Effects of temperature on ventilatory response to hypercapnia in newborn mice heterozygous for transcription factor Phox2b

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First published August 22, 2007; doi:10.1152/ajpregu.00349.2007.—Congenital central hypoventilation syndrome (CCHS) is a rare disease with variable severity, generally present from birth and chiefly characterized by impaired chemosensitivity to hypercapnia. The main cause of CCHS is a mutation in the PHOX2B gene, which encodes a transcription factor involved in the development of autonomic medullary reflex pathways. Temperature regulation is abnormal in many patients with CCHS. Here, we examined whether ambient temperature influenced CO2 sensitivity in a mouse model of CCHS. A weak response to CO2 at thermoneutrality (32°C) was noted previously in 2-day-old mice with an invalidated Phox2b allele (Phox2b+/−), compared with wild-type litters. We exposed Phox2b+/− pups to 8% CO2 at three ambient temperatures (TAs): 29°C, 32°C, and 35°C. We measured breathing variables and heart rate (HR) noninvasively using a novel whole body flow plethysmograph equipped with contact electrodes. Body temperature and baseline breathing increased similarly with TA in mutant and wild-type pups. The hypercapnic ventilatory response increased linearly with TA in both groups, while remaining smaller in mutant than in wild-type pups at all TAs. The differences between the absolute increases in ventilation in mutant and wild-type pups become more pronounced as temperature increased above 29°C. The ventilatory abnormalities in mutant pups were not associated with significant impairments of heart rate control. In both mutant and wild-type pups, baseline HR increased with TA. In conclusion, TA strongly influenced the hypercapnic ventilatory response in Phox2b+/− mutant mice. These findings suggest that abnormal temperature regulation may contribute to the severity of respiratory impairments in CCHS patients.

CENTRAL CONGENITAL HYPOVENTILATION syndrome (CCHS or Ondine’s curse) is a rare disease, generally present from birth and characterized by hypventilation during sleep with apneas and cyanotic episodes of variable severity, in the absence of primary neuromuscular or lung disease (1). Throughout life, patients with CCHS have absent or markedly reduced ventilatory responses to hypercapnia (33). All patients require lifelong nocturnal mechanical ventilation. The ventilatory deficit is generally ascribed to impaired integration of chemosensory inputs at the brain stem level (18, 23, 42). Over 90% of patients with CCHS have a heterozygous Phox2b gene mutation consisting in a polyalanine-repeat expansion (2, 27, 49, 50). PHOX2B is a master regulator of the noradrenergic phenotype and of all neuronal relays of autonomic medullary reflex pathways (34, 45).

In addition to chemosensitivity disorders, abnormal temperature regulation occurs in CCHS (47). Sporadic profuse sweating with cool extremities or absence of fever during infections is common (47). Poor tolerance of even moderate increases in ambient temperature (TA) by children with CCHS may require daytime ventilatory support (21). The anterior hypothalamus, which is the main integration center for thermoregulation (9, 10, 51) and exerts direct control over respiratory brain stem structures (29), does not express PHOX2B (12). However, morphological defects of the anterior hypothalamus were documented recently in patients with CCHS (21). Taken together, these data suggest that TA may influence chemosensitivity disorders in patients with CCHS and may explain, at least in part, their considerable intraindividual and interindividual variability. This clinically important issue had not been examined previously.

The aim of this study was to investigate the effects of TA on CO2 sensitivity in a mouse model of CCHS. Newborn mice heterozygous for Phox2b invalidation (Phox2b+/−) exhibit two main features of CCHS at thermoneutrality (32°C): longer sleep-apnea times (11) and weaker responses to CO2, compared with wild-type littermates (12). To examine the effects of TA on these respiratory abnormalities, we measured ventilatory responses to hypercapnia in unrestrained 2-day-old Phox2b+/− mice at three TAs (thermoneutrality and 3°C in each direction). We anticipated that increasing TA would potentiate the hypercapnic ventilatory response in wild-type Phox2b+/+ pups, as previously described in newborns of other mammalian species (29, 40). We made no hypothesis regarding the effects of TA in Phox2b+/− pups. The effects of TA on breathing in mutant and wild-type pups were interpreted in the light of concomitant effects on body temperature, heart rate (HR), and movement.

METHODS

Animals. Phox2b+/− mutant mice were produced and phenotyped, as described previously (13). Pups were tested at 2 days of postnatal age (P2, day of birth: P0). In a preliminary experiment (study 1), we measured body temperature at different TAs in restrained pups. We used 21 heterozygous mutant pups (Phox2b+/−) and 43 wild-type littermates (Phox2b+/+) obtained by mating 12 wild-type females...
with mutant Phox2b+/− males. In the main experiment (study 2), we studied the effects of TA on the hypercapnic ventilatory response. We used 101 Phox2b+/− and 100 Phox2b+/+ pups obtained by mating 31 wild-type females with mutant males. In both studies, each pup was randomly extracted from the litter and assigned to a TA group (29°C, 32°C, or 35°C) without previous knowledge of phenotype.

**Body temperature.** In both study 1 and study 2, after extracting the pup from the litter, interscapular skin temperature was measured immediately by placing a thermocouple probe on the skin at the level of the interscapular region. This region contains brown adipose tissue, the main heat-producing organ in mouse pups, and is the site where skin temperature is highest (6). In study 1, body temperature during normoxia and hypercapnia in the plethysmograph was continuously monitored at each TA using a previously described method (35). Briefly, each pup was lightly anesthetized with isoflurane using a method that is followed by recovery in 10 min in mouse pups (14). A thermocouple probe was positioned through a 2- to 3-mm incision in the interscapular region. The interscapular region contains brown adipose tissue, the main heat-producing organ in mouse pups, and is the site where skin temperature is highest (6). Interscapular probes were preferred over rectal probes, which are poorly tolerated in newborn mice during prolonged recordings (36 min). Previous studies in newborn rats showed that colonic and interscapular temperatures were closely correlated over TAs ranging from 22°C to 37°C (43). Each pup was placed in a restraining device in the plethysmograph to ensure that the temperature probe position remained unchanged. Temperature measurements were initiated 10 min after anesthesia. Because the restraining device disrupted breathing measurements, breathing variables were not investigated in study 1. In study 2, body temperature was not continuously monitored during ventilatory measurements, as this would have required restraining the pups. We measured interscapular skin temperature immediately after extraction from the litter, using a thermocouple probe.

**Whole body flow plethysmography.** Breathing variables were measured noninvasively using whole body flow barometric plethysmography, as previously described (11, 28, 35) (Fig. 1A). The plethysmograph was composed of two Plexiglas cylinders serving as the animal (40 ml) and reference (70 ml) chambers, respectively, immersed in a thermostatically regulated water bath set that maintained TA at 29°C, 32°C, or 35°C. A 100-ml/min flow of dry air (Bronkhorst Hi-Tec airflow stabilizer, Urlo, Holland) was divided into two 50 ml/min flows through the chambers to avoid CO2 accumulation and water vapor condensation. Hypercapnia was achieved by switching the airflow through the plethysmograph to a hypercapnic mixture (8% CO2, 21% O2, and 71% N2) at the same flow rate. The time needed to flush the chamber was about 1 min. The pressure difference 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 (18 bits, PCI 6284, National Instruments, Austin, TX) at a sampling rate of 100 Hz, and processed using custom-written software (Labview, National Instruments, Austin, TX). The time constant of the pressure decay within the system (2 s) 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 microsyringe (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. Tidal volume (VT) was calculated using Drorbaugh and Fenn’s equation (15). In study 2, the alveolar temperatures used in this equation were the mean interscapular temperatures over time obtained with each TA in study 1. Because of the limitations of flow barometric plethysmography (44), especially in newborn mice (28), the absolute values of VT and minute ventilation (Ve) reported here are indicative only, whereas breath duration (T(res)), absolute values, and apnea durations are reliable.

**Design.** Each pup was randomly assigned to a TA group and tested once on P2 to avoid the effects of repeated tests. Study 1 and study 2 had the same design (Fig. 1B). In study 1, body temperature was continuously monitored in restrained pups after 10 min of familiarization to allow recovery from anesthesia; breathing variables were not measured. In study 2, breathing variables were continuously monitored in unrestrained pups after 2 min of familiarization; body temperature was not measured. Baseline breathing variables were measured for 12 min. Then, each pup was exposed to 6 min of hypercapnia followed by 6 min of normocapnia, and then the same sequence again (for 36 consecutive minutes in all). We performed the sequence twice to increase statistical power.

**Breathing variables.** Breathing variables were measured without previous knowledge of genotypes. TTOT (in seconds), respiratory rate (RR; breaths/min), VT (μl/g BTPS), and Ve (calculated as VT/TTOT and expressed in μl·s−1·g−1 BTPS) were calculated on apnea-free periods (see apnea determination below). Breathing variables were averaged over consecutive 30-s periods. The baseline normocapnic levels for these variables were calculated as the mean values over the 12 min of air-breathing before the first hypercapnic exposure. The ventilatory response to hypercapnia was assessed by the Ve increase

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**Figure 1.** A: simultaneous recording of breathing and ECG in unrestrained 2-day-old mice. A recording platform containing four identical rectangular gold electrodes was embedded into the floor of the flow-through barometric plethysmograph chamber. P, pressure. B: examples of respiratory and ECG recordings in a Phox2b+/− pup and a wild-type littermate at 32°C. Baseline breathing and heart rate were not significantly different between the two pups. C: experimental design. Each pup underwent one experimental session. The recording began after at least 2 min of familiarization with the apparatus.
from normocapnia to hypercapnia, designated ΔVE hereafter. Normocapnic V˙E levels were calculated as the mean values over the 5 min preceding the hypercapnic exposure (the longest baseline duration available in both tests; we discarded the 1-min transition from CO2 to air after the first test). Hypercapnic V˙E values were calculated as the mean values over the last 4 min of hypercapnic exposure (we discarded the 1-min transition from air to CO2). We used the same approach to compute ΔTTOT and ΔVT.

Apneas were determined using an automatic classification method based on spectral analysis (28). Apneas were defined as ventilatory pauses longer than twice the duration of the preceding breath (36, 41). This definition takes into account interindividual differences in resting breathing frequency in newborn mice. Movement artifacts (MVT) were detected based on changes in the baseline respiratory signal, using a previously validated criterion: (Vi−V˙e)/(Vi+V˙e), where Vi and Ve, were the magnitudes of the inspiratory and expiratory limbs of the volume signal, respectively (28). Movement durations were summed over successive 30-s periods. The movement response to hypercapnia (ΔMVT) was calculated as the difference: MVTCO2 − MVTair, where MVT0 and MVTair were the total movement durations during hypercapnia (last 4 min of CO2 exposure) and air (last 4 min preceding CO2 exposure), respectively.

Heart rate. Noninvasive ECG recordings were obtained in the measurement chamber of the plethysmograph, simultaneously with breathing variables, for the first time in unrestrained rodent pups (Fig. 1A). A recording platform composed of four identical rectangular gold electrodes (15 mm by 3 mm) located 1 mm apart and insulated from one another was embedded in the floor of the measurement chamber. Conduction was enhanced using electrode gel (Sigma gel; Parker Laboratories, Fairfield, NJ). The electrodes were connected by a shielded lead wire through the wall of the chamber to a built-in differential amplifier circuit powered by a 3.6 V lithium battery (overall gain: 5000; bandwidth at -3 dB: 0.5–800 Hz; input impedance: 100–250 KOhms; power consumption <1 mA). The signals were digitized at a sample rate of 1,000 Hz (18 bits, PCI 6284, National Instruments, Austin, TX). An ECG signal was obtained when the pup lay on the floor over three electrodes. HR was determined from the R-R wave peaks after visual selection of continuous, 20-s or longer ECG segments with clearly defined QRS waves. HR values were calculated over normocapnic and hypercapnic periods at each TA.

Statistics. ΔVE, ΔTTOT, and ΔVT responses were evaluated using repeated-measures ANOVA with genotype (mutant Phox2b+/− vs. wild-type Phox2b+/+) and TA (29°C, 32°C, or 35°C) as the between-subject factors and test number (two levels) as a repeated factor. Statview 5 and Superanova Software (SAS, Cary, NC) were used to perform the statistical tests.

RESULTS

Body temperature. Neither body weight nor interscapular skin temperature, measured immediately after extraction from the litter in studies 1 and 2, differed significantly between the two genotypes (Table 1). Body temperatures remained closely similar in both genotype groups throughout breathing-variable recordings (Fig. 2). In both groups, and at all TAs, interscapular temperature increased with TA and showed no influence from hypercapnia (Fig. 2).

Baseline normocapnic breathing. Large proportions of the 36-min recordings were free of movement artifacts in both the mutant and the wild-type pups at all TAs (Table 2). Movement durations during normocapnia were closely similar in the two groups and were not affected by TA (hypercapnia-induced movements are analyzed below).

Baseline V˙E levels were not significantly different in mutant and wild-type pups at any of the three TAs (Table 2). Baseline V˙E increased with TA in both groups (main effect for TA, P = 0.024, genotype-by-TA interaction: not significant; Table 2) due to smaller TTOT values (P < 0.0001, Table 2). The effects of TA on V˙E were not significant.

Total apnea durations under baseline normocapnic conditions were not significantly different in mutant and wild-type pups

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**Table 1. Body weight and temperature in 2-day-old Phox2b+/− pups and wild-type littermates**

<table>
<thead>
<tr>
<th>Study</th>
<th>Groups</th>
<th>Number</th>
<th>Body Weight, g</th>
<th>Body Temperature, °C</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>29°C</td>
<td>6</td>
<td>1.57 (0.30)</td>
<td>33.7 (0.8)</td>
</tr>
<tr>
<td>2</td>
<td>29°C</td>
<td>36</td>
<td>1.66 (0.34)</td>
<td>34.3 (1.0)</td>
</tr>
<tr>
<td></td>
<td>35°C</td>
<td>9</td>
<td>1.75 (0.30)</td>
<td>33.6 (1.2)</td>
</tr>
<tr>
<td>3</td>
<td>32°C</td>
<td>36</td>
<td>1.90 (0.30)</td>
<td>34.0 (1.5)</td>
</tr>
<tr>
<td></td>
<td>35°C</td>
<td>11</td>
<td>1.77 (0.24)</td>
<td>34.3 (0.8)</td>
</tr>
</tbody>
</table>

Values are group means (SD). Weight and interscapular skin temperature were measured immediately after taking the pup from the litter. Genotype-related differences and temperature group-related differences were not significant.

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**Fig. 2. Interscapular temperature in 2-day-old Phox2b+/− pups (solid circles) and wild-type littermates (open circles) exposed to two hypercapnic (8% CO2) challenges (shaded areas) at 29°C, 32°C, and 35°C. Values are group means (error bars show means ± SE). Genotype effects are not significant as a main effect or via interaction with other factors.**
pups (Table 2). Apnea durations tended to decrease with increasing TA in both groups (main effect for TA: \( P < 0.041 \); genotype-by-TA interaction: not significant). At all TAs, apnea duration correlated negatively with HR, in both genotypes (correlation coefficients (\( R \)) at 29°C: \( R = 0.57, P = 0.027 \); 32°C: \( R = 0.62, P = 0.003 \); 35°C: \( R = 0.48, P = 0.020 \); data from mutant and wild-type pups were pooled for these calculations). Thus, the Phox2b mutation did not significantly affect the baseline breathing pattern or HR at any of the three TAs.

**Ventilatory responses to hypercapnia.** Mutant and wild-type pups responded to hypercapnia by increasing \( V \dot{E} \), chiefly via an increase in \( V_T \) (Figs. 3 and 4). \( V \dot{E} \) tended to be larger in test 2 (main effect for test, \( P < 0.006 \); test-by-genotype and test-by-TA interactions: not significant). The \( V \dot{E} \) response to CO2 was significantly smaller in the mutant pups at all TAs (main effect for genotype on \( V \dot{E} \), \( P = 0.0001 \); partial comparisons shown in Fig. 4B). In both genotype groups, \( V \dot{E} \) increased linearly with TA (main effect for TA: \( P < 0.0001 \) within each group and correlation analyses, not shown), revealing potentiation of the hypercapnic ventilatory response by TA. This potentiation of \( V \dot{E} \) by TA was similar in mutant and wild-type pups. For example, when TA increased from 29°C to 35°C, mean \( V \dot{E} \) for the two tests increased by 213% in mutants compared with 205% in wild-type pups. Importantly, the absolute \( V \dot{E} \) response deficit in mutants, compared with wild-type pups, increased with TA (genotype-by-TA interaction: \( P = 0.040 \); and partial comparisons shown in Fig. 4B).

TA had opposite effects on the \( T_{TOT} \) and VT responses to hypercapnia (\( \Delta T_{TOT} \) and \( \Delta V_T \), Table 3). On the one hand, the \( \Delta T_{TOT} \) difference between mutant and wild-type pups was significant at 29°C but not at 32°C or 35°C (genotype-by-TA interaction: not significant). In contrast, the absolute \( V_T \) deficit in mutants, compared with wild-type pups, increased with TA (genotype-by-TA interaction: \( P = 0.004 \); and partial comparisons shown in Fig. 4B). Furthermore, this effect of temperature was confirmed by the time course of the percent \( V \dot{E} \) response to hypercapnia in mutant and wild types (The online version of this article contains a supplemental Fig. 1).

### Table 2. Baseline breathing during normocapnia in 2-day-old Phox2b+-/- and wild-type littermates at three ambient temperatures

<table>
<thead>
<tr>
<th>Ambient Temperature, °C</th>
<th>29</th>
<th>32</th>
<th>35</th>
</tr>
</thead>
<tbody>
<tr>
<td>Percent Signal</td>
<td>74 (8)</td>
<td>76 (8)</td>
<td>74 (8)</td>
</tr>
<tr>
<td>Apneas, s</td>
<td>100.05 (92.21)</td>
<td>97.85 (24.80)</td>
<td>97.85 (24.80)</td>
</tr>
<tr>
<td>( T \text{TOT}, ) s</td>
<td>0.65 (0.14)</td>
<td>0.63 (0.16)</td>
<td>0.63 (0.16)</td>
</tr>
<tr>
<td>RR, breaths/min</td>
<td>97.85 (24.80)</td>
<td>103.18 (33.16)</td>
<td>101.74 (23.24)</td>
</tr>
<tr>
<td>( V_T, ) μl/g</td>
<td>5.70 (1.47)</td>
<td>5.80 (1.29)</td>
<td>6.00 (1.71)</td>
</tr>
<tr>
<td>( V \dot{E}, ) μl·g⁻¹·s⁻¹</td>
<td>9.67 (4.18)</td>
<td>10.15 (3.82)</td>
<td>10.80 (5.50)</td>
</tr>
</tbody>
</table>

Values are group means (SD). Data from study 2. The durations of free-from-artifacts signal expressed as the percentage of the total ventilatory recording (%signal), calculated over the entire 36 min, were not significantly different in mutants and wild-type pups. Baseline breathing variables were averaged over the 12-min period preceding the first hypercapnic test. Total apnea duration was also calculated over this 12-min period. Genotype-related differences were not significant. RR, respiratory rate; \( T \text{TOT} \), breath duration; \( V_T \), tidal volume; \( V \dot{E} \), minute ventilation.

![Fig. 3. Examples of respiratory recordings in 2-day-old Phox2b+-/- pups and wild-type littermates at three ambient temperatures (TAs). At all TAs, hypercapnia caused smaller increases in breathing rate and amplitude in mutants than in wild-type pups.](http://ajpregu.physiology.org/)

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interaction for $\Delta T_{TOT}; P < 0.0228$; partial comparisons between mutant and wild-type pups on average on the two tests: 29°C; $P < 0.007$; 32°C and 35°C; not significant; Table 3; test effects on $\Delta T_{TOT}$ were not significant). On the other hand, a significant genotype-related difference in $\Delta V_T$ emerged at 32°C and 35°C (genotype-by-TA interaction for $\Delta V_T; P < 0.099$; and partial comparisons between mutants and wild-type pups on average on the two tests: at 29°C: NS; at 32°C: $P < 0.003$; and at 35°C: $P < 0.001$; Table 3; test effects on $\Delta V_T$ were not significant). Thus, with increasing TA, the $T_{TOT}$ response deficit in mutant pups diminished, whereas the $V_T$ response deficit increased significantly. Because the effects of TA predominated on the $V_T$ response, the overall effect of increasing TA was an increase in the $V_e$ deficit in mutant pups.

Movements. The motor responses to hypercapnia decreased with increasing TA in both mutant and wild-type pups (main effect for TA: $P < 0.0001$; genotype-by-TA interaction: not significant). Test had no significant effects on the motor response to hypercapnia. On average, for the two tests, in both mutant and wild-type pups, hypercapnia increased the total movement duration at 29°C ($P < 0.0001$), whereas it had no effect at 32°C, and it decreased movement duration at 35°C ($P < 0.0001$, Table 3). The total duration of movements was closely similar in mutant and wild-type pups at all TAs (Tables 2 and 3), suggesting similar durations of wakeful periods.

Heart rate. HR values were obtained for at least one of the two tests at all three TAs in a subsample of mutant and wild-type pups (Table 4). In normocapnia, HR increased with TA in both groups (main effect for TA: $P < 0.0001$; genotype-by-TA: not significant; Table 4). In contrast with $\Delta V_e$, the HR response to hypercapnia ($\Delta HR$) displayed a quadratic trend, with higher values at 29°C and 35°C than at 32°C (main effect for TA: $P < 0.010$; quadratic contrast for TA: $P < 0.007$; and 32°C vs. 35°C: $P < 0.003$; $\Delta HR$ between 29°C and 32°C was not statistically significant, Table 4). Differences between mutant and wild-type pups were not significant. Thus, the ventilatory abnormalities in mutant pups were not associated with significant impairments of heart rate control.

**DISCUSSION**

In this study, we showed that the hypercapnic ventilatory response was smaller in mutant Phox2b+/− pups than in their wild-type littermates at all studied TAs. The ventilatory response to hypercapnia was potentiated by TA in both mutant and wild-type pups. The difference in the absolute increases in ventilation between mutant and wild-type pups became more pronounced above 29°C. Body temperature and baseline breathing increases with TA were similar in mutant and wild-type pups.

**Limitations of plethysmographic measurements.** Whole body plethysmography is the only available method for measuring breathing in unrestrained newborn mice (30). We assumed that the physical principle underlying whole body plethysmography (15, 17) (pressure changes in the measurement chamber are due to heating and humidification of inspired gas) extended to newborn mice. Whereas heating probably had limited effects at TAs close to body temperature, the humidification of inspired gas caused pressure changes, because the flow through the chamber was dry air at all TAs. However, the present absolute values of VT and $V_e$ derived from Drorbaugh and Fenn’s equation (15, 17) should be considered with caution. These values have not been validated against a reference method, due to the lack of miniaturized reference devices (pneumotachographs or spirometers). The main sources of error lie in two parameters of this equation, namely, alveolar temperature and humidity, which are used in computing the calibration constant but cannot be directly measured (17). Thus, the absolute values of $V_T$ (and therefore $V_e$) derived from Drorbaugh and Fenn’s equation may be biased by systematic errors induced by these parameters. However, these systematic errors in absolute values of $V_T$ (and thus $V_e$) did not invalidate group comparisons between mutant and wild-type pups, which constitute the core of the study, given that mutant and wild-type pups had closely similar body temperatures. Moreover, the relative changes in $V_e$, expressed as percent changes from baseline, are devoid of most of the systematic
Table 3: Ventilatory responses to hypercapnia in 2-day-old Phox2b+/− pups and wild-type littermates at three ambient temperatures

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Phox2b+/−</th>
<th>Wild-Type</th>
<th>Phox2b+/−</th>
<th>Wild-Type</th>
<th>Phox2b+/−</th>
<th>Wild-Type</th>
</tr>
</thead>
<tbody>
<tr>
<td>29°C</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Test 1</td>
<td>20.7 (28.3)</td>
<td>13.5 (19.2)</td>
<td>14.2 (14.4)</td>
<td>14.0 (19.0)</td>
<td>10.3 (13.0)</td>
<td>13.3 (7.0)</td>
</tr>
<tr>
<td>Test 2</td>
<td>20.7 (28.3)</td>
<td>13.5 (19.2)</td>
<td>14.2 (14.4)</td>
<td>14.0 (19.0)</td>
<td>10.3 (13.0)</td>
<td>13.3 (7.0)</td>
</tr>
<tr>
<td>32°C</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Test 1</td>
<td>20.7 (28.3)</td>
<td>13.5 (19.2)</td>
<td>14.2 (14.4)</td>
<td>14.0 (19.0)</td>
<td>10.3 (13.0)</td>
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<tr>
<td>Test 2</td>
<td>20.7 (28.3)</td>
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<td>14.2 (14.4)</td>
<td>14.0 (19.0)</td>
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<td>35°C</td>
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<tr>
<td>Test 1</td>
<td>20.7 (28.3)</td>
<td>13.5 (19.2)</td>
<td>14.2 (14.4)</td>
<td>14.0 (19.0)</td>
<td>10.3 (13.0)</td>
<td>13.3 (7.0)</td>
</tr>
<tr>
<td>Test 2</td>
<td>20.7 (28.3)</td>
<td>13.5 (19.2)</td>
<td>14.2 (14.4)</td>
<td>14.0 (19.0)</td>
<td>10.3 (13.0)</td>
<td>13.3 (7.0)</td>
</tr>
</tbody>
</table>

All responses are expressed as absolute differences from baseline levels (see text). Test had no significant effects, except for total movement duration (over 30-s periods, movement artifacts) which was significantly shorter in test 2 (p < 0.001).

Errors due to calibration inaccuracy. In the present study, the genotype-related differences noted for absolute V̇E values were confirmed by relative V̇E values.

**Body temperatures.** Our results suggest normal body temperature control in mutant pups. Body temperatures were closely similar in mutant and wild-type pups, whether measured by subcutaneous probes during normocapnia and hypercapnia at various TAs (study 1) or measured at the skin surface immediately after extraction from the litter (study 2). In both genotype groups, and at all TAs, interscapular temperature increased with TA and showed no influence from hypercapnia. The pups’ body temperatures fell rapidly after extraction from the litter, exposure to the ambient temperature in the laboratory, and handling for measurement of temperatures and weights, as previously reported in newborn mice (20). Once the pups were placed in the plethysmograph, their body temperature increased gradually toward TA. At 29°C, baseline body temperature was never reached, due to the limited ability of newborn mice to respond to cold by increasing heat production (48). The relatively long recording duration (40 min) was not sufficient for body temperature to stabilize. This duration was not extended to avoid problems related to prolonged separation from the mothers. In both groups, interscapular temperature tended toward a plateau that was slightly higher than TA, in agreement with data obtained in 1- to 2-day-old rat pups (22). Body temperature was not influenced by hypercapnia, as previously reported in newborn mice and rats (26, 32, 40). Thus, our results do not support the possibility that the Phox2b mutation affects temperature control, at least within TA range used in our study.

**Heart rates.** HR measurement in freely moving mouse pups, with concomitant measurements of breathing variables, is a novel technique that requires further development to increase the amount of collected data. This might be achieved by increasing the number of contact electrodes embedded in the plethysmograph-chamber floor. With the current device, we obtained a limited amount of ECG data, which precluded evaluation of HR variability [a variable of considerable relevance in CCHS (46)]. Despite these limitations, we found that HR increased with increasing TA in both mutant and wild-type pups, as previously reported in 2-day-old rats exposed to increasing TA from 21°C to 36°C (7). There were no significant genotype-related differences. HRs during specific respiratory events could not be analyzed. However, the overall negative correlation between apnea duration and HR may denote bradycardia, which is known to occur during severe apneas in preterm infants (25).

**TA effects on baseline breathing.** Normocapnic V̇E levels were closely similar in mutant and wild-type pups. In both genotype groups, increasing TA led to decreases in ṪTOT (i.e., increased breathing rate) and increases in V̇L (that should be interpreted with caution as indicated above). The effects of TA on breathing rate confirmed the major role for breathing rate in thermoregulation, as reported in other newborn mammals (31). Also, the V̇E increase caused by TA elevation was associated with a decrease in total apnea duration. In human near-term infants, apneas occur more frequently at higher TA values (3, 4). However, this effect was not seen in more immature neonates (8, 39). Our study was conducted in 2-day-old mice, whose brain development corresponds roughly to 25- to 30-wk gestational age in humans (24). Thus, the decrease in apneas...
with increasing TA observed here may reflect processes occurring in very immature neonates.

**TA effects on the hypercapnic ventilatory response.** Mutant and wild-type pups increased their \( V_T \) in response to hypercapnia chiefly by increasing \( V_T \), as previously reported in newborn mice (38). Newborns of larger mammalian species, which do not pose the technical difficulties for \( V_T \) measurement by whole body plethysmography seen with mouse pups, also showed this \( V_T \)-based hypercapnic response (32, 38). \( V_T \) responses to hypercapnia were smaller in mutants than in wild-type littersates at all TAs, that is, beyond the thermoneutral range. Of note, mutant and wild-type pups displayed highly similar movement durations at all TAs, which probably reflected wakefulness periods. Therefore, it is unlikely that the smaller \( V_T \) response to hypercapnia in mutant pups was secondary to differences in wakefulness duration during plethysmographic recordings. These findings extend our previous results obtained at 32°C (12). In both genotype groups, the TA increase produced an increase in the \( V_T \) response to hypercapnia. This potentiation of the hypercapnic response by higher TA, which has not been previously described in newborn mice, extends results obtained in newborns of various mammalian species (29, 40).

Importantly, elevating TA above 29°C amplified the absolute \( V_T \) difference between mutant and wild-type pups. Whereas the deficit of the \( V_T \) response to hypercapnia was small at 29°C, it was considerably larger at 32°C and 35°C. The strong effect of TA on the hypercapnic response in newborn mice suggests that higher TAs may amplify relatively small genotype-related differences observed at lower TAs. Of note, this effect was mainly accounted for by the TA effects on the VT response to hypercapnia.

**Putative mechanisms.** The impaired ventilatory response to hypercapnia in Phox2b +/- mice at all TAs constitutes functional evidence that Phox2b controls neuronal circuits involved in chemosensitivity. Phox2b is expressed by chemosensitive neurons located in the retrotrapezoid nucleus and the area postrema (45). Furthermore, the carotid body (which contains carbon dioxide sensors in addition to oxygen sensors), the petrosal ganglion (which innervates the carotid body), and the NTS (which integrates chemosensory stimuli) also depend on Phox2b for their development (12).

Both mutant pups and wild-type pups at 2 days of age had long apnea durations under basal conditions, without significant genotype-related differences. Ventilatory instability is a known characteristic of breathing control in newborn mammals that is usually ascribed to immaturity of the neural circuitry subserving respiratory rhythmogenesis and peripheral and central chemosensitivity. In a previous study in 5-day-old Phox2b +/- pups, we found that this apneic pattern was practically absent in wild-type pups but was prominent in mutant pups (16). Between 2 and 5 days of age, impairments of CO₂ sensitivity tended to resolve (12), suggesting that mutant pups had impaired development of respiratory rhythm generation. The main respiratory rhythm generator, namely, the pre-Bötzinger complex (preBoTC) (37), does not express Phox2b (5). However, Phox2b is present in a recently identified respiratory rhythm generator, the retrotrapezoid nucleus/parafacial group (20, 45). Therefore, Phox2b mutations may impair respiratory rhythm generation. The time course of this possible developmental defect in respiratory rhythm generation cannot be determined from the present data. In contrast, the rostral hypothalamus, which is the primary integration center for thermoregulation (9, 10, 51) and directly controls the respiratory brain stem structures (29), does not express Phox2b (12). This pattern of Phox2b expression is consistent with our results, suggesting relative sparing of thermoregulation in mutant pups having impaired CO₂ sensitivity.

**Implications for CCHS.** The respiratory abnormalities in Phox2b +/- mice are milder than the manifestations of CCHS, and they are short-lived. Furthermore, we showed here that Phox2b +/- pups had apparently normal thermoregulation, in contrast with the thermoregulation disorders frequently encountered in patients with CCHS. This difference is probably due to the fact that in contrast to the Phox2b-targeted mutation in the mouse model investigated here (a null mutation), the alanine expansions generally found in the PHOX2B genes of CCHS patients exert a toxic gain of function or dominant-negative effect. Despite this limitation, phenotypic studies of Phox2b +/- mice shed light on the role for Phox2b in the development of autonomic reflexes at the level of the organism. We found that a Phox2B mutation impaired the hypercapnic ventilatory response beyond the thermoneutral range. Furthermore, TA influenced this response and amplified the genotype-related differences. Abnormal temperature control is common in children with CCHS (47). Parents often report poor tolerance to increases in ambient temperature (21), and many patients exhibit episodes of profuse sweating and cool extremities (43%) or absence of fever during infections (22%). Episodes of intermittent hypothermia occurred shortly after birth in a full-term infant with CCHS (19). However, these symptoms are often associated with a broad spectrum of manifestations attributable to dysfunction of the

### Table 4. Heart rate responses to hypercapnia in 2-day-old Phox2b +/- pups and wild-type littermates at three ambient temperatures

<table>
<thead>
<tr>
<th>Ambient Temperature, °C</th>
<th>29</th>
<th>32</th>
<th>35</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Phox2b +/-</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline HR, bpm</td>
<td>336 (53)</td>
<td>325 (70)</td>
<td>420 (54)</td>
</tr>
<tr>
<td>ΔHR, bpm</td>
<td>25 (28)</td>
<td>19 (34)</td>
<td>-3 (21)</td>
</tr>
<tr>
<td><strong>Wild-Type</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline HR, bpm</td>
<td>325 (70)</td>
<td>419 (64)</td>
<td>447 (59)</td>
</tr>
<tr>
<td>ΔHR, bpm</td>
<td>19 (34)</td>
<td>10 (25)</td>
<td>48 (44)</td>
</tr>
</tbody>
</table>

Values are group means (SD). HR, heart rate (study 2). Baseline normocapnic values (mean values over the 3-min period preceding each hypercapnic stimulus) increased significantly with ambient temperature (TA). ΔHR, difference between mean HR during the last 3 min of hypercapnia (8% CO₂) and baseline. Temperature effects on ΔHR were not significant.

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autonomic nervous system, which may confound the effects of temperature. In our study, the depression of the hypercapnic response at 29°C, which is cold for newborn mice, suggests that a cold ambient temperature may be particularly harmful in infants with CCHS.

**Conclusion.** At all three TAs investigated in our study, mutant pups showed smaller hypercapnic ventilatory responses than their wild-type littermates. The interaction between TA and the hypercapnic ventilatory response suggests that abnormal temperature control may further impair respiratory control and contribute to the high inter- and intra-individual variability of the respiratory phenotype in patients with CCHS.

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