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

Frédéric S. Dumont and Richard Kinkead

Department of Pediatrics, Centre de Recherche du Centre Hospitalier Universitaire de Québec, Université Laval, Québec, Canada

Corresponding author:
Frédéric Dumont, PhD Candidate

Centre de recherche du CHUQ, hôpital St-François d'Assise
10, rue de l'Espinay, local D0-707
Québec, QC
G1L 3L5

Tel: (418) 525-4444 ext. 53232
Fax: (418) 525-4195
E-mail: frederic.dumont.1@ulaval.ca

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Abstract

Neonatal maternal separation (NMS) is a form of stress which 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 FiO₂ 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 3h/day from post-natal days 3 to 12. Control rats were undisturbed. At adulthood, rats were anesthetised (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 anesthetised 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.

Key words: Control of breathing, development, stress, central integration
INTRODUCTION

In humans, rats, and non-human primates, the tactile, olfactory, and auditory stimuli provided by the mother to her offspring have a strong influence on CNS development (17, 18, 38, 40, 50, 56). Neonatal maternal separation (NMS) is a well established, clinically relevant stress model that disrupts mother-offspring interactions to reproduce environmental conditions experienced by infants deprived from adequate parental stimulation due to special medical interventions associated with prematurity or inadequate maternal care (e.g. parental neglect, mother suffering from depression, orphanage) (14, 38, 50). Because this stress typically occurs during a critical period of development, NMS has long lasting consequences on central nervous system development and affects maturation of basic homeostatic functions such as the neuroendocrine regulation of stress responses (18, 61) and the respiratory control system (20, 21, 37).

With regards to respiratory regulation, several recent reviews have highlighted the profound effects of excessive stimulation of chemosensory pathways (e.g. intermittent hypoxia, chronic hypoxia, etc.) 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; F\textsubscript{e}CO\textsubscript{2} = 0.05; 20 minutes) 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). Based on indirect evidence indicating that NMS augments carotid body’s responsiveness to hypoxia (35, 36), it was proposed that the relative hypoxia that occurs during hypercapnic hyperpnea (~20 mm Hg increase (34)) is sufficient to attenuate the CO\textsubscript{2} 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 mechano-sensitive 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 chemo- and baroreceptor afferents)(35). Therefore, we proposed that NMS can potentiate the baroreflex. During hypercapnia, CO\textsubscript{2} 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 which 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 O\textsubscript{2} 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, St-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 amongst experimental series and the number of animals per group (n) are detailed in Table 1.

**Neonatal maternal separation procedures**

The neonatal maternal separation (NMS) protocol started on postnatal day 3 (P3). Pups were separated from their mother and isolated from each other during three hours (9 AM to noon) each day for ten consecutive days (P3 to P12). Pups were placed in a temperature (32°C) and humidity (45%) controlled incubator. Based on 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 two weeks 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, USA) 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 (FiO₂ = 0.3) to maintain the PaO₂ 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 Instrument, Sarasota, FL, USA) and withdrawal of blood samples (70μL) for analysis of arterial blood-gases (model ABL-5, Radiometer, Copenhagen, Denmark), corrected for the rat's body temperature. Rats were slowly converted from isoflurane to urethane anaesthesia (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, model PHD2000, Holliston, MA, USA). A pressure controlled ventilator was also used during the transition to urethane to ensure adequate ventilation during the procedure (Kent Scientific, Litchfield, CT, USA). 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 cardio-respiratory 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
diaphragm, one centimeter apart. The electrodes were placed as laterally as possible to reduce electrical interference from the heart. Electrical 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 (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, USA). Once the surgical procedures were completed, a 60 minutes “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- 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 cardio-respiratory 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 ten minutes 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 O$_2$ was either unchanged (FIO$_2$ = 0.3; poikilo-oxic experiments) or reduced (FIO$_2$ = 0.23) to maintain PaO$_2$ constant, within 3 Torr of baseline values (iso-oxic experiments). A second measurement of arterial blood gases confirmed that the target PaCO$_2$ (and PaO$_2$, when appropriate) had been reached (see Tables 2 and 3). Hypercapnia (under poikilo- or iso-oxic conditions) was then maintained for five minutes during which cardio-respiratory 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 mV) 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 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 seconds bins. In this series, a mean baseline value was obtained by averaging 5 minutes of recording prior to hypercapnic exposure. Similarly, a mean hypercapnic value was obtained by averaging the 5 minutes 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 normalised 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 the afferent signal. The same method was used on the contra-lateral side. At the end of the experiment (~ 15 minutes 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 was measured continuously with an O₂ analyzer (Teledyne analytical instruments, TED-60T, Industry, CA, USA). The target FIO₂ was achieved within 30 sec; this dynamic was consistent between trials. The magnitude of the minute activity response measured after 1 minute of hypoxia was recorded and compared to that of animals with their carotid sinus nerves intact (Fig. 5).

Experimental protocol and data analysis: Once the animal recovered from surgery and the cardio-respiratory 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 hypo- and hypertensive responses, respectively. Both drugs were obtained from Sigma Aldrich (St-Louis, MO, USA) and prepared at a concentration of 5mg/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). Based on preliminary experiments, the doses were selected for their ability to induce both physiological and supra-physiological changes in arterial blood pressure to test the baroreflex. Prior to drug injections, baseline arterial blood gases and cardio-respiratory variables were recorded for 10 minutes and then the effect of sham injection (saline, 8µL) was tested. The drug injection protocol began by injecting NP; three volumes were used: 2µL, 4µL and 8µL. This was followed by PE injections; five volumes were used: 1µL, 2µL, 3µL, 4µL and 8µL. Each bolus injection was performed over a 1 minute period. Each injection was followed by 15 minutes recuperation. For each 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, cardio-respiratory 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 (20µL, 40µL, and 80µL) of PE to ensure that the same range of blood pressure changes was tested between studies (intact versus 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 seconds bins. Prior to injections, a mean baseline value was obtained by averaging 1 minute of recording. To compare baroreflex sensitivity between groups (NMS versus control) and procedures (intact versus 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 was generated for each animal and then group data were plotted (Figs. 6 and 7, respectively).

**Statistical analysis**

The equality of variance and the normal distribution were tested for all parameters of 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 PLSD 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 cardio-respiratory 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 cardio-respiratory parameters (dependent variables) were statistically significant. Slopes were calculated for each animal and group data were compared using ANOVA. Differences were considered significant at $p \leq 0.05$ 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 standard deviation (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: PaCO₂, 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). PaO₂ 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 (Figure 1, Tables 2 and 3; treatment effect: p > 0.09 for all variables). Comparison of baseline values between O₂ conditions (poikilo- versus iso-oxic) showed that PaO₂, pHa, heart rate, and breathing frequency were similar across studies (Figure 1, Tables 2 and 3: O₂ effect: p > 0.3); however, resting mean arterial blood pressure and PaCO₂ of iso-oxic rats were lower than those involved in the poikilo-capnic experiments (Tables 2 and 3; O₂ effect: p < 0.001 for both).

Hypercapnia: After five minutes of moderate hypercapnia (whether poikilo- or iso-oxic), PaCO₂ 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, PaO₂ increased in poikilo-oxic rats only (Tables 2 and 3; CO₂ × O₂ effect: p < 0.0001); in these experiments, the relative hyperoxia was the same for NMS and control animals (Table 2; treatment: p = 0.28). Under poikilo-oxic conditions, the relative increase in diaphragmatic minute activity observed in NMS rats was 34% less than control (Fig. 2A, treatment: p = 0.019). This response contrasts with the one observed under iso-oxic conditions during which the hyperventilatory response of NMS rats was 27% greater than controls (Fig 2A; treatment: p = 0.006; treatment × O₂ effect: p = 0.0008). It is noteworthy that
the effect of iso-oxia was greater in control than NMS rats and that this change was mediated exclusively by a greater breathing frequency response (Fig 2B; treatment × O2 effect: p = 0.004). Statistical analysis of the absolute breathing frequency data support these results (Fig. 1; CO2 effect × treatment × O2 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 PaO2 levels (Fig. 3A; p = 0.49 and 0.64, respectively), the hypertension was influenced by both variables (Fig. 3B; treatment × 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 × treatment × O2 effect: p = 0.05).

Hypercapnic ventilatory response following carotid sinus nerve section (CSX) in NMS and control rats

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

Hypercapnia: Following five minutes of moderate poikilo-oxic hypercapnia, PaCO2 increased by 10 Torr in both groups (Table 4; CO2 effect: p < 0.0001); an acidosis was observed and PaO2 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 carotid sinus nerve section (CSX): Figure 5 shows that the minute activity response to hypoxia (FI,O₂: 0.12; 1 minute) 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 anesthetised animals) (19, 20, 33, 35, 37). These results confirm the effectiveness of the chemodenervation.

Effects of neonatal maternal separation on baroreflex sensitivity in intact and CSX rats.

Baseline: Analysis of arterial blood gases measured under baseline conditions revealed no significant effect of CSX or NMS treatment for any of these variables (Table 5).

Drug injections: In intact and CSX rats, blood gas samples obtained following drug injections show that PaO₂, pH and PaCO₂ remained stable over the course of the injection procedures (data not shown; drug effect: p = 0.08, 0.67, and 0.44, respectively). In intact and CSX rats, nitroprusside and phenylephrine injections decreased and increased blood pressure, respectively (Fig. 6 A and B, drug effect: p < 0.0001 for both). Saline injections did not induce any changes in mean arterial blood 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 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 analysis of covariance (ANCOVA) to determine whether these factors affect the relationship between change in arterial blood pressure and the related changes in selected cardio-respiratory variables. In intact rats, ANCOVA showed that NMS affects the relationship between blood pressure and heart rate responses significantly (treatment × 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: Δ pressure (mmHg) versus Δ 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, r² = 0.68; control slope: -0.27, r² = 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 were significant regardless of the status of the carotid sinus nerve (intact or sectioned; Fig. 6C and D, p < 0.0001 for all correlations).
Interaction between MBP response and ventilatory parameters

Figures 7A and 7B 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 × 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, in comparison with the "standard" (poikilo-oxic) response, iso-oxia reduces the HCVR of control rats only. Though 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 regulation 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⁺ chemodetection and the initiation of hyperpnea contribute 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 a non-respiratory stress can have a persistent impact on a crucial aspects of cardio-respiratory regulation which may, in turn, contribute to the emergence of disease state at adulthood.

Critique of the method: validity of the anesthetized rat preparation

Neonatal maternal separation affects development of the neuroendocrine response to stress (1, 6, 39). At adulthood, male NMS rats are hyper-responsive to stress and exhibit elevated
indices of anxiety (27). Considering that CO$_2$ 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 anesthetised 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 CO$_2$ 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 anesthetised rats. Nevertheless, it is clear that NMS affects breathing pattern differently between studies: in the anesthetised 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 versus* tidal volume) to ventilatory stimuli between awake and urethane-anesthetised rodents have been reported previously (26). Nevertheless, data from this and previous studies, addressing the consequences of 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 validate (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 to the use of anesthesia are non 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 PO₂ reduces the carotid body's response to CO₂ in vitro (22, 52). Based on these results, it is unlikely that the relative hyperoxia achieved during the 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 (iso-oxic condition) attenuated the HCVR of control (but not NMS) rats by 39% were unexpected. Although hypo-additive interactions have been reported by others (11, 53) the experimental approach used in our study did not provide sufficient control over specific levels of chemoreceptor stimulation (central versus 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). Though 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. hypo- 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- versus poikilo-oxic conditions within a physiologically relevant PaO₂ range, proper comparisons are difficult. Regardless, it is important to keep in mind that 1) under "standard" (poikilo-oxic) conditions, the HCVR of NMS rats is lower than controls (whether the animal is awake or anesthetised), and 2) that lack of effect of PaO₂ on the HCVR of NMS rats indicates a problem with the hypo-additive relation between peripheral and central chemoreceptors. More precisely, the fact that rats subjected to carotid sinus nerve section prior to hypercapnic exposure had the same phenotype as intact rats suggests that NMS interferes with central mechanisms involved in the integration of sensory afferent signals. Previous studies using carotid sinus nerve stimulation (35) or combining immunohistochemistry, GABA₄ receptor autoradiography with microinjections of pharmacological agents within the nucleus tractus solitarius (33) (the main site of central projection for the carotid bodies (15)) support that interpretation.

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%. Based on the interaction slopes between MBP and respiratory rate of each group (controls: -0.1791; NMS: -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. Based on 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. Though 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.

Based on the present data, it would also be very interesting to address this issue in females. However, the fact 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 though 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 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 cardio-respiratory 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.

ACKNOWLEDGMENTS

The authors would like to thank Mélanie Pelletier, Sylvie Viger, and Évelyne Vachon (animal care specialists) for their help with the planning and maintenance of the animal colony; Dr. Cécile Julien for helpful discussion. The authors also acknowledge the advice of Mr. Merlin Mbuembue Njoya, consultant in statistics. This research was supported by the CIHR and the Canada Research Chair in Respiratory Neurobiology (RK). FD held a graduate scholarship from la Fondation des Étoiles for children's health research.
REFERENCES


33. **Kinkead R, Balon N, Genest SE, Gulemetova R, Laforest S, and Drolet G.** Neonatal maternal separation and enhancement of the inspiratory (phrenic) response to hypoxia in adult


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

<table>
<thead>
<tr>
<th></th>
<th>Series I and II: Hypercapnic response</th>
<th>Series III: Baroreflex</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Intact</td>
<td>CSX</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>NMS</td>
</tr>
<tr>
<td>Age (days)</td>
<td>77 (17)</td>
<td>72 (20)</td>
</tr>
<tr>
<td>Weight (g)</td>
<td>457(51)</td>
<td>473 (80)</td>
</tr>
<tr>
<td>number of animals</td>
<td>9</td>
<td>9</td>
</tr>
</tbody>
</table>

Values are reported as mean (SD). † indicates a value statistically different from corresponding control value at p ≤ 0.05.
Table 2: Arterial blood gases and selected cardio-vascular variables measured in under baseline conditions and during exposure to poikilo-oxic hypercapnia.

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>+10 Torr CO₂</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>NMS</td>
</tr>
<tr>
<td>PaCO₂ (mmHg)</td>
<td>48 (2)</td>
<td>49 (4)</td>
</tr>
<tr>
<td>ΔPaCO₂ (mmHg)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>pH</td>
<td>7.37 (0.03)</td>
<td>7.36 (0.03)</td>
</tr>
<tr>
<td>PaO₂ (mmHg)</td>
<td>116 (11)</td>
<td>122 (15)</td>
</tr>
<tr>
<td>ΔPaO₂ (mmHg)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Heart rate (bpm)</td>
<td>356 (47)</td>
<td>352 (41)</td>
</tr>
<tr>
<td>Mean blood pressure (mmHg)</td>
<td>109 (7)</td>
<td>110 (10)</td>
</tr>
<tr>
<td>Breathing frequency (bpm)</td>
<td>113 (13)</td>
<td>103 (14)</td>
</tr>
</tbody>
</table>

Data are reported as means (SD). * indicates a value statistically different from corresponding baseline (normocapnic) value at p ≤ 0.05. † indicates a value statistically different from corresponding control value at p ≤ 0.05.
**Table 3:** Arterial blood gases and selected cardio-vascular variables measured in under baseline conditions and during exposure to iso-oxic hypercapnia.

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>Hypercapnia</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>NMS</td>
</tr>
<tr>
<td>PaCO₂ -mmHg</td>
<td>41 (5)</td>
<td>40 (4)</td>
</tr>
<tr>
<td>∆PaCO₂ -mmHg</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>pHa</td>
<td>7.40 (0.01)</td>
<td>7.34 (0.01)</td>
</tr>
<tr>
<td>PaO₂ -mmHg</td>
<td>106 (12)</td>
<td>124 (10) †</td>
</tr>
<tr>
<td>∆PaO₂ -mmHg</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Heart rate -bpm</td>
<td>385 (54)</td>
<td>388 (70)</td>
</tr>
<tr>
<td>Mean blood pressure -mmHg</td>
<td>98 (13)</td>
<td>84 (7) †</td>
</tr>
<tr>
<td>Breathing frequency (bpm)</td>
<td>96 (14)</td>
<td>105 (17)</td>
</tr>
</tbody>
</table>

Data are reported as means (SD). * indicates a value statistically different from corresponding baseline (normocapnic) value at p ≤ 0.05. † indicates a value statistically different from corresponding control value at p ≤ 0.05.
Table 4: Arterial blood gases and selected cardio-vascular variables measured in under baseline conditions and during exposure to poikilo-oxic hypercapnia following carotid sinus nerve section (CSX).

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>Hypercapnia (poikilo-oxic)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>NMS</td>
</tr>
<tr>
<td>PaCO₂ (Torr)</td>
<td>41 (6)</td>
<td>45 (4)</td>
</tr>
<tr>
<td>ΔPaCO₂ (Torr)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>pHₐ</td>
<td>7.36 (0.02)</td>
<td>7.33 (0.04)</td>
</tr>
<tr>
<td>PaO₂ (Torr)</td>
<td>110 (13)</td>
<td>110 (15)</td>
</tr>
<tr>
<td>ΔPaO₂ (Torr)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Heart rate (bpm)</td>
<td>355 (27)</td>
<td>360 (57)</td>
</tr>
<tr>
<td>Mean blood pressure (mmHg)</td>
<td>108 (11)</td>
<td>104 (15)</td>
</tr>
<tr>
<td>Breathing frequency (bpm)</td>
<td>86 (11)</td>
<td>95 (20)</td>
</tr>
</tbody>
</table>

Data are reported as means (SD). * indicates a value statistically different from corresponding baseline (normocapnic) value at p ≤ 0.05. † indicates a value statistically different from corresponding control value at p ≤ 0.05.
Table 5: Arterial blood gases measured under baseline conditions, prior to injection of hypo- and hypertensive drugs in intact and CSX rats.

<table>
<thead>
<tr>
<th></th>
<th>INTACT Control</th>
<th>INTACT NMS</th>
<th>CSX Control</th>
<th>CSX NMS</th>
</tr>
</thead>
<tbody>
<tr>
<td>PaCO₂ (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 (mm Hg)</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). † indicates a value statistically different from corresponding control value at p ≤ 0.05.
FIGURE LEGENDS

Figure 1. Comparison of the breathing frequency response to hypercapnia under iso-oxic (black symbols) and poikilo-oxic (white symbols) conditions between control rats (panel A; circles) and rats previously subjected to neonatal maternal separation (NMS, panel B; triangles). Data are expressed as means and error bars represent standard deviations. * Indicates a value statistically different from corresponding baseline value ($p \leq 0.05$). § Indicates a value statistically different from corresponding poikilo-oxic value ($p \leq 0.05$). † Indicates a value statistically different from corresponding control value ($p \leq 0.05$).

Figure 2. Relative hypercapnic ventilatory response of control animals (white bars) and rats previously subjected to neonatal maternal separation (NMS; black 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. § Indicates a value statistically different from corresponding poikilo-capnic value ($p \leq 0.05$). † Indicates a value statistically different from corresponding control value ($p \leq 0.05$).

Figure 3. Relative cardiovascular response to hypercapnia of control rats (white bars) and animals rats exposed to neonatal maternal separation (NMS; black bars) under poikilo-oxic and iso-oxic conditions. The heart rate (A) and the blood pressure (B) values represented in this figure are expressed as a percentage of increase from baseline. Data are reported as means and error bars represent standard deviations. § Indicates a value statistically different from poikilo-capnic value ($p \leq 0.05$).
**Figure 4.** Comparison of the relative hypercapnic ventilatory response (poikilo-capnic condition) between control rats (white bars) and rats previously subjected to neonatal maternal separation (NMS; black bars) after carotid sinus nerve section. Results for the main component of the response (dEMG minute activity, breathing frequency and dEMG amplitude) are expressed as a percentage change from baseline values. Data are expressed as means and error bars represent standard deviations. † Statistically different from the corresponding control value ($p \leq 0.05$).

**Figure 5.** Comparison of the dEMG minute activity response to acute hypoxia ($F_iO_2= 0.12; 1$ minute) between animals with their carotid sinus nerve intact and following carotid sinus nerve section (CSX). Note that unlike intact animals, CSX data were obtained in control rats (white bars) and rats subjected to neonatal maternal separation (black bars). Data are expressed as means and error bars represent standard deviations. * Indicates a value statistically different from intact rats ($p \leq 0.05$).

**Figure 6. Top panels:** Blood pressure responses to hypo- and hyper-tensive agents (nitroprusside and phenylephrine, respectively) in rats with (A) carotid sinus nerves intact and (B) following bilateral carotid sinus nerve section (CSX). Experiments were performed on control animals (open circles) and rats previously subjected to neonatal maternal separation (NMS; black triangles). **Lower panels:** Comparison of the heart rate responses to changes in blood pressure (baroreflex sensitivity) between control and NMS rats. Experiments were performed on (C) intact rats and (D) following bilateral carotid sinus nerve section. Data are expressed as a percentage change from pre-injection (baseline) value. † Statistically different from control ($p \leq 0.05$).
**Figure 7. Top panels:** Comparison of the breathing frequency responses to pharmacologically induced changes in blood pressure (baroreflex sensitivity) between control rats (open circles) and rats subjected to neonatal maternal separation (NMS; black triangles). Experiments were performed on (A) intact rats and (B) following bilateral carotid sinus nerve section. **Lower panels** present dEMG minute activity response for (C) intact and (D) CSX rats. Data are expressed as a percentage change from pre-injection (baseline) value. † Statistically different from control ($p \leq 0.05$).
Figure 1

A  Control

B  Neonatal maternal separation

Breathing frequency (bpm)

<table>
<thead>
<tr>
<th>Baseline</th>
<th>Hypercapnia</th>
</tr>
</thead>
<tbody>
<tr>
<td>Poikilo-oxic</td>
<td>Iso-oxic</td>
</tr>
</tbody>
</table>
Figure 2

A

Frequency response (% Change from baseline)

Poikilo-oxic

Iso-oxic

Control

NMS

B

Frequency response (% Change from baseline)

Poikilo-oxic

Iso-oxic

C

dEMG amplitude response (% Change from baseline)

Poikilo-oxic

Iso-oxic
Figure 4

**Carotid sinus nerve section**

The figure shows the hypercapnic response (% Change from baseline) for both control and NMS conditions. The y-axis represents the hypercapnic response, while the x-axis categorizes the variables as dEMG minute activity, breathing frequency, and dEMG amplitude. The control group is indicated by white bars, and the NMS group by black bars. The data points are accompanied by error bars, indicating the variability. The figure includes symbols to denote statistical significance. The title of the figure is 'Carotid sinus nerve section.'
Effectiveness of carotid sinus nerve section: Hypoxic test

Figure 5
Figure 6

Blood pressure responses to drug injections

A  Intact rats

B  CSX rats

Baroreflex: Heart rate response

C  Intact rats

D  CSX rats

Regression of CTRL
NMS
Regression of NMS

slope = -0.06
r² = 0.44
p < 0.0001

slope = -0.15
r² = 0.60
p < 0.0001

NMS slope = -0.10
r² = 0.44
p < 0.0001

CTRL slope = -0.09
r² = 0.32
p < 0.0001

†
Figure 7

**Baroreflex: Breathing frequency response**

**A**  
Intact rats  
- **Control**  
- **Regression of CTRL**  
- **NMS**  
- **Regression of NMS**  

**B**  
CSX rats  
- **CTRL slope = -0.31**  
- **r² = 0.51**  
- **p < 0.0001**  
- **NMS slope = -0.33**  
- **r² = 0.70**  
- **p < 0.0001**

**Baroreflex: dEMG minute activity response**

**C**  
Intact rats  
- **Control**  
- **Regression of control**  
- **NMS**  
- **Regression of NMS**  

**D**  
CSX rats  
- **CTRL slope = -0.31**  
- **r² = 0.42**  
- **p < 0.0001**  
- **NMS slope = -0.25**  
- **r² = 0.71**  
- **p < 0.0001**