Abstract—Previous studies indicate that catheter-based renal denervation reduces blood pressure and renal norepinephrine spillover in human resistant hypertension. The effects of this procedure onafferent sensory and efferent sympathetic renal nerves, and the subsequent degree of reinnervation, have not been investigated. We therefore examined the level of functional and anatomic reinnervation at 5.5 and 11 months after renal denervation using the Symplicity Flex catheter. In normotensive anesthetized sheep (n=6), electric stimulation of intact renal nerves increased arterial blood pressure from 99±3 to 107±3 mm Hg (afferent response) and reduced renal blood flow from 198±16 to 85±20 mL/min (efferent response). In a further group (n=6), immediately after denervation, renal sympathetic nerve activity was absent and the responses to electric stimulation were abolished. At 11 months after denervation (n=5), renal sympathetic nerve activity and the responses to electric stimulation were at normal levels. Immunohistochemical staining for renal efferent (tyrosine hydroxylase) and renal afferent nerves (calcitonin gene–related peptide), as well as renal norepinephrine levels, was normal 11 months after denervation. Findings at 5.5 months after denervation were similar (n=5). In summary, catheter-based renal denervation effectively ablated the renal afferent and efferent nerves in normotensive sheep. By 11 months after denervation the functional afferent and efferent responses to electric stimulation were normal. Reinnervation at 11 months after denervation was supported by normal anatomic distribution of afferent and efferent renal nerves. In view of this evidence, the mechanisms underlying the prolonged hypotensive effect of catheter-based renal denervation in human resistant hypertension need to be reassessed. (Hypertension. 2015;65:393-400. DOI: 10.1161/HYPERTENSIONAHA.114.04176.) ● Online Data Supplement

Key Words: cardiovascular diseases ■ catheter ablation ■ electric stimulation

There is extensive evidence that renal afferent and efferent nerves play a critical role in the control of renal function and in setting the level of arterial blood pressure.¹ This includes the findings that in experimental and human hypertension there are increases in renal sympathetic nerve activity (RSNA) and renal norepinephrine spillover, respectively. In addition, surgical renal denervation (RDN) reduces blood pressure in hypertensive animals and patients,¹–⁴ although in patients this was associated with several side effects.⁵ The development of catheter-based radiofrequency RDN has resulted in a resurgence of interest in RDN as a treatment for resistant hypertensive patients. Initial trials demonstrated reductions in office systolic blood pressure⁶,⁷ and in the 36-month follow-up from the first trial 93% of patients showed reductions in office systolic blood pressure of ≥10 mm Hg after RDN.⁷ In contrast, the recent Symplicity HTN-3 trial did not demonstrate reductions in systolic blood pressure beyond that observed in sham control patients 6 months after RDN,⁸ although there is still debate on factors that may have led to the lack of effect, such as procedural and population variability. It would be expected that destruction of the renal nerves reduces blood pressure because the efferent renal nerves play a major role in stimulating renin release, causing renal vasoconstriction and inducing sodium retention.¹ It is also plausible that in hypertension, increased afferent renal nerve activity may cause a reflex increase in sympathetic outflow and worsening hypertension.⁹,¹⁰ Such actions are supported by findings that in some hypertensive patients, catheter-based RDN reduced the level of muscle SNA¹¹,¹² and plasma norepinephrine.¹³ Despite these proposed mechanisms, it is unknown how
effectively catheter-based RDN ablates the renal afferent and efferent nerves, a central question in the ongoing debate of the results of the HTN-3 trial. Furthermore, it has not been established whether the renal nerves regrow and functionally reinnervate the kidney after catheter-based RDN.

After surgical renal RDN in rats there is immunohistochemical evidence of reinnervation within 12 weeks\(^{14}\) and functional reinnervation after 8 weeks.\(^{15}\) Considering the differences in body size and technique, such studies may not predict the rate or degree of reinnervation after catheter-based RDN in humans. Studies in humans after renal transplants have found variable rates of reinnervation,\(^{16,17}\) probably because human tissue was often derived from failed transplants. In recent studies in porcine\(^{18}\) and canine\(^{19}\) models, RDN using Symplicity and alternative catheters was associated with 81%, 59%, and 47% reductions in kidney norepinephrine content at 1, 4, and 8 weeks after denervation, respectively. Although these studies suggest significant denervation followed by potential reinnervation early after RDN, functional responsiveness and anatomic distribution of afferent and efferent renal nerves were not assessed.

In view of the controversy on the effectiveness of catheter-based RDN, we used functional, anatomic, and biochemical techniques to establish the degree of denervation of renal afferent and efferent nerves immediately after catheter-based RDN using the Symplicity Flex catheter in anesthetized normotensive sheep. Furthermore, considering the lack of information on reinnervation after catheter-based RDN, we investigated the degree of reinnervation at 5.5 and 11 months after RDN. This study focussed on the level of denervation and reinnervation after catheter-based RDN, not the effect of this procedure on blood pressure.

**Methods**

**Surgical Procedures**

Experimental procedures were approved by the Animal Ethics Committee of the Florey Institute of Neuroscience and Mental Health under guidelines laid down by the National Health and Medical Research Council of Australia. Experiments were performed in 4 groups of normotensive Merino ewes (A, nondenervated controls \(n=6\); B, acutely denervated \(n=6\); C, 5.5 months after RDN \(n=5\); and D, 11 months after RDN \(n=5\)). Sheep were individually housed and given free access to water and oat chaff once a day. In all surgeries, anesthesia was induced with sodium thiopentone (15 mg/kg IV) and, after endotracheal intubation, was maintained with 1.5% to 2.0% isoflurane-O\(_2\)/air mixture. Sheep were treated with intramuscular antibiotics (900 mg, procaine penicillin, Troy Laboratories, NSW) at surgery and 1 day postoperatively. Analgesia was maintained with intramuscular flunixin meglumine (1 mg/kg; Troy Laboratories) at surgery.

Anesthetized sheep the facial artery was cannulated to measure mean arterial pressure (MAP) and heart rate (HR). A transit-time flow probe (Transonics Systems, Ithaca, NY) was use to measure renal blood flow (RBF) and intrafascicular electrodes were placed in the renal nerve to record RSNA.\(^{20}\) The electrodes were removed after a 5-minute recording was obtained. In addition, in group A only, a double-lumen polyethylene catheter (outer diameter, 1.7 mm) was secured in the ureter for intrapelvic infusions.

**Renal Nerve Stimulation**

Renal nerves were placed on a pair of hooked stimulating electrodes and stimulated for 30 seconds at 10 V at 3 frequencies (1, 3, and 5 Hz). All variables were allowed to return to prestimulus baseline levels before the next stimulation.

**Unilateral RDN**

In anesthetized sheep, after a cut down on the right femoral artery, a 6F catheter was introduced and heparin (3000 IU) administered. A Symplicity Flex catheter (Symplicity, Medtronic Ardian Inc, CA) was introduced into renal arteries using radiopaque contrast under fluoroscopic guidance and 5 to 6 two-minute radiofrequency ablations were delivered in a helical configuration along the renal artery, starting as close to the kidney as anatomically possible (see online-only Data Supplement). The catheter system (Medtronic Ardian Inc, CA) monitored tip impedance and temperature and altered energy delivery in response to an algorithm used in humans.\(^{4}\) After the experiments the catheter was withdrawn, the femoral wound was closed and sheep were recovered from anesthesia.

At the end of experiments sheep were euthanized with intravenous pentobarbitone (100 mg/kg). Kidneys were taken for histological analysis and norepinephrine content (see online-only Data Supplement). For details of data recording and analysis, see online-only Data Supplement.

**Experimental Groups**

**Group A: Control, Nondenervated Sheep \(n=6\)**

After baseline measurements of MAP, HR, and RSNA, capsaicin (1 µg/ml in 1 mL) was infused into the renal pelvis for 1 minute to stimulate the renal afferent nerves. The whole renal nerve was then stimulated as described above. After recovery, the renal nerve was transected and the proximal and distal ends were separately stimulated (10 V, 5.0 Hz, 30 s) to determine the specific effects of renal afferent and efferent stimulation, respectively. Sheep were euthanized at the end of the protocol.

**Group B: Acute RDN With the Symplicity Catheter System \(n=6\)**

Sheep underwent unilateral RDN as described above and then intact renal nerve stimulation (10 V, 5.0 Hz, 30 s). Sheep were recovered from anesthesia and were euthanized after 1 week.

**Groups C and D: Electric Stimulation 5.5 and 11 Months After RDN \(n=5\) per Group.**

Unilateral RDN was completed and sheep were recovered from anesthesia and returned to the farm. At 5.5 months (group C) and 11 months (group D) after RDN sheep were returned to the laboratory and renal nerve stimulation protocol performed. Sheep were euthanized at the end of the protocol.

**Renal Norepinephrine Content and Immunohistochemistry**

The levels of tyrosine hydroxylase (TH) and calcitonin gene-related peptide (CGRP) in the renal cortex, medulla and pelvis were determined by immunohistochemistry using an anti-TH primary antibody (Merck Millipore) and a rabbit anti-CGRP primary antibody (from Ingrid Nylander, Uppsala University, Sweden),\(^{21}\) with fluorescent images acquired blind and imaged using Image J (National Institutes of Health, Bethesda, MD; see online-only Data Supplement). Tissue levels of norepinephrine were determined in renal cortex and medulla as previously described (see online-only Data Supplement).\(^{22}\)

**Statistical Analysis**

Statistical analysis was performed in Rcmdr\(^{23,24}\) using absolute values.

To compare responses in proximal and distal transected nerve stimulation with those from whole nerve stimulation, simple linear regression models were fitted to each animal during the 30-second stimulation period and slopes compared using Mann–Whitney test.\(^{25}\) Changes after acute RDN were tested using Friedman test of the 30-second stimulation period. Differences between controls, 5.5 and 11 months after denervation were tested by fitting a linear mixed effects model of the responses against group, frequency, and baseline (10 second before stimulation), adjusting for repeated observations.
within animal. This allowed animals to be considered under each frequency condition. Differences between immunohistochemical staining and norepinephrine concentration between the groups were tested using 1-way ANOVAs. Post hoc analysis was performed using Tukey multiple comparison test. Data sets were assessed for normality and square root transformed as appropriate. Statistical significance was accepted when $P<0.05$. Data are mean±SEM.

Results

Physiological Data

**Group A: Intrapelvic Capsaicin and Electric Stimulation of Renal Nerves in Anesthetized Nondenervated Sheep**

In nondenervated sheep, there was a high level of RSNA, one burst of activity with every heart beat, which is the normal level in anesthetized sheep (see online-only Data Supplement).26,27 Intrapelvic infusion of capsaicin (1 μg/1 mL for 1 minute) had no significant effects on MAP, HR or RSNA, with a trend toward a small increase in RBF (−6±3 mL/min; $P<0.09$). In view of this lack of treatment effect this was not repeated in the other groups. Electric stimulation (5 Hz, 10 V) of intact renal nerves in nondenervated sheep increased MAP and reduced HR, RBF, and renal vascular conductance (RVC; Figures 1 and 2; Table). Stimulation of the proximal end of the nerve, to stimulate afferent fibers, increased MAP (not significant compared with whole nerve), decreased HR (not significant compared with whole nerve), and caused no change in RBF ($P<0.005$ compared with whole nerve; Figure 1). In contrast, stimulation of the distal end of the cut nerve, to stimulate sympathetic efferent fibers, decreased RBF and RVC (not significant compared with whole nerve), but did not change MAP or HR ($P<0.05$ compared with whole nerve stimulation; Figure 1). Electric stimulation of the whole nerve with lower frequencies (1 and 3 Hz) caused less pronounced changes in RBF, RVC, and HR ($P<0.0001$ for all three variables; Figure 3; Table), whereas the changes in MAP across frequencies were not significantly different.

**Group B: Electric Stimulation Immediately After RDN**

Stimulation of renal nerves directly after RDN caused no changes in MAP, HR, RBF, or RVC (Figure 2; not significant compared with baseline). We were unable to record RSNA in any animals acutely after RDN (see Results in the online-only Data Supplement).

**Groups C and D: Electric Stimulation 5.5 and 11 Months After Catheter-Based Denervation**

The gross anatomy was variable between animals, but there was consistently more fibrotic tissue around the renal artery at 5.5 months than at 11 months after denervation. The main nerve bundle from the renal plexus to the renal artery was located in all but 1 animal (5.5-month group). The kidney levels of norepinephrine and TH in this animal, however, indicated that reinnervation had occurred. In addition to the main nerve bundle, in both the 5.5- and 11-month groups, there were wide-spread smaller nerve branches, indicating nerve regeneration. Following the path of these new nerves was difficult because of fibrosis and dissection was not attempted to

![Figure 1](http://hyper.ahajournals.org/)

**Figure 1.** Electric stimulation (10 V, 5 Hz, 0–30 s) of the whole renal nerve (whole nerve), renal afferents (proximal stimulation), and renal efferents (distal stimulation). Data are 5-s averages±SE. HR indicates heart rate; MAP, mean arterial blood pressure; RBF, renal blood flow; and RVC, renal vascular conductance.
avoid damaging the nerve. By 11 months after RDN the level of RSNA recorded in the renal nerve close to the renal artery was similar to that in the control nondenervated sheep (see Results in the online-only Data Supplement).

Electric stimulation (1, 3, and 5 Hz) of the renal nerves close to the renal artery at 5.5 (n=4) and 11 (n=5) months after denervation resulted in increases in MAP and decreases in RBF and RVC. There were no significant differences between the responses in the nondenervated control group and the groups that had been denervated 5.5 and 11 months previously (Figure 3; Table). There were differences in the levels of MAP between the nondenervated and the 5.5-month denervated group (P<0.01) and in RBF between the nondenervated and the 11-month denervated group (P<0.05), but whether this was a treatment effect or because of the variability in MAP and RBF that occurs under anesthesia is unclear.

Renal Norepinephrine Content and Immunohistochemistry for TH and CGRP

One week after RDN, the levels of TH were significantly lower around the intrarenal vessels, capillary beds and pelvic wall compared with the nondenervated group (Figure 4; Figure S5 in the online-only Data Supplement). In the 5.5- and 11-month denervated groups the levels of TH had significantly increased and were not significantly different from the control group. The changes in norepinephrine content after RDN paralleled the changes in TH staining (Figure 5). One week after RDN the mean renal medullary and cortical levels of NE were 13.5% and 20.1%, respectively, of those in nondenervated sheep (Figure 5). The mean medullary and cortical levels of NE were 63.4% and 88.9% of control levels, respectively, at 5.5 months after RDN and 77.1% and 131.0%, respectively, at 11 months after RDN.

The level of CGRP staining was greater in the pelvic wall than around intrarenal vessels and glomeruli (Figure 4; Figure...
The CGRP staining in the pelvic wall was significantly reduced in the acutely denervated group compared with non-denervated sheep ($P<0.005$). In the 11-month group the level of CGRP staining was significantly greater than in the acute group and not different from the innervated control group (Figure 4).

Around the intrarenal vessels, CGRP was significantly lower in the acutely denervated group compared with the 11-month group ($P<0.001$). Around the glomeruli CGRP levels were low in all groups (see online-only Data Supplement).

**Discussion**

We investigated the functional and anatomic reinnervation of the kidney in a large animal after RDN using the Symplicity Flex System with the same algorithm used in humans. The principal findings were that immediately after a standard clinical protocol of 5 to 6 ablations per renal artery, RSNA was not present in the renal nerve and the afferent and efferent responses to electric stimulation of the nerve were abolished, demonstrating the effectiveness of this technique. Eleven months after RDN, functional reinnervation was demonstrated by the presence of RSNA and normal afferent and efferent responses to nerve stimulation. Anatomic evidence of reinnervation of the afferent and efferent nerves 5.5 and 11 months after RDN was indicated by the normal levels of TH and CGRP staining, respectively, as well as the normal levels of tissue norepinephrine.

It was originally hypothesized that RDN reduced blood pressure primarily by destroying efferent sympathetic nerves, resulting in reduced vascular resistance, renin release, and sodium retention. Increasingly it is proposed that denervation of the afferent renal nerves contributes to the hypotensive effect of RDN because of removal or alteration of the renal afferent reflex, which has been shown to activate important central cardiovascular nuclei. Our findings indicate that catheter-based RDN destroys renal efferent nerves as shown by reduced TH staining, tissue norepinephrine content, absence of RSNA, and responses to electric stimulation. These findings corroborate recent studies showing significant reductions in renal norepinephrine 8 weeks after RDN in obese hypertensive dogs and 1 and 4 weeks after RDN in normal pigs. In addition, after RDN we found inhibition of the afferent response to electric stimulation and decreased levels of CGRP staining.

Interestingly, we found that by 5.5 months after RDN there was almost complete functional and anatomic reinnervation, and by 11 months after denervation there were no differences in the responses to electric stimulation, renal distribution of TH and CGRP, or renal norepinephrine levels compared with non-denervated controls. Importantly, by demonstrating unambiguously different responses to stimulation of the proximal and distal ends of the cut renal nerve we were able to demonstrate normalization of both the afferent and efferent responses to stimulation of the intact renal nerves. To the best of our knowledge this is the first examination of functional and anatomic reinnervation of the kidney after catheter-based RDN. To date, there has been no examination of kidneys from.
patients who have undergone catheter-based RDN, only a recent case study investigating perivascular nerves 12 days after RDN showing that renal nerve damage was limited to an area 2 mm from the vascular lumen. In a study in rats, 12 weeks after surgical denervation there was immunohistochemical evidence of reinnervation of renal afferent and efferent nerves and functional reinnervation has been shown 8 weeks after surgical denervation in rats. These studies concur with our findings that reinnervation of afferent and efferent renal nerves after catheter-based RDN is almost complete by 5.5 months and seems to be complete by 11 months after denervation.

Strengths and Limitations

The strength of this study is that RDN was completed in a large mammal with the Symplicity RDN System used clinically, making it likely that the findings are relevant to the clinical setting. We determined the level of functional reinnervation by recording RSNA and determining the afferent and efferent responses to electric nerve stimulation. The degree of anatomic reinnervation was established using specific markers for the efferent sympathetic nerves (TH and norepinephrine) and the afferent sensory nerves (CGRP). Importantly, the investigations of reinnervation were completed over long, clinically relevant time frames (5.5 and 11 months). Although the HTN3 trial demonstrated the importance of a sham-denervation group to control for regression to the mean of blood pressures and increased adherence to antihypertensive treatment, these factors were not relevant in the current study. A weakness of the study is that although we showed return of the vascular response to supramaximal electric stimulation after denervation, we did not investigate whether there were changes in RSNA in the conscious state or in the ability of physiologically induced changes in RSNA to alter RBF, renin release, or sodium excretion. In addition, using intrapelvic capsaicin we were unable to stimulate the renal afferent sensory nerves in nondenervated sheep, so we were unable to test whether the renal afferent sensory response was normal after RDN. We were, however, able to demonstrate normalization of the afferent response (increase in MAP) to electric stimulation and a return of CGRP staining. These studies were conducted in healthy, normotensive young animals and it is possible that age, hypertension, and other comorbidities alter the rate of nerve regrowth. Finally, whether our findings in normotensive sheep can be directly translated into the human situation remains unclear, but because we denervated with the same catheter and algorithm used in patients it is likely that this model has some clinical relevance. In fact, estimates of efferent RDN based on renal norepinephrine spillover in patients suggest

Figure 4. Efferent and afferent nerve distribution as shown by tyrosine hydroxylase (TH; green) and calcitonin gene–related peptide staining (CGRP; red). Cell nuclei are stained with 4′,6-diamidino-2-phenylindole DAPI (blue). Left, Representative images from nondenervated sheep (Con), acutely denervated sheep (1 week after renal denervation [RDN]) and 5.5 and 11 months after RDN around the intrarenal vessels at the corticomedullary junction, around the glomeruli (G) and in the renal pelvis. Right, Density of nerve distribution in control (n=6), acute (n=6), 5.5-month (n=5), and 11-month (n=5) groups, showing median and individual data points. Scale bar, 100 µm. *P<0.05, **P<0.005, and ***P<0.001; Post hoc Tukey test.
that we produced a comparable, if not superior, degree of denervation.

**Perspectives**

Numerous studies have demonstrated hypotensive responses after surgical RDN in many forms of experimental hypertension and after catheter-based RDN in hypertensive patients. It was therefore surprising that the recent HTN-3 trial did not show a reduction in systolic blood pressure at 6 months. Whether effective denervation was achieved in these patients remains problematic as there were no measures indicating the level of denervation. The finding in the present study that catheter-based RDN effectively ablated the renal nerves demonstrates the effectiveness of the technique if applied appropriately. The finding of complete functional and anatomic reinnervation 11 months after catheter-based RDN in sheep is consistent with the finding of anatomic reinnervation after surgical RDN in rats. Although it is unknown whether a similar degree of reinnervation occurs in patients, these findings challenge the current presumption that a permanent loss of sympathetic nerves reinnervate the kidney in a similar time-dependent fashion.

**Acknowledgments**

We acknowledge the expert technical assistance of Anthony Dornorn and Alan McDonald, Dr. Karen Lamb for statistical consultation and Sarah Phillips and Dr Nina Eikelis for assistance in catecholamine determination. We thank Medtronic who provided the Symplicity RDN System in kind.

**Sources of Funding**

This work was supported by National Health and Medical Research Council of Australia (NHMRC; 1012100) and the Victorian Government’s Operational Infrastructure Support Program. Dr Booth was the recipient of a NHMRC Early Career Fellowship (1054619). E.E. Nishi was supported by Research Internships Abroad Fellowship of São Paulo Research Foundation and Drs May, Schlaih, and Lambert were supported by NHMRC Research Fellowships.

**Disclosures**

Dr May has received honoraria and travel support for presentations from Medtronic, Dr Schlaih from Abbott, Servier, Novartis, and Medtronic and Dr Lambert from Medtronic, Pfizer, Wyeth Pharmaceuticals, and Servier. Dr Schlaih serves on scientific advisory boards for Abbott (formerly Solvay) Pharmaceuticals, Novartis Pharmaceuticals, and Medtronic. Dr Lambert has acted as a consultant for Medtronic. The laboratories of Drs Schlaih and Lambert currently receive research funding from Medtronic, Abbott (formerly Solvay) Pharmaceuticals, Servier Australia, and Allergan. The other authors report no conflicts.

**References**


What Is New?

• Catheter-based radiofrequency renal denervation was shown to effectively ablate the afferent and efferent nerves within the kidney in normotensive sheep.
• By 11 months after renal denervation, reinnervation of the afferent and efferent renal nerves was shown by the return of renal sympathetic nerve activity, normal responses to electric stimulation of the nerves, and normal distribution of markers of the afferent and efferent nerves in the kidney.

What Is Relevant?

• These findings that the afferent and efferent renal nerves reinnervate after catheter-based renal denervation in a normotensive sheep model challenge the proposed mechanisms determining the long-term reduction in blood pressure in hypertensive patients.

Summary

Catheter-based renal denervation effectively ablated the renal afferent and efferent nerves, but functional and anatomic reinnervation occurred within months.
Reinnervation of Renal Afferent and Efferent Nerves at 5.5 and 11 Months After Catheter-Based Radiofrequency Renal Denervation In Sheep
Lindsea C. Booth, Erika E. Nishi, Song T. Yao, Rohit Ramchandra, Gavin W. Lambert, Markus P. Schlaich and Clive N. May

Hypertension. 2015;65:393-400; originally published online November 17, 2014;
doi: 10.1161/HYPERTENSIONAHA.114.04176
Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2014 American Heart Association, Inc. All rights reserved.
Print ISSN: 0194-911X. Online ISSN: 1524-4563

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://hyper.ahajournals.org/content/65/2/393

Data Supplement (unedited) at:
http://hyper.ahajournals.org/content/suppl/2014/11/17/HYPERTENSIONAHA.114.04176.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Hypertension can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Hypertension is online at:
http://hyper.ahajournals.org/subscriptions/
SUPPLEMENTARY MATERIAL: REINNERVATION OF RENAL AFFERENT AND EFFERENT NERVES AT 5 ½ AND 11 MONTHS AFTER CATHETER-BASED RADIO-FREQUENCY RENAL DENERVATION IN SHEEP

Short title: Reinnervation following catheter denervation

Lindsea C. Booth,* PhD, Erika E. Nishi,* MSc, Song T. Yao, PhD, Rohit Ramchandra, PhD, Gavin W. Lambert, PhD, Markus P. Schlaich, PhD MD, Clive N. May, PhD

*Both authors contributed equally to this study.

From the Florey Institute of Neuroscience and Mental Health, Melbourne (LCB, EEN, STY, RR, CNM), Baker IDI Heart and Diabetes Institute and Central Clinical School Monash University (MPS, GWL), Melbourne, Victoria, Australia and Department of Physiology, Cardiovascular Division, Federal University of São Paulo, São Paulo, Brazil (EEN).

CORRESPONDENCE TO:

Dr Lindsea C. Booth,
Florey Institute of Neuroscience and Mental Health,
University of Melbourne, Parkville, Victoria 3010, Australia.
Phone: +613 83440182
Fax: +613 93481707
Email: lindsea.booth@florey.edu.au
Supplementary methods

Data collection
Data including mean arterial blood pressure (MAP; 100 Hz), heart rate (HR; 100 Hz), renal blood flow (RBF; 100 Hz), renal sympathetic nerve activity (RSNA; 5000 Hz) and stimulation signal (5000 Hz) were recorded on computer using a CED Micro1401 interface and Spike2 software (Cambridge Electronic Design, Cambridge, UK). RSNA was recorded differentially between the pair of electrodes. The signal was amplified (x20,000) and filtered (band pass, 300–1,000 Hz), displayed on an oscilloscope, and passed through an audio amplifier and loud speaker. Renal vascular conductance (RVC) was calculated by dividing RBF by MAP. In the control group and 11 month denervation group burst frequency was determined on the raw RSNA signal.

Immunohistochemistry
Following euthanasia, the kidneys were removed quickly and cut transversally. One quarter of each kidney was stored in 20% formalin at room temperature for a week before dehydration and embedding in paraffin. Sections containing renal cortex, medulla and pelvis were cut at 4 µm and mounted on glass slides (Histology Core Services, Florey Institute, Melbourne). Slides were deparaffinized with a series of xylenes, cleared in graded alcohols and rehydrated. Endogenous peroxidase was quenched using 0.03 % H2O2 for 30 min and then the sections were incubated at room temperature for 1 hour and then at 4°C overnight in a rabbit anti-Tyrosine hydroxylase (TH) primary antibody (1:1000 dilution, Merck Millipore) or a rabbit anti-calcitonin gene-related peptide (CGRP) primary antibody (1:16,000 dilution, Ingrid Nylander, Uppsala University, Sweden). The sections were incubated in blocking solution for 30 min to minimize non-specific binding and then incubated at room temperature for 1 hour in a horseradish peroxidase-conjugated anti-rabbit secondary antibody (1:500 dilution, DAKO). For amplification of the signal, a tyramide signal amplification kit (Perkin Elmer, Waltham, MA, USA) was applied. The sections were incubated in the fluorogenic substrate Alexa Fluor 488 tyramide (1:100) for 10 min and then coverslipped with mounting medium with DAPI (Sigma-Aldrich, St. Louis, MO, USA).

Image analysis was undertaken using a fluorescent microscope (Leica DMLB2); slides were read blinded and pictures were captured at x10 or x20 magnification. The percentage of TH or CGRP area staining from each image was quantified with Image J software (NIH, USA). The level of TH staining was quantified in four kidney regions that are well established to be high in sympathetic innervation2; namely 1) arcuate arterial/venous complex in the border area between the medulla and cortex, 2) capillary plexus/vascular bundles in the outer medulla, 3) glomerulus/arteriole complex and 4) renal pelvic wall3. CGRP was quantified in three kidney regions; 1) arcuate arterial/venous complex at the border of the medulla and cortex, 2) glomerulus/arteriole complex and 3) renal pelvic wall, which had the highest density of sensory nerve innervation3. Two images were taken for each defined region from each kidney slice and the percentage of staining averaged for each animal. Areas were chosen using the DAPI fluorescent channel from similar areas in each kidney slice. For pelvic regions fluorescent staining was calculated as a percentage of pelvic area. For all other regions fluorescent staining was calculated as percentage of whole image.

Norepinephrine content
Following euthanasia, the kidneys were removed quickly and cut transversally. Sections of medulla and cortex (0.5 g) were snap frozen in liquid nitrogen and stored at -80°C. On the day of norepinephrine analysis, kidney samples were thawed and accurately weighed before being homogenized on ice in 0.5 mL of 0.4 M perchloric acid containing 0.01 % EDTA using
a glass-glass hand held homogeniser as previously described\textsuperscript{4}. The homogenate was then rapidly centrifuged, and the supernatant was collected for subsequent neurochemical analysis. Catecholamines were extracted from the perchloric acid supernatant, and also from plasma, with alumina adsorption, separated by high-performance liquid chromatography, and the amounts were quantified by electrochemical detection according to previously described methods.

**Supplementary Results**

**Renal sympathetic nerve activity:** In anesthetised non-denervated, control sheep there was a high level of RSNA with a burst of activity with every heart beat (S4). In contrast, in the acutely denervated group, we were unable to record RSNA from the renal nerves close to the renal artery immediately after renal denervation (RDN).

In sheep investigated at 5.5 months after RDN, RSNA was evident in four of the animals, in the other animal we were unable to locate the renal nerve. In these four sheep, the RSNA signal-to-noise was low and the level of activity could not be accurately qualified (S4). It should be noted that although the low signal-to-noise ratio may indicate low nerve signal transduction, the quality of the nerve signal is highly dependent on the electrode position within the nerve and therefore caution must be taken when interpreting these data. For this reason we did not attempt to quantify the nerve signal in µV in any of the groups. In the one animal in the 5.5 month group where we could not identify the renal nerve, analysis of renal norepinephrine levels and immunohistochemistry of the kidney demonstrated that reinnervation had occurred. At eleven months after RDN, the burst frequency of RSNA (101 ± 3 bursts/100 heart beats; n=5) was not statistically different from that in non-denervated control animals (102 ± 2 bursts/100 heart beats; n=5; P=0.69 tested using Two-sample Wilcoxon test).

Due to the fibrosis following renal denervation we were only able to dissect out a sufficiently long section of nerve to cut and stimulate both ends in a few of the denervated sheep (n=3). In these animals the responses to stimulation of the proximal and distal ends of the nerve showed a similar clear distinction of afferent and efferent responses, exactly as seen in the control group.

**Intrapelvic capsaicin infusion:** Intrapelvic infusions of capsaicin were administered in non-denervated control sheep. Intrapelvic infusion of capsaicin (1 µg/1 mL over 1 min) had no significant effects on MAP, HR or RSNA, with a trend towards a small increase in RBF (6 ± 3 mL/min, P<0.09).
References:


<table>
<thead>
<tr>
<th>Group (time since denervation)</th>
<th>Ablations/artery (W)</th>
<th>Max power delivery (W)</th>
<th>Max change in impedance (% change)</th>
<th>Max temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group B (1 hour)</td>
<td>5.4 ± 0.4</td>
<td>8 ± 0</td>
<td>-17 ± 2</td>
<td>57 ± 2</td>
</tr>
<tr>
<td>Group C (5.5 months)</td>
<td>6.4 ± 0.2</td>
<td>8 ± 0</td>
<td>-14 ± 1</td>
<td>57 ± 1</td>
</tr>
<tr>
<td>Group D (11 months)</td>
<td>6.4 ± 0.2</td>
<td>8 ± 1</td>
<td>-13 ± 2</td>
<td>58 ± 2</td>
</tr>
</tbody>
</table>

**Figure S1:** Technical details of the two minute ablation procedures applied using the Symplicity Flex catheter in Groups B, C and D.
Figure S2: Experimental timeline showing the control, non-denervated group, acutely denervated group and 5.5 month and 11 month groups. CAP: Intrapelvic infusion of 1 µg capsaicin in 1 mL measuring blood pressure, heart rate and renal blood flow, Nerve stimulation: Renal sympathetic nerve recordings and electrical stimulation of renal nerve (10 V, 30 s) at 1, 3 and 5 Hz. Cut nerve stim: Transect renal nerve and stimulate afferent and efferent ends separately (10V, 30 s, 5 Hz). PM: After the end of experiments sheep were euthanised and kidneys taken for immunohistochemical analysis and measurement of norepinephrine content.
Figure S3: Mean arterial blood pressure (MAP), heart rate (HR), renal blood flow (RBF) and renal vascular conductance (RVC) in non-denervated sheep during electrical stimulation of the intact renal nerve (10 V, 0-30 sec) at 1 Hz (crosses), 3 Hz (empty circles) and 5 Hz (filled circles). Data are 5 sec averages ± S.E., n=6.
Figure S4: Examples of raw renal sympathetic nerve activity (RSNA; µV) and arterial blood pressure (AP; mmHg) from a non-denervated control sheep, an acutely denervated sheep and sheep denervated 5.5 and 11 months previously. Data are sampled at 5000 Hz.
Figure S5: Efferent and afferent nerve distribution as shown by tyrosine hydroxylase (TH; green) and calcitonin gene-related peptide staining (CGRP; red). Cell nuclei are stained with DAPI (blue). Left panels: Representative images from non-denervated sheep (Control), sheep one week post RDN (Acute) and 5.5 and 11 months post-RDN in the renal pelvic wall, capillary beds (white arrows) and around the glomeruli (G). Right panel: Density of nerve distribution in control (n=6), acute (n=6), 5.5M (n=5) and 11M (n=5) groups, showing the median, maximum and minimum points. Scale bar=100µm. *P<0.05, **P<0.005; Posthoc Tukey test.