Neonatal maternal separation induces sex-specific augmentation of the hypercapnic ventilatory response in awake rat

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Genest S-E, Gulemetova R, Laforest S, Drolet G, Kinkead R. Neonatal maternal separation induces sex-specific augmentation of the hypercapnic ventilatory response in awake rat. J Appl Physiol 102: 1416–1421, 2007. First published December 21, 2006; doi:10.1152/japplphysiol.00454.2006.—Neonatal maternal separation (NMS) is a form of stress that exerts persistent, sex-specific effects on the hypoxic ventilatory response. Adult male rats previously subjected to NMS show a 25% increase in the response, whereas NMS females show a response 30% lower than controls (8). To assess the extent to which NMS affects ventilatory control development, we tested the hypothesis that NMS alters the ventilatory response to hypercapnia in awake, unrestrained rats. Pups subjected to NMS were placed in a temperature- and humidity-controlled incubator 3 h/day for 10 consecutive days (P3 to P12). Control pups were undisturbed. At adulthood (8 to 10 wk old), rats were placed in a plethysmography chamber for measurement of ventilatory parameters under baseline and hypercapnic conditions (inspired CO2 fraction = 0.05). After 20 min of hypercapnia, the minute ventilation response measured in NMS males was 47% less than controls, owing to a lower tidal volume response (22%). Conversely, females previously subjected to NMS showed minute ventilation and tidal volume responses 63 and 18% larger than controls respectively. Although a lower baseline minute ventilation contributes to this effect, the higher minute ventilation/CO2 production response observed in NMS females suggests a greater responsiveness to CO2/H+ in this group. We conclude that NMS exerts sex-specific effects on the hypercapnic ventilatory response and that the neural mechanisms affected by NMS likely differ from those involved in the hypoxic chemoreflex.

THE NEONATAL ENVIRONMENT is critical to proper development of neurophysiological function. The formation and fine tuning of neural circuits during early life require adequate sensory guidance, and conditions providing excessive or insufficient levels of stimulation can disrupt system development and compromise their subsequent performance throughout life. The olfactory, tactile, and auditory stimuli that the mother provides her offspring following birth are among the most potent environmental factors contributing to the “neonatal programming” of neural circuits (12, 22). Although the life-long consequences of disrupting mother-pup interactions have been mainly associated with behavioral and neuroendocrine dysfunction (1, 2, 20), less is known about the impact of mother-pup interaction on other homeostatic functions such as cardiorespiratory regulation. Accordingly, we showed that neonatal maternal separation (NMS) disrupts cardiorespiratory responses to moderate hypoxia in a persistent and sex-specific fashion (8, 16). In addition to eliciting the well described enhancement of basal hypothalamo-pituitary-adrenal axis function in rats (34; for review see Ref. 5), we showed that NMS also augmented the hypoxic ventilatory response of adult male rats by 25%. These animals were also characterized by a mean arterial blood pressure 20% higher than controls (8). Together, these data show that early life exposure to a nonrespiratory stress such as NMS disrupts the neural mechanisms involved in cardiorespiratory regulation.

Detailed analysis of the time course of the hypoxic ventilatory response strongly suggests that NMS elicits sex-specific enhancement of carotid body function because the onset of the frequency response of male NMS rats (whether awake or anesthetized) was more rapid and greater than that observed in controls (8, 17). RT-PCR analysis of mRNA encoding for tyrosine hydroxylase or dopamine D2 receptor expression indicates that NMS likely affects dopaminergic neurotransmission in the carotid body (18). However, the effects of NMS on respiratory control development are not limited to peripheral chemoreceptors because male (but not female) rats previously subjected to NMS produce greater tidal volume (VT) and phrenic responses to hypoxia than controls (8, 16).

We still have a limited understanding of the neural mechanisms underlying the effects of NMS on the respiratory control system. To better understand the extent to which NMS affects respiratory control development, the main objective of the present study was therefore to test the hypothesis that NMS disrupts the ventilatory response to moderate normoxic hypercapnia [inspired CO2 fraction (FiCO2) = 0.05] in a sex-specific manner in awake rats.

METHODS

Animals

Experiments were performed on 22 male and 30 female Sprague-Dawley rats (Charles River Canada, St. Constant, Quebec, Canada). 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 0600 and off at 1800). Laval University Animal Care Committee approved the experimental procedures described in this manuscript, and the protocols used were in accordance with the guidelines detailed by the Canadian Council on Animal Care.

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Mating and NMS Procedures

Virgin females were mated and delivered 10–15 pups. Two days after delivery, litters were culled to 12 pups, when necessary, with a roughly equal number of males and females. The NMS protocol, inspired from that of Wigger and Neumann (34), was identical to the one used in our previous studies (8, 17, 18). Briefly, the entire litter was separated daily from their mother for 3 h/day (0900–1200) from days 3 to 12. Separated pups were placed in a temperature (35°C)- and humidity (45%)-controlled incubator and isolated from each other by a cardboard partition. On day 21, rats were weaned and housed under standard animal care conditions until adulthood (8–10 wk old; see Table 1 for between-group comparison of age and weight data), at which time ventilatory measurements were performed.

The ventilatory data obtained from this experimental group were then compared with those of animals not subjected to the NMS procedure and continuously maintained under standard animal care. These animals are the most desirable control group for investigations of the effects of maternal separation on central nervous system development (21).

Respiratory Measurements

Measurements of minute ventilation (V̇E), breathing frequency (f), and V̇t in unrestrained rats were obtained by whole body flow-through plethysmograph (model PLY3223, Buxco Electronics, Sharon, CT) according to our method described previously (8, 15). Briefly, the system consisted of a 4.5-liter Plexiglas experimental chamber. The flow of air or hypercapnic gas mixture delivered to the chamber was kept constant and ranged between 2.0 and 2.5 l/min. Rectal temperature was measured before and after each experiment. Barometric pressure, chamber temperature, and humidity were also measured to express V̇t in milliliters (BTPS) per 100 g. Part of the gas mixture flowing out of the chamber was aspirated by a flow-through capnograph and analyzed (Novametrix, Wallingford, CT) for subsequent calculation of CO2 production (V̇CO2) with an open system (23). Based on the work of Strough and colleagues on various rat strains, we assumed that our Sprague-Dawley rats had a respiratory quotient value of 0.74 (31).

Table 1. Effects of neonatal maternal separation on selected variables including body temperature and CO2 production in male and female rats under normoxic (baseline) and hypercapnic conditions

<table>
<thead>
<tr>
<th>Body weight, g</th>
<th>Control</th>
<th>Neutropenic Maternal Separation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline</td>
<td>Hypercapnia</td>
</tr>
<tr>
<td>Male</td>
<td>368±44‡</td>
<td>356±35‡</td>
</tr>
<tr>
<td>Female</td>
<td>252±30</td>
<td>264±36†</td>
</tr>
<tr>
<td>Age, days</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>67±6</td>
<td>61±4†</td>
</tr>
<tr>
<td>Female</td>
<td>64±9</td>
<td>70±10</td>
</tr>
<tr>
<td>Body temperature, °C</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>37.7±0.5</td>
<td>36.8±0.4*</td>
</tr>
<tr>
<td>Female</td>
<td>38.0±0.7</td>
<td>37.0±0.7*</td>
</tr>
<tr>
<td>V̇CO2, ml min⁻¹·100 g⁻¹</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>3.0±0.3‡</td>
<td>4.0±0.6*‡</td>
</tr>
<tr>
<td>Female</td>
<td>3.7±0.7</td>
<td>5.1±1.0*</td>
</tr>
</tbody>
</table>

Values are expressed as means ± SD. Baseline values were obtained in quiet but awake rats at least 1 h after the animal acclimated to the plethysmography chamber. Hypercapnic values were obtained after 20 min of exposure to moderate hypercapnia (inspired CO2 fraction = 0.05). *Statistically different from baseline (P < 0.05); †Statistically different from corresponding control value (P < 0.05); ‡Statistically different from corresponding female value (P < 0.1).

The rat was placed in the chamber with room air flowing through. The rat was allowed to acclimatize to the chamber for roughly 1 h, and baseline (normocapnic) measurements were made when the animal was quiet but awake and the ventilatory variables were stable (8). The baseline values obtained were representative of the data recorded over the preceding 30–45 min. Then a gas mixture of 5% CO2 in air was delivered to the chamber for 20 min, and the recording chamber was opened for a final body temperature measurement. This CO2 level was chosen because we wanted a stimulus that was physiologically relevant. All measurements were performed between 1000 and 1200 to minimize changes in endocrine and respiratory activity associated with the circadian rhythm.

For ventilatory measurements, groups were distributed as follows: controls, 8 males and 11 females; NMS, 10 males and 16 females. Note that, for each group, rats originated from at least three different litters to ensure that treatment-related differences were not due to a litter-specific effect.

Data Analysis

Respiratory measurements. Baseline measurements of ventilatory variables were obtained by averaging the last 10 min of stable recording, whereas a 5-min average was taken for each variable at the end of the hypercapnic exposure. Unlike the hypoxic response, the temporal dynamics of the frequency response to hypercapnia are not commonly analyzed in detail. However, a significant statistical interaction between time and separation (see RESULTS) brought us to analyze the time course of the frequency response to hypercapnia on a minute-by-minute basis and in two distinct segments (early and late phases), according to criteria similar to those established for the hypoxic response (8). Briefly, the early phase was defined as the period from the onset of hypercapnia [time (t) = 0 min] to the time at which f of NMS group reached steady state and became similar to that of controls (t = 12 min). The late phase (t > 12 min) was defined as the period during which the frequency response of both groups reached steady state until the end of the hypercapnic stimulus (t = 20 min; Fig. 1, A and B). Note that the time course of the V̇t (and thus V̇E) data could not be obtained since the body temperature measurements necessary for correcting these results were not measured continuously during the experiments.

The effects of sex and treatment on the hypercapnic ventilatory response were assessed both on absolute data and data expressed as a percentage change from baseline. With such normalization procedure, a 0% value represents no change from baseline, whereas a 100% change represents a twofold increase above baseline value.

Statistical Analysis

Respiratory data were analyzed using three-way ANOVA for repeated measures (hypercapnic stimulus × sex × separation; Statview 5.0, SAS Institute, Cary, NC). Hypercapnic ventilatory responses were analyzed using a two-way ANOVA (sex × separation). These analyses were followed by a post hoc Fisher’s test when appropriate (P < 0.05). ANOVA results are typically indicated by specifying the factor(s) of interest; P values otherwise reported in the text indicate the results of the post hoc test. All data are presented as means ± SD, according to the American Physiological Society guidelines for reporting statistics (4).

RESULTS

Sex, NMS, and Basal V̇E

Baseline (normocapnic) ventilatory measurements of control animals were comparable to those reported in other studies using male and female Sprague-Dawley rats under similar conditions (Figs. 1 and 2) (3, 6–8, 14, 24, 26, 27, 30). Females subjected to NMS showed a lower resting V̇E compared with
controls (treatment effect: \( P = 0.04 \); Fig. 2A), owing to a lower \( \dot{V}_T \) (treatment effect: \( P = 0.003 \); Fig. 2C). These results contrast with those reported for males in which baseline \( \dot{V}_E \) was the same for NMS and controls (Fig. 2B) and from our previous study in which NMS females had a higher baseline \( \dot{V}_E \) than controls (8). During normoxia, neither body temperature nor \( \dot{V}_CO_2 \) (Table 1) were affected by NMS. Overall, \( \dot{V}_CO_2 \) was higher in females than in males (sex effect: \( P = 0.03 \)). Despite suggestive trends, sex-related differences in body temperature were not statistically significant (sex effect: \( P = 0.12 \); Table 1).

Sex, NMS, and Time Course of the Hypocapnic Ventilatory Response

Temporal analysis of the frequency response to hypocapnia showed that the initial phase (0–12 min) varied according to sex and separation (bifactorial interaction: \( P = 0.07 \)). In females, there is no between-group difference during the hypocapnic ventilatory response (\( P = 0.6 \); Fig. 1A). At the onset of hypocapnia, male rats subjected to NMS showed a stronger increase in \( f \) than controls (\( P = 0.01 \); Fig. 1B), which then decreased slightly to reach steady state at a rate similar to that of the control group at 12 min. During the late phase of the response (13–20 min), there was no between-group difference, either in females (Fig. 1A) or in males (Fig. 1B).

Sex, NMS, and Hypocapnic Ventilatory Response

\( \dot{V}_E \) increased during hypocapnia (stimulus effect: \( P < 0.0001 \)), but the response measured at the end of hypocapnic exposure was higher in females than in males (stimulus \( \times \) sex: \( P = 0.014 \); Fig. 2, A and B). NMS reduced the magnitude of the response in males but had no significant effect in females (stimulus \( \times \) treatment: \( P = 0.008 \) and \( P = 0.7 \) for males and females, respectively; Fig. 2, A and B). These treatment-related differences in the response were mainly related to differences in the \( \dot{V}_T \) increase (stimulus \( \times \) treatment: \( P = 0.008 \) and \( P = 0.8 \) for males and females, respectively; Fig. 2, C and D). Expressing these data as a percent change from baseline values yielded similar results for most variables, except \( \dot{V}_E \) in females. Statistical analysis of the responses (% change from baseline) showed a significant interaction between sex and separation for \( \dot{V}_E, \dot{V}_T, \) inspiratory flow, and the convective requirement for \( CO_2 \) (\( \dot{V}_E/\dot{V}_CO_2 \); \( P = 0.003, P = 0.0007, P = 0.02, \) and \( P = 0.04 \), respectively; Fig. 2). Compared with controls, the \( \dot{V}_E \) response of NMS adult female rats was increased by 63% due to the augmentation of \( \dot{V}_T \) response (18%; Fig. 2A). For females, the difference between absolute and normalized results is related to the lower baseline values. The inspiratory flow and \( \dot{V}_E/\dot{V}_CO_2 \) responses were greater than controls also (by 61 and 117%, respectively). These results contrast with those of NMS males in which \( \dot{V}_E \) and \( \dot{V}_T \) responses were decreased by 27 and 32%, respectively (Fig. 2B). Furthermore, the inspiratory flow and \( \dot{V}_E/\dot{V}_CO_2 \) responses of males were not affected by NMS. The \( f \) response was unchanged by NMS in both sexes. Hypocapnia had a significant effect on the variables reported in Table 1 with the exception of \( \dot{V}_CO_2 \) in females. After 20 min of hypocapnia, body temperature decreased in all groups (stimulus effect: \( P < 0.0001 \); Table 1).

DISCUSSION

This study expands on our previous findings by showing that NMS alters development of the hypocapnic ventilatory response in a persistent and sex-specific fashion. Male NMS rats initially showed a greater frequency increase at the onset of hypocapnia compared with controls, a result consistent with the hypothesis that NMS augments overall carotid body function in male rats (Ref. 18, present data). However, this response was not sustained, and the \( \dot{V}_E \) response measured at the end of hypocapnia was lower than controls. These results contrast with those obtained in female rats in which NMS increased the \( \dot{V}_E \) response measured at the end of the hypocapnic exposure, suggesting a greater responsiveness to \( CO_2/H^+ \). These results, combined with our previous work (8), show that NMS affects both the hypocapnic and hypoxic chemoreflexes in a distinct and sex-specific manner.

NMS and Sex-Specific Plasticity of the Hypocapnic Ventilatory Response

The time domains of the hypoxic ventilatory response are well described, and the rapid increase in \( f \) at the onset of hypoxia is commonly attributed to carotid body activation (29). Although carotid bodies have been shown to contribute to the rapid response to \( CO_2 \) in dogs (25), the mechanisms underlying the temporal dynamics of the hypocapnic ventilatory response have received little attention. Short-term depression of \( f \) is a phenomenon that characterizes the hypoxic ventilatory re-
response since it does not normally occur during hypercapnic challenge. Adenosine, cellular acidification, a change in the balance between excitatory (glutamate) and inhibitory (GABA) inputs, and activation of the platelet derived growth factor β-receptor at the level of the NTS are mechanisms that likely contribute to the frequency ‘roll-off’ during hypoxia (11). Since this is the first time that an experimental treatment appears to elicit f depression during a hypercapnic challenge, it is difficult to explain why, unlike all other groups, NMS males did not maintain a constant f throughout the hypercapnic period. Although it is possible that NMS affects one or several of the aforementioned neural mechanisms, the larger increase in f at the onset of hypercapnia could be linked to carotid body responsiveness and/or a higher state of vigilance in this group.

At the end of the hypercapnic stimulus, the Ve response of male NMS rats was lower than controls, owing to an attenuation of the Vt response. This effect differs from the enhancement of Vt and inspiratory efforts observed in male NMS rats during an acute hypoxic challenge or carotid sinus nerve stimulation (8, 17). The mechanisms underlying the effects of NMS on the hypercapnic chemoreflex in males are unclear but are similar to the attenuation of the hypercapnic (but not hypoxic) ventilatory response observed in rats previously subjected to NMS (vc; males, n = 10; females, n = 16). These data show that NMS reduced the Ve response to hypercapnia in males (B) but not females (A) (stimulus × treatment: P = 0.008 and P = 0.7 for males and females, respectively). However, NMS augments the Ve/VCO2 response to hypercapnia in females (E) but not in males (stimulus × treatment: P = 0.07 and P = 0.7 for males and females, respectively). Values are expressed as means ± SD. *Statistically different from baseline (P < 0.05). †Statistically different from corresponding control value (P < 0.05). ‡Statistically different from corresponding control value (P < 0.1).
Marginal. Since the hyperventilation that occurs during acute exposure to 5% CO₂ typically augments arterial Po₂ (PaO₂) from ~105 to ~125 Torr (15), we propose that the lower V̇E observed in male NMS rats is related to their higher sensitivity and responsiveness to changes in PaO₂ than controls (8, 17). This increase in PaO₂ may not be sufficient to reduce carotid body discharge frequency under normocapnic conditions (33) but could reduce carotid body activity during hypercapnia (especially in NMS rats) since O₂ and CO₂ levels interact in a multiplicative fashion to determine the overall level of carotid body activity (9). Accordingly, we propose that the PaO₂ increase observed during hypercapnia attenuates carotid body-mediated respiratory drive in this group. Although this hypothesis remains to be addressed with a more direct (electrophysiological) approach, the fact that there is no evidence suggesting that NMS alters carotid body function in females is consistent with this interpretation.

These data contrast remarkably with those observed in females in which proportional enhancement of the V̇T response accounts for the larger increase in V̇E. However, these data must be interpreted carefully because treatment-related differences in baseline V̇T (and thus V̇E) contribute the enhancement of the response seen with normalized data (see Fig. 3). Moreover, despite rigorously controlled experimental conditions, NMS affected baseline V̇E in females in a way that was opposite from that reported in our previous study (8). The factors underlying this puzzling difference between the two studies are still unclear to us, but since baseline ventilatory data obtained in males were nearly identical between these two studies, such effect could be related to differences in the predominant stage of the oestrus cycle during which experiments were performed. Nonetheless, the higher V̇E/V̇CO₂ response observed in NMS females is consistent with the enhanced responsiveness to CO₂/H⁺. This observation, combined with the lack of evidence suggesting that NMS affects carotid body function in females, suggests that NMS increases central chemosensitivity in a sex-specific fashion. However, a more direct experimental approach is necessary to test this hypothesis adequately, especially since NMS rats showed no V̇CO₂ increase during hypercapnic exposure, thereby suggesting that regulation of metabolism may contribute also. But in the present context, it is interesting to note that these results contrast substantially with the effects of NMS on the hypoxic chemoreflex in which females showed a depression of the f response to hypoxia (8). In light of the present data, the decrease in arterial Pco₂ during hypoxia combined with the higher CO₂/H⁺ responsiveness in NMS females may explain why these animals showed a lower response during hypoxia.

Other than the work by Strohl and collaborators (31), few studies allow direct comparisons of the hypercapnic ventilatory response between male and female rats. These authors reported no sex-related differences in ventilatory activity, but the fact that their experiments were performed under hypoxic conditions (inspired O₂ fraction = 0.93) makes it difficult to determine whether these results truly contrast with ours. Regardless, the present data suggest that the sex-based increase in CO₂/H⁺ responsiveness observed in NMS rats is related to the effect of sexual steroids on V̇T regulation. Moreover, our data indicate that, in females, NMS affects the neural mechanisms regulating the hypoxic and hypercapnic reflexes differently. During hypercapnia, NMS females increased their V̇E response by augmenting the V̇T response, whereas during hypoxia the lower V̇E response seen in NMS females is due to frequency component of the response. This interpretation is supported by the fact that females see their hypoxic (8) and hypercapnic ventilatory responses affected by NMS in the opposite way of males, thus suggesting that the capacity to modulate ventilatory chemoreflexes in response to changes in arterial O₂ and CO₂ levels shows a great sexual dimorphism.

Together, these data reinforce the notion that adverse early life experiences that do not pose a direct threat to respiratory homeostasis have long-lasting consequences on respiratory control development (16). The mechanisms at the basis of these alterations in neonatal programming are still unclear, but the sex-specific nature of this manifestation of respiratory plasticity suggest that gonadal hormones contribute to the expression of the effect of NMS. Moreover, these results indicate that ovarian hormones do not necessarily protect against the effects of early life stress on respiratory control development. In light of the present data, we propose that, with such enhancement of the CO₂ chemoreflex, NMS rats may be more susceptible to develop respiratory disorders associated with neural control dysfunction such as respiratory instability during sleep or panic attacks (10, 13, 19, 28, 32). Should data support this hypothesis, NMS may be a valuable model to understand these pathologies.

Fig. 3. Effects of NMS on hypercapnic ventilatory response of adult female (A) and male (B) rats. For each group, selected ventilatory variables (V̇E, V̇T, breathing frequency, inspiratory flow, and V̇E/V̇CO₂) were measured after 20 min of exposure to moderate hypercapnia (FICO₂ = 0.05) and expressed as a percentage change from normoxic baseline values. Data are compared between controls (filled bars); males: n = 8 and females: n = 11) and rats previously subjected to NMS (open bars); males, n = 10; females, n = 16). Values are expressed as means ± SD. †Statistically different from corresponding control value (P < 0.05). ‡Statistically different from corresponding female value (P < 0.05).
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GRANTS

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REFERENCES


