Behavioral/Systems/Cognitive

Phox2a Gene, A6 Neurons, and Noradrenaline Are Essential for Development of Normal Respiratory Rhythm in Mice

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Although respiration is vital to the survival of all mammals from the moment of birth, little is known about the genetic factors controlling the prenatal maturation of this physiological process. Here we investigated the role of the *Phox2a* gene that encodes for a homeodomain protein involved in the generation of noradrenergic A6 neurons in the maturation of the respiratory network. First, comparisons of the respiratory activity of fetuses delivered surgically from heterozygous *Phox2a* pregnant mice on gestational day 18 showed that the mutants had impaired *in vivo* ventilation, *in vitro* respiratory-like activity, and *in vitro* respiratory responses to central hypoxia and noradrenaline. Second, pharmacological studies on wild-type neonates showed that endogenous noradrenaline released from pontine A6 neurons potentiates rhythmic respiratory activity via α1 medullary adrenoceptors. Third, transynaptic tracing experiments in which rabies virus was injected into the diaphragm confirmed that A6 neurons were connected to the neonatal respiratory network. Fourth, blocking the α1 adrenoceptors in wild-type dams during late gestation with daily injections of the α1 adrenoceptor antagonist prazosin induced *in vivo* and *in vitro* neonatal respiratory deficits similar to those observed in *Phox2a* mutants. These results suggest that noradrenaline, A6 neurons, and the *Phox2a* gene, which is crucial for the generation of A6 neurons, are essential for development of normal respiratory rhythm in neonatal mice. Metabolic noradrenaline disorders occurring during gestation therefore may induce neonatal respiratory deficits, in agreement with the catecholamine anomalies reported in victims of sudden infant death syndrome.

Key words: **prenatal maturation of the respiratory network; *Phox2a* gene; fetal mice; *in vivo* ventilation; *in vitro* respiratory activity of brainstem; spinal cord preparations; noradrenaline; A6 neurons**

Introduction

The neural network responsible for rhythmic respiratory muscle contractions in mammals must function at birth for ventilation, blood oxygenation, and survival to be possible. In neonatal rodents, the respiratory rhythm generator (RRG) has been located in the rostral ventrolateral medulla, and a special set of respiratory neurons has been thought to play a key role (Smith et al., 1991; Rekling and Feldman, 1998; Koshiya and Smith, 1999). Their stimulation, inhibition, and lesion trigger and abolish the respiratory rhythm (Arata et al., 1990; Errchidi et al., 1990, 1991; Smith et al., 1991; Funk et al., 1993; Hilaire et al., 1997; Ballanyi et al., 1999), and these neurons mediate the modulation of respiratory rhythm by various systems, including noradrenaline (NA) (Onimaru et al., 1997; Arata et al., 1998; Hilaire and Duron, 1999; Bou-Flores et al., 2000; Thoby-Brisson and Ramirez, 2000; Gray et al., 2001). Little information is available, however, concerning prenatal maturation of the RRG (Jansen and Chernick, 1991; Di Pasquale et al., 1992, 1994; Greer et al., 1992; Blanchi et al., 2003; Viemari et al., 2003) and the role of several early expressed neuromodulators such as NA and serotonin, which are known to affect CNS maturation (Lauder, 1993; Thomas et al., 1995; Levitt et al., 1997; Weiss et al., 1998). It has been shown recently in mice that a main maturational step in RRG maturation occurs between gestational day (E) 16 and E18: the RRG is immature and highly sensitive to NA by E16 but behaves as the neonatal RRG by E18 (Viemari et al., 2003). The availability of *Phox2a* transgenic mice with known genetic alterations of the NA system (Morin et al., 1997) provides a good opportunity for investigating the role of the *Phox2a* gene and NA in the RRG prenatal maturation by comparing the respiratory activity of wild-type (WT) and *Phox2a* mutant mice before birth, at E18.

Phox2a encodes for a homeodomain protein that is expressed in all ganglia of the peripheral autonomic system, some cranial sensory ganglia, and hindbrain nuclei, including all of the noradrenergic centers (Morin et al., 1997). *Phox2a* is essential for the proper development of the noradrenergic A6 neurons, but its deletion does not affect other NA neurons (Pattyn et al., 1997, 2000). At birth, *Phox2a* mutant mice are of normal size and show no gross anatomical abnormalities, but they have no A6 neurons, do not feed, and die on the day of birth (Morin et al., 1997).

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Herein, experiments performed before birth at E18 established that *Phox2a* mutant fetuses have disrupted ventilation *in vivo* and altered RRG activity *in vitro*, as well as altered responses to central hypoxia and NA. In WT neonates we show that the A6 neurons are connected to the respiratory network and facilitate RRG activity, whereas blockade of α1 adrenoceptors during late gestation alters ventilation and RRG activity at birth. Development of normal respiratory rhythm and rhythm-generating networks therefore may require *Phox2a* gene, A6 neurons, and their facilitatory actions on the maturing RRG.

Materials and Methods

The experimental procedures were performed in keeping with the European guidelines for the care and use of laboratory animals (Council Directive 86/6009/EEC). *Phox2a* heterozygous mice, maintained on a C57BL6 genetic background, were intercrossed to produce mutant fetuses (Jacob et al., 2000). Pregnant mice ($n = 14$) were decapitated on E18, the fetuses were delivered by cesarean section, and the umbilical cords were transected as described previously (Di Pasquale et al., 1992; Greer et al., 1992; Viemari et al., 2003). The fetuses were kept in a warm environment until the *in vivo* and *in vitro* experiments, which were performed within 10–30 min of delivery. Fetuses were subsequently genotyped by PCR as null-mutants ($n = 16$), WT ($n = 17$), or heterozygous ($n = 26$). Neonatal studies were performed on C57BL6 WT pups aged from postnatal day (P) 0–P4 (Iffa-Credo Breeding Center, Saint Germain sur l'Arbresle, France).

In vivo *recording of breathing parameters.* The breathing movements of surgically delivered fetuses were recorded as described previously (Burnet et al., 2001; Blanchi et al., 2003; Viemari et al., 2003), using the whole-body plethysmography technique in 25 ml thermostated chambers equipped with a differential pressure transducer connected to a sine-wave carrier demodulator (Validyne CD15, Northbridge, CA). Mouth temperature was monitored with a miniature thermistor nylon-coated probe (YSI 555; Cole-Parmer, Vernon Hills, IL). In some experiments, we measured the respiratory frequency changes induced when replacing air (21% O_2) with a hypoxic mixture (90% N_2, 10% O_2) for 3 min.

In vitro *recording of medullary respiratory network activity.* As described previously (Bou-Flores et al., 2000; Viemari and Hilaire, 2002; Blanchi et al., 2003; Viemari et al., 2003), the isolated medullary–spinal cord preparations of either P0–P2 neonatal or E18 fetal mice were placed in a 2 ml chamber superfused (2 ml/min) with artificial CSF (aCSF) containing (in mM): 129 NaCl, 3.35 KCl, 1.26 $CaCl_2$, 1.15 $MgCl_2$, 21.0 $NaHCO_3$, 0.58 NaH_2PO_4, 30 glucose, gassed (95% O_2–5% CO_2, pH 7.4), and kept at 27 ± 1°C. The C4 phrenic root activity was recorded with suction electrodes, filtered, amplified, and integrated (time constant, 50 msec). Pontomedullary transections were performed manually with thin microscissors. A tungsten microelectrode was lowered into the pons using a micromanipulator, and direct current was applied (3 sec; 100–200 μA) to make electrolytic lesions in area A5 or A6. Histological controls were performed on 100 μm transversal slices (Nissl coloration).

Pharmacological experiments. In some *in vitro* experiments, aCSF was replaced for 5 min by either nonoxygenated aCSF (95% N2 + 5% CO_2, pH 7.4) or aCSF containing drugs (Sigma, St. Louis, MO) such as NA (25 μM), the α1 adrenoceptor agonist 6-fluoro-noradrenaline (40 μM), the monoamine oxidase inhibitor pargyline (50 μM, 10 min), or the α1 adrenoceptor antagonist prazosin (50 μM, 15 min). A partition was occasionally placed at the pontomedullary junction to apply the various aCSF solutions to the medulla and normal aCSF to the pons. Two pregnant dams were given daily subcutaneous injections of prazosin (100 mg/kg in 0.5 ml saline) from gestational day E16 to delivery. At birth, the respiratory activity of the pups was studied using both *in vivo* and *in vitro* approaches.

Transynaptic tracing with rabies virus. As reported previously (Burnet et al., 2001; Gaytan et al., 2002), the challenge virus standard (CVS) rabies virus is amplified across the synapses and can be used to transynaptically label the respiratory network. After cold anesthesia of three P1 neonates, 1 μl of CVS rabies virus solution (2.5×10^7 PFU/ml) was injected via a 10 μl Hamilton syringe inserted into the left part of the diaphragm. After a 2 d survival period, the pups were killed, and the brainstem and medulla were dissected out and placed in paraformaldehyde in saline (PBS). The brainstems were cut into 70 μm sections in the coronal plane using a freezing microtome. The virus was detected using pre-embedding immunohistochemical procedures with a mouse monoclonal primary antibody specifically recognizing the phosphoprotein and a fluorescein (FITC)-conjugate (Jackson ImmunoResearch, West Grove, PA) as the secondary antibody to identify the labeled neurons. Using a Zeiss Axiophot fluorescence microscope, the green fluorescent FITC-labeled neurons were detected by means of a barrier filter system H (450–490 nm excitation wavelength).

Biochemical analyses. The pons and medulla of exteriorized E18 fetuses were dissected out as reported above and placed in 100 μl of 0.1 M perchloric acid/2.7 mM Na_2-EDTA. The chilled structures were homogenized in an ultrasound device (1 min, 20 kHz, 40 W). The homogenates were centrifuged (5 min, 8800 × *g*), and a 10 μl aliquot of the supernatant was injected into a reverse-phase column (Spherisorb ODS2; 5 μm, 125 mm × 4 mm; Macherey-Nagel, Hoerdt, France) with a mobile phase consisting of (in mM): 27 citric acid; 50 sodium acetate; 1 EDTA-Na_2; 0.8 sodium octyl sulfonate, and 6% methanol. A flow rate of 0.9 ml/min was set up with a Shimazu LC 10 AD pump. Monoamines were measured as described previously (Cransac et al., 1996) at +670 mV versus Ag^+/AgCl electrode (detector Eldec 102; Chromatofield, Chateauneuf-les-Martigues, France). The detection limits were calculated by doubling the background noise level and expressed in term of picomoles of injected amounts; they were <0.03 pmol with both compounds, and the intra-assay coefficients were 0.2%. The data are expressed in picomoles.

Statistics. The data were analyzed using SigmaStat software (SPSS, ASC Gmbh, Erkrath, Germany). The first step of the analysis was to verify the assumptions of normality and homoscedasticity of the samples. Respiratory frequency (plethysmography) or phrenic burst (PB) frequency (brainstem–spinal cord preparations) were compared in WT and mutant mice by the nonparametric Mann–Whitney *U* test. Respiratory frequency or phrenic burst frequency of neonatal mice born from control dams and from prazosin-treated dams were compared by Student's *t* test. To estimate the cycle duration variability, we calculated the coefficients of variability of cycle duration defined as the ratio between the SD and the mean cycle duration measured during 30–60 successive cycles (Bou-Flores et al., 2000; Viemari et al., 2003). In WT neonates, the changes induced in phrenic burst frequency by aCSF containing drugs were analyzed by one-way factor (experimental conditions: control and first to fifth minutes of drug application) ANOVA for repeated measures in the same subjects, followed by Tukey's tests as multiple-comparisons procedure. The effect of hypoxic aCSF on phrenic burst frequency was analyzed by a two-way factors ANOVA [factors are strain (WT or mutant mice and experimental conditions), control, hypoxic aCSF, and recovery] for repeated measurements in the same subjects with only one repeated factor (experimental conditions) followed by Tukey's tests as multiple comparisons procedure. The effect of aCSF containing drugs on phrenic burst frequency was analyzed by the same statistical procedure. For all tests, statistical significance was taken at $p \leq 0.05$. Data are expressed as means ± SEM. As reported previously for normal maturing mice (Viemari et al., 2003), coefficients of variability that express sample variability relative to the mean of the sample are given but not compared statistically. Because SD and mean have identical units, coefficient of variability has no units at all; thus these are relative values.

Results

Abnormal *in vivo* breathing and *in vitro* activity in the isolated respiratory network in Phox2a mutant fetuses

In 14 pregnant *Phox2a* heterozygous mice, 59 preterm neonates were surgically delivered at E18 and genotyped as 16 mutant, 17 WT, and 26 heterozygous fetuses. No significant differences in mean weight (1.19 ± 0.05 gm) or mouth temperature (32.8 ± 0.5°C) were observed among the three groups. As reported previously (Blanchi et al., 2003; Viemari et al., 2003), all of the E18

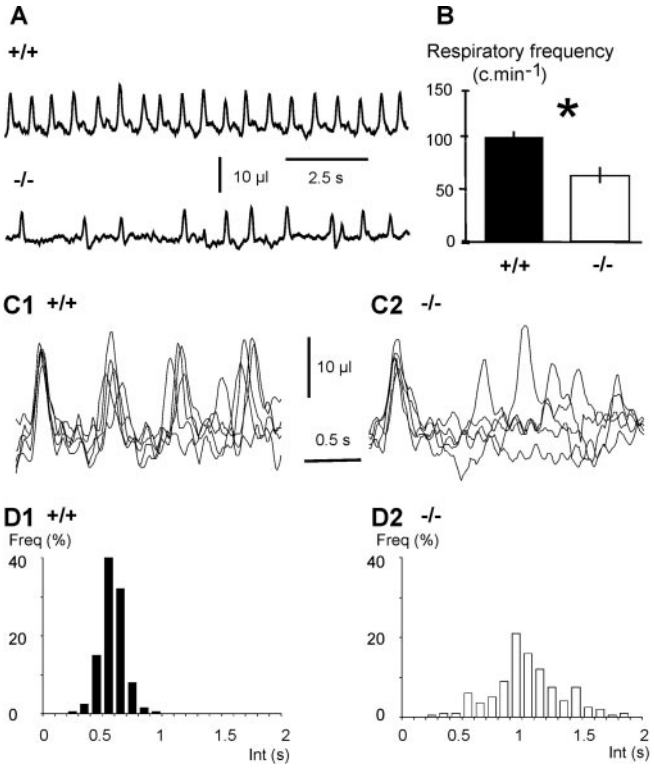

Figure 1. Altered *in vivo* respiratory activity in *Phox2a* mutant fetuses. *A*, Plethysmographic recordings of ventilation (inspiration upward) in WT and mutant fetuses (+/+, top trace, and −/−, bottom trace, respectively) exteriorized from heterozygous mice on gestational day 18. *B*, Black and gray columns show the mean respiratory frequency (±SEM) of 14 WT and 7 mutant fetuses, respectively; asterisk indicates significant difference between WT and mutant at $p < 0.05$. *C1*, *C2*, Five superimposed traces from plethysmographic recordings in a WT fetus and a mutant fetus (*C1* and *C2*, respectively); calibration time as indicated. *D1*, *D2*, The columns in the histograms show the distribution of respiratory cycle duration in the WT and mutant fetuses illustrated above (*D1* and *D2*, respectively). The frequency of occurrence [Freq (%); 100% = 60] of a given duration is expressed in 20 classes of 0.1 sec for 60 successive respiratory cycles. Note the large variability of cycle duration in *D2*.

fetuses were breathing only in gasps just after their surgical delivery, i.e., deep, irregular inspiratory movements with a low frequency (one to two cycles per minute) involving the whole body and a wide open mouth. The gasps persisted for only 2–3 min and most of the fetuses produced normal respiratory movements within 3–5 min of their delivery, but one WT and three mutant fetuses did not survive.

The breathing parameters of 7 mutant and 14 WT E18 fetuses were analyzed by performing *in vivo* plethysmography during the first 30 min after exteriorization (Fig. 1*A*). Exteriorized fetuses from both groups could occasionally produce one to two gasps during the recording session, but the frequency of occurrence of gasps was not greater in mutant than WT fetuses. As shown in Figure 1*B*, however, the mean respiratory frequency was significantly lower in mutant than in WT fetuses [65 ± 11 and 104 ± 6 cycles per minute (c/min), respectively]. The tidal volume was ~10 µl in both genotypes, and the low respiratory frequency of mutant mice therefore lead to a significantly weaker minute ventilation in mutant than in WT mice (689 ± 110 and 1150 ± 95 µl/min, respectively). As shown in Figure 1, *C1* and *C2*, the inspiratory duration was rather constant and ranged 100 msec in both groups (109 ± 20 and 123 ± 15 msec for mutant and WT fetuses, respectively), whereas the expiratory duration was irregular, especially in mutants. The respiratory cycle duration was found to be more variable in mutant than in WT fetuses (Fig. 1*D1*,*D2*), leading to mean coefficients of variability of cycle duration of 0.79 ± 0.09 and 0.52 ± 0.08 for mutant and WT fetuses, respectively.

It has been reported previously that exteriorized E18 fetuses respond to hypoxic conditions by increasing their respiratory frequency but not their tidal volume (Viemari et al., 2003). The effect of breathing a hypoxic mixture for 3 min (10% O_2 instead of 21% O_2) was tested in four mutant and eight WT fetuses at E18. Hypoxia did not affect the mean tidal volume but significantly increased the mean respiratory frequency of three of the four mutant fetuses to 122 ± 7% of the control value under air and of all the WT fetuses to 129 ± 7% of the control (no significant difference between WT and mutant fetuses). In the remaining mutant, the resting respiratory frequency, which was very slow under normoxia (~30 c/min), was not significantly affected by hypoxia.

Because a wide range of peripheral inputs could affect the RRG activity in awake animals, no definite conclusions about the RRG maturation in mutants could be reached despite their slow respiratory frequency and high variability of cycle durations observed *in vivo*. We therefore further analyzed the respiratory command exerted by the fetal medullary respiratory network under conditions in which all of the peripheral inputs were eliminated, i.e., in *in vitro* en bloc medullary preparations (Fig. 2). As reported previously (Suzue, 1984; Di Pasquale et al., 1992; Greer et al., 1992; Blanchi et al., 2003; Viemari et al., 2003), the completely deafferented medullary respiratory network of neonatal and fetal rodents remains able to produce rhythmic PBs from the cervical C4 ventral roots for several hours *in vitro*. Rhythmic PBs occurred in 10 of 15 mutant and 14 of 14 WT preparations (Fig. 2*A*); however, the mean frequency of the PBs was significantly lower in mutant than in WT preparations (5.2 ± 1.1 and 10.4 ± 0.5 c/min, respectively) (Fig. 2*B*). No obvious differences were found in the integrated PBs of mutant and WT preparations (Fig. 2*C1*,*C2*). The PBs had a decrementing envelope in both groups with a mean total duration of 675 ± 62 and 640 ± 37 msec for mutant and WT preparations, respectively (NS), with a rising time that was shorter than the decay time (mean rising time 112 ± 25 and 95 ± 15 msec, for mutant and WT preparations, respectively; NS). Although no definite conclusions can be reached about PB amplitude because it depends on the fit of the suction electrode, the PB amplitude was not consistently smaller or larger between groups. The duration of the individual PB cycles produced by fetal medullary preparations was variable (Di Pasquale et al., 1994; Bou-Flores et al., 2000; Viemari et al., 2003), but the variability of the interburst interval was found to be particularly high here in mutant preparations (Fig. 2*C2*), with short and long-lasting respiratory cycles occurring in a random pattern (Fig. 2*D2*). The mean coefficient of variation was almost twice as high in mutant as in WT preparations (0.59 ± 0.06 vs 0.37 ± 0.02).

Finally, we measured the endogenous levels of bioamines in brainstems from 12 WT and 9 mutant E18 fetuses (Fig. 2*E*). The pontine NA levels were significantly lower in mutant than in WT E18 mice (0.49 ± 0.17 and 2.12 ± 0.20 pmol, respectively), confirming the lack of A6 neurons in the *Phox2a* mutants. The medullary NA levels were 0.96 ± 0.42 and 1.90 ± 0.22 pmol for mutant and WT mice, respectively, but the difference did not reach the significance level ($p = 0.052$), in agreement with the fact that *Phox2a* is not crucial for the development of medullary NA neurons (Pattyn et al., 1997, 2000). The levels of endogenous serotonin in the pons (2.10 ± 0.13 and 2.04 ± 0.32 pmol, respec-

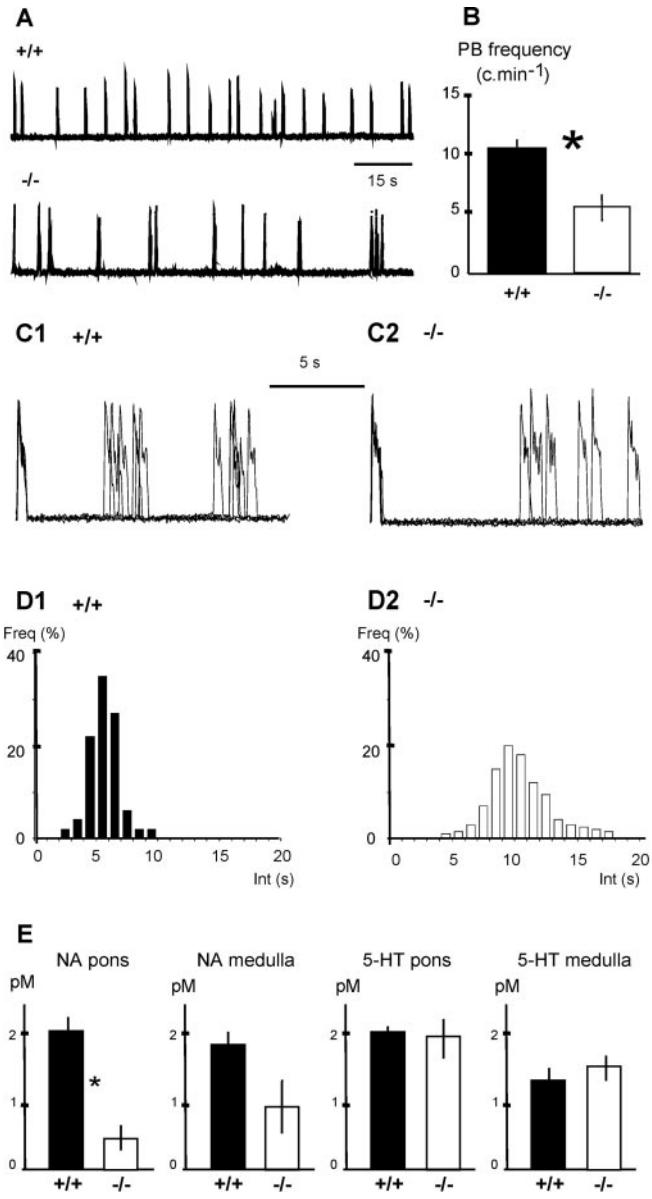

Figure 2. Altered *in vitro* respiratory activity in *Phox2a* mutant fetuses. *A*, Integrated PBs produced by the isolated respiratory network in medullary preparations from +/+ and −/− E18 fetuses (top and bottom traces, respectively). *B*, Black and gray columns show the mean PB frequency (±SEM) of 14 WT and 10 mutant preparations, respectively; asterisk indicates significant difference between WT and mutant at $p < 0.05$. *C1*, *C2*, Five superimposed traces of integrated PBs recorded in a WT preparation and a mutant preparation (*C1* and *C2*, respectively); calibration time as indicated. *D1*, *D2*, The columns in the histograms show the distribution of the PB cycle duration in the WT and mutant preparations illustrated above (*D1* and *D2*, respectively). The frequency of occurrence [Freq (%)] of a given duration is expressed in 20 classes of 0.1 sec for 60 successive PBs. Note the large variability of cycle duration in *D2*. *E*, Histograms show NA and serotonin (5-HT) mean levels (±SEM) expressed in picomoles in the pons and the medulla of +/+ and −/− fetuses (black and gray columns for 12 WT and 9 mutant fetuses, respectively; asterisk indicates significant difference between WT and mutant at $p < 0.05$).

tively) and the medulla (1.38 ± 0.12 and 1.58 ± 0.17 pmol, respectively) were not statistically different in mutant and WT fetuses.

In heterozygous E18 fetuses, the *in vivo* respiratory frequency (116 ± 5 c/min; $n = 11$), the *in vitro* PB rhythm (10.9 ± 0.8 c/min; $n = 10$), the cycle duration variability (0.61 ± 0.12 and 0.42 ± 0.03 for 7 and 10 *in vivo* and *in vitro* experiments, respectively), and the pontine and medullary levels of NA (1.69 ± 0.23 and 1.64 ± 0.28 pmol, respectively; $n = 9$) and serotonin (2.07 ± 0.16 and 1.73 ± 0.24 pmol, respectively; $n = 9$) were in the range of those of their WT littermates.

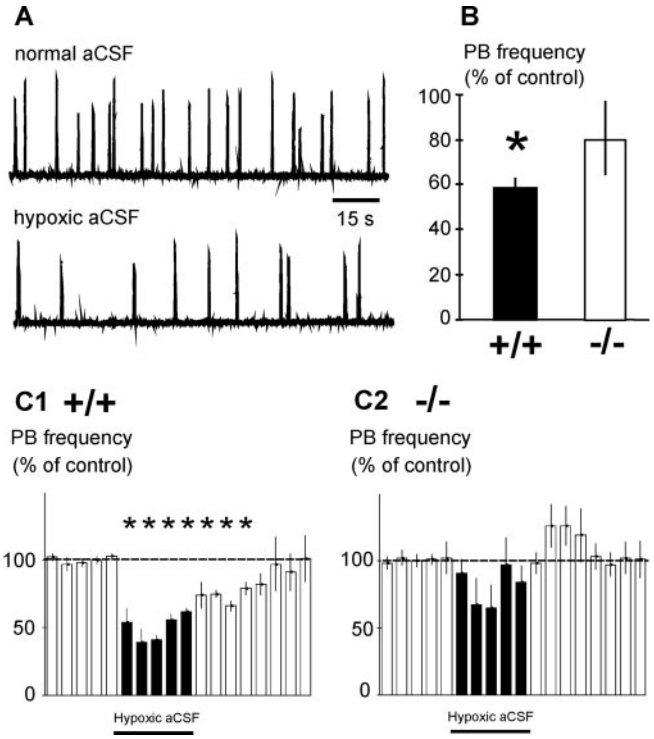

Figure 3. Altered responses of the RRG to hypoxic aCSF applications in medullary preparations from Phox2a mutant fetuses. *A*, Traces show integrated PBs recorded from the same WT medullary preparation under normal aCSF (top trace) and nonoxygenated aCSF applied for 5 min (bottom trace). *B*, Black and gray columns show the mean PB frequency (±SEM) under 5 min of hypoxic aCSF application expressed as percentages of the control values under normal aCSF for eight WT and five mutant fetuses, +/+ and −/−, respectively; asterisk indicates a significant decrease versus control at $p < 0.05$. *C1*, *C2*, The columns in the histogram show the mean changes (±SEM) in the PB frequency (expressed every minute as percentages of the control values) with time when the normal aCSF (white columns) was replaced by hypoxic aCSF for 5 min (black columns above horizontal black bar) in eight WT and five mutant preparations (*C1*, +/+, and *C2*, −/−, respectively); asterisk indicates significant changes versus control values at $p < 0.05$

Deletion of the *Phox2a* gene alters the responses of the *in vitro* RRG to NA and hypoxic aCSF

The low respiratory frequency observed *in vivo*, the lack of rhythmic PBs in some *in vitro* preparations, the low PB frequency in active preparations, and the great variability of the PB cycle duration suggested that the *Phox2a* gene deletion affected the prenatal maturation of the RRG or a source of tonic excitatory drive. We checked in E18 medullary preparations whether some central processes affecting the RRG activity might have been altered in mutants. First, we observed that the response of the mutant RRG to imposed central hypoxia was altered. Although central hypoxia has been reported to transiently induce an initial facilitation and then a depression of the respiratory rhythm produced by the RRG in brainstem slices (Thoby-Brisson and Ramirez, 2000), central hypoxia routinely depresses the PB frequency of neonatal and fetal en bloc preparations (Kawai et al., 1995; Kato et al., 2000; Blanchi et al., 2003; Viemari et al., 2003). We compared the effects of replacing oxygenated aCSF with nonoxygenated aCSF for 5 min in mutant and WT medullary preparations (Fig. 3*A*). As shown in Figure 3*B*, the mean PB frequency calculated for the 5

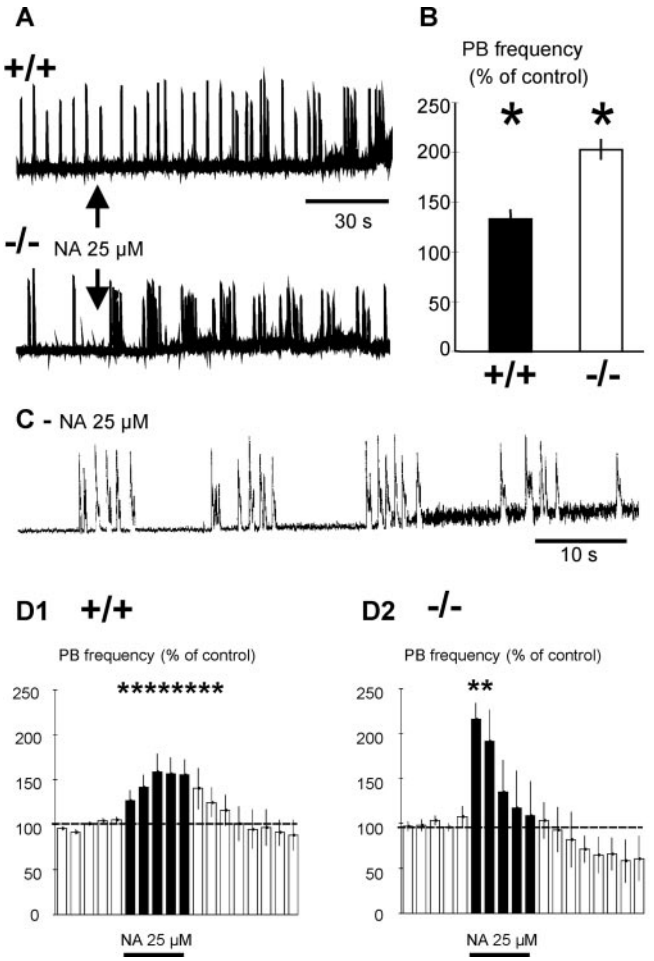

Figure 4. Altered responses of the RRG to aCSF containing NA application in medullary preparations from *Phox2a* mutant fetuses. *A*, Traces show integrated PBs recorded from the same medullary preparation under normal aCSF and the changes induced by application of aCSF containing NA for 5 min (25 μM, at the arrow) in WT and mutant preparations (top and bottom traces, respectively). Note the increased PB frequency and the grouping of the PBs in both preparations under NA. *B*, Black and gray columns show the mean PB frequency (±SEM) during the first 2 min of NA application expressed as percentages of the control for seven WT and five mutant fetuses (+/+ and −/−, respectively); asterisk indicates significant changes versus control values at $p < 0.05$. *C*, Trace illustrates the grouping of integrated PBs and the occurrence of the tonic discharge in a mutant preparation under NA (NA application started 1 min before). *D1*, *D2*, The columns in the histogram show the mean changes (±SEM) in the PB frequency (expressed every minute as percentages of the control values) with time when the normal aCSF (white columns) was replaced by aCSF containing NA for 5 min (black columns above the horizontal black bar) in seven WT and five mutant preparations (*D1*, +/+, and *D2*, −/−, respectively); asterisks indicate significant changes versus control values at $p < 0.05$.

min period of hypoxic aCSF application did not significantly change in mutant preparations (80 ± 17% of the control value; $n = 5$), whereas it decreased significantly in WT preparations (58 ± 5% of the control value; $n = 8$). The difference in response is confirmed by analyzing the time course of the PB frequency changes every minute under hypoxic aCSF. During the second and third minute of application, no significant depression in the PB frequency was observed in any of the mutant preparations (Fig. 2*C2*), whereas the PB frequency decreased to 40% of control in WT preparations (Fig. 2*C1*).

Second, we noted that the response of the mutant RRG to application of exogenous NA was also altered. NA increases the PB frequency in medullary preparations from neonatal and fetal mice (Viemari and Hilaire, 2002; Viemari et al., 2003). As shown in Figure 4*A*, replacing aCSF by aCSF containing NA (25 μM; 5 min) increased the PB frequency in both mutant ($n = 5$) and WT ($n = 7$) preparations, but some quantitative and qualitative differences were found to exist between the two. During the first 2 min of NA application, the increase in the mean PB frequency was significantly larger in mutant than in WT preparations (Fig. 4*B*). NA increased the mean PB frequency from 6.3 ± 0.9 to 12.8 ± 1.3 c/min in mutant preparations (203 ± 10% of the control value) and from 9.9 ± 0.6 to 13.2 ± 0.9 c/min in WT preparations (133 ± 8% of the control value). Therefore, the PB frequency of both mutant and WT preparations was above 12 c/min for a while; however, the NA facilitation was transient only in all of the mutant preparations (Fig. 4*D1*,*D2*), and their mean PB frequency decreased during the last 3 min of NA application, reaching the control levels (7.1 ± 0.8 c/min; 113 ± 15% of the control value; NS), whereas it remained significantly higher than control in WT preparations (15.3 ± 1.5 c/min; 154 ± 10% of the control). Replacing the aCSF containing NA by normal aCSF depressed the mean PB frequency below the control levels in mutants for 5–10 min (4.7 ± 0.5 c/min; 75 ± 10% of the control), whereas it restored the control values in WT preparations (10.9 ± 2.2 c/min; 110 ± 22% of the control). In both mutant and WT preparations, a special PB time course was observed during the NA-induced increase in PB frequency (Fig. 4*C*). The PBs fired in rhythmical groups of three to five cycles (at an intragroup frequency of 20–30 c/min) separated by long-lasting expiratory pauses (lasting ~7–10 sec). In addition, a tonic activity appeared in the cervical recordings in both mutant and WT preparations, with a 2–3 min latency as reported previously (Viemari et al., 2003).

In the heterozygous fetuses ($n = 9$), the PB frequency was depressed by nonoxygenated aCSF as in the WT fetuses, with mean PB frequency falling to 48 ± 9% of the control frequency during the 5 min application of hypoxic aCSF. The responses of the heterozygous fetuses to NA application, however, resembled the WT response in magnitude but the mutant response in time course. As in WT fetuses, the PB frequency increased significantly during the first 2 min of NA application, reaching 148 ± 12% of the control value. As in the case of the mutant fetuses, the increase in PB frequency was only transient, and the mean PB frequency recorded during the last 3 min of NA application did not differ significantly from the control value (104 ± 8%). Resuming the control aCSF then depressed the PB frequency to values that were lower than the control (70 ± 8%), as with the mutant fetuses.

Pontine A6 neurons exert a facilitatory modulation on the medullary neonatal respiratory network via α1 medullary receptors

The results presented above clearly indicated that the respiratory rhythmogenesis and the effects of central hypoxia and NA applications were abnormal in *Phox2a* mutant fetuses lacking A6 neurons. We therefore performed additional pharmacological experiments to determine the mechanisms whereby NA acted on the RRG of WT neonatal mice.

First, in P0–P2 neonatal medullary preparations (pons resected), we noted that both the NA facilitation of the PB frequency and the PB group effects were mediated by medullary α1 adrenoceptors, because the NA effects (1) were mimicked by applying the α1 adrenoceptor agonist 6-fluoro-NA (30 μM; $n = 5$), which significantly increased the PB frequency up to 141 ± 11% of the control value, and (2) were prevented by pretreating the preparations with the α1 adrenoceptor antagonist prazosin (50 μM; 5 min; $n = 7$; 105 ± 10% of the control value; NS). In addition, we observed that applying the α1 adrenoceptor antag-

onist prazosin to preparations where the pons was removed did not affect the PB frequency, which means that none of the medullary adrenergic nuclei modulated the activity of the RRG via α1 adrenoceptor activation by releasing endogenous NA. Second, we wondered whether the effects of exogenous NA might reflect some facilitatory modulation exerted on the RRG by endogenous NA released from the pontine A6 group. In neonatal rat, the pontine A5 group is known to exert a potent inhibition on the medullary RRG via medullary α2 adrenoceptors (Errchidi et al., 1990, 1991). In mice, this inhibition is strong enough to prevent the RRG from producing rhythmic PBs in preparations still containing the pons in neonates (Viemari and Hilaire, 2002) and fetuses (Viemari et al., 2003). To abolish this inhibition and trigger rhythmic PBs in preparations still containing the pontine A6 nuclei, we performed bilateral electrolytic lesions of A5 areas in the ventral pons (Fig. 5*D1*) (Viemari et al., 2003). Then, we used a partition placed at the pontomedullary level to apply aCSF containing drug to the medulla while the pons was being exposed to normal aCSF. In five preparations, applying the monoamine oxidase A inhibitor pargyline (50 μM; 10 min) to block the degradation of endogenous NA within the medulla slowly but significantly increased the mean PB frequency, which reached 124 ± 2% of the control value in the last 5 min of pargyline application (Fig. 5*A*). Washing off the aCSF containing pargyline restored PB frequency to control values within 10 min. In seven other pontomedullary preparations retaining A6 but not A5 nuclei, applying the α1 adrenoceptor antagonist prazosin to the medulla (50 μM; 5 min) to block the medullary α1 receptors (normal aCSF to the pons) significantly decreased the PB frequency to 80 ± 5% of the control frequency within 2–3 min (Fig. 5*B*). Washing off the aCSF containing prazosin restored the control PB frequency within 3–4 min. As shown in Figure 5*C*, however, when bilateral electrolytic lesions were performed on A6 nuclei (Fig. 5*D2*), prazosin applied to the medulla did not significantly alter the PB frequency (95 ± 5% of the control value; $n = 4$). The fact that pargyline increased the PB frequency and prazosin decreased the PB frequency when and only when the A6 nuclei were intact indicates that A6 nuclei exert a facilitatory modulation on the RRG via medullary α1 adrenoceptors. Finally, we examined whether there is anatomical support for this modulation, i.e., if any synaptic relationships exist between pontine A6 neurons and the medullary respiratory network. We transynaptically labeled the pontomedullary respiratory network by administering diaphragmatic injections of rabies virus, as described previously in adults (Burnet et al., 2001; Gaytan et al., 2002). Rabies virus was injected into the diaphragm of three P1 neonatal mice, and the pups were killed 48 hr later. In all the pups, rabies-infected neurons were detected in well known medullary and pontine respiratory-related areas (data not shown). In addition, rabies-infected neurons were also observed in the pontine A6 groups (Fig. 6). One to two A6-infected neurons were found in every 70 μm slice. The A6 rabies-infected neurons were located at the limits of the A6 nuclei; they were similar in shape and size to the noninfected A6 neurons, with round-shaped soma ~25 μm in diameter and large nuclei. Thus, the anatomical support for the facilitation of the RRG by endogenous NA released from the A6 neurons does exist.

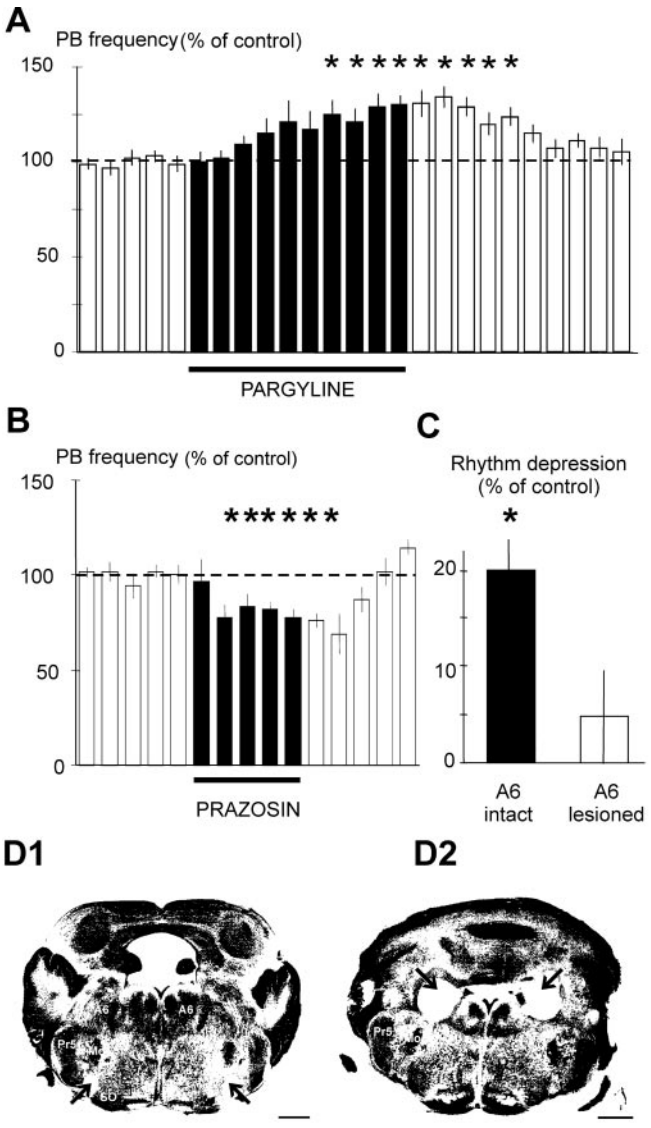

Figure 5. A6 neurons exert a permanent facilitation on the PB frequency via medullary α1 adrenoceptors in preparations from WT neonatal mice. *A*, The columns in the histogram show the mean changes (±SEM) in the PB frequency (expressed every minute as percentages of the control values) with time when the aCSF perfusing the medulla (white columns) was replaced by aCSF containing 50 μM pargyline for 10 min (black columns; normal aCSF to the pons). Asterisks significant increase in PB frequency under pargyline versus control aCSF ($n = 5$). In these pontomedullary preparations, drug application was performed after bilateral electrolytic lesion of A5 groups (*D1*). *B*, Same as in *A* (different preparations) but the aCSF perfusing the medulla (white columns) was replaced by aCSF containing 50 μM prazosin for 5 min (black columns; normal aCSF to the pons). Asterisks indicate significant decrease in PB frequency under prazosin versus control aCSF ($n = 7$). In these pontomedullary preparations, drug application was performed after bilateral electrolytic lesion of A5 groups (*D1*). *C*, The black and gray columns in the histogram show the depression of the PB frequency expressed in percentage of control values recorded after 5 min application of prazosin to the medulla (normal aCSF to the pons) when the A6 groups were intact (black column; $n = 7$) and when the A6 groups were destroyed by performing bilateral electrolytic lesions (gray column; $n = 4$). *D1*, *D2*, Histological controls showing the extent of the bilateral electrolytic lesions performed on the A5 groups (*D1*, arrows) and on the A6 groups (*D2*, arrows). Mo5, Trigeminal motor nucleus; Pr5, principal sensory trigeminal nucleus; A6, locus coerueleus, SO, superior olive. Scale bars, 500 μm.

α1 facilitatory neuromodulation is required during gestation for normal respiratory rhythm to occur at birth

To test the hypothesis that the lack of A6 neurons in *Phox2a* mutants and the resulting lack of A6 facilitation on the maturing RRG may have been responsible for the respiratory deficits described above, we prevented the α1 adrenoceptor facilitation during the late gestational period. WT pregnant mice were given daily injections of the α1 antagonist prazosin (100 mg/kg; $n = 2$) from gestational day E16 to delivery. On the day of birth, plethysmography measurements were performed on the ventilation of

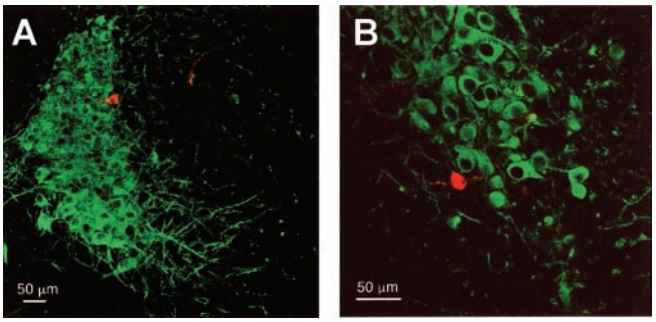

Figure 6. A6 contains neurons that are connected to the respiratory network. *A* and *B* show that the A6 nucleus of two different neonatal mice contain rabies-infected neurons 2 d after injection of 1 μl of rabies virus solution into the diaphragm.

neonates born from control and treated dams (Fig. 7*A*). They revealed that the respiratory frequency was significantly lower in the treated than in the control group (73 ± 11 and 120 ± 9 c/min in 18 treated and 12 control neonates, respectively) (Fig. 7*B*). In addition, the cycle duration was more variable in treated than control neonates (mean coefficient of variability: 0.47 ± 0.08 and 0.25 ± 0.04). Thereafter we analyzed the *in vitro* respiratory activity produced in medullary preparations (Fig. 7*C*). The mean PB frequency was significantly lower in the treated than in the control group (4.7 ± 0.7 and 9.8 ± 0.8 c/min in 12 treated and 7 control neonates, respectively) (Fig. 7*D*), and the duration of the PB cycle of the treated group was twice as variable as that of the control group (mean coefficient of variability: 0.49 ± 0.04 and 0.21 ± 0.03, respectively). Finally, we tested the effect of NA application (25 μM; 5 min) on the PB frequency produced by medullary preparations from control and treated neonates (Fig. 7*E1*,*E2*). NA significantly increased the mean PB frequency in both groups (151 ± 6 and 142 ± 7% of the control frequency in 11 treated and 8 control preparations). In addition, the time course of the NA effects was similar. Thus, the α1 adrenoceptors were not blocked by prazosin when the experiments were performed (~20–24 hr after the last prazosin injection), and even if more complex effects cannot be excluded, the respiratory alterations of the treated neonates could result, at least partly, from the lack of NA facilitation on the maturing RRG during the late gestational period.

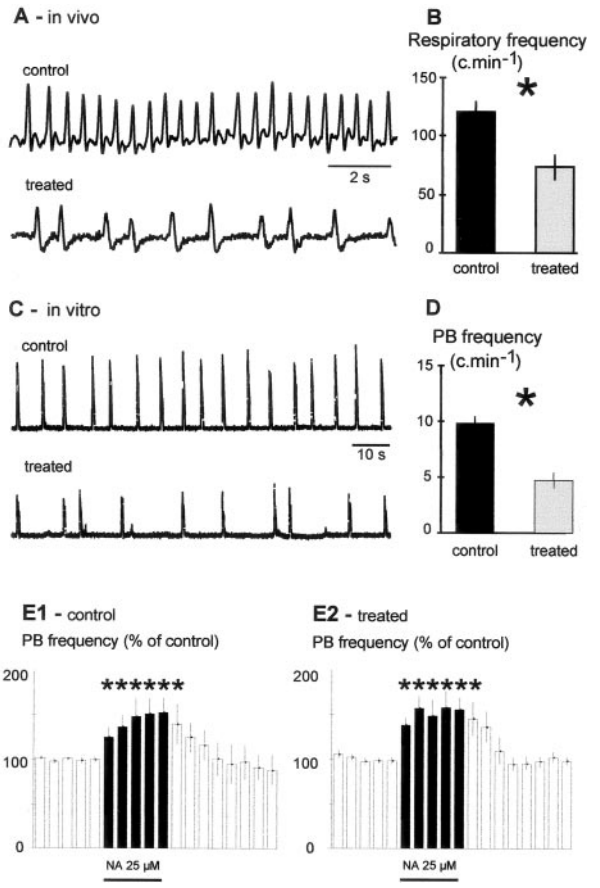

Figure 7. Altered *in vivo* and *in vitro* respiratory activity in neonatal mice born from prazosin-treated dams. *A*, Plethysmographic recordings of ventilation (inspiration upward) in neonatal mice born from control dams (top trace) and from dams to which daily injections of prazosin (100 mg/kg) were administered from gestational day 16 to birth (bottom trace). Note the variability of respiratory cycle duration in the neonate born from treated dam. *B*, Black and gray columns show the mean respiratory frequency (±SEM) of 12 control and 18 treated neonates, respectively; asterisks indicate significant difference between WT and mutant at $p < 0.05$. *C*, Integrated PBs produced by the isolated respiratory network in medullary preparations obtained from neonates born from control and prazosin-treated dams (top and bottom traces, respectively). Note the variability of PB cycle duration in the preparation from neonate born from treated dam. *D*, Black and gray columns show the mean PB frequency (±SEM) of 7 control and 12 treated neonates, respectively; asterisks indicate significant difference between WT and mutant at $p < 0.05$. *E1*, *E2*, The columns in the histogram show the mean changes (±SEM) in the PB frequency (expressed every minute as percentages of the control values) with time when the normal aCSF (white columns) was replaced by aCSF containing 25 μM NA for 5 min (black columns above the black horizontal bar) in 7 control and 12 treated neonates (*E1* and *E2*, respectively); asterisks indicate significant difference versus control at $p < 0.05$.

Discussion

Possible contribution of the *Phox2a* gene to RRG prenatal maturation

The present *in vivo* and *in vitro* data show that the *Phox2a* deletion altered both the *in vivo* ventilation and the *in vitro* activity produced by the isolated respiratory network of E18 fetuses. After delivery, the breathing parameters may be regulated by several peripheral inputs, including those arising from carotid bodies and the lungs and reaching the RRG via the IX and X cranial nerves. Because these ganglia are atrophic in *Phox2a* mutants (Morin et al., 1997), changes in these peripheral systems of regulation might alter neonatal breathing. Central deficits in respiratory rhythmogenesis are nevertheless involved, however, because the mutant RRG produced rhythmic PBs *in vitro* with an abnormally low frequency and a highly variable cycle duration. These anomalies could result from altered prenatal maturation of the RRG or from the general health of the embryo. On the one hand, the fact that 5 of 15 mutant preparations did not produce rhythmic PBs could suggest that these mutants were already in the process of dying and that the general health of all of the mutants was weak. On the other hand, the facts that some fetuses exteriorized at E18 do not survive even in WT mice (Viemari et al., 2003), that *Phox2a* mutants are able to survive several hours after birth (Morin et al., 1997), and that gasps are not more frequent in *Phox2a* mutants than in their WT littermates at E18 (present study) do not favor the hypothesis of a generally bad health with extended neurovegetative defects inducing the respiratory deficits that we observed at E18. We observed alteration of respiratory rhythmogenesis without concomitant anomalies in tidal volume and PB envelope only at E18. Thus, although other neurovegetative disorders may develop in *Phox2a* mutants within the hours after birth, contributing to their rapid death, it is likely that the disrupted respiratory rhythm of *Phox2a* mutants just

after their exteriorization at E18 reflects altered maturation of the RRG.

Possible contribution of A6 neurons to RRG prenatal maturation

Our pharmacological results in WT neonates showed that the RRG activity underwent a facilitatory modulation via the activation of medullary α1 adrenoceptors by endogenous NA released from pontine A6 neurons. In addition, our rabies virus transynaptic tracing experiments provided anatomical data supporting the existence of this pathway in neonates, in agreement with previous studies in adult rats and mice (Dobbins and Feldman, 1994; Gaytan et al., 2002). In WT mice, NA activates the fetal RRG as early as E16 (Viemari et al., 2003). Blocking fetal α1 adrenoceptors during gestation by applying prazosin treatment to their dams showed that the activation of α1 adrenoceptors by A6 neurons is required for the expression of normal respiratory rhythm at birth. In *Phox2a* mutant fetuses, endogenous levels of NA are abnormally low in the pons but not in the medulla, confirming the implication of *Phox2a* gene in the differentiation of the A6 group but not that of the other NA neurons (Pattyn et al., 2000). Therefore the respiratory impairments observed both *in vivo* and *in vitro* in the *Phox2a* mutant fetuses mean that A6 neurons and α1 receptor activation are required for normal respiratory rhythm to emerge at birth. It cannot be determined, however, whether the *Phox2a* gene is directly implicated in the maturation of some neural elements of the RRG as reported for MafB transcription factor (Blanchi et al., 2003) or whether it is indirectly implicated via disruption of the balance between inhibitory and excitatory inputs to the RRG.

Phox2a gene, A6 neurons, and RRG responses to hypoxia and NA

In adult mammals, A6 neurons send extensive axonal processes to the whole brain, receive inputs from a large number of regions, and may participate in several functions such as sleep, nociception, cardiovascular regulation, anxiety, etc. (Foote et al., 1983; Aston-Jones et al., 1986, 1991). It is worth noting that A6 neurons may also play a role in respiratory control because they display a respiratory-modulated firing, are sensitive to hypoxia, and may contribute to long-term adaptation to hypoxia (Guyenet et al., 1993; Dobbins and Feldman, 1994; Oyamada et al., 1998; Ballantyne and Scheid, 2000; Roux et al., 2000a, 2000b). Along these lines, the impaired A6 effect on the medullary RRG in *Phox2a* mutant mice may result in respiratory deficits at birth. Indeed, *Phox2a* deletion has affected the RRG responses to central hypoxia and NA. It is generally recognized that the O_2 levels affect respiratory rhythm by acting at both the peripheral and central levels. First, any peripheral blood hypoxia is rapidly detected by the carotid body receptors, which in turn activate the RRG. This activation already exists by E18 because peripheral hypoxia significantly increases the breathing frequency of exteriorized WT fetuses (Viemari et al., 2003; present results). In *Phox2a* mutants, despite the reported atrophia of cranial sensory ganglia (Morin et al., 1997), the respiratory response to peripheral hypoxia is preserved in three of four fetuses. Second, if the respiratory response to peripheral hypoxia fails to restore blood normoxia, central hypoxia develops and depresses the RRG (Eden and Hanson, 1987; Neubauer et al., 1990; Gauda and Lawson, 2000). This depression has been studied mainly in neonatal (Kawai et al., 1995; Kato et al., 2000; Thoby-Brisson and Ramirez, 2000) and fetal *in vitro* preparations (Viemari et al., 2003). Here we show that central hypoxia induced by application of nonoxygenated aCSF to the isolated respiratory network at E18 depresses the PB frequency in WT mice but has no significant effects in *Phox2a* mutants. Concerning the effect of NA, we confirmed the finding that exogenous NA increases the PB rhythm via α1 adrenoceptors in neonatal and fetal mice (Viemari and Hilaire, 2002; Viemari et al., 2003). In addition, we show that the NA effect is altered in all of the *Phox2a* mutant preparations. The inability of the mutant RRG to react correctly to changes in O_2 and NA central levels may contribute, at least in part, to their rapid death after birth.

Developmental and pathological aspects

The early expressed and widely distributed neuromodulators NA and serotonin are known to affect prenatal maturational processes in the CNS (Lauder, 1993; Thomas et al., 1995; Levitt et al., 1997; Weiss et al., 1998). The very high endogenous serotonin levels occurring in monoamine oxydase A-deficient mice (Cases et al., 1995; Lajard et al., 1999) affect respiratory network maturation, but the resulting alterations are not drastic and do not affect pups' survival (Bou-Flores et al., 2000; Burnet et al., 2001), whereas the lack of A6 neurons in *Phox2a* mutants significantly alters the respiratory rhythmogenesis at birth and is lethal. The differentiation of NA central neurons during CNS development is controlled by several homeobox genes such as the two closely related members of the Phox/aristaless family of paired-class homeobox genes, the Phox2a and Phox2b transcription factors, and the homeodomain protein Rnx/Tlx3 and bHLH transcription factor Mash1 (Tiveron et al., 1996; Pattyn et al., 1997, 1999, 2000; Hirsch et al., 1998; Lo et al., 1998; Qian et al., 2001, 2002). All of these genes are linked in transcriptional cascades controlling the NA phenotype, but their respective positions differ from one NA cell group to another; *Rnx* deletion compromises the formation of most NA groups but partly preserved that of A6, whereas *Phox2a* deletion leads to the agenesis of A6 but does not affect the other NA groups. The *Rnx*- and *Phox2a*-deficient mice die within 24 hr but show opposite respiratory deficits; *Rnx* mutants have an abnormally high respiratory rate interrupted by apneas (Shirasawa et al., 2000), whereas *Phox2a* mutants have a low respiratory rate. These findings tend to confirm the idea that endogenous NA plays a crucial role in the prenatal maturation of the CNS (Thomas et al., 1995) and that the proper development of the NA brainstem groups is required for normal activity of the RRG to occur.

Among various clinical breathing disorders (Gaultier and Guilleminault, 2001), sudden infant death syndrome (SIDS) is the leading cause of postneonatal infant death in the industrial world. The pathogenesis of SIDS is still unknown, but SIDS may be caused by environmental risk factors and genetic susceptibilities affecting the development of three functions in which NA neurons are involved: i.e., respiratory, circulatory, and sleep–wake regulation (Takashima and Becker, 1991; Filiano and Kinney, 1994). In victims of SIDS, several authors have noted catecholaminergic anomalies such as altered activity of the NA synthesis enzyme tyrosine hydroxylase in the brain (Ozand and Tildon, 1983) and in the ventrolateral reticula, vagal nuclei, and basal ganglia (Obonai et al., 1998; Ozawa et al., 1999), lack of adrenergic neurons in the dorsal medulla (Chigr et al., 1989; Kopp et al., 1993), abnormal morphology of medullary NA neurons (Takashima and Becker, 1991), and altered cerebrospinal fluid bioamine metabolites (Cann-Moisan et al., 1999). Although these authors did not clearly establish that one particular NA group is affected in SIDS victims, they all suggest that impaired NA neurons are involved. Our results show that the proper development of the A6 group is required for normal RRG activity to

occur in mice. Keeping in mind the fact that interspecies differences may well exist in prenatal maturation of mouse and human RRG and that several genes are involved in transcriptional cascades controlling NA phenotype in various nuclei, analysis of the expression of the genes required for NA neuron development in SIDS victims should shed some light on the genetic alterations underlying this tragic syndrome.

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