ERJ Express. Published on September 27, 2006 as doi: 10.1183/09031936.00111705

Inhibitory Effects of Repeated Hyperoxia on Breathing in Newborn Mice

Frédéric Lofaso,[1,2] Stéphane Dauger,[1,3] Boris Matrot,[1] Guy Vardon,[4] Claude Gaultier,[1] Jorge Gallego[1]

[1]INSERM U676, Hôpital Robert-Debré, 48 Boulevard Sérurier, 75019 Paris, France

[2]AP-HP, Hôpital Raymond Poincaré, Service de Physiologie, 104 av Raymond Poincaré, 92380 Garches, France

[3] AP-HP, Hôpital Robert-Debré, Service de Réanimation Pédiatrique, 48 Boulevard Sérurier, 75019 Paris, France

[4]Faculté de Médecine d'Amiens, 3 rue des Louvels, Amiens 80036, France

Running head: Repeated Hyperoxia in Newborn Mice

Send all correspondence including reprint requests to

Dr F. LOFASO, Service de Physiologie-Explorations Fonctionnelles, Hôpital Raymond Poincaré, 92380 GARCHES, FRANCE

Tel: (33-1) 47 10 79 41; Fax: (33-1) 47 10 79 43; E-mail: f.lofaso@rpc.ap-hop-paris.fr

Copyright 2006 by the European Respiratory Society.

ABSTRACT

Question of the study: Brief oxygen therapy is commonly used for resuscitation at birth or for preventing hypoxemia before procedures in neonatal period in term and premature newborn infants. However, O_2 may severely depress breathing, especially when administered repeatedly. The aim of this study was to test the effects of repeated hyperoxia on breathing control in newborn mice.

Materials: We randomly assigned 97 Swiss mice to O_2 or air on postnatal day 0, 1, or 2. Each pup in the O_2 group was subjected to four hyperoxic tests (100% O_2 for 3 min followed by 12 min normoxia), whereas pups in the air group were maintained in normoxia. Breathing variables were measured using flow–through barometric plethysmography.

Results: Oxygen significantly decreased V_E because of a decrease in respiratory rate. This decrease became significantly larger with repeated exposure ($P<0.0005$) and ranged from –17% to –26% for all ages combined. Furthermore, hyperoxia increased total apnoea duration, as compared to the baseline value ($P<0.03$).

Answer to the question: In newborn mice, repeated hyperoxia increasingly depressed breathing. This finding further supports a need for stringent control of O_2 therapy, most notably repeated oxygen administration, in neonatal period in term and premature newborn infants.

Key words: oxygen, respiration, neonate

BACKGROUND

Oxygen (O_2) is the most commonly used treatment at birth and is an integral part of the respiratory support provided in neonatal units. Approximately 5% to 10% of neonates require resuscitation at birth (1), and most of these neonates are born prematurely (2). Furthermore, short periods of oxygen therapy are used preventively in critically ill neonates to prevent hypoxemia when mechanical ventilation is not available, especially during transfer to the neonatal intensive care unit and before lumbar puncture (3), tracheal suction (4, 5), bronchoscopy (6, 7), and bottle feeding (5, 8).

The use of 100% O_2 instead of room air has been challenged (1) based on studies showing cerebral blood flow reduction (9), generation of free O_2 radicals that cause or worsen brain injury (2, 10-12), and increased rates of bronchopulmonary dysplasia (13) and retinopathy (14). In addition, the inhibitory effects of hyperoxia on breathing may compromise oxygenation after O_2 administration, particularly in preterm humans, who are more susceptible to apnoeas (15). Although this inhibitory effect of hyperoxia may be considered minor compared to other adverse effects of oxygen therapy, it may become worrisome when O_2 is administered repeatedly, for instance to non-ventilated preterm infants with recurrent cyanosis after O_2 withdrawal. We reasoned that repeated hyperoxia, which alternately inhibits and stimulates chemoreceptors (upon return to normoxia), may induce potentiation, which is commonly observed with a variety of respiratory stimuli such as hypoxia (16, 17). For ethical reasons, these effects are difficult to investigate in human neonates.

The aim of this study was to assess the inhibitory effects of repeated hyperoxia (administration of 100% O_2) on ventilation in newborn mice. Breathing variables were measured non-invasively in freely moving pups to mimic human infants. The newborn mice were tested from birth (postnatal day 0, P0) to P2. The postnatal resetting of peripheral

chemoreceptors occurs during this period in mice (18). P2 is a period of high vulnerability for white matter injury, which is a major consequence of hypoxia in the newborn brain, and corresponds to the high-risk period from 23 to 32 weeks postconceptional age in humans (19).

METHODS

Animals

Mice pups from Swiss female mice (IFFA-CREDO, L'Arbresle, France) were housed at 24°C with a 12h/12h light/dark cycle and fed *ad libitum*. The experimental protocols complied with the animal research guidelines established by the Institut National de la Santé et de la Recherche Médicale (French national institute for health and medical research). Pups were examined on the first day of life (P0), with a 12-hour uncertainty regarding time from birth to testing, on P1, or on P2. At each age, the pups were randomly assigned to O_2 or air with a 2:1 ratio. The weight differences between the O_2 and air groups were not significant at any of the three ages. We ran two complementary experiments in 2-day old pups (see below).

Whole-body flow plethysmography

Respiratory variables in unrestrained newborn mice were measured non-invasively using whole-body flow barometric plethysmography as previously described (20). The mice were left unrestrained because restraint may affect baseline ventilation and ventilatory responses to chemical stimuli, as previously shown in adult mice (21).

Movements were detected based on changes in the baseline respiratory signal, using a previously validated criterion: ($|Vi-Ve|/(Vi+Ve)$ where Vi and Ve were the magnitudes of the inspiratory and expiratory limbs of the volume signal, respectively (20).

The plethysmograph was composed of two Plexiglas cylinders serving as the animal (40 ml) and reference (70 ml) chambers, respectively, immersed in a thermoregulated water-bath set that maintained their temperature at 32.8 °C. A 100 $ml \cdot min^{-1}$ flow of dry air (Bronkhorst

Hi-Tec airflow stabilizer, Uurlo, Holland) was divided into two 50 ml·min^{-1} flows through the chambers, thus avoiding carbon dioxide (CO_2) and water accumulation. The differential pressure between the two chambers (DRUCK-EFFA transducer, Asnières, France; range±0.1 mb) was filtered (bandwidth, 0.05-15 Hz at -3 dB), converted to a digital signal (Instrunet model 200, 14-bits converter, GW-INSTRUMENTS, Somerville, MA) at a sample rate of 100 Hz, and processed by custom-written software (Software Superscope II, GW-INSTRUMENTS). The time constant of the pressure decay within the system (0.35 seconds) was measured by injecting 2 µl into the measurement chamber. This allowed measurement of breathing frequencies within the 0.5 Hz-10 Hz range at -3dB. Calibration was done before each session by injecting 2 µl of air into the animal chamber from a microsyringe (Ito Corporation, Japan). The pressure rise induced by this injection was of similar magnitude to that induced by a pup. Body temperature was not continuously recorded during ventilatory measurements but was measured immediately after the plethysmographic recordings. Considering the limitations of flow barometric plethysmography in newborn mice, the absolute values of tidal volume (V_T) and minute ventilation (V_E) presented here should be considered with caution, whereas the absolute total respiratory time (T_{TOT}) values are valid.

Design

Each pup was tested once, on P0, P1, or P2. At each age, the pups were randomly assigned to oxygen or air with ratios of 3:1 (P0 and P1) or 2:1 (P2). Mean weights (SD) in the O_2 group were 1.49 g (SD 0.26) on P0 (n=22), 1.72 g (SD 0.11) on P1 (n=11), and 1.95 g (SD 0.27) on P2 (n=26); in the air group, mean weights were 1.62 g (SD 0.11) on P0 (n=17), 1.74 g (SD 0.17) on P1 (n=8), and 2.01 g (SD 0.28) on P2 (n=13). The weight differences between the O_2 and air groups were not significant at any of the three ages.

The experimental design is summarized in Figure 1 of the text. After an about 3-minute waiting time for adaptation to the chamber, we recorded baseline V_E for 3 min. In the

O_2 group, the airflow through the plethysmograph was then switched to a 100% O_2 flow for 3 minutes and back to air for the next 12 minutes. This sequence was repeated three times (total duration, 63 min). The normoxic control pups were constantly maintained in normoxia in the plethysmograph to look for possible effects of isolation on breathing pattern and for drifts in breathing variables. In preliminary experiments, we checked that breathing variables were identical when pups were exposed to continuous airflow from the hospital compressed air system or to alternation of compressed air from the hospital system and an air bottle (to mimic O_2 administration). Simultaneous measurements of breathing variables and body temperature were done in 3 additional pups on P2 (mean weight, 2.20 g (SD 0.16)).

Furthermore, we ran two complementary experiments in 2-day old pups. First, in a follow-up experiment we exposed an independent sample of pups (n=10; weight: 1.84±.09 g) to an additional O_2 test 1 hour after completing the 100% O_2 protocol (Fig. 1, B). Second, to examine the effects of repeated exposures to lower O_2 concentrations, we repeatedly exposed an independent sample of pups (n=16; 2.13±0.21 g) to 30% O_2, instead of 100% O_2 (Fig. 1C).

Ventilatory response to hyperoxia

Breathing variables and apnoeas were determined using a recently developed automatic classification method (20). Briefly, apnoeas were defined as ventilatory pauses longer than twice the duration of the preceding breath, whereas T_{TOT} (s), V_T (µl g^{-1}), and V_E (calculated as V_T T_{TOT}^{-1} and expressed in µl s^{-1} g^{-1}) were calculated on apnoea-free periods. Breathing variables were averaged over consecutive 30-s periods. For each test, baseline values for these variables were calculated as the mean value over the 3 min of air exposure preceding the test (or the corresponding period in the air group).

We evaluated the ventilatory response to hyperoxia based on the maximal V_E decline, i.e., the minimal V_E value (min V_E) over the 3 min of O_2 exposure expressed as the percentage of the baseline level (22), according to the formula: 100 x (min V_E-baseline

V_E)/baseline V_E, where min V_E was the minimum V_E value over the 3-min period of O_2 exposure. This formula took into account possible interindividual differences in the time course of the V_E response to O_2. We determined the V_T and the T_{TOT} responses to O_2 using the same formula with the values of V_T and T_{TOT} corresponding to min V_E. The same calculations were done in the air group. This yielded a negative value for the V_E response because min V_E was smaller than the sample mean.

Total apnoea duration was calculated as the total apnoeic time during each 3-min period, i.e., before, during, and after O_2 exposure in the O_2 group (or air in the air group). The apnoea response to O_2 was calculated as the difference between air and O_2; (percentages could not be calculated, because some pups had no apnoeas during air breathing, i.e., had an apnoea duration of 0).

Statistics

Breathing variables and apnoeas were subjected to analyses of variance with group (O_2 versus air) and age (P0, P1, and P2) as the between-subject factors and test (from 1 to 4) as the within-subject factor (Superanova and Statview 5 Software, Abacus Concepts, Berkeley, CA). To take into account the heterogeneous correlations among the repeated time measurements, we adjusted the degrees of freedom using the Greenhouse and Geisser factor, which is a conservative downward correction to the degrees of freedom (23). Within-subject main effects and interactions are presented, together with *P* values based on these adjusted degrees of freedom. When the interaction analysis was significant, contrast analyses were conducted to detect where the significant differences lay. Values are means (standard deviations, SD) in the text and tables and means (standard errors of the means, SEM) in the figures.

RESULTS

Baseline breathing variables

Baseline V_E levels were similar in the two groups (Table 1). These values did not show any significant group or age effects. However, in both groups we found a small but significant V_E increase from the first to the fourth test ($P<0.0016$) (the group differences were not significant). This drift was ascribable to a V_T increase (main effect for test: $P<0.0001$, not shown), whereas the corresponding change in T_{TOT} was not significant (not shown).

Ventilatory response to hyperoxia

Large proportions of the 63-minute recordings were free of movement artefacts in both the O_2 (P0: 90% (SD 4); P1: 86% (SD 7); P2: 92% (SD 3)) and the air group (P0: 92% (SD 4); P1: 90% (SD 5); P2: 92% (SD 3)).

At all ages, hyperoxia caused a significant V_E decline on average over the four tests, compared to air (Table 2). The V_E decline in the O_2 group was chiefly ascribable to a T_{TOT} increase, whereas changes in V_T were small (Table 2). Because of the time need to flush the chamber with O_2, min V_E was achieved during the second 30-s period of O_2 exposure. The V_E decline was reversed within a 3-min period upon return to normoxia: the analysis of post-hyperoxic values (calculated over successive 3-min periods from the return to normoxia) did not indicate significant differences between the O_2 group and the air group.

The comparison between the O_2 and air groups (about 10% difference between groups on P0 and P1 and 20% on P2) showed a significant increase in the O_2-induced V_E decline on P1 and P2. This effect was supported by a significant group-by-age interaction and by the between-group and within-group pairwise comparisons described in detail in Table 2. The group differences were not accounted for by changes in baseline levels, which were small and

similar in the two groups, as previously mentioned (Table 1). Thus, a significant response to hyperoxia, consisting mainly in T_{TOT} changes, was present on the day of birth and increased over the next 2 days. This effect was abolished by the return to normoxia, at all ages and for all test numbers (not shown). As for V_E, reversal of the apnoea effect occurred within 3 min after the return to normoxia. Post-hypoxic values of apnoeas calculated over successive 3-min periods did not show significant differences between the O_2 group and the air group.

Finally, we determined whether V_T and V_E values were influenced by body temperature changes during hyperoxia. Body temperatures (measured in a separate sample of 3 pups subjected to the same protocol as the O_2 group) showed remarkable stability throughout the experiment. Individual ranges of temperature changes were 0.7, 0.5, and 0.7°C, respectively, and variation coefficients were 0.5%, 0.3%, and 0.4%, respectively.

Apnoeas

In normoxia, total apnoea duration was significantly longer on P1, compared to P0 and P2, with no significant difference between the O_2 and air groups (Table 3). Hyperoxia significantly increased mean total apnoea duration at all ages (example in Figure 2), as compared to the pre-oxygen period (group-by-period interaction, $P<0.0001$, Fig. 3). The increase in mean apnoea duration was not significantly affected by age (group-by-period interaction non-significant). When we considered the mean values for all four tests combined (test effects are examined below), the mean increase in apnoea duration from air to O_2 was 8.7 s (SD 10.8) on P0, 8.4 s (SD 16.4) on P1, and 5.0 s (SD 8.9) on P2 (non-significant differences). In the air group, there were virtually no changes at any of the three ages. The apnoea increase was reversed by the return to normoxia, at all ages and for all test numbers (not shown).

Finally, neither the linear correlation coefficient nor the Spearman rank correlation between the total apnoea duration and the mean V_E decline was significant overall or in any of the age groups.

Effect of repeated exposure to oxygen

The V_E decline caused by O_2 (expressed as percent baseline) increased significantly with repeated exposure in the O_2 group (group-by-test interaction, $P<0.0005$, Fig. 3A). In the O_2 group, the V_E drop was about 70% larger during the fourth test than during the first test, with no significant effect of age. The small changes in baseline normoxic V_E did not account for the V_E decline (Table 2).

The increase in apnoea duration caused by O_2 exposure was significantly greater during the second test than during the first test in the O_2 group and remained elevated thereafter (group-by-test interaction, $P<0.03$; no significant effect of age; Fig. 3B). This increase was not ascribable to differences in baseline levels, which were closely similar for the first and second tests (12.5 (16.9) s and 11.7 (16.9) s, respectively; Fig. 3B).

Follow-up

We replicated the protocol of the main experiment (i.e., four successive 100% O_2 tests) in a smaller, independent sample of P2 pups (n=10), and we re-exposed the pups to a follow-up test 1 hour after completing the last O_2 test. This experiment confirmed the repetition effects on V_E ($P<0.030$, effects on apnoeas were not significant). After 1 hour, the V_E decline was partially reversed, and its value was not significantly different from the baseline value on Test 1 (Fig. 4).

30% O_2 effects

To determine whether repeated exposure to lower concentrations of O_2 also magnified the V_E decline, we exposed an independent sample of P2 pups (n=16) to the same protocol after replacing the 100% O_2 stimulus by 30% O_2. The V_E declines were -22.1 % (SD 14.1), -23.9 % (SD 13.2), -24.2 % (SD 7.1), and -22.2 % (SD 13.3) from Test 1 to Test 4, respectively. In contrast to the 100% O_2 tests, repeated exposure had no significant effect on the V_E decline, which remained the same over the four tests. As with the 100% O_2 test, the V_E decline was chiefly ascribable to an increase in T_{TOT} (27.0 % (SD 14.4) on average over the four tests), whereas changes in V_T were small (-2.1 % (SD 4.5). The VE decline was slightly smaller than that caused by 100% O_2, but the difference was not significant.

The 30% O_2 stimulus slightly but significantly increased mean total apnoea duration at all ages, as compared to the pre-oxygen period ($P<0.008$). The mean increase in apnoea duration from air to O_2 was 1.9 s (SD 4.9), 2.6 s (SD 4.9), 1.5 s (SD 4.0), 2.5 (SD 5.8), and 2.1 s (SD 2.7). The repeated-exposure effect was not significant.

DISCUSSION

Repeated exposure to 100% O_2 was associated with increasing inhibitory effects of hyperoxia on V_E, as a result of increases in T_{TOT} and in total apnoea duration. These findings are consistent with potentiating effects.

Methodological considerations

Whole-body plethysmography is the only method for measuring breathing variables in unrestrained newborn mice. However, this method has been validated against pneumotachography in larger animals but not in newborn mice, due to the lack of pneumotachographs designed for small animals. Furthermore, body temperature, which is in

the equations for calculating V_T and V_E, was not continuously measured during plethysmographic recordings, as this would have required restraining the pups. Thus, the absolute V_T and V_E values in the present study should be considered with caution. However, these limitations do not invalidate our results, because the ventilatory depression caused by hyperoxia was explained by increases in T_{TOT} and apnoea duration, two variables known to be reliably measured by plethysmography (20). Furthermore, the body temperatures measured in three animals during plethysmography showed very small variations.

To evaluate the ventilatory response to hyperoxia, we used min V_E over the 3-min O_2 exposure period instead of the mean decrease over the total O_2 exposure period (22). Min V_E during hyperoxia is thought to depend only on suppression of the peripheral chemoreceptor drive. However, the increase in metabolism caused by hyperoxia (24) may elicit excitatory inputs to breathing that may *partially* counteract the ventilatory fall caused by peripheral chemoreceptor silencing. Thus, the mean VE value calculated over the entire hyperoxic period may confound excitatory and inhibitory inputs to breathing. Furthermore, our method for assessing the ventilatory response to hyperoxia took into account possible interindividual differences in the time-course of the VE response to O_2.

The study period (from P1 to P3) encompassed major postnatal changes in respiratory sensitivity to hypoxia. Previous studies showed that significant changes in ventilatory responses to chemical stimuli take place around 12 hours after birth in mice (18). In human infants, postnatal resetting of peripheral chemoreceptors occurs within days to weeks after birth (25). The effects of hyperoxia beyond this critical period were not examined in the present study. Oxygen causes ventilatory depression in mature mammals (see (26) for a review) and may yield similar potentiation effects to those found here. However, in this study, we focused on the early postnatal period during which ventilatory depression may be aggravated by other sources of respiratory instability, in particular immaturity of the central

chemoreceptors (27).

Development of the hyperoxic response

Hyperoxia induced depression of ventilation in our animals, as previously reported in mouse pups between 1 and 2 days after birth (28), as well as in other newborn mammals including humans (29). The present results establish that the V_E decline is present on P0 and is accompanied by apnoeas. Previous work by our group suggested that the postnatal resetting of peripheral chemoreceptors occurred within 6-12 hours after birth in mice (18). This maturation effect may account for the increase in the V_E decline after P0 in the present study.

The V_E decline in the O_2 group was chiefly ascribable to a T_{TOT} increase, whereas V_T changes were small. In a study of human preterm infants, the V_E decline after 3 min of O_2 exposure was due to a significant decrease in respiratory rate with little or no change in V_T on P2, whereas the opposite breathing pattern changes were found on P6 (15) and in term infants between P2 and P6 (29). Taken together, these results suggest that the breathing strategy during hyperoxia undergoes rapid developmental changes and that newborn mice resemble preterm infants shortly after birth in this respect. This similarity further supports the validity of newborn mice as a model for studying early breathing disorders in preterm human infants and in a less stage in neonatal period in term.

Effects of repeated O_2 exposure

The VE decline and total apnoea duration tended to increase with repeated 100% O_2 exposure, whereas both VE and apnoea duration returned to baseline levels between 100% O_2 exposures. These effects were transient: after 1 hour, re-exposure to 100% O_2 did not induce a significantly larger VE decline compared to the first test.

Finally, the increase in ventilatory depression observed with repeated 100% O_2 was not found with a lower O_2 concentration (30%). Exposure to 30% O_2 significantly inhibited ventilation, but this effect did not become stronger with repeated exposure.

Repeated hyperoxia was associated with alternating inhibition (during hyperoxia) and stimulation of peripheral chemoreceptors (upon return to normoxia). However, the effects shown in our study departed from potentiation effects induced by repeated hypoxia in that the V_E changes induced by repeated hyperoxia did not outlast the exposure to the stimulus (16). Sustained changes in metabolic rate from one test to the other may also contribute to the increase in ventilatory depression with repeated hyperoxia. Hyperoxia has previously been found to increase O_2 consumption and CO_2 production in newborn mammals (24, 30). However, the rapid return of V_E and apnoea duration to pre-O_2 baseline levels upon return to normoxia after each O_2 exposure in our experiments militates against a sustained increase in metabolic rate.

CONCLUSION

Repeated O_2 administration exacerbated O_2-induced ventilatory depression by increasing the VE decline and apnoea duration in newborn mice. These findings support that systematic preventive oxygenotherapy should be avoided. They may indicate a need to revise current resuscitation strategies for premature infants. Other studies are warranted to know the age at which this detrimental effect disappears.

Acknowledgements: This study was supported by the Institut National de la Santé et de la Recherche Médicale, the Centre National de la Recherche Scientifique, and the Chancellerie des Universités de Paris en Sorbonne (Legs Poix).

REFERENCES

1. Davis PG, Tan A, O'Donnell CP, Schulze A. Resuscitation of newborn infants with 100% oxygen or air: a systematic review and meta-analysis. *Lancet* 2004: 364(9442): 1329-1333.

2. Wiswell TE. Neonatal resuscitation. *Respir Care* 2003: 48(3): 288-294; discussion 294-285.

3. Fiser DH, Gober GA, Smith CE, Jackson DC, Walker W. Prevention of hypoxemia during lumbar puncture in infancy with preoxygenation. *Pediatr Emerg Care* 1993: 9(2): 81-83.

4. Chen CH, Wang TM, Chang HM, Chi CS. The effect of breast- and bottle-feeding on oxygen saturation and body temperature in preterm infants. *J Hum Lact* 2000: 16(1): 21-27.

5. Thoyre SM, Carlson J. Occurrence of oxygen desaturation events during preterm infant bottle feeding near discharge. *Early Hum Dev* 2003: 72(1): 25-36.

6. Niggemann B, Haack M, Machotta A. How to enter the pediatric airway for bronchoscopy. *Pediatr Int* 2004: 46(2): 117-121.

7. Soong WJ, Hwang B. Intratracheal oxygen administration during bronchoscopy in newborns: comparison between two different weight groups of infants. *Zhonghua Yi Xue Za Zhi (Taipei)* 2000: 63(9): 696-703.

8. Chow LC, Wright KW, Sola A. Can changes in clinical practice decrease the incidence of severe retinopathy of prematurity in very low birth weight infants? *Pediatrics* 2003: 111(2): 339-345.

9. Lundstrom KE, Pryds O, Greisen G. Oxygen at birth and prolonged cerebral vasoconstriction in preterm infants. *Arch Dis Child Fetal Neonatal Ed* 1995: 73(2): F81-86.

10. Kelly FJ. Free radical disorders of preterm infants. *Br Med Bull* 1993: 49(3): 668-678.

11. Niermeyer S, Vento M. Is 100% oxygen necessary for the resuscitation of newborn infants? *J Matern Fetal Neonatal Med* 2004: 15(2): 75-84.

12. Saugstad OD, Ramji S, Vento M. Resuscitation of depressed newborn infants with ambient air or pure oxygen: a meta-analysis. *Biol Neonate* 2005: 87(1): 27-34.

13. Rehan V, Torday J. Hyperoxia augments pulmonary lipofibroblast-to-myofibroblast transdifferentiation. *Cell Biochem Biophys* 2003: 38(3): 239-250.

14. Prieto-Lloret J, Caceres AI, Obeso A, Rocher A, Rigual R, Agapito MT, Bustamante R, Castaneda J, Perez-Garcia MT, Lopez-Lopez JR, Gonzalez C. Ventilatory responses and carotid body function in adult rats perinatally exposed to hyperoxia. *J Physiol* 2004: 554(Pt 1): 126-144.

15. Rigatto H, Brady JP, de la Torre Verduzco R. Chemoreceptor reflexes in preterm infants: I. The effect of gestational and postnatal age on the ventilatory response to inhalation of 100% and 15% oxygen. *Pediatrics* 1975: 55(5): 604-613.

16. Powell FL, Milsom WK, Mitchell GS. Time domains of the hypoxic ventilatory response. *Respir Physiol* 1998: 112(2): 123-134.

17. Gozal D. New concepts in abnormalities of respiratory control in children. *Curr Opin Pediatr* 2004: 16(3): 305-308.

18. Renolleau S, Dauger S, Autret F, Vardon G, Gaultier C, Gallego J. Maturation of baseline breathing and of hypercapnic and hypoxic ventilatory responses in newborn mice. *Am J Physiol Regul Integr Comp Physiol* 2001: 281(5): R1746-1753.

19. Craig A, Ling Luo N, Beardsley DJ, Wingate-Pearse N, Walker DW, Hohimer AR, Back SA. Quantitative analysis of perinatal rodent oligodendrocyte lineage progression and its correlation with human. *Exp Neurol* 2003: 181(2): 231-240.

20. Matrot B, Durand E, Dauger S, Vardon G, Gaultier C, Gallego J. Automatic classification of activity and apneas using whole body plethysmography in newborn mice. *J Appl Physiol* 2005: 98(1): 365-370.

21. Dauger S, Nsegbe E, Vardon G, Gaultier C, Gallego J. The effects of restraint on ventilatory responses to hypercapnia and hypoxia in adult mice. *Respir Physiol* 1998: 112(2): 215-225.

22. Bouferrache B, Filtchev S, Leke A, Marbaix-Li Q, Freville M, Gaultier C. The hyperoxic test in infants reinvestigated. *Am J Respir Crit Care Med* 2000: 161(1): 160-165.

23. Keselman HJ, Algina J, Kowalchuk RK. The analysis of repeated measures designs: a review. *Br J Math Stat Psychol* 2001: 54(Pt 1): 1-20.

24. Mortola JP, Frappell PB, Dotta A, Matsuoka T, Fox G, Weeks S, Mayer D. Ventilatory and metabolic responses to acute hyperoxia in newborns. *Am Rev Respir Dis* 1992: 146(1): 11-15.

25. Donnelly DF. Developmental aspects of oxygen sensing by the carotid body. *J Appl Physiol* 2000: 88(6): 2296-2301.

26. Gautier H. Hypoxia,hyperoxia and breathing. *J Biosci* 2006: 31(2): 185-190.

27. Katz-Salamon M. Delayed chemoreceptor responses in infants with apnoea. *Arch Dis Child* 2004: 89(3): 261-266.

28. Mortola JP, Tenney SM. Effects of hyperoxia on ventilatory and metabolic rates of newborn mice. *Respir Physiol* 1986: 63(3): 267-274.

29. Hertzberg T, Lagercrantz H. Postnatal sensitivity of the peripheral chemoreceptors in newborn infants. *Arch Dis Child* 1987: 62(12): 1238-1241.

30. Frappell PB, Dotta A, Mortola JP. Metabolism during normoxia, hyperoxia, and recovery in newborn rats. *Can J Physiol Pharmacol* 1992: 70(3): 408-411.

FIGURE LEGENDS

Figure 1. Study protocols.

Panel A: Breathing variables were recorded for 63 min. 100% O_2 group: after 3 minutes of normoxia, the gas in the plethysmograph was changed to 100% O_2 for 3 min then returned to normoxia for 12 min. This cycle was repeated three more times (four cycles in all). Air group: the pups were continuously maintained in normoxia.

Panel B: In an independent sample of pups an additional 100% O_2 test was performed 1 hour after completing the 100% O_2 protocol.

Panel C: An independent sample of pups was exposed to repeated 30% O_2 stimuli instead of 100% O_2.

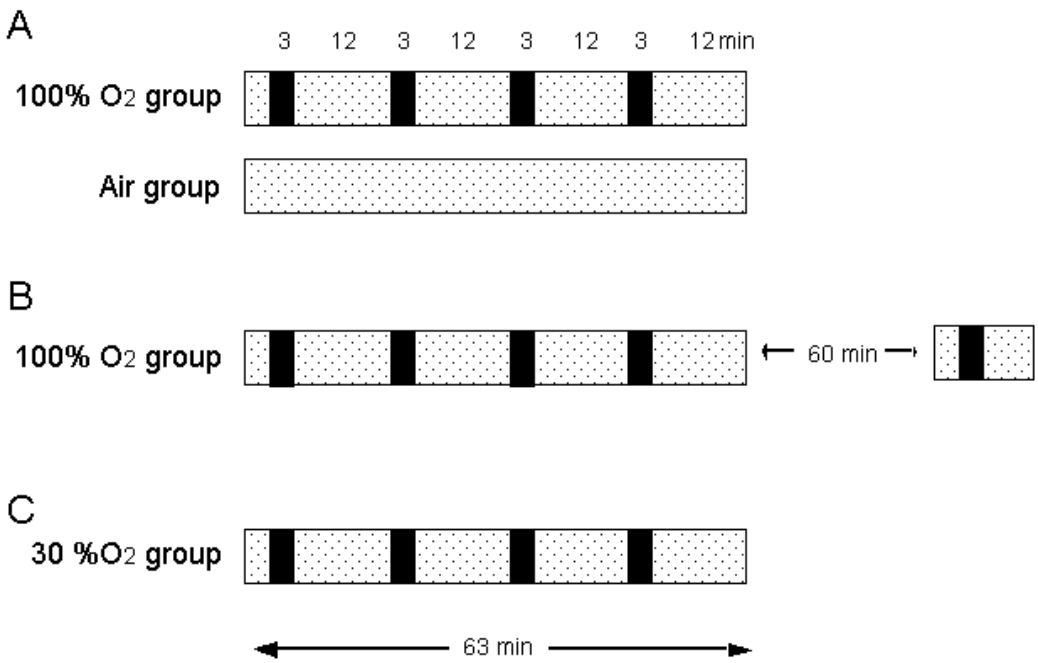

Figure 2. Illustrative respiratory traces in a 2-day-old mouse during air exposure (top trace) and 100% O_2 exposure (bottom trace). Thirty seconds after the switch to O_2, apnoeas and a slight increase in breath duration occurred.

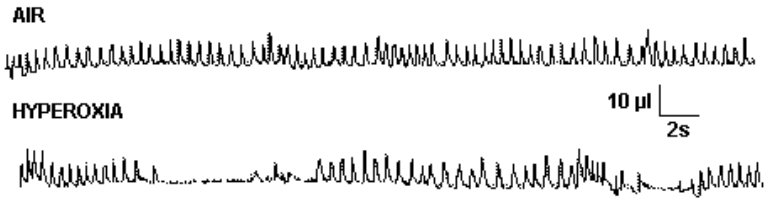

Figure 3. Effects of repeated 3-min O_2 exposures on V_E and apnoea duration. Values were averaged over age groups. A: V_E drop expressed as percent baseline calculated over the 3-min period of air exposure preceding O_2 exposure. *: significant changes from the preceding test. The V_E drop increased significantly from one test to the next in the O_2 group (Test 1 versus Test 2, $P<0.0093$; Test 3 versus Test 4, $P<0.0112$). B: apnoea duration increase from baseline calculated over the 3-min period of air exposure preceding O_2 exposure. The increase in total apnoea duration was significantly more marked after the first test in the O_2 group (Test 1 versus Test 2, $P<0.0397$). Repeated-exposure effects on V_E and apnoeas were not influenced by age. Values are means (SEM).

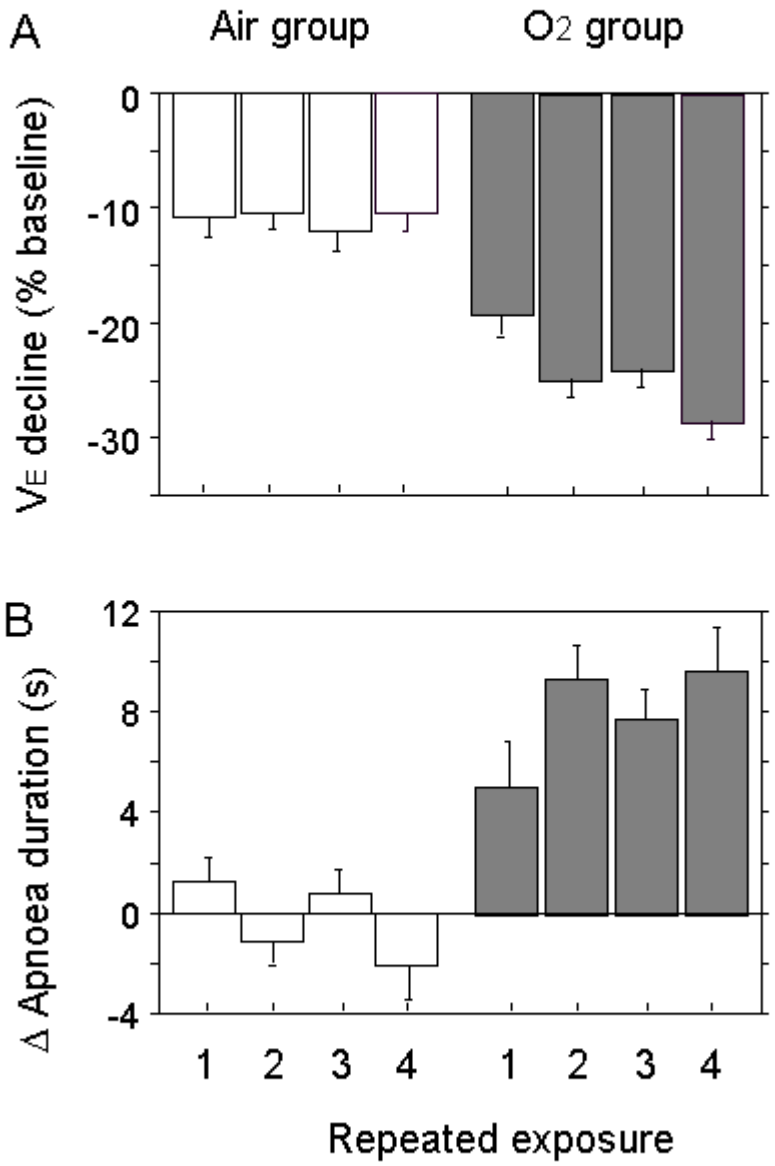

Figure 4. Resolution of the repeated-exposure effects. Two-day old pups were exposed to a follow-up test 1 hour after completing the last 100% O_2 test. The V_E decline induced by this test was not significantly different from the baseline value on Test 1.

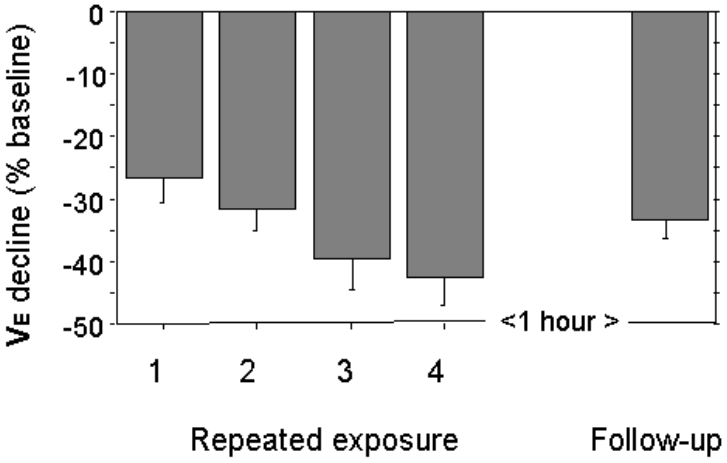
0
-10
-20
-30
-40
-50
VE decline (% baseline)
<1 hour >
1
2
3
4
Repeated exposure
Follow-up

Table 1. Baseline V_E in air (3 min) before each 3-min exposure to 100% O_2 (O_2 group) or air (air group). Baseline V_E increased significantly from Test 1 to Test 4 ($P<0.0009$), by about 9% in the O_2 group and 6% in the air group (non-significant difference). Group and age had no significant effects on V_E, neither as the main effect or as an interaction with the remaining factors. Values are group means (SD).

	Baseline V_E (µl/g/s)									
Age	O_2 group					Air group				
(days)	Test 1	Test 2	Test 3	Test 4	Mean over tests	Test 1	Test 2	Test 3	Test 4	Mean over tests
0	9.7 (3.4)	10.4 (3.6)	10.6 (3.6)	10.5 (3.4)	10.3 (3.5)	10.3 (2.6)	10.0 (2.5)	10.4 (2.7)	10.5 (3.4)	10.3 (2.8)
1	11.1 (3.7)	11.1 (2.5)	12.3 (2.7)	13.1 (3.3)	11.9 (3.1)	9.2 (4.4)	9.5 (3.3)	9.8 (3.3)	10.0 (3.2)	9.6 (3.5)
2	10.1 (2.6)	10.6 (2.6)	10.7 (3.0)	10.6 (2.8)	10.5 (2.7)	9.9 (3 .2)	10.8 (2.8)	10.9 (3.1)	11.1 (3.2)	10.7 (3.1)
Mean	10.1 (3.1)	10.6 (3.0)	11.0 (3.2)	11.0 (3.2)	10.3 (3.0)	10.0 (3.2)	10.2 (2.8)	10. 6 (3.0)	10.6 (3.2)	10.3 (3.1)

Table 2. Peak changes in breathing variables in newborn mice exposed to 100% O_2 (oxygen group) or air (air group) for 3 min. Values are expressed as percentage of baseline values calculated over the 3 min in air before each test. *P* values correspond to pairwise comparisons between the oxygen and air groups at a given age. NS: not significant. All pairwise comparisons were conducted after checking that age-by-group interactions were significant for V_E ($P<0.0053$), T_{TOT} ($P<0.0004$), and V_T ($P<0.0248$). Values are group means (SD).

		V_E (% baseline)			T_{TOT} (% baseline)			V_T (%baseline)		
Age (days)	Test	100% O_2 group	air group	*P* value	100% O_2 group	air group	*P* value	100% O_2 group	air group	*P* value
0	1	-13.0 (8.6)	-7.3 (8.7)	NS	13.9 (11.8)	9.2 (8.1)	NS	-0.9 (11.3)	2.4 (8.6)	NS
	2	-18.8 (12.8)	-7.9 (4.8)	0.0002	17.1 (15.5)	3.4 (8.2)	0.0064	-5.6 (10.2)	-3.1 (8.0)	NS
	3	-20.2 (13.4)	-13.0 (8.6)	0.0430	22.3 (17.0)	10.4 (13.0)	0.0134	-2.0 (9.8)	-3.8 (6.6)	NS
	4	-22.7 (13.5)	-11.0 (7.2)	0.0002	23.3 (14.1)	9.5 (10.2)	0.0110	-4.8 (12.9)	-1.6 (7.7)	NS
1	1	-23.9 (17.2)	-19.7 (7.6)	NS	16.5 (22.1)	12.7 (10.9)	NS	-6.1 (19.8)	7.2 (2.5)	NS
	2	-25.5 (9.2)	-16.4 (5.7)	0.0275	18.8 (17.6)	10.3 (10.0)	NS	-10.5 (11.2)	-5.0 (9.7)	NS
	3	-27.7 (13.5)	-15.8 (10.0)	0.0206	23.0 (17.6)	8.7 (14.7)	0.0396	-12.0 (10.4)	-5.6 (6.9)	NS
	4	-36.3 (11.9)	-14.6 (10.2)	0.0001	24.8 (21.3)	-9 (15.1)	0.0011	-20.1 (7.5)	-5.3 (8.1)	0.0010
2	1	-20.1 (13.0)	-7.2 (8.3)	0.0009	23.0 (16.4)	2.3 (8.9)	NS	-0.9 (7.4)	1.3 (5;1)	NS
	2	-27.3 (6.5)	-8.9 (9.5)	0.0001	31.4 (20.3)	0.1 (10.7)	0.0001	-4.1 (9.4)	5.0 (16.7)	0.0148
	3	-25.7 (10.5)	-4.9 (7.1)	0.0001	24.7 (14.4)	4.7 (9.7)	0.0001	-8.1 (10.2)	10.1 (21.0)	0.0001
	4	-29.2 (8.8)	-5.3 (4.1)	0.0001	31.4 (22.0)	-4.0 (6.3)	0.0001	-7.9 (8.8)	0.2 (6.9)	0.0124

Table 3. Mean baseline total apnoea duration before 100% O2 (oxygen group) or air (air group). Group differences were not significant. Values were significantly higher on P1 than on P0 ($P<0.0098$) and P2 ($P<0.0002$), in both groups. Values are group means (SD).

	Baseline apnoea duration (s)									
Age	100% O_2 group					Air group				
	Test 1	Test 2	Test 3	Test 4	mean over tests	Test 1	Test 2	Test 3	Test 4	mean over tests
0	14.4 (21.1)	13.1 (16.3)	12.2 (18.3)	13.1 (18.0)	13.2 (18.2)	4.0 (4.6)	9.8 (7.7)	10.2 (10.2)	10.6 (9.4)	8.6 (8.5)
1	21.9 (14.0)	20.0 (16.0)	17.0 (12.3)	20.1 (16.4)	19.8 (14.3)	17.8 (19.3)	28 (22.4)	22.3 (26.9)	25.7 (25.2)	23.4 (23.2)
2	7.0 (11.8)	7.1 (16.9)	7.9 (17.0)	9.5 (19.9)	7.8 (16.4)	3.0 (4.0)	2.9 (4.7)	1.1 (1.5)	3.2 (5.4)	2.6 (4.1)
mean	12.5 (16.9)	11.7 (17)	11.2 (16.9)	12.8 (18.7)	12.1 (17.3)	6.6 (11.0)	11.3 (14.6)	9.6 (15.6)	11.3 (16.0)	9.7 (12.1)