Vulnerability of neonatal respiratory neural control to sustained hypoxia during a uniquely sensitive window of development

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Mayer CA, Di Fiore JM, Martin RJ, MacFarlane PM. Vulnerability of neonatal respiratory neural control to sustained hypoxia during a uniquely sensitive window of development. J Appl Physiol 116: 514–521, 2014. First published December 26, 2013; doi:10.1152/japplphysiol.00976.2013.—The first postnatal weeks represent a period of development in the rat during which the respiratory neural control system may be vulnerable to aberrant environmental stressors. In the present study, we investigated whether sustained hypoxia (SH; 11% O2) exposure starting at different postnatal ages differentially modifies the acute hypoxic (HVR) and hypercapnic ventilatory response (HCVR). Three different groups of rat pups were exposed to 5 days of SH, starting at either postnatal age 1 (SH1–5), 11 (SH11–15), or 21 (SH21–25) days. Whole body plethysmography was used to assess the HVR and HCVR the day after SH exposure ended. The primary results indicated that 1) the HVR and HCVR of SH11–15 rats were absent or attenuated (respectively) compared with age-matched rats raised in normoxia; 2) there was a profoundly high (~84%) incidence of unexplained mortality in the SH11–15 rats; and 3) these phenomena were unique to the SH11–15 group with no comparable effect of the SH exposure on the HVR, HCVR, or mortality in the younger (SH1–5) or older (SH21–25) rats. These results share several commonalities with the risk factors thought to underlie the etiology of sudden infant death syndrome, including 1) a vulnerable neonatal; 2) a critical period of development; and 3) an environmental stressor.

hypoxia; development; respiratory control; SIDS

THE RESPIRATORY NEURAL CONTROL system undergoes considerable adjustment throughout the early postnatal period. Maturation of key brain stem respiratory control regions, for example, involves changes in the expression of several neurochemical factors, particularly during the first 2 wk of life in the rat (43). The neonatal period, therefore, may represent a transitional stage of development in which the respiratory control system is vulnerable to specific environmental stressors. In the present study, we investigated whether neonatal sustained hypoxia (SH) exposure during specific stages of postnatal development differentially modifies the acute hypoxic (HVR) and hypercapnic ventilatory response (HCVR) in rats.

Postnatal development of the peripheral and central respiratory neural control systems mediating the acute HVR and HCVR has been well characterized in the rat. The primary peripheral O2 chemoreceptor, the carotid body, matures during the first weeks of life and involves synthesis of brain and glial derived neurotrophic factors for its full functional development (66). Over a similar time course, the carotid body also increases sensitivity to hypoxia (2, 11, 35), which coincides with maturation of the HVR (1, 18). Neurochemical changes, which include GABA, glutamate, and serotonin (44), also take place in key brain stem respiratory control regions, including the rhythmogenic network (pre-Botzinger complex) and the nucleus of the solitary tract, where carotid body chemoeffersents terminate (20). Changes in the serotonergic system may be particularly relevant since it appears to be heavily involved in modulating the HVR (36, 60) and HCVR (29, 30, 38, 55). Although the role of glutamate, GABA, and 5-HT in modulation of the acute HVR and HCVR has been well studied, the specific purpose of the postnatal changes in their expression is unknown.

It is perhaps not surprising that such a period of development in which the respiratory system is gradually changing is particularly sensitive to environmental stressors, some of which can impose permanent changes that persist into adulthood. Specifically, neonatal (from birth) hyperoxia (3, 4, 39) or hypoxia (5, 23, 27, 49, 53, 54) exposure irreversibly attenuates the HVR, whereas chronic intermittent hypoxia (CIH) can (but not always) enhance it (34, 56, 57) via mechanisms that can involve changes at the level of the carotid body (6, 12, 19, 64). Furthermore, several days of neonatal-maternal separation (15, 25) or SH (46) exposure cause a long-term enhancement of the HCVR. Neonatal SH (17, 57) exposure during the first days to weeks also attenuated the HVR in rats. Similar SH exposures during adulthood, however, enhanced the HVR (58), suggesting that, at some (unknown) time, there is a transitional period of development where the effects of SH on the neonatal respiratory system contrasts markedly from its effects on the adult. Not only do these data indicate the neonatal period is likely to be sensitive to prolonged (days) exposure to various stressors, there is also a consistent observation that critical periods of development exist in which the respiratory control system is possibly at a heightened level of vulnerability (67).

Brain stem neurochemical expression changes abruptly and, in some cases, transiently toward the end of the second postnatal week in rats. Glutamate, GABA, and 5-HT (41, 42, 44) expression (and their receptors) in key brain stem respiratory control regions change dramatically at approximately postnatal days 12 and 13 in the rat, and the HVR seems to transiently disappear at approximately the same age (40). However, whether such a critical and brief stage of development exhibits a uniquely heightened level of vulnerability to any environmental stressors has not been explored. In the present study, therefore, we investigated whether SH exposure encompassing the postnatal day 12 and 13 period uniquely modifies the respiratory neural control system compared with the same exposure paradigm applied at earlier or later time points. We hypothesized that SH exposure during (but not before or after) this critical period of development impairs the ventilatory response to acute hypoxia and hypercapnia.
EXPERIMENTAL PROCEDURES

Experiments were performed on neonatal male Lewis rats (Charles River, colony PO6). All procedures were carried out in accordance with the National Institutes of Health guidelines for care and use of laboratory animals and were approved by the Animal Care and Use Committee at Case Western Reserve University.

Hypoxia exposures. Following the day of birth (P0), the dam (and her pups) were assigned to one of three groups that received SH (11% O2) for 5 consecutive days (24 h/day), beginning either at postnatal age 1 (P1), 11 (P11), or 21 days (P21; see Fig. 1). Five days of SH exposure beginning at P11 was chosen to encompass neurotransmitter changes known to be taking place around a similar time point (see DISCUSSION). P1 and P21 age groups were then chosen to represent younger and older age groups, respectively, that were considerably outside of this potentially vulnerable window. SH was achieved by placing the mother and pups inside a Plexiglas chamber connected to adjustable rotameters for mixing air and nitrogen (N2). Oxygen levels were monitored (TED 60T, Teledyne Analytical Instruments) and adjusted if necessary to maintain appropriate level of SH. Airflow through the chamber was maintained at ~4 l/min, and carbon dioxide (CO2) levels in the airflow exiting the chambers were monitored to ensure flow was adequate to prevent CO2 accumulation. At the end of the 5th day of exposure (at ~5 PM), the rats were removed from the chamber and allowed to recover in room air overnight and then assessed for acute HVR and HCVR using whole body plethysmography (Fig. 1). Since the 5 days of SH exposure began at different time points (P1, P11, or P21), these rats are designated using the following nomenclature: SH1–5 (n = 11), SH11–15 (n = 10), and SH21–25 (n = 10), respectively. The HVR and HCVR of SH-treated rats were compared with age-matched normoxic-raised rats [designated using the following nomenclature: NX6 (n = 10), NX16 (n = 16), and NX26 (n = 10)]. After plethysmography measurements, rats were allowed to survive through to P30 after weaning at P27. An additional group of rats was exposed to SH 13% O2 between P11 and P15 (n = 10) to test the effects of less severe hypoxia. Furthermore, to ensure the attenuated HVR/HCVR and mortality observed in the rat pups (see RESULTS) were not the result of an effect of SH on the dam per se, we cross-fostered three separate SH11–15 dams with three normoxia dams at equivalent stages of lactation in the middle of the SH exposure period (P13). Although there was a brief interruption of SH and unavoidable exposure to normoxia associated with opening the chambers, exchanging an SH-exposed dam with a normoxic one did not appear to affect our observations. The remaining experiments were performed on litters in which the natural mother of the pups was also exposed to SH.

Plethysmography and rates of metabolism. The day after the rat pups were removed from SH, individual pups were removed from the litter and placed inside a custom-made Perspex plethysmograph chamber. Different sized chambers were used to accommodate rats at various ages and body size. Temperature inside the chamber was maintained (~28°C) at all ages by adjusting a water bath (Isotemp 3013S, Fisher Scientific) that circulated water to a heat pad positioned underneath the plethysmograph. Airflow through the chamber was held constant (450 ml/min) using a mass flow controller (0–2 l/min, Aalborg), and hypoxia (10% O2) was administered using a gas mixer (Bird Blender 2003, Pneumatic Services). Before each experiment, the chamber was assessed for adequate seal by observing stability of the square pressure change following injection of a calibration volume (50 μl) using a glass microsyringe (Hamilton, Harvard Apparatus). The same injection volume was used later for calibration of tidal volume (VT) changes associated with breathing (see below). Rectal temperature was monitored continuously throughout the experiment with a fine temperature thermocouple (Physitemp), which was held securely in place with tape adhered to the base of the tail. O2 and CO2 in the gas that passed through the chamber were also measured continuously using gas analyzers (ADInstruments, Gas Analyzer ML206).

The plethysmograph enabled accurate measurement of the rates of ventilation (Ve) and metabolism (oxygen consumption, VO2; carbon dioxide production, VCO2) during baseline, hypoxia, and hypercapnia. Rats were allowed ~25 min to acclimatize to the plethysmograph before receiving 10% O2 (5 min), followed by hypercapnia (5% CO2, 5 min). Ve and metabolism were measured when the plethysmograph was sealed during the last 30 s of each exposure. Chambers were sealed by turning stopcocks upstream and downstream of the plethysmograph to bypass airflow, which was also used to determine the incremental fractional concentrations of O2 and CO2 for calculating the rates of metabolism. The corresponding pressure signal associated with breathing during the time the chamber was sealed and calibrated for volume using a glass syringe (50 μl injection) for calculating VT (14, 22, 48).

Similarly, the air that bypassed the chamber corresponded to incremental fractional concentrations of O2 and CO2 of each gas, which were used in the calculation of VO2 and VCO2. Excurrent fractional

![Treatment Groups:](image)

Fig. 1. Schematic illustrating the postnatal protocol for exposure to sustained hypoxia (SH). The three primary groups consist of rats born in room air, then exposed to SH for 5 consecutive days (11% O2; shaded arrows), starting at either postnatal age 1 (group 1: SH1–5), 11 (group 2: SH11–15) or 21 days (group 3: SH21–25). Plethysmography (P) was performed the day after the rats were removed from SH [i.e., at postnatal day 6 (P6), P16, or P26 for each of the respective groups]. Rats were then allowed to continue developing in normoxia (dashed lines) through to 30 days of age. Note, however, that considerable mortality was observed in SH1–5, rats several days after plethysmography measurements (see Table 2). All rats were weaned (W) at P27. Metabolic and ventilatory values collected from plethysmography measurements for each SH-exposed group of rats were compared with corresponding age-matched rats raised in normoxia (i.e., NX6, NX16, and NX26). B, baseline.
concentrations of O₂ and CO₂ were determined for the 10 s immediately before turning the stop cocks to flush the chamber with incumbent gas. V̇O₂ was calculated according to the equations of Frappell and colleagues (21). We also calculated the coefficient of variation (CV) of breathing frequency (fB) as a measure of ventilatory variability.

Statistical analysis. Statistical comparisons were made between treatment groups and within plethysmography measurements using two-way, repeated-measures ANOVA. Comparison of baseline conditions between groups was performed using a one-way ANOVA. Differences were considered significant at P < 0.05. All values are expressed as means ± 1 SE.

RESULTS

Rates of \( \dot{V}e \) and metabolism in normoxia. Body weights and values for baseline levels of \( \dot{V}e \) and metabolism in SH1-5, SH11-15 (11% and 13% O₂), SH21-25, and corresponding age-matched control rats raised in normoxia (NX6, NX16, and NX26, respectively) are provided in Table 1. Body weights of SH1-5 and SH11-15 rats exposed to 11% O₂ were significantly less than those of control rats, whereas SH had no significant effect in the oldest age group (SH21-25). Furthermore, less severe SH exposure (SH11-15, 13% O₂) had no significant effect on body weight compared with controls.

SH (11% O₂) exposure tended to increase baseline V̇O₂ and V̇CO₂ in all three age groups, except V̇CO₂ in SH21-25 was similar to that of age-matched control rats (Table 1). Baseline V̇e was also higher in SH11-15 and SH21-25 11% O₂-exposed rats compared with controls, whereas it was no different from controls in the younger (SH1-5) age group. The higher V̇e in SH1-5 rats was the result of a deeper breathing pattern, despite a significant reduction in fB. The higher baseline V̇e of SH21-25 rats was the result of a faster, deeper breathing pattern; SH1-5 rats also breathed faster than control rats, but failed to result in a significant change in V̇e.

13% O₂ exposure resulted in a significant increase in V̇O₂, whereas V̇E was reduced because of a slower breathing pattern. Baseline V̇e/V̇O₂ and V̇e/V̇CO₂ values of SH1-15 and SH21-25 rats were similar to those of control rats, whereas SH1-5 rats tended to hypoventilate with respect to V̇O₂ (reduced V̇e/V̇O₂) and V̇CO₂ (reduced V̇e/V̇CO₂). Less severe SH exposure in the intermediate age group also tended to result in a hypventilation.

Rates of metabolism and \( \dot{V}e \) during acute hypoxia. The magnitude of the acute HVR [expressed as a delta (Δ) baseline] following 11% O₂ exposure was similar to that of age-matched control rats in both SH1-5 and SH21-25 groups, whereas the HVR of SH11-15 rats was significantly reduced (Fig. 2A). The ΔV̇CO₂ during acute hypoxia was not different between control or SH-exposed rats in any age group (Fig. 2B). The magnitude of the hypoxic hyperventilation (increase in V̇e/V̇CO₂) in SH1-5 and SH21-25 was similar to that of control rats following 11% O₂ exposure, whereas SH11-16 rats failed to hyperventilate at all (Fig. 2C). In contrast, the magnitude of the HVR (Fig. 2A, cross-hatched bar) and the hyperventilatory response to acute hypoxia (Fig. 2C) in SH11-15 rats exposed to 13% O₂ were similar to those of age-matched controls and also significantly higher than 11% O₂-exposed rats.

The reduced HVR (Fig. 2) of SH11-15 rats following 11% O₂ exposure was the result of a significantly reduced V̇r (Fig. 3A) and fB (Fig. 3B) response. In contrast, the V̇r and fB response to acute hypoxia in SH1-5 and SH21-25 rats was similar to that of controls. Similarly, 13% O₂ exposure (SH11-15) also had no effect on the V̇r and fB response to acute hypoxia compared with control rats.

To determine whether SH modified variability of the respiratory pattern, we calculated the CV for fB during acute hypoxia. The CV of fB was elevated in SH1-5 and SH11-15 rats compared with corresponding control rats (Fig. 4), whereas SH21-25 rats were not different than controls. The CV of fB in 13% SH-exposed rats during acute hypoxia was less than that of 11% SH-exposed rats and also comparable to control rats (Fig. 4A, cross-hatched bar).

Ventilatory response to acute hypercapnia. The magnitude of the acute HCVR was enhanced in SH1-5 rats compared with controls (Fig. 5A); the larger HCVR was the result of a greater V̇r (Fig. 5B), whereas changes in fB during hypercapnia were similar between SH1-5 and control animals (Fig. 5C). The magnitude of the HCVR in SH21-25 rats was comparable to that of control animals (Fig. 5A), whereas it was attenuated in SH11-15 (Fig. 5A). Despite the attenuated HCVR in SH11-15 rats, there was a significantly increased V̇r and a reduced fB response (Fig. 5, B and C). Similarly, 13% SH exposure (P11-15) also resulted in a significantly smaller HCVR compared with control rats.

Mortality following SH exposure. We noted a considerable degree of unexplained mortality in SH11-15 rats, but not in the other age groups (Table 2). The majority of the rat pups spontaneously died at ~P18, corresponding to ~3 days after

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Body Weight, g</th>
<th>Tb, °C</th>
<th>V̇O₂, ml O₂·g⁻¹·min⁻¹</th>
<th>V̇CO₂, ml CO₂·g⁻¹·min⁻¹</th>
<th>V̇e, ml·g⁻¹·min⁻¹</th>
<th>fB, breaths/min</th>
<th>V̇r, ml/g</th>
<th>V̇e/V̇O₂</th>
<th>V̇e/V̇CO₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>NX6</td>
<td>13.3 ± 0.4</td>
<td>35.5 ± 0.3</td>
<td>0.037 ± 0.0003</td>
<td>0.020 ± 0.002</td>
<td>1.44 ± 0.09</td>
<td>157.9 ± 8.3</td>
<td>0.0092 ± 0.0006</td>
<td>39.3 ± 2.4</td>
<td>75.5 ± 6.6</td>
</tr>
<tr>
<td>SH1-5 (11%)</td>
<td>7.5 ± 0.4*</td>
<td>35.3 ± 0.4</td>
<td>0.081 ± 0.006*</td>
<td>0.040 ± 0.003*</td>
<td>1.54 ± 0.08</td>
<td>180.8 ± 8.9*</td>
<td>0.0086 ± 0.005</td>
<td>20.6 ± 2.3*</td>
<td>41.6 ± 4.8*</td>
</tr>
<tr>
<td>NX16</td>
<td>31.6 ± 1.5</td>
<td>36.5 ± 0.2</td>
<td>0.043 ± 0.002</td>
<td>0.024 ± 0.002</td>
<td>1.39 ± 0.08</td>
<td>142.3 ± 3.3</td>
<td>0.0098 ± 0.0005</td>
<td>32.6 ± 1.8</td>
<td>61.6 ± 4.7</td>
</tr>
<tr>
<td>SH11-15 (11%)</td>
<td>14.6 ± 0.3*</td>
<td>33.0 ± 0.7</td>
<td>0.057 ± 0.006*</td>
<td>0.031 ± 0.005*</td>
<td>1.59 ± 0.18</td>
<td>116.8 ± 12.0*</td>
<td>0.0138 ± 0.0009</td>
<td>29.4 ± 2.0</td>
<td>59.8 ± 6.4</td>
</tr>
<tr>
<td>SH11-15 (13%)</td>
<td>28.2 ± 1.3</td>
<td>37.4 ± 0.4</td>
<td>0.056 ± 0.004*</td>
<td>0.026 ± 0.002</td>
<td>1.17 ± 0.07*</td>
<td>131.8 ± 3.1*</td>
<td>0.0089 ± 0.0006</td>
<td>21.4 ± 1.3*</td>
<td>45.6 ± 2.7*</td>
</tr>
<tr>
<td>NX26</td>
<td>55.8 ± 4.6</td>
<td>37.3 ± 0.1</td>
<td>0.049 ± 0.007</td>
<td>0.030 ± 0.004</td>
<td>1.07 ± 0.08</td>
<td>141.6 ± 6.6</td>
<td>0.0075 ± 0.0002</td>
<td>23.7 ± 1.9</td>
<td>37.6 ± 2.6</td>
</tr>
<tr>
<td>SH21-25</td>
<td>51.9 ± 1.0</td>
<td>36.9 ± 0.2</td>
<td>0.057 ± 0.005*</td>
<td>0.032 ± 0.003</td>
<td>1.21 ± 0.05*</td>
<td>153.7 ± 2.1*</td>
<td>0.0079 ± 0.0003</td>
<td>22.7 ± 2.2</td>
<td>40.4 ± 2.2</td>
</tr>
</tbody>
</table>

Values are means ± 1 SE. Plethysmography was performed the day after their last day of sustained hypoxia (SH) exposure [postnatal day 6 (P6), 16, or P26; see Fig. 1]. Values are compared with corresponding age-matched rats raised in normoxia (i.e., NX6, NX16, and NX26). Values for SH11-15 rats exposed to less severe hypoxia (13% O₂) are also provided. Tb, body temperature; V̇O₂, O₂ consumption; V̇CO₂, CO₂ production; V̇e, ventilation; fB, breathing frequency; V̇r, tidal volume. *Significant difference from NX-raised rats (P < 0.05) for corresponding age group. Chamber temperature (~28°C) and humidity were maintained constant throughout the experiment.
being removed from SH and 2 days after measurements of plethysmography (P16). Approximately 83.8% (31 of 37 pups) of the SH11–15 rats died, whereas only 2.6% of SH1–5 rats (1 of 39 pups) died, but there was zero mortality in SH21–25 rats. The mortality consisted of both male and female rats, and, on one occasion, we observed an individual SH11–15 male pup die without any apparent reason during plethysmography. Death rarely occurred during the SH exposure, although a small percentage (7.7%) of the SH1–5 rats died during the SH exposure; a similar degree of mortality (~5%) was observed in normoxic rats at approximately the same age. Less severe SH exposure (13% O₂), however, did not result in any unexpected deaths. The cause of the high mortality in SH11–15 group remains unexplained.

The primary results of the present study indicate that neonatal SH exposure between P11 and P15 (SH11–15) attenuates the HVR and HCVR compared with normoxic-raised rats, whereas the same exposure paradigm in younger (SH1–5) or older (SH21–25) rats had no comparable effect. The most striking finding, however, was the substantial degree of mortality uniquely associated with the SH11–15 group. The mortality was spontaneous, inexplicable, and did not occur during the SH exposure period per se, but after ~3 days return to room air (~P18). Collectively, these data reveal a critical window of neonatal development in which the respiratory neural control system exhibits a heightened level of vulnerability to SH exposure. However, the vulnerability of the respiratory system and extent of mortality was dependent on the severity of SH exposure. The respiratory defects (which include a greater degree of respiratory variability) and mortality in these rats following SH exposure resemble several characteristics associated with sudden infant death syndrome (SIDS), including 1) impaired (cardio) respiratory function; 2) evidence of hypoxia; 3) a critical window of development; and 4) spontaneous, unexplained death (37). Here we propose a likely model of SIDS encompassing all three of the proposed risk factors thought to underlie the etiology of the syndrome.
1) a vulnerable neonate; 2) a critical period of postnatal development; and 3) a heightened sensitivity to an environmental stressor (37). The results of this study may provide insight into the interaction between sustained periods of hypoxia exposure and a vulnerable stage of postnatal development. Below we discuss the significance of these findings in regard to the effects of SH on development of the respiratory neural control system.

**Attenuation of the HVR and HCVR following neonatal SH.** In the present study, SH11–15 rats failed to increase V˙E during acute hypoxia, even if V˙E was normalized to metabolic V˙CO2 (V˙E/V˙CO2; Fig. 2). Since metabolic V˙CO2 was unchanged during acute hypoxia in SH11–15 treated rats, the lack of a HVR is likely the consequence of an SH-induced disturbance in normal neurodevelopmental processes, rather than a consequence of changes in metabolic rate that can sometimes occur in small newborn animals during acute hypoxia (21, 23). The first weeks of life represent an important period of development for the respiratory neural control system. The HVR matures during the first postnatal weeks (1, 18), which involves an increase in the carotid body sensitivity to acute hypoxia (11, 35) and is dependent on brain and glial derived neurotrophic factor synthesis (66). Previous studies have shown neonatal hypoxia exposure starting within days of birth attenuates the HVR, and the effects seem to persist into adulthood (5, 23, 27, 49, 53, 54). An attenuated HVR following neonatal SH could be related to developmental impairment of O2-sensing mechanisms (64). Neonatal SH exposure may represent an inflammatory stressor, leading to a cytokine-mediated inhibition of hypoxic chemosensory signaling in carotid body glomus cells (24). A disturbance in the carotid body sensitivity to hypoxia would interfere with an ability to mount an appropriate HVR. The precise effects of SH exposure on the respiratory control system in the present study, however, requires further investigation.

Substantial changes in brain stem neurochemistry (GABA, N-methyl-D-aspartate, glutamate, 5-HT, see below) also occurs during the first postnatal weeks in the rat (41, 42, 44) and might also be sensitive to SH exposure. Rats born at high altitude exhibit a marked (and transient) reduction in brain stem tyrosine hydroxylase activity between 2 and 3 wk of age that was not seen in sea level control rats (33). It was hypothesized that the SH associated with high altitude may cause a metabolic challenge to neural activity, thereby interfering with protein synthesis and activity in developing neurons. Indeed, there was also a transient reduction in brain metabolites during acute hypoxia that occurred in 9- to 13-day old rats, but not in older or younger rats (32). Interestingly, we did observe an increased baseline aerobic metabolism in SH11–15 rats compared with age-matched normoxic rats (Table 1), but it is unknown whether SH-induced changes in whole body metabolism also reflects changes in brain metabolism. The postnatal changes in the serotoninergic system (41, 42) may also be particularly relevant since we observed an attenuated HCVR (Fig. 5). Brain stem 5-HT raphe neurons are CO2/pH sensitive (59, 65) and...
SH1–5 rats compared with age-matched normoxic rats, which is an exception, however, the HCVR was actually enhanced in the intermediate age group. In contrast to the neonate, SH exposure impaired the HVR and HCVR of only the neonatal age group. SH exposure following neonatal SH also differentially affected the mortality rates for different age rats following NX exposure (Table 2). Less severe SH exposure (13% O2) during the same period, however, did not result in any mortality. Also, although plethysmography was only performed on male rats, the mortality rates here included the number of female rats in each litter.

Table 2. Mortality rates for different age rats following NX or SH exposure

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Pups, no.</th>
<th>Litters, no.</th>
<th>Mortality During Exposure, no.</th>
<th>Mortality After Exposure, no.</th>
<th>Mortality After Exposure, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>NX4</td>
<td>20</td>
<td>2</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>SH1–5 (11%)</td>
<td>39</td>
<td>5</td>
<td>3</td>
<td>0</td>
<td>2.6</td>
</tr>
<tr>
<td>NX16</td>
<td>32</td>
<td>4</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>SH11–15 (11%)</td>
<td>37</td>
<td>4</td>
<td>0</td>
<td>31</td>
<td>83.8</td>
</tr>
<tr>
<td>SH11–15 (13%)</td>
<td>17</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>NX26</td>
<td>24</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>SH21–25 (11%)</td>
<td>27</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Note the high percentage of rats that died after exposure to SH (11% O2) between P11 and P15 (SH11–15). In this group, 83.8% of rats died within 3 days of SH exposure being terminated. Less severe SH exposure (13% O2) during the same age period, however, did not result in any mortality. Also, although plethysmography was only performed on male rats, the mortality rates here included the number of female rats in each litter.

The mortality we observed in the SH11–15 group of rats did not occur during the exposure period per se, but rather occurred spontaneously several days after recovery in room air (~P18). Although it was associated with an attenuated HVR and HCVR (at P16), the cause of the mortality is unclear, and it is difficult to pinpoint whether impairment in hypoxia (e.g., carotid body or hypercapnia) sensing mechanisms can by themselves explain the mortality. Other than smaller body weights, the only noticeable result of SH was an apparently smaller amount of milk in the stomach compared with normoxia rats. However, since all pups exposed to SH, regardless of age, contained a reduced amount of milk in their stomach, it is difficult to determine whether the lack of adequate nutritional requirements could explain the mortality or the respiratory disturbances observed in the present study. It is also difficult to determine whether there was a primary effect of SH exposure on the dams, although we still observed an attenuated HVR, HCVR, and mortality in two SH11–15 litters in which the dams were rotated with normoxia-exposed dams midway into the exposure period.

A critical window. The attenuation of the HVR and HCVR in the SH11–15 group (but not the other age groups that received SH) could be the result of a disturbance in the naturally occurring, abrupt, developmental changes in brain stem neurochemistry that occurs at ~P12 (i.e., within the critical window of SH exposure in the present study; Refs. 41–44, 67). Disruption of brain stem neurochemical signaling during this key period of development could modify HVR and HCVR. An effect of SH on the serotonergic system is an attractive hypothesis, since we observed an attenuated HCVR in the SH11–15 group, and there is a dramatic change in serotonergic expression that occurs in the brain stem at a similar time point (67). Interestingly, brain stem deficits in the serotonergic system have been implicated in SIDS (16, 37), which may be particularly pertinent to the present study, since we also observed a substantial degree of unexplained mortality in the SH11–15 rats (see below).

The neurochemical adjustments also roughly correspond with expression of functional phenomena related to respiratory control. The ventilatory response to acute hypoxia is suddenly and transiently lost at ~P13 in rats, but returns to “normal” levels the following day (40a). The peak HVR at P10 doubled in magnitude by P15, and the N-methyl-D-aspartate receptor blocker (MK-801) injected into the nucleus of the solitary tract of neonatal rats did not affect baseline Ve until P15 (52). Furthermore, CIH exposure from P10–25 significantly impairs spatial learning and causes hyperactivity in rats (61). Thus the consistent observation that critical periods of development exist in which the respiratory (and other) neural control system may be at a heightened level of vulnerability (67) may explain why SH exposure during such a period elicits unique responses not seen at earlier or later stages of development.

Mortality. Perhaps the most striking result of the present study was the substantial degree of mortality in the SH11–15 rats compared with all other age and treatment groups. The high mortality was not observed in the younger (SH1–5) or older (SH21–25) rats exposed to the same stimulus of hypoxia. To our knowledge, this is the first reported example of an animal model in which mortality was associated with SH exposure specifically during a unique period of development. Indeed, several studies have investigated the effects of SH exposure in animal models starting early after birth, and some even extend the exposure period into adulthood without any appreciable incidences of mortality. In a previous study, however, SH exposure that started at P1 resulted in a higher degree of mortality than if it were implemented at P5; incidentally, the incidence of mortality was fairly minimal until at ~P13, at which time the survival rates dropped precipitously (47). In contrast to that study, however, we did not observe any alarming amount of mortality in SH1–5 rats.

The mortality we observed in the SH11–15 group of rats did not occur during the exposure period per se, but rather occurred spontaneously several days after recovery in room air (~P18). Although it was associated with an attenuated HVR and HCVR (at P16), the cause of the mortality is unclear, and it is difficult to pinpoint whether impairment in hypoxia (e.g., carotid body or hypercapnia) sensing mechanisms can by themselves explain the mortality. Other than smaller body weights, the only noticeable result of SH was an apparently smaller amount of milk in the stomach compared with normoxia rats. However, since all pups exposed to SH, regardless of age, contained a reduced amount of milk in their stomach, it is difficult to determine whether the lack of adequate nutritional requirements could explain the mortality or the respiratory disturbances observed in the present study. It is also difficult to determine whether there was a primary effect of SH exposure on the dams, although we still observed an attenuated HVR, HCVR, and mortality in two SH11–15 litters in which the dams were rotated with normoxia-exposed dams midway into the exposure period.
contributes to sustained hypoxia and enhances respiratory drive, contributing physiological impairments, although there is some data suggesting removal of chemoreflex control of breathing may be lethal in some instances (45). Denervation of the rat carotid body at P7–8 resulted in a higher incidence of mortality than if older animals were denervated (62). A similar scenario was observed in carotid body denervated pigs (9, 26) and lambs (8). There was a higher degree of mortality in neonatal piglets denervated at P15 vs. P10, and death occurred within 4–7 days after CB denervation (26). Surgical denervation of the carotid bodies also profoundly disturbed the arousal response to acute hypoxia in sleeping dogs (7), and surviving denervated piglets exhibited pronounced apnea and hypoven-
tilation (13). The latter could be consistent with our observation of an increased respiratory variability (CV) in the SH1,1–15 rats before they died. However, respiratory variability may not be a primary determinant that would explain the mortality, since SH1,5 rats also had a more variable respiratory pattern, but survived through to, and beyond, weaning. Finally, loss of central nervous system serotonin in mice has also been shown to be postnatally lethal (31). Whether the effects of SH during the critical period of development (P11–15) in the present study results in impaired carotid body function and/or a deficit in brain stem neurochemistry is an attractive hypothesis that warrants further investigation.

**Summary and significance.** We have shown that a critical period of development in the rat (P11–15) seems to be at a heightened level of vulnerability to SH exposure, and it coincides with a period during which key stages of respiratory and central nervous development are taking place. The unexplained mortality and impaired respiratory responses to acute hypoxia and hypercapnia challenge are additional characteristics that share similarities to the proposed risk factors thought to underlie the etiology of SIDS. These data may also begin to offer an understanding of the consequences of prolonged hypoxia exposure during certain stages of development that can occur with cyanotic heart disease and high-altitude exposure.

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**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the author(s).

**AUTHOR CONTRIBUTIONS**

Author contributions: C.A.M. and P.M.M. performed experiments; C.A.M., J.M.D.F., R.J.M., and P.M.M. edited and revised manuscript; C.A.M., J.M.D.F., R.J.M., and P.M.M. approved final version of manuscript; C.A.M., J.M.D.F., R.J.M., and P.M.M. edited and revised manuscript; C.A.M., and P.M.M. conception and design of research; P.M.M. analyzed data; J.M.D.F., R.J.M., and P.M.M. approved final version of manuscript; J.M.D.F., J.M.D.F., R.J.M., and P.M.M. edited and revised manuscript; C.A.M.

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