Carotid sinus nerve stimulation, but not intermittent hypoxia, induces respiratory LTF in adult rats exposed to neonatal intermittent hypoxia

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Julien CA, Niane L, Kinkead R, Bairam A, Joseph V. Carotid sinus nerve stimulation, but not intermittent hypoxia, induces respiratory LTF in adult rats exposed to neonatal intermittent hypoxia. Am J Physiol Regul Integr Comp Physiol 299: R192–R205, 2010. First published April 21, 2010; doi:10.1152/ajpregu.00707.2009.—We tested the hypothesis that exposure to neonatal intermittent hypoxia (n-IH) in rat pups alters central integrative processes following acute and intermittent peripheral chemoreceptor activation in adults. Newborn male rats were exposed to n-IH or normoxia for 10 consecutive days after birth. We then used both awake and anesthetized 3- to 4-mo-old rats to record ventilation, blood pressure, and phrenic and splanchnic nerve activities to assess responses to peripheral chemoreceptor activation (acute hypoxic response) and long-term facilitation (LTF, long-term response after intermittent hypoxia). In anesthetized rats, phrenic and splanchnic nerve activities and hypoxic responses were also recorded with or without intact carotid body afferent signal (bilateral chemodenervation) or in response to electrical stimulations of the carotid sinus nerve. In awake rats, n-IH alters the respiratory pattern (higher frequency and lower tidal volume) and increased arterial blood pressure in normoxia, but the ventilatory response to repeated hypoxic cycles was not altered. In anesthetized rats, phrenic nerve responses to repeated hypoxic cycles or carotid sinus nerve stimulation were not altered by n-IH; however, the splanchnic nerve response was suppressed by n-IH compared with control. In control rats, respiratory LTF was apparent in anesthetized but not in awake animals. In n-IH rats, respiratory LTF was not apparent in awake and anesthetized animals. Following intermittent electrical stimulation, however, phrenic LTF was clearly present in n-IH rats, being similar in magnitude to controls. We conclude that, in adult n-IH rats: 1) arterial blood pressure is elevated, 2) peripheral chemoreceptor responses to hypoxia and its central integration are not altered, but splanchnic nerve response is suppressed, 3) LTF is suppressed, and 4) the mechanisms involved in the generation of LTF are still present but are masked most probably as the result of an augmented inhibitory response to hypoxia in the central nervous system.

neonatal intermittent hypoxia; long-term facilitation

DEVELOPMENT OF THE RESPIRATORY control system is tightly regulated by complex interactions between genetic and environmental factors that contribute to shape the mature phenotypic expression of this neural network (4). Over the past years, several studies have documented that alterations in environmental oxygen levels during development lead to important changes of the respiratory control system (4). A particular importance has been given to intermittent hypoxic exposure (38, 47, 50) because it may be used in laboratory animals to reproduce some of the effects encountered in human newborn suffering from apneas, which remains one of the major causes of morbidity in preterm neonates (10). Recent studies have shown that neonatal intermittent hypoxia (n-IH) exposure for 10 days after birth enhances the carotid body response to hypoxia as recorded using the in vitro carotid body/carotid sinus nerve (CSN) preparation in 2-mo-old rats (47). It has also been reported that rats raised during 30 days from birth under IH have elevated minute ventilation, but showed a blunted respiratory response to hypoxia and reduced expression of respiratory plasticity such as the one evoked by successions of hypoxic episodes (50) [long-term facilitation (LTF); see Refs. 40 and 41], which might seem contradictory with an enhanced response of peripheral chemoreceptor. However, these apparently conflicting results are difficult to reconcile because of the different protocols of hypoxic exposure used. From a mechanistic perspective, we do not know whether the long-term consequences of n-IH also involve central integration processes.

We have recently reported in rat pups that n-IH during 10 days from birth increases ventilatory response to brief (50 s) hypoxic exposure (25), strongly suggesting higher sensitivity of peripheral chemoreceptors as reported by in vitro recordings (47), while the ventilatory LTF was suppressed (25), as reported by Reeves et al. (50) in 2-mo-old rats raised under n-IH for 30 days from birth. Accordingly, this model might be useful to determine whether n-IH-related alterations in respiratory regulation persist into adulthood and alter the central integration of peripheral chemoreceptor inputs. We also hypothesized that n-IH induces long-lasting alterations of sympathetic and hemodynamic control.

To address these issues, male rats were exposed to n-IH for 10 days from birth and were then raised under normoxia until reaching adulthood (3–4 mo). By recording respiratory, hemodynamic, and sympathetic (splanchnic nerve) activity in both awake and anesthetized adult rats, we asked whether n-IH alters: 1) acute responses to brief and intense hypoxia, as an index of peripheral chemoreceptor activation and central integration processes and 2) LTF induced by repeated exposures to brief hypoxic cycles, as an index of “plasticity” of the central respiratory control system. Next, in anesthetized rats, we asked whether n-IH exposure induces long-term alterations of respiratory and sympathetic responses 3) to acute electrical stimulation of the CSN and 4) to induction of LTF by repeated electrical stimulation of the CSN.

MATERIALS AND METHODS

Animals and General Experimental Design

All experimental protocols were approved by the local committee for animal ethics of Laval University and were in accordance with the guidelines of the Canadian Council of Animal Care. Sixty-four male Sprague-Dawley rats born in our animal care facility were exposed to either n-IH or ambient air (control) for 10 consecutive days starting at postnatal day 1 as previously described (25). We used adult male rats.
because they showed stronger hemodynamic and respiratory alterations than adult females after n-IH exposure (26).

The profile of n-IH exposure is depicted in Fig. 1. It consisted of six consecutive hypoxic cycles (every 10 min for a total of 1 h) during which oxygen in the chamber dropped from 21 to 5% in 100 s and then rapidly returned to 21%. This was followed by 1 h in normoxia. This profile was repeated 24 h/day between postnatal days 1–10. Control rats were placed in an adjacent chamber that was left open to room air.

At the end of the postnatal exposure, rats were returned to the animal care facility and raised under normal conditions. After weaning (at 22 days), rats were housed with two animals per cage until reaching 3 mo of age. Next, we recorded respiratory, hemodynamic, and sympathetic responses to acute and intermittent hypoxic (to evaluate LTF; see Fig. 1) exposures in awake or anesthetized rats. In anesthetized rats, the role of peripheral chemoreceptors in these responses was evaluated using bilateral chemodenervation or CSN stimulation (without hypoxia).

**Specific Experimental Details**

**Ventilatory and hemodynamic recordings in awake adult rats.** We used 28 male rats (15 control and 13 n-IH) from seven virgin females (Charles Rivers, St. Constant, Québec, Canada). Before the recordings (5–7 days), adult rats were anesthetized with 3% isoflurane in 30% O2 balanced with nitrogen. A mixed PE-10/PE-50 polyethylene catheter containing heparinized saline (200 UI/ml) was inserted in the abdominal aorta via the left femoral artery. Lidocaine was applied topically for its anesthetic and vasodilator effects. The catheter was exteriorized at the back of the skull and fixed to the skin. Body temperature was maintained constant (37°C) throughout the surgery using a homeothermic blanket (Harvard Instruments, Holliston, MA). Postsurgery analgesia (0.2 mg/kg buprenorphine 0.2 mg/kg 2 times/day) and antibiotic (5 mg/kg baytril in lactate ringer, one time/day) were injected subcutaneously for 3 days following surgery. The animals were housed individually in regular cages after surgery, and the antibiotics (5 mg/kg buprenorphine 0.2 mg/kg 2 times/day) and analgesia (0.2 mg/kg buprenorphine 0.2 mg/kg 2 times/day) were injected subcutaneously for 3 days following surgery. The animals were housed individually in regular cages after surgery, and the catheter was flushed daily with saline to maintain patency. On the day of recordings, the arterial catheter was connected to a pressure transducer via a PE-50 polyethylene catheter filled with saline. The signal from the pressure transducer was amplified (gain = 100; Transbridge TBM4M-B; World Precision Instrument, Sarasota, FL) before acquisition.

Ventilation was recorded using a double-chamber plethysmograph (model PLY 3023; Buxco Electronics, Sharon, CT). The gas flows through the front (head) and rear chambers were set at ≈1 l and 0.5 l/min, respectively. The respiratory flow trace, recorded from the rear chamber, was calibrated by injecting a known volume of air in the chamber. Air flow through the front chamber was continuously measured with a mass flowmeter (TSI series 4140; TSI, Shoreview, MN), and outflowing oxygen (O2 out) was monitored with a gas analyzer (AEI technologies, Pittsburgh, PA) for calculation of oxygen consumption (V˙O2) with an open system.

The measurements were digitized (ventilation and blood pressure at 500 Hz; gas and flow at 10 Hz) with on-line calculations (IOX software; Emka Technologies, Paris, France) by a computer (PC compatible) of respiratory parameters throughout the experiments [minute ventilation (V˙E), respiratory frequency (fR), and tidal volume (VT)], hemodynamic parameters [systolic, diastolic (DAP), and mean (MAP) arterial blood pressure and heart rate], and V˙O2 = 20.9% O2 out × flow.

Rats were left undisturbed in normoxia (21% of O2) during at least 30 min for stabilization before starting the experiment. After 5 min of baseline recording, rats were exposed to 10 consecutive poikilocapnic hypoxic cycles by switching air to nitrogen during 60 s. During each cycle, O2 dropped from 21 to 5% in 60 s and then returned to 21% O2 in 100 s. This was followed by 5 min in normoxia before the next cycle (see Fig. 1). After the completion of the 10 cycles, rats were left undisturbed under normoxia for 2 h (recovery period). Each recording was visually examined to ensure that the animal was quiet, and recordings were analyzed second by second by IOX. We report the average of V˙E, mean arterial pressure, and heart rate responses for the last five cycles (during cycles 1–5, body movements impeded correct analysis) at inspired O2 concentrations of 20, 15, 12, 10, 8, and 5% (averaged over 1 s for each level) and then during recovery at 0, 15, 30, 60, and 120 min (averaged over 5 min for each time). This protocol allowed assessing ventilatory and cardiovascular responses to both acute hypoxic cycles and during the recovery period.

**Recordings of inspiratory and sympathetic activity in anesthetized adult rats.** Phrenic and splanchnic nerve activities were recorded in 36 anesthetized rats (18 control and 18 n-IH) born from 10 virgin females (Charles Rivers, St. Constant, Quebec, Canada). They were anesthetized using isoflurane (3.5% in 35% O2 balanced nitrogen), tracheotomized, mechanically ventilated (model 683; Harvard Instruments; 193).
frequency: 55 ± 1 beats/min, volume: 2.92 ± 0.04 ml), and bilaterally vagotomized to prevent entrainment of inspiratory motor output with the ventilator. During all of the experiments, rectal temperature (measured with a rectal probe for rats) was maintained at 37°C using a homeothermic blanket (Harvard Instruments). The end-tidal partial pressure of carbon dioxide (PETCO₂) was measured in the expired line of the ventilatory circuit using a capnograph (model 1265; Novametrix, Wallingford, CT). The left femoral artery was catheterized for blood pressure monitoring and blood sampling for arterial blood gas analysis [PaO₂, PaCO₂, arterial pH (pHₐ), HCO₃⁻; corrected for measured rectal temperature; ABL-5; Radiometer, Copenhagen, Denmark]. The left femoral vein was catheterized for drug injection. The right postganglionic splanchnic nerve was isolated unilaterally using a retroperitoneal approach, placed on a bipolar silver recording electrode (tefloned silver 0.10 in. diameter, stripped at the point of contact of the wire with the splanchnic nerve; World Precision Instruments), and enrobed with silicone cement (World Precision Instruments). The electrode was exteriorized, and the abdomen was sutured. The right phrenic nerve was isolated unilaterally using a dorsal approach, cut distally, placed on a bipolar silver recording electrode, and enrobed with silicone cement. The signals from the phrenic and splanchnic nerves were amplified (gain = 10,000; model 1700; AM-System, Everett, WA), band-pass filtered (0.1–10 and 0.01–10 kHz, respectively), and fed to a moving averager (100 and 200 ms, respectively; model MA-821RSP; CWE, Ardmore, PA). The signal from the blood pressure transducer was amplified (gain = 100; Transbridge TBM4M-B; World Precision Instrument). Next, all signals were digitized and recorded with a data acquisition system (model DI-720; Dataq Instruments, Akron, OH).

After the surgical preparation, the rats were slowly converted from isoflurane to urethane anesthesia (1.6 g/kg iv in saline, duration 10–20 min) and paralyzed with pancuronium bromide (2.5 mg/kg iv). The depth of anesthesia was evaluated by assessing arterial blood pressure responses to hind paw pinches, and supplemental urethane was injected if necessary (0.4 g/kg). The lungs were hyperinflated roughly once every 30 min to prevent alveolar atelectasis. The carbon dioxide apneic threshold for inspiratory phrenic activity was determined by mechanically decreasing ventilator frequency until the phrenic signal appeared. All experiments were performed while maintaining PETCO₂ 3 mmHg above this threshold by adjusting ventilator frequency. Phrenic and splanchnic electroneurograms and arterial blood pressure were allowed to stabilize under 50% O₂ in nitrogen for at least 60 min before initiating the baseline recording for 5 min. Rats were then exposed to 10 consecutive hypoxic cycles by replacing oxygen by nitrogen for 60 s. During each cycle, O₂ rapidly dropped from 50 to

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**Fig. 2.** Respiratory, metabolic, and hemodynamic values in awake controls (n = 15) and n-IH (n = 13) adult male rats. A: minute ventilation (V˙E), respiratory frequency (fₐ), and tidal volume (V₉). bpm, Beats/min. B: oxygen consumption (V˙O₂) and oxygen convection ratio (V˙E/V˙O₂). C: mean arterial blood pressure (MAP), diastolic arterial blood pressure (DAP), systolic arterial blood pressure (SAP), and heart rate (HR). All values are means ± SE. *P < 0.05 vs. controls.
12% in 60 s and then returned to 50% in 60 s, followed by 5 min at 50% before the next cycle (see Fig. 1; control vs. n-IH; n = 6/group).

In a separate group of animals (6 control, 6 n-IH), each carotid bifurcation was exposed during the surgical preparation, and the sinus nerves were cut at the junction with the glossopharyngeal nerve for bilateral chemodenervation. The rats were then exposed to 10 consecutive hypoxic challenges followed by 1 h of recovery. Finally, in six control and six n-IH rats, one of the sinus nerves was cut unilaterally proximal to the carotid body and was placed on a stimulating electrode. Responses to 10 consecutive stimulations of the CSN under 50% O2 were then recorded. Each stimulation lasted 45 s at 20 Hz followed by 5 min before the next stimulation. Simulation current was determined as being three times higher than the threshold that elicits a phrenic response, as previously described (28). This was followed by recovery for 1 h under 50% O2.

We analyzed the integrated burst frequency and amplitude for phrenic nerve activity, the integrated amplitude for splanchnic nerve activity, and the MAP. For intact and chemodenervated rats, responses to repeated hypoxic cycles were assessed by measuring each parameter before the peak, at peak response, and at return to the basal value for each hypoxic challenge. Similar data analysis was performed in rats exposed to electrical stimulation, for each stimulation. In all groups, 5-min epochs were analyzed at baseline and at 0, 15, 30, and 60 min during the recovery period. At each time point, a blood sample was taken for measurement of arterial blood gases to verify isocapnia. For phrenic and splanchnic amplitudes, background noise was estimated by the integrated value measured in animals after death and subtracted to analyze data. Phrenic and splanchnic amplitudes were expressed in relative values as a percent of the baseline value.

**Statistics**

All results were expressed as means ± SE. Data analysis was performed using Sigmaplot software (version 11.0; Systat Software, Chicago, IL). Because of small sample sizes, we used nonparametric tests to avoid potential errors due to reduced accuracy and stringency of parametric tests in these conditions. n-IH effects on baseline parameters were assessed by a Mann-Whitney U-test (rank sum test). For repeated measures, we used a Friedman repeated-measures ANOVA on ranks. When the Friedman analysis gave a P value <0.05, we used a Wilcoxon signed rank test to compare each individual time point vs. baseline. Differences between control and
n-IH rats at each time point were tested using the Mann-Whitney U rank sum test. The significance level was set at \( P < 0.05 \).

When values are presented as percent changes from baseline, we applied the following: \((\text{value/baseline value}) \times 100\). For arterial blood pressure and heart rate (Fig. 4), baseline is the value at 20% \( O_2 \) averaged over the five last hypoxic cycles (see above). Otherwise, baseline is the value in normoxia before the hypoxic cycles.

**RESULTS**

At weaning (21 days old) n-IH rats had lower body weight than control rats \((49 \pm 1 \text{ vs. } 61 \pm 1 \text{ g}; P < 0.001)\), but this difference was not present at 60 days \((361 \pm 5 \text{ vs. } 372 \pm 5 \text{ g}; P = 0.12)\) and 90 days \((509 \pm 10 \text{ vs. } 494 \pm 8 \text{ g}; P = 0.49)\) of age.

**Awake Adult Rats**

Baseline. Respiratory and hemodynamic parameters recorded under baseline conditions are shown in Fig. 2. Although \( V_{\text{E}} \) was similar between control and n-IH rats, n-IH rats had a different ventilatory pattern with a higher \( f_R \) and a lower \( V_T \) compared with control rats. \( V_{O_2} \) rate was lower in n-IH rats compared with controls, but the oxygen convection ratio was similar between groups. In n-IH rats, mean blood pressure was significantly higher than control rats \((P < 0.01)\) because of higher diastolic blood pressure, whereas heart rate was reduced in n-IH rats compared with controls (Fig. 2).

Responses to repeated hypoxic cycles. Respiratory and cardiac responses to repeated hypoxic cycles are reported for five control and six n-IH rats; in other animals, body movements during exposures impeded proper analysis. The profile of hypoxic exposure in the plethysmograph is represented in Fig. 3A. This, however, is \( O_2 \)% in the outflowing line from the chamber and is slightly delayed compared with the actual inspired \( O_2 \) level. The ventilatory response in awake rats was similar between control and n-IH (Fig. 3, B–D). \( V_{\text{E}}, f_R, \) and \( V_T \) increased as \( O_2 \) decreased, with a maximal value observed 30–35 s after the beginning of hypoxia. \( V_{\text{E}} \) then decreased gradually in both groups. During the decrease in \( O_2 \), mean arterial pressure increased sharply and then dropped below baseline (Fig. 3E). Peak arterial pressure response occurred 40–50 s after the beginning of hypoxia, i.e., at a lower \( O_2 \) level than the peak for \( V_{\text{E}} \). Mean arterial pressure returned to the baseline value during return to normoxia in both control and n-IH rats. Heart rate increased progressively in control rats, reaching a peak at the lowest \( O_2 \) level (Fig. 3F); contrastingly, heart rate decreased slightly (although not significantly) in n-IH rats and rose significantly at the lowest \( O_2 \) level. Arterial pressure and heart rate responses were also reported as normalized values (Fig. 4, A and B; in this case, baseline being the value at 20% \( O_2 \)). There was no group difference in the arterial pressure and heart rate responses when analyzing these data.

Recovery period. During recovery, there was no significant modification of \( V_{\text{E}} \) compared with baseline in either control or n-IH rats (Friedman tests: \( P = 0.94 \) and \( P = 0.22 \), respectively; data not shown). Similarly, \( f_R, V_T, \) and \( V_{O_2} \) remained stable during this period in both groups (Friedman tests in control and n-IH: \( P = 0.37 \) and 0.12 for \( f_R; P = 0.98 \) and 0.65 for \( V_T; P = 0.32 \) and 0.28 for \( V_{O_2} \), respectively; data not shown). Mean arterial pressure was higher at each time point during recovery in n-IH compared with control rats (Mann-Whitney test \( P < 0.05 \); Fig. 5); however, it decreased progressively in n-IH rats with a slope of \(-0.67 \text{ mmHg/min} (r^2 = 0.93)\) to reach 125 ± 6 mmHg at the end of the recovery period (Friedman test \( P < 0.01 \)). In parallel, heart rate also decreased throughout the recovery period with a slope of \(-1.05 \text{ beats/min} (r^2 = 0.82)\). In control rats, blood pressure (Friedman tests \( P = 0.12 \)) and heart rate (\( P = 0.32 \)) remained unchanged during recovery.

**Anesthetized Animals**

Arterial blood gas. \( P_{O_2}, P_{CO_2}, pH_a, \) and \( HCO_3^- \) were measured during baseline and at 0, 15, 30, and 60 min during the recovery period in anesthetized rats exposed either to hypoxic challenges with intact (Table 1) or denervated (Table 2) carotid bodies or to CSN stimulations (Table 3). In CSN-intact n-IH animals, \( pH_a \) and \( HCO_3^- \) rose above baseline levels during recovery, reaching higher levels than controls (Table 1), which may indicate alterations of blood and/or kidney buffering function. In the other experiments, \( P_{O_2}, pH_a, \) and \( HCO_3^- \) were not
significantly different between control and n-IH rats throughout the experiment. As expected based on our control of the end-tidal CO₂, PaCO₂ did not change during the recovery period in any groups despite the fact that baseline PaCO₂ in the stimulation experiment was higher in n-IH than in control rats (Table 3).

**Basal arterial pressure.** Mean arterial pressure was significantly higher in n-IH rats (92 ± 4 mmHg, n = 6) compared with control (83 ± 4 mmHg, P = 0.04, n = 6). This difference was not observed in anesthetized control or n-IH rats following bilateral chemodenervation (96 ± 7 vs. 99 ± 3 mmHg, n = 6 for both groups) or unilateral carotid body denervation for electrical stimulations of the CSN (96 ± 6 vs. 95 ± 6 mmHg, n = 6).

**Response to repeated hypoxic cycles.** Throughout the hypoxic cycles, phrenic burst frequency increased in n-IH and control intact rats (Fig. 6A; Friedman P < 0.01 in both groups), with no difference observed between groups. During return to normoxia in control rats, phrenic burst frequency decreased below the baseline value, corresponding to a posthypoxic frequency decline (6). This response was not apparent in n-IH rats. Integrated phrenic amplitude increased also during hypoxic cycles (Fig. 6B; Friedman P < 0.01 in both groups), with no significant differences between control and n-IH. Note that, in control animals, normoxic integrated phrenic amplitude increased slightly toward the end of the hypoxic cycles (although this is only significant between cycles 7 and 8), likely representing the onset of LTF. The integrated splanchnic nerve amplitude increased during hypoxic exposure in control rats, but this response was drastically attenuated in n-IH rats (Fig. 6C; Friedman P < 0.01 in both groups). Mean blood pressure decreased during hypoxic cycles with similar amplitude (from 93 ± 4 to 59 ± 5 in control, and 99 ± 5 to 55 ± 4 mmHg in n-IH rats; P = 0.15, Mann-Whitney n-IH vs. control in hypoxia).

**Recovery period.** Figure 7, A–C, shows the experimental profile and typical raw recordings, in control and n-IH rats, of integrated phrenic and splanchnic nerve activities and blood pressure. As apparent on the phrenic neurogram, integrated phrenic amplitude increased gradually during recovery in intact control rats (Fig. 7B), but this was not observed in intact n-IH rats (Fig. 7C). As shown in Fig. 7D, at the end of the recovery period, phrenic nerve amplitude was 75 ± 26% higher than at baseline in controls but remained unchanged in n-IH. Integrated splanchnic amplitude in intact control rats remained slightly (but not significantly) above the baseline level during recovery (Fig. 7, B and D). In n-IH rats, however, the integrated splanchnic amplitude decreased gradually, reaching 59 ± 9% of the baseline value at the end of the recovery period (Fig. 7, C and D). Mean blood pressure remained at the baseline level during recovery, with no difference between control and n-IH rats (Fig. 7, B–D).

To take into account the entrainment of splanchnic nerve activity by the respiratory network (17, 19, 42, 43), we also analyzed cycle-triggered histograms by dividing splanchnic activity according to the phase of the respiratory cycle into first and second halves of inspiration and first and second halves of expiration (8), such as previously used to demonstrate a coupling of splanchnic activity to phrenic LTF (9).

In control rats, splanchnic nerve activity was at its lowest level during inspiration and reached a peak during the first half of expiration (cf. Fig. 8A). At the end of the recovery period, the expiratory peak of splanchnic activity of n-IH was specifically increased compared with baseline conditions (Fig. 8, A and C). In n-IH rats contrastingly, under baseline conditions, the expiratory peak was of lower magnitude than in controls.

![Graph](http://ajpregu.physiology.org/)

**Fig. 5.** MAP during the recovery period in awake n-IH or control adult male rats. All values are averaged over 5 min and expressed as means ± SE. P < 0.05 vs. control (*) and vs. baseline (†).

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**Table 1. Arterial blood gas, pH, and base excess during baseline and recovery period after 10 hypoxic challenges in anesthetized adult male rats with intact carotid body**

<table>
<thead>
<tr>
<th>Arterial Blood Gas</th>
<th>Recovery Period Time, min</th>
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<tbody>
<tr>
<td></td>
<td>Baseline</td>
</tr>
<tr>
<td>PaO₂, mmHg</td>
<td></td>
</tr>
<tr>
<td>CONT</td>
<td>195 ± 10</td>
</tr>
<tr>
<td>n-IH</td>
<td>209 ± 13</td>
</tr>
<tr>
<td>PaCO₂, mmHg</td>
<td></td>
</tr>
<tr>
<td>CONT</td>
<td>45 ± 2</td>
</tr>
<tr>
<td>n-IH</td>
<td>44 ± 3</td>
</tr>
<tr>
<td>pHₐ</td>
<td></td>
</tr>
<tr>
<td>CONT</td>
<td>7.323 ± 0.010</td>
</tr>
<tr>
<td>n-IH</td>
<td>7.327 ± 0.011</td>
</tr>
<tr>
<td>HCO₃⁻, mmol/l</td>
<td></td>
</tr>
<tr>
<td>CONT</td>
<td>22 ± 1</td>
</tr>
<tr>
<td>n-IH</td>
<td>23 ± 1</td>
</tr>
</tbody>
</table>

Data are represented as means ± SE in rats exposed to neonatal intermittent hypoxia (n-IH; n = 6) or ambient air (CONT; n = 6) during the postnatal period. PaO₂, arterial pressure in oxygen; PaCO₂, arterial pressure in carbon dioxide; pHₐ, arterial pH; HCO₃⁻, arterial bicarbonate concentration. P < 0.05 vs. control (*) and vs. baseline (†).
anesthetized adult male rats with denervated carotid body (Fig. 9, in control and n-IH rats following bilateral chemodenervation recordings of integrated phrenic and splanchnic nerve activities. Carotid Body Denervation and CSN Stimulation: significant (Fig. 8).

In chemodenervated control and n-IH rats, as apparent from the neurograms, the phrenic responses to repeated hypoxic cycles and expiratory splanchnic activity at the end of the recovery period, integrated phrenic amplitude did not change in n-IH and control rats (Fig. 10, A and B). In n-IH rats, this response was not observed, as reported after hypoxic stimulation. The splanchnic response to hypoxia was suppressed in control chemodenervated rats (Fig. 10E), and integrated splanchnic amplitude decreases during CSN electrical stimulation with similar magnitude in n-IH and control rats (Fig. 10, E and F).

Recovery period. In chemodenervated rats during the recovery period, integrated phrenic nerve amplitude did not change in either control or n-IH rats (Fig. 11, A and B; Friedman P = 0.19 and 0.10, respectively). Following electrical CSN stimulations, integrated phrenic amplitude increased with similar magnitude in control and n-IH rats.

Splanchnic nerve activity was unaltered during recovery in chemodenervated n-IH and control rats (Fig. 11, C and D; Friedman P = 0.96 and 0.09, respectively). Following CSN stimulations, integrated splanchnic amplitude remained stable in control rats (Fig. 11C; Friedman P = 0.39). In n-IH rats, it decreased at the onset of the recovery period and then increased in control and n-IH rats. The phrenic response produced by CSN electrical stimulation appears slightly lower than hypoxic stimulation, but this was not significant in either group. Of note, the post-CSN stimulation frequency decline was still present after CSN electrical stimulation in control but was also slightly reduced compared with hypoxic stimulation (Fig. 10, A and B). In n-IH rats, this response was not observed, as reported after hypoxic stimulation.

Figure 9 shows the experimental profiles and typical raw recordings of integrated phrenic and splanchnic nerve activities in control and n-IH rats following bilateral chemodenervation (Fig. 9, A–C) or in response to CSN stimulation (Fig. 9, D–F). In chemodenervated control and n-IH rats, as apparent from the neurograms, the phrenic responses to repeated hypoxic cycles and LTF were both suppressed (Fig. 9, B–C). On the contrary, electrical stimulation of the CSN restored acute responses and LTF both in control and n-IH rats (Fig. 9, E and F). Detailed group data are provided in Figs. 10 (responses to acute stimuli) and 11 (recovery).

Response to Acute Repeated Stimuli

Responses of phrenic burst frequency (Fig. 10, A and B) and amplitude (Fig. 10, C and D) to CSN simulation were similar in control and n-IH rats.

Table 2. Arterial blood gas, pH, and base excess during baseline and recovery period after 10 hypoxic challenges in anesthetized adult male rats with denervated carotid body

<table>
<thead>
<tr>
<th>Arterial Blood Gas</th>
<th>Baseline</th>
<th>0</th>
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<th>30</th>
<th>60</th>
</tr>
</thead>
<tbody>
<tr>
<td>PaO₂, mmHg</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CONT</td>
<td>215 ± 8</td>
<td>168 ± 9†</td>
<td>204 ± 12</td>
<td>188 ± 15</td>
<td>161 ± 3†</td>
</tr>
<tr>
<td>n-IH</td>
<td>179 ± 13</td>
<td>162 ± 10</td>
<td>185 ± 15</td>
<td>214 ± 14†</td>
<td>181 ± 8</td>
</tr>
<tr>
<td>PaCO₂, mmHg</td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>CONT</td>
<td>40 ± 3</td>
<td>41 ± 2</td>
<td>39 ± 3</td>
<td>39 ± 3</td>
<td>41 ± 3</td>
</tr>
<tr>
<td>n-IH</td>
<td>43 ± 3</td>
<td>44 ± 3</td>
<td>45 ± 2</td>
<td>44 ± 3</td>
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</tr>
<tr>
<td>pHa</td>
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<tr>
<td>CONT</td>
<td>7.348 ± 0.018</td>
<td>7.365 ± 0.021†</td>
<td>7.373 ± 0.024†</td>
<td>7.378 ± 0.024</td>
<td>7.357 ± 0.022</td>
</tr>
<tr>
<td>n-IH</td>
<td>7.300 ± 0.017</td>
<td>7.318 ± 0.020</td>
<td>7.320 ± 0.021</td>
<td>7.333 ± 0.014†</td>
<td>7.327 ± 0.016†</td>
</tr>
<tr>
<td>HCO₃⁻, mmol/l</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CONT</td>
<td>22 ± 1</td>
<td>23 ± 1</td>
<td>22 ± 1</td>
<td>22 ± 1</td>
<td>22 ± 1</td>
</tr>
<tr>
<td>n-IH</td>
<td>21 ± 1</td>
<td>21 ± 1</td>
<td>21 ± 1</td>
<td>21 ± 1</td>
<td>21 ± 1</td>
</tr>
</tbody>
</table>

Data are represented as means ± SE in n-IH rats (n = 6) or control rats (n = 6) during the postnatal period. †P < 0.05 vs. baseline.

Table 3. Arterial blood gas, pH, and base excess during baseline and recovery period after 10 electrical stimulations of carotid sinus nerve in anesthetized adult male rats

<table>
<thead>
<tr>
<th>Arterial Blood Gas</th>
<th>Baseline</th>
<th>0</th>
<th>15</th>
<th>30</th>
<th>60</th>
</tr>
</thead>
<tbody>
<tr>
<td>PaO₂, mmHg</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CONT</td>
<td>198 ± 18</td>
<td>177 ± 9</td>
<td>188 ± 13</td>
<td>204 ± 16</td>
<td>205 ± 17</td>
</tr>
<tr>
<td>n-IH</td>
<td>162 ± 8</td>
<td>169 ± 6</td>
<td>172 ± 9</td>
<td>183 ± 10</td>
<td>200 ± 12</td>
</tr>
<tr>
<td>PaCO₂, mmHg</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CONT</td>
<td>39 ± 1</td>
<td>40 ± 3</td>
<td>39 ± 1</td>
<td>41 ± 2</td>
<td>41 ± 1</td>
</tr>
<tr>
<td>n-IH</td>
<td>44 ± 2*</td>
<td>43 ± 1</td>
<td>45 ± 3</td>
<td>45 ± 2</td>
<td>44 ± 2</td>
</tr>
<tr>
<td>pHa</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CONT</td>
<td>7.325 ± 0.026</td>
<td>7.338 ± 0.005</td>
<td>7.347 ± 0.006</td>
<td>7.342 ± 0.010</td>
<td>7.327 ± 0.007</td>
</tr>
<tr>
<td>n-IH</td>
<td>7.305 ± 0.012</td>
<td>7.327 ± 0.012</td>
<td>7.324 ± 0.013†</td>
<td>7.315 ± 0.012†</td>
<td>7.315 ± 0.008</td>
</tr>
<tr>
<td>HCO₃⁻, mmol/l</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CONT</td>
<td>20 ± 1</td>
<td>21 ± 1</td>
<td>21 ± 1</td>
<td>22 ± 1</td>
<td>22 ± 1</td>
</tr>
<tr>
<td>n-IH</td>
<td>21 ± 1</td>
<td>22 ± 1</td>
<td>23 ± 1</td>
<td>23 ± 1</td>
<td>22 ± 1</td>
</tr>
</tbody>
</table>

Data are represented as means ± SE in n-IH rats (n = 6) or control rats (n = 6) during the postnatal period. P < 0.05 vs. control (*) and vs. baseline (†).
creased again (Fig. 11D; Friedman $P < 0.01$) to reach levels similar to those reported in control rats, and well above the level reached in intact n-IH animals.

Mean blood pressure during recovery was not significantly altered by either chemodenervation or CSN stimulations in control rats (Friedman $P = 0.19$ and $0.48$, respectively) but decreased in chemodenervated n-IH rats (Friedman $P < 0.05$), an effect that was not observed in intact or following CSN stimulation (Friedman $P = 0.09$ and $0.32$, respectively).

**DISCUSSION**

This study was designed to better delineate the mechanisms underlying the long-term alterations of cardiorespiratory responses to hypoxia induced by n-IH. To this aim, we recorded respiratory and sympathetic (splanchnic nerve) activity and hemodynamic parameters in both awake and anesthetized adult rats. We specifically tested whether n-IH alters respiratory responses to brief and intense hypoxia, or CSN stimulation, as an index of peripheral chemoreceptor activation, and LTF induced by repeated exposure to brief hypoxic cycles, or CSN stimulation, as an index of plasticity of the central respiratory control system. Our results show that n-IH does not affect the acute respiratory responses to either hypoxia or repeated electrical stimulation of the CSN. Respiratory LTF induced by hypoxia was suppressed in anesthetized n-IH rats. Interestingly, in n-IH rats, following repeated cycles of CSN stimulation, phrenic LTF was completely restored and was of similar magnitude as in controls. This indicates that, in rats raised under n-IH, the mechanisms involved in the generation of LTF
are still present and can be revealed by CSN stimulation in the absence of hypoxia.

The results also show that n-IH drastically reduced the splanchnic nerve response to brief hypoxia. Furthermore, while in control rats the pattern of splanchnic activity following the repeated hypoxic cycles was similar to the one described by Dick et al. (increased expiratory peak activity) (9), this was not observed in n-IH rats. This indicates that the responses of the sympathetic system to brief or repeated hypoxic episodes are altered in rats raised under n-IH.

Finally, it is worth noting that, in n-IH rats, blood pressure was elevated compared with control. The fact that this elevated blood pressure is no longer present following CSN denervation in anesthetized rats suggests that n-IH specifically alters...
baroreflex control and may involve changes in peripheral baroreceptor functions.

Methodological Considerations

In awake control rats, we did not observe an increase in ventilation during recovery, i.e., ventilatory LTF was absent. However, in 10-day-old rats, we have reported a marked ventilatory LTF using whole body plethysmography (25). In adult humans, LTF is modulated by sleep/wake states, being apparent during non-rapid eye movement sleep (1, 5) but not during wakefulness (24). Although we did not assess sleep/wake state, the use of the double-chamber plethysmograph restrains the animal, which remains awake throughout the exposure. This, along with the potential stress associated with contention, may contribute to prevent ventilatory LTF. Finally, ventilatory LTF expression in adult rats is larger under iso- and hypercapnic conditions (18, 45). Because our experiments were done under poikilocapnic hypoxia, this may further limit LTF.

In anesthetized rats, cortical inputs are reduced, and isocapnia is maintained, which likely facilitates the expression of phrenic LTF. One of the drawbacks of this approach is the bilateral vagotomy (necessary to prevent entrainment of inspiratory motor output with the ventilator) that suppresses barosensory inputs from the aorta and cardiopulmonary receptors. In these conditions, the baroreflex depends on the inputs from carotid sinus baroreceptors whose sensory fibers travel through the CSN. In such conditions, and as previously reported (49), electrical stimulation of the CSN inhibits sympathetic activity as expected as a classical output of the baroreflex response (baroreceptor activation decreases sympathetic output, thus leading to vasodilatation and reduced arterial pressure).

Finally, one should keep in mind that our experimental design involved poikilocapnic IH during development, with the aim to gain insights on pathological situations associated with apneas in neonates. Obviously, hypoxia is only one of the alterations of apneas, which also produces bouts of hypercapnia. However, the effects of intermittent hypercapnic exposures are not well known and warrant proper investigation.

Respiratory and Hemodynamic Variables in Normoxia in Control and n-IH Rats

Under baseline conditions, the respiratory pattern of awake n-IH rats was altered compared with control, with a slight elevation of $f_R$ and reduced $V_T$, whereas $V_E$ was similar between groups. A much larger difference between groups was found for mean blood pressure, which was higher in n-IH rats compared with controls. This is strikingly similar to the effects of chronic exposure to IH in adults, which also induces hypertension (12). In adults, this depends on the recurrent activation of peripheral chemoreceptors and is prevented by bilateral carotid body denervation during exposure (12, 30), and following chemical sympathetic denervation (13). This is accompanied by an enhanced activity of peripheral chemoreceptors (23, 48), chronic sympathetic activation (11, 29, 31, 44), blunted baroreflex responses (3, 31), and functional changes in the vascular bed (7). Whether similar changes are also induced by exposure to neonatal hypoxia in newborns and have long-lasting effects remains to be documented. The fact that, in anesthetized rats, chemodenervation suppressed the blood pressure difference between groups and that the acute ventilatory response to hypoxia was similar between groups points toward a neural mechanism involving arterial baroreceptors.

Respiratory Responses to Repeated Hypoxic Cycles in Control and n-IH Rats

The hypoxic ventilatory response was similar between groups, both in awake and anesthetized rats. In anesthetized animals, electrical stimulation of the CSN elicits phrenic responses of similar amplitude in control and n-IH rats, showing that n-IH does not alter the central translation of the carotid body inputs to respiratory neurons. Because the profile of hypoxic exposure was very brief, we are quite confident that the acute response to hypoxia under these conditions represents a reliable output of the activation of the peripheral chemoreceptors. Accordingly, our results strongly suggest that hypoxic sensitivity in peripheral chemoreceptors was not altered in n-IH rats. Contrastingly, in rat pups, hypoxic ventilatory response (25) and carotid body response to hypoxia (47) were increased following chronic intermittent hypoxic exposure. On the other hand, a similar model of n-IH induced a persistent...
enhancement in the hypoxic response of the superfused in vitro carotid body/CSN preparation from 60-day-old rats (47) while we studied in vivo rats at 90 days of age. These different effects of n-IH at different ages strongly suggest that early alterations of peripheral chemoreceptor functions are not permanent but are rather gradually restored to normal during subsequent development in room air.

During the return to normoxia following each hypoxic exposure, phrenic nerve activity decreases as classically reported (6, 21, 32, 52). This pattern is called posthypoxic frequency decline and is an active neural process involving noradrenergic pathways in the ventrolateral pons, particularly the A5 nucleus (6, 52). This response is not present in adult rats exposed to n-IH, which is consistent with previous reports showing that it is also suppressed in adult rats exposed for 1 wk to nocturnal IH (32). This may suggest that n-IH decreases the A5 response to hypoxia. The fact that neonatal hypoxia reduces catecholamine turnover in A5 neurons (53) is consistent with this interpretation.

Phrenic LTF is observed in control but not in n-IH rats. This is in agreement with another study that demonstrated an attenuation of the phrenic LTF in adult rats exposed to n-IH during the first month of life (50). Interestingly, it has been reported that other stimulus, such as fetal alcohol (27) or nicotine (14) exposures, also reduces the expression of LTF in rat pups. This pattern is therefore not specific to IH. In the particular case of IH, however, the suppression of LTF appears specific to exposure in the neonatal period, since a similar exposure in adults enhances LTF (32, 36, 39). Nonetheless, the induction of LTF in n-IH rats following electrical stimulation of the CSN suggests that the fundamental mechanisms underlying LTF expression are still present but cannot be expressed.

An important body of literature shows that LTF depends on activation of brain stem raphe neurons, leading to spinal serotonin release and serotonin receptor activation (15), which leads to de novo brain-derived neurotrophic factor synthesis, strengthening synaptic efficiency on spinal respiratory motoneurons (2, 34). Other mechanisms involve spinal adenosine, and adenosine A2A receptors, which enhances phrenic activity in the absence of hypoxia (16) but reduces the magnitude of LTF induced by IH (22). Generation of reactive oxygen species, which are dependent on cyclic hypoxic exposure rather than CSN activation, are also involved in this response (33). Accordingly, one of the potential differential effects of n-IH may involve at least one of these elements.

Fig. 10. Respiratory and sympathetic responses to repeated hypoxic cycles or CSN stimulation in CSN-intact and chemodenervated control and n-IH rats. A and B: phrenic burst frequency. C and D: integrated phrenic amplitude. E and F: integrated splanchnic amplitude. All values are means ± SE, reported as % changes vs. baseline. P < 0.05 n-IH vs. control (*) and vs. baseline (†).
Hemodynamic and Sympathetic Responses to Hypoxia in Control and n-IH Rats

The splanchnic nerve response to repeated hypoxic cycles is drastically attenuated in n-IH rats. It is unlikely that this alteration is because of reduced peripheral chemoreceptor responses, since the phrenic nerve response is similar between n-IH and control rats (see above). The lower splanchnic nerve response to hypoxia may be because of modifications of the central integration processes toward sympathetic centers downstream of the peripheral chemoreflex inputs. This may involve alterations of neurochemicals such as evoked above for the reported respiratory alterations. Again, evidence suggests that A5 neurons are likely affected by n-IH. The fact that the splanchnic response to hypoxia is reduced in n-IH rats may have important functional implications. The splanchnic nerve innervates the mesenteric blood reservoir, which upon mobilization may facilitate blood delivery to several important territories (20). How blood flow is distributed in these conditions and how an organism may sustain its activity under hypoxic exposures lasting more than a few seconds are questions that require further studies.

To further delineate the respiratory modulation on splanchnic nerve activity under baseline conditions and during recovery, we used cycle-triggered average of the integrated splanchnic signal, which is particularly useful to increase the signal-to-noise ratio of the respiratory modulation of sympathetic nerve activity (8, 42, 43). As previously described, we observed a peak of sympathetic activity during the expiratory phase. This respiratory modulation originates from respiratory neurons, providing phasic inputs that increase firing of sympathetic neurons in the ventrolateral medulla during expiration, whereas the nucleus tractus solitarius provides a direct tonic activation related to peripheral chemoreceptor activity (35). The coupling of these systems appears to be quiet efficient, as seen by the close positive correlation between the amplitude of the peak of sympathetic activity during expiration and the
phrenic nerve activity under hypercapnia (17), acute hypoxia (8), or following induction of phrenic LTF by IH (9). Accordingly, in control rats, phrenic LTF was accompanied by an enhanced sympathetic activity during expiration. In intact n-IH rats under baseline conditions, the peak of activation during expiration is of reduced magnitude, which may be a consequence of hypertension, as previously reported (17). Furthermore, concomitantly with the suppression of phrenic LTF during recovery, sympathetic activation during expiration is suppressed, whereas overall splanchnic nerve activity decreases gradually. It is striking to observe these concomitant alterations, and we speculate that the suppression of the respiratory LTF in n-IH rats unmasks a general inhibitory tone of the sympathetic activity induced by the repeated hypoxic cycles. Of note, the reduced splanchnic nerve activity during recovery in n-IH rats likely explains the drop of arterial blood pressure reported in anesthetized and in awake rats.

Perspectives and Significance

This study shows that exposure to n-IH in rat pups modifies the respiratory and hemodynamic responses to hypoxia in adult rats. Although we have previously shown that this pattern of n-IH exposure enhances the ventilatory response to repeated hypoxic cycles (i.e., involving peripheral chemoreceptors) in 10-day-old rats (25), our actual results show that this altered response is not persistent in adults. Contrarily, responses involving central integration of the cardiorespiratory control system (posthypoxic frequency decline and phrenic LTF) are altered in adult rats exposed to n-IH. However, in n-IH rats, the mechanisms for phrenic LTF can be elicited by electrical stimulation of the CSN and are therefore apparently blunted by an inhibitory effect of hypoxia on central integrative structures.

Although the physiological function of LTF is still debated (37), it has been suggested to play a protective mechanism against the development of apneic disorders, particularly when a mild form of apnea/hypopnea during sleep is already apparent (34). In this case, stronger coordinated activity of the diaphragm and upper airway dilator muscles may help to prevent collapses and the resulting apnea. Accordingly, recurrent IH during the neonatal period may contribute to the establishment of sleep apnea later in life through a suppression of respiratory LTF. This would be in line with recent studies showing that preterm birth is a risk factor for sleep-disordered breathing in 8- to 11-yr-old infants (51) and young adults (46). In addition, in n-IH adult rats, high arterial blood pressure is associated with attenuated splanchnic nerve response during hypoxia and altered hemodynamic regulation following IH. These impairments could represent a threat to the perfusion of vital tissues and contribute to enhance cardiovascular morbidity in adults.

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Disclosures

No conflicts of interest are declared by the authors.

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