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Angiotensinergic Neurons in the Neonatal Rat Heart and in Atrial Cell Culture

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Abstract

The renin-angiotensin system (RAS) and its main effector peptide angiotensin II (Ang II) are known to play a pivotal role in the regulation of heart function, development of cardiac remodeling, arrhythmia, and heart failure. Recently, a novel population of sympathetic neuronal cells containing Ang II was described in the adult rat heart.

The aim of the current study was to investigate the presence of Ang II positive neurons in the neonatal rat heart and to develop methods to maintain these neurons in primary atrial cell cultures.

The immunohistochemical characterization of angiotensinergic neurons in whole rat hearts and in primary cell culture preparations was based on colocalization studies of Ang II with other components of the RAS and known neuronal cell markers. The survival of Ang II positive neurons in primary cell culture was studied under different culture conditions. Single cell electrophysiological studies were performed using patch clamp recording on isolated neurons in cell culture. Viral transduction using a neuron specific promoter served to investigate the neuronal identity of angiotensinergic cells.

In the atria of whole neonatal rat heart preparations, neuronal cell bodies and fibers of intracardiac ganglia were found to contain Ang II that colocalized with neuron specific beta III tubulin (NM III), synaptophysin and calcitonin gene related peptide (CGRP). Ang II and the vesicular acetylcholine transporter (VAChT) displayed an intriguing staining pattern with VAChT positive structures encircling angiotensinergic ganglion cells, which may indicate a functional link between the parasympathetic nervous system and the angiotensinergic system. Labeling of the sympathetic nervous system with antibodies against dopamine-β-hydroxylase (DβH) resulted in strong staining of neuronal cell processes while cell bodies were only weakly stained. Neither renin nor cathepsin D could be detected immunohistochemically.

In primary atrial cell cultures, neuron-like cells with long and slender processes exhibiting colocalization of Ang II with NM III were found. Similar to intact tissue, these neurons were renin negative. The finding that VAChT staining was unspecific and DβH was negative suggests that neither sympathetic nor parasympathetic neuronal cells were present in cell culture. The quantity of angiotensinergic neurons in primary culture was unaffected by addition of nerve growth factor, Ang II or aliskiren to the cell culture medium. Adding pepstatin A at high concentration reduced the number of Ang II positive neurons relative to control conditions. Patch clamp recordings of Ang II positive neurons revealed these cells to be excitable. Resting membrane potentials averaged -64.45 mV ± 2.83 mV (n=6). The threshold potential for eliciting action potentials was -47.43 mV ±

6.38 mV (n=6). Action potential duration was ~5 ms and the maximal spike frequency observed was ~20 Hz. Viral transduction experiments showed expression of the enhanced yellow fluorescent protein (eYFP) under the calcium/calmodulin-dependent protein kinase IIa (CaMKIIa) promoter in Ang II positive cells thereby underlining the neuronal identity of the angiotensinergic cells under investigation.

Overall, the results show that rat hearts contain an early developed intracardiac angiotensinergic nervous system. When isolated and kept under appropriate culture conditions, Ang II positive cells show the typical electrophysiological characteristics of neurons. Further investigation will be needed to elucidate the specific physiological role of angiotensinergic neurons in heart function.

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Non-Standard Abbreviations

ACE	angiotensin converting enzyme
Ang II	angiotensin II
AV	atrioventricular
CaMKIIa	calcium/calmodulin-dependent protein kinase IIa
CGRP	calcitonin gene related peptide
Cy3, Cy5	cyanine 3, cyanine 5
DAPI	4', 6-diamidino-2-phenylindole
DAR	donkey anti-rabbit
DβH	dopamine-β-hydroxylase
DMEM	Dulbecco's Modified Eagle Medium
eYFP	enhanced yellow fluorescent protein
FBS	fetal bovine serum
GAM	goat anti-mouse
HBSS	Hanks' balanced salt solution
LY	Lucifer yellow
NBM	Neurobasal® Medium
NCS	neonatal calf serum
NGF	nerve growth factor
NM III	neuron specific beta III tubulin
n. s.	not significant
PFA	paraformaldehyde
PBS	phosphate buffered saline
Pep	pepstatin A
qRT-PCR	quantitative real-time polymerase chain reaction
SA	sinoatrial
SD	standard deviation
TGF-β_1	transforming growth factor-β_1
TH	tyrosine hydroxylase
VAChT	vesicular acetylcholine transporter

Introduction

The intracardiac nervous system importantly contributes to the regulation of heart function. Whereas sympathetic and parasympathetic modulation is well established, there is increasing evidence for additional types of intracardiac neurons that form extensive networks with extracardiac neurons involving efferent and afferent nerves and interconnecting neurons [1] [2]. This intracardiac nervous system, comparable to the enteric nervous system, is supposed to modulate heart function under both physiological as pathological conditions [1] [2]. Intracardiac ganglia in the adult human heart have mainly been detected near the sinoatrial (SA) and atrioventricular (AV) nodes, on the superior left atrial surface, the interatrial septum and the atrial appendage-atrial junctions, as well as at the base of the great vessels and at the base of the ventricles [3]. By contrast, the right atrial free wall, atrial appendages, trunks of the great vessels and most of the ventricular myocardium are devoid of cardiac ganglia (see Figure 1, cf. also [3]).

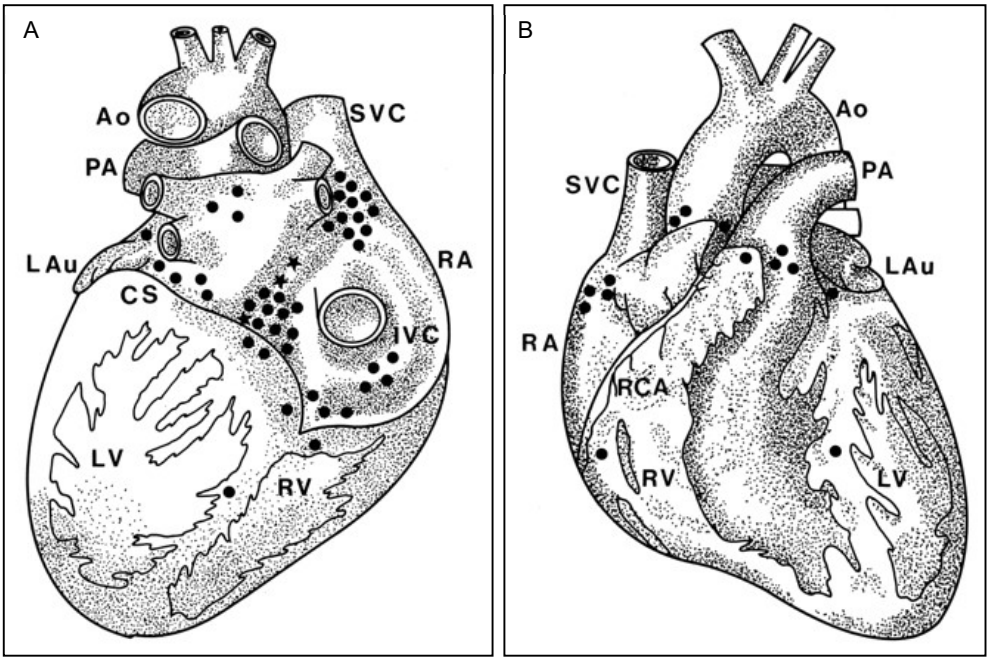

Fig. 1: Topography of intracardiac ganglia in the adult human heart.

A, Posterior view: Para-SA nodal ganglia are concentrated primarily lateral to the right pulmonary veins. The para-AV nodal ganglia are on the epicardial surface superior to the coronary sulcus *(CS)* (●) and within the interatrial septum (★). **B,** Anterior view: Ganglia are concentrated primarily at base of the aorta *(Ao)* and the pulmonary artery *(PA)*. Ganglia from the para-SA nodal collection can be seen extending to the right atrial *(RA)* and atrial-appendage junction. *LAu* left atrial appendage, *LV* left ventricle, *RV* right ventricle, *SVC* superior vena cava, *IVC* inferior vena cava, *RCA* right coronary artery. See text for more detailed information. Figures and comments from [3].

The cellular composition of intracardiac ganglia was shown by immunohistochemistry to consist of various subgroups of neurons that stain positive for neuronal cell markers like protein gene product 9.5 but with different staining patterns for tyrosine hydroxylase (TH), dopamine-β-hydroxylase (DβH), vasoactive intestinal peptide, calcitonin gene related peptide (CGRP), substance P, pituitary adenylate cyclase-activated peptide, synaptophysin and vesicular acetylcholine transporter (VAChT) [4] [5] [6].

Another recently discovered group of intracardiac neurons in the adult rat and human heart stains strongly positive for angiotensin II (Ang II) [6]. These neurons presumably contain the machinery that allows them to produce Ang II de-novo. The finding of colocalization studies showing that Ang II containing nerve cells also express DβH (an enzyme required for norepinephrine production) suggests that Ang II, apart from acting as a hormone, might constitute a neurotransmitter of the sympathetic nervous system [6]. These angiotensinergic neurons have not only been detected in the heart, but also in sympathetic coeliac ganglia, in trigeminal ganglia and in the dorsal root ganglia [7] [8] [9]. Their significance for physiology and pathophysiology is still unknown.

In general, the renin-angiotensin system (RAS) has endocrine characteristics. Its main effector, the octapeptide angiotensin II, regulates the release of aldosterone from the adrenal cortex and induces vasoconstriction by interacting with Ang II receptors on vascular smooth muscle cells [10]. The substrate of the RAS is angiotensinogen, an α-glycoprotein that is released from the liver and cleaved by the enzyme renin (secreted by the juxtaglomerular apparatus) to result in the decapeptide angiotensin I (Ang I). Further cleaving by angiotensin converting enzyme (ACE), a membrane-bound metalloproteinase that is mainly expressed on endothelial cells in the pulmonary circulation, results in the active octapeptide Ang II [10]. Different angiotensin receptors (AT_1, AT_2, AT_4) and signal transduction pathways have been discovered, as well as further enzymes in Ang II formation like cathepsins (as an alternative to renin, cf. to [11]), tonin, chymase or the zinc metalloproteinase ACE2 that generates alternative angiotensin peptides by conversion of Ang II to Ang-(1-7), and Ang I to Ang-(1-9) [10]. The heptapeptide Ang-(1-7) binds to specific receptors (neither AT_1 nor AT_2) on cardiac fibroblasts, where it induces antifibrotic and antitrophic effects and, thus, acts as an antagonist of Ang II [10] [12] [13]. In addition, other angiotensins like the heptapeptide Ang-(2-8) (Ang III) and the hexapeptide Ang-(3-8) (Ang IV) have been found to be biologically active [14].

The RAS also plays an important role in cardiac pathologies. Following binding to the AT_1 receptor, Ang II can cause cardiac hypertrophy by inducing protein synthesis in cardiomyocytes which is not dependent on increased blood pressure. In primary cultures of cardiomyocytes, this effect is enhanced in presence of cardiac fibroblasts [15] [16]. Moreover, Ang II modulates the expression

of transforming growth factor-β_1 (TGF-β_1) that stimulates fibrosis in infarcted and noninfarcted myocardia [17]. Another AT_1 mediated Ang II effect consists of promoting the phenotype switch from fibroblasts to myofibroblasts [18]. Myofibroblasts ("activated fibroblasts") play an important role in tissue repair, myocardial fibrosis and in the development of cardiac arrhythmias [19]. Besides being a target of the RAS, myofibroblasts in turn participate in the RAS by their intrinsic capability to synthesize angiotensin peptides de novo [20] [21]. The activation of the intracardiac RAS and aldosterone are involved in cardiac arrhythmogenesis by promoting structural remodeling of cardiac tissue [22] [23] [24] [25] [26] [27]. Whether the AT_2-receptor promotes growth-inhibiting effects [28] or hypertrophic effects on the heart in response to pressure overload [29] is still under debate. Aldosterone, released in response to Ang II, contributes in part to Ang II induced cardiovascular remodeling [30]. Blocking ACE or AT_1 receptors inhibits the profibrotic and protein stimulating characteristics of Ang II, ameliorates the remodeling process of the heart, and enhances long-term survival in animal models of cardiac hypertrophy and heart failure [17] [18] [28] [31] [32] [33] [34].

The long-standing view of the RAS as a systemic endocrine system has been extended during the last decades by the discovery of "local" or "tissue" renin angiotensin systems. Such local self-contained RAS have been found in organs as different as the heart, the vasculature, the nervous system, the reproductive tract, the skin, digestive and sensory organs as well as lymphatic and adipose tissues. This led to the hypothesis that, apart from systemic actions of the RAS, there might exist locally confined RAS with equally local actions based on Ang II [10].

During recent years, it was additionally suggested that there exist complete RAS within individual cells, referred to as intracellular RAS [35]. In addition to the intracellular presence or synthesis of Ang II, it is thought to exert "intracrine", functionally relevant biological effects [35]. Both intracellular synthesis and receptor-mediated internalization of Ang II are believed to contribute to the specific levels of intracellular Ang II observed [35].

Even though there is ample evidence for an important role of circulating Ang II in the heart, little is known about the precise localization and the development of the local RAS and, in particular, about the possible link of the local RAS to the intracardiac nervous system. In this context, a discovery with potentially far reaching consequences has recently been made as it was found that the nervous system of adult rat and human hearts contains a distinct population of neurons that stains positive for Ang II. Confirmation that these Ang II positive cells were in fact neurons was based on both their typical morphology and on immunohistochemistry demonstrating that Ang II in these cells colocalized with neuronal markers like DβH, synaptophysin and VAChT [6].

Based on these intriguing findings, it was the goal of the current study to investigate whether Ang II positive neurons (angiotensinergic nerves) are also present in neonatal rat hearts and whether they can be propagated in cell culture with the aim to conduct functional electrophysiological studies. We found an early developed intracardiac angiotensinergic nervous system consisting of Ang II positive cells mainly located in atrial ganglia. Furthermore, we found that Ang II positive cells show the typical electrophysiological characteristics of neurons.

Materials and Methods

Experimental Preparations

Whole Heart

Sections of a whole neonatal rat heart were used for colocalization studies. A single one day old male Wistar Kyoto rat was purchased from the Central Animal Facilities of the University of Bern. Adequate measures were taken to minimize pain or discomfort in accordance with Swiss and State Veterinary laws and protocols. The rat was sacrificed by decapitation. The thoracic cavity was opened by a right parasternal incision and the heart was carefully dissected.

The heart was washed twice in phosphate buffered saline (PBS) and fixated by immersion in 2% paraformaldehyde (PFA) for three days at 4°C. At day 4, the heart was immersed for 14 hours in PBS containing 18% sucrose at 4°C. The heart was frozen in isopentane at -50°C and 30 µm thin sections were cut on a cryostat and subsequently used as free-floating sections for immunohistochemistry.

Cell Culture Preparation

Animals

Neonatal Wistar Kyoto rats (1-2 days old) were purchased from the Central Animal Facilities of the University of Bern. Adequate measures were taken to minimize pain or discomfort in accordance with Swiss and State Veterinary laws and protocols. 10 to 12 animals were used per individual cell culture. Rats were sacrificed by decapitation. Thoracic cavities were opened by a right parasternal incision and hearts were carefully dissected. Ventricle and adhering vessels were removed from the atria under microscopic control.

Tissue Dissociation

Atrial tissue was minced with scissors. Resulting tissue pieces were immersed in Hanks' balanced salt solution (HBSS without Ca^{2+} and Mg^{2+}; Bioconcept) containing trypsin (0.12 %, Roche Diagnostics), pancreatin (120 µg/mL, Sigma) and penicillin/streptomycin (20'000 U/L, 34 µmol/L; Biochrom AG). The tissue containing dissociation solution was stirred for 15 min in a water bath at 35°C before performing a mechanical dissociation by trituration. The supernatant was collected in a tube, placed on ice, fresh dissociation solution was added to the tissue pieces and incubation was restarted. Usually, five incubation periods were required to dissociate the tissue entirely. The collected cells from dissociation cycles 2-5 were sedimented by centrifugation at 1000 rpm at room temperature for 5 min.

The resulting cell pellets were re-suspended in culture medium consisting of M199 with Hanks' salts (Sigma), penicillin/streptomycin (20'000 U/L, 34 µmol/L; Biochrom AG), vitamin B12 (1.5 µmol/L; Sigma), L-glutamine (680 µmol/L, Sigma) and 10% neonatal calf serum (Bioconcept), and strained through a 70 µm filter (Falcon).

Preplating and Seeding

After cell counting with a C-Chip Neubauer improved cell counter, cell suspensions were seeded on glass coverslips that had been spin-coated with agar and subsequently coated with either collagen type I or matrigel and sterilized under UV light. Cells were seeded at densities between 300 and 1200 cells/mm^2 according to experimental needs and subsequently put in the incubator. After 2 hours, the supernatant was removed and decanted into a culture flask. Fresh M199 culture medium supplemented with 10% neonatal calf serum (NCS), epinephrine (10 µmol/L, Sigma) and ascorbic acid (18 µmol/L, Sigma) was added to attached cells in multiwells. The supernatant containing mostly cardiomyocytes were used for other experiments in the laboratory.

Washing

Cell cultures were washed 16-24 hours after seeding to remove non-adherent cells and debris and were incubated with M199 culture medium containing a reduced serum content (5%). The M199 culture medium was exchanged every other day thereafter.

Optimization of Culture Conditions

Glass Coverslip Coating

Two coating substances were compared in regard to their ability to support cell attachment. Cells cultivated on collagen type I or matrigel coated coverslips grew evenly and formed a cell monolayer. The density of angiotensinergic neurons was assessed after fixation and immunocytochemical staining for Ang II. On collagen coated coverslips, we found 35.6 ± 8.7 Ang II positive cells per 500 mm^2 (mean ± SD, n=7). A slightly higher number was found on matrigel coated coverslips (45.1 ± 27.3 per 500 mm^2, n=7). Because these numbers were not significantly different, substrates used in the experiments were routinely coated with collagen type I.

Culture Media

Effects of different media and supplements on the growth and survival of neurons was investigated by comparing the density of angiotensinergic nerves grown in control medium (M199, serum free) to that in serum supplemented medium. In the few experiments done, the density of angiotensinergic nerves in serum supplemented medium was slightly higher than that in serum-free conditions.

Additionally, Neurobasal® Medium (NBM, Gibco) was tested as an alternative to M199 culture medium to optimize growth conditions for neurons. As shown in Figure 2, cells grown in NBM showed a much lower density and smaller size than cells cultivated in supplemented M199. It seemed that cells were compromised in their ability to attach to the substrate in presence of NBM. Also, there were no Ang II positive cells left in these cultures. Accordingly, NBM was abandoned and standard serum supplemented M199 culture medium was used for all experiments.

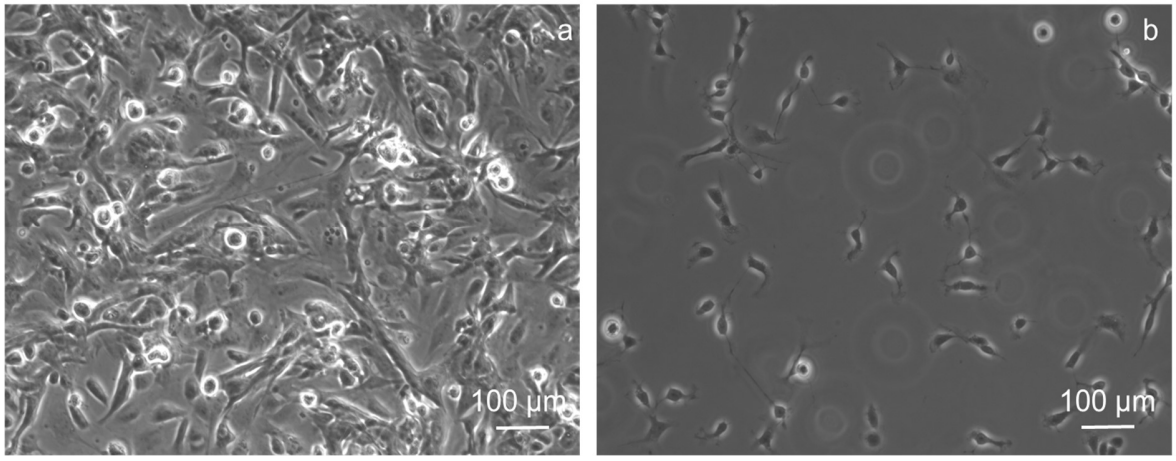

Fig. 2: Phase contrast images of cells grown in standard M199 and in Neurobasal® medium.

a, Cells grown in serum supplemented M199 medium, **b,** Cells grown in Neurobasal® Medium.

Survival of Angiotensinergic Neurons under Variable Culture Conditions

To investigate whether freshly dissociated neurons were attaching to the substrate within the 2-hour lasting preplating period ('fast adhering fraction') or only during the subsequent culturing period ('slowly adhering fraction'), both fractions were kept in culture for up to 7 days. Cell numbers and type were determined at different time points after fixation and staining of the preparations. The results shown in Figure 3 demonstrate that most of the angiotensinergic neurons were found in the fast (≤ 2 hours) adhering cell fraction while only a few neurons survived in the supernatant fraction. Thus, neurons behaved similarly to the fast adhering mesenchymal cells and unlike the cardiomyocytes that remained mostly in suspension during the 2 hours preplating period. The number of angiotensinergic neurons per preparation decreased with time in culture as shown in Figure 3C.

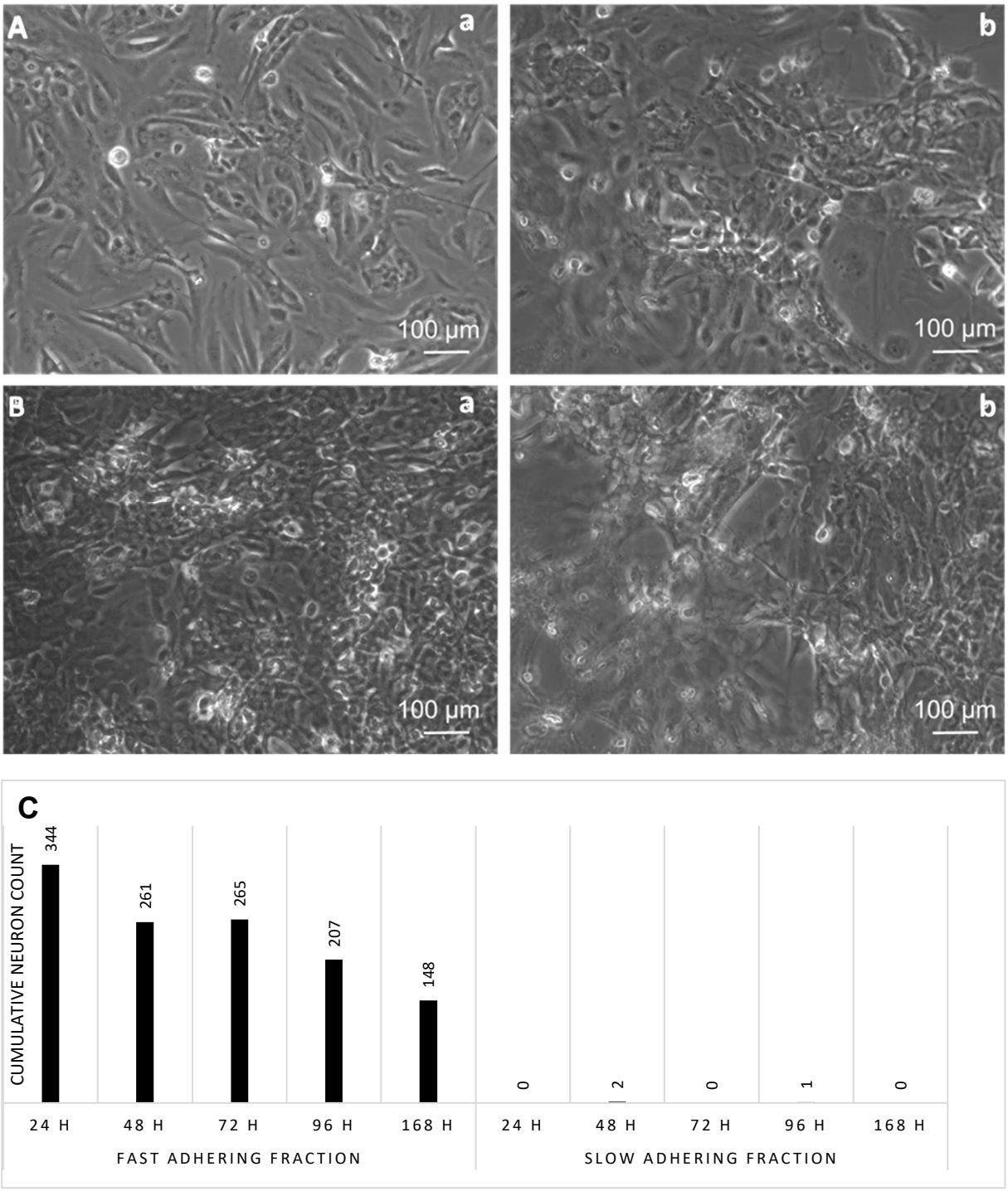

Fig. 3: Ang II positive neurons in cultures of fast vs. slowly adhering cells.

A, Phase contrast images of cell culture from slow adhering fraction (supernatant) 96 h after seeding that shows a cell monolayer consisting mostly of cardiomyocytes (**a**) which starts to detach with increasing time in culture (168 h, **b**). **B,** Phase contrast images of the fast adhering cell fraction that forms a dense monolayer 96 h after seeding (**a**) and shows little change with increasing culture age (168 h, **b**). **C,** Number of angiotensinergic neurons in fast (n=5) and slowly adhering (n=5) cultures at different time points after seeding.

As preplating for 2 hours led to preferential mesenchymal and neuronal cell attachment (see above), we investigated the possibility of shorter preplating time to enhance the density of neurons in atrial cell cultures. Preparations undergoing preplating for 1 hour contained 34.8 ± 5.3 (n=4) Ang II positive neurons per 500 mm^2. Preplating for 2 hours instead led to a slightly higher amount of angiotensinergic neurons (47 ± 12.4 per 500 mm^2, n=4; n. s. versus short preplating time). Because the number of neurons after preplating for 2 hours tended to be slightly higher, this protocol was subsequently used for all experiments.

Angiotensinergic neurons from the fast adhering fraction are shown in Figure 4. Typically, they showed neuron-like cell bodies with long, slender, and branched extensions. The cytoplasm of cell bodies and fibers exhibited colocalized staining for Ang II and NM III. Ang II positive neurons appeared alone or grouped and were distributed randomly over the preparations. As mentioned above, their number declined with time in culture.

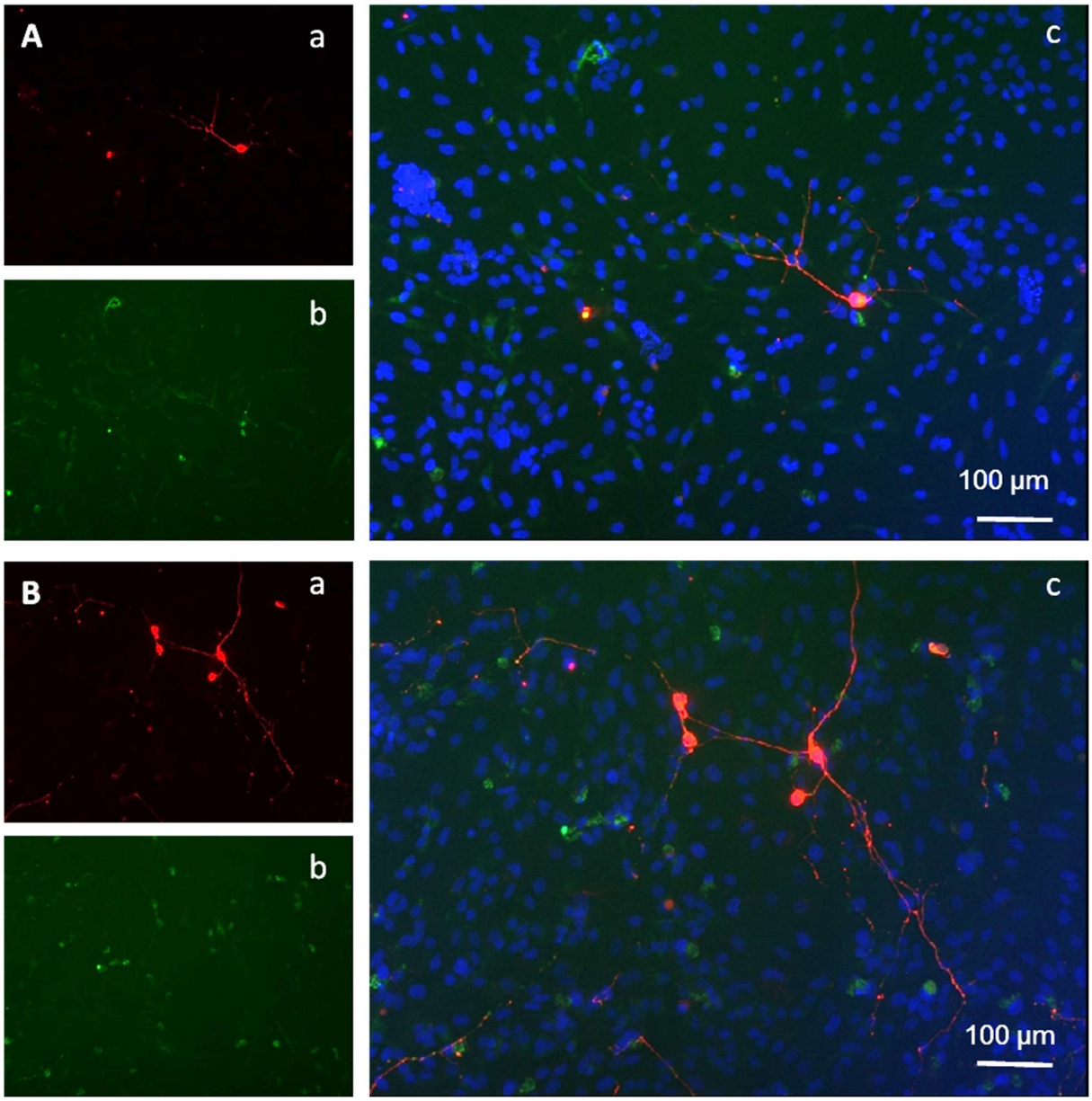

Fig. 4: Morphology of angiotensinergic neurons of the fast adhering fraction.

A, 24h old culture stained for angiotensin (red, a) and NM III (green, b) and overlay (c) including DAPI staining. **B,** Same for 48 h old culture.

Experimental substances

To investigate the source of Ang II in angiotensinergic neurons and to modulate neuronal growth/proliferation, the following substances were added to the cell culture medium 2h after preplating:

Substance	Concentration	Incubation Time
Angiotensin II	1 nmol/L; 1 µmol/L	24 h
Fluorescein labeled Angiotensin II	1 nmol/L; 1 µmol/L	24 h
Aliskiren	10 nmol/L; 100 nmol/L; 1 µmol/L; 100 µmol/L	48 h
Pepstatin A	10 µmol/L; 100 µmol/L	24 - 72 h
Nerve Growth Factor	10 ng/mL	48 h

Patch Clamp Recording

Standard whole-cell recordings of the patch-clamp techniques were used to investigate basic electrophysiological characteristics of Ang II positive cells [36]. All experiments were conducted at room temperature. The patch pipette filling solution contained (in mmol/L): K-aspartate 120, NaCl 10, MgATP 3, $CaCl_2$ 1, EGTA 10, Hepes 5 (pH 7.2) with a free cytosolic Ca^{2+} concentration of 10^{-8} mmol/L. A HEKA EPC-10 patch clamp amplifier system was used to amplify, digitize (2.9 kHz) and filter (1 kHz, lowpass) the signals that were sent to a computer for offline analysis using both the PatchMaster and Fitmaster software (HEKA Instruments). Experimental protocols were controlled by the PatchMaster software. Pipette resistances ranged from 4 to 6 MOhm and pipette potentials were zeroed before cell contact. The liquid junction potential was corrected by 12.4 mV as calculated by the pCLAMP software (Axon Instruments). For experiments, cultures were placed in a superfusion chamber mounted on the stage of an inverted microscope (Nikon eclipse, TE2000-E). Preparations were superfused with Hanks' balanced salt solution containing 1% neonatal calf serum (pH 7.40, buffered with 10 mmol/L Hepes). The patch electrode was positioned with a micromanipulator (MP-225, Sutter Instrument Company) and brought in close contact with the cells to establish a high resistance seal (2-10 GOhm). After rupturing the cell membrane, access resistances typically ranged from 2-15 MOhm. Series resistances and whole cell capacitances were compensation. Cell capacitance was measured using a sinusoidal voltage stimulus and processing the resulting current using a lock-in amplifier implemented either in HEKA hardware or software. Input resistance was calculated from voltage changes in response to 5 pA hyperpolarizing current steps from a holding potential of -60 mV.

Action potentials of single neurons were elicited by current clamping the cells for 500 ms to increasingly depolarized membrane potentials using inward current steps of 2 pA until firing of action potentials was observed. After recording, preparations were fixated and stained for Ang II and NM III in order to prove that the cells investigated by patch clamp were indeed angiotensinergic neurons.

Patch clamp experiments were performed by Dr. Nicolò Salvarani who consents to including the respective data in this manuscript.

Viral Transduction

Virus Production Protocol

Day 0: HEK 293 cells were seeded in a T-175 tissue culture flask in DMEM (Dulbecco's Modified Eagle Medium, Cambrex) containing 10% FBS (fetal bovine serum, HyClone), 0.1% penicillin/streptomycin (20'000 U/L, 34 µmol/L; Biochrom AG), and 1% sodium pyruvate (Cambrex). Under these conditions, cells nearly reached confluency the next day.

Day 1: A Fugene 6 transfection was performed within 24 hours after plating of the cells. 132 µL of Fugene 6 transfection reagent (Promega) was added to 4 mL of DMEM. In another tube filled with 0.5 mL DMEM, the DNA mix consisting of 22 µg lentiviral gene carrier *pLenti-CaMKIIa-eNpHR2.0-EYFP-WPRE*, 15 µg pDelta 8.74 (packaging plasmid), and 7 µg pMD2.G (VSVg, coat protein) was prepared. The DNA mix was added to the Fugene-DMEM mix and served to transfect the HEK cells.

Day 2: 24 hours post-transfection, the transfection medium was exchanged for the virus production medium comprising UltraCULTURE (Cambrex), 0.1% penicillin/streptomycin (20'000 U/L, 34 µmol/L; Biochrom AG), 1% sodium pyruvate (Cambrex), and 1% sodium butyrate (Sigma).

Day 3-4: 48-72 hours post-transfection, the virus supernatant was collected, spun at 1000 rpm for 5 min in a tabletop centrifuge to pellet cellular debris, and then filtered through a 0.45 µm filter mesh that was pre-wetted with a small amount of virus production medium to reduce protein binding.

Day 5: The filtered and virus-containing supernatant was divided into six previously sterilized centrifuge tubes. To the bottom of each tube, 2 mL of 20% sucrose solution was added to make a sucrose cushion that prevented debris to accumulate at the bottom of the ultracentrifuge tube.

Tubes were then centrifuged (Beckman SW-28 rotor) for 2 hours at 25'000 rpm or 2.5 hours at 22'000 rpm. The supernatant was removed, and the pelleted virus was re-suspended in 25 µL cold PBS. Solution was let to settle for 1-2 hours at 4°C and then triturated by gently pipetting up and down. The final virus solution was divided into aliquots and frozen at -80°C.

Virus production was performed by Dr. Sarah S. Moyle who consents to including the respective data in this manuscript.

Transduction Protocol

Day 0: Primary cell cultures containing Ang II positive neurons were prepared according to the protocols described above.

Day 1: The concentrated virus solution was added at 1:400 (initial experiments) or 1:2000 (later experiments) to the culture transduction medium consisting cell culture medium supplemented with 5% neonatal calf serum (NCS) and 5 µg/mL polybrene. 1 mL of this solution was then added to each culture disk.

Day 2: After 24 hours of virus incubation, the transduction medium was replaced with normal 5% NCS containing M199 medium.

Day 4: Medium exchange.

Day 7: Medium exchange and start of evaluation.

Immunohisto- and cytochemistry

Whole Heart Staining Protocol

Double staining: 30 µm thin cryostat sections of the frozen heart (cf. above) were incubated free floating for 36 hours at 4°C with one murine and one rabbit primary antibody (see below for antibody combinations and complete description of primary antibodies). Sections were then washed and incubated with both secondary antibodies for 90 min at room temperature. Finally, sections were counterstained with DAPI (4', 6-diamidino-2-phenylindole). After the last wash steps, sections were mounted on gelatin-coated slides, allowed to air-dry, and preserved with Glycergel (DAKO) before final placement on a coverslip. Control stainings with secondary antibodies only showed no staining.

(Whole heart preparations were made by Prof. Hans Imboden. Methods were like in [6]; except for the acquisition of the heart and the choice of antibodies.)

The following combinations of primary antibodies were used:

Murine antibodies targeted to	**Rabbit antibodies targeted to**	**Aim**
Angiotensin II	NM III *(neuron specific beta III tubulin, neuronal marker)*	- Investigation of colocalization of Ang II with neuronal markers and enzymes of the RAS
	Synaptophysin *(neuronal protein in synapses)*	
	TH *(tyrosine hydroxylase, enzyme of the sympathetic nervous system)*	
	Renin *(RAS-component)*	
	Cathepsin D *(alternative for renin in Ang II generation)*	
	CGRP *(calcitonin gene related peptide, protein of sensory nerves)*	
	VAChT *(vesicular acetylcholine transporter, protein of the parasympathetic nervous system)*	
AT_1 *(Ang II receptor)*	NM III	- Investigation of the presence of AT_1 receptors in neurons
	Synaptophysin	
	TH	
Dopamine-β-hydroxylase *(enzyme of the sympathetic nervous system)*	NM III	- Investigation of colocalization with Ang II
	Synaptophysin	
	TH	
	CGRP	
	VAChT	
Renin	DβH	- Investigation of colocalization of RAS components with the sympathetic nervous system
	TH	

Cell Culture Staining Protocol

Cell culture preparations were washed with HBSS and fixated either with methanol at -20°C or with 2% PFA at room temperature. After two washing steps with PBS, preparations were blocked with 20% goat serum in PBS for 20 minutes at room temperature. Following three wash steps, preparations were incubated with one murine and one rabbit primary antibody in PBS containing 1% goat serum and 0.15% triton X for 2 hours at room temperature and for another 24 hours at 4°C. After another three wash steps, preparations were incubated with secondary antibodies in PBS for 20 minutes at room temperature. After an additional wash step, preparations were mounted in Fluoroshield mounting medium (ImmunoBioScience) on glass slides.

The following combinations of primary antibodies were used:

Murine antibodies targeted to	**Rabbit antibodies targeted to**	**Aim**
Angiotensin II	NM III	-Investigation of colocalization of Ang II with neuronal markers and enzymes of the RAS -Comparison to whole rat heart
	Synaptophysin	
	VAChT	
	Renin	
Dopamine-β-hydroxylase	NM III	-Comparison to the staining pattern of Ang II -Comparison to whole rat heart
	Synaptophysin	
	VAChT	
	Renin	

Antibodies

Primary Antibodies

Ang II antibody

Immunohistochemistry was performed with a protein G purified murine monoclonal antibody (Mab 4B3) targeted to human Ang II (Mab-Trap G II column, Amersham Sciences) at a concentration of 0.3 µg/mL in buffer solution. The anti-Ang II antibody was raised in the laboratory of Hans Imboden by immunization of Balb/cJ female mice with Ang II cross-linked with glutaraldehyde to keyhole limpet hemocyanin. The specificity of Mab 4B3 has been documented in detail (refer to [37]) with the final Mab 4B3 antibody producing stainings comparable to a rabbit polyclonal affinity-purified Ang II antibody used in rat adrenal glands [38]. In dot blot assay, Mab 4B3 fully cross-reacted with the C-terminal metabolites Ang III [Ang-(2-8)], Ang-(3-8), Ang-(4-8) and Ang-(5-8) and it showed no cross reaction with human plasma angiotensinogen, Ang I (1-10) and Ang-(1-7). In addition to protein G purification of Mab 4B3, it was also affinity-purified by CH-Sepharose-4B that contained N-terminally linked Ang II. Staining with affinity-purified and with protein G-purified Mab 4B3 were identical. The absence of staining was documented by using mouse pre-immune serum or Sepharose-4B fall-through from Mab 4B3. No staining was observed with secondary goat anti-mouse Cy3 antibody when applied without the primary antibody (taken from [6] with permission of Hans Imboden).

List of other primary antibodies used in this study

Used in whole heart preparations	**Used in cell culture preparations**	**Antibodies**
X	**X**	Rabbit polyclonal antibody to neuron specific beta III tubulin (NM III, ab18207, 1:2000, Abcam)
X	**X**	Rabbit monoclonal anti-synaptophysin (C-term) antibody (YE269, 1:1200, Epitomics)
X	**X**	Rabbit polyclonal anti-vesicular acetylcholine transporter (VAChT) antibody (ab68984, affinity isolated, 1:1250, Abcam)
X	**X**	Murine monoclonal anti-DβH antibody (4F9/9; diluted 1:600, Biotrend)
X	**X**	Rabbit anti-human renin antibody (L22, 1:5000 a generous gift by J. Menard, Paris, refer to [39])
X		Rabbit polyclonal anti-calcitonin gene related peptide (CGRP) antibody (Cat. T-4032, 1:1000, Peninsula)
X		Murine monoclonal anti-AT1 receptor antibody (4H2, diluted 1:1250, affinity purified, raised in the laboratory of Hans Imboden, refer to [37])
X		Rabbit monoclonal anti-tyrosine hydroxylase (TH) antibody (EP1533Y (pS71), 1:400, Epitomics)
X		Rabbit monoclonal anti-cathepsin D antibody (Cat. 2431-1, 1:600, Epitomics)
X		Murine monoclonal anti-rat renin antibody (ascites No118, 1:500, Swant)
X		Rabbit polyclonal anti-DβH antibody (DZ 1020, 1:500, Biomol)

Secondary Antibodies

Whole heart preparations

-Goat anti-mouse Cy3 (GAM^{Cy3}), Jackson ImmunoResearch

-Donkey anti-rabbit Cy5 (DAR^{Cy5}), Jackson ImmunoResearch

-4', 6- diamidino-2-phenylindole (DAPI), diluted 1:20'000

Cell culture preparations

-Alexa Fluor 488, goat anti-mouse green, Molecular Probes, diluted 1:200

-Alexa Fluor 546, goat anti-mouse red, Molecular Probes, diluted 1:200

-Alexa Fluor 555, goat anti-rabbit red, Molecular Probes, diluted 1:200

-4', 6- diamidino-2-phenylindole (DAPI), diluted 1:20'000

Microscopy

Images of whole heart preparations were taken either with a widefield epifluorescence microscope (LEICA DM6000B) using a digital camera or with a LEICA SP2 (with acousto-optical beam splitter)

confocal laser scanning microscope. Leica software was used for three-dimensional reconstruction or to create monoplane overlay views from multiple optical z-stack layers.

Stained cell culture preparations were examined with an inverted microscope equipped for epifluorescence (Axiovert 35 M, Zeiss) using a slow-scan high-sensitivity camera (Spot RT, Diagnostic Instruments).

Data Analysis

Neuron counting was done manually in fixated and stained preparations that were blinded for counting. All analysis was performed using GraphPad InStat 3.01 (Graph-Pad Software, San Diego, California, USA). Values are expressed as mean ± standard deviation. Variables were analyzed for normal distribution using the Kolmogorov-Smirnov test. Parametric variables were compared using the unpaired student t-test. A Welch correction was applied if appropriate. Non-parametric variables were compared using the Mann-Whitney test. A two-tailed p-value < 0.05 was considered statistically significant.

Results

Ang II Positive Neurons in the Intact Neonatal Rat Heart

Colocalization of Ang II with Neuronal Markers

Widefield immunohistological images offering an overview of the atrial and ventricular tissues in respect to angiotensinergic nerves are shown in Figure 5. They reveal ganglionic structures with colocalized Ang II and NM III staining which suggests that angiotensinergic cells were of neuronal origin. As shown by the overlay views from multiple optical z-stack layers acquired with the confocal laser scanning microscope in Figure 5B, colocalized staining was observed in the cytoplasm but not in the nucleus as indicated by the arrows in the transverse section shown in panel 5Bc.

Figure 6 shows two atrioseptal ganglia containing angiotensinergic ganglion cells. Synaptophysin staining was used to reveal synaptic contacts. While the cytoplasm of neurons stained positive for Ang II (red), synaptophysin positive dots (green) encircled the cell somata and extending processes (Figure 6B, 6C). As illustrated by Figure 6B by arrows, a punctate synaptophysin staining surrounding cell somata was also found in Ang II negative cells. Further to neuronal cells, Ang II positive staining was also present in erythrocytes (see asterisks in Figures 5 and 6).

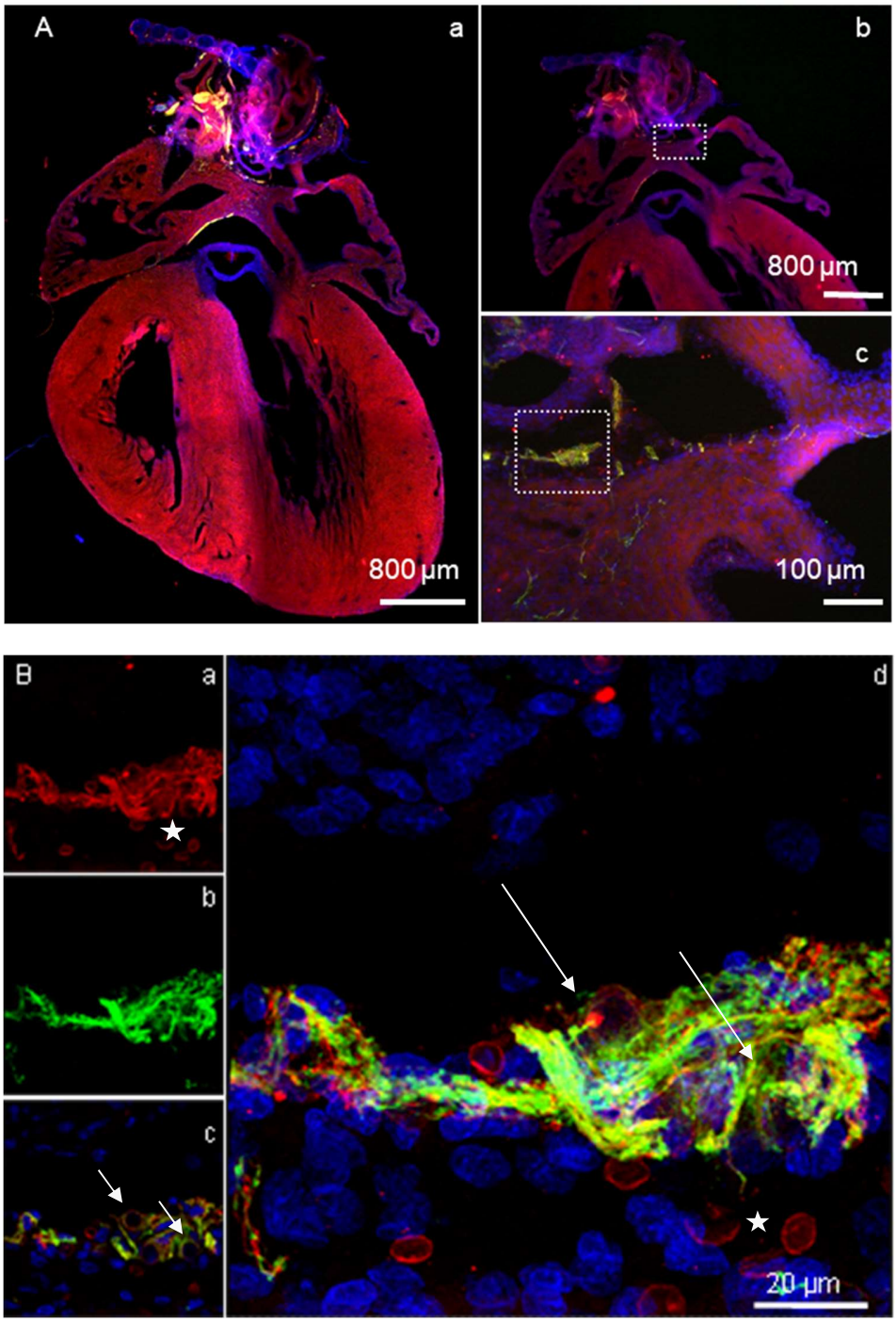

Fig. 5: Angiotensin II staining in the neonatal rat heart.

A, Longitudinal section of a neonatal rat heart (a) stained for Ang II (red), NM III (green) and counterstained with DAPI (blue). A ganglionic structure in the region of the interatrial septum is shown at higher magnification in b and c. **B,** Ganglion indicated with a dashed box in Ac at higher magnification: Staining for Ang II (a: red) and NM III (b: green) reveals a high degree of cytoplasmic colocalization (c, d: yellow, see arrows). Ang II positive staining in erythrocytes (a, d: red, see asterisks) [40].

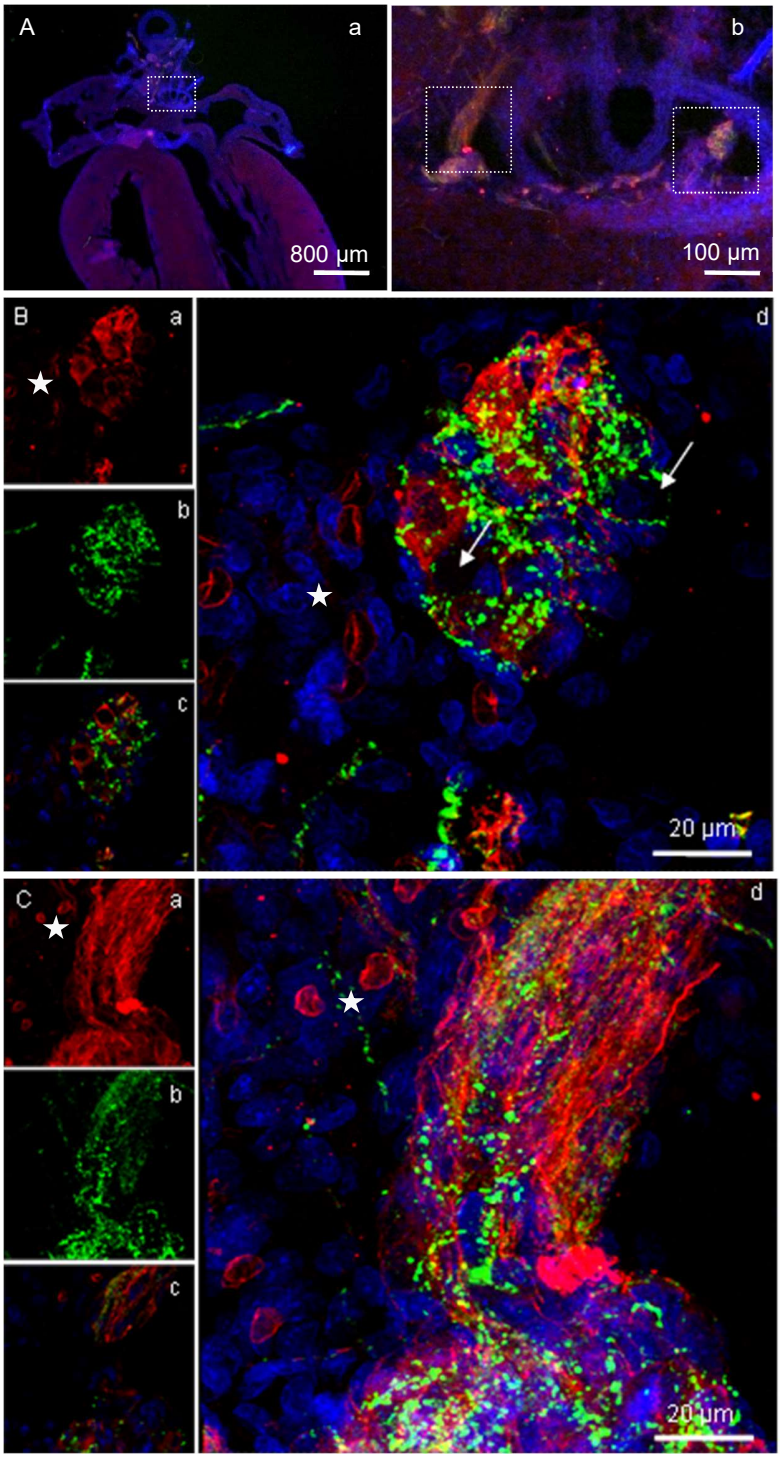

Fig. 6: Two atrioseptal ganglia demonstrating colocalization of Ang II with synaptophysin.

A, Overview of the localization of anteroseptal ganglia (a) with two ganglia marked by dashed boxes in the higher magnification image (b). **B,** Confocal images of the right dashed region in Ab showing angiotensin-positive neurons (a; red) and synaptophysin positive structures (b: green). The overlay shown in c and d indicates that angiotensinergic neurons are surrounded by the synaptophysin positive structures (b: green) suggesting presence of synapses. The arrows in Bd indicate Ang II negative cells surrounded by synaptophysin staining. Ang II positive staining in erythrocytes (a, d: red, see asterisks). **C,** Same as B for the left dashed region indicated in Ab. [40]

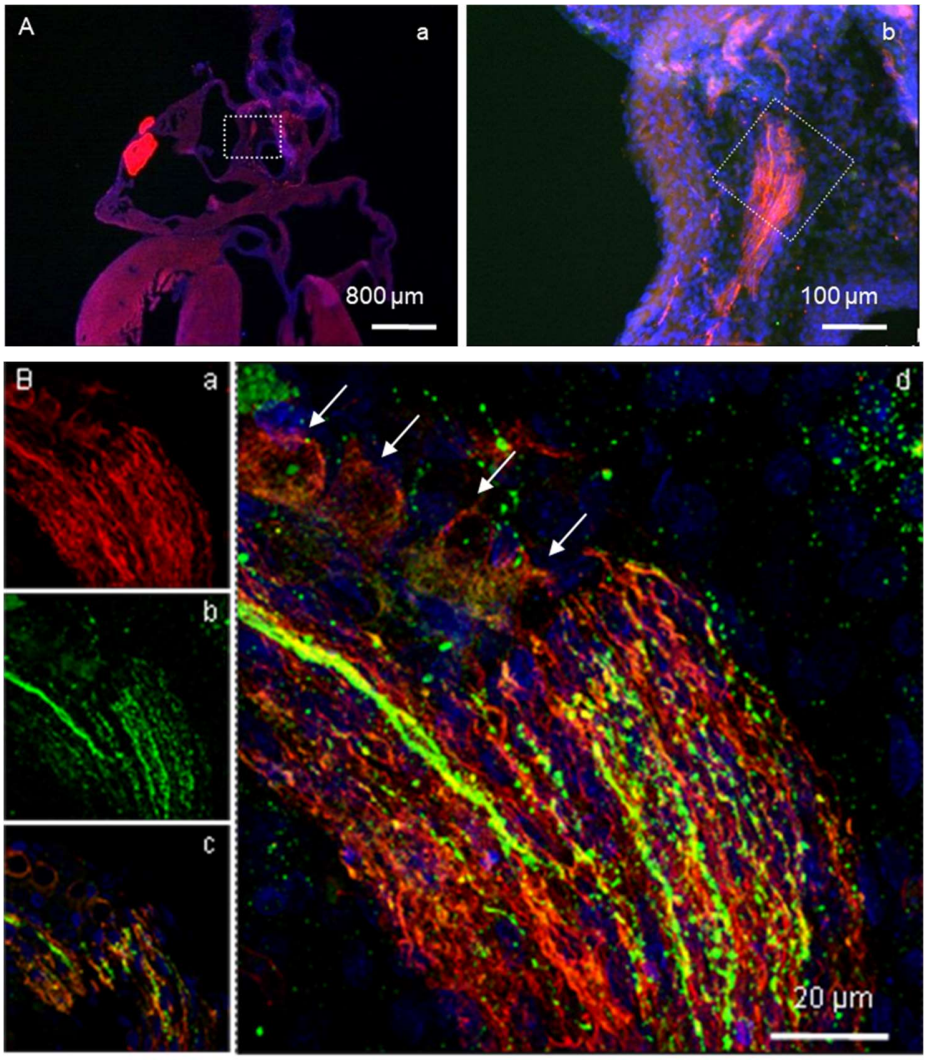

Fig. 7: Colocalization of Ang II with CGRP in right atrial ganglion with fiber bundle.

A, Localization of an angiotensinergic ganglion with thick fiber bundle at low (a) and higher (b) magnification. **B,** Colocalization of Ang II (a: red) and CGRP (b: green) is seen in fibers (c: yellow). The multistack composite image (d) of neuronal cell bodies shows a robust Ang II signal but only a modest CGRP staining (arrows) [40].

Figure 7 depicts colocalization of Ang II and CGRP in a fiber bundle emanating from a right atrial ganglion. In the bundle shown in panels Ba-d of Figure 7, some fibers stain positive for either CGRP or Ang II while others exhibit a clear colocalization of the two markers. The ganglion cell bodies adjacent to the fiber bundle show a robust Ang II staining while the CGRP signal, even though distinct, is less pronounced (Figure 7d, arrows).

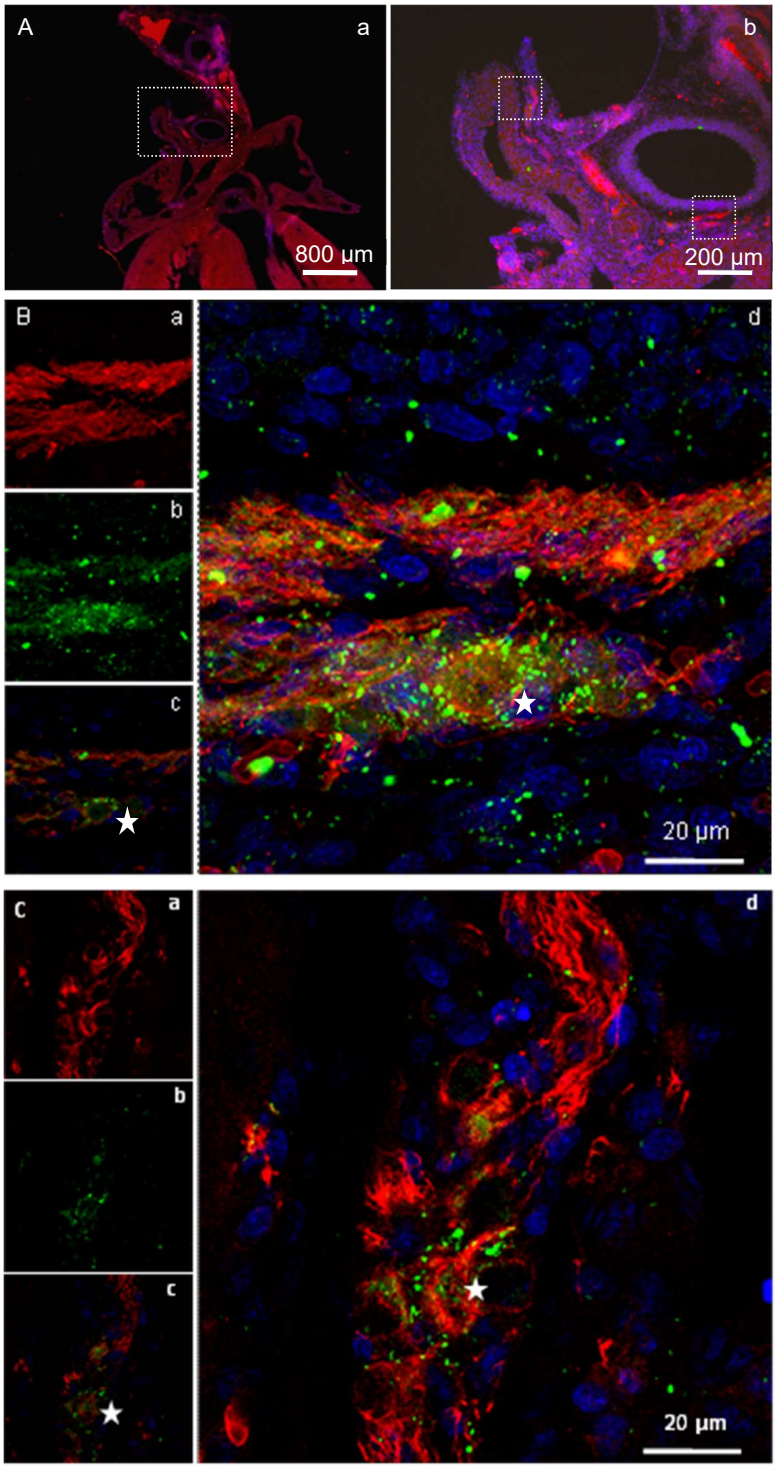

Fig. 8: Angiotensin and cholinergic neuronal markers in right atrial ganglia.

A, Localization of two angiotensinergic ganglia (a, b) in the right atrium. **B,** Enlarged view of right dashed box in Ab with angiotensin staining (a: red), VAChT staining (b: green) and overlay (c, d) showing an angiotensinergic ganglion cell surrounded by VAChT positive structures (asterisks in c and d). **C,** Enlarged view of left dashed box in Ab presenting Ang II positive neurons (a: red) and another example of VAChT positive structures (b: green) surrounding angiotensinergic ganglion cells (see asterisks in overlay images c and d) [40].

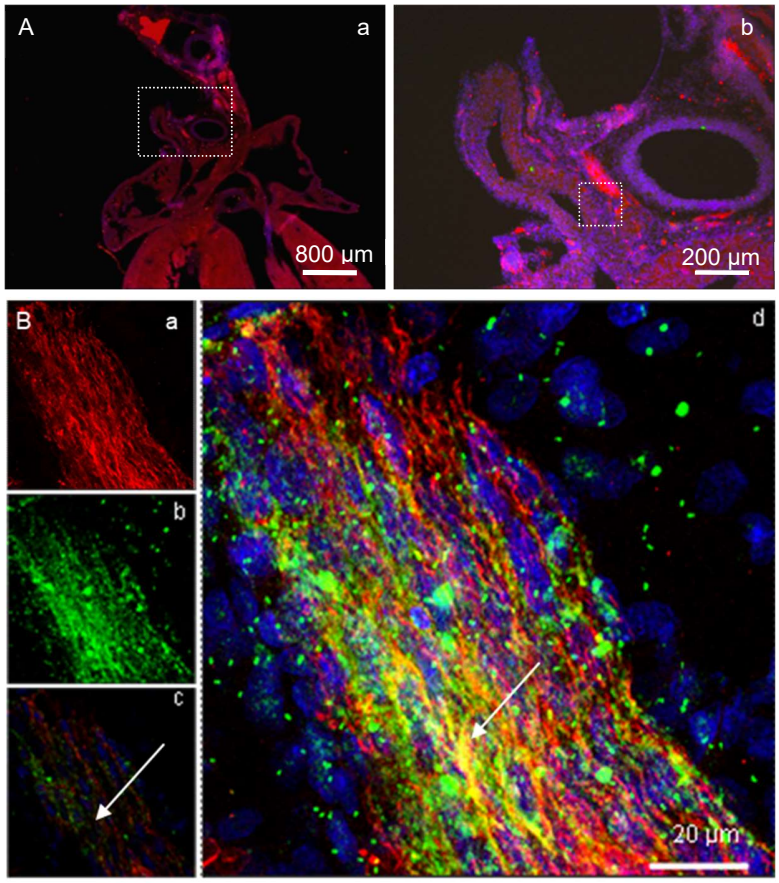

Fig. 9: Angiotensin and cholinergic neuronal markers in thick fiber bundle in the right atrium.

A, Localization of fiber bundle in the right atrium at low (a) and higher (b) magnification. **B,** Confocal images of a fiber bundle stained for angiotensin (a: red) and VAChT (b: green). The overlay shown in c indicates presence of either Ang II (red) or VAChT (green) positive fibers (arrow). As fibers change direction in the tissue, Ang II and VAChT positive structures are artifactually overlaid in multistack composite image (d: yellow; arrow) [40].

The distribution of VAChT, a cholinergic specific neuronal marker, is shown in Figures 8 and 9. While cholinergic synapses could be identified at cell bodies of an angiotensinergic ganglion (cf. asterisks in Figure 8Bc, Bd, Cc, and Cd), there were no detectable signals for VAChT along the processes of the Ang II positive neurons (Figure 8Bc, Bd, Cc, and Cd). Fibers were either positive for Ang II or VAChT (cf. arrows in Figure 9Bc and Bd). In the sections examined, we failed to find aggregations of VAChT stained cell bodies that would indicate presence of parasympathetic ganglia.

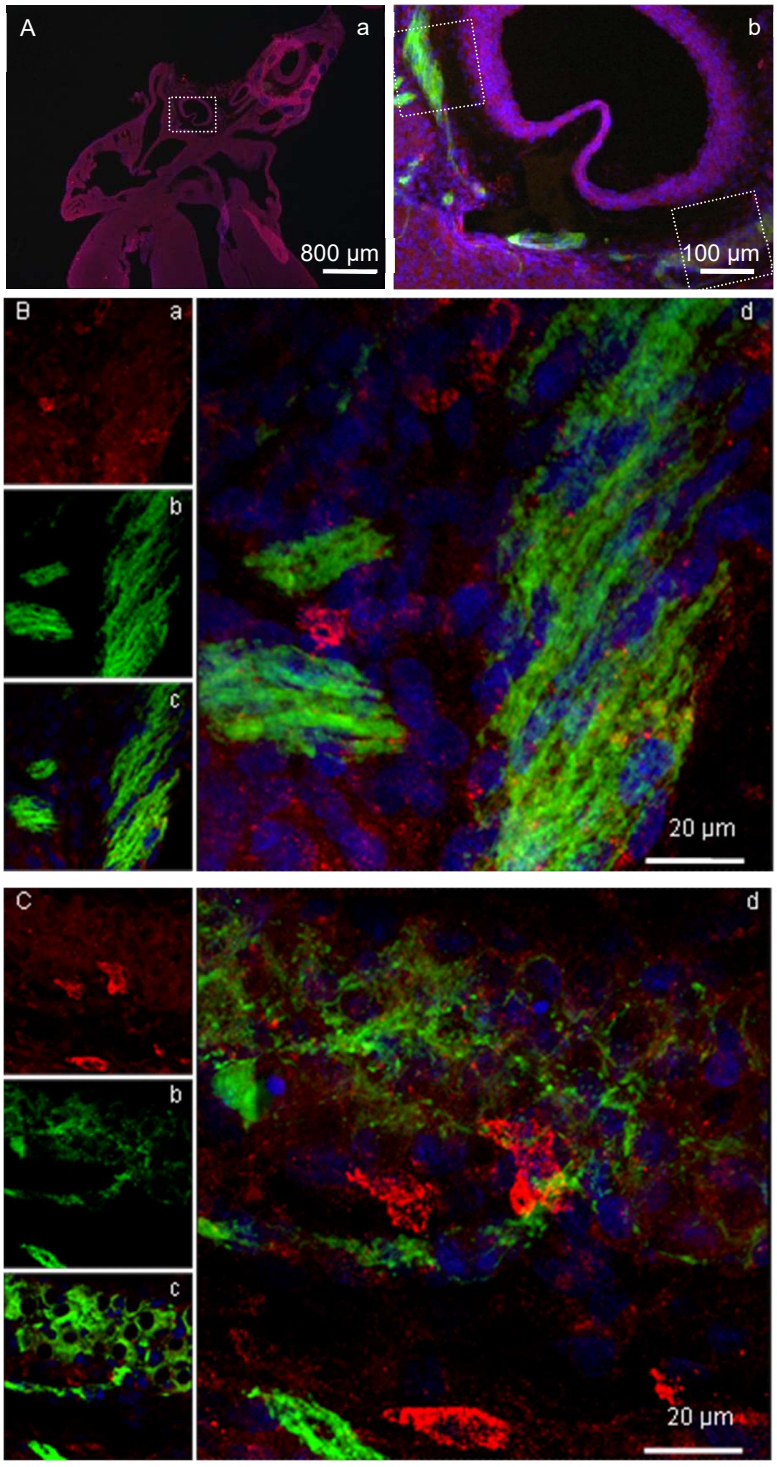

Fig. 10: Staining of right atrial fiber bundle and atrioseptal ganglion for AT_1 receptors and NM III.

A, Localization of atrial neuronal structures at low (a) and higher (b) magnification next to the superior vena cava (dashed boxes). **B,** Confocal image of fiber bundle (left dashed box in Ab), stained for AT_1 receptor (a: red), NM III (b: green), overlay (c), and multistack layers (d). **C,** Enlarged view of a ganglion (right dashed box in Ab); staining for AT_1 receptor (a: red), produces a substantial background signal. Neuronal cells and fibers stain strongly positive for NM III (b: green), showing some colocalization with AT_1 receptor (overlay images c and d) [40].

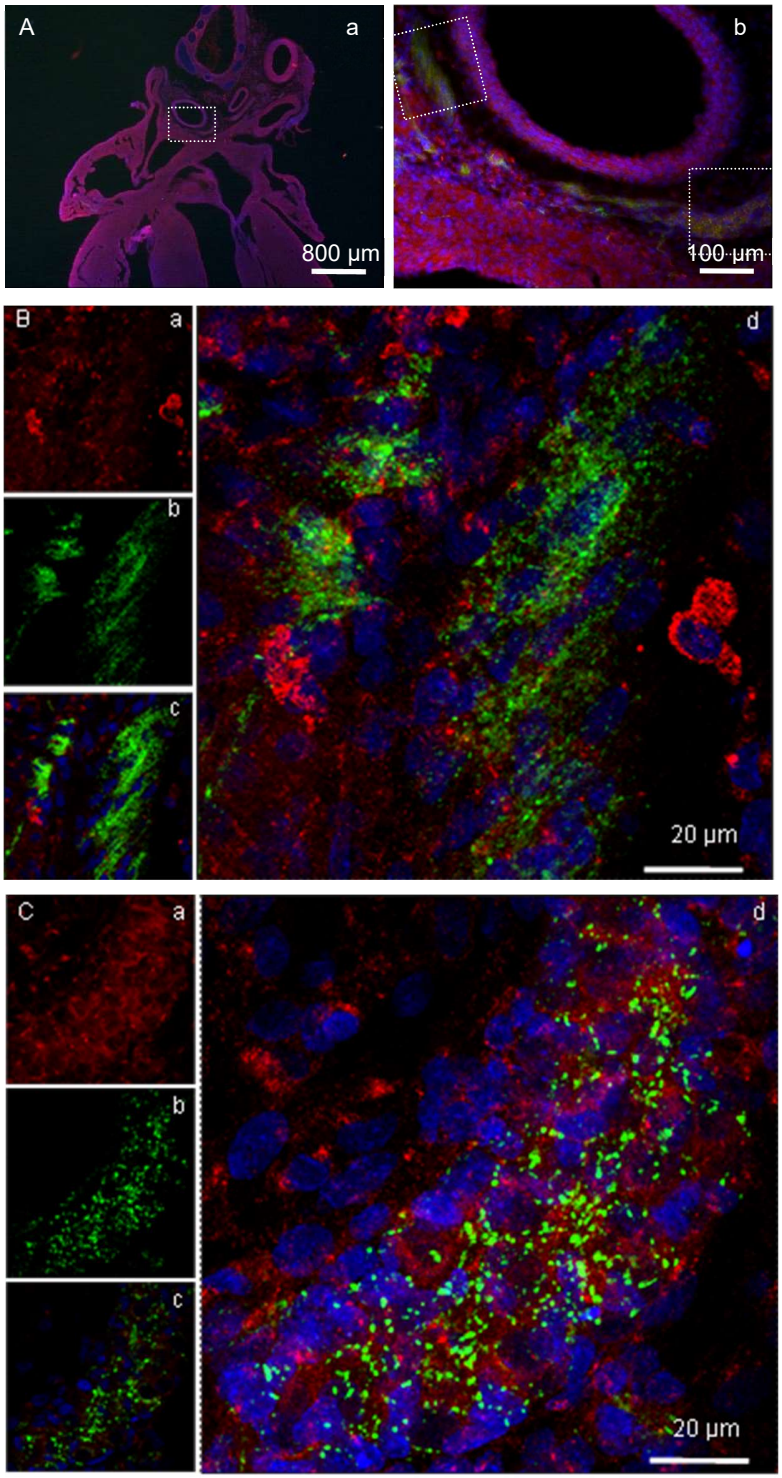

Fig. 11: Right atrial ganglion and fiber bundle, stained for AT_1 receptor and synaptophysin.

A, Localization of atrial neuronal structures at low (a) and higher (b) magnification next to the superior vena cava (dashed boxes). **B,** Confocal image of left dashed box in Ab, staining for AT_1 receptor (a: red) and synaptophysin (b: green). The neuronal fiber bundle (c, d, green, on the right) stains positive for synaptophysin, colocalization with AT_1 receptor is difficult to assess because of ample background staining. **C** Image of right dashed box in Ab, neuronal cell bodies positive for AT_1 receptor (a: red) and synaptophysin staining (b: green). Overlay projections (c, d) indicate the presence of synapses around neuronal cell bodies [40].

Colocalization of the AT_1 Receptor with Neuronal Markers

Because AT_1 antibodies produced a substantial background staining, the assessment of a possible colocalization with either NM III or synaptophysin was difficult. As shown in Figures 10 and 11, a fiber bundle and a ganglion in the region of the interatrial septum close to the superior vena cava stained positive for both neuronal markers and seemed to show some degree of colocalization with AT_1.

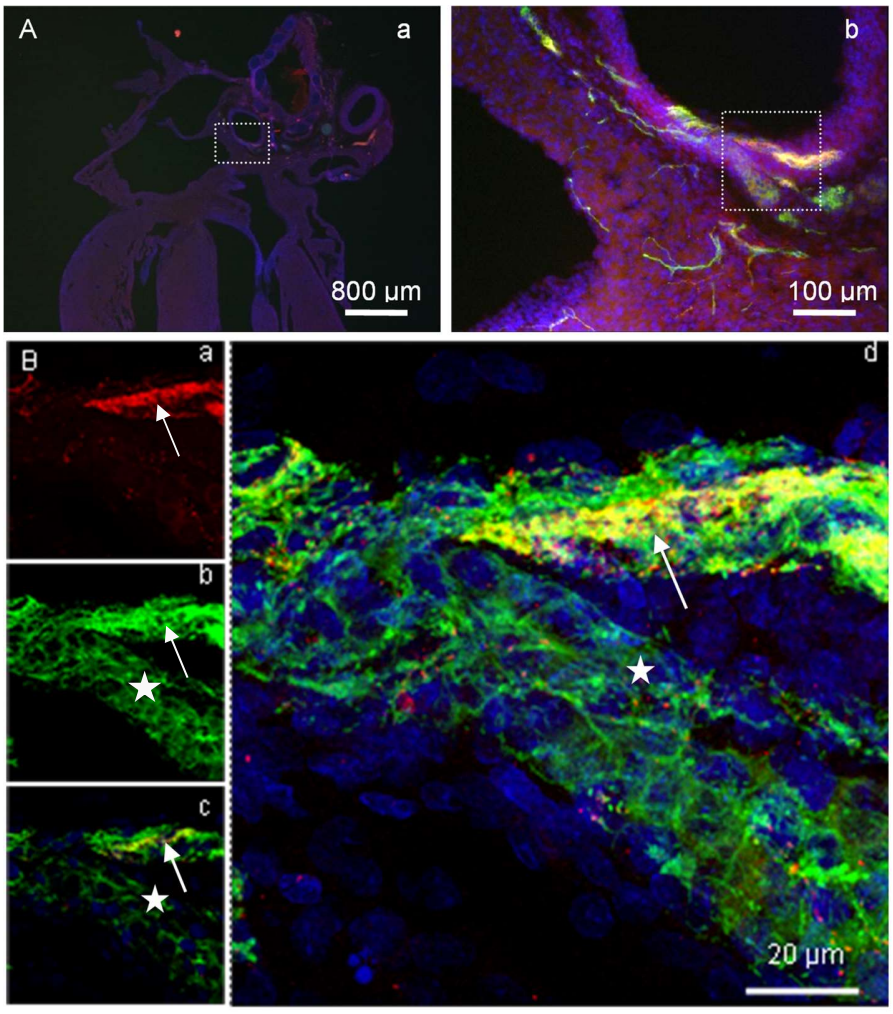

Fig. 12: Intracardiac ganglion and fibers stained for dopamine-β-hydroxylase and NM III.

A, Localization of NM III positive right atrial ganglion with fibers at lower (a) and higher (b) magnification. **B,** DβH (a: red) positive staining is seen in some of the fibers (a: arrow). Positive staining for NM III (b: green) in somata (b, asterisk) and fibers (b, arrow).DβH colocalized with NM III in fibers (c, d, yellow, see arrows) but only very weak or not at all in the cell bodies (c, d, asterisks) [40].

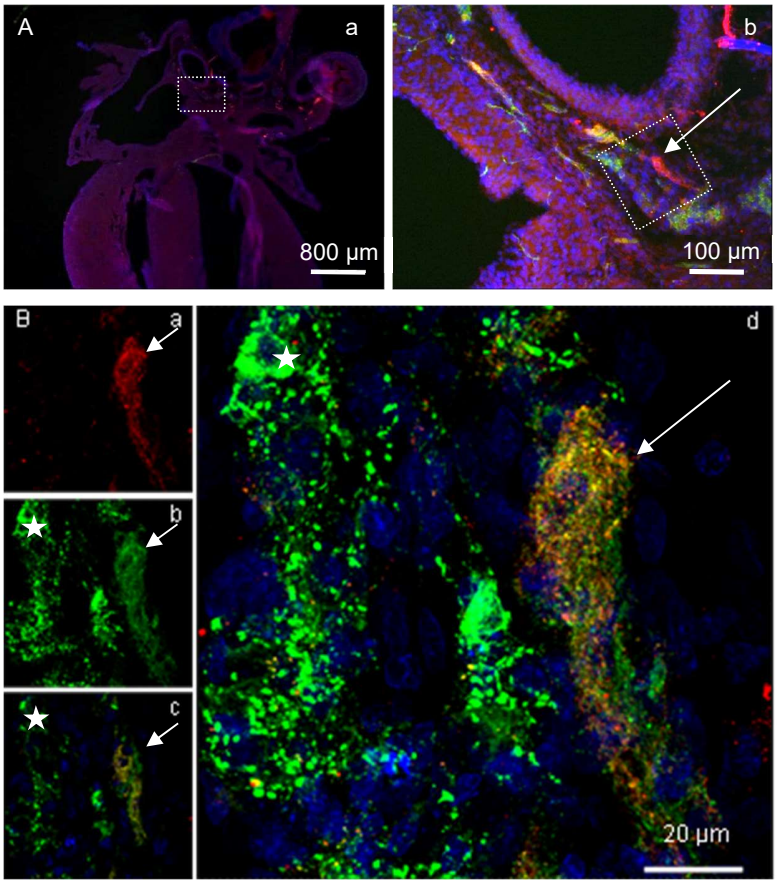

Fig. 13: Right atrial neurons stained for DβH and synaptophysin.

A, Localization of a ganglion and fiber bundle in the right atrium at lower (a) and higher (b) magnification. **B,** Fiber bundle staining positive for DβH (a: red, arrow). The staining pattern of synaptophysin (b: green) indicates a ganglion on the left side (b, asterisk). As depicted in the overlay images, these ganglion cell bodies do not contain DβH (c, d, asterisks). The fiber bundle shows colocalization of DβH and synaptophysin (c, d, arrow) [40].

Colocalization of Dopamine-β-Hydroxylase with Neuronal Markers

Tissue sections were stained with dopamine-β-hydroxylase (DβH) to visualize sympathetic neurons. DβH is an enzyme required for the production of norepinephrine and is therefore present in neurons and neuronal processes of the sympathetic nervous system. Double staining with the same neuronal markers as used with Ang II (NM III and synaptophysin, CGRP as a sensory neuron marker, and VAChT as a parasympathetic neuron marker) were performed to investigate the staining pattern of DβH in the neonatal rat heart and compare it to that of Ang II. In contrast to published findings made in the adult rat heart, where clear colocalization of DβH and Ang II was demonstrated in ganglion cell bodies [6], we found only weak or no DβH in cell bodies of atrial ganglia that, otherwise, showed strong NM III staining (cf. asterisks in Figure 12). Distinct DβH staining that colocalized with NM III was found in fiber bundles, however (see arrows, Figure 12).

A similar pattern was found when combining DβH with synaptophysin staining. Again, and as shown in Figure 13, DβH stained the fiber bundle (arrows) but not the neuronal cell somata which were girdled by the punctate synaptophysin staining (asterisks).

When investigating the intensity of DβH and CGRP signals in a right atrial ganglion, we found that fibers stain strongly positive for DβH (cf. arrows in Figure 14) while cell bodies exhibit only a weak signal for DβH and CGRP (cf. asterisks, same figure).

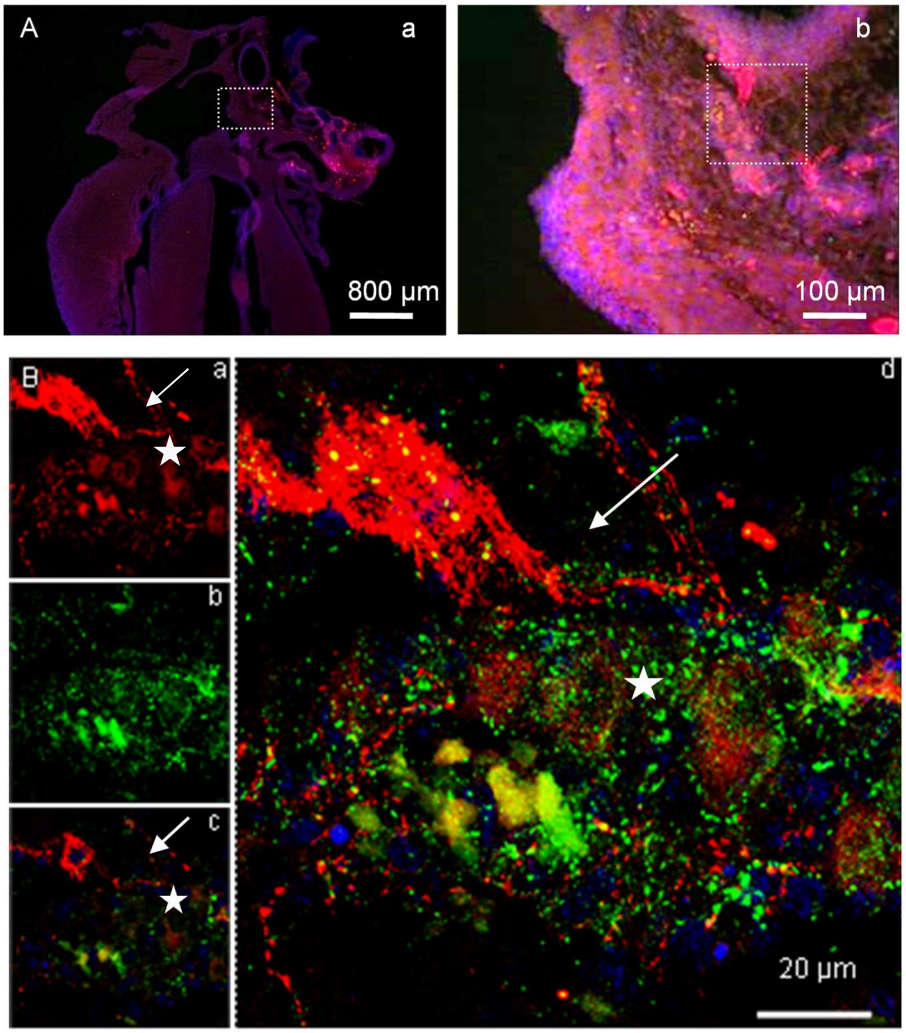

Fig. 14: Right atrial ganglion stained for DβH and CGRP.

A, Localization of a right atrial ganglion at lower (a) and higher (b) magnification. **B,** Robust positive DβH staining (a: red) in fibers (arrows) is observed with weak staining of the neuronal cell bodies (asterisks). Weak staining of CGRP (b: green) can be detected. Overlay views could indicate a possible colocalization of the two markers in cell bodies (c, d, see asterisks) [40].

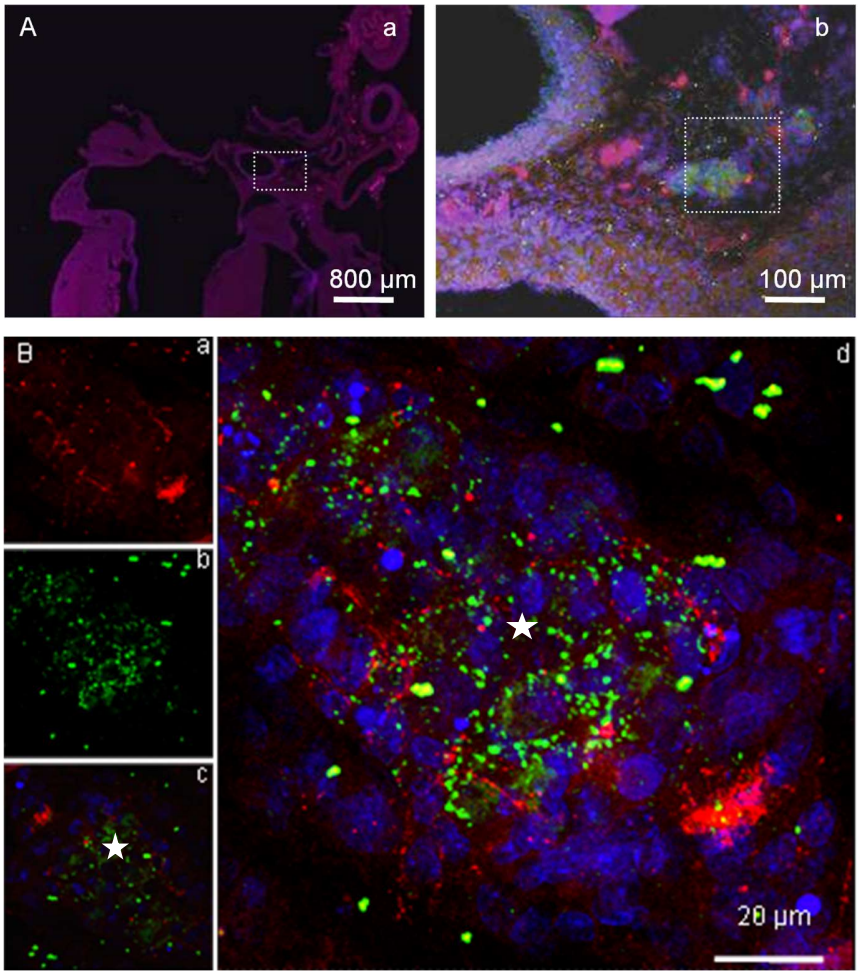

Fig. 15: Staining of an atrioseptal ganglion for DβH and VAChT.

A, Localization of an atrioseptal sympathetic ganglion at lower (a) and higher (b) magnification. **B,** Sympathetic fibers strongly stain for DβH (a: red), whereas the neuronal cell bodies show weak DβH staining (a: red). The punctate VAChT staining pattern (b: green) indicates synaptic contacts (c, d, see asterisks in overlays) [40].

The right atrial ganglion shown in Figure 15 contains strongly stained sympathetic fibers with neuronal cell bodies staining weakly for DβH surrounded by punctate VAChT positive structures (asterisks).

Enzymes of the RAS

Because the production of Ang II is renin or, alternatively, cathepsin D dependent [11], we investigated whether these enzymes are expressed by Ang II positive neurons [7] [8] [9]. However, using both murine and rabbit antibodies against the two enzymes, we failed to detect presence of these enzymes in the sections investigated (data not shown).

Summary

In the neonatal rat heart, Ang II was detected in the cytoplasm and processes of a subpopulation of ganglionic neurons. Ganglia that contained Ang II positive neurons were found in the right and left atrium and in the region of the interatrial septum. Ang II positive neurons were absent in the auricles. While the ventricular myocardium was devoid of Ang II positive cell bodies, it contained angiotensinergic fibers that displayed colocalized staining for neuron specific beta III tubulin (NM III), synaptophysin, calcitonin gene related peptide (CGRP), vesicular acetylcholine transporter (VAChT), AT_1 receptor, and dopamine-β-hydroxylase (DβH). These fibers were mainly located at the epicardium (data not shown). Their density was substantially lower than what has previously been reported for adult rat ventricles [6].

Atrial Cell Culture Experiments

Ang II Positive Neurons in Primary Atrial Cell Cultures

In order to further characterize and experimentally manipulate Ang II positive cardiac neurons, we tried to isolate these cells and propagate them in primary cell cultures. After optimizing cell culture conditions (cf. Method section), we detected Ang II positive neurons in our cultures as displayed in Figure 16. The general morphology of Ang II positive cells recapitulated that of neurons with polyhedral somata, round to oval nuclei and long, slender, and branched processes.

The molecular makeup of cultured neurons was compared to that of neurons in intact hearts by staining for Ang II, DβH, NM III, synaptophysin, renin, and VAChT (cf. Method section for combinations). As shown in Figure 16, colocalized Ang II and NM III staining could be demonstrated in several neurons. Cells with neuron-like morphology staining positive for Ang II were discovered in each culture preparation. Hardly any NM III positive cells staining negative for Ang II were found. The punctate synaptophysin staining was difficult to interpret and the VAChT staining was, unlike in intact tissue, unspecific. Similar to the intact heart, renin and DβH stainings were negative.

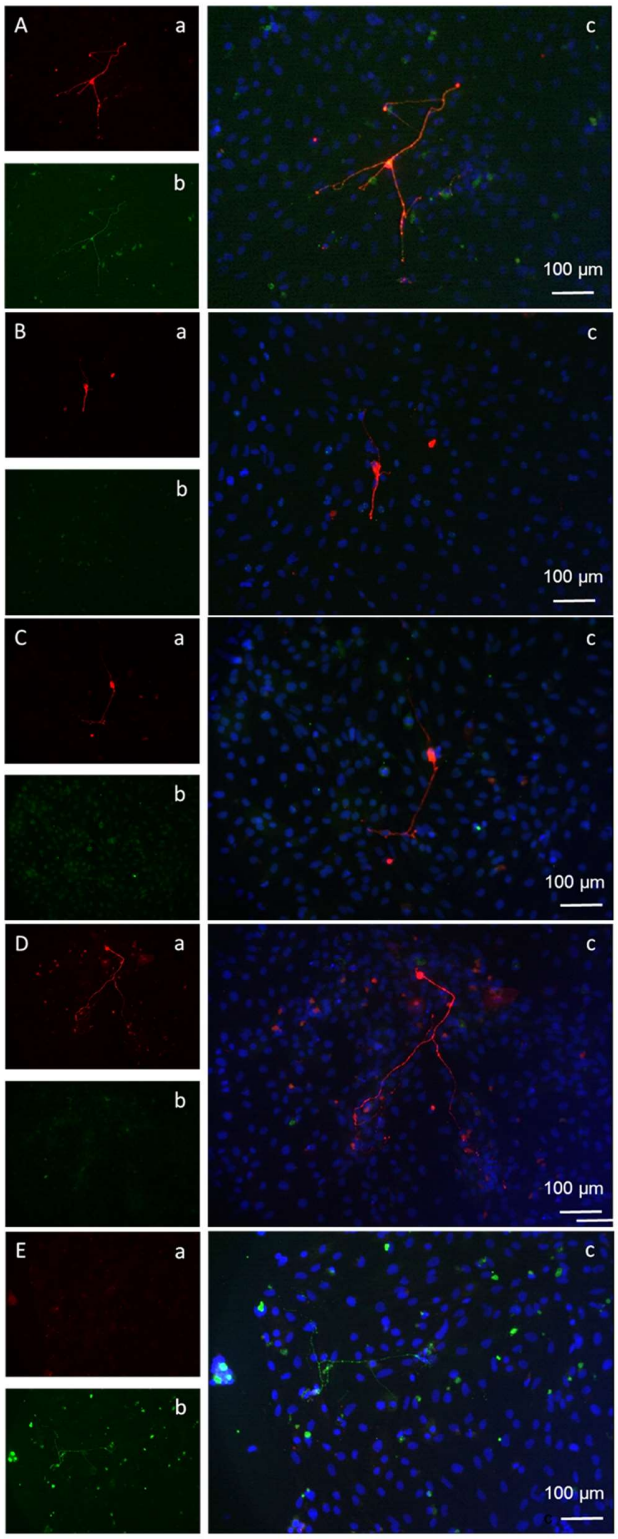

Fig. 16: Colocalization of Ang II with neuronal markers in atrial cell culture.

A, Ang II in red (a), NM III in green (b), both colocalized (c) in neuron cell body and deflections. **B,** Ang II in red (a), synaptophysin in green (b), no colocalization. **C,** Ang II in red (a), VAChT in green (b), no colocalization. **D,** Ang II in red (a), renin in green (b), no colocalization. **E,** DβH in red (a), NM III in green (b), no colocalization. **a** Murine primary antibody, **b** rabbit primary antibody, **c** merged image with DAPI in blue.

Effects of Modified Cell Culture Media on Ang II Positive Neurons

Angiotensin II

The absence of renin and cathepsin D staining (the latter was tested in whole rat heart preparations only) in Ang II positive neurons suggests that these cells may take up Ang II instead of producing it themselves. If correct, addition of Ang II to the cell culture medium should result in an increased staining intensity and/or an increase in number of Ang II positive cells. By adding 1 nmol/L or, alternatively, 1 µmol/L Ang II to the cell culture medium for 24 hours, we tested the possibility that Ang II positive neurons may take up angiotensin instead of producing it themselves. The average number of Ang II positive neurons per 500 mm^2 under control conditions was 18.5 ± 12.02 (n=2). Following addition of 1 nmol/L or 1 µmol/L Ang II for 24 hours, the count remained virtually unchanged (1 nmol/L: 19 ± 1.41; 1 µmol/L: 22 ± 2.83; n=2 each; n. s. vs. control). Also, as judged qualitatively by eye, the staining intensity of Ang II was not affected by exposition to Ang II. Overall, and respecting the caveat of a low number of experiments, the findings suggest that uptake of Ang II by neurons is unlikely.

Fluorescein labeled Angiotensin II

In order to directly test the hypothesis of cellular Ang II uptake, we exposed cell cultures to 1 nmol/L and 1 µmol/L of fluorescein labeled Ang II for 24 hours. Apart from unspecific staining of cellular debris, there was no Ang II related fluorescence detectable in cells with neuronal morphology which, after fixation and staining for Ang II and NM III, proved to be Ang II positive neurons (Figure 17). Unless the tagging of Ang II with a fluorophore prevented this molecule from being taken up by neurons, the findings demonstrate that angiotensinergic neurons are likely capable of synthesizing Ang II themselves.

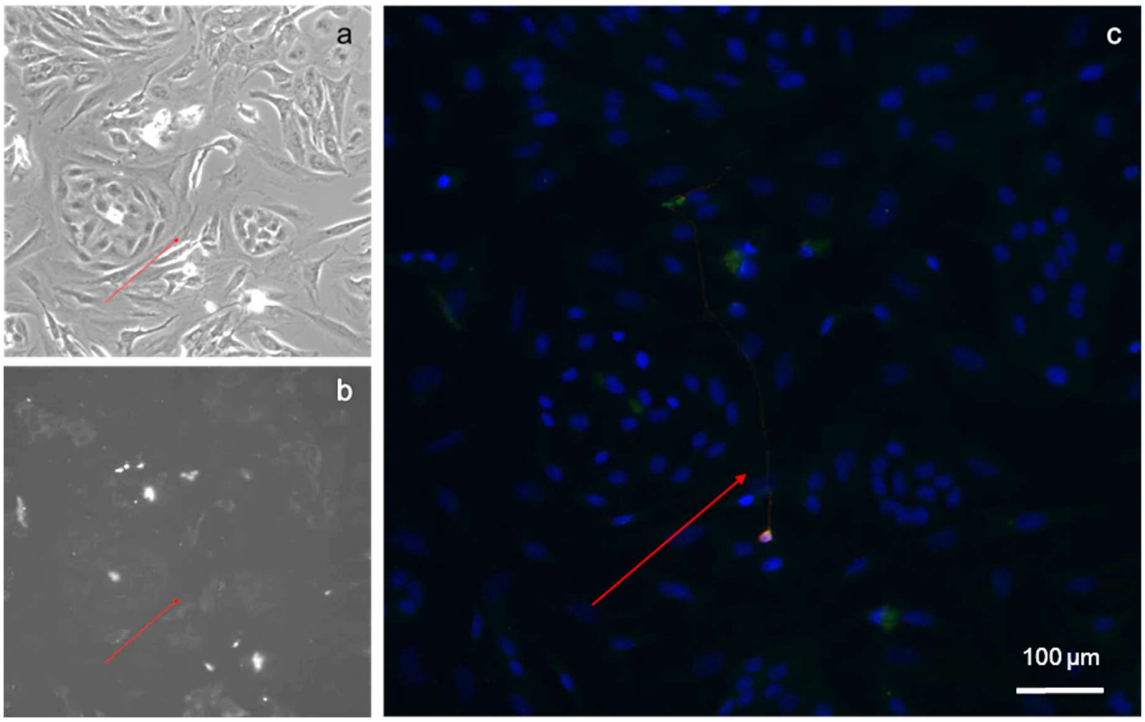

Fig. 17: Addition of 1 µmol/L fluorescein labeled Ang II to culture medium.

A, Living culture under phase contrast microscopy, arrow indicating a neuron. **B,** Living culture under fluorescent microscopy, arrow at the same position, no fluorescence seen. **C,** Same neuron after fixation, showing colocalized Ang II staining in red and NM III staining in green, DAPI in blue.

NGF

To investigate whether neuronal cell number and/or size may be increased by appropriate growth factors, 10 ng/mL nerve growth factor (NGF, concentration according to manufacturer instruction) was added to the culture medium for 48 hours. As shown in Figure 18, treated cultures showed no change in cell morphology and cell density when compared to controls. The size of the neuronal cell somata and the lengths of extensions were not affected by NGF treatment and cells exhibited the typical colocalization of Ang II with NM III (Figure 18B, C). The number of Ang II positive neurons showed large variations under both control and experimental conditions. Between 1 and 38 angiotensinergic neurons per 500 mm^2 growth area were found in control preparations (12.7 ± 17.3; n=7), whereas, in NGF treated cultures, the number varied between 8 and 41 angiotensinergic neurons per 500 mm^2 growth area (21.8 ± 15.7; n=4). Differences were not significant suggesting that, at least at the concentrations used, NFG had no substantial effect on cell number and size.

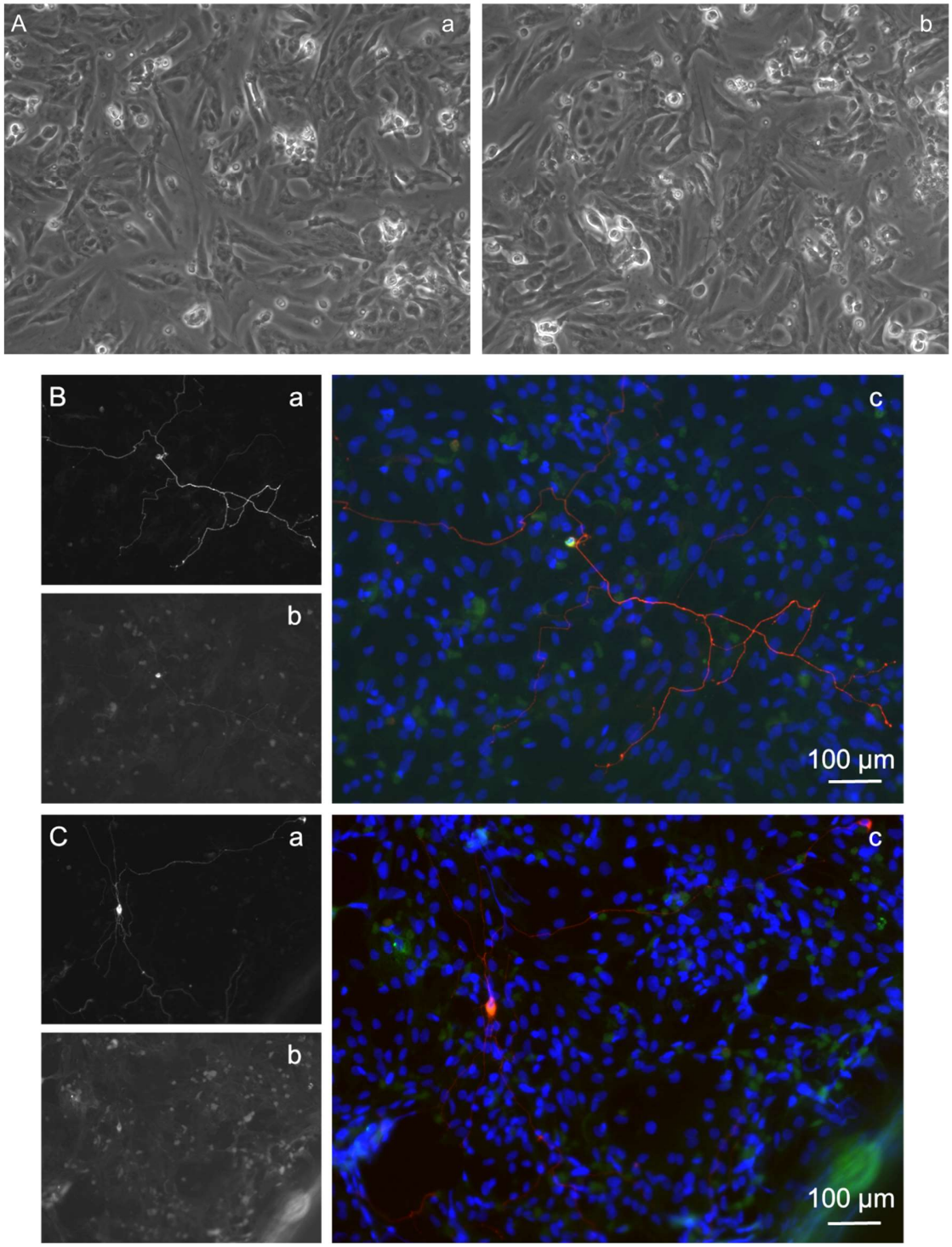

Fig. 18: Morphology of preparations cultured in presence of NGF.

A, Living culture under phase contrast microscopy, day 2, (a) 10 ng/mL NGF added, (b) control conditions. **B, C,** Immunocytochemically stained preparations. Neurons in culture with 10 ng/mL NGF added, showing colocalized Ang II staining in red (a) and NM III staining in green (b), DAPI in blue and merged image (c).

Aliskiren

Because it could not be ruled out that the failure to visualize renin by immunocytochemistry may have been due to shortcomings of the antibodies used, we thought it worthwhile to investigate whether functional block of this enzyme by aliskiren may change appearance of angiotensinergic neurons in culture. Aliskiren was added to the culture medium for 48 hours at concentrations ranging from 10 nmol/L to 100 µmol/L before staining and counting the angiotensinergic neurons. At 10 and 100 nmol/L aliskiren, cell cultures showed a normal morphology. At higher concentrations, aliskiren seemed to exert cytotoxic effects as all cells did not adhere properly, lost their typical shape, and tended to detach from the substrate.

The number of Ang II positive neurons under control conditions averaged 10.3 ± 6.3 (n=4). This number was not significantly affected by any of the aliskiren concentrations used (10 nmol/L: 13.5 ± 14.9 (n=2); 100 nmol/L: 5.3 ± 5.3 (n=6); 1 µmol/L: 1.5 ± 0.7 (n=2); 100 µmol/L 2.5 ± 5 (n=4)) suggesting that it is rather unlikely that Ang II production in the investigated neurons is dependent on renin.

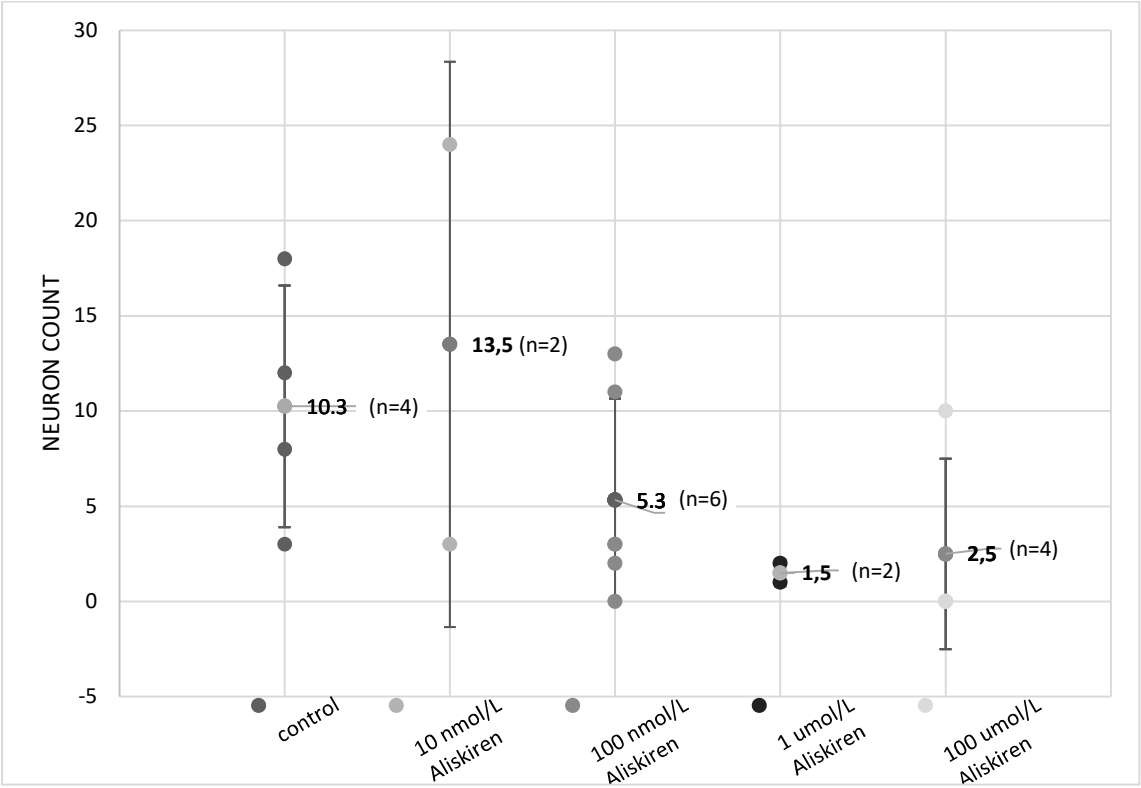

Fig. 19: Angiotensinergic neuron count after aliskiren addition to culture medium.

Number of Ang II positive neurons under control conditions (n=4) and following addition of aliskiren at increasing concentrations.

Pepstatin A

It was the goal of this experiment to investigate whether pepstatin A (Pep) could inhibit the production of Ang II. Pep is an inhibitor of cathepsin D (acid protease) which, in turn, is an established alternative to renin in cleaving angiotensinogen into angiotensin I. Pep was added to culture medium at concentrations of 10 µmol/L and 100 µmol/L for 24, 48, and 72 hours. After fixation, cells were immunocytochemically stained against Ang II and NM III and counted in blinded preparations. Again, the number of angiotensinergic neurons per 500 mm^2 showed large variation under both control and experimental conditions. The number of Ang II positive neurons under control conditions was 34.3 ± 24.3 (n=10). At 10 µmol/L Pep, Ang II positive neurons averaged 22.2 ± 13.9 (n=11; n. s. vs. control). At 100 µmol/L Pep, however, the number of angiotensinergic neurons decreased to 13.9 ± 8.9 (n=11), which was significantly lower than control ($p < 0.05$). Whether this decrease was due to inhibition of cathepsin D or to cytotoxic effects of Pep was not further investigated. To do this, one could have determined the number of NM III positive neurons in presence of Pep; in case of inhibition of cathepsin D dependent Ang II production, that number would have stayed constant, whereas, if cytotoxic effects prevailed, it would have declined.

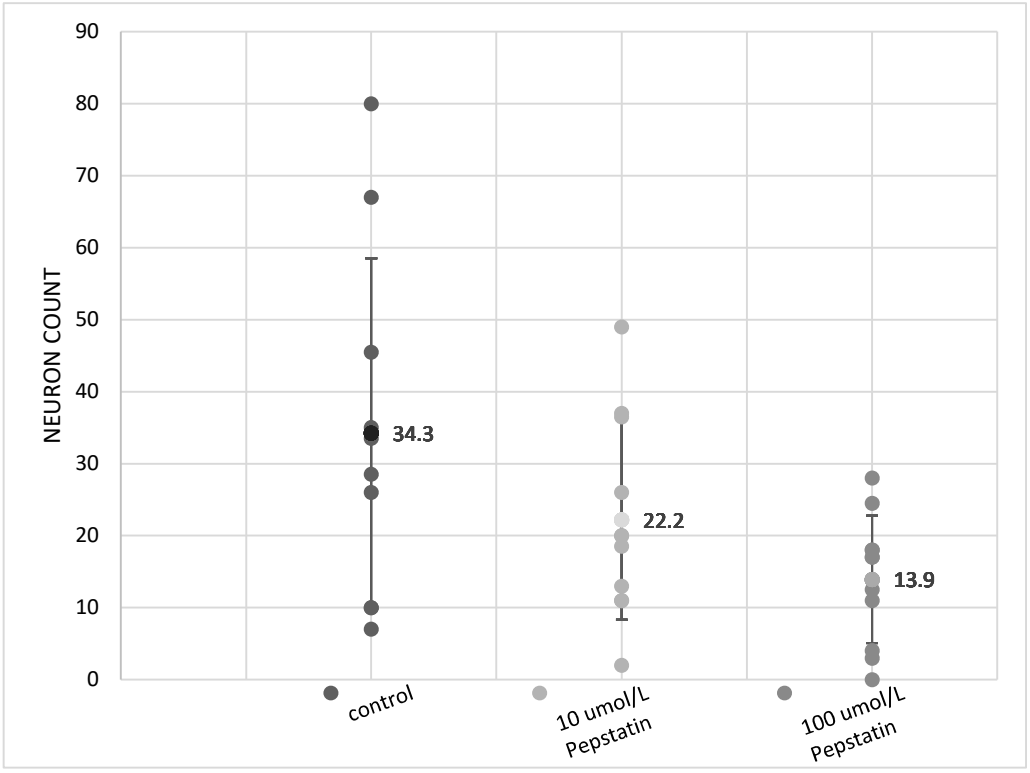

Fig. 20: Effect of pepstatin addition to culture medium on quantity of Ang II positive neurons.

Number of angiotensinergic neurons in 32 immunocytochemically stained preparations under control conditions (n=10) and following addition of 10 µmol/L pepstatin (n=11) and 100 µmol/L pepstatin (n=11) to culture medium. Numbers normalized to seeding density.

Cellular Electrophysiology of Angiotensinergic Neurons

In primary atrial cell cultures, whole cell patch clamp recording was performed to study the electrophysiological properties of angiotensinergic cells. A total of 6 cells having an average membrane capacity of 14.97 ± 13.28 pF was studied. Resting polarization of these cells ranged from -60 mV to -70 mV (average ± SD: -64.45 mV ± 2.83 mV). Depolarization of the putative neurons above a mean threshold potential of -47.43 mV ± 6.38 mV (n=6) precipitated bursts of action potentials of ~5 ms duration and ~20 Hz spiking frequency (Figure 21). Figure 22 depicts whole cell currents of a neuron recorded during voltage clamp steps to the potentials indicated.

In order to assure that the cells undergoing patch clamp experiments were indeed angiotensinergic neurons, preparations were fixated and immunocytochemically stained after the experiment. Whereas staining of the cell somata was difficult to judge because of their destruction following retraction of the patch electrode, cell extensions emanating from the destroyed somata stained positive for both Ang II and NM III in all neurons measured as shown in Figure 23.

In one experiment, Lucifer yellow (LY) was added to the patch pipette to visualize the dimensions of the neuronal extensions and to investigate possible gap junctional coupling of angiotensinergic neurons to other neurons, cardiomyocytes or mesenchymal cells (LY permeates through gap junctions). As shown in Figure 24, LY successfully labeled the somata and extensions of the patched neuron but failed to stain neighboring cells thereby excluding presence of substantial gap junctional coupling.

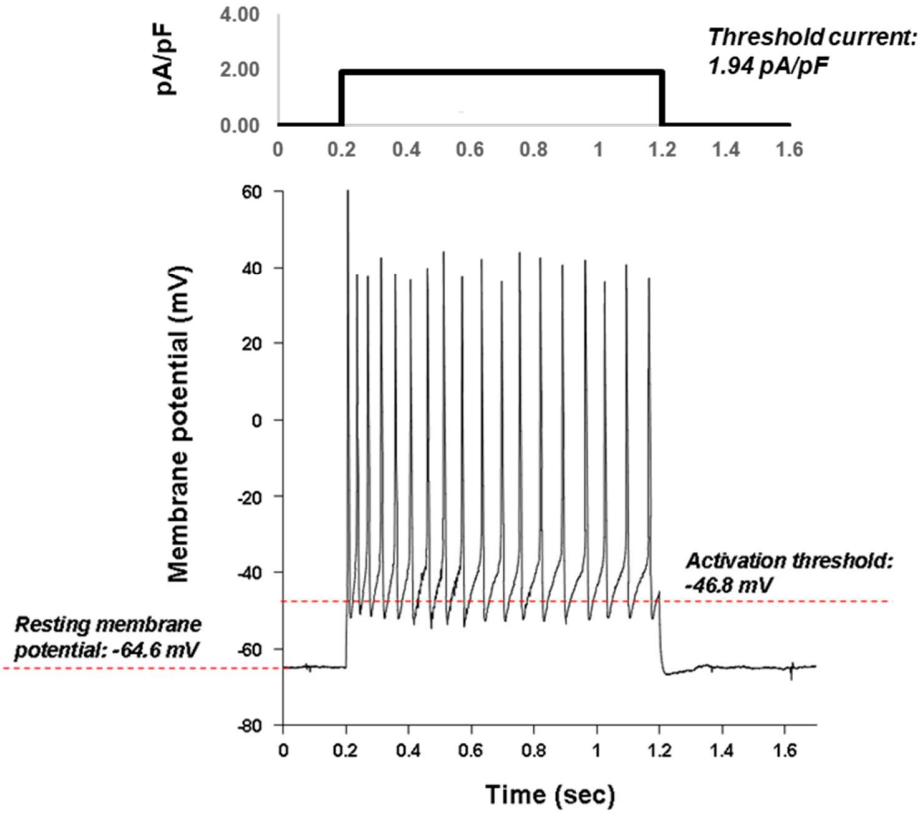

Fig. 21: Action potential burst of an angiotensinergic neuron.

Current injection (1.94 pA/pF) into a quiescent Ang II positive neuron kept in whole cell patch clamp recording configuration induces a burst of action potentials.

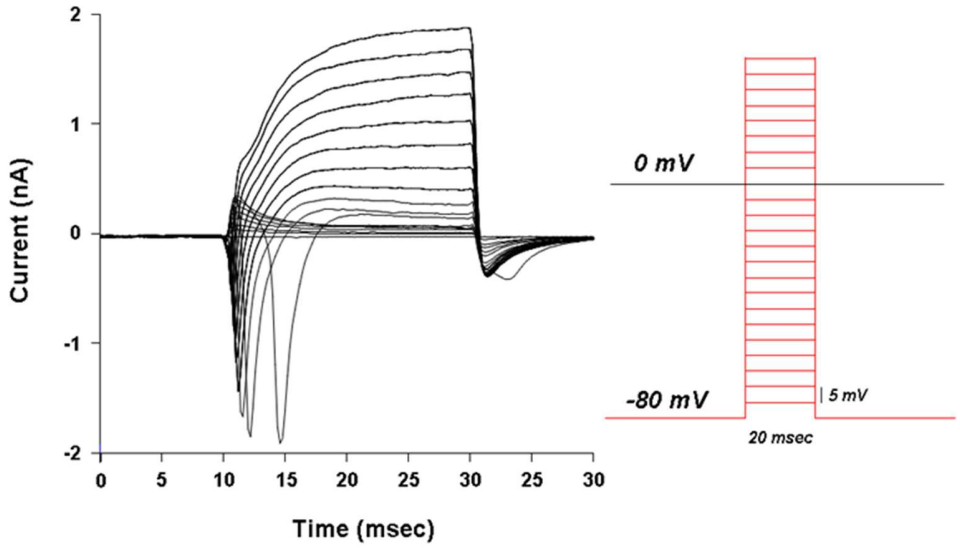

Fig. 22: Whole cell current recording in an angiotensinergic neuron.

Stepping an angiotensinergic neuron for 20 ms to the potentials indicated by the panel on the right induced the whole cell currents depicted by the left panel.

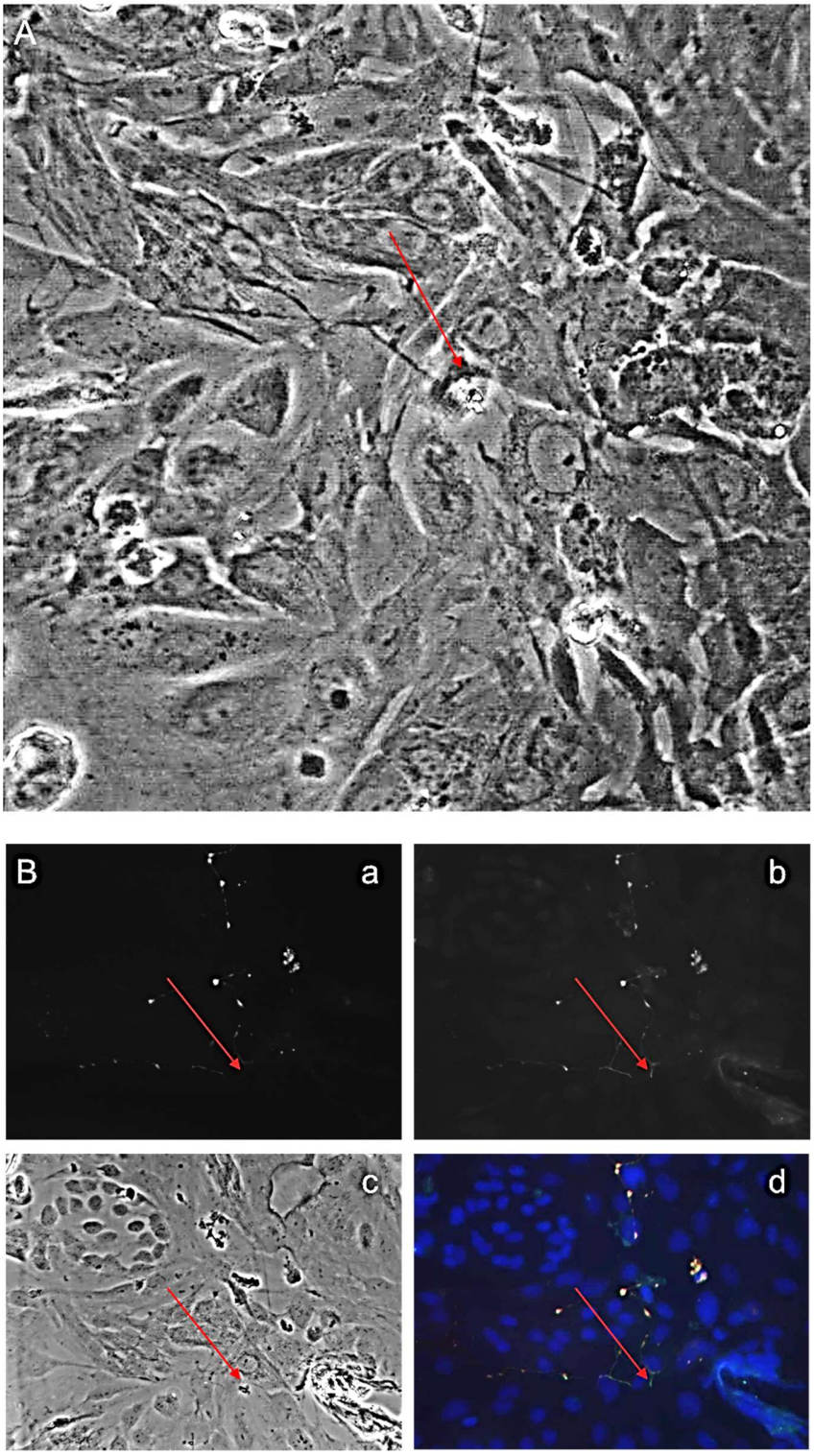

Fig. 23: Immunofluorescent characterization of patched cell.

A, Phase contrast image of a living cell culture undergoing patch clamp recording (arrow on patched neuron). **Ba,** Ang II stained. Positive staining cell deflections. **b** NM III stained. Positive staining cell deflections. **c** Phase contrast image after fixation (arrow on patched neuron). **d** Merged image, colocalized Ang II staining in red and NM III staining in green. DAPI in blue. Arrow on patched neuron cell body (not stained), deflections show colocalization (d, yellow) of Ang II and NM III.

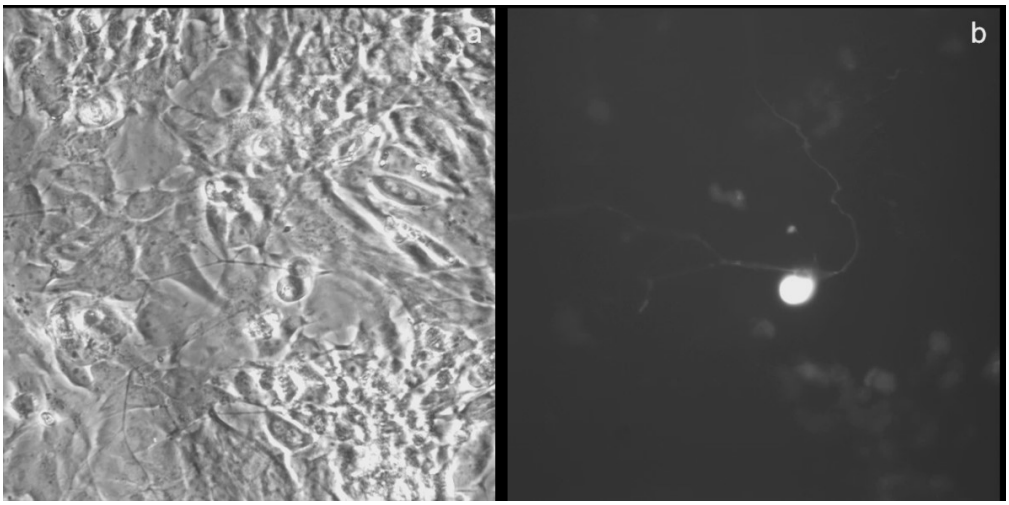

Fig. 24: Probing gap junctional coupling of neurons with adjacent cells.

a Phase contrast image of living culture. **b** Lucifer yellow diffusing from the patch pipette into the neuron causes labeling of the cell body and dendrite-like extensions.

Viral Transduction

Because the search for neurons suitable for patch clamp studies using phase contrast microscopy proved challenging due to high density of mesenchymal cell cultures present, we explored whether it is feasible to express a fluorescent protein specifically in neurons. For these pilot experiments, we used a lentivirus carrying a CaMKIIa (neuron specific, refer to [41]) promoter coupled to eYFP (enhanced yellow fluorescent protein). After finding adequate transduction conditions, several neurons expressing eYFP were found to stain positive for Ang II when fixated and immunostained (Figure 25). However, because there was a substantial loss of cells in all transduction experiments, we finally abandoned this approach aimed at an easier identification of neuronal cells for patch clamp experiments.

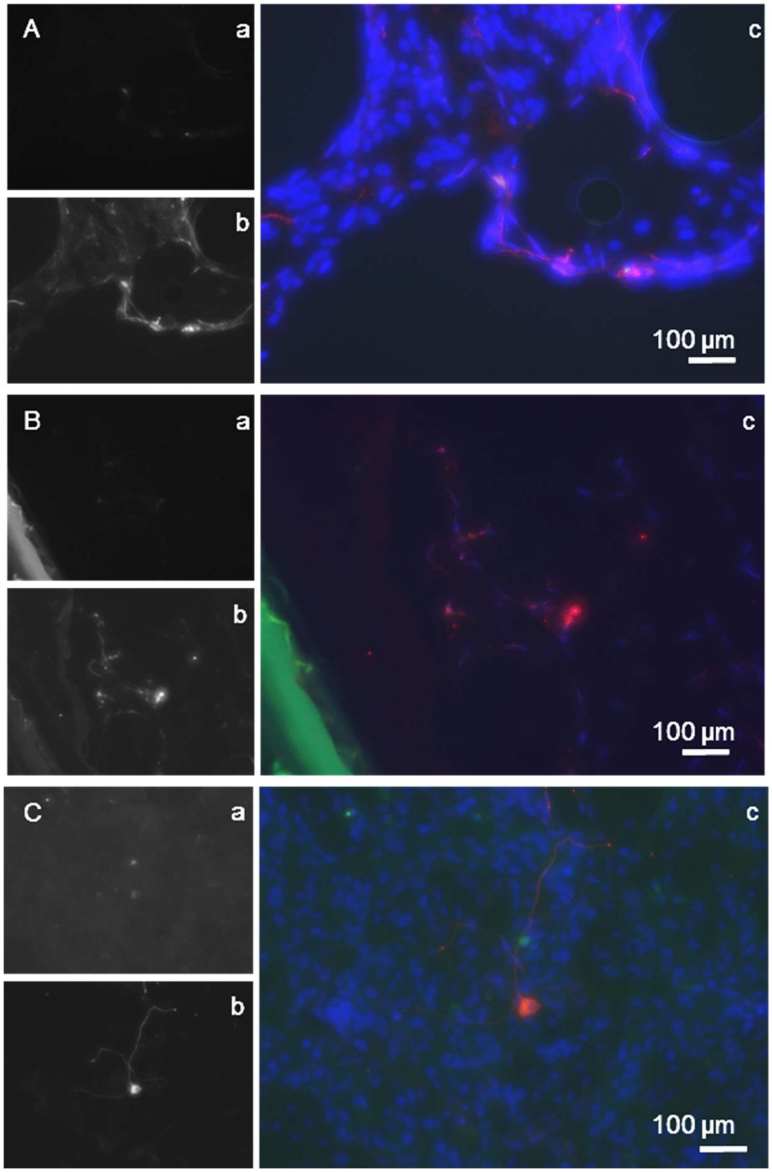

Fig. 25: Viral transduction experiment.

A, B, C, Immunocytochemically identified neurons on different culture disks. **a** Fixated culture, neuron with eYFP expression. **b** Neuron staining strongly positive for Ang II. **c** Merged image with eYFP in green, Ang II in red, DAPI in blue. Colocalization of eYFP expression and Ang II staining.

Discussion

Ang II Positive Neurons in the Intact Neonatal Rat Heart

Based on immunohistological evidence using an array of neuron-specific antibodies, the results of this study demonstrate that, similar to hearts of adult rats, Ang II expressing neurons are also present in hearts of neonates. This nervous system consists of intraatrial angiotensinergic ganglia in contact with other intracardiac neurons belonging to the parasympathetic nervous system, the sensory nervous system, and various intracardiac fiber pathways. Ang II positive cells represent a subpopulation of neurons in ganglia that are, as indicated by the synaptophysin staining, in synaptic contact with other neurons [40].

As demonstrated by previous work of Patil *et al*. [6], Ang II positive nerve fibers show colocalization with DβH, suggesting that Ang II – besides its endocrine functions – acts as a possible neurotransmitter of the sympathetic nervous system in the heart. Interestingly and in contrast to analogous experiments in adult rat hearts [6], ganglion cell bodies of neonatal rats showed no or only a weak staining for DβH in the present study [40]. This difference may imply that the development of the local angiotensinergic nervous system is faster than that of the sympathetic nervous system which takes a few weeks of postnatal development to reach adult norepinephrine levels [42]. In line with this interpretation, we also failed to detect substantial VAChT signals in neuronal cell bodies which suggests that the development of the parasympathetic nervous system was equally delayed. Sympathetic fiber bundles observed might belong to the central nervous system that influences the intracardiac neuronal network.

The possibility that Ang II may act as a neurotransmitter in the sympathetic nervous system is supported by specific patterning of VAChT staining that encircles angiotensinergic ganglion cells, indicating the possibility of synaptic crosstalk between the cholinergic and the angiotensinergic nervous system. The same VAChT staining pattern was observed around ganglion cell shaped structures staining weakly positive for DβH, which may represent, in analogy, intracardiac synaptic contacts between the sympathetic and the parasympathetic nervous system [40].

CGRP is known to be a strong vasodilator and a possible marker of sensory neurons comparable to substance P [8] [9]. CGRP has been found to colocalize with Ang II in the human dorsal root ganglia before [9]. In analogy to these results, we found intracardiac Ang II positive neurons to stain positive for CGRP. Colocalization of Ang II with CGRP possibly suggests a role of Ang II in the sensory nervous system (also proposed by Imboden *et al*. [8], Patil *et al*. [9], and [40]).

Moreover, a colocalization of the AT_1 receptor with neuronal markers was found, demonstrating that ganglion cells contain this receptor and may therefore represent a target for Ang II. This finding, however, was confounded by the heavy positive staining of cardiomyocytes and other cardiac cells ubiquitously expressing the AT_1 receptor, that precluded to draw an unequivocal conclusion [40].

Interestingly, neonatal erythrocytes stained positive for Ang II as well (c. f. to Figure 5 and 6). Confocal microscopy revealed that Ang II positive staining was, unlike staining of neuronal cell somata, restricted to the cell surface. This suggests that, in erythrocytes, circulating Ang II binds to plasma membrane but is neither taken up nor synthesized by an intra-erythrocytic RAS. So far, erythrocytes have been found to metabolize Ang II to Ang-(1-7) and Ang IV, what may be related to cell immunology [43].

Atrial Cell Culture Experiments

Ang II Positive Neurons in Atrial Cell Culture

To compare the staining behavior of angiotensinergic neurons kept in primary culture to those in intact neonatal rat heart, immunocytochemical investigations were performed with identical primary antibodies. As in whole heart preparations, Ang II and NM III demonstrated a clear colocalization. In the many atrial cultures prepared and used for immunocytochemistry, there were practically no Ang II negative neurons, i.e., cells that stained positive for NM III but negative for Ang II. No DβH positive cells were found in the cultures which is in line with absence of these neuronal subtypes in the intact neonatal rat heart. This raises evidence for an early developed specialized angiotensinergic nervous system that precedes the final development of the sympathetic nervous system. In contrast to whole heart preparations, cell somata of Ang II positive cells were not surrounded by ‘baskets’ of VAChT positive synapse-like contacts, which may be due to the low neuron density or insufficient time in culture to develop this feature.

Origin of Ang II in Neurons

When looking at Ang II positive neurons, the central question as to the source of Ang II arises. Two major possibilities exist: (I) uptake of Ang II into the cells where it contributes to an intracellular RAS [35], and (II) intraneuronal synthesis of Ang II or its metabolites as proposed by Patil *et al.* [6].

In the present study, Ang II and fluorescein-labeled Ang II were added to culture medium to test the first hypothesis. Clearly, labeled Ang II was not taken up into angiotensinergic cells. Also, the supplementation with unlabeled Ang II neither increased the number nor the visually quantified

staining intensity of angiotensinergic neurons when compared to control conditions. Under the caveat that the number of experiments was low, these results suggest that uptake of Ang II is less likely. Concerning the second hypothesis, aliskiren was added to the culture medium to investigate possible changes in the development of angiotensinergic neurons in presence of renin inhibition. When used at sub-toxic concentrations, renin-inhibition with aliskiren did not alter the number of angiotensinergic neurons suggesting that angiotensin production in these cells is likely not dependent on renin. In line with this conclusion, it was found by Patil *et al.* [6] before that renin mRNA is untraceable by qRT-PCR in any tested part of the rat heart which suggests a renin-independent pathway of Ang II production in the rat heart. By contrast to renin mRNA, cathepsin D mRNA reached comparable levels to those found in rat liver in all the rat hearts investigated by Patil *et al*. [6]. Cathepsin D is an acid peptidase cleaving, similar to renin, angiotensinogen to angiotensin I and is thought to participate in non-renin pathways of Ang II-formation [7] [8] [9]. While we found no immunohistochemical evidence for renin or cathepsin D expression in the present study, the possibility that this may have been due to limitations of the antibodies made us perform an additional functional test. For this purpose, we added Pepstatin A (Pep), a potent inhibitor of cathepsin D [44],to the culture medium to examine its effect on angiotensinergic neurons. Compared to control preparations, we found that Pep at 100 µmol/L significantly reduced the number of angiotensinergic neurons which supports the hypothesis of de-novo Ang II generation in angiotensinergic neurons by a renin independent pathway requiring cathepsin D as cleaving enzyme. This interpretation, however, is relativized by the finding that the decline of angiotensinergic neurons during Pep treatment was not accompanied by the expected parallel increase in angiotensin-negative neurons.

Overall and in agreement with a former study showing that angiotensinergic nerves are capable of producing Ang II [6], our findings in cell culture suggest that angiotensinergic neurons produce Ang II themselves but the evidence is ambiguous and further investigations into this question are clearly warranted.

Survival and Growth of Ang II Positive Cells

Despite optimization of culture conditions, the number of Ang II positive neurons in culture showed a large variance and the search for neurons suitable for electrophysiological studies using phase contrast microscopy proved challenging due to low angiotensinergic cell density compared to mesenchymal cells. Therefore, we investigated (I) whether Ang II positive cell number or size may be increased by means of an appropriate growth factor like NGF, and (II) whether it would be possible to express a fluorescent protein specifically in neurons by using viral transduction to facilitate patch clamp experiments.

NGF addition, in contrast to former published studies [45], did not appreciably affect cell morphology, cell size or cell number. This may be due to confounding additional NGF production by non-neuronal cells in mixed atrial cultures. Other possibilities include the low concentration of NGF used or insufficient incubation time and/ or sample size to arrive at significant differences.

Viral transduction experiments were unsuccessful because transduction led to clumping and detachment of the cultured primary cells. When the viral load was reduced, neurons expressing eYFP could be sporadically detected. These cells showed colocalized Ang II staining. Successful expression of eYFP in Ang II positive cells under the neuron specific promoter CaMKIIa strengthens the evidence that the angiotensinergic cells were in fact neurons [46] [47].

Cellular Electrophysiology of Ang II Positive Neurons

Limitations of former studies of angiotensinergic neurons in the rat heart concern the lack of proof that these cells exhibit, apart from immunohistological evidence, electrophysiological properties of neurons [6]. Whereas it is not feasible to probe the electrophysiological characteristics of these cells in intact tissue in vivo with presently available methodologies, isolated angiotensinergic cells in culture offered the unique possibility to assess electrical function using the whole cell recording configuration of the patch clamp technique. These experiments showed that the cells exhibit a resting potential typical for neurons (-60 to -70 mV) and that critical depolarization to a threshold potential of ~ -45 mV elicited bursts of action potentials at frequencies up to 20 Hz with action potential durations measured at 90% repolarization amounting to ~5 ms. Taken together, these findings represent unequivocal proof the Ang II positive cells investigated are indeed of neuronal origin.

Conclusion

The results of the current study demonstrate presence of an early developed intracardiac angiotensinergic nervous system in rats, consisting mainly of Ang II positive ganglion cells targeted to the atria. Cells containing Ang II can be kept and tested in primary culture and were found to exhibit neuron-like electrophysiological characteristics. The origin of Ang II accumulation in the neurons remains unclear as angiotensinergic neurons could either synthesize Ang II or its metabolites or take up Ang II from the circulation. The significance of these cells in the context of the intracardiac nervous system, the possible role of Ang II as a neurotransmitter, and the importance of angiotensinergic neurons for the function of the heart in health and disease remains to be further elucidated. Of particular interest in this context may future experiments also explore

in greater detail the type and classification of cells that the Ang II positive neuronal cells have contact with.

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