An Intraneuronal Angiotensinergic System in the Rat and Human Peripheral Nervous System

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Submitted by

Jaspal Patil

(India)

Thesis advisor

Prof. Dr. Hans Imboden

Institute of Cell Biology

Faculty of the University of Bern

Bern, Switzerland

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Bern, Dean of the Faculty of Medicine

Bern, Dean of the Faculty of Science

Bern, Dean of the Vetsuisse Faculty Bern

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List of abbreviations

ACE	Angiotensin converting enzyme
ACTH	Adrenocortico-trophic hormone
Ang I	Angiotensin I
Ang II	Angiotensin II
Ang-N	Angiotensinogen
ANS	Autonomic nervous system
AT_1	Angiotensin receptor type 1
AT_2	Angiotensin receptor type 2
AT_{1A}	Angiotensin receptor type 1A
AT_{1B}	Angiotensin receptor type 1B
BCIP	5-Bromo-4-chloro-3-indolyl phosphate
CLSM	Confocal laser scanning microscopy
CNS	Central nervous system
DAPI	4',6-diamidino-2-phenylindole
DAR	Donkey anti-rabbit
DEPC	Diethylpyrocarbonate
DRG	Dorsal root ganglia
DβH	Dopamine-β-hybroxylase
GAM	Goat anti-mouse
GPCR	G-protein coupled receptor
HPLC	High performance liquid chromatography
ISH	*In situ* hybridization
LH	Luteinizing hormone
MAP	Mitogen-activated protein
NBT	nitro blue tetrazolium
NE	Norepinephrine
NTS	Nucleus tractus solitarii
PBS	Phosphate-buffered saline
PISHIC	Post *in situ* hybridization immunocytochemistry
PNS	Peripheral nervous system
PSNS	Parasympathetic nervous system
qRT-PCR	Quantitative real time PCR
RT-PCR	Reverse transcriptase PCR
RAS	Renin angiotensin system
RIA	Radioimmunoassay
SNS	Sympathetic nervous system
Sp5	Spinal trigeminal tract
VSMCs	Vascular smooth muscle cells
WKY	Wistar Kyoto

Abstract

The pivotal aim of my study was to investigate a possible intraneuronal angiotensinergic system by means of localization of renin angiotensin system (RAS) components in the sympathetic (SNS) and the sensory nervous system of Wistar Kyoto rat and normotensive human tissues.

Experiments were performed at extract level by using quantitative real time PCR (qRT-PCR) and high performance liquid chromatography-radioimmunoassay (HPLC-RIA), while at cellular level we used *in situ* hybridization (ISH), immunocytochemistry and post *in situ* hybridization immunocytochemistry (PISHIC) to detect RAS components. As a part of immunocytochemical work, optical sections and movies were made at advanced level by confocal laser scanning microscopy (CLSM).

In the current study, angiotensinogen (Ang-N) - and angiotensin converting enzyme-mRNA's were detected by using qRT-PCR in total RNA extracts obtained from the rat coeliac ganglia, trigeminal ganglia, dorsal root ganglia (DRG) and heart, while renin mRNA was untraceable in all the tissues studied. Instead of renin mRNA, we detected in all the investigated tissues, expression of cathepsin D mRNA, a protease responsible for cleavage beneath other substrates also Ang-N to angiotensin I. ISH performed with the rat coeliac ganglia, trigeminal ganglia, DRG and in the intracardiac ganglia of heart, confirmed the presence of Ang-N mRNA in the cytoplasm of neurons of these ganglia.

Angiotensins were quantitated by RIA with and without prior separation by HPLC, in the extracts of the rat and human coeliac ganglia, trigeminal ganglia, DRG and different parts of heart.

Immunoreactivity for Ang II was demonstrated in rat and human coeliac ganglia as well as with mesenteric resistance blood vessels. By using CLSM we were able to demonstrate the presence of angiotensinergic synapses en passant along side of vascular smooth muscle cells. In the sensory system, a number of neurons and their processes in both the rat and human trigeminal ganglia

were stained for Ang II. PISHIC reveals that in the rat trigeminal ganglia some, but not all Ang-N mRNA-positive neurons marked for Ang II. In some neurons Substance P was found colocalized with Ang II. Intracellular Ang II staining could be shown in number of neurons and their processes in both the rat and human DRG. Interestingly we observed neuronal processes with angiotensinergic synapses en passant within the human DRG. In the DRG, we also identified by qRT PCR, expression of Ang II-receptor AT_{1A}- and AT_2 - mRNA while AT_{1B} - mRNA was not traceable. Number of neurons in the atria and their processes in both the rat and human heart were stained for Ang II and DβH. In most of the neurons DβH was found together with Ang II, indicating a partial colocalization of the sympathetic and angiotensinergic system. We observed angiotensinergic synapses en passant in rat and human heart. The colocalization of neuronal Ang II with the sympathetic nervous system, these intracardiac neurons may be the basis for a participation and function of Ang II in the blood pressure regulation locally and the adjustment of the contractile force of the atria and the ventricles.

In conclusion, our findings indicate that Ang II is synthesized inside the neurons of sympathetic coeliac ganglia and may act as an endogenous neurotransmitter locally with the mesenteric resistance blood vessels. In sensory nervous system, we demonstrate that the Ang II may be produced locally in the neurons of the rat and human trigeminal ganglia and DRG, and act as a neurotransmitter. The colocalization of neuronal Ang II with Substance P in the trigeminal ganglia and DRG neurons may be the basis for a participation and function of Ang II in the regulation of nociception. In the heart study, our results suggest that Ang II could be produced intrinsically in the rat and human intracardiac neurons and may act as a neurotransmitter within the heart. In addition, the results of my thesis provide strong evidence for the existence of a local renin-independent angiotensinergic system in the peripheral nervous system.

1. General introduction

1.1 What is the RAS?

The discovery of the renin-angiotensin-system (RAS) was initially done by Bright who established a link between renal diseases and hypertension (**Bright, 1836**). The RAS is a peptidergic system having endocrine properties. The only known precursor of the system, angiotensinogen (Ang-N), is highly produced in the liver (**Menard et al., 1983; Deschepper, 1994; Hall, 2003**). To form the decapeptide angiotensin I (Ang I), in the circulation Ang-N is cleaved by an enzyme called renin, mainly secreted from juxtaglomerular apparatus of the kidney (**Sealey et al., 1977; Hackenthal et al., 1990; Hall, 2003; Persson et al., 2004**). In the pulmonary circulation, the inactive decapaptide Ang I is then cleaved to form the active octapeptide angiotensin II (Ang II) by the action of angiotensin converting enzyme (ACE). ACE is a membrane-bound metalloproteinase, which is predominantly expressed in high concentrations on the surface of endothelial cells in the lung (**Ng and Vane, 1967; Soubrier et al., 1993(a); Soubrier et al., 1993(b); Corvol et al., 1995; Costerousse et al., 1998; Wei et al., 1999; Hall, 2003**).

Ang II is the main active peptide of the RAS, and acts on his specific Ang II receptors and shows various actions. It is the most potent vasoconstrictor known to our body. Ang II interacts with the angiotensin receptors on vascular smooth muscle cells (VSMCs) as well as with the adrenal cortex where it stimulates the release of the enzyme aldosterone (**Quinn and Williams, 1988; Hall, 2003**). In summery, the RAS, with circulating Ang II as its active principle, is a key regulatory factor in blood pressure control and fluid balance (**Paul et al., 2006**). There are evidences available for the existence of tissue RAS's which are independent from the circulating RAS.

1.2 Components of the RAS

1.2.1 Angiotensinogen

The majority of angiotensinogen (Ang-N) is synthesized in the liver. Ang-N is not stored in hepatocytes, but is constitutively secreted into the blood stream (**Stock et al., 1995**). It is a 58,000 Da glyco-serum protein and it is the only known precursor for the synthsis of Ang II. The human Ang-N cDNA is 1,455 nucleotides long and codes for a 485-amino acid protein (**Corvol and Jeunemaitre, 1997**). Ang-N serves as a substrate for renin. Regulation of Ang-N gene is influenced by hormones, glucocorticoids and estrogens. Ang-N protein belongs to the serpine family (**Lynch and Peach, 1991**). Hepatocytes and adipocytes in the liver are the major source of Ang-N, however the synthesis of Ang-N is also reported in fibroblasts and cardiomyocytes (**Singh et al., 2008**), in liver, fat, and brain, even though in small amounts, in a variety of tissues including lung, kidney, ovary, adrenal gland (**Ohkubo et al., 1986**) heart, spinal cord, and testes (**Campbell and Habener, 1986**). Ang-N mRNA is also reported in sections of rat mesenteric vessels (**Campbell and Habener, 1987**). By using *in situ* hybridization, localization of Ang-N mRNA in the neuronal cytoplasm of coeliac ganglia and trigeminal ganglia has been reported recently (**Patil et al., 2008; Imboden et al., 2009**).

1.2.2 Renin

Renin was discovered, characterized and named in 1898 by Robert Tigerstedt (**Tigerstedt, 1898**). Renin is a proteolytic enzyme belonging to the family of the aspartic proteases (**Fig**. **1**). Mature renin contains 340 amino acids and has a mass of 37,000 Da (**Imai et al., 1983**). Renin has very high substrate specificity to Ang-N. The kidney is main

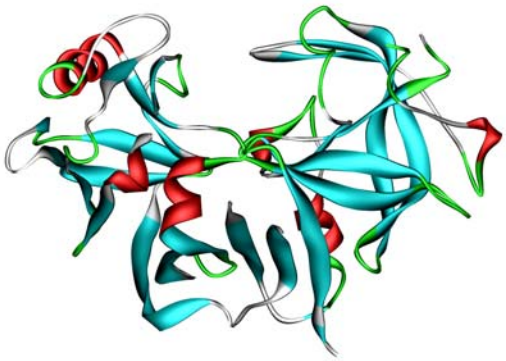

Fig. 1 Band model of the aspartic protease Renin. *(Swiss PDB, 2REN)*

source of the renin synthesis. It is produced and stored in the juxtaglomerular cells in its inactive form called pro-renin (**Persson, 2003**). Prorenin is converted to renin by the proteolytic cleavage of the prosegment in the myoepithelioid cells of the glomerular afferent artery (**Nguyen, 2008**). Renin can also be produced in larger arteries outside the kidney to support renin production within the kidney (**Fuchs et al., 2002**). Renin is a very important enzyme involved in regulation of blood pressure and several other physiological functions, hence there are lots of approaches to invent renin inhibitors and other antagonists (**Persson, 2003**).

1.2.3 Angiotensin I

In the circulation, renin cleaves Ang-N to form an inactive decapeptide Ang I. Ang I represents an intermediate product of the RAS and has a molecular mass of 1296 Da (**Fig. 2**).

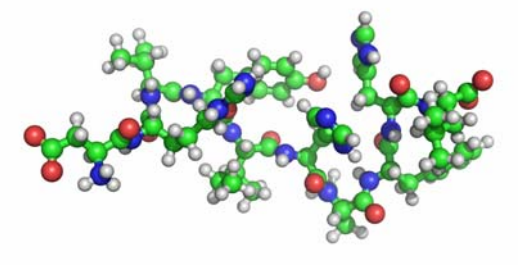

Fig. 2 Ball and stick modell of the decapeptide angiotensin I *(Swiss PDB, 1N9U)*.

Recently it is discussed that Ang I could be further cleaved by angiotensin converting enzyme typ 2 and other peptidases to Ang (1-7) (**Santos et al., 2003**). Ang (1-7) is known as a vasodilator and can act as ligand for G-proteins coupled receptors (GPCR`s). Ang (1-7) also has roles like regulation of the growth of cardiovascular muscle cells and it also seems to protect blood vessels by decreasing the growth rate of injured cells (**Tallant et al., 1999**).

1.2.4 Angiotensin converting enzyme

Skeggs and coworkers discovered the angiotensin converting enzyme (**Fig. 3**) in 1956 (**Skeggs et al., 1956**). Angiotensin converting enzyme (ACE) is produced by pulmonary and renal endothelial cells and catalyzes the conversion of the decapeptide Ang I to octapeptide Ang II, in a substrate concentration dependent manner (**Zhang et al., 2000**). The α-isoform of ACE

resides mainly on the endothelial cells of the lung, whereas the β- and γ-ACE isoforms occurs in the endothelial layers of kidney vessels. (**Balyasnikova et al., 2005**). The two amino acids histidine and leucine from the decapeptide Ang I will be cleaved by ACE as Zn^{2+} carbopeptidase. ACE can also cleave peptide hormones like bradykinin, which is nine amino acids in length and very similar in structure of Ang II. Bradykinin has vasodilatory effects and acts like histamine, by increasing the pain perception (**Shiota et al., 1992**). In patients with hypertension, ACE inhibitors are of special importance to reduce the blood pressure. There are several ACE inhibitors available like enalapril, captopril etc. It has been demonstrated that binding of an ACE inhibitor to ACE causes to activation of signal events that are likely to affect the expression of several proteins (**Fleming et al., 2005**). The therapeutic effects of ACE inhibitors could be because of the activation of a different ACE signaling cascade rather than to the changes in Ang II and bradykinin levels (**Fleming et al., 2005**).

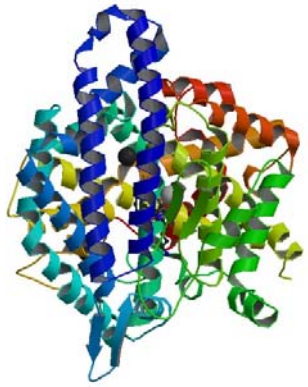

Fig. 3 Band model of the ACE *(Swiss PDB)*.

1.2.5 Angiotensin II and AT_1- and AT_2- receptors

Angiotensin II (Ang II) is an octapeptide with eight amino acids, Asp-Arg-Val-Tyr-Ile-His-Pro-Phe and represents the most important peptide of the RAS with a molecular weight of 1046 Da (**Fig. 4**). As mentioned previously, renin catalyses Ang-N to form Ang I and through pulmonary circulation ACE cleaves Ang I to form Ang II. Ang II acts on angiotensin receptor type 1 (AT_1) and angiotensin receptor type 2 (AT_2) and shows variety of actions, like thirst stimulation within different centers

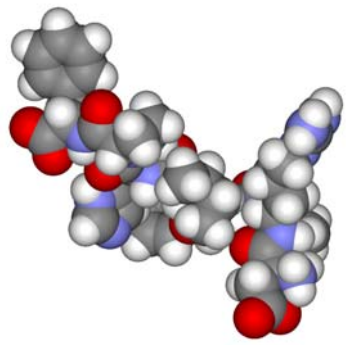

Fig. 4 Calotte model of the angiotensin II *(Swiss PDB) 1N9V)*.

in the brain (**Fitzsimons, 1998**), release of vasopressin, ACTH (adrenocortico-trophic hormone), luteinizing hormone (LH) and prolaktin from central nervous system (CNS). Action of Ang II leads to release of aldosterone and catecholamines from the adrenal medulla (**Mazzocchi et al., 1998**), which futher leads to retension of sodium and body fluid from kidneys. Ang II is the most potent vasoconstrictor known to human body, hence it greatly influences blood pressure, on heart Ang II exerts positive chronotopic effect (**Li et al., 1996**). Ang II is synthesized in circulation as well as intracellularly (**Paul et al., 2006, Singh et al., 2008**). Interaction between Ang II and sympathic nervous system (SNS) has been discussed (**Reid, 1992**). Localization of Ang II in the neurons of coeliac ganglia innervating mesenteric resistance arteries and in the neurons of trigeminal ganglia has been reported recently (**Patil et al., 2008, Imboden et al., 2009**). Ang II is also involved in developmental processes, cell proliferations, tissue regeneration and apoptosis, via its action on AT_2 receptors (**Fitzsimons, 1998**).

As discussed above, the seven transmembrane receptors AT_1 and AT_2 mediate the actions of Ang II. The rat and mouse AT_1 receptors exist as two distinct subtypes, termed AT_{1A} and AT_{1B}, that are 95% identical in their amino acid sequences. Although these two subtypes of AT_1 receptor differ in their tissue distribution, chromosomal localization, genomic structure, and transcriptional regulation, they have similar ligand binding and activation properties. These gly-coproteins share about 30% sequence homology and AT_1 is expressed by two similar AT_{1A} and AT_{1B} genes in rodents (**de Gasparo et al., 2000**). The signals mediated by AT_1 receptors are further conducted via small G-proteins, phospholipase C, A, B, ending in Ca^{2+} dependent responses or in Ras/Raf-MAP-kinase pathways, to switch genes on/off for neuromodulations. In contrast only few is known about the signalling pathway followed by AT_2 activation, but it was discovered, that in this cascades G-proteins and MAP-kinases are involved as well (**de Gasparo et al., 2000**).

Ang II is also known for his local action within cardiac tissues to influence protein synthesis and cellular growth (**Lindpainter and Ganten, 1991; Baker et al., 1992; Dostal and Baker, 1992; Dostal et al., 1992, 1996**). The AT_2 receptor seems to open a delayed rectifier potassium channel at least in hypothalamic neuronal tissues (**Kang et al., 1994, 1995**), to close a T-type Ca21 channel (**Buisson et al., 1992, 1995**), to suppress tissue and cellular growth (**Nakajima et al., 1995; Meffert et al., 1996; Munzenmaier and Greene, 1999; Stoll et al., 1995; Tsuzuki et al., 1996a,b**), to induce (neuronal) cell differentiation (**Laflamme et al., 1996; Meffert et al., 1996; Gallinat et al., 1997; Stroth et al., 1998; Coté et al., 1999; Gendron et al., 1999**) and to support apoptosis (**Yamada et al., 1996; Chamoux et al., 1999; Gallinat et al., 1999**).

By using single cell RT-PCR, it has been demonstrated that in the adult rat about 50% of cardiomyocytes contain the AT_1 receptor, whereas only about 10% cardiomyocytes were carring the AT_2 receptor (**Busche et al., 2000**). AT_1 and AT_2 receptor expression in various regions of the brain has been determined, and its age dependence in 2- and 8-week-old rat brains. Although AT_1 receptor expression did not show marked age dependence, the AT_2 receptor showed a significant decrease from 2 to 8 weeks of age (**Saavedra, 1992; de Gasparo et al., 2000**). Even though it is well known that the AT_1 receptor, present in vascular smooth muscle, the presence of the AT_2 receptor in the vasculature in vivo was seen only by autoradiography. The AT_1 receptors are abundantly expressed in small cortical arteries, glomeruli, proximal tubules, and interstitium, while the AT_2 receptor are also expressed in the kidney (**de Gasparo et al., 2000**).

1.2.6 Alternate pathways for Ang II formation

There are different alternative pathways of Ang II generation in the brain (**Fig. 5**).

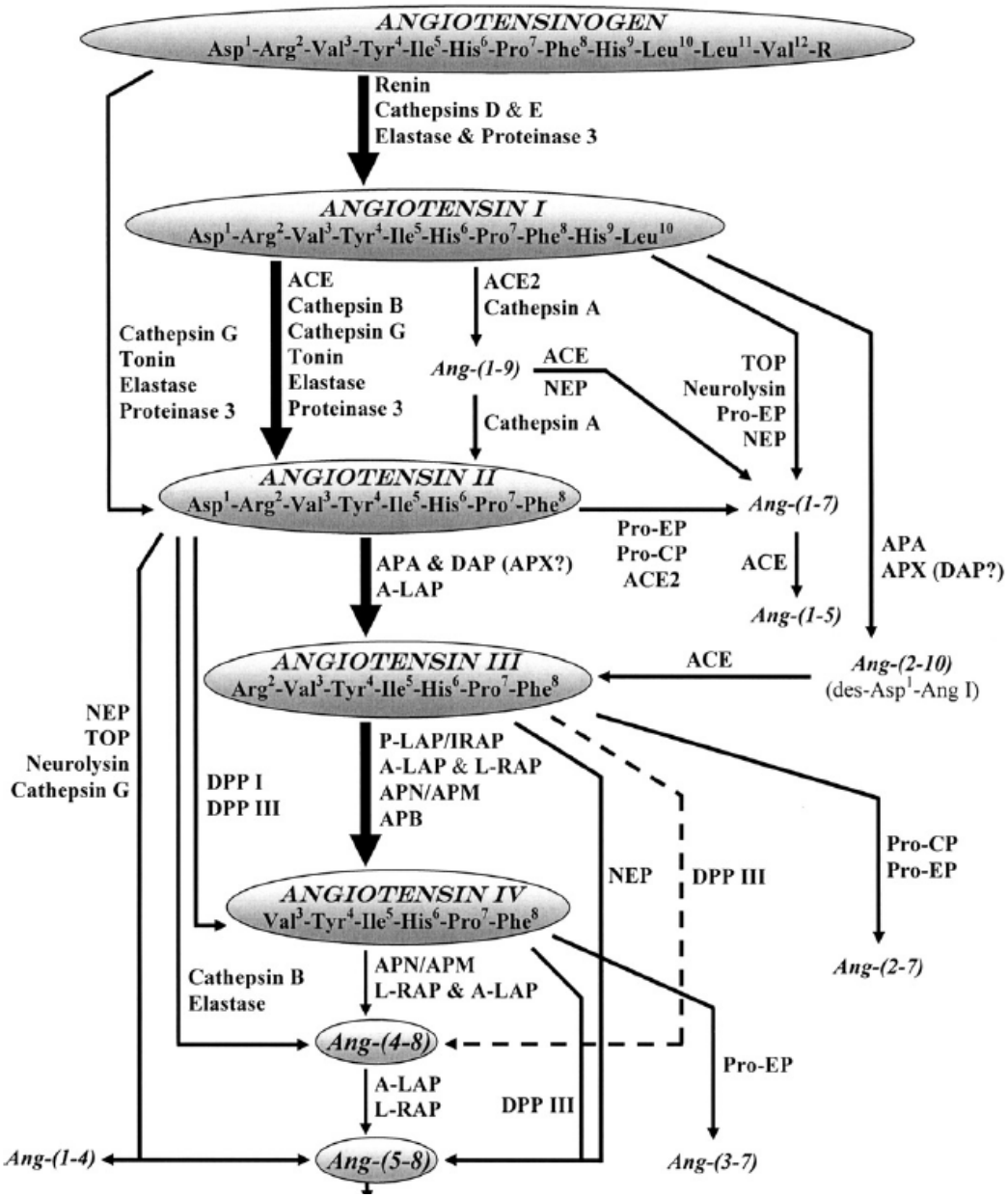

Fig. 5 Summary of alternative pathways involved in this processing mechanism of RAS in the brain. Abbreviations used: ACE - angiotensin converting enzyme; ACE2 - human homolog of angiotensin converting enzyme; APA - aminopeptidase A; A-LAP - adipocyte derived leucine-aminopeptidase; L-RAP - leukocyte-derived arginine aminopeptidase; NEP - neutral endopeptidase; TOP - thimet endopeptidase; Pro-EP - prolyl-endopeptidase; Pro-CP - prolyl-carboxypeptidase; APX - aminopeptidase X; DAP - aspartyl aminopeptidase; P-LAP/IRAP - placental leucine-aminopeptidase/ insulin-regulated aminopeptidase; APN/APM - aminopeptidase N/M; APB- aminopeptidase B; DPP I - dipeptidyl peptidase I; DPP III - dipeptidyl peptidase III. Numbering of amino acid residues in all fragments is based on the numbering in angiotensinogen. Larger sized arrows indicate the “classical” metabolic pathways for angiotensin peptides (**Karamyan and Speth, 2007**).

1.3 Overview of mammalian nervous system

The mammalian nervous system has two main divisions: the central nervous system (CNS) and the peripheral nervous system (PNS) see **Fig. 6**.

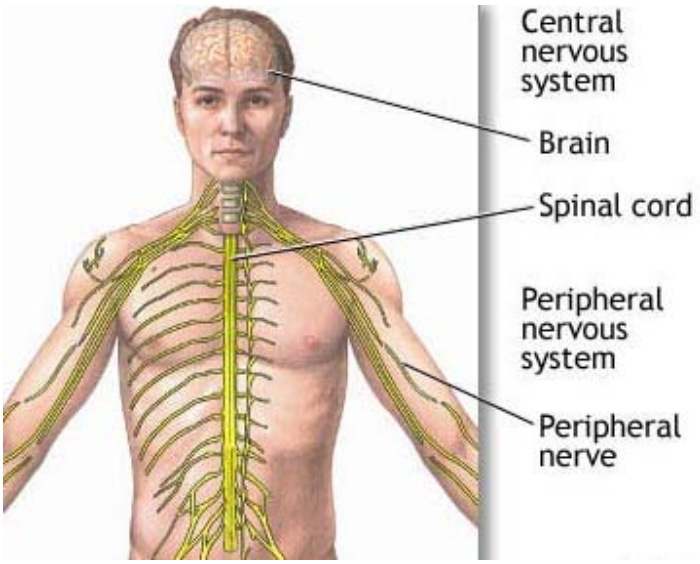

Fig. 6 Human nervous systems (ADAM)

The brain and spinal cord are the two parts of the CNS. Brain stores and process the information in memory centers and is also involved in sending signals to different parts of the body via the spinal cord. The CNS is highly responsible for processing information and issuing commands from different sense organs. The PNS is made up of nerves, associated nervous tissues as well as ganglia. Ganglia are a group of nerve tissue containing several nerve cell bodies and synapses (nerve endings). PNS forms a large communication network of sensory and motor pathways. Somatic nervous system contains sensory neurons those transmit signals from peripheral sensory receptors to CNS, also includes motor neurons which transmit nerve signals to skeletal muscles. As many of the actions of the Somatic nervous system are under conscious control it is also known as voluntary nervous system. The autonomic nervous system (ANS) is also know as involuntary nervous system because this system is active without out consciousness. ANS is further divided into parasympathetic nervous system (PSNS) and sympathetic nervous system

(SNS). PSNS is involved in "rest and digest" action while SNS is involved in "fight or flight response". PSNS contains acetylecholine as a neurotransmitter, some of the actions of PSNS involves - reducing heart rate, constrict the pupils, dilatation of blood vessels, etc. Norepinephrine is the main neurotransmitter used in the SNS. Stimulation of SNS leads to increase in heart rate, dilatation of pupils, vasoconstriction, etc indicating that PSNS and SNS have opposite actions (**Kent, 2000**).

1.4 RAS and the peripheral nervous system

1.4.1 RAS and the sympathetic coeliac ganglia

Sympathetic coeliac ganglia are defined as any of the largest and highest group of sympathetic prevertebral ganglia, on the upper part of the abdominal aorta on either side of the celiac artery, containing the sympathetic neurons whose unmyelinated postganglionic axons innervate the stomach, liver, gallbladder, spleen, kidney, small intestine, and the ascending and transverse colon, also called as semilunar ganglion, solar ganglion (**The American Heritage Medical Dictionary, 2007**). Blood pressure control is predominantly regulated by the SNS and the RAS. Circulating Ang II in the blood has been reported to interact with the SNS at different sites and to directly stimulate the sympathetic activity (**Lewis and Reit, 1965; Reid, 1992; Campese et al., 2002; Paul et al., 2006**). Furthermore, it has been proposed that circulating Ang II functions at synaptic nerve endings for example with blood vessels by enhancing norepinephrine release and thereby facilitating sympathetic neurotransmission (**Levens et al., 1981; Suter and Coote, 1987; Paul et al., 2006**). The presence of RAS components in circulation and at tissue level has been reported (**Paul et al., 2006)**, but the question of endogenous RAS in the neurons of the sympathetic coeliac ganglia has not been addressed so far. Sympatheitc coeliac ganglia are know to innervate mesenteric resistance arteries (**Hsieh et al.,**

2000), resistance arteris are major effector organ of cardiovascular system, and have been known as precapilary vessels that contribute significantly both passively to resting resistance and actively to the blood flow control during altered demands (**Christensen and Mulvany, 2001**). We tested our hypothesis of an endogenous synthesis of Ang II in neurons of sympathetic coeliac ganglia and further their angiotensinergic innervation with mesenteric resistance blood vessels under normal physiological conditions, in normotensive rat and human tissues.

1.4.2 RAS and the trigeminal ganglia

The trigeminal ganglion, a cranial analog of the dorsal root ganglia in the peripheral nervous system (**Fig. 7**), is the site of primary sensory neurons involved in the regulation of nociception, thermoreception, proprioception and mechanoreception in the facial skin, eye, nasal and oral cavities, teeth and periodontal tissue and vibrissae (**Lazarov, 2002**) and provides sensory afferents to the cerebral blood vessels (**Dostrovsky et al., 1991**).

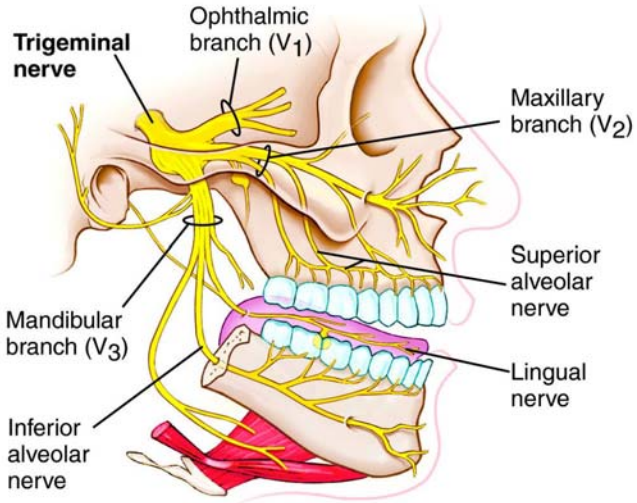

Fig. 7 Trigeminal ganglia (Mosby's Medical Dictionary, 2009)

A multitude of transmitters and neuromodulators have been described in the trigeminal ganglion and its primary afferent neurons exhibit pathway-specific patterns of neurochemical expression and transmitter colocalization (**Lazarov, 2002**). However, the presence and specific localization of RAS components and role of Ang II has not been studied, in spite of the association of Ang II with central and peripheral sensory systems (**Buck et al., 1982; Tsutsumi and Saavedra, 1991; Wu et al., 2000; Burkhalter et al., 2001**) and its proposed role in the regulation of pain (**Pelegrini-da-Silva et al., 2005; Fusayasu et al., 2007**), cerebrovascular inflammation (**Zhou et al., 2005; Ando et al., 2004**) and migraine (**Schrader et al., 2001; Tronvik et al., 2003**).

Here in this study, we addressed the issue of the possible formation and localization of Ang II in the trigeminal ganglion by determination of Ang-N-, renin-, ACE- and cathepsin D-mRNA by qRT-PCR in rat trigeminal ganglia. By using *in situ* hybridization of the Ang II precursor Ang-N mRNA in rat trigeminal ganglia and the expression of Ang II by immunocytochemistry in rat as well as in human trigeminal ganglia. The rat and human trigeminal extracts were investigated by high performance liquide chromatography and radioimmunoassy (HPLC-RIA) for the presence of Ang II and its metablites. Several studies substantiated the role of substance P in the regulation of sensory transmission in the trigeminal ganglion (**Edvinsson, 1991; Harrison and Geppetti, 2001; Lazarov, 2002; Hou et al., 2003**) and the involvement of Ang II in the regulation of Substance P release (**Diz et al., 1986; Kopp et al., 2003**). Hence, we attempted to determine the possible formation and localization of Ang II in the neurons of trigeminal ganglion and colocalization with Substance P in the same neurons.

1.4.3 RAS and the dorsal root ganglia

Spinal ganglia are located on the posterior root of each spinal nerve, composed of unipolar nerve cell bodies of the sensory neurons (**Fig. 8**), also called as dorsal root ganglion (DRG)

(**Dorland's Medical Dictionary, 2007**). In the nervous system, Ang II is involved in the regulation of multiple functions, including sympathetic and hormonal control (**Tsutsumi and Saavedra, 1991; Saavedra, 1992; Saavedra, 1999; Saavedra, 2005; Bader and Ganten, 2008**). In addition, there is substantial evidence that Ang II contributes to regulate the central and peripheral components of sensory systems (**Buck et al., 1982; Saavedra, 1992; Wu et al., 2000; Burkhalter et al., 2001; Schrader et al., 2001; Tronvik et al., 2003; Pelegrini-da-Silva 2005; Fusayasu et al., 2007**).

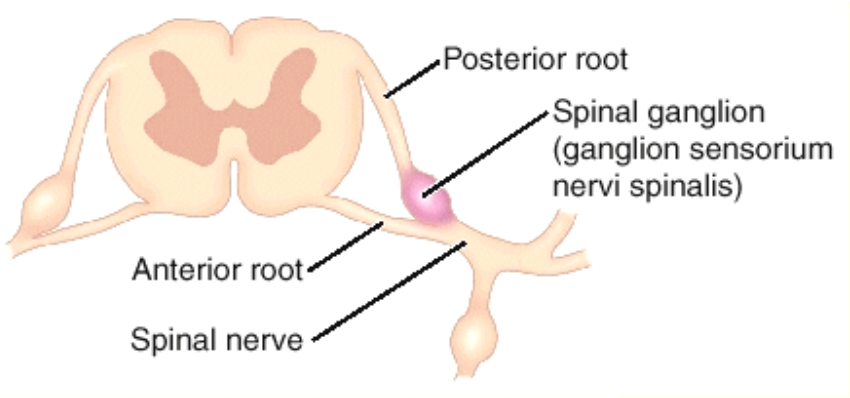

Fig. 8. Dorsal root ganglia (Dorland's Medical Dictionary, 2007)

Ang II may exert local regulatory effects in DRG (**Wu et al., 2000; Kawasaki et al., 2003**), structures containing cell bodies of primary afferent neurons that are involved in the regulation of blood pressure and in many sensory modalities (**Gibson et al., 1984; Wilmalawansa, 1996**). Neuronal localization of RAS components has been reported in bilaterally ovariectomized rat DRG (**Chakrabarty et al., 2008**), in normotensive rat and human sympathetic coeliac ganglia (**Patil et al., 2008**) as well as in normotensive rat and human sensory trigeminal ganglia (**Imboden et al., 2009**). Substance P is colocalized with Ang II in neurons of the trigeminal ganglia (**Imboden et al., 2009**), whereas other studies show that Substance P release is modulated by Ang II (**Diz et al., 1986; Kopp et al., 2003**). Here we addressed the intracellular

localization of RAS components in rat and human DRG, and the relationship of Ang II and Substance P expression in these sensory tissues.

1.4.4 RAS and the heart

According to many reports localization and function of RAS components in the heart is still an issue of controversies, because of the circulating RAS and local generation of Ang II (**Singh et al., 2008; Paul et al., 2006**). The RAS and the SNS both contribute in the progress and maintenance of high blood pressure, as well as interaction between circulating RAS and SNS has also been reported (**Rupp and Jäger, 2001**). The heart is innervated by sympathetic and parasympathetic nerves (**Kukanova and Mravec, 2006**), the cardiac nervous system also influences heart rate, coronary blood flow, arterial and ventricular behavior and is essential for proper function of the cardiac valve systems (**Roper and Taylor, 1982**). The existence of an endogenous angiotensinergic system in the neurons of rat and human sympathetic coeliac ganglia has been reported recently (**Patil et al., 2008**). The sensory neurons of the trigeminal ganglia also synthesize Ang II (**Imboden et al., 2009**). Although the RAS is widely studied, the specific localization of RAS components in the heart is still missing. In our current work we provide strong evidence for the existence of novel, local angiotensinergic system in the rat and human heart, especially in neurons of intracardiac ganglia with the atria. By using different molecular and cell biological research tools like quantitative real time PCR, HPLC-RIA, *in situ* hybridization and immunocytochemistry, we investigated the existence of Ang II components in the neurons of intracardiac ganglia and their processes in different parts of the rat and human heart. We also studied the probable colocalization of the noradrenergic and the angiotensinergic systems in the neurons of intracardiac ganglia in the atria and their processes in the heart.

2. Angiotensinergic neurons in sympathetic coeliac ganglia innervating rat and human mesenteric resistance blood vessels

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Angiotensinergic neurons in sympathetic coeliac ganglia innervating rat and human mesenteric resistance blood vessels

Jaspal Patil [a], Eva Heiniger [a], Thomas Schaffner [b], Oliver Mühlemann [a], Hans Imboden [a,*]

[a] *Institute of Cell Biology, University of Bern, Bern, Switzerland*
[b] *Institute of Pathology, University of Bern, Bern, Switzerland*

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Abstract

In contrast to the current belief that angiotensin II (Ang II) interacts with the sympathetic nervous system only as a circulating hormone, we document here the existence of endogenous Ang II in the neurons of rat and human sympathetic coeliac ganglia and their angiotensinergic innervation with mesenteric resistance blood vessels. Angiotensinogen - and angiotensin converting enzyme-mRNA were detected by using quantitative real time polymerase chain reaction in total RNA extracts of rat coeliac ganglia, while renin mRNA was untraceable. Cathepsin D, a protease responsible for cleavage beneath other substrates also angiotensinogen to angiotensin I, was successfully detected in rat coeliac ganglia indicating the possibility of existence of alternative pathways. Angiotensinogen mRNA was also detected by *in situ* hybridization in the cytoplasm of neurons of rat coeliac ganglia. Immunoreactivity for Ang II was demonstrated in rat and human coeliac ganglia as well as with mesenteric resistance blood vessels. By using confocal laser scanning microscopy we were able to demonstrate the presence of angiotensinergic *synapses en passant* along side of vascular smooth muscle cells. Our findings indicate that Ang II is synthesized inside the neurons of sympathetic coeliac ganglia and may act as an endogenous neurotransmitter locally with the mesenteric resistance blood vessels.

Keywords: RAS; Angiotensin II; SNS; *Synapses en passant*

1. Introduction

Blood pressure control is predominantly regulated by the sympathetic nervous system (SNS) and the renin angiotensin system (RAS). The octapeptide angiotensin II (Ang II) is the effector component of the RAS and well known for his potent vasoconstriction action, which leads to increased blood pressure and release of aldosterone from the adrenal cortex. Local Ang II production at tissue level has been discussed in many different tissues [1,11–13]. Circulating Ang II in the blood has been reported to interact with the SNS at different sites and to directly stimulate the sympathetic activity [1,3,6,15]. Furthermore, it has been proposed that circulating Ang II functions at synaptic nerve endings for example with blood vessels by enhancing norepinephrine release and thereby facilitating sympathetic neurotransmission [1,4,5]. Controversially, it is also reported in some studies that Ang II has no or nearly negligible effect on the enhancement of neurotransmission at the postjunctional level [25,26]. Immunoreactive Ang II release and angiotensinogen mRNA expression in cardiac sympathetic ganglia has been studied [23]. The presence of RAS components in circulation and at tissue level has been reported [1], but the question of endogenous RAS in the neurons of sympathetic coeliac ganglia has not been addressed so far. We tested our hypothesis of an endogenous synthesis of Ang II in neurons of sympathetic coeliac ganglia and further their angiotensinergic innervation with mesenteric resistance blood vessels under normal physiological conditions, in normotensive rat and human tissues.

* Corresponding author. University of Bern, Institute of Cell Biology, Baltzerstrasse 4, 3012 Bern, Switzerland. Tel.: +41 31 631 48 99; fax: +41 31 631 38 66.
E-mail address: hans.imboden@izb.unibe.ch (H. Imboden).

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2. Methods

2.1. Tissue preparation

Wistar Kyoto (WKY) male rats were purchased (8-week-old, approximately 200 g body weight) from the Central Animal Facilities of the University of Bern. According to the European Communities Council Directive of 24 November 1986 (86/609/EEC) and in accordance with Animal Protocols approved by the Animal Care and Use Committee, NIMH, NIH, USA, adequate measures were taken to minimize pain or discomfort. Rats were anaesthetized intraperitoneally with 100 mg/kg thiopentane sodium (Pentothal, Abbott Laboratories, Germany) and were perfused transcardially with 150 ml ringer solution containing 1000 U heparin at 37 °C followed by 300 ml 2% freshly prepared formaldehyde at 4 °C. Coeliac ganglia and mesenteric resistance blood vessels were carefully removed and incubated by immersion fixation in 2% formaldehyde for 28 h at 4 °C. Later, coeliac ganglia and mesenteric blood vessels were immersed for 14 h in phosphate-buffered saline containing 18% sucrose at 4 °C. These tissues were frozen in isopentane at −50 °C and cryosections of 30 μm thickness were subsequently used free floating for immunocytochemistry. After perfusion and immersion fixation, both rat tissues were also embedded in paraffin to perform additional experiments. 6 μm thick paraffin sections were used for immunocytochemical and for *in situ* hybridization. Human coeliac ganglia and mesenteric resistance blood vessels were procured from human individuals for whom a permit for clinical autopsy (informed written consent by next of kin) had been obtained according to state law, in accordance with the Code of Ethics of the World Medical Association (Declaration of Helsinki). Human coeliac ganglia were fixed by immersion in freshly prepared 2% formaldehyde for 3 days and then used for cryosectioning or embedded in paraffin.

2.2. RNA isolation and quantitative realtime RT-PCR (qRT-PCR)

Fresh rat coeliac ganglia were dissected as mentioned above and instantly transferred into RNA later (Ambion), frozen in liquid nitrogen, and then processed for total RNA extraction (Ambion). RNA integrity was confirmed for each sample on the Agilent Bioanalyzer using the RNA 6000 Nano kit (Agilent Technologies). 1 μg of total RNA was reverse transcribed using Superscript II (Invitrogen) and random hexamers according to the manufacturer's protocol. For realtime PCR, reverse transcribed material corresponding to 40 ng RNA was amplified with the TaqMan assays described below in 25 μl Universal PCR Master Mix, No AmpErase UNG on the SDS 7000 (Applied Biosystems) using the standard thermal protocol.

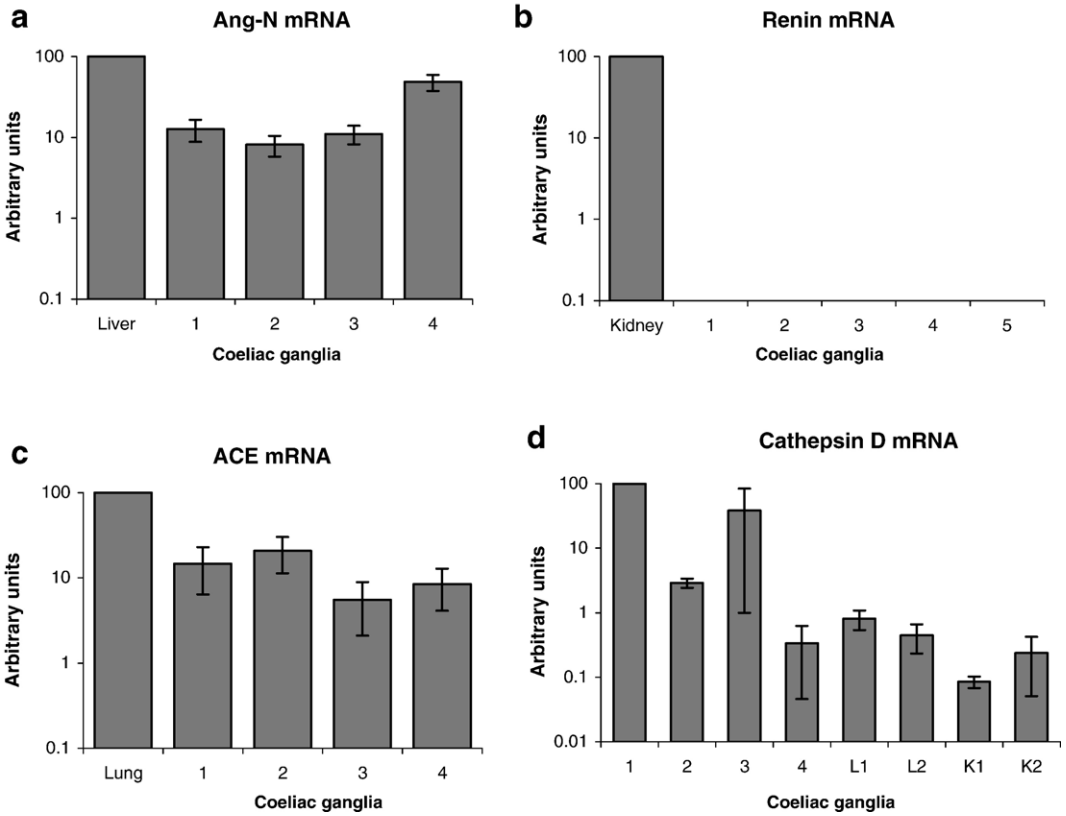

Fig. 1. Determination of relative levels of Ang-N-, renin-, ACE- and cathepsin D- mRNA by qRT-PCR in rat coeliac ganglia. Reverse transcribed total RNA extracts of rat coeliac ganglia from different rats were tested by qRT-PCR for the presence of Ang-N mRNA (a), for renin mRNA (b), for ACE mRNA (c) and for cathepsin D mRNA (d). Total RNA from liver (a), kidney (b), and lung (c) were used as a reference, respectively and liver (L1, L2), kidney (K1, K2) samples were used as reference for cathepsin D expression. All relative mRNA values were normalized to 18S rRNA levels. Average values and standard deviations of 4 qRT-PCR measurements are shown.

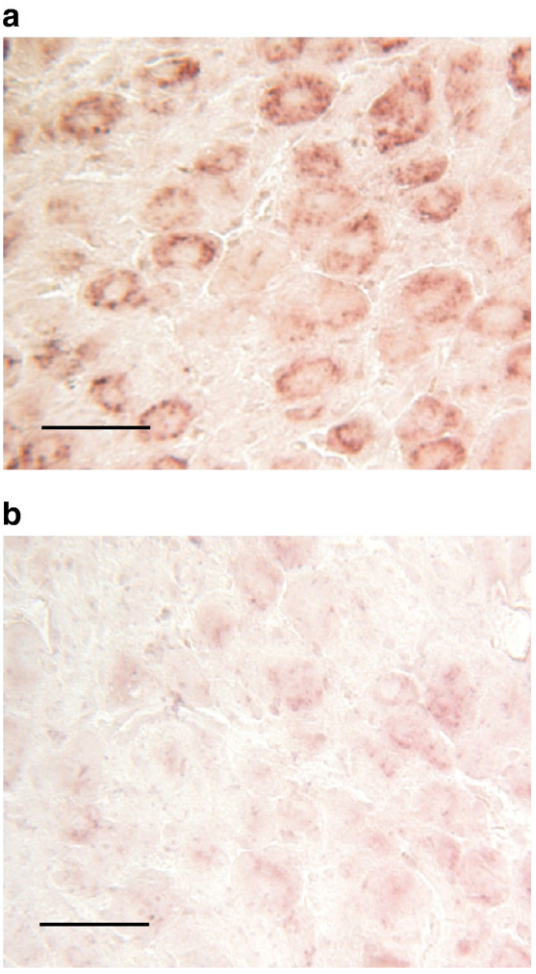

Fig. 2. *In situ* hybridization with rat coeliac ganglion for detection of Ang-N mRNA, 6 μm thick consecutive paraffin sections were processed as described in methods. a) antisense probe. b) control with sense probe, revealing no staining. Bar: 50 μm.

Average values and standard deviations of relative mRNA levels of each sample, normalized to relative 18S rRNA levels, are from four measurements and were calculated using the relative differences.

The following TaqMan assays were used for qRT-PCR at a final concentration of 250 nM TaqMan probe and 900 nM of each primer:

Angiotensinogen Forward primer 5′-CACGACTTCCTGACTTGGATAAAGA-3′; reverse primer 5′-CTGCGGCAGGGTCAGA-3′; TaqMan probe 5′-FAM-CTCCTCGGGCCATCCG-MGB-3′; manufactured as Assays-by-Design (RATG-EJ3) by Applied Biosystems.
Renin Assay-on-demand Rn00561847_m1 from Applied Biosystems.
ACE Assay-on-demand Rn00561094_m1 from Applied Biosystems.
Cathepsin D Assay-on-demand Rn00592528_m1 from Applied Biosystems.
18S rRNA Predeveloped Assay Reagent 431-9413E from Applied Biosystems.

2.3. In situ hybridization

2.3.1. DIG-labelled RNA probe preparation

By using an appropriate cDNA template for Ang-N [14], a 403 bp long fragment corresponding to nucleotides 221–623 was generated by digestion with restriction enzymes Eco RV and Bam HI. The obtained fragment was cloned between Stu I and Bam HI into pBluescript I KS+ (Stratagene). Digoxigenin-labelled probes were prepared using the DIG-RNA-labelling Mix (Roche) according to the manufacturer's protocol. T7 RNA polymerase was used to generate antisense riboprobe using the Hind III linearized template, and the sense strand (used as a control) was generated by T3 RNA polymerase using the same template linearized with Bam HI. The RNA concentration was estimated comparing dot-blot tests and NanoDrop measurements. Filter hybridization with RNA extracts from coeliac ganglia (RNaqueous-4PCR kit from Ambion) was used for binding tests.

2.3.2. Procedure for in situ hybridization

For *in situ* hybridization, 6 μm thick paraffin sections were rehydrated in a graded alcohol series (2 times Xylol for 10 min, EtOH 100% (2 times), 96%, 70%, 50%, each step 5 min) using DEPC-treated H_2O for the dilution of all reagents and solutions. The sections were equilibrated in proteinase K buffer (100 mM Tris, 50 mM EDTA, pH 7.5) for 5 min and then treated with proteinase K (19 μg/ml) at 37 °C for 2 min, after a wash with DEPC-treated H_2O. Following a wash in DEPC-water, sections were post fixed with freshly prepared 4% formaldehyde for 5 min, followed by two subsequent washes in DEPC-water for 5 min each. Sections were then incubated in prehybridization solution (SIGMA) at 45 °C for 2 h, followed by incubation with heat denatured sense and antisense riboprobes (5–10 ng/μl) in 30 μl hybridization mix (SIGMA) for 48 h at 45 °C in a humid chamber (saturated with 2× SSC). Subsequently, the sections were incubated with 2× SSC for 30 min at room temperature, followed by 1 h in 2× SSC at 45 °C and 1 h in 0.1× SSC at 45 °C. Sections were equilibrated for 5 min with buffer 1 (100 mM Tris, 150 mM NaCl, pH 7.5), then incubated with alkaline phosphatase-coupled anti-digoxigenin antibody (Roche) 1:500 diluted in buffer 2 (10× blocking solution diluted with buffer 1) for 2 h at room temperature, followed by two 5 min washes with buffer 1. Finally, after 5 min equilibration with buffer 3 (100 mM Tris, 100 mM NaCl, 50 mM $MgCl_2$, pH 9.5), the color reaction containing NBT and BCIP in buffer 3 was completed according to manufacturer's protocol (Sigma).

2.4. Immunocytochemistry

For immunocytochemical incubations, a protein G purified murine monoclonal antibody against Ang II was used at a concentration of 0.3 μg/ml in buffer solution. This is a self-generated monoclonal antibody against the synthetic peptide Ang II. The specificity for this monoclonal antibody against Ang II has been previously documented and it produced the same staining as a polyclonal antibody against Ang II in rat adrenal glands [2]. Goat anti-mouse immunoglobulins (GAM ^{Cy3};

Jackson ImmunoResearch) pre-incubated with rat serum prior to immunocytochemistry were used as secondary antibodies. Sections were incubated free floating for 36 h at 4 °C with the primary antibody, washed and incubated with the secondary antibody for 90 min at room temperature and counterstained with DAPI. After washing, cryosections were mounted on gelatine-coated slides and air-dried. The stained sections were embedded with Glycergel (DAKO) and cover-slipped. The same procedure as for free floating cryosections has been used for paraffin sections mounted on glass slides. Pictures were taken by a normal fluorescent light microscope (LEICA DM 6000B) using a digital camera or by a confocal laser scanning microscope (LEICA SP2 with AOBS).

3. Results

3.1. Quantitative real time RT-PCR

We investigated the presence of key components of the RAS, in order to demonstrate the synthesis of Ang II in the coeliac ganglion. Both, Ang-N mRNA (Fig. 1a) and ACE mRNA (Fig. 1c) were readily detected in total RNA extracts of rat coeliac ganglia by qRT-PCR, whereas no renin mRNA was traced (Fig. 1b). To investigate the possibility of existence of an alternate pathway for Ang II production we studied cathepsin D mRNA (Fig. 1d). RNA from tissues with previously reported high levels of the respective mRNA were used as references, all mRNA values were normalized to 18S rRNA.

3.2. In situ hybridization

At single cell resolution, Ang-N mRNA was detected in nearly all of the neurons in the rat coeliac ganglion by *in situ* hybridization with the antisense probe (Fig. 2a), but not with the sense probe used as a control (Fig. 2b).

3.3. Immunocytochemistry

Immunocytochemical studies using our previously characterized monoclonal antibody against Ang II [2], detected Ang II immunoreactivity in both the neuronal cytoplasm as well as in their projections of rat coeliac ganglia (Fig. 3a). In a transverse section of mesenteric resistance blood vessels, we can find strong angiotensinergic innervation (Fig. 3b). With confocal laser scanning microscopy (LSM), we were able to illustrate angiotensinergic *synapses en passant* (sympathetic varicosities) alongside the outer layer of vascular smooth muscle cells from rat (Fig. 3c–d). Similarly, by immunocytochemical studies we could also show the presence of Ang II in coeliac ganglia neurons and in their projections and with

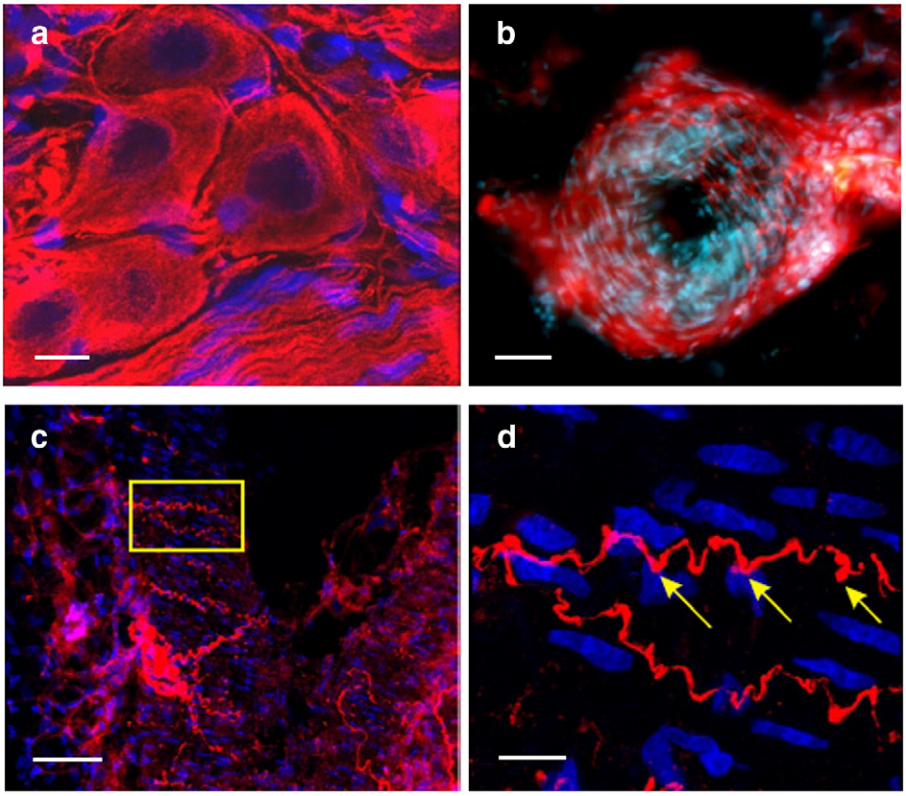

Fig. 3. Ang II immunoreactivity with rat coeliac ganglion, mesenteric resistance blood vessels and the presence of angiotensinergic *synapses en passant*. Cryosections of 30 μm thickness were incubated with our murine monoclonal anti Ang II antibody [2], stained with goat anti-mouse (GAM^{Cy3}) in red and DAPI in blue for nuclei. a) Ang II staining in the cytoplasm of neurons and their projections within the sympathetic coeliac ganglion. Bar: 10 μm. b) Ang II staining with a mesenteric resistance blood vessel, transverse section. Bar: 50 μm. c) Ang II staining with mesenteric resistance blood vessels, longitudinal section. Bar: 50 μm. d) Zoomed inset of panel c. Visualization of angiotensinergic *synapses en passant* (indicated by arrows). Bar: 10 μm. See supplemented material for movies.

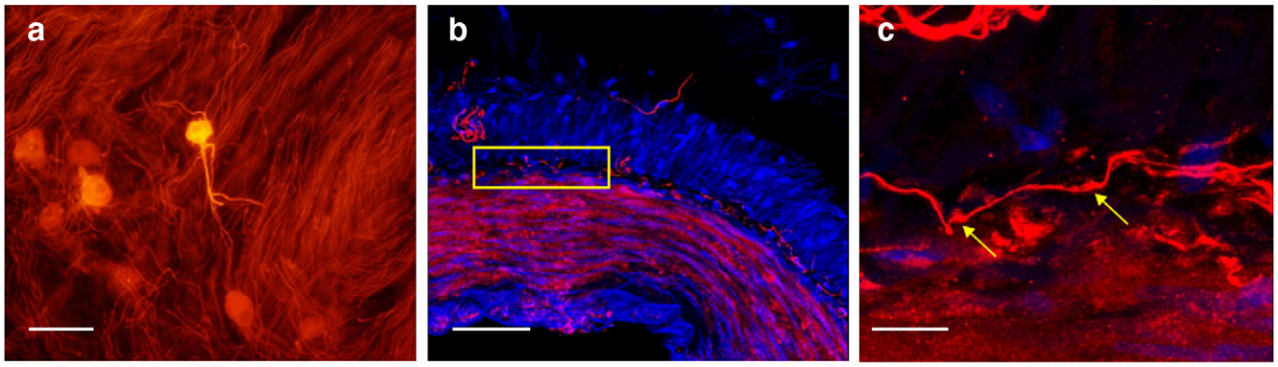

Fig. 4. Ang II immunoreactivity with human coeliac ganglion, mesenteric resistance blood vessels and presence of angiotensinergic *synapses en passant*. Cryosections of 30 μm thickness were stained as in Fig. 3. a) Ang II staining in the cytoplasm of neurons and their projections within the sympathetic coeliac ganglion. Bar: 50 μm. b) Ang II staining with mesenteric resistance blood vessels. Bar: 50 μm. c) Zoomed inset of panel b. Visualization of angiotensinergic *synapses en passant* (indicated by arrows). Bar: 10 μm. See supplemented material for movies.

mesenteric resistance blood vessels from human postmortem tissue (Fig. 4a–c).

4. Discussion

Previous studies have established the presence of Ang II in circulation and at tissue level [1]. We have investigated a novel concept of synthesis of Ang II in neurons of the sympathetic coeliac ganglion. In the current study, our results demonstrate the expression of Ang-N mRNA and ACE mRNA in neurons of rat coeliac ganglia and the presence of Ang II in the cytoplasm of these neurons and in their projections, which suggests intracellular production of Ang II. The Ang-N mRNA was quantitatively detected by qRT-PCR by using liver samples as a reference (Fig. 1a). Lung samples were used as a reference for ACE mRNA detection (Fig. 1c). The Ang-N mRNA and ACE mRNA have indicated minor physiological variation in individual samples of coeliac ganglia. We could not detect any renin expression in these ganglia (Fig. 1b), the enzyme necessary for cleaving Ang-N into the Ang I precursor of angiotensin II [1]. This would be similar to renin studies in cardiac tissue, where strong evidence exists for uptake of extracellular renin from the circulation [1,7–9]. The presence of renin in cardiac tissue is still a matter of controversial debate but some investigators have provided evidence for presence of cardiac renin mRNA by Northern blot analysis [16], solution hybridization assays [17], and by qRT-PCR [18] in different species. The amount of renin mRNA detected in all different species by different experiments was very low, moreover very high concentration of total mRNA was needed to be used to bring the renin signal to level of detection. On the otherside, some researchers were not able to find local renin mRNA expression in cardiac tissue and claimed that the local renin mRNA expression results were based on artifacts [19]. This investigation was supported by findings that cultured cardiomyocytes or fibroblasts did not synthesize renin [20,21]. Hence we postulate that coeliac ganglion neurons take up circulating renin to support synthesis of endogenous Ang II or renin can be replaced by the presence of other unknown enzyme(s) [10]. Cathepsin D is such a candidate protease, an enzyme which is capable of cleaving apart other proteins, Ang-N into angiotensin I [24]. In our experiments, detected expression levels of Cathepsin D (Fig. 1d) in coeliac ganglia were high in comparison with expression in rat liver and kidney. Detection of Cathepsin D mRNA with coeliac ganglia indicates the existence of possible alternate pathways for Ang II synthesis in these sympathetic neurons. Ang-N mRNA was additionally identified at cellular level in nearly all neurons of coeliac ganglion by *in situ* hybridization, with the antisense probe (Fig. 2a), but not with the sense probe used as a control (Fig. 2b).

Fibers originating from neurons of sympathetic coeliac ganglia are known to innervate mesenteric resistance blood vessels [22]. By using our preestablished monoclonal antibody against Ang II [2], we have detected strong immunoreactivity for Ang II in the cytoplasm of neurons and their projections of rat coeliac ganglia (Fig. 3a) and further angiotensinergic innervation with mesenteric resistance blood vessels (Fig. 3b–c). We

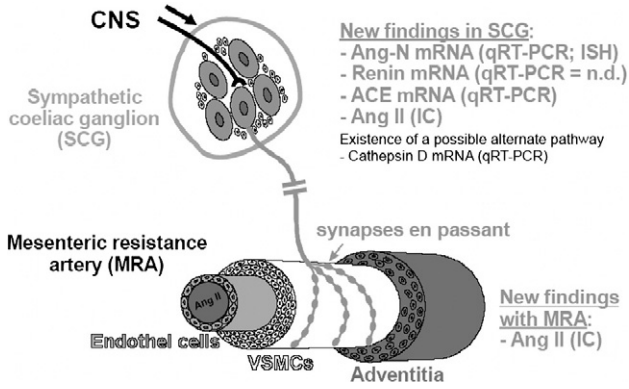

Fig. 5. Schematic presentation of the new angiotensinergic system in neurons and their projections within the rat and human sympathetic coeliac ganglia. Innervation of neurons within sympathetic coeliac ganglion by central nervous system (CNS) and their fibres innervating mesenteric resistance blood vessels. This figure indicates the new findings, existence and synthesis of endogenous angiotensinergic system components in coeliac ganglion, studied by different techniques, like quantitative real time PCR (qRT-PCR), *in situ* hybridization (ISH) and immunocytochemistry (IC). Mesenteric resistance artery showing different cell layers, including presence of angiotensinergic *synapses en passant* on vascular smooth muscle cells (VSMCs). Untraceable results are indicated by n.d. (not detected).

could successfully find the same immunoreactivity for Ang II in the neurons of human coeliac ganglia (Fig. 4a) and their projections with mesenteric resistance blood vessels (Fig. 4b) indicating the existence of same system in rat and human tissue.

With the use of confocal laser scanning microscopy we were able to show discrete Ang II staining in fibers and moreover fibers building "*synapse en passant*" (sympathetic varicosities) with vascular smooth muscle cells (VSMCs) (see Fig. 3c and d for rat and Fig. 4c for human). In conclusion, our findings strongly indicate the existence of an endogenous synthesis of Ang II in the neurons of sympathetic coeliac ganglia and further their angiotensinergic innervation with mesenteric resistance blood vessels (Fig. 5). Since the presence of Ang II type 1 receptors on VSMCs has been reported [1], our findings support the hypothesis that neuronally produced Ang II is released through *synapses en passant* and plays an important role in the regulation of blood pressure.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.regpep.2008.01.006.

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3. Endogenous angiotensinergic system in neurons of rat and human trigeminal ganglia.

Hans Imboden, **Jaspal Patil**, Juerg Nussberger, Françoise Nicoud, Benno Hess, Nermin Ahmed, Thomas Schaffner, Maren Wellner, Dominik Müller, Tadashi Inagami, Takaaki Senbonmatsu, Jaroslav Pavel, Juan M. Saavedra.

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Endogenous angiotensinergic system in neurons of rat and human trigeminal ganglia

Hans Imboden [a,*], Jaspal Patil [a], Juerg Nussberger [f], Françoise Nicoud [f], Benno Hess [a], Nermin Ahmed [a], Thomas Schaffner [b], Maren Wellner [c], Dominik Müller [c], Tadashi Inagami [d], Takaaki Senbonmatsu [d], Jaroslav Pavel [e], Juan M. Saavedra [e]

[a] Institute of Cell Biology, University of Bern, Bern, Switzerland
[b] Institute of Pathology, University of Bern, Bern, Switzerland
[c] Max Delbrück Center, Molecular Medicine, Berlin, Germany
[d] Vanderbilt Department of Biochemistry, Nashville, USA
[e] Section on Pharmacology, National Institutes of Mental Health, Bethesda, USA
[f] Department of Internal Medicine, University Hospital, Lausanne, Switzerland

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ABSTRACT

To clarify the role of Angiotensin II (Ang II) in the sensory system and especially in the trigeminal ganglia, we studied the expression of angiotensinogen (Ang-N)-, renin-, angiotensin converting enzyme (ACE)- and cathepsin D-mRNA, and the presence of Ang II and substance P in the rat and human trigeminal ganglia. The rat trigeminal ganglia expressed substantial amounts of Ang-N- and ACE mRNA as determined by quantitative real time PCR. Renin mRNA was untraceable in rat samples. Cathepsin D was detected in the rat trigeminal ganglia indicating the possibility of existence of pathways alternative to renin for Ang I formation. *In situ* hybridization in rat trigeminal ganglia revealed expression of Ang-N mRNA in the cytoplasm of numerous neurons. By using immunocytochemistry, a number of neurons and their processes in both the rat and human trigeminal ganglia were stained for Ang II. Post *in situ* hybridization immunocytochemistry reveals that in the rat trigeminal ganglia some, but not all Ang-N mRNA-positive neurons marked for Ang II. In some neurons Substance P was found colocalized with Ang II. Angiotensins from rat trigeminal ganglia were quantitated by radioimmunoassay with and without prior separation by high performance liquid chromatography. Immunoreactive angiotensin II (ir-Ang II) was consistently present and the sum of true Ang II (1-8) octapeptide and its specifically measured metabolites were found to account for it. Radioimmunological and immunocytochemical evidence of ir-Ang II in neuronal tissue is compatible with Ang II as a neurotransmitter. In conclusion, these results suggest that Ang II could be produced locally in the neurons of rat trigeminal ganglia. The localization and colocalization of neuronal Ang II with Substance P in the trigeminal ganglia neurons may be the basis for a participation and function of Ang II in the regulation of nociception and migraine pathology.

1. Introduction

The trigeminal ganglion, a cranial analog of the dorsal root ganglia in the peripheral nervous system, is the site of primary sensory neurons involved in the regulation of nociception, thermoreception, proprioception and mechanoreception in the facial skin, eye, nasal and oral cavities, teeth and periodontal tissue and vibrissae [30] and provides sensory afferents to the cerebral blood vessels [15]. The trigeminovascular system plays an essential role in the pathophysiology of migraine [6,18], in the cerebrovascular vasospasm occurring after subarachnoid hemorrhage [23] and in chronic pain and inflammatory syndromes [13].

A multitude of transmitters and neuromodulators have been described in the trigeminal ganglion and its primary afferent neurons exhibit pathway-specific patterns of neurochemical expression and transmitter colocalization [30]. However, the presence and specific localization of renin-angiotensin system (RAS) components and role of angiotensin II (Ang II) has not been studied, in spite of the association of Ang II with central and peripheral sensory systems [4,5,52,54] and its proposed role in the regulation of pain [22,43], cerebrovascular inflammation [2,57] and migraine [49,51].

Formation of circulating and local Ang II is mediated through activation of the RAS. The RAS includes a precursor, angiotensinogen (Ang-N) cleaved by the enzyme renin to produce the decapeptide angiotensin I (Ang I). In turn, Ang I is cleaved by angiotensin converting enzyme (ACE) to generate the active RAS principle, the octapeptide Ang II [11].

Existence of an endogenous angiotensinergic system in the neurons of rat and human sympathetic coeliac ganglia innervating mesenteric

* Corresponding author. Institute of Cell Biology, University of Bern, Baltzerstrasse 4, 3012 Bern, Switzerland. Tel.: +41 31 631 48 99; fax: +41 31 631 46 16.
E-mail address: hans.imboden@izb.unibe.ch (H. Imboden).

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resistance arteries has been reported recently [37]. Here we address the issue of the possible formation and localization of Ang II in the trigeminal ganglion by determination of Ang-N-, Renin-, ACE- and Cathepsin D-mRNA by qRT-PCR in rat trigeminal ganglia, by using *in situ* hybridization of the Ang II precursor Ang-N mRNA in rat trigeminal ganglia and the expression of Ang II by immunocytochemistry in rat as well as in human trigeminal ganglia. Several studies substantiated the role of substance P in the regulation of sensory transmission in the trigeminal ganglion [17,24,25,30] and the involvement of Ang II in the regulation of Substance P release [14,28]. Hence we attempted to determine the possible formation and localization of Ang II in the neurons of trigeminal ganglion and colocalization with Substance P in the same neurons.

2. Methods

2.1. Rat and human trigeminal ganglia

We purchased 8-week-old, male Wistar Kyoto (WKY) rats (approximately 200 g body weight) from the Central Animal Facilities of the University of Bern. Adequate measures were taken to minimize pain or discomfort, in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC) and in accordance with Animal Protocols approved by the Animal Care and Use Committee, NIMH, NIH, USA. Rats were anaesthetized intraperitoneally with 100 mg/kg thiopentane sodium and were perfused transcardially with 150 ml Ringer solution containing 1000 U heparin at 37 °C followed by 300 ml 2% freshly prepared formaldehyde at 4 °C. Trigeminal ganglia were carefully dissected and incubated by immersion fixation in 2% formaldehyde for 28 h at 4 °C. Subsequently, ganglia were immersed for 14 h in phosphate-buffered saline (PBS-Dulbecco) containing 18% sucrose at 4 °C. Fixed ganglia were frozen in isopentane at −50 °C and 30 μm thick sections were cut on a cryostat and subsequently used as free-floating sections for immunocytochemistry. For some experiments after perfusion and immersion fixation rat ganglia were embedded in paraffin. Paraffin sections, 7 μm thick, were used for immunocytochemical as well as for *in situ* hybridization experiments.

For extraction of total RNA and angiotensin components rats were shortly anesthetized with ether and subsequently sacrificed by decapitation. Fresh rat trigeminal ganglia were dissected and instantly transferred into RNA later (Ambion), frozen in liquid nitrogen, and then processed for total RNA extraction (Ambion). For angiotensin component extraction, trigeminal ganglia were rapidly removed, rinsed with cold Ringer solution, blotted by filter paper and wet weight was measured. The ganglia were frozen in liquid nitrogen and stored at −70 °C.

Human trigeminal (semilunar) ganglia were procured from three adult individuals for whom a permit for clinical autopsy (informed written consent by next of kin) had been obtained according to state law, in accordance with the Code of Ethics of the World Medical Association (Declaration of Helsinki). After removal of the brain with transection of the cranial nerve roots along the brainstem and harvest of the pituitary gland from the sella, the superficial dura of the middle cranial fossa was removed by traction with forceps. The semilunar ganglion was then easily detached from its bed (Meckle's cave) and the three trigeminal branches transected at their passage through the foramina. Human trigeminal ganglia were fixed by immersion in freshly prepared 2% formaldehyde for 3 days and then used for cryosectioning or embedded in paraffin.

2.2. RNA isolation and quantitative realtime RT-PCR (qRT-PCR)

Total 6 trigeminal ganglia from different WKY rats were taken for total RNA extraction. RNA integrity was confirmed for each sample on the Agilent Bioanalyzer using the RNA 6000 Nano kit (Agilent Technologies). 1 μg of total RNA was reverse transcribed using Superscript II (Invitrogen) and random hexamers according to the manufacturer's protocol. For realtime PCR, reverse transcribed material corresponding to 40 ng RNA was amplified with the TaqMan assays described below in 25 μl Universal PCR Master Mix, No AmpErase UNG on the SDS 7000 (Applied Biosystems) using the standard thermal protocol. Average values and standard deviations of relative mRNA levels of each sample, normalized to relative 18S rRNA levels, are from four measurements and were calculated using the relative differences. The following TaqMan assays were used for qRT-PCR at a final concentration of 250 nM TaqMan probe and 900 nM of each primer:

Angiotensinogen
Forward primer 5′-CACGACTTCCTGACTTGGATAAAGA-3′;
Reverse primer 5′-CTGCGGCAGGGTCAGA-3′;
TaqMan probe 5′-FAM CCTCGGGCCATCC GMGB- 3′;
manufactured as Assays-by-Design (RATG-EJ3) by Applied Biosystems.
Renin Assay-on-demand Rn00561847_m1 from Applied Biosystems.
ACE Assay-on-demand Rn00561094_m1 from Applied Biosystems.
Cathepsin D Assay-on-demand Rn00592528_m1 from Applied Biosystems.
18S rRNA Predeveloped Assay Reagent 431-9413E from Applied Biosystems.

2.3. In situ hybridization

2.3.1. DIG-labelled RNA probe preparation

By using an appropriate cDNA template for Ang-N [31], a 403 bp long fragment corresponding to nucleotides 221–623 was generated by digestion with restriction enzymes *EcoRV* and *Bam HI*. The obtained fragment was cloned between *Stu I* and *Bam HI* into pBluescript I KS+ (Stratagene). Digoxigenin-labelled probes were prepared using the DIG-RNA-labelling Mix (Roche) according to the manufacturer's protocol. T7 RNA polymerase was used to generate antisense riboprobe using the *Hind III* linearized template, and the sense strand (used as a control) was generated by T3 RNA polymerase using the same template linearized with *Bam HI* (for gel images please see online supplementary method). The RNA concentration was estimated comparing dot-blot tests and NanoDrop measurements. Filter hybridization with RNA extracts from trigeminal ganglia (RNaqueous-4PCR kit from Ambion) was used for binding tests.

2.3.2. Procedure for in situ hybridization

For *in situ* hybridization, 6 μm thick paraffin sections were rehydrated in a graded alcohol series (2 times Xylol for 10 min, EtOH 100% (2 times), 96%, 70%, 50%, DEPC H_2O each step 5 min) using DEPC-treated H_2O for the dilution of all reagents and solutions. The sections were equilibrated in proteinase K buffer (100 mM Tris, 50 mM EDTA, pH 7.5) for 5 min and then treated with proteinase K (19 μg/ml) at 37 °C for 2 min, after a wash with DEPC-treated H_2O. Following a wash in DEPC-water, sections were post fixed with freshly prepared 4% formaldehyde for 5 min, followed by two subsequent washes in DEPC-water for 5 min each. Sections were then incubated in prehybridization solution (SIGMA) at 45 °C for 2 h, followed by incubation with heat denatured sense and antisense riboprobes (5–10 ng/μl) in 30 μl hybridization mix (SIGMA) for 48 h at 45 °C in a humid chamber (saturated with 2x SSC). Subsequently, the sections were incubated with 2x SSC for 30 min at room temperature, followed by 1 h in 2x SSC at 45 °C and 1 h in 0.1x SSC at 45 °C. Sections were equilibrated for 5 min with buffer 1 (100 mM Tris, 150 mM NaCl, pH 7.5), then incubated with alkaline phosphatase-coupled anti-digoxigenin antibody (Roche) 1:500 diluted in buffer 2 (10x blocking solution diluted with buffer 1) for 2 h at room temperature, followed by two 5 min washes with buffer 1. Finally, after 5 min equilibration with buffer 3 (100 mM Tris, 100 mM NaCl, 50 mM $MgCl_2$, pH 9.5), the

color reaction containing NBT and BCIP in buffer 3 was completed according to manufacturer's protocol (Sigma).

2.4. Immunocytochemistry

For immunocytochemical incubations, a protein G purified murine monoclonal antibody against Ang II (Mab-Trap G II column, Amersham Sciences) was used at a concentration of 0.3 μg/ml in buffer solution. This is a self generated monoclonal antibody against the synthetic peptide to human Ang II. The specificity for the monoclonal antibody against Ang II (4B3) has been previously documented and it produced the same staining as a polyclonal antibody against Ang II in rat adrenal glands [20]. For immunization of the mice, Ang II peptide was cross-linked with glutaraldehyde to keyhole limpet hemocyanin. In dot blot assay, the monoclonal antibody against Ang II (4B3) showed total cross reactivity with Ang III (2–8), Ang 3–8, Ang 4–8 and Ang 5–8. It showed no cross reaction with human plasma angiotensinogen, Ang I (1–10) and angiotensin 1–7. Pre-absorption was performed by using a batch procedure with the synthetic Ang II-peptide that was covalently linked through its N-terminus to CH-Sepharose 4 B. The gel was loaded into a glass column and the monoclonal anti Ang II-antibody solution effluent was used for immunocytochemical preabsorption controls. Further controls were done with mouse non-immune serum and for the secondary goat anti-mouse Cy3 antibody without the primary antibody. All these additional controls showed absolute no staining within the sections. The pre-absorbed monoclonal antibody after low pH-elution revealed the same staining as the Mab-Trap G II purified antibody.

Substance P was detected with a mouse monoclonal antibody to the synthetic peptide Substance P from Novus Biologicals (SP-DE4-21). Goat anti-mouse immunoglobulins (GAM Cy3; Jackson ImmunoResearch) pre-incubated with rat serum prior to immunocytochemistry were used as secondary antibodies. Sections were incubated free floating for 36 h at 4 °C with the primary antibody, washed and incubated with the secondary antibody for 90 min at room temperature and counterstained with DAPI (4′,6-diamidino-2-phenylindole). After washing, cryosections were mounted on gelatin-coated slides and air-dried. The stained sections were embedded with Glycergel (DAKO) and cover-slipped. The same procedure as for free floating cryosections has been used for paraffin sections mounted on glass slides. Pictures were taken by a normal fluorescent light microscope (LEICA DM 6000B) using a digital camera or by a confocal laser scanning microscope (LEICA SP2 with AOBS).

2.5. Colocalization studies

Directly adjacent free-floating cryosections of rat trigeminal ganglia were immunostained for Ang II and Substance P, as mentioned under methods (2.4 Immunocytochemistry). Pictures were taken by a normal fluorescent light microscope (LEICA DM 6000B) using a digital camera LEICA-DFC350FX. The total number of neurons was counted for each adjacent section, and nuclei which were counterstained with DAPI in blue. Ang II and Substance P immunopositive neurons were counted in respective adjacent sections. For colocalization studies Substance P staining was digitally changed to yellow from red, taking the advantage of directly adjacent sections we superimposed the Ang

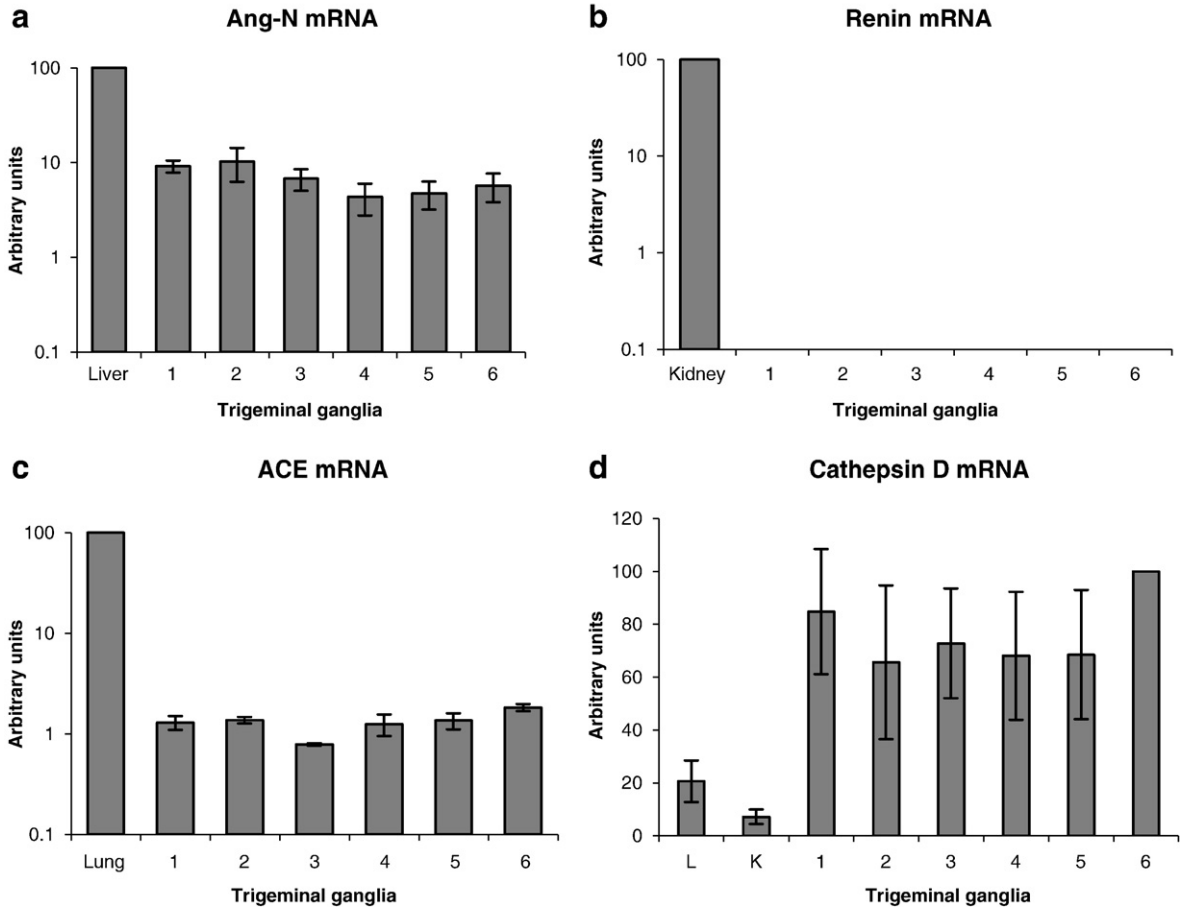

Fig. 1. Determination of relative mRNA levels in rat trigeminal ganglia. Reverse transcribed total RNA extracts of rat trigeminal ganglia from different rats were tested by qRT-PCR for the presence of Ang-N mRNA (a), for renin mRNA (b), for ACE mRNA (c) and cathepsin D mRNA (d). Total RNA extracts from liver (a), kidney (b), lung (c) and liver–kidney (d) were used as a reference samples expression. All relative mRNA values are normalized to 18S rRNA levels. Average values and standard deviations of 4 qRT-PCR measurements are shown.

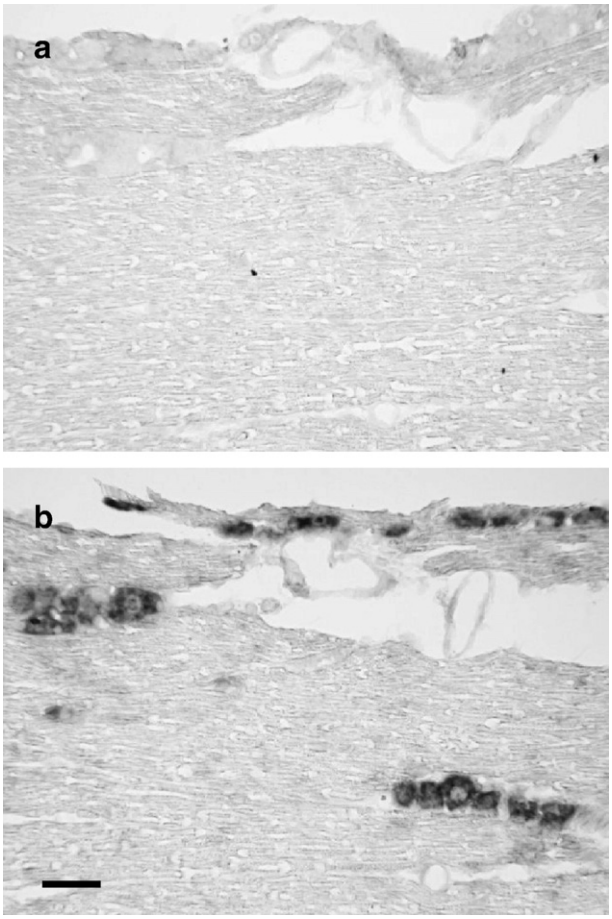

Fig. 2. *In situ* hybridization in rat trigeminal ganglion for detection of Ang-N mRNA, 6 μm thick consecutive paraffin sections were processed as described in methods. a) sense probe. b) with antisense probe, revealing positive staining in the cytoplasm of numerous neurons within the ganglion. Bar: 100 μm.

II and Substance P sections by using Corel Draw and colocalized neurons where counted.

2.6. Measurement of angiotensin peptides

Immunoreactive Ang II (ir-Ang II) as well as Ang-(1–8) octapeptide, Ang-(2–8) heptapeptide, Ang-(3–8) hexapeptide, Ang-(4–8) pentapeptide, Ang-(1–10) decapeptide and Ang-(2–10) nonapeptide were measured in rat trigeminal ganglia by radioimmunoassay without and with prior separation by high performance liquid chromatography (HPLC) [34]. Measurement of true angiotensin was applied also for Ang I, and peptide extraction from tissue was adapted for additional freeze-thaw procedures to ensure the lysis of cells [35,36]. Briefly, trigeminal ganglia weighing 14.8–21.9 mg were homogenized in 1.5 ml water, shock frozen in ethanol/dry ice, thawed in a 37 °C water bath. The freeze-thaw procedure was repeated twice. Samples were sonicated for 10 min at 37 °C, centrifuged at 3500 g and the supernatant in an albumin-coated polypropylene tube was dried at 37 °C under nitrogen. The residue was dissolved in 2.2 ml Tris buffer (0.1 M, pH 7.5) containing bovine serum albumin (5 g/l). Solid-phase extraction on phenylsilylsilica, HPLC and radioimmunoassay were performed according to the routine procedures using very sensitive antisera for Ang II and Ang I with cross-reactivities of 53% for Ang-(2–8) heptapeptide as well as for Ang-(3–8) hexapeptide, 52% for Ang-(4–8) pentapeptide, and 33% for Ang-(2–10) nonapeptide, respectively [35,36]. Recoveries for the different Ang peptides and for ir-Ang II were consistently above 67% except for the nonapeptide (34 ± 2%, mean ± SD). Therefore, no corrections were made for recovery losses. Results are presented as fmol Ang per gram wet weight. Detection limits were variable according to the various weights of individual ganglia. Results below detection limits were taken as zero. All measurable Ang concentrations were at least at 150% of the detection limit except for one hexapeptide result at the detection limit. Instead of homogenates, controls of 1.5 ml water were extracted and quantitated with every analysis and any traces of Ang peptides found in these "blanks" were subtracted to obtain final ganglial concentrations. In 3 rats, ir-Ang II of one trigeminal ganglion was compared with the sum of the specifically measured Ang peptides of the contra lateral trigeminal ganglion. Immunoreactivity of the metabolites was calculated using the cross-reactivity as a factor.

3. Results

3.1. Quantitative real time RT-PCR

In order to demonstrate the existence of Ang II in the trigeminal ganglion we investigated the presence of different RAS components. We detected substantial amounts of Ang-N- and ACE-mRNA in the rat (Fig. 1a and c) trigeminal ganglion by qRT-PCR. No renin mRNA was detected (Fig. 1b). We have discovered cathepsin D mRNA, a possible alternative pathway for Ang I formation (Fig. 1d). RNA from tissues with previously reported high levels of the respective mRNAs were used as positive controls, and all mRNA values were normalized to 18S rRNA.

3.2. In situ hybridization and post in situ hybridization immunocytochemistry

With paraffin sections, Ang-N mRNA was identified in most, if not all the neurons studied in the rat trigeminal ganglion (Fig. 2a and b). Ang II immunoreactivity was present in both the neuronal cytoplasm and neuronal processes in the rat trigeminal ganglion (Fig. 3). However some neurons were not stained for Ang II (Fig. 3). Additionally we demonstrate Ang II staining in the rat central nervous system (CNS) in the spinal trigeminal tract (Fig. 4a and b).

In the human trigeminal ganglion Ang II immunoreactivity was observed in the cytoplasm (see movie 2, as online supplement) of many neurons and in their processes with both paraffin and free-floating cryosections (Fig. 5a and b).

We used rat paraffin sections for post *in situ* hybridization immunocytochemistry to determine the colocalization of Ang-N mRNA and

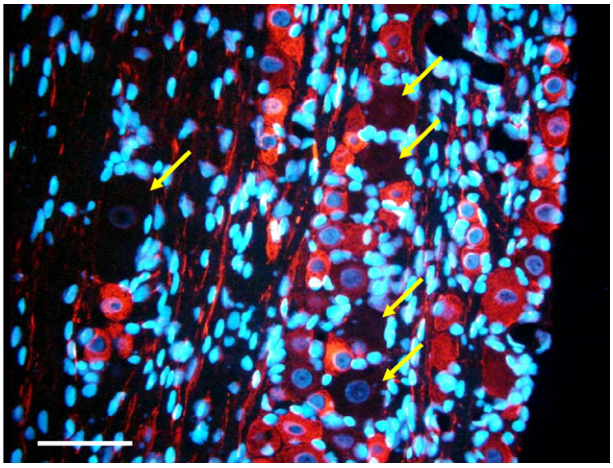

Fig. 3. Ang II immunocytochemistry in rat trigeminal ganglion. 30 μm thick free-floating cryosections were prepared and incubated with our murine monoclonal antibody against Ang II [20], stained with goat anti-mouse (GAM^{Cy3}) in red and DAPI in blue for nuclei, as described under methods. Note, the intensity of the staining for Ang II varies among the neurons and their projections, some neurons are not stained for Ang II (see arrows). Bar: 50 μm. See movie 1 in supplementary material.

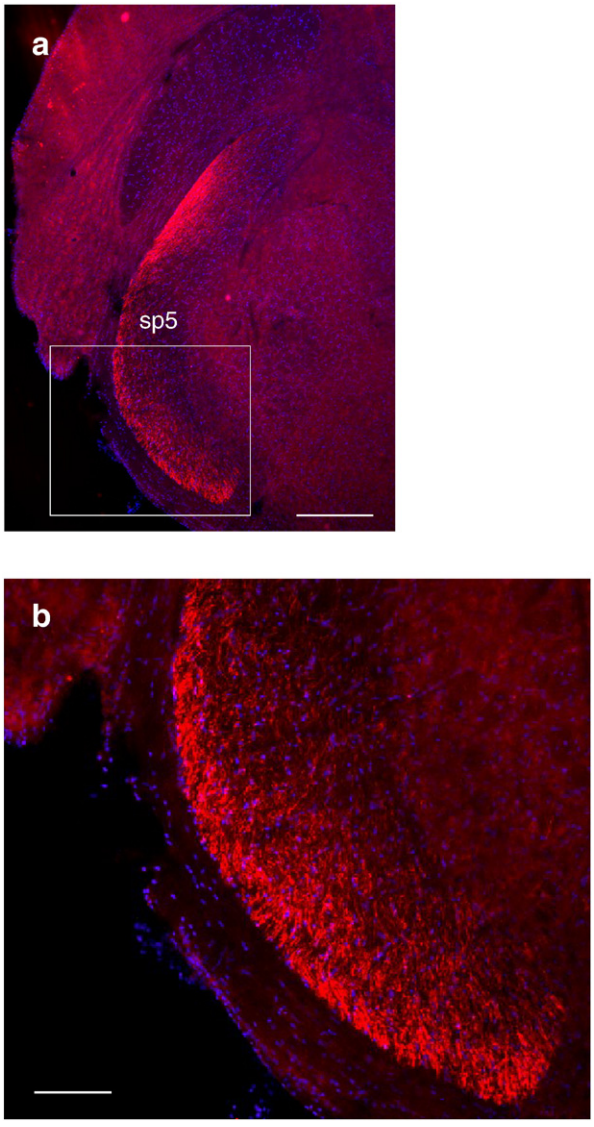

Fig. 4. Angiotensinergic neuronal processes within the central nervous system in the rat spinal trigeminal tract (sp5) [41]. a. Bar: 200 μm. b. Bar: 100 μm.

Ang II in trigeminal ganglia neurons. In the rat, we found that some, but not all the Ang-N mRNA positive neurons (Fig. 6a) were stained for Ang II (Fig. 6b) as revealed in Fig. 6c.

3.3. Immunocytochemistry and colocalization studies

In directly adjacent free-floating cryosections of rat trigeminal ganglia, we detected a number of Ang II (Fig. 7a) and of Substance P-positive neurons (Fig. 7b) and their respective processes. In consecutive paraffin sections we found that out of total neurons, approximately 25% neurons were immunopositive for Ang II (Fig. 8a) and 7 to 8% for Substance P (Fig. 8b). In sum of Ang II and Substance P positive neurons approximately 7% neurons indicated Ang II colocalized with Substance P. The number of Ang II neurons was higher than that of Substance P neurons, and some Ang II positive cells did not contain Substance P (Fig. 8c).

3.4. Concentration of angiotensin peptides in trigeminal ganglia

Immunoreactive Ang II was found in 9 of 10 extracted trigeminal ganglia (76 ± 53 fmol/g, mean $\pm$ SD, median 53 fmol/g). Ang I or Ang-(1–10) decapeptide was never ($n = 10$) found above any detection limit (≥ 4 fmol/g) and no Ang-(2–10) nonapeptide was found in a single experiment. Ang-(1–8) octapeptide was measurable only in one of eight individual ganglia, but in 2 out of 3 pooled ganglia extracts. Only 3 of 6 ganglia contained Ang-(2–8) heptapeptide (33 ± 28 fmol/g, median 27 fmol/g), but 5 of 6 ganglia contained Ang-(3–8) hexapeptide (32 ± 22 fmol/g, median 22 fmol/g). Significant amounts of Ang-(4–8) pentapeptide were present in 4 of 6 ganglia (55 ± 49 fmol/g, median 87 fmol/g). Table 1 presents levels of Ang I, Ang II, Ang III, Ang IV and Ang V and corresponding detection limits measured in trigeminal ganglia extracts pooled from 2 rats: All Ang peptides except Ang I were found well above detection limits. For individually examined rats, the sum of true Ang II (Ang-1–8) and specifically measured metabolites Ang III, Ang IV and Ang V of the left sided trigeminal ganglion corresponded well with ir-Ang II extracted from the right sided trigeminal ganglion (Fig. 9).

4. Discussion

The proposal of important roles of Ang II in peripheral sympathetic and sensory ganglia [37,38] and our clear demonstration of the

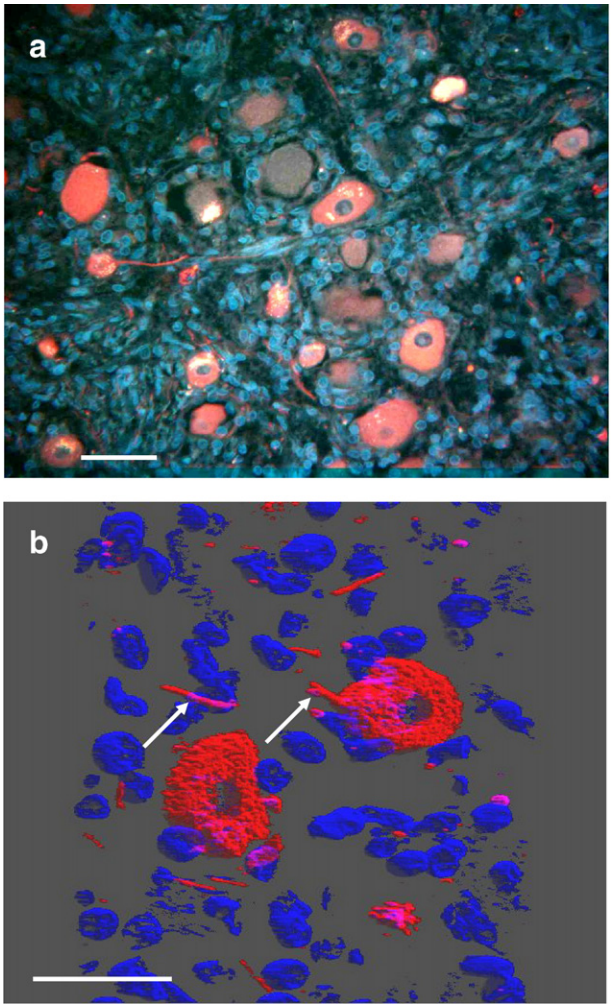

Fig. 5. Ang II immunocytochemistry in human trigeminal ganglion. a. A 6 μm thick paraffin section, incubated with murine monoclonal anti Ang II antibody [20], stained with goat anti-mouse (GAM^{Cy3}) in red and DAPI in blue for nuclei. Note, Ang II staining in neuronal cytoplasm and projections and the intensity of the staining varies among the neurons. Autofluorescence of lipofuscin granules is shown in yellow, Bar: 50 μm. b. A free floating 30 μm thick cryosection, incubated with murine monoclonal anti Ang II antibody stained with goat anti-mouse (GAM^{Cy3}) in red and DAPI in blue, as described under methods. Ang II staining in trigeminal neurons is cytoplasmic and in processes (see arrows). The picture was taken with a confocal laser scanning microscope. Bar: 50 μm. See movie 2 in supplemented material.

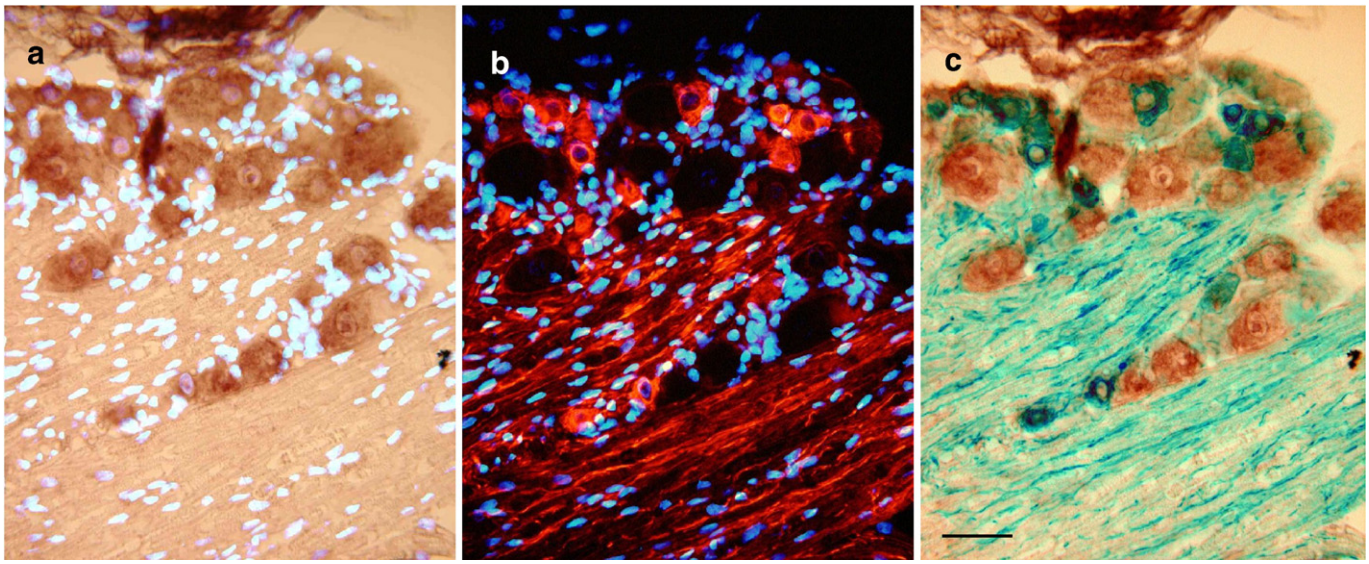

Fig. 6. Colocalization of Ang-N mRNA and Ang II immunoreactivity in the rat trigeminal ganglion. A 7 μm thick paraffin section was processed for *in situ* hybridization with the antisense probe to detect Ang-N mRNA (a) followed by immunocytochemistry for Ang II as described in methods (b) post *in situ* hybridization immunocytochemistry (c) Here angiotensinergic staining in neurons and projections is in greenish-blue color. Note that not all Ang-N mRNA-expressing neurons stain for Ang II to the same extent. Bar: 100 μm.

intraneuronal expression of Ang II in the trigeminal ganglia, raises the issue of the possibility of local Ang II formation in these neuronal tissues.

The existence of an endogenous angiotensinergic system in the neurons of rat and human sympathetic coeliac ganglia has been reported recently [37]. In our current work we provide evidence for local formation of Ang II in sensory neurons of trigeminal ganglia. First, we report with the use of qRT-PCR, the presence of Ang-N mRNA and ACE mRNA in the tissue extracts of trigeminal ganglia from rats. Second, *in situ* hybridization confirmed the existence of Ang-N mRNA at cellular level in the cytoplasm of rat trigeminal ganglia neurons. Ang-N mRNA detection by *in situ* hybridization demonstrates that Ang-N could be locally formed in this tissue, indicating that Ang-N uptake from the circulation is not necessary for the local formation of Ang II. With the exception of one earlier communication on the presence of Ang-N mRNA in the rat trigeminal ganglion extracts [7] ours is the first report on the presence of angiotensin system components at cellular resolution in this structure.

We did not detect the expression of mRNA for renin, the enzyme classically considered necessary for cleaving Ang-N into the Ang I precursor of angiotensin II [38], in the rat trigeminal ganglion. Instead, we detected expression of cathepsin D mRNA in the trigeminal ganglia, at levels higher in comparison with those found in rat liver and kidney, but lower in comparison to those recently reported in rat coeliac ganglia [37]. Cathepsin D is a protease capable of cleaving Ang-N into angiotensin I [48], a non-renin alternative pathway for Ang I formation. Our results are in agreement with an earlier report of the existence of cathepsin D enzymatic activity in rat trigeminal ganglion neurons [1]. These results indicate the existence of an alternate pathway for Ang II synthesis in trigeminal ganglion sensory neurons. Alternatively, neurons of trigeminal ganglion may take up circulating renin to support local synthesis of endogenous Ang II [21]. These mechanisms have been proposed for other tissues such as the heart, where strong evidence exists for uptake of extracellular renin from the circulation [38,10,53,44], while the presence of renin in cardiac tissue is still a matter of controversial debate [16,38–40,45,50,53,56].

In the brain, Ang-N is localized to multiple cell types, predominantly in astrocytes but also in neurons [26]. The expression of the Ang-N gene in selective groups of neurons has been conclusively demonstrated in the murine central nervous system [55]. Our *in situ* hybridization studies demonstrate the presence of Ang-N mRNA specifically in most, but not all, the neuronal cell bodies of the trigeminal ganglia, and not in surrounding cells (Fig. 6a). Not all Ang-N mRNA containing neurons stained for the same extent for Ang II (Fig. 6c). There are several

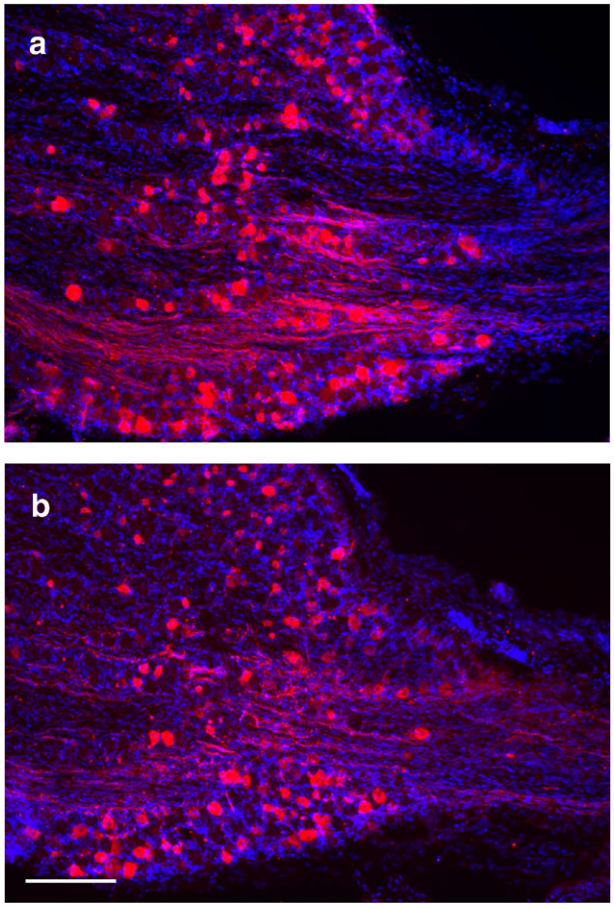

Fig. 7. Ang II and Substance P immunocytochemistry in rat trigeminal ganglion. Directly adjacent, 30 μm free-floating cryosections were incubated with Ang II (a) or Substance P (b) antibodies and stained with goat anti-mouse (GAM^{Cy3}) as secondary antibodies in red and DAPI in blue as nuclear stain, as described under methods. Bar: 200 μm.

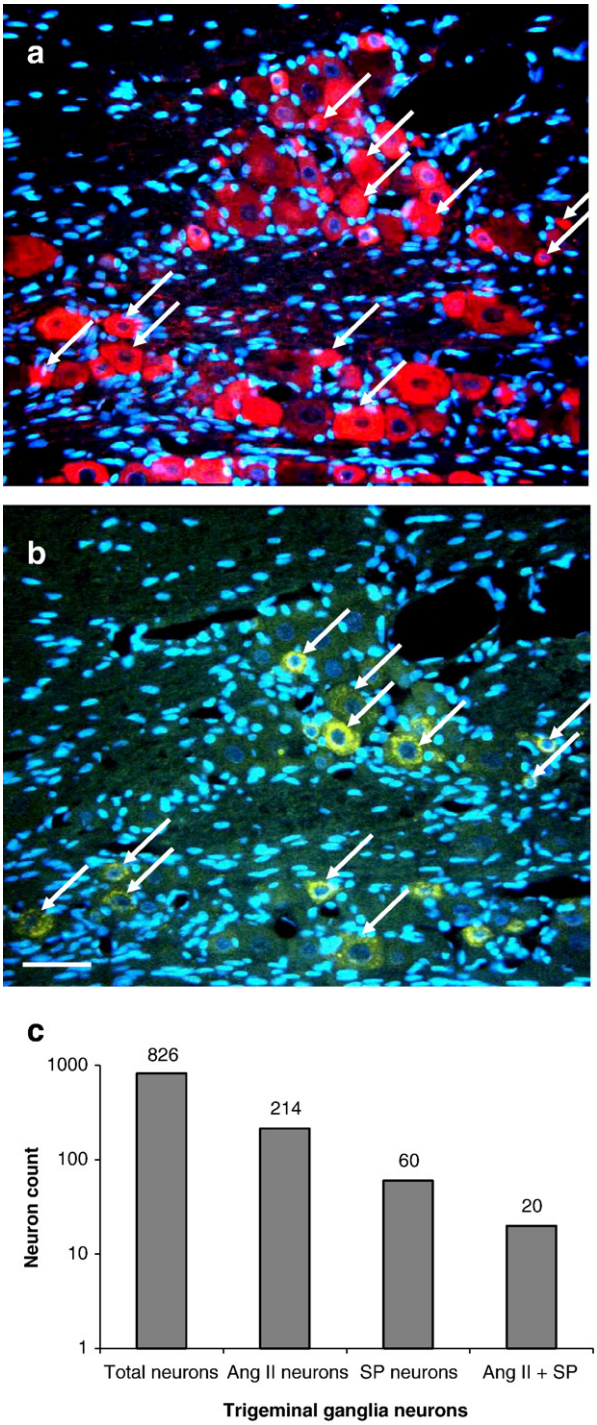

Fig. 8. Colocalization of Ang II and Substance P (SP) immunoreactivity in rat trigeminal ganglion neurons. Directly adjacent, 6 μm thick paraffin sections were incubated with Ang II (a) or Substance P (b) antibodies and then stained with goat anti-mouse (GAM^{Cy3}) as secondary antibodies in red, DAPI for nuclei in blue. In order to perform the colocalization studies Substance P originally stained in red, was digitally changed to yellow. Arrows indicate colocalized, positive neuronal staining Bar: 50 μm. Note that more neurons were stained for Ang II than for Substance P, while some neurons show colocalization of Ang II with Substance P. Statistical analysis showing total neurons of trigeminal ganglia sections, Ang II-, Substance P-positive neurons and their colocalization (c).

possibilities for this result, including lack of Ang II internalization or storage in these neurons, Ang II production in amounts too low to be detected with our immunocytochemical methods, or to different roles for Ang-N, not related to Ang II formation [9,29].

We have detected Ang II-like immunoreactivity in the cytoplasm of neurons and their processes (Figs. 3, 5 and 7a) within the trigeminal ganglia. Ang II immunoreactivity was found in higher concentration in small and medium sized neurons as compared to large neurons of the rat and human trigeminal ganglia. The use of polyclonal, affinity-purified, monospecific antibodies to Ang II (BODE 1) [5] have revealed similar distribution of Ang II immunoreactivity within the neurons of the rat trigeminal ganglia as with the applied monoclonal anti Ang II (4B3) antibody. We could also detect angiotensinergic processes in the rat CNS in the spinal trigeminal tract (sp5), indicating that Ang II may act as a neurotransmitter in the CNS (Fig. 4a and b). Hence, we can hypothesize that locally formed Ang II may be transported to the terminal fields innervated by the trigeminal ganglion in order to act as a neurotransmitter for sensory transmission, a function similar to that demonstrated in the sympathetic nervous system [37].

Table 1
Angiotensin concentration of rat trigeminal ganglia using HPLC-RIA (fmol/g wet weight; 4 ganglia of 2 rats were pooled).

Peptide name	Ang-(1–10) decapeptide	Ang-(1–8) octapeptide	Ang-(2–8) heptapeptide	Ang-(3–8) hexapeptide	Ang-(4–8) pentapeptide
Trivial name	Ang I	Ang II	Ang III	Ang IV	Ang V
Peptide content (fmol/g)	<4.0	19.7	8.2	13.4	17.9
Detection limit (fmol/g)	4.0	2.0	3.8	3.8	3.8

Our results demonstrate the presence of Ang II and its metabolites in extracts of trigeminal ganglia of Wistar–Kyoto rats (Table 1) at concentrations well comparable with other tissue concentrations of Ang II established in Wistar rats with similar methodology [36]: These tissue levels were depending on the organ about tenfold higher than plasma concentrations (4.4 ± 0.4 fmol/ml), in heart (10.1 ± 1.2 fmol/g), muscle ($30.3. \pm 3.5$ fmol/g), liver (69.7 ± 6.9 fmol/g), lung (79.5 ± 9.7 fmol/g), kidney tissue (192 ± 23 fmol/g), but low in whole brain (3.2 ± 0.5 fmol/g) and very high in adrenal gland (3061 ± 159 fmol/g). The Ang II concentration in WKY rat trigeminal ganglia is thus six fold higher than whole brain Ang II in Wister rats. Interestingly, brain Ang I (decapeptide) levels (3.0 ± 0.7 fmol/g) in Wister rats were comparable to Ang II levels, but similar concentrations in trigeminal ganglia would have been below detection limit (<3.95 fmol/g) of the present work since maximally 121 mg pooled ganglial tissue was available. It cannot be concluded that Ang I is absent in trigeminal ganglia, but if it would be present it had to be fivefold below Ang II levels and less than half of all other measured Ang peptides.

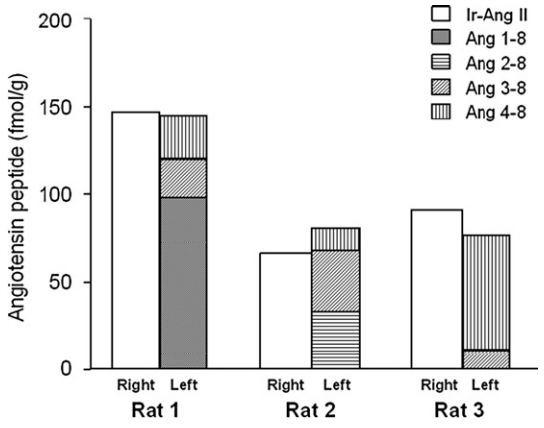

Fig. 9. Concentration of ir-Ang II (RIA) and specifically measured Ang peptides (HPLC-RIA) in trigeminal ganglia of 3 rats: The sum of individually measured Ang peptides of one trigeminal ganglion parallels levels of ir-Ang II as measured in the contralateral trigeminal ganglion.

The reliability of our Ang measurements is well documented by the close relation between ir-Ang II of one-sided ganglia extract and the sum of immunoreactivities of all specifically measured Ang peptides of the contra lateral trigeminal ganglion. There can be little doubt that the Ang II visualized by immunocytochemistry in trigeminal ganglia is indeed Ang II or its metabolites. In the context of perivascular Ang II immunocytochemical stainings and visibility of corresponding varicosities as synapses en passant, a role for angiotensin as a neurotransmitter is very likely [37].

Additionally, we attempted to determine whether Ang II was associated with other neuropeptides in the trigeminal ganglion. From the multiple neurotransmitters and neuromodulators identified in this ganglion, we chose Substance P, a neuropeptide established as a key regulator of sensory transmission in the trigeminal ganglion [17,25,30]. We found that Ang II was colocalized with Substance P in trigeminal ganglion neurons (Fig. 8), supporting the hypothesis of a close integration between these two systems, and the previous reports of regulation of Substance P release by Ang II [14,28,22]. We found that in the total of 826 neurons of trigeminal ganglia 214 were stained for Ang II, 60 were stained for Substance P, whereas 20 neurons were showing colocalization of Ang II and Substance P (Fig. 8c). Neuronal colocalization suggests the possible interaction of Ang II with Substance P and further involvement of Ang II in trigeminal neuralgia and nociception [12,42,43,46]. Angiotensin receptor type 1 have been reported in the nucleus tractus solitarri (NTS) of rat brain [19,47], moreover Substance P receptors are also localized in NTS of rat brain [32].

Our results are not without clinical interest. The trigeminal ganglion provides sensory afferents to the cerebral blood vessels [15] and the trigeminovascular system is involved in the pathophysiology of migraine, cerebrovascular vasospasm, chronic pain and inflammatory syndromes [6,13,23]. RAS inhibition through ACE inhibitors or Ang II type 1 receptor blockade appears to be effective for the prevention of migraine, independently of their blood pressure lowering effects [8,49,51], to increase resistance to brain ischemia [27,33] and to reduce cerebrovascular inflammation [2,57]. It is reasonable to propose that Ang II, formed in the neurons of trigeminal ganglia, plays an important role in cerebrovascular pathophysiology either directly and/or in association with other transmitter systems such as Substance P or calcitonin gene-related peptide [30]. Ang II has an antinociceptive effect during high pain sensitivity [42]. Interactions between the Substance P and RAS are also proposed in the pineal gland, which receives Substance P innervation from the trigeminal ganglia [46] and expresses a local RAS system [3].

In conclusion, our findings strongly indicate the presence of a local angiotensinergic system in neurons of the trigeminal ganglia, where Ang II may act as a neurotransmitter. The roles of Ang II in the trigeminal ganglia possibly include the regulation of sensory pathways including pain and the functions of other local neurotransmitters.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.regpep.2009.02.002.

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4. Intraneuronal angiotensinergic system in the rat and human dorsal root ganglia

Jaspal Patil, Alexander Schwab, Thomas Schaffner, Juerg Nussberger, Hans Imboden.

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Intraneuronal angiotensinergic system in the rat and human dorsal root ganglia

Jaspal Patil[a], Alexander Schwab[a], Juerg Nussberger[b], Thomas Schaffner[c],
Juan M. Saavedra[d], Hans Imboden[a]*

[a]Institute of Cell Biology, University of Bern, Bern, Switzerland
[b]Department of Internal Medicine, University Hospital, Lausanne, Switzerland
[c]Institute of Pathology, University of Bern, Bern, Switzerland
[d]Section on Pharmacology, Division of Intramural Research Programs,
National Institute of Mental Health, National Institutes of Health, Bethesda, Maryland USA

* **Corresponding author**:
Prof. Dr. Hans Imboden
Institute of Cell Biology, University of Bern
Baltzerstrasse 4, 3012 Bern, Switzerland
Phone: +41-31-631 48 99
Fax: +41-31-631 46 16
Email: hans.imboden@izb.unibe.ch

Abstract

To elucidate the local formation of angiotensin II (Ang II) in the neurons of sensory dorsal root ganglia (DRG), we studied the expression of angiotensinogen (Ang-N)-, renin-, angiotensin converting enzyme (ACE) - and cathepsin D-mRNA, and the presence of Ang II and Substance P in the rat and human thoracic DRG. Quantitative real time PCR (qRT-PCR) studies revealed that rat DRG expressed substantial amounts of Ang-N- and ACE mRNA, while renin mRNA was untraceable. Cathepsin D was detected in the rat DRG indicating the possibility of existence of pathways alternative to renin for Ang I formation. Angiotensin peptides were successfully detected with high performance liquid chromatography and radioimmunoassay in human DRG extracts. *In situ* hybridization in rat DRG confirmed additionally expression of Ang-N mRNA in the cytoplasm of numerous neurons. Intracellular Ang II staining could be shown in number of neurons and their processes in both the rat and human DRG. Interestingly we observed neuronal processes with angiotensinergic synapses en passant within the DRG. In the DRG, we also identified by qRT-PCR, expression of Ang II-receptor AT_{1A} and AT_2- mRNA while AT_{1B}-mRNA was not traceable. In some neurons Substance P was found colocalized with Ang II. The intracellular localization and colocalization of Ang II with Substance P in the DRG neurons may indicate a participation and function of Ang II in the regulation of nociception. In conclusion, these results suggest that Ang II may be produced locally in the neurons of rat and human DRG and act as a neurotransmitter.

Keywords:

Renin-angiotensin system, angiotensin II, neurotransmitter, neuronal angiotensin, sensory system.

1. Introduction

The renin-angiotensin system (RAS), with circulating angiotensin II (Ang II) as its active principle, is a key regulatory factor in blood pressure control and fluid balance (**Fyhrquist and Saijonmaa, 2008**). Ang II effects are produced by stimulation of selective receptor types, the AT_1 and AT_2 receptors. While humans express a single type of AT_1 receptors, rodents express two AT_1 receptor subtypes, the AT_{1A} and AT_{1B} receptors (**Sasamura et al., 1992; Timmermans et al., 1993; De Gasparo et al., 2000**). Formation of circulating Ang II is mediated through cleavage of the precursor angiotensinogen (Ang-N) by the enzyme renin to produce the decapeptide angiotensin I (Ang I). In turn, Ang I is cleaved by angiotensin converting enzyme (ACE) to generate Ang II (**De Gasparo M, 2000**). However, more recent studies suggest significant roles for Ang II related peptides in addition to those of Ang II (**Fyhrquist and Saijonmaa, 2008**), the presence of multiple local organ and cellular RAS systems where Ang II plays multiple regulatory roles (**Paul et al., 2006**) and alternative pathways for Ang II formation, in particular for the intracellular RAS (**Kumar et al., 2008; Kumar and Boim, 2009**).

In the nervous system, Ang II is involved in the regulation of multiple functions, including sympathetic and hormonal control (**Tsutsumi and Saavedra, 1991; Saavedra, 1992; Saavedra, 1999; Saavedra, 2005; Bader and Ganten, 2008**). In addition, there is substantial evidence that Ang II contributes to regulate the central and peripheral components of sensory systems (**Buck et al., 1982; Saavedra, 1992; Wu et al., 2000; Burkhalter et al., 2001; Schrader et al., 2001; Tronvik et al., 2003; Pelegrini-da-Silva 2005; Fusayasu et al., 2007**). Ang II may exert local regulatory effects in dorsal root ganglia (DRG) (**Wu et al., 2000; Kawasaki et al., 2003**), structures containing cell bodies of primary afferent neurons that are involved in the regulation of blood pressure and in many sensory modalities (**Gibson et al., 1984; Wilmalawansa, 1996**).

Neuronal localization of RAS components has been reported in bilaterally ovariectomized rat DRG (**Chakrabarty et al., 2008**), in normotensive rat and human sympathetic coeliac ganglia (**Patil et al., 2008**) as well as in normotensive rat and human trigeminal ganglia (**Imboden et al., 2009**). Recent studies characterized the presence and transport of Ang II AT_1 receptors between the DRG and the spinal cord (**Pavel et al., 2008; Tang et al., 2009**). In the DRG, Ang II may act directly and/or modulating the effects of additional local peptidergic systems. Of particular interest is Substance P, a regulator of sensory transmission in the trigeminal ganglion and other DRG (**Edvinsson, 1991; Harrison and Geppetti, 2001; Lazarov, 2002; Hou et al., 2003**). This peptide has been proposed as an important neurochemical mediator of certain kinds of noxious peripheral stimuli (**Buck et al., 1982**). Substance P is colocalized with Ang II in neurons of trigeminal ganglia (**Imboden et al. 2009**), whereas other studies show that Substance P release is modulated by Ang II (**Diz et al., 1986; Kopp et al., 2003**).

Here we address the intracellular localization of RAS components in rat and human DRG, and the relationship of Ang II and Substance P expression in these sensory tissues.

2. Methods

2.1 Rat and human dorsal root ganglia and sampling procedures

We purchased 8-week-old, male Wistar Kyoto (WKY) rats (approximately 200 g body weight) from the Central Animal Facilities of the University of Bern. Adequate measures were taken to minimize pain or discomfort, in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC) and in accordance with Animal Protocols approved by the Animal Care and Use Committee, NIMH, NIH, USA. Rats were anaesthetized intraperitoneally with 100 mg/kg thiopentane sodium and were perfused transcardially with 150 ml Ringer solution containing 1000 U heparin at 37 °C followed by 300 ml 2 % freshly prepared formaldehyde at 4 °C. Thoracic dorsal root ganglia (DRG) were carefully removed and incubated by immersion fixation in 2 % formaldehyde for 28 h at 4 °C. Subsequently, tissues were immersed for 14 h in phosphate-buffered saline (PBS-Dulbecco) containing 18 % sucrose at 4 °C, embedded with M-1 embedding matrix (Thermo Shandon). Then 30 μm thick sections were cut on a cryostat (at –15 ^{o}C) and subsequently used as free-floating sections for immunocyto-chemistry. For some experiments after perfusion and immersion fixation the rat ganglia were embedded in paraffin. Paraffin sections, 7 μm thick, were used for immunocytochemical as well as for *in situ* hybridization experiments.

For extraction of total RNA, rats were shortly anesthetized with halothane and subsequently sacrificed by decapitation. For extraction of total RNA, fresh rat thoracic DRG, liver, lung, adrenal glands and kidneys were dissected and instantly transferred into RNA later (Ambion), frozen in liquid nitrogen, and then processed for total RNA extraction (Ambion).

For specific measurement of different angiotensin peptides separated by high performance liquid chromatography (HPLC) prior to highly sensitive radioimmunoassay, rat thoracic DRG

were rapidly removed, rinsed with cold Ringer solution, blotted by filter paper and wet weight was measured. The ganglia were frozen in liquid nitrogen and stored at –70 °C.

Human thoracic DRG were procured from adult human individuals for whom a permit for clinical autopsy (informed written consent by next of kin) had been obtained according to state law, in accordance with the Code of Ethics of the World Medical Association (Declaration of Helsinki). Human DRG were fixed by immersion in freshly prepared 2 % formaldehyde for 3 days and then used for cryosectioning or embedded in paraffin. To perform HPLC-RIA the same method as described for rats was used.

2.2 RNA isolation and quantitative real time PCR (qRT-PCR)

Fresh rat DRG were dissected as mentioned above and instantly transferred into RNA later (Ambion), frozen in liquid nitrogen, and then processed for total RNA extraction (Ambion). RNA integrity was confirmed for each sample on the Agilent Bioanalyzer using the RNA 6000 Nano kit (Agilent Technologies). 1 µg of total RNA was reverse transcribed using Superscript II (Invitrogen) and random hexamers according to the manufacturer's protocol. For qRT-PCR, reverse transcribed material corresponding to 40 ng RNA was amplified with the TaqMan assays described below in 25 µl Universal PCR Master Mix, No AmpErase UNG on the SDS 7000 (Applied Biosystems) using the standard thermal protocol. Average values and standard deviations of relative mRNA levels of each sample, normalized to relative 18S rRNA levels, are from four measurements and were calculated using the relative differences. Values were expressed as percent of values obtained from liver for Ang-N and cathepsin D, kidney for renin, lung for ACE, and adrenal gland for angiotensin receptors.

The following TaqMan assays were used for qRT-PCR at a final concentration of 250 nM TaqMan probe and 900 nM of each primer:

Angiotensinogen

Forward primer 5'-CACGACTTCCTGACTTGGATAAAGA-3';

Reverse primer 5'-CTGCGGCAGGGTCAGA-3';

TaqMan probe 5'-FAM CCTCGGGCCATCC G MGB- 3';

manufactured as Assays-by-Design (RATG-EJ3) by Applied Biosystems.

Renin Assay-on-demand Rn00561847_m1 from Applied Biosystems.

ACE Assay-on-demand Rn00561094_m1 from Applied Biosystems.

Cathepsin D Assay-on-demand Rn00592528_m1 from Applied Biosystems.

AT_{1A} Assay-on-demand Rn01435427_m1 from Applied Biosystems.

AT_{1B} Assay-on-demand Rn02132799_s1 from Applied Biosystems.

AT2

Forward primer 5' GTGGGAAGCTCAGTAAGCTGATTTA -3';

Reverse primer 5' GTCAGAGACTCCCAATCCTTACAC -3';

TaqMan probe 5'FAM ACACTGGCAACTAAAAGA MGB- 3'

manufactured as Assays-by-Design (RATG-EJ3) by Applied Biosystems.

18S rRNA Predeveloped Assay Reagent 431-9413E from Applied Biosystems.

2.3 *In situ* hybridization

2.3.1 DIG-labelled RNA probe preparation

By using an appropriate cDNA template for Ang-N (**Lynch et al., 1986**), a 403 bp long fragment corresponding to nucleotides 221-623 was generated by digestion with restriction enzymes *EcoRV* and *Bam HI*. The obtained fragment was cloned between *Stu I* and *Bam HI* into pBluescript I KS+ (Stratagene). Digoxigenin-labelled probes were prepared using the DIG-RNA-labelling mix (Roche) according to the manufacturer's protocol. T7 RNA polymerase was used to

generate antisense riboprobe using the *Hind III* linearized template, and the sense strand (used as a control) was generated by T3 RNA polymerase using the same template linearized with *Bam HI*. The RNA concentration was estimated comparing dot-blot tests and NanoDrop measurements. Filter hybridization with RNA extracts from DRG (RNaqueous-4PCR kit from Ambion) was used for binding tests.

2.3.2 Procedure for *in situ* hybridization

For *in situ* hybridization, 6 µm thick paraffin sections from rat DRG were rehydrated in a graded alcohol series (2 times Xylol for 10 min, EtOH 100 % (2 times), 96 %, 70 %, 50 %, each step 5 min) using DEPC-treated H_2O for the dilution of all reagents and solutions. The sections were equilibrated in proteinase K buffer (100 mM Tris, 50 mM EDTA, pH 7.5) for 5 min and then treated with proteinase K (19 µg/ml) at 37 ºC for 2 min, after a wash with DEPC-treated H_2O. Following a wash in DEPC-water, sections were post fixed with freshly prepared 4 % formaldehyde for 5 min, followed by two subsequent washes in DEPC-water for 5 min each. Sections were then incubated in prehybridization solution (Sigma) at 45 °C for 2 h, followed by incubation with heat denatured sense and antisense riboprobes (5-10 ng/µl) in 30 µl hybridization mix (Sigma) for 48 h at 45 °C in a humid chamber (saturated with 2x SSC). Subsequently, the sections were incubated with 2x SSC for 30 min at room temperature, followed by 1 h in 2x SSC at 45 °C and 1 h in 0.1x SSC at 45 °C. Sections were equilibrated for 5 min with buffer 1 (100 mM Tris, 150 mM NaCl, pH 7.5), then incubated with alkaline phosphatase-coupled anti-digoxigenin antibody (Roche) 1:500 diluted in buffer 2 (10x blocking solution diluted with buffer 1) for 2 h at room temperature, followed by two 5 min washes with buffer 1. Finally, after 5 min equilibration with buffer 3 (100 mM Tris, 100 mM NaCl, 50 mM $MgCl_2$, pH 9.5), the color

reaction containing nitro blue tetrazolium (NBT) and 5-Bromo-4-chloro-3-indolyl phosphate (BCIP) in buffer 3 was completed according to manufacturer's protocol (Sigma).

2.4 Immunocytochemistry

For immunocytochemical incubations for rat and human DRG, a protein G purified murine monoclonal antibody against Ang II (Mab-Trap G II column, Amersham Sciences) was used at a concentration of 0.3 µg/ml in buffer solution. This is a self generated monoclonal antibody against the synthetic peptide to Ang II. The specificity for the monoclonal antibody against Ang II (4B3) has been previously documented and it produced the same staining as a polyclonal antibody against Ang II in rat adrenal glands (**Frei et al., 2001**). For immunization of the mice, Ang II peptide was cross-linked with glutaraldehyde to keyhole limpet hemocyanin. In dot blot assay, the monoclonal antibody against Ang II (4B3) showed total cross reactivity with Ang III (2-8), Ang 3-8, Ang 4-8 and Ang 5-8. It showed no cross reaction with human plasma Ang-N, Ang I (1-10) and Ang 1-7. Pre-absorption was performed by using a batch procedure with the synthetic Ang II-peptide that was covalently linked through its N-terminus to CH-Sepharose 4 B. The gel was loaded into a glass column and the monoclonal anti Ang II-antibody solution effluent was used for immunocytochemical preabsorption controls. Further controls were done with mouse non-immune serum and for the secondary goat anti mouse Cy3 (GAM^{Cy3}) antibody without the primary antibody. All these additional controls showed absolute no staining within the sections. The pre-absorbed monoclonal antibody after low pH-elution revealed the same staining as the Mab-Trap G II purified antibody.

Substance P was investigated with a mouse monoclonal antibody to the synthetic peptide Substance P from Novus Biologicals (SP-DE4-21). Goat anti-mouse immunoglobulins (GAM Cy3; Jackson Immuno Research) pre-incubated with rat serum prior to immunocytochemistry

were used as secondary antibodies. Sections were incubated free floating for 36 h at 4 ºC with the primary antibody, washed and incubated with the secondary antibody for 90 min at room temperature and counterstained with 4',6-diamidino-2-phenylindole (DAPI). After washing, cryosections were mounted on gelatin-coated slides and air-dried. The stained sections were embedded with Glycergel (DAKO) and cover-slipped. The same procedure as for free floating cryosections has been used for paraffin sections mounted on glass slides. Pictures were taken by a normal fluorescent light microscope (LEICA DM 6000B) using a digital camera or by a confocal laser scanning microscope (LEICA SP2 with AOBS).

2.5 Post *in situ* hybridization immunocytochemistry

Paraffin sections of rat DRG underwent *in situ* hybridization incubation and staining. Afterwards the same sections were processed for immunocytochemistry as mentioned above.

2.6 Colocalization studies for Ang II and Substance P

Directly adjacent free-floating cryosections of rat DRG were immunostained for Ang II and Substance P, as mentioned under Methods. Pictures were taken by a normal fluorescent light microscope (LEICA DM 6000B) using a digital camera LEICA-DFC350FX.

2.7 Measurement of angiotensin peptides

Ang-(1-8) octapeptide (Ang II) , Ang-(2-8) heptapeptide, Ang-(3-8) hexapeptide and Ang-(4-8) pentapeptide were measured in rat and human DRG by RIA after prior separation by HPLC (Nussberger et al., 1986). Measurement of true angiotensin was applied also for Ang-(1-10) decapeptide (Ang I), and peptide extraction from tissue was adapted for additional freeze-thaw procedures to ensure the lysis of cells (**Nussberger et al., 1985; Nussberger, 2000**). Briefly,

DRG were homogenized in 1.5 ml water, shock frozen in ethanol/dry ice, thawed in a 37 °C water bath. The freeze-thaw procedure was repeated twice. Samples were sonicated for 10 min at 37 °C, centrifuged at 3500 g and the supernatant in an albumin-coated polypropylene tube was dried at 37 °C under nitrogen. The residue was dissolved in 2.2 ml Tris buffer (0.1 M, pH 7.5) containing bovine serum albumin (5 g/l). Solid-phase extraction on phenylsilylsilica, HPLC and radioimmunoassay were performed according to the routine procedures using very sensitive antisera for Ang II and Ang I with cross-reactivities of 53 % for Ang-(2-8) heptapeptide as well as for Ang-(3-8) hexapeptide and 52 % for Ang-(4-8) pentapeptide (**Nussberger et al., 1986; Nussberger, 2000**). Recoveries for the different angiotensin peptides and for ir-Ang II were consistently above 67 %. Therefore, no corrections were made for recovery losses. Results are presented as fmol Ang per gram wet weight. Detection limits were variable according to the various weights of individual ganglia. Instead of homogenates, controls of 1.5 ml water were extracted and quantitated with every analysis and any traces of Ang peptides found in these “blanks” were subtracted to obtain final ganglial concentrations.

3. Results

3.1 Quantitative determination of RAS components in the rat and human dorsal root ganglia

3.1.1 Quantitative real time RT-PCR

We detected significant amounts of Ang N-, ACE and cathepsin D mRNA in the rat DRG (**Fig. 1a**, **c** and **d**, respectively). No renin mRNA was detected in the same DRG extracts (**Fig. 1b**).

Receptor studies revealed AT_{1A} receptor mRNA expression in rat DRG (**Fig. 2a**) whereas no expression of AT_{1B} receptor mRNA could be discovered (**Fig. 2b**). Also the presence of AT_2 receptor mRNA could be shown (**Fig. 2c**).

3.1.2 HPLC and RIA of angiotensin peptides

Quantitative measurement of Ang II and related peptides in individual DRG was possible for human DRG, but not for the tiny rat DRG (detection limit too high). Ang II and related peptides (Ang 1-10, Ang 2-8, Ang 3-8 and Ang 4-8) were extracted, separated by HPLC and quantitated by RIA as described in Methods. Results of 4 human DRG are summarized in table 1. They are expressed as fmol/g wet weight. Ang I was found to be twice and three times above detection limit in two DRG but absent in the remaining DRG. Ang II was consistently found at 1.3-, 9-, 13- and 42-fold above detection limit, respectively. No metabolites of Ang II were found (all below detection limits).

In rat DRG, only after pooling of six individual samples, merely Ang II was detected at 1.3-fold above detection limit (data not shown).

3.2 Localization of Ang-N mRNA and Ang II in rat dorsal root ganglia

3.2.1 *In situ* hybridization

Ang-N mRNA was detected in most, if not all the neurons studied in the rat DRG with the antisense probe, indicating intracellular localization of Ang-N mRNA (**Fig. 3b**). No staining was produced using the sense probe as a control (**Fig. 3a**).

3.2.2 Immunocytochemistry

Ang II immunoreactivity was present in many, but not all, neurons of the rat DRG, with a cytoplasmic localization and a variable intensity (**Fig. 4**).

In the human DRG we detected Ang II immunoreactivity in the cytoplasm of many neurons showing the intracellular localization of Ang II (**Fig. 5a**) and in their projections (**Fig. 5b**) with free-floating cryosections. Interestingly we could also detect the presence of synapses en passant in fibers within the DRG (**Fig. 5c and 5d**; Supplemental material, **movie 2** and **3**).

3.2.3 Post *in situ* hybridization immunocytochemistry

We used rat DRG paraffin sections for *in situ* hybridization followed by immunocytochemistry to determine the intracellular colocalization of Ang-N mRNA (**Fig. 6a**) and Ang II (**Fig. 6b**). We found that most but not all the Ang-N mRNA positive neurons were stained with the antibody recognizing Ang II as indicated by "*".

3.2.4 Colocalization studies for Ang II and Substance P

In directly adjacent free-floating cryosections of rat DRG, we detected a number of neurons revealing intracellular colocalization of Ang II with Substance P (**Fig. 7**). A higher number of neurons expressed immunoreactive Ang II (**Fig. 7a**) than immunoreactive Substance P (**Fig. 7b**).

4. Discussion

The major findings of our study are the demonstration and quantitation of intraneuronal Ang II in rat and/or human sensory DRG and the presence of Ang-N, ACE and cathepsin D mRNA, Ang II and related peptides, Ang II AT_{1A} and AT_2 receptor mRNAs; the neuronal colocalization of Ang-N mRNA and Ang II, and the neuronal colocalization of Ang II and Substance P in rat DRG.

We have detected significant amounts of Ang II in both the rat and human DRG, a clear localization of intraneuronal Ang II. In addition, we detected mRNA for the Ang II precursor Ang-N, and for ACE, in the rat DRG. Furthermore, combination of *in situ* hybridization and immunohistochemistry revealed colocalization of Ang-N mRNA and Ang II immunoreactivity in most DRG neurons of the rat. The coexistence of Ang II and mRNA for Ang-N, the only known precursor for Ang I production and Ang II in the same neurons demonstrates that Ang-N uptake from the circulation may not be necessary for the local intraneuronal formation of Ang II. While in the brain, Ang-N is localized and transcribed in multiple cell types, predominantly in astrocytes but also in neurons and cerebral endothelial cells (**Imboden et al., 1987; Stornetta et al., 1988; Yang et al., 1999; Zhou et al., 2006**), our study demonstrates the presence of Ang-N mRNA specifically in the cytoplasm of neuronal cell bodies of the DRG, and not in surrounding cells.

It is of note that Ang-N mRNA in addition to its colocalization with neuronal Ang II was present in neurons without certain Ang II immunoreactivity. It is possible that in some neurons Ang II may be produced in amounts too low to be detected with our immunocytochemical methods. Alternatively, Ang-N may participate, in some neurons, in functions unrelated to the RAS system (**Sernia et al., 1997; Morgan et al., 1996**).

Surprisingly, and in contradiction to a previous report in bilaterally ovariectomized rat DRG (**Chakrabarty et al., 2008**) we did not detect mRNA for renin, the enzyme classically considered necessary for cleaving Ang-N into the Ang I precursor of Ang II (**Paul et al., 2006**) in our wild type rat DRG samples. Instead, we found substantial expression of cathepsin D mRNA, at levels higher in comparison with those found in rat liver and kidney, but lower in comparison to those recently reported in rat coeliac and trigeminal ganglia (**Patil et al., 2008; Imboden et al., 2009**). Cathepsin D is a protease capable of cleaving Ang-N into angiotensin I (**Saye et al., 1993**), a non-renin alternative pathway for Ang II formation. Our results are compatible with an alternate pathway for Ang II synthesis in DRG sensory neurons. Such and alternate pathway for neuronal Ang II generation would allow to maintain the hypothesis that kidney renin is the main, if not the only source of renin in mammals (**Von Lutterotti et al., 1994**). Nevertheless, local Ang II formation through a renin-dependent pathway may still occur, if circulating renin and (pro) renin are taken up and activated by the recently discovered renin-receptor (**Danser, 2009**), but the presence of (pro)renin receptors in DRG has not been reported so far.

Taken together, our results suggest local Ang II generation and storage in the DRG similar to earlier published evidence for the rat and human sympathetic coeliac and sensory trigeminal ganglia (**Patil et al., 2008; Imboden et al., 2009**). Our findings suggest that in the DRG, Ang II may be a principal effector for neuronal functions and that a non-renin enzyme like cathepsin D may cleave Ang-N to form Ang I. Neuronal Ang II demostrated to occur intracellularly may take part in an angiotensinergic system, i.e. Ang II may act as a neurotransmitter.

We detected the expression of Ang II receptor mRNA in our DRG samples. The physiological receptor for Ang II is the AT_1 receptor type (**Iwai et al., 1992**). In rodents, there are two AT_1 receptor subtypes, differentially expressed and regulated (**Iwai et al., 1992**). AT_{1A} receptors are widely distributed and their stimulation carries out most of the physiological effects

of Ang II in rodents (**Llorens-Cortes et al., 1994; Burson et al., 1994; Jöhren et al., 1995**). AT_{1B} receptor distribution is more restricted, principally to the pituitary and adrenal glands and the hippocampus in adult rodents (**Llorens-Cortes et al., 1994; Burson et al., 1994; Jöhren et al., 1995**). The AT_{1A} and AT_{1B} receptor subtypes cannot be distinguished pharmacologically because of the high homology of their translated regions and similar agonist affinities; however they can be identified by qRT-PCR or *in situ* hybridization with the use of selective primers or probes for untranslated, non-homologous regions (**Llorens-Cortes et al., 1994; Burson et al., 1994; Jöhren et al., 1995**). Using specific primers with qRT-PCR, we detected AT_{1A} mRNA, but not AT_{1B} mRNA expression in our rat DRG samples. Receptor expression in DRG is an indication that Ang II, either circulating or locally formed, may exert effects in this tissue. Our results confirm previous observations of high AT_{1A} expression in selective DRG neurons, as determined by *in situ* hybridization (**Pavel et al., 2008**), and high AT_1 receptor immunoreactivity in DRG neurons (**Tang et al., 2009**).

In our samples we detected low expression of Ang II AT_2 receptor mRNA, a receptor type whose function is still controversial (**Saavedra, 1999; De Gasparo and Siragy, 1999; Saavedra, 2005; Porrello et al., 2009**). Expression of AT_2 receptor mRNA in DRG, as reported recently by *in situ* hybridization and receptor binding, was very variable and much lower than that of AT_{1A} receptor mRNA (**Pavel et al., 2008**). Nevertheless, the presence of specific AT receptors in DRG increases the likelihood of a neuronal function of Ang II.

We have detected Ang II-like immunoreactivity not only in neuronal cell bodies but also in neuronal projections within the rat DRG, and intense immunoreactivity in fibers with synapses en passant in the human DRG. There, neuronal Ang II may act as a neurotransmitter at presynaptic sites to regulate sensory transmission, a function similar to that demonstrated in the sympathetic nervous system and in sensory system of trigeminal ganglia (**Patil et al., 2008; Imboden et al.,**

2009). The presence and importance of presynaptic Ang II receptors in sympathetic nerves, spinal cord motor neurons, the neuromuscular junction and central sensory fibers has long been recognized (**Starke, 1977; Huang et al., 2003; Oz et al., 2005; Oliveira et al., 2007**).

Although the functions of Ang II in the DRG system have not been extensively studied and therefore are not fully clarified, our results are not without clinical interest. Ang II has been proposed to be involved in the regulation of sensory information, and in particular nociception (**Takai et al., 1996; Irvine and White, 1997; Pelegrini-da-Silva et al., 2005; Pechlivanova and Stoynev, 2007; Marques-Lopes et al., 2009**). Hence, we attempted to determine whether Ang II was associated with other nociceptive-controlling neuropeptides in the DRG. We selected Substance P, a neuropeptide firmly established as a key regulator of sensory transmission, and in particular nociception, in the DRG (**Otten et al., 1982; Badalamente et al., 1987; Suarez-Roca and Maixner, 1995; Nichols et al., 1999; DeVane, 2001**). A relationship between Substance P and Ang II was proposed earlier, since Ang II was shown to regulate Substance P release (**Diz et al., 1986; Kopp et al., 2003; Fusayasu et al., 2007**). We found that Ang II was colocalized with Substance P in DRG neurons, supporting the hypothesis of a close integration between these two systems.

Sensory denervation exacerbates the development of hypertension and impairs renal excretory function when a suppressor dose of Ang II is given. These results indicate that activation of sensory nerves, either by Ang II or by other hormonal or hemodynamic factors, plays a compensatory role in promoting urine and sodium excretion and attenuating elevated blood pressure initiated by Ang II (**Wu et al., 2000**). Thus, the role of Ang II in sensory function may also be related to its well-known regulation of cardiovascular function.

In conclusion, our results support the hypothesis of intraneuronal Ang II formation in the rat and human DRG, and are compatible with an interaction between Ang II and Substance P. We

hypothesize that these two peptides interact in the DRG in the management of sensory, and particularly nociceptive information.

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Figure legends

Fig. 1. Determination of relative levels of Ang-N-, renin-, ACE- and cathepsin D mRNA by qRT-PCR in rat thoracic DRG. Reverse transcribed total RNA extracts of rat thoracic DRG were studied by qRT-PCR for the presence of Ang-N mRNA (a), for renin mRNA (b), for ACE mRNA (c) and cathepsin D mRNA (d) and values expressed as percent of those obtained from liver, kidney and lung as described in Methods. Total RNA extracts from liver (a), kidney (b), lung (c) and liver (d) were used as a reference samples expression. All relative mRNA values are normalized to 18S rRNA levels. Average values and standard deviations of four qRT-PCR individual measurements are shown.

Fig 2. Determination of relative levels of Ang II AT_{1A} (a), AT_{1B} (b) and AT_2 (c) receptor mRNA in rat DRG. Reverse transcribed total RNA extracts of rat thoracic DRG were considered by qRT-PCR for the presence of Ang II AT_{1A}, AT_{1B} and AT_2 receptor mRNA as described in Methods. Values are expressed as percent of those obtained from adrenal gland. All relative mRNA values are normalized to 18S rRNA levels. Average values and standard deviations of four qRT-PCR individual measurements are shown.

Fig. 3. *In situ* hybridization in rat thoracic DRG for detection of Ang-N mRNA. 6 µm thick consecutive paraffin sections were processed as described in Methods. a) sense probe. b) antisense probe, revealing positive staining in the cytoplasm of numerous neurons within the ganglion. Bar: 100 µm.

Fig. **4**. Angiotensin II immunocytochemistry in rat thoracic DRG. 30 µm thick free-floating cryosections were prepared and incubated with our murine monoclonal anti Ang II antibody (**Frei et al., 2001**), stained with GAM^{Cy3} in red and DAPI in blue for nuclei, as described under Methods. Note, the intensity of the staining for intracellular Ang II varies among the neurons. Some neurons are not stained for Ang II (see **x**). Bars: 100 µm (a) and 50 µm (b).

Fig. **5**. Ang II immunocytochemistry in human DRG. 30 µm thick free-floating cryosections were prepared and incubated with our murine monoclonal anti Ang II antibody (Frei et al., 2001), stained with GAM^{Cy3} in red and DAPI in blue for nuclei, as described under Methods. Note, intracellular Ang II staining in neuronal cytoplasm (a) and projections (b). Autofluorescence of lipofuscin granules is marked with an asterisk (a). Additionally to thick fiber pathways there can be seen fine fibers with synapses en passant (c) and synapses en passant zoomed in of panel c (d). Arrows are pointing to synapses en passant. The pictures were taken with a confocal laser scanning microscope. Bars: in (a) 10 µm; in (b) 50 µm; in (c) 25 µm and in (d) 10 µm. For better illustration of figures, see movie 1 for Fig. 5a and see movie 2 and 3 for Fig. 5c and Fig. 5d in supplemented material.

Fig. **6**. Intracellular colocalization of Ang-N mRNA and Ang II immunoreactivity in the rat DRG. A 7 µm thick paraffin section was processed for *in situ* hybridization with the antisense probe to detect Ang-N mRNA (a) followed by immunocytochemistry for Ang II (b) as described in Methods. Not all cells expressing Ang-N mRNA are colocalized with Ang II positive cells, see asterisks in (b). Bar: 20 µm.

Fig. **7**. Colocalization of Ang II and Substance P immunocytochemistry in rat thoracic DRG. Directly adjacent, 30 µm thick free-floating cryosections were incubated with our murine monoclonal Ang II (a) or anti Substance P (b) antibodies and stained with GAM^{Cy3} as secondary antibodies in red and DAPI in blue as nuclear stain, as described under Methods. Arrows show colocalization, “x” indicate absence of colocalization while asterisks indicate absence of staining for Ang II as well as for Substance P. Bar: 50 µm.

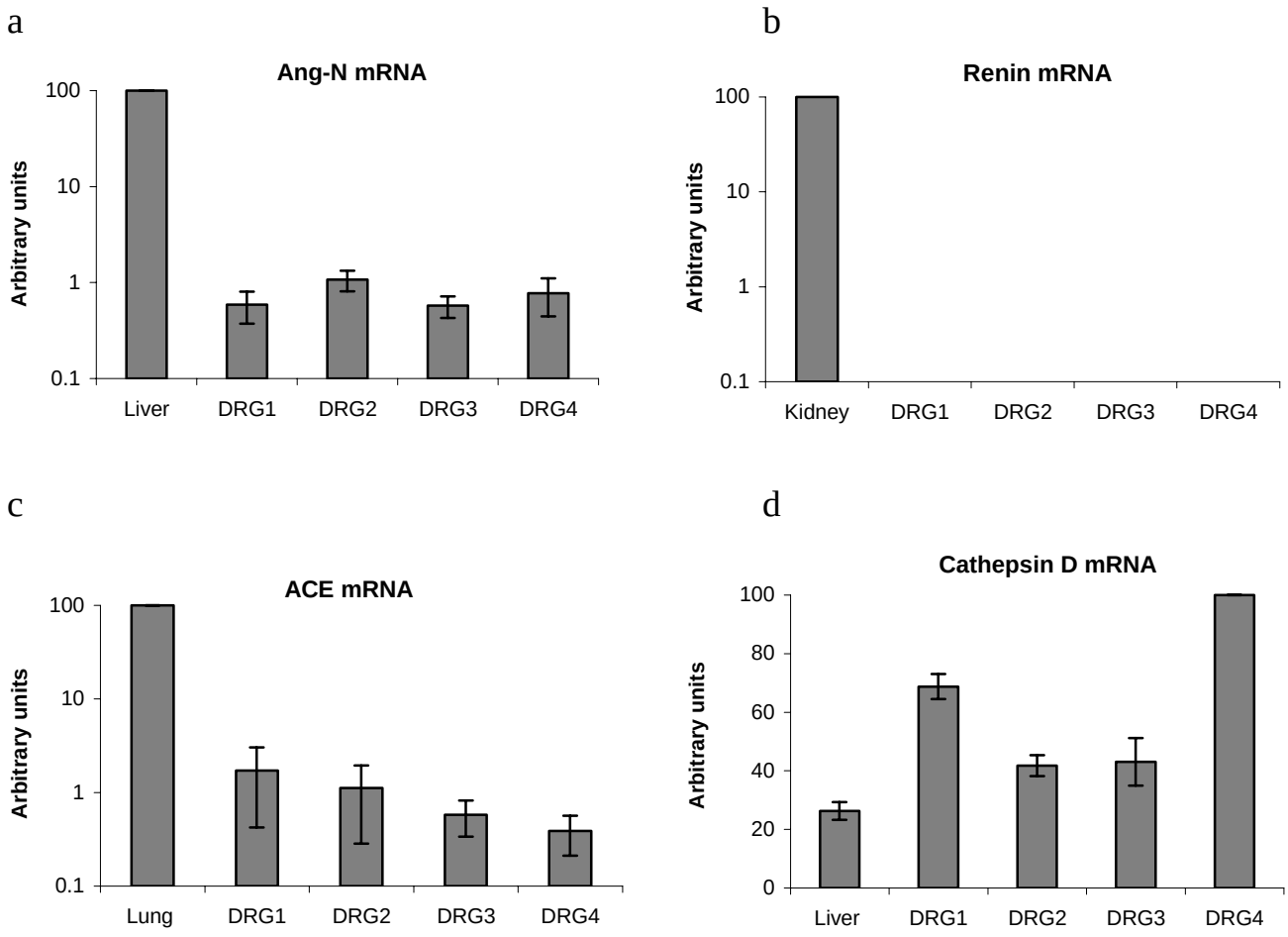
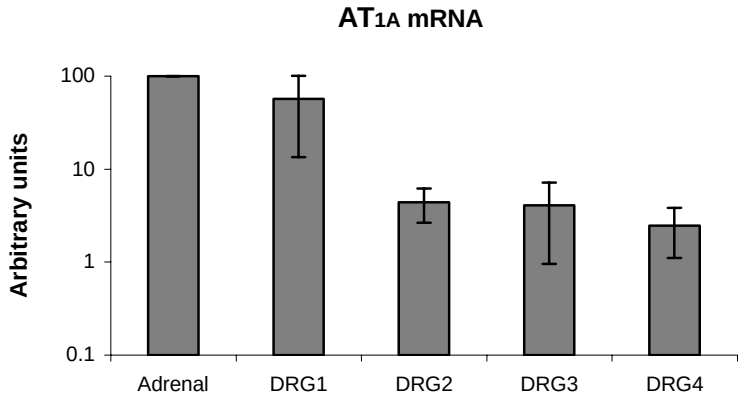
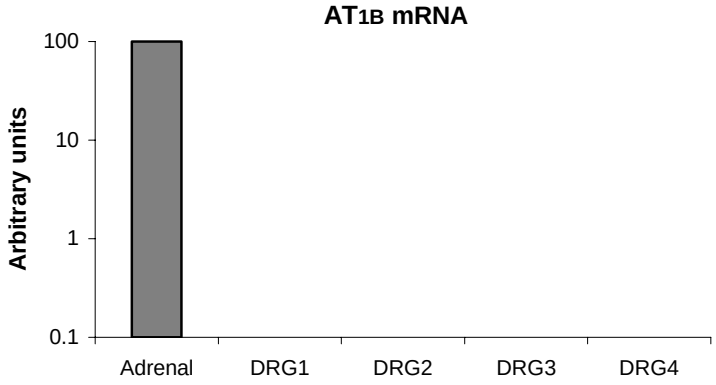

a
Ang-N mRNA
Arbitrary units
100
10
1
0.1
Liver
DRG1
DRG2
DRG3
DRG4
b
Renin mRNA
Arbitrary units
100
10
1
0.1
Kidney
DRG1
DRG2
DRG3
DRG4
c
ACE mRNA
Arbitrary units
100
10
1
0.1
Lung
DRG1
DRG2
DRG3
DRG4
d
Cathepsin D mRNA
Arbitrary units
100
80
60
40
20
0
Liver
DRG1
DRG2
DRG3
DRG4

Figure 1

a


AT1A mRNA
Arbitrary units
100
10
1
0.1
Adrenal
DRG1
DRG2
DRG3
DRG4

b


AT1B mRNA
Arbitrary units
100
10
1
0.1
Adrenal
DRG1
DRG2
DRG3
DRG4

c

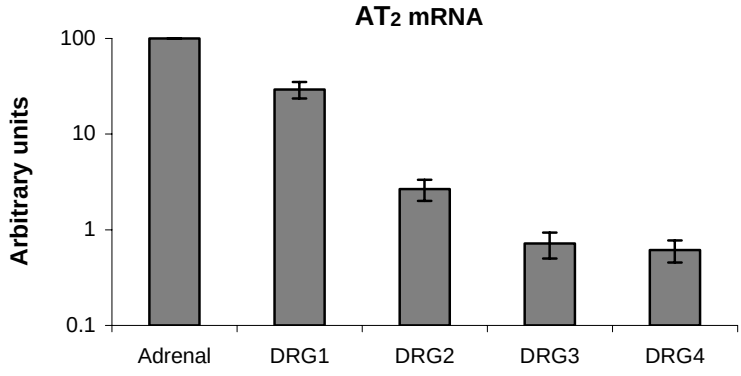

AT2 mRNA
Arbitrary units
100
10
1
0.1
Adrenal
DRG1
DRG2
DRG3
DRG4

Figure 2

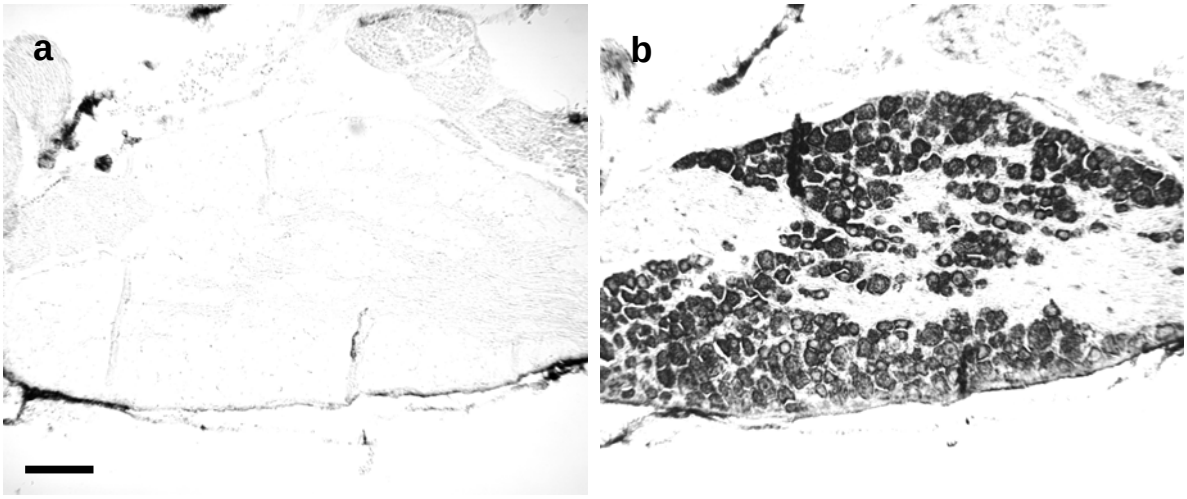


Figure 3

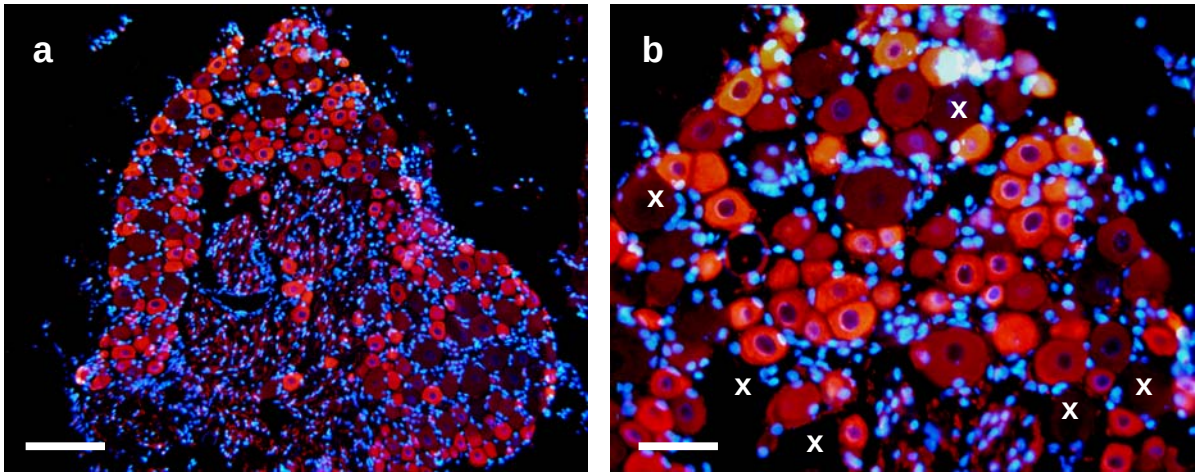


Figure 4

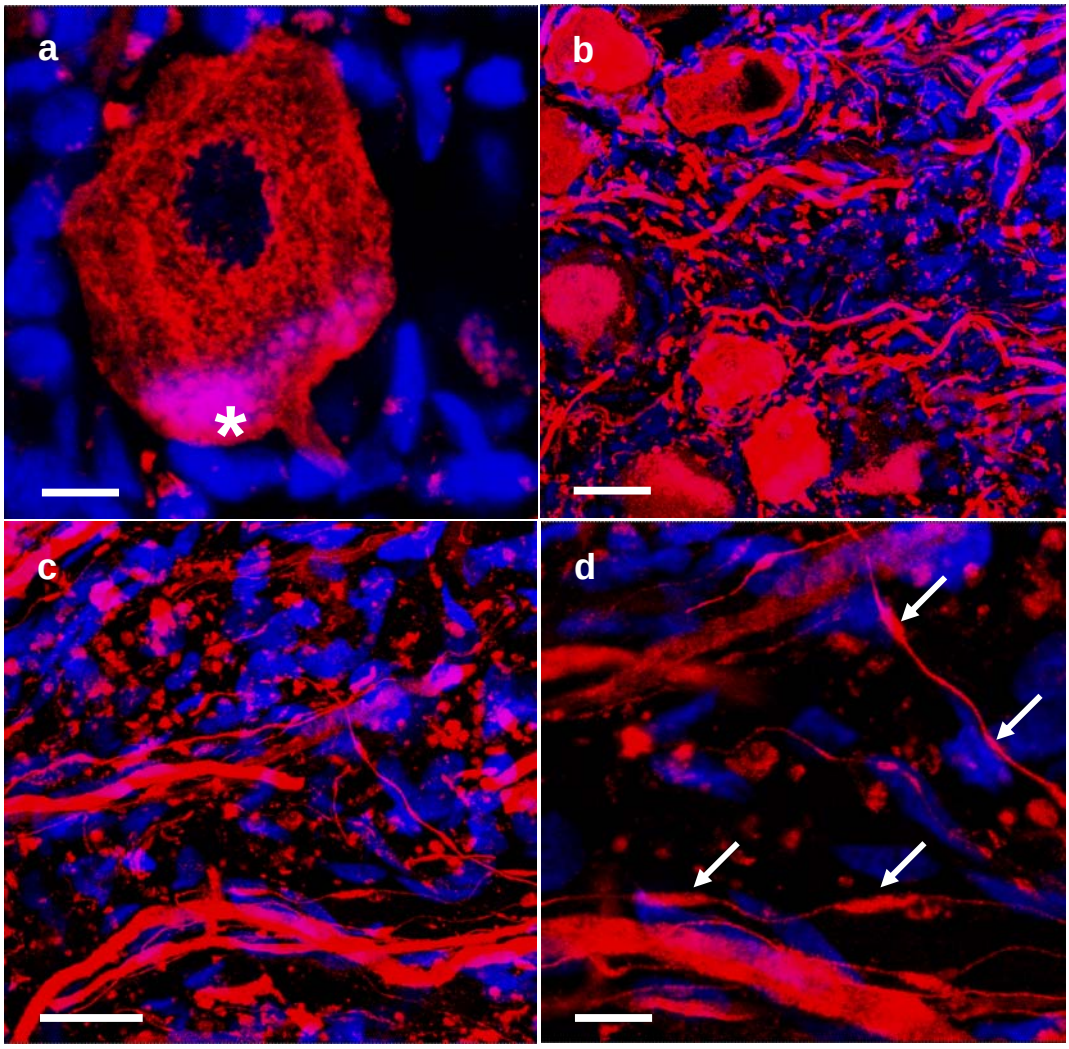


Figure 5

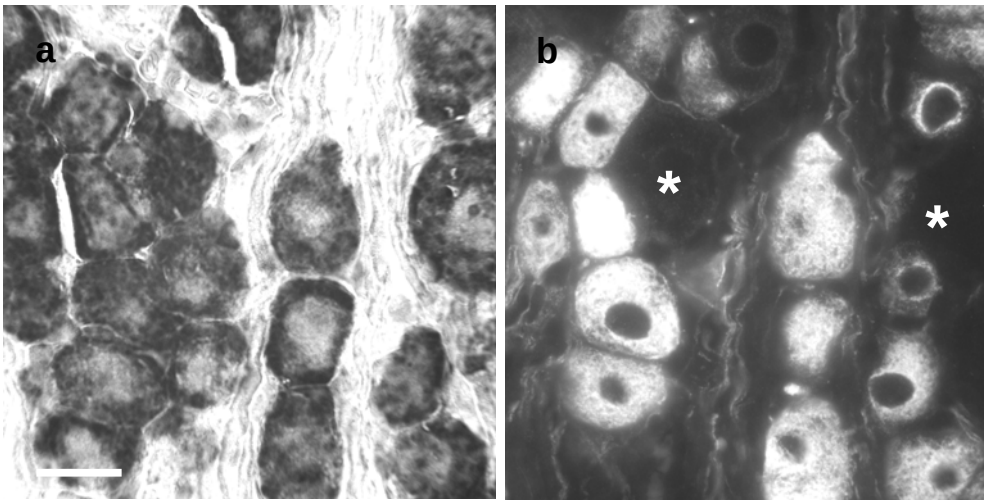


Figure 6

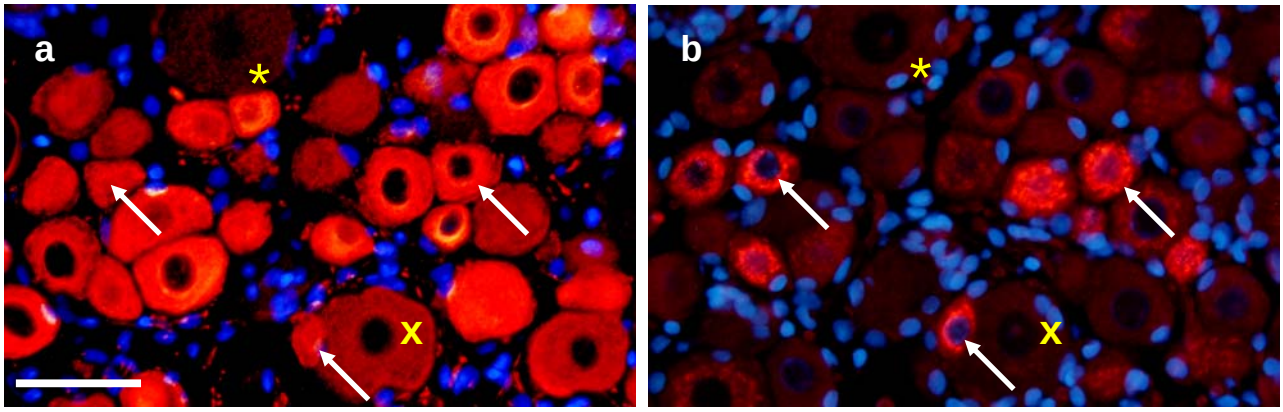


Figure 7

Angiotensin peptides in human spinal ganglia (fmol/gram wet weight)

Spinal Ganglia	Weight	Ang-(1-10)*	Ang-(1-8)**	Ang-(2-8)	Ang-(3-8)	Ang-(4-8)
1	200.4 mg	< 2.4	50.6 (<1.2)	< 2.3	< 2.3	< 2.3
2	76.2 mg	< 6.3	4.1 (<3.2)	< 6.0	< 6.0	< 6.2
3	132.6 mg	7.2 (<3.6)	23.2 (<1.8)	< 3.4	< 3.4	< 3.5
4	102.4 mg	12.3 (<4.7)	20.2 (<2.3)	< 4.3	< 4.3	< 4.4

Detection limit given in brackets. * Angiotensin I. **Angiotensin II

Table 1

5. An intrinsic, neuronal angiotensinergic and noradrenergic system in the rat and human heart

Jaspal Patil, Silvan Stucki, Thomas Schaffner, Juerg Nussberger, Hans Imboden.

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An intrinsic, neuronal angiotensinergic and noradrenergic system in the rat and human heart

Jaspal Patil[a], Silvan Stucki[a], Juerg Nussberger[b], Thomas Schaffner[c], Hans Imboden[a]*

[a]Institute of Cell Biology, University of Bern, Bern, Switzerland
[b]Department of Internal Medicine, University Hospital, Lausanne, Switzerland
[c]Institute of Pathology, University of Bern, Bern, Switzerland

* Corresponding author:
Prof. Dr. Hans Imboden
Institute of Cell Biology, University of Bern
Baltzerstrasse 4, 3012 Bern, Switzerland
Phone: +41-31-631 48 99
Fax: +41-31-631 46 16
Email: hans.imboden@izb.unibe.ch

Abstract

To elucidate the existence of Angiotensin II (Ang II) in tissue homogenate and at cellular resolution in the heart, we studied the expression of different components of the renin angiotensin system (RAS) in different parts of the rat and human heart. We investigated angiotensinogen (Ang-N)-, renin-, angiotensin converting enzyme (ACE)-, cathepsin D-, angiotensin receptor AT_{1A}-, angiotensin receptor AT_{1B}- and angiotensin receptor AT_2-mRNA as well as the presence of Ang II and dopamine β hydroxylase (DβH). The rat heart expressed substantial amounts of Ang-N and ACE mRNA as determined by quantitative real time PCR. Renin mRNA was untraceable in rat heart samples. Cathepsin D was detected in the rat heart indicating the possibility of existence of pathways alternative to renin for Ang II formation. In situ hybridization in rat heart revealed expression of Ang-N mRNA in the cytoplasm of numerous intracardiac neurons in the atria. By using immunocytochemistry, a number of neurons in the atria and their processes in both the rat and human heart were stained for Ang II and DβH. In most of the neurons DβH was found together with Ang II, indicating a partial colocalization of the sympathetic and angiotensinergic system. We observed angiotensinergic synapses en passant in rat and human heart. Angiotensins from rat and human heart were quantitated by radioimmunoassay with and without prior separation by high performance liquid chromatography, showing angiotensin peptides in different parts of rat and human hearts. The colocalization of neuronal Ang II with DβH, these intracardiac neurons may be the basis for a participation and function of Ang II in the blood pressure regulation locally and the adjustment of the contractile force of the atria and the ventricles. In conclusion, our results suggest that Ang II could be produced intrinsically in the rat and human intracardiac neurons and may act as a neurotransmitter within the heart.

Key words – neuronal angiotensin II, noradrenergic system, intracardiac ganglia, heart

1. Introduction

The renin angiotensin system (RAS) has been studied widely in the heart (**Paul et al., 2006**). According to many researchers localization and function of RAS components in the heart is still an issue of controversies, because of the circulating and local generation of Ang II (**Singh 2008, Paul et al., 2006**). Under supraphysiological conditions, Ang II acts as an inotropic agent in heart (**Danser and Saris, 2002**). In human preparations, in vitro studies done under physiological conditions in right atrial and right and left ventricular myocardial preparations demonstrated positive inotropic action of Ang II only in atrial preparations (**Holubarsch et al., 1993**).

The RAS and the sympathetic nervous system (SNS) both contribute in the progress and maintenance of high blood pressure, as well as interaction between circulating RAS and SNS has also been reported (**Rupp and Jäger, 2001**). The heart is innervated by sympathetic and parasympathetic nerves (**Kukanova and Mravec, 2006**). In addition to the conducting system, the cardiac nervous system also influences heart rate, coronary blood flow, arterial and ventricular behavior and is essential for proper function of the cardiac valve systems (**Roper and Taylor, 1982**). The cardiac nervous system is comprised of spatially distributed sensory, interconnecting and motor (adrenergic and cholinergic) neurons. Explantation of donor heart for the heart transplantation leads to autonomic denervation as axonal degeneration (**Überfuhr et al., 2000**). Several evidences are reported for sympathetic and parasympathetic reinnervation of heart after transplantation (**Tio et al., 1997, Armour, 2008**).

In addition to the circulating RAS, strong evidence exists for the existence of local RAS which is entirely independent from circulating RAS (**Paul et al., 2006**). The existence of an endogenous angiotensinergic system in the neurons of rat and human sympathetic coeliac ganglia has been reported recently (**Patil et al., 2008**). Sensory neurons of the trigeminal ganglia also

synthesize Ang II (**Imboden et al., 2009**). Although the RAS is widely studied, the specific localization of RAS components in the heart is still missing.

In our current work we provide strong evidence for the existence of novel, local angiotensinergic system in the rat and human heart, especially in neurons of intracardiac ganglia with the atria. By using different molecular and cell biological research tools like quantitative real time PCR, high performance liquid chromatography and radio immunoassay (HPLC-RIA), *in situ* hybridization and immunocytochemistry, we investigated the existence of Ang II components in the neurons of intracardiac ganglia and their processes in different parts of the rat and human heart. We also studied the probable colocalization of the noradrenergic and the angiotensinergic systems in the neurons of intracardiac ganglia.

2. Methods

2.1 Rat and human heart

We purchased 8-week-old, male Wistar Kyoto (WKY) rats (approximately 200 g body weight) from the Central Animal Facilities of the University of Bern. Adequate measures were taken to minimize pain or discomfort, in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC) and in accordance with Animal Protocols approved by the Animal Care and Use Committee, NIMH, NIH, USA. Rats were anaesthetized intraperitoneally with 100 mg/kg thiopentane sodium and were perfused transcardially with 150 ml Ringer solution containing 1000 U heparin at 37 °C followed by 300 ml freshly prepared 2 % formaldehyde at 4 °C. Hearts were carefully dissected and incubated further by immersion fixation in 2 % formaldehyde for 28 h at 4 °C. Subsequently, hearts were immersed for 14 h in phosphate-buffered saline (PBS-Dulbecco) containing 18 % sucrose at 4 °C. Fixed hearts were frozen in isopentane at -50 °C and 30 μm thick sections were cut on a cryostat and subsequently used as free-floating sections for immunocytochemistry. For some experiments after perfusion and immersion fixation rat hearts were embedded in paraffin. Paraffin sections, 7 μm thick, were used for immunocytochemical as well as for *in situ* hybridization experiments.

For extraction of total RNA and angiotensin components rats were shortly anesthetized with halothane and subsequently sacrificed by decapitation. Fresh rat hearts were dissected, different parts of heart were prepared and instantly transferred into RNA later (Ambion), frozen in liquid nitrogen, and then processed for total RNA extraction (Ambion). For angiotensin component extraction, hearts were rapidly removed, rinsed with cold Ringer solution, blotted by filter paper and wet weight was measured. The hearts were frozen in liquid nitrogen and stored at -70 °C.

Human heart tissue were procured from three adult individuals for whom a permit for clinical autopsy (informed written consent by next of kin) had been obtained according to state law, in accordance with the Code of Ethics of the World Medical Association (Declaration of Helsinki). Human heart were fixed by immersion in freshly prepared 2 % formaldehyde for 3 days and then used for cryosectioning or embedded in paraffin.

2.2 RNA isolation and quantitative real time RT-PCR (qRT-PCR)

Total 4 hearts from different WKY rats were taken for RNA extraction. Each heart was divided into four parts as : 1. right atrium, 2. right ventricle, 3. left atrium and 4. left ventricle. RNA integrity was confirmed for each sample on the Agilent Bioanalyzer using the RNA 6000 Nano kit (Agilent Technologies). 1 µg of total RNA was reverse transcribed using Superscript II (Invitrogen) and random hexamers according to the manufacturer's protocol. For quantitative real time PCR, reverse transcribed material corresponding to 40 ng RNA was amplified with the TaqMan assays described below in 25 µl Universal PCR Master Mix, No AmpErase UNG on the SDS 7000 (Applied Biosystems) using the standard thermal protocol. Average values and standard deviations of relative mRNA levels of each sample, normalized to relative 18S rRNA levels, are from four measurements and were calculated using the relative differences. The following TaqMan assays were used for qRT-PCR at a final concentration of 250 nM TaqMan probe and 900 nM of each primer:

Angiotensinogen

Forward primer 5'-CACGACTTCCTGACTTGGATAAAGA-3';

Reverse primer 5'-CTGCGGCAGGGTCAGA-3';

TaqMan probe 5'-FAM CCTCGGGCCATCC GMGB- 3';

manufactured as Assays-by-Design (RATG-EJ3) by Applied Biosystems.

Renin Assay-on-demand Rn00561847_m1 from Applied Biosystems.

ACE Assay-on-demand Rn00561094_m1 from Applied Biosystems.

Cathepsin D Assay-on-demand Rn00592528_m1 from Applied Biosystems.

AT_{1A} Assay-on-demand Rn01435427_m1 from Applied Biosystems.

AT_{1B} Assay-on-demand Rn02132799_s1 from Applied Biosystems.

AT_2

Forward primer 5' GTGGGAAGCTCAGTAAGCTGATTTA -3';

Reverse primer 5' GTCAGAGACTCCCAATCCTTACAC -3';

TaqMan probe 5'FAM ACACTGGCAACTAAAAGA MGB- 3'

manufactured as Assays-by-Design (RATG-EJ3) by Applied Biosystems.

18S rRNA Predeveloped Assay Reagent 431-9413E from Applied Biosystems.

2.3 *In situ* Hybridization

2.3.1 DIG-labelled RNA probe preparation

By using an appropriate cDNA template for Ang-N (**Lynch et al., 1986**), a 403 bp long fragment corresponding to nucleotides 221-623 was generated by digestion with restriction enzymes *EcoRV* and *Bam HI*. The obtained fragment was cloned between *Stu I* and *Bam HI* into pBluescript I KS+ (Stratagene). Digoxigenin-labelled probes were prepared using the DIG-RNA-labelling Mix (Roche) according to the manufacturer's protocol. T7 RNA polymerase was used to generate antisense riboprobe using the *Hind III* linearized template, and the sense strand (used as a control) was generated by T3 RNA polymerase using the same template linearized with *Bam HI*. The RNA concentration was estimated comparing dot-blot tests and NanoDrop measurements.

2.3.2 Procedure for *in situ* hybridization

For *in situ* hybridization, 6 µm thick paraffin sections were rehydrated in a graded alcohol series (2 times Xylol for 10 min, EtOH 100 % (2 times), 96 %, 70 %, 50 %, DEPC H_2O each step 5 min) using DEPC-treated H_2O for the dilution of all reagents and solutions. The sections were equilibrated in proteinase K buffer (100 mM Tris, 50 mM EDTA, pH 7.5) for 5 min and then treated with proteinase K (19 µg/ml) at 37 ºC for 2 min, after a wash with DEPC-treated H_2O. Following a wash in DEPC-water, sections were post fixed with freshly prepared 4 % formaldehyde for 5 min, followed by two subsequent washes in DEPC-water for 5 min each. Sections were then incubated in prehybridization solution (SIGMA) at 45 °C for 2 h, followed by incubation with heat denatured sense and antisense riboprobes (5-10 ng/µl) in 30 µl hybridization mix (SIGMA) for 48 h at 45 °C in a humid chamber (saturated with 2x SSC). Subsequently, the sections were incubated with 2x SSC for 30 min at room temperature, followed by 1 h in 2x SSC at 45 °C and 1 h in 0.1x SSC at 45 °C. Sections were equilibrated for 5 min with buffer 1 (100 mM Tris, 150 mM NaCl, pH 7.5), then incubated with alkaline phosphatase-coupled anti-digoxigenin antibody (Roche) 1:500 diluted in buffer 2 (10x blocking solution diluted with buffer 1) for 2 h at room temperature, followed by two 5 min washes with buffer 1. Finally, after 5 min equilibration with buffer 3 (100 mM Tris, 100 mM NaCl, 50 mM MgCl2, pH 9.5), the colour reaction containing nitro blue tetrazolium (NBT) and 5-Bromo-4-chloro-3-indolyl phosphate (BCIP) in buffer 3 was completed according to manufacturer's protocol (Sigma).

2.4 Immunocytochemistry

For immunocytochemical incubations for rat and human heart tissue, a protein G purified murine monoclonal antibody against Ang II (Mab-Trap G II column, Amersham Sciences) was used at a concentration of 0.3 µg/ml in buffer solution. This is a self generated monoclonal

antibody against the synthetic peptide to human Ang II. The specificity for the monoclonal antibody against Ang II (4B3) has been previously documented and it produced the same staining as a polyclonal antibody against Ang II in rat adrenal glands (**Frei et al., 2001**). For immunization of the mice, Ang II peptide was cross-linked with glutaraldehyde to keyhole limpet hemocyanin. In dot blot assay, the monoclonal antibody against Ang II (4B3) showed total cross reactivity with Ang III (2-8), Ang 3-8, Ang 4-8 and Ang 5-8. It showed no cross reaction with human plasma angiotensinogen, Ang I (1-10) and angiotensin 1-7. Pre-absorption was performed by using a batch procedure with the synthetic Ang II-peptide that was covalently linked through its N-terminus to CH-Sepharose 4 B. The gel was loaded into a glass column and the monoclonal anti Ang II-antibody solution effluent was used for immunocytochemical preabsorption controls. Further controls were done with mouse non-immune serum and for the secondary goat-anti-mouse Cy3 antibody without the primary antibody. All these additional controls showed absolute no staining within the sections. The pre-absorbed monoclonal antibody after low pH-elution revealed the same staining as the Mab-Trap G II purified antibody.

Sections were incubated free floating for 36 h at 4 ºC with the primary antibody, washed and incubated with the secondary antibody for 90 min at room temperature and counterstained with DAPI (4',6-diamidino-2-phenylindole). After washing, cryosections were mounted on gelatin-coated slides and air-dried. The stained sections were embedded with Glycergel (DAKO) and cover-slipped. The same procedure as for free floating cryosections has been used for paraffin sections mounted on glass slides. Pictures were taken by a normal fluorescent light microscope (LEICA DM 6000B) using a digital camera or by a confocal laser scanning microscope (LEICA SP2 with AOBS).

For double staining, sections were incubated free floating for 36 h at 4 ºC with both the primary antibodies, murine monoclonal anti Ang II (4B3) and rabbit polyclonal anti-dopamine-

beta-hydroxylase (DβH, DZ1020 BIOMOL). Then washed and incubated with both the secondary antibodies, goat anti mouse Cy3 (GAM^{Cy3}) and donkey-anti-rabbit Cy5 (DAR^{Cy5}) for 90 min at room temperature and counterstained with DAPI (4',6-diamidino-2-phenylindole). Some incubation were done with a murine monoclonal anti-DβH antibody (4F9/9 ANAWA). After washing, same procedure as mentioned above has been used.

2.5 Measurement of angiotensin peptides

Immunoreactive Ang II (ir-Ang II) as well as Ang-(1-8) octapeptide, Ang-(2-8) heptapeptide, Ang-(3-8) hexapeptide, Ang-(4-8) pentapeptide, Ang-(1-10) decapeptide and Ang-(2-10) nonapeptide were measured in rat and human heart by radioimmunoassay without and with prior separation by high performance liquid chromatography (HPLC) (**Nussberger et al., 1986**). Measurement of true angiotensin was applied also for Ang I, and peptide extraction from tissue was adapted for additional freeze-thaw procedures to ensure the lysis of cells (**Nussberger et al., 1985**; **Nussberger, 2000**).

3. Results

3.1 Quantitative determination of RAS components in the rat and human heart

3.1.1 Quantitative Real time RT-PCR

We detected significant amounts of Ang-N-, ACE- and cathepsin D- mRNA in different parts of the rat heart (**Fig**. **1a**, **c** and **d**, respectively). No renin mRNA was detected in the same heart extracts (**Fig**. **1b**). In the extracts of the right atrium consistently Ang-N mRNA could be detected the most in comparison to the other heart parts. Receptor studies revealed AT_{1A}- and AT_2- receptor mRNA expression in different parts of the rat heart (**Fig**. **2a** and **Fig**. **2c** respectively) whereas no expression of AT_{1B} receptor mRNA could be discovered (**Fig**. **2b**). RNA from tissues with previously reported high levels of the respective mRNAs were used as positive controls, and all mRNA values were normalized to 18S rRNA.

3.1.2 HPLC and RIA of angiotensin peptides.

Data obtained from HPLC-RIA experiment shows existence of substantial amount of Ang I and Ang II peptides in the rat (**Table 1**) and human (**Table 2**) hearts.

3.2 Localization of Ang-N mRNA and Ang II in rat dorsal root ganglia

3.2.1 *In situ* hybridization

Ang-N mRNA was detected in most, if not all the neurons studied in the rat right atrium with the antisense probe, indicating intracellular localization of Ang-N mRNA (**Fig. 3b**). No staining was produced using the sense probe as a control (**Fig. 3a**).

3.2.2 Immunocytochemistry and colocalization studies

Ang II immunoreactivity was present in the neuronal cytoplasm as well as in their processes in the rat intracardiac ganglia of both atria. Angiotensinergic neurons in an intracardiac ganglion in the right atrium (**Fig. 4a** and **Fig. 4b** zoomed illustration of **Fig. 4a**) and in the left atrium (**Fig. 4c** and **Fig. 4d** zoomed illustration of **Fig. 4c**). Angiotensinergic neuronal processes were detected in the right ventricle wall (**Fig. 5a**), in the left ventricle wall (**Fig. 5b**) and in the septum wall (**Fig. 5c**).

In the human heart Ang II immunoreactivity was observed in the cytoplasm of many neurons and in their processes in right atrium (**Fig. 6a**, **Fig. 6b** is zoomed illustration of **Fig. 6a** and **Fig. 6c** is one single optical transverse section of neuron from **Fig. 6a**). A neuronal fiber pathway observed in right atrium (**Fig. 7a**) and very fine neuronal processes detected in left atrium are indicated by arrows (**Fig. 7b**). Angiotensinergic fibers and angiotensin stained cardiomyocytes can be explored in the human right ventricle (**Fig. 7c**). Zoomed illustration of striated cardiomyocytes of **Fig. 7c** indicating presence of Ang II (**Fig. 7d**) with presumably transverse tubules (**Kessel and Kardon, 1979**). **Fig. 7e** shows very fine angiotensinergic fibers (arrows) and synapses en passant (arrowheads) in the left ventricle, while **Fig. 7f** demonstrates Ang II staining in processes in the septum wall.

A possible colocalization of Ang II and dopamine-β-hydroxylase (DβH) was also studied in different regions of the rat heart. **Fig. 8a** specifies the staining for Ang II and **Fig. 8b** for DβH, merged image shows the partial colocalization of Ang II and DβH (stained in yellow) in the cytoplasm of the intracardiac neurons and their processes in the right atrium (**Fig**. **8c**). Costaining for Ang II (**Fig. 8d**) and DβH (**Fig. 8e**) was also done with pericardium of rat heart, where as **Fig. 8f** show the colocalization of fiber pathways with pericardium in yellow. Partial colocalization of Ang II and DβH (stained in yellow), in fiber pathways and synapses en passant in the right

ventricle wall (**Fig**. **8g**) and with a blood vessel in the septum (**Fig**. **8h**). In **Fig. 8g** and **Fig. 8h** the synapses en passant stained for DβH are highlighted by arrowheads.

3.3 Supplemented material will be available in online publication.

4. Discussion

The localization and the function of a renin angiotensin system (RAS) in the heart is an issue of many controversies, because of the complexity of the systemic and the local RAS (**Singh et al., 2008; Paul et al., 2006**). Our study reveals an existence of a local angiotensinergic system in rat and human heart, in the neurons of the intracardiac ganglia in both of the atria and their processes in the heart.

The expression of Ang-N mRNA has been demonstrated in the murine central nervous system (**Yang et al., 1999**). In the brain, Ang-N was localized to multiple cell types predominantly in astrocytes but also in neurons (**Imboden et al., 1987**). In the peripheral nervous system, predominant expression of Ang-N mRNA in neurons of the sympathetic coeliac ganglia (**Patil et al., 2008**) and the trigeminal ganglia (**Imboden et al., 2009**) has been shown. Ang-N mRNA in the rat (**Hellmann et al., 1988; Lindpaintner et al., 1993**) and human (**Paul et al., 1993**) heart has been studied. Studies done in the isolated perfused rat hearts suggested that the majority of the cardiac angiotensinogen is taken up from the circulation (**De Lannoy et al., 1997**). Despite of the several studies published, a definite location of Ang-N synthesis in the heart is completely missing. The mammalian heart is composed of different chambers with variation in their function. Hence, to study the specific localization of RAS components the rat and human hearts were divided into four chambers as right atrium, left atrium, right ventricle and left ventricle.

By using qRT-PCR we could detect higher amount of Ang-N mRNA in the right and left atrium of rat heart than in the ventricles (**Fig**. **1a**). In comparison as with liver, the major source of Ang-N production (**Dzau et al., 1987**), Ang-N mRNA levels were low.

Meiklejohn and Walker reported that the ganglia were found only in the right and left atrium of the rat heart (**Meiklejohn, 1914**). In order to confirm the presence of Ang-N mRNA

revealed in extracts by qRT-PCR we performed *in situ* hybridization at the cellular resolution in rat heart. *In situ* hybridization confirmed the existence of Ang-N mRNA in the cytoplasm of the neurons of the rat intracardiac ganglia of the atria. Ang-N mRNA detection by in situ hybridization demonstrates the local formation of Ang-N in this tissue, indicating that Ang-N uptake from the circulation is not necessary for the local formation of Ang II. In this experiment with sense probe for Ang-N mRNA revealed no staining (**Fig. 3a**), while with antisense probe uncovered Ang-N mRNA in the cytoplasm of the neurons (**Fig. 3b**) and not in surrounding cell types. This fact further confirms that Ang-N is synthesized locally in neurons and not taken up from the circulation (**Paul et al., 2006**). Ours is the first report on the presence of Ang-N mRNA at cellular resolution in the neurons of intracardiac ganglia.

The expression of renin in the heart is a matter of controversial debate, some investigators were able to detect renin mRNA in the heart of various species by Northern blotting (**Dzau et al., 1987**), RT-PCR and solution hybridization assays (**Paul et al., 1988**). However, some investigators claimed these renin measurements as artifacts (**Von Lutterotti et al., 1994**). Renin expression studied in transgenic rat and mice indicated that in some species heart is a site of extra-renal renin synthesis (**Yan et al., 1998; Pinto et al., 1997**). Studies also show that under pathophysiological conditions renin gene expression may be turned on (**De Mello et al., 2000**). In the present study, renin expression was not detected in any part of the rat heart by qRT-PCR. Even after using high concentrations of the cDNA (200 ng/sample), renin mRNA was untraceable when compared to renin mRNA levels in kidney, the major source of renin production (**Persson, 2003**).

Instead, we detected expression of cathepsin D mRNA in all parts of rat heart, at levels higher than in comparison with those found in rat liver, but lower in comparison to those recently reported in rat coeliac (**Patil el al., 2008**) and trigeminal ganglia (**Imboden et al., 2009**).

Cathepsin D is a protease capable of cleaving Ang-N into angiotensin I (**Saye et al., 1993; Katwa et al., 1999**), a non-renin alternative pathway for Ang II formation. These results indicate the possibility of the existence of an alternate pathway for the local synthesis of Ang I in the heart. Alternatively, cardiac cells may take up circulating renin to support local synthesis of endogenous Ang I. These mechanisms have been proposed by several investigators, where strong evidence exists for the uptake of extracellular renin from the circulation (**Paul et al., 2006; Danser et al., 1997; Von Lutterotti et al., 1994; Peters et al., 2002**). The presence of renin in cardiac tissue is still a matter of controversial debate (**Dzau et al., 1987; Paul et al., 1988; Paul et al., 1993; Von Lutterotti 1994**; **Pinto et al., 1997**; **Yan et al., 1998; Paul et al., 2006; Singh et al., 2008**).

In comparison to lung the major source of ACE production (**Pueyo et al., 2004**), we detected substantial amounts of ACE mRNA in all the parts of rat heart by qRT-PCR (**Fig. 1c**).

Angiotensin receptor type 1 (AT_1) - and Angiotensin receptor type 2 (AT_2) - mRNA expression in the heart has already been reported (**Paul et al., 2006**). AT_1 receptors lead to proliferation of cardiac cells and are stimulator of hypertrophy, while AT_2 is mediating the opposite effects (**Widdop et al., 2003**). AT_{1A} and AT_{1B} are the receptor subtypes of AT_1 in rodents (**Paul et al., 2006**). Ang II receptor studies are reported either in whole heart or in cardiac cell culture, hence we investigated, AT_{1A}-, AT_{1B}- and AT_2- mRNA expression in different parts of the rat heart by using qRT-PCR. AT_{1A} mRNA (**Fig. 3a**) is expressed in all the parts of the rat heart while AT_{1B} mRNA (**Fig. 3b**) expression was not traceable. In order to detect AT_2 mRNA (**Fig. 3c**) in the heart we had to use higher concentration of cDNA (200 ng/sample), while 40 ng of cDNA per sample was used for all other mRNA measurements.

Data obtained from high performance liquide chromatography (HPLC) and radioimmunoassay (RIA) experiments demonstrate the presence of Ang I and Ang II peptides in the extracts of human and Wistar-Kyoto rat hearts (**Table 1**) and human (**Table 2**).

Additionally at cellular level we report here the existence of Ang II especially in the rat and human intracardiac ganglia, which are also known as a cardiac nervous system (**Armour, 2008**). Intracardiac neurons play a crucial role in several heart diseases. During cardiac disease conditions the abnormal interaction of intracardiac nervous system can lead to alterations in cardiomyocyte function (**Armour, 2008**). Intracardiac neurons may potentially participate in the induction of arrhythmia after myocardial infarct (**Kukanova and Mravec, 2006**). It has been shown that some treatments for arrhythmias may produce beneficial effect via the modification of intracardiac neuronal activity (**Scherlag et al., 2005**). After coronary bypass surgery, a significant reduction of post-operative arrhythmia was observed when anterior epicardial fat, a region rich in intracardiac ganglia was preserved (**Cummings et al., 2004**).

Rat and human tissue samples from the four different chambers of hearts and septum were used for Ang II detection by immunocytochemistry. We have detected Ang II like immunoreactivity in the neurons of the intracardiac ganglia of rat and human hearts. Intracardiac ganglia in right atrium of rat heart (**Fig. 4a**) showing neurons and their processes stained for Ang II, **Fig. 4b** a zoomed illustration of **Fig. 4a**, the red staining for Ang II in the cytoplasm of the neurons and nuclei stained in blue for DAPI. Angiotensinergic intracardiac ganglia were also detected in the left atrium of rat heart (**Fig. 4c**, **Fig 4d** a zoomed illustration of **Fig. 4c**).

The heart is innervated by sympathetic and parasympathetic fibers of the autonomic nervous system (**Armour, 2008; Chow et al., 2001**). Also the sympathetic nerves innervate sinoatrial node and atriovantricular node of the heart (**Steele et al., 1996**). Intracardiac ganglia are innervated by the fibers from central nervous system (**Kukanova and Mravec, 2006**). It is

certainly important to study the autonomic innervation of the heart as numerous evidences are reported for sympathetic and parasympathetic functional reinnervation of heart after transplantation surgery (**Tio et al., 1997; Überfuhr et al., 1997; Armour, 2008**). Loss of sympathetic and parasympathetic influences on heart rate is apparent from the complete denervation of the heart after transplantation surgery (**Pope et al., 1980; Quigg et al., 1989; Rudas et al., 1991**). Moreover after heart transplant, functional recovery of the sympathetic (**Schwaiger et al., 1991; Wilson et al., 1991**; **Kaye et al., 1993**; **De Marco et al., 1995**) and parasympathetic (**Fallen et al., 1988**; **Fitzpatrick et al., 1993; Le Guludec et al., 1994**) cardiac innervation has been described. In our study we could find angiotensinergic fibers in the right ventricle wall (**Fig. 5a**), left ventricle wall (**Fig. 5b**) and in septum wall (**Fig. 5c**) of the rat heart. Cardiomyocytes seen in the septum wall (**Fig. 5c**) are know for the production of Ang II (**Singh et al., 2008**) are also stained for Ang II. There is little doubt that the Ang II visualized by immunocytochemistry in intracardiac ganglia and their fibers is indeed Ang II or its metabolites. In the context of perivascular Ang II immunocytochemical stainings and visibility of corresponding varicosities as synapses en passant, a role for angiotensin as a neurotransmitter is very likely (**Patil et al., 2008**).

To confirm also the presence of Ang II peptide in human heart at cellular level, we performed immunohistochemistry for different parts of this tissue. Ang II immunoreactivity was discovered in the right atrium of human heart in the intracardiac neurons and their fibers (**Fig. 6a**). **Fig. 6b** is a zoomed illustration from **Fig. 6a**. One single optical transverse section of an intracardiac neuron (**Fig. 6c**) evidently specifies the staining for Ang II in the cytoplasm of the neuron and their fibers but not in the nucleus. In our study angiotensinergic fiber pathways were detected in right atrium (**Fig. 7a**), and Ang II immunoreactivity in very fine neural fibers in the left atrium of the human heart (**Fig. 7b**). Also both the ventricle walls and the septum of the

human heart were studied for Ang II immunoreactivity. In the right ventricle we found Ang II staining in neuronal processes and in the cardiomyocytes (**Fig**. **7c**). **Fig**. **7d** is a zoomed illustration of **Fig**. **7c**, showing Ang II staining in striated cardiac fibers presumably with transverse tubules (**Kessel and Kardon, 1979**). Left ventricle innervated by very fine angiotensinergic fibers indicated by arrows and synapses en passant highlighted by arrowheads (**Fig**. **7e**). Moreover Ang II staining in the neuronal processes was also found in septum of the human heart (**Fig**. **7f**). Axons from intracardiac ganglia as well as from the extracardiac origin innervate different heart regions (**Kukanova and Mravec, 2006; Steele et al., 1996**), leading to cardiac innervation by neurons of multiple origin. Hence, the origin of angiotensinergic innervation other than intracardiac ganglia, innervating different heart regions has to be investigated further.

Localization of tyrosine hydroxylase, dopamine-β-hydroxylase (DβH) and norepinephrine in the human heart has been shown by immunohistochemistry (**Singh et al., 1998**), in our study we explored further the presence of DβH in the intracardiac neurons and their processes and the possible colocalization within the angiotensinergic system. Rat right atria containing intracardiac ganglia, were double stained for Ang II (**Fig**. **8a**) and DβH (**Fig**. **8b**), indicating partial colocalization, most neurons are stained positive for both Ang II and DβH while some are not (**Fig. 8c**). Partial colocalization of Ang II (**Fig. 8d**) with DβH (**Fig. 8e**) can be seen in fiber pathways with pericardium of rat heart. Merged image shows yellow staining where Ang II and DβH are colocalized (**Fig. 8f**). Colocalization studies done by confocal microscopy at the nerve terminals specify that Ang II is also partially colocalized with the SNS in synapses en passant. In the right ventricle wall (**Fig. 8g**) and with blood vessels in the septum (**Fig. 8h**) of the rat heart, red staining represents only Ang II while green staining reveals DβH containing synapses en passant and colocalized structures are seen in yellow. As several evidences indicates that the

neurons of intracardiac ganglia can considerably regulate the heart activity (**Armour, 2008; Kukanova and Mravec, 2006**), localization, and colocalization of Ang II with DβH in the intracardiac neurons significantly supports the involvement of Ang II in the regulation of heart activity.

In conclusion, our findings strongly indicate the presence of an endogenous, neuronal angiotensinergic system in the neurons of rat and human intracardiac ganglia. Ang II and its partial colocalization with DβH in intracardiac neurons and in synapses en passant with blood vessels and cardiomyocytes in the ventricles indicate that Ang II and norepinephrine act as neurotransmitters locally in the heart.

Figure legends

Fig. **1**. Determination of relative levels of Ang-N-, renin-, ACE- and cathepsin D-mRNA by qRT-PCR in different parts of the rat heart. Heart was divided into right atrium (RA), left atrium (LA), right ventricle (RV) and left ventricle (LV). Reverse transcribed total RNA extracts of heart from four rats were tested by qRT-PCR for the presence of Ang-N mRNA (a), for renin mRNA (b), for ACE mRNA (c) and cathepsin D mRNA (d). Total RNA extracts from liver (a), kidney (b), lung (c) and liver (d) were used as a positive control samples. All relative mRNA values are normalized to 18S rRNA levels. Average values and standard deviations of four qRT-PCR measurements are shown.

Fig. **2**. Determination of relative levels of Ang II AT_{1A}- (a), AT_{1B}- (b) and AT_2- (c) receptor mRNA in different parts of the rat heart. Reverse transcribed total RNA extracts of heart from four rats as mentioned before, were considered by qRT-PCR for the presence of Ang II AT_{1A}, AT_{1B} and AT_2 receptor mRNA as described in Methods. Total RNA extracts from adrenal gland (Ad) was used as a positive control sample. All relative mRNA values are normalized to 18S rRNA levels. Average values and standard deviations of four qRT-PCR individual measurements are shown.

Fig. **3**. *In situ* hybridization in right atrium of rat heart for detection of Ang-N mRNA, 6 μm thick consecutive paraffin sections were processed as described in Methods. a) Sense probe. b) With antisense probe, revealing positive staining in the cytoplasm of numerous neurons within the intracardiac ganglion. Bar: 50 μm.

Fig. **4**. Ang II immunocytochemistry in the neuronal cytoplasm and projections in atria of rat heart. a) Ang II staining in the neurons of intracardiac ganglia and their processes in right atrium, bar: 50 μm. b) Zoomed inset of panel a), bar: 30 μm. c) Ang II staining in the neurons of intracardiac ganglia in the left atrium, bar: 50 μm. d) Zoomed inset of panel c), bar: 30 μm. A 30 μm thick free-floating cryosection was prepared and incubated with our murine monoclonal anti Ang II antibody (**Frei et al., 2001**), stained with goat-anti-mouse (GAM^{Cy3}) in red and DAPI in blue for nuclei, as described under methods. See movie 1 for right atrium and movie 2 for left atrium in supplemented material.

Fig. **5**. Angiotensinergic processes in the rat heart. a) Ang II staining within fibers in the right ventricle, bar: 20 μm. b) Ang II staining in processes and fine fibers in the left ventricle, bar: 20 μm and c) Ang II staining processes as well as with cardiomyocytes in the septum, bar: 50 μm. 30 μm thick free-floating cryosections were prepared and incubated with murine monoclonal anti Ang II antibody (**Frei et al., 2001**), stained with goat-anti-mouse (GAM^{Cy3}) in red and DAPI in blue for nuclei, as described under methods.

Fig. **6**. Ang II staining in the neuronal cytoplasm and projections in the right atrium of human heart. a) Ang II staining in the neurons of intracardiac ganglia and their processes in right atrium, b) Zoomed inset of panel a), c) One optical transverse section of the human intracardiac neuron from a) and b) indicating Ang II staining specifically in the neuronal cytoplasm (not in the nucleus) and not in surrounding cells. In b), angiotensinergic synapses en passant can be observed around the angiotensinergic neurons, highlighted by arrows. A 30 μm thick free-floating cryosection was prepared and incubated with our murine monoclonal anti Ang II antibody (**Frei et al., 2001**), stained with goat-anti-mouse (GAM^{Cy3}) in red and DAPI in blue for

nuclei, as described under methods, bar: 30 μm. See movie 3 for better illustration of Fig 6a, in supplemented material.

Fig. **7**. Ang II staining in the human heart. a) An angiotensinergic fibre pathway in the right atrium, bar: 30 μm. b) Very fine processes indicated by arrows are stained for Ang II in the left atrium, in the region of blood vessels (shown by arrows), bar: 50 μm. c) Angiotensinergic fibers (indicated by arrows) and myocytes in right ventricle, for better illustration see movie 4, bar: 30 μm. d) Zoomed inset of panel c), bar: 15 μm. e) Ang II staining in the fibers with synapses en passant in left ventricle, indicated by arrows, bar: 50 μm. f) Ang II staining in the fibre pathway in septum wall, bar: 100 μm. A 30 μm thick free-floating cryosections were prepared and incubated with murine monoclonal anti Ang II antibody (**Frei et al., 2001**), stained with goat-anti-mouse (GAM^{Cy3}) in red and DAPI in blue for nuclei.

Fig. 8. Colocalization of Ang II and Dopamine-beta-hydroxylase (DβH) in various regions of rat heart. Double staining for Ang II in red (a) and DβH in green (b) in intracardiac neurons in the right atrium, the merged image is shown in (c), bar: 25 μm. In Fig. 8a and Fig 8b, colocalized neurons are indicated by arrows while individual neurons are highlighted with asterisks (*). Double staining for Ang II in red (d) and DβH in green (e) with the pericardium, the merged image is shown in (f), bar: 15 μm. Merged image of colocalization of Ang II (in red) and DβH (in green) in neuronal processes in right ventricle, partial colocalization is observed in yellow (g), bar: 10 μm and in blood vessel of septum (h) in rat heart, bar: 10 μm. Synapses en passant stained with DβH in right ventricle are indicated by arrowheads in (g and h). Free-floating cryosections were prepared and incubated with murine monoclonal anti Ang II antibody (**Frei et al., 2001**), stained with goat-anti-mouse (GAM^{Cy3}) in red and polyclonal anti rabbit DβH, stained with

donkey-anti-rabbit (DAR^{Cy5}) in green and DAPI in blue for nuclei. For additional figures showing DβH staining in rat heart, see online supplementary **Fig**. **S1**, in supplemented material. See movie 5 in supplemented material.

Table 1. Concentration of specifically measured Ang I and Ang II peptides (HPLC-RIA) in rat hearts.

Table 2. Concentration of specifically measured Ang I and Ang II peptides (HPLC-RIA) in human heart.

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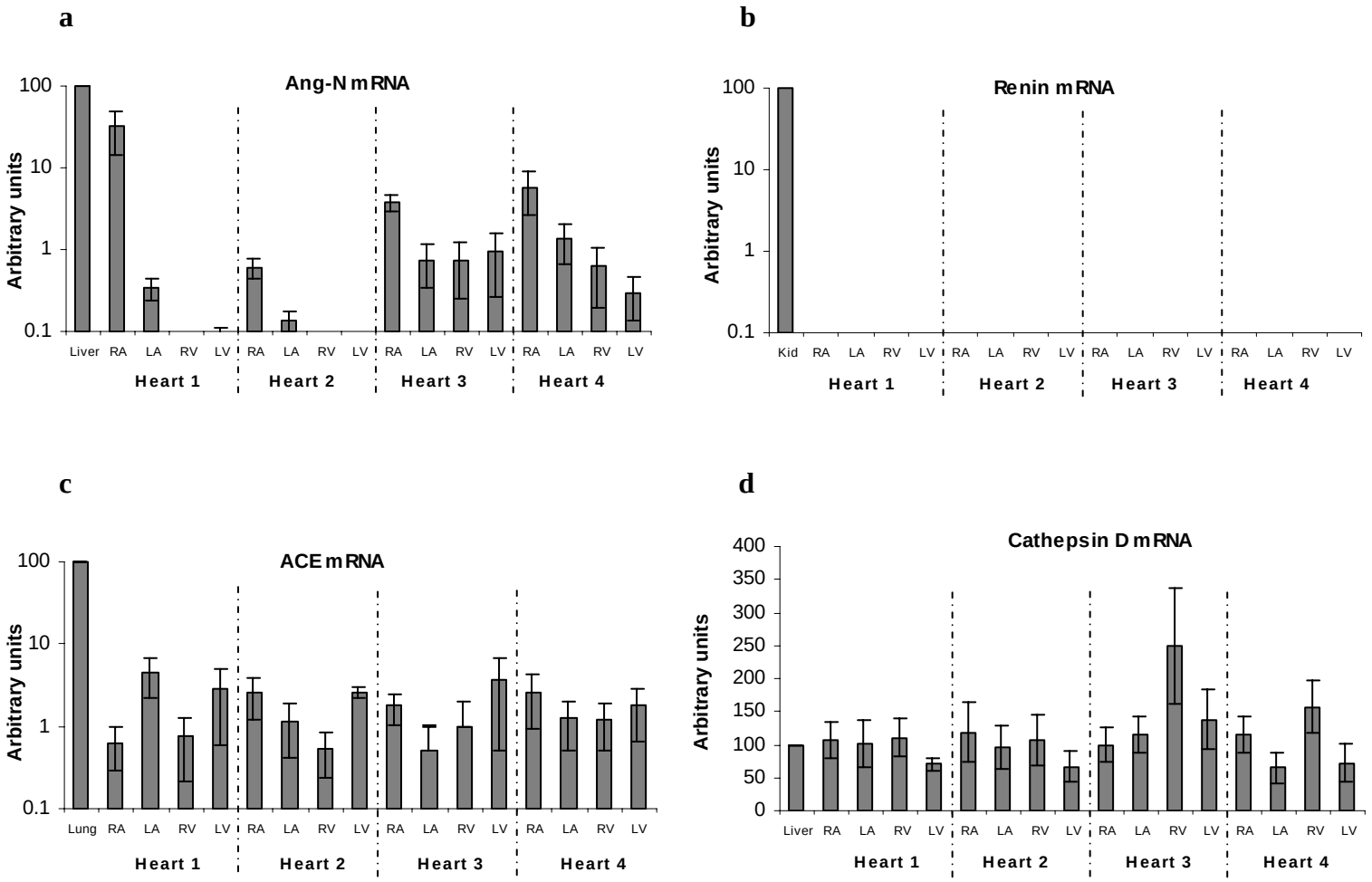
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ACE mRNA
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d
Cathepsin D mRNA
Arbitrary units
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Figure 1

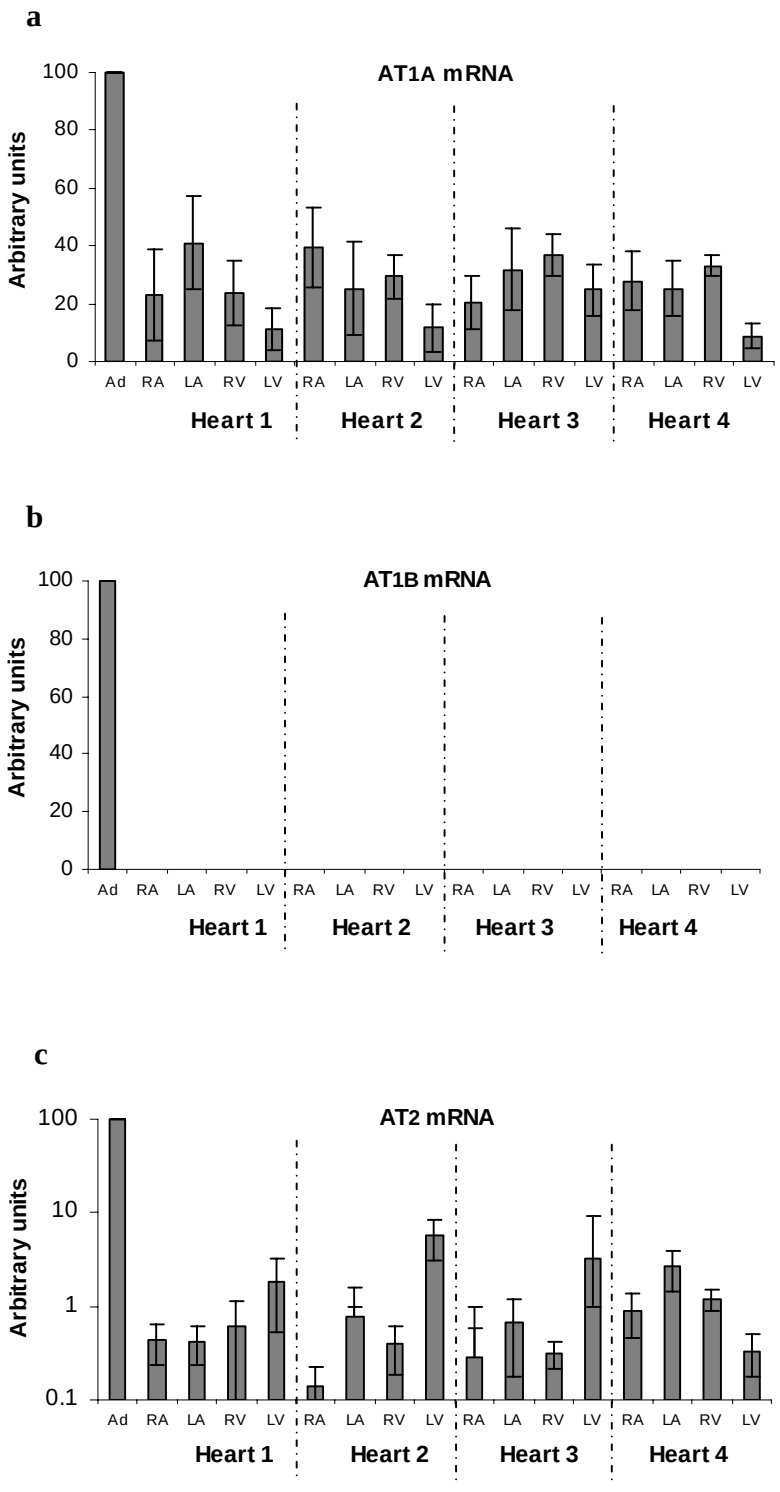
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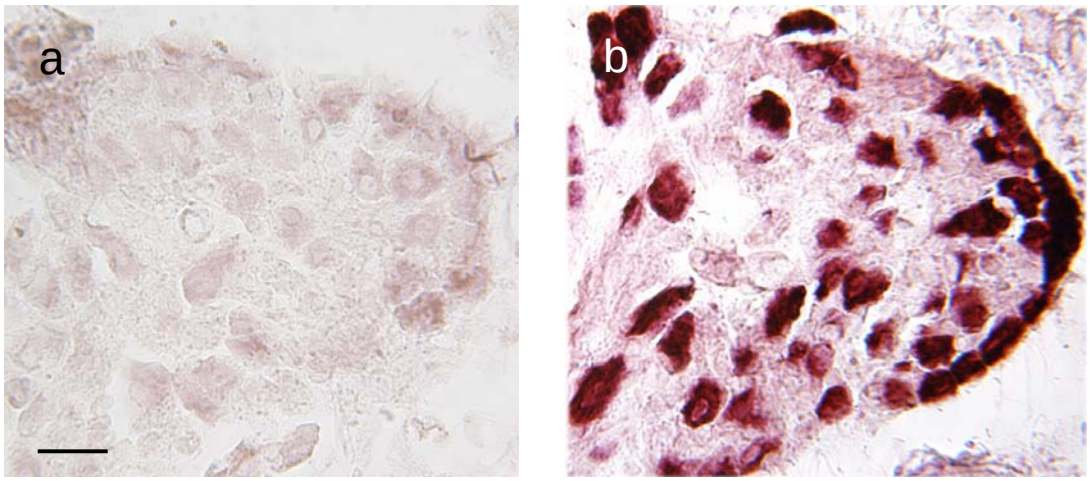
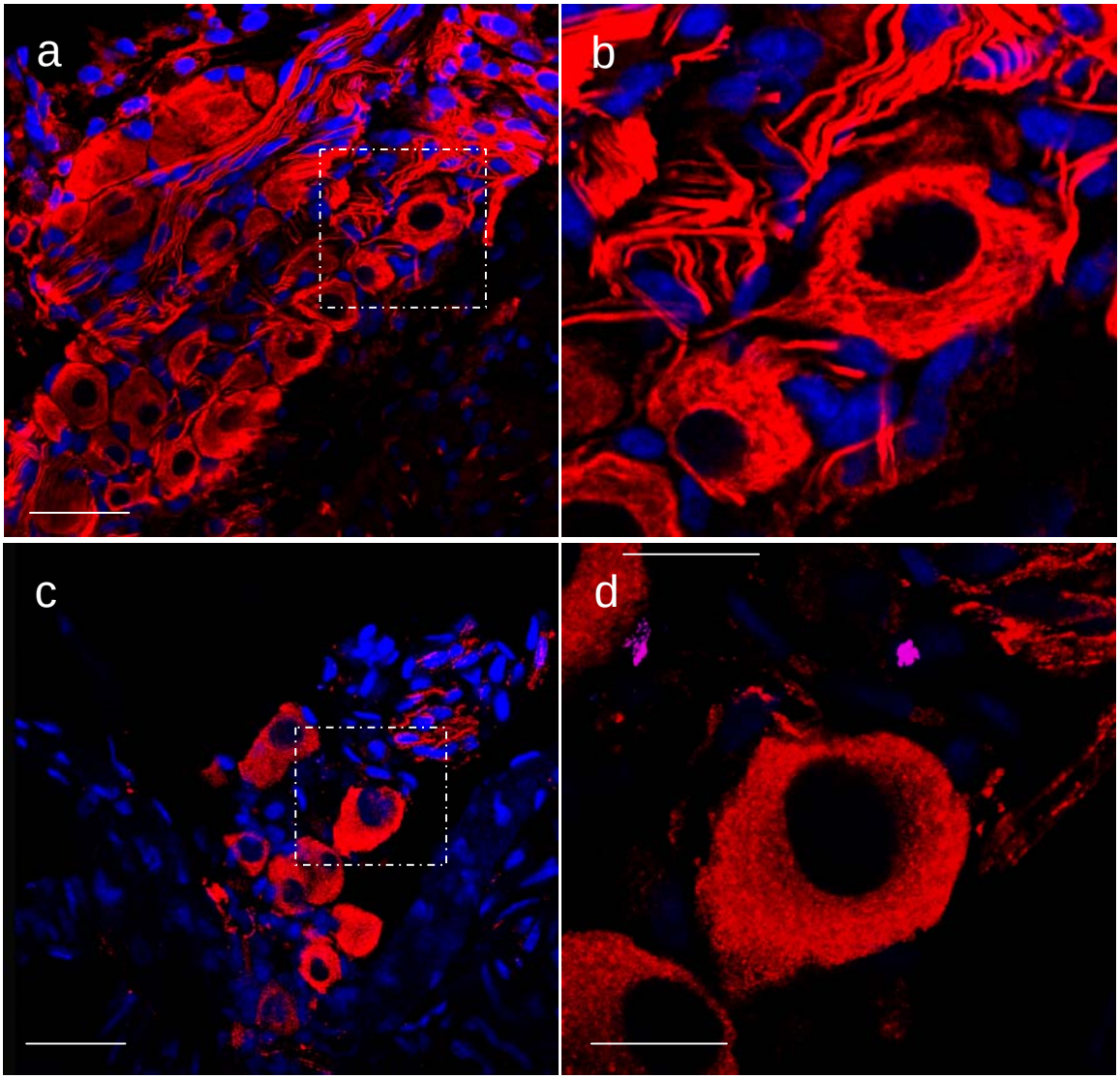
Figure 2



sense antisense

Figure 3



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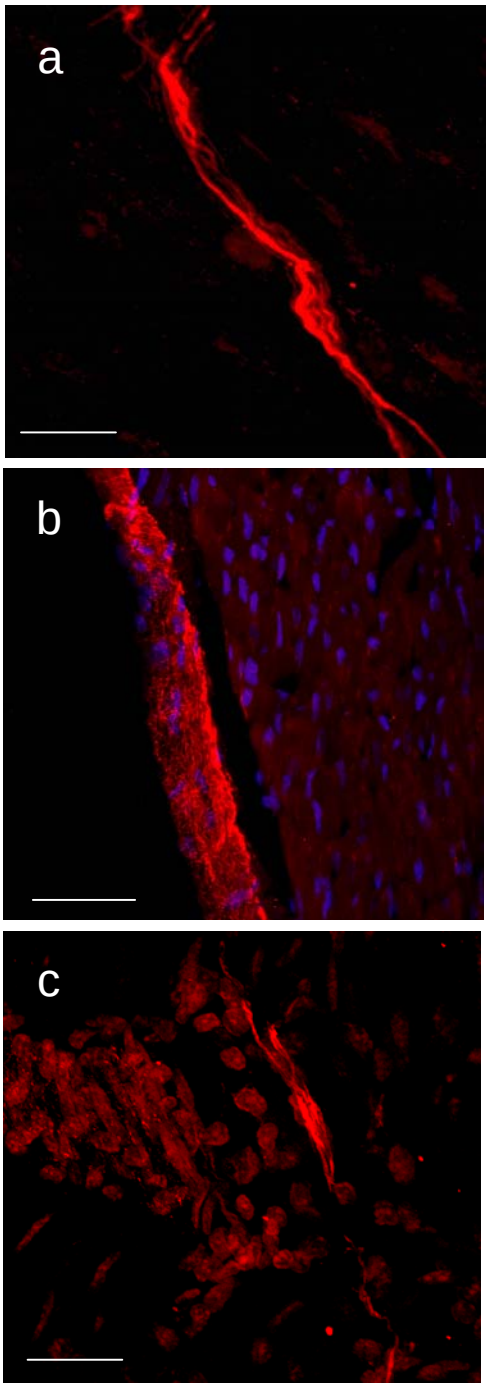

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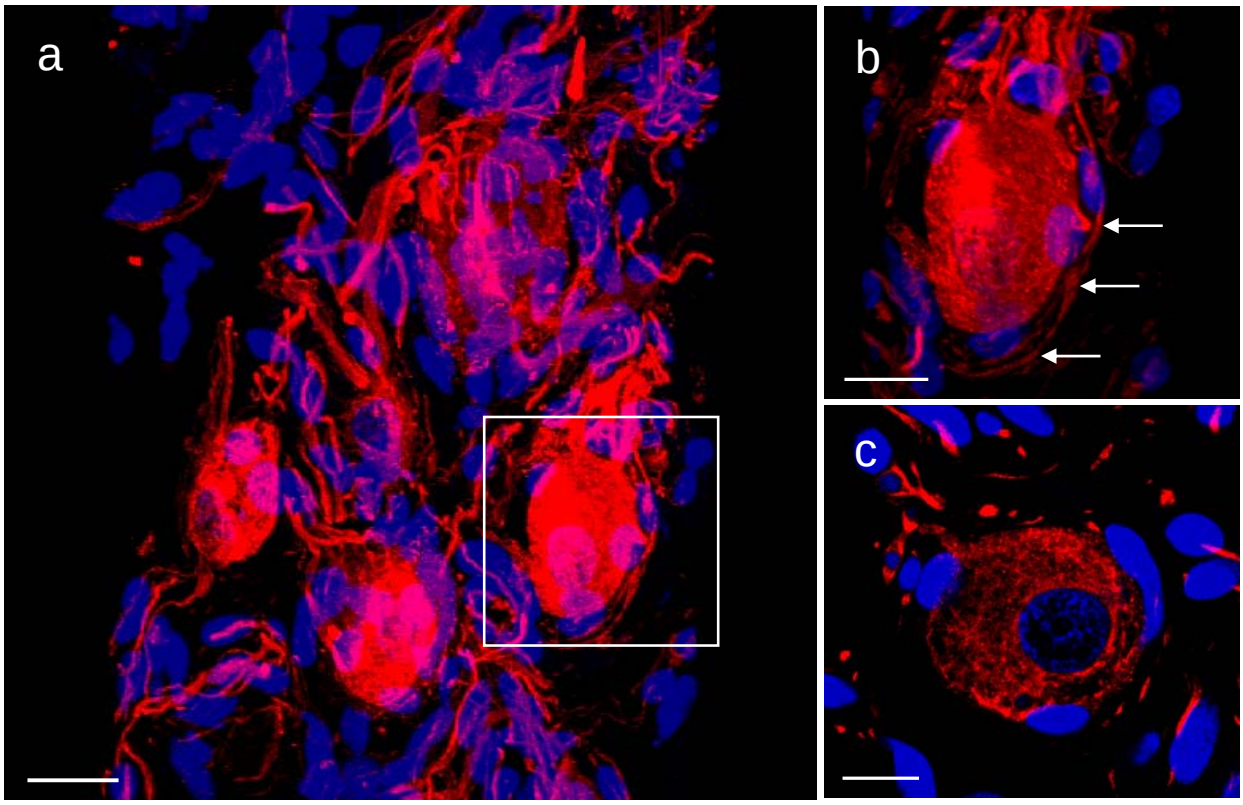

Figure 6

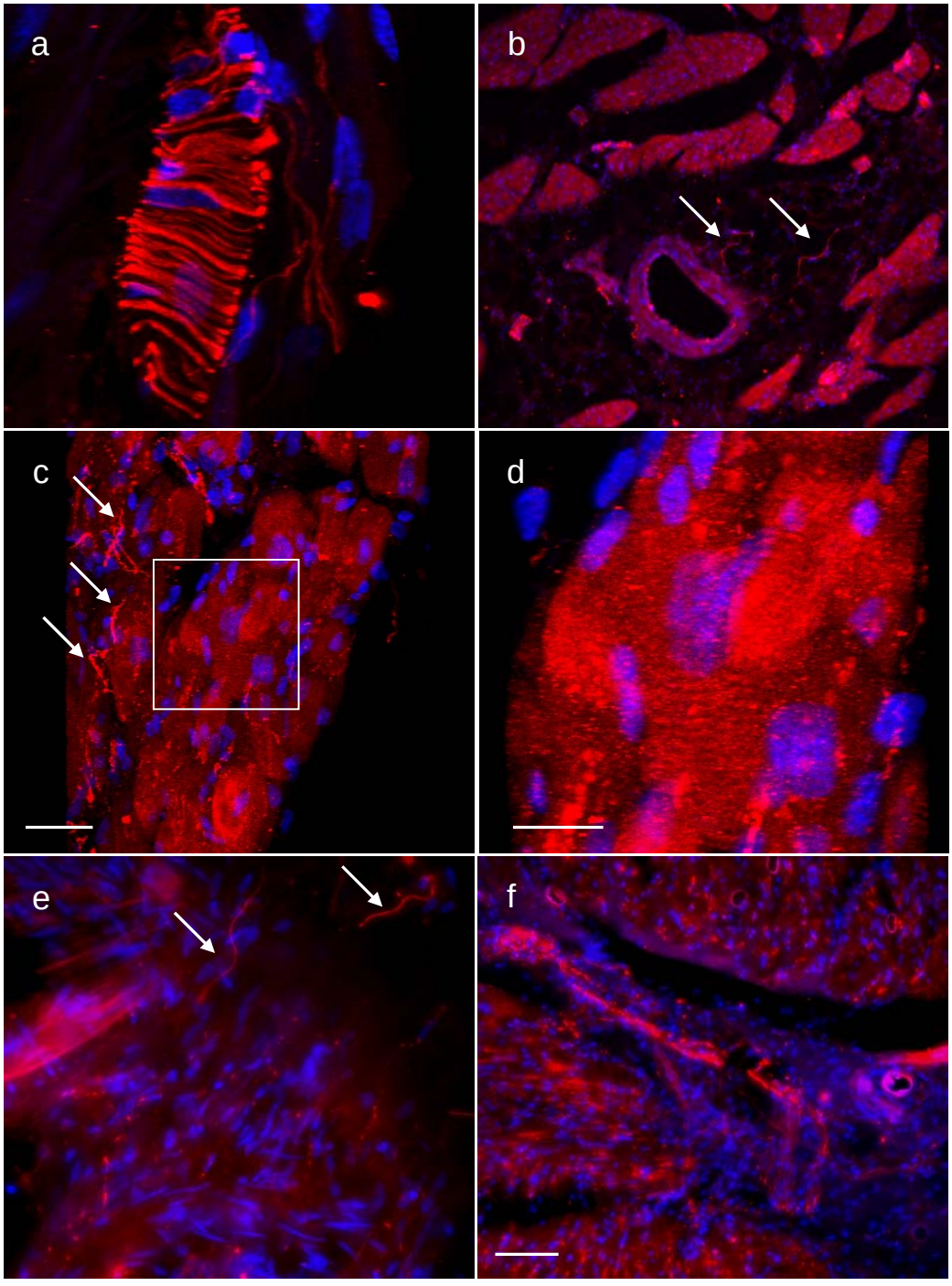

Figure 7

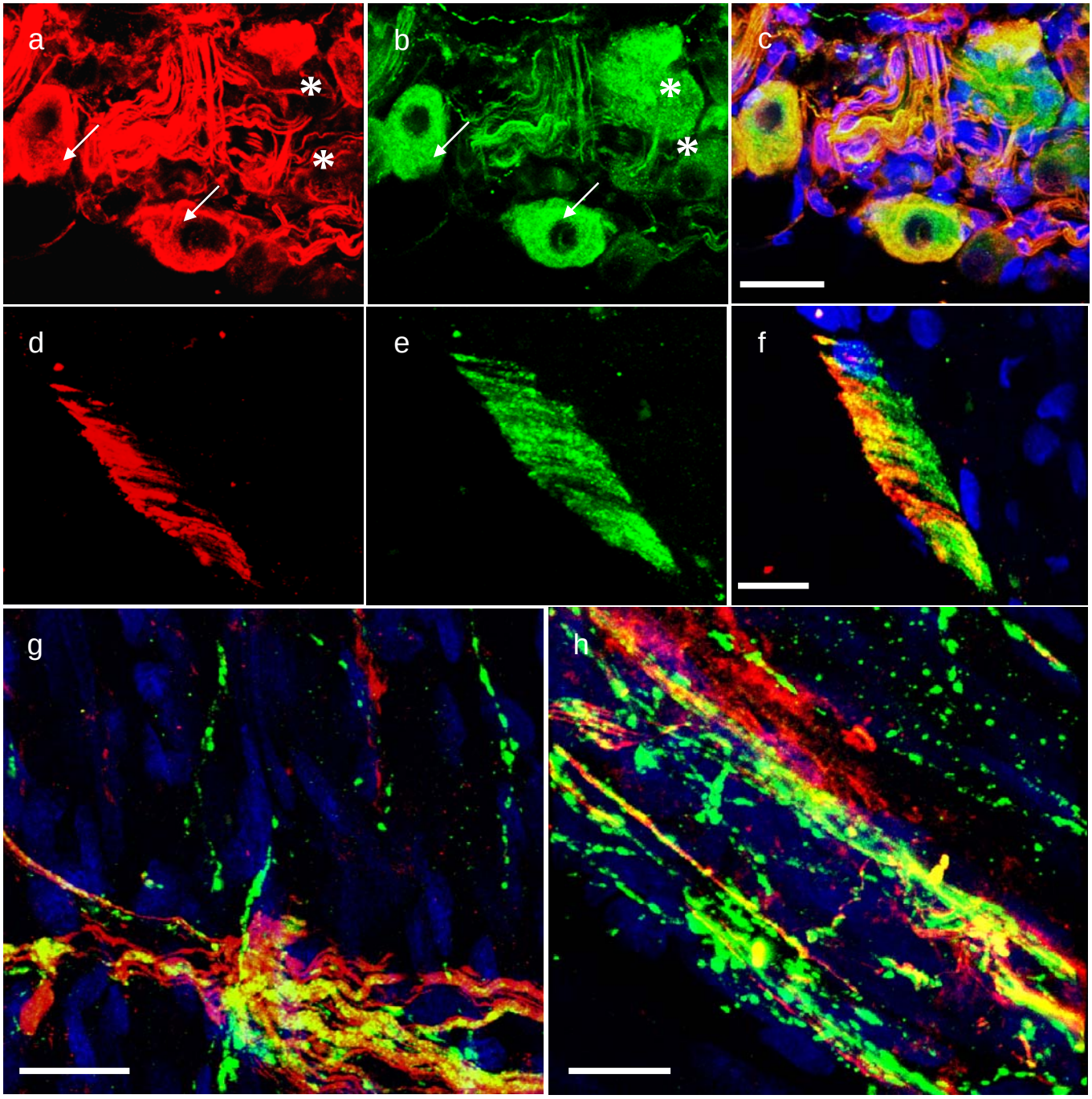

Figure 8

Angiotensin peptides in rat heart (fmol/gram wet weight)

Angiotensin II (fmol/g)

Heart	Right Atrium	Left Atrium	Right Ventricle	Left Ventricle	Septum
Rat 1	8.4	< 8.7	< 1.7	1.45	13.4
Rat 2	21.9	14.4	4.2	1.1	2.0
Rat 3	< 5.6	< 9.2	7.9	2.1	< 1.9
Rat 4	< 5.1	< 11.2	< 2.1	1.8	1.9
Rat 5	6.6	8.4	2.4	4.5	10.8
Rat 6	4.5	10.7	< 1.9	< 1.5	< 1.7
Rat 1	< 8.5	< 13.0	< 2.1	< 1.4	< 5.8
Rat 2	11.3	< 14.3	< 3.5	11.5	8.5
Rat 3	19.9	28.4	2.9	4.3	5.6
Rat 4	6.8	< 17.4	< 2.1	< 2.5	< 5.6
Rat 5	< 6.3	< 13.1	< 2.8	< 1.4	< 5.9
Rat 6	< 10.7	< 14.2	< 3.1	< 1.9	< 5.9

Angiotensin I (fmol/g)

Heart	Right Atrium	Left Atrium	Right Ventricle	Left Ventricle	Septum
Rat 1	61.7	< 17.3	20	< 2.2	< 5.7
Rat 2	< 9.9	< 17.6	< 3.8	< 1.9	< 3.9
Rat 3	17.5	< 18.3	21.2	11.6	10.7
Rat 4	< 10.2	< 22.3	< 4.1	< 3.2	< 2.4
Rat 5	10.2	< 16.7	15.5	8.3	9.7
Rat 6	< 7.8	< 16.6	< 3.8	< 3.0	< 3.3
Rat 1	< 16.9	25.8	< 4.2	3.1	< 11.6
Rat 2	47.3	50.4	8.2	35.7	24.5
Rat 3	< 12.8	< 29.8	< 4.7	4.3	< 10.6
Rat 4	< 13.6	< 34.6	10.4	< 5.0	11.1
Rat 5	18.4	25.9	< 5.6	3.6	27.2
Rat 6	< 21.3	28.2	18.6	< 3.7	15.4

Table 1

Angiotensin peptides in human heart (fmol/gram wet weight)

Heart Tissue	Weight	Angiotensin I	Angiotensin II
Right Atrium	143.2 mg	4.9 (<3.3)	2.0 (<1.7)
Left Atrium	125.5 mg	5.6 (<3.8)	< 1.9
Right Ventricle	149.5 mg	3.2 (<3.2)	7.5 (<1.6)
Left Ventricle	115.7 mg	< 4.1	2.5 (<2.1)
Septum	128.2 mg	3.7 (<3.7)	< 1.9

Detection limit given in brackets.

Table 2

7. General discussion

The presence of Ang II as a main effector molecule of the RAS has been established in the circulation and at tissue level (**Paul et al., 2006**). We have investigated a novel concept of the existence of an intracellular neuronal angiotensinergic system in the rat and human sympathetic as well as sensory ganglia.

7.1 Angiotensinergic system in the sympathetic coeliac ganglia

In the current study, we investigated the intracellular production of Ang II in the neurons of rat and human coeliac ganglia, and in their innervation to mesenteric resistance arteries (**Chapter 2**). The Ang-N-, ACE-mRNA was quantitatively detected by qRT-PCR in these ganglia. We could not find any renin expression in this tissue, the enzyme necessary for cleaving Ang-N into the Ang I precursor of Ang II (**Paul et al., 2006**). Instead we discovered cathepsin D, is such a candidate protease, an enzyme which is capable of cleaving apart other proteins, Ang-N into Ang I (**Saye et al., 1993**). Finding of cathepsin D mRNA with coeliac ganglia indicates the existence of possible alternate pathways for Ang II synthesis in these sympathetic neurons. By using *in situ* hybridization, we could confirm the synthesis of Ang-N mRNA at cellular level in nearly all neurons of the rat coeliac ganglion.

Fibers originating from neurons of sympathetic coeliac ganglia are known to innervate mesenteric resistance blood vessels (**Hsieh et al., 2000**). By using our preestablished monoclonal antibody against Ang II (**Frei et al., 2001**), we have detected strong immunoreactivity for Ang II in the cytoplasm of neurons and their projections of rat coeliac ganglia and further angiotensinergic innervation with mesenteric resistance blood vessels. We could successfully find the same immunoreactivity for Ang II in the neurons of human coeliac ganglia and their projections with mesenteric resistance blood vessels indicating the existence of the same system

in rat and human tissue. With the use of confocal laser scanning microscopy we were able to show discrete Ang II staining in fibers and moreover fibers building "synapse en passant" (sympathetic varicosities) with VSMCs.

7.2 Angiotensinergic system in the trigeminal ganglia

In our present study we provide evidence for local formation of Ang II in the sensory neurons of trigeminal ganglia (**Chapter 3**). We report with the use of qRT-PCR, the presence of Ang-N mRNA and ACE mRNA in the tissue extracts of trigeminal ganglia from rats. *In situ* hybridization confirmed the existence of Ang-N mRNA at cellular level in the cytoplasm of rat trigeminal ganglia neurons. We did not detect the expression of mRNA for renin in the rat trigeminal ganglion. Instead, we found expression of cathepsin D mRNA in the trigeminal ganglia.

Data obtained from HPLC-RIA experiments demonstrate the presence of Ang II and its metabolites in the extracts of rat trigeminal ganglia.

We have discovered Ang II-like immunoreactivity in the cytoplasm of neurons and their processes within the trigeminal ganglia. Ang II has been proposed to be involved in the regulation of sensory information, and in particular nociception (**Takai et al., 1996; Irvine and White, 1997; Pelegrini-da-Silva et al., 2005; Pechlivanova and Stoynev, 2007; Marques-Lopes et al., 2009**). Hence, we attempted to determine whether Ang II was associated with other nociceptive-controlling neuropeptides in the trigeminal ganglia. From the multiple neurotransmitters and neuromodulators identified in this ganglion, we chose Substance P, a neuropeptide established as a key regulator of sensory transmission in the trigeminal ganglion (**Edvinsson, 1991; Lazarov, 2002; Hou et al., 2003**). We found that Ang II was colocalized with Substance P in trigeminal ganglion neurons, supporting the hypothesis of a close integration

between these two systems, and the previous reports of regulation of Substance P release by Ang II (**Kopp et al., 2003; Fusayasu et al., 2007**).

Angiotensin receptor type 1 (AT_1) have been reported in the nucleus tractus solitarii (NTS) of rat brain (**Fior-Chadi and Fuxe, 1998; Rocha et al., 2003**), moreover Substance P receptors are also localized in NTS of rat brain (**Mazzone et al., 1997**). We could also detect angiotensinergic processes in the rat CNS in the spinal trigeminal tract (sp5), indicating that Ang II may act as a neurotransmitter in the CNS. Hence, we can hypothesize that locally formed Ang II may be transported to the terminal fields innervated by the trigeminal ganglion in order to act as a neurotransmitter for sensory transmission, a function similar to that demonstrated in the SNS (**Patil et al., 2008**).

The trigeminal ganglion provides sensory afferents to the cerebral blood vessels (**Dostrovsky et al., 1991**). RAS inhibition through ACE inhibitors or AT_1 receptor blockade appears to be effective for the prevention of migraine, independently of their blood pressure lowering effects (**Schrader et al., 2001; Tronvik et al., 2003; Charles et al., 2006**), to increase resistance to brain ischemia (**Nishimura et al., 2000; Ito et al., 2002**) and to reduce cerebrovascular inflammation (**Ando et al., 2004; Zhou et al., 2005**). These studies indicate involvement of Ang II in migraine and other associated pathology.

7.3 Angiotensinergic system in the sensory dorsal root ganglia

The major findings of our study are the demonstration and quantitation of components of RAS in the rat and/or human sensory dorsal root ganglia (DRG) see **Chapter 4**. We have detected mRNA for the Ang II precursor Ang-N, and for ACE, in the rat DRG. Furthermore, combination of *in situ* hybridization and immunohistochemistry revealed colocalization of Ang-N mRNA and Ang II immunoreactivity in most DRG neurons of the rat. The coexistence of Ang II

and mRNA for Ang-N, the only known precursor for Ang I production, in the same neurons demonstrates that Ang-N uptake from the circulation may not be necessary for the local intraneuronal formation of Ang II. Surprisingly, and in contradiction to a previous report in bilaterally ovariectomized rat DRG (**Chakrabarty et al., 2008**) we did not detect mRNA for renin in our wild type rat DRG samples. Instead, we found substantial expression of cathepsin D mRNA.

Data obtained from HPLC- RIA experiments demonstrate the presence of Ang I and Ang II peptides in the extracts of rat and human DRG samples.

In rodents, there are two AT_1 receptor subtypes: AT_{1A} and AT_{1B}, differentially expressed and regulated (**Iwai et al., 1992**). Using specific primers with qRT-PCR, we detected AT_{1A} mRNA, but not AT_{1B} mRNA expression in our rat DRG samples. Receptor expression in DRG is an indication that Ang II, either circulating or locally formed, may exert effects in this tissue. In our samples we noticed low expression of angiotensin type II (AT_2) receptor mRNA, a receptor type whose function is still controversial (**Saavedra, 1999; Saavedra, 2005; Porrello et al., 2009**). Nevertheless, the presence of specific angiotensin receptors in DRG increases the likelihood of a neuronal function of Ang II.

We have noticed Ang II-like immunoreactivity not only in neuronal cell bodies but also in neuronal projections within the rat and human DRG, and intense immunoreactivity in fibers with synapses en passant in the human DRG. As mentioned previously, Ang II has been proposed to be involved in the regulation of sensory information, and particularly in nociception, moreover Substance P is one of the neuropeptides established as a key regulator of sensory transmission. Hence, we attempted to determine whether Ang II was associated with Substance P in the DRG. We found that Ang II was colocalized with Substance P in DRG neurons, supporting the hypothesis of a close integration between these two systems.

7.4 Angiotensinergic system in the heart, intracardiac ganglia

The localization and the function of the RAS in the heart is an issue of many controversies, because of the complexity of the systemic and the local RAS (**Paul et al., 2006; Singh et al., 2008**). The mammalian heart is composed of different chambers with variation in their function. Hence, to study the specific localization of RAS components in the rat and human hearts, these tissues were divided into four chambers as right atrium, left atrium, right ventricle and left ventricle (**Chapter 4**).

Despite of several published studies, a definite location of Ang-N synthesis in the heart is completely missing. By using qRT-PCR we could detect higher amount of Ang-N mRNA in the right and left atria of rat heart than in the ventricles, in comparison as with liver, the major source of Ang-N production (**Dzau et al., 1987**).

Meiklejohn and Walker reported that the ganglia were found only in the right and left atrium of the rat heart (**Meiklejohn, 1914**). In order to confirm the presence of Ang-N mRNA revealed in extracts by qRT-PCR we performed *in situ* hybridization at the cellular resolution in rat heart. *In situ* hybridization confirmed the existence of Ang-N mRNA in the cytoplasm of the neurons of the rat intracardiac ganglia of the atria. Ours is the first report on the presence of Ang-N mRNA at cellular resolution in the neurons of intracardiac ganglia.

In the present study, renin expression was not traced in any part of the rat heart by qRT-PCR. Even after using high concentrations of the cDNA (200 ng/sample), renin mRNA was untraceable when compared to renin mRNA levels in kidney, the major source of renin production (**Persson, 2003**). Instead, we detected expression of cathepsin D mRNA in all parts of the rat heart. These results indicate the possibility of the existence of an alternate pathway for the local synthesis of Ang I in the heart.

In comparison to lung the major source of ACE production (**Pueyo et al., 2004**), we detected substantial amounts of ACE mRNA in all the parts of rat heart by qRT-PCR.

AT_1- and AT_2- mRNA expression in the heart has already been reported, AT_{1A} and AT_{1B} are the receptor subtypes of AT_1 in rodents (**Paul et al., 2006**). Ang II receptor studies are reported either in whole heart or in cardiac cell culture, hence we investigated, AT_{1A}-, AT_{1B}- and AT_2-mRNA expression in different parts of the rat heart by using qRT-PCR. AT_{1A} mRNA is expressed in all the parts of the rat heart while AT_{1B} mRNA expression was not traceable. In order to detect AT_2 mRNA in the heart we had to use higher concentration of cDNA (200 ng/sample), while 40 ng of cDNA per sample was used for all other mRNA measurements.

Data obtained from HPLC-RIA experiments demonstrate the presence of Ang I and Ang II peptides in the extracts of WKY rat as well as human hearts.

Rat and human tissue samples from the four different chambers of hearts and septum were used for Ang II detection by immunocytochemistry. At cellular level we have noticed the existence of Ang II especially in the rat and human intracardiac ganglia, which are also known as a cardiac nervous system (**Armour, 2008**). Angiotensinergic intracardiac ganglia were located in the right and left atria of the rat heart, as well as in their processes.

The heart is innervated by sympathetic and parasympathetic fibers of the autonomic nervous system (**Chow et al., 2001; Armour, 2008**). In the present study, we could find angiotensinergic fibers in both atria, in the right ventricle wall, left ventricle wall and in septum wall of the rat heart. Cardiomyocytes in all parts of the heart are known for the production of Ang II (**Singh et al., 2008**), in our study these cells are also stained for Ang II.

Ang II immunoreactivity was discovered in the right atrium of the human heart in the intracardiac neurons and their fibers. In the present study angiotensinergic fiber pathways were detected in the right atrium as well as in very fine neural fibers in the left atrium of the human

heart. In the right ventricle we found Ang II staining in neuronal processes and in the cardiomyocytes, showing Ang II staining in striated cardiac fibers presumably with transverse tubules *(***Kessel and Kardon, 1979**). Moreover Ang II staining in the neuronal processes was also found in septum of the human heart. Axons from intracardiac ganglia as well as from the extracardiac origin innervate different heart regions (**Steele et al., 1996; Kukanova and Mravec, 2006**), leading to cardiac innervation by neurons of multiple origin. Hence, the origin of the angiotensinergic innervation other than intracardiac ganglia, innervating different heart regions has to be investigated further.

Localization of tyrosine hydroxylase, dopamine-β-hydroxylase (DβH) and norepinephrine in the human heart has been shown by immunohistochemistry (**Singh et al., 1998**). In current study, we explored further the presence of DβH in the intracardiac neurons and their processes and the possible colocalization within the angiotensinergic system. Rat right atria containing intracardiac ganglia, were double stained for Ang II and DβH, indicating partial colocalization, most neurons are stained positive for both Ang II and DβH while some are not. As several evidences indicate that the neurons of intracardiac ganglia can considerably regulate the heart activity (**Kukanova and Mravec, 2006; Armour, 2008**), localization, and colocalization of Ang II with the SNS in the intracardiac neurons significantly supports the involvement of Ang II in the regulation of heart activity.

7.5 Conclusion

In conclusion, our findings strongly indicate the existence of an endogenous synthesis of Ang II in the neurons of sympathetic coeliac ganglia and further their angiotensinergic innervation with mesenteric resistance blood vessels. Localization of Ang II in sensory trigeminal ganglia and dorsal root ganglia, and its neuronal colocalization with Substance P suggests the possible interaction of Ang II with Substance P. Furthermore, Ang II could be involved in the regulation of sensory pathways including pain and the functions of other local neurotransmitters. Our findings in the heart demonstrate the presence of an endogenous, neuronal angiotensinergic system in the rat and human intracardiac ganglia. Ang II, as norepinephrine, may act as a neurotransmitter locally in the heart. Absence of renin mRNA in all the tissues studied and on the other side the existence of cathepsin D mRNA in the same tissue samples, strongly indicate existence of an endogenous renin independent angiotensinergic system.

7.6 Outlook

As mentioned before, even though the heart is innervated by fibers from CNS we still have no clue of the origin of the angiotensinergic neuronal fibers present in different parts of the heart. Hence, we have extended our studies to investigate the angiotensinergic system in denervated and infracted rat heart models.

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"*Guru Brahma Guru Vishnu, Guru Devo Maheshwara,*
Guru Saakshaat Param Brahma, Tasmai Shree Guruve Namaha"

Meaning:
Guru Is Brahma (Who plants the qualities of goodness),
Guru Is Vishnu (Who nurtures and fosters the qualities of goodness),
Guru Is Maheshwara (Who weeds out the bad quality),
Guru Is Supreme Brahma Itself…My Salutations are to that Guru.

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Curriculum vitae

Personal Data

Last name:	Patil
First name:	Jaspal Jaysing
Date of birth:	9th May 1983
Birthplace:	Kolhapur, India
Nationality:	Indian
Civil status:	Married
Languages known:	Mother language: Marathi Fluent in English and Hindi. Beginner in German and Swedish.
Addresses: Permanent:	Sr. No. 71/2/6, Ganeshnagar, Godoli, Satara. Maharashtra, India.
Official:	Institute of Cell Biology Baltzerstrasse 4 Bern, 3012 Switzerland Email: jaspal.patil@izb.unibe.ch jaspalpatil@gmail.com

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Academic degrees

1999	Completed higher secondary studies from Y.C Institute of Science, Satara, India
2000-2004	Graduated as Bachelor of Pharmacy, University of Pune, Pune, India
2005-2006	Completed, Masters of Science in Molecular Biology from University of Skövde, Skövde, Sweden.
Feb-July 2006	Masters thesis at Institute of Clinical Neuroscience, Würzburg, Germany in research group of Prof. Dr. Michael Sendtner.
November 2006 - Present	PhD student in research group of Prof. Dr. Hans Imboden in Institute of Cell Biology, University of Bern, Bern, Switzerland.

Professional experience

June – November 2004	Worked as a Medical Representative in Cipla Pharmaceuticals, Pune, India.

Publications

Jaspal Patil, Eva Heiniger, Thomas Schaffner, Oliver Mühlemann, Hans Imboden. Angiotensinergic neurons in sympathetic coeliac ganglia innervating rat and human mesenteric resistance blood vessels. *Regulatory Peptides*. 2008 Apr 10;147(1-3):82-7.

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Jaspal Patil, Silvan Stucki, Thomas Schaffner, Juerg Nussberger, Hans Imboden. Angiotensinergic system in the mamalian heart, especially in the rat and human intracardiac ganglia. Expected submission in October, 2009

Invited talks in conferences

Graduate School Symposium(3rd) January 2009, Bern, Switzerland. Endogenous angiotensinergic system in neurons of rat and human trigeminal ganglia

Clinical Neuroscience Meeting (4th) November 2008, Bern, Switzerland. Endogenous angiotensinergic system in neurons of rat and human trigeminal ganglia

Cardiovascular Research Conference June 2008, Medical Faculty, Department of Clinical Research, Insel Hospital, Bern, Switzerland. Angiotensinergic neurons in sympathetic coeliac ganglia innervating rat and human mesenteric resistance blood vessels.

Institute of Cell biology November 2007, Baltzerstrasse 4, Bern, Switzerland. Angiotensinergic innervation of Rat and Human blood vessels a novel concept for renin angiotensin system.

Poster presentations

Swiss Society for Microcirculation (SSM), October 2009, Bern, Switzerland. Angiotensin II an endogenous neurotransmitter with rat and human mesenteric resistance blood vessels

Angiotensin, February 2008 - Gordon Research Conference, Ventura, CA, United States. Angiotensinergic neurons in sympathetic coeliac ganglia innervating rat and human mesenteric resistant blood vessels.

Federation of European Neurosciences Societies July, 2008;4:194.12, Geneva, Switzerland. Angiotensinergic neurons in sympathetic coeliac ganglia innervating rat and human mesenteric resistance blood vessels.

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Indian Pharmaceutical Congress (54th) Pune, India. Patil J.J Golden opportunity for Indian pharmaceutical R & D. in scientific views of *Express Pharma Pulse*. 2002;32.

Declaration of Originality

Last name, first name: **Patil, Jaspal**

Matriculation number: **07-106-164**

I hereby declare that this thesis represents my original work and that I have used no other sources except as noted by citations.
All data, tables, figures and text citations which have been reproduced from any other source, including the internet, have been explicitly acknowledged as such.
I am aware that in case of non-compliance, the Senate is entitled to divest me of the doctorate degree awarded to me on the basis of the present thesis, in accordance with the "Statut der Universität Bern (Universitätsstatut; UniSt)", Art. 20, of 17 December 1997.

Place, date

Bern,
18 September 2009

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Signature

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