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Gender considerations in ventilatory and metabolic development in rats: Special emphasis on the critical period

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ABSTRACT

In rats, a critical period exists around postnatal day (P) 12–13, when an imbalance between heightened inhibition and suppressed excitation led to a weakened ventilatory and metabolic response to acute hypoxia. An open question was whether the two genders follow the same or different developmental trends throughout the first 3 postnatal weeks and whether the critical period exists in one or both genders. The present large-scale, in-depth ventilatory and metabolic study was undertaken to address this question. Our data indicated that: (1) the ventilatory and metabolic rates in both normoxia and acute hypoxia were comparable between the two genders from P0 to P21; thus, gender was never significant as a main effect; and (2) the age effect was highly significant in all parameters studies for both genders, and both genders exhibited a significantly weakened response to acute hypoxia during the critical period. Thus, the two genders have comparable developmental trends, and the critical period exists in both genders in rats.

1. Introduction

Postnatal respiratory development in rats does not follow a smooth path, but rather, a critical period exists around postnatal days (P) 12–13, when a striking and transient imbalance between enhanced inhibition and suppressed excitation is evident both neurochemically within the respiratory network (Liu and Wong-Riley, 2002, 2005, 2010c; Wong-Riley and Liu, 2005) as well as electrophysiologically in hypoglossal motoneurons (Gao et al., 2011). Other concomitant changes include a precipitous fall in the expression of several serotonergic neurochemicals (Liu and Wong-Riley, 2008, 2010a,b), a switch in $GABA_A$ receptor subunit dominance (Liu and Wong-Riley, 2004, 2006), a switch in dominance from a chloride importer (NKCC1) to a chloride exporter (KCC2) (Liu and Wong-Riley, 2012), and a significant reduction in the expression of brain-derived neurotrophic factor (BDNF) and its high-affinity tyrosine receptor kinase B (TrkB) (Liu and Wong-Riley, 2013) in multiple respiratory-related nuclei. During this time, the animals' ventilatory and metabolic responses to hypoxia are also at their weakest (Liu et al., 2006, 2009; Wong-Riley and Liu, 2008). These findings, in which male and female data were combined, have special implication for Sudden Infant Death Syndrome (SIDS), whose peak incidence is between the 2nd and 4th postnatal months, strongly implicating a critical period of postnatal development (Filiano and Kinney, 1994; Mage and Donner, 2009).

An open question that deserves some attention is whether both male and female animals follow the same or different developmental trends and whether the critical period exists in one or both genders. This is of clinical relevance, as the prevalence of SIDS is higher in male than in female infants (Mage and Donner, 2009). A recent report (Holley et al., 2012) stated that "P10–15 includes a critical developmental period in male but not female rats". As the study grouped animals into two-day pairs and concentrated only on P10–15 animals, the question remains as to whether the two genders differ throughout the first 3 postnatal weeks or whether the disparity occurs only during the critical period. Based on our initial analysis of a lack of gender difference, we deemed it necessary to rigorously test our hypothesis that the two genders exhibit comparable developmental trends throughout the first 3 postnatal weeks and that the critical period exists in both genders.

Our goal was to undertake a large-scale, in-depth study to compare the ventilatory and metabolic responses of male versus female rats in both normoxia and acute hypoxia *daily* during the entire first three postnatal weeks. In addition to segregating our previous large-scale data (Liu et al., 2009) into the two genders and analyzing them each day from P0 to P21, we added many more animals to increase the *N* of each gender at each age and to substantially strengthen the statistical power.

2. Methods

2.1. Animals

All experiments and animal procedures were performed in accordance with the Guide for the Care and Use of Laboratory

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Table 1
Animal and litter numbers.

Postnatal day (P)	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
M(N)	16	22	19	20	19	14	11	16	14	15	20	22	26	22	23	21	20	22	14	17	14	21
M(L)	14	16	16	18	16	12	8	11	12	12	17	15	19	19	18	17	14	16	12	14	11	14
F(N)	16	20	20	23	23	11	10	17	15	16	19	23	22	23	24	20	16	16	12	17	12	19
F(L)	12	16	17	18	16	9	8	14	12	11	15	19	19	17	17	16	13	13	11	14	11	18

M(N), number of male rats in each age group; M(L), number of litters (to which male rats belonged) in each age group; F(N), number of female rats in each age group; F(L), number of litters (to which female rats belonged) in each age group.

Animals (National Institutes of Health), and all protocols were approved by the Medical College of Wisconsin Animal Care and Use Committee.

A total of 302 Sprague-Dawley rats (parents purchased from Harlan, Indianapolis, IN) from 30 litters were used in this study, including data of 139 rats from 16 litters published previously (Liu et al., 2009) in which male and female data were combined but are now segregated by gender and re-analyzed, as well as 163 new rats from 14 new litters. The litter size was typically 10–15 pups. All rat pups used in the current study were born between 10 am and 4 pm, and all ventilatory and metabolic data were collected between those two time points during the day. The test days were staggered among the animals such that every single day between P0 (day of birth) and P21 was covered. For *each* of the days from P0 to P21, animal numbers ranged from 10 rats from 8 litters to 26 rats from 19 litters for each gender (see Table 1). Ten male and 10 female rats from 3 litters starting at P10–11 and exposed to acute hypoxia only once were compared with other litters (starting at P0–4 and exposed to hypoxia every 5th day) to determine if repeated hypoxic exposure (every 5th day for 7 min) would affect the latter's hypoxic ventilatory and metabolic responses. Another 17 male rats from 11 litters and 15 female rats from 12 litters were studied daily under normoxia to serve as a reference for the normoxic data obtained from pups examined every fifth day under normoxia followed by 7 min of hypoxia. For younger animals, gender distinction was based on the distance between the anus and the urethra (with the distance being further apart in males than in females) as well as on markings of developing nipples in female pups.

2.2. Ventilatory and metabolic measurements during normoxia and acute hypoxia

Ventilatory and metabolic measurements were performed according to a protocol described previously (Liu et al., 2009). Briefly, O_2 fraction, CO_2 fraction, and ventilation (including respiratory frequency (f), tidal volume (V_T), and their product minute ventilation ($\dot{V}_E$)) were determined in awake rat pups placed in an airtight 150 ml plastic syringe with flow through at 150 ml/min. The O_2 fraction, CO_2 fraction, pressure, relative humidity (RH), and temperature (T) signals were digitized with a data acquisition device. Each animal was tested first in room air for 6 min and then subjected to 7 min of hypoxia (10% O_2 balanced with 90% nitrogen N_2). Each pup was exposed to a 7-min hypoxia only every fifth day to prevent adverse cumulative effect of hypoxia (Liu et al., 2006, 2009).

Before and during each experiment, the animals' body temperature was maintained at a level comparable to their starting temperature when they were first taken out of the cage, i.e., 34–38.5 °C (depending on the age). This was achieved by placing the animals on a moderately heated heating pad on the floor of the plethysmograph and their body temperature was monitored before and after each experiment. The ambient temperature of the chamber was maintained at 26–28 °C. Gas calibration was taken before and after each animal's experiment with known gas mixture. For recording of ventilation, the system was calibrated by applying 0.1 ml of air into the plethysmographic chamber ten times before and after each animal's recording (Liu et al., 2009). Rectal temperature of each animal was measured before and after each recording. Adjustments of calibration with different breathing frequency and body volume were extensively addressed in our previous study (Liu et al., 2009).

2.3. Data collection and statistical analyses

Metabolic rates, oxygen consumption ($\dot{V}_{O_2}$) and carbon dioxide production ($\dot{V}_{CO_2}$) were calculated by applying the Fick Principle (Fishman et al., 1952). V_T, f, and $\dot{V}_E$ were calculated by using the formulae of Drorbaugh and Fenn (1955), which takes into account the RH, temperature, and pressure of the chamber. Thus, the ratios of $\dot{V}_E/\dot{V}_{O_2}$ and $\dot{V}_E/\dot{V}_{CO_2}$ as well as respiratory quotient ($\dot{V}_{CO_2}/\dot{V}_{O_2}$) were calculated. The values were grouped into 30-s bins during the 5th and 6th min in normoxia and 6th and 7th min in hypoxia, when breathing and gas concentrations were more stable (see Liu et al., 2009, Fig. 1A and B), and expressed as the mean of the last 2 min either in 6 min normoxia or 7 min hypoxia ± standard error of the mean (SEM). A total of 35 parameters (f, V_T, $\dot{V}_E$, $\dot{V}_{O_2}$, $\dot{V}_{CO_2}$ [except for f, each of the parameters were non-normalized as well as normalized to body weight], $\dot{V}_E/\dot{V}_{O_2}$, $\dot{V}_E/\dot{V}_{CO_2}$, and respiratory quotient [RQ] in normoxia and in hypoxia, the ratio of each parameter in hypoxia versus that in normoxia, body weight, and body temperature in normoxia and hypoxia) and 22 ages (P0 to P21) were analyzed.

For statistical analysis, a generalized linear mixed model analysis was used to account for clustering in the data and to control for repeated observations of each animal and multiple testing. A random effect was used to account for repeated observations of the same animals, and fixed effects were used to model the effects of litter, age (categorical, with 21 degrees of freedom), gender, and age-by-gender interaction. Examination of residuals showed non-normality, with a tendency toward larger error for larger values, and a log-transform was applied to the data to correct the residual error to normality. Least-square means estimates were used to generate plots and numerous post hoc comparisons. Results were exponentiated (reverse-transformed) and reported on the original scale. Multiple testing was controlled by applying a 5% false discovery rate (FDR, which expects that 5% of all significant results will be falsely significant; Benjamini and Hochberg, 1995), including 770 comparisons of Gender at each Age (22 age groups times 35 parameters), 735 comparison of day-to-day changes (gender averaged; 21 pairs of adjacent age groups times 35 parameters), 735 day-to-day Gender interactions (21 pairs of adjacent age groups times 35 parameters). This last group could also be described as the simple age-by-gender interaction-effects on any two consecutive days. Applying the FDR to these 2240 comparisons, raw P-values less than 0.00126 met the threshold for a 5% FDR. A 5% significance level was used. The analysis was performed using SAS version 9.3 (The SAS Institute, Cary, NC).

All figures shown represented the raw data (mean ± SEM), but all statistical significance shown in the figures was based on the mixed model analysis with FDR.