Neonatal stress and attenuation of the hypercapnic ventilatory response in adult male rats: the role of carotid chemoreceptors and baroreceptors

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Dumont FS, Kinkead R. Neonatal stress and attenuation of the hypercapnic ventilatory response in adult male rats: the role of carotid chemoreceptors and baroreceptors. Am J Physiol Regul Integr Comp Physiol 299: R1279–R1289, 2010. First published September 1, 2010; doi:10.1152/ajpregu.00446.2010.—Neonatal maternal separation (NMS) is a form of stress that disrupts respiratory control development. Awake adult male rats previously subjected to NMS show a ventilatory response to hypercapnia (HCVR; FICO₂ = 0.05) 47% lower than controls; however, the underlying mechanisms are unknown. To address this issue, we first tested the hypothesis that carotid bodies contribute to NMS-related attenuation of the HCVR by using carotid sinus nerve section or FICO₂ manipulation to maintain PaO₂ constant (iso-oxic) during hypercapnic hyperpnea. We then determined whether NMS-related augmentation of baroreflex sensitivity contributes to the reduced HCVR in NMS rats. Nitroprusside and phenylephrine injections were used to manipulate arterial blood pressure in both groups of rats. Pups subjected to NMS were separated from their mother 3 h/day from postnatal days 3 to 12. Control rats were undisturbed. At adulthood, rats were anesthetized [urethane (1g/kg) + isoflurane (0.5%)], and diaphragmatic electromyogram (dEMG) was measured under baseline and hypercapnic conditions (PaCO₂: 10 Torr above baseline). The relative minute activity response to hypercapnia of anesthetized NMS rats was 34% lower than controls. Maintaining PaO₂ constant during hypercapnia reversed this phenotype; the HCVR of NMS rats was 45% greater than controls. Although the decrease in breathing frequency during baroreflex activation was greater in NMS rats, the change observed within the range of pressure change observed during hypercapnia was minimal. We conclude that NMS-related changes in carotid body sensitivity to chemical stimuli and/or its central integration is a key mechanism in the attenuation of HCVR by NMS.

With regard to respiratory regulation, several recent reviews have highlighted the profound effects of excessive stimulation of chemosensory pathways (e.g., intermittent hypoxia, chronic hypoxia) on the developmental trajectory of the neural circuits that regulate breathing (2, 7). Depending on the severity of the stimulation protocols used, these models may activate the neuroendocrine response to stress, but the contribution of these hormones to the final respiratory phenotype remains unknown. In contrast, NMS does not have any direct effect on chemosensory pathways but exposes pups to daily surges of stress hormones. This relatively novel approach, therefore, gives the opportunity to gain better insight into the contribution of stress hormones in the development of various respiratory phenotypes.

In awake animals, we previously showed that the hypercapnic ventilatory response (HCVR; FICO₂ = 0.05; 20 min) of adult male rats previously subjected to NMS is 47% less than controls (21). The sexual dimorphism of this effect is striking because females previously subjected to NMS showed a HCVR 63% larger than controls, a result that suggests that ovarian hormones offer no protection against the consequences of NMS. The origins and clinical significance of the respiratory phenotype observed in male NMS rats are still unclear, but abnormal (or incomplete) maturation of the HCVR observed in male rats may contribute to the respiratory instability observed in NMS rats during sleep and wakefulness (37).

The hypercapnic chemoreflex is a complex interplay between sensory afferents and central integration of somatic and cognitive signals. Since NMS affects both the central and peripheral components of the respiratory control system (19, 35), several mechanisms have been evoked to explain the HCVR attenuation. Although the relative contribution of each component may vary between species and experimental approaches, it is clear that both central chemoreceptors and carotid bodies contribute significantly to the HCVR (54, 28, 51, 58). On the basis of indirect evidence indicating that NMS augments carotid body’s responsiveness to hypoxia (35, 36), it was proposed that the relative hyperoxia that occurs during hypercapnic hyperpnea [~20 mmHg increase (34)] is sufficient to attenuate the CO₂ response of NMS rats (21). However, the recent evidence indicating that central and peripheral chemoreceptors are interdependent and influence each other’s sensitivity (4, 10, 11, 57) raises the possibility that NMS disrupts this interaction.

Besides the carotid bodies, the carotid bifurcation is also the site of mechanosensitive nerve endings that constitute the arterial baroreceptors. Much like the carotid bodies (15), these structures convey their sensory afferents via the carotid sinus...
nerve and project centrally to the nucleus tractus solitarius (NTS) (42). NMS disrupts the NTS function in a way that augments the ventilatory response to hypoxia and the response to carotid sinus nerve stimulation (which conveys both chemoreceptor and baroreceptor afferents) (35). Therefore, we proposed that NMS can potentiate the baroreflex. During hypercapnia, CO₂ increases mean arterial blood pressure by a direct effect on vessels and by sympathetic activation (13, 23, 32). This increase in blood pressure activates baroreceptors that modulate respiratory activity and decreases breathing frequency (9, 25). Therefore, the baroreflex potentiation is another mechanism by which NMS could attenuate the HCVR.

The main objective of the present study was to determine whether disruption of peripheral chemoreflex and/or baroreflex is responsible for the attenuation of the HCVR in adult male rats previously subjected to neonatal stress. To do so, we used an anesthetized rat preparation to manipulate chemoafferent input during hypercapnic exposure either by decreasing inspired O₂ levels to maintain rats iso-oxic during hypercapnic hyperpnea or by acute carotid sinus nerve section (CSX). Responses to these experimental interventions were compared between NMS and control rats. In a separate series of experiments, we determined whether NMS potentiates the baroreflex by comparing the breathing frequency and heart rate responses of NMS and control rats to pharmacological manipulations of systemic blood pressure with hypertensive (phenylephrine) and hypotensive (nitroprusside) agents. Parts of these results have been published in abstract form (12).

**MATERIALS AND METHODS**

Adult male Sprague-Dawley rats (Charles River Canada, Saint-Constant, QC, Canada) were used in this study. Rats were supplied with food and water ad libitum and maintained in standard laboratory and animal care conditions (21°C, 12:12-h dark-light cycle: lights on between 6 AM and 6 PM). Protocols were in accordance with the guidelines detailed by the Canadian Council on Animal Care and approved by the Laval University Animal Care Committee. Distribution of the ages and weights among experimental series and the number of animals per group (n) are detailed in Table 1.

**NMS Procedures**

The NMS protocol started on postnatal day 3 (P3). Pups were separated from their mother and isolated from each other during 3 h (9 AM to noon) each day for 10 consecutive days (P3 to P12). Pups were placed in a temperature (32°C)- and humidity (45%)-controlled incubator. On the basis of the work of Malik and Fewell (45), this ambient temperature is within the thermoneutral zone (near minimal in O₂ consumption) for rat pups of this age group. In contrast, control rats were not disturbed during the first 2 wk postpartum. On day 21, rats were weaned and housed two per cage under standard animal care conditions until adulthood, at which time, experiments were performed. Each experimental group was composed of pups originating from at least two litters to avoid litter-specific effects.

**Surgical Procedures**

Anesthesia was first induced with isoflurane (3.5%) in a closed chamber and then maintained via a nose cone. The rat was placed on a homeothermic blanket (Harvard Apparatus, Holliston, MA), and rectal temperature was maintained at 37°C. The trachea was cannulated, the nose cone was removed, and a “T”-shaped tube was placed on the cannula and connected to the breathing circuit. Rats were breathing spontaneously a mixture of 30% O₂ and 70% N₂ (P\(\text{O}_2 = 0.3\)) to maintain the P\(\text{O}_2\) within normoxic range (see Tables 2–5). A venous femoral catheter was inserted for anesthetic and fluid administration. Arterial femoral catheter was inserted for blood pressure monitoring (Transbridge TBM4M-B, World Precision Instruments, Sarasota, FL) and withdrawal of blood samples (70 μl) for analysis of arterial blood-gases (model ABL-5, Radiometer, Copenhagen, Den-

### Table 1. Age, weight, and number of animals amongst experimental groups

<table>
<thead>
<tr>
<th></th>
<th>Series I &amp; II: Hypercapnic Response</th>
<th>Series III: Baroreflex</th>
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<tbody>
<tr>
<td></td>
<td>Control</td>
<td>NMS</td>
</tr>
<tr>
<td>Age, days</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Weight, g</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number of animals</td>
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</table>

Values are reported as means ± SD. CSX, carotid sinus nerve section; NMS, neonatal maternal separation. *Statistically significant difference from corresponding control value at \(P \leq 0.05\).

Data are reported as means ± SD. *Statistically significant difference from corresponding baseline (normocapnic) value at \(P \leq 0.05\). †Statistically significant difference from corresponding control value at \(P \leq 0.05\).

Table 2. Arterial blood gases and selected cardiovascular variables measured under baseline conditions and during exposure to poikilo-oxic hypercapnia

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>NMS</th>
<th>Change from baseline (mmHg)</th>
<th>Control</th>
<th>NMS</th>
</tr>
</thead>
<tbody>
<tr>
<td>PaCO₂, mmHg</td>
<td>48 (2)</td>
<td>49 (4)</td>
<td>0</td>
<td>58 (3)*</td>
<td>59 (3)*</td>
</tr>
<tr>
<td>pH</td>
<td>7.37 (0.03)</td>
<td>7.36 (0.03)</td>
<td>0</td>
<td>7.31 (0.03)*</td>
<td>7.30 (0.03)*</td>
</tr>
<tr>
<td>PaO₂, mmHg</td>
<td>116 (11)</td>
<td>122 (15)</td>
<td>0</td>
<td>159 (12)*</td>
<td>159 (9)*</td>
</tr>
<tr>
<td>Heart rate, bpm</td>
<td>356 (47)</td>
<td>352 (41)</td>
<td>0</td>
<td>387 (39)</td>
<td>388 (36)</td>
</tr>
<tr>
<td>Mean blood pressure, mmHg</td>
<td>109 (7)</td>
<td>110 (10)</td>
<td>0</td>
<td>132 (10)*</td>
<td>126 (13)*</td>
</tr>
<tr>
<td>Breathing frequency, bpm</td>
<td>113 (13)</td>
<td>103 (14)</td>
<td>0</td>
<td>157 (14)*</td>
<td>126 (17)*†</td>
</tr>
</tbody>
</table>
EFFECTS OF NEONATAL STRESS ON CHEMO- AND BAROREFLEXES

mark), corrected for the rat’s body temperature. Rats were slowly converted from isoflurane to urethane anesthesia (1.0 g/kg). The isoflurane level within the inspired gas mixture was decreased progressively, while urethane was infused slowly (0.125 ml/min) with a motorized pump (Harvard Instruments, Holliston, MA; model PHD2000). A pressure-controlled ventilator was also used during the transition to urethane to ensure adequate ventilation during the procedure (Kent Scientific, Litchfield, CT). 0.5% of isoflurane was kept throughout the experiment.

We used 1.0 g/kg of urethane because this dose has been shown to have little effect on cardiorespiratory function in rats (43, 44). However, at this low dose, variations of the electroencephalographic (EEG) activity have been observed and can influence the HCVR (5). The use of isoflurane stabilizes the EEG activity (55), and little effect of isoflurane on the HCVR and hypoxic ventilatory response has been observed at this dose (29, 59). Then, by combining these low doses of anesthetics we aimed to minimize the impact of anesthesia on ventilation.

Electromyographic activity from the diaphragm (dEMG) was recorded as a correlate of inspiratory motor output and compared between control and NMS rats. To do so, a ventral incision was performed to reach the diaphragm, and two stainless-steel electrodes were sewn into the diaphragm, 1 cm apart. The electrodes were placed as laterally as possible to reduce electrical interference from the heart. Electrical activity was amplified (gain = 10,000; model no. 1700, AM Systems, Everett, WA), band-pass filtered (100 Hz to 10 kHz), and fed to a moving averager (time constant: 100 ms; CWE, model MA-821, Ardmore, PA, USA) before being digitized and recorded with a data acquisition system (IOX software, EMKA Technologies, Falls Church, VA). Once the surgical procedures were completed, a 60-min “stabilization” period was allowed before the onset of the experiments. At the end of the experiment, euthanasia was performed by urethane overdose.

Series I: Effects of Neonatal Maternal Separation on the Hypercapnic Ventilatory Response: Poikilo-Oxic Versus Iso-Oxic Conditions

After the stabilization period, the hypercapnia protocol described below was initiated with two distinct approaches. To reproduce conditions used previously in awake intact rats (21), hypercapnia was first performed without regulating PaO2 (poikilo-oxic conditions). In separate groups of rats, hypercapnic exposure was performed while reducing FiO2 to prevent PaO2 from increasing during hypercapnic hyperpnea (iso-oxic conditions). Unlike some animals in series II and III, no surgical interventions were performed on the carotid sinus nerve (intact animals).

Experimental protocol. Once the animal recovered from surgery and the cardiorespiratory parameters were stable, the experiment began by taking an arterial blood sample to measure blood gases and pH under baseline (normocapnic) conditions. Basal dEMG, blood pressure, and heart rate were recorded for 10 min under baseline conditions. Hypercapnia was then induced by adding CO2 to the inspired gas mixture (FiCO2 ≈ 0.075) to increase the PaCO2 by 10 Torr above the baseline level. During that procedure, the inspired O2 was either unchanged (FiO2 = 0.3; poikilo-oxic experiments) or reduced (FiO2 = 0.23) to maintain PaO2 constant, within 3 Torr of baseline values (iso-oxic experiments). A second measurement of arterial blood gases confirmed that the target PaCO2 (and PaO2, when appropriate) had been reached (see Tables 2 and 3). Hypercapnia (under poikilo-oxic or iso-oxic conditions) was then maintained for 5 min, during which cardiorespiratory variables were recorded.

Data analysis. dEMG bursts were detected by the acquisition system, and their amplitude was calculated as the difference between the peak and baseline activity. Owing to differences in contact efficiency and/or electrode placement, raw dEMG activity (in millivolts) was variable between experiments. To address this issue, the dEMG amplitude was expressed as a percentage change from baseline. As a result, the minute activity corresponds to the product of the relative dEMG amplitude (% of baseline) and the breathing frequency (contractions per minute), and it is a measure of the activity developed by the diaphragm each minute. Throughout the experiment, the dEMG amplitude, breathing frequency, mean arterial blood pressure, and heart rate data were averaged in 15-s bins. In this series, a mean baseline value was obtained by averaging 5 min of recording prior to hypercapnic exposure. Similarly, a mean hypercapnic value was obtained by averaging the 5 min of recording under hypercapnic conditions. For each respiratory variable, the response to hypercapnic stimulation was assessed both on absolute data (repeated measures design) and on normalized data expressed as a percentage change from baseline (see statistical analysis below).

Series II: Effects of Neonatal Maternal Separation on the (Poikilo-Oxic) Hypercapnic Ventilatory Response Following Carotid Sinus Nerve Section

Carotid sinus nerve section (CSX) was performed after urethane infusion by accessing the carotid bifurcation from a ventral approach. The carotid body was then located between the internal and external carotid arteries and under the occipital artery. The glossopharyngeal nerve was identified, and the branch reaching the carotid sinus area was isolated from the surrounding tissue. The carotid sinus nerve was cut, and a piece of 1 mm was removed to ensure complete interruption of theafferent signal. The same method was used on the contralateral side. At the end of the experiment (~15 min after completion of the hypercapnic protocol), the effectiveness of the CSX procedure was confirmed by subjecting rats to an acute hypoxic test induced by progressively reducing O2 of the inspired gas mixture (FiO2 = 0.12). During that test, O2 levels delivered to the animal were measured continuously with an O2 analyzer (TED-60T; Teledyne Analytical Instruments, Industry, CA). The target FiO2 was achieved within 30 s; this dynamic was consistent between trials. The magnitude of the minute activity response measured after 1 min of hypoxia was recorded and compared with that of animals with their carotid sinus nerves intact (Fig. 5).

Experimental protocol and data analysis. Once the animal recovered from surgery and the cardiorespiratory parameters were stable, the experiment began following the same protocol described for the poikilo-oxic series described previously. The procedure for data analysis was the same as the previous series. Arterial blood gases and cardiovascular parameters for these experiments are reported in Table 4.

Series III: Effects of Neonatal Maternal Separation on Heart Rate and Breathing Frequency Responses to Baroreflex Stimulation

Baroreflex stimulation protocol. Rats were prepared with the same protocol described in the "surgical procedures"; experiments were performed on intact and CSX rats. Intravenous injections of sodium nitroprusside (NP) and phenylephrine hydrochloride (PE) were used to evoke hypotensive and hypertensive responses, respectively. Both drugs were obtained from Sigma Aldrich (St. Louis, MO) and prepared at a concentration of 5 mg/ml (molarity: NP: 16.8 mM; PE: 24.5 mM). The choice of this concentration was based on its ability to elicit adequate changes in blood pressure using volumes ranging between 1 and 80 μl (see below). On the basis of preliminary experiments, the doses were selected for their ability to induce both physiological and supraphysiological changes in arterial blood pressure to test the baroreflex. Prior to drug injections, baseline arterial blood gases and cardiorespiratory variables were recorded for 10 min, and then the effect of sham injection (saline, 8 μl) was tested. The drug injection protocol began by injecting NP; three volumes were used: 2, 4, and 8 μl. This was followed by PE injections; five volumes were used: 1, 2, 3, 4, and 8 μl. Each bolus injection was performed over a 1-min period. Each injection was followed by 15-min recuperation. For each

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**Table 3. Arterial blood gases and selected cardiovascular variables measured in under baseline conditions and during exposure to iso-oxic hypercapnia**

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>NMS</th>
<th>Control</th>
<th>NMS</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>PaCO₂, mmHg</strong></td>
<td>41 (5)</td>
<td>40 (4)</td>
<td>51 (5)*</td>
<td>50 (5)*</td>
</tr>
<tr>
<td><strong>DPaCO₂, mmHg</strong></td>
<td>0</td>
<td>0</td>
<td>10 (1)*</td>
<td>10 (2)*</td>
</tr>
<tr>
<td><strong>pH</strong></td>
<td>7.40 (0.01)</td>
<td>7.34 (0.01)</td>
<td>7.28 (0.05)*</td>
<td>7.24 (0.03)*</td>
</tr>
<tr>
<td><strong>PaO₂, mmHg</strong></td>
<td>106 (12)</td>
<td>124 (10)*</td>
<td>109 (22)</td>
<td>123 (8)*</td>
</tr>
<tr>
<td><strong>ΔPaO₂, mmHg</strong></td>
<td>0</td>
<td>0</td>
<td>3 (8)</td>
<td>−1 (6)</td>
</tr>
<tr>
<td><strong>Heart rate, bpm</strong></td>
<td>385 (54)</td>
<td>388 (70)</td>
<td>412 (32)</td>
<td>427 (28)</td>
</tr>
<tr>
<td><strong>Mean blood pressure, mmHg</strong></td>
<td>98 (13)</td>
<td>84 (7)*</td>
<td>116 (16)*</td>
<td>109 (10)*</td>
</tr>
<tr>
<td><strong>Breathing frequency, bpm</strong></td>
<td>96 (14)</td>
<td>105 (17)</td>
<td>112 (23)*</td>
<td>138 (13)†</td>
</tr>
</tbody>
</table>

Data are reported as means ± SD. *Statistically significant difference from corresponding baseline (normocapnic) value at \( P < 0.05 \). †Statistically significant difference from corresponding control value at \( P < 0.05 \).

drug, the order of the injected volume differed between experiments except for the largest PE, which was always performed at the end; preliminary experiments showed that, unlike all other injections, cardiorespiratory parameters did not fully recover from this injection. Because CSX reduced hypertensive responses to drug injections significantly, three CSX rats in each group received higher doses (20, 40, and 80 μl) of PE to ensure that the same range of blood pressure changes was tested between studies (intact vs. CSX).

After each injection, a blood sample was taken to ensure that blood gases were unchanged by the procedure (see Table 5). Note that no more than 10 samples (700 μl total) were taken from each rat to avoid hypovolemia. This protocol was performed both in intact and CSX animals.

**Data analysis.** As in the previous series, the EMG amplitude, breathing frequency, mean arterial blood pressure, and heart rate data were recorded continuously throughout the experiments, but data were averaged in 10-s bins. Prior to injections, a mean baseline value was obtained by averaging 1 min of recording. To compare baroreflex sensitivity between groups (NMS vs. control) and procedures (intact vs. CSX), the maximum mean arterial blood pressure, heart rate, and breathing frequency response to each drug injection was measured. A linear regression between blood pressure response and heart rate or breathing frequency response to each drug injection was measured. A correlation coefficient was calculated for each animal, and then group data were plotted (Figs. 6 and 7, respectively).

**Statistical analysis.** The equality of variance and the normal distribution of all parameters for this study using the equality of variance F-test and the Shapiro-Wilk test. Results from these tests validated the use of parametric analyses. Data were analyzed using multifactorial ANOVA; a repeated-measures design was used, when appropriate. When ANOVA results showed a significant effect of a factor, Fisher’s paired least significant difference test was used as a post hoc test to determine which means were statistically different. In series III, the effects of NMS and carotid sinus nerve section on cardiorespiratory responses to changes in mean blood pressure (baroreflex) were first assessed using analysis of covariance (ANCOVA). Correlation Z test was then used to determine whether the regressions between blood pressure response (independent variable) and cardiorespiratory parameters (dependent variables) were statistically significant. Slopes were calculated for each animal, and group data were compared using ANCOVA. Differences were considered significant at \( P < 0.05 \) unless otherwise mentioned. Statistical analyses were performed using Statview 5.0 (SAS Institute, Cary, NC). In accordance with American Physiological Society guidelines (8), all data are presented as means, and variability is expressed as SD. Note that P values reported in the text are results from ANOVA and ANCOVA or correlation analyses. Results from post hoc tests are reported on the figures and tables only.

**RESULTS**

**Modulation of the Hypercapnic Ventilatory Response by Arterial O₂ Levels: NMS Versus Control Rats**

**Baseline.** \( \text{PaCO}_2 \), pHa, mean arterial blood pressure, and heart rate did not differ between NMS and control animals (Tables 2 and 3; treatment effect: \( P > 0.05 \) for all variables). \( \text{PaCO}_2 \) of control rats was lower than NMS; however, this effect was significant only in the iso-oxic experiments (Table 3; treatment effect: \( P = 0.008 \)). At rest, breathing frequency, heart rate, and mean arterial blood pressure did not differ between groups (Fig. 1, Tables 2 and 3; treatment effect: \( P > 0.09 \) for all variables). Comparison of baseline values between O₂ conditions (poikilo-oxic vs. iso-oxic) showed that \( \text{PaO}_2 \), pHa, heart rate, and breathing frequency were similar across studies (Fig. 1, Tables 2 and 3; \( O_2 \) effect: \( P > 0.3 \); however, resting mean arterial blood pressure and \( \text{PaCO}_2 \) of iso-oxic rats

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![Fig. 1. Comparison of the breathing frequency response to hypercapnia under iso-oxic (solid symbols) and poikilo-oxic (open symbols) conditions between control rats (A; circles) and rats previously subjected to neonatal maternal separation (NMS) (B; triangles). Data are expressed as means ± SD. *Statistically significant difference from corresponding baseline value (\( P < 0.05 \)). †Statistically significant difference from corresponding poikilo-oxic value (\( P < 0.05 \)). ‡Statistically significant difference from corresponding control value (\( P < 0.05 \)).](http://ajpregu.physiology.org/)}
were lower than those involved in the poikilo-capnic experiments (Tables 2 and 3; O2 effect: \( P < 0.001 \) for both).

**Hypercapnia.** After 5 min of moderate hypercapnia (whether poikilo- or iso-oxic), \( \Delta P_{CO_2} \), increased by 10 Torr in all groups (Tables 2 and 3; CO2 effect: \( P < 0.0001 \) and an acidosis was observed (CO2 effect: \( P < 0.0001 \)). As expected, \( \Delta P_{O_2} \) increased in poikilo-oxic rats only (Tables 2 and 3; CO2 effect: \( P < 0.0001 \)); in these experiments, the relative hyperoxia was the same for NMS and control animals (Table 2; treatment effect: \( P = 0.28 \)). Under poikilo-oxic conditions, the relative increase in diaphragmatic minute activity observed in NMS rats was 34% less than control (Fig. 2; treatment: \( P = 0.019 \)). This response contrasts with the one observed under iso-oxic conditions during which the hyperventilatory response of NMS rats was 37% less than controls (Fig. 4; treatment: \( P = 0.004 \)). Statistical analysis of the absolute breathing frequency data support these results (Fig. 1; CO2 effect: \( P = 0.0009 \)). The diaphragmatic EMG amplitude response was not affected by either factor (Fig. 2C; treatment and O2 effects: \( P = 0.71 \) and 0.36, respectively).

Heart rate and mean arterial blood pressure increased during hypercapnia (Tables 2 and 3; CO2 effect: \( P = 0.001 \) and \( P < 0.0001 \), respectively). While the heart rate response was not influenced either by NMS or \( \Delta P_{O_2} \) levels (Fig. 3A; \( P = 0.49 \) and 0.64, respectively), the hypertension was influenced by both variables (Fig. 3B; treatment \( \times \) O2 effect: \( P = 0.02 \)). Specifically, maintaining rats iso-oxic augmented the hypertensive response of NMS but not control rats. Statistical analysis of the absolute blood pressure data confirmed these results (Fig. 3B; CO2 effect \( \times \) treatment \( \times \) O2 effect: \( P = 0.05 \)).

**Hypercapnic Ventilatory Response Following CSX in NMS and Control Rats**

**Baseline.** In CSX rats, comparison of baseline arterial blood gases and cardiorespiratory parameters revealed no significant differences between NMS and controls (Table 4).

**Hypercapnia.** Following 5 min of moderate poikilo-oxic hypercapnia, \( \Delta P_{CO_2} \), increased by 10 Torr in both groups (Table 4; CO2 effect: \( P < 0.0001 \)); an acidosis was observed and \( \Delta P_{O_2} \), increased (Table 4; CO2 effect: \( P < 0.0001 \) for both variables). In this series, the increase in diaphragmatic minute activity observed in NMS rats was 37% less than controls (Fig. 4; treatment effect: \( P = 0.02 \)). This effect was mediated exclusively by the breathing frequency response (Fig. 4; treatment effect: \( P = 0.04 \)). Despite a suggestive trend, attenuation of the diaphragmatic EMG response in NMS rats was not statistically significant (Fig. 4; treatment effect: \( P = 0.28 \)). Hypercapnic exposure increased heart rate and mean arterial blood pressure in this series also (Table 4; CO2 effect: \( P < 0.0001 \)); however, post hoc analyses did not detect significant effects in individual groups.

**Hypoxic test and effectiveness of CSX.** Fig. 5 shows that the minute activity response to hypoxia (FIO2: 0.12; 1 min) observed following bilateral CSX is significantly lower than in intact control rats (CSX effect: \( P < 0.0001 \)). A hypoxic test was not performed in intact NMS rats to reduce the number of animal used considering that their hypoxic ventilatory response is even greater than controls (showed in awake, sleeping, and

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**Fig. 2.** Relative hypercapnic ventilatory response of control animals (open bars) and rats previously subjected to NMS (solid bars) under poikilo-oxic and iso-oxic conditions. The dEMG minute activity (A), the breathing frequency (B), and the dEMG amplitude (C) are represented in this figure as a percentage of increase from baseline. Data are expressed as means, and error bars represent standard deviations. †Statistically significant difference from corresponding control value (\( P \leq 0.05 \)). §Statistically significant difference from corresponding poikilo-capnic value (\( P \leq 0.05 \)).
Saline injections did not induce any changes in mean arterial pressure. Yohimbine injections decreased and increased blood pressure, respectively. In intact and CSX rats, nitroprusside and phenylephrine injections were used, with nitroprusside decreasing blood pressure and phenylephrine increasing it. The effect of these drugs was not significantly different between intact and CSX rats (data not shown; drug effect: \( P > 0.05 \)).

Baseline. Analysis of arterial blood gases measured under baseline conditions revealed no significant effect of NMS on any of these variables (Table 5).

Drug injections. In intact and CSX rats, blood gas samples obtained following drug injections showed that \( \text{PaO}_2, \) pH, and \( \text{PaCO}_2 \) remained stable over the course of the injection procedures (data not shown; drug effect: \( P = 0.08, 0.62, \) and 0.44, respectively). In intact and CSX rats, nitroprusside and phenylephrine injections decreased and increased blood pressure, respectively (Fig. 6A and B, drug effect: \( P < 0.0001 \) for both). Saline injections did not induce any changes in mean arterial pressure in both groups (data not shown). Note that in this analysis, direct comparison between intact and CSX rats was not possible because the drug doses used were not always the same (see MATERIALS AND METHODS for details). Neonatal maternal separation had no effect on the blood pressure responses observed in each condition (Fig. 6A, intact: treatment effect: \( P = 0.22 \); Fig. 6B, CSX: treatment effect: \( P = 0.74 \)).

Interaction between MBP response and heart rate. The effect of NMS on baroreflex sensitivity in the intact and CSX rats was first assessed by ANCOVA to determine whether these factors affect the relationship between change in arterial blood pressure and the related changes in selected cardiorespiratory variables. In intact rats, ANCOVA showed that NMS affects the relationship between blood pressure and heart rate responses significantly (treatment \( \times \) blood pressure: \( P = 0.01 \)). Specifically, the slope of the linear regression between these variables was greater in NMS (2.4X) than in control rats (Fig. 6C; \( P < 0.0001 \)). To ensure that this effect was not biased by expressing the results as a percentage of change (normalization artifact), this analysis was repeated on data expressed as absolute change: \( \Delta \) pressure (mmHg) vs. \( \Delta \) heart rate (bpm). ANCOVA revealed the same difference (\( P < 0.0001 \)). With this analysis, the slope of the regression line of NMS rats was 2.3 times greater than controls (NMS slope: \(-0.62, r^2 = 0.68\); control slope: \(-0.27, r^2 = 0.44\)). In CSX animals, ANCOVA revealed no effect of NMS on the inversely proportional relationship between heart rate and blood pressure responses (Fig. 6D; treatment effect: \( P = 0.659 \)). The regression lines between those variables represent significant coefficients of the status of the carotid sinus nerve (intact or sectioned; Fig. 6, C and D, \( P < 0.0001 \) for all correlations).

Interaction between MBP response and ventilatory parameters. Figs. 7, A and B show the interaction between the changes in mean arterial blood pressure and the related breathing frequency response (both expressed as \% change from baseline). Each regression line is statistically significant (\( P < 0.0001 \)) and represents the inversely proportional relationship between those parameters. In intact rats, the slope of NMS rats is 34% greater than controls (Fig. 7A; treatment effect: \( P = 0.026 \)). Conversely, CSX eliminated the effect of NMS on this relationship (Fig. 7B; treatment effect: \( P = 0.74 \)). The effects of CSX did not differ significantly between groups (CSX \( \times \) treatment: \( P = 0.35 \)). In intact animals, the relationship between MBP and minute activity was similar between control and NMS (Fig. 7C; treatment effect: \( P = 0.31 \)). Similarly, in CSX animals, the relation between MBP and minute activity was not different between groups (Fig. 7D; treatment effect: \( P = 0.49 \)).

DISCUSSION

Our results show that maintaining rats iso-oxic during hypercapnia “reverses” the respiratory phenotype of adult male rats previously subjected to neonatal stress since under those conditions, the hyperpneic response of NMS rats is no longer lower than controls. However, this result mainly arises from the fact that, compared with the “standard” (poikilo-oxic) response, iso-oxia reduces the HCVR of control rats only. Although these data indicate that neonatal stress disrupts development of the interdependent relationship between peripheral and central chemoreceptor functions, the consequences of this effect may differ between cardio- and respiratory regula-
tion because maintaining rats iso-oxic during hypercapnia augmented the hypertensive response of NMS but not control animals. The demonstration that NMS potentiates the baroreflex is an important finding also, but given that the respiratory depression observed over a physiologically relevant range of hypertension was marginal, its contribution to NMS-related attenuation of the HCVR is marginal. We, therefore, conclude that NMS disrupts the carotid body’s function and/or its interdependence with other structures involved in CO2/H+ detection, and the initiation of hyperpnea contributes to the relative HCVR attenuation that characterizes male rats previously subjected to NMS. Nevertheless, these results are important because they show how early life exposure to a nonrespiratory stress can have a persistent impact on crucial aspects of cardio-respiratory regulation which may, in turn, contribute to the emergence of a disease state at adulthood.

Critique of the Method: Validity of the Anesthetized Rat Preparation

NMS affects development of the neuroendocrine response to stress (1, 6, 39). At adulthood, male NMS rats are hyperresponsive to stress and exhibit elevated indices of anxiety (27). Considering that CO2 is a potent panic-inducing agent in patients suffering from panic and anxiety disorders (46), the “emotional” or fearful perception of the hypercapnic challenge (rather than respiratory control dysfunction per se) could contribute to the respiratory phenotype of male rats subjected to NMS. However, the poikilo-oxic HCVR of anesthetized NMS rats was 34% lower than controls, a result that compares favorably with the 47% decrease observed in awake male rats previously subjected to NMS (21). Given that anesthesia likely attenuates the associative/emotional perception of the CO2 challenge, these results indicate that the respiratory phenotype of NMS is mainly due to neural control dysfunction.

The necessity to normalize the diaphragmatic EMG within and between subjects precludes direct comparisons of respiratory activity between experiments performed on awake and anesthetized rats. Nevertheless, it is clear that NMS affects breathing pattern differently between studies: in the anesthetized rat, a reduced breathing frequency response was the main variable responsible for the HCVR attenuation in NMS rats, whereas in the awake rat, the tidal volume response was attenuated. Although not well understood, such differences in breathing pattern response (frequency vs. tidal volume) to ventilatory stimuli between awake and urethane-anesthetized rodents have been reported previously (26). Nevertheless, data from this and previous studies, addressing the consequences of

<table>
<thead>
<tr>
<th>Table 4. Arterial blood gases and selected cardiovascular variables measured in under baseline conditions and during exposure to poikilo-oxic hypercapnia following CSX</th>
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<tbody>
<tr>
<td><strong>Baseline</strong></td>
</tr>
<tr>
<td><strong>Control</strong></td>
</tr>
<tr>
<td>PacO2, Torr</td>
</tr>
<tr>
<td>ΔPacO2, Torr</td>
</tr>
<tr>
<td>pH</td>
</tr>
<tr>
<td>PacO2, Torr</td>
</tr>
<tr>
<td>ΔPacO2, Torr</td>
</tr>
<tr>
<td>Heart rate, bpm</td>
</tr>
<tr>
<td>Mean blood pressure, mmHg</td>
</tr>
<tr>
<td>Breathing frequency, bpm</td>
</tr>
</tbody>
</table>

Data are reported as means ± SD. *Statistically significant difference from corresponding baseline (normocapnic) value at P ≤ 0.05. †Statistically significant difference from corresponding control value at P ≤ 0.05.
NMS on the hypoxic ventilatory response (20, 33, 35), show that the respiratory phenotype of male NMS rats is preserved under urethane anesthesia. The sum of these data validates (albeit indirectly) the use of this preparation for studies aiming to investigate the mechanisms by which NMS disrupts the respiratory control system. Although the influence of urethane on cardiovascular functions is lower than barbiturates or halothane (24), the limitations inherent in the use of anesthesia are not negligible and must be considered carefully while interpreting our data.

Neonatal Stress and Its Consequences on PaO₂ Modulation of the Hypercapnic Ventilatory Response

The interactions between O₂/CO₂ in setting carotid bodies’ activity and responsiveness are well described. Data from isolated carotid body preparations from rats and cats show that elevating P₀₂ reduces the carotid body's response to CO₂ in vitro (22, 52). On the basis of these results, it is unlikely that the relative hyperoxia achieved during hypercapnic hyperpnea is sufficient to reduce carotid body discharge frequency significantly during hypercapnic stimulation (22, 60). Nevertheless, the indirect evidence from previous studies suggesting that NMS augments peripheral chemoreceptor sensitivity (20, 35, 36) brought us to propose that the relative hyperoxia that takes place during hypercapnic hyperpnea is responsible for HCVR attenuation in NMS rats (21). Therefore, our results showing that preventing this relative hyperoxia (isoxic condition) attenuated the HCVR of control (but not NMS) rats by 39% were unexpected. Although hypoadditive interactions have been reported by others (11, 53), the experimental ap-

Table 5. Arterial blood gases measured under baseline conditions, prior to injection of hypotensive and hypertensive drugs in intact and CSX rats

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>NMS</th>
<th>Control</th>
<th>NMS</th>
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</thead>
<tbody>
<tr>
<td>PCO₂, Torr</td>
<td>34 (6)</td>
<td>37 (11)</td>
<td>38 (11)</td>
<td>41 (6)</td>
</tr>
<tr>
<td>pHa</td>
<td>7.33 (0.02)</td>
<td>7.30 (0.03)</td>
<td>7.38 (0.05)</td>
<td>7.35 (0.04)</td>
</tr>
<tr>
<td>PaO₂, Torr</td>
<td>108 (13)</td>
<td>106 (9)</td>
<td>119 (33)</td>
<td>128 (4)</td>
</tr>
<tr>
<td>Heart rate, bpm</td>
<td>392 (38)</td>
<td>384 (42)</td>
<td>384 (37)</td>
<td>363 (42)</td>
</tr>
<tr>
<td>Mean blood pressure, mmHg</td>
<td>97 (11)</td>
<td>108 (20)</td>
<td>98 (15)</td>
<td>98 (15)</td>
</tr>
<tr>
<td>Breathing frequency, bpm</td>
<td>83 (5)</td>
<td>79 (12)</td>
<td>87 (9)</td>
<td>73 (9)*</td>
</tr>
</tbody>
</table>

Data are reported as means ± SD. *Significant difference indicates a value statistically different from corresponding control value at P ≤ 0.05.

Fig. 6. Top: blood pressure responses to hypotensive and hypertensive agents (nitroprusside and phenylephrine, respectively) in rats with carotid sinus nerves intact (A) and following bilateral CSX (B). Experiments were performed on control animals (○) and rats previously subjected to NMS (▲). Bottom: comparison of the heart rate responses to changes in blood pressure (baroreflex sensitivity) between control and NMS rats. Experiments were performed on intact rats (C) and following bilateral CSX (D). Data are expressed as a percentage change from preinjection (baseline) value. †Statistically different from control (P ≤ 0.05).
proach used in our study did not provide sufficient control over specific levels of chemoreceptor stimulation (central vs. peripheral) to determine with certainty whether this model explains the reduced HCVR during iso-oxia. Nevertheless, these data suggest that results obtained in isolated organs, such as the in vitro carotid body preparation may not always apply to the ventilatory response observed at the whole animal level.

There is growing evidence indicating that peripheral and central chemoreceptors are interdependent, such that the sensitivity of the medullary chemoreceptors is highly determined by input from carotid bodies and perhaps by other sensory afferents (4, 10, 11, 57). Although difficult to explain and reconcile with current knowledge, one must keep in mind that the nature of the interdependence between central and peripheral chemoreceptors remains controversial as various types of interactions (e.g., hypoadditive and hyperadditive) have been reported, owing, in part, to differences in experimental approaches used (57).

Since to the best of our knowledge, few studies (if any) have compared the HCVR of rats under iso-oxic vs. poikilo-oxic conditions within a physiologically relevant PaO2 range, proper comparisons are difficult. Regardless, it is important to keep in mind that the nature of the interdependence between central and peripheral chemoreceptors remains controversial as various types of interactions (e.g., hypoadditive and hyperadditive) have been reported, owing, in part, to differences in experimental approaches used (57).

Neonatal Stress Potentiates the Baroreflex of Adult Rats

Comparing the inverse relationship between the changes in mean arterial blood pressure and the concomitant changes in heart rate and breathing frequency (the main respiratory variable accounting for the reduced HCVR in NMS rats) showed that early life exposure to stress augments baroreflex sensitivity, an effect that persists well into adulthood. Since carotid sinus nerve section eliminated the effects of NMS on baroreflex sensitivity, these results strongly suggest that, unlike the HCVR, NMS interfered with peripheral receptor function rather than on the processes responsible for central integration of barosensory afferents. While these data could argue for a role of baroreceptors in HCVR attenuation in NMS rats, we must keep in mind that the hypertension observed during hypercapnia is about 20%. On the basis of the interaction slopes between MBP and respiratory rate of each group (controls: slope = −0.1791; NMS: slope = −0.2716), the theoretical reduction...
during hypercapnia of the respiratory rate is estimated at 3.6% for controls and 5.4% in NMS rats. Clearly, this difference alone (1.8%) is not sufficient to explain the differences in HCVR between groups reported here. On the basis of the concept of receptor interdependence described previously, performing these experiments under hypercapnic conditions could reveal more profound effects on NMS on the baroreflex sensitivity. But given that maintaining rats normoxic helped explain most of the effects of NMS on HCVR, this possibility was not addressed experimentally. Although the physiological relevance of NMS-related enhancement of baroreflex sensitivity by NMS is limited, it is interesting to note that there is a high prevalence of spontaneous periodic breathing in human patients with hypertension and a markedly higher baroreceptor gain (3). It raises the possibility that this pathological phenotype may share its origins with those of NMS rats.

**Perspectives and Significance**

At this stage, we have little information explaining the mechanisms by which NMS affects the hypercapnic ventilatory response. We know, however, that in male rats, chronic injection of dexamethasone (a synthetic glucocorticoid) reduces the tyrosine hydroxylase activity in the NTS, the A5, and A7 neurons (30). The fact that reducing the number of these CO2/H⁺-sensitive neurons by injections of beta-hydroxylase-saporin decreases the HCVR in awake (28%) and sleeping rats (26%) (41), raises the possibility that the chronic increase in corticosterone levels observed in male NMS rats (20) is responsible for the reduced HCVR in these animals. Considering that chronic elevation of corticosterone alone augments the hypoxic ventilatory response of males (but not female) rats in a way that is similar to what has been reported in NMS rats (16), the effects of corticosterone per se on the hypercapnic chemoreflex is worth further investigation.

On the basis of the present data, it would also be very interesting to address this issue in females. However, the fact that female rats exhibit a higher HCVR in poikilo-oxic condition brings us to propose that other mechanisms are implicated in this exaggerated response (21). In that regard, we are currently determining whether NMS interferes with the endocrine mechanisms involved in the perception of the hypercapnic stress in females.

**Conclusion**

The present study aimed to elucidate the mechanisms responsible for attenuation of the HCVR of adult rats previously subjected to NMS. Data show that although enhancement of the baroreceptor function may contribute to this effect, the disruption of the central mechanisms involved in the integration of sensory afferent signals has a much stronger influence on HCVR. Results from manipulating PaO2 during hypercapnia strongly suggest that NMS-related disruption of the neural circuits integrating carotid body chemoafferent signal plays an important role. At this stage, however, we cannot exclude the possibility that integration of other sensory afferents (e.g., pulmonary stretch receptors) contribute to this intriguing respiratory phenotype.

The physiological significance of NMS-related attenuation of the hypercapnic ventilatory response is still unclear; however, abnormal chemoreflexes are observed in several cardiorespiratory disorders (obstructive sleep apnea, panic disorders, heart failure) (31, 46–49) and as such, they likely represent good indicators of the overall “health” of the neural circuits that help maintain homeostasis. The sum of the data gathered to date demonstrates the importance of early life environment on programming of vital homeostatic system and could contribute to the emergence of disease state at adulthood.

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