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An animal model for chorioamnionitis at term

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Abstract

OBJECTIVE—The purpose of this study was to develop an animal model for intrapartum inflammation at term to investigate the interactions between maternal and fetal inflammatory responses and adverse neurologic outcome.

STUDY DESIGN—Lipopolysaccharide (160, 320, or 640 $\mu\text{g/kg}$) was administered intraperitoneally to day 20 term-pregnant Sprague Dawley rat dams 2, 4, and 6 hours before sample collection. Maternal outcomes included dam core temperature and plasma interleukin 6 (IL-6). Fetal outcomes included plasma IL-6, brain IL-6 messenger RNA expression, and brain IL-6 protein expression. Primary cortical cell cultures were prepared to examine neuronal morphologic condition. Neurite counts were obtained with the use of automated Sholl analysis.

RESULTS—Maternal plasma IL-6 levels peaked 2 hours after lipopolysaccharide stimulus and rapidly resolved, except for an observed low level persistence at 6 hours with 640 $\mu\text{g/kg}$. Fetal plasma and placental IL-6 expression also peaked rapidly but only persisted in placental samples. Fetal brain IL-6 RNA and protein expression was significantly higher than control litters at 6 hours after the exposure to both 320 $\mu\text{g/kg}$ ($P = .05$) and 640 $\mu\text{g/kg}$ ($P = .01$). Cortical cells from fetuses that were exposed for 6 hours to maternal systemic inflammation showed reduced neurite number and neurite length ($P < .001$) with increasing lipopolysaccharide dose.

CONCLUSION—Our results demonstrate that fetal brain injury follows isolated systemic maternal inflammation and that fetal brain inflammation lags after maternal stimulus, which creates a potential 4-hour clinical window for therapeutic intervention.

Keywords

brain injury; cerebral palsy; chorioamnionitis

Cerebral palsy affects 1-3 of every 1000 newborn infants after term birth, with distinct pathologic conditions that are related to the stage of brain development at the time of

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insult.^{1,2} In the immature human brain, the periventricular white matter of the motor cortex is the most susceptible to injury; the basal ganglia and gray matter are more likely to be affected at term.³⁻⁴ Intrapartum chorioamnionitis significantly increases the risk of neonatal encephalopathy and subsequent cerebral palsy; this association is most robust at term.⁵⁻⁹ A growing body of evidence suggests that clinical chorioamnionitis at term is largely attributable to maternally driven, noninfectious inflammatory causes rather than ascending infection.¹⁰⁻¹⁵ Fever at term is associated with low rates of placental infection by detection of bacterial RNA (5.4%).¹⁰ Further, 40% of patients with the diagnosis of clinical chorioamnionitis at term do not have evidence of bacteria in the amniotic cavity with the use of cultivation or molecular microbiologic techniques.¹¹ Therefore, it is important that experimental models adequately reproduce the likely maternal-to-fetal transmission of inflammation that occurs with term chorioamnionitis.

Rodent models of fetal inflammation have been used widely to investigate the consequences of maternal inflammation on preterm delivery, fetal development and the mechanisms that are involved in preterm brain damage.¹⁶ In addition, 1 model of infectious term chorioamnionitis has been described previously.^{17,18} Because the clinical cause of inflammation varies with gestational age, the models that are used to study the mechanisms of perinatal brain injury should also vary with respect to inflammatory agent, dose, and delivery route. Lipopolysaccharide commonly is used as a nonspecific inducer of inflammation. In studies that are designed to mimic chorioamnionitis at term, it is critical to model maternal systemic inflammation with subsequent fetal inflammation, rather than the preterm mechanisms of ascending intrauterine infection. Therefore, we used an intraperitoneally administered lipopolysaccharide rodent model to induce maternal systemic to fetal inflammation.

The risk of fetal brain injury that is associated with inflammation at term increases synergistically with other adverse exposures, especially hypoxia and hyperthermia.¹⁹⁻²² Therefore, it is vital to understand the timing of maximal fetal brain inflammation after a maternal inflammatory stimulus so that secondary insults and interventions can be timed effectively in experimental investigations.

Materials and Methods

Animals

Twenty-four timed-pregnant Sprague Dawley rats (Harlan, Indianapolis, IN) were housed in our animal facility with a 12-hour light cycle (6 AM to 6 PM) and ad libitum access to food and water during both housing and experimental time. All experiments were performed on day 20 of pregnancy. To avoid any circadian effect, all experimental procedures were started at the same time of the day. The experimental protocol was reviewed and approved by the Institutional Animal Care and Use Committees at Medical University of South Carolina and Temple University. Before each experiment, a baseline sample of maternal blood (0.5 mL) was withdrawn from the tail vein and collected in a microcentrifuge tube that contained a solution of disodium dihydrate EDTA (1.8 mg/mL blood) and centrifuged (1300g, 10 minutes, 4°C). A Lidoderm 5% lidocaine patch (2.5 cm²; ENDO Pharmaceuticals, Malvern, PA) was applied on the dorsum between the shoulder blades 1 hour before the injection of

an implantable glass-encapsulated passive transponder that was inserted in a dedicated stainless-steel syringe-type applicator (Implantable Programmable Temperature Transponder, IPTT-300; Bio Medic Data Systems Inc, Seaford, DE). Animals were then returned to their cages for temperature stabilization. After 20 minutes, temperature was recorded with a wireless receiver (DAS-6007; Bio Medic Data Systems Inc) every 2 minutes until at least 5 consecutive recordings were obtained that differed by no $>0.3^{\circ}\text{C}$.

At this time, either 0.9% sodium chloride (Hospira, Inc, Lake Forest, IL) or increasing doses (0, 160, 320, and 640 $\mu\text{g/kg}$; $n = 6$ dams per dose) of *Escherichia coli* O55:B5 lipopolysaccharide (Calbiochem, San Diego, CA) resuspended and sonicated in normal saline (Hospira, Inc) was injected (500 μL) intraperitoneally. Injection time was counted as time 0 hour.

Core body temperature was then recorded every 2 minutes, and 0.5-mL blood samples were collected from the tail vein every 2 hours until the end of the experiment. Euthanasia was performed by decapitation at 2, 4, or 6 hours after intraperitoneal injection, and all the pups were collected by hysterectomy. From each litter, 3 pups were collected aseptically in ice-cold Ca^{2+} and Mg^{2+} free Hanks' balanced salt solution (Gibco, Gaithersburg, MD) to prepare cortical cell cultures. From the remaining pups in each litter, brains and placentas were divided into 2 symmetrical halves; 1 was snap-frozen in liquid nitrogen, and the other was placed in RNAlater at 4°C for 24 hour and stored at -80°C until use. Blood was collected from the vessels of the neck after decapitation with an EDTA-Minivette POCT (Sarstedt, Nümbrecht, Germany).

Enzyme-linked immunosorbent assay

Quantification of interleukin-6 (IL-6) from plasma samples was performed with Quantikine rat IL-6 immunoassay kit (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions. Because of the limited volumes that were obtained, plasma that was collected from the pups was pooled for IL-6 quantification. Sample absorbance was quantified by a microplate reader (Bio-Tek Instruments, Winooski, VT) at 450 nm with a correction wavelength set at 570 nm. Values were calculated by nonlinear regression with the use of the GraphPad Prism (version 6; GraphPad Software Inc, La Jolla, CA). The sensitivity of the assay was 21 pg/mL. The coefficient of variation was 8.1%.

Quantitative polymerase chain reaction

Total RNA was extracted from brain and placenta samples by homogenization in TRIzol reagent (1 mL/ 100 mg tissue; Ambion Life Technologies, Austin, TX) according to the manufacturer instructions. Extracted RNA was resuspended in 100 μL of nuclease-free water with the addition of RNasin ribonuclease inhibitor (Promega Corp, Madison, WI) and stored at -80°C . RNA was further purified with the RNeasy Mini Kit combined with the RNase-Free DNase Set (Qiagen, Valencia, CA) according to manufacturer's protocol. RNA was analyzed by spectrophotometry (NanoVue Plus; GE Healthcare, Piscataway, NJ) and by gel electrophoresis (6 V/cm). Each sample (2–4 μg) was loaded onto a 1.2% agarose gel with 0.7% formaldehyde added as a denaturing agent.

One-step quantitative polymerase chain reaction (qPCR) was performed with StepOne Plus (Applied Biosystems, Foster City, CA) reverse transcription PCR system with the GoTaq 1-Step RT-qPCR System (Promega Corp). One hundred nanograms of RNA and 500 nmol/L of carboxy-X-rhodamine reference dye (Promega Corp) were added to each 20 μ L reaction. The housekeeping gene primer pair (β actin) was diluted to 300 nmol/L, and the IL-6 primer pair was added according to manufacturer instructions. QPCR cycles were set as recommended by the manufacturer, with minor modifications (15 minutes at 37.5°C; 10 minutes at 95°C, and 40 cycles of 15 seconds at 95°C and 1 minute at 60°C). Final melting curves were run by 0.5°C increments (15 seconds at 95°C; 1 minute at 60°C) to 95°C (15 seconds). Single peak melting curves were obtained from all qPCR reactions, which is indicative of specific product amplification (data not shown).

Primer sequences for β actin were obtained from NCBI Blast (F: 5'-AGC CAT GTA CGT AGC CAT CC -3'; R: 5'- CTC TCA GCT GTG GTG GTG AA -3'). The primer pair for rat IL-6 was a RT2 qPCR Primer Assay (Qiagen). For each experimental group, 8 samples from 2 litters were run in triplicate. Relative quantification of gene expression was calculated by 2^{-C_t} and are shown as folds of increased messenger RNA transcription after normalization over the control values.²³

Electrophoresis

QPCR product size was confirmed by gel electrophoresis on Criterion (Bio-Rad Laboratories, Hercules, CA) 15% tris/boric acid/ethylenediaminetetraacetic acid precast gels run for 3 hours in a Criterion Cell (Bio-Rad Laboratories), according to manufacturer instructions. Diluted β -actin samples (1:2) and undiluted IL-6 reactions were run together with a GeneRuler Ultra Low Range DNA ladder (Thermo Scientific, Waltham, MA) were used as reference, according to manufacturer protocol. Gels were poststained for 45 minutes on a rocker in a $\times 3$ solution of RedGel (Biotium, Inc, Haywood, CA) diluted in water. Images were acquired with a Gel Doc EZ system (Bio-Rad Laboratories).

Quantitative western blot

Protein extracts were prepared from rat brain tissues. Cellular debris was removed by centrifugation for 5 minutes at 4°C; the supernatant was assayed for protein content by Bradford analysis (Bio-Rad Laboratories). Fifty micrograms of proteins were diluted with Laemmli sodium dodecylsulfate sample-reducing buffer, (6X), heated at 95°C for 10 minutes, and separated by gradient sodium dodecylsulfate sample-polyacrylamide gel electrophoresis in 1X Tris-Glycine-Sodium Dodecyl Sulfate buffer and transferred to reinforced supported nitro-cellulose membranes (Whatman, Piscataway, NJ) for 2 hours at 4°C. Membranes were blocked with Odyssey Blocking Buffer (LI-COR, Inc, Lincoln, NE) for 1 hour at room temperature and incubated with specific primary antibodies for 2 hours in Odyssey Blocking Buffer with 0.1% Tween-20. The blots subsequently were washed 3 times and incubated with IRDye 800CW goat anti-mouse and IRDye 680RD goat anti-rabbit secondary antibodies and visualized with an Odyssey CLx Imaging System (LI-COR, Inc), with the use of Odyssey software (LI-COR Biosciences). Anti-IL-6 mouse monoclonal antibody (ab9324) was purchased from Abcam (Cambridge, MA). Anti-GAPDH rabbit

polyclonal (# PA1-987), which was purchased from Pierce Chemical Co (Rockford, IL), served as a loading control.

Cortical cell cultures

Cultures were prepared as previously described.¹⁷ Briefly, brains were removed from the skull and sectioned into ice-cold Hank's Balanced Salt Solution (Gibco) to discard olfactory bulbs, brain stem, cerebellum, and meninges. Cortices from each litter were stored separately until being processed in ice-cold Hibernate-E complete media (5 mL/pair; Gibco) supplemented as indicated by manufacturer with GlutaMAX-I and B27 supplement (Gibco). Tissues were trypsinized for 15 minutes in 0.05% Trypsin-EDTA (Gibco) diluted in Neurobasal media (Gibco) (1:80; 4 mL/pair of cortices) in a cell culture incubator (37°C; 5% CO₂; 95% humidity). Fragments of tissue were then added to neurobasal media that were enriched with 10% heat-inactivated fetal bovine serum (Sigma Chemical Company, St. Louis, MO). Samples were pooled together and further dissociated by mechanical trituration for 1 minute. The cell suspension was collected for cell counting and seeding.

Immunofluorescence

Cells were seeded (40,000 cells/well) into 12-well plates on 0.01% poly-L-lysine coated (Sigma Chemical Company) 18-mm glass coverslips in neurobasal media that were supplemented with GlutaMAX and B27 supplement (neuronal selective media; Gibco). On in vitro day 3, the cells were rinsed with warm phosphate-buffered saline solution (PBS) and fixed with ice-cold 4% paraformaldehyde for 30 minutes at room temperature.

Cultures were costained with DAPI (Sigma-Aldrich, St. Louis, MO) and anti-MAP2 antibody. Briefly, cell membranes were permeabilized with 0.5% Triton X-100 in PBS (Sigma) and nonspecific binding sites were saturated with 2% BSA (Fisher Scientific, Waltham, MA) in PBS. Samples were incubated overnight at 4°C with the primary antibody (ab11267, 1:500) in 1% goat serum (Sigma-Aldrich) in PBS. A secondary antibody solution that was prepared in the aforementioned manner (ab96882, 1:100) was incubated for 1.5 hours in the dark at room temperature. Nuclei were stained for 5 minutes at room temperature with 1 µg/mL DAPI in PBS. Coverslips were mounted with Prolong Gold anti-fade reagent (Invitrogen, Carlsbad, CA), according to manufacturer instructions. Images were acquired with a Nikon i80 microscope (Nikon Instruments Inc, Melville, NY) equipped with a Roper Scientific camera (Q Imaging, Surrey, Canada) with a ×100 objective.

Three brains from 2 litters were used to prepare cell cultures; from each culture, 3 coverslips were stained. From each coverslip, the images of at least 10 single nontouching neurons were acquired randomly (n = 60 per experimental condition). The total number of neurites and the length of each neurite were obtained with the use of automated software for Sholl analysis (ImageJ; National Institutes of Health, Bethesda, MD).

Statistical analysis

Ordinal-by-ordinal testing or 1-way analysis of variance with Least Significant Difference post-hoc testing was performed (SPSS Statistics for Windows, version 20.0; IBM, Armonk, NY). A probability value of < .05 was considered statistically significant. Forty neurons

were counted from 6 pooled cortical cultures for each experimental condition (80% power to detect a 33% decrease in mean number of neurites compared with the control group in pairwise analysis). Eight brains were prepared for analysis of inflammatory cytokines for each experimental condition (80% power to detect a 60% increase in mean IL-6 compared with the control group in pairwise analysis).

Results

Lipopolysaccharide reduces maternal core temperature

The lowest dose of lipopolysaccharide that was administered (160 $\mu\text{g/kg}$) did not have any effect on maternal core temperature. At 320 $\mu\text{g/kg}$ and 640 $\mu\text{g/kg}$, lipopolysaccharide significantly reduced body core temperature with dose-dependent latency, duration, and magnitude (Figure 1). At the median dose, the effect lasted up to 4 hours after a latency of 1.5 hours; at the higher dose, the effect lasted up to 5.5 hours after a latency of 1 hour. The mean decrease in temperature was $-1.0 \pm 0.3^\circ\text{C}$ (1.5 hours to 4 hours) after 320 $\mu\text{g/kg}$ and $-1.6 \pm 0.3^\circ\text{C}$ (1.5 hours to 5.5 hours) after 640 $\mu\text{g/kg}$.

Maternal and fetal plasma IL-6 concentrations after lipopolysaccharide

Peak maternal plasma IL-6 cytokine response occurred within the first 2 hours, and the magnitude of response did not differ with dose of lipopolysaccharide, except for a persistent low-level maternal response at 6 hours after the highest dose (Figure 2, A). Maternal plasma levels declined rapidly to baseline by 6 hours at lower doses. Control group dams had constant undetectable IL-6 plasma levels. Fetal plasma IL-6 levels also peaked at 2-4 hours, but the magnitude of the response did not appear to be dose dependent (Figure 2, B). Fetal plasma IL-6 levels returned to baseline by 6 hours at all doses.

Placental and fetal IL-6 messenger RNA expression

Placental IL-6 RNA expression peaked at 2 hours (Figure 3, A). At 6 hours, placental IL-6 RNA expression remained elevated compared with controls at all doses. Fetal brain IL-6 RNA expression was increased significantly only at the 6-hour time point and only at doses of 320 $\mu\text{g/kg}$ and 640 $\mu\text{g/kg}$ (Figure 3, B). Fetal brain IL-6 RNA expression for individual pups is shown in Figure 3, C. These data indicate that fetal brain response is largely uniform in this intraperitoneal administration model.

IL-6 protein in fetal brain

Fetal brain IL-6 protein expression was quantified in fetal brain at 6 hours (Figure 4). Given that IL-6 RNA expression was not increased at 160 $\mu\text{g/kg}$, only specimens from pups that were exposed to 320 or 640 $\mu\text{g/kg}$ were assayed. Protein data confirmed our gene data. IL-6 protein expression was increased significantly at both 320 $\mu\text{g/kg}$ and 640 $\mu\text{g/kg}$ but was highest in the 640 $\mu\text{g/kg}$ group. Further, the data spread again suggests a relatively uniform pup brain inflammatory response to maternal lipopolysaccharide treatment.

Neuronal cell damage assessed by neurite sprouting

To assess fetal brain injury, we used an established method to quantify neuronal cell damage in cortical primary cultures.¹⁶ A representative image from each maternal dose is shown with a superimposed Sholl template (Figure 5, A). The number of neurites per cell body and the length of each individual neurite was quantified by automated Sholl analysis in 40 neurons. There was a significant decrease in both neurite length with an increasing lipopolysaccharide dose (Figure 5, B) and the number of neurites per cell body after the 320 $\mu\text{g/kg}$ and 640 $\mu\text{g/kg}$ doses (Figure 5, C).

Comment

Principal study findings

Our data suggest a rapid and consistent maternal, placental, and fetal in-inflammatory response after maternal intraperitoneal lipopolysaccharide administration. The mechanism of the fetal response to maternal inflammation is not well understood either in animal models or in humans in vivo. Lipopolysaccharide does not appear to cross the placenta^{24,25}; direct placental transfer of maternal cytokines such as IL-6 is limited.²⁶⁻²⁸ We and others have demonstrated that the placenta can be a source of inflammatory cytokines and may be the link that propagates the fetal inflammatory response.^{27,29}

After a fetal plasma cytokine response, it is unknown to what extent cytokines permeate the fetal blood-brain barrier (BBB). In adult rodents, cytokines are transported through the BBB in low amounts.^{30,31} In rat fetuses, contrasting opinions have been reported regarding the maturity of BBB that may permit for more permissive transfer.³²⁻³⁴ An other potential source of inflammatory cytokines is the brain itself; neurons, astrocytes, microglial, and endothelial cells are all capable of producing cytokines. It is still unclear from available data whether the damaging fetal neuroinflammatory cascade originates from the fetal,³⁵⁻³⁷ placental,^{29,38,39} or maternal⁴⁰ compartment or a combination of these sources.

The effect of systemic inflammation on maternal temperature

Previous investigations in rat models after lipopolysaccharide administration have reported altered thermoregulatory responses. Core temperature depression appears to follow a decrease in the central nervous system thermoregulatory set-point.⁴¹ Brain nitric oxide⁴² and endogenous glucocorticoids⁴³ have been shown to act as mediators. Because lipopolysaccharide passage across the BBB does not occur in adult rats,²⁵ the changes induced on dam core temperature are likely indirect effects of lipopolysaccharide and involve cytokines and second messengers such as prostaglandins.

Harre et al⁴⁴ showed no gestation-related changes in the activation of signal transducer and activator of transcription 3, which is an IL-6 activated transcription factor, and suggests that signal transducer and activator of transcription 3 is not involved in the hypothermic response. Fofie et al⁴⁵ hypothesized that hypothermia that occurs in term pregnant rats is due to an imbalance of cryogenic cytokines (IL-1 receptor antagonist) compared with pyrogenic cytokines (IL-6 and interleukin 1 beta). Fofie et al⁴⁵ based this hypothesis on findings that lipopolysaccharide O111:B4 induced IL-1 receptor antagonist and tumor

necrosis factor- α in all rats, but that IL-6 and IL-1 β increased only in nonpregnant rats. In contrast, our data with lipopolysaccharide O55:B5 demonstrate a robust increase in IL-6 after lipopolysaccharide administration in pregnant dams. Further, peak maternal serum IL-6 levels correspond to the nadir in maternal temperature (2 hours).

The effect of systemic maternal lipopolysaccharide on maternal and fetal cytokines and fetal IL-6

Our data suggest a rapid and consistent maternal, placental, and fetal inflammatory response after maternal intraperitoneal lipopolysaccharide administration. The timeline in maternal plasma, placenta, and fetal plasma suggests that the inflammatory response in these compartments is rapid and essentially synchronous. Of note, neonatal cytokine production is biased towards IL-6 (compared with other inflammatory cytokines such as tumor necrosis factor- α),⁴⁶ and IL-6 predominates in the fetal inflammatory response syndrome.⁴⁷ In the fetal brain compartment, the inflammatory response is delayed until 6 hours after maternal stimulus. Fetal brain levels lag 4 hours behind fetal plasma levels; therefore, permissive transfer across an immature BBB is not a compelling mechanism. Taken together, these data suggest a fetal neuroinflammatory response with in situ cytokine production.

The effect of systemic lipopolysaccharide on fetal neuronal injury

Our data demonstrate that there is dose-dependent fetal neuronal injury after isolated maternal lipopolysaccharide injection. The injury we demonstrate would be expected primarily to affect communications between neurons and other surrounding cells through a decrease in length of communicating neurites or a decrease in their number. Our data are consistent with that of Gilmore et al,⁴⁸ who found that inflammatory cytokines affect the development of primary dendrites and the complexity of developing cortical neurons prepared from embryonic Sprague-Dawley rats. These findings suggest a possible impairment consequent to maternal inflammation in the absence of infection, hyperthermia, or hypoxia. The functional significance of these findings is not understood fully at this time. It is not known whether neuronal plasticity can compensate for early injury and/or to what degree.

Research implications

The current investigation establishes a reliable model of chorioamnionitis at term and delineates several important variables. First, we have determined that the ideal dose of *E coli* O55:B5 lipopolysaccharide is 640 μ g/kg, which induces maximal fetal brain inflammation without precipitating parturition. This is critical to allow investigators a sufficient window to administer either additional intrauterine insults or targeted protective interventions. The lipopolysaccharide serotype, the dose within the O55:B5 lipopolysaccharide serotype, and the timing of sampling can have a profound effect on experimental results.⁴⁹⁻⁵³ We acknowledge that lipopolysaccharide mimics an infectious stimulus for inflammation. Although much of term chorioamnionitis is now believed to be sterile, the signature maternal and fetal cytokine of term fever is IL-6.⁵⁴

Lipopolysaccharide O55:B5 appears to reproduce the time course and elevation of IL-6 that has been observed in human serum and cord blood after term chorioamnionitis. We chose

not to assess multiple other cytokines because the maternal/fetal cytokine patterns of infectious vs sterile chorioamnionitis overlap and have not been reported discretely. Until these patterns are better understood, it is impossible to assess whether the lipopolysaccharide sero-type O55:B5 is the best stimulus to mimic term chorioamnionitis. However, the key take-away point is that sterile maternal inflammation, with or without fever, results in inflammation in the fetal brain and placenta and increased measures of fetal brain injury and may affect the fetal immune system.

Second, we delineate the time course for fetal brain inflammation after maternal systemic inflammation to allow subsequent studies to time secondary insults accurately (ie, ischemia-hypoxia and hyperthermia). Given the relatively small number of fetal brain samples in each subgroup, we may have had limited power to detect smaller increases in fetal brain IL-6 before the peak at 6 hours. Therefore, we cannot rule out some in-flammatory insult at 2 or 4 hours. Reduced maternal core temperature during systemic inflammation may have a neuroprotective effect on fetal brain that is supported by the clinical use of hypothermia for infants who are born with complications such as perinatal asphyxia and ischemic encephalopathy. Hypothermia has been shown to improve outcomes if administered within the first 6 hours of life.⁵⁴⁻⁵⁷ Therefore, lipopolysaccharide-based rat models that seek to investigate in utero fetal brain injury after term chorioamnionitis should stabilize explicitly and/or increase dam temperature to avoid inadvertent neuroprotection. We have developed and tested a heated air system to increase dam core temperature (data not shown) to mimic fully term chorioamnionitis. Despite this limitation, our results underscore that neuronal injury can result from the maternal-to-fetal transmission of inflammation without the additional insult of hyperthermia.

Clinical implications of this research

Our findings may have important clinical implications. If human responses are similar, the onset of clinical chorioamnionitis (maternal fever) coincides with maternal and placental inflammation and follows shortly after maternal inflammatory stimuli (eg, epidural analgesia). There may be a 4-hour therapeutic window between the inflammatory stimulus and the onset of significant fetal brain inflammation. During this window, the potential deleterious effects on the fetal brain may be limited to hyperthermia. It is critical to note that our results do not suggest that cesarean delivery for chorioamnionitis is beneficial; indeed, available data suggest no benefit of a shortened duration of neonatal exposure.⁵⁸ More encouraging is the concept of intrauterine neuroprotection. The ideal neuroprotective agent could be given after the identification of maternal fever and rapidly cross the placenta and fetal BBB before the onset of fetal brain inflammation. One proposed neuroprotectant with adequate and rapid placental transfer is N-acetyl cysteine⁵⁹; however, no adequate clinical trials have been reported. In addition, several promising neonatal interventions have been described and are under development, which include dendrimer-based N-acetyl cysteine therapy.⁶⁰ The experimental model we describe can enhance the investigation of new, clinically relevant, therapeutic windows to reduce the incidence of neonatal encephalopathy and cerebral palsy back to background levels from the approximately 9-fold increased risk that has been observed after inflammation/hyperthermia at term.⁵⁻⁹

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FIGURE 1. Dose-dependent effect of lipopolysaccharide on dam core temperature

Lipopolysaccharide dose effect on dam core temperature was monitored up to 6 hours at 640 µg/kg (dotted/-dashed line), 320 µg/kg (dashed line), 160 µg/kg (dotted line), and 0 µg/kg (continuous line). Data are expressed as mean $\pm$ SD (n = 2-6 animals per time point). One-way analysis of variance with Least Significant Difference Post-Hoc test was performed with repeated measures taken into account; a probability value of .05 was set as a significance threshold. The *single asterisk* indicates a probability value of .05; the *double asterisk* indicates a probability value of .01 vs control. The *apostrophe* indicates minutes.

FIGURE 2. Time-course of maternal and fetal plasma IL-6 levels after exposure to increasing doses of LPS

LPS was injected at time 0 hours at the doses of 640 µg/kg (*squared bars*), 320 µg/kg (*vertical line bars*), 160 µg/kg (*horizontal line bars*), and 0 µg/kg (*open bars*). Data are expressed as mean $\pm$ SD. One-way analysis of variance with Least Significance Difference Post-Hoc test was performed; a probability value of .05 was set as a significance threshold. **A**, Values that were obtained at time 0 hours before LPS administration were below the lowest limit of detection of the assay in maternal plasma (mean sensitivity = 21 pg/mL). At 2 hours, 320 µg/kg and 640 µg/kg was associated with increased IL-6 compared with control ($P < .05$). At 4 and 6 hours, only 640 µg/kg significantly increased compared with control ($P < .05$). Control group dams had constant basal IL-6 levels at all time points. **B**, Values at 2 and 4 hours increased at all doses compared with controls ($P < .05$); there was no significant difference in fetal response by dose. At 6 hours, fetal levels were comparable with controls at all doses. Control fetuses had constant basal IL-6 levels at all time points.

FIGURE 3. Time-course of placental and fetal brain IL-6 RNA levels after dam exposure to increasing doses of LPS

Representative gel of amplicons that were obtained from **A**, placenta tissue or **B**, fetal brain 2 hours after maternal LPS injections. In each gel, products that were obtained from amplification reactions with IL-6 primers are shown on the *left*; products that were obtained with β -actin primers are shown on the *right*. Lanes are labeled *c*(controls) and *i*, *ii*, *iii* (160, 320 and 640 μ g/kg LPS), respectively. Graphs depict IL-6 RNA expression levels in placenta and fetal brain relative to expression at 0 hours with LPS doses of 640 μ g/kg (*squared bars*), 320 μ g/kg (*vertical line bars*), 160 μ g/kg (*horizontal line bars*) and 0 μ g/kg (*open bars*). Data are expressed as mean $\pm$ SD. One-way analysis of variance with Least Significant Difference Post-Hoc test was performed; a probability value of .05 was set as a significance threshold. **A**, The *single asterisk* indicates a probability value of .05; the *double asterisk* indicates a probability value of .01 vs saline solution; the *triple asterisk* indicates no significance compared with 640 μ g at 2 hours or 160 μ g at 6 hours. **B**, The *single asterisk* indicates a probability value of .05 vs saline solution. **C**, Distribution of fold IL-6 expression normalized to β -actin in individual pup brains from the 640 μ g/kg and control groups is shown.

FIGURE 4. IL-6 protein expression in fetal brains 6 hours after maternal LPS injection
 Representative protein blots from control, 320 $\mu\text{g}/\text{kg}$, and 640 $\mu\text{g}/\text{kg}$ with control (GAPDH).
 Relative fluorescent intensity normalized to GAPDH shown from individual pup brains.
 One-way analysis of variance with Least Significant Difference Post-Hoc test was performed; a probability value of .05 was set as a significance threshold.

FIGURE 5. LPS dose-dependent reduction in neurite sprouting by automated Sholl analysis

A, A representative neuron is shown from primary neuronal cultures at each dose with an overlay, **B**, to demonstrate quantification of neurite length. Categories of neurite length by LPS dose are shown. **C**, Ordinal-by-ordinal analysis was performed for categories of neurite length ($P = .0006$). The number of neurites per cell body is shown. One-way analysis of variance with Least Significant Difference Post-Hoc test was performed; a probability value of $.05$ was set as a significance threshold. The number of neurites per cell body was significantly related to dose (overall analysis of variance: $P < .001$). Post-hoc pairwise analysis compared with control: $P < .06$ vs $160 \mu\text{g/kg}$; $P < .001$ vs $320 \mu\text{g/kg}$; $P < .001$ vs $640 \mu\text{g/kg}$.