1-1-2017

Pathophysiological Relevance of Astroglial Angiotensin and the Endocannabinoid Signaling Systems in SHRs

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PATHOPHYSIOLOGICAL RELEVANCE OF ASTROGLIAL ANGIOTENSIN AND THE ENDOCANNABINOID SIGNALING SYSTEMS IN SHRs

by

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A dissertation submitted in partial fulfillment of the requirements for the degree of
Doctor of Philosophy

College of Pharmacy

Nova Southeastern University

Fort Lauderdale, Florida 33328

June, 2017

Dissertation Advisor: Dr. Michelle A. Clark, Ph.D. (HPD-PHR)

Acknowledgements

This dissertation is dedicated to my advisor, Dr. Michelle A. Clark. My advisor has been, and will continue to be my strongest inspiration. Under Dr. Clark's unparalleled training and leadership, I was able to acquire the confidence and the skills needed to pursue a career in research. The past 5 years in the lab was a fabulous and a life altering experience, and I cannot thank my advisor enough for her support and encouragement, even in the most testing of times. I will try to pay it forward, if I ever have an opportunity to do so. This dissertation is also dedicated to the late Dr. Hugh McLean. His words of wisdom and advice always meant a lot to me. He truly embodied the virtues of prudence and compassion, and was an amazing mentor to several graduate students here at NSU.

The discussions that I had with my committee members, Dr. Luigi Cubeddu, Dr. Anastasios Lymperopoulos and Dr. Umadevi Kandalam, both in person as well as in the committee meetings, helped greatly in shaping this project. My committee members, including my advisor, were always open to my ideas, and offered excellent advice and input on every aspect of this project. I must also thank them for their courses, since the classes on hypertension, GPCRs and clinical pharmacology helped me immensely in investigating pertinent research questions.

My colleagues in the lab have always been there to support and help me at every step of this amazing journey. Having had the opportunity to work closely with Shmuel Negussie and Ahmed Alanazi, both quality researchers and amazing friends, has made this a memorable experience for me. Also, I am extremely grateful to Michael Dressler, Dr. Ann Tenneil O'Connor and Waad Samman for their help and support over the last few years. The discussions that I had with all of them has helped me immensely to fine-tune my

project. Shmuel helped me with several aspects of my project, which includes developing protocols as well as interpreting results, and I am very grateful for having had the opportunity to work with him on several experiments. Also, I thank Dr. Francisco Puerta, Dr. Yugandhar Gowrisankar, and Dr. Umadevi Kandalam again, for sharing their wealth of knowledge on molecular biology techniques with me. Additionally, it was an amazing learning experience to work as a teaching assistant, under the supervision of Dr. Ana Maria Castejon and my advisor, Dr. Clark, during my first year at NSU. I greatly appreciate the opportunity to learn from the best teachers here at NSU.

I have also relied on several of my friends, colleagues and professors here for advice, and guidance. The bonds that I have with my peers and professors have helped me immensely over the course of my education here at NSU. Although I cannot mention everyone's name, I am very grateful for their help here at NSU. Srinath Muppalaneni especially offered me some amazing advice all throughout this period, and I value his help immensely. My parents, Dr. Aruna and Dr. Giridhar, and my sister, Misha have been robust support systems over the duration of my program. Thank you for the support and love.

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List of Abbreviations

2-AG	2-arachidonoylglycerol
AKT	Protein kinase-b
ACEA	Arachidonyl-2-chloroethylamide
ACEIs	Angiotensin converting enzyme inhibitors
ADHD	Attention deficit hyperactivity disorder
AGT	Angiotensinogen
Ang II	Angiotensin II
AT1R	Ang type 1 receptor
AT2R	Ang type 2 receptor
ATP	Adenosine triphosphate
BBB	Blood brain barrier
BCA	Bicinchoninic acid
BIM I	Bisindolylmaleimide I
CB1R	Cannabinoid Type 1 Receptor
CHO	Chinese hamster ovary
CNS	Central nervous system
Ct	Cycle threshold
CVLM	Caudal ventrolateral medulla
DAG	Diacylglycerol
DAGL	Diacylglycerol lipase
DMEM/F12	Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12
ERK	Extracellular signal–regulated kinase
FAAH	Fatty acid amide hydrolase
FBS	Fetal bovine serum
GABA	Gamma aminobutyric acid

GFAP	Glial fibrillary acidic protein
GPCRs	G protein coupled receptors
IL-10	Interleukin 10
IL-1β	Interleukin 1β
IL-6	Interleukin 6
JAK	Janus Kinase
JAM-1	Junctional adhesion molecule 1
JNK	c-Jun N-terminal kinase
MAGL	Monoacylglycerol lipase
MAPKs	Mitogen activated protein kinases
MCP-1	Monocyte chemotactic protein 1
NAT	N-acetyltransferase
NF-KB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NTS	Nucleus tractus solitaris
PI3K	Phosphoinositide 3-kinase
PKC	Protein kinase C
PLC	Phospholipase C
PLD	Phospholipase D
PMA	Phorbol 12-myristate 13-acetate
PVN	Paraventricular nucleus
qPCR	quantitative PCR
RAS	Renin Angiotensin System
ROS	Reactive oxygen species
RVLM	Rostral ventrolateral medulla
SHR	Spontaneously hypertensive rat
STAT-5	Signal Transducer and Activator of Transcription

TBS	Tris buffered saline
THC	Delta-9-tetrahydrocannabinol
TRPV1	Transient receptor potential cation channel subfamily V member 1
VSMCs	Vascular smooth muscle cells
WKY	Wistar Kyoto rats

Abstract

An Abstract of a Dissertation Submitted to Nova Southeastern University in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy

PATHOPHYSIOLOGICAL RELEVANCE OF ASTROGLIAL ANGIOTENSIN AND THE ENDOCANNABINOID SIGNALING SYSTEMS IN SHRs

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BACKGROUND: Spontaneously hypertensive rats (SHRs), an essential hypertension model, are characterized by pro-inflammatory states in brainstems. While Angiotensin (Ang) II, via activation of glial Ang Type 1 receptors (AT1Rs), has been shown to trigger a significant upsurge in pro-inflammatory cytokines, activation of astroglial Cannabinoid Type 1 Receptors (CB1Rs), elicits a potent anti-inflammatory response in the brain. Both brainstem AT1Rs and CB1Rs have also been reported to alter cardiovascular parameters in SHRs. Evidence of crosstalk between CB1Rs and AT1Rs has also emerged, further highlighting a need to understand their signaling interaction in cardiovascular diseases.

APPROACH: The purpose of this study was twofold- first, to investigate the downstream consequences of AT1R and CB1R activation in astrocytes under early hypertensive and normotensive conditions, and second, to explore potential crosstalk mechanisms between the two receptors. The proposed studies were carried out in brainstem and cerebellar

astrocytes isolated from SHRs and their normotensive controls, the Wistar rats. Alterations in activation patterns of Mitogen activated protein kinases (MAPKs), and/or the levels of the total and inactive (phosphorylated) forms of the receptor, especially CB1R in the latter case, in SHR astrocytes, were employed as indices of receptor functionality. Additionally, changes in the levels of pro- and/or anti-inflammatory cytokines in response to Ang II and CB1R agonist, both alone and in combination, were also employed to assess the immunomodulatory effects of the two systems in SHR and Wistar rat astrocytes.

RESULTS: Altered neuroinflammatory states were observed in brainstem astrocytes, but not cerebellar astrocytes of SHRs when compared to Wistar rats. While Ang II triggered potent activation of MAPKs and elicited prominent pro-inflammatory effects in brainstem astrocytes of both models, its activation in cerebellar astrocytes resulted in an increase in both pro- and anti-inflammatory states in both models. A reduction in CB1R expression, and CB1R-mediated anti-inflammatory effects were observed in brainstem astrocytes of SHRs when compared to Wistar rats. Although CB1R expression in cerebellar astrocytes was similar in both models, its downstream effects were partially reduced in cerebellar astrocytes of SHRs when compared to Wistar rats. While CB1R activation diminished Ang II-mediated pro-inflammatory effects in brainstem astrocytes of Wistar rats, its effects were not abated in SHRs. Interestingly, Ang II not only reduced CB1R expression in brainstem astrocytes of SHRs, but also triggered phosphorylation of CB1Rs in cerebellar astrocytes of both models.

CONCLUSION: A dysregulated neuroinflammatory status, along with a dampened brainstem astroglial endocannabinoid tone, could well be important factors in the etiology of hypertension. Since AT1R activation results in downregulation and phosphorylation of

CB1Rs, counteracting the effects of renin angiotensin system (RAS) could serve as a viable strategy to indirectly elevate/preserve the basal endocannabinoid tone. This is especially applicable to hypertension, which is characterized by hyperactivity of the RAS and a potential dysregulation of the endocannabinoid system.

Chapter 1

Background

Hypertension has the dubious distinction of being termed the silent killer. In addition to being a peerless risk factor for cardiovascular diseases, hypertension is associated with minimal, if any, visible symptoms during the initial stages of disease progression. About 1 billion people are currently diagnosed with hypertension worldwide, and the numbers are expected to rise in the future (Kearney *et al,* 2005). Antihypertensive drug therapy is hailed as one of the most significant medical breakthroughs to come out of the 20th century, since its impact on mortality rates, is second only to antibiotics (Kaplan, 1980). Since the introduction of antihypertensive drug therapy, death rates associated with cardiovascular disease have dropped by 72% in the United States (https://www.nhlbi.nih.gov/files/docs/research/2012_ChartBook_508.pdf). In spite of the progress made, more than 30% of the United States population who are currently receiving anti-hypertensive drug therapy, continue to remain hypertensive (Calhoun *et al.* 2008). Identification of novel therapeutic targets that can lead to the generation of effective treatment strategies for resistant hypertensive cases, is the need of the hour. Since a multifactorial, polygenic disorder like hypertension may have multiple blood pressure regulatory mechanisms compromised at different stages of the disorder (Sleight 1971), the identification of trigger mechanisms becomes an uphill task. Identification of effective

therapeutic targets hinges on understanding the mechanisms that are impaired at the very early stages of hypertension. These may well be the drivers of hypertension, and treating them at the earliest stages would result in a better response to drug therapy.

Augmented sympathetic activity is identified as one of the earliest mechanisms to be impaired in essential hypertension (Guyenet 2006). In borderline hypertensive individuals, an elevation in sympathetic activity was reported in several landmark studies (Julius *et al.* 1991) (Anderson *et al.* 1989). Research in spontaneously hypertensive rats (SHRs), an animal model of hypertension that is widely employed in studying the disease, further corroborated the importance of autonomic dysfunction that is centrally mediated (Folkow 1982) (Saavedra *et al.* 1976). This model is also characterized with an elevation in sympathetic activity at very early stages of hypertension (Saavedra *et al.* 1976) (Dickhout and Lee 1998). A dysregulation of neurotransmitters in the cardiovascular centers of the brainstem, has been theorized, as a cause of an imbalance between the two arms of the autonomic system (Reis 1981) (Sved and Gordon 1994) (Takemoto and Yumi 2012). The brain renin angiotensin system (RAS) has been implicated as being the most important driver of sympathetic activity from the central nervous system (CNS) (Carlson and Wyss 2008). An overactive brain RAS has been reported by several studies (Veerasingham and Raizada 2003). Angiotensin (Ang) II, the primary effector peptide of RAS, is able to elicit its deleterious effects via the activation of the Ang type 1 receptor (AT1R). Several *in vitro* and *in vivo* studies have highlighted the emerging role of brain inflammation in the pathogenesis of hypertension (Waki *et al.* 2008a). An elevation in the levels of pro-inflammatory cytokines, and a reduction in the levels of anti-inflammatory cytokines, have been reported in the cardiovascular centers of the brainstem and the hypothalamus of SHRs

when compared to their normotensive controls (Agarwal *et al.* 2011). Ang II has been demonstrated to alter neuroinflammatory and redox states in different cell types (Ogawa *et al.* 2011) (Kandalam and Clark 2010). Since glial cells have a role in regulating inflammatory states in the brain, the glial RAS was proposed as an important contributor to the pathogenesis of essential hypertension. Shi et al, hypothesized that glial AT1R activation by Ang II can cause an upsurge in the levels of reactive oxygen species (ROS) and pro-inflammatory cytokines via activation of glial AT1Rs (Shi *et al.* 2010a). These pro-hypertensive mediators, in a paracrine fashion, can alter neuronal functions by activating surface receptors, modifying ion channels and intracellular proteins (Shi *et al.* 2010a). It was later demonstrated that chronic infusion of Ang II in the paraventricular nucleus (PVN), leads to an elevation in pro-inflammatory cytokines via activation of microglial AT1Rs (Shi *et al.* 2010b).

It is well known that both microglial and astroglial cells, via a host of different mechanisms, can contribute to the resolution of pro-inflammatory states in the brain (Shastri *et al.* 2013). Recently, it has been reported that astroglial AT1Rs in the brainstem play critical roles in the regulation of sympathetic nervous system activity (Marina *et al.* 2016). Ablation of the astroglial AT1R in the brainstem is associated with a decrease in sympathetic nervous system activity in a model of heart failure (Isegawa *et al.* 2014a). Data from our laboratory indicated that Ang II has potent pro-inflammatory and pro-oxidant effects on brainstem astrocytes (Gowrisankar and Clark 2016c). In addition, Ang II can also upregulate a host of pro-hypertensive markers from brainstem astrocytes, making this an important cell type in the context of hypertension (Gowrisankar and Clark 2016b). Interestingly, Ang II was able to elicit similar effects in astrocytes from other regions of the brain, such as cerebellum

(Clark *et al.* 2013)(Gowrisankar and Clark 2016c). The RAS has been demonstrated to have a role in cerebellar development (Côté *et al.* 1999). In the last decade or so, several studies have highlighted the utility of targeting components of the RAS in the treatment of disorders characterized by cognitive decline (Bodiga and Bodiga 2013) (Gao *et al.* 2013). In a recent study, anti-hypertensive drugs were also demonstrated to improve symptoms of autism in children (Zamzow *et al.* 2016). The incidence of neurodevelopmental disorders such as ADHD has been identified to be far greater in children with hypertension, than without (Adams *et al.* 2010).

Cannabinoids are well-known to exert protective effects in the brain, by their crucial role in the regulation of homeostasis (Di Marzo 2009). Their ability to neutralize pro-inflammatory states in the brain makes them therapeutically invaluable for the treatment of neurological disorders (Bisogno and Di Marzo 2007). A plethora of pre-clinical and clinical data support the beneficial role of cannabis or cannabinoid-based drugs in alleviating symptoms associated with neurological disorders (Consroe 1998). Interestingly, cannabinoids have been demonstrated to have tremendous promise as a potential treatment strategy for ADHD based on numerous positive pre-clinical and clinical outcomes (Adriani *et al.* 2003) (Milz & Grotenhermen, 2015). Endocannabinoid hypofunction/deficiency has been hypothesized to contribute significantly to the etiology of ADHD (Adriani *et al.* 2003). Since dysregulated neuroinflammatory states are hypothesized to be key contributors to the biological basis of ADHD (Costantino *et al.* 2009), the immunosuppressive effects elicited by the cannabinoid type 1 receptor (CB1R) activation may well be crucial therapeutic targets. Key to its role may be the astroglial CB1R, as its

activation has been demonstrated to result in neutralization of pro-inflammatory conditions (Molina-Holgado *et al.* 2003) (Nagarkatti *et al.* 2009).

Endocannabinoid system activation is associated with an improvement in cardiovascular parameters by several mechanisms that encompass both peripheral and central sites of action (Mendizábal and Adler-Graschinsky 2007). In SHRs, the ability of cannabinoids to dampen centrally-mediated sympathoexcitation, was impaired (Brozoski *et al.* 2009), while their cardioregulatory and vasodilatory properties were potentiated (Bátkai *et al.* 2004). Several reports of crosstalk between AT1Rs and CB1Rs has come to the fore in the recent past, resulting in an alteration of AT1R's actions by cannabinoids (Turu *et al.* 2009; Rozenfeld *et al.* 2011). It could well be that the homeostatic role of CB1R to negate some of Ang II's deleterious functions, may be impaired, leading to a potentiation of Ang II-mediated neuroinflammatory and pro-oxidant states in SHRs.

The purpose of this study was to investigate the downstream consequences of AT1R and CB1R activation in astrocytes under pre-hypertensive and normotensive conditions. *We hypothesize that a dysregulation of CB1R functions in SHR astrocytes, alters its ability to modulate Ang II-mediated effects.* In order to test our hypothesis, we employed primary astrocytes isolated from the brainstems of SHRs and their normotensive controls, the Wistar rats. Neonatal rats were used for this study since they serve as a model for prehypertension. We employed indices of receptor functionality, activated mitogen activated protein kinases (MAPKs), and total and inactive forms of receptor, in order to determine preservation or dampening of CB1Rs role under prehypertensive conditions. Additionally, we also explored the possibility of AT1R hyperactivity under prehypertensive conditions. Finally, we explored their neuroprotective or neurotoxic

effects by investigating the roles of the aforementioned receptors in the regulation of pro- and anti-inflammatory cytokines. Our three specific aims are as follows:

Specific Aim 1: Determine whether Ang II alters CB1R expression in astrocytes isolated from SHRs and Wistar rats

Specific Aim 2: a) Determine the consequences of RAS and endocannabinoid system activation, both alone and in combination, on MAPK signaling pathways in astrocytes isolated from SHRs and Wistar rats. b) Determine the effect of Ang II on triggering phosphorylation of CB1R in astrocytes isolated from SHRs and Wistar rats.

Specific Aim 3: Determine the consequences of RAS and endocannabinoid system activation, both alone and in combination, on neuroinflammatory cytokines in astrocytes isolated from SHRs and Wistar rats.

While our primary focus was on understanding the roles of the systems under investigation in brainstem astrocytes, we also employed astrocytes isolated from cerebellum as a reference model. Astrocytes from cerebellum were chosen as a model for two reasons. Firstly, cerebellum, unlike brainstem, is characterized by high levels of CB1R, and hence would make an ideal cell model to investigate crosstalk mechanisms between AT1R and CB1R. But more importantly, SHRs are characterized by astroglial dysfunction in the cerebellum, which results in a neurotoxic microenvironment (Yun *et al.* 2014). The SHR is also a widely employed model for studying ADHD (Adriani *et al.* 2003). Considering that there is a strong correlation between hypertension and neurodevelopmental disorders (Adams *et al.* 2010), evidence of potential augmentation of pro-hypertensive systems such as RAS, and dampening of protective systems such as the endocannabinoid system in SHR

cerebellar astrocytes, could aid in designing better treatment strategies for neurodevelopmental disorders such as ADHD.

The major strength of this study is the use of prehypertensive rats as opposed to adult SHRs. This allows us to draw causal relationships between the results of our study to the etiology of hypertension. Also, the conclusions that we make from our results, will be based on several different indices for a single outcome. For instance, in addition to measuring CB1R expression levels, we are also employing two MAPKs and two cytokines, to examine receptor hyper/hypo- functionality. The limitation of this study is that it is purely an *in vitro* study. Hence the results must be interpreted with caution as there is a possibility that a change in receptor functionality or cytokine levels, observed in prehypertensive SHR astrocytes, may have only limited impact on the overall progression of the disease.

The results from this study would serve two vital purposes. Firstly, it would be a step forward in expanding our understanding of astroglial functions in cardiovascular and neurodevelopmental disorders. Secondly, these results would add tremendously to our knowledge of the astroglial CB1Rs, which are theorized to play a vital role in both physiological and pathological conditions.

Chapter 2

Introduction

Overview

This chapter will provide a brief synopsis of topics, that are core to the main idea of the project. Section 1 provides a detailed account of two brain regions, brainstem and cerebellum, in the pathogenesis of hypertension, and neurodevelopmental disorders such as ADHD, respectively. Sections 2 and 3 introduce the importance of astroglial dysfunction, specifically neuroinflammation, in the etiology of hypertension and ADHD. Sections 4 and 5 are focused on the role of RAS and endocannabinoid systems in the aforementioned pathological conditions, respectively. In all the above sections, the emphasis is on SHRs, since it is widely believed to be the best disease model to study hypertension and ADHD. Section 6 provides a brief description of the studies that have investigated crosstalk mechanisms between AT1Rs and CB1Rs. Section 7 provides a brief synopsis of all of the topics and also the rationale for the hypothesis and the specific aims of the project.

2.1 A brief introduction of the significance of brainstem and cerebellum impairment in pathological conditions: Emphasis on the SHR model.

i) Brainstem

a) Current status of hypertension

Although anti-hypertensive medications have made rapid strides in drastically reducing mortality and morbidity rates due to cardiovascular diseases, the most alarming statistic, is that a significant percentage of hypertensive individuals that are currently on anti-hypertensive therapy, remain hypertensive (Calhoun *et al.* 2008). A lack of effectiveness of traditional anti-hypertensive medications, necessitates identification of therapeutic targets that are fundamental to the pathogenesis of hypertension (Fisher and Fadel 2010). Hence understanding the mechanisms that become dysregulated and eventually lead to an elevation in blood pressure, remains the focal point of cardiovascular research.

b) Role of sympathetic hyperactivity in the pathogenesis of hypertension

Since regulation of blood pressure is orchestrated by multiple mechanisms encompassing both neurogenic and non-neurogenic origins, the task of isolating hypertension trigger mechanisms remains a challenge. Evidence of autonomic dysfunction contributing to the development of hypertension came from studies conducted in borderline hypertensive individuals, where an elevated cardiac output was observed in young hypertensive individuals (Widimsky *et al.* 1957). By employing direct and also indirect parameters of sympathetic activation in humans (Julius and Esler 1975) (Safar *et al.* 1974), researchers were able to identify autonomic dysfunction as being a crucial mechanism for

the development of hypertension. A meta-analysis of plasma catecholamine levels in hypertensive individuals revealed a high correlation between younger hypertensive individuals, rather than older hypertensive individuals with hypertension (Goldstein., 1983). But seminal works of Anderson and Julius, published in the late 1980's and early 1990's, not only established the importance of sympathetic overactivation as a significant factor in the initiation of hypertension (Julius *et al.* 1991), but also provided convincing evidence of CNS involvement (Anderson *et al.* 1989). In addition, studies conducted in animal models of hypertension, such as SHRs, revealed prominent autonomic dysfunction. Similar to borderline hypertensive individuals, sympathetic tone was augmented during the early stages of hypertension in SHRs (Fisher and Paton 2012) (Korner *et al.* 1993). Blockade of sympathetic activity was demonstrated to be more effective in young prehypertensive SHRs, when compared to adult SHRs (Weiss *et al.* 1974). Thus, evidence from prehypertensives and borderline hypertensive humans, in addition to the studies in hypertensive animal models, led to the development of the neuroadrenergic hypothesis for hypertension put forth by Grassi, which underscored the role of sympathetic hyperactivity in triggering and in perpetuating hypertensive conditions (Grassi *et al.* 2010). Traditional antihypertensives, other than centrally acting sympatholytics, have been demonstrated to have a minimal/neutral (Grassi *et al.* 1998) and also an exacerbatory effect (Fu *et al.* 2005) on central sympathetic outflow. Since an elevated central sympathetic outflow is a key underlying determinant of several cardiovascular diseases as well, it is essential that we explore the molecular mechanisms that leads to an impairment in cardiovascular parameters. The search for promising therapeutic strategies for lowering an augmented sympathetic drive, that is centrally mediated, has resulted in investigating the pleiotropic

effects of statins. They have been demonstrated to have potent antioxidant and anti-inflammatory actions resulting in a decrease in sympathetic outflow in heart failure (McGowan *et al.* 2013).

c) Brainstem cardiovascular centers and sympathetic activity

Elevation in sympathetic drive was theorized to originate from an impairment of the CNS cardioregulatory centers, especially the brainstem (Saavedra *et al.* 1976) (Julius and Esler 1975). In the CNS, the cardioregulatory centers of the brainstem and hypothalamus play a critical role in the homeostatic regulation of blood pressure over a short duration or a longer period of time (Osborn 2005) (Guyenet 2006). Brainstem comprises of the nucleus tractus solitaris (NTS), which is the major command center for the integration of inputs from arterial baroreceptors, chemoreceptors, and also cardiopulomonary receptors (Zanutto *et al.* 2010). It also receives inputs from amygdala and the PVN located in the hypothalamus. Optimum functioning of the peripheral baroreceptors and the NTS, and the higher brain regions such as hypothalamus, have a crucial role in the regulation of baroreflex. Resetting of the baroreflex is a characteristic feature of neurogenic hypertension (Folkow 1982) (Fisher and Paton 2012). Activation of the peripheral baroreceptors serves to depress the rostral ventrolateral medulla (RVLM) activity, the major pressor center in the brain, leading to a decrease in sympathetic activity (Dembowsky and McAllen 1990). This action is achieved through baroreceptor afferents that terminate into the NTS, whose primary function is to keep check on the RVLM, via its activity on the major inhibitory center, the caudal ventrolateral medulla (CVLM). Additionally, afferent inputs from chemoreceptors and projections from PVN also terminate into the RVLM (Dampney *et al.* 2002). RVLM neurons, which are primarily of

the C1 type, terminate in the intermediolateral cell column, from which arises the sympathetic preganglionic neurons. Its activation has been demonstrated to result in an increase in sympathetic efferent activity to heart, arteries and kidneys, resulting in an increase in heart rate, vasoconstriction, and an increase in renin release (Kumagai *et al.* 2012). Projections from several regions of the brain terminate into the RVLM. Hence elevation of sympathetic activity that is observed in hypertensive states could be either due to an impairment in baroreceptor and chemoreceptor afferent input to the brainstem, or a processing error in brainstem cardiovascular centers (Guyenet 2006).

d) SHR- A model of essential hypertension

The SHR is one of the most widely used models to study essential hypertension, as it exhibits remarkable similarities to human hypertension. These similarities are:

1. Similar to the development of hypertension in humans, hypertension in SHRs develops over a period of time, and it is preceded by a pre-hypertensive stage. Similar to pre-hypertensive individuals, SHRs are also characterized by an elevated sympathetic activity that is centrally mediated (Fisher *et al.* 2009) (Judy *et al.* 1979).

2. A study examining the effects of gender on hypertension, reported that the severity of hypertension is greater in men when compared to women. In SHRs as well, a greater severity of hypertension in males when compared to females is observed (Sandberg and Ji 2012).

3. Similar to humans, anti-hypertensive medications demonstrated greater efficacy in the treatment of hypertension at the earlier stages, than at the later stages in SHRs (Weiss 1974) (Harrap *et al.* 1990).

Both *in vitro* and *in vivo* studies in SHRs, have greatly contributed to our understanding of the role of the brain in the pathogenesis of hypertension (Folkow 1982). An impairment of neuronal activity in the cardiovascular centers of the brainstem (Ito *et al.* 2000) and hypothalamus (Allen 2002) is theorized to be intricately connected to an augmentation of sympathetic activity. The role of the neurohumoral systems in long-term alterations of blood pressure, has also been highlighted by several studies (Dampney *et al.* 2002). Pro-hypertensive hormonal systems such as the RAS are overactive in the brains of SHRs (Veerasingham and Raizada 2003). In RVLM, where the AT1R is expressed at relatively high levels (Hu *et al.* 2002), activation of the receptor has been demonstrated to result in an increased firing frequency in SHRs (Matsuura *et al.* 2002). In addition to RVLM, hypothalamic cardiovascular regulatory regions such as PVN was also demonstrated to have a crucial role in mediating sympathetic hyperactivity observed in SHRs (Allen 2002). The PVN also receives direct inputs from the subfornical organ (SFO), which have been demonstrated to be sensitive to Ang II treatment (Okuya *et al.* 1987) (Dampney *et al.* 2002). The AT1Rs in the PVN have a critical role in sympathetic overactivity observed in SHRs, as experimentally-induced reduction of AT1R levels in SHRs, resulted in a lowering of blood pressure (Shan *et al.* 2011).

e) Molecular mechanisms of elevated central sympathetic drive

Evidence from several studies have identified elevations in pro-oxidant states (Gao *et al.* 2005), and pro-inflammatory states (Guggilam *et al.* 2008), to be candidate mechanisms by which Ang II via neuronal AT1R activation can cause an elevation in sympathetic activity in SHRs. While most of the studies have been geared towards unraveling the dysregulated molecular mechanisms of established hypertension, by

employing adult SHRs, very few studies have investigated the molecular mechanisms that become impaired at early stages (Doggrell and Brown 1998). Considering that these mechanisms may well be the drivers of hypertension, it becomes imperative that we identify the genes and proteins that either exhibit altered expression patterns or altered functions or both, in the brainstem and hypothalamus of SHRs. Only recently, an altered inflammatory status was identified in the NTS of prehypertensive, as well as hypertensive SHRs (Paton and Waki 2009) (Waki *et al.* 2008a). This discovery was instrumental in highlighting the importance of neuroinflammation in the development of hypertension. Studies from our laboratory and others, have further confirmed the existence of an altered inflammatory state in the brainstem and hypothalamus of SHRs (Gowrisankar and Clark 2016c) (Agarwal *et al.* 2011). While there has been some headway into understanding the role of hormones and secreted factors that regulate neuroinflammatory states in SHRs, this area of research has not been well investigated. A better understanding of potential regulators of neuroinflammatory states would enable us to leverage the anti-inflammatory potential of brain cells to achieve resolution of neuroinflammatory conditions. A condition like hypertension is characterized by neuroinflammatory and pro-oxidant states, as well as aberrant neuronal activity. Targeting multifunctional regulatory systems such as the endocannabinoid system may well prove as an attractive therapeutic target, since neuromodulatory and immunomodulatory effects, could be crucial in regulating dysregulated neuronal activity and inflammatory states (Di Marzo 2009).

ii) Cerebellum

a) Cerebellar impairment and ADHD

While the prefrontal cortex and basal ganglia have been extensively investigated in neurodevelopmental disorders such as ADHD, less importance has been given to cerebellum. The cerebellum was widely believed to have a central role in motor functions exclusively (Goetz *et al.* 2014b). The importance of purkinje cells in motor functions has been reported by several studies (Paulin 1993). In the recent past however, cerebellum has been theorized to have a critical role in non-motor functions such as cognition (Stoodley 2012). The cerebellum sends and receives extensive inputs to the basal ganglia and cortex- the cerebellar dentate nucleus connects to the prefrontal cortex and the striatum. These projections that arise from the non-motor and motor regions of the cerebellum, may be critical in regulating several functions that are often intrinsic to the frontal lobe (Strick *et al.* 2009). Morphometric analysis conducted on different brain regions of boys with ADHD revealed significant reduction in the size of different brain regions, including the cerebellum, when compared to the control brains (Castellanos *et al.* 1996a). Cerebellar volumetric reductions have been reported to be even more evident than prefrontal cortex reductions in individuals with ADHD (Castellanos *et al.* 2002). Evidence of alterations in specific regions of the cerebellum that connects to brain regions with higher brain functions has also been reported (Bledsoe *et al.* 2011). Functional magnetic resonance imaging studies revealed that different functional domains of the cerebellum, involved in motor functions or attention, have mutually independent roles (Allen *et al.* 1997), which may be selectively altered in neurodevelopmental disorders (Allen and Courchesne 2003). Morphological changes observed in the structure of the cerebellum in neurological and

neurodevelopmental disorders, that are not associated with motor impairments, also highlights the role of cerebellum in functions such as cognition and attention. Additionally, motor and cognitive deficits are observed in several neurodevelopmental disorders (Goetz *et al.* 2014b) (Rogers *et al.* 2013). Impairment in cerebellar functioning has been observed in neurodevelopmental disorders such as autism (Allen *et al.* 2004) and ADHD (Valera *et al.* 2005). Along with the morphometric findings, these studies provide convincing evidence of cerebellar impairment in neurodevelopmental disorders, such as ADHD (Schneider *et al.* 2006), and other anxiety disorders which are not typically associated with motor functional deficits, such as posttraumatic stress disorder (Phillips *et al.* 2015). Since several structural and functional alterations of cerebellum are associated with neurodevelopmental disorders from a young age, cerebellar hypoplasia may well be a key feature of disorders such as autism and ADHD (Basson and Wingate 2013).

b) The SHR as a disease model for ADHD

Traits that are often observed in ADHD individuals such as shorter attention spans, inability to focus, and hyperexcitability, are also observed in SHRs, making it an ideal model to investigate the etiology of ADHD (Adriani *et al.* 2003). Astrogliosis, a hallmark of neurodegenerative disorders, is also reported in SHRs (Tomassoni *et al.* 2004). Similar to ADHD individuals, SHRs are also characterized by cerebellar atrophy and cerebellar impairment (Yun *et al.* 2014). An increase in the levels of apoptotic factors and astrogliosis was reported in the cerebellum of SHRs when compared to their normotensive controls (Yun *et al.* 2014). While the exact molecular mechanisms have not been investigated, one study has implicated the beneficial effects of cannabinoids on the symptoms of ADHD (Adriani *et al.* 2003).

iii) Hypertension and neurological impairments

A strong correlation has already been demonstrated between hypertension and cognitive deterioration, and also brain RAS and cognitive function (Nelson *et al.* 2014) (Tzourio 2007) (Bodiga and Bodiga 2013). Although the SHR has been employed as a model for studying ADHD, the role of the RAS in the development of ADHD has not been investigated. Evidence of a greater incidence of learning disabilities in ADHD children diagnosed with primary hypertension, than those without, has been reported (Adams *et al.* 2010). An observational case-control study assessing the efficacy of angiotensin-converting-enzyme inhibitors (ACEIs) on cognitive functions in the elderly, also had favorable results (Gao *et al.* 2013). Since hyperactivation of brain RAS is associated with elevated pro-inflammatory and pro-oxidant states, neutralization of the RAS could result in resolution of neuroinflammatory conditions, leading to improvement of various neurological impairments.

2.2 Physiological and pathophysiological roles of astroglia: Emphasis on cardiovascular and neurodevelopmental disorders

i) History of astrocytes

Following the discovery of glial cells in the mid-1800's, several theories of their possible roles in normal brain functioning and also in brain disorders were put forth during the late 19th and early 20th century (Hubbard *et al.* 2016). Lack of sophisticated tools to study glial cells were major hindrances during that era (Somjen 1988). It was only in the mid-1950's that glial cell functioning was experimentally determined (Hubbard *et al.* 2016). The availability of microelectrode recordings for studying electrophysiological properties of neuronal cells (Hodgkin and Huxley 1952), gave a major boost to

understanding glial cell physiology. The notion that astrocytes were electrically neutral, and hence were not capable of eliciting an electrophysiological response, was dispelled as researchers observed a hyperpolarized membrane in these cells (Hild *et al.* 1958). This was followed by studies that provided evidence of astroglial's ability to buffer excess potassium (Orkand *et al.* 1966). In spite of these landmark experiments, astrocytes were mostly relegated to have secondary roles when compared to their prized counterparts, neurons. This may well be due to their perceived inability to transmit information (Volterra and Meldolesi 2005). Landmark studies in the late 1980's and early 1990's, provided the first evidence of astroglial communication, which is mediated via mobilization of neurotransmitters (Hatton 1988) and transmission of calcium waves (Cornell-Bell *et al.* 1990) (Nedergaard 1994). This was believed to be the turning point in astroglial research, and also in neuroscience research (Ndubaku and de Bellard 2008). Their ability to communicate with neurons, function as secretory cells, in addition to their housekeeping roles, made them vital from a physiological and pathological standpoint. Interestingly, several of these experiments were based on hypotheses that were stated by researchers close to a century earlier (Hubbard *et al.* 2016) (Somjen 1988). While discrepancies still persist over the ratio of astrocytes to neurons (Hilgetag and Barbas 2009) (Herculano-Houzel 2009), astrocytes are one of the most abundant brain cell types. Their ability to function as mediators of communication between neurons (Araque *et al.* 2001), surveyors of brain bioenergetics (Brown and Ransom 2007), and bidirectional regulators of inflammation (Bélanger and Magistretti 2009), are important to ensure normal brain functions.

ii) Classification

Astrocytes are classified mainly into two types, protoplasmic and fibrous astrocytes. Protoplasmic astrocytes (Type 1) are the most abundant type, and they are found in grey matter. They are closely associated with synapses and blood vessels and hence have an integral role in synaptic transmission, and formation of the blood brain barrier (BBB) (Molofsky *et al.* 2012). Fibrous astrocytes (Type 2) are present in the white matter. Along with oligodendrocytes, they have a role in the formation of myelin (Molofsky *et al.* 2012). In addition to these two types, glial cells with specialized functionality have also been identified. The Bergmann glia or Golgi epithelial cells, which are found in great numbers in the cerebellum, perform highly specialized functions encompassing neuroprotection and optimum functioning of the cerebellar circuit (Korbo *et al.* 1993) (De Zeeuw and Hoogland 2015). The other specialized glial cell type is the Muller cell, which are present in the retina. Their functions resemble those of protoplasmic astrocytes in the adult CNS (Börner *et al.* 2007). In addition to the above cells, progenitor cells possessing astrocyte like properties, known as radial glial cells, have also been identified.

iii) Physiological roles of astrocytes

a) Role in neurodevelopment

Radial glial cells are theorized to arise from precursor neural epithelial cells during embryogenesis (Götz and Barde 2005). Radial glia cells are one of three types of progenitor cells which are involved in the generation of neurons and astrocytes during the development of the nervous system (Götz and Barde 2005) (Freeman 2010). Astrocytes are also involved in guiding newly formed axons to their correct destinations thereby contributing to the functional specialization of neurons. Postnatally, there is an expansion in the number of glial cells, followed by maturation and specialization (Freeman 2010).

Mature astrocytes are then able to take part in synaptogenesis, neurite outgrowth, selective apoptosis of neurons (synaptic pruning), and at later stages they are involved in neurogenesis in select brain regions (Reemst *et al.* 2016) (Reemst *et al.* 2016).

b) Supportive and specialized functions

Astrocytes express a large number of ion channels. Due to their hyperpolarized resting state, they are able to participate in the redistribution of ions (Olsen *et al.* 2015). Ion channels such as Kir4.1, which are abundantly expressed in astrocytes, are involved in regulating the levels of potassium ions in the neuronal milieu (Olsen *et al.* 2015). Astrocytes also possesses multiple secretory and reuptake mechanisms for neurotransmitters (Malarkey and Parpura 2008) (Anderson and Swanson 2000). Their ability to modulate neurotransmitter levels in the synaptic cleft, is an essential aspect of their homeostatic and neuroprotective repertoire (Markowitz *et al.* 2007). Their role as homeostatic regulators hinges on their ability to regulate the levels of ions and neurotransmitters present in the neuronal milieu and the synaptic cleft. Additionally, astrocytes express an array of G protein coupled receptors (GPCRs), such as metabotropic glutamate receptors and purinergic receptors. These GPCRs along with connexins 43 and 30, are critical players in astrocyte-mediated calcium signaling (Olsen *et al.* 2015) (Bradley and Challiss 2012). Mobilization of neurotransmitters such as glutamate and Adenosine triphosphate (ATP), enables astrocytes to not only communicate with neighboring neurons and astrocytes, but also distant neurons as well (Fellin 2009) (Navarrete and Araque 2008). This is primarily due to an elevation in calcium which triggers mobilization of a second wave of gliotransmitters (Fellin 2009). Their ability to communicate with neurons and with each other, was the basis of the tripartite synapse hypothesis put forth by Araque (1999).

Additionally, astrocytes can regulate other glial cell's functions by either physically interacting with them, as in the case of microglia (Orthmann-Murphy *et al.* 2008), or by secretion of factors and cytokines (Shih *et al.* 2006) (Claycomb *et al.* 2013). Astrocytes form connections not just with neurons and other glial cells, but also with neighboring blood vessels. Astrocytes supply neurons with energy metabolites, such as lactate, after taking up glucose from the neighboring blood vessels (Stobart and Anderson 2013). Astrocytes along with capillary endothelial cells form the major constituents of the BBB. The BBB serves to isolate the brain from circulating macromolecules that are present in the peripheral circulation (Abbott 2002). Hence by forming a physical barrier, and by neutralizing foreign pathogens, astrocytes are part of a formidable CNS defense.

iv) Role in pathological states

a) Aberration in astroglial functions

Based on astroglial's ability to perform highly specialized functions, in addition to their supportive roles in the brain, it has become evident that they have crucial roles in mediating pathological states. As mentioned earlier, astrocytes were incorrectly believed to have an insignificant role in pathological conditions. While astrocytes have both supportive and independent roles under physiological states, they undergo radical transformation when they sense an invasion of a pathogen in the CNS, or an injury to the CNS (Sofroniew 2009). This sentinel like function is crucial for warding off a pathogen or limiting CNS injury. This response of astrocytes where they undergo distinctive morphological and molecular changes, is termed reactive astrogliosis, Morphological changes such as hypertrophy, and molecular changes such as high glial fibrillary acidic protein (GFAP) expression are observed during reactive astrogliosis (Sofroniew 2009).

The other protective functions of astrocytes include an uptake of excitotoxic neurotransmitters, counteracting neuroinflammatory states, and protection from oxidative stress (Ndubaku and de Bellard 2008) (Bélanger and Magistretti 2009). Astrocytes possess high levels of glutathione, that enables them to neutralize threats associated with excess ROS such as superoxides and hydrogen peroxide during cellular stress (Dringen et al., 2000). Alterations in astroglial functions, either a loss of function or an erroneous regulation, has been reported in multiple pathological conditions (Verkhratsky *et al.* 2012) (Parpura *et al.* 2012). Central to this idea is the dichotomy of reactive astrogliosis. While the major purpose of reactive astrogliosis is to confer CNS protection, diffused and persistent reactive astrogliosis is deleterious (Sofroniew and Vinters 2010). Scientific literature is rife with both beneficial and deleterious effects of reactive astrogliosis (Sofroniew 2009). Inhibition of reactive astrogliosis was demonstrated to have beneficial effects in the long run in different models of CNS injury, inspite of compromising on regeneration capabilities at the earlier stage (Pekny and Pekna 2014). Akin to inflammation, the outcome is strongly dictated by the rate of resolution. A persistent and diffused reactive astrogliosis is not only an impediment to stable neuronal activity, but on a larger scale, can trigger neuronal damage (Sofroniew and Vinters 2010). Severe CNS insults like ischemia, chronic neurodegeneration and injury, not only triggers reactive astrogliosis, but can often lead to the formation of glial scar (Sofroniew 2009). While this serves to primarily isolate the site of damage from the rest of the healthy brain, collateral damage such as gross diminishment of regenerative capabilities of axons also takes place (Sofroniew and Vinters 2010) (Pekny and Pekna 2014) (Yuan and He 2013). In addition, reactive astrocytes are characterized by an impairment in its neuroprotective functionality,

such as reuptake disruption and barrier disintegration (Sofroniew and Vinters 2010) (Sofroniew 2009). As a result, understanding their functions in pathological states, would not only aid to strengthen our understanding of several pathological conditions, but may also reveal several additional drug targets and therapeutic opportunities that may have been overlooked so far (Scuderi *et al.* 2013) (Colangelo *et al.* 2014). Several of the drugs that are currently available to treat neurological impairments, are designed based on receptors, ion channels and signaling systems that are impaired in neurons, and not astrocytes or other glial cells (Verkhratsky *et al.* 2012).

b) Neurodevelopmental disorders

While significant progress has been made in astroglial pharmacology, there are still avenues that have not been completely explored. Knowledge of potential aberrations in astroglial functions and development during embryogenesis, and its relation to neurodevelopmental disorders, still remains a fairly unknown area of research. Considering that their roles in synaptogenesis and neuronal survival have been fairly well established (Reemst *et al.* 2016) (Molofsky *et al.* 2012), astroglial dysfunction may well be intertwined with inaccuracies in synaptic activity, the latter being a characteristic of multiple neurodevelopmental disorders. Interestingly, in support of this notion is a study investigating the brains of autistic individuals. Autism, a neurodevelopmental disorder which usually manifests at a very early stage, is characterized by behavioral and cognitive deficits (Happé 1999). A high degree of astrogliosis was reported in the cerebellar cortex of brains from post-mortem autistic individuals (Yang *et al.* 2013). In other neurodevelopmental disorders such as ADHD, astroglial dysfunction was also reported. In a rat model of ADHD, the SHR, an increase in cerebellar astrogliosis and an increase in

apoptotic markers were observed when compared to their controls (Yun *et al.* 2014). An impairment in astroglial development in neurodevelopmental disorders, may well be contributing to the overall pathogenesis of disorders such as autism and ADHD (Molofsky *et al.* 2012).

c) Hypertension and cardiovascular disorders

Essential hypertension and several cardiovascular diseases, such as heart failure, are characterized by an enhanced sympathetic tone (Malpas 2010). As mentioned in the earlier sections, this enhanced sympathetic tone is theorized to not be a consequence of the disorder, but contributes to the progression of it. Overt excitatory impulses from the RVLM presympathetic neurons, are ascribed as a major cause for sympathoexcitation that is observed in essential hypertension and cardiovascular diseases (Kumagai *et al.* 2012). Several theories have been attributed to this phenomenon. Imbalances in the levels of activating and restraining inputs to the RVLM (Smith and Barron 1990) (Ito *et al.* 2000), pro-oxidant and pro-inflammatory states in the RVLM (Wu *et al.* 2012), and imbalances between the excitatory and inhibitory neurotransmitters in the RVLM (Kishi *et al.* 2002), are some theories that have been highlighted in the literature. NTS via the CVLM has a restraining effect on RVLM activity (Guyenet 2006). ATP has been demonstrated to be an extremely important neurotransmitter in mediating the excitatory components of RVLM (Marina *et al.* 2013) and the chemoreflex input of the NTS neurons (Braga *et al.* 2007). Purinergic signaling is a key signaling system that regulates cardiovascular function by exerting autonomic modulatory influences (Gourine *et al.* 2009). Purinergic signaling is also crucial for astrocytes to communicate not only with other astrocytes, but also with neurons (Fields and Burnstock 2006). Brainstem hypoxia, which is commonly observed in

hypertension and cardiovascular diseases, is also known to be a favorable environment for ATP release from astrocytes (Marina *et al.* 2015) (Marina *et al.* 2016). Using rat models of heart failure and hypertension, the role of astroglial ATP were demonstrated to have exceedingly important roles in the pathogenesis of the aforementioned disorders (Marina *et al.* 2016). Interestingly, neuroinflammatory cytokines were also reported to be elevated in the cardiovascular centers of the brainstem and hypothalamus (Agarwal *et al.* 2011). Not only do astrocytes and microglial cells have fundamental roles in the homeostatic regulation of cytokines in the brain, but cytokines themselves can influence glial cell functions (John *et al.* 2003). Impaired resolution of neuroinflammatory conditions, could well lead to an impairment of cardioregulatory regions of the brain. In support of this view, SHRs treated with an inhibitor of S100B, a pro-inflammatory and an apoptotic marker of astrocytes (Higashino *et al.* 2009), and minocycline, an inhibitor of microglial activation (Shi *et al.* 2010b), resulted in a significant reduction of blood pressure. Prominent astrogliosis was reported in SHRs by multiple groups, further highlighting the role of astrocytes in the pathogenesis of hypertension (Tomassoni *et al.* 2004).

v) Conclusion

While the role of astrocytes has been explored in neurodegenerative disorders, their role in neurodevelopmental disorders, such as ADHD, and cardiovascular diseases, is still relatively unknown. As mentioned earlier, astrocytes are involved in a multitude of CNS functions, which encompasses crucial CNS development and cardioregulatory roles. Several disorders have already been directly linked to astrocyte malfunction (Borrett and Becker 1985) (Furnari *et al.* 2007) (Verkhratsky *et al.* 2012). Since most of the currently employed CNS drugs are designed based on neuronal receptors and functions, it is

plausible that investigation of these cells, in pathological conditions such as ADHD and hypertension, may reveal new insights into the pathogenesis of these diseases.

2.3 Brain inflammation in neurodevelopmental and cardiovascular disorders

i) Physiological roles of cytokines

Cytokines are a group of polypeptides that are fundamental for the initiation, perpetuation and termination of neuroinflammatory responses. Along with other peptides, these soluble factors are crucial for mediating both acute and chronic phases of inflammation (Ramesh *et al.* 2013). Cytokines are not only critical for the regulation of inflammatory responses, but they can also elicit a variety of cellular functions. In the CNS, cytokines are secreted by all brain cells, but microglia and astrocytes are the two major sources (Benveniste 1992). Once secreted, they can activate and transform glial cells to reactive cells (John *et al.* 2003). Cytokines can also alter the structural integrity of the BBB, resulting in entry of circulating immune cells (Pan *et al.* 2011). Additionally, glial cells can also produce anti-inflammatory cytokines that can terminate the inflammatory response (Vitkovic *et al.* 2001). Therefore, cytokines such as IL (Interleukin)-10 and IL-1 receptor antagonist (IL-1RA) can orchestrate an immune response to a CNS injury or an invading pathogen, and constitute key elements of the anti-inflammatory machinery of glial cells. In addition to their neuroprotective functionality, cytokines also have potent neuromodulatory roles (Vitkovic *et al.* 2000). Under normal conditions, cytokines such as IL-6, and other pro-inflammatory cytokines, once secreted from glial cells can activate neuronal cytokine receptors in a paracrine fashion (Vezzani and Viviani 2015). Recently, their roles in early CNS development has also come to the fore (Deverman and Patterson 2009). Neuropoietic cytokines, mostly belonging to the IL-6 family, along with neurotrophic factors, are key

regulators of neuronal development during the embryonic stage, and gliogenesis during post-natal stages (Stolp 2013). This highly regulated process, controls cell-fate decisions, and also the specialization of progenitor cells into different cell types in the mature brain.

ii) Pathological roles

a) Aberrant levels

Under chronic pathological conditions, excessive production of pro-inflammatory cytokines, can lead to impaired firing of neurons, excitotoxicity and neurodegeneration (Wilcox and Vezzani 2014). These effects result in neurological and cardiovascular impairments (Waki and Gouraud 2014). Evidently, neurodegenerative disorders are characterized by a marked alteration in the levels of neuroinflammatory cytokines (Frank-Cannon *et al.* 2009) (Li *et al.* 2011) (Wang *et al.* 2015). Also, aberration in cytokine levels at prenatal stages, has been linked to psychosis and several neurodevelopmental disorders (Stolp 2013).

b) Role of cytokines in the etiology of neurodevelopmental disorders

Neurodevelopmental disorders such as autism are characterized by inflammatory states, in the periphery as well as the CNS (Onore *et al.* 2012). Post mortem studies, conducted on the brains of individuals with autism, revealed an elevated pro-inflammatory state in the cortex and the cerebellum, as well as a marked glial cell activation (Vargas *et al.* 2005). Additionally, another study reported an imbalance between the levels of pro- and anti-inflammatory cytokines in post-mortem brain specimens of individuals with autism (Li *et al.* 2009). A balance between pro and anti-inflammatory cytokines is essential for the normal development of the brain. Since an imbalance in the levels at prenatal stages

can result in significant deviations in neuronal circuit development (Meyer *et al.* 2009), the concept of fetal programming, has gained credence in the field of neurodevelopmental disorders (Szatmari 2011) (Madore *et al.* 2016). Cytokine imbalances were also reported in ADHD by two different studies (Oades *et al.* 2010) (Mittleman *et al.* 1997). A slight increase in the levels of IL-16 and the anti-inflammatory cytokine IL-13, and glial cell dysfunction were observed (Oades *et al.* 2010). Interestingly, our laboratory reported an altered neuroinflammatory state in cerebellar astrocytes isolated from SHRs when compared to Wistar rats (Gowrisankar and Clark 2016c). Dysfunction of SHR cerebellar astrocytes have been reported to have a crucial role in the development of ADHD in that model (Yun *et al.* 2014). Cerebellar astrogliosis has been shown to contribute to ADHD symptoms (Yun *et al.* 2014). Impaired functioning of cerebellar astrocytes in SHRs, warrants further investigation of this cell type in neurodevelopmental disorders.

c) Role of cytokines in the etiology of cardiovascular disorders

Chronic inflammation has often resulted in a deterioration of several cardiovascular functions, both in the periphery as well as the brain (Dinh *et al.* 2014) (Pauletto and Rattazzi 2005). An augmentation of centrally-mediated sympathetic activity, is theorized to be a crucial mechanism for the pathogenesis of essential hypertension and cardiovascular diseases such as heart failure (Grassi *et al.* 2010). An impairment in the functions of the presympathetic neurons arising from the RVLM, and regulatory inputs into the RVLM from other cardiovascular centers, is ascribed as being a major factor for centrally-mediated sympathetic augmentation (Dampney 1994) (Wainford 2014). The idea that dysregulated neuroinflammatory states, in the cardiovascular centers of the brainstem, could contribute

to sympathoexciation, was suggested by Paton and colleagues, less than a decade ago (Waki *et al.* 2008b). This was based on gene expression studies in the NTS of SHRs, where they observed altered inflammatory states, specifically in the levels of junctional adhesion molecule 1 (JAM-1), monocyte chemotactic protein 1 (MCP-1) and IL-6 (Waki *et al.* 2008b). Following their study, multiple groups have provided evidence of a neuroinflammatory involvement in Ang II-mediated sympathoexcitation (Shi *et al.* 2011) (Shan *et al.* 2011) (Shi *et al.* 2010b) (Wu *et al.* 2012) (Winklewski *et al.* 2015). An elevation in the levels of IL-1β and IL-6, and a decrease in the anti-inflammatory cytokine IL-10 were observed in the cardiovascular centers of the hypothalamus and brainstem of SHRs when compared to their normotensive controls (Agarwal *et al.* 2011). Evidence of a direct link between neuroinflammation and hypertension was established when it was demonstrated that intracerebroventricular administration of the pro-inflammatory cytokine IL-1β into the PVN resulted in an elevation in blood pressure (Shi *et al.* 2011). A disruption in BBB has also been demonstrated in hypertensive conditions; this may result in infiltration of peripheral immune cells into the CNS (Marvar *et al.* 2011). Also, blockade of Toll like receptor 4 in the PVN, resulted in restoring the balance between pro- and anti-inflammatory cytokines, suggesting the crucial role of neuroinflammation in the regulation of various cardiovascular parameters (Dange *et al.* 2015). The latter underscores the importance of microglia in the exacerbation of neuroinflammatory states in the PVN of SHRs. In support of this finding, minocycline, an inhibitor of microglial activation, neutralized Ang II-mediated elevation in pro-inflammatory cytokines and elevated sympathetic activity in Wistar rats (Shi *et al.* 2010b). The above studies indicate the importance of glial cells and neuroinflammation in the elevation of sympathetic activity.

iii) Conclusion

Since astroglial dysfunction and altered inflammatory states have been identified in hypertensive rat models, it is plausible that brainstem astrocytes contribute to the impaired neuroinflammatory and pro-oxidant state observed in SHRs. This altered neuroinflammatory state, may well be contributing to a dysfunction of the cardiovascular centers in the brain. Our laboratory has observed significant differences in IL-6 mRNA levels in brainstem astrocytes isolated from SHRs when compared to their normotensive controls (Gowrisankar and Clark 2016c). Additionally, treatment of brainstem astrocytes with Ang II, the major pro-hypertensive peptide, as well as Ang III caused a spike in the levels of IL-6 (Kandalam and Clark 2010) (Kandalam *et al.* 2015). Whether other inflammatory cytokines, such as IL-1β and IL-10, are altered as well, will be the one of the aspects that were investigated in this study.

2.4 RAS and neuroinflammation

i) Classical and non-classical RAS

The RAS is a cardioregulatory, peptidergic, hormonal system. It is composed of several different components which encompasses precursor and active peptides, enzymes and receptors. In response to a drop in blood pressure, low salt concentration, or low blood volume, the juxtaglomerular apparatus in the kidneys triggers the RAS cascade, which ultimately culminates in a significant anti-natriuretic, anti-diuretic and vasoconstrictive effect (Montani and Van Vliet 2004) (Hall 1986). Renin, secreted from the kidneys, converts Angiotensinogen (AGT), secreted from the liver, to Ang I. Ang I is converted into Ang II by the action of ACE, an enzyme produced in the lungs and blood vessels (Montani and Van Vliet 2004). Ang II, the major effector peptide of RAS, is a circulating hormone,

that has multiple physiological functions. While most of their functions converge to have one singular outcome, that is an elevation in blood pressure, RAS also has a role to play in several other functions such as digestion, reproduction and prenatal development (Paul *et al.* 2006). The widely studied and documented actions of Ang II such as aldosterone secretion, vasoconstriction and ionotropic effects are due to its ability to interact with the AT1R (Fyhrquist *et al.* 1995). Ang II via activation of the AT1R, a pertussis toxin-insensitive GPCR, causes a spike in the intracellular calcium levels. Elevation in calcium triggers activation of kinases, signaling pathways and transcription factors, and consequently causes several physiological actions such as smooth muscle contraction and aldosterone synthesis (de Gasparo *et al.* 2000). By interacting with the AT1R on the renal, cardiac and vascular cells, Ang II is able to increase aldosterone levels, elevate salt intake, sympathetic nervous system hyperactivation, have a positive ionotropic effect, and is also known to elicit potent vasoconstriction (Fyhrquist *et al.* 1995). Ang II is also known to interact with the AT2R, which is antagonistic to AT1R functions (Stoll *et al.* 1995). Its activation results in vasodilatory and cardioprotective effects (Li *et al.* 2012b). AT2R expression is higher during prenatal stages when compared to adulthood (de Gasparo *et al.* 2000), although this view has been challenged (Yu *et al.* 2010). Several other peptides that have functional similarity as well as dissimilarity to Ang II have also been identified, characterized and studied (Paul *et al.* 2006). Ang III, Ang IV and Ang (1-7) are physiologically active degradation products of Ang II. Ang III interacts with the AT1R, while Ang IV and Ang (1-7) interacts with their own cognate receptors, the Ang type 4 receptor (AT4R) and the Mas receptor, respectively (Varagic *et al.* 2008). Ang II is degraded by Aminopeptidases to Ang III and Ang IV, and by ACE2 to Ang (1-7) (Paul *et*

al. 2006). Alternatively, Ang (1-7) can also be synthesized from Ang I by the action of neprilysin (Paul *et al.* 2006). Ang III exhibits functional similarity to Ang II, while Ang (1-7) counteracts the deleterious effects of Ang II (Ferrario *et al.* 1991). The schematic representation of RAS pathway is in Fig 2.1. It is now widely accepted that Ang II is not only produced exclusively by a select group of tissues, but also by several other cell types (Paul *et al.* 2006). Optimal functioning of both systemic and local RAS, is critical for overall cardiovascular homeostasis (Lavoie and Sigmund 2003).

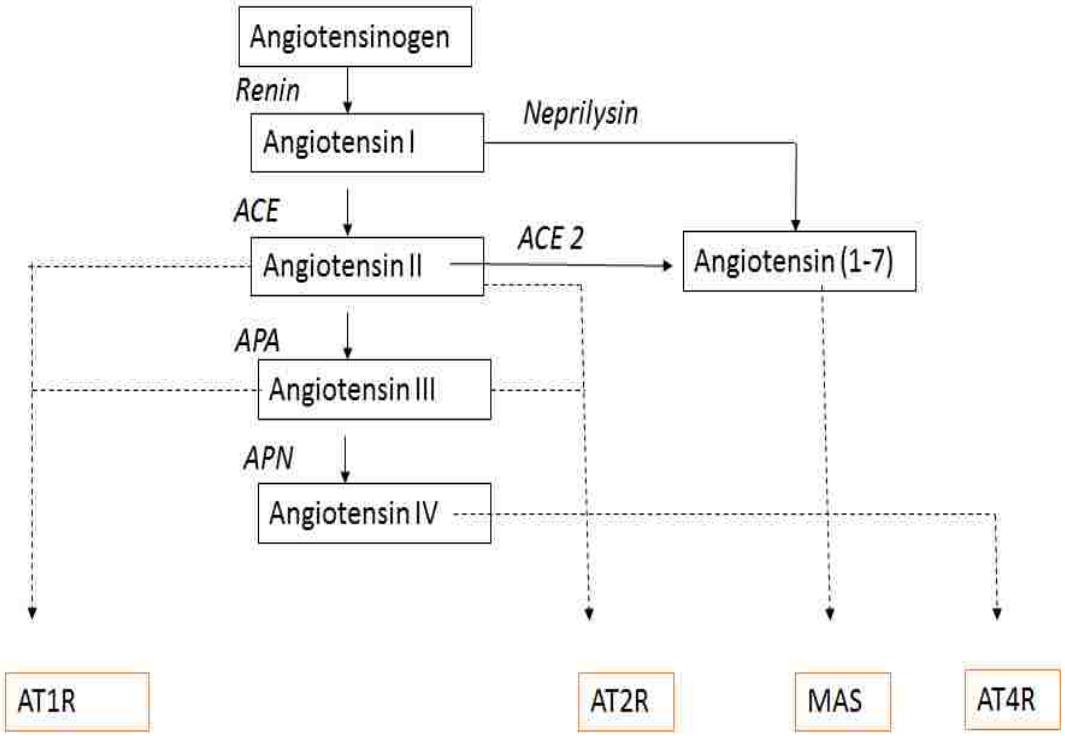

Fig 2.1: RAS pathway

ii) Significance in cardiovascular diseases

A dysregulated RAS is one of the hallmarks of cardiovascular diseases (Veerasingham and Raizada 2003). A hyperactive RAS is strongly correlated with several risk factors of cardiovascular diseases (Fyhrquist *et al.* 1995). Since both Ang II synthesis and AT1R activity is fundamental to RAS-mediated elevation in blood pressure, drugs that impede synthesis of Ang II, or those that antagonize the deleterious effects of AT1Rs, are the mainstay in the pharmacological management of numerous cardiovascular diseases, risk factors and their complications (Atlas 2007) (Burnier and Zanchi 2006). In addition to its augmented ability to elevate blood pressure by multiple mechanisms in cardiovascular diseases, Ang II can also cause extensive damage to the heart, kidneys and the vasculature (Wang *et al.* 2014) (Long *et al.* 2004) (Montezano *et al.* 2014). AT1R blockers and ACE inhibitors are routinely employed in several cardiovascular disorders and their risk factors, and are considered to be an extremely valuable therapeutic strategy (Aranda and Conti 2003) (Atlas 2007). At a molecular level, elevation in ROS and inflammatory cytokines is identified as a single overarching paradigm for AT1R-mediated effects (Mehta and Griendling 2006). Some of the physiological and pathological roles of the AT1R are visually represented in Fig 2.2.

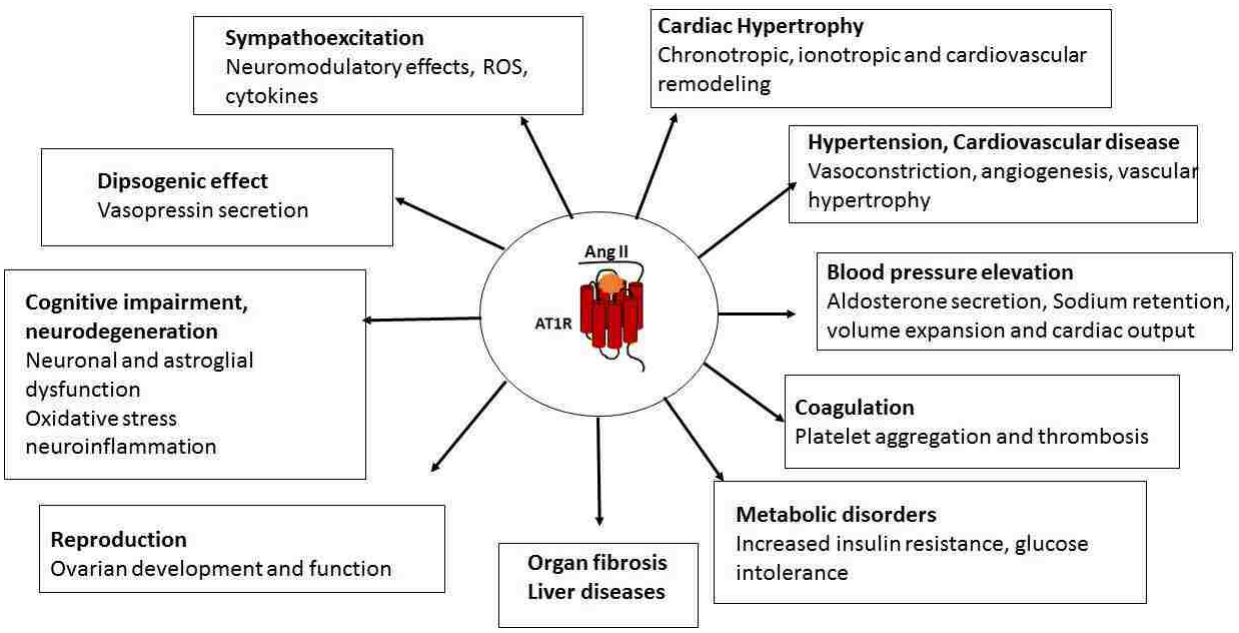

Fig 2.2: Physiological and pathological roles of AT1R activation

iii) Brain RAS and its role in blood pressure control

Although functional AT1Rs in the brain were identified in the 1960's, the notion that brain cells could produce Ang II, was suggested much later (Bickerton and Buckley 1961). Evidence of Ang II synthesizing enzymes and Ang II precursors, in brain cells, provided the necessary impetus for the idea of an independently functioning brain RAS (Brooks and Malvin 1979) (Phillips 1983) (Campbell *et al.* 1984). This proved to be an unheralded discovery in the late 1970's and early 1980's. Following the discovery of brain RAS, several research groups reported its involvement in the development of cardiovascular diseases and their risk factors (Veerasingham and Raizada 2003) (Huang and Leenen 2009) (Campos *et al.* 2012). In the CNS, the AT1R levels are particularly greater in the

cardiovascular centers of the hypothalamus and brainstem; this is indicative of the importance of brain RAS in the regulation of cardiovascular parameters (Lenkei *et al.* 1995) (Phillips *et al.* 1993). Significant evidence for its role in cardiovascular disorders came from studies on animal models of essential hypertension, such as SHRs. The brain RAS was demonstrated to be overactive in SHRs when compared to their normotensive controls (Veerasingham and Raizada 2003). Both Ang II as well as AT1R levels were observed to be higher in brainstems of SHRs when compared to their normotensive controls, Wistar Kyoto Rats (WKY) (Veerasingham and Raizada 2003). Microinjection of Ang II, or modulation of RAS, in the cardioregulatory regions of the brainstem and hypothalamus, was demonstrated to result in an increase in mean arterial pressure (Casto and Phillips 1985) (Zhu *et al.* 1998) (Matsuda *et al.* 1987) (Ito *et al.* 2002). (Stadler *et al.* 1992). RVLM neurons treated with Ang II resulted in greater firing rates in SHRs when compared to WKY neonatal pups (Matsuura *et al.* 2002). In addition, the signal transduction pathway, phosphoinositide 3-kinase (PI3K), was demonstrated to be a critical mechanism for AT1R-mediated elevation of RVLM neuronal activity in SHRs, but not in their normotensive controls (Veerasingham *et al.* 2005) (Seyedabadi *et al.* 2001). The brain Ang II has potent neuromodulatory effects. By acting on the neuronal AT1R, Ang II can modulate impulses generated by several neurotransmitters such as glutamate, GABA and norepinephrine and thereby alter synaptic strength and plasticity (Tsuda 2012). Ang II was demonstrated to decrease baroreflex sensitivity by increasing GABAergic input into the NTS (Wang *et al.* 2006). Elevation in ROS and endothelial nitric oxide synthetase, and a decrease in neuronal nitric oxide synthetase were described as crucial mediators of AT1R-mediated impairment of baroreflex gain in the NTS (Cheng *et al.* 2010) (Wang *et al.* 2006).

iv) Astroglial RAS and hypertension

Components of the RAS are not only expressed in neuronal cells, but also glial cells, and both these brain cells have a role in regulating cardiovascular functions (Morimoto *et al.* 2002). Astrocytes are theorized to be the major source of AGT in the brain (Deschepper *et al.* 1986). In SHRs, an increase in the levels of AGT, prior to the development of hypertension, was identified in the brain (Tamura *et al.* 1996). This further underscores the role of astroglial AGT in the development of hypertension. Interestingly, our laboratory has demonstrated an increase in the levels of AGT from brainstem astrocytes in response to Ang II (Gowrisankar and Clark 2016b). Additionally, we observed an increase in ACE and a decrease in ACE2 in response to Ang II treatment (Gowrisankar and Clark 2016a). Hence, the balance between the synthesizing and degradative enzymes could be altered, favoring Ang II synthesis. The consequence may well be an elevation or a renewal of brain Ang II, leading to an enhancement in AT1R activity. This self-replenishment of Ang II, could be the reason for an elevation in the levels of Ang II, that is observed in the brainstems of SHRs. Also, ablation of astroglial AT1Rs in the brainstem, has been demonstrated to result in an improvement in the symptoms of heart failure, by normalization of sympathetic activity (Isegawa *et al.* 2014a).

While the importance of the astroglial RAS has been underscored by several studies, the molecular mechanisms underlying their effects have not been well investigated. Several groups have demonstrated the ability of pro-inflammatory cytokines to regulate sympathetic activity (Winklewski *et al.* 2015). Neuroinflammatory states were identified at very early stages of hypertension in SHRs, indicating a causal role in the development of essential hypertension (Waki *et al.* 2008a). Whether an increase in pro-inflammatory

cytokines, in the cardiovascular centers of the brain, was an important mediator of Ang II-mediated sympathoexcitation, was investigated by Kang et al. (2008). They demonstrated that chronic infusion of Ang II in Sprague-Dawley rats resulted in an elevated sympathoexcitatory state, that was characterized by a pro-inflammatory and a pro-oxidant state in the PVN. Blockade of AT1Rs and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-KB) was also demonstrated to normalize pro-inflammatory cytokines as well as sympathetic activity, further authenticating the role of neuroinflammation and RAS in sympathoexcitation (Kang *et al.* 2009). Also chronic infusion of Ang II led to a prominent inflammatory state in the brain vasculature via an increase in ROS (Zhang *et al.* 2010). The role of glial RAS in elevating neuroinflammatory states was theorized in a review by Shi P et al (2010). They conceptualized that glial AT1R activation results in an increase in the levels of pro-inflammatory cytokines, which can then act as neuromodulators and regulate synaptic activity (Shi *et al.* 2010b). It is plausible that mobilization of cytokines from glial cells can alter neuronal activity, since low levels of cytokines have been theorized to alter neuronal activity (Waki and Gouraud 2014). Definitive evidence of the role of inflammatory cytokines and Ang II-mediated elevation in sympathetic nervous system activity came from studies in hypothalamus by the same group (Shi *et al.* 2010a). Chronic Ang II infusion in the PVN resulted in an increase in pro-inflammatory cytokines and a decrease in anti-inflammatory cytokines, which then caused an elevation in blood pressure (Shi *et al.* 2010a). This effect could be blocked by minocycline, indicating that this effect was mediated by microglial AT1Rs (Shi *et al.* 2010a). Studies have shown that microglial cell activation, is followed by astroglial activation (Liu *et al.* 2011b). Hence, microglia may initiate the inflammatory response, and

astrocytes may aid in perpetuating the inflammatory condition. A synergistic effect between pro-inflammatory cytokines and Ang II has also been reported in the PVN on sympathetic activity (Shi *et al.* 2011). Additionally, PVN astrocytes isolated from SHRs, when treated with prorenin, resulted in an augmented increase in proinflammatory cytokines (Rodríguez *et al.* 2015) (Rodríguez *et al.* 2016). Our group observed an elevation of pro-oxidant and pro-inflammatory states in brainstem astrocytes, isolated from SHRs and Wistar rats, in response to Ang II treatment (Gowrisankar and Clark 2016c). Ang II via AT1R has also been demonstrated to elevate ROS as well as activate key signal transduction pathways, such as extracellular signal–regulated kinase (ERK), p38 and Janus kinase (JAK)-signal transducer and activator of transcription (STAT) pathway, key pathways that are critical to several astroglial functions (Clark *et al.* 2013) (Kandalam *et al.* 2015) (Z. Alanazi *et al.* 2014) (Gowrisankar and Clark 2016c). Activation of these pathways, along with an elevation in ROS levels, has been demonstrated to lead to cell proliferation and mobilization of inflammatory cytokines in astrocytes (Kandalam and Clark 2010) (Clark *et al.* 2013) (Gowrisankar and Clark 2016c).

v) Brain RAS and neurological disorders

Due to their pro-oxidant and pro-inflammatory effects, the role of brain RAS in the development of disorders characterized by neurological impairments and neurodegeneration, has also been investigated (Mascolo *et al.* 2017). Since astrocytes are involved in mediating homeostasis by regulating levels of cytokines and ROS in the brain, hyperreactive astroglial AT1R may well be a prominent feature of not just cardiovascular, but also neurological disorders. Astrocytes from brain regions other than brainstem, such as cerebellum, are also responsive to Ang II treatment (Clark *et al.* 2013)(Gowrisankar and

Clark 2016b). Ang II caused a significant increase in the pro-inflammatory cytokine, IL-6, and ROS levels, in astrocytes isolated from cerebellum from both Wistar and SHRs (Gowrisankar and Clark 2016c). Ang II-mediated elevation in ROS and pro-inflammatory states have been demonstrated to be associated with neurodegeneration, and also astrocyte senescence (Liu *et al.* 2011a) (Lanz *et al.* 2010) (Min *et al.* 2011). Further, several studies have also demonstrated the beneficial effects of RAS blockade in neurological diseases (Mogi and Horiuchi 2009). Evidence linking ACEIs or AT1R blockers with improvement of cognitive function was also reported (Davies *et al.* 2011). Favorable outcomes of ACEIs and AT1R blockers on improving rates of cognitive function in elderly individuals have also been reported (Gao *et al.* 2013) (Saxby *et al.* 2008). It could well be that RAS hyperactivity also plays a role in the etiology of neurodevelopmental disorders. As mentioned earlier, SHRs are employed as a rat model for ADHD. Considering that RAS is a premium hormonal system that is augmented in the SHR brains, it is surprising to observe a paucity of studies investigating the effects of RAS in ADHD. Nevertheless, the ability of Ang II to promote a pro-inflammatory state in different regions of the brain, may lead to significant alteration in brain functions, eventually leading to neurological disorders or an exacerbation of several neurological conditions.

2.5 Endocannabinoid system in physiological and pathological conditions

i) History

The therapeutic potential of cannabis, commonly known as marijuana, has been a subject of great interest for several centuries. Its anxiolytic and euphoric properties were acknowledged in religious scriptures that date back to several millennia (Touw 1981). Several cultures and civilizations have used cannabis preparations to treat a variety of

ailments, ranging from rheumatism, inflammatory disorders in the previous millennia, to dysentery and malaria in the current millennia (Zuardi 2006). While evidence of therapeutic utility of cannabis was known in Asia and Africa, its therapeutic utility was relatively unknown to the western world until the 19th century (Di Marzo 2006). The first scientific report on cannabis was published by the Irish physician, William 'O Shaughnessy, which marked the first traces of cannabis globalization. By providing evidence of its therapeutic efficacy and safety for pathological conditions such as infantile convulsions and cholera, he was instrumental in laying the foundation for cannabis research (O 'Shaughnessy, 1838-1840) (Di Marzo 2006). Pioneering works from the groups of Cahn, Todd, Adams and Mechoulam, in the 20th century, led to a better understanding of the chemical makeup of cannabis (Di Marzo 2006). However, the mechanism of its action, remained a mystery. Three decades later came the first report of the existence of the cannabinoid receptor, termed cannabinoid receptor type 1 (CB1R), in the brain, which was reported by Howlett's group in the late 1980's in rat brain (Devane *et al.* 1988). The discovery of CB1R was followed by the identification of the second cannabinoid receptor, termed cannabinoid receptor type 2 (CB2R), and their endogenous ligands, termed endocannabinoids (Bisogno *et al.* 2005) (Pertwee 2009b). Unlike other GPCRs which usually have only one endogenous ligand, the cannabinoid receptors could be activated by two endogenous ligands, anandamide and 2-arachidonoylglycerol (2-AG). These ligands have complementary as well as divergent functions (Di Marzo and De Petrocellis 2012). Endocannabinoids not only interact with the two cannabinoid receptors, but they are capable of interacting with an array of different receptors, channels and proteins (Di Marzo and De Petrocellis 2012). The identification of the enzymes that are involved in the

degradation and biosynthesis of endocannabinoids, fatty acid amide hydrolase (FAAH) and diacylglycerol lipase (DAGL), in the following years, made up the classical endocannabinoid system (Bisogno *et al.* 2005).

ii) Localization of CB1R

The CB1R is highly expressed in the CNS, with densities that rivals other neurotransmitter and neuromodulatory receptors (Herkenham *et al.* 1991). Their expression however is region dependent; CB1Rs are highly expressed in the cerebellum, hippocampus and basal ganglia, while markedly reduced in the brainstem (Herkenham *et al.* 1991). In contrast to opioids, another commonly abused drug, marijuana possess a very high therapeutic index. This has been attributed to a sparse distribution of CB1R in the human brainstem. Its propensity to regulate motor, memory and emotional functions, were attributed to the greater CB1R distribution in the cerebellum and limbic system (Herkenham *et al.* 1991). The CB1R is also found in peripheral regions of the body, such as the peripheral nervous system, as well as in the cardiovascular, metabolic, renal, and reproductive organs (Pacher *et al.* 2005a) (Szekeres *et al.* 2012). Although the CB2R expression is considered to be much higher in lymphoid organs, CB1R transcripts have also been detected in primary and secondary lymphoid cells (Galiègue *et al.* 1995). Owing to their ubiquitous expression in the body, CB1R has been investigated in a multitude of physiological functions. Endocannabinoid synthesis and degradation, along with their receptor functions are shown in Fig 2.3.

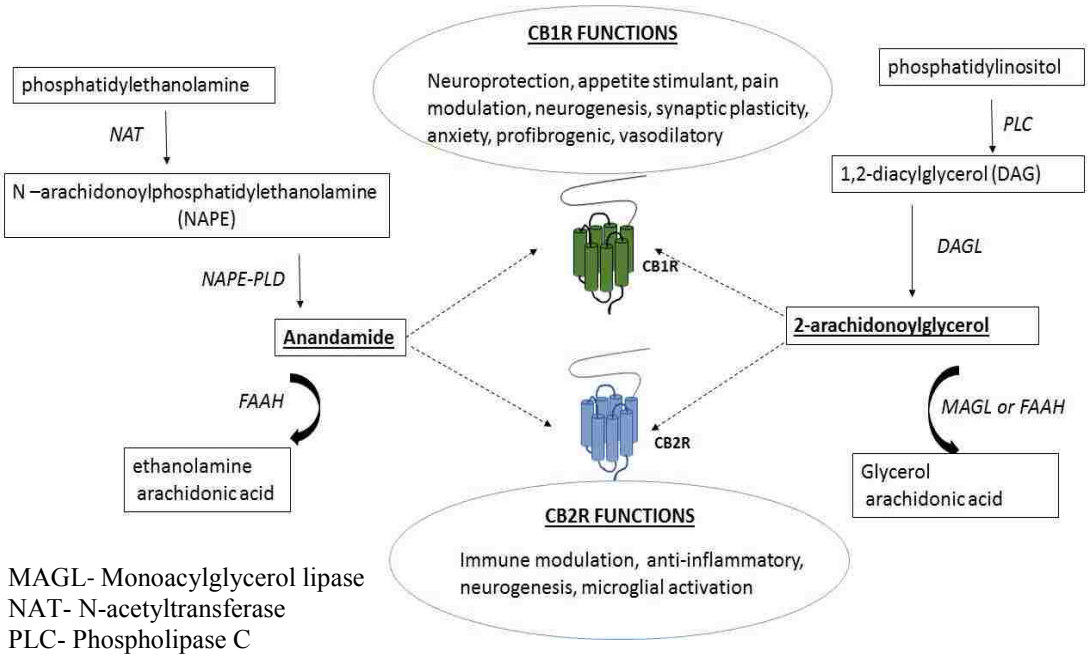

Fig 2.3: Endocannabinoid system

CB1R is a Class A GPCR that couples to the pertussis toxin-sensitive G-protein. It is theorized to exhibit high constitutive activity (Turu and Hunyady 2010). The majority of CB1Rs have been reported to be present intracellularly by several groups (Leterrier *et al.* 2004) (Rozenfeld and Devi 2008) (McIntosh *et al.* 1998). Its localization in astrocytes however has not been well documented. Similar to its localization in other cells, we observed high intracellular staining for the CB1R and its phosphorylated form in astrocytes. While astrocytes displayed staining for CB1R over the entire cell, the staining was of higher intensity in the intracellular region, predominantly in the nuclear and the perinuclear regions (Fig 2.4-Panel A). In the case of phosphorylated CB1R (p-CB1R), we not only observed nuclear staining, albeit a less intense one to that observed for the total CB1R, but also a localized punctate cytoplasmic staining. The latter that may be indicative of receptors that have been endocytosed (Fig 2.4- Panel B).

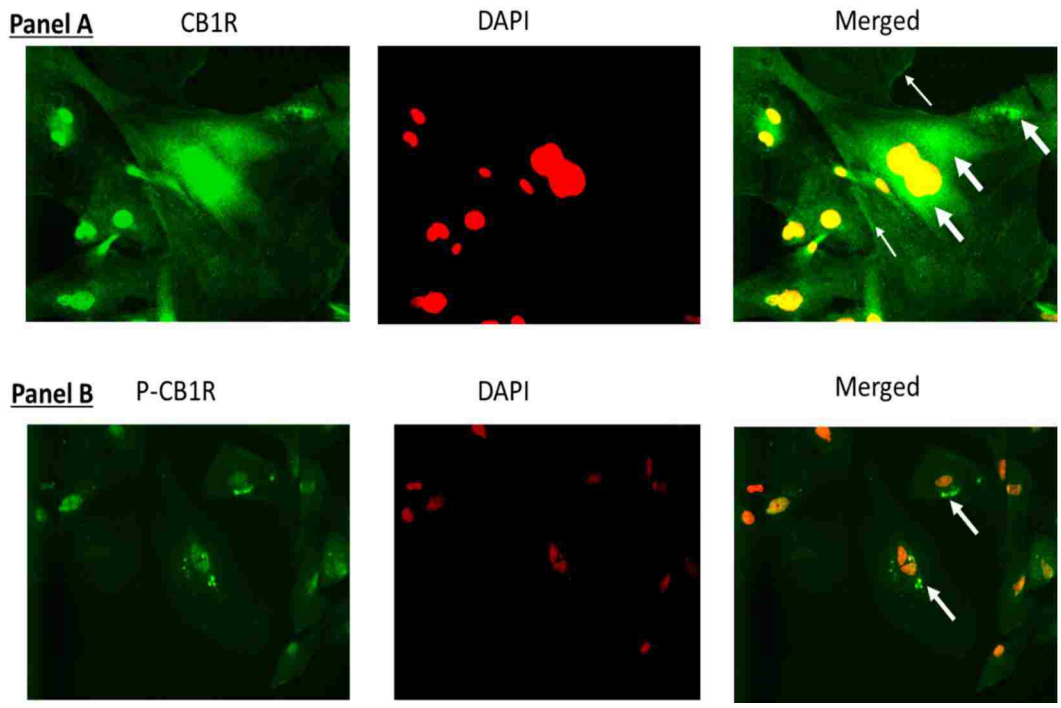

Fig 2.4: CB1R and p-CB1R localization in astrocytes: Immunofluorescence technique was employed to determine the localization of CB1R and p-CB1R localization in astrocytes. **Panel A** denotes the cells that were stained with an antibody for CB1R. Thin arrows denotes surface expressed receptors and thick arrows denotes receptors that are found in cytoplasm. **Panel B** denotes the cells that were stained with antibody for the p-CB1R. Thick arrows denotes cytoplasmic staining. Refer to chapter 4 for details about staining procedures and antibodies.

iii) Summary of CB1R's physiological roles

a) Neurodevelopment

The CB1R is of paramount importance in cell to cell communication. Although evidence of endocannabinoid synthesis can be traced back to unicellular organisms (Elphick 2012), the origins of CB1R closely parallels the evolution of multicellular organisms (Elphick and Egertová 2005), which underscores its importance in mediating cell interactions. The ubiquitous expression of DAGL (Bisogno *et al.* 2003), and the high density of CB1Rs in the brain, makes this system especially important in brain functions that are tightly regulated by endocannabinoid levels. Elements of this system are detected

at virtually every stage of brain development and maturation (Galve-Roperh *et al.* 2009). Endocannabinoids acting through the CB1R, regulate progenitor cell proliferation, cell specialization and cell survival (Maccarrone *et al.* 2014). Cell fate decisions such as neurogenesis and axonal elongation, synaptogenesis and regulation of synaptic strength are tightly regulated by the levels of endocannabinoids (Aguado *et al.* 2005) (Harkany *et al.* 2008).

b) Neuromodulatory Roles

Fundamental to CB1R's neuroprotective roles, is its ability to fine tune and regulate synaptic transmission via retrograde signaling (Ohno-Shosaku *et al.* 2001) (Alger 2002). The CB1R is highly localized in presynaptic neurons (Egertová and Elphick 2000). Although originally identified on the presynaptic GABAergic neurons (Katona *et al.* 1999), evidence of CB1R localization has also been observed on the presynaptic glutamatergic (Robbe *et al.* 2002), cholinergic (Degroot *et al.* 2006) and nonadrenergic (Oropeza *et al.* 2007) neurons. Excessive postsynaptic receptor activation results in mobilization of endocannabinoids into the synaptic cleft. They can then traverse across the synaptic cleft, from the postsynaptic neuron to the presynaptic neuron, where they activate the CB1R, which couples to the pertussis toxin-insensitive G-protein. Activation of CB1R attenuates neurotransmitter release into the synaptic cleft, resulting in dampened synaptic activity (Castillo *et al.* 2012). Hence, the CB1R can either suppress excitatory neuronal activity (Kreitzer and Regehr 2001), termed depolarization-induced suppression of excitation (DSE), or dampen the activity of inhibitory neurons (Ohno-Shosaku *et al.* 2001), termed depolarization-induced suppression of inhibition (DSI). This 'circuit breaker' like

functionality is a crucial modus operandi of the CB1R by which it influences synaptic plasticity.

c) Functions of Astroglial CB1R

Glial cell lineages develop postnatally, and the CB1R is demonstrated to have a key role in regulating the transformation of progenitor cells into astrocytes (Aguado *et al.* 2006). Since the emergence of the concept of the tripartite synapse, several groups have shown that the paracrine signaling of endocannabinoids form the crucial bridge between brain cells (Araque *et al.* 1999) (Navarrete *et al.* 2014). Endocannabinoid-mediated bidirectional communication between astrocytes and neurons (Navarrete and Araque 2008), has been demonstrated to significantly impact synaptic plasticity (Navarrete and Araque 2010) and memory formation (Han *et al.* 2012), further underpinning the importance of the astroglial endocannabinoid system in regulating physiological functions that were earlier believed to be exclusively neuronal. In addition to astroglial signaling, glial cells are known to have immunomodulatory roles in the brain (Bélanger and Magistretti 2009). Activation of the astroglial CB1R has been demonstrated to promote an anti-inflammatory state by elevating anti-inflammatory cytokines, and simultaneously lowering the levels of pro-inflammatory cytokines (Molina-Holgado *et al.* 2003) (Sheng *et al.* 2005a) (Nagarkatti *et al.* 2009). Also, one study has highlighted the role of astroglial CB2R, along with CB1R, in mediating the anti-inflammatory effects of cannabinoids (Molina-Holgado *et al.* 2002a). Further, cannabinoids have been demonstrated to have anti-oxidant effects as well (Lipina and Hundal 2016). Activation of astroglial CB1R protects astrocytes against insults which induce apoptosis via an elevation in free radicals (Gómez Del Pulgar *et al.* 2002) (Carracedo *et al.* 2004).

iv) Endocannabinoid system and pathological conditions

a) Neurological impairment

Cannabinoid-based therapies have shown tremendous potential, at both pre-clinical and clinical stages, for several neurological impairments (Giacoppo *et al.* 2014). *In vitro* and *in vivo* studies in animal models of neurological and neurodevelopmental diseases, along with human data, helped to greatly expand our understanding of the endocannabinoid system's role in brain disorders (Scotter *et al.* 2010). Several of these disorders are characterized by an alteration of the levels of various components of the endocannabinoid system (Di Marzo 2008). Neurological and neurodegenerative disorders are often characterized by an impairment in long and short term synaptic plasticity and/or excitotoxicity resulting in neuronal cell death (Parpura *et al.* 2012). Elevation in the levels of glutamate has been observed in disorders such as ischemia and epilepsy (Stobart and Anderson 2013). The ability of CB1R to offer considerable protection against excitotoxic lesions, has been a compelling reason to view CB1R agonists as a bona fide therapeutic option for neurological and neurodegenerative disorders (Bisogno and Di Marzo 2007) (Scotter *et al.* 2010). CB1R agonists have been demonstrated to have immense therapeutic utility in numerous neurological conditions that are characterized with excitotoxic neuronal damage, such as Huntington's disease and epilepsy (Chiarlone *et al.* 2014) (Alger 2004). While the activation of neuronal CB1R corrects for synaptic errors (Katona and Freund 2008) (Alger 2014), glial CB1R activation leads to neutralization of free radicals and pro-inflammatory cytokines (Nagarkatti *et al.* 2009). Both of them essentially work to reestablish homeostasis in the brain. Persistent pro-oxidant and pro-inflammatory states can be a major factor in the development of debilitating brain disorders (Baker *et al.* 2009),

in addition to exacerbation of symptoms of neurodegeneration. Targeting the endocannabinoid system in neurological disorders may well be a judicious therapeutic strategy, since cannabinoids have demonstrated great therapeutic efficacy in clinical trials for several neurological impairments such as neuropathic pain and multiple sclerosis (Nagarkatti *et al.* 2009) (Pertwee 2002). Impairment of the endocannabinoid system has also been observed in neurodegenerative disorders, such as Parkinson's disease, that are characterized by an imbalance in excitatory and inhibitory neurotransmitter levels (More and Choi 2015) (Di Marzo 2008).

b) Neurodevelopmental disorders

The endocannabinoid system plays a fundamental role in the development and maturation of the nervous system during pre- and postnatal stages (Basavarajappa *et al.* 2009). In addition, the high density of CB1Rs in brain regions that regulate movement, memory, executive decisions and emotions is strongly indicative of their involvement in the progression of multiple neurodevelopmental disorders (Strohbeck-Kuehner *et al.* 2008). Modulation of the endocannabinoid system has been investigated for neurodevelopmental disorders such as Fragile X syndrome, autism and ADHD (Busquets-Garcia *et al.* 2013) (Chakrabarti *et al.* 2015) (Strohbeck-Kuehner *et al.* 2008). Depending on the pathological conditions, CB1R agonism can bring about beneficial or deleterious effects. CB1R-mediated enhanced suppression of GABA release is described as playing a vital role in the pathogenesis of Fragile X syndrome (Zhang and Alger 2010). This finding suggests that hyperactivation of the CB1R may well be contributing to the etiology of Fragile X syndrome. Dysfunctional FAAH enzyme activity was reported in peripheral lymphocytes of ADHD individuals (Centonze *et al.* 2009). In a rat model of ADHD,

hypoactivation of CB1Rs in the prefrontal cortex was observed (Adriani *et al.* 2003). Administration of cannabinoids resulted in improvement of symptoms associated with ADHD in that rat model (Adriani *et al.* 2003), indicative of a potential beneficial effect of CB1R agonism. Since several neurodevelopmental disorders are characterized by neuroinflammatory and pro-oxidant states, the strategy of leveraging the anti-inflammatory potential of the cannabinoid receptors to reestablish homeostasis of the neuronal milieu, may be an attractive strategy for treating these disorders (Wu *et al.* 2012) (Stolp 2013) (El-Ansary and Al-Ayadhi 2012)

c) Hypertension

Hypotensive effects of cannabinoids were known well before the identification of the endocannabinoid system. Studies in the 1970's assessed the impact of long-term effects of cannabinoids on cardiovascular parameters (Pacher *et al.* 2005a). Prolonged use, either marijuana inhalation, or delta-9-tetrahydrocannabinol (THC) consumption, resulted in a significant fall in heart rate and blood pressure (Benowitz and Jones 1975) (Rosenkrantz and Braude 1974). THC was demonstrated to have a potent hypotensive effect in hypertensive individuals when compared to normotensive individuals (Crawford and Merritt 1979). Evidence of central sympathoinhibition in response to cannabinoids, was also demonstrated (Vollmer *et al.* 1974) (Pacher *et al.* 2005a). But the complex cardioregulatory mechanisms of cannabinoids began to unravel, only after the investigation of the cannabinoid receptors in hypertensive animal models. By using SHRs and other rat models of hypertension, several studies in the late 1990's were able to elucidate the mechanisms by which cannabinoids regulate blood pressure. Cannabinoids were demonstrated to normalize blood pressure elevation by both central and peripheral

mechanisms (Mendizábal and Adler-Graschinsky 2007) (Malinowska *et al.* 2012). Centrally, administration of cannabinoids into the NTS resulted in sympathoinhibition via dampening of GABAergic neurons. This neuromodulatory effect of CB1R results in an improved baroreflex sensitivity (Seagard *et al.* 2004). In the periphery, enhancement of basal endocannabinoid tone resulted in improving cardiovascular parameters, such as heart rate and vascular resistance, of SHRs and other models of hypertension (Bátkai *et al.* 2004). Interestingly, while the myocardial and endothelial CB1R was elevated in SHRs, leading to enhancement of the peripheral endocannabinoid tone (Bátkai *et al.* 2004), reduced density of CB1R and consequently, a dampened endocannabinoid tone was identified in the CNS (Brozoski *et al.* 2009). Cannabinoid administration resulted in a marked reduction of blood pressure in SHRs, but not in WKY. Considering that the CB1R expression has been lowered in NTS of SHR, this effect may well be mostly mediated via peripheral CB1R. Although a possible involvement of the peripheral transient receptor potential cation channel subfamily V member 1 (TrpV1) channels cannot be disregarded (Li *et al.* 2003). Endocannabinoid hyperactivity in the periphery could be an adaptive or a compensatory mechanism in response to an elevation in blood pressure in established hypertension. In that case, hypofunctional endocannabinoid system in the NTS leading to an elevated sympathetic activity in SHRs (Brozoski *et al.* 2009), may be a crucial causative mechanism in the development of hypertension. This is a plausible theory since sympathetic hyperactivity is theorized to be one of the earliest cardioregulatory modifications in the etiology of hypertension (Anderson *et al.* 1989).

d) Positive and negative cardiovascular outcomes of endocannabinoid system activation

While favorable results were observed with CB1R agonists, several unanswered questions remain. As most of the studies employed an established hypertensive model, the claim that endocannabinoid dysfunction triggers hypertension, can neither be confirmed or denied, at the present moment. It should also be noted that some studies have reported sympathoexcitatory effects of endocannabinoid system activation (Niederhoffer and Szabo 2000) (Padley *et al.* 2003). This apparent discrepancy in results may be attributed to one of many reasons. The data generated from employing normotensive models (Padley *et al.* 2003); or monogenetic models of hypertension, as opposed to a polygenetic hypertension model (Schaich *et al.* 2014), may not be representative of a potential deleterious or beneficial role in hypertension. Other reasons such as inter-species variability (Niederhoffer and Szabo 2000); and the site of drug administration, RVLM or NTS (Padley *et al.* 2003), may also be a factor. However, there is evidence to support the therapeutic utility of CB1R antagonists, as CB1R agonism was demonstrated to worsen the symptoms of endothelial dysfunction and hepatic cirrhosis (Cooper and Regnell 2014) (Tiyerili *et al.* 2010). Although Rimonabant, the CB1R antagonist, has been demonstrated to have beneficial outcomes in obesity and metabolic syndrome (Pi-Sunyer *et al.* 2006), several psychiatric problems have also been reported (Mendizábal and Adler-Graschinsky 2007). Association of neurological complications with Rimonabant treatment, is a major impediment for the use of CB1R antagonists as a therapeutic strategy (Topol *et al.* 2010) (Boekholdt and Peters 2010). However, no differences in blood pressure was noted when Rimonabant treated groups were compared with the control groups over a period of 2 years (Pi-Sunyer *et al.* 2006). Evidence of beneficial effects of endocannabinoid activation in

cardiovascular diseases have also been reported. In a recent analysis of NHANES data, a lower incidence of metabolic syndrome, in individuals with marijuana use, was reported (Vidot *et al.* 2016). Additionally, due to the neuroprotective effects of cannabinoids, such as normalization of aberrant neuronal firing and an elevation in antioxidant defense mechanisms, CB1R agonism is theorized to be a viable therapeutic strategy for ischemic conditions such as stroke and also myocardial reperfusion injury (Mendizábal and Adler-Graschinsky 2007) (Hillard 2008).

e) CB1R dysregulation

It is evident that the endocannabinoid system has a major role in the etiology of neurological and cardiovascular disorders. Identification of a strong correlation between the severity of pathological conditions, and an elevated level of circulating endocannabinoids, has led to several research groups investigating the potential utility of endocannabinoids as potential biomarkers (Hillard *et al.* 2012) (Pacher and Kunos 2013) (Matias *et al.* 2012). In addition to endocannabinoids, alterations in CB1R expression and its homeostatic functionality has also been identified in pathological states (Miller and Devi 2011) (Chen 2015). A loss of endocannabinoid protective function, often manifests as a reduction in endocannabinoid levels or receptor expression (Miller and Devi 2011). Since the CB1R plays an integral role in the regulation of homeostasis in the brain, several neurological conditions have been characterized with a downregulation of CB1R, and consequentially, a loss of protective functions (Horne *et al.* 2013). For some pathological conditions, an upregulation of CB1R is observed, where its activation has been demonstrated to improve (Pertwee 2009a) as well as worsen (Teixeira-Clerc *et al.* 2006) symptoms. Also, depending on the stage of the pathogenesis, endocannabinoid system

activation can oppose, as well as contribute to pathological conditions (Di Marzo 2008). Factors that drive chronic pathological conditions can also alter CB1R expression or function via several multiple mechanisms, such as transcriptional regulation and receptor crosstalk (Miller and Devi 2011).

v) Alternative strategy to target endocannabinoid system

Both cannabinoid agonists as well as antagonists have been associated with severe adverse effects, the latter especially led to a heightened suicidal risk (Topol *et al.* 2010). The use of indirect drugs that can enhance one's own endocannabinoid system, by inhibiting enzymes that degrade endocannabinoids, was suggested as an alternative therapeutic strategy (McPartland *et al.* 2014) (Di Marzo 2008). However, this strategy is also not devoid of life threatening adverse effects. A recently held clinical trial that employed this strategy to test the safety profile of a FAAH inhibitor, resulted in lethal adverse effects, with one patient reported to be brain dead from neurological complications associated with the use of the highest dose (Kerbrat *et al.* 2016). Also suggested is the strategy of multi-drug therapy, which is to employ a low dose partial CB1R agonist as an adjunct therapy (Pertwee 2009a). Since the CB1R has a high tendency to crosstalk with several different GPCRs, the ability of this receptor to synergize or antagonize the effects of other GPCRs could be employed to maximize the beneficial effects, and possible blunt the adverse effects associated with cannabinoid drugs.

2.6 Crosstalk between RAS and endocannabinoid system: emphasis on AT1Rs and CB1Rs

i) Crosstalk mechanisms

The concept of paracrine transactivation of pre-synaptic CB1R by postsynaptic Gq GPCRs, was leveraged by the Hunyady group to investigate the crosstalk mechanisms between the AT1R, a Gq GPCR, and CB1Rs on non-neuronal cells. In chinese hamster ovary (CHO) and other commercial cell lines, the CB1R was demonstrated to be transactivated, both in an autocrine and paracrine fashion, by Ang II via the AT1R-DAGL axis (Turu *et al.* 2007) (Turu *et al.* 2009). The physiological consequences of AT1R to CB1R transactivation was evaluated by employing one of the AT1R's fundamental functions, Ang II-mediated vasoconstriction. The CB1R has opposing roles to AT1R in the vasculature. It was observed that Ang II-mediated vasoconstriction was partially dampened by a simultaneous transactivation of CB1R. By employing a CB1R antagonist, they observed an augmentation of AT1R-mediated vasoconstriction by Ang II (Szekeres *et al.* 2012), which demonstrated that the CB1R have a restraining role over AT1R functions. In the brain, there is evidence of CB1R involvement in AT1R-mediated elevation in blood pressure. An increase in mean arterial pressure from the administration of Ang II into PVN of WKY was blunted by simultaneous infusion of the CB1R inverse agonist, AM251 (Gyombolai *et al.* 2012). This however is indicative of a potentiation of AT1R-mediated effects by CB1R in the CNS. The interaction is not limited to a transactivation of the receptors. Heterodimers of the AT1R and CB1R have also been identified, and the downstream significance was investigated in a neuroblastoma cell line and in hepatic cells (Rozenfeld *et al.* 2011). Co-treatment of Ang II with CB1R agonist, HU-210, led to an increase in AT1R-mediated activation of ERK in a neuroblastoma cell line, suggestive of possible synergism between the two receptors. Further, there was a potentiation of AT1R-mediated pro-fibrogenic activity in hepatic cells by CB1R activation (Rozenfeld *et al.*

2011). The CB1R antagonist, Rimonabant, was demonstrated to improve symptoms of endothelial dysfunction, and it also downregulated AT1Rs in the vasculature (Tiyerili *et al.* 2010). It is evident that CB1R activation could both potentiate, as well as neutralize AT1R-mediated effects. The type of crosstalk and the consequence of it, is cell-type specific. Variations in receptor expression, level of basal endocannabinoid tone, and the downstream consequence of receptor activation, could well be crucial determinants of the type and nature of the crosstalk.

ii) Contrasting roles of astroglial AT1Rs and CB1Rs

AT1Rs and CB1Rs have opposing roles in regulating astroglial functions. AT1R activation is associated with astroglial senescence (Liu *et al.* 2011a), an increase in factors that promote neuronal damage (Min *et al.* 2011), and its activation also worsens the outcome of cardiovascular diseases (Isegawa *et al.* 2014a) (Fig 2.5A). CB1R activation on the other hand is described as having an 'astroprotective' role (Gómez Del Pulgar *et al.* 2002), whereby its activation results in a secretion of factors that are capable of inducing neuroprotection in neurodegenerative disorders and cardiovascular diseases such as stroke (Aguirre-Rueda *et al.* 2015) (Hillard 2008) (Fig 2.5B). While AT1R activation leads to an increase in pro-oxidant and pro-inflammatory states (Rodríguez *et al.* 2016), CB1R activation is associated with an elevation in anti-oxidant and anti-inflammatory states (Molina-Holgado *et al.* 2003) (Gómez Del Pulgar *et al.* 2002). Possible mechanisms by which CB1R activation can negate AT1R mediated deleterious effects, are shown in Fig 2.6.

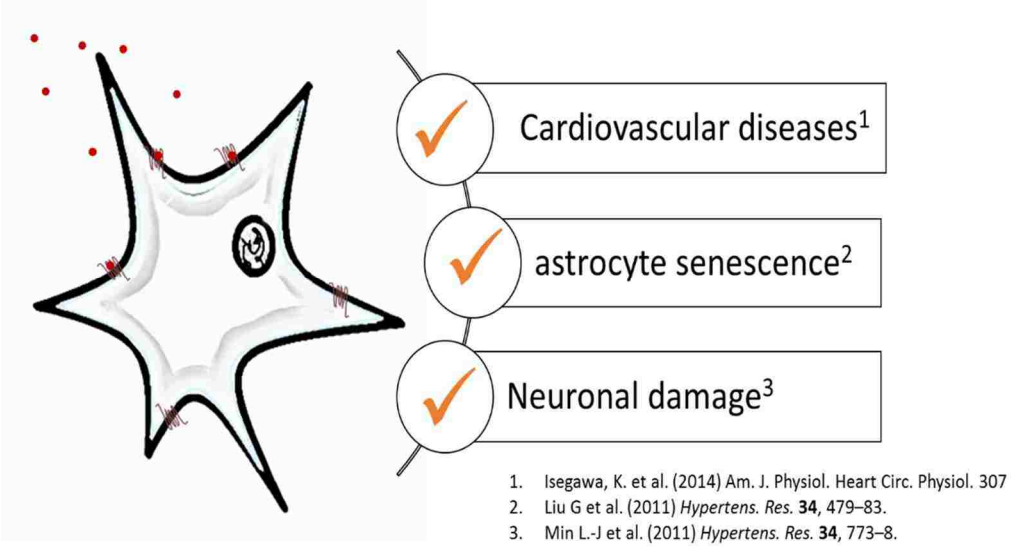

Fig 2.5A: AT1R-mediated deleterious effects

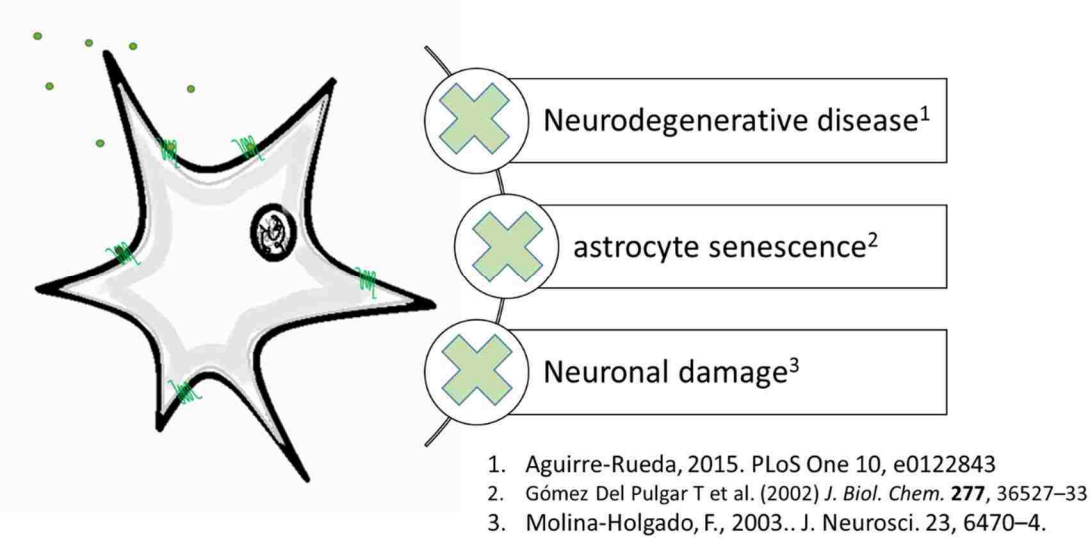

Fig 2.5B: CB1R-mediated protective effects

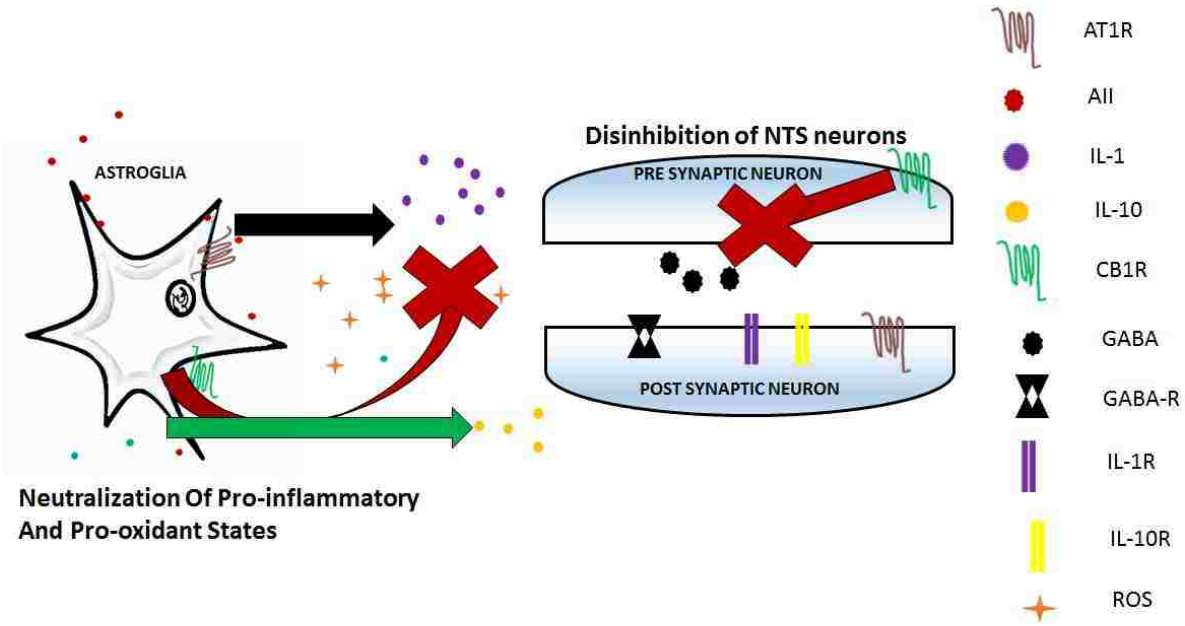

Fig 2.6: Potential mechanisms of CB1R-mediated neutralization of deleterious effects of AT1R: Ang II via astroglial AT1R activation results in an elevation of pro-inflammatory cytokines and ROS, that can generate greater GABAergic input to the NTS, leading to the disinhibition of RVLM neurons. This ultimately results in sympathoexcitation, as the restraining influence is diminsihed. Astroglial CB1R activation can lead to neutralization of Ang II-mediated elevation in pro-inflammatory cytokines and ROS. Also, presynaptic CB1R activation results in inhibition of GABAergic input, resulting in a greater baroreflex sensitivity.

iii) Conclusion

While several groups have demonstrated multiple modes of crosstalk between the AT1R and CB1R, with conflicting results, the ability of the CB1R to alter AT1R-mediated effects has not been investigated in hypertensive rats. Recently, CB1R antagonism was demonstrated to normalize sympathetic activity in a monogenic model of hypertension, characterized by an overactive RAS (Schaich *et al.* 2014). However, to our knowledge no such study exists in SHRs. Pathological conditions can also significantly alter the levels of the CB1R (Miller and Devi 2011). Factors that are responsible for the progression of the disease, are usually the key regulators of CB1R dysregulation and dysfunction in

pathological conditions. As mentioned earlier, CB1R dysregulation in the form of either a reduction in receptor density or hyofunctionality, or both have been reported in pathological conditions such as hypertension and ADHD (Brozoski *et al.* 2009) (Adriani *et al.* 2003). However, the role of Ang II in regulating CB1R expression has not been well investigated. In the same vein, CB1R-mediated alterations in AT1R functions, has also not been well investigated in SHRs. Since the CB1R and the AT1R have been demonstrated to crosstalk at several different levels, it is important to determine the consequences of their crosstalk in brain cells such as astrocytes.

2.7 Rationale and Specific Aims

i) Research in our laboratory

Astrocytes serve to regulate several functions in the brain, and their roles in neurodevelopmental and cardiovascular disorders have come to the fore only in the recent past (Sofroniew and Vinters 2010) (Marina *et al.* 2016). Understanding the molecular aspects of astroglial functions, that result in an altered neuronal activity, such as an augmentation of ATP-mediated purinergic signaling, or triggering an upsurge in pro-inflammatory and pro-oxidant states, are crucial to gain a better understanding of cardiovascular and neurological disorders (Marina *et al.* 2016). Ongoing research in our laboratory is geared towards unraveling the role of astroglial RAS in the development of hypertension, and to a lesser extent the development of neurodevelopmental disorders such as ADHD. While several studies have investigated the pro-inflammatory and pro-oxidant effects of Ang II on astrocytes (Winklewski *et al.* 2015), either from commercial sources or from normotensive rats, our laboratory has investigated Ang II effects in cerebellar and brainstem astrocytes from a well-established model of hypertension and ADHD, the SHR.

In addition to ROS and neuroinflammatory cytokines, other pro- and anti-hypertensive markers, were also investigated (Gowrisankar and Clark 2016a) (Gowrisankar and Clark 2016b). With few exceptions, Ang II treatment resulted in an augmentation of pro-hypertensive markers, and a reduction of anti-hypertensive markers investigated (Gowrisankar and Clark 2016a) (Gowrisankar and Clark 2016b). In most cases, the effects of Ang II was found to be greater in SHRs when compared to Wistar rats.

ii) Rationale for investigating crosstalk between AT1Rs and CB1Rs

Independent groups have demonstrated the ability of Ang II to downregulate Peroxisome proliferator-activated receptor gamma, in the periphery, and the anti-inflammatory cytokine IL-10, in the brain (Tham *et al.* 2002a) (Shi *et al.* 2010a). Whether Ang II could alter other modulatory systems that have potent anti-inflammatory effects in the brain, is an area of research that has not been investigated. Strong evidence of protective effects in the brain by endocannabinoid system activation, in the form of its neuromodulatory and immunomodulatory effects, has been reported under pathological conditions (Nagarkatti *et al.* 2009) (Katona and Freund 2008). Whether CB1R activation leads to a neutralization of the deleterious effects that are associated with AT1R activation, has not been well investigated in brain cells. In the periphery however, evidence of both an elevation and also a reduction in Ang II's effects, by CB1R activation, has been reported (Rozenfeld *et al.* 2011) (Szekeres *et al.* 2012).

iii) Hypothesis and specific aims

While several studies have investigated the functional relevance of activating or antagonizing the two aforementioned systems in astrocytes, there are very few studies investigating the molecular aspects of these two systems in these cells. Understanding their roles in the regulation of neuroinflammatory cytokines or in the activation of key signal transduction pathways, prior to the development of hypertension, is essential as it may lead to the identification of novel and superior therapeutic targets for treating pathological conditions. In addition, the role of astroglial CB1R in cardiovascular and neurodevelopmental disorders has not been investigated, in spite of the relevance of the endocannabinoid system to sympathetic nervous system activity and brain development. *We hypothesize that a dysregulation of CB1R functions in SHR astrocytes, alters its ability to modulate Ang II-mediated effects.* Here we aim to not only understand the consequences of activation of these systems on key astroglial functions, but also delineate the crosstalk mechanisms that may exist between the two key receptors, AT1Rs and CB1Rs. Astrocytes were isolated from the brainstems of SHRs since the brainstem has been implicated to play a key role in the integration and processing of sympathoexcitatory/sympathoinhibitory signals from peripheral baroreceptors and the hypothalamus. Cerebellar astroglial impairments have been documented in SHRs. As cerebellar impairments are characteristic of ADHD, cerebellar astrocytes from SHRs were also employed. The specific aims of this study are as follows,

Specific Aim 1**:** Determine whether Ang II alters CB1R expression in astrocytes isolated from SHRs and Wistar rats

Rationale: CB1R expression serves as a crucial indicator of endocannabinoid hypo/hyperfunctionality in pathological conditions. Instances of an alteration of CB1R

expression levels in pathological conditions, have been ascribed to factors or mediators that often are involved in the progression of the disorder (Miller and Devi 2011). Investigating the basal CB1R expression in both rat models, and investigating the effect that Ang II has on CB1R expression, was the purpose of this aim. Potential mechanisms by which Ang II can alter CB1R expression, are shown in Fig 2.7.

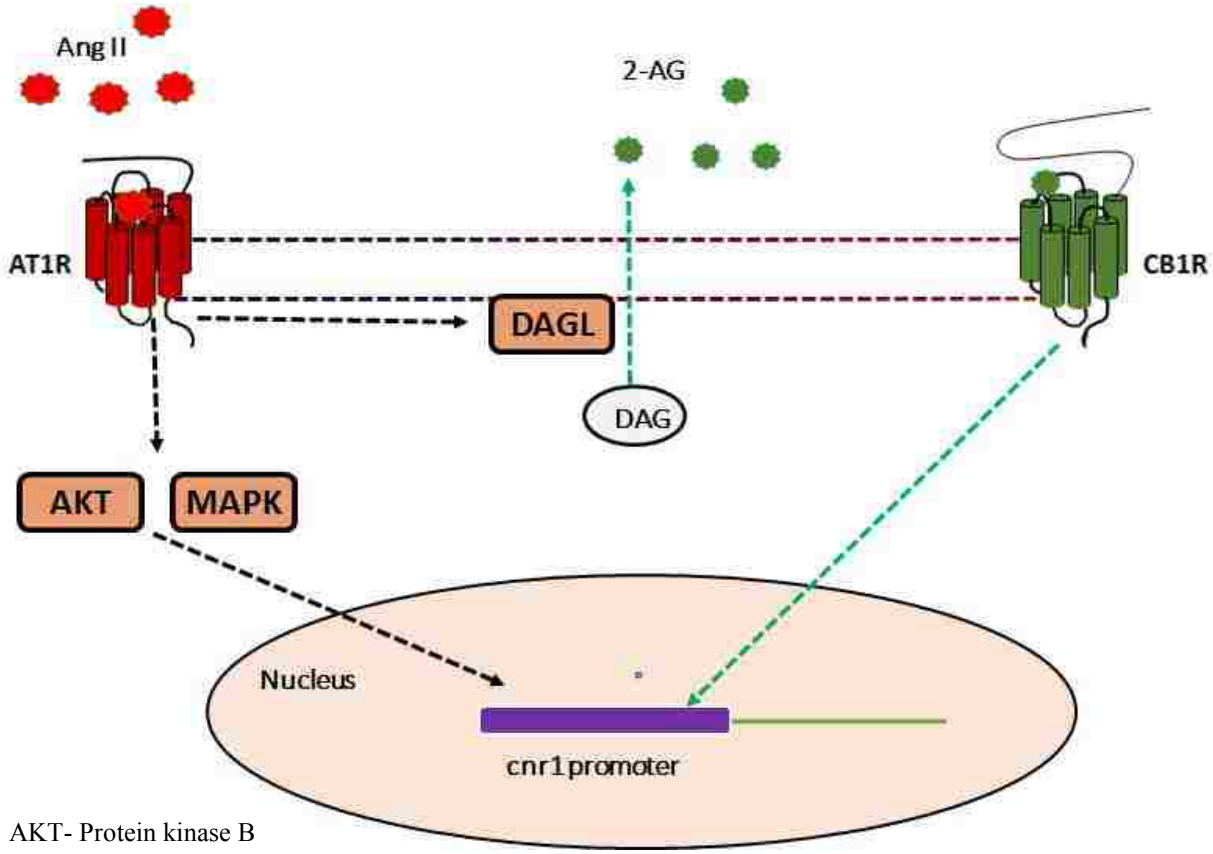

Fig 2.7: Potential mediators of Ang II mediated changes in CB1R expression: MAPKs and AKT signaling pathways have been demonstrated to regulate the transcription of CB1R (Miller and Devi 2011). Also CB1R activation has been demonstrated to result in an elevation of CB1R transcription (autoinduction) (Laprairie *et al.* 2013). Since Ang II via the AT1R has been demonstrated to activate a diverse array of signal transduction pathways, and it also transactivates the CB1R, it is functionally capable of regulating CB1R transcription.

Specific Aim 2: a) Determine the consequences of RAS and endocannabinoid system activation, both alone and in combination, on MAPK signaling pathways in astrocytes

isolated from SHRs and Wistar rats. b) Determine the effects of Ang II on triggering phosphorylation of CB1Rs in astrocytes isolated from SHRs and Wistar rats.

Rationale: a) Activation of the RAS and the endocannabinoid system leads to the activation of MAPKs, such as ERK and p38, which serve as critical cellular switches for long term alterations in cellular activity, by regulating transcription and translation (Clark *et al.* 2008) (Turu and Hunyady 2010). These pathways are often of immense physiological and pathological relevance. Although crosstalk between the two receptors, the AT1R and the CB1R has been reported by us and several other groups, the consequences of this interaction at the level of MAPK activation, in primary cells, has not been investigated. b) Activation of protein kinase C (PKC) has been demonstrated to inactivate CB1R by a phosphorylation mechanism (Garcia *et al.* 1998). The mechanism of Ang II-induced phosphorylation of CB1R is showed in Fig 2.8. Since the AT1R-PKC axis is the dominant mechanism by which Ang II is able to elicit its deleterious effects, we investigated the ability of Ang II to phosphorylate CB1R in astrocytes isolated from both models.

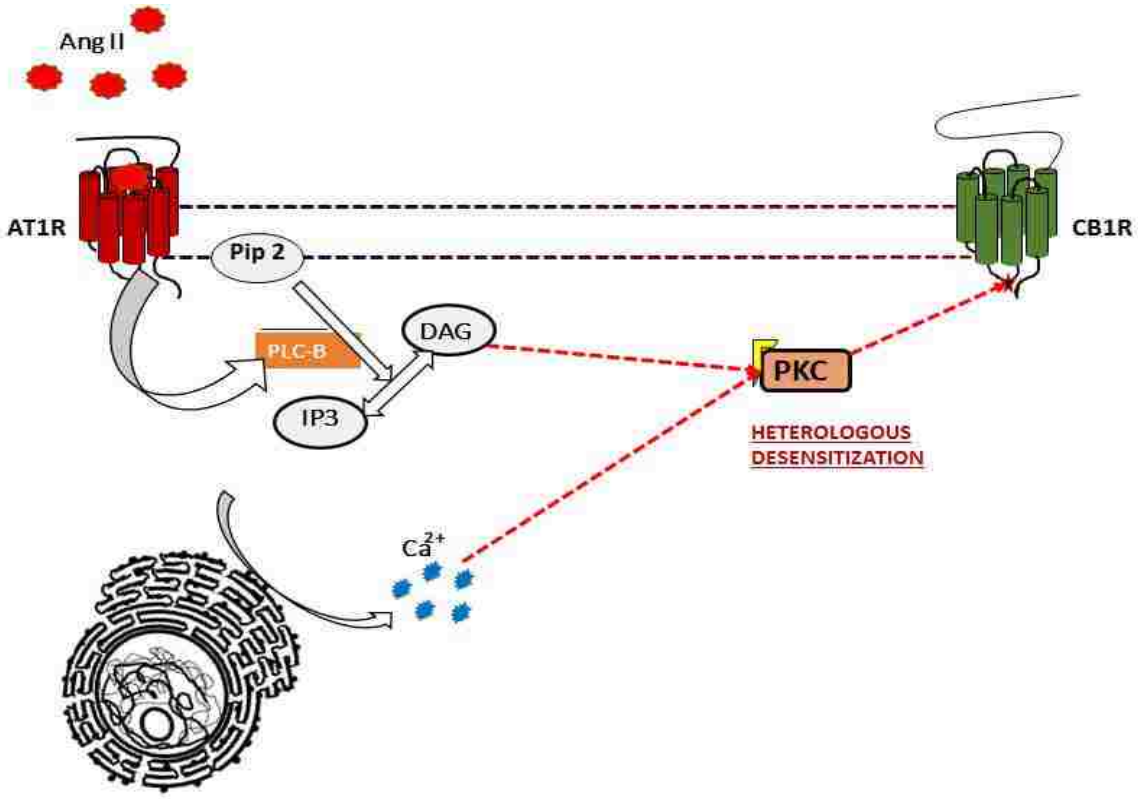

Fig 2.8: Mechanism of Ang II induced phosphorylation of CB1R: We propose that a potential candidate mechanism of unidirectional crosstalk may exist from AT1R to CB1R, which may disrupt the actions of CB1R activation in astrocytes. Activation of PKC was demonstrated to disrupt the neuromodulatory effects of CB1R (Garcia *et al.* 1998). It was demonstrated that PKC phosphorylates residues in the third intracellular loop leading to desensitization of the receptor (Garcia *et al.* 1998). Gq GPCRs, such as the AT1R, are capable of activating PKC via PLC. Hence Ang II is functionally capable of inactivating CB1R.

Specific Aim 3: Determine the consequences of RAS and endocannabinoid system activation, both alone and in combination, on inflammatory cytokines in astrocytes isolated from SHRs and Wistar rats.

Rationale: While it is evident that activation of glial RAS and endocannabinoid system may have opposing roles in the regulation of neuroinflammatory cytokines, whether an interplay between the two systems at the levels of pro- and anti-inflammatory cytokines

exists, has not been investigated. Interestingly, the consequences of astroglial CB1R activation on regulation of neuroinflammatory states, in cardiovascular or neurodevelopmental disorders, have also not been investigated. Since dampening of pro-inflammatory states, and elevation of anti-inflammatory states is an integral part of CB1R-mediated neuroprotection (Molina-Holgado *et al.* 2002b) (Molina-Holgado *et al.* 2003), we investigated the effect of CB1R agonist on both IL-1β and IL-10, in the presence and absence of Ang II.

Overall, the findings from this study will reveal new insights on the role of the astroglial endocannabinoid system in the etiology of cardiovascular and neurodevelopmental disorders. By investigating variations in CB1R functionality and expression, under baseline conditions as well as a function of AT1R crosstalk, would lay the foundation for the identification of novel, yet viable therapeutic targets for the aforementioned disorders.

Chapter 3

Heterologous Regulation of the Cannabinoid Type 1 Receptor by Angiotensin II in Astrocytes of Spontaneously Hypertensive Rats

(See Appendix 4 for license agreement)

Abstract

Brainstem and cerebellar astrocytes have critical roles to play in hypertension and ADHD, respectively. Ang II, via the astroglial AT1R, has been demonstrated to elevate pro-inflammatory mediators in the brainstem and the cerebellum. The activation of astroglial CB1R, a master regulator of homeostasis, has been shown to neutralize inflammatory states. Factors that drive disease physiology, are known to alter the expression of CB1Rs. In the current study, we investigated the role of Ang II in regulating CB1R protein and mRNA expression in astrocytes isolated from the brainstem and the cerebellum of SHRs. The results were then compared with the normotensive counterpart, Wistar rats. Not only was the basal expression of CB1R protein and mRNA significantly lower in SHR brainstem astrocytes, but treatment with Ang II resulted in lowering it further in the initial 12 hours. In the case of cerebellum, Ang II upregulated the CB1R protein and mRNA in SHR astrocytes. While the effect of Ang II on CB1R protein was predominantly mediated via the AT1R in SHR brainstem; both AT1R and AT2R mediated Ang II's effect in the SHR cerebellum. This data is strongly indicative of a potential new mode of cross talk between components of the RAS and the endocannabinoid system in astrocytes. The

consequence of such a crosstalk could be a potential reduced endocannabinoid tone in brainstem in hypertensive states, but not in the cerebellum under the same conditions.

3.1 Introduction

Since the seminal work in the late 1980's, which first provided evidence of cannabinoid receptors in the brain (Devane *et al.* 1988), several other noteworthy findings soon followed which confirmed their existence (Herkenham *et al.* 1991) (Matsuda *et al.* 1990). Subsequently, the endogenous ligands for the receptors, anandamide and 2-AG, and their metabolizing enzymes were also discovered (Devane *et al.* 1992) (Sugiura *et al.* 1995) (Pacher *et al.* 2006). The consequence was the unearthing of an ancient, yet highly important, physiological system which we now know as the endocannabinoid system. Understanding the complexities of the endocannabinoid system has not only paved the way for the identification of novel therapeutic targets, but it has also significantly aided in furthering our understanding of brain physiology. The CB1R is one of the most abundant G protein-coupled receptors in the brain, and the CB2R is mostly expressed on immune cells in the periphery (Munro *et al.* 1993). While the functionality of the endocannabinoid system has been extensively studied in neuronal cells, several studies have highlighted their role in regulating glial cell functions as well (Stella 2004) (Massi *et al.* 2008). Astrocytes isolated from both mice and rats not only express the CB1R, but also generate endocannabinoids (Walter *et al.* 2002). The endocannabinoid system is involved in regulating several functions of astrocytes such as energy balance (Bosier *et al.* 2013), neuron-astrocyte communication (Navarrete and Araque 2008), and modulation of inflammatory conditions (Molina-Holgado *et al.* 2002a) (Sheng *et al.* 2005a). As high levels of calcium also act as one of the triggers to generate endocannabinoids (Freund *et*

al. 2003), this system is well placed to re-establish equilibrium in conditions where homeostatic processes have gone awry.

It is appreciated that neuronal CB1Rs and glial CB1Rs have diverse roles. The neuronal CB1R is activated in response to excessive neurotransmitter release, while the glial CB1R serves an important immunomodulatory role. The ability of the CB1R to serve both neuromodulatory and neuroprotective functions, lends itself to be an attractive target for research of several neurological impairments (Pacher *et al.* 2006). Its upregulation in pathological conditions has a protective (Lim *et al.* 2003), as well as a detrimental effect (Teixeira-Clerc *et al.* 2006). Hence it is imperative to not only identify the pathological conditions where CB1R is dysregulated, but to also understand the causes for it. Factors that play a key role in mediating disease conditions are the most likely candidates for regulating CB1R expression levels (Jean-Gilles *et al.* 2015) (Miller and Devi 2011). Several signaling pathways, such as ERK (Chiang *et al.* 2013), protein kinase b (AKT) (Laprairie *et al.* 2013), and STAT5 (Börner *et al.* 2007) have been proposed to play key roles in the transcriptional regulation of the CB1R. Cannabinoids have also been demonstrated to have a role to play in CB1R regulation (Laprairie *et al.* 2013) (Miller and Devi 2011).

A dysregulated brain RAS is one of the hallmarks of essential hypertension (Veerasingham and Raizada 2003). The RAS comprises of the effector peptide Ang II, its cognate receptors Ang type 1 and type 2 receptors (AT1R and AT2R, respectively), and the enzymes involved in Ang II synthesis and degradation. The SHR, one of the most widely used genetic models of essential hypertension, is characterized by an overactive brain RAS (Veerasingham and Raizada 2003). The notion that chronic inflammation in the

brainstem contributes to an augmented sympathetic drive has received attention only in the last decade (Shi *et al.* 2010b). Our lab has previously reported the presence of functional astroglial AT1Rs in the brainstem and cerebellum of normotensive rats (Kandalam and Clark 2010) (Clark *et al.* 2013) (Clark *et al.* 2008). Ang II via the AT1R can activate several signaling pathways that are critical to several astrocyte functions such as regulation of inflammation (Kandalam and Clark 2010) and proliferation (Clark *et al.* 2008). The pro-inflammatory effects of the AT1R are not restricted to the brainstem and hypothalamus, but several other regions of the brain are also susceptible to its deleterious effects. In the cerebellum, the AT1R has been demonstrated to oppose the beneficial effect of AT2R activation (Côté *et al.* 1999). Ang II is able to induce neuronal damage via activation of astroglial AT1R by increasing levels of pro-inflammatory cytokines (Lanz *et al.* 2010), or ROS (Liu *et al.* 2011a), the latter associated with astrocyte senescence.

Considering the role of the RAS in perpetuating neuroinflammatory states, several studies have emphasized the positive effects of Ang receptor blockers (ARBs) in neurological and neurodegenerative disorders (Wolozin *et al.* 2008) (Mogi and Horiuchi 2009). The SHR has also been employed to study ADHD as several distinct behavioral traits of this disorder, such as impulsivity, are exhibited by this animal model (Adriani *et al.* 2003). Recent studies highlight the importance of research in cerebellum to further our understanding of the pathophysiology of ADHD (Goetz *et al.* 2014a). Not only was a reduced cerebellar volume reported in children diagnosed with ADHD (Castellanos *et al.* 1996b), but an increase in an astrocytic marker was also observed in the cerebellum of SHRs (Yun *et al.* 2014). As there is a dearth of information available on the brainstem and cerebellum astroglial CB1R and its potential regulators, this study becomes vital.

Inflammatory cytokines have been demonstrated to induce CB1R expression (Jean-Gilles *et al.* 2015). An increase in the levels of pro-inflammatory mediators are observed in the brainstem of SHRs (Waki *et al.* 2008a). Not only can Ang II elevate pro-inflammatory mediators, but it can also downregulate anti-inflammatory mechanisms that could help to perpetuate its inflammatory prowess in pathological conditions (Tham *et al.* 2002a). Whether Ang II, a major driver of neuroinflammatory conditions, possesses the ability to alter a key neuroprotective regulator, CB1R, in hypertensive conditions is unknown. The ability of AT1Rs to generate endocannabinoids (Turu *et al.* 2009), to activate signaling pathways that play a role in CB1R transcriptional regulation and also to elevate pro-inflammatory cytokines, led us to postulate that Ang II can regulate CB1R expression in astrocytes. In this study, we employed cerebellar and brainstem astroglial cells from SHRs and compared the results with its normotensive counterpart, the Wistar rats. We believe that the presence of a hypertensive background could significantly alter the effect that AT1R activation could have on neuroprotective regulators such as the CB1R. Hence, we not only investigated the changes in basal CB1R expression in brainstem and cerebellar astrocytes isolated from SHR and Wistar rats, but also investigated whether Ang II alters CB1R protein and mRNA levels in the aforementioned regions and rat models. The objectives of this study were three fold; firstly, to determine the basal expression of astroglial CB1R in hypertensive conditions and non-hypertensive conditions. Secondly, to investigate the effect of Ang II on CB1R expression under hypertensive and non-hypertensive conditions. And lastly, to determine the receptor, either AT1R or AT2R or both, through which this Ang II effect is mediated.

3.2 Materials and methods

i) Materials

Ang II was obtained from Bachem (Torrance, CA). PD123319, the selective AT2R antagonist was obtained from Sigma (St. Louis, MO), and Losartan (AT1R antagonist) was kindly provided by Du Pont Merck (Wilmington, DE). Western blotting supplies were purchased from Bio-Rad Laboratories (Hercules, CA) or VWR International (Suwanee, GA). The CB1R antibody (209550) was purchased from Calbiochem (San Diego, CA), and the beta-actin antibody (A2066) was purchased from Sigma (St. Louis, MO). Anti-glial fibrillary acidic protein (GFAP) antibody [EP672Y] (ab33922) and Goat anti-Rabbit IgG H&L (Alexa Fluor® 488) (ab150077) were purchased from Abcam (Cambridge, MA). Rat cerebellum extract (sc-2398) was purchased from Santa Cruz Biotechnology (Dallas, Texas). The Bicinchoninic acid (BCA) protein kit was obtained from Pierce Biotechnology (Rockford, IL). Quantitative PCR (qPCR) products including the Taqman primer sets for CB1R (Rn02758689_s1), GFAP (Rn00566603_m1), Itgam (Rn00709342_m1), Pecam1 (Rn01467262_m1), and beta-actin (Rn00667869_m1) were obtained from Applied Biosystems (Foster City, CA). All other chemicals were purchased from either VWR international (Suwannee, GA), Fisher Scientific (Waltham, MA) or Sigma (St. Louis, MO).

ii) Isolation and culture of primary astrocytes

Timed pregnant Wistar rats and SHRs were obtained from Charles River Laboratories (Wilmington, MA) and maintained in the ALAAC-accredited animal facility of Nova Southeastern University. All animal protocols were approved by the University Institutional Animal Care and Use committee and complied with the ethical treatment of animals as outlined in the NIH Guide for Animal Care and Use. The brainstem and

cerebellar astrocyte cultures were prepared using mechanical dissociation as previously described (Tallant and Higson 1997). 2-3 day old rat pups were sacrificed by CO_2 deprivation, followed by decapitation. Whole brains were then isolated from rat pups by cutting the skull open using micro dissecting scissors. An incision was made at the occipital bone at the base of the skull. The skull was then split open by extending rostrally, along the midline fissure, and terminating at the frontal bone near the orbital cavity. The whole brains were then detached from the underlying cranial nerves, and removed from the skull using curved forceps. The brains were quickly immersed in media DMEM/F12 culture media containing 10% FCS, 10,000 I.U/mL penicillin, 10,000 µg/mL streptomycin and 25 µg/mL amphotericin B. The procedure was repeated for the remainder of the rat pups. Individual brains were then placed longitudinally on a sterile gauze. Using sterile micro-dissecting forceps and scalpel blades, a section were made caudal to the cerebral hemispheres. The cerebral cortex was then separated from the cerebellum and the brainstem. A slant section was made rostral to the pons, in order to obtain the cerebellum. The remaining part of the brain is the brainstem. This procedure was repeated for all the rat pups. Cerebellums and brainstems from one litter of pups were then pooled separately, and were subjected to physical dissociation, in order to obtain a cell suspension. This suspension was then passed through two different sets of filters, 100 mm and 60 mm, to remove tissue debris, and to obtain a pure cell suspension. The cells were grown in DMEM/F12 culture media containing 10% FBS, 10,000 I.U/mL penicillin, 10,000 µg/mL streptomycin and 25 µg/mL amphotericin B at 37°C in a humidified incubator (5% CO_2 and 95% air). The cell cultures were fed every 3-4 days. On attaining confluency, the cells were subjected to vigorous shaking overnight which resulted in the detachment of

microglia and oligodendrocytes. Subsequently the cell cultures were detached with trypsin/EDTA (0.05% trypsin, 0.53mM EDTA) and replated at a ratio of 1:10. The astrocyte-enriched cultures were fed once every 3 days until they were about 90% confluent. Before all cell treatments, the cultures were made quiescent by treating with media, devoid of serum, for 48 hours. All subsequent treatments were conducted in serum free DMEM/F12 culture media containing 10,000 I.U/mL penicillin, 10,000 µg/mL streptomycin and 25 µg/mL amphotericin B.

iii) Purity of astrocyte cultures

The purity of the astrocyte cultures was assessed using qPCR, western blotting and flow cytometry. In order to detect the presence of astrocytes, microglia, and endothelial cells, we employed Taqman primers for GFAP (astrocytes), Itgam (microglia) and Pecam1 (endothelial cells). For western blotting, we used a monoclonal antibody for GFAP at a concentration of 1: 1000. The steps are described in detail under the western blotting section. In order to test the percentage of astrocytes present in our culture, cells were analyzed using a BD C6 Accuri^{TM}Flow cytometer (BD Bioscience, San Jose CA). Briefly, untreated cells were fixed with 2% formaldehyde followed by permeabilization using 0.1% triton in phosphate buffer saline (PBS). The reagents and the amounts employed for preparing PBS, are listed in the supplementary section (Appendix 3- Table 3S (C)). Cells were then probed with a monoclonal antibody for GFAP at a concentration of 1:150. Then they were treated with a secondary antibody conjugated with Alexa Fluor 488 at a concentration of 1:200. A total of 10,000 events were analyzed.

iv) Cell treatments

Astrocytes were treated with 100 nM Ang II for varying time periods ranging from 1 hour to 48 hours. For CB1R protein estimation, the time periods were 1, 4, 8, 12, 16, 24 and 48 hours. For CB1R mRNA estimation, the time periods were 4, 8, 12, 16 and 24 hours. For the inhibitor studies, the cells were pretreated with inhibitors for the AT1R (10 μM Losartan) and the AT2R (10 μM PD123319) for 30 mins before the addition of Ang II for varying times. The times for Ang II treatments for the inhibitor studies were chosen based on the earliest common point, where the difference observed with respect to its control, was statistically significant. For all experiments, cells that received no treatments were used as the control.

v) Cell lysate preparation

Immediately following treatments, cell lysates were prepared by washing cells with Tris buffered saline (TBS) followed by the addition of supplemented lysis buffer (100 mM NaCl, 50 mM NaF, 5 mM EDTA, 1% Triton X-100, 50 mM Tris-HCl, 0.01 mM NaVO4, 0.1 mM PMSF and 0.6 μM leupeptin, pH 7.4). The reagents and the amounts employed for preparing TBS, are listed in the supplementary section (Appendix 3-Table 3S (A)). The supernatant was subjected to centrifugation (12,000xg for 10 min, 4°C) and the protein concentrations of the cell lysates were measured using the BCA method.

vi) Total RNA extraction and mRNA expression

Total RNA was extracted from astrocytes using the trizol method and subjected to a DNA cleaning step before determining the RNA concentrations using a Bio-Rad SmartSpec^{TM} spectrophotometer (Bio-Rad Laboratories, Hercules, CA). Reverse transcription from total RNA (2μg) to complementary strand DNA was done using a high

capacity reverse transcription reagent kit (Applied Biosystems). qPCR was performed using the TaqMan Universal master mix, and the TaqMan gene expression primers (Applied Biosystems) for the CB1R gene (cnr1), GFAP, Itgam and Pecam1. Samples were analyzed in 96-well plates using the StepOne^{TM}plus Real time PCR system from Applied Biosystems (Foster City, CA). The relative fold difference of Ang II treated samples over/under the control, was calculated for each target gene after normalization to levels of the housekeeping control gene, beta-actin. Data are expressed as fold change in gene of interest expression (Pecam or Itgam or CB1R) in treated/ untreated cells, as compared with the reference gene (GFAP or CB1R) in untreated cells.

vii) Western blotting

Volumes equivalent to 30μg of solubilized proteins were loaded into 10% polyacrylamide gels, and subsequently transferred to nitrocellulose membranes. The reagents and the amounts employed for preparing transfer buffer for western blotting, are listed in the supplementary section (Appendix 3-Table 3S (B)). The membranes were then blocked with 5% non-fat dry milk prepared in TBS containing 0.1% tween (TBS-T). The membranes were then subjected to 3 washes, 5 min each using TBS-T followed by incubation with an anti-CB1R rat polyclonal antibody or a GFAP monoclonal antibody at a concentration of 1:1000, prepared in TBS-T containing 5% milk, at 4°C overnight. The membranes were then subsequently washed and probed with an anti-rabbit secondary antibody for 1 hour at room temperature. After another round of washes, the bands were then visualized using ECL reagent (Pierce Biotechnology, Rockford, IL) and quantified using the Image J software (National Institute of Health (NIH), Bethesda, MS, USA). The

membranes were then stripped and reprobed with a beta-actin antibody at a concentration of 1:5000. The CB1R or GFAP bands were then normalized to beta-actin.

viii) Statistical analysis

A 2x2 mixed ANOVA was employed to determine if there were any significant differences in the basal values between SHRs and Wistar rats. This was followed by a Bonferoni T test to determine differences between groups. A two-way ANOVA was employed for testing the effect of Ang II on CB1R in SHRs as compared to Wistar rats. A Bonferoni T test was employed to determine significant differences between treatments and the respective control in different strains. In order to make comparisons between identical time points from different rat models, a student t test was employed. All data is expressed as mean± SEM for 7 or more experiments.

3.3 Results

i) Determination of the purity of the cell culture

In order to test the purity of our cultures, we employed qPCR and flow cytometry. qPCR results revealed a high level of mRNA for GFAP, which is the astrocytic marker, while negligible levels of mRNA transcripts were detected for Itgam and Pecam1, which are markers for microglia and endothelial cells, respectively (Fig. 3.1A). Western blotting revealed higher levels of GFAP in astrocytes and negligible levels were detected in aortic vascular smooth muscle cells (VSMC) which were employed as a negative control (Fig. 3.1B). The percentage of cells that were positive for the astrocyte marker, GFAP, was determined using flow cytometry. The proportion was estimated to be between 85-90% as indicated by the peak (Fig. 3.1C).

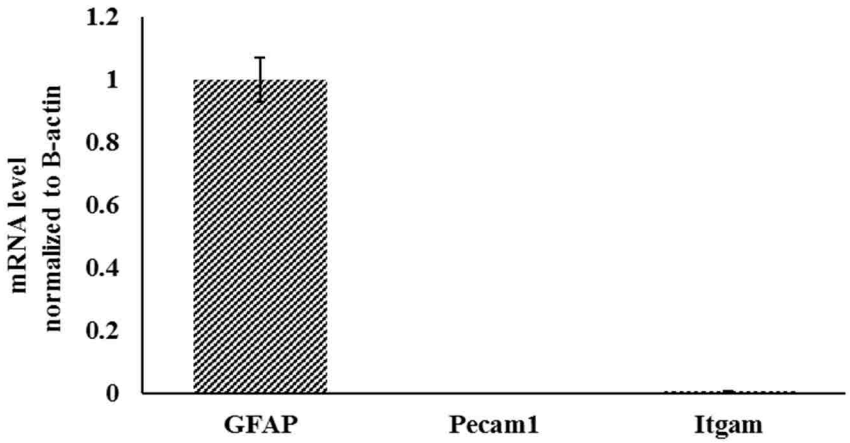

Fig 3.1A: A comparison of mRNA levels for markers of astrocytes, endothelial cells and microglia in our cell culture: A comparison of mRNA levels for GFAP, Itgam and Pecam1 was made by employing qPCR. The data is represented as arbitrary units that were obtained when the cycle threshold (Ct) values for the markers were normalized to the Ct values of beta-actin using the $2^{-\Delta\Delta C}{}_T$ method (Livak and Schmittgen 2001). Each value represents the mean ± SEM of preparations of astrocytes isolated from at least 4 litters of neonatal rat pups.

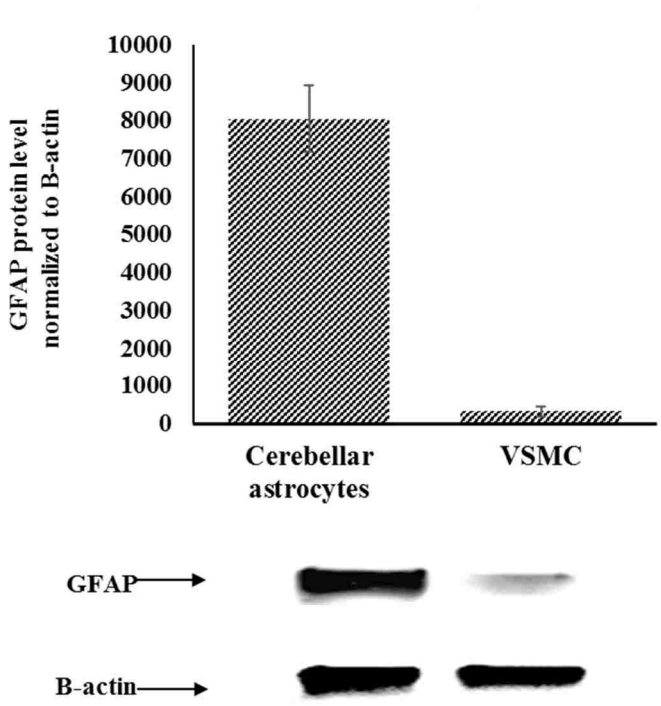

Fig 3.1B: A comparison of GFAP protein levels between astrocytes and VSMCs: A comparison of GFAP levels in cerebellar astrocytes and VSMCs was made by employing western blotting. Cell lysates from cerebellar astrocytes were loaded in lanes 1 and 2, and VSMC lysates were loaded in lanes 3 and 4. Each value represents the mean ± SEM of preparations of astrocytes and VSMCs isolated from at least 4 litters of neonatal rat pups.

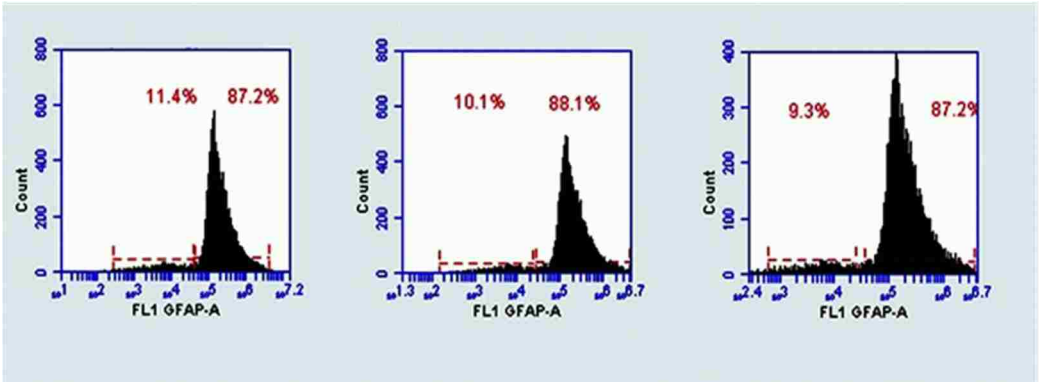

Fig 3.1C: Determination of GFAP+ cells in our culture: Flow cytometry was employed to determine the proportion of cells that expressed GFAP in our cell culture.

ii) CB1R basal expression in SHR and Wistar astrocytes

We used western blotting to detect CB1R protein levels in astrocytes isolated from the brain. The antibody employed was previously validated using a CB1R knock out model by another research group (Parmentier-Batteur *et al.* 2002). As CB1R is highly expressed in brain cells and expressed at relatively lower levels in peripheral tissues, we compared cell lysates prepared from cerebellar astrocytes with rat cerebellum extract and VSMC. The strongest band in all three samples was at ~64 kDa which denotes the glycosylated fraction of the receptor (Song and Howlett 1995) (Fig. 3.2A). The unglycosylated fraction or the native receptor (~53 kDa) was present only in whole cell extract, but weakly expressed or absent in cerebellar astrocytes and VSMCs. While the band intensity was the greatest in cerebellar whole cell extract, it was lowest in VSMC lysate. The band intensity in astroglial cell lysate was significantly greater than VSMC. This is indicative of a higher CB1R expression in the brain than in the periphery.

In untreated astrocytes isolated from the brainstem, the basal levels of CB1R protein in the SHR samples were significantly lower than in the Wistar rat samples. Although the levels were higher in SHR cerebellum, the difference was not statistically significant. The CB1R was also expressed to a higher level in cerebellum than in brainstem astrocytes isolated from both normotensive and hypertensive rats (Fig 3.2B). The mRNA levels followed an identical pattern to the CB1R protein expression, whereby lower levels were observed in brainstem astrocytes isolated from SHRs when compared to Wistar brainstem samples (Fig 3.2C). The mean Ct values for SHR brainstem and Wistar brainstem were 33.3 and 31.9, respectively. In the case of cerebellum, the difference observed between the

SHR and the Wistar rat was not statistically significant (Fig 3.2D). Mean Ct values for SHR cerebellum and Wistar cerebellum were 31.5 and 31.7, respectively.

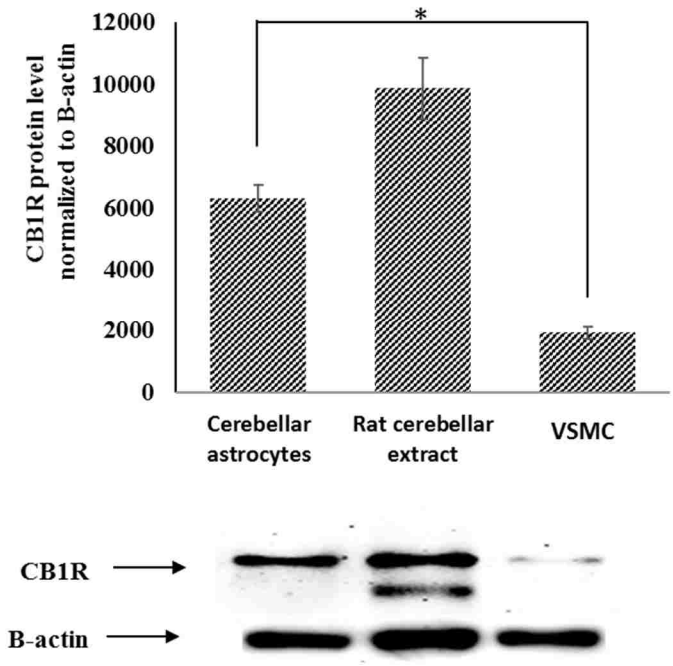

Fig 3.2A: Comparison of CB1R Protein in CNS and peripheral cells: A comparison of CB1R protein in Cerebellar astrocytes, rat cerebellum extract and VSMC was made by employing Western blotting. Cerebellar astroglial cell lysates in lane 1, rat cerebellar extract was loaded in lane 2, and VSMC cell lysate is loaded in lane 3. The data is represented as arbitrary units after normalization. (*denotes $p < 0.05$ and **denotes $p < 0.01$).

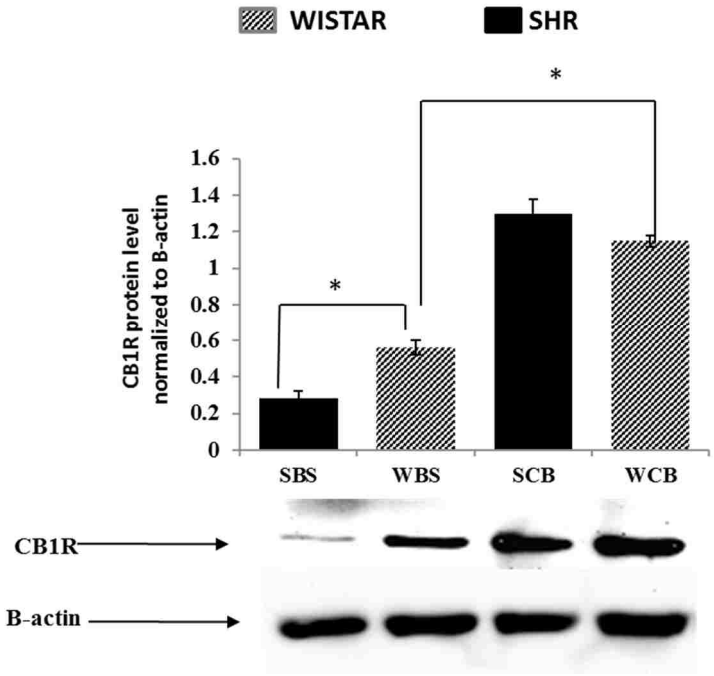

Fig 3.2B: Basal CB1R Protein in brainstem and cerebellar astrocytes of SHRs and Wistar rats: A comparison of CB1R basal protein expression in brainstem and cerebellum astrocytes, isolated from SHRs and Wistar rats, was made by employing the Western blotting technique. Cell lysates prepared from astrocytes of Wistar brainstem (WBS), SHR brainstem (SBS), Wistar cerebellum (WCB) and SHR cerebellum (SCB) were loaded in lanes 1, 2, 3 and 4, respectively. The data is represented as arbitrary units after normalization. (*denotes $p < 0.05$ and **denotes $p < 0.01$).

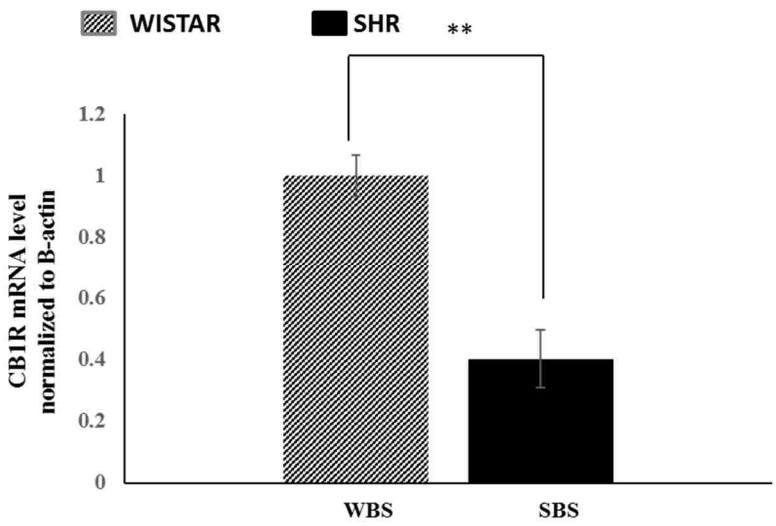

Fig. 3.2C: A comparison of Basal CB1R mRNA in brainstem astrocytes between SHRs and Wistar rats: A comparison of CB1R mRNA expression in brainstem astrocytes, isolated from SHRs and Wistar rats was made using qPCR. The data is represented as arbitrary units that are obtained after normalization (Livak and Schmittgen 2001). Each value represents the mean ± SEM of preparations of astrocytes isolated from six or more litters of neonatal rat pups. (*denotes $p < 0.05$ and **denotes $p < 0.01$).

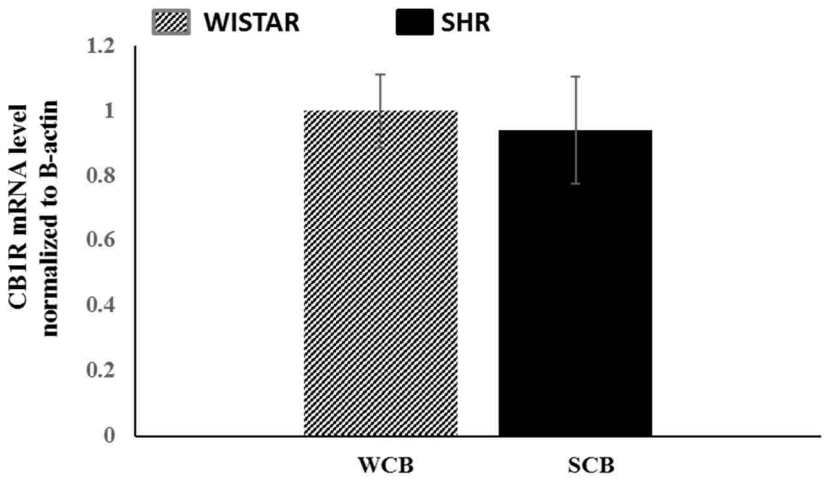

Fig. 3.2D: A comparison of Basal CB1R mRNA in cerebellar astrocytes between SHRs and Wistar rats: A comparison of CB1R mRNA expression in cerebellum astrocytes, isolated from SHRs and Wistar rats was made using qPCR. The data is represented as arbitrary units that are obtained after normalization (Livak and Schmittgen 2001). Each value represents the mean ± SEM of preparations of astrocytes isolated from six or more litters of neonatal rat pups. (*denotes $p < 0.05$ and **denotes $p < 0.01$).

iii) Effect of Ang II on CB1R protein expression in brainstem astrocytes

In order to determine if Ang II has any effect on CB1R expression in astrocytes isolated from the brainstem, we treated quiescent astrocytes with 100 nM Ang II for different time periods (1 hour to 48 hours). This concentration was optimal for activation of the astroglial AT1R as previously reported by our laboratory (Clark *et al.* 2008). We observed that Ang II caused an increase in CB1R protein expression from 4 hours onwards and this increase was sustained till 24 hours (Fig 3.3A). In the case of SHR brainstem astrocytes, Ang II caused a biphasic effect, where it downregulated the CB1R initially (maximum downregulation at 8 hours) and then upregulated the receptor at later time points (from 16 hours onwards). In both cases, the difference at 1 hour was not statistically significant. When individual time points from SHR samples were compared with its respective Wistar time points, the difference was statistically significant from 4 to 12 hours. This is the period

where downregulation was observed in SHR brainstem samples while upregulation was seen in Wistar brainstem samples. At the later time points however, the difference was not statistically significant.

For the inhibitor studies, Wistar and SHR brainstem astrocytes were treated with 100 nM Ang II for 4 hours, in the presence and absence of inhibitors, before harvesting them for proteins. As shown in Fig 3.3B, pretreating the cells with either the AT1R inhibitor or the AT2R inhibitor had no significant effect on Ang II-mediated CB1R protein expression. However, Losartan (AT1R inhibitor) completely prevented Ang II's effects on the CB1R in both SHR and Wistar brainstem astrocytes. PD123319 (AT2R inhibitor) was ineffective in preventing Ang II's effects on these cells.

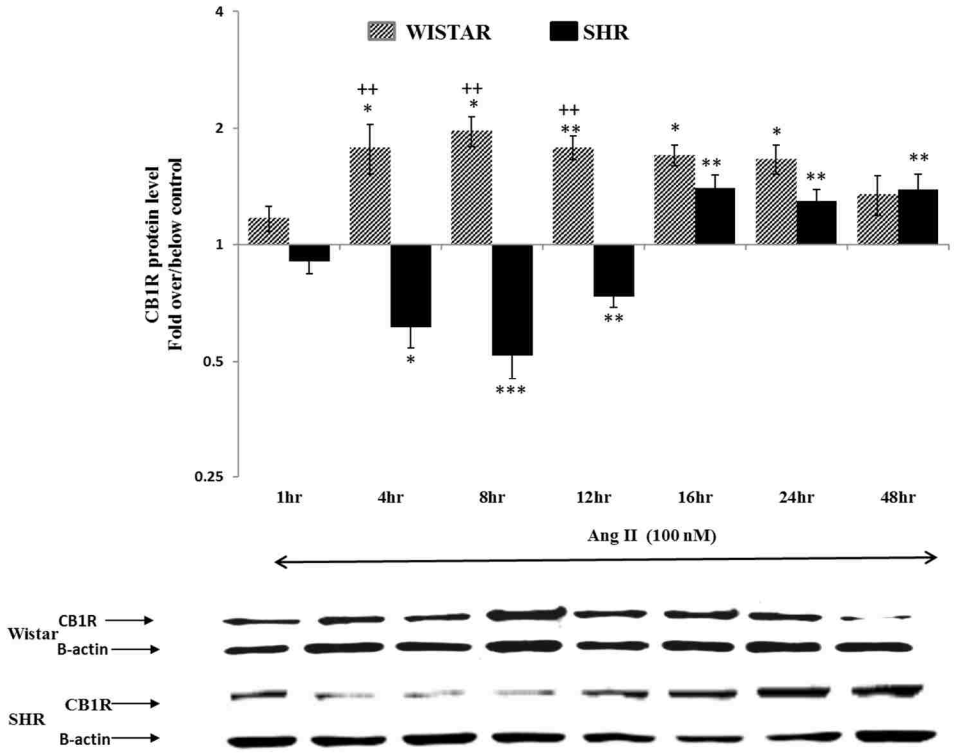

Fig 3.3A: Ang II Effects on CB1R Protein Expression in Brainstem Astrocytes Isolated from SHRs and Wistar Rats: Western blotting was employed to compare CB1R protein levels from Wistar and SHRs brainstem astrocytes, which were pretreated with 100 nM Ang II for varying time periods. Each value represents the mean ± SEM of preparations of astrocytes isolated from six or more litters of neonatal rat pups. The data is represented as fold over/under control that were obtained when CB1R bands were normalized to beta-actin bands and further normalized to its control (*denotes $p < 0.05$ and **denotes $p < 0.01$ ***$p < 0.001$ compared to its basal values; $^{+}$ denotes $p < 0.05$, $^{++}$ denotes $p<0.01$ compared to its corresponding Wistar time point).

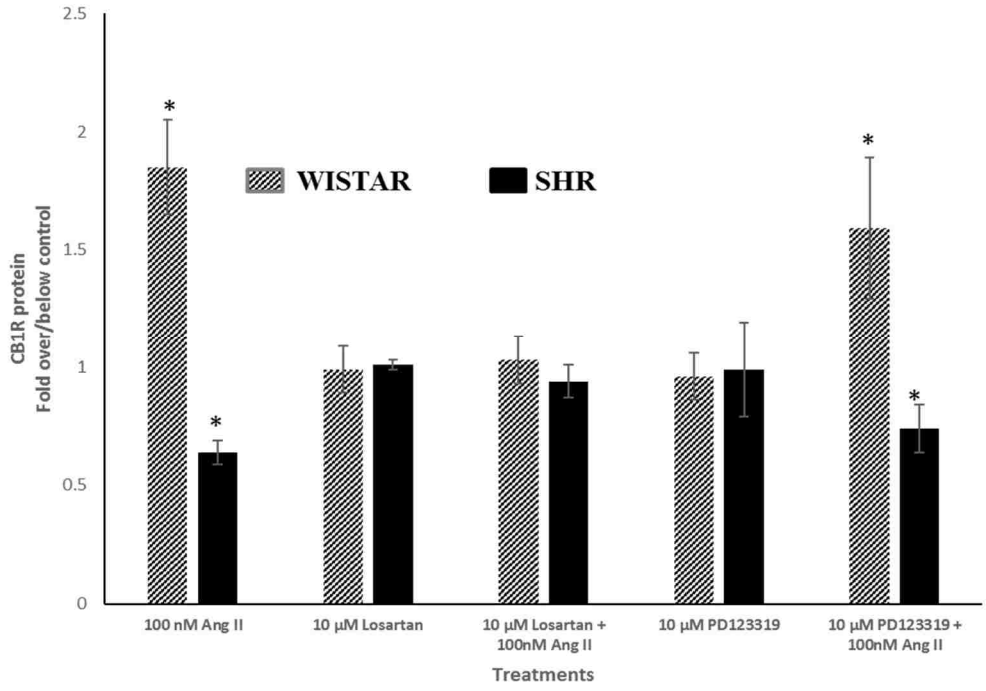

Fig 3.3B: Effect of Angiotensin Receptor Blockers on Ang II-mediated effects on CB1R Protein levels in brainstem astrocytes isolated from SHRs and Wistar Rats: CB1R protein levels in brainstem astrocytes from Wistar and SHRs that have been pretreated with 100 nM Ang II alone, inhibitors (10 µM) alone, or a combination of Ang II and inhibitors, was measured using Western blotting technique. Each value represents the mean ± SEM of preparations of astrocytes isolated from six or more litters of neonatal rat pups. *Denotes $p < 0.05$ as compared to basal CB1R protein expression.

iv) Effect of Ang II on CB1R mRNA expression in brainstem astrocytes

Ang II effects on CB1R mRNA levels were also examined in brainstem astrocytes. As shown in Fig 3.4A, Ang II downregulated CB1R mRNA expression in Wistar brainstem astrocytes at all the time points examined (maximum was at 8 hours). In SHR brainstem astrocytes, Ang II had a biphasic response on CB1R mRNA levels, an effect similar to that observed for CB1R protein expression (see Fig 3.3A). However, maximum downregulation was observed at 12 hours and the peak effect occurred at 24 hours (over a 4-fold increase). Upon comparison of individual time points of SHR with Wistar samples, the differences were found to be statistically significant at 4, 8, 16 and 24 hours. The

difference at 24 hours was the greatest because upregulation was observed in SHR brainstem astrocytes, while downregulation was seen in its normotensive counterpart.

To determine the Ang receptor involved in Ang II-mediated effects on the CB1R mRNA levels, Wistar and SHR brainstem astrocytes were treated with 100 nM Ang II for 4 hours, in the presence and absence of inhibitors, before harvesting them for mRNA. As shown in Fig 4B, pretreating brainstem astrocytes with the inhibitors alone had no effect on the mRNA levels of the CB1R. But pretreatment with Losartan inhibited most of the Ang II effect on CB1R mRNA expression. PD123319, the AT2R blocker however was ineffective in preventing the actions of Ang II.

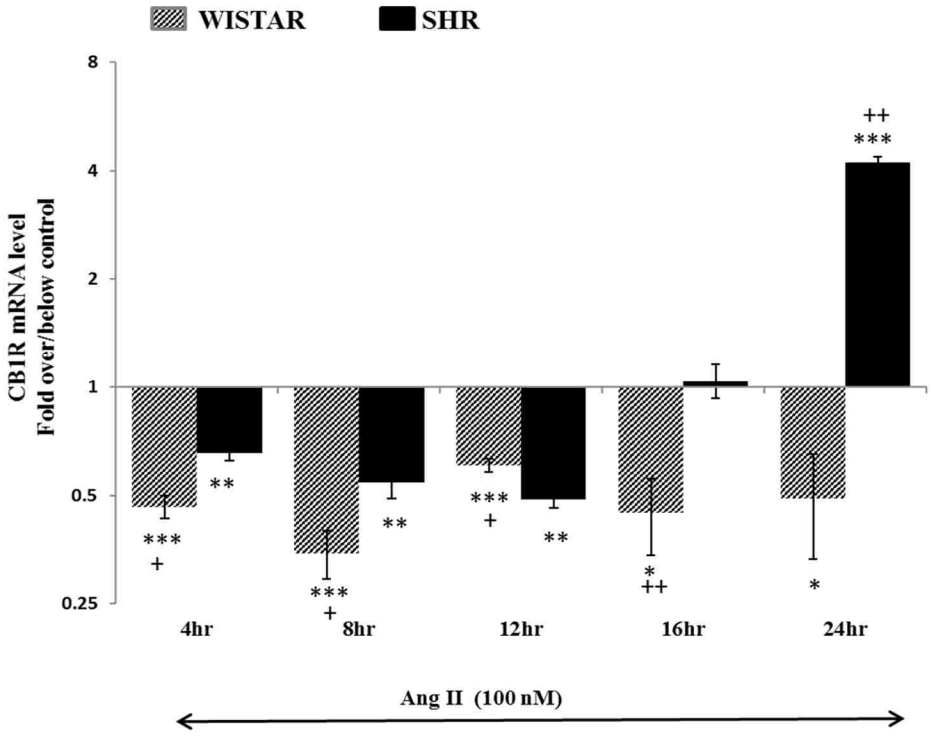

Fig 3.4A: Ang II Effects on CB1R mRNA Expression in Brainstem Astrocytes Isolated from SHRs and Wistar Rats: qPCR was employed to compare CB1R mRNA levels from Wistar and SHRs brainstem astrocytes, which were pretreated with 100 nM Ang II for varying time periods. Each value represents the mean ± SEM of preparations of astrocytes isolated from six or more litters of neonatal rat pups. The data is represented as fold over/under control that were obtained when CB1R Ct values were normalized to beta-actin Ct values and further normalized to its control (*denotes $p < 0.05$ and **denotes $p < 0.01$ ***$p < 0.001$ compared to its basal values; $^{+}$denotes $p < 0.05$, $^{++}$denotes $p<0.01$, compared to its corresponding Wistar time point).

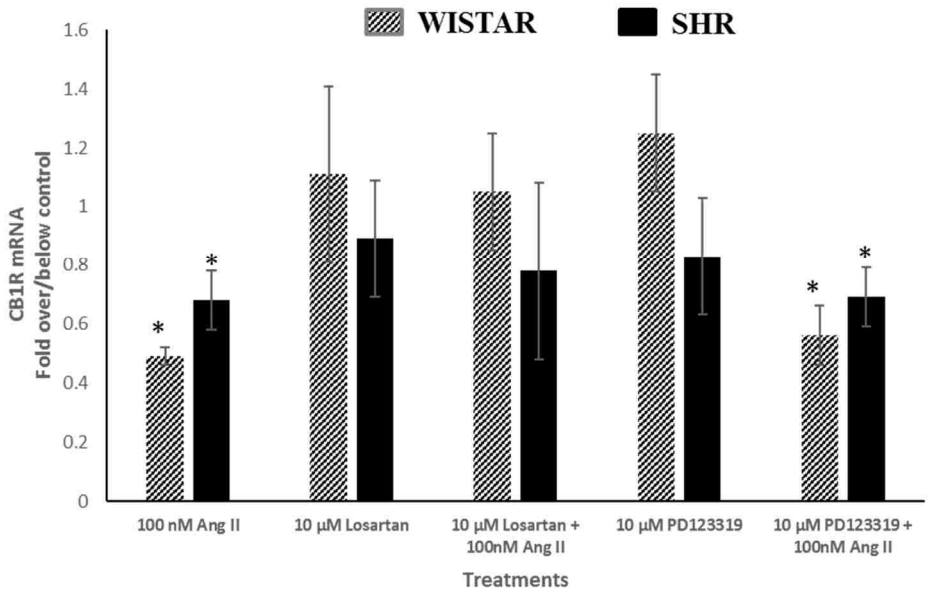

Fig 3.4B: Effect of Angiotensin Receptor Blockers on Ang II-mediated effects on CB1R mRNA levels in brainstem astrocytes isolated from SHRs and Wistar rats: qPCR was employed to compare CB1R mRNA levels in Wistar and SHR brainstem astrocytes that have been pretreated with 100 nM Ang II alone, inhibitors (10 µM) alone, or a combination of both Ang II and inhibitors. Each value represents the mean ± SEM of preparations of astrocytes isolated from six or more litters of neonatal rat pups. *Denotes $p < 0.05$ as compared to basal CB1R protein expression.

v) Effect of Ang II on CB1R protein expression in cerebellum astrocytes

In astrocytes isolated from Wistar cerebellums, Ang II caused downregulation of the CB1R protein at the higher time points (12 to 48 hours), while it had no effect at time points prior to 12 hours (Fig 3.5A). In SHR cerebellum samples, Ang II caused an upregulation of the CB1R protein. The difference was found to be significant from 4 to 48 hours. Similar to brainstem samples, Ang II had no effect on CB1R protein at 1 hour in both strains of rats. Except for the 1 hour treatment point, differences observed for SHR samples when compared with Wistar samples, were statistically significant.

To determine the Ang receptor involved in this effect, Wistar and SHR cerebellum astrocytes were treated with 100 nM Ang II for 12 hours and 4 hours, respectively in the presence and absence of inhibitors, before harvesting the cells for protein estimations (Fig 3.5B). Pretreating with the Ang AT1R and AT2R blockers had no significant effect on the basal protein expression of the CB1R. Both Losartan and PD123319 partially prevented Ang II-mediated CB1R protein expression in SHR cerebellum astrocytes. In contrast, while PD123319 had no effect on Ang II-mediated downregulation of CB1R protein expression in the Wistar cerebellum samples, Losartan was effective in abolishing its effect.

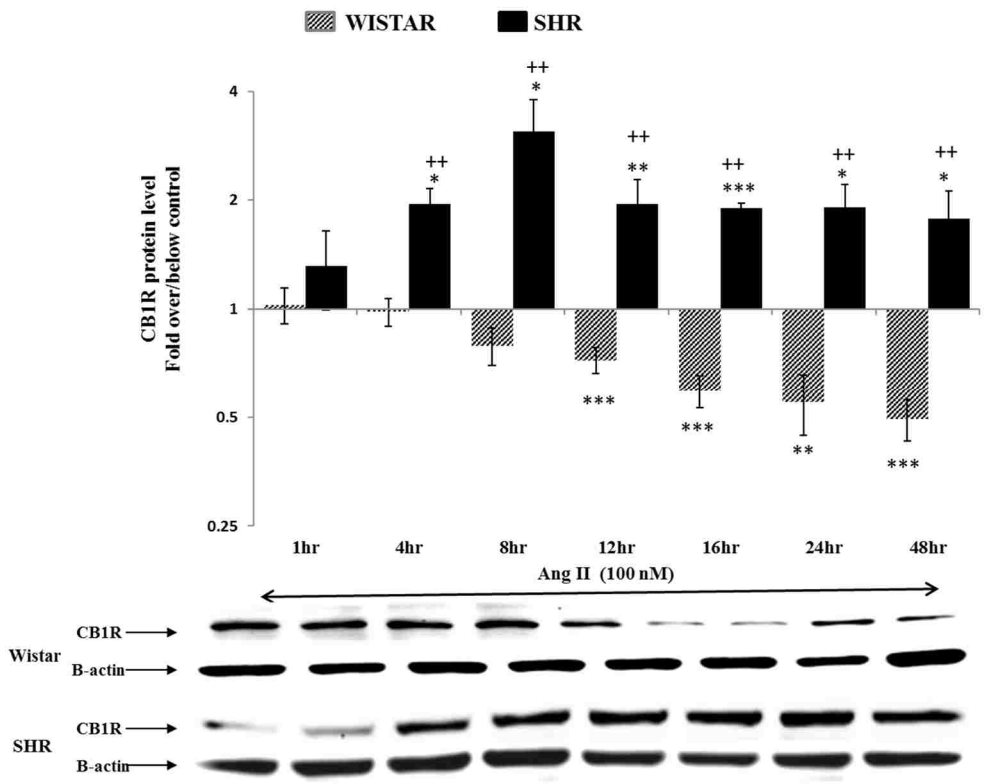

Fig 3.5A: Ang II Effects on CB1R Protein Expression in Cerebellum Astrocytes Isolated from SHRs and Wistar Rats: Western blotting was employed to compare CB1R protein levels from Wistar and SHRs cerebellum astrocytes, which were pretreated with 100 nM Ang II for varying time periods. Each value represents the mean ± SEM of preparations of astrocytes isolated from six or more litters of neonatal rat pups. The data is represented as fold over/under control that were obtained when CB1R bands were normalized to beta-actin bands and further normalized to its control (*denotes $p < 0.05$ and **denotes $p < 0.01$ ***$p < 0.001$ compared to its basal values; $^{+}$denotes $p < 0.05$, $^{++}$denotes $p<0.01$ compared to its corresponding Wistar time point).

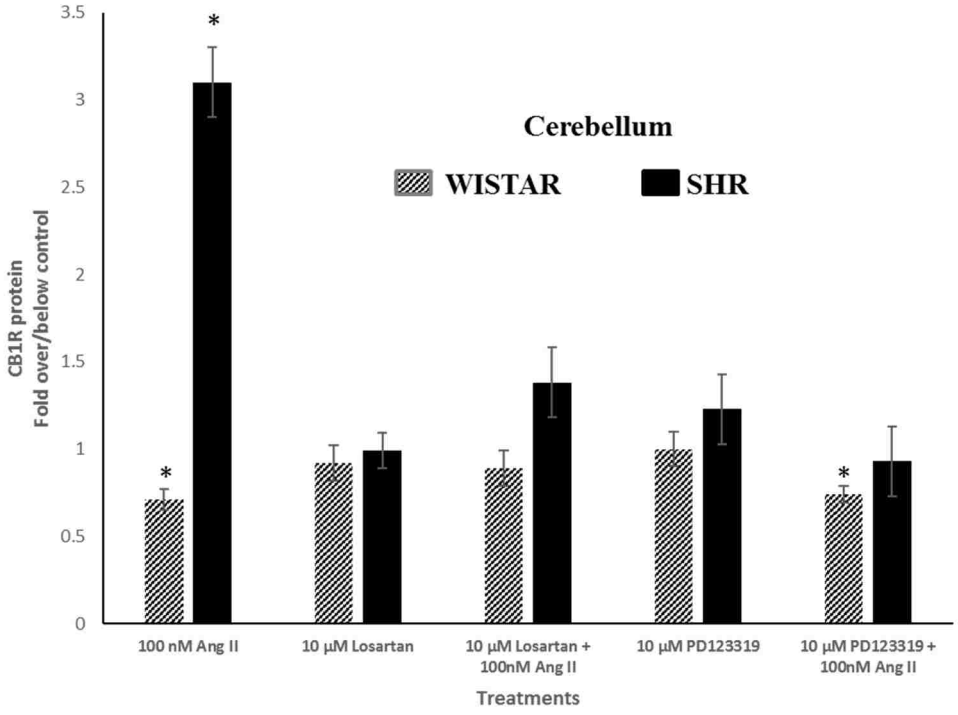

Fig 3.5B: Effect of Angiotensin Receptor Blockers on Ang II-mediated effects on CB1R Protein levels in cerebellar astrocytes isolated from SHRs and Wistar Rats: Western blotting technique was employed to compare CB1R protein levels in Wistar and SHR cerebellar astrocytes that have been pretreated with 100 nM Ang II alone, inhibitors (10 µM) alone, or a combination of both Ang II and inhibitors. Each value represents the mean ± SEM of preparations of astrocytes isolated from six or more litters of neonatal rat pups. *Denotes $p < 0.05$ as compared to basal CB1R protein expression.

vi) Effect of Ang II on CB1R mRNA expression in cerebellum astrocytes

The major effect of Ang II on CB1R mRNA expression in Wistar samples was down-regulation, while upregulation was observed in SHR cerebellar astrocytes (Fig 3.6A). Although exceptions to this trend was observed at the 8 and 12 hour time points in Wistar samples, the difference was not found to be statistically significant. In the case of SHR cerebellum samples, down-regulation was observed at 4 hours (Fig 3.6A). In this case however, the difference was found to be significantly different. Upon comparison of SHR

time points with their respective Wistar time points, the differences observed from 8 to 24 hours were statistically significant.

To ascertain which Ang receptor involved in this effect, Wistar and SHR cerebellar astrocytes were treated with 100 nM Ang II for 4 hours, respectively in the presence and absence of inhibitors, before harvesting them for mRNA. As shown in Fig 3.6B, treating the cells with just the Ang receptor inhibitors alone, had no effect. While Ang II alone was able to significantly alter CB1R mRNA in both Wistar and SHR cerebellar astrocytes, pretreatment with Losartan resulted in termination of its effect. However, pretreating the astrocytes with the PD123319 was ineffective in preventing the Ang II-mediated effects on the CB1R.

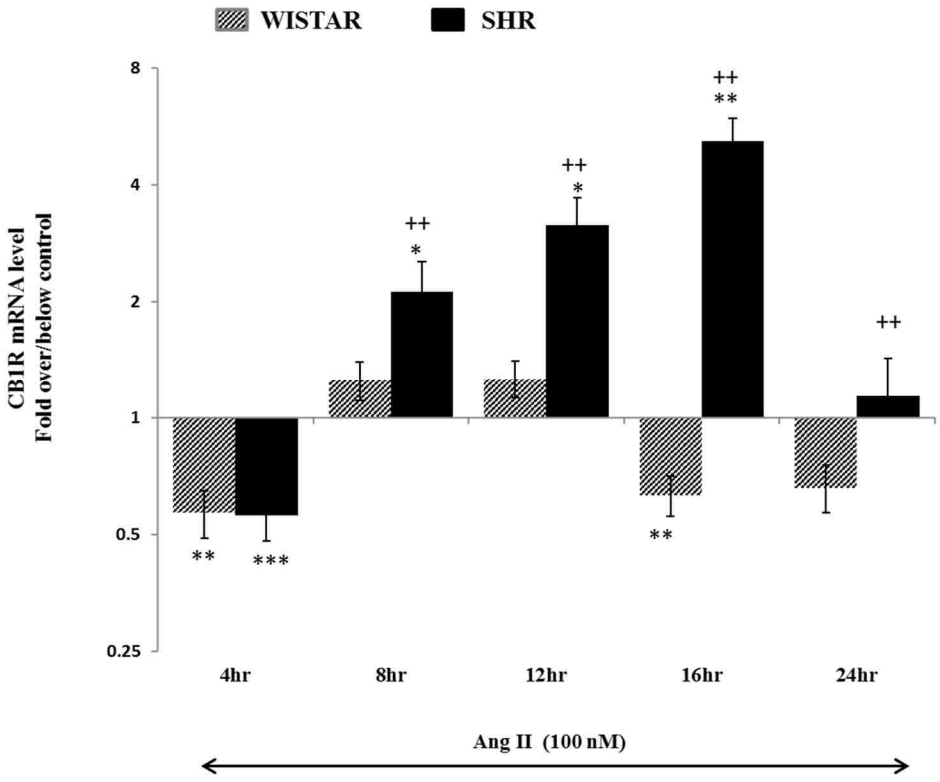

Fig 3.6A: Ang II Effects on CB1R mRNA Expression in Cerebellum Astrocytes Isolated from SHRs and Wistar Rats: qPCR was employed to compare CB1R mRNA levels from Wistar and SHR cerebellar astrocyte samples, which were pretreated with 100 nM Ang II for varying time periods. Each value represents the mean ± SEM of preparations of astrocytes isolated from six or more litters of neonatal rat pups. The data is represented as fold over/under control that were obtained when CB1R Ct values were normalized to beta-actin Ct values and further normalized to its control (*denotes $p < 0.05$ and **denotes $p < 0.01$ ***$p < 0.001$ compared to its basal values; $^{+}$denotes $p < 0.05$, $^{++}$denotes $p<0.01$, compared to its corresponding Wistar time point).

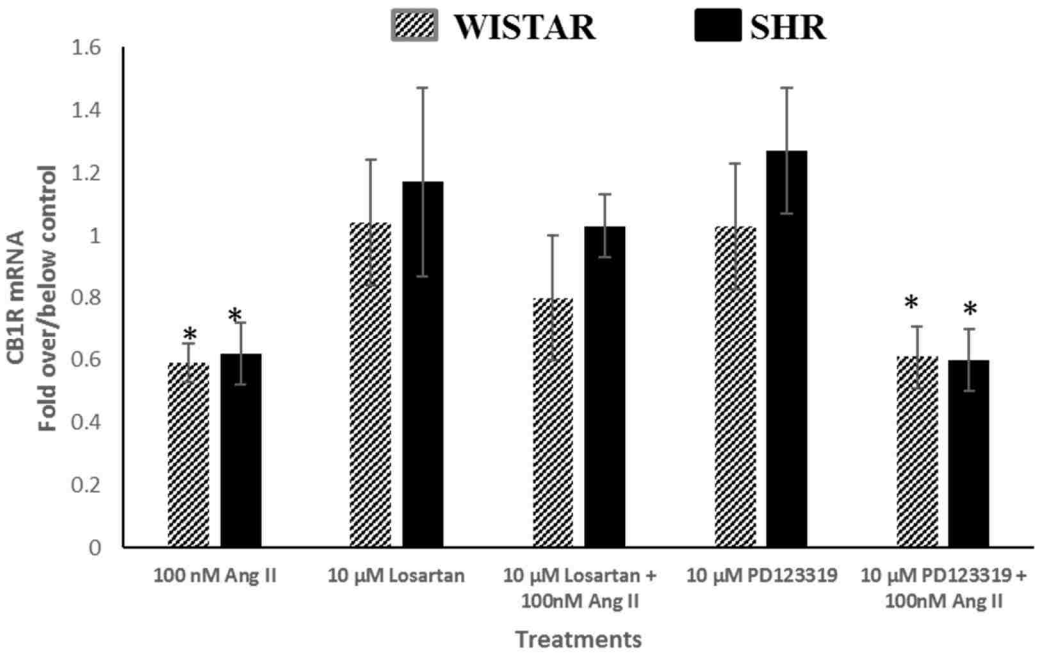

Fig 3.6B: Effect of Angiotensin Receptor Blockers on Ang II-mediated effects on CB1R mRNA levels in Cerebellar Astrocytes isolated from SHRs and Wistar rats: qPCR was employed to compare CB1R mRNA levels in astrocytes of both Wistar and SHRs that have been pretreated with 100 nM Ang II alone, inhibitors (10 µM) alone, or in combination with both Ang II and inhibitors. Each value represents the mean ± SEM of preparations of astrocytes isolated from six or more litters of neonatal rat pups. *Denotes $p < 0.05$ as compared to basal CB1R protein expression

3.4 Discussion

The most significant finding of this study is that Ang II, mostly via the AT1R, is capable of altering CB1R expression in astrocytes isolated from both cerebellum and brainstem under both hypertensive and non-hypertensive conditions. The direction and magnitude of change however, is not only different based on the presence/absence of a pathological state, but also dependent on the brain regions that the astrocytes were isolated from. Interplay between a triad of factors, namely Ang II, presence or absence of hypertension, and the brain region, may well be responsible for CB1R expression to be either elevated or lowered.

A higher basal CB1R protein expression was observed in cerebellar astrocytes when compared to brainstem astrocytes in both Wistar and SHRs, which is in congruence with other studies that have reported a higher CB1R expression in cerebellum compared to brainstem (Herkenham *et al.* 1991) (Tsou *et al.* 1998). Although expressed at lower levels in brainstem, cannabinoids can elicit anti-nociceptive (Manzanares *et al.* 2006) and anti-emetic (Van Sickle *et al.* 2001) effects, in addition to regulation of sympathetic activity via the brainstem CB1R (Seagard *et al.* 2004). Interestingly, a significant reduction in the levels of the CB1R, both protein and mRNA, in brainstem astrocytes isolated from the SHR when compared to its normotensive counterpart, the Wistar rat were observed in this study. However, in cerebellar astrocytes, both CB1R protein and mRNA levels were not significantly different between the two rat models. Reduced CB1R expression in SHR brains have been previously reported wherein researchers observed a reduction of CB1R levels in the prefrontal cortex of SHR (Adriani *et al.* 2003). Reduced expression in SHR brainstem astrocytes may be suggestive of a dampened endocannabinoid tone in blood pressure regulation under hypertensive conditions. The endocannabinoid tone in SHR cerebellum however could still be functional in pathological states such as ADHD. Administration of the CB1R agonist WIN-55,212, was demonstrated to improve symptoms of ADHD in SHRs (Adriani *et al.* 2003). It could well be that the unchanged CB1R density in SHR cerebellum is mediating some of the positive effects of cannabinoids in this case. While CB1R protein levels were remarkably higher in cerebellar when compared to brainstem astrocytes, a comparison of CB1R mRNA levels between the two regions yielded no significant differences. This indicates that any possible difference observed in the basal expression of CB1R cannot be solely attributed to transcriptional efficiency

across different brain regions in different rats, but additional factors such as translational efficiency, post translational modifications and/or protein stability could be contributing to it (Maier *et al.* 2009).

When brainstems were treated with Ang II for increasing time periods, CB1R protein levels were significantly elevated in normotensive conditions, but not in hypertensive states during the earlier time points. The AT1R was the major Ang receptor responsible for these effects. This effect was most prominent at the earlier (until 8 hours) than the later time points. This suggests that the relatively early elevation of CB1R, which is observed in response to a hypertensive stimulus (Ang II) in Wistar brainstem astrocytes, is lost in SHR brainstem astrocytes. A plausible theory could be that an elevation in CB1R, in response to AT1R activation, may be a homeostatic mechanism to negate the pro-inflammatory nature of Ang II under normal physiological conditions. This is possibly disrupted under pathological conditions in brainstem astrocytes. Not only is an elevated level of pro-inflammatory cytokines reported in SHR brainstem (Agarwal *et al.* 2011), but Ang II via the brainstem AT1R has been demonstrated to have a pressor effect that is significantly more dominant in SHRs when compared to Wistar rats (Seyedabadi *et al.* 2001). While the brainstem astroglial AT1R has been shown to play an important role in augmenting sympathetic outflow (Isegawa *et al.* 2014a), CB1R activation in the brainstem has been demonstrated to lower blood pressure (Seagard *et al.* 2004) (Lake *et al.* 1997b) (Bátkai *et al.* 2004). Although there are studies that demonstrate the ability of CB1R to increase blood pressure, these have not been done in SHRs (Ibrahim and Abdel-Rahman 2011) (Schaich *et al.* 2014). Excessive production of pro-inflammatory cytokines in the cardiovascular centers of the brain is tightly intertwined with the progression of

hypertensive conditions (Shi *et al.* 2010b). As the glial CB1R is known to elevate levels of anti-inflammatory cytokines (Molina-Holgado *et al.* 2003), it is conceivable that the downregulation of the CB1R, during the early phase of AT1R activation, may be contributing to the hypertension phenotype. With regards to the correlation between mRNA and protein data in brainstem astrocytes, the CB1R trend in response to Ang II was similar in SHRs, but not in Wistar rats. This suggests that under normal physiological conditions, the elevation of CB1R protein in response to Ang II in brainstem astrocytes, may not be linked to transcriptional regulation of the receptor (Figs. 3A and 4A). However, this may be a dominant mechanism in hypertensive conditions. An alternative mechanism could be that Ang II is affecting the stability of CB1R mRNA which may result in a drop in corresponding protein levels.

In contrast to brainstem astrocytes, where Ang II treatment had a significant impact on CB1R expression within the first 8 hours, the earliest effect in Wistar cerebellum astrocytes was observed at the 12 hours' time point. In SHR cerebellum samples, CB1R protein was elevated in response to Ang II from 4 hours onwards. This suggests that in cerebellar astrocytes, CB1R protein is either elevated (SHR), or remains unaltered (Wistar), in response to early AT1R activation. While the elevation is persistent even at later time points in SHR cerebellum astrocytes, CB1R protein falls appreciably from 12 hours onwards in Wistar cerebellum astrocytes. It is possible that the role of the CB1R may be preserved in cerebellum astrocytes in pathological conditions. Although the CB1R protein followed similar suit to that of the CB1R mRNA levels in response to Ang II, the receptor mediating this effect was not identical. While the effect of Ang II on CB1R protein expression was predominantly via the AT1R in Wistar cerebellar astrocytes, both AT1R

and AT2R effects were observed in SHR cerebellum astrocytes. Although several studies have reported an absence of AT2R in astrocytes (Li *et al.* 2012c), there have been others that have reported functional AT2R in astrocytes (Downie *et al.* 2009) (Park *et al.* 2013), such as neutralizing pro-inflammatory mediators (Steckelings *et al.* 2011). This data points to a potential role of the AT2R, in conjunction with AT1R, in elevating neuroprotective regulators, such as CB1R protein, in SHR cerebellar astrocytes. As CB1R is elevated in response to Ang II in SHR cerebellar astrocytes, its activation could be explored as a possible therapeutic strategy in diseases, such as ADHD, where cerebellar functions are dysregulated due to neuroinflammatory mediators and astrogliosis (Yun *et al.* 2014). Although a strong correlation between hypertension and various learning (Adams *et al.* 2010), cognitive (Nade *et al.* 2015), and motor disabilities (Qian *et al.* 2010) has already been reported, the role of brain RAS has not been well investigated in disorders such as ADHD. More research on the cross-talk between the two systems, RAS and the endocannabinoid system, in cerebellum could shed some light on disorders that are linked to cerebellar dysfunction.

The crosstalk between the CB1R and the AT1R has already been explored at both a mechanistic and functional level in peripheral tissues. Ang II via the AT1R generates endocannabinoids which can transactivate the CB1R in a paracrine manner, in both *in vitro* (Turu *et al.* 2009), and *in vivo* (Szekeres *et al.* 2012) conditions. Activation of the vascular CB1R has been shown to play a role in mitigating some of the AT1R effects on promoting vasoconstriction (Szekeres *et al.* 2012) suggesting a possible protective role of CB1R during hypertensive conditions. Although our study reported a reduction in the basal CB1R expression in astrocytes of SHR brainstem, CB1R expression was shown to be elevated in

the heart and blood vessels of SHRs (Bátkai *et al.* 2004). It could well be that while the peripheral endocannabinoid system is functioning at a higher degree in hypertensive states, the central endocannabinoid system may not be able to counteract the effects of Ang II in the brainstem under the same conditions. While an elevation of CB1R is thought to elicit a protective role, there have been cases where an increase is linked to a worsening of disease progression (Di Marzo 2008). Studies have also reported on the ability of CB1R to further enhance AT1R actions in the periphery, thereby hastening the process of disease progression (Rozenfeld *et al.* 2011), (Tiyerili *et al.* 2010). This is suggestive of the fact that the outcome of the interaction between the two systems, RAS and the endocannabinoid system is not only tissue specific, but may depend on the disease model. Further studies in our laboratory are underway to determine the downstream effects and functional significance of CB1R activation alone, and also in conjunction with Ang receptor activation in astrocytes isolated from SHR.

The observed differential regulation pattern of CB1R by Ang II in this study underscores a potential region specific dampening of the endocannabinoid tone by one of the key drivers of hypertension. As glial AT1R and CB1R have opposing roles in regulating inflammatory states (Winklewski *et al.* 2015) (Sheng *et al.* 2005a) (Molina-Holgado *et al.* 2002a) (Molina-Holgado *et al.* 2003), this interaction represents a potential therapeutic target not just for hypertension, but other diseases that have a neuroinflammatory component. A tendency for an increase in CB1R protein in cerebellar astrocytes and a decrease in brainstem astrocytes, in response to Ang II, suggests that homeostatic systems, such as the endocannabinoid system, may be functioning at suboptimal levels only in certain brain regions under hypertensive conditions. Ang II by downregulating an already

small pool of CB1R in the brainstem astrocytes under hypertensive states, may be involved in mediating a drop in endocannabinoid regulation of astroglial functions. A possible therapeutic strategy could be to elevate the brainstem CB1R in order to circumvent the pro-inflammatory effects of Ang II in key cardiovascular centers. Centrally acting ARBs could well be a possible route to prevent Ang II from downregulating CB1R expression in the brainstem astrocytes. As AT1R is highly elevated in the cardiovascular centers in SHR (Reja *et al.* 2006), this strategy could aid in preventing CB1R from being downregulated in specific brain regions that are associated with blood pressure regulation. Administration of CB1R agonists has been demonstrated to negatively impact cerebellar functions (Patel and Hillard 2001), suggesting that direct agonism may be detrimental. Modulating the components, and possibly the functions, of the endocannabinoid system by targeting such indirect modulators, would help in circumventing the undesirable adverse effects elicited by direct activation of the receptor (Di Marzo 2008).

Chapter 4

MAPK Activation Patterns of AT1R and CB1R in SHR versus Wistar Astrocytes: Evidence of CB1R Hypofunction and Crosstalk between AT1R and CB1R.

Abstract

Background: Ang II and cannabinoids are able to regulate physiologically relevant astroglial functions via receptor-mediated activation of MAPKs. In this study, we investigated the consequences of astroglial AT1R and CB1R activation, alone and in combination on MAPK activation, in the presence and absence of hypertensive states. In addition, we also investigated a novel unidirectional crosstalk mechanism between AT1R and CB1R, that involves PKC-mediated phosphorylation of CB1R. Methods: Astrocytes were isolated from the brainstem and cerebellum of SHRs and normotensive Wistar rats. The cells were treated with either 100 nM Ang II or 10 nM Arachidonyl-2'-chloroethylamide (ACEA), both alone and in combination, for varying time periods and the extent of phosphorylation of MAPKs, ERK and p38, and the phosphorylated forms of CB1R (p-CB1R), were measured using western blotting. Results: Ang II treatment resulted in a greater activation of MAPKs in SHR brainstem astrocytes, but not SHR cerebellar astrocytes when compared to Wistar rats. ACEA mediated MAPK activation was significantly lower in brainstem astrocytes of SHRs when compared to Wistar rats. ACEA negatively modulates AT1R-mediated MAPK activation in both cerebellar and brainstem astrocytes of both models. The effect however was diminished in brainstem astrocytes. Ang II caused a significant increase in phosphorylation of CB1R in cerebellar astrocytes, while a significantly lesser effect was observed in brainstem astrocytes of both models.

Conclusion: Both Ang II and ACEA-induced MAPK activation were significantly altered in SHR astrocytes when compared to Wistar astrocytes. A possible reduction in CB1R functionality, coupled with a hyperfunctional AT1R in the brainstem, could well be significant factors in the development of hypertensive states. AT1R-mediated phosphorylation of CB1R could be critical for impaired cerebellar development characterized by a hyperactive RAS.

4.1 Introduction

Astrocytes play critical roles in several pathological conditions. Their ability to serve as mediators of communication between neurons (Araque *et al.* 2001), to alter neuroinflammatory states (Bélanger and Magistretti 2009), to be a major source for AGT in the brain (Stornetta *et al.* 1988), and to regulate energy stores (Brown and Ransom 2007), lends these cells to be an ideal model for studying cardiovascular and neurological disorders. The SHR is a well-established animal model for essential hypertension (Badyal *et al.* 2003). In addition to being a model of choice to study hypertension and several other cardiovascular disorders, they have also been used to study neurodevelopmental disorders such as ADHD (Adriani *et al.* 2003). While astrocytes from the brainstem have been implicated in augmenting sympathoexcitatory drive (Marina *et al.* 2013), increased astrogliosis has been reported in the SHR cerebellum (Goetz *et al.* 2014b) which further illustrates the necessity of investigating the potential dysregulated molecular machinery in these cells.

An overactive brain RAS is one of the most prominent characteristics of SHRs (Veerasingham and Raizada 2003). Astroglial AT1R in the brainstem has a critical role in elevating sympathetic activity in cardiovascular diseases (Isegawa *et al.* 2014a). In

astrocytes from the brainstem and cerebellum, Ang II via activation of the AT1R results in an increase in pro-hypertensive and a decrease in anti-hypertensive markers (Gowrisankar and Clark 2016b) (Gowrisankar and Clark 2016a). Several reports of crosstalk between the angiotensin system and the endocannabinoid system have emerged in the recent past (Turu *et al.* 2009) (Rozenfeld *et al.* 2011). CB1R is one of the most abundant GPCRs in the brain and its impairment has been linked to a multitude of neurological disorders (Miller and Devi 2011). CB1R antagonism has been demonstrated to both potentiate (Szekeres *et al.* 2012), as well as neutralize Ang II-mediated effects (Rozenfeld *et al.* 2011). In the brain, both exogenous and endogenous cannabinoids (endocannabinoids) are claimed to have prominent neuroprotective and anti-inflammatory effects via activation of the CB1R, and the CB_2R activation (Miller and Devi 2011). Interestingly, astroglial AT1Rs and CB1Rs have seemingly opposing roles in the regulation of several astroglial functions. For instance, while astroglial AT1R can cause astrocyte senescence (Liu *et al.* 2011a) and an increase in proinflammatory cytokines (Gowrisankar and Clark 2016c), astroglial CB1R activation confers protection against apoptosis (Gómez Del Pulgar *et al.* 2002) and an elevation in anti-inflammatory cytokines (Molina-Holgado *et al.* 2003). Neuroinflammation is not only critically implicated in neurological disorders, but also cardiovascular disorders since an increase in pro-inflammatory cytokines from glial cells has been shown to elevate sympathetic activity (Shi *et al.* 2010a).

Activation of cell surface receptors, such as AT1Rs and CB1Rs, evokes cellular responses that are tightly regulated by key signal transduction pathways. Some of these key intracellular pathways associated with AT1Rs and CB1Rs in astrocytes fall under the umbrella of MAPKs. MAPKs serve as critical linking points between receptor activation

and cellular functions. MAPK activation in astrocytes is associated with a diverse array of functions, which have both physiological and pathophysiological consequences. Activation of astroglial MAPKs such as extracellular signal regulated kinase (ERK), p38 and c-Jun N-terminal kinase (JNK) regulate proliferation (Clark *et al.* 2001), astrogliosis and mobilization of neuroinflammatory cytokines (Gadea *et al.* 2008) (Winklewski *et al.* 2015). These functions make them important targets of neurological and cardiovascular diseases. Studies from our laboratory and others have shown that MAPKs are involved in Ang II-mediated proliferative (Clark *et al.* 2013) and pro-inflammatory effects (Zhang *et al.* 2017). Cannabinoids also employ the ERK MAPK pathway in mediating their protective effects in astrocytes (Galve-Roperh *et al.* 2002). In brainstem neurons, ERK and other signal transduction pathways such as PI3K are crucial for CB1R-mediated regulation of cardiovascular parameters (Ibrahim and Abdel-Rahman 2012). While AT1R signaling mechanisms have been studied in SHR neurons (Yang and Raizada 1998), both AT1R- and CB1R-mediated effects on MAPK activation has not been well investigated in SHR astrocytes.

Several studies have reported alterations in CB1R levels in SHR cells when compared to their normotensive counterparts, a decrease was observed in brains cells (Adriani *et al.* 2003) and an increase in myocardial cells (Bátkai *et al.* 2004). Consequently, a change in CB1R expression levels has also been demonstrated to correlate strongly with an alteration in CB1R-mediated downstream effects (Miller and Devi 2011). Previously, we have reported a reduction in the CB1R expression levels in brainstem, but not cerebellar, astrocytes of SHRs when compared to Wistar rats (Haspula and Clark 2016b). Whether this reduction translates into a dampened CB1R tone, has not been investigated. MAPK

activation is governed by many factors, one of the primary factors being receptor density and trafficking (Murphy and Blenis 2006). Comparing signal transduction pathways of the CB1R and the AT1R, between the two strains would enable us to have a better understanding of potential endocannabinoid or Ang receptor dysregulation in pathological conditions, respectively. Interestingly, crosstalk between the two receptors at the level of ERK was also investigated in a neuroblastoma cell line (Rozenfeld and Devi 2008). Co-treatment of Ang II with a CB1R agonist, HU-210, led to an increase in AT1R-mediated activation of ERK.

Therefore the focus of our study was three-fold. First to investigate the effects of Ang II and a potent CB1R agonist, ACEA, individually on MAPK activation in SHR and Wistar rat astrocytes. This would enable us to understand the patterns of MAPK activation by RAS and endocannabinoid systems in astrocytes. Secondly, we investigated crosstalk between the two systems. This was assessed by investigating the effect of co-treatments with Ang II and ACEA on MAPKs. Whether ACEA treatment neutralizes or potentiates Ang II-mediated activation of MAPKs, when compared to Ang II alone would then help us to understand the mode of crosstalk that exists between these two systems in these primary cells. Lastly, we explored a novel mode of crosstalk between AT1R and CB1R, which involves PKC activation. Garcia et al., (1998) has earlier demonstrated that PKC activation by Phorbol 12-myristate 13-acetate (PMA) results in phosphorylation of the CB1R in the third intracellular loop. This phosphorylation event diminishes CB1R's ability to elicit its functions. As the AT1R is a Gq GPCR, Ang II is functionally capable of activating PKC and thereby phosphorylating CB1R. Hence, both basal and Ang II-mediated phosphorylation of CB1R, was also investigated in this study.

4.2 Materials and methods

i) Materials

Ang II was obtained from Bachem (Torrance, CA). PD123319, the selective AT_2R antagonist was obtained from Sigma (St. Louis, MO), and Losartan (AT1R antagonist) was kindly provided by Du Pont Merck (Wilmington, DE). ACEA, the specific CB1R agonist, was purchased from Tocris (Bristol, UK) and Bisindolylmaleimide I, Hydrochloride #9841 (BIM I), the potent PKC inhibitor, was obtained from Cell signaling Technology (Beverly, MA). Orlistat, the DAGL inhibitor and PMA, the PKC activator were purchased from Sigma (St. Louis, MO). Western blotting supplies were purchased from Bio-Rad Laboratories (Hercules, CA) or VWR International (Suwanee, GA). The polyclonal antibody that detects the phosphorylated forms of CB1R, p-CB1 Antibody (Ser 316) [sc-17555], was purchased from Santa Cruz (Dallas, TX), and the monoclonal phospho CB1R antibody, Anti-Cannabinoid Receptor I (phospho S316) antibody [EPR2223(N)], was purchased from Abcam. The phospho-p38 (P-p38) and the phospho ERK (P-ERK) antibodies were purchased from Cell Signaling Technology (Beverly, MA). Beta-actin antibody (A2066) was purchased from Sigma (St. Louis, MO). The BCA protein kit was obtained from Pierce Biotechnology (Rockford, IL). All other chemicals were purchased from either VWR International (Suwannee, GA), Fisher Scientific (Waltham, MA) or Sigma (St. Louis, MO).

ii) Isolation and culture of primary astrocytes

Timed pregnant Wistar rats and SHRs were obtained from Charles River Laboratories (Wilmington, MA) and maintained in the ALAAC-accredited animal facility of Nova Southeastern University. All animal protocols were approved by the University

Institutional Animal Care and Use committee. The brainstem and cerebellar astrocyte cultures were prepared using mechanical dissociation as previously described (Tallant and Higson 1997). Briefly, brains from 3-day old rat pups were isolated and the cerebellum and brainstem were carefully separated from each brain. These regions are visible to the naked eye and can be clearly differentiated from each other. Astrocyte cultures were then prepared from the pooled brainstem and the pooled cerebellum by mechanical dissociation. The cells were grown in DMEM/F12 culture media containing 10% FBS, 10,000 I.U/mL penicillin, 10,000 µg/mL streptomycin and 25 µg/mL amphotericin B at 37°C in a humidified incubator (5% CO_2 and 95% air). The cell cultures were fed every 3-4 days.

On attaining confluency, the cells were subjected to vigorous shaking overnight which resulted in the detachment of microglia, oligodendrocytes and cell debris. Subsequently, the cell cultures were detached with trypsin/EDTA (0.05% trypsin, 0.53mM EDTA) and replated at a ratio of 1:10. The astrocyte enriched cultures were fed once every 3 days until they were about 90% confluent. Before all cell treatments, the cultures were made quiescent by treating with serum free media, for 48 hours. All subsequent treatments were conducted in serum free DMEM/F12 culture media containing 10,000 I.U/mL penicillin, 10,000 µg/mL streptomycin and 25 µg/mL amphotericin B. The purity of the enriched astrocyte cultures were determined using Flow cytometry, western blotting and qPCR as shown previously (Haspula and Clark 2016b).

iii) Cell treatments

Astrocytes were treated with 100 nM Ang II and/or with 10 nM ACEA, a potent CB1R agonist, for varying time periods ranging from 1 min to 1 hour. For P-ERK and P-p38 protein estimation, the time periods of Ang II or ACEA treatments were 1, 5, 10, 15,

30, 45 and 60 mins. Select time points (5, 15, 30, 45 and 60 mins) were employed to compare the effect of co-treatment on the two MAPKs with Ang II. For p-CB1R protein estimation, the time periods of Ang II treatments were 1, 5, 10, 15, 30, 45 and 60 mins and for 50 nM PMA treatment, the exposure time was 15 mins. For the inhibitor studies, the cells were pretreated with inhibitors for the AT1R (10 μM Losartan), the AT_2R (10 μM PD123319), PKC (50 nM BIM I), or DAGL (100 nM Orlistat) for 30 mins before the addition of 100 nM Ang II for 15 mins.

iv) Cell lysate preparation for western blotting

Immediately following treatments, cell lysates were prepared by washing cells with TBS followed by direct lysis with 200 μL of 1x Laemmli sample buffer (125 mM Tris-HCl, pH 6.8, 1% SDS, 0.5% glycerol, 5 mM EDTA, 5 mM EGTA, 0.01 mM $NaVO_4$, 0.1 mM PMSF and 0.6 μM leupeptin) per well. Protein concentrations were determined using the BCA assay as per the manufacturer's instructions. After protein determinations, β-mercaptoethanol (5%) and bromophenol blue (0.01%) were added to the samples, and the cell lysates were stored at -80°C until further processing.

v) Western blotting

Volumes equivalent to 20-50 μg of solubilized proteins were loaded into 10% polyacrylamide gels, and subsequently transferred to nitrocellulose membranes. The membranes were then blocked with 5% BSA prepared in TBS containing 0.05% tween TBS-T. The membranes were then subjected to 3 washes, 5 mins each using TBS-T followed by incubation with a P-ERK, P-p38 or with a p-CB1R rat polyclonal antibody. P-ERK was prepared in TBS-T containing 5% milk, while P-p38 was prepared in TBS-T containing 5% BSA. Both were prepared at a concentration of 1:5000. The p-CB1R

antibody was prepared at a concentration of 1:100, in TBS-T containing 5% BSA. All incubations with primary antibodies were done overnight, at 4°C. The membranes were then subsequently washed and probed with an anti-rabbit secondary antibody for 1 hour at 37°C. After another round of washes, the bands were visualized using ECL reagent (Pierce Biotechnology, Rockford, IL) and quantified using the Image J software (National Institute of Health (NIH), Bethesda, MD, USA). The membranes were subsequently stripped and reprobed with a β-actin antibody at a concentration of 1:5000 in TBS-T, containing 5% milk. The bands were then normalized to β-actin.

vi) Statistical analysis

A 2x2 mixed ANOVA was employed to determine if there were any significant differences in the basal p-CB1R levels between SHRs and Wistar rats. This was followed by a Bonferoni T test to determine differences between groups. A two-way ANOVA was employed for testing the effect of treatments on MAPKs and p-CB1R in SHRs as compared to Wistar rats. A Bonferoni T test was employed to determine significant differences between treatments and the respective control. In order to make comparisons between identical time points from different rat models, a two-tailed student t test was employed. All data is expressed as mean± SEM for 5 or more experiments.

4.3 Results

i) Effect of Ang II and ACEA alone on MAPK activation in brainstem astrocytes

An alteration in Ang and endocannabinoid system's activities in brainstem astrocytes from SHR may well be relevant to pathological conditions that have a dysregulated cardiovascular component. Since both Ang II and ACEA are potent activators of MAPKs, the extent of activation of these signaling pathways would presumably be a measure of the

level at which it is functioning. In other words, a lower activation of the MAPK by the agonists may correspond to a dampened effect. In order to investigate this, samples prepared from brainstem astrocytes, earlier treated with either Ang II or ACEA, were subjected to western blotting and probed with antibodies that detect P-ERK or P-p38.

Ang II caused a greater activation of P-ERK and P-p38 at most time points examined in SHRs when compared to Wistar rat samples (Fig 4.1A and 4.1B respectively). Ang II's effect however on P-ERK was greater than on P-p38. This effect of Ang II was mediated by the AT1R in brainstem astrocytes (Appendix 1- Fig 4.1S and 4.2S). Unlike Ang II, ACEA caused a greater activation of P-ERK and P-p38 in Wistar rats when compared to SHR samples (Fig 4.1C and 4.1D, respectively). The effect of ACEA on P-ERK was similar to that of P-p38.

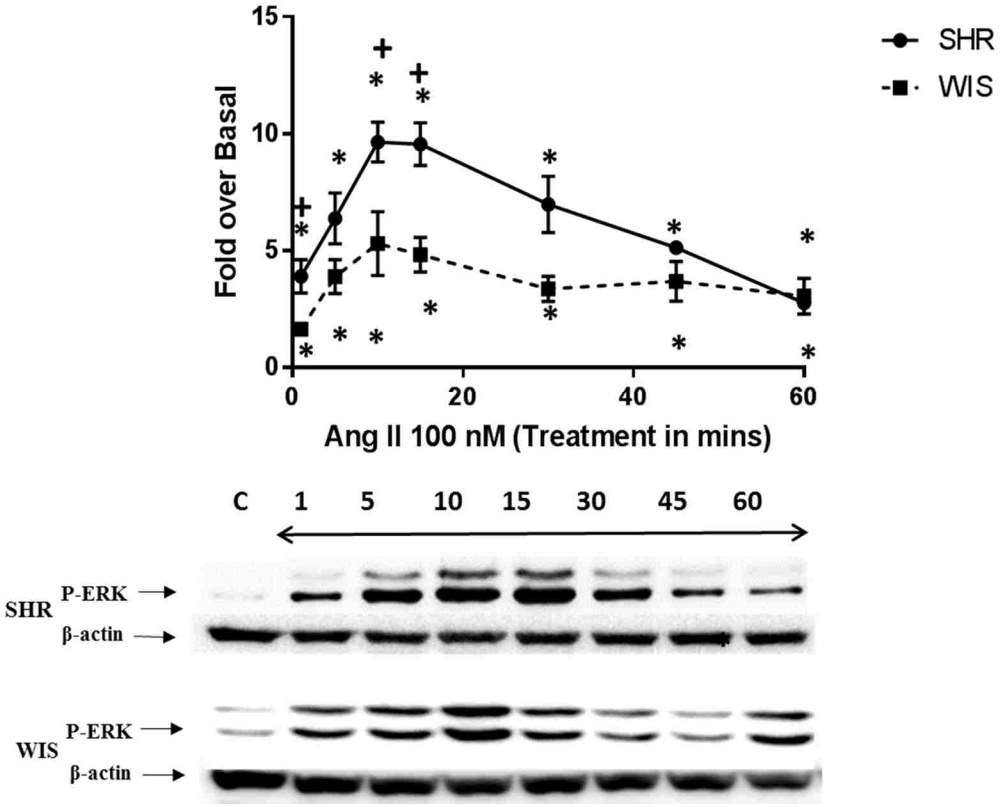

Fig 4.1A: Effect of Ang II on ERK activation in brainstem astrocytes: Brainstem astrocyte samples, pretreated with 100 nM Ang II for varying times, were subjected to western blotting technique and probed with a P-ERK antibody as described. Each value represents the mean ± SEM of 5 or more litters of neonatal rat pups. (* $p < 0.05$- treated versus untreated samples; $^{+}$ $p < 0.05$- SHR versus Wistar samples).

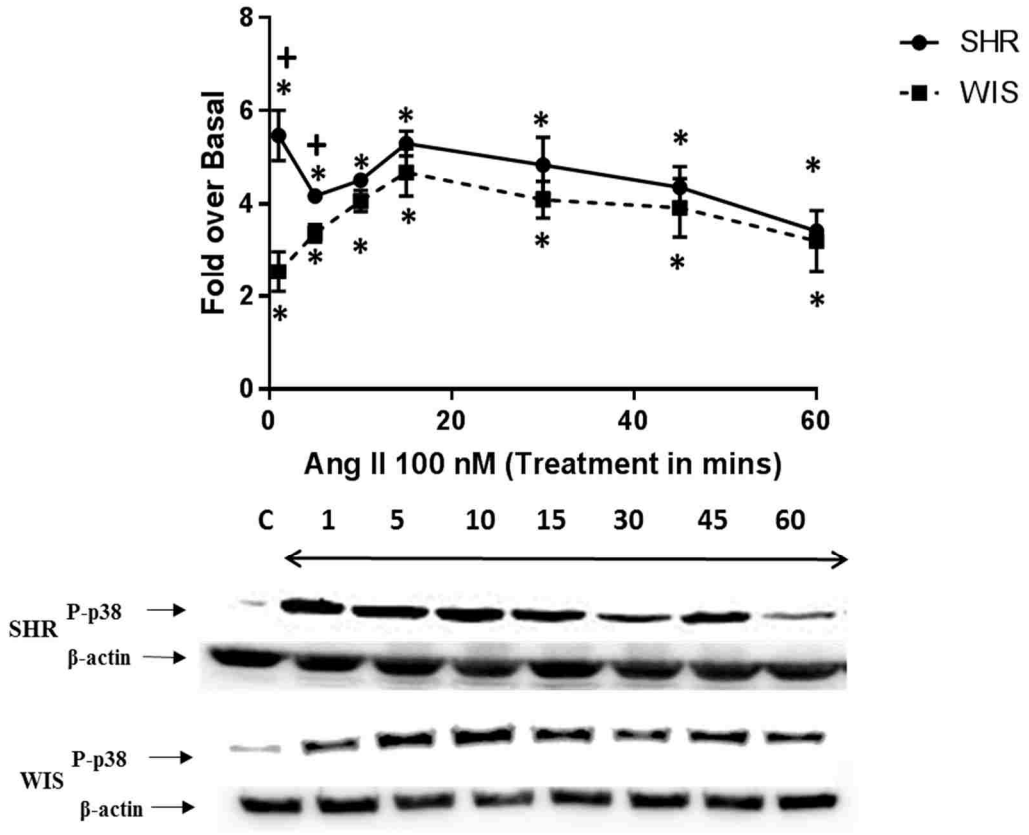

Fig 4.1B: Effect of Ang II on p38 activation in brainstem astrocytes: Brainstem astrocyte samples, pretreated with 100 nM Ang II for varying times, were subjected to western blotting technique and probed with a P-p38 antibody as described. Each value represents the mean ± SEM of 5 or more litters of neonatal rat pups. (* $p < 0.05$- treated versus untreated samples; $^{+}$ $p< 0.05$- SHR versus Wistar samples).

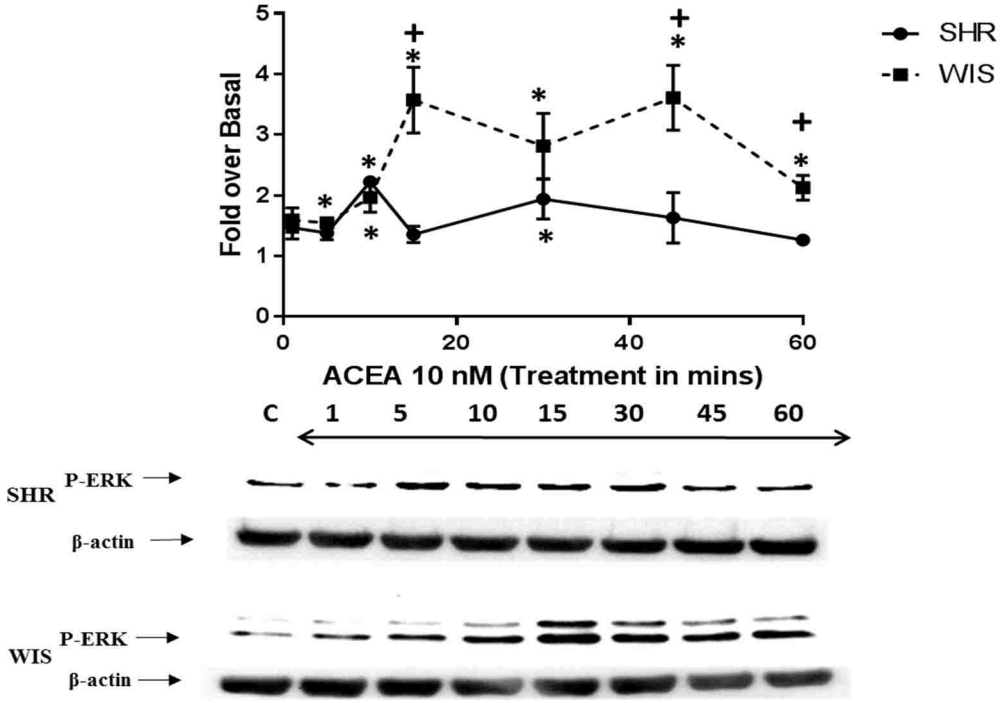

Fig 4.1C: Effect of ACEA on ERK activation in brainstem astrocytes: Brainstem astrocyte samples, pretreated with 10 nM ACEA for varying times, were subjected to western blotting technique and probed with a P-ERK antibody. Each value represents the mean ± SEM of 5 or more litters of neonatal rat pups. (* $p < 0.05$- treated versus untreated samples; $^{+}$ $p < 0.05$- SHR versus Wistar samples).

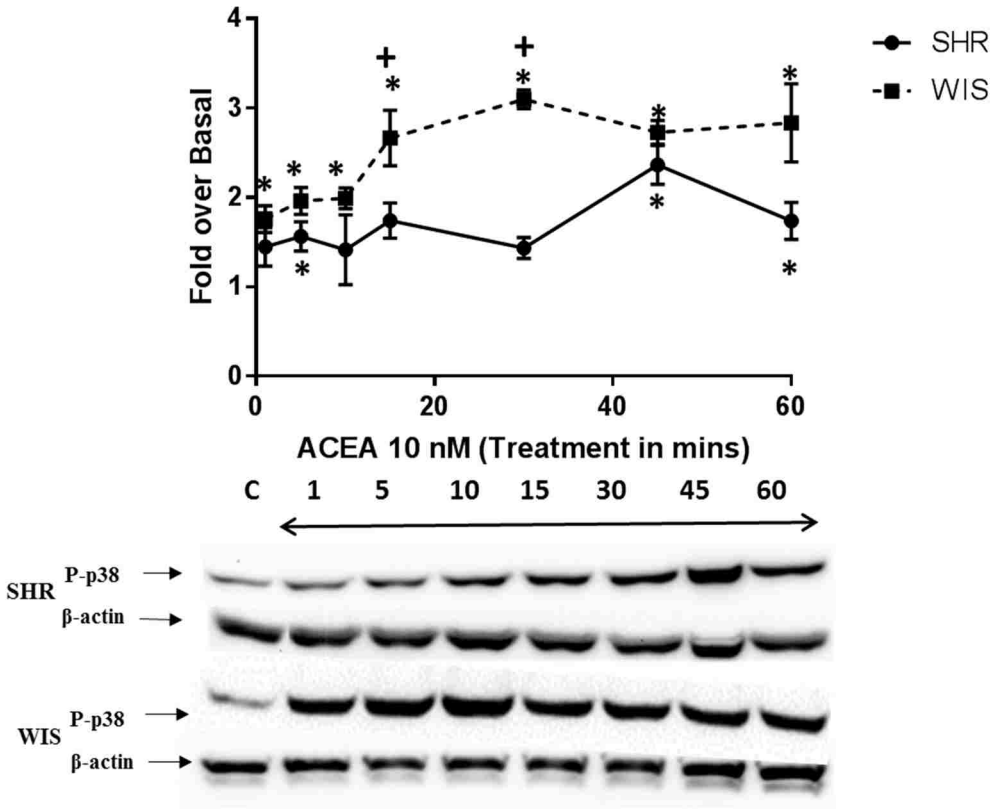

Fig 4.1D: Effect of ACEA on p38 activation in brainstem astrocytes: Brainstem astrocyte samples, pretreated with 10 nM ACEA for varying times, were subjected to western blotting technique and probed with a P-p38 antibody. Each value represents the mean ± SEM of 5 or more litters of neonatal rat pups. (* $p < 0.05$- treated versus untreated samples; $^{+}$ $p < 0.05$- SHR versus Wistar samples).

ii) Effect of Ang II and ACEA in combination on MAPK activation in brainstem astrocytes

Since CB1R agonism/antagonism was demonstrated to significantly alter AT1R-mediated effects, we explored the consequences of co-activation of both receptors in our model system on MAPK activation. In order to explore this, samples prepared from brainstem astrocytes were treated with Ang II and ACEA simultaneously. They were then subjected to western blotting and probed with antibodies that detect P-ERK or P-p38. Comparison of select time points were done between the combination treatment (Ang II+ACEA), with Ang II alone.

Co-treatment with Ang II and ACEA resulted in a slight reduction of MAPK activation in both SHR and Wistar brainstem astrocytes than treatment with Ang II alone (Fig 4.2A-4.2D). The effect was statistically significant for P-ERK, at the higher time points. Although both Ang II and ACEA treatments resulted in MAPK activation, when employed as treatments alone, the peak effect produced by ACEA alone was significantly lower than Ang II. The decrease in MAPK activation observed when employed together, could reflect a CB1R-mediated reduction in AT1R's effect.

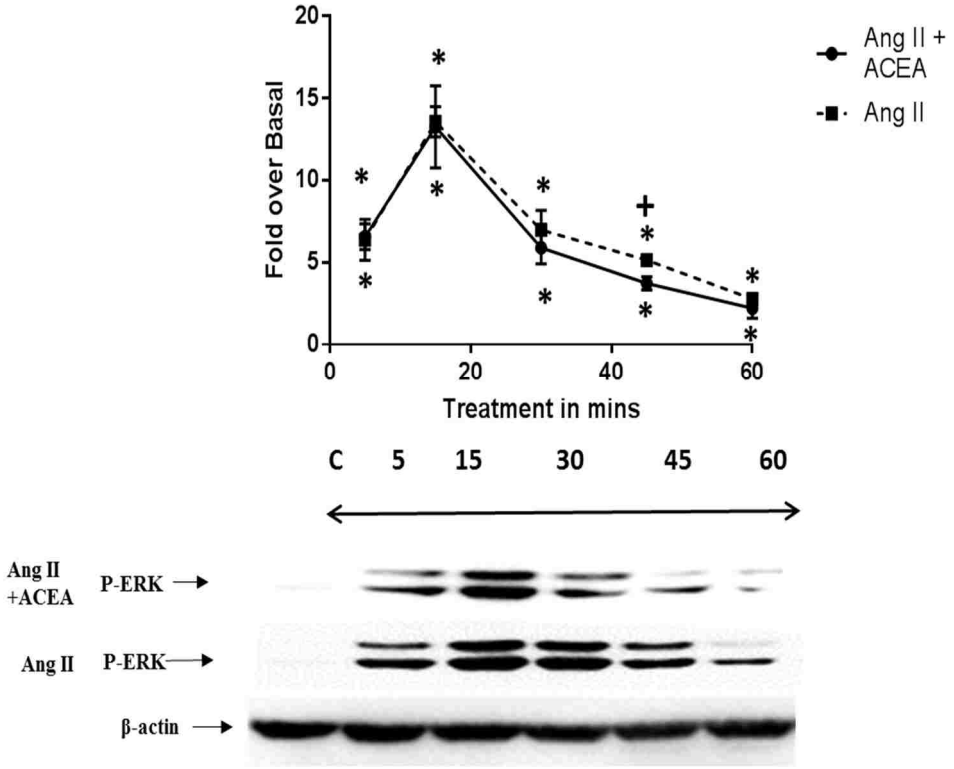

Fig 4.2A: Effect of Ang II and ACEA in combination on ERK activation in SHR brainstem astrocytes: SHR brainstem astrocyte samples, pretreated with 100 nM Ang II alone or in combination with 10 nM ACEA for varying times, were subjected to western blotting technique and probed with a P-ERK antibody. Each value represents the mean ± SEM of 5 or more litters of neonatal rat pups. (* $p < 0.05$- treated versus untreated samples; $^{+}$ $p < 0.05$- Co-treatment versus Ang II alone).

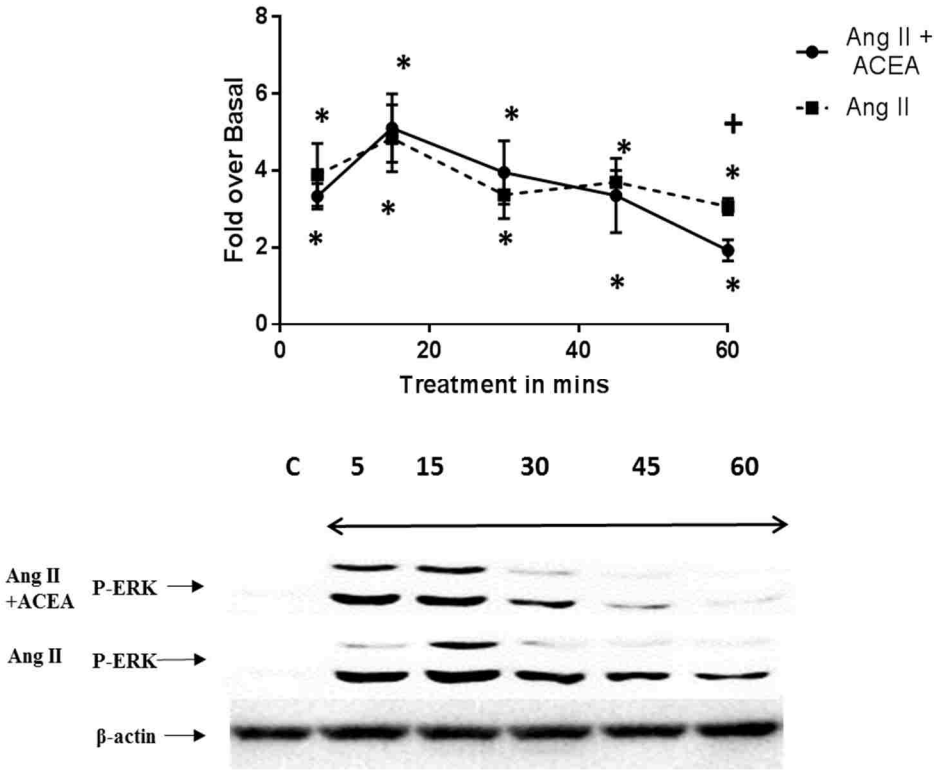

Fig 4.2B: Effect of Ang II and ACEA in combination on ERK activation in Wistar brainstem astrocytes: Wistar brainstem astrocytes, pretreated with 100 nM Ang II alone or in combination with 10 nM ACEA for varying times, were subjected to western blotting technique and probed with a P-ERK antibody. Each value represents the mean ± SEM of 5 or more litters of neonatal rat pups. (*$p < 0.05$- treated versus untreated samples; $^{+}$ $p< 0.05$- Co-treatment versus Ang II alone).

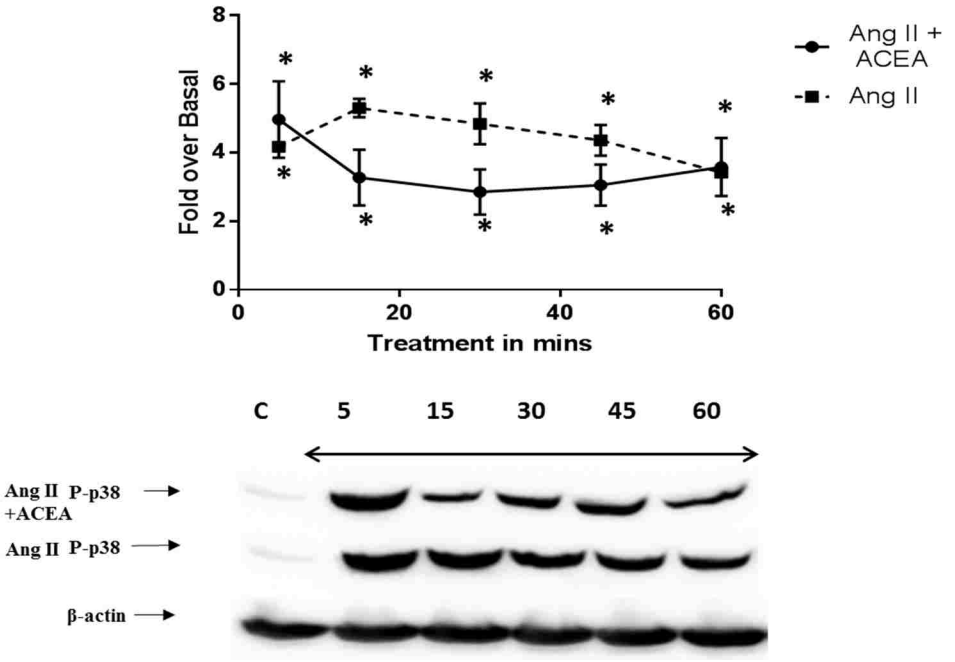

Fig 4.2C: Effect of Ang II and ACEA in combination on p38 activation in SHR brainstem astrocytes: SHR brainstem astrocyte samples pretreated with 100 nM Ang II alone or in combination with 10 nM ACEA for varying times, were subjected to western blotting technique and probed with a P-p38 antibody. Each value represents the mean ± SEM of 5 or more litters of neonatal rat pups. (* $p < 0.05$- treated versus untreated samples).

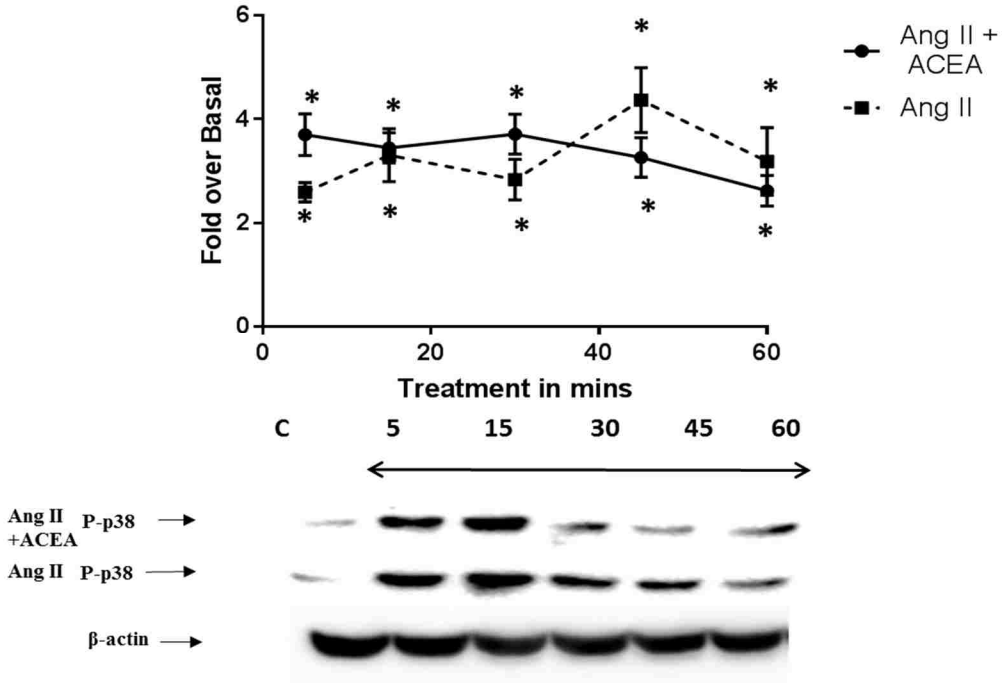

Fig 4.2D: Effect of Ang II and ACEA in combination on p38 activation in Wistar brainstem astrocytes: Wistar brainstem astrocytes (Fig 4.2D), pretreated with 100 nM Ang II alone or in combination with 10 nM ACEA for varying times, were subjected to western blotting technique and probed with a P-p38 antibody. Each value represents the mean ± SEM of 5 or more litters of neonatal rat pups. (* $p < 0.05$- treated versus untreated samples).

iii) Effect of Ang II and ACEA alone on MAPK activation in cerebellar astrocytes

Administration of exogenous cannabinoids was demonstrated to improve symptoms of ADHD in SHRs (Adriani *et al.* 2003). Several studies have demonstrated a link between an impairment in cerebellar functions with symptoms of ADHD (Goetz *et al.* 2014b). Neutralization of prominent cerebellar apoptosis and astrogliosis in SHRs, was also shown to improve symptoms of ADHD in that rat model (Yun *et al.* 2014). Considering that the CB1R has protective effects in astrocytes (Gómez Del Pulgar *et al.* 2002), and the AT1R has deleterious effects (Liu *et al.* 2011a), a comparison of key signaling pathways in these cells would enable us to better understand the consequences of receptor dysregulation (either upregulation or downregulation) in ADHD. Using P-ERK and P-p38 as indicators

of receptor activation, we explored the possibility of an alteration in AT1R and CB1R signaling in cerebellar astrocytes of SHRs.

Interestingly, Ang II caused a greater activation of both P-ERK and P-p38 in Wistar cerebellar astrocytes when compared to SHR cerebellar astrocytes (Fig 4.3A and 4.3B, respectively). This Ang II effect was entirely different from that observed in brainstem astrocytes (Fig 4.1A and 4.1B). The effect of ACEA in cerebellar astrocytes however, did resemble its effect in brainstem astrocytes. The ACEA effect was greater in Wistar astrocytes when compared to SHR astrocytes isolated from the cerebellum (Fig 4.3C and 4.3D). This indicates a possible impairment of both systems under investigation in SHR cerebellar astrocytes when compared to Wistar rat cerebellar astrocytes.

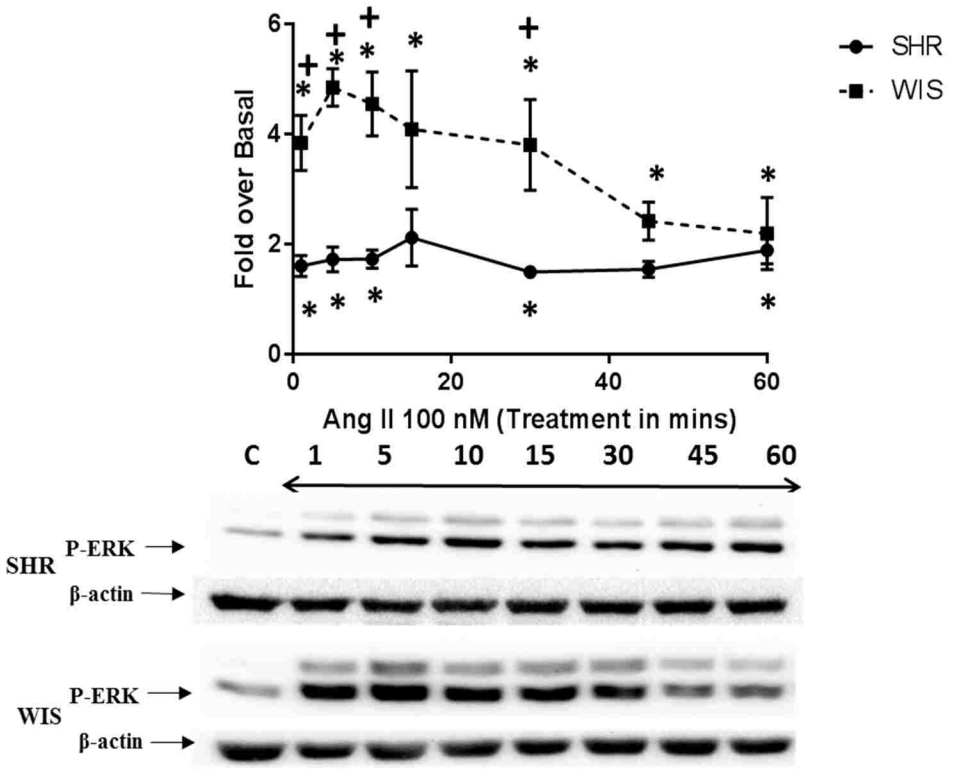

Fig 4.3A: Effect of Ang II on ERK activation in cerebellar astrocytes: Cerebellar astrocyte samples, pretreated with 100 nM Ang II for varying times, were subjected to western blotting technique and probed with a P-ERK antibody as described. Each value represents the mean ± SEM of 5 or more litters of neonatal rat pups. (* $p < 0.05$- treated versus untreated samples; $^{+}$ $p< 0.05$- SHR versus Wistar samples).

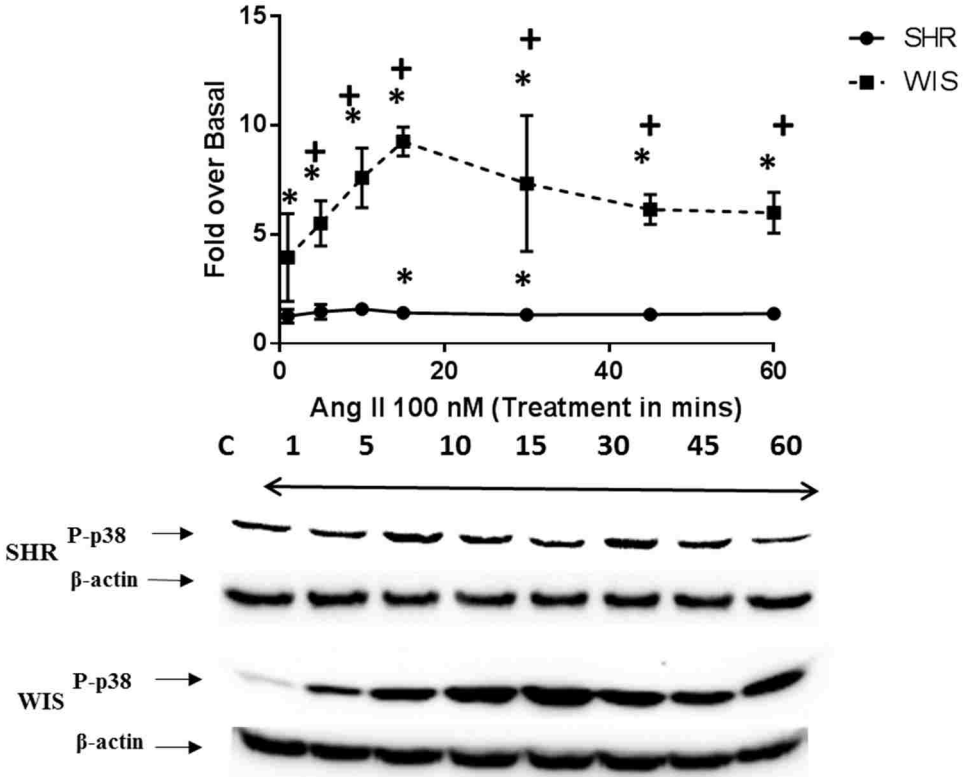

Fig 4.3B: Effect of Ang II on p38 activation in cerebellar astrocytes: Cerebellar astrocyte samples, pretreated with 100 nM Ang II for varying times, were subjected to western blotting technique and probed with a P-p38 antibody as described. Each value represents the mean ± SEM of 5 or more litters of neonatal rat pups. (* $p < 0.05$- treated versus untreated samples; $^{+}$ $p < 0.05$- SHR versus Wistar samples).

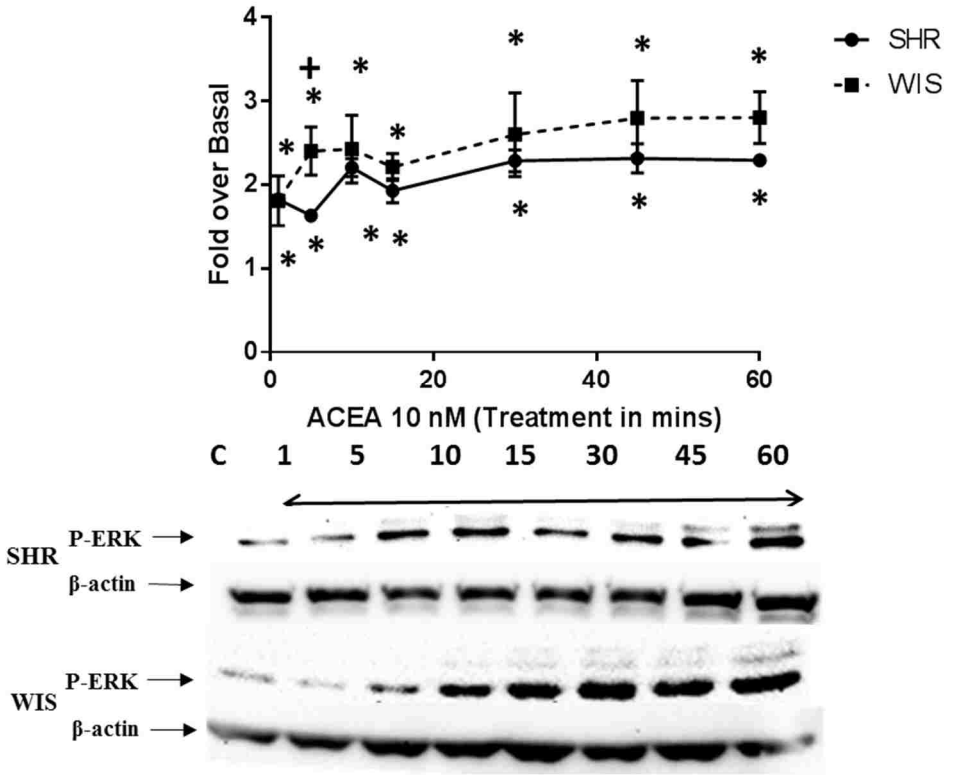

Fig 4.3C: Effect of ACEA on ERK activation in cerebellar astrocytes: Cerebellar astrocyte samples, pretreated with 10 nM ACEA for varying times, were subjected to western blotting technique and probed with a P-ERK antibody. Each value represents the mean ± SEM of 5 or more litters of neonatal rat pups. (* $p < 0.05$- treated versus untreated samples; + $p< 0.05$- SHR versus Wistar samples).

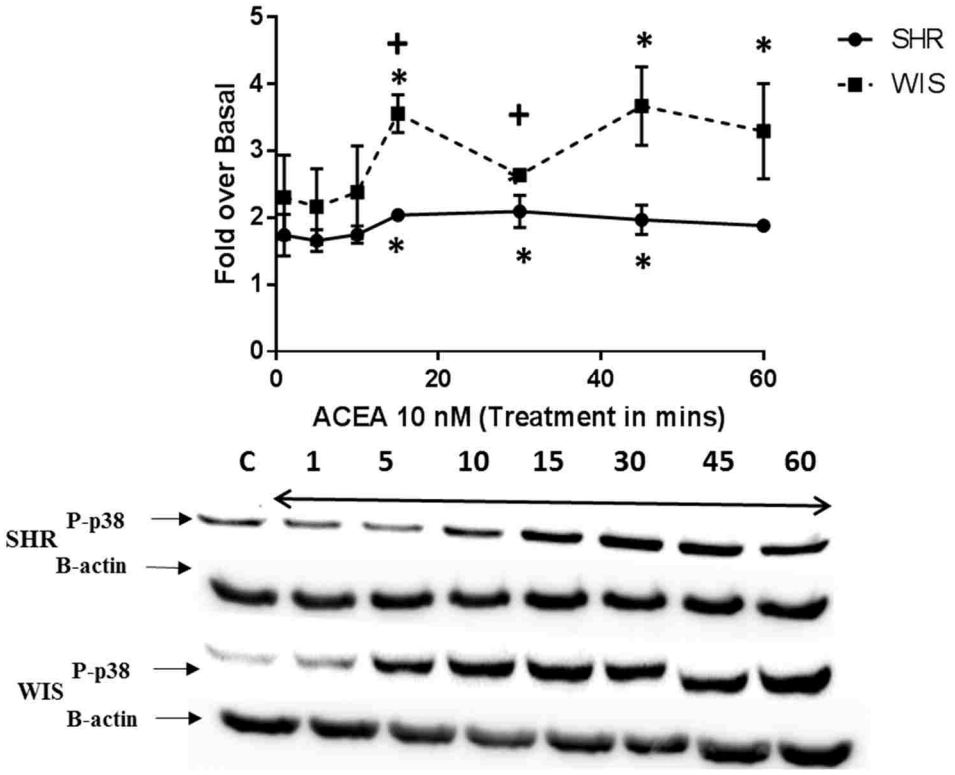

Fig 4.3D: Effect of ACEA on p38 activation in cerebellar astrocytes: Cerebellar astrocyte samples, pretreated with 10 nM ACEA for varying times, were subjected to western blotting technique and probed with P-p38 antibody. Each value represents the mean ± SEM of 5 or more litters of neonatal rat pups. (* $p < 0.05$- treated versus untreated samples; $^{+}$ $p< 0.05$- SHR versus Wistar samples).

iv) Effect of Ang II and ACEA in combination on MAPK activation in cerebellar astrocytes

Unlike brainstem astrocytes, CB1Rs are expressed in relatively higher levels in cerebellar astrocytes (Haspula and Clark 2016b). Low CB1R expression in brainstem astrocytes, could well be a significant factor in obscuring CB1R-mediated alterations of Ang II's activity. We again explored the consequence of co-activation of both the receptors in cerebellar astrocytes of the two models. Of particular interest was Wistar cerebellar astrocytes, where both systems had a considerable effect on MAPK activation.

Co-treatment with Ang II and ACEA, resulted in a significant reduction in P-ERK and P-p38 signal, when compared to Ang II alone (Fig 4.4A-D). While the effect was observed in both rat models, the difference was statistically significant in Wistar cerebellar astrocytes only (Fig 4.4B and 4.4D). ACEA-mediated inhibition of Ang II's effect on MAPK was also observed to be more robust in cerebellar astrocytes when compared to brainstem astrocytes.

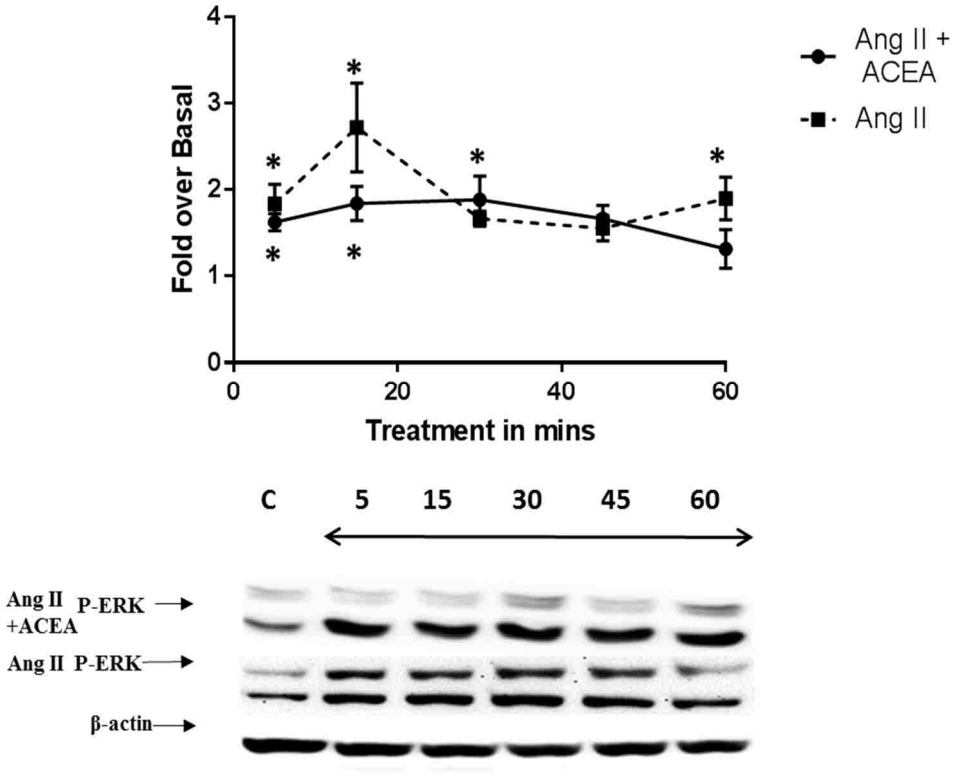

Fig 4.4A: Effect of Ang II and ACEA in combination on ERK activation in SHR cerebellar astrocytes: SHR cerebellar astrocyte cell lysates, pretreated with 100 nM Ang II alone or in combination with 10 nM ACEA for varying times, were subjected to western blotting technique and probed with a P-ERK antibody. Each value represents the mean ± SEM of 5 or more litters of neonatal rat pups. (* $p < 0.05$- treated versus untreated samples).

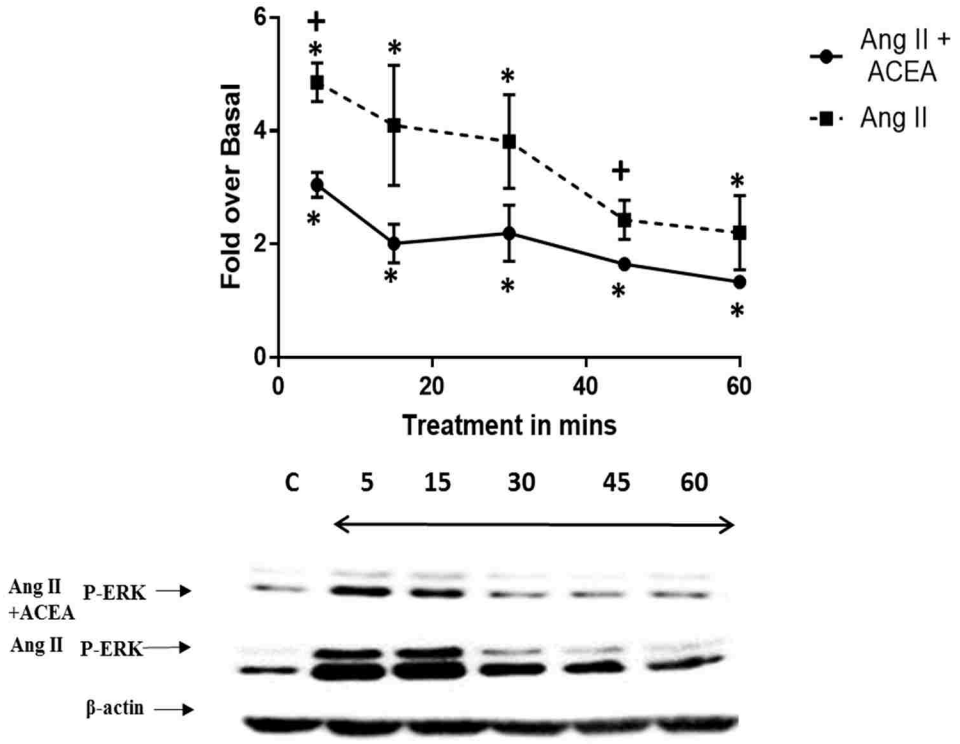

Fig 4.4B: Effect of Ang II and ACEA in combination on ERK activation in Wistar cerebellar astrocytes: Wistar cerebellar astrocyte cell lysates, pretreated with 100 nM Ang II alone or in combination with 10 nM ACEA for varying times, were subjected to western blotting technique and probed with a P-ERK antibody. Each value represents the mean ± SEM of 5 or more litters of neonatal rat pups. (* $p < 0.05$- treated versus untreated samples; $^{+}$ $p < 0.05$- Co-treatment versus Ang II alone).

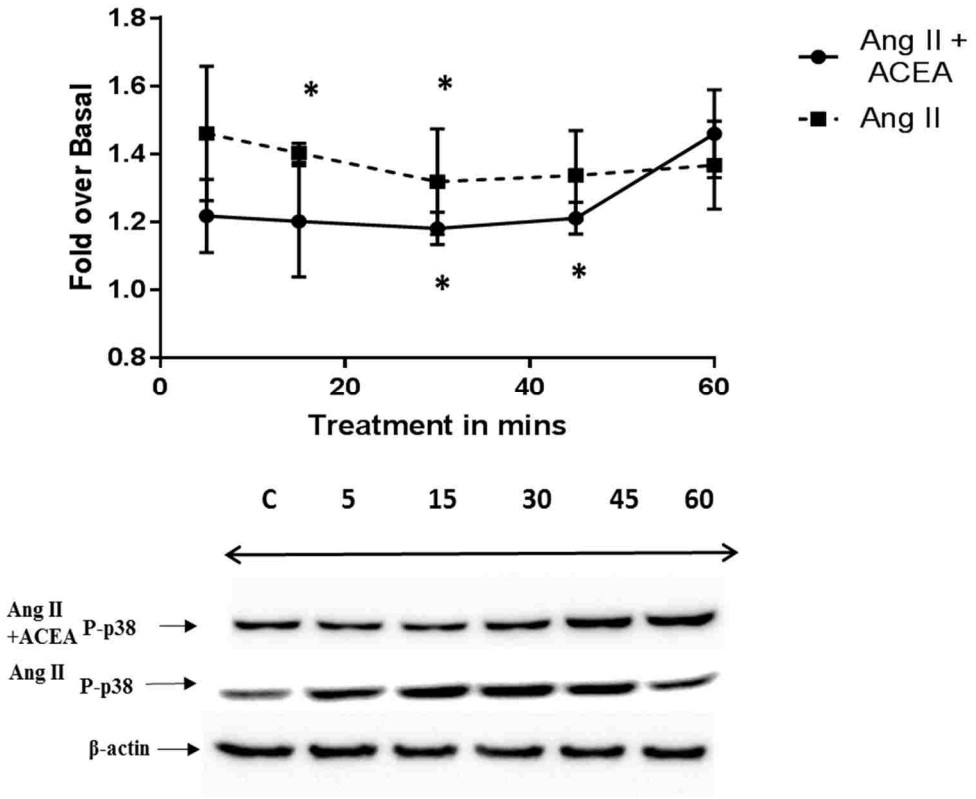

Fig 4.4C: Effect of Ang II and ACEA in combination on p38 activation in SHR cerebellar astrocytes: SHR cerebellar astrocyte cell lysates, pretreated with 100 nM Ang II alone or in combination with 10 nM ACEA for varying times, were subjected to western blotting technique and probed with a P-p38 antibody. Each value represents the mean ± SEM of 5 or more litters of neonatal rat pups. (* $p < 0.05$- treated versus untreated samples).

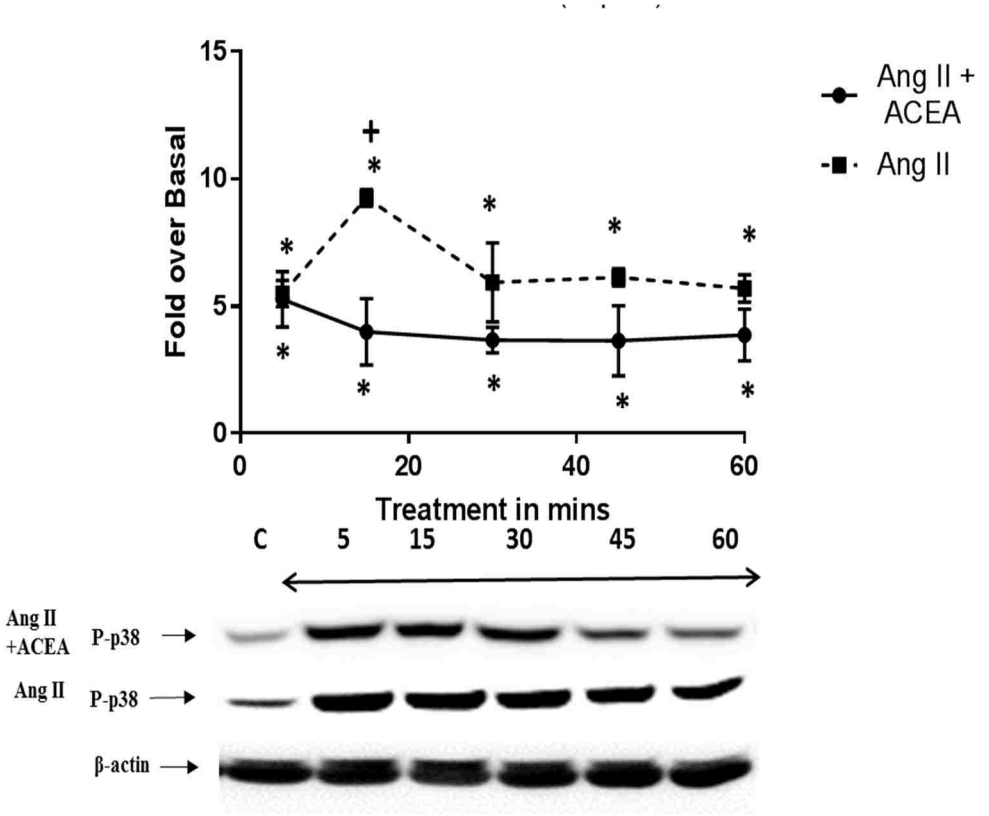

Fig 4.4D: Effect of Ang II and ACEA in combination on p38 activation in cerebellar astrocytes: Wistar cerebellar astrocyte samples, pretreated with either 100 nM Ang II alone or in combination with 10 nM ACEA for varying times, were subjected to western blotting technique and probed with a P-p38 antibody. Each value represents the mean ± SEM of 5 or more litters of neonatal rat pups. (* $p < 0.05$- treated versus untreated samples; $^{+}$ $p< 0.05$- Co-treatment versus Ang II alone).

v) PKC-mediated p-CB1R expression in brainstem and cerebellar astrocytes

Since the phosphorylated form of the CB1R is an indicator of an inactive receptor, we compared the levels of p-CB1R in both brainstem and cerebellar astrocytes of the two rat models. For this experiment, we employed the polyclonal phospho-CB1R antibody (p-CB1R) (Santa Cruz) that detects phosphorylated serine in the third intracellular loop. In order to determine the specificity of the p-CB1R polyclonal antibody employed, we measured p-CB1R levels in samples obtained from astrocytes that were pretreated with increasing concentrations of ACEA, a potent CB1R agonist, thereby triggering

phosphorylation of the receptor by a homologous mechanism. We found that increasing concentrations of ACEA resulted in an increase in p-CB1R levels which is indicated as a single band at ~49 kDa (Appendix 1- Fig 4.3S). This is in agreement with the results obtained from the Anti-CB1R monoclonal antibody (Abcam) where again a single band at ~49 kDa was observed (Appendix 1- Fig 4.4S) (http://www.abcam.com/Cannabinoid-Receptor-I-phospho-S316-antibody-EPR2223N-ab186428/reviews/49381). We observed that the basal p-CB1R levels were significantly higher in cerebellar astrocytes when compared to brainstem astrocytes (Fig 4.5A), which is most likely due to a higher CB1R expression in cerebellar astrocytes when compared to brainstem astrocytes (Haspula and Clark 2016b). When the two models were compared, the levels of p-CB1R were greater in brainstem astrocytes of SHRs than from Wistar rats (Fig 4.5A). The difference between the two models in cerebellar astrocytes however was not statistically significant. An elevation of p-CB1R levels, in SHR brainstem astrocytes could be a possible explanation for a decreased ACEA-mediated MAPK activation, that was observed in our previous experiments.

In order to determine whether PKC is involved in triggering the phosphorylation of CB1R, PMA-treated samples, were analyzed for p-CB1R expression. We observed a significantly higher PMA-induced p-CB1R elevation in cerebellar astrocytes, when compared to brainstem astrocytes (Fig 4.5B). Upon comparison between the two models, the levels of PMA-induced p-CB1R was slightly greater in brainstem astrocytes isolated from SHRs when compared to Wistar rats. However in cerebellar astrocytes, the PMA-induced p-CB1R elevation, was not significantly different between the two models (Fig 4.5B). The above results indicate that PKC-mediated CB1R phosphorylation is more prominent in

astrocytes isolated from the cerebellum when compared to brainstem. The presence of hypertension alters the levels in brainstem astrocytes, but not in cerebellar astrocytes.

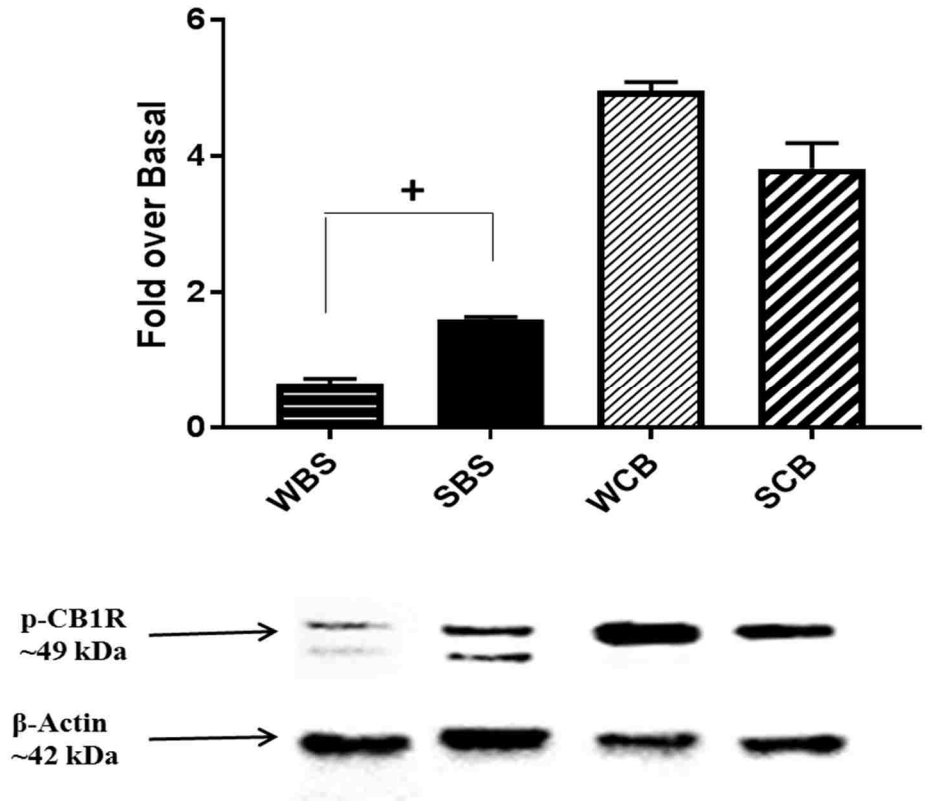

Fig 4.5A: Basal p-CB1R expression in brainstem and cerebellar astrocytes of SHRs and Wistar rats: Basal p-CB1R levels from brainstem and cerebellar astrocytes of Wistar rats were compared with p-CB1R levels of SHRs using western blotting. Lanes 1 and 2 denote Wistar brainstem (WBS) and SHR brainstem astrocyte (SBS) samples, while lanes 3 and 4 denotes Wistar cerebellar (WCB) and SHR cerebellar astrocyte (SCB) samples, respectively. Each value represents the mean ± SEM of 5 or more litters of neonatal rat pups. ($^{+}$ $p < 0.05$- SHR versus Wistar samples).

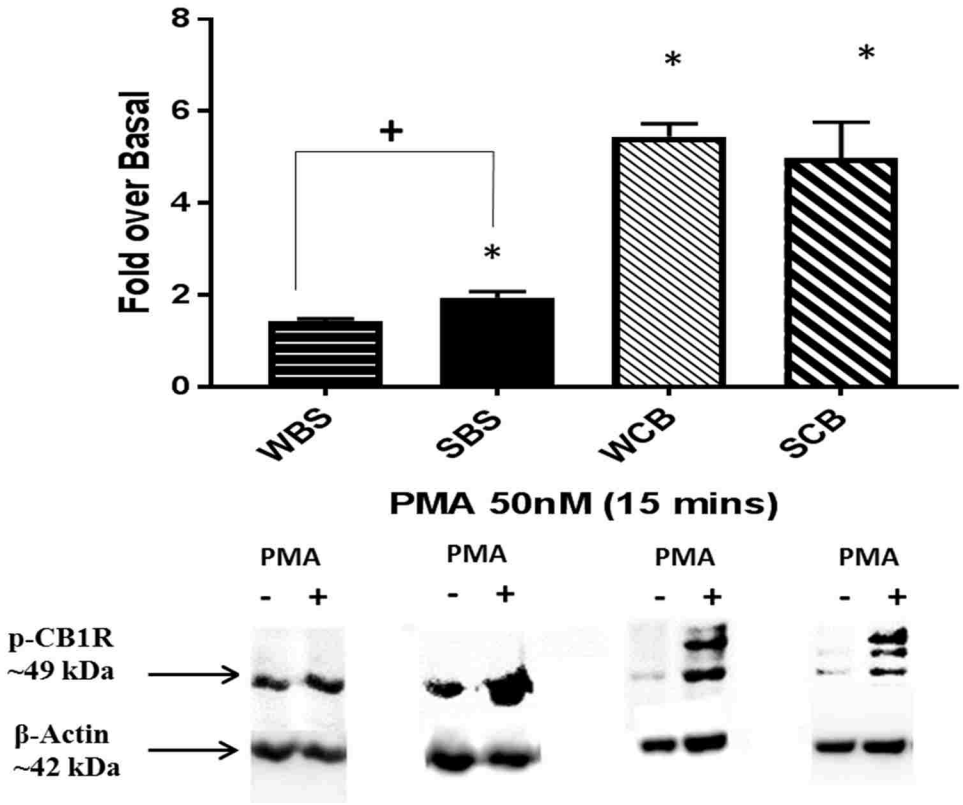

Fig 4.5B: PMA-induced p-CB1R expression in brainstem and cerebellar astrocytes: p-CB1R levels from untreated astrocytes were compared from astrocytes that were treated with 50 nM PMA for 15 mins. The four bars represent the elevation of p-CB1R levels in PMA-treated samples over that of the untreated samples, and the second lane denotes treated samples. The first lane in each of the 4 representative blots denotes untreated samples. The four representative blots from left to right are astrocyte samples from Wistar brainstem (WBS), SHR brainstem (SBS), Wistar cerebellum (WCB) and SHR cerebellum (SCB). Each value represents the mean ± SEM of 5 or more litters of neonatal rat pups. (* $p < 0.05$- treated versus untreated samples; $^{+}$ $p < 0.05$- SHR versus Wistar samples).

vi) Effect of Ang II on p-CB1R expression in brainstem and cerebellar astrocytes

Our earlier experiments were designed to investigate the effects of ACEA on modulating AT1R-induced MAPK activation. Unidirectional crosstalk from AT1R to CB1R has been shown to enhance the endocannabinoid tone by CB1R transactivation (Turu *et al.* 2009). However, possible inactivation of CB1R by Ang II, has not been investigated. Whether Ang II can induce phosphorylation of CB1R in both brainstem and cerebellar astrocytes, was the primary focus of this experiment. The peak effect produced by Ang II on p-CB1R

levels in cerebellar astrocytes was observed to be much greater than that produced in brainstem astrocytes (Fig 4.6A and 4.6B). However, a comparison between the two models for the same brain region indicated no significant differences in the peak effect produced by Ang II on p-CB1R levels (Fig 4.6A and 4.6B). Regarding temporal activation, Ang II initiated the phosphorylation event much earlier in SHR cerebellar astrocytes than in Wistar cerebellar astrocytes (Fig 4.6A).

The Ang receptor involved in Ang II-mediated phosphorylation of the CB1R, was also investigated. As observed in Fig 4.6C, Ang II elicited this response by activation of the AT1R in Wistar cerebellar astrocytes. Losartan, the AT1R antagonist, prevented Ang II-mediated elevation of p-CB1R levels. On the other hand, the AT2R antagonist, PD123319, was unsuccessful in preventing Ang II-mediated phosphorylation of the CB1R. Treatment with inhibitors alone had no significant effect on p-CB1R expression (Fig 4.6C).

Phosphorylation of the CB1R can be mediated via PKC or DAGL. Gq GPCRs were demonstrated to transactivate CB1R via activation of DAGL (Turu *et al.* 2009). Hence, phosphorylation of CB1R could well be the consequence of CB1R transactivation by AT1R. We employed inhibitors for PKC (BIM I) and DAGL (Orlistat) to determine the most prominent mechanism among the two. Since the effect of Ang II and PMA on p-CB1R levels was diminished in brainstem astrocytes of both models, when compared to cerebellar astrocytes, we employed cerebellar astrocytes from Wistar rats to investigate the mechanisms involved in Ang II-mediated phosphorylation of CB1R. As observed in Fig 4.6D, PKC was exclusively contributing to Ang II-mediated CB1R phosphorylation in cerebellar astrocytes of Wistar rats, suggesting that this effect is not a consequence of receptor transactivation, but is through a desensitization mechanism.

A complete list of inhibitor data is provided in the supplementary section (tables 1S and 2S). PKC was the most dominant mechanism in SHR cerebellar astrocytes as well. Although PKC is again a key mediator of Ang II-induced phosphorylation of CB1R in brainstem astrocytes, we also observed DAGL involvement in Ang II-mediated phosphorylation of CB1R in the case of Wistar brainstem astrocytes (Appendix 2- Table 1S and 2S).

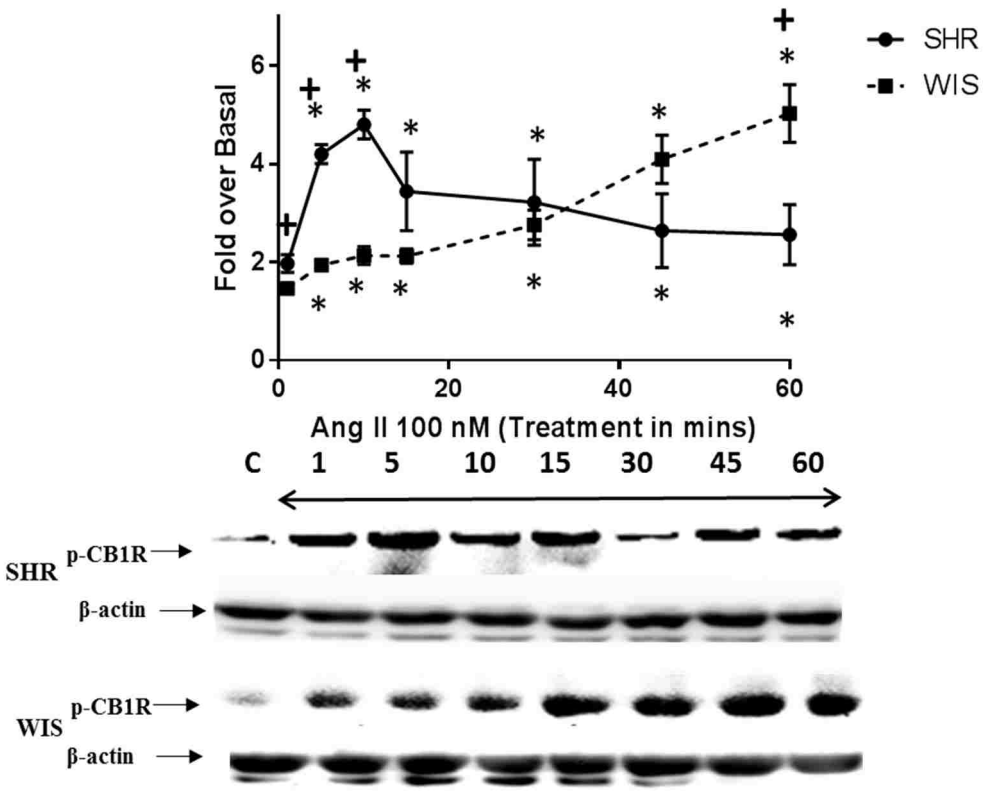

Fig 4.6A: Effect of Ang II on p-CB1R expression in cerebellar astrocytes: Wistar and SHR cerebellar astrocytes were treated with 100 nM Ang II for times ranging from 1 min to 60 mins. p-CB1R levels from treated samples were then compared with the untreated sample. Each value represents the mean ± SEM of 5 or more litters of neonatal rat pups. (* $p < 0.05$- treated versus untreated samples; + $p < 0.05$- SHR versus Wistar samples).

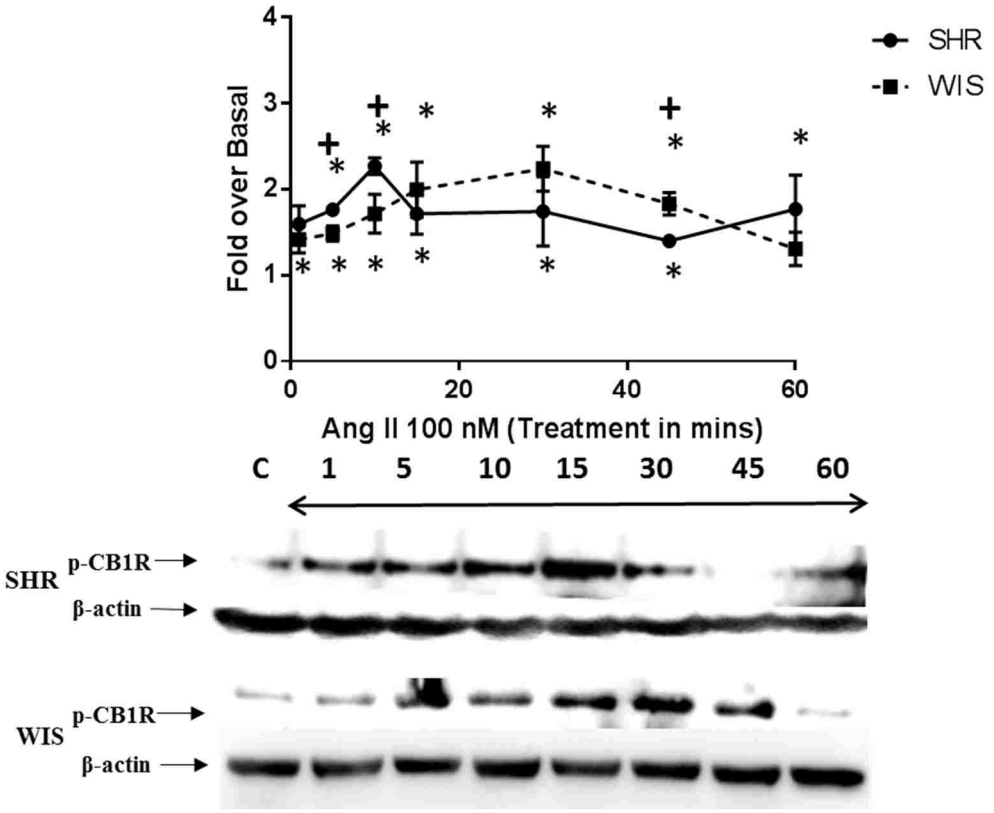

Fig 4.6B: Effect of Ang II on p-CB1R expression in brainstem astrocytes: Wistar and SHR brainstem astrocytes were treated with 100 nM Ang II for times ranging from 1 min to 60 mins. p-CB1R levels from treated samples were then compared with the untreated sample. Each value represents the mean ± SEM of 5 or more litters of neonatal rat pups. (* $p < 0.05$- treated versus untreated samples; + $p < 0.05$- SHR versus Wistar samples).

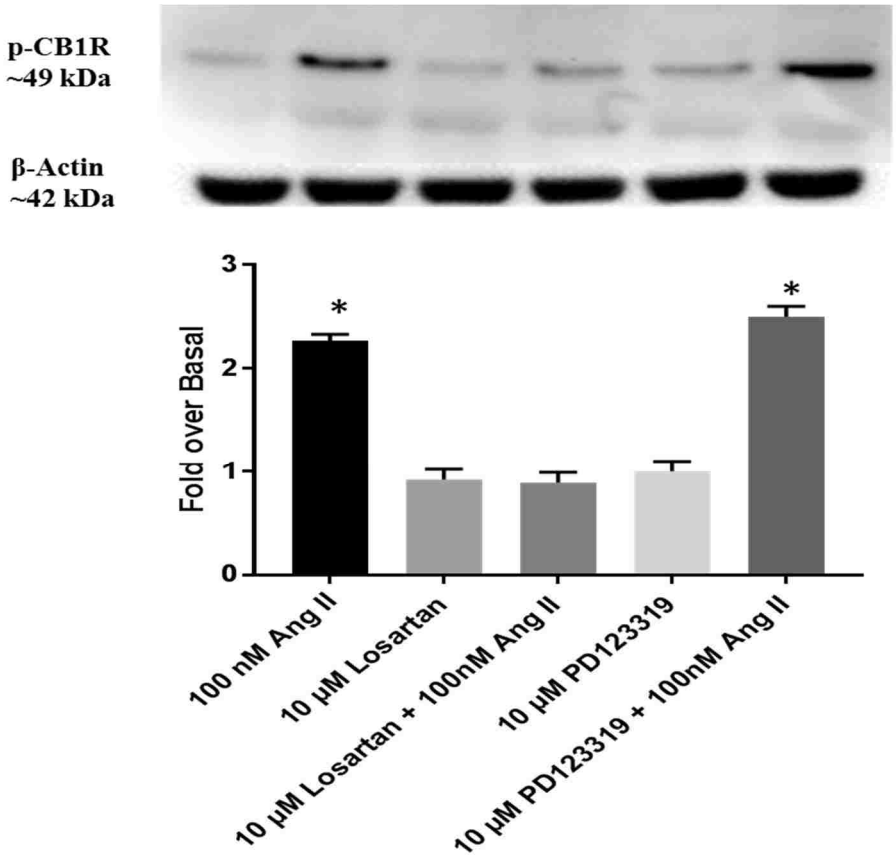

Fig 4.6C: Effect of Ang II with or without Ang receptor inhibitors on p-CB1R expression in cerebellar astrocytes: Wistar cerebellar astrocyte samples, treated with Ang II with or without 10 µM Losartan (AT1R inhibitor) or 10 µM PD123319 (AT_2R inhibitor), represent lanes 2-6. Lane 1 is the untreated sample and lane 2 is sample that was treated with 100 nM Ang II alone. Each value represents the mean ± SEM of 5 or more litters of neonatal rat pups. (* $p < 0.05$- treated versus untreated samples).

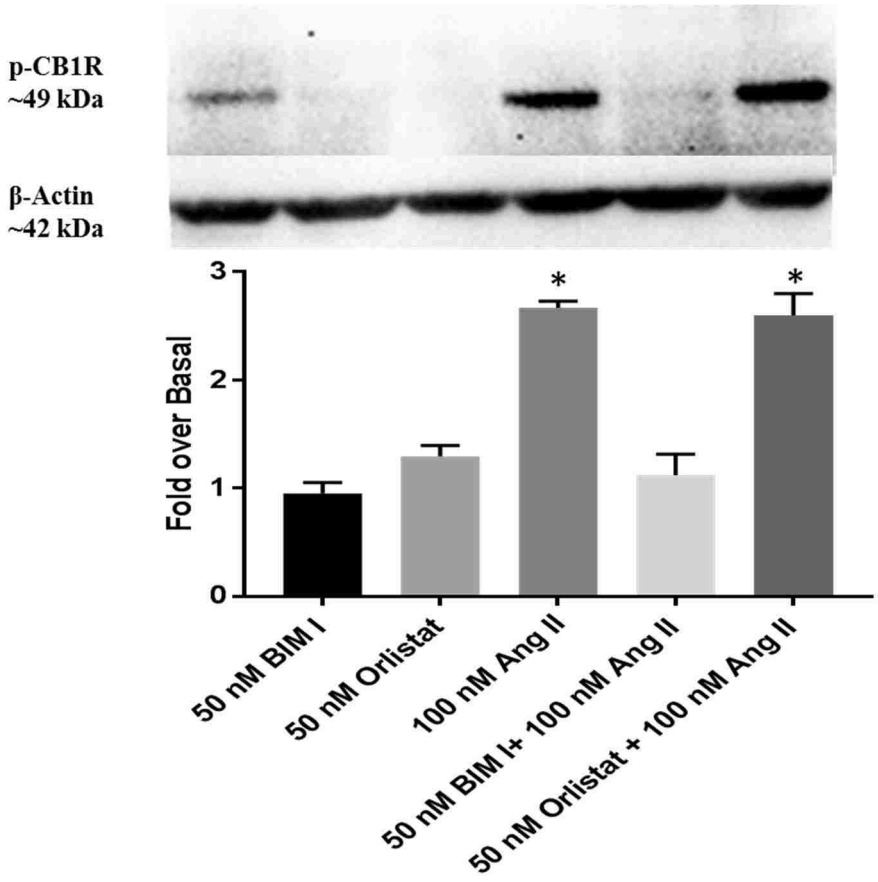

Fig 4.6D: Effect of Ang II with or without inhibitors for PKC and DAGL on p-CB1R expression in cerebellar astrocytes: Wistar cerebellar astrocyte samples treated with either 50 nM BIM I (PKC inhibitor) and 50 nM orlistat (DAGL inhibitor) are loaded in lanes 2 and 3 respectively. Lane 4 is sample that is treated with 100 nM Ang II alone, while lanes 5 and 6 are inhibitors with 100 nM Ang II. Lane 1 is the untreated sample. Each value represents the mean ± SEM of 5 or more litters of neonatal rat pups. (* $p < 0.05$- treated versus untreated samples).

4.4 Discussion

Although several findings were reported in this study, the most significant ones are related to CB1R dysfunction in SHR astrocytes, and phosphorylation of CB1R via the AT1R-PKC axis in cerebellar astrocytes of both models. Reduced ability of ACEA to activate MAPK, coupled with an increase in basal levels of p-CB1R, in SHR brainstem when compared to Wistar brainstem astrocytes, is suggestive of a potential dampening of

CB1Rs effects under hypertensive conditions. Regarding receptor crosstalk, we have identified heterologous phosphorylation of CB1R via PKC as a candidate mechanism for Ang II to phosphorylate, and potentially inactivate the CB1R. This form of crosstalk between AT1R and CB1R is more prevalent in cerebellar than brainstem astrocytes of both models.

Treatment with either Ang II or ACEA resulted in contrasting effects on MAPK activation in brainstem astrocytes isolated from SHRs when compared to Wistar rats. While Ang II-mediated activation of MAPKs was greater in brainstem astrocytes isolated from SHRs when compared to Wistar rats, ACEA effect on MAPKs activation was more prominent in astrocytes isolated from normotensive rats. Several studies have reported an augmentation of RAS activity in SHR brain cells such as RVLM neurons when compared to their normotensive controls (Matsuura *et al.* 2002). More robust activation of MAPKs by Ang II in SHR brainstem astrocytes, when compared to Wistar brainstem astrocytes, could be an indicator of an overactive AT1R in astrocytes. Ang II, a potent mitogen, is capable of causing prominent ERK activation, the latter being a key signaling pathway in cell proliferation (Clark *et al.* 2013). Regarding the endocannabinoid system, our laboratory and others have reported a reduction in the levels of CB1R in SHRs when compared to their normotensive controls (Adriani *et al.* 2003) (Brozoski *et al.* 2009) (Haspula and Clark 2016b). In the current study, we observed an elevation in the levels of p-CB1R in SHR brainstem astrocytes when compared to Wistar brainstem astrocytes. In addition, we also observed a reduction in MAPK activation by ACEA in brainstem astrocytes of SHRs, when compared to Wistar rats. Both findings are indicative of a dampened astroglial endocannabinoid system in brainstem under hypertensive conditions. Hypofunctional

CB1R has also been reported by others in SHRs (Adriani *et al.* 2003) (Brozoski *et al.* 2009) . It could well be that a reduction of protective systems such as the endocannabinoid system, and a potentiation of pro-hypertensive systems such as the RAS, could be critical in the etiology of hypertension.

In contrast to brainstem astrocytes, Ang II treatment did not elicit stronger MAPK activation in SHR cerebellar astrocytes, when compared to Wistar rat cerebellar astrocytes. Interestingly, a reduction in cerebellar volume is observed in children with ADHD (Bledsoe *et al.* 2011). Ang II is capable of triggering significant ERK activation under normal physiological conditions in cerebellar astrocytes (Clark *et al.* 2013). But an impairment in SHR cerebellar astrocytes, suggests a possible disruption in the gliogenic machinery in SHR cerebellum. ERK is a key player in gliogenesis, the latter a prominent feature of CNS development in postnatal stages (Li *et al.* 2012a). The same findings were also observed for p38. This MAPK pathway is a key signal transduction pathway involved in regulating inflammatory responses from glial cells (Guo *et al.* 2010). Previously, we observed that Ang II had a greater effect on elevating secreted IL-6 levels in cerebellar astrocytes isolated from Wistar rats when compared to SHRs (Gowrisankar and Clark 2016c). As activation of p38 and ERK is purely an AT1R-mediated effect, AT1R expression or functionality could be reduced in cerebellar astrocytes of SHRs when compared to Wistar rats. Unpublished data from our laboratory indicates a significant reduction in the levels of AT1R mRNA in SHR cerebellar astrocytes, which supports our current findings. Similar to brainstem astrocytes, ACEA had a slightly reduced effect on MAPKs activation in SHR cerebellar astrocytes when compared to Wistar cerebellar astrocytes. This suggests a possible dampening of the endocannabinoid system functions

in cerebellar astrocytes of SHRs when compared to Wistar rats. We have previously reported that the CB1R expression levels in cerebellar astrocytes in SHRs, was similar to that of Wistar rats (Haspula and Clark 2016b). In the current study, p-CB1R levels were similar in both models. Therefore, there may be other factors at play that we have not accounted for in this study.

Results from our co-treatment experiments indicate that ACEA was able to decrease Ang II-mediated activation of MAPKs in both cerebellar and brainstem astrocytes. The effect however was much more prominent in cerebellar astrocytes. AT1R and CB1R couple to Gq and Gi G proteins, respectively. Crosstalk between Gi and Gq GPCRs have been demonstrated to potentiate Gq-mediated downstream effects (Rozenfeld *et al.* 2011) (Carroll *et al.* 1995) (Rives *et al.* 2009). Interestingly, co-stimulation of GPCRs, that are both capable of MAPK activation, have been reported to cancel out excessive MAPK activation by inactivating one of the receptor's signaling pathway (Hanke *et al.* 2001). Since CB1R activation results in a reduction of AT1R-mediated MAPK activation in both cerebellar and brainstem cells, we can conclude that CB1R agonists can offset AT1R-mediated deleterious effects which are mediated by MAPK activation. Owing to CB1Rs neuroprotective role, the receptor is known to have important homeostatic regulatory functions in astrocytes (Carracedo *et al.* 2004). The CB1R could well be keeping a check on incessant MAPK activation that may come about by prolonged agonist treatment.

In addition to identifying astroglial CB1R impairment in SHR brains, we have identified a new mode of crosstalk between AT1R and CB1R, that is CB1R phosphorylation by Ang II. Heterologous desensitization of CB1R may also serve as a new paradigm for Gq GPCR-mediated inactivation of CB1R. Activation of Gs-coupled GPCRs such as Adenosine

receptors, have been demonstrated to trigger inactivation of CB1R in presynaptic neurons (Ferreira *et al.* 2015). However, activation of Gq GPCRs has been shown to transactivate CB1R (Turu *et al.* 2009). Whether the balance between inactivation or transactivation of the CB1R is altered under certain pathological conditions and in certain brain regions, needs further investigation. Owing to relatively low CB1R expression in brainstem, the extent of Ang II-mediated CB1R phosphorylation was greater in cerebellar astrocytes when compared to brainstem astrocytes. The consequence of such an inactivation mechanism could be relevant to cerebellar impairments that are characterized by excessive RAS activation. It is well known that counteracting the deleterious effects of RAS activation in the brain is a valuable therapeutic strategy for not only cardiovascular diseases, but also for neurological disorders (Gao *et al.* 2013). This could well be a mechanism by which an overactive RAS, that is observed in hypertensive conditions, is able to dampen protective regulatory mechanisms such as the endocannabinoid system in regions of the brain that are not linked to blood pressure regulation. Disturbances in normal physiological processes, such as repeated stress, have also been shown to elevate phosphorylated forms of the CB1R in rat cerebellum, but not in rat brainstem (Xing *et al.* 2011). Since cerebellum has higher CB1R expression than the brainstem (Moldrich and Wenger 2000) (Haspula and Clark 2016b), AT1R-mediated phosphorylation of CB1R could well be a prominent endocannabinoid inactivation mechanism in regions of the brains with high CB1R expression. Whether this or other inactivation mechanisms are prevalent in those brain regions where CB1R expression is greater, remains to be investigated.

The findings from this study highlight the potential interplay between AT1Rs and CB1Rs in astrocytes under normal and pathological conditions. Not only can CB1R activation lead

to inhibition of AT1R-mediated MAPK activation, but Ang II can also induce significant phosphorylation of CB1R in cerebellar astrocytes, and consequentially trigger potential inactivation of CB1R. However, the functional consequences of these interactions need to be evaluated in pathological conditions such as hypertension, and neurological impairments such as ADHD.

Chapter 5

Regulation of Neuroinflammatory Cytokines by Angiotensin II and ACEA in SHR Astrocytes

Abstract

Background: An imbalance in the levels of pro- and anti-inflammatory cytokines has been reported in the brains of SHRs. Neuroinflammation in the cardioregulatory regions of brainstem and hypothalamus has been theorized to be a major contributor to the development of hypertension. Although cannabinoids exert potent neuroprotective and anti-inflammatory effects, their role in regulation of cytokines in hypertension has not been investigated. Methods: Astrocytes were isolated from the brainstem and cerebellum of Wistar rats and SHRs, a model of hypertension and ADHD. Astrocytes were then treated with 100 nM Ang II or 10 nM ACEA, both alone and in combination, for 12 and 24 hours. Following treatments, IL-1β and IL-10 mRNA and secreted protein levels, were measured using qPCR and ELISA, respectively. Results: Both IL-1β and IL-10 levels were significantly higher in brainstem astrocytes, but not cerebellar astrocytes, of SHRs when compared to Wistar rats. The ability of Ang II and ACEA to alter IL-10, and not IL-1β levels, were significantly different in brainstem astrocytes of SHRs when compared to Wistar rats. Neither treatments had potent effects on altering the secreted cytokine fractions. The effect of ACEA on IL-10 mRNA levels was greater in brainstem and cerebellar astrocytes of Wistar rats when compared to SHRs. This is indicative of potential hypofunction of CB1Rs in SHR brainstem astrocytes. Co-treatment with Ang II and ACEA

resulted in a greater neutralization of Ang II-mediated increases of IL-10 protein and mRNA levels in brainstem astrocytes of SHRs when compared to Wistar rats. Conclusion: Differences in basal cytokine levels are indicative of a dysregulated neuroinflammatory state in brainstem astrocytes of SHRs when compared to Wistar rats. Both astroglial angiotensin and endocannabinoid systems had seemingly opposing roles in regulating neuroinflammatory cytokines in these cells. The inability of ACEA to elicit prominent anti-inflammatory effects in SHR astrocytes is indicative of a potential CB_1R hypofunction. Impairment of neuroprotective systems and an exaggerated neuroinflammatory response in early stages of hypertension, could well be major contributors to the pathogenesis of hypertension.

5.1 Introduction

SHR is a widely employed model for studying essential hypertension (Veerasingham and Raizada 2003). Several parallels can be drawn between development of hypertension in humans with SHRs. During early stages of hypertension, SHRs are characterized by an augmented sympathetic response - a characteristic also seen in borderline hypertensives (Julius *et al.* 1991) (Mancia and Grassi 2014). In fact autonomic dysfunction, observed at early stages of hypertension, has been implicated to have a causal role in the development of essential hypertension (Mancia *et al.* 1999). Moreover, inflammation in the cardiovascular centers of the brain, is deemed to be a major contributor to an augmented sympathetic drive observed in hypertensive conditions (Winklewski *et al.* 2015). Neuroinflammation is not only a hallmark of several neurological disorders, but is also a feature of cardiovascular diseases (Shi *et al.* 2010b) (Waki and Gouraud 2014). An imbalance in the levels of pro- and anti-inflammatory cytokines, was observed in the brains

of SHRs when compared to their normotensive controls (Agarwal *et al.* 2011) (Waki *et al.* 2008a). Direct injection of IL-1β into the PVN, a cardiovascular center in the hypothalamus, resulted in an increase in renal sympathetic nerve activity and mean arterial pressure, which is strong evidence of causality between an elevation in pro-inflammatory cytokines in the brain and hypertension (Shi *et al.* 2011).

An overactive RAS has been reported in SHR brains (Veerasingham and Raizada 2003). Ang II, the primary effector peptide of the RAS, is able to elicit prominent pro-inflammatory (Shi *et al.* 2010a), and pro-oxidant effects (Liu *et al.* 2011a) in the brain via activation of the AT1R. Ang II has been demonstrated to alter the balance of inflammatory cytokines in the PVN, by shifting it towards a pro-inflammatory state, via activation of the microglial AT1R (Shi *et al.* 2010a). This shift has been shown to be a major mechanism by which Ang II elevates sympathetic activity. But a neuroinflammatory state is also observed in the cardiovascular centers in brainstems of young prehypertensive and adult SHRs (Waki *et al.* 2008a) (Paton and Waki 2009). Interestingly in the brainstem, both neuronal and astroglial AT1Rs are key players in the regulation of sympathetic outflow from the brain. Ablation of the astroglial AT1R in the brainstem has been demonstrated to cause a decrease in sympathetic activity in a heart failure model, further highlighting astroglial AT1R as a necessary component in the elevation of sympathetic activity (Isegawa *et al.* 2014a). It is well known that astrocytes along with microglia are important in regulating neuroinflammatory states in the brain in several neurological disorders. Considering that Ang II has pro-hypertensive effects via the activation of brainstem astroglial AT1Rs, it becomes necessary to investigate the possible role of neuroinflammatory cytokines in the development of hypertension.

Interestingly, the presence of prominent astrogliosis (Tomassoni *et al.* 2004) and a neuroinflammatory state (Agarwal *et al.* 2011) in SHRs, makes it an ideal model to investigate neurological disorders. Apart from hypertension, SHRs are also a validated model of ADHD (Adriani *et al.* 2003). Several personality traits that are present in ADHD individuals such as hyperreactivity to external stimuli, shorter attention spans and a predisposition towards impulsive behavior, are also observed in SHRs (Adriani *et al.* 2003). In the recent past, the cerebellum has received considerable attention in the pathogenesis of ADHD (Goetz *et al.* 2014b). Cerebellar functions are compromised in ADHD individuals (Goetz *et al.* 2014a) (Goetz *et al.* 2014b). In addition, a significant reduction in the size of cerebellar lobes are also observed (Bledsoe *et al.* 2011). SHRs also have compromised cerebellar functions (Goetz *et al.* 2014b). Prominent astrogliosis is observed in the SHR cerebellum, which indicates that astroglial dysfunction is a major contributor to this phenotype (Yun *et al.* 2014). Although significant correlation has been shown between hypertension and ADHD, the possible role of the RAS in cerebellar dysfunction in SHRs has not been investigated. Interestingly, multiple studies have demonstrated that cannabinoids can cause significant improvement in symptoms associated with ADHD (Strohbeck-Kuehner *et al.* 2008) (Milz and Grotenhermen., 2015) (Adriani *et al.* 2003).

In the context of neuroinflammation, the endocannabinoid system has been extensively investigated in several neurological disorders (Di Marzo 2008). The ability of this system to elicit prominent protection against excitotoxicity (Maresz *et al.* 2007) and pro-inflammatory states (Molina-Holgado *et al.* 2003), makes it a prime therapeutic target in disorders that have a major neuroinflammatory component. Astrocytes not only express

CB1R, but are capable of synthesizing endocannabinoids as well (Walter *et al.* 2002). Cannabinoids exert their protective effects via the activation of the CB1R (Molina-Holgado *et al.* 2003) (Molina-Holgado *et al.* 2002a). CB1Rs have been demonstrated to crosstalk with several receptors (Turu and Hunyady 2010), and thereby can potentiate or suppress the receptor's functions. By employing drugs that modulate CB1R actions as adjunct therapies, it would enable us to design better therapeutic strategies. Not only does CB1Rs crosstalk with AT1Rs at multiple levels, that is heterodimerization or Ang II-mediated changes in CB1R expression (Turu *et al.* 2009) (Rozenfeld *et al.* 2011) (Haspula and Clark 2016b), but cannabinoids have been demonstrated to exert both functionally similar (Rozenfeld *et al.* 2011) and functionally diametric effects (Szekeres *et al.* 2012) in response to Ang II. For instance, in hepatic cells, CB1R inhibition was demonstrated to decrease AT1R-mediated deleterious effects in hepatic cells and reduce AT1R expression in vascular cells (Rozenfeld *et al.* 2011) (Tiyerili *et al.* 2010). However, another study reported that CB1R inhibition resulted in a decrease in AT1R-mediated vasoconstriction (Szekeres *et al.* 2012). In astrocytes however, certain critical functions of AT1Rs and CB1Rs have been demonstrated to be antagonistic to each other. Activation of AT1Rs is associated with astrocyte senescence (Liu *et al.* 2011a) and exacerbation of pro-inflammatory states (Gowrisankar and Clark 2016c). CB1R activation on the other hand, exerts prominent astro-protective (Gómez Del Pulgar *et al.* 2002), neuroprotective and anti-inflammatory states (Molina-Holgado *et al.* 2003). Intriguingly, studies investigating the role of CB1R in the regulation of neuroinflammatory states in hypertension, are not available. We and others have already demonstrated several levels of crosstalk between these two receptors (Haspula and Clark 2016a) (Haspula and Clark 2016b). The

consequence could well be potentiation or antagonism of AT1R-mediated deleterious effects. This study aims at understanding the functional consequences of AT1R and CB1R activation, both alone and in combination, in the regulation of inflammatory cytokines, IL-1β and IL-10, in primary astrocytes prepared from the brainstem and cerebellum of SHR brains. The ability of CB1R activation to neutralize (or potentiate) Ang II-mediated pro-inflammatory effects, will be addressed from the results of this study.

5.2 Materials and methods

i) Materials

Ang II was obtained from Bachem (Torrance, CA). ACEA or Arachidonyl-2'-chloroethylamide, the specific CB_1R agonist, was purchased from Tocris (Bristol, UK). Sodium deoxycholate (DOC) (89904) was purchased from Thermo Fischer (San Diego, CA). qPCR products including the Taqman primer sets for IL-1β (Rn00580432_m1), IL-10 (Rn00563409_m1), and beta-actin (Rn00667869_m1) were obtained from Applied Biosystems (Foster City, CA, USA). ELISA kits for IL-1β (BMS630) and IL-10 (BMS629) were purchased from eBioscience (San Diego, CA). The BCA protein kit was obtained from Pierce Biotechnology (Rockford, IL). All other chemicals were purchased from either VWR International (Suwannee, GA), Fisher Scientific (Waltham, MA) or Sigma (St. Louis, MO).

ii) Isolation and culture of primary astrocytes

Timed pregnant Wistar rats and SHRs were obtained from Charles River Laboratories (Wilmington, MA) and maintained in the ALAAC-accredited animal facility of Nova Southeastern University. All animal protocols were approved by the University Institutional Animal Care and Use committee. The brainstem and cerebellar astrocyte

cultures were prepared using mechanical dissociation as previously described (Tallant and Higson 1997). Briefly, brains from 3-day old rat pups were isolated and the cerebellum and brainstem were carefully separated from each brain. These regions are visible to the naked eye and can be clearly differentiated from each other. Astrocyte cultures were then prepared from the pooled brainstem and the pooled cerebellum by physical dissociation. The cells were grown in DMEM/F12 culture media containing 10% FBS, 10,000 I.U/mL penicillin, 10,000 µg/mL streptomycin and 25 µg/mL amphotericin B at 37°C in a humidified incubator (5% CO_2 and 95% air). The cell cultures were fed every 3-4 days.

On attaining confluency, the cells were subjected to vigorous shaking overnight which resulted in the detachment of microglia, oligodendrocytes and cell debris. Subsequently, the cell cultures were detached with trypsin/EDTA (0.05% trypsin, 0.53mM EDTA) and replated at a ratio of 1:10. The astrocyte-enriched cultures were fed once every 3 days until they were about 90% confluent. Before all cell treatments, the cultures were made quiescent by treating for 48 hours with DMEM/F12 culture media containing 10,000 I.U/mL penicillin, 10,000 µg/mL streptomycin and 25 µg/mL amphotericin B. All subsequent treatments were conducted in serum free media. The purity of the enriched astrocyte cultures were determined using Flow cytometry, western blotting and qPCR as shown previously (Haspula and Clark 2016b).

iii) Cell treatments

For determining the effect of RAS activation on IL-1β and IL-10 levels, astrocytes were treated with 100 nM Ang II and 10 nM ACEA for 12 and 24 hours alone. In order to determine the ability of the CB_1R agonist, ACEA, to neutralize or potentiate Ang II's

effect, astrocytes were co-treated with both Ang II (100 nM) and ACEA (10 nM) for 12 and 24 hours.

iv) Total protein extraction and measurement from conditioned medium

Immediately following treatments, the conditioned medium was collected and subjected to centrifugation at 1,500 rpm for 10 min at 4°C. The supernatant was collected and stored at -80°C until further use. Owing to low amounts of secreted IL-1β and IL-10 in the conditioned medium, the protein was concentrated using the DOC-TCA precipitation method as previously described (Chevallet *et al.* 2007). The protein was then measured using the BCA assay as per the manufacturer's instructions. Equal concentrations of proteins samples (10 ug) were then employed for ELISA. ELISA for IL-1β and IL-10 was then performed as per the manufacturer's protocol.

v) Total RNA extraction and mRNA expression

Total RNA was extracted from astrocytes using the trizol method and subjected to a DNA cleaning step before determining the RNA concentrations using a Bio-Rad SmartSpec^{TM} spectrophotometer. Reverse transcription from total RNA (2 µg) to complementary strand DNA was done using a high capacity reverse transcription reagent kit (Applied Biosystems). qPCR was performed using the TaqMan Universal master mix, and the TaqMan gene expression primers (Applied Biosystems) for the IL-1β and IL-10 genes. Samples were analyzed in 96-well plates using the StepOne^{TM}plus Real time PCR system from Applied Biosystems. For the experiments estimating the basal expression of cytokines, data are expressed as fold change in the expression of genes of interest (IL-1β and IL-10) in SHR samples, as compared with reference genes (IL-1β or IL-10) in Wistar samples. For the experiments determining the effect of Ang II and/or ACEA, data are

expressed as fold-change in the expression of genes of interest (IL-1β or IL-10) in treated cells, as compared with the reference gene (IL-1β or IL-10) in untreated cells. β-actin was employed as the housekeeping gene.

vi) Statistical analysis

A 2x2 mixed ANOVA was employed to determine if there were any significant differences in the basal expression of IL-1β and IL-10 alone, between SHRs and Wistar rats. This was followed by a Bonferoni T test to determine differences between groups. A two-way ANOVA was employed for testing the effect of treatments on IL-1β and IL-10 alone, in SHRs as compared to Wistar rats. A Bonferoni T test was employed to determine significant differences between treatments and the respective control. In order to make comparisons between identical time points from different rat models, a two-tailed student t test was employed. All data is expressed as mean± SEM for 6 or more experiments.

5.3 Results

i) Basal expression of IL-1β and IL-10 in brainstem astrocytes

Both IL-1β and IL-10 levels were reported to be different in adult SHR brains when compared to their normotensive controls (Agarwal *et al.* 2011). Distinct neuroinflammatory states have been reported in the brainstem cardiovascular centers of prehypertensive as well as hypertensive SHRs (Waki *et al.* 2008a) (Waki *et al.* 2008b). In order to determine whether brainstem astrocytes contribute to the inflammatory states observed in hypertension, we employed astrocytes isolated from the brainstems of neonatal SHRs and Wistar rats.

In brainstem astrocytes, both ELISA (Fig 5.1A) and qPCR (Fig 5.1B) data indicates that IL-1β levels were significantly higher in SHRs when compared to Wistar rats. Interestingly, IL-10 levels were also significantly higher in SHR brainstem astrocytes (Fig 5.1C, 5.1D). The mean Ct values for SHR brainstem for IL-1β was 25 as opposed to 31 in Wistar brainstem samples. In the case of IL-10, the Ct value for SHR brainstem was 32 as opposed to 36 in Wistar brainstem.

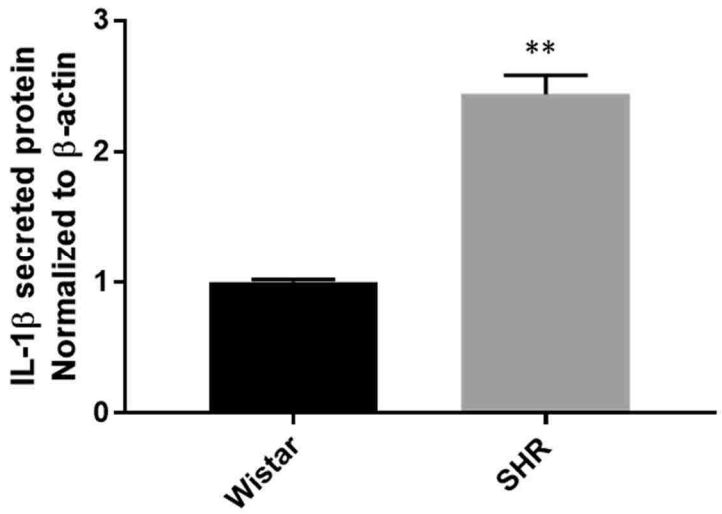

Fig 5.1A: Basal expression of IL-1β secreted protein in brainstem astrocytes: ELISA was employed to compare levels of secreted IL-1β in brainstem astrocyte samples between Wistar and SHRs. Each value represents the mean ± SEM of preparations of astrocytes isolated from six or more litters of neonatal rat pups. (**denotes $p < 0.01$ for SHR versus Wistar samples).

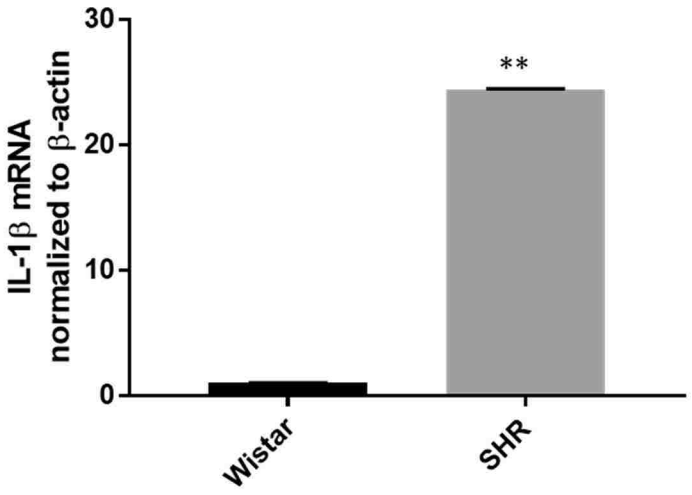

Fig 5.1B: Basal expression of IL-1β mRNA in brainstem astrocytes: qPCR was employed to compare levels of IL-1β mRNA in brainstem astrocyte samples between Wistar and SHRs. Each value represents the mean ± SEM of preparations of astrocytes isolated from six or more litters of neonatal rat pups. (**denotes $p < 0.01$ for SHR versus Wistar samples).

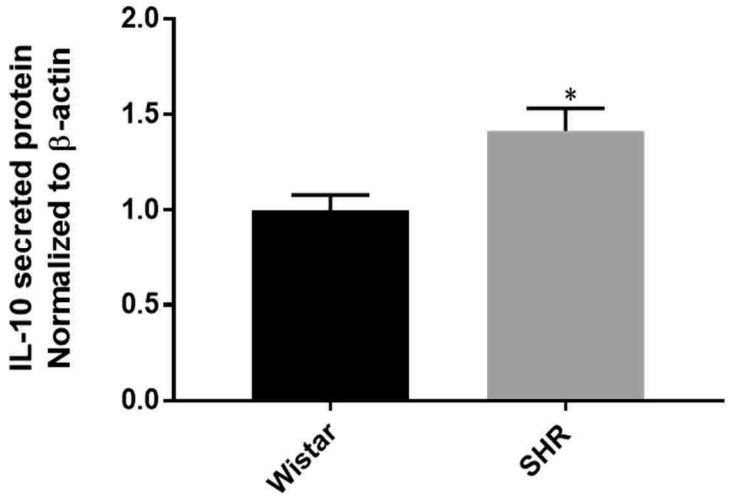

Fig 5.1C: Basal expression of IL-10 secreted protein in brainstem astrocytes: Elisa was employed to compare levels of secreted IL-10 in brainstem astrocyte samples between Wistar and SHRs. Each value represents the mean ± SEM of preparations of astrocytes

isolated from six or more litters of neonatal rat pups. (*denotes $p < 0.05$ for SHR versus Wistar samples).

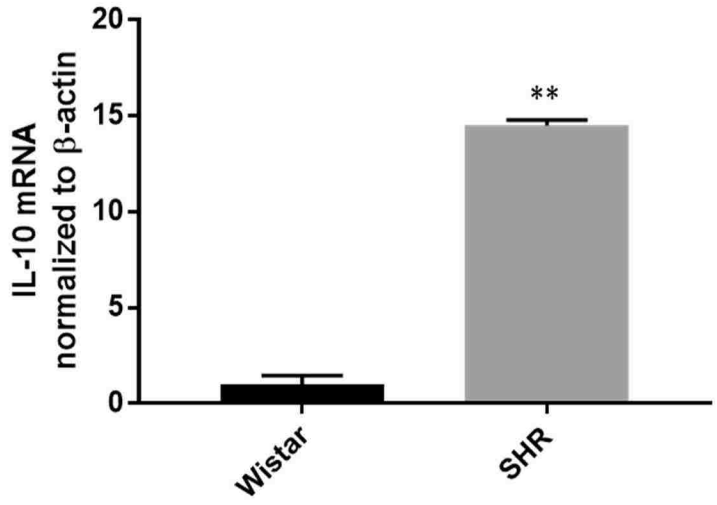

Fig 5.1D: Basal expression of IL-10 mRNA in brainstem astrocytes: qPCR was employed to compare levels of IL-10 mRNA in brainstem astrocyte samples between Wistar and SHRs. Each value represents the mean ± SEM of preparations of astrocytes isolated from six or more litters of neonatal rat pups. (**denotes $p < 0.01$ for SHR versus Wistar samples).

ii) Basal expression of IL-1β and IL-10 in cerebellar astrocytes

In cerebellar astrocytes as well, the basal levels of IL-1β, both secreted protein (Fig 5.2A) and mRNA (Fig 5.2B), were higher again in SHRs when compared to Wistar rats. The differences however were not as apparent as that observed in brainstem samples. The Ct value for IL-1β in SHR cerebellum was 29 and in Wistar cerebellum it was 30.5. Secreted basal IL-10 protein levels were higher in Wistar rats when compared to SHRs (Fig 5.2C). IL-10 mRNA levels on the other hand were not significantly different (Fig 5.2D). The Ct value for IL-10 in SHR cerebellum samples was 34 and in Wistar cerebellum it was 35.

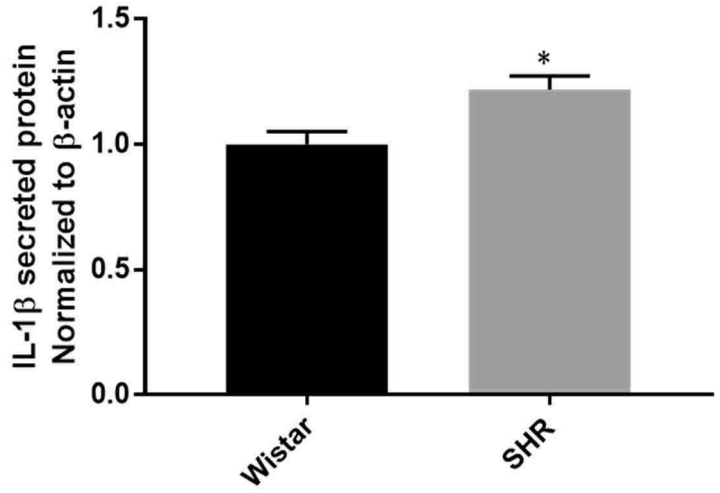

Fig 5.2A: Basal expression of IL-1β secreted protein in cerebellar astrocytes: ELISA was employed to compare levels of secreted IL-1β in cerebellar astrocyte samples between Wistar and SHRs. Each value represents the mean ± SEM of preparations of astrocytes isolated from six or more litters of neonatal rat pups. (*denotes $p < 0.05$ for SHR versus Wistar samples).

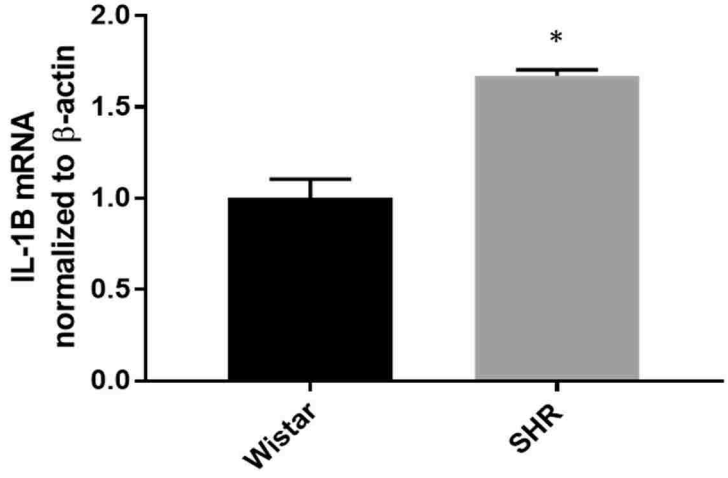

Fig 5.2B: Basal expression of IL-1β mRNA in cerebellar astrocytes: qPCR was employed to compare levels of IL-1β mRNA in cerebellar astrocyte samples between Wistar and SHRs. Each value represents the mean ± SEM of preparations of astrocytes isolated from six or more litters of neonatal rat pups. (*denotes $p < 0.05$ for SHR versus Wistar samples).

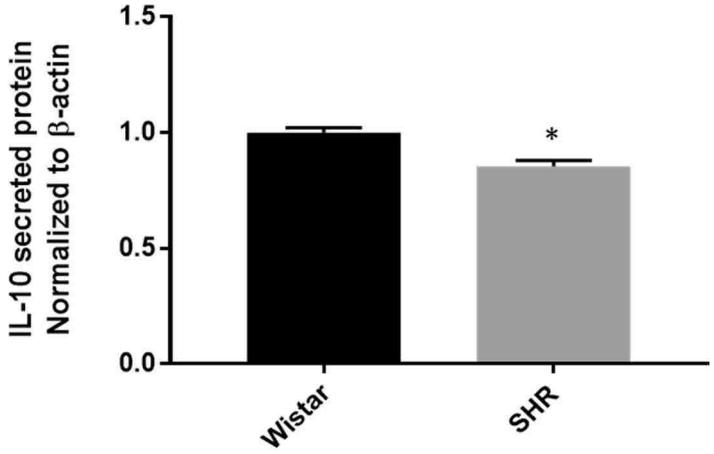

Fig 5.2C: Basal expression of IL-10 secreted protein in cerebellar astrocytes: ELISA was employed to compare levels of secreted IL-10 in cerebellar astrocyte samples between Wistar and SHRs. Each value represents the mean ± SEM of preparations of astrocytes isolated from six or more litters of neonatal rat pups. (*denotes $p < 0.05$ for SHR versus Wistar samples).

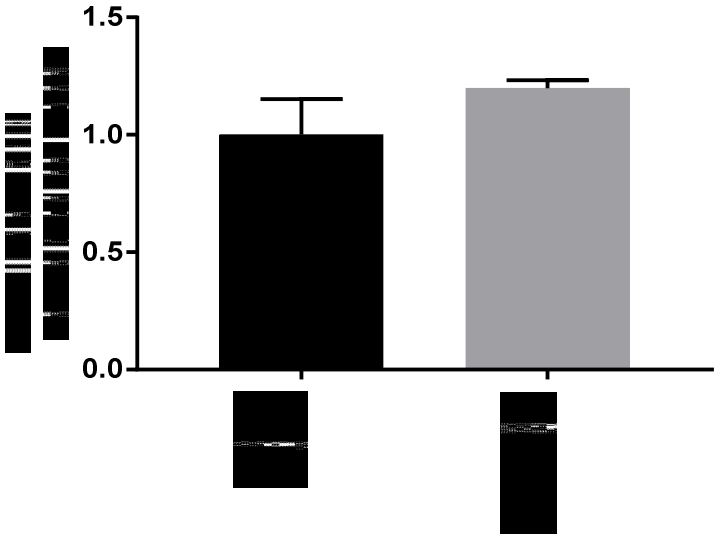

Fig 5.2D: Basal expression of IL-10 mRNA in cerebellar astrocytes: qPCR was employed to compare levels of IL-10 mRNA in cerebellar astrocyte samples between Wistar and SHRs. Each value represents the mean ± SEM of preparations of astrocytes isolated from six or more litters of neonatal rat pups.

iii) Effect of treatments on IL-10 levels in brainstem astrocytes

To determine the roles of astroglial angiotensin and cannabinoid systems in regulating IL-10 levels in brainstem, we employed brainstem astroglial samples that were pretreated with either Ang II or ACEA, both alone and in combination. These were subjected to ELISA and qPCR measurements in order to determine the secreted protein fraction and mRNA transcript levels of IL-10, respectively. Both Ang II and ACEA had significant effects on the mRNA levels, while their effects on the secreted fraction was not as prominent. Nevertheless, we observed that Ang II treatment decreased both IL-10 protein (Fig 5.3A, 5.3C) and IL-10 mRNA (Fig 5.3B, 5.3D) levels, in SHR and Wistar rat brainstem astrocytes. Its effect on reducing the secreted IL-10 fraction however was more prominent in SHRs than in Wistar rat (Fig 5.3A, 5.3C) brainstem astrocytes. ACEA had a prominent effect on elevating IL-10 protein (Fig 5.3A, 5.3C) and mRNA (Fig 5.3B, 5.3D) levels, in Wistar when compared to SHR brainstem astrocytes. Treating astrocytes with a combination of Ang II and ACEA resulted in a neutralization of Ang II mediated decreases in Wistar (Fig 5.3A, 5.3B), but not in SHR brainstem astrocytes levels (Fig 5.3C, 5.3D).

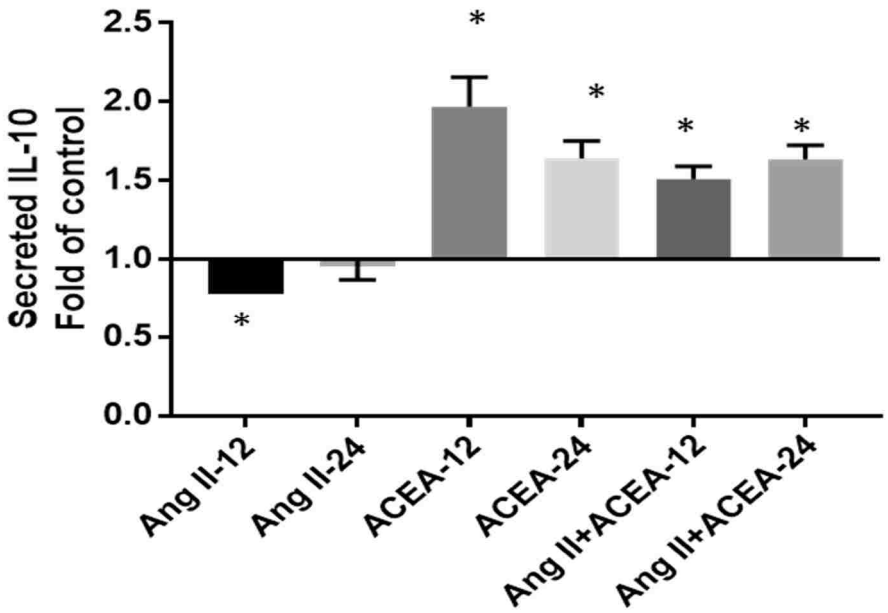

Fig 5.3A: Effect of treatments on IL-10 secreted protein levels in Wistar brainstem astrocytes: ELISA was employed to compare levels of secreted IL-10 in Wistar brainstem astrocyte samples treated with Ang II, ACEA and a combination of Ang II and ACEA. The treatment times were 12 and 24 hours. Each value represents the mean ± SEM of preparations of astrocytes isolated from six or more litters of neonatal rat pups. (*denotes $p < 0.05$ for treated versus untreated samples).

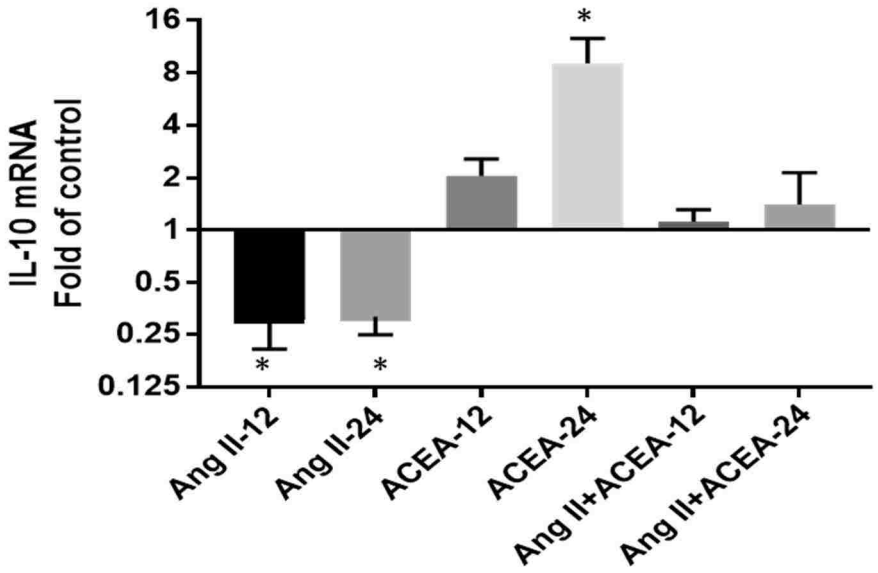

Fig 5.3B: Effect of treatments on IL-10 mRNA levels in Wistar brainstem astrocytes: qPCR was employed to compare levels of IL-10 mRNA in Wistar brainstem astrocyte samples treated with Ang II, ACEA and a combination of Ang II and ACEA. The treatment times were 12 and 24 hours. Each value represents the mean ± SEM of preparations of astrocytes isolated from six or more litters of neonatal rat pups. (*denotes $p < 0.05$ for treated versus untreated samples).

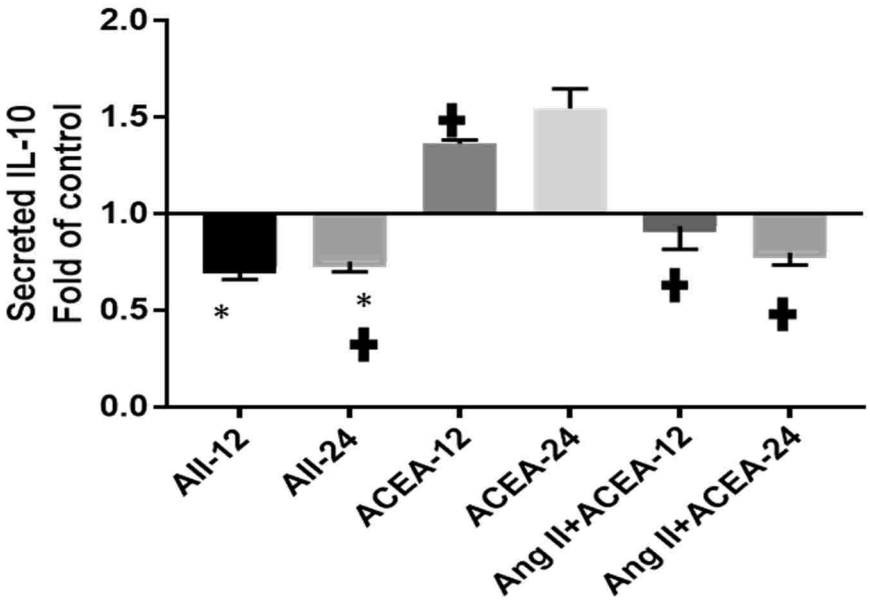

Fig 5.3C: Effect of treatments on IL-10 secreted protein levels in SHR brainstem astrocytes: ELISA was employed to compare levels of secreted IL-10 in SHR brainstem astrocyte samples treated with Ang II, ACEA and a combination of Ang II and ACEA. The treatment times were 12 and 24 hours. Each value represents the mean ± SEM of preparations of astrocytes isolated from six or more litters of neonatal rat pups. (*denotes $p < 0.05$ for treated versus untreated samples, and + denotes $p < 0.05$ for SHR versus Wistar samples- see fig 5.3A).

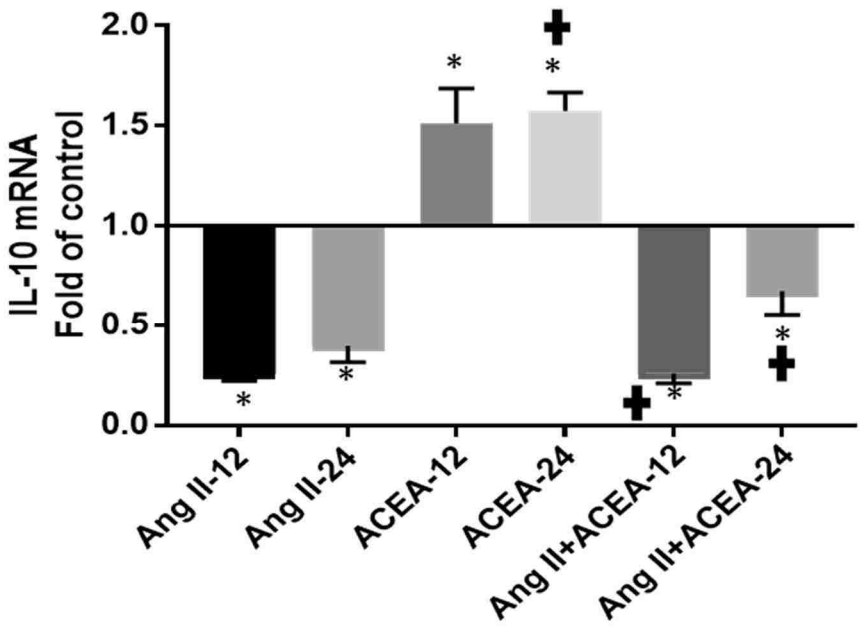

Fig 5.3D: Effect of treatments on IL-10 mRNA levels in SHR brainstem astrocytes: qPCR was employed to compare levels of IL-10 mRNA in SHR brainstem astrocyte samples treated with Ang II, ACEA and a combination of Ang II and ACEA. The treatment times are 12 and 24 hours. Each value represents the mean ± SEM of preparations of astrocytes isolated from six or more litters of neonatal rat pups. (*denotes $p < 0.05$ for treated versus untreated samples, and + denotes $p < 0.05$ for SHR versus Wistar samples- see fig 5.3B).

iv) Effect of treatments on IL-10 levels in cerebellar astrocytes

The immunomodulatory roles of both angiotensin and endocannabinoid systems are not well understood in cerebellum, under both physiological as well as in pathological conditions. As mentioned earlier, gross anatomical and functional changes have been reported in ADHD individuals and SHRs, a model of ADHD (Goetz *et al.* 2014b) (Bledsoe *et al.* 2011) (Yun *et al.* 2014). Since RAS hyperactivity (Veerasingham and Raizada 2003) and endocannabinoid hypofunctionality (Adriani *et al.* 2003) have been both reported in SHR brains, we investigated the roles of these two systems in regulating the levels of neuroinflammatory cytokines in SHR and Wistar rat cerebellar astrocytes. Ang II treatment resulted in a decrease in IL-10 secreted protein and mRNA levels again in cerebellar

astrocytes of both rat models (Fig 5.4A-D). The effect was more prominent at the level of mRNA in both SHRs and Wistar rat cerebellar astrocytes. Similar to what we observed in brainstem astrocytes, ACEA treatment in cerebellar astrocytes resulted in a significant increase in the levels of IL-10 mRNA, and only a partial increase in the levels of secreted IL-10 in both rat models (Fig 5.4A-D). Although ACEA was able to increase the levels of IL-10 mRNA, a combination of Ang II and ACEA on cerebellar astrocytes did not result in an increase in IL-10 levels (Fig 5.4B and 5.4D).

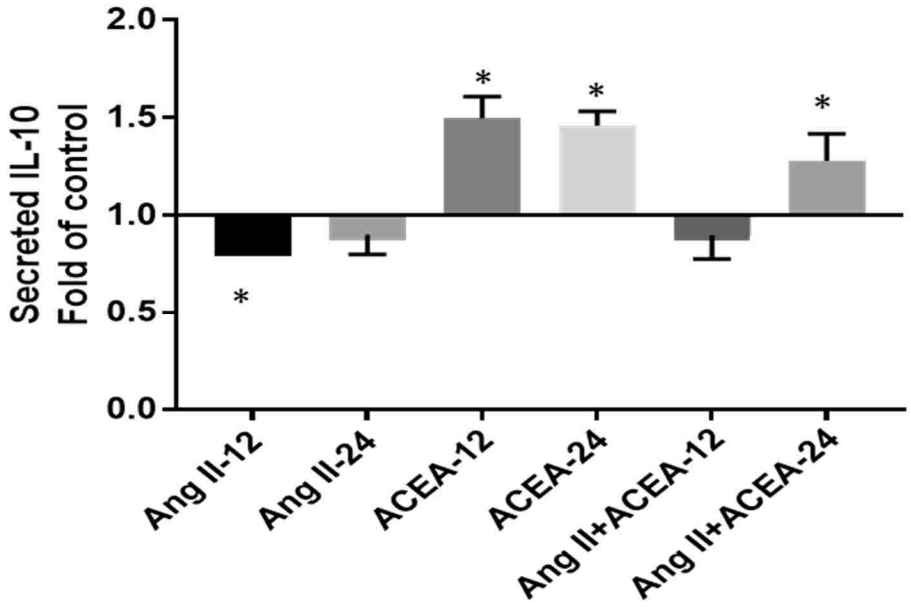

Fig 5.4A: Effect of treatments on IL-10 secreted protein levels in Wistar cerebellar astrocytes: ELISA was employed to compare levels of secreted IL-10 in Wistar cerebellar astrocyte samples treated with Ang II, ACEA and a combination of Ang II and ACEA. The treatment times are 12 and 24 hours. Each value represents the mean ± SEM of preparations of astrocytes isolated from six or more litters of neonatal rat pups. (*denotes $p < 0.05$ for treated versus untreated samples).

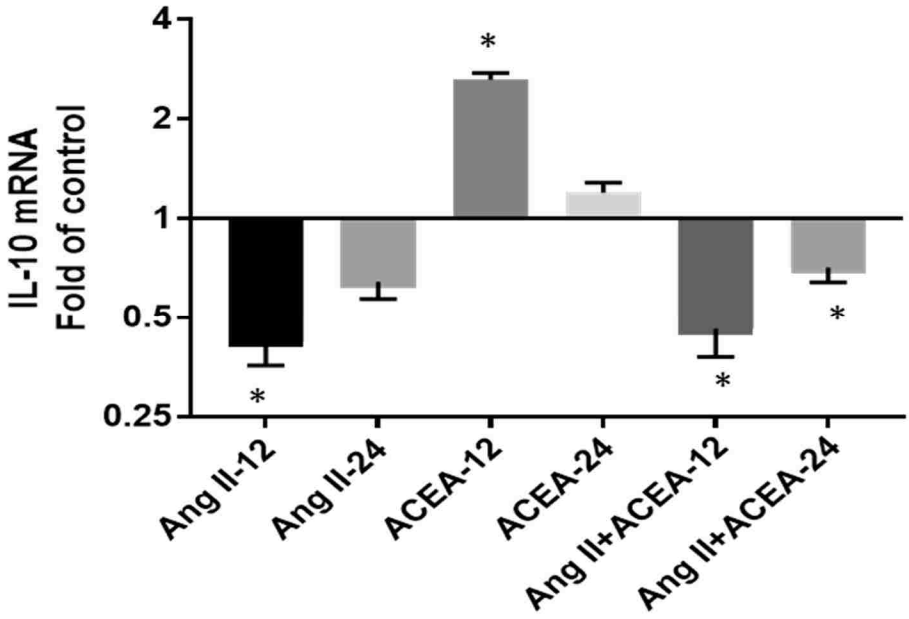

Fig 5.4B: Effect of treatments on IL-10 mRNA levels in Wistar cerebellar astrocytes: qPCR was employed to compare levels of IL-10 mRNA in Wistar cerebellar astrocyte samples treated with Ang II, ACEA and a combination of Ang II and ACEA. The treatment times are 12 and 24 hours. Each value represents the mean ± SEM of preparations of astrocytes isolated from six or more litters of neonatal rat pups. (*denotes $p < 0.05$ for treated versus untreated samples).

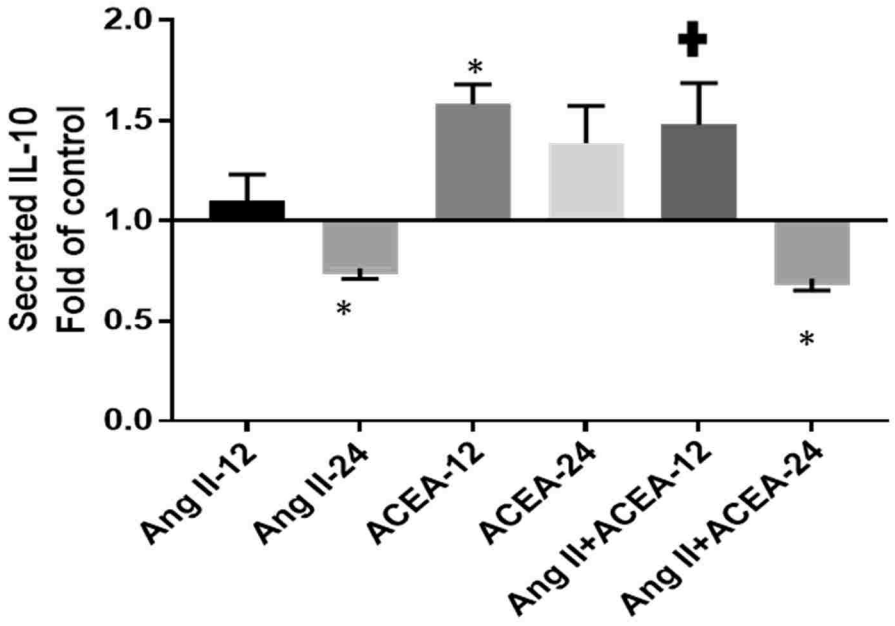

Fig 5.4C: Effect of treatments on IL-10 secreted protein levels in SHR cerebellar astrocytes: ELISA was employed to compare levels of secreted IL-10 in SHR cerebellar astrocyte samples treated with Ang II, ACEA and a combination of Ang II and ACEA. The treatment times are 12 and 24 hours. Each value represents the mean ± SEM of preparations of astrocytes isolated from six or more litters of neonatal rat pups. (*denotes $p < 0.05$ for treated versus untreated samples, and $^{+}$ denotes $p < 0.05$ for SHR versus Wistar samples- see Fig 5.4A).

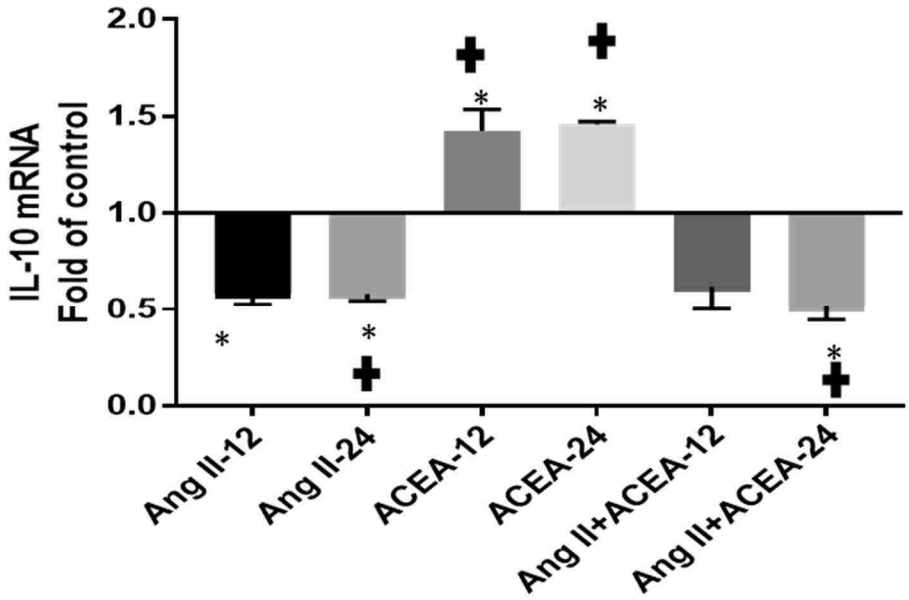

Fig 5.4D: Effect of treatments on IL-10 mRNA levels in SHR cerebellar astrocytes: qPCR was employed to compare levels of IL-10 mRNA in SHR cerebellar astrocyte samples treated with Ang II, ACEA and a combination of Ang II and ACEA. The treatment times were 12 and 24 hours. Each value represents the mean ± SEM of preparations of astrocytes isolated from six or more litters of neonatal rat pups. (*denotes $p < 0.05$ for treated versus untreated samples, and $^{+}$ denotes $p < 0.05$ for SHR versus Wistar samples- see Fig 5.4B).

v) Effect of treatments on IL-1β levels in brainstem astrocytes

An increase in IL-1β is associated with an elevation in sympathetic activity (Shi *et al.* 2011). Chronic Ang II infusion resulted in an elevation of IL-1β levels in PVN via glial cell activation (Shi *et al.* 2010a). Interestingly, cannabinoids, via the activation of the glial CB1R, has been demonstrated to neutralize IL-1β surges as a consequence of inflammatory insults (Molina-Holgado *et al.* 2003). Whether activation of CB_1R or AT_1R has a role in altering IL-1β levels, has not been studied yet. Brainstem astrocytes treated with Ang II resulted in only a moderate increase in IL-1β levels, both protein and mRNA, in both models (Fig 5.5A-D). Interestingly, the effect of Ang II in brainstem astrocytes was partially greater in Wistar rats (Fig 5.5A, 5.5B) when compared to SHRs (Fig 5.5C, 5.5D).

ACEA did not have a significant effect on IL-1β mRNA levels in both models (Fig 5.5B, 5.5D). A partial reduction in IL-1β levels in SHR brainstem astrocytes (Fig 5.5C, 5.5D) was observed in response to ACEA treatment. Interestingly, treatment of Wistar brainstem astrocytes with ACEA, resulted in an increase in IL-1β protein levels (Fig 5.5A), albeit the increase was less than 50%. The combination treatment of Ang II with ACEA resulted in an increase in IL-1β protein and mRNA levels, and this effect was similar to Ang II alone in Wistar brainstem astrocytes (Fig 5.5A, 5.5B). ACEA however was effective in partially neutralizing Ang II-mediated increases in IL-1β protein and mRNA levels in SHR brainstem astrocytes (Fig 5.5C, 5.5D).

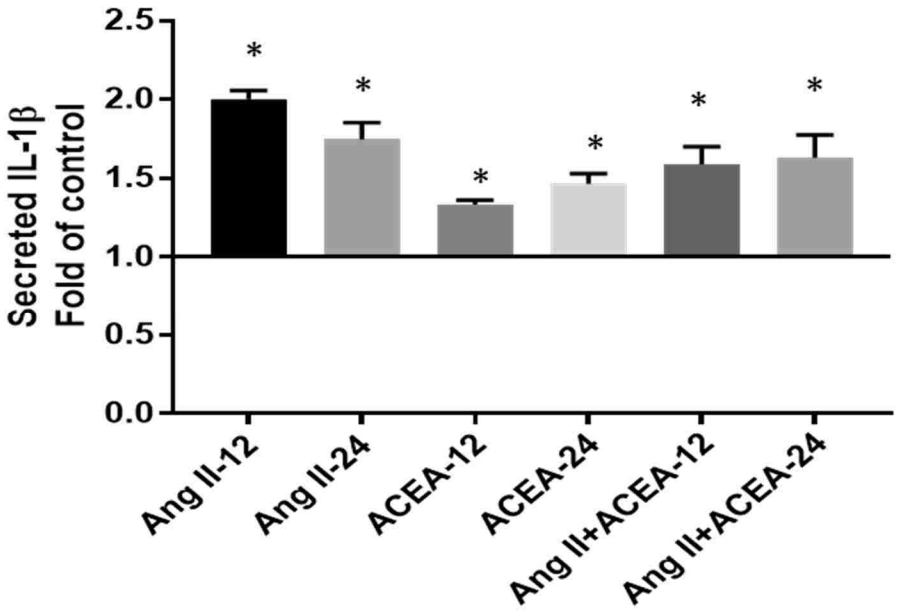

Fig 5.5A: Effect of treatments on IL-1β secreted protein levels in Wistar brainstem astrocytes: ELISA was employed to compare levels of secreted IL-1β in Wistar cerebellar astrocyte samples treated with Ang II, ACEA and a combination of Ang II and ACEA. The treatment times were 12 and 24 hours. Each value represents the mean ± SEM of preparations of astrocytes isolated from six or more litters of neonatal rat pups. (*denotes $p < 0.05$ for treated versus untreated samples).

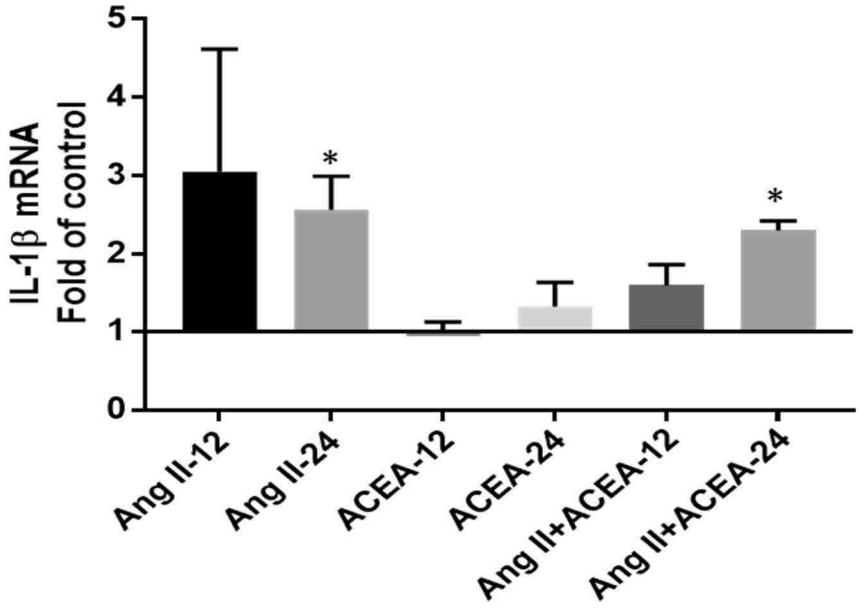

Fig 5.5B: Effect of treatments on IL-1β mRNA levels in Wistar brainstem astrocytes: qPCR was employed to compare levels of IL-1β mRNA in Wistar cerebellar astrocyte samples treated with Ang II, ACEA and a combination of Ang II and ACEA. The treatment times were 12 and 24 hours. Each value represents the mean ± SEM of preparations of astrocytes isolated from six or more litters of neonatal rat pups. (*denotes $p < 0.05$ for treated versus untreated samples).

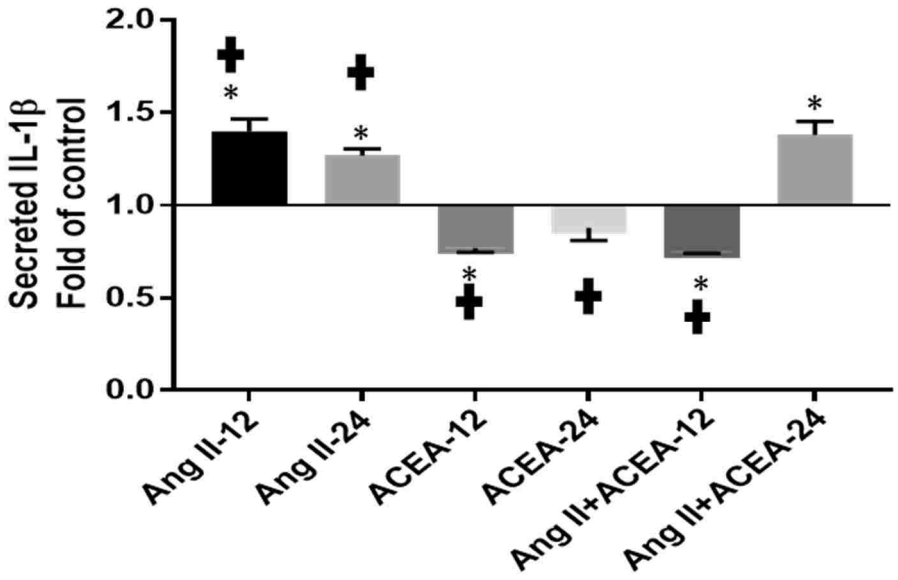

Fig 5.5C: Effect of treatments on IL-1β secreted protein levels in SHR brainstem astrocytes: ELISA was employed to compare levels of secreted IL-1β in SHR cerebellar astrocyte samples treated with Ang II, ACEA and a combination of Ang II and ACEA. The treatment times were 12 and 24 hours. Each value represents the mean ± SEM of preparations of astrocytes isolated from six or more litters of neonatal rat pups. (*denotes $p < 0.05$ for treated versus untreated samples, and $^{+}$ denotes $p < 0.05$ for SHR versus Wistar samples- see Fig 5.5A).

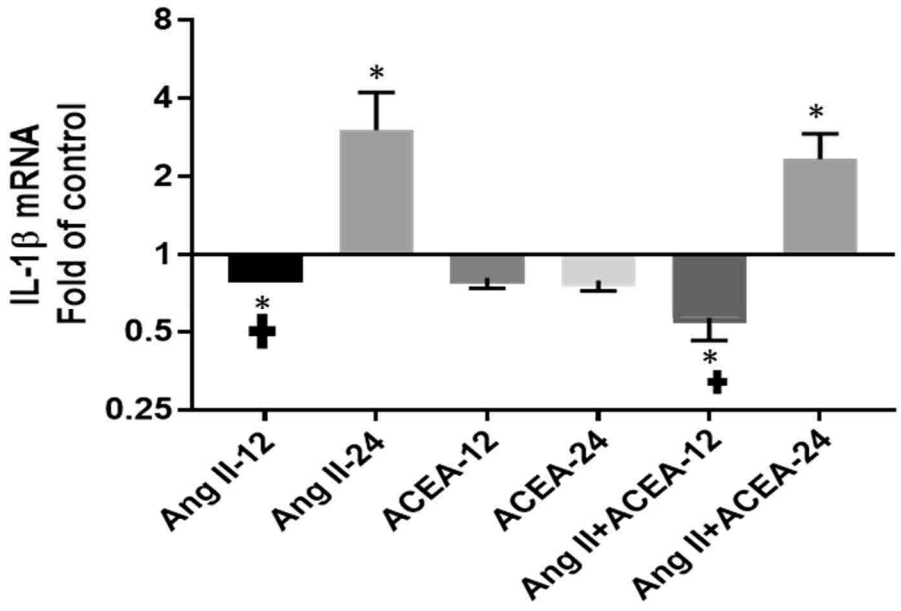

Fig 5.5D: Effect of treatments on IL-1β mRNA levels in SHR brainstem astrocytes: qPCR was employed to compare levels of IL-1β mRNA in SHR cerebellar astrocyte samples treated with Ang II, ACEA and a combination of Ang II and ACEA. The treatment times were 12 and 24 hours. Each value represents the mean ± SEM of preparations of astrocytes isolated from six or more litters of neonatal rat pups. (*denotes $p < 0.05$ for treated versus untreated samples, and $^{+}$ denotes $p < 0.05$ for SHR versus Wistar samples- see Fig 5.5B).

vi) Effect of treatments on IL-1β levels in cerebellar astrocytes

Both Ang II and ACEA were observed to significantly alter the levels of IL-10 mRNA in cerebellar astrocytes. Whether these systems can elicit an effect on the pro-inflammatory cytokine, IL-1β, is the focus of this experiment. Interestingly, Ang II treatment resulted in a pronounced decrease in IL-1β mRNA levels in both Wistar and SHR cerebellar astrocytes (Fig 5.6B, 5.6D). In the case of secreted data, Ang II again did not significantly change the levels of IL-1β from astrocytes (Fig 5.6A, 5.6C). Unlike brainstem astrocytes, ACEA was effective in lowering the secreted IL-1β fraction in cerebellar astrocytes of both rat models (Fig 5.6A, 5.6C). ACEA treatment was ineffective in

significantly modulating Ang II-mediated changes either at the level of secreted IL-1β or at the level of IL-1β mRNA (Fig 5.6A-D).

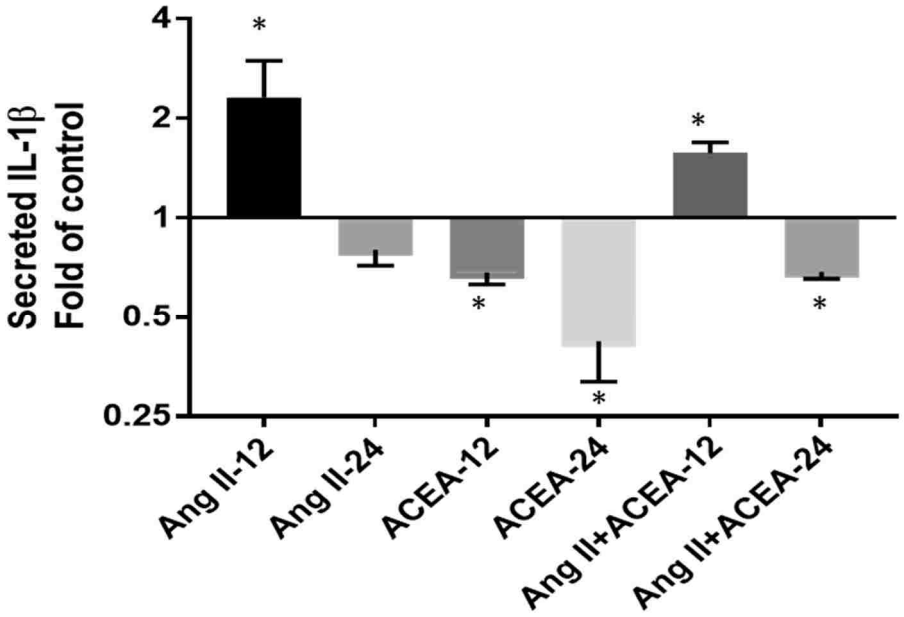

Fig 5.6A: Effect of treatments on IL-1β secreted protein levels in Wistar cerebellar astrocytes: ELISA was employed to compare levels of secreted IL-1β in Wistar cerebellar astrocyte samples treated with Ang II, ACEA and a combination of Ang II and ACEA. The treatment times were 12 and 24 hours. Each value represents the mean ± SEM of preparations of astrocytes isolated from six or more litters of neonatal rat pups. (*denotes $p < 0.05$ for treated versus untreated samples).

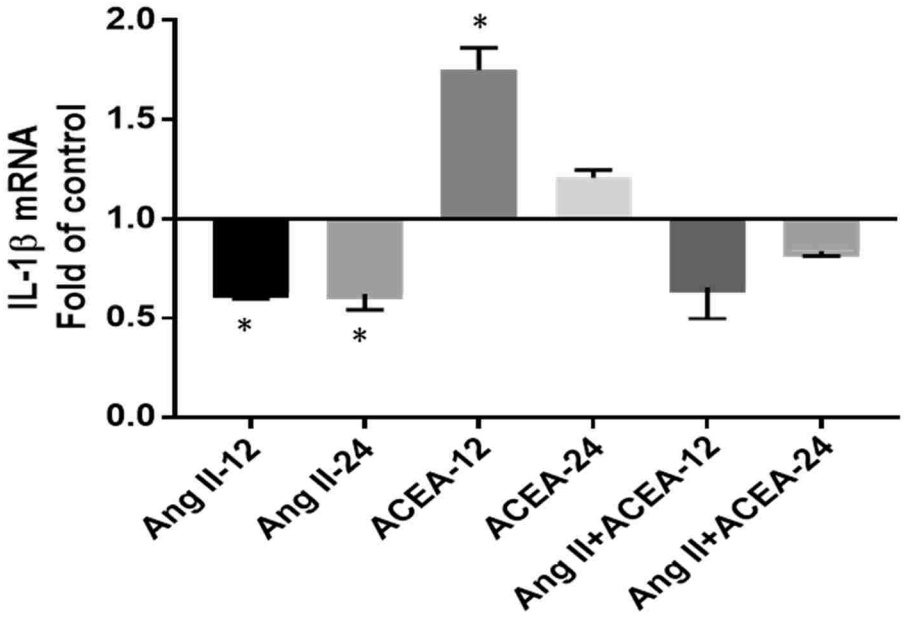

Fig 5.6B: Effect of treatments on IL-1β mRNA levels in Wistar cerebellar astrocytes: qPCR was employed to compare levels of IL-1β mRNA in Wistar cerebellar astrocyte samples treated with Ang II, ACEA and a combination of Ang II and ACEA. The treatment times were 12 and 24 hours. Each value represents the mean ± SEM of preparations of astrocytes isolated from six or more litters of neonatal rat pups. (*denotes $p < 0.05$ for treated versus untreated samples).

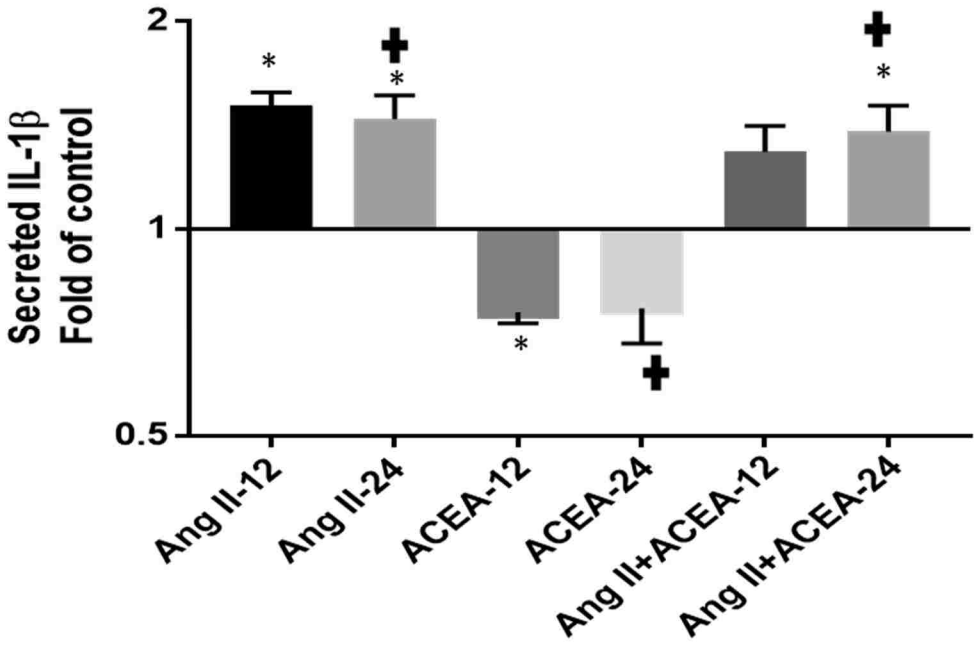

Fig 5.6C: Effect of treatments on IL-1β secreted protein levels in SHR cerebellar astrocytes: ELISA was employed to compare levels of secreted IL-1β in SHR cerebellar astrocyte samples treated with Ang II, ACEA and a combination of Ang II and ACEA. The treatment times were 12 and 24 hours. Each value represents the mean ± SEM of preparations of astrocytes isolated from six or more litters of neonatal rat pups. (*denotes $p < 0.05$ for treated versus untreated samples, and $^{+}$ denotes $p < 0.05$ for SHR versus Wistar samples- see fig 5.6A).

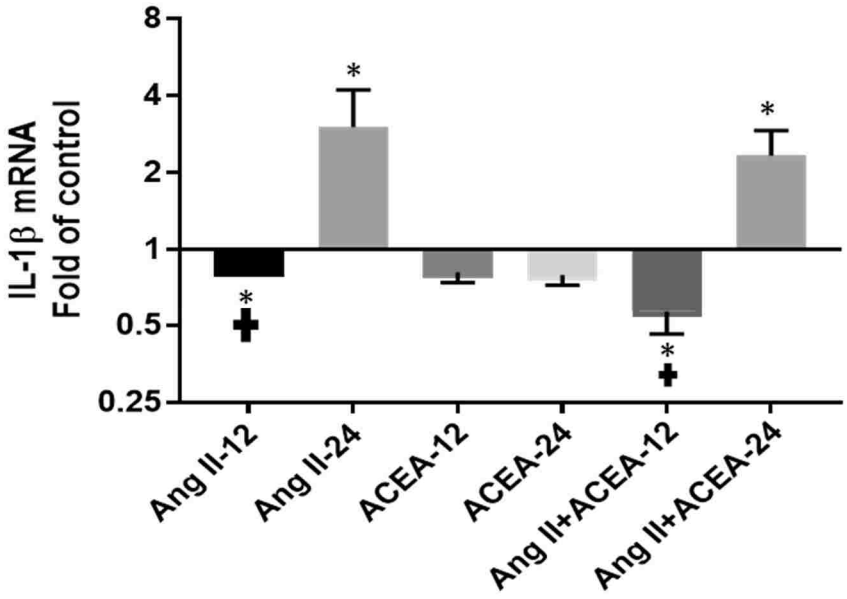

Fig 5.6D: Effect of treatments on IL-1β mRNA levels in SHR cerebellar astrocytes: qPCR was employed to compare levels of IL-1β mRNA in SHR cerebellar astrocyte samples treated with Ang II, ACEA and a combination of Ang II and ACEA. The treatment times were 12 and 24 hours. Each value represents the mean ± SEM of preparations of astrocytes isolated from six or more litters of neonatal rat pups. (*denotes $p < 0.05$ for treated versus untreated samples, and $^{+}$ denotes $p < 0.05$ for SHR versus Wistar samples- see fig 5.6B).

5.4 Discussion

Although both the RAS and the endocannabinoid system have well-established roles in modulating neuroinflammatory states, their ability to alter neuroinflammatory markers in SHRs, has not been well-investigated. The current study not only reinforces the immunomodulatory roles of the two systems, but also highlights their importance in pathological conditions such as hypertension. In addition to investigating these systems, the role of astrocytes in regulating neuroinflammatory states, under physiological and pathological conditions, is also reported in this study.

Our findings suggest that both pro-inflammatory and anti-inflammatory states are elevated in SHR brainstem astrocytes when compared to Wistar brainstem astrocytes. An increase in both IL-1β and IL-10 mRNA and protein levels, is observed in SHR brainstem astrocytes when compared to Wistar brainstem astrocytes. It should be noted that the cells employed in the current study are from neonatal pups which have not yet attained hypertensive states, but serves as a model for understanding the factors that contribute to genetic programming of hypertension. By employing cells from pre-hypertensive neonatal rat pups, we are able to identify candidate mechanisms that may contribute to the etiology of hypertension, and not mechanisms that have been dysregulated as a cause of hypertension. This principle has been employed by others as well (Ferrari *et al.* 2009). Protective systems are upregulated in certain hypertensive rat models (Mirabito *et al.* 2014). It could be that at these early stages, a compensatory increase in anti-inflammatory cytokines, such as IL-10, is able to neutralize the deleterious effects of the pro-inflammatory cytokine, IL-1β. Since we did not observe a similar large difference in the levels of cytokines in cerebellar astrocytes between the two models, the change observed in brainstem astrocytes may well have a consequential role in augmentation of cardiovascular functions that are relevant to hypertensive conditions, such as elevation of sympathetic activity.

The ability of Ang II to elevate levels of ROS (Liu *et al.* 2011a) and pro-inflammatory cytokines (Kandalam and Clark 2010) (Gowrisankar and Clark 2016c) in astroglial cultures, has already been demonstrated. Whether Ang II can induce pro-inflammatory cytokines, and neutralize anti-inflammatory cytokines, to a greater extent in SHR brainstems when compared to Wistar brainstems, has not been studied. In our study, we observed that Ang II significantly lowered IL-10, and elevated IL-1β, mRNA levels in SHR

brainstem astrocytes. The Ang II effect on secreted fraction of both cytokines however was not pronounced. When the two models were compared, Ang II-mediated changes on IL-10 protein levels was greater than its effect on elevating IL-1β levels in SHRs. Previously, we have reported that Ang II has a greater effect on the pro-inflammatory cytokine IL-6 levels in Wistar brainstem astrocytes when compared to SHR brainstem astrocytes prepared from neonatal pups (Gowrisankar and Clark 2016c). Based on this data and our current findings, we can conclude that Ang II's major mechanism of action, during the early stages of hypertension, could well involve dampening of anti-inflammatory cytokines, such as IL-10, rather than elevating pro-inflammatory cytokines, such as IL-1β and IL-6. Ang II was reported to enhance vascular inflammation by downregulating protective/anti-inflammatory systems such as PPAR-γ (Tham *et al.* 2002b)**.** Ang II was able to downregulate the CB_1R in brainstem astrocytes isolated from SHRs, but not Wistar rats (Haspula and Clark 2016b). Based on these findings, we can theorize that Ang II could be eliciting its deleterious effects in the early stages of hypertension by dampening protective or anti-inflammatory systems in astrocytes.

Several studies have highlighted the roles of the CB_1R in promoting anti-inflammatory responses in astrocytes (Molina-Holgado *et al.* 2002a) (Molina-Holgado *et al.* 2003). In our study, ACEA treatment neutralized the Ang II-mediated decrease in IL-10 levels in brainstem astrocytes isolated from Wistar rats. But its effect on neutralizing Ang II-mediated changes in IL-10 levels in brainstem astrocytes of SHRs was not as prominent as that observed in brainstem astrocytes isolated from Wistar rats. This could well be due to a hyperactive RAS, coupled with a hypofunctional CB1R effect in SHR brainstem astrocytes. The ability of ACEA to elicit a greater anti-inflammatory effect in brainstem

astrocytes from Wistar rats when compared to SHRs, suggests a dampened CB_1R response in hypertensive conditions in brainstem. CB1R dysregulation has been reported in several pathological conditions, that encompass both cardiovascular and neurological disorders (Pacher *et al.* 2005b) (Di Marzo 2008) (Miller and Devi 2011). Our laboratory and others have reported a decrease in CB_1R expression in SHR brainstem when compared to their normotensive controls (Haspula and Clark 2016b) (Brozoski *et al.* 2009), and an increase in the levels of inactivated forms of CB1R (Haspula and Clark 2016b). Dampening of protective systems, such as the endocannabinoid system, observed during prehypertensive conditions, could well be a significant factor in the transformation of prehypertension to an established hypertensive state. However, cannabinoids were demonstrated to not only promote anti-inflammatory states, but can also result in elevation of pro-inflammatory effects (Nagarkatti *et al.* 2009). ACEA treatment did result in a slight elevation of secreted IL-1β protein in brainstem astrocytes under normotensive conditions.

While Ang II mostly reduced both mRNA and protein levels of the anti-inflammatory cytokine, IL-10, in brainstem astrocytes, the same was not observed in cerebellar astrocytes. In the case of cerebellar astrocytes, Ang II lowered IL-1β mRNA levels and partially elevated IL-1β protein levels. A lack of correlation between mRNA and protein may be attributed to several factors (Maier *et al.* 2009). But a decrease in IL-1β mRNA levels by Ang II highlights a possible role of protective systems such as the AT2R and possible crosstalk with CB1R in cerebellar astrocytes. We previously observed an increase in CB1R expression as well in SHR cerebellar astrocytes, which have been pretreated with Ang II (Haspula and Clark 2016b). This effect was mediated by both AT1Rs and AT2Rs, suggesting a possible protective role of the AT2R in cerebellum. AT2R activation has been

demonstrated to increase IL-10 and suppress pro-inflammatory effects (Dhande *et al.* 2013). Unpublished data from our laboratory suggests that AT2R levels were increased in cerebellar astrocytes of SHRs when compared to Wistar rats. An increase in AT2R levels, observed in SHR cerebellar astrocytes, could well be one of the many protective mechanisms that are evident at the early stages of hypertension. Ang II however is not devoid of pro-inflammatory effects in cerebellar astrocytes. A significant decrease in IL-10 mRNA and protein levels, in response to Ang II, is observed in cerebellar astrocytes from both models. It could well be that some of Ang II's pro-inflammatory effects in cerebellar astrocytes is neutralized by its anti-inflammatory effects, the latter being mediated by the AT2R. Further investigations are underway to determine the receptor involved in mediating this effect.

The effects of ACEA in cerebellar astrocytes was similar to that observed in brainstem astrocytes at the level of IL-10 mRNA. Following ACEA treatment, the peak increase in IL-10 mRNA was significantly greater in cerebellar astrocytes of Wistar rats when compared to SHRs. Interestingly, in the case of cerebellar astrocytes, ACEA was able to again elevate IL-1β levels, on this occasion however it was at the level of mRNA. This data confirms a possible low level pro-inflammatory effects of CB_1R activation under normal physiological conditions, since partial pro-inflammatory effects were also observed in both brainstem and cerebellar astrocytes under normotensive conditions. ACEA was mostly ineffective in counteracting Ang II-mediated alteration in IL-1β and IL-10 levels in cerebellar astrocytes. An increase in the phosphorylated forms of the CB1R, has been demonstrated to result in desensitization and inactivation of CB1R (Garcia *et al.* 1998). Ang II triggered potent CB1R phosphorylation in cerebellar astrocytes of both SHRs and

Wistar rats (Haspula and Clark 2016a). Ang II via AT_1R not only alters neuroinflammatory states, but could also inactivate the endocannabinoid system by elevating the phosphorylated forms of the CB1R. It could well be that in cerebellar astrocytes, ACEA is ineffective in neutralizing some of Ang II's effects since Ang II via AT1R is able to desensitize CB_1R more so in cerebellar than in brainstem astrocytes.

As the peak effects for the secreted cytokine fraction were less than 2-fold for both Ang II and ACEA, either alone or in combination, we can conclude that neither Ang II no ACEA can cause a significant spike in the secretion profile of neuroinflammatory cytokines from astrocytes. It can however, significantly alter the levels of IL-1β and IL-10 mRNA levels in brainstem and cerebellar astrocytes isolated from both models. Factors secreted from microglial cells, in response to inflammatory or neurotoxic insults, were reported to act as triggers to mobilize cytokines from astrocytes (Shinozaki *et al.* 2014). Since our cell culture is devoid of microglial cells, it could well be that the translational and secretory machinery is regulated by these other factors. Nevertheless, both astrocytes and microglia are major players in regulating neuroinflammatory states in the brain. But most of the available data regarding Ang II-mediated neuroinflammation, is mainly restricted to microglial cells (Shi *et al.* 2010a). In addition to highlighting the contrasting roles of the two systems, RAS and endocannabinoid system, in the regulation of neuroinflammatory cytokines, the findings reported in this study provide clear evidence of an inflammatory response that is generated from an astrocyte enriched culture. An exaggerated basal inflammatory response observed in SHR brainstem astrocytes, underscores the importance of brainstem astrocytes in the development of hypertension. Whether CB_1R hypofunction and Ang II-mediated neuroinflammation in brainstem astrocytes, is an integral component of the dysregulated

molecular machinery that contributes to the pathogenesis of hypertension, remains to be investigated

Chapter 6

Conclusion

6.1 Overview

Imbalances of the levels of inflammatory cytokines in the brain are ascribed as being a key factor in the etiology of cardiovascular and neurological disorders (Winklewski *et al.* 2015) (Stolp 2013) (Deverman and Patterson 2009) (Frank-Cannon *et al.* 2009). SHR, a widely-used model to investigate hypertension and ADHD, is characterized by RAS hyperactivity and astroglial dysfunction (Matsuura *et al.* 2002) (Tomassoni *et al.* 2004) (Veerasingham and Raizada 2003). While Ang II-induced pro-oxidant and pro-inflammatory effects in the CNS have been reported by several groups (Kang *et al.* 2009) (Carlson and Wyss 2008) (Zhang *et al.* 2010), the importance of the glial AT1R in mediating the deleterious effects of Ang II, has come to the fore only in the recent past (Liu *et al.* 2011a) (Lanz *et al.* 2010) (Gowrisankar and Clark 2016c) (Isegawa *et al.* 2014b). Since astroglial dysfunction is a feature of pathological conditions that encompass etiologies relevant to both neurological and cardiovascular abnormalities, identifying molecular mechanisms that can neutralize AT1Rs, or help to revert astroglial function back to normalcy, is critical for the identification of viable therapeutic targets (Verkhratsky *et al.* 2012) (Reemst *et al.* 2016). The endocannabinoid system, and especially CB1Rs, is widely accepted as a crucial homeostatic regulator (Di Marzo 2009). Activation of the presynaptic CB1R dampens hyperactivity of neurons, while astroglial CB1R activation aids in resolution of neuroinflammatory and neurotoxic states in the CNS (Katona and

Freund 2008) (Massi *et al.* 2008) (Walter *et al.* 2004) (Gómez Del Pulgar *et al.* 2002). In the recent past, multiple groups have provided evidence of altered CNS endocannabinoid system activity, in both hypertension and ADHD (Brozoski *et al.* 2009) (Bátkai *et al.* 2004) (Adriani *et al.* 2003). Activation of the CB1R is associated with an improvement in indices of cardiovascular as well as attentional processing in SHRs (Brozoski *et al.* 2009) (Bátkai *et al.* 2004) (Adriani *et al.* 2003). Additionally, CB1R agonists and antagonists have been demonstrated to alter AT1R's functionality (Rozenfeld *et al.* 2011) (Szekeres *et al.* 2012) (Tiyerili *et al.* 2010). Owing to CB1R's anti-inflammatory and sympathoinhibitory effects (Sheng *et al.* 2005b) (Lake *et al.* 1997a) (Mendizábal and Adler-Graschinsky 2007), the endocannabinoid system is ideally placed to neutralize AT1R's deleterious effects in pathological conditions.

Hence, identification of potential hyper/hypofunctionality of RAS and endocannabinoid system, in SHR astrocytes, was the major focus of this study. Although RAS hyperactivity has been reported in SHR brainstems (Veerasingham and Raizada 2003), a potential neuroinflammatory link in brainstem astrocytes, has not been investigated. Another important aspect of this study was to investigate the consequences of RAS activation on CB1R expression and function. It was important to us to identify the regulators of a potential CB1R dysregulation in SHR astrocytes. Since the regulators of CB1R expression and functions, are routinely identified as being the same factors that are instrumental in the progression of the disorder (Miller and Devi 2011), we explored the possibility of Ang II being a critical factor in the alteration of endocannabinoid tone in SHR astrocytes.

Our data strongly suggests a prominent neuroinflammatory state, and a reduction in CB1R activity in SHR brainstem astrocytes. However, activation of the astroglial AT1Rs in SHR

brainstem astrocytes, shifts the balance between pro- and anti-inflammatory cytokines towards the former. In the case of cerebellar astrocytes, Ang II treatment resulted in an elevation in both pro-inflammatory and anti-inflammatory cytokines in SHR cerebellar astrocytes. Albeit, the effect was only at the level of mRNA in the latter. While we did observe neuroinflammatory states, and a slight alteration in CB1R's expression and functions in cerebellar astrocytes, the difference was not as marked as that observed in brainstem astrocytes of SHRs when compared to Wistar astrocytes. CB1R activation predominantly opposes AT1R-mediated activation of MAPKs and an elevation in pro-inflammatory states, in both brainstem and cerebellar astrocytes of both rat models. Additionally, Ang II treatment is associated with a decrease in CB1R levels in SHR brainstem astrocytes, and an increase in p-CB1R levels in cerebellar astrocytes of both models. Our data is strongly indicative of a genetically programmed CB1R dampening, that could result in dysregulation of neuroinflammatory cytokines, eventually leading to prominent pro-inflammatory states that are characteristic of hypertensive states. Additionally, Ang II-induced minimization of CB1R activity in astrocytes, either via receptor downregulation or receptor phosphorylation, could be of significance for pathological conditions characterized by RAS hyperactivity and/or astroglial dysfunction.

6.2 Summary of results

i) Astrocytes and neuroinflammatory cytokines

Multiple studies have reported the presence of a markedly distinct neuroinflammatory state in SHRs when compared to their normotensive controls (Waki *et al.* 2008a) (Agarwal *et al.* 2011). Distinct neuroinflammatory states have been reported in the cardiovascular centers of prehypertensive as well as hypertensive SHRs (Waki *et al.*

2008a) (Waki *et al.* 2008b). In our study, we observed a significant elevation of both pro- and anti-inflammatory cytokines IL-1β and IL-10, respectively, in astrocytes isolated from the brainstems of prehypertensive SHRs when compared to Wistar rats. An increase in IL-10 levels may serve as a compensatory mechanism that is functional at prehypertensive stages, but may well be lost at later stages of hypertension. Nevertheless, an augmented level of inflammatory cytokines at early stages of hypertension in the brainstem, is strongly suggestive of a causal role for neuroinflammation in the pathogenesis of hypertension. In the case of cerebellar astrocytes, differences in the levels of neuroinflammatory cytokines were not as drastic as in the case of brainstem astrocytes, between the two models. However, significant increases in IL-1β mRNA, and a slight reduction in the levels of secreted IL-10 levels, does indicate the presence of a dysregulated neuroinflammatory state in SHR cerebellum as well. As the magnitude of difference was not great, their contribution to the pathogenesis of disorders characterized by SHR cerebellar impairments, such as ADHD, remains debatable.

ii) Astroglial CB1R and p-CB1R expression

Despite definitive evidence of its neuroprotective and anti-inflammatory effects (Nagarkatti *et al.* 2009), the role of astroglial CB1Rs in the development of cardiovascular diseases and their risk factors has not been well explored. Alterations in CB1R expression and functions have been reported in the CNS, heart and vasculature of adult SHRs (Brozoski *et al.* 2009) (Bátkai *et al.* 2004) (Adriani *et al.* 2003). However, these variations could be secondary to hypertension, with little or no causal effect on the development of hypertension. Our results indicate that CB1R expression, both protein and mRNA, was significantly lowered in brainstem astrocytes, but not in cerebellar astrocytes, of SHRs.

Also, a moderate increase in the level of p-CB1R was observed in SHR brainstem astrocytes. The levels of p-CB1R observed were remarkably higher in cerebellar when compared to brainstem astrocytes of both models. While differences in p-CB1R and CB1R levels were also observed in cerebellar astrocytes, the difference was not statistically significant. This is indicative of a hypofunctional CB1R in SHR brainstem astrocytes. Since brain cells from prehypertensive SHRs were employed, there could be a causal relationship between the observed basal CB1R changes in brainstem and the development of hypertension.

iii) Ang II-mediated effects in astrocytes from SHRs and Wistar Rats

a) Ang II-induced neuroinflammatory changes

Chronic infusion of Ang II has been reported to elevate neuroinflammatory states (Kang *et al.* 2009) (Shi *et al.* 2010b). Elevation of neuroinflammatory cytokines and ROS are deemed critical intermediary steps in Ang II-mediated sympathoexcitation (Kang *et al.* 2009) (Shi *et al.* 2010b). However, it is unknown whether Ang II elicits a pronounced neuroinflammatory effect in brainstem astrocytes isolated from a prehypertensive rat model. Ang II was effective in lowering IL-10 levels, both mRNA and secreted protein, in astrocytes isolated from brainstems of both SHRs and Wistar rats. Its effect however, was greater in SHRs when compared to Wistar brainstem astrocytes. The effect of Ang II on IL-1β however was slightly greater in brainstem astrocytes isolated from Wistar rats when compared to SHRs. This suggests that Ang II-induced elevation of pro-inflammatory cytokines is not augmented under prehypertensive conditions, but its effect on lowering anti-inflammatory cytokines is potentiated. In the case of cerebellum astrocytes, Ang II lowered both IL-10 and IL-1β mRNA levels, while it had negligible effects on the secreted

fraction in both rat models. However, the effect in cerebellar astrocytes of SHRs was similar to Wistar rats. This is indicative of an absence of compensatory mechanisms in brainstem astrocytes under prehypertensive states, but not in cerebellar astrocytes. A possible AT2R involvement may well be responsible for Ang II-mediated anti-inflammatory effects in cerebellar astrocytes.

b) Ang II-induced MAPK activation

MAPKs serve as vital interceding points between receptor activation and receptor function. AT1R stimulation was earlier reported to lead to a pronounced activation of MAPKs in astrocytes (Clark *et al.* 2001) (Clark *et al.* 2008) (Clark *et al.* 2013) (Nemoto *et al.* 2015). We have employed two MAPKs, ERK and p38, as potential indicators of AT1R's activity in astrocytes from SHRs and Wistar neonatal rat pups. Our results indicate that the activation patterns of both ERK and p38 by Ang II were nearly identical, and they are driven exclusively by the AT1R. In SHR brainstem astrocytes, Ang II caused an augmented increase in the activation of P-ERK and P-p38. Interestingly, in cerebellar astrocytes, Ang II had a prominent effect on MAPKs in Wistar rats, while its effect in SHRs was greatly diminished. Unpublished data from our laboratory suggests that AT1R mRNA expression is lowered and AT2R mRNA is elevated in cerebellar astrocytes of SHRs when compared to Wistar rats. It could well be that the AT1R-MAPK axis is amplified in SHR brainstem astrocytes, but diminished in cerebellar astrocytes from the same model.

c) Ang II-induced changes in CB1R and p-CB1R

In order to ascertain whether RAS activation leads to endocannabinoid dampening under prehypertensive conditions, we measured CB1R and p-CB1R levels in SHR astrocytes treated with Ang II. A decrease in CB1R expression or an increase in phosphorylation of the CB1R represents a fall in endocannabinoid function.

Treatment of brainstem astrocytes with Ang II for 12 hours resulted in a moderate increase in CB1R protein expression in Wistar rats. In brainstem astrocytes isolated from SHRs however, a decrease was observed in response to Ang II treatment for the first 8 hours, followed by a normalization to baseline levels by the 12^{th} hour. Interestingly in Wistar cerebellar astrocytes, Ang II treatment resulted in no significant effect for the first 8 hours, which was then followed by a decrease. In SHRs however, Ang II caused an increase in CB1R expression within the first 8 hours. With the exception of SHR cerebellar astrocytes, the effect was exclusively mediated by AT1Rs. The decrease in CB1R expression in SHR brainstem astrocytes treated with Ang II, could represent a loss in protective/compensatory mechanisms in prehypertensive states. This may well be preserved in other regions of the SHR brains, as we did observe an increase in response to Ang II in cerebellar astrocytes.

Phosphorylation of CB1R represents another novel and significant mode of CB1R dampening by PKC activation (Garcia *et al.* 1998). Ang II-induced phosphorylation of the CB1R was similar in astrocytes isolated from normotensive as well as prehypertensive rats, suggesting that Ang II mediated inactivation of CB1R, via phosphorylation, may not be a contributing factor for the development of hypertension. However, substantial differences were observed when different regions were compared. In the case of cerebellar astrocytes, Ang II induced significantly higher phosphorylation of CB1R, with a peak effect of ~5 fold over basal. Only about a 2-fold over basal peak effect was observed in brainstem astrocytes.

This suggests that Ang II-induced inactivation of CB1R, via phosphorylation of the receptor, may be of prominence in brain regions with high CB1R expression, and in pathological conditions associated with high brain RAS activity. This however may not contribute to an impairment in cerebellar functions in SHRs since basal p-CB1R expression was not found to be different in SHRs when compared to Wistar rats in cerebellar astrocytes.

iv) CB1R-mediated effects in astrocytes from SHRs and Wistar rats

a) ACEA-induced neuroinflammatory changes

Apart from neutralizing excitotoxicity, cannabinoids are also known to elicit their neuroprotective effect by their anti-inflammatory and anti-oxidant actions (Nagarkatti *et al.* 2009). Activation of the astroglial CB1R has been demonstrated to protect against neurotoxic, pro-oxidant and pro-inflammatory stimuli (Gómez Del Pulgar *et al.* 2002) (Aguirre-Rueda *et al.* 2015). This is due to an increase in the levels of anti-inflammatory cytokines, as well as a reduction in the levels of pro-inflammatory cytokines in response to astroglial CB1R activation. Whether astroglial CB1R's immunomodulatory effects are preserved in prehypertensive conditions, was the focus of these experiments. ACEA treatment elevated IL-10 levels and also reduced IL-1β levels in both brainstem and cerebellar astrocytes of SHRs. However, its effect on IL-10 levels in brainstem astrocytes was significantly dampened in SHR astrocytes when compared to Wistar astrocytes. In the case of cerebellar astrocytes, the effect on IL-10 was slightly greater again in Wistar rat astrocytes when compared to SHR's, albeit the difference was not as marked as that observed in brainstem. This suggests that ACEA could elicit anti-inflammatory effects in both SHR brainstem and cerebellar astrocytes, in spite of changes in CB1R expression,

phosphorylation and a reduced ability to activate MAPK. However, its effect on altering IL-10 levels were markedly diminished in SHR brainstem astrocytes, suggesting that a potential hypofunction of CB1Rs could contribute to the development of pro-inflammatory conditions in brainstem, a state that is observed in hypertension.

b) ACEA-induced MAPK activation

Other groups have reported hypofunctional CB1Rs in SHR brainstems and prefrontal cortex when compared to their normotensive controls (Brozoski *et al.* 2009) (Adriani *et al.* 2003). Our results indicate an alteration of CB1R expression and function in SHR brainstem astrocytes, and to a lesser extent in SHR cerebellar astrocytes as well. In order to ascertain whether changes in basal CB1R expression and phosphorylation have any effect on its downstream targets, we measured activation of MAPKs, ERK and p38, as downstream molecular indicators. ACEA treatment resulted in pronounced ERK and p38 activation in both Wistar brainstem and cerebellar astrocytes. The effect however, was markedly diminished in SHR brainstem astrocytes. This could be due to a reduction in CB1R expression and an elevation in phosphorylated forms of the CB1R. This effect is highly indicative of CB1R hypofunction. Since this finding was observed in the brainstems of prehypertensive SHRs, it is possible that this reduced endocannabinoid tone is contributing to the initiation of hypertension. Despite no marked variations in CB1R and p-CB1R levels in cerebellar astrocytes of SHRs, a partial dampening of CB1R-mediated ERK and p38 activation was observed. Perhaps, the CB1R-MAPK axis dampening in the cerebellum may not be associated with CB1R-mediated anti-inflammatory effects, and may be linked to other astroglial functions.

v) Co-activation of both RAS and endocannabinoid system in astrocytes from SHRs and Wistar rats

a) Effect of Co-treatment of Ang II with ACEA on neuroinflammatory cytokines

Several groups have reported evidence of crosstalk between AT1Rs and CB1Rs, resulting in either an enhancement or a reduction of AT1R's effects (Szekeres *et al.* 2012) (Rozenfeld *et al.* 2011). The CB1R has been shown to be vital to Ang II-mediated deleterious effects (Schaich *et al.* 2014) (Szekeres *et al.* 2012). Our results reveal that ACEA, a potent CB1R agonist, did not potentiate Ang II effects, but was capable of limiting Ang II's neurotoxic effect, specifically on IL-10 levels in Wistar brainstem astrocytes. This effect however was greatly diminished in SHR brainstem astrocytes. In cerebellar astrocytes, ACEA was capable of reducing Ang II-mediated effects on inflammatory cytokines. In either case, we did not observe synergistic or additive effects on co-stimulation of Ang receptors and CB1Rs. We conclude that activation of CB1R may well be a viable strategy for pathological conditions characterized by Ang II-mediated neuroinflammatory conditions.

b) Effect of co-treatment of Ang II with ACEA on MAPK activation

Crosstalk between Gi and Gq GPCRs potentiates Gq GPCRs, either by a heterodimerization-independent, or a heterodimerization-dependent interaction (Rozenfeld *et al.* 2011) (Carroll *et al.* 1995) (Rives *et al.* 2009). We investigated whether co-stimulation of CB1R with AT1R, a Gi- and a Gq- coupled GPCR, respectively results in a synergistic effect when compared to AT1R activation alone. Co-stimulation of AT1R and CB1R with Ang II and ACEA respectively, resulted in a decrease in Ang II-induced MAPK effect in brainstem astrocytes isolated from both neonatal SHRs and Wistar rats. In the case

of cerebellar astrocytes however, the decrease was much more prominent. This suggests a possible dampening of Ang II's effect on MAPK activation by cannabinoids.

6.3 Strengths and limitations

i) Strengths

The biggest strength of this study was that we employed multiple parameters to assess a single outcome. For instance, we investigated two signaling pathways, both CB1R and p-CB1R levels, as well as neuroinflammatory cytokines, to ascertain a potential CB1R dysregulation in brainstem astrocytes of SHRs. By employing prehypertensive SHRs, we are able to establish potential causality between the results of our study and the development of hypertension, which has been a severe drawback of several studies, including those that have employed adult SHRs (Doggrell and Brown 1998). In addition to brainstem astrocytes, parallel experiments were run in cerebellar astrocytes of both models. These experiments allowed us to compare the consequences of AT1R-CB1R crosstalk in regions of low CB1R, such as brainstem, with regions of relatively higher CB1R expression, such as cerebellum.

ii) Limitations

The major drawback of this study is the lack of *in vivo* data. Hence, we cannot confirm the therapeutic utility of targeting CB1R in hypertension at this moment. Also, in most of the cases, both Ang II and ACEA, had a much greater effect on cytokine mRNA levels, than at the level of the secreted fraction. It could well be that factors secreted from microglia or neurons, both of which are extremely low in our cell culture (Haspula and Clark 2016b), have a role in translation and secretion of interleukins.

6.4 Future perspectives

Longitudinal studies keeping track of inflammatory cytokines in SHRs brains, over different stages of hypertension, will help in developing a better understanding of the contribution of neuroinflammation to the development of hypertension. Although we did not observe a significant difference in the levels of inflammatory cytokines in cerebellar astrocytes, other neurotoxic mediators should also be investigated, especially since high levels of apoptotic factors and astrogliosis have been reported in SHR cerebellum (Yun *et al.* 2014). *In vivo* studies investigating the potential of cell type specific CB1R upregulation, would help in determining the impact of restoring astroglial CB1R functions in brainstem on the progression of hypertension. In addition, the role of centrally administered anti-inflammatory drugs in the brainstems of SHRs on potential improvement of cardiovascular parameters, could also be investigated. Possible synergistic effects of partial CB1R agonists and AT1R antagonists could well be a therapeutically viable option, and their synergistic or antagonistic effects need to be evaluated in both *in vitro* and *in vivo* conditions.

6.5 Significance

This is the first study to demonstrate dampening of the endocannabinoid system by Ang II, either by receptor downregulation, and/or by receptor phosphorylation (inactivation) in prehypertensive SHRs. Additionally, we observed a hypofunctional endocannabinoid system in the brainstem of SHRs, a region which is characterized by hyperactive RAS in SHRs. These mechanisms of endocannabinoid dampening by Ang II could be extremely important in pathological conditions that are characterized by an overactive brain RAS, as the counterregulatory mechanisms are reduced. This

encompasses not just cardiovascular disorders and their risk factors, but also several neurological impairments that have responded favorably to drugs that counteract the brain RAS, such as centrally acting ACEIs (Gao *et al.* 2013). Endocannabinoid dampening is a crucial link between RAS and neurological impairments, especially since CB1R hypofunction is a characteristic feature of several neurological diseases.

6.6 Therapeutic significance

Our findings suggest that Ang II can reduce the endocannabinoid tone by multiple mechanisms. This suggests that in instances of high RAS activity, neutralizing the effects of RAS could be a useful alternative strategy to boost basal endocannabinoid system activity. This would also isolate basal endocannabinoid tone elevation to only those regions of the brain that are characterized with excessive RAS activity. This is especially important since direct CB1R agonists, results in CB1R activation in unwanted brain regions and are therefore associated with several adverse effects. Thus, indirect activation of CB1R would bypass the need for direct CB1R agonists.

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APPENDIX 1

Supplementary Figures

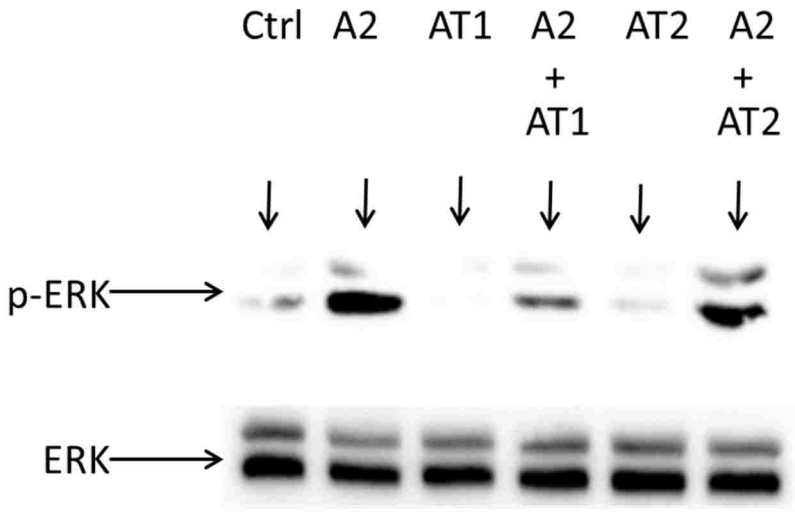

Fig 1S: Effect of Inhibitors on Ang II-mediated ERK activation in Wistar brainstem astrocytes: First lane (Ctrl) represents the untreated sample. Second lane (A2) represents the Ang II-treated sample. Third and fifth lanes represents samples that have been treated with 10 μM of AT1R inhibitor, Losartan (AT1), and AT2R inhibitor, PD123319 (AT2), each for 30 mins respectively. Fourth and Sixth lanes represents samples that have been treated with 10 μM of AT1 and AT2, each for 30 mins respectively, followed by A2 treatment for 15 mins.

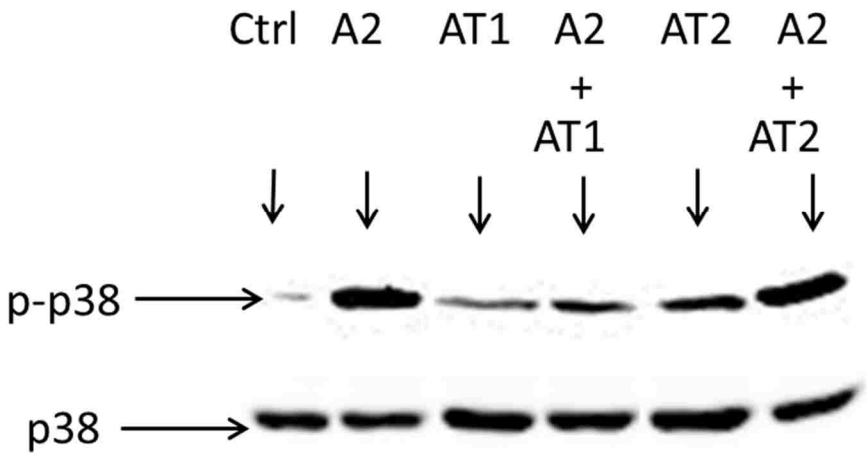

Fig 2S: Effect of Inhibitors on Ang II-mediated p38 activation: First lane (Ctrl) represents the untreated sample. Second lane (A2) represents the Ang II-treated sample. Third and fifth lanes represents samples that have been treated with 10 μM of AT1R inhibitor, Losartan (AT1), and AT2R inhibitor, PD123319 (AT2), each for 30 mins respectively. Fourth and Sixth lanes represents samples that have been treated with 10 μM of AT1 and AT2, each for 30 mins respectively, followed by A2 treatment for 15 mins.

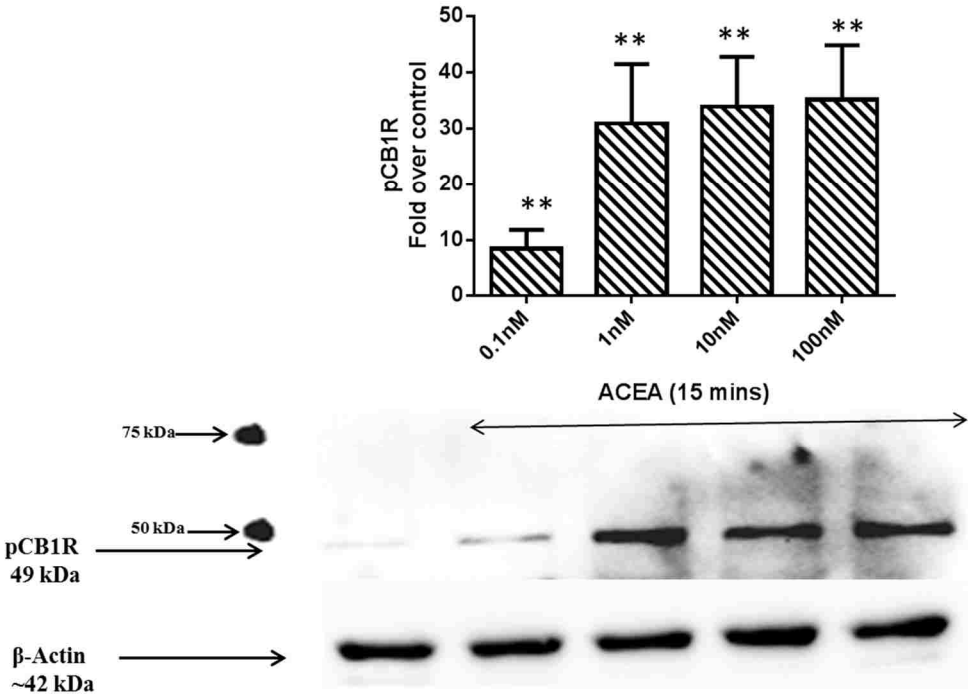

Fig 3S: Polyclonal p-CB1R antibody employed to compare p-CB1R levels in ACEA-treated samples. Lane 1 represents untreated sample. Lanes 2-5 represent samples that have been pretreated with increasing concentrations of ACEA. Concentrations range from 0.1 nM to 100 nM.

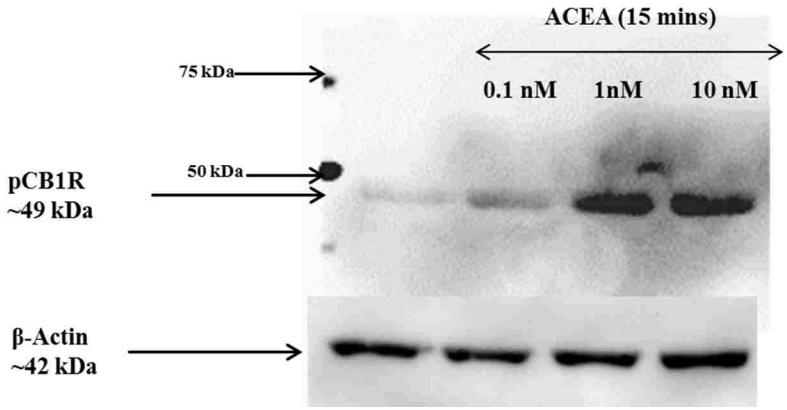

Fig 4S: Monoclonal p-CB1R antibody employed to compare p-CB1R levels in ACEA-treated samples. Lane 1 represents untreated sample. Lanes 2-4 represent samples that have been pretreated with increasing concentrations of ACEA. Concentrations range from 0.1 nM to 10 nM.

APPENDIX 2

Supplementary Tables

Treatment	Wistar (Fold over Basal)		SHR (Fold over Basal)	
	Brainstem	Cerebellum	Brainstem	Cerebellum
100 nM Ang II	2.25 ± 0.2*	2.27 ± 0.06*	2.28 ± 0.3*	2.3 ± 0.2*
10 µM Losartan	0.99 ± 0.1	0.92 ± 0.1	1.1 ± 0.1	1.3 ± 0.1
10 µM Losartan + 100nM Ang II	1.03 ± 0.1	0.89 ± 0.1	1.0 ± 0.2	1.1 ± 0.2
10 µM PD123319	0.96 ± 0.1	1.0 ± 0.1	1.0 ± 0.2	1.0 ± 0.2
10 µM PD123319 + 100nM Ang II	2.15 ± 0.2*	1.9 ± 0.2*	2.21± 0.05*	1.7 ± 0.1*

Table 1S: Effect of Angiotensin receptor blockers on Ang II-induced phosphorylation of CB1R. Each value represents the mean ± SEM of 5 or more litters of neonatal rat pups. (* $p < 0.05$- treated versus untreated samples).

Treatment	Wistar (Fold over Basal)		SHR (Fold over Basal)	
	Brainstem	Cerebellum	Brainstem	Cerebellum
100 nM Ang II	2.62 ± 0.2*	2.67 ± 0.06*	2.25 ± 0.2*	2.3 ± 0.1*
50 nM BIM I+ 100 nM Ang II	1.1 ± 0.1	1.12 ± 0.2	1.1 ± 0.3	1.2 ± 0.1
50 nM Orlistat + 100 nM Ang II	1.23 ± 0.2	2.1 ± 0.2*	1.4 ± 0.1*	1.8 ± 0.2*
50 nM BIM I	1.2 ± 0.2	0.95 ± 0.1	0.99 ± 0.1	1.3 ± 0.1
50 nM Orlistat	0.99 ± 0.1	1.3 ± 0.1	1.1 ± 0.2	1.3 ± 0.1

Table 2S: Effect of BIM I and orlistat on Ang II-induced phosphorylation of CB1R. Each value represents the mean ± SEM of 5 or more litters of neonatal rat pups. (* $p < 0.05$- treated versus untreated samples).

APPENDIX 3

Buffer Solutions

Tris	**4.85 g**
Sodium chloride	**58.44 g**
pH	*7.5*
Total volume	*2 liters with distilled water*

Table 3S (A): TBS for western blotting

Tris	**3 g**
Glycine	**14.4 g**
Methanol	**200 ml**
Total volume	*1 liter with distilled water*

Table 3S (B): Transfer buffer for western blotting

Sodium dihydrogen phosphate	**3.4 g**
Sodium monohydrogen phosphate	**10.2 g**
Sodium chloride	**17.5 g**
pH	*7.5*
Total volume	*2 liters with distilled water*

Table 3S (C): PBS for cell culture

APPENDIX 4

License agreement for chapter 3

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