Role of Circulating Angiotensin II in Activation of Aldosterone production in the Central Nervous System

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To those who dream and make a difference

Abstract

Elevated circulating Ang II activates neurons in the forebrain cardiovascular regulatory areas to cause sympatho-excitation and hypertension. We hypothesized that circulating Ang II causes neuronal activation in the SFO and thereby activates efferent pathways to the PVN, and chronically causes activation of aldosterone production in magnocellular neurons in PVN and SON, which amplifies neuronal activation in the PVN and central sympatho-excitatory pathways. The aim of the present study was to determine the pattern of neuronal activation in forebrain nuclei by circulating Ang II and to elucidate where in the hypothalamus Ang II may stimulate aldosterone biosynthesis. Dose related effects of circulating Ang II on BP were first assessed. Wistar rats instrumented with telemetry probes were infused subcutaneously with Ang II 150 and 500 ng/kg/min for 14 days. The subcutaneous (sc) infusion of Ang II at 150 ng/kg/min increased blood pressure gradually up to 20 mmHg and at 500 ng/kg/min up to 60 mmHg. Ang II at 500 ng/kg/min increased plasma Ang II by 4-fold. To assess effects of circulating Ang II on CNS pathways, Wistar rats were implanted subcutaneously with minipumps infusing 150 and 500 ng/kg/min Ang II for 1, 4 and 14 days. Three patterns of neuronal activation were observed by sc infusion of Ang II. The SFO was activated during the first day and remained activated for 4 days, but at 14 days showed diminished activation. MnPO did not show significant activation during the first day but, after several days the activation was high and then less by 14 days. Parvocellular PVN (pPVN), magnocellular PVN (mPVN) and SON showed an initial activation that increased over time. Chronic intracerebroventricular infusion of an aldosterone synthase inhibitor or a mineralocorticoid receptor (MR) blocker attenuated the increase in Fra expression in

PVN but not SON, and prevented the decrease in SFO after 14 days infusion of Ang II. A significant increase in mRNA expression of steroidogenic acute regulatory protein (StAR), a rate limiting enzyme in aldosterone production was found in glia cells of PVN and SFO assessed by rt-PCR after 3 days subcutaneous infusion of Ang II at 500 ng/kg/min. Total expression of aldosterone synthase (CYP11B2) mRNA was increased in SFO, MnPO, SON and PVN after 3 days of infusion of Ang II. After 14 days no significant changes were observed in the expression of StAR or CYP11B2 mRNA. In comparison, in adrenal StAR mRNA expression increased after 3 days but no longer after 14 days. In contrast, CYP11B2 mRNA expression in adrenal increased after both 3 and 14 days of infusion. These findings support our hypothesis that chronic elevation of circulating Ang II increases neuronal activity in CVOs, presumably leading to activation of the PVN and SON to induce an increase in aldosterone production in magnocelular PVN and SON. In the second phase activation of CVOs appears to diminish, but an aldosterone-dependent amplifying mechanisms, causes sustained activation of the PVN and thereby hypertension.

Table of content

List of Tables

List of Figures

Figure R7. Photomicrograph of coronal sections through the SON.

Figure R8. Time related changes in Fra expression in the SON.
Figure R9. Photomicrograph of coronal sections through the PVN.

Figure R10. Time related changes in Fra expression in the pPVN.

Figure R11. Time related changes in Fra expression in the mPVN.

Figure R12. Effects of central blockade of MR or aldosterone synthase on Fra expression in the mPVN, pPVN, SFO, SON and MnPO.

Figure R13. Time course of changes in mRNA expression for StAR in adrenal.

Figure R14. Time course of changes of mRNA expression for CYP11B2 and CYP11B1 in adrenal.

Figure R16. Changes in mRNA expression for GFAP in hypothalamic nuclei by Ang II.

Figure R17. The relative amount of neuron versus glia in hypothalamic nuclei.

Figure R18. Time course of changes of mRNA expression for StAR in SFO.

Figure R19. Time course changes of mRNA expression for CYP11B2 and CYP11B1 in SFO by Ang II.

Figure R20. Time course of changes of mRNA expression for StAR in OVLT by Ang II.

Figure R21. Time course of changes of mRNA expression for CYP11B1 and CYP11B2 in OVLT by Ang II.

Figure R22. Time course of changes of mRNA expression for StAR in MnPO by Ang II.

Figure R23. Time course of changes of mRNA expression for CYP11B2 and CYP11B1 in MnPO by Ang II.

Figure R24. Time course of changes of mRNA expression for StAR in SON by Ang II.

Figure R25. Time course changes of mRNA expression for CYP11B2 and CYP11B1 in the SON.

Figure R26. Time course of changes of mRNA expression for StAR in PVN by Ang II.

Figure R27. Time course of changes of mRNA expression for CYP11B2 and CYP11B1 in PVN.

Figure R28. Time course of changes of mRNA expression for StAR and CYP11B1 in hippocampus by Ang II.
Figure R29. Summary of the time course of changes of mRNA expression for StAR by Ang II.

Figure R30. Summary of the time course of changes of mRNA expression for StAR in glia of hypothalamic nuclei by Ang II.

Figure R31. Summary of the time course of changes of mRNA expression for CYP11B2 by Ang II.

Figure D1. Proposed pathways for dual activation of pPVN.

List of Abbreviations

3β-HSD:	3β-hydroxysteroid dehydrogenase
ACE:	Angiotensin Converting Enzyme
aCSF:	Artificial CerebroSpinal Fluid
ACTH:	Adrenocoritotropic Hormone
Ang II:	Angiotensin II
AP:	Area Postrema
AP-1:	Activating Protein-1
APA:	Aminopeptidase A
APB:	Aminopeptidase B
APN:	Aminopeptidase N
AS:	Aldosterone Synthase
AT_1-R:	Angiotensin Receptor Type 1
AV3V:	Anteroventral Third Ventricle
AVP:	Arginin Vasopressin
BBB:	Blood Brain Barrier
BNST:	Bed Nucleus of Stria Terminalis
BP:	Blood Pressure
cAMP:	Cyclic Adenosine Monophosphate
CHF:	Chronic Heart Failure
CNS:	Central Nervous System
CSAR:	Cardiac Sympathetic Afferent
CVOs:	Circumventricular Organs

CYP11B1: Cytochrome P450 11 B1, 11-β hydroxylase

CYP11B2: Cytochrome P450 11 B2, Aldosterone Synthase

DOC: 11-deoxycorticosterone

ELISA: Enzyme-Linked ImmunoSorbent Assay

ENaC: Epithelial Sodium Channel

Fra: Fos Related Antigen

GABA: Gamma-AminoButyric Acid

GFAP: Glial Fibrillary Acidic Protein

GR: Glucocorticoid Receptor

HPLC: High Pressure Liquid Chromatography

ICV: Intracerebroventricular

IEG: Immediate Early Gene

ILM: Intermediolateral

IV: Intravenous

LV: Left Ventricle

MAP: Mean Arterial Pressure

MI: Myocardial Infarction

MnPO: Median Preoptic Nucleus

MR: Mineralocorticoid Receptor

NE: Norepinephrine

NMDA: N-Methyl-D-Asparate Receptor

NO: Nitric Oxide

NOS: Nitric Oxide Synthase

NTS: Nucleus Tractus Solitarius

OVLT: Organum Vasculosum of Lamina Terminalis

$P450_{scc}$: P450 Side Chain Cleavage

PGE_2: Prostaglandin E_2

PGK: Phosphoglycerate Kinase

PGP: Protein Gene Product

PVN: Paraventricular Nucleus

RAAS: Renin Angiotensin Aldosterone System

ROS: Reactive Oxygene Species

RSNA: Renal Sympathetic Nerve Activity

RVLM: Rostral Ventrolateral Medulla

SBP: Systolic Blood Pressure

SC: Subcautenous

SFO: Subfornical Organ

SNS: Sympathatic Nervous System

SON: Supraoptic Nucleus

StAR: Steroidogenic Acute Regulatory Protein

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1. Introduction

Overview

Heart Failure and central nervous system (CNS)

Following myocardial infarction (MI) with loss of cardiac myocytes, a series of responses, such as apoptosis and fibrosis occurs that affect the non-infarcted zone of the left ventricle and the right ventricle. These ventricular remodeling processes lead eventually to ventricular dilation, infarct expansion and loss of viable myocardium, all in the long term contributing to progressive left ventricular (LV) dysfunction and CHF (Lal et al. 2004; Sun et al. 2000).

A number of local and systemic mechanisms have been implicated in myocardial remodeling and dysfunction. Sympathetic activity increases in parallel to LV dysfunction (Rundquvist et al. 1997). Sympathetic hyperactivity contributes by hemodynamic effects, by increasing renal renin release and thereby plasma angiotensin II and aldosterone and by direct cardiac effects (Packer 1992). There is still limited knowledge related to the central factors determining activation of the sympathetic nervous system post MI. Our group has reported that activation of an aldosterone-ouabain neuromodulatory pathway and resulting increase in angiotensin receptors type 1 (AT_1-R) stimulation is responsible for the chronic increase in sympathetic activity and the increase in activity of circulating and cardiac renin angiotensin aldosterone system (RAAS) in chronic phase post MI.

Firstly, in rats, chronic central infusion of aldosterone increases “ouabain” (digitalis-like substances) in the hypothalamus (Wang et al. 2003), as well as increases AT_1-R mRNA and protein expression (Zhang et al. 2008), which can be prevented by central infusion of fab fragments to bind ouabain (Huang et al. 2008). The sympathetic hyperactivity caused

by central infusion of aldosterone or ouabain can be prevented by central AT_1-R blocker (Huang et al. 1996, Gabor et al. 2009).

Secondly, blockade of aldosterone synthase, mineralocorticoid receptors (MR), "ouabain" and AT_1-R specifically in the CNS similarly prevent sympathetic hyperactivity (Huang et al. 2005, 2008; Lal et al. 2004) and attenuate cardiac remodeling and LV impairment post MI (Wang et al. 2004; Lal et al. 2005).

Altogether, these findings have demonstrated that in rats post MI aldosterone produced locally in the brain binds MR and increases release of "ouabain". Increased "ouabain" enhances Ang II release and/or AT_1 receptor responsiveness in the CNS leading to sympathetic hyperactivity and progressive cardiac remodeling (Huang et al. 2005).

However, the mechanisms by which aldosterone and "ouabain" production in the CNS are increased post MI, are not clear yet.

Stimuli for Activation of central pathways Post MI

The CNS acts as a conductor receiving and integrating peripheral inputs from various sources in the body, which activate CNS pathways and increase peripheral outputs (Leenen 2007).

Post MI, circulatory RAAS becomes rapidly activated (Leenen et al. 1999). Circulating Ang II may activate neurons in the forebrain circumventricular organs (subfornical organs (SFO) and organum vasculosum of lamina terminalis (OVLT)) that project to paraventricular nucleus (PVN) and supraoptic nucleus (SON) (Rowland et al. 1995). The relative role of circulating Ang II in activating the CNS post MI has not yet been assessed.

Post MI, cardiac vagal or sympathetic afferent fibers (CSAR) conveying mechanosensitive and chemosensitive information to the CNS are activated and through AT_1-receptors in the PVN contribute to sympathetic excitation (Zhu et al. 2004). CSAR sensitivity becomes enhanced in dogs with pacing-induced CHF and bilateral microinjection of AT_1-receptor antagonist losartan normalized the enhanced CSAR in rats.

As a third mechanism, afferent fibers from arterial baroreceptors located in aortic arch and carotid sinuses convey mechano-sensitive and baro-sensitive information to the CNS that terminates in nucleus tractus solitarius (NTS). In response to hypotension induced by LV dysfunction, disinhibition of presympathetic neurons in the rostral ventrolateral medulla (RVLM) due to reduced baroreceptor inhibition results in excitatory inputs to preganglionic spinal neurons (Zhu et al. 2004). However, arterial baroreceptors reset and this mechanism may only play initially post MI a role.

Recent studies by Felder's group suggest that increased levels of circulating cytokines post MI inform the brain of the cardiac injury. Cytokines are too large to cross the brain-blood barrier (BBB), but may activate the vasculature of the BBB of the PVN to induce COX-2 activity and the synthesis of prostaglandin E_2 (PGE_2). PGE_2 may enter the brain resulting in enhanced pro-inflammatory cytokines in the PVN and sympathoexcitation (Rivest et al. 2000).

We hypothesize that circulating Ang II is a major factor for activation of CNS mechanisms post MI. Acute response may be mediated by a rapid pathway starting from the SFO that projects to PVN and eventually activate presympathetic neurons in RVLM.

Chronically, this pathway may be enhanced/amplified by activation of the aldosterone-"ouabain" pathway.

The following aspects will be discussed in this literature review:

1. Cardiovascular regulatory centers (anatomy and connections)
2. Neurosteroid and neuropeptide production in forebrain nuclei.
3. Central effects of circulating Ang II on neuronal activation, blood pressure (BP) and sympathetic activity
4. Aldosterone synthesis in CNS

1.1. Cardiovascular regulatory centers

Overview

Autonomic centers are protected behind the BBB, but neurons in these centers are influenced by circulating regulatory peptides such as angiotensin II, acting on neurons in nuclei outside the BBB. The anatomical features of the BBB consist of endothelial cells of cerebral microvessels joined together by tight junction. Also the end feet of astrocytes wrap around the brain side of the endothelial cell membrane. The BBB limits movements across the cell membrane to lipophilic substances (that readily diffuse across the lipid bilayer) or peptides that have specific transport systems. Large hydrophilic molecules such as peptides and proteins that do not have a specific transport system are excluded from the CNS (Abbott et al. 2006).

1.1.1. Circumventricular organs

CVOs are a group of specialized CNS structures that are characterized by the lack of the normal BBB. These structures can therefore sense and respond to circulating lipophobic substances. In addition to fenestrated capillaries, CVOs contain dense aggregations of a variety of different receptors for peripheral signals including regulatory peptides (Mendelsohn et al. 1984, Pulman et al. 2006, Smith et al. 2009). CVOs implicated in blood pressure control include OVLT, SFO and the area postrema (AP) (Brody et al. 1980). The OVLT and SFO are located within the anteroventral third ventricle (AV3V) region which plays an important role in neuronal and neuro-hormonal control of body fluid and cardiovascular homeostasis (Johnson et al. 2003).

1.1.1.A. Subfornical Organ (SFO)

SFO is located in the forebrain on the midline wall of the third ventricle at the top of the lamina terminalis. The SFO comprises two distinct regions, a core region and a peripheral outer zone, which have different neuronal projections (McKinley et al. 1998, Fry and Ferguson 2007). SFO consists of neuronal cell bodies that have compact dendritic trees and do not receive extensive neural inputs (Dellman and Simpson 1979) which is consistent with the role of this region in receiving afferent information from the circulation. Limited afferent inputs to the SFO originate from the same areas that receive SFO efferents, including median preoptic nucleus (MnPO), lateral hypothalamus (Lind et al. 1984), OVLT (Cottrell et al. 2004), as well as the NTS (Tanaka et al. 1993, Shioya et al. 1989). SFO sends direct efferent projections to hypothalamic autonomic and neuroendocrine centers, such as PVN and SON (Lind et al. 1982). Specific excitatory projections have been found in vasopressin and oxytocin secreting magnocellular neurons

in the SON and PVN, as well as parvocellular neurons of the PVN which project to median eminence, medulla or spinal cord (Ferguson et al. 1996). The SFO also sends efferents to neurons in the MnPO and to the OVLT both of which send additional projections to the SON and the PVN (Miselis et al. 1979).

Double-labeled neurons showed about 60% of the neurons express AT_1 receptors in the core of the SFO and 34% in the periphery of the SFO (Grob et al. 2004). Neurons in the core of the SFO have the highest densities of the AT_1R (Giles et al. 1999, Lenkei et al. 1997) and respond to relatively low concentrations of Ang II (35 ng/kg per min, iv) (McKinley et al. 1998). AT_1R in the outer part (periphery) of the SFO are in fewer numbers and are responsive to higher concentrations of circulating Ang II (58 ng/kg per min) (McKinley et al. 1992). This part of the SFO has numerous efferent connections to the SON, PVN and MnPO and lateral hypothalamus (McKinley et al. 1998, Sunn et al. 2001, Oldfield et al. 1994). Angiotensin-sensitive neurons in the periphery of the SFO have direct efferent axonal projections to magnocellular neurons of SON which mediate Ang II-stimulated vasopressin release (Oldfield et al. 1994). There is also electrophysiological evidence for the existence of a pathway from SFO to magnocellular vasopressin secreting neurons via synapse in MnPO (Tanaka et al. 1989).

The PVN receives projections originating from the outer shell of the SFO while OVLT as well as bed nucleus of stria terminalis (BNST) receive fibers from the core part of the SFO (Swanson and Lind 1986, McKinley et al. 2003). More recent retrograde and anterograde tracing studies showed that the SFO projects to both magnocellular PVN and parvocellular PVN and indicate that not only the periphery of SFO but also the core of SFO project to the PVN. However, the core of SFO preferentially projects to the

parvocellular PVN and the periphery of the SFO to the magnocellular PVN (Kawano et al. 2010).

1.1.1.B. Organum vasculosum of lamina terminalis (OVLT)

The OVLT is located at the bottom of the lamina terminalis. Tracer studies indicate that most inputs to the OVLT are from the MnPO, SFO and NTS (Phillips et a. 1987, Armstrong et al. 1996). The SFO and OVLT share a number of efferent targets. The two CVOs are also connected reciprocally with each other. The OVLT also sends efferent fibres to both magnocellular and parvocellular regions of the PVN, and to the SON (McKinley et al. 1999, Miselis et al. 1981).

The information from CVOs may relay to downstream nuclei directly or via synapses in MnPO. Immunoelectronmicroscopy and anterograde tracing studies have shown neuronal links from SFO and OVLT to the SON and PVN through MnPO (Oldfield et al. 1990).

OVLT is rich in AT_1R. Neurons around the lateral margins of the OVLT have the highest densities of the AT_1R and appear to be most responsive to Ang II (McKinley et al. 1998). Neurons in this region have been shown by retrograde tracing studies to send efferent projections to BNST. These pathways may mediate central effects of circulating Ang II in stimulation of sodium appetite (Johnson et al 1999, Zardetto et al. 1994). AT_1 receptors have a lower density in the dorsal part of the OVLT. Neurons in dorsal cap project to the SON and PVN (Oldfield et al. 1994), either direct or indirect through synapses in MnPO (Tanaka et al. 1989).

1.1.2. Median preoptic nucleus (MnPO)

The MnPO occupies the mid part of the lamina terminalis region, being interrupted half way along its length by the anterior commissure (McKinley et al. 1999). MnPO receives

afferents inputs from both SFO and OVLT and sends efferents fibers to both magnocellular and parvocellular region of PVN and SON (Miselis et al. 1981).

AT_1R mRNA expression in MnPO has been shown by in situ hybridization (Lenkei et al. 1998, Bunnemann et al. 1992) and the presence of AT_1 receptors in MnPO was demonstrated by immunohistochemical staining (Mollenhoff et al. 2001, Phillips et al. 1993).

1.1.3. Supraoptic nucleus (SON)

The SON is a paired nucleus located rostrally in the anterior hypothalamus in a ventrolateral position above the optic chiasm (Angel et al. 1992). The SON is a compact group of magnocellular neurons with projections through the lateral and basal hypothalamus to the neuronal lobe of the pituitary gland for release of neurohormones into the circulation. In rats it contains more magnocellular neurons than the PVN.

Most studies have shown that neurons of SON project only to the neurohypophysis. However more recent electrophysiological studies have found that stimulation of SON neurons provokes spikes in OVLT and suprachiasmatic nucleus (Trudel et al. 2003, Inyushkin et al. 2009). This may indicate possible efferent projections from the SON to these nuclei. Immunocytochemical studies have demonstrated the presence of AT_1R in the cytoplasm and at the cell membrane of magnocellular neurons in the SON (Pfister et al. 1997). Thomas et al found immunohistochemical staining of AT_1R in magnocellular vasopressin and oxytocin releasing neurons. However the majority of the neurons had faint or no staining at all (Thomas et al. 2006).

1.1.4. Paraventricular nucleus (PVN)

The PVN is part of a group of midline thalamic nuclei adjacent to anterior wall of the third ventricle. Efferent inputs to the PVN from many important nuclei such as SFO, MnPO and the NTS and RVLM have been identified (Swanson et al. 1980, Coote et al. 1995). The PVN is a bilateral periventricular structure in the hypothalamus that contains magnocellular and parvocellular neurons. Magnocellular neurons send their axonal projections through the lateral and basal hypothalamus to the neurohypophysis (posterior pituitary) (Tasker et al. 1991, Swanson and Sawchenco 1980). Parvocellular subgroup consists of neurons subdivided into dorsal, ventral, medial and the periventricular subdivisions of the PVN (Figure 1.) Ventral and medial parvocellular neurons project to the median eminence and release various signalling peptides into the median eminence. These peptides referred as releasing and release-inhibiting hormones are carried by the pituitary portal system to anterior pituitary where they regulate the release of pituitary hormones that control endocrine tissues. The dorsal parvocellular subdivision contains a population of neurons that send their axons to preautonomic cells in medulla (RVLM) or spinal cord (intermediolateral cells) that regulate autonomic control centers (Saper et al. 1976, Swanson et al. 1977, Ferguson et al. 2008). Retrograde neuronal labelling showed that the NTS receives extensive inputs from the PVN. The greatest concentrations of neurons that project to NTS are in medial parvocellular neurons and lesser amounts are in dorsal parvocellular subdivisions. A few labelled neurons were also present in magnocellular subdivision (Geerling et al. 2010).

High immunoreactivity of Ang II receptors in PVN has been found (Obermuller et al.1991, Lenkei et al. 1997). The AT_1R are predominantly in the parvocellular

subdivision of PVN. However, the AT_1 receptors were not found on parvocellular neurons projecting to RVLM and spinal cord but were found strongly on neurons in the anterior parvocellular division of the nucleus which direct axons to the median eminence (Oldfield et al. 2001). Although AT_1 receptors were not detected on spinally projecting neurons, electrophysiological studies showed that Ang II significantly increases the excitability of spinally projecting neurons which is eliminated by GABA receptor antagonist. Attenuation of the inhibitory GABAergic synaptic input is likely mediated by activation of AT_1 receptors on GABAergic interneurons located presynaptically to spinally projecting parvocellular neurons. Attenuation of GABA release from the terminals of GABAergic interneurons results in activation of glutamatergic interneurons that through glutamate release activates spinally projecting parvocelular neurons (Li et al. 2003, Li et al. 2005). AT_1 receptors were found to a lesser degree in magnocellular vasopressin and oxytocin releasing neurons of PVN (Thomas et al. 2006). Ang II induced depolarization of magnocellular neurons is abolished by a glutamate antagonist, suggesting that AT_1 receptor activation stimulates glutamate release from interneurons (Latchford and Ferguson 2004).

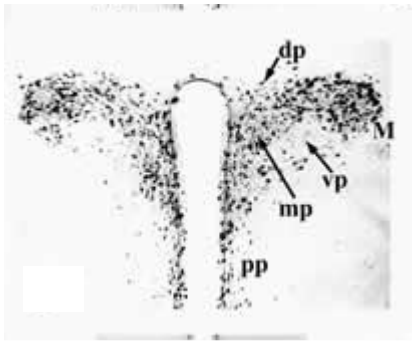


Figure 1. Subdivisons of the PVN. M = magnocellular division of the PVN, dp = dorsal parvocellular subdivision, mp = medial parvocellular subdivision, pp = periventricular parvocellular subdivision, vp = ventral parvocellular subdivision of the PVN. *Journal of Comparative Neurology 1980.*

1.2. Neurosteroids and neuropeptides in forebrain nuclei

1.2.1. Angiotensinergic pathways

The first and rate limiting step in the production of all subsequent angiotensin peptides is the enzymatic cleavage of angiotensinogen by renin (Fischer-Ferraro et al. 1971). Renin activity and immunoreactivity are high in the hypothalamus (Bader et al. 2001) but renin mRNA is present in very low concentrations (Baltatu et al. 1998, Karamyan and Speth 2007). Renin and angiotensin expressing cells have been demonstrated in SFO, PVN and RVLM (Lavoie et al. 2004). Renin expression in brain in transgenic mouse was shown in neurons in hypothalamus, hippocampus, cortex and medulla (Allen et al. 2008, Castrop et al. 2006). Receptors of renin and its precursor prorenin, the (pro)renin receptor, have been identified with high levels of expression in neurons of rat hypothalamus and brainstem (Shan et al. 2008, Nguyen et al. 2002). The prorenin receptor by binding to prorenin activates the enzyme activity of renin resulting in generation of Ang II at the cell surface (Danser et al. 2007, Ichihara et al. 2007, Nguyen et al. 2007). In the adult mouse brain the (pro)renin receptor is widely expressed in the SFO, PVN, AP, NTS and the RVLM (Contrepas et al. 2009).

Angiotensinogen mRNA was first reported to colocalize with glial fibrillary acidic protein (GFAP) positive astrocytes but not with neurons in rat brain (Davisson et al. 1998) and constitutively secreted by astrocytes into the interstitial space and cerebrospinal fluid (Deschepper et al. 1986, Thomas et al. 1988, Stornetta et al. 1988). However, neurons in the CNS also produce angiotensinogen. Angiotensinogen production in pure rat neuronal cell cultures has been reported (Thomas et al. 1992). Immunohistochemical analysis demonstrated the presence of angiotensinogen in both cell types in the rat brain (Thomas et al. 1988). Transgenic mouse models demonstrated that

the angiotensinogen gene is expressed not only in astrocytes but also in neurons. Angiotensinogen is however, predominantly synthesized in astrocytes (Yang et al 1999). The presence of angiotensinogen has been demonstrated in magnocellular neurons of the PVN, SON as well as in NTS and SFO (Aronsson et al. 1988, Fleix and Michelini 2007, Maximino et al. 2006).

Renin metabolizes its only substrate, angiotensinogen, to liberate the decapeptide angiotensin (Ang I). Ang I is rapidly converted by angiotensin-converting enzyme (ACE) to octapeptide Ang II. In rats, rabbits and humans ACE mRNA, protein and binding were found in SON, PVN, MnPO, SFO, OVLT (Chai et al. 1990, Chai et al. 1987, Yu et al. 2007, Lu et al. 2009, Ahmad et al. 2008, Tan et al.2004) NTS and RVLM (Kar et al. 2010). ACE binding densities were high in SFO and to a lesser degree in PVN and OVLT. MnPO showed the least density for ACE (Tan et al. 2004, Cheung et al. 2006). Ang II can be produced in neuron cytoplasm and is stored in vesicles for axonal transport to act at the axonal terminals (Pan et al.2004). It has also been suggested that Ang II produced in the interstitium can be taken up in nerve terminals and then released as a neurotransmitter (McKinley et al. 2003). Ang II can be converted to 2-8 fragment of Ang II called Ang III via the enzyme aminopeptidase A (APA) and Ang III further to a shorter fragment 3-8 called Ang IV by aminopeptidase N, aminopeptidase B (APB) or both (Figure 2) (Vauquelin et al. 2002). In addition, ACE can convert Ang I directly to Ang III (Wright and Harding 1997, Speth et al. 2003). A number of studies suggest that the active form of angiotensin with respect to central control of cardiovascular functions is Ang III, and that Ang II must be converted to Ang III to activate the AT_1 receptor subtype (Wright and harding 1995, Reaux et al. 1999). Icv administration of an inhibitor of APA, reduced

Ang II-induced pressor responses, presumably by inhibiting its conversion to Ang III. In contrast, pretreatment with an inhibitor of APB, potentiated pressor responses to Ang II (Wright and Harding 1990). ACE2 cleaves phenylalanine from the carboxyterminal end of the octapeptide Ang II to form the heptapeptide Ang-(1-7) with an affinity 400-fold greater than for Ang II (Vickers et al. 2002). Both ACE2 mRNA and protein are present in regions involved in the central regulation of cardiovascular function (Doobay et al. 2007). Ang-(1-7) through activation of nitric oxide synthase (NOS) increases nitric oxide (NO) release in the brain (Kalka et al. 1994) and thereby decreases sympathetic activity and BP (Paton et al. 2002, Alzamora et al. et al. 2002, Kishi et al. 2003).

Neuronal cell bodies showing Ang-like immunoreactivity have been observed in only a few brain regions such as the SFO, NTS, and the PVN (Chappell et al. 1987, Lind et al. 1984). Ang II immunoreactive nerve fibers have been identified in the SFO, MnPO, SON and PVN. Ang II immunoreactive cell bodies were most prominent in magnocellular parts of the PVN and SON but were also found in parvocellular parts of the PVN (Lind et al. 1985). The RVLM contains Ang II–immunoreactive nerve terminals and high density of AT_1 receptors (Song et al. 1991, 1992, Aldred et al. 1993) and Ang II sensitive neurons that are excited by Ang II via AT_1 receptors (Li and Guyenet 1996).

Studies combining immunohistochemistry with retrograde transport identified the perifornical zone of the lateral hypothalamus, as the source of Ang II stained inputs to the SFO, and the region of the MnPO as a recipient of Ang II immunoreactive SFO efferents (Lind et al. 1984). Ang II was also localized in SFO neurons projecting to the PVN (Lind et al. 1985, Osborn et al. 2007). Electrophysiological studies demonstrated that activation of this pathway resulted in increases in excitability of both magnocellular and

parvocellular neurons in the PVN (Ferguson et al. 1984). Microinjection of Ang II into the SFO significantly raises Ang II release in the PVN (Wright et al. 1993). AT_1R antagonist blocked these excitatory effects confirming the role of Ang II as a neurotransmitter (Li et al. 1993). Measurements of NAD(P)H activity and superoxide production in vivo in anaesthetized rats, showed that superoxide production via NAD(P)H oxidase mediates the enhancement of sympathetic outflow in response to central Ang II via AT_1 receptors in the RVLM, the PVN and the SFO (Erdos et al. 2006). NADPH inhibitor blocked the pressor response to central Ang II. Chronic icv infusion of Ang II in rabbits increased AT_1 receptor density, NADPH oxidase levels and superoxide production in the RVLM, suggesting that NADPH-derived superoxide production in the RVLM contributes to AT_1 receptor-mediated sympathoexcitation in the RVLM (Gao et al. 2005).

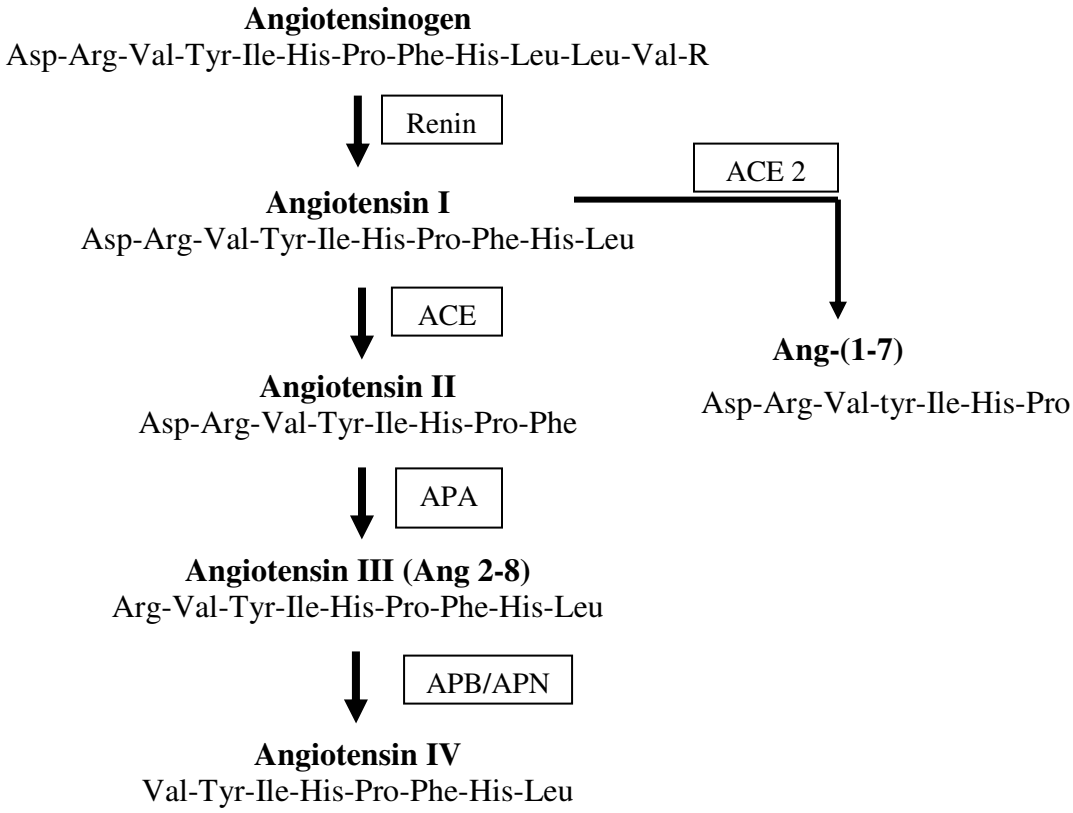


Figure 2. Metabolic pathways of angiotensin peptides formation and degradation. *Heart Failure Review 2008.*

1.2.1.A. Regulation of sympathetic activity by forebrain nuclei

Both neuroanatomical and electrophysiological studies have shown direct efferent projections to the PVN from the SFO and OVLT and the hypertensive effect of stimulation of the SFO is prevented by ablation of the PVN (Ferguson and Renaud 1981). Electrophysiological techniques demonstrated that excitatory effects of SFO stimulation on PVN neurons projecting to posterior pituitary, median eminence and intermediolateral spinal, were all inhibited by icv pre-treatment with AT_1 receptor antagonist, losartan (Li and Ferguson 1993, Bains and Ferguson 1995). The PVN projects directly to the intermediolateral cell columns of spinal cord and RVLM in the brainstem. The PVN is a

major source of input into the RVLM and is involved in the central control of sympathetic outflow (Fig. 3). Activation of PVN neurons elicits an increase in BP, heart rate and sympathetic activity. These responses are reduced by AT_1 receptor blockade in the RVLM, suggesting that AT_1 receptors in the RVLM mediate excitatory synaptic inputs from the PVN to the RVLM (Tagawa et al. 1999). Presympathetic neurons from the RVLM region send projections to sympathetic preganglionic neurons in the intermediolateral (IML) column of the spinal cord, and function as the major source of sympathoexcitatory activities from the brain (Kantzides et al. 2005, Pan 2004).

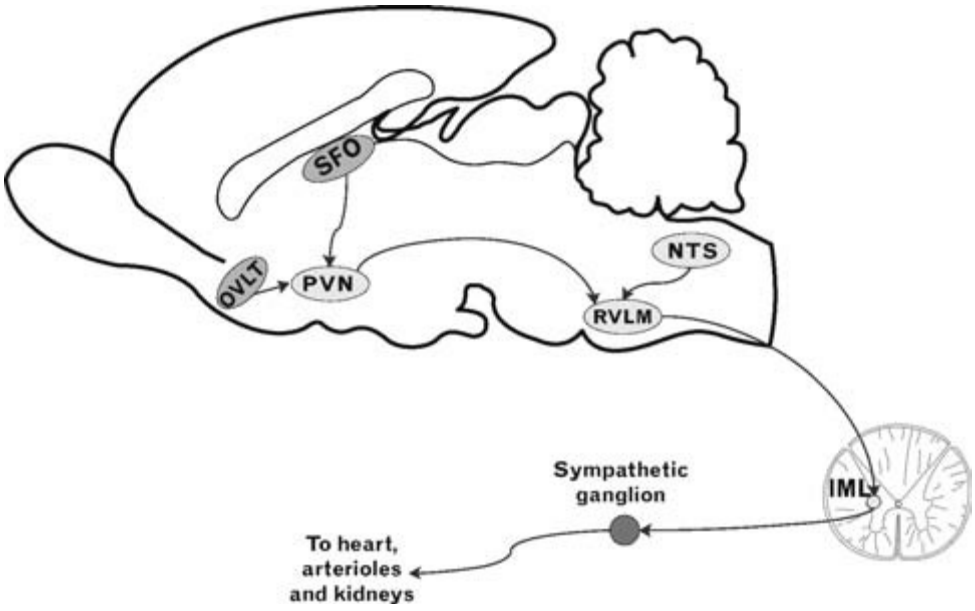


Figure 3. Central regulation of the sympathetic tone. *Journal of Hypertension 2010*

1.2.2. Vasopressin and oxytocin pathways

Neurosecretory magnocellular neurons in the SON and PVN synthesize vasopressin and oxytocin (Silverman and Zimmerman 1983). Bilateral pretreatment of the SON with losartan markedly inhibited the vasopressin release by icv Ang II. The increase in vasopressin release after Ang II injections into the SON was also inhibited by losartan

pretreatment into the SON. These studies suggest that AT_1 receptors in the SON can contribute to vasopressin release after periventricular Ang II receptor stimulation (Qadri et al. 1993). Studying lamina terminalis innervation to magnocellular neurons revealed a complex organization. SFO neurons directly (monosynaptically) innervate magnocellular neurons and this projection utilizes Ang II as neurotransmitter (Jahamandas et al. 1989). Inputs to both vasopressin and oxytocin secreting SON magnocellular neurons from the MnPO appear to be predominantly inhibitory. Electrophysiological data indicate that both inhibitory and excitatory inputs to vasopressin magnocellular neurons in the SON arise from the OVLT and SFO (Yang et al. 1994, Nissen et al. 1994).

PVN neurons express vasopressin. In situ hybridization and immunohistochemical labelling of retrograde tracing studies have identified vasopressinergic neurons and vasopressin mRNA in spinally projecting neurons. More than 40% of the spinally projecting neurons in PVN of Sprague-Dawley rats express vasopressin mRNA. The lateral parvocellular subdivision and the ventral part of the medial parvocellular subdivision contain the densest distribution of spinal cord-projecting vasopressin mRNA-expressing neurons. The magnocellular subdivisions display large numbers of vasopressin mRNA expressing neurons, but very few of those project to the spinal cord. The dorsal parvocellular subdivision contains a large number of spinally projecting neurons, but very few of those express vasopressin mRNA (Hallbeck et al. 1999). Fibers immunoreactive to vasopressin have projections to spinal cord, intermediolateral cell column (Nilaver et al.1980), and spinal vasopressin has been associated with sympathetic mechanisms such as renal sympathetic nerve activity (RSNA) (Malpas and Coote, 1994).

1.2.3. Neurosteroid pathways (for aldosterone see section "Aldosterone in CNS")

Digitalis-like substances ("ouabain") contain a steroid nucleus with one to four sugar moieties. It binds the α-subunit of the Na^+, K^+-ATPase enzyme inhibiting the enzyme's activity. Inhibition of this enzyme affects many Na^+-gradient dependent transport processes (Blaustein et al. 1993). Neurons express the α3-isoform, the most ouabain-sensitive isoform of the α-subunits and are very sensitive to ouabain (Antonelli et al. 1995). Ouabain binds to Na^+, K^+-ATPase, causing a gradual decrease in membrane potential and may thereby sensitize neurons to stimuli such as Ang II, enhancing responses to AT_1 receptor activation (Gabor et al. 2009).

"Ouabain" immunoreactivity is present in the neurons of several hypothalamic areas in rat and macaque. In both species "ouabain" immunoreactive neurons were observed in the magnocellular neurons of the PVN and SON and its accessory nuclei (Yamada et al. 1987). In these nuclei "ouabain" was mostly observed in vasopressinergic and to a lesser extent in oxytocinergic neurons in rats. Dense immunoreactivity of "ouabain" was also observed in OVLT, SFO and the MnPO. Positive nerve fibers projecting to the OVLT were found with few fibers detected around SFO. "Ouabain" immunoreactive neurons were also scattered in the periventricular, perifornical, lateral hypothalamic, anterior hypothalamic and preoptic areas. In these nuclei nerve "ouabain" immunoreactive nerve fibers were also observed. "Ouabain" immunoreactive nerve fibers with varicosities ran from the PVN through the lateral hypothalamic area to the infundibulum (pituitary stalk). "Ouabain" immunoreactivity nerve fibers were also observed in pituitary gland, PVN and SON (Yamada et al. 1992). "Ouabain" immunoreactivity showed a large number of axons originating from PVN directed toward SON. These fibers together with axons from

SON were found to project to the infundibulum and a lesser number from infundibulum to posterior pituitary lobe (Yamada et al. 1992).

1.3. CNS Pathways and mechanisms mediating effects of circulating Ang II in the brain on blood pressure

Overview

Apart from its vasoconstrictor and other peripheral effects, circulating Ang II acts on the brain to elicit a number of responses including a centrally mediated pressor response. Ang II may directly activate neurons in regions that lack the BBB, specifically the CVOs such as the OVLT and SFO. Projections from CVOs to downstream hypothalamic nuclei eventually activate presympathetic neurons as well as neurosecretory neurons (Potts et al. 1999). In this section, studies will be reviewed that present a model of neural control that contributes to elevation of BP induced by elevated levels of circulating Ang II.

1.3.1. Blood pressure pattern in response to circulating Ang II

Long term infusion of Ang II in low doses, raises BP in a single-phasic gradually developing manner. Infusion of Ang II at low rates (10 ng/kg/min, iv), increased BP within the first 24 h by 10 mmHg and by 20 mm Hg after 3-5 days (Kanagy et al. 1990, Cowley et al. 1981) and in recent studies by 15 mmHg after 10 days (Hendel and Collister 2005, Vieira et al. 2010). Infusion of Ang II at 200 ng/kg/min sc for 10 days increased systolic BP (SBP) by 30 mmHg within the first 3 days and 52 mm Hg by 10 days (Griffin et al. 1991). Chronic infusion of Ang II in high doses increases BP in a biphasic manner (Fink 1997, Li et al. 1996). Ang II infusion at 200 ng/kg/min iv induced a rapid increase (by 40 mmHg) over the first few hours of infusion followed by a further increase (total of 60 mmHg) after 7 days (Li et al. 1996). Infusion of Ang II at 350

ng/kg/min sc for 2 weeks increased systolic BP by 20 mmHg by day 2 and by 90 mmHg by day 10 (Luft et al. 1989).

BP response to infusion of Ang II in mice is different than that observed in rats. In mice sc infusion of Ang II (600 ng/kg/min, 14 days or 400 ng/kg/min, 12 days) induced a delayed increase (in MAP by 10 mmHg or in SBP by 20 mmHg respectively) that started at day 9 and by 15 mmHg or 25 mmHg by 14 or 12 days respectively (Capone et al. 2010, Gonzalez-Villalobos et al. 2008*).* In contrast, higher dose (1000 ng/kg/min, sc) of Ang II induced a rapid increase in SBP by 30 mmHg by day 3 and by total of 40 mmHg by day 12 (Gonzalez-Villalobos et al. 2008).

1.3.2. CNS pathways activated by circulating Ang II

1.3.2.A. Fra expression as marker for chronic neuronal activation

c-fos, fos-B, fos-related antigen (fra) 1 and fra 2 are proto-oncogens which belong to the immediate early gene (IEG) family of transcription factors. IEGs play a role in response of neurons to trans-synaptic stimulation and metabolic responses to electrical excitation. In resting cells c-fos mRNA and Fos protein are present at very low concentration (Hoffman et al. 1993, Mogran and Curran 1989). Under a variety of stimulated conditions Fos is synthesized dramatically in neurons. mRNA level reaches the maximum within 30 minutes after stimulation and the protein product reach the maximum 60-90 minutes after stimulation and last for 2-5 hours (Sharp et al. 1991). The pathway that links electrical stimulation at cell surface to transcriptional responses in the nucleus is suggested to occur through transcriptional activation of c-fos by depolarization and Ca^{+} influx (Sheng et al. 1990). Like their mRNA Fos proteins are short-lived and have a half life of 2 hours. Immunocytochemical localization of Fos shows staining in the cell nucleus. These

proteins are translocated to the nucleus where they form a heterodimeric protein complex with other oncogene products mostly with members of Jun family (c-Jun, Jun-B and Jun D). The Fos protein has a leucin zipper motif, promoting dimerization and binding of the dimers occurs at activating protein-1 (AP-1) (Morgan and Curran 1989). Many genes coding for neuropeptide and neurotransmitter, have AP-1 sequences in their promoters suggesting the involvement of Fos in regulation of synthesis of these molecules (Sonnenberg et al. 1989). Fos upregulates transcription of genes involved in neuron activity (Hoffman et al. 1993). Genes regulated by depolarization encode neuropeptide and neurotransmitters biosynthetic enzymes (Morris et al. 1988, Black et al. 1987).

Fos immunohistochemistry has been used to detect acutely activated neurons in the CNS (Dampney et al. 1995). However, Fos downregulates quickly during chronic exposure to a variety of stimuli and therefore is not a useful marker for detection of chronic neural activation (Morgan et al. 1989). Some investigators have suggested that Fos acts as a negative regulator of its own promoter, and is a repressor of its own expression (Schbntal et al. 1988, Sassone-Corsi et al. 1988) and Fos cannot be fully reinduced despite further stimulation (Morgan et al., 1987). Fos may repress its own transcriptional activation (Tratner et al. 1995) and transactivate the expression of genes like fra-1 (Braselmann et al. 1992).

Other IEG proteins, Fos-related antigens (Fra 1 and Fra 2), are coded by different genes. Fra proteins have a longer half-life than Fos but act in a similar way. Fras show long-term induction, which lasts for chronic periods with persistent stimulation. (Morgan et al. 1989, Hoffman et al. 1993). The protein encoded by fra-1 shows a high degree of sequence similarity with Fos in the leucine zipper region (Landschulz et al. 1988). Fras

also possess leucin zipper motifs and form heterodimers with Jun proteins and bind to AP-1 binding site of DNA (Gentz et al.1989, Cohen et al. 1989). The promoter region of fra-2 resembles that of fos. Most of the fos gene enhancers are also present in the fra-2 gene in the same order. Furthermore, involvement of AP-l in fra-2 gene expression suggests that there are autoregulatory systems and/or cross-regulatory systems among Fos/Jun family proteins for the transcriptional induction or subsequent repression of the fra-2 gene (Yoshida et al. 1993). The difference in the induction kinetics of these two genes after stimulation suggests they have distinct roles in transcriptional regulation in response to extracellular signals (Nishina et al. 1990). The levels of Fra proteins rise with a delayed kinetic and persist far longer than Fos. Thus, there is a cascade of Fos and Fras. The phase of c-fos repression correlates with expression of Fras (Sonnenberg et al. 1989). Antibodies have been generated against the N-terminal (amino acid 4-17) where Fras do not share sequence homology with Fos. This antibody tends to be more specific for Fos (Hoffman et al. 1993). Another antibody detects all members of the Fos family including Fos, Fos-B, Fra 1 and Fra 2 by cross-reacting with these molecules. This polyclonal antibody c-fos K-25 recognizes amino acids 128-152 in the NH2 terminal region of Fos, Fos-B, Fra-1 and Fra-2 proteins and is a useful tool for detection of both acute and chronic activation (Hoffman et al. 1993). These antibodies are generated against the portion of Fos that includes the leucin zipper that is essential for the formation of dimers and most likely detect Fos and Fras equally well. This antibody has been used for anatomical mapping of chronic neuronal activation in many studies including those in our lab (Budzikowski et al. 1998, Veerasingham et al. 2000, Vahid-Ansari et al. 1998).

Ang II upon binding to its receptor, AT_1-R, acts as an excitatory stimuli of neurons inducing expression of a variety of AP-1 subunits (Fos, FosB, JunB, JunD, and Jun) in the SFO, MnPO, PVN, SON, and OVLT (Blume et al. 1999). Ang II triggers mitogen-activated protein kinase (MAPK) signalling pathways. The MAPK family members p44/42 and JNK are predominantly responsible for the induction of Fos and Jun, the two major components of AP-1 (Angel et al. 1991, Chiu et al. 1988) (Figure 4). AP-1 binding sequences have been identified in the regulatory region of the AT_1-R gene (Murasava et al. 1993) and activation of AP-1 has been implicated in upregulation of the AT_1-R in HF (Liu et al. 2006). Sustained MAPK activation is required for the induced expression of Fra-1, Fra-2, Jun, and JunB (Cook et al. 1999).

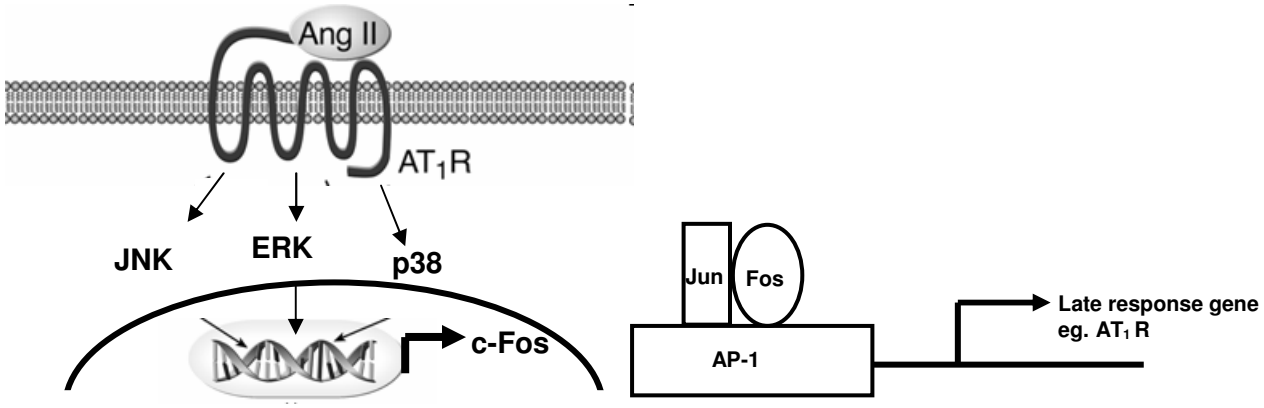


Figure 4. MAPK signalling pathway of fos induction and transcription regulation. *Current Opinion in Cell Biology. 1994*

1.3.2.A.i. Fos expression by short term infusion of Ang II

In rabbits, iv infusion of Ang II at 40 ng/kg/min for 1 hour increased Fos immunoreactivity in the SFO and OVLT (by 10-15 fold) (Badoer et al. 1997), whereas iv infusion of Ang II for 60 min at 22 ng/kg/min increased number of Fos-positive neurons

in OVLT (by 7 fold), MnPO (by 3 fold), NTS (by 2 fold) (Potts et al. 1999). In rats relatively low infusion rates of Ang II (35 ng/kg/min, iv) for 2 hours increased Fos expression in the core of SFO and lateral region of the OVLT. Higher rates of Ang II infusion (58 ng/kg/min) resulted in expression of c-Fos in the periphery and core of SFO as well as dorsal cap and lateral part of OVLT (McKinley et al. 1992, 2003). Iv infusion of Ang II (36 ng/kg/min) for 10 min increased Fos expression of neurons of both dorsal and ventral part of the MnPO with axonal projections to the PVN (Oldfield et al. 1994, Stocker and Toney, 2005). Ang II infusion (50 ng/kg/min iv) for 2-18 hours in rats significantly increased number of Fos-positive neurons in the NTS. This increase was similar (by 4 fold) at 2 and 18 hours of infusion. In the RVLM this infusion increased number of Fos-positive neurons by 2 fold only at 18 hours (Li et al. 1998). Electrolytic lesions in the SFO prevented the increase in Fos-like immunoreactivity in MnPO, SON and PVN by iv infusion of Ang II at 100 ng/kg/min for 6 hours. (Rowland et al. 1994). MnPO lesions markedly attenuated Fos expression in the parvocellular and magnocellular PVN and in SON but not in the SFO, in response to iv infusion of 100 ng/kg/min for 60 min (Xu and Herbert, 1995). These studies suggest that the SFO is the nucleus through which via the MnPO circulating Ang II activates downstream forebrain nuclei such as SON and PVN.

1.3.2.A.ii. Fra expression by chronic infusion of Ang II

In rabbits chronic iv infusion of Ang II at 50 ng/kg/min induced Fra expression in the CVOs and hypothalamic nuclei inside the BBB such as the PVN, SON (Davern et al. 2007). Iv infusion of Ang II activated neurons in the OVLT and SFO by approximately 150-fold as early as 3 hours, but activation decreased to 10- to 15-fold of control in these

nuclei by day 14. In contrast, Fra expression in downstream hypothalamic nuclei such as the PVN and SON increased 6-8 fold by 3 hours and this increase persist over the 14 days of infusion of Ang II. Despite receiving diminished inputs from the SFO and OVLT, neuronal activation in the PVN and SON remained high suggesting that other CNS mechanisms are activated by the initial signals from the CVOs, causing continuous neuronal activation of the PVN and SON (Davern et al. 2007).

In dogs infusion of Ang II at 5 ng/kg/min for 2h significantly increased the number of Fos positive cells in parvocellular PVN, but not in the magnocellular PVN. Chronic infusion of Ang II for 5 days produced sustained Fra like immunoreactivity in the NTS, CVLM, magnocellular PVN and SON. There was a greater Fra like immunoreactivity in the PVN during chronic infusion than acute infusion (Lohmeier et al. 2002).

Chronic iv infusion of Ang II in rats at 10 ng/kg/min for 4 weeks significantly increased Fra like immunoreactivity by 2 fold in PVN (Kang et al. 2009).

However, so far, the time course of changes (initial effects followed by chronic effects) in forebrain neuronal activity by systemic infusion of Ang II in rats has not been elucidated.

1.3.2.B. Central pathways involved in BP response to circulating Ang II

In rats, lesions disrupting the AV3V region including OVLT, SFO and MnPO, prevent pressor action of circulating Ang II (Knuepfer et al. 1984, Brody et al. 1978, Mangagapane and Simpson. 1980, Haywood et al. 1983).

In rats iv infusion of Ang II 10 ng/kg/min for 10 days increased BP by ~5 mmHg by day 2 in both SFO lesion and control group. However after 5 days, MAP increased 12 mmHg in sham but by only 4 mmHg in rats with SFO lesion. This attenuation of BP continued throughout the 10 days infusion (Collister and Hendel 2005).

Electrolytic lesion of the OVLT blocked the hypertension produced by 10 days of iv infusion of Ang II (10 ng/kg/min). MAP increased 16 mmHg in sham but only 4 mmHg in rats with OVLT lesion (Vieira et al. 2010).

After electrolytic lesion of both the dorsal and ventral part of the MnPO in rats, iv infusion of Ang II at 10 ng/kg/min increased MAP 40 mm Hg after day 7 in sham rats, but by only 18 mm Hg in rats with MnPO lesion (Ployngam and Collister 2007).

After reducing AT_1 receptors in the PVN by microinjection of Ad-AT_1 shRNA adenovirus, iv infusion of Ang II at 30 ng/kg/min for 60 min increased MAP by ~10 mmHg compared to 30 mmHg in sham rats (Ad-LacZ). These studies indicate that circulating Ang II activates an excitatory pathway starting from SFO and OVLT which through MnPO activates AT_1 receptors in the PVN leading to increase in BP (Northcott et al. 2010).

1.3.3. Central mechanisms involved in BP responses to circulating Ang II

1.3.3.A. Sympathetic activity in response to circulating Ang II

Several studies have assessed the effects of circulating Ang II on sympathetic activity. Sc infusion of Ang II (350 ng/kg/min) for 2 wks increased the resting splanchnic sympathetic nerve activity by ~2 fold in rats (Luft et al. 1989). The splanchnic sympathetic nervous system (SNS) is the most important regulator of venous tone, and splanchnic veins and venules are the most active and responsive and are richly innervated by the SNS (Greenway 1986, 1983). Iv infusion of Ang II (10 ng/kg/min) for 4 weeks increased renal sympathetic activity (RSNA), and plasma norepinephrine (PNE) in rats (Kang et al. 2009). In rabbits iv infusion of Ang II at 50 ng/kg/min reduced plasma PNE and RSNA levels acutely at day 1 but increased chronically (2-9 days) (Cox and Bishop 1991). In contrast, in rats infusion of Ang II 10 ng/min iv for 10 days increased BP by ~

30 mmHg but did not significantly change NE turnover (an index of sympathetic activation) in heart, kidney, skeletal muscle, or intestine (Kline et al. 1990).

1.3.3.A.i. Role of SNS in pressor responses to circulating Ang II

Several studies have shown the contribution of sympathetic activity in the hypertension caused by acute or chronic infusion of Ang II using ganglion blockade. Infusion of Ang II at 50 ng/kg/min iv for 24 hours in rats, increased MAP by 30 mmHg and depressor responses to ganglion blocking drug (trimethaphane) were significantly reduced at 1 and 5 hours time points: the fall in MAP by ganglion blocker (35 mmHg), was significantly less than before Ang II infusion (52 mmHg) and the minimal MAP in response to trimethaphan was significantly higher than that before Ang II (Li et al.1996). Similarly in rabbits iv Ang II infusion (50 ng/kg/min) elevated MAP by 20 mmHg at day 1 and the fall in arterial pressure by ganglion blockade was attenuated (22 mmHg) as compared to rabbits with vehicle infusion (38 mmHg) (Cox and Bishop 1991), consistent with a direct vasoconstrictor action of Ang II and withdrawal of sympathetic tone. However, in rats with infusion of Ang II at 50 ng/kg/min, at 10, 15 and 24 hours the depressor response to trimethaphane was significantly enhanced (trimethaphan decreased MAP to pre-Ang II levels), indicating that the neural component was fully active after only 10 h of infusion (Li et al.1996). In conscious rats infused with Ang II (10-12 ng/kg/min, iv) for 7-10 days, ganglion blockade produced a significantly large decrease in MAP (by ~75 mmHg) compared to rats infused with vehicle (by 38-47 mm Hg) (Kline et al. 1990, LaGrange et al. 2003). Chronic infusion of Ang II (50 ng/kg/min or 200ng/kg/min, sc) up to 7 days resulted in a dose related increase in MAP that at both doses was completely reversed by iv Trimethaphane (Li et al.1996). In rabbits iv Ang II infusion (50 ng/kg/min) for 10 days

elevated MAP by 20 mmHg. The depressor response to ganglion blockade was significantly enhanced on days 5 and 7 (by 52 mmHg) as compared to vehicle infused rabbits (by 38 mmHg) (Cox and Bishop 1991). In contrast, after chronic sc infusion of Ang II 50 ng/kg per/min for 5 weeks in rabbits the depressor responses to ganglion blockade were similar in Ang II treated and control rabbits during the first week of infusion but by 5 weeks were 54% greater in Ang II treated rabbits (by 34 mmHg) than in vehicle treated rabbits (by 22mmHg) (Burke et al. 2008). These studies indicate that chronic hypertension induced by Ang II is mediated centrally through contribution of SNS and the direct vasoconstrictor action of Ang II in hypertension appears to predominate acutely. The delay in contribution of SNS in chronic hypertension is Ang II-dose dependent and at higher rates of Ang II, central mechanisms start to mediate the hypertension earlier, particularly in rats.

1.3.3.A.ii. RSNA in Ang II induced pressor responses

The role of the renal nerves in Ang II induced hypertension is somewhat controversial. 7 day infusion of Ang II reduced RSNA in rabbits (Barrett et al. 2003) and renal spillover of NE in dogs (Carroll et al. 1984). However, renal denervation delays or blunts the development of hypertension during chronic infusion of Ang II in rats (Ichihara et al. 1997, Osborn and Camara et al. 1997). In rats iv infusion of Ang II (10 ng/kg/min) for 16 days caused an increase in MAP by 65 mmHg in sham operated rats and by 35 mmHg in renal denervated rats at day 10 (Hendel and Collister et al. 2006). In rabbits 5 weeks after sc infusion of Ang II (50 ng/kg/min) or vehicle renal sympathetic nerve activity in Ang II and control groups were similar. After denervation or sham denervation of both kidneys, MAP was lower 4 days after denervation (~ 6 mmHg) in vehicle-treated rabbits, but

remained unchanged in Ang II infused rabbits (Burke et al. 2008). These studies show that renal denervation does not affect an already developed (established) chronic Ang II hypertension and renal sympathetic nerves do not contribute to maintenance of chronic Ang II hypertension. However, renal denervation blunts the development of hypertension indicating the involvement of renal sympathetic activity initially in onset of Ang II hypertension.

1.3.3.B. Vasopressin release

Ang II stimulates arginine vasopressin (AVP) secretion in humans and animals. Ang II (4 ng/kg/min iv for 60 min) caused a significant increase (by 75%) in serum vasopressin level in normal men (Chiodera et al. 1998). In rats infusion of 100 ng/kg/min Ang II for 45 min increased plasma vasopressin level by 2 fold (Stocker et al. 2004).

Lesions of the SFO diminished 50 % of the increase in vasopressin release induced by iv infusion of Ang II 192 ng/kg/min for 20 min (Iovino and Steardo 1984).

So far, the contribution of plasma vasopressin in the elevation in BP by circulating Ang II has not been elucidated. However, studies have investigated the role of central vasopressin release in mediating effects of central Ang II on BP.

Central blockade of V1 receptors in rats or dogs, attenuated by 70% the pressor response to icv infusion of Ang II (Gruber et al. 1986, Unger 1981, Lon et al. 1996), suggesting that the central Ang II-mediated pressor responses are mediated partially by a central AVP mechanism (Shi et al. 2004).

1.4. Aldosterone in CNS

Overview

The genomic effects of aldosterone starting from binding of the agonist to MR lead to increase in epithelial sodium channel (ENaC) expression and activity on the cell membrane (Garty et al. 1994). Aldosterone binds to the MR, a member of the nuclear receptor family of ligand-dependent transcription factors (Arriza et al. 1987). The activated receptor–hormone complex binds to hormone response elements and regulates gene transcription. This hormone can influence both epithelial and non-epithelial tissues including the brain. A central pressor action of aldosterone was first demonstrated by studies showing that small doses (ineffective when infused systemically) elevate BP after chronic infusion into cerebral ventricle (Gomez-Sanchez 1986, Kageyama et al. 1988). Central administration of an MR antagonist blocks the central pressor effect of aldosterone (Gomez Sanchez et al. 1990). In this section, studies will be reviewed for the pathway of aldosterone synthesis, the presence of the enzymes involved in aldosterone biosynthesis in the CNS, regulation of aldosterone synthesis and role of aldosterone in activation of CNS pathways by circulating Ang II or post MI.

1.4.1. Aldosterone synthesis

The initial step in steroidogenesis is the translocation of cholesterol from the outer mitochondrial membrane to the inner membrane. This process is mediated by the steroidogenic acute regulatory protein (StAR), which forms a core through the membrane. StAR plays a crucial role in cytosolic cholesterol transfer into the mitochondrial inner membrane and is a rate limiting step for steroid biosynthesis. StAR is present in all steroidogenic tissues (Stocco 2001a, Stocco 2001b). Conversion of the

cholesterol to the first steroid, pregnenolone occurs by the cytochrome P450 side chain cleavage enzyme ($P450_{scc}$), present in the inner mitochondrial membrane in steroidogenic cells (Farkash et al. 1986). $P450_{scc}$ enzyme is encoded by the CYP11A1 gene (Liberman & Lin 2001). Pregnenolone is released into the cytosol and converted to progesterone by 3ß-hydroxysteroid dehydrogenase (3ß-HSD), located on the membrane of the smooth endoplasmic reticulum (Mason 1993; Simard et al 1993). CYP21A enzyme, located on the cytoplasmic surface of the smooth endoplasmic reticulum, produces 11-deoxycorticosterone (DOC) by 21-hydroxylation of the progesterone (Shinzawa et al. 1988). The conversion of DOC to aldosterone involves three reactions. 11-ßhydroxylation of DOC forms corticosterone. Further, 11-ßhydroxylation gives 11-ßhydroxycorticosterone (18-OH-B). Finally 18- methyloxidation yields aldosterone. These reactions are catalyzed by aldosterone synthase, located on the inner mitochondrial membrane and encoded by the CYP11B2 gene (Figure2.) (Chua et al. 1987, Mornet et al. 1989).

1.4.1.A. Steroid synthesis in the CNS

1.4.1.A.i. Presence of enzymes

The genes for all of the enzymes required for production of aldosterone de novo from cholesterol are transcribed in the CNS including those early in the pathway such as StAR, $P450_{scc}$ and 3ß-HSD (Furukawa et al. 1998) and those of terminal stages, namely aldosterone synthase (Mackenzie et al. 2000).

StAR is constitutively expressed in the brain at low levels (Kim et al. 2003). StAR mRNA transcripts were identified in several nuclei of rat hypothalamus such as MnPO, PVN and arcuate nucleus (Kim et al 2003, Furukawa et al. 1998). In mice, dual-labeling

immunohistochemical analysis using antibodies against glial (GFAP) and neuronal (NeuN) markers showed the expression of StAR protein in neurons of cerebellum, hippocampus, medial preoptic area, arcuate nucleus as well as glia of fornix and cortex. In astrocyte culture mRNA expression of StAR was shown by rt-qPCR and mitochondrial StAR protein by western blot (King et al. 2002). Studies by Kimoto et al (2001) in rats showed StAR immunoreactivity in hippocampus in neurons only and the presence of StAR in mitochondria was shown by western blot analysis in hippocampus. Sierra et al. (2003) found astrocytes immunoreactive for both GFAP and StAR and neurons immunoreactive for both NeuN and StAR in the hippocampus and cerebral cortex. Hypothalamic nuclei all showed weak staining of StAR in neurons. StAR staining appeared as punctuate staining in soma of cells with staining always being excluded from the nucleus consistent with StAR being located in mitochondria (Sierra et al. 2003, King et al. 2002).

In situ analysis showed the presence of StAR mRNA with the mRNA for $P450_{scc}$ and 3ß-HSD in purkinje cells (Furukawa et al. 1998). Cultures of glial cells expressed the mRNA of $P450_{scc}$ and 3ß-HSD (Jung-Testas et al. 1989, Mellon & Deschepper 1993). In mice, $P450_{scc}$ immunoreactivity was shown in neurons of cerebral cortex and glia of lateral hypothalamus (King et al. 2002). In rats immunoreactivity of $P450_{scc}$ double stained with GFAP or NeuN was found in hippocampus and western blot analysis showed presence of P450 in mitochondria of cerebellum and hippocampus (Kimoto et al. 2001). Colocalization of StAR and $P450_{scc}$ immunoreactivity was shown in anterior hypothalamus (King et al. 2002). So far not much is known about the expression or presence of these enzymes involved in early steps of aldosterone biosynthesis,

specifically in hypothalamic nuclei as these enzymes have not been well studied in hypothalamic nuclei.

The brain is a major extra-adrenal site of 11β-hydroxylase (CYP11B1), which converts DOC to corticosterone, and of aldosterone synthase (CYP11B2), which catalyses the steps between DOC and aldosterone (Gomez-Sanchez et al. 2010, Ye et al. 2008, Huang et al. 2006). mRNA of aldosterone synthase and 11-βhydroxylase was demonstrated in the hypothalamus of rats (Stromstedt et al. 1995; Pitt et al. 1999). The transcript level of CYP11B1 was found to be higher in hypothalamus and brainstem than in cerebellum and hippocampus (Ye et al. 2003). CYP11B2 mRNA has been measured in the whole hypothalamus (Zhang et al. 2006, Ye et al. 2008) as well as in specific nuclei such as the SON, PVN, and SFO (Wang et al. 2010). In rat brain, the highest CYP11B2 transcript levels were found in the hypothalamus and brainstem (Gomez-Sachez et al. 2010, Ye et al. 2008). The levels of CYP11B1 and CYP11B2 transcripts detected in brain are much lower than those in the adrenal cortex. In rats, CYP11B1 and CYP11B2 mRNA expression levels in the hypothalamus are about 1/500 and 1/1000 levels in the adrenal gland, respectively (Ye et al. 2003, Gomez-Sanchez et al. 2009). So far protein expression of CYP11B1 and CYP11B2 in hypothalamus has not been studied.

Unlike those enzymes acting earlier in the pathway, CYP11B1 and CYP11B2 could not be detected by RT-PCR in glia (Jung-Testas et al. 1989, Mellon & Deschepper 1993, MacKenzie et al. 2001). RT-PCR and southern blot analysis showed that CYP11B1 and CYP11B2 genes were transcribed within primary cultures of fetal rat hippocampal neurons and immunostaining of these cultures showed that the genes were subsequently translated to form immunoreactive 11-βhydroxylase and aldosterone synthase

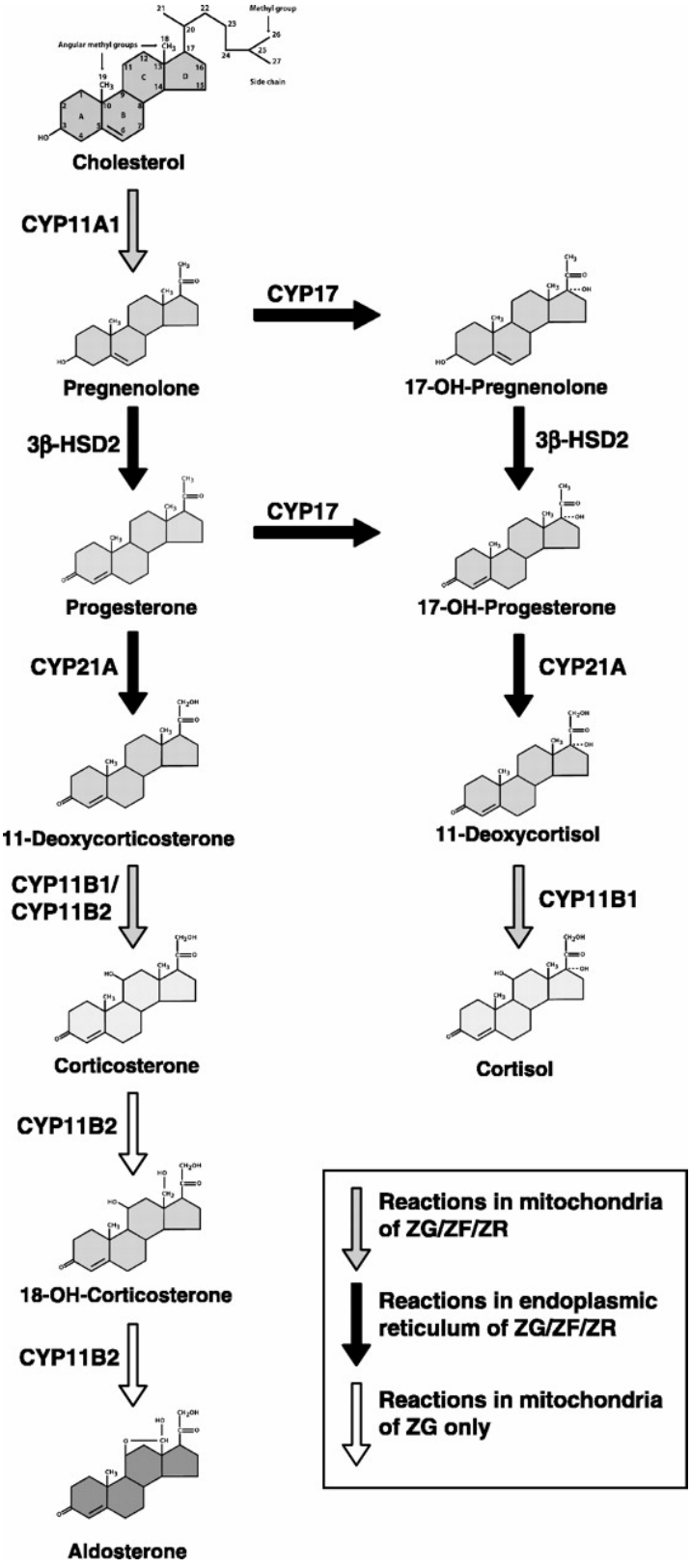
(MacKenzie et al. 2002). Immunohistochemical localization studies in rat cerebellum showed the presence of both 11β-hydroxylase and aldosterone synthase in the cerebellar cortex particularly within the Purkinje cells. 11β-Hydroxylase and aldosterone synthase immunoreactivity were also detected in the hippocampus, with greatest intensity in the dentate gyrus and CA3 cells (MacKenzie et al. 2000). MacKenzie in 2002 suggested that expression of CYP11B1 and CYP11B2 does not take place in glia but rather in neurons (MacKenzie et al. 2002). Studies by Wang et al (2010) have shown the presence of CYP11B2 and CYP11B1 transcripts in rat hypothalamic nuclei such as PVN, SON and SFO. However there is no direct evidence that these low mRNA levels translate into enzymatic activity that contributes to local aldosterone synthesis in brain.

1.4.1.A.ii. Evidence for functional production pathway in the CNS

Pregnenolone biosynthesis from cholesterol in intact mitochondria of the whole brain was assessed. Incubations of 200 mM cholesterol with 0.25 mg of mitochondrial protein showed that pregnenolone was formed in brain mitochondria and the pregnenolone concentration per unit mitochondria protein increased as a function of incubation time suggesting that the side-chain cleavage of cholesterol occurs (Tsutsui et al. 1999). Incubation of purkinje cells with the enzymes' common substrate, DOC, resulted in the production of their respective products, corticosterone and aldosterone, suggesting the presence of enzymatic activity for 11-βhydroxylase and aldosterone synthase within these cells (MacKenzie et al. 2000a).

To investigate aldosterone synthase activity in the brain, minces of hippocampus, hypothalamus, and cerebellum from intact rats and rats adrenalectomized for 5 days were incubated. Both intact and adrenalectomized rats secreted similar amounts of aldosterone

into the medium, suggesting that aldosterone is formed *de novo* in the brain from endogenous precursors. Conversion of ^{3}H-DOC to aldosterone, corticosterone, 18-hydroxy-DOC, and 11-dehydrocorticosterone from incubations with minces from various brain areas of intact rats was examined. Aldosterone formation was confirmed by HPLC (Gomez-sanchez et al. 1997). Aldosterone and corticosterone were formed in hippocampus, hypothalamus, brain stem, and cerebellum with corticosterone (and its metabolite 11-dehydrocorticosterone) formation predominating. Both aldosterone and corticosterone production in the hippocampus were ~2 fold higher than in the hypothalamus.



Aldosterone biosynthesis pathway. Connell JM, Davies E.J Endocrinol. 2005 Jul;186(1):1-20. Review

1.4.2. Mechanisms regulating aldosterone synthesis

1.4.2.A. Aldosterone in adrenal cortex

Three histological zones with functionally distinct role in steroid hormone production are present in mammalian adrenal cortex; zona glomerulosa synthesizes mineralocorticoids (e.g. aldosterone), zona fasiculata produces glucocorticoids and zona reticularis produces so-called adrenal androgens (Arnold 1866, Ogishima et al. 1992). The regulation of aldosterone synthesis can be divided into two phases. Acutely (hours) aldosterone production is controlled by the movement of cholesterol into the mitocondria which is mediated by increased expression of StAR (Cherradi et al. 1998). Chronically (hours to days) aldosterone production is regulated at the level of expression of aldosterone synthase (Bassett et al. 2004).

Steroidogenic cells store minimal amounts of hormone and the regulation of steroid secretion is primarily at the level of steroid hormone synthesis. The ability to induce steroidogenesis 10–100-fold rapidly is regulated at the level of cholesterol flow from the outer mitochondrial membrane to the inner mitochondrial membrane. Rapid increase in cholesterol translocation (10- to 100-fold) requires StAR. An increase in cleaved and active StAR (30K) translocated into the inner mitochondrial membrane and an increase in accumulated pregnenolone secreted from COS-1 cells after 30 min incubation with cholesterol was shown (Bose et al. 2002). Regulation of StAR transcript levels may also be involved in the long-term regulation of steroid production. In situ hybridization and immunostaining of adrenal cortex showed that a high-potassium diet for 5 days increased the expression of StAR mRNA and protein prominently in the zona glomerulosa. This increase in StAR expression was associated with 8 fold increase in plasma aldosterone (Peters et al. 2007).

In vitro studies have shown that the effect of Ang II on StAR mRNA expression is biphasic. In human adrenocortical NCI-H295R cells, Ang II increased StAR mRNA and protein in a time-dependent way, with a maximum at 12 h, with a 3 fold increase in aldosterone secretion. By 24 hours StAR expression was at level of control (Krug et al. 2007). Time-course effect of Ang II on bovine adrenocortical cells showed a peak within 3 h, followed by a rapid decline to very low level (12% of control) mRNA and protein of StAR (Christine Le Roy et al. 2000). In H295R cells, Ang II treatment resulted in a dose dependent increase in CYP11B2 expression and aldosterone synthesis. Treatment of H295R cells with Ang II resulted in a time-dependent increase in aldosterone secretion over 72 h. The increase started within 1 h of treatment and continued to increase up to 10 fold by 72 hr (Bird et al. 1993). A significant increase in CYP11B2 mRNA expression was found in H295R cells treated with Ang II for 24 hours (Bassett et al. 2000). In human (H295R), rat and hamster (NCI-H295) glomerulosa cells Ang II treatment for 48-60 hr induced a significant increase in expression of CYP11B2 mRNA (Spät and Hunyady 2004, Capponi 2004). After sc infusion of Ang II at 200 ng/kg/min for 55 hr in Sprague-Dawley rats in situ hybridization studies showed no significant change in StAR mRNA in the outer adrenal cortex, while the signal per cell for CYP11B2 mRNA became more intense and the number of positive cells increased. Plasma aldosterone increased 3 fold (Peters et al. 1998). 7 days sc infusion of Ang II in rats showed a significant increase (by ~ 8 fold) in CYP11B2 mRNA expression in the adrenal cortex with 2 fold increase in plasma aldosterone (Ye et al. 2003).

Potassium also can increase adrenal CYP11B2 expression (Okubo et al. 1997, Chen et al 1997). Potassium signalling in glomerulosa cells involves depolarization of membrane

leading to calcium influx. In primary cultures of rat adrenal cells and in the H295R adrenal cell line, K^+ increased aldosterone synthase mRNA and protein levels (Denner et al. 1996). In rats high potassium diet for 5 days increased CYP11B2 mRNA expression in adrenal cortex together with 8 fold increase in plasma aldosterone level (Peters et al. 2007).

ACTH causes an acute increase in production of aldosterone both in vivo and in isolated cells but chronically decreases plasma aldosterone levels in human and lowers adrenal expression of CYP11B2 in animal models. In rats ACTH administration caused an increase in adrenal mRNA of aldosterone synthase at 3 hours but at 24 hours decreased the mRNA level (Holland and Carr 1993). Repeated ACTH injections for 14 days in rats inhibited aldosterone secretion and decreased aldosterone synthase mRNA level in adrenal zona glomerulosa (Aguilera et al. 1996).

1.4.2.B. Aldosterone in CNS

1.4.2.B.i. Regulation of enzymes

Not much is known about the mechanisms regulating StAR expression and steroidogenesis in brain. Stressors (such as injuries, aging and alcohol) caused 2 fold increase in the mRNA expression of StAR and $P450_{scc}$ in hypothalamus. In hippocampus StAR was also upregulated but P450scc mRNA expression did not change (Kim et al 2003). A potent inductor of StAR expression in steroidogenic tissues is cyclic AMP (cAMP) (Stocco et al. 2001). cAMP was shown to upregulate StAR expression in astrocytes culture (King et al. 2002, Hu et al. 1987). In contrast, StAR expression was downregulated by cAMP in Schwann cells (Benmessahel et al. 2002, 2004), suggesting that the mechanisms controlling StAR may differ between different cell types. Activation

of *N*-methyl-D-aspartate (NMDA) receptors in hippocampal neurons resulted in the processing of full-length StAR (37-kDa) to the truncated 30-kDa form parallel with an increase in the synthesis of pregnenolone (Kimoto et al. 2001) suggesting that the processing of StAR may coincide with the cholesterol transfer from the outer to the inner membranes of the mitochondria and the initiation of steroidogenesis. The time course of pregnenolone was investigated in the hippocampal tissues that were incubated in the presence of NMDA. The pregnenolone concentration reached a maximal value at 30 min after the NMDA application and decreased gradually thereafter. A specific inhibitor of cytochrome P450scc completely blocked the pregnenolone production induced by NMDA stimulation, indicating that NMDA-induced pregnenolone production in the hippocampus is due to the P450scc enzyme.

These findings suggest that StAR expression may be regulated in neurons by synaptic activity and the consequent activation of NMDA receptors. Furthermore, since the activation of NMDA receptors induces the influx of Ca^{2+} in hippocampal neurons, inhibition of Ca^{2+} influx through NMDA receptors, suppressed the processing of StAR, suggesting that Ca^{2+} signaling drives the neuronal steroidogenic reactions (Kimoto et al. 2001). However, so far there is no evidence on the regulation of StAR expression in hypothalamus.

Low-sodium intake for 12 days in Wistar rats increased CYP11B2 mRNA expression by 10 fold in the hippocampus and cerebellum compared to normal sodium diet. However, low sodium diet showed no significant effect on CYP11B2 expression in the hypothalamus and brainstem. High sodium diet did not affect CYP11B2 expression in the brain regions. Sodium intake did not affect CYP11B1 expression brain regions either (Ye

et al. 2003). Wistar rats infused with ACTH (40 ng/day, sc) for 7 days showed a significant increase in expression of CYP11B2 mRNA by ~ 5 fold in hypothalamus and hippocampus as well as ~3 fold in brain stem and cerebellum but not in cortex. CYP11B1 mRNA expression increased in hypothalamus and cortex (Ye et al. 2008).

Investigating expression of enzymes involved in aldosterone production in hypothalamic nuclei could possibly be an indicator of production of aldosterone. Infusion of Ang II at 200 ng/kg/min for 7 days in rats caused a significant increase by ~ 8 fold in CYP11B2 expression in the adrenal but no change in the expression in the hippocampus, hypothalamus, brain stem and cortex (Ye et al 2003). Failure to detect changes in the expression of enzymes in a whole region may reflect that aldosterone may be synthesized in few cells of a particular cell type within hypothalamus and analyzing the whole region may mask or underestimate the differences in expression level in specific cell type.

1.4.2.B.ii. Regulation of aldosterone levels in the CNS

Despite its lipophilicity BBB penetration by aldosterone is poor compared to corticosterone, due to a protein transporter located within the BBB known as P-glycoprptein that pumps aldosterone back across the cerebral vascular endothelium into the blood (Pardridge and Mietus 1979, Geerling et al. 2009).

Aldosterone was first demonstrated in the brain by autoradiographic studies in neurons of the hippocampus and brain stem (Birmingham et al. 1979, 1984). Aldosterone measured by radioimmunoassay showed highest concentrations in the hypothalamus and hippocampus of rats (Yongue and Roy 1987). Recent studies found aldosterone in hypothalamus in adrenal intact rats at levels of ~ 150 pg/g tissue measured by ELISA (Yu et al. 2008). Aldosterone was also shown in hypothalamus (200-400 pg/g tissue) and

hippocampus (~100 pg/g tissue) in control rats measured by radioimmunoassay (Huang et al. 2009, 2010). In 2 weeks adrenalectomized rats aldosterone in the hypothalamus was present at very low levels (~15 pg/g) (Yu et al. 2008, Gomez-Sanchez 2005). These low levels persisting in the hypothalamus may reflect local synthesis.

Adrenalectomized rats infused sc with aldosterone (0.1 μg/h or 0.5 μg/h) for 2 weeks showed a significant increase in hypothalamic aldosterone levels (~ 50 ng/g tissue or ~ 100 ng/g tissue respectively). The increase in hypothalamic aldosterone correlated with the increase in plasma aldosterone (Yu et al. 2008). In contrast, in studies in intact rats sc infusion of aldosterone at 0.5 and 2.5 μg/h for 2 weeks increased plasma aldosterone concentrations by ~ 400% and 600% but caused minor increases in hypothalamic aldosterone at both infusion rates. In hippocampus aldosterone content increased by 150 % at 2.5 μg/h infusion rate and did not change at 0.5 μg/h rate (Huang et al. 2010). The absence of competition for uptake with corticosterone in adrenalectomized rats, (Pardridge and Mietus 1979, Geerling et al. 2009) may possibly explain the difference in findings in adrenalectomized rats and intact rats.

Sc infusion of Ang II at 500 ng/kg/min for 2 weeks increased aldosterone content in hypothalamus and hippocampus by ~ 3 fold but plasma aldosterone by 25 fold. Central infusion of aldosterone synthase inhibitor significantly attenuated the increase in hypothalamic aldosterone but had no effect on plasma aldosterone (Huang et al. 2010). This study suggests that sc infusion of Ang II may increase hypothalamic aldosterone independent of periphery.

In studies in rats post MI, significantly higher plasma (by 70%) and hypothalamic (by 60%) aldosterone was found 4 weeks post MI as compared to sham rats (Yu et al. 2008).

Our lab found a modest increase in aldosterone in the whole hypothalamus and a significant increase in hippocampus with no changes in plasma aldosterone level in rats 4 weeks post MI. Chronic blockade of aldosterone synthesis by icv infusion of an aldosterone synthase inhibitor significantly lowered aldosterone in hypothalamus and hippocampus without affecting the plasma level (Huang et. al 2008). The central increase in aldosterone post MI may therefore reflect an increase in local aldosterone production in the hypothalamus. However, it is not clear yet what causes the increase in production of aldosterone post MI and where specifically in the hypothalamus.

1.4.3. Role for aldosterone in activation of CNS pathways

In rats, levels of the corticosterone in the hippocampus and hypothalamus are 100-fold to 200-fold higher than those of aldosterone (Huang et al. 2008, 2009, 2010, Yu et al. 2008) and considering its high affinity for the MR which is three fold higher than aldosterone (De Kloet et al. 2000), corticosterone could be expected to occupy and block aldosterone binding to MR. However, 11ß -hydroxysteroid dehydrogenase type 2 (11β-HSD-2) is expressed abundantly in the hypothalamus. 11ß–HSD-2 converts corticosterone to inactive 11-dehydrocorticosterone and thus protects MR from exposure to corticosterone (Funder et al. 1988, Edward et al. 1988). Enzyme activity, estimated as the percentage conversion of [^{3}H]corticosterone to [^{3}H] 11-dehydrocorticosterone was highest in hippocampus and hypothalamus, and lower in brain stem and spinal cord. Northern blots analysis and in situ hybridization showed highest expression in hippocampus, cortex, hypothalamic medial preoptic area, arcuate nuclei and NTS (Moisan et al. 1990). rt-PCR analysis showed expression of 11ß–HSD2 mRNA in PVN (Zhang et al. 2006). However,

whether the presence of 11β-HSD-2 provides aldosterone selectivity for MR in specific brain areas is still under debate.

MR immunoreactivity has revealed the distribution of this receptor in many areas of the brain. In rats, MR immunoreactivity was distributed in the PVN in both the parvocellular and magnocellular regions in both nucleus and cytoplasm of neurons. Glucocorticoid receptors (GR) were also localized in the parvocellular region of the PVN. In the magnocellular region of the PVN only MR immunoreactivity was observed. MR immunoreactivity was mainly localized in the cytoplasm of the magnocellular neurons in the PVN (Han et al. 2005). High levels of MR were found in hippocampus, SON, magnocellular PVN, periventricular nucleus. MR immunoreactivity was also present in MnPO, OVLT and SFO (Amin et al. 2005). Taken together, studies by Zhang et al. (2006) showing the expression of 11ß–HSD2 mRNA in the PVN and other studies by Amin et al. (2005) showing the presence of MR immunoreactivity in PVN may confer aldosterone specificity in this nucleus. However, there is no evidence for colocalization of the MR and 11ß–HSD-2 in the same cell type within this nucleus.

1.4.3.A. Exogenous aldosterone

In unilaterally nephrectomized rats, sc infusion of aldosterone at 1 μg/hour increased BP by 40 mm Hg on day 3 which was prevented by concomitant icv infusion of an MR blocker at a dose that was ineffective when infused subcutaneously (Gomez-Sánchez et al. 1990). In Sprague-Dawley rats, sc infusion of aldosterone at 1.8 μg/kg per hour plus 1% NaCl drinking fluid for 28 days increased BP by about 30 mm Hg and this increase was prevented by icv infusion of the MR antagonist spironolactone or RU28318 (Xue et al. 2010).

Central infusion of aldosterone at doses that are ineffective when administered peripherally causes sympathoexcitation and hypertension. In wistar rats, icv infusion of aldosterone at 20-25 ng/hour with slightly elevated sodium of 0.15 M for 2 weeks increased MAP by 20 mm Hg (Wang et al. 2003). Icv infusion of aldosterone at the low rate of 5 ng/hour (in aCSF with 0.152 M Na^+) increased BP by 20 mm Hg after 4 weeks and MR blocker attenuated the increase in BP by 10 mm Hg (Gomez-Sanchez 1986). In Sprague-Dawley rats, icv infusion of aldosterone for one week at 22.5 ng/hour (in aCSF with unidentified sodium concentration) increased MAP by 20 mm Hg and increased Fra like immunoreactivity in the PVN and plasma norepinephrine. These effects were ameliorated by icv infusion of MR blocker RU-28318 (Zhang et al. 2008).

1.4.3.B. Endogenous aldosterone

Icv infusion of MR blocker eplerenone or spironolactone significantly attenuated the increase in MAP (by 70-80 % or 50 % respectively) induced by 500 ng/kg/min sc infusion of Ang II (Huang et al. 2010). Icv infusion of MR antagonist RU28318 significantly reduced the increase in MAP (by ~ 60 %) induced by 100 ng/kg/min sc infusion of Ang II (Xue et al. 2010).

Icv infusion of MR blocker RU28318 attenuated (by 100%) the 2 fold increased neuronal activity in PVN assessed by Fra like immunoreactivity, 4 weeks post MI, with greater effect in parvocellular regions. Icv spironolactone significantly reduced PVN neuronal discharge rate 75% in rats 4-6 post MI (Zhang et al. 2002). The 4 fold increase in plasma norepinephrine in MI rats was attenuated 50 % suggesting that aldosterone possibly contributes to excitation of presympathetic neurons (Yu et al. 2008). In rats at 4 weeks post MI the 100-120 % increase in MAP and RSNA in response to air-jet stress, was

prevented by icv spironolactone (Huang et al. 2004). Icv infusion of aldosterone synthase inhibitor FAD286 significantly attenuated cardiac tissue remodelling such as cardiac fibrosis and cardiomyocyte diameter and hyperthrophy (by ~ 50%) at 4 weeks post MI (Huang et al. 2008). In rats, icv infusion of MR blocker, spironolactone, at doses (5-6 μg/kg/day) 10000-15000 lower than doses used orally (20-80 mg/kg/day) for 6 weeks post MI caused equal effects in improving cardiac remodelling to that achieved when administered peripherally: prevention of the increase of cardiomyocyte diameter, interstitial and perivascular fibrosis and plasma aldosterone (Lal et al. 2004). These studies suggest that aldosterone possibly activates CNS pathways through binding to MR and contributes to sympathoexcitation and pressor responses and thereby influences peripheral mechanisms involved in cardiac remodelling and dysfunction.

1.5. Rationale

Post MI local aldosterone content in the hypothalamus increases (Huang et al. 2009, Yu et al. 2008). However, an increase in aldosterone content in brain does not necessarily reflect an increase in local synthesis. Evidence for local production of aldosterone in brain has been provided by studies using icv infusion of aldosterone synthase inhibitor, FAD286, which is a selective inhibitor of CYP11B2 (Menard et al. 2006), to block aldosterone synthesis without affecting corticosterone synthesis. In Wistar rats, sc infusion of Ang II increases hypothalamic aldosterone but not corticosterone content, and icv infusion of FAD286 prevents the increase in hypothalamic aldosterone without affecting corticosterone or the increase in plasma aldosterone concentration (Huang et al. 2010). In rats post MI, icv infusion of FAD286 prevents the increase in hypothalamic

aldosterone content without affecting hypothalamic corticosterone levels (Huang et al. 2009). These finding suggest that aldosterone may be locally produced in brain.

However, it is not clear yet what causes the increase in production of aldosterone post MI and where specifically in the brain this occurs. Circulating Ang II increases post MI (Leenen et al. 1999) and Ang II may activate the SFO that lies outside BBB. Since sc infusion of Ang II increases the aldosterone content of hypothalamus and icv infusion of aldosterone synthase inhibitor attenuates this increase (Huang et al. 2010), circulating Ang II might be a possible factor in central production of aldosterone. It is not known where specifically in the hypothalamus circulating Ang II induces aldosterone production. Investigating the pattern of neuronal activation and the expression of enzymes involved in aldosterone biosynthesis in hypothalamic nuclei may be a possible approach clarifying the local production of aldosterone in these nuclei.

1.6. Hypothesis

In rats, chronic elevated levels of circulating Ang II cause a biphasic response in neuronal activity of forebrain nuclei: the SFO shows an initial activation whereas the PVN and SON show a progressive activation over time.

Circulating Ang II causes neuronal activation in the SFO and thereby activates efferent pathways to the PVN, and in addition causes chronic activation of a neuro-modulatory mechanism starting with aldosterone production in magnocellular neurons in PVN and SON, which amplifies neuronal activation in the PVN and central sympatho-excitatory pathways.

1.7. Specific objectives

Ang II was infused subcutaneously in rats in order to simulate the post MI condition.

To investigate the role of circulating Ang II in activation of CNS in terms of aldosterone production, the following approach was taken:

1. To find the regions of forebrain that are possibly involved in aldosterone production: i) Fra-like immunoreactivity was used to determine the pattern of neuronal activation in forebrain during chronic sc infusion of Ang II. ii) To assess the role of central aldosterone in chronic neuronal activation, central aldosterone synthase inhibitor or MR blocker was used concomitant with sc Ang II infusion.
2. In order to elucidate where in the hypothalamus Ang II may stimulate aldosterone biosynthesis, gene expression of the enzymes known in the adrenal cortex to be involved in aldosterone production, such as StAR and aldosterone synthase (CYP11B2) was investigated in activated forebrain areas SON, PVN, MnPO, SFO and OVLT. To analyze the expression of steroid enzymes specifically per neuron, protein gene product PGP 9.5, a specific cytoplasmic neuronal marker that is highly expressed in neurons, was used as a neuron specific normalization standard. Glial fibrillary acidic protein (GFAP) was used a glia specific normalization standard in order to assess the expression level of the genes in glia.

2. Materials and Methods

Animals: Male Wistar rats weighing 200–250 g were purchased from Charles River (Montreal, QC, Canada). All rats were housed in a climatized room on a 12:12-h light-dark cycle at constant room temperature (22 ºC) and humidity (30 %) and given standard laboratory chow (120 µmol Na+/g) and tap water ad libitum. All studies were carried out in accordance with the guidelines of the Canadian Council on Animal Care, which conform to National Institutes of Health guidelines, and were approved by the University of Ottawa Animal Care Committee. All surgeries were performed under sterile conditions and isoflurane inhalation. Several types of osmotic minipumps were used. Alzet models 2001D (8 µl/h, 1 effective day), 2001 (1µl/h, 7 effective days), and 2002 (0.5 µl/h, 14 effective days) were used for subcutaneous infusions over various periods, whereas Alzet model 2004 (0.25 µl/h, 28 effective days) was used for all icv infusions.

2.1. Experimental protocols

2.1.1. Experiment i) Activation of CNS pathways by circulating angiotensin II

To determine the pattern of neuronal activation in forebrain nuclei during chronic infusion of Ang II:

Treatments: Animals were subjected to subcutaneous infusion for 1, 4 or 14 days. For each time point animals were divided into four groups as follows (n=4/group):

Group I: sc infusion of Ang II 500 ng/kg/min

Gropu II: sc infusion of Ang II 150 ng/kg/min

Group III: sc infusion of 0.9% physiologic saline

Group IV: control, no treatment

At the end of the infusion periods animals were perfused transcardially and brain tissues were collected for Fra like immunoreactivity.

2.1.2. Experiment ii) Assessment of role of aldosterone in chronic neuronal activation

In order to assess whether aldosterone in the CNS is involved in the chronic neuronal activation, Fra expression was assessed in Wistar rats infused sc with Ang II and treated with chronic icv infusion of aldosterone synthase inhibitor FAD 286 or MR blocker spironolactone. For this purpose 20 Wistar rats were divided into four groups (n=5/group):

Group I: icv infusion of aldosterone synthase inhibitor FAD 286 (100µg/kg/day) and sc infusion of Ang II (500 ng/kg/min).

Group II: icv infusion of MR blocker spironolactone (10µg/kg/day) and sc infusion of Ang II (500 ng/kg/min).

Group III: icv infusion of aCSF and sc infusion of Ang II (500 ng/kg/min).

Group IV: icv infusion of aCSF and sc infusion of 0.9 % physiologic saline.

In four groups of rats, icv cannula and osmotic minipumps were implanted for the chronic icv infusion of the AS inhibitor (FAD286) and MR antagonist (spironolactone), or aCSF. Two days after the start of the icv infusions, a sc infusion of Ang II at 500 ng/kg/min in groups I-III and vehicle (0.9% NaCl) in group IV was added for 14 days.

Surgery plan:

Day 1	Day 3	Day 16 (after 14 days sc infusion)
Start of icv infusion	**Start of sc infusion**	**Tissue collection**

Body weights were measured at day 1, 7 and 14 after the sc implantation.

Treatment	Day 1 sc	Day 7 sc	Day 14 sc
Ang II, FAD	323 ± 17	293 ± 20	298 ± 23 *
Ang II, Spiro	332 ± 10	308 ± 9	333 ± 22*
Ang II, aCSF	343 ± 19	324 ± 20	358 ± 29 ‡
Saline, aCSF	356 ± 22	362 ± 19	399 ± 23

Table M1. Body weights of rats during sc infusion of Ang II and icv blockers. The group with sc Ang II and icv FAD or icv Spiro treatment had a significant lost in body weight compared to the saline, aCSF group after 14 days sc infusion. The group with sc Ang II and icv FAD had a significant lost in body weight also compared to the group sc Ang II and icv aCSF. Minor changes in body weights were observed in each group during the infusion period. two-way ANOVA was used for the comparisons. (n=5/group). Treatment (F=6.3, P=0.001),* P< 0.05 vs saline, aCSF, ‡ P< 0.05 vs Ang II, a CSF

At the end of the infusion periods animals were perfused transcardially and brain tissues were collected for Fra like immunoreactivity.

2.1.3. Experiment iii) Assessment of effects of sc Ang II on expression of steroidogenic enzymes

The animals were divided into two groups (n=6/group):

Group I: sc infusion of Ang II 500 ng/kg/min (n=6)

Group II: control group treated with sham operation (n=6)

Animals were infused with Ang II for 3 or 14 days. Animals were weighted on the day of implantation and by the end of infusion period. At the end of the infusion period blood pressure was measured with a Millar Catheter. Animals were perfused and brain and adrenal tissues were collected for rt-PCR analysis.

Treatment	Implantation day	14 days
Sham	269 ± 5	344 ± 9*
Ang II	282 ± 6	318 ± 8 *

Table M2. Body weights of rats during sc infusion of Ang II. By the end of the infusion period animals in both group gained a significant amount of weight compared to the implantation day. Two-way anova was used for the comparison. (n=6/group). Day (F=52.47, P=0.001), treatment x day (F=6.5, P=0.019). * P <0.05 vs implantation day.

Surgical procedures

Placement of intracerebroventricular cannula: Animals were placed in a stereotaxic head frame, and the skull was leveled between the bregma and lambda. A small burr hole was made on the skull, and a right-angled guide cannula (23 gauge) was lowered into the left cerebral ventricle according to the rat atlas of Paxinos and Watson, 0.5 mm posterior to the bregma, 1.4 mm lateral to the bregma, and 3.5 mm ventral from the dura. Polymerizing dental orthodontic resin (Dentsply International, Milford, DE) was applied to the surface of the skull, and three protective screws were placed around the cannula. The cannula was fixed again with dental orthodontic resin. PE-50/60 tube connected to the upper end of cannula was fixed to an osmotic minipump implanted subcutaneously at the back of the neck. The skin incisions were sutured after the cannulation was done.

Placement of osmotic minipump: Under sterile conditions osmotic minipumps were filled using a small syringe (1.0 ml) and the blunt-tipped, 27 gauge filling tube provided by the company , Alzet. The pumps were incubated in sterile saline overnight. When the osmotic minipump was used for an icv infusion the osmotic minipump containing dissolved drugs was positioned subcutaneously at the back of the neck and connected with sterilized tubing to the free end of the cannula. Penicillin G (30 000 IU IM Derapen, Ayerst Laboratories, Montreal, Canada) was injected after surgery. The skin incisions were sutured after the implantation was done.

Proper location of the icv cannula was visualized after brain tissue collection and at the time of sectioning of brain. Data from animals with incorrectly placed cannula were excluded from the analyses.

Drugs infused

Ang II was obtained from Sigma (St. Louis, MO) and was dissolved in 0.9% saline to concentrations shown in table below for the infusion rates of 150 or 500 ng/kg/min. Saline 0.9% was used as control (vehicle). Aldosterone synthase (AS) inhibitor FAD-286 (100 μg/kg/day) dissolved in artificial cerebrospinal fluid (aCSF) and MR antagonist spironolactone (a kind gift from Pfizer) (10 μg/kg/day) dissolved in aCSF with 2% ethanol were used for icv infusion. These doses of AS inhibitor or MR blocker have been shown to be ineffective when infused peripherally in case of leakage of these drugs to periphery (Francis et al. 2003, Huang et al. 2009). Both AS inhibitor and MR antagonist were infused at an infusion rate of 0.25μl/h. Considering the secretion rate of CSF in rats (120-320 μl/h) the low rate of icv infusion unlikely affects CSF pressure. FAD-286 hydrogen tartrate (Novartis Institutes for BioMedical Research) was used because it is soluble in aCSF. Each 1.67 mg of FAD-286 hydrogen tartrate provides 1 mg of FAD-286 free base. As the amount of the drug needed in the pump was 14.4 mg for each rat, a total of 24 mg of FAD-286 hydrogen tartrate was filled in the pump to acquire the desire 14.4 mg of FAD-286 free base.

Alzet models	Pumping rates	Concentrations for infusion rates			
		Sc Ang II at 150 ng/kg/min	Sc Ang II at 500 ng/kg/min	Icv FAD	Icv spironolactone
2001D, effective1 day	8 μl/h	0.28 mg/ml	0.9 mg/ml	------	-------
2001, effective 7 days	1μl/h	2.25 mg/ml	7.5 mg/ml	-------	-------
2002, effective 14 days	0.5 μl/h	4.5 mg/ml	15 mg/ml	-------	------
2004, effective 28 days	0.25 μl/h	-----	-----	144 mg/ml	14.4 mg/ml

Table M3. Concentration of drugs prepared for the respective sc or icv infusion rates.

Blood pressure measurement: A laparotomy was performed and abdominal aorta was exposed by blunt dissection. The tip of telemetry transmitter catheter (model TA11PA-C40, DSI) filled with special gel Cusion, was inserted via a needle puncture into abdominal aorta below the renal artery. The puncture site was sealed with tissue glue. The transmitter was secured by 3 non absorbable sutures to abdominal wall. The transmitter was placed into the abdominal cavity and secured to the ventral abdominal wall with the catheter inserted into the abdominal aorta. The abdomen was then closed in layers leaving transmitter inside the abdominal cavity. After recovery, the telemetry signal was obtained and evaluated using an analog adapter and data-acquisition system, which was set to calculate and store the mean values of resting BP and HR with a 3-s interval over a 1-min period each hour. Continuous recordings of BP and HR during the day (7 AM–7 PM) and the night (7 PM–7 AM) were started 3 days after the probe implantation. After 3 days of control recordings of BP and HR, osmotic minipumps were implanted subcutaneously for a 2-wk infusion of vehicle (0.9% saline) or Ang II at 150 or 500 ng/kg/min at 0.5 μl/h.

Blood pressure measurement for molecular biology studies: By the end of 14 days sc infusion of Ang II, under general anesthesia with isoflurane abdominal aorta was canulated and BP was measured with Millar catheter.

Collection of Blood: The night before the blood collection rats were moved to a quite room. The next morning with care being taken not to necessarily disturb the rats, they were taken very carefully (to minimize the stress) one by one to necropsy room and were decapitated. The trunk blood was collected to a precilled wide mouth 50 ml falcon tube

(few drops of heparin were added to the falcon tube before pouring the blood into it). The blood was rapidly mixed with the heparin in the falcon tube. For plasma Ang II measurements, 2 ml of blood were collected into a prechilled microcentrifuge tube containing 1,10-phenanthroline and EDTA-Na2 at final concentrations of 0.8 mM and 1.2 mM in blood, respectively. The blood was centrifuged for 10-15 min at 8,000 rpm and 4°C, using an Eppendorf model 5415 C microcentrifuge.

Plasma Ang II assay: The plasma for Ang II measurements was immediately applied to preconditioned Waters Sep-Pak C18 cartridges. The cartridges were washed with 10 ml of 0.1% trifluoroacetic acid (TFA), and then the Ang peptides were eluted with 2 ml methanol-water-TFA at a ratio of 80:19.9:0.1 vol/vol/vol, and the eluates were evaporated to dryness using a Savant SpeedVac and stored at -20°C till analysis. Plasma Ang II was measured by radioimmunoassay after separation by HPLC. Plasma extracts were first redissolved in mobile phase, centrifuged, and the supernatant was injected onto a CSC (Montreal, Canada) Select 100A/ODS2 column, 15 × 0.46 cm, 5-µm particle size, with a Rheodyne 7125 manual injector. Gradient elution was used, starting with 100:40% vol/vol methanol in 10 mM acetate buffer at *time 0*, changing to 100:80% vol/vol methanol in 10 mM acetate buffer at 38 min (slope −4), at a flow rate of 1.0 ml/min (Waters models 501 and 510 HPLC pumps,controlled by Maxima 820 workstation software, Millipore).Eluate fractions were collected at 1-min intervals (with a Spectrum Spectra/Chrom CF-1 Fraction Collector) and evaporated to dryness in the SpeedVac. Ang II radioimmunoassay was performed on the individual tubes from approximately 8–20 min which contained the Ang II peak. 0.05M Tris, pH 7.4, with 1 mg/mL RIA-grade BSA and 0.02% sodium azide was used to dilute the Ang II antisera (to yield 40% to 45%

specific binding after 16-24 hours incubation at 4°C) and I-125 labelled Ang II (to 4000-4500 cpm total counts) 300µl of antiserum and 50µl of tracer were added to each dried eluate tube. After overnight incubation, 500µl dextran-coated charcoal (nonspecific binding <1%) was added to each tube to separate bound and free tracer After centrifugation (30 min at 4° C and 3500rpm in a Sorvall RT benchtop model), the radioactivity of the bound fraction was counted for 3 min using a Canberra-Packard Cobra II Auto-Gamma Counter, and the Ang II concentration in the unknowns determined from a standard curve included in the RIA assay. The Ang II antibody had 100% cross-reactivity with Ang II. The ANG II antibody had 55% cross-reactivity with Ang III, and <0.1% cross-reactivity with ANG I. Only the tubes corresponding to the Ang II peak were included in the calculations for the concentration of this peptide. The ANG III peak, which is well separated, was not included.

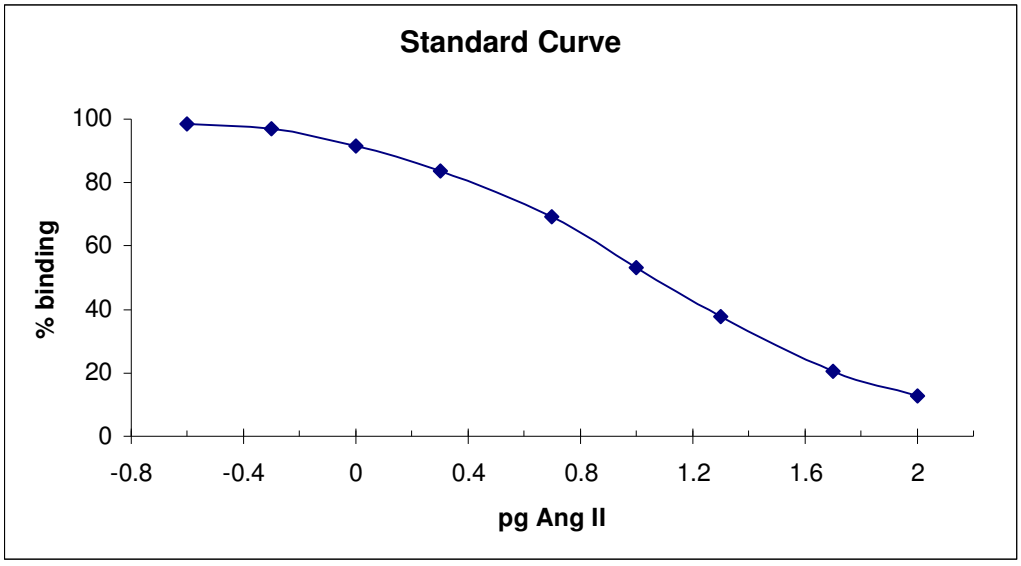


Figure M1. Standard curve of Ang II. Estimated pg at 20 % binding (ED20)= 51.8, ED50= 11.4, ED80= 2.7. Total binding (%ref/total) = 46.6
Non-specific binding = 1.7%

2.2. Fra-like Free-Floating Immunoassay

At the end of the infusion period, animals were deeply anesthetized by injection of pentobarbital sodium (100mg/kg) intra peritoneally. When animals lost the response to toe pinch, animals were perfused transcardially with 200ml of 0.9% saline followed by 150 ml of freshly prepared 0.1 M phosphate buffer (PH 7.4) containing 4% paraformaldehyde (PFA) at room temperature. Brains were removed immediately and postfixed in 4% PFA for 24-48 h at 4 ºC. If longer storage was needed, the brains were incubated in PBS at 4 ºC until the sectioning.

Brains were cut in 40µm coronal sections on a vibratome, and placed in 0.1 PBS for immediate immunohistochemistry or kept in 30% ethylene glycol with sucrose in 0.1 M PBS at -70ºC for Free-Floating immunohistochemistry.

Brain sections were placed in 0.1 PBS for immediate immunostaining or kept in 30% ethylene glycol with sucrose in 0.1 M PBS at -70ºC for storage.

Coronal sections of brain were transferred to the 12 well-plate inserts and rinsed 3 x 5 minutes in 0.1 M PBS under gentle agitation on a shaker. Endogenous peroxidase were deactivated/blocked by washing sections in 0.01 M PBS containing 0.3% hydrogen peroxidase for 10 min at room temperature with gentle agitation. Sections were then washed three times in PBS at room temperature. Sections were then preincubated in 0.1 M PBS and blocking serum (1.5% Donkey serum, abcam (ab7475) and 1% BSA) for 2 hours with gentle agitation.

Then sections were incubated for 48 hours at 4ºC with gentle agitation, with PBS containing 0.3% Triton x-100, 0.02% sodium azide and primary antibody using affinity purified rabbit polyclonal antibody c-fos K-25 (Santa Cruz Biotechnology, Santa Cruze,

CA) (1:2000), recognizing amino acids 128-152 in the NH_2 terminal region of Fos, Fos-B, Fra-1, Fra-2 proteins. Sections were then washed three times with PBS (Alternate section without primary antibody are negative control).

Sections were then incubated with Biotin-SP-conjugated AffinityPure Donkey Anti-Rabbit secondary antibody (H+L), (1:500) for 16 hours at 4ºC with gentle agitation.

Sections were washed then three times with PBS and were incubated for 3 hours with PBS containing 0.3% Triton X-100 and Streptavidin Horseradish Perxoxidase Conjugate at room temperature (1:300, Amersham Canada, Oakville, ON, Canada, Code # RPN1231) with gentle agitation.

For visualizing the reaction diaminobenzidine-nickel method was used (Vector DAB Kit). The reaction was terminated by washing the sections in distilled water.

Subsequently Sections were mounted on gelatin-coated slides, dried, dehydrated through a graded series of alcohols and two changes of Xylene and coverslips for microscopic observation. The dark dots representing Fra positive neuron were counted in 3-4 sections for each nucleus (Figure M2). Fra positive neurons were quantified in SFO, MnPO, SON and PVN (Figure M3,4).

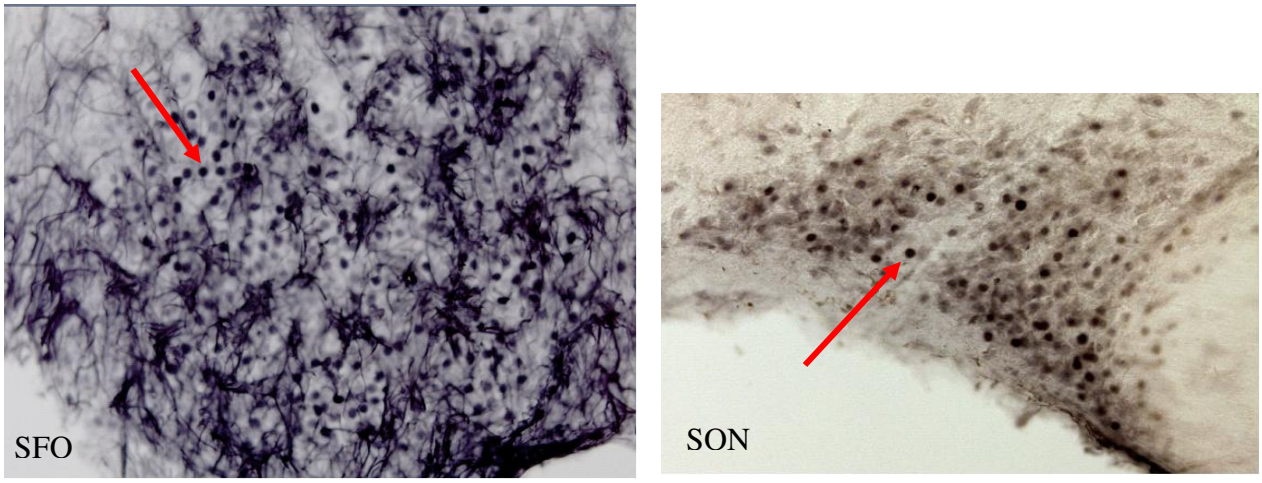


Figure M2. Fra staining in SFO and SON. Each dark dot showed by arrows represents one Fra positive neuron cell.

Limitation of the sectioning technique by vibratome: Intact OVLT region could not be collected in the sections. Brain tissues were fixed with PFA and sectioned at room temperature in a container filled with PBS by vibratome. By this technique collected sections were damaged in OVLT region. However, when using a cryostat for sectioning and following punches for molecular biology studies, the tissue is frozen fixed and intact OVLT could be easily collected.

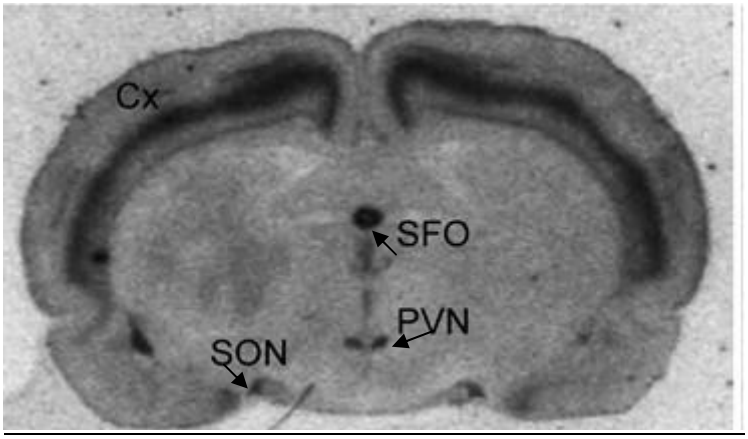


Figure M3. Coronal section of forebrain. SFO, PVN and SON are shown by arrows as dark stained regions. Level of the section: Bregma -1.40 mm

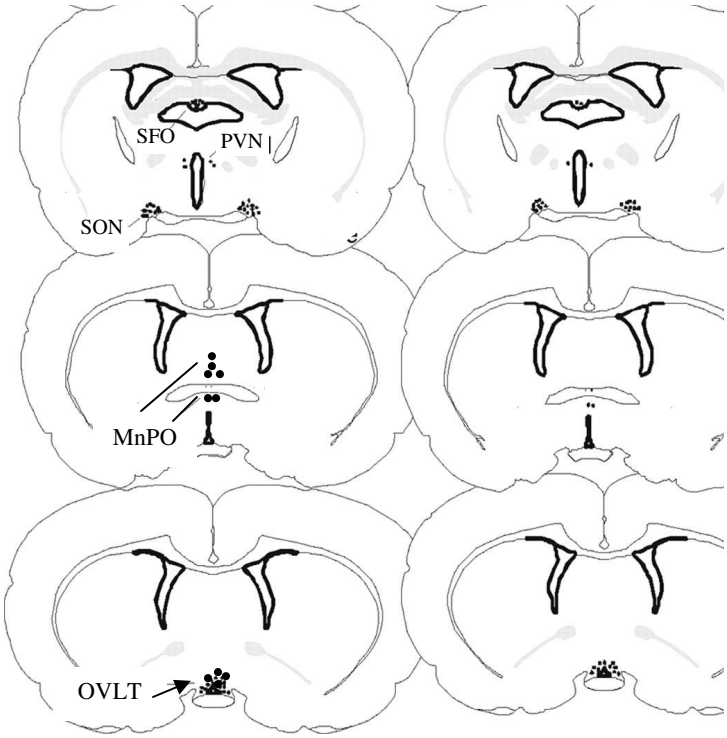


Figure M4. Schematic drawing of coronal sections of brain. Dark dots represent Fra positive neurons in the nuclei shown

2.3. Molecular Biology Assays

Tissue Collection: Animals were anesthetized by injection of pentobarbital sodium(100 mg/kg, intra peritoneal). Animals were transcardially perfused with chilled diethyl pyrocarbonate-treated (DEPC-treated dd-water) 0.1 M phosphate buffer (PBS) (pH 7.4) under pentobarbital anesthesia. The brains and adrenals were removed immediately frozen in isopentane with dry ice at ~ -20°C to -30 °C, placed on dry ice, and then stored at -80°C until use.

Brain punch procedure

Serial 80-µm-thick coronal slices were cryosectioned, and the brain micropunches of all activated forebrain areas (OVLT, SFO, MnPO, SON and PVN and also hippocampus as a

control) were taken with prechilled Drummond microdispensers (Drummond Scientific Broomall, PA). The microdispensers are microtubes with 1.5 mm diameter. The nucleus was collected by placing the microtube centered over the nucleus in the section and punch to pick up the nucleus. PVN and SON were collected bilaterally. MnPO was collected from both the dorsal and ventral part. For the SFO some immediate surrounding tissue was included. The tissue pellet was homogenized in 0.2-ml Trizol Reagent (Invitrogen, Burlington, ON, Canada) by using a pestle (Bel-Art-Products, Pequannock, NJ) driven by a pellet pestle motor, and 0.3 ml Trizol reagent was then added.

2.3.1. Total RNA isolation

Total RNA was isolated according to the manufacturer's protocol. Following the homogenization, additional Trizol was added to reach a total volume of 1ml for adrenal and 500 ml for brain punches. Homogenized samples were then incubated in room temperature for 5 min to permit a complete dissociation of nucleoprotein complexes. Chloroform was then added at 0.2 ml per 1ml of Trizol to separate the RNA in an aqueous phase. Samples in tubes were shaken vigorously for 30 seconds and followed by centrifugation at 12000 x g for 20 minutes at 4 °C. Following centrifugation the mixture separated into a lower red phenol-chloroform phase an interphase and a colorless upper aqueous phase where total RNA remained in exclusively. The aqueous phase containing dissolved RNA was collected. RNA was then precipitated from aqueous phase by 0.5 ml isopropyl alcohol per 1 ml Trizol used for the initial homogenization. Samples were incubated at room temperature for 15 min followed by centrifugation for 20 minutes at 12000 x g at 4 °C. The RNA pellet where then washed by 800 ml 75% ethanol and centrifuged for 10 min at 7500 x g. RNA pellet was air dried and/or vacuum dried for 10

min. RNA was then dissolved in RNase free water (20 µl for brain punches and 50 µl for adrenal). The integrity of RNA was verified on agarose gel (Figure M5) and the quantity and purity of RNA was measured by Nano-drop Spectrophotometer (ND-1000, USA). The dissolved RNA was stored at -80 °C.

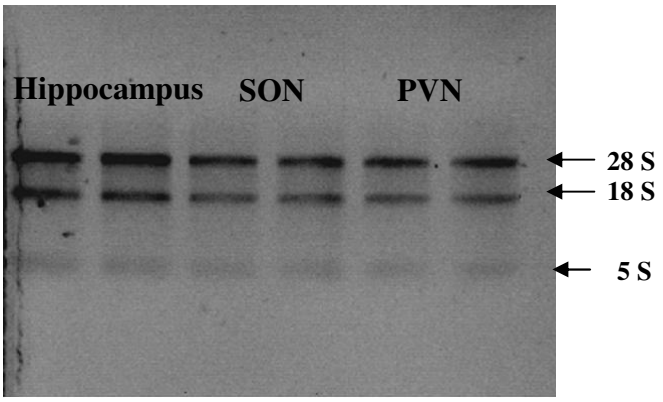


Figure M5. RNA native agarose gel. RNA extracted from brain nuclei (duplicate samples) were run on agarose gel for quality control.

2.3.2. DNase I treatment

Ambion's DNA-*free*^{TM} kit was used for removal of contaminating genomic DNA from RNA preparations. DNase I treatment was performed on 10µg RNA of adrenal or the total amount of RNA from the brain punches. The recombinant DNase I (rDNase) (Ambion, Austin, TX) (0.5 µl for brain punches and 1 µl for adrenal) was used with 0.1 volume of 10 x DNase I buffer for a total volume of 50 µl. Samples were then incubated at 37 °C for 30 minutes. To stop the reaction, 0.1 volume of DNase Inactivation Reagent (Ambion, Austin, TX) was then added and incubated in room temperature with occasionally vortex. After centrifuge at 12,000g for 2 min at room temperature, the supernatants were collected into a new 1.5 ml eppendorff tube. To concentrate the RNA, 2 volume of 100% ethanol and 1/10 volume of sodium acetate (NaAc, 3M PH 5.3) was added and the mixture was incubated overnight at -20 °C. The following day samples

were centrifuged at 12000 x g for 20 minutes at 4 °C. Supernatant were discarded and the RNA pellet were washed with 75 % ethanol as described above. The concentration of dissolved RNA in RNAase-free water was measured by UV absorbance (OD260, Nano-drop) (Figure M6).

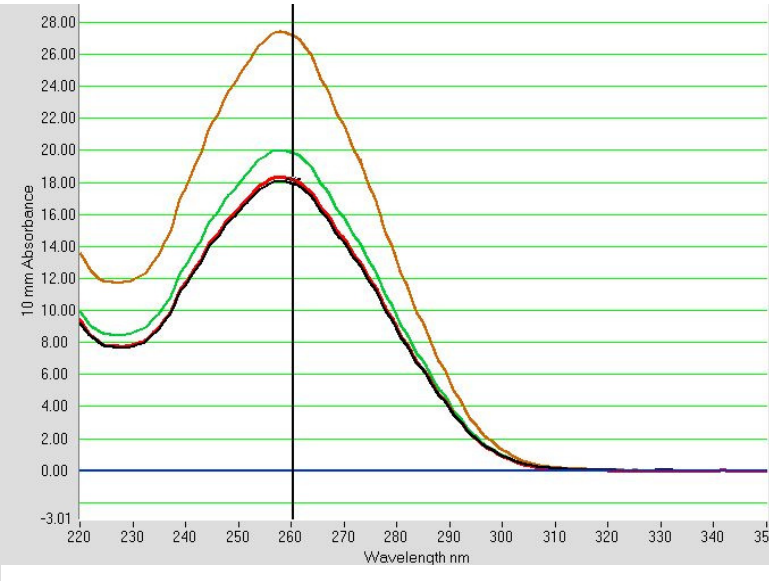


Figure M6. RNA absorbance curve. RNA absorbance curve obtained from UV absorbance at OD260, Nano-drop measurement.

All the reagents were purchased from Invitrogen, Burlingtone, ON, Canada.

For the first strand cDNA synthesis, maximum of 1µg DNase I treated RNA was used. Oligo(dT)$_{12\text{-}18}$ primers (1 µl, 500µg/ml) and dNTP Mix (1 µl, 10 mM) were mixed with 1µg of RNA to a total volume of 12 µl. The mixture was heated to 65 °C for 5 min and quickly chilled on ice. 5x First-Strand Buffer (4 µl), DDT (2 µl, 0.1 M) and RNaseOUT (1 µl, 40 units/ µl) was added to the mixture and warmed up for 2 minutes at 42 °C. The mixture was then incubated with 200 U SuperScript ™ II RNase H- Reverese Transcriptase at 42ºC for 50 minutes. To inactivate the reverse transcriptase samples were incubated for 15 minutes at 70 ºC. Samples were then stored at -20 °C till the following PCR.

2.3.3. Primer design

We used the same primers of CYP11B1 as described elsewhere (Ye et al., 2003) where primers were designed to amplify a 324 segment corresponding to positions: 552–875 of CYP11B1 gene. For the aldosterone synthase gene, CYP11B2, primers and standard had already been developed by Dr. Wang in our lab (Wang et al. 2010) where primers were designed to amplify a 203-bp fragment corresponding to positions: 558–881 of CYP11B2 gene. Specific primers for CYP11B1 and CYP11B2 were selected in the region of the lowest homology in DNA sequences by alignment analysis of those two genes (see alignment analysis). For the StAR gene primers were used as described by Peters et al. 2007 where a 600 bp fragment corresponds to positions at 2701-3301. For the GFAP gene primers used were designed to amplify a 356 bp fragment corresponding to positions at 1360-1715. All primers were ordered from Integrated DNA Technologies. The primer sequences were as follows:

Gene	Sequence	Amplicon Length
CYP11B1	Forward: 5'- GTCTATAAACATTCAGTCCAA-3' Reverse: 5'- ATCTCCGATATGACACTCC-3'	324 bp
CYP11B2	Forward: 5'- CCCTGGTAGCCTGAAGTTCATC -3' Reverse: 5'- TCTGAGAGCTGCCGAGTCTG -3'	203 bp
StAR	Forward: 5'- GCTCCTGATGGCTGTGTGTAC-3' Reverse: 5'- GAATGGGGATAACAGCTCAGA-3'	600 bp
GFAP	Forward: 5'- TTGTTTGCTAGGCCCAATTC-3' Reverse: 5'- CCTCGGGATCTTTTCCTTTC-3'	356 bp
PGK1	Forward: 5'- GCTGCAGAACTCAAATCTCT-3' Reverse: 5'- TGTGTGCAGTCCCAAAAGCA-3'	263 bp
PGP	Forward: 5'- CCAGTTCAGAGGACTCTCTG-3' Reverse: 5'- GCCTCTGAAGTTCACACTGG-3'	297 bp

Table M4. Primer sequences for the genes of interest

Conventional PCR for optimization of annealing temperature

To generate the standards, cDNA prepared from Wistar rat adrenal (StAR) or brain (GFAP) RNA was used as the template. To amplify the cDNA conventional PCR were run several times to optimize the annealing temperature. cDNA template, specific primers, dNTPs, Taq Polymerase (Genescript, Inc), appropriate 10x PCR buffer containing 15mM $MgCl_2$ and a thermocycler was used for conventional PCR. Optimized conventional PCR profiles were: an initial denaturation step at 94^o C for 3 min and followed by 30 cycles of 94^o C for 45 sec, 50^o C (for CYP11B1) or 53^o C (for CYP11B2), 54^o C (for StAR) and 55^o C (for GFAP) for 45 sec, and 72^o C for 1 min. PCR products were verified on an agarose gel where a single band was visualized/obtained (Figure M7).

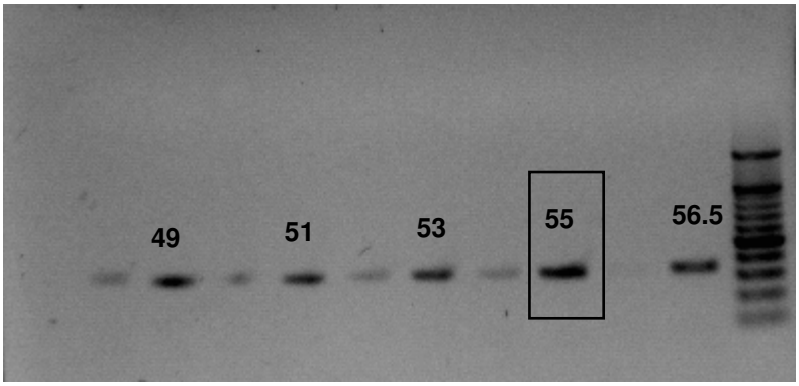


Figure M7. Conventional PCR product of GFAP cDNA with gradient annealing temperature. Duplicated samples (10 µl and 5 µl) were run on agarose gel. A clear single band at 350 bp was found with highest efficiency for PCR amplification at annealing at 55 ^{o}C.

2.3.4. Subcloning

The specific amplifications of conventional PCR products were purified by PCR purification kit (Roche, Germany, Mannheim) (Figure M8). PCR products concentration was determined by both Nano-drop OD 260 and agarose gel compared to the density of λDNA/Hind III marker. Ligation was done overnight at 14^{o}C by incubate PCR product

with ampicillin-resistant pCR2.1 TA cloning vector from the Invitrogen TA Cloning Kit ® at a ratio of 1:3 (vector:insert). The ligation reactions were transformed into One Shot® Competent Cells (E. coli cells, Invitrogen, Burlington, ON, Canada). Transformed cells were then plated on LB agar plates containing 160 μg/ml X-Gal and 100 μg/ml ampicillin for blue/white screening. Plates were then incubated overnight at 37° C. White colonies were picked up and grown overnight in 2 mL LB broth medium containing 100 μg/ml ampicillin. Plasmid DNA was extracted from transformed bacterial culture by the GeneScript QuickClean Miniprep Kit ®. As a verification test digestion/restriction reaction were occurred on plasmids using EcoRI enzyme to separate the DNA insert from the plasmid (Figure M9). The identity of the inserts was confirmed by DNA sequencing analysis. Two subsequent conventional PCR were run to amplify the positive plasmid vector, with M13 forward or reverse primer, Big dye and 5x sequencing buffer (Applied Biosystem, USA, CA). The PCR products were purified by Qiagen spin column followed by a heat dry. Formamide was added to dissolve PCR product and PCR product was loaded into sequencing machine (AB-Applied Biosystem, ABI PRISM, 3130X/ Genetic Analyzer, US). Sequencing results indicated that the cDNA of the gene of interest contained the same sequence as a portion of original gene obtained from the database of GeneBank (Appendix A, B).

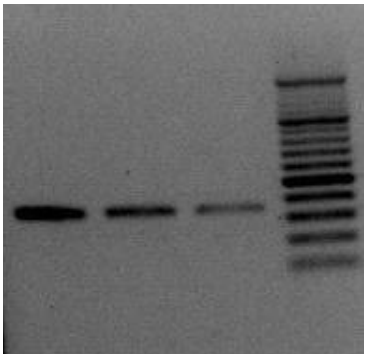


Figure M8. Agarose gel of Purified PCR product for GFAP
Triplicate samples of amplified GFAP by conventional PCR (from left to right: 8 μl, 4 μl and 2 μl of PCR product)

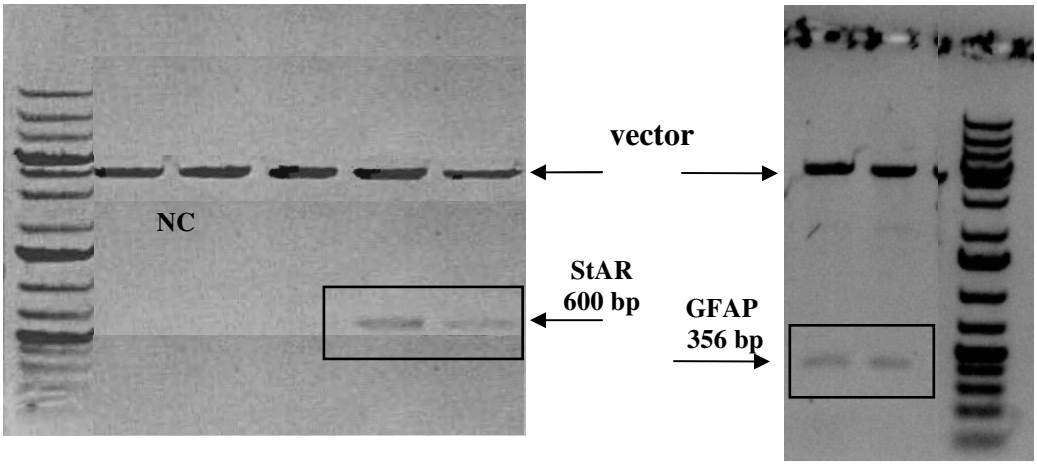


Figure M9. Agarose gel of digested GFAP and StAR plasmid. Duplicated samples of GFAP plasmid and of StAR plasmid digested with EcoRI was run on agorose gel. The lower bands show the GFAP or StAR fragment cleaved from the vector (the upper bands). NC, negative control

Real-time qPCR optimization

Real-time qPCR was performed using the Roche LightCycler and fast-start DNA Master SYBR Green I dye (Roche Diagnostics, Laval, QC, Canada). The experimental program for PCR was set up according to Instruction Manual (Roche Diagnostics, Laval, QC, Canada) as follows:

Parameter	**Value**
Denaturation temperature (^{o}C)	95
Pre-incubation time ("activation" of the Fast-Start Taq DNA polymerase (min)	10
Denaturation time (s)	10
Annealing temperature (^{o}C)	Primer dependent (optimized from conventional PCR + 5)
Annealing time (s)	0-10
Elongation temperature (^{o}C)	72
Elongation time	Length of product (bp)/25

Several PCR with gradient concentration of Mg^{++} were run to optimize the proper concentration. Afterward several PCR were run to optimize more precisely the annealing

temperature and annealing time with the proper concentration of Mg^{++}. The PCR products were run on agarose gel and a single band was observed (Figure M10).

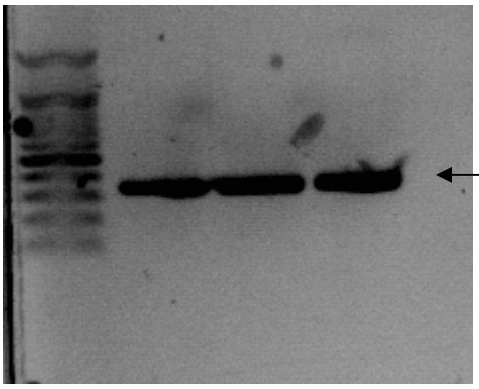


Figure M10. Agarose gel of PCR product for GFAP.
Triplicate samples of amplified GFAP by optimized PCR

Optimization of PCR program for CYP11B1, CYP11B2, StAR and GFAP resulted in the following conditions:

For CYP11B1 the PCR condition was set as follows: first, 95ºC for 10 min to activate Taq polymerase, followed by 45 cycles of denaturation at 95ºC for 5 sec, annealing at 55ºC for 5 sec and extension at 72ºC for 14 sec. The PCR condition for CYP11B2 was set as follows: first, 95ºC for 5 min to activate Taq polymerase, followed by 45 cycles of denaturation at 95ºC for 10 sec, annealing at 65ºC for 5 sec and extension at 72ºC for 10 sec. For StAR the primers were as mention above and the PCR condition was set as follows: first, 95ºC for 10 min to activate Taq polymerase, followed by 45 cycles of denaturation at 95ºC for 5 sec, annealing at 65ºC for 5 sec and extension at 72ºC for 25 sec. For GFAP the primers were as mention above and the PCR condition was set as follows: first, 95ºC for 10 min to activate Taq polymerase, followed by 45 cycles of denaturation at 95ºC for 5 sec, annealing at 62ºC for 5 sec and extension at 72ºC for 12 sec. Melting curve analysis was used to confirm the unique specific PCR for gene of interest (Figure M11).

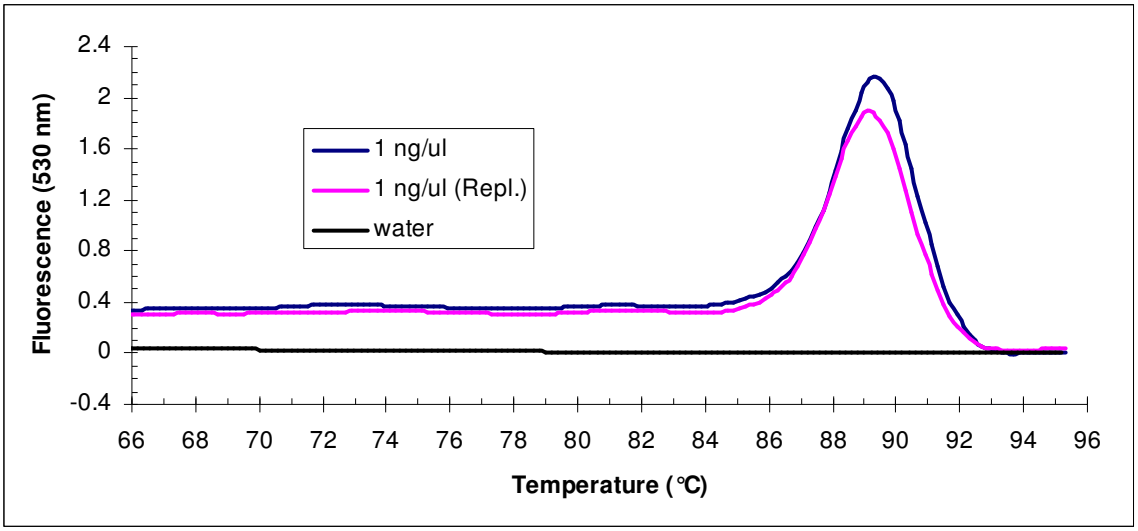


Figure M11. Melting curve. Melting cutve of a duplicated GFAP PCR product at 1000 pg/µl cDNA

Generation of standard

The concentrations of plasmids were measured by UV absorbance (OD260, Nano-drop). A serial 10 fold dilution from 1000 pg/µl to 0.001 pg/µl of the DNA plasmids containing cDNA of the gene of interest was used to generate a standard curve. The optimized PCR program for respective gene was used for generation of the standard curves (Figure M12-15).

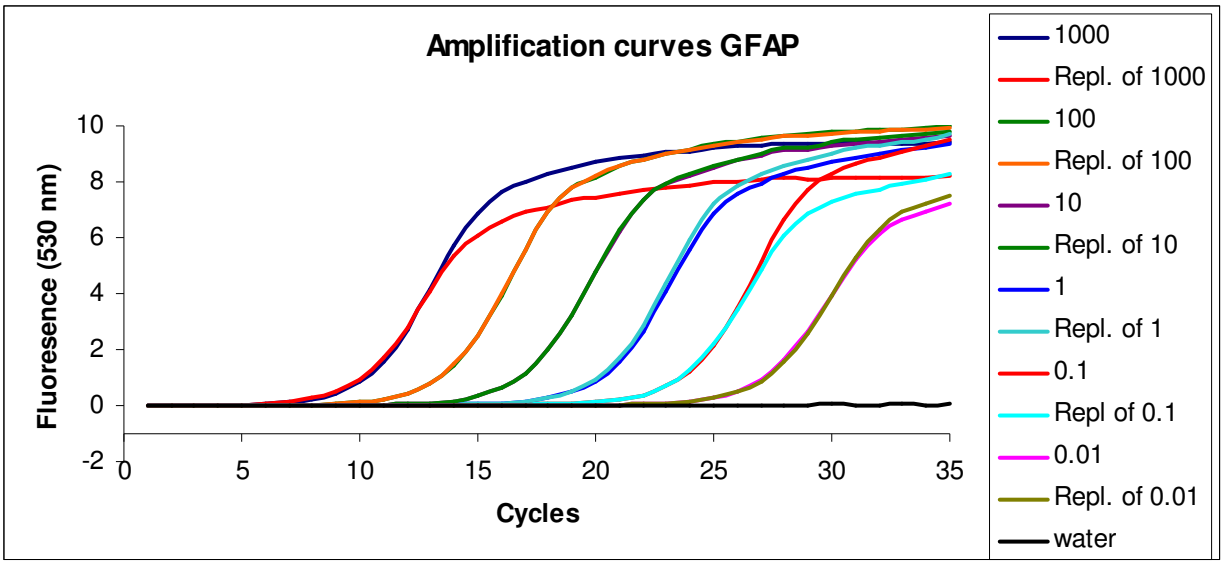


Figure M12. GFAP standard amplification curve. A serial 10 fold dilution from 1000 pg/µl to 0.01 pg/µl of the DNA plasmids containing the GFAP DNA fragment was used to generate a standard curve.

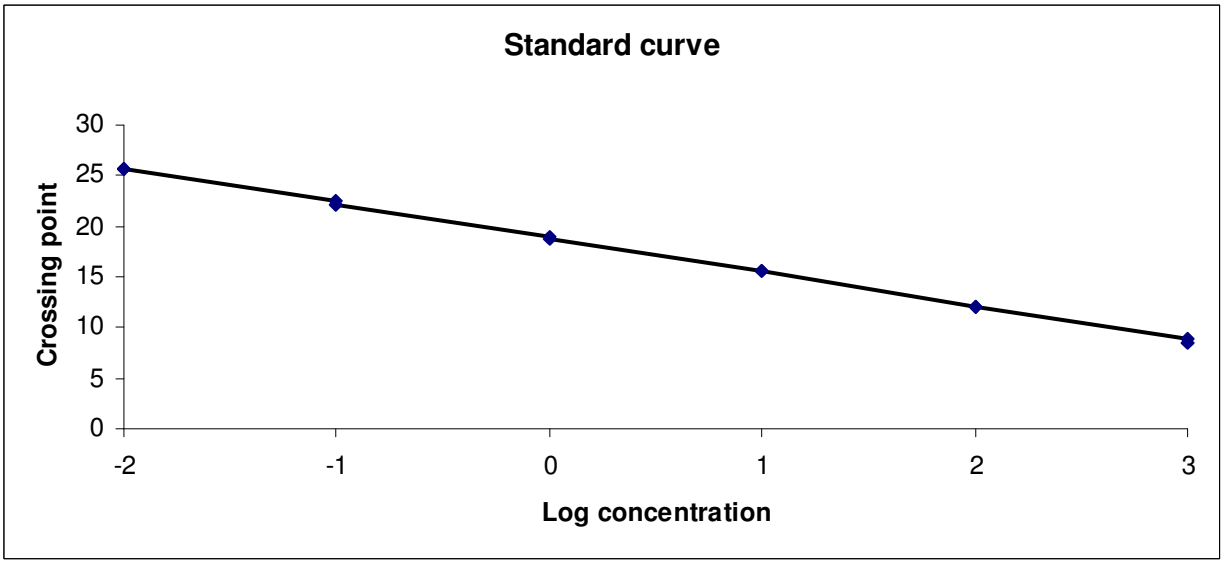


Figure M13. GFAP standard curve. A linear standard curve was obtained from the serial dilutions of cDNA of the PGK. Efficiency 1.96.

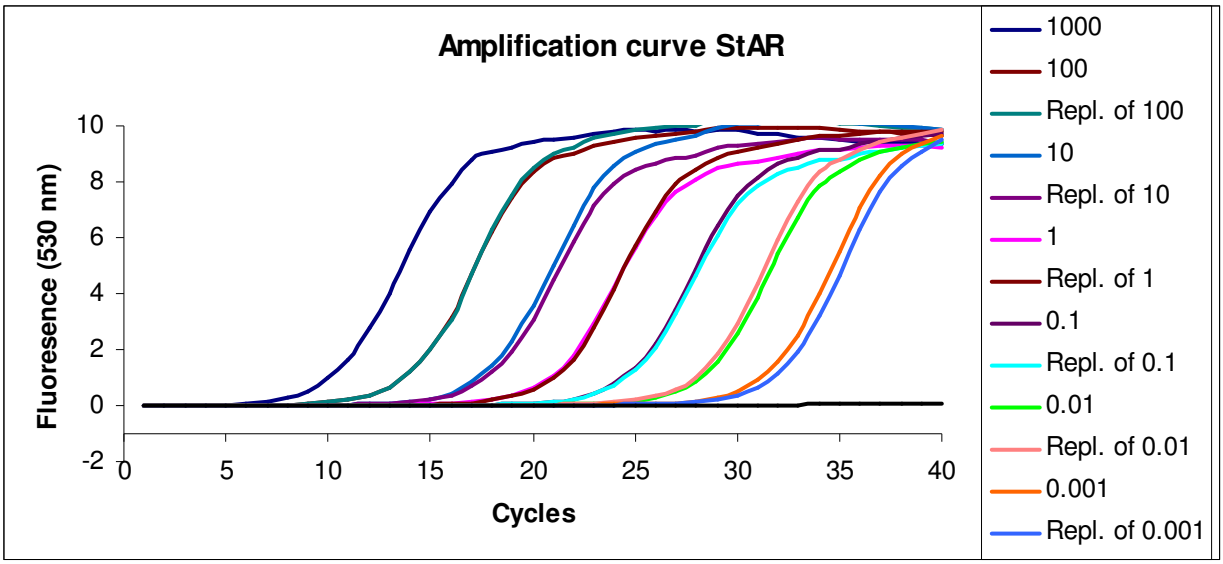


Figure M14. StAR standard amplification curve. A serial 10 fold dilution from 1000 pg/µl to 0.001 pg/µl of the DNA plasmids containing the StAR DNA fragment was used to generate a standard curve.

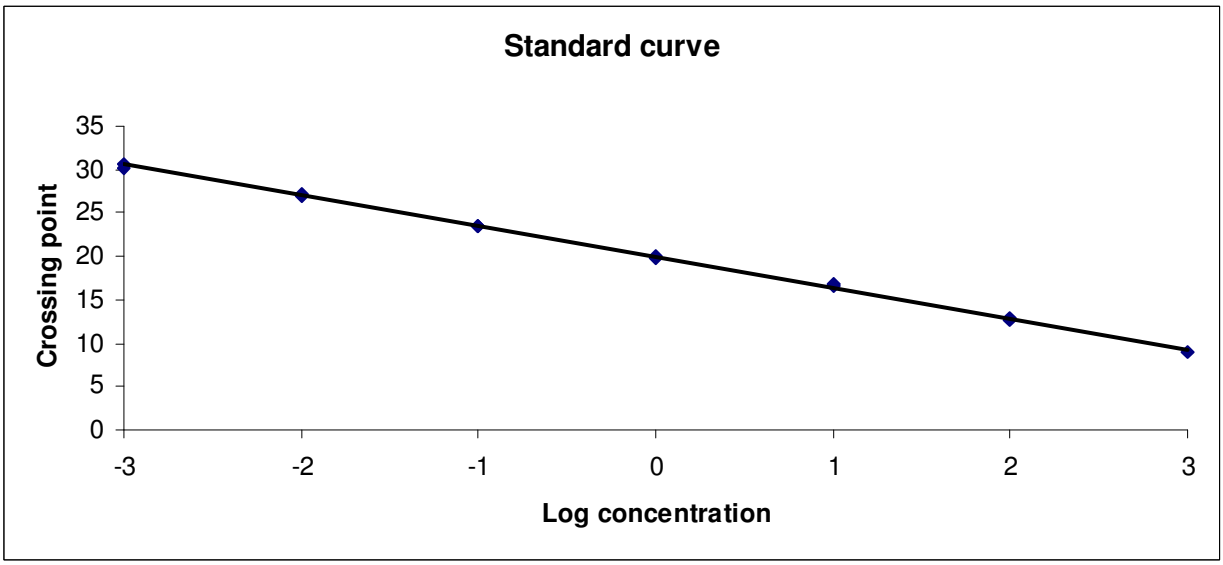


Figure M15. StAR standard curve. A linear standard curve was obtained from the serial dilutions of cDNA of the PGK. Efficiency 1.9.

2.3.5. Quantification of mRNA by real-time qPCR

Quantitative normalization of CYP11B1, CYP11B2 and StAR mRNA expression were achieved using the expression of the constitutively expressed reference gene, phosphoglycerokinase1 (PGK1), as an internal control. Protein gene product PGP 9.5 and glial fibrillary acidic protein (GFAP) was used as a neuron specific and glia specific marker gene for normalization, respectively. External standards were generated by using serial dilution of CYP11B1, CYP11B2 and StAR plasmids as mentioned above followed by respective real-time PCR conditions. Quantitative normalization of CYP11B1, CYP11B2 and StAR mRNA expression were achieved using the expression of phosphoglycerokinase 1(PGK1) as an internal control (Amin et al., 2005).

2.4. Statistical analysis

One way repeated-measures ANOVA analysis of variance was performed for the comparison of blood pressure data obtained from telemetry. For analysis of Fra immunostatining data one way ANOVA was used. When the F values were significant for main effect, a Duncan's method was used for multiple comparisons as a post-hoc procedure. For data with a failed normality test, one-way ANOVA on ranks was performed followed by Duncans's method for multiple comparisons. In PCR data for the comparison of the Ang II treated group and sham control group, t-test was performed. Statistical significance for all tests was defined as $p < 0.05$.

BLAST Analysis for StAR primer at Genebank

2701 **g ctcctgatgg ctgtgtgtac**
2761 tcacatctgc actaagtatc ttacacacaa atactggaat ccctctgctg aataagggtg
2821 aaacaattat aacaacctaa agatcttcct tctagacatc tttaaaagaa taatgtaatg
2881 tctctgtgag tgtgtgtgtg tgccaccgca tacacgtgaa ggtcagaggg caacttcagg
2941 agtgtgcccc gccccgtcct ggtcttgggt tgtcaggtta gacatcaagt acttttatct
3001 gcactgccat cttgctggcc cacttttctg tccctccgag taaacggtct tagtcgtctg
3061 ggtccccatc acctgcaagg actgcgctta tttatgaaca gaacaacgtg gaacgcgtgt
3121 ttatttattg aagtctgaag acttaacagc tggtatttat tgtaattcat tctgactttc
3181 tcagtctact ctgacagaga gtactgttgc tcctcgctgg tttcagaagc tgacagtgaa
3241 ggacagggcc tgcagcaagc actgtgtggc tgtaacggga acaccccggc tccctacaca
3301 ctgtaactgt ctctccaccg tc**tgagctgt tatccccattc**

Sequences producing significant alignments:

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
AB001349.1	Rattus norvegicus mRNA for steroidogenic acute regulatory protein,	42.1	42.1	100%	0.023	100%
AF044081.1	Rattus norvegicus steroidogenic acute regulatory protein (StAR) mRI	42.1	42.1	100%	0.023	100%

>dbj|AB001349.1| Rattus norvegicus mRNA for steroidogenic acute regulatory protein, complete cds
Length=3396

GENE ID: 25557 Star | steroidogenic acute regulatory protein
[Rattus norvegicus] **(Over 10 PubMed links)**

Score = 42.1 bits (21), Expect = 0.023
Identities = 21/21 (100%), Gaps = 0/21 (0%)
Strand=Plus/Plus

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             |||||||||||||||||||||
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```

>gb|AF044081.1|AF044081 Rattus norvegicus steroidogenic acute regulatory protein (StAR) mRNA, complete cds
Length=3402

GENE ID: 25557 Star | steroidogenic acute regulatory protein
[Rattus norvegicus] **(Over 10 PubMed links)**

Score = 42.1 bits (21), Expect = 0.023
Identities = 21/21 (100%), Gaps = 0/21 (0%)
Strand=Plus/Plus

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             |||||||||||||||||||||
Sbjct  2723  GCTCCTGATGGCTGTGTGTAC  2743
```

BLAST analysis for GFAP primer at Genebank

1321 atacagtgtg agggcctaaa gctccctcct cagatagtc**t tgtttgctag gcccaattc**c
1381 catccacacc agtgctcccc ttccttctgt ttttatgccc acggctcggt cagtgcggag
1441 tctcatggac ggcacagacc accctgcatc tccaactaac aggatactca ccccaaaggg
1501 gcaatcagga ggggaggacc cccctccccc cagctgggtt agaactggaa gaaagaggaa
1561 agacaggggc agggagactt aacaaatccc ttccttcatc cttgttgtta tggaaaccgt
1621 tgccagagct ggaggtctct gggaactgga ctttgagttt tcataggctg ctggagcaag
1681 acaaacattc agaca**gaaag gaaaagatcc cgagg**

Sequences producing significant alignments:

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
NM_017009.2	Rattus norvegicus glial fibrillary acidic protein (Gfap), mRNA >qb|BCC	40.1	40.1	100%	0.060	100%
AF028784.1	Rattus norvegicus glial fibrillary acidic proteins alpha and delta (GFAI	40.1	40.1	100%	0.060	100%
L27219.1	Rattus norvegicus glial fibrillary acidic protein (GFAP) mRNA sequenc	40.1	40.1	100%	0.060	100%
U03700.1	Rattus norvegicus Spraque Dawley glial fibrillary acidic protein mRNA	40.1	40.1	100%	0.060	100%

>ref|NM_017009.2| Rattus norvegicus glial fibrillary acidic protein (Gfap), mRNA
qb|BC088851.1| Rattus norvegicus glial fibrillary acidic protein, mRNA (cDNA clone MGC:105466 IMAGE:7315338), complete cds
Length=2714

GENE ID: 24387 Gfap | glial fibrillary acidic protein [Rattus norvegicus]
(Over 10 PubMed links)

Score = 40.1 bits (20), Expect = 0.060
Identities = 20/20 (100%), Gaps = 0/20 (0%)
Strand=Plus/Plus

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             ||||||||||||||||||||
Sbjct  1360  TTGTTTGCTAGGCCCAATTC  1379
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Length=8797

GENE ID: 24387 Gfap | glial fibrillary acidic protein [Rattus norvegicus]
(Over 10 PubMed links)

Score = 40.1 bits (20), Expect = 0.060
Identities = 20/20 (100%), Gaps = 0/20 (0%)
Strand=Plus/Plus

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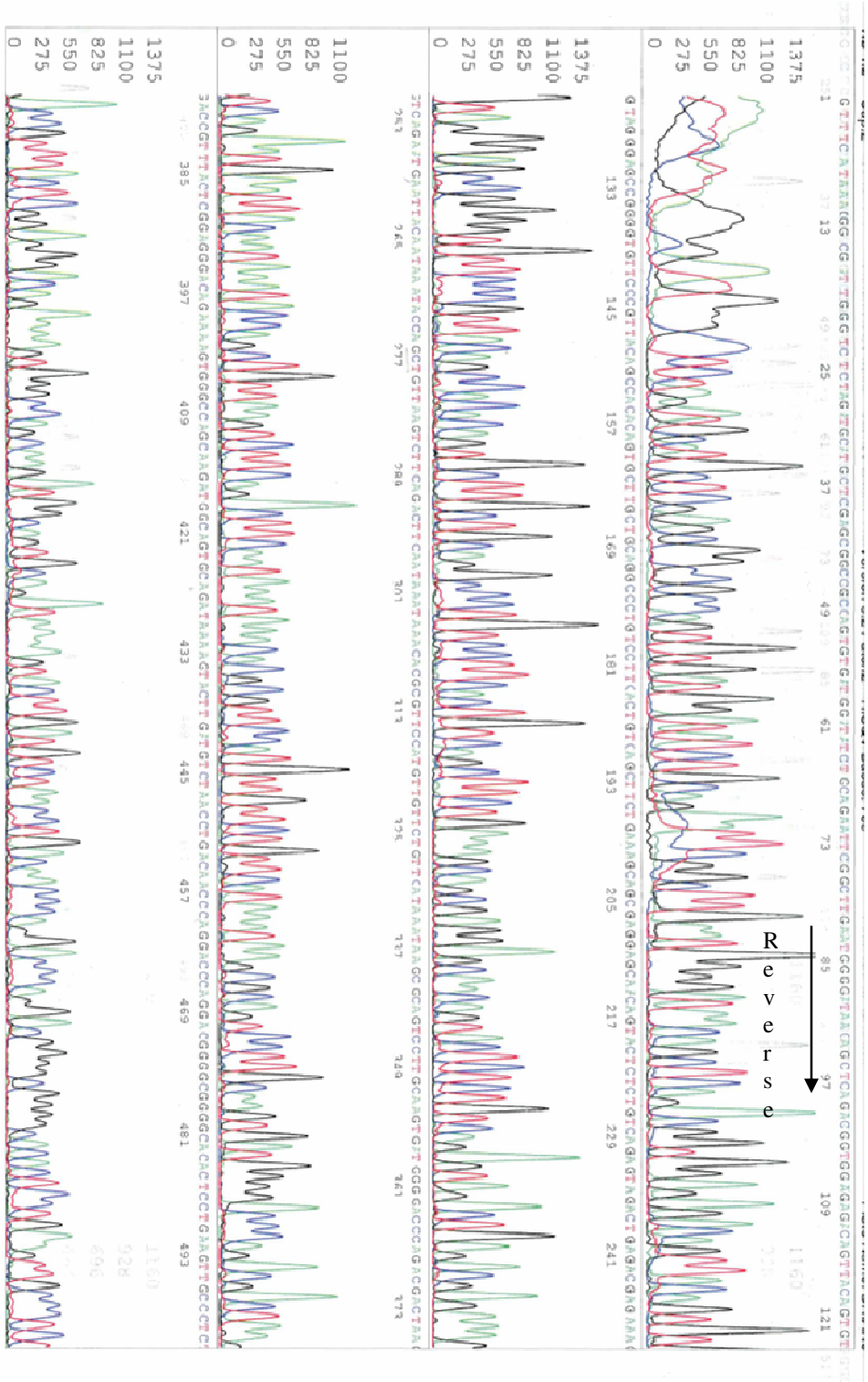
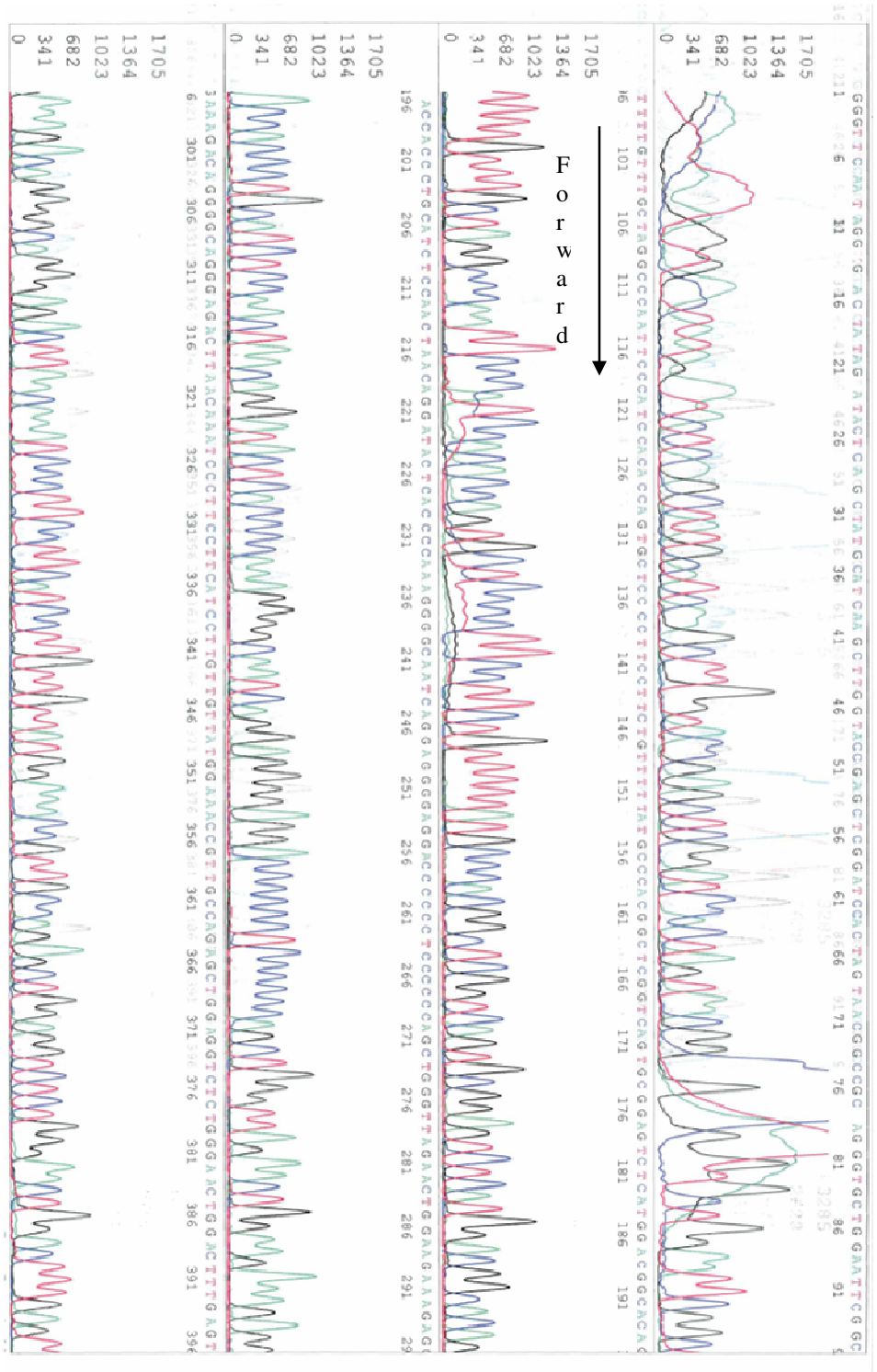
Appendix A. Sequences of LacZα gene of pCR2.1 vector containing StAR insert



Appendix B. Sequences of LacZα gene of pCR2.1 vector containing GFAP insert



Rattus norvegicus glial fibrillary acidic protein (Gfap), mRNA

NCBI Reference Sequence: NM_017009.2

Rattus norvegicus glial fibrillary acidic protein (Gfap), mRNA

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GGGTAGACTTCTCCCTGGCCGGGGCGCTCAATGCCGGCTTCAAAGAGACTCGGGCCAGCGAGCGTGCGGA
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Rattus norvegicus mRNA for steroidogenic acute regulatory protein, complete cds

GenBank: AB001349.1

Rattus norvegicus mRNA for steroidogenic acute regulatory protein, complete cds

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Alignment analysis for StAR and GFAP sequences at Genebank

StAR:

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Score = 1051 bits (569),  Expect = 0.0
 Identities = 596/608 (98%), Gaps = 6/608 (1%)
 Strand=Plus/Minus
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Sbjct  687
GCTCCTGATGGCTGTGTGTACTCACATCTGCACTAAGTATCTTACACACAAATACTGGAA  628

Query  61
TCCCTCTGCTGAATAAGGGTGAAACAATTATAACAACCTAAAGATCTTCCTTCTAGACAT  120
|||||  ||||||||| ||||||||||||||||||||||||||||||||||||||||||||
Sbjct  627
TCCCTTTGCTGAATATGGGTGAAACAATTATAACAACCTAAAGATCTTCCTTCTAGACAT  568

Query  121
CTTTAAAAGAATAATGTAATGTCTCTGTGAgtgtgtgtgtgtgtgCCACCGCATACACGTGA  180

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Query  181
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Sbjct  507
AGGTCAGAGGGCAACTTCAGGAGTGTGCCCCGCCCCGTCCTGGGTCCTGGGTTGTCAGGT  448

Query  240
TAGACATCAAGTACTTTTATCTGCACTGCCATCTTGCTGGCCCACTTTTCTGTCCCTCCG  299

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Query  300
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AGTAAACGGTCTTAGTCGTCTGGGTCCCCATCACTTGCAAGGACTGCGCTTATTTATGAA  328

Query  360
CAGAACAACGTGGAACGCGTGTTTATTTATTGAAGTCTGAAGACTTAACAGCTGGTATTT  419
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Query  475
TCGCTGGTTTCAGAAGCTGACAGTGAAGGACAGGGCCTGCAGCAAGCACTGTGTGGCTGT  534
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Sbjct  207
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Query  535
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Sbjct  147
AACGGGAACACCCCGGCTCCCTACACACTGTAACTGTCTCTCCACCGTCTGAGCTGTTAT  88

Query  595
CCCCATTCA  603
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Sbjct 87
CCCCATTCA 79
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http://blast.ncbi.nlm.nih.gov/Blast.cgi

GFAP:

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Score =  658 bits (356),  Expect = 0.0
 Identities = 356/356 (100%), Gaps = 0/356 (0%)
 Strand=Plus/Plus

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TTGTTTGCTAGGCCCAATTCCCATCCACACCAGTGCTCCCCTTCCTTCTGTTTTTATGCC  157

Query  61
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Sbjct  158
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Query  121
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||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  218
CAGGATACTCACCCCAAAGGGGCAATCAGGAGGGGAGGACCCCCCTCCCCCCAGCTGGGT  277

Query  181
TAGAACTGGAAGAAAGAGGAAAGACAGGGGCAGGGAGACTTAACAAATCCCTTCCTTCAT  240

||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  278
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Query  241
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||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  338
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Query  398
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||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  301
TTCATAGGCTGCTGGAGCAAGACAAACATTCAGACAGAAAGGAAAAGATCCCGAGG  453
```

Alignment analysis for CYP11B2 and CYP11B1

Since there is too high homology between the CYP11B1 and CYP11B2 in their whole sequence the alignment alanysis were done. The alignment results showed relatively low homology in gene sequence.

```
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                                              |   | |||    | | |
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||  |||||||||||  ||||   |||  |||   ||||   |||||  ||  ||  ||||||||||||
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|||||||||||||||  ||||||||||||   ||||||||||| |||||||||||||  ||||
TCCACCACACAGCTCATGTTCTTACCCAAGAGCTTGACTCGTTGGACAAGCACCCGGGTG

TGGAAAGAACATTTTGATGCCTGGGATGTCATCTCTGAGTATGCCAACAGATGTATCTGG
||||||||||| |||||| ||||||||  ||||||||||||||||  ||   | |||||||   |
TGGAAAGAACACTTTGATTCCTGGGATATCATCTCTGAGTATGTCACAAAATGTATCAAG

AAGGTGCACCAGGAACTCAGACTCGGCAGCTCTCAGACCTACAGTGGCATTGTGGCAGCACTAAT
||  |||  | |   |||||    |    ||   ||    |||  |||   ||||  |||     |
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3. Results

3.1. Blood pressure response and plasma Ang II levels to sc infusion of Ang II

Wistar rats instrumented with telemetry probes were infused subcutaneously with Ang II at 150 and 500 ng/kg/min for 14 days. The baseline MAP was not significantly different among groups (Table R1). The sc infusion of Ang II at 150 ng/kg/min induced a slow progressive increase in BP: a significant increase by ~8mmHg in the first few days and by ~ 20mmHg on days 10-14. Sc infusion of Ang II at 500ng/kg/min increased the BP by ~20mmHg within the first few days and by ~60mmHg on days 10-14. ($P < 0.05$ for all vs corresponding baseline) (Figure R1). After 14 days of infusion, trunk blood was collected and plasma Ang II levels measured. Ang II at 150 ng/kg/min did not change plasma Ang II level, whereas infusion of Ang II at 500 ng/kg/min induced a 4 fold increase in plasma Ang II (Figure R2).

Groups	**MAP (mmHg)**
Vehicle	110±4
Ang II at 150 ng/kg/min	116±5
Ang II at 500 ng/kg/min	114±3

Table R1. The 24 h baseline MAP before start of sc infusion of Ang II in different groups of rats. See Fig. R2 for n values. Values are mean±SE.

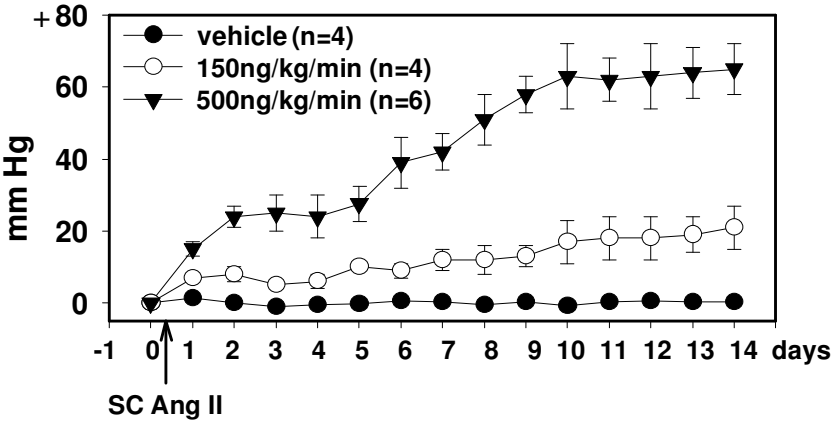


Figure R1. Effects of sc infusion of Ang II on blood pressure. Effects of sc infusion of Ang II at 150 or 500 ng/kg/min or vehicle (0.9% saline) on 24-h MAP as assessed with telemetry in Wistar rats. Continuous recording of resting BP was started 3 days after the probe implantation. After 3 days of control recordings of resting BP, sc osmotic minipumps were implanted for a 2-wk infusion (day 0). Data are means ± SE. Average MAP over 24 h was used for comparisons. One-way repeated-measures ANOVA was performed. F=107.2, P=0.0001

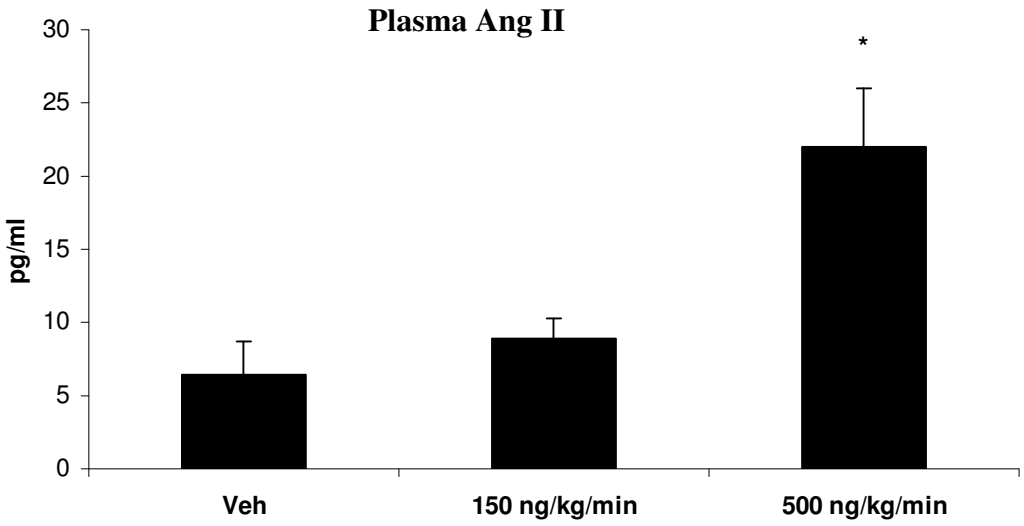


Figure R2. Plasma Ang II level after sc infusion of Ang II. Plasma Ang II concentrations in Wistar rats after a 14 day sc infusion of Ang II at 150 or 500 ng/kg/day, or 0.9% saline (Veh). Data are means ± SE. one-way ANOVA test was used. See Fig. R2 for n values. F=17.0, p=0.0009, * P<0.05 vs. Vehicle.

For the assessment of effects of sc infusion of Ang II on central and adrenal aldosterone biosynthesis (molecular biology studies) rats were infused with Ang II 500ng/kg/min sc for 3 or 14 days. Control rats were sham operated. At day 14 prior to termination of the infusion under general anesthesia with isoflurane BP and heart rate were measured. Sc infusion of Ang II increased systolic BP by ~50 mmHg, diastolic BP by ~20 mmHg, mean arterial pressure (MAP) by 25 mmHg and heart rate by 40 bpm compared to sham operated rats (Figure R3). Blood pressure and heart rates were not measured for the 3 days infusion of Ang II.

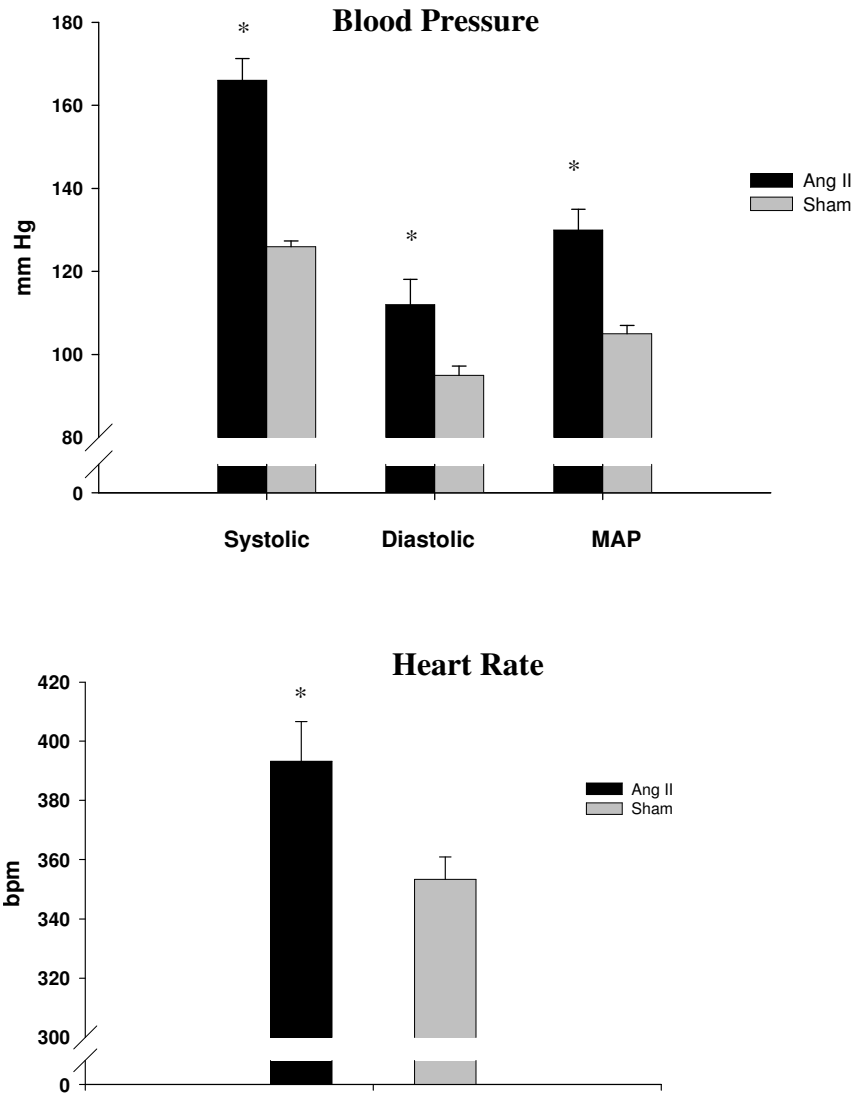


Figure R3. **Blood pressure and heart rate**. Systolic and diastolic pressure and heart rate in rats infused with Ang II 500 ng/kg/min for 14 days as compared to sham operated rats. Data are presented as means ± SE. t-test was used for analysis. (n= 6/group). * $P \leq 0.05$.

3.2. Time-Related Changes in Fra expression by sc infusion of Ang II

Ang II was infused at 150 and 500 ng/kg/min for 1, 4 or 14 days to assess the time course of neuronal activation. Neuronal activation in various hypothalamic nuclei was assessed by counting the number of Fra positive neurons. Table R2 summarizes the number of Fra positive neurons in the hypothalamic nuclei.

Infusion of saline at day 1 induced a 50% increase in Fra expression in pPVN and a 100% increase in mPVN. By 14 days infusion of saline increased Fra expression by 2 fold in MnPO, as compared to the control group that did not receive any treatment. In other nuclei infusion of saline did not significantly change Fra expression as compared to the controls group. This indicates that the implantation of osmotic minipumps and the infusion by itself induces some stress and thereby neuronal activation. Therefore, saline groups (vehicle) were considered as control rather than the groups that did not receive any treatment.

	SFO	MnPO	pPVN	mPVN	SON
Day 1					
Intact	11±0	16±2	43±5	11±3	40±5
Vehicle	15±1	20±2	61±3‡	26±2‡	47±2
Ang II (150)	28±5*	25±2	100±7*	46±7*	71±6*
Ang II (500)	51±2*†	32±4	115±3*	56±5*	83±8*
Day 4					
Vehicle	14±1	23±4	45±3	21±2	35±6
Ang II (150)	52±8*	51±8*	120±10*	49±6*	93±4*
Ang II (500)	70±5*	80±6*†	150±8*†	70±7*†	104±12*
Day 14					
Intact	10±1	12±1	45±1	28±1	36±9
Vehicle	13±1	32±3‡	51±9	35±3	52±8
Ang II (150)	13±1	28±3	117±17*	82±4*	97±10*
Ang II (500)	26±5*†	32±2	195±17*†	85±5*	134±9*†

Table R2. Fra expression in various brain nuclei in intact rats and rats after 1, 4 or 14 day of sc infusion of vehicle or Ang II at 150 or 500 ng/kg/min. Values are means±SE, for numbers of Fra positive cells as average of 3-4 sections per nucleus from each rat. (n=4 rats/group). One-way ANOVA was performed followed by a Duncan's test as a post hoc procedure.
*: $p<0.05$, vs. Vehicle ; †: $p<0.05$, vs. Ang II (150). ‡: $p<0.05$, vs. intact.
SFO : subfornical organ; MnPO : median preoptic nucleus
pPVN: parvocellular part of paraventricular nucleus; mPVN : magnocellular part of paraventricular nucleus; SON: supraoptic nucleus.

SFO: Figure R4 shows the Fra positive neurons as dark dots, and the changes in Fra expression after 1, 4 or 14 days of sc infusion Ang II. Ang II infusion at 150 and 500 ng/kg/min induced an initial increase (by ~2 or ~3 fold respectively) of Fra expression in the SFO which persisted after 4 days (by ~ 4 - 5 fold). After 14 days, Ang II at 150 ng/kg/min no longer had an effect and at 500 ng/kg/min still increased Fra expression (by 2 fold), but less than after 1 and 4 days of infusion. Fra expression at 1 and 14 days of infusion of Ang II at 500 ng/kg/min showed a significant further increase as compared to the expression by 150 ng/kg/min Ang II (Figure R5).

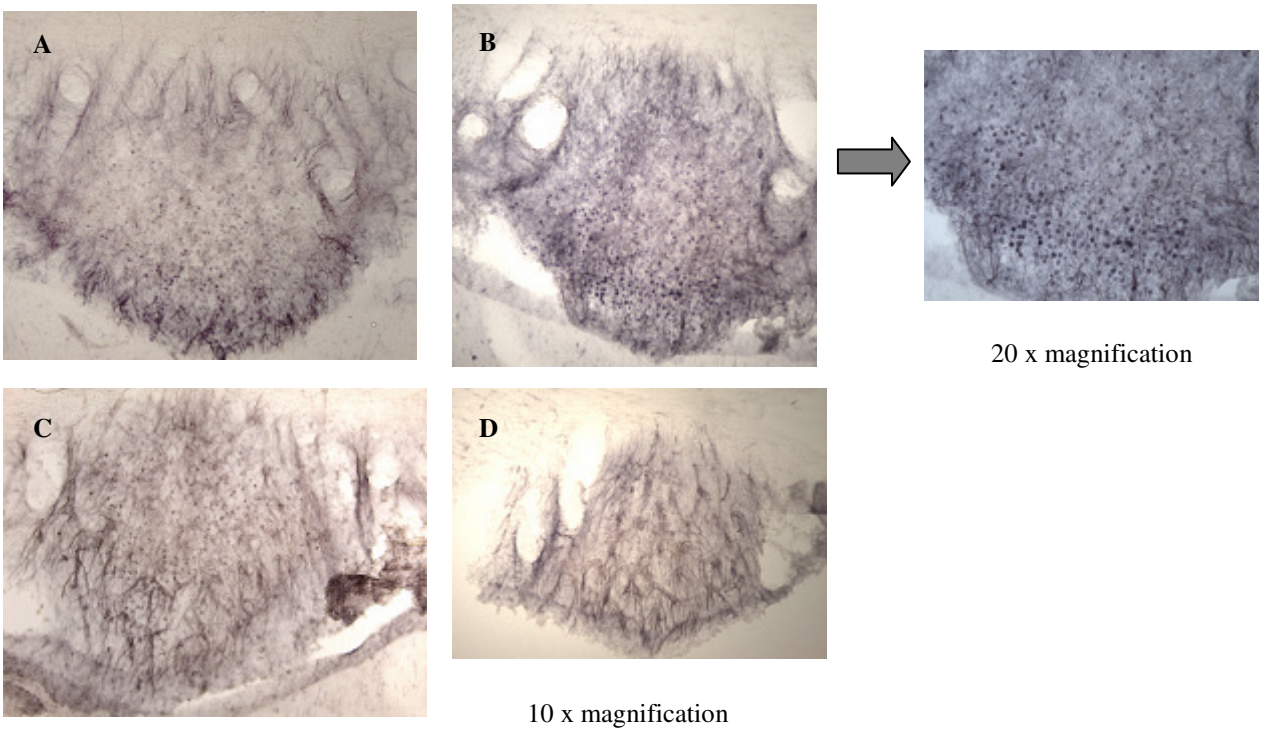


Figure R4. Photomicrograph of coronal sections through the SFO.
Ang II infusion at 500 ng/kg/min for A)1 day B) 4 days C) 14 days
D) sc saline infusion for 14 days

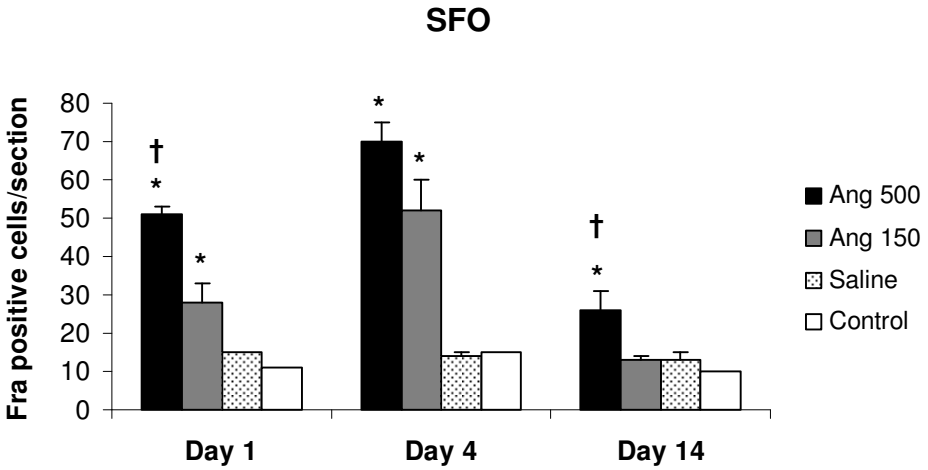


Figure R5. Time related changes in Fra expression in the SFO. Number of Fra-like immunoreactivity-positive neurons in SFO after sc infusion of Ang II for 1, 4 and 14 days. Data are presented as means ± SE. One-way ANOVA was performed followed by a Duncan's test as a post hoc procedure. (n=4 for Ang 500, Ang 150, saline groups and, n=2 for control). 1 day (F= 29.4, P= 0.0001),
4 days (F= 44.4, P= 0.001), 14 days (F= 5.1, P= 0.03). *P<0.05 vs saline. † P<0.05 vs Ang II (150)

MnPO: Infusion of Ang II at 150 or 500 ng/kg/min for 1 day did not increase Fra expression in the MnPO. After 4 days Ang II infusion at 150 or 500 ng/kg/min caused a dose related increase in Fra expression by (~2 or 3.5 respectively). Infusion of Ang II at 500 ng/kg/min showed a further increase in Fra expression as compared to the Fra expression induced at 150 ng/kg/min. After 14 days infusion of Ang II at both rates no longer increased Fra expression (Figure R6).

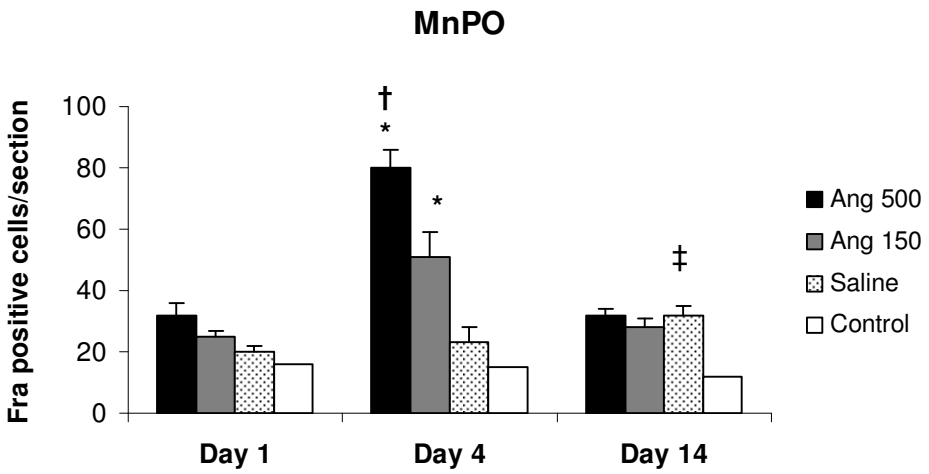


Figure R6. Time related changes in Fra expression in the MnPO. Number of Fra-like immunoreactivity-positive neurons in MnPO after sc infusion of Ang II for 1, 4 and 14 days. Data are presented as means ± SE. One-way ANOVA was performed followed by a Duncan's test as a post hoc procedure. (n=4 for Ang 500, Ang 150, saline groups and, n=2 for control). 4 day (F= 14.3, P= 0.002), 14 days (F= 6.3, P= 0.01). *P<0.05 vs saline, † P<0.05 vs Ang II (150), ‡ P<0.05 vs control.

SON: Figure R7 shows the changes in Fra expression in SON by sc infusion of Ang II for 1, 4 or 14 days. Ang II infusion at both 150 and 500 ng/kg/min induced an initial increase (by ~ 1.5 fold) of Fra expression in SON which persisted after 4 days (by ~2.5 fold) and 14 days (1.5 or 2 fold respectively). Only at 14 days Fra expression at 500 ng/kg/min Ang II was significantly higher than the expression by 150 ng/kg/min Ang II (Figure R8).

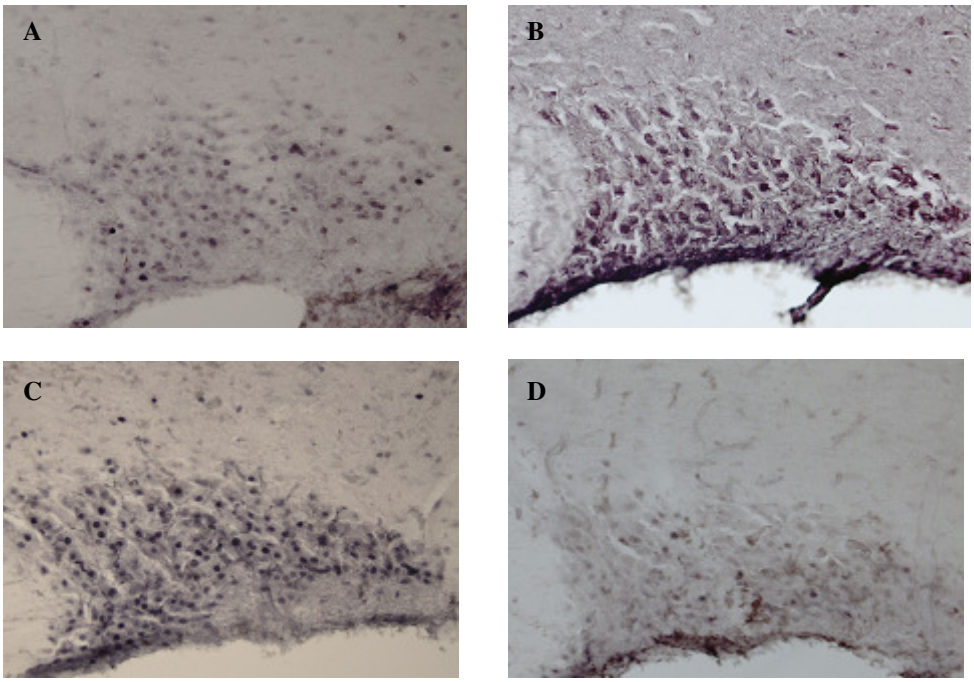


Figure R7. Photomicrograph of coronal sections through the SON.
Infusion of Ang II at 500 ng/kg/min for A)1 day B) 4 days C) 14 days
D) sc saline infusion for 14 days

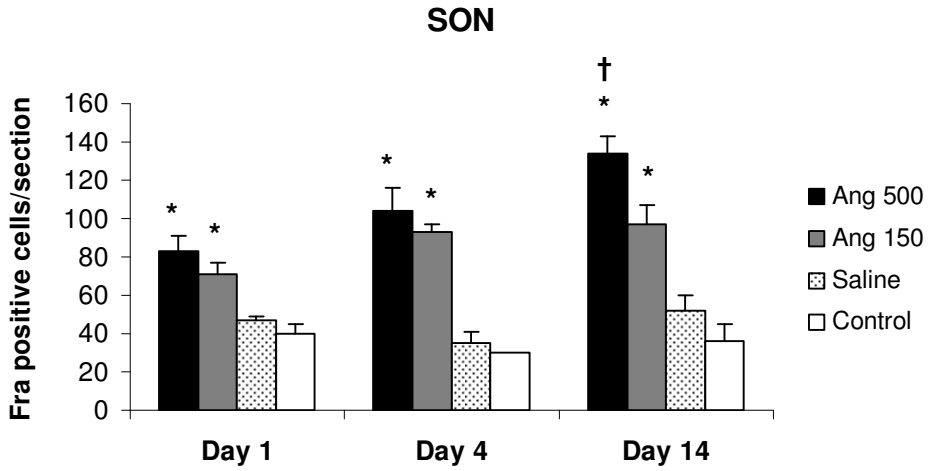
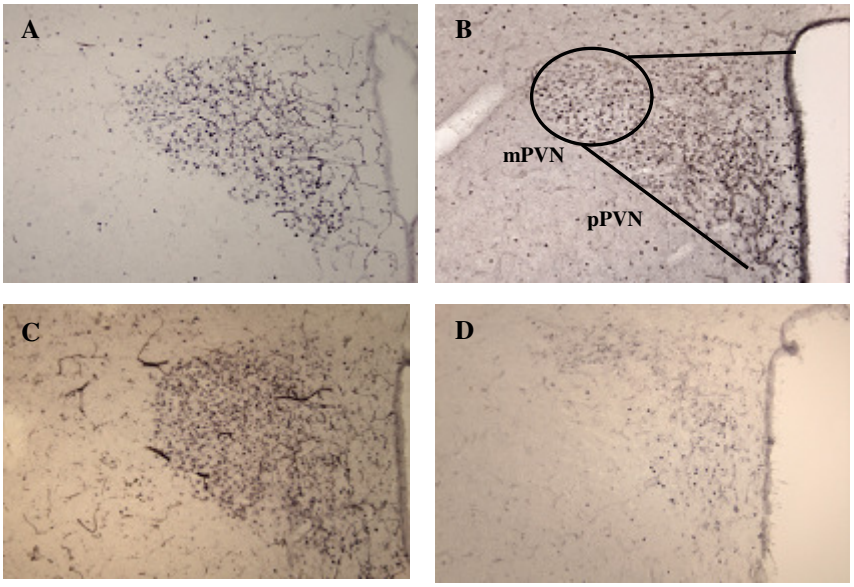


Figure R8. Time related changes in Fra expression in the SON. Number of Fra-like immunoreactivity-positive neurons in SON after sc infusion of Ang II for 1, 4 and 14 days. Data are presented as means ± SE. One-way ANOVA was performed followed by a Duncan's test as a post hoc procedure (n=4 for Ang 500, Ang 150, saline groups and, n=2 for control). 1 day (F= 9.0, P= 0,003), 4 days (F=20.9, P=0.0004), 14 days (F=16.4, P= 0.0003). *P<0.05 vs saline, † P<0.05 vs Ang II (150)

PVN: Figure R9 shows the changes in Fra expression in the parvocellular and magnocellular subdivisions of PVN by sc infusion of Ang II for 1, 4 or 14 days. In both parvocellular and magnocellular PVN infusion of Ang II increased number of Fra positive cells as compared to the vehicle (Figure R10 and R11). In parvocellular PVN infusion of Ang II at 150 and 500 ng/kg/min significantly increased the Fra expression by ~1.5 fold at day 1. The increase in Fra expression persisted after 4 days (by ~2.5 or ~3 fold respectively) and after 14 days (~2.5 or ~4 fold respectively) (Figure R10). Fra expression at 500 ng/kg/min at day 4 and 14 was significantly higher than the expression by 150 ng/kg/min.



Distribution of Fra expression in subdivisions of PVN
mPVN: magnocellular neurons
pPVN: parvocellular neurons

Figure R9. Photomicrograph of coronal sections through the PVN.
Infusion of Ang II at 500 ng/kg/min for A)1 day B) 4 days C) 14 days
D) sc saline infusion for 14 days

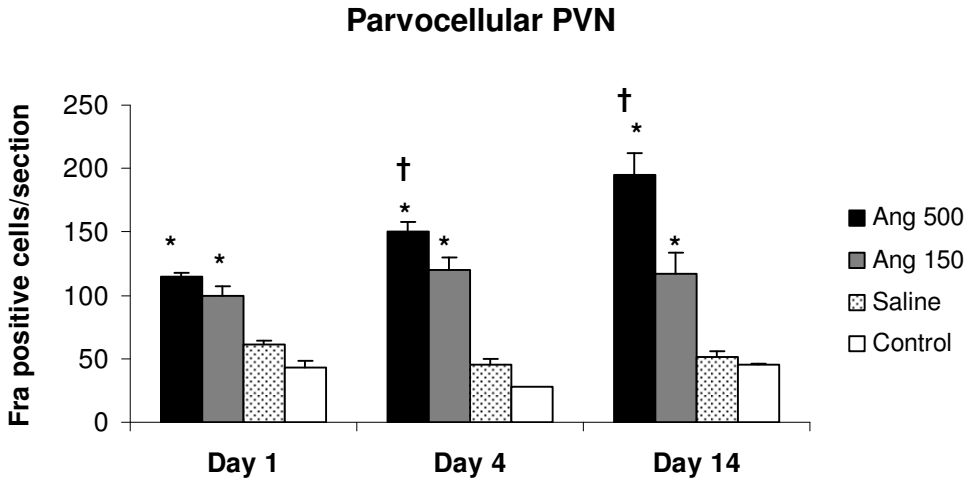


Figure R10. Time related changes in Fra expression in the pPVN. Number of Fra-like immunoreactivity-positive neurons in pPVN after sc infusion of Ang II for 1, 4 and 14 days. Data are presented as means ± SE. One-way ANOVA was performed followed by a Duncan's test as a post hoc procedure. (n=4 for Ang 500, Ang 150, saline groups and, n=2 for control). 1day (F= 35.0, P= 0.0001), 4 days (F= 72.5, P= 0.0001), 14 days (F=18.0, P=0.0006). *P<0.05 vs saline, † P<0.05 vs Ang II (150).

In the magnocellular PVN infusion of Ang II at 150 and 500 ng/kg/min significantly increased Fra expression by ~ 2 fold at day 1. The increase in Fra expression persisted after 4 days (2 or 3 fold respectively) and 14 days (by ~ 2.5 fold) (Figure R11). Only at 4 days Fra expression at 500 ng/kg/min was significantly higher than the expression induced by 150 ng/kg/min.

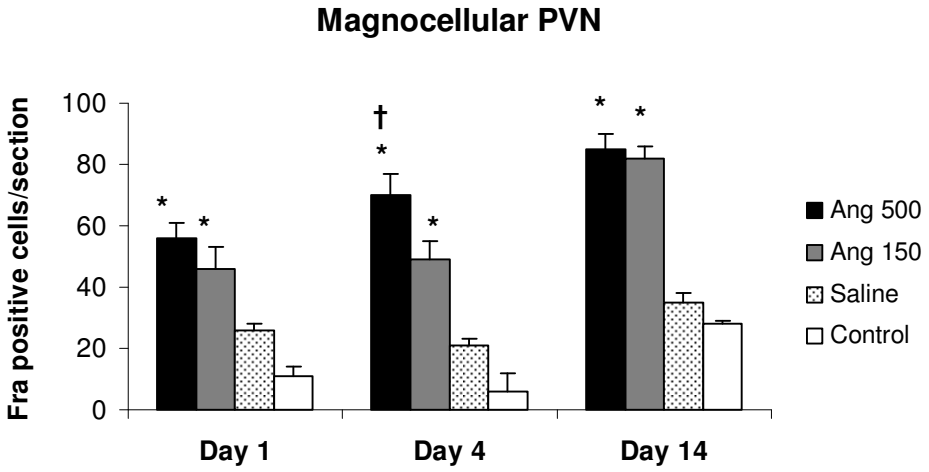


Figure R11. Time related changes in Fra expression in the mPVN. Number of Fra-like immunoreactivity-positive neurons in mPVN after sc infusion of Ang II for 1, 4 and 14 days. Data are presented as means ± SE. One-way ANOVA was performed followed by a Duncan's test as a post hoc procedure . (n=4 for Ang 500, Ang 150, saline groups and, n=2 for control). 1 day (F= 10.6, P= 0.002), 4 days (F=19.8, P= 0.0005), 14 days (F= 55.5, P= 0.0001). *P<0.05 vs saline, † P<0.05 vs Ang II (150).

3.2.1. Effects of central blockades on neuronal activation by sc infusion of Ang II

Sc infusion of Ang II at 500 ng/kg/min combined with icv infusion of vehicle for 14 days significantly increased Fra expression in the mPVN, pPVN, and SON, compared to rats with both icv and sc infusion of vehicles. This pattern of Fra expression was similar to the pattern observed with sc infusion of Ang II alone. Icv infusion of either FAD286 (100 μg/kg/day), or spironolactone (10 μg/kg/day) attenuated Fra expression in pPVN by 50% and in mPVN by 80%. However, Fra expression remained higher than the Fra expression in the vehicle group. In contrast, icv infusion of FAD286 or spironolactone did not affect the neuronal activation in the SON, MnPO and further increased Fra expression in the SFO by 2 fold, compared with rats with sc Ang II and icv vehicle. (Figure R12).

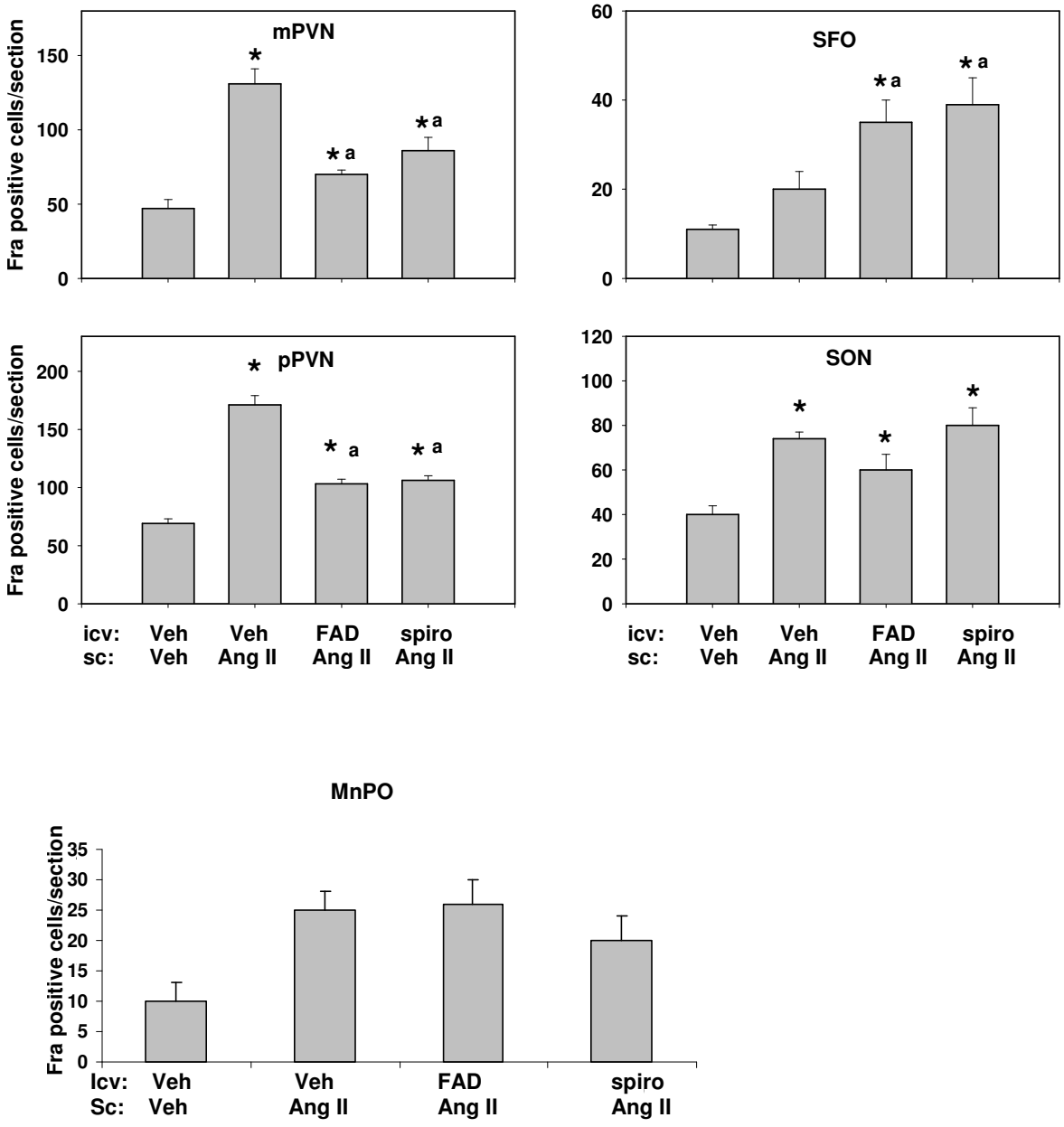


Figure R12. Effects of central blockade of MR or aldosterone synthase on Fra expression in the mPVN, pPVN, SFO, SON and MnPO. Wistar rats received a sc infusion of Ang II at 500 ng/kg/min or 0.9% saline (Veh) for 14 days with icv infusion of aldosterone synthase inhibitor FAD286 (FAD) at 100μg/kg/day, MR blocker spironolactone (spiro) at 10 μg/kg/day, or vehicle for spironolactone (Veh). Data are mean ± SE (n=5-6). Data are presented as means ± SE. One-way ANOVA was performed followed by a Duncan's test as a post hoc procedure.
pPVN (F= 43.2, P= 0.0001), mPVN (F= 24.8, P= 0.0001), SON (F= 7.9, P= 0.002),
SFO (F= 7.6, P= 0.003), MnPO (F= 3.27, P= 0.053) *p<0.05, vs. Veh/veh; a:p<0.05, vs. Veh/Ang II.

3.3. Effects of sc infusion of Ang II on adrenal steroidogenesis

Baseline levels: mRNA expression levels for the steroidogenic enzymes are shown in table R3. In adrenals of sham rats StAR mRNA level was 26 fold higher than CYP11B2 mRNA and 3 fold higher that CYP11B1. mRNA levels of steroidogenic enzymes were markedly higher in adrenal than in forebrain nuclei (StAR 1000 fold and CYP11B1 and B2 10 000 fold higher) (Table R3 vs. R8-R12).

Adrenal	B1/PGK		B2/PGK		StAR/PGK	
	Sham	Ang II	Sham	Ang II	Sham	Ang II
3 days	3.6 ± 0.37	3.7 ± 0.26	0.4 ± 0.17	**4.8 ± 0.88** [a]	10.5 ± 1.40	**18.2± 1.33** [a]
14 days	6.4 ± 0.83	6.7 ±0.75	0.9 ± 0.20	**10.3 ± 1.90** [a]	31.0 ± 6.90	27.0 ± 5.70

Table R3. mRNA expression of enzymes involved in steroidogenesis (StAR, CYP11B2, CYP11B1) in adrenal. (n=6/group). Data are mean values ± SE. t-test was used for comparison. ***(a) P<0.01***

The effects of sc infusion of Ang II at 500 ng/kg/min for 3 and 14 days on mRNA expression of steroidogenic enzymes were assessed. After 3 days sc infusion of Ang II increased expression of StAR mRNA by 73%. However, after 14 days no changes were found in StAR mRNA expression (Table R3, Figure R13).

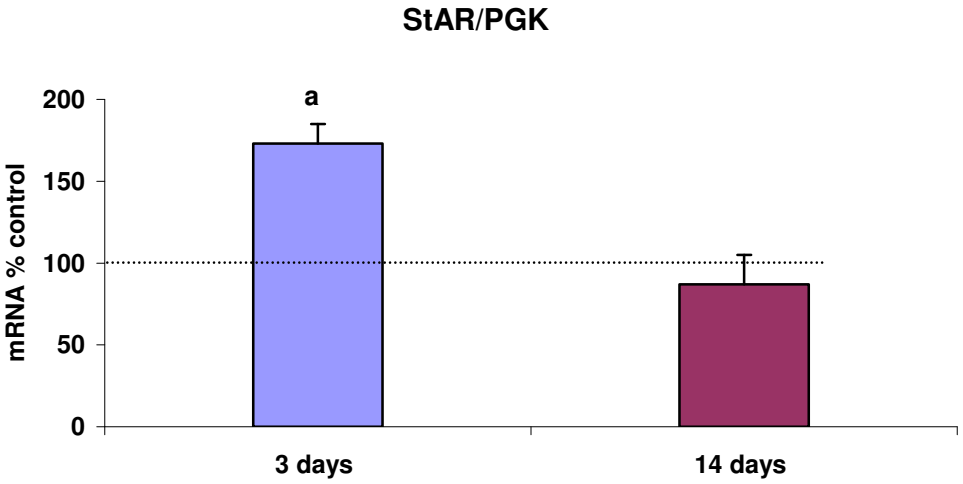


Figure R13. Time course of changes in mRNA expression for StAR in adrenal. mRNA expression for StAR in rats infused for 3 or 14 days sc with Ang II 500 ng/kg/min as compared to vehicle. Data is shown as percentage of control group (100%). (n=6/group), a P<0.01

sc infusion of Ang II significantly increased CYP11B2 mRNA expression by 12 fold after both 3 and 14 days. No changes were observed in expression of CYP11B1 mRNA after either 3 or 14 days of infusion of Ang II (Table R3, Figure R14).

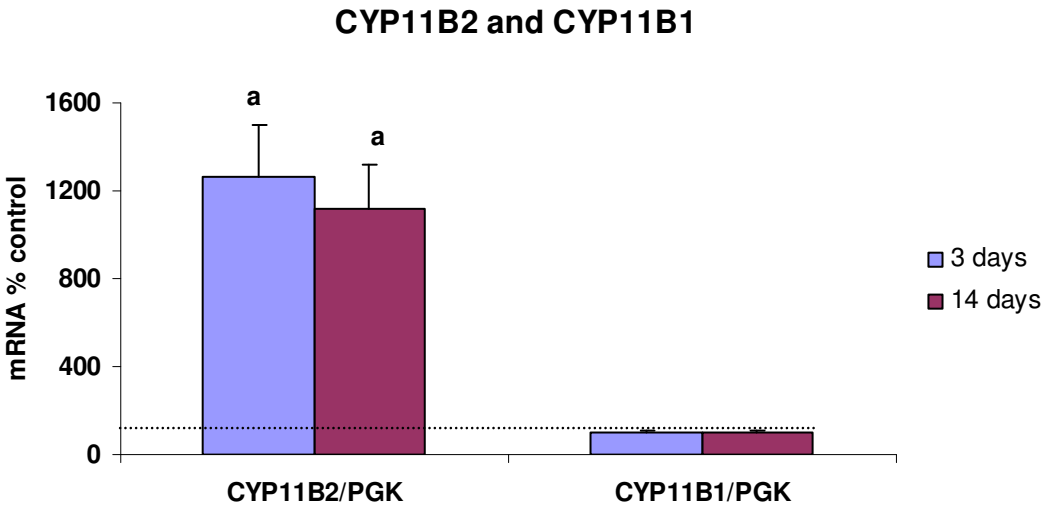


Figure R14. Time course of changes of mRNA expression for CYP11B2 and CYP11B1 in adrenal. mRNA expression for CYP11B2 and CYP11B1 in rats infused for 3 or 14 days sc with Ang II at 500 ng/kg/min as compared to vehicle. Data is shown as percentage of control group (100%). (n=6/group), a P<0.01

3.4. Effects of sc infusion of Ang II on central steroidogenesis

Neurons and glia express different steroidogenic enzymes and it was therefore considered necessary to measure the expression versus the respective cell type. The relative amount of neurons and glia in different nuclei as indicated by PGP/PGK and GFAP/ PGK was assessed first.

3.4.1. mRNA expression of neuronal marker PGP in hypothalamic nuclei, and effects of infusion of Ang II

Baseline level: Table R4 summarizes the mRNA expression of PGP over PGK in hypothalamic nuclei. Expression of PGP mRNA as a neuron specific marker reflects the amount of neurons present in each nucleus. In sham groups OVLT had lower expression of PGP mRNA than the other nuclei indicating that OVLT contained lowest amount of neurons among the nuclei.

PGP/PGK, $x10^{-1}$	3 days		14 days	
	Sham	Ang II	Sham	Ang II
SON	6.8 ± 0.96	**4.5 ± 0.23** *	11.5 ± 1.00	**6.4 ± 0.30** a
PVN	12.8 ± 0.08	**9.7 ± 0.05** a	7.2 ± 0.37	**5.5 ± 0.30** a
MnPO	7.6 ± 0.72	**5.0 ± 0.45***	13.7 ± 1.20	**6.2 ± 0.86** a
OVLT	4.7 ± 0.13	**2.4 ± 0.07** a	3.9 ± 0.39	**2.7 ± 0.18** *
SFO	14.8 ± 1.37	**9.1 ± 0.58** a	8.8 ± 1.28	**4.2 ± 0.53** a

Table R4. **mRNA expression of the protein gene product 9.5 (PGP) in hypothalamic nuclei.** (n=6/group). Data are mean values ± SE. t-test was used for comparison. ***P< 0.05, (a) P<0.01***

Infusion of Ang II for 3 or 14 days reduced mRNA expression of PGP in all nuclei studied by ~ 30-40% (Figure R15).

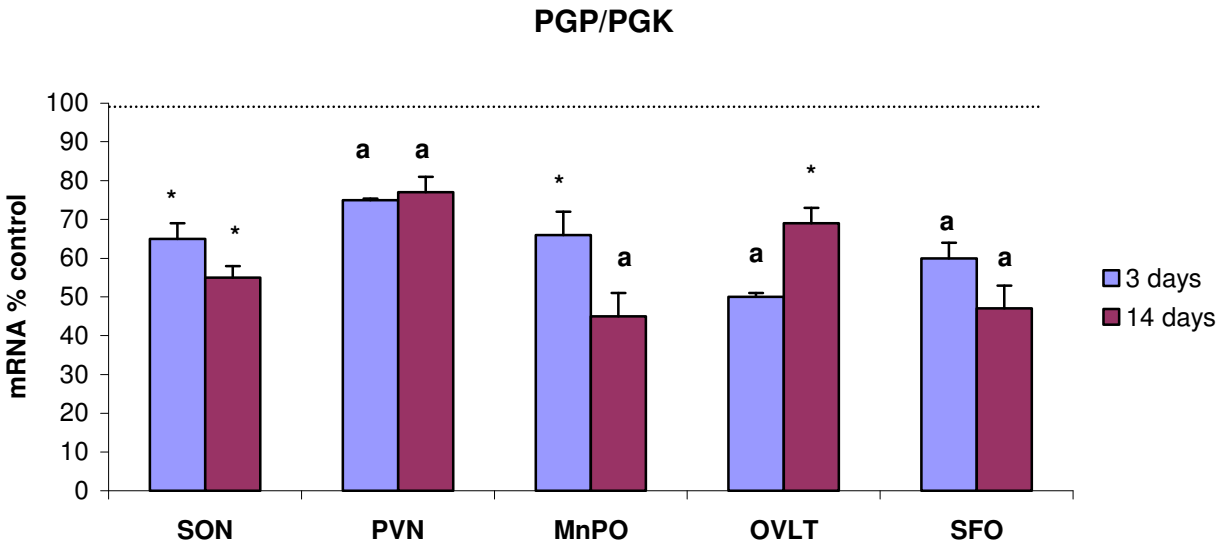


Figure R15. Changes in mRNA expression for PGP in hypothalamic nuclei by Ang II. mRNA expression for PGP in rats infused for 3 or 14 days sc with Ang II 500 ng/kg/min as compared to sham operated rats. Data is shown as percentage of control group (100%). (n=6/group), * $P \leq 0.05$, a $P < 0.01$

3.4.2. Ang II on mRNA expression of glia marker GFAP in hypothalamic nuclei

To take into account possible differences in the ratio of glia cells versus neurons in different brain punches the expression level of GFAP mRNA, a marker for glia cell, was measured for each nucleus.

Baseline levels: Table R5 summarizes the mRNA expression of GFAP over PGK in hypothalamic nuclei. In sham groups, GFAP mRNA expression was lower in PVN and MnPO than the other nuclei indicating that the two nuclei contain lower amount of glia cells.

GFAP/PGK, x10^{-1}	3 days		14 days	
	Sham	Ang II	Sham	Ang II
SON	1.0 ± 0.14	1.1 ± 0.16	1.3 ± 0.18	**2.1 ± 0.31***
PVN	0.2 ± 0.03	0.2± 0.05	0.3 ± 0.01	0.4 ± 0.08
MnPO	0.4 ± 0.04	**0.2 ± 0.02***	0.5 ± 0.07	0.6 ± 0.08
OVLT	0.9 ± 0.07	1.1 ± 0.19	0.5 ± 0.07	0.8 ± 0.18
SFO	0.8 ± 0.05	0.6 ± 0.11	1.3 ± 0.17	1.6 ± 0.20

Table R5. **mRNA expression of the glial fibrillary acidic protein (GFAP) in hypothalamic nuclei.** (n=6/group). Data are mean values ± SE. t-test was used for comparison. **** P< 0.05***

Ang II infusion for 3 days did not change GFAP mRNA expression except for a 50 % reduction in the MnPO after 3 days and a significant increase in SON by 60 % after 14 days (Figure R16).

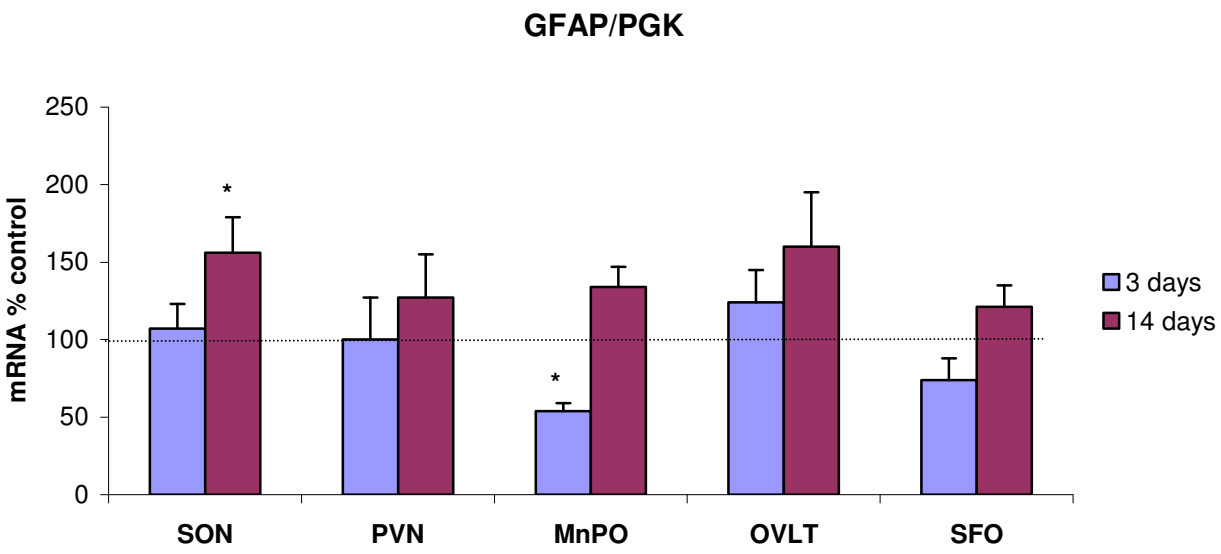


Figure R16. Changes in mRNA expression for GFAP in hypothalamic nuclei by Ang II. mRNA expression for GFAP in rats infused for 3 or 14 days sc with Ang II 500 ng/kg/min as compared to vehicle Data is shown as percentage of control group (100%). (n=6/group), * P ≤ 0.05

3.4.3. Relative amount of neurons versus glia

As the expression of the mRNA of the neuron specific marker PGP is changed by sc Ang II, we are not able to assess the relative amount of neurons versus glia in Ang II treated groups. The relative amount of neurons versus glia was therefore only assessed in sham groups. Relative amount of neurons versus glia was lowest (<1) in SON and highest (~ 3) in PVN and MnPO (Figure R17). The 3 day PVN data may reflect a technical "outliner". However, PGK mRNA expression, indicating the total amount of cells, was fairly consistent at the two time point and similar across the nuclei (Table R6).

Sham	PGP/GFAP		PGK	
	3 days	14 days	3 days	14 days
SON	0.8 ± 0.17	0.9 ± 0.11	0.7 ± 0.06	0.6 ± 0.03
PVN	5.6 ± 0.63	2.5 ± 0.18	1.0 ± 0.07	0.8 ± 0.03
MnPO	2.8 ± 0.46	2.7 ± 0.37	0.9 ± 0.08	0.5 ± 0.03
OVLT	2.8 ± 0.27	0.8 ± 0.13	0.7 ± 0.04	0.95 ± 0.07
SFO	1.9 ± 0.15	0.8 ± 0.20	0.7 ± 0.07	0.7 ± 0.09

Table R6. mRNA expression of the neuron specific marker (PGP) versus glial fibrillary acidic protein (GFAP) in hypothalamic nuclei. (n=6/group), Data are mean values ± SE.

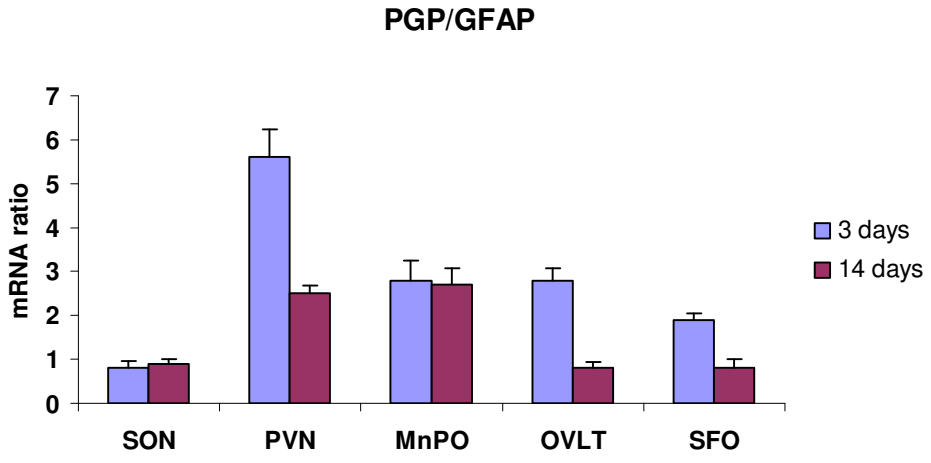


Figure R17. The relative amount of neuron versus glia in hypothalamic nuclei. Relative amount of neuron (PGP) versus glia (GFAP) in sham groups of hypothalamic nuclei at 3 or 14 days. (n=6/group),

3.4.4. Effects of sc infusion of Ang II on steroidogenesis in nuclei of lamina terminalis.

mRNA expression of steroidogenic enzymes was assessed in both neurons and glia or glia alone depending on the cell type that expresses the specific type of enzyme. StAR mRNA expression was assessed in both glia and neurons (StAR/PGK) as the total expression of StAR, as well as in glia alone (StAR/GFAP). CYP11B1 and CYP11B2 mRNA expression was assessed versus PGK only, as the two latter enzymes are expressed by neurons only. As PGP mRNA expression is affected by sc Ang II we were not able to assess the mRNA expression of these two enzymes in neurons alone (versus PGP) but rather in total cells (versus PGK).

Baseline level: Expression of StAR mRNA in sham rats was 10 fold higher than expression of CYP11B1 and CYP11B2 in SFO, OVLT and MnPO.

mRNA expression of CYP11B2 was 20 fold higher in MnPO than the expression in SFO and OVLT. CYP11B1 expression in MnPO was 10 fold higher than the expression in OVLT and 4 fold higher than that in SFO (Table R7-R9).

SFO: Changes in expression of StAR mRNA induced by sc infusion of Ang II 500 ng/kg/min were measured versus a glia specific standard, GFAP, or a general normalization standard PGK. Infusion of Ang II for 3 days did not change the total expression of StAR mRNA in both glia and neuron, as indicated by ratios versus PGK. However, a significant increase by 86 % in the expression of the StAR mRNA in glia cells was found as indicated by ratios versus GFAP. By 14 days, a modest increase was found in the total expression of StAR, but the expression remained unchanged in glia cells (Figure R18).

SFO	3 days		14 days	
	Sham	Ang II	Sham	Ang II
B1/PGK, x10 $^{-4}$	5.7 ± 1.16	**8.8 ± 0.99** ^{b}	5.5 ± 0.90	**9.1 ± 1.43** ^{b}
B2/PGK, x 10^{-4}	0.5 ± 0.14	**1.5 ± 0.13***	1.7 ± 0.36	3.1 ± 0.69
StAR/PGK, x10^{-3}	6.9 ± 0.95	8.1 ± 0.77	4.5 ± 0.48	**5.6 ± 0.50** ^{b}
StAR/GFAP, x10^{-2}	1.3 ± 0.11	**2.4 ± 0.36***	0.4 ± 0.05	0.4 ± 0.07

Table R7. **mRNA expression of the enzymes involved in steroidogenesis (StAR, CYP11B2, CYP11B1) in the SFO.** (n=6/group). Data are mean values ± SE. t-test was used for comparison. **** P< 0.05, (b) 0.1> P >0.05***

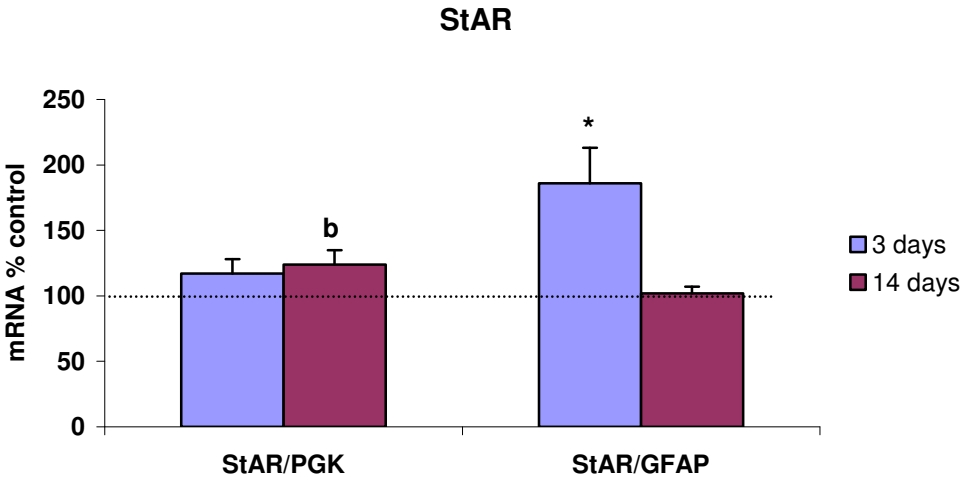


Figure R18. Time course of changes of mRNA expression for StAR in SFO. mRNA expression for StAR in rats infused for 3 or 14 days sc with Ang II 500 ng/kg/min as compared to vehicle. Data is shown as percentage of control group (100%). (n=6/group) * P < 0.05, b 0.1>P>0.05

Infusion of Ang II for 3 days increased CYP11B2 mRNA expression 3 fold which was no longer apparent after 14 days. CYP11B1 mRNA expression showed a modest (P<0.1) increase at both 3 and 14 days of infusion (Figure R19).

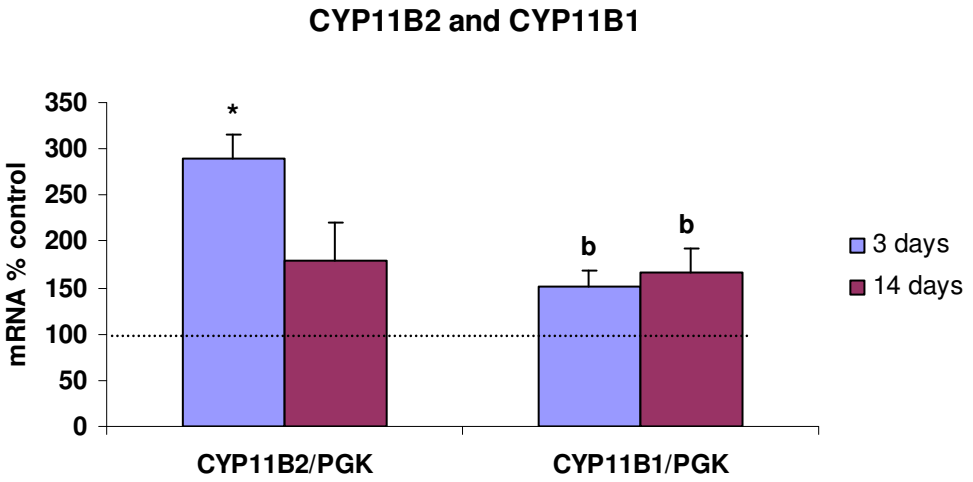


Figure R19. Time course changes of mRNA expression for CYP11B2 and CYP11B1 in SFO by Ang II. mRNA expression for CYP11B2 in rats infused for 3 or 14 days sc with Ang II 500 ng/kg/min as compared to vehicle. Data is shown as percentage of control group (100%). (n=6/group) * $P < 0.05$, b $0.1>P>0.05$

OVLT: The total expression of StAR mRNA in both glia and neurons as indicated by ratios versus PGK showed a modest increase after infusion of Ang II for 3 days which was no longer present after 14 days. The expression of StAR mRNA in glia cells as indicated by ratios versus GFAP remained unchanged at both time points (Figure R20).

OVLT	3 days		14 days	
	Sham	Ang II	Sham	Ang II
B1/PGK, $x10^{-4}$	1.7 ± 0.25	2.1 ± 0.18	2.1 ± 0.32	**3.3 ± 0.63** [b]
B2/PGK, $x\ 10^{-4}$	0.4 ± 0.15	0.6 ± 0.25	0.8 ± 0.13	0.9 ± 0.26
StAR/PGK, $x10^{-3}$	8.1 ± 0.41	**9.7 ± 0.61**[b]	12.5 ± 1.16	12.0 ± 1.26
StAR/GFAP, $x10^{-2}$	0.1 ± 0.10	0.1 ± 0.10	2.5 ± 0.25	2.5 ± 0.55

Table R8. mRNA expression of the enzymes involved in steroidogenesis (StAR, CYP11B2, CYP11B1) in the OVLT. (n=6/group). Data are mean values ± SE. t-test was used for comparison. ***(b) 0.1> P >0.05***

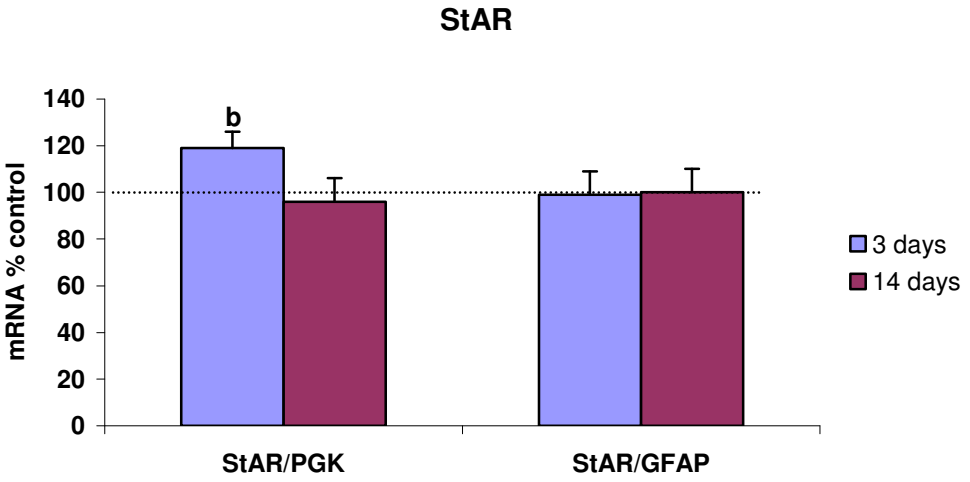


Figure R20. Time course of changes of mRNA expression for StAR in OVLT by Ang II. mRNA expression for StAR in rats infused for 3 or 14 days sc with Ang II 500 ng/kg/min as compared to vehicle. Data is shown as percentage of control group (100%). (n=6/group), b 0.1>P>0.05

CYP11B2 mRNA expression did not change at either 3 or 14 days infusion of Ang II. No changes were observed in CYP11B1 expression in OVLT after 3 days of infusion and a modest increase (P<0.1) was found after 14 days of infusion of Ang II (Figure R21).

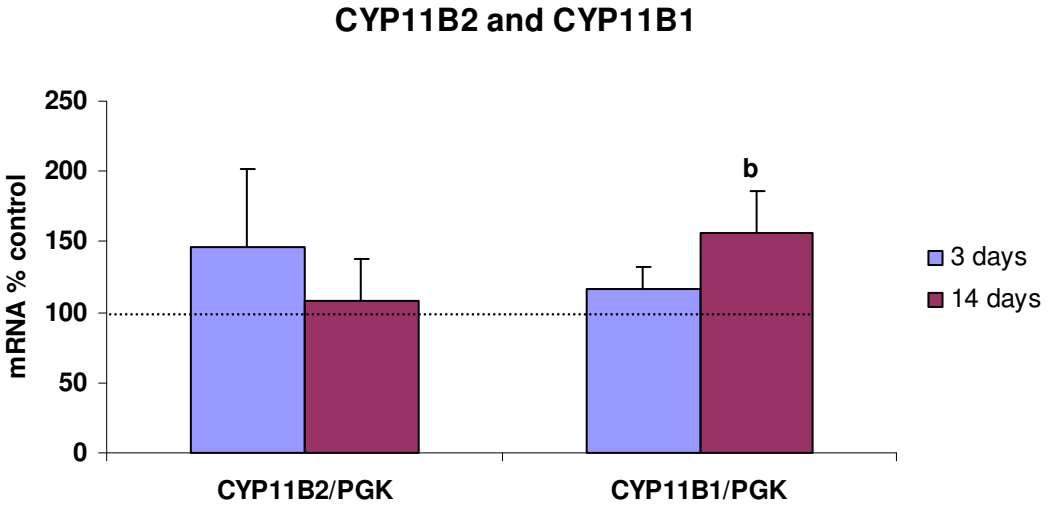


Figure R21. Time course of changes of mRNA expression for CYP11B1 and CYP11B2 in OVLT by Ang II. mRNA expression for CYP11B1and CYP11B2 in rats infused for 3 or 14 days sc with Ang II 500 ng/kg/min as compared to vehicle. Data is shown as percentage of control group (100%). (n=6/group), b 0.1>P>0.05

MnPO: Infusion of Ang II did not change StAR mRNA expression in both glia and neurons as indicated by the ratios versus the expression of the general normalization standard PGK. No changes were observed in the expression specifically in the glia cells, as indicated by ratios versus expression of glia specific standard GFAP (Figure R22).

MnPO	3 days		14 days	
	Sham	Ang II	Sham	Ang II
B1/PGK, $x10^{-4}$	23.8 ± 1.30	24.7 ± 3.00	22.0 ± 0.90	22.0 ± 1.60
B2/PGK, $x\ 10^{-4}$	8.5 ± 1.47	**14.2 ± 1.63***	11.4 ± 1.18	12.0 ± 1.07
StAR/PGK, $x10^{-3}$	9.3 ± 0.55	10.2 ± 0.56	16.5 ± 1.28	18.3 ± 1.96
StAR/GFAP, $x10^{-2}$	———	———	4.3 ± 0.82	3.4 ± 0.71

Table R9. mRNA expression of the enzymes involved in steroidogenesis (StAR, CYP11B2, CYP11B1) in the MnPO. (n=6/group). Data are mean values ± SE. t-test was used for comparison. **** P< 0.05***

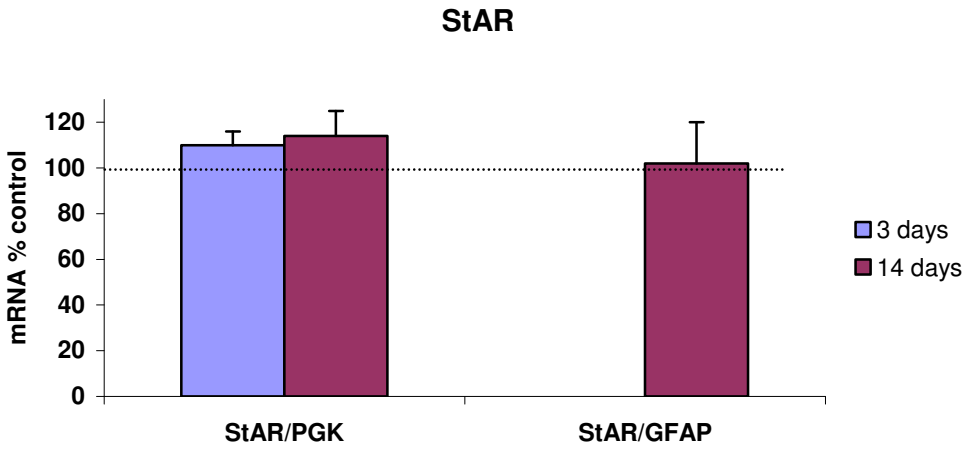


Figure R22. Time course of changes of mRNA expression for StAR in MnPO by Ang II. mRNA expression for StAR in rats infused for 3 or 14 days sc with Ang II 500 ng/kg/min as compared to vehicle. Data is shown as percentage of control group (100%). (n=6/group)

Ang II infusion for 3 days induced a significant increase by 65% in CYP11B2 mRNA expression which was no longer present after 14 days. CYP11B1 did not change in the MnPO at either 3 or 14 days of Ang II infusion (Figure R23).

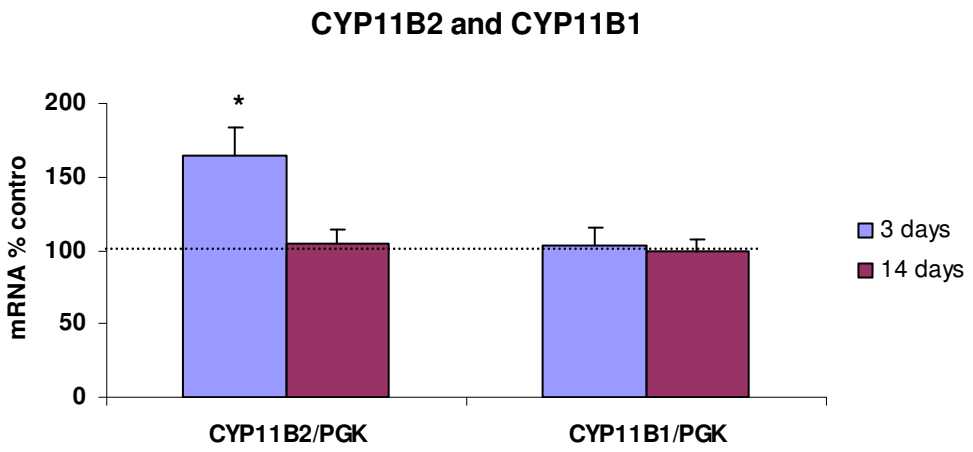


Figure R23. Time course of changes of mRNA expression for CYP11B2 and CYP11B1 in MnPO by Ang II. mRNA expression for CYP11B2 and CYP11B1 in rats infused for 3 or 14 days sc with Ang II 500 ng/kg/min as compared to vehicle. Data is shown as percentage of control group (100%). (n=6/group)
* $P < 0.05$

3.4.5. Effects of sc infusion of Ang II on steroidogenesis in SON and PVN

Baseline levels: StAR mRNA expression in sham rats was 10 fold higher than the mRNA expression for CYP11B1 and CYP11B2 in the SON and PVN. CYP11B1 mRNA expression was 2 fold higher in SON than in the PVN. CYP11B2 was 3 fold higher in PVN than the expression in SON. mRNA expression of CYP11B1 and CYP11B2 was 10000 fold lower and mRNA expression of StAR was 1000 fold lower in SON and PVN than expression levels in adrenal (Table R11,R12).

SON: Infusion of Ang II did not change in the total StAR mRNA expression in both glia and neurons as indicated by the ratios versus the expression of the general normalization standard PGK. No changes were observed in the expression specifically in the glia cells, as indicated by ratios versus expression of glia specific standard GFAP (Figure R 24).

Ang II infusion for 3 days induced a significant increase by 2.7 fold in CYP11B2 mRNA expression in the SON, but no longer by 14 days. CYP11B1 mRNA expression at both 3 and 14 days of infusion of Ang II remained unchanged (Figure R25).

SON	3 days		14 days	
	Sham	Ang II	Sham	Ang II
B1/PGK, x10 $^{-4}$	17.3 ± 3.43	22.1 ± 4.33	21.8 ± 5.17	16.8 ± 1.88
B2/PGK, x 10^{-4}	0.4 ± 0.16	**1.2 ± 0.12** [a]	1.5 ± 0.25	1.42 ± 0.38
StAR/PGK, x10^{-3}	10.5 ± 0.89	10.9 ± 0.92	12.8 ± 0.80	14.1 ± 0.48
StAR/GFAP, x10^{-2}	0.75 ± 0.13	0.74 ± 0.13	———	———

Table R11. mRNA expression of enzymes involved in steroidogenesis (StAR, CYP11B2, CYP11B1) in the SON. Data are mean values ± SE. t-test was used for comparison. (***a) P< 0.01***, ***(b) 0.1> P >0.05***

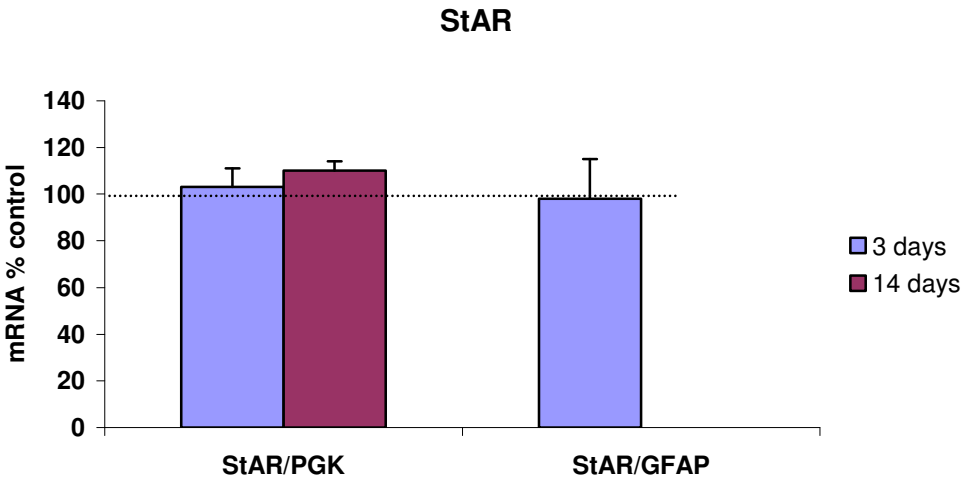


Figure R24. Time course of changes of mRNA expression for StAR in SON by Ang II. mRNA expression for StAR in rats infused for 3 or 14 days sc with Ang II 500 ng/kg/min as compared to vehicle. Data is shown as percentage of control group (100%). (n=6/group)

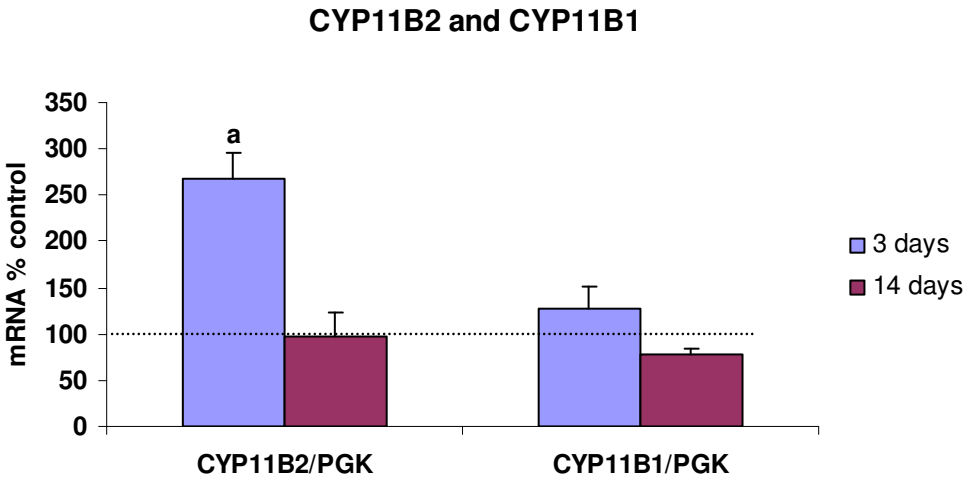


Figure R25. Time course changes of mRNA expression for CYP11B2 and CYP11B1 in the SON. mRNA expression for CYP11B2 and CYP11B1 in rats infused for 3 or 14 days sc with Ang II 500 ng/kg/min as compared to vehicle. Data is shown as percentage of control group (100%). (n=6/group)
a $P < 0.01$

PVN: Expression of StAR mRNA in both glia and neurons showed minor changes after 3 or 14 days of infusion of Ang II as indicated by ratios of StAR mRNA versus PGK mRNA. In glia StAR mRNA expression showed a significant increase by 60 % at 3 days but not after 14 days as indicated by ratios of StAR mRNA versus glia specific marker, GFAP mRNA (Figure R 26).

PVN	3 days		14 days	
	Sham	Ang II	Sham	Ang II
B1/PGK, x10 $^{-4}$	6.2 ± 0.51	6.6 ± 0.19	10.0 ± 1.82	**5.9 ± 0.49** ^{b}
B2/PGK, x 10^{-4}	3.8 ± 0.31	**4.9 ± 0.36***	3.4 ± 0.41	2.8 ± 0.35
StAR/PGK, x10^{-3}	8.0 ± 0.86	11.0 ± 1.10	15.0 ± 0.68	13.5 ± 0.43
StAR/GFAP, x10^{-2}	5.4 ± 0.78	**8.7 ± 1.27***	5.2 ± 0.32	3.8 ± 0.92

Table R12. mRNA expression of the enzymes involved in steroidogenesis (StAR, CYP11B2, CYP11B1) in the PVN. Data are mean values ± SE. t-test was used for comparison.
**** P< 0.05*, *(b) 0.1> P >0.05***

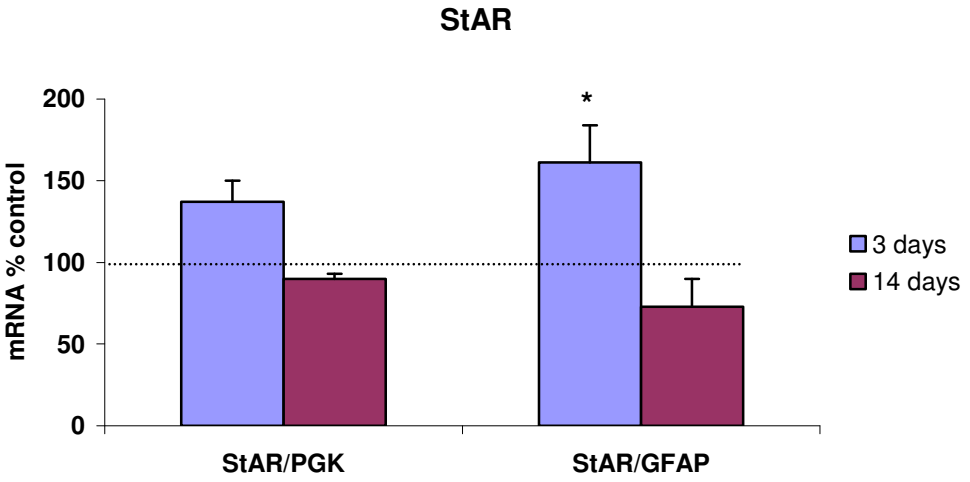


Figure R26. Time course of changes of mRNA expression for StAR in PVN by Ang II. mRNA expression for StAR in rats infused for 3 or 14 days sc with Ang II 500 ng/kg/min as compared to vehicle. Data is shown as percentage of control group (100%). (n=6/group), * P < 0.05

CYP11B2 mRNA expression in PVN showed a significant increase by 30 % after 3 days of Ang II infusion but not after 14 days. Ang II infusion for 3 days did not change CYP11B1 mRNA expression but by 14 days a modest decrease was found in the expression of CYP11B1 mRNA (P = 0.08) (Figure R27).

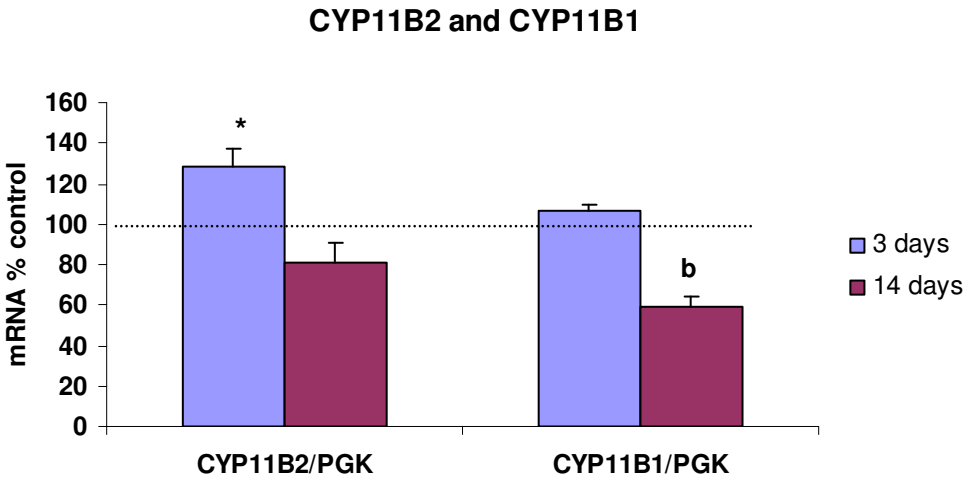


Figure R27. Time course of changes of mRNA expression for CYP11B2 and CYP11B1 in PVN. mRNA expression for CYP11B2 and CYP11B1 in rats infused for 3 or 14 days sc with Ang II 500 ng/kg/min as compared to vehicle. Data is shown as percentage of control group (100%). (n=6/group)
* P < 0.05, b 0.1>P>0.05

3.4.6. Effects of sc infusion of Ang II on steroidogenesis in hippocampus

Baseline levels: In sham rats StAR mRNA expression was 10 fold higher than the expression for CYP11B1. Expression of CYP11B2 was below detection level in the hippocampus (Table R13). Ang II infusion did not change mRNA expression of StAR or CYP11B1 in the hippocampus at either 3 or 14 days infusion (Figure R28).

Hippocampus	B1/PGK, x 10^{-4}		B2/PGK		StAR/PGK, x 10^{-3}	
	Sham	Ang II	Sham	Ang II	Sham	Ang II
3 days	2.9 ± 0.51	4.0 ± 0.92	ND	ND	7.4 ± 0.77	8.9 ± 0.88
14 days	9.3 ± 2.8	9.9 ± 3.7	ND	ND	9.0 ± 0.85	9.2 ± 0.45

Table R13. mRNA expression of the enzymes involved in steroidogenesis (StAR, CYP11B2, CYP11B1) in the hippocampus. Data are mean values ± SE. t-test was used for comparison

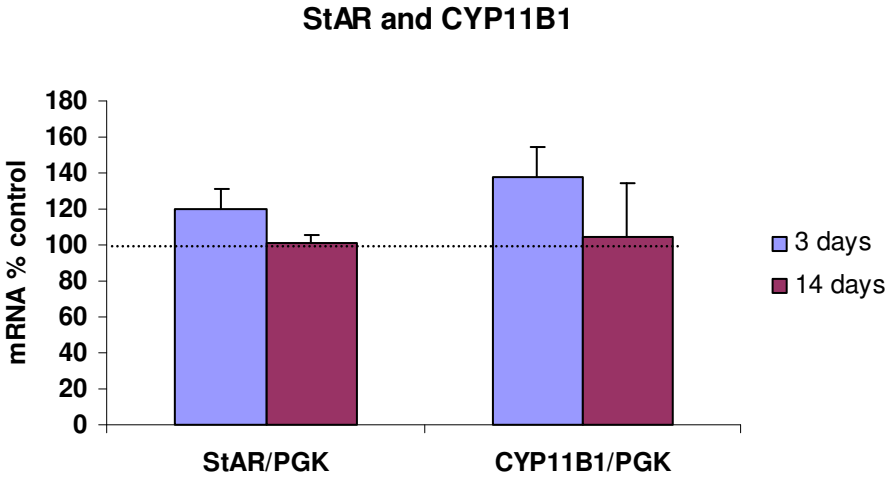


Figure R28. Time course of changes of mRNA expression for StAR and CYP11B1 in hippocampus by Ang II. mRNA expression for StAR and CYP11B1 in rats infused for 3 or 14 days sc with Ang II 500 ng/kg/min as compared to vehicle. Data is shown as percentage of control group (100%). (n=6/group)

3.5. Summary of the effects of sc infusion of Ang II on steroidogenesis in adrenal, hippocampus and hypothalamic nuclei.

Figure R29 summarizes the changes in StAR mRNA expression induced by sc Ang II. StAR mRNA expression in adrenal showed a transient increase at 3 days whereas in hippocampus and hypothalamic nuclei no changes were found except for minor increases in OVLT and SFO.

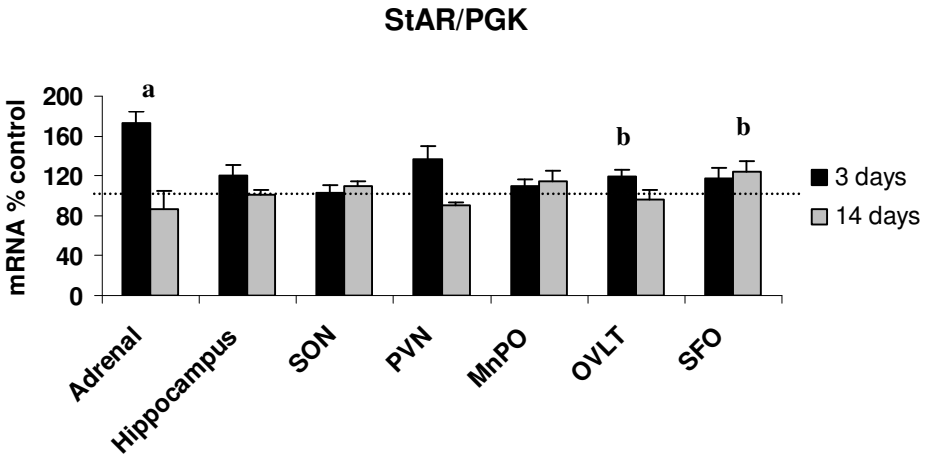


Figure R29. Summary of the time course of changes of mRNA expression for StAR by Ang II. mRNA expression for StAR in rats infused for 3 or 14 days sc with Ang II 500 ng/kg/min as compared to vehicle. Data is shown as percentage of control group (100%). (n=6/group). a $P < 0.01$, b $0.1>P>0.05$

Figure R30 summarizes the StAR mRNA expression in glia of forebrain nuclei, by sc infusion of Ang II. StAR mRNA expression showed a significant increase at 3 days in SFO and PVN but remained unchanged in other nuclei.

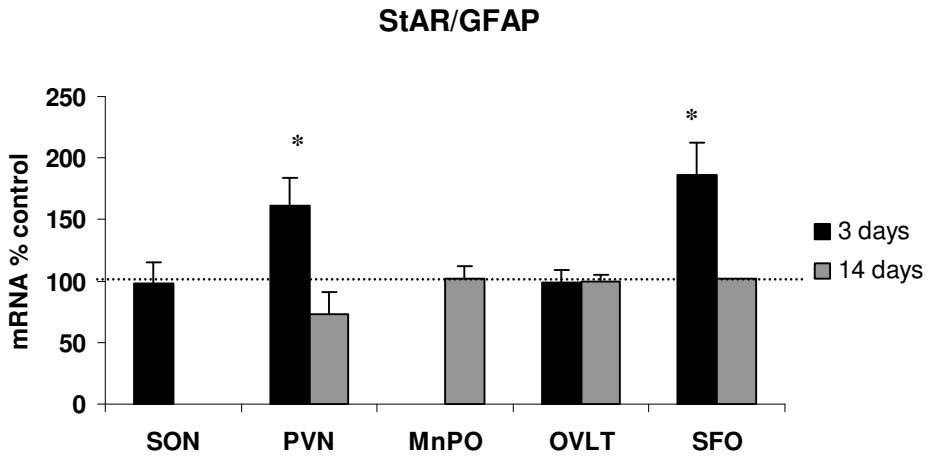


Figure R30. Summary of the time course of changes of mRNA expression for StAR in glia of hypothalamic nuclei by Ang II. mRNA expression for StAR in rats infused for 3 or 14 days sc with Ang II 500 ng/kg/min as compared to vehicle. Data is shown as percentage of control group (100%). (n=6/group). * $P < 0.05$

Figure R31 summarizes the changes in CYP11B2 mRNA expression induced by sc infusion of Ang II. CYP11B2 mRNA expression showed a marked and persistent increase in adrenal. Hypothalamic nuclei (except for OVLT) also showed a significant increase in the expression of CYP11B2 but only initially at 3 days and the increases in expression were much less than in adrenal.

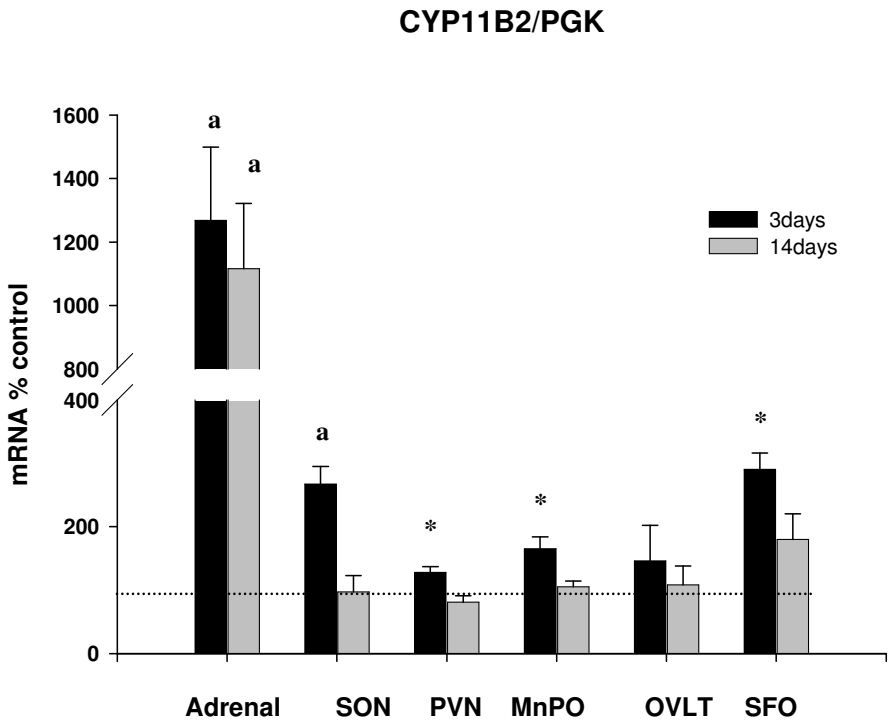


Figure R31. Summary of the time course of changes of mRNA expression for CYP11B2 by Ang II. mRNA expression for CYP11B2 in rats infused for 3 or 14 days sc with Ang II 500 ng/kg/min as compared to vehicle. Data is shown as percentage of control group (100%). (n=6/group). * $P < 0.05$, a $P<0.01$

Figure R32 summarizes the expression level of CYP11B1 induced by sc infusion of Ang II. No significant changes were observed in CYP11B1 mRNA expression in the adrenal hippocampus or hypothalamic nuclei.

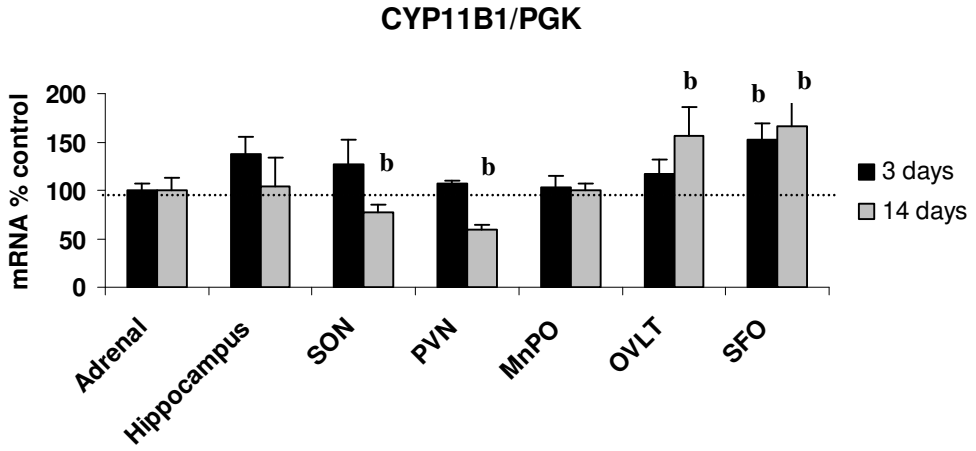


Figure R32. Summary of the time course of changes of mRNA expression for CYP11B1 by Ang II. mRNA expression for CYP11B1 in rats infused for 3 or 14 days sc with Ang II 500 ng/kg/min as compared to vehicle. Data is shown as percentage of control group (100%). (n=6/group). b 0.1>P>0.05

4. Discussion

4.1. Summary of main findings

14 days sc infusion of Ang II at a modest rate (150 ng/kg/min) induces minor increases in plasma Ang II and a gradual pressor response. Ang II at a higher rate (500 ng/kg/min) causes a significant increase in plasma Ang II and a marked pressor response.

Three patterns of neuronal activation are observed by sc infusion of Ang II. The first pattern of Fra expression is in the forebrain CVO, SFO. The SFO is activated during the first day and remains activated for 4 days, but at 14 days shows diminished activation. The second pattern of Fra expression is in MnPO that does not show significant activation during the first day but, after several days the activation is high and then less by 14 days. The third pattern is in downstream hypothalamic nuclei, PVN and SON. Parvocellular PVN (pPVN), magnocellular PVN (mPVN) and SON shows an initial activation that increases over time. Central infusion of aldosterone synthase inhibitor, or MR blocker, attenuates the increase in Fra expression in PVN but not SON, and prevents the decrease in SFO after 14 days infusion of Ang II.

Sc infusion of Ang II at the high rate increases StAR mRNA expression in glia cells in PVN and SFO at 3 days but no longer at 14 days. mRNA expression of aldosterone synthase increases significantly at 3 days in PVN, SON, MnPO and SFO but no longer after 14 days. Sc infusion of Ang II at high rates induces a significant increase in CYP11B2 mRNA expression in adrenal at both 3 and 14 days. StAR mRNA expression in adrenal increases at 3 days but no longer at 14 days.

PGP mRNA expression in SON, PVN, MnPO, SFO and OVLT decreases by sc Ang II at both 3 and 14 days.

4.2. Pressor responses to Ang II

We found a gradual pressor response (~ 8 mmHg in the first few days and ~ 20 mm Hg by 14 days) at low rate of sc infusion of Ang II. Similar pattern of BP was also found in earlier and recent studies where long term infusion of Ang II in low doses raised BP in a gradually developing manner. Infusion of Ang II at low rates (10 ng/kg/min, iv), increased BP within the first 24 h by 10 mmHg and by 20 mm Hg after 3-5 days (Kanagy et al. 1990, Cowley et al. 1981) and in recent studies by 15 mmHg after 10 days (Hendel and Collister 2005, Vieira et al. 2010). This rate of Ang II infusion caused only minor changes in plasma level of Ang II, and persistent neuronal activation in SON and PVN. Therefore we suggest that the increase in BP could be related to central effects of Ang II. Consistent with the role of central mechanisms in hypertension caused by infusion of Ang II, ganglion blockade produced a significantly large decrease in MAP (by ~75 mmHg) compared to vehicle infused rats (by 38-47 mm Hg) (Kline et al. 1990, LaGrange et al. 2003). Furthermore, studies in our lab showed that icv infusion of aldosterone synthase inhibitor FAD-286 at 100 μg/kg/day fully prevented the increase in MAP throughout the 14 days of infusion of Ang II at 150 ng/kg/min (Huang et al. 2010) suggesting role of central aldosterone in activating CNS pathways by circulating Ang II leading to hypertension.

Higher rates of infusion of Ang II (500 ng/kg/min) caused a 4 fold increase in plasma Ang II and a ~ 20 mmHg increase in BP within the first few days and ~ 60 mmHg by 14 days. This pattern is consistent with other studies using chronic infusion of Ang II in high doses. Li in 1996 found a rapid increase (by 40 mmHg) over the first few hours followed

by a further increase (total of 60 mmHg) after 7 days of Ang II infusion at 200 ng/kg/min iv. Luft in 1989 showed that infusion of Ang II at 350 ng/kg/min sc for 2 weeks increased systolic BP by 20 mmHg by day 2 and by 90 mmHg by day 10 (Luft et al. 1989).

The initial increase in BP is consistent with a direct vasoconstrictor action of Ang II and withdrawal of sympathetic tone. Li in 1996 found that infusion of Ang II at 50 ng/kg/min, iv for 24 hours in rats, increases MAP by 30 mmHg and depressor responses to ganglion blocking drug significantly reduces at 1 and 5 hours time points. However, the increase in MAP in chronic infusion of Ang II (200 ng/kg/min, sc) up to 7 days was completely reversed by ganglion blocker drugs (Li et al.1996). Studies in our lab showed that icv infusion of FAD-286 (100 μg/kg/day) prevented 70–80% of the increase in MAP induced by ANG II at 500 ng/kg/min for 14 days. Also icv infusion of eplerenone at 10 and 20 μg/kg/day significantly attenuated the increases in MAP by Ang II at 500 ng/kg//min for 14 days by 70% and 80%, respectively and icv infusion of spironolactone attenuated the increase in MAP by ~ 50%. Central blockers caused minor attenuation in MAP already at day 1 of infusion of Ang II and significant attenuation of MAP by central blockers started already at day 2 of infusion of Ang II (Huang et al. 2010).

Our findings together with other studies mentioned above suggest that chronic infusion of low rates Ang II raises BP in a gradual developing manner that involves predominantly the central neurogenic component. Infusion of Ang II at high rates raises BP in a biphasic manner in which the central mechanisms contribute already within the first few days and become more prominent during chronic infusion.

4.3. Neuronal activation in the forebrain by sc Ang II

Pattern of forebrain activation by Ang II infusion was revealed by Fra-immunoreactivity. SFO is directly influenced by changes in circulating Ang II because of lack of the BBB in this nucleus (Johnsson et al. 1993, 1996). The SFO remained activated up to 4 days but at 14 days showed diminished activation. Davern and Head (2007) found similar pattern in SFO in rabbits and suggested that the transient nature of activation of SFO by Ang II may indicate desensitization of AT_1-R in this nucleus. In vitro studies with cultures of rat neurons of OVLT and SFO demonstrated rapid desensitization of AT_1R in these nuclei as shown by reduced intracellular Ca^+ concentration induced by high concentration of Ang II (Gebke et al. 1998). However recent in vivo studies have shown that sc infusion of Ang II at 35 ng/kg/min over 4 weeks upregulates AT_1R expression in both PVN and SFO (Wei et al. 2009). Therefore it is unlikely that the diminished neuronal activation in the SFO is due to downregulation or internalization of receptors.

MnPO did not show significant activation during the first day, but after several days the activation was high and reduced by 14 days. This finding may indicate that activated neurons in SFO at first day bypass the MnPO and activate the PVN and SON by direct projections. However at 4 days activated SFO may activate PVN and SON via projections to MnPO. These findings are consistent with earlier studies where immunoelectronmicroscopy and anterograde tracing studies links SFO through MnPO (Oldfield et al. 1990) or directly (Lind et al. 1982) to the SON and PVN. At 14 days MnPO showed no activation which is consistent with the diminished activation of SFO. This may indicate that at 14 days SFO may no longer activate PVN and SON via MnPO.

PVN and SON showed sustained neuronal activation over time. In dogs chronic infusion of Ang II for 5 days produced sustained Fra like immunoreactivity in the magnocellular PVN and SON (Lohmeier et al. 2002) and in rats chronic iv infusion of Ang II at 10 ng/kg/min for 4 weeks significantly increased Fra like immunoreactivity by 2 fold in PVN (Kang et al. 2009). The time course of changes (initial effects followed by chronic effects) in forebrain neuronal activity by systemic infusion of Ang II in rats had not yet been elucidated. In rabbits Davern and Head found the similar pattern of neuronal activity in SON and PVN. They suggested that the sustained neuronal activation in the PVN and SON despite the reduced input from SFO may indicate that an "unknown mechanism" is activated initially by SFO that causes progressive activation of the SON and PVN.

We hypothesized that an increase in circulating Ang II increases neuronal activity in CVOs presumably leading to activation of the MnPO, pPVN and to a lesser extent mPVN and SON. In the second phase activation of CVOs appears to diminish, but aldosterone production in the mPVN and SON may increase and contribute to further increase in neuronal activity in pPVN.

4.4. Neuronal activity by sc Ang II and central blockers

To further examine whether aldosterone contributes to chronic neuronal activation, Fra expression was assessed in Wistar rats infused sc with Ang II and treated with chronic icv infusion of an aldosterone synthase (AS) inhibitor or a MR blocker. These studies showed that AS inhibitor and MR blocker both attenuate the increase in neuronal activation in the pPVN (by 50 %) and mPVN (by 80 %) after 14 days infusion of Ang II, but do not prevent the increase in Fra expression in SON.

Based on these findings we proposed a dual activation of PVN. Direct projections from SFO to the PVN possibly activate pPVN. Activation of SFO by circulating Ang II also activates a neuromodulatory pathway, i.e. increases aldosterone production in magnocellular neurons in the SON which via MR causes further excitation of mPVN and pPVN. These findings may support our hypothesis that chronic elevation of circulating Ang II causes neuronal activation in the SFO and thereby activates efferent pathways to the PVN, and in addition causes chronic activation of a neuro-modulatory mechanism starting with aldosterone production in magnocellular neurons in SON, which amplifies neuronal excitation of mPVN and presympathetic neurons in the pPVN (Figure D1).

In the SFO, icv infusion of the AS inhibitor or MR blocker for 14 days prevented the decrease in neuronal excitation. This finding is interesting as it may indicate an indirect negative feedback mechanism induced by high BP causing inhibition of neuronal activation in SFO. Anatomical tracing studies have revealed that excitatory noradrenergic neurons in the NTS, the primary site of termination of cardiovascular afferent fibers (Kalia, 1981, Bousqet et al.1982), innervate the SFO (Ciriello et al, 1996, Kawano and Masuko, 2001). Activation of peripheral baroreceptors, by raising arterial blood pressure with an intravenous infusion of phenylepherine, suppressed the activity of neurons in the NTS that project to the SFO (Tanaka et al. 2001). It is possible that increases in BP by chronic sc infusion of Ang II activate peripheral baroreceptors decreasing activity in excitatory NTS neurons projecting to the SFO. This mechanism may explain the findings that neuronal activity in the SFO was diminished after 14 days infusion of Ang II when BP was chronically elevated.

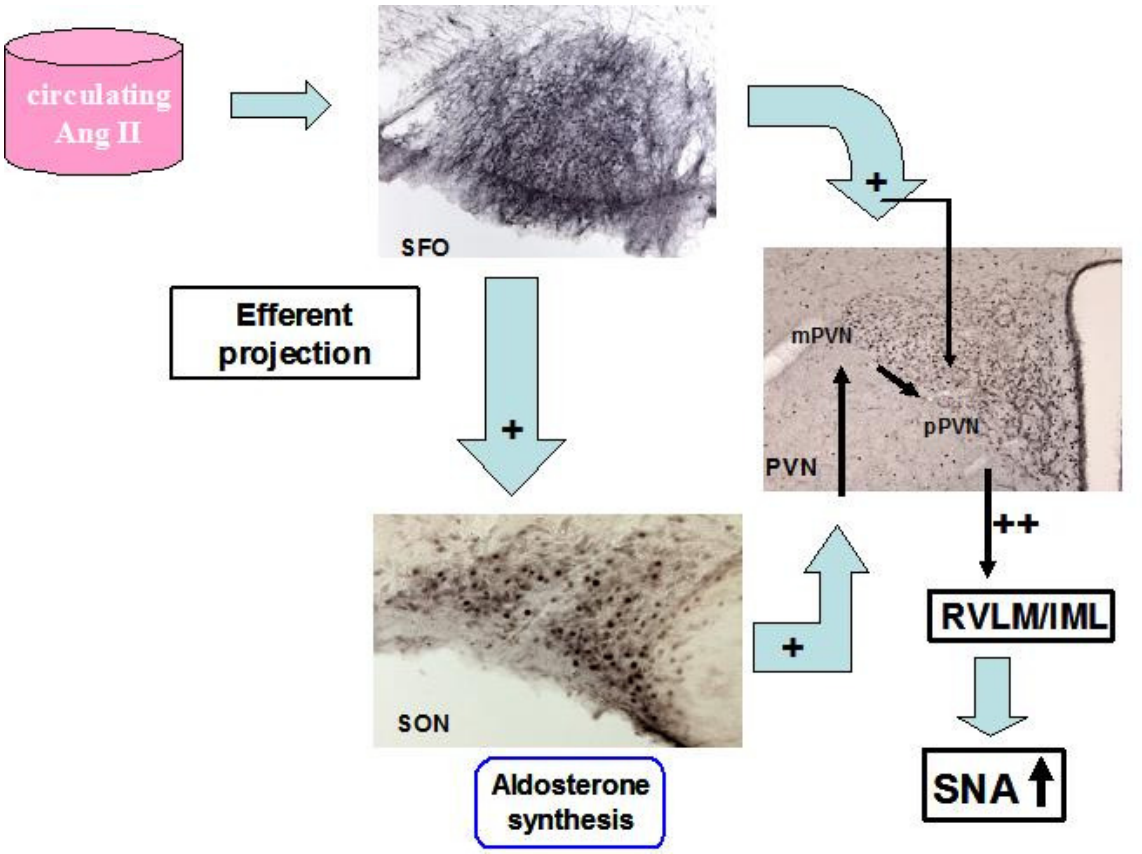


Figure D1. Proposed pathways for dual activation of pPVN

4.5. Steroidogenesis in adrenal by sc Ang II

mRNA expression of StAR, aldosterone synthase (CYP11B2) and 11-beta hydroxylase (CYP11B1) in adrenal cortex by sc Ang II infusion at the high rate of 500 ng/kg/min was analyzed. StAR mRNA expression increased significantly after 3 days. Immunohistochemistry studies on adrenal cortex in our lab using antiStAR antibody showed also a significant increase in StAR protein expression in zona glomerulosa after 3 days of Ang II infusion (unpublished data 2011). However by 14 days of infusion no changes in the expression level of StAR mRNA were found. These findings are consistent with previous in vitro studies showing a biphasic time-dependent kinetic in

expression of StAR mRNA by Ang II. In human adrenocortical NCI-H295R cells, Ang II increased StAR protein and mRNA in a time-dependent way, with a maximum at 12 h. By 24 hours StAR expression was at level of control (Krug et al. 2007). Time-course effect of Ang II on bovine adrenocortical cells showed a peak within 3 h, followed by a rapid decline to very low level (12% of control) mRNA and protein of StAR (Christine Le Roy et al. 2000). However the biphasic effect of Ang II on StAR expression in vivo had not been reported before. In a study by Peter et al (1998) sc infusion of Ang II at the lower dose of 200 ng/kg/min for 55 hr in Sprague-Dawley rats showed no significant change in StAR mRNA in the outer adrenal cortex. This finding is not consistent with our data of 72 hr infusion of Ang II at 500 ng/kg/min, and the differences in infusion rates of Ang II may explain these differences in StAR expression.

In contrast, a significant increase in aldosterone synthase mRNA expression in adrenal of Ang II infused rats after both 3 and 14 days was observed. There were no changes in the level of 11-beta hydroxylase mRNA expression by infusion of Ang II. Our findings are consistent with studies by Ye et al. (2003) showing that sc Ang II for 7 days up-regulated expression of CYP11B2 mRNA in adrenal by ~ 8 fold, but did not affect the expression of CYP11B1 (Ye et al. 2003). After sc infusion of Ang II at 200 ng/kg/min for 55 hr in Sprague-Dawley rats the outer adrenal cortex showed an increased in the number of positive cells for CYP11B2 mRNA as assessed by in situ hybridization (Peters et al. 1998).

4.6. Effects of sc Ang II on gene expression in forebrain

To further investigate where in the hypothalamus Ang II may stimulate de novo aldosterone biosynthesis, gene expression of the enzymes known in the adrenal cortex to be involved in aldosterone production, such as StAR and CYP11B2, was assessed by rt-PCR. This was done after 3 or 14 days of Ang II infusion at 500 ng/min/kg.

For the assessment of aldosterone biosynthesis in specific cell type PGP was used as neuron specific marker and GFAP as glia specific marker. PGP mRNA expression level had not been assessed in hypothalamic nuclei before. In sham rats OVLT had lower expression of PGP mRNA than the other nuclei indicating that OVLT contained lowest amount of neurons among the nuclei. Surprisingly PGP mRNA expression, primarily used as a neuron specific normalization standard, was affected by sc Ang II infusion. Thus, a reduction in PGP mRNA expression (versus PGK as a general normalization standard) was found in all hypothalamic nuclei at the two time points by sc Ang II. Therefore PGP could not be used as a normalization standard. PGP 9.5 is a stress responsive gene that can be downregulated by oxidative stress (Shen et al. 2006). Ang II increases the NAD(P)H-dependent superoxide anion production and the intracellular generation of ROS mediates downstream effects of Ang II (Wei et al. 2008). Considering that ROS are mediators of Ang II signalling, the significant decrease in mRNA expression of PGP in all nuclei may relate therefore to the activation of these nuclei by angiotensinergic projections starting from activation of SFO by sc Ang II. The effects of Ang II on PGP expression in brain had not been studied before. It is unlikely that the reduction in PGP mRNA expression would indicate a reduction in number of neurons as there is no evidence for Ang II inducing death of neurons. Also our Fra-expression

studies showing a high number of activated neurons by Ang II does not support the reduction in number of neurons by Ang II.

GFAP mRNA expression in hypothalamus (Nichols et al. 1995) and SON (Laly et al. 2005) was shown by in situ hybridization. However the relative comparison in expression level of GFAP mRNA in hypothalamic nuclei had not been studied before. GFAP mRNA expression in the sham group was lower in PVN and MnPO than the other nuclei indicating that these two nuclei contain a lower amount of glia cells. GFAP mRNA expression remained unchanged by sc Ang II except for a significant increase at 14 days in SON and a significant decrease at 3 days in MnPO. Therefore, for the nuclei and time points where GFAP remained unaffected by Ang II we used the GFAP mRNA expression as a glia marker to analyse the expression of the genes specifically in glia.

4.6.1. StAR

StAR mRNA transcripts were previously identified in several nuclei of rat hypothalamus such as MnPO, PVN and arcuate nucleus (Kim et al 2003, Furukawa et al. 1998). However relative comparison in the level of expression of StAR mRNA in different nulclei of hypothalamus was not studied. Expression of StAR mRNA in sham rats was 10 fold higher than expression of CYP11B1 and CYP11B2 in all the nuclei studied and 1000 fold lower than the expression of StAR in adrenal. The higher expression of StAR compared to CYP11B1 and CYP11B2 in forebrain nuclei may result from StAR being a common enzyme in all steroid synthesis. Sierra et al. (2003) and King et al. (2002) showed that StAR is expressed in both neuron and glia cells in contrast to CYP11B1 and CYP11B2 which may be expressed in neurons only (MacKenzie et al. 2002). This may also explain the abundance of StAR mRNA as compared to CYP11B1 and CYP11B2.

Little is known about the regulation of StAR mRNA expression in brain. Some studies relate an acute expression of StAR in brain induced by stressors (Kim et al. 2004). In the present study sc Ang II increased StAR mRNA expression transiently in glia cells in PVN and SFO. This pattern of expression of StAR is similar to that observed in adrenal.

The total expression of StAR in neurons and glia as indicated by StAR versus PGK did not change in the nuclei studied, except for minor increases in OVLT at 3 days and in SFO at 14 days. These increases could be related to expression in neurons as no changes were found in glia in these nuclei. No changes were found in the expression level of StAR mRNA in the SON and MnPO. We could not measure the expression level in glia in MnPO at 3 days and SON at 14 days as the GFAP mRNA expression was affected by sc Ang II.

No changes were observed in the total expression of StAR mRNA in both glia and neurons in hippocampus as assessed by StAR mRNA versus PGK mRNA (StAR/PGK). However we did not assess the expression level of GFAP in hippocampus and therefore not the expression of StAR in glia.

Taken together, our finding may indicate that sc infusion of Ang II induce mRNA expression in the glia of SFO and PVN but not in neurons.

4.6.2. CYP11B2

CYP11B2 mRNA has been measured in the whole hypothalamus (Ye et al. 2003, 2008) as well as in specific nuclei such as the SON, PVN, and SFO (Wang et al. 2010). In rat brain, the highest CYP11B2 transcript levels are found in the hypothalamus and brainstem (Gomez-Sanchez et al. 2010, Ye et al. 2008). CYP11B2 mRNA expression levels in the hypothalamus were about 1/1000 levels in the adrenal gland (Ye et al. 2003,

Gomez-Sanchez et al. 2009). mRNA expression of CYP11B2 in forebrain nuclei was 10000 fold lower than the expression in adrenal which may indicate lower aldosterone production in the nuclei than in the adrenal. mRNA expression of CYP11B2 in sham rats was 20 fold higher in MnPO than the expression in other nuclei. This may indicate higher aldosterone production in the MnPO. CYP11B2 was modestly higher in PVN than the expression in SON, whereas Wang et al. (2010) found similar expression of CYP11B2 in the two nuclei. SON and PVN may therefore have similar level of aldosterone production. However for a relative comparison of the level of aldosterone production in the nuclei, the actual aldosterone content or protein expression of the enzymes in these nuclei needs to be assessed. The expression level in hippocampus was below detection level. Studies by MacKenzie et al. (2002) using RT-PCR and southern blot analysis showed that CYP11B2 gene was transcribed in primary cultures of fetal rat hippocampal neurons and immunostaining of these cultures showed that the gene was subsequently translated to form immunoreactive aldosterone synthase. In vivo studies have also localized aldosterone synthase immunostaining in the hippocampus (MacKenzie et al. 2000). There is no in vivo evidence for the expression of aldosterone synthase mRNA in the hippocampus. As we did not detect CYP11B2 mRNA expression in our in vivo study in hippocampus, the immunoreactivity of CYP11B2 in hippocampus in the study by MacKenzie et al. (2000) may suggest that the enzyme is transcribed and synthesized possibly in the hypothalamus and then via axonal projections secreted or released to the hippocampus. Retrograde axonal transport techniques show projections from hypothalamic nuclei to hippocampus (Onat et al. 2002). One other possibility is that the

mRNA expression of the aldosterone synthase in hippocampus is very low, and therefore RT-PCR combined with southern blotting is required for its demonstration in this region.

Ye et al. (2003) studied the effect of 7 days sc infusion of Ang II at 200 ng/kg/min on mRNA expression for CYP11B2 in several brain regions and found no changes in the level of expression in hypothalamus, hippocampus and brain stem. In contrast, CYP11B2 mRNA expression showed a significant increase at 3 days in all the hypothalamic nuclei except for the OVLT. The expression was no longer increased after 14 days. Our finding suggests that sc infusion of Ang II induces only transiently mRNA expression of CYP11B2 in the SFO, MnPO, PVN and SON.

As the Fra like immunoractivity showed highest neuronal activity at 14 days in SON and PVN, these two nuclei were assumed to be the putative sites of aldosterone production. We were expecting to observe the induction of the enzymes mRNA by sc Ang II infusion by 14 days as at this time point neuronal activity was highest in SON and PVN. However, we found no significant changes in the expression of the mRNA for the enzymes at 14 days. A recent study showed that 14 days infusion of Ang II at 400 ng/kg/min via oxidative stress significantly affected the BBB, inducing 3.8 times higher permeability. Oral treatment with Tempol significantly attenuated the increased permeability and protected the BBB integrity on day 14 of Ang II infusion (Zhang et al. 2010). CYP11B2 are located in mitochondria which is a major source of superoxide and other reactive oxygen species (ROS), generating ~ 85% of total cellular oxygen radicals (Droge et al. 2002). The free radicals formed can lead to excessive mitochondrial ROS production and release (Zorov et al. 2005). Many enzymes may undergo self-inactivation by ROS (Bernhardt et al. 1996, Huang et al. 2010) and then cause deregulation of steroid

secretion as well as downregulation of CYP11B2 by ROS. mRNA levels of 3beta-HSD which are not located in mitochondria but in endoplasmic reticulum was up-regulated (Huang et al. 2010). These studies may explain our findings why after 14 days CYP11B2 mRNA was no longer changed by sc Ang II. Ang II over time may increase the permeability of BBB and amplify its effects in superoxide production leading to down-regulation of these enzymes. On the other hand, CYP11B2 mRNA expression remained elevated in adrenal. Moreover, our group has recently shown that chronic sc infusion of Ang II for 14 days at 500 ng/kg/min increases aldosterone content in the whole hypothalamus (Huang et al. 2010) and this increase is attenuated by central aldosterone synthase inhibitor which does not support the downregulation of CYP11B2 expression. However, we did not analyse the aldosterone content in specific hypothalamic nuclei but rather in the whole hypothalamus. One other possibility would be that Ang II does not chronically increase mRNA expression of CYP11B2 but induces posttranscriptional modifications in terms of upregulation of protein synthesis of the enzyme or the enzyme activity. Assessment of the protein expression and enzyme activity in hypothalamic nuclei by sc Ang II would possibly explain more clearly these events. Therefore it is still unclear where specifically aldosterone content increases in the hypothalamus and whether the CYP11B2 expression downregulates.

4.6.3. CYP11B1

CYP11B1 mRNA has been measured in the whole hypothalamus (Ye et al. 2003, 2008) as well as in specific nuclei such as the SON, PVN, and SFO (Wang et al. 2010). In rats, CYP11B1 mRNA expression levels in the hypothalamus are about 1/500 levels in the adrenal gland (Ye et al. 2003, Gomez-Sanchez et al. 2009). In our study mRNA expression of CYP11B1 was 10000 fold lower in forebrain nuclei than the expression levels in adrenal. This may reflect the lower corticosterone production in the forebrain nuclei than in adrenal. In hypothalamus CYP11B1 mRNA expression was 10 fold and in hippocampus 100 fold higher than the expression of CYP11B2 (Ye et al. 2003). In sham rats CYP11B1 expression in MnPO was higher than in OVLT and in SFO suggesting possibly higher corticosterone production in MnPO. CYP11B1 mRNA expression was 2 fold higher in SON than in the PVN. Similar pattern of expression was observed in the SON and PVN by Wang et al. (2010). This may suggest that corticosterone production is higher in SON than in PVN. CYP11B1 mRNA expression in SON and MnPO was ~ 10 fold higher than the expression in hippocampus. Ye et al. (2003) found ~ 10 fold higher expression of CYP11B1 mRNA in hypothalamus than in hippocampus. Therefore, the higher expression of CYP11B1 in SON and MnPO than in hippocampus may reflect higher corticosterone production in these nuclei than in hippocampus. However for a relative comparison of the corticosterone production in these regions, assessment of the protein expression of these enzymes and the actual corticosterone content has to been done. Only minor changes were observed in CYP11B1 mRNA expression at the two time points of sc infusion of Ang II. In PVN and SON a modest decrease after 14 days and in SFO and OVLT a modest increase after 14 days and a modest increase after 3 days in

SFO was found. As no significant change was observed in the expression level of CYP11B1 mRNA, we can specifically relate the induction of expression of CYP11B2 to sc Ang II infusion.

4.7. Conclusion

The present study shows that central mechanisms are involved in chronic Ang II induced hypertension. Elevated levels of circulating Ang II induce a chronic increase in BP as well as increase neuronal activity in SFO, presumably leading to activation of PVN and SON. In chronic phase, an aldosterone dependent neurogenic mechanism appears to be activated that through MR activation sustains neuronal activation in PVN. Blockade of central aldosterone synthase or MR results in attenuation of neuronal activity in PVN and BP responses to Ang II. This supports our hypothesis that central aldosterone synthesis is a major factor in sustained Ang II induced hypertension. The pattern of neuronal activation induced by sc Ang II is similar to the pattern observed Post MI in which, circulating Ang II rapidly increases (Leenen et al. 1999), and neurons in the SFO, SON, and PVN are activated (Vahid-Ansari et al. 1998). Central blockade of aldosterone synthase or MR prevents sympathetic hyperactivity and LV dysfunction and remodeling (Huang et al. 2005, 2009, Francis et al. 2001) which indicates activation of the central aldosterone pathway also in rats after MI. This suggests that circulating Ang II may be a major factor in induction of a neuromodulatory pathway leading to chronic sympathetic activity post MI and progression of heart failure. However, our assessment of local aldosterone production induced by circulating Ang II is restricted to mRNA expression

levels and further studies of protein expression and enzyme activity need to be done to draw a firm conclusion.

4.8. Limitation of our studies

Brain punches or dissection of the brain into specific regions before isolating RNA is technically demanding and cross contaminations between brain regions is possible.

We used neuron and glia specific markers to be able to detect the expression level of enzyme mRNA in a specific cell type. However finding a cell specific marker that would not be affected with Ang II was a challenge and the neuron specific marker could not be used as a normalization standard. The glia specific marker at two different nuclei at two different time points (SON at 14 days and MnPO at 3 days) was also affected by Ang II. However as SON showed an increase and MnPO a reduction in expression level of GFAP by Ang II we could not explain the changes of GFAP mRNA in these nuclei.

It is not clear yet to what extent the glia content of the brain punches reflects glia cells located within the nuclei or in tissues surrounding the nuclei. Double immunostaining or in situ hybridization would enable us to localize these cells.

4.9. Future studies

In order to investigate the effects of sc Ang II on aldosterone production in the hypothalamus further studies need to be done. First of all we need to analyse the expression of StAR in hypothalamic nuclei at protein level in order to verify the PCR findings. This would allow us to examine whether the regulation of the enzymes by Ang II is at the translation level as well. In situ hybridization for CYP11B2 and

immunohistochemistry for StAR possibly with double staining are further approaches in order to localize and identify the type of cells responsible for the expression of these genes. A follow up approach would be to assess effects of Ang II on the enzyme activity. One further approach would be to measure levels of aldosterone in specific hypothalamic nuclei in rats infused with Ang II.

5. References

Ahmad M, White R, Tan J, Huang BS, Leenen FH. Angiotensin-converting enzyme inhibitors, inhibition of brain and peripheral angiotensin-converting enzymes, and left ventricular dysfunction in rats after myocardial infarction. J Cardiovasc Pharmacol 2008;51:565–572

Allena AM, O'Callaghana EL, Hazelwooda L, Germainb S, Castropc H, Schnermannc J, Bassia JK. Distribution of cells expressing human renin-promoter activity in the brain of a transgenic mouse. Brain Res. 2008; 1234: 78-85

Dupont AG and Brouwers S. Brain angiotensin peptides regulate sympathetic tone and blood pressure. J Hypertens. 2010; 28:1599–1610

Amin MS, Wang HW, Reza E, Whitman SC, Tuana BS, Leenen FH. Distribution of epithelial sodium channels and mineralocorticoid receptors in cardiovascular regulatory centers in rat brain. Am J Physiol Regul Integr Comp Physiol 2005;289:R1787-1797.

Antonelli MC, Costa Lieste M, Baskin DG, Stahl WL, Schwartz MW. Localization of Na, K-ATPase isoforms in the hypothalamus of the rat. Cell Mol Biol 1995;41:79-85.

Angel P and Karin M. The role of Jun, Fos and the AP-1 complex in cell-proliferation and transformation. Biochim. Biophys. Acta. 1991; 1072: 129-157.

Angel C, Machin C, Sanchez-Toscano F. Cytoarchitectonic and quantitative Golgi study of the hedgehog supraoptic nucleus. J. Anal 1992; 180:31-39

Baltatu O and Bader M. Brain Renin-Angiotensin System. Neuroendocrin. 2003;78:253–259

Bader M. Tissue Renin-Angiotensin-Aldosterone Systems: Targets for Pharmacological Therapy. Annu. Rev. Pharmacol. Toxicol 2010; 50:439–65

Bunnemann B, Iwai N, Metzger R, Fuxe K, Inagami T, Ganten D. The distribution of angiotensin II ATI receptor subtype mRNA in the rat brain. Neurosci. Lett. 1992; 142: 155-158.

Baulieu EE. Neurosteroids: a new function in the brain. Biol Cell 1991;71:3-10.

Birmingham MK, Sar M, Stumpf WE. Localization of aldosterone and corticosterone in the central nervous system, assessed by quantitative autoradiography. Neurochem Res 1984;9:333-350.

Blaustein MP. Physiological effects of endogenous ouabain: control of intracellular Ca2+ stores and cell responsiveness. Am J Physiol 1993;264:C1367-1387.

Brooks VL and Osborn JW. Hormonal-sympathetic interactions in long-term regulation of arterial pressure: an hypothesis. Am. J. Physiol. 1995; 268: R1343-R1358, 1995

Burke SL, Evans RG, Moretti JH, Head GA. Levels of renal and extrarenal sympathetic drive in Angiotensin II–induced hypertension. Hypertension. 2008;51:878-883

Budzikowski AS, Vahid-Ansari F, Leenen FH. Chronic activation of brain areas by high-sodium diet in Dahl salt-sensitive rats. Am J Physiol 1998;274:H2046-2052.

Bose HS, Lingappa VR, Miller WL. Rapid regulation of steroidogenesis by mitochondrial protein import. Nat. 2002; 417

Campese VM, Shaohua Y, Huiquin Z. Oxidative stress mediates Angiotensin II–dependent stimulation of sympathetic nerve activity. Hypertension. 2005;46:533-539.

Chen Y, Chen H, Hoffmann A, Cool DR, Diz DI, Chappell MC, Chen A, Morris M. Adenovirus-mediated small-interference RNA for in vivo silencing of Angiotensin AT1a receptors in mouse brain. Hypertension. 2006;47:230-237

Cherradi N and Capponi AM. The acute regulation of mineralocorticoid biosynthesis: scenarios for the StAR system. Trensd Endocrinol Metab 1998; 9:412-8

Cheung WJ, Kent MA, El-Shahat E, Wang H, Tan J, White R, and Leenen FH. Central and peripheral renin-angiotensin systems in ouabain-induced hypertension. Am J Physiol Heart Circ Physiol 2006; 291: H624–H630.

Chiu R, Boyle WJ, Meek J, Smeal T, Hunter T, Karin M. The c-Fos protein interacts with c-Jun/AP-1 to stimulate transcription of AP-1 responsive genes. Cell 1988;54:541-552.

Chua SC, Szabo P, Vitek A, Grzeschik KH, John M, White PC. Cloning of cDNA encoding steroid 11 beta-hydroxylase (P450c11). Proc Natl Acad Sci U S A 1987;84:7193-7197.

Cohen DR and Curran T. fra-1: a Serum-Inducible, Cellular immediate-early gene that encodes a Fos-related antigen. Mol. Cell. Biol. 1988; 8; 2063-2069

Collister JP, Hendel MD. Role of the subfornical organ in the chronic hypotensive response to losartan in normal rats. Hypertension. 2003;41:576-582.

Coleman CG, Anrather J, IAdecola C, Pickel VM. Angiotensin II type 2 receptors have a major somatodendritic distribution in vasopressin-containing neurons in the mouse hypothalamic paraventricular nucleus. Neurosci. 2009; 163: 129–142.

Cook SJ, Aziz N, McMahon M. The repertoire of Fos and Jun proteins expressed during the G1 Phase of the cell cycle is determined by the duration of mitogen-activated protein kinase activation. Mol. Cell. Biol. 1999; 19: 330–341

Crestani CC, Busnardo C, Tavares RF, Alves FHF, Correa FMA. Involvement of hypothalamic paraventricular nucleus non-N-methyl-d-aspartate receptors in the pressor response to noradrenaline microinjected into the bed nucleus of the stria terminalis of unanesthetized rats. Eur J Neurosci. 2009; 29: 2166–2176.

Correa FM, Plunkett LM, Saavedra JM. Quantitative distribution of angiotensin-converting enzyme (kininase II) in discrete areas of the rat brain by autoradiography with computerized microdensitometry. Brain Res 1986;375:259-266.

Cox BF and Bishop VS. Neural and humoral mechanisms of angiotensin-dependent hypertension. Am. J. Physiol. 1991; 261: H1284-H1291,1991

Dampney RA, Li YW, Hirooka Y, Potts P, Polson JW. Use of c-fos functional mapping to identify the central baroreceptor reflex pathway: advantages and limitations. Clin Exp Hypertens 1995;17:197-208.

Davern PJ, Head GA. Fos-related antigen immunoreactivity after acute and chronic angiotensin II-induced hypertension in the rabbit brain. Hypertension 2007;49:1170-1177.

Dragunow M. Presence and induction of Fos B-like immunoreactivity in neural, but not non-neural, cells in adult rat brain. Brain Res. 1990; 533: 324-328

Dunn JD and Williams TJ. Effect of sinoaortic denervation on arterial pressure changes evoked by bed nucleus stimulation. Brain Res. Bull. 1998; 46(4): 361–365.

Farkash Y, Timberg R, Orly J. Preparation of antiserum to rat cytochrome P-450 cholesterol side chain cleavage, and its use for ultrastructural localization of the immunoreactive enzyme by protein A-gold technique. Endocrinol. 1986;118:1353-1365.

Fink GD. Long-term sympathoexcitatory effec of angiotensin II: A mechanims of spontaneous and renovascular hypertension . Clin Exp Pharmacol Physiol 1997; 24: 9 1-95

Foletta V. Transcription factor AP-1, and the role of Fra-2. Immunol. Cell. Biol. 1996; 74: 121-133. Review

Francis J, Weiss RM, Johnson AK, Felder RB. Central mineralocorticoid receptor blockade decreases plasma TNF-alpha after coronary artery ligation in rats. Am J Physiol Regul Integr Comp Physiol 2003;284:R328-335.

Furukawa A, Miyatake A, Ohnishi T, Ichikawa Y. Steroidogenic acute regulatory protein (StAR) transcripts constitutively expressed in the adult rat central nervous system: colocalization of StAR, cytochrome P450scc and 3β hydroxysteroid dehydrogenase in the rat brain. J. Neurochem, 1998;71:2231-2238.

Ganten D, Boucher R, Genest J. Renin activity in brain tissue of puppies and adult dogs. Brain Res 1971;33:557-559.

Ganten D, Marquez-Julio A, Granger P, Hayduk K, Karsunky KP, Boucher R, Genest J. Renin in dog brain. Am J Physiol 1971;221:1733-1737.

Ganten D, Minnich JL, Granger P, Hayduk K, Brecht HM, Barbeau A, Boucher R, Genest J. Angiotensin-forming enzyme in brain tissue. Science 1971;173:64-65.

Garcia-Segura LM.Lavaque E, Sierra A, Azcoitia. Steroidogenic acute reguylatory protein in the brain. Neurosci. 2006; 138: 741–747

Gao L, Wang W, Li YL, Schultz HD, Liu D, Cornish KG, Zucker IH. Sympathoexcitation by central ANG II: roles for AT1 receptor upregulation and NAD(P)H oxidase in RVLM. Am J Physiol Heart Circ Physiol 2005;288:H2271-2279.

Gao, L., Wang, W.Z., Wang, W. & Zucker, I.H. Imbalance of angiotensin type 1 receptor and angiotensin II type 2 receptor in the rostral ventrolateral medulla: potential mechanism for sympathetic overactivity in heart failure. Hypertension. 2008;52: 708–714.

Gao L, Wang W, Li YL, Schultz HD, Liu D, Cornish KG Zucker IH. Superoxide mediates sympathoexcitation in heart failure: roles of angiotensin II and NAD(P)H oxidase. Circ Res ;2004:95 , 937–944.

Gebke E, Muller AR, Jurzak M, Gerstberger R. Angiotensin II-induced calcium signaling in neurons and astrocytes of rat circumventricular organs. Neurosci.1998; 85: (2) 509–520.

Gomez-Sanchez EP, Gomez-Sanchez CE. Is aldosterone synthesized in the CNS regulated and functional? Endocrinology 1986 118: 819-823,

Gomez-Sanchez EP. Intracerebroventricular Infusion of Aldosterone Induces Hypertension in Rats . Trends Endocrinol Metab. 2003;14:444-6.

Gomez-Sanchez EP, Fort C, Thwaites D. Central mineralocorticoid receptor antagonism blocks hypertension in Dahl S/JR rats. Am J Physiol 1992;262:E96-99.

Gomez-Sanchez EP, Fort CM, Gomez-Sanchez CE. Intracerebroventricular infusion of RU28318 blocks aldosterone-salt hypertension. Am J Physiol 1990;258:E482-484.

Gomez-Sanchez EP, Venkataraman MT, Thwaites D, Fort C. ICV infusion of corticosterone antagonizes ICV-aldosterone hypertension. Am J Physiol 1990;258:E649-653.

Gomez-Sanchez CE, Zhou MY, Cozza EN, Morita H, Eddleman FC, Gomez-Sanchez EPCorticosteroid synthesis in the central nervous system. Endocr Res. 1996 Nov;22:463-70.

Gomez-Sanchez Celso E. Gomez-Sanchez, Ming Yi Zhou, Eduardo N. Cozza, Hiroyuki Morita, Mark F. Foecking and Elise P. Aldosterone Biosynthesis in the Rat Brain. Endocrinology 1997 138: 3369-3373.

Gottlieb SS, Rogowski AC, Weinberg M, Krichten CM, Hamilton BP, Hamlyn JM. Elevated concentrations of endogenous ouabain in patients with congestive heart failure. Circulation 1992;86:420-425.

Griffin SA, Brown WCB, MacPherson F, McGrath JC, Wilson VG, Korsgaard N, Mulvany MJ, Lever AF. Angiotensin II Causes Vascular Hypertrophy in Part by a Non-pressor Mechanism. Hypertension 1991;17:626-635

Guggilam A, Haque M, Kerut EK, McIlwain E, Lucchesi P, Seghal I Francis J. TNF-α blockade decreases oxidative stress in the paraventricular nucleus and attenuates sympathoexcitation in heart failure rats. Am J Physiol Heart Circ Physiol; 2007;293: H599–H609.

Hallbeck M and Blomquvist A. Spinal Cord-Projecting Vasopressinergic Neurons in the Rat Paraventricular Hypothalamus. J Comp. Neurol. 1999; 411:201–211

Hatae T, Kawano H, karptiskiy A, Krause JE and Masuko S. Arginin-Vasopressin neurons in the rat hypothalamus produce neurokinin B and co-express the tachykinin NK-3 receptor and angiotensin II type 1 receptor. Arch. Histol. Cytol. 2001; 641: 37-44

Hendel MD and Collister JP. Contribution of the subfornical organ to angiotensin II-induced hypertension. Am J Physiol Heart Circ Physiol 288: H680–H685, 2005.

Hendel MD and Collister JP. Renal denervation attenuates long-term hypertensive effects of angiotensin II in the rat. Clin Exp Pharmacol Physiol. 2006; 33: 1225–1230

Henegar JR, Brower GL, Kabour A, Janicki JS. Catecholamine response to chronic ANG II infusion and its role in myocyte and coronary vascular damage. Am. J. Physiol. 1995; 269: H1564-H1569, 1995

Hoffman GE, Smith MS, Verbalis JG. c-Fos and related immediate early gene products as markers of activity in neuroendocrine systems. Front Neuroendocrinol 1993;14:173-213.

Huang BS, Cheung WJ, Wang H, Tan J, White RA, Leenen FH. Activation of brain renin-angiotensin-aldosterone system by central sodium in Wistar rats. Am J Physiol Heart Circ Physiol 2006;291:H1109-1117.

Huang BS, Leenen FH. Brain ouabain and central effects of dietary sodium in spontaneously hypertensive rats. Circ Res 1992;70:430-437.

Huang BS, Leenen FH. Sympathoexcitatory and pressor responses to increased brain sodium and ouabain are mediated via brain ANG II. Am J Physiol 1996;270:H275-280.

Huang BS, Leenen FH. Brain renin-angiotensin system and ouabain-induced sympathetic hyperactivity and hypertension in Wistar rats. Hypertension 1999;34:107-112.

Huang BS, Van Vliet BN, Leenen FH. Increases in CSF [Na+] precede the increases in blood pressure in Dahl S rats and SHR on a high-salt diet. Am J Physiol Heart Circ Physiol 2004;287:H1160-1166.

Huang BS, Wang H, Leenen FH. Chronic central infusion of aldosterone leads to sympathetic hyperreactivity and hypertension in Dahl S but not Dahl R rats. Am J Physiol Heart Circ Physiol 2005;288:H517-524.

Huang BS, White RA, Ahmad M, Tan J, Jeng AY, Leenen FH. Central infusion of aldosterone synthase inhibitor attenuates left ventricular dysfunction and remodelling in rats after myocardial infarction. Cardiovasc Res 2008;81:574-81.

Huang BS, Yuan B, Leenen FH. Blockade of brain "ouabain" prevents the impairment of baroreflexes in rats after myocardial infarction. Circulation 1997;96:1654-1659.

Huang BS, Leenen FH. The brain-renin-angiotensin-aldosterone system: A major mechanism for sympathetic hyperactivity and left ventricular remodeling and dysfunction after myocardial infarction. Curr Heart Fail Rep. 2009, 6:81-88.

Inyushkin AN, Orlans HO, Dyball REJ. Secretory cells of the supraoptic nucleus have central as well as neurohypophysial projections. J. Anat. 2009; 215: 425–434.

Iovino M and Steardo L. Vasopressin release to central and peripheral angiotensin II in rats with lesions of the subfornical organ. Brain Res. 1984; 322:365-368.

Johren O, Imboden H, Hauser W , Maye I, Sanvitto GL, Saavedra JM. Localization of angiotensin-converting enzyme, angiotensin II, angiotensin II receptor subtypes, and vasopressin in the mouse hypothalamus. Brain Res. 1997; 757: 218–227.

Johansen H, Strauss B, Arnold JM, Moe G, Liu P. On the rise: The current and projected future burden of congestive heart failure hospitalization in Canada. Can J Cardiol 2003;19:430-435.

Jung-Testas I, Alliot F, Pessac B, Robel P, Baulieu EE. Immunocytochemical localization of cytochrome P-450scc in cultured rat oligodendrocytes. C R Acad Sci III 1989;308:165-170.

Jung-Testas I, Hu ZY, Baulieu EE, Robel P. Steroid synthesis in rat brain cell cultures. J Steroid Biochem 1989;34:511-519.

Kang YM, Ma Y, Elks C, Zheng JP, Yang ZM, Francis J. Cross-talk between cytokines and renin-angiotensin in hypothalamic paraventricular nucleus in heart failure: role of nuclear factor-kappaB. Cardiovasc Res 2008;79:671-678.

Kang YM, Zhang ZH, Johnson RF, Yu Y, Beltz T, Johnson AK, Weiss RM, Felder RB. Novel effect of mineralocorticoid receptor antagonism to reduce proinflammatory cytokines and hypothalamic activation in rats with ischemia-induced heart failure. Circ Res 2006;99:758-766.

Kang YM, Zhang ZH, Xue B, Weiss RM, Felder RB. Inhibition of brain proinflammatory cytokine synthesis reduces hypothalamic excitation in rats with ischemia-induced heart failure. Am J Physiol Heart Circ Physiol 2008;295:H227-236.

Kang1 YM, Ma Y, Zheng JP, Elks C, Sriramula S, Yang ZM, Francis J. Brain nuclear factor-kappa B activation contributes to neurohumoral excitation in angiotensin II-induced hypertension. Cardiovasc Res 2009; 82: 503–512

Kawano H and Masuko S. Region specific projections from the subfornical organ to the paraventricular hypothalamic nucleus in the rat. Neurosci. 2010; 169: 1227–1234.

Kanagy NL, Pawloski CM, Fink GD. Rol of aldosterone in angiotensin II-induced hypertension in rats. Am. J. Physiol. 1990; 259 (28) :R102-R109.

Khne RL, Chow KY, Mercer PF. Does enhanced sympathetic tone contribute to angiotensin II hypertension in rats? Eur J Pharmacol. 1990; 184 109-118.

Kim HJ ,Ha M, Park CH, Park SJ, Youn SM, Kanga SS, Choa GJ, Choia WS . StAR and steroidogenic enzyme transcriptional regulation in the rat brain: effects of acute alcohol administration. Mol. Brain Res. 2003; 115: 39–49

King SR, Manna PR, Ishii T, Syapin PJ, Ginsberg SD, Wilson K, Walsh LP, Parker KL, Stocco DM, Smith RG, Lamb DJ. An essential component in steroid synthesis, the steroidogenic acute regulatory protein, is expressed in discrete regions of the brain. J Neurosci 2002;22:10613-10620.

King AJ, Osborn JW, Fink JD. Splanchnic circulation is a critical neural target in angiotensin II salt hypertension in rats. Hypertension. 2007;50:547-556

Kim HJ, Kang SS, GJ Cho, W S Choi . Steroidogenic acute regulatory protein, Its presence and function in brain neurosteroidogenesis. Arch Histol Cytol. 2004; 67: 383-392.

Khne RL, Chow KY, Mercer PF. Does enhanced sympathetic tone contribute to angiotensin II hypertension in rats? Eur J Pharmacol 1990; 184 :109-118.

Kimoto T, Tsurugizawa T, Ohta Y, Makino J, Tamura HO, Hojo Y, Takata N and Kawato S. Neurosteroid Synthesis by Cytochrome P450-Containing Systems Localized in the Rat Brain Hippocampal Neurons: *N*-Methyl-D-Aspartate and Calcium-Dependent Synthesis. Endocrinology 2001; 142:3578–3589

Krug1 AW, Vleugels1 K, Schinner S, Lamounier-Zepter1V, Ziegler1 CG, Bornstein1 SR, Ehrhart-Bornstein1 M. Human adipocytes induce an ERK1/2 MAP kinasesmediated upregulation of steroidogenic acute regulatory protein (StAR) and an angiotensin II sensitization in human adrenocortical cells. Int J Obes. 2007; 31:1605–1616.

Lal A, Veinot JP, Ganten D, Leenen FH. Prevention of cardiac remodeling after myocardial infarction in transgenic rats deficient in brain angiotensinogen. J Mol Cell Cardiol 2005;39:521-529.

Lal A, Veinot JP, Leenen FH. Critical role of CNS effects of aldosterone in cardiac remodeling post-myocardial infarction in rats. Cardiovasc Res 2004;64:437-447.

Lally BE, Albrecht PJ, Levison SW, Salm AK. Divergent glial fibrillary acidic protein and its mRNA in the activated supraoptic nucleus. Neurosci Lett. 2005; 380:295-9.

Latchford KJ and Ferguson AV. Ang II induced excitation of paraventricular nucleus magnocellular neurons: a role of glutamate interneurons. Am J Physiol Regul Integr Com Physiol 2004; 286: R894R902.

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

Leenen FH. Brain mechanisms contributing to sympathetic hyperactivity and heart failure. Circ Res. 2007;101:221-223.

Leenen FH, Huang BS, Yu H, Yuan B. Brain 'ouabain' mediates sympathetic hyperactivity in congestive heart failure. Circ Res. 1995; 77:993-1000.

Leenen FH, Skarda V, Yuan B, White R. Changes in cardiac ANG II postmyocardial infarction in rats: effects of nephrectomy and ACE inhibitors. Am J Physiol 1999;276:H317-325.

Levy D, Kenchaiah S, Larson MG, et al. Long-term trends in the incidence of and survival with heart failure. N Engl J Med 2002;347:1397-1402.

Li YW, Dampney RA. Expression of Fos-like protein in brain following sustained hypertension and hypotension in conscious rabbits. Neuroscience 1994;61:613-634.

Li DP, Chen SR, Pan HL. Angiotensin II stimulates spinally projecting paraventricular neurons through presynaptic disinhibition. The Journal of Neuroscience 2003; 23 :5041-5049.

Lieberman S and Lin YY. Reflections on sterol sidechain cleavage process catalyzed by cytochrome P450(scc). J Steroid Biochem Mol Biol 2001;78:1-14.

Lind RW, Swanson LW, Ganten D. Angiotensin II immunoreactive pathways in the central nervous system of the rat: evidence for a projection from the subfornical organ to the paraventricular nucleus of the hypothalamus. Clin Exp Hypertens A 1984;6:1915-1920.

Liu D, Gao L, Roy SK, Cornish KG, Zucker IH. Neuronal angiotensin II type 1 receptor upregulation in heart failure: activation of activator protein 1 and Jun N-terminal kinase. Circ Res 2006;99:1004-1011.

Li, Qian, William E. Dale, Eileen M. Hasser, Edward H. Blaine. Acute and chronic angiotensin hypertension: neural and nonneural components, time course, and dose dependency. Am. J. Physiol. 271 (Regulatory Integrative Comp. Physiol. 40): R200-R207, 1996

Lu B, Yang XJ, Chen K, Yang JQ. Dietary sodium deprivation evokes activation of brain regional neurons and downregulation of angiotensin II type 1 receptor and angiotensin-convertion enzyme mRNA expression. Neuroscience 2009;164:1303-11.

Luft FC, Wilcox CS, Unger T, Kiihn R, Demmert G, Rohmeiss P, Ganten D, Sterzel RB. Angiotensin-Induced Hypertension in the Rat Sympathetic Nerve Activity and Prostaglandins. Hypertension 1989;14:396-403.

Lohmeier TE, Lohmeier JR, Warren S, May PJ, Cunningham JT. Sustained activation of the central baroreceptor pathway in angiotensin hypertension. Hypertension 2002;39:550-556.

Llorens-Cortes C and Kordon C. The neuroendocrine view of the angiotensin and apelin systems.
J Neuroendocrinol. 2008; 20: 279–289.

MacKenzie1 SM, Clark1 CJ, Fraser1 R, Gómez-Sánchez CE, Connell JMC, E Davies. Expression of 11β-hydroxylase and aldosterone synthase genes in the rat brain. J Mol Endocrinology 2000; 24: 321–328.

MacKenzie SM, Clark CJ, Ingram MC, Lai M, Seckl J, Gomez-Sanchez CE, Fraser R, Connell JM, Davies E. Corticosteroid production by fetal rat hippocampal neurons. Endocr Res. 2000 Nov; 26:531-5.

MacKenzie SM, Lai1M, Clark CJ, Fraser R, Gómez-Sánchez CE, Seckll JR, Connell JMC, Davies E. 11β-Hydroxylase and aldosterone synthase expression in fetal rat hippocampal neurons. J Mol Endocrinol 2002; 29: 319–325.

MacKenzie SM, Dewar1D, Stewart W, Fraser R, Connell JMC. The transcription of steroidogenic genes in the human cerebellum and hippocampus: a comparative survey of normal and Alzheimer's tissue and Eleanor Davies. J Endocrinol. 2008; 196: 123–130.

Mason JI. Cytochrome P-450 as a target for therapeutic inhibitors. Biochem Soc Trans 1993;21:1057-1060.

McKinley MJ, Badoer E, Oldfield BJ. Intravenous angiotensin II induces Fos-immunoreactivity in circumventricular organs of the lamina terminalis. Brain Res. 1992; 594: 295-300

McKinley MJ, Allen AM, Burns P, Colvill LM, Oldfield BJ. Interaction of circulating hormones with the brain: the roles of the subfornical organ and the organum vasculosum of the lamina terminalis. Clin Exp Pharmacol Physiol 1998; 25: (Suppl.), S61-S67.

McKinley MJ, Allen AM, May CN, McAllen RM, Oldfield BJ, Sly D, Mendelsohn FAO. Neuronal pathways from the lamina terminalis influencing cardiovascular and body fluid hemostasis. Clin Exp Pharmacol Physiol. 2001; 28: 990–992.

McKinley MJ, Mathai ML, McAllen RM, McClear RC, Miselis RR, Pennington GL, Vivast L, Wade JD and Oldfield BJ. Vasopressin secretion: osmotic and hormonal regulatio by the lamina terminalis. J Neuroendocrinol. 2004 ; 16: 340-347.

McKinley MJ, Badoer E, Oldfield B. Intravenous angiotensin II induces Fos-immunoreactivity in circumventricular organs of the lamina terminalis. Brain Res. 1992; 594: 295-300

Mellon SH and Deschepper CF. Neurosteroid biosynthesis: genes for adrenal steroidogenic enzymes are expressed in the brain. Brain Res. 1993;629:283-292.

Mellon SH and Griffin LD. Neurosteroids: biochemistry and clinical significance. Trends Endocrinol Metab 2002;13:35-43.

Mellon SH and Griffin LD, Compagnone NA. Biosynthesis and action of neurosteroids. Brain Res. Rev. 2001; 37: 3–12.

Mendelsohn FA, Quirion R, Saavedra JM, Aguilera G, Catt KJ. Autoradiographic localization of angiotensin II receptors in rat brain. Proc Natl Acad Sci U S A 1984;81:1575-1579.

Mitchell LD, Barron K, Brody MJ, Johnson AK. Two possible actions for circulating Angiotensin II in the control of vasopressin release I. Peptides. 1982; 3: 503-507

Moellenhoff E, Blume A, Culman J, Chatterjee B, Herdegen T, Lebrun CJ, Unger T. Effect of repetitive icv injections of ANG II on c-Fos and AT(1)-receptor expression in the rat brain. Am J Physiol Regul Integr Comp Physiol 2001;280:R1095-1104.

Morgan JI and Curran T. Stimulus-transcription coupling in neurons: role of cellular immediate-early genes. Trends Neurosci 1989;12:459-462.

Mornet E, Dupont J, Vitek A, White PC. Characterization of two genes encoding human steroid 11 beta-hydroxylase (P-450(11) beta). J Biol Chem 1989;264:20961-20967.

Murasawa S, Matsubara H, Urakami M, Inada M. Regulatory elements that mediate expression of the gene for the angiotensin II type 1a receptor for the rat. J Biol Chem 1993;268:26996-27003.

Murrell JR, Randall JD, Rosoff J, Zhao JL, Jensen RV, Gullans SR, Haupert GT Jr. Endogenous ouabain: upregulation of steroidogenic genes in hypertensive hypothalamus but not adrenal. Circulation 2005;112:1301-1308.

Northcott CA, Watts S, Chen Y, Morris M, Chen A, Haywood JR. Adenoviral inhibition of AT1a receptors in the paraventricular nucleus inhibits acute increases in mean arterial blood pressure in the rat. Am J Physiol Regul Integr Comp Physiol 299: R1202–R1211, 2010.

Nichols NR, Finch CE, Nelson JF. Food restriction delays the age-related increase in GFAP mRNA in rat hypothalamus. Neurobiol Aging. 1995 16:105-10

Nishina H, Sato H, Suzuki T, Satot Moriyuki, Ibat H. Isolation and characterization offra-2, an additional member of the fos gene family. Cell Biol. 1990; 87: 3619-3623.

Obermuller N, Unger T, Culman J, Gohlke P, de Gasparo M, Bottari SP. Distribution of angiotensin II receptor subtypes in rat brain nuclei. Neurosci Lett 1991;132:11-15.

Oldfield BJ, Badoer E, Hards DK, Mckinley MJ. Fos production in retrogradely labelled neurons of the lamina terminalis following intravenous infusion of either hypertonic saline or agiotensin II. Neuroscience 1994; 60 (1): 255-262.

Oldfeld BJ, Davern PJ, Giles ME ,Allen AM, Badoer E McKinley MJ. Efferent Neural Projections of Angiotensin Receptor (AT1) Expressing Neurones in the Hypothalamic Paraventricular Nucleus of the Rat. J Neuroendocrinol. 2001; 13: 139-146.

Oldfield BJ, Miselis RR, McKinley MJ. Median preoptic nucleus projections to vasopressin-containing neurones of the supraoptic nucleus in sheep. A light and electron microscopic study. Brain Res. 1991; 542: 193-200.

Osborn JW, Fink GD, Sved AF, Toney GM, Raizada MK. Circulating angiotensin II and dietary salt: converging signals for neurogenic hypertension. Curr Hypertens Rep 2007;9:228-235.

Packer M. The neurohormonal hypothesis: a theory to explain the mechanism of disease progression in heart failure. J Am Coll Cardiol 1992;20:248-254.

Peters B, Yeubner PH, Clausmeryer S, Puschner T, Maser-Gluth Ch, Wrede H J, Kränzlin B, Jörj P. StAR expression and the long term aldosterone response to high potassium diet in Wistar-Kyoto and spontaneously hypertensive tars. Am J Physiol Endocrinol Metab. 2007; 292: E16-E23

Peters B, Clausmeyer S, Obermüller N, Woyth A, Kränzlin B, Gretz N, Peters J. Specific Regulation of StAR Expression in the Rat Adrenal Zona Glomerulosa: an In Situ Hybridization Study. J histochem Cytochem 1998; 46: 1215–1221.

Pitt B, Zannad F, Remme WJ, Cody R, Castaigne A, Perez A, Palensky J, Wittes J. The effect of spironolactone on morbidity and mortality in patients with severe heart failure. Randomized Aldactone Evaluation Study Investigators. N Engl J Med 1999;341:709-717.

Pfister J, Spengler C, Grouzmann E, Raizada MK, D. Felix D, Imboden H. Intracellular staining of angiotensin receptors in the PVN and SON of the rat. Brain Res. 1997; 754 : 307–310.

Ployngam T and Collister JP. An Intact Median Preoptic Nucleus is Necessary for Chronic Angiotensin II-Induced Hypertension. Brain Res. 2007;1162: 69–75.

Potts PD, Polson JW, Hirooka Y, Dampney RA. Effects of sinoaortic denervation on fos expression in the brain evoked by hypertension and hypotension in conscious rabbits. Neuroscience 1997; 77: 503-520.

Potts PD, Hirooka Y, Dampney RA. Activation of brain neurons by circulating angiotensin II: direct effects and baroreceptor mediated secondry effects. Neuroscience 1999; 90: 581–594.

Qadri F, Culman J, Veltmar A, Maas K, Rascher W, Unge T. Angiotensin Il-Induced Vasopressin Release is Mediated Through Alpha-i Adrenoceptors and Angiotensin II AT1 Receptors in the Supraoptic Nucleus. J Pharmacol Exp Therap 1993; 267: 567-74.

Pyner S, Coote JH. Identification of branching paraventricular neurons of the hypothalamus that project to the rostroventrolateral medulla and spinal cord. Neuroscience 2000;100:549-556.

Robert C. Speth Æ Vardan T. Karamyan.The significance of brain aminopeptidases in the regulation of the actions of angiotensin peptides in the brain. Heart Fail Rev 2008; 13:299–309

Roy CL, Li JY, Stocco DM, Langlois D, Saez JM Regulation by adrenocorticotropin (ACTH), Angiotensin II, Transforming growth factor Factor-ß, and Insulin-Like Growth Factor I of Bovine Adrenal Cell Steroidogenic Capacity and Expression of ACTH Receptor, Steroidogenic Acute Regulatory Protein, Cytochrome P450c17, and 3ß-Hydroxysteroid Dehydrogenase. Endocrinology 2000; 141: 1599-1607.

Rivest S, Lacroix S, Vallieres L, Nadeau S, Zhang J, Laflamme N. How the blood talks to the brain parenchyma and the paraventricular nucleus of the hypothalamus during systemic inflammatory and infectious stimuli. Proc Soc Exp Biol Med 2000;223:22-38.

Reid IA. Interactions between ANG II, sympathetic nervous system, and baroreceptor reflexes in regulation of blood pressure. Am. J. Physiol. 262: E763-E778, 1992.

Roland BL, Li KX, Funder JW. Hybridization histochemical localization of 11 beta-hydroxysteroid dehydrogenase type 2 in rat brain. Endocrinology 1995; 136: 4697-4700

Rowland NE, Fregly MJ, Li BH, Smith GC. Action of angiotensin converting enzyme inhibitors in rat brain: interaction with isoproterenol assessed by Fos immunocytochemistry. Brain Res. 1994; 654: 34-40

Rowland NE, Li BH, Krozelle AK, Fregly MJ, Garcia M, Smith GC. Localization of Changes in Immediate Early Genes in Brain in Relation to Hydromineral Balance: Intravenous Angiotensin II Emin. Res Bull.1994; 33: 427-436.

Rowland NE, Li BH, Fregly MJ, Smith GC. Fos induced in brain of spontaneously hypertensive rats by angiotensin II and co-localization with AT-1 receptors. Brain Res. 1995;675:127-134.

Renaud LP. CNS pathways mediating cardiovascular regulation of vasopressin. Clin. Exp. Pharmacol Physiol. 1996; 23: 157-160.

Saka K and Sigmund CD. Molecular Evidence of Tissue Renin-Angiotensin Systems: A Focus on the Brain. Curr. Hypertens. Rep. 2005; 7:135–140.

Samson WK. Hypothalamic localization of steroidogenic acute regulatory protein: the missing piece of the puzzle is found . Trends Endocrinol. Metabol. 2003; 14: 99-100

Sanne JL and Krueger KE. Expression of Cytochrome P450 Side-Chain Cleavage Enzyme and 3,ß-Hydroxysteroid Dehydrogenase in the Rat Central Nervous System: A Study by Polymerase Chain Reaction and In Situ Hybridization. J. Neurochem 1995; 65: 528-536.

Sgro S, Ferguson AV, Renaud LP. Subfornical organ--supraoptic nucleus connections: an electrophysiologic study in the rat. Brain Res. 1984; 303:7-13.

Shan Z, Cuadra AD, Sumners C, Raizada MK. Characterization of a functional (pro)renin receptor in rat brain neurons. Exp Physiol 2008; 93: 701–708.

Sharp FR, Sagar SM, Hicks K, Lowenstein D, Hisanaga K. c-fos mRNA, Fos, and Fos-related antigen induction by hypertonic saline and stress. J Neurosci 1991;11:2321-2331.

Sheng M, McFadden G, Greenberg ME. Membrane depolarization and calcium induce c-fos transcription via phosphorylation of transcription factor CREB. Neuron. 1990;4:571-582.

Shen H, Sikorska M, LeBlanc J, Walker PR, Liu QY. Oxidative stress regulated expression of Ubiquitin Carboxyl-terminal Hydrolase-L1: Role in cell survival. Apoptosis 2006; 11(6):1049-59.

Scopinho AA, Tavares RF, Busnardo C, Aguiar Correa FMA. Non-N-Methyl-D-Aspartate Glutamate Receptors in the Paraventricular Nucleus of Hypothalamus Mediate the Pressor Response Evoked by Noradrenaline Microinjected Into the Lateral Septal Area in Rats. J Neurosci. Res 2008; 86:3203–3211.

Schwartz JA, Reilly NS, Knuepfer MM. Angiotensin and NMDA receptors in the median preoptic nucleus mediate hemodynamic response patterns to stress. Am J Physiol Regul Integr Comp Physiol 2008; 295: R155–R165, 2008.

Shinzawa K, Ishibashi S, Murakoshi M, Watanabe K, Kominami S, Kawahara A, Takemori S. Relationship between zonal distribution of microsomal cytochrome P-450s (P-450(17)alpha,lyase and P-450C21) and steroidogenic activities in guinea-pig adrenal cortex. J Endocrinol 1988;119:191-200.

Sierra A. The StAR Protein in the Brain. J Neuroendocrinol. 2004, Vol 16, 787–793

Sierra A, Lavaque E, Perez-Martin M, Azcoitia I, Hales DB, Garacia-Segura LM. Steroidogenic acute regulatory protein in the rat brain, cellular distribution, developmental regulation and overexpression after injury. Eur J Neurosci. 2003, 18, 1458-1467.

Sinnayah P, Lazartigues E, Sakai K, Sharma RV, Sigmund CD, Davisson RL. Genetic Ablation of Angiotensinogen in the Subfornical Organ of the Brain Prevents the Central Angiotensinergic Pressor Response. Circ Res. 2006;99:1125-1131.

Simard J, Couet J, Durocher F, Labrie Y, Sanchez R, Breton N, Turgeon C, Labrie F. Structure and tissue-specific expression of a novel member of the rat 3 beta-hydroxysteroid dehydrogenase/delta 5-delta 4 isomerase (3 beta-HSD) family. The exclusive 3 beta-HSD gene expression in the skin. J Biol Chem 1993;268:19659-19668.

Sonnenberg JL, Mitchelmore C, Macgregor-Leon PF, Hempstead J, Morgan JI, Curran T. Glutamate receptor agonists increase the expression of Fos, Fra, and AP-1 DNA binding activity in the mammalian brain. J Neurosci Res 1989;24:72-80.

Sonnenberg J, Macgregor-Leon PF, Curran T, Morgan JI. Dynamic Alterations Occur in the levels and Composition of Transcription Factor AP-1 Complexes after Seizure. Neuron. 1989; 3: 359-365.

Stocco DM. StAR protein and the regulation of steroid hormone biosynthesis. Annu Rev Physiol 2001;63:193-213.

Stocco DM. Tracking the role of a star in the sky of the new millennium. Mol Endocrinol 2001;15:1245-1254.

Stromstedt M, Waterman MR. Messenger RNAs encoding steroidogenic enzymes are expressed in rodent brain. Brain Res Mol Brain Res 1995;34:75-88.

Sun Y and Weber KT. Infarct scar: a dynamic tissue. Cardiovasc Res. 2000;46:250-256.

Swanson LW. An autoradiographic study of the efferent connections of the preoptic region in the rat. J. Comp. Neur. 167: 227-256. 227

Tan J, Wang H, Leenen FH. Increases in brain and cardiac AT1 receptor and ACE densities after myocardial infarct in rats. Am J Physiol Heart Circ Physiol 2004;286:H1665-1671.

Torres M and Forman HJ. Redox signaling and the MAP kinase pathways. Biofactors 2003; 17 (1-4): 287–296.

Thomas MA, Lemmer B. The use of heat-induced hydrolysis in immunohistochemistry on angiotensin II (AT1) receptors enhances the immunoreactivity in paraformaldehyde-fixed brain tissue of normotensive Sprague–Dawley rats. Brain Res. 2006; 1119: 150-164.

Tribollet E, Armstrong WE, Dubois-Dauphin M, Dreifu JJ. Extra hypothalamic afferent inputs to the supraoptic nucleus area of the rat as determined by retrograde and anterograde tracing techniques. Neuroscience 1985; 1S (1): 135-148.

Tomaschitz A, Pilz S, Ritz E, Obermayer-Pietsch B and Pieber TR. Aldosterone and arterial hypertension. Endocrinol. Rev. 2010; 6, 83–93.

Trudel E, Bourque CW. A rat brain slice preserving synaptic connections between neurons of the suprachiasmatic nucleus, organum vasculosum lamina terminalis and supraoptic nucleus. J Neurosci. Methods. 2003;128: 67-77

Tsutsui K, Ukena K, Takase M, Kohchi C, Lea RW. Neurosteroid biosynthesis in vertebrate brains. Comp Biochem. Physiol. 1999; 124 : 121–129

Vahid-Ansari F and Leenen FH. Pattern of neuronal activation in rats with CHF after myocardial infarction. Am J Physiol 1998;275:H2140-2146.

van Houten M, Schiffrin EL, Mann JF, Posner BI, Boucher R. Radioautographic localization of specific binding sites for blood-borne angiotensin II in the rat brain. Brain Res. 1980;186:480-485.

Veerasingham SJ, Vahid-Ansari F, Leenen FH. Neuronal Fos-like immunoreactivity in ouabain-induced hypertension. Brain Res. 2000;876:17-21.

Wang H, Huang BS, Ganten D, Leenen FH. Prevention of sympathetic and cardiac dysfunction after myocardial infarction in transgenic rats deficient in brain angiotensinogen. Circ Res. 2004;94:843.

Wang H, Huang BS, Leenen FH. Brain sodium channels and ouabainlike compounds mediate central aldosterone-induced hypertension. Am J Physiol Heart Circ Physiol 2003; 285:H2516-2523.

Wang H, Amin A, El-Shahat E, Huang BS, Tuana BS, Leenen FH. Effects of central sodium on epithelial sodium channels in rat brain. Am J Physiol Regul Integr Comp Physiol 299: R222–R233, 2010.

Wang, W.Z., Gao, L., Wang, H.J., Zucker, I.H., Wang, W. Interaction between cardiac sympathetic afferent reflex and chemoreflex is mediated by the NTS AT1 receptors in heart failure. Am. J. Physiol. Heart Circ. 2008; 295: H1216–H1226.

Wei S-G, Yu Y, Zhang Z-H, Weiss RM, Felder RB. Angiotensin II-triggered p44/42 mitogen-activated protein kinase mediates sympathetic excitation in heart failure rats. Hypertension 2008; 52: 342–350.

Wei S-G, Yu Y, Zhang Z-H, Felder RB. Angiotensin II upregulates hypothalamic AT_1 receptor expression in rats via the mitogen-activated protein kinase pathway. Am J Physiol Heart Circ Physiol 2009; 296: H1425–H1433.

Wielbo D, Sernia C, Gyurko R, Phillips MI. Antisense inhibition of hypertension in the spontaneously hypertensive rat. Hypertension 1995;25:314-319.

Yamada H, Naruse M, Naruse K, Demura H, Takahashi H, Yoshimura M, Ochi J. Histological study on ouabain immunoreactivities in the mammalian hypothalamus. Neurosci Lett 1992;141:143-146.

Yamada H, Ihara N, Takahashi H, Yoshimura M, Sano Y. Distribution of the endogenous digitalis-like substance (EDLS)-containing neurons labeled by digoxin antibody in hypothalamus and three circumventricular organs of dog and macaque. Brain Res. 1992; 584: 237-243.

Ye P, Kenyon CJ, MacKenzie SM, Seckl JR, Fraser R, Connell JM, Davies E. Regulation of aldosterone synthase gene expression in the rat adrenal gland and central nervous system by sodium and angiotensin II. Endocrinology 2003;144:3321-3328.

Ye P, Kenyon1 CJ, MacKenzie SM, Nichol1 K, Seckl1 JR, Fraser R, Connell JM, Davies E. Effects of ACTH, dexamethasone, and adrenalectomy on 11beta-hydroxylase (CYP11B1) and aldosterone synthase (CYP11B2) gene expression in the rat central nervous system. J Endocrinol. 2008; 196: 305–311

Yongue RG and Roy EJ. Endogenous aldosterone an corticosterone in brain cell nuclei of adrenal-intact rats: regional distribution an effects of physiological variations in serum steroids. Brain Res, 1987; 436: 49-6 1.

Vieira AA, David B. Nahey, and John P. Collister. Role of the organum vasculosum of the lamina terminalis for the chronic cardiovascular effects produced by endogenous and exogenous ANG II in conscious rats. Am J Physiol Regul Integr Comp Physiol 2010; 299: R1564–R1571, 2010.

Weinberg MS, Girotti M, Spencer RL. Restraint-induced fra-2 and c-fos expression in the rat forebrain: relationship to stress duration. Neuroscience. 2007; 150: 478–486.

Xu Z and Herbert J. Regional supression by lesions in the anterior third ventricle of c-fos expression induced by either angiotensin II or hypertonic saline. Neuroscience 1995; 67: 135-147.

Yang G, Gray TS, Sigmund CD, Cassell MD. The angiotensinogen gene is expressed in both astrocytes and neurons in murine central nervous system. Brain Res. 1999; 817:123–131

Yu Y, Wei SG, Zhang ZH, Gomez-Sanchez E, Weiss RM, Felder RB. Does aldosterone upregulate the brain renin-angiotensin system in rats with heart failure? Hypertension 2008;51:727-733.

Yu L, Romero DG, Gomez-Sanchez CE, Gomez-Sanchez EP. Steroidogenic enzyme gene expression in the human brain. Mol Cell Endocrinol. 2002; 190:9-17.

Zhang ZH, Kang YM, Yu Y, Wei SG, Schmidt TJ, Johnson AK, Felder RB. 11beta-hydroxysteroid dehydrogenase type 2 activity in hypothalamic paraventricular nucleus modulates sympathetic excitation. Hypertension 2006;48:127-133.

Zhang ZH, Yu Y, Kang YM, Wei SG, Felder RB. Aldosterone acts centrally to increase brain renin-angiotensin system activity and oxidative stress in normal rats. Am J Physiol Heart Circ Physiol 2008;294:H1067-1074.

Zhu GQ, Gao L, Patel KP, Zucker IH, Wang W. ANG II in the paraventricular nucleus potentiates the cardiac sympathetic afferent reflex in rats with heart failure. J Appl Physiol 2004;97:1746-1754.

Zimmerman MC, Lazartigues E, Lang JA, Sinnayah P, Ahmad IM, Spitz DR, Davisson RL. Superoxide mediates the actions of angiotensin II in the central nervous system. Circ Res 2002;91:1038-1045.

Zimmerman MC, Sharma RV, Davisson RL. Superoxide mediates angiotensin II-induced influx of extracellular calcium in neural cells. Hypertension 2005;45:717-723.