N-acetylcysteine attenuates lipopolysaccharide-induced impairment in lamination of Ctip2- and Tbr1- expressing cortical neurons in the developing rat fetal brain

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Oxidative stress and inflammatory insults are the major instigating events of bacterial intrauterine infection that lead to fetal brain injury. The purpose of this study is to investigate the remedial effects of N-acetyl-cysteine (NAC) for inflammation-caused deficits in brain development. We found that lipopolysaccharide (LPS) induced reactive oxygen species (ROS) production by RAW264.7 cells. Macrophage-conditioned medium caused noticeable cortical cell damage, specifically in cortical neurons. LPS at 25 μg/kg caused more than 75% fetal loss in rats. An increase in fetal cortical thickness was noted in the LPS-treated group. In the enlarged fetal cortex, laminar positioning of the early born cortical cells expressing Tbr1 and Ctip2 was disrupted, with a scattered distribution. The effect was similar, but minor, in later born Satb2-expressing cortical cells. NAC protected against LPS-induced neuron toxicity in vitro and counteracted pregnancy loss and alterations in thickness and lamination of the neocortex in vivo. Fetal loss and abnormal fetal brain development were due to LPS-induced ROS production. NAC is an effective protective agent against LPS-induced damage. This finding highlights the key therapeutic impact of NAC in LPS-caused abnormal neuronal laminar distribution during brain development.
Results

NAC protected primary cortical neurons against LPS-induced neurotoxicity.  
In utero events, such as intrauterine inflammation (also known as chorioamnionitis) trigger significant systemic inflammatory responses at the maternal-fetal interface and increase the incidence of inflammation in the central nervous system of the fetus. Activated macrophages elevate cytotoxic molecules, such as free radical species and proinflammatory cytokines, which contribute to neurotoxicity in coculture systems and in a murine model. NAC is a known ROS scavenger that not only ameliorates LPS-induced ROS generation and inflammatory effects in amniotic fluid and placenta, but also increases fetal viability and reduces white matter injury. Therefore, we tested the protective effect of NAC against LPS-induced neurotoxicity in an in vitro model. Mouse macrophage cells, RAW264.7, were treated with various doses of LPS for 24 h, and the LPS-pretreated RAW264.7 culture medium (LCM) was collected and presented to heterogeneous primary cortical cells for 48 h. The RAW264.7 cells mimic simple maternal immune effectors, and the heterogeneous primary culture mimics the natural fetal brain with various neuronal and glial cells. LPS application increased LCM ROS levels up to 180% at 24 h (Fig. 1a). MTS assay results show heterogeneous cortical cell viability decreased in a dose-dependent manner (Fig. 1b). Cortical cultures were then immunostained with anti-MAP2 for localization analysis of only cortical neurons and to determine neuronal viability with DAPI staining. The result showed that neurons were killed 48 h after LCM treatment (Fig. 1d). Furthermore, we found that NAC blocked ROS production at lower LPS doses, but not at higher doses (Fig. 1a). Interestingly, cell viability was restored by NAC when applied with the LCM in heterogeneous cortical cultures at all doses (Fig. 1b). Neuronal number was restored in the presence of NAC, which suggests that the survival of neurons was enhanced (Fig. 1d). These data clearly show that NAC resolved LPS-stimulated maternal intrauterine oxidative and inflammatory responses.

NAC inhibited maternal physiological changes in response to LPS exposure.  
Our in vitro data show that NAC ameliorated LPS-induced cytotoxicity and ROS up-regulation. Therefore, we injected NAC and LPS maternally to analyze the effect of NAC in LPS-treated pregnant rats at GD14. Maternal organs, including spleen, placenta, and blood, were harvested to evaluate LPS-induced inflammation. We found that although all the parameters changed in the LPS group, there were no significant differences compared to controls at low doses of LPS (0.25–12.5 μg/kg) (Table 1). However, the weights of the spleen and placenta increased dramatically at a dose of 25 μg/kg LPS. At this dose, LPS not only reduced RBC number and hemoglobin (Hb) value, but also significantly increased WBC number (Table 1). We found that NAC rescued spleen and placenta weights, Hb level, and RBC and WBC numbers in LPS-treated samples. Another antioxidant, ascorbic acid, was less effective in most cases (Table 1). Furthermore, the concentrations of both amniotic fluid IL-6 (Fig. 2a) and ROS (Fig. 2b) were increased in the group that was treated with 25 μg/kg LPS. NAC reduced these concentrations to control levels. These data clearly show that NAC resolves LPS-stimulated maternal intrauterine oxidative and inflammatory responses.
Figure 1. NAC rescued the LPS caused consequentially cortical neurotoxicity by inactivated macrophages secreting extracellular ROS. Primary cortical neurons were plated on 24 well plastic plates pre-coated with poly-D-lysine. At GD10, indicated LPS-conditioned media (LCM) with or without 5 mM NAC were applied for 48 hours followed by CytoTox- MTS Homogeneous Integrity Assay or fixation in 4% paraformaldehyde. Immunocytochemistry for MAP2 was conducted in fixed cells, and DAPI staining was performed to determine neuron survival. (a) Cm-H$_2$DCFDA detection kit was used to determine the LPS-induced ROS generation in cultural medium of RAW264.7 cells. RAW264.7 cells were induced to release extracellular ROS in response to 24 h LPS exposure, and NAC decreased the level of ROS in lower dose of LPS treated group. *p < 0.05; **p < 0.01; ***p < 0.001 by ANOVA with Kruskal–Wallis test followed by Dunn's multiple comparisons test. n = 3 assays, each performed in duplicate. (b) The cell viability decreased in a dose dependent manner and NAC inhibited the LPS-induced cell lost. n = 3 assays, each performed in duplicate. (c) Quantitative results from twenty different objective views were randomly selected from two to three independent experiments, and live cells were counted at each concentration. Significantly neuronal toxicity of LCM and dramatically rescued effect of NAC were observed. *Statistically different from vehicle condition without NAC. #Statistically different from each LPS dosage compared to NAC. *p < 0.05; **p < 0.01; ***p < 0.001 by ANOVA with Kruskal–Wallis test followed by Dunn's multiple comparisons test. n = 20. (d) Representative images of MAP2 positive neurons after LCM exposure in the presence/absence of NAC were generated from (c). Scale bar, 50 μm.

| Parameters                  | Vehicle | LPS (0.25 μg/kg) | LPS (2.5 μg/kg) | LPS (12.5 μg/kg) | LPS (25 μg/kg) | LPS (25 μg/kg) + NAC | LPS (25 μg/kg) + Asc |
|-----------------------------|---------|------------------|-----------------|-----------------|----------------|-----------------------|----------------------|
| Spleen Weight (%)           | 100 ± 14.8 | 82 ± 0.1 | 106 ± 12.6 | 107 ± 2.5 | 202 ± 21.7*** | 108 ± 1.9 | 112 ± 9.8* | 154 ± 29.1*** |
| Placenta Weight (g)         | 0.35 ± 0.06 | 0.31 ± 0.04 | 0.36 ± 0.05 | 0.32 ± 0.05 | 0.42 ± 0.09* | 0.38 ± 0.05 | 0.32 ± 0.06** | 0.41 ± 0.10*** |
| Placenta Area (cm$^2$)      | 1.36 ± 0.10 | 1.31 ± 0.11 | 1.34 ± 0.11 | 1.29 ± 0.13 | 1.31 ± 0.11** | 1.30 ± 0.07 | 1.31 ± 0.16 | 1.34 ± 0.20 |
| Embryo Weight (g)           | 0.94 ± 0.13 | 0.95 ± 0.13 | 0.95 ± 0.16 | 0.91 ± 0.09 | 1.09 ± 0.22** | 0.99 ± 0.13 | 0.94 ± 0.13 | 0.90 ± 0.08*** |
| Hemoglobin (g/dL)           | 16.4 ± 2.68 | 15.3 ± 0.52 | 14.0 ± 0.93 | 12.5 ± 5.67 | 13.5 ± 2.96*** | 14.5 ± 0.84 | 15.1 ± 1.05** | 11.8 ± 1.60 |
| RBC (10$^9$/mL)             | 6.48 ± 2.27 | 6.59 ± 0.57 | 4.78 ± 1.26 | 4.66 ± 0.60 | 3.62 ± 1.31*** | 6.91 ± 2.08 | 5.81 ± 0.37* | 5.23 ± 2.21** |
| WBC (10$^9$/mL)             | 8.94 ± 2.22 | 8.90 ± 1.03 | 8.62 ± 4.95 | 9.86 ± 3.11 | 14.4 ± 3.98 | 10.8 ± 4.22 | 8.53 ± 2.98 | 12.3 ± 1.55 |

Table 1. Maternal and embryo physiological conditions changed in response to LPS plus or minus NAC exposure on GD 18. The results are presented as means ± SD of three independent experiments and duplicate measurements. *p < 0.05, indicates that the mean is significantly different from the control by ANOVA with Kruskal–Wallis test followed by Dunn’s multiple comparisons test. *p < 0.05, indicates that the mean is significantly different from the LPS (25 μg/kg) group by ANOVA with Kruskal–Wallis test followed by Dunn’s multiple comparisons test.
NAC counteracted the LPS-induced decrease in embryo number and up-regulation of antioxidative enzymes in embryonic brain. Since NAC mitigated the LPS-caused maternal intrauterine inflammation and amniotic fluid ROS concentration in our in vivo model, we then asked whether NAC could alleviate LPS-induced fetal death. We found that the number of embryos was significantly decreased in the 25-μg/kg LPS group. NAC restored embryo number to normal levels (Fig. 2c). In addition, ascorbic acid showed less protection (3.75 ± 3.5 embryos, p > 0.05 compared to the 25-μg/kg LPS group). Similarly, embryo weight was increased in the 25-μg/kg LPS group, and NAC returned this level close to control (Table 1). Previous studies suggest that LPS-induced oxidative stress and ROS contribute to fetal death. NAC may have the capacity to attenuate LPS-induced cerebral white matter injury. However, little is known about the role of NAC with regard to LPS-induced oxidative damage to the embryonic brain. Accordingly, we determined the levels of antioxidative enzymes in fetal brains. As shown in Fig. 2d to 2g, antioxidative enzymes (SOD, HO-1, and catalase) were significantly increased in fetal brains of the 25-μg/kg LPS group. In NAC-treated fetuses, these enzyme levels are normal. Our findings show that the antioxidative machinery is dramatically activated against ROS production in...
Fetal brain even at 3 days after LPS application. These oxidative events were attenuated by NAC. This suggests that continuous oxidative stress can be relieved by NAC and thereby prevent fetal loss. 

NAC mitigated LPS-induced abnormal fetal brain cytoarchitecture. Previous studies offer inconsistent results regarding changes in fetal brain morphology after prenatal immune activation. For example, Carpentier and colleagues found that cortical thickness was decreased in LPS-treated animals relative to saline controls. However, increased thickness of the cortical plate and hippocampus was reported by Ghiani and colleagues. This discrepancy may be due to differences in infection models, target animal species, and infection protocol and time. To evaluate the effect of LPS on the morphology of fetal brain in our \textit{in vivo} model, we analyzed coronal sections of LPS-exposed fetal brains using H&E staining. LPS caused brain swelling particularly in the high-LPS-dose group (Fig. 3a). Application of NAC ameliorated the effects. We examined frontal neocortex thickness in both dorsal and lateral regions (location shown in Fig. 3a marked with open rectangle). The data show a significant 20% increase in the depth of lateral and dorsal neocortex in the group treated with a high dose of LPS (Fig. 3c,d). Application of NAC reduced these effects, and the thickness of the neocortex in this group was comparable to control levels (Fig. 3c,d). Furthermore, we found that the cellular structure displayed a swollen nucleus and unregulated vascular proliferation in response to LPS (Fig. 3b). The data imply a white matter injury morphology in the LPS groups. These characteristics are obvious at 25 μg/kg LPS, and NAC inhibited these changes. In addition, a previous report shows that glial activation is associated with maternal \textit{E. coli}-induced white matter injury. We then analyzed the activation of microglia by immunostaining for CD11b/c, a microglia marker. As shown in Fig. 2e, 25 μg/kg LPS induced microglial activation in the fetal cortical plate (CP), intermediate zone (IZ), and sub-ventricular and ventricular zone (SVZ/VZ). Microglia were activated in LPS treated animals, which were reduced in the presence of NAC. Scale bar, 50 μm.

NAC counteracted the LPS-induced aberrant lamination of embryonic cortex. Prenatal exposure to LPS causes a decrease in neurogenesis, myelination deficits, and abnormal neuronal morphology in developing brains. As shown in our \textit{in vivo} model, NAC could prevent LPS-induced damage to fetal brain morphology (Fig. 3). We next asked whether NAC can improve LPS-induced reductions in neurogenesis. When the ratio of bromodeoxyuridine (BrdU)-positive cells in each condition was assessed after normalizing to the vehicle, we
found no difference in neurogenesis with or without LPS treatment (Fig. 4). Moreover, application of NAC did not affect the number of BrdU-labeled cells (Fig. 4). However, we found BrdU-positive cells distributed close to the ventricles at high doses of LPS compared to the vehicle exposed group, which BrdU-positive neurons were localized mostly in the cortical plate. The effects of NAC returned the abnormal laminar characteristic to control levels. Scale bar, 50 μm. (b) displays the quantitative results of BrdU+ and DAPI+ cells in the indicated dorsal area of cortex (dotted square in (a)). Four embryo brains were randomly selected from at least three different pregnant rats. In each brain, two coronal sections near the site around 2380 μm from the front of olfactory bulb were analyzed. Ratio of BrdU+/DAPI in each condition was normalized to the PBS-exposed group. LPS treatment had no effects on the neurogenesis, determined by ANOVA followed by Tukey-Kramer Multiple Comparisons Test.

![Figure 4](image)

**Figure 4.** Maternal LPS and NAC exposure had no effects on embryonic neurogenesis. BrdU (50 mg/kg/day) were injected intradermally from GD 15 to GD 18 after exposure to LPS. Animals were sacrificed at GD 18 and immunohistochemistry was followed. Coronal sections of indicated GD 18 rat embryonic brains were immunostained for BrdU and DAPI. Merged image is composed of BrdU (red) and DAPI (green). (a) Representative images of the sections at cortical dorsal area from three independent experiments are shown. BrdU+ cells were displayed closest to ventricles in high doses of LPS treated group compared to vehicle exposed group, which BrdU+ neurons were localized mostly in cortical plate. The effects of NAC returned the abnormal laminar characteristic to control levels. Scale bar, 50 μm. (b) displays the quantitative results of BrdU+ and DAPI+ cells in the indicated dorsal area of cortex (dotted square in (a)). Four embryo brains were randomly selected from at least three different pregnant rats. In each brain, two coronal sections near the site around 2380 μm from the front of olfactory bulb were analyzed. Ratio of BrdU+/DAPI in each condition was normalized to the PBS-exposed group. LPS treatment had no effects on the neurogenesis, determined by ANOVA followed by Tukey-Kramer Multiple Comparisons Test.
Ctip2 were significantly decreased in the 25-μg/kg LPS-exposed group (Fig. 5e,f). Satb2 was not affected (Fig. 6d). The reductions of Tbr1- and Ctip2-labeled cells were restored by NAC. These data suggest that LPS-induced ROS damage dramatically affects lamination in the fetal cortex.

**Prolonged effects of LPS-induced intrauterine inflammation in fetal brain.** To determine whether the LPS-induced abnormal laminar pattern in developing brains is a temporally delayed action or an irreversible outcome, we examined LPS-treated fetal brains at 20 d of gestation with the Tbr1 and Ctip2 cortical laminae layer markers. We found that Tbr1- and Ctip2-labeled cells overlapped each other in the LPS-treated group (Fig. 7a). In the control group, layer V is clearly distinguished from layer VI (Fig. 7a), and the subventricular/ventricular zone is thicker (Fig. 7b). However, the trend is different in GD18 fetal brains with the proportion of cortical plate increased and intermediate zone reduced in the LPS-treated group (Fig. 7c). No significant difference was found in the subventricular/ventricular zone. NAC resolved the abnormal distribution to control levels.
except in the intermediate zone (Fig. 7c). Interestingly, the total thickness of the fetal cortex was decreased by LPS treatment at GD20 (Fig. 7d), but increased at GD18 (Fig. 3c,d). These data suggest that maternal infection causes an irreversible shift in the architecture of the fetal brain during development.

**Discussion**

Intrauterine infection induces serious complications in both the mother and the fetus. For the fetus, one major complication is preterm labor with a high risk for fetal death. Organ infections or abnormal development are common in premature infants. Perinatal brain injury is also a significant clinical problem caused by intrauterine infection due to the inflammatory mediators, which further lead to acute or chronic brain disease. These inflammatory mediators include ROS and cytokines. Previous studies have shown increased ROS production and associated antioxidant events in preterm infants. Systemic administration of LPS mimics maternal infection and induces macrophage activation. ROS and proinflammatory cytokine secretion contribute to neurotoxicity. Boksa and colleagues have compiled reports from 2001 to 2009 on the effects of LPS on the fetal brain, but controversy remains. Antibiotics, NSAIDs and CSAIDs, are used to treat maternal infections, but have some undesirable side effects. The development of innovative therapies therefore remains essential for progress in the prevention and treatment of intrauterine infections. In our study reported herein, using both *in vitro* and...
in vivo models, we demonstrated that NAC reduced LPS-induced oxidative damage in cultured neurons and in fetal brain, counteracted an irreversible abnormality in neuron laminar positioning during development, and decreased fetal death.

Macrophages are one of the primary maternal immune cells that respond to intrauterine infection. LPS triggers ROS elevation in macrophages, which contributes to the inflammation and fetal complications. Increased ROS also result in LPS-induced intrauterine fetal death, intrauterine growth restriction, and preterm labor. In the present study, we did not observe LPS-induced fetal growth restriction after prenatal exposure to LPS. This may be due to the lower LPS dose (25 μg/kg) at a later embryonic day, 14 d gestation, compared with other findings. However, we did observe significantly decreased embryo number (Fig. 2c) and increased ROS and IL-6 levels in amniotic fluid, consistent with previous findings. Furthermore, treatment with NAC significantly reduced embryo loss and amniotic fluid ROS and IL-6 levels (Fig. 2a–c), which is supported by other reports showing that NAC improved survival of offspring after LPS injection in rat dams at GD16-18, provided protection against fetal death and preterm labor induced by maternal inflammation, and lowered the inflammatory cytokine response in amniotic fluid and placenta in rats. A previous publication reported that NAC has potential life-threatening effects on a fetus that has been exposed to endotoxins, which worsens LPS-induced fetal hypoxemia and hypotension. However, our data show no reduction of hemoglobin and
LPS exposure. All of the aberrant LPS-induced changes in brain morphology were attenuated by administration and activation of microglia, which subsequently decreases or returns to normal. Indeed, we found that failure as discussed below. The alteration of cortex or cortical plate thickness may be due to the initial proliferation showed no notable differences (Fig. 6d) either with regard to neurogenesis or in the whole cortex (Fig. 3b). These findings indicate that endogenous tissue antioxidants respond to the increased oxidative level of antioxidant enzymes three days after LPS injection (Fig. 2d–g), similar to other published reports. Herein we show that NAC attenuated deleterious effects caused by LPS, including fetal loss, oxidative and peroxisomes, rescues cerebral oligodendroglial precursor cells, restores myelination, decreases caspase 1 and peroxisomal protein expression, and promotes corticospinal motor neuron projection. The significant reduction in specific layers of the cortical plate leads to an abnormality that alters axon projections. Such alterations in neocortical development may demonstrate how adverse perinatal events predispose the damage in the developing brain toward later behavioral abnormalities. Importantly, all lamination deficits were prevented by NAC. Other factors should not be ruled out in the case of morphological changes in the developing fetal brains. For example, a reduction in brain-derived neurotrophic factor or nerve growth factor caused by LPS affects neurogenesis in the fetal brain; reelin expression is decreased in fetal brain; lower activation and expression of Pax6 in cortical progenitors was found in the polyriboinosinic-polyribocytidylic acid infection model. We found the level of GSH, SOD, and catalase in fetal brain increased in a LPS dose-dependent manner (Fig. 2d–f). These findings indicate that endogenous tissue antioxidants respond to the increased oxidative levels induced by LPS, also noted in a previous study. NAC is an endogenous thiol-containing amino acid that scavenges ROS by interactions with its thiol redoxing group. We show that NAC promoted detoxification and prevented redox failure. NAC is able to cross the placenta and the blood-brain barrier, where NAC preserves peroxisomes, rescues cerebral oligodendroglial precursor cells, restores myelination, decreases caspase 1 and 3 expression in fetal brains with hypoxia ischemia, increases the glutathione level in fetal liver, and regulates antioxidant machinery to restore it to normal levels. However, our results showed that LPS increased the expression level of antioxidant enzymes three days after LPS injection (Fig. 2d–g), similar to other published reports. This suggests that LPS evoked temporally specific oxidative stress, which could be regulated by NAC.

Herein we show that NAC attenuated deleterious effects caused by LPS, including fetal loss, oxidative and inflammatory responses, and abnormal neocortical lamination. This strongly suggests that NAC is a potential neuroprotective factor against maternal bacterial infection.
Materials and Methods

Animals and chemicals application. All experiments were conducted in accordance with the principles of animal care and experimentation in the Guide for the Care and Use of Laboratory Animals. The Institutional Animal Care and Use Committee of the Chung Yuan Christian University approved the use of animals in this study. Six to eight weeks old Sprague Dawley rats of both sexes were purchased from BioLASCO Taiwan. The rats were put as a pair for mating once a pregnant rat was planned for the experiment. For LPS treatment, pregnant rats were injected intraperitoneally with 0, 0.25, 2.5, 12.5 or 25 μg/kg/day LPS (Sigma-Aldrich) with or without pretreated 20 mg/kg/day NAC or ascorbic acid 1 hour before LPS at GD14. For bromodeoxyuridine (BrdU) labeling, pregnant rats were injected with a single dose of 5-bromodeoxyuridine solution (50 mg/kg/day in PBS) (Sigma-Aldrich) three days from GD 15 to GD 17. At GD 18 or GD 20, the mother was sacrificed with CO2 inhalation, the blood, liver, spleen and embryo was collected immediately for analysis.

Cell culture and treatment. The mouse macrophage-like RAW264.7 cell line was obtained from the American Type Culture Collection (ATCC, Rockville, MD). They were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with penicillin, streptomycin and 10% FBS in a humidified atmosphere with 5% CO2 at 37°C. Cells were subcultured when confluence reached 90%. For primary cortical cell culture, the cultures were prepared from cortices of rat embryos of both sexes at GD 18. Cortex was dissociated and the cells were plated on glass coverslips (12 mm in diameter) coated with poly-D-lysine at a density of 1200–1600 cells/mm². The cells were cultured in Neurobasal media supplemented with B27, penicillin, streptomycin, and GlutaMax (Invitrogen, Carlsbad, CA) for 10 days prior to the treatments. For LPS application, RAW264.7 cells were treated with LPS for 24 h. The LPS-pretreated RAW264.7 cultured media (LCM) thereafter was collected and placed into the cortical cultures for additional 48 h.

Analysis of extracellular and amniotic ROS production. ROS detection studies were performed using a Cm-H2DCFDA detection kit (Invitrogen). After exposure to LPS ± NAC, the cultured media LCM or the amniotic fluid was collected and pipetted into 96-well plate and mixed with 10 μL Hank's Buffered Salt Solution (HBSS) containing Cm-H2DCFDA reagent (25 μM final concentration in each well). ROS concentration was determined immediately at fluorescence 485/530 nm by using a microreader. The ROS production values were normalized and presented as percentage of the control.

Amniotic fluid IL-6 detection. IL-6 was evaluated using a Rat IL-6 ELISA Kits (R&D Systems, Minneapolis, MN, USA), following the manufacturer’s instructions. The levels of fluid IL-6 was determined by comparison to a standard curve, which was prepared by analyzing 2-fold serial dilutions of each cytokine.

Determination of cytotoxicity in cortical cells. Cytotoxicity was determined by the commercial CytoTox- MTS Homogeneous Integrity Assay Kit (Promega, Madison, WI). The live cortical cells were determined with quantifying their mitochondrial enzyme activity via reductive conversion of the tetrazolium salt MTS to a soluble formazan dye. The amount of the dye was measured spectrophotometrically at absorbance 490 nm. Values were normalized and expressed as percentage of the control.

Neurons viability. The LPS ± NAC treated cortical cells were fixed with 4% paraformaldehyde in PBS for 15 min at room temperature, followed by PBS washes. The fixed neurons were then immunostained with mouse anti-MAP2 (neuron marker) (1:500, Abcam, Cambridge, MA). MAP2 positive neurons were then visualized with FITC or Alexa Fluor 488 anti-mouse secondary antibodies (Jackson ImmunoResearch Inc.) under a 20X objective. Nuclei were stained using DAPI (Sigma) to determine cell survival. At least twenty different objective views were randomly selected from two to three independent experiments. The experimenter was blinded to the condition when taking images and counting. Neurons with positive MAP2 immunostaining, and even and intact DAPI staining were considered alive.

Immunoblot. The tissue lysates were prepared in SDS containing sample buffer, and equal volumes of lysates were separated by 12% SDS-PAGE gel. Proteins were transferred to nitrocellulose membrane and the blots were probed with the following primary antibodies: mouse anti-SOD (1:1000, Abcam); rabbit anti-catalase (1:2500, Abcam); mouse anti-HO-1 (1:10000, Abcam), and mouse anti-α-tubulin (1:5000, Abcam). Appropriated HRP-conjugated secondary antibodies were then applied to the blots. Blots were visualized by enhanced chemiluminescence (Promega™ ECL Western Blotting Substrate) and analyzed on the Odyssey Infrared imaging system (LI-COR Biosciences) (Lincoln, NE).

Immunohistochemistry and analysis of fetal brain development. The fetal brain were harvested at GD 18 or GD 20 in some experiments, and was perfused and fixed in 4% paraformaldehyde for at least 12 h, sunk in 30% sucrose/PBS and embedded in OCT and frozen at −80 °C. The fixed samples were sectioned at 10 μm with a cryostat. A GD 18 fetal brain was carried out approximately 780 slices and a GD 20 fetal brain was approximately 1090 slices. In each quantification, four to five fetal brains from different pregnant rats were collected. In each brain, two coronal sections near the site, around 2380 μm for GD18 and 3330 μm for GD20, from the front of olfactory bulb were analyzed. The sections were dried overnight. Nonspecific reactivity was blocked and sequentially incubated with primary rabbit anti-TBR1 (1:500, Abcam), rat anti-Ctip2 (1:500, Abcam), mouse anti-SATB2 (1:400, Abcam) and mouse anti-BrdU (1:500) (Sigma-Aldrich). The slides were transferred to secondary antibodies labeled with Alexa Fluor 488 and 594 (Jackson ImmunoResearch Inc.) and covered with an anti-fade mounting media with DAPI. Images were observed on an IX51 Olympus Microscope. For laminar analysis, dorsal area of coronal cortical section was analyzed using NIH Image J software based on a previous
and 2 was analyzed. The ratio of Ctip2+/DAPI staining from the front of olfactory bulb were analyzed. There were approximately 780 sections in a GD18 fetal brain.

In each condition is normalized to the vehicle. The depth of cortex was measured along a line orthogonal to the superficial pia and callosal surfaces. At least five images from two to three independent experiments were counted at each group. For neurogenesis, the ratio of BrdU+/DAPI, Satb2+/DAPI, Ctip2+/DAPI or Tbr1+/DAPI cells were counted at an oblong area of dorsal cortex with fixed width (108.1 μm) over entire thickness of cortex. The ratio of cortical plate, intermediate zone, and subventricular/ventricular zone to the depth of cortex was analyzed. At least five images from two to three independent experiments were counted at each group.

H&E (hematoxylin and eosin) staining and analysis. The coronal sections of LPS-exposed fetal brain were stained with H&E staining based on published report with some modification. Briefly, the slides were post-fixed with 4% PFA, followed by distilled water washes. Slides were stained with Hematoxylin for 5 min and washed out with distilled water and 95% ethanol. Eosin were then applied for 3 min. Sequential dehydration steps (50 to 100% ethanol and xylene) were followed and mounting solution was applied. The slides were visualized by general phase contrast microscope. The thickness of dorsal or lateral neocortex were obtained from presumptive motor area above the middle body of the corpus callosum. Total dorsal cortical thickness were measured along a line orthogonal to the superficial pia and callosal surfaces. Lateral cortex measurements were obtained from the presumptive boundary in between central neocortex and insular cortex. Total lateral cortical thickness were measured along a line orthogonal to the superficial pia and ventricle surfaces between striatal, subventricular zone and neocortical subventricular zone. Five embryo brains were randomly chosen from at least three different pregnant rats in each condition. In each brain, two coronal sections near the site around 2380 μm from the front of olfactory bulb were analyzed. There were approximately 780 sections in a GD18 fetal brain.

Statistics. Statistical significance (p < 0.05) was determined using ANOVA followed by the appropriate post hoc test or Student's t-Tests using GraphPad InStat software.

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Author Contributions

M.-W.C. and C.-P.C. contribute equally to the work. M.-W.C. and C.-P.C. conceived of the study, and participated in the study design and helped to draft the manuscript. Y.-H.Y. performed the research and analyzed the data. JC carried out the macrophage culture and its related experiments. CL participated in primary cortical neuron viability assay. T.-Y.C. and JC analyzed amniotic fluid and its analysis. CL contributed in primary cortical neuron viability assay. Y.-C.C. performed the western blotting and its analysis. CL performed in primary cortical neuron viability assay. T.-Y.C. and JC analyzed amniotic fluid ROS production and immunostaining. C.-Y.T. designed, drafted the manuscript, and revised the manuscript. All authors read and approved the final manuscript.

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