polarization. Therefore, prolonged and unresolved inflammation harm neurons and synapses, which results in chronic dysregulation of glial cells followed by chronic deterioration in the brain structure and function (Denver and McClean, 2018). Furthermore, a recent study showed that higher Ang II levels are associated with smaller total gray matter, hippocampal, rostral middle frontal, and supramarginal parietal volumes which are related to cognitive domains that may decline in preclinical AD (Yasar et al., 2020).

A complementary explanation suggests the relation among increased oxidative stress, mitochondrial impairment, and alterations in the antioxidant system as a contributor to AD (Padurariu et al., 2013). As described in Section "Prelude to the Renin–Angiotensin System Signaling and Functions," over activity of the Ang II/AT_1R axis causes increased ROS and oxidative stress. First, oxidative stress causes damage by lipid peroxidation of the mitochondrial membrane as well as oxidation of structural and enzymatic proteins and nucleic acids. Increased oxidation of mitochondrial DNA impairs mitochondrial integrity and decreases ATP production, thus potentially resulting in mitochondrial dysfunction (Mecocci et al., 2018). As mitochondrial function declines, it leads to cellular alterations observed in AD, such as amyloid-β (Aβ) production, tau phosphorylation, synaptic degeneration, and oxidative stress (Swerdlow, 2011, 2012). Secondly, oxidative stress-associated modifications of the proteins can result in aggregation of Aβ and phosphorylation of tau protein, which could induce a vicious cycle of pathogenesis in AD (Kim et al., 2015). The amount of antioxidants is also an essential factor determining the extent of oxidative damage in the pathogenesis of AD (Mecocci et al., 2018). Their importance is supported by data showing neuroprotective effects of some anti-oxidants such as γ-tocopherol and lycopene (Yin et al., 2014; Morris et al., 2015).

Amyloid-β accumulation is another important aspect of AD pathogenesis. Ang II enhances the γ-secretase activity and Aβ production (Zhu et al., 2011). Aβ production leads to AT_2R oligomerization, which is associated with enhanced neurodegeneration (AbdAlla et al., 2009). In addition to the Ang II/AT_1R axis, ACE has also been investigated for its possible role in Aβ degradation (Miners et al., 2011). Although ACE was shown to degrade Aβ peptide (Hemming and Selkoe, 2005), it can also degrade neprilysin, an Aβ-degrading enzyme that may contribute to Aβ aggregation (Carson and Turner, 2002). Moreover, it has been shown in brain tissue autopsy of AD patients that ACE levels are elevated in the hippocampus, frontal cortex, and caudate nucleus regardless of hypertension and that

these levels have been reported to correlate with AD pathology (Miners et al., 2008, 2009). Similarly, it was shown in a recent study that cerebrospinal fluid (CSF) ACE activity was elevated in AD (Kehoe et al., 2019). Furthermore, the same study showed that RAS overactivity is correlated with CSF markers of capillary damage in AD, including elevated CSF soluble platelet-derived growth factor receptor β indicating pericyte damage and elevated CSF albumin indicating BBB breakdown.

Vascular disease may also contribute to the pathogenesis of AD. This is supported by the fact that many risk factors for vascular disease such as hypertension and diabetes are associated with Alzheimer's dementia (de La Torre, 2004). These relationships are complex and the association is likely due in part to mixed pathologies (Abner et al., 2016; Pruzin et al., 2017; Arvanitakis et al., 2018). The progressive degeneration of brain capillaries, such as thickened basement membrane, cerebral atrophy, reduced vessel elasticity, or genetic predisposition, disturbs the blood flow to the brain (de la Torre and Mussivand, 1993). The hypothesis proposes the impaired blood flow in conjunction with neuroinflammation as the central reason for Aβ aggregates and neuronal damage in the AD (de la Torre, 2002). RAS can modulate both of these underlying factors. In this regard, Ang II/AT_1R axis causes vasoconstriction of the cerebral vessels, vascular remodeling, impaired cerebrovascular autoregulation, and endothelial dysfunction (Iadecola and Davisson, 2008; Pires et al., 2013). As mentioned so far, Ang II/AT_1R axis has pro-inflammatory and pro-oxidant effects that can damage the BBB, increase its permeability, and reduce CBF, thus contributing to the pathogenesis of the AD (Miners et al., 2011). Consistent with this, blockade of AT_1R and activation of AT_2R reverse the hypertension-induced cerebrovascular dysfunction and improve the barrier function of endothelial cells and diabetes-associated cerebral endothelial dysfunction (Alhusban et al., 2013; Gallego-Delgado et al., 2016; Fouda et al., 2019).

Interestingly, there is a relationship between AD and diabetes mellitus type 2, which are both age-associated diseases (Denver and McClean, 2018). IR increases the risk of developing cognitive decline, and IR has been shown in postmortem brain tissue of AD patients without diabetes (Talbot et al., 2012; Arvanitakis et al., 2020). Also, impaired neuronal insulin signaling was demonstrated in the AD brain (Denver and McClean, 2018). In the brain, normally functioning insulin signaling is very important for proliferation, differentiation, and neurite growth. Insulin plays an essential role in learning and memory (Blázquez et al., 2014). In this regard, it was shown in rats in a water-maze task that long-term fructose-drinking causes IR, impaired insulin signaling, oxidative stress, neuroinflammation, the down-regulated activity of the cholinergic system, cognitive decline, impairments of spatial memory and learning (Yin et al., 2014). IR also causes endothelial dysfunction, which is important for AD development (Aronis and Mantzoros, 2012). Therefore, another impact of RAS in the aspect of AD might be impaired insulin signaling and its contribution to IR (Folli et al., 1999) through mechanisms mentioned in Sections "Neurons" and "Oxidative Stress." Interestingly, a significant increase in intracellular ACE was shown in high glucose conditions despite no change in extracellular ACE under the same circumstances (Cristovam et al., 2008).

Finally, b-RAS has important learning and memory-related effects (Ho and Nation, 2018). Among these effects, the overactivation of the Ang II/AT_1R axis is known to decrease acetylcholine release (Barnes et al., 1992; Tota et al., 2013). This might complement the role of cholinergic dysfunction in AD, proposing the loss of acetylcholine in the central nervous system as an important determinant of the cognitive decline in AD (Bartus et al., 1982; Kehoe, 2018). It was also shown that Ang II injection inhibits LTP in the hippocampus. On the other hand, the protective arm of the RAS has memory-enhancing effects. AT_2R activation through Ang II/III initiate cellular proliferation and differentiation accompanied by regenerative processes. Ang (1–7)/MasR axis enhances the release of NO and facilitates LTP, thus resulting in improved memory (Wright and Harding, 2019). Ang IV enhances dopamine release in the striatum and acetylcholine release in the hippocampus, thus facilitating LTP and neuroprotection (Lee et al., 2001; Lew et al., 2003; Stragier et al., 2004; Davis et al., 2006). Ang IV increases concentrations of cognition-enhancing peptides such as vasopressin and oxytocin by inhibiting aminopeptidase activity of ATR4 (Tomizawa et al., 2003; Bielsky et al., 2005). Ang IV can stimulate angiogenesis and enhance NMDA currents and synaptic plasticity in the hippocampus by binding the c-Met receptor (Akimoto et al., 2004). Ang IV could increase CBF without significant changes in systemic blood pressure (Wright and Harding, 2019).

Renin–angiotensin system lies at the intersection of pathologies of AD through many mechanisms mentioned so far, thus being a potentially critical component of AD pathogenesis. In the light of this, angiotensin hypothesis was suggested for further research (Kehoe, 2018). Finally, the ready availability of RAS-modifying drugs makes RAS more attractive as those drugs can be repurposed for the prevention and treatment of AD.

Parkinson's Disease

Parkinson's disease is a common neurodegenerative disease affecting more than 6 million individuals globally according to a recent study (Dorsey et al., 2018). It is characterized by dysregulation of the dopaminergic pathways and neuronal death as with other neurodegenerative diseases. Aging and neuroinflammation are two critical factors that have roles in the development and progression of PD. Brain RAS modulates both of these factors in SN (Valenzuela et al., 2010; Garrido-Gil et al., 2013b). The relationship between RAS and PD was first defined by Allen et al. (1992). It has been shown in several studies that b-RAS has a significant role in the progression of dopaminergic neuron degeneration in PD models (Grammatopoulos et al., 2007; Zawada et al., 2011; Sonsalla et al., 2013; Labandeira-Garcia et al., 2017). Moreover, increased CSF ACE activity in PD patients and an association between genetic polymorphism of the ACE gene and PD were also shown (Almeida-Santos et al., 2017).

The Ang II/AT_1R axis acting presynaptically in the SN and striatum enhances dopamine release (Mendelsohn et al., 1993; Brown et al., 1996). However, overactivation of this axis surprisingly shows a reverse effect, contributing to the loss of

dopaminergic neurons and progression of neurodegeneration in PD models through oxidative stress induced by NOX complex activation and enhanced neuroinflammation mostly via microglia activation (Costa-Besada et al., 2018). Interestingly, Ang II/AT_1R axis did not lead to dopaminergic neuronal death in the absence of microglia (Joglar et al., 2009). These findings show the importance of microglia in PD. Finally, in PD, low dopamine levels further increase neuroinflammation and neurodegeneration via the upregulation of the AT_1R/NADPH-oxidase axis (Rodriguez-Perez et al., 2019).

Renin–angiotensin system also has protective roles against the progression of PD. The Ang II/AT_2R axis can lead to actions opposing those of the Ang II/AT_1R axis in SN (Costa-Besada et al., 2018). Besides, Ang IV stimulates c-Met, and activation of the HGF/c-Met pathway inhibits the dopaminergic neuron loss in the SN in rats. Furthermore, maintenance of AT_1R and AT_2R balance via external modulations with AT_1R blockage or hormonal replacement therapy-based AT_2R upregulation was shown to be beneficial in PD (Farag et al., 2017). In addition, it has been shown that RAS blockade can reduce motor and non-motor symptoms of PD and neuronal damage (Almeida-Santos et al., 2017). For example, the AT_1R antagonist candesartan decreased the expression of nigral proinflammatory cytokines and dopaminergic cell vulnerability to neurotoxins in aged rats (Villar-Cheda et al., 2012). Finally, a similar modulation was obtained with estrogen replacement therapy, which led to a reduced nigral RAS and oxidative stress in young surgically menopausal rats than in aged menopausal rats. However, a remarkable reduction in dopaminergic neuron loss in both groups of menopausal rats was seen with the AT_1R antagonist candesartan (Rodriguez-Perez et al., 2012).

Vascular Cognitive Impairment

Cerebrovascular disease is common and often followed by brain dysfunction, further leading to cognitive loss, which is called vascular cognitive impairment (Gorelick et al., 2011; Arvanitakis et al., 2016). Cognitive impairment is seen in nearly 30–40% of stroke survivors and progresses slowly even after a single-stroke lesion (Wiesmann et al., 2013; Levine et al., 2015). Two major risk factors of both stroke and the subsequent cognitive impairment are aging and hypertension, both of which affect CBF through vascular dysfunctionalities discussed in Section "Vascular Dysfunction" (Ivan et al., 2004; Ahmed et al., 2019). Diminished CBF leads to hypoperfusion and hypoxia by creating a pro-oxidative and pro-inflammatory environment in the brain, which eventually results in neuronal death, thus contributing to the development of cognitive impairments (Ahmed et al., 2019).

Because of its pro-oxidative and pro-inflammatory properties, AT_1R overactivity plays an essential role in vascular cognitive impairment (Arroja et al., 2016). In this regard, AT_1R overactivity promotes vasoconstriction, reduces CBF, and increases oxidative stress, inflammation, and vulnerability to ischemia (Labandeira-Garcia et al., 2014). It was also shown that AT_1R-induced astrocyte senescence exacerbates cerebral ischemic injury (Liu et al., 2011). By contrast, the protective arm of RAS counteracts these effects. Six hours after ischemic injury in rodents, the MasR was found to be upregulated in the peri-infarct cortex (Arroja et al., 2016). Another study, reporting a similar overexpression of ACE2 and MasR in ischemic tissues, suggested that the Ang (1–7)/MasR axis potentially plays a pivotal role in the regulation of acute neuron injury in ischemic cerebrovascular diseases (Lu et al., 2013). MasR activation and ACE2 overexpression reduced both inducible NOS and the production of pro-inflammatory cytokines in the peri-infarct cortex (Bedecs et al., 1997; Xia and Lazartigues, 2008; Gwathmey et al., 2009; Arroja et al., 2016). Because of its anti-oxidative, anti-inflammatory, and neuroprotective properties, the protective arm of RAS has been studied as a therapeutic option for vascular cognitive impairment. In this regard, the Ang (1–7)/MasR axis was shown to have proangiogenic properties; administration of Ang (1–7) for 4 weeks promoted brain angiogenesis (Jiang et al., 2014). MasR expression was found in large amounts in hippocampus, perirhinal cortex, and vascular endothelial cells, and it has been shown that Ang (1–7)/MasR axis facilitate LTP (Hay et al., 2017). Glycosylated Ang (1–7)/MasR agonist improved object recognition and spatial memory impairment and reduced ROS and inflammation in mouse models of VCI and dementia (Hay et al., 2019). Interestingly, in a study comparing wild-type mice, MasR KO mice, AT_2R KO mice, and AT_2R/MasR double KO mice, it was found that cognitive status was unchanged in MasR KO mice despite decreased cerebral blood flow after bilateral carotid artery stenosis (Higaki et al., 2018).

Internal carotid artery administration of increasing doses of Ang IV significantly decreased cerebral infarct size in rats 24 h following embolic stroke, possibly due to Ang IV-facilitated the redistribution of blood flow to ischemic areas within a few minutes as indicated by cerebral arteriography (Faure et al., 2006). Furthermore, it was shown that C21, an AT_2R agonist, prevented the development of cognitive impairment after stroke in aged animals and animal models of vascular dementia (Iwanami et al., 2015; Ahmed et al., 2019). The use of ARBs showed both vascular and neuroprotective effects after ischemic stroke and preserved cognitive function in aged animals with chronic cerebral hypoperfusion (Alhusban et al., 2015; Ahmed et al., 2018). Lastly, the TROPHY (TRial Of Preventing Hypertension) study also showed protective effects of valsartan against ischemic brain injury after middle cerebral artery occlusion in mice given non-hypotensive doses (Li et al., 2008).

RENIN-ANGIOTENSIN SYSTEM-ACTING DRUGS

As of today, an effective risk reduction, prevention, or treatment strategy is not available for AD. Over 20 years, two categories of drugs have been used for the treatment of AD. One of them is cholinesterase inhibitors that extend the half-life of acetylcholine, whereas the other is memantine, an NMDA receptor antagonist, that limits glutamate excitotoxicity and neuronal damage (Wright and Harding, 2019). With the discovery of b-RAS and its multidimensional effects on the nervous system beyond its well-known hypertensive effect, RAS-acting drugs have been considered as a potential preventive and therapeutic intervention for neurodegenerative diseases. There

are three types of RAS-acting drugs: ARBs, ACEIs, and direct renin inhibitors (DRI), each of which act at different steps of the RAS process. While ARBs (e.g., losartan, valsartan, telmisartan, and candesartan) block binding of Ang II to the AT_1R, ACEIs (e.g., captopril, enalapril, lisinopril, and perindopril) block the hydrolysis of Ang I to Ang II. The blockade of AT_1R results in increased Ang II levels and consequently increases stimulation of AT_2R. In contrast, ACEI causes lower Ang II levels and consequently reduces stimulation of both AT_1R and AT_2R (Düsing, 2016). It was hypothesized that ARBs have potential advantages over ACEIs in the prevention of cognitive impairment because of more specific concomitant blocking of harmful effects while possibly enhancing beneficial effects such as vasodilatation and endothelial modulation (Fournier et al., 2004; Anderson et al., 2011). DRIs directly inhibit generation of Ang I from angiotensinogen, acting upstream of both ARBs and ACEIs (Riccioni, 2013). RAS inhibitors can reduce Aβ deposition and its consequences, and also suppress inflammation, oxidative stress, vascular damage/ischemia, and increase acetylcholine release and glutamate uptake (Gebre et al., 2018; Lebouvier et al., 2020). Especially, ARBs can prevent impairment of the BBB and reduce infiltration of inflammatory mediators observed in many neurodegenerative disease such as AD (Gebre et al., 2018). A summary of studies examining RAS-acting drugs in cognition and dementia is given in **Table 1**.

Angiotensin Receptor Blockers

Studies Showing Positive Effects on Cognition and Neural Protection

Early studies investigated cognitive effects of ARBs in animals. It was shown that pretreatment with losartan reduced chronic ethanol-induced cognitive deficits (Tracy et al., 1997) and improved both spatial and short-term working memory in animals (Raghavendra et al., 1998; Royea et al., 2020). It was shown that telmisartan has a long term anti-inflammatory effect by modulating microglia *in vitro* and *in vivo* (Torika et al., 2016). Chronic intranasal administration of losartan decreased plaque number in an AD mouse model (Danielyan et al., 2010) and also attenuated Ang II-induced cognitive impairment and tau phosphorylation (Tian et al., 2012). Candesartan was found to be more effective than perindopril in blunting the neuroinflammation in both astroglial and microglial cells of the rat brain (Bhat et al., 2016). An *in vitro* study screening 55 commonly prescribed antihypertensive drugs with respect to their anti-Aβ properties found that only valsartan and losartan are capable of both lowering Aβ and decreasing oligomerization of Aβ peptides in primary neuronal cultures, while candesartan was effective on oligomerization only (Wang et al., 2007; Zhao et al., 2009). Preventive treatment with valsartan significantly reduced brain Aβ deposits and Aβ-mediated cognitive deterioration, independently of blood-pressure lowering in AD mice model (Wang et al., 2007). Interestingly, it was shown that olmesartan and losartan protect cognitive function and cerebrovascular activity independently from blood pressure changes through decreasing oxidative stress in brain microvessels without decreasing Aβ levels (Takeda et al., 2009; Ongali et al., 2014). Some ARBs especially telmisartan were found to be partial agonists of peroxisome proliferator-activated receptor-γ (PPAR-γ) which is a target in AD with its anti-inflammatory, anti-amyloidogenic and insulin-sensitizing effects. In this regard, it was hypothesized that beneficial effects of telmisartan are attributed not only to its AT_1R antagonist properties but also to the activation of PPAR-γ (Heneka et al., 2007; Torika et al., 2016; Lebouvier et al., 2020). In line with this, it was shown that diabetes-induced increases in BBB permeability was attenuated by telmisartan through PPAR-γ activation to improve diabetes-induced cognitive decline (Min et al., 2012).

One of the earliest human studies was a double-blind randomized control trial which compares losartan and hydrochlorothiazide in 69 elderly patients. It was shown that losartan has a positive effect not only on blood pressure but also on impaired cognitive function, reversing even minimal cognitive deficits induced by hypertension (Tedesco et al., 1999). Similarly, losartan was found to improve memory in a comparative study between losartan and atenolol (Fogari et al., 2003). The Study on COgnition and Prognosis in the Elderly (SCOPE) is a double-blind and placebo-controlled study of candesartan conducted in 4937 mild-to-moderate hypertensive patients, aged between 70 and 89 years with a mean follow-up of 3.7 years. It was found that in patients with low cognitive function, decline of the mini-mental state exam score over time was less in the candesartan group (Skoog et al., 2005). Furthermore, candesartan was shown to be associated with less decline in attention and episodic memory during a follow up period of 44 months in a substudy of the SCOPE (Saxby et al., 2008). Consistent with the results of SCOPE trial, another study showed that trajectories of cognitive decline evaluated with periodic mini-mental state exam were less steep with antihypertensive administration, and interestingly, patients taking ARBs even had improved cognitive scores (Hajjar et al., 2005). On the other hand, ARBs were found to reduce the incidence and progression of AD and dementia when compared to ACEIs and other cardiovascular drugs in 819,491 predominantly male participants (98%) aged 65 or older with cardiovascular disease (Li et al., 2010). A double-blind randomized controlled clinical trial called antihypertensives and vascular, endothelial, and cognitive function (AVEC) trial compared 1-year treatment of lisinopril, candesartan, or hydrochlorothiazide with regard to their effects on memory, executive function, CBF, and central endothelial function. This study found that ARBs are associated with improvement in executive function in hypertensive older adults with early executive cognitive impairment (Hajjar et al., 2009, 2012). A secondary longitudinal data analysis of the Ginkgo Evaluation of Memory Study showed that diuretic, ARB, and ACEI uses were, in addition to and/or independently of mean systolic blood pressure, associated with reduced risk of AD in older adults with normal cognition (n = 1,928) while only diuretic use was associated with reduced risk in participants with MCI (n = 320) (Yasar et al., 2013). Furthermore, a meta-analysis of studies on AD and aging showed that ARBs have a protective role in the risk of cognitive impairment of aging and AD, while both ACEIs and ARBs have benefits on prevention of

TABLE 1 | Summary of research studies evaluating the effects of RAS-acting agents on cognition and dementia.

Author/year	Study design	Number of patients	Mean age (years)	Follow-up (years)	Treatment	Disease	Results
Tzourio et al., 2003	Double-blind RCT	6,105	64	3.9	ACEI	Dementia	Active treatment (perindopril with or without indapamide) was associated with reduced risk of dementia (relative risk reduction, 12% [95% CI, −8% to 28%]; $P = 0.2$). Cognitive decline was seen in 9.1% of the actively treated group and 11.0% of the placebo group (risk reduction, 19% [95% CI, 4% to 32%]; $p = 0.01$).
Ohrui et al., 2004a	Cohort study	4,124	69	8	ACEI	AD	Central-acting captopril and perindopril were associated with a significantly lower incidence of AD than the use of those that cannot inhibit brain ACE (imidapril or enalapril) (odds ratio = 0.25, 95% CI = 0.08–0.75; $p = 0.014$).
Ohrui et al., 2004b	RCT	162	76	1	ACEI	AD	The mean 1-year decline in MMSE scores in the participants of brain-penetrating ACEIs (perindopril or captopril) was lower than those in the participants of non-brain-penetrating ACEIs (imidapril or enalapril) and CCBs ($p < 0.001$).
Khachaturian et al., 2006	Cohort study	3,217	74.9	3	ACEI	AD	In the analyses of AD risk among different types of antihypertensive medications, ACEIs (HR, 1.13; 95% CI, 0.60–1.98) and CCBs (HR, 0.86; 95% CI, 0.45–1.53) showed no impact on AD risk.
Li et al., 2010	Prospective cohort analysis	819,491	74	4	ARB/ACEI	AD	A significant reduction in the occurrence of AD was identified with ARBs compared to lisinopril (HR 0.81, 95% CI 0.68–0.96, $P = 0.016$).
Yasar et al., 2013	*Post hoc* analysis of RCT	1,928	78.6	6.1	ARB/ACEI	AD	HR for AD occurrence among participants with normal cognition was found 0.51 in diuretic (95% CI 0.31–0.82), 0.31 in ARB (95% CI 0.14–0.68), 0.50 in ACEI (95% CI 0.29–0.83), 0.62 in CCB (95% CI 0.35–1.09), and 0.58 in BB (95% CI 0.36–0.93) users.
Barthold et al., 2020	Retrospective cohort study	694,672	77.3	7	ARB/ACEI	AD	The annual AD and related dementias incidence rate was found 2.07% among persons using a RAS-acting antihypertensive and any statin, and 2.64% among persons using a non-RAS-acting antihypertensive and any statin. ACEI + pravastatin OR = 0.942 (CI: 0.899–0.986, $p = 0.011$), ACEI + rosuvastatin OR = 0.841 (CI: 0.794–0.892, $p < 0.001$), ARB + pravastatin OR = 0.794 (CI: 0.748–0.843, $p < 0.001$), ARB + rosuvastatin OR = 0.818 (CI: 0.765–0.874, $p < 0.001$).

ACEI, angiotensin-converting enzyme inhibitor; ARB, angiotensin receptor blocker; CCB, calcium-canal blocker; BB, beta-blocker; AD, Alzheimer's disease; RCT, randomized controlled trial; HR, hazard ratio; CI, confidence interval; OR, odds ratio.

AD (Zhuang et al., 2016). A more recent retrospective cohort study found that ARBs combined with statins reduced AD more than statins combined with non-RAS-acting antihypertensives (Barthold et al., 2020). Consistent with previous studies, ARBs were found to be more effective than ACEIs in reducing dementia risk. Similarly, a meta-analysis of 2 case-control studies and 7 cohort studies showed that ARB use was associated with a reduced risk of incident AD (Oscanoa et al., 2020). There are also ongoing studies which aim to further elucidate the role of ARBs on AD (Kehoe et al., 2018; Hajjar, 2020).

Studies Showing Neutral or Negative Effects on Cognition and Neural Protection

In contrast to many promising results of ARBs, there are also conflicting animal and human studies that did not demonstrate any beneficial effects of RAS-acting drugs. For example, some animal studies showed that losartan alone did not cause any changes in learning and memory (Kułakowska et al., 1996) whereas valsartan abolished the intracerebroventricular Ang II-induced improvement of memory retrieval and consolidation in animals (Braszko, 2005). Similar dissociation results were obtained in human studies as well. The SCOPE trial mentioned in the previous section did not show any difference between candesartan and placebo groups with regard to incidence of dementia (Lithell et al., 2003). The Ongoing Telmisartan Alone and in Combination with Ramipril Global Endpoint Trial (ONTARGET), a double-blind randomized controlled trial conducted on 25,620 participants, and the parallel Telmisartan Randomized Assessment Study in ACE Intolerant Subjects with Cardiovascular Disease (TRANSCEND) trial in 5,926 participants did not show any significant effect of ARBs on cognitive outcomes (Anderson et al., 2011). A quantitative meta-analysis of longitudinal studies was conducted to compare subjects with (n = 32,658) and without (n = 36,905) antihypertensive medication use (Chang-Quan et al., 2011). The study found that the risk of AD is unchanged even though risk of vascular dementia decreased with antihypertensive medication use.

Angiotensin Converting Enzyme Inhibitors

Studies Showing Positive Effects on Cognition and Neural Protection

The ACEIs can improve basal learning performance, and antagonize scopolamine-induced learning deficits in animals (Barnes et al., 1992). Different cognitive effects of ACEIs in animal models were studied over the years. In the setting

of diabetic rats with learning impairment, enalapril treatment improved water maze performance and hippocampal LTP (Manschot et al., 2003). Cilazapril improved memory in aged rats when administered at low doses without antihypertensive effects (Hirawa et al., 1999). Bhat et al. (2016) showed the anti-inflammatory effects of perindopril in rodent glial cells. Captopril slowed the accumulation of Aβ plaques and hippocampal ROS in a mouse model of AD (AbdAlla et al., 2013). Intranasal captopril treatment regulated microglial activation and decreased Aβ burden in an AD mouse model (Asraf et al., 2018). Although ACEIs have effects on varied brain functions, their activities within the brain were found to be different: enalapril and ramipril produced no significant inhibition of ACE at any time while captopril and zofenopril had modest, short-lasting effects, and lisinopril had long-lasting inhibitory effects (Cushman et al., 1989). In this regard, ACEIs are classified as central-acting and non-central-acting. Central-acting ACEIs are perindopril, captopril, fosinopril, lisinopril, trandolapril, and zofenopril, while benazepril, enalapril, moexepril, quinapril, and ramipril are non-central-acting (Sink et al., 2009).

There are also many human studies showing positive effects of ACEI use. A review by Amenta et al. (2002) suggested that calcium channel blockers and ACEIs had positive effects on cognitive domains of hypertension compared to diuretics and beta-blockers. The Perindopril Protection Against Recurrent Stroke Study (PROGRESS), a randomized, double-blind, placebo-controlled trial, showed reduced risk of dementia and cognitive decline with perindopril and indapamide in patients with history of prior stroke or transient ischemic attack (Tzourio et al., 2003). Furthermore, among patients with vascular disease or diabetes plus an additional risk factor in the Heart Outcomes Prevention Evaluation (HOPE) study, ramipril reduced the incidence of stroke and was associated with lower prevalence of cognitive impairment (Bosch et al., 2002). In a study evaluating effects of different antihypertensives drug classes in elderly patients with a blood pressure of less than 150/90 mmHg, it was shown that central-acting ACEIs were associated with a significantly lower incidence of AD compared to non-central-acting ACEIs, calcium channel blockers, beta-blockers, and diuretics (Ohrui et al., 2004a). Ohrui et al. (2004b) designed a randomized study to investigate the use of ACEIs as a possible treatment for mild-to-moderate AD, in addition to cholinesterase inhibitor. In this study, mini-mental state examination score decline was significantly reduced in AD patients treated with central-acting ACEI compared to other drug classes (Ohrui et al., 2004b). Consistent with these results, the prescription of ACEIs was found to be independently associated with the stability of cognitive function after 1-year follow-up of mild cognitive impairment patients (Rozzini et al., 2006).

These findings were further expanded by a substudy of the Cardiovascular Health Study showing that central-acting ACEIs are associated with lower risk of cognitive decline while non-central acting ACEIs were associated with a greater risk of incident dementia (Sink et al., 2009). As an explanation, it was hypothesized that central-acting ACEIs act via mechanisms other than blood pressure control (Sink et al., 2009). Additionally, centrally active ACEIs were associated with a reduced rate of cognitive decline in dementia patients and improved cognitive scores in the first 6 months after treatment (Gao et al., 2013). Interestingly, a recent pharmacogenetic study suggested that ACEIs can slow cognitive decline independently of blood pressure variations in patients with AD, particularly for APOE4-carriers of specific ACE genotypes (de Oliveira et al., 2018).

Studies Showing Neutral or Negative Effects on Cognition and Neural Protection

As with ARBs, there are studies reporting conflicting results with respect to effects of ACEIs on cognition. In one of the earliest studies, no cognitive and CBF changes were seen in patients treated with ceranapril for 4 weeks (Weiner et al., 1992). The Hypertension Old People in Edinburgh (HOPE) study evaluated cognitive function after treatment with ACEI (captopril) or diuretic (bendrofluazide) and found no difference in cognitive function after 24 weeks (Starr et al., 1996). Similar results were seen after 18 weeks of perindopril treatment (Louis et al., 1999). Although the double-blind placebo-controlled Systolic Hypertension in Europe (Syst-Eur) trial showed that antihypertensive treatment was associated with a lower incidence of dementia in elderly people (Forette et al., 1998), its follow-up study showed that calcium channel blocker therapy was effective protection against dementia in older patients regardless of enalapril use (Forette et al., 2002). Consistent with previous studies, the Cache County Study showed that ACEIs have no specific effects on AD risk apart from the general positive effects of antihypertensive medications on incidence of AD (Khachaturian et al., 2006). In the Rotterdam study, subjects (n = 2015) taking antihypertensive medication at baseline had a reduced incidence of dementia that was significant for vascular dementia and not significant for AD (in't Veld et al., 2001). However, follow-up study of it showed that antihypertensive use reduced risks of all dementia including AD, with duration of hypertensive use being an important determinant (Haag et al., 2009). The same study did not find any apparent differences among different types of antihypertensive drugs (Haag et al., 2009). Finally, an observational study showed that ACEIs increase the risk of mortality in AD patients (Kehoe et al., 2013). However, this finding was not replicated in other cohorts (Lebouvier et al., 2020).

Renin Inhibitors

Another potential modulator of the RAS-system is the class of drugs that directly inhibit renin. Aliskiren, a renin inhibitor, blocks the catalytic effects of renin, which is the first and rate-limiting step of the RAS. Besides its anti-hypertensive effects, it also promotes neuroprotection, improving functional outcomes in a model of ischemic stroke (Panahpour et al., 2019). Aliskiren pretreatment attenuated oxidative stress, glial activation, white matter lesion, and spatial working memory deficits by inhibiting brain renin (Dong et al., 2011). Additionally, aliskiren was shown to counteract pathophysiological mechanisms of AD in animal models. In particular, suppressed Aβ neurotoxicity and attenuated Aβ-induced intra-neuronal renin expression in rat cortical neurons (Chen et al., 2012). Furthermore, aliskiren

also improved scopolamine-induced amnesia and increased acetylcholine through a decrease in acetylcholinesterase activity (Anil Kumar et al., 2015). However, a double-blind placebo-controlled trial showed that the incidence rate of dementia did not change in people over 80 years old who were treated with aliskiren for high blood pressure (Peters et al., 2008). Aliskiren is a promising agent for prevention or treatment of dementia (O'Caoimh et al., 2014). However, more clinical trials with longer follow-up periods are needed to investigate effects of DRIs on cognition comprehensively.

CONCLUSION

Alzheimer's disease and frailty are interrelating age-related disorders with a high level of morbidity and mortality. Although epidemiological and observational studies have shown close associations of frailty with AD, the biological mechanisms responsible for linking these two conditions remain elusive. Mitochondrial dysfunction, chronic inflammation, and oxidative stress constitute primary theories of aging and have been implicated as major contributors to the pathogenesis of both frailty and AD. The renin–angiotensin system is a central hormonal system that contributes to both inflammation and mitochondrial dysfunction. Many important studies have emerged that suggest that angiotensin system blocking drugs, commonly used in clinical practice for hypertension and heart failure, can favorably impact many chronic disease states, tissues and organ systems that are negatively impacted by age and inflammation. The choice of RAS-blocking drug and deciding on the onset of treatment remain uncharted territory.

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All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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Peripheral Levels of Renin-Angiotensin System Components Are Associated With Cognitive Performance in Huntington's Disease

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The renin-angiotensin system (RAS) has proven to be involved in the pathophysiology of neurodegenerative diseases, such as Parkinson's disease (PD) and Alzheimer's disease (AD), serving as a potential therapeutic target and a disease burden marker. Studies have associated negative clinical outcomes with the activation of the classical RAS arm composed of the angiotensin-converting enzyme (ACE) and angiotensin (Ang) II, while suggested positive outcomes with the activation of the counter-regulatory RAS arm involving ACE2 and Ang-(1–7). Huntington's disease (HD) shares many pathological and clinical outcomes with AD and PD, but the evidence of direct involvement of RAS components in the pathophysiology of HD is still limited and needs further investigation. Herein, we investigated peripheral levels of the RAS components Ang II, Ang-(1–7), ACE, and ACE2 in controls, premanifest, and manifest HD gene carriers and their relationship with clinical outcomes. Peripheral blood samples were collected via phlebotomy, and plasma concentrations of RAS components were measured by Enzyme-Linked Immunosorbent Assay. Clinical evaluation included a questionnaire about socio-demographic characteristics, motor, and cognitive assessments. Results showed (1) no significant group differences in plasma concentrations of RAS components; (2) positive correlations between ACE2 and Verbal Fluency Test (VFT) scores; and (3) negative correlations between Ang II and Mini–Mental State Examination scores. These results corroborate the proposed balance between the classical (ACE/Ang II) and the counter-regulatory [ACE2/Ang-(1–7)] arms of the RAS, with the former associated with negative clinical outcomes and the latter with positive effects in HD.

Keywords: Huntington's disease, renin-angiotensin system, angiotensin, biomarker, cognition

INTRODUCTION

The renin-angiotensin system (RAS) is a peptidergic system with endocrine characteristics, well known for its pivotal role in the regulation of water and sodium homeostasis and blood pressure (Paul et al., 2006b). It involves the enzymatic conversion of angiotensinogen to angiotensin (Ang) I by renin. Through the classical pathway, Ang I may be converted to Ang II via the angiotensin-converting enzyme (ACE). Ang II is an active component involved in vasoconstriction, effective circulatory volume maintenance, thirst stimulation, and adrenal gland aldosterone release. Most of Ang II actions are exerted via its binding to angiotensin type 1 receptors (AT_1; Ferrario and Chappell, 2004). Through an alternative pathway, Ang II may be converted into Ang-(1–7) via ACE2, and this counter-regulatory arm works in opposition to the ACE-AngII-AT_1 systemic effects. For instance, Ang-(1–7) has cardioprotective effects via reduction of fibrosis and cardiac hypertrophy (Grobe et al., 2007). In addition, Ang-(1–7) has shown beneficial anti-inflammatory effects in hepatic and renal diseases and inhibition of fibrogenesis through the G protein coupled-receptor Mas (Santos et al., 2003; Simoes e Silva et al., 2013; Prestes et al., 2016). The systemic balance between these two RAS branches is of significant physiological importance.

It is now recognized that the RAS is involved in several physiological functions other than cardiovascular and renal homeostasis. In this regard, the RAS has been implicated in several brain functions, including motor control, cognition, and behavior [for a review see Paul et al. (2006a)]. The RAS has proven to be involved in the pathophysiology of neurodegenerative diseases, including Parkinson's (PD) and Alzheimer's disease (AD; Munoz et al., 2006; Rocha et al., 2016b, 2018). For instance, Ang II increased neuronal death through activation of oxidative and inflammatory responses (Griendling et al., 2000; Chabrashvili et al., 2003; Rodriguez-Pallares et al., 2008). The neurodegenerative effects of Ang II have been confirmed by experiments with animal models of PD, which have shown a reduction in dopaminergic neuron degeneration with ACE inhibitor treatment (Kurosaki et al., 2005; Munoz et al., 2006). The deleterious effects of Ang II-AT_1 receptor binding were further confirmed by studies showing the neuroprotective effects of AT_1 receptor antagonists in animal models of PD (Grammatopoulos et al., 2007; Rey et al., 2007). Although experimental data support a role for the RAS in neurodegeneration, data obtained from human samples are still scarce. We have previously shown that patients with PD have reduced plasma levels of Ang I, Ang II, and Ang-(1–7), which correlated with depressive symptoms (Rocha et al., 2016b). In patients with AD, cerebrospinal fluid (CSF) levels of ACE were reduced in comparison with controls and correlated with reduced amyloid-β $(Aβ)_{42}$ levels, which indicates increased Aβ amyloid accumulation in the brain, a known indicator of AD burden (Rocha et al., 2018). These studies highlight the participation of RAS components in the pathophysiology of neurodegenerative diseases, also indicating possible associations with clinical outcomes. Despite the evidence of RAS-related mechanisms in AD and PD, the role of this system in Huntington's disease (HD), which shares many neurodegenerative pathways and clinical outcomes with AD and PD (Ross and Tabrizi, 2011), must be explored further.

HD is an autosomal dominant neurodegenerative disease with cognitive, motor, and behavioral symptoms. The HD mutation is described as an expanded CAG trinucleotide repeat in the huntingtin gene (*HTT)* on chromosome 4 encoding huntingtin (HTT), a protein that has proven to be necessary for embryonic development and maintenance. Manifestation age in HD is highly variable and primarily influenced by the length of the CAG trinucleotide repeat with an average age of onset around 45 years (Langbehn et al., 2010). Manifestation or clinical diagnosis of HD has historically been defined as the onset of an "unequivocal presence of an otherwise unexplained extrapyramidal movement disorder (e.g., chorea, dystonia, bradykinesia, rigidity) in a subject at risk for HD" (Hogarth et al., 2005). Subjects with a confirmed expanded CAG trinucleotide repeat but without motor symptoms of HD are classified as premanifest HD gene carriers. HD is regarded as an interesting model to study the pathophysiological mechanisms associated with neurodegeneration because it allows the assessment of patients in a preclinical stage of the disease.

While the cause of HD is known, the mechanisms resulting in neurodegeneration in HD are not fully understood. Similar to AD and PD, inflammation and oxidative stress have been regarded as key-players in HD pathophysiology (Kumar and Ratan, 2016; Rocha et al., 2016a). As well as in other neurodegenerative diseases, the RAS may be an important player in the neurodegenerative outcomes of HD. To the best of our knowledge, no studies have evaluated RAS components in HD gene carriers. Therefore, this study was designed to elucidate whether HD gene carriers present with changes in the RAS. In addition, we sought to explore the potential association between peripheral levels of RAS components and clinical symptoms.

MATERIALS AND METHODS

Subject Evaluation and Biological Sample Collection

Fifty-seven participants (18 premanifest HD gene carriers, 23 manifest HD, and 16 controls) underwent a comprehensive clinical interview and a blood draw. Genetic diagnosis of HD was confirmed by a genotype larger CAG allele $\geq$36. A movement disorder specialist evaluated all patients, and the clinical diagnosis of HD (manifest HD) was based on the motor signs certainty, i.e., a Diagnostic Confidence Level (DCL) set to 4 in the Unified HD Rating Scale (UHDRS; Ross et al., 2014). All subjects provided written informed consent before admission to the study. The Research Ethics Committees of UTHealth approved this study.

The clinical evaluation included a questionnaire about socio-demographic characteristics, motor, and cognitive assessments. HD gene carriers were subjected to a motor function assessment with the UHDRS. The motor section of the UHDRS assesses motor features of HD with standardized ratings of oculomotor function, dysarthria, chorea, dystonia, gait, and postural stability.

It is comprised of 31 items with a 5-point ordinal scale ranging from 0–4, and the total motor score is the sum of all the individual motor ratings, with higher scores indicating more severe motor impairment (Huntington Study Group, 1996). All individuals underwent a brief cognitive examination using the Mini–Mental State Examination (MMSE; Folstein et al., 1975), the Symbol Digit Modalities Test (SDMT), and the Verbal Fluency Test (VFT; Huntington Study Group, 1996). The MMSE is a 30-point questionnaire for cognitive screening, comprising items from different domains such as orientation, attention, memory, and language (Folstein et al., 1975). The VFT and SDMT are part of the UHDRS – cognitive assessment proposed by the Huntington Study Group (Huntington Study Group, 1996). The SDMT is a simple substitution task. Using a reference key, the examinee has 90 s to pair specific numbers with given geometric figures. The score is the number of correct responses achieved in 90 s. The VFT assesses the ability to spontaneously produce words orally within a fixed time span (60 s). For category fluency, words must be produced according to semantic constraints. The measure of performance used is the number of correctly generated words within 60 s (CHDI Foundation, 2011).

Ten milliliters of blood were drawn by venipuncture in vacuum tubes containing heparin on the same day of the clinical assessment. Whole blood samples were used for plasma obtaining within 2 h of having been drawn. These samples were centrifuged at 3,000 *g* for 10 min, 4°C, twice. Plasma was collected and stored at −80°C.

Biochemical Analysis

For the evaluation of the RAS components, samples were thawed and plasma levels of Ang II, Ang- (1–7), ACE, and ACE2 were measured by Enzyme-Linked Immunosorbent Assay (ELISA), according to the procedures supplied by the manufacturer (MyBioSource, San Diego, CA, United States). Ang-(1–7; MBS084052) and ACE2 (MBS723213) assays employed the quantitative sandwich ELISA technique. Ang II (MBS764273) and ACE (MBS727096) assays were based on the competitive ELISA detection method. Briefly, for the sandwich-ELISA-based assays [Ang-(1–7) and ACE2], standards and samples were pipetted into the wells of a plate that had been pre-coated with antibodies specific for each marker to be analyzed. Any Ang-(1–7)/ACE2 present was bound by the immobilized antibody. After removing any unbound substances, a biotin-conjugated antibody specific for Ang-(1–7)/ACE2 was added to the wells. After washing, avidin conjugated Horseradish Peroxidase (HRP) was added to the wells. Following a wash to remove any unbound avidin-enzyme reagent, a substrate solution (3,3′,5,5′-Tetramethylbenzidine, TMB) was added to the wells, and the color developed in proportion to the amount of Ang-(1–7)/ACE2 bound in the initial step. The color development was stopped by adding sulfuric acid solution and the intensity of the color was measured spectrophotometrically at a wavelength of 450 nm. The procedure for the competitive ELISA assays (Ang II and ACE) was very similar, except for the fact that the sample was incubated together with Ang II-/ACE-HRP conjugate. Ang II/ACE and Ang II-/ACE-HRP conjugates competed for the antibodies binding sites, and the final color intensity was inversely proportional to the Ang II/ACE concentration. The concentrations were calculated based on a standard curve in which the absorbance was plotted against the standard concentration. The sensitivity of the assays was 1.0 pg/mL for ACE and ACE2; 2.0 pg/mL for Ang- (1–7); and <0.094 ng/mL for Ang II.

Statistical Analysis

Association between dichotomous variables was assessed with the Chi-Square test. All variables were tested for Gaussian distribution by the Shapiro–Wilk normality test. Comparisons between three groups (i.e., controls vs. premanifest HD vs. manifest HD) were made by Kruskal–Wallis or ANOVA tests, according to the non-Gaussian or Gaussian distribution of the variables, respectively. *Post Hoc* analyses were used to determine significant differences between pairs of groups. Spearman's correlations were performed to examine the relationship between plasma levels of RAS components and: (i) the scores in the clinical scales (UHDRS – Total motor score, SDMT, VFT, and MMSE), and (ii) CAG repeat length. All statistical tests were two-tailed and were performed using a significance level of $\alpha = 0.05$. Statistical analyses were performed using SPSS software version 26.0 (SPSS Inc., Chicago, IL, United States) and GraphPad Prism version 5.0 (GraphPad Software, Inc., La Jolla, California, United States).

RESULTS

The demographic and clinical characteristics of the study participants are shown in **Table 1**. The three groups presented comparable age, sex distribution, and educational level. The groups were significantly different regarding ethnicity. HD gene carriers' groups (both premanifest and manifest) were mostly comprised of White individuals, while the controls were mostly Hispanics/Latinos. This result is in line with the literature showing higher rates of HD among White/Caucasian populations (Rawlins et al., 2016), and controls were recruited from the local community (Houston, TX, United States), which has a high percentage of Hispanics/Latinos.

As expected, individuals with manifest HD had worse cognitive performance than premanifest HD gene carriers and controls, as evidenced by the lower scores on the MMSE, SDMT, and VFT. Premanifest HD gene carriers' scores on the MMSE and SDMT were similar to controls, while the performance of the premanifest group was worse than controls on the VFT (**Table 1**). Plasma concentrations of RAS components [Ang-(1–7), Ang II, ACE, and ACE2] were determined in controls, premanifest, and manifest HD gene carriers. No significant differences were found when comparing the three groups (**Figure 1**). There were significant correlations between ACE2 levels and VFT scores (rho = 0.329, $p = 0.017$); and between Ang II levels MMSE scores (rho = -0.341, $p = 0.012$; **Figure 2**). We did not find any significant correlation between plasma levels of RAS components and SDMT scores, CAG length, or motor score.

TABLE 1 | Demographic and clinical characteristics of study participants.

Variable	Controls (*N* = 16)	Premanifest HD (*N* = 18)	Manifest HD (*N* = 21)	*P* value
Age in years [mean ± SD (Min – Max)]	48.12 ± 11.02 (32.7–64.0)	44.30 ± 11.34 (24.5–66.6)	49.88 ± 12.64 (21.5–72.1)	0.336[1]
Female sex, N (%)	10 (62.5)	12 (66.7)	14 (66.7)	0.957[2]
Ethnicity, N (%)				
Hispanic or Latino	13 (81.3%)	2 (11.1%)	3 (14.3%)	<0.001[2]
White	3 (18.7%)	16 (88.9%)	17 (81.0%)	
Black or African American	0 (0%)	0 (0%)	1 (4.8%)	
Antihypertensive drugs				
ACE inhibitors	0	2 (11.1%)	1 (4.8%)	
AT_1 receptor antagonists	2 (12.5%)	2 (11.1%)	1 (4.8%)	N/A[3]
Others*	1 (6.25%)	4 (22.2%)	3 (14.3%)	
Educational Level in years [mean ± SD (median)]	17.28 ± 3.93 (18)	15.17 ± 3.49 (14.5)	15.20 ± 2.07 (15)	0.098[1]
CAG repeats [mean ± SD (median)]	–	41.88 ± 1.80 (41)	44.63 ± 4.14 (43)	0.010[4]
UHDRS – Total motor score [mean ± SD (median)]	–	4.61 ± 4.80 (3)	29.67 ± 12.91 (30)	<0.001[4]
SDMT (total correct) [mean ± SD (median)]	50.88 ± 9.62 (52)[a]	48.00 ± 11.43 (49)[a]	30.55 ± 11.83 (30.5)[b]	<0.001[1]
VFT (category) – number of correct responses in1 min [mean ± SD (median)]	22.38 ± 4.46 (22)[a]	18.39 ± 5.25 (18)[b]	12.55 ± 4.36 (12)[c]	<0.001[1]
MMSE [mean ± SD (median)]	28.13 ± 1.78 (29)[a]	28.33 ± 1.72 (29)[a]	25.85 ± 2.76 (26.5)[b]	0.002[5]

*[1]ANOVA followed by Tukey's multiple comparisons test. Significant differences between groups are indicated by different letters. [2]Pearson Chi-Square test. [3]Due to the low expected frequencies, chi-square analysis was not appropriate. [4]Mann–Whitney test. [5]Kruskal–Wallis followed by Dunn's multiple comparisons test. Significant differences between groups are indicated by different letters. *Other antihypertensive drugs include diuretics, calcium channel blockers and beta-adrenergic receptor antagonists. Abbreviations: ACE, angiotensin-converting enzyme; AT1, Angiotensin II type I receptor; HD, Huntington's disease; MMSE, Mini–Mental State Examination; SD, Standard deviation; SDMT, Symbol Digit Modalities Test; UHDRS, Unified HD Rating Scale; and VFT, Verbal Fluency Test.*

DISCUSSION

This study aimed to evaluate peripheral levels of RAS components in HD gene carriers in comparison with controls, and the potential association between the RAS and clinical outcomes in HD. No significant differences were found between controls, premanifest, and manifest HD gene carriers when evaluating plasma concentrations of Ang-(1–7), Ang II, ACE, and ACE2. However, there were significant correlations between ACE2 levels and VFT scores, and between Ang II levels and MMSE scores.

There are a few studies implicating the RAS in HD pathophysiology. Early studies have found a decrease in ACE activity in brain samples of patients with HD in comparison with controls. The reductions were reported in brain regions involved in HD pathophysiology, such as caudate nucleus (Arregui et al., 1977; Butterworth, 1986), putamen, and globus pallidus (Arregui et al., 1977), in addition to substantia nigra (Arregui et al., 1978). Later, increased ACE activity was reported in the CSF of patients with HD in comparison with controls (Schweisfurth et al., 1987). Decreased ACE activity in the brain may be a result of neuronal death as ACE is expressed by neurons. On the other hand, the increased ACE activity in the CSF can reflect compensatory mechanisms associated with the reduced ACE availability in the central nervous system. The ACE activity can also be influenced by the availability of its substrates and products, and by other RAS components, including ACE2, and angiotensin receptors. Using radioligands, one study described a 35% reduction in AT_1 receptor levels in the putamen of patients with HD in comparison with controls (Ge and Barnes, 1996). Altogether, these data point to a role of the RAS in HD. Our findings partially corroborate these previous data. While the lack of difference in RAS components between HD gene carriers and controls undermines our hypothesis of the involvement of this system in HD pathophysiology, the observed correlations support it.

The RAS is also known for its role in inflammatory mechanisms. While the classical axis of the RAS (ACE/Ang II/AT_1 receptor) activates several pathways related to inflammation, the counter-regulatory axis [ACE2/Ang-(1–7)/Mas receptor] exerts anti-inflammatory responses (Rodrigues Prestes et al., 2017). Immune/inflammatory mechanisms are involved in HD pathophysiology (Rocha et al., 2016a) and studies are needed to elucidate how the RAS and inflammatory/immune mechanisms are linked to HD. In this regard, one study reported that anti-AT_1 receptor antibodies are more frequent in individuals with HD than in controls. The anti-AT_1 antibody titers correlated with the age of HD onset and disease burden scores and were also linked to smoking and infection. These data suggest a dysfunction of the adaptive immune system in HD, which may be triggered by different stimuli including autoimmune responses, infection, and possibly smoking (Lee et al., 2014).

We found positive correlations between ACE2 and VFT scores, and thus, higher concentrations of ACE2 were associated with better verbal function. Noteworthy, ACE2 activity has been reported to be reduced in AD patients in comparison with controls (Liu et al., 2014; Kehoe et al., 2016). The ratio of Ang II to Ang-(1–7; an indirect estimate of ACE2 activity) was elevated

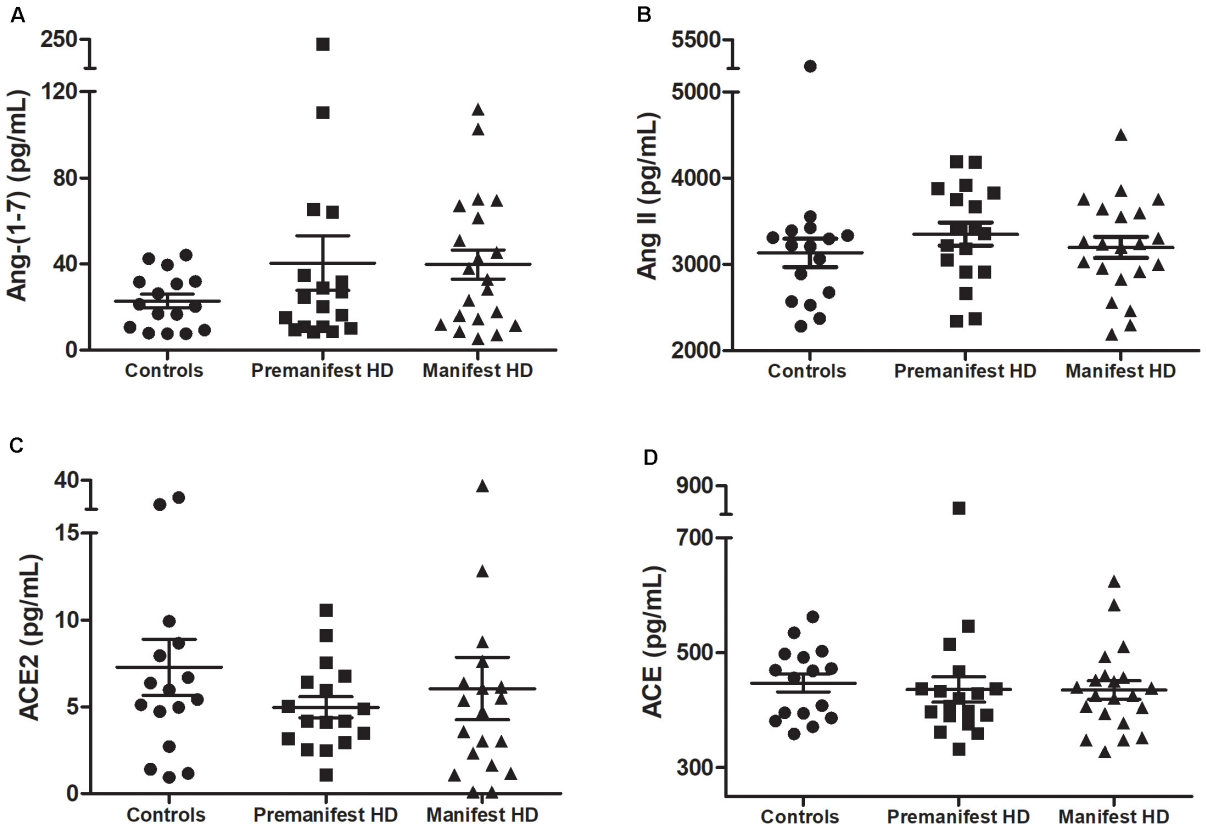

FIGURE 1 | Plasma levels of **(A)** angiotensin-(1–7; Ang-1–7), **(B)** angiotensin II (Ang II), **(C)** ACE2 in controls, premanifest, and manifest Huntington's disease (HD) individuals and **(D)** angiotensin-converting enzyme (ACE). There were no significant differences between the three groups [Kruskal–Wallis test, as data were determined to not follow a normal distribution]. Horizontal bars indicate mean and standard error of the mean for each set of values.

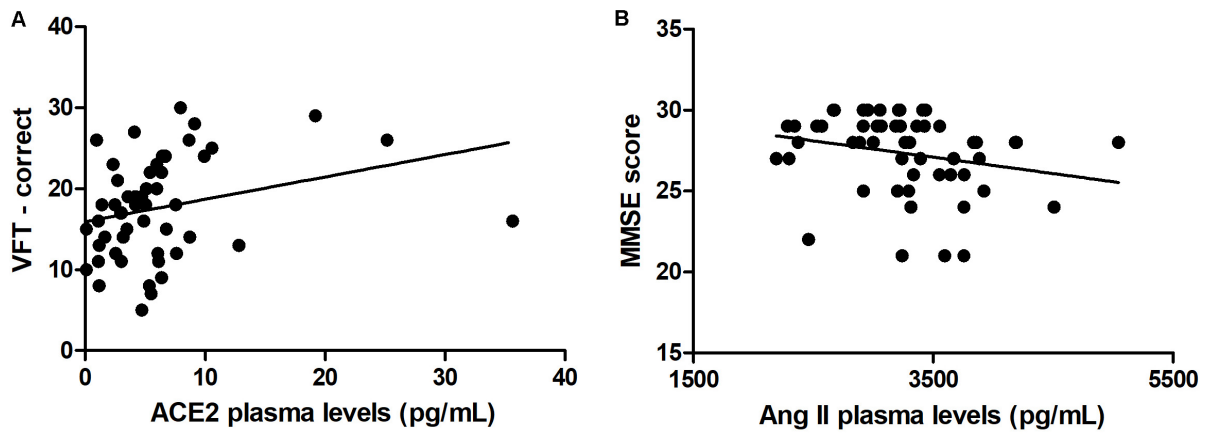

FIGURE 2 | Correlations between renin-angiotensin components and clinical scores. **(A)** Plasma levels of angiotensin-converting enzyme 2 (ACE2) correlated positively with verbal fluency test (VFT) scores. **(B)** Plasma levels of angiotensin II (Ang II) correlated negatively with Mini-Mental State Examination (MMSE) scores. Spearman correlation analyses were performed and a p-value < 0.05 was considered significant.

in AD patients, suggesting reduced conversion of Ang II to Ang (1–7; Kehoe et al., 2016). Together, these studies suggest that cognitive decline is associated with reduced RAS counter-regulatory axis components. Our study supports these findings by extrapolating them to HD patients, yet more studies are still necessary to fully determine the beneficial effects of increased ACE2 activity in HD patients. In addition, we found negative correlations between Ang II and MMSE scores, a measure of

general cognition. Thus, higher levels of Ang II were associated with worsening cognition. As mentioned before, many studies have revealed the potential neurodegenerative effects of Ang II, and our data is consistent with those reports of negative effects of Ang II (or the classical RAS axis) on cognition.

The deleterious effects of Ang II were further confirmed by an observational study evaluating the effects of anti-hypertensive drugs on HD progression and outcomes. Untreated hypertensive HD patients suffered from more depressive symptoms, worsening cognition, and a more rapid decline in total functional capacity (TFC) and motor function when compared to normotensive and treated hypertensive patients (Steventon et al., 2020). Increasing evidence of the negative effects of untreated hypertension in HD adds value to our proposals of RAS involvement in disease outcomes. Our sample population had a negligible number of participants simultaneously treated with antihypertensive drugs, and thus, no analysis was performed in this regard. To our knowledge, no studies have evaluated the specific effects of RAS-targeted antihypertensive drugs in normotensive HD patients. Yet, the demonstrated protective effects of such medication in hypertensive HD patients is evident and creates an area of scientific curiosity around the role these drugs would play in HD. One study analyzed the use of RAS blockade (RASB) and the incidence of cognitive impairment in AD (Zhuang et al., 2016). Their results demonstrated that RASB was associated with a 35% risk reduction in the incidence of cognitive impairment and 20% risk reduction in the incidence of AD (Zhuang et al., 2016). In addition, the treatment with AT_1 receptor antagonists attenuates cognitive impairment in a blood pressure-independent manner through reductions in blood-brain barrier permeability in Ang II- and salt-dependent hypertensive patients (Pelisch et al., 2013). These results further emphasize the positive cognitive benefits associated with RAS-targeted antihypertensive drugs in neurodegenerative diseases. More studies must be performed to evaluate their potential protective effect in HD patients.

Our study has several limitations that must be acknowledged. Our sample size was relatively small, preventing us to control for potential confounding factors. The cross-sectional nature of the study also limits inferences of causality. While associations have been made, further research using longitudinal studies following RAS components concentrations throughout the life of HD patients and correlating them with clinical symptoms would be of value to determining the relationship between RAS and HD pathophysiology. In addition, our study did not measure the activity of RAS enzymes (only plasma concentrations) or levels of angiotensin receptors. Therefore, future studies should focus on measures of enzyme activity and receptor levels, which may yield valuable information.

In conclusion, although the RAS components concentrations did not differ among controls, premanifest, and manifest HD gene carriers, there were meaningful correlations between RAS components and cognitive performance in HD. Besides implicating RAS in the pathophysiology of HD, our results corroborate the emerging evidence on the balance between classical and counter-regulatory arms of RAS in neurodegeneration and neuroprotection, respectively.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Committee for the Protection of Human Subjects – UTHealth. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

NR and AT worked on the design and conceptualization of the study. NR, GC, and EF executed the research project. NR and CC designed and executed the data analysis and interpretation. EF and AT reviewed data analysis. NR and CC wrote the first draft of the manuscript. All authors reviewed the manuscript and approved the submitted version.

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Epilepsy Seizures in Spontaneously Hypertensive Rats After Acoustic Stimulation: Role of Renin–Angiotensin System

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Hypertension is a common comorbidity observed in individuals with epilepsy. Growing evidence suggests that lower blood pressure is associated with reduced frequency and severity of seizures. In this study, we sought to investigate whether the renin–angiotensin system (RAS), which is a critical regulator of blood pressure, is involved in the pathogenesis of audiogenic epilepsy-related seizures in a hypertensive rat model. Spontaneously hypertensive rats (SHRs) were given RAS inhibitors, angiotensin-converting enzyme (ACE) inhibitor or angiotensin II type I receptor (AT1R) antagonist, for 7 days prior to inducing epileptic seizures by acoustic stimulation. After the pretreatment phase, blood pressure (BP) of SHRs normalized as expected, and there was no difference in systolic and diastolic BP between the pretreated SHRs and normotensive rat group (Wistar). Next, treated and untreated SHRs (a high BP control) were individually subjected to acoustic stimuli twice a day for 2 weeks. The severity of tonic–clonic seizures and the severity of temporal lobe epilepsy seizures (product of forebrain recruitment) were evaluated by the mesencephalic severity index (Rossetti et al. scale) and the limbic index (Racine's scale), respectively. Seizures were observed in both untreated (a high BP control) SHRs and in SHRs treated with AT1R antagonist and ACE inhibitor. There was no statistical difference in the mesencephalic severity and limbic index between these groups. Our results demonstrate that SHRs present seizure susceptibility with acoustic stimulation. Moreover, although RAS inhibitors effectively reduce blood pressure in SHR, they do not prevent developing epileptic seizures upon acoustic stimulation in SHR. In conclusion, our study shows that RAS is an unlikely link between hypertension and susceptibility to epileptic seizures induced by acoustic stimulation in SHRs, which is in contrast with the anticonvulsant effect of losartan in other animal models of epilepsy.

Keywords: hypertension, epilepsy, renin–angiotensin system, ACE inhibitors, AT1 antagonist, SHR

INTRODUCTION

Epilepsy, affecting 50 million people worldwide (World Health Organization, 2019), is a chronic neurological disorder characterized by unprovoked recurrent seizures more than 24 h apart (Fisher et al., 2014). Individuals with epilepsy are reported to have a higher prevalence of psychiatric and cardiovascular comorbidities (Keezer et al., 2016). A prevalence of heart disease in adult patients with a history of epilepsy is reported to be about 9% higher than in individuals without a history of epilepsy (Zack and Luncheon, 2018). Interestingly, hypertension and hyperlipidemia, two classic cardiovascular risk factors, are more prevalent than a psychiatric diagnosis in epileptic adult populations (Wilner et al., 2014). This suggests that high blood pressure may be associated with cardiovascular events and sudden death in patients with epilepsy (Szczurkowska et al., 2019). Conversely, sudden deaths observed in this group of patients may be partially related to cardiovascular abnormalities (Ngugi et al., 2013).

Renin–angiotensin system (RAS), a hormone complex involved in blood pressure control, regulates cardiovascular functions; however, when dysregulated it contributes to pathological processes leading to hypertension and cardiovascular disease (de Gasparo et al., 2000; Bader and Ganten, 2008; Becari et al., 2011, 2017). Classically, RAS is described as a cascade in which angiotensinogen (AGT) is cleaved by renin to circulating angiotensinogen-forming angiotensin I (Ang I) and next to angiotensin II (Ang II) by angiotensin-converting enzyme (ACE). AngII, the strongest effector of RAS, exerts effects by activating angiotensin 1 (AT1Rs) and angiotensin 2 (AT2R) receptors. It is widely recognized that RAS components are also expressed in other organs and tissues such as the brain. Local RAS in the central nervous system has been shown to be involved (directly or indirectly) in the modulation of some cognitive functions such as memory and learning (Farag et al., 2017; Huber et al., 2017; Mascolo et al., 2017).

The spontaneously hypertensive rat (SHR) is a classical hypertension model (Okamoto and Aoki, 1963) and also has been considered to model other diseases such as attention-deficit hyperactivity disorder (ADHD) with over activity, impulsiveness, deficient sustained attention, depression (Tchekalarova et al., 2011), and more recently epilepsy (Vorobyov et al., 2011; Tchekalarova et al., 2014, 2015, 2016; Russo et al., 2017; Atanasova et al., 2018). The first description of SHR presenting kindling with fewer electroencephalogram (EEG) after discharges was by amygdala or/and pyriform cortex stimulation (Greenwood et al., 1989). Other studies have shown that SHR are susceptible to epilepsy induced by kainate (Tchekalarova et al., 2016), as well as by pilocarpine (Mello et al., 1993; Scorza et al., 2005; Grosser et al., 2020). However, the response of SHR to acoustic stimulation with audiogenic seizures has not been shown.

Our group has shown previously that the RAS system is involved in epileptogenesis in the Wistar audiogenic rat (WAR) experimental model (Garcia-Cairasco et al., 2017). Treatment with RAS inhibitors not only lowered blood pressure levels but importantly reduced the severity of tonic–clonic seizures and blocked limbic seizures during sound stimulation (audiogenic kindling) (Pereira et al., 2010). We also demonstrated that both a local non-classical pathway was involved in Ang1–7 generation in the hippocampus of rats, suggesting a functional relevance of local RAS in the pathophysiology of epilepsy (Pereira et al., 2013). Although WARs have been genetically developed initially for the epilepsy phenotype, they display, in addition to neuropsychiatric comorbidities (Garcia-Cairasco et al., 2017), systemic cardiovascular alterations (Fazan et al., 2015) and impaired central respiratory chemoreflex (Totola et al., 2017). Other groups have shown RAS contribution to epilepsy using animal models such as the kainate (KA) model of temporal lobe epilepsy (TLE) and the pilocarpine in Wistar and SHR (Mello et al., 1993; Scorza et al., 2005; Ivanova et al., 2015; Tchekalarova et al., 2016; Atanasova et al., 2018; Grosser et al., 2020). Interestingly, the long-term treatment with losartan after kainate (KA)-induced *status epilepticus* (SE) exerted a disease-modifying effect on spontaneous seizure activity and neuronal damage in a SHR (Tchekalarova et al., 2014, 2015, 2016). Interestingly, losartan and enalapril treatment in SHR submitted to acoustic stimulation with audiogenic seizures has not been investigated yet.

Based on evidence presented above, we hypothesized that the elevated blood pressure, via RAS, is involved in the development of epilepsy induced by acoustic stimulation and that a pretreatment with RAS inhibitors would reduce epileptic seizures in SHR.

MATERIALS AND METHODS

Animals

Experiments were conducted on 9–12 week old male SHR and Wistar rats. Animals were maintained in a controlled environment with a constant 12:12 h light–dark cycle and provided with food and water *ad libitum*. All surgical procedures were performed under tribromoethanol anesthesia (250 mg/kg, ip). All experiments were conducted following the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Every effort was done in order to avoid unnecessary suffering and stress to the animals.

Protocol Design

In the first protocol group, blood pressure measurements, acoustic stimulation, and seizure evaluation were performed in SHR and in Wistar rats as a control. The aim of this protocol was to determine whether SHR would develop epileptic seizures when submitted to acoustic stimulation following behavioral evaluation protocols by Garcia-Cairasco et al. (1992) and Pereira et al. (2010), developed for genetically developed Wistar audiogenic rat (WAR) strain (Doretto et al., 2003). The acoustic stimulation and seizure evaluation were described ahead.

In the second protocol group, blood pressure measurements were performed before the treatment initiation and, after 7 days of enalapril treatment (Galena Company), losartan treatment (Galena Company) or vehicle treatment (water) in SHR and

in Wistar rats as a control, as described by Pereira et al. (2010). The aim of this protocol was to analyze whether targeting RAS pathway attenuates epileptic seizures induced by acoustic stimulation.

Systolic Blood Pressure Measurements

Systolic blood pressure (SBP) was measured using a tail-cuff plethysmography method (MK-II; E&M Instrument) in conscious SHR and Wistar rats in a prewarmed (10 min at 38°C) and thermostatically controlled heating cage box. In the first protocol group, blood pressure measurements were performed before the start of acoustic stimulation in Wistar rats and in SHRs. In the second protocol group, blood pressure measurements were performed before the start of the treatment and after 7 days of a given treatment. Final SBP values were obtained by averaging three successful consecutive readings.

Acoustic Stimulation and Seizure Evaluation

Acoustic stimulation was performed twice a day (kindling protocol as described by Pereira et al., 2010) at fixed times (between 08:00–09:00 h and 16:00–17:00 h) during 10 consecutive days after 7 days of pretreatment with enalapril, losartan, or vehicle. An acoustically isolated box was used to expose each animal to a high-intensity sound, until tonic seizure appearance or for a maximum time of 1 min (Pereira et al., 2010). The sound of a ringing bell (120 dB) was digitized with a high-pass filter (N500 Hz) and reproduced with a personal computer coupled to amplifiers and tweeters under the top of the cage. Rat's behavior was recorded during the stimulus duration and for 1 min after exposure. The severity of tonic–clonic seizures was evaluated using the mesencephalic severity index (Rossetti et al., 2006). Racine's scale (limbic index) was used to assess the severity of temporal lobe epileptic seizures (Racine, 1972; Galvis-Alonso et al., 2004). Repeated audiogenic seizures were named audiogenic kindling by Marescaux et al. (1987), and we reproduced this phenomenon in WARs (Garcia-Cairasco et al., 1996a; Dutra Moraes et al., 2000; Pereira et al., 2010). Briefly, development of chronic seizures is characterized by an increase in the mesencephalic index, followed by a decrease in the mesencephalic index values, and a concomitant increase in limbic index values. Observed changes are due to the recruitment of different brain regions, mostly prosencephalic and limbic, which leads to different behavioral alterations in animals with epileptic background (Garcia-Cairasco et al., 1996b, 2017; Dutra Moraes et al., 2000; Romcy-Pereira and Garcia-Cairasco, 2003; Pereira et al., 2010). The treatment groups were subjected to induction of audiogenic seizures (Garcia-Cairasco et al., 1992), and data were collected in a blind manner.

Pharmacological Treatment

SHRs were pretreated by gavage with ACE inhibitor (enalapril, 10 mg kg^{-1} of body weight·day^{-1}), AT_1R antagonist (losartan, 50 mg kg^{-1} of body weight·day^{-1}), and vehicle once a day (between 12:00 and 14:00 h) for 7 days prior to acoustic stimulation and for 10 consecutive days during acoustic stimulation. The efficacy of the doses was validated *in vivo* in SHRs, such as both enalapril and losartan showed a significant antihypertensive effect (reduction of approximately 30% in mean BP).

Statistical Analyses

Data are presented as mean ± SEM of the indicated number of independent experiments. Statistical analyses were done using one-way analyses of variance (ANOVAs) or unpaired Student's *t*-tests, as appropriate. $P < 0.05$ were considered as statistically significant. Statistical analysis and graph plotting were performed in GraphPad Prism software program (San Diego, CA, United States).

RESULTS

First, we demonstrated that SHR showed seizure susceptibility with acoustic stimulation. The seizure severity was described based on indexes recorded for tonic–clonic seizures (mesencephalic index, **Figures 1A,B**) and limbic seizures (limbic index, **Figures 1C,D**). The first day of the audiogenic kindling protocol of SHR presented seizure behavior characterized by wild running and jumping (mesencephalic index equal 2) and, after day 4, the appearance of limbic behavior such as myoclonus (limbic index equal 2). No seizures were observed in Wistar (control) rats.

As expected, SBP measured prior to the first acoustic stimulus was markedly increased in SHR when compared to Wistar control (210 ± 2 vs. 107 ± 5), **Figure 1E**). Next, we investigated further whether a treatment with RAS inhibitors (ACE inhibitor and AT_1 receptor antagonist) would prevent seizures in the SHR group, submitted to the same chronic acoustic stimulation protocol. As shown in **Figures 2A,B**, enalapril or losartan treatment did not significantly suppress the tonic–clonic seizures (mesencephalic index) in SHR, when compared to SHR treated with vehicle. Similarly, limbic seizures (limbic index, **Figures 2C,D**) were not affected after enalapril or losartan treatment. Despite having no effect in the seizure severity, as shown in **Figure 2E**, both enalapril and losartan were able to decrease blood pressure in SHR. Furthermore, Wistar rats treated with the same dose of both enalapril or losartan or vehicle had no effects in the blood pressure or seizure behavior (not shown).

DISCUSSION

Our study investigated the involvement of RAS in epileptic seizures behavior evoked by chronic acoustic stimulation (audiogenic kindling) in animals of the SHR strain. Although chronic treatment with the RAS inhibitors, such as ACE inhibitors and AT1 receptor antagonist, reduced blood pressure in SHR, the same treatment was not associated with reduction in epileptic seizures, as detected by the mesencephalic index (acute audiogenic seizures) and forebrain index (chronic audiogenic seizures/audiogenic kindling). This suggests that the classical systemic RAS components are not linked directly to audiogenic

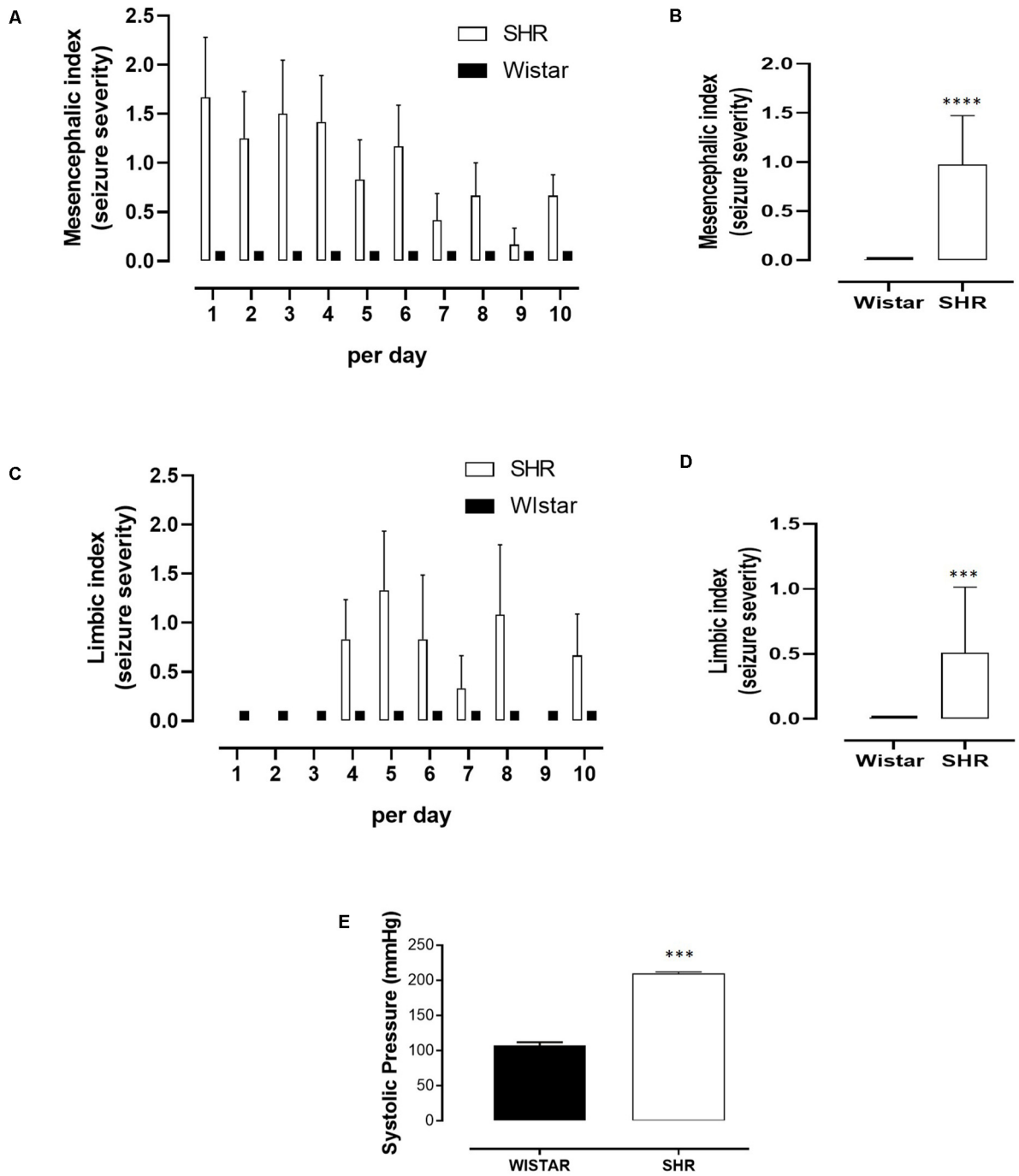

FIGURE 1 | Seizure severity during acoustic stimulation and systolic blood pressure (SBP) measure comparing Wistar rats and spontaneous hypertensive rats (SHR). **(A)** Severity indexes for tonic–clonic seizures (mesencephalic index) per day. **(B)** Mesencephalic indexes mean for tonic–clonic seizures. **(C)** Severity indexes for temporal lobe epilepsy seizures (limbic index) per day. **(D)** Limbic indexes mean for temporal lobe epilepsy seizures. **(E)** Systolic pressure of Wistar and SHR rats measured prior to the first acoustic stimulus. **(A,C)** Data are expressed as mean ± SEM for each group (Wistar or SHR), per day. **(B,D,E)** Data are expressed as mean ± SEM for each group (Wistar or SHR), and Student's *t*-test was used to compare between groups. ***$p < 0.001$. ****$p < 0.0001$. For acoustic stimulation, Wistar ($n = 10$) and SHR ($n = 10$). For SBP measure, Wistar ($n = 15$) and SHR ($n = 23$).

seizure susceptibility in animals of the SHR strain. This was in contrast to other studies, where the same treatment was able to decrease the seizure severity in the Wistar audiogenic rat (Pereira et al., 2010) strain and in SHR after kainate-induced SE (Tchekalarova et al., 2016). These comparative controversial results may be explained by the fact that epilepsy is not a specific

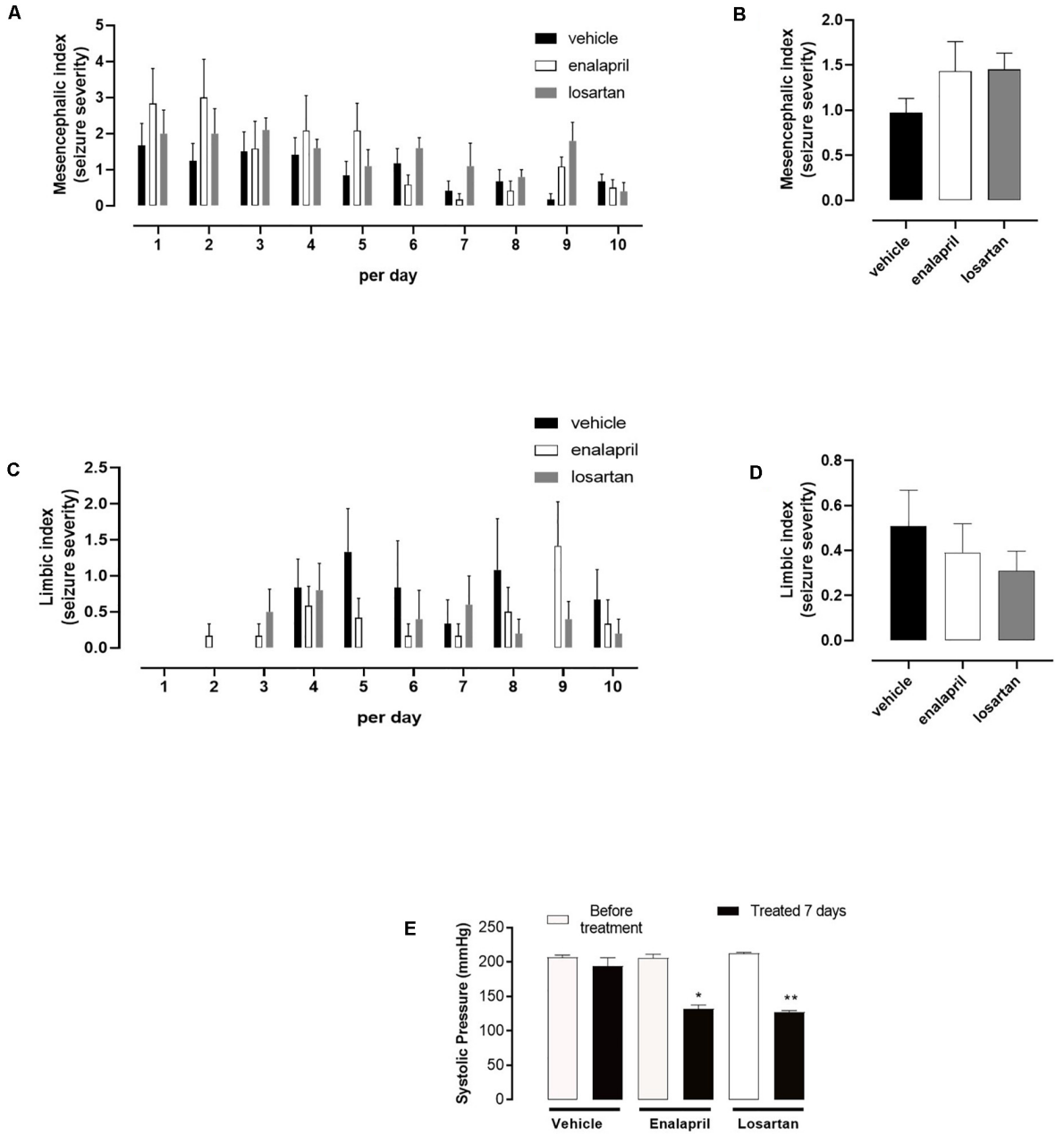

FIGURE 2 | Seizure severity and systolic blood pressure (SBP) measure for spontaneous hypertensive rats (SHR) administered with vehicle (water) or enalapril (10 mg kg^{-1}) or losartan (50 mg kg^{-1}). **(A)** Severity indexes for tonic–clonic seizures (mesencephalic index) per day. **(B)** Mesencephalic indexes mean for tonic–clonic seizures. **(C)** Severity indexes for temporal lobe epilepsy seizures (limbic index) per day. **(D)** Limbic indexes mean for temporal lobe epilepsy seizures. **(E)** SBP measured in conscious SHR before treatment and on the seventh day of pharmacological treatment. **(A,C)** Data are expressed as mean ± SEM for each group (vehicle, enalapril, or losartan), per day. **(B,D)** Data are expressed as mean ± SEM for each group (vehicle, enalapril, or losartan), and analysis of variance (ANOVA) was used to compare between groups. **(E)** Data are expressed as mean ± SEM for each group (before treatment and treated 7 days for vehicle, enalapril, or losartan) and Student's *t*-test was used to compare before treatment and treated 7 days groups. $^{*}p < 0.05$. $^{**}p < 0.01$. For acoustic stimulation, vehicle ($n = 10$), enalapril ($n = 8$), and losartan ($n = 8$). For SBP measure, vehicle ($n = 10$), enalapril ($n = 8$), and losartan ($n = 8$).

disease. In fact, epilepsies are a heterogeneous group of disorders resulting from altered brain functions. The triggers that induce seizures in rats could involve different pathways, sometimes secondary to some pathological processes, such as hypertension and cardiovascular disturbances (Szczurkowska et al., 2019).

In the current study, we show that SHR animals subjected to audiogenic kindling developed epileptic seizures of low-level severity, when compared to those displayed by WARs (Garcia-Cairasco et al., 2017). For comparison, the highest levels found in the current study were of mesencephalic index 3

(see **Figure 1**), and the maximum of the mesencephalic index is 8. Moreover, while RAS inhibitors effectively lowered blood pressure in SHR, they were not able to block even low levels of audiogenic seizures in the same animals. This result was unexpected in the light of our previous study showing that the RAS inhibitors were able to reduce the severity of tonic–clonic seizures and block limbic seizures during audiogenic kindling in WARs, as well as to reduce their blood pressure levels (Pereira et al., 2010). However, it is important to highlight that SHR were selected for high blood pressure phenotype and WARs for high seizure severity phenotype. Susceptibility to seizures are in fact a comorbidity for SHR (Tchekalarova et al., 2015), and hypertension or ectopic beats are comorbidities for WARs (Garcia-Cairasco et al., 2017). In the context of our findings, it is of great interest to verify whether patients with epilepsy will improve their excitability control with blood pressure control. Conversely, it will be also important to see if controlling epileptic seizures may improve blood pressure levels/control. Such a type of interaction has been demonstrated in the scenario of epilepsy and depression (Kanner et al., 2018) and hypertension and depression (Polanka et al., 2018).

Similar to our results, it has been shown that SHR exhibited higher susceptibility to SE after the kainate (KA) model of temporal lobe epilepsy (TLE) (Tchekalarova et al., 2011) and after amygdala and piriform cortex stimulation (Greenwood et al., 1989). The treatment with AT1R antagonists did not prevent the development of SE in SHRs or WKY rats (Tchekalarova et al., 2015). It was hypothesized that non-responsiveness to treatment to AT1R antagonists may be associated with plasticity induced by hippocampal extracellular noradrenaline, serotonin, and dopamine levels. Further, the data by Atanasova et al. (2018) showed that AT1 receptor expression was increased in the amygdala of epileptic SHRs model by kainate but not of epileptic Wistar rats. In addition, long-term treatment with losartan was capable of suppressing the AT1 receptor expression in SHRs, when compared to controls. Losartan showed neuroprotection in the hippocampus and of the dentate gyrus in SHRs after kainate. However, the AT1 receptor antagonist did not exert a substantial influence on epilepsy behavioral (Tchekalarova et al., 2016).

Even though RAS inhibitors showed no effect on reducing epileptic seizures in some comorbidities models, the observation that losartan potentiates the anticonvulsant effects of other drugs could justify further investigation. Lukawski et al. (2010), for example, demonstrated that losartan potentiates the anticonvulsant effects of carbamazepine and lamotrigine in the electroshock model.

CONCLUSION

In conclusion, this study demonstrates that animals of the SHR strain, phenotypically selected for hypertension, are susceptible to audiogenic seizures of low-level severity. Still, these seizures appear not to be related to the blockade of the traditional components of the RAS when induced by acoustic stimulation.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

All experiments were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were reviewed and approved by the Animal Care and Use Committee of the Ribeirão Preto Medical School, University of São Paulo.

AUTHOR CONTRIBUTIONS

CB and MP conducted all the experiments and wrote the manuscript. JO and NG-C provided rats from the breeding colony. KP analyzed all the results and corrected the English version. GP and JO conducted part of the experiments regarding audiogenic kindling and wrote part of the manuscript. CB, CC-N, NG-C, and MP conceived the idea of the experimental design and analyzed all the results. All authors have approved the final version of the manuscript and agreed to be accountable for all aspects of the work.

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BRIEF RESEARCH REPORT
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Decreased Plasma Levels of Angiotensin-Converting Enzyme Among Patients With Bipolar Disorder

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Background: Dysfunctions in the renin-angiotensin system (RAS) seem to be involved in the pathophysiology of several mental illness, including schizophrenia and mood disorders. We carried out a cross-sectional study assessing the levels of RAS-related molecules among bipolar disorder (BD) patients compared to healthy controls.

Methods: our sample consisted of 30 outpatients with BD type 1 (10 males, 20 females, age = 35.53 $\pm$ 10.59 years, 14 euthymic, 16 experiencing mood episodes) and 30 healthy controls (10 males, 20 females, age = 34.83 $\pm$ 11.49 years). Plasma levels of angiotensin-converting enzyme (ACE), angiotensin-converting enzyme 2 (ACE2), angiotensin-II (Ang II), and angiotensin (1–7) [Ang-(1–7)] were determined by ELISA.

Results: BD patients experiencing ongoing mood episodes had significantly lower ACE levels compared to controls (median: 459.00 vs. 514.10, $p < 0.05$). There was no association between the levels of these biomarkers and clinical parameters.

Conclusion: Our findings support the involvement of RAS dysfunction in the pathophysiology of BD. Considering the potential therapeutic implications linked to a better understanding of the role of RAS dysfunction in BD, studies allowing a better characterization of RAS-related molecules level and activity across different mood states are of high interest.

Keywords: bipolar disorder, mood disorders, depression, renin-angiotensin system, angiotensin II, angiotensin converting enzyme

INTRODUCTION

Bipolar disorder (BD) is a chronic and potentially severe mental illness that affects 2–5% of the American population (Merikangas et al., 2007). It is implicated in a high rate of morbidity and important functional impact (Kessler et al., 2006).

The pathophysiology of BD has not yet been completed elucidated but seems to involve multiple dimensions, including dysfunctions in the brain circuits associated with the processing of emotions,

neurodevelopmental disruptions, and systemic processes (Brambilla et al., 2008; Sanches et al., 2008; Goldstein et al., 2009; Barbosa et al., 2014; Sanches and Soares, 2016). More recently, it has been hypothesized that dysfunctions in the renin-angiotensin system (RAS) might play a role in the pathophysiology of BD (de Góis Queiroz et al., 2013; Mohite et al., 2020).

Systemically, the RAS plays a crucial role in blood pressure regulation and in the maintenance of homeostasis (Ito et al., 1995). The existence of a brain RAS is well-established, and angiotensin-II receptors can be found in different areas of the brain, including amygdala, hippocampus, and prefrontal cortex (Reinecke et al., 2018). Angiotensin II (Ang II) is produced through the conversion of Angiotensin I by the action of the angiotensin-converting enzyme (ACE). Abnormal levels of ACE in the cerebrospinal fluid (CSF) and plasma of patients with schizophrenia (Wahlbeck et al., 1998; Baskan et al., 2010; Mohite et al., 2018), have been previously reported (Mohite et al., 2018). These findings seem to be of particular importance, in light of the possible role of the RAS in regulating the inflammatory response and the large amount of evidence supporting the involvement of inflammation in the pathogenesis of different mental illnesses, such as major depressive disorder (MDD), schizophrenia, and BD (Bauer and Teixeira, 2019). Furthermore, in Alzheimer's disease, decreased ACE levels in plasma and CSF have been previously described (Jochemsen et al., 2014), and it has been hypothesized that these decreases are directly related to the accumulation of amyloid plaques and, ultimately, neuronal damage (Rocha et al., 2018b).

Specifically with respect to mood disorders, retrospective data point to lower levels of depressive and anxious symptoms among hypertensive patients receiving ACE inhibitors, as well as to lower rates of antidepressant use among patients with hypertension or diabetic nephropathy treated with ACE inhibitors or angiotensin receptor antagonists (Braszko et al., 2003; Rocha et al., 2018a). On the other hand, decreased ACE serum levels have been previously described among patients with MDD (Stelzhammer et al., 2014).

Considering the potential involvement of the RAS in the pathophysiology of BD and its potential therapeutic implications, we carried out a cross-sectional study analyzing the levels of RAS-related molecules- ACE, angiotensin-converting enzyme 2 (ACE2), angiotensin-II (Ang II), and angiotensin (1–7) [Ang-(1–7)]- among patients with BD compared to healthy controls (HC).

METHODS

Participants

The individuals who participated in the present study were recruited from the outpatient clinics of the Department of Psychiatry of the University of Texas Health Science Center at Houston, as well as through flyers placed on the community. Our sample was composed by 30 BD patients (10 males, 20 females, age = 35.53 $\pm$ 10.59 years) and 30 matched healthy controls (10 males, 20 females, age = 34.83 $\pm$ 11.49 years). For both groups, the established inclusion criteria were: age equal or superior to 18 years, no family history of hereditary neurological disorder, no neurological or major medical condition, no current substance abuse or dependence, and a negative urine drug screening. In addition, specifically in the case of healthy controls (HC), individuals with a positive family history of mental disorders in first-degree relatives were excluded.

The diagnosis of BD among patients and the absence of psychiatric disorders in HC was established through the administration of the Structured Clinical Interview for DSM-IV Axis I Disorders-SCID (First et al., 1996). All patients met DSM-IV criteria for BD type I. At the time of their inclusion in the study, 14 BD patients were euthymic, while eight were depressed, five were manic, one was hypomanic, and two met criteria for a mixed mood state. The participants' mood state were defined according to their answers to the SCID. Euthymic mood was defined as an absence of criteria for an acute mood episode at the time of the assessment, based on the SCID and, in addition, according to the scores of the MADRS and YMRS. The following cut offs were adopted: MADRS score of at least 7 (for the characterization of depressive mood) and YMRS of at least 12 (for mania/hypomania). Most patients (n = 26) were receiving one or more psychiatric medications at the time of their inclusion in the study (seven patients were on lithium, 16 on anticonvulsant mood stabilizers, 12 on antidepressants, and 17 on antipsychotics). There were no statistically significant differences between euthymic and non-euthymic patients with respect to medication status. This study was approved by the respective Institutional Review Board (HSC-MS-09-0340). Informed consent was obtained from all participants.

Measurement of the Levels of RAS-Related Molecules

Blood samples were collected in heparin-coated collection tubes and centrifuged twice at ∼20°C for 10 min, one at 1,800 rpm and the other one at 3,000 rpm. Plasma samples were stored at −80 °C for further processing. The levels of plasma ACE (catalog # MBS727096), ACE2 (catalog # MBS723213), Ang II (catalog # MBS764273), and Ang-(1–7) (catalog # MBS084052) were assessed using Enzyme-Linked Immunosorbent Assay (ELISA), according to the manufacturer's instructions (MyBioSource, Inc., SanDiego, CA, United States). A competitive ELISA method was used for ACE, while a sandwich ELISA procedure was used for ACE2, Ang II, and Ang-(1–7). Concentrations were measured in pg/mL (ACE, ACE2, and Ang II) and in ng/ml [Ang (1–7)]. The sensitivity of the assays was 1.0 pg/mL for ACE and ACE2, 18.75 pg/mL for Ang II, and 0.02 ng/mL for Ang (1–7). The analyses were performed blind to subject group (patients vs. controls).

Statistical Analysis

The statistical analyses were performed using IBM SPSS statistical software (IBM SPSS, version 19, Armonk, NY) and STATA (StataCorp, 2013). The sociodemographic features of the groups were compared using exact chi-square tests (for categorical variables) and the Student's t-test (for continuous variables). The levels of Angiotensin II, ACE, and other markers of RAS activity in patients and controls were compared using the Mann–Whitney U test. Among patients, we also looked into

TABLE 1 | Sociodemographic features and plasma levels of renin-angiotensin system (RAS)-related molecules in bipolar disorder patients (BD) and healthy controls.

	BD (n = 30)	HC (n = 30)	p
Gender (males/females)	10/20	10/20	1.00[a]
Age (year = mean ± SD)	35.53 ± 10.59	34.83 ± 11.49	0.81[b]
Education (years)	14.67 ± 1.88	15.10 ± 1.88	0.38[b]
Ethnicity (Hispanic/Non-Hispanic)	7/23	7/23	1.00[a]
SBP (mean ± SD)	123 ± 17	116 ± 19	0.18[b]
DBP (mean ± SD)	82 ± 12	78 ± 14	0.34[b]
RAS molecules (Median)			
ACE[d]	504.01	514.10	0.29[c]
ACE2[d]	18.82	15.91	0.71[c]
Ang II[d]	408.61	415.10	0.59[c]
Ang (1–7)[e]	0.30	0.25	0.24[c]

[a] *Chi-square test.*
[b] *Student's t-test.*
[c] *Mann-Whitney U test. SBP, systolic blood pressure in mmHg; DBP, diastolic blood pressure in mmHg; ACE, angiotensin-converting enzyme; ACE2, angiotensin-converting enzyme 2; Ang II, angiotensin-II; Ang-(1–7), angiotensin (1–7).*
[d] *Levels presented in pg/ml.*
[e] *Levels presented in ng/ml.*

possible correlations (using the Spearman correlation coefficient) between the levels of RAS-related molecules and mood-rating scales, namely the Montgomery–Åsberg Depression Rating Scale (MADRS) and the Young Mania Rating Scale (YMRS).

RESULTS

Both groups were matched according to age and sex, and did not differ with respect to other sociodemographic features (**Table 1**). No statistically significant differences between groups were observed with regards to the plasma levels of ACE, ACE2, Ang-(1–7), and Ang II (**Table 1**). However, a secondary analysis, including only non-euthymic BD patients and HC (**Figure 1**) revealed significantly lower ACE levels among patients compared to controls (median:459.00 vs. 514.10, $p < 0.05$). Differences regarding ACE2 (median: 20.73 vs. 15.91, p = 043), Ang II (median: 374.90 vs. 415.10, p = 0.35), and Ang-(1–7) [median: 0.34 vs. 0.25, p = 0.12) remained non-significant. Similarly, among bipolar patients, we found no statistically significant correlations between the plasma levels of the molecules of interest and the scores of the MADRS [ACE: r = 0.12, p = 0.53; ACE2: r = −0.01, p = 0.94; Ang II: r = −0.17, p = 0.37); Ang (1–7): r = 0.21, p = 0.26] or YMRS [ACE: r = −0.16, p = 0.40; ACE2: r = −0.14, p = 0.45; Ang II: r = 0.08, p = 0.69); Ang (1–7): r = 0.12, p = 0.59].

DISCUSSION

Our results indicate that patients with BD in non-euthymic state had lower plasma levels of ACE when compared to healthy controls. No statistically significant differences were observed with respect to the levels of ACE2, Ang II, and Ang-(1–7). While these results may be, at least in part, interpreted as evidence against the involvement of the RAS in the pathophysiology of BD, their putative pathophysiological significance needs to be carefully discussed.

The relationship between RAS activity and mental disorders seems to be complex, with evidence suggesting a close association between RAS, inflammation, and psychiatric disorders (Saavedra, 2012; Rocha et al., 2018a; Ren et al., 2019). It has been proposed that the RAS is composed by two arms, which have opposite actions in terms of inflammatory activity and effects on the pathophysiology of mental illnesses. The first arm, composed by ACE, Angiotensin II, and Angiotensin receptor type I (AT1), displays proinflammatory actions. Increments in this pathway are hypothesized to contribute to the development of mental disorders (Rocha et al., 2018a). On the other hand, the second arm, comprised of ACE2, Ang (1–7), and Angiotensin receptor type II (AT2) seems to have anti-inflammatory effects and a putatively protective effect against the development of neuropsychiatric disorders (Mohite et al., 2018). Nevertheless, there are inconsistent patterns of findings on the possible involvement of the RAS across different psychiatric conditions.

While part of these conflicting findings may be secondary to methodological issues, including the characteristics of the subjects, it is possible that these discrepancies are related to variations in the pathophysiological factors involved in different subtypes of mood disorders. For example, some patients with mood disorders seem to have a more prominent involvement of immune factors and stress-related hyperactivation of the HPA axis. The relationship between the RAS and HPA axis is well-described and seems to be bidirectional, with the ACE contributing to the activation of the HPA axis and, in contrast, elevated cortisol levels leading to compensatory decreases in ACE levels (Stelzhammer et al., 2014). Therefore, while our finding of decreased ACE levels in BD patients may sound counterintuitive, it may suggest that, in certain groups of psychiatric patients (including patients with mood disorders and schizophrenia), abnormalities in the RAS system may occur as a downstream consequence of increases in inflammatory and HPA activity. This possibility might also explain the fact that we found significantly decreases in ACE levels among non-euthymic patients but not in euthymic patients. In other words, according to this hypothesis, decreased ACE might represent a potential state biomarker for BD but not an endophenotype/marker of vulnerability for that condition.

Our study has some methodological limitations that need to be acknowledged. First, our small sample size may, in part, explains the lack of statistically significant differences between groups regarding other RAS-related molecules. Second, our sample included only outpatients, and it is unclear whether similar findings would be applicable to a sample of patients with more severe forms of the disease. Third, most of the bipolar patients in our sample were medicated, and literature data indicates that antipsychotics are associated with increases in the CSF levels of ACE (Wahlbeck et al., 1998). While it is uncertain whether other drugs, such as lithium, other mood stabilizers, and antidepressants have similar effect, this possibility exists. Finally, we measured ACE and ACE 2 levels but not enzyme activity.

Moreover, RAS abnormalities may be involved in the pathophysiology of other mental disorders, whose symptoms

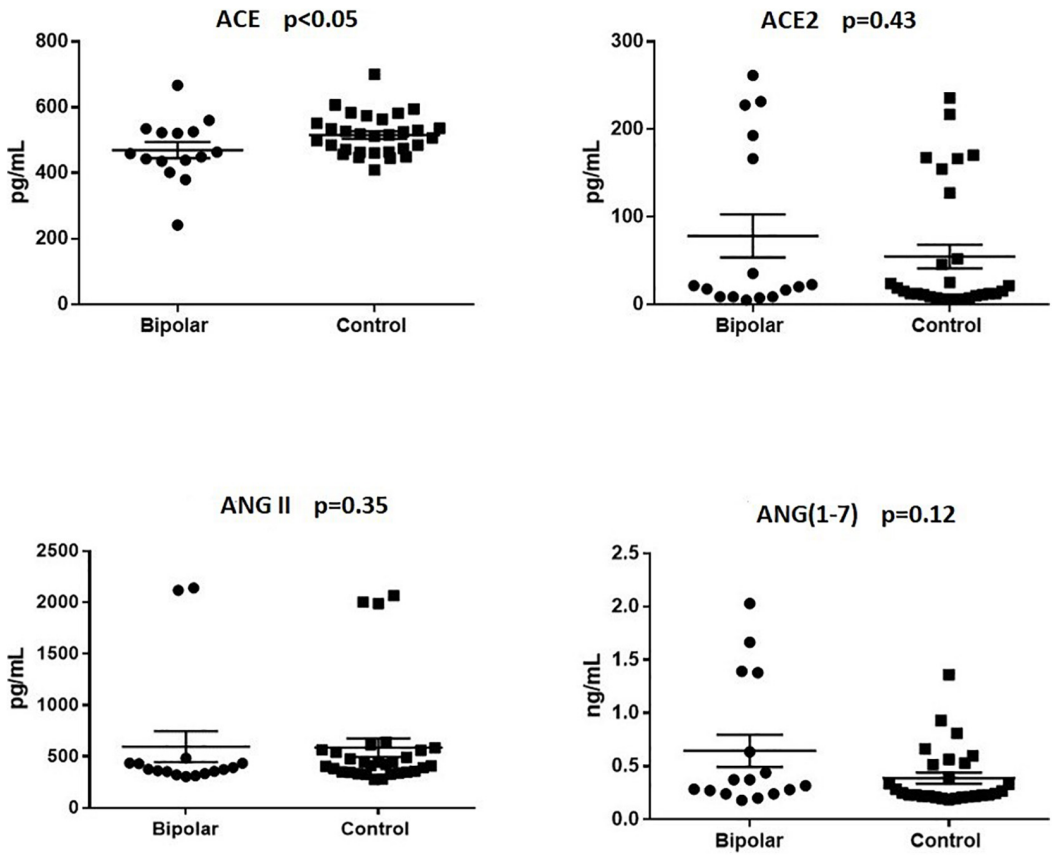

FIGURE 1 | Plasma levels of renin-angiotensin system (RAS)-related molecules in non-euthymic bipolar patients and healthy controls. Non-euthymic bipolar disorder patients (n = 16) presented with significantly lower plasma levels of angiotensin-converting enzyme (ACE) than healthy controls (n = 30). Differences regarding the levels of angiotensin-converting enzyme 2 (ACE2), angiotensin-II (Ang II), and angiotensin (1–7) [Ang-(1–7)] were not statistically significant.

might potentially overlap with the ones from BD. In spontaneous hypertensive rats, which show behavioral features correlated with hyperactivity and impulsivity and have been proposed as an animal model for attention-deficit disorder (ADHD) (Cho et al., 2014; Natsheh and Shiflett, 2018), associations between RAS and HPA activity have previously been described (Raasch et al., 2006). These finds suggest that RAS dysfunctions might play a role in the pathogenesis of ADHD. Given the commonly observed challenges involved in the differentiation between BD and ADHD due to phenotypical overlap between both conditions, one could hypothesize that some of our negative findings are related to the inadvertent inclusion of ADHD patients in our sample. While the possibility in question cannot be completely ruled out, it is unlikely that was the case, as the diagnosis of BD was confirmed by a structured psychiatric interview (SCID). Furthermore, we recruited only BD I patients, whose higher severity in terms of lifetime psychopathological features allows for a more clear distinction between BD and ADHD.

One last methodological limitation of our study is related to potential concerns about the accuracy and specificity of commercial ELISAs for the measurement of Ang II, and Ang-(1–7), which can produce results at range values distinct from those obtained through other approaches (Chappell et al., 2021). That issue may create difficulties in the comparison of our results with the ones obtained by other groups. Our findings should be considered preliminary and must be confirmed and validated by future studies utilizing different methods.

In summary, our results raise some hypothesis about the potential involvement of RAS dysfunction in the pathophysiology of BD, particularly as a putative marker of activity of that illness. The molecular mechanisms underlying this involvement, as well as its possible modulation by clinical factors such as mood state, illness severity, and treatment status among patient with BD, correspond to a promising and relatively unexplored area of study. Given the potential therapeutic implications linked to a better understanding of the role of RAS dysfunction in BD, studies with larger samples and longitudinal designs, allowing the measurement of RAS-related molecules level and activity across different mood states are of high interest.

DATA AVAILABILITY STATEMENT

The datasets for this article are not publicly available. Requests to access the datasets should be directed to MS.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the CPHS-UT Health Science Center at Houston. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

MS participated in the research design, data collection and analysis, data interpretation and in the drafting, revision, and approval of the final manuscript. GC participated in the data analysis and interpretation and in the drafting, revision, and approval of the final manuscript. VC and TB participated in the data collection and in the revision and the drafting, revision, and approval of the final manuscript. DR participated in the drafting, revision, and approval of the final manuscript. JS participated in the research design, data interpretation, and in the drafting, revision, and approval of the final manuscript. AT participated in the research design, data analysis and interpretation, and in the drafting, revision, and approval of the final manuscript. All authors contributed to the article and approved the submitted version.

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Transcriptomic Analysis of Mouse Brain After Traumatic Brain Injury Reveals That the Angiotensin Receptor Blocker Candesartan Acts Through Novel Pathways

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Traumatic brain injury (TBI) results in complex pathological reactions, where the initial lesion is followed by secondary inflammation and edema. Our laboratory and others have reported that angiotensin receptor blockers (ARBs) have efficacy in improving recovery from traumatic brain injury in mice. Treatment of mice with a subhypotensive dose of the ARB candesartan results in improved functional recovery, and reduced pathology (lesion volume, inflammation and gliosis). In order to gain a better understanding of the molecular mechanisms through which candesartan improves recovery after controlled cortical impact injury (CCI), we performed transcriptomic profiling on brain regions after injury and drug treatment. We examined RNA expression in the ipsilateral hippocampus, thalamus and hypothalamus at 3 or 29 days post injury (dpi) treated with either candesartan (0.1 mg/kg) or vehicle. RNA was isolated and analyzed by bulk mRNA-seq. Gene expression in injured and/or candesartan treated brain region was compared to that in sham vehicle treated mice in the same brain region to identify genes that were differentially expressed (DEGs) between groups. The most DEGs were expressed in the hippocampus at 3 dpi, and the number of DEGs reduced with distance and time from the lesion. Among pathways that were differentially expressed at 3 dpi after CCI, candesartan treatment altered genes involved in angiogenesis, interferon signaling, extracellular matrix regulation including integrins and chromosome maintenance and DNA replication. At 29 dpi, candesartan treatment reduced the expression of genes involved in the inflammatory response. Some changes in gene expression were confirmed in a separate cohort of animals by qPCR. Fewer DEGs were found in the thalamus, and only one in the hypothalamus at 3 dpi. Additionally, in the hippocampi of sham injured mice, 3 days of candesartan treatment led to the differential expression of 384 genes showing that candesartan in the absence

of injury had a powerful impact on gene expression specifically in the hippocampus. Our results suggest that candesartan has broad actions in the brain after injury and affects different processes at acute and chronic times after injury. These data should assist in elucidating the beneficial effect of candesartan on recovery from TBI.

Keywords: traumatic brain injury, angiotensin, RNA seq, hippocampus, candesartan, transcriptomic (RNA-Seq)

INTRODUCTION

Traumatic brain injury (TBI) can result in permanent difficulties with sleep, concentration, memory and mood, even from a seemingly minor injury (Katz et al., 2015; Cole and Bailie, 2016; Giza et al., 2018; Paredes et al., 2020). Morbidity increases with the severity of injury (LoBue et al., 2019; Svingos et al., 2019). Damage from the initial impact, the primary injury, occurs even without external signs, or abnormal CT scans in patients (Schweitzer et al., 2019; Lefevre-Dognin et al., 2020). However, the primary lesion may cause significant internal injuries including axonal shearing and blood brain barrier damage (Laskowski et al., 2015; Ichkova et al., 2017). This injury also initiates secondary cascades of inflammation, oxidative stress, apoptosis and excitotoxicity that result in glial reactivity, further axonal damage, activation of the innate and acquired immune systems, dysfunction of neuronal circuitry, and interruption of cerebrovascular flow (Ling et al., 2015; Ichkova et al., 2017; Sullan et al., 2018; Sta Maria et al., 2019). These secondary cascades worsen the initial impact and provide a therapeutic window to intervene in order to reduce pathology and improve recovery (Diaz-Arrastia et al., 2014). However, despite many years of research, and over 30 clinical trials, there is no FDA approved therapy to treat TBI (Armstead and Vavilala, 2020; Figaji et al., 2017; Xiong et al., 2015). TBI impacts all cell types in the area of injury, so therapies that are broadly acting may have greater potential to succeed.

The renin angiotensin system (RAS) is predominantly recognized as a major regulator of systemic blood pressure and fluid homeostasis (Miller and Arnold, 2019; Mirabito Colafella et al., 2019). However, the brain expresses all components of the RAS, and it is not acknowledged that the brain RAS influences many aspects of brain function that are independent of the regulation of systemic blood pressure, including the stress response, limbic function, sensory responses, regulation of cerebral circulation and sympathetic activity (Wright and Harding, 2004; Krause et al., 2011; Bali and Jaggi, 2013; Kangussu et al., 2013; Marvar et al., 2014; Hurt et al., 2015; Wang et al., 2016a; Miller and Arnold, 2019; Yu et al., 2019). Angiotensin II, acting through the AT1 Receptor in neurons, astrocytes, microglia and endothelial cells, stimulates oxidative stress, inflammatory signaling, apoptosis and vasoconstriction (Saavedra, 2005; Saavedra et al., 2006; Pavel et al., 2008; Benicky et al., 2009; Villapol and Saavedra, 2015). As there are many similarities between some of the cascades activated after traumatic brain injury, and those initiated by AT1R signaling, it was proposed that AT1R signaling in different cell types could contribute to some of the adverse reactions after TBI (Thone-Reineke et al., 2006; Timaru-Kast et al., 2012). In support of this, mice that lack the AT1R have a smaller lesion after controlled cortical impact injury (Villapol et al., 2015). Further, drugs that block signaling through the AT1R can improve recovery after TBI in the mouse (Timaru-Kast et al., 2012; Villapol et al., 2012; Villapol et al., 2015; Janatpour and Symes, 2020). These drugs, called angiotensin receptor blockers (ARBs) have also been shown to be neuroprotective in rodent models of other disorders of the CNS including Parkinson's disease, Alzheimer's disease, stroke, cerebral hemorrhage and cerebral edema initiated by systemic inflammation (Kehoe, 2009; Villapol and Saavedra, 2015; Saavedra, 2017). Clinical trials have shown reduced incidence of stroke in patients on ARBs (Thone-Reineke et al., 2006; Sandset et al., 2010; Regenhardt et al., 2014). ARBs have also been shown to reduce the progression from mild cognitive impairment towards Alzheimer's disease, and to reduce the incidence of Alzheimer's disease in patients on long term ARB therapy for hypertension (Davies et al., 2011; Zhuang et al., 2016; Ho and Nation, 2017; Kehoe, 2018). This seems to be distinct from the general protective effects of controlling hypertension (Saavedra, 2016). Further, retrospective studies have shown that ARB therapy leads to lower symptoms of post-traumatic stress disorder (PTSD), and reduced markers for depression in specific populations (Khoury et al., 2012; Boal et al., 2016; Hurt et al., 2015). Patients with TBI often have overlapping symptoms with PTSD, including depression and sleep disruptions (Stein and McAllister, 2009; Howlett and Stein, 2016), and TBI increases the overall risk of Alzheimer's disease (Chauhan, 2014; Crane et al., 2016; LoBue et al., 2019). Thus, treatment of TBI with ARBs has the potential to reduce both the acute and chronic consequences of the injury.

Many of the neuroprotective effects of ARBs have been attributed to their anti-inflammatory effects in CNS tissues acting through both AT1R dependent and independent mechanisms (Benicky et al., 2011; Saavedra, 2011; Villapol et al., 2015). A major AT1R independent effect of ARBs is the ability of some specific ARBs to act as PPARγ partial agonists (Schupp et al., 2004; Miura et al., 2011). Telmisartan, candesartan, losartan and irbesartan have this dual ability to differing effects (Benson et al., 2004; Schupp et al., 2004; An et al., 2010). Stimulating PPARγ in addition to AT1R antagonism provides a dual mechanism to act in an anti-inflammatory manner and broadens the range of cells that ARBs will reach to improve recovery. Indeed, PPARγ activity is prominent in microglia and oligodendrocytes whereas there is some dispute about AT1R signaling in these cells *in vivo* (Bernardo and Minghetti, 2006; Kapadia et al., 2008; McCarthy et al., 2013; Wu et al., 2013; Xu et al., 2015). Additionally, some ARBs also can activate AMPK signaling within microglia, and potentially have other effects on different signaling pathways (Xu et al., 2015). We have previously shown that the ARB,

candesartan, can improve behavioral and pathologic recovery after controlled cortical impact injury in mice (Villapol et al., 2012, 2015). Candesartan, administered 6 hours after injury, increases cognitive function four weeks after injury, reduces lesion volume, and reduces astrocyte and microglial activation (Villapol et al., 2012, 2015).

In order to understand better the mechanisms through which candesartan acts after TBI, we have performed RNA seq analysis on different brain regions at 3 and 29 days post injury in order to determine how candesartan treatment alters the response to injury. We examined gene expression in the hippocampus, the region immediately under the lesion, the thalamus where the cortical thalamic neurons have their cell bodies and the hypothalamus, the site of the most dense expression of AT1R in the brain (Lenkei et al., 1997). We used a low subhyptotensive dose of candesartan, that we and others have previously shown to enhance recovery in this mouse model of TBI (Timaru-Kast et al., 2012; Villapol et al., 2012, 2015). We found that the clearest gene expression differences were found between different brain regions independent of injury status. Injury had stronger effects at a more acute time point [3 days post injury (dpi)], and the effects of candesartan were more muted. Nevertheless, we were able to determine that candesartan influenced numerous pathways after injury in mice at both 3 and 29 dpi, with the largest effects found in the hippocampus. Understanding the molecular mechanisms of these treatments in animal models may assist in determining pathways that are critical to drug efficacy and provide biomarkers of drug target engagement.

MATERIALS AND METHODS

Animals

All animal studies were approved by the Uniformed Services University of the Health Sciences (USUHS) Institutional Animal Care and Use Committee and were conducted in accordance with the NRC guide to the Care and Use of Laboratory Animals. Adult male age-matched (8-10 week old) C57BL/6 mice weighing 20–25g were obtained from Charles River Laboratories (Frederick, MD, United States). All mice were kept under 12:12 h light and dark cycle with access to food and water *ad libitum*. Five mice were housed in each cage. After arrival, mice were acclimatized for one week before use. Animals were randomly assigned to receive a controlled cortical impact (CCI) injury or sham injury as well as treatment with candesartan or vehicle.

Controlled Cortical Impact Injury

Animals in the injury group were given a moderate brain injury utilizing a CCI. The CCI was performed on the animals as previously described (Villapol et al., 2012). In brief, animals were anesthetized with isoflurane (3% induction, 2% maintenance) and placed into a stereotactic mount. The animal's scalp was shaved and the head secured with ear bars. A 3 mm craniectomy was made (2 mm lateral (left), 2 mm caudal to bregma) over the location of the impact site. A pneumatic impactor (Impact One stereotaxic impactor Leica Microsystems, Buffalo Grove, IL, United States) with a 2mm rounded impact tip was used to deliver the CCI (3.6 m/s, 1.5 mm depth, 100 ms dwell time, 12° angle to the dura mater). The scalp was then sutured closed and the animal allowed to recover prior to placing back in the cage. Sham animals received the same exposure to anesthesia and scalp excision, but without a craniectomy or injury.

Drug Treatment

Candesartan can cross the blood brain barrier (Nishimura et al., 2000) so we administered it peripherally either through intraperitoneal (i.p.) injection (3 day cohort), or osmotic minipump (29 day cohort). Candesartan was resuspended in 0.1N Na_2CO_3, pH 7.4, and administered at 0.1 mg/kg/day. All mice received their first dose of either candesartan (Tocris Bioscience, Minneapolis, MN, United States # 4791) or vehicle (0.9% saline and 0.1N Na_2CO_3 at pH = 7.4) six hours after the CCI or sham procedure through i.p. injection. Mice in the 3 day cohort received two subsequent i.p. injections at 24- and 48-h after CCI, before sacrifice at 3 dpi. Mice in the 29-day cohort were surgically implanted with an osmotic pump (#1004, Alzet, Cupertino, CA, United States) 24 h after the CCI or sham procedure. Pumps were placed in the lower back through a tunneled incision at the base of the neck under isoflurane anesthesia. Prior to implantation the osmotic pumps were primed with candesartan or vehicle at 37°C overnight.

Brain Region Isolation and RNA Extraction

At either 3 or 29 dpi, mice were anesthetized with ketamine and xylazine and perfused with ice-cold filtered 0.9% saline solution and decapitated. Brains were removed and individual brain regions dissected. Tissue was placed into TRI reagent (Zymo Scientific, Tustin, CA, United States # R2050-1-50) and triturated through a 22G needle followed by a 25G needle. RNA was extracted utilizing the Direct-zol RNA MiniPrep kits (Zymo Research, # R2025) according to the manufacturer's instructions. RNA was quantified by UV spectrometry with a NanoDrop^{TM} UV-Vis Spectrophotometer (Thermo Scientific, United States). RNA quality for the RNA sequencing was assessed using the BioRad Experion Automated Electrophoresis System (BioRad, Hercules, CA, United States). RNA quality for qPCR was checked by agarose gel electrophoresis.

RNA Sequencing

The four highest quality RNA samples per group were identified, aliquoted, given unique identifiers, and sent to the Collaborative Health Initiative Research Program (CHIRP) American Genome Center at USUHS for processing. Total RNA integrity was assessed using automated capillary electrophoresis on a Fragment Analyzer (Roche). For all samples RQI > 8.0, a total RNA amount of > 75 ng was used as input for library preparation using the TruSeq Stranded mRNA Library Preparation Kit (Illumina, San Diego, CA, United States). Sequencing libraries were quantified by PCR using KAPA Library Quantification Kit for NGS (Kapa, Wilmington, MA, United States) and assessed for size distribution on a Fragment Analyzer. Sequencing libraries were pooled and sequenced on a HiSeq 3000 System (Illumina) using a

HiSeq 3000/4000 PE Cluster Kit and SBS Kit (150 cycles) with run conditions of paired-end reads at 75 bp length. Raw sequencing data were demuxed using bcl2fastq2 conversion software 2.20.

Bioinformatics Analysis

RNA-seq samples were aligned to the mouse genome (mm10) using MapSplice (v. 2.2.1), expression quantification of individual genes was performed using HTSeq (v. 0.9.1), and differential gene expression was performed using DESeq2 (v 1.16.1). Individual groups were compared to sham mice and differentially expressed genes (DEGs) were identified with a false discovery rate of 0.05 and an absolute log2 fold-change (abs log2 FC) > 0.32.

Unique Gene ID and Gene Ontology Analysis

Differentially expressed genes that were unique to either the TBI-candesartan or the TBI-vehicle group at each time point within the hippocampal data were identified for both up-regulated and down-regulated DEGs. Gene Ontology (GO) analysis was performed on unique up- and down-regulated DEGs altered by candesartan and vehicle after TBI at 3 dpi and 29 dpi in the hippocampus, using the PANTHER Overrepresentation Test (Release 20190429) with the Mus musculus GO database (Released 2020-06-01[1]). GO biological process terms were identified using a Fisher's Exact test and a false discovery rate of 0.05 (Ashburner et al., 2000; Mi et al., 2017).

Pathway Enrichment Analysis

Pathway enrichment analysis was performed on unique up- and down-regulated DEGs altered by candesartan and vehicle after TBI at 3 dpi in the hippocampus, using Molecular Signatures Database v7.1 (MSigDB v7.1[2]) (Subramanian et al., 2005; Liberzon et al., 2015). DEGs were converted to Mouse Ensemble IDs (species: Mus musculus), which were used as the input gene list. The analyses included the selected canonical pathway databases: BIOCARTA[3], KEGG[4], PID[5], and REACTOME[6]. Pathways were identified using a Fisher's exact test with a false discovery rate of less than 0.05. A minimum gene set size of 5, and a maximum gene set size of 350 were used as additional filters.

Functional Interaction Network

A functional interaction network was created using Cytoscape (v3.8.0) with the ReactomeFIViz plugin (v.7.2.3) (Shannon et al., 2003; Wu and Haw, 2017). To create the network, the up-regulated DEGs in the TBI candesartan group at 3 dpi in the hippocampus were used as input for the gene set analysis feature (Reactome FI Network Version 2019). Unlinked genes were not included in the analysis. The network was then clustered into functional modules and were further analyzed for pathway enrichment using a false discovery rate of less than 0.05.

qPCR

cDNA was synthesized using SuperScript III (Life Technologies) and qPCR performed with PerFecTa SYBR Green mastermix (QuantaBio, Beverly, MA, United States) in a CFX96 Thermocycler (Bio-Rad). The following primers were used: Vim - forward 5′-GCCAGATGCGTGAGATGGA-3′, reverse 5′-GGC GATCTCAATGTCCAGGG-3′; Lyz2 (lysozyme C-2 precursor) - forward 5′-AGCACTGTACCCCACCATTT-3′, reverse 5′-CTT TTCCTTCTCAGGGGTGTG-3′, Gpnmb (glycoprotein NMB) - forward 5′-CTGCTTTAAAGACCCAGACTCC-3′, reverse 5′-ACTTACTTGTACAGCAAGATGGTAA-3′; C4b (complement C4B) - forward 5′-ACTTACTTGTACAGCAAGATGGTAA-3′, reverse 5′-ACACTGTGCTCTGGAGATGT-3′, Ywhaz forward 5′-CTTTCTGGTTGCGAAGCATT-3′, reverse 5′-TTGAGCAG AAGACGGAAGGT-3′; Rpl13 forward 5′-CTTTTCCCAG ACGAGGATATTCC-3′, reverse 5′-CCAGCCGTTTAGGCA CTCT-3′. Target gene expression was normalized to the housekeeping genes Rpl13 or Ywhaz using the delta threshold cycle ($\Delta\Delta$Ct) method (Susarla et al., 2011) and analyzed with Bio-Rad CFX Manager 2.0 software.

Statistical Analysis for qPCR

All data are expressed as mean $\pm$ SEM. Statistical analysis was performed utilizing Prism software (version 8.1). Relative gene expression determined by qPCR was compared between sham and injured groups, and between vehicle and drug treated groups using a two-way ANOVA with Holm's Sidak multiple comparisons correction. $P < 0.05$ was considered statistically significant.

RESULTS

We determined gene expression in three different brain regions of mice at either 3 days or 29 days after controlled cortical impact or sham injury. Mice were treated either with candesartan (0.1 mg/kg/day) or vehicle, starting 6 hours after injury (**Figure 1**). Gene expression was determined in the ipsilateral hippocampus, thalamus or hypothalamus by bulk mRNA seq analysis.

Semi-Supervised Hierarchical Clustering and Principal Component Analysis

After quality analysis of samples, the genes were mapped utilizing semi-supervised hierarchical cluster according to the median absolute deviation (MAD) of the gene transcripts per million (TPM) (**Figure 2**) for both 3 and 29 dpi cohorts. The hierarchical clustering showed the greatest differentiation among the samples by brain region at either time point. The second greatest differentiation in clustering occurred only within the hippocampus in the 3 dpi samples and showed differentiation between RNA taken from TBI and Sham mouse brains. We did not observe defined clustering according to candesartan treatment in either time point or

[1]http://www.GeneOntology.org
[2]http://www.gsea-msigdb.org
[3]http://www.biocarta.com
[4]http://www.genome.ad.jp
[5]http://www.ndexbio.org
[6]http://www.reactome.org

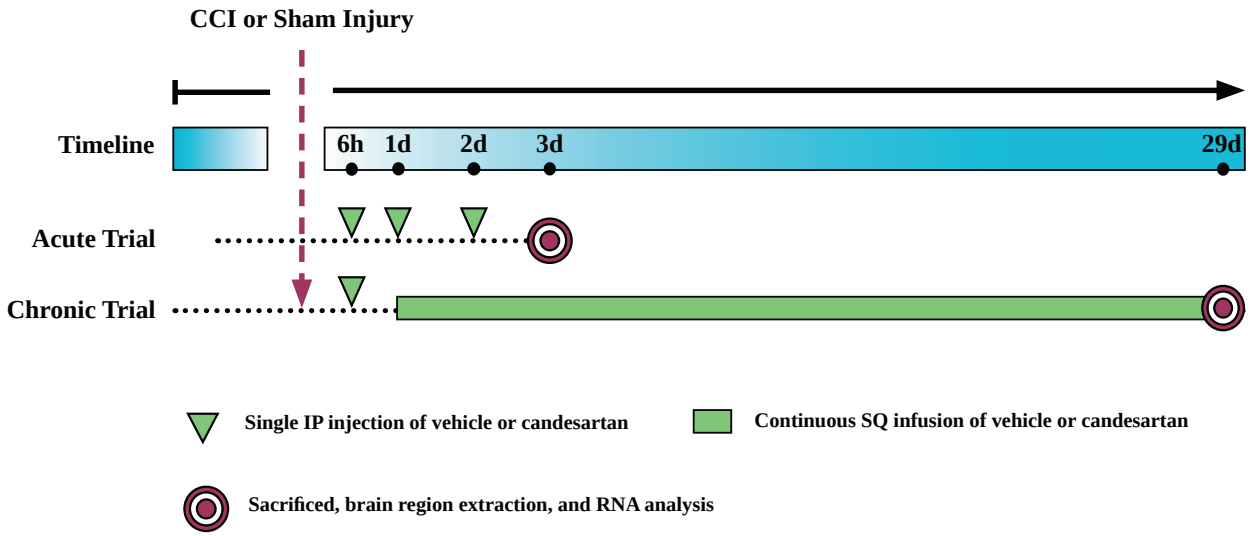

FIGURE 1 | Experimental Design. Mice were subjected to controlled cortical impact (CCI) or sham injury and sacrificed at either 3 days post-injury (dpi) (acute) or 29 dpi (chronic). Mice in the acute trial received three 0.1 mg/kg intraperitoneal (IP) injections of candesartan or vehicle at 6, 24, and 48 h post-injury. Mice in the chronic trial received an initial dose of 0.1 mg/kg candesartan or vehicle at 6 h post-injury and then at 1 dpi, were implanted with osmotic pumps with continuous SQ infusion of candesartan or vehicle (0.1 mg/kg/day) until sacrifice. Brains were removed and the hippocampus, thalamus, and hypothalamus dissected, and RNA isolated from these regions.

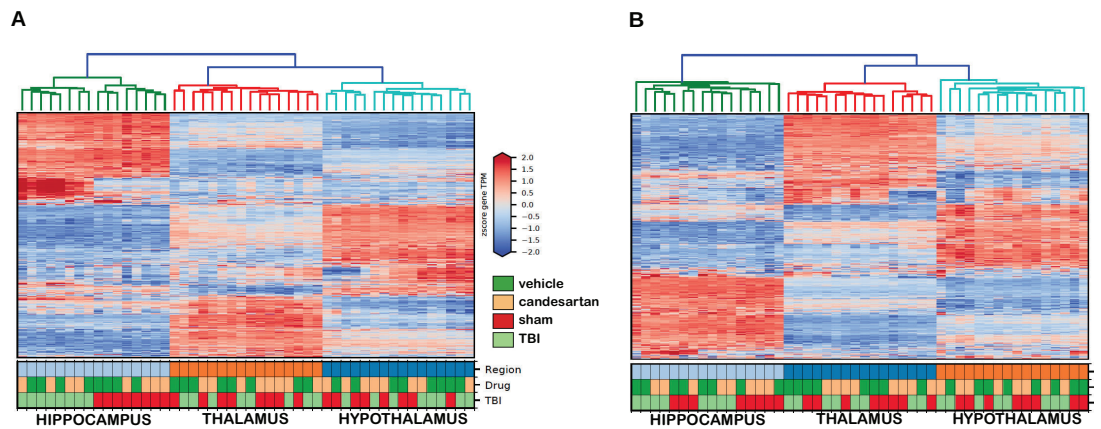

FIGURE 2 | Semi-Supervised Hierarchical Clustering of Genes at 3 and 29 dpi. The top 5,000 genes according to median absolute deviation (MAD) of the gene transcripts per million (TPM) across all samples underwent semi-supervised hierarchical clustering (*n* = 4/group). Samples clustered primarily by brain region at 3 dpi **(A)** and at 29 dpi **(B)**.

in any brain region. These observations were confirmed by principal component analysis (PCA) (**Figure 3**). The largest observed variation was between samples taken from the different brain regions regardless of injury status or candesartan treatment at either time point (**Figures 3A,B**). The second largest variation was observed between the samples taken from the hippocampus at 3 dpi after TBI and sham injury. This difference had almost disappeared at 29 dpi in the hippocampal samples and was not detectable in the samples taken from the thalamus or hypothalamus at either 3 or 29 dpi.

Differential Gene Expression

Differential gene expression analysis was performed within each brain region at each time point, comparing the gene expression after TBI +/− candesartan treatment to that in the sham injury

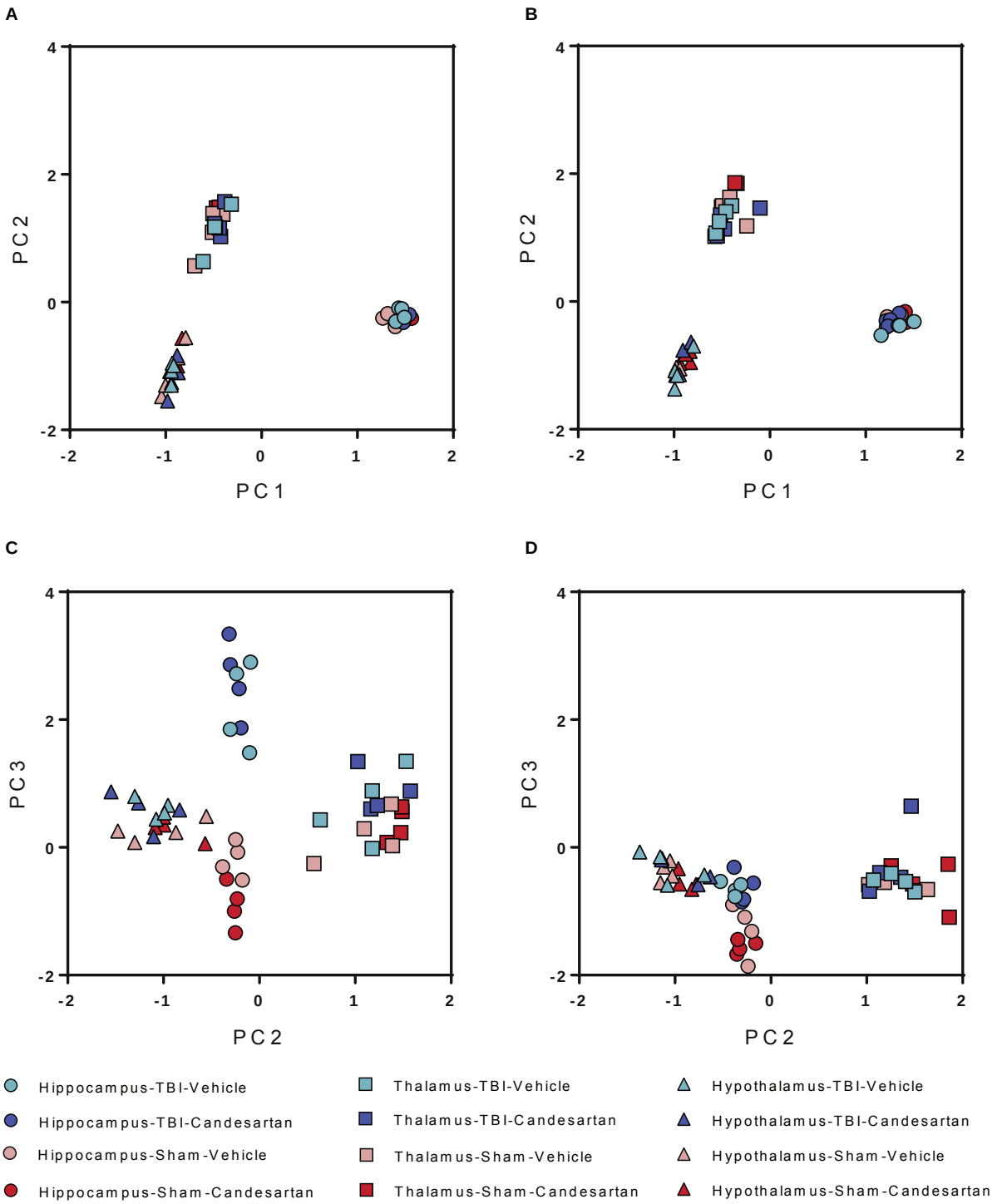

FIGURE 3 | Principal Component Analysis (PCA) of Differentially Expressed Genes at 3 and 29 dpi. PCA analysis shows the largest differences between brain regions at both **(A)** 3 dpi and **(B)** 29 dpi for all conditions. Differences are also seen between TBI and sham in the hippocampus at **(C)** 3 dpi but not at **(D)** 29 dpi (n = 4/group).

and vehicle treated group (Sham + VH). As the intent of the project was to identify changes in gene expression due to candesartan treatment, we chose to identify DEGs with a log2 > 0.32 as larger cut offs were associated only with changes caused by TBI. The number of differentially expressed genes (DEGs) for each condition are listed in **Table 1**.

TABLE 1 | Total Differentially Expressed Genes.

3DPI

	Hippocampus		Thalamus		Hypothalamus	
	Sham	TBI	Sham	TBI	Sham	TBI
VH		1540		56		0
CD	384	1732	3	66	0	1

29DPI

	Hippocampus		Thalamus		Hypothalamus	
	Sham	TBI	Sham	TBI	Sham	TBI
VH		89		0		2
CD	1	40	0	5	1	1

Total number of differential expressed genes found in the hippocampus, thalamus, and hypothalamus after TBI +/− candesartan (CD) treatment in comparison with genes expressed in those regions in sham + vehicle (VH) mice at 3 and 29 dpi. Differentially expressed genes (DEGs) were identified with a false discovery rate of 0.05 and an absolute log2 fold change > 0.32. VH, vehicle; CD, candesartan.

The largest number of DEGs were found in the hippocampus at 3 dpi. This region is closest to the cortical lesion and therefore the most altered by the injury. At 3 dpi, the hippocampus showed a large effect in both TBI groups with 1732 DEGs identified in the TBI with candesartan treated group (TBI + CD) and 1540 DEGs identified in the TBI and vehicle treated group (TBI + VH) (**Supplementary Table 1**). A TBI effect was identifiable within the thalamus but fewer DEGs were noted compared to those in the hippocampus. This included 56 DEGs in the TBI + VH group and 66 DEGs with candesartan treatment (TBI + CD) (**Supplementary Table 1**). At 29 dpi, the largest number of DEGs was again observed in the hippocampus. However, their number was far smaller than at 3 dpi with 89 DEGs in vehicle alone (TBI + VH) and 40 DEGs with candesartan treatment (TBI + CD). The effect of TBI in the thalamus at 29 dpi had nearly completely dissipated and the hypothalamus showed little effect at either 3 or 29 dpi.

Interestingly, at 3 dpi, there were a large number of DEGs identified in the hippocampus of mice after candesartan treatment in the sham group (Sham + CD) compared to the Sham + VH group. A total of 384 genes were differentially expressed in response to candesartan treatment in the sham mice at this time point. 358 DEGs were down-regulated whereas only 26 were up-regulated (**Supplementary Table 1**). GO analysis of the down-regulated unique DEGs identified common GO terms related to cilium organization, cilium assembly, and cell adhesion (**Supplementary Table 2**). GO analysis within the up-regulated unique DEGs identified GO terms associated with voltage gated cation channel activity (**Supplementary Table 3**). The thalamus and the hypothalamus had few DEGs with candesartan treatment in sham mice. By 29 dpi there was only 1 DEG identified in the hippocampus and hypothalamus with candesartan treatment and sham injury.

Unique Gene Analysis

A direct statistical comparison between the transcriptomes of the TBI + CD and the TBI + VH mouse hippocampi only produced one differentially expressed gene (Kcnh7), presumably because of the large effect of TBI on gene expression. Therefore, to identify DEGs that were only found in either the TBI + CD or the TBI + VH group, we carried out a unique gene analysis and performed GO analysis on these "unique DEGs." Analysis was focused on the DEGs found in the hippocampus as this was the brain region with the most changes in gene expression. DEGs that were shared in both conditions were attributed to a common TBI effect (1166 DEGs). The remaining genes were determined to be the unique up- and down-regulated genes within that condition (**Figure 4**).

Analysis of the DEGs that were up-regulated relative to Sham + VH at 3 dpi in the hippocampus, showed that there were 281 DEGs unique to the TBI + CD group, and 102 DEGs unique to the TBI + VH group (**Figure 4**). Gene Ontology (GO) analysis of the unique up-regulated genes in the TBI + CD group identified GO terms associated with the regulation of stress, stress responses and wounding, as well as cell adhesion (**Table 2**). GO analysis of unique up-regulated genes in the TBI + VH group identified no GO terms.

Unique gene analysis of DEGs that were down-regulated relative to Sham + VH showed that there were 152 DEGs unique to the TBI + CD group and 138 downregulated DEGs unique to the TBI + VH group (**Figure 4** and **Supplementary Table 4**). GO analysis of the unique down-regulated DEGs in the TBI + CD group identified GO terms associated with cellular structure, in particular related to cilium organization, and assembly and movement. These GO categories were also identified in the down-regulated DEGs in the hippocampus of the Sham + CD mice (**Supplementary Table 2**), showing similar mechanisms of candesartan action in sham or TBI mice. In comparison, unique down-regulated DEGs in the TBI + VH group showed a weak association with GO terms associated with anion transport and reproductive processes.

At 29 dpi in the hippocampus the number of DEGs identified was much reduced compared to those at 3 dpi (**Figure 4**). A majority of those were unique up-regulated DEGs within the TBI + VH group (**Supplementary Table 4**). All other groups had relatively few DEGs altered and no GO terms identified. GO analysis of the unique up-regulated DEGs within the vehicle group identified GO terms associated with responses to stress, as well as immune processes (**Table 3**).

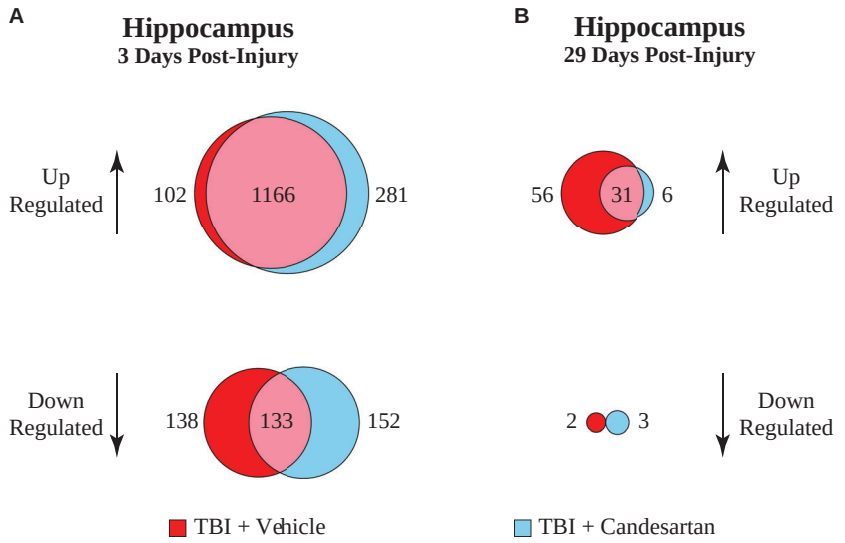

FIGURE 4 | Unique gene analysis in the hippocampus at 3 and 29 dpi. Differentially expressed genes (DEGs) that were either upregulated or down-regulated after TBI relative to those in the sham + vehicle group, were compared between vehicle and candesartan treatments at **(A)** 3 and **(B)** 29 dpi in the hippocampus. DEGs were identified with a false discovery rate of 0.05 and an absolute log2 fold-change (abs log2 FC) > 0.32.

Pathway Analysis

Out of 281 up-regulated DEGs in the TBI + CD group at 3 dpi in the hippocampus, 261 genes were mapped and 90 significant pathways were identified (FDR < 0.05). The top 20 enriched pathways are presented (**Table 4**). The enriched pathways were largely involved in the following: (1). DNA replication (chromosome maintenance, lagging strand synthesis, telomere c-strand synthesis, and polymerase switching), (2). extracellular matrix organization (integrin-1 pathway, integrin cell surface interaction, focal adhesions, and integrins in angiogenesis), (3). platelet activation, signaling, and aggregation (response to elevated platelet cytosolic Ca2+), and (4). interferon signaling (interferon alpha/beta signaling). Other pathways such as signaling by moderate kinase activity BRAF mutants and MAP2K and MAPK activation, were also enriched. Out of 152 down-regulated DEGs in the TBI + CD group at 3 dpi in the hippocampus, 139 genes were mapped and no significant pathways were enriched. Out of 102 up-regulated DEGs in the TBI + VH group at 3 dpi in the hippocampus, 97 genes were mapped and no significant pathways were enriched. Out of 139 down-regulated DEGs in the TBI + VH group at 3 dpi in the hippocampus, 131 genes were mapped and 20 significant pathways were identified (FDR < 0.05) (**Table 5**). The pathways enriched in this group were largely involved in extracellular matrix organization. These pathways include collagen degradation, collagen biosynthesis and modifying enzyme, assembly of collagen fibrils and chain trimerization, laminin interactions, integrin cell surface interaction, and integrins in angiogenesis. Other pathways enriched include response to elevated platelet cytosolic Ca^{2+}, SLC-mediated transmembrane transport, cargo concentration in ER, transport of bile salts, organic acids, metal ions and amine compounds, and retinoid cycle disease events. There were three pathways in common between the up-regulated DEGs in the TBI + CD group and down-regulated DEGs in the TBI + VH groups: extracellular matrix organization, integrins in angiogenesis, and response to elevated platelet cytosolic Ca^{2+}.

Functional Interactive Network

A functional interactive network was created using an input of the 261 mapped DEGs that were uniquely up-regulated in the TBI + CD group at 3 dpi (**Figure 5**). The network included 71 nodes and 108 edges. Unlinked genes were not included in the network. The network was clustered into 15 functional modules. The genes with the highest degrees, included ACTG1 (Degree = 14), PCNA (Degree = 12), VCL (Degree = 10), ITGA1 (Degree = 9), FN1 (Degree = 8), RFC3 (Degree = 7), COL3A1 (Degree = 7), and FLNA (Degree = 7). Modules 0, 1, 2, and 3 had the greatest amount of nodes which were 13, 12, 9, and 8, respectively. Pathway enrichment was performed on the modules with 8 or greater nodes. The top enriched pathways were the following: Module 0- DNA replication, Telomere Maintenance, Synthesis of DNA, and S Phase. Module 1- Extracellular matrix organization, Beta1 integrin cell surface interactions, Integrin signaling pathway, and Focal adhesion. Module 2- Salmonella infection, Pathogenic E. coli infection, west nile virus, and apoptotic signaling in response to DNA damage. Module 3- Extracellular matrix organization, phospholipase c-epsilon pathway, cystic fibrosis transmembrane conductance regulator, and beta 2 adrenergic receptor pathway, and corticosteroids and cardioprotection (**Supplementary Table 5**).

qPCR Confirmation

For confirmation of the RNA-seq data, we ran an additional cohort of mice through the identical experimental TBI and treatment paradigm as before. We isolated RNA and performed

TABLE 2 | Gene Ontology Analysis of Unique Genes at 3 dpi in the Hippocampus.

Up or Down	Treatment	GO Biological Process	FDR
Up-Regulated	Candesartan	Response to Stress (GO:0006950)	1.23E-05
		Regulation of Cell Adhesion (GO:0030155)	6.44E-05
		Response to Chemical (GO:0042221)	9.95E-04
		Positive Regulation of Cell Adhesion (GO:0045785)	1.05E-03
		Cellular Response to Nitrogen Compound (GO:1901699)	1.18E-03
		Cellular Response to Chemical Stimulus (GO:0070887)	1.34E-03
		Response to Wounding (GO:0009611)	1.38E-03
		Cellular Response to Stress (GO:0033554)	2.36E-03
		Response to Stimulus (GO:0050896)	2.98E-03
		Circulatory System Development (GO:0072359)	4.79E-03
		Tube Development (GO:0035295)	4.85E-03
		Wound Healing (GO:0042060)	4.91E-03
		Positive Regulation of Biological Process (GO:0048518)	5.16E-03
		Regulation of Programmed Cell Death (GO:0043067)	5.22E-03
		Regulation of Apoptotic Process (GO:0042981)	7.02E-03
		Response to Nitrogen Compound (GO:1901698)	7.67E-03
		Blood Vessel Development (GO:0001568)	7.84E-03
		Positive Regulation of Cellular Process (GO:0048522)	7.99E-03
		Metabolic Process (GO:0008152)	8.11E-03
		Regulation of Developmental Process (GO:0050793)	8.40E-03
	Vehicle	No GO Families Identified	
Down-Regulated	Candesartan	Cilium Assembly (GO:0060271)	3.46E-07
		Cilium Organization (GO:0044782)	3.83E-07
		Microtubule Bundle Formation (GO:0001578)	4.08E-07
		Axoneme Assembly (GO:0035082)	7.29E-07
		Cilium Movement (GO:0003341)	3.29E-06
		Plasma Membrane Bounded Cell Projection Assembly (GO:0120031)	1.30E-05
		Cell Projection Assembly (GO:0030031)	1.96E-05
		Organelle Assembly (GO:0070925)	1.79E-04
		Microtubule-Based Process (GO:0007017)	2.88E-04
		Cell Projection Organization (GO:0030030)	1.42E-03
		Plasma Membrane Bounded Cell Projection Organization (GO:0120036)	2.20E-03
		Microtubule Cytoskeleton Organization (GO:0000226)	2.74E-03
		Microtubule-Based Movement (GO:0007018)	6.10E-03
		Cilium or Flagellum-Dependent Cell Motility (GO:0001539)	6.53E-03
		Cilium-Dependent Cell Motility (GO:0060285)	6.99E-03
		Cytoskeleton Organization (GO:0007010)	7.02E-03
		Sperm Motility (GO:0097722)	3.19E-02
	Vehicle	Anion Transport (GO:0006820)	2.76E-02
		Reproduction (GO:0000003)	4.29E-02
		Organic Anion Transport (GO:0015711)	4.48E-02
		Reproductive Process (GO:0022414)	5.31E-02
		Multicellular Organismal Homeostasis (GO:0048871)	6.27E-02

Gene ontology analysis (biological process) of genes altered by candesartan or vehicle treatment after TBI at 3 dpi in the hippocampus after unique gene analysis. GO biological process terms were identified using a Fisher's Exact test and false discovery rate of 0.05.

qPCR on four genes that the RNA seq data showed were elevated by TBI but were down-regulated by candesartan treatment at 29 dpi (**Figure 6C**). These genes are mainly expressed in microglia and astrocytes (Kamphuis et al., 2015; Kawahara et al., 2016; DePaula-Silva et al., 2019). All four genes were strongly up-regulated at 3 dpi in the hippocampus in both TBI + VH and TBI + CD groups. Candesartan treatment did not reduce expression of these genes at 3 dpi by either RNA seq or qPCR (**Figures 6A,B**). However, by 29 dpi, candesartan treatment reduced the expression of the genes Lyz2, C4b, and vimentin by both RNA seq and qPCR analysis (**Figures 6C,D**). GPNMB expression was reduced by candesartan in the samples that were analyzed by RNA-seq, but not by qPCR. Overall, the qPCR of an independent cohort of mice validated the RNA-seq analysis and indicated that the DEG analysis identified specific genes whose expression

TABLE 3 | Gene Ontology Analysis of Unique Genes at 29 dpi in the Hippocampus.

Up or Down	Treatment	GO Biological Process	FDR
Up-regulated	Candesartan	No GO Families Identified	
	Vehicle	Response to External Stimulus (GO:0009605)	1.19E-13
		Immune System Process (GO:0002376)	1.21E-13
		Defense Response (GO:0006952)	2.95E-13
		Response to Stress (GO:0006950)	3.45E-11
		Response to External Biotic Stimulus (GO:0043207)	1.60E-10
		Response to Other Organism (GO:0051707)	1.81E-10
		Interspecies Interaction Between Organisms (GO:0044419)	1.91E-10
		Response to Biotic Stimulus (GO:0009607)	2.17E-10
		Regulation of Localization (GO:0032879)	7.18E-10
		Inflammatory Response (GO:0006954)	8.79E-10
		Regulation of Multicellular Organismal Process (GO:0051239)	1.58E-09
		Immune Response (GO:0006955)	3.67E-09
		Response to Chemical (GO:0042221)	4.09E-09
		Regulation of Transport (GO:0051049)	1.34E-08
		Regulation of Immune Response (GO:0050776)	3.10E-08
		Defense Response to Other Organism (GO:0098542)	3.93E-08
		Positive Regulation of Biological Process (GO:0048518)	4.16E-08
		Positive Regulation of Cellular Process (GO:0048522)	1.18E-07
		Regulation of Cytokine Production (GO:0001817)	1.27E-07
		Negative Regulation of Multicellular Organismal Process (GO:0051241)	1.54E-07
Down-regulated	Candesartan	No GO Families Identified	
	Vehicle	No GO Families Identified	

Gene ontology analysis (biological process) of unique genes altered by candesartan or vehicle treatment after TBI at 29 dpi in the hippocampus after unique gene analysis. GO biological process terms were identified using a Fisher's Exact test and false discovery rate of 0.05.

was reduced by candesartan treatment at 29dpi. Many of these genes, as the GO analysis showed (**Table 3**), encode proteins involved with immune and inflammatory processes (**Supplementary Table 4**).

DISCUSSION

This study focused on a transcriptomic analysis of individual mouse brain regions after TBI and after treatment with the angiotensin receptor blocker candesartan. We and others have previously shown that candesartan improves functional and morphological recovery in pre-clinical models of TBI (Villapol et al., 2012, 2015; Villapol and Saavedra, 2015). In this study we sought to understand the molecular mechanisms underpinning candesartan's beneficial effects. Our data point to a role for candesartan in altering many different aspects of the response to TBI, particularly those involved with cellular response to stress, extracellular matrix alterations and the innate immune response. We found a more pronounced effect at an earlier (3 dpi) rather than a later time point (29 dpi) and in the area closer to the lesion (hippocampus) rather than further away (thalamus, hypothalamus). This transcriptomic analysis indicates several novel pathways that are altered by candesartan after brain injury that should assist in determining the molecular effects of candesartan's beneficial actions in treating TBI.

The most pronounced differences within this transcriptomic analysis were the differences in gene expression between brain regions (**Figures 2**, **3**). Surprisingly, these differences were greater than the differences after TBI at either time point or between candesartan and vehicle treated animals. This large difference in the transcriptome between the hippocampus, thalamus and hypothalamus indicates the very specialized functions that each of these brain regions has, and is similar to what others have found, in the mouse, and in higher organisms (Ng et al., 2009; Kasukawa et al., 2011; DiCarlo et al., 2017; Zhu et al., 2018). The effect of TBI on the transcriptome was most apparent in the hippocampus at 3 dpi, with over 1500 genes differentially expressed in the hippocampus after TBI in either vehicle or candesartan treated mice (**Table 1**). The pronounced effect of TBI within the hippocampus was expected given the proximity of the hippocampus to the location of the TBI. The effect of TBI on the transcriptome dissipated with distance from the lesion with a reduced effect in the thalamus and only one DEG in the hypothalamus at 3 dpi (**Table 1**). The TBI-induced differences in the transcriptome were much reduced at 29 dpi, with the hippocampus having the most DEGs at this later time point also.

We designed this study to identify candesartan-mediated changes in the brain transcriptome in response to TBI, in order to identify novel pathways through which candesartan could act to improve recovery from injury. Although the magnitude of the candesartan response was lower than expected, we did find many genes and pathways that were regulated by candesartan particularly in the hippocampus at 3dpi (**Tables 2**, **4** and **Supplementary Table 1**). Interferon signaling was one immune related pathway that was altered by candesartan at 3 dpi in

TABLE 4 | Pathway Analysis of Unique Up-Regulated Genes at 3 dpi in the Hippocampus.

Pathway Database	Pathway Description	Genes	FDR *q*-value
Reactome	Extracellular matrix organization	VCAM1, FN1, THBS1, VWF, COL3A1, ITGA1, F11R, COL12A1, ADAM8, MMP2, BMP1, CASP3, FBLN5, LOXL1, BMP4, LRP4	7.41E-07
Reactome	Interferon signaling	VCAM1, FLNA, OAS1, ISG15, USP18, SAMHD1, IFI35, ISG20, BST2, DDX58, TRIM21, SP100, TRIM34	1.74E-06
Reactome	Chromosome maintenance	PCNA, POLA2, PRIM2, RFC3, LIG1, H2BC14, CENPK, CENPO, CENPX	8.60E-05
Reactome	Lagging strand synthesis	PCNA, POLA2, PRIM2, RFC3, LIG1	1.09E-04
Reactome	Response to elevated platelet cytosolic Ca2+	FN1, THBS1, VWF, FLNA, F13A1, VCL, PFN1, PF4, LAMP2	1.25E-04
PID	Integrin-1 pathway	VCAM1, FN1, THBS1, COL3A1, ITGA1, F13A1, MDK	1.25E-04
Reactome	Interferon alpha/beta signaling	OAS1, ISG15, USP18, SAMHD1, IFI35, ISG20, BST2	1.61E-04
Reactome	Telomere c-strand (lagging strand) synthesis	PCNA, POLA2, PRIM2, RFC3, LIG1	3.31E-04
Reactome	Integrin cell surface interactions	VCAM1, FN1, THBS1, VWF, COL3A1, ITGA1, F11R	4.02E-04
Reactome	Polymerase switching	PCNA, POLA2, PRIM2, RFC3	4.02E-04
Reactome	Platelet activation, signaling and aggregation	FN1, THBS1, VWF, FLNA, F13A1, VCL, PFN1, PF4, LAMP2, PIK3R6, GNG10	4.02E-04
Reactome	DNA strand elongation	PCNA, POLA2, PRIM2, RFC3, LIG1	4.43E-04
Reactome	Processive synthesis on the lagging strand	PCNA, POLA2, PRIM2, LIG1	4.53E-04
KEGG	DNA replication	PCNA, POLA2, PRIM2, RFC3, LIG1	6.78E-04
Reactome	Polymerase switching on the C-strand of the telomere	PCNA, POLA2, PRIM2, RFC3	6.78E-04
Reactome	MAP2K and MAPK activation	FN1, VWF, VCL, ACTG1, NRAS	1.04E-03
KEGG	Focal adhesion	FN1, THBS1, VWF, COL3A1, ITGA1, FLNA, VCL, ACTG1, ILK	1.27E-03
PID	Integrins in angiogenesis	FN1, COL3A1, F11R, COL12A1, VCL, ILK	1.41E-03
Reactome	Extension of telomeres	PCNA, POLA2, PRIM2, RFC3, LIG1	1.41E-03
Reactome	Signaling by moderate kinase activity BRAF mutants	FN1, VWF, VCL, ACTG1, NRAS	1.50E-03

Pathway analysis of unique up-regulated genes altered by candesartan after TBI at 3 dpi in the hippocampus. Pathways were identified using a Fisher's exact test with a false discovery rate of < 0.05.

the hippocampus. We found enrichment of genes involved with interferon signaling, including type 1 interferon (**Table 4**) at this early time point in the TBI + CD group. IFN signaling activates innate and adaptive immunity in response to infections and cell injury (Ivashkiv and Donlin, 2014). Activation of the type 1 IFN pathway in response to TBI induces neuro-inflammation, neuronal cell death, and glial reactivity (Karve et al., 2016). We observed up-regulation of the IFN signaling genes USP18 and ISG15 in the hippocampi of mice in the TBI + candesartan group at 3 dpi (**Table 4**). USP18 acts as a type 1 IFN receptor modulator, decreasing IFN responsiveness through blocking the interaction between JAK1 and the IFN receptor and therefore inhibiting downstream signaling events (Malakhova et al., 2006; Francois-Newton et al., 2011; Burkart et al., 2013; Wilmes et al., 2015; Honke et al., 2016; Basters et al., 2018). Induction of USP18 gene expression by candesartan would therefore inhibit interferon signaling in the cells in which this gene is expressed. ISG15 acts on USP18, stabilizing USP18 and preventing it from degradation. Induction of ISG15 by candesartan may therefore also reduce type 1 IFN signaling (Bihl et al., 2015). Some studies have shown that USP18 may act as a negative regulator of microglia activation through modulating IFNAR2, signaling, thus having a protective role on microglia function (Goldmann et al., 2015). Although both USP18 and ISG15 are both expressed in microglia, their expression in the brain is the strongest in endothelial cells (Zhang et al., 2014). Thus, candesartan may be interfering with interferon signaling in microglia and endothelial cells after TBI.

Pathway analysis of the effects of candesartan at 3 dpi in the hippocampus identified nine genes associated with chromosome maintenance and other processes associated with DNA replication (**Table 4**). These were also part of the reactome (**Figure 5**). The majority of these genes encode non-specific regulators of DNA synthesis. Angiotensin II has been shown to promote DNA replication in vascular smooth muscle and other cell types (Makita et al., 1995; Pawlikowski et al., 1999; Touyz et al., 1999; Chiu et al., 2003) and candesartan can mitigate angiotensin II induced DNA damage (Schmid et al., 2008). DNA replication and repair are important components of the response to TBI, with problems in DNA repair, and enduring DNA damage contributing to functional deficits after TBI (Davis and Vemuganti, 2020). Candesartan's actions in regulating DNA repair and synthesis may indicate an additional pathway through which it has beneficial action after TBI.

Both GO (**Table 2**) and pathway analysis (**Table 4**) identified multiple genes associated with blood vessel and circulatory system development up-regulated in the hippocampi of the TBI + CD mice, suggesting a possible role for candesartan in the regulation of vascular endothelial cells within the

TABLE 5 | Pathway Analysis of Unique Down-Regulated Genes at 3 dpi in the Hippocampus.

Pathway Database	Pathway Description	Genes	FDR *q*-value
Reactome	Core matrisome	COL9A3, COL8A2, COL14A1, COL18A1, HSPG2, FBN1, NID2, PRELP, FNDC1	1.05E-03
Reactome	SLC-mediated transmembrane transport	SLC44A5, SLC31A1, SLC22A8, SLC13A4, SLC4A5, SLC4A2, SLCO2A1, SLC16A2	2.75E-03
PID	Integrins in angiogenesis	COL9A3, COL8A2, COL14A1, VEGFA, ADGRA2	3.54E-03
Reactome	Degradation of the extracellular matrix	COL9A3, COL8A2, COL14A1, COL18A1, HSPG2, FBN1	3.54E-03
Reactome	Extracellular matrix organization	COL9A3, COL8A2, COL14A1, COL18A1, HSPG2, FBN1, NID2, TTR	3.54E-03
Reactome	Retinoid cycle disease events	TTR, ABCA4, STRA6	3.54E-03
Reactome	Integrin cell surface interactions	COL9A3, COL8A2, COL18A1, HSPG2, FBN1	3.54E-03
NABA	Collagens	PCOL9A3, COL8A2, COL14A1, COL18A1	3.85E-03
Reactome	Collagen chain trimerization	COL9A3, COL8A2, COL14A1, COL18A1	3.85E-03
Reactome	Assembly of collagen fibrils and other multimeric structures	COL9A3, COL8A2, COL14A1, COL18A1	1.27E-02
Reactome	The canonical retinoid cycle in rods (twilight vision)	TTR, ABCA4, STRA6	1.28E-02
Reactome	Collagen degradation	COL9A3, COL8A2, COL14A1, COL18A1	1.28E-02
Reactome	Collagen biosynthesis and modifying enzymes	COL9A3, COL8A2, COL14A1, COL18A1	1.42E-02
Reactome	Response to elevated platelet cytosolic Ca2+	VEGFA, F5, ACTN2, IGF2, PHACTR2	1.47E-02
Reactome	Laminin interactions	COL18A1, HSPG2, NID2	2.16E-02
Reactome	Cargo concentration in the ER	F5, CD59, FOLR1	2.69E-02
Reactome	Transport of bile salts and organic acids, metal ions and amine compounds	SLC44A5, SLC31A1, SLC22A8, SLC13A4	2.86E-02
Reactome	Collagen formation	COL9A3, COL8A2, COL14A1, COL18A1	3.21E-02
NABA	Basement membranes	COL18A1, HSPG2, NID2	4.04E-02
Reactome	Visual phototransduction	HSPG2, TTR, ABCA4, STRA6	4.32E-02

Pathway analysis of unique down-regulated genes altered by vehicle after TBI at 3 dpi in the hippocampus. Pathways were identified using a Fisher's exact test with a false discovery rate of < 0.05.

hippocampus after injury. Additionally, pathway analysis of down-regulated unique genes in the TBI + VH condition identified a reduction in integrins of angiogenesis (**Table 5**). AT1 receptor blockade utilizing candesartan stimulates angiogenesis through the regulation of vascular endothelial factor (VEGF) and offers protection in animal models of ischemic retinopathy (Shanab et al., 2015) and stroke (Guan et al., 2011). There are many adverse effects of TBI on the cerebral vasculature, including edema and chronic inflammation (Salehi et al., 2017). Candesartan mediated improvements in angiogenesis and vascular function after TBI are an obvious target as the AT1 receptor is expressed throughout the vasculature. These transcriptomic data suggest that candesartan is functioning through such mechanisms after TBI.

Genes associated with the extracellular matrix (ECM) were also highly represented in our analysis of candesartan activity at 3dpi in the hippocampus. Candesartan administration after TBI resulted in the up-regulation of GO families associated with cell adhesion (**Table 2**), and pathway analysis identified the ECM organization as the most significantly up-regulated reactome (FDR < 0.05) (**Table 4**). The angiotensin system's modulation of the ECM has been studied extensively in its role in cardiac disease (Weber, 1997; Singh et al., 2008; Dab et al., 2012) and suggested to play a role in neural plasticity (Wright and Harding, 2004). The maintenance of the ECM plays an important role in modulating inflammation and is a potentially important factor in repair after TBI (Gaudet and Popovich, 2014).

Candesartan treatment for 3 days in sham injured mice resulted in significant differential gene expression (FDR 0.05, abs log2 FC > 0.32) in the hippocampus relative to that in sham injured mice treated with vehicle (**Table 1**). Interestingly, this effect was not noted in any of the other brain regions suggesting that this effect is hippocampal-specific. As these mice received a skin incision with isoflurane administration without a craniotomy it is surprising that this differential expression is not seen in either the hypothalamus or the thalamus, particularly given the high level of expression of AT1R in the hypothalamus (Nishimura et al., 2000; de Kloet et al., 2015; Wang et al., 2016b). These data suggest therefore that either candesartan has greater access to the hippocampus, than the thalamus or hypothalamus, perhaps indicating region specific differences in the blood brain barrier; or that candesartan has different effects within specific brain regions. Analysis of the candesartan effects in the hippocampus of sham mice shared some gene ontology categories with those from unique DEGs identified in the hippocampus of mice treated with candesartan after TBI. Specifically, these included genes involved with cilium organization and structure, and ion transport (**Table 2** and **Supplementary Table 2**). To

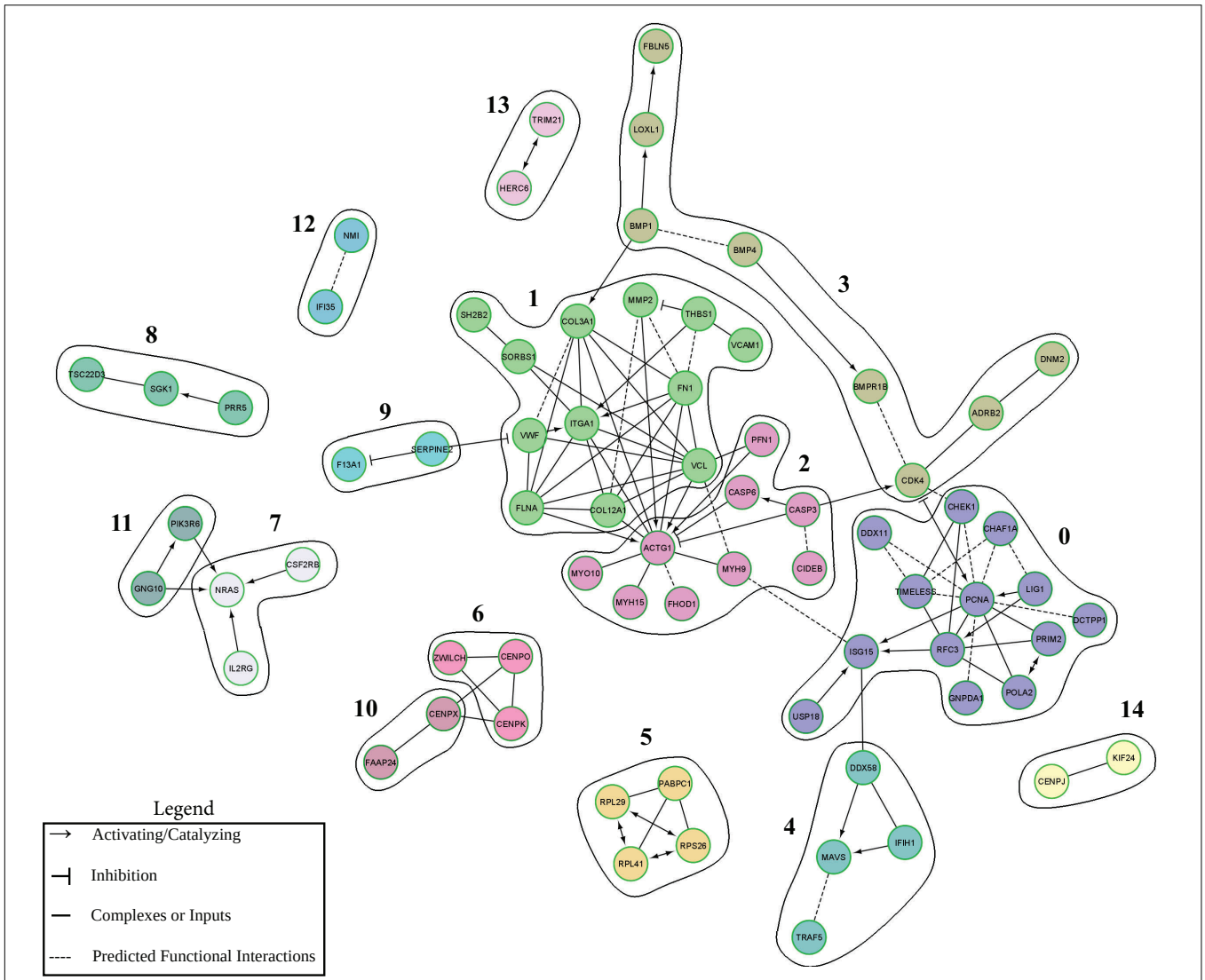

FIGURE 5 | ReactomeFIViz-Derived Cluster Functional Interaction Network. Functional modules of unique up-regulated DEGs altered by candesartan treatment after TBI at 3 dpi in the hippocampus. Nodes in different network modules are shown in different colors. The modules listed are described in **Supplementary Table 5**.

our knowledge this is the first report of candesartan-specific gene regulation in the hippocampus of mice without brain injury. As several different behavioral effects have been noted for candesartan and related ARBs (Wright and Harding, 2004; Khoury et al., 2012; Nylocks et al., 2015), our data provide useful information as to their potential effects in specific brain regions. This hippocampal-specific effect of candesartan in sham mice was no longer detectable in mice treated for 29 dpi. It is possible that longer term treatment led to desensitization of receptors or other signaling molecules to reduce this effect, particularly as the 29 dpi mice had candesartan administered through an osmotic minipump, providing constant low dose administration in contrast to the daily injections received by the 3 dpi group mice.

The overall effect of candesartan treatment in mice after TBI was smaller than expected. We used a sub-hypotensive dose of candesartan (0.1 mg/kg/day) that we and others have previously shown to be effective for improving recovery from CCI in mice (Timaru-Kast et al., 2012; Villapol et al., 2012, 2013, 2015). However, this low dose did not produce robust changes in gene expression, even in the hippocampus. The changes in gene expression with candesartan treatment after TBI were swamped by those produced in response to the TBI, particularly in the hippocampus. Thus, while there were 1732 DEGs in the hippocampus in the TBI + CD group in comparison to the Sham + VH group, 1166 of these genes were also found in the TBI + VH group. Nonetheless, candesartan treatment did produce important changes in gene expression in specific pathways, even at the acute 3 dpi time point. The magnitude of these changes were not as great as those produced by TBI. However, as this dose of candesartan has therapeutic efficacy in this model of TBI, it is possible that these smaller changes in gene expression, or

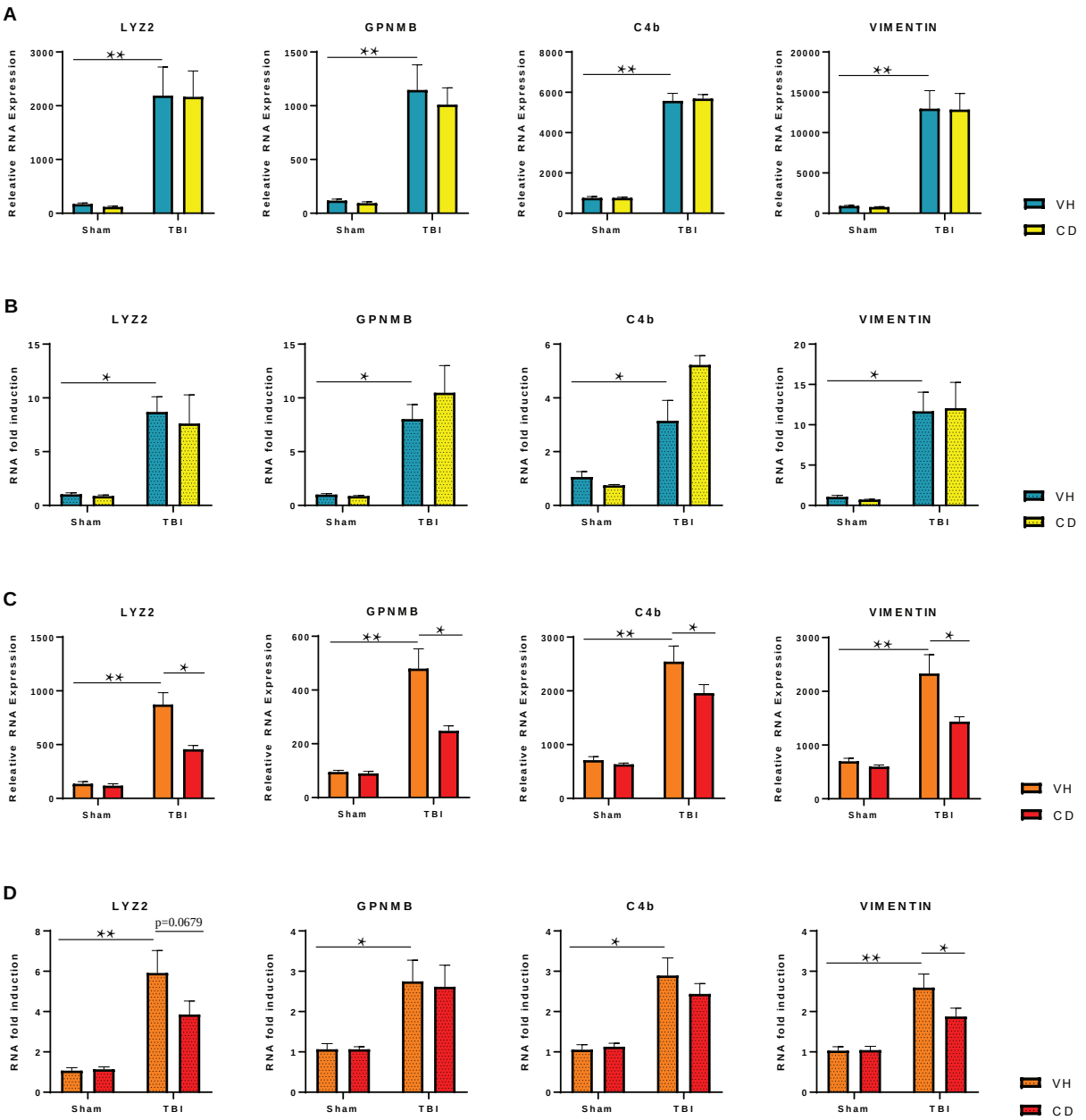

FIGURE 6 | RT-qPCR Verification of Selected Differentially Expressed Genes in the Hippocampus at 3 and 29 dpi. **(A)** RNA-seq analysis shows highly elevated expression of specific genes in the ipsilateral hippocampus at 3 dpi that is not reduced on candesartan treatment. **(B)** RT-qPCR of RNA isolated from an independent cohort of injured mice confirmed changes seen in RNA-seq of injured hippocampi at 3 dpi. **(C)** RNA-seq analysis shows candesartan reduced the TBI mediated induction of RNA expression of these genes at 29 dpi. **(D)** RT-qPCR confirmed candesartan mediated reduction in expression of elevated gene Vimentin at 29 dpi, but not all investigated genes. C4b (complement C4B), Gpnmb (Glycoprotein Nmb), Lyz2 (Lysozyme 2) Vim (Vimentin) VH, vehicle; CD, candesartan. Mean +/– S.E.M., $n = 4$ **(A–C)**, $n = 8$ **(D)**. $^{*}p < 0.05$, $^{**}p < 0.0001$.

those that were more variable in our hands, may be relevant to its mechanism of action.

An additional explanation for the relatively small effects of candesartan on gene expression after TBI, is the potentially small effect of blocking the AT1 angiotensin receptor after injury. The number of cells that have high level expression of this receptor in the hippocampus and thalamus is quite small (Lenkei et al., 1997, 1998), and the effect of angiotensin II after TBI is not known. Examination of gene expression changes in specific cell types that are known to express the AT1R, such as endothelial cells and neurons, may have produced stronger gene expression changes. In the present study, some significant cell-specific alterations in gene expression by candesartan may have been masked by unchanged expression in other cell types.

We have previously shown that AT1aR KO mice are partially protected from TBI, with a smaller lesion and reduced GFAP expression after injury (Villapol et al., 2015), suggesting that signaling through the AT1R does play some role in the response to TBI in mice. Additionally, candesartan efficacy in improving recovery in a mouse model of TBI is partially also due to its partial agonism of the PPARγ receptor (Villapol et al., 2012, 2015). As PPARγ is more widely expressed than the AT1R (Bernardo and Minghetti, 2006), stimulating PPARγ receptor signaling expands the cellular repertoire of candesartan. However, in this study we were not able to differentiate between expression changes that were a result of antagonism of the AT1R or agonism of PPARγ .

Another limitation of our study was that our sham group of animals was anesthetized with a skin incision only without craniectomy. It has been shown that even a very careful craniectomy can cause minor damage to the brain parenchyma, simulating a mild brain injury (Cole et al., 2011; Lagraoui et al., 2012). Therefore, many groups, including ours, have switched to using mice without a craniectomy as a sham. Our original studies that showed candesartan efficacy in the mouse CCI model were run using this sham (Villapol and Saavedra, 2015; Villapol et al., 2015), and as we replicated those studies here, we used the same controls. Nonetheless, there are limitations in comparing the transcriptome in brain regions after craniectomy and TBI with those in a brain without either. Potential damage to the bone or meninges could influence gene expression in the underlying brain. As the brain regions investigated here do not lie immediately below the skull, this may be less of a concern. A potential alternate control could have been the contralateral, uninjured corresponding brain region. However, it has also been shown that the contralateral side is not completely unaltered by the injury (Rola et al., 2006; Urrea et al., 2007), and the contralateral regions were not considered good controls for the comparisons with the injured brain run here. We therefore feel that the sham injured animals we used as controls were the most appropriate for our specific experiments, although there are limitations associated with their use.

We and others have previously shown that administration of ARBs after brain injury has anti-inflammatory activity, including reduction of reactive astrocytosis and microgliosis, and reduction of cytokines in the peri-lesional area (Timaru-Kast et al., 2012; Villapol et al., 2012, 2015) even at acute times after injury. However, in this study candesartan administration did not reduce TBI-induced expression of multiple pro-inflammatory genes at 3 dpi in the hippocampus (**Figure 6** and data not shown). We interpret these data to show the considerable strength of the TBI-mediated induction of inflammatory gene expression at this early time point. This discrepancy in candesartan action with similar dose and route of administration, between our prior findings, and those of this transcriptomic analysis is likely not explainable by the difference between the induction of RNA, in this paper, and protein in our prior publications. However, the anti-inflammatory action of candesartan was detectable in the RNA seq data from the hippocampi of mice at 29 dpi where candesartan did reduce expression of several inflammatory genes and genes associated with reactive gliosis including GFAP (**Figure 6** and **Supplementary Table 4**). This action was also reflected in the GO analysis of unique genes that were up-regulated in the TBI + VH group but not in the TBI + CD group at 29dpi in the hippocampus. These GO terms include immune response, regulation of the immune response, and inflammatory response amongst other terms (**Table 3**).

The reduction in inflammatory gene expression at 29 dpi is probably the result of candesartan action on several different cell types, with an important microglial component. Interestingly, we observed that some genes associated with the transcriptomic signature for Disease Associated Microglia (DAM) were shown in our unique gene analysis at 29 dpi in the hippocampus (Keren-Shaul et al., 2017; Deczkowska et al., 2018). These genes, including Trem2, Itgax and Spp1 were upregulated only in the TBI + VH group, implying that candesartan reduced their expression. There were many mainly microglial-specific genes, associated with the unique gene analysis in the TBI + VH group at 29dpi, including Clq, Tlr7, Itgb2, C3ar1 and Csfr1 adding to the evidence that candesartan had a specific effect on activated microglia at this time point (Zhang et al., 2014). One gene we identified as being reduced by candesartan treatment after TBI at 29dpi in the hippocampus was the Lyz2 gene, despite this gene not being part of the unique gene analysis at this time point. The Lyz2 gene encodes the lysosomal enzyme lysozyme M (Orthgiess et al., 2016) which in mice is highly expressed within peripheral macrophages and monocytes (Cross et al., 1988), and whose promoter use in LysM-Cre mice has driven many macrophage-specific mouse knockouts (Dosch et al., 2019). Although once thought of as a microglial gene, Lyz2 now forms part of the transcriptomic signature for infiltrating monocytes into the CNS (Izzy et al., 2019; Ronning et al., 2019; Ellwanger et al., 2021), so its reduction by candesartan may indicate reduced infiltration of these monocytes at this later time point. A closer examination of the effect of candesartan treatment on microglial and macrophage lineage cells in the CNS after injury will require cell sorting and scRNA seq analysis on these different cellular populations.

Although the effects of candesartan in the hippocampus could be mediated by either/both antagonism at the AT1R or agonism at the PPARγ receptor, it is also possible that a third receptor mediates some of the changes in gene expression specifically in the hippocampus. The Mas receptor is very highly expressed in the dentate gyrus of the hippocampus (Freund et al., 2012), and our RNA seq data indicate that at least at the level of RNA, is much more highly expressed than the AT1a receptor in the hippocampus (**Supplementary Figure 3**). As candesartan has been shown to induce activity and expression of ACE2, the enzyme that converts Ang II to the Mas receptor ligand, Ang-(1-7), it has been postulated that some of candesartan's beneficial effects may be mediated by enhancing activation of the ACE2/Ang-(1-7)/MasR axis (Pernomian et al., 2015). Indeed, we have previously shown that Ang-(1-7) treatment after TBI can also enhance recovery (Janatpour et al., 2019). Further delineation of which receptor mediates candesartan's regulation of gene expression will await experiments involving specific receptor knockout mice.

The data we present here provide an array of information on the response to TBI in different brain regions at different time points, and the effects of candesartan on these responses. We have shown that candesartan can alter multiple pathways including interferon signaling, extracellular matrix alterations, DNA replication and manipulation of cerebrovascular repair and function. Some of these functions are in agreement with a prior microarray study of candesartan action on primary cerebellar granular neurons in response to glutamate treatment (Elkahloun et al., 2016), suggesting that candesartan can act directly on neurons in the intact brain. However, we also propose that much of the beneficial effects of candesartan after TBI will be on glia and the cerebrovasculature. This study will serve as a starting point for a more detailed and granular examination of candesartan action on specific cell types, and pathways to delineate the molecular actions of this drug after TBI. Further understanding of the complex interactions through which candesartan can improve the pathophysiology of TBI may help provide future targets for treatment.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: GEO database, GSE163415.

ETHICS STATEMENT

The animal study was reviewed and approved by Uniformed Services University Institutional Animal Care and Use Committee.

AUTHOR CONTRIBUTIONS

PA, CD, and AJS planned the experiments. PA, MR, and AI performed the experiments. PA, AS, MW, and DS analyzed the data. PA, MW, AS, CD, DS, and AJS interpreted the analysis. PA, DS, and AJS wrote the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnins.2021.636259/full#supplementary-material

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Circulating Angiotensin-(1–7) Is Reduced in Alzheimer's Disease Patients and Correlates With White Matter Abnormalities: Results From a Pilot Study

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Introduction: Alzheimer's disease (AD) is the leading cause of dementia worldwide. Despite the extensive research, its pathophysiology remains largely unelucidated. Currently, more attention is being given to the disease's vascular and inflammatory aspects. In this context, the renin-angiotensin system (RAS) emerges as a credible player in AD pathogenesis. The RAS has multiple physiological functions, conducted by its two opposing axes: the classical, led by Angiotensin II (Ang II), and the alternative, driven by Angiotensin-(1–7) [Ang-(1–7)]. These peptides were shown to interact with AD pathology in animal studies, but evidence from humans is scarce. Only 20 studies dosed RAS molecules in AD patients' bloodstream, none of which assessed both axes simultaneously. Therefore, we conducted a cross-sectional, case-control exploratory study to compare plasma levels of Ang II and Ang-(1–7) in AD patients vs. age-matched controls. Within each group, we searched for correlations between RAS biomarkers and measures from magnetic resonance imaging (MRI).

Methods: We evaluated patients with AD (n = 14) and aged-matched controls (n = 14). Plasma Ang II and Ang-(1–7) were dosed using ELISA. Brain MRI was performed in a 3 Tesla scan, and a three-dimensional T1-weighted volumetric sequence was obtained. Images were then processed by FreeSurfer to calculate: (1) white matter hypointensities (WMH) volume; (2) volumes of hippocampus, medial temporal cortex, and precuneus. Statistical analyses used non-parametrical tests (Mann-Whitney and Spearman).

Results: Ang-(1–7) levels in plasma were significantly lower in the AD patients than in controls [median (25th–75th percentiles)]: AD [101.5 (62.43–126.4)] vs. controls [209.3 (72–419.1)], $p = 0.014$. There was no significant difference in circulating Ang II. In the AD patients, but not in controls, there was a positive and significant correlation between Ang-(1–7) values and WMH volumes (Spearman's rho = 0.56, $p = 0.038$). Ang-(1–7) did

not correlate with cortical volumes in AD or in controls. Ang II did not correlate with any MRI variable in none of the groups.

Conclusion: If confirmed, our results strengthen the hypothesis that RAS alternative axis is downregulated in AD, and points to a possible interaction between Ang-(1–7) and cerebrovascular lesions in AD.

Keywords: Alzheimer's disease, renin angiotensin system, Angiotensin-(1–7), angiotensin II, white matter hypointensities, cerebrovascular lesions

INTRODUCTION

Worldwide, more than 50 million people suffer from dementia, in 60–70% of the cases caused by Alzheimer's disease (AD) (World Health Organization (WHO), 2018). The social burden posed by AD places it amid the top priorities for medical research: studies on the subject receive billions of dollars each year in the United States alone (Alzheimer's association, 2020). Even with the extensive scientific efforts taking place, AD's pathophysiology remains far from elucidated. Currently, our comprehension of the disease's mechanisms may be about to face a turning point. So far, most attempts to explain AD's onset and progression have focused on the brain deposits of beta-amyloid (Aβ) protein. Lately, though, amyloid-targeting drugs have failed to show clinical benefits in successive trials. Such mounting high-quality evidence fuel an active debate around the "amyloid hypothesis" of AD and the limits of its explanatory power (Makin, 2018). Ever more attention is shifting to the disease's vascular and inflammatory features, encouraging new models to come forward. For instance, one theory suggests that concurrent cerebrovascular dysfunction could prompt AD onset, or synergistically contribute to its progression (Solis et al., 2020). Another hypothesis points to neuroinflammation as a major component of AD's cognitive decline (Heneka et al., 2015). In this context, the renin-angiotensin system (RAS) emerges as a credible player in AD's pathogenesis, particularly the RAS' components involved in cerebrovascular regulation and brain inflammation (Kehoe, 2018).

Primarily remembered as a blood pressure controller, the RAS is in fact a multifaceted system for homeostasis, carrying out diverse and intricate functions. In the past decades, important discoveries transformed the way we think about the RAS. First, active peptides were described and added to the angiotensins' family, leading to the RAS' division in two main components: the classical axis, led by its main effector molecule, angiotensin II (Ang II), and the alternative axis, driven by angiotensin (1–7) [Ang-(1–7)]. Second, the concept of "local RAS" (in opposition to systemic RAS) was coined after RAS compounds were found in different organs and tissues, including the central nervous system (CNS) (Mascolo et al., 2017). Following these developments, the RAS has been implicated in medical conditions outside the heart and the kidneys, including neuropsychiatric disorders, AD among them (Rocha et al., 2018). Brain RAS is present to some extent in the hippocampus and other areas affected by AD pathology, where an interaction could take place. More significant, though, are the postulated relations between systemic RAS and AD. At the neurovascular unit level, circulating Ang II deregulates the cerebral blood flow, weakens the blood-brain barrier, and promotes neuroinflammation—all actions that might contribute to AD onset and progression. Plasma Ang-(1–7), on the contrary, might protect against AD-related damages, once it increases cerebral blood flow, reduces blood-brain barrier permeability, and inhibits inflammation. These theoretical perspectives are extensively discussed in our recent review (Ribeiro et al., 2020).

Literature on RAS-AD interaction is profuse and goes beyond theoretical speculation. Experiments with animal models help to build the case for a distinct role for RAS in AD pathogenesis, many even testing RAS as drug target in AD (Saavedra, 2016). Evidence from humans is contrastingly scarce. A recent review found 20 reports of RAS' molecules measured in AD subjects (Ribeiro et al., 2020). Most of these studies were investigating RAS' components in the CNS and thus examined brain tissue or cerebrospinal fluid (CSF), with conflicting results. The state of systemic RAS in AD is still largely unexplored. Less than ten studies dosed RAS molecules in AD patients' blood samples, none of which assessed both RAS axes. More often the focus has been the classical axis, especially the angiotensin-converting enzyme (ACE), and even its role remains unclear. One exception is worth noting: in a case-control study with 228 participants, plasma Ang-(1–7) was significantly lower in the AD group (Jiang et al., 2016a). However, such interesting finding only describes the alternative axis, as other molecules have not been analyzed in the same sample.

Here, we aimed to help shed more light on the complex relationship between AD and systemic RAS. Hence, we have conducted a cross-sectional exploratory study, comparing plasma levels of Ang II and Ang-(1–7) in AD patients vs. cognitively healthy age-matched subjects. Within each group, we searched for correlations between RAS biomarkers and relevant neuroimaging variables, particularly the cortical areas most hit by AD, and markers of cerebrovascular lesions. With our results, we expect to generate hypotheses about the state of both systemic RAS axes in AD, which may help to build inferences about potential mechanisms of interaction.

MATERIALS AND METHODS

Participants

We included 14 patients with mild to moderate AD evaluated at the Neurology Outpatient Clinic of a University Hospital

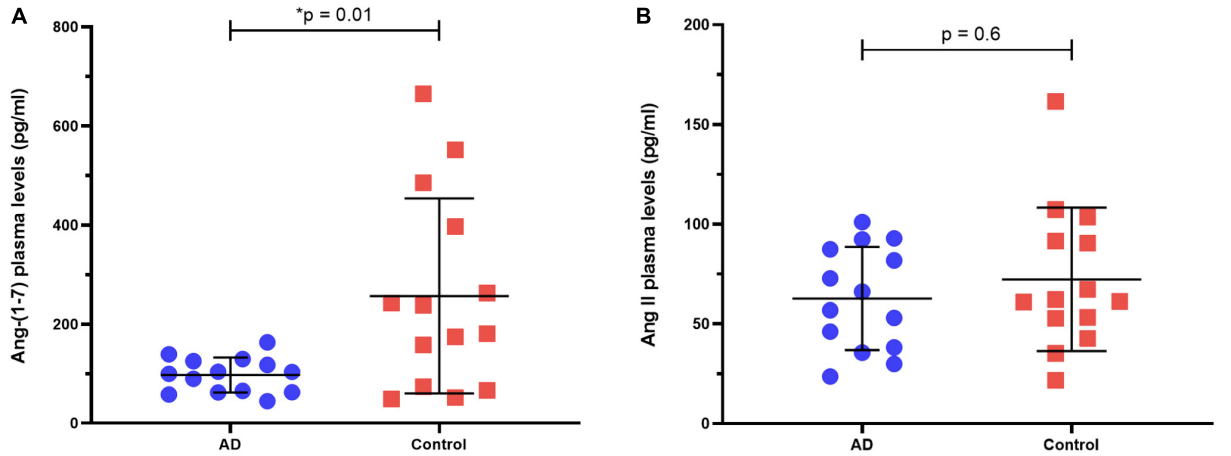

FIGURE 1 | Ang-(1–7) and Ang II plasma levels in AD patients vs. controls. **(A)** AD patients had smaller levels of Ang-(1–7) compared to controls. **(B)** AD patients and controls had similar levels of Ang II. Horizontal bars represent the upper quartile, median, and lower quartile. *P*-values from Mann-Whitney *U*-test.

(Hospital das Clínicas da Universidade Federal de Minas Gerais, Belo Horizonte, MG, Brazil). All patients presented with a typical history of progressive episodic memory deficits and showed medial temporal atrophy in brain magnetic resonance imaging (MRI), meeting the AD diagnostic criteria (McKhann et al., 2011). Experienced neurologists and psychiatrists carefully evaluated all patients, to rule out conditions that may mimic AD cognitive impairment. In addition, 10 out of the 14 patients had their CSF analyzed for amyloid beta 42 (Aβ 42), total tau (t-tau) and phosphorylated tau (p-tau). CSF samples were collected by lumbar puncture and biomarkers were measured with a double-sandwich enzyme-linked immunosorbent assay (ELISA) kit (Innogenetics, Gent, Belgium), as previously described (Magalhães et al., 2015). Patients with marked cerebrovascular lesions on brain MRI (Fazekas grade 3) were not included. To further ensure diagnostic accuracy, we followed the participants for at least 24 months after data were collected. In all of them, the disease progressed as expected given the baseline diagnosis.

To compose the control group, 14 older adults without cognitive complaints were recruited within the local community. All participants (AD and controls) underwent a neurological assessment, which included versions of the Mini-Mental State Examination (MMSE) validated in Brazil (Folstein et al., 1975; Brucki et al., 2003). All controls scored 28 or higher in the MMSE. In the clinical interview, participants or family members were asked about time of disease, comorbidities (including hypertension, diabetes, heart failure) and use of medications, especially ACE inhibitors (ACEi) and angiotensin receptor blockers (ARBs). Additional neuropsychological assessment included the Figure Memory Test from the Brief Cognitive Screening Battery (BCSB) for visual episodic memory (Nitrini et al., 2004), the Frontal Assessment Battery (FAB) for executive functions (Beato et al., 2012), and category fluency test (animals in 1 min) for verbal fluency (Machado et al., 2009).

Exclusion criteria for both groups were: (a) history or signs of previous stroke; (b) past neurosurgical procedures; (c) history of other neuropsychiatric conditions, including epilepsy, traumatic brain injury, demyelinating diseases, schizophrenia, bipolar disorder; (d) current or recent (past month) infections; (e) unstable clinical diseases. The Local Research Ethics Committee approved this study's protocol. All controls and patients (or their legal representatives) were informed about the study and agreed to participate, providing their written informed consent.

Measurement of Angiotensin Molecules

Peripheral blood was collected from all participants. Blood samples were drawn in vacuum tubes with heparin, centrifuged twice at 1,800 × g for 10 min at 4°C. Plasma samples were then obtained and stored at −70°C until further processing. A quantitative sandwich ELISA was performed to assess plasma levels of Ang-(1–7) (catalog # MBS084052) and Ang II (catalog # MBS028394), following manufacturer's instructions (MyBioSource, San Diego, CA, United States). Concentrations were measured in pg/ml. The reported sensitivity of the ELISA kits is 2.0 pg/ml for both analytes. All samples were measured in a single assay to avoid inter-assay variability. Our intra-assay variability was lower than 3%. To estimate the balance between RAS alternative and classical axes, the Ang-(1–7)/Ang II ratio was calculated for each subject (Mohite et al., 2018).

Neuroimages Acquisition and Processing

For all participants, brain MRI was performed in a 3 Tesla Intera-Achieva (Philips, Netherlands) scan. Three-dimensional 1 mm isometric T1-weighted (T1w) volumetric sequence images were acquired with the following parameters: TR: 8.13 ms, TE: 3.71 ms, 256 × 256 matrix, coronal field of view, and slice thickness of 1 mm. Fluid-attenuated inversion recovery (FLAIR) sequence was obtained in all AD subjects (n = 14) as well as controls (n = 14). FLAIR axial images were evaluated by a neuroradiologist, blinded to subjects' identity and diagnosis, who classified the deep white matter lesions (WMLs) in the Fazekas' scale, as described (Fazekas et al., 1987; Kim et al., 2008). Briefly, Fazekas scores are assigned as 0 (absence of WMLs), 1 (punctate

WMLs), 2 (early confluent WML), and 3 (large confluent areas of lesion in the white matter).

Before processing, all MRIs were manually assessed for quality control. MRIs with low quality were excluded, e.g., significant presence of motion artifacts, blurring, ringing/truncation, susceptibility phenomenon and bad contrast to noise ratio. On this basis, we excluded from further analysis the MRI data from one of the controls (and none of the AD patients). MRI T1 images were processed for cortical reconstruction and volumetric segmentation using the Freesurfer image analysis suite version 6.0 (FreeSurfer 6.0, 2017). The technical details of these procedures are described in prior publications (Dale and Sereno, 1993; Dale et al., 1999; Fischl et al., 1999a,b, 2001, 2002, 2004a,b; Fischl and Dale, 2000). Cortical areas are anatomically labeled by an automated system (Desikan et al., 2006). Using intensity and continuity information from the entire three-dimensional MR volume, the software processes it (through segmentation and deformation) and calculates cortical thickness, and then cortical volumes. The method has been validated against histological analysis (Rosas et al., 2002) and manual measurements (Han et al., 2006). Following initial automated analysis, manual inspection of the accuracy of post-processing steps was performed. Identifiable errors were corrected through the Freeview visualization tool (from the Freesurfer image analysis tool)[1]. Following manual inspection and any necessary edits, each subject was re-processed through the automated pipeline to account for manual intervention and then manually re-inspected for correction accuracy.

Neuroimaging variables of interest were pre-determined according to their relevance to AD. Hippocampus, entorhinal cortex, and parahippocampal cortex volumes were chosen considering the relevance of medial temporal atrophy for AD (Frisoni et al., 2010). Entorhinal and parahippocampal cortices were combined to compose the medial temporal cortex, as previously defined (de Souza et al., 2012). Precuneus' cortical volume was also selected given the area's relevance for disease progression: for instance, this region shows the earliest decline in cerebral perfusion in AD patients (Miners et al., 2016). Finally, the extent of cerebral small vessel disease was assessed using the volume of white matter hypointensities on T1-w images. On T1w sequences, WMLs of presumed vascular origin can appear hypointense, especially when more severe (Wardlaw et al., 2013). Using a probabilistic procedure (Fischl et al., 2002), FreeSurfer differentiates between normally appearing white matter and encompassed white matter signal abnormalities (i.e., hypointensities). The volume of T1w WM hypointensities strongly correlates with distinguished markers of WMLs, such as the Fazekas scale and white matter hyperintensities on T2w and FLAIR sequences (Dadar et al., 2018; Cedres et al., 2020). T1w WM hypointensities may underestimate the true extent of WMLs (Olsson et al., 2013), but have been nonetheless consistently used to measure white matter damage in AD patients and healthy elders (Burns et al., 2005; Salat et al., 2009; Jacobs et al., 2013; Leritz et al., 2014; Fischer et al., 2015; Dadar et al., 2019; Wei et al., 2019; Nemy et al., 2020). All volumes are given in mm^3 and reported as the sum of left and right hemispheres' measurements for each individual.

[1]https://surfer.nmr.mgh.harvard.edu/fswiki/FreeviewGuide

Statistical Analysis

Statistical analyses were performed using GraphPad Prism 8.0.2 (GraphPad Prism 8.0.2, 2019; GraphPad Software, San Diego, California, United States). To assess normality, we visually inspected the distributions of all continuous variables and run Shapiro-Wilk test. Since data were not normally distributed, non-parametrical tests were used in further analyses. Regarding continuous variables, the two groups (AD and controls) were compared using Mann-Whitney *U*-test. Fisher's exact test was used to compare categorical (binary) variables among groups. Correlations between variables were calculated using Spearman's coefficient. Due to the exploratory nature of the study, we have chosen not to adjust for multiple comparisons (Bender and Lange, 2001). When the study sample was divided in three categories, they were compared by Kruskal–Wallis one-way analysis of variance.

RESULTS

Clinical Parameters

AD patients and controls were similar in age [(mean ± standard deviation)]: AD (69.5 ± 8.8 years-old) v. controls (66.0 ± 11.0 years-old), $p = 0.59$. As shown in **Table 1**, AD and control groups were also balanced regarding sex, rates of hypertension and diabetes, and use of ACEi or ARB. Time since first symptoms was, on average, 3.3 years in those with AD (3.3 ± 1.3). As expected, AD patients scored less than controls in MMSE (24.8 ± 2.2 vs. 28.8 ± 0.8, $p < 0.0001$). The results of AD patients were also lower in Figure Memory, categorical fluency (animals) and FAB tests (see **Table 1**).

Angiotensins

Ang-(1–7) levels in plasma were significantly lower in the AD patients than in controls [median (25th–75th percentiles)]: AD [101.5 (62.43–126.4)] vs. controls [209.3 (72–419.1)], $p = 0.014$ (**Figure 1A**). There was no significant difference in circulating Ang II between AD patients [61.45 (37.52–88.6)] and controls [61.7 (50.3–94.5)], $p = 0.602$ (**Figure 1B**). The difference in Ang-(1–7) levels between groups reflected in the Ang-(1–7)/Ang II ratio, which was significantly lower in AD patients ($p = 0.044$). All results are detailed in **Table 1**.

To evaluate whether ACEi and ARB use by few patients have influenced our results, we divided the whole sample (AD and controls included) between ACEi/ARB users and non-users. These groups have shown no significant difference in Ang-(1–7) and Ang II levels (see **Supplementary Table 1**).

Neuroimaging Variables

MRI variables were first compared between groups. As expected, AD patients presented lower cortical volumes than controls in the hippocampus, medial temporal cortex, and precuneus (all $p < 0.01$). Values are reported in **Table 1**. Differences in Fazekas grades between the groups were not significant ($p = 0.21$). White

TABLE 1 | AD patients vs. controls: clinical characteristics, plasma angiotensins and neuroimaging.

	AD (*n* = 14)	Controls (*n* = 14)	*p*-value
Clinical data			
Age in years—mean ± SD	69.5 ± 8.8	66.0 ± 11.0	0.594[a]
Sex (female)—n (%)	6 (42)	8 (57)	0.706[b]
Hypertension—n (%)	8 (57)	5 (35)	0.449[b]
ACEi or ARB use—n (%)	5 (35)	2 (14)	0.384[b]
Diabetes—n (%)	2 (14)	1 (7)	>0.999[b]
Time of disease (years)—mean ± SD	3.3 ± 1.3	–	–
CSF biomarkers			
Aβ 42 (pg/ml)—mean ± SD	572 ± 109*	–	–
t-tau (pg/ml)—mean ± SD	742 ± 271*	–	–
p/tau (pg/ml)—mean ± SD	96 ± 37*	–	–
Cognitive tests			
MMSE—median (25th–75th percentile)	24.5 (24–26)	29 (28–30)	**<0.0001**[a]
FMT—5 min Delayed Recall (/10)—median (25th–75th percentile)	4 (2.75–5)	9 (7.75–10)	**<0.0001**[a]
Categorical Fluency (Animals)—median (25th–75th percentile)	12.5 (9.75–14.5)	17.5 (14–19.75)	**<0.001**[a]
FAB—median (25th–75th percentile)	14 (11–15.25)	15.5 (14–17)	**0.035**[a]
Plasma molecules			
Ang II pg/ml, median (25th–75th percentile)	61.4 (37.5–88.6)	61.7 (50.3–94.5)	0.602[a]
Ang-(1–7) pg/ml, median (25th–75th percentile)	101.5 (62.4–126.4)	209.3 (72.0–419.1)	**0.014**[a]
Ang-(1–7)/Ang II ratio, median (25th–75th percentile)	1.62 (1.24–2.12)	2.67 (1.63–6.17)	**0.044**[a]
MRI measures			
Hippocampus volume† mm^3 median (25th–75th percentile)	5,523 (5,183–6,504)	7,771 (7,303–8,241)[#]	**<0.0001**[a]
Medial temporal cortex volume ǂ † mm^3 median (25th–75th percentile)	5,283 (4,322–5,575)	6,858 (6,452–7,258)[#]	**<0.0001**[a]
Precuneus cortical volume†, mm^3 median (25th–75th percentile)	14,454 (13,800–15,029)	16,692 (15,471–17,702)[#]	**0.004**[a]
White matter hypointensities volume, mm^3 median (25th–75th percentile)	1,912 (1,409–3,436)	1,025 (645–1,697)[#]	**0.007**[a]

ACEi, Angiotensin-converting enzyme inhibitors; ARB, Angiotensin Receptor Blockers; FAB, Frontal Assessment Battery; FMT, Figure Memory Test; MMSE, Mini Mental State Examination; MRI, Magnetic Resonance Imaging; SD, standard deviation.

[a] *Mann Whitney U-test.*

[b] *Fisher's exact test.*

**n = 10.*

† *Regions reported as the sum of left and right hemisphere volumes in each subject.*

ǂ *Medial temporal cortex defined as the combination of entorhinal and parahippocampal cortices.*

[#] *n = 13.*

Significant findings (p < 0.05) are in bold.

matter hypointensities (WMHs) showed a significantly higher volume in AD patients (2,565 ± 1,775 mm^3) compared to controls (1,204 ± 675 mm^3), $p = 0.007$.

To verify whether T1w WMHs reflected WMLs in our sample, we grouped all participants (AD and controls) and evaluated the correlation between the Fazekas scale and WMHs volume. Confirming previous findings (Cedres et al., 2020), there was a significant correlation between WMHs and Fazekas grade in our cohort (Spearman's rho = 0.62, $p < 0.001$). Dividing subjects in three categories according to Fazekas' grade (0, 1, and 2), we showed WMHs volume was significantly different across three groups (Kruskal-Wallis test, $p < 0.01$). Results of this proof of concept are depicted in **Supplementary Figure 1**.

Correlations Between Neuroimaging Variables and Angiotensins

In the AD group, no significant correlation was found between plasma Ang II and MRI variables, namely hippocampus, medial temporal cortex, precuneus, and WMHs (see **Table 2**). In AD patients, Ang-(1–7) levels were not associated with any cortical measure of interest. In contrast, there was a positive and significant correlation between Ang-(1–7) values and WMHs volumes in AD (Spearman's rho = 0.56, p =0.038). The same relationship was not observed in controls (see **Figure 2**). In fact, controls did not present any significant correlations between MRI variables and angiotensins. All analyses are detailed in **Table 2**, whereas the main findings are shown in **Figure 2**.

DISCUSSION

To find whether RAS systemic axes were unbalanced in AD, we compared Ang II and Ang-(1–7) levels between AD patients and cognitively healthy controls. Our results showed that Ang-(1–7) was reduced in AD patients, whereas no difference was found in Ang II levels. Ang-(1–7)/Ang II ratio was lower in AD patients simply reflecting the difference in Ang-(1–7). Then, to investigate if systemic RAS is linked to brain pathology, we looked for correlations between plasma angiotensins and MRI variables. In AD patients, but not in controls, plasma levels of Ang-(1–7) correlated with WMHs. No association was found between angiotensins and selected cortical volumes.

It is noteworthy that all our significant findings regarded the RAS alternative axis' main peptide, Ang-(1–7). Before our study, Ang-(1–7) was already found to be reduced in AD patients (Jiang et al., 2016a), and mice models (Jiang et al., 2016b). The notion that AD patients may lack Ang-(1–7) is consistent with the peptide's alleged neuroprotective properties (Farag et al., 2017). To our knowledge, no prior study has evaluated Ang-(1–7) in relation to MRI measurements, neither in AD patients nor in controls. As for Ang II, however, our results contrast with a recent report by Yasar et al. (2020), who found an association between Ang II levels and hippocampal atrophy in cognitively healthy elders. The size of our sample and the dissimilar demographics may account for this difference. Another study (Zhuang et al., 2016) described a higher ACE activity in AD patients compared to controls. This would presumably result in higher Ang II levels, which were not verified in our sample. Differences in the target population may help explain the disparities, as Zhuang and colleagues selected subjects with moderate-to-severe AD.

In our results, it was unsurprising that WMHs' volume was higher in AD patients. In fact, MRI and post-mortem pathological studies reveal that cerebrovascular lesions

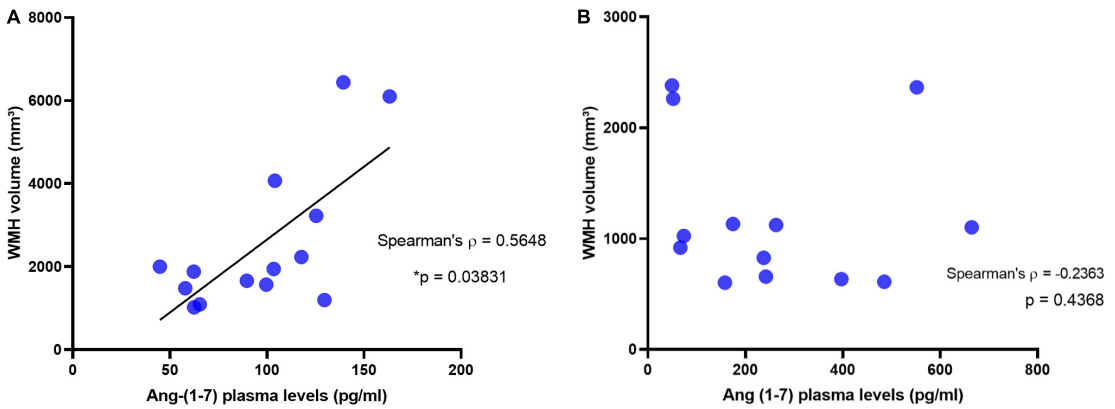

FIGURE 2 | Correlation between White Matter Hypointensities and Plasma Levels of Ang-(1–7) in AD patients and in controls. White matter hypointensities volumes positively correlated with Ang-(1–7) in AD patients **(A)**, but not in controls **(B)**.

TABLE 2 | Correlations between MRI variables and plasma angiotensins in AD patients and controls.

	Plasma Ang II	Plasma Ang-(1–7)	Ang-(1–7)/Ang II ratio
	Spearman's rho coefficient (95% confidence interval)		
AD patients			
Hippocampus volume	−0.05 (−0.57 to 0.5), p =0.868	0.13 (−0.44 to 0.63), p = 0.64	0.19 (−0.39 to 0.66), p = 0.512
Medial temporal cortex volume	−0.21 (−0.67 to 0.37), p = 0.464	0.02 (−0.52 to 0.56), p = 0.928	0.26 (−0.32 to 0.7), p = 0.357
Precuneus cortical volume	−0.43 (−0.79 to 0.14), p = 0.124	−0.21 (−0.67 to 0.37), p = 0.463	0.3 (−0.28 to 0.72), p = 0.295
White matter hypointensities volume	0.464 (−0.1 to 0.8), p = 0.097	**0.56 (0.03 to 0.84), p = 0.038**	−0.05 (−0.58 to 0.49), p = 0.844
Controls			
Hippocampus volume	0.09 (−0.49 to 0.62), p = 0.751	−0.36 (−0.77 to 0.24), p = 0.217	−0.38 (−0.77 to 0.22), p = 0.196
Medial temporal cortex volume	−0.02 (−0.57 to 0.54), p = 0.945	−0.45 (−0.8 to 0.15), p = 0.123	−0.36 (−0.77 to 0.24), p = 0.214
Precuneus cortical volume	0.22 (−0.39 to 0.69), p = 0.47	−0.09 (−0.62 to 0.49), p = 0.751	−0.32 (−0.75 to 0.29), p = 0.28
White matter hypointensities volume	−0.16 (−0.66 to 0.43), p = 0.591	−0.23 (−0.70 to 0.37), p = 0.437	−0.4 (−0.78 to 0.21), p = 0.176

Significant findings ($p < 0.05$) are in bold.

are more frequent in AD (Schneider et al., 2004, 2007; Jellinger and Attems, 2005; Attems and Jellinger, 2014; Suemoto et al., 2017; Hase et al., 2018). Classically, AD and cerebrovascular disease are considered independent entities, which often happen together only because the prevalence of both increase with age. Assuming such independence, cerebrovascular lesions would contribute to cognitive decline in AD patients only by reducing the "brain reserve," thus allowing symptoms to manifest earlier (Kapasi and Schneider, 2016; Raz et al., 2016). This notion has been challenged by mounting evidence of interaction between cerebrovascular disease and AD amyloid and tau pathologies (van Norden et al., 2012; Nucera and Hachinski, 2018; Solis et al., 2020). For instance, cerebral blood flow is dysregulated in AD (Jagust et al., 1997; Roher et al., 2012). Traditionally, ensuing brain hypoperfusion is interpreted as a late consequence of AD pathology, when neurodegeneration diminishes cerebral metabolism, and thus reduces the brain's need for blood. But there are clinical, radiological and pathological findings suggesting that the mechanisms are likely more complex: control of cerebral perfusion can be disrupted early in AD, and the reduction in blood supply may exceed the decline in metabolic demand (Ruitenberg et al., 2005; Binnewijzend et al., 2014; Hays et al., 2016; Love and Miners, 2016a). Some hypotheses go as far as to state that hypoperfusion can precede (or even induce) other key pathological events in AD (Niedermeyer, 2006; Hays et al., 2016; de la Torre, 2018). Moreover, cerebral blood flow dysregulation is possibly caused by functional changes, rather than a result of structural vascular abnormalities (e.g., atherosclerosis, cerebral amyloid angiopathy) (Kelleher and Soiza, 2013; Love and Miners, 2016b).

These changes take place at the level of the neurovascular unit, which adjusts the vascular tone so as that blood supply matches energy demand in the brain (a process named neurovascular coupling). In AD, a malfunctioning neurovascular unit fails to adequately regulate cerebral blood flow and weakens the blood-brain barrier (Benarroch, 2007; Zlokovic, 2011; Iadecola, 2017; Kisler et al., 2017).

If AD pathophysiology is actually influenced by vascular pathology and neurovascular unit dysfunction, then systemic RAS is likely an important player, including its alternative axis (Kangussu et al., 2020). Acting upon the Mas receptor, Ang-(1–7) mediates the alternative axis' anti-inflammatory, anti-oxidative and vasodilatory properties (Santos et al., 2018). Evidence from preclinical studies suggest that Ang-(1–7) is especially important in brain response to ischemia-hypoxia, increasing cerebral blood flow and preventing blood-brain barrier breakdown (Lu et al., 2008; Zhang et al., 2008; Wu et al., 2015). In animal models of chronic cerebral hypoperfusion, Ang-(1–7) induces tolerance to ischemia and improves cognitive function (Jiang et al., 2014; Xie et al., 2014). Ang-(1–7) has also been studied in mice models of AD. In SAMP8 mice, Ang-(1–7) was reduced and inversely correlated with Tau hyperphosphorylation (Jiang et al., 2016b). When constantly given to SAMP8 mice, Ang-(1–7) counteracted Ang II and prevented cognitive decline (Cao et al., 2019). In Tg2576 mice, upregulating RAS alternative axis reduced amyloid pathology and restored cognition (Evans et al., 2020). It is worth mentioning that no mice model credibly reproduces all the key features of sporadic AD (LaFerla and Green, 2012; Neha et al., 2014). Hence, pathological inferences from animal studies should be interpreted with caution. With such caveat in mind, we can state that preclinical data support the hypothesis of an Ang-(1–7) downregulation contributing to AD.

Against this background, we risk extrapolating our findings to hypothesize that AD patients produce less Ang-(1–7), which may contribute to their disease by diminishing the magnitude of Ang-(1–7) neuroprotective effects. We also speculate that, if confirmed, the positive correlation between plasma levels of Ang-(1–7) and cerebrovascular lesions in AD might result from some sort of response mechanism: for instance, Ang-(1–7) could be upregulated in an effort to counteract the underlying cerebrovascular disease and increase tolerance to ischemia. We assume that such attempt, however, would not raise Ang-(1–7) concentrations to the same levels seen in healthy individuals.

We are aware of this study's many limitations, starting with the sample size. Besides reducing power and generalizability, having a small sample limited our capacity to adjust results for possible confounders (e.g., hypertension, ACEi or ARB use). To minimize the chance of spurious results, we tried to keep groups balanced concerning variables that might interfere. We still recognize that when few subjects are analyzed, statistical positives can arise only by chance. Regarding the reduced Ang-(1–7) in AD, however, this possibility seems less likely in light of existing data (Jiang et al., 2016a). We also acknowledge that the study would benefit from having a second control group, ideally of patients with another dementia (e.g., vascular dementia). If such third group had been added, it would help to determine whether our findings are specific of AD or common to different dementias. Moreover, one of the inherent disadvantages of the cross-sectional design, not being able to establish causality constrained our attempts to explain mechanistically our main results. Likewise, the lack of histopathological data restricts the consistency of pathophysiological inferences we made. It should also be noticed that in this study, even the biochemical assessment of the RAS pathways was far from complete. To understand why AD patients have lower Ang-(1–7), we would have to look at the protein that produces it, angiotensin-converting enzyme 2 (ACE2). If ACE2 activity was found to be reduced in AD patients, it would explain the immediate mechanism behind their lack of Ang-(1–7). It would also be useful to measure ACE2 concentration together with its activity. Especially if the correlation between ACE2 levels and activity was strong, dosing both would guide future studies about which assays to perform (Chappell, 2015). Despite these limitations, we believe that, for an exploratory study, our methods were appropriate and achieved the goal of generating hypotheses about RAS-AD interaction.

CONCLUSION

In conclusion, our study strengthens the hypothesis that RAS alternative axis is downregulated in AD. It also points to a possible interaction between Ang-(1–7) and cerebrovascular lesions in AD patients. We hope these hypotheses will be addressed in the future by larger studies, with longitudinal follow-up and a more comprehensive assessment of the RAS molecules. We believe that, as AD pathogenesis remains largely unelucidated, it is important to follow every lead that may help to explain the disease. If confirmed, our findings corroborate the view that the RAS is a possible player in Alzheimer's disease pathophysiology.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Comitê de Ética em Pesquisa da Universidade Federal de Minas Gerais. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

VR, AS, and LS designed the study and wrote the protocol. TC proposed the neuroimaging protocol and conducted the MRI analysis. LP and RF planned and conducted the biomarker assays. LS, PC, and AT enrolled participants and performed neurological evaluation. VR undertook the statistical analysis, reviewed by AS and LS. All authors contributed to and have approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnins.2021.636754/full#supplementary-material

Supplementary Figure 1 | Validation of T1-weighted White Matter Hypointensities volume against Fazekas scale in the whole sample. White matter hypointensities volume correlated with Fazekas scale **(A)** and was different across groups defined by Fazekas score **(B)** - $p = 0.005$ (Kruskal-Wallis).

Supplementary Table 1 | Ang-(1–7) and Ang II plasma levels in ACEi/ARB users vs. non-users.

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Angiotensin-(1-7) Central Mechanisms After ICV Infusion in Hypertensive Transgenic (mRen2)27 Rats

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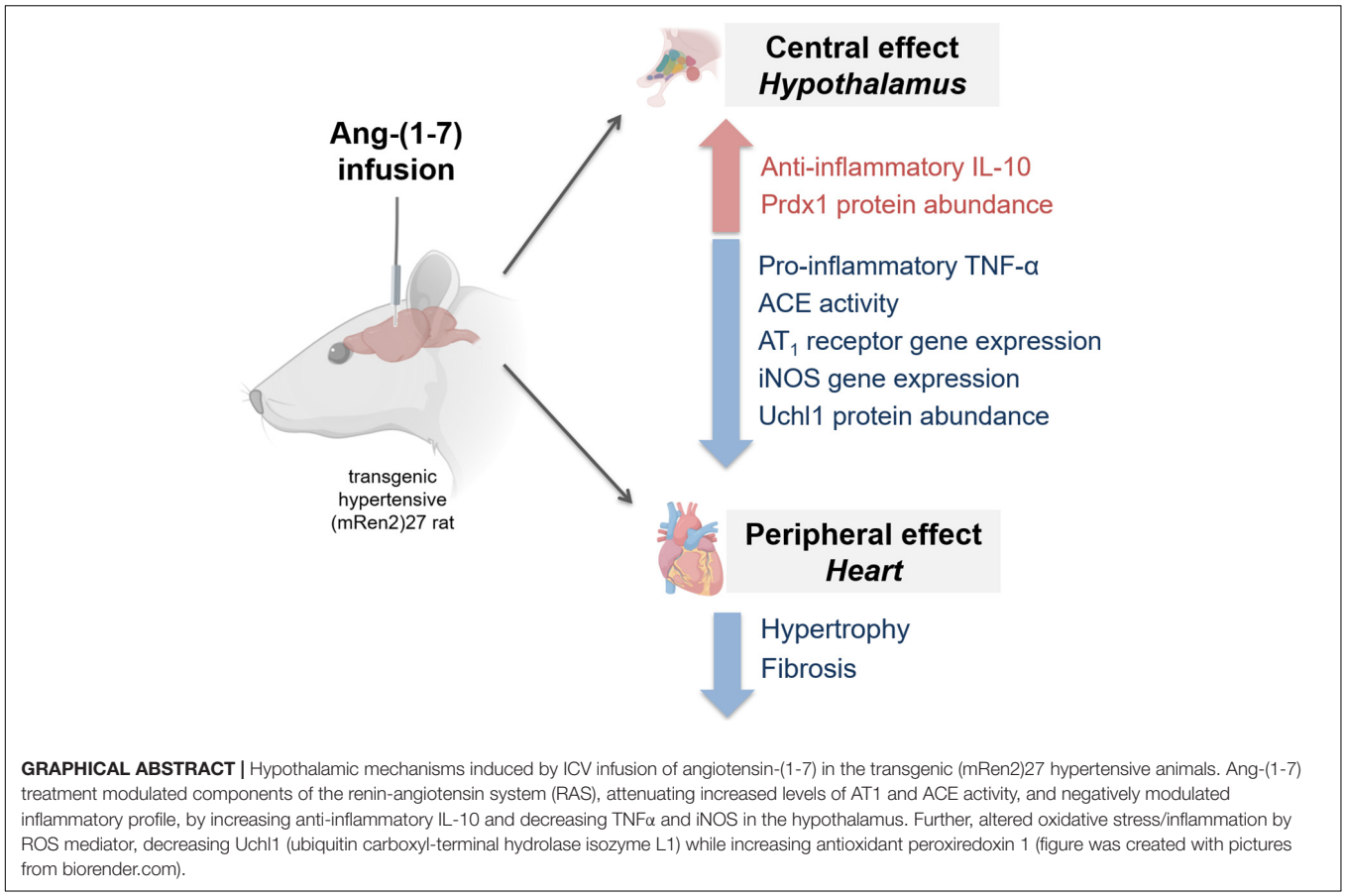
Previous data showed hypertensive rats subjected to chronic intracerebroventricular (ICV) infusion of angiotensin-(1-7) presented attenuation of arterial hypertension, improvement of baroreflex sensitivity, restoration of cardiac autonomic balance and a shift of cardiac renin-angiotensin system (RAS) balance toward Ang-(1-7)/Mas receptor. In the present study, we investigated putative central mechanisms related to the antihypertensive effect induced by ICV Ang-(1-7), including inflammatory mediators and the expression/activity of the RAS components in hypertensive rats. Furthermore, we performed a proteomic analysis to evaluate differentially regulated proteins in the hypothalamus of these animals. For this, Sprague Dawley (SD) and transgenic (mRen2)27 hypertensive rats (TG) were subjected to 14 days of ICV infusion with Ang-(1-7) (200 ng/h) or 0.9% sterile saline (0.5 μl/h) through osmotic mini-pumps. We observed that Ang-(1-7) treatment modulated inflammatory cytokines by decreasing TNF-α levels while increasing the anti-inflammatory IL-10. Moreover, we showed a reduction in ACE activity and gene expression of AT1 receptor and iNOS. Finally, our proteomic evaluation suggested an anti-inflammatory mechanism of Ang-(1-7) toward the ROS modulators Uchl1 and Prdx1.

Keywords: angiotensin-(1-7), hypothalamus, hypertensive transgenic (mRen2)27 rats, cytokines, iNOS, ROS modulators

INTRODUCTION

Angiotensin-(1-7) [Ang-(1-7)] is a key component of the renin-angiotensin system (RAS) and an important modulator of cardiovascular function (Santos et al., 2018). Its actions mainly counterbalance those effects of Angiotensin II (Ang II) in different tissues and several pathophysiological conditions (Santos et al., 2018). Of note, it is important to highlight that Ang-(1-7) actions go beyond modulation of the cardiovascular system and include effects on inflammation, stress coping behaviors, and neurodegenerative diseases (Kangussu et al., 2013;



GRAPHICAL ABSTRACT | Hypothalamic mechanisms induced by ICV infusion of angiotensin-(1-7) in the transgenic (mRen2)27 hypertensive animals. Ang-(1-7) treatment modulated components of the renin-angiotensin system (RAS), attenuating increased levels of AT1 and ACE activity, and negatively modulated inflammatory profile, by increasing anti-inflammatory IL-10 and decreasing TNFα and iNOS in the hypothalamus. Further, altered oxidative stress/inflammation by ROS mediator, decreasing Uchl1 (ubiquitin carboxyl-terminal hydrolase isozyme L1) while increasing antioxidant peroxiredoxin 1 (figure was created with pictures from biorender.com).

Rodrigues-Machado et al., 2013; Almeida-Santos et al., 2017; Magalhaes et al., 2018; Santos et al., 2018).

We have shown Ang-(1-7), upon Intracerebroventricular (ICV) infusion, improves baroreflex control of heart rate (HR), especially its bradycardic component, both in normotensive or hypertensive animals (Campagnole-Santos et al., 1992; Oliveira et al., 1996; Britto et al., 1997; Heringer-Walther et al., 2001; Guimaraes et al., 2012). Additionally, ICV infusion of Ang-(1-7) inhibited sympathetic and increased vagal drive to periphery in rabbits with chronic heart failure, thus contributing to improve baroreflex gain also in this condition (Kar et al., 2011). In addition, ICV infusion of Ang-(1-7) for 4 weeks significantly reduces the expression of Ang II and AT1 receptors in the brain of spontaneously hypertensive rats (Jiang et al., 2013). Besides, ICV administration of Ang-(1-7) (Dobruch et al., 2003) or the delivery of an Ang-(1-7) fusion protein in the cisterna magna (Garcia-Espinosa et al., 2012) attenuated high blood pressure of the hypertensive (mRen2)27 rats. Of note, the actions of Ang-(1-7) in the brain seem to be mostly mediated by Mas receptor (Kangussu et al., 2015; Santos et al., 2018), which was shown to be expressed in different areas (Becker et al., 2007; Freund et al., 2012).

Findings from our laboratory showed that ICV infusion of Ang-(1-7) attenuated hypertension, normalized baroreflex control of arterial pressure and the autonomic tone to the heart, and also prevented the increase in cardiac collagen type I mRNA expression in DOCA-salt hypertensive rats (Guimaraes et al., 2012). Furthermore, we also showed that Ang-(1-7)/Mas activation in the brain is capable of reducing cardiac hypertrophy and pre-fibrotic lesions and decreasing the altered imbalance of Ang II/Ang-(1-7) in the heart of hypertensive transgenic rats (mRen2)27 (Kangussu et al., 2015). These effects occurred in association with the improvement of baroreflex control of HR and cardiac autonomic control and decreased blood pressure. Although several brain effects were described, the central mechanisms triggered by Ang-(1-7) are still not fully understood.

In the present study, we evaluated whether a chronic increase in Ang-(1-7) in the brain could modulate inflammatory cytokines and expression/activity of RAS components in the hypothalamus of normotensive and hypertensive transgenic rats (mRen2)27. Furthermore, we have performed a proteomic analysis to evaluate the mechanisms involved in the central beneficial effects of Ang-(1-7) in arterial hypertension.

MATERIALS AND METHODS

Animals

Male heterozygous (mRen2)27 rats (TG; 10 to 12 weeks old) and age-matched normotensive control Sprague-Dawley (SD) rats were obtained from the animal facility of the Laboratory of Hypertension, Institute of Biological Sciences, Federal University

of Minas Gerais (UFMG), Brazil. Rats were housed in the animal facility and kept at controlled room temperature (22–24°C) and 12/12 h light/dark cycle. (mRen2)27 transgenic rat, that overexpress a mouse renin gene, is an interesting model of experimental hypertension, in which kidney and plasma renin activity are suppressed (Mullins et al., 1990) and the development and maintenance of hypertension has been attributed to the high activity of renin, and perhaps angiotensin II, in extrarenal tissues, such as, the adrenal glands, heart, brain and blood vessels (Bader et al., 1992; Campbell et al., 1995).

All procedures used in this study were approved and strictly followed the ethical principles of animal experimentation adopted by the Ethics Committee on Animal Use of Federal University of Minas Gerais and institutionally approved under protocol number 49/2013.

Chronic Intracerebroventricular (ICV) Infusion

Rats were anesthetized with tribromoethanol (25 mg/100 g of b.w., i.p.), for ICV infusion, a metallic cannula (guide cannula) was implanted into the right lateral ventricle (from the bregma: AP −1.0 mm; LL 1.5 mm; and DV −4.5 mm and cemented with three anchoring screws to the skull). ICV cannula was connected via vinyl tubing to an osmotic mini-pump (ALZET, model 2004), which was implanted subcutaneously between the scapulae. The infusion rate was 0.5 μl/h for 14 days. After surgery, rats received a poly-antibiotic (20U; Pentabiotico®, Fort Dodge, Brazil) and flunixin meglumine (1 mg/Kg, s.c.; Banamine®, Schering Plough, Brazil) for post-operation analgesia. The control groups normotensive (SD saline or SD CT) and transgenic hypertensive rats (mRen2)27 (TG saline or TG CT) received sterile isotonic saline. SD A7 and TG A7 groups received Ang-(1-7) at 200 ng/h. The site of infusion was verified postmortem by the presence of Alcian blue dye (5%), injected through the ICV cannula (2 μL), only in the ventricular system. Ang-(1-7) was purchased from Bachem, Germany.

Heart Histological Analysis

In a sub-group of animals, the heart beat was stopped in diastole using KCl (10%, i.v.). The heart was fixed in 10% neutral-buffered formalin solution and stained with hematoxylin and eosin for cell morphometry. Three sections (5 μm; with 10 μm intervals between each section) from each animal were visualized in a light microscope (BX41®; Olympus, Center Valley, PA, United States) photographed (Q-Color3™; Olympus, Center Valley, PA, United States) under 400x magnification and analyzed using the ImageJ software. Cardiomyocytes diameters of the left ventricular wall (∼50 cardiomyocytes for each animal) were measured across the region corresponding to the nucleus. Only cardiomyocytes cut longitudinally with nuclei and cellular limits visible were considered for analysis. All analyses were performed in a double-blind way by the same researcher. Levels of hydroxyproline in heart tissue were measured using the hydroxyproline assay kit (Sigma-Aldrich, St. Louis, MO, United States) according to the manufacturer instructions.

Evaluation of Cytokines in the Brain

Fourteen days After ICV infusion, all groups Had the hypothalamus extracted and homogenized (100 mg/mL of extraction solution). Brain homogenate Was centrifuged at 3,000 × *g* for 10 min at 4°C. the supernatant Was collected and stored at −20°C. Concentrations of interleukin-1α (IL-1α), interleukin-6 (IL-6), tumor necrosis factor-alpha (TNF-α) and interleukin-10 (IL-10) Were measured using commercially available antibodies according to the manufacturer (R&D Systems, Minneapolis, MN, United States) by enzyme-linked immunosorbent assay (ELISA). Results Were expressed as pg/100 mg of tissue.

Measurement of Angiotensinergic Receptors and Inducible Nitric Oxide Synthase (iNOS) mRNA Expression

Total RNA was obtained following the TRIzol reagent method (Invitrogen, Life Technologies, United States) according to the manufacturer's protocol. RNA samples (2 μg) were treated with DNase to eliminate genomic DNA present in the samples. mRNA expression was assessed by qRT-PCR after reverse transcription with MML-V (Moloney murine leukemia virus) (Invitrogen Life Technologies, United States). The cDNA for endogenous S26 ribosomal (endogenous control) and AT_1, AT_2, Mas receptors and iNOS were amplified using specific primers using SYBR Green reagent (Applied Biosystems, Foster City, NY, United States). The reactions were performed using 40 cycles and annealing temperature of 60°C (ABI Prism 7000, Applied Biosystems, Foster City, NY, United States). Gene expression was quantified using the comparative Ct (threshold cycle) method. Primers sequence of AT_1: 5′-GGT GGG AAT ATT GGA AAC AG-3′ (forward) and 5′-AAG AAG AAA AGC ACA ATC GCC-3′ (reverse); AT_2: 5′-GCT GAG TAA GCT GAT TTA TG-3′ (forward) and 5′-TTA AGA CAC AAA GGT GTC CA-3′ (reverse); Mas receptor: 5′-CCC ACC CAT TCC CAT AGT GC-3′ (forward) and 5′-CCG AGA GGA GAG ATG CTC ATG-3′ (reverse); iNOS: 5'-CCT TGT TCA GCT ACG CCT TC-3' (forward) and 5'-GGT ATG CCC GAG TTC TTT CA-3' (reverse); endogenous control S26: 5′-CGA TTC CTG ACA ACC TTG CTA TG-3′ (forward) and 5′-CGT GCT TCC CAA GCT CTA TGT-3′ (reverse).

Measurement of ACE and ACE2 Activity

Hypothalamus ACE activity was measured as previously described (Santos et al., 1985). Briefly, the hypothalamus homogenate was incubated at 37°C with the ACE substrate hippuryl-His-Leu (1 mM; Sigma-Aldrich) in a total volume of 500 μl buffer (0.4 M sodium borate buffer, 0.3 M NaCl, pH 8.3) in the presence or absence of the ACE-specific inhibitor captopril (10 μM), for 30 min. Following incubation, 120 μl of 0.3 N NaOH and 10 μl o-phthaldialdehyde (20 mg/ml in methanol) were added. After 10 min at room temperature, 20 μl of 3 N HCl was added and the tubes were centrifuged at 16,000 × *g* in a tabletop microcentrifuge for 5 min. Supernatants were transferred to black 96-well microplate. Fluorescence (excitation wavelength of 355 nm, emission wavelength of 485 nm) was measured using a

FLUOstar Optima plate reader (BMG Labtechnologies, Durham, NC, United States). The rate of substrate cleavage was determined by comparison with a standard curve of the His-Leu product.

The enzymatic activity of ACE2 was determined using a fluorogenic substrate (fluorogenic peptide VI; R&D Systems, United States). Enzymatic activity was measured with a Spectra Max Gemini EM Fluorescence Reader (Molecular Devices, United States), as previously described (Huang et al., 2003; Huentelman et al., 2004). Samples were read every minute for 60 min, beginning immediately after adding the fluorogenic peptide substrate at 37°C. The result of each sample was expressed as arbitrary units (a.u.) corresponding to the average of the fluorescence measured in the maximum velocity of the reaction, corrected for mg of protein measured by the Bradford method (Bradford, 1976).

Proteomic Analysis Based on Two-Dimensional Gel Electrophoresis (2DE)

Proteomic analysis was performed in hypothalamus from independent biological replicates of each group, SD CT (n = 4), SD A7 (n = 5), TG CT (n = 5), and TG A7 (n = 6). The first step was the removal of fat from the sample by washes with chloroform/methanol/water (4:8:3) v/v, for 1 h under stirring followed by centrifugation at 1,500 × g for 10 min at room temperature (Folch et al., 1957; Bligh and Dyer, 1959). This washing procedure was repeated three times. After this, the sample was allowed to dry at 4°C for 16 h. Then, to extract protein, the hypothalamus was resuspended in lysis buffer (8M urea, 2M thiourea, 4% CHAPS, 65 mM DTT, 40 mM Tris base, and a protease inhibitor mix (GE Healthcare, United States). The sample was incubated under agitation for 2 h and then centrifuged at 10,000 × g for 30 min. The soluble fraction was obtained and maintained at −80°C until use. According to the manufacturer's instructions, protein content was measured using the 2D-Quant kit (GE Healthcare, United States).

The experiment was conducted as described by Lima et al. (2017). Briefly, we first analyzed all independent biological replicates from each group with Coomassie 2D gels. We confirmed a high reproducibility (coefficient of variation ≤10%) regarding the total number of spots, their relative positions, and intensities (data not shown). Next, we performed the DIGE analysis with 150 μg of a pool containing biological replicates of each four animal groups labeled with 400 pmol fluorophore CyDye^{TM} (Cy2, Cy3, and Cy5) (GE Healthcare, United States), according to the experimental design (**Table 1**). A mixture of protein extracts from the four groups was labeled with Cy2 as an internal standard. The experiments were performed in triplicate. Then, the samples were loaded onto IPG strips (18 cm, pH 4–7; GE Healthcare, United States) for overnight rehydration at room temperature, following to IEF on an Ettan IPGphor system (GE Healthcare, United States) at 20°C and a maximum current of 50 μA/strip (see more detail in Lima et al., 2017). After reduction and alkylation, the strips were applied to a 12% SDS-PAGE within low-fluorescence glass plates (GE Healthcare, United States). The 2D-gel electrophoresis was performed in the dark using an Ettan DALT 6 unit (GE Healthcare, United States). Gels images were obtained on a Typhoon Trio laser imager (GE Healthcare, United States) and analyzed using DeCyder 2D software, Version 7.0 (GE Healthcare, United States). The statistic t-test with false discovery rate correction was used with $\alpha < 0.05$ of significance. Protein spots that showed high abundance in each animal group (p-value < 0.01) or qualitatively (detected in only one condition in a specific comparison) were selected for MS identification. The DIGE gels were also stained with colloidal CBB G-250 following procedures previously described (Neuhoff et al., 1988) to improve manually excised of selected spots. Spots were processed as described by Costa et al. (2011). Tryptic peptides were analyzed with a MALDI-ToF-ToF AB Sciex 5800 (AB Sciex, Foster City, CA, United States) mass spectrometer, following protein search using MASCOT as previously described (Lima et al., 2017).

TABLE 1 | DIGE experimental design.

Gel	Cy3	Cy5	Cy2
1	TG A7	TG CT	Internal standard
2	SD A7	SD CT	
3	SD A7	TG A7	
4	TG CT	SD A7	
5	TG A7	SD CT	
6	SD CT	TG CT	

Schematic Cy Dye labeling for each gel that contains a specific pool of protein extract from hypothalamus for each group.

Statistical Analysis

Data are expressed as mean ± SEM. Differences among groups were assessed by one-way ANOVA followed by Newman-Keuls *post hoc* test and were performed with GraphPad Prism software (version 6.0). All values were expressed as mean ± standard error of the mean (SEM). Statistical significance was assumed for all values at $p < 0.05$.

RESULTS

Chronic Ang-(1-7) ICV Infusion Decreased Cardiac Hypertrophy in TG Rats

In order to confirm our previous result, we have first evaluated alterations on cardiac structures, hypertrophy and fibrosis, in hypertensive rats subjected to chronic Ang-(1-7) ICV infusion by histology. **Figure 1A–D** shows representative images of the histology of the different groups. As expected, morphometric analysis of the images showed that TG animals presented cardiomyocytes hypertrophy (12.6 ± 0.16 μm, n = 6; **Figures 1C,E**) when compared to SD group (10.9 ± 0.13 μm, n = 6; **Figures 1A,E**). As we showed in previous work (Guimaraes et al., 2012; Kangussu et al., 2015), hypertensive animals treated with Ang-(1-7) showed significant attenuation of cardiac hypertrophy (11.6 ± 0.11 μm, n = 6; **Figures 1D,E**). Similar results were obtained when the level of hydroxyproline, a biochemical marker of collagen deposition, was evaluated

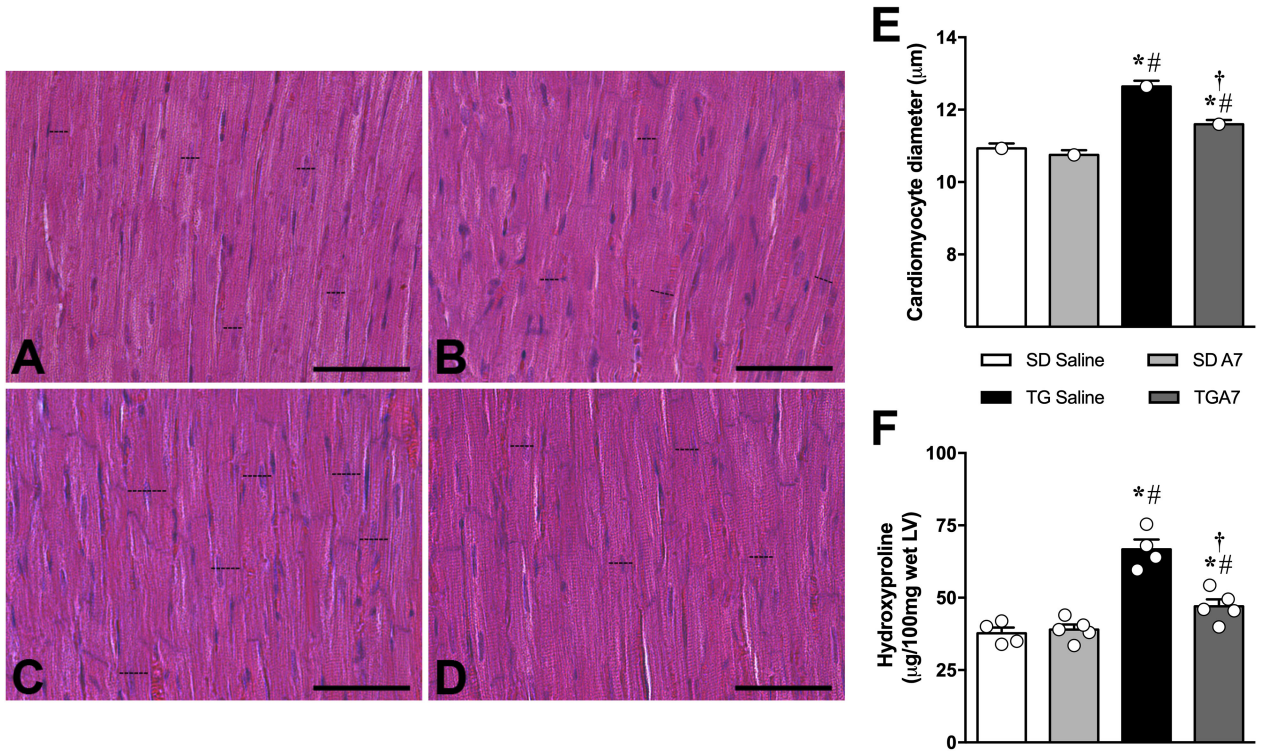


FIGURE 1 | Representative photomicrographs of sections of the left ventricle of sections control (SD saline, $n = 5$); **(A)**, control treated with Ang-(1-7) (SD A7, $n = 4$); **(B)**, hypertensive rats control (TG saline, $n = 4$); **(C)** and hypertensive rats treated with ICV Ang-(1-7) (TG A7, $n = 5$); **(D)**. Bar = 20 μm. Stain: hematoxylin/eosin. The cardiomyocyte diameter **(E)** of the free wall LV and Hydroxyproline as a fibrotic marker **(F)**. $^{*}p < 0.05$ vs. SD saline; $^{\#}p < 0.05$ vs. SD A7; $^{\dagger}p < 0.05$ vs. TG saline (One-way ANOVA followed by Newman-Keuls *post hoc* test).

(**Figure 1F**). TG CT group revealed a remarkable increased hydroxyproline level (66.7 ± 1.3 μg, $n = 5$; **Figure 1F**) in the LV when compared to SD CT group (37.7 ± 1.9 μg, $n = 5$; **Figure 1F**) while ICV treatment with Ang- (1-7) attenuated hydroxyproline levels in TG animals (47.0 ± 2.37 μg, $n = 5$; **Figure 1F**).

Chronic Ang-(1-7) ICV Infusion Modulated Cytokines in the Hypothalamus of TG Rats

One of our hypotheses for the central mechanism associated with the anti-hypertensive effect of Ang-(1-7) in transgenic hypertensive rats was a modulation of inflammatory mediators levels, as cytokines in the hypothalamus. As expected, TG animals showed higher levels of different pro-inflammatory cytokines in comparison to SD control group: IL-1α (48 ± 3.5 vs. 23 ± 1.8 pg/mg – **Figure 2A**), IL-6 (61 ± 2.7 vs. 38 ± 3.4 pg/mg – **Figure 2B**) and TNF-α, (76 ± 4.3 pg/mg vs. 39 ± 3.5 pg/mg – **Figure 2C**). No change was observed for the anti-inflammatory cytokine, IL-10 (**Figure 2D**). ICV infusion of Ang-(1-7) mitigated TNF-α levels in TG group (48 ± 3.4 pg/mg, $n = 6$; **Figure 2C**) when compared to untreated TG rats (76 ± 4.3 pg/mg, $n = 6$; **Figure 2C**). Moreover, ICV Ang-(1-7) significantly increased IL-10 levels in TG rats (32 ± 2.6 pg/mg vs. 19 ± 1.2 pg/mg, untreated TG, $n = 6$ each; **Figure 2D**). Ang-(1-7) did not change IL-1α and IL-6 levels in the hypothalamus of TG rats (**Figures 2A,B**, respectively).

Evaluation of Renin-Angiotensin System (RAS) Components in the Hypothalamus of TG Rats

Among the main pathophysiological mechanisms of arterial hypertension is the hyperactivity of the renin-angiotensin system (RAS), characterized by an increase in Ang II in plasma and tissue concentration, as previously reported in these hypertensive transgenic rat model (Bader et al., 1992; Campbell et al., 1995; Nakagawa and Sigmund, 2017). Therefore, we evaluated the alteration in gene expression of main angiotensinergic receptors. Hypertensive untreated TG rats showed increased gene expression of AT_1 (4.5 ± 0.5 a.u.; **Figure 3A**) and AT_2 receptor (3.2 ± 0.3 a.u., **Figure 3B**) when compared to SD group (1.2 ± 0.1 a.u. and 1.3 ± 0.2 a.u., respectively; $n = 5$ each; **Figures 3A,B**). Interestingly, chronic administration of Ang-(1-7) was able to attenuate AT_1 receptor mRNA expression in the hypothalamus of hypertensive animals (2.5 ± 0.25 a.u., $n = 5$; **Figure 3A**) without altering the increased AT_2 receptor gene expression (3.2 ± 0.2 a.u., $n = 5$; **Figure 3B**). Gene expression of Mas receptor was not altered in untreated or Ang-(1-7) treated TG rats (**Figure 3C**).

Regarding the activity of ACE, TG hypertensive untreated rats showed increased activity (21 ± 2.5 nmoles of His-Leu/min/mg

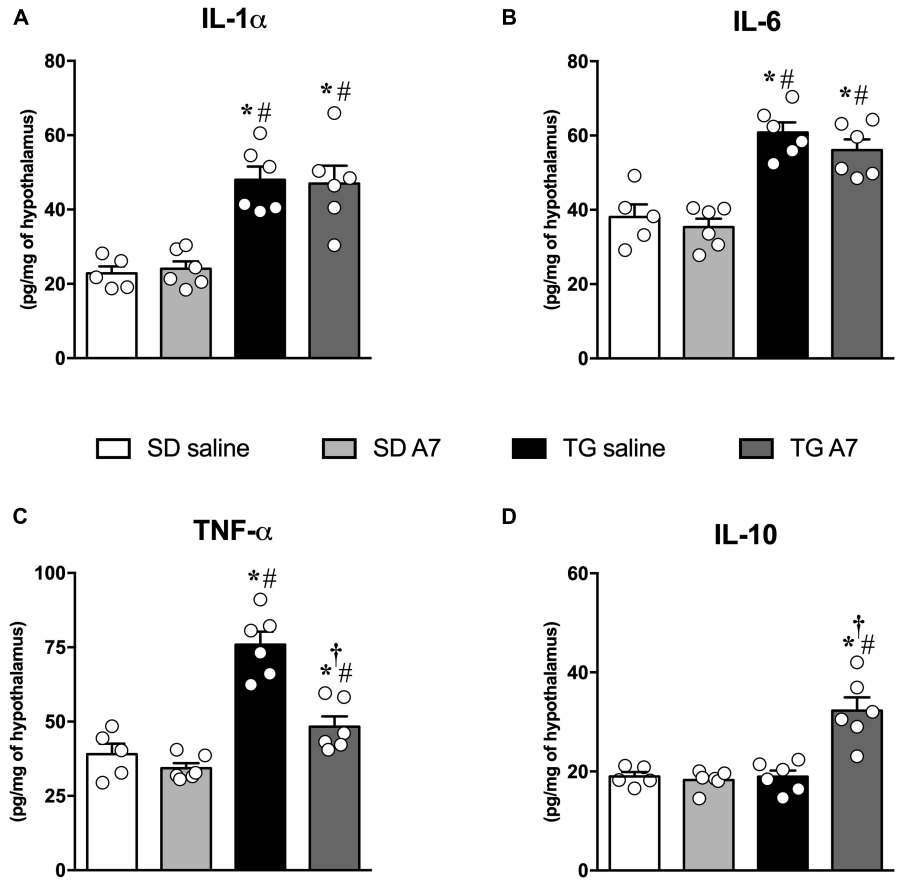


FIGURE 2 | Concentrations of pro-inflammatory cytokines in the hypothalamus in SD and hypertensive rats treated with Ang-(1-7) ICV. interleukin-1 alpha (IL-1α) **(A)**, interleukin-6 (IL-6) **(B)**, tumor necrosis factor-alpha (TNF-α) **(C)** and anti-inflammatory cytokine, interleukin-10 (IL-10) **(D)** were measured using commercially available antibodies by enzyme-linked immunosorbent assay (ELISA). Results are expressed as mean ± SEM. $n = 5$ or 6. $^{*}p < 0.05$ vs. SD saline; $^{\#}p < 0.05$ vs. SD A7; $^{\dagger}p < 0.05$ vs. TG saline (One-way ANOVA followed by Newman-Keuls *post hoc* test).

of protein, $n = 5$; **Figure 3D**) when compared to normotensive control animals (17 ± 1.3 nmoles of His-Leu/min/mg of protein; $n = 4$; **Figure 3D**). Chronic Ang-(1-7) ICV infusion induced a pronounced reduction in ACE activity in the hypothalamus of TG rats to the level of the normotensive control group (13 ± 0.7 nmoles of His-Leu/min/mg of protein, $n = 5$; **Figure 3D**). In addition, there was no alteration in ACE2 activity in the hypothalamus among all groups (**Figure 3E**).

Evaluation of iNOS Expression in the Hypothalamus

Another important protein in the pathophysiology of hypertension is iNOS (Oliveira-Paula et al., 2014). Therefore, we have measured iNOS gene expression in the hypothalamus of TG rats. As can be seen in **Figure 4**, hypertensive untreated TG rats showed increased gene expression of iNOS (5.1 ± 0.4 a.u., $n = 5$) when compared to SD group (1.1 ± 0.1 a.u.; 1.3 ± 0.2 a.u., $n = 5$). Ang-(1-7) ICV was able to attenuate the increased iNOS gene expression in the hypothalamus of TG animals (3.7 ± 0.2 a.u., $n = 5$).

Proteomic Analysis of Hypothalamus After Chronic ICV Infusion of Ang-(1-7)

Next, the effect of Ang-(1-7) ICV infusion in transgenic (mRen2)27 hypertensive animals was evaluated at the proteome level by combining 2D electrophoresis and MALDI-TOF/TOF approaches. **Figure 5** shows a representative 2-DE gel indicating protein spots, which had matched identification between the groups in the different comparison aimed in this work (TG CT vs. TG A7, SD CT vs. SD A7, and SD CT vs. TG CT). Our results revealed a highly similar profile of all four analyzed conditions (SDCT, SDA7, TGCT and TGA7), containing few qualitative and significant quantitative differences, as shown in **Table 2**. Treatment with Ang-(1-7) in TG hypertensive rats negatively modulated three proteins (Uchl1, Prdx2 and Pebp1, also known as HCNP) and one proteoform (Uchl1), while positively modulated Prdx1. In the SD animals, ICV Ang-(1-7) positively regulated three proteins (Prdx2, Prdx1, and Ppia, also known as CypA) and negatively regulated one protein, ubiquitin carboxyl-terminal hydrolase isozyme L1 (Uchl1) and its proteoform. Notably, treatment with Ang-(1-7) decreased the expression level of two proteoforms of Uchl1 while increased

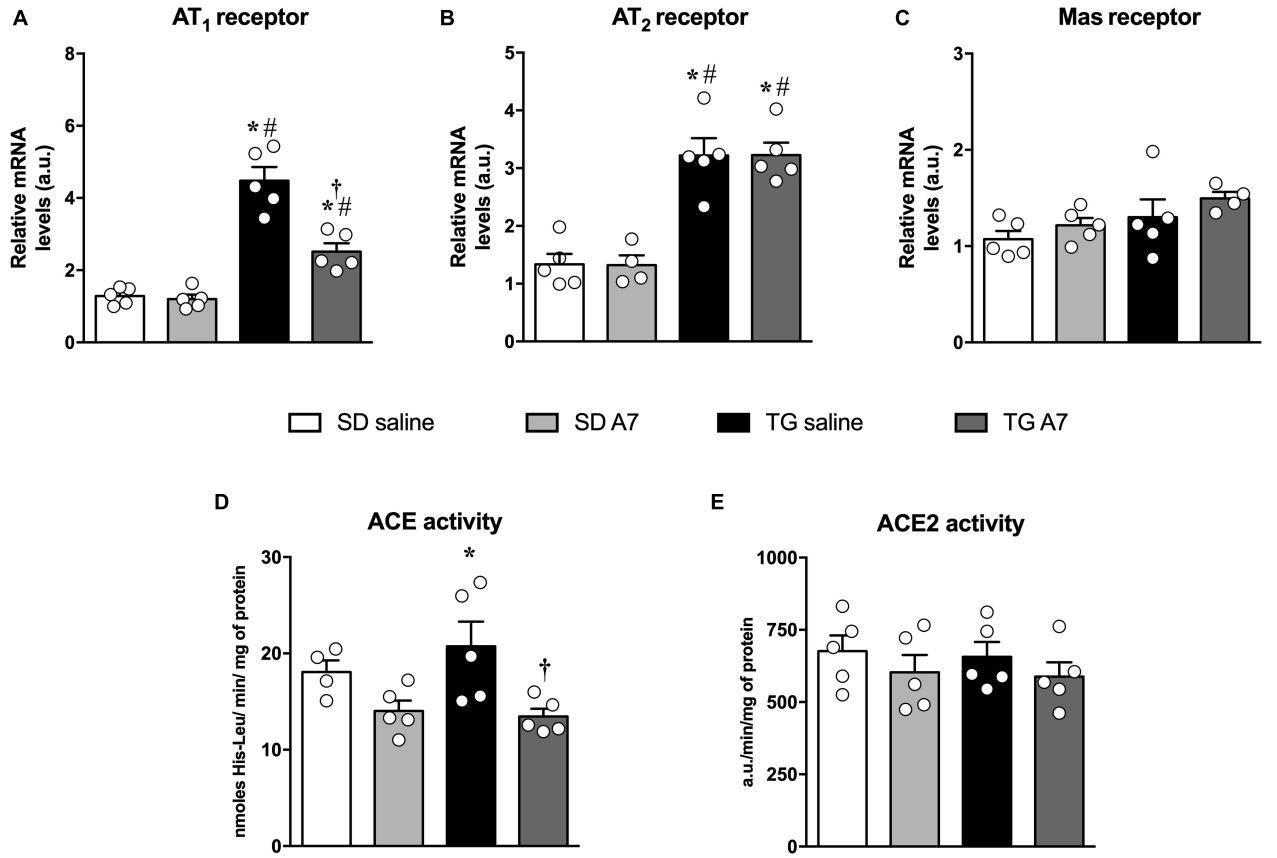


FIGURE 3 | Expression or activity of renin-angiotensin components in the hypothalamus in SD and hypertensive rats treated with Ang-(1-7) ICV. AT_1 receptor **(A)**, AT_2 receptor **(B)**, Mas receptor **(C)**, ACE activity **(D)**, and ACE2 activity **(E)**. Results are expressed as mean ± SEM. $n = 4$ or 5. $^{*}p < 0.05$ vs. SD saline; $^{\#}p < 0.05$ vs. SD A7; $^{\dagger}p < 0.05$ vs. TG saline (One-way ANOVA followed by Newman-Keuls *post hoc* test).

the abundance of Prdx1 in both normotensive and hypertensive groups. On the other hand, we observed opposing expression levels for Prdx2 in SD and TG groups after chronic infusion of Ang-(1-7), i.e., upregulation in the former and downregulation in the latter group. However, it seems to be two different proteoforms, since they were identified with different mass and pI (**Table 2**). Moreover, three proteins were differentially regulated in TG CT in comparison to SD CT (Pdia3, Prdx1, and Ppia; **Table 2**).

DISCUSSION

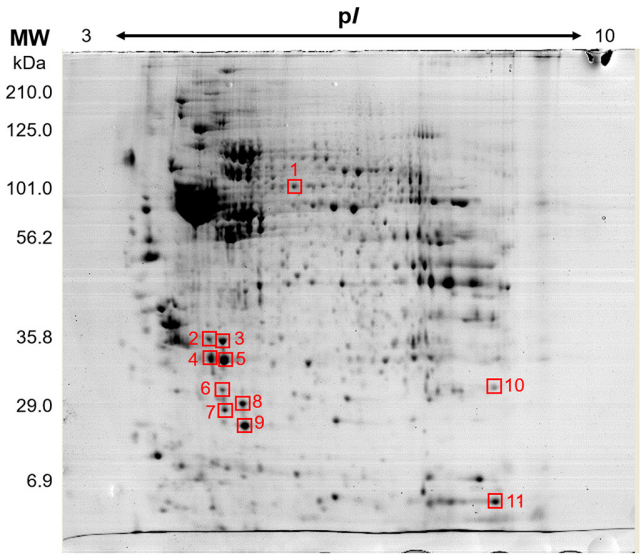
Ang-(1-7) mechanisms of action in the heart have been extensively studied and its protective role is well recognized (Santos et al., 2018). We have previously shown that Ang-(1-7) ICV treatment lowered blood pressure and improved cardiac function in hypertensive rats (Guimaraes et al., 2012; Kangussu et al., 2015), as well as attenuated metabolic syndrome induced by fructose intake (Guimaraes et al., 2014). However, the mechanisms triggered by Ang-(1-7) in the brain are yet not very well understood. Here, we present the effect of ICV infusion with Ang-(1-7) on mediators in the hypothalamus of hypertensive transgenic (mRen2)27 rats. These effects include a decrease in TNF-α, decrease in ACE activity, reduction in gene expression of Ang II AT_1 receptor and iNOS, an increase in the anti-inflammatory cytokine, IL-10, and alteration in proteins related to ROS modulation with decreased abundance of Uchl1 and increased abundance of Prdx1.

It has been shown that neurogenic models of hypertension present increase reactive oxygen species, activation of NF-κB and production of TNF-α in the brain. These effects indicate an activation of glial cells and the production of pro-inflammatory cytokines, thus contributing to the neurohumoral excitation observed in hypertension (Kang et al., 2008; Sriramula et al., 2008). The hypothalamus is one important site in the CNS where inflammatory signals are involved in pathophysiology of hypertension. Studies have shown that main neuroactive cytokines involved in hypothalamic inflammatory mechanisms related to cardiovascular diseases are TNF-α, IL-6, IL1-α/IL-1β, and IL-10. TNF-α and IL-1-β can increase the activity of cyclooxygenase-2 in perivascular macrophages to generate prostaglandin E2, increasing the discharge of PVN neurons, which in turn regulate adrenocorticotropic hormone release, sympathetic outflow, and ultimately blood pressure elevation (Khor and Cai, 2017). Following this research line,

FIGURE 4 | Expression of mRNA for iNOS in the hypothalamus in SD and hypertensive rats treated with Ang-(1-7) ICV. Results are expressed as mean ± SEM. $n = 4$ or 5. $^{*}p < 0.05$ vs. SD saline; $^{\#}p < 0.05$ vs. SD A7; $^{\dagger}p < 0.05$ vs. TG saline (One-way ANOVA followed by Newman-Keuls *post hoc* test).

Kang et al. (2009) have investigated the involvement of RAS components and brain cytokines in the induction of heart failure by ligation of the anterior descending coronary artery. The authors found that ICV infusion with losartan, SN50 (NF-κB nuclear translocation inhibitor) or tempol (superoxide anion scavengers) significantly attenuated gene expression of AT_1, NADPH oxidase, NF-κB-p50 receptors, as well as the production of pro-inflammatory cytokines in the PVN. Moreover, Shi et al. (2010) showed that microglial activation and production of pro-inflammatory cytokines in PVN contributes to the neurogenic hypertension pathophysiology. In addition, inhibition of microglial activation (reduced production of pro-inflammatory cytokines) or overexpression of IL-10 (an anti-inflammatory cytokine) in PVN through viral transfection attenuates Ang II-induced hypertension (Shi et al., 2010).

Interesting observations were made by Cardinale et al. (2012), among the pathophysiological mechanisms of Ang II-induced hypertension, an important modulation of NF-κB in PVN. Chronic treatment with a sequence of inhibition of NF-κB bilaterally in PVN for 14 days was able to reduce blood pressure, reduce the activity of the p65 subunit of NF-κB, pro-inflammatory cytokines, reactive oxygen species, AT_1 receptor expression and ACE activity in PVN (Cardinale et al., 2012). Additionally, Sriramula et al. (2013) demonstrated that the infusion of Ang II for 28 days in Sprague-Dawley rats induced a significant increase in mean arterial pressure and cardiac hypertrophy. Interestingly, hypertension and cardiac hypertrophy were alleviated by ICV administration of Etanercept (TNF-α inhibitor). Infusion of Ang II also induced an increase in the expression of ACE and the AT_1 receptor and reduced the expression of ACE2 and the Mas and AT_2 receptors in PVN. Treatment with the TNF-α inhibitor was able to reverse all these changes (Sriramula et al., 2013). Among its central actions, Ang-(1–7) can exert direct effects on microglia lowering the release pro-inflammatory cytokines, as well as, by attenuating the



Identified spots

TG CT	TG A7	SD CT	SD A7	Gene
#1	#54	-	-	Pdia3
#2	#59	#42	#18	Uchl1
#3	#60	#43	#19	Uchl1
#4	-	#44	-	Uchl1
#5	-	#45	-	Uchl1
#6	-	#46	#20	Prdx2
#7	#61	#47	-	Prdx2
#8	-	#48	#21	Pebp1/HCNP
#9	#62	#49	-	Pebp1/HCNP
#10	#63	-	#24	Prdx1
#11	#64	-	#25	Ppia/CypA

FIGURE 5 | Representative 2-DE gel image of DIGE-stained proteins (gray view). The represented gel is from the TG CT group and the squares indicate the identified spots. The panel on the right shows the number of the matched spots identified in each analyzed group, where (-) means not detected. The panel also include the gene from the identified protein. Pdia3, protein disulfide-isomerase A3; Uchl1, ubiquitin carboxyl-terminal hydrolase isozyme L1; Prdx2, peroxiredoxin-2; Pebp1/HCNP, phosphatidylethanolamine-binding protein 1; Prdx1, peroxiredoxin-1; Ppia/CypA, peptidyl-prolyl *cis-trans* isomerase A/cyclophilin A.

TABLE 2 | Identification of differentially expressed proteins in the hypothalamus of Sprague-Dawley (SD) and TGR (mRen2)27 (TG) rat treated with 14 days of Ang-(1–7) (A7) or saline ICV infusion the control group (CT).

Spot	Protein	Accession	Gene	Theor. pI	Theor. Mass (Da)	Obs. pI	Obs. Mass (Da)	Abundance
TG CT vs. TG A7								
4	Ubiquitin carboxyl-terminal hydrolase isozyme L1	gi| 61098212	Uchl1	5.14	24838.26	4.31	32000	↓ A7 (qualitative)
5	Ubiquitin carboxyl-terminal hydrolase isozyme L1	gi| 61098212	Uchl1	5.14	24838.26	4.56	32000	↓ A7 (qualitative)
6	Peroxiredoxin-2	gi| 8394432	Prdx2	5.34	21783.69	4.49	29000	↓ A7 (qualitative)
8	Phosphatidylethanolamine-binding protein 1	gi| 8393910	Pebp1/HCNP	5.47	20801.39	4.87	25000	↓ A7 (qualitative)
10	Peroxiredoxin-1	gi| 6435547	Prdx1	8.27	22109.41	9.39	30000	↑ A7 (3.47*)
SD CT vs. SD A7								
17	Peroxiredoxin-2	gi| 8394432	Prdx2	5.34	21783.69	5.14	35000	↑ A7 (qualitative)
24	Peroxiredoxin-1	gi| 6435547	Prdx1	8.27	22109.41	9.3	30000	↑ A7 (qualitative)
25	Peptidyl-prolyl *cis-trans* isomerase A/cyclophilin A	gi| 8394009	Ppia/CypA	8.34	17874.33	9.31	6000	↑ A7 (qualitative)
44	Ubiquitin carboxyl-terminal hydrolase isozyme L1	gi| 61098212	Uchl1	5.14	24838.26	5.02	32000	↓ A7 (qualitative)
45	Ubiquitin carboxyl-terminal hydrolase isozyme L1	gi| 61098212	Uchl1	5.14	24838.26	5.18	32000	↓ A7 (qualitative)
TG CT vs. SD CT								
1	Protein disulfide-isomerase A3	gi| 1352384	Pdia3	5.88	56623.37	5.69	94000	↑ TG (qualitative)
10	Peroxiredoxin-1	gi| 6435547	Prdx1	8.27	22109.41	9.39	30000	↑ TG (qualitative)
11	Peptidyl-prolyl *cis-trans* isomerase A/cyclophilin A	gi| 8394009	Ppia/CypA	8.34	17874.33	9.12	6000	↑ TG (qualitative)

gi, NCBI database accession number; Theor, theoretical; pI, isoelectric point; Obs, observed.
**Fold change TG A7/TG CT (%volume), $p < 0.01$.*
Table shows only the proteins that were differentially changed in the 3 comparisons presented, i.e., TG-A7 vs. TG, SD-A7 vs. SD and TG vs. SD. Qualitative means that using our approach and considering its limit of detection, the specific protein/proteoform was detected (up red arrow) or not detected (down blue arrow) in one of the group of the comparison (A7 or TG).

prorenin-induced increases in pro-inflammatory cytokines (Liu et al., 2016). In addition, PVN overexpression of ACE2 attenuates the increase in TNF-α, IL-1β, and IL-6 (Sriramula et al., 2011).

Therefore, taken together, our results are in line with these findings showing that our transgenic hypertensive animals have higher levels of pro-inflammatory cytokines (e.g., TNF-α, IL-1α, and IL-6), higher ACE activity and higher expression of AT_1 receptor. Further, we now advanced these observations by showing, for the first time, the chronic ICV infusion of Ang-(1-7) in (mRen2)27 rats modulate the RAS components (decreasing AT_1 receptor and ACE activity) and inflammatory mediators (diminishing TNF and increasing IL-10) in the hypothalamus. It is possible the reduction in ACE activity may also contribute to increase hypothalamic level of Ang-(1-7), since, ACE not only reduce Ang II generation but also decrease Ang-(1-7) degradation. These effects, combined with the reduction of AT_1 expression, positively contributes to ultimately decrease BP and fibrotic cardiac effect of Ang-(1-7). Although, Ang-(1-7) treatment did not alter the high levels of IL-1α and IL-6 in (mRen2)27 rats, the shift in the hypothalamic inflammatory condition caused by the alteration in the relationship between the important pro- and anti-inflammatory mediators, TNF-α and IL-10, have certainly contributed to lower blood pressure and to mitigate cardiac remodeling in (mRen2)27 rats. Of note, IL-10 is a potent anti-inflammatory and immune regulatory cytokine that contributes importantly to curtailing the inflammatory response, and more importantly to promoting resolution of inflammation. In addition to reduce the level of pro-inflammatory cytokine production by activated CNS cells (Moore et al., 2001), IL-10 can alter microglial phenotype polarization from the predominantly inflammatory "M1" phenotype to a more immunoregulatory "M2" phenotype that expresses protective and/or repairing factors (Deng et al., 2012; Mingomataj and Bakiri, 2016). Thus, IL-10 is generally considered to be the quintessential immunosuppressive cytokine produced within the CNS (Burmeister and Marriott, 2018).

In hypertensive states, elevated levels of reactive oxygen species (ROS) or reactive nitrogen species such as superoxide anion (O_2^-) and nitric oxide (NO), respectively, may be altered in medullary areas related to cardiovascular control, such as rostral ventrolateral medulla (RVLM) (Tai et al., 2005), caudal (CVLM) (Braga et al., 2011) and hypothalamic area (Khor and Cai, 2017). While relatively small amounts of NO plays an important role in cardiovascular homeostasis, high NO levels may have detrimental consequences to the cardiovascular system and contribute to hypertension (Oliveira-Paula et al., 2014). NO in the CNS, including the brainstem and hypothalamus, plays an important role in the regulation of blood pressure via the sympathetic nervous system. Its enzymatic formation is derived from three types of NO synthase (NOS): neuronal, endothelial and inducible. The latest, also known as iNOS, is not usually expressed in cells, but its expression can be induced by cytokines, for example, and the exaggerated expression of iNOS can lead to hypertension (Zanzinger, 1999; Patel et al., 2001; Kimura et al., 2005). Kimura et al. (2005, 2009) showed that overexpression of iNOS in the rostral ventrolateral medulla (RVLM) activates the sympathetic nervous system, inducing hypertension, probably by an increase in oxidative stress in this area. Furthermore, iNOS levels in the RVLM were significantly higher in SHR than in Wistar-Kyoto rats (WKY). Furthermore, a decreased BP and heart rate in SHR, but not in WKY, was observed after bilateral microinjection of aminoguanidine (iNOS

inhibitor) into the RVLM. Our present results show that, as expected, (mRen2)27 rats presented high levels of iNOS in the hypothalamus. Interestingly, ICV Ang-(1-7) infusion mitigates this increase, consequently attenuating the hypertension effects.

In the present study, we also observed a higher protein abundance of Ppia/CypA in (mRen2)27 hypertensive compared to normotensive rats. Ppia/CypA facilitates protein folding, and under inflammatory conditions, such as oxidative stress, Ppia/CypA can be secreted. Its extracellular form stimulates pro-inflammatory mediators in endothelial cells and vascular smooth muscle cells (VSMC) (Nigro et al., 2013). In fact, others have shown that Ppia/CypA enhanced NF-κB activity by promoting its stability and nuclear translocation (Sun et al., 2014). Besides, Ppia/CypA is also involved in the ROS production, vascular remodeling, cardiac hypertrophy, matrix degradation, and it has been shown that Ang II induces Ppia/CypA release (Satoh et al., 2009; Nigro et al., 2013).

Also comparing hypertensive and normotensive rats, our findings showed an increased level of Ang II AT_2 receptor gene expression, protein abundance of antioxidant Peroxiredoxin-1 (Prdx1) and protein folding disulfide-isomerase A3 (Pdia3). These results indicate a compensatory fight response mechanism against the higher stress situation on (mRen2)27 rats to preserve cell function and survival. The peroxiredoxins (Prdx) are antioxidant enzymes that protect the organism against hydrogen peroxide (H_2O_2)-induced oxidative stress. The balance of the redox system is essential since high levels of ROS induce apoptosis (Finkel, 1998). Therefore, peroxiredoxins have an important role in cellular homeostasis by decreasing H_2O_2 levels and consequent downstream responses (Chae et al., 1999). Several molecules induce the production of H_2O_2, such as TNF-α and the downstream effects include the expression of pro-inflammatory molecules as IL-1 and IL-6 (Kang et al., 2004). Also, cytosolic peroxiredoxins have been suggested to be an important regulator of TNF signaling pathways (Kang et al., 2004). Therefore, the increase in Pdrx1 expression may counterbalance H_2O_2 and consequently reduce the expression of pro-inflammatory effectors such as TNF-α to avoid apoptosis and cell death. However, the redox balance still favors the pro-inflammatory side. The other compensatory mechanism related to higher ROS levels is associated with Pdia3, a chaperon protein involved in reconstructing misfolded proteins by disulfide bond formation. Pdia3 has a neuroprotective role against the aggregation of ROS-induced misfolding proteins (Andreu et al., 2012). A pro-apoptotic function has been related to Pdia3, providing a link between unfolded protein and apoptotic signaling (Zhao et al., 2015).

Our proteomic data also revealed that treatment with Ang-(1-7) decreased the expression level of two proteoforms of the ubiquitin carboxyl-terminal hydrolase isozyme L1 (Uchl1) in both normotensive and hypertensive groups. Uchl1 is highly expressed in the brain and is required for axonal integrity maintenance. It has also been suggested to be a biomarker for traumatic brain injury (Papa et al., 2010). This protein belongs to the ubiquitin system and has a significant role in the regulation of several cellular processes, controlling protein activity and abundance. Misfolding proteins are usually ubiquitinated and then degraded via the 26S proteasome or by lysosomal degradation. The dimer form of Uchl1 has ubiquitin-ligase activity, while its monomer form functions as a deubiquitylating enzyme (Bishop et al., 2016). Moreover, Uchl1 promotes H_2O_2 production by upregulating NADPH oxidase 4 (NOX4) activity through deubiquitination in the migration process of cancer cells (Kim et al., 2015), as well as in angiogenesis (Song et al., 2018). Furthermore, Ang-(1-7) treatment increased the levels of the antioxidant Prdx1 in both groups while increased the level of antioxidant peroxiredoxin-2 (Prdx2) only in the SD treated group. As mentioned before, these proteins are important regulators of oxidative stress and inflammation by decreasing the H_2O_2 levels and consequent its downstream responses. Therefore, one of the anti-inflammatory mechanisms triggered by Ang-(1-7) seems to be decreasing the levels of ROS, such as H_2O_2, by decreasing the abundance of ROS generation modulator Uchl1 while increasing ROS scavengers Prdx1 and Prdx2.

Surprisingly, we found a decreased abundance of the Prdx2 after Ang-(1-7) infusion in the hypertensive group. As mentioned before, this proteoform is different from the other identified with higher abundance after Ang-(1-7) infusion in the normotensive group. Therefore, we believe they may have different functions. Furthermore, the decreased protein level of Prdx2 in TG treated could be an indirect effect of Ang-(1-7) that leads to a reduced level of inflammatory TNF-α and a higher level of anti-inflammatory IL-10, suggesting a lower level of ROS by upstream molecules as the already mentioned Uchl1 and Prdx1. Ang-(1-7) treatment also decreased the expression level of phosphatidylethanolamine-binding protein 1 (Pebp1 or HCNP), also called Raf kinase inhibitor protein (RKIP), in hypertensive animals. Pebp1 is the precursor of hippocampal cholinergic neurostimulating peptides (HCNP) (Ling et al., 2014). RKIP inhibits the transcription factor NF-κB and MAPK signaling independently. It is a regulatory mechanism of NF-κB activation in response to pro-inflammatory cytokines stimulation as TNF-α and IL-1β (Yeung et al., 2001). As mentioned before, the abundance of these cytokines is lower in TG treated group suggesting NF-κB reduced activity. The higher level of Pebp1 in the TG control group may also be a compensatory mechanism to counterbalance the remarkable inflammation observed in these animals.

Finally, we would like to point out some of the limitations of the present study. First, the hypothalamus is intricately involved in the development and maintenance of arterial hypertension. However, it comprises different nuclei and areas when activated were shown to either raise or lower BP, mainly by altering sympathetic nervous activity. The hypothalamic nuclei are closely interconnected and also communicate with many other areas in the central nervous system both rostral and caudally. Thus, future evaluations of the protein profile expressed in each nucleus/region may extend the observations made in the present study, which were not possible at this study due to the amount of total protein in each sample and the method available for proteomic evaluation. Second, the study was carried out only in male rats, however, there are important gender differences in this TG rat related to the RAS, such as, prorenin, Ang I,

and Ang II were higher only in transgenic males compared with females (Lee et al., 1996). Thus, it is crucial and perhaps even mandatory, that in future studies gender comparisons are done. Knowing better the effect of treatments or maneuvers on female animals and women will certainly improve the treatment and control of high blood pressure. Third, we have not validate the results of protein expression with Western blotting. Although some debate exists in the literature regarding the value of this type of validation, future studies should evaluate the expression of the specific proteins altered. Furthermore, future studies should also reproduce these protein changes in experimental models to provide more insights on the role of these proteins for the pathophysiology of hypertension.

CONCLUSION

We provide evidence that the chronic infusion of Ang-(1-7) in the brain modulates inflammatory mediators, RAS components and iNOS in the hypothalamus, suggesting a possible additional anti-hypertensive mechanism for Ang-(1-7) in the CNS. Moreover, we highlight one of the anti-inflammatory mechanisms of Ang-(1-7) could be by decreasing Uchl1 abundance while increasing Prdx 1 and, subsequently decreasing ROS production in the oxidative stress and inflammation (Graphical abstract). Although proteins evaluation by Western blotting are still required to confirm the proteomic analysis, the data of this study reinforce that pharmacological strategies leading to brain accumulation of Ang-(1-7) may become alternative therapies to treat arterial hypertension, especially those of neurogenic or resistant nature.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

All experimental protocols were approved by the Institutional Committee that regulates the use of laboratory animals – Comitê de Ética no Uso de Animais (CEUA/UFMG, protocol #49/2013) and were conducted in accordance with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals.

AUTHOR CONTRIBUTIONS

LK contributed in conception and design of the research, performed the experiments, analyzed the data, prepared the figures, interpreted the results of experiments, writing, review, and editing the manuscript, and approved the final version of the manuscript. MM-B analyzed the data, prepared the figures, interpreted the results of experiments, writing, review, and editing the manuscript, and approved the final version of the manuscript. BS performed the experiments, analyzed the data, prepared the figures, interpreted the results of experiments, and approved the final version of the manuscript. RS interpreted the results of experiments, reviewed the manuscript, and approved the final version of the manuscript. HA contributed in conception and design of the research, analyzed the data, interpreted the results of experiments, reviewed the manuscript, and approved the final version of the manuscript. MC-S contributed in conception and design of the research, analyzed the data, prepared the figures, interpreted the results of experiments, writing, review, and editing the manuscript, and approved the final version of the manuscript. All authors contributed to the article and approved the submitted version.

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