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Postnatal development of eupneic ventilation and metabolism in rats chronically exposed to moderate hyperoxia

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Abstract

Newborn rats chronically exposed to moderate hyperoxia (60% O₂) exhibit abnormal respiratory control, including decreased eupneic ventilation. To further characterize this plasticity and explore its proximate mechanisms, rats were exposed to either 21% O₂ (Control) or 60% O₂ (Hyperoxia) from birth until studied at 3 – 14 days of age (P3 – P14). Normoxic ventilation was reduced in Hyperoxia rats when studied at P3, P4, and P6-7 and this was reflected in diminished arterial O₂ saturations; eupneic ventilation spontaneously recovered by P13-14 despite continuous hyperoxia, or within 24 h when Hyperoxia rats were returned to room air. Normoxic metabolism was also reduced in Hyperoxia rats but could be increased by raising inspired O₂ levels (to 60% O₂) or by uncoupling oxidative phosphorylation within the mitochondrion (2, 4-dinitrophenol). In contrast, moderate increases in inspired O₂ had no effect on sustained ventilation which indicates that hypoventilation can be dissociated from hypometabolism. The ventilatory response to abrupt O₂ inhalation was diminished in Hyperoxia rats at P4 and P6-7, consistent with smaller contributions of peripheral chemoreceptors to eupneic ventilation at these ages. Finally, the spontaneous respiratory rhythm generated in isolated brainstem-spinal cord preparations was significantly slower and more variable in P3-4 Hyperoxia rats than in age-matched Controls. We conclude that developmental hyperoxia impairs both peripheral and central components of eupneic ventilatory drive. Although developmental hyperoxia diminishes metabolism as well, this appears to be a regulated hypometabolism and contributes little to the observed changes in ventilation.

Keywords

developmental plasticity; control of breathing; hypometabolism; perinatal hyperoxia; brainstem-spinal cord preparation

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1. Introduction

The developing respiratory control system often exhibits substantial functional plasticity in response to chronic environmental perturbations (Carroll, 2003; Bavis and Mitchell, 2008). Previous studies have demonstrated long-lasting attenuation of the hypoxic ventilatory response (HVR) in mammals exposed to moderate hyperoxia (30-60% O₂) during perinatal development (e.g., Ling et al., 1996; Fuller et al., 2002; Bavis et al., 2011a). This plasticity is linked to abnormal development of the carotid body, the organ which serves as the principal O₂ sensor for the respiratory control system (Bavis et al., 2013). Specifically, chronic hyperoxia inhibits postnatal growth of the carotid body (Erickson et al., 1998; Wang and Bisgard, 2005; Dmitrieff et al., 2012), causes degeneration of carotid chemoafferent neurons (Erickson et al., 1998; Chavez-Valdez et al., 2012), and diminishes carotid chemoreceptor O₂ sensitivity (Hanson et al., 1989; Bavis et al., 2011b; Kim et al., 2013). These phenotypic changes begin to appear by the fourth day of hyperoxia in rats (Donnelly et al., 2009; Bavis et al., 2011b; Dmitrieff et al., 2012), and the morphological plasticity may be permanent (Fuller et al., 2002).

Despite strong and consistent evidence for the impairment of hypoxic ventilation, the influence of chronic postnatal hyperoxia on eupneic ventilation has proven to be more variable. Several studies have examined the normoxic ventilation of adult rats that had been reared in hyperoxia (60% O₂) for the first 1-4 postnatal weeks and subsequently maintained in room air (i.e., after 1-4 months of normoxic recovery). Normoxic ventilation was similar to that of age-matched control rats in some groups of rats (Wenninger et al., 2006; Bavis et al., 2007, 2011a), while the rats in other studies exhibited a mild hyperpnea and/or hyperventilation (generally <15% increase relative to controls; Ling et al., 1996; Bavis et al., 2007, 2008). Normoxic minute ventilation also tends to be normal in adult mice reared in hyperoxia (Dauger et al., 2003; Bavis et al., 2011a), but the respiratory pattern was altered in the mice in one study (i.e., slower, deeper breathing pattern following postnatal exposure to 65% O₂; Dauger et al., 2003). In contrast to adult rats and mice, Bavis et al. (2010) found that neonatal rats exposed to hyperoxia from birth exhibit markedly reduced ventilation shortly after being returned to room air; this effect was most apparent in the youngest rats studied (–39% decrease relative to controls at 4 days of age (P4)) and diminished with age (i.e., no longer significantly different by P13-14). The decreased ventilation in these neonatal rats reflected both lower tidal volumes (at P4, P6-7, and P13-14) and lower respiratory frequencies (at P4 only). Hyperoxia-treated rats also had reduced metabolic rates, but the reduction in O₂ consumption appeared to be too small to account for the observed reduction in ventilation (Bavis et al., 2010).

In the present study, we first sought to confirm the observation that neonatal rats exposed to chronic hyperoxia hypoventilate shortly after return to room air through measurements of ventilation, metabolic rate, and arterial O₂ saturation (as a proxy for blood gases). To further characterize this plasticity, additional measurements were made to determine whether this hypoventilation persists during a period of normoxic “recovery” and whether blood O₂ transport is enhanced to compensate for diminished ventilation (e.g., increased blood O₂ carrying capacity and/or cardiac output). Finally, we investigated the mechanistic basis for the observed respiratory and metabolic plasticity. Since both the peripheral and central

nervous systems contribute to eupneic ventilatory drive (Dejours, 1963; Teppema and Dahan, 2010), we evaluated the peripheral and central contributions to the observed hypoventilation. Specifically, the ventilatory response to acute O₂ inhalation was used to probe peripheral chemoreceptor involvement and an *in vitro* brainstem-spinal cord preparation was used to assess spontaneous respiratory rhythm generation. A parallel set of experiments considered whether decreased O₂ consumption reflects O₂ limitation (secondary to inadequate alveolar ventilation) versus a state of regulated hypometabolism (e.g., Steiner and Branco, 2002; Mortola, 2004).

2. Methods

2.1 Experimental animals

Timed-pregnant Sprague-Dawley rats were obtained from Charles River Laboratories. For hyperoxic exposures ("Hyperoxia"), timed-pregnant dams were placed into a custom-built acrylic chamber maintained at 60% O₂, or into a Biospherix Oxycycler chamber maintained at 50-60% O₂ (for *in vitro* brainstem-spinal cord experiments, section 2.7), 1-2 days prior to parturition; gas flow rate through the chambers were adjusted to maintain CO₂ <0.4%. Individual Hyperoxia pups were kept with their mothers until studied between 3 and 14 days of age (i.e., between P3 and P14, where the day of birth = P0); the specific ages and sample sizes studied varied by experiment and are provided in the text and/or figure legends. Age-matched control litters ("Control") were reared in identical chambers maintained at 21% O₂ (CO₂ <0.4%). Chambers were opened briefly to remove animals for measurements and to clean cages as needed.

Control and Hyperoxia rat pups were typically studied within 15-20 min (brainstem-spinal cord experiments) or within 30-45 min (all other experiments) after removal from their respective chambers. In one experiment, Control and Hyperoxia litters were returned to the animal colony after ventilation measurements were made at P3 and subsequently housed in room air until re-studied at P4 and P6 (i.e., after 1 or 3 days of normoxic recovery).

Rats were housed under a 12:12 light:dark cycle and provided food and water *ad libitum* throughout the study. All experimental procedures were approved by the Institutional Animal Care and Use Committee at Bates College, or at the University of Wisconsin (brainstem-spinal cord experiments).

2.2. Ventilation measurements

Ventilation was measured using a custom-built head-body plethysmograph chamber, as previously described (Bavis et al., 2010). The chamber was separated into head and body compartments using a flexible collar; petroleum jelly was spread around the neck to seal any gaps in the collar. Test gases (21-99% O₂, balance N₂; see below) were delivered to the head compartment at 1 L/min using a gas mixing mass flow controller (MFC-4; Sable Systems) and valves (series 840; Sierra Instruments). Respiratory-induced airflows in and out of the body compartment were detected using a pneumotach coupled to a differential pressure transducer (MLT1L and ML141; ADInstruments); the pneumotach was calibrated prior to placing each animal into the plethysmograph by injecting 0.5 ml of air into the sealed body

compartment. Respiratory airflows were recorded to a computer at a sampling rate of 1000 Hz, integrated, and digitally filtered (high-pass, 0.1 Hz) to obtain respiratory volumes (PowerLab 8SP and LabChart 6 or 7 software; ADInstruments). Air temperature in the body compartment was monitored continuously with a T-type thermocouple probe (Physitemp Instruments) and maintained at 32–34°C (i.e., within the thermoneutral zone of neonatal rats; Malik and Fewell, 2003) with the aid of an incubator.

Two different experimental protocols were used. In the first set of experiments, ventilation was measured in Control rats (from 12 litters) and Hyperoxia rats (from 9 litters) at P4, P6–7, and P13–14; individuals were studied only once. After being sealed into the plethysmograph, rats were given 10–15 minutes to adjust to the chamber before commencing the ventilation measurements. During this adjustment period, rats were exposed to either 21% O₂ (approximately half of the Control group) or 60% O₂ (remainder of the Control group and all Hyperoxia rats); subsequent analysis revealed that inspired O₂ levels during this adjustment period had no effect on the ventilation of Control rats, so data for all Control rats were pooled. Once the rat appeared calm (based on stability of the respiratory pattern), respiratory airflows were recorded during exposures to 60% O₂ (10 min), 21% O₂ (10 min), and 99% O₂ (1 min), presented in that order; changeover of gases in the plethysmograph head chamber from 21% O₂ to 99% O₂ took approximately 30 sec. Ventilatory parameters were analyzed on a breath-by-breath basis and averaged over 45–60 sec at the end of the 60% O₂ and 21% O₂ exposures and over the final 10–15 sec of the 99% O₂ exposure, excluding movement artifacts and sighs; usable data were obtained for only a subset of rats during the 99% O₂ trials because of excessive movement artifacts during the final 10–15 seconds for some individuals (compare sample sizes in Figs. 1 and 2).

In a separate set of experiments, ventilation was measured for Control (from 2 litters) and Hyperoxia (from 2 litters) rats at P3, and then re-measured for the same individuals at P4 and P6 (i.e., after 1 and 3 days of recovery in room air). After being sealed into the plethysmograph chamber, rats were given 10–15 minutes to adjust to the chamber while breathing 21% O₂ before commencing the ventilation measurements. Ventilation was then recorded for approximately 7 min in 21% O₂; ventilatory parameters were analyzed on a breath-by-breath basis and averaged over 45–60 sec at the end of the exposure, excluding movement artifacts and sighs.

2.3. Metabolism and body temperature measurements

O₂ consumption and CO₂ production were measured by open-system respirometry. Rats were sealed into clear plastic boxes (chamber volume: 180 ml for P4–5 rats or 420 ml for P14 rats); rats were able to move freely within the chamber. The respirometer chamber was placed into an incubator so that air temperature could be maintained at 32–34°C as described above. Airflow through the chamber was set at 200 (P4–5) or 300 (P14) ml min⁻¹ STPD using a gas mixing mass flow controller (MFC-4; Sable Systems) and valves (840 series; Sierra Instruments) or with gas mixing rotameters (7300 series; Matheson Tri-Gas) and a mass flow monitor (G265; Qubit Systems). Fractional concentrations of O₂ and CO₂ in air entering and exiting the respirometer chamber were measured (S-3A and CD-3A analyzers; AEI Technologies, Pittsburgh, PA) and recorded to computer at a sampling rate of 20 Hz

(PowerLab 8SP and LabChart 6 software; ADInstruments); air was dried (Drierite, W.A. Hammond Drierite Co.) prior to passing through the gas analyzers.

Even at the low gas flow rates used, it was necessary to place two P4-5 rats (from the same litter) in the respirometer chamber simultaneously to achieve a sufficient change in the gas concentrations between the air entering and exiting the chamber; the O₂ consumption and CO₂ production of the pair was divided by their combined mass and used as a single replicate. P14 rats were studied individually.

Two different experimental protocols were used. In the first round of experiments, P4-5 and P14 Control (from 14 litters) and Hyperoxia (from 15 litters) rats were injected with 2,4-dinitrophenol (2,4-DNP), a drug that stimulates cellular O₂ consumption by uncoupling oxidative phosphorylation within the mitochondrion (Brody, 1955). 2,4-DNP (Aldrich) was prepared in phosphate buffered saline (PBS, pH 7.4; Sigma) at a concentration of 17 mg ml⁻¹, and this was administered by intraperitoneal injection (1-2 ml kg⁻¹) to achieve final dosages of 0 (i.e., PBS only), 17, or 34 mg kg⁻¹ of 2,4-DNP; 2,4-DNP dosages were selected based on the work of Saiki and Mortola (1997). Following injection, rats were sealed into the respirometer chamber for 30 min while breathing 21% O₂. O₂ consumption and CO₂ production were calculated using the average gas concentrations over the final 5 min of each exposure. At the end of the trial, rats were removed from the respirometer chamber and the rectal temperature was determined immediately for each individual (IT-21 thermocouple coupled to a BAT-12 microprobe thermometer; Physitemp Instruments). Animals were then killed by decapitation to collect blood for determination of lactate (all rats) and hemoglobin (only rats receiving 0 mg kg⁻¹ of 2,4-DNP) concentrations (see section 2.5). Rectal temperature, lactate, and hemoglobin measurements were recorded separately for P4-5 individuals.

To determine the metabolic rates for rats breathing 60% O₂, additional groups of Control (from 6 litters) and Hyperoxia (from 5 litters) rats were studied at P4-5; no injections were administered to these rats. Pairs of rats were sealed into the respirometer chamber as described above and maintained on 21% O₂ for a 10 min adjustment period. The inspired gas was then switched to 60% O₂ for 20 min, and O₂ consumption and CO₂ production were calculated using the average gas concentrations over the final 5 min of the hyperoxic exposure.

2.4. Pulse oximetry

Arterial O₂ saturation and heart rate measurements were made on conscious rat pups using a rodent pulse oximeter (MouseOx; STARR Life Sciences). “Collar clip” sensors (small and medium sizes for P4 and P13-14 rats, respectively) were placed dorsolaterally, with the tips situated near the carotid arteries; a depilatory cream (Nair; Church and Dwight Co.) was used to remove fur from the necks of the P13-14 rats prior to placing the sensors. Individual rats were placed into a clear plastic box (550 ml) with the sensor cable exiting through the lid; the bottom of the chamber was covered with corncob bedding. The animal chamber was then placed into an incubator so that air temperature could be maintained at 32-34°C as described above. Airflow through the chamber was set at 1.5 l min⁻¹ STPD using a gas mixing mass flow controller (MFC-4; Sable Systems) and valves (840 series; Sierra

Instruments). Output from the pulse oximeter was recorded to a computer at a sampling rate of 1 Hz.

Two different experimental protocols were used. In the first round of experiments, P4 and P13-14 Control (from 6 litters) and Hyperoxia (from 3 litters) rats were exposed to 21% O₂ for 15-20 min. O₂ saturation and heart rate values were averaged for 40-50 sec of data near the end of each exposure, excluding values flagged by the software as potentially inaccurate (typically caused by movement artifacts). At the end of the trial, rats were killed to collect blood for hematocrit measurements and/or to collect hearts (see section 2.5).

In the next round of experiments, additional groups of Control (from 2 litters) and Hyperoxia (from 3 litters) rats were studied at P4 and P13-14 while breathing 60% O₂. Individual rats were exposed to 60% O₂ for 15 min, and O₂ saturation and heart rate values were averaged for 40-50 sec of data near the end of the trial.

2.5. Blood analysis for hematocrit, hemoglobin, and lactate

Blood samples were collected from subsets of rats used in the metabolism (section 2.3) and pulse oximetry (section 2.4) experiments. Blood was sampled from the severed neck vessels immediately following decapitation. For hematocrit, blood was collected into heparinized hematocrit tubes (HematoClad; Drummond) and centrifuged for 5 min (RCF 3,900 × g; ZIPocrit; LW Scientific). The hematocrit was then estimated from the ratio of packed red cell volume to the total sample volume. Hematocrit was determined in the same manner for a small number of adult rats, except that adult rats were killed by CO₂ inhalation prior to decapitation. For hemoglobin concentration, blood was analyzed with an Hb 201⁺ analyzer (HemoCue). For lactate concentration, blood was collected directly onto test strips and analyzed using a Lactate Pro analyzer (Arkray).

2.6. Heart mass

Hearts were collected from a subset of rats from the pulse oximetry studies (section 2.4). After the major vessels were removed, the heart was rinsed in saline and dried for 48 h at 90°C. Whole hearts were then weighed to the nearest 0.0001 g.

2.7. In vitro brainstem-spinal cord preparation

An *in vitro* brainstem-spinal cord preparation was used to investigate the effects of developmental hyperoxia on intrinsic respiratory rhythmogenesis. Rat pups were derived from two Control litters and three Hyperoxia litters; for comparison, additional rat pups were obtained from four litters housed in room air outside of the environmental chambers. P3-4 rat pups (i.e., 88-102 h old) of either sex were anesthetized with 5% isoflurane (balance O₂) and decerebrated. The remaining tissue was placed in ice-cold artificial cerebrospinal fluid (aCSF) composed of (in mM): 120 NaCl, 26 NaHCO₃, 20 glucose, 2 MgSO₄, 1 CaCl₂, 3 KCl, and 1.25 Na₂HPO₄. The brainstem and spinal cord from the pontomedullary border to cervical spinal segment C8 was removed *en bloc* in ice-cold aCSF and pinned ventral side up in a standard recording chamber (0.6 ml volume). The tissue was continuously bathed (4.0-4.5 ml min⁻¹) with oxygenated aCSF solution (26°C, aerated with 5% CO₂ and 95% O₂, pH = 7.4).

Spontaneously produced respiratory motor output was recorded by attaching glass suction electrodes to cervical (C4-C5) nerve roots. Signals were acquired at 50 Hz, amplified (10,000x) and band-pass filtered (0.1-500 Hz) using a differential AC amplifier (model 1700; A-M Systems) before being rectified and integrated (time constant = 50 ms) using a moving averager (MA-821/RSP; CWE, Inc.). Data were collected using Axoscope hardware and software (Molecular Devices).

Recordings were started within 2-3 min of placing the tissue in the bath (time 0 = time when tissue was placed in the bath) and continued for at least 70 min. Voltage traces of spinal respiratory motor output or “bursts” were analyzed using Clampfit software (Molecular Devices) and data were averaged into 5-min bins. Respiratory motor burst intervals and frequency were calculated based on the timing of the respiratory motor burst peaks. Motor burst interval coefficient of variation was calculated as the standard deviation divided by the mean during the 5-min bin.

2.8. Statistical analysis

Preliminary analysis indicated that developmental hyperoxia had similar effects on the breathing of male rats and female rats, so data were pooled across sexes for statistical analysis; both sexes were represented approximately equally in each treatment group. For P4, P6-7, and P13-14 rats, ventilation in 21% O₂ and 60% O₂ were compared between treatment groups (i.e., Hyperoxia and Control) and among age groups using two-way analysis of variance (ANOVA) (factors = treatment group, age). Ventilatory responses to acute O₂ inhalation (99% O₂) was assessed separately at each age by two-way repeated measures ANOVA (factors = treatment group, inspired O₂), or expressed as a percentage change from baseline and compared across ages within a treatment group (one-way ANOVA). For P3 rats that were restudied after return to room air, ventilatory parameters were compared between treatment groups and among ages by two-way repeated measures ANOVA (factors = treatment group, age). Metabolic variables (e.g., O₂ consumption, body temperature, blood lactate, etc.) were compared between treatment groups and among 2,4-DNP dosages by two-way ANOVA (factors = treatment group, drug). For *in vitro* brainstem-spinal cord preparations, respiratory bursting characteristics across time were compared among treatment groups by two-way repeated measures ANOVA (factors = treatment group, time). Where significant differences were detected among groups by ANOVA, Tukey's test was used for *post hoc* multiple comparisons. For all other variables, comparisons between Hyperoxia and Control groups were made by unpaired t-tests, or by Mann-Whitney U tests (arterial O₂ saturation) where equal variance assumptions were not met.

Statistical tests were run using SigmaStat 3.11 (Systat Software) or Prism 6.00 (GraphPad Software), and *P* 0.05 was considered significant. For parametric tests, equal variance among groups was confirmed using Levene's test (SigmaStat) or an F test (Prism); data were assumed to be normally distributed. Unless noted otherwise, values in the text are reported as mean±SEM.

3. Results

3.1. Effects of developmental hyperoxia on body mass

Body mass was generally similar between Control and Hyperoxia rats, with no differences in mass observed between these treatment groups at P3, P6-7, or P13-14 (Table 1).

Hyperoxia rats weighed less than Control rats at P4-5 ($P=0.02$; Table 1), but this amounted to only a 3.6% difference in body mass.

3.2. Effects of developmental hyperoxia on ventilation

3.2.1. Ventilation in normoxia (21% O₂)—Normoxic ventilation varied by developmental treatment group and age (treatment $\times$ age, $P<0.001$; Fig. 1A). At P4 and P6-7, Hyperoxia rats exhibited significantly reduced minute ventilation compared to Control rats (36% and 21% less, respectively). However, no difference was detected in minute ventilation between treatment groups at P13-14. The convergence between the treatment groups with advancing age primarily reflected an age-dependent decrease in ventilation in Control rats between P4 and P6-7 ($P<0.001$) that was not observed in the Hyperoxia rats.

The reduced ventilation in Hyperoxia rats reflected lower tidal volume and lower respiratory frequency. Tidal volume was reduced in Hyperoxia rats at all ages (main effect for treatment, $P<0.001$; treatment $\times$ age, $P=0.07$). In contrast, respiratory frequency was reduced in Hyperoxia rats only at P4 (128 ± 4 vs. 157 ± 5 breaths min⁻¹ in Control) (treatment $\times$ age, $P<0.01$).

3.2.2. Ventilation in moderate hyperoxia (60% O₂)—Differences in minute ventilation were also evident between Hyperoxia and Control rats during a 10-min exposure to 60% O₂ (treatment $\times$ age, $P<0.001$; Fig. 1B). In fact, minute ventilation was similar to the values measured in 21% O₂, with the exception that ventilation was generally lower at P13-14 in Hyperoxia rats. As a result, Hyperoxia rats exhibited lower ventilation than Controls while breathing 60% O₂ at all three ages studied. As in normoxia, the difference in minute ventilation was greatest at P4 (i.e., 37% less than in Controls) and somewhat smaller at P6-7 and P13-14 (i.e., 13% and 19% less than in Controls, respectively).

3.2.3. Ventilation during acute O₂ inhalation—Immediately following normoxia measurements, rats were administered 99% O₂ to breathe for one minute. The general response to acute O₂ inhalation was a reduction in ventilation (Fig. 2). At both P4 and P6-7, however, the ventilatory response was attenuated in Hyperoxia rats compared to Controls (treatment $\times$ inspired O₂, $P<0.001$ and 0.01, respectively); this is evident in the different slopes of the ventilatory responses presented in Figures 2A and 2B. In contrast, the effect of acute O₂ inhalation was similar between Hyperoxia and Control rats at P13-14 (treatment $\times$ inspired O₂, $P=0.52$; Fig. 2C). Overall, the magnitude of the ventilatory response decreased with advancing age in the Control group: Control rats decreased ventilation by $27 \pm 3\%$ at P4 and $24 \pm 3\%$ at P6-7, but by only $14 \pm 3\%$ at P13-14 (one-way ANOVA, $P=0.015$; *post hoc* comparisons revealed that only P4 and P13-14 responses differed from one another). In comparison, Hyperoxia rats decreased ventilation by $11 \pm 3\%$, $13 \pm 6\%$, and $11 \pm 4\%$ at these ages.

3.2.4. Recovery of normoxic ventilation after return to room air—Normoxic ventilation was measured in additional groups of Control and Hyperoxia rats at P3 and re-measured after return to room air (Fig. 3). There was a significant interaction between treatment group and age for respiratory frequency ($P=0.002$), tidal volume ($P=0.013$), and minute ventilation ($P<0.001$). Similar to P4 (Fig. 1A), minute ventilation was reduced in Hyperoxia rats at P3 owing to both lower respiratory frequency and tidal volume (Fig. 3). Upon return to room air, however, there was a significant increase in resting minute ventilation in Hyperoxia rats by P4 ($P<0.001$ vs. P3) that was not observed in Control rats; consequently, ventilation no longer differed between Control and Hyperoxia rats by P4. Mass-specific ventilation decreased between P4 and P6 to a similar extent in both treatment groups (both $P<0.001$).

3.3. Effects of developmental hyperoxia on metabolism and body temperature

3.3.1. Metabolic responses to 2,4-dinitrophenol— O_2 consumption, CO_2 production, and rectal temperature were measured in P4-5 and P14 rats following injection with PBS or the metabolic stimulant 2,4-dinitrophenol (2,4-DNP); measurements were made while rats were breathing normoxic gas mixtures (21% O_2).

At P4-5, O_2 consumption was ~17% lower in Hyperoxia rats than in Controls (treatment, $P<0.001$; Fig. 4A); this effect was evident independent of drug treatment (treatment $\times$ drug, $P=0.26$). Importantly, 2,4-DNP increased O_2 consumption in both treatment groups (drug, $P<0.001$). In fact, O_2 consumption in Hyperoxia rats administered 34 mg kg^{-1} of 2,4-DNP exceeded the O_2 consumption rates measured in PBS-injected Control rats; thus, Hyperoxia rats were capable of sustaining higher rates of aerobic metabolism than observed in Control rats under normal resting conditions. CO_2 production was also lower in Hyperoxia rats, but this was only significant after injection with 2,4-DNP (treatment $\times$ drug, $P=0.04$; *post hoc* comparison between Hyperoxia and Control at 0 mg kg^{-1} (i.e., PBS), $P=0.08$) (Fig. 4C). Taken together, it appears that aerobic metabolism was somewhat reduced in Hyperoxia rats but that this effect was exacerbated following administration of 2,4-DNP. This pattern was also reflected in body temperature: Hyperoxia rats exhibited lower rectal temperatures than Control rats whether injected with PBS or 2,4-DNP (Fig. 4G), but the difference between groups increased following 2,4-DNP (treatment $\times$ drug, $P=0.02$). Lower body temperature in Hyperoxia rats is consistent with lower metabolic heat production while breathing normoxic gas mixtures.

At P14, O_2 consumption was similar between Hyperoxia and Control rats injected with PBS, but Hyperoxia rats exhibited a smaller increase in O_2 consumption after receiving 34 mg kg^{-1} of 2,4-DNP (treatment $\times$ drug, $P<0.01$; Fig. 4B); rats were not exposed to the lower dosage of 2,4-DNP at this age. Metabolic CO_2 production (Fig. 4D) and rectal temperature (Fig. 4H) were significantly reduced in Hyperoxia rats given either PBS or 2,4-DNP (main effect for treatment, $P<0.01$ and 0.001, respectively), but these effects appear to be driven by differences among rats injected with 2,4-DNP rather than among those injected with PBS.

At both P4-5 and P14, the ratio of CO_2 production to O_2 consumption (i.e., the respiratory exchange ratio) was increased in rats after 2,4-DNP (drug, $P<0.001$ and 0.012, respectively; Figs. 4E and 4F); this response did not differ between treatment groups (treatment and

treatment $\times$ drug, all $P > 0.05$). Although this ratio did not exceed 1.0, the rising value could reflect increased minute ventilation and/or an increased reliance on anaerobic metabolism after 2,4-DNP. Immediately following metabolism measurements, rats were sacrificed and blood samples were analyzed for lactate concentration. Blood lactate levels did not differ between Hyperoxia and Control rats injected with PBS, but Hyperoxia rats had larger increases in blood lactate following administration of 2,4-DNP at both P4-5 and P14 (treatment $\times$ drug, $P < 0.001$ and $P = 0.04$, respectively; Figs. 4I and 4J).

3.3.2. Metabolism in moderate hyperoxia (60% O₂)—Resting O₂ consumption was measured for a separate group of P4-5 rats ($n = 15$ Hyperoxia, 19 Control) during a 20-min exposure to 60% O₂; these rats were not injected with PBS or 2,4-DNP. In contrast to normoxia, the rates of O₂ consumption were similar between Hyperoxia and Control rats while breathing 60% O₂ (3.60 ± 0.13 and 3.56 ± 0.59 ml O₂ min⁻¹ 100g⁻¹; $P = 0.95$). No parallel group (without injection) was studied in 21% O₂, but the O₂ consumption rates determined in the Hyperoxia and Control groups in 60% O₂ were approximately equal to those measured for PBS-injected Control rats in 21% O₂ (i.e., 3.60 ± 0.21 ml O₂ min⁻¹ 100g⁻¹; Fig. 4A). To the extent that these data sets can be directly compared, these data suggest that Hyperoxia rats increased O₂ consumption when exposed to 60% O₂ (with no such change in metabolic rate in the Control rats).

3.4. Effects of developmental hyperoxia on blood O₂ transport

3.4.1. Hemoglobin O₂ saturation and heart rate in normoxia (21% O₂)—Pulse oximetry measurements were performed on conscious neonatal rats at P4 and P14. At both ages, hemoglobin O₂ saturation values in normoxia (21% O₂) were more variable in Hyperoxia rats than in Control rats. The median O₂ saturation was 92.8% in Hyperoxia rats at P4, much lower than the median O₂ saturation of 99.0% observed in age-matched Controls ($P < 0.01$; Fig. 5A); this pattern was also evident in the mean O₂ saturation values (93.0 ± 1.3 vs. $98.8 \pm 0.2\%$). The magnitude of this difference was reduced by P14, but again Hyperoxia rats exhibited somewhat lower median (97.5 vs. 98.8% in Controls; $P < 0.001$; Fig. 5B) and mean (97.0 ± 0.4 vs. $98.7 \pm 0.1\%$ in Controls) O₂ saturations.

Resting heart rate was lower in Hyperoxia rats than in Controls at P4 ($P < 0.001$; Fig. 5C), but slightly higher than in Controls at P14 ($P = 0.03$; Fig. 5D).

3.4.2. Hemoglobin O₂ saturation and heart rate in moderate hyperoxia (60% O₂)—Pulse oximetry measurements were completed on additional groups of Hyperoxia ($n = 15$ P4 and 9 P13-14) and Control ($n = 9$ P4 and 6 P13-14) rats as they breathed 60% O₂. Mean hemoglobin O₂ saturation values were nearly identical among all four groups (99.3-99.5%) and no significant differences were detected between Hyperoxia and Control rats ($P > 0.05$ at both ages). As in normoxia, heart rate was lower in Hyperoxia rats than in Controls at P4 (319 ± 9 vs. 359 ± 12 beats min⁻¹; $P = 0.01$), but not at P13-14 (471 ± 12 vs. 428 ± 18 beats min⁻¹; $P = 0.06$).

3.4.3. Heart mass, hematocrit, and hemoglobin concentrations—Dry heart mass was 5% smaller in Hyperoxia rats compared to Control at P4-5 ($P = 0.03$; Table 1), but this

difference was no longer apparent at P14 ($P=0.19$); right and left sides of the heart were not measured separately. Hematocrit and blood hemoglobin concentration (measured in separate groups of rats) were also reduced in Hyperoxia rats at both P4-5 and P14 (all $P<0.05$ vs. Control; Table 1). The relatively low hematocrits measured for the Control rats in this study ($\sim 32\%$) likely reflects “neonatal anemia”, a normal decline in hematocrit in newborn rats.

For comparison, we also sampled blood from a small number of dams ($n=3$ Hyperoxia and 6 Control) at the end of the two-week exposures (i.e., after collecting their pups at P14). Adult female rats exposed to 60% O_2 had lower hematocrits than females maintained in 21% O_2 (43 ± 1 vs. $52\pm 1\%$; $P<0.01$). Thus, the effects of chronic hyperoxia on hematocrit are not specific to development. Hemoglobin concentration was not measured for adult rats.

3.5 Effects of developmental hyperoxia on in vitro respiratory rhythm

Spontaneous respiratory motor output was recorded from cervical (C4-C5) nerve roots of isolated brainstem-spinal cord preparations. Respiratory bursts increased in frequency (Fig. 6) and amplitude (data not shown) over the first 30-35 minutes that the preparation was in the recording chamber and stabilized thereafter. Accordingly, statistical comparisons among treatment groups were limited to the second half of the experimental period (i.e., minutes 35 through 70, represented by bins 40-70); preliminary analysis revealed that the results and conclusions would be qualitatively similar if all data were included.

There was a significant interaction between developmental treatment group and time for both interburst interval ($P<0.001$; Fig. 6A) and burst frequency ($P=0.02$; Fig. 6B). Specifically, P3-4 Hyperoxia rats exhibited a significantly longer interburst interval and a correspondingly lower respiratory burst frequency compared to Control rats at every time point. Overall, the timing of respiratory bursts was more variable in Hyperoxia rats, as shown by a significantly greater coefficient of variation for interburst interval (main effect for treatment, $P<0.001$; Fig. 6C).

Although the chamber-reared Control group is the more appropriate comparison for the Hyperoxia rats in this study (i.e., similar developmental conditions apart from inspired O_2), an additional group of rats ($n=8$) was studied in which pups were housed in room air outside the environmental chamber. The interburst intervals were longer (and burst frequency shorter) for these rats than in the chamber-reared Control rats (e.g., interburst interval: 5.9 ± 0.4 s vs. 4.7 ± 0.3 s; burst frequency: 10.5 ± 0.6 bursts min^{-1} vs. 13.2 ± 0.9 bursts min^{-1} , averaged over the 40-70 min bins). However, respiratory bursts still occurred at shorter intervals and higher frequencies than in Hyperoxia rats (interburst interval: 7.9 ± 0.6 s; burst frequency: 8.1 ± 0.6 bursts min^{-1}) over the same time period.

4. Discussion

It was previously reported that neonatal rats chronically exposed to moderate hyperoxia (60% O_2) for the first 4-7 postnatal days exhibit reduced minute ventilation when returned to room air, but that ventilation recovered by 14 days of age despite continued exposure to hyperoxia (Bavis et al., 2010). The present study confirmed these results: normoxic ventilation was markedly reduced in Hyperoxia rats when studied at P3, P4, and P6-7, but

not at P13-14. Moreover, Hyperoxia rats exhibited lower arterial hemoglobin O₂ saturations while breathing normoxic gas mixtures, indicating that they were hypoventilating despite their reduced metabolic rates. Since Hyperoxia rats also had lower hemoglobin concentrations at P4-5 and P13-14, as well as lower heart rates and somewhat smaller hearts at P4-5, it does not appear that individuals compensate for this hypoventilation through changes in cardiovascular function. In other words, O₂ delivery to tissues appears to be compromised in the youngest neonatal rats shortly after return to room air.

In contrast to life-long changes in the hypoxic ventilatory response caused by developmental hyperoxia (Fuller et al., 2002), plasticity in eupneic ventilation recovered over a fairly short time course following return to room air. Hyperoxia rats exhibited normoxic ventilation comparable to age-matched Control rats one day (16-24 h) after return to room air. The present study did not rigorously assess shorter periods of recovery, but the typical time between removal of an animal from hyperoxia and recording ventilation measurements was 30-60 min in this and previous experiments in our laboratory (e.g., Bavis et al., 2010; Roeser et al., 2011). Since rats were exposed to room air during this period, hyperoxia-induced hypoventilation persists for at least one hour but for less than one day.

4.1. Mechanisms underlying hypoventilation after developmental hyperoxia

The reduced normoxic ventilation observed in young rats after developmental hyperoxia is accompanied by lower rates of O₂ consumption (Bavis et al., 2010), as confirmed in the present study (Fig. 4A). Although lower metabolic demand could lead to lower ventilatory drive, this does not appear to be the case in Hyperoxia rats. First of all, the reduction in minute ventilation was greater than one would predict based on the observed reduction in O₂ consumption: ventilation was 36% lower than that of age-matched Control rats at P4, while O₂ consumption was only 17% lower. Moreover, O₂ consumption increased when Hyperoxia rats were acutely returned to 60% O₂ (to a level comparable to that of age-matched Control rats) but minute ventilation remained low; in other words, reduced ventilation could be dissociated from reduced metabolism. Finally, Hyperoxia rats exhibited lower arterial hemoglobin O₂ saturations at P4 compared to Control rats; this suggests that alveolar ventilation was inadequate relative to metabolic demands. Collectively, these data indicate that reduced metabolism does not explain the reduced ventilation observed in Hyperoxia rats at P3-P7.

Tonic input from peripheral chemoreceptors also contributes to normoxic ventilatory drive in neonatal and adult mammals (Dejours, 1963; Teppema and Dahan, 2010), as demonstrated by the reduction in ventilation during an abrupt increase in inspired O₂ to inhibit peripheral arterial chemoreceptors (i.e., the “Dejours test”). In the present study, rats reared in hyperoxia were less responsive to O₂ inhalation at P4 and P6-7, but not at P13-14, compared to Control rats. Thus, normoxic hypoventilation in the younger age groups could result, in part, from reduced drive from peripheral chemoreceptors. Indeed, Hyperoxia rats have smaller carotid bodies with fewer glomus cells and fewer chemoafferent neurons projecting to the brainstem (Erickson et al., 1998; Chavez-Valdez et al., 2012; Dmitrieff et al., 2012; Bavis et al., 2013). Furthermore, the remaining glomus cells are less sensitive to O₂ (Donnelly et al., 2005, 2009; Bavis et al., 2011b), and the spontaneous activity recorded

from single-unit chemoafferent neurons may be reduced (or even silenced) at normoxic partial pressures of O₂ in Hyperoxia rats (Donnelly et al., 2005).

Abnormal carotid body function persists through P14 in rats continuously exposed to hyperoxia from birth (Donnelly et al., 2005; Bavis et al., 2011b), so it is somewhat surprising that normoxic ventilation no longer differed between Hyperoxia and Control rats at P13-14. One possibility is that brainstem mechanisms gradually compensate for inadequate sensory input (i.e., homeostatic plasticity), perhaps through the enhancement of excitatory modulation or the reduction of inhibitory modulation of respiratory neurons. Alternatively, this pattern might be explained by an age-dependent decrease in the relative contribution of peripheral chemoreceptors to eupneic ventilatory drive. In rats, the contribution of the peripheral chemoreceptors to normoxic ventilation appears to diminish with postnatal age, at least over the age range studied here. In the present study, for example, O₂ inhalation depressed ventilation more at P4 than at P13-14 in untreated Control rats. Similarly, the ventilatory response to acute O₂ inhalation decreased between P3 and P8 in one study of neonatal rats (Bamford and Carroll, 1999), and it decreased between P3 and P18 in another study (Huang et al., 2010) if the ventilatory response is expressed as a percentage of baseline ventilation. Thus, the relatively normal eupneic ventilation of P13-14 Hyperoxia rats could reflect postnatal maturation of the balance between peripheral and central ventilatory drive.

Despite the evidence that abnormal carotid body function contributes to the diminished normoxic ventilation observed after developmental hyperoxia, this appears insufficient to explain the observed hypoventilation. For example, morphological changes to the carotid body persist after return to room air (Fuller et al., 2002; Bisgard et al., 2003; Bavis et al., 2011a) even though the present study found that normoxic ventilation recovers within one day. It is possible that diminished O₂ sensitivity of the remaining carotid body cells is an important factor, and O₂ sensitivity does recover after return to room air; recovery appears mostly complete within 3 days, but shorter periods of recovery have not been studied. In addition, week-old neonatal rats exposed to 60% O₂ for 23-28 hours exhibit reduced normoxic ventilation as well (Roeser et al., 2011). Previous studies have shown enhanced glomus cell O₂ sensitivity after 24-hour hyperoxic exposures in week-old rats (Donnelly et al., 2009), and it may take several days for hyperoxia to cause measurable changes to carotid body size (Dmitrieff et al., 2012). Thus, developmental hyperoxia likely influences normoxic ventilation by eliciting plasticity/injury at additional levels of the respiratory control system.

In the present study, we used an *in vitro* brainstem-spinal cord preparation to assess potential changes in the CNS respiratory network during developmental hyperoxia. Although there is debate as to whether respiratory bursts generated by the isolated brainstem reflect eupneic respiratory activity versus gasping, this preparation is useful to explore plasticity in respiratory rhythm-generating circuitry since the respiratory network is largely intact (Johnson et al., 2012). Consistent with the lower respiratory frequency observed in conscious, Hyperoxia rats at P3-4 (Bavis et al., 2010; Bierman et al., 2014; this study), respiratory bursts occurred at a lower frequency in the brainstems from Hyperoxia rats. Bierman et al. (2014) recently reported similar findings for brainstem-spinal cord

preparation from rats reared in 60% O₂ through P4-5. This agreement between laboratories, as well as the consistent difference between treatment groups across the entire 70-min protocol in the present study, is important given the experimental variation inherent to this preparation. In addition to the slower burst frequency after developmental hyperoxia, the greater variability in the timing of respiratory bursts observed in the present study may help to explain increased breath-to-breath variability in neonatal Hyperoxia rats (Bavis et al., 2010). Whether lower respiratory frequencies and greater respiratory instability reflect changes at the pre-Botzinger complex, recognized as the principal driver of inspiratory rhythm (Feldman et al., 2013), or at other components of the rhythm-generating network remains to be determined.

Collectively, these findings point to multiple contributors to the hypoventilation observed in Hyperoxia rats, including deficits in both peripheral respiratory drive and CNS rhythmogenesis. Additional plasticity downstream of the rhythm-generating network (e.g., respiratory motoneurons and/or respiratory muscles) cannot be excluded.

4.2. Hypometabolism following developmental hyperoxia

The present study confirmed an earlier report (Bavis et al., 2010) that rats reared in hyperoxia have lower O₂ consumption rates in normoxia than their age-matched Control rats at P4-5 but not at P14. Although decreased ventilation in itself could lower O₂ consumption (i.e., reduced activity of respiratory muscles), the observation that O₂ consumption is restored to normal levels during 60% O₂ exposure while ventilation remains low indicates that some additional explanation is needed. Importantly, tissue O₂ delivery appears to be reduced following developmental hyperoxia which could depress aerobic metabolism. Hyperoxia rats have lower hematocrits and lower blood hemoglobin concentrations than age-matched Control rats. When combined with decreased O₂ saturations, these findings suggest that blood O₂ content is reduced even while breathing normoxic gas mixtures. Moreover, heart rate and heart mass are reduced at P4-5; to the extent that heart mass correlates with stroke volume, this suggests that cardiac output is also reduced in these rats.

There are at least two ways that tissue hypoxia could alter aerobic metabolism: O₂ limitation or a regulated decrease in metabolism. While O₂ limitation represents an inability to sustain aerobic metabolism, the latter response would be an adaptive process in which whole-body metabolism is regulated at a lower level in order to match O₂ demand to diminished O₂ availability. For example, many vertebrates exhibit reduced metabolism during acute exposure to low inspired O₂ (i.e., “hypoxic hypometabolism”) (Mortola, 2004; Atchley et al., 2008). This response is associated with a controlled decrease in body temperature (anapyrexia) and, thus, a reduced demand for metabolic heat production for thermoregulation (Steiner and Branco, 2002; Mortola, 2004). Several lines of evidence have been used to demonstrate that hypoxic hypometabolism is a regulated process rather than simply the result of O₂ limitation. These include: (1) no compensatory increase in anaerobic metabolism at levels of hypoxia that elicit hypometabolic responses (Frappell et al., 1991; Atchley et al., 2008) and (2) the ability for hypoxic animals to increase O₂ consumption when challenged by cold or pharmacological stimulants of metabolism (Saiki and Mortola, 1997; Rohlicek et al., 1998).

In both scenarios (i.e., O₂ limitation or regulated hypometabolism), O₂ consumption would be expected to increase when P4 Hyperoxia rats are exposed to 60% O₂ (which enhances arterial O₂ content by increasing O₂ saturation to nearly 100%), as observed. However, in the case of O₂ limitation, one might expect Hyperoxia rats to rely more heavily on anaerobic metabolism in an attempt to sustain the same overall metabolic rate. This was not seen in neonatal rats under resting conditions: blood lactate levels and the respiratory exchange ratio were not different between Hyperoxia and Control rats during normoxic exposures. On the other hand, Hyperoxia rats were able to increase O₂ consumption when challenged with 2,4-DNP. In fact, Hyperoxia rats treated with 2,4-DNP were able to sustain rates of O₂ consumption greater than those exhibited by saline-injected Control rats; this would not be expected if the capacity for O₂ delivery constrained resting O₂ consumption. It should also be noted that Hyperoxia rats had lower rectal temperatures than their age-matched Control rats (i.e., mild hypothermia). Thus, the lower normoxic metabolic rate observed in Hyperoxia rats resembles the hypoxic hypometabolism previously described for newborn rats and other small animals. It remains to be determined, however, whether the stimulus for the hypometabolic response in these rats is the absolute lower level of O₂ (i.e., tissue hypoxia *per se*) or the perception of relative hypoxia (i.e., a shift in the “normoxic” setpoint to higher O₂ partial pressures in rats reared in hyperoxia).

Although O₂ limitation does not explain the lower O₂ consumption rates observed in Hyperoxia rats at rest, Hyperoxia rats may experience some degree of O₂ limitation as metabolic demand increases. Hyperoxia and Control rats each increased lactate production and the respiratory exchange ratio after administration of 2,4-DNP. However, Hyperoxia rats exhibited greater increases in blood lactate levels than observed in the Control rats, suggesting a greater increment in anaerobic metabolism. It is possible that the lower blood O₂ carrying capacity (i.e., lower hematocrit and hemoglobin concentration) contributes to this apparent O₂ limitation. In addition, chronic exposure to hyperoxia is known to damage developing lungs and may impair gas exchange (Buczynski et al., 2013; Kallet and Matthay, 2013). Two-week exposures to 60% O₂ do not appear to cause persistent gas exchange impairment in rats (Bavis et al., 2007), but we cannot exclude the possibility of short term effects. Indeed, mild lung injury could help to explain the slight reduction in arterial O₂ saturations observed in Hyperoxia rats at P14 despite their apparently normal pulmonary ventilation in normoxia. The reduction in median O₂ saturation was quite modest at P14 (97.5 vs. 98.8%), however, and the dose-dependence of hyperoxic lung injury makes it unlikely that lung injury contributes substantially to the much lower normoxic O₂ saturations observed at P4 (i.e., after a much shorter hyperoxic exposure).

4.3. Significance

Supplemental O₂ 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 (BPD), retinopathy of prematurity (ROP)), preterm infants are hyperoxic relative to their intended O₂ saturation limits for 30-40% of the time that they are receiving O₂ therapy (Hagadorn et al., 2006; Claure and Bancalari, 2009; Finer and Leone, 2009). Whether these exposures are sufficient to impair respiratory control development in humans has not yet been established, but there is suggestive evidence that this could be the

case. For example, preterm infants that received supplemental O₂ exhibit blunted ventilatory responses to changes in inspired O₂ (Calder et al., 1994; Katz-Salamon and Lagercrantz, 1994; Katz-Salamon et al., 1996). In particular, these infants have an attenuated ventilatory response to acute O₂ inhalation, similar to the rats in the present study. Although it is impossible to attribute abnormal ventilatory responses directly to hyperoxia (versus other factors associated with prematurity) in these human studies, this effect is related to the duration of the infant's O₂ treatment (Katz-Salamon and Lagercrantz, 1994).

The impact of developmental hyperoxia on normoxic ventilation appears to be relatively short-lived in neonatal rats, both in terms of spontaneous recovery during continuous hyperoxia and recovery after return to room air (Bavis et al., 2010; present study). If hyperoxia has similar effects in human infants, however, even a transient period of hypoventilation may be clinically significant. The clinical use of supplemental O₂ in preterm infants is intended to treat desaturations associated with immature lungs and/or apnea of prematurity. Thus, if infants exhibit low saturations after supplemental O₂ is discontinued (analogous to returning Hyperoxia rats to room air), the likely response would be to reinstate supplemental O₂. Continued O₂ therapy could cause further impairment to respiratory control while also increasing the risk for BPD, ROP, and other O₂-dependent morbidities.

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Highlights

- Newborn rats reared in hyperoxia hypoventilate shortly after return to normoxia.
- Smaller hyperoxic ventilatory responses suggest reduced carotid body contribution.
- Respiratory burst frequency is also slower in brainstem-spinal cord preparations.
- The decreased minute ventilation cannot be explained by decreased metabolic rate.
- Diminished metabolic rates appear to be regulated, akin to hypoxic hypometabolism.

Fig. 1.

Minute ventilation (mean±SEM) for neonatal rats reared in 21% O₂ (Control) or 60% O₂ (Hyperoxia) while breathing gas mixtures containing 21% O₂ (A) or 60% O₂ (B). Sample sizes (P4, P6-7 and P13-14) are 24, 24 and 21 for Control rats, and 19, 19 and 21 for Hyperoxia rats; different individuals were studied at each age, but the same individuals were studied at both levels of inspired O₂. * $P < 0.05$ vs. Control within the same age group; † $P < 0.05$ vs. 4 days of age within the same developmental treatment group.

Fig. 2.

Ventilatory responses to acute O₂ inhalation (99% O₂) for neonatal rats reared in 21% O₂ (Control) or 60% O₂ (Hyperoxia) and studied at P4 (A), P6-7 (B) and P13-14 (C). Minute ventilation (mean±SEM) data were obtained from the same individuals reported in Figure 1, but these data represent only the subset of rats for which 99% O₂ ventilation was successfully measured. Sample sizes (P4, P6-7 and P13-14) are 20, 21 and 18 for Control rats, and 18, 15 and 18 for Hyperoxia rats. The switch to 99% O₂ caused a significant decrease in ventilation in both treatment groups at all ages (all $P < 0.05$), but note that the slope is most severe for Control rats at P4 (see text for details). * $P < 0.05$ vs. Control at the same inspired O₂ level.

Fig. 3.

Spontaneous recovery of eupneic ventilation following return to room air. Respiratory frequency (A), tidal volume (B), and minute ventilation (C) are presented for neonatal rats while breathing gas mixtures containing 21% O₂. Rats were reared in 21% O₂ (Control) or 60% O₂ (Hyperoxia) from birth through P3. Breathing was then measured immediately after return to room air (P3) and again after 1 and 3 days recovery in room air (i.e., at P4 and P6, respectively); note that the same individuals were studied at each time point (n=16 per treatment group). Values are mean±SEM. * $P < 0.05$ vs. Control at the same age.

Fig. 4.

The influence of the uncoupling agent 2,4-dinitrophenol (2,4-DNP) on the metabolism of neonatal rats reared in 21% O₂ (Control) or 60% O₂ (Hyperoxia). Rats were studied at either P4-5 (left panels) or P14 (right panels); different individuals were studied at each dosage of 2,4-DNP and at each age. For gaseous metabolism (panels A-F), sample sizes are 12-14 per treatment group per dosage at each age. For rectal temperature (panels G-H) and blood lactate concentration (panels I-J), sample sizes are 24-28 per treatment group per dosage at P4-5 and 14 per treatment group per dosage at P14. Where significant treatment × drug

interactions were detected, * $P < 0.05$ vs. Control at the same drug dosage. Where interactions were not significant, † denotes a significant main effect for developmental treatment (i.e., Control vs. Hyperoxia, $P < 0.05$). For clarity, differences among drug dosages (i.e., main effects for drug) are not illustrated.

Fig. 5.

Arterial O₂ saturation (A, B) and heart rate (C, D) for neonatal rats reared in 21% O₂ (Control) or 60% O₂ (Hyperoxia) while breathing normoxic gas mixtures. Rats were studied at either P4 (left panels) or P14 (right panels). For arterial O₂ saturation, each dot represents an individual rat and the horizontal line represents the median value; sample sizes are P4: 11 Control, 13 Hyperoxia and P14: 15 Control, 15 Hyperoxia. For heart rate, values are mean $\pm$ SEM; sample sizes are P4: 14 Control, 14 Hyperoxia and P14: 15 Control, 15 Hyperoxia. * $P < 0.05$ vs. Control.

Fig. 6.

Spontaneous respiratory motor activity of isolated brainstem-spinal cord preparations from neonatal rats reared in 21% O₂ (Control; n=7) or 60% O₂ (Hyperoxia; n=10). The interburst interval (A), burst frequency (B), and coefficient of variation for interburst intervals (C) are reported for respiratory bursts recorded from cervical (C4-C5) nerve roots while superfused with aCSF equilibrated with 95% O₂ / 5% CO₂ (pH 7.4, 26°C). Values (mean±SEM) are reported for the entire 70-minute protocol in 5-min bins (time 0 = time when tissue was placed in the bath), but data from the first 35 min were excluded from the statistical analysis (shaded region; see text for details). Where significant treatment × time interactions were detected, * *P* < 0.05 vs. Control at the same time point. Where interactions were not significant, † denotes a significant main effect for developmental treatment (i.e., Control vs. Hyperoxia, *P* < 0.05).

Table 1

Body mass (g) for rat pups reared in 21% O₂ (Control) or 60% O₂ (Hyperoxia) from birth. Data are pooled across all experiments in the present study. Values are means $\pm$ SEM (n).

	P3	P4-5	P6-7	P13-14
Control	8.5 $\pm$ 0.3 (29)	11.0 $\pm$ 0.1 (200)	13.8 $\pm$ 0.4 (40)	31.1 $\pm$ 0.4 (71)
Hyperoxia	8.6 $\pm$ 0.2 (23)	10.6 $\pm$ 0.1 * (179)	14.4 $\pm$ 0.4 (35)	30.6 $\pm$ 0.5 (78)

*
P<0.05 vs. Control at same age.

Table 2

Heart mass and hematological properties of rats reared in 21% O₂ (Control) or 60% O₂ (Hyperoxia) from birth. Values are means $\pm$ SEM (n).

	P4^a		P14	
	Control	Hyperoxia	Control	Hyperoxia
Dry Heart Mass (mg g ⁻¹)	0.92 $\pm$ 0.01 (17)	0.87 $\pm$ 0.02 [*] (14)	0.85 $\pm$ 0.02 (16)	0.82 $\pm$ 0.02 (17)
Hematocrit (%)	32 $\pm$ 0.4 (22)	28 $\pm$ 0.6 [*] (14)	33 $\pm$ 0.5 (16)	31 $\pm$ 0.4 [*] (20)
Hemoglobin (g dL ⁻¹)	7.9 $\pm$ 0.1 (26)	6.7 $\pm$ 0.2 [*] (26)	7.6 $\pm$ 0.2 (14)	7.1 $\pm$ 0.2 [*] (14)

^{*} $P < 0.05$ vs. Control at same age.

^a For P4 hemoglobin concentrations, the sample includes data from P4 and P5 individuals.