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Subclinical exposure to low-dose endotoxin impairs EEG maturation in preterm fetal sheep

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1Department of Physiology, University of Auckland, Auckland, New Zealand; 2Institute of Genetic Medicine, International Centre for Life, Newcastle University, Newcastle Upon Tyne, United Kingdom; 3Howard Florey Institute, University of Melbourne, Melbourne, Australia; and 4Liggins Institute, University of Auckland, Auckland, New Zealand

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Keogh MJ, Bennet L, Drury PP, Booth LC, Mathai S, Naylor AS, Fraser M, Gunn AJ. Subclinical exposure to low-dose endotoxin impairs EEG maturation in preterm fetal sheep. Am J Physiol Regul Integr Comp Physiol 303: R270–R278, 2012. First published June 13, 2012; doi:10.1152/ajpregu.00216.2012.—Exposure to chorioamnionitis is strongly associated with neurodevelopmental disability after premature birth; however, it remains unclear whether subclinical infection affects functional EEG maturation. Chronically instrumented 103–104-day-old (0.7 gestational age: term 147 days) fetal sheep in utero were randomized to receive either gram-negative LPS by continuous low-dose infusion (100 ng iv over 24 h, followed by 250 ng/24 h for 4 days; n = 6) or the same volume of normal saline (n = 9). Arterial plasma cortisol, ACTH, and IL-6 were measured. The delta (0–3.9 Hz), theta (4–7.9 Hz), alpha (8–12.9 Hz), and beta (13–22 Hz) components of the EEG were determined by power spectral analysis. Brains were taken after 10 days for histopathology. There were no changes in blood gases, cardiovascular variables, or EEG power during LPS infusion, but a transient rise in plasma cortisol and IL-6 (P < 0.05). LPS infusion was associated with loss of the maturational increase to higher frequency activity, with reduced alpha and beta power, and greater delta power than saline controls from 6 to 10 days (P < 0.05). Histologically, LPS was associated with increased numbers of microglia and TNF-α-positive cells in the periventricular white matter and frontoparietal cortex, increased caspase-3-positive cells in white matter, but no loss of CNPase-positive oligodendrocytes, Nurr-1 subplate cells, or gyrality complexity. These data suggest that low-dose endotoxin exposure can impair EEG maturation in preterm fetal sheep in association with neural inflammation but without hemodynamic disturbances or cortical injury.

NEURODEVELOPMENTAL DISABILITY is very common after premature birth (5). Although the etiology is complex, there is increasing evidence that exposure to in utero infection at critical stages of brain development can significantly increase the risk of neurodevelopmental abnormalities, as recently reviewed by Hagberg et al. (24). Strongly supporting a causal linkage, in fetal sheep, high-dose exposure to LPS, a purified polysaccharide from the outer wall of gram-negative bacteria, is associated with significant cardiovascular instability, including hypotension, and mortality, followed by significant neural injury (10, 11, 15, 18–20, 37). For example, Dean et al. (15) recently showed that a single bolus of LPS in preterm fetal sheep was associated with acute brain injury after 3 days (13), followed by impaired brain growth and loss of the normal maturational increase in cortical EEG amplitude 10 days later.

In contrast, there are relatively few data on how, or indeed whether, subclinical insults that do not cause cardiovascular or placental compromise affect the developing brain (34, 35). Subclinical infection is common and also highly associated with adverse outcomes (23, 47). The purpose of this study was, therefore, to investigate the hypothesis that subclinical exposure of the preterm fetal sheep at 0.7 gestation to low-dose LPS would be associated with impaired electrophysiological maturation, as assessed by quantitative EEG analysis (29). Brain development at this age is broadly consistent with 28 to 32 wk in humans, before the development of cortical myelination (33). The fetal EEG at this age is consistent with that of the preterm human infant, with discontinuous mixed frequency activity with periods of quiescence alternating with periods of high-amplitude, slow-wave activity (3, 12).

MATERIALS AND METHODS

Experimental Preparation

All procedures were approved by the Animal Ethics Committee of Auckland University, New Zealand. Fifteen Romney/Suffolk fetal sheep (gestation 98–99 days; term = 147 days) were operated on using sterile techniques (4). Ewes were given 5 ml of Strepcin [procaine penicillin (250,000 IU/ml) and dihydrostreptomycin (250 mg/ml), Stockguard Laboratories, Hamilton, New Zealand] intramuscularly for prophylaxis 30 min prior to surgery. Anesthesia was induced by intravenous (iv) injection of Alfaxan (alphaxalone, 3 mg/kg, Jurox, Rutherford, Australia), and general anesthesia was maintained using 2–3% isoflurane in oxygen. Following a maternal midline abdominal incision and exteriorization of the uterus, fetal catheters were placed in the left femoral artery and vein, right brachial artery and vein, and the amniotic sac, for the recording of blood pressure, blood sampling, and drug infusions. A reversible inflatable silicone occluder was placed around the umbilical cord (In Vivo Metric, Healdsburg, CA). Electrocardiograph (ECG) electrodes were sewn across the chest to record fetal heart rate (FHR). Two pairs of EEG electrodes (AS633–7SSF, Cooner Wire, Chatsworth, CA) were placed on the dura bilaterally over the parasagittal parietal cortex (5
mm and 10 mm anterior to bregma and 5 mm lateral). A reference electrode was sewn over the occiput. The fetus was returned to the uterus, and all leads were exteriorized through the maternal flank. The maternal long saphenous vein was catheterized for postoperative maternal care and euthanasia. Gentamicin (80 mg; Pharmacia and Upjohn, Perth, Australia) was administered into the amniotic sac prior to uterus closure. Following surgery, sheep were housed together in separate metabolic cages with access to water and food ad libitum, in a temperature-controlled room (16 ± 1°C, humidity 50 ± 10%) with a 12:12-h light-dark cycle. Antibiotics were administered daily for 4 days iv to the ewe, comprising 2 days of gentamicin, and 4 days of benzylpenicillin (600 mg benzylpenicillin sodium; Novartis, Auckland, New Zealand; 80 mg gentamicin). Fetal arterial blood was taken daily for blood gas analysis for assessment of fetal condition. Fetal catheters were maintained patent by continuous heparinized isotonic saline infusion (20 IU/ml at 0.2 ml/h).

Recordings

All signals were acquired at 512 Hz, processed online, and stored to disk as 1-min averaged intervals by custom software (LabView for Windows, National Instruments, Austin, TX). Fetal mean arterial pressure (MAP), corrected for maternal movement by subtraction of amniotic fluid pressure (Novatrans II MX860, Medex, Hilliard, OH), FHR, and EEG were recorded continuously from 12 h before the start of the saline or LPS infusions, and continued for a further 10 days. The EEG signal was high-pass filtered at 1.6 Hz and low-pass filtered at 50 Hz; then it was stored for analysis of seizures at a sampling rate of 64 Hz. Total EEG power was calculated from the intensity spectra and normalized by log transformation (dB, 20 log [power]), and presented as a change from the 12-h baseline period. EEG spectral edge frequency (SEF) was calculated as the frequency below which 90% of the EEG intensity was present (40). Quantitative EEG measurements for each waveform band were performed by automated analysis techniques, and each band is presented as the frequency below which 90% of the EEG intensity was present (40). Power was calculated from the intensity spectra and normalized by log transformation (dB, 20 log [power]), and presented as a change from the 12-h baseline period. EEG spectral edge frequency (SEF) was calculated as the frequency below which 90% of the EEG intensity was present (40).

Experimental Protocol

Experiments were conducted at 103–104 days gestation following 4 to 5 days postoperative recovery. Fetuses were randomly assigned to either saline infusion (n = 9) or LPS infusion (n = 6). LPS (Escherichia coli 055:B5; Sigma-Aldrich New Zealand, Auckland, New Zealand) 100 ng/kg iv was infused over 24 h (50 ng/ml at 83 μl/h) beginning at 5 days after instrumentation. The infusion rate was then increased to 250 ng·kg⁻¹·24 h⁻¹ for the next 92 h (50 ng/ml at 207.5 μl/h). Saline control fetuses received with sterile normal saline at the same rate as the LPS infusion group.

In all groups, fetal arterial blood was taken at 5 min before the start of infusions, 5 min before the increase in infusion rate, and 2 and 6 h after the increase in infusion rate. Once infusions had finished, arterial samples were taken every 24 h until the end of the experiment. Blood samples were tested for pH and blood gas determination, acid-base balance (Ciba-Corning Diagnostics 845 Blood Gas Analyzer/CO-oximeter, East Walpole, MA), and glucose/factate measurements (YSI 2300; Yellow Springs Instruments, Yellow Springs, OH). Arterial blood samples were taken for cortisol, ACTH, and IL-6 analysis.

Cortisol and ACTH Analysis

Fetal plasma cortisol levels were measured using triple quadrupole mass spectrometry. One-hundred microliters of internal standard (20 ng/ml cortisol-d4 in water) was added to 200 μl of plasma. Steroids were extracted using 1 ml of ethyl acetate (Merck, Darmstadt, Germany). After removal of the organic supernatant, samples were dried by vacuum concentration (Savant SC250EXP, Thermo Scientific, Asheville, NC), resuspended in 60 μl of mobile phase 72% methanol (Merck) and 28% water, and transferred to HPLC injector vials. Twelve microliters were injected onto an HPLC mass spectrometer system consisting of an Accela MS pump and autosampler followed by an Ion Max APCI source on a Finnigan TSQ Quantum Ultra AM triple quadrupole mass spectrometer all controlled by Finnigan Xcalibur software (Thermo Electron, San Jose, CA). The mobile phase was isocratic, flowing at 250 μl/min through a Luna HST 2.6 μm C18(2) 100 × 3.0 mm column at 40°C (Phenomenex, Auckland, New Zealand). Retention time was 3.1 min for both cortisol and cortisol-d4. Ionization was in positive mode, and Q2 had 1.2 mTorr of argon. The mass transitions followed were cortisol-d4 367.2 → 121.2 at 28 V and cortisol 363.2 → 122.2 at 28 V. Mean interassay and intra-assay CV values for cortisol were 5.8% and 6.0%, respectively.

Immunoreactive concentrations of ACTH were measured in duplicate using a commercially available 125I RIA kit (24130; DiaSorin, Stillwater, MN) previously validated for use with both fetal and maternal ovine plasma. The intra-assay and interassay coefficients of variation were 9.7 and 12.8%, respectively. The mean sensitivity of the ACTH assay was 9.7 pg/ml; samples containing <9.7 pg/ml were given this value for analysis.

IL-6 concentrations in the plasma were measured using an in-house ELISA, using ovine specific antibodies (Epitope Technologies, Melbourne, Australia). Standards used were ovine recombiant IL-6 (Protein Express, Cincinnati, OH). The standard series ranged from 0 to 5 ng/ml. The assay sensitivity was 0.097 ng/ml, and internal quality controls were included in each assay. IL-6 concentrations were within the detection limit in all samples.

Tissue Preparation

Ten days after the start of LPS infusion, the fetus was killed with pentobarbital sodium (9 g iv to ewe; Pentobarb 300; Provet, Christchurch, NZ). Total fetal weight and brain weights were recorded. Fetal brains were perfusion-fixed in situ with 0.9% saline solution then 10% phosphate-buffered formalin (500 ml), then removed and fixed for a further 3 days before processing and paraffin embedding (2). Serial coronal sections (10 μm) of the forebrain were taken beginning at 26 mm anterior to stereotaxic zero (level of the midbrain-thalamus) and 17 mm anterior to stereotaxic zero, level of the dorsal horn of the anterior hippocampus and the midthalamus). For all analyses described below, numbers of cells were determined for both hemispheres at each level, and the average was used for analysis.

Histology and Immunohistochemistry

Basic histological evaluation was performed using thionine and acid-fuchsin-stained slides under a Nikon Eclipse 80i microscope with a motorized stage and Stereo Investigator software V.8 (Microbrightfield, Williston, VT). An experimenter blinded to different treatments assessed these slides for structural damage, tissue infarction, and necrosis. In the periventricular white matter (PVWM; Fig. 1), numbers of oligodendrocytes (CNPase positive), activated microglia [isolectin B4 (IB4) positive], and cells positive for tumor necrosis factor-alpha (TNF-α), or the apoptotic marker cleaved caspase-3 were quantified. IB4, TNF-α, Nurr-1, and cleaved caspase-3-positive cells were quantified in the cortex.

Lectin immunohistochemistry. Reactive microglia was labeled with biotin-conjugated lectin from Lycopersicon esculentum (Sigma, St. Louis, MO). Sections from the frontoparietal cortex were deparaffinized, hydrated, and subjected to antigen unmasking in 0.1 M citrate buffer (pH 6.0) at 95°C for 20 min in a water bath and left for cooling at room temperature (RT). Endogenous peroxidase was quenched with 1% H2O2 in methanol. Sections were then incubated with lectin (diluted 1:100) in Tris-buffered saline at 4°C overnight and later incubated with ExtrAvidin peroxidase (Sigma; diluted 1:200) for 3 h at RT before developing color with diaminobenzidine tetrahydrochloride (DAB).
Fig. 1. Photomicrograph showing an example of the coronal section at 26 mm anterior to stereotaxic zero used for histological analysis. Nurr-1 counts were taken from the three highlighted sulci on the left and right. ×1.25 magnification. Scale bar = 2.5 mm.

CNPase, TNF-α, and caspase-3. Mouse monoclonal antibody to CNPase (2′3′-cyclic nucleotide 3′-phosphodiesterase, 11–58) was used to label myelinated fibers and myelinating oligodendrocytes (Abcam: www.abcam.com, diluted 1:200). To label cells undergoing apoptotic cell death in the PVWM, polyclonal cleaved rabbit anti-caspase-3 (ASP 175) was used (Cell Signaling Technology, Beverly, MA; diluted 1:200). Monoclonal mouse anti-TNF-α was used to label TNF-α (1:200; AbD Serotec) expression. Dewaxed, hydrated, and antigen unmasked sections were treated with 1% H2O2 in methanol for 30 min. Sections were then treated with 5% normal horse serum (for CNPase) or normal goat serum (for caspase-3) for 1 h for blocking nonspecific binding prior to incubating with primary antibody at 4°C overnight. Sections were incubated with biotinylated horse anti-mouse/goat anti-rabbit secondary body (Vector Laboratories, Burlingame, CA; diluted 1:200) for 3 h. These sections were subsequently incubated with extravidin peroxidase (Sigma; diluted 1:200) for 3 h at RT. DAB was used as a chromogen.

Rabbit polyclonal antibody to Nurr-1 was used to label subplate neurons in cortical layers adjacent to periventricular white matter ([15] Santa Cruz Biotechnology, Santa Cruz, CA; diluted 1:200). Sections were incubated with biotinylated goat anti-rabbit secondary antibody (1:200 dilution).

The density (cells/mm²) of oligodendrocytes and TNF-α-positive cells were estimated in the PVWM and cortex using Stereo Investigator. Total counts of capase-3-positive cells in the PVWM and cortex were obtained after marking the boundary with Stereo Investigator. Nurr-1-positive cells were counted in 3 sulci from both the left and right cortex at 17 and 26 mm from stereotaxic zero, as shown in Fig. 1 and the estimated cell counts were then averaged for each animal.

Sampling for counting was based on stereology principles. The area to be counted was initially traced around each region of interest and then randomly translated to a grid onto the sections. We then applied a fractionator probe consisting of a counting frame for object inclusion and exclusion. Cell counts for the region of interest were converted to density (cells/mm²) by applying the equation [(total markers counted/number of sampling sites × counting frame area) × 106 (14), and the average value from the left and right, and three sections per animal were used for analysis.

**Gyri assessment.** To assess whether LPS infusion influenced gyral development, thionine-acid fuchsin-stained sections at +17 and 26 mm from stereotaxic zero were analyzed. A contour was traced,

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Data are expressed as means ± SE. C, saline control group; LPS, LPS group; BE, base excess; Glu, glucose; Lac, lactate.

Table 1. Blood gases, acid-base status, glucose, and lactate values over the duration of the experiment.
horizontal to the midline of the hemispheres touching the upper limit of the lateral ventricle using Stereo Investigator. A comparable area in the whole hemisphere dorsal to this line (A) and the total length of the boundary (L) was measured for calculating the gyral surface folding index (GSFI) (15). The mathematical formula for calculating GSFI is $L^2/A$ (38). GSFI is not affected by shrinkage of tissue and since the SFI of a circle is 12.56, higher values suggest greater expansion of the surface relative to total volume (6).

Data analysis. All data were originally processed in minute averages for the whole 10-day period and subsequently averaged into time periods as outlined in the RESULTS section. The effect of the infusions on EEG intensity, spectral edge frequency, delta, theta, alpha, or beta power, blood pressure, carotid blood flow, or heart rate were evaluated.

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**Fig. 2.** Time sequence of changes in mean arterial pressure (MAP), carotid artery blood flow (CaBF), change in EEG power, and spectral edge frequency, with saline (○) or LPS infusion (●). All data represent 8-h averages from 20 h before infusions commenced until day 10 of the experiment. Data are expressed as means ± SE. *P < 0.05, ANOVA with post hoc least significant difference (LSD) tests. Light-shaded area indicates low-dose infusion (100 ng over 24 h), while dark-shaded area indicates increased infusion (250 ng over 24 h).

**Fig. 3.** Time sequence of changes in the percentage of power in the delta, theta, alpha, and beta bands (delta activity, % of EEG power), theta activity (theta activity, % of EEG), beta activity (beta activity, % of EEG), with saline infusion (○), or LPS infusion (●). All data represent 8-h averages from 20 h before infusions until day 10 of the experiment. Data are expressed as means ± SE. *P < 0.05, ANOVA with post hoc LSD tests. Light-shaded area indicates low-dose infusion (100 ng over 24 h), while dark-shaded area indicates increased infusion (250 ng over 24 h).
by ANOVA with time as a repeated-measure (ANOVA, SPSS v15, SPSS, Chicago, IL) followed by Fisher’s protected least-significant difference (LSD) post hoc test when a significant overall effect was found. Blood gas analysis, hormone, and cytokine results were assessed by one-way ANOVA at each time point with subsequent LSD post hoc tests. Where baseline differences were found, an ANCOVA was used for the remainder of those data. Nonparametric data were tested with Mann-Whitney U-tests. Statistical significance was accepted when \( P < 0.05 \). Data are expressed as means ± SE.

RESULTS

Blood Composition Measurements, Blood Pressure, and Fetal Weight

There was no difference in baseline \( \text{PO}_2 \), \( \text{PCO}_2 \), acid base status, glucose, and lactate concentrations between groups prior to the infusions, or at any point during or after the infusions (Table 1). There was no change in fetal blood pressure or carotid artery blood flow during or after LPS infusion (Fig. 2). There was no difference in fetal weight at post mortem (LPS 2.2 ± 0.1 vs. saline 2.1 ± 0.1 kg).

EEG Maturation in Saline Control Group

There was no significant change in EEG power over the 10 days from the start of infusion in saline controls, but a marked increase in SEF (repeated-measures ANOVA, \( P < 0.001 \), Fig. 2). There was a progressive fall in delta power (Fig. 3) and an increase in the proportion of theta, alpha, and beta power (\( P < 0.05 \), repeated-measures ANOVA).

Fig. 4. Time sequence of changes in cortisol, ACTH, and IL-6 levels over the 10-day experimental period in the saline infusion (○), or LPS infusion (●) groups. Data are expressed as means ± SE. Each time point was tested by one-way ANOVA. \( *P < 0.05 \), ANOVA with post hoc LSD tests. Light-shaded area indicates low-dose infusion (100 ng over 24 h), while dark-shaded area indicates increased infusion (250 ng over 24 h).

Fig. 5. Changes in numbers of activated microglia (isolectin B4), TNF-\( \alpha \) positive (+) cells, apoptotic cells (cleaved caspase-3), and CNPase positive oligodendrocytes in periventricular white matter in saline controls and fetuses exposed to a 5-day infusion of LPS. \( *P < 0.05 \), **\( P < 0.001 \).
Effect of LPS on EEG maturation

There was no difference in changes in EEG power between groups, before, during, or after LPS infusion; however, SEF increased less in the LPS group over the 10 days and was significantly lower than saline controls from day 6 to day 10 ($P < 0.05$; Fig. 2). This corresponded with a reduced proportion of alpha and beta power in the LPS group than controls from day 6 onward ($P < 0.05$, Fig. 3), with, reciprocally, partial loss of the maturational fall in delta power ($P < 0.05$). There was no significant change in theta power.

Endocrine Response

LPS infusion was associated with a significant increase in plasma levels of cortisol at 2 and 6 h after the start of infusion compared with baseline; the increase was significant compared with saline controls at 2 h ($P < 0.05$, Fig. 4). There was an increase in IL-6 levels at 2 and 6 h compared with controls that resolved by 24 h. The increase in infusion rate after 24 h was associated with a small increase in IL-6 but not cortisol at 6 h. There were no further significant differences between groups despite continued infusion. ACTH showed an apparent trend to be transiently higher after the start of infusion ($P = 0.051$).

Histology and Immunohistochemistry

There was no difference in brain weights between groups at post mortem (LPS 31.2 ± 2.4 vs. saline 29.7 ± 2.3 g). No structural injury of the brain was seen on thionine/acid fuchsin-stained coronal sections in either group.

Fig. 6. Photomicrographs showing examples of isolecitin B4 (IB4), TNF-α, CNPase-positive oligodendrocytes, cleaved caspase-3 positive cells (arrows) in periventricular white matter, and Nurr-1-positive subplate neurons in saline controls (left) and after LPS infusion (right). Scale bar is 40 μm.
Periventricular white matter. In saline-treated control brains, resting microglia, identified by their characteristic ramified appearance (45), were sparsely distributed in the white matter. The low-dose infusion of LPS was associated with extensive infiltration of reactive (amoeboid) microglia staining for IB4 in the PVWM (Figs. 5 and 6, \( P < 0.001 \)). TNF-\( \alpha \)-labeled cells were present in PVWM in saline controls, with a marked increase in numbers of cells after LPS infusion (\( P < 0.001 \)). Oligodendrocytes labeled with anti-CNP-ase were seen in the superficial, middle, and deep cerebral white matter. There was no significant effect of LPS compared with saline controls (Fig. 5). Few caspase-3 positive oligodendrocytes were present in the PVWM in saline controls, and there was a modest increase after LPS infusion (\( P = 0.04 \)).

Cortex. No neurons showing ischemic cell change with acidophilic cytoplasm were seen in the hippocampus, striatum, or thalamus in any group. There was no effect of LPS on the GSFI (LPS 47.7 ± 6.0 vs. saline 49.6 ± 4.1). Activated microglia were sparsely distributed throughout the cortex, with a moderate increase after LPS infusion (\( P < 0.05 \), Fig. 7). Similarly, TNF-\( \alpha \) cells were widely expressed in both groups, and were increased after LPS infusion (\( P < 0.01 \)). There was no significant effect of LPS on numbers of cleaved caspase-3-positive cells within the parietal cortex (\( P = 0.84 \)), and there was no significant difference in numbers of Nurr-1-positive subplate neurons in the sulci after LPS infusion (\( P = 0.53 \)).

DISCUSSION

This study shows that a stable, low-dose exposure to LPS over 5 days that was not associated with any change in fetal blood gases, perturbation of carotid blood flow, or arterial blood pressure, still markedly impairs the normal maturation of the EEG. This impaired maturation developed in the presence of a low-grade systemic and cerebral inflammatory response, with modestly increased white matter apoptosis, but no significant loss of CNPase-positive oligodendrocytes, and no changes in gray matter gyration or apoptosis, or loss of Nurr-1-positive subplate neurons. These data strongly infer that subclinical exposure to infection can impair or delay functional maturation without significant pathological injury.

The 103–113 -day period of gestation in fetal sheep, the equivalent of weeks 27–32 of human gestation, is a key period of neural development (1), when myelination of the cortex and subcortical white matter has just begun (36). Brain stem-evoked potentials have not yet emerged (8, 9), consistent with the increase in numbers of cortical synapses from thalamocortical afferent axons at a similar stage in humans that enables the progressive vertical organization of the cortical plate (44). In turn, the development of visible EEG waveforms such as delta, theta, alpha and beta requires more developed neural networks with signal generators in deep cerebral structures (42). The emergence of fast synchronous rhythms appears to be related to maturation of inhibitory interneuron activity (31).

The present observations are consistent with clinical evidence that lower frequency of the EEG may be associated with adverse prognosis in preterm infants (27), although others have not reproduced this association (46). Potentially, this inconsistency could reflect relatively small cohorts and, in light of the experimental data discussed below, variable exposure to chorioamnionitis. More broadly, qualitative clinical studies have shown that a “dysmature” EEG, with features of maturational arrest, strongly predicts subsequent cognitive impairment (25, 41).

Experimentally, two previous studies in fetal sheep have also suggested long-term effects on EEG development after acute, severe LPS exposure. Experimentally, Dean et al. (15) found that in preterm fetal sheep, at a similar gestation to the
present study, a single intravenous bolus of LPS triggered loss of a maturational increase in overall EEG amplitude, although changes in frequency were not reported. Further, Gavilanes et al. (21) showed that a large intra-amniotic bolus of LPS given to older fetuses (111 days) was associated with increased delta activity on a 5-min EEG recording 14 days after exposure. Both of these paradigms are associated with substantial white and gray matter injury (13, 21, 22, 39) with loss of cortical volumes (15). Dean et al. (15) found marked loss of the Nurr-1-positive subplate neurons, which may deprive the brain of one substrate of ongoing cortical development.

In contrast, the present study found no loss of gyral complexity, no increase in cortical apoptosis, and no loss of Nurr-1 cells, suggesting that functional maturation of the brain may be directly related to exposure to endotoxins in utero and is not necessarily a consequence of overt gray matter damage. The changes in EEG maturation were seen somewhat earlier in the present study than after bolus LPS (15), despite the much milder, subclinical insult, and lack of change in total EEG power, most likely reflecting greater sensitivity of continuous power spectral analysis to detect functional changes. There was a modest increase in apoptotic cells in the periventricular white matter but no change in numbers of the immature/mature myelinating oligodendrocytes that are labeled by CNPase. Thus, this low-dose exposure to LPS appears to have had relatively limited impact on the developing white matter. Potentially, the relatively mild effects of LPS infusion might reflect development of tolerance to endotoxin as suggested by attenuation of both cardiovascular responses (16), as well as the reaction of isolated monocytes to endotoxin in vitro (28, 30) after repeated exposure to LPS in fetal sheep. This possibility is supported by the transient elevations of IL-6 and cortisol, despite continuing LPS exposure in the present study.

The specific mechanisms of impaired EEG maturation after subclinical exposure to LPS are not clear. The transient increase in serum IL-6, induction of microglia, and greater expression of TNF-α-positive cells in the frontoparietal cortex 5 days after the end of LPS exposure support the possibility that proinflammatory cytokines directly contribute to the impaired maturation of the EEG. Similar to the present study, TNF-α and IL-1β are present in the developing sheep neocortex, at a time of intense synaptogenesis (17). In vitro studies show that proinflammatory cytokines mediate synaptic dysfunction, in part, indirectly through adenosine and GABA (26, 32). These effects appear to be dose-dependent, with synaptic inhibition at lower concentrations (7, 32, 43). Further, there is evidence that in slice cultures, chronic LPS infusions have a greater inhibitory effect on synaptic function than acute exposure (26). Thus, it is plausible that chronic low levels of cerebral cytokines during and after LPS exposure may have led to reduced neural excitability and synaptogenesis in the cortex, thus impairing EEG maturation.

**Perspectives and Significance**

Although severe infection is associated with a high risk of cerebral palsy in survivors of preterm birth, milder or subclinical infection is more common and also associated with adverse outcomes (23, 47). The present study demonstrates that a relatively short-lived and subclinical inflammatory state, with only a mild, transient increase in fetal cortisol and cytokines, that would be unlikely to be recognized in routine practice, can affect functional brain maturation. This strongly supports the hypothesis that low-grade inflammation in the fetus may contribute to cognitive impairments in preterm infants (5). Further studies are needed to confirm whether these functional changes persist into postnatal life.

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