Maturation of baseline breathing and of hypercapnic and hypoxic ventilatory responses in newborn mice

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Renolleau, Sylvain, Stéphane Dauger, Fanny Autret, Guy Vardon, Claude Gaultier, and Jorge Gallego. Maturation of baseline breathing and of hypercapnic and hypoxic ventilatory responses in newborn mice. Am J Physiol Regulatory Integrative Comp Physiol 281: R1746–R1753, 2001.—Breathing during the first postnatal hours has not been examined in mice, the preferred mammalian species for genetic studies. We used whole body plethysmography to measure ventilation (Ve), breath duration (TTOT), and tidal volume (Vr) in mice delivered vaginally (VD) or by cesarean section (CS). In experiment 1, 101 VD and 100 CS pups aged 1, 6, 12, 24, or 48 h were exposed to 8% CO2 or 10% O2 for 90 s. In experiment 2, 31 VD pups aged 1, 12, or 24 h were exposed to 10% O2 for 5 min. Baseline breathing maturation was delayed in CS pups, but Ve responses to hypercapnia and hypoxia were not significantly different between VD and CS pups [at postnatal age of 1 h (H1): 48 ± 44 and 18 ± 32%, respectively, in VD and CS pups combined]. The Ve increase induced by hypoxia was greater at H12 (46 ± 27%) because of TTOT response maturation. At all ages, hypoxic decline was ascribable mainly to a Ve decrease, and posthypoxic decline was ascribable to a TTOT increase with apneas, suggesting different underlying neuronal mechanisms.

POSTNATA LLY, RAPID CHANGES occur in the relative contributions to breathing of wakefulness, temperature, mechanoreceptor, and chemoreceptor drives, the ultimate result being predominance of the chemoreceptor drive (20). Hyperpnea in response to hypoxia increases sharply after birth because of peripheral chemoreceptor resetting (9, 38). During sustained hypoxia, newborn mammals exhibit a decrease in ventilation (Ve), i.e., the hypoxic ventilatory decline (HVD), whereas adults can maintain their Ve above the baseline level (23, 25). The early maturation of breathing is of major clinical relevance (14, 15).

Mice are the preferred mammalian species for manipulating genes and characterizing physiological phenotypes (30). Data obtained soon after birth are crucial.

Null mutants for most genes investigated in respiratory studies of newborn mice survived only a few hours (e.g., Refs. 1, 6, 31). Some of the survivors breathed normally after a period of impaired breathing at birth (6, 31). Data on breathing maturation soon after birth are meager. Two studies in mice with the same genetic background showed a twofold Ve increase with 8% CO2 within 1 h of birth (6) and a fivefold increase with 10% CO2 within 1 day of birth (1), respectively. Small Ve increases in response to 10% O2 have been reported (6, 18, 19), but in one study, mice failed to respond to 10% O2 24 h after birth (1). Paton and Richter (27) found that 3-day-old mice increased their Ve by 75% when exposed to 10% O2 (5, 6, 36). HVD has been observed within 12 h of birth (32), but early HVD changes have not been investigated. These studies suggest that ventilatory control undergoes rapid maturation during the first 2 days of life in mice but do not provide data on the timing of this process.

Our first experiment investigated Ve responses to hypercapnia and hypoxia in mice at several postnatal ages (PNAs) during the first 2 days of life. The breathing of older mice has been characterized in previous studies (27, 32). Our first hypothesis was that the response to brief (90 s) hypoxia, which is determined mainly by peripheral excitatory input, would increase sharply because of peripheral chemoreceptor resetting but that no sharp increase would occur in the response to hypercapnia, which is generally mature at birth. Because cesarean section (CS) is used to improve the accuracy of PNA determination (6, 18, 31), we looked for effects of CS on ventilatory maturation by comparing pups delivered vaginally (VD) to pups delivered by CS.

Our second experiment investigated HVD maturation by using a longer period of hypoxia (5 min). Robinson et al. (32) reported that HVD was less marked in juvenile and adult mice than in newborns. Our second hypothesis was that HVD may be present in newborn mice. We made no hypothesis regarding changes in HVD during the 48-h study period.

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**METHODS**

**Mice**

In experiment 1, we studied 201 Swiss-IOPS newborn mice of both genders (IFFA-CREDO, L’Arbresle, France), including 101 VD pups and 100 CS pups, divided into five balanced groups with PNAS of 1, 6, 12, 24, or 48 h (H1, H6, H12, H24, and H48, respectively). In experiment 2, 31 Swiss-IOPS VD pups were divided into three balanced age groups, H1, H12, and H24. In both experiments, we used independent age groups to avoid possible effects on ventilatory control maturation of repeated exposure to chemical stimuli (22, 29). Mean group weights are indicated in Table 1.

The morning after mating was defined as embryonic day 0 (E0). The mice were housed at 24°C with a normal 12:12-h light-dark cycle and food and water ad libitum. Experimental protocols met animal research guidelines established by the Institut National de la Santé et de la Recherche Médicale (National Institute for Health and Medical Research).

**CS**

Pregnant mice were killed on E18.5 (normal delivery is on E19) by cervical dislocation. Previously described procedures were used to deliver (6) the pups and to stimulate the CS pups (33). All CS pups survived. Those tested at H1 were placed in a box that contained litter from their mother’s box and was kept at a constant temperature of 32°C (the mean temperature in 1-h-old litters with the mother). CS newborns tested at H6, H12, H24, and H48 were with foster mothers.

**Ventilatory Measurements**

We used noninvasive, whole body plethysmography based on Drorbaugh and Fenn’s principle (7, 10, 11, 24, 26) to measure breath duration (T_BREATHTOT, ms), tidal volume (V_T, μl), and V_E (calculated as V_TV_BREATHTOT, in μl/s). V_T and V_E were divided by body weight and expressed in microliters per gram and microliters per gram per second [body temperature, pressure, and saturation (BTPS)], respectively. Hereafter, V_T and V_E designate these weight-normalized variables.

The plethysmograph has been described previously (6, 31). Calibration was done before each test by injecting 2 μl of air into the measurement chamber from a syringe and by introducing the corresponding pressure into Drorbaugh and Fenn’s equation. Hypercapnic (8% CO₂, 21% O₂, and 71% N₂) and hypoxic (10% O₂, 3% CO₂, and 87% N₂) mixtures were obtained commercially. The hypoxic mixture contained 3% CO₂ to maintain near-normal arterial P_CO₂ (Paco2) values during hypoxia (28).

**Procedure**

**Experiment 1.** Each animal underwent two hypercapnic or two hypoxic tests. The first test was used to assess responses to hypercapnia and hypoxia. To study repeatability of ventilatory responses, the tests were run in close succession. Each test was started after 1 min of familiarization inside the measurement chamber and consisted of five steps: 1) the chamber was flushed with 60 ml of air, 2) breathing variables were recorded for 90 s, 3) the chamber was flushed with 60 ml of air, 4) the hypercapnic or hypoxic mixture was injected, and 5) breathing variables were recorded for 90 s. Each gas injection took ~45 s. Because of a transient pressure signal disturbance, valid pressure signals became available ~15 s after the end of each gas injection. After the last recording, the mouse was removed from the chamber and subjected to measurements of body weight and mouth temperature. Each animal spent 11–12 min in the chamber.

**Experiment 2.** Each animal underwent a single hypoxic test (10% O₂ with 3% CO₂ in N₂) with exposure to air for 3 min, to hypoxia for 5 min, and to air again for 3 min. To shorten the pressure signal disturbance caused by the gas injections, we decreased the output of the measuring chamber and reference chambers during the injections. This reduced the injection time to 10 s (45 s in experiment 1). The measurement chamber was too small (30 ml) to allow determination of gas concentrations. However, based on CO₂ production by rats aged 7 to 9 days (Ref. 34; corresponding values in newborn mice are unknown), we estimated that CO₂ in the chamber remained below 1.6% after 5 min (the longest time between 2 gas changes). Finally, body temperature was recorded continuously throughout the test in three mice (H1, H6, and H12) using a PT100 platinum temperature probe attached to the cervical skin and connected through the wall of the plethysmograph to an external amplifier.

**Data Selection**

Ventilatory data free from movement artifacts were selected visually by discarding trace segments without individualized breaths or with drifts larger than twice the mean volume signal amplitude. This was done by two investigators, each of whom processed half the VD and CS pups and half the pups within each age group. In experiment 1, T_BREATHTOT, V_T, and V_E were averaged over each continuous sequence of valid breaths. The overall mean of these averaged values weighted for the number of breaths in each sequence was calculated. The numbers of breaths (and cumulative duration on 90-s recordings) were 111 ± 56 (56 ± 21 s) during air breathing and 134 ± 65 (62 ± 21 s) during hypercapnia or hypoxia. In experiment 2, we averaged valid data over successive 1-min periods. Sequences of valid breaths were used after exclusion of apneas (defined as ventilatory pauses longer than twice the duration of the preceding valid breath). The number of apneas was calculated in segments free from movement artifacts.

**Statistics**

Analysis of variance for each stimulus and each variable (T_BREATHTOT, V_T, and V_E, and apnea number and duration) was done using Superanova Software (Abacus Concepts, Berkeley, CA). We used the percentage change from normoxia to gas stimulus to assess ventilatory responses to hypoxia and hypercapnia [i.e., 100 × (V_Estimulus – V_Eair)/V_Eair], hereafter.
called the $\dot{V}E$, $V_T$, and $T_{TOT}$ responses. Data are summarized as the group means ± SDs in the text and Table 1 and as the means ± SEs in Figs. 1–5.

In experiment 1, delivery mode and PNA were between-subject factors, and the test (1st vs. 2nd) was a within-subject factor. In experiment 2, PNA was a between-subject factor, and time (5 levels during hypoxia, from minute 1 to minute 5, or 3 levels during air breathing, from minute 1 to minute 3) was a within-subject (repeated) factor. In both experiments, we analyzed HVD by comparing breathing variables during the air periods before and after hypoxia. To take into account the heterogeneous correlations among the repeated time measurements in experiment 2, we adjusted the degrees of freedom using the Huynh and Feldt factor (4). The effects of time at testing (morning, midday, and afternoon) and investigator who performed data selection were found to have no significant effects and are not discussed in this article.

RESULTS

Baseline Breathing in Air

VD and CS pups had similar $\dot{V}E$ at H1, but the $\dot{V}E$ increase with PNA ($P < 0.0001$) was delayed in CS pups (PNA by group interaction: $P < 0.0015$, Fig. 1A). $T_{TOT}$ changes mirrored $\dot{V}E$ changes (Fig. 1B). $V_T$ was smaller in CS than VD pups ($P < 0.033$, Fig. 1C).

Ventilatory Response to Hypercapnia

The $\dot{V}E$ response to hypercapnia (1st test of experiment 1), although weaker in CS pups, was not significantly different between CS and VD pups (for example, 20 ± 31 and 32 ± 27% at H6, 45 ± 32 and 45 ± 31% at H24; main effect for delivery mode and interaction with PNA not significant). Therefore, we pooled data from CS and VD pups (Fig. 2A, left). $\dot{V}E$ response increased with PNA ($P < 0.027$), mainly because of a $V_T$ response increase (Fig. 2C, left; $P < 0.003$), with no significant $T_{TOT}$ response change (Fig. 2B, left).

The second hypercapnic test of experiment 1 confirmed that the $\dot{V}E$ response was independent from delivery mode and increased with PNA from 26 ± 37% at H1 to 57 ± 37% at H48. $\dot{V}E$ and $V_T$ responses were weaker in the second than in the first test ($P < 0.0001$) because of higher $\dot{V}E$ levels during the second air period ($P < 0.0001$) but not during hypercapnia (Fig. 3A).

Ventilatory Response to Hypoxia

Hyperpneic response to hypoxia. The $\dot{V}E$ response to hypoxia (1st test of experiment 1), although weaker in CS pups, was not significantly different between CS and VD pups (for example, 13 ± 25% in CS vs. 18 ± 16% in VD at H6; 46 ± 22 vs. 53 ± 22% at H24; main effect for delivery mode and interaction with PNA not significant). Therefore, we pooled the data from CS and VD pups (Fig. 2A, right). Most of the $\dot{V}E$ response increase occurred around H12 (main effect of PNA: $P < 0.0009$; H1 and H6 vs. H12: $P < 0.014$, Fig. 2A, right) and was ascribable to $T_{TOT}$ response maturation (Fig. 2B, right; main effect for PNA: $P < 0.0003$; H1 and H6 vs. H12: $P < 0.005$). The $V_T$ response did not change significantly with PNA (Fig. 2C, right).

The second hypoxic test of experiment 1 confirmed that the $\dot{V}E$ response was independent from delivery mode and increased with PNA (from 38 ± 82% at H1 to 74 ± 35% at H48). The $\dot{V}E$ and $V_T$ responses were larger in the second than in the first test ($P < 0.0001$) because of smaller $\dot{V}E$ levels during the second air period ($P < 0.0001$) but not during hypoxia (Fig. 3B).

The sharp increase in $\dot{V}E$ responses to hypoxia around H12 was confirmed in experiment 2 (Fig. 4A). The significant main effect for PNA ($P < 0.013$) was accounted for by the difference between H1 and the other two ages (H12 and H24).

HVD. $\dot{V}E$ during hypoxia displayed a significant linear decrease at H12 and H24 ($P < 0.0001$ and $P <
0.002, respectively, Fig. 4A), indicating an HVD, but was still significantly greater than baseline at the end of the stimulus \( (P < 0.001 \) and \( P < 0.005 \), respectively). VE decreased between the first and second minute of hypoxia \( (P < 0.0015 \), Fig. 4A). \( T_{TOT} \) did not change significantly over time (Fig. 4B), whereas VT decreased linearly at H12 and H24 \( (P < 0.001 \) and \( P < 0.0001 \), respectively; Fig. 4C). The additional tests in three animals at H1, H6, and H12 showed that temperature changes during hypoxia were 0.5, 0.2, and 0.6°C, respectively, producing about 4% error in VT measurements (11).

Posthypoxic ventilatory decline. Posthypoxic ventilatory decline (PHVD) was caused mainly by a longer \( T_{TOT} \) after than before hypoxia in experiment 1 \( (P < 0.0006 \), Fig. 3B); VT levels were similar before and after hypoxia. Similarly, in experiment 2, VE at H1 and H24 was significantly below baseline after hypoxia \( (P < 0.002 \) and \( P < 0.005 \), respectively; nonsignificant difference at H12; Fig. 4A), and PHVD was driven by \( T_{TOT} \) \( (P < 0.003 \), Fig. 4B). The differences for VT were not significant (Fig. 4C). Another manifestation of PHVD was a greater number of apneas after vs. before hypoxia in both experiments \( (P < 0.002 \) in experiment 1, Fig. 5A, and \( P < 0.0001 \) in experiment 2, not shown) in all age groups except for the H48 group in experiment 1, leading to a small PNA by pre-post factor interaction \( (P = 0.046 \); we considered this effect marginal and pooled the PNA groups in Fig. 5A). Apneas were longer after than before hypoxia \( (P < 0.002 \) in experiment 1, Fig. 5B, and \( P < 0.004 \) in experiment 2, not shown). This effect was present in all age groups in both experiments and was not significantly influenced by PNA. Neither the number nor the duration of apneas was significantly different after vs. before hypercapnia (Fig. 5).

DISCUSSION

This is, to our knowledge, the first description of breathing control maturation during the first 48 h of life in mice. The main results were as follows: 1) despite normal baseline VE levels at H1, the postnatal VE increase was delayed in CS pups because of a delayed \( T_{TOT} \) decrease; 2) delivery mode had no significant influence on VE responses to hypercapnia or hypoxia during the 48-h study period; 3) a VE response to hypercapnia was present at H1 and ascribable mainly...
4) the $\dot{V}_E$ response to hypoxia was small until H12, then increased sharply as a result of $TTOT$ response maturation; and 5) HVD and PHVD were present in all age groups.

Plethysmographic Measurements

Contrary to plethysmography in adult mice (10, 26), plethysmography in newborns has not been validated against pneumotachography. Therefore, the validity of absolute $V_t$ and $\dot{V}_E$ values can be questioned. However, the percent increases from baseline in response to hypercapnia and hypoxia reliably assess responsiveness to chemical stimuli. Head-out plethysmography (1, 32) was not used in this study because it requires restraining the animals. In adult mice, restraint strongly stimulates baseline $\dot{V}_E$ without significantly affecting the $\dot{V}_E$ increase caused by chemical stimuli (5). The two studies that used head-out plethysmography in newborn mice (1, 32) found very strong $\dot{V}_E$ responses to hypercapnia (500% increase in response to 10% CO$_2$ + 30% O$_2$) or hypoxia (140% increase in response to 7.4% O$_2$) within 1 day of birth, compared with the present and previous data. These differences may be ascribable to $\dot{V}_E$ measurement methods, but no strong interpretation can be offered in the absence of...
associated with profound changes in plasma cat-

environment, including breathing air after delivery, is

groups, and weight was slightly higher in CS pups.

differences in gestational age between CS and VD pups

ing pattern and weight was probably not ascribable to

CS pups). The delayed maturation of baseline breath-

ing delayed maturation. This was perhaps ascribable

to differences in maternal care and feeding between

effects in VD pups between H6 and H12, a finding not reported previously. Norepi-
nephrine can increase or decrease respiratory fre-

quency in brain stem-spinal cord preparations of new-

born rats depending on whether its main action is on

the pons or medulla (12). The higher TTOT in CS pups

might be ascribable to their presumably smaller nor-

epinephrine release.

Despite these differences in baseline breathing, re-
sponses to hypercapnia or hypoxia were similar in CS

and VD mice, in line with evidence from infants that

CS does not affect the peripheral chemoreflex (39).

Thus CS may affect ventilatory function maturation

without significantly modifying responsiveness to

chemical stimuli.

Maturation of Ventilatory Responses to Hypercapnia

The VE response to hypercapnia was vigorous from

H1 and dependent mainly on a VT increase. At H48,

this response (74 ± 48%) was lower than in previously

studied adult Swiss mice exposed to the same hyper-

capnic stimulus with or without restraint [163 ± 100

and 118 ± 40%, respectively (5)]. Thus the VE response
to hypercapnia may continue to increase after 48 h of

PNA.

Maturation of Ventilatory Responses to Hypoxia

Hyperpneic response to hypoxia. The increase in the

VE response to hypoxia after 12 h was probably caused

by postnatal resetting of the chemoreceptors. Peak

responses to hypoxia at H12 and H24 were stronger in

experiment 2. In experiment 1, the longer gas injection

and the subsequent pressure signal instability delayed

occurrence of a valid respiratory signal, so that some of

the movement-free data used for VE determination

were collected after the VE peak, possibly leading to

underestimation of the response to hypoxia. However,

both experiments showed a sharp hypoxic response

increase around H12.

We added 3% CO2 to the hypoxic mixture to main-
tain near-normal PaCO2 levels during hypoxia (28).

Because at H1 and H6 the hypercapnic response is

mature and the hypoxic response is immature, 3% CO2

may have stimulated breathing at H1 and H6, causing

the 19 and 16% VE increases, respectively. In support

of this, the VE increases were related to VT increases

rather than to TTOT decreases, a pattern characteristic

of the response to hypercapnia. Furthermore, assum-
ing rough proportionality between inspired CO2 frac-

comparative studies of whole body vs. head-out pleth-
ysmography in newborn mice.

Effects of CS on Ventilatory Control

CS was associated with delayed maturation of the

baseline breathing pattern. In VD pups, VE increased

more frequent and longer after than before hypoxia (***P < 0.002 and

***P < 0.0002, respectively). The number and duration of apneas

were not significantly different before and after hypercapnia. Values

before hypoxia and hypercapnia were not significantly different.

PNA had a marginal effect on the pre-post comparisons of apnea

numbers and no significant effect on apnea duration; therefore, we

pooled these variables over the 5 age groups of

experiment 1. Pre-post comparisons for hypoxia in experi-

ment 2 yielded similar results. Values are means ± SE; n = 100 for

hypercapnia, and n = 101 for hypoxia.

Fig. 5. Number (A) and duration (B) of apneas before (pre) and after
(post) hypoxia and hypercapnia over all PNA groups. Apneas were

more frequent and longer after than before hypoxia (***P < 0.002 and

***P < 0.0002, respectively). The number and duration of apneas

were not significantly different before and after hypercapnia. Values

before hypoxia and hypercapnia were not significantly different.

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pooled these variables over the 5 age groups of

experiment 1. Pre-post comparisons for hypoxia in experi-

ment 2 yielded similar results. Values are means ± SE; n = 100 for

hypercapnia, and n = 101 for hypoxia.


Conclusions

The present results have practical implications regarding the analysis of ventilatory control in newborn mice. First, CS delays baseline V̇E maturation but has no significant effect on ventilatory responses to hypercapnia and hypoxia. Second, the response to hypercapnia can be assessed at birth, whereas the response to hypoxia is weak within 12 h of birth and should be evaluated later. Third, the hyperpneic responses to hypoxia and HVD undergo different maturation processes within 48 h after birth. Both are relevant to the development of breathing control and should be evaluated based on the time course of ventilatory variables during hypoxia. Fourth, hypercapnia stimulates and hypoxia inhibits baseline V̇E after stimulus cessation. Therefore, V̇E measured immediately after the stimulus may not reflect baseline breathing.

Perspectives

This study acknowledges the growing importance of mice as a model for studying organ physiology (30, 32). Because strain differences may affect breathing pattern in newborn mice, as in adults (35–37), only values from wild-type litters can serve as reference values for breathing variables in mutant (homozygous or heterozygous) mice of a given strain. However, the developmental milestones of breathing control in outbred mice described in this study provide a basis for designing future experiments in inbred newborn mice. Until now, most work on genetic factors in physiological functions has been conducted in adult mice. However, the phenotypic expression of a given mutation may recover with time, masking the potential role of the mutation. Postnatal physiological function studies designed with maturation profiles in mind can be expected to benefit the search for genotype-phenotype relationships.

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