Complex reinnervation pattern after unilateral renal denervation in rats

Kristina Rodionova,1 Christian Fiedler,1 Franziska Guenther,3 Eric Grouzmann,5 Winfried Neuhuber,2 Michael J. M. Fischer,3 Christian Ott,1 Peter Linz,1 Wolfgang Freisinger,1 Sonja Heinlein,1 Stephanie T. Schmidt,1 Roland E. Schmieder,1 Kerstin Amann,4 Karie Scrogin,6 Roland Veelken,1 and Tilmann Ditting1

1Department of Internal Medicine 4, Nephrology and Hypertension, Friedrich-Alexander University Erlangen-Nuremberg, Erlangen, Germany; 2Department of Anatomy I, Friedrich-Alexander University Erlangen-Nuremberg, Erlangen, Germany; 3Department of Physiology 1, Friedrich-Alexander University Erlangen-Nuremberg, Erlangen, Germany; 4Department of Pathology, Friedrich-Alexander University Erlangen-Nuremberg, Erlangen, Germany; 5Service de Biomédecine, Laboratoire des Catéchoalamines et Peptides, Centre Hospitalier Universitaire Vaudois CHUV, Lausanne, Switzerland; and 6Department of Pharmacology and Experimental Therapeutics, Loyola University Chicago, Stritch School of Medicine, Maywood, Illinois

Submitted 2 June 2014; accepted in final form 10 February 2016

An increasing number of studies have reported the beneficial effects of interventional renal nerve ablation in patients with treatment-resistant hypertension (18, 30, 32, 44a). Though early results were promising, more recent findings have left the benefit of renal nerve ablation in some doubt (5). The rationale for renal denervation as a therapeutic intervention for treatment (40) has evolved from a broad base of experimental (9) and clinical studies (43), originating from the last century. The most successful clinical denervation studies (18, 30, 32, 44a) recorded blood pressure and other clinical outcomes but did not focus on pathophysiological mechanisms. The degree of denervation or reinnervation was not addressed or considered, given the dramatic blood pressure-lowering effects of the procedure. However, all of these studies lacked truly blinded observations or sham surgical controls (5).

Outcomes demonstrating successful denervation, such as catecholamine spillover, have only been provided in a few studies. However, it was reported that renal catecholamine spillover was not completely abolished after renal nerve ablation in humans, indicating incomplete denervation (39). Despite only reduced renal catecholamine spillover, hypertension was successfully reduced. Thus, it seems that a complete and long-lasting reduction of renal nerve innervation is not necessary for the blood pressure lowering effects of renal denervation. Moreover, there is some debate about the role of sympathetic- vs. peptidergic afferent nerves, and whether afferents provide sympatho-inhibitory or sympatho-excitatory responses (3, 8, 21).

An unresolved question is to what degree autonomic and sensory nerves regrow along the renal vessels following denervation (9). Reinnervation has previously been described in transplanted kidneys in humans and rodents (22, 23). Recently, a systematic morphological study in rats demonstrated that sympathetic efferent nerves reappear in the renal pelvis and parenchyma within 3 mo of unilateral denervation. Peptidergic afferent nerve fibers reappeared in the renal pelvis, but were not described in the renal parenchyma (34).

We recently provided functional evidence that intrarenal afferent nerves exert tonic inhibition on contralateral sympathetic nerve activity, most likely via a substance P-dependent pathway. Renal pelvic and intrarenal afferent compartments could be functionally distinguished by pharmacologic blockade of pelvic transient receptor potential vanilloid, type 1 (TRPV1) receptors. However, afferent-mediated sympathoinhibition was conserved following blockade of renal pelvic nerves (10). Thus, there is evidence for a functionally relevant peptidergic innervation of the renal pelvis (19), as well as the renal parenchyma (10).

We hypothesized that intrarenal afferent and efferent perivascular nerves regrow after renal denervation. Here, we used the method of Mulder et al. (34) to evaluate the impact of unilateral renal denervation on both the denervated- and con-
trilateral nondenervated kidney with a special focus on intrarenal perivascular nerve fibers.

Since we were interested in the reinnervation potential rather than long-lasting functional denervation effects, we did not combine the surgical denervation with topically applied phenol as it has been done in prior studies to ensure complete denervation (26, 34). Moreover, renal nerves are located nearly exclusively in the adventitial and perivascular space (36, 42), where they are readily accessible for subtotal renal denervation by a mere surgical procedure around the renal artery. Thus, we attempted to better model the reinnervation potential that develops after subtotal clinical denervation procedures (39, 40) by foregoing the use of additional neurotoxin. However, we also assessed the impact of additional phenol application on innervation.

In this study, immunohistochemical analysis of renal innervation was made 1, 4, and 12 wk after unilateral surgical denervation in male Sprague-Dawley rats. Renal pelvic fibers were not investigated in the present study. Additional experiments were done to determine the effect of denervation on renal tissue content of norepinephrine and the afferent neuronal marker, calcitonin gene-related peptide (CGRP).

MATERIALS AND METHODS

Animals. Male Sprague-Dawley rats (Charles River, Kisslegg, Germany) weighing 170–200 g were maintained in cages at 24 ± 2°C and fed a standard rat diet (no. C-1000; Altromin, Lage, Germany) containing 0.2% sodium by weight and were allowed free access to tap water. All procedures were performed on animals in accordance with the National Institutes of Health (NIH) “Guide for the Care and Use of Laboratory Animals” and were approved by the local government agency (Regierung von Mittelfranken, Ansbach, Germany).

Unilateral surgical renal denervation. Twelve rats were assigned to three timed groups (n = 4 each), which underwent the denervation procedure 12, 4, and 1 wk before immunohistological examination of the kidneys. This reverse time schedule was chosen to avoid time- and storage-derived sample errors. Tissue could be processed in a short period of time and in randomized order.

Unilateral surgical denervation of the left renal artery was performed as follows, while the right kidney was left intact: Rats were anesthetized with a mixture of O₂, 50% N₂O, and ~1.5% isoflurane. A left flank incision was made, and the renal denervation was performed by surgically stripping the renal arteries and veins of adventitia and cutting all renal nerve bundles visible under a dissection microscope (×25 to ×40). This procedure not only removed sympathetic but also sensory fibers that course in the same nerve bundles as the sympathetic axons. In this set of experiments, we did not coat the vessels with the neurotoxin phenol, as described by others (26), because we were attempting to model the subtotal denervation that is normally achieved in clinical procedures, rather than complete renal denervation. However, we were very careful to meticulously remove the adventitial tissue from the renal vessels, since the bulk of renal nerve fibers travel through the adventitia of the renal artery and the perivascular tissue to the renal hilus (28, 36, 37, 42). The wound was closed in layers, and following surgery, the animals were allowed to recover in the vivarium, where they were kept under observation over several hours. They were then given buprenorphine (0.05 mg/kg sc), and the kidneys were perfused via the abdominal aorta. Briefly, the abdominal cavity was opened, and the abdominal aorta was cannulated. The inferior caval vein was cut, after which heparinized saline (150 ml NaCl 0.9% + heparin 1000 IE) was infused into the aorta at a rate of 25 ml/min. Saline perfusion was followed by 350 ml of fixation solution [1% paraformaldehyde + 0.8% picric acid (pH 7.2–7.4)] at the same rate. Both kidneys were removed and rinsed in phosphate buffer for 24 h, and then transferred to a 15% sucrose-phosphate buffer before they were frozen and stored at −20°C in Tissue Tek OCT Compound (4583; Sakura-Finetech, Germany).

Immunohistochemistry. Sympathetic and sensory-peptidergic structures, as well as vascular structures, were labeled in the renal tissue with antibodies targeting tyrosine hydroxylase (TH; sheep anti-TH, 1:2,000, NB 300-110; Novus Biologicals, Cambridge, UK), CGRP (rabbit anti-CGRP, 1:1,000, T-4032; Peninsula Laboratories, San Carlos, CA), and smooth muscle actin (SMA; mouse anti-SMA, 1:2,500, A2547; Sigma, St. Louis, MO) (4, 47). Briefly, perfusion-fixed kidneys were cut with a cryostat (50 µm) and incubated with primary antibodies diluted in TBS (0.05 M) with 1% BSA, 0.5% Triton X 100 for 24 h followed by incubation with appropriate fluorochrome-tagged secondary antibodies for 1 h (Alexa Fluor 488 donkey anti-sheep IgG, 1:1,000, A-11015; Alexa Fluor 555 donkey anti-rabbit IgG, 1:1,500, A-31572; Alexa Fluor 647 donkey anti-mouse IgG, A-31571; Invitrogen, Carlsbad, CA). Staining of TH and SMA was performed simultaneously, while CGRP staining was performed subsequently in the same samples to optimize triple-labeling. Negative control tissue was similarly treated except that primary antibodies were omitted from the first incubation step. The specificity (Fig. 1) of the primary antibodies and the lack of interaction was established previously by preadsorption and preincubation with the respective antigens in separate experiments.

Sections were examined with a Nikon Eclipse E1000M microscope, equipped with a confocal system (Nikon Digital Eclipse C1). A 488-nm argon laser, a 543-nm helium-neon laser (Melles Griot, Carlsbad, CA), and a 638-nm diode laser (Coherent, Santa Clara, CA) were used for excitation of Alexa Fluor 488, 555, and 647, respectively, resulting in green (TH), red (CGRP), and blue (SMA) fluorescence of the labeled structures. Unless otherwise stated, for laser scanning of images, a Nikon 20 × 0.75 microscope lens was used, resolution was set to 1.024 × 1.024 pixels, resulting in a sample area of 635 × 635 µm. Z-axis steps were set to 0.5 µm.

Since afferent and efferent nerve fibers are only sparsely and randomly dispersed in the renal interstitium, we focused on the perivascular innervation, where renal nerve fibers are found in larger bundles. Representative images were taken from the entire cross section, but emphasis was placed on the cortical and cortico-medullary zone. Images were taken from areas with best labeling and the least number of artifacts. Crystalline condensation and formation of wrinkles were the most common problems. We focused on small, representative, and homogenous areas of interest within the sections instead of whole cross sections. The area of interest was chosen visually and subjectively by a skilled examiner since automated randomizing algorithms could potentially provide inaccurate data.

The anatomical classification of each vessel in the area of interest was based on anatomical map within whole kidney cross sections and by the diameter of the vessel. For the illustrative figures, single- and double-channel or merged three-channel confocal images were adjusted for contrast and brightness using FIJI Image J (38) and Photoshop CS5.

To more easily correlate vessel size and type, e.g., interlobar, arcuate, and interlobular arteries, RECA-1 antibodies (MCA970R mouse anti-rat; AbD Serotec, Germany) were used to label endothelial cells (12, 45) in separate adjacent sections. Panorama stacks of multiple images (×4 optical magnification) were generated with a KEYENCE BZ-9000 digital microscope (Keyence, Neu-Isenburg, Germany).
Image analysis. In a preliminary qualitative approach, an estimation of nerve density was performed visually in 183 image stacks by four investigators blinded to the denervation status and time point. TH and CGRP labeling was scored in each stack (0 = none; 1 = weak; 2 = strong; and 3 = very strong). Labeling was assessed from single-channel images (see Fig. 2).

Fiji Image J 1.48e was used for morphometric analysis (38). Z-projections of maximum pixel intensity were made, which yielded a two-dimensional image (1,024 × 1,024 pixel, i.e., 635 × 635 μm), in which each XY-pixel coordinate was represented by that pixel of all Z-steps that had the highest intensity. This image was converted into a composite file of the three color channels, which allowed for splitting of the three color channels for thresholding and to remove artifacts if necessary. To approach optical intensity, a brightness threshold was applied on the basis of exposure values, and the resulting histograms for each color channel, which yielded a binary information (i.e., positive or negative). Artifacts were removed using the region of interest (ROI) function. The resulting positive pixels were counted for each color channel (green = TH; red = CGRP; blue = SMA). Positive TH and CGRP labels were normalized to vascular areas (SMA). Label density per ROI was determined by normalizing the TH or CGRP label to the area of the image or the vascular area (TH/SMA, CGRP/SMA). Nondimensional ratios are a reasonable approach, although the affinity-related staining results might vary between the respective primary and secondary antibodies.

Determination of tissue CGRP content. Twenty-four rats were subjected to the same unilateral surgical denervation procedure as described above. Half of the rats were treated with topical application of phenol during denervation, and the other half were denervated without phenol. After 12, 4, and 1 wk, rats were anesthetized (methohexitol, 40 mg/kg ip, + buprenorphine 0.05 mg/kg sc), and the kidneys were removed and divided in half and weighed. For determination of tissue CGRP content, tissue was placed in 1 ml of acetic acid (2 M) at 95°C for 10 min, homogenized (Ultra-Turrax, IKA, Staufen, Germany) and further incubated for 10 min at 95°C in a preheated water bath. The suspension was centrifuged for 30 min at 10,000 g, and 100 μl of the supernatant was mixed with 35 μl of five-fold concentrated enzyme immuno-assay buffer. The pH was adjusted to

Fig. 1. Vessel wall sector (Arteria renalis; 100 × 160 μm, 100 × oil immersion) with perivascular innervation. A: tyrosine hydroxylase (TH)-positive nerve fibers. B: CGRP-positive nerve fibers. C: immediate vicinity of sympathetic (TH-positive) and afferent (CGRP-positive) nerve fibers yields yellowish or light green color. No nerve fibers are visible in the vessel wall. Scale bar = 40 μm.

Fig. 2. Representative image stack (renal cortex 635 μm × 635 μm) from a right nondenerervated kidney (the corresponding triple-channel image is shown in Fig. 5A). A: “green channel” stained for TH denotes efferent sympathetic. B: “red channel” stained for CGRP denotes peptidergic afferent. CGRP staining was always much weaker than TH staining (score = 3, both channels). Scale bar = 200 μm.
7.0–7.4 with 46 μl sodium hydroxide (3 M), and CGRP was detected by ELISA (SPI bio, Bertin Pharma, Montigny le Bretonneux, France; detection limit of 5 pg/ml) (1, 20). To verify specificity of the ELISA, CGRP levels were compared in kidneys from three eCGRP knockout mice and three C57BL/6 littermates. CGRP values were 18.2 ± 0.7 ng/g and 2.5 ± 0.3 ng/g for a wild-type and knockout mouse, respectively. Nonspecific binding was determined as the percent difference from wild-type (i.e., 14%). All values were corrected by 14% and expressed as nanograms per gram wet weight.

Determination of tissue noradrenaline content. Tissue was homogenized using an Ultra-Turrax and sonicated with a microtipped sonifier (Sonopuls HD70, Bandelin Electronic, Berlin, Germany) for 10 s, twice in 1 M HClO4 (2 ml for 500 mg tissue), then centrifuged at 14,000 g (4°C). The catecholamines were then extracted from the supernatant using aluminum oxide adsorption. Norepinephrine (NE), epinephrine, and dopamine were separated and quantified using ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) (13). Catecholamine content was expressed as picomoles per milligram wet weight.

Affenter neuronal tracing. Unilateral tracing of affenter renal nerves was performed in four rats, as previously described (11). Briefly, rats were anesthetized as described above for the renal denervation procedure, and the dicarbocyanine dye DiI (A9-DiI, 50 mg/ml in DMSO; Molecular Probes, Eugene, OR) was applied to the subcapsular space of either the left or right kidney. Rats were allowed to recover for 1 wk to permit retrograde transport of the tracer to cell bodies in dorsal root ganglia T11-L2. The ganglia were excised bilaterally and focus-stacked with a fluorescent microscope (Keyence BZ-9000). Immediately thereafter, the right and left DRGs were dissociated with collagenase (1 mg/ml), trypsin (1 mg/ml), and DNase (0.1 mg/ml). The cells were resuspended in modified L-15 medium that contained 5% rat serum, 2% chick embryo extract, as well as necessary inorganic salts, amino acids, and vitamins for 1 h at 37°C (Sigma-Aldrich, Munich, Germany). Enzymatic activity was terminated by the addition of soybean trypsin inhibitor (2 mg/ml), BSA (1 mg/ml), and CaCl2 (3 mmol/l). The ganglia were triturated using sterile siliconized Pasteur pipettes to dissociate individual cells. After centrifugation, the cells were resuspended in the modified L-15 medium and plated on poly-L-lysine-coated glass. Fluorescently labeled DRG cells were counted in all samples.

Statistics. All image data were tested for normality using the Kolmogorov-Smirnov-test. Nonparametric tests were used when data failed normality testing. Analyses of observation groups (R1, R4, L1, K1) were done using the Holm-Sidak method. Tissue transmitter content data were analyzed by two-way ANOVA. All pairwise comparisons were performed using the Holm-Sidak method. Tissue transmitter content data showed a normal distribution and were analyzed in the same manner. Statistical significance was defined as P < 0.05 (two-tailed). Results are presented either as means ± SE (bar plots with error bars) or as medians (first to third quartile) (box-whisker plots), according to their distribution. For the sake of clarity, medians in box-whisker plots are shown as vertical bars. The box boundaries denote the first and third quartiles, whiskers indicate the 90th and 10th percentiles, small crosses indicate outliers. "R" denotes the right nondenervated kidney, "L" denotes the left denervated kidney. The numerals 1, 4, and 12 indicate the weeks after the denervation procedure when the tissue was harvested from the respective groups of rats.

RESULTS

All rats recovered from the renal denervation procedure and gained weight during the observation period (Table 1). Kidney weights also increased over time. No statistical difference could be detected between the groups of rats that were used for the different experiments, i.e., immunohistology or transmitter content measurements with or without phenol.

A total of 183 image stacks containing 702 vessel cross sections were included in the analyses. Each stack contained two to seven vessel cross sections ranging from 8 to 665 μm in diameter. The frequency distribution of vessel diameters did not differ significantly between the observation groups (Fig. 3, Table 2). Furthermore, the vessel area indicated by the area of SMA label was similar between groups (data not shown).

In nine RECA-1-labeled kidney cross sections randomly selected from all three time points, 193 circumferential vessel cross sections were measured. Interlobar artery diameters ranged from 298 to 816 μm [median 470 μm (425–661); mean 526 μm; n = 22]. Arcuate artery diameters ranged from 102 to 304 μm [median 165 μm (148–192); mean 176 μm; n = 52], and interlobular artery diameters ranged from 40 to 162 μm [median 85 μm (72–103), mean 88 μm, n = 119]. Brackets denote first to third quartile. As shown in Fig. 3, ~95% of the vessels had a diameter of less than 300 μm, 90% less than 160 μm, and 64% less than 40 μm. Thus, most of the observed vessels were likely downstream of interlobular and arcuate arteries, and only 5% were large vessels in the range of interlobular arteries.

Visual analysis. Qualitative visual analysis demonstrated decreased TH- and CGRP-positive labeling 1 wk after denervation [L1_THscore 1.33 (1.00–2.00) vs. R12_THscore 2.33 (2.00–3.00), P < 0.05; L1_CGRPscore 0.67 (0.00–1.33) vs. R12_CGRPscore 2.33 (1.08–2.67), P < 0.05], which appeared to completely recover by week 12 [L12_THscore 2.33 (1.67–3.00) vs. R12_THscore 2.67 (2.33–3.00), P = ns; L12_CGRPscore 2.00 (1.33–2.67) vs. R12_CGRPscore 2.33 (1.67–2.67), P = ns]. Representative micrographs are shown in Figs. 4 and 5.

Morphometric analysis (TH). Results of the morphometric analysis of TH+ staining in pooled observations are shown in Fig. 6. Decreased TH+ pixel counts were found on the denervated side, 1 wk after surgery. TH+ label on the denervated side had recovered to R1_TH levels by 4 wk and remained there at 12 wk postsurgery. However, at 12 wk, TH+ pixel counts on the denervated side remained lower than the nondenervated side.

Table 1. Mean body and kidney weights of all rats in the study

<table>
<thead>
<tr>
<th>Group</th>
<th>Week 1</th>
<th>Week 4</th>
<th>Week 12</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight</td>
<td>267 ± 7</td>
<td>375 ± 6</td>
<td>528 ± 21</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>Kidney weight</td>
<td>1.17 ± 0.12</td>
<td>1.32 ± 0.08</td>
<td>1.40 ± 0.06</td>
<td>&lt; 0.05</td>
</tr>
</tbody>
</table>

Values are expressed as means ± SE. Body weight, as well as kidney weight, increased significantly between each observation time point.
The analyses based on averaged observations similarly showed reduced TH\(^+\) pixel counts (given as absolute pixel count/sample area, i.e., 1024\(^2\) pixels) 1 wk after surgery in denervated kidneys compared with nondenervated kidneys (R1\_TH: 2,886 \pm 482 vs. L1\_TH: 374 \pm 109; \textit{P} = 0.019; two-way ANOVA). By week 4 and week 12, the TH\(^+\) pixel counts in denervated kidneys reached the R1\_TH level (R1\_TH vs. L4\_TH: 3,093 \pm 498 and L12\_TH: 4,437 \pm 1,613; \textit{P} = 0.562; two-way ANOVA). The nondenervated kidneys showed increased TH\(^+\) pixel counts 4 and 12 wk after denervation (R1\_TH vs. R4\_TH: 7,866 \pm 1,383 and R12\_TH: 6,725 \pm 1,693; \textit{P} < 0.027; two-way ANOVA, one-way ANOVA). However, the denervated TH\(^+\) pixel counts at week 12 tended to be lower than the nondenervated side, but no statistical difference between right and left kidneys was observed in week 12 (R12\_TH vs. L12\_TH; \textit{P} = 0.175; two-way ANOVA).

Morphometric analysis (CGRP). The analysis of CGRP\(^+\) pixel counts based on pooled observations is shown in Fig. 7. Briefly, a significant decrease in CGRP\(^+\) label was observed on the denervated side, which recovered to the R1\_CGRP levels within 4 wk. Furthermore, an increase on the nondenervated side was also observed. However, no statistical difference was detected between the nondenervated and the denervated side in week 12.

The CGRP\(^+\) staining based on averaged observations in week 1 similarly showed decreased CGRP\(^+\) pixel counts in the denervated kidneys compared with the nondenervated kidneys (R1\_CGRP: 766 \pm 149 vs. L1\_CGRP: 43 \pm 12; \textit{P} = 0.024; two-way ANOVA). By weeks 4 and 12, the CGRP\(^+\) pixel counts in the denervated kidneys reached and tended to surpass the R1\_CGRP level (R1\_CGRP vs. L4\_CGRP: 718 \pm 137 and L12\_CGRP: 1,638 \pm 520; \textit{P} = 0.271; two-way ANOVA, one-way ANOVA). The nondenervated kidneys showed increased CGRP\(^+\) pixel counts in weeks 4 and 12 (R1\_CGRP vs. R4\_CGRP: 2,174 \pm 437 and R12\_CGRP: 1,874 \pm 417; \textit{P} < 0.027; two-way ANOVA, one-way ANOVA). No differences in CGRP\(^+\) pixel counts were observed between right and left kidneys in week 12 (R12\_CGRP vs. L12\_CGRP; \textit{P} = 0.627; two-way ANOVA).

**Table 2. Observation groups of the study**

<table>
<thead>
<tr>
<th>Weeks After Surgery</th>
<th>Right Kidneys (Nondenervated)</th>
<th>Left Kidneys (Denervated)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Group</td>
<td>Image Stacks (n)</td>
</tr>
<tr>
<td>1</td>
<td>R1</td>
<td>31</td>
</tr>
<tr>
<td>4</td>
<td>R4</td>
<td>23</td>
</tr>
<tr>
<td>12</td>
<td>R12</td>
<td>26</td>
</tr>
</tbody>
</table>

The number of image stacks taken from each group of kidneys and the cumulative number of vessel cross sections contained in these image stacks are given.

The analyses based on averaged observations similarly showed reduced TH\(^+\) pixel counts (given as absolute pixel count/sample area, i.e., 1024\(^2\) pixels) 1 wk after surgery in denervated kidneys compared with nondenervated kidneys (R1\_TH: 2,886 \pm 482 vs. L1\_TH: 374 \pm 109; \textit{P} = 0.019; two-way ANOVA). By week 4 and week 12, the TH\(^+\) pixel counts in denervated kidneys reached the R1\_TH level (R1\_TH vs. L4\_TH: 3,093 \pm 498 and L12\_TH: 4,437 \pm 1,613; \textit{P} = 0.562; two-way ANOVA). The nondenervated kidneys showed increased TH\(^+\) pixel counts 4 and 12 wk after denervation (R1\_TH vs. R4\_TH: 7,866 \pm 1,383 and R12\_TH: 6,725 \pm 1,693; \textit{P} < 0.027; two-way ANOVA, one-way ANOVA). However, the denervated TH\(^+\) pixel counts at week 12 tended to be lower than the nondenervated side, but no statistical difference between right and left kidneys was observed in week 12 (R12\_TH vs. L12\_TH; \textit{P} = 0.175; two-way ANOVA).

Morphometric analysis (CGRP). The analysis of CGRP\(^+\) pixel counts based on pooled observations is shown in Fig. 7. Briefly, a significant decrease in CGRP\(^+\) label was observed on the denervated side, which recovered to the R1\_CGRP levels within 4 wk. Furthermore, an increase on the nondenervated side was also observed. However, no statistical difference was detected between the nondenervated and the denervated side in week 12.

The CGRP\(^+\) staining based on averaged observations in week 1 similarly showed decreased CGRP\(^+\) pixel counts in the denervated kidneys compared with the nondenervated kidneys (R1\_CGRP: 766 \pm 149 vs. L1\_CGRP: 43 \pm 12; \textit{P} = 0.024; two-way ANOVA). By weeks 4 and 12, the CGRP\(^+\) pixel counts in the denervated kidneys reached and tended to surpass the R1\_CGRP level (R1\_CGRP vs. L4\_CGRP: 718 \pm 137 and L12\_CGRP: 1,638 \pm 520; \textit{P} = 0.271; two-way ANOVA, one-way ANOVA). The nondenervated kidneys showed increased CGRP\(^+\) pixel counts in weeks 4 and 12 (R1\_CGRP vs. R4\_CGRP: 2,174 \pm 437 and R12\_CGRP: 1,874 \pm 417; \textit{P} < 0.027; two-way ANOVA, one-way ANOVA). No differences in CGRP\(^+\) pixel counts were observed between right and left kidneys in week 12 (R12\_CGRP vs. L12\_CGRP; \textit{P} = 0.627; two-way ANOVA).

**Table 2. Observation groups of the study**

<table>
<thead>
<tr>
<th>Weeks After Surgery</th>
<th>Right Kidneys (Nondenervated)</th>
<th>Left Kidneys (Denervated)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Group</td>
<td>Image Stacks (n)</td>
</tr>
<tr>
<td>1</td>
<td>R1</td>
<td>31</td>
</tr>
<tr>
<td>4</td>
<td>R4</td>
<td>23</td>
</tr>
<tr>
<td>12</td>
<td>R12</td>
<td>26</td>
</tr>
</tbody>
</table>

The number of image stacks taken from each group of kidneys and the cumulative number of vessel cross sections contained in these image stacks are given.
Morphometric analysis (TH/SMA). For pooled observations, we found decreased TH/SMA on the denervated side 1 wk after denervation [R1_TH/SMA: 0.071 (0.037–0.141) vs. L1_TH/SMA: 0.007 (0.002–0.014); P < 0.0001; 2W-ANOVA-aRT], which recovered to R1_TH/SMA levels by week 4 and remained there at 12 wk [L4_TH/SMA: 0.052 (0.042–0.083); L12_TH/SMA: 0.106 (0.079–0.153); P = 0.253; 2W-ANOVA-aRT, 1W-ANOVA-oR]. The TH/SMA on the denervated side at weeks 4 and 12 (L4_TH/SMA and L12_TH/SMA) were lower than that on the nondenervated side [R4_TH/SMA: 0.147 (0.100–0.246); R12_TH/SMA: 0.159 (0.094–0.206); P = 0.024; 2W-ANOVA-aRT]. An increase in TH/SMA on the nondenervated side was observed (R1_CGRP vs. R4_CGRP and R12_CGRP; P = 0.007; 1W-ANOVA-oR).

The averaged observations analysis yielded similar results, which are displayed in Fig. 8. Briefly, a significant decrease in TH/SMA was found 1 wk after denervation, which recovered to R1_TH/SMA levels within 4 to 12 wk. At 12 wk, the TH/SMA ratio on the denervated side tended to be lower than on the nondenervated side.

Morphometric analysis (CGRP/SMA). On the basis of pooled observations, a decrease in the CGRP/SMA ratio was found 1 wk after denervation [R1_CGRP/SMA: 0.019 (0.007–0.051) vs. L1_CGRP/SMA: 0.00062 (0.00006–0.00187); P < 0.0001; 2W-ANOVA-aRT], which recovered to the R1_CGRP/SMA level by week 4 [L4_CGRP/SMA: 0.0140 (0.0082–0.262); P = 0.411; 2W-ANOVA-aRT] and even surpassed the R1_CGRP/SMA level by week 12 [L12_CGRP/SMA: 0.039 (0.028–0.055); P = 0.024; 2W-ANOVA-aRT, 1W-ANOVA-oR]. The CGRP/SMA on the denervated side in week 4 (L4_CGRP/SMA) was lower than on the nondenervated side [R4_CGRP/SMA: 0.045 (0.029–0.054); P < 0.0001; 2W-ANOVA-aRT] but was no longer different from the nondenervated side by week 12 [R12_CGRP/SMA: 0.0455 (0.0305–0.0670); P = 0.255; 2W-ANOVA-aRT]. There was an increase in CGRP/SMA on the nondenervated side (R1_CGRP vs. R4_CGRP and R12_CGRP; P = 0.007; 1W-ANOVA-oR).

The analysis based on averaged observations for the CGRP/SMA yielded similar results (Fig. 9). One week after denervation, a significant decrease of CGRP/SMA was found, which recovered to the R1_CGRP/SMA level by week 4 and surpassed the R1_CGRP/SMA level by week 12. The CGRP/SMA on the denervated side in week 4 (L4_CGRP/SMA) was lower than on the nondenervated side in week 4, but no longer different from the nondenervated side in week 12. Furthermore, there was an increase of CGRP/SMA on the nondenervated side at weeks 4 and 12.

Morphometric analysis (CGRP/TH). On the basis of pooled observations, the CGRP/TH of the denervated kidneys was shifted toward the CGRP-positive label [L1_CGRP/TH: 0.147 (0.0527–0.440) vs. L4_CGRP/TH: 0.219 (0.103–0.542) vs. L12_CGRP/TH: 0.402 (0.280–0.528); P = 0.019; 1W-ANOVA-oR]. No such shift was observed on the nondenervated side [R1_CGRP/TH: 0.276 (0.0856–0.462) vs. R4_CGRP/TH: 0.309 (0.171–0.392) vs. R12_CGRP/TH: 0.318 (0.256–0.463); P = 0.194; 1W-ANOVA-oR]. On the basis of averaged observations, the results were similar (Fig. 10).
Tissue transmitter content. Tissue analysis revealed a decrease of CGRP content by \(-72\%\) 1 wk after surgery in denervated kidneys compared with nondenervated kidneys. By week 12, the CGRP content in denervated kidneys was no longer significantly different from innervated kidneys.

A decrease in NE of \(-78\%\) was found in denervated kidneys 1 wk after surgery. Norepinephrine values in the denervated kidneys remained lower throughout the 12-wk study. No significant levels of epinephrine or dopamine were detected. The addition of phenol to the denervation procedure did not significantly influence tissue levels of CGRP or NE (Fig. 11 and 12).

Afferent neuronal tracing. No evidence of contralateral labeling by DiI was identified in either whole ganglia or in cultured DRG cells. In accordance with previous studies (11), cell cultures from the ipsilateral side showed that 14 \(\pm\) 1% of cells were labeled by DiI.

DISCUSSION

Here, we investigated the reinnervation of afferent peptidergic and efferent sympathetic nerves in a rat model of unilateral surgical renal denervation. On the basis of a purely morphological analysis, we found strong evidence that the denervation procedure in male Sprague-Dawley rats decreased both efferent and the afferent innervation of small renal vessels within 1 wk of surgery, indicating subtotal denervation, and that both regrew within 4 wk of denervation. Furthermore, by 12 wk after denervation, we found evidence that TH immunoreactivity surrounding small vessels of the intact kidney was greater than that observed 1 wk after surgery, suggesting that the area of the vessel covered by neural tissue increased in the intact kidney as a consequence of denervation of the contralateral kidney. A similar outcome was found for CGRP immunoreactivity, suggesting that both sympathetic and sensory afferents are influenced by denervation in a parallel manner. Furthermore, within 12 wk, CGRP immunoreactivity of the denervated kidney surpassed the basal level of the intact kidney, indicating overshooting afferent innervation.

Interestingly, data comparing TH and CGRP area suggest that CGRP regrowth outpaced that of the sympathetic nerves. This latter interpretation was confirmed by assessments of tissue neurotransmitter content, which showed deficits in NE throughout the postsurgical assessment period, while CGRP content had recovered by 12 wk postdenervation.

The immunostaining approach used here for determination of nerve regrowth is limited in that it does not provide information about the structure or function of new nerve fibers. It is not possible, for instance, to determine whether the increase in immunoreactivity is due to a greater density of axons or dendrites, a higher degree of branching of the regrown axons, or an increased number of varicosities containing the respective labeled antigens. However, constrictive nerve injury has been shown to increase the density of peptidergic and sympathetic fibers, possibly through migration and branching of sprouting nerve fibers (49). Our evidence that NE and CGRP content in the intact kidney did not increase over time seemingly contradicts our histological evidence of hyperinnervation of the intact kidney by both the sensory afferent and sympathetic efferent systems and the overshooting reinnervation of

![Fig. 5. Representative images of small peripheral renal vessels labeled for TH (green), CGRP (red), and SMA immunoreactivity (blue). A: right, nondenervated, week 1 (R1). B: left, denervated, week 1 (L1). C: right, nondenervated, week 12 (R12). D: left, denervated, week 12 (L12). Hardly any nerves are visible in L1. L12 looks very similar to R1 and R12. Scale bar = 200 μm.](http://ajpregu.physiology.org/)

R812 AFFERENT RENAL REINNERVATION

AJP-Regul Integr Comp Physiol • doi:10.1152/ajpregu.00227.2014 • www.ajpregu.org
the denervated kidney by sensory afferent nerves. Although both the NE and CGRP content were reduced to a similar extent in the denervated kidney, only CGRP concentrations appeared to return to levels close to baseline. These findings also seem to contradict our morphological evidence of reinnervation of denervated kidney by sympathetic efferents. Our findings are instead consistent with immunohistochemical evidence reported by others that have shown the neuronal marker PGP9.5 returning to baseline levels within 6 mo of syngeneic rat renal transplantation, indicating reinnervation, while NE content in the transplanted kidney remained low until 9 mo after transplant (23). Although reliable functional conclusions cannot be drawn from these findings, it seems that the sympathetic efferent reinnervation is functionally inadequate.

It is not clear why neurotransmitter content was not increased in the nondenervated kidney despite histological evidence of hyperinnervation, a finding that was more robust for the afferent nerves. It is possible that an apparent hyperinnervation in intact kidneys was due to the continued maturation and growth of the sympathetic and sensory innervation of the kidney between weeks 1 and 12 postdenervation. However, a previous report indicates that in normotensive rats, the distribution of sympathetic and sensory innervation of the kidney is similar to the adult by 21 days of age (31). Nevertheless, we cannot rule out maturation as a cause for increased area of innervation in the nondenervated kidneys at 12 wk due to the lack of an untreated time-matched control group.

Indeed, our histological methods are limited by our inability to obtain measures of TH and CGRP intensity throughout the full thickness of the vessel, as only surface areas could be appropriately imaged. Furthermore, we utilized thresholding and Z-projection to compress volume information to a two-dimensional area. Using this method, we are limited to the determination of a two-dimensional area of visible vessels covered by immunoreactive components of the nerve. Because we used a thresholding method, we are only describing the area of TH expression, rather than the intensity of expression. Moreover, our antibody detected total TH rather than phosphorylated forms of the enzyme, so we were unable to measure indices of TH activity in regrown nerves. We cannot make inferences about the level of TH expression or its activity and certainly not norepinephrine content. Thus, a clear-cut corre-
lation between NE tissue content and TH-positive label cannot be expected.

In contrast, CGRP tissue content would more likely correlate with the cumulative fiber volume of sensory nerves because sensory neuropeptides, such as CGRP, are stored and secreted along the length of the axon (10, 25, 46). Nevertheless, the label observed using our methods is related only to the diameter of the vessel given that thresholding and Z-projection reduces the measurement to a two-dimensional area. The volume of the nerve cannot be determined. Given that the fibers are more or less cylindrical in shape, changes in diameter will lead to quadratic changes of volume. Thus, many small fibers, which represent the same surface information as a single large fiber, will contain far less transmitter than the single large fiber. Therefore, sprouting of very small and highly branched nerve fibers (49) will not necessarily be associated with an increased tissue transmitter content. Predicting the influence of denervation on NE neurotransmission using two-dimensional TH immunolabel is further complicated by the fact that NE production is presumably amplified from the amount of TH through the enzymatic cascade, whereas CGRP label provides a more direct indication of the amount of neurotransmitter present.

An additional complication lies in the fact that we chose areas for image analysis on the basis of consistent and reliable labeling, whereas the NE and CGRP content represent neurotransmitter available in the whole kidney. It is possible that the discrepancy lies in the fact that we examined areas with the most robust label. It is possible that regrowth had not yet reached areas where reliable label was more difficult to obtain. Although we cannot conclude with certainty that an apparent increase in innervation of the intact kidney was dependent on the denervation procedure due to the lack of timed control experiments, it is clear that peptidergic reinnervation of the denervated kidney dominated sympathetic reinnervation. No such dominance in peptidergic growth was found on the nondenervated kidneys. These latter findings are in accordance with our tissue measurements.

Functional interpretations of our findings remain speculative. Although there is evidence for both inhibitory (8, 10, 44)
but recovered to the “R1_CGRP/SMA-level” by week 12 and surpassed the “R1_CGRP/SMA-level” by P 0.049, using two-way ANOVA and one-way ANOVA). In weeks 4 and 12; the TH/SMA ratio 4 wk after DNX was lower than on the non-DNX side (*P = 0.011, using two-way ANOVA) and still tended to be lower in week 12 (R12 vs. L12; P = 0.069, using two-way ANOVA). The TH/SMA ratio significantly increased on the non-DNX side (R1 vs. R4 and R12; P < 0.001, using one-way ANOVA).

and excitatory effects (3, 21) of renal peptidergic afferent innervation, it is tempting to suggest that a net surplus of afferent activity could contribute to an overall decrease in sympathetic activity (10). In patients, we noted that renal denervation did not affect renal vascular resistance or perfusion, but did lower indices of systemic vascular resistance (35). Renal hemodynamics have been shown to be relatively free from the influence of the renal sympathetic nerves. However, this might be more apparent than real, as the renal vasculature has powerful autoregulatory mechanisms that come into play, perhaps offsetting the vasoconstrictor action of the renal sympathetic nerves (15, 16, 24). Interestingly, sensory afferents have been reported to play a key role in myogenic contraction of blood vessels and, thus, in autoregulation (41). In a separate study, renal denervation in hypertensive patients was found to lower blood pressure and muscle sympathetic nerve activity (MSNA), as well as whole body catecholamine spillover (39). Moreover, the hypotensive effects appeared to be long-lasting (17).

An additional limitation of the study was our focus on the intrarenal vasculature in the periphery of the kidney downstream of interlobular and arcuate arteries, rather than the interstitial tissue, the latter of which might have a more significant impact on the hypotensive effects of denervation. The innervation of the interstitium is not homogenous, but is instead patchy and focally distributed. Thus, morphometric analysis of interstitial innervation is much more difficult to standardize. Here, we focused on the perivascular nerves due to our recent finding that stimulation of intrarenal afferents by injection of the TRPV1 receptor agonist capsaicin into the renal artery, resulted in a tonic inhibition of contralateral renal sympathetic nerve activity (10). Although the renal parenchyma is far less densely innervated than the renal pelvis, there exists a reasonable density of intrarenal innervation in the perivascular and adventitial space. The density of the afferent peptidergic fibers is far less than that of the sympathetic nerves, even around the vessels. In the study by Mulder et al. (34), intrarenal afferents were not even mentioned. We found the ratio of afferent-to-efferent fiber density ranged from 0.15 to 0.4 in the nondenervated kidney, which is in accordance with recently published data (33). This ratio was shifted toward sensory innervation on the denervated side. Such a shift has not yet been described. It is not yet clear whether this complex reinnervation pattern identified in intrarenal perivascular nerves also occurs in the renal pelvic nerves, because our study exclusively focused intrarenal innervation, and recent studies did not investigate this question (6, 34).

**Fig. 8.** TH⁺/SMA⁺ ratios (averaged observations). White bars denote non-DNX kidneys, while gray bars denote DNX kidneys. On the DNX side, the CGRP⁺/TH⁺ ratio was shifted toward CGRP (L1 vs. L12; P = 0.009, using one-way ANOVA). No such shift was observed on the non-DNX side (R1 vs. R12; P = 0.314, using one-way ANOVA). In week 1, the ratio on the DNX side was significantly lower (**P = 0.019, using a Student’s t-test), and in week 12, it was significantly higher (**P = 0.044, using a Student’s t-test) compared with the non-DNX side. No statistical difference could be detected in week 4 (ns; P = 0.465, using a Student’s t-test).

**Fig. 9.** CGRP⁺/SMA⁺ ratios (averaged observations). White bars denote the non-DNX kidneys, while gray bars denote DNX kidneys. One week after DNX, the CGRP/SMA ratio decreased (*P < 0.009, using two-way ANOVA) but recovered to the “R1_CGRP/SMA-level” by week 4 (R1 vs. L4, P = 0.244) and surpassed the “R1_CGRP/SMA-level” by week 12 (R1 vs. L12, P = 0.049, using two-way ANOVA and one-way ANOVA). In week 4, the CGRP/SMA ratio was significantly lower on the DNX side (*P = 0.009, using two-way ANOVA) but not in week 12 (R12 vs. L12; P = 0.931, using two-way ANOVA). A significant increase in CGRP/SMA ratio on the non-DNX-side was observed (R1 vs. R4 and R12; P = 0.037, using one-way ANOVA).

**Fig. 10.** CGRP⁺/TH⁺ ratios (averaged observations). White bars denote the non-DNX kidneys, while gray bars denote DNX kidneys. One week after DNX, the CGRP⁺/TH⁺ ratio was shifted toward CGRP (L1 vs. L12; P = 0.009, using one-way ANOVA). No such shift was observed on the non-DNX side (R1 vs. R12; P = 0.314, using one-way ANOVA). In week 1, the ratio on the DNX side was significantly lower (**P = 0.019, using a Student’s t-test), and in week 12, it was significantly higher (**P = 0.044, using a Student’s t-test) compared with the non-DNX side. No statistical difference could be detected in week 4 (ns; P = 0.465, using a Student’s t-test).
The thermophysical endovascular denervation procedure used for human hypertension therapy exerts its main effects in the perivascular space (36, 37, 42). Although our unilateral subtotal denervation model does not perfectly simulate the clinical procedures, our approach does provide new insight into renal reninnervation processes.

Our morphological findings support the work recently published by Mulder et al. (34), which showed a complete re-growth of the renal pelvic afferent and intrarenal efferent innervation 12 wk after unilateral renal denervation. In their work, the renal denervation procedure included the use of phenol application to the surface of the vasculature after surgical stripping of the adventitia. Our work extends these findings by describing intrarenal afferent and efferent innervation without the use of phenol. We purposely omitted the use of phenol for the denervation procedure to investigate patterns of reinnervation after subtotal denervation in an effort to better mimic the subtotal denervation achieved in clinical renal denervation procedures. Our evidence suggests that the use of phenol does not have much effect on reinnervation properties, as both CGRP and NE content in the denervated kidney was similar whether denervation included phenol or not. Furthermore, we could not detect any intramural nerves in the renal artery that would definitely require the use of phenol (Fig. 1).

To our knowledge this is the first study that indicates a contralateral effect of unilateral denervation of the kidney. We ruled out the possibility of bilateral innervation of the kidney from the same source. Our neuronal tracing experiments failed to identify any monosynaptic innervation from the kidney to the contralateral dorsal root ganglia in the healthy rat. The exact mechanisms for altered contralateral innervation are not clear. The release of humoral factors at the lesion site or some communication to the contralateral side by neuronal mechanisms is speculated to play a role (27). A recent study in sheep reported complete morphological and functional reinnervation, the latter of which was tested by electrical stimulation. How-

Fig. 11. Renal tissue CGRP content. Left: one week after unilateral surgical DNX, CGRP content was reduced, but it recovered with reinnervation (black bars denote non-DNX, while white bars denote DNX). *P < 0.005 non-DNX vs. DNX, using two-way ANOVA. Right: application of phenol had no significant effect; CGRP just tended to be lower in week 1 (gray denotes non-DNX, while white-striped denotes DNX+Phenol). *P < 0.002 non-DNX vs. DNX+Phenol; ns ≥ 0.138.

Fig. 12. Renal tissue norepinephrine (NE) content: Left: one week after unilateral surgical DNX, NE content was reduced, but the tissue NE levels did not recover (black bars denote non-DNX, while open bars denote DNX). *P < 0.005 non-DNX vs. DNX. Right: application of phenol had no significant effect; NE just tended to be lower in weeks 1 and 4 (gray bars denote non-DNX, while white-striped bars denote DNX+Phenol). *P < 0.001; non-DNX vs. DNX+Phenol, using two-way ANOVA.
ever, in this study, no interaction between the denervated and un-denervated kidney was described and no distinction was made between intralrenal and pelvic reinnervation.

Finally, even the source of reinnervation is not completely clear. It has been reported that the removal of postganglionic cell bodies by celiac ganglionectomy could not permanently ablate sympathetic innervation of splanchic organs. In fact, sympathetic fibers innervating the kidney demonstrated the most prominent regrowth after ganglionectomy in rats (29, 48). After cell body removal (ganglionectomy), sympathetic reinnervation may occur via sprouting from other ganglia. After axotomy (as was done here), it is not quite clear whether the source of reinnervation is from the same ganglia, or via sprouting from other sources, or both.

**Perspectives and Significance**

Although the functional significance of our indirect measures of reinnervation has yet to be defined, our data provide important insight into the complexity of the renal reinnervation process. A complex reinnervation pattern with a shift toward afferent innervation seems to take place. Our study provides the framework for well-designed in vivo studies that are necessary to further substantiate our understanding of renal innervation and its function in health and disease. Because renal reinnervation seems to be a multispecies phenomenon, it must also be expected in humans over months after percutaneous renal denervation. Long-term effects on blood pressure and sympathetic control described in humans might rather be due to a regenerative remodeling of renal nerves than complete and long-lasting loss of innervation.

**DISCLOSURES**

R. Veelken and T. Ditting received funding by Medtronic. Other funding was provided by Deutsche Forschungsgemeinschaft, SFB 423 (to R. Veelken).

**AUTHOR CONTRIBUTIONS**


**REFERENCES**


29. Li M, Galligan J, Wang D, Fink G.


