

The Journal of Physiology

<http://jp.msubmit.net>

JP-RP-2016-272708R2

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Author Conflict: Julian Paton: J.F.R.P. is a consultant for Cibiem. The remaining authors declare no competing financial interests.

Author Contribution: Wioletta Pijacka: Conception and design; Collection and assembly of data; Data analysis and interpretation; Manuscript Writing; Final approval of manuscript (required) Fiona McBryde: Conception and design; Collection and assembly of data; Data analysis and interpretation; Manuscript Writing Paul Marvar: Collection and assembly of data; Data analysis and interpretation; Manuscript Writing; Final approval of manuscript (required) Gisele Lincevicius: Data analysis and interpretation; Final approval of manuscript (required) Ana Abdala: Data analysis and interpretation; Manuscript Writing; Final approval of manuscript (required) Lavinia Woodward: Collection and assembly of data Dan Li: Collection and assembly of data; Data analysis and interpretation; Final approval of manuscript (required) David Paterson: Data analysis and interpretation; Manuscript Writing; Final approval of manuscript (required) Julian Paton: Conception and design; Financial

Disclaimer: This is a confidential document.

Support; Administrative support; Data analysis and interpretation; Manuscript Writing;
Final approval of manuscript (required)

Running Title: Carotid Body in Renovascular Hypertension

Dual Publication: No

Funding: British Heart Foundation (BHF): Julian F. R. Paton, RG/12/6/29670; NIH-NCCIH: Ana Paula Abdala, 1R01AT008632-01; Royal Society: Fiona D McBryde, 0; CiBiem Ltd: Julian F. R. Paton, 0

Carotid sinus denervation (CSD) ameliorates renovascular hypertension in adult Wistar rats.

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Key Points Summary

- Peripheral chemoreflex sensitization is a feature of renovascular hypertension.
- Carotid sinus nerve denervation (CSD) has recently been shown to relieve hypertension and reduce sympathetic activity in other rat models of hypertension.
- We show that CSD in renovascular hypertension halts further increases in blood pressure.
- Possible mechanisms include improvements in baroreceptor reflex sensitivity and renal function, restoration of cardiac calcium signalling towards control levels and reduced neural inflammation.
- Our data suggest that the peripheral chemoreflex may be a viable therapeutic target for renovascular hypertension.

Key words: Renovascular hypertension, carotid sinus denervation, chemoreceptor reflex

Abstract:

The peripheral chemoreflex is known to be hyper-responsive in both spontaneously hypertensive (SHR) and Goldblatt hypertensive (2 kidney 1 clip; 2K1C) rats. We have previously shown that carotid sinus nerve denervation (CSD) reduces arterial blood pressure (ABP) in SHR. Here, we show that CSD ameliorates 2K1C hypertension and reveal potential underlying mechanisms. Adult Wistar rats were instrumented to record ABP via telemetry, then underwent CSD (n=9) or sham CSD (n=9) five weeks after renal artery clipping, versus normal Wistar (n=5). After 21 days renal function was assessed, and tissue collected to assess sympathetic postganglionic intracellular calcium transients ($[Ca^{2+}]_i$) and immune cell infiltrates. Hypertensive 2K1C rats showed a profound elevation in ABP (Wistar: 98 ± 4 mmHg vs. 2K1C: 147 ± 8 mmHg; $p < 0.001$), coupled with impairments in renal function and baroreflex sensitivity, increased neuro-inflammatory markers and enhanced $[Ca^{2+}]_i$ in stellate neurons ($p < 0.05$). CSD reduced ABP in 2K1C+CSD rats and prevented the further progressive increase in ABP seen in 2K1C+sham CSD rats, with a between-group difference of 14 ± 2 mmHg by Week 3 ($p < 0.01$), accompanied by improvements in both baroreflex control and spectral indicators of cardiac sympatho-vagal balance. Furthermore, CSD improved protein and albuminuria, decreased $[Ca^{2+}]_i$ evoked responses from stellate neurons, and reduced indicators of brainstem inflammation. In summary, CSD in 2K1C rats reduces the hypertensive burden and improves renal function. This may be mediated by improvements in autonomic balance, functional remodelling of post-ganglionic neurones and reduced inflammation. Our results suggest that the peripheral chemoreflex may be considered as a potential therapeutic target for controlling renovascular hypertension.

Abbreviations:

2K1C – two kidney, one clip hypertension; CSD – carotid sinus denervation; SHR – spontaneously hypertensive rat; ABP – arterial blood pressure; MAP – mean arterial pressure; HR – heart rate; Ang II – angiotensin II; AT1-R – angiotensin type 1 receptor; NaCN – sodium cyanide.

Introduction

Clinical renovascular hypertension occurs when the renal artery is narrowed, predominantly due to atherosclerosis (70-90% of cases) or less commonly due to dysplasia. It usually involves the ostium and proximal third of the main renal artery and the peri-renal aorta (Safian & Textor, 2001; Lopez-Novoa *et al.*, 2011). Renovascular hypertension affects approximately 6% of the elderly population, regardless of race or sex (Hansen *et al.*, 2002; Piecha *et al.*, 2012; Weber, 2014). For many years the Goldblatt or two kidney-one clip (2K1C) experimental model has been used to study renovascular hypertension (Goldblatt *et al.*, 1934) and has been adopted herein.

In both the 2K1C model, and in human patients, renal artery stenosis is associated with a decrease in renal perfusion pressure, activation of the renin-angiotensin system, suppression of the baroreceptor reflex and increased sympathetic nerve activity (Johansson *et al.*, 1999; Oliveira-Sales *et al.*, 2014). Angiotensin II (Ang II) has been shown to drive increases in reactive oxygen species (ROS) production and inflammatory markers in renovascular hypertension (Ruiz-Ortega *et al.*, 2006), and inhibition of these pathways can mitigate the increase in arterial pressure in the 2K1C model (Bivol *et al.*, 2008). Previous studies in renovascular hypertensive rats have found evidence of elevated inflammatory markers in the heart (Nicoletti *et al.*, 1996) and kidneys (Bivol *et al.*, 2008; Cheng *et al.*, 2009). We have previously shown that carotid sinus nerve denervation (CSD) in spontaneously hypertensive rats (SHR) reduces T-cell infiltration in the aorta and brainstem (McBryde *et al.*, 2013), but it is currently unknown whether a similar benefit might be seen in renovascular hypertension.

The carotid body has attracted considerable interest as a potential new treatment target for sympathetically-mediated cardiovascular disease (Paton *et al.*, 2013a; Paton *et al.*, 2013b). We have recently shown that removal of peripheral chemoreceptor afferents, by CSD, ameliorates hypertension in the spontaneously hypertensive rat (Abdala *et al.*, 2012), in conjunction with an improvement in cardiac baroreceptor reflex gain and a profound (~50%) reduction in renal sympathetic activity (McBryde *et al.*, 2013). In other conditions where the renin-angiotensin system is activated, Ang II has been shown to facilitate the release of noradrenaline from sympathetic nerves (Maruyama *et al.*, 2000; Fabiani *et al.*, 2001). Given our recent finding that peripheral chemoreceptor reflex sensitivity to stimulation sodium cyanide is greatly increased in 2K1C rats (Oliveira-Sales *et al.*, 2016), we hypothesised that CSD would alleviate hypertension and improve renal function in experimental renovascular hypertension. We further hypothesized that the mechanisms underlying the responses to CSD may involve improvements in baroreceptor reflex function, reductions in inflammation and sympathetic post-ganglionic remodelling.

Methods

Ethical Approvals

Procedures were carried out according to the United Kingdom Home Office Guidelines Scientific Procedures Act of 1986. Rats were housed individually, given normal rat chow and drinking water *ad libitum*, and kept on a 14/10hour light/dark cycle. Animals were divided into three experimental groups: (1) two kidney one clip sham carotid sinus denervated (2K1C+sham CSD; n=9), (2) two kidney one clip carotid sinus denervated (2K1C+CSD; n=9) and (3) age-matched normotensive Wistar controls (Wistar; n=5). All surgeries were conducted under aseptic conditions, with animals anaesthetised with ketamine (60mg/kg, Vetalar, Zoetis, London, UK) and medetomidine (250 µg/kg, Elanco Animal Health, Hampshire, UK) via intramuscular injection. The level of anaesthesia was checked frequently by testing limb withdrawal reflexes. Body temperature was regulated using a feedback-controlled heating pad (Harvard Apparatus, Cambridge, UK). Post-operatively non-steroidal anti-inflammatory pain relief was given (0.004ml/100g of Metacam, Boehringer Ingelheim, Germany) for 3 days following surgery. At the completion of the experimental protocol, animals were anaesthetised with isoflurane, then euthanized with an intraperitoneal injection of sodium pentobarbital (100mg/kg).

Goldblatt Model of Hypertension (two kidney one clip, 2K1C)

Male Wistar rats (150-180g) were anaesthetized. The left renal artery was accessed via a retroperitoneal incision, and a silver clip with an internal width of 0.2mm was used to partially obstruct the artery, as described previously (Oliveira-Sales *et al.*, 2014). On completion of the surgery, anaesthesia was reversed with a subcutaneous injection of atipamezole (1mg/kg, Antisedan, Zoetis, London, UK), and the animals returned to a warm recovery box.

Carotid sinus nerve denervation (CSD)

Bilateral carotid sinus nerve denervation was performed five weeks after renal artery clipping, using a surgical approach as described previously (Abdala *et al.*, 2012; McBryde *et al.*, 2013). Briefly, the carotid sinus was accessed via a ventral midline neck incision, the carotid sinus nerve branches removed and the adventitia of the bifurcation and the internal carotid artery carefully stripped. Sham-operated rats underwent the same surgical procedures but the carotid sinus nerves were left intact.

Experimental design

Four weeks after the implantation of renal clips, rats were instrumented to record systemic arterial pressure via wireless telemetry (Data Sciences International Ltd), and a catheter was inserted into

the femoral vein and externalized between the scapulae, as described previously (Waki *et al.*, 2006; McBryde *et al.*, 2013). After five days of recovery, a baseline period was recorded. Carotid sinus denervation (CSD) or Sham CSD was performed five weeks after renal artery clipping. Renal function was tested in each group 21 days after CSD (2K1C+CSD) or sham surgery (2K1C+sham CSD) and in aged matched control animals (Wistar). Chemoreflex sensitivity was assessed before and after CSD or sham CSD, with an i.v. bolus infusion of sodium cyanide (NaCN; 120 µg/kg i.v.).

Cardiac baroreflex sensitivity analysis

Bradycardic and tachycardic reflex responses produced by bolus infusion of phenylephrine (0.1 mg ml⁻¹, I.V.) and sodium nitroprusside (0.1 mg ml⁻¹, I.V.) (Sigma-Aldrich Co, UK) were measured to generate baroreflex function curves, as previously reported (Abdala *et al.*, 2012; Lincevicius *et al.*, 2015). Values of matching SBP variations with reflex heart rate (HR) responses were plotted separately for each vasoactive drug to create linear regression curves for each group, and their slopes were compared to evaluate changes in baroreflex sensitivity (bpm/mmHg).

Renal function

Renal function was studied as previously described (McBryde *et al.*, 2013; Pijacka *et al.*, 2015). Briefly, animals were housed in metabolic cages with free access to food and water for 24h. Blood was collected from the tail vein, and was kept on ice until centrifugation. Plasma and urine creatinine were measured by the improved Jaffe method (Jaffe, 1886), with the commercially available QuantiCrom Creatinine Assay Kit (DICT-500, Universal Biologicals, Cambridge, UK). Creatinine clearance was estimated as: urinary creatinine [µmol/L] x urine volume produced in 24 h [mL]/(plasma creatinine [µmol/L] x 1440 [min]) (Cornock *et al.*, 2010). Urinary albumin was measured by the improved bromocresol green (BCG) method, with the commercially available Albumin Assay (Randox Laboratories Ltd, Crumlin, UK), and total protein was measured by the Lowry method (Lowry *et al.*, 1951) with the DC Protein Assay Kit (Bio-Rad Laboratories Ltd., Hemel Hempstead, UK).

Primary Cultures of Dissociated Sympathetic Neurons

Sympathetic neurons were isolated using a previously published method (Shanks *et al.*, 2013). Following euthanasia, the stellate and mesenteric ganglia were removed from rats and isolated enzymatically with collagenase and trypsin. Experiments were performed 2-3 days after plating. In addition to the 2K1C+sham CSD and 2K1C+CSD groups mentioned above, neurones were also cultured from separate 2K1C (n=8) and normotensive Wistar (n=8) groups.

Measurement of Free Intracellular Calcium Concentration

[Ca²⁺]_i was determined in single neurons using Fura-2 acetoxymethyl ester (Fura-2/AM, 2μmol/L) as previously described (Li *et al.*, 2012). Loaded neurons were imaged with a QIClick digital CCD camera (Photometrics) connected to an OptoLED fluorescence imaging system (Cairn Research Ltd) housed on an inverted Nikon microscope equipped with a 40×, oil-immersion objective. The cover slip containing the neurons was placed into a temperature-controlled (36±0.5 °C), gravity fed, perfusion chamber (volume: 100 μl), perfused with Tyrode solution at a flow rate of 2 ml/min. The evoked [Ca²⁺]_i transient was evaluated by 30 s exposure to 50 mmol/L KCl (with equimolar reduction in NaCl) in the Tyrode solution. Fura-2AM was excited alternately at 355 and 380 nm and the emitted at 510 nm. Fluorescence excitation ratios were calculated.

FACS analysis of cellular inflammation

Analyses of T cells and macrophages in tissue homogenates of the aorta and brainstem were performed as recently described (McBryde *et al.*, 2013) 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 70μ 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. Antibodies (BD Biosciences) used for staining were as follows: V450 anti-CD45; anti-CD3 PerCP-eFluor® 710 and anti-macrophage markers. After immuno-staining, cells were re-suspended in FACS buffer and analysed immediately on a LSR-II flow cytometer with DIVA software (Becton Dickinson). Data were analysed with FlowJo software (Tree Star Inc., Ashland, Oregon, USA) and an initial gate was applied to exclude cell debris from the analysis. CD45 positive cells were identified as leukocytes within the tissue cell suspension and T cells and macrophages were identified with above antibodies.

Data Acquisition and Analysis

Blood pressure telemetry data were acquired and analysed using purpose-written scripts in Spike2 (CED Ltd, Cambridge, UK). Heart rate (HR), respiratory rate (RR) and spectral parameters, including spontaneous cardiac baroreflex gain (sBRG) were obtained from the arterial waveform data, as described previously (McBryde *et al.*, 2013). Briefly, power spectral density was computed using purpose-written scripts in Spike2. The following frequencies were calculated in normalized units: <0.27 Hz (very low frequency), 0.27–0.75 Hz (LF) and 0.75–3.3 Hz (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. Averaged data were expressed as the daily average mean or as the within-animal change from baseline.

Statistical analysis

Data were analysed by GraphPad Prism (Version 6.05). Once normal distribution was confirmed, between groups statistical differences were analysed using a one-way ANOVA, with Newman-Keuls post-hoc comparisons. Within-group analyses were performed as the comparison between Baseline (Days -5 to 0) and Week 3 (Days 14-21) by repeated measures ANOVA and the Holm-Sidak multiple comparisons test. The type of post-hoc analysis performed is indicated in each figure legend. The level of statistical significance was defined as $P < 0.05$. All results are presented as mean $\pm$ SEM.

Results

Cardiovascular Responses to Carotid Sinus Nerve Denervation (CSD)

Prior to CSD/sham surgeries, baseline mean arterial pressure (MAP) was significantly elevated after 5 weeks of renal artery clipping in both 2K1C groups compared to normotensive control rats (Wistar: 98 ± 4 mmHg vs. 2K1C+CSD: 142 ± 8 mmHg and 2K1C+sham CSD: 147 ± 8 mmHg; $p < 0.001$) and there was no significant difference in the level of hypertension between the two 2K1C rat groups. At this time rats were either sham operated or underwent CSD. In the three weeks following sham-CSD surgery arterial pressure continued to gradually increase, reaching a level 8 ± 2 mmHg above baseline ($p < 0.01$). In contrast, 2K1C+CSD animals showed a decrease in MAP by day 21 (-6 ± 2 mmHg; $p = 0.02$). Thus, a between-group comparison found that MAP was 14 ± 2 mmHg lower in 2K1C+CSD versus 2K1C+sham CSD animals (Figure 1; $p < 0.01$). The day/night difference was similar between Wistar and 2K1C rats (6.4 ± 0.8 vs 3.8 ± 2 mmHg; $p = 0.28$), and was not changed after CSD (4.6 ± 2 vs 3.7 ± 1.6 mmHg; $p = 0.83$).

Respiration rate was not significantly different at baseline between groups (75 ± 1 vs 76 ± 2 breaths.min⁻¹), but fell by 9 ± 1 breaths.min⁻¹ around day 5 in 2K1C+CSD but not 2K1C+sham CSD rats ($p < 0.01$). Respiration recovered to baseline levels by day 8, with no significant difference from either baseline, or between groups for the remainder of the experiment (Figure 1).

Baseline heart rate was not significantly different between 2K1C and normotensive rats (Wistar: 345 ± 7 bpm vs. 2K1C+sham CSD: 346 ± 7 bpm and 2K1C+CSD: 352 ± 8 bpm). There were no statistically significant changes in heart rate after CSD.

Spontaneous baroreflex gain (sBRG) was significantly reduced in 2K1C hypertension compared to Wistar rats (-1.3 ± 0.13 vs -2.3 ± 0.08 bpm/mmHg), but did not significantly differ within or between groups following CSD or sham-CSD surgery (Figure 1). The low frequency: high frequency ratio of

pulse interval (LF:HF) was significantly lower in both 2K1C groups compared to Wistar control rats (0.23 ± 0.02 and 0.25 ± 0.03 vs 0.39 ± 0.04 ; $p > 0.01$). Over the 21 day recording window the LF:HF ratio increased in 2K1C+sham CSD rats (0.23 ± 0.02 to 0.32 ± 0.03 ; $p < 0.05$), but not 2K1C+CSD rats (0.25 ± 0.02 to 0.25 ± 0.03 ; $p = 0.69$), leaving a significant between groups difference by Week 3 (Figure 1; $p < 0.05$). This indicates that CSD may prevent further deterioration in cardiac sympatho-vagal balance in 2K1C rats. The low frequency component of SBP decreased from baseline by Day 21 in 2K1C+CSD rats (2.9 ± 0.3 to 2.1 ± 0.3 mmHg², Figure 1; $p < 0.05$).

Chemoreflex and baroreflex responses to CSD

The success of CSD was confirmed with 2K1C+CSD rats showing little or no arterial pressure response and no bradycardia response (Δ HR: 2K1C+sham CSD -191.8 ± 30.5 ; 2K1C+CSD -4.4 ± 3.6 ; Wistar -182.9 ± 48.0) to transient sodium cyanide activation of the chemoreflex, compared to the powerful pressor response seen in sham CSD rats ($P < 0.001$; Figure 2A). 2K1C+CSD rats also exhibited an improved tachycardic response to induced falls in blood pressure, compared to 2K1C+sham CSD animals (Figure 2B; $p < 0.05$). However, the bradycardic response to induced increases in blood pressure was not significantly different between 2K1C+CSD and 2K1C+sham CSD rats (Figure 2B; $p = 0.1$).

Renal Function after CSD

2K1C hypertension was associated with increased plasma creatinine, decreased creatinine clearance, elevated albuminuria and proteinuria and increases in both water intake and urine production (Figure 5; $p < 0.05$ Wistar vs sham CSD). CSD did not change plasma creatinine or creatinine clearance, but did normalise urinary protein and albumin levels (Figure 5; $p < 0.05$ CSD vs Wistar and sham CSD). Following CSD, water intake and urine production tended to decrease slightly to an intermediate level not significantly different to either Wistar or sham CSD groups (Figure 5).

Inflammatory changes in the aorta and brainstem

When compared to 2K1C+Sham CSD rats, no differences were observed in the percent of vascular aortic immune cell infiltrates (CD45⁺, CD3⁺ cells and Macrophages) in 2K1C+CSD rats. However, the percentage of macrophages in brainstem homogenates was significantly reduced in 2K1C+CSD rats (Figure 4, $P < 0.05$).

Intracellular Free Calcium Transients in Sympathetic Neurons

During depolarization with high K⁺, neurones cultured from the stellate ganglia of 2K1C rats showed a greater increase in [Ca²⁺]_i (2.71 ± 0.26 , $n = 10$) when compared with those from Wistar animals (2.08 ± 0.13 , $n = 20$, $P < 0.05$). This heightened [Ca²⁺]_i response evoked was decreased by 24% in

2K1C+CSD rats (Figure 3 C&D; $P < 0.05$). In contrast, no significant differences in $[Ca^{2+}]_i$ were observed from neurones cultured from the mesenteric ganglia (Figure 3E) of Wistar, 2K1C or 2K1C+CSD rats.

Discussion

Our results are the first ever demonstration that renovascular hypertension can be ameliorated by CSD, in the two kidney one clip rat (2K1C) model. We found that resecting carotid sinus nerves bilaterally five weeks after renal artery clipping reduced arterial pressure, thereby preventing the further increases in arterial pressure seen in sham-operated animals where the carotid nerves were left intact. Our observed reduction in the hypertensive burden of 2K1C rats was accompanied by some improvements in baroreflex control and renal function, a restoration of stellate calcium signalling and a reduction in neural inflammatory markers. This work is an important extension of our previous work demonstrating the efficacy of CSD in another experimental model of hypertension (Abdala *et al.*, 2012; McBryde *et al.*, 2013), which has identified the carotid bodies as a potential therapeutic target for treating refractory or essential hypertension in human patients (Paton *et al.*, 2013b; Ratcliffe *et al.*, 2014). Our current study suggests that the carotid body may be a viable therapeutic target for renovascular hypertension.

We observed a significant impairment in renal function assessed after 8 weeks of renal artery clipping, with reduced creatinine clearance, and increased plasma creatinine, albuminuria and proteinuria, suggesting a breakdown in the filtration barrier. Carotid sinus nerve resection at 5 weeks post clipping, was observed to normalize albuminuria and proteinuria, but not other indicators of renal function (Figure 5). While a presumed decrease in renal sympathetic drive may contribute, we suggest that this may also have occurred secondary to the decrease in arterial pressure, which could itself reduce the excessive pressure in glomerular capillaries and reduce leakage from the intact kidney. The lack of improvement in other indicators of renal function such as creatinine clearance may be explained by an already prolonged exposure to protein in the filtrate, which can cause inflammation and scarring in the renal tubules, with subsequent nephron loss, and an ensuing fall in renal filtration rate which may be more difficult to rescue (reviewed in (Abbate *et al.*, 2006). Thus we conclude that changes in renal function are not likely to be the primary mechanism for the anti-hypertensive effect of CSD in renovascular hypertension.

Using both direct and indirect measures, our group has previously suggested that 2K1C hypertension is associated with an increase sympathetic drive, which precedes the increase in arterial pressure (Oliveira-Sales *et al.*, 2014; Campos *et al.*, 2015; Oliveira-Sales *et al.*, 2016). Consistent with earlier work (Oliveira-Sales *et al.*, 2014; Campos *et al.*, 2015; Oliveira-Sales *et al.*, 2016), our current results

show 2K1C hypertension to be associated with impaired cardiac baroreflex sensitivity and impaired cardiac sympatho-vagal balance (Figure 2B), which has previously been reported to contribute to the development and maintenance of essential hypertension (Abdala *et al.*, 2012; McBryde *et al.*, 2013). Interestingly, neither the bradycardia response to an increase in arterial pressure, nor the net spontaneous baroreflex gain was observed to change with CSD. Importantly, despite the complete bilateral resection of the carotid sinus nerves and the removal of carotid baroreceptor input, no impairment in baroreflex function was seen. This result may initially be counterintuitive, but it is known that the aortic baroreceptors play a dominant role in mediating cardiac baroreflex in the rat (Pickering *et al.*, 2008), and is furthermore consistent with our previous work, where CSD was found to improve baroreflex gain in the SHR model (Abdala *et al.*, 2012). We suggest that the improvements in baroreceptor reflex function may be due to central remodelling following the removal of the antagonism of peripheral chemoreceptor overactivity.

Analysis of the calcium transients in post-ganglionic mesenteric and stellate ganglia revealed an increase in stellate $[Ca^{2+}]_i$ transients in 2K1C rats. These results indicate that CSD may be causing remodelling of post-ganglionic sympathetic neuronal function. This is consistent with previous results reported in the SHR, which also showed at the level of the end organ, abnormalities in post-ganglionic intracellular calcium signalling (Li *et al.*, 2013) may result in the enhanced exocytosis and impaired re-uptake of noradrenaline from sympathetic neuronal terminals (Li *et al.*, 2012; Shanks *et al.*, 2013). We have shown previously that activation of the peripheral chemoreflex can evoke powerful increases in inferior cardiac sympathetic activity (Boscan *et al.*, 2001). Interestingly, although in the current study we observed attenuation of stellate $[Ca^{2+}]_i$ transients with CSD, we did not see any corresponding reduction in heart rate after CSD. We suggest that any functional effects of cardiac post-ganglionic sympathetic remodelling are thus likely to be iono- or dromo-trophic rather than chrono-trophic; this is an area which warrants further investigation. It is unclear whether our observed effects of CSD on calcium signalling are due to a direct resetting of the arterial chemoreceptor cardiac sympathetic axis, or may have occurred secondary to the differences in arterial blood pressure itself – although the within-subject fall in blood pressure after CSD was modest, hypertension in the sham operated group continued to increase with time, such that at the time tissue was removed for testing, the between group difference was ~14mmHg. Our finding that calcium signalling in the mesenteric ganglia was not affected by CSD should be taken with the caveat that our tissue collection protocol was not able to discriminate between the clipped and unclipped kidneys. Early work has shown that renal denervation can mitigate hypertension and reduce sympathetic tone in the 2K1C rat, but only when the nerves from the clipped kidney are ablated (Katholi *et al.*, 1982). Subsequent work has shown a similar fall in arterial pressure after selective

renal afferent denervation by dorsal rhizotomy, but again, no effect is seen with a contralateral denervation (Wyss *et al.*, 1986). This suggests that the clipped and unclipped renal nerves are likely to play quite different roles in the development and maintenance of renovascular hypertension. Thus it is perhaps not surprising that our 'mixed approach' did not yield any significant results, and any future work should attempt to tease out these differences.

CSD was associated with reduced brainstem macrophage infiltration compared to sham operated animals, consistent with our previously findings in the spontaneously hypertensive rat (McBryde *et al.*, 2013). We suggest that there are several possible explanations for this observation. Firstly, the reduction in inflammation may simply occur secondary to the reduction in the prevailing level of pressure. Alternatively, given that the brainstem is a key region responsible for the regulation of arterial blood pressure and sympathetic activity, if the removal of carotid body input itself reduces brainstem inflammation, this may contribute causally to the reduction in arterial pressure. In favour of the latter, it has been recently reported that the generation of the sympathetic vasomotor tone and maintenance of hypertension in 2K1C rats is mediated by increases in reactive oxygen species production in the rostroventrolateral medulla (RVLM) (Oliveira-Sales *et al.*, 2010). We speculate that an increase in brainstem reactive oxygen species may contribute to brainstem inflammation, or vice versa. Alternatively, brainstem inflammation may be triggered via an afferent feedback mechanism from the ischemic kidney, and/or high circulating levels of ANG II (Marvar *et al.*, 2010; Oliveira-Sales *et al.*, 2014). Further characterization of the inflammatory cells following CSD are required to determine the potential neuro-immune modulation in the 2K1C model of renovascular hypertension, and we recognise the importance of assessing whether CSD altered the inflammatory state of the kidney in future studies.

Both renovascular and essential hypertension are associated with sympathetic overactivity (Johansson *et al.*, 1999; Carthy, 2014; Grassi *et al.*, 2014). Recent evidence has demonstrated that the carotid bodies contribute to increased sympathetic drive in several models of cardiovascular disease (McBryde *et al.*, 2013; Marcus *et al.*, 2014a). In the present study on rats with renovascular hypertension, CSD improved indicators of cardiac sympatho-vagal balance compared to sham operated animals. Although the commonly-used (Kuwahara *et al.*, 1994; Waki *et al.*, 2006) indirect methods used herein to assess autonomic tone are not universally accepted (Billman, 2013), our current results are consistent with both our present finding that CSD reduced [Ca]²⁺ in stellate cells from 2K1C rats and our previous finding that CSD reduces renal sympathetic nerve activity in another model of hypertension (McBryde *et al.*, 2013). Thus, we suggest that there is a strong likelihood that our observed fall in arterial pressure after the removal of peripheral chemoreceptor

input is due, at least in part, to a reduction in sympathetic drive. Although the purpose of the current study was to examine the impact of CSD on established renovascular hypertension, it would be an interesting future study to test whether CSD can prevent the onset of renovascular hypertension, as we have previously shown to be the case in the pre-hypertensive young SHR (Abdala *et al.*, 2012). We predict that while CSD is unlikely to fully prevent renovascular hypertension, given that both chemoreflex sensitivity and sympathetic outflow appear to pre-date the increase in blood pressure in 2K1C hypertension, the pre-emptive removal of the carotid bodies may limit the degree of hypertension.

Following CSD, we observed a transient fall in respiratory rate which returned to baseline rates within 5 days. We have previously observed a similar response after CSD in spontaneously hypertensive, but not Wistar rats (Abdala *et al.*, 2012; McBryde *et al.*, 2013). We interpret this result to indicate that there is tonic drive from the peripheral chemoreceptors in both the SH and 2K1C rat, the interruption of which is responsible for the temporary dip in the rate of breathing, until compensatory central remodelling occurs. This is a further indicator of abnormal peripheral chemoreceptor function in the 2K1C hypertensive rat.

In terms of possible mechanisms linking renal stenosis to carotid body function, we speculate that these effects may be mediated in glomus cells by angiotensin II (Ang II) via type 1 angiotensin II receptors (AT₁R), which have been shown to increase carotid body (CB) activity (Allen, 1998). In pathological conditions where Ang II production is chronically increased, CB chemoreceptor sensitivity to hypoxia and/or cyanide is enhanced, and has been linked to AT₁R up-regulation (Li & Schultz, 2006; Li *et al.*, 2006; Marcus *et al.*, 2010; Peng *et al.*, 2011; Oliveira-Sales *et al.*, 2016). Since 2K1C hypertension is known to be Ang II-dependent, it is possible that increased Ang II production could contribute to the excitation of the glomus cells in this model. Furthermore, the actions of Ang II on the AT₁R have also been shown to drive inflammation (Chabrashvili *et al.*, 2003) and facilitate the release of noradrenaline from sympathetic nerves (Maruyama *et al.*, 2000; Fabiani *et al.*, 2001), which is consistent with our present findings. The increased sympathetic tone seen in renovascular hypertension has also recently been linked to an up-regulation of AT₁R in the RVLM (Lincevicius *et al.*, 2015). Thus, it is possible that brainstem inflammation and sympathetic overactivity in renovascular hypertension may be driven by elevated ANG II signalling. Healy *et al* found that sino-aortic denervation was associated with a decrease in brainstem AT₁-R in normal Wistar rats (Healy *et al.*, 1989). This suggests that interrupting the neural reflex arcs controlling arterial pressure can directly impact brainstem Ang II signalling, although no attempt was made to resolve chemoreceptor versus baroreceptor pathways. We speculate that CSD may act, at least in part, via reducing central

AT₁ receptors to reduce brainstem inflammation. This putative relationship between Ang II/AT₁-R signalling and carotid body function in renovascular hypertension should be examined in future studies.

Perspectives

The results presented herein give support to the concept that the carotid body should be considered as a potential therapeutic target in renovascular hypertension. This extends our previously published results showing benefit in an animal model of neurogenic hypertension (Abdala *et al.*, 2012; McBryde *et al.*, 2013), and adds to the growing body of evidence suggesting that the carotid body may be a suitable target in a range of cardiovascular diseases characterised by parallel changes in peripheral chemoreceptor function and autonomic imbalance (Del Rio *et al.*, 2013; McBryde *et al.*, 2013; Niewinski *et al.*, 2013; Paton *et al.*, 2013a; Paton *et al.*, 2013b; Marcus *et al.*, 2014b; Del Rio *et al.*, 2015; Moraes *et al.*, 2015). Recent studies have evaluated the feasibility of both surgical resection (Narkiewicz *et al.*, 2016), and a novel pharmacological compound (Pijacka *et al.*, 2016) to selectively target the carotid body in resistant hypertension. Notably, renal artery stenosis remains difficult to identify and treat clinically. Renal function studies show that only 5-10% of patients with renal artery stenosis exhibit increased plasma creatinine, yet most have high arterial pressure (Safian & Textor, 2001); thus many renovascular pathology patients may be initially misdiagnosed with essential hypertension. Since removing chemoreflex drive is effective in animal models of both neurogenic and renovascular hypertension, we suggest that the carotid body presents a viable therapeutic target independent of these types of hypertension. This, together with improved selective targeting of the carotid body, perhaps via pharmacological modulation, awaits testing.

Additional Information:

Disclosures: None.

Author Contributions: All authors contributed to the writing of this study. JFRP was responsible for acquisition of funding, administrative support, conception and experimental design and data interpretation. FDM, WP, LW and APA designed experiments, collected, analysed and interpreted data for the main study, performed at the University of Bristol. PJM was responsible for the inflammatory markers component during a fellowship at the University of Bristol, and GSL, DL and DJP for the calcium imaging study performed at Oxford University, on tissue collected from Bristol.

Funding: This work was supported by funding from the British Heart Foundation (RG/12/6/29670), Royal Society, NIH (1R01AT008632-01), European Commission Research Executive Agency Marie Curie International Incoming Fellowship (P.J.M) (MC-IIF – 276147); NIH R00 HL107675-03 (P.J.M); American Heart Association 15CSA24340001 (P.J.M) and a gift from CiBiem NC1 to JFRP.

Acknowledgement: We acknowledge the assistance of Dr Elizabeth Oliviera-Sales in establishing the 2K1C model of hypertension.

Figure 1 - Cardiovascular response to carotid sinus nerve denervation (CSD) or sham surgery (sham-CSD) 5 weeks after induction of 2K1C renovascular hypertension. Top panels shows the temporal responses in mean arterial blood pressure (MAP), respiratory frequency (Resp.), heart rate (HR) and heart rate variability (LF:HF) responses 5 days before and 21 days after CSD or sham CSD surgery, (grey dashed vertical line; n=9 per group). Bottom panels show the averaged responses of Baseline (days -5 to 0) vs Week 3 (Days 14-21) in Wistar, 2K1C+sham CSD and 2K1C+CSD animals for low frequency power of systolic blood pressure (LF of SBP) and spontaneous baroreflex gain (sBRG). (Differences were evaluated using one-way ANOVA with Holm-Sidak posthoc comparisons; # p<0.05, between-groups comparison; * p<0.05 repeated measures within-subject comparison).

Figure 2 - Assessment of chemoreflex and baroreflex sensitivity in 2K1C+sham CSD and 2K1C+CSD rats. (A) Chemoreflex-induced increases in SBP by bolus infusion of sodium cyanide (NaCN; 120 µg/kg i.v.) were abolished after CSD; P<0.001. **(B)** Cardiac baroreceptor reflex curves produced with i.v. infusions of vasoactive drugs (sodium nitroprusside and phenylephrine) showed an increase in sensitivity during sodium nitroprusside infusion in 2K1C+CSD vs. 2K1C+sham CSD rats, P<0.05. Note that the responses in the 2K1C+CSD occurred despite loss of carotid sinus baroreceptor inputs and mediated by aortic baroreceptors. Data were analysed by one-way ANOVA with Newman-Keuls multiple comparisons post hoc test, n=4, *p<0.05, **p<0.01, ***p<0.001. Data are presented as mean±SEM.

Figure 3 - Intracellular Free Calcium Transients in Sympathetic Neurons from the 2 kidney-1 clip-sham carotid sinus denervation (2K1C+sham CSD), 2 kidney-1 clip-carotid sinus denervation (2K1C+CSD) and age matching controls (Wistar). A: (i) All neurons imaged were sympathetic as evidenced by tyrosine hydroxylase immuno-positivity (green) and (ii) were localised in relation to other nuclei stained in blue by DAPI. B: Pseudocolour-coded ratio images of Fura-2-loaded neurons were obtained by conventional fluorescence microscopy. Ca^{2+} concentrations were colour-coded with a basal Ca^{2+} concentration in blue and a high Ca^{2+} concentration in red. C: An example recording from a stellate neuron exposed to 50 mmol/L of KCl (30 seconds) depolarization resulting in a rise in intracellular free calcium concentration ($[\text{Ca}^{2+}]_i$). Note that CSD attenuated this response. D, E: Quantitative data showing the difference in the peak evoked $[\text{Ca}^{2+}]_i$ between Wistar and Goldblatt hypertensive rats with CSD (2K1C+ CSD) or sham CSD (2K1C+sham CSD), obtained from stellate ganglia (D) and mesenteric ganglia (E) neurons collected and cultured from >3 rats per group. The numbers inside the bars refers to the number of neurons tested, with each response per neuron

representing one K exposure. ** $p < 0.01$, *** $p < 0.001$, 1-way ANOVA with Holm-Sidak post hoc comparisonst test.

Figure 4 - FACS data showing immune cell infiltration of vascular aortic and brainstem tissue in the 2 kidney-1 clip (2K1C+sham CSD), 2 kidney-1 clip-carotid sinus denervation (2K1C+CSD) and age matching control Wistars. CSD reduced macrophage infiltration in the brainstem but had no effect on CD45 and CD3 expressing T cells. One-way ANOVA with Newman-Keuls multiple comparisons test, * $p < 0.05$. Data are presented as mean $\pm$ SEM.

Figure 5

Effect of carotid sinus nerve denervation or sham surgery on renal function. Renal function was assessed at 21 days after carotid sinus denervation (CSD, $n=10$) or sham CSD ($n=14$) in 2K1C hypertensive rats, as well as in age-matched Wistar controls ($n=11$). 2K1C hypertension was associated with multiple impairments in renal function, compared to Wistar controls. CSD normalised proteinuria and albuminuria ($p < 0.05$). Data were analysed by one-way ANOVA with Newman-Keuls multiple comparisons tests, * $p < 0.05$, ** $p < 0.01$. Data are presented as mean $\pm$ SEM.

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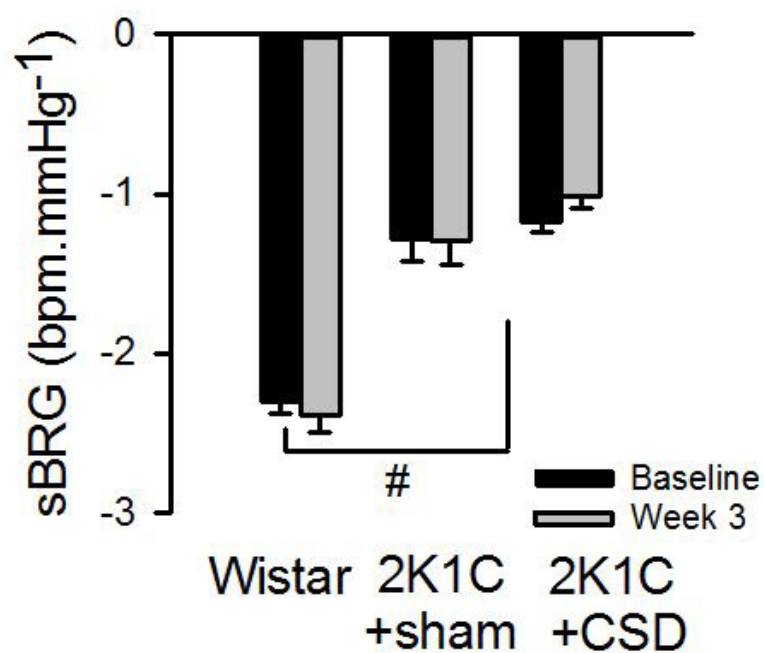
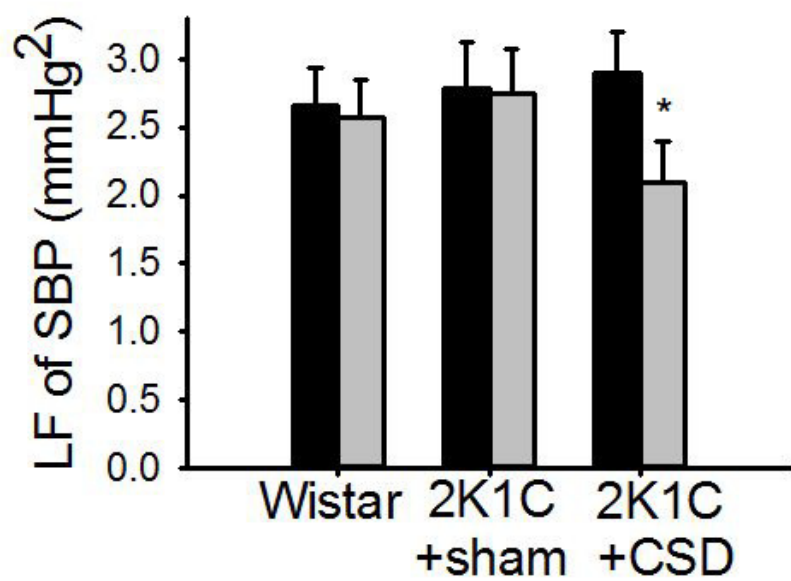
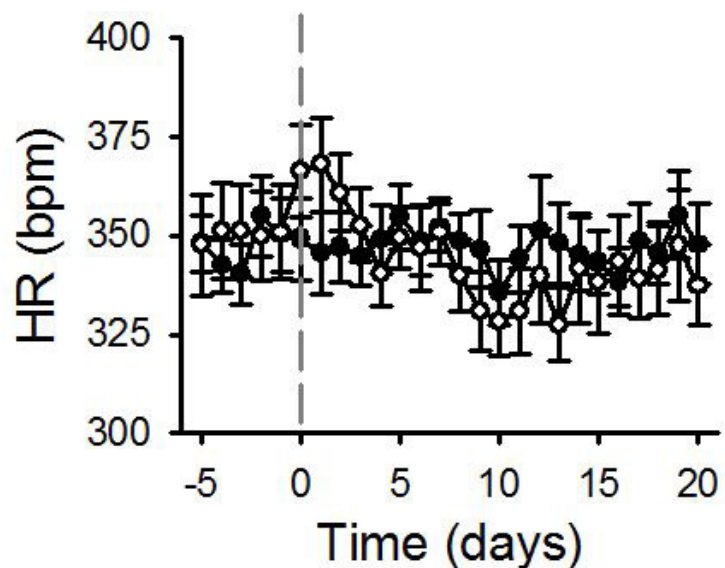
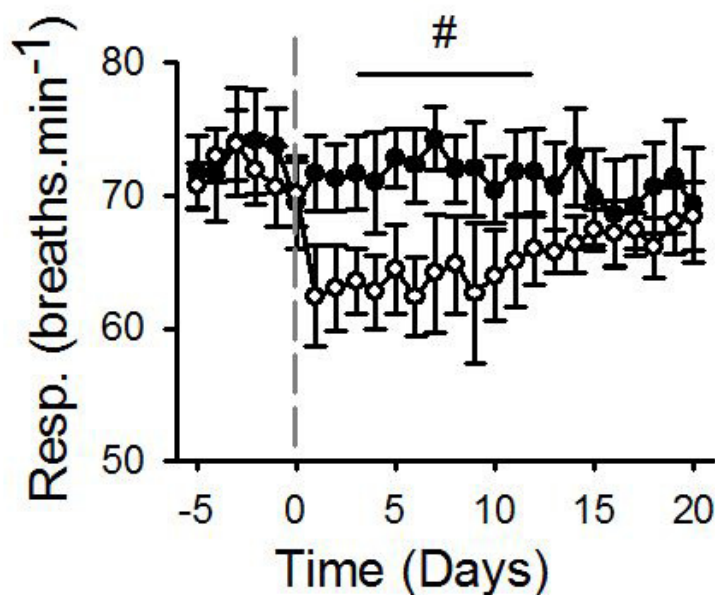
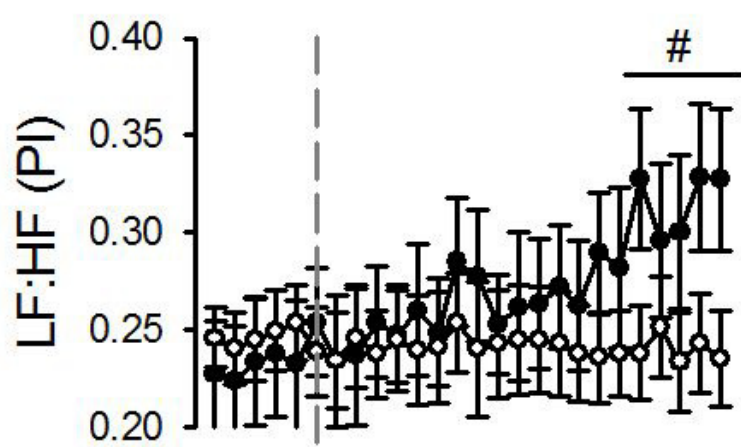
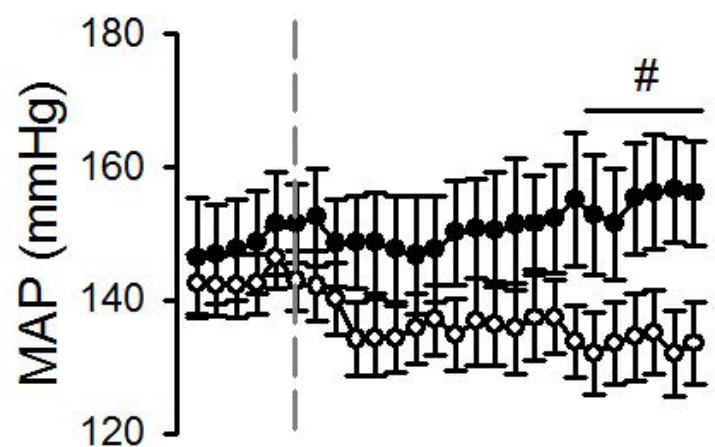
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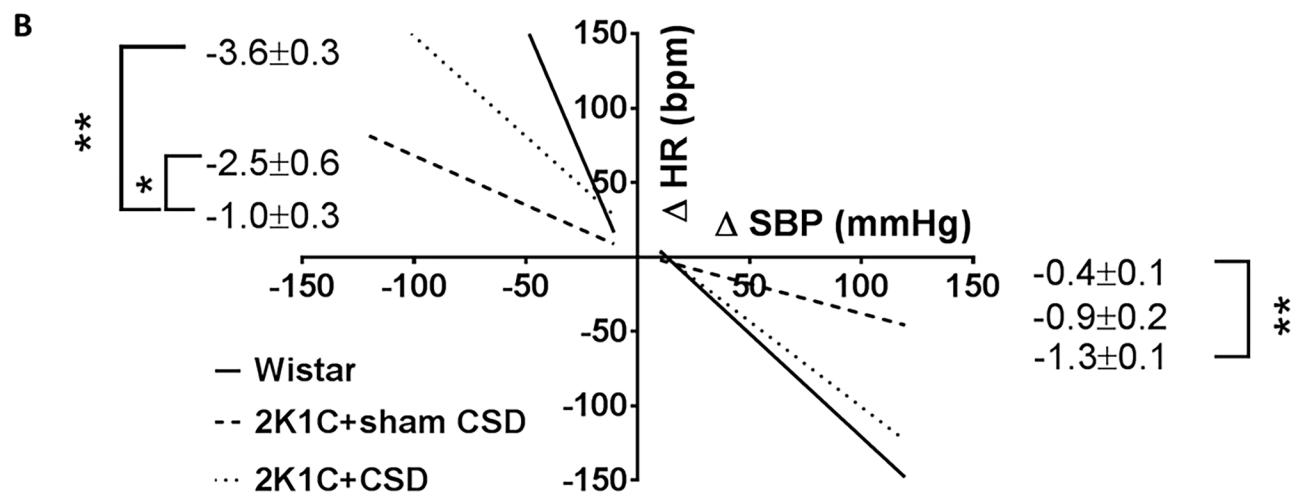
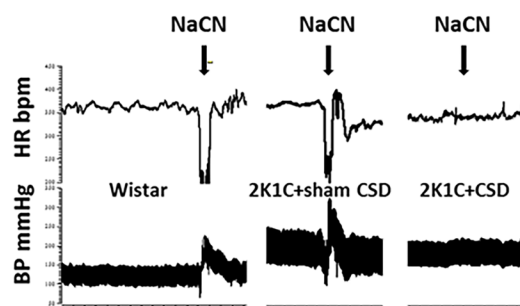
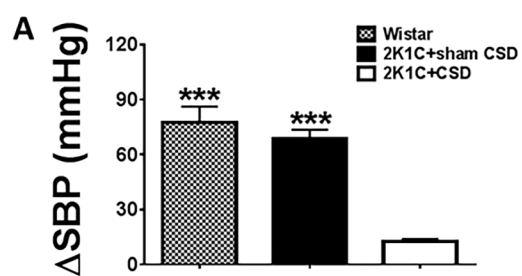
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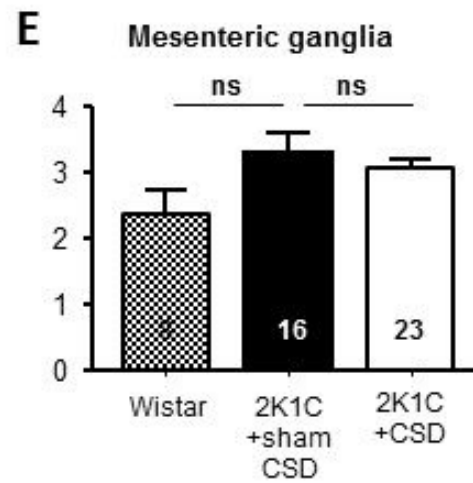
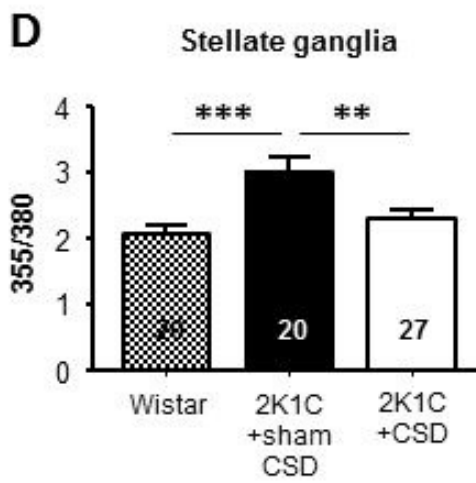
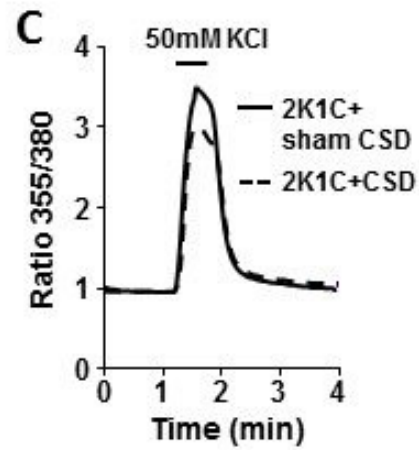
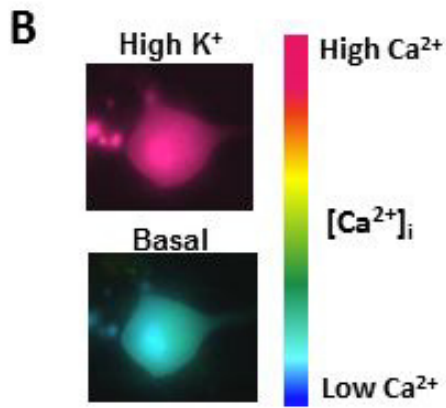
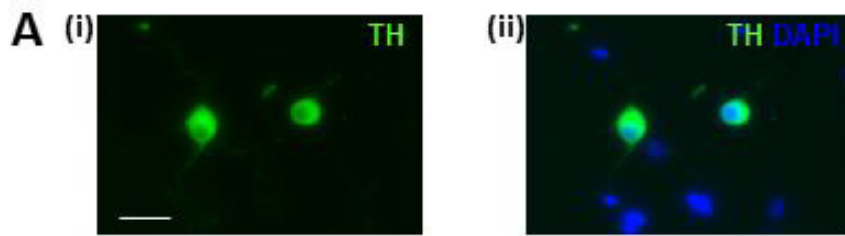
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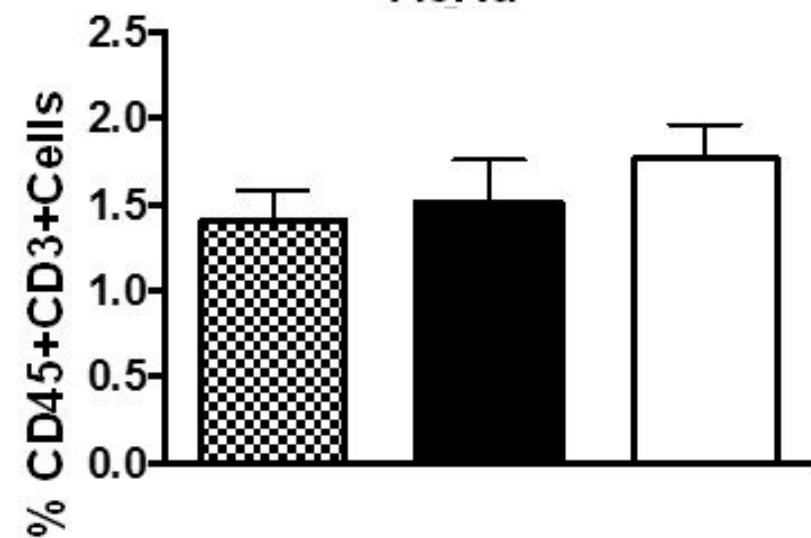
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Aorta**Brainstem**