Ventilatory Response to Hyperoxia in Newborn Mice

Heterozygous for the Transcription Factor Phox2b

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ABSTRACT

Heterozygous mutations of the transcription factor PHOX2B have been found in most patients with central congenital hypoventilation syndrome (CCHS), a rare disease characterized by sleep-related hypoventilation and impaired chemosensitivity to sustained hypercapnia and sustained hypoxia. PHOX2B is a master regulator of autonomic reflex pathways, including peripheral chemosensitive pathways. In the present study, we used hyperoxic tests to assess the strength of the peripheral chemoreceptor tonic drive in Phox2b+/- newborn mice. We exposed 69 wild-type and 67 mutant mice to two hyperoxic tests (12 min air followed by 3 min 100% O2) 2 days after birth. Breathing variables were measured noninvasively using whole-body flow plethysmography. The initial $\dot{VE}$ decrease was larger in mutant pups than in wild-type pups: -37% (SD 13) and -25% (SD 18), respectively, $P<0.0001$. Furthermore, $\dot{VE}$ remained depressed throughout O2 exposure in mutants, possibly because of their previously reported impaired CO2 chemosensitivity, whereas it returned rapidly to the normoxic level in wild-type pups. Hyperoxia considerably increased total apnea duration in mutant compared to wild-type pups ($P=0.0001$). A complementary experiment established that body temperature was not influenced by hyperoxia in either genotype group and therefore did not account for genotype-related differences in the hyperoxic ventilatory response. Thus, partial loss of Phox2b function by heterozygosity did not diminish the tonic drive from peripheral chemoreceptors.

Keywords: control of breathing, chemosensitivity, apnea.
Introduction

Central congenital hypoventilation syndrome (CCHS or Ondine's curse) is a rare disease, generally present from birth and characterized by hypoventilation during sleep in the absence of primary neuromuscular or lung disease or of brainstem lesions (1). Throughout life, patients with CCHS have absent or markedly reduced ventilatory responses to sustained hypercapnia (29) and to a lesser extent, to sustained hypoxia (29). These respiratory impairments are generally ascribed to impaired central integration of chemosensory inputs at the brainstem level, rather than to failure of chemoreceptor activity, which is at least partially present (15, 22, 37). The genetic basis for CCHS was discovered recently: most patients with CCHS have a heterozygous mutation of the \textit{PHOX2B} gene (3, 23, 41, 43). \textit{PHOX2B} is a master regulator of the noradrenergic phenotype and of all neuronal relays of autonomic medullary reflex pathways (30), including peripheral chemosensitive pathways (9).

Mice with a single functional \textit{Phox2b} allele (i.e. \textit{Phox2b}+/− mice) provide a unique opportunity to investigate the genotype-phenotype relationship in CCHS. Our previous studies showed that \textit{Phox2b}+/− mice had longer sleep-apnea times than their wild-type littermates on postnatal day 5 (P5) (11) and a weaker response to CO$_2$ on P2 followed by normalization before P10 (9). These ventilatory impairments were reminiscent of CCHS. However, the hyperpneic
response to sustained hypoxia (5% O₂) on P2 was normal, which is not the

The aim of the present study was to further examine the functional deficits
caused by loss of a single Phox2b allele. To this end, we investigated the
ventilatory response to hyperoxia, which provides a functional estimate of the
tonic chemoreceptor drive (19, 28, 40). Previous studies in newborn mammals
showed divergences between the results of hypoxic and hyperoxic tests (5, 25),
raising the possibility that a decrease in tonic chemoreceptor drive might coexist
with normal ventilatory responses to hypoxia. The present study tested this
possibility. As previously, the mice were studied soon after birth (on P2), to
avoid possible confounding effects of recovery processes (9).

METHODS

Animals

The generation and genotyping of Phox2b mutant mice have been reported
elsewhere (31). Pups were tested at 2 days of postnatal age (P2). Mating 23
wild-type females with mutant males yielded 67 heterozygous mutant pups
(weight: 1.73±0.30 g) and 69 wild-type littermates (1.77±0.34 g) that served as
controls. In a complementary experiment investigating the effects of hyperoxia
on body temperature, we used 10 mutant and 11 wild-type pups (weight:
1.52±0.18 g and 1.68±0.12 g, respectively, p<0.028) born to 4 wild-type
females. The mice were housed at 24 °C with a 12h/12h light/dark cycle. The
experimental protocols complied with the animal research guidelines established by the Institut National de la Santé et de la Recherche Médicale (French national institute for health and medical research).

Whole-body flow plethysmography

Respiratory variables were measured noninvasively using whole-body flow barometric plethysmography as previously described (11, 12, 24). The plethysmograph was composed of two Plexiglas cylinders serving as the animal (40 ml) and reference (70 ml) chambers, respectively, immersed in a thermoregulated water-bath set that maintained their temperature at 32.8 °C. A 100-ml min⁻¹ flow of dry air (Bronkhorst Hi-Tec airflow stabilizer, Urlo, Holland) was divided into two 50-ml min⁻¹ flows through the chambers, to avoid CO₂ accumulation and water vapor condensation. The differential pressure between the two chambers (DRUCK-EFFA transducer, Asnières, France; range±0.1 mb) was filtered (bandwidth, 0.05-15 Hz at -3 dB), converted to a digital signal (Instrunet model 200, 14-bit converter, GW-INSTRUMENTS, Somerville, MA) at a sample rate of 100 Hz, and processed using custom-written software (Software Superscope II, GW-INSTRUMENTS). The time constant of the pressure decay within the system (2 seconds) was measured by injecting 2 µl into the measurement chamber. This allowed measurement of breathing frequencies within the 0.5 Hz-10 Hz range at -3 dB. Calibration was done before each session using a built-in pump incorporating a micro-syringe (Ito
corporation, Fuji, Japan), which injected a sinusoidal airflow with a maximal amplitude of 2 µl and a frequency of 8 Hz into the animal chamber. Previous tests indicated that calibration coefficients did not vary as a function of pump frequency within the 2-8 Hz range. The pressure rise induced by this injection was of similar magnitude to that induced by the pup's breathing. Considering the limitations of flow barometric plethysmography (39), especially in newborn mice (24), the absolute values of VT and $\dot{V}_E$ presented here should be considered indicative only, whereas $T_{TOT}$ absolute values and apnea duration are reliable.

**Design**

After at least 2 minutes' adaptation to the chamber, each pup was exposed to two consecutive poikilocapnic hyperoxic tests (Test 1 and Test 2) consisting of 12 min air followed by 3 min O₂. To increase statistical power, we performed two tests. We performed poikilocapnic tests because available techniques fail to control normocapnia in freely moving newborn mice. Hyperoxia was achieved by switching the airflow through the plethysmograph to 100% O₂ flow at the same flow rate (50 ml/min per chamber). Thus, the time needed to flush the chamber (40 ml) was about 1 min. After the second O₂ exposure, the flow was switched back to air and breathing was measured for 12 minutes (total duration of the session: 42 min). In a complementary experiment, we used the same
design in restrained pups subjected to continuous body temperature measurement (see measurement technique below).

*Ventilatory response to O₂*

Breathing variables were analyzed without previous knowledge of genotypes. Breath duration ($T_{TOT}$, s), tidal volume ($VT$, $\mu l \; g^{-1}$), and minute ventilation $\dot{V}_E$ (calculated as $VT \times T_{TOT}^{-1}$ and expressed in $\mu l \; s^{-1} \; g^{-1}$) were calculated on apnea-free periods (see apnea determination below). Breathing variables were averaged over consecutive 30-sec periods. The baseline levels for these variables for each test were calculated as the mean value over the 3-min of air-breathing preceding the test. We expressed the O₂-induced $\dot{V}_E$ decrease as the percentage of baseline, using the formula $100 \times (\text{min } \dot{V}_E - \text{baseline } \dot{V}_E)/\text{baseline } \dot{V}_E$, where min $\dot{V}_E$ was the minimum $\dot{V}_E$ value over the 3-min period of O₂ exposure. This method took into account possible interindividual differences in time to the $\dot{V}_E$ response to O₂. We determined the $VT$ and $T_{TOT}$ responses to O₂ using the same formula with the $VT$ and $T_{TOT}$ values measured at min $\dot{V}_E$.

Apneas were determined using an automatic classification method based on spectral analysis (24). Apneas were defined as ventilatory pauses longer than twice the duration of the preceding breath (32, 36). This definition takes into account the large interindividual differences in resting breathing frequency in newborn mice. Total apnea duration was calculated during each 3-min period,
i.e., before and during O$_2$ exposure. The apnea response to O$_2$ was calculated as the difference between air and O$_2$ (percentages could not be calculated because some subjects had no apneas during normoxia, so that baseline apnea duration was zero). Movements were detected based on changes in the baseline respiratory signal, using a previously validated criterion: \( \frac{|V_i - V_e|}{V_i + V_e} \) where \( V_i \) and \( V_e \) were the magnitudes of the inspiratory and expiratory limbs of the volume signal, respectively (24).

**Body temperature**

In the main experiment, body temperature was not continuously recorded during ventilatory measurements, as this would have required restraining the pups; instead, body temperature was measured immediately after the plethysmographic recordings. To evaluate whether body temperature changes during hyperoxia might affect \( \dot{V}_E \) and \( V_T \) measurements, we measured body temperature in 10 mutant and 11 wild-type pups exposed to the above-described protocol. Each pup was lightly anesthetized with isoflurane using a method previously shown to be associated with rapid recovery in newborn mouse pups (10). Measurements were initiated 4-5 min after anesthesia, which is sufficient for recovery from anesthesia on P2 (10). A thermocouple probe was positioned through a 2-3 mm incision in the interscapular region, which is the area of highest skin temperature (6). Then, the pup was placed in the plethysmograph in a restraining device to ensure that temperature probe
position remained unchanged. Previous studies in newborn rats showed that
colonic and interscapular temperatures were closely correlated over
temperatures ranging from 22.5 °C to 37 °C (38). Because the restraining
device disrupted breathing measurements in several pups, we did not analyze
the respiratory data.

Statistics

Breathing variables were subjected to analyses of variance with genotype
(mutant Phox2b+/- versus wild-type Phox2b+/+) as the between-subject factor
and time (nine levels: six 30-sec means during hyperoxia followed by three 30-
sec means during normoxia) and test number (two levels) as repeated factors
(Superanova Software, Abacus Concepts, Berkeley, CA). Normoxic values of
breathing variables and apnea durations were analyzed with genotype and
periods (four levels: baseline, pre-hyperoxia 1, pre-hyperoxia 2, and final
values) as the factors. To take into account the heterogeneous correlations
among the repeated time measurements, we adjusted the degrees of freedom
using the Greenhouse and Geisser factor, which is a very conservative
downward correction to the degrees of freedom (18). Within-subject main
effects and interactions are presented, together with P values based on these
adjusted degrees of freedom. When the overall analysis was significant,
multiple pairwise post-hoc analyses using the Bonferroni/Dunn method were
conducted to determine where the significant differences lay. In all tests, the critical significance level was set at 0.05.

RESULTS

Breathing pattern during normoxia

Large proportions of the 42-minute recordings were free of artifacts in both the mutant pups (83 % (SD 9)) and the wild-type pups (86% (SD 5)). Breathing variables were averaged over four 3-min periods of normoxia (initial baseline levels, pre-hyperoxic levels for Test 1 and Test 2, and final values, Table 1). Normoxic \( {\dot{V}_E} \) values tended to increase from baseline to final values in both groups, although this trend was more marked in mutants than in wild-type pups (genotype-by-period interaction: \( P<0.007 \)): 43% (SD 75) in mutant pups versus 21% (SD 58) in wild-type pups. In particular, pre-hyperoxic \( {\dot{V}_E} \) values were slightly but significantly larger in Test 2 than in Test 1 in mutant pups, due to significantly higher VT values (Table 1). This trend was mainly due to \( T_{TOT} \), which decreased significantly in mutants but remained unchanged in wild-type pups (genotype by period interaction: \( P<0.012 \), Table 1) and, to a lesser extent, to VT (genotype by period interaction: \( P<0.006 \), Table 1).

Ventilatory response to hyperoxia

In both tests, hyperoxia caused significant \( {\dot{V}_E} \) depression in mutants and wild-type pups (Fig. 1 and Fig. 2A) due to the \( T_{TOT} \) increase (Fig. 2B), whereas VT changes were small (less than 5%) and nonsignificant (Fig. 2C). The initial \( {\dot{V}_E} \)
decrease (the early response to O₂) generally occurred within 1 min of O₂ exposure and was followed by an increase (the late response to O₂) toward baseline levels (Fig. 1 and Fig. 2A).

The time courses of the \( \dot{V}_E \) and T\text{TOT} responses to O₂ were different in mutants and wild-type pups (genotype by time interaction for \( \dot{V}_E \) and T\text{TOT}: \( P<0.0001 \); VT: nonsignificant, Fig. 2A, B, C). In wild-type pups, \( \dot{V}_E \) rapidly returned to baseline levels during hyperoxia (Fig. 2A); a single mean \( \dot{V}_E \) value (30 s after O₂ onset) was lower than the pre-O₂ value (paired t-tests: Test 1, \( P<0.0007 \), Test 2: \( P<0.0001 \); all other differences were nonsignificant). In contrast, in mutants, \( \dot{V}_E \) remained lower than the pre-O₂ level throughout O₂ exposure and for 1 minute following the return to normoxia (Fig. 2A, Test 1 and Test 2: \( P<0.0001 \) for all comparisons with pre-O₂ levels, except for the first 30-s period). Furthermore, in Test 2, \( \dot{V}_E \) was significantly lower than the pre-O₂ level as soon as the first 30-second period (\( P<0.0001 \), Fig. 2A).

The min \( \dot{V}_E \) values, expressed as percentages of pre-hypoxic levels, and the corresponding T\text{TOT} values were larger in mutants than in wild-type pups (\( \dot{V}_E \): -37% (SD 13) and -25% (SD 18), respectively, \( P<0.0001 \); T\text{TOT}: 61% (SD 45) and 36% (SD 39); respectively, \( P<0.0001 \); VT: nonsignificant, Fig. 2D, E, F). Finally, min \( \dot{V}_E \) values were lower in Test 2 than in Test 1, irrespective of genotype, confirming the potentiation effect mentioned above. Repetition differentially affected VT in mutants and wild-type pups, but the changes were
small in both tests (test by genotype interaction, \( P<0.038 \), Fig. 2F). The \( \dot{V}_E \) decreases were not significantly correlated to body weight or to body temperature measured after plethysmography.

Thus, mutant pups showed a larger and more sustained ventilatory decrease in response to hyperoxia than their wild-type littermates.

*Hyperoxia induced apneas*

Total apnea duration during normoxia was not significantly different in mutant and wild-type pups (Table 1). As with \( \dot{V}_E \) and TTOT, the increase in total apnea duration (relative to normoxic levels) displayed a biphasic pattern during hyperoxia, although with greater interindividual variability (main effect for group: \( P<0.0001 \), genotype by time interaction: \( P<0.017 \) and partial comparisons, Fig. 3A). The increases in total apnea duration during hyperoxia were significantly larger in mutants than in wild-type pups in both tests (Test 1: \( P<0.0001 \); Test 2: \( P<0.0001 \), genotype by test interaction: NS, Fig. 3B). These increases were not significantly correlated to body weight in either genotype group. Thus, the evaluation of apneas confirmed that the hyperoxia-induced ventilatory depression was stronger in mutants than in wild-type pups. In both groups, the increase in apnea duration was weakly but significantly correlated with the \( \dot{V}_E \) decrease (mutants: Test 1, \( r^2=0.267 \); Test 2, \( r^2=0.276 \); wild-type pups: Test 1, \( r^2=0.337 \); Test 2: \( r^2=0.246 \), all values significant at \( P<0.0001 \), group differences nonsignificant).
Body temperature varied within very narrow limits throughout the recording session, with no significant effect of genotype (Table 2). This showed that the reported changes in \( V_E \) and \( V_T \) during hyperoxia and the genotype-related differences in breathing pattern were not due to body temperature differences.

**DISCUSSION**

In this study, we showed that the ventilatory decrease caused by hyperoxia was larger in newborn \( \text{Phox2b}+/− \) mutant mice than in their wild-type littermates. Furthermore, mutant pups showed a more sustained ventilatory decrease, outlasting the return to normoxia, compared to wild-type pups. In mutants, the depressed ventilation was further aggravated by a longer apnea duration in response to hyperoxia.

*Limitations of whole-body plethysmography in newborn mice*

Whole-body plethysmography is the only available method for measuring breathing variables in unrestrained newborn mice. However, it has been validated against pneumotachography in larger animals (26) but not in newborn mice. This limitation stems from the lack of miniaturized reference devices (spirometers and pneumotachographs) and from difficulties in obtaining accurate body temperature measurements. Thus, the absolute \( V_T \) and \( \dot{V}_E \) values in the present study should be considered with caution. However, this limitation does not invalidate our results, because our main finding, i.e., the
genotype-related difference in ventilatory response to hyperoxia, is based on $TTOT$ and apneas, which are reliably measured by plethysmography.

**Physiological chemodenervation in newborn mice**

The initial $V_E$ decrease caused by hyperoxia was chiefly ascribable to a $TTOT$ increase, whereas $VT$ changes were small and nonsignificant, in line with previous studies in mice pups of similar ages (28). In humans, the ventilatory response to hyperoxia undergoes developmental changes. In preterm infants, $V_E$ decreased after 3 min of $O_2$ exposure 2 days after birth, as a result of a decrease in respiratory rate with little or no change in $VT$, whereas the opposite breathing pattern was observed 6 days after birth (35) and in term infants between P2 and P6 (16). Thus, the breathing strategy of 2-day-old mice pups in the present study resembled the one reported in preterm infants shortly after birth. This similarity further supports the use of newborn mice as a model of respiratory disorders in preterm infants, who contribute 20% of patients with CCHS (11). Furthermore, hyperoxia also increased apnea duration, as previously reported in human (term or preterm) infants (2). The weak correlation between $V_E$ decrease and apnea duration suggested that these two indices of ventilatory depression might be complementary. The $V_E$ decrease and the increase in apnea duration were larger during the second than the first test, suggesting potentiation of the hyperoxic response. However, this effect was not significantly related to genotype.
Augmented tonic drive of peripheral chemoreceptors in Phox2b+/- mutant pups

The $\dot{V}E$ decrease caused by hyperoxia was stronger in mutants than in wild-type pups, suggesting that the tonic activity of oxygen-sensitive peripheral chemoreceptors was greater in mutant pups. This augmented peripheral tonic input may be ascribable to low arterial PO$_2$ levels, which, unfortunately, cannot be measured in newborn mice using current techniques. Our data did not indicate whether the ventilatory control disorders previously reported in mutant pups (low CO$_2$ chemosensitivity (9) and sleep-related apneas (11)) were associated with low PaO$_2$ values. In human term or preterm infants, periodic breathing was associated with low PaO$_2$ values and with greater decreases in ventilation and longer apnea times in response to 100% O$_2$ (2, 33, 34). Mutant pups may display a similar pattern of breathing disorders. The present finding that mutant pups displayed a vigorous $\dot{V}E$ response to hyperoxia extend our previous result that the phasic response to acute hypoxia is spared in Phox2b+/- mutant pups.

The augmented ventilatory decrease in response to hyperoxia in mutant pups may also reflect impairment of excitatory effects on the respiratory central pattern generator indirectly exerted by sustained (as opposed to short-lived) hyperoxia, considering that 1 minute of O$_2$ was needed to flush the chamber and that O$_2$ was maintained for 2 additional minutes. These excitatory effects, some involving CO$_2$ chemosensitivity, progressively offset the initial $\dot{V}E$
decrease (13, 27). Among these, cerebral vasoconstriction and decreased hemoglobin transport capacity for tissue CO\textsubscript{2} (Haldane effect (17)) are thought to decrease CO\textsubscript{2} elimination and to increase brain tissue PCO\textsubscript{2} at the central chemoreceptor level (20, 27). Genotype-related differences in the hyperoxic response may stem from impaired CO\textsubscript{2} chemosensitivity in mutant pups (9). Previous experiments performed on the same colony, at the same postnatal age (P2), and using the same experimental setup showed that the ventilatory response to 8\% CO\textsubscript{2} was about twice as small in mutant as in wild-type pups (9). This impairment may account for the larger and more sustained ventilatory decrease shown by mutant pups. In line with this possibility, we found that impairment of the T\textsubscript{TOT} response explained the greater ventilatory depression in mutant pups, as well as their impaired ventilatory response to CO\textsubscript{2}, compared to wild-type pups (9). Finally, genotype-related differences in the metabolic response to hyperoxia may also account for the ventilatory differences. O\textsubscript{2} consumption and CO\textsubscript{2} production were not measured in the present study, but the stability of body temperature during hyperoxic tests did not support a major role for metabolic changes.

Implications for the pathogenesis of CCHS

We previously reported that newborn Phox2b+/− mutant mice showed a weaker response to CO\textsubscript{2} (9), a longer sleep-apnea time (11), and a normal hyperpneic response to hypoxia followed by an increased post-hypoxic decline, compared
to their wild-type littermates (9). The present study shows that the hyperoxic response was preserved. In CCHS children who were able to sustain adequate ventilation during wakefulness, peripheral chemosensitivity to oxygen was intact (15). However, the breathing frequency response to 100% O₂ (ventilation was not measured) occurred earlier and was stronger in CCHS children than in controls (21). Taken together, these studies suggest that tonic drive to breathing from oxygen-sensitive chemoreceptors may be impaired only in the most severe cases of CCHS and that Phox2b+/- newborn mice may exhibit impairments akin to CCHS in its mildest form (9, 11).

The milder phenotype of Phox2b+/- mice compared to CCHS may be due to functional differences between the Phox2b-targeted mutation in mice (a null mutation) and the alanine expansion generally found in the PHOX2B gene of CCHS patients. Alanine expansions may result in a protein that can bind to the correct targets in the genome but is unable to carry out its normal regulatory function and therefore competes with the product of the wild-type allele (4). In contrast, a single functional Phox2b allele may ensure correct protein function, leading to a less severe phenotype (8). Furthermore, alanine-expanded proteins may be toxic to vulnerable cells that express them, due to aggregate formation (7).

Alternatively, the more severe phenotype in CCHS patients may be due to genetic factors. Heterozygous mutations affecting genes involved in important
neural crest development pathways have been found in a subset of CCHS patients (14, 42). The phenotype in mice heterozygous for mutations in Phox2b and another gene previously found mutated in CCHS, which have not been described so far, may more closely resemble CCHS than the phenotype produced by a single mutation of Phox2b.

**Conclusion**

The ventilatory decrease caused by hyperoxia was larger and more sustained in mutant Phox2b+/- pups than in their wild-type littermates. Thus, the tonic drive from oxygen-sensitive peripheral chemoreceptors was not disrupted, but was augmented, in mutant pups. Taken together, this and previous studies (9, 11) suggest that the impaired chemosensitivity to oxygen in CCHS may not be ascribable to PHOX2B loss of function related to heterozygosity.
Acknowledgments

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REFERENCES


FIGURE LEGENDS

Figure 1: Examples of respiratory recordings in a 2-day-old Phox2b+/+ mouse (panel A) and a 2-day-old Phox2b+- mouse (panel B). In both pups, hyperoxia caused an initial decrease in breathing frequency (early phase) followed by an increase toward pre-hypoxic levels (late phase). The late phase in the Phox2b+- mutant pup was characterized by sustained ventilatory depression with apneas (arrows).

Figure 2: Ventilatory response to 3-min O₂ exposure in 2-day-old Phox2b+/+ pups (empty circles, n=69) and Phox2b+- pups (filled circles, n=67). Values are group means (error bars represent the standard error of the mean, SEM). Breathing variables are expressed as percentages of baseline levels calculated over the 3-min period of normoxia preceding each O₂ exposure. Panel A: Hyperoxia (shaded area) caused a larger and more sustained $\dot{V}E$ decrease in mutant than in wild-type pups. This effect was due to a larger increase in $TTOT$ (panel B), whereas differences in $VT$ were not significant (panel C). Min-$\dot{V}E$, calculated individually throughout O₂ exposure, was lower in mutant than in wild-type pups in both tests (**: $P<0.003$) but the genotype-related differences were not significant.

Figure 3: Total apnea duration in 2-day-old Phox2b+/+ pups (n=69) and Phox2b+-/+ pups (n=67). Values are means±SEM. Panel A: Time course of
apnea durations. Δ Apnea: \( O_2 \) values (over successive 30-sec periods) minus pre-\( O_2 \) values (over 30 sec). Total apnea durations during hyperoxia (shaded areas) displayed a biphasic pattern in both tests (\( *: P<0.05 \)). Panel B: Δ Apnea: \( O_2 \) values (over 3 min \( O_2 \)) minus pre-\( O_2 \) values (over 3 min). The increases in total apnea duration were larger in mutant than in wild-type pups (\( ***: P<0.0001 \), \( *: P<0.05 \)).
Table 1: Breathing variables during normoxia in 2-day-old Phox2b+/+ (N=69) and Phox2b+/- (N=67) pups. See text for statistical analyses. *, †, ‡: significantly different from baseline, from pre-hyperoxia 1 and from pre-hyperoxia 2, respectively, at the P<0.05 level. Group differences were not significant at either period. Values are means (SD). Mean body temperature (after plethysmography) was not significantly different in mutant and wild-type pups (33.47 °C (SD 1.18) and 33.24 °C (SD 1.28), respectively).

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<td>13.94 (11.75)</td>
<td>13.94 (11.75)</td>
<td>13.94 (11.75)</td>
<td>13.94 (11.75)</td>
<td>13.94 (11.75)</td>
<td>13.94 (11.75)</td>
</tr>
<tr>
<td><strong>呼吸暂停次数</strong></td>
<td>13.86 (20.22)</td>
<td>16.68 (23.35)</td>
<td>14.07 (17.57)</td>
<td>15.29 (21.69)</td>
<td>14.02 (21.96)</td>
<td>12.75 (17.17)</td>
<td>14.02 (21.96)</td>
<td>12.75 (17.17)</td>
</tr>
<tr>
<td><strong>VT</strong></td>
<td>7.17 (1.69)</td>
<td>7.81 (1.69)</td>
<td>7.94 (1.54)</td>
<td>7.94 (1.54)</td>
<td>7.66 (1.54)</td>
<td>7.94 (1.54)</td>
<td>7.94 (1.54)</td>
<td>7.94 (1.54)</td>
</tr>
<tr>
<td><strong>VE</strong></td>
<td>17.21 (8.62)</td>
<td>15.19 (7.79)</td>
<td>18.36 (8.25)</td>
<td>17.64 (7.67)</td>
<td>18.36 (8.25)</td>
<td>17.64 (7.67)</td>
<td>18.36 (8.25)</td>
<td>17.64 (7.67)</td>
</tr>
<tr>
<td><strong>VE</strong></td>
<td>8.21 (1.82)</td>
<td>7.81 (1.69)</td>
<td>8.32 (1.80)</td>
<td>8.32 (1.80)</td>
<td>8.21 (1.82)</td>
<td>7.81 (1.69)</td>
<td>8.32 (1.80)</td>
<td>8.32 (1.80)</td>
</tr>
<tr>
<td><strong>VTOT</strong></td>
<td>0.54 (0.30)</td>
<td>0.54 (0.30)</td>
<td>0.50 (0.26)</td>
<td>0.50 (0.26)</td>
<td>0.54 (0.30)</td>
<td>0.54 (0.30)</td>
<td>0.50 (0.26)</td>
<td>0.50 (0.26)</td>
</tr>
<tr>
<td><strong>RTOT</strong></td>
<td>14.02 (11.75)</td>
<td>14.02 (11.75)</td>
<td>13.94 (11.75)</td>
<td>13.94 (11.75)</td>
<td>13.94 (11.75)</td>
<td>13.94 (11.75)</td>
<td>13.94 (11.75)</td>
<td>13.94 (11.75)</td>
</tr>
</tbody>
</table>

Statistical analyses: *, †, ‡: significantly different from baseline, from pre-hyperoxia 1 and from pre-hyperoxia 2, respectively.
Table 2: Body temperature during hyperoxic tests in 2-day-old Phox2b+/+ (N=11) and Phox2b+-/ (N=10) pups. Hyperoxia did not induce significant temperature changes. Genotype-related differences were not significant. Values are means (SD).

<table>
<thead>
<tr>
<th>Body temperature (°C)</th>
<th>Test 1</th>
<th>Test 2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pre-O2</td>
<td>O2</td>
</tr>
<tr>
<td>Phox2b+/+</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>31.1 (0.6)</td>
<td>31.0 (0.8)</td>
</tr>
<tr>
<td>Phox2b+-/</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>30.9 (0.5)</td>
<td>30.8 (0.5)</td>
</tr>
</tbody>
</table>
Figure 1

**Phox2b+/+**

**Phox2b+-/-**
Figure 2

A) 

B) 

C) 

D) 

E) 

F)
Figure 3