The effect of prenatal maternal infection on respiratory function in mouse offspring: evidence for enhanced chemosensitivity

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Running Title: Prenatal maternal infection and respiratory function

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ABSTRACT:

Systemic maternal inflammation is implicated in preterm birth and bronchopulmonary dysplasia (BPD) and may induce morbidities including reduced pulmonary function, sleep-disordered breathing and cardiovascular disorders. Here we test the hypothesis that antenatal maternal inflammation per se causes altered alveolar development and increased chemoreflex sensitivity that persists beyond infancy. Pregnant C57BL/6 mice were administered lipopolysaccharide (LPS; 150 µg/kg; i.p.) to induce maternal inflammation or saline (SHAM) at embryonic day 16 (randomized). Pups were weighed daily. On days 7, 28 and 60 (D07, D28 and D60) unrestrained whole-body plethysmography quantified ventilation and chemoreflex responses to hypoxia (10%), hypercapnia (7%) and asphyxia (hypoxic hypercapnia). Lungs were harvested to quantify alveolar number, size and septal thickness. LPS pups had reduced baseline ventilation per unit bodyweight (~40%, \(P<0.001\)) versus SHAM. LPS increased ventilatory responses to hypoxia (Day 7: 66% vs 28% increase in ventilation; \(P<0.001\)) hypercapnia (170% vs. 88%, \(P<0.001\)) and asphyxia (249% vs 154%; \(P<0.001\)); hypersensitive hypoxic responsiveness persisted until D60 (\(P<0.001\)). LPS also increased apnea frequency (\(P<0.01\)). LPS caused thicker alveolar septae (D07, \(P<0.001\)), diminished alveolar number (D28, \(P<0.001\)) versus SHAM, but effects were minimal by D60. Pups delivered from mothers exposed to antenatal inflammation exhibit deficits in lung structure and hypersensitive responses to respiratory stimuli that persist beyond the newborn period. Antenatal inflammation may contribute to impaired gas exchange and unstable breathing in newborn infants and adversely affect long-term health.

Keywords: Respiratory function, Chemosensitivity, Prenatal Infection
INTRODUCTION

Preterm birth (PTB), defined as delivery occurring at <37 completed weeks of gestation, is a major risk factor for neonatal mortality and morbidity. Of the four million neonatal deaths world-wide each year, an estimated 27% are attributable to PTB (45). Although we have a poor understanding of the aetiology of PTB, it is strongly associated with advanced maternal age (42), poor socio-economic status (2, 23), maternal smoking (72), a previous history of PTB, and multiple gestations (44). Animal studies have shown that infection is implicated in PTB, as blockade of maternal inflammation substantially reduces PTB in the mouse (71). Yet it remains unclear whether maternal inflammation is directly responsible for two of the most common sequelae of PTB, namely bronchopulmonary dysplasia (BPD) and unstable breathing (periodic breathing and apnea), two conditions that by promoting hypoxemia reduce survival in the newborn period (10) and increase morbidity thereafter (30, 57, 66).

Intrauterine inflammation is present in 25% of all babies who develop severe and chronic lung disease in the form of BPD (40) a condition that causes significant morbidity and mortality in preterm babies. Acute and chronic intrauterine infection/inflammation associated with PTB can adversely influence lung development by exposing the developing fetus to cytokines, chemokines and lipid mediators (24, 46). In a number of experimental models (29, 33, 36, 43), antenatal inflammation caused by intra-amniotic lipopolysaccharide (LPS) influences lung development and maturation via elevated expression of pro-inflammatory cytokines resembling what is seen in premature infants (25). An acute infection of the fetal and maternal membranes predisposes to bronchopulmonary dysplasia (BPD), which leads to the infant being given supplemental oxygen, a treatment that itself adversely affects lung development by creating a phenotype with fewer and larger alveoli (3, 32).

Unstable breathing in the form of apnea and periodic breathing is extremely common in preterm infants and has long been considered to be the consequence of immature ventilatory
control (82, 83). Despite a high prevalence of inflammation in pregnancy, how it affects ventilatory control over extended periods of infancy is unknown. What is known is that the acute effect of intravenous infusion of LPS in anesthetized cats is to diminish hypoxic ventilatory responses mediated by the carotid body (19). Likewise, in newborn mice, rats and piglets, pro-inflammatory cytokine such as IL-1β acutely depress respiration, prolong the duration of apnea and modify autoresuscitation (21, 52, 73). In infants, acute respiratory infection is commonly associated with apnea and unstable breathing (5, 8, 49, 58). Mechanistically, unstable breathing reflects an elevated “loop gain” (sensitivity of the feedback loop controlling ventilation) due to hypersensitive ventilatory chemoreflex responses to hypoxia and hypercapnia. Indeed, increased loop gain via exaggerated chemoreflexes is implicated in periodic breathing and apnea in infants (1), newborn animals (17, 18) and adults (20, 31, 84).

We aimed to improve our understanding of the effects of prenatal maternal infection on postnatal respiratory structure and function in offspring. We hypothesised that newborn mice exposed to an in utero maternal inflammatory insult have enhanced chemosensitivity and impaired lung development. Accordingly, we administered pregnant mice with lipopolysaccharide (LPS) at a dose that elevates expression of pro-inflammatory cytokines similar to that present in preterm human infants (51). We then compared the ventilatory responses to hypoxia, hypercapnia and asphyxia of pups from LPS-treated and saline-injected pregnancies. Finally, we quantified the propensity of the two sets of pups for spontaneous apneas and post-sigh apneas during recovery from hypoxic, hypercapnic and asphyxic inspired gas.
MATERIALS AND METHODS:

Animals

All experimental procedures conformed to the guidelines established by the National Health and Medical Research Council of Australia and had the approval of the standing committee in Ethics in Animal Experimentation of Monash University, Melbourne, Victoria, Australia. Time mated C57BL/6 mice were used for the experiments. The morning of finding the vaginal plug was considered day 1 of pregnancy. All animals were maintained under standard environmental conditions (12:12hr light:dark cycle) with free access to food and water.

Experimental Design

At embryonic day 16, C57BL/6 pregnant mice randomly received a single intraperitoneal injection of either lipopolysaccharide (LPS; Escherichia coli serotype 0127:B8: Sigma-Aldrich, Castle Hill, NSW) at a dose of 150 µg/kg, or an equal volume of saline. Mice were then allowed to deliver naturally and the pups randomly allocated to one of three experimental study points: day 07 (D07), day 28 (D28) or day 60 (D60). The pups were monitored daily for weight and general well-being.

Ventilatory measurement

The ventilatory responses to hypoxia (10% O₂), hypercapnia (7% CO₂) and asphyxia (10% O₂ +7% CO₂) were measured by whole-body-plethysmography as previously described (12). Air was drawn through the measurement chamber at 2 Lmin⁻¹ using a bias flow regulator (Buxco Electronics) in a circuit connected to vacuum. The pressure difference between the chamber containing the pup and the reference chamber was measured using a differential pressure transducer (range, ±0.1 mbar, EFFA, Asnières, France). Care was taken to minimise the impact of random pressure fluctuations in the laboratory, by isolating the chamber from environmental vibrations and by incorporating a capacitative filter in series within the bias-
flow circuit. Gas leaving the chamber passed through an O₂ analyser (model S-3A/I, Ametek Process Instruments, Pittsburgh, PA) and a CO₂ analyser (Novametrix NICO CO₂ Monitor, USA) to determine fractional inspired concentrations of O₂ (FIO₂) and CO₂ (FICO₂). Signals from the pressure transducer and gas analyzers were recorded at 400Hz (LabChart 7.0, ADInstruments, Sydney, Australia). The differential pressure signal was bandpass filtered (0.4-15Hz) and used to calculate respiratory frequency (f), tidal volume (Vₜ) and minute ventilation (Vₑ=Vₜ×f) for each breath as described previously (14). The system was calibrated prior to each study by repetitive injection of 50 μl and then 100 μl of air into the chamber with a precision syringe (Hamilton Bonaduz AG, Switzerland); volume deflections made at the respiratory frequency were used to calibrate pressure measurements.

On the day of the experiment, mice were placed inside the measurement chamber. We assumed body temperature was constant at 37°C (67) and the measurement chamber was maintained within the thermoneutral range for young, adult mice as described previously (11). After 15 min acclimatisation, a 30 min period of baseline breathing was recorded, followed by exposure to hypoxia, hypercapnia and asphyxia for 10 min each. Each challenge was presented in random order and separated by a 15 min recovery period (air).

Data Analysis

Ventilation and ventilatory responses: Analysis of ventilatory parameters was performed using MATLAB (The MathWorks, Natick, MA, USA). Ventilatory variables VT, f, and Vₑ were calculated by carrying out breath-by-breath analysis of traces free from movement artefact. The baseline values of ventilatory variables were obtained by averaging 10 min of stable recording in the control period at the start of the experiment. Earlier measurement established that gas within the chamber takes 2.5 min to reach a steady-state once the inspired gas is changed; thus the first 2.5 min of respiratory data for each test was discarded, leaving
the last 7.5 min for quantitation of responses to hypoxia, hypercapnia and asphyxia. The ventilatory response data are presented as a percent increase from baseline since the relative changes are most important for blood gas homeostasis and also due to distinct changes in ventilatory responses across a range of ages where baseline V_E changed markedly. The time taken to reach 50% of the peak ventilatory response was also calculated.

**Apnea Analysis:** Apnea was defined as a cessation of ventilatory airflow for at least two respiratory cycles (50) and was classified as either a spontaneous (SA) or post-sigh apnea (PSA). To be adjudged a PSA, an apnea had to be preceded by a breath with double the amplitude of resting tidal volume (Figure 1), whereas an apnea that was not preceded by a sigh was scored as SA. SA and PSA frequencies were determined from the number of apneas during the 10 min baseline period, during the last 7.5 min of the three challenge phases, and during the 15 min recovery phase between challenges. All apnea numbers were divided by the number of minutes in the sample period. Apneas were assessed during the recovery phase from hypoxic, hypercapnic and asphyxic challenges due to their prevalence at this time (and absence at baseline and during the challenges). The recovery phase from hypoxia is an established period of instability (27, 62, 81), likely due to the presence of arterial hypoxia (which increases chemoreflex responsiveness) and an enhanced PO_2 gradient for gas exchange (inspired–alveolar PO_2; due to a lower alveolar PO_2) (38, 69). Likewise, the recovery phase from hypercapnia predisposes to instability possibly via an increased PCO_2 gradient for gas exchange (while PICO_2 is zero and alveolar PCO_2 is still elevated).

**Histology:** Pups were humanely killed by an intra-peritoneally injected overdose - a mixture of a ketamil (100mg/ml) xylazine (100mg/ml). A catheter was then inserted into the trachea to fill the lung with 10% formalin at a constant pressure of 20 cmH_2O for 5 min before the trachea was ligated distal to the catheter tip. The lungs were excised and further fixed by immersion in 10% formalin for 48h followed by storage in PBS. Lungs were processed,
embedded in paraffin cassettes and sectioned at 5μm thickness before staining with hemotoxylin and eosin (H&E) using standard techniques.

**Digital Imaging:** H&E stained slides were digitally scanned at 400x magnification by Aperio ScanScope XT (Aperio Technologies, USA) to produce high-resolution whole-slide images. The images were analysed using Aperio ScanScope software (Aperio Technologies, USA). In each lung, five non-overlapping fields of view in different sections, representing an area of 2000 X 2000 μm², were used for counting alveolar number and estimating alveolar area. Septal thickness was assessed by linear measurements of the septum, using fifteen measurements per field, and five fields per lung. Total alveolar number, alveolar area and septal thickness were measured using ImageJ software (1.47v, NIH, USA). Image J measurements such as perimeter and surface area were used to calculate a surrogate measure of diffusion capacity.

**STATISTICAL ANALYSIS:**

All results are presented as mean±SEM. Statistical analyses were performed on raw data for all variables using SigmaPlot 12 (SyStat software Inc., San Jose, CA, USA). The impact of LPS on body weight, growth rate, baseline physiological values, chemoreflex responses, apnea frequency, apnea duration and lung histology parameters were assessed using a two-way ANOVA with Student-Newman-Keuls post-hoc test. $P<0.05$ was considered significant.
RESULTS:

Body weight

Pups from 3-5 separate litters in LPS and SHAM groups were used at each time point for birth weight analysis. Despite the trend towards reduced body weight in LPS pups at D07 there were no significant differences in body weight at any age. However LPS exposed pups had a significantly greater growth rate than SHAM pups at D28 (ANOVA main effect; Table 1).

Baseline ventilation

Baseline $V_E$ was lower in LPS pups versus SHAM pups across all three age groups (ANOVA main effect $P<0.001$; Table 1).

Response to hypoxia

Pups exposed to antenatal LPS exhibited a larger $V_E$ increase than SHAMs in response to hypoxia ($10\% O_2$) across all three age groups (ANOVA main effect $P<0.001$; Figure 2A). At D07, SHAM pups ($n=19$) had a peak $V_E$ increase of $28\pm6\%$ above baseline, whereas in pups exposed to LPS ($n=22$) the increase was $65\pm6\%$ (Figure 2A, 3A). At D07 the speed of hypoxic response, as measured by time to reach 50% of the peak $V_E$, was not significantly different between groups (LPS: $115.8\pm16.3s$, SHAM: $183.2\pm35.4s$; $P=0.074$). At D28, SHAM pups ($n=16$) lowered ventilation in hypoxia ($-8.4\pm6.6\%$) whereas LPS pups ($n=12$) had an increase in $V_E$ ($16.2\pm7.7\%$; Figure 2A, 3A). A hypoxia-mediated increase in $V_E$ was present in both groups at D60, being $35.9\pm9.4\%$ in LPS pups ($n=8$) and $7.5\pm7.7\%$ in SHAM pups ($n=12$) (Figure 2A, 3A). The time to 50% peak response did not differ between LPS and SHAM pups at D28 and D60.
Response to hypercapnia

LPS significantly increased the ventilatory response to hypercapnia (ANOVA main effect $P=0.006$), which was most evident at D07 (Figure 2B, 3B). While the speed of the hypercapnic response was similar in SHAM and LPS groups at each age, there was a significant difference in the size of $V_E$ responses of the LPS group (169±15%) compared to the SHAM at D07 (87±16%) (Figure 2B, 3B). Responses to the hypercapnic challenge were similar in LPS and SHAM pups at D28 and D60.

Response to asphyxia

Asphyxia caused an increase in $V_E$ at all three ages (Figure 2C). LPS increased the magnitude of $V_E$ response (ANOVA main effect $P=0.002$), which was significantly greater at D07 and D28 (Figure 2C, 3C). LPS increased the speed of response evident exclusively at D07 (LPS: 41.1±4.9s, SHAM; 63.5±10.0s, $P<0.05$).

Spontaneous and post-sigh apnea

During baseline breathing, and during exposure to hypoxia, hypercapnia and asphyxia, SA and PSA were infrequent and similar between groups at the three ages studied. Following return to room air after hypoxia, LPS pups exhibited a greater frequency of spontaneous apneas (SA; ANOVA group×age $P=0.002$; Figure 4) and post-sigh apneas (PSA; ANOVA main effect $P=0.047$; Figure 4) at D07 compared to SHAM pups. Following hypercapnia, LPS increased the frequency of PSA (ANOVA main effect $P<0.001$) but not SA, an effect that again was most evident at D07 (Figure 4). Following asphyxia, LPS increased the frequency of SA (ANOVA group×age $P=0.03$), most clearly at D28 (Figure 4), and increased PSA frequency (ANOVA main effect $P<0.001$), which was most evident at D07 and D28 (Figure 4).
At D07, LPS pups exhibited longer SA duration vs. SHAM during hypoxic (ANOVA main effect $P=0.01$) and hypercapnic recovery (ANOVA main effect $P=0.02$). At D28, SA durations were significantly longer in LPS than SHAM pups in the post-asphyxia period ($P<0.05$). At D60, PSA duration was significantly longer in LPS pups than shams during post asphyxia (ANOVA main effect $P=0.045$; Figure 5).

*Lung Histology and Morphometric Analysis*

Compared to SHAM, LPS pups had a reduced alveolar number (ANOVA main effect $P=0.02$; Figure 6A). Alveolar size was similar between groups with a trend towards larger alveoli with LPS ($P=0.08$ at D28; Figure 6B). LPS pups had thicker septae (ANOVA main effect $P<0.001$), which was most evident at D07 (Figure 6C). Example lung sections are shown in Figure 7.

*Alternative Analysis*

The effect of LPS on the ventilatory responses to hypoxia ($P<0.001$ ANOVA main effect), hypercapnia ($P<0.001$), and asphyxia ($P=0.004$) maintained significance when responses were reported in absolute change from baseline (units of $\mu l/s/g$) rather than as a percentage of baseline.
DISCUSSION:

For the first time we demonstrate that experimentally-induced prenatal inflammation in the last days of gestation in the mouse markedly increases ventilatory responses to hypoxia, hypercapnia and asphyxia in the offspring, particularly at 7 days postnatal age. Our finding of enhanced chemical controller sensitivity is expected to increase the propensity for unstable ventilatory patterns once air breathing begins after delivery. Our finding of greater apnea frequency in LPS mice and duration at D07 and D28 confirms our proposal that the LPS-exposed ventilatory control system is indeed less stable. We also show that offspring affected by LPS exhibit evidence of disrupted lung development, with substantially increased septal thickness and alveolar size and reduced alveolar number. These anatomical features promote reduced gas transfer across the lung and may limit oxygenation of arterial blood. Together, our data demonstrate that prenatal inflammation causes ventilatory instability and structural lung impairment that could lead to intermittent and chronic hypoxia which is common in neonates and can have major adverse effects on long-term outcome (26, 30, 57, 66).

In mathematical models, unstable breathing is expected when the overall sensitivity, or loop gain (LG), of the feedback loop governing ventilation, exceeds a critical value of 1.0. Any random disturbance to resting ventilation, such as a sigh, causes a disturbance to arterial O₂ and CO₂ which is sensed by peripheral and central chemoreceptors whose altered discharge then elicits a compensatory change in ventilation (e.g. reduction in ventilation to apnea); ultimately the ventilatory response to the initial ventilatory disturbance depends on loop gain (controller gain × plant gain). The current study clearly demonstrates that maternal inflammation enhances the chemosensitivity to hypoxia/hypercapnia (increased “controller gain”) and thereby favors instability (particularly at D07), a prediction borne out by the increased incidence of SA and PSA in pups exposed to prenatal LPS (see Figure: 4). We also reason that the reduction in resting ventilation with LPS, in the presumed absence of a decline
in metabolism, must lead to an increase in resting arterial PCO$_2$; of note, greater PCO$_2$
renders ventilatory disturbances more effective at changing PCO$_2$ (raising “plant gain”),
further acting to destabilize breathing (16, 68).

There are several possibilities for the reduction in ventilation observed with LPS. First
we consider the possibility that ventilation was lowered due to changes in metabolic rate. The
allometric relationship between metabolic rate and body mass ($y = aM^{0.7}$) suggests that a
smaller pup should have a greater metabolic rate (and thus greater ventilation) per unit mass
than a larger control (80); however there was only a ~10% (non-significant) reduction in
body weight in LPS pups at 7 days which would not account for the 37% reduction in
ventilation (per unit body weight) at this time. Growth rates were also not reduced by LPS
(Table 1), suggesting that a substantial reduction in metabolic rate is unlikely. Second, it is
possible that the impaired or delayed lung function observed with LPS was accompanied by
increased lung elastance; thus ventilation may have been reduced due to greater mechanical
impedance to respiration. However, the effect of LPS on ventilation persisted after day 7
when lung function largely normalized. Third, ventilation may have been suppressed by a
reduction in ventilatory drive (lowered brainstem output). A combination of reduced
ventilatory drive and increased chemosensitivity is theoretically destabilizing and has been
observed in infants exhibiting periodic breathing (61).

Our study shows that prenatal LPS exposure results in respiratory instability.
Specifically, we observed: 1) The incidence of apneas, both SA and PSA, was greater in LPS
versus SHAM pups at D07 during hypoxic, hypercapnic and asphyxic recovery phases. This
increased frequency remained evident at D28 post asphyxia. 2) Apneas were longer in
duration in LPS (D07 and D28). These findings of increased incidence and duration of apneas
with LPS, combined with increased chemosensitivity and depressed mean ventilation, clearly
demonstrate that perinatal inflammation destabilizes ventilatory control. That post-stimulus
apneas diminished in LPS by D60 is consistent with the reduced frequency of periodic breathing with age seen in infants (4, 83). However an enhanced response to hypoxia persisted at D60; while not sufficient to cause overt central apneas in pups, increased chemosensitivity has been implicated in the later development of hypertension (56) and obstructive sleep apnea (15, 60, 65, 79). Our results suggest that measures to avert maternal inflammation may mitigate these lasting adverse effects.

Prenatal infections adversely affect lung development and evidence to date suggests that intra-amniotic injections of endotoxin in sheep cause fetal lung inflammation thereby reducing alveolar septation and vascularization (35) and in rodents; it has been shown to induce pulmonary inflammation and delayed alveolar maturation (9). In the mouse, lung development at birth is at the saccular phase, and alveolarization is initiated around postnatal day 5 and is completed during the first 2-3 wks of life (78). Because much of the lung development occurs postnatally in mice, we focused on the morphological differences in our two treatment groups across three time points. Increases in septal wall thickness have been reported previously in response to hyperoxia, with the phenomenon attributed to inhibition of pathways that lead to septal thinning (54, 63). Additionally, Velten and colleagues, examined the ratio of total perimeter per high-power field to septal wall thickness as a measure of surface area available for gas exchange in rat pups exposed to a combination of hyperoxia and prenatal LPS. Both these insults exhibited a remarkable increase in septal wall thickness (77). While confirming an effect of LPS, our study indicated that exposure to prenatal LPS had modest effects on postnatal respiratory structure. Differences in alveolar number were observed in LPS pups, primarily at D28. Thicker septae were observed in LPS pups at D07 (28% increase in septal thickness compared to SHAM pups). According to Fick’s Law of diffusion, thicker septae will reduce lung diffusing capacity and potentially impede gas exchange unless a compensating rise in ventilation increases alveolar $O_2$; our observation that
ventilation is less in LPS-treated pups shows that no such compensation occurred. Accordingly, the alterations in lung structure we induced via maternal inflammation may translate into reduced gas exchange, and may contribute to the requirement for supplemental oxygen in many preterm infants to achieve adequate oxygenation (10).

The impairments we observed in lung structure and ventilatory stability due to maternal inflammation/infection may contribute to the increased risk of SIDS in preterm infants. There is a wealth of supportive evidence linking SIDS to inflammation/infection, which includes autopsy findings of infection and inflammatory markers in SIDS victims (6, 28, 47, 55) and an increased risk of SIDS in winter months (13). Unrecognized hypoxemia has also been firmly implicated in SIDS, with evidence including brain stem gliosis (39, 48, 74, 76), elevated hypoxanthine in vitreous humor (53, 64), and increased VEGF in cerebral spinal fluid (34) of SIDS victims. Dominant causes of hypoxemia in infancy are, in general, abnormalities of ventilatory control and of respiratory function. In particular, ventilatory control instability has long been suspected to play an important role in SIDS (70, 75); near-miss SIDS infants have been found to have a greater predisposition to apnea and periodic breathing (7, 37), SIDS risk is elevated with residence at higher altitude where respiratory disturbances are common (22, 41), and a high proportion of SIDS victims exhibited abnormal carotid body glomus cell mass (59). Importantly, infection and ventilatory instability are also associated and may combine to yield profound hypoxemia (5, 58). A link between SIDS and maternal inflammation/infection, while speculative, is highly feasible now that we have shown that delayed/impaired ventilatory control and lung structure are consequences of maternal inflammation/infection.

This study had limitations due to the absence of metabolic rate and arterial PCO₂ measurements. We chose to employ unrestrained whole-body plethysmography to assess the primary respiratory control effects of LPS longitudinally under maximally natural conditions;
the most accurate metabolic rate measurement requires a sealed flow-through system and animal constraint which is a considerably less natural setting (and may alter metabolic rate due to stress responses to the experimental conditions).

In summary, our study shows that prenatal fetal inflammation has a significant impact on the developing lung and on the sensitivity of respiratory chemoreflexes. LPS leads to changes in lung structure in the first month of postnatal life in the mouse that is likely to reduce gas exchange efficiency, as reported for human premature infants. The reduced resting ventilation and enhanced chemoreflexes could explain the high prevalence of periodic breathing and apnea in many preterm human infants. Since apnea and periodic breathing promote hypoxemia, prenatal maternal inflammation may provide a clue to the higher incidence of SIDS in preterm infants.

**Acknowledgements**

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FIGURE LEGENDS:

Figure 1: Classic example of a spontaneous apnea (A) and a post-sigh apnea (B).
Respiratory recording was obtained from a C57BL/6 pup at D28. Arrowheads in panel A indicate the apneic pause for more than two breathing cycles. The respiratory interruptions were classified as spontaneous apneas (SA). Panel B arrowheads indicate a post sigh apnea (PSA), classified when the amplitude of the breath exceeds twice the resting V\textsubscript{T}. RESP, respiration recorded by whole body plethysmography.

Figure 2: Changes in ventilatory parameters during (A) hypoxic (10% O\textsubscript{2}) (B) hypercapnic (7% CO\textsubscript{2}) (C) asphyxic challenge (10% O\textsubscript{2} + 7% CO\textsubscript{2}) for 15 min.
Left-hand panels: responses at D07. Middle panels: responses at D28. Right-hand panels: responses at D60. All values are calculated as percentage change from baseline and expressed as mean ± SEM. The serrated box (3A) indicate the minute ventilation over 7.5 min of the challenge that has been used for analysis in conditions of hypoxia, hypercapnia and asphyxia. The graph represents SHAM pups (black circles) and LPS pups in (open circles).

Figure 3: Average Minute Ventilation (V\textsubscript{E}) through D07-D60.
The bar graph represent the changes in minute ventilation (V\textsubscript{E}) from baseline to the average of the last 7.5 min of 10 min exposures to (A) hypoxia (10% O\textsubscript{2}) (B) hypercapnia (7% CO\textsubscript{2}) (C) asphyxia (10% O\textsubscript{2} + 7% CO\textsubscript{2}) in SHAM and LPS pups through D07-D60. SHAM pups are denoted by black bars and LPS pups by open bars). All data represented as mean ± SEM
* P<0.05, ** P<0.01, *** P<0.001 LPS versus sham, post-hoc comparisons.
Figure 4: The effects of inspired gas on spontaneous apnea (SA) and post sigh apnea (PSA) frequency.

SA and PSA events expressed per minute. In SHAM pups SA are expressed in black bars, PSA in grey bars. In pups exposed to LPS, SA is indicated by open bars and PSA are indicated by serrated open bars. With the abrupt return to room air following gas challenges, the pups exposed to LPS had higher incidence of SA and PSA at D07. Modest effects were observed in the LPS group at D28 in the incidence of SA and PSA upon the transition from asphyxia. Values are mean ± SEM. *P<0.05, **P <0.01 LPS versus sham, post-hoc comparisons.

Figure 5: The effects of inspired gas on spontaneous apnea (SA) and post sigh apnea (PSA) duration.

SA and PSA events expressed per minute. In SHAM pups SA are expressed in black bars, PSA in grey bars. In pups exposed to LPS, SA is indicated by open bars and PSA are indicated by serrated open bars. Pups exposed to prenatal LPS exhibited longer SA at D07 during hypoxic and hypercapnic recovery phases. At D28, with the abrupt return to room air following asphyxic challenge; LPS pups demonstrated longer SA duration. N: apneas not detected. Values are mean ± SEM. *P<0.05, **P <0.01, LPS versus sham, post-hoc comparisons.

Figure 6: Effects of prenatal LPS on postnatal lung structure at D07-D60.

Morphometric analyses of lung tissue samples obtained from SHAM pups (black bars) and LPS pups (open bars). Panel (A) alveolar number, (B) alveolar size (µm²) (C) septal thickness (µm): n=5-10 per group. At D28, LPS pups had a decreased alveolar number, particularly at D28. LPS pups also had thicker septae at D07. Values are mean ± SEM. *P<0.05, ***P <0.001 LPS versus sham, post-hoc comparisons.
Figure 7: Histological lung sections of pups in SHAM and LPS groups.

Fixed lung tissues were stained with hematoxylin and eosin. n=5-10 per group. Stained slides scanned at a magnification X400. Internal scale bar 20µm. Top: histological sections from SHAM and LPS pups at D07. Middle: Sections from pups at D28. Bottom: Sections from the pups at D60.

Table 1: Baseline respiratory parameters in SHAM and LPS mice from D07-D60

<table>
<thead>
<tr>
<th>Parameter</th>
<th>DAY 07 SHAM (19)</th>
<th>LPS (22)</th>
<th>DAY 28 SHAM (16)</th>
<th>LPS (12)</th>
<th>DAY 60 SHAM (12)</th>
<th>LPS (8)</th>
<th>LPS vs. SHAM</th>
<th>Age×LPS Interaction</th>
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</thead>
<tbody>
<tr>
<td>Body weight (g)†</td>
<td>3.7±0.1</td>
<td>3.3±0.1</td>
<td>11.6±0.5</td>
<td>12.6±0.3</td>
<td>22.0±0.8</td>
<td>24.0±0.8</td>
<td>p=0.003</td>
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<td>Growth rate (g/day)</td>
<td>0.34±0.03</td>
<td>0.43±0.03</td>
<td>0.48±0.06</td>
<td>0.73±0.05***</td>
<td>0.15±0.02</td>
<td>0.16±0.03</td>
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<tr>
<td>Ti (s)</td>
<td>0.10±0.005</td>
<td>0.09±0.001***</td>
<td>0.07±0.001</td>
<td>0.07±0.002</td>
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<tr>
<td>Te (s)</td>
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<td>0.13±0.003</td>
<td>0.09±0.005</td>
<td>0.12±0.011***</td>
<td>0.07±0.004</td>
<td>0.09±0.001</td>
<td>p&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>Ttot (s)</td>
<td>0.23±0.007</td>
<td>0.22±0.004</td>
<td>0.16±0.007</td>
<td>0.19±0.014***</td>
<td>0.14±0.005</td>
<td>0.16±0.002</td>
<td>p&lt;0.006</td>
<td></td>
</tr>
<tr>
<td>VT (µl/g)</td>
<td>7.9±0.7</td>
<td>5.5±0.8***</td>
<td>8.3±0.5</td>
<td>6.6±0.2</td>
<td>4.8±0.1</td>
<td>3.2±0.1</td>
<td>p&lt;0.001</td>
<td>p=0.03</td>
</tr>
<tr>
<td>VE (µl/s/g)</td>
<td>28.6±2.1</td>
<td>17.9±1.2***</td>
<td>49.6±2.6</td>
<td>37.3±2.6***</td>
<td>35.1±0.8</td>
<td>19.5±1.0***</td>
<td>p&lt;0.001</td>
<td></td>
</tr>
</tbody>
</table>

The data are presented as means ± SEM. Ti: inspiratory time; Te: expiratory time; VT: tidal volume; VE: minute ventilation; Ttot: breath duration. Numbers are calculated over a 10 min control period from > 2000 breaths for each variable. *p<0.05, **p<0.01, ***p<0.001 LPS versus sham, post-hoc comparisons. †Log-transformed to provide equal variance before statistical comparisons. Growth rate was calculated using body weight over a week before the study time-point calculated as the difference/day. Age had a significant ANOVA main effect P<0.001 on all variables.
REFERENCES:


65. Rosen CL, Larkin EK, Kirchner HL, Emancipator JL, Bivins SF, Surovec SA, Martin RJ, and Redline S. Prevalence and risk factors for sleep-disordered breathing


