Received Date : 07-Jul-2016

Revised Date : 08-Aug-2016

Accepted Date : 09-Aug-2016

Article type : Original Article

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

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Ang II effects on CB1Rs in astrocytes

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Abbreviations: ADHD, Attention Deficit Hyperactivity Disorder; AKT, Protein kinase b; ALAAC, Association for Assessment and Accreditation of Laboratory Animal Care International; Ang, Angiotensin; AT1R; Ang type 1 receptor; AT2R, Ang type 2 receptor; BCA,

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1111/jnc.13776

Bicinchoninic acid; CB1R, Cannabinoid Type 1 Receptor; CT, cycle threshold; DMEM/F12, Dulbecco's Modified Eagle Medium/ Nutrient Mixture F-12; ERK; FBS, Fetal Bovine Serum; GFAP, Glial Fibrillary Acidic Protein; NaCl, Sodium chloride; NaF, Sodium fluoride; $NaVO_4$, Sodium orthovanadate; PMSF, Phenylmethylsulfonyl fluoride; qPCR, quantitative PCR; RAS, Renin Angiotensin System; SHR, spontaneously hypertensive rat; STAT-5, Signal Transducers and Activator of Transcription-5; VSMC, vascular smooth muscle cells.

Abstract

Brainstem and cerebellar astrocytes have critical roles to play in hypertension and attention deficit hyperactivity disorder (ADHD), respectively. Angiotensin (Ang) II, via the astroglial Ang Type 1 receptor (AT1R), has been demonstrated to elevate pro-inflammatory mediators in the brainstem and the cerebellum. The activation of astroglial Cannabinoid Type 1 Receptor (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 Spontaneously Hypertensive Rats (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 renin angiotensin system 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.

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 (AEA) and 2-arachidonyl glycerol (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 type 1 cannabinoid receptor (CB1R) is one of the most abundant G protein-coupled receptors in the brain, and the type 2 cannabinoid receptor (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.* 2002) (Sheng *et al.* 2005). 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 the extracellular regulated kinase (ERK) (Chiang *et al.* 2013), protein kinase b (AKT) (Laprairie *et al.* 2013), and signal transducers and activators of transcription-5 (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 renin angiotensin system (RAS) is one of the hallmarks of essential hypertension (Veerasingham and Raizada 2003). The RAS comprises of the effector peptide angiotensin (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 spontaneously hypertensive rat (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.* 2010). 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 reactive oxygen species (Liu *et al.* 2011), 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 attention deficit hyperactivity disorder (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.* 2014). Not only was a reduced cerebellar volume reported in children diagnosed with ADHD (Castellanos *et al.* 1996), 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.* 2008). 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.* 2002). 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.

Materials and Methods

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).

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). Briefly, brains from 3-day old male and female rat pups were isolated and the cerebellum and brainstem were carefully separated from each brain. 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 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.

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). 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.

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.

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 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.

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™ 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™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.

Western Blotting

Volumes equivalent to 30µg of solubilized proteins were loaded into 10% polyacrylamide gels, and subsequently transferred to nitrocellulose membranes. 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.

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.

Results

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. 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. 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. 1C).

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. 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. 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 2C). The mean cycle threshold (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 2D). Mean Ct values for SHR cerebellum and Wistar cerebellum were 31.5 and 31.7, respectively.

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. 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 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.

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 Figure 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. 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.

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. 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. As shown in Fig 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.

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. 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. 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 was 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 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.

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 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.* 2014), CB1R activation in the brainstem has been demonstrated to lower blood pressure (Seagard *et al.* 2004) (Lake *et al.* 1997) (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.* 2010). 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.* 2012), 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 and/or downregulation 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.* 2005) (Molina-Holgado *et al.* 2002) (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).

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ARRIVE guidelines have been followed:
Yes
=> if No, skip complete sentence
=> if Yes, insert "All experiments were conducted in compliance with the ARRIVE
guidelines."

Conflicts of interest:  None
=> if 'none', insert "The authors have no conflict of interest to declare."
=> otherwise insert info unless it is already included
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Acknowledgments

We acknowledge the technical assistance of Ms. Nour Samra, Ms. Jenny Lee and Mr. Michael Dressler. This work was supported by a President's Faculty Research & Development Grant (#335309) and a Health Professions Grant (335585) from Nova Southeastern University.

Conflict of Interest:

The authors have no conflicts of interest to declare.

Figure Legends

Figure 1: Determination of the Purity of the Astrocyte Cell Culture.

In **Fig. 1A**, 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 cerebellum astrocytes isolated from at least 4 litters of neonatal rat pups. In **Fig. 1B**, a comparison of GFAP levels in cerebellar astrocytes was made with VSMC. Cell lysates from cerebellar astrocytes were loaded in lanes 1 and 2, and VSMC lysates were loaded in lanes 3 and 4. **Fig. 1C**, shows representative peaks obtained by flow cytometry showing the proportion of cells that expressed GFAP in our cell culture.

Figure 2: Basal CB1R Protein and mRNA Expression Levels in Brainstem and Cerebellum Astrocytes Isolated from SHRs and Wistar Rats.

In **Fig. 2A**, 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. In **Fig. 2B**, 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. Protein samples from 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. In **Fig. 2C**, a comparison of CB1R mRNA expression in brainstem astrocytes, isolated from SHRs and Wistar rats was made using qPCR. In **Fig. 2D**, 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 $p < 0.01$).

Figure 3:

a) Ang II Effects on CB1R Protein Expression in Brainstem Astrocytes Isolated from SHRs and Wistar Rats.

A comparison of CB1R protein levels from Wistar and SHRs brainstem astrocytes, which were pretreated with 100 nM Ang II for varying time periods, was made by employing Western blotting. 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).

b) Effect of Angiotensin Receptor Blockers on Ang II-mediated CB1R Protein Levels in Wistar and SHRs Brainstem Astrocytes.

A comparison of CB1R protein 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, was made by employing Western blotting. 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.

Figure 4:

a) Ang II Effects on CB1R mRNA Expression in Brainstem Astrocytes Isolated from SHRs and Wistar Rats.

A comparison of CB1R mRNA levels from Wistar and SHRs brainstem astrocytes, which were pretreated with 100 nM Ang II for varying time periods, was made by employing qPCR. 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).

b) Effect of Angiotensin Receptor Blockers on Ang II-mediated CB1R mRNA Levels in Wistar and SHRs Brainstem Astrocytes.

A comparison of 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, was made by employing qPCR. 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.

Figure 5:

a) Ang II Effects on CB1R Protein Expression in Cerebellum Astrocytes Isolated from SHRs and Wistar Rats.

A comparison of CB1R protein levels from Wistar and SHRs cerebellum astrocytes, which were pretreated with 100 nM Ang II for varying time periods, was made by employing Western Blotting. 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).

b) Effect of Angiotensin Receptor Blockers on Ang II-mediated CB1R Protein Levels in Wistar and SHRs Cerebellum Astrocytes.

A comparison of CB1R protein 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, was made by employing Western blotting. 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.

Figure 6:

a) Ang II Effects on CB1R mRNA Expression in Cerebellum Astrocytes Isolated from SHRs and Wistar Rats.

A comparison of CB1R mRNA levels from Wistar and SHRs cerebellum astrocytes, which were pretreated with 100 nM Ang II for varying time periods, was made by employing qPCR. 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).

b) Effect of Angiotensin Receptor Blockers on Ang II-mediated CB1R mRNA Levels in Wistar and SHRs Cerebellum Astrocytes.

A comparison of 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, was made by employing qPCR. 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.

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Figure 1: Determination of the Purity of the Astrocyte Cell Culture.

1A: GFAP, Itgam and Pecam1 mRNA levels

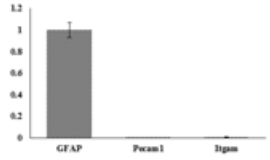

Figure 1: Determination of the Purity of the Astrocyte Cell Culture.

1B: GFAP Levels in Cerebellum Astrocytes and VSMC

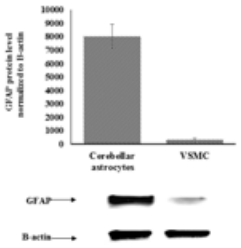

Figure 1: Determination of the Purity of the Astrocyte Cell Culture.

Figure 1C: GFAP Expression

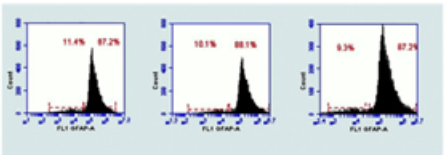

Figure 2: Basal CB1R Protein and mRNA Expression Levels in Brainstem and Cerebellum Astrocytes Isolated from SHRs and Wistar Rats.

2A: CB1R Protein in Rat VSMC, Cerebellum Astrocytes and Extract

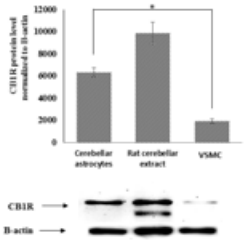

Figure 2: Basal CB1R Protein and mRNA Expression Levels in Brainstem and Cerebellum Astrocytes Isolated from SHRs and Wistar Rats.

2B: CB1R Basal Protein Expression in Brainstem and Cerebellum Astrocytes

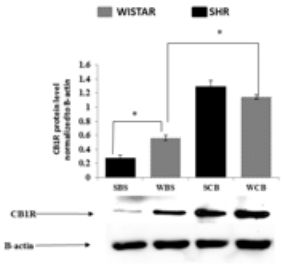

Figure 2: Basal CB1R Protein and mRNA Expression Levels in Brainstem and Cerebellum Astrocytes Isolated from SHRs and Wistar Rats.

2C: CB1R mRNA Expression in Brainstem Astrocytes

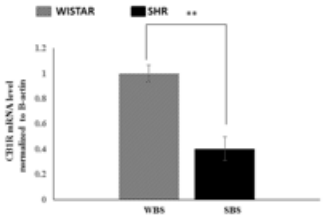

Figure 2: Basal CB1R Protein and mRNA Expression Levels in Brainstem and Cerebellum Astrocytes Isolated from SHRs and Wistar Rats.

2D: CB1R mRNA Expression in Cerebellum Astrocytes

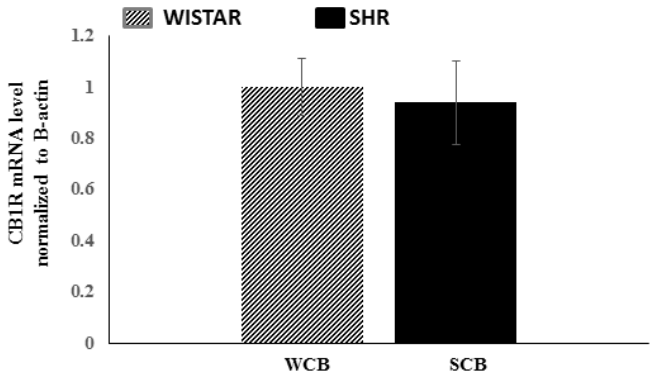

Figure 3A: Ang II Effects on CB1R Protein Expression in Brainstem Astrocytes Isolated from SHRs and Wistar Rats.

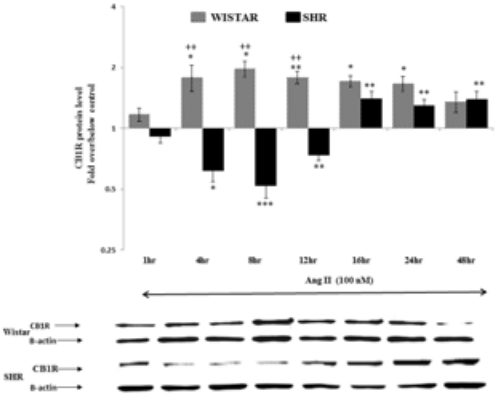

Figure 3B: Effect of Angiotensin Receptor Blockers on Ang II-mediated CB1R Protein Levels in Wistar and SHRs Brainstem Astrocytes.

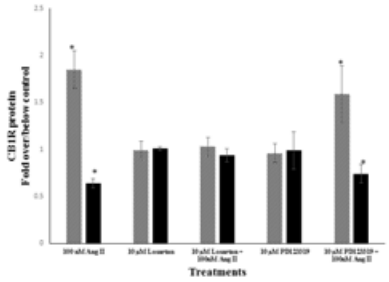

Figure 4A: Ang II Effects on CB1R mRNA Expression in Brainstem Astrocytes Isolated from SHRs and Wistar Rats.

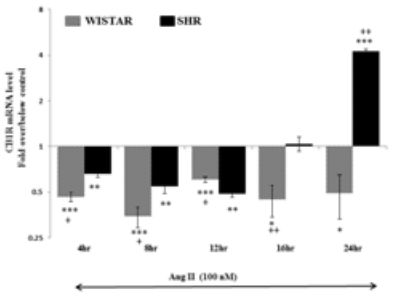

Figure 4B: Effect of Angiotensin Receptor Blockers on Ang II-mediated CB1R mRNA Levels in Wistar and SHRs Brainstem Astrocytes.

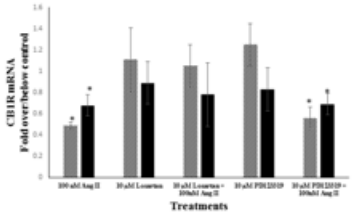

Figure 5A: Ang II Effects on CB1R Protein Expression in Cerebellum Astrocytes Isolated from SHRs and Wistar Rats.

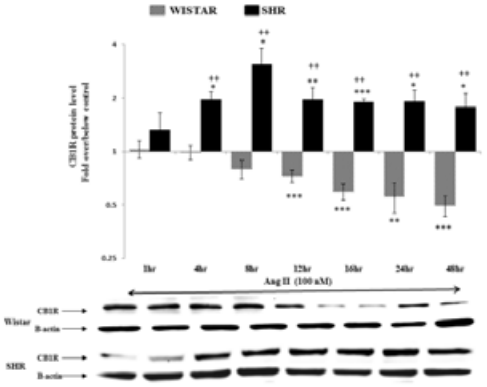

Figure 5B: Effect of Angiotensin Receptor Blockers on Ang II-mediated CB1R Protein Levels in Wistar and SHRs Cerebellum Astrocytes.

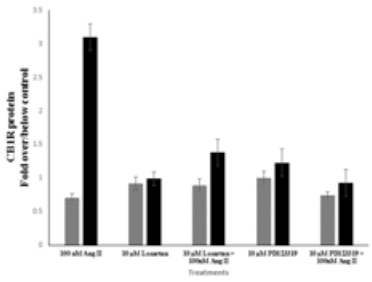

Figure 6A: Ang II Effects on CB1R mRNA Expression in Cerebellum Astrocytes Isolated from SHRs and Wistar Rats.

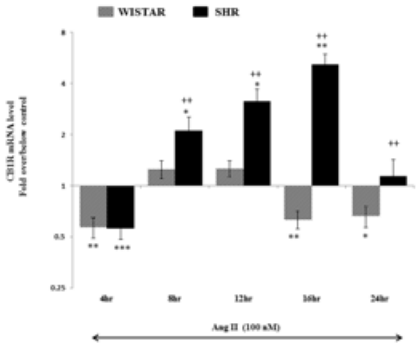

Figure 6B: Effect of Angiotensin Receptor Blockers on Ang II-mediated CB1R mRNA Levels in Wistar and SHRs Cerebellum Astrocytes.