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Research paper

Chronic intermittent hypoxia disrupts cardiorespiratory homeostasis and gut microbiota composition in adult male guinea-pigs

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ABSTRACT

Background: Carotid body (peripheral oxygen sensor) sensitisation is pivotal in the development of chronic intermittent hypoxia (CIH)-induced hypertension. We sought to determine if exposure to CIH, modelling human sleep apnoea, adversely affects cardiorespiratory control in guinea-pigs, a species with hypoxia-insensitive carotid bodies. We reasoned that CIH-induced disruption of gut microbiota would evoke cardiorespiratory morbidity.

Methods: Adult male guinea-pigs were exposed to CIH (6.5% O_2 at nadir, 6 cycles.hour^{-1}) for 8 h.day^{-1} for 12 consecutive days.

Findings: CIH-exposed animals established reduced faecal microbiota species richness, with increased relative abundance of Bacteroidetes and reduced relative abundance of Firmicutes bacteria. Urinary corticosterone and noradrenaline levels were unchanged in CIH-exposed animals, but brainstem noradrenaline concentrations were lower compared with sham. Baseline ventilation was equivalent in CIH-exposed and sham animals; however, respiratory timing variability, sigh frequency and ventilation during hypoxic breathing were all lower in CIH-exposed animals. Baseline arterial blood pressure was unaffected by exposure to CIH, but β-adrenoceptor-dependent tachycardia and blunted bradycardia during phenylephrine-induced pressor responses was evident compared with sham controls.

Interpretation: Increased carotid body chemo-afferent signalling appears obligatory for the development of CIH-induced hypertension and elevated chemoreflex control of breathing commonly reported in mammals, with hypoxia-sensitive carotid bodies. However, we reveal that exposure to modest CIH alters gut microbiota richness and composition, brainstem neurochemistry, and autonomic control of heart rate, independent of carotid body sensitisation, suggesting modulation of breathing and autonomic homeostasis via the microbiota-gut-brainstem axis. The findings have relevance to human sleep-disordered breathing.

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1. Introduction

Exposure to chronic intermittent hypoxia (CIH) mimicking the recurring episodic oxygen fluctuations that are characteristic of human sleep apnoea causes sympathetic nervous system hyperactivity and systemic hypertension [1–5]. There is considerable evidence that exposure to CIH sensitises the carotid bodies—the dominant peripheral oxygen sensors—increasing chemo-afferent traffic to the brainstem, with consequential changes in autonomic outflow that serve to increase blood pressure [6–8]. Fletcher and colleagues were the first to describe CIH-induced hypertension, highlighting the pivotal roles of carotid body chemoreceptors and sympatho-excitation in driving the hypertensive phenotype [3,9]. The hypoxic sensitivity of the carotid body is elevated after exposure to CIH, which manifests increased hypoxic ventilatory and sympathetic responsiveness to chemo-stimulation [10,11]. Hypertension is a consistent feature of various models of CIH in several

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Research in context

Evidence before this study

Chronic intermittent hypoxia (CIH) is the primary pathogenic factor associated with obstructive sleep apnoea. Carotid body sensitisation is widely implicated in the development of CIH-induced cardiorespiratory morbidity, including hypertension.

Added value of this study

We provide evidence of residual cardiorespiratory dysfunction in CIH-exposed guinea-pigs, a mammalian species with hypoxia-insensitive carotid bodies. Exposure to CIH alters microbiota richness and composition, brainstem neurochemistry and cardiorespiratory control, independent of carotid body sensitisation, suggesting modulation of central cardiorespiratory control via the microbiota-gut-brainstem axis.

Implications of all the available evidence

Our findings have implications for human sleep-disordered breathing and contribute to an emerging interest in manipulation of the microbiota as an adjunctive therapy in human disease.

mammalian species [12–15], including human subjects [16]. The significance of these findings is that enhanced peripheral sensory facilitation may serve to destabilise cardiorespiratory control, with resultant sympathetic nervous over-activity, leading to diurnal hypertension. Moreover, respiratory stability decreases in rats exposed to CIH, with evidence of increased propensity for central apnoea [17–19], which disrupts respiratory-sympathetic coupling [20] potentially establishing a vicious cycle of disrupted breathing and blood pressure in people with sleep apnoea.

Carotid body denervation or ablation blocks CIH-induced persistent elevations in blood pressure, with studies revealing that an intact peripheral chemosensory network appears essential for the development of respiratory plasticity, sympathetic nervous system hyperactivity and systemic hypertension after exposure to CIH [1,3,9,18,21,22]. However, interruption of peripheral sensory input can give rise to central respiratory network reconfiguration and can establish a persistent hypoventilation [23]. Thus, whereas carotid bodies are implicated in the development and maintenance of CIH-induced cardiorespiratory morbidity, it is not clear if they are obligatory. There is clear evidence of CIH-induced plasticity in other key sites of the cardiorespiratory control network, including cells of the nucleus tractus solitarius [24], respiratory-related cells of the medullary network [4] and rhythmogenic cells of the pre-Bötzinger complex [25]. Prabhakar and colleagues have revealed that epigenetic changes in the medulla oblongata dependent on increased chemoafferent input arising from exposure to CIH result in redox-dependent persistently elevated sympathetic outflow to target tissues implicated in the control of blood pressure [8]. However, neurons of the rostral ventrolateral medulla that govern spinal vasomotor neurons are excited by systemic hypoxia in carotid body denervated rats demonstrating that the carotid body is not obligatory to the hypoxic recruitment of sympathetic neurons per se [26]. There is also evidence of direct effects of CIH on respiratory-related neurons within the medulla, and altered respiratory-autonomic coupling with implications for cardiovascular control [20,25]. Indeed, it is well recognised that exposure to CIH evokes adverse effects in various tissues, including end-effector organs of the cardio-respiratory system i.e. respiratory muscles [27,28] and cardiomyocytes [29], which predisposes to cardiorespiratory morbidity. Moreover, there is the possibility that exposure to CIH indirectly affects host physiology. Intriguingly, exposure to CIH alters the gut microbiome [15,30] and metabolome [30] and CIH-induced alterations in microbiota composition remain perturbed even during extended normoxic recovery periods [31].

The microbiota are increasingly recognised as orchestrators of host physiology, brain and behaviour extending to cardiovascular homeostasis [32–34]. Transplant of gut microbiota from spontaneously hypertensive rats into normotensive, microbiome-compromised control rats triggers the development of a hypertensive phenotype with evidence of a positive correlation between systolic blood pressure and *Lactobacillus* bacteria [35]. Similarly, gut microbiota samples transferred from hypertensive donors (animal model of sleep apnoea fed a high-fat diet) to normotensive recipient rats caused gut microbiome disruption and hypertension [36]. Reduced microbiota diversity and richness is established in hypertensive patients compared with normotensive controls [37]. Furthermore, faecal transplant from hypertensive human donors into germ-free mice establishes higher blood pressures than faecal transplants from normotensive human donors [37]. Collectively, these findings provided the rationale for our hypothesis that CIH-induced alterations in gut microbiota modulate cardiorespiratory control via altered microbe-gut-brainstem signalling, independent of carotid body plasticity.

> For a large number of problems there will be some animal of choice or a few such animals on which it can be most conveniently studied.
>
> [August Krogh, [38] —The Progress of Physiology, 1929.]

Examination of the putative effects of CIH-induced alterations to the microbiome and resultant influences on cardiorespiratory control in mammals are complicated by the dominant effect of exposure to CIH on carotid body function. Ablation of the carotid bodies to remove their contribution to CIH-induced cardiorespiratory morbidity [1] is confounded by the potential for redundancy in hypoxia-sensing and central reconfiguration of the respiratory network in the absence of a major afferent drive to breathe. However, the carotid bodies of the guinea-pig are structurally and biochemically 'normal' [39] and yet the guinea-pig carotid body is generally insensitive to hypoxia [40]. Of interest, the carotid body of the guinea-pig is responsive to cyanide, severe hypoxia and to hypercapnia and as such is 'functional' but lacks the inherent capacity to respond to moderate hypoxaemia; moreover, it appears that the contribution of the carotid body to eupnoeic drive to breathe in the guinea-pig is negligible [40]. Because of these unique characteristics, we reasoned that guinea-pigs serve as a useful experimental model to explore the effects of exposure to CIH on cardiorespiratory control, as all afferent and efferent pathways maintain their physiological connections, therefore putative CIH-induced effects on cardiorespiratory control can be explored without a dominant influence of the carotid bodies.

Our major aim was to explore the effects of exposure to CIH on blood pressure in guinea-pigs, mammals with hypoxia-insensitive carotid bodies. We reasoned that exposure to CIH, utilising a paradigm that causes hypertension in rats [41], would not evoke hypertension supporting suggestions of an obligatory role for carotid body sensitisation in the manifestation of CIH-induced hypertension. We also explored the potential for residual cardiorespiratory effects of exposure to CIH with a focus on the gut microbiota as a potential protagonist in CIH-related cardiorespiratory dysfunction.

2. Materials and methods

2.1. Ethical approval

All procedures on live animals were performed under licence from the Government of Ireland Department of Health (B100/4498) in accordance with National and European Union legislation, with prior ethical

approval by University College Cork (AEEC #2013/035). Experiments were carried out in accordance with guidelines laid down by University College Cork Animal Welfare Body, and conform to the principles and regulations described by Grundy [42].

2.2. Chronic intermittent hypoxia animal model

Adult male Duncan Hartley guinea pigs ($n = 16$; purchased from Envigo, UK) were randomly assigned to one of two groups: sham (810 ± 66 g; mean ± SD; $n = 8$) and CIH exposed (816 ± 60 g; n = 8). Animals were housed in standard cages in a temperature (20.4 ± 0.6 °C) and humidity-controlled (46.8 ± 6.6%) environment on a 12 h light:12 h dark cycle, with ad libitum access to standard guinea-pig chow and water. The CIH group was placed in environmental chambers wherein ambient oxygen concentration was tightly-controlled using a dynamic oxygen/nitrogen controller (Oxycycler™; Biospherix, New York, NY, USA). Animals assigned to the CIH group were exposed to repeated 10-min cycles consisting of 5 min of exposure to hypoxia (nadir, $FiO_2 = 0.065$, balance N_2) and 5 min of normoxia ($FiO_2 = 0.21$; balance N_2) for eight hours.day^{-1} for twelve consecutive days during the light phase. Contemporaneously, sham animals were exposed to room air in the same room with similar environmental cues for the duration of the study. Cardiorespiratory parameters were assessed on day 13, the day subsequent to the final episodes of CIH or sham exposure.

2.3. Assessment of respiratory flow in awake guinea-pigs

2.3.1. Whole-body plethysmography

Respiratory flow recordings were recorded in unrestrained, unanaesthetised awake guinea-pigs during quiet rest using the technique of whole-body plethysmography (Buxco Ltd., St. Paul, Minneapolis, USA). CIH-exposed and sham guinea-pigs were introduced into custom plethysmograph chambers with room air flushed through each chamber (4 L/min) ensuring the maintenance of O_2 and CO_2 environmental conditions. Animals were allowed to settle during an acclimation period of 40 min to ensure the animals were habituated to the new environment.

Following the acclimation period, baseline respiratory parameters were assessed during a 10 to 15 min steady-state normoxia period ($FiO_2 = 0.21$; balance N_2). This was followed by a 10 min poikilocapnic hypoxia challenge ($FiO_2 = 0.10$; balance N_2). Following hypoxia, each animal was re-exposed to normoxia and after an adequate recovery period of at least 10 min a second baseline period was established. Animals were subsequently exposed to a 10 min hypercapnia challenge ($FiCO_2 = 0.05$; balance O_2). Respiratory variables including respiratory frequency (f_R), minute ventilation (V_E), tidal volume (V_T), inspiratory time (Ti), expiratory time (Te) and mean inspiratory flow (V_T/Ti) were recorded on a breath-by-breath basis for offline analysis. Concentrations of O_2 and CO_2 in gas entering and exiting the plethysmograph chambers were constantly measured (O_2 and CO_2 analyser; ADInstruments, Colorado Springs, CO, USA). O_2 consumption (VO_2) and CO_2 production (VCO_2) in sham and CIH-exposed animals were determined.

2.3.2. Data analysis for whole-body plethysmography

FinePointe software (Buxco Research Systems, Wilmington, NC, USA) generated breath-by-breath analysis of respiratory flow signals. Artefacts related to movement, exploratory behaviour and sniffing were excluded from analysis by software algorithm and by visual inspection of the recording sessions designated for analyses. Baseline breathing and metabolic analyses were averaged from the 10–15 min period of stable normoxia. Data are reported for the final 5 min of each of the hypoxic and hypercapnic challenges allowing for steady-state assessment of respiratory and metabolic parameters. V_T, V_E, V_T/Ti, VO_2 and VCO_2 were normalised for body mass (per 100 g). Additionally, respiratory flow signals were analysed for the occurrence of apnoeas and augmented breaths (sighs) during normoxic breathing. The criterion for apnoea was a pause in breathing greater than two missed breaths, expressed as either a spontaneous or post-sigh apnoea as previously described [18]. Sighs were defined as an augmented breath twice the amplitude of the average V_T. Poincaré plots expressing breath-to-breath (BBn) versus next breath-to-breath interval (BBn + 1) for 200 consecutive breaths were extrapolated for sham and CIH-exposed guinea-pigs. Respiratory timing variability in total breath duration and expiratory time were analysed for short-term (SD1) and long-term (SD2) variability.

2.4. Assessment of cardiorespiratory parameters under urethane anaesthesia

2.4.1. Surgical protocol

Subsequent to plethysmography challenges, animals were anaesthetised via an intraperitoneal injection of 20% *w/v* urethane (1.5 g.kg^{-1}), placed in the supine body position and core body temperature was maintained at 38.5 °C using a rectal temperature probe and homeothermic blanket system (Harvard Apparatus, Holliston, MA, USA). Depth of anaesthesia was regularly monitored by absence of the pedal withdrawal to noxious pinch; supplemental doses of anaesthetic were administered as required. A mid-cervical tracheotomy was performed and the right jugular vein was cannulated for intravenous (i.v.) infusion of supplemental anaesthetic and drugs. The left femoral artery was cannulated for the recording of arterial blood pressure as well as withdrawal of arterial blood gas samples. Arterial blood gas, pH and electrolyte assessment was performed using specialised cartridges (EG6+; Abbott Laboratories Ltd., Dublin Ireland) and a portable blood gas analyser (I-STAT; Abbot Laboratories Ltd). All animals were maintained with a bias flow of supplemental O_2 to maintain an arterial oxygen saturation (SaO_2; Starr Life Sciences, PA, USA) above 95% during basal conditions. A pneumotachometer was connected to a tracheal cannula to determine tracheal flow (Hans Rudolph Inc., KS, USA). A side arm of the tracheal cannula was connected to a CO_2 analyser (microCapStar End-Tidal CO_2 analyser; CWE Inc., USA), which provided measurement of tracheal end-tidal CO_2. All data were digitised and displayed using LabChart v7 (ADInstruments).

2.4.2. Experimental protocol

Following a 30-min period of stabilisation following surgical procedures, an arterial blood gas sample was acquired and baseline parameters were assessed. The bias flow was manipulated using an electronic gas mixer (GSM-3 Gas Mixer; CWE Inc.) to administer chemostimulation challenges to the animals. A graded hypercapnic challenge was performed in which animals were exposed to increasing levels of inspired carbon dioxide: $FiCO_2 = 0.05$ and 0.10 (supplemental O_2; balance N_2) consecutively for 5 min each with no recovery period between hypercapnic stimuli. A minimum of 10 min was allowed for the recovery of stable parameter recordings between all challenges. Animals were subsequently challenged with a 5 min hypoxia challenge ($FiO_2 = 0.10$; balance N_2), followed by a 5 min asphyxic challenge ($FiO_2 = 0.10$, $FiCO_2 = 0.05$, balance N_2). Subsequent to the inspired gas challenges and after recovery, sodium cyanide (NaCN; 200 μg.kg^{-1}) was administered i.v. to stimulate the type 1 glomus cell of the carotid body. Some guinea-pigs presented with an apnoea upon NaCN infusion and were excluded from data analysis as no hypoxic ventilatory response was elicited. Successively, intravenous administrations of phenylephrine (PE; 50 μg.kg^{-1}), propranolol (PROP; 1 mg.kg^{-1}) and hexamethonium (25 mg.kg^{-1}) were used to assess cardiovascular responses to pharmacological manipulation. Due to the intrinsic hypotensive phenotype of guinea-pigs (Table 3), some animals failed to recover from the otherwise transient hypotensive state induced pharmacologically by propranolol (sham, $n = 2$; CIH, $n = 3$) and hexamethonium (sham, $n = 1$; CIH, n = 1). Animals were euthanised by i.v. anaesthetic overdose and various organs were collected, weighed and frozen at −80 °C for

subsequent analysis. Lungs were weighed wet, dried at 37 °C for a minimum of 48 h and reweighed.

2.4.3. Data analysis of cardiorespiratory parameters in anaesthetised guinea-pigs

Baseline parameters were averaged over 10 min of stable recording and data are presented as absolute units. Cardiorespiratory responses during graded hypercapnic, hypoxic and asphyxic challenges were averaged in 10 s time bins and were compared with the respective 1-min pre-challenge baseline. To assess dynamic responses to drug administration (NaCN, PE, PROP and hexamethonium), cardiorespiratory responses were averaged into 1-s time bins and were compared with the respective 1-min pre-challenge baseline. Cardiorespiratory responses to chemostimulation and drug administration are expressed as a percent change from the preceding baseline value.

2.5. Analysis of brainstem homogenates and urine

2.5.1. Brainstem monoamine concentrations determined by high-performance liquid chromatography (HPLC) coupled to electrochemical detection

Brainstems were removed from the guinea-pigs immediately after euthanasia, snap frozen in isopentane cooled in liquid nitrogen and transferred to −80 °C for long-term storage. Each brainstem was separated into two distinct regions, pons and medulla oblongata at −20 °C. The dissected pons and medulla oblongata tissues were sonicated in 1 ml of chilled mobile phase, spiked with 2 ng/20 μl of a *N*-methyl 5-HT (internal standard) (Bandelin Sonolus HD 2070) for six by six second bursts. The mobile phase contained 0.1 M citric acid (Alkem/Reagecon), 0.01 mM ethylenediaminetetraacetic acid (EDTA), 0.1 M sodium dihydrogen phosphate (Alkem/Reagecon), 5.6 mM octane-1-sulphonic acid (Alkem/Reagecon) and 9% (*v*/v) methanol, adjusted to pH 2.8 using 4 N sodium hydroxide. Samples were centrifuged at 14,000*g* for 20 min at 4 °C (MIKRO 22R refrigerated centrifuge). Cell pellets were stored at −80 °C. The supernatant (20 μl) was injected onto the HPLC system coupled to electrochemical detection. The HPLC system consisted of a SCL 10-Avp system controller, LC-10AS pump, SIL-10A autoinjector (with sample cooler maintained at 4 °C), CTO-10A oven, LECD 6A electrochemical detector and an online Gastorr Degasser. A reverse-phase column (kinetex 2.6u C18 100Amm X 4.6 mm), maintained at 30 °C, was employed in the separation (flow rate 0.9 ml/min). The monoamines, dopamine, noradrenaline (NA), serotonin (5-HT), and monoamine metabolite 5-hydroxyindoleacetic acid (5-HIAA), 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) were assessed. The monoamines were identified by their characteristic retention times, which were determined by standard injections run at regular intervals during the sample analysis. Chromographs were processed using Class-VP5 software and allowed the identification of the desired monoamines. Concentrations were calculated using analyte:internal standard peak response ratios.

2.5.2. Urine immunoassays

Urine samples collected from anaesthetised animals were stored at −80 °C until use. After storage, samples were thawed and concentrations of corticosterone (ENZO Life Sciences, UK), noradrenaline (Abnova) and creatinine (Sigma-Aldrich, Ireland.) were determined (sham $n = 6$; CIH $n = 6$) using commercially available enzyme-linked immunosorbent assay kits according to the manufacturers' instructions. A spectrophotometer (SpectraMax M3, Molecular devices) was used in reading absorbance for each assay. Urine samples were diluted 50-fold to determine corticosterone concentrations and 100-fold to determine creatinine and catecholamine concentrations. Urinary catecholamine and corticosterone concentrations were normalised to creatinine concentration of the urine, which provided a biomarker of urine production.

2.6. Microbiota composition analysis of faecal content

2.6.1. Faecal content DNA extraction

Faecal content was removed from the guinea-pig rectum and snap frozen in liquid nitrogen for long-term storage. DNA was extracted from the faecal material using the QIAamp DNA Stool Mini Kit (Qiagen, Venlo, Netherlands) according to the manufacturer's instructions, but including an additional bead-beating step at the beginning of the procedure for mechanical lysis. 200 mg of each faecal sample was homogenised in the extraction buffer and transferred to 2 ml screw cap tubes containing 2 × 2.3 mm, 0.2 g of 1.0 mm and 0.2 g of 0.1 mm zirconia/silica beads before being subjected to a 3-min bead-beating step using a Mini-Beadbeater-16 (Bispec, Bartlesville, USA). The following steps were in accordance with the manufacturer's instructions. The obtained faecal genomic DNA was quantified using the Qubit broad range DNA kit (Qubit, London, UK), measured on a Qubit 3.0 fluorometer (Qubit, London, UK), normalised to 5 ng/μl before following the Illumina 16S rRNA Metagenomic Sequencing Library protocol.

2.6.2. 16S rRNA Gene sequence-based microbiota analysis

The V3-V4 hypervariable region of the 16S rRNA gene was amplified and prepared for sequencing, the amplicon products were purified with Agencourt AMPure XP kit (Backman Coulter, Pasadena, USA). Amplicon PCR negative controls were utilised and verified through a 1.5% agarose gel made up with a tris base, acetic acid and EDTA buffer with Midori green stain (100 V for 30 mins; Nippon genetics, 52,351 Dueren, Germany). In the next step the purified product underwent an index PCR step using the Nextere XT index primer kit (Illumina, San Diego, USA). Following this a second purification step occurred with Agencourt AMPure XP. The index PCR products were quantified using the Qubit high sensitivity DNA kit (Qubit, London, UK) and measured on Qubit 3.0. The PCR products were normalised and pooled before being sequenced on the MiSeq sequencing platform (Illumina, San Diego, USA) using a 2 × 250 bp cycle kit, according to standard Illumina sequencing protocols at the Teagasc sequencing facility, Teagasc Food Research Centre, Fermoy, County Cork, Ireland.

2.6.3. Bioinformatic sequence analysis

Briefly, 250 base paired-end sequences were assembled using FLASH (FLASH: fast length adjustment of short reads to improve genome assemblies). Further processing of paired-end reads including quality filtering based on a quality score > 25 and removal of mismatched barcodes and sequences below length thresholds was completed using QIIME version 1.9.0 (Quantitative Insights Into Microbial Ecology). Sequences were quality checked (denoising, chimera detection) and the remaining sequences were clustered into operational taxonomic units (OTUs) using USEARCH v7 (64-bit). OTUs were aligned using PyNAST (PyNAST; a flexible tool for aligning sequence to a template alignment) and taxonomy was assigned using BLAST against the SILVA SSURef database release 123. Data was rarefied to 60,000 reads. Alpha diversity indices were generated in QIIME and the beta diversity Bray Curtis index was calculated in R software package. Relative abundance of bacterial taxa was expressed as % of identified sequences.

2.7. Statistical analysis

Statistical analysis on microbiota data was carried out in SPSS version 24 and R statistical environment (R version 3.4.4). GraphPad Prism Software (GraphPad Software, San Diego, CA, USA) was used for all other statistical analysis and for the generation of graphs. Data are presented as mean ± SD, median (IQR) or box and whisker plots (median, IQR, minimum and maximum values). Data were checked for a normal distribution with a Shapiro-Wilk test and for homogeneity of variances with Levene's test. Two-tailed Student's unpaired *t*-test and non-parametric Mann-Whitney *U* test were used, where appropriate, unless otherwise stated. Brainstem monoamine concentrations were

assessed using two-way ANOVA (treatment and brainstem region as two independent factors) with Bonferroni post-hoc tests. Pearson correlation coefficients were used to assess associations between brainstem monoamines and physiological parameters.

Bacterial taxa that were detected in fewer than three samples in each group were omitted from the subsequent analysis. Principal coordinate analysis (PCoA) plots were generated from Bray-Curtis dissimilarity matrices using the Vegan and Car packages in R. Differences in Bray-Curtis dissimilarity on the PCoA were assessed by PERMANOVA with the vegan package in R. The most substantially changed bacterial genera ($p < .053$) were chosen for the construction of the *Z*-score heatmap with the ggplot2 package in R. LEfSe analysis [43] was carried out using Calypso (v8.72) [44] with Chloroplast and Cyanobacteria counts removed. Hierarchical All Against All (HAllA) was used to detect correlations between microbiota data and the physiological measurements. Statistical significance was accepted at $p < .05$. The Benjamini-Hochberg adjustment procedure was applied with the false discovery rate (FDR) set at 20% to correct for multiple testing.

3. Results

3.1. Baseline ventilation and ventilatory responses to chemostimulation in sham and CIH-exposed awake guinea-pigs

3.1.1. Baseline ventilation and metabolism in sham and CIH-exposed awake guinea-pigs

Baseline minute ventilation (Fig. 1e; $p = .545$) and the ventilatory equivalent (V_E/VCO_2) during normoxia (Fig. 1h; $p = .201$) were equivalent in sham and CIH-exposed guinea-pigs. Basal O_2 consumption and CO_2 production (Table 1) were similar in sham and CIH-exposed guinea-pigs. Exposure to acute hypoxia ($FiO_2 = 0.10$) failed to evoke an excitatory ventilatory response (Fig. 1f; $p = .798$); CO_2 production during exposure to hypoxia was higher in CIH-exposed guinea-pigs (1.00 ± 0.09 vs 1.15 ± 0.19 ml.min^{-1}.100 g^{-1}; $p = .067$), such that (V_E/VCO_2) during hypoxia was lower in CIH-exposed guinea-pigs compared with sham guinea-pigs (Fig. 1i; $p < .001$). Minute ventilation during hypercapnia (Fig. 1g; $p = .180$) and V_E/VCO_2 during exposure to CO_2 (Fig. 1j; $p = .613$) were equivalent in sham and CIH-exposed guinea-pigs.

3.1.2. Respiratory timing indices in sham and CIH-exposed awake guinea-pigs

Fig. 2 displays Poincaré plots of breath-to-breath period (BBn) and subsequent interval (BBn + 1) during baseline breathing both for expiratory duration (T_e; Fig. 2a) and total breath duration (T_{tot}; Fig. 2b). Assessment of the short-term (SD1) and long-term (SD2) variability of respiratory timing revealed lower values in CIH-exposed guinea-pigs compared with sham guinea-pigs. Long-term variability for T_e (Fig. 2d; $p = .026$), and both short-term (Fig. 2e; $p = .007$) and long-term T_{tot} (Fig. 2f; $p = .048$) were each lower in CIH-exposed guinea-pigs compared with sham animals.

3.1.3. Expression of sighs and apnoeas in sham and CIH-exposed awake guinea-pigs

Sigh frequency was lower in CIH-exposed animals compared with sham animals (Fig. 3b; $p = .015$). Lower sigh frequency was also observed during hypoxic (Fig. 3c; $p = .016$) and hypercapnic ventilation (Fig. 3d; $p = .026$) in CIH-exposed guinea-pigs compared with sham guinea-pigs. Sigh amplitude was equivalent in both groups during normoxia, hypoxia and hypercapnia ($p > .05$). Spontaneous apnoeas were absent in sham and CIH-exposed guinea pigs. Post-sigh apnoeas were infrequent, however they were more prevalent in sham guinea-pigs ($n = 5$) compared with CIH-exposed ($n = 2$) guinea-pigs. Owing to the low apnoea count, statistical analysis was not performed on post-sigh apnoea data.

3.2. Cardiorespiratory parameters in sham and CIH-exposed anaesthetised guinea-pigs

3.2.1. Baseline respiratory parameters in sham and CIH-exposed anaesthetised guinea-pigs

Baseline arterial blood gases were similar in both groups (Table 2). Arterial blood bicarbonate concentration was lower ($p = .023$) in CIH-exposed guinea-pigs compared with sham animals, consistent with a trend towards lower values for $PaCO_2$ (Table 2). Higher f_R and V_E was evident in CIH-exposed guinea-pigs compared with sham guinea-pigs (Table 3), but $ETCO_2$ values were similar in CIH-exposed and sham animals (Table 3).

3.2.2. Cardiovascular responsiveness in sham and CIH-exposed anaesthetised guinea-pigs

Guinea pigs exposed to 12 consecutive days of CIH did not develop systemic hypertension (Table 3); however, a significant tachycardia was evident in CIH-exposed guinea-pigs compared with sham animals. Left ventricular weights tended to be heavier in CIH-exposed guinea-pigs compared with sham guinea-pigs suggesting the onset of development of left ventricular hypertrophy (Table 4) in the absence of hypertension (no increase in afterload). There was a trend towards an increased haemoglobin concentration in CIH-exposed animals compared with sham animals (Table 2). Urinary concentrations of noradrenaline and corticosterone were not different in CIH-exposed animals compared with sham animals (Table 4).

IV injection of phenylephrine produced a pressor response equivalent in CIH-exposed and sham guinea-pigs (Fig. 4a; $p = .327$), however the associated reflex bradycardia was significantly blunted in CIH-exposed guinea-pigs compared with sham guinea-pigs (Fig. 4b $p = .032$). β-adrenoceptor blockade with iv propranolol evoked a greater bradycardia in CIH-exposed guinea-pigs compared with sham guinea pigs (Fig. 4d; $p = .023$). Interestingly, the propranolol-induced hypotensive response was blunted in CIH-exposed animals compared with sham controls (Fig. 4c; $p = .047$). IV administration of hexamethonium produced similar hypotensive responses in CIH-exposed and sham guinea-pigs ($-38 \pm 7\%$ vs. $-35 \pm 9\%$, % change from baseline MAP; sham, $n = 5$; CIH, $n = 5$; $p = .582$).

3.2.3. Cardiorespiratory responses to chemostimulation in sham and CIH-exposed anaesthetised guinea-pigs

Blood pressure and heart rate reflex responses to chemostimulation challenges were equivalent in CIH-exposed and sham animals in all instances ($p > .05$). The hypercapnic ventilatory response in anaesthetised CIH-exposed guinea-pigs was significantly blunted compared with sham guinea-pigs (Table 5), particularly during the 10% inspired CO_2 challenge. Respiratory parameters were unaffected by exposure to acute hypoxia, however there was a trend for a blunted ventilatory response to asphyxia in CIH-exposed guinea-pigs (Table 5). There was a trend towards a blunted ventilatory response to iv NaCN administration in CIH-exposed guinea-pigs, arising predominantly due to a significantly reduced respiratory frequency response compared with sham guinea-pigs (Table 5).

3.3. Brainstem monoamine analysis

Pontine and medulla oblongata noradrenaline concentrations were significantly lower in CIH-exposed guinea-pigs compared with sham guinea-pigs (Fig. 5a). Brainstem dopamine, 5-HIAA and 5-HT concentrations were equivalent in CIH-exposed and sham samples (Fig. 5). Correlation analysis was performed for pontine and medullary noradrenaline concentrations and breathing variability, sigh frequency, heart rate and heart rate responsiveness to drug administration. No significant correlations were evident between medullary noradrenaline concentrations and all physiological parameters ($p > .05$; see supplementary data; Fig. S1). However, there were positive correlations between pontine

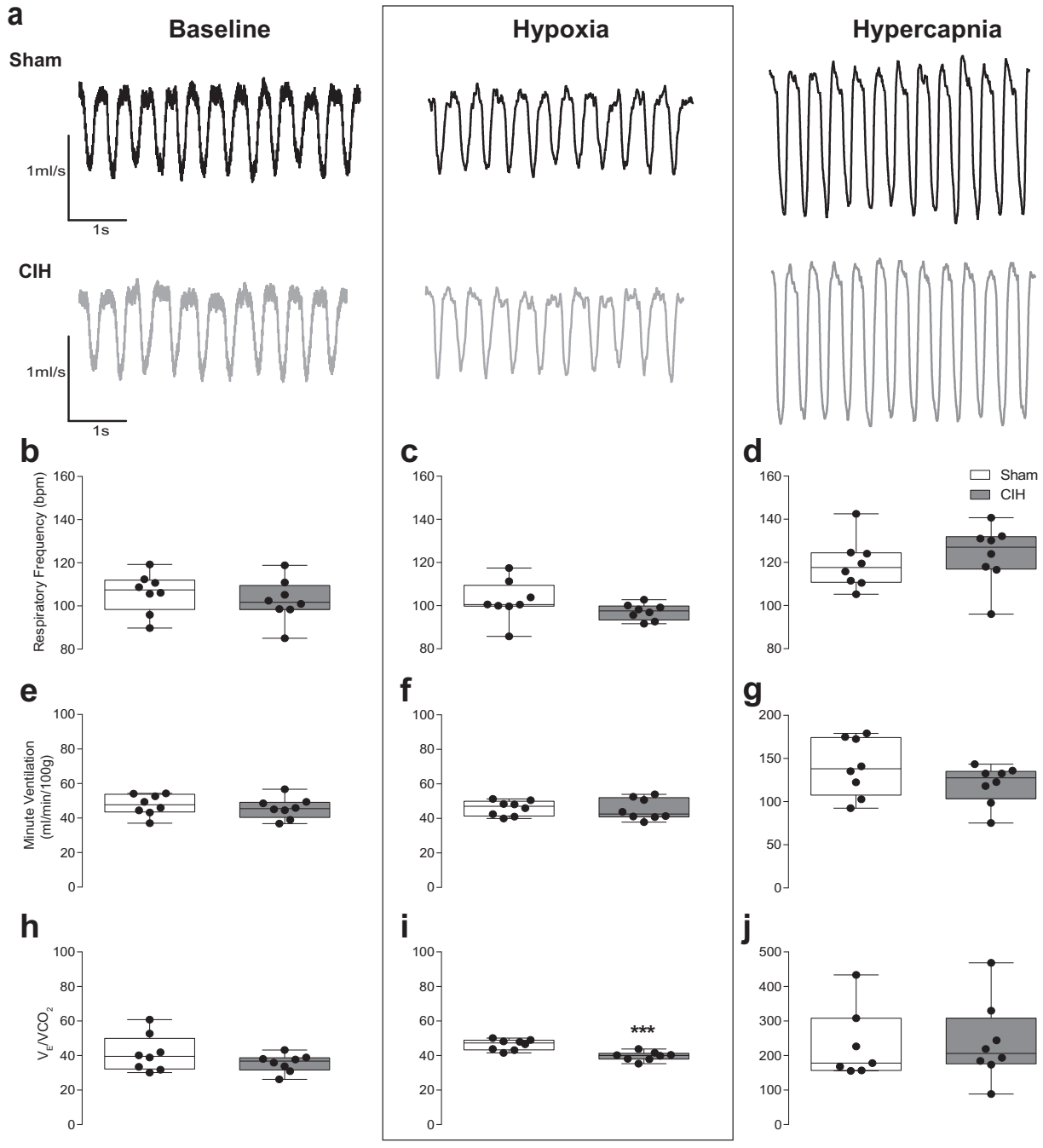


Fig. 1. CIH does not significantly affect baseline, hypoxic or hypercapnic ventilation in guinea-pigs. (a) Representative respiratory airflow traces during normoxic, hypoxic and hypercapnic ventilation in Sham (black) and CIH-exposed (grey) guinea-pigs; inspiration represented by downward deflections. b-j) Group data for respiratory frequency (b-d) minute ventilation (e-g) and the ventilatory coefficient (h-j) in sham and CIH-exposed guinea-pigs during normoxic (b, e, h), hypoxic (c, f, i) and hypercapnic (d, g, j) ventilation. Values are expressed as box and whisker plots (median, 25–75 percentiles and minimum and maximum values). Groups were statistically compared by unpaired Student's *t*-tests. ***$p < .001$ compared with sham.

noradrenaline concentrations and T_e SD2 ($p = .032$; Fig. 6b), T_{total} SD1 ($p = .047$; Fig. 6d), T_{total} SD2 ($p = .049$; Fig. 6d e) and respiratory frequency under urethane anaesthesia ($p = .017$; Fig. 6f).

3.4. Microbiota richness and composition

The species richness of faecal microbiota was reduced in CIH-exposed guinea-pigs (Chao1 index, $p = .011$; total number of species observed, $p = .053$; Fig. 7A-B). However, the difference in other metrics of alpha-diversity, which take into account the evenness of species abundance (Simpson and Shannon), was modest between groups (Fig. 7C-E). Principal coordinate analysis revealed a strong trend towards separation of microbiota communities between sham and CIH-exposed animals ($p = .059$; Fig. 7f). To address in more detail the structural changes of faecal microbiota, we compared the relative abundance of individual bacterial taxa at the phylum, family and genus level. Significant differences between CIH-exposed and sham guinea-pigs were uncommon at the family and genus level (see supplementary data; Fig. S2). However, at the phylum level, the relative abundance of Firmicutes bacteria was significantly lower ($p = .007$), whereas Bacteroidetes phylum was significantly more abundant in CIH-exposed animals compared with sham animals ($p = .038$; Fig. 8a and Supplementary Table S1). As a result, there was a trend for the Firmicutes:Bacteroidetes ratio to be lower in CIH-exposed guinea-pigs

Table 1
Baseline ventilation and metabolic parameters in sham and CIH-exposed awake guinea-pigs.

	Sham ($n = 8$)	CIH (n = 8)	P Value
V_T (ml/100 g)	0.462 ± 0.502	0.454 ± 0.066	0.781
Ti (ms)	227 ± 23	239 ± 25	0.351
Te (ms)	341 ± 57	363 ± 38	0.384
V_T/Ti (ml/s/100 g)	2.03 ± 0.23	1.91 ± 0.28	0.341
VO_2 (ml/min/100 g)	1.33 ± 0.61	1.75 ± 0.31	0.103
VCO_2 (ml/min/100 g)	1.25 ± 0.27	1.32 ± 0.20	0. 603
VCO_2/VO_2	1.15 ± 0.57	0.76 ± 0.11	0.038

V_T, tidal volume; Ti, Inspiratory time; Te, expiratory time; V_T/Ti, inspiratory flow; VO_2, oxygen consumption; VCO_2, carbon dioxide production. Data are shown as mean ± SD and were statistically compared using unpaired two-tailed Student's *t*-tests.

compared with sham guinea-pigs ($p = .068$). The increase in Bacteroidetes in CIH-exposed samples was mainly driven by dominant *Bacteroidales S24–7 family* (Fig. 8b and Supplementary Table S2). These increases occurred at the expense of some Firmicutes taxa, such that bacterial genera from the *Lachnospiraceae* and *Ruminococcaceae* families were decreased in CIH-exposed faecal samples compared with sham faecal samples (Fig. 8b and Supplementary Table S2), although the changes in the relative abundance of bacterial genera were no longer significant after the Benjamini-Hochberg adjustment ((i/m) *Q < p; Supplementary Table S3).

The linear discriminant analysis (LDA) effect size method (LEfSe) was employed to identify bacterial genera associated with exposure to CIH, which might explain the underlying differences in the microbiota between sham and CIH-exposed animals (Fig. S3). At the genus level, LEfSe analysis identified 7 genera associated with exposure to CIH and thus over-represented in the CIH-exposed group were: an uncultured bacterium belonging to the *Bacteroidales S24–7* family and *Butyricimonas* (both from the Bacteroidetes phylum), *Bifidobacterium* and *Coriobacteriaceae UCG002* (Actinobacteria), *Coprococcus 2* and *Flavonifractor* (Firmicutes), *Methanobrevibacter* (Eurvarchaeota). In contrast, 6 genera were associated with sham guinea-pigs and were therefore under-represented in CIH-exposed guinea-pigs. These genera were: *Blautia*, *Anaerotruncus*, *Lachnospiraceae UCG005*, *Intestinimonas*, *Ruminiclostridium 5* and *Lachnoclostridium 10* (all from the Firmicutes phylum). Fig. 8c represents significant correlations identified between various physiological parameters and identified bacterial genera.

4. Discussion

There is considerable interest in the mechanisms driving CIH-induced respiratory plasticity and hypertension. This interest stems from the potential clinical relevance of the findings to an understanding of cardiorespiratory and autonomic function in persons with sleep-disordered breathing, a disorder characterised by recurrent apnoeic episodes during sleep manifesting in exposure to CIH. We sought to explore the putative obligatory role of carotid body sensitisation in the expression of aberrant cardiorespiratory control following exposure to CIH by use of a unique rodent model—the guinea-pig. If carotid body sensitisation is essential for aberrant respiratory plasticity and the development of hypertension following exposure to CIH, then little change should be expected in cardiorespiratory control in guinea-pigs exposed to CIH, based on the premise that the guinea-pig carotid body is essentially insensitive to hypoxia [40,45,46]. Conversely, if exposure to CIH disrupts cardiorespiratory control in guinea-pigs, this would provide evidence of sites beyond the carotid body that are sufficient to drive aberrant cardiorespiratory control in rodents, and perhaps humans. Our findings are generally supportive of the hypothesis that the carotid body is essential for the expression of CIH-induced aberrant control of breathing. In rodents with hypoxia-sensitive carotid bodies, CIH has

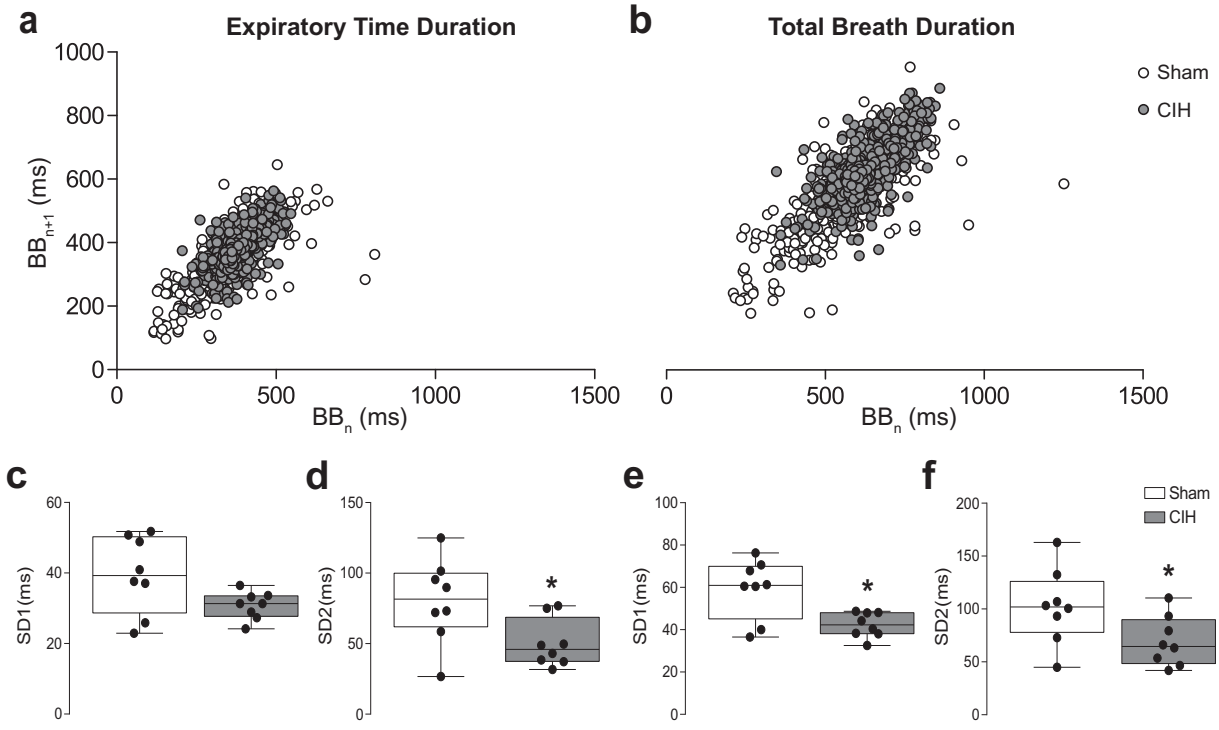


Fig. 2. Normoxic breathing variability is reduced in CIH-exposed awake guinea-pigs. Poincaré plots of breath-to-breath (BBn) and next breath-to-breath interval (BBn + 1) of time to expire (a) and total breath duration (b) during normoxic breathing in Sham ($n = 8$) and CIH-exposed (n = 8) guinea-pigs. Group data for average expiratory time short-term (c) and long-term (d) variability of breathing in sham and CIH-exposed guinea-pigs. Average total breath duration short-term (e) and long-term (f) variability of breathing in sham and CIH-exposed guinea-pigs. Values (c-f) are expressed as box and whisker plots (median, 25–75 percentiles and minimum and maximum values). Groups were statistically compared by unpaired Student's *t*-tests. *$p < .05$ compared with sham.

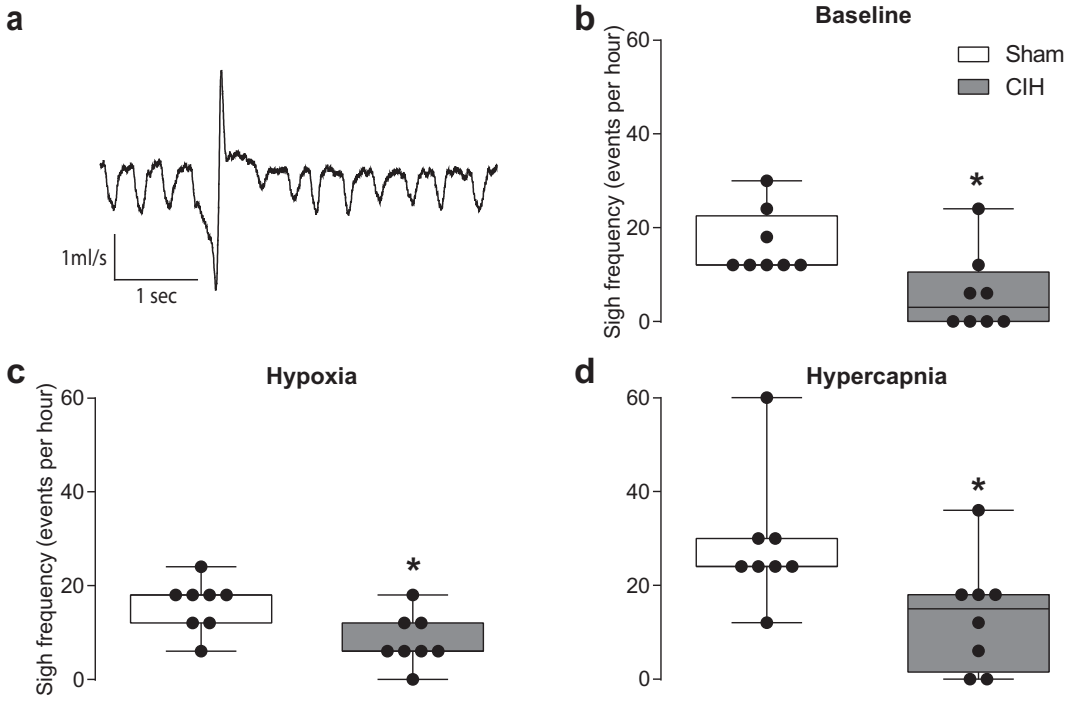


Fig. 3. Sigh frequency is reduced in CIH-exposed awake guinea-pigs. (a) Representative trace of respiratory airflow demonstrating an augmented breath (sigh); inspiration represented by downward deflections. Group data for sigh frequency during normoxic (b), hypoxic (c) and hypercapnic ventilation (d) in Sham ($n = 8$) and CIH-exposed (n = 8) guinea pigs. Values (b-d) are expressed as box and whisker plots (median, 25–75 percentiles and minimum and maximum values). Groups were statistically compared by unpaired Student's t-tests or non-parametric Mann-Whitney *U* test where appropriate. *p < .05 compared with sham.

been shown to cause hyperventilation and augmented responsiveness to hypoxic chemostimulation [47]. Whereas elevated ventilatory responses to hypoxia have not been universally observed in studies of rodents exposed to CIH [48], Morgan et al. [47] provided the definitive study by measurement of breathing and metabolism (which are both affected by hypoxia in rodents), and careful consideration of the stimulus-response relationship by way of concurrent assessment of oxygen saturation during hypoxic challenge. Assessing the relationship of all three parameters reveals that exposure to CIH increases the ventilatory response to hypoxia in rats [47]. Additionally, several groups have reported increased ventilatory drive in rats exposed to CIH [28,47].

Exposure to CIH in guinea-pigs did not affect baseline ventilation or metabolism or ventilatory responses to hypoxia and hypercapnia in awake animals. Indeed, the ventilatory equivalent (V_E/VCO_2) during hypoxic ventilation was depressed, not augmented, in CIH-exposed guinea-pigs. Unaltered ventilation and ventilatory responsiveness to chemo-stimulation in CIH-exposed guinea-pigs supports the contention that carotid body sensory facilitation is wholly necessary for CIH-mediated enhancement of hypoxic ventilatory responses. Likewise, in CIH-exposed anaesthetised guinea-pigs, there was no evidence of potentiated chemoreceptor responsiveness. Indeed, responses were generally less in CIH-exposed animals, with a significant blunting of the ventilatory response to 10% inspired CO_2. Of interest, in a recent study in guinea-pigs, utilising a more severe CIH protocol, consisting of 30 cycles.hour^{-1} (5% O_2 at nadir), 8 h.day^{-1}, for 30 days, V_E/VO_2 during severe hypoxia ($FiO_2 = 0.07$) and hypercapnia ($FiCO_2 = 0.05$) was blunted compared with sham, principally owing to CIH-induced increases in oxygen consumption [45]. Whereas a modest

Table 2
Baseline arterial blood gas and electrolyte concentrations in sham and CIH-exposed guinea-pigs.

	Sham (n = 8)	CIH (n = 8)	P Value
pH	7.36 ± 0.04	7.36 ± 0.06	0.820
$PaCO_2$ (mmHg)	46.1 ± 5.4	42.3 ± 3.7	0.137
PaO_2 (mmHg)	110±15	111 ± 14	0.896
$[HCO_3^-]$ (mmol/L)	26.2 ± 2.3	24.1 ± 2.4	**0.041**
$[TCO_2]$ (mmol/L)	27.1 ± 2.9	25.3 ± 2.2	0.293
SaO_2 (%)	97.8 ± 0.9	98 ± 1	0.681
$[Na^+]$ (mmol/L)	143 ± 3	143 ± 4	0.745
$[K^+]$ (mmol/L)	3.43 ± 0.47	3.53 ± 0.34	0.338
Hct (%)	42.9 ± 4.8	45.4 ± 2.5	0.104
[Hb] (g/dl)	14.6 ± 1.6	15.5 ± 0.9	0.088

$PaCO_2$, arterial blood partial pressure of CO_2; PaO_2, arterial blood partial pressure of O_2; $[HCO_3^-]$, bicarbonate concentration; $[TCO_2]$, total CO_2 concentration; SaO_2, arterial oxygen saturation; $[Na^+]$, sodium concentration; $[K^+]$, potassium concentration; Hct, haematocrit; [Hb], haemoglobin concentration. Data are shown as mean ± SD. Haematocrit and haemoglobin parameters were statistically compared using one-tailed unpaired Student's *t*-tests. All other parameters were statistically compared using two-tailed unpaired Student's *t*-tests. Bolded numbers highlight statistical significance (P < 0.05)

Table 3
CIH induces tachycardia and hyperventilation in anaesthetised guinea-pigs.

	Sham (n = 8)	CIH (*n* = 8)	P Value
MAP (mmHg)	37.3 ± 9.4	35.6 ± 6.9	0.346
SBP (mmHg)	51.0 ± 18.3	48.3 ± 11.1	0.362
DBP (mmHg)	27.9 ± 5.2	27.8 ± 4.7	0.483
HR (bpm)	279 ± 27	307 ± 36	**0.048**
f_R (brpm)	50.4 ± 12.1	62.4 ± 12.4	0.071
V_T (ml/100 g)	0.184 ± 0.024	0.177 ± 0.014	0.515
V_E (ml/min/100 g)	9.1 ± 1.4	11.0 ± 1.9	**0.036**
$ETCO_2$ (%)	6.26 ± 0.78	6.16 ± 0.92	0.818

MAP, mean arterial blood pressure; SBP, systolic blood pressure; DBP, diastolic blood pressure; HR, heart rate (bpm, beats.min^{-1}); f_R, respiratory frequency (brpm, breaths.min^{-1}); V_T, tidal volume; V_E, minute ventilation; $ETCO_2$, end-tidal carbon dioxide; CIH, chronic intermittent hypoxia. Data are shown as mean ± SD. Blood pressure and heart rate parameters were statistically compared using one-tailed unpaired Student's *t*-tests. All other parameters were statistically compared using two-tailed unpaired Student's *t*-tests. Bolded numbers highlight statistical significance (P < 0.05)

Table 4
Body organ weights and urinary stress profile of sham and CIH-exposed guinea-pigs.

	Sham (n = 8)	CIH (n = 8)	*P* Value
LV (mg/100 g)	170 ± 12	179 ± 13	0.075
RV (mg/100 g)	54.9 ± 4.0	57.5 ± 5.4	0.144
RV + LV (mg/100 g)	225 ± 15	237 ± 17	0.074
RV:LV	0.324 ± 0.018	0.322 ± 0.027	0.409
Lung wet weight (mg/100 g)	427 ± 26	487 ± 67	**0.032**
Lung dry weight (mg/100 g)	95 ± 5	111 ± 13	**0.006**
Lung fluid content (%)	77.8 ± 1.4	77.2 ± 0.9	0.353
Spleen (mg/100 g)	0.804 ± 0.163	0.678 ± 0.124	0.102
	(n = 6)	(n = 6)	
Urinary noradrenaline (ng/mg creatinine)	215 ± 156	190 ± 196	0.404
Urinary corticosterone (ng/mg creatinine)	2.56 ± 2.40	1.38 ± 0.34	0.410

LV, left ventricle; RV right ventricle; CIH, chronic intermittent hypoxia. Data are shown as mean ± SD. Ventricular, noradrenaline and corticosterone parameters were statistically compared using one-tailed unpaired Student's *t*-tests. All other parameters were statistically compared using two-tailed unpaired Student's *t*-tests. Bolded numbers highlight statistical significance (P < 0.05)

hyperventilation can be evoked by severe hypoxia in sham guinea-pigs, these effects are not potentiated, but instead are diminished, following exposure to CIH [45]. Moreover, exposure to CIH failed to potentiate intracellular calcium or catecholamine release from carotid body type 1 cells during acute activation by hypoxia or high K^+, revealing convincingly that exposure to CIH fails to sensitise carotid bodies of the guinea-pig [45]. Carotid bodies of the guinea-pig lack O_2-sensitive K^+ channels [46] and exposure to CIH does not evoke a capacity for hypoxia-dependent type 1 cell depolarisation and neurosecretory response [45], which is essential to chemo-afferent signalling from the carotid body. However, the type 1 cells retain the ability to excite sensory nerve endings as evidenced by cyanide mitochondrial poisoning. These elegant data provide evidence that exposure to CIH does not potentiate carotid body activity in guinea-pigs (unlike rats), and confirm the characteristic quiescent nature of the guinea-pig carotid body in normoxia and hypoxia. In our study, exposure to CIH did not evoke alterations to breathing in normoxia or hypoxia, whereas similar paradigms disrupt respiratory control in rats [18,28,47].

In rodents with hypoxia-sensitive carotid bodies, CIH increases the propensity for central apnoeas and respiratory instability [17–19]. Interestingly, CIH-exposed guinea-pigs exhibited reduced respiratory variability concurrent with decreased sigh frequency and qualitative observations of fewer central apnoeas. We observed altered brainstem neurochemistry evidenced by lower noradrenaline concentrations in CIH-exposed pons and medulla. Noradrenaline drives breathing irregularities in IH-exposed anaesthetised mice [49]. Thus, lower sigh/apnoea frequency and lower brainstem noradrenaline concentrations in CIH-exposed guinea-pigs are congruent observations, especially when one considers that CIH-exposed rats show evidence of increased central noradrenergic terminals [50] and elevated apnoea index [18]. Whereas, we observed no evidence of a significant correlation between sigh frequency and medullary or pontine noradrenaline concentrations, significant correlations between respiratory timing variability and pontine noradrenaline concentrations were evident.

CIH impairs baroreflex control [51] and drives the development of hypertension in rodents with hypoxia-sensitive carotid bodies [41,52]. Hypertension did not manifest in our guinea-pig model of CIH, which seemingly supports the hypothesis that carotid body sensitisation is essential for cardiovascular maladaptation, as previously suggested by carotid body denervation studies [3,21]. Exposure to CIH, using a profile similar to the present study evokes hypertension in rats [41]. Indeed, hypertension can manifest after as few as two days of exposure to CIH [52]. Thus, we are confident that the CIH protocol utilised in this study would be sufficient to induce hypertension in a rodent model with hypoxia-sensitive carotid bodies. Remarkably, exposure to CIH (30 cycles.hour^{-1} for 8 h.day^{-1} for 30 days) caused hypertension in guinea-pigs [47], with tachycardia and increased concentrations of circulating noradrenaline, indicative of sympathetic nervous hyperactivity, the hallmark of carotid body-dependent sympatho-excitation in rat models of CIH. It is therefore apparent that exposure to CIH has the capacity to

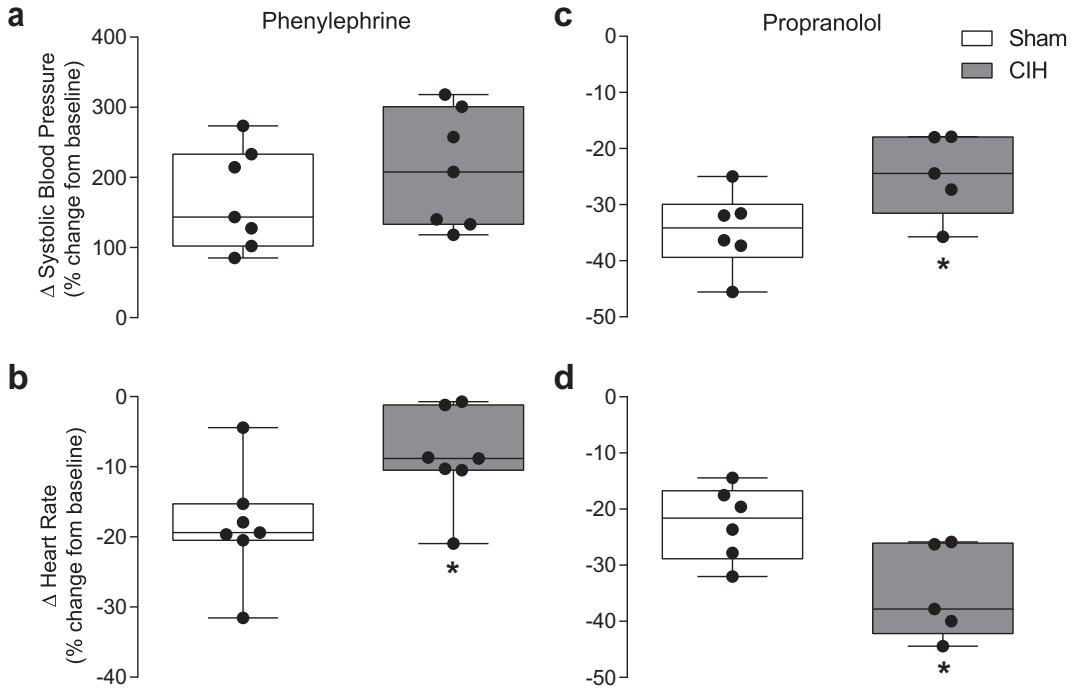


Fig. 4. Autonomic control of heart rate is altered in CIH-exposed anaesthetised guinea-pigs. Group data for systolic blood pressure (a, c), heart rate (b, d) in response to intravenous infusion of the β-adrenoceptor blocker propranolol (a-b) and α-adrenoceptor agonist phenylephrine (c-d). Values (a-d) are expressed as box and whisker plots (median, 25–75 percentiles and minimum and maximum values). Groups were statistically compared by unpaired Student's *t*-tests. *p < .05 compared with sham.

Table 5
Respiratory responsiveness to chemostimulation in sham and CIH-exposed anaesthetised guinea-pigs.

	Sham (n = 8)	CIH (n = 8)	P Value
% change from baseline	Hypercapnia ($FiCO_2 = 0.05$)		
f_R	20 ± 13	3 ± 14	**0.027**
V_T	70 ± 14	74 ± 20	0.643
V_E	104 ± 30	79 ± 29	0.124
	Hypercapnia ($FiCO_2 = 0.10$)		
f_R	13 ± 26	−14 ± 18	**0.034**
V_T	171 ± 40	113 ± 71	0.064
V_E	206 ± 92	82 ± 69	**0.009**
	Hypoxia ($FiO_2 = 0.10$)		
	(n = 8)	(n = 7)	
f_R	1 ± 12	5 ± 18	0.286
V_T	5 ± 6	2 ± 13	0.298
V_E	6 ± 17	7 ± 20	0.451
	Asphyxia ($FiCO_2 = 0.05$ & $FiO_2 = 0.10$)		
	(n = 8)	(n = 8)	
f_R	25 ± 21	9 ± 19	0.064
V_T	71 ± 17	69 ± 17	0.413
V_E	114 ± 34	85 ± 42	0.086
	Sodium cyanide (200 μg/kg i.v.)		
	(n = 7)	(n = 5)	
f_R	41 ± 19	22 ± 6	**0.043**
V_T	63 ± 25	63 ± 47	0.9922
V_E	101 ± 46	89 ± 36	0.086

f_R, respiratory frequency; V_T, tidal volume; V_E, minute ventilation; CIH, chronic intermittent hypoxia. Data are shown as mean ± SD and were statistically compared using unpaired Student's *t*-tests. Bolded numbers highlight statistical significance ($P < 0.05$)

evoke cardiovascular morbidity in guinea-pigs, through a carotid body-independent mechanism. We posit that our modest paradigm (6 cycles. hour^{-1} for 8.hours day^{-1} for 12 days), sufficient to evoke hypertension in rats [41], was most likely insufficient to provoke sensory facilitation of carotid chemoafferent input to the brainstem, but was also insufficient to evoke sympathetic overactivity, by whatever means, and hence hypertension. This is supported by urinary measures of noradrenaline and corticosterone in our study, which were unchanged in CIH-exposed guinea-pigs.

Of interest, we observed a CIH-induced tachycardia, which is also reported in some studies in CIH-exposed rats [14,53], and consistent with observations in the rat that CIH-induced tachycardia was amenable to β-adrenoceptor blockade with propranolol [14]. Of note, enhanced heart rate responses to sympathetic nervous stimulation was observed in an isolated atria/right stellate ganglion preparation in CIH-exposed guinea-pigs [54]. Tachycardia was also evident in guinea-pigs exposed to CIH for 30 days [45], accompanied by evidence of sympathetic overactivity and hypertension. Our finding of a β-adrenoceptor-mediated tachycardia suggests activation of the cardiac sympathetic branch with resultant chronotropic effects without elevation in blood pressure. Blunted bradycardia responses to phenylephrine-induced elevations in blood pressure (which were equivalent in magnitude between groups), suggests impaired baroreflex responsiveness in CIH-exposed guinea-pigs, perhaps secondary to CIH-induced central remodelling at the level of the nucleus tractus solitarius and paraventricular nucleus [55] or related to increased inhibitory neurotransmission to cardiac vagal neurons of the nucleus ambiguous and dorsal motor nucleus of the vagus resulting in blunted heart rate responsiveness, which has been reported following exposure to CIH [56]. We conclude that these effects are independent of carotid body sensitisation, revealing a capacity for CIH to alter autonomic control of the heart through actions at one or more extra-carotid sites. Whereas our modest paradigm of CIH was insufficient to evoke hypertension in guinea-pigs, at least over the timeframe of our study, Docio et al. [45] have clearly demonstrated a capacity for more severe exposures to CIH to elicit sympathetic overactivity and hypertension in guinea-pigs, without carotid body sensitisation.

Regulation of blood pressure is complex with multiple contributory mechanisms over varying time domains. Increasing evidence supports the symbiotic gut microbiota as a major contributor to long-term blood pressure regulation [35,36]. New evidence implicates sympathetic neuronal communication between the hypothalamic paraventricular nucleus and the gut in the regulation of blood pressure [57]. In recent years, it has become apparent that various hypertensive animal models develop gut dysbiosis, including spontaneously hypertensive rats [35,58] and animal models of sleep apnoea [15,36,59]. A variety of systemic stressors are capable of inducing alterations in the gut microbiota, including prenatal stress [34], maternal separation [60], social disruption [61,62] and sleep fragmentation [61]. Hyper-activation of the sympathetic nervous system appears to be fundamental to this process. As such, exposure to CIH could conceivably disrupt gut microbiota through autonomic dysregulation. In addition, CIH is a systemic insult that may directly influence microbiota function and microbiota/host interaction. Repeated bouts of hypoxia may favour the proliferation of obligate anaerobes. Moreover, exposure to CIH induces oxidative stress and inflammation and decreases the expression of tight junction proteins [63], all of which contribute to altered microbiota composition and diversity. Spontaneously hypertensive rats have decreased expression of several tight junction proteins in the gut, which increases intestinal permeability [58]. We did not examine the effects of exposure to CIH on intestinal permeability in this study, but others have reported that exposure to CIH results in a late-onset persistent low-grade endotoxaemia in mice, with positive correlations between plasma LPS and the abundance of *Mucispirillum* and *Desulfovibrio* [31]. Also, bacteria-derived metabolites such as short-chain fatty acids are influenced by CIH exposure [35,57] and these bacterial metabolites, in addition to others such as hydrogen sulphide [64] and indole [65], are associated with blood pressure control.

A pivotal role for microbiota in blood pressure regulation is revealed by demonstration that faecal transplant from spontaneously hypertensive rats into normotensive, microbiome-compromised control rats induces hypertension and gut dysbiosis [35]. Alterations in the composition of the gut microbiota coincide with increases in blood pressure, since changes in the microbiota are not evident in pre-hypertensive rats, whereas expansion of Bacteroidetes and a reduction in Firmicutes, as well as increased gut barrier permeability are evident once hypertension is established [58,66]. Changes in the relative proportions of Firmicutes and Bacteroidetes bacteria have been reported in animal models of hypertension and sleep apnoea, although the results are inconsistent across the studies [15,36,59]. Indeed, six weeks of recovery in normoxia following exposure to CIH is insufficient for recovery of the microbiota [31], a potential mechanism underlying failed interventional therapy for hypertension despite prevention of CIH in persons with sleep apnoea, in addition to suggestions of persistent epigenetic redox changes driving sympathetic over-activity [8].

In the present study, the relative abundance of Firmicutes was significantly reduced and the relative abundance of Bacteroidetes was significantly increased. As a consequence, the Firmicutes:Bacteroidetes ratio tended to decrease, as did the alpha diversity indices following exposure to CIH. Post-hoc analysis revealed that this comparison was underpowered to detect a significant difference, based upon the effect size determined in our study and sample size, which we acknowledge is a limitation of our study. LEfSe analysis detected statistically significant over-representation of Actinobacteria (*Bifidobacterium* and *Coriobacteriaceae UCG-002*) and Bacteroidetes species (*Butyricimonas* and *Bacteroidales S24–7* group) in the CIH-exposed group, while Firmicutes bacteria (*Blautia*, *Anaerotruncus*, *Ruminiclostridium*, *Lachnoclostridium*) were largely under-represented in CIH-exposed guinea pigs. The LEfSe results largely overlap with the differential abundance data presented on the heatmap (Fig. 8b), thus supporting the observed shift towards an increase in the relative abundance of

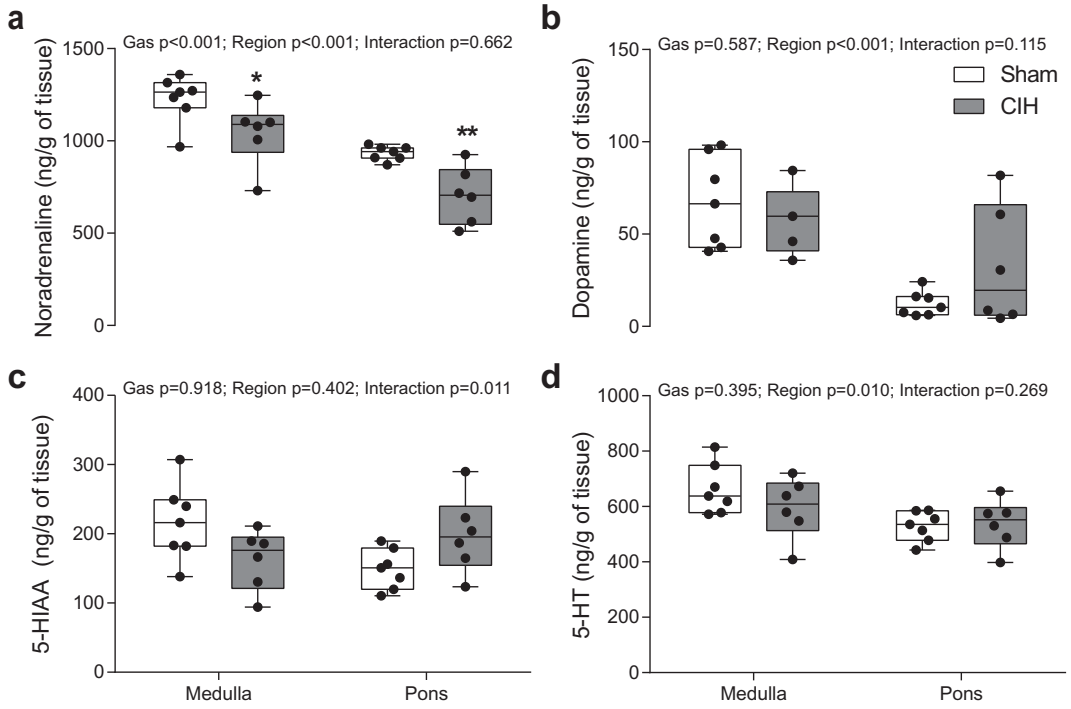


Fig. 5. Pontine and medulla oblongata noradrenaline concentrations are reduced in CIH-exposed guinea-pigs. Group data for noradrenaline (a), dopamine (b), 5-HIAA (c) and 5-HT (d) in pontine and medulla oblongata tissue homogenates from sham ($n = 7$) and CIH-exposed ($n = 6$) guinea-pigs. Values (a-d) are expressed as box and whisker plots (median, 25–75 percentiles and minimum and maximum values). 5-HIAA, 5-Hydroxyindole acetic acid; 5-HT, serotonin. Groups were statistically compared by two-way ANOVA with Bonferroni post hoc test. *sham pons vs. CIH pons, $p < .05$; **sham medulla vs. CIH medulla, $p < .01$.

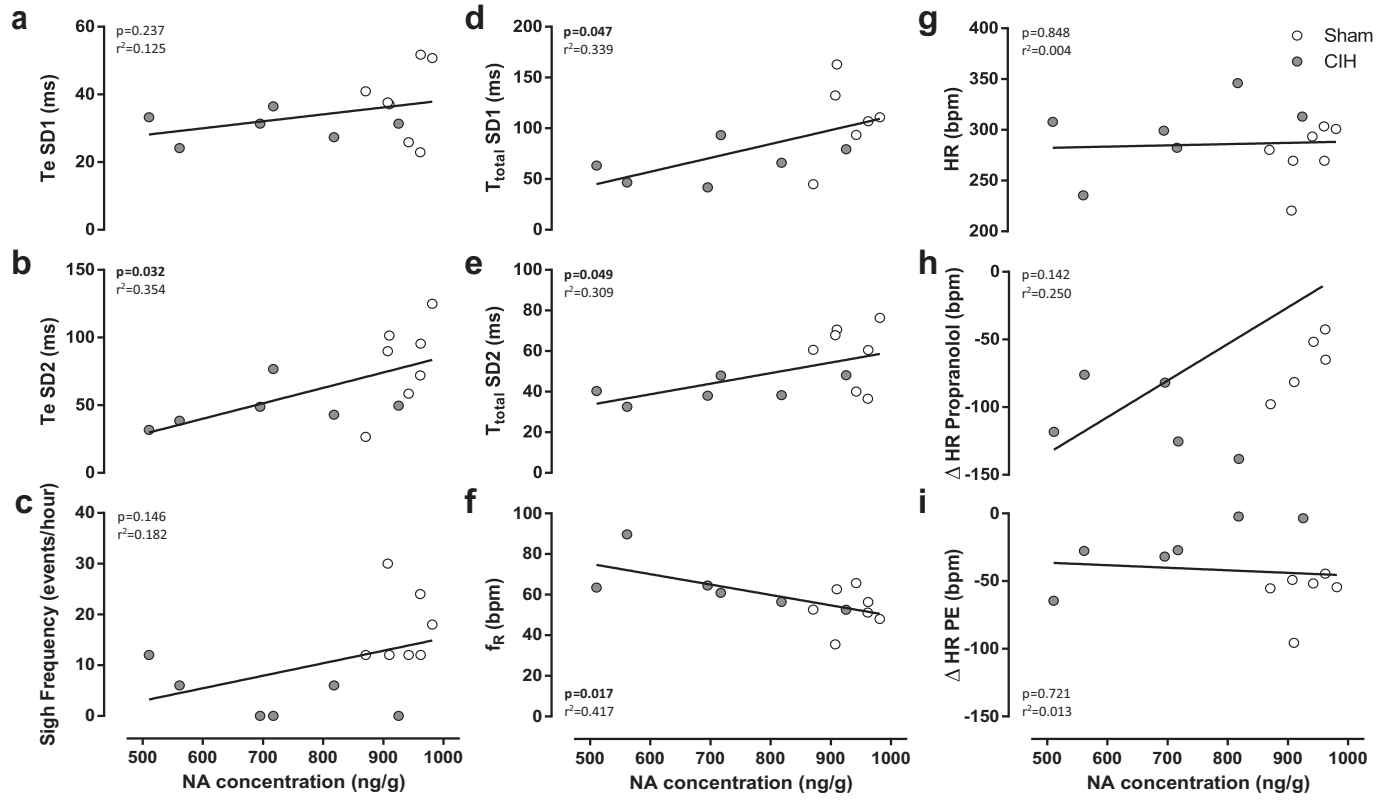


Fig. 6. Significant correlations are evident between pontine noradrenaline concentrations and various cardiorespiratory parameters. Individual data points for correlations between pontine noradrenaline (NA) concentrations and time to expire (T_e) short-term variability (SD1; a), T_e long-term variability (SD2; b), sigh frequency (c), total breath duration (T_{total}) SD1 (d), T_{total} SD2 (e) in sham and CIH-exposed awake guinea-pigs, and respiratory frequency (f_R; f), heart rate (HR; g), change in HR in response to propranolol administration (h) and change in HR in response to phenylephrine (PE) administration (i) in sham and CIH-exposed anaesthetised guinea-pigs. Solid black lines represent linear regression analysis for each parameter.

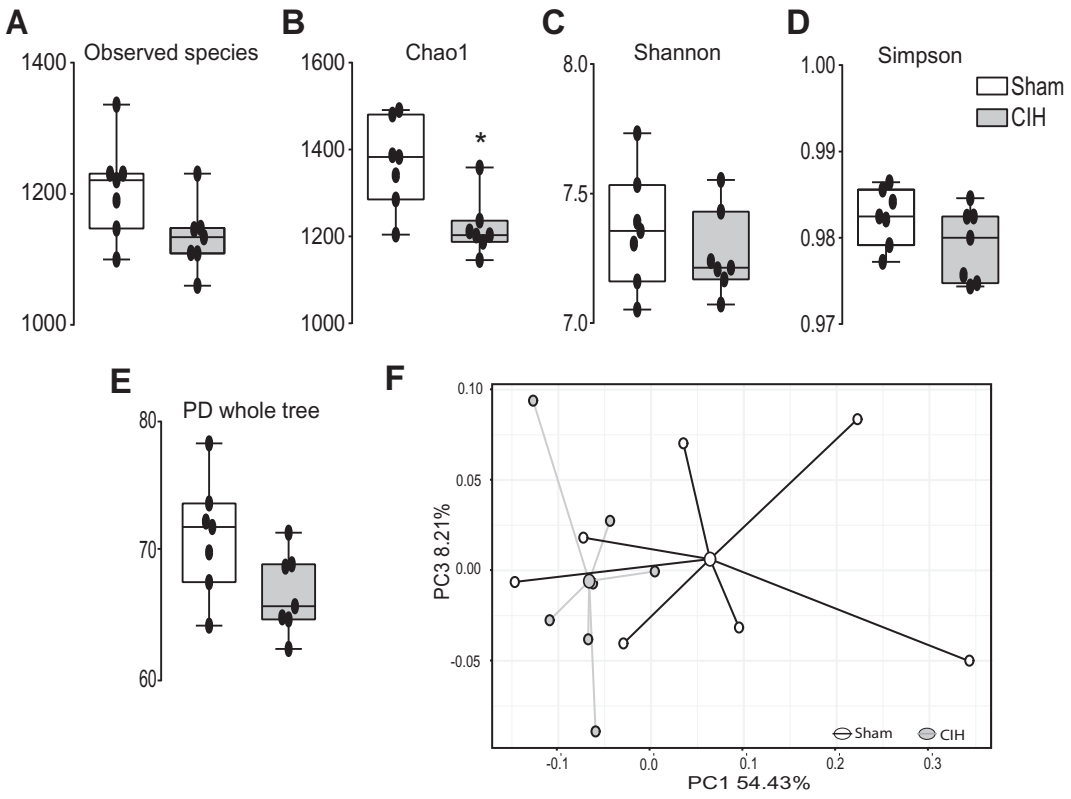


Fig. 7. Faecal microbiota richness is reduced in CIH-exposed guinea-pigs. Group data for alpha-diversity indices (a-e) and principal coordinate analysis (PCoA) plot based on Bray-Curtis dissimilarity matrices (f) for Sham and CIH-exposed animals. Values (a-e) are expressed as box and whisker plots (median, 25–75 percentiles and minimum to maximum values) and were statistically compared by non-parametric Mann-Whitney U test. Benjamini-Hochberg adjustment with Q = 0.2 was used to correct *p*-values for multiple testing (f). PD, phylogenetic diversity. Sham (n = 7), CIH (n = 7). *p < .05 compared with sham.

Bacteroidetes and a reduction in Firmicutes bacteria in CIH-exposed microbiota. Similar to our findings, an increase in the abundance of Bacteroidetes bacterial species was observed in deoxycorticosterone acetate (DOCA)-salt mice fed with a high fibre diet. This shift in the microbiota was accompanied by reduced systolic and diastolic blood pressures, as well as reduced cardiac weight index, cardiac fibrosis and improved cardiac function [67], suggesting beneficial effects of increased abundance of Bacteroidetes on cardiovascular function in the DOCA model. An additional common feature of disrupted microbiota is reduced microbial diversity. Hypertensive animals have reduced Shannon diversity and Chao1 richness [33,37,66]. We observed a decrease in Chao1 richness in CIH-exposed animals in our study, with trends towards decreases in other measures of microbiota diversity.

We posit that CIH-induced reduction in microbiota richness, in tandem with changes in microbiota composition demonstrate the physiologically significant influence of exposure to CIH on microbiota populations. These are likely direct effects of exposure to CIH, independent of sympathetic nervous activation, notwithstanding that putative links between CIH-induced hypertension and alterations to the gut microbiome are dependent upon hyper-activation of the sympathetic nervous system, which in turn serves to perpetuate cardiorespiratory dysregulation. CIH in rodents with hypoxia-sensitive carotid bodies is associated with increased catecholamine secretions from the adrenal glands [68], hormones which act to promote epithelial barrier dysfunction [58,69] as well as the proliferation of specific bacteria [70]. It is plausible to suggest that exposure to CIH characterised by more frequent and/or severe and/or longer durations than those used in the present study, which have been shown to cause hypertension in guinea-pigs [45], could cause severe disruption to gut microbiota, and indeed may be the basis for the observations of CIH-induced hypertension and sympathetic hyper-activity in guinea-pigs [45].

Our study reveals the capacity for CIH to alter microbiota composition, richness and beta-diversity which may have contributed to altered autonomic control of the heart, effects independent both of carotid body sensitisation and sympathetic over-activity, therefore purportedly a result of direct actions of CIH on the microbiota. It is also possible that direct actions of CIH on the microbiota, in the absence of sympathetic over-activity, may have conferred cardiovascular protection in respect of blood pressure responses to exposure to CIH.

The afferent vagal nerve pathway is proposed as an essential link between the gut microbiota and the central nervous system [32,71,72]. Alterations in microbiota diversity/populations and their production of metabolites could influence vagal afferent traffic directed towards the cardiorespiratory centres of the brainstem. Positive correlations between disruption of the microbiota and respiratory timing variability have been described in adult rats exposed to pre-natal stress [34]. Exposure to CIH in adult guinea-pigs caused a reduction in the frequency of sighs, a critical component of the normal respiratory control repertoire, important in the control of respiratory timing variability. Moreover, as a result of the mechanical consequences of augmented breaths, sighs defend the respiratory gas exchange units against collapse (atelectasis). CIH-associated alterations to the microbiota and/or direct effects on the central neural circuitry within the brainstem demonstrably interfered with the protective sigh reflex, revealing a CIH-induced respiratory-related morbidity unrelated to carotid body sensitisation.

In conclusion, hallmark features of cardiorespiratory morbidity evoked by exposure to CIH in rats, principally apnoea and high blood pressure, were not evident in guinea-pigs. There were nevertheless residual aberrant phenotypes including decreased respiratory timing variability and frequency of sighs as well as tachycardia and decreased heart rate responsiveness. Exposure to CIH altered brainstem neurochemistry, revealed as a significant reduction in the concentration of

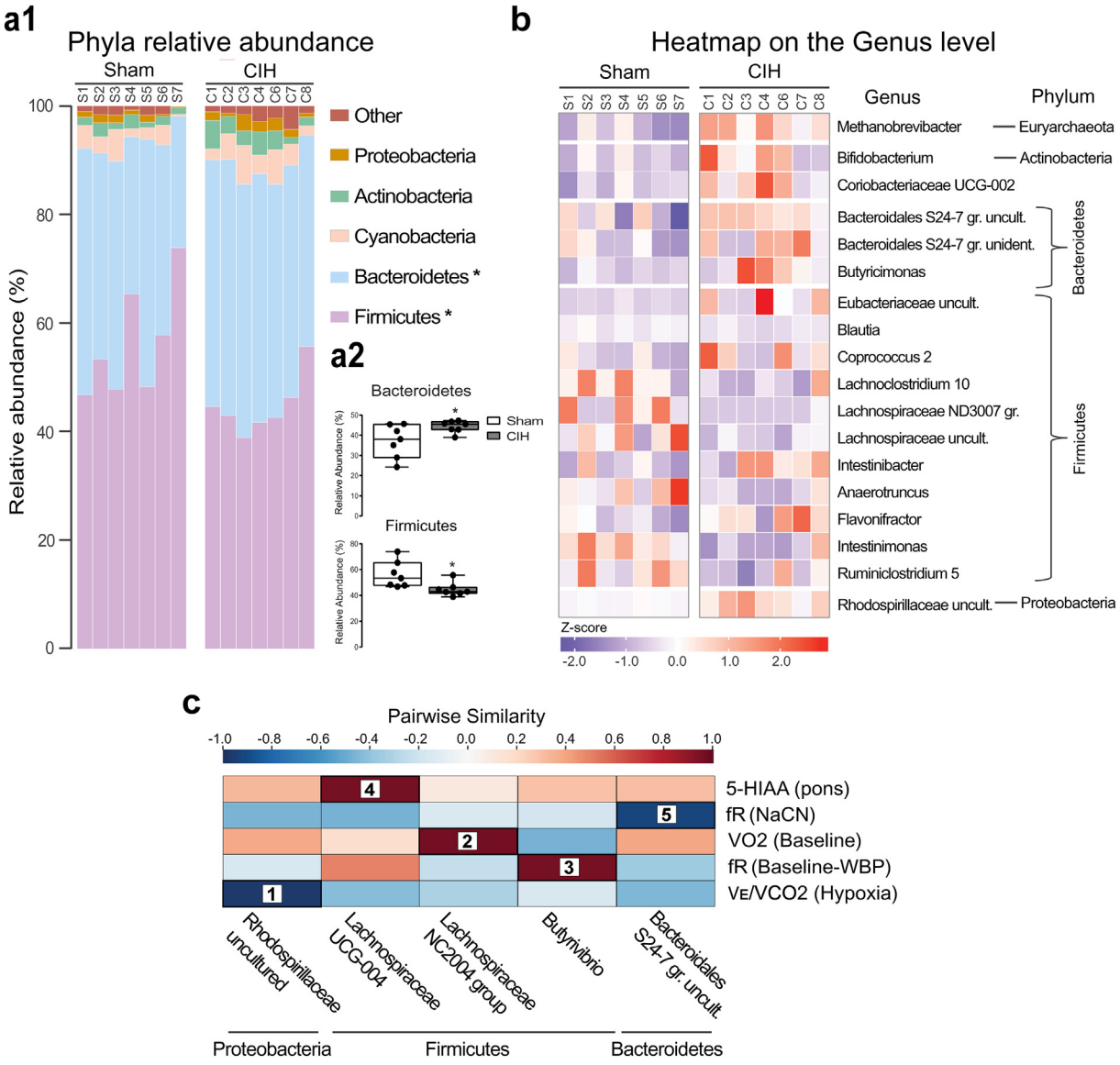


Fig. 8. CIH reduces Firmicutes and increases Bacteroidetes relative abundance in guinea-pig faecal samples. Stacked barplot (a1) at the phylum level of faecal samples taken from each guinea-pig with group data (a2) showing significant changes in the relative abundance of Bacteroidetes and Firmicutes phyla in CIH-exposed microbiota. Heatmap of *Z*-scores at the genus level (b) showing increases (red) and decreases (purple) in the relative abundance of bacterial genera in each guinea-pig; sham (left-side columns) and CIH (right-side columns). (c) Hallagram depicting Spearman correlations between the relative abundance of bacterial genera and physiological readouts. Genera are ordered taxonomically. Significant correlations are ranked 1–5 (strongest-weakest): positive associations are shown in red, negative – in blue. Benjamini-Hochberg adjustment p-value with Q = 0.2 was used to correct p-values for multiple testing. 5-HIAA, 5-hydroindole acetic acid; f_R. respiratory frequency; NaCN, sodium cyanide; V_E/VCO_2, ventilatory equivalent; VO_2, oxygen consumption; WBP, whole-body plethysmography. Sham ($n = 7$), CIH (n = 7). *p < .05, CIH compared with sham.

noradrenaline in the pons and medulla oblongata. In the likely absence of sympathetic over-activity, exposure to CIH decreased gut microbiota species richness, and altered microbiota composition, decreasing the relative abundance of Firmicutes and increasing the relative abundance of Bacteroidetes.

Our study reveals obligatory roles for the carotid body in CIH-induced cardiorespiratory morbidity. Moreover, the gut microbiota are potential players contributing to central network remodelling during exposure to CIH through potential alterations in microbiota-gut-brain axis signalling. Our findings have implications for human sleep-disordered breathing and contribute to an emerging interest in manipulation of the microbiota as a potential adjunctive therapy in human disease [36,73].

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Declaration of interests

The authors have no professional, personal or financial conflicts of interests relating to this publication.

Author contributions

EFL: experimental design; acquisition of data; data and statistical analysis; interpretation of data; drafting of the original manuscript; KMO'C: acquisition of data; data and statistical analysis and interpretation of data; drafting of the original manuscript; EFL and KMO'C contributed equally to the study; CRS: 16S rRNA sequencing: acquisition of data; data and statistical analysis; FF: 16S rRNA sequencing: acquisition of data; data and statistical analysis; TFSB: Interpretation of data; Data analysis; Statistical analysis; DPB: experimental design; AVG: interpretation of data; statistical analysis; critical revision of the manuscript; CS: critical revision of the manuscript; GC: HPLC studies: experimental design; interpretation of data; critical revision of the manuscript; JFC: critical revision of the manuscript; KDO'H: experimental design; interpretation of physiological data; drafting and critical revision of the manuscript.

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