Neonatal caffeine induces sex-specific developmental plasticity of the hypoxic respiratory chemoreflex in adult rats

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1Department of Physiology, University of Toronto, Ontario; and 2Department of Pediatrics, Laval University, Centre de Recherche Hôpital St-François d’Assise, Québec, Canada

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Montandon G, Bairam A, Kinkead R. Neonatal caffeine induces sex-specific developmental plasticity of the hypoxic respiratory chemoreflex in adult rats. Am J Physiol Regul Integr Comp Physiol 295: R922–R934, 2008. First published July 2, 2008; doi:10.1152/ajpregu.00059.2008.—Caffeine is widely used to treat apneas of prematurity during the neonatal period; however, the potential consequences of administering a neonatal caffeine treatment (NCT) during a critical period for respiratory control development are unknown. The present study therefore determined whether NCT in rats alters the hypoxic respiratory chemoreflex measured at adulthood. Newborn rats received either caffeine (15 mg/kg) or water (control) each day from postnatal day 3 to 12. The ventilatory response to a hypoxic challenge (inspired O2 fraction = 0.12) was first evaluated in awake adult male and male rats using whole body plethysmography. Results showed that NCT increased the initial phase of the breathing frequency response to hypoxia in males only. This result was confirmed in anesthetized and artificially ventilated adult male rats where NCT also increased the phrenic burst frequency response to hypoxia. RT-PCR assessment of mRNA encoding for adenosine A1A and A2A receptors, dopamine D2 receptors, and tyrosine hydroxylase in the rat carotid bodies showed that NCT enhanced mRNA expression levels of adenosine A2A, dopamine D2, and tyrosine hydroxylase of males but not females. Subsequent experiments on awake male rats showed that injection of the adenosine A2A receptor antagonist ZM2413855 (1 mg/kg ip) before ventilatory measurements abolished, in NCT rats, the enhanced respiratory frequency response observed during the early phase of hypoxia. We propose that NCT elicits a sex-specific increase in the hypoxic respiratory chemoreflex, which is related, at least partially, to an enhancement in adenosine A2A receptors in the rat carotid body.

Caffeine; control of breathing; development; sexual dimorphism

IN THE CLINIC, CAFFEINE ADMINISTRATION is acknowledged as an effective treatment for apneas commonly seen in premature newborn infants (42). Caffeine is an adenosine receptor antagonist, which is a relatively safe ventilatory stimulant in this population, even when administered at high doses (7, 11, 41). However, the use of such pharmacological agents during a critical period of development is a matter of concern for clinicians given its potential impact on central nervous system (CNS) maturation (19). In rats, for instance, neonatal caffeine administration was shown to change adenosine receptor expression in the brain (27). Although several studies have addressed the consequences of perinatal caffeine exposure on numerous aspects of brain development and function (23, 27), relatively little is known about the possible consequences of this treatment on the developmental trajectory of the respiratory control system.

Despite the limitations inherent to animal research, the newborn rat remains a valuable model for developmental studies because, at birth, the degree of CNS maturation of rats is less advanced than a full-term human and corresponds well to that of a premature baby (10, 20). Using a protocol that, in newborn rats, reproduced plasma caffeine levels measured in the clinic, we showed that chronic neonatal caffeine treatment (NCT; 15 mg·kg−1·day−1 from postnatal day 3 to 12) has no significant effect on “resting” ventilatory activity but increases CO2 chemosensitivity in young (20 days old) male rats (45). This effect changed with maturation since, in adult rats, NCT no longer augments the magnitude of the hypercapnic ventilatory response but modifies its pattern (tidal volume vs. frequency) in males but not in females (45). Subsequent results showed that NCT decreases ventilatory responsiveness to selective adenosine receptor antagonists, thereby suggesting that changes in adenosinergic neurotransmission are involved (46). However, our understanding of the persistent effects of NCT on respiratory control development remains limited since other functional and mechanistic aspects of this system have not been explored.

The ventilatory response to hypoxia is the result of a complex interplay between peripheral chemosensory afferent signals and their integration at the CNS levels. Adenosine is an important modulator of this chemoreflex since hypoxia elicits adenosine release both at the central and peripheral (carotid body) level (12, 26). Although the precise role of adenosine in regulating the hypoxic ventilatory response at the CNS level is still unclear (26, 37), data consistently show that adenosine stimulates carotid body activity (13, 44) and that A2A and A2B receptors contribute to this effect (13). These results, combined with our previous work, brought us to test the hypothesis that NCT alters the hypoxic ventilatory response in adult rats. Since the functional (respiratory) consequences of neonatal interventions on rat pups show important sexual dimorphism (24, 45), this hypothesis was tested on adult male and female rats. To this aim, we first used whole body plethysmography to compare the hypoxic ventilatory response of adult, freely behaving animals between control and NCT rats, and data show that NCT increased the breathing frequency response during the early phase of hypoxia. To complement this approach, we then compared phrenic (inspiratory) responses to hypoxia using an anesthetized, paralyzed, and artificially ventilated rat preparation, which allows better control

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of arterial blood gases and eliminates potential caveats associated with behavioral interference. Based on plethysmography results, these electrophysiological experiments were performed on males only. Results obtained with this preparation are consistent with those obtained in the awake rats since they show that the acute phrenic burst frequency response to hypoxia of NCT rats is greater than control rats. Finally, the impact of NCT on selected indicators of carotid body regulation, such as adenosine receptor, dopamine receptor, and tyrosine hydroxylase mRNA expressions, was assessed using RT-PCR analysis on carotid bodies harvested from adult male and female rats. These genes were chosen because caffeine is a nonspecific adenosine receptor antagonist and because of the functional interaction between adenosine and dopamine receptors at the level of CNS and carotid bodies (13, 22). Enhancements of adenosine A2A receptor mRNA levels, combined with results from pharmacological experiments, indicate that A2A receptors are likely involved in the enhanced hypoxic response observed in NCT rats. Preliminary results were reported in abstract form (47).

METHODS

Ethical Information

The experimental protocols were approved by the Animal Care Committee at Laval University in accordance with the Canadian Council on Animal Care guidelines.

Mating and NCT

The study was performed on 94 male and 52 female adult (3- to 5-mo-old) Sprague-Dawley rats. Mating and NCT were performed according to our protocol (45). Dam and male Sprague-Dawley rats were obtained from Charles Rivers Canada (St. Constant, Canada). All rats used in this study were born in our animal care facility. Rats were supplied with food and water ad libitum and maintained in standard laboratory conditions (21°C, 12:12-h dark-light cycle: lights on at 0800 and off at 2000). Briefly, caffeine was administered by gavage to half the pups of the litter (NCT group) each day from postnatal day 3 to 12 at 15 mg/kg (caffeine citrate 30 mg/kg) in a volume of 0.05 ml/10 g body wt. The other half of the litter received water (control group) at the same volume. This caffeine dose results in pharmacological levels of caffeine and dopamine receptors at the level of CNS and carotid bodies (13, 22). Enhancements of adenosine A2A receptor mRNA levels, combined with results from pharmacological experiments, indicate that A2A receptors are likely involved in the enhanced hypoxic response observed in NCT rats. Preliminary results were reported in abstract form (47).

Study 1: Effects of Neonatal Caffeine on the Hypoxic Ventilatory Response

These experiments were conducted on 10 control male, 10 control female, 10 NCT male, and 10 NCT female adult (4- to 5-mo-old) rats. Measurements of inspiratory duration (Ti), breathing frequency (fR), and tidal volume (VT) in unrestrained rats were obtained by whole body flow-through plethysmography (model PLY3223, Buxco Electronics, Sharon, CT) as described previously (33, 45). Body temperature was measured by telemetry with a transponder (E-mitter, Mini Mitter, Bend, OR) implanted intraperitoneally 1 wk before experiments (45). Surgical implantation of transponder was done under anesthesia with ketamine/xylazine (10/50 mg/kg) injected intraperitoneally. Barometric pressure, flow rate, chamber temperature, humidity, and body temperature were used to express VT in milliliters (ml BTPS) per 100 grams of body weight according to standard equations (15, 50). Minute ventilation (VE) was calculated as the product of fR and VT and normalized according to body weight. Oxygen consumption (VO2) and carbon dioxide production (VCO2) were measured using an oxygen analyzer (model S-3A, Ametek, Pittsburgh, PA) and carbon dioxide analyzer (model CD-3A, Ametek) and calculated according to the Fick principle and formulas ((O2in - O2out) × flow and (CO2out - CO2in) × flow, respectively, used for an open system (49). VO2 and VCO2 were expressed in milliliters per minute per 100 grams standard temperature and pressure, dry (STPD).

Each rat was acclimated to the plethysmographic chamber for ~30 min before experimental recordings. Resting ventilatory and metabolic measurements were made when the rat was quiet but awake and breathing room air. However, should the animal appear to go to sleep (no active behavior for several minutes), we gently knocked on the chamber to ensure that the animal was awake during measurements. After 10 min of normoxic measurements (rest), a hypoxic gas mixture [inspired O2 fraction (FiO2) = 0.12; balance N2] was delivered to the chamber for 20 min. Measurements of O2 levels within the chamber (at the level of the animal) over the course of the hypoxic protocol are shown in Figs. 1B and 2B.

Study 2: Impact of Neonatal Caffeine on Phrenic Nerve Activity

To compare the neural correlate of inspiratory motor output between control (n = 7) and NCT (n = 7) adult male rats (3–4 mo-old), phrenic nerve activity was recorded as previously described (34). Anesthesia was induced using isoflurane (3%, FiO2 = 0.5, balance N2). Rat’s temperature was maintained between 37 and 38°C with a thermoregulated blanket (Harvard Apparatus, Holliston, MA). The trachea was cannulated, and bilateral vagotomy was performed at midcervical level. The rat was then artificially ventilated with a rodent ventilator (Harvard Apparatus). A venous femoral catheter was inserted for anesthetic and fluid administration, and an arterial femoral catheter was placed for blood pressure monitoring (Transbridge TBM4M-B, World Precision Instruments, Sarasota, FL) and withdrawal of blood samples for arterial blood-gas analysis (model ABL-5, Radiometer Copenhagen, London, Canada). Rats were slowly converted (~10 min) from isoflurane to urethane anesthesia (1.6 g/kg iv in distilled water) and were then paralyzed with pancuronium bromide (2.5 mg/kg iv). Proper anesthesia was confirmed by monitoring changes in heart rate and blood pressure in response to toe pinch. The end-tidal expiratory PCO2 was measured using an in-line CO2 analyzer (model 1265, Novametrix-Respironics, Murrysville, PA). The phrenic nerve was isolated unilaterally, using a left dorsal approach, cut distally, and desheathed. The nerve was submerged in mineral oil and placed on a bipolar silver recording electrode. Nerve activity was amplified (gain = 10,000; model 1700, AM-Systems, Everett, WA), band-pass filtered (100 Hz to 10 kHz), and fed to a moving averager (model MA-821, CWE, Ardmore, PA) before being digitized and recorded with a data acquisition system (IOX, EMKA Technologies).

After surgery, CO2 apneic threshold for inspiratory (phrenic) activity was then determined by mechanically hyperventilating the rats until phrenic nerve activity ceased. At this point, a blood sample was taken for blood gas measurement. This procedure allowed us to determine whether NCT affects the apneic threshold. Rate of the ventilator was then decreased progressively until phrenic activity returned and arterial PCO2 (Paco2) was set 2–3 Torr above the apneic threshold. This procedure served to standardize baseline phrenic activity relative to its threshold rather than an arbitrarily predeter-
every 15 s and reported both in absolute values and expressed as a percentage change from baseline. Euthanasia was performed at the end of protocol by urethane overdose.

Study 3: Effect of Neonatal Caffeine on Adenosine A2A, Dopamine D2, and Tyrosine Hydroxylase mRNA Expression in the Rat Carotid Body

Real-time RT-PCR was used for relative quantification of mRNA expression levels for adenosine A1, A2A, dopamine D2 receptors, and tyrosine hydroxylase (TH) mRNAs from carotid bodies harvested from male and female adult rats. Procedures for animal preparation, surgery, organ collection, and mRNA level estimations were similar to those described previously (4, 35). Experiments were realized on 32 male and 32 female adult rats of 3–4 mo old (n = 16 for control and n = 16 for NCT). Two pools each containing 16 carotid bodies from 8 rats were collected for control and NCT groups and for each sex. Briefly, each rat was anesthetized with a mixture of ketamine/xylazine (10/50 mg/kg) injected intraperitoneally. Rats were rapidly tracheotomized and artificially ventilated with air. The carotid bodies were quickly removed and were then immediately frozen on dry ice and stored at −80°C. Total RNA extraction as well as RT using random decamer primers for transcripts amplifications were done as previously described (35). Aliquots of 2 µl from the resulting single-stranded cDNA products were used along with the appropriate primers for the A1, A2A, D2, TH, or for 18S ribosomal RNA amplification (Table 1). Each amplification was run in separate wells with 2×
antagonist enhanced breathing frequency response observed at the onset of hypoxia in NCT rats, the selective adenosine A2A receptor antagonist measured ventilation at rest, metabolism, and baseline phrenic nerve activity between control and NCT rats were made using a one-way ANOVA (JMP 7, SAS Institute, Cary, NC). Comparisons of the hypoxic responses between control and NCT rats were done with analysis of variance for repeated measures using a full-factorial standard least square model (interaction: hypoxia × treatment, with hypoxia as repeated factor, JMP 7). This allowed us to compare the slope of the response between control and NCT rats. To determine sex-specific impacts of NCT on the hypoxic response, we applied three-way ANOVAs with a full factorial standard least square model (interaction: hypoxia × treatment × sex, hypoxia as repeated factor). These tests were followed by least-significant mean difference Student’s t-tests (indicated with * in Figs. 1–3).

mRNA data were obtained from a small sample size, and tissue from carotid bodies had to be pooled to have enough material for RT-PCR analysis. Since the data did not follow Gaussian distribution and variances were not equal, statistical analysis was performed using distribution-free Wilcoxon rank-sum test to compare mRNA levels between control and NCT rats (67). However, this approach does not allow comparison between male and female rats. If not specified, P values reported in the text indicate ANOVA results of standard least square models or Wilcoxon rank-sum tests. Data were considered significantly different when P < 0.05 and were expressed using means ± SE.

RESULTS

Study 1: Neonatal Caffeine Enhances Hypoxic Chemoreflex in Freely Behaving Adult Rats

To determine whether neonatal caffeine modifies the hypoxic chemoreflex in awake freely behaving adult male and female rats, we first measured metabolism and breathing at rest and during hypoxia using whole body plethysmography.

Metabolism. Table 2 summarizes data in control and NCT male and female adult rats at rest. In males, NCT did not change body weight, VO₂, VCO₂, minute ventilation (VE) VE/VO₂, VE/VCO₂, and body temperature at rest (Table 2) as well as during hypoxic exposure (data not shown). In females, NCT did not change body weight and VO₂ but increased VCO₂ by 20% (Table 2), decreased VE/VO₂ at rest, and did not change VE/VCO₂. NCT did not change body temperature in females. During hypoxia in females, there were no changes due to NCT in any of the indicators of metabolism (VO₂, VCO₂, and body temperature).

Breathing. Representative plethysmographic recordings of ventilatory activity at rest and during moderate hypoxia (FIO₂ = 0.12) in a control and a NCT male rat are presented in Fig. 1A, respectively. In males, hypoxia increased respiratory frequency in control and NCT rats (P < 0.001; Fig. 1C). The respiratory frequency in response to hypoxia was significantly

Table 1. Oligonucleotide primers selected for real-time PCR, expected length of the PCR products

<table>
<thead>
<tr>
<th>Oligonucleotide primers</th>
<th>Forward Primers</th>
<th>Reverse Primers</th>
<th>Length, bp</th>
</tr>
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<tbody>
<tr>
<td>A₁</td>
<td>5'-GGGAAAGGAGGAAGGACTGA-3'</td>
<td>5'-GGCAGATCGAACCTGCG-3'</td>
<td>77</td>
</tr>
<tr>
<td>A₂A</td>
<td>Residues 1,103-1,123</td>
<td>Residues 1,103-1,123</td>
<td>57</td>
</tr>
<tr>
<td>D₂ common segment</td>
<td>Residues 780-790</td>
<td>Residues 791-813</td>
<td>144</td>
</tr>
<tr>
<td>TH</td>
<td>Residues 421-440</td>
<td>Residues 467-483</td>
<td>73</td>
</tr>
<tr>
<td>18S</td>
<td>Residues 467-483</td>
<td>Residues 453-473</td>
<td>67</td>
</tr>
<tr>
<td></td>
<td>Residues 407-425</td>
<td>Residues 497-513</td>
<td>67</td>
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</tbody>
</table>

SYBR Green Universal PCR Master Mix containing 100 nM of each of specific forward and reverse gene primers (total 25 µl). Primers were chosen using the Primer Express software program (version 2.0, Applied Biosystems, Foster City, CA; Table 1). PCR amplification was performed on an ABI Prism 7000 Sequence Detector System (Applied Biosystems) according to manufacturer’s instruction. The 18S ribosomal RNA amplification was used to normalize the data for the difference in starting material and to test the efficiencies of RNA extraction and RT reactions for each sample. A total of six RTs were prepared from the two different RNA pools. Then, two PCRs were performed from each RT giving a total of 12 PCR reactions for each amplified gene. The relative expression level of A₁, A₂A, D₂, or TH transcript was first normalized to 18S expression level obtained from the same cDNA sample following the standard curve method instructions as given by the manufacturer (User Bulletin, version 2, ABI PRISM 7700 sequence detection system, Applied Biosystem). Hence, an average normalized gene expression and standard error of the mean for each transcript studied for each group was calculated. Differences of <10% were considered in the range of variability related to RT-PCR amplification and were not considered different even though statistical analysis was <5% (P < 0.05) (4).

Study 4: Effect of IP Injection of Adenosine A₂A Receptor Antagonist ZM241385 on the Hypoxic Ventilatory Response in Control and NCT Rats

To determine whether adenosine A₂A receptors contribute to the enhanced breathing frequency response observed at the onset of hypoxia in NCT rats, the selective adenosine A₂A receptor antagonist ZM241384 or vehicle was administered (1 mg/kg ip) 30 min before plethysmographic measurements in 14 control and 14 NCT male rats. ZM241385 was first diluted into DMSO and Cremophor EL (46). Before intraperitoneal injection, the solution was diluted into saline with final concentration of 1 mg/kg, 5%, and 5% for ZM241385, DMSO, and Cremophor EL, respectively. This ZM241385 concentration was sufficient to modify ventilation in freely behaving juvenile rats (46). Measurements of the hypoxic ventilatory response were identical to study 1.

Data Analysis

For plethysmographic measurements, average values of ventilatory variables were obtained on a minute-by-minute basis using DataAnalyst software (EMKA). For phrenic nerve measurements, average values of phrenic nerve frequency were obtained every 15 s (DataAnalyst, EMKA) and were also expressed as a percentage change (relative change) from baseline to assess phrenic nerve response to hypoxia. Phrenic burst amplitude was analyzed in the same way. Showing both forms of data (absolute and % change from baseline) ensures that data interpretation is not biased by normalization artifacts. Comparisons of ventilation at rest, metabolism, and baseline phrenic nerve activity between control and NCT rats were made using a one-way ANOVA (JMP 7, SAS Institute, Cary, NC). Comparisons of the hypoxic responses between control and NCT rats were done with analysis of variance for repeated measures using a full-factorial standard least square model (interaction: hypoxia × treatment, with hypoxia as repeated factor, JMP 7). This allowed us to compare the slope of the response between control and NCT rats. To determine sex-specific impacts of NCT on the hypoxic response, we applied three-way ANOVAs with a full factorial standard least square model (interaction: hypoxia × treatment × sex, hypoxia as repeated factor). These tests were followed by least-significant mean difference Student’s t-tests (indicated with * in Figs. 1–3).

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TH, tyrosine hydroxylase. Genebank accession number: aNM_017155; bNM_053294; cNM_012547; dNM_012740; eX01117.

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higher in NCT compared with control rats (hypoxia × treatment, \( P < 0.001 \); Fig. 1C), and post hoc tests demonstrated that this between-group difference was present during the first 9 min of hypoxia only (Fig. 1C). A similar effect was observed for \( T_1 \), which was significantly lower in NCT rats compared with controls over the first 10 min of hypoxia (hypoxia × treatment, \( P < 0.001 \); Fig. 1D). Finally, \( V_{E} \) and \( V_T \) were not changed by NCT either at rest or during hypoxia (Fig. 1, E and F, respectively). In adult females, representative plethysmographic recordings of ventilation at rest and during hypoxia (\( P_{O_2} = 0.12 \)) in a control and a NCT rat are presented in Fig. 2A. Similar to what was obtained in males, hypoxia increased \( f_R \) in both control and NCT groups (hypoxia effect: \( P < 0.0001 \); Fig. 2C). However, neither \( f_R \), \( T_1 \), \( T_E \), nor \( V_T \) were modified by NCT (Fig. 2, C–F, respectively). The effects of NCT on the \( f_R \) and \( T_i \) responses to hypoxia were sex specific (hypoxia × treatment × sex, \( P = 0.001 \) and \( P = 0.004 \), respectively).


tudy 2: Neonatal Caffeine Increases Phrenic Nerve Activity in Anesthetized Rats

To determine whether neonatal caffeine elicits changes in phrenic nerve activity similar to those reported in unanesthetized, freely behaving rats, inspiratory (phrenic) motor output was compared between NCT and control under “baseline” and hypoxic conditions. Since in study 1 NCT had no effect on the hypoxic ventilatory response measured in females, these experiments were performed on anesthetized (urethane 1.6 g/kg iv) and paralyzed (pancuronium bromide, 2.5 mg/kg iv) male rats only. No additional doses of anesthetics were necessary.

\textbf{Blood gases.} Table 3 presents arterial blood gases, pH, and body weight obtained in this series. \( P_{O_2} \) apneic threshold for phrenic activity did not differ between control and NCT rats (data not shown). Accordingly, baseline \( P_{ACO_2} \), arterial \( P_{O_2} \) (\( P_{O_2} \)), and \( pH \) values were similar in both groups (Table 3). During hypoxia, \( P_{AaO_2} \) decreased to the same level for both groups (hypoxia effect: \( P < 0.0001 \)), whereas \( P_{ACO_2} \) and \( pH \) remained at baseline levels for both groups, indicating that isocapnia was maintained during hypoxic stimulation. Finally, as observed in the previous series of experiments, NCT did not affect adult body weight (Table 3).

\textbf{Phrenic nerve activity.} Representative recordings of phrenic nerve activity during baseline (\( P_{O_2} = 0.5 \)) and moderate hypoxia (\( P_{O_2} = 0.12 \)) for a control and a NCT anesthetized adult male rat are shown in Fig. 3, A and B, respectively. During baseline and hypoxia, absolute phrenic burst frequency did not differ between control and NCT groups (Fig. 3C). Relative change of phrenic nerve frequency was increased by NCT during the first minute of hypoxia (Fig. 3D). The phrenic nerve frequency responses to hypoxia (both absolute and normalized) were higher in NCT than in control rats (treatment × hypoxia, \( P = 0.049 \) and \( P = 0.026 \); Fig. 3, C and D, respectively); these differences were only present during the first minute of hypoxia. During the following phase (minutes 1–6), however, phrenic frequency and relative change did not differ between control and NCT rats, demonstrating that NCT increased the hypoxic response during the onset of hypoxia only. Absolute phrenic burst amplitude recorded from NCT rats was greater than controls from baseline to minute 1 of hypoxia (Fig. 3E) and from minute 3 to 6. However, relative change of phrenic nerve amplitude did not differ during hypoxia (Fig. 3F). The hypoxic response of amplitude and relative change of amplitude were not changed by NCT (\( P = 0.076 \) and \( P = 0.10 \)). Absolute minute activity (product of frequency and amplitude) was higher in NCT compared with control rats from minute 0.5 to 1 (Fig. 3G). Relative change of minute activity did not differ between control and NCT rats during baseline and hypoxia (Fig. 3H). However, NCT increased the minute activity response to hypoxia (both absolute and normalized, hypoxia × treatment, \( P = 0.016 \) and \( P = 0.025 \), respectively; Fig. 3, G and H).

\textbf{Cardiovascular parameters.} Representative blood pressure recordings are shown in Fig. 4, A and B for a control and a NCT rat. At rest, mean arterial blood pressure was lower in NCT rats compared with controls (\( P = 0.040 \); Fig. 4C) but was identical during hypoxia. Systolic blood pressure was not changed by NCT (Fig. 4D) at baseline or during hypoxia, whereas diastolic blood pressure was lower in NCT than in control rats at rest (\( P = 0.012 \); Fig. 4E) and during the last 2 min of hypoxia (\( P < 0.006 \)). Heart rate did not differ between control and NCT rats (Fig. 4F) during baseline and hypoxia.

\textbf{Study 3: Neonatal Caffeine Augments mRNA Levels of Adenosine A\textsubscript{2A} Receptor, Dopamine D\textsubscript{2} Receptor, and Tyrosine Hydroxylase in the Rat Carotid Body

To determine whether neonatal caffeine affects indicators of adenosinergic and dopaminergic neurotransmission within the carotid bodies of adult rats, we compared expression levels of mRNA transcripts encoding for adenosine A\textsubscript{1} and A\textsubscript{2A} receptors, dopamine D\textsubscript{2} receptors, and tyrosine hydroxylase (TH) between control and NCT rats. In male rats (Fig. 5A), there were clear increases of mRNA levels of A\textsubscript{2A}, D\textsubscript{2} receptors, and TH in NCT compared with control rats. Wilcoxon rank sum tests confirmed that NCT increased mRNA expression levels

Table 2. Resting ventilatory, metabolic, and body weight data in control and NCT adult male and female rats

<table>
<thead>
<tr>
<th></th>
<th>Male Rats</th>
<th>Female Rats</th>
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<tbody>
<tr>
<td></td>
<td>Control (( n = 10 ))</td>
<td>NCT (( n = 10 ))</td>
</tr>
<tr>
<td>Body weight, g</td>
<td>522 ± 31</td>
<td>494 ± 15</td>
</tr>
<tr>
<td>( V_{O_2} ) (STPD), ml/min ( \cdot )100 g( ^{-1} )</td>
<td>2.5 ± 0.2</td>
<td>2.7 ± 0.1</td>
</tr>
<tr>
<td>( V_{CO_2} ) (STPD), ml/min ( \cdot )100 g( ^{-1} )</td>
<td>1.7 ± 0.1</td>
<td>1.8 ± 0.1</td>
</tr>
<tr>
<td>( V_{E}/V_{O_2} )</td>
<td>44 ± 4</td>
<td>41 ± 3</td>
</tr>
<tr>
<td>( V_{E}/V_{CO_2} )</td>
<td>62 ± 6</td>
<td>62 ± 5</td>
</tr>
<tr>
<td>( T_{body},{^\circ} )C</td>
<td>37.0 ± 0.2</td>
<td>37.3 ± 0.4</td>
</tr>
</tbody>
</table>

Values are means ± SE. NCT, neonatal caffeine treatment; \( V_{O_2} \), oxygen consumption; STPD, standard temperature and pressure, dry; \( V_{CO_2} \), carbon dioxide production; \( V_{E}/V_{O_2} \) and \( V_{E}/V_{CO_2} \), oxygen and carbon dioxide convection ratios, respectively; \( T_{body} \), body temperature. *\( P < 0.05 \) vs. control.
for adenosine A2A receptors, dopamine D2 receptors, and TH by 24%, 43%, and 36%, respectively (P < 0.0015, P < 0.0007, P < 0.0002, respectively) but did not change levels of adenosine A1 receptor mRNA. In female rats (Fig. 5B), NCT increased adenosine A2A receptor mRNA level by only 14% (P < 0.0102) and did not change A1, D2 receptor, and TH mRNA expression levels.

**Study 4: Systemic Inactivation of Adenosine A2A Receptors Suppressed the Enhanced Breathing Frequency Response Observed in NCT Male Rats**

In vehicle-treated animals, NCT rats presented higher respiratory frequencies during the first 10 min of hypoxia (Fig. 6A; 28%, P < 0.001) compared with control rats. No differences in VT or VE were observed between control and NCT rats at room air or during hypoxia (Fig. 6, B and C). However, administration of ZM241385 before the experiment abolished the NCT-related enhancement of the respiratory frequency response observed during the first 10 min of hypoxia (Fig. 6A; two-way ANOVA, drugs × treatment, P = 0.019). Effects of ZM241385 on VT and VE were similar in control and NCT rats (Fig. 6, B and C).

**DISCUSSION**

This study shows that chronic NCT leads to long-lasting, sex-specific (male only) enhancement of the breathing frequency response to moderate hypoxia in adult rats. Despite
important methodological differences, this effect was observed both in freely behaving and anesthetized adult rats. Although more experiments are required to fully understand the effects of NCT on the respiratory control system, the rapid dynamics and the fact that, in both experimental approaches, treatment-related difference were mainly observed on the frequency component of the response initially suggested that peripheral hypoxic chemosensitivity is enhanced by NCT (52). This interpretation was then supported by RT-PCR data showing that NCT increases adenosine A2A receptor, dopamine D2 receptor, and TH mRNA expression levels in the carotid bodies in male rats. Subsequently, results showing that inactivation of adenosine A2A receptors by a specific antagonist abolished the enhanced hypoxic response observed in NCT rats allowed us to demonstrate a functional link between RT-PCR data and the respiratory phenotype observed in NCT male rats. The fact that NCT did not affect the VE response significantly raises questions about the potential functional significance of these effects. However, these data nonetheless show that NCT affects respiratory control development in a way that, in adult males, may compromise breathing efficiency and stability, especially during sleep.

### Table 3. Arterial blood gases and pH during baseline and moderate hypoxia in control and NCT anesthetized adult male rats

<table>
<thead>
<tr>
<th></th>
<th>Control Rats (n = 7)</th>
<th>NCT Rats (n = 7)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline</td>
<td>Hypoxia</td>
</tr>
<tr>
<td>PaCO₂, Torr</td>
<td>38.6 ± 2.1</td>
<td>37.1 ± 2.4</td>
</tr>
<tr>
<td>PaO₂, Torr</td>
<td>152 ± 8</td>
<td>41 ± 4*</td>
</tr>
<tr>
<td>pH</td>
<td>7.34 ± 0.01</td>
<td>7.26 ± 0.02</td>
</tr>
<tr>
<td>Body weight, g</td>
<td>625 ± 26</td>
<td>656 ± 23</td>
</tr>
</tbody>
</table>

Values are means ± SE. PaCO₂, arterial PCO₂; PaO₂, arterial PO₂. *P < 0.05 vs. baseline.
Critique of Methods

The use of whole body plethysmography to measure ventilatory activity in freely behaving rats has several limitations that preclude the conclusion that NCT persistently modifies the hypoxic ventilatory response. For instance, sleep-wake states were not determined during the recordings, and the ventilatory response to hypoxia could be affected by a change in behavior (change of sleep-wake states, increase of sniffing, movements, or other behavior-related artifacts) rather than a change in respiratory output per se (60). Furthermore, arterial blood gases were not controlled in freely behaving animals, and the enhanced hypoxic response observed above could be due to hypocapnia associated with hyperventilation rather than exposure to hypoxia itself (14). Although whole body plethysmography remains the best (least invasive) method currently available to measure ventilatory activity in awake rodents (50), its validity and accuracy are the subject of much debate (16, 38, 61). Many of the limitations associated with whole body plethysmography were addressed by measuring phrenic activity in anesthetized male adult rats for which many of these variables were better controlled. Results obtained with this approach confirmed data obtained in awake rats as we showed that NCT increases phrenic burst frequency response to hypoxia. However, we also observed that the minute activity response to hypoxia was higher in NCT compared with control rats; an enhancement was not observed in freely behaving rats. These discrepancies between anesthetized and freely behaving rats might be due to the fact that isocapnia was maintained and vagotomy was performed in anesthetized rats. Despite these differences, both approaches suggest that the enhancement of the breathing frequency in response to hypoxia is likely due to an increase of the hypoxic chemoreflex rather than to sleep or behavioral and/or technical artifacts.

NCT-Related Enhancement of the Acute Frequency Response to Hypoxia in Adult Male Rats: Putative Mechanisms

Peripheral vs. central mechanism. The initial phase of ventilatory response to hypoxia involves distinct but overlapping
Activation of adenosine A2A receptors, dopamine D2 receptors, and tyrosine hydroxylase (TH) in the carotid body of control (white) and NCT (black) adult male (A) and female (B) rats. Values are means ± SE. *Significantly different from control (P < 0.05).

peripheral and central mechanisms (52). The immediate augmentation of ventilation at the onset of hypoxia (within a few breaths) reflects rapid detection of low PaO2 by carotid body (52), and the stronger frequency response seen in NCT rats suggests that carotid body function is affected by NCT. However, central brain stem structures, such as the nucleus tractus solitarius (NTS), that receive afferent information from peripheral chemoreceptors (21, 28) also play a significant role in the initiation and maintenance of the cardio-respiratory responses to hypoxia. The NTS is a brain stem region rich in adenosine receptors (9, 23, 54, 62) where adenosine is naturally released during hypoxia and could facilitate neurotransmitter release [e.g., norepinephrine and glutamate (59)]. Consequently, changes in adenosinergic neurotransmission within the NTS could also contribute to enhancement of the frequency response seen in NCT rats. However, adenosine release in the NTS occurs at least 2 min after the onset of the hypoxic stimulus (26), suggesting that adenosine is not implicated in the initial phase of the ventilatory response to hypoxia but rather during the late phase (roll-off) of the hypoxic response (37). Although these observations do not exclude the possibility that other CNS structures relevant to respiratory control were affected by NCT, they prompted us to focus on the potential role of the carotid bodies.

**Adenosine and carotid body.** Adenosine is among the numerous neurotransmitters involved in the regulation of carotid body function during hypoxia. In vitro studies have shown that hypoxia increases endogenous adenosine release from rat carotid body (12, 25). Activation of adenosine A2A receptors stimulates catecholamine release from in vitro rat carotid body (13), increases carotid sinus nerve activity of adult cats in vitro (55), and increases breathing in anesthetized rats (48). Together, these results indicate that adenosine has a stimulatory role in the carotid body, which likely contributes to the increase in activity during a hypoxic challenge. The fact that systemic injection of adenosine accentuates the hypoxic ventilatory response in humans supports this interpretation (43, 66). Adenosine A2A receptors have been identified in the rat carotid body using either immunohistochemistry (13, 25), Northern blot (2, 5, 35), or RT-PCR analysis (5, 36). Since adenosine A1 receptors were only detected using RT-PCR (2, 5), this indicates that its expression level is very low in this structure. However, given that NCT had no effect on A1 receptor mRNA expression level, its potential involvement at the peripheral level will not be addressed in the following discussion. Adenosine A2B receptors have also been detected in the carotid body (13), but adenosine has low affinity for this receptor subtype, suggesting that its role in the hypoxic chemoreflex is minimal (59). Thus adenosine A2A receptors appear as the major excitatory adenosine receptor subtype in the carotid body since its activation increases the chemo- sensory response to hypoxia (13) even though studies using the nonspecific antagonist caffeine have shown contradictory effects on chemosensory activity. Caffeine has no effect on chemo- sensory activity under basal condition of normoxia (3, 13), but it increases the chemosensory response to hypoxia of rat carotid body in vitro (13), whereas it has no effect on the in vivo carotid body responses measured in newborn and adult cats (3). Methodological approaches, species differences, and type of caffeine administration may account for these differences.

In the present study, NCT rats showed an increase in mRNA expression levels of adenosine A2A receptors, which may increase the final protein expression. It also suggests that an increase of adenosine A2A receptor expression in the carotid body might contribute to the enhancement of the breathing frequency observed in NCT rats (freely behaving or anesthe- tized). However, this interpretation had to be confirmed. In awake male rats, inactivation of adenosine A2A receptors by ZM241385 abolished the NCT-related enhancement of respiratory frequency observed during the first 10 min of hypoxia. Although this result argues in favor of a direct role of adeno- sine A2A receptors in the enhancement of the hypoxic response in NCT rats, we must consider that ZM241385 was administered systematically and that the sites of action of this drug may be numerous. However, peripheral chemoreceptors are likely candidates since adenosine A2A receptor mRNA expression level measured in the carotid bodies of NCT rats was higher than in controls. Moreover, NCT-related enhancement of the breathing frequency response was observed during the initial phase of hypoxia, an aspect of the response attributed to carotid body activation (52).

**Dopamine and carotid body.** Regardless of the experimental approach used (in vivo vs. in vitro), it is generally admitted that dopamine inhibits the chemosensory activity (2, 25, 29). Both dopamine D2 receptors and TH (rate-limiting enzyme for dopamine synthesis) mRNAs are increased in the carotid body of
NCT male rats. It is likely that this increase helps reduce or control the stimulatory role of adenosine A2A receptors on carotid body function. This interpretation is consistent with the "push-pull mechanism" recently proposed to explain the role of excitatory and inhibitory transmitters that help regulate carotid body activity (53). Finally, we are not neglecting the fact that adenosine A2A receptor gene was also increased in NCT females. However, this increase was more modest and was not associated with any changes in carotid body dopaminergic system indicators or in the ventilatory response to hypoxia. If any effect should be present, it seems minimal compared with what was observed in male NCT rats.

**Cardiovascular Changes due to NCT**

Unlike in the freely behaving model, anesthetized rats allowed us to measure blood pressure without imposing further stress on the animal. We observed a significant decrease of mean arterial blood pressure at baseline owing to a reduced diastolic blood pressure. As mentioned previously, adenosine

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**Fig. 6.** Respiratory activity after intraperitoneal injection of vehicle or adenosine A2A receptor antagonist ZM241385 (1 mg/kg) in control (open bars, *n* = 7 for vehicle, *n* = 7 for ZM241385) and NCT (filled bars, *n* = 7 for vehicle and *n* = 7 for ZM241385) adult male rats. Mean values are for *f*<sub>R</sub> (A), *V*<sub>T</sub> (B), and *V*<sub>E</sub> (C) at room air (normoxia) and during the first 10 min and last 10 min (11–20 min) of hypoxia (F<sub>O</sub><sub>2</sub> = 0.12). Values are means ± SE. *Significantly different from control (*P* < 0.05).
plays a critical role in cardiovascular regulation both at the central and peripheral levels (56, 58). Injections of a specific adenosine A2A receptor agonist within the NTS decreases mean arterial blood pressure as well as sympathetic nerve activity (6, 56, 57). Thus an increase of adenosine A2A receptor expression in the NTS and/or periphery due to NCT could potentiate the depressing effect of adenosine on blood pressure. In the present study, the fact that NCT reduces diastolic pressure suggests that peripheral resistance is reduced in these rats, which is consistent with the effects of adenosine on sympathetic nerve activity described previously (57).

Sex Specificity

The present study is not the first to report sex-specific changes in ventilatory control following NCT. We previously showed that NCT augments the hypercapnic ventilatory response of young (20 days old) in a sex-specific (males only) fashion (45). The fact that this sexual dimorphism was observed at such a developmental early stage suggests that, in this model, the hormonal milieu during the neonatal period, rather than the surge of gonadal hormones that takes place during puberty, is a stronger determinant of the effects of NCT on respiratory control. Although ovarian hormones could play an important “protective” role against the effects of NCT, the neuroendocrine mechanisms underlying this sex-specific plasticity are complex and still poorly understood. Clearly, understanding this phenomenon is a highly promising research avenue, but such investigation is well beyond the scope of this study. However, in the present context, it is noteworthy that a similar, sex-specific sensitivity to caffeine exposure has been observed in humans. For instance, high consumption of caffeine during pregnancy leads to a higher number of small-for-gestational-age boys but not girls (63).

Putative Mechanisms of Caffeine-Induced Plasticity

Chronic exposure to adenosine receptor antagonist caffeine in mice increases adenosine receptor binding in the whole brain (8, 40). More precisely, chronic exposure to caffeine enhances adenosine A1 receptor binding in the mouse hippocampus and adenosine A2A receptor binding in the mouse striatum (31). Chronic exposure to theophylline, another adenosine receptor antagonist, in adult rats increases adenosine A1 receptor binding in the cortex (18, 51) and in the cerebellum, the hippocampus, and the thalamus (39). This suggests that chronic inactivation of adenosine receptors enhances functional expression of these receptors. During the neonatal period, chronic caffeine exposure modifies adenosine A1 receptor expression in adult rat cortex, cerebellum, and hippocampus (17, 27). It has been proposed that when caffeine occupies the adenosine receptor’s binding site the number of receptors increases to maintain the efficiency of adenosinergic systems (8). Here, we reported an increase of adenosine A2A receptor mRNA expression in the carotid bodies of NCT male rats. Although we are aware that an increase in mRNA expression does not necessarily lead to an increase in protein receptor level, the increase in breathing frequency response observed at the onset of hypoxia is consistent with a greater expression in protein level (A2A receptors) in the carotid body. Also, it is possible that NCT changes the cell-type composition of the carotid bodies (more type I cells in NCT rats) instead of modifying adenosine A2A receptor expression. This effect might result in an increase of adenosine A2A receptor mRNA expression in the whole carotid body. In mice, however, chronic caffeine administration enhances mRNA level of adenosine A2A receptors, as well as binding of agonists to adenosine A2A receptors in specific brain areas (31), suggesting that the functional role of adenosine A2A receptors was affected by chronic caffeine. Despite several experimental differences with the above-mentioned studies, our results suggest that NCT preferentially increases adenosine A2A receptor expression in the rat carotid body, an effect that persists until adulthood.

Perspectives and Significance

The respiratory control system is tightly regulated to respond appropriately to arterial blood gas fluctuations. If breathing increases too quickly and vigorously during hypoxia, the ensuing hypocapnia could lead to destabilization of ventilation and might, therefore, provoke respiratory instabilities (32, 65). Previous studies demonstrate that exaggerated ventilatory responses to hypoxia or hypocapnia are initiators and perpetuators of breathing instabilities, such as obstructive (64) and central sleep apneas (30). We demonstrated that neonatal caffeine increases hypoxic chemosensitivity, and we previously showed that it modifies the pattern of the hypercapnic ventilatory response (45, 46). Cumulative, long-term changes of chemosensitivities due to neonatal caffeine might therefore predispose to respiratory instabilities, especially during sleep. Although this hypothesis remains to be addressed, the sum of these results nonetheless raises important questions regarding the potential consequences of neonatal caffeine administration on the performance of the respiratory control system later in life.

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NEONATAL CAFFEINE AND CONTROL OF BREATHING IN ADULT RAT


