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Breathing without CO_2 Chemosensitivity in Conditional *Phox2b* Mutants

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Breathing is a spontaneous, rhythmic motor behavior critical for maintaining O_2, CO_2, and pH homeostasis. In mammals, it is generated by a neuronal network in the lower brainstem, the respiratory rhythm generator (Feldman et al., 2003). A century-old tenet in respiratory physiology posits that the respiratory chemoreflex, the stimulation of breathing by an increase in partial pressure of CO_2 in the blood, is indispensable for rhythmic breathing. Here we have revisited this postulate with the help of mouse genetics. We have engineered a conditional mouse mutant in which the toxic *PHOX2B*^{27Ala} mutation that causes congenital central hypoventilation syndrome in man is targeted to the retrotrapezoid nucleus, a site essential for central chemosensitivity. The mutants lack a retrotrapezoid nucleus and their breathing is not stimulated by elevated CO_2 at least up to postnatal day 9 and they barely respond as juveniles, but nevertheless survive, breathe normally beyond the first days after birth, and maintain blood PCO_2 within the normal range. Input from peripheral chemoreceptors that sense PO_2 in the blood appears to compensate for the missing CO_2 response since silencing them by high O_2 abolishes rhythmic breathing. CO_2 chemosensitivity partially recovered in adulthood. Hence, during the early life of rodents, the excitatory input normally afforded by elevated CO_2 is dispensable for life-sustaining breathing and maintaining CO_2 homeostasis in the blood.

Introduction

Ever since Haldane and Priestly wrote a century ago that "the regulation of the rate of alveolar ventilation depends under normal conditions exclusively on the CO_2 pressure in the respiratory center" (Haldane and Priestley, 1905), the stimulation of breathing by CO_2 has been considered as an indispensable drive to breathe. Cells that sense CO_2 (PCO_2) and O_2 (PO_2) partial pressure in the blood and interstitial fluid provide excitatory input to the respiratory rhythm generator (RRG) (Nattie, 1999). Elevated PCO_2, through the attendant decrease in pH, is thought to be sensed principally by chemosensors in the brain while the carotid bodies (CBs) in the periphery are the main sensors of PO_2 and provide a substantial fraction of the CO_2 response in some species (Forster et al., 2000). In man, dysfunction of respiratory control is seen in a variety of genetic diseases. It is most common in preterm infants, and immaturity of the CO_2-sensing mechanisms has been regarded as a major cause of central apnea in neonates (Gaultier and Gallego, 2005). In anesthetized and mechanically ventilated rats, apnea results if PCO_2 falls below a critical threshold (Boden et al., 1998), suggesting that CO_2 chemical drive is indeed essential for breathing. Evidence for the Haldane and Priestley postulate in conscious animals and under physiological conditions has been hard to obtain and is nonexistent in rodents. In conscious dogs, goats, and lambs, artificially lowering PCO_2 caused life-threatening apneas (Mitchell et al., 1966; Phillipson et al., 1981; Praud et al., 1997; Nakayama et al., 2003), with the caveat that in two of the studies, PCO_2 is lowered by mechanical hyperventilation, which can inhibit respiration by itself through stimulation of lung stretch receptors (Hayashi et al., 1996; Kubin et al., 2006; Subramanian et al., 2007).

Recent evidence from studying a human genetic disease and from mouse genetics seems also to support Haldane's statement (Haldane and Priestley, 1905). In man, congenital central hypoventilation syndrome (CCHS) is characterized by a lack of ventilatory response to inhaled CO_2 and to elevated PCO_2. Most patients require respiratory assistance from birth; as they age, they still need respiratory assistance during sleep to avoid lethal sleep apneas (Weese-Mayer et al., 2010). Mice harboring the most frequent CCHS-causing mutation, an expansion of a 20-residue poly-alanine stretch in *PHOX2B* (termed *PHOX2B*^{27Ala} mutation) (Amiel et al., 2009) do not respond to elevated CO_2 and die at birth from respiratory failure, as do several other mouse mutants with loss of CO_2 chemosensitivity (Goridis et al.,

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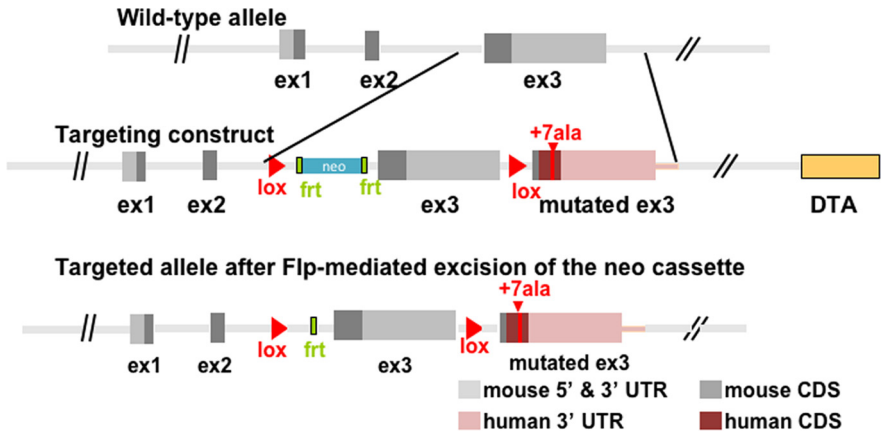

Figure 1. Schematic of the wild-type *Phox2b* gene, the targeting construct, and the targeted *Phox2b* locus. From 5′ to 3′, the targeting vector contained a 5′ homology arm of 3.7 kb, a loxP site inserted into the second intron followed by a neomycin resistance cassette flanked by frt sites and mouse exon 3, a loxP site inserted 3′ of the mouse polyA signal followed by the mutated human *PHOX2B* exon 3, and a 3′ homology arm of 6.9 kb followed by a diphtheria toxin A chain (DTA) cassette. CDS, coding sequence; Ex1, exon1; UTR, untranslated region.

2010). However, in none of these cases can one exclude additional defects that contribute to the fatal apnea, and there still is no definitive answer to the question of whether CO_2 chemosensitivity is crucial for life-sustaining respiration. In this study, we have reexamined the Haldane and Priestley postulate using a conditional mouse mutation that targets the CCHS-causing $PHOX2B^{27Ala}$ allele to the retrotrapezoid nucleus (RTN), a major brain site for integrating chemosensory information (Guyenet et al., 2009).

Materials and Methods

Mice. To generate a mutant line expressing a CCHS-causing expansion of the 20-residue poly-alanine stretch in Phox2b, we flanked the normal mouse exon 3 with *loxP* sites and inserted 3′ of the polyA signal the human *PHOX2B* exon 3 bearing the seven-residue expansion. Upon cre recombinase-mediated recombination, mouse *Phox2b* exon 3 is replaced by the mutated human exon 3. Because human and mouse Phox2b proteins are identical in sequence, the encoded protein is identical to mouse Phox2b except for the extension of the poly-alanine stretch. The targeting vector (Fig. 1), constructed by conventional cloning methods, was electroporated into 129S2/SvPas ES cells. Correctly targeted ES cells were injected into C57BL/6 blastocysts. ES cell manipulation and blastocyte injection were done at the Mouse Clinical Institute (Illkirch, France). Chimeric founders giving germ line transmission were mated with *Flpe* deleter mice (Lallemand et al., 1998) to remove the neomycin cassette. After crossing with C57BL/6 × DBA/2 F1 mice for several generations, the resulting $Phox2b^{27Alacki/+}$ ($P2b^{27Alacki/+}$) mice were intercrossed to produce $P2b^{27Alacki/27Alacki}$ homozygotes. Offspring with the recombined locus were produced by crossing $Egr2^{cre/+}$ (Voiculescu et al., 2000), *Pgk::cre* (Rodríguez et al., 2000), or *Brn4::cre* (Zechner et al., 2003) males with $P2b^{27Alacki/27Alacki}$ females. The day on which a vaginal plug was detected was considered as embryonic day 0.5 (E0.5). Genotyping was done on tail DNA. To detect the presence of the $P2b^{27Alacki}$ allele, the primers GCCCACAGTGCCTCTTAACT and CTCTTAAACGGGC GTCTCA were used, yielding bands of 330 kb for the wild-type, 474 kb for the mutated, and 380 kb for the recombined allele. To detect *cre*, the primers TGATGGACATGTTCAGGGATC and GAAATCAGTGCGTT CGAACGCTAGA were used, yielding a band of 347 kb. For the estimation of mutant survival, we counted the number of mutants that survived beyond the first week and compared this number to the Mendelian proportion expected from the crossings from which there were derived. All animal studies were done in accordance with the guidelines issued by the French Ministry of Agriculture and have been approved by Direction Départementale des Services Vétérinaires de Paris.

Histology. The methods for immunohistochemistry and *in situ* hybridization combined with immunohistochemistry on transverse 16 μm cryosections have been described (Hirsch et al., 1998; Dubreuil et al., 2009). Riboprobes for *Atoh1* were synthesized using a DIG RNA labeling kit (Roche) as specified by the manufacturer. The primary antibodies used were as follows: rabbit anti-Phox2b (Pattyn et al., 1997), guinea pig anti-Phox2b (Dubreuil et al., 2009), mouse anti-Islet1,2 (40.2D6 and 39.4D5; Developmental Studies Hybridoma Bank), rabbit anti-tyrosine hydroxylase (TH; Millipore Bioscience Research Reagents), rabbit anti-peripherin (Abcam). The primary antibodies were revealed for fluorescent staining by Alexa 488-, Cy3-, or Cy5-labeled secondary antibodies of the appropriate specificity (Jackson ImmunoResearch) or, for bright field observation, by biotin-labeled secondary antibodies and Vectastain ABC kit (Vector) revealed with 3,3′-diaminobenzamide. Pictures were captured with either a Hamamatsu ORCA-ER or a Leica DFC420C camera mounted on a Leica DM5500B microscope for observation through fluorescence or bright field optics, respectively. RTN neurons, defined either as $Atoh1^{+}$;$Phox2b^{+}$ (Dubreuil et al., 2009) or as $Phox2b^{+}$, TH-negative, Islet1,2-, or peripherin-negative cells (Takakura et al., 2008), were counted throughout an area delimited by the ventral borders of the facial nucleus (nVII) visualized by Islet1,2 or peripherin labeling and the medullary surface, starting rostrally with the first section containing nVII neurons and stopping 64 μm (at E14.5) or 96 μm [at postnatal day 1 (P1)] caudal to the caudal end of nVII. Cells responding to these criteria were counted on every fourth section on both sides. The Abercrombie correction was applied to cell ($Atoh1^{+}$;$Phox2b^{+}$) or nuclear ($Phox2b^{+}$, $Islet1,2^{-}$, TH^{-}) profile counts to estimate total cell numbers essentially as described previously (Lazarenko et al., 2009). Statistical analysis was done using a two-tailed *t* test and XLSTAT software with α set at 0.05.

Electrophysiology. Brainstem–spinal cord preparations and transverse medullary slices were obtained from E16.5 embryos and incubated in oxygenated artificial CSF at pH 7.4 and 7.2, as described previously (Dubreuil et al., 2009). The methods used for calcium imaging and for recording phrenic nerve and local population activity have been described previously (Dubreuil et al., 2009; Bouvier et al., 2010). Calcium imaging was done on brainstem–spinal cord preparations and transverse medullary slices loaded with Calcium Green-1 AM. Phrenic nerve activity was recorded using suction electrodes positioned at the C4 roots and local population activity was recorded on the surface of transverse slices through the pre-Bötzinger complex (pre-BötC) region. Values are given as mean ± SEM. Statistical significance was tested using a difference Student's *t* test to compare frequencies obtained from different mutants and a paired difference Student's *t* test to compare the same preparation in two different conditions.

Plethysmography. Breathing variables of P2–P9 pups were measured noninvasively in unanaesthetized, unrestrained pups using whole-body flow barometric plethysmography as described previously (Matrot et al., 2005; Ramanantsoa et al., 2006). Breathing variables of P22 and adult mice were measured using a larger whole-body flow barometric plethysmograph (Dauger et al., 2003). Gas flow through the plethysmograph was adjusted to deliver highly similar hypercapnic challenges in adult and newborn mice. After 10 min in room air, the pups were exposed to hypercapnic air ($8\%CO_2/21\%O_2/71\%N_2$) for 5 min, followed by 10 min in room air. Breathing variables were continuously monitored after a 7 min familiarization period in the plethysmograph chambers. Activity periods, which typically included exploratory movements, grooming, and sniffing, were detected based on large disturbances in the respiratory signal caused by the combined effects of positional changes and changes in breathing pattern (Matrot et al., 2005). Breathing variables were analyzed over activity-free periods, which represented >80% of recording

time in both groups. Apneas, defined as ventilatory pauses longer than twice the duration of the preceding breath, were determined using an automatic classification method (Matrot et al., 2005). To measure the ventilatory responses to hypoxia and hyperoxia, after 10 min in room air, P4 pups were first exposed to 100% O_2 for 3 min, then after 10 min in room air they were exposed to hypoxic air (10%O_2/90%N_2) for 3 min, and finally to room air again for 10 min. Breathing variables [breath duration (TTOT), tidal volume (VT), and ventilation (VE) calculated as VT/TTOT] were measured in apnea-free periods, except when stated otherwise. Statistical analysis was done using standard ANOVAs with genotype group as a between-subject factor. The total significance was set at $p < 0.05$ with individual tests corrected for multiple comparisons using Bonferroni adjustment.

Blood gas analysis. PCO_2 and pH were analyzed in P9 mutant and control mice. The pups were decapitated and mixed blood samples collected from the neck in heparinized capillary tubes as previously described for infant rats (Dingley et al., 2008). PCO_2 and pH were measured immediately using a clinical blood gas analyzer (ABL 80; Radiometer).

Results

Loss of retrotrapezoid nucleus neurons is compatible with survival

The RTN, a loose collection of neurons that reside in the rostral medulla close to the medullary surface, ventral and immediately caudal of nVII, has emerged as a site crucial for CO_2 sensing in the brain (Guyenet et al., 2009). These neurons are glutamatergic and express the Phox2b and Atoh1 transcription factors but, in contrast to other Phox2b$^+$ neurons nearby, they express neither catecholaminergic (TH) nor motoneuronal (Islet1,2 or peripherin) markers (Stornetta et al., 2006; Takakura et al., 2008; Dubreuil et al., 2009). Initially identified in cats (Smith et al., 1989) and then in adult rats (Nattie and Li, 1994; Mulkey et al., 2004), chemosensitive neurons with similar properties have since been identified at the same location in neonatal rats (Onimaru et al., 2008) and in mouse embryos (Dubreuil et al., 2009; Thoby-Brisson et al., 2009).

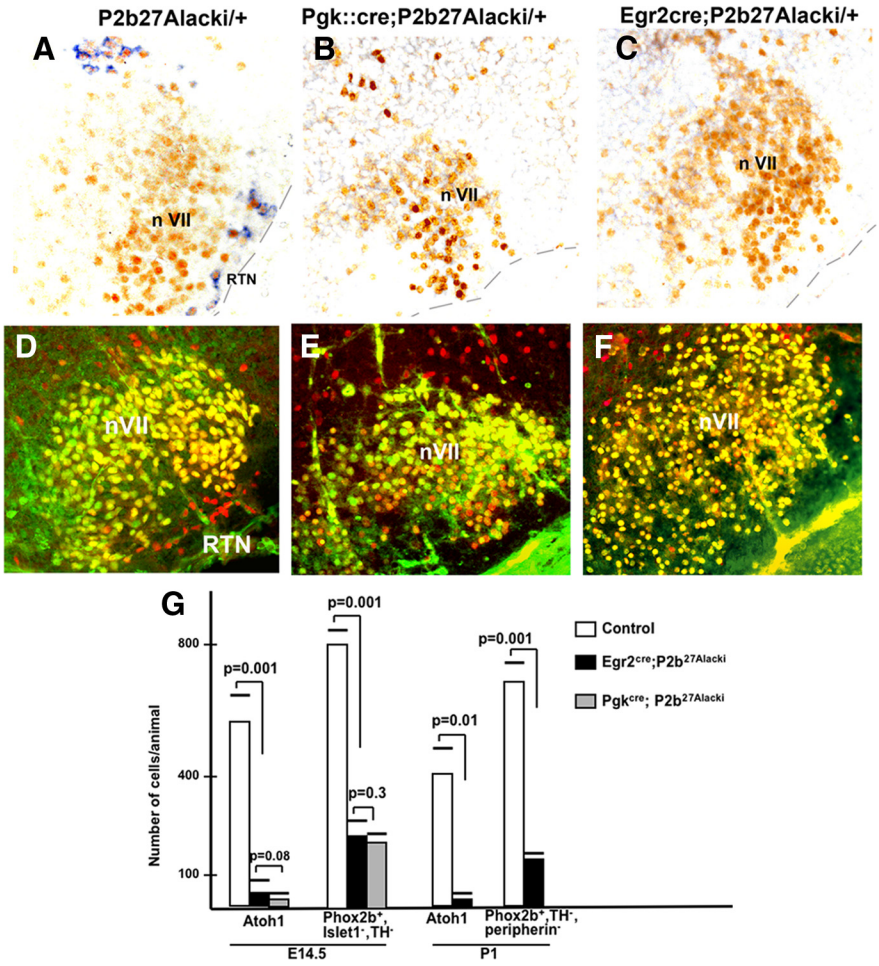

Figure 2. Loss of RTN neurons in conditional *Phox2b*^{27Alacki} mutants. ***A–C***, Combined *in situ* hybridization with an *Atoh1* probe and immunohistochemistry with anti-Phox2b antibodies on transverse sections showing the loss of differentiated RTN neurons in control *P2b*^{27Alacki}, *Pgk:cre;P2b*^{27Alacki}, or *Egr2*^{cre}*;P2b*^{27Alacki} E14.5 embryos as indicated. A population of equally *Atoh1;*Phox2b double-positive cells dorsal of nVII (Dubreuil et al., 2009; Rose et al., 2009) has also disappeared in the mutants. ***D–F***, Triple labeling for Phox2b (red), Islet1,2 and TH (green) showing depletion of Phox2b$^+$, Islet1,2$^-$, and TH$^-$ cells in the RTN region in *Pgk:cre; P2b*^{27Alacki} or *Egr2*^{cre}*;P2b*^{27Alacki} E14.5 embryos, as indicated. ***G***, Quantification of RTN neuron loss in E14.5 and P1 *P2b*^{27Alacki} mutants expressing either *Egr2*^{cre} or *Pgk::cre* as cre drivers. RTN neurons were identified either by *Atoh1* expression or by positivity for Phox2b and absence of Islet1,2 or peripherin and TH. The horizontal lines above the bars indicate +SD of the means.

Massive depletion of RTN neurons was the only discernable defect found in *Phox2b*$^{27Ala/+}$ mice that express the most frequent CCHS-causing mutation, an expansion of a poly-alanine stretch by seven residues, and the likely cause of their lack of CO_2 chemosensitivity (Dubreuil et al., 2008). The heterozygous offspring of chimeric founders all died at birth from respiratory failure. To derive a mutant mouse line, we generated mice expressing the *PHOX2B* exon 3 harboring the human mutation conditionally upon cre recombinase action (*P2b*^{27Alacki} mice) (Fig. 1). In the absence of cre recombinase, the homozygous mutants had a normal life span and did not display any overt defects. However, like the constitutive *Phox2b*$^{27Ala/+}$ mutants, *Pgk::cre;P2b27*$^{Alacki/+}$ mice, in which recombination occurs in the germ line, did not survive beyond the first day after birth (no survivors instead of the expected number of 49 mutants). *Pgk::cre;P2b*$^{27Alacki/+}$ embryos also shared with the constitutive mutants a massive depletion of RTN neurons (Fig. 2*B*,*E*,*G*). Thus, the un-recombined *P2b*^{27Alacki} allele is functionally equivalent to the wild-type allele and the recombined allele is functionally equivalent to the constitutive *Phox2b*^{27Ala} allele. Similarly, *Brn4::cre;P2b*$^{27Alacki/+}$ mice, in which recombination is limited to the CNS (Zechner et al., 2003), did not survive (zero instead of the expected number of 15.5 mutants). The *Egr2*^{cre} (*Krox20*^{cre}) line drives cre expression specifically in rhombomeres 3 (r3) and 5 of the embryonic neural tube (Voiculescu et al., 2000). Previous studies have shown that virtually all RTN neurons are descended from the *Egr2* lineage and that RTN neurons are depleted in animals conditionally null for *Phox2b* in this lineage (Dubreuil et al., 2009). This cre driver thus provided the opportunity to target the *P2b*^{27Ala} mutation to RTN neurons with greater specificity. To our surprise, the great majority of *Egr2*$^{cre/+}$*;P2b*$^{27Alacki/+}$ mice survived to adulthood. Of a total of 131 births, 48 mutants survived beyond the first week instead of the expected 52 from a Mendelian proportion, and all reached adulthood except for 12 that were killed at P9 for blood gas analysis.

As in the constitutive *Phox2b*$^{27Ala/+}$ mutants or when *Pgk::cre* was used as cre driver, there was a massive loss of RTN neurons in

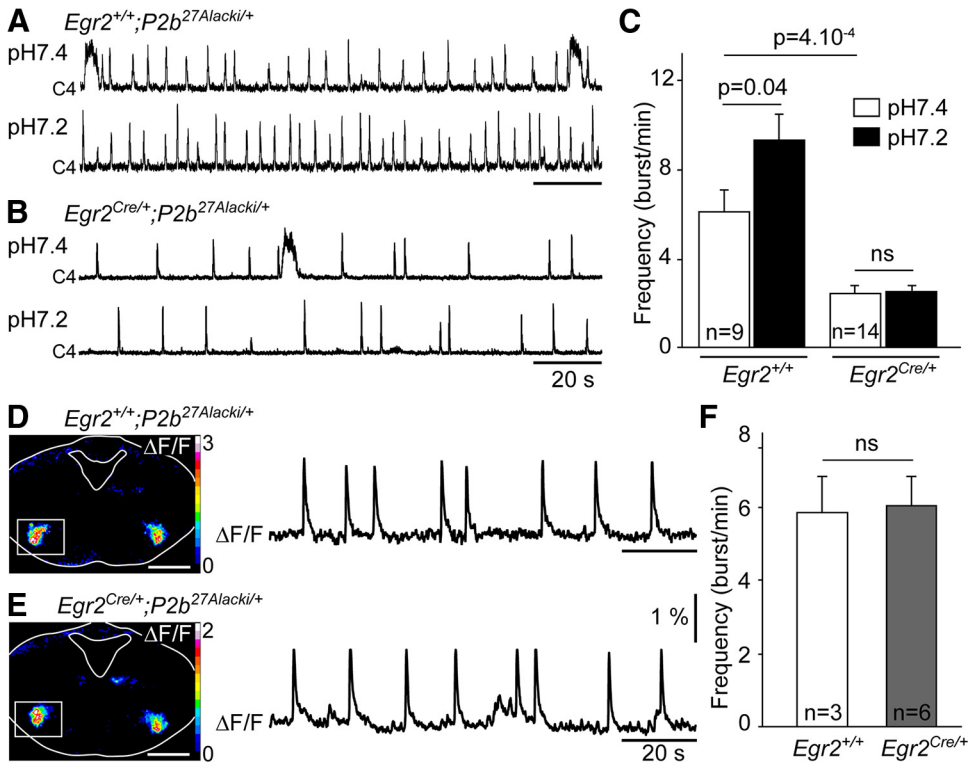

Figure 3. Slowed down respiratory-like rhythm unresponsive to a low pH challenge but a functional pre-BötC in hindbrain–spinal cord preparations from E16.5 $Egr2^{cre}$;$Phox2b^{27Alacki}$ embryos. ***A***, Integrated phrenic nerve discharges (C4) at pH 7.4 and pH 7.2 for an $Egr2^{+/+}$;$P2b^{27Alacki/+}$ control embryo. ***B***, Same as ***A*** for an $Egr2^{cre/+}$;$P2b^{27Alacki/+}$ mutant embryo. ***C***, Quantification of the burst frequencies for $Egr2^{+/+}$;$P2b^{27Alacki/+}$ ($Egr2^{+/+}$) and $Egr2^{cre/+}$;$P2b^{27Alacki/}$ ($Egr2^{Cre/+}$) embryos as indicated. ***D***, Left, Calcium imaging showing bilateral peak fluorescence changes during one burst of activity of the pre-BötC in a $Egr2^{+/+}$;$P2b^{27Alacki/+}$ transverse medullary slice loaded with Calcium Green-1 AM. Scale bar, .25 mm. Right, Trace showing rhythmic relative fluorescence changes (ΔF/F). ***E***, Same as ***D*** for an $Egr2^{cre/+}$;$P2b^{27Alacki/+}$ mutant embryo. ***F***, Quantification of the frequency of rhythmic bursts in the control ($Egr2^{+/+}$) and mutant ($Egr2^{cre/+}$) pre-BötC as indicated. ns, Not significant.

$Egr2^{cre/+}$;$P2b^{27Alacki/+}$ embryos. Expression of *Atoh1*, which marks 85% of RTN neurons (Dubreuil et al., 2009), had virtually disappeared from the RTN region at E14.5 (Fig. 2*C*,*G*). To make sure that RTN neurons are absent and do not merely lack expression of *Atoh1*, we used positivity for Phox2b and absence of Islet1,2 or peripherin and TH as alternative criterion. The great majority of the neurons that met this criterion were lost from the RTN region in $Egr2^{cre}$;$P2b^{27Alacki}$ embryos (Fig. 2*F*,*G*). Residual $Phox2b^{+}$, $Islet1,2^{-}$, and TH^{-} cells were depleted to a very similar extent whether *cre* was driven by *Pgk* or *Egr2*. The same proportion of RTN neurons was lost in $Egr2^{cre}$;$P2b^{27Alacki}$ neonates (Fig. 2*G*), excluding the possibility that their differentiation was merely delayed.

Slowed-down respiratory-like rhythm and lack of response to acidification in brainstem–spinal cord preparations from $Egr2^{cre}$;$P2b^{27Alacki}$ embryos

A population of spontaneously rhythmic neurons in the parafacial region termed e-pF constitutes the embryonic forerunner of the RTN (Thoby-Brisson et al., 2009). These neurons share location, marker gene expression, and origin from $Egr2^{+}$ precursors with RTN neurons and are activated by a pH challenge (Dubreuil et al., 2009; Thoby-Brisson et al., 2009). The e-pF appears to entrain the pre-BötC when the latter becomes active at E15 (Thoby-Brisson et al., 2009). Mutants in which RTN development or function is compromised have a slowed-down fetal respiratory rhythm, which, when tested, has been found unresponsive to acidification used as proxy for elevated CO_2 (Pagliardini et al., 2008; Dubreuil et al., 2009; Rose et al., 2009; Caubit et al., 2010). By contrast, the pre-BötC, a core component of the RRG (Gray et al., 2001; Feldman et al., 2003; Bouvier et al., 2010), functioned normally when tested in isolation. Hence, a slower than normal respiratory-like rhythm and unresponsiveness to CO_2/pH seems a hallmark of a nonfunctional RTN. To investigate this in $Egr2^{cre/+}$;$P2b^{27Alacki/+}$ embryos, we recorded phrenic nerve root activity as a measure of the motor outflow of the RRG in brainstem–spinal cord preparations. In control embryos, the rhythmic bursts recorded from C4 roots were accelerated by lowering the pH. In the mutant littermates, the frequency of rhythmic phrenic discharges was reduced and did not respond to acidification (Fig. 3*A–C*). As in other mutants with impaired RTN function (Dubreuil et al., 2009; Rose et al., 2009; Caubit et al., 2010), pre-BötC oscillator activity in transverse medullary slices was not affected (Fig. 3*D–F*). Hence, $Egr2^{cre}$;$P2b^{27Alacki}$ embryos share with the other mutants lacking a functional RTN a slower than normal respiratory-like rhythm and unresponsiveness to CO_2/pH in brainstem–spinal cord preparations.

$Egr2^{cre}$; $P2b^{27Alacki}$ mice lack CO_2 chemosensitivity at least up to postnatal day 9

We assessed breathing parameters in infant mice with whole-body plethysmography of unrestrained pups (Matrot et al., 2005; Ramanantsoa et al., 2006). In normoxia, mean ventilation (VE) of P2 $Egr2^{cre/+}$;$P2b^{27Alacki/+}$ pups (henceforth called mutants) was reduced by 42 ± 13% ($n = 10$) compared with control $Egr2^{+/+}$; $P2b^{27Alacki/+}$ littermates (henceforth called controls) because of longer breath duration (TTOT) (Fig. 4*A*,*C*). A striking defect was the complete absence of the normal response to hypercapnia, an

increase in ambient CO_2. Control littermates markedly increased VE when breathing a hypercapnic (8%CO_2) mixture, mainly because of an increase in breath volume (tidal volume VT). In the mutants instead, ventilation had a slight tendency to decline because of an increase in TTOT (Fig. 4*C*). Despite their breathing defects and the absence of a normal CO_2 response, perinatal lethality of mutant pups was very low (see above). At P9, mutant VE had increased to 77 ± 8% ($n = 9$) of that of the controls. However, the mutants still completely lacked the normal response to hypercapnia (Fig. 4*B*,*D*).

Although the P9 mutant pups did not respond to CO_2, PCO_2 in the blood was within the normal range (40.0 ± 4.5 and 39.8 ± 3.5 mmHg for mutants and controls, respectively) (Table 1). A potential caveat is that handling the pups before decapitation may have caused hyperventilation and an attendant drop in PCO_2 resulting in apparently normal values. However, the PCO_2 of the control littermates handled in exactly the same fashion was normal, implying that only the mutants but not the control littermates hyperventilate in response to handling, an unlikely possibility. The slight, but statistically significant increase in pH suggests that the mutants may use a mild metabolic alkalosis as a means to keep blood PCO_2 and pH within the normal range.

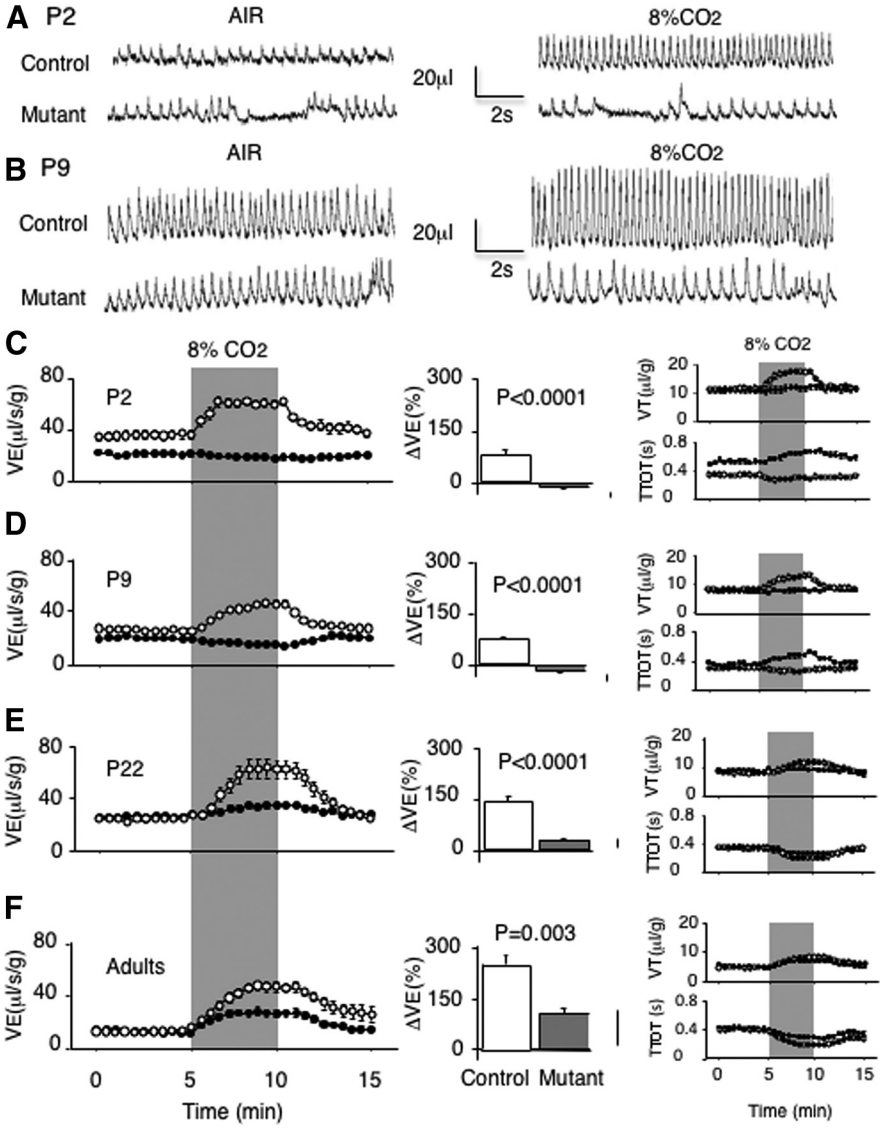

Figure 4. Lack of CO_2 chemosensitivity in young postnatal *Egr2*^{cre};*P2b*^{27Alacki} mice and partial recovery in adulthood. ***A***, Representative examples of plethysmographic recordings of P2 mutant and control mice in air and in response to 8% CO_2. ***B***, Same as ***A*** for P9 mice. ***C–F***, Left, Mean values of ventilation (VE) in air or in response to 8% CO_2 (shaded area) in mutant mice (black circles) and their control littermates (white circles) on P2 ($n = 10$ and $n = 13$ for mutants and controls, respectively), P9 ($n = 9$ and $n = 13$, respectively), P22 ($n = 12$ and $n = 8$, respectively), and adulthood (4 months old, $n = 9$ for both conditions). Each circle represents the mean ± SEM over a 30 s period. Middle, Ventilatory responses to hypercapnia expressed as the percentage VE change relative to baseline average VE, using the formula 100 × (peak VE − baseline VE)/ baseline VE. The peak VE response to hypercapnia was determined over the entire hypercapnic exposure. Right, Mean values of tidal volumes (VT) and breath durations (TTOT) in air or in response to 8% CO_2 (shaded area) in mutant mice (black circles) and their control littermates (white circles) from which the VE values in the left panels have been calculated. At P2, baseline ventilation was lower in mutants than in controls because of longer breath duration, but had almost normalized at P9. Mutants did not increase VE in response to hypercapnia until a small response appeared on P22 and consolidated in adulthood, still blunted compared with controls.

Partial recovery of the CO_2 response in adult mutants

At 3 weeks of age, the mutants displayed normal baseline ventilation in room air. When breathing a hypercapnic mixture, they responded with an increase in ventilation, but it amounted to a mere 20% of that of their control littermates (Fig. 4*E*). At 4 months, adult mutants had recovered a substantial, albeit incomplete (40% of that of the controls) CO_2 response (Fig. 4*F*). The possibility exists that the residual 15–20% Phox2b$^+$;TH$^-$; Islet1,2$^-$;peripherin$^-$ cells in the RTN region are able to compensate for the missing neurons in later life. However, not all such neurons are chemosensitive in the first place (Onimaru et al., 2008). Double-positivity for Phox2b and *Atoh1* seems a more reliable criterion for chemoresponsive RTN neurons. It was virtually absent in the RTN area from *Egr2*$^{cre/+}$;*Phox2b*$^{27Alacki/+}$ mutants. A more plausible scenario thus is that other chemosensitive sites inactive or without strong connections with the RRG in infant mice overcome, in part, the complete lack of a CO_2 response.

Input from PO_2-sensing peripheral chemoreceptors appears to compensate for the lost CO_2 response

One reason why the mutant pups can maintain adequate ventilation in the absence of CO_2 chemosensitivity could be that input from the CBs that sense blood PO_2 compensates for the lack of a CO_2 response. The CBs should be intact in the conditional mutants, in which the mutation is targeted to the hindbrain. We verified this point by subjecting P4 pups to a decrease in ambient O_2. Hypoxia caused the biphasic response characteristic for newborn mammals (Saetta and Mortola, 1987) in both mutant and control pups (Fig. 5). Not only did the mutants respond to hypoxia, showing that PO_2-sensing chemoreceptors were functional, but they did so more vigorously. In the mutants, the increase in VE was more pronounced and lasted longer than in the controls because of a larger and more sustained increase in respiratory

Table 1. Determination of PCO_2 and pH in the blood of P9 *Egr2*$^{+/+}$;*Phox2b*$^{27Alacki/+}$ (control) and *Egr2*$^{cre/+}$;*Phox2b*$^{27Alacki/+}$ (mutant) pups

	Control ± SD ($n = 15$)	Mutant ± SD ($n = 14$)	p value
PCO_2 (mmHg)	39.8 ± 3.5	40.0 ± 4.5	0.895
pH	7.47 ± 0.04	7.52 ± 0.05	0.008
Body weight (g)	5.01 ± 0.66	4.04 ± 0.06	0.0001
Body temperature (°C)	34.2 ± 0.6	33.6 ± 0.6	0.01

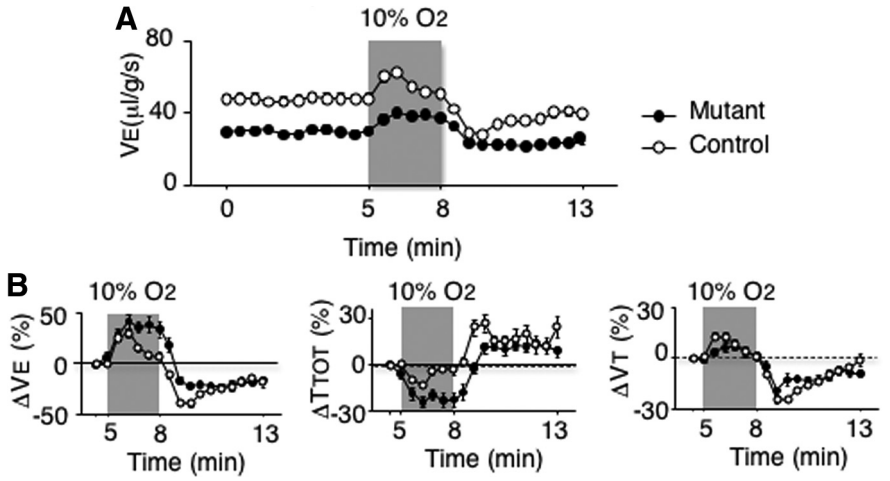

Figure 5. Ventilatory responses of *Egr2*^{cre};*P2b*^{27Alacki} mice to hypoxia. ***A***, Mean values of ventilation (VE) in air and in response to hypoxia (10% O_2, shaded area) of P4 mutant mice (black circles; $n = 10$) and their control littermates (white circles; $n = 13$). Each circle represents the mean ± SEM over a 30 s period. ***B***, VE, TTOT, and VT values expressed as percentage change relative to baseline ventilation as indicated. The mutants show a more vigorous and sustained ventilatory response, mainly due to a decrease in breath duration.

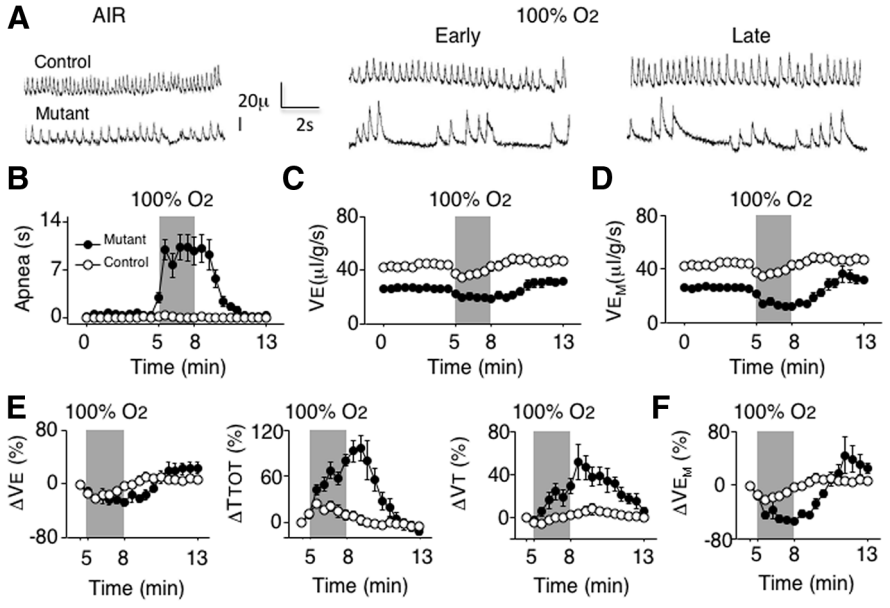

Figure 6. Enhanced dependence of *Egr2*^{cre};*P2b*^{27Alacki} mice on O_2 chemosensitivity. ***A***, Representative examples of plethysmographic recordings of P4 mutant and control mice in air and in response to 100% O_2. Early and late phases correspond to the first and the second half of the 3 min 100% O_2 exposure, respectively. ***B***, Total apnea time in mutants (black circles) and controls (white circles) in air or in response to 100% O_2 (shaded area). Each circle represents the mean ± SEM over a 30 s period. ***C***, Mean values of ventilation in air or in response to 100% O_2 measured in apnea-free periods (noted as VE). ***D***, Mean values of ventilation in air or in response to 100% O_2 averaged over the entire 30 s period, including apneas (VE_M). ***E***, ΔVE, ΔTTOT, and ΔVT values expressed as percentage of baseline values in apnea-free periods. ***F***, ΔVE_M values when including apnea periods. In 100% O_2, the mutants, but not the controls, showed a massive increase in the time spent in apnea. The ventilatory depression caused by 100% O_2 was more sustained in the mutants than in the controls in apnea-free periods. This difference was greatly enhanced when the calculation of mean ventilation included apnea periods.

frequency (Fig. 5*B*). The latter result suggested already that the mutants rely more on input from the CBs than the controls do. This interpretation was amply confirmed when we tested their response to pure O_2, known to inhibit peripheral chemoreceptor input (a manipulation called physiological chemodenervation) (Dejours, 1962; Hertzberg et al., 1990; Blain et al., 2010). Strikingly, this maneuver triggered periodic breathing—a series of deep breaths interrupted by prolonged apneas (Darnall, 2010)—in all mutants but not in controls. As a result, apnea duration increased massively in the mutants breathing pure O_2, but remained negligible in the control littermates (Fig. 6*A*,*B*). Mean ventilation of the controls showed the normal biphasic response to hyperoxia, an initial decrease followed by a return to baseline levels. The mutants also responded with decreased VE caused by a large increase in TTOT, which lasted throughout and even beyond the hyperoxic episode (Fig. 6*C*,*E*). Inspection of the individual traces revealed that the increased VT of the mutants in hyperoxia reflected the deep breaths caused by periodic breathing (Fig. 6*A*,*E*). The decrease in VE measured in apnea-free periods greatly underestimated the breathing deficit of the mutants because of the vast increase in time spent in apnea. Taking into account the periods spent in apnea revealed a 46 ± 11% drop of VE for the mutants compared with 21 ± 12% for control littermates ($n = 10$ and $n = 13$, respectively; $p = 0.00001$) and equivalent decreases in ΔVE during the first minute in 100% O_2. Together, these results indicate that the newborn mutants rely on peripheral chemosensation as tonic respiratory drive much more than the control littermates do.

One may argue that a 42% decrease in ventilation in normoxia (Fig. 4*A*,*C*) will not drop PO_2 in the blood sufficiently to activate the O_2 sensors in the CBs. However, CB sensitivity to O_2 is highly plastic and substantially enhanced by intermittent (Peng et al., 2004) and chronic (Powell, 2007) hypoxia. In addition, the CBs may exert a tonic excitatory drive to the RRG even in normoxia. This is suggested both by our results showing an immediate drop in VE after switching to 100% O_2, which was stronger in the mutants (Fig. 6*E*,*F*), and by evidence from the literature obtained in rat pups after intermittent hypoxia or lung injury (Peng et al., 2004; Jacono et al., 2006), after CB resection in man (Dahan et al., 2007), or by extracorporeal CB perfusion in dogs (Blain et al., 2009).

Discussion

All existing models of respiratory control postulate that to oscillate properly, the

RRG requires excitatory inputs, among which that afforded by CO_2 has been considered as crucial (Phillipson et al., 1981; Takakura et al., 2008; Guyenet et al., 2009; Spyer and Gourine, 2009). In previous work, we have produced mice with different mutations in *Phox2b*—either the toxic *Phox2b*^{27Ala} mutation that causes CCHS in man or conditional null mutations—both of which compromise RTN neuron differentiation (Dubreuil et al., 2008, 2009). In line with the eminent role proposed for the RTN in central chemosensitivity, CO_2/pH sensitivity was abrogated when tested in fetal hindbrain preparations or at birth *in vivo*. A common trait of these mutants was neonatal lethality because of respiratory failure. Our previous results could thus be interpreted as evidence in favor of the vital importance of the CO_2 response. However, the possibility remained that as yet undetected defects in other sites involved in respiratory control contributed to the lethal breathing deficits.

We have now produced an additional genetic background, in which RTN development is disrupted. This was made possible by the engineering of a mouse mutant that expresses the CCHS-causing *Phox2b*^{27Ala} mutation conditionally upon cre recombinase action. Partnering the *Egr2*^{cre} and *P2b*^{27Alacki} alleles allowed us to target the RTN more specifically than has been possible with the constitutive *Phox2b*^{27Ala}, but also with a conditional *Phox2b*-null mutation because of the selective toxicity of the *Phox2b*^{27Ala} mutation for certain types of neurons (Amiel et al., 2009). As anticipated, differentiated RTN neurons were depleted in the resulting *Egr2*^{cre};*Phox2b*^{27Alacki} mice to the same extent as in constitutive mutants. In contrast to the latter, however, the *Egr2*^{cre};*Phox2b*^{27Alacki} mutants survived to adulthood and maintained physiological levels of CO_2 in the blood, despite the fact that they lacked the respiratory drive exerted by acidification of fetal brainstem preparations or by inhaled CO_2. Hence, neither the RTN nor a functional respiratory chemoreflex are necessary to maintain CO_2 homeostasis in the blood. Like other mutants with defective RTN development, *Egr2*^{cre};*P2b*^{27Alacki} mutants had a slowed-down respiratory rhythm as neonates. This could reflect the loss of CO_2 chemical drive, but also the lack of entrainment of the pre-BötC by the embryonic RTN (Thoby-Brisson et al., 2009), as indicated by the slower than normal respiratory-like rhythm in late gestation. The breathing deficit normalized with age, probably because of maturation or plasticity of the respiratory system (Mitchell and Johnson, 2003). Similarly, CO_2 chemosensitivity had partly recovered in adult mutants. This recovery was a protracted process, since as juveniles, the mutants, whose baseline ventilation was completely normal, increased their ventilation only slightly in hypercapnia. These results raise several major questions that will be discussed in turn.

First, which mechanisms enable the mutants to breathe and to maintain normal blood PCO_2 in the absence of the excitatory input by elevated CO_2? One possibility is that CB stimulation by hypoxia compensates for the lack of CO_2 chemosensitivity. When the excitatory input from the CBs was inhibited by pure oxygen breathing, *Egr2*^{cre};*Phox2b*^{27Alacki} pups, but not control littermates, switched to periodic breathing with long apneic pauses. In mechanically ventilated rodents, apnea results when the PCO_2 falls below the apneic threshold, i.e., the PCO_2 below which excitatory input from the CO_2 sensors to the respiratory network ceases (Boden et al., 1998; Takakura et al., 2008). The underlying cause of the apneas may be the same in these animals mechanically hyperventilated with a hyperoxic gas mixture and in our mutants breathing pure oxygen: the cessation of all chemosensory drive to the respiratory network by silencing the input from both PCO_2 and PO_2 sensors. In any case, our results strongly suggest that tonic stimulation by the peripheral chemoreceptors compensates for the missing CO_2 response to maintain rhythmic breathing. Less clear, however, is how the animals are able to preserve normal levels of CO_2 in the blood. The near normal ventilation achieved by the input from the PO_2-responsive CBs may suffice, but nonrespiratory mechanisms, e.g., modulation of HCO_3^- retention or acid excretion by the kidneys (Cogan, 1984; Widener et al., 1986), may also be involved.

Second, why are the CBs unable to compensate for the lack of central CO_2 sensitivity, despite the fact that they contribute a still debated, but probably substantial fraction to the overall CO_2 response (Forster et al., 2000; Blain et al., 2009)? One possibility is that in the newborn period, the CBs still respond poorly to CO_2/pH while the response to hypoxia is already well developed, although in the rat, there is evidence for a well developed peripheral CO_2 chemosensitivity at birth (Saetta and Mortola, 1987). A perhaps more plausible explanation emerges by considering that RTN neurons are strongly activated by CB stimulation and provide powerful excitatory input to the RRG (Takakura et al., 2006). They may thus be obligatory intermediates for relaying the CO_2 response of the CBs, hence the loss of it when RTN neurons are missing. This hypothesis entails that the excitatory input afforded by hypoxia, but not that caused by hypercapnia, is also transmitted by pathways that bypass the RTN. In fact, such connections between the CBs and the respiratory neurons in the lower medulla have been identified (Finley and Katz, 1992) that may become strengthened in the mutants. They may convey information about PO_2, but not PCO_2 in the blood, although the existence of separate afferences for the two modalities is controversial (Bisgard et al., 1986; Niu et al., 1990).

Third, what are the mechanisms that lead to the improved CO_2 response in adults? Possibilities include strengthening of the CO_2-responsive input from the CBs or compensation by one or the other of the multiple sites postulated to function as CO_2 sensors in the brain (Nattie and Li, 2009; Nattie, 2011), which may be inactive or without strong enough connections with the RRG in early life. Another possibility is that the RTN is an obligatory intermediate for funneling input from other central chemoreceptors to the RRG early but not later in life, although experimental evidence for this is still lacking. Candidate chemoreceptor sites are serotonergic neurons that have been reported to be virtually pH-insensitive at birth and to become fully operational only beyond the newborn period (Wang and Richerson, 1999; Corcoran et al., 2009) but also glial cells (Gourine et al., 2010; Wenker et al., 2010). The latter respond to low pH already in young postnatal rats and may act mainly via RTN neurons, but additional pathways may come into play in adulthood.

Fourth, the tight correlation between impaired RTN development and CO_2 chemosensitivity shown by previous work (Goridis et al., 2010) is amply confirmed in the mutants analyzed here that completely lack any response to elevated CO_2 at least up to P9, and provide the first evidence that genetic ablation of the RTN abolishes the hypercapnic response beyond the first day after birth.

Finally, it becomes clear that loss of CO_2 chemosensitivity alone cannot be the cause of the fatal central apnea of the other *Phox2b* mutants with impaired RTN development, either the conditional *Phox2b*-null mutants or the *Phox2b*^{27Ala} mutants that express the CCHS-causing human mutation constitutively (Dubreuil et al., 2008, 2009). Why, then, do the other *Phox2b* mutants die at birth of respiratory failure? At present, all we can say is that the defect must be in neurons that are located in the CNS, express *Phox2b*, and depend on it for proper development.

In mice expressing the *Phox2b*^{27Ala} mutation constitutively, the only anatomical defect found was loss of RTN neurons, but in principle, any *Phox2b*-expressing cell could be affected by the toxic mutation, with the restriction that the lethal breathing defect of the *Brn4cre::P2b*$^{27Alacki/+}$ mice shows that the critical deficiency is in the CNS. More telling is a comparison between the *Egr2*^{cre}*;P2b*$^{27Alacki/+}$ animals analyzed here that survive and *Egr2*^{cre}*;Phox2b*$^{lox/lox}$ mice that do not (Dubreuil et al., 2009). In both cases, the mutation, either the *Phox2b*^{27Ala} or a *Phox2b*-null mutation, has been targeted to r3/5-derived cells. In the *Egr2*^{cre}*; Phox2b*$^{lox/lox}$ mutants, the additional defect must be in neurons with a history of *Egr2*^{cre} expression that are not affected by the *Phox2b*^{27Ala} mutation, but depend on *Phox2b* for proper differentiation.

A key tenet in respiratory physiology states that arterial PCO_2 is the essential drive to breathe, and sentences abound in the recent literature such as "CO_2 provides the major tonic drive to breathe" (Spyer and Gourine, 2009) or "we have known for more than a century that the levels of CO_2 in the systemic circulation and in the brain are the major determinants of breathing in humans and other mammals" (Blain et al., 2010). Here we have revisited this dogma using a new mouse mutant. Our results do not support the notion that CO_2 chemosensitivity is indispensable for breathing. We show that a complete lack of the respiratory chemoreflex, classically revealed as ventilatory stimulation by an increase in inspired CO_2, is compatible with breathing adequate for survival and maintaining normal CO_2 levels in the blood. There are precedents for life-sustaining breathing in the absence of CO_2 chemical drive in some situations. In rodents, some (Serra et al., 2001; Stunden et al., 2001) but not all (Wickström et al., 2002) investigators reported a blunted hypercapnic response in 1-week-old rats, but a similar transient drop in CO_2 responsiveness has not been found in mice (Renolleau et al., 2001; this paper). In humans, pronounced hypocapnia induces apnea in sleep, but not in wakefulness during which a still enigmatic "wakefulness drive" is supposed to replace that afforded by CO_2 (Dempsey, 2005; Duffin, 2010).

The breakdown of rhythmic breathing in mutant mice breathing pure O_2, i.e., when both the O_2 and CO_2 response are missing, reveals that, in accordance with virtually all models of respiratory control, proper functioning of the RRG requires some form of chemosensory input. Our results suggest that the response to hypoxia of the CBs provides the required stimulation. Beyond the first days after birth, this drive appears sufficient for near-normal baseline respiration. The fact that most CCHS patients harboring the *PHOX2B*^{27Ala} mutation require life-long ventilatory assistance during sleep in order not to die from central apnea suggests that additional defects caused by the constitutive *PHOX2B*^{27Ala} mutation are involved. Revealing such defects in our mouse models may lead to a better understanding of the etiopathology of CCHS and perhaps to new modes of therapeutic intervention.

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