

ANESTHESIOLOGY

Breathing under Anesthesia

A Key Role for the Retrotrapezoid Nucleus Revealed by Conditional *Phox2b* Mutant Mice

Thomas Bourgeois, M.Eng., Maud Ringot, M.Eng.,
Nelina Ramanantsoa, M.Eng., Boris Matrot, M.Eng.,
Stéphane Dager, M.D., Ph.D., Christophe Delclaux, M.D., Ph.D.,
Jorge Gallego, Ph.D.

ANESTHESIOLOGY 2019; 130:995–1006

EDITOR'S PERSPECTIVE

What We Already Know about This Topic

- Many if not all drugs used in anesthesia and analgesia can produce potentially severe respiratory depression
- Maintenance of breathing under anesthesia is linked to the drive exerted by the retrotrapezoid nucleus on the respiratory central pattern generator
- The retrotrapezoid nucleus neurons that stimulate breathing during anesthesia are carbon dioxide–sensitive noncatecholaminergic neurons that express *Phox2b*, a master gene for the development of autonomic neurons
- The conditional mouse model with the +7Ala repeat mutation targeted to the retrotrapezoid nucleus (*Phox2b*^{27Ala/+/+} mice) present a massive selective loss of retrotrapezoid nucleus neurons and lack carbon dioxide chemosensitivity at birth but survive normally and partially recover carbon dioxide chemosensitivity in adulthood

What This Article Tells Us That Is New

- Ketamine, propofol, and fentanyl caused lethal respiratory failure in most mice with selective genetic loss of retrotrapezoid nucleus neurons, at doses that were safe in their wild type littermates

Respiratory depression associated with sedation and anesthesia is a persistent and puzzling problem in anesthesiology.^{1–4} In the last decade, particular attention has been paid to the respiratory depressant effects of opioids,

ABSTRACT

Background: Optimal management of anesthesia-induced respiratory depression requires identification of the neural pathways that are most effective in maintaining breathing during anesthesia. Lesion studies point to the brainstem retrotrapezoid nucleus. We therefore examined the respiratory effects of common anesthetic/analgesic agents in mice with selective genetic loss of retrotrapezoid nucleus neurons (*Phox2b*^{27Ala/+/+} mice, hereafter designated “mutants”).

Methods: All mice received intraperitoneal ketamine doses ranging from 100 mg/kg at postnatal day (P) 8 to 250 mg/kg at P60 to P62. Anesthesia effects in P8 and P14 to P16 mice were then analyzed by administering propofol (100 and 150 mg/kg at P8 and P14 to P16, respectively) and fentanyl at an anesthetic dose (1 mg/kg at P8 and P14 to P16).

Results: Most mutant mice died of respiratory arrest within 13 min of ketamine injection at P8 (12 of 13, 92% vs. 0 of 8, 0% wild type; Fisher exact test, $P < 0.001$) and P14 to P16 (32 of 42, 76% vs. 0 of 59, 0% wild type; $P < 0.001$). Cardiac activity continued after terminal apnea, and mortality was prevented by mechanical ventilation, supporting respiratory arrest as the cause of death in the mutants. Ketamine-induced mortality in mutants compared to wild types was confirmed at P29 to P31 (24 of 36, 67% vs. 9 of 45, 20%; $P < 0.001$) and P60 to P62 (8 of 19, 42% vs. 0 of 12, 0%; $P = 0.011$). Anesthesia-induced mortality in mutants compared to wild types was also observed with propofol at P8 (7 of 7, 100% vs. 0 of 17, 0%; $P < 0.001$) and P14 to P16 (8 of 10, 80% vs. 0 of 10, 0%; $P < 0.001$) and with fentanyl at P8 (15 of 16, 94% vs. 0 of 13, 0%; $P < 0.001$) and P14 to P16 (5 of 7, 71% vs. 0 of 11, 0%; $P = 0.002$).

Conclusions: Ketamine, propofol, and fentanyl caused death by respiratory arrest in most mice with selective loss of retrotrapezoid nucleus neurons, in doses that were safe in their wild type littermates. The retrotrapezoid nucleus is critical to sustain breathing during deep anesthesia and may prove to be a pharmacologic target for this purpose.

(ANESTHESIOLOGY 2019; 130:995–1006)

which are particularly strong compared to those of other agents. Nonetheless, many if not all drugs used for anesthesia and analgesia can produce potentially severe respiratory depression.^{5,6} Although we probably cannot fully prevent respiratory depression during anesthesia,⁷ active research is underway to identify respiratory stimulants capable of minimizing this effect without overly affecting anesthesia.⁴ Knowledge about the toxicity and efficacy of these drugs is still insufficient to allow clinical use.⁴ Identifying the cell types and neural pathways that sustain breathing during deep anesthesia may facilitate the development of effective respiratory stimulants.

Supplemental Digital Content is available for this article. Direct URL citations appear in the printed text and are available in both the HTML and PDF versions of this article. Links to the digital files are provided in the HTML text of this article on the Journal's Web site (www.anesthesiology.org). Part of the work presented in this article has been presented at the joint meeting of the Federation of European Physiological Societies and the French Physiological Society in Paris, France, June 29 to July 1, 2016.

Submitted for publication July 24, 2018. Accepted for publication February 1, 2019. From Institut National de la Santé et de la Recherche Médicale (INSERM, National Institute of Health and Medical Research, UMR 1141), Paris Diderot University, Paris, France (T.B., M.R., N.R., B.M., S.D., C.D., J.G.); and the Pediatric Intensive Care Unit (S.D.) and Physiology Department (C.D.), Robert Debré Hospital, Paris, France.

Copyright © 2019, the American Society of Anesthesiologists, Inc. Wolters Kluwer Health, Inc. All Rights Reserved. Anesthesiology 2019; 130:995–1006

Lesion studies suggest that breathing maintenance during anesthesia may involve the drive supplied by the retrotrapezoid nucleus to the respiratory central pattern generator.^{5,8,9} The retrotrapezoid nucleus is a small group of glutamatergic neurons located in the rostral ventrolateral medulla, whose activation by arterial carbon dioxide stimulates breathing.¹⁰ In anesthetized cats and rats, neuronal lesions produced in the retrotrapezoid nucleus region by the neurotoxin kainic acid or by electrolysis caused apnea.^{11–13} However, both these lesion-induction techniques lack selectivity, as recently pointed out by Souza *et al.*¹⁴ Studies of saporin–substance P injections showed that the retrotrapezoid nucleus neurons responsible for stimulating breathing during anesthesia were carbon dioxide–sensitive noncatecholaminergic neurons that expressed *Phox2b*,¹⁴ a master gene for the development of autonomic neurons.¹⁵ In anesthetized rats, 70% destruction of these retrotrapezoid–nucleus carbon dioxide–sensitive chemoreceptors substantially raised the carbon dioxide apneic threshold, thereby predisposing to apnea.¹⁶

To look for further evidence that the retrotrapezoid nucleus is pivotal in breathing maintenance during anesthesia, we examined breathing patterns in anesthetized mice with selective genetic depletion of the retrotrapezoid nucleus neurons. Loss of retrotrapezoid nucleus neurons has been reported in mouse models of congenital central hypoventilation syndrome, a life-threatening disorder characterized by hypoventilation during sleep and absence of the ventilatory response to carbon dioxide.^{17,18} The disease-causing mutations in congenital central hypoventilation syndrome are generally polyalanine repeat expansion mutations of *PHOX2B*.¹⁹ Knock-in mice bearing the 7-alanine expanded allele (*i.e.*, 27Ala) of *PHOX2B* (the most frequent mutation in patients¹⁹) exhibit massive loss of retrotrapezoid nucleus neurons, lack carbon dioxide chemosensitivity, and die within hours after birth.²⁰ In contrast, in the conditional mouse model with the +7Ala repeat mutation targeted to the retrotrapezoid nucleus (*Phox2b*^{27Ala/+/+} mice, hereafter designated “mutants”), despite massive selective loss of retrotrapezoid nucleus neurons and absence of carbon dioxide chemosensitivity at birth, survival is normal and carbon dioxide chemosensitivity recovers partially in adulthood.²¹ We hypothesized that mice with selective genetic depletion of retrotrapezoid nucleus neurons would be prone to severe respiratory failure during anesthesia.

Materials and Methods

Mice

All experimental protocols were approved by local and national ethics committees (Ministry of Higher Education and Scientific Research, Directorate-General for Research and Innovation, Paris, France), in accordance with the European Communities Council Directive 2010/63/EU for animal care. The experiments in the main study were conducted in mutant mice and their wild type littermates

at postnatal day (P) 8, which approximately corresponds to term in humans,²² and P14 to P16, corresponding to infancy in humans.²² Complementary studies were conducted at P29 to P31, corresponding to adolescence,²³ and at P60 to P62, corresponding to early adulthood. The mice that survived anesthesia were killed at P8 by decapitation and beyond P8 by cervical dislocation, without anesthesia. The experiments were conducted in mice of both sexes. The proportions of males and females at birth were not significantly different in the wild types (125 males and 97 females in total; male-to-female ratio, 1.29) and mutants (86 males and 87 females in total; male-to-female ratio, 0.99, Fisher exact test; $P = 0.192$).

We generated conditional, tissue-specific, knock-in *Phox2b*^{27Ala/+/+} mice as previously described.²¹ Briefly, upon *cre* recombinase-mediated recombination, mouse *Phox2b* exon 3 was replaced by the mutated human exon 3 bearing the seven-residue expansion. Because human and mouse *Phox2b* protein sequences are identical, the encoded protein is identical to mouse *Phox2b* except for the extension of the polyalanine stretch. Offspring with the recombined locus were produced by crossing *Egr2*^{cre/+} males with *Phox2b*^{27Ala/+/+} females. Genotyping was performed with tail DNA after completion of the protocol and data processing. To detect the presence of the *Phox2b*^{27Ala} allele, the primers GCCCAGTGCCTCTTAAC and CTCTTAAACGGGCGTCTCAC were used, yielding bands of 330bp for the wild type gene, 474bp for the mutated gene, and 380bp for the recombined allele. To detect *cre*, the primers AAATTTGCCTGCATTACCG and ATGTTTAGCTGGCCCAAATG were used, yielding a band of 200bp.

Treatments

In the main study, ketamine anesthesia was administered to mice at P8 (8 wild types, 13 mutants) and P14 to P16 (59 wild types, 42 mutants) and in complementary studies at P29 to P31 (45 wild types, 36 mutants) and P60 to P62 (12 wild types, 19 mutants). We chose ketamine as an anesthetic/analgesic agent with minimal effects on central respiratory drive.²⁴ We also tested propofol and fentanyl at P8 (propofol: 17 wild types, 7 mutants; fentanyl: 13 wild types, 16 mutants) and P14 to P16 (propofol: 10 wild types, 10 mutants; fentanyl: 11 wild types, 7 mutants). Both propofol and fentanyl markedly depress respiration.²⁴ Ketamine, propofol, and fentanyl are widely used for pediatric and adult anesthesia/sedation and act mainly by different mechanisms.^{5,25}

All agents were administered by intraperitoneal injection in the lower left quadrant of the abdomen, near the midline and umbilicus, taking care to avoid any visible milk spots. A 19-mm/30-gauge needle was inserted at an angle of 45° to the abdominal wall. The maximal volume administered was 20 μ l/g. All injections were performed by the same experimenter.

The doses were as follows: ketamine, 100 mg/kg at P8, 150 mg/kg at P14 to P16, and 250 mg/kg at P29 to P31 and P60 to P62; propofol, 100 and 150 mg/kg at P8 and P14 to P16, respectively; and fentanyl, 1 mg/kg at P8 and P14 to P16. These doses were previously shown to rapidly produce deep anesthesia at all studied ages, as assessed by the loss of righting and tail-pinch responses,²⁶ with no or minimal mortality in wild type mice. They were at the upper end of the intraperitoneal ketamine,²⁷ propofol,²⁸ and fentanyl²⁹ dose ranges previously used in mice. The investigators were blinded to genotype, which was visually indiscernible. The genotypes were determined after completion of each protocol and therefore, cardiorespiratory data processing and mortality recordings were performed without previous knowledge of genotypes.

Pharmacologic Manipulation of Serotonin Neuronal Function

In a separate experiment, we analyzed the possible involvement of the serotonergic system (which is not affected by *Phox2b* mutation²⁰) in the response to anesthesia in mutants and wild type littermates. In rodents, serotonin produces an excitatory effect on the pre-Bötzinger complex, mediated by 5-hydroxytryptamine 2 receptors.³⁰ We tested serotonin system involvement by using the 5-hydroxytryptamine 2_{A/C} antagonist ketanserin at P29 to P31 (10 mg/kg intraperitoneal, combined with 150 mg/kg ketamine in 25 wild types, 16 mutants, *vs.* ketamine alone, in 22 wild types, 7 mutants). The litters were alternately assigned to the ketanserin and control groups.

Plethysmography

Breathing variables were measured noninvasively by using a battery of four custom-made, whole-body flow barometric plethysmographs (fig. 1, A–D), as previously described.²¹ Data were collected in all P8 mice (38 wild types, 36 mutants) and a large subset of P14 to P16 mice (69 wild types, 47 mutants). Each plethysmograph was composed of two 100-ml Plexiglas chambers (fig. 1B) immersed in a thermoregulated water bath to maintain the temperature at 32°C to prevent anesthesia-induced hypothermia. A 200 ml · min⁻¹ flow of dry air (Brooks airflow stabilizer, Urlo, The Netherlands) was injected in each chamber. The differential pressure between the chambers (GE Sensing transducer, France; range, \pm 0.1 millibar) was converted into a digital signal at a sampling rate of 100 Hz and processed by using Labview software (National Instruments, USA). The apparatus was calibrated before each session by using a built-in pump incorporating a microsyringe (Ito Inc., Japan), which injected a sinusoidal airflow into the animal chamber with maximal amplitude 2 μ l and frequency 6 Hz. We measured breath duration (s) and tidal volume (ml · g⁻¹) on a breath-by-breath basis to calculate minute ventilation (tidal volume · breath duration⁻¹ · 60, ml · min⁻¹ · g⁻¹). The limitations of

the plethysmographic method in newborn mice have been discussed elsewhere.³¹ Briefly, plethysmography is effective for measuring breath duration (or frequency) and apneas but only provides semiquantitative measurements of tidal volume and minute ventilation because of gas-compression effects related to airway resistance.³¹ In particular, upper airway obstruction may affect tidal volume and ventilation values. By allowing simultaneous measurements in the same pup, and in four pups simultaneously, our experimental setup contributed to reduce experimental variability and sample size, thereby meeting the ethical requirements of reducing and refining animal use in research.

Electrocardiography

The plethysmograph chambers were equipped with electrocardiography recording platforms composed of four rectangular gold electrodes insulated from one another and embedded in the floor of the chamber (fig. 1, B–E). Conduction was enhanced by using electrode hydrogel (Sekisui Plastics, Japan). Signals were digitized at a sampling rate of 1,000 Hz (16 bits, PCI-6229, National Instruments, USA). An electrocardiograph signal was obtained when at least three paws were in contact with three different electrodes or, occasionally, when the pup was lying on the floor.

Mechanical Ventilation

In a separate study, P14 to P16 mice anesthetized with 150 mg/kg ketamine (22 wild types, 7 mutants) were mechanically ventilated with air until the anesthesia wore off. The negative pressure ventilator comprised a set of chambers connected to a common negative-pressure generator allowing for simultaneous ventilation of up to 60 mice. Each mouse was placed for 2 h in a custom setting that allowed mechanical ventilation by intermittent extra-thoracic negative pressure. The head of the animal was carefully placed in a thin latex ring, which was then affixed to a polycarbonate chamber so that the body was inside and the head outside. An intermittent negative-pressure generator alternately connected the chamber to the atmosphere or to a low-pressure vacuum source. Ventilation frequency was set at 3 Hz, duty cycle at 0.2, and peak negative pressure in the chamber at 14 cm H₂O. This pressure value was chosen to generate respiratory movements whose amplitude and frequency approximately corresponded to those during spontaneous breathing in mice of the same strain and age, according to preliminary pneumotachography measurements (not shown).

Design

The study design is summarized in figure 1F. In Phase 1 (preanesthesia carbon dioxide test, 15 min), mice were randomly taken from each litter in successive groups of four, weighed, and placed together in the plethysmograph chambers. After 5 min of familiarization with the chamber, baseline

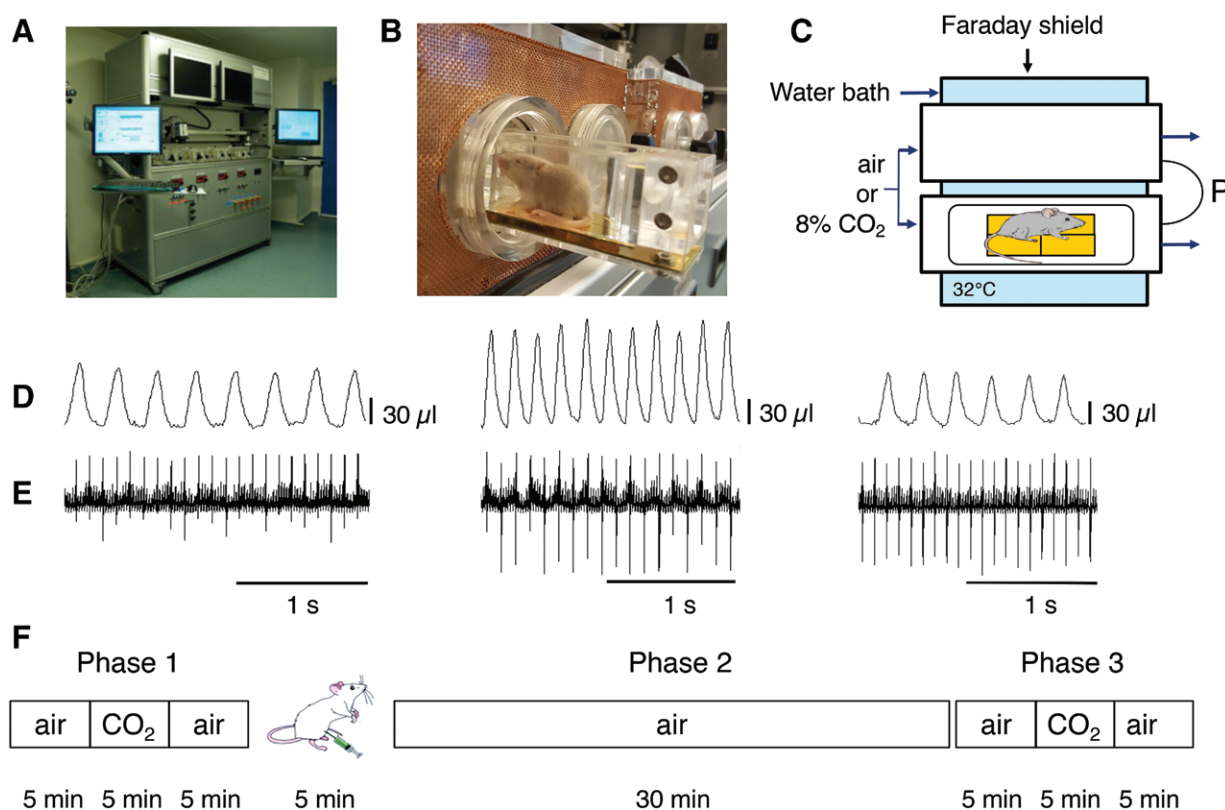


Fig. 1. Experimental setup and protocol. (A) General view of the cardiorespiratory recording system for simultaneous and noninvasive measurements of ventilation and electrocardiography. Four pups were tested simultaneously. (B) Each pup was installed in the plethysmograph chamber in which it could move freely. Electrocardiography was recorded *via* four rectangular gold electrodes embedded in the floor of each chamber. (C) Breathing variables were derived from the difference in pressure (P) between the two thermoregulated chambers. (D and E) Examples of ventilatory and electrocardiography traces during air, hypercapnic challenge, and air again in a postnatal day 15 mice before anesthesia. (F) Graphical representation of the experimental protocol in postnatal day 8 and postnatal day 14 to postnatal day 16 mice. Each mouse was initially placed in the plethysmograph for a 15-min recording comprising an 8% carbon dioxide (CO₂) challenge (Phase 1). Then, the mice were extracted from the chamber to receive an intraperitoneal injection of ketamine, propofol, or fentanyl. Within a 4-min delay, mice were re-placed in the plethysmograph for a 30-min physiologic recording (Phase 2) followed by a second 8% CO₂ challenge (Phase 3, identical to Phase 1). Mortality was assessed after completion of Phase 3.

breathing and heart rate were recorded over 5 min. Then, the airflow through the plethysmograph was switched to 8% CO₂ + 21% O₂ + 71% N₂ at the same flow rate (200 ml/min per chamber) for 5 min, after which the flow was switched back to normoxia for 5 min. In Phase 2 (air breathing under anesthesia, 30 min), the four mice were extracted from the plethysmograph chambers, given anesthetics, and replaced simultaneously in the plethysmograph chambers within 4 min after the injection, for a 30-min baseline recording. Phase 3 (posttreatment carbon dioxide test, 15 min) was identical to Phase 1. The primary outcome of the study was mortality caused by anesthesia in the mutants compared to the wild types. Mortality was assessed immediately after completion of Phase 3, based on cardiorespiratory signal monitoring while the mice were in the plethysmograph and on immobility, unresponsiveness to pinch, and discoloration. Survivors were

reunited with their dams. Adolescent and adult mice (P29 to P31 and P60 to P62) did not undergo plethysmography; they were anesthetized with ketamine, and mortality was then assessed for 60 min. All the experiments were performed during the day, between 9:00 AM and 6:30 PM.

Statistical Analysis

Anesthesia-induced mortality was compared between mutant and wild type mice, at each age and with each anesthetic agent, by applying the two-tailed Fisher exact test. The experimental unit was the animal (n refers to the number of animals in each group). Weights and baseline cardiorespiratory variables were compared by two-tailed independent *t* tests. The time-course of the ventilatory response to hypercapnia was analyzed by averaging minute ventilation over consecutive 30-s

periods throughout the 15-min plethysmographic recording (5 min air, 5 min hypercapnia, 5 min air) at P8 and P14 to P16. These data were compared between mutant and wild type mice using two-way repeated measures ANOVA with genotype (wild types *vs.* mutants) as a between-subject factor and time period (1 to 30) as a repeated factor. Bonferroni *post hoc* tests were performed to compare time-matched minute ventilation data between genotypes. For all parametric tests, normality of data was assessed by the Shapiro–Wilk test. Data are mean \pm SD in the text, tables, and figures. No statistical power calculation was conducted before the study. The sample size was based on our previous experience with respiratory variability in this genetic model. All analyses were done using R Studio v1.1.243 and JMP Pro 13 (SAS Institute, Inc., USA). Differences were considered statistically significant if $P < 0.05$. Sex had no statistically significant effect in any analyses and will not be mentioned further.

Results

Disruption of Carbon Dioxide Sensitivity in Mutant Mice

At P8 and P14 to P16, the mutants ($n = 36$ and $n = 47$, respectively) weighed slightly less than their wild type littermates ($n = 38$ and $n = 69$, respectively; table 1) but were normal in general appearance and behavior. First, we verified that the *Phox2b*^{27Alacki/+} mutation effectively disrupted the carbon dioxide-sensitive drive, as previously shown,²¹ by performing whole-body plethysmography at P8 and P14 to P16. Mutant and wild type mice at P8 did not differ significantly regarding baseline breathing variables (*i.e.*, before anesthesia, Phase 1, table 1). At P14 to P16, mutant mice showed significantly longer breath durations (*i.e.*, lower breathing frequencies) and marginally smaller ventilation values than wild type mice. As expected, at P8, mutants lacked any ventilatory response to carbon dioxide, in contrast to wild type mice (genotype-by-time interaction, $P < 0.001$; fig. 2A). A slight response was present at P14 to P16 but was significantly smaller than in the wild type mice (genotype-by-time interaction, $P < 0.001$; fig. 2B). This disruption of carbon dioxide

sensitivity in mutant mice confirms previous results.²¹ Wild type mice sustained a regular breathing pattern and a vigorous response to hypercapnia (fig. 2, A and B).

Ketamine-induced Mortality in Mutant Mice

Ketamine anesthesia produced high mortality rates in mutant mice at all studied ages, including adulthood, whereas all or most wild type mice survived (table 2). The sedative effect extended beyond completion of Phase 3 in all surviving mice, including mutants.

Ketamine-induced Mortality Was Due to Respiratory Arrest

The cause of death was first determined by analyzing the cardiorespiratory recordings. Figure 3 shows a typical sequence of respiratory events in a P14 to P16 mutant mouse given ketamine. A period of eupneic breathing (fig. 3A) was followed by a sequence of short clusters of gasps, in pairs (“double gasps”) or triplets (“triple gasps”), separated by apneas (fig. 3B). This sequence was followed by a series of gasps of decreasing amplitude ending with terminal apnea (fig. 3C), usually during Phase 2 (within 13 min after ketamine administration). However, the time to terminal apnea varied widely across individuals, with some mice dying before being placed in the plethysmograph (*i.e.*, within 4 min after ketamine administration). The electrocardiograph signal consistently outlasted the terminal gasps, although the heart rate was reduced (fig. 4). These findings support respiratory arrest as the cause of death, as opposed to cardiac arrest.

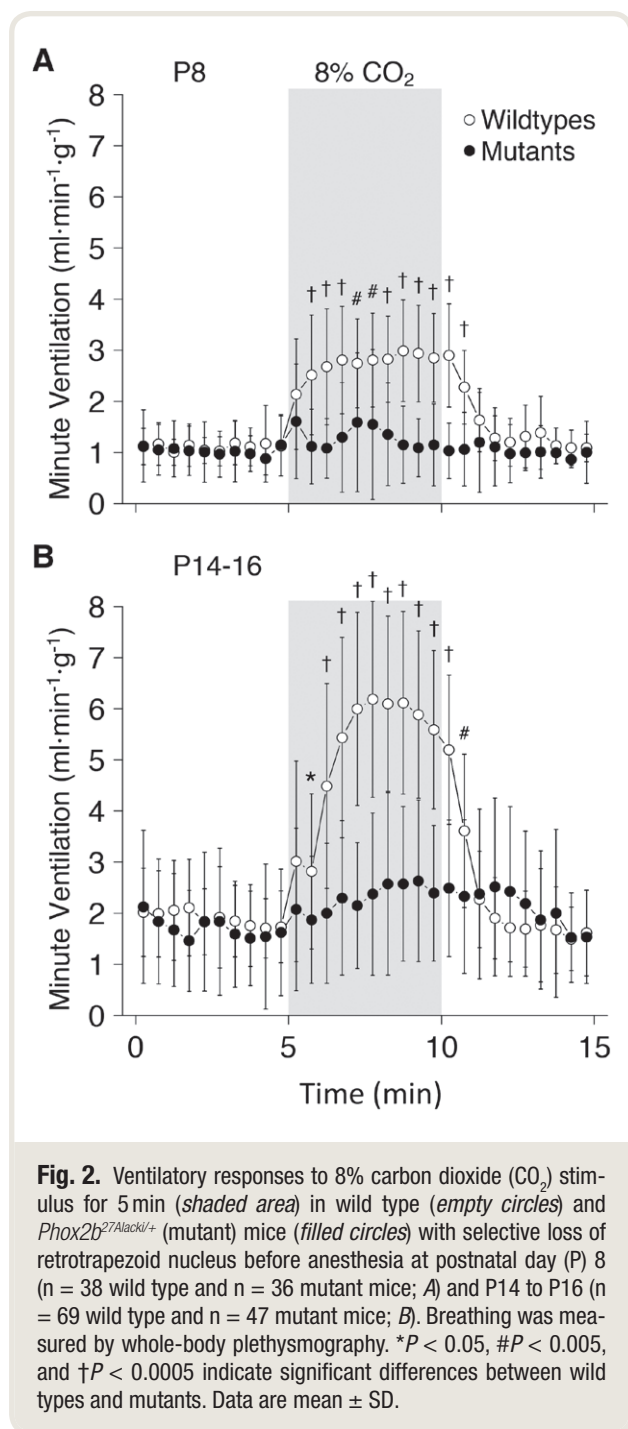
That death was due to respiratory arrest in ketamine-exposed mutants was further confirmed by exposing a separate group of P14 to P16 mutants ($n = 7$) and wild type littermates ($n = 22$) to mechanical ventilation immediately after a 150-mg/kg ketamine injection. The mice were ventilated until the effects of the anesthesia wore off, as reflected by gross body movements. The end of anesthesia varied widely among mice, from 60 min to 90 min after ketamine injection. In contrast to the high mortality rate

Table 1. Baseline Respiratory Variables for Wild Type and *Phox2b*^{27Alacki/+} Mutant Mice at P8 and P14 to P16

Variables	P8		<i>P</i> Value	P14 to P16		<i>P</i> Value
	Wild Type <i>n</i> = 38	Mutant <i>n</i> = 36		Wild Type <i>n</i> = 69	Mutant <i>n</i> = 47	
Weight (g)	4.53 \pm 0.49	4.02 \pm 0.49	< 0.001*	7.91 \pm 1.27	6.92 \pm 1.00	< 0.001*
Breath duration (s)	0.28 \pm 0.04	0.30 \pm 0.04	0.130	0.25 \pm 0.07	0.31 \pm 0.09	0.002*
Tidal volume (ml·g ⁻¹ ·10 ⁻³)	5.1 \pm 0.8	4.9 \pm 0.9	0.432	7.5 \pm 1.3	7.2 \pm 1.5	0.263
Minute ventilation (ml·min ⁻¹ ·g ⁻¹)	1.1 \pm 0.2	1.0 \pm 0.3	0.158	1.9 \pm 0.6	1.6 \pm 0.8	0.047*

Data are mean \pm SD calculated over the 5 min preceding the onset of hypercapnia (see fig. 1).

* $P < 0.05$ by two-tailed independent *t* test. Respiratory recording could not be obtained because of technical failure in one wild type postnatal day (P) 14 to P16 mouse.



caused by 150-mg/kg ketamine in nonventilated mutants (32 of 42, 76%; table 2), all ventilated mutants survived anesthesia (exact Fisher test, ventilated *vs.* nonventilated mutants, $P < 0.001$), as did all wild type mice, confirming respiratory arrest as the cause of ketamine-induced death in nonventilated mutant mice. Monitoring for 3 days after anesthesia confirmed that all ventilated mice survived and appeared normal, ruling out any major delayed effect of ketamine anesthesia.

To further analyze the respiratory effects of ketamine in mutants, we first compared breathing variables in mutants and their wild type littermates throughout the recording session (see Supplemental Digital Content 1, <http://links.lww.com/ALN/B908>, showing breathing variables in wild types and mutants from Phase 1 to Phase 3, and distinguishing surviving and nonsurviving mutants in Phase 1). Before ketamine treatment, the mutants had no ventilatory response to carbon dioxide. In both genotypes, ketamine depressed minute ventilation, mainly by decreasing tidal volume and increasing breath duration. However, the wild types tended to recover pre-ketamine levels for all variables, whereas ventilation in the mutant survivors remained depressed throughout Phases 2 and 3. The inspiratory-to-expiratory ratio was also markedly affected by ketamine in mutant survivors, suggesting impaired respiratory phase timing. Then, we analyzed individual plots of nonsurviving mutants (Supplemental Digital Content 2, <http://links.lww.com/ALN/B909>, which is a zoom of Phase 2, including wild types, mutant survivors, and mutant nonsurvivors with available respiratory data until the onset of gasping). No clear differences in respiratory variables were observed between surviving and nonsurviving mutants.

Effect of the Serotonin System on Ketamine-induced Mortality

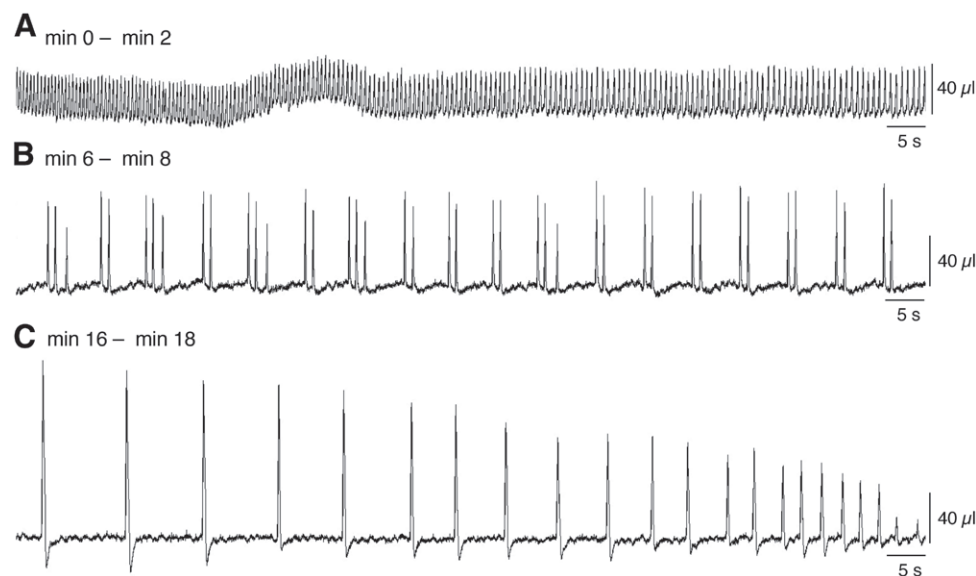
We then examined whether survival of a subgroup of ketamine-exposed mutants was due to the serotonergic drive to the respiratory central pattern generator. Serotonergic terminals are found throughout brainstem respiratory regions, and both serotonin and the peptides released by serotonergic cells modify the activity of many types of respiratory neurons.⁸ In a separate experiment, we addressed the contribution of the serotonergic drive (which is not affected by *Phox2b* gene mutation²⁰) by combining 10 mg/kg ketanserin, a serotonin 5-hydroxytryptamine $2_{A/C}$ antagonist, with 150 mg/kg ketamine, administered to P29 to P31 mice (25 wild types, 16 mutants), and comparing the findings to those in a group given only ketamine (22 wild types, 7 mutants).

The 150 mg/kg ketamine dose was nonlethal in P29 to P31 mutants (table 3). However, when combined with ketanserin, ketamine caused death in 11 of 16 (69%) mutants (exact Fisher test, ketanserin-treated *vs.* untreated mutants, $P = 0.005$). All but one ketanserin-treated wild type mouse survived (table 3). These results support a role for the serotonin system in protecting mutants against ketamine-induced respiratory arrest. However, our attempt to decrease mortality in ketamine-exposed mutants by using the 5-hydroxytryptamine 2 agonist 1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane in doses of 0.75 to 1.5 mg/kg did not yield statistically significant results (0.75 mg/kg: $P > 0.999$; 1 mg/kg: $P = 0.121$; 1.5 mg/kg: $P = 0.184$).

Table 2. Ketamine-induced Mortality Rates in *Phox2b*^{27Alacki/+} Mutant and Wild Type Mice

Age (days)	Ketamine Dose (mg/kg)	Genotype	No.	No. Alive	No. Dead	Mortality Rate (%)	95% CI	P Value*
8	100	Wild type	8	8	0	0	(0–32)	< 0.001
		Mutant	13	1	12	92	(67–99)	
14–16	150	Wild type	59	59	0	0	(0–6)	< 0.001
		Mutant	42	10	32	76	(61–86)	
29–31	250	Wild type	45	36	9	20	(11–34)	< 0.001
		Mutant	36	12	24	67	(50–80)	
60–62	250	Wild type	12	12	0	0	(0–24)	0.011
		Mutant	19	11	8	42	(23–64)	

*Fisher exact test

**Fig. 3.** Respiratory arrest in a *Phox2b*^{27Alacki/+} mutant mouse at postnatal day 15 after ketamine administration. Breathing pattern, initially normal at the onset of recording (A), was followed after 3- to 4-min recording by apneas interspersed with clusters of gasps (B), and after 16-min recording by a series of gasps gradually diminishing in size until terminal apnea (C).

Extrapolation to Propofol and Fentanyl

We then examined whether the lethal effects of ketamine anesthesia in mutants extended to other anesthetic/analgic agents with different mechanisms of action. We exposed mutants and their wild type littermates to propofol (P8: 17 wild types, 7 mutants, 100 mg/kg; and P14 to P16: 10 wild types, 10 mutants, 150 mg/kg) or fentanyl (P8: 13 wild types, 16 mutants, 1 mg/kg; and P14 to P16, 11 wild types, 7 mutants, 1 mg/kg; table 3). Both agents produced very high mortality rates in mutants but no mortality in wild type mice (table 4), further supporting the mutants' vulnerability to anesthesia previously suggested by ketamine-induced mortality. The respiratory traces in propofol-exposed mice displayed similar patterns of gasps and apneas as did those

seen with ketamine (see Supplemental Digital Content 3, <http://links.lww.com/ALN/B910>, showing respiratory arrest in a P15 mutant mouse after propofol administration). Fentanyl-exposed mice exhibited severely disrupted ventilation from the onset of the recording, also followed by a sequence of gasps that gradually diminished in size until terminal apnea (see Supplemental Digital Content 4, <http://links.lww.com/ALN/B911>, showing respiratory arrest in a P15 mutant mouse after fentanyl administration).

Discussion

In this study, ketamine, propofol, and fentanyl caused respiratory arrest in most mice with selective genetic loss of retrotrapezoid nucleus neurons, in doses that were safe in their

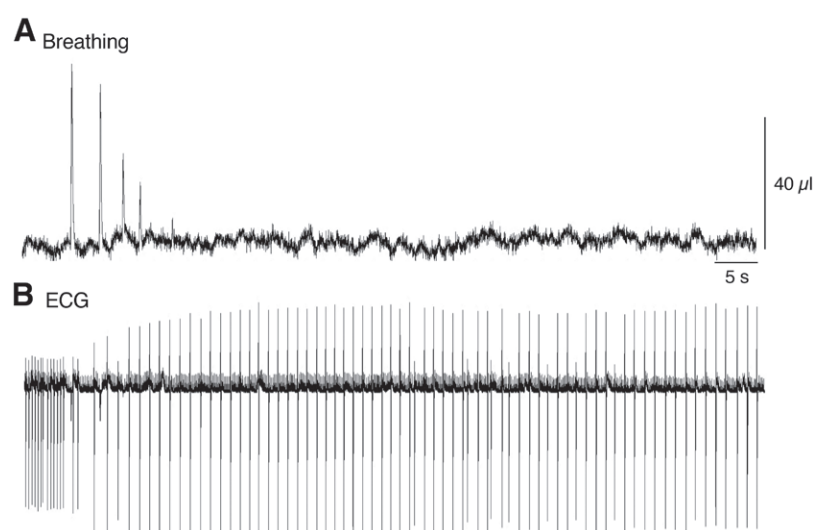


Fig. 4. Illustrative cardiorespiratory traces in a *Phox2b*^{27Alackl/+} mutant mouse at postnatal day (P) 8 exposed to ketamine. (A) The end of the series of gasps gradually diminishing in size followed by terminal apnea. (B) Cardiac activity outlasted the terminal apnea, showing that death was caused by respiratory arrest. P14 to P16 mice presented similar patterns of lethal respiratory arrests. ECG, electrocardiography.

Table 3. Effect of Ketanserin on Ketamine-induced Mortality in Postweaning *Phox2b*^{27Alackl/+} Mutant Mice

Age (days)	Treatment (dose) (mg/kg ip)	Genotype	No.	No. Alive	No. Dead	Mortality Rate (%)	95% CI	P Value*
29–31	Ketamine (150)	Wild type	22	22	0	0	(0–15)	> 0.999
		Mutant	7	7	0	0	(0–35)	
29–31	Ketamine (150) + ketanserin (10)	Wild type	25	24	1	4	(0–20)	< 0.001
		Mutant	16	5	11	69	(44–86)	

ip, Intraperitoneal.

*Fisher exact test.

wild type littermates. These results support a pivotal role for the retrotrapezoid nucleus in maintaining spontaneous breathing during deep anesthesia. The serotonin system probably contributed to sustain breathing during anesthesia in retrotrapezoid nucleus neuron-depleted mice.

Central Respiratory Failure

That the high mortality in the ketamine-exposed mutants was due to respiratory arrest was first established by examining the order of respiratory and cardiac arrests and was subsequently confirmed by showing that the mutants survived if given mechanical ventilation. In mutant mice, ketamine anesthesia produced apneas interspersed with clusters of gasps, a common marker of hypoxia. Gasps (and sighs) are generated by the pre-Bötzinger complex,³² which does not express *Phox2b* and therefore is spared by *Phox2b* gene mutations.³³ Clusters of double or triple gasps have never

been reported in ketamine-anesthetized animals; there is a single report in piglets sedated with 20 mg/kg intraarticular pentobarbital sodium administration.³⁴ However, this pattern is often reported in dying preterm infants and in infants who subsequently experienced sudden infant death syndrome.³⁵ Thus, in both these human cases and our mutant mice, the lack of successful autoresuscitation was not related to an inability to gasp.^{35,36} Rather, the anesthetized mutants probably died of hypoxic hypoventilation caused by disruption of the excitatory drive sent by the retrotrapezoid nucleus to the central pattern generator.

The recurrent apnea in anesthetized mutants suggested that absence of the retrotrapezoid nucleus may have caused an increase in the carbon dioxide apneic threshold. In an earlier study, the carbon dioxide apneic threshold was increased by 70% destruction of carbon dioxide-sensitive chemoreceptors in the retrotrapezoid nucleus of anesthetized rats.¹⁶ Our mutants exhibited similar or higher loss

Table 4. Propofol and Fentanyl-induced Mortality in *Phox2b*^{27Alacki/+} Mutant Mice

Treatment	Age (days)	Dose (mg/kg)	Genotype	No.	No. Alive	No. Dead	Mortality Rate (%)	95% CI	P Value*
Propofol	8	100	Wild type	17	17	0	0	(0–18)	< 0.001
			Mutant	7	0	7	100	(65–100)	
	14–16	150	Wild type	10	10	0	0	(0–28)	< 0.001
			Mutant	10	2	8	80	(49–94)	
Fentanyl	8	1	Wild type	13	13	0	0	(0–23)	< 0.001
			Mutant	16	1	15	94	(72–99)	
	14–16	1	Wild type	11	11	0	0	(0–26)	0.002
			Mutant	7	2	5	71	(36–92)	

*Fisher exact test.

rates.²¹ Furthermore, ketamine, propofol, and μ -opioid agonists also increase the carbon dioxide apneic threshold, in a dose-dependent manner.^{25,37} Breathing was not impaired by anesthesia alone (as indicated by the breathing pattern in anesthetized wild types) or neuronal loss alone (as indicated by the breathing pattern in non-anesthetized mutants). However, when combined in anesthetized mutants, these two factors caused respiratory arrest, probably by increasing the carbon dioxide apneic threshold.

Interindividual Variability

Few mutant mice survived ketamine, propofol, or fentanyl anesthesia, and mortality was highest in the youngest animals. Individual differences in the vulnerability to anesthesia possibly reflected variations in retrotrapezoid nucleus cell loss caused by the *Phox2b*^{27Alacki/+} mutation.²¹ In previous studies, the number of residual cells in the retrotrapezoid nucleus of mutant mice was estimated at 15 to 20% of the mean value in wild types.²¹ This range is close to the threshold at which pharmacologic destruction of the retrotrapezoid nucleus disrupted phrenic nerve discharge in rats.¹⁶ In anesthetized and ventilated rats, bilateral 70% destruction of retrotrapezoid nucleus carbon dioxide-sensitive chemoreceptors (*i.e.*, 30% spared neurons) substantially raised the carbon dioxide apneic threshold.¹⁶ Also, in rats with unilateral 70% retrotrapezoid nucleus neuron destruction, acute inhibition of the contralateral intact retrotrapezoid nucleus with muscimol instantly abolished phrenic nerve discharge.¹⁶ Cell counting was not done in the current study, but the mutant mice that survived anesthesia may have been those with more numerous spared cells in the retrotrapezoid nucleus and, therefore, with a stronger residual excitatory drive to the central pattern generator.¹⁴

Serotonergic Contribution to Breathing

The key role of the retrotrapezoid nucleus in sustaining breathing during anesthesia does not preclude contributions of other neuronal systems. In particular, activation of the serotonergic raphe neurons stimulates ventilation, and

the full effects of carbon dioxide on breathing require activity of these neurons.¹⁰ Therefore, we examined whether the serotonin system contributed to sustain breathing during anesthesia in mutants. We found that the 5-hydroxytryptamine 2 receptor antagonist ketanserin caused death in mutants despite being used in doses that were safe in wild types when administered alone. This finding supports a role in breathing maintenance during anesthesia of the excitatory drive from serotonergic neurons to the respiratory network (especially the pre-Bötzing complex), *via* 5-hydroxytryptamine 2 receptors.

This analysis, however, has several limitations. First, the 5-hydroxytryptamine 2 receptor agonist 2,5-dimethoxy-4-iodoamphetamine failed to significantly reduce ketamine-induced mortality in P14 to P16 mutants. Second, the role of the serotonin system revealed by ketanserin may reflect a compensatory adaptation to retrotrapezoid nucleus loss specific to mutant mice. Third, a previous report that knock-out mice with disrupted 5-hydroxytryptamine 2_A receptor signaling survived anesthesia with ketamine 150 mg/kg³⁸ would seem at variance with our findings. We focused on the 5-hydroxytryptamine 2 receptor because of its excitatory effect on respiratory rhythm generation, but the effects of 5-hydroxytryptamine 1,4 receptor agonists, which counteract opioid-induced ventilatory depression,^{1,4,39} also deserve attention. Finally, we cannot discount that ketanserin increased ketamine-induced respiratory depression and mortality *via* its α 1-adrenergic receptor blocking properties in residual retrotrapezoid nucleus cells⁴⁰ or in the pre-Bötzing complex.⁴¹ This possible effect might explain why the 5-hydroxytryptamine 2 agonist 1-(2,5-dimethoxy-4-iodophenyl)-2-amino-propane did not decrease ketamine-induced mortality. The role for serotonin pathways in sustaining breathing during anesthesia clearly requires further investigation.

Mono- versus Polysynaptic Inputs

The critical role of the retrotrapezoid nucleus during deep anesthesia probably reflects its strategic location and function in the central pattern generator. One important

characteristic of retrotrapezoid nucleus neurons is their monosynaptic input to the respiratory central pattern generator,⁴² which contrasts with the chiefly polysynaptic inputs from other sources (e.g., the peripheral chemosensory drive, arousal-related suprapontine activation, and exercise-related central command). Polysynaptic pathways are strongly affected by anesthetics due to cumulation of effects along the signaling chain.⁴³ Furthermore, the pontine parabrachial-Kölliker-Fuse complex, which controls expiratory duration and the inspiratory on-switch,^{44,45} has strong reciprocal connections with the retrotrapezoid nucleus.⁴⁶ The loss of retrotrapezoid input in mutants may have contributed to the disruption of respiratory phase timing in this group. Therefore, the cumulative effect of anesthetic agents on synaptic transmission, combined with retrotrapezoid neuron depletion, may account for the vulnerability of mutants to all anesthetics studied, despite their different cellular targets and properties.

The most parsimonious explanation of anesthesia-induced respiratory arrest in mutant mice is that massive loss of retrotrapezoid nucleus drive combined with silencing of polysynaptic respiratory pathways fully abolished the respiratory drive to the central pattern generator. However, our data do not rule out the possibility that respiratory arrest in the mutants involved mechanisms related to the specific cellular targets and respiratory central pattern generator effects of each anesthetic.^{5,25} Ketamine is an *N*-methyl-D-aspartate receptor antagonist that also depresses ventilation *via* γ -aminobutyric acid type A and opioid receptors.⁴⁷ Propofol depresses ventilation *via* the γ -aminobutyric acid type A receptor. The glutamatergic pre-Bötzing complex and the pontine parabrachial-Kölliker-Fuse complex, which, as noted above, control expiratory duration and the inspiratory on-switch,^{44,45} express opioid receptors. Possibly, activation of one of these pathways may be sufficient to disrupt the function of a system made inherently unstable by retrotrapezoid neuronal loss.

Finally, *in vitro* studies recently showed that isoflurane exposure aggravated the toxic effects of the mutated *PHOX2B* gene (i.e., *PHOX2B* protein misfolding, aggregation, and loss of nuclear localization) in cultured cells with the +7Ala expansion.⁴⁸ Further research is needed to determine how these effects may affect the function of residual retrotrapezoid nucleus neurons in mutant mice.

The current results may explain why patients with congenital central hypoventilation syndrome, most of whom carry *PHOX2B* polyalanine expansions, are prone to major anesthesia-related complications that require profound perioperative precautions.⁴⁹ They also suggest that the often used combination of opioids and propofol, which act additively on anesthesia induction,⁵⁰ may be particularly detrimental to patients with congenital central hypoventilation syndrome.

Conclusions

Mice with selective loss of retrotrapezoid nucleus neurons were highly vulnerable to deep anesthesia induced by

ketamine, propofol, or fentanyl. Most of them died of respiratory arrest when anesthetized, in contrast to their wild type littermates. These results confirm the pivotal role of the retrotrapezoid nucleus in breathing maintenance during deep anesthesia. They suggest that drugs capable of selectively activating retrotrapezoid nucleus neurons may hold promise for preventing anesthesia-induced respiratory depression.

Research Support

Support was provided solely from institutional sources, Association Française du Syndrome d'Ondine (French Association of Ondine Syndrome; Paris, France) and Legs Poix (Poix Legacy Fund, Chancellery of Paris Universities; Paris, France).

Competing Interests

The authors declare no competing interests.

Correspondence

Address correspondence to Dr. Gallego: Institut National de la Santé et de la Recherche Médicale (INSERM), Robert-Debré Hospital, 48 Bd Sérurier, 75019 Paris, France. jorge.gallego@inserm.fr. Information on purchasing reprints may be found at www.anesthesiology.org or on the masthead page at the beginning of this issue. ANESTHESIOLOGY's articles are made freely accessible to all readers, for personal use only, 6 months from the cover date of the issue.

References

1. Ren J, Ding X, Greer JJ: 5-HT_{1A} receptor agonist Befiradol reduces fentanyl-induced respiratory depression, analgesia, and sedation in rats. *ANESTHESIOLOGY* 2015; 122:424–44
2. Linz K, Schröder W, Frosch S, Christoph T: Opioid-type respiratory depressant side effects of cebranopadol in rats are limited by its nociceptin/orphanin FQ peptide receptor agonist activity. *ANESTHESIOLOGY* 2017; 126:708–15
3. van der Schier R, Roozkrans M, van Velzen M, Dahan A, Niesters M: Opioid-induced respiratory depression: Reversal by non-opioid drugs. *F1000Prime Rep* 2014; 6:79
4. Dahan A, van der Schier R, Smith T, Aarts L, van Velzen M, Niesters M: Averting opioid-induced respiratory depression without affecting analgesia. *ANESTHESIOLOGY* 2018; 128:1027–37
5. Teppema LJ, Baby S: Anesthetics and control of breathing. *Respir Physiol Neurobiol* 2011; 177:80–92
6. Dahan A, Roozkrans M, van der Schier R, Smith T, Aarts L: Primum non nocere or how to resolve

- drug-induced respiratory depression. *ANESTHESIOLOGY* 2013; 118:1261–3
7. Dahan A, Teppema LJ: Influence of anaesthesia and analgesia on the control of breathing. *Br J Anaesth* 2003; 91:40–9
 8. Guyenet PG: The 2008 Carl Ludwig Lecture: Retrotrapezoid nucleus, CO₂ homeostasis, and breathing automaticity. *J Appl Physiol* (1985) 2008; 105:404–16
 9. Smith JC, Abdala AP, Borgmann A, Rybak IA, Paton JF: Brainstem respiratory networks: Building blocks and microcircuits. *Trends Neurosci* 2013; 36:152–62
 10. Guyenet PG, Bayliss DA: Neural control of breathing and CO₂ homeostasis. *Neuron* 2015; 87:946–61
 11. Nattie EE, Li AH: Fluorescence location of RVLM kainate microinjections that alter the control of breathing. *J Appl Physiol* (1985) 1990; 68:1157–66
 12. Nattie EE, Li AH, St John WM: Lesions in retrotrapezoid nucleus decrease ventilatory output in anesthetized or decerebrate cats. *J Appl Physiol* (1985) 1991; 71:1364–75
 13. Nattie EE, Li A: Retrotrapezoid nucleus lesions decrease phrenic activity and CO₂ sensitivity in rats. *Respir Physiol* 1994; 97:63–77
 14. Souza GMPR, Kanbar R, Stornetta DS, Abbott SBG, Stornetta RL, Guyenet PG: Breathing regulation and blood gas homeostasis after near complete lesions of the retrotrapezoid nucleus in adult rats. *J Physiol* 2018; 596:2521–45
 15. Pattyn A, Morin X, Cremer H, Goridis C, Brunet JF: The homeobox gene *Phox2b* is essential for the development of autonomic neural crest derivatives. *Nature* 1999; 399:366–70
 16. Takakura AC, Moreira TS, Stornetta RL, West GH, Gwilt JM, Guyenet PG: Selective lesion of retrotrapezoid *Phox2b*-expressing neurons raises the apnoeic threshold in rats. *J Physiol* 2008; 586:2975–91
 17. Weese-Mayer DE, Berry-Kravis EM, Ceccherini I, Keens TG, Loghmanee DA, Trang H; ATS Congenital Central Hypoventilation Syndrome Subcommittee: An official ATS clinical policy statement: Congenital central hypoventilation syndrome: Genetic basis, diagnosis, and management. *Am J Respir Crit Care Med* 2010; 181:626–44
 18. Gallego J: Genetic diseases: Congenital central hypoventilation, Rett, and Prader-Willi syndromes. *Compr Physiol* 2012; 2:2255–79
 19. Amiel J, Laudier B, Attié-Bitach T, Trang H, de Pontual L, Gener B, Trochet D, Etchevers H, Ray P, Simonneau M, Vekemans M, Munnich A, Gaultier C, Lyonnet S: Polyalanine expansion and frameshift mutations of the paired-like homeobox gene *PHOX2B* in congenital central hypoventilation syndrome. *Nat Genet* 2003; 33:459–61
 20. Dubreuil V, Ramanantsoa N, Trochet D, Vaubourg V, Amiel J, Gallego J, Brunet JF, Goridis C: A human mutation in *Phox2b* causes lack of CO₂ chemosensitivity, fatal central apnea, and specific loss of parafacial neurons. *Proc Natl Acad Sci USA* 2008; 105:1067–72
 21. Ramanantsoa N, Hirsch MR, Thoby-Brisson M, Dubreuil V, Bouvier J, Ruffault PL, Matrot B, Fortin G, Brunet JF, Gallego J, Goridis C: Breathing without CO(2) chemosensitivity in conditional *Phox2b* mutants. *J Neurosci* 2011; 31:12880–8
 22. Semple BD, Blomgren K, Gimlin K, Ferriero DM, Noble-Haesslein LJ: Brain development in rodents and humans: Identifying benchmarks of maturation and vulnerability to injury across species. *Prog Neurobiol* 2013; 106–107:1–16
 23. Brust V, Schindler PM, Lewejohann L: Lifetime development of behavioural phenotype in the house mouse (*Mus musculus*). *Front Zool* 2015; 12(suppl 1):S17
 24. Dahan A: General anesthesia and control of respiration. *Semin Anesth* 1996; 15: 328–34
 25. Stuth EA, Stucke AG, Zuperku EJ: Effects of anesthetics, sedatives, and opioids on ventilatory control. *Compr Physiol* 2012; 2:2281–367
 26. Drobac E, Durand E, Laudénbach V, Mantz J, Gallego J: A simple method for short-term controlled anesthesia in newborn mice. *Physiol Behav* 2004; 82:279–83
 27. Zuurbier CJ, Koeman A, Janssen BJ: Letter to the editor: Ketamine-only versus isoflurane effects on murine cardiac function: Comparison at similar depths of anesthesia? *Am J Physiol Heart Circ Physiol* 2015; 309:H2160
 28. Kubo K, Nishikawa K, Hardy-Yamada M, Ishizeki J, Yanagawa Y, Saito S: Altered responses to propofol, but not ketamine, in mice deficient in the 65-kilodalton isoform of glutamate decarboxylase. *J Pharmacol Exp Ther* 2009; 329:592–9
 29. Kendall LV, Hansen RJ, Dorsey K, Kang S, Lunghofer PJ, Gustafson DL: Pharmacokinetics of sustained-release analgesics in mice. *J Am Assoc Lab Anim Sci* 2014; 53:478–84
 30. Peña-Ortega F: Tonic neuromodulation of the inspiratory rhythm generator. *Front Physiol* 2012; 3:253
 31. Ren J, Lenal F, Yang M, Ding X, Greer JJ: Coadministration of the AMPAKINE CX717 with propofol reduces respiratory depression and fatal apneas. *ANESTHESIOLOGY* 2013; 118:1437–45
 32. Lieske SP, Thoby-Brisson M, Telgkamp P, Ramirez JM: Reconfiguration of the neural network controlling multiple breathing patterns: Eupnea, sighs and gasps [see comment]. *Nat Neurosci* 2000; 3:600–7
 33. Pattyn A, Morin X, Cremer H, Goridis C, Brunet JF: Expression and interactions of the two closely related homeobox genes *Phox2a* and *Phox2b* during neurogenesis. *Development* 1997; 124:4065–75

34. Sanocka UM, Donnelly DF, Haddad GG: Autoresuscitation: A survival mechanism in piglets. *J Appl Physiol* (1985) 1992; 73:749–53
35. Sridhar R, Thach BT, Kelly DH, Henslee JA: Characterization of successful and failed autoresuscitation in human infants, including those dying of SIDS. *Pediatr Pulmonol* 2003; 36:113–22
36. Tomori Z, Donic V, Benacka R, Gresova S, Peregrim I, Kundrik M, Pallayova M, Jakus J: Reversal of functional disorders by aspiration, expiration, and cough reflexes and their voluntary counterparts. *Front Physiol* 2012; 3:467
37. Dahan A, Nieuwenhuijs D, Olofsen E: Influence of propofol on the control of breathing, *Advances in Modelling and Clinical Application of Intravenous Anaesthesia*. Edited by Vuyk J, Schraag S. Boston, MA, Springer, 2003, pp 81–94
38. Morici JF, Ciccio L, Malleret G, Gingrich JA, Bekinschtein P, Weisstaub NV: Serotonin 2a receptor and serotonin 1a receptor interact within the medial prefrontal cortex during recognition memory in mice. *Front Pharmacol* 2015; 6:298
39. Czick ME, Waldman JC, Gross JB: Sources of inspiration: A neurophysiologic framework for understanding anesthetic effects on ventilatory control. *Curr Anesthesiol Rep* 2014; 4:67–75
40. Kuo FS, Falquetto B, Chen D, Oliveira LM, Takakura AC, Mulkey DK: *In vitro* characterization of noradrenergic modulation of chemosensitive neurons in the retrotrapezoid nucleus. *J Neurophysiol* 2016; 116:1024–35
41. Viemari JC, Ramirez JM: Norepinephrine differentially modulates different types of respiratory pacemaker and nonpacemaker neurons. *J Neurophysiol* 2006; 95:2070–82
42. Bochorishvili G, Stornetta RL, Coates MB, Guyenet PG: Pre-Bötzinger complex receives glutamatergic innervation from galaninergic and other retrotrapezoid nucleus neurons. *J Comp Neurol* 2012; 520:1047–61
43. Banoub M, Tetzlaff JE, Schubert A: Pharmacologic and physiologic influences affecting sensory evoked potentials: Implications for perioperative monitoring. *ANESTHESIOLOGY* 2003; 99:716–37
44. Zuperku EJ, Stucke AG, Hopp FA, Stuth EA: Characteristics of breathing rate control mediated by a subregion within the pontine parabrachial complex. *J Neurophysiol* 2017; 117:1030–42
45. Zuperku EJ, Stucke AG, Krolikowski JG, Tomlinson J, Hopp FA, Stuth EA: Inputs to medullary respiratory neurons from a pontine subregion that controls breathing frequency. *Respir Physiol Neurobiol* 2018 [Epub ahead of print]
46. Rosin DL, Chang DA, Guyenet PG: Afferent and efferent connections of the rat retrotrapezoid nucleus. *J Comp Neurol* 2006; 499:64–89
47. Sarton E, Teppema LJ, Olivier C, Nieuwenhuijs D, Matthes HW, Kieffer BL, Dahan A: The involvement of the mu-opioid receptor in ketamine-induced respiratory depression and antinociception. *Anesth Analg* 2001; 93:1495–500, table of contents
48. Coghlan M, Richards E, Shaik S, Rossi P, Vanama RB, Ahmadi S, Petroz C, Crawford M, Maynes JT: Inhalational anesthetics induce neuronal protein aggregation and affect ER trafficking. *Sci Rep* 2018; 8:5275
49. Basu SM, Chung FF, AbdelHakim SF, Wong J: Anesthetic considerations for patients with congenital central hypoventilation syndrome: A systematic review of the literature. *Anesth Analg* 2017; 124:169–78
50. Ben-Shlomo I, Finger J, Bar-Av E, Perl AZ, Etchin A, Tverskoy M: Propofol and fentanyl act additively for induction of anaesthesia. *Anaesthesia* 1993; 48:111–3