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Perinatal Inflammation Results in Decreased Oligodendrocyte Numbers in Adulthood

Amanda E. Graf^{1,3,*}, Krista M. Haines^{1,3,*}, Christopher R. Pierson^{2,5}, Brad Bolon⁴, Ronald H. Houston², Markus Velten⁵, Kathryn M. Heyob¹, and Lynette K. Rogers^{1,3}

¹Center for Perinatal Research, at Nationwide Children's Hospital, Columbus, Ohio

²Pathology and Laboratory Medicine at Nationwide Children's Hospital, at The Ohio State University, Columbus, Ohio

³Departments of Pediatrics, Pathology, at The Ohio State University, Columbus, Ohio

⁴Comparative Pathology and Mouse Phenotyping Shared Resource at The Ohio State University, Columbus, Ohio

⁵Department of Anesthesiology and Intensive Care Medicine, Rheinische Friedrich-Wilhelms-University, University Medical Center, Bonn, Germany

Abstract

Aims—Maternal inflammation is a risk factor for preterm birth, and premature infants are often exposed to supplemental oxygen as a life-sustaining therapy. While more immature neonates are surviving, rates of neurodevelopmental impairment are not improving. We developed a novel mouse model with clinically relevant exposures to test the hypothesis that systemic maternal inflammation with transient neonatal hyperoxia exposure will induce a phenotype similar to diffuse periventricular leukomalacia (PVL) like that observed in premature human infants.

Main Methods—Timed-pregnant C3H/HeN mice received intraperitoneal injections of lipopolysaccharide (LPS) or saline on embryonic day 16. Newborn pups were placed in room air (RA) or 85% oxygen (O₂) for 14 days, followed by 14 days in RA recovery. Oligodendroglial and microglial populations were evaluated at 14 and 28 days.

Key Findings—Brain weight to body weight ratios were lower in mice exposed to LPS. Oligodendrocyte numbers were decreased significantly in cerebral cortex and hippocampus in groups exposed to LPS or LPS/O₂ at 14 days, and persisted in cerebral cortex at 28 days for LPS/O₂ mice. At day 14, cleaved caspase 3 was increased and numbers of microglia were elevated in the cerebral cortex and hippocampus of LPS/O₂ animals.

Significance—These data indicate that combining systemic maternal LPS and neonatal hyperoxic exposure impairs myelination, and suggest that this novel mouse model may represent a

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Send correspondence and reprint requests to: Lynette K. Rogers, PhD, Center for Perinatal Research, The Research Institute at Nationwide Children's Hospital, 575 Children's Cross Road, Columbus, Ohio 43215, Phone: (614) 355-6715, Lynette.Rogers@nationwidechildrens.org.

*the first two authors share authorship and contributed equally to the project

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There are no conflicts of interest, financial or otherwise, to disclose by the authors.

subtle, diffuse form of periventricular white matter injury that could provide a clinically relevant platform for further study of perinatal brain injury.

Keywords

Lipopolysaccharide; inflammation; hyperoxia; oligodendrocyte; microglia; white matter injury; premature brain injury; development; periventricular leukomalacia

INTRODUCTION

Advances in neonatal care have resulted in smaller and more immature babies surviving the perinatal period. However, adverse neurologic outcomes remain a significant consequence of premature birth, with as many as 25–50% of very low birth weight (VLBW) babies, born weighing less than 1500 grams, experiencing some degree of neurodevelopmental impairment [1–3]. Historically, cerebral palsy was a major defining factor of adverse neurologic outcomes from the perinatal period. However, it is increasingly recognized that long term impairments related to brain injury in preterm neonates represent a spectrum of type and severity, involving mechanisms outside of classic intraventricular hemorrhage or cystic periventricular leukomalacia (PVL) [4,5]. This shift is encapsulated by descriptions of non-cystic PVL, characterized by diffuse periventricular white matter injury in the cerebrum involving a constellation of damage to vulnerable premyelinating oligodendrocytes, microglial activation, astrogliosis, and neuronal injury [4,6,7].

Translational science including investigation of animal models is necessary to understand the mechanisms and develop preventive and treatment strategies for perinatal brain injury in preterm infants [8]. Such animal models need to show brain pathology consistent with that seen in human neonates and should be induced by clinically relevant stimuli. While existing animal models have studied the impact of neonatal hypoxia-ischemia, chorioamnionitis, neonatal infection, and neonatal hyperoxia on brain development and injury [9–12], the effects of combined exposures have been reported only recently [13].

Maternal inflammation and infection are risk factors for both preterm birth and subsequent cerebral palsy [9,14,15]. Systemic maternal inflammation in the absence of chorioamnionitis or other infection is commonly seen in mothers with chronic disease, obesity, preeclampsia, and diabetes [16]. Animal studies have demonstrated that multiple infectious and inflammatory stimuli may induce a range of effects on the developing brain [17–20]. Premature neonates commonly require ventilator support and supplemental oxygen therapy at a time when decreased antioxidant capacities make them most vulnerable to oxidative stress. Injurious effects of hyperoxic conditions on the developing brain have been reported in existing animal studies [21–25].

In this study, we investigated a novel mouse model of maternal systemic lipopolysaccharide (LPS) administration followed by transient neonatal hyperoxia exposure. Prior work in our laboratory indicates that this model is well suited to examining the effects of neonatal inflammation on developing systems. We have observed an inflammatory lung phenotype similar to severe bronchopulmonary dysplasia [26–28] as well as significant functional and structural alterations in cardiac development that persist into adulthood [29].

The combination of systemic maternal inflammation and neonatal hyperoxia has overwhelming potential for injury in the neonatal brain during a critical developmental period. However, the neuropathology after these combined exposures has not been elucidated, even though it is one of the most common courses experienced by VLBW infants in Neonatal Intensive Care Units worldwide. Our hypothesis was that the combination of

systemic maternal inflammation and transient neonatal hyperoxia exposure would result in patterns of injury consistent with pathologic changes observed in preterm infants.

MATERIALS AND METHODS

Animal Model—Animal study protocols were approved by the Institutional Animal Care and Use Committee at the Research Institute at Nationwide Children's Hospital, Columbus, OH. Adult C3H/HeN mice (8 – 10 weeks old) were purchased from Harlan Sprague Dawley (Indianapolis, IN). Mice were housed in our facility for at least 7 days prior to breeding. Male and females were paired, and the presence of a vaginal plug was designated as embryonic day 1 (E1). On E16, dams received intraperitoneal injections of LPS (serotype 0111:B4, catalog no. 437627; Calbiochem, Gibbstown, NJ) in saline (approximately 0.1 mL), or saline only. The LPS dose (80 µg/kg) was chosen based on preliminary studies that consistently resulted in a viable litter [27]. Newborn mice from saline- or LPS-injected dams were pooled and redistributed randomly (yielding mixed litters of 6 to 7) to the two dams in separate cages within 24 hours of birth. One litter of pups was exposed to 85% oxygen (O₂) for 14 days in a Plexiglas chamber which was regularly calibrated (chamber calibrated every other day with an Oxygen Analyzer, Hudson RCI, model 5577) while the litter of pups from the corresponding maternal E16 treatment was maintained in room air (RA) for the same period. Day 1 was defined as the first 24 hours of O₂ or RA exposure. Litters were either sacrificed at day 14 (after hyperoxia exposure) or maintained in RA for 14 days of recovery and sacrificed at day 28. In this fashion, four treatment groups were created: saline/RA, saline/O₂, LPS/RA, LPS/O₂. To avoid oxygen toxicity in the dams and reduce confounding maternal effects between groups, the nursing dams from the same E16 treatment groups were rotated between their RA and O₂ litters every 24 hours.

Histology Processing—At days 14 and 28, two animals from each litter were anesthetized with intraperitoneal administration of ketamine/xylazine (150 mg/kg; 15 mg/kg, respectively). Whole-body perfusion was performed with ice cold saline followed by freshly prepared ice cold neutral buffered 4% paraformaldehyde. Brains were removed immediately and immersed in fresh fixative for 24 hours, then washed 3× in phosphate buffered saline (PBS) and transferred into PBS until processed and embedded into paraffin blocks. Coronal sections cut serially at 4 µm were placed on positively charged slides and then either stained with hematoxylin and eosin to evaluate general tissue architecture or labeled by indirect immunohistochemistry to examine the distribution of selected cell populations (Table 1). All immunohistochemical methods were visualized with a polymer detection system and the chromogen 3,3'-diaminobenzidine (DAB; kit DS9800; Leica Microsystems, Buffalo Grove, IL). Sections were counterstained with hematoxylin.

Glial Cell Counts—For both days 14 and 28, oligodendrocyte and microglial counts were performed on selected brain regions (cerebral cortex, hippocampus) of the middle cerebrum at the level of the internal capsule and thalamus. Intracortical oligodendrocytes, defined by their immunoreactivity with 2', 3' cyclic nucleotide 3' phosphodiesterase (CNPase), and microglia, identified by their immunoreactivity with ionizing calcium-binding adaptor molecule 1 (Iba1), were counted between the cingulum and rhinal fissure in four non-overlapping high-power fields (i.e., 100×). Additionally, tissue counts were performed on hippocampal neurons within the dentate gyrus in two non-overlapping high-power fields. Microglial cell bodies were also quantified in four non-overlapping high-power fields in the cerebral cortex and hippocampus. All counts were performed by a single investigator (oligodendrocytes by KMH, microglia by AEG) for consistency.

Caspase 3 Expression—Whole brains were extracted from randomly selected pups (2 per litter) at postnatal days 3, 7, and 14. Whole brains were selected as the sample due to the

small size of the brains at the early time points, which precluded regional microdissection. Specimens were frozen in liquid nitrogen and maintained at -80°C until analyses. Frozen whole brains were homogenized, and protein concentrations were determined by Bradford assay. For western blots, proteins were separated on SDS-PAGE gels and transferred to PVDF membranes, which were then probed with rabbit monoclonal anticaspase 3 primary antibody (Catalog# 9664; Cell Signaling, Boston, MA) at a 1:500 dilution. Antibody binding was detected using anti-rabbit secondary antibody (Catalog #170-6515, Bio-Rad Laboratories, Hercules, CA) at 1:12,000 and then developed using enhanced chemiluminescence (ECL Prime Western Blotting Detection, GE Healthcare, UK). Expression levels were quantified using Image Quant Software, Version 5.0 (Molecular Dynamics, Sunnyvale, CA). The density of the band for the protein was normalized to the density of β -actin protein (Catalog# ab6276; Abcam, Cambridge, MA).

Statistics—Statistical analyses were performed by two-way analysis of variance (ANOVA) followed by least significant difference (LSD) or Tukey's tests (corrected for multiple analyses). All data are presented as means $\pm$ standard error of the mean (SEM), and the results of the two-way ANOVA are indicated. Significance was defined as $p < 0.05$. All analyses were performed with GraphPad PRISM 5 (La Jolla, CA).

RESULTS

Brain and body weight growth patterns differ after LPS or O_2 exposures—At 14 days, hyperoxia exposure caused a modest decrease in body weight with preservation of brain weight (Table 2) leading to an effect of hyperoxia on the brain:body weight ratios (Table 2). At day 28, after 14 days for RA recovery, there was “catch-up” growth of the body weight in the saline/ O_2 -exposed group and even greater gain in the LPS/ O_2 group. There was evidence of excessive weight gain in the mice exposed to LPS which resulted in brain to body weight ratios that were lower in the LPS-treated groups than in the saline-treated groups at day 28 (Table 2).

Gross pathological changes were not observed across treatment groups—Hematoxylin and eosin stained brain sections were evaluated at days 14 and 28. These sections were examined for gross pathological changes including infarction, focal areas of necrosis, cyst formation, significant hemorrhage, or parenchymal abnormalities visible at low magnification. No overt neuropathological abnormalities were observed in any of the treatment groups at either time point (data not shown).

Cortical early myelinating oligodendrocyte cell number persistently decreased after LPS and O_2 —Numbers of CNPase-positive cortical oligodendrocytes were decreased by both LPS and O_2 exposures. At 14 days, cortical oligodendrocytes were fewer in LPS/RA and saline/ O_2 and LPS/ O_2 compared to saline/RA (Figure 1A and C). The lower numbers of cortical oligodendrocytes persisted at 28 days in the LPS/ O_2 group compared to LPS/RA and saline/RA (Figure 1B and D). Qualitatively, staining with myelin basic protein (MBP, a marker of mature myelinating oligodendrocytes) at 28 days was reflective of the quantitative results from CNPase (Figure 1E). The intensity of staining and progression of myelination from the corpus callosum through the neocortex was decreased in LPS- and O_2 -treated groups at 28 days, and was most pronounced in the hyperoxia groups.

Hippocampal early oligodendrocytes decreased at 14 days—Numbers of CNPase-positive hippocampal oligodendrocytes in the dentate gyrus were significantly decreased by treatment with LPS and O_2 . At 14 days, hippocampal oligodendrocytes were decreased in the LPS-treated groups compared to the saline-treated groups, with the lowest

numbers in the LPS/O₂-treated group (Figure 2A and C). Following the 14-day RA recovery, at day 28 there was no significant difference in hippocampal oligodendrocyte numbers (Figure 2B and D).

Combined LPS/O₂ exposure increased cleaved caspase 3 levels—Cleaved caspase 3 levels measured by western blot in whole brain homogenates were similar across treatment groups at day 3 and day 7 (data not shown). At day 14, increased levels of cleaved caspase 3 were observed in the LPS/O₂ treatment group (Figure 3).

Microgliosis, but not astrocytosis, observed at 14 days after combined LPS/O₂ exposure—Pups exposed to LPS and O₂ had increased numbers of Iba1-positive microglia in both the cerebral cortex (Figure 4 A and C) and the hippocampus at day 14 (Figure 4 B and D) compared to all other groups. However, numbers of Iba1-positive cells were no longer elevated in either region by day 28 (data not shown). Qualitative analysis of sections stained for GFAP-positive astrocytes showed no differences among treatment groups at either day 14 or 28 (data not shown).

DISCUSSION

The complex interaction between the maternal environment and fetal development directly influences the spectrum of neurodevelopmental deficits in VLBW infants [30]. Those neonates who experience seemingly benign hospital courses are still at risk for impaired neurodevelopment, despite normal-appearing routine head ultrasonography [31]. Our findings in this study demonstrate a pattern of cellular injury consistent with aspects of diffuse PVL as described in human neonates. The phenotype is subtle compared to others previously described, enhancing the potential for clinical relevance of our model.

In examination of the brain:body ratios, there were lower brain weights associated with the 14 days of hyperoxic exposure, thus leading to increased ratios in the O₂-treated groups at day 14 (Table 2). Unexpectedly at day 28, after 14 days of room air recovery, the brain:body weight ratios were lower as a result of a significant increase in body weight in the LPS-exposed groups, independent of O₂ exposure. These findings suggest a lasting effect of the maternal inflammatory response. Velten et al. reported that birth weights of LPS-exposed pups were decreased compared to saline control. In the same study, body weights of LPS/O₂ exposed animals were significantly lower than saline/RA controls at 8 weeks of age, when impaired cardiac function was identified [26]. Based upon these combined findings, we could speculate that the LPS-exposed pups develop metabolic disturbances related to intrauterine growth restriction associated with maternal inflammation, an aspect of this model that will require further investigation.

The complexity of understanding and preventing brain injury in VLBW infants requires a translational and comprehensive approach [8]. As such, several animal models have been developed that vary in insult type, timing, and severity. Current models provide a system for studying one or more of the pathophysiologic components of human PVL or gray matter injury which include loss of oligodendrocyte progenitors, impaired myelination, increased microglial activation, varying degrees of focal necrosis, reactive astrocytosis, neuronal injury, and a range of functional deficits [8–10,12]. Similar to other animal models examining LPS or hyperoxia as single interventions [9,10,21,32], our work also found decreased numbers of cortical and hippocampal early myelinating oligodendrocytes with persistently impaired myelination at 28 days (Figures 1 and 2). In contrast to neuropathologic studies of human PVL and existing animal models, we did not identify significant macro or microscopic foci of necrosis nor astrocytosis or glial scarring, suggesting a more subtle pattern of white matter injury. These findings may be due to a less

severe insult in our model, or possibly the timing of the interventions and analysis precluded us from observing additional abnormalities that may be present either prior to the conclusion of the hyperoxic exposure at day 14 or further into adulthood. Understanding the functional implications of exposure to maternal inflammation and neonatal hyperoxia in the setting of a diffuse histopathological injury could further enhance the clinical relevance of this model.

The additive effect of maternal inflammation with hyperoxia exposure on oligodendrocyte cell populations was particularly impressive in the LPS/O₂ treatment group. While apparent recovery of both early CNPase-positive oligodendrocytes and MBP-positive cell numbers were appreciated at 28 days compared to 14 days after maternal LPS exposure alone, this recovery was not seen in the combined LPS/O₂ exposure group. Recovery of oligodendrocyte cell number is been reported in animal models of PVL. Back et al. showed an loss of oligodendrocyte progenitors soon after hypoxic-ischemic injury, followed by increased oligodendrocyte cell number but persistently impaired myelination, suggesting a maturational or functional defect of remaining oligodendrocytes [11]. This may suggest that prolonged exposure to hyperoxia during a critical period in development induces further cell death rather than allowing for a reactive oligodendrogliosis. A model of systemic neonatal inflammation (LPS on day 3) and 24 hour hyperoxia exposure (day 6) has been reported by Brehmer, et al [13]. In comparison to the model presented here, there was an increase in caspase 3 protein associated with hyperoxia and a decrease in MBP after single LPS or O₂ or combined exposure. In contrast to our findings associated with maternal inflammation and 14 days of neonatal hyperoxic exposure, they observed a “protective effect” of LPS exposure on early oligodendrocyte survival and report an increase in oligodendrocyte apoptosis with O₂ exposure alone. These data suggest that while hyperoxia induces cell death, LPS exposure may impair oligodendrocyte maturation [13]. Both effects would impair myelination as evidenced by the decrease in MBP. The differences between models may be related to both the timing of LPS administration and contribution of maternal inflammatory response, as well as the difference in length of hyperoxic exposure. The finding that hyperoxia is associated with increased oligodendrocyte cell death would also support the lack of recovery seen at 28 days after combined LPS/O₂ exposure.

As mentioned, one possible mechanism for the decrease in oligodendrocytes in LPS/O₂-treated mice is apoptosis. To examine this possibility, western blots for cleaved caspase 3 protein levels were performed on whole brain homogenates at early time points (Figure 3). The increased level of caspase 3 in the LPS/O₂-exposed mice up to day 14 indicates that a substantial amount of apoptosis was taking place, and that the combined exposures had a more profound effect on apoptosis than either the LPS or O₂ exposures alone. This observation, indicating ongoing cell loss during the hyperoxic period, could explain the persistence of decreased oligodendrocyte numbers in the cerebral cortex even after a recovery period in room air. It is important to note that, because of the small brain size at early time points, caspase 3 protein levels were determined from whole brain homogenates. Analyses of specific brain regions may reveal areas of increased vulnerability and are the subject of ongoing investigation.

Another potential mechanism for the decrease in oligodendrocytes is the increased numbers of microglia present in both the cerebral cortex and hippocampus at day 14 (Figure 4) in LPS/O₂ exposed pups. Microglia are present in abundance during critical periods in the developing brain [7] and would be activated by the inflammatory stimuli imposed in this model. Although these cells do not persist after room air recovery, their presence and increased activation during early critical developmental stages may contribute to the decrease in oligodendrocyte numbers by either causing direct injury with release of reactive oxygen and nitrogen species and inflammatory cytokines, or by indirectly altering the neural

microenvironment in such a fashion as to impair oligodendrocyte survival and maturation [33–35].

The decreased numbers of cortical and hippocampal oligodendrocytes and transient microgliosis that we observe in our model aligns with recent descriptions of ‘encephalopathy of prematurity’ [5]. The finding of persistently impaired myelination may be in part related to recent reports in the magnetic resonance imaging literature in which former premature infants continue to exhibit decreased cerebral volumes into early childhood and adulthood [36,37]. Oligodendrocytes are immature and susceptible to injury at 23 to 32 weeks in a human infant [4]. In the current experiment, the timing of the maternal and neonatal interventions represents a comparable period in mouse brain development [38]. Thus, the clinical relevance of our novel “two-hit” model, LPS *in utero* combined with subsequent postnatal hyperoxia, is compelling in a biological sense and offers a plausible representation of the pathophysiology observed in VLBW human infants. The two stimuli recapitulate a common clinical scenario and are associated with histologic neuropathology that is subtle when compared to existing models. Further study of this novel model may add insight into the complex interactions between the maternal environment, fetal brain development, and inflammatory insults during critical periods. In the future, this model could potentially be used as a platform to investigate novel preventive and therapeutic strategies to improve neurodevelopmental outcomes in VLBW survivors.

CONCLUSION

Vulnerable premature infants often experience multiple inflammatory insults perinatally, including maternal inflammation or infection, followed by neonatal oxygen exposure as a life-saving medical intervention. In the current animal model, exposure antenatally to maternal inflammation and subsequent neonatal hyperoxia results in significant alterations of glial cell populations in the developing mouse brain. Further interrogation of this model may provide a mechanism to identify preventive and therapeutic targets.

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Figure 1. Oligodendrocyte numbers are decreased in cerebral cortex by treatment with LPS and/or O₂

A and B, representative images of saline/RA, saline/O₂, LPS/RA and LPS/O₂ treatment groups, labeled with CNPase as a marker for early myelinating oligodendrocytes, are shown (100×). C and D, labeled cortical oligodendrocyte cell bodies were counted per high-power field in the cerebral cortex between the cingulum and rhinal fissure. Significant effect of LPS and an effect of O₂ were indicated by two-way ANOVA at both day 14 and day 28, $p < 0.05$, $n = 2-4$ litters, $n = 1-2$ pups per treatment group. LSD *post hoc* analyses indicated: * different than saline/RA; # different than LPS/RA; ^ different than saline/O₂. Arrowheads

provide orientation toward the white matter at the base of the cortex. Small arrows identify representative CNPase-positive cell bodies.

Figure 2. Oligodendrocyte numbers are decreased in dentate gyrus of hippocampus by treatment with LPS and/or O₂

A and B, representative images of saline/RA, saline/O₂, LPS/RA and LPS/O₂ treatment groups, labeled with CNPase as a marker for early myelinating oligodendrocytes, are shown (100×). C and D, labeled hippocampal oligodendrocyte cell bodies were counted per high-power field in the dentate gyrus. Significant effect of LPS and an effect of O₂ were indicated by two-way ANOVA at day 14, $p < 0.05$, $n = 2-4$ litters, $n = 1-2$ pups per treatment group. LSD *post hoc* analyses indicated: * different than saline/RA; #different than LPS/RA; ^different than saline/O₂. No statistical differences were indicated at day 28.

Figure 3. Cleaved caspase 3 protein levels were elevated in the brains of animals exposed to LPS, with or without O_2

Proteins from whole brain homogenates obtained from saline/RA, saline/ O_2 , LPS/RA and LPS/ O_2 treatment groups at day 14 were analyzed for cleaved caspase 3 by western blot. An effect of O_2 and an interaction between O_2 and LPS were indicated at both day 14 and 28 by two-way ANOVA, $p < 0.05$, $n = 2-4$ litters, $n = 1-2$ pups per treatment group. LSD post hoc analyses indicated: *different than saline/RA; #different than LPS/RA; ^different than saline/ O_2 .

Figure 4. Immunohistochemical staining and quantification of cortical and hippocampal microglia

A, representative images of saline/RA, saline/O₂, LPS/RA and LPS/O₂, treatment groups of at day 14 are shown (100×). B, cortical and hippocampal microglial cell bodies were counted per high-power field in the the cingulum and rhinal fissure and the dentate gyrus, respectively. A significant effect of LPS was observed by two-way ANOVA, $p < 0.05$, $n = 2-4$ litters, $n = 3-4$ pups per treatment group. LSD *post hoc* analyses indicated: *different than saline/RA.

Table 1

Antibodies for immunohistochemistry analyses.

Antibody	Clone	Dilution	Antigen Retrieval	Catalog #	Supplier
CNPase (Oligodendrocytes)	11-5B mouse anti-human	1:800	citrate	ab6319	Abcam Cambridge, MA
MBP (Oligodendrocytes, myelin)	7H11 mouse anti-human	1:200	citrate	NCL-MBP	Leica Microsystems Buffalo Grove IL
Iba-1 (microglia, macrophages)	rabbit anti-human polyclonal	1:1000	EDTA	PP290-AA	Biocare Medical Concord, CA
GFAP (reactive astrocytes)	rabbit anti-guinea pig polyclonal	Predilute	None	RB-087-R7	Thermo LabVision Kalamazoo MI
<i>All antibodies were diluted with Antibody Diluent, Catalog# S3022, Dako, Carpinteria CA</i>					

Abbreviations: CNPase - 2', 3' cyclic nucleotide 3' phosphodiesterase; MBP - myelin basic protein; Iba-1 - ionizing calcium-binding adaptor molecule 1; GFAP - glial fibrillary acidic protein

Table 2

Brain weights and brain to body weight ratios.

treatment	N		day 14 [†]			day 28		
	litters d14/d 28	pups d14/d 28	brain weight [†] g	body weight [†] g	brain: body weight ^{d†}	brain weight [†] g	body weight [†] g	brain: body weight ^{d+}
Saline/RA	6/5	11/10	0.44±0.01	8.56±0.11	5.01±0.05	0.44±0.01	13.94±0.59	3.16±0.11
Saline/O ₂	6/5	11/10	0.41±0.01 [#]	8.05±0.15 ^{**#}	5.28±0.17 [#]	0.43±0.02 [#]	13.39±0.61 [#]	3.24±0.14 ^{**#}
LPS/RA	4/4	9/7	0.46±0.01	9.55±0.20 [*]	4.77±0.08	0.47±0.02	16.88±0.82 [*]	2.64±0.10 [*]
LPS/O ₂	4/4	9/7	0.42±0.02	8.33±0.47 [#]	5.15±0.17	0.46±0.01	16.64±0.40 ^{**^}	2.77±0.08 ^{**^}

^a data are expressed as g/100 g animal.

An effect of LPS (+); effect of O₂ (†); and interaction between LPS and O₂ (‡) were indicated by two-way ANOVA, p<0.05. LSD *post hoc* analyses indicated the following significant findings: *different than saline/RA; # different than LPS/RA; ^ different than saline/O₂.

Abbreviations: LPS = lipopolysaccharide, O₂ = hyperoxia, RA = room air