LPS-Induced Microglial Secretion of TNFα Increases Activity-Dependent Neuronal Apoptosis in the Neonatal Cerebral Cortex

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During the pre- and neonatal period, the cerebral cortex reveals distinct patterns of spontaneous synchronized activity, which is critically involved in the formation of early networks and in the regulation of neuronal survival and programmed cell death (apoptosis). During this period, the cortex is also highly vulnerable to inflammation and in humans prenatal infection may have a profound impact on neurodevelopment causing long-term neurological deficits. Using in vitro and in vivo multi-electrode array recordings and quantification of caspase-3 (casp-3)-dependent apoptosis, we demonstrate that lipopolysaccharide-induced inflammation causes rapid alterations in the pattern of spontaneous burst activities, which subsequently leads to an increase in apoptosis. We show that these inflammatory effects are specifically initiated by the microglia-derived pro-inflammatory cytokine tumor necrosis factor α and the chemokine macrophage inflammatory protein 2. Our data demonstrate that inflammation-induced modifications in spontaneous network activities influence casp-3-dependent cell death in the developing cerebral cortex.

Keywords: cortex, cytokines, development, inflammation, programmed cell death

Introduction

During the perinatal period, the developing brain is most vulnerable to inflammation (Hagberg and Mallard 2005). Prenatal infection or exposure to inflammatory factors may disturb fetal neurodevelopment and cause long-term neurological deficits (Volpe 2003; Deverman and Patterson 2009). Inflammation in the brain is characterized by activation of resident immune cells, especially microglia (Mcgeer et al. 1993; Block et al. 2004; Allan et al. 2005) and astrocytes (Allan et al. 2005). These cell types express, release and respond to pro-inflammatory mediators such as cytokines (Wyss-Coray and Mucke 2002; Allan et al. 2005), which are critically involved in the immune response to infection (Hagberg and Mallard 2005) and directly influence neuronal function, for example, the pro-inflammatory cytokine tumor necrosis factor α (TNFα) enhances excitatory synaptic function by increasing the surface expression of α-amino-3-hydroxy-5-methyl-4-isoxazolpropionic acid (AMPA) receptors (Beattie et al. 2002). In an activity-dependent manner, TNFα homeostatically regulates the balance between neuronal excitation and inhibition (Stellwagen and Malenka 2006). Since TNFα is also involved in neuronal cell death (Araki et al. 2001; Block et al. 2004; Lucin and Wyss-Coray 2009), the interplay between neuronal activity and TNFα may control the process of cell death and cell survival in developing neuronal networks.

An increasing body of evidence suggests that neuronal activity is important in the regulation of neuronal survival and programmed cell death (apoptosis) during early development (Mennerick and Zorumski 2000). In our study, we investigated the impact of inflammation on neuronal activity and cell survival during early cortical development in vivo and in vitro. Since caspase-3 (casp-3) activation represents a key event in the apoptosis process (Lakhani et al. 2006), we used an antibody directed against cleaved casp-3 to detect apoptotic neurons. Inflammation was experimentally induced by application of the endotoxin lipopolysaccharide (LPS), which initiates a rapid and well-characterized immune response (Hagberg and Mallard 2005). Using cytokine antibody arrays, we identified microglia-derived pro-inflammatory factors mediating LPS-induced alterations in neuronal network activity and apoptosis.

In detail, we addressed the following questions: 1) What are the consequences of LPS-induced inflammation on spontaneous network activity and apoptotic cell death in the neonatal cerebral cortex in vivo? 2) What is the effect of LPS-induced inflammation on spontaneous neuronal network activity and apoptotic cell death in the developing cerebral cortex in vitro? 3) Which factors are released from LPS-treated microglia influencing network activity and neuronal survival and what is their time course of action? 4) Does neutralization of the identified cytokines lead to re-establishment of physiological activity patterns and to cell survival?

Materials and Methods

Surgical Preparation and In Vivo Recordings

All experiments were conducted in accordance with the national laws for the use of animals in research and approved by the local ethical committee (Landesuntersuchungsamt Rheinland-Pfalz 23.177-07/G 10-1-010). Extracellular recordings were performed in the barrel cortex of postnatal days (P) P3–P5 Wistar rats using experimental protocols as described previously (Yang, Hanganu-Opatz, et al. 2009). Briefly, under deep hypothermia combined with initial light intraperitoneal urethane anesthesia (0.5–1 g/kg, Sigma-Aldrich, Steinheim, Germany), the head of the pup was fixed and the bone, but not the dura mater, above the barrel cortex was carefully removed. Animals were kept at a constant temperature of 37°C by placing them on a heating blanket and covering their bodies with cotton. These procedures kept the pups in very light urethane anesthesia and the pups stayed calm in the recording system for >5 h. After 30–60 min recovery, a 4-shank 16-channel electrode (125 µm horizontal shank distance and 50 µm vertical inter-electrode distance, 1–2 MΩ, NeuroNexus Technologies, Ann Arbor, MI, United States of America) was inserted perpendicularly into the cortical layer II/III and layer IV in a depth of 200–400 µm to obtain field-potential (FP) recordings from these layers (Fig. 1A). To investigate the LPS effect in different cortical layers, a 1-shank 16-channel Michigan electrode (100 µm vertical inter-electrode distance) was inserted perpendicularly into the cortex from the cortical surface to the white matter. Electrodes were labeled with 1,1-diocadecyl-3,3,3’,3’-tetramethyl indocarbocyanine
(Dil, Molecular Probes, Eugene, OR, United States of America) to enable postmortem reconstruction of the electrode tracks in Nissl-stained coronal sections (Figs 1A ii and 2A i). An intracortical injection of phosphate-buffered saline (PBS), LPS (5 mg/mL in PBS), or LPS + anti-TNFα (Millipore, Bedford, MA, United States of America; 0.4 µg/mL in PBS) was performed with a glass pipette (tip diameter 30–40 µm) attached to a syringe. The tip of the pipette was positioned intracortically at a distance of 0.2–0.5 mm from the Michigan recording.

Figure 1. LPS-induced inflammation in the newborn rat somatosensory cortex in vivo leads to alterations in spontaneous neuronal network activity. (A) A schematic illustration of a 4-shank 16-channel electrode (i). Image of a Nissl-stained coronal section superimposed with the Dil track of a glass pipette and a multi-electrode shank2 (S2) from one P3 rat (ii). The same photograph of the Nissl-stained coronal section in (ii) shows no obvious damage induced by the glass pipette or multi-electrode (iii). (B) Continuous extracellular field-potential recordings of spontaneous activity in a P4 rat under control conditions (i) and 3 h after LPS injection (ii). Lower traces show spindle bursts (s) (5–30 Hz filtered) and gamma oscillations (g) (30–80 Hz filtered) boxed in the upper panel at higher temporal resolution. (C) LPS-induced modifications in the relative occurrence (i) and duration (ii) of spontaneous spindle bursts (left) and gamma oscillations (right) when compared with PBS-treated control animals. The number of PBS-treated controls and LPS-injected animals is 13 and 18, respectively. In this and the following figures data are presented as mean ± SEM and the number of experiments is indicated in the bars.
electrode at a depth of ∼600 µm (Figs 1Aii and 2Aii). No obvious morphological changes related to electrode penetration could be observed (Fig. 1Aiii). After 40 min baseline control recording, 2 µL PBS, LPS, or LPS + anti-TNFα was injected for 5–10 min and spontaneous activity was recorded for 3 h following injection. The spontaneous activity recorded in the last 10 min of this 3-h recording period was normalized to the control spontaneous activity recorded for 10 min before application of PBS, LPS, or LPS + anti-TNFα. For statistical analyses, values of the different groups were compared with each other in percentage.
In vivo data were digitally filtered, imported, and analyzed off-line using the MATLAB software. Spontaneous events were detected as FP deflections exceeding 5 times the baseline standard deviation (SD). Only events lasting >100 ms and containing >3 cycles were considered for analysis. Spontaneous oscillatory events were separated into spindle bursts and gamma oscillations (Yang, Hangjani-Opatz, et al. 2009, Yang, An, et al. in press) and analyzed in their occurrence, duration, peak-to-peak amplitude, and maximal frequency within each event (Table 1).

### Western Blot Analysis

Following the same surgical procedure as described above in “Surgical preparation and in vivo recordings” without inserting Michigan-type recording electrodes, the somatosensory cortex was injected with either PBS or LPS dissolved in PBS (5 mg/mL). Six hours later, tissue was removed with a skin puncher (3 mm) and western blot analysis was performed. Three control and 3 LPS-treated tissue samples were pooled and lysed in 125 μL extraction buffer [50 mM Tris–HCl pH 7.4; 1% (v/v) Triton X-100; 150 mM NaCl; 1 mM ethylenediaminetetraacetic acid (EDTA)] for 2 h on ice. Nuclei and cellular debris were pelleted by centrifugation at 5000 × g for 5 min at 4°C and the protein concentrations of the supernatants were determined using the bicinchoninic acid test (Pierce) according to the manufacturer’s instructions. Organotypic slice cultures (8 days in vitro [DIV]) of P0 mouse brains, treated with staurosporine (100 nM for 24 h), were used as positive control.

### Dissociated Neuronal Cell Cultures

Cultures of neocortical neurons were prepared from P0–P1 C57Bl/6 mice, following the same procedure as described above for

### Organotypic Neocortical Slice Cultures

Organotypic neocortical slices were prepared from newborn P0–P1 C57Bl/6 mice and cultured according to the method described by Stoppini et al. (Stoppini et al. 1991; Heck et al. 2008). Animals were killed by decapitation and the brain was quickly removed. All subsequent procedures were performed in Minimal Essential Medium (MEM; Invitrogen GmbH, Darmstadt, Germany) supplemented with 2 mM glutamine, pH 7.4 at 4°C. Neocortical hemispheres were isolated from the hippocampus, thalamus, and striatum and 350-μm-thick coronal slices containing the parietal cortex were cut with a tissue chopper (McClellan, Mickie Laboratory Engineering, Surrey, United Kingdom). Parietal cortical slices were isolated with a microscalpel and transferred onto Millicell-CM membranes (Millipore). Slices were kept at 37°C, 5% CO2 in medium containing neurobasal (NB) medium supplemented with 2% B27 (Invitrogen), 2 mM glutamax (Invitrogen), and 10 μg/mL penicillin/streptomycin (NB/B27 medium). After 24 h, 1 μM arabinofuranoside cytidine (AraC; Sigma-Aldrich) was added to the medium. Thereafter, culture medium with AraC was renewed every 2 days.

### Table 1

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<th>Table 1</th>
<th>The influence of intracortical injection of PBS or LPS on spontaneous occurring spindle bursts and gamma oscillations in the newborn rat somatosensory cortex in vivo</th>
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<tr>
<td><strong>Spindle bursts</strong></td>
<td><strong>Gamma oscillations</strong></td>
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<tr>
<td><strong>Occurrence (min⁻¹)</strong></td>
<td>PBS Control</td>
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<tr>
<td>PBS</td>
<td>Control</td>
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<td></td>
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<tr>
<td>LPS</td>
<td>Control</td>
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<td>Control</td>
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<tr>
<td><strong>Duration (s)</strong></td>
<td>PBS Control</td>
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<td>Control</td>
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<tr>
<td>LPS</td>
<td>Control</td>
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<td><strong>Peak-to-peak amplitude (μV)</strong></td>
<td>PBS Control</td>
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<td>LPS</td>
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<td><strong>Frequency (Hz)</strong></td>
<td>PBS Control</td>
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<td>LPS</td>
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</table>

Note: Data are expressed as mean ± standard error (n = 13 and 18 animals for PBS and LPS groups, respectively). The occurrence decreases and duration becomes longer both in spindle bursts and gamma oscillations after LPS injection when compared with the PBS control injection group (***P < 0.01 and **P < 0.001, Mann–Whitney test).
organotypic slice cultures. The dissociation of the cerebral cortex was achieved by incubation in modified PBS without Ca²⁺ and Mg²⁺ containing 0.25% trypsin (Sigma-Aldrich) at a temperature of 37°C for 12 min. Trypsinization was finally blocked by addition of basal medium eagle (Invitrogen) containing 10% fetal calf serum (BME/10% FCS). Cortices were further dissociated in 0.05% DNase by sequential repeated passages through a fire-polished Pasteur pipette. Three million cells were preplated in a 3-cm Petri dish in BME/10% FCS at 37°C, 5% CO₂ for 45 min. This treatment allowed astrocytes to attach to the bottom of the Petri dish whereas neurons remained in the medium. For electrophysiological recordings, the resulting cell suspension, which was enriched in neurons compared with glial cells (Supplementary Fig. 1), was then plated at a density of 1.0 × 10⁶ cells/mL on polyornithine (Sigma) coated multi-electrode arrays (MEAs; Multi Channel Systems, Reutlingen, Germany). For analyses of the neuronal survival rate, cells were plated on 96-well plates at a density of 1.0 × 10⁵ cells/well. Neurons were cultured in NB/B27 medium at 37°C, 5% CO₂. After 1 DIV, the medium was completely changed and after 7 days by 33%. Neurons were cultured for 2 weeks.

**BV-2 Microglial Cell Cultures**

Cells of the murine microglial cell line BV-2 were maintained in 25 cm² culture flasks in Roswell park memorial institute medium (RPMI) + l-glutamine supplemented with 10% FCS (Invitrogen) at 37°C in a humidified atmosphere of 5% CO₂. The culture medium was changed to NB medium before each experiment. To obtain conditioned medium, BV-2 cells were cultured in 25 cm² culture flasks for 2 days in RPMI + 10% FCS and treated with various factors for 6 h to obtain control-conditioned medium (C-CM), LPS-conditioned medium (LPS-CM), and heat-inactivated LPS-conditioned medium + anti-TNF (Invitrogen) medium at 37°C, 5% CO₂ for 2 days prior to experiments.

**Cortical Astrocyte Cultures**

Astrocytes were prepared from P3 C57Bl/6 mice. After decapitation, scalp and skullcap were removed and the brain was placed in ice cold Hank’s balanced salt solution (HBSS). Both hemispheres were isolated and the meninges and the bulbi olfactory were removed. The cortices were separated from the hippocampus, striatum, and thalamic nuclei. The dissociation of the cortex was achieved by incubating the hemispheres in 0.05% trypsin/EDTA at a temperature of 37°C, 5% CO₂ and humidity of 95% for 20 min, after mechanical dissociation. Trypsinization was blocked by addition of HBSS containing 10% horse serum. Cortices were further dissociated by repeated passages through a blue pipette tip cut to a pore size 2 µm to remove remaining cells and stored at −20°C until use. For analyses of the cell survival rate, cells were plated on 24-well plates at a density of 4 × 10⁴ cells/well in RPMI + l-glutamine supplemented with 10% FCS (Invitrogen) at 37°C, 5% CO₂ for 2 days prior to experiments.

**Table 2**

<table>
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<tr>
<th>Abbreviation</th>
<th>Full name</th>
<th>Condition</th>
<th>Antibodies</th>
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<tr>
<td>C-CM</td>
<td>Control conditioned medium</td>
<td>6 h in NB-medium</td>
<td>–</td>
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<tr>
<td>LPS-CM</td>
<td>LPS-conditioned medium</td>
<td>6 h in NB-medium containing</td>
<td>–</td>
</tr>
<tr>
<td>LPS-CM + LPS</td>
<td>LPS-conditioned medium + LPS</td>
<td>6 h in NB-medium containing + injection</td>
<td>LPS + BME</td>
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<tr>
<td>LPS-CM heat</td>
<td>LPS-conditioned medium heat</td>
<td>6 h in NB-medium containing heat</td>
<td>LPS + BME</td>
</tr>
<tr>
<td></td>
<td>medium heat</td>
<td>6 h in NB-medium containing heat</td>
<td>LPS + BME</td>
</tr>
<tr>
<td>LPS-CM + anti-TNFα</td>
<td>LPS-conditioned medium anti-TNFα</td>
<td>6 h in NB-medium containing anti-TNFα</td>
<td>Anti-TNFα</td>
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<tr>
<td>LPS-CM + anti-MIP-2</td>
<td>LPS-conditioned medium anti-MIP-2</td>
<td>6 h in NB-medium containing anti-MIP-2</td>
<td>Anti-MIP-2</td>
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**In Vitro Electrophysiological Recordings**

Electrophysiological recordings of neuronal network activity in 5–7 DIV organotypic slice cultures were performed with 3D MEAs (200 µm inter-electrode distance) in artificial cerebrospinal fluid that resembled the ionic and molecular composition of the NB medium without B27 (Brewer et al. 1993) (in millimolar): 51.3 NaCl, 26 NaHCO₃, 0.9 NaH₂PO₄·H₂O, 0.812 MgCl₂·6H₂O, 1.8 CaCl₂, 5.36 KCl, 25 g glucose, 10 (N-[2-hydroxyethyl]-piperazine-N’-[2-ethanesulfonicacid]) sodium salt, 0.23 sodium pyruvate, 0.4 l-glutamine, 0.4 isocitric acid, 0.02 l-alanine, 0.028 choline chloride, and 0.2 µM Fe(NO₃)₃·9H₂O equilibrated with 95% O₂/5% CO₂ (pH 7.4; osmolality 205 mOsm). Slices were continuously perfused at a rate of 2–3 mL/min at a temperature of 28°C. Spontaneous network activity was recorded in control slices and in LPS-treated slices, which was exposed to 10 µg/mL LPS after recording network activity for 1.5 h under control condition. Field potentials were recorded simultaneously with 60 extracellular electrodes at a sampling rate of 1 kHz using the MC_RACK software (Multi Channel Systems, Reutlingen, Germany). Data were imported to a custom-written program in Matlab version 7 (Mathworks, Natick, MA, United States of America) with datasrmm and nextdata.m (MC_Rack, Multi Channel Systems). Spontaneous network oscillations were quantified in their occurrence and duration in recording intervals of 1.5 h at control conditions and compared with 1.5 h recordings following LPS or PBS injection.

Electrophysiological recordings of dissociated neuronal cultures were performed after 14 DIV in NB/B27 medium using the 60 channel planar MEA (30 µm diameter with 200 µm spacing) with a sampling rate of 25 kHz and a 100 Hz high-pass filter. Spikes were detected with MC_RACK software using a threshold-based detector set to a threshold of 7 times the SD of noise level. After 1 h recording under control condition, 100 µL of the NB/B27 medium was exchanged with 100 µL of the conditioned medium (Table 2) and the network activity was recorded for another hour. Dissociated cultures were perfused with 95% O₂/5% CO₂ during the whole experiment and kept at 28°C. Spike activity was recorded as described above and network bursts and spike number were quantified in epochs of 10 min. Binary data of the spike trains were divided into 2 s in size and the number of spikes in each bin was summed up. Bins were sorted by their values. The fraction, f₅₃ was derived from the total number of spikes by the number of spikes in the first 15% of sorted bins. If most spikes occurred in bursts, this fraction, f₅₃ was close to one. Conversely, if spikes were sparsely distributed in time, f₅₃ was close to 0.15. The burst index was defined as BI = (f₅₃ − 0.15)/0.85 (Wagenaar et al. 2005) and amounted from 0 (no burst) to 1 (burst discharge).

To detect synchronized bursting events, we scanned the binary data in consecutive windows of 2 s. Each window was divided into bins of 200 ms. Only events with >5 active neurons and a delay of >5 s between the previously detected synchronized burst event were analyzed (Raichman and Ben Jacob 2008). Spike activity was quantified by counting the number of spikes per 10 min recording epoch and normalized to the 1 h control measurement preceding the experimental condition.

**Immunostaining for Cleaved Caspase-3 and Quantification of Apoptotic Cell Death**

A primary rabbit polyclonal antibody directed against the cleaved active form of casp-3 was used (Asp175, Cell Signaling Technology...
Inc.; Marin-Teva et al. 2004). For analysis of in vivo experiments brains were removed, fixed with 4% paraformaldehyde (PFA), and stored in 30% sucrose overnight. A freezing microtome was used to collect 200 µm slices. Those containing Dil staining were chosen for further analysis, were rinsed in PBS, and treated either for 2 h with 0.8% Triton X-100 in 7% normal goat serum or with 1000 µg/mL digi-tonin in 7% normal goat serum. Immunostainings with anti-cleaved casp-3 antibody (1/400) were performed. In vivo slices and organo-

tic slices were analyzed using a confocal system (QLC100; Visitec, Sunderland, United Kingdom) attached to an upright Olympus micro-
scope (BX51; Olympus, Tokyo, Japan). For analysis of the in vivo slices, 6 regions of interest along the injection site were counted for cleaved casp-3-positive cells. Slices from at least 5 different rats were analyzed. Organotypic slice cultures were fixed with 4% PFA, rinsed in PBS, treated for 30 min with 0.1% Triton X-100 in PBS, and prein-cubated in 5% bovine serum albumin in PBS for 2 h. Immunostainings with anti-cleaved casp-3 antibody (1/400) and/or rabbit polyclonal antibody directed against Glial fibrillary acidic protein (GFAP) (1/400, Dako, A/S, Denmark) were performed (Golbs et al. 2011; Wagner-

Golbs and Luhmann 2012). Immunostained whole cortex slices of or-

ganotypic slices were observed at ×600 magnification which corre-
sponded to a field of view of 125 µm². Apoptotic cells were counted in 18 fields of view that were assigned to cover all cortical layers in 3 different regions of each slice. Because no significant differences between the regions and between the layers could be observed, the number of apoptotic cells from the 18 fields of view was averaged. Slices from at least 6 independent cultures were analyzed. For in vivo and in vitro analyses, each field of interest was automatically counted for apoptotic cells with the Metamorph program in combination with ImageJ. The results for both methods are shown as percentage of the respective control condition (control is 100%).

Resazurin-Based In Vitro Cell Viability Assay Kit (Alamar Blue Assay)

To measure cell viability of dissociated neuronal cultures, BV-2 cells or astrocytes, culture medium was replaced by conditioned medium for 1, 2, or 6 h. After incubation time, medium was replaced with 200 µL Alamar Blue stock solution diluted 1:60 with HBSS++. The blue non-fluorescent oxidized form becomes pink and fluorescent upon reduction through metabolic active cells in the cultures. Alamar Blue and cultures were incubated for various periods at 37°C, 5% CO₂. Cell viability was measured 1, 3, and 6 h after Alamar Blue application in a Tecan plate reader (Tecan GmbH, Crailsheim, Germany) with excitation wavelength of 540 nm and the emission at 595 nm. Since the survival rate was not altered between 3 and 6 h, only data measured at 3 h are shown. The relative survival rate was normalized to control conditions.

Measurements of Cytokine Levels

The release of cytokines in C-CM and LPS-CM was measured with a mouse cytokine array panel A array kit (R&D System, MN, United States of America). The assay was performed according to the manu-

facturer’s protocol. Briefly, capture antibodies directed against various cytokines were spotted in duplicate on nitrocellulose mem-

branes. Cell culture supernatants were diluted and mixed with a cock-
tail of biotinylated detection antibodies. The sample/antibody mixture was then incubated with the mouse cytokine array membrane. Any cytokine/detection antibody complex present was bound by its cognate immobilized capture antibody on the membrane. Following a wash to remove unbound material, streptavidin-HRP and chemilumi-

nescence detection reagents were added sequentially. Light was pro-
duced at each spot in proportion to the amount of cytokine bound.

Statistics

For statistical analysis, Student’s t-test was used when distribution was Gaussian and Mann–Whitney test for non-Gaussian distribution. For comparing different groups, one way analysis of variance was used followed by the Tukey test when the distribution was Gaussian and

Kruskal–Wallis test followed by Dunns test when the distribution was not Gaussian. All data are expressed as mean ± standard error of the mean (SEM) and statistically significant differences are indicated by *P < 0.05, **P < 0.01, and ***P < 0.001.

Results

LPS Induces Modifications in Spontaneous Neuronal Activity and an Up-Regulation of Activated Casp-3 in the Immature Neocortex In Vivo

The consequences of LPS-induced inflammation on neuronal activity in the developing cerebral cortex in vivo were studied with extracellular field-potential recordings in the barrel cortex of P3–P5 rats, using a 4-shank 16-channel electrode (Fig. 1A1) or a 1-shank 16-channel electrode (Fig. 2A1). In agreement with previous reports on somatosensory cortex of newborn rats (Minlebaev et al. 2007; Yang, Hanganu-Opatz, et al. 2009, Yang, An, et al. in press) and preterm human neo-

nates (Milh et al. 2007), spontaneous network activity was characterized by distinct neuronal activity patterns (Fig. 1B) namely spindle bursts and gamma oscillations (see lower traces in Fig. 1B1). The short-term effects of inflammation on these activity patterns were studied by recording the spontaneous network activity 3 h after intracortical injection of LPS (10 µg in 2 µL; Rodgers et al. 2009). A control group of animals received an intracortical injection of PBS.

To further elucidate the effects of inflammation on activity patterns, the occurrence (min⁻¹), peak-to-peak amplitude (µV), duration (s), and frequency (Hz) values were analyzed for spindle bursts and gamma oscillations before and after LPS or PBS treatment (Table 1). In LPS-treated animals, the occurrences of spindle bursts and gamma oscillations were significantly reduced when compared with PBS-treated rats (Fig. 1B1i,Gi, Table 1; n = 13 for PBS, 18 for LPS, P = 0.0001 and P = 0.0001, respectively), while the mean duration of spindle bursts and gamma oscillations increased (see lower traces in Fig. 1B1ii,Gi, Table 1; n = 13 for PBS, 18 for LPS P = 0.0001 and 0.0043, respectively). However, the amplitude and frequency did not show significant differences both in spindle bursts and gamma oscillations comparing LPS- with PBS-treated animals (Table 1). In addition, the cross-correlation analysis also showed that the spatiotemporal characteristics of spindle bursts and gamma oscillations were not changed after LPS injection (Supplementary Fig. 2).

To address the question whether the LPS-induced changes occur in all cortical layers, we used a 1-shank 16-channel elec-

trode (Fig. 2A1), which was inserted perpendicularly from the cortical surface to the white matter. We found that LPS signi-

ficantly reduced the occurrence of spindle bursts in all cortical layers, while their mean duration was significantly increased in all layers (Fig. 2A). LPS also significantly reduced the occurrence and increased the duration of gamma oscillations in layers II/III and IV (Fig. 2B). Neither the amplitude nor the frequency of spindle bursts and gamma oscillations were changed by LPS application. Furthermore, no significant difference of these parameters between 2 age groups, P3 and P5, could be observed (Supplementary Fig. 3).

To address the question whether LPS injection also causes a rapid increase in casp-3-mediated apoptosis in vivo, the barrel cortex of 6 P4–P5 rats was injected with either PBS
(n = 3) or LPS (n = 3) following the same experimental procedure as described above, but without inserting recording electrodes. Six hours later, the neocortical tissue was removed with a skin puncher and western blot analysis was performed. Western blots revealed a band at 17 kDa corresponding to cleaved casp-3 (Fig. 2C). After LPS treatment, cleaved casp-3 protein levels showed a 3.3-fold increase in comparison with PBS control (Fig. 2Ci). Furthermore, the laminar distribution of casp-3–positive cells revealed that the effect of LPS in vivo was localized to a depth of 600–900 µm (Fig. 2Di), corresponding to the location of LPS injection through the glass pipette (Fig. 2Ai).

**LPS Modifies Spontaneous Synchronized Network Activity and Causes Increased Casp-3–Dependent Neuronal Cell Death in Organotypic Slice Cultures**

The consequences of LPS-induced inflammation on spontaneous neuronal network activity and casp-3–dependent cell death were further studied in organotypic neocortical slice cultures, in which cortical architecture, synaptic connectivity and microenvironment of the cells strongly resemble the in vivo situation. In agreement with our previous observations (Heck et al. 2008), we recorded in 5–7 DIV organotypic slice cultures with 3D MEAs (Fig. 3A) spontaneous neuronal activity consisting of network oscillations, which occurred at an average rate of 18.3 ± 1.6 per hour (n = 6 slices). These oscillations had an average duration of 10.4 ± 1.6 s, a frequency of 1.14 ± 0.3 Hz, and an amplitude of 473.2 ± 90.1 µV, and were synchronized over the whole organotypic neocortical slice (Fig. 3B). When compared with untreated control organotypic slice cultures (n = 6), cultures treated for 1.5 h with LPS (10 µg/mL) revealed a significant decrease in the occurrence (10.1 ± 2.5 per hour, n = 6, P = 0.0029; Fig. 3C) and an increase in the relative duration of spontaneous network oscillations (from 100 ± 2.5% to 108.2 ± 2.23%, n = 6, P = 0.0125; Fig. 3D). These results are in good agreement with our in vivo recordings described above.

In a next step, we studied casp-3–dependent apoptosis in organotypic slice cultures. In agreement with our previous report (Heck et al. 2008), developmentally regulated casp-3–dependent apoptosis could be detected under normal conditions in all cortical layers (Fig. 4A) and casp-3–positive cells were identified as neurons based on their clear somatodendritic morphology (Fig. 4B). Comparisons of untreated control slices (Fig. 4C) with LPS-treated (10 µg/mL) slices (Fig. 4D) revealed a prominent (P < 0.0001) increase in the percentage of casp-3–positive cells in 6 DIV (n = 10) or 12 DIV (n = 6) cultures after 24 h LPS treatment (Fig. 4E) and in 4–5 DIV cultures after 6 h treatment (n = 9, P < 0.0001; Fig. 4F).

**Figure 3.** Effects of LPS on synchronized spontaneous network activity in neocortical slice cultures. (A) Image of a 5 DIV organotypic neocortical slice culture placed on a 60-channel MEA. Black dots represent extracellular recording electrodes separated by 200 µm. (B) A simultaneous 60-channel MEA recording of synchronized network oscillations in a control organotypic neocortical slice culture. Five channels are damaged and were switched off. (C and D) Treatment of 5–7 DIV organotypic cultures with LPS for 1.5 h causes a significant decrease in the occurrence of spontaneous network oscillations (C) and an increase in their duration (D) when compared with untreated controls.
Immunostainings showed that casp-3-positive cells were not GFAP positive, demonstrating that LPS treatment does not induce apoptosis in astrocytes (data not shown). LPS application for 6 h on BV-2 microglial cells or primary astrocytes had no effect on the survival rate as measured by Alamar Blue assays (Supplementary Fig. S4). These data demonstrate that transient LPS application to organotypic neocortical slice cultures induces rapid changes in spontaneous network activity and casp-3-dependent cell death in neurons, while microglial BV-2 cells and primary astrocytes are not affected.

**LPS-Induced Release of Inflammatory Factor(s) Causes an Increase in the Firing Rate, but Disrupts Neuronal Network Synchronization**

To further investigate the molecular mechanisms underlying the LPS effect on spontaneous network activity, we used neuron-enriched primary dissociated cell cultures (Supplementary Fig. S1), which on MEAs also revealed a distinct pattern of spontaneous synchronized network activity (Fig. 5Ai; Sun et al. 2010). Spontaneous network bursts appeared on average 1.8 ± 0.5 times/min (n = 9 cultures) and single bursts had a duration of 4.6 ± 0.9 s. Dissociated cultures were treated with conditioned media from LPS-activated microglial BV-2 cells (LPS-CM) and C-CM. Furthermore, we applied C-CM + LPS to study the effects of LPS without microglial contact and LPS-CM pretreated with heat to denature the proteins in this conditioned medium (LPS-CM heat; Table 2). In cultures treated with C-CM (Fig. 5Ai), the average relative BI amounted to 1.12 ± 0.13 (n = 9; Fig. 5B). In LPS-CM (Fig. 5Ai), the BI decreased significantly to 0.563 ± 0.055 (n = 11, P = 0.0006; Fig. 5B), indicating a desynchronization of spontaneous activity. In cultures treated with C-CM + LPS (Fig. 5Ai), the BI amounted to 0.937 ± 0.085 (n = 5), but this effect was not significantly different when compared with the C-CM controls (P = 0.354; Fig. 5B). As another parameter for neuronal network activity, the relative spike number was determined in these 4 experimental groups (Fig. 5C). In C-CM cultures, this value
amounted to 0.58 ± 0.11 (n = 9). Cultures in LPS-CM revealed a 3-fold increase to 1.72 ± 0.5 (n = 11), but this difference was not significant (P = 0.063). In C-CM + LPS (1.022 ± 0.09, n = 5) and LPS-CM heat (0.47 ± 0.19, n = 8) treated cultures, the spike number was close to the control values of C-CM cultures and they revealed no significant difference. These results indicate that one or several soluble heat-sensitive factor(s), most likely proteins, are released from BV-2 cells after LPS treatment, which induce an overall increase in firing rate, but a decrease in synchronized network activity.

LPS Induces Fast Release of Rapidly Acting Inflammatory Factor(s)

To study the time course of the release of inflammatory factors and LPS-induced cell death, we tested cell survival in dissociated neuronal cultures at various time points. Dissociated cultures treated with LPS-CM for 6 h revealed a significant decrease in the relative survival rate measured in Alamar Blue assays (see Material and Methods) to 86.4 ± 1.2% (n = 18, P < 0.0001) when compared with the relative survival rate of control C-CM cultures (100 ± 1.3%, n = 18; Fig. 6A). In agreement with our electrophysiological data, cultures treated with C-CM + LPS (99.4 ± 3.5%, n = 4, P = 0.85) or heat-inactivated LPS-CM cultures (105 ± 2.6%, n = 12, P = 0.76) showed no significant difference in their survival rate when compared with C-CM control cultures (Fig. 6A). To estimate the time course of LPS-induced cell death, we treated the dissociated cultures for only 1 or 2 h with conditioned medium (C-CM or LPS-CM). Whereas 1 h LPS-CM treatment had no significant effect on survival rate (n = 6, P = 0.39), 2 h LPS-CM treatment induced a significant decrease in the survival rate to 80.3 ± 1.6% (n = 5, P < 0.0001) when compared with C-CM controls (100 ± 1.5%, n = 5; Fig. 6B). To clarify how fast LPS-induced microglial release of (unknown) factor(s) occurs, BV-2 cells were treated with LPS for 2 h only. Neuronal cultures treated for 6 h with this 2 h conditioned LPS-CM revealed a significant decrease in the survival rate to 81 ± 2.3% (n = 3, P < 0.0001) when compared with C-CM controls (100 ± 2.6%, n = 3; Fig. 6C). These data indicate that heat-sensitive soluble factor(s) are released from BV-2 cells within 2 h after LPS addition, which cause a decrease in the neuronal survival rate within 2 h.

TNFα and MIP-2 are Significantly Up-Regulated in LPS-Conditioned Medium

LPS-CM treatment led to a decreased bursting activity and a decreased survival rate and both effects were mediated by heat-sensitive factor(s). An increase in pro-inflammatory factors was observed in LPS-CM, with significant up-regulation of TNFα and MIP-2 compared to C-CM.
factor(s) in LPS-CM compared with C-CM could be shown by using cytokine antibody arrays. The levels of various cytokines and chemokines were significantly increased in LPS-CM compared with C-CM (Fig. 7A, B): G-CSF (granulocyte-colony stimulating factor), KC (keratinocyte-derived cytokine), RANTES (regulated on activation normal T cell expressed), GM-CSF (granulocyte macrophage colony-stimulating factor), IL-1ra (interleukin-1 receptor antagonist), TNFα, IL-6 (interleukin-6), and MIP-2 (macrophage inflammatory protein 2) (all n = 3). Only JE/monocyte chemoattractant protein-1 (mouse homolog of human monocyte chemoattractant protein 1), a factor responding to mitogenic stimuli, was down-regulated. G-CSF is hypothesized to increase the activation of resident microglia and mobilization of marrow-derived microglia (Sanchez-Ramos et al. 2009). KC, also called CXCL1, and RANTES are chemoattractants for a variety of inflammatory immune cells like neutrophils (van Rossum et al. 2008) and can be excluded as potential factors in our in vitro model systems. GM-CSF induces proliferation and activates microglia (Reddy et al. 2009) and IL-1ra inhibits IL-1 activity (Sims 2002). TNFα, MIP-2, and IL-6 are important cytokines primarily involved in inflammation. It has been demonstrated that IL-6 causes reactive astrogliosis, but no neuronal damage (Fattori et al. 1995; Raivich et al. 1999). Therefore, we focused on TNFα and MIP-2 as the most likely
downstream molecules mediating LPS-induced modifications in spontaneous neuronal activity and cell death.

Neutralization of TNFα or MIP-2 Prevents LPS-Induced Modifications in Spontaneous Network Activity and Cell Death In Vitro

To study the consequences of TNFα and MIP-2 neutralization, anti-TNFα, or anti-MIP-2 antibodies were added to the BV-2 cell medium and the effects on neuronal network synchronization, relative spike number, and cell survival were studied (Fig. 5B–D). Application of anti-MIP-2 prevented the LPS-induced network desynchronization (Fig. 5B) and the increase in relative spike numbers (Fig. 5C). The effect of anti-MIP-2 application on the neuronal cell survival rate showed no significant difference when compared with the LPS-CM cultures although a tendency of increase could be observed (Fig. 5D). In contrast, treatment with anti-TNFα enhanced the BI (Fig. 5B) and survival rate (Fig. 5D) in LPS-treated cultures and led to a normalization of the relative spike number (Fig. 5C), demonstrating that TNFα is critically involved in the regulation of neuronal network activity and cell survival in vitro. Neither the survival rate, nor the BI recovered completely in anti-TNFα conditions, suggesting the participation of additional factor(s) in both processes.

Neutralization of TNFα Prevents LPS-Induced Modifications in Spontaneous Network Activity and Cell Death In Vivo

Next, we addressed the significance of our in vitro results for the in vivo situation. First, we checked TNFα protein levels in response to intracortical LPS injections by western blotting. A strong increase in the levels of soluble TNFα (17kDa) could be detected in LPS-treated tissue compared with the PBS control (Fig. 8Ai), while levels of the loading control α-tubulin (55 kDa) are similar in both samples. We then injected an anti-TNFα antibody into LPS-treated cortices.

Figure 8. LPS-induced inflammation in the newborn rat somatosensory cortex in vivo can be blocked by treatment with anti-TNFα (aTNFα) antibodies. (A) Western blot analyses show an increase of soluble TNFα protein in LPS-treated tissue when compared with PBS-treated controls. The levels of the loading control α-tubulin are similar in both samples (i). LPS-induced modifications in the relative occurrence (ii) and duration (iii) of spontaneous spindle bursts (left) and gamma oscillations (right) when compared with PBS-treated control animals and LPS + anti-TNFα–treated animals. (iv) A comparison of relative multi-unit activity (MUA) and relative BI reveals no significant difference between the 3 groups. (B) Image of Nissl-stained coronal section shows the regions selected for quantification, a region marked by rectangle in the non-injected left hemisphere serves as a control, while the region marked by rectangle in the right hemisphere is close to the injection site. (ii) Images show cleaved casp-3–positive cells in the non-injected site (left), and cleaved casp-3–positive cells in the PBS, LPS, and LPS + anti-TNFα–injected site (right). (iii) PBS injection has no effect on the number of apoptotic cells. LPS treatment leads to a significant increase in casp-3–positive cells, which is blocked by injection of LPS + anti-TNFα. Data are based on 14 slices from 5 rats in the PBS group, 15 slices from 7 rats in the LPS group, and 13 slices from 5 rats in the LPS + aTNFα group.
Blocking TNFα action in vivo significantly prevented the LPS-induced decrease in the occurrence of spindle bursts and gamma oscillations (n = 13 for PBS, 18 for LPS, 5 for LPS + anti-TNFα, P = 0.0081 and 0.01, respectively, Fig. 8ii). Similarly, treatment with anti-TNFα antibodies prevented the prolongation of the duration for spindle bursts as well as gamma oscillations (n = 13 for PBS, 18 for LPS, 5 for LPS + anti-TNFα, P = 0.0009 and 0.0052, respectively, Fig. 8iii). Similar with the in vitro results (Fig. 5C), the amount of spike activity showed a slight but not significant increase after PBS treatment, and treatment with anti-TNFα led to a normalization of the relative amount of multi-unit activity (MUA) (Fig. 8iv). However, the relative BI did not change after LPS application or treatment with PBS combined with anti-TNFα (Fig. 8iv).

Furthermore, to study whether blocking TNFα action can diminish the LPS effect on the increase of casp-3-dependent apoptosis in vivo, the barrel cortex of P4–P5 rats was injected with either PBS, LPS, or LPS + anti-TNFα antibody. Six hours later, rats were sacrificed and the brain was removed and fixed in PFA. Brains were cut and the level of casp-3-positive cells was measured by immunohistochemistry (Fig. 8j). The number of casp-3-positive cells from the PBS-injected hemisphere showed no significant increase (113.7 ± 13.74%, n = 14 slices from 5 animals, P = 0.4) when compared with non-injected control side (100 ± 8.17%). While LPS injection led to a significant increase of casp-3-positive cells (174.8 ± 14.61%, n = 15 slices from 7 animals, P = 0.0001), injection of LPS + anti-TNFα antibody prevented this increase of casp-3-positive cells (127.3 ± 12.51%, n = 13 from 5 animals P = 0.056; Fig. 8iii). These data demonstrate that TNFα is critically involved in the regulation of neuronal network activity and cell survival in vivo.

**Discussion**

Our study demonstrates for the first time a direct link between inflammation-induced modifications in neuronal activity and the control of cell survival in a developing neuronal network in vitro and in vivo. Our data may be of important clinical relevance, since spontaneous synchronized activity is also a hallmark of the developing human brain (Mihl et al. 2007; Tolonen et al. 2007) and inflammation-induced alterations in this early network activity may have a critical impact on the survival of immature neurons and the formation of neuronal networks.

During the earliest stages of development, neuronal networks in various brain regions generate spontaneous oscillatory activity patterns (Khazipov and Luhmann 2006; Allene and Gossart 2010; Kilb et al. 2011) and GABAergic interneurons play an important role in the generation of these synchronized discharges by activating gamma-aminobutyric acidergic A (GABA-A) receptors on pyramidal cells (Mann and Paulsen 2007; Blankenship and Feller 2010). Distinct patterns of synchronized burst activity, rather than a steady rate of single action potential discharge, is most effective in triggering the release of neurotrophic survival factors, such as brain-derived neurotrophic factor (BDNF) (Lessmann et al. 2003).

Such spontaneous network bursts have been demonstrated in the cerebral cortex of newborn rodents in vitro (Sun and Luhmann 2007; Sun et al. 2010), in vivo (Minlebaev et al. 2007; Yang, Hanganchi-Opatz, et al. 2009, Yang, An, et al. in press) and in preterm human babies (Mihl et al. 2007), suggesting that this spontaneous activity may play an important general role in early cortical development, such as control of cell survival versus programmed cell death. We have previously shown in developing neocortical slice cultures that spontaneous burst activity supports neuronal survival by activation of the TrkB neurotrophin receptor, which is activated by BDNF (Heck et al. 2008). Our present in vivo data demonstrate that inflammation induces a rapid change in the pattern of spontaneous synchronized burst discharges. Since LPS selectively activates microglial cells via the toll-like receptor-4 receptor, which is not expressed on astrocytes and neurons (Wright et al. 1990; Lehhardt, Lachance, et al. 2002; Lehndt, Massillon, et al. 2003; Block et al. 2004), we suggest that activated microglia are the initial mediators in inflammation-induced modifications of spontaneous network activity. By cytokine array analysis, MEA recordings, and cell survival assays, we identified TNFα and MIP-2 as the major pro-inflammatory factors in our model. Activated microglia release TNFα (Hanisch 2002), which subsequently induces the expression and release of MIP-2 (Otto et al. 2001; Hanisch 2002; Glaubinski et al. 2003; Kobayashi 2008). Stimulation with LPS may also cause a direct production and release of MIP-2 in microglia (Hanisch 2002). Other cytokines related to LPS-induced immune response like IL-1 (Rodgers et al. 2009) were not significantly increased in our array, and therefore not further tested. TNFα released from activated microglia has a direct effect on glutamatergic and GABAergic synaptic function. TNFα causes an enhancement in the synaptic strength of glutamatergic synapses by a PI3 kinase-mediated increased surface expression of AMPA receptors (Beattie et al. 2002) and also an endocytosis of GABA-A receptors, resulting in a decrease in inhibitory synaptic strength (Stellwagen et al. 2005). This inflammation-induced imbalance in excitatory-inhibitory synaptic function causes an increase in the overall firing rate of the neuronal network activity (Fig. 5ii). The decrease in GABAergic function also induces a desynchronization of the network associated with a decrease of the BI (Fig. 5ii), since an intact inhibition is required for the generation of synchronized burst activity (Mann and Paulsen 2007). Previous studies suggested that altered levels of TNFα may ultimately endanger the viability of neurons. While pro-inflammatory cytokines like IL-1 and TNFα are under intense investigation for possible contributions to epileptogenesis (Rodgers et al. 2009), no epileptic discharges could be seen in any of our experiments. Both our in vivo recordings and in vitro data from organotypic slice cultures demonstrated a fast LPS-induced reduction in the occurrence of spontaneous oscillatory activity (Figs 1iii and 3C). Both in vivo and in vitro recordings demonstrated a prolongation of spontaneous activity (Figs 1iii and 3D) and in vivo this effect was highly significant for spindle bursts and gamma oscillations. The effect of LPS on the BI and MUA did not show the same effect in vivo (Fig. 8iv) compared with our in vitro experiments (Fig. 5B), indicating that certain factors only present under in vivo conditions (e.g. systemic factors, up- and down-states, neuromodulatory influences, blood flow) may have an effect here. However, overall our in vivo experiments provided very similar results as our different in vitro models.

Our data indicate that LPS-induced inflammation causes fast (<2 h) modifications in neuronal network activity. Spontaneous oscillatory activity patterns, predominantly gamma oscillations, are disturbed, the release of survival factors is diminished and casp-3–dependent apoptotic cell death
increases. Our experiments with specific antibodies demonstrate (Figs 5B–D and 8A,B), that TNFα and to a lesser extent the chemokine MIP-2 are the main inflammatory mediators causing activity-dependent neuronal cell death in the developing brain. In vivo LPS + anti-TNFα antibody injection confirms these in vitro results. TNFα was neutralized via an antibody and prevented LPS-induced alterations in network activity and cleaved casp-3 immunoreactivity. Our results indicate that TNFα is an important glia-mediated pro-inflammatory cytokine participating in network alterations and casp-3-dependent cell death since anti-TNFα antibody prevented the effects of LPS. However TNFα is only one of several pro-inflammatory cytokines participating in LPS-induced inflammation and additional factors are probably involved.

Our data provide new experimental evidence for the role of spontaneous synchronized burst activity, which transiently occur in developing neuronal networks (Khazipov and Luhmann 2006; Allene and Cossart 2010; Blankenship and Feller 2010; Kilb et al. 2011). Electroencephalograph recordings from the somatosensory cortex of human preterm babies have demonstrated spontaneous and stimulus-evoked network activity classified as spontaneous activity transients (Tolonen et al. 2007) or delta-brush oscillations (Mihl et al. 2007; Colonnese et al. 2010) with a frequency up to 25–30 Hz. Similar spontaneous activity patterns can be found in the cerebral cortex of newborn rodents (Khazipov and Luhmann 2006) and it has been postulated that these early network oscillations play an important role in the formation of topographic maps (Khazipov et al. 2004) and in coupling developing neocortical networks to functional columns (Dupont et al. 2006). Here, we provide evidence that inflammation induces rapid alterations in the pattern of spontaneous spindle bursts and gamma oscillations, which subsequently leads to increased apoptotic cell death in the developing cerebral cortex in vivo and in vitro. Since the immature human brain is most vulnerable to inflammation (Hagberg and Mallard 2005), prenatal infection, or exposure to inflammatory factors may modify spontaneous neocortical activity causing disturbances in programmed cell death.

Supplementary Material
Supplementary material can be found at: http://www.cercor.oxfordjournals.org/.

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