

# Angiotensinergic innervation of the kidney: Localization and relationship with catecholaminergic postganglionic and sensory nerve fibers

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**Summary.** We describe an angiotensin (Ang) II-containing innervation of the kidney. Cryosections of rat, pig and human kidneys were investigated for the presence of Ang II-containing nerve fibers using a mouse monoclonal antibody against Ang II (4B3). Co-staining was performed with antibodies against synaptophysin, tyrosine 3-hydroxylase, and dopamine beta-hydroxylase to detect catecholaminergic efferent fibers and against calcitonin gene-related peptide to detect sensory fibers. Tagged secondary antibodies and confocal light or laser scanning microscopy were used for immunofluorescence detection. Ang II-containing nerve fibers were densely present in the renal pelvis, the subepithelial layer of the urothelium, the arterial nervous plexus, and the peritubular interstitium of the cortex and outer medulla. They were infrequent in central veins and the renal capsule and absent within glomeruli and the renal papilla. Ang II-positive fibers represented phenotypic subgroups of catecholaminergic postganglionic or sensory fibers with different morphology and intrarenal distribution compared to their Ang II-negative counterparts. The Ang II-positive postganglionic fibers were thicker, produced typically fusiform varicosities and preferentially innervated the outer medulla and periglomerular arterioles. Ang II-negative sensory fibers were highly varicose, prevailing in the pelvis and scarce in the renal periphery compared to the rarely varicose Ang II-positive fibers. Neurons within renal microganglia displayed angiotensinergic, catecholaminergic, or combined phenotypes. Our results

suggest that autonomic fibers may be an independent source of intrarenal Ang II acting as a neuropeptide co-transmitter or neuromodulator. The angiotensinergic renal innervation may play a distinct role in the neuronal control of renal sodium reabsorption, vasomotion and renin secretion.

**Key words:** Kidney, Innervation, Angiotensin, Noradrenaline, Microganglion

## Introduction

The kidney has a rich sympathetic innervation by postganglionic neurons located in the celiac and prevertebral sympathetic ganglia. These neurons receive their higher central input mainly via spinal segments Th 12 to L2. Additionally, there is an important sensory-afferent renal innervation through dorsal root ganglionic neurons with distinct chemo-, mechano- and nociceptive properties (Barajas et al., 1991; Ammons, 1992). Afferent and efferent renal fibers both constitute complex autonomic reflexes placed under the integrative control of the central nervous system. They provide a comprehensive neuronal control of renal salt- and water excretion, blood pressure adaptation, and also renin release. Sympathetic nervous activity is furthermore responsible for the fine tuning of the tubulo-glomerular feed-back mechanism maintaining overall external sodium balance constant at different levels of arterial blood pressure (DiBona and Kopp, 1997). Chronic dysfunction of the kidney's neuronal control may result in abnormal renal salt and water handling, a reduced ability to compensate for circulatory challenges, and facilitate renal disease and arterial hypertension

(Schlaich et al., 2009; DiBona and Esler, 2010). An abnormal overactivity of the sympathetic nervous system, for instance, causes increased spillover of catecholamines into the renal, and also systemic circulation, and is a common feature of human essential hypertension (Grassi, 2009). Global or regional sympathetic overactivity is also a characteristic of animal models of genetic or experimentally induced hypertension (Katholi et al., 1980; Lundin et al., 1984; Osborn and Fink, 2010). In the clinical setting, patients with resistant hypertension can be treated successfully by catheter-based radio-frequency ablation of renal nerves, which is an emerging new therapeutic option for otherwise medically unresponsive cases and underscores the pathophysiological relevance of renal sympathetic innervation for maintaining chronically elevated blood pressure (Krum et al., 2009).

Sympathetic postganglionic fibers have a wide intrarenal distribution. They were found by immunohistological methods to innervate the pelvic wall, the entire arterial tree, the larger central veins, the juxtaglomerular (JG) cells and the renal tubular compartment. The kidney's sensory innervation instead has a more limited anatomical range with a marked central to peripheral gradient and highest density in the pelvic region. Neurotransmission in the postganglionic sympathetic renal fibers is essentially noradrenergic and involves co-release of neuropeptide Y (NPY) as a neuromodulator (Liu and Barajas, 1993a; DiBona and Kopp, 1997). Renal sensory fibers in contrast may release calcitonin gene-related peptide (CGRP) and substance P (SP), which for instance plays a pathogenetic role during tissue inflammatory responses (Knight et al., 1991; Kopp et al., 2003; Grant et al., 2004).

Angiotensin II is another neuropeptide long known to be an important synaptic co-transmitter in the brain, where it modulates central sympathetic outflow, baroreflex activity, thirst and neuroendocrine secretion (Ferguson et al., 2001). Its expression in peripheral neurons located in celiac, dorsal root, and trigeminal ganglia, however, has only recently been reported by our laboratory using a sensitive monoclonal Ang II antibody with unique immunohistological properties to detect Ang II at the subcellular level (Patil et al., 2008, 2010; Imboden et al., 2009). We also subsequently demonstrated an extensive angiotensinergic innervation of the heart and arterial blood vessels and further characterized its phenotype (Patil et al., 2008, 2011). The data implicate that, as in the brain, Ang II may be released as a neuropeptide co-transmitter from peripheral autonomic nerves where it probably has a neuromodulatory effect on target cell function or influences synaptic neurotransmission (Bohlender and Imboden, 2012).

Extending these initial studies we here report the presence of an important angiotensinergic innervation of the kidney. We provide its anatomical distribution and analyze its relationship with postganglionic efferent and

sensory afferent fibers using immunohistological methods. Our results suggest that Ang II is also a neuropeptide co-transmitter in large subsets of both efferent and afferent autonomic fibers in the kidney, with distinct anatomical distributions and possibly new physiological functions

## Materials and methods

### *Kidney specimens*

Human kidney specimens from two male adult individuals were provided by the Bern University Hospital's Department of Urology. The specimens were obtained intraoperatively for use during routine pathological examinations. Additionally, fresh kidney specimens from two male minipigs and from normotensive male Wistar Kyoto rats (n=20) were provided by the Bern University Hospital's central animal facility. All experimentation was in accordance with American Society of Physiology guidelines and complied with local animal legislation.

### *Immunocytochemistry*

Human and pig kidney specimens were immersion fixed in 2% formaldehyde contained in 0.1 M phosphate buffer (pH 7.4) for 7 days at 4°C immediately after sampling. Rat kidneys were from animals anaesthetized intraperitoneally with 100 mg/kg thiopentane sodium and perfused transcardially with 150 ml Ringer solution containing 1000 U heparin at 37°C followed by perfusion with 300 ml freshly prepared 2% formaldehyde at 4°C. The kidneys were then harvested, carefully dissected, and incubated in 2% formaldehyde for another 28 h at 4°C. All kidney specimens were then rinsed for 14 h in 18% sucrose diluted with 0.015 M Dulbecco's phosphate buffered saline (pH 7.4, 4°C) and frozen embedded in matrix solution (Shandon M-1; No. 114496-SHN, Thermo Scientific). Adjacent tissue sections with a thickness of 20 or 25 µm were prepared on a Leica cryostat microtome (-15°C) and stained immunocytochemically using a free-floating microwell incubation technique. All primary antibodies were of mouse or rabbit origin and diluted for use in 0.05 M TRIS buffered saline (0.81% NaCl, 0.1% T-X-100, 0.01% sodium azide, pH 7.6). The kidney sections were first incubated with the primary antibodies for 36 h at 4°C. After repeated washing the sections were then incubated for another 90 minutes with either Cy<sup>3</sup>-labeled goat anti-mouse IgG (GAM-Cy<sup>3</sup>, No. 115-165-146 Jackson ImmunoResearch; 1:600) or Cy<sup>5</sup>-labeled donkey anti-rabbit IgG (DAR-Cy<sup>5</sup>, Jackson ImmunoResearch No. 711-175-152; 1:300) to allow for immunofluorescence detection of the primary antibodies. Staining of nuclear desoxyribonucleic acid (DNA) was with 4',6'-diamidino-2-phenylindole (DAPI) added to the incubation medium. The sections were air dried, mounted on gelatine-coated glass slides, embedded with

Glycergel (DAKO) and finally cover-slipped.

Detection of Ang II was with a protein G purified mouse monoclonal antibody (Mab 4B3) generated by our laboratory and used at 0.3  $\mu\text{g/ml}$  final concentration. The characteristics of this antibody have been published elsewhere (Frei et al., 2001; Patil et al., 2010). The specificity of Mab 4B3 has previously been tested and documented in detail. The monoclonal antibody produced the same staining as a rabbit polyclonal affinity-purified anti-Ang II control antibody (BODE) in experiments with rat adrenal gland tissue (Frei et al., 2001). Furthermore, using dot blot assays, Mab 4B3 fully cross-reacted with the C-terminal metabolites Ang 2-8, Ang 3-8, Ang 4-8 and Ang 5-8 but not with human plasma Ang-N, Ang I, and Ang (1-7). In a separate test, Mab 4B3 had also been purified by CH-Sepharose-4B that contained N-terminally linked Ang II. Stainings with protein G purified and with Sepharose purified Mab 4B3 gave identical results. There was no staining when mouse pre-immune serum or Sepharose 4B fall-through from Mab 4B3 purification was used. Similarly, there was no staining when the secondary goat anti-mouse antibody tagged with Cy3 was used without Mab 4B3 pre-treatment of tissue sections. Extraction of Ang peptides from human and rat ganglionic tissue with quantification by high pressure liquid chromatography and specific radioimmunoassay confirmed the presence of true Ang II antigen as detected immunohistochemically by Mab 4B3 (Imboden et al., 2009; Patil et al., 2010).

Staining of renal tissue sections for Ang II was also performed with a polyclonal rabbit anti-Ang II antiserum at final dilutions between 1:100 and 1:10,000 (Huang et al., 1989). Catecholaminergic nerve fibers were visualized using a purified rabbit monoclonal anti-tyrosine 3-hydroxylase (TH) antibody (clone EP1533Y, Epitomics No. 2129-1; 1:200) or a polyclonal rabbit anti-dopamine  $\beta$ -hydroxylase (DBH) antibody (No. DZ1020-0050, Biotrend; 1:500). Synaptophysin staining was with a purified mouse (clone SY38, Fitzgerald No. 10R-S124A; 1:600) or rabbit C-terminal monoclonal antibody (clone YE269, Epitomics No. 1485-1; 1:400) and renin staining with a polyclonal rabbit anti-human renin antiserum "L22" at a final antiserum dilution of 1:5000 (Guyenne et al., 1980), which like the polyclonal Ang II-antibody was kindly provided by J. Ménard, Université Descartes, Paris, France. The renin antibody also crossreacts with rat and pig renin. Calcitonin gene-related peptide (CGRP), a marker of renal afferent fibers, was detected with a purified rabbit polyclonal antibody (Peninsula No. T-4032). Co-staining experiments were performed for Ang II using the monoclonal antibody and TH or DBH, synaptophysin and CGRP antibodies. Control sections without primary or secondary antibodies or both were examined in parallel each time to control for artefacts. Immunofluorescence detection was with a Leica DM6000B digital confocal fluorescent light microscope or a Leica SP2 laser scanning microscope (LSM).

Monoplane views from up to 20 optical z-layers representing 2-6  $\mu\text{m}$  thick sample volumes were generated with Leica computerized imaging software as necessary.

## Results

### General pattern

Overview micrographs from rat and human kidneys (Figs. 1, 2) show stainings for Ang II using the monoclonal mouse anti-Ang II antibody 4B3. They revealed the presence of thick bundles and numerous smaller fibers with Ang II-staining throughout the pelvic region. Ang II-positive fibers were seen in the renal nerves and their branches (Figs. 1a, 2a), as well as in the sympathetic nerve plexus surrounding arterial blood vessels, including pre- and postglomerular arterioles (Figs. 1a-c, 2a), in the adventitia of the urothelium (Fig. 1a,d), and around tubules in the outer medulla and renal cortex (Figs. 1e, 2a). A scarce angiotensinergic innervation was also seen in the adventitia of central veins and the renal capsule (not shown). Ang II-positive nerve fibers were not detected within glomerula, the renal papilla or penetrating into the vascular smooth muscle cell layer of blood vessels and the urothelium. Various other cell types, such as tubular epithelium (Fig. 1a,e), the urothelium (Fig. 1a,d) and smooth vascular muscle cells (Fig. 1b) also contained immunoreactive Ang II and were similarly stained by the monoclonal antibody at varying intensity. Rat, pig and human kidneys all gave the same result.

Similar Ang II stainings concerning the presence of Ang II-positive fibers in various regions of the kidney were obtained with the polyclonal anti-Ang II antiserum. The overall luminescence and spatial resolution of the nervous structures, however, were low and there was troublesome non-specific background staining (not shown). All further studies shown and discussed here were therefore performed with the monoclonal anti-Ang II antibody only.

### Co-staining of Ang II and TH or DBH

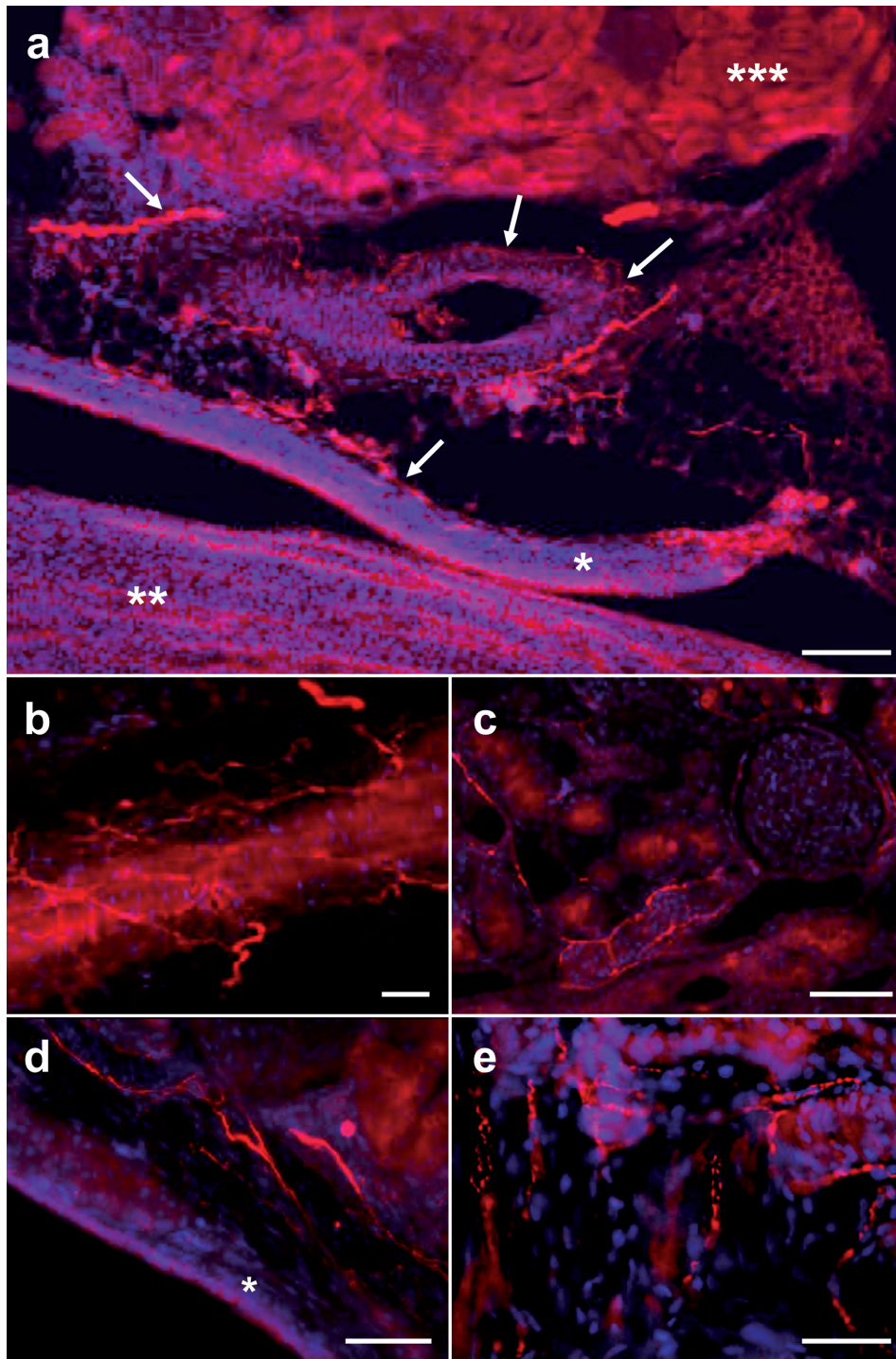
Co-staining of kidney sections for Ang II and for TH or DBH (rabbit monoclonal antibodies EP1533Y and DZ1020) showed Ang II-positive and catecholaminergic (sympathetic efferent) fibers sharing the same intrarenal distribution (Fig. 2). Studies with TH or DBH-staining yielded identical results in all three species investigated. Figures 3 and 4 show intrapelvic fiber bundles co-stained for Ang II and TH at high resolution. These bundles showed numerous Ang II-positive nerve fibers (Fig. 3a, 4a). Most fibers in the bundles, however, were catecholaminergic (Fig. 3b, 4b) which frequently co-localized Ang II (Fig. 3c, 4c). There were fibers with a purely angiotensinergic or catecholaminergic phenotype, and fibers with a combined phenotype. The same three fiber types were also found elsewhere in the kidney (Fig.



5).

Ang II-containing fibers, including those co-localizing TH or DBH, did not display visible synaptic enlargements in the nerve plexus surrounding arteries or, when occasionally present, they were relatively small and asymmetric. It appeared that many such Ang II-positive fibers in the arterial plexus had their terminals

and targets elsewhere in the periphery. Their exclusively catecholaminergic companions in the plexus, however, showed innumerable small and polymorphic varicosities, sometimes in local clusters (Fig. 5b). The diameters of Ang II-positive fibers were greater than those of the exclusively catecholaminergic fibers, usually more than 2-fold (Fig. 5a,b). Purely catecholaminergic varicose



**Fig. 1.** Angiotensin II-staining of rat and human kidney sections with the monoclonal antibody 4B3 (red). **a.** Pelvic region of a rat kidney. Numerous Ang II-positive fibers are visible next to a branch of the renal artery (center), within the connective tissue and adjacent to the urothelium (arrows). The urothelium (\*) and tubular epithelia in the renal papilla (\*\*) and cortex (\*\*\*) also stain strongly positive for Ang II. **b-e.** Close-up views of rat and human (**c** and **e**) kidney sections. Ang II-positive fibers are seen in the arterial plexus of an arteriole (**b**), in the afferent arteriole (**c**), adjacent to the urothelium (**d**), and in the tubular compartment of the outer medulla (**e**). Fluorescent light microscopy; DNA staining in blue. Scale bars: a, 150  $\mu$ m; b, d, e, 50  $\mu$ m; c, 100  $\mu$ m.

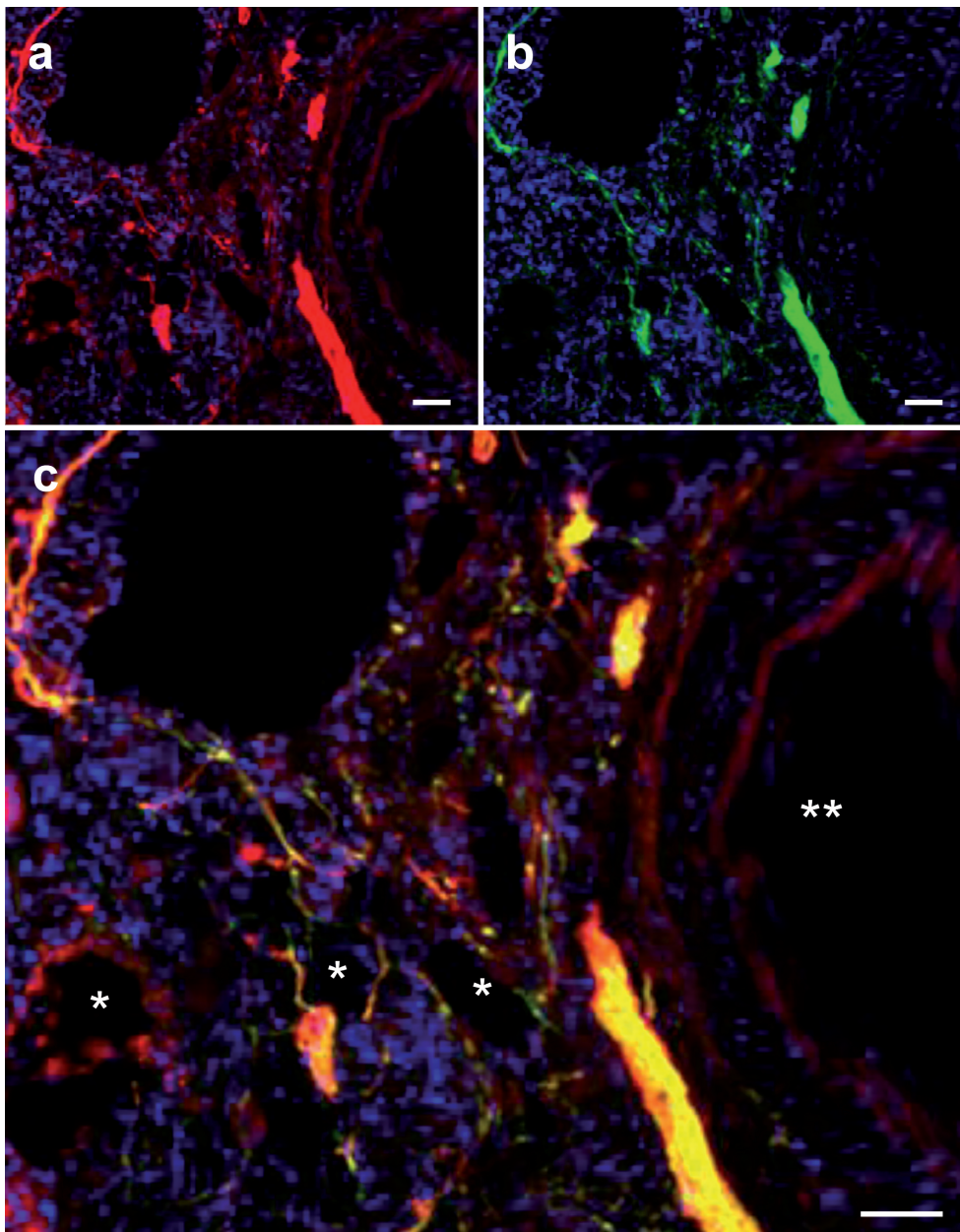


# Angiotensinergic kidney innervation

fibers were the prevailing fiber type in the proximal arterioles where they outnumbered Ang II-positive fibers (Fig. 5c). The purely angiotensinergic fibers without a catecholaminergic co-phenotype became increasingly rare in the periphery of the arterial tree. They were not seen in the afferent or efferent arterioles but detected in the cortical interstitium where they continued to be without visible varicosities over long distances (Fig. 5d-f).

Ang II-containing fibers with a catecholaminergic co-phenotype changed their appearance in the renal periphery. Near tubuli and in the afferent arterioles these fibers now displayed many large, spindle-shaped

varicosities in a row, sometimes with a dented appearance (Fig. 5d-i). These enlargements were much greater, sometimes up to 5  $\mu\text{m}$  in pigs and humans, than those of purely catecholaminergic fibers. Their intervaricose spacing was greater. The fiber diameters also became somewhat thinner at the terminals. Occasionally, dot-like smaller varicosities were interspersed (Fig. 5d-f). In the afferent arterioles all visible fibers showed a combined catecholaminergic and angiotensinergic phenotype. In human and pig kidneys they were present as a circular network with multiple fusiform and sometimes dot-like enlargements. In the rat kidney the fibers stayed mainly within fascicles running

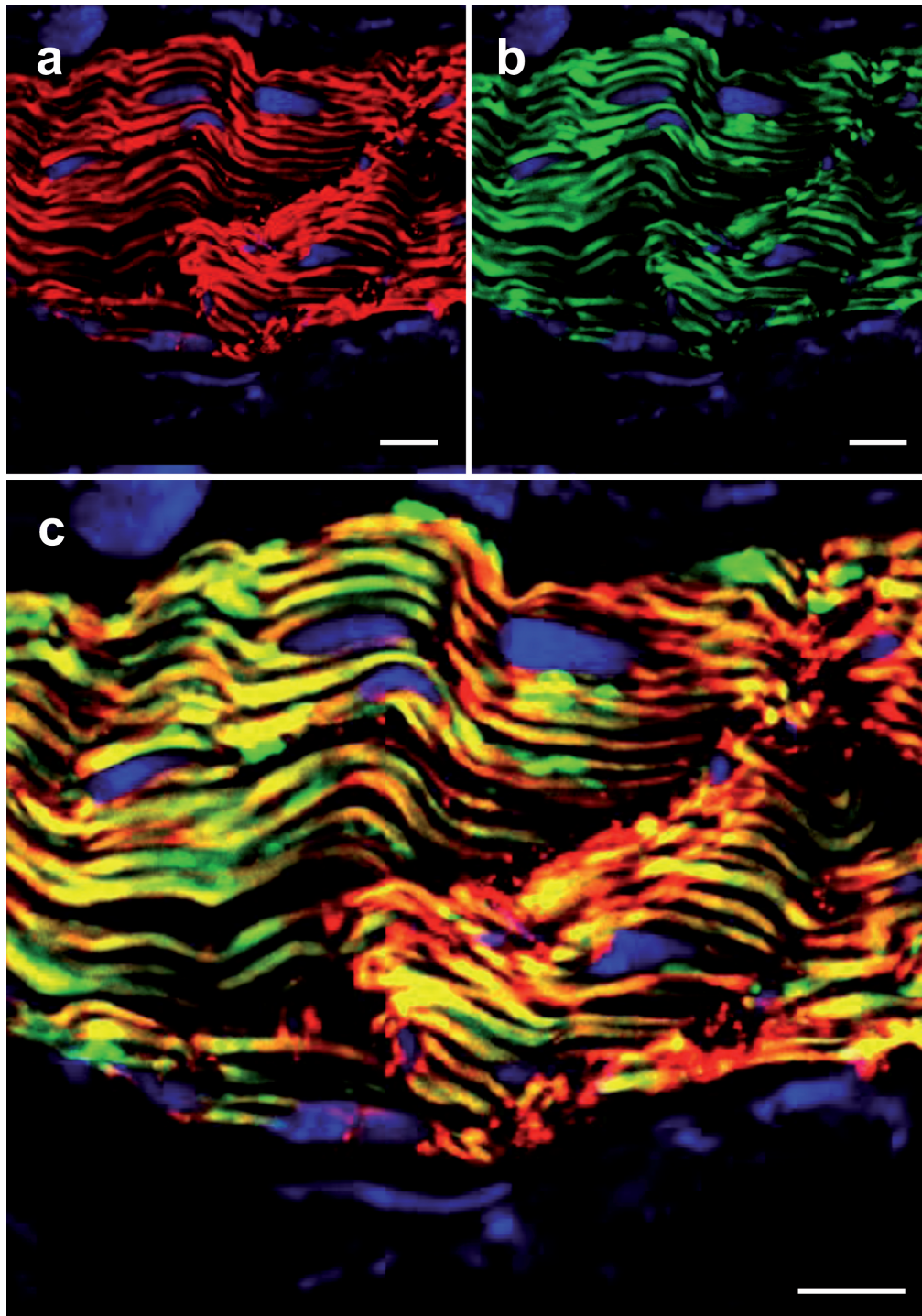


**Fig. 2.** A human kidney section co-stained for Ang II (red) and tyrosine hydroxylase (TH, green). Nerve fibers staining for Ang II (a) and for TH (b) have similar distributions as shown in the merged picture (c) where co-localization is indicated in yellow. Tubuli are indicated by (\*) and a local artery branch by (\*\*). Fluorescent light microscopy; DNA staining in blue. Scale bars: 50  $\mu\text{m}$ .

along the vessel axis (Fig. 1c). Fibers with a double catecholaminergic and angiotensinergic phenotype were occasionally seen to extend through the glomerular pole and to innervate the efferent arteriole, or were in touch with Bowman's capsule.

Similar to the afferent arteriole, fibers in the outer

renal medulla were almost exclusively catecholaminergic and Ang II-positive (Fig 5g-i). They were varicose, ran often in parallel with the tubuli over long distances  $>200\ \mu\text{m}$  and displayed frequent fusiform enlargements (Fig. 1e, 5g-i). Catecholaminergic fibers co-expressing Ang II instead were relatively scarce in



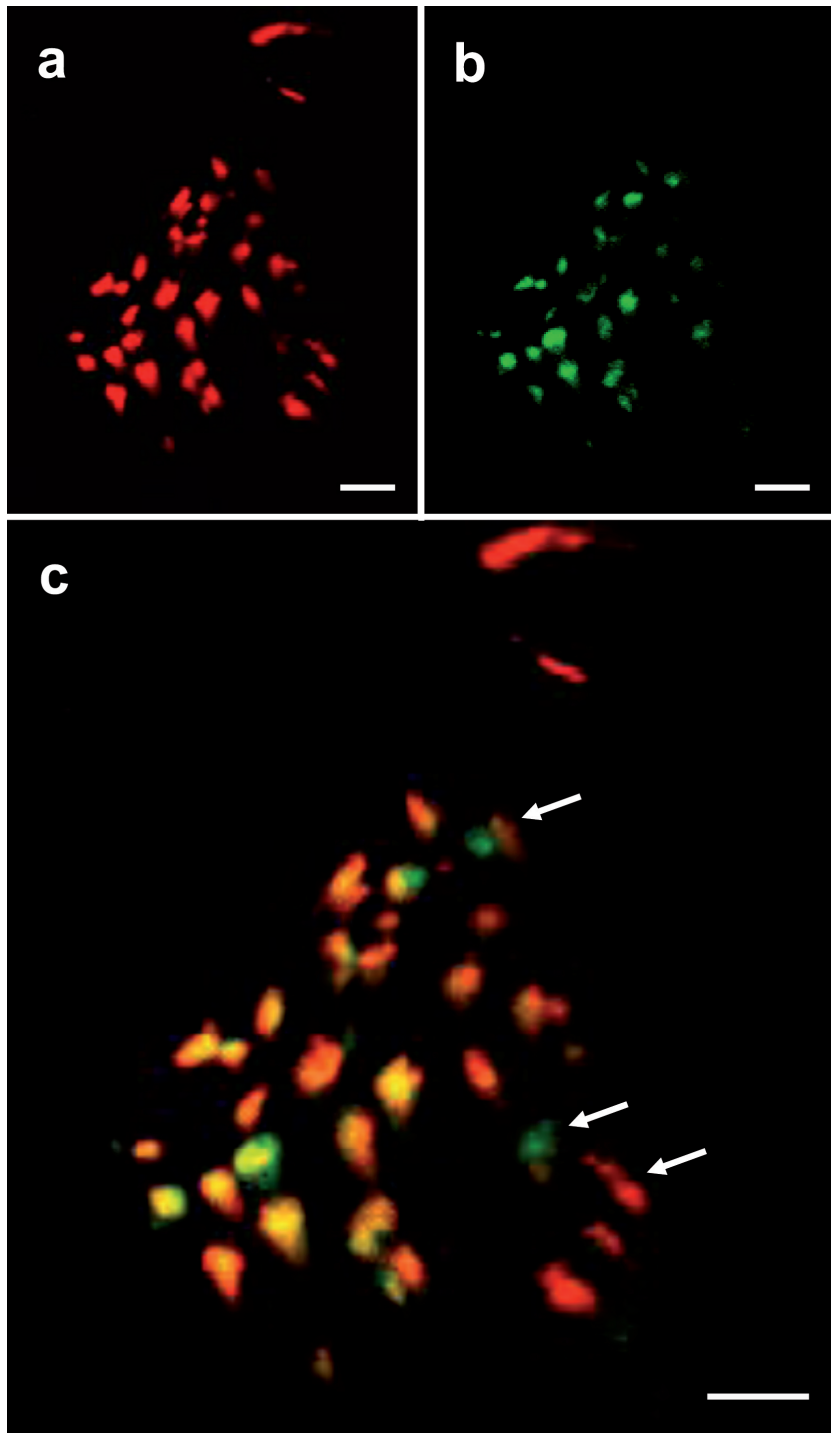
**Fig. 3.** A pelvic fiber bundle in a human kidney co-stained for Ang II (red) and tyrosine hydroxylase (TH, green). Almost all fibers in the bundle co-localized Ang II (a) and TH (b) as shown by the merged picture (c) in yellow colour. Laser scanning microscopy; DNA staining in blue. Scale bars: 10  $\mu\text{m}$ .

the renal cortical interstitium where Ang II-negative efferent fibers were prevailing.

#### *Co-staining of Ang II and synaptophysin*

Co-staining of Ang II (mouse monoclonal antibody 4B3) and synaptophysin (rabbit monoclonal antibody

YE269) gave essentially the same results as for co-staining of Ang II with TH or DBH concerning intrarenal fiber distribution and their phenotypic appearance. There were fibers exclusively positive for Ang II or synaptophysin and fibers expressing both antigens (not shown). There were no significant interspecies differences.



**Fig. 4.** Cross-section of a smaller fiber bundle seen in a rat kidney co-stained for Ang II (red) and tyrosine-hydroxylase (TH, green). Most fibers co-localize both antigens as shown by the merged picture (c) in yellow. There are also a few fibers exclusively positive for Ang II or TH (arrows). LSM; staining of nuclear deoxyribonucleic acid in blue. Scale bars: 2.5  $\mu$ m.



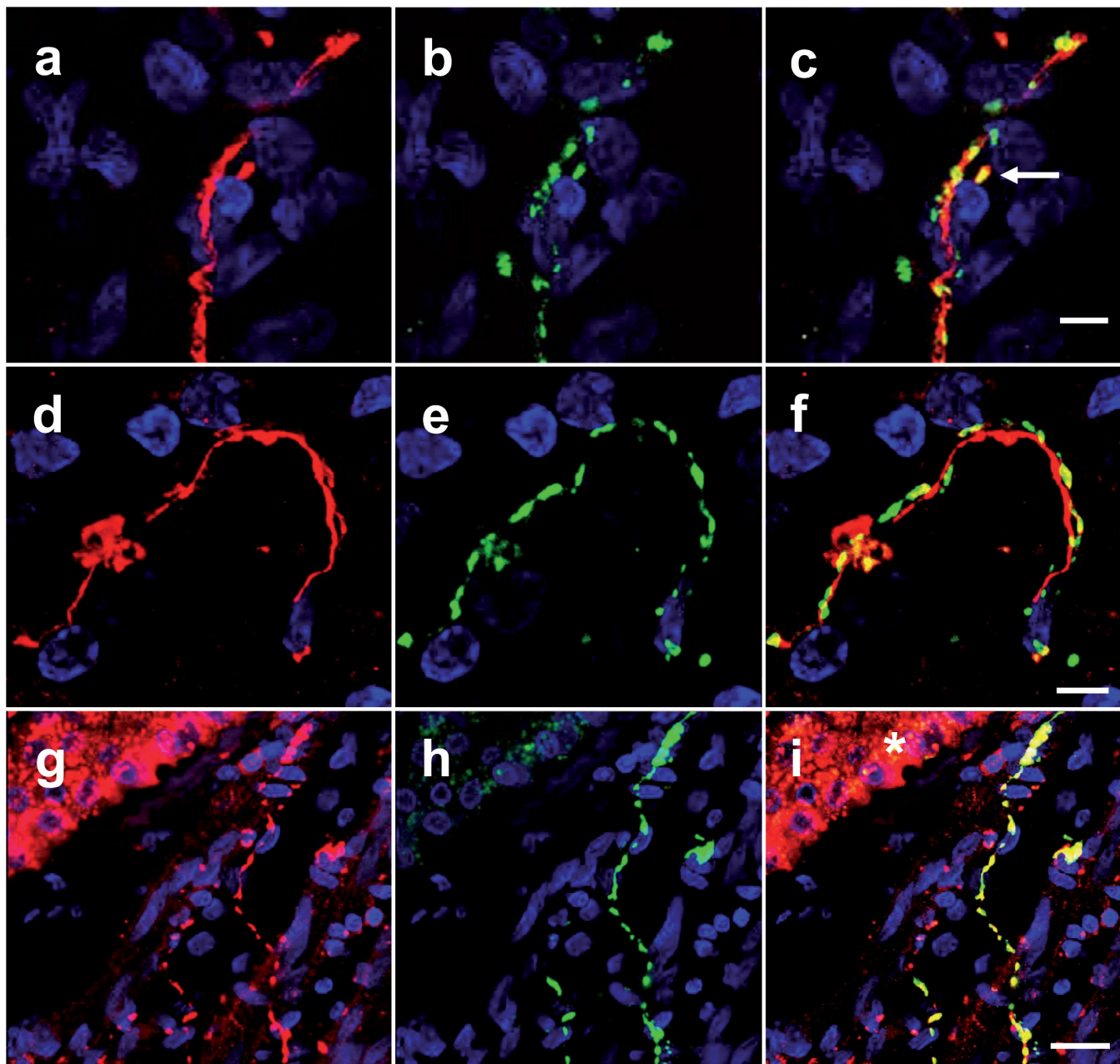
*Co-staining of TH or DBH and synaptophysin*

All fibers staining for TH or DBH (rabbit monoclonal antibodies EP1533Y or DZ1020) also showed co-staining for synaptophysin (mouse monoclonal antibody SY38). No exclusively

synaptophysin positive fibers were identified.

*Co-staining of Ang II and CGRP*

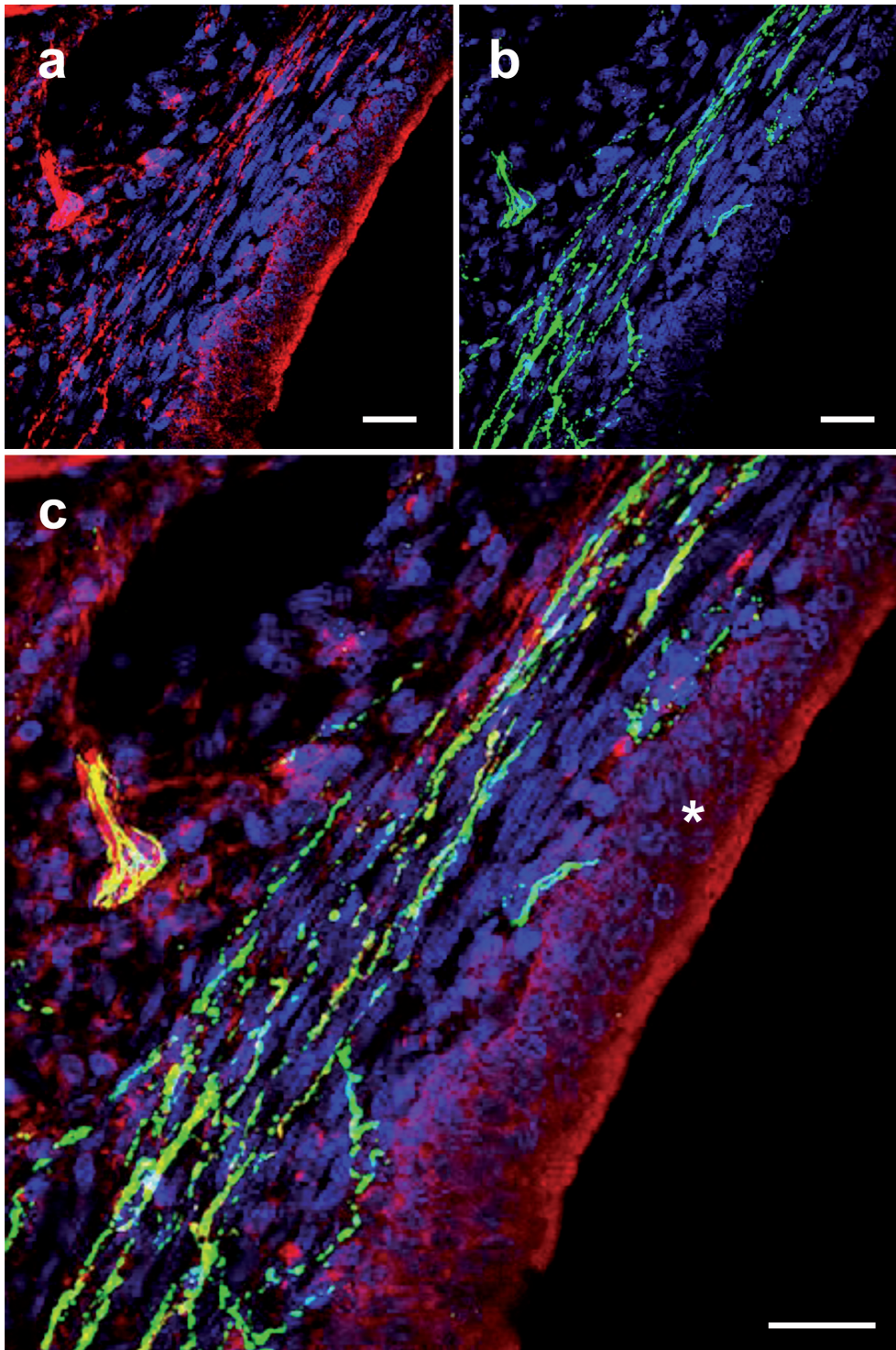
Numerous CGRP-positive sensory nerve fibers with a varicose appearance were found abundantly in the



**Fig. 5.** Nerve fibers in the rat arteriolar adventitia (**a-c**), human renal cortex (**d-f**), and in the human outer renal medulla (**g-i**) after co-staining for Ang II (red) and tyrosine hydroxylase or dopamine  $\beta$ -hydroxylase (DBH) as catecholaminergic markers (green). Merged pictures are shown in panels **c**, **f** and **i** with co-localization in yellow. **a-c** show varicose and non-varicose Ang II-positive fibers (**a**), and highly varicose TH-positive fibers (**b**) within the same fascicle in the adventitia of an artery (**c**). TH and Ang II co-localized in some varicose fibers (arrow); the other fibers in the bundle were purely catecholaminergic and varicose or angiotensinergic and non-varicose. **d-f** show a varicose and a non-varicose Ang II-containing fiber running closely together in the cortical interstitium (**d**). The varicose Ang II-positive fiber co-localized also DBH (**f**). **g-h** show varicose fibers co-localizing Ang II and DBH in the outer medulla. The asterisk indicates tubular epithelium strongly staining for Ang II. LSM; DNA staining in blue. Scale bars: a-c, 10  $\mu$ m; d-f, 5  $\mu$ m; g-i, 25  $\mu$ m.

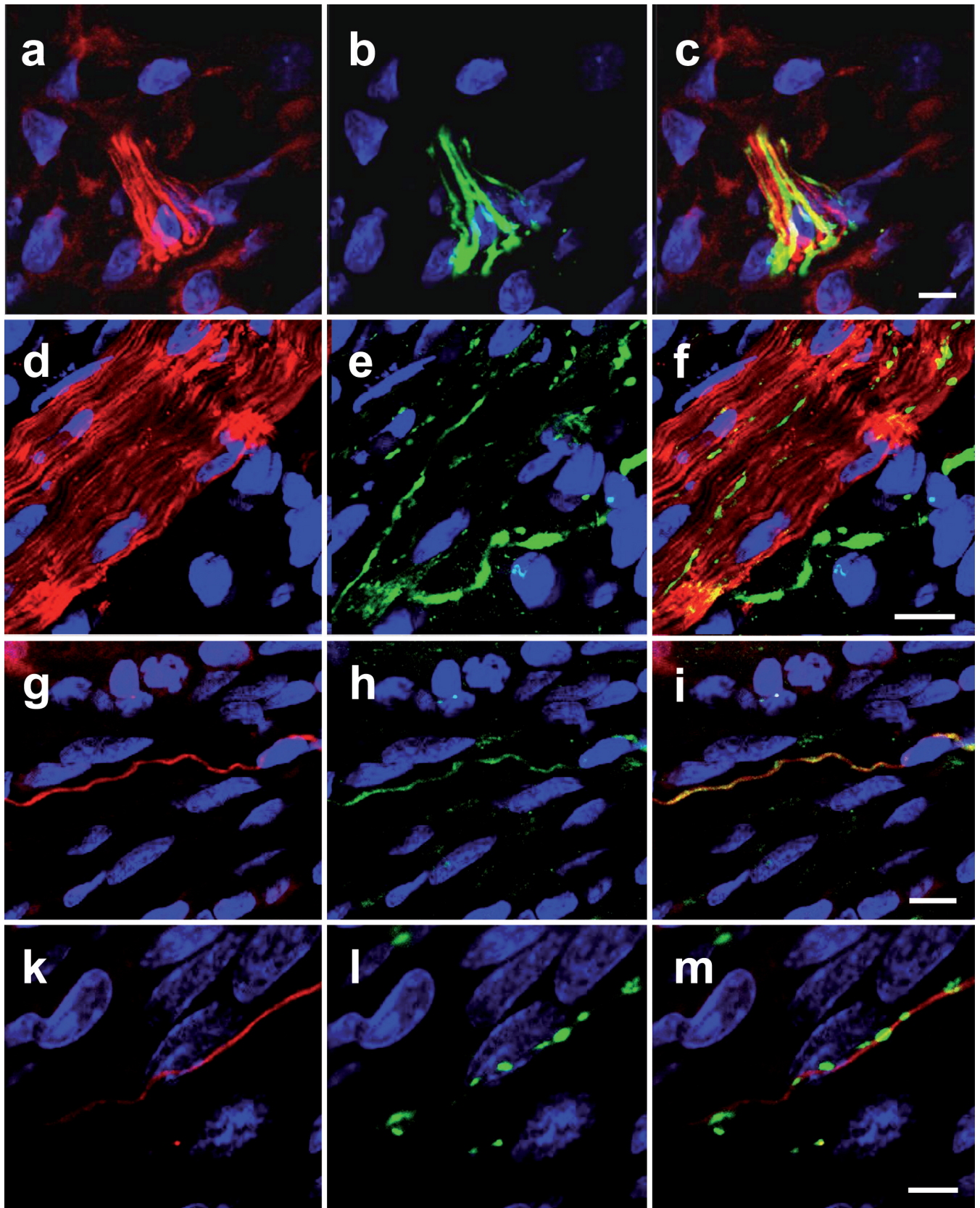
pelvic region. These fibers showed an overlapping distribution with those positive for Ang II (Fig. 6). CGRP-positive sensory fibers were furthermore found within renal nerves and their local branches (Figs. 6b, 7a-f), in the periarterial nerve plexus, and also in the

renal cortical interstitium. A dense innervation by CGRP-positive fibers was present in the connective tissue adjacent to the urothelium (Fig. 6b). The density of CGRP-containing fibers rapidly decreased in the arterial periphery and the renal cortex. CGRP-containing



**Fig. 6.** Co-staining of a rat kidney section for Ang II (red) and calcitonin gene-related peptide (CGRP, green), a marker of sensory fibers. Shown is the pelvic region. Both Ang II-positive (a) and CGRP-positive fibers (b) share the same regional distribution and fascicles as shown by the merged picture (c). CGRP-positive fibers were highly varicose. A nerve trunk with Ang II-positive and CGRP-positive fibers is seen to the left. The urothelium is indicated by the asterisk and also stains for Ang II. Laser scanning microscopy; DNA staining in blue. Scale bars: 25  $\mu$ m.





**Fig. 7.** Close-up views of rat kidney sections co-stained for Ang II (red) and calcitonin gene-related peptide (CGRP, green), a sensory marker. **a-c** and **d-e** show two nerve bundles in the pelvic area containing both Ang II-positive and CGRP-positive fibers. The bundle in **a-c** is that seen in Fig. 6. The number and morphology of CGRP-positive fibers was different in the two bundles. Merged pictures are shown in **c, f, i** and **m** with co-localization in yellow. There were CGRP-positive fibers that co-localized Ang II (**a-c**) while others did not (**d-f**). The fibers without Ang II-expression were highly varicose with many and sometimes large fusiform enlargements (**e, l**). The Ang II-containing fibers not co-localizing CGRP were most likely postganglionic efferent. **g-i** show a single, non-varicose fiber co-localizing Ang II and CGRP. **k-m** show two closely adjacent fibers, one exclusively positive for Ang II and non-varicose and one positive only for CGRP and varicose with fusiform enlargements in a row. Laser scanning microscopy; DNA staining in blue. Scale bars: a-i, 10  $\mu$ m; k-m, 5  $\mu$ m.



fibers were not found in the periglomerular arterioles, glomerula or the renal papilla.

Fig. 7 shows results at a higher resolution. Fibers exclusively positive for CGRP or Ang II and fibers with a combined phenotype could be distinguished. Fibers positive only for CGRP were varicose and showed many large, spindle-shaped enlargements (Fig. 7e,l) at their terminals. Intervaricose diameters were smaller than those of Ang II-containing fibers (Fig. 7d-f,k-m). Fibers with a combined CGRP and angiotensinergic phenotype (Fig. 7g-i) were often without varicosities over long distances. In the pelvic region, these double-staining fibers were significantly less frequent than the exclusively CGRP positive fibers. The prevalence of double-staining fibers rapidly decreased in the arterial periphery. They were not identified in periglomerular arterioles but occasionally present in the cortex. The number of CGRP-positive fibers in the larger pelvic fiber bundles was small compared to the number of angiotensinergic fibers (Fig. 7d-f). Such sensory fibers were varicose.

#### *Co-staining of Ang II and renin*

Ang II-containing nerve fibers were detected next to patchy intracellular renin staining of juxtaglomerular cells (Fig. 8a). The fibers also produced fusiform enlargements next to areas with renin staining, suggesting the presence of synaptic junctions with juxtaglomerular cells (Fig. 8b). There was a very weak

granular Ang II-staining present together with the renin staining.

#### *Intrarenal autonomic microganglia*

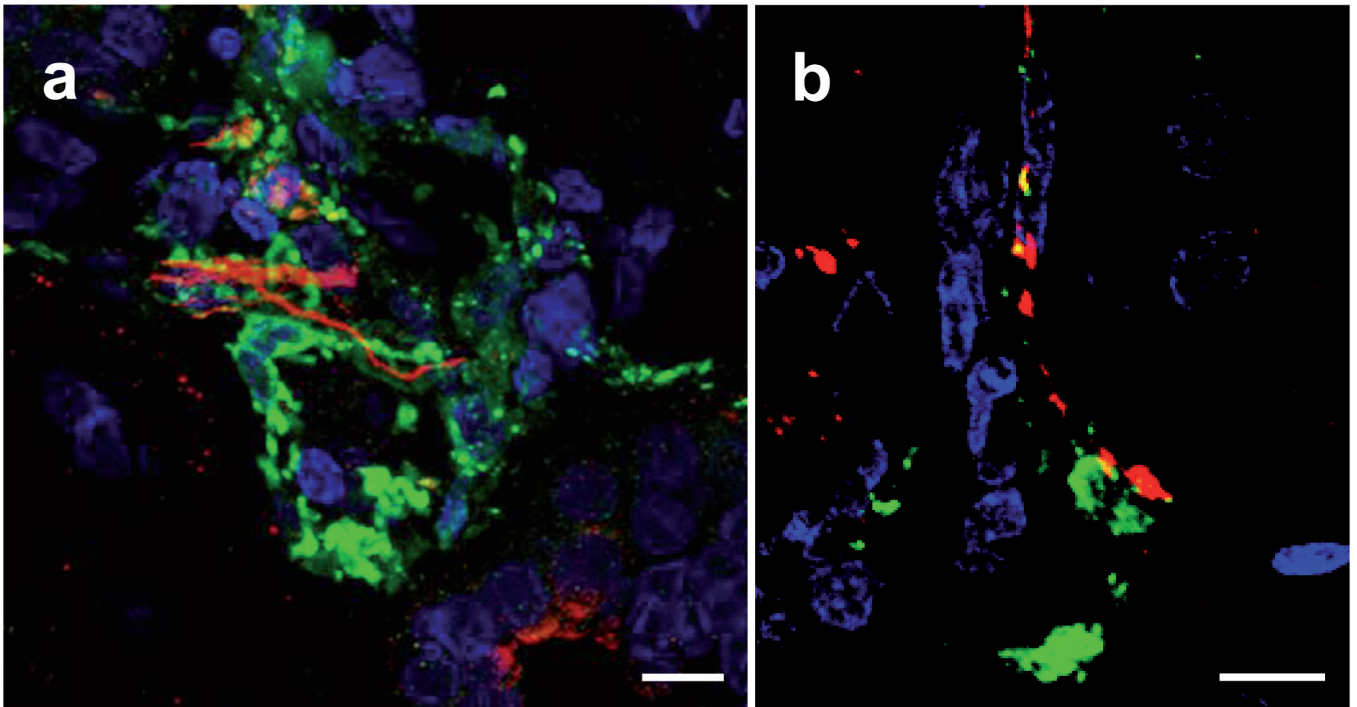
Various microganglia from rat and pig kidneys could be identified. They were located in the pelvic region or adjacent to the pelvic renal artery and its branches, while all contained Ang II-positive neurons. The human kidney specimens did not contain a microganglion or identifiable single neuronal pericarya. Fig. 9 shows a bipartite microganglion from a pig kidney with Ang II-positive neurons. Co-staining for Ang II and TH revealed three distinct neuronal cell phenotypes. There were neurons exclusively positive for Ang II or TH (Fig. 9a,b), and neurons co-localizing both antigens (Fig. 9c). Ang II-positive neurons were the largest group.

#### *Interspecies differences*

There were no significant interspecies differences for these findings except for the shape of the axons in the periglomerular arterioles in rats where synapses showed a greater morphological variability.

#### **Discussion**

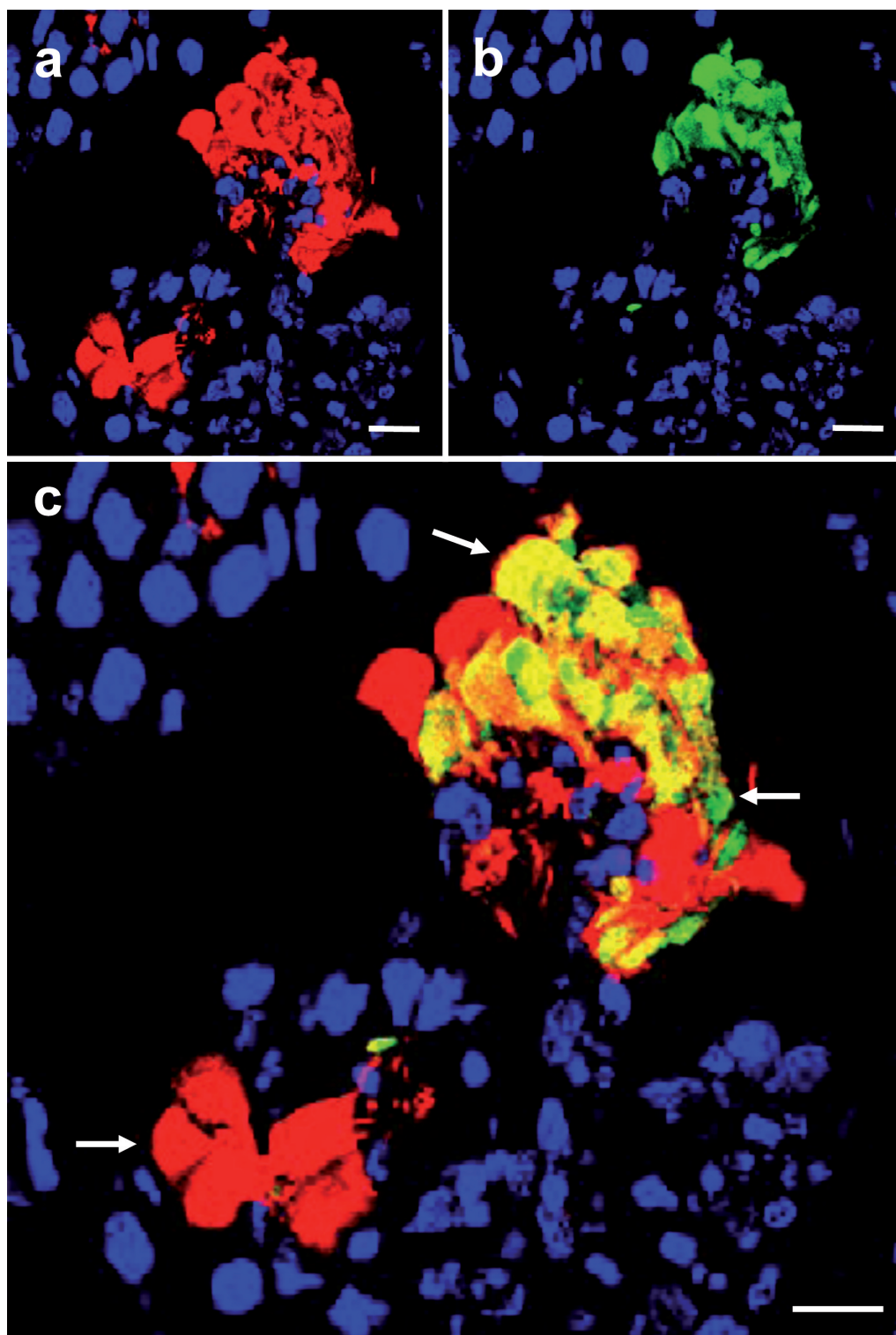
This report describes the presence of an extensive angiotensinergic innervation of the adult rat, pig and human kidney which, to the best of our knowledge, has



**Fig. 8.** Co-staining of rat kidney sections for Ang II (red) and renin (green). The vascular pole with patchy renin staining of juxtaglomerular cells is seen. Ang II-positive nerve fibers are running on their surface (a). b shows two closely adjacent Ang II-containing axons with fusiform varicosities next to renin immunoreactivity. Laser scanning microscopy; DNA staining in blue. Scale bars: 10  $\mu$ m.

not yet been recognized so far. We have already reported on the presence of Ang II-containing sympathetic nerve fibers in the adventitia of rat and human mesenteric arteries and provided evidence for an extensive

angiotensinergic innervation in the heart (Patil et al., 2008, 2011). Our present investigations extend these earlier findings and we now describe a similarly dense angiotensinergic innervation of the kidney with probably



**Fig. 9.** A pig renal microganglion co-stained for Ang II (red, **a**) and tyrosine hydroxylase (TH, green) is shown (**b**). The merged picture (panel **c**) shows co-localization in yellow. There were neurons exclusively positive for Ang II or TH and also neurons co-localizing both antigens (**a**) as indicated by arrows. The size of the neurons was between  $\sim 10$  and  $25 \mu\text{m}$ . Laser scanning microscopy; DNA staining in blue. Scale bars:  $25 \mu\text{m}$

a particular functional role. The high number of Ang II-containing axons present in nerve cross-sections and elsewhere in the kidney indicates its overall physiological importance. Our results suggest that Ang II is released from these fibers as a neuropeptide co-transmitter with noradrenaline (NA) and other peptides, such as NPY or CGRP, to modulate sympathetic neurotransmission or to influence target cell function via pre- and postjunctional angiotensin receptors (Bohlender and Imboden, 2012).

The kidney has been shown to contain a predominantly noradrenergic sympathetic innervation (Barajas et al., 1991; DiBona and Kopp, 1997). Our immunocytochemical stainings with antibodies directed against the catecholaminergic markers TH and DBH revealed an anatomical distribution and morphological appearance of these fibers in accordance with previous reports (Ferguson et al., 1988; Knight et al., 1989; Liu and Barajas, 1993a; Tiniakos et al., 2004). Attempts to localize Ang II at the cellular level in the kidney, however, have so far failed to provide evidence for an Ang II-containing renal innervations, despite the use of sensitive visualization techniques or electron microscopy. Such earlier investigations provided in part conflicting results, for instance concerning the presence of Ang II in the vasculature (Celio and Inagami, 1981; Brooks et al., 1982; Naruse et al., 1982; Taugner et al., 1983; Hunt et al., 1992). These studies were based on the use of polyclonal antisera against Ang II. It is an undisputed fact that the specificity and immunological characteristics of such antisera clearly determine the ability to detect intracellular Ang II reliably, as exemplified by our own polyclonal antiserum, which produced only modest results. We therefore relied on a monoclonal mouse antibody (4B3) with high specificity and sensitivity to detect Ang II at the cellular level, which finally provided superior immunohistological results when combined with confocal fluorescent light or laser scanning microscopy (Frei et al., 2001). Our results with the polyclonal anti-Ang II antibody served as a positive control and further confirmed the intrarenal presence of Ang II-containing nerve fibers.

Our co-staining of kidney sections for Ang II and the catecholaminergic markers TH and DBH revealed two distinct subtypes of noradrenergic postganglionic fibers, depending on the presence or absence of Ang II. Also a third type of fiber positive for Ang II but not for catecholaminergic markers could be identified. To further classify these fibers we performed co-staining experiments of Ang II with synaptophysin, a synaptic vesicular protein and non-specific marker of postganglionic efferent neurons. Synaptophysin is typically not expressed by sensory fibers supplying arteries or viscera of rodents (Luff et al., 2000; Morris et al., 2005). This alternative approach to identify efferent fibers gave the same results as with the catecholaminergic markers and showed two types of synaptophysin-positive fibers, with and without Ang II-staining, and one additional type exclusively positive for

Ang II. In these two different staining experiments the Ang II-containing fibers without a catecholaminergic phenotype and also without detectable synaptophysin expression were most likely identical and represented sensory-afferent fibers expressing Ang II. This conclusion was further supported by compatible findings obtained earlier by Luff et al. staining the nervous plexus of rat mesenteric arteries for synaptophysin, TH, CGRP and substance P (Luff et al., 2000).

We subsequently confirmed this interpretation using CGRP staining as a general marker of sensory kidney fibers (Liu and Barajas, 1993a) to visualize the entire afferent fiber population including those possibly negative for Ang II. The intrarenal distribution of CGRP-positive fibers seen in these experiments was consistent with previously published data (Ferguson and Bell, 1988; Knight et al., 1991; Liu and Barajas, 1993a). Co-staining for Ang II identified two different groups of CGRP-expressing sensory fibers, with and without colocalization of Ang II, similar to the efferent fibers. All CGRP-negative fibers staining positive for Ang II could be classified as postganglionic-efferent by exclusion. Together these experiments clearly showed that Ang II-containing fibers did not constitute a separate network of its own but could be classified as distinct phenotypic subgroups of sympathetic postganglionic or sensory fibers. This was in agreement with our previous observations that Ang II-containing neurons in celiac and dorsal root ganglia represent only subgroups of the ganglionic neuron populations, respectively (Patil et al., 2008, 2010). The absence of Ang II-expressing sensory fibers in periglomerular arterioles was consistent with other reports (Ferguson and Bell, 1988).

Postganglionic efferent and sensory-afferent fibers with an angiotensinergic phenotype were next found to have different morphological appearances and also anatomic distributions compared to their Ang II-negative counterparts. Postganglionic fibers with a combined catecholaminergic and angiotensinergic phenotype producing fusiform synapses were the prevalent if not exclusive fiber type in the afferent arterioles and around tubuli of the outer medulla. In contrast, Ang II-negative catecholaminergic fibers with small polymorphic beaded varicosities were abundant in the sympathetic plexus of proximal arteries and also in the interstitium of the cortex. Concerning sensory fibers, Ang II-negative axons were frequent in the pelvic region but appeared scarce in the renal cortex, where fibers co-expressing Ang II were more frequently encountered. A classification of renal nerve fibers according to our results is shown in Table 1. Luff et al. using electron microscopy further subdivided postganglionic fibers in rat periglomerular arterioles based on morphological criteria (Luff et al., 1991). Theoretically, a few nociceptive fibers may also be non-peptidergic and therefore could not be visualized in our study (Silverman and Kruger, 1988). Some other cell types in the kidney, such as vascular smooth muscle cells, tubular epithelia, and renin-containing JG cells are known to contain Ang



II and were accordingly also stained for Ang II at varying intensity during our experiments (Taugner et al., 1983; Hunt et al., 1992; Zhuo and Li, 2007). In fact, tubular epithelia are additional sites of intrarenal angiotensinogen and renin expression. Furthermore, there is also receptor-mediated cellular Ang II uptake in the kidney (Navar et al., 2011) and vascular smooth muscle cells may similarly synthesize Ang II (Kumar et al., 2008), which explains these observations.

Sympathetic neurons have been found to innervate intrarenal cell compartments sequentially. Nevertheless, there may also be target cell specialization among subgroups of neurons. Electrophysiological studies have shown that axons with larger diameters and high conduction velocity innervate the tubular compartment, while smaller axons with a lower conductivity innervate the arterial tree (DiBona, 2000). In line with these findings we found noradrenergic fibers with small diameters to innervate the proximal arterioles abundantly, while thicker fibers with an angiotensinergic co-phenotype were targeting tubules in the outer medulla. Our data thus provide evidence for an anatomical and functional sub-specialization of sympathetic neurons projecting to the kidney depending on their angiotensinergic phenotype.

Several arguments speak in favour of a role of neuronal Ang II as a co-transmitter or neuromodulator, including its presence in varicose axons, its colocalization with synaptophysin or CGRP and its independent intraneuronal production (Bohlender and Imboden, 2012). Our earlier studies suggested that neurons in the sympathetic and dorsal root ganglia may generate Ang II autonomously from angiotensinogen using a renin-free enzymatic pathway that possibly relies on cathepsin D as the relevant enzyme. Cellular uptake of Ang II from the interstitium, for instance, by receptor internalization is another possibility, but this appears less likely to represent the key mechanism in neurons (Navar

et al., 2002; Salio et al., 2006). Based on the known effects of humoral Ang II, neuronal Ang II acting on renal angiotensin type 1 receptors would be expected to synergistically potentiate NA-induced arterial vasoconstriction, inhibit NA-induced renin-secretion by juxtaglomerular cells and enhance NA-induced tubular sodium reabsorption (Navar et al., 2002; DiBona and Esler, 2010). In support of these assumptions, various electron microscopic studies have demonstrated the presence of synaptic junctions between sympathetic fibers and vascular smooth muscle cells, tubuli, and also renin-producing JG cells (Simpson and Devine, 1966; Barajas and Wang, 1979; Luff et al., 1991). However, most sympathetic axons release their neurotransmitters and neuropeptides at non-synaptic sites from where they reach their distant targets by interstitial diffusion across a concentration gradient (Luff et al., 2000; Burnstock, 2008). The same mechanism may also apply to neuronal Ang II-release at autonomic neuroeffector junctions in the kidney. Ang II of neuronal origin therefore is possibly in direct competition with humoral Ang II for pre- and postjunctional binding sites, whereas it does not depend on renal renin secretion. Neuronal Ang II thus may control similar physiological effects but with a different regulatory purpose.

NPY is the characteristic peptide co-transmitter of NA in renal postganglionic nerve fibers, while Ang II appears to be a co-transmitter only in a subset of these fibers. Apart from NPY, CGRP, SP and Ang II, a few other neuropeptides have been identified in the renal innervation albeit only in a small number of fibers (Reinecke and Forssmann, 1988). In rats, a few noradrenergic fibers may also contain vasoactive intestinal peptide instead of NPY (Knight et al., 1989). At the functional level, neuronal NPY co-transmission may potentiate the vasoconstrictor effects of NA via postjunctional  $Y_1$  receptors while conversely inhibiting synaptic NA release via prejunctional  $Y_2$  receptors (Oellerich and Malik, 1993; Eppel et al., 2005). NPY may furthermore inhibit renal renin secretion by JG cells or stimulate tubular sodium reabsorption showing similarity to Ang II (Bischof and Michel, 1998). In contrast, CGRP released from sensory-afferent fibers has a strong vasodilatory effect by stimulation of CGRP1 receptors on arterial vascular smooth muscle cells and, by this mechanism, it may also antagonize NPY- or endothelin-induced vasoconstriction (De Mey et al., 2008; Meens et al., 2009). Ang II and NPY were experimentally found to be inhibitors of prejunctional CGRP-release (Kawasaki et al., 1991, 1998). Such effects could be those mediated, for instance, by neuronal Ang II-release. Under pathological circumstances, an abnormally increased neuronal release of Ang II in the kidney may hypothetically also facilitate arterial hypertension by stimulation of tubular sodium reabsorption.

Very little information is available on neurons localized in renal microganglia or occasionally encountered in the pelvis, parenchyma or along central

**Table 1.** Classification of intrarenal autonomic nerve fibers according to our staining results for Ang II and other neuronal markers\*.

|                                       | Fiber type                                    |                                                  |           |                                        |
|---------------------------------------|-----------------------------------------------|--------------------------------------------------|-----------|----------------------------------------|
|                                       | postganglionic efferent A                     | postganglionic efferent B                        | sensory A | sensory B                              |
| <i>Staining</i>                       |                                               |                                                  |           |                                        |
| TH or DBH                             | +                                             | +                                                | -         | -                                      |
| Synaptophysin                         | +                                             | +                                                | -         | -                                      |
| Ang II                                | -                                             | +                                                | -         | +                                      |
| CGRP                                  | -                                             | -                                                | +         | +                                      |
| <i>Distribution</i>                   |                                               |                                                  |           |                                        |
| Localization/<br>main target<br>areas | central arteries,<br>cortical<br>interstitium | afferent<br>arteriole, outer<br>medulla (tubuli) | pelvis    | pelvis and<br>cortical<br>interstitium |

\* tyrosine hydroxylase (TH), dopamine beta-hydroxylase (DBH), synaptophysin, angiotensin II (Ang II) and calcitonin gene-related peptide (CGRP).

arteries in the kidney (Schwalew, 1964; Liu and Barajas, 1993b; Shannon et al., 1998). Such neurons may display a catecholaminergic phenotype or express nitric oxide synthase (Liu and Barajas, 1993b; Liu et al., 1995). According to our own results, microganglionic neurons are frequently Ang II positive. They may display an angiotensinergic, a noradrenergic or a combined phenotype with presumably the same afferent and efferent distinctions as discussed before. Analogous to the intramural ganglionated plexus in the heart, neurons in the renal microganglia may constitute local oligosynaptic reflexes with only indirect regulatory input from the extrinsic renal innervation (Armour, 2010). The projections and functions of these reflexes in the kidney nevertheless remain obscure. The number of these independent neurons in the kidney was relatively small compared to the number of fibers in the renal nerves and also to the number of such independent neurons in the heart.

In conclusion, we here document an important angiotensinergic innervation of the kidney which comprises distinct angiotensinergic subpopulations of noradrenergic postganglionic and sensory-afferent fibers. Ang II-containing neurons were also found in renal microganglia. The findings suggest that neuronal Ang II is an independent source of Ang II in the kidney where it is probably released as a neuropeptide to modulate sympathetic neurotransmission and renal target cell function. Our results will serve as a base for future functional studies and will help generate new hypotheses on the neuronal pathogenesis of renal diseases, including arterial hypertension.

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*Conflict of interest:* None

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