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Combined effects of intermittent hyperoxia and intermittent hypercapnic hypoxia on respiratory control in neonatal rats

Ryan W. Bavis*, Alexandra H. Millström, Song M. Kim, Carolyn A. MacDonald, Caitlin A. O'Toole, Kendra Asklof, Amy B. McDonough

Department of Biology, Bates College, Lewiston, ME 04240 USA

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ABSTRACT

Chronic exposure to intermittent hyperoxia causes abnormal carotid body development and attenuates the hypoxic ventilatory response (HVR) in neonatal rats. We hypothesized that concurrent exposure to intermittent hypercapnic hypoxia would influence this plasticity. Newborn rats were exposed to alternating bouts of hypercapnic hypoxia (10% O_2/6% CO_2) and hyperoxia (30–40% O_2) (5 cycles h^{-1}, 24 h d^{-1}) through 13–14 days of age; the experiment was run twice, once in a background of 21% O_2 and once in a background of 30% O_2 (i.e., "relative hyperoxia"). Hyperoxia had only small effects on carotid body development when combined with intermittent hypercapnic hypoxia: the carotid chemoafferent response to hypoxia was reduced, but this did not affect the HVR. In contrast, sustained exposure to 30% O_2 reduced carotid chemoafferent activity and carotid body size which resulted in a blunted HVR. When given alone, chronic intermittent hypercapnic hypoxia increased carotid body size and reduced the hypercapnic ventilatory response but did not affect the HVR. Overall, it appears that intermittent hypercapnic hypoxia counteracted the effects of hyperoxia on the carotid body and prevented developmental plasticity of the HVR.

1. Introduction

Although mammals generally respond to hypoxia by increasing pulmonary ventilation, the magnitude of this hypoxic ventilatory response (HVR) depends both on an individual's genetic background and the environmental conditions experienced during its lifetime (Mitchell and Johnson, 2003; Bavis and MacFarlane, 2017). The perinatal environment is particularly consequential in this respect as the capacity of the respiratory phenotype to exhibit phenotypic plasticity often diminishes with age (Bavis and MacFarlane, 2017).

Hyperoxia has been shown to have a variety of effects on the respiratory control of developing animals. For example, sustained exposure to moderate hyperoxia (30–60% O_2) causes neonatal rats to hypoventilate and leads to a progressive blunting of the HVR (Bavis et al., 2003, 2010, 2014). Rats reared in hyperoxia for the first 1–4 postnatal weeks continue to exhibit reduced HVR as adults (Ling et al., 1996, 1997; Bavis et al., 2003), and the effects may be permanent (Fuller et al., 2002). The impaired HVR reflects abnormal development of the carotid body, including reduced O_2 sensitivity of individual glomus cells, smaller carotid bodies (with fewer glomus cells), and fewer chemoafferent axons in the carotid sinus nerve (CSN) (Erickson et al., 1998; Prieto-Lloret et al., 2004, 2015; Donnelly et al., 2005; Bavis et al., 2011; Dmitrieff et al., 2012). This plasticity is unique to hyperoxic exposures occurring during perinatal development (Ling et al., 1997; Erickson et al., 1998; Bavis et al., 2002, 2013) and may result from hyperoxia's suppression of normal carotid body activity during a critical developmental period (i.e., the first two postnatal weeks in rats) (Bavis et al., 2002, 2007).

Chronic exposure to sustained hyperoxemia is rare in clinical settings, but intermittent hyperoxemia is common. Supplemental O_2 is among the most common therapies for preterm and very low birth weight infants. Despite awareness of the potential dangers of hyperoxia (e.g., bronchopulmonary dysplasia, retinopathy of prematurity) and attempts to maintain patients within a narrow range of oxygenation, measured O_2 saturations fluctuate and preterm infants have been found to be hyperoxemic relative to target levels for 30–40% of the time (or more) while receiving supplemental O_2 and/or mechanical ventilation (Hagadorn et al., 2006; Claure and Bancalari, 2009; Finer and Leone, 2009; Jain et al., 2018). Moreover, fetal development occurs at relatively low O_2 levels *in utero*, so preterm birth entails an earlier rise in arterial O_2 partial pressure (PO_2); thus, intermittent hyperoxemia may be superimposed upon a background of "relative hyperoxia" (cf.

* Corresponding author at: Department of Biology, Bates College, 44 Campus Ave., Carnegie Science Hall, Lewiston, ME 04240 USA.
E-mail address: rbavis@bates.edu (R.W. Bavis).

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Carroll, 2003). Importantly, intermittent exposure to either 30% or 60% O_2 decreases carotid body size and attenuates the HVR in neonatal rats (Bavis et al., 2007; Logan et al., 2016), similar to the effects of sustained hyperoxia; intermittent exposure to 60% O_2 also transiently reduces carotid chemoreceptor responses to acute hypoxia (Logan et al., 2016).

On the other hand, preterm and very low birth weight infants may also experience intermittent bouts of hypercapnic hypoxemia as a result of immature lungs, hypoventilation, and apnea of prematurity (Claure and Bancalari, 2009; Finer and Leone, 2009; Jain et al., 2018). Many studies have investigated the effects of chronic intermittent hypoxia on cardiorespiratory function in neonatal and adult animals (reviewed in Mitchell and Johnson, 2003; Teppema and Dahan, 2010; Prabhakar et al., 2015). Although most of these studies reveal plasticity in eupneic ventilation and hypoxic chemoreflexes, the specific effects vary considerably and may be influenced by the species studied and by the pattern and duration of intermittent hypoxia. A common finding is that chronic intermittent hypoxia augments resting ventilation (Peng et al., 2004; Reeves and Gozal, 2006a, b) and that this hyperpnea persists for weeks after return to normoxia (Reeves et al., 2006). Another common finding is that chronic intermittent hypoxia during early postnatal life potentiates the acute HVR. For example, 16 h – 10 d of intermittent hypoxia increases the peak HVR in neonatal rats while the hypercapnic ventilatory response (HCVR) remains unchanged (Peng et al., 2004; Julien et al., 2008, 2011). Although central contributions should not be ruled out (Ling et al., 2001), postnatal chronic intermittent hypoxia increases carotid body activity through a mechanism involving reactive oxygen species and endothelin-1 (Peng et al., 2004; Pawar et al., 2008, 2009; Prabhakar et al., 2015).

However, there have also been studies in which chronic intermittent hypoxia attenuated the HVR. Reeves and Gozal (2006b) found that ventilation was greater in absolute terms after 15 or 30 days of chronic intermittent hypoxia during the postnatal period, but the HVR (expressed as the change from baseline) was blunted; the HVR remained blunted for at least several weeks after return to normoxia (Reeves et al., 2006). In a much different paradigm, Waters et al. (1997) exposed piglets to 10% O_2 for 30 min on each of five consecutive days. The piglets had substantially smaller hypoxic responses (measured by diaphragm EMG activity) after the repeated bouts of hypoxia. This effect is opposite that observed in piglets subjected to daily bouts of hypercapnic hypoxia which exhibited augmented HVR (Waters and Tinworth, 2001); thus, the presence of hypercapnia (which accompanies intermittent hypoxia during hypoventilation, recurrent apnea, and rebreathing) may influence the plasticity elicited by chronic intermittent hypoxia.

Given that preterm and very low birth weight infants may experience both hypercapnic hypoxia and hyperoxia and that each stimulus is known to elicit respiratory plasticity independently, we investigated the effects of combined exposures to intermittent hypercapnic hypoxia and intermittent hyperoxia on the developing respiratory control system. Since hypoxia and hyperoxia often produce opposite effects on carotid body function and the associated HVR, we hypothesized that alternating exposures to these stimuli might offset and yield relatively normal respiratory control. Alternatively, since at least some studies report blunted HVR after chronic intermittent hypoxia, reciprocal bouts of hypercapnic hypoxia and hyperoxia might exacerbate functional deficits.

Two sets of experiments were run. In the first set, O_2 and CO_2 levels were cycled around a normoxic (21% O_2) background. The pattern of intermittent hyperoxia was identical to that used by Logan et al. (2016), except short periods of hypercapnic hypoxia were introduced between the bouts of hyperoxia. In the second set of experiments, O_2 and CO_2 levels were cycled around a mildly hyperoxic background (30% O_2) to simulate the "relative hyperoxia" that might be experienced by preterm infants. Ventilation, metabolism, and carotid body size and function were measured after approximately two weeks of exposure (i.e, at

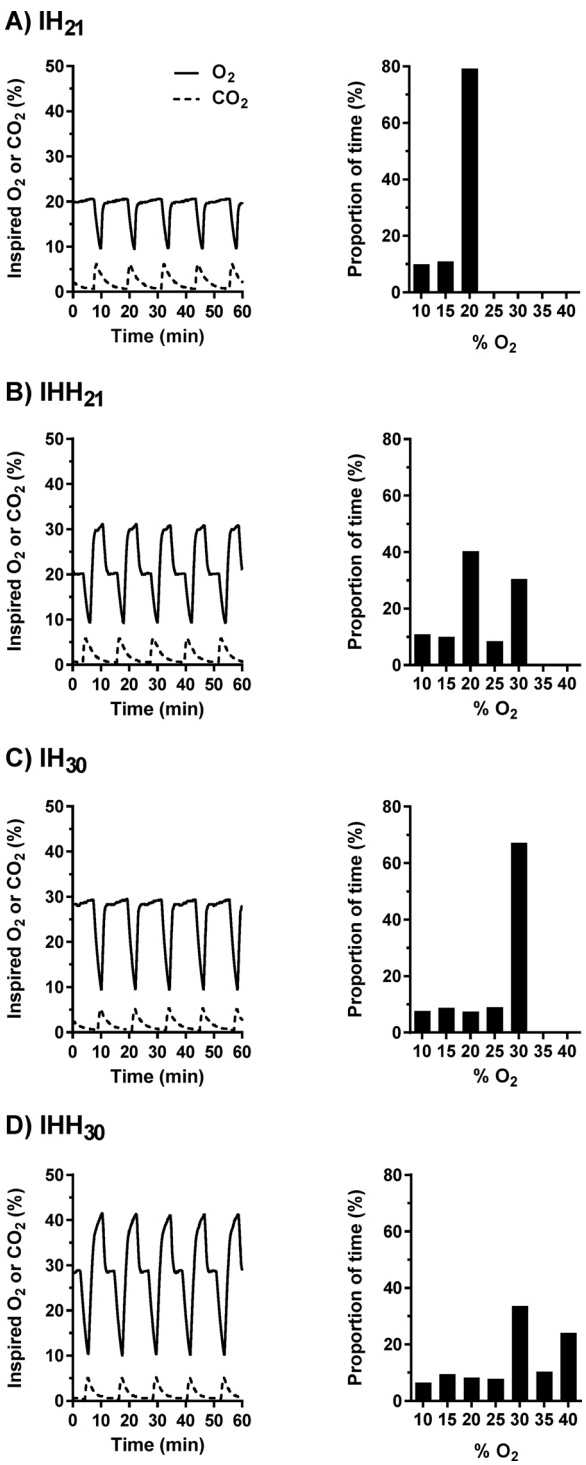

Fig. 1. Neonatal rats were exposed to intermittent hypercapnic hypoxia (IH) or intermittent hypercapnic hypoxia + intermittent hyperoxia (IHH) through P13-14 (5 cycles h^{-1}, 24 h d^{-1}). O_2 levels cycled from a background of 21% O_2 (IH_{21} and IHH_{21}) in Experiment #1 or from a background of 30% O_2 (IH_{30} and IHH_{30}) in Experiment #2. *Left* panels are representative chamber O_2 and CO_2 profiles measured over the course of one hour. *Right* panels show the proportion of time rats were exposed to different levels of O_2; the center of each bin is labeled and bin width = 5% units. Additional groups of rats were exposed to room air (21% O_2; Control) or sustained hyperoxia (30% O_2; SH) (not shown).

13–14 days of age).

2. Methods

2.1. Experimental animals

All experimental protocols were approved by the Bates College Institutional Animal Care and Use Committee. Timed-pregnant Sprague-Dawley rats were obtained from a commercial supplier (Charles River Laboratories) and housed in room air until birth. The resulting litters were assigned to experimental groups 2–3 h after parturition.

In Experiment #1 (background = 21% O_2), rats were assigned to one of three treatment groups: room air (Control), intermittent hypercapnic hypoxia (IH_{21}), or intermittent hypercapnic hypoxia + intermittent hyperoxia (IHH_{21}). The IH_{21} and IHH_{21} litters (housed with their mothers) were placed into chambers programmed to cycle between set O_2 and CO_2 levels at a rate of 5 cycles h^{-1} (OxyCycler A420C controller with A-30274 A chambers; BioSpherix) for 24 h d^{-1} (Fig. 1). For IH_{21}, the settings for each 12-min cycle were 2.4 min at 10% O_2 / 6% CO_2 and 9.6 min at 21% O_2 / 0% CO_2. For IHH_{21}, the settings were 2.4 min at 10% O_2 / 6% CO_2, 4.5 min at 30% O_2 / 0% CO_2, and 5.1 min at 21% O_2 / 0% CO_2. The actual time spent in each gas mixture was less than programmed due to the gas exchange dynamics of the system, but both groups spent approximately 20% of their time in hypoxia (< 17.5% O_2) and the IHH_{21} group also spent approximately 30% of their time in hyperoxia (> 27.5% O_2) (Fig. 1). Age-matched Control rats were housed in a large acrylic chamber (310 l) flushed with room air at flow rates sufficient to maintain approximately 21% O_2 (and less than 0.4% CO_2); the Control chamber sat adjacent to the IH_{21} and IHH_{21} chambers.

In Experiment #2 (background = 30% O_2), rats were assigned to one of four treatment groups: room air (Control), sustained hyperoxia (30% O_2; SH), intermittent hypercapnic hypoxia (IH_{30}), or intermittent hypercapnic hypoxia + intermittent hyperoxia (IHH_{30}). As in Experiment #1, the IH_{30} and IHH_{30} newborns (housed with their mothers) were placed into chambers programmed to cycle between set O_2 and CO_2 levels at a rate of 5 cycles h^{-1} for 24 h d^{-1} (Fig. 1). For IH_{30}, the settings for each 12-min cycle were 2.4 min at 10% O_2 / 6% CO_2 and 9.6 min at 30% O_2 / 0% CO_2. For IHH_{30}, the settings were 2.4 min at 10% O_2 / 6% CO_2, 4.5 min at 40% O_2 / 0% CO_2, and 5.1 min at 30% O_2 / 0% CO_2. Again, the actual time spent in each gas mixture was less than programmed for the intermittent treatment groups due to the dynamics of the system; both groups spent approximately 16% of their time in hypoxia (< 17.5% O_2) and approximately 68% of their time in hyperoxia (> 27.5% O_2) (Fig. 1). Age-matched Control and SH rats were reared in chambers flushed with room air or 30% O_2, respectively; the chambers sat adjacent to the IH_{30} and IHH_{30} chambers.

Rats were exposed to a 12-h light : 12-h dark cycle and were provided food and water *ad libitum*. Chambers were opened briefly when animals were removed for study and for routine cage-cleaning as needed. All physiological and morphological measurements were made when rats were 13–14 days of age (i.e., P13-14, where P0 is the day of birth). Both male and female neonatal rats were used in this study. Sample sizes for each subset of measurements are provided in the tables and figure legends.

2.2. Ventilation measurements

Ventilation was measured in neonatal rats at P14 (Experiment #1) or P13-14 (Experiment #2) with a custom-built head-body plethysmograph; measurements began within one hour after pups were removed from the chronic exposure chambers. The plethysmograph consisted of two cylindrical compartments, a head compartment (4.75 cm ID; 70 ml) and a body compartment (3.1 cm ID; internal volume adjusted for the size of the animal with modeling clay). After weighing, the rat was sealed into the plethysmograph chamber with a flexible collar constructed of layers of latex film and Parafilm (Pechiney Plastic Packaging Co.) separating the head and body compartments; petroleum jelly was applied around the animal's neck to ensure an airtight seal. Gas mixtures were passed through the head compartment using a mass flow controller (MFC-4; Sable Systems) and valves (840 series; Sierra Instruments) at a flow rate of 1.5 l min^{-1}. A pneumotach and differential pressure transducer (MLT1L and ML141, respectively; ADInstruments) monitored air flowing in and out of the body compartment during breathing. Prior to placing the animal into the plethysmograph, 0.5 ml of air was injected into the body compartment to calibrate the pneumotach. Output from the pressure transducer was recorded at a sampling rate of 1000 Hz, passed through a high pass filter (0.1 Hz), and integrated to obtain respiratory volumes (PowerLab 8SP and LabChart 7 software with the spirometry extension; ADInstruments). Air temperature within the body compartment was monitored with a T-type thermocouple probe (IT-18; Physitemp Instruments) and maintained at 32–34 °C (i.e., within the thermoneutral zone for neonatal rats; Malik and Fewell, 2003) by placing the plethysmograph chamber in a temperature-controlled incubator.

Following a 10–15 min adjustment period (21% O_2, balance N_2), baseline ventilation was recorded for approximately 5 min. Gas concentrations were then switched to 12% O_2 (balance N_2) for 10 min to assess the HVR, returned to 21% O_2 (balance N_2) for 5 min of recovery, and then switched to 5% CO_2 (21% O_2, balance N_2) for 10 min to assess the HCVR. Respiratory parameters (tidal volume, respiratory frequency, and minute ventilation) were calculated from 40 to 60 s of the record during the baseline and recovery periods, and from 10 to 15 s of the record during hypoxia (minutes 1, 5, and 10) and hypercapnia (minute 10); multiple time points were analyzed for hypoxia to look for evidence of a biphasic HVR.

2.3. Metabolism measurements

The gas flow rate through the plethysmograph head chamber was too great to simultaneously measure metabolic O_2 consumption and CO_2 production with sufficient resolution. Therefore, flow-through respirometry was performed on a separate groups of P14 rats (Experiment #1) or P13-14 rats (Experiment #2). After weighing, the rat was sealed into a clear plastic box (420 ml) that was supplied with a continuous flow of air. The respirometer chamber was placed into an incubator so that it could be maintained at 32–34 °C; air temperature was monitored with a T-type thermocouple (IT-18; Physitemp Instruments). Airflow through the chamber was set at 300 ml min^{-1} STPD using a gas mixing mass flow controller (MFC-4; Sable Systems) and valves (840 series; Sierra Instruments); the gas was humidified by bubbling through approximately 40 ml of deionized water upstream of the respirometry chamber. Fractional concentrations of O_2 and CO_2 in the air exiting the respirometer chamber were measured (ML206 gas analyzer; ADInstruments) and recorded to computer at a sampling rate of 20 Hz (PowerLab 8SP and LabChart 7 software; ADInstruments); air was dried (Direrite, W.A. Hammond Drierite Co.) prior to passing through the gas analyzer. The concentrations of O_2 and CO_2 in the air entering the chamber were determined immediately after 20 min in 21% O_2 (balance N_2), and then the incurrent gas was switched to 12% O_2 (balance N_2) for 15 min. O_2 and CO_2 measurements were repeated after 15 min. O_2 consumption and CO_2 production rates were calculated from the average incurrent and excurrent gas concentrations recorded over the final minute of each exposure (i.e., 21% or 12% O_2) with equations from Frappell et al. (1992).

2.4. Single-unit carotid chemoafferent activity

Rats were deeply anesthetized with CO_2 and decapitated at P14 (Experiment #1) or P13-14 (Experiment #2). The carotid bifurcation and petrosal-nodose ganglion complex were removed together and

placed in an ice-cold, oxygenated (95% O_2, 5% CO_2) saline solution containing (in mM): 125 NaCl, 5 KCl, 2 $CaCl_2$, 1 Na_2HPO_4, 1 $MgSO_4$, 24 $NaHCO_3$, and 5 dextrose. The carotid body preparation was exposed to a solution of 0.10-0.13% collagenase (collagenase P; Roche Diagnostics) and 0.02-0.03% protease (type XIV; Sigma-Aldrich) in oxygenated saline for 30 min at 37 °C and then manually cleaned until only the carotid body, carotid sinus nerve (CSN), glossopharyngeal nerve, and petrosal ganglion remained. The cleaned preparation was held in ice-cold, oxygenated saline solution until being transferred to a perfusion chamber (RC-22C; 140 µL; Warner Instruments) on the stage of an inverted microscope (Eclipse TE-300; Nikon). The chamber was perfused with warm (~37 °C) saline solution that was bubbled with 21% O_2 (5% CO_2, balance N_2) and delivered at a rate of approximately 3 ml min^{-1} through stainless steel tubing (0.030 in. I.D.; Upchurch Scientific).

A suction electrode was used to record single-unit nerve activity from soma of chemoafferent neurons within the petrosal ganglion. An extracellular amplifier (EX-1; Dagan Instruments) was employed to amplify pipette potential (2000–5000 ×), which was passband-filtered (0.1–2 kHz), digitized (10 kHz sample rate), and recorded to a computer (Powerlab 8/30 and LabChart 7 software; AD Instruments). To identify individual chemoafferent neurons under baseline conditions (21% O_2, 5% CO_2, balance N_2), the carotid body was stimulated (~200 µA × 0.05 ms pulse duration) at 0.5–1 Hz (Isostim A320; World Precision Instruments) using a glass electrode (filled with 1 M NaCl). If a stimulus elicited an orthodromic action potential and the unit exhibited spontaneous activity, the stimulator was turned off and activity was recorded for 2 min under baseline conditions followed by 2 min in acute hypoxia (5% O_2, 5% CO_2, balance N_2). One recording was made for each carotid body-petrosal ganglion complex. Single-unit chemoreceptor activity was discriminated off-line (Spike Histogram Module v.2; ADInstruments). The number of action potentials was counted for the final 1 min of baseline and in 1-s bins throughout the duration of acute hypoxia. The peak response to hypoxia was determined from a 3-s moving average of action potential frequency.

2.5. Carotid body histology

Rats were euthanized with CO_2 at P14 (Experiment #1) or P13-14 (Experiment #2) and then decapitated for dissection of the carotid bifurcations. The bifurcations were then fixed with 4% paraformaldehyde in 0.1 M phosphate buffered saline (PBS; pH 7.4) for at least 1 h, cyoprotected in 30% sucrose in PBS (24 h at 4 °C), and embedded in Tissue-Tek O.C.T. compound (Sakura Finetek). Once embedded, samples were flash frozen on dry ice and stored at -80 °C. Samples were sectioned at 12 µm with a cryostatic microtome, mounted onto slides, and stained with hematoxylin and eosin. The carotid bifurcations were imaged with a Nikon Eclipse 80i microscope attached to a 2 megapixel camera and NIS Elements Software. Carotid body area within each section was calculated using Image J software (version 1.43 u; National Institutes of Health). Carotid body volume was calculated from the area of the carotid body in each section, section thickness, and total number of sections containing the carotid body.

2.6. Lung mass and blood hemoglobin concentration

To confirm that the brief hypoxic stimulus employed in this study was physiologically relevant, we assessed whether intermittent hypercapnic hypoxia was sufficient to produce increases in lung mass and hemoglobin concentration as observed after chronic sustained hypoxia (e.g., Mortola et al., 1986; Okubo and Mortola, 1988). Following ventilation or metabolism measurements, or while harvesting carotid bodies for electrophysiology, a subset of individuals were deeply anesthetized with CO_2 and decapitated. Blood was sampled from the severed neck vessels and immediately analyzed for hemoglobin concentration (Hb 201^+ analyzer, HemoCue). Lungs were dissected free and rinsed in saline. After removing upper airways and vessels, the lungs were dried for 48 h at 85–90 °C and weighed to the neared 0.0001 g. Since lungs were not perfused prior to drying, differences in residual blood mass (i.e., due to changes in hematocrit and/or hemoglobin concentration) may contribute to observed differences in lung mass among treatment groups.

2.7. Statistical analysis

Changes in ventilation or metabolism in hypoxia were compared among treatment groups using two-way repeated measures ANOVA (factor 1: developmental treatment, factor 2: time / level of inspired O_2) followed by Student-Newman-Keuls *post hoc* tests. Due to the non-normal distribution of the data, baseline carotid body activity was compared among groups using Kruskal-Wallis ANOVA on Ranks followed by Dunn's *post hoc* tests. Comparisons of all other variables among treatment groups were made using one-way ANOVA followed by Tukey's *post hoc* tests. Preliminary analysis showed that male and female rats had similar body sizes and ventilatory responses, so data were pooled across sexes to increase statistical power. It was necessary to transform some data to meet the equal variance assumption and/or normality assumptions for parametric tests prior to running the appropriate ANOVA (i.e., square root transformation for HCVR in Experiment #1 and HVR in Experiment #2; logarithmic transformation for carotid body size in Experiment #1). All statistical tests were performed using SigmaStat 4.0 (Systat Software) with significance determined at $P \leq 0.05$. Values are presented as mean ± SEM.

3. Results

3.1. Experiment #1: background 21% O_2

3.1.1. Body mass

Body mass data were pooled for individuals from the ventilation, metabolism, and electrophysiology studies (Table 1). There were statistically significant differences among treatment groups (Treatment, $P < 0.001$). IH_{21} weighed 6% less and IHH_{21} weighed 19% less than Control rats ($P = 0.03$ and $P < 0.001$, respectively).

3.1.2. Ventilation

Baseline minute ventilation was similar among Control, IH_{21}, and IHH_{21} treatment groups (Fig. 2A) despite a slightly greater tidal volume in IHH_{21} rats at both levels of inspired O_2 (main effect for Treatment, $P = 0.003$) (Table 2). All groups exhibited a sustained increase in ventilation in 12% O_2; although tidal volume decreased between the 1st and 10th minute of hypoxia when pooled across treatment groups (Time, $P < 0.001$), this was compensated by a nonsignificant trend toward increased respiratory frequency over the same period. The magnitude and shape of the HVR was similar among groups when expressed either in absolute units (Treatment, $P = 0.11$ and Treatment × Time, $P = 0.60$) or as a percentage increase from baseline (Treatment,

Table 1
Body mass for P13-14 rats pooled across ventilation, metabolism, and electrophysiology studies.

Treatment	# litters	# individuals	Mass (g)
Background 21% O_2			
Control	7	50	30.6 ± 0.4 ^{A}
IH_{21}	6	43	28.9 ± 0.5 ^{B}
IHH_{21}	6	44	24.8 ± 0.6 ^{C}
Background 30% O_2			
Control	5	37	30.6 ± 0.5 ^{A}
SH	5	42	30.2 ± 0.4 ^{A}
IH_{30}	4	37	27.8 ± 0.4 ^{B}
IHH_{30}	4	39	26.0 ± 0.6 ^{C}

Groups with different letters are significantly different ($P < 0.05$).

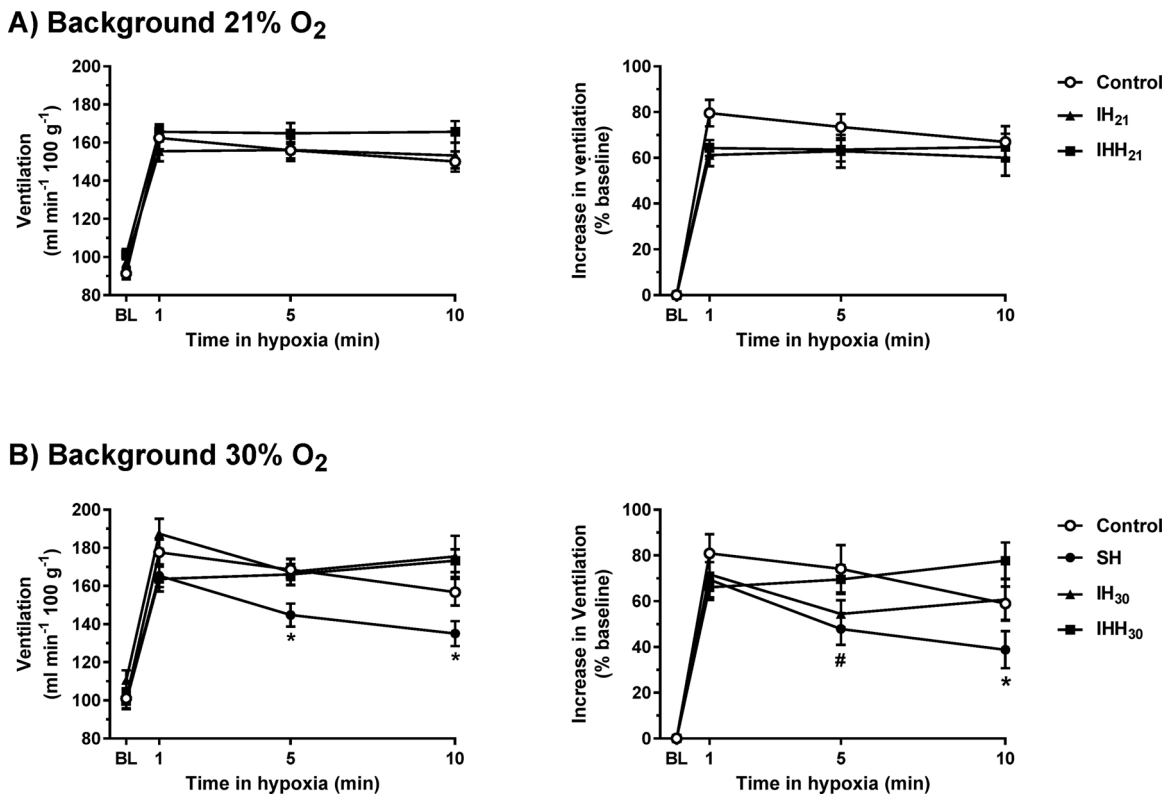

Fig. 2. Hypoxic ventilatory responses for (A) Control (n = 24), IH_{21} (n = 19), and IHH_{21} (n = 23) rats from Experiment #1 and (B) Control (n = 18), SH (n = 18), IH_{30} (n = 20), and IHH_{30} (n = 19) rats from Experiment #2; see Fig. 1 for a description of the treatment groups. Ventilation was measured in 21% O_2 (baseline; BL) and during a 10 min exposure to 12% O_2 and is expressed in raw units (*left* panels) and as a percentage increase from baseline (*right* panels). Values are means ± SEM. Where the treatment × time interaction term was significant, * $P < 0.05$ vs. all other treatment groups at the same time point and # $P < 0.05$ vs. Control and IHH_{30} groups at the same time point.

$P = 0.18$ and Treatment × Time, $P = 0.39$) (Fig. 2A).

In contrast, the HCVR was significantly lower in IH_{21} compared to Control rats (Treatment, $P = 0.037$) (Fig. 3A). Whereas Control rats increased ventilation by 104% when acutely exposed to 5% CO_2, IH_{21} rats increased ventilation by only 79% ($P = 0.045$ vs. Control). A similar trend toward a reduced HCVR was observed for IHH_{21} rats, but this did not reach statistical significance ($P = 0.12$ vs. Control).

3.1.3. Metabolic responses to hypoxia

Metabolic O_2 consumption varied among treatment groups (main effect for Treatment, $P < 0.001$). Across both inspired O_2 levels, IH_{21} and IHH_{21} groups exhibited 21–22% greater O_2 consumption rates than Control (both $P < 0.001$) (Fig. 4A). All three treatment groups responded to 12% O_2 by decreasing O_2 consumption (main effect for Time, $P < 0.001$), and the magnitude of this response was similar across groups (approximately 17% reduction; Treatment × Time, $P = 0.10$).

The respiratory exchange ratio (RER; i.e., CO_2 production / O_2 consumption) was significantly lower in IH_{21} rats than in either of the other groups during normoxia (0.70 ± 0.01 vs. 0.73 ± 0.01 in Control and IHH_{21}) and during hypoxia (0.76 ± 0.01 vs. 0.79 ± 0.01 in Control and 0.80 ± 0.02 in IHH_{21}) (Treatment, $P < 0.001$). The increase in RER during hypoxia was significant (Time, $P < 0.001$) but did not differ among groups (Treatment × Time, $P = 0.99$)

Table 2
Respiratory frequency and tidal volume during baseline (BL; 21% O_2) and during the 1st, 5th, and 10th minutes of hypoxia (12% O_2) for P13-14 rats.

		Respiratory Frequency (breaths min^{-1})				Tidal Volume (ml $100\,g^{-1}$)			
Treatment	n	BL	1 min	5 min	10 min	BL	1 min	5 min	10 min
Background 21% O_2									
Control	24	127 ± 3	174 ± 5	174 ± 4	179 ± 6	0.71 ± 0.02 ^{A}	0.93 ± 0.02 ^{A}	0.90 ± 0.02 ^{A}	0.84 ± 0.02 ^{A}
IH_{21}	19	144 ± 4	184 ± 5	179 ± 5	185 ± 6	0.68 ± 0.02 ^{A}	0.85 ± 0.02 ^{A}	0.88 ± 0.03 ^{A}	0.83 ± 0.03 ^{A}
IHH_{21}	23	137 ± 3	175 ± 4	172 ± 4	178 ± 4	0.75 ± 0.02 ^{B}	0.96 ± 0.02 ^{B}	0.97 ± 0.03 ^{B}	0.93 ± 0.03 ^{B}
Background 30% O_2									
Control	18	137 ± 5	192 ± 6	197 ± 8	193 ± 10	0.74 ± 0.03 ^{A}	0.93 ± 0.03 ^{A}	0.87 ± 0.03 ^{A}	0.82 ± 0.03 ^{AB}
SH	18	141 ± 6	187 ± 6	177 ± 6	174 ± 7	0.72 ± 0.03 ^{A}	0.89 ± 0.03 ^{A}	0.83 ± 0.03 ^{A}	0.78 ± 0.03 ^{A}
IH_{30}	20	146 ± 4	191 ± 6	188 ± 7	195 ± 7	0.76 ± 0.03 ^{A}	0.98 ± 0.03 ^{A}	0.90 ± 0.02 ^{A}	0.90 ± 0.04 ^{B}
IHH_{30}	19	141 ± 4	185 ± 5	187 ± 5	194 ± 5	0.71 ± 0.02 ^{A}	0.89 ± 0.03 ^{A}	0.89 ± 0.03 ^{A}	0.90 ± 0.03 ^{B}

Groups with different letters are significantly different ($P < 0.05$). For experiment #1 (Background 21% O_2), tidal volume differed across all time points (i.e., main effect for Treatment). There was a significant Treatment × Time interaction for tidal volumes in experiment #2 (Background 30% O_2), so pairwise comparisons between treatment groups are made separately at each time point. Comparisons across time are not shown for clarity (see text).

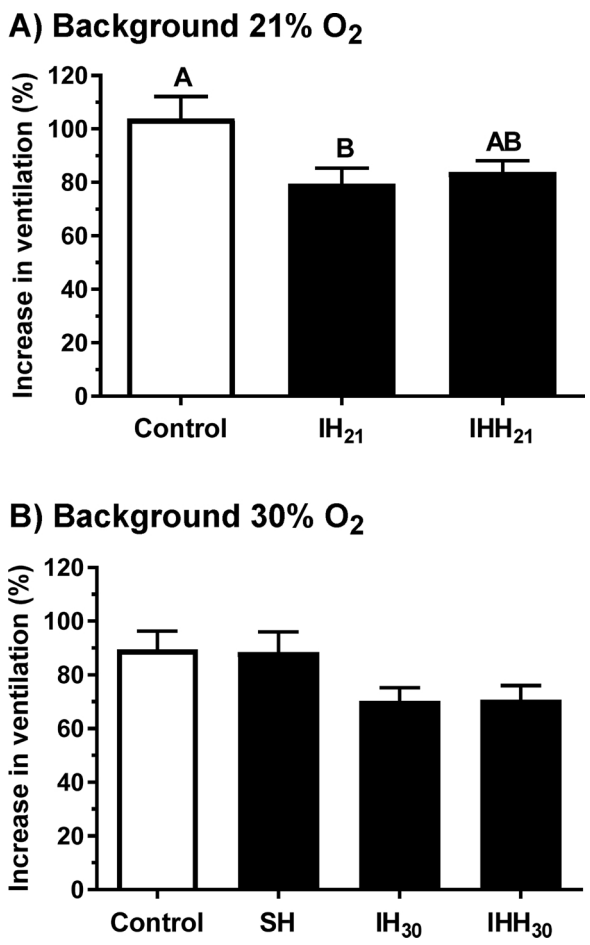

Fig. 3. Hypercapnic ventilatory responses (5% CO_2) for (A) Control (n = 24), IH_{21} (n = 18), and IHH_{21} (n = 23) rats and (B) Control (n = 18), SH (n = 18), IH_{30} (n = 20), and IHH_{30} (n = 19) rats. Values are means ± SEM. In panel A, groups with different letters are significantly different ($P < 0.05$). In panel B, *post hoc* testing did not identify which groups differed despite a significant overall treatment effect ($P = 0.038$).

3.1.4. Carotid responses to hypoxia

Baseline (21% O_2) and hypoxic (5% O_2) carotid chemoafferent activity both varied among groups (Treatment, $P = 0.001$ and 0.04, respectively) (Fig. 5A). Specifically, median baseline discharge rate was approximately 4-fold greater in IH_{21} rats than in the Control and IHH_{21} groups ($P = 0.007$ and 0.004, respectively) (Fig. 5A, *left* panel). When acutely exposed to 5% O_2, peak activity was slightly lower in the IHH_{21} group compared to the Control group ($P = 0.045$) (Fig. 5A, *right* panel); a similar trend was observed for IH_{21} rats but it did not reach statistical significance ($P = 0.12$). However, given the differences in baseline activity among treatment groups, we also compared carotid body responses calculated as the change in discharge rate from baseline (Treatment, $P = 0.003$). When expressed in this manner, hypoxic responses were significantly attenuated in both IHH_{21} ($\Delta = 8.8 \pm 0.6$ impulses s^{-1}; $P = 0.01$) and IH_{21} ($\Delta = 8.4 \pm 0.8$ impulses s^{-1}; $P = 0.005$) compared to Control ($\Delta = 12.2 \pm 1.0$ impulses s^{-1}).

3.1.5. Carotid body size

Mean carotid body size nearly doubled (+93%) in IH_{21} rats compared to Control (Fig. 6A). Carotid body size was variable in the IH_{21} group, so it was necessary to transform the data prior to running parametric ANOVA (i.e., unequal variance among groups). This analysis detected an overall treatment effect ($P = 0.01$), with carotid body size being significantly larger in IH_{21} than in Control ($P = 0.01$). Although carotid body sizes were similar between Control and IHH_{21} rats, the comparison between IH_{21} and IHH_{21} rats did not quite reach statistical significance ($P = 0.053$).

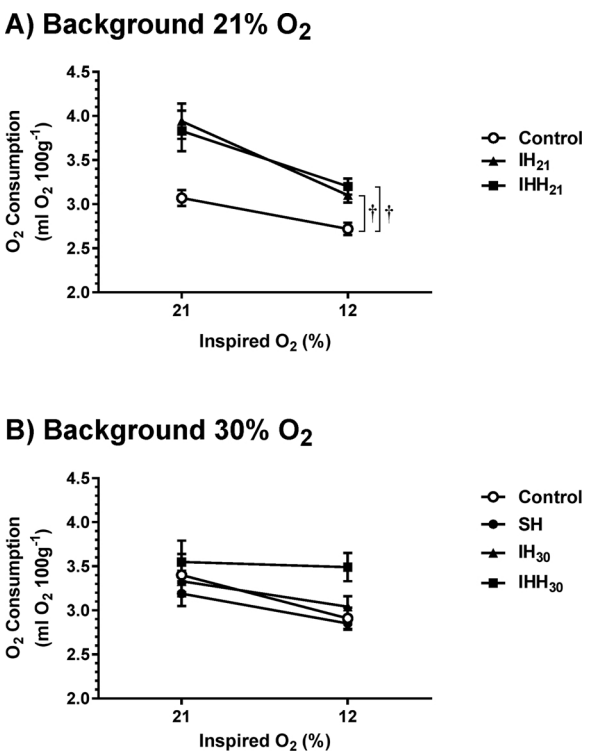

Fig. 4. Metabolic responses to hypoxia for (A) Control (n = 18), IH_{21} (n = 15), and IHH_{21} (n = 13) rats and (B) Control (n = 8), SH (n = 9), IH_{30} (n = 8), and IHH_{30} (n = 9) rats. O_2 consumption (mean ± SEM) was measured in 21% O_2 and 12% O_2. † $P < 0.05$ vs. Control (*post hoc* test for main effect of treatment).

3.1.6. Lung mass and hemoglobin concentration

IH_{21} and IHH_{21} treatments significantly increased lung mass (Treatment, $P < 0.001$) (Table 3). Mass-specific dry lung mass was 18% greater in IH_{21} rats ($P = 0.002$ vs. Control) and 15% greater in IHH_{21} rats ($P = 0.003$ vs. Control). No difference was detected between the IH_{21} and IHH_{21} groups. We had not previously studied the effects of developmental hyperoxia on lung mass, so we opportunistically harvested lungs from 22 rats that were reared from the day prior to birth through P14 in 60% O_2 in a concurrent study in our lab; these samples were processed alongside the samples from this study. Dry lung mass was 18% less in these rats than in the Control group (3.04 ± 0.04 vs. 3.69 ± 0.10 mg g^{-1}; unpaired t-test, $P < 0.001$). Thus hypoxia and hyperoxia had opposite effects on lung mass.

IH_{21} and IHH_{21} treatments also significantly increased blood hemoglobin concentration (Treatment, $P = 0.003$) (Table 3). Hemoglobin concentrations increased 12–13% in rats from both groups ($P = 0.007$ and 0.013 vs. Control, respectively). Again, no difference was detected between the IH_{21} and IHH_{21} groups. We previously showed that sustained 60% O_2 decreases hemoglobin concentration in neonatal rats (Bavis et al., 2014).

3.2. Experiment #2: background 30% O_2

3.2.1. Body mass

Body mass differed among treatment groups at P13-14 (Treatment, $P < 0.001$) (Table 1). Although Control and SH rats had similar body mass, IH_{30} weighed 9% less and IHH_{30} weighed 15% less than Control rats (both $P < 0.001$, respectively). IHH_{30} rats also weighed slightly less than IH_{30} rats ($P = 0.049$).

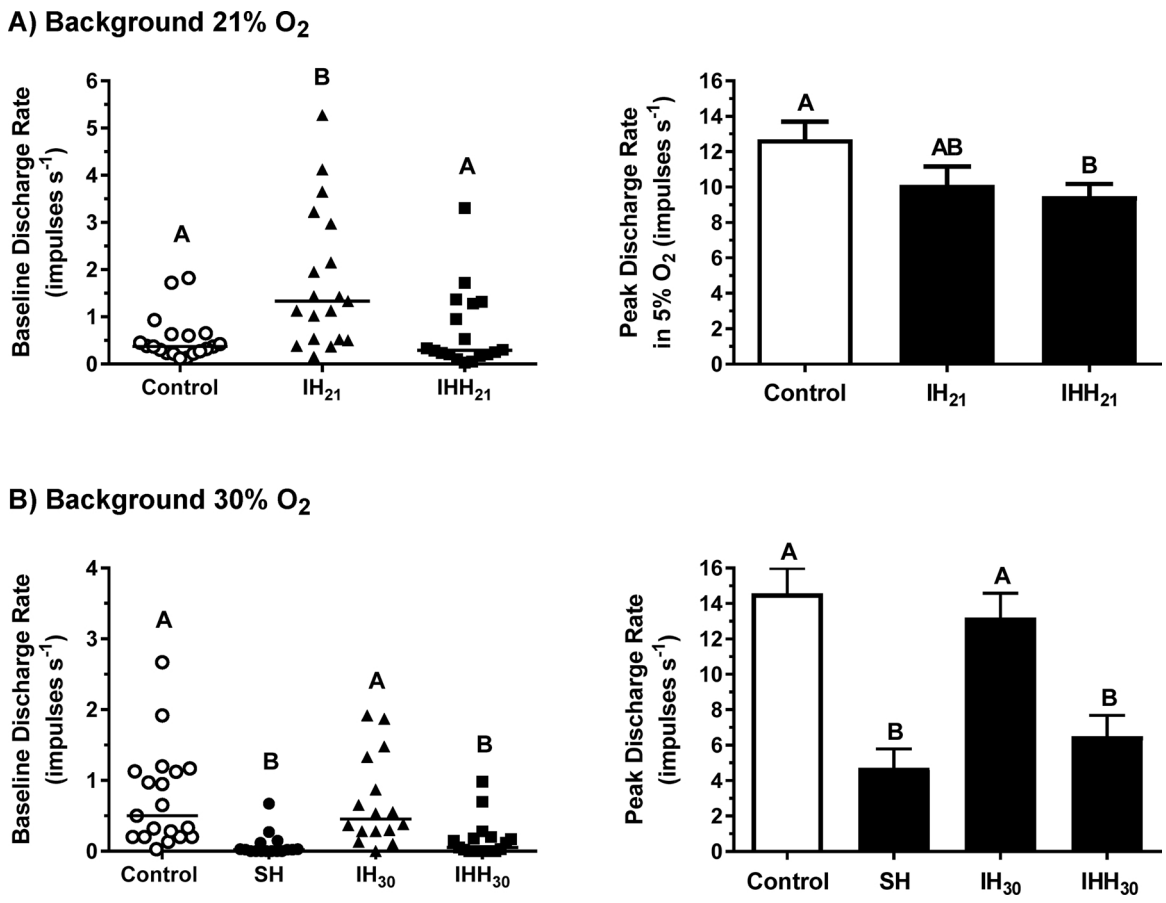

Fig. 5. Single-unit chemoafferent activity recorded from carotid bodies *in vitro*. *Left* panels show spontaneous activity under baseline conditions (21% O_2); each data point represents one chemoafferent unit and horizontal lines represent median values. Note the difference in y-axis scales. *Right* panels show the peak activity (mean ± SEM) observed in response to 5% O_2. Sample sizes are (A) 19 Control, 19 IH_{21}, 18 IHH_{21} and (B) 19 Control, 16 SH, 16 IH_{30}, 17 IHH_{30}. Groups with different letters are significantly different ($P < 0.05$).

3.2.2. Ventilation

There was a significant effect of developmental treatment on the HVR of P13-14 rats (Treatment × Time, $P < 0.001$) (Fig. 2B, *left* panel). Although no differences were detected among groups under baseline (21% O_2) conditions, minute ventilation for SH rats during 12% O_2 exposure was significantly reduced compared to the Control, IH_{30}, and IHH_{30} groups after 5 min ($P = 0.049$, 0.039, and 0.019, respectively) and after 10 min ($P = 0.016$, < 0.001, and < 0.001, respectively). Neither IH_{30} nor IHH_{30} differed from the Control group. The lower ventilation of SH rats during hypoxia appeared to reflect both lower tidal volume and lower respiratory frequency (Table 2), but only the tidal volume response differed significantly among groups (Treatment × Time, $P = 0.003$). Specifically, SH rats had lower tidal volumes in the 10^{th} minute of hypoxia than IH_{30} and IHH_{30} rats. With the exception of IHH_{30}, all groups showed a mildly biphasic HVR owing to a decrease in tidal volume between the 1^{st} and 10^{th} minutes of hypoxia; however, ventilation remained well above baseline in all groups throughout the hypoxic challenge (Fig. 2B).

Expressing the hypoxic ventilatory response as a percentage increase from baseline yielded similar results (Fig. 2B, *right* panel): the magnitude of the HVR varied among treatment groups (Treatment × Time, $P < 0.001$), but SH was the only group that differed from Control. Specifically, the ventilatory response was reduced in SH rats compared to Control and IHH_{30} after 5 min for hypoxia ($P = 0.018$ and 0.009, respectively) and was reduced compared to all three other groups (Control, IH_{30}, and IHH_{30}) after 10 min ($P = 0.011$, 0.015, and < 0.001, respectively).

The HCVR (5% CO_2) was also affected by developmental exposure (Treatment, $P = 0.038$) (Fig. 3B). *Post hoc* testing failed to identify which treatment groups differed ($P > 0.10$ for all pairwise comparisons), but visual inspection of Fig. 3B reveals that Control and SH rats had similar responses (~90% increase in ventilation) whereas IH_{30} and IHH_{30} rats had somewhat lower responses (only ~70% increase in ventilation).

3.2.3. Metabolic responses to hypoxia

Metabolic O_2 consumption was similar among treatment groups (main effect for Treatment, $P = 0.08$) (Fig. 4B). All groups exhibited a decrease in O_2 consumption during hypoxia (Time, $P = 0.002$). The magnitude of the hypometabolic response did not differ significantly among the groups (Treatment × Time, $P = 0.40$), although there appeared to be a trend toward a blunted response in IHH_{30} rats (Fig. 4B).

The respiratory exchange ratio (RER) was similar among treatment groups and increased to a similar extent in hypoxia (Treatment, $P = 0.157$, Treatment × Time, $P = 0.180$). RER was 0.71 ± 0.01 in Control, 0.69 ± 0.02 in SH, 0.72 ± 0.02 in IH_{30}, and 0.72 ± 0.02 in IHH_{30} in normoxia, and increased to 0.76 ± 0.01, 0.81 ± 0.01, 0.83 ± 0.03, and 0.82 ± 0.02, respectively (Time, $P < 0.001$).

3.2.4. Carotid chemoafferent responses to hypoxia

Baseline (21% O_2) and hypoxic (5% O_2) carotid chemoafferent activity both varied among groups (Treatment, both $P < 0.001$) (Fig. 5B). Baseline carotid body activity was very low in SH and IHH_{30} rats when measured *in vitro* (Fig. 5B, *left* panel), with median discharge frequencies of only 0.02 and 0.05 impulses s^{-1}, respectively. This activity was much lower than in the Control and IH_{30} groups (~0.5

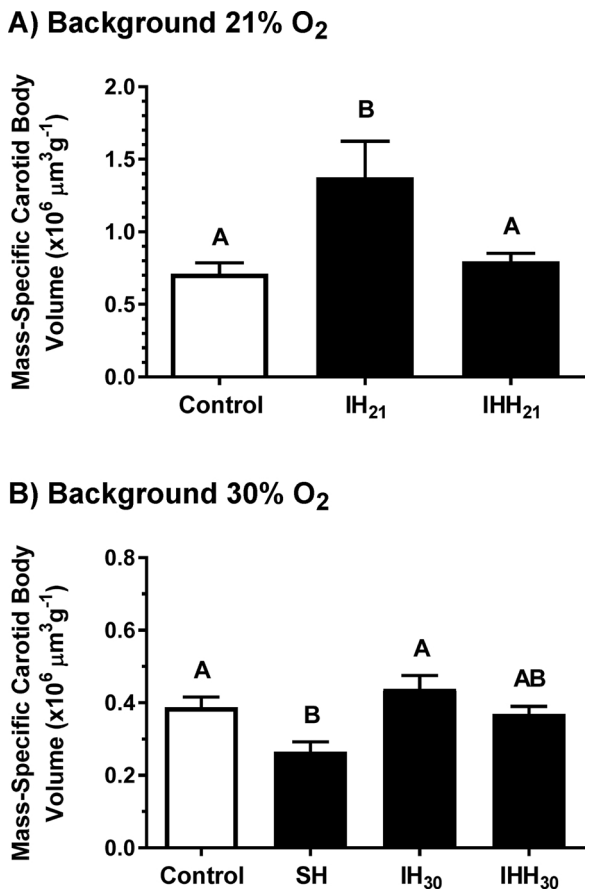

Fig. 6. Carotid body volumes for (A) Control (n = 9), IH_{21} (n = 9), and IHH_{21} (n = 10) rats and (B) Control (n = 12), SH (n = 10), IH_{30} (n = 11), and IHH_{30} (n = 12) rats. Note the difference in y-axis scales. Values are means ± SEM. Groups with different letters are significantly different ($P < 0.05$).

Table 3
Lung mass and blood hemoglobin concentration for P13-14 rats.

Treatment	n	Mass-specific Dry Lung Mass ($mg\,g^{-1}$)	n	Hemoglobin ($g\,dl^{-1}$)
Background 21% O_2				
Control	20	3.69 ± 0.10 ^{A}	15	8.2 ± 0.1 ^{A}
IH_{21}	20	4.36 ± 0.16 ^{B}	14	9.3 ± 0.2 ^{B}
IHH_{21}	31	4.26 ± 0.10 ^{B}	15	9.2 ± 0.2 ^{B}
Background 30% O_2				
Control	24	3.58 ± 0.10 ^{A}	19	7.7 ± 0.1 ^{A}
SH	28	3.19 ± 0.08 ^{B}	28	7.6 ± 0.1 ^{A}
IH_{30}	22	4.38 ± 0.14 ^{C}	16	8.5 ± 0.1 ^{B}
IHH_{30}	24	4.31 ± 0.11 ^{C}	23	8.4 ± 0.1 ^{B}

Groups with different letters are significantly different ($P < 0.05$).

impulses s^{-1} in both groups). Peak activity in 5% O_2 was also reduced in SH and IHH_{30} rats (both $P < 0.001$ vs. Control), with mean discharge rates less than half of those observed in Control and IH_{30} rats (Fig. 5B, *right* panel). Peak activity was similar between Control and IH_{30} rats ($P = 0.87$).

Expressing the hypoxic response as the change in carotid chemoafferent activity between baseline and 5% O_2 produced similar results (Treatment, $P < 0.001$): Control and IH_{30} rats had strong hypoxic responses (Δ = 13.8 ± 1.4 and 12.5 ± 1.4 impulses s^{-1}, respectively) whereas SH and IHH_{30} rats exhibited relatively weak responses (Δ = 4.6 ± 1.1 and 6.3 ± 1.1 impulses s^{-1}, respectively). Only the responses of SH and IHH_{30} rats were significantly different from Control (both $P < 0.001$).

3.2.5. Carotid body size

Carotid body size varied among treatment groups (Treatment, $P = 0.002$) (Fig. 6B). SH rats had significantly smaller carotid bodies than either Control rats ($P = 0.026$) or IH_{30} rats ($P = 0.001$). On average, SH carotid bodies were 32% smaller than in the age-matched Control group; neither IH_{30} nor IHH_{30} rats differed significantly from Control.

3.2.6. Lung mass and hemoglobin concentration

Developmental treatment affected both lung mass and blood hemoglobin concentration (Treatment, both $P < 0.001$) (Table 3). Mass-specific dry lung mass was 11% lower in SH rats ($P = 0.047$) and approximately 20% heavier in IH_{30} and IHH_{30} rats (both $P < 0.001$) than Control. Hemoglobin concentration was approximately 10% greater in IH_{30} ($P = 0.001$) and IHH_{30} rats ($P = 0.004$) compared to Control. However, hemoglobin concentration did not differ between Control and SH rats.

4. Discussion

Chronic intermittent hyperoxia from birth decreases carotid body size and attenuates the HVR in neonatal rats (Logan et al., 2016). The present study considered whether concurrent exposure to intermittent hypercapnic hypoxia would alter this plasticity. Although hypercapnic hypoxia might oppose the effects of hyperoxia on the developing carotid body (e.g., Peng et al., 2004; Julien et al., 2008, 2011), other studies suggest that intermittent hypercapnic hypoxia might further impair the HVR (e.g., Waters et al., 1997; Reeves and Gozal, 2006b; Reeves et al., 2006). Therefore, the intermittent hyperoxia protocol from Logan et al. (2016) was modified to include brief periods of hypercapnic hypoxia between bouts of hyperoxia. Neonatal rats exposed to these alternating bouts of intermittent hypercapnic hypoxia and intermittent hyperoxia exhibited diminished carotid body activity, particularly when this treatment was superimposed onto a relatively hyperoxic background (i.e., O_2 levels cycling around 30% O_2 rather than room air). However, intermittent hypercapnic hypoxia prevented chronic hyperoxia from decreasing carotid body size and preserved the HVR. In contrast, neonatal rats exposed continuously to 30% O_2 exhibited blunted HVR associated with reductions in carotid body size and carotid body activity. Overall, it appears that intermittent hypercapnic hypoxia counteracted the effects of intermittent hyperoxia and largely prevented developmental plasticity in the HVR.

It is interesting that neonatal rats with diminished carotid body O_2 sensitivity but normal carotid body sizes (i.e., IHH_{21} and IHH_{30}) retained normal HVR. The lack of a blunted HVR cannot be explained by altered metabolic responses to hypoxia since hypoxic hypometabolism was similar among treatment groups. Instead, there may be sufficient redundancy in the carotid body and/or its chemoafferent pathways to compensate for modest reductions in chemoafferent input. For example, acute, unilateral carotid body denervation (i.e., 50% reduction in chemoafferent input) may not alter the HVR even though bilateral carotid body denervation nearly abolishes the HVR (Busch et al., 1983; Cragg and Khrisanapant, 1994). Thus, more dramatic decreases in carotid body O_2 sensitivity, carotid body hypoplasia, or a combination of both (e.g., SH rats) may be required to reduce the HVR measurably.

Rats in the IHH_{21} and IH_{30} treatment groups experienced similar O_2 exposures (oscillating between 10% O_2 and 30% O_2), except that the IHH_{21} rats spent a portion of each cycle steady at 21% O_2 and thus less total time at 30% O_2 (Fig. 1). It is surprising, therefore, that the IHH_{21} treatment diminished carotid body responses to hypoxia while IH_{30} did not. The reduction in chemoafferent activity was small, so perhaps a similar trend in the IH_{30} group was missed in this sample. All other morphological and physiological measurements were similar between the IHH_{21} and IH_{30} groups.

4.1. Interaction between intermittent hypercapnic hypoxia and intermittent hyperoxia

Concurrent exposure to intermittent hypercapnic hypoxia substantially reduced the effects of hyperoxia on the HVR of neonatal rats. This likely reflects the fact that hypoxia and hyperoxia have opposite effects on many cellular processes. There are several proximate mechanisms by which chronic hyperoxia could influence carotid body development, including hyperoxia-induced carotid body inactivity (i.e., less frequent depolarization of glomus cells and afferent neurons at higher PO_2), oxidative stress, and altered expression of O_2-sensitive genes or gene products (reviewed in Bavis et al., 2013). Studies using dietary antioxidant supplementation failed to prevent hyperoxia-induced carotid body plasticity and thus do not support the oxidative stress hypothesis (Bavis et al., 2008; Prieto-Lloret et al., 2015). We cannot rule out important changes in O_2-dependent gene expression, but the results of the present study are consistent with the first hypothesis: intermittent hypercapnic hypoxia should have increased carotid body activity between bouts of hyperoxia, and this may have normalized activity-dependent developmental pathways. Similarly, Bavis et al. (2007) reared rats in continuous 60% O_2 for the first two postnatal weeks but superimposed intermittent hypercapnia (alternating 1-h bouts of 0% and 7.5% CO_2). Although breathing was not measured in neonates in the earlier study, the adult HVR was less impaired in rats that received intermittent hypercapnia during developmental hyperoxia than in those reared in hyperoxia alone (Bavis et al., 2007). Since the rats were not exposed to hypoxia, the protective effect of intermittent hypercapnia cannot be explained by changes in O_2-dependent gene expression. In contrast, periodic activation of the developing carotid bodies (by hypercapnic hypoxia or hypercapnia) can explain the results of both Bavis et al. (2007) and the present study.

Hyperoxia-induced carotid body plasticity may reflect, at least in part, abnormal expression of neurotrophic factors that regulate cell growth, proliferation, and survival. The carotid body expresses many neurotrophic factors, and brain-derived neurotrophic factor (BDNF) in particular is critical to carotid body growth and the survival of carotid chemoafferent neurons during early postnatal development (Hertzberg et al., 1994; Erickson et al., 2001; Bavis et al., 2015). The transcription, translation, and release of BDNF are regulated in an activity-dependent manner in many neurons (Brady et al., 1999; Balkowiec and Katz, 2000; Lau et al., 2010), providing a potential mechanism for developmental hyperoxia to alter carotid body BDNF levels. Indeed, chronic hyperoxia reduces BDNF protein levels in the carotid body and nucleus tractus solitarius (nTS), the central synapse for carotid chemoafferents, in neonatal rats (Dmitrieff et al., 2011; Chavez-Valdez et al., 2012). On the other hand, acute hypoxia increases BDNF mRNA expression in the nTS (Chavez-Valdez et al., 2012), presumably reflecting increased carotid chemoafferent activity; this might explain the protective effect of intermittent hypercapnic hypoxia on carotid body growth in the hyperoxic rat pups.

Although the opposing effects of hyperoxia and hypercapnic hypoxia on carotid body activity offer a compelling explanation for the protective effect of intermittent hypercapnic hypoxia on carotid body development and the HVR, it is also possible that intermittent hypercapnic hypoxia elicits compensatory CNS plasticity. Chronic intermittent hypoxia (Ling et al., 2001) and chronic sustained hypoxia (Dwinell and Powell, 1999) both increase the gain of the CNS response to carotid chemoafferent input in adult rats. If the same is true in neonatal rats, the increased CNS gain could yield relatively normal respiratory motor output despite less responsive carotid bodies or any potential loss of O_2-sensitive glomus cells / chemoafferent neurons. However, intermittent hypercapnic hypoxia alone (i.e., IH_{21}) did not enhance the HVR (see section 4.2, below), so there is no evidence for increased CNS gain in the present study.

4.2. Developmental exposure to intermittent hypercapnic hypoxia

Chronic intermittent hypoxia has been shown previously to elicit respiratory plasticity in neonatal and adult rats, often increasing the HVR through a combination of increased carotid body activity and increased CNS gain (e.g., Ling et al., 2001; Peng et al., 2004; Julien et al., 2008, 2011). Moreover, neonatal rats appear to be more susceptible to chronic intermittent hypoxia than adults. Pawar et al. (2008) noted that (1) carotid body sensitization is easier to elicit in neonates (i.e., observed after fewer hypoxic episodes), (2) chronic intermittent hypoxia promotes carotid body hyperplasia in neonates but not in adult rats, and (3) carotid body O_2 sensitivity remains enhanced at least 50 days after neonates are returned to normoxia but recovers in less than 10 days in adults. Similarly, there is at least some evidence that chronic intermittent hypercapnia alone can increase the HVR of neonatal rats (Steggerda et al., 2010); the mechanisms underlying this effect have not been studied.

In the present study, intermittent hypercapnic hypoxia caused changes in carotid body morphology and function but did not alter either normoxic or hypoxic ventilation. The most noticeable effect of chronic intermittent hypercapnic hypoxia was carotid body hypertrophy in the IH_{21} rats (93% increase in carotid body volume). In addition to these morphological changes, intermittent hypercapnic hypoxia increased baseline chemoafferent activity as previously reported after chronic intermittent hypoxia (Peng et al., 2004; Pawar et al., 2008, 2009). However, the increased baseline chemoafferent activity did not translate to increased activity in hypoxia. If anything, chronic intermittent hypercapnic hypoxia caused a modest decrease in the carotid body response to hypoxia; the peak response tended to be lower, but the reduction was only statistically significant when expressed as the change from baseline. In contrast, other groups have reported increased carotid body O_2 sensitivity (Peng et al., 2004; Pawar et al., 2008, 2009) or no change in sensitivity (Mayer et al., 2015) in neonatal rats treated with intermittent hypoxia.

There are several differences among studies that likely contribute to these divergent results. First of all, the present study administered less frequent and less severe bouts of hypoxia than commonly used (5 cycles h^{-1}, nadir 10% O_2 vs. 9–12 cycles h^{-1}, nadir 5% O_2 in Pawar et al. (2008, 2009) and Mayer et al. (2015)). It is possible that frequent, severe O_2 fluctuations produce more reactive oxygen species (ROS), and ROS are critical to carotid body sensitization during intermittent hypoxia (Pawar et al., 2009). Our protocol also ran 24 h d^{-1} (vs. 8 h d^{-1}), so the cumulative time in hypoxia was greater. Interestingly, chronic sustained hypoxia diminishes carotid body responses to hypoxia in neonatal rats (Sterni et al., 1999; Carroll, 2003). This seems unlikely to explain the current results since intermittent hypercapnic hypoxia appeared to oppose, rather than exacerbate, the effects of developmental hyperoxia (see section 4.1). Finally, the presence of CO_2 (i.e., hypercapnic hypoxia vs. poikilocapnic, and thus hypocapnic, hypoxia) may alter the expression of respiratory plasticity as previously reported for other models of acute intermittent hypoxia (Mateika et al., 2018) and chronic intermittent hypoxia (Waters and Tinworth, 2001). Additional experiments would be required to explore this possibility.

All treatment groups that received intermittent hypercapnic hypoxia either had blunted HCVR (IH_{21}, IH_{30}, and IHH_{30}) or tended to have blunted HCVR (IHH_{21}); Waters and Tinworth (2001) reported reduced HCVR in piglets after cyclical hypercapnic hypoxia as well. This effect was independent of exposure to hyperoxia, which is not surprising: chronic sustained hyperoxia does not blunt the hypercapnic ventilatory response (Ling et al., 1996; Prieto-Lloret et al., 2004, 2015; Bavis et al., 2017). On the other hand, chronic sustained hypercapnia is known to blunt the HCVR in humans and other animals, including neonatal and adult rats (Lai et al., 1981; Rezzonico and Mortola, 1989; Bavis et al., 2006). This blunting is usually attributed to changes in H^+ buffering capacity reducing the effects of acute hypercapnia on pH (i.e., partial metabolic compensation), thus reducing the stimulus at central

chemoreceptors. Intermittent hypercapnia likely affects the HCVR through the same mechanism, but blood pH and bicarbonate concentrations were not measured here. Although relatively short daily exposures to intermittent hypercapnia did not alter the HCVR of neonatal rats in a previous study (only 18 cycles d^{-1}; Steggerda et al., 2010), more continuous exposures to intermittent hypercapnia (as employed in the present study) should be a stronger stimulus for metabolic compensation.

4.3. Critique of model

Developing clinically relevant models of intermittent hyperoxia and intermittent hypercapnic hypoxia is challenging given seemingly infinite potential combinations of O_2 levels, durations, and frequencies of bouts. The models used in the present study were developed by reviewing published examples of arterial O_2 saturation profiles experienced by preterm infants during routine O_2 therapy (Claure and Bancalari, 2009; Claure et al., 2009) and reports that infants often spend approximately 20% of their time hypoxemic and 30–40% of their time hyperoxemic relative to target O_2 saturations (Hagadorn et al., 2006; Claure and Bancalari, 2009); the models employed in the present study achieved comparable frequencies and cumulative durations of hypoxia and hyperoxia (Fig. 1). The specific ranges for inspired O_2 (i.e., 10 to 30–40% O_2) were similar to those used in retinopathy of prematurity studies to translate O_2 profiles observed clinically to rats (e.g., 8–30% O_2, Penn et al., 1995; 10 to > 40% O_2, Cunningham et al., 2000; 5–50% O_2, Winners-Mendizabal et al., 2014). Since hypoxia is usually accompanied by hypercapnia in clinical situations, and since hypoxia and hypercapnia can have synergistic effects on carotid body activity (e.g., Kumar and Bin-Jaliah, 2007), a hypercapnic stimulus (6% CO_2) was included during the hypoxic bouts. Relatively few studies have examined the respiratory effects of intermittent hypercapnia (or hypercapnic hypoxia) in developing rodents, but those few have used 5–7.5% CO_2 (e.g., Bavis et al., 2007; Douglas et al., 2010; Steggerda et al., 2010). Finally, intermittent exposures were presented continuously (24 h d^{-1}) rather than for only ≤8 h d^{-1} as commonly done in other studies. Although shorter periods of intermittent hypoxia are appropriate for modeling obstructive sleep apnea in adult animals, preterm infants experience intermittent hypercapnic hypoxemia and intermittent hyperoxemia around the clock (e.g., Jain et al., 2018). Given that the prevalence of hyperoxemia and hypoxemia can differ between the day and the night in clinical settings (Jain et al., 2018), it might be appropriate to incorporate circadian variation into the intermittent hyperoxia and intermittent hypercapnic hypoxia protocols used in future studies.

The duration of each bout of hypercapnic hypoxia was relatively short in our models, but it was sufficient to elicit systemic hypoxemia. We used a rodent pulse oximeter (MouseOx; Starr Life Sciences) to determine arterial O_2 saturation for six P14 rat pups (three IH_{21} and three IHH_{21}) while in the chronic exposure chamber, with each individual being exposed to four complete cycles of hypercapnic hypoxia. In this sample, the minimum O_2 saturation achieved during hypercapnic hypoxia averaged 84.8 ± 1.6% (vs. 98–99% while exposed to 21% O_2), with a lowest recorded value of 76.9%. Moreover, intermittent hypercapnic hypoxia increased lung mass and blood hemoglobin concentrations. Since both of these effects are also observed after chronic sustained hypoxia during development (e.g., Mortola et al., 1986; Okubo and Mortola, 1988), our model appears to have produced physiologically relevant hypoxia at the cellular level.

Neonatal rats are developmentally similar to preterm human infants in some respects (Darnall, 2010), but what constitutes “normoxia” differs; room air (21% O_2) represents a normal O_2 level for a newborn rat whereas it is relatively hyperoxic for a preterm infant (Carroll, 2003). Therefore, experiments were conducted twice in the present study: once with O_2 levels cycling from a background of 21% O_2 and once with O_2 levels cycling from a background of 30% O_2. Neither background (21% or 30% O_2) will perfectly represent conditions of preterm infants, but the goal was to bracket a realistic range of postnatal oxygenation. Importantly, the overall conclusions were similar between the two sets of experiments so the choice of background ultimately was not a factor.

4.4. Significance

Intermittent hyperoxemia is common in preterm and very low birth weight infants, but our data suggest that concurrent hypercapnic hypoxemia may (partially) protect the developing respiratory control system from maladaptive plasticity. This is an encouraging result, but this might not always be the case. Since hyperoxia and hypoxia are opposing stimuli that each can elicit plasticity, the outcome of combined exposures will likely reflect the frequency, duration, and magnitude of each. The greater the hyperoxic exposure (or lesser the hypercapnic hypoxic exposure), the more likely the balance will tip toward impaired carotid body function and diminished HVR. This tipping point may vary for different phenotypes. Indeed, the hypoxic stimulus seemed to “win out” over hyperoxia in the case of lung mass and hemoglobin concentration, but hyperoxia seemed to overcome hypoxia in the case of carotid chemoafferent activity and (to a lesser extent) carotid body size during experiments conducted in the 30% O_2 background.

This observation is particularly relevant given the tremendous variation in oxygenation observed in clinical settings within and across patients (e.g., Hagadorn et al., 2006; Claure and Bancalari, 2009; Claure et al., 2009). Whereas the exposures employed in the present study were modeled after “average” conditions reported for preterm infants on supplemental O_2 (e.g., hyperoxemic 30–40% of the time), some individuals experience greater cumulative durations of hyperoxemia (e.g., 90% of the time; Hagadorn et al., 2006) and arterial PO_2 can be quite high (e.g., 316 mmHg; Castillo et al., 2008). This could explain why some preterm infants exhibit reduced peripheral chemoreceptor function after receiving supplement O_2 (e.g., Katz-Salamon and Lagercrantz, 1994) and may continue to have blunted HVR as adults (Bates et al., 2014) despite their likely exposure to postnatal hypercapnic hypoxia.

Declarations of interest

None.

Acknowledgments

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