Sympathoexcitation following intermittent hypoxia in rat is mediated by circulating angiotensin II acting at the carotid body and subfornical organ

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Brief Biography

Seung Jae Kim is a PhD candidate at the University of Sydney (Sydney Medical School & Heart Research Institute). He is currently co-supervised by Prof Paul M Pilowsky (University of Sydney) and Dr Stephen BG Abbott (University of Virginia). His work focuses on studying neural mechanisms that cause sympathetic nerve activity to elevate, which later progresses into hypertension. Here, he

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demonstrates that the enhancement of sympathetic outflow following intermittent hypoxia is mediated by circulating angiotensin II acting at the carotid bodies and the subfornical organ. The two areas equally contribute to the overall sympathoexcitatory output.

Key points

- In anesthetized rats, acute intermittent hypoxia increases sympathetic nerve activity (SNA), sympathetic peripheral chemoreflex sensitivity, and central sympathetic-respiratory coupling.
- Renin-angiotensin system inhibition prevents the sympathetic effects of intermittent hypoxia, and intermittent injections of angiotensin II into the systemic circulation replicate these effects.
- Bilateral carotid body denervation reduces the sympathetic effects of AIH, and eliminates the increases in chemoreflex sensitivity and sympathetic-respiratory coupling.
- Pharmacological inhibition of the subfornical organ also reduces the sympathetic effects of AIH, but has no effect on the increase in chemoreflex sensitivity and central sympathetic-respiratory coupling.
- Combining both interventions eliminates the sympathetic effects of both intermittent hypoxia and angiotensin II.

Abstract

Circulating angiotensin II (AngII) is vital for arterial pressure elevation following intermittent hypoxia in rats, but its importance in the induction of sympathetic changes is unclear. We tested the contribution of the RAS to the effects of acute intermittent hypoxia (AIH) in anesthetized ventilated adult rats. AIH caused a 33.7±2.9% increase in sympathetic nerve activity (SNA), and a one-fold increase in sympathetic peripheral chemoreflex sensitivity and central sympathetic-respiratory

coupling. The sympathetic effects of AIH were prevented by blocking angiotensin type 1 receptors with systemic losartan and intermittent systemic injections of AngII (Int.AngII) elicited similar sympathetic responses to AIH. To identify the neural pathways responsible for the effects of AIH and Int.AngII, we performed carotid body denervation (CBD), which reduced the increase in SNA by 56% and 45% respectively. Conversely, pharmacological inhibition of the subfornical organ (SFO), an established target of circulating AngII, reduced the increase in SNA following AIH and Int.AngII by 65% and 59% respectively, but did not prevent the sensitization of the sympathetic peripheral chemoreflex, or the increase in central sympathetic-respiratory coupling. Combined CBD and inhibition of the SFO eliminated the sympathetic SNA following AIH and Int.AngII. Intermittent systemic injections of phenylephrine cause an elevation in SNA similar to AIH, and this effect is prevented by renin-inhibitor, aliskiren. Our findings show that the sympathetic effects of AIH are due to RAS-mediated activation of the CB and SFO.

ABBREVIATIONS LIST

AIH, acute intermittent hypoxia;
AngII, angiotensin II;
AP, arterial pressure;
AT_1R, angiotensin II type 1 receptor;
AUC, area under curve;
CB, carotid body;
CBD, carotid body denervation;
CIH, chronic intermittent hypoxia;
HR, heart rate;
Int.AngII ; intermittent systemic injections of angiotensin II;
PNA, phrenic nerve activity;
RAS, renin-angiotensin system;
SFO, subfornical organ;
SNA, sympathetic nerve activity.

INTRODUCTION

Chronic elevations in the activity of the sympathetic nerves controlling the cardiovascular system are a pathophysiological characteristic of neurogenic hypertension (Esler, 2010). In patients with obstructive sleep apnea, there is evidence suggesting that recurrent hypoxia during sleep contributes to the elevation in daytime sympathetic nerve activity (SNA) and hypertension associated with this condition (Peppard *et al.*, 2000; Taylor *et al.*, 2016). Similarly, adult rodents exposed to chronic intermittent hypoxia (CIH) develop increased SNA, hypertension (Fletcher *et al.*, 1992a; Fletcher *et al.*, 1992b; Fletcher *et al.*, 1999; Zoccal *et al.*, 2007; Del Rio *et al.*, 2016), and increased sensitivity of sympathoexcitatory reflexes (Huang *et al.*, 2009; Silva & Schreihofer, 2011). The processes responsible for the effects of intermittent hypoxia on the SNA are not fully understood (Prabhakar *et al.*, 2015; Kim *et al.*, 2016a).

Experimental data suggests peripheral renin-angiotensin system (RAS) activation and circulating angiotensin II (AngII) contributes to hypertension in CIH-treated rodents (Shell *et al.*, 2016). For example, plasma renin is elevated in CIH-treated rats (Fletcher *et al.*, 1999) and prophylactic treatment with losartan, an angiotensin II type 1 receptor antagonist (AT_1R) prevents hypertension caused by CIH (Fletcher *et al.*, 1999; Marcus *et al.*, 2010; Marcus *et al.*, 2012). Interestingly, AT_1R antagonism does not normalize arterial pressure (AP) once hypertension is established in CIH-treated rats (Zoccal *et al.*, 2007), which suggests that AT_1R binding does not support elevated AP in CIH-treated rats.

Currently, the neural mechanisms by which RAS activation in CIH causes elevated SNA and hypertension are unclear. Normally, the neural effects of circulating AngII are mediated largely by neural structures that lie outside the blood-brain barrier (Smith & Ferguson, 2010), notably the carotid bodies (CB) and the subfornical organ (SFO) in the hypothalamus. The CB, which is critical for the cardio-respiratory response to hypoxia (Prabhakar *et al.*, 2015) and contributes to hypertension in CIH-treated rats (Del Rio *et al.*, 2016), expresses AT_1Rs and is activated by AngII *in vitro* (Allen, 1998; Peng *et al.*, 2011). The SFO is arguably the most important neural structure for the autonomic and behavioural effects of circulating AngII, and siRNA knockdown of AT_1R in the SFO reduces hypertension in CIH-treated rats (Saxena *et al.*, 2015).

In order to identify the mechanisms of the sympathetic changes caused by intermittent hypoxia, we and others have used acute intermittent hypoxia (AIH) in anesthetized adult rats (Dick *et al.*, 2007; Xing & Pilowsky, 2010; Yamamoto *et al.*, 2015; Kim *et al.*, 2016b). In the AIH model, there

is a highly reproducible increase in mean SNA in the absence of changes in arterial pressure (AP), and inconsistent changes in central respiratory drive (Dick *et al.*, 2007; Xing & Pilowsky, 2010). Like CIH, the underlying processes that lead to elevated SNA in AIH remain unclear. Considering the evidence implicating RAS signalling in cardiovascular effects of CIH, we examined the contribution of RAS signalling to the sympathetic effects of AIH, and subsequently to determine the contribution of the CB and the SFO to the sympathetic effects of AIH.

Methods

Ethical approval

All experimental procedures were executed in strict accordance with the guidelines set in the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes, endorsed by the National Health and Medical Research Council of Australia. This study was approved by the Sydney Local Health District Animal Welfare Committee (2015-013). All experimental procedures conform to the principles and regulations as described in Grundy (2015).

Subjects and Preparation

Male Sprague-Dawley rats (n = 114; 250–400 g) were acquired from Rat Resource Centre (WA, Australia) and were group housed and provided *ad libitum* water and standard rodent chow at the Heart Research Institute Biological Facilities prior to experiments. On the day of the experiment, rats were anesthetized with urethane (1.3 $g \cdot kg^{-1}$ i.p., with additional doses of 30 mg in 10% solution as required). An absence of the hind-paw withdrawal reflex and corneal reflex was used to confirm adequate anaesthesia prior to beginning surgery. Core temperature was maintained throughout the surgery and experimental procedures using a servo-controlled heating pad set at 37.0 ± 0.5°C (CWE, Inc., Ardmore, PA). Following completion of experiments, rats were given an additional 60 mg of urethane intravenously followed by 1 ml of 3M potassium chloride to stop the heart.

Surgical Procedure

The right carotid artery and jugular vein were cannulated using polyethylene tubing (PE50; Microtube Extrusions, North Rocks, Australia) for recording arterial blood pressure and infusing drugs and fluids, respectively. Electrocardiogram was recorded from leads implanted in the forepaws to determine heart rate. A tracheostomy was performed, and a 14-gauge cannula was sutured into the trachea to allow connection to a ventilator (Ugo Basile, Varese, Italy). Rats were artificially ventilated with medical oxygen (Ventilation parameters: 70 cycles/min, 1 mL/100 gm). Expired CO_2 was monitored (CapStar 100 CO_2 analyser; CWE, Inc., Ardmore, PA) and arterial blood gases and

electrolytes (VetStat; IDEXX Laboratories, Westbrook, ME) were intermittently measured (Table 1 and 2). Ventilation was adjusted to maintain a constant expired CO_2 and a P_aCO_2 of 40.0 ± 5 mmHg and pH between 7.35 and 7.45.

Following ventilation, rats were bilaterally vagotomised and paralysed using Pancuronium Bromide (0.4 mg administered as a 0.2 ml bolus i.v. injection, followed by an infusion of 10% Pancuronium bromide diluted in 0.9% saline, infused at a rate of 2 ml·hour^{-1}; AstraZeneca, London, United Kingdom). Following paralysis, depth of anaesthesia was determined by observing MAP changes in response to a strong tail pinch stimulus. Additional doses of urethane (30 mg in 10% solution) were delivered intravenously in cases where MAP increased or decreased by more than 10 mmHg. A retroperitoneal approach was used to access to the left greater splanchnic nerve at a site proximal to the coeliac ganglion, and a dorsal approach was used to access the left phrenic nerve. Nerve activities were recorded using bipolar silver electrodes.

Bilateral Carotid Body Denervation

To access the carotid body, the sternohyoid and sternocleidomastoid muscles were carefully retracted. The carotid bifurcations were exposed on both sides, the carotid body identified, and the carotid sinus nerve was resected. Sham-operated rats underwent the same surgical procedures to expose the carotid body, but the carotid sinus nerves were left intact.

Subfornical Organ Microinjection

A midline incision was made through the skin of the rat skull and a craniotomy was performed to provide access to the SFO (Co-ordinates: 1.35 mm caudal of bregma, midline, 4.2-4.3 mm ventral from the surface of the brain). To preserve the superior sagittal venous sinus, the initial penetration of the micropipette was performed 0.5 mm lateral of midline and advanced 3.0 mm ventrally before being shifted back to the midline, and advanced to final target of the SFO. To inhibit the SFO we injected the $GABA_a$ receptor agonist, Isoguvacine (10 mM in phosphate buffer saline, PBS; Sigma-Aldrich, Australia), 10-min prior to the start of AIH or intermittent AngII and then again 1 hour later after the completion of the protocol. Two injections were performed to ensure the SFO was inhibited for the duration of the experiment. Injection volume was 100 nl per injection and the site was injection was marked by adding 1% Chicago Blue dye (Sigma-Aldrich, Australia) to the injectate. PBS injections were performed to control for the effects of microinjection. Following these experiments, the brain was extracted and fixed in 10% neutral-buffered formalin (Sigma-Aldrich, Australia) for at least 72 hours. The brain was coronally sectioned (80µm) and mounted on slides and cover slipped before being examined to confirm the correct placement of injections in the SFO.

Gas delivery

Prior to the commencing the AIH protocol, and between periods of hypoxia, rats were ventilated with 100% O_2. The protocol for AIH was adopted from previous studies (Xing & Pilowsky, 2010; Kim *et al.*, 2016b). Rats are subject to 10 cycles of hypoxia lasting 45 seconds with 5 minutes of recovery between hypoxia cycles breathing 100% oxygen. The hypoxia gas consists of 10% oxygen balanced with nitrogen (Coregas, Yennora, Australia). All gases were administered through the ventilator's passive air intake.

Drug Administration

Intermittent (Int.) intravenous injections of Angiotensin II (35 pmol per injection; Abcam, Melbourne, Australia), Phenylephrine-Hydrochloride (2.5 μg per injection; Sigma-Aldrich, Castle Hill, Australia) and Sodium Nitroprusside-Dihydrate (2.5 μg per injection; Sigma-Aldrich, Castle Hill, Australia) were administered in 10 boluses of 0.1 ml delivered with 5-min recovery intervals. Both AngII and phenylephrine (350 pmol and 25 μg in 0.1 ml saline, respectively) were delivered as a single bolus or as an infusion over 10 minutes as dose and injection control groups respectively. In experiments involving pre-treatment with Losartan-Potassium (2 $mg \cdot kg^{-1}$; Sapphire Bioscience, Redfern, Australia) or Aliskiren-Hemifumarate (1 $mg \cdot kg^{-1}$; Selleckchem, Australia), drugs were delivered as an intravenous bolus 10 minutes before the initiation of AIH or Int.AngII. To control for the potential changes in basal SNA owing to changes in depth of anaesthesia or pre-treatment with Losartan or Aliskiren, we performed "time control", "vehicle control", and "pre-treatment drug control" experiments where rats were subjected to no intervention, vehicle injections (saline for intravenous; PBS for brain), or drug pre-treatment alone respectively.

Data Acquisition and Analysis

Data were obtained using an ADC system (CED 1401; Cambridge Electronic Design) and Spike 2 acquisition and analysis software (version 8.07; Cambridge Electronic Design). The neurograms were amplified 10-fold by a very low-noise preamplifier (CWE, Inc.), band-pass filtered (0.1–3 kHz), and amplified a further 1000 times for phrenic nerve activity and 2000 times for splanchnic nerve activity by a scaling amplifier (BMA-400 AC/DC Bioamplifier; CWE Inc.). The analog signal was then digitized (A/D converter 1401; Cambridge Electronic Design, Cambridge, United Kingdom) and sampled at 5 kHz.

Splanchnic Sympathetic Nerve Activity

For analysis, splanchnic SNA was rectified and smoothed (τ =1 second) and death level of SNA following euthanasia was subtracted from all values. In all experiments SNA was evaluated 2 hours from the protocol onset. Values of SNA in text refer to the mean activity over a 1-min interval taken immediately before AIH or Int.AngII (baseline) and then again 2 later (i.e. 60 minutes after the completion of AIH or Int.AngII). The final value in the text refers to the change in SNA normalized to the baseline period calculated as follows: SNA in μV (A) baseline before AIH or Int.AngII, and (B) 60 mins after AIH or Int.AngII:

$$\frac{B - A}{A} \times 100$$

Sympathetic Chemoreflex response

The Sympathetic chemoreflex response was calculated as the area under the curve (AUC) of the sympathetic response to hypoxia (45-sec of 10% O_2 balanced in N_2) above the resting level of SNA prior to any intervention, and again at the end of a 2-hour recording period. As with basal SNA, we normalised the sympathetic chemoreflex to the response observed prior to any treatment. The equation used is given as:

$$\frac{[\text{Hypoxia AUC (post stimulus)}] - [\text{Hypoxia AUC (baseline)}]}{[\text{Hypoxia AUC (baseline)}]} \times 100$$

Phrenic Nerve Activity

Phrenic nerve activity (PNA) was rectified and smoothed (τ 0.05 seconds). Phrenic nerve peak amplitude on the waveform across 10 respiratory bursts and frequency across this period were all measured at baseline before AIH or Int.AngII protocol and 60-min after the termination of the protocol. Values for MAP and HR (mean value across a 1-minute interval) were recorded at baseline and 60-min after the protocol to observe for any changes. If the antagonist was used, the baseline was taken following the injection of treatment.

Respiratory Modulation of Sympathetic Nerve Activity

Respiratory modulation of SNA was assessed using cycle-triggered averages of SNA triggered from the onset of integrated phrenic nerve bursts (minimum 10 cycles). Under hyperoxic normocapnic conditions in this prep, the most consistent pattern of respiratory-sympathetic coupling we observe is a post-inspiratory peak followed by a trough during the expiratory phase. We measured the difference between the post-inspiratory peak and following trough under hyperoxic conditions as an index of respiratory modulation. We also measured PNA amplitude to assess central

respiratory drive. These values were acquired prior to and 1 hour after the AIH protocol and expressed as a percentage change from baseline.

Statistics

Statistical analyses were performed using GraphPad Prism software (version 7; GraphPad Software, San Diego, CA). Following tests for normality (Shapiro-Wilk test), statistical significance was determined by either unpaired student's t-test where necessary, and a one-way analysis of variance using Holm-Šidák *post-hoc* test with correction for multiple comparisons. A two-way analysis of variance using Holm-Šidák *post-hoc* test with correction for multiple comparisons was used where stated. Differences were considered statistically significant if $P < 0.05$. Grouped data is presented as mean ± S.E.M unless noted.

RESULTS

Blood gases, electrolytes, and arterial pressure remain unchanged for all experimental conditions

Variables for AP and HR, and blood pCO2, pH and electrolytes were unchanged by AIH or any other procedure used in this study (Tables 1 and 2). Oxygen saturation was measured during a 45s hypoxia challenge (N=12 challenges); rats were hyperoxic at rest, and reached an oxygen saturation nadir of 87.3±0.7% during hypoxia trials (Table 3).

Increased SNA and peripheral chemoreflex sensitivity following AIH are dependent on AT_1Rs and renin activity

To evaluate the involvement of AT_1Rs in the elevation of SNA following AIH, rats were administered either saline or losartan 10 minutes before the AIH protocol (Figure 1A and B). Rats pre-treated with saline exhibited a significant increase in mean SNA 60 minutes after AIH (AIH+Saline vs. Time control, 33.7±2.9% vs 2.6±2.2%, P<0.0001, Figure 1C), which was completely prevented by pre-treating rats with losartan (-0.9±4.1%, P<0.0001 vs. AIH+Saline, Figure 1A, B and C); this dose of losartan significantly reduced the pressor response caused by intravenous 35 pmol of AngII (21.2±0.9 vs. 8.2±0.8 mmHg, P<0.0001, AngII vs. AngII+Losartan),. Furthermore, the sympathetic response to hypoxia was approximately doubled following AIH in saline-treated rats (AIH+Saline vs. Time control, 114.0±19.7% vs. 11.5±5.4%, P=0.0003, Figure 1D and E), and this too was completely prevented by pre-treating rats with losartan (11.4±10.4%, P=0.0003 vs. AIH+Saline, Figure 1D and E). For a more

direct confirmation that the sympathetic effects of AIH is mediated by the RAS, we used aliskiren (1 $mg \cdot kg^{-1}$), a drug that binds to renin preventing the conversion of angiotensinogen to angiotensin I, to inhibit the RAS. Administration of aliskiren fully abolished the elevation in SNA (-0.8±4.6%, P=0.0003 vs. AIH+Saline, Figure 1F and G) and sympathetic chemoreflex sensitivity (6.0±9.3%, P=0.0027 vs. AIH+Saline, Figure 1H) following AIH.

Intermittent systemic injections of AngII increases SNA and peripheral chemoreflex sensitivity

Next, we examined the effect of intermittent intravenous injections of AngII delivered in a pattern replicating AIH (abbreviated to Int.AngII; 35 pmol in 0.1ml repeated 10 times in 5 minute intervals). Int.AngII produced an increase in mean SNA that was not observed when saline was administered in the same fashion (Int.AngII vs. Int.Saline, 31.4±5.7% vs 1.2±2.7%, P=0.0003, Figure 2A and B). The overall kinetic and magnitude of the elevation in SNA after Int.AngII was comparable to AIH (compare Figure 1A, C and 2A, B), while AP was unchanged from baseline (Table 1). Similar to AIH, Int.AngII potentiated the sympathetic response to hypoxia (93.1±13.1%, P<0.0001 vs. Int.Saline, Figure 2C, D). Similar to AIH, pre-treating rats with losartan prior to Int.AngII prevented the elevation in mean SNA (0.8±3.7%, P=0.0003 vs. Int.AngII, Figure 2A and B), and the sensitization of the sympathetic response to hypoxia (6.7 ±10.9%, P<0.0001 vs. Int.AngII; Figure 2C and D). Finally, an equivalent dose of AngII (i.e. 350 pmol) given as a single injection or infused over a 10-min period had no effect on mean SNA (0.9±2.6% and -0.9±3.0% respectively, n=4 for both, P<0.001 vs. Int.AngII for both, Figure 2B).

Confirmation of successful carotid body denervation and SFO microinjections

Bilateral denervation of the carotid body (CBD) was confirmed by the absence of PNA (49.4±7.1% vs. 1.5±1.7%, P=0.0006, Sham vs. CBD), phrenic frequency (PNf; 58.3±9.9% vs. 1.4±2.1%, P=0.0013, Sham vs. CBD), and SNA (65.0±5.4 vs. 12.2±1.3%, P<0.0001, Sham vs. CBD, n=10 for Sham and n=11 for CBD) responses to hypoxia (Figure $3A_i$-A_{iv}). Baroreflex function was confirmed by the presence of SNA fluctuations associated with pulsatile blood pressure (0.50±0.1 µV vs. 0.59±0.2µV, P=0.6881, Sham vs. CBD, n=10 for Sham and n=11 for CBD, Figure $3B_i$ and B_{ii}). Microinjections of the GABA receptor agonist isoguvacine or saline mixed 1% Chicago Blue dye (N=22 with isoguvacine and N=10 with saline) resulted in injection sites that covered the rostro-caudal extent of the SFO and included the ventral hippocampal commissure and in some cases the corpus callosum (example of the injection site shown in Figure 3C). We did not observe dye labelling in the median preoptic

nucleus or dorsal thalamic nuclei below the 4th ventricle. In the course of establishing accurate stereotaxic coordinates for the SFO, we performed injections in the 4th ventricle as indicated by the absence of a discrete injection spot and diffuse light staining of ependymal cells lining the 3rd and 4th ventricle and the choroid plexus. We grouped these cases separately (SFO_{miss}) from microinjections that successfully targeted the SFO.

Elevation of SNA following AIH requires functional CBs and SFO

Following CBD, the elevation in mean SNA following AIH was approximately half the magnitude of sham-operated rats (14.0±1.2% vs. 32.2±3.2%, CBD vs. Sham, P<0.001; Figure 4A and B). Conversely, inhibition of the SFO with the $GABA_A$ agonist, isoguvacine (SFO_{iso}), resulted in a significantly smaller increase in mean SNA following AIH compared to rats injected with vehicle and then subject to AIH ($SFO_{vehicle}$ vs SFO_{iso}; 17.0±1.8% vs. 48.1±3.3%; P<0.0001; Figure 4A and C). Inhibition of the SFO did not abrogate the enhanced sympathetic response to hypoxia observed in AIH-treated rats ($SFO_{vehicle}$ vs SFO_{iso}; 106.8±15.7% vs. 108.0±13.2%; Figure 4D). When injections of isoguvacine were placed in the 4th ventricle (SFO_{miss}), we observed elevations in mean SNA following AIH (42.4±5.8%) equivalent to rats injected with vehicle prior to AIH (P=0.3101; Figure 4C). Notably, mean SNA following AIH in rats with CBD or SFO inhibition was still significantly elevated compared to rats not subject to AIH (AIH+CBD vs. Time control, P=0.0209; AIH+SFO_{iso} vs. Time control, P=0.0059, Figure 4C). However, combining CBD and SFO inhibition eliminated the elevation in mean SNA following AIH (5.9±3.0%, P=0.4493 vs. Time control, Figure 4A and C).

Intermittent AngII increases SNA via the CBs and the SFO pathways

As the sympathetic effect of AIH requires the CBs and the SFO, we speculated that the sympathetic effects of Int.AngII may utilise similar pathways. We found CBD reduced the elevation in SNA following Int.AngII by 45% (CBD vs. Sham; 16.3±1.9% vs. 28.8±2.1%, P=0.0001; Figure 5A and B), and inhibition of the SFO in CB intact rats reduced the elevation in SNA following Int.AngII by 59% (12.1±1.0%, P<0.0001 vs. Sham (also microinjected with PBS), Figure 5A and B). Combined CBD and SFO inhibition in a separate series of rats fully eliminated the elevation in mean SNA caused by Int.AngII (1.8±0.5%, P<0.0001 vs. CBD; P=0.001 vs. SFO_{iso}; Figure 5A and B).

Enhanced central sympathetic-respiratory coupling following AIH requires AT_1R and functional CBs, but not the SFO

Previous studies have demonstrated that AIH increases the coupling between central respiratory drive and SNA (Dick *et al.*, 2007; Xing & Pilowsky, 2010). Consistent with these studies, we found that AIH approximately doubles central sympathetic-respiratory coupling in rats pre-treated with saline (98.6±24.0% increase in modulation; Figure 6A and B). Contrastingly, AIH did not increase central sympathetic-respiratory coupling in rats pre-treated with losartan (7.0±15.2%, P=0.0017 vs. AIH+Saline) or after CBD (-3.3±7.7%, P=0.0027 vs. Sham). Inhibition of the SFO did not prevent the increased central sympathetic-respiratory coupling following AIH (83.5±12.6%, P=0.6826 vs. AIH+S; Figure 6B). Phrenic amplitude was also compared between groups; AIH did not produce a noticeable change in phrenic amplitude (13.2±2.3% vs. 19.3±3.9%, Time control vs. AIH+Saline, P>0.9999), while antagonising AT_1Rs caused a substantial reduction in phrenic nerve amplitude (19.3±3.9% vs. -38.4±10.5%, AIH+Saline vs. AIH+Losartan, P=0.0009), whereas all other interventions had no discernible effects (Figure 6C).

Intermittent injections of phenylephrine systemically cause SNA to enhance, which is renin-dependent

One of the effects of acute hypoxia and systemic AngII in CB intact rats is peripheral vasoconstriction. A transient increase in AP during acute hypoxia may cause restricted blood flow into the kidney, triggering the release of renin. To mimic this effect, we administered intravenous phenylephrine in a pattern matching AIH in CB intact rats (abbreviated as Int.Phenylephrine, 2.5μg in 0.1 ml repeated 10 times in 5-minute intervals). Phenylephrine caused a transient elevation in AP that was similar in magnitude to acute hypoxia and AngII (Hypoxia vs. AngII vs. Phenylephrine; 30±5 mmHg vs. 24±2 mmHg vs. 25±6 mmHg respectively, P>0.5 for all comparisons, Figure 7A_i and A_{ii}). As hypothesised, Int.Phenylephrine produced a progressive increase in mean SNA that had a similar kinetic and magnitude to AIH and Int.AngII (35.9±4.2%, Figure 7B and C), and could be prevented by pre-treating rats with aliskiren (1 mg· kg^{-1}, IV; -1.1±2.2%, P<0.0001 vs. Int.Phenylephrine; Figure 7B and C). Finally, a continuous infusion of phenylephrine for 10 minutes did not increase mean SNA (-5.1±6.1%, n=4, P=0.9655 vs. Int.Saline, data not shown in figure).

Elevation of SNA following AIH in CBD rats is not caused by hypotension

In the absence of the CBs, hypoxia causes hypotension and a modest increase in SNA presumably due to unloading of arterial baroreflex (Figure $7A_{iii}$). We considered the possibility that intermittent unloading of the baroreceptors may be responsible for the increase in mean SNA after AIH in CB denervated rats. To test this possibility, we administered intravenous sodium nitroprusside (Int.Sodium nitroprusside) in a pattern matching AIH in CB intact rats (2.5µg in 0.1 ml repeated 10 times in 5-minute intervals). Injections of sodium nitroprusside in CB intact rats produced similar falls in AP as hypoxia in CBD rats (Hypoxia + CBD vs. SNP, -12±2 mmHg vs. -15±1 mmHg, P=0.1905, Figure $7A_{iii}$ and A_{iv}) but failed to produce a sustained elevation in mean SNA 60 minutes after this protocol (2.2±3.2% vs. 1.2±2.7%, Int.Sodium nitroprusside vs. Int.Saline, P=0.9966, Figure 7B and C).

DISCUSSION

We demonstrate that AIH in anesthetized adult rats causes an elevation in SNA, sensitization of the sympathetic peripheral chemoreflex, and an increase in central sympathetic-respiratory coupling that are all prevented by pre-treatment with an AT_1R antagonist or inhibition of renin activity. We also show that the sympathetic effects of AIH can be recapitulated by injecting exogenous AngII in a pattern matching AIH. The elevation in SNA following AIH and intermittent AngII is the result of two distinct neural mechanisms involving a CB-mediated increase in central sympathetic-respiratory coupling and enhanced chemoreflex sensitivity, and a SFO-mediated increase in tonic SNA. Finally, we show that intermittent sodium nitroprusside does not enhance sympathetic discharge, whereas experimentally-induced vasoconstriction with phenylephrine produces a similar elevation in SNA as AIH and intermittent AngII, and this effect is also dependent on RAS activity.

The sympathetic effects of AIH are mediated by the renin-angiotensin system

We demonstrate that the sympathetic effects of AIH are dependent on activation of RAS. First, we show that AT_1Rs and renin activity are necessary for the increase in SNA following AIH. Secondly, we show that systemic AngII delivered in an AIH-like pattern is sufficient to replicate the sympathetic effects of AIH, and this also requires AT_1Rs. As has been shown before (Peng *et al.*, 2011), the intermittent pattern of AngII administration is important for the effects we observed because an equivalent dose of AngII delivered by infusion, or as a single injection, did not produce

any lasting effects on SNA. Together, these experiments suggest that the sympathetic effects of AIH are caused by transient surges in AngII, and these surges engage mechanisms that cause a protracted increase in SNA via actions in the CNS. A similar pattern of RAS activation may be present in CIH because prophylactic losartan reduces or prevents hypertension in CIH-exposed rats (Fletcher *et al.*, 1999; Marcus *et al.*, 2010; Marcus *et al.*, 2012), as well as in healthy humans exposed to 6 hours of recurring hypoxia (Foster *et al.*, 2010).

Whether or not AIH causes a chronic elevation in RAS activity that supports elevated SNA was not evaluated in this study, though this is possible. However, studies have shown that administering AT_1R antagonists after AIH in anesthetised rats does not normalise mean SNA (Yamamoto *et al.*, 2015), and losartan does not correct hypertension in CIH-exposed rats (Zoccal *et al.*, 2007). Furthermore, losartan application in superfused CBs effectively prevents the sensory facilitation caused by intermittent hypercapnic hypoxia, but does not reverse this effect when applied afterwards (Roy *et al.*, 2017). This evidence suggests that AngII surges and AT_1R activation during intermittent hypoxia activates a cascade of signalling that is subsequently insensitive to acute AT_1R blockade. Intermittent hypoxia and AngII cause transcriptional changes in the CB (Prabhakar *et al.*, 2015) and SFO (Zimmerman *et al.*, 2002; Lob *et al.*, 2013) that are associated with the production of ROS, which may explain the persistence of changes in SNA following AIH and Int.AngII. Furthermore, several neuropeptides have been suggested to contribute to the sympathetic effects of AIH, such as orexin (Kim *et al.*, 2016b), serotonin (Bautista *et al*., 2012; Dick *et al.*, 2007), vasopressin (Kc *et al.*, 2010), and PACAP (Kakall *et al.*, 2017); the release of these peptides during AIH would produce long-lasting changes in CNS excitability that are unresponsive to acute AT_1R blockade.

Chemosensory CB and SFO mediate distinct components of the sympathetic effects of AIH

We show that the CB and the SFO are required for the elevation of SNA following AIH and intermittent AngII. Our supposition that the CB and SFO are the site of action for AngII in AIH is supported by evidence that AT_1Rs are expressed by glomus cells of the CB (Allen, 1998; Lam *et al.*, 2014) and neurons in the SFO (Mendelsohn *et al.*, 1984; Castrén & Saavedra, 1989). Nevertheless, the site of action of AngII in our model may be in the periphery, for example, the blood vessel endothelial cells or the kidney (Marcus *et al.*, 2012; Emans *et al.*, 2016), and the activation of the CBs and SFO in AIH may be mediated by secondary messengers released from these tissues in response to AT_1R binding, such as endothelin-1 (Kanagy *et al.*, 2001; Rey *et al.*, 2006). Another outstanding

issue not addressed in this study is whether AngII is derived from peripheral RAS activation, or from endogenous mechanisms for AngII production in the SFO (Grobe *et al.*, 2008) and CBs (Leung *et al.*, 2000; Lam & Leung, 2002, 2003; Lam *et al.*, 2014). Notably, previous studies indicate that AngII production in the CB is renin-independent (Lam & Leung, 2002). We show that the sympathetic effects of AIH are prevented by inhibition of renin activity with aliskiren, which supports a role for peripheral RAS in AIH.

An interesting finding in this study is that CBD eliminated the increase in sympathetic chemoreflex sensitivity and central sympathetic-respiratory coupling following AIH, and inhibition of the SFO had no effect on these parameters. It is important to note that unlike models used to induce phrenic long-term facilitation (Mitchell & Johnson, 2003), we do not regularly observe increased phrenic nerve activity following AIH, likely due to differences in AIH protocol. As such increased central sympathetic-respiratory coupling following AIH is not caused by enhanced central respiratory drive, but relates to changes in coupling of respiratory and sympathetic neurons; and our data shows that the CB drives this effect. Conversely, the SFO likely elevates SNA through hypothalamic pathways that engage sympathetic neurons in the brainstem or spinal cord that are largely independent of the central respiratory network, such as descending inputs from neurons in the paraventricular nucleus of the hypothalamus (Holbein & Toney, 2015).

There is evidence that elevated SNA precedes the onset of hypertension in rodent models (Cabassi et al., 1998; Simms et al., 2009), as such, we speculate that the increase in SNA following AIH reflects the early stages of the sympathetic changes that cause hypertension in CIH; our finding that the elevation in SNA after AIH is mediated by the CBs and SFO is consistent with this notion because hypertension in CIH is also dependent on the CBs and SFO. Del Rio *et al.* (2016) showed that CBD in CIH-exposed rats with established hypertension normalises AP and autonomic function, proving that CIH has modest effects in the absence of the CBs. While an earlier study demonstrated that central administration of losartan or knockdown of AT_1Rs in the SFO reduces the elevation of AP in CIH-exposed rats (Saxena *et al.*, 2015). Collectively, these studies support the notion that the effect of CIH on AP is mediated by recurrent activation of the CBs, leading to peripheral RAS activation and subsequent activation of the SFO and possibly other neurons expressing AT_1Rs (Faulk *et al.*, 2016; Shell *et al.*, 2016). This model largely accommodates our data, however, we provide evidence that hypoxia causes RAS-dependent sympathetic effects in the absence of the CBs.

Whether CB-independent RAS activation during hypoxia is relevant in CIH models is not clear, though the report by Del Rio *et al.* (2016) suggests that this is unlikely. There are considerable technical differences between the AIH and the CIH model that may influence the underlying

processes that cause SNA to increase, most notably, our use of anaesthetics, mechanical ventilation, and supplemental oxygen. Reporting on oxygen saturation during CIH is limited; one report indicates that oxyhemoglobin falls to 60-80% after less than 6 sec of exposure to FiO_2 of 3-5% (Fletcher *et al.*, 1992b). Del Rio *et al.* (2016) exposed rats to FiO_2 of 5% for 20 sec, suggesting that these rats were probably subject to oxygen desaturations less than 60%. Oxygen desaturations in our AIH model is comparably mild (nadir of 87%), but our rats were ventilated at all other times with oxygen resulting in a stark elevation in baseline pO_2 (≈470mmHg). Thus, differences between the CIH and the AIH model could be related to more severe oxygen desaturations during CIH, or a hyperoxia baseline in AIH. Hyperoxia has broad ranging effects on the CNS including inducing oxidative stress and cerebrovascular acidosis (Dean *et al.*, 2004). Most significantly, hyperoxia silences the CB, which eliminates the CB as a source of drive for the sustained increase in SNA following AIH. Thus performing experiments over a background of hyperoxia may under-emphasise the involvement of the CB in the sympathetic effects of intermittent hypoxia. Nevertheless, it is clear that the sympathetic effects of AIH and CIH share a dependency on RAS activation.

Systemic injections of phenylephrine produced a similar sympathoexcitatory effect to AIH

We provide evidence that peripheral vasoconstriction induced by activating α_1-adrenergic receptors with phenylephrine causes a protracted increase in SNA similar to AIH. The elevation in SNA following repetitive administrations of phenylephrine was prevented by blocking renin activity, thus is probably mediated by the same pathways as AIH. It is noteworthy that injections of phenylephrine cause inhibitions of sympathetic neurons in the rostral ventrolateral medulla and the spinal cord, which is the opposite to the excitatory effects that is elicited by hypoxia. Conversely, intermittent sodium nitroprusside had no lasting effects on mean SNA even though it strongly activates the central sympathetic network controlling the cardiovascular system. Collectively, these evidence show that renin activity induced by peripheral vasoconstriction alone is sufficient to reproduce the sympathetic effects of AIH, while repeated sympathetic activation in the presence of exogenous nitric oxide (sodium nitroprusside) has no discernible effect on SNA.

Conclusion

The present study demonstrates the importance of the RAS for the sympathetic effects of AIH, and suggests that transient repeated surges in AngII causes immediate increases in SNA. The relationship between recurrent hypoxia and transient RAS activation may contribute to the

association between apnea/hypopnea index and daytime SNA in humans with OSA (Peppard *et al.*, 2000; Taylor *et al.*, 2016). We also show that the sympathetic effects of AIH require both the CB and SFO, and AIH has sympathetic effects in CB denervated rats. Considering that CBD is increasingly considered as viable treatment for severe and resistant hypertension in humans (Paton *et al.*, 2013), and the modulation of CB activity is proposed as a treatment for autonomic dysfunction in OSA patients (Del Rio *et al.*, 2016), it will be important to fully understand the underlying mechanism responsible for sympathetic activation by hypoxia in the absence of the CBs.

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AUTHOR CONTRIBUTIONS

S.J.K, A.Y.F, P.M.P. and S.B.A. conceived and designed the study;
S.J.K. and A.Y.F performed experiments;
S.J.K. and S.B.A. analyzed the data, interpreted the results, prepared figures, and drafted the manuscript
S.J.K, P.M.P. and S.B.A. edited and revised the manuscript;
All authors approved the final version of the manuscript

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CONFLICT OF INTEREST/DISCLOSURE

None.

TABLES

Table 1. Values for sympathetic nerve activity, mean arterial pressure, and heart rate at before (i.e. baseline) and after the termination of the protocol (i.e. 60 mins after recording).

Treatment groups	*n*	SNA (µV)	MAP (mmHg)		HR (bpm)	
		Baseline	Baseline	Post-	Baseline	Post-
Time control	5	3.55±0.36	99±6	97±6	457±18	449±17
Losartan control	4	3.44±0.57	89±6	88±9	477±12	467±12
AIH+Saline	5	3.34±0.25	101±9	107±8	446±10	463±11
AIH+Losartan	5	3.43±0.24	88±11	90±6	439±18	437±9
AIH+Aliskiren	4	3.98±0.50	98±4	95±4	431±10	427±8
Int.Saline	4	3.21±0.11	110±8	109±4	436±3	436±7
Int.AngII	5	3.18±0.15	100±5	110±4	433±5	448±7
Int.AngII+Losartan	4	3.25±0.46	90±5	91±7	441±16	432±11

Single AngII	4	3.17±0.19	102±5	105±6	414±19	414±20
Continuous AngII	4	3.17±0.18	99±8	101±5	429±7	432±11
AIH+Sham	4	3.34±0.40	101±8	105±6	463±8	468±11
AIH+CBD	6	3.21±0.34	96±4	99±5	486±7	476±10
AIH+$SFO_{vehicle}$	4	3.49±0.33	92±8	85±9	439±8	444±10
AIH+SFO_{miss}	4	3.19±0.13	102±1	96±3	471±7	476±10
AIH+SFO_{iso}	6	3.28±0.25	112±5	105±7	472±5	471±8
AIH+CBD·SFO_{iso}	6	3.71±0.48	96±3	91±4	478±7	460±10
Int.AngII+Sham	6	3.00±0.27	104±6	106±5	450±20	449±18
Int.AngII+CBD	5	3.50±0.27	110±3	114±4	458±10	461±11
Int.AngII+SFO_{iso}	6	4.10±0.35	102±4	100±5	484±10	479±11
Int.AngII+CBD·SFO_{iso}	4	3.80±0.54	91±6	92±5	464±9	468±13
Int.Phenylephrine	5	3.32±0.41	101±8	101±13	426±8	427±3
Int.Phenylephrine+Aliskiren	4	3.10±0.17	108±6	112±6	431±6	428±4
Continuous Phenylephrine	4	3.21±0.13	103±7	102±8	410±3	407±6
Int.Sodium nitroprusside	6	3.31±0.26	115±6	115±7	433±5	432±8

Values are mean ± SEM. Comparisons using one-way ANOVA with Holm-Šidák correction for multiple comparisons between groups for baseline sympathetic nerve activity (SNA), mean arterial pressure (MAP), and heart rate (HR) values. Comparisons using Two-way ANOVA with Holm-Šidák correction for multiple comparisons between baseline and post- values for MAP and HR values. There were no differences between all comparisons.

Table 2. Values for pulmonary carbon dioxide (PCO_2),), sodium (Na^+), potassium (K^+), and chloride (Cl^-) levels prior to the start of the protocol (i.e. baseline conditions) and after the termination of the protocol (i.e. 60 mins after recording).

Hypoxia groups	PCO_2 (mmHg)		Na^+(mmolL^{-1})		K^+(mmolL^{-1})		Cl^-(mmolL^{-1})	
	Baseline	Post-	Baseline	Post-	Baseline	Post-	Baseline	Post-
Time control	43.0 ±1.0	42.4±0.5	145.4 ±1.5	143.6±1.5	4.2±0.2	4.2±0.2	112.4± 1.2	111.8 ±1.0
Losartan control	42.3 ±1.2	42.8±0.9	142.0 ±1.1	142.8±1.3	4.1±0.2	4.1±0.1	110.5± 0.9	111.3 ±1.1
AIH+Saline	41.2 ±0.7	42.8±0.7	142.2 ±0.7	140.6±0.7	3.9±0.4	3.9±0.3	109.4± 0.5	110.2 ±0.4
AIH+Losartan	40.0 ±0.7	41.0±1.0	140.8 ±0.9	141.2±1.0	4.3±0.2	4.2±0.1	111.4± 1.2	112.8 ±0.8
AIH+Aliskiren	41.5 ±1.7	40.8±1.6	142.0 ±1.5	141.3±0.8	3.8±0.2	3.7±0.1	109.5± 0.6	110.5 ±0.9
AIH+Sham	41.3 ±1.5	41.8±1.4	141.3 ±0.9	141.4±0.7	3.7±0.2	3.7±0.2	110.0± 1.2	111.7 ±1.2
AIH+CBD	40.3 ±0.9	41.3±1.3	139.8 ±1.0	140.1±0.7	4.2±0.1	4.2±0.1	112.0± 1.3	111.3 ±0.6
AIH+$SFO_{vehicle}$	41.0 ±1.6	42.0±0.9	139.3 ±1.5	139.3±0.5	4.1±0.2	4.1±0.2	109.0± 0.9	109.5 ±0.9
AIH+SFO_{miss}	41.4 ±1.4	41.0±1.5	140.8 ±1.1	141.2±0.7	4.0±0.1	4.0±0.1	112.2± 1.0	111.4 ±0.4
AIH+SFO_{iso}	39.0 ±1.5	40.5±0.8	139.4 ±1.5	140.3±0.9	4.1±0.2	4.2±0.1	111.4± 1.4	111.0 ±0.7
AIH+CBD·SFO_{iso}	41.3 ±1.5	42.3±0.7	141.2 ±1.0	143.2±0.9	4.1±0.2	4.1±0.1	110.2± 1.0	109.5 ±1.0

Drug treatment groups	PCO_2 (mmHg)		Na^+(mmolL^{-1})		K^+(mmolL^{-1})		Cl^-(mmolL^{-1})	
	Baseline	Post-	Baseline	Post-	Baseline	Post-	Baseline	Post-

Int.Saline	41.5±1.7	40.8±1.5	140.8±0.8	140.3±0.3	4.1±0.2	4.1±0.5	111.3±1.7	110.3±0.5
Int.AngII	41.4±1.1	40.4±0.5	143.8±2.4	141.6±1.4	3.7±0.5	3.7±0.3	114.4±2.8	111.6±1.6
Int.AngII+Losartan	42.3±1.3	41.0±0.7	145.3±**2.4**	144.0±1.5	3.6±0.6	3.6±0.3	111.5±2.1	111.8±1.5
Int.AngII+Sham	40.6±0.9	39.8±0.6	141.8±0.8	141.4±0.6	4.0±0.3	4.1±0.2	108.6±1.5	108.6±1.4
Int.AngII+CBD	40.8±1.6	40.8±0.7	141.2±1.2	141.6±0.7	4.0±0.3	4.1±0.2	111.8±2.0	111.6±1.3
Int.AngII+SFO_{iso}	39.3±1.1	39.8±0.9	142.0±0.7	141.5±0.6	4.4±0.0	4.4±0.0	110.3±1.1	109.8±0.9
Int.AngII+CBD·SFO_{iso}	40.5±0.9	40.5±0.8	142.0±1.4	141.3±1.1	4.0±0.2	4.0±0.1	110.8±1.3	111.3±0.9
Int.Phenylephrine	40.7±1.8	39.7±0.9	139.3±2.2	140.7±0.3	4.4±0.2	4.3±0.0	109.0±3.8	110.3±2.9
Int.Phenylephrine+Aliskiren	42.7±0.9	42.7±0.6	139.0±2.5	139.8±2.4	4.5±0.1	4.5±0.1	111.3±0.3	111.7±0.5
Int.Sodium nitroprusside	40.0±2.5	40.0±1.3	142.3±0.3	142.2±0.5	4.4±0.2	4.4±0.2	110.7±1.8	109.6±1.9

Values are mean ± SEM. Comparisons using one-way ANOVA with Holm-Šidák correction for multiple comparisons between groups for baseline pulmonary carbon dioxide (PCO_2), sodium (Na^+), potassium (K^+), and chloride (Cl^-) levels. Comparisons using Two-way ANOVA with Holm-Šidák correction for multiple comparisons between baseline and post- values for PCO_2 values. There were no differences between all comparisons.

Table 3. Values for partial oxygen, plasma haemoglobin, and saturated oxygen levels at baseline, during acute hypoxia, and following re-oxygenation.

	Baseline	**Hypoxia**	**Re-oxygenation**
Partial O_2 (mmHg)	482.2±11.8	67.6±2.3****	475.6±8.1††††
Plasma Haemoglobin (g/dL)	21.0±0.4	24.2±0.3****	20.7±0.2††††
Saturated O_2 (%)	100.0±0.0	87.3±0.7****	100.0±0.0††††

Values are mean ± SEM. Comparisons using one-way ANOVA with Holm-Šidák correction for multiple comparisons. ****P<0.0001 vs. baseline, ††††P<0.0001 vs. Hypoxia, N=12.

FIGURE LEGENDS

Figure 1. Enhancement of sympathetic nerve activity following acute intermittent hypoxia (AIH) is mediated by the renin-angiotensin system.
Effect of AIH on arterial pressure (AP) and sympathetic nerve activity (SNA) in ***A,*** saline- and ***B,*** losartan-treated rats. ***C,*** Group data for the change in SNA 60-min post-AIH (****P<0.0001 vs. Time control, ††††P<0.0001 vs. AIH+Saline). ***D,*** Effect of losartan on the changes in the acute sympathetic chemoreflex caused by AIH. ***E,*** Group data for the change in acute sympathetic chemoreflex (****P<0.0001 vs. Time control, ††††P<0.0001 vs. AIH+Saline). ***F,*** Effects of AIH on AP and SNA in a rat pre-treated with aliskiren. ***G,*** Group data for the change in SNA 60-min post-AIH in aliskiren treated rats (***P<0.001 vs. AIH+Saline). ***H,*** Grouped data of the effect of aliskiren on the changes in the acute sympathetic chemoreflex caused by AIH. (**P<0.01 vs. AIH+Saline).

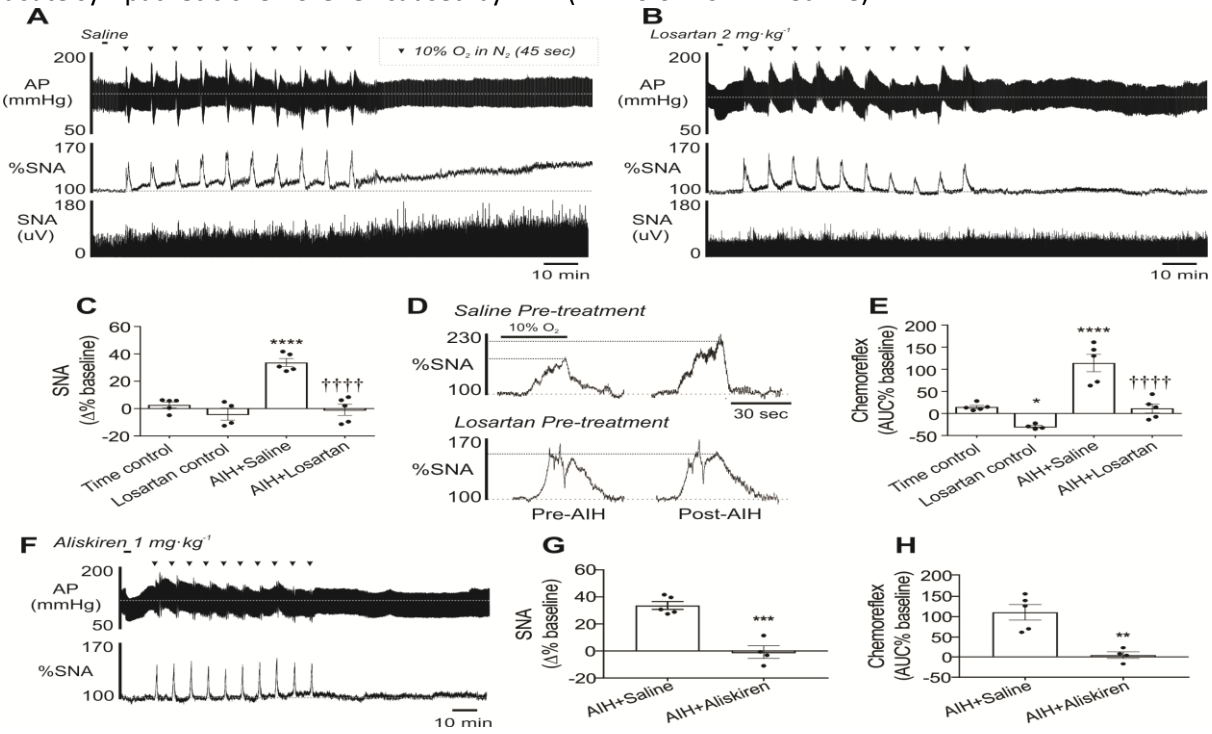

Figure 2. Intermittent intravenous injections of Angiotensin II causes AT_1R-dependent SNA facilitation.

A, Effects of intermittent intravenous injections of AngII (Int.AngII) on AP and SNA in rats untreated (upper trace) or pre-treated (lower trace) with losartan. ***B,*** Group data of the change in SNA 60-min after Int.AngII (***P<0.001 vs. Intermittent saline (Int.Saline), †††P<0.001 vs. Int.AngII). ***C,*** Changes in the acute sympathetic chemoreflex caused by AngII and saline injection paradigms. ***D,*** Group data for the change in acute sympathetic chemoreflex (****P<0.0001 vs. Int.Saline, ††††P<0.0001 vs. Int.AngII).

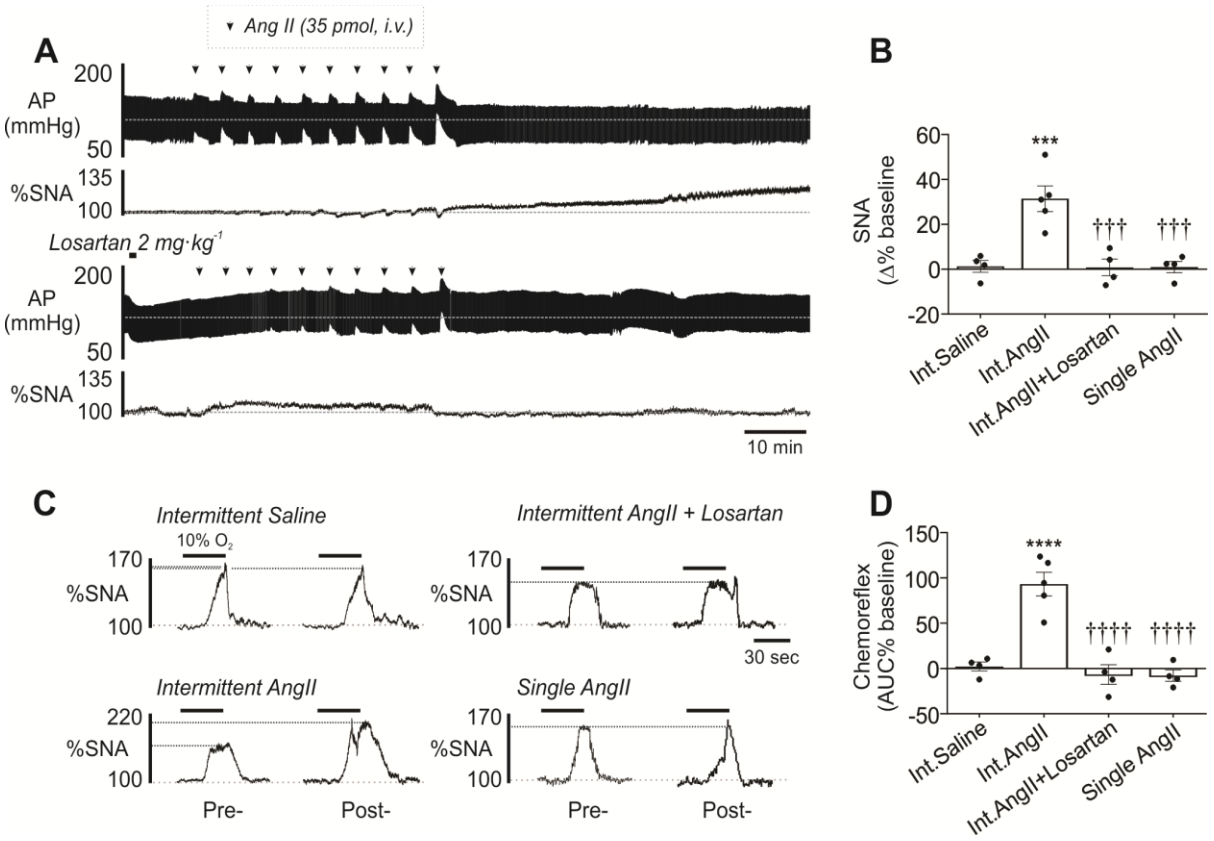

Figure 3. Confirmation of carotid body denervation (CBD) and microinjection site in the subfornical organ (SFO).

A_i, A 45-sec hypoxia challenge on AP, phrenic nerve frequency (PNf), phrenic nerve activity (PNA), and SNA in a sham-operated (left panel) and CB denervated (right panel) rat. Grouped data quantifying the percentage change from baseline for (***A_{ii}***) PNA, (***A_{iii}***) PNf, and (***A_{iv}***) SNA in sham-operated vs. CB denervated rat (****P<0.0001, ***P<0.001, **P<0.01 vs. Sham). ***B_i,*** Baroreflex modulation of SNA in sham and CBD rats. ***B_{ii},*** Grouped data showing SNA fluctuation in sham and

CBD rats. ***C,*** An example of a successful injection in the SFO marked with Chicago blue dye in a coronal section of brain at Bregma level -1.33mm. Scale bar: 1 mm.

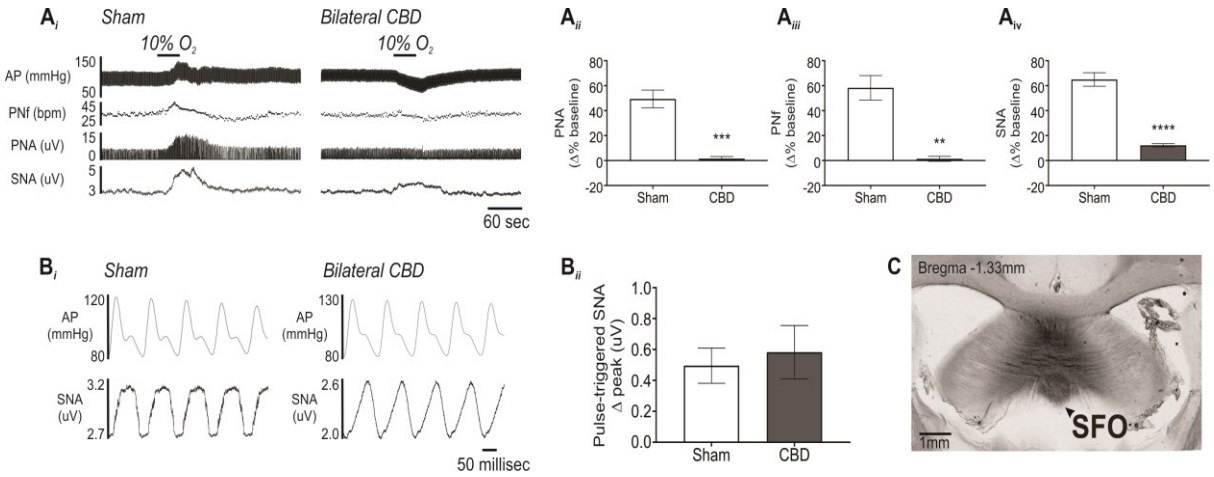

Figure 4. SNA elevation caused by AIH requires the CBs and SFO.

A, Overlaid SNA from animals subjected to AIH with SFO microinjections of vehicle ($SFO_{vehicle}$), isoguvacine (SFO_{iso}), CBD, or combined CBD and SFO inhibition ($CBD{\cdot}SFO_{iso}$). ***B,*** Group data for the change in SNA 60-min post-AIH in sham-operated and CBD rats ($^{***}P<0.001$ vs. AIH+Sham). ***C,*** Group data for the change in SNA 60-min post-AIH in rats with vehicle and isoguvacine injected in the SFO (including cases in which the SFO was not successfully targeted, $AIH+SFO_{miss}$), and rats with CBD and injections of isoguvacine ($^{****}P<0.0001$, $^{\dagger\dagger\dagger}P<0.001$, $^{\ddagger\ddagger}P<0.01$). ***D,*** Example of acute sympathetic chemoreflex before and after AIH in $SFO_{vehicle}$ (upper) and SFO_{iso} (lower) rat. ***E,*** Group data for the change in acute sympathetic chemoreflex.

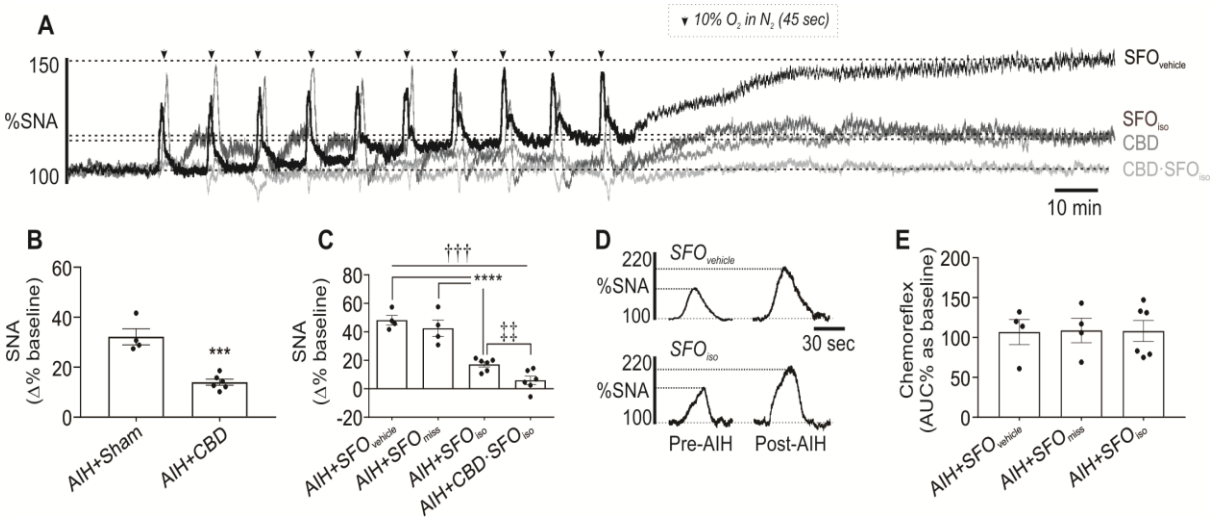

Figure 5. SNA elevation caused by Int.AngII requires the CBs and SFO.

A, Overlaid SNA from animals subjected to Int.AngII that were either sham-operated (Sham), CB-denervated (CBD), SFO inhibition with isoguvacine (SFO_{iso}), or combined CBD and SFO inhibition ($CBD{\cdot}SFO_{iso}$). ***B,*** Group data for the change in SNA 60-min post-Int.AngII ($^{****}P<0.0001$ vs. Sham, $^{***}P<0.001$ vs. Sham, $^{\dagger\dagger\dagger\dagger}P<0.0001$, $^{\dagger\dagger}P<0.01$ vs. $CBD{\cdot}SFO_{iso}$).

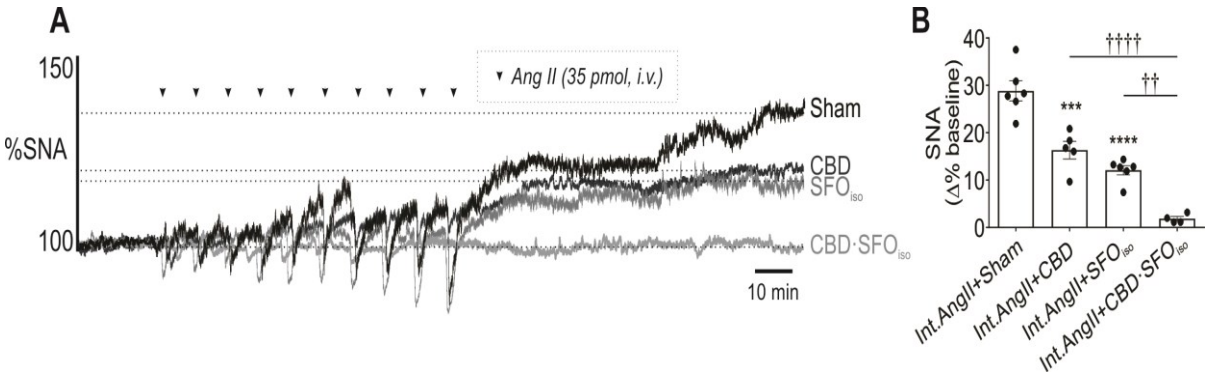

Figure 6. Enhanced central sympathetic-respiratory coupling following AIH is dependent on AT_1Rs and the CBs.

A, Method for determining the respiratory modulation index. ***B,*** Grouped data for the change in the respiratory modulation index 60-min after AIH ($^{**}P<0.01$ vs. AIH+Saline, $^{\dagger\dagger}P<0.01$ vs. Sham). ***C,*** Change in PNA 60-min after AIH ($^{***}P<0.001$ vs. AIH+Saline).

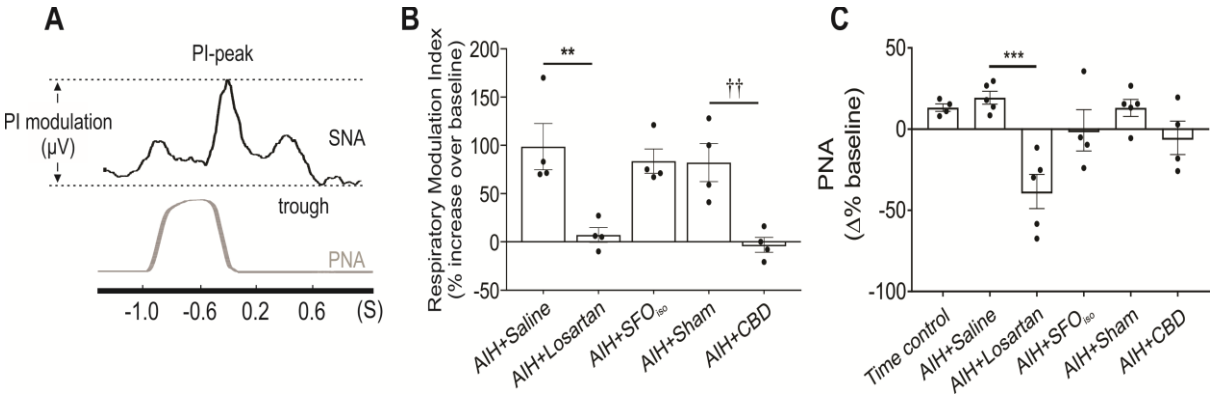

Figure 7. Intermittent intravenous injections of phenylephrine, but not sodium nitroprusside, causes a renin activity-dependent elevation in SNA.

A_i, AP and SNA changes induced by hypoxia, AngII (35 pmol, i.v.), and phenylephrine (2.5 μg, i.v.). ***A_{ii},*** Grouped data comparing the peak increase in AP from baseline following hypoxia, AngII, and phenylephrine. ***A_{iii},*** AP and SNA changes induced by hypoxia, hypoxia in CBD, and sodium nitroprusside (2.5 μg, i.v.). ***A_{iv},*** Grouped data comparing the peak decrease in AP from baseline

following hypoxia, hypoxia (CBD), and sodium nitroprusside. ***B,*** Effects of Intermittent intravenous injections of sodium nitroprusside (upper), phenylephrine (lower, black tace), and phenylephrine with aliskiren pre-treatment (lower, gray trace). ***C,*** Group data for the change in SNA 60-min post-AIH (****P<0.0001 vs. Int.Saline, ††††P<0.0001 vs. Int.Phenylephrine).