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# The carotid body as a putative therapeutic target for the treatment of neurogenic hypertension

Carotid body, renal nerves and hypertension

*Fiona D. McBryde PhD<sup>1</sup>, Ana P. Abdala PhD<sup>1</sup>, Emma B. Hendy PhD<sup>1</sup>, Wioletta Pijacka PhD<sup>1</sup>, Paul Marvar PhD<sup>1</sup>, Davi J.A. Moraes PhD<sup>2</sup>, Paul A. Sobotka MD<sup>3</sup>, Julian F.R. Paton PhD<sup>1</sup>*

<sup>1</sup>School of Physiology & Pharmacology, Bristol Heart Institute, Medical Sciences Building, University of Bristol, Bristol BS8 1TD, England

<sup>2</sup>Department of Physiology, School of Medicine of Ribeirão Preto, University of São Paulo, Ribeirão Preto, SP, Brazil

<sup>3</sup>Coridea NC1., 134 W 26th St., Suite 1011, New York, NY 10001, USA

Corresponding author:

**Julian F.R. Paton**

School of Physiology & Pharmacology, Bristol Heart Institute, Medical Science Building, University of Bristol, Bristol, United Kingdom. BS8 1TD

Tel: +44- (0)117 331 2275

FAX: +44- (0) 117 331 2288

Email: [Julian.F.R.Paton@Bristol.ac.uk](mailto:Julian.F.R.Paton@Bristol.ac.uk)

## **Abstract**

In the spontaneously hypertensive (SH) rat, hyperoxic inactivation of the carotid body produces a rapid and pronounced fall in both arterial pressure and renal sympathetic nerve activity (RSA). Here we show that carotid body de-afferentation through carotid sinus nerve denervation (CSD) reduces the overactive sympathetic activity (SA) in (SH) rats, providing an effective anti-hypertensive treatment. We demonstrate that CSD lowers renal (R) SA chronically and that this is accompanied by a depressor response in SH but not normotensive rats. The drop in blood pressure is not dependent on renal nerve integrity, but mechanistically accompanied by a resetting of the RSA-baroreflex function curve, sensitisation of the cardiac baroreflex, changes in renal excretory function and reduced T-lymphocyte infiltration. We further show that, combined with renal denervation, CSD remains effective, producing a summative response indicative of an independent mechanism. Our findings indicate that carotid body de-afferentation is an effective means for robust and sustained sympathoinhibition, which is ripe for translating to patients with neurogenic hypertension.

## Introduction

The causes of human essential hypertension remain unknown. It is established that in many patients, sympathetic nerve activity increases proportionately as hypertension develops and this may be a causative factor<sup>1-5</sup>, but it is unclear what triggers heightened sympathetic traffic (reviewed in<sup>6</sup>). Despite good pharmacological control of arterial pressure in most patients with essential hypertension, a significant proportion remain drug resistant or intolerant to medication<sup>7,8</sup> and this has been highlighted as a growing problem<sup>9,10</sup>. This presents a significant clinical challenge, and provides the motivation to discover novel ways to control arterial pressure.

Recently, the role of the carotid body (CB) chemoreceptors in the pathophysiology of cardiovascular disease is gaining considerable interest<sup>11-15</sup>. The sympathoexcitation in both sleep apnoea and heart failure may originate from enhanced activity of the CB chemoreceptors<sup>16-20</sup>. In both human patients and animal models of hypertension the CB chemoreflex-evoked sympathoexcitatory responses are enhanced<sup>15,21-23</sup>. However, the hypothesis that CB chemoreceptor drive plays an important role in the pathogenesis and/or maintenance of high arterial pressure was undetermined until most recently. We found that in spontaneously hypertensive (SH) rats, carotid sinus denervation (CSD) lowered arterial pressure by ~17 mmHg and that this was well maintained and tolerated<sup>11</sup>. These data are comparable to those in human hypertensive patients where inactivation of the CB with 100% oxygen reduced both arterial pressure and sympathetic activity<sup>24</sup> supporting a causal role for peripheral chemoreceptors in the aetiology of neurogenic hypertension. Based on these data, and that CB resection has been performed and well tolerated for the treatment of dyspnoea in ~5,600 patients with asthma and chronic obstructive pulmonary disease (reviewed in<sup>14</sup>), we recently proposed that CB ablation may be an effective interventional strategy to control blood pressure in drug resistant hypertensive patients<sup>11,14</sup>.

To assess the mechanisms underlying the hypotensive effect of CSD, we have made direct long-term simultaneous recordings of blood pressure and renal sympathetic activity (RSA) before and after CSD in conscious SH and Wistar rats. Given the current clinical interest and application of renal denervation (RD) in the treatment of drug resistant hypertension<sup>25-29</sup>, we have characterised the interaction between CSD and RD in combination to establish the type of interaction: summative, occlusive or facilitatory. Given that T lymphocytes have been shown to contribute to both angiotensin II and neurally mediated chronic hypertension<sup>30,31</sup> we have assessed immune function following CSD. Unlike Wistar rats, we find that the CB of the SH rat has resting tone driving hypertension, which is not dependent on renal nerves, depresses both the cardiac and sympathetic vasomotor baroreflex, and attenuates the adaptive immune response.

## **Results**

All numerical data is reported as mean±standard error margin (SEM), with statistical significance ( $P<0.05$ ) determined using two-way ANOVA (repeated measures for within-subject comparison), followed where appropriate with the Holm-Sidak post hoc test.

### **Transient inactivation of CB activity with hyperoxia**

In SH rats, exposure to 100% oxygen to inactivate the CB produced falls in both SBP ( $-12\pm 4$  mmHg,  $n=5$ ,  $P=0.047$ ) and RSA ( $-2.9\pm 0.4$   $\mu$ V,  $n=5$ ,  $P=0.019$ ) but no consistent change in heart rate (Fig 1). In stark contrast, pure oxygen was without significant effect on these variables when delivered to Wistar rats or SH rats after CSD (Supplementary Fig S1). These data provide evidence that in SH rats, tonic afferent activity emanating from the CB provides a potent drive for neurogenically mediated hypertension.

### **Peripheral chemoreflex sensitivity in Wistar and SH rats**

Whilst previous studies have found increased reflex peripheral chemosensitivity in anaesthetised and decerebrate SH versus Wistar rats<sup>15</sup>, there are no data in conscious animals. Compared to Wistar rats, SH rats showed a more profound peripheral chemoreceptor reflex evoked bradycardia ( $-194\pm 5$  vs.  $-126\pm 54$  bpm,  $n=6$ ,  $P=0.046$ ) and RSA excitatory responses ( $13.4\pm 0.8$  vs.  $9\pm 0.6$   $\mu$ V,  $n=6$ ,  $P=0.021$ ), but not a pressor response ( $54\pm 8$  vs.  $48\pm 21$  mmHg;  $p=0.811$ ) following an i.v. sodium cyanide bolus.

### **Time profile of RSA response to CSD in SH and Wistar rats**

Following the surgery related increase in RSA, there was a prompt and profound reduction in RSA below pre-operative baseline after CSD in SH rats ( $-1.94\pm 0.3$   $\mu$ V,  $-56\pm 12\%$ ;  $n=6$ ,  $P=0.027$ ; Fig 2a,b & c). The peak fall occurred 5 days post-CSD, and showed no signs of recovery throughout the 3 week experimental period. The fall in RSA was correlated temporally with a fall in MAP of  $-17\pm 3$  mmHg from a baseline of  $137\pm 7$  mmHg ( $n=6$ ,  $P=0.009$ ). Spectral analysis also revealed a reduction in LF SBP from  $3.5\pm 0.2$  to  $2.5\pm 0.2$

mmHg<sup>2</sup> ( $P < 0.012$ ; Table 1) suggesting a global reduction in sympathetic traffic. In these animals, we also tested efficacy of CSD: Fig 2d shows that the sympathoexcitatory reflex response following i.v. sodium cyanide was blunted severely. Despite the substantial fall in basal RSA after CSD, a stressful stimulus (high frequency noise) continued to evoke a significant ( $P = 0.013$ ) sympatho-excitatory response in SH rats, the magnitude of which was not different to those evoked prior to CSD (before:  $139 \pm 28\%$ , after:  $153 \pm 13\%$ ,  $n = 6$ ,  $P = 0.529$ ; Fig 4e). This test served to both validate the viability of our chronic recordings and showed that despite lower resting levels of RSA, alerting/arousal responses remained intact ensuring that fundamental visceral reactions were preserved. In Wistar rats, although there was no significant change in MAP after CSD ( $-6 \pm 5$  mmHg,  $n = 6$ ), there was a small fall in RSA ( $-0.9 \pm 0.1$   $\mu$ V,  $-18 \pm 7\%$ ,  $n = 6$ ,  $P = 0.045$ ; Fig 2a,b,c), which was much less than that seen in SH rats ( $P < 0.01$ ; Fig 2a,b,c).

### ***Baroreflex function improvement following CSD***

Cardiac baroreceptor reflex function curves were constructed recently after CSD<sup>11</sup> and showed an increase in sensitivity, which might contribute to the hypotensive effect of CSD; hence, we have analysed this in more detail. Consistent with this was the new finding that spontaneous cardiac baroreflex gain increased from  $-1.16 \pm 0.09$  to  $-1.53 \pm 0.27$  bpm.mmHg<sup>-1</sup> after CSD ( $n = 7$ ,  $P < 0.01$ ). We also found an improvement in the sympathetic baroreflex sensitivity after CSD in SH rats (Fig 3) where the curve was shifted leftwards ( $n = 5$ ,  $P = 0.032$ ) and the operating point moved to the left and downward ( $P = 0.026$ ; Fig 3). An increase in the sensitivity of the curve was revealed by increases in both the upper and lower curvature parameters (see Supplementary Table S1) which translated into an increase in maximum gain ( $-6.8 \pm 1.7$  to  $-9.7 \pm 1.9\%$ .mmHg<sup>-1</sup>,  $P = 0.028$ ) towards that seen in Wistar rats ( $13.4 \pm 3.1\%$ .mmHg<sup>-1</sup>). No significant changes in cardiac or sympathetic baroreflex function were found in Wistar rats post CSD (Supplementary Fig S2).

### ***Reduced T cell infiltration into the aorta after CSD and RD***

Inflammation, in particular the adaptive immune response, has been shown to play a significant role in the pathogenesis of hypertension<sup>30-32</sup>. We therefore examined the effects of CSD and RD on the percentage of total aortic leukocytes (CD45+ average total cells  $1.9 \times 10^4$  / aorta) and T lymphocytes (CD3+ average total cells  $2.5 \times 10^3$  / aorta). The brainstem has also been identified as a site of increased inflammatory molecules during hypertension<sup>33,34</sup>, so we have examined the percentage of CD45+ CD3+ cells (average total cells  $3.6 \times 10^2$  / brainstem) within this tissue. As shown in figure 3b, compared to the surgical shams there were no significant differences in the percentage of aortic CD45+ cells following CSD or RD procedures. However, when we examined the number of aortic infiltrates of CD3+ cells, there was a significant decrease in both the CSD (Figure 3c-d;  $p < 0.01$ ) and RD groups (Figure 3c-d;  $p < 0.01$ ). Similarly, in single cell suspensions of whole brainstem homogenates, a significant reduction in percentage of CD3+ cells was determined following RD (Supplementary Figure S3;  $p < 0.05$ ) and a trend, that did not reach statistical significance, for a reduction in CD3+ cells in the CSD group. These data suggest that both CSD and RD procedures can reduce T cell mediated infiltration of tissue that has been associated with many forms of hypertension.

### ***Renal Excretory Function after CSD***

Given the alterations in arterial pressure and RSA, we looked to see if renal function was altered. Despite our observed reduction in arterial pressure, SH rats showed an increase in 24-hour urine volume at 12 days following CSD (from  $4.25 \pm 0.28$  to  $5.57 \pm 0.32$  ml/24h/100g body weight;  $p < 0.05$ ), which was sustained at days 21 and 28. This was accompanied by an increase in creatinine clearance ( $0.94 \pm 0.03$  to  $1.15 \pm 0.07$  ml/min;  $p < 0.01$ ), indicating an increase in glomerular filtration rate (GFR). The total protein in urine decreased significantly ( $10.5 \pm 1.0$  to  $8.7 \pm 0.8$  mg/ml;  $p < 0.05$ ), albuminuria was lower after CSD but not significant ( $0.57 \pm 0.05$  to  $0.45 \pm 0.05$  mg/ml;  $p = 0.067$ ), although water intake also increased significantly ( $10 \pm 1.2$  to  $12.8 \pm 1.6$  ml/24h/100g body weight;  $P < 0.01$ ). For comparison, Wistar rats had a lower total protein and albumin level in their urine compare to SH rats before denervation:  $9.75 \pm 0.84$  vs  $10.49 \pm 0.77$  mg/ml and  $0.40 \pm 0.06$  vs  $0.57 \pm 0.05$  mg/ml, respectively. The water



intake and creatinine clearance was similar between Wistar and SH rats before denervation:  $30.81 \pm 2.13$  versus  $30.49 \pm 2.13$  ml/24h and  $0.83 \pm 0.07$  versus  $0.94 \pm 0.03$  ml/min, respectively. Taken together these data indicate that the progression of glomerular kidney damage/leakage in SH rats may have been retarded by CSD.

### **Time to sympatho-inhibition after CSD in SH rats**

In conscious SH rats it was not possible to assess the precise time to onset of the depressor and sympatho-inhibition following CSD, due to the side effects of surgery and anaesthesia. To this end, we recorded internal cervical and lumbar sympathetic post-ganglionic nerves simultaneously before and after CSD in *in situ* unanesthetised decerebrated SH rats. Figure 4 shows that by 40 min there was already a significant decrease in sympathetic nerve activity in all outflows and that this was due, in part, to a decrease in the amplitude of respiratory-modulated discharge ( $P < 0.05$ ; Fig 4b). The peak response was achieved at 60 min post CSD as activity levels at 75 or 90 min were not different to the level recorded at 40 min post CSD but were different to their own baseline (both  $P < 0.01$ ; Fig 4c). Consistent with the reduction of respiratory-sympathetic modulation, we found a decrease in HF SBP in conscious SH rats after CSD (from  $4.52 \pm 0.3$  to  $3.9 \pm 0.3$  mmHg<sup>2</sup>;  $n=7$ ,  $P < 0.05$ ).

### **Comparing uni- versus bilateral CSD in SH rats**

We wished to test any difference in the sympathoexcitatory drive from the left versus the right CB as well as their individual responses. Figure 5 shows the time profile of the response in SBP following unilateral and subsequent bilateral CSD. Unilateral CSD, whether targeting the left ( $n=4$ ) or right side ( $n=4$ ) produced no change in SBP (Supplementary Table S2). The fall in SBP achieved when bilateral CSD was staggered in time by 15 days (i.e. denervating left then right CB, or denervating right then left CB) was no different to the response when both the CBs were denervated at the same time ( $-16 \pm 2$  vs.  $-17 \pm 3$  mmHg, respectively,  $p=0.831$ ). In our *in situ* preparation which allowed high temporal resolution of

changes in sympathetic activity (see above), unilateral CSD did not produce a measurable change in any of the recorded variables.

### **Systolic blood pressure response to combined CSD and RD**

Given the current interest in RD<sup>25-29</sup>, the relative potency and interaction (occlusive, summative, facilitatory) of RD versus CSD and was deemed scientifically and clinically important. All sham-operated SH rats showed a time related increase in SBP ( $4\pm 4$  and  $3\pm 2$  mmHg for sham CSD-RD and sham RD-CSD respectively; Figs 6 & 7). The efficacy of RD was confirmed by measuring renal noradrenaline content after RD (with or without concurrent CSD), which showed a significant reduction compared to sham controls (RD alone:  $45\pm 6$ ; RD with CSD:  $31\pm 9$ ; sham:  $443\pm 54$  pg/100 $\mu$ g protein;  $n=18$ ,  $P<0.01$ ). The data reported herein are all expressed relative to the pre-operative control level.

Figure 6 shows the time profile of the SBP response to RD followed by CSD (Fig 6a) and CSD followed by RD (Fig 6b). In SH rats, performing RD first produced a fall of SBP of  $-9\pm 2$  mmHg from a baseline of  $166\pm 5$  mmHg ( $n=6$ ,  $P<0.013$ ). A further fall of  $-12\pm 3$  mmHg ensued after subsequent CSD ( $P<0.001$ ) and showed no recovery over the observational period. In SH rats undergoing the CSD-RD surgical sequence, basal SBP was  $160\pm 8$  mmHg, CSD alone evoked a prompt fall of  $-14\pm 2$  mmHg ( $n=7$ ,  $P<0.001$ ) with a further fall of  $-6\pm 2$  mmHg ( $P<0.001$ ) after RD; no recovery was observed over the time course of the study. The total fall ( $-17\pm 2$  mmHg) was not different to that measured with the reversed sequence of RD-CSD ( $-20\pm 3$  mmHg;  $p=0.363$ ). These data are summarised in Figure 7 and show an additive interaction between RD and CSD that this is similar irrespective of the order in which the surgical procedures are carried out in SH rats. CSD, but not RD, produced significant bradycardia ( $P=0.018$ ) and a small reduction in respiratory frequency ( $P<0.01$ ) in SH rats (Table 1). Table 1 documents spectral frequency analysis of both pulse interval and SBP after RD-CSD and CSD-RD. RD alone resulted in an increase in spontaneous baroreflex gain ( $-1.73\pm 0.4$  bpm.mmHg<sup>-1</sup> mmHg;  $p<0.01$ ; Table 1) and decrease in CD3+

cells (Figure 3b-d;  $p < 0.01$ ). Figure 6c demonstrates that the reflex bradycardia and pressor responses to sodium cyanide (i.v.), used to activate peripheral chemoreceptors in conscious SH rats, were abolished after bilateral CSD.

## Discussion

Our data demonstrate that the CB in the conscious SH rat is constitutively active and provides a tonic pro-hypertensive drive. Mechanistically, the anti-hypertensive effects of CSD in SH rats include: a substantial reduction in sympathetic nerve discharge including that to the kidney involving a reduction of respiratory modulation; a resetting of the sympathetic-baroreflex over lower arterial pressures; an improvement in spontaneous cardiac-baroreflex gain; possible changes in renal excretory function; and a reduction in vascular T cell infiltration. Unilateral CSD, whether left or right side, was ineffective in reducing arterial pressure, but subsequent resection of the contralateral side remained an effective anti-hypertensive therapy. In conscious SH rats, we show that CSD is more potent as an anti-hypertensive intervention than RD, but that the renal nerves are not necessary for the hypotensive effect of CSD. Combining RD with CSD, or reversing the sequence (CSD then RD), gives an additive response that is similar in magnitude irrespective of the order in which the surgical procedures are performed.

RD in young SH rats prevents the full development of hypertension<sup>35,36</sup>. The fall in arterial pressure of ~9 mmHg we report herein following RD in Wistar rats is consistent with previous studies in Sprague Dawley rats<sup>37</sup> but not with Wistar Kyoto rats where arterial pressure was unaltered<sup>35,38</sup>. Previous reports in SH rats demonstrated larger falls in arterial pressure after RD (~19 mmHg<sup>36,38</sup>) relative to our results (~9 mmHg). The difference may reflect the use of tail cuff/indwelling catheters rather than radio-telemetry, and between-group versus within-animal analyses. Despite these quantitative differences, renal nerves (afferent and/or efferent) play a tonic role in regulating arterial pressure in some strains of normotensive and SH rats, as reviewed recently<sup>39</sup>.

In conscious SH rats, our finding that the CB chemoreflex sensitivity is elevated relative to the Wistar animal is consistent with prior data from animals and humans<sup>15,21-23</sup>. For the first time, we describe heightened CB *tonicity* in SH rats as revealed by a depressor and sympatho-inhibitory response to hyperoxia in SH but not Wistar rats. This suggests that the CB has elevated activity in the SH rat providing excitatory drive to the generation of sympathetic vasomotor tone that in turn contributes to the hypertension, as recently described in hypertensive human patients<sup>24</sup>. It may also explain why CSD is effective in producing a long-term anti-hypertensive response. The tonic activity in SH rats may result from increased expression of ASIC3 and TASK1 - two acid-sensing non-voltage gated channels<sup>15</sup>. Others suggest that the balance between carbon monoxide and hydrogen sulphide within the CB and the activity of HIF-1 $\alpha$  versus HIF-2 $\alpha$  play a role<sup>40</sup>. Animal models of chronic heart failure have revealed that CB chemoreceptor activity is augmented by: angiotensin II receptors<sup>41</sup>, impaired nitric oxide synthase activity<sup>20</sup>, reduced CB blood flow<sup>42</sup>, enhanced adenosine monophosphate-activated protein kinase<sup>43</sup> and inflammation<sup>44,45</sup>. Heightened sympathetic drive to the arterioles of the CB itself may also contribute to its hyperactivity due to hypoperfusion (via vasoconstriction and vascular remodelling<sup>46</sup>) and inflammation.

Because of the complications of anaesthesia and surgical recovery, we were unable to determine the precise time-dependent effects following CSD in conscious SH rats. Using our *in situ* rat preparation, a fall in perfusion pressure and sympathetic activity occurred at 40 min post CSD indicating a relatively rapid response. Presumably 40 min permits a reconfiguration of the cardio-respiratory brainstem network in the *in situ* rat, as reflected by a reduction in both respiratory-sympathetic coupling and the magnitude of the Traube-Hering wave amplitude. Consistent with reduced respiratory-sympathetic coupling, we saw a decrease in HF SBP spectra in conscious SH rats after CSD. Given that respiratory-sympathetic coupling contributes significantly to vascular resistance and arterial pressure

development the SH rat<sup>47</sup>, this could be a major mechanism for the CSD evoked anti-hypertensive effect.

It was evident that removal of a single CB, whether the left or right, *in vivo* or *in situ*, was ineffective in lowering arterial pressure in SH rats, although there was a brief reduction in respiratory frequency perhaps indicative of a transient effect. Given that CB afferents terminate in the commissural NTS with axons traversing the midline providing ample innervation of contralateral targets<sup>48</sup>, it perhaps is not surprising that unilateral CSD was not effective long-term.

On subsequent removal of the second CB from a SH rat with chronic, unilateral CSD, arterial pressure fell precipitously; the level reached was not different to that recorded following a procedure where both CBs were removed at the same time. This suggests that any compensation occurring after loss of a single CB does not affect the efficacy of response following contralateral CB denervation. Whether these findings relate to humans with hypertension is unknown, but provides the following predictions: (i) unilateral CSD may be effective transiently in lowering arterial pressure and compensated for with time; (ii) subsequent CB resection following a unilateral procedure will cause a potent hypotensive effect. For safety reasons, a clinical study should start with unilateral CB ablation; thus, knowing that a hypotensive effect persists following staggered bilateral CSD (i.e. not ameliorated by a prior unilateral intervention) is important pre-clinical information.

Previously, we have shown that CSD in young, pre-hypertensive SH rats prevented the full manifestation of hypertension, and lowered arterial pressure in adult SH rats<sup>11</sup>. Using both power spectral analysis and hexamethonium to block ganglionic transmission, we provided indirect evidence that the hypotension was caused, in part, by a generalised reduction in sympathetic activity<sup>11</sup>. Here, we demonstrate for the first time that the CSD induced hypotension was accompanied with a substantial reduction in RSA that fell with a similar

time course. Despite this robust reduction of ~55% in RSA, we showed that animals were not left sympathetically incompetent and, importantly, could respond vigorously to a stressor stimulus. Given that lumbar and cervical sympathetic outflows also fell (as shown acutely), we suspect that there is widespread sympathoinhibition following CSD and that it is not confined to the kidneys. This is supported by a chronic reduction in low frequency spectra of SBP. This has important clinical implications regarding the applicability of CSD beyond hypertension, to conditions such as heart failure, sleep apnoea and insulin resistance where there is excessive and pathological sympathetic activity<sup>14</sup>.

The mechanisms for blood pressure reduction following CSD are likely to be numerous, and operate over different time scales giving a persistent effect. Our hyperoxia data clearly shows that removal of CB tone in SH rats can, at least acutely, provide sympathoinhibition. This may suggest a hypoxic drive within the CB of the SH rat, which might be the stimulus for the increased density of small vessels in the CB of SH versus normotensive rats<sup>46</sup>. However, we do not rule out non-hypoxic intra-CB drives to explain the tonicity within the SH rat CB. Other mechanisms by which CSD lowers blood pressure include an improvement in both cardiac and sympathetic vasomotor limbs of the baroreflex after CSD (see<sup>11</sup>). Because our method of CSD removes innervation of the carotid sinus baroreceptors, the improvement in baroreceptor reflex function must be of aortic baroreceptor origin. Based on this observation, it is expected that selective removal of the carotid body, sparing carotid sinus baroreceptors, would be predicted to enhance their gain also and produce a greater anti-hypertensive effect. The known antagonistic interaction between chemo- and baro- afferents both centrally (at a neuronal level within nucleus tractus solitarius<sup>49</sup>) and of their respective reflex responses<sup>50</sup> could explain this beneficial result as enhanced baroreflex function can contribute to the long-term lowering of blood pressure and sympathetic activity in dogs<sup>51</sup> and humans<sup>52</sup>. We do not rule out improved compliance of the aorta, central plasticity and/or improved cardiac vagal transmission to explain better baroreflex function after CSD in SH rats. The finding of reduced internal cervical sympathetic activity may improve brainstem

blood flow; given that the SH rat brainstem is hypoperfused, improving flow may contribute to reducing arterial pressure<sup>53</sup>. Our observation of increased urine production and GFR in the face of decreased systemic arterial pressure, indicates that CSD may be associated with active improvements in renal excretory function. This may be mediated at least in part directly through the substantial inhibition of renal sympathetic activity, and may contribute to the hypotensive effect of CSD through fluid excretion. The reduced percentage of vascular and brainstem infiltrates of T lymphocytes is unlikely to contribute to the prompt fall in arterial pressure because of its rapid time course but may assist in the long term maintenance of the hypotensive effect of CSD especially since T lymphocytes have been shown to contribute to both angiotensin II and neurally mediated chronic hypertension<sup>54,55</sup>. We speculate that the sympathoinhibitory effect of CSD contributes to reduced T cell infiltration in the aorta and brainstem described herein, which could improve vascular compliance including aortic baroreceptor sensitivity, and improved baroreflex transmission centrally<sup>33,34</sup>. The location of the vascular immune cell infiltrates is unknown, but we speculate based on previous studies<sup>56</sup> that these cells predominantly accumulate in the adventitia and perivascular adipose tissue of blood vessels. Given the sympathetic innervation of immune system organs such as the spleen, thymus and bone marrow<sup>26</sup>, we suggest that reductions in sympathetic activity may be the trigger for the anti-inflammatory response thereby breaking this positive (and pathological) feedback loop<sup>6</sup>.

One successful new anti-hypertensive interventional approach is renal denervation (RD), which appears to be a safe treatment in some humans with drug-resistant hypertension<sup>25-29</sup>. In one study blood pressure was lowered by >10 mmHg with a high success rate (84%), although all patients remained hypertensive and their anti-hypertensive medication was maintained<sup>25</sup>. In contrast, an 84% success rate was not confirmed by other groups who reported no overall change in arterial pressure, although some individuals did respond<sup>27</sup> or around a 40-50% blood pressure response rate<sup>26,28,29</sup>. Given these findings, and the recent success of unilateral CB resection in a heart failure patient<sup>57</sup>, CB ablation to control arterial

pressure in drug resistant hypertensive patients could be considered. Thus, the following scenarios are imagined and include patients in which RD has: (i) not lowered arterial pressure to target; (ii) not circumvented prescribed and poorly tolerated medication; (iii) failed and (iv) not been possible due to renal artery stenosis or bifurcation. Thus, it becomes critical to study any interaction on arterial pressure following combined RD and CSD as well as CSD alone procedures.

The finding that unilateral CB resection in a heart failure patient was effective in correcting partially both cardiac autonomic balance and cardiac baroreflex gain<sup>57</sup> yet ineffective in altering these variables in SH rats is curious. Clearly, heart failure and hypertension have distinct pathologies, which could explain this difference. Equally, there may be differences in CB signalling mechanisms between SH rats and humans that could contribute to response differences between unilateral versus bilateral CB resection. This remains an open question.

In conclusion, there is an additive anti-hypotensive interaction between CSD and RD, which is unaffected by either the order or timing of the procedures. Bilateral CSD provides a potent and persistent anti-hypertensive response in SH rats accompanied by substantial sympathoinhibition in multiple outflows, improved baroreflex and renal function, and reduced T lymphocyte infiltration in the aorta and brainstem. Whether CSD offers therapeutic benefit to drug resistant/intolerant human patients with sympathetically mediated diseases is currently undergoing clinical trial.



## **Methods**

Animal experiments were conducted in accordance with the UK Animals (Scientific Procedures) Act 1986 and associated guidelines and approved by the University of Bristol Ethics Committee. Adult male (12 weeks; n=38) SHR and Wistar rats (12 weeks; n=6) bred in the University of Bristol Animal Services Unit were used. For all surgeries rats were anaesthetised with ketamine ( $60 \text{ mg kg}^{-1}$ ; i.m.) and medetomidine ( $250 \text{ } \mu\text{g kg}^{-1}$ , i.m.) and aseptic techniques used.

### ***Renal and carotid body (CB) surgery***

For CSD, the CB was approached via an anterior midline neck incision and the carotid sinus nerve branches sectioned<sup>11</sup>. Sham-operated rats underwent the same surgical procedures to expose the CB but the carotid sinus nerves were left intact. Unilateral CSD rats (SH, n=8) were randomised to have either their left (n=4) or right (n=4) carotid sinus denervated first, followed by contralateral denervation 14 days later. On denervating the second CB a transient apnoea occurred in all SH rats with some requiring resuscitation. This apnoea was not observed in Wistar rats. No post-surgical mortalities occurred suggesting that there were no lethal apnoeic episodes post-CSD, and all rats gained weight normally. To assess the efficacy of CSD, arterial pressure was recorded (and from this heart rate and respiratory frequency were derived) during an injection of sodium cyanide (NaCN,  $100 \text{ } \mu\text{l}$  0.04% i.v.), 1-2 weeks post-CSD.

Bilateral renal denervation (RD) was achieved via a retroperitoneal incision to allow retraction of each kidney and exposure of the renal artery. The nerves and adventitia were stripped from the renal artery and renal plexus, which were then painted with a dilute (10% in ethanol) phenol solution. Sham-operated rats had both kidneys exposed via the retroperitoneal incision and the renal nerves visualised but left intact.

### **Renal sympathetic activity response to CSD**

In rats (SHR n=6, Wistar n=6) following implantation of the blood pressure catheter into the abdominal aorta and closure of the midline incision<sup>33</sup>, the right kidney was exposed via a retroperitoneal incision. Using a fine round-tipped glass hook the renal nerve was freed from the surrounding connective tissue, and a small patch of parafilm slipped underneath to prevent fluid ingress. The nerve was passed over the electrodes from a sympathetic nerve activity telemeter (Model TR46SP, Telemetry Research Ltd, Auckland, New Zealand), carefully fixed in place using sutures, then isolated from surrounding tissue with silicone elastomer (Kwik-Sil, WPI Inc.). A polyurethane catheter (0.033 in (~0.84 mm) OD; 0.014 in (~0.36 mm) ID; Micro-Renathane, Braintree Scientific) was implanted into the left femoral vein, connected with to a transcutaneous port between the scapulae and flushed every 2-3 days with saline containing heparin (100 U ml<sup>-1</sup>) and penicillin G (2000 U ml<sup>-1</sup>) solution<sup>11</sup>. On days -2 and 8, the venous lines were connected and solutions of phenylephrine (0.1 mg/ml) and sodium nitroprusside (0.1 mg/ml) were infused i.v. to obtain ramp increases in arterial pressure of 1-2 mmHg/s between ~60-180mmHg. These data were then used to generate 5-parameter sigmoidal regression baroreflex function curves. A brief sodium cyanide bolus (NaCN, 100 µl 0.04% i.v.) was given to stimulate peripheral chemoreceptors. The 'zero' level of renal sympathetic activity (RSA) was assessed after ganglionic blockade using hexamethonium tartrate (100 µl, 10mg/ml i.v.). To assess any tonic activity generated by the CB, hyperoxia was used to 'silence' the CBs by briefly placing their cages into a plexiglass box connected to a 100% oxygen source on day -1. Arterial pressure was recorded 24 hours per day while RSA was recorded for 60 minutes at approximately the same time each day when animals were observed to be resting quietly.

Arterial pressure and RSA signals were sampled at 500Hz using an analog-digital data acquisition card (PCI 6024E National Instruments, Austin, Texas) and continuously displayed by a data acquisition program (Universal Acquisition 11, University of Auckland, Auckland, New Zealand). Heart rate (HR) was derived from the arterial pressure waveform.

The RSA signal was amplified, filtered between 50-5000 Hz, full-wave rectified and integrated using a low pass filter with a 20ms time constant. At least 7 days were allowed for recovery before a 3 day baseline period was recorded. Arterial pressure and heart rate were recorded for 24 hours a day. To avoid movement artefact, RSA was recorded for an hour at the same time each day while the rats undisturbed in their home cage were observed to be quiescent. On days -2 and 7, rats were exposed to a brief burst of high frequency noise, which produces a stress increase in RSA. The magnitude of this response was used to check that the RSA signal remained viable and responsive throughout the experiment.

### ***Renal Excretory Function***

Rats were housed in metabolic cages with free access to water and food for 24h. Blood was collected via withdrawn from tail vein. Plasma and urine creatinine were measured by improved Jaffe method with the commercially available QuantiCrom Creatinine Assay Kit (DICT-500, Universal Biologicals, Cambridge, UK)<sup>58</sup>. Creatinine clearance was estimated as (urinary creatinine [ $\mu\text{mol/l}$ ]  $\times$  volume urine produce in 24 hours [mls])/(plasma creatinine [ $\mu\text{mol/l}$ ]  $\times$  1440 [minutes]). Urinary albumin was measured by the improved BCG method<sup>59</sup> with the commercially available Albumin Assay (Randox Laboratories Ltd, Crumlin, UK) and the total protein was measured by the Lowry method<sup>60</sup> with the DC Protein Assay Kit (Bio-Rad Laboratories Ltd, Hemel Hempstead, UK). Urinary sodium was measured using an ion-selective electrode (Cole-Parmer, London ,UK). Renal excretory function was assessed over a 24 h period before (Day -2) and after (Day 28) CSD (n=6) or sham (n=6) surgery.

### ***Renal noradrenaline content***

The left kidneys were crushed in liquid nitrogen and homogenised in an extraction buffer (0.01M HCl, 1mM EDTA, 4mM sodium metabisulfate). Samples were centrifuged (8000 rpm for 30 min) and the supernatant protein concentration determined by DC-Protein assay (BioRad, UK, No 500-0112). Tissue noradrenaline content was assessed by ELISA (Alpco Diagnostics, USA, No 17-NORHU-E01-RES) and normalised to protein concentration.

### ***FACS analysis of cellular inflammation***

Analysis of T cells in vascular homogenates of the aorta and brainstem was performed using fluorescence-activated cell sorting (FACS). To analyze leukocytes in the aorta and brain, tissue was digested using collagenase type IX (125u/ml); collagenase type IS (450U/ml) and hyaluronidase IS (60U/ml) dissolved in 20 mM HEPES-PBS buffer for 30 minutes at 37° C, while constantly agitated. The dissolved tissue was then passed through a 70 m sterile filter (Falcon, BD), yielding a single cell suspension. An additional step was applied for brain tissue using a 30% / 70% percoll gradient to separate out the mononuclear cell layer. All cells were then washed twice with FACS buffer (0.5% bovine serum albumin in PBS) then counted, stained and analyzed using multi-color flow cytometry<sup>61</sup>. Antibodies (BD Biosciences) used for staining were as follows: V450 anti-CD45; PE anti-CD3. After immunostaining, cells were re-suspended in FACS buffer (0.5% bovine serum albumin in PBS) and analyzed immediately on a LSR-II flow cytometer with DIVA software (Becton Dickinson). Data were analyzed with FlowJo software (Tree Star Inc., Ashland, Oregon, USA).

### ***In situ working heart-brainstem preparation***

As described originally<sup>62</sup>, animals were anaesthetized deeply (halothane), heparinised (1000 units i.p.), bisected below the diaphragm and decerebrated pre-collicularly. Anaesthesia was terminated. Preparations were transferred to a recording chamber and a double lumen cannula was placed into the descending aorta for retrograde perfusion with a Ringer solution containing in mM: NaCl (125), NaHCO<sub>3</sub> (24), KCl (3), CaCl<sub>2</sub> (2.5), MgSO<sub>4</sub> (1.25), KH<sub>2</sub>PO<sub>4</sub> (1.25), and D-glucose (10). Ficoll (MWt 20,000; 1.25%) was added as an oncotic agent and the perfusion solution was saturated with 95% O<sub>2</sub>-5% CO<sub>2</sub> (pH 7.35-7.4; osmolality 290±5 mosm±kg H<sub>2</sub>O<sup>-1</sup> at 31°C). Perfusion pressure was maintained between 60-80 mmHg with additions of arginine vasopressin (200–400 pM, Sigma). A muscle relaxant, vecuronium bromide (4 µg/ml; Organon Teknica, Cambridge, UK) was added to the perfusate.

Recordings from the phrenic, lumbar and internal cervical sympathetic nerves were made simultaneously using custom bipolar glass suction electrodes. Signals were amplified (x20k), filtered (60-3000 Hz), rectified and integrated (50 ms time constant) and the noise level (obtained at the end of the experiment) subtracted. Phrenic triggered averaging of integrated sympathetic activity (over 10 phrenic cycles) allowed peak-to-trough amplitudes of the respiratory related sympathetic burst to be measured. The Traube-Hering waves were also analyzed using phrenic triggered averaging.

### ***Cardiovascular responses to CSD and RD***

Radio-telemeters (PA-C40, Data Sciences Inc, St Paul, Minnesota, USA) were implanted as described before<sup>33,34</sup>. After seven full days of recovery, four days of baseline arterial pressure was recorded prior to the first surgical intervention (All SH rats: CSD, n=7; sham-CSD n=7; RD, n=6; sham-RD n=6). 21 days after CSD/sham-CSD, RD surgery was performed; conversely, 14 days after RD/sham-RD, CSD surgery was performed. Arterial pressure was recorded for 5 min every hour, 24 hours per day using Hey Presto software, and heart rate and respiratory rate were derived from the arterial pressure waveform<sup>33</sup>. The data shown for cardiovascular parameters and ventilatory frequency represent daily averages from these 5 minute periods. On the final day of the experiment, rats were euthanized (sodium pentobarbital 40mg/kg i.p.) and the kidneys extracted, weighed and frozen in liquid nitrogen for a noradrenaline assay. The aorta was removed for fluorescence-activated cell sorting (FACS) and analysis of cellular inflammation (see below).

### ***Power spectral analysis***

Power spectral density was computed using purpose-written software<sup>33</sup>. The following frequencies were calculated in normalized units: <0.27 Hz (very low frequency; VLF), 0.27-0.75 Hz (low frequency; LF) and 0.75-3.3 Hz (high frequency; HF). The ratio of the LF to the HF component was used as an indicator of cardiac sympatho-parasympathetic balance.

Spontaneous baroreflex gain was computed and respiratory rate was inferred from the peaks of respiratory modulation of the systolic pressure frequency spectrum.

### ***Data analysis***

From the arterial pressure waveform, values for systolic, diastolic, mean pulse pressure, heart rate and respiratory rate were derived. RSA is expressed as a percentage, where 100% is the average integrated value across the baseline period. Values are presented as mean  $\pm$  SEM except where stated. Data were compared between baseline and CSD, and where applicable, RD time points, by repeated measures two-way ANOVA, followed where appropriate by the Holm-Sidak post hoc multiple comparison test. Differences within or between groups with p values of  $<0.05$  were considered significant.

## **Acknowledgments**

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## **Author Contribution**

FDM performed the majority of experiments and analysis assisted by APA and EBH. WP performed the renal function and noradrenaline assays. PM performed FACS analysis. DJAM contributed to all data in Fig 6. PAS provided intellectual input. JFRP designed/conceived the experiments, provided funding, wrote the manuscript with FDM and PM.

## **Conflict of interest**

JFRP is a consultant for Cibiem, and PAS is the Chief Medical Officer for Cibiem.

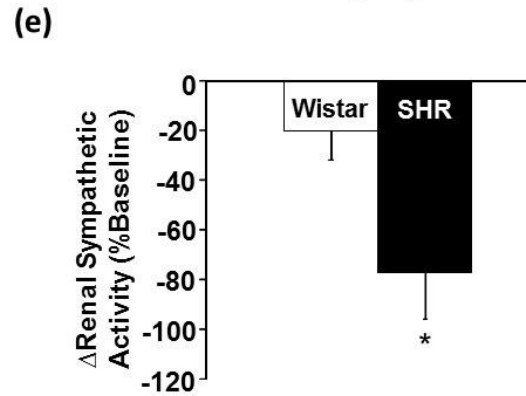
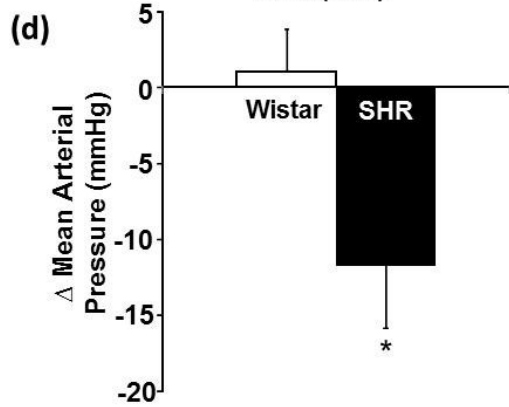
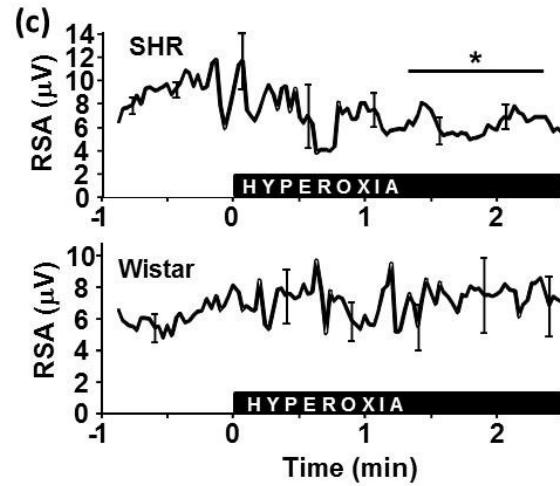
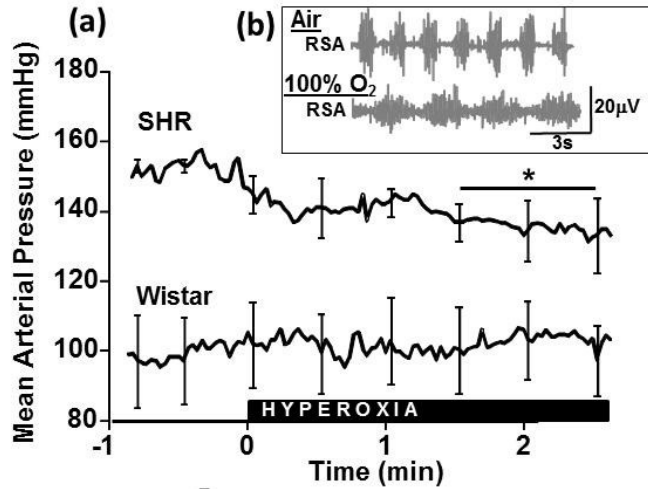
CSD + RD (n=7)		MBP (mmHg)	HR (beats.min <sup>-1</sup> )	sBRG (ms.mmHg <sup>-1</sup> )	RR (breaths.min <sup>-1</sup> )	LF:HF (PI)	VLF (SBP - mmHg <sup>2</sup> )	LF (SBP - mmHg <sup>2</sup> )	LF:HF (SBP)
<b>Absolute</b>	Baseline	134±6	333±5	0.66±0.05	71±2	0.21±0.01	5.3±0.6	3.5±0.2	0.72±0.03
	CBD	120±6**#	314±6*	0.97±0.14**#	64±2**#	0.22±0.01	5.8±0.05	2.5±0.2*	0.60±0.06**
<b>Change</b>	RD	117±5*#	317±7	1.1±0.19*#	68±2	0.23±0.01	5.7±0.04	2.4±0.2	0.52±0.05*
	Diff CBD	-14±3**#	-19±7*	0.30±0.12**#	-7±1**#	0.014±0.009	0.45±0.28	-0.99±0.16*#	-0.13±0.04**
	Diff RD	-4±3*	5±4	0.10±0.07*	5±2	0.012±0.008	-0.36±0.58	-0.04±0.14	-0.07±0.02
	Total Fall	-17±3	-16±5	0.44±0.09	-2±1	0.025±0.008	0.40±0.43	-0.52±0.24	-0.20±0.03
<b>Sham + RD (n=7)</b>									
<b>Absolute</b>	BASELINE	132±8	326±4	0.87±0.18	76±5	0.20±0.01	5.3±0.6	3.2±0.3	0.65±0.03
	Sham	135±5	306±7*	0.81±0.12	75±4	0.21±0.01	5.8±0.5	3.3±0.3	0.62±0.03
	RD	122±9*	300±2	0.821±0.08	69±4	0.23±0.02	5.7±0.4	2.6±0.2	0.57±0.03
<b>Change</b>	Diff Sham	3±3	-19±7*	-0.06±0.16	0±1	0.01±0.01	0.48±0.28	0.11±0.18	-0.03±0.01
	Diff RD	-8±7*	-6±10	-0.082±0.09	-6±4	0.01±0.02	-0.36±0.58	-0.63±0.30	-0.04±0.02
<b>RD + CSD (n=6)</b>									
<b>Absolute</b>	BASELINE	143±5	325±3	0.67±0.04	86±4	0.21±0.01	5.9±0.3	3.4±0.2	0.58±0.01
	RD	137±5*#	324±7	0.72±0.04	88±4	0.21±0.01	5.6±0.1	3.2±0.2	0.56±0.01
	CBD	126±4*	313±5*	0.78±0.04	76±2**	0.21±0.01	5.6±0.3	2.9±0.1*	0.50±0.02**
<b>Change</b>	Diff RD	-6±2*#	-1±3	0.06±0.03	2±1	0.005±0.012	-0.35±0.14	-0.15±0.13	-0.02±0.01
	Diff CBD	-11±3*	-11±5*	0.06±0.03	-11±2**	0.001±0.007	0.03±0.21	-0.35±0.02*	-0.07±0.01**
	Total Fall	-17±2	-12±4	0.12±0.03	-9±2	0.005±0.011	-0.33±0.17	-0.50±0.08	-0.09±0.01
<b>Sham + CSD (n=6)</b>									
<b>Absolute</b>	BASELINE	128±8	329±9	0.74±0.07	74±4	0.19±0.01	5.9±0.3	3.5±0.2	0.65±0.03
	Sham	132±9	323±5	0.69±0.06	77±5	0.20±0.01	5.5±0.3	3.1±0.2	0.65±0.02
	CBD	122±9*	313±9*	0.85±0.06	65±2**	0.20±0.01	5.7±0.3	2.9±0.15	0.59±0.03*
<b>Change</b>	Diff Sham	4±2	-6±5	-0.05±0.02	3±1	0.004±0.007	-0.39±0.32	-0.35±0.27	0.01±0.01
	Diff CBD	-11±6*	-10±5*	0.16±0.04	-10±3**	-0.005±0.005	0.23±0.24	-0.20±0.17	-0.06±0.03*



**Table 1**

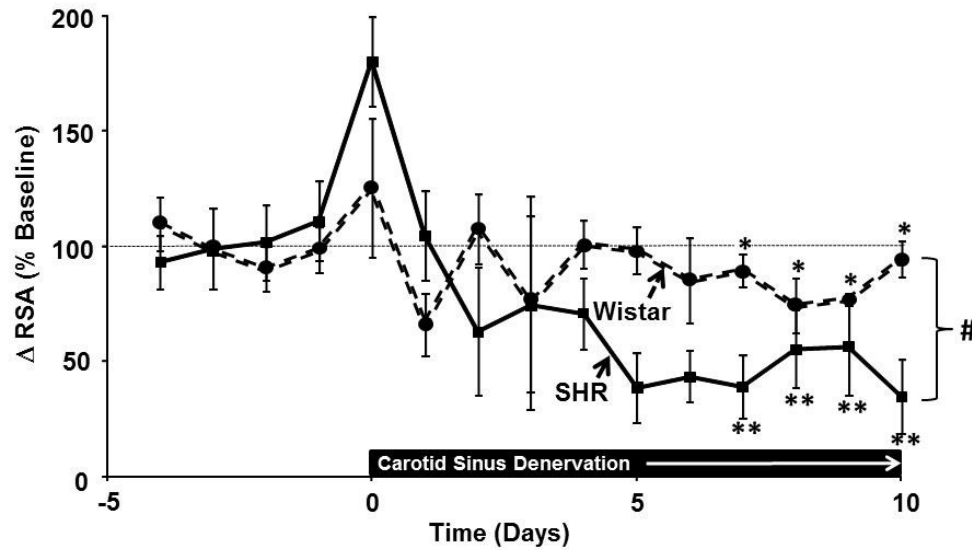
Mean arterial pressure (MAP), heart rate (HR), spontaneous baroreceptor reflex gain (sBRG), respiratory rate (RR) and spectral analyses of pulse interval (PI) and systolic blood pressure (SBP; HF, high frequency; LF, low frequency; VLF, very low frequency) after carotid sinus nerve denervation (CSD) and renal denervation (RD) and combinations of these interventions. Sham operated data are given for each procedure.

**Figure 1**



**Figure 1**

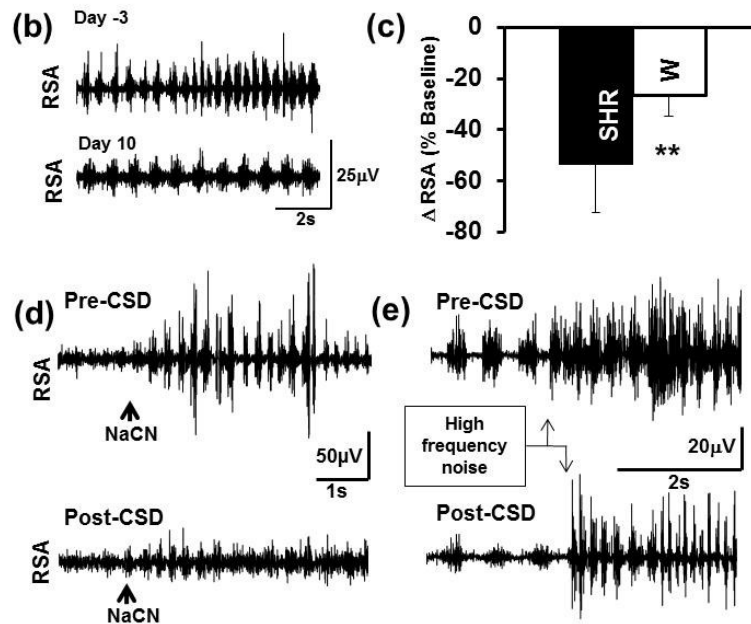
The carotid bodies in hypertensive rats are tonically active. Comparing the effect of 100% oxygen to depress tonic carotid body (CB) activity in Wistar (n=6) and SH (n=6) rats on SBP (a) and RSA (b, c). The CB in SH rats, but not Wistar rats, exhibits tonic activity that is functionally relevant and contributes to basal arterial pressure (d) and sympathetic activity (e). Data shown as mean±sem \*p<0.05, repeated measures ANOVA.



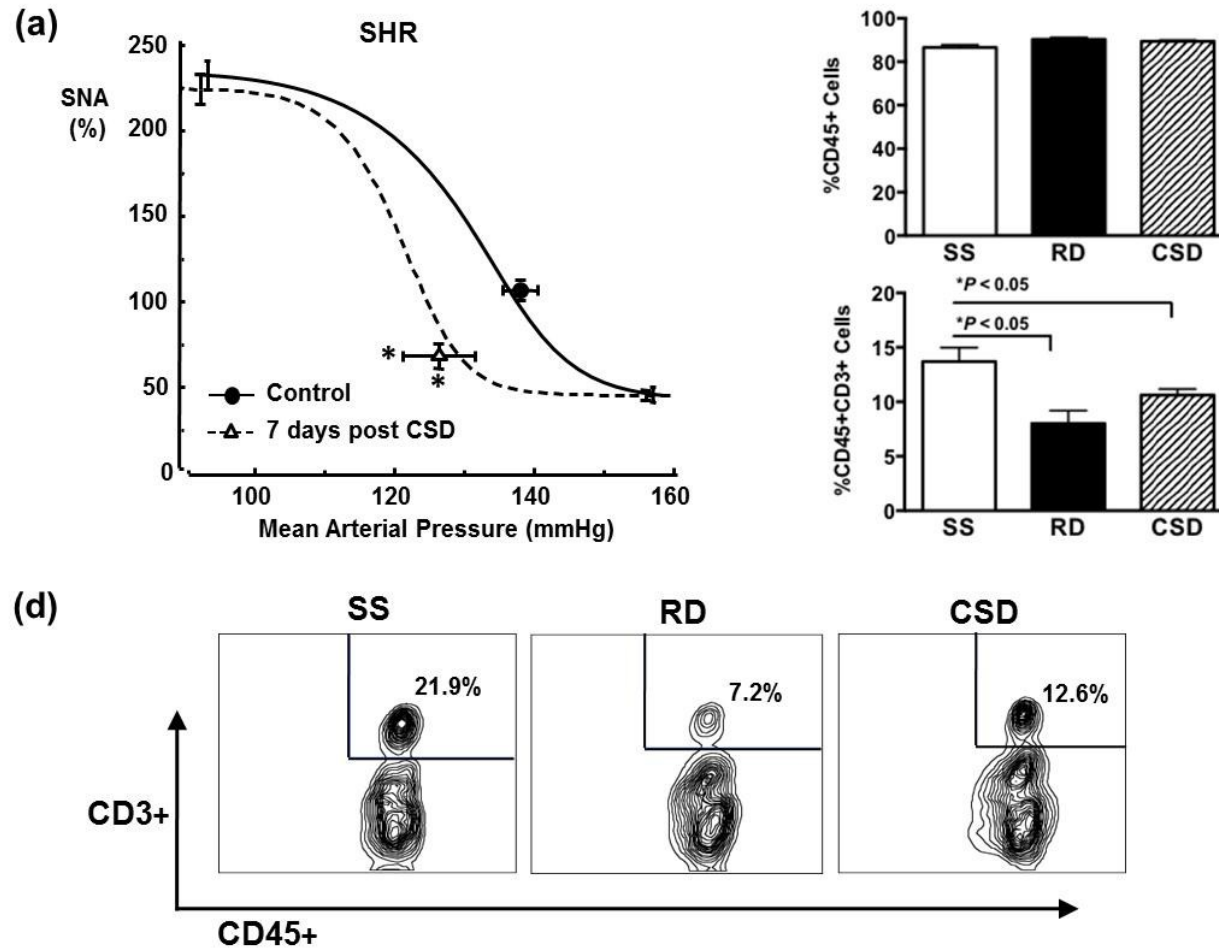
**Figure 2**

Carotid sinus denervation causes substantial renal sympathoinhibition.

Time profile of renal sympathetic nerve activity (RSA) response to bilateral carotid sinus nerve denervation (CSD) in SH (n=6) and Wistar (n=6) rats (a). Inset (b) shows the raw activity levels of RSA before and after CSD in one SH rat sitting quietly. The fall in RSA was ~55% relative to baseline in SH rats and significantly greater than in Wistar rats (c). Efficacy of CSD is shown in (d) where the sodium cyanide (i.v.) evoked RSA excitatory response was greatly attenuated. (e) A CSD SH rat showing that it is able to generate a robust RSA response to a stressful stimulus. Data shown as mean±sem \*P<0.05, \*\*P<0.01; between groups ANOVA, #P<0.05 repeated measures ANOVA.



**Figure 3**

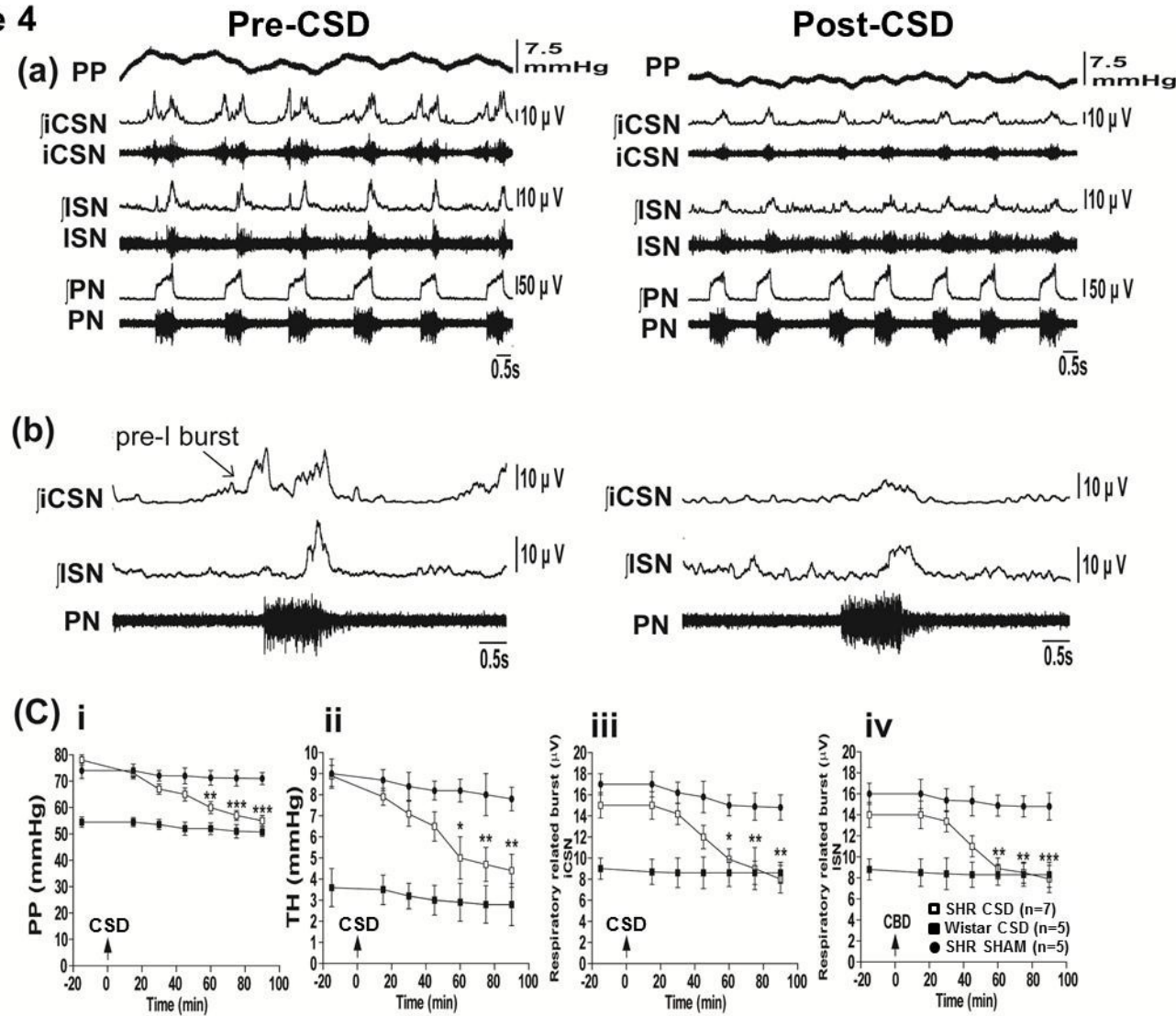


**Figure 3**

Effects on the sympathetic baroreflex and vascular infiltration.

(a) The renal sympathetic activity baroreflex function curve is rightward shifted over lower pressure ranges after carotid sinus nerve denervation (CSD) in SH rats (n=6) but no changes were observed in Wistar rats (n=6, Supplementary Fig S3). Percent total leukocytes (CD45+ cells) in the aorta of surgical sham (SS), RD and CSD animals (b). There was reduced vascular T cell infiltration following RD and CSD in the SH rats as the percentage of T lymphocytes (CD45+CD3+ cells) in the aorta of RD and CSD animals was less relative to SS (n= 6-7/group); c). Representative flow cytometry contour plot showing T lymphocytes (CD45+CD3+) in aortic samples of each rat group (d). All data shown as mean±sem, ANOVA. \*P<0.05 ANOVA.

**Figure 4**

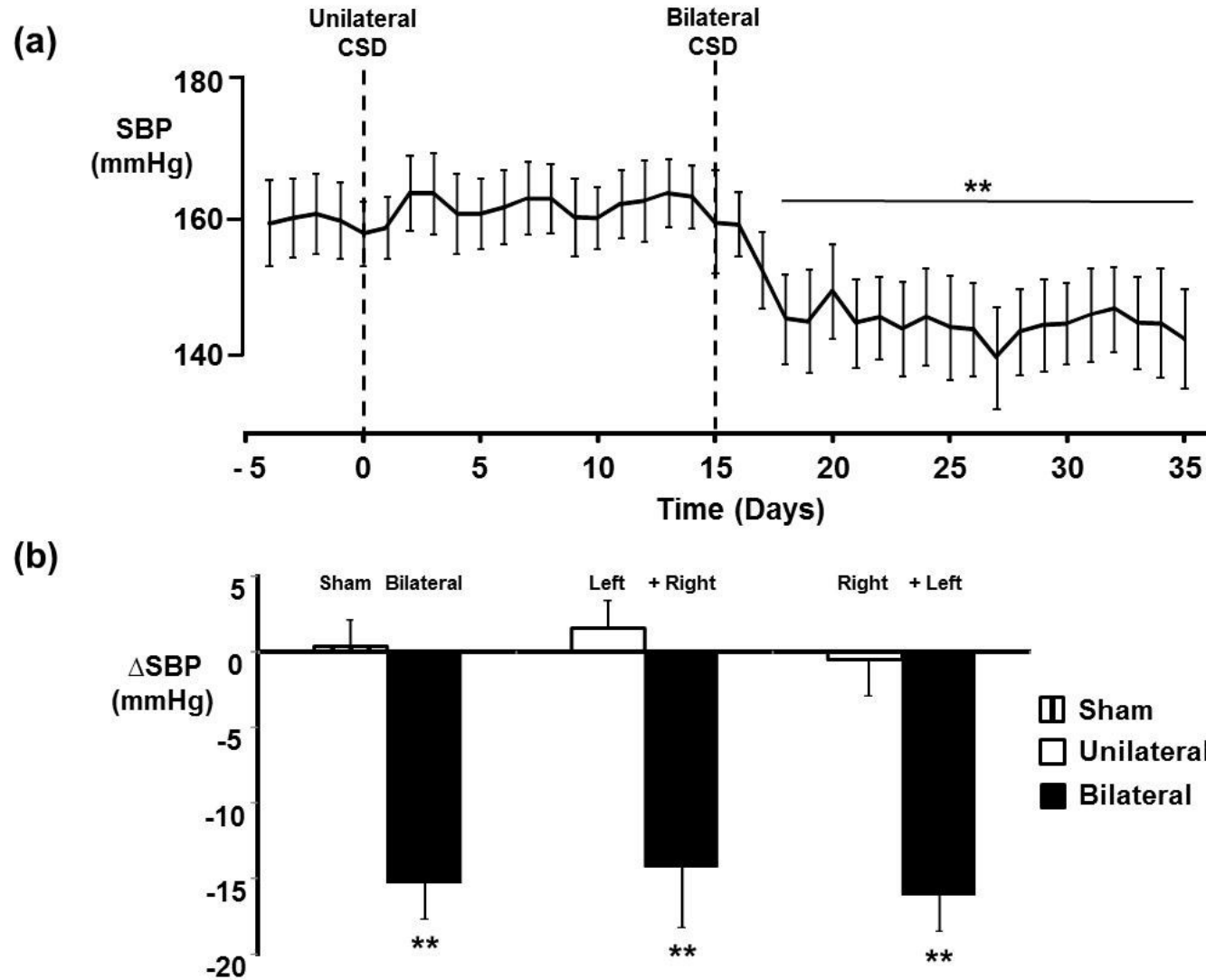


**Figure 4**

Immediate effects of carotid sinus denervation.

The time course of the fall in sympathetic nerve activity (raw and integrated) after carotid sinus nerve denervation (CSD) was compared for the internal cervical (iCSN) and lumbar (ISN) sympathetic post-ganglionic nerves *in situ*. By 40 min there was a significant decrease in sympathetic nerve activity in both outflows (a). Note the decrease in the amplitude of respiratory-modulated discharge ( $P < 0.05$ ; b). The peak response was achieved at 60 min post CSD as activity levels at 75 or 90 min were not different to the level recorded at 40 min post CSD but were different to their own baseline (c). Data shown as mean  $\pm$  sem, \* $P < 0.05$ , \*\* $P < 0.01$ ; \*\*\* $P < 0.001$  repeated measures ANOVA,  $n \geq 5$ . Abbreviations: PN, phrenic nerve; PP, perfusion pressure (aortic); Pre-I, pre-inspiratory; TH, Traube-Hering arterial pressure waves.

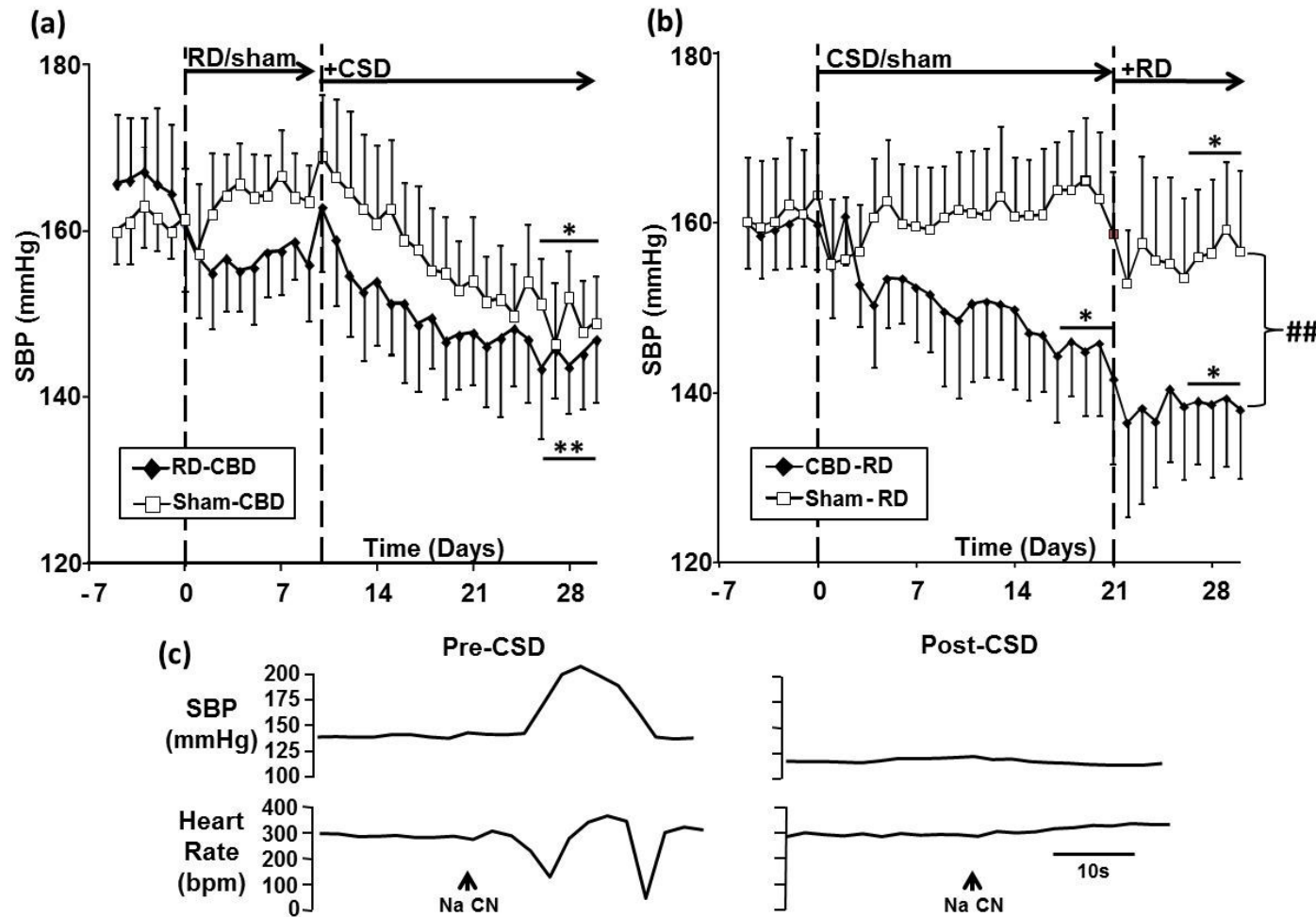
**Figure 5**



**Figure 5**

Unilateral carotid sinus denervation does not lower blood pressure in the rat. Unilateral carotid sinus nerve denervation (CSD) was ineffective at lowering SBP in SH rats whether the left or right CB was removed (n=8, a). Irrespective of whether the left or right CB was removed first, there was no difference in the fall in SBP observed on removal of the contralateral CB (b). Note that time staggered unilateral CB denervation does not affect the magnitude of the fall in SBP, which is the same as that seen when both left and right CB are denervated simultaneously (see Fig. 2 & 3). Data shown as mean  $\pm$  sem, \*\*P < 0.01 within group ANOVA.

**Figure 6**



**Figure 6**

Carotid sinus and renal denervation interaction time course.

Time profile of the SBP response to (a) renal nerve denervation (RD) followed by carotid sinus nerve denervation (CSD) (n=6) and (b) CSD followed by RD resection in SH rats (n=7). Note the time-dependent increase in SBP in shams (n=6 and 7 respectively).

The completeness of the CSD is shown by the absence of a reflex pressor and bradycardic response following i.v. injections of sodium cyanide (NaCN; c). Data shown as mean±sem, repeated measures ANOVA: \*P<0.05, \*\*P<0.01; between group ANOVA ##P<0.01.

Figure 7

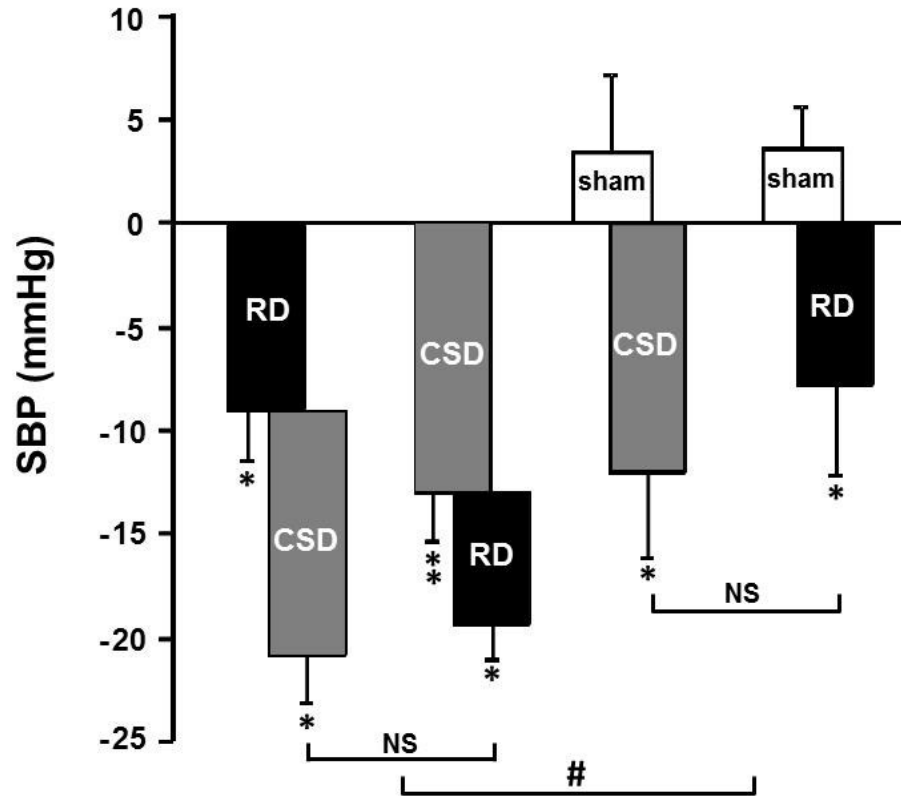


Figure 7

Carotid sinus and renal denervation interaction summary.

Group data show the interaction between carotid sinus nerve denervation (CSD; n=6) and renal nerve denervation (RD; n=7) in SH rats. There was an additive effect of CSD and RD, irrespective of the order in which the intervention was made. The combined intervention produced a greater fall in SBP than each RD or CSD alone. Data shown as mean±sem, repeated measures ANOVA: \* P<0.05, \*\*P<0.01; between group ANOVA, #P<0.05.



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