The Paradoxical Effects of Chronic Intra-Amniotic *Ureaplasma parvum* Exposure on Ovine Fetal Brain Development

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Keywords

Ureaplasma parvum · Intra-amniotic inflammation · Chorioamnionitis · Fetal brain · Sheep · Preterm birth

Abstract

Chorioamnionitis is associated with adverse neurodevelopmental outcomes in preterm infants. *Ureaplasma* spp. are the microorganisms most frequently isolated from the amniotic fluid of women diagnosed with chorioamnionitis. However, controversy remains concerning the role of *Ureaplasma* spp. in the pathogenesis of neonatal brain injury. We hypothesize that reexposure to an inflammatory trigger during the perinatal period might be responsible for the variation in brain outcomes of preterms following *Ureaplasma*-

driven chorioamnionitis. To investigate these clinical scenarios, we performed a detailed multimodal study in which ovine neurodevelopmental outcomes were assessed following chronic intra-amniotic *Ureaplasma parvum* (UP) infection either alone or combined with subsequent lipopolysaccharide (LPS) exposure. We show that chronic intra-amniotic UP exposure during the second trimester provoked a decrease in astrocytes, increased oligodendrocyte numbers, and elevated 5-methylcytosine levels. In contrast, short-term LPS exposure before preterm birth induced increased microglial activation, myelin loss, elevation of 5-hydroxymethylcytosine levels, and lipid profile changes. These LPS-induced changes were prevented by chronic preexposure to UP (preconditioning). These data indicate that chronic UP exposure has dual effects on preterm brain development in utero. On

the one hand, prolonged UP exposure causes detrimental cerebral changes that may predispose to adverse postnatal clinical outcomes. On the other, chronic intra-amniotic UP exposure preconditions the brain against a second inflammatory hit. This study demonstrates that microbial interactions and the timing and duration of the inflammatory insults determine the effects on the fetal brain. Therefore, this study helps to understand the complex and diverse postnatal neurological outcomes following UP driven chorioamnionitis.

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Introduction

Neonatal brain injury acquired during pregnancy remains a major cause of adverse neurodevelopmental outcomes throughout life [1, 2]. Chorioamnionitis, which is defined as a microbial invasion and infection of the amniotic cavity, is one of the most important risk factors for adverse neurodevelopmental outcomes of the newborn [3, 4]. *Ureaplasma* spp. are the most common isolated microorganisms associated with chorioamnionitis [5]. Clinical recognition of amniotic fluid (AF) infections is challenging given its asymptomatic course despite sustained fetal exposure to intrauterine inflammation, particularly during the critical period of fetal brain development [6].

Intra-amniotic exposure to *Ureaplasma* spp. is associated with development of fetal and neonatal brain injury [7–10]. Clinical data show that there is an increased risk of intraventricular hemorrhage and impaired neurodevelopmental outcomes later in life after intra-amniotic *Ureaplasma* spp. exposure [7, 9, 10]. This association was confirmed by Normann et al. [11] who showed that intra-amniotic *Ureaplasma parvum* (UP) exposure resulted in increased microglial activation, delayed myelination, and disturbed cortical development of the fetal murine brain. In contrast, clinical studies have reported that antenatal exposure to *Ureaplasma* spp. and brain injury do not correlate [12, 13]. Diversity in microbial interplay and the timing, duration, and severity of the inflammatory response after the onset of chorioamnionitis are considered to determine the neurodevelopmental outcome, which most likely explains the considerable differences in antenatal UP exposure and brain injury incidences among studies [6, 14]. In particular, the onset of cerebral inflammation during the brain's most vulnerable period from 23 to 32 weeks of gestation can have detrimental consequences for the fetal brain, particularly white matter damage. Multiple animal models have demonstrated that the brain becomes more (i.e., sensitization) or less (i.e., pre-

conditioning) susceptible to a second injurious hit following preexposure to inflammation [15, 16]. Besides cerebral inflammation, epigenetic mechanisms (such as DNA methylation and DNA hydroxymethylation) may mediate the processes leading to brain injury in response to environmental challenges in utero [17]. In line with this, DNA methylation levels in genes involved in growth and development are found to be increased in premature infants with chorioamnionitis compared to infants without chorioamnionitis [18].

Moreover, alterations of phospholipids, which are highly abundant in the brain and play important functions in cell membrane formation, as energy reservoirs and as precursors for second messengers (i.e., arachidonic acidAA) [19] have been implicated in multiple brain pathologies. In particular, changes in lipid metabolism, as seen in lysosomal storage diseases, can cause severe impaired brain function, with lipids accumulating within the brain [20].

Detailed investigations of the interactions between different infectious triggers and the timing and duration of inflammatory exposures in the context of a polymicrobial syndrome such as chorioamnionitis are essential to understanding the complex and diverse neurodevelopmental outcomes after birth. We therefore investigated the effects of chronic intra-amniotic UP exposure in the presence or absence of a second (acute) inflammatory stimulus on fetal neurodevelopment. We used a well-established translational ovine model of intrauterine inflammation in which fetuses were chronically exposed to intra-amniotic UP, followed by acute exposure to *Escherichia coli*-derived lipopolysaccharide (LPS). Cerebral outcome was studied by analyzing inflammation, structural injury, epigenetic markers, and the lipid profile composition of the fetal brain.

Methods

Animal Experiments

The animal procedures were performed with the approval of the Animal Ethics Committee of The University of Western Australia (Perth, WA, Australia).

Thirty-seven date-mated merino ewes were randomly assigned to study groups of 5–7 animals. Fetuses of either sex were used. Ewes received an ultrasound-guided intra-amniotic injection of culture media (2 mL) as the control or strain HPA5 of UP serovar 3 (2×10^5 colony-changing units [CCU]) at 80 days of gestation (term ~150 days). To minimize any inflammatory effects from culture media, both UP and control injections were created from stock cultures/sterile media diluted 1:100 in sterile saline. To assess the effect of an additional inflammatory hit following long-term preexposure with

Fig. 1. Experimental groups. Animals were intra-amniotically exposed to *Ureaplasma parvum* (UP; red arrow) for 42 days with ($n = 11$) or without ($n = 6$) subsequent lipopolysaccharide (LPS; black arrow) exposure at 2 ($n = 5$) or 7 ($n = 5$) days before preterm delivery at 122 days of gestation (GA) and sacrificed immediately after birth ($\dagger$). Control animals received intra-amniotic injection with saline (SAL; open arrow).

UP, both groups received a second intra-amniotic injection of 10 mg *E. coli*-derived LPS (O55:B5; Sigma-Aldrich, St. Louis, MO, USA) at 2 or 7 days before preterm delivery at 122 ± 2 days of gestation or an equivalent dose of saline (SAL; controls) (Fig. 1).

Data Acquisition and Analysis

All fetuses were surgically delivered via Cesarean section at 122 ± 2 days of gestation (equivalent to 32–34 weeks of human gestation) and euthanized with intravenous pentobarbitone (100 mg/kg) immediately after birth. AF, blood, and cerebrospinal fluid were collected at delivery and cultures for UP were performed. Brains were immersion fixed in 4% paraformaldehyde.

Culture Analysis of UP Infection

Samples of AF (1 mL) collected by amniocentesis at LPS or control saline injection, as well as plasma, cerebrospinal fluid, and AF collected at Cesarean section delivery, were cultured for UP growth as previously described [21]. For each animal, 20 μ L biological fluid was serially diluted 1:10 in *Ureaplasma* selective medium (Mycoplasma Experience plc., Reigate, UK) in triplicate for each sample and incubated at 37°C. Assays were performed in 96-well plates and bacterial growth was quantified via titration of the urease activity (conversion of urea to ammonium ions leading to a pH color change). Plates were observed until the bacterium-mediated color change ceased and the titration of the bacteria present was determined.

Analysis of the Interleukin-6 Concentration

The proinflammatory cytokine interleukin (IL)-6 was measured in fetal plasma as a marker for systemic inflammation using a sheep-specific sandwich enzyme-linked immunosorbent assay (ELISA). Briefly, a mouse anti-ovine monoclonal antibody (MAB1004, working concentration 1:200; Millipore, Darmstadt, Germany) was the coating antibody. Plasma samples (100 μ L) were loaded per well in duplicate and incubated for 2 h at room temperature. Incubation with the detection antibody (rabbit anti-ovine IL-6, AB1839, working concentration 1:500; Millipore) was performed for 60 min, followed by incubation for 30 min with 100 μ L goat anti-rabbit HRP (working concentration 1:500; Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA). After washing, incubation with 3,3',5,5'-tetramethylbenzidine substrate solution was performed for 15 min. The reaction was stopped by addition of 50 μ L 2 N sulfuric acid. Optical density was measured using a microplate reader at 450 nm. Concentrations were expressed relative to a standard curve of ovine IL-6 recombinant protein (ImmunoChemistry Technologies, Bloomington, MN, USA).

Histology and Immunohistochemistry

After fixation, the right hemisphere was divided into 4 defined anatomical regions. The region of the posterior hippocampus/mid-thalamus was embedded in paraffin and serial coronal sections (4 μ m) were cut with a Leica RM2235 microtome. At this level, we analyzed the hippocampus and cerebral white matter for inflammatory and structural changes since these regions are most affected following intrauterine infection at this developmental stage [22]. Four slides per staining per animal were used (i.e., every 10th consecutive slide) for immunohistochemical analysis. Hematoxylin and eosin (H&E) staining was performed for morphological and anatomical analysis. Adjacent sections were stained as previously described using a rabbit anti-ionized calcium binding adaptor molecule 1 (IBA-1) antibody (Wako Pure Chemical Industries, Osaka, Japan) for microglia, a rabbit anti-glial fibrillary acidic protein (GFAP) antibody (Z0334; DAKO) for astrocytes, a rat anti-myelin basic protein (MBP) antibody (MAB386; Merck Millipore) for myelin sheaths, a rabbit anti-oligodendrocyte transcription factor 2 (Olig2) antibody (AB9610; Millipore) for oligodendrocyte lineage cells, rabbit anti-myeloperoxidase (MPO) (A0398; DAKO) for neutrophils, mouse anti-cluster of differentiation (CD) 68 (M0718; DAKO) for active microglia/phagocytizing macrophages, and rabbit anti-CD3 (A0452; DAKO) for T lymphocytes, and mouse anti-5-methylcytosine (5-mc) (GWB-CB561B; Genway) and rabbit anti-5-hydroxymethylcytosine (5-hmc) (39769; Active Motif) were used as epigenetic markers.

Endogenous peroxidase activity was inactivated with 0.3% H_2O_2 treatment (or 1% H_2O_2 for 5-mc and 5-hmc). Antigen retrieval was performed by microwave boiling of tissue sections in citrate buffer (pH 6.0). Nonspecific binding was blocked by incubation with bovine, goat, or donkey serum in PBS. Sections were incubated overnight at 4°C with the diluted primary antibody (5-hmc 1:2,500; IBA-1, GFAP, and MBP 1:1,000; 5-mc 1:500; and Olig2 and MPO 1:200). The following day, sections were incubated with the specific secondary antibody and staining was enhanced with a Vectastain ABC Peroxidase Elite Lit (Vector Laboratories Inc., Burlingame, CA, USA) and (nickel) 3,3'-diaminobenzidine staining. If required, appropriate background staining was performed.

Matrix-Assisted Laser Desorption Ionization Mass Spectrometry Imaging

A more detailed molecular analysis of the cerebral tissue was done by matrix-assisted laser desorption ionization mass spectrometry imaging (MALDI-MSI) to map variations in lipid profiles of the white and grey matter. MALDI-MSI to image the lipid distribution can be invaluable in understanding complex lipid

changes, and it has been used to study these molecular patterns in models of brain injury [23]. With MALDI-MSI we avoid all extraction and purification steps for lipid analysis while retaining their spatial distribution. For this technique, postfixation tissues of controls, 42UP, 2LPS, and UP&LPS groups were frozen in liquid nitrogen and subsequently samples were cryo-sectioned (10- μ m thickness) in a cryostat (Leica CM3050S), deposited on indium tin oxide high-conductive slides (Delta Technologies, USA), and stored at -20°C . Subsequently, the matrix solution consisting of norharman (7 mg/mL) in 2:1 chloroform:methanol was sprayed on top of the tissue section by a vibrational sprayer (Suncollect; SunChrom, Germany) for positive ion mode and 9-aminoacridine (10 mg/mL) in 70% ethanol for negative ion mode MALDI-MSI analysis. Digital optical scans of all of the tissue sections were obtained prior to MALDI-MSI experiments using a 2,400-dots-per-inch desktop scanner. The resulting digital images were imported into MALDI Imaging HDI software v1.4 (Waters Corporation). A MALDI-quadrupole time-of-flight SYNAPT HDMS G2Si system (Waters Corporation) operating with a 200-Hz Nd:YAG laser was configured to acquire data in positive and negative V-reflectron modes. Data were acquired at a raster size of $100 \times 100 \mu\text{m}$. Instrument calibration was performed using a standard calibration solution of red phosphorus.

Principal components analysis (PCA) and discriminant analysis (DA) were used to investigate spectral similarities and differences between all samples. PCA was performed as a data compression and noise-filtering step before the application of DA, and only one fourth of the functions ($n = 216$) were used as input for DA. In short, PCA is an unsupervised statistical method that aims to pool a maximum amount of variance in a minimum number of independent variables. Data pretreatment, PCA, and DA were performed using our in-house built ChemomeTricks toolbox for MATLAB version 2014a (MathWorks, Natick, MA, USA). Peak assignments were performed according to the bibliography and LIPID MAPS software (<http://www.lipidmaps.org/>).

Qualitative Analysis

H&E-stained sections were analyzed by 2 independent investigators and a neuropathologist, blinded to the experimental set-up, to assess overall brain structure and inflammatory changes. The sections were examined for the presence of gliosis, hemorrhages, (cytotoxic) edema and structural damage, including cyst formation.

Immunohistochemical Analysis

Immunohistochemical stainings were analyzed using a light microscope (Leica DM2000) equipped with Leica QWin Pro version 3.4.0 software (Leica Microsystems, Mannheim, Germany). Regions of interest of the white matter and hippocampus were defined as previously described [24]. These regions were chosen since they are most affected by intrauterine infections at this developmental stage. In the white matter 4–6 adjacent images were taken at $\times 100$ magnification and from the hippocampus 1 image at $\times 20$ magnification was taken. To assess regional vulnerability within the hippocampus, separate images were taken at $\times 200$ magnification of the cornu amonis 1 and 2, 3, and 4 and the dentate gyrus. Area fractions of IBA-1, GFAP, MBP, 5-mc, and 5-hmc expression were determined using a standard threshold to detect positive staining with Leica QWin Pro V 3.5.1 software (Leica, Rijswijk, The Netherlands). Regions of interest were delineated in the image

Fig. 2. *Ureaplasma* titer at the time of subsequent lipopolysaccharide (LPS) or saline injection. Establishment of chronic infection was confirmed in amniocentesis samples taken at the time of LPS or saline injection. Mean and standard error of the mean are shown for each group (culture titration of viable *Ureaplasma parvum* [UP] determined in triplicate for each animal). No statistical difference was found by one-way ANOVA testing. CCU, colony-changing units.

with large blood vessels and artefacts excluded from analysis. Since the level of DNA methylation and hydroxymethylation can differ per cell, the integrated density of 5-mc and 5-hmc was calculated by multiplying the area fraction by the mean grey value, and these values were normalized to the data of the control group as previously described by Lardenoije et al. [25]. In addition to area fraction analysis, IBA-1- and GFAP-positive cells were counted in 3 fields of view within the regions of interest at a magnification of $\times 400$. The Olig2-positive cells were counted using Qwin software and expressed as cells/ mm^2 . MPO-positive cells were counted focusing on the cerebral vasculature, meninges, and choroid plexus. To quantify the density (cells/ mm^2) of MPO-, CD68-, and CD3-positive cells, representative images of the choroid plexus present in the lateral ventricles aligning the hippocampus were counted using ImageJ software. The images were acquired and analyzed by an independent observer who was blinded to the experimental groups.

Data Analysis

All values are shown as means with 95% CI or SD and $p < 0.05$ was considered statistically significant. Comparison between different experimental groups was performed using analysis of variance (ANOVA) or with a random intercept mixed model in case of repeated measurements per animal (e.g., different sections per brain). We applied log-transformation to obtain normal distributed data when data or variables were positively skewed before statistical testing. Statistical analysis was performed using IBM SPSS Statistics version 22.0 (SPSS, RRID: SCR_002865; IBM Corp., Armonk, NY, USA). Considering the relatively low number of animals per group, we depicted the exact p values in Figure 4–6.

Results

UP Cultures and Detection

Chronic UP infection in animals inoculated at 80 days of gestation was confirmed by culture of AF at the time of subsequent LPS or saline injections by amniocentesis (Fig. 2). No significant differences in UP levels were observed among the 3 groups (42UP, $1.1 \pm 0.8 \times 10^6$ CCU/mL; UP&2LPS, $1.1 \pm 0.8 \times 10^7$ CCU/mL; UP&7LPS, $9.4 \pm 0.7 \times 10^6$ CCU/mL). Cultures of AF at the time of delivery were positive for UP in all experimentally infected animals, except for 1 of the animals in the UP&7LPS group. No endogenous UP growth was observed in the AF of animals that were not inoculated with UP (SAL, 2LPS, and 7LPS groups). No UP growth in cerebrospinal fluid or plasma was observed in any animal.

Animal Characteristics

Overall, no sex differences in susceptibility were observed in any of the readouts. LPS exposure for 2 days decreased the body weight (SAL vs. 2LPS, $p = 0.002$) and increased the brain-to-body ratio (SAL vs. 2LPS, $p = 0.038$) compared to controls (Table 1). These significant changes were not observed in animals that were chroni-

cally exposed to UP prior to 2 days of LPS exposure (UP&2LPS). Moreover, no change in brain weight or brain-to-body ratio was observed in the animals of the 42UP, 7LPS, and UP&7LPS groups compared to the control animals.

Fig. 3. Systemic immune activation was ascertained by measuring circulatory interleukin (IL)-6 concentrations. For statistical analysis, undetectable values were assigned an arbitrary value of 1 pg/mL. No statistical difference was found by one-way ANOVA testing.

Table 1. Animal characteristics

	SAL (n = 7)	42UP (n = 6)	2LPS (n = 5)	7LPS (n = 5)	UP&2LPS (n = 5)	UP&7LPS (n = 5)
Body weight, g	3,021 ± 328	2,765 ± 312	2,256 ± 305*	3,040 ± 285	2,580 ± 285	2,652 ± 396
Brain weight, g	52.2 ± 4.5	50.8 ± 4.9	46.0 ± 2.8**	51.7 ± 7.0	47.9 ± 2.1	51.7 ± 4.1
Brain/body ratio, %	1.7 ± 0.02	1.9 ± 0.02	2.1 ± 0.02*	1.7 ± 0.02	1.9 ± 0.02	2.0 ± 0.02

Values are expressed as means ± SD. One-way ANOVA with Dunnett's multiple comparisons testing was performed. SAL, control; UP, *Ureaplasma parvum*; LPS, lipopolysaccharide. * $p < 0.05$ vs. control; ** $p < 0.1$ vs. control.

Fig. 4. Intra-amniotic exposure to *Ureaplasma parvum* (UP) induces a decrease in astrocytes and preconditions the fetal brain against reexposure to lipopolysaccharide (LPS). A significant decrease ($p < 0.05$) in the area fraction of glial fibrillary acidic protein (GFAP) immunoreactivity (IR) was observed in animals of the chronic UP group (42UP) compared to controls (SAL) (SAL vs. 42UP, $p = 0.020$) (a, c). GFAP-positive cells tended to be decreased following chronic UP exposure (SAL vs. 42UP, $p = 0.100$) (e). No changes in GFAP IR were found in animals of the LPS exposed groups regardless of the presence or absence of UP preexposure. A significant increase ($p < 0.05$) in the area fraction of IBA-1 IR and IBA-1-positive cells was observed in animals exposed to LPS for 2 days (2LPS) (SAL vs. 2LPS, $p = 0.008$ and $p = 0.036$, respectively),

which was prevented by preexposure to UP (UP&2LPS) (b, d, f). No changes in IBA-1 IR or cell numbers were found in animals exposed to LPS for 7 days (SAL vs. 7LPS, $p = 0.342$ and $p = 1.00$, respectively) (d, f). Representative histological figures of the GFAP-positive astrocytes and IBA-1-positive microglia are shown in a and b, respectively. Morphological analysis showed a higher density of amoeboid microglia present in the white matter after 2 days of LPS exposure (b, insets). Figures for animals exposed to LPS for 7 days (7LPS) and animals exposed to UP for 42 days and LPS for 7 days (UP&7LPS) are not depicted. GFAP and IBA-1 IR are represented in the graphs as means (% area fraction) ± 95% CI. Images were taken at ×100 magnification (insets at ×400 magnification). Scale bar, 200 μm.

(For figure see next page.)

Circulatory Proinflammatory Cytokine Levels

Elevated plasma IL-6 concentrations were found in 50% (3 out of 6) of the animals exposed to LPS for 2 days and in 20% (1 out of 5) of the animals exposed to LPS for 42 days compared to controls (Fig. 3). Plasma IL-6 concentrations in the SAL, 42UP, 7LPS, and UP&7LPS animals were not detectable.

Structural Analysis of the Brain

Qualitative analysis of the H&E-stained sections revealed increased cell densities in the gyral crest of the white matter which primarily consisted of glial cells. These gliotic foci were most prominent in 3 out of 6 (50%) of the animals exposed to LPS for 2 days. Furthermore, in 1 out of 6 (16%) of the animals exposed to LPS for 42 days and in 1 out of 6 (16%) of the animals exposed to LPS for 7 days, these gliotic foci were present. Control animals and animals of the UP&LPS combined groups had mild to no gliotic foci. No evidence of structural changes including intraventricular hemorrhages and cystic lesions was present in any of the experimental groups.

Dual Effects of Chronic UP Infection on Cerebral Development

The neuroinflammatory changes, as indicated by the more pronounced presence of gliotic foci in the animals exposed to LPS for 2 days, were further evaluated by cell counts and area fraction analysis of the microglial marker IBA-1 and the astrocytic marker GFAP in the cerebral white matter and hippocampus. Chronic intra-amniotic exposure to UP decreased GFAP immunoreactivity (IR) (SAL vs. 42UP, $p = 0.020$) and the number of astrocytes (SAL vs. 42UP, $p = 0.100$) compared to controls (Fig. 4). IBA-1 IR- and IBA-1-positive cell numbers remained unaltered following chronic intra-amniotic UP exposure (Fig. 4). In contrast, acute exposure to LPS increased IBA-1 IR (SAL vs. 2LPS, $p = 0.008$) and the number of IBA-1-positive cells (SAL vs. 2LPS, $p = 0.036$) in the cerebral white matter (Fig. 4). In addition, morphological analysis revealed a higher density of amoeboid microglia present in the white matter after 2 days of LPS exposure (Fig. 4 insets). However, if the animals were chronically exposed to UP prior to LPS, no IBA-1 IR or IBA-1-positive cell increase was observed in the cerebral white matter at 2 or 7 days post-LPS challenge (Fig. 4d, f). Equally, LPS administration did return GFAP IR in chronically UP-infected animals to control levels (Fig. 4c). This preconditioning effect of UP was also found in the hippocampus, in which an increase in IBA-1 IR was found at 2 and 7 days following LPS exposure, but not in the groups

with preexposure to UP (SAL vs. 2LPS, $p = 0.002$; SAL vs. 7LPS, $p = 0.000$) (data not shown). No changes in GFAP IR or GFAP-positive cell numbers following LPS exposure were found in the white matter (Fig. 4) or in the hippocampus (data not shown).

Since the choroid plexus is primarily dominated by macrophages, T lymphocytes and dendritic cells for continuous immune surveillance, and the resolution of cerebral inflammation [26], we analyzed the distribution of CD68+ macrophages, CD3+ lymphocytes, and MPO+ neutrophils within the choroid plexus. MPO-positive cells tended to be increased following 7-day LPS exposure compared to control animals and this increase was prevented by preexposure to UP for 42 days (SAL vs. 7LPS, $p = 0.086$; Table 2). In line, this increase in MPO-positive cells at 7 days post-LPS exposure was accompanied by a decrease in IBA-1 IR in the cerebral white matter compared to animals exposed to LPS for 2 days. No differences in CD68- or CD3-positive cells were found in the choroid plexus. No CD3- and MPO-positive cells were detected in the brain parenchyma.

White matter injury was studied by assessing the densities of mature (MBP) and overall (Olig2) oligodendrocytes in the cerebral white matter. An apparent increase in Olig2-positive cells was found for all groups relative to control levels; however, this only reached significance for chronically UP-infected animals (SAL vs. 42UP, $p = 0.012$) and animals exposed to LPS for 2 days (SAL vs. 2LPS, $p = 0.037$) (SAL vs. 7LPS, $p = 0.211$; SAL vs. 42UP&2LPS, $p = 0.558$; SAL vs. 42UP&7LPS, $p = 0.467$; Fig. 5c). In addition, MBP IR tended to be decreased at 42 days following UP exposure (SAL vs. 42UP, $p = 0.097$; Fig. 5). Short-term LPS exposure for 2 days resulted in a decrease in MBP IR within regions of overt microgliosis (SAL vs. 2LPS, $p = 0.001$) which was prevented by preexposure to UP. At 7 days of LPS exposure no changes in MBP IR were found (2LPS vs. 7LPS, $p = 0.000$).

We analyzed 5-mc and 5-hmc integrated density as epigenetic markers for DNA-methylation in the dentate gyrus of the hippocampus. We focused our analysis on the dentate gyrus since this is the region in the hippocampus where neurogenesis takes place and DNA methylation and demethylation are important contributors to this process [27, 28]. Both short-term and chronic UP exposure resulted in an increase in the gene repression marker 5-mc integrated density compared to controls (SAL vs. 2LPS, $p = 0.008$; SAL vs. 7LPS, $p = 0.002$; SAL vs. 42UP, $p = 0.008$) (Fig. 6). The increase in 5-mc following LPS exposure tended to be prevented by preexposure to UP. An increase in transcription activation marker 5-hmc in-

Fig. 5. Intra-amniotic exposure to *Ureaplasma parvum* (UP) induces changes in white matter development and preconditions the fetal brain upon reexposure to a second inflammatory hit with lipopolysaccharide (LPS). A significant increase ($p < 0.05$) in Olig2-positive cells was observed in animals in the chronic UP group (SAL vs. 42UP, $p = 0.012$) and those exposed to LPS for 2 days (SAL vs. 2 LPS; $p = 0.037$) compared to controls (**b, c**). This increase in

Olig2-positive cells was accompanied by a decrease in MBP (SAL vs. 42UP, $p = 0.097$; SAL vs. 2LPS, $p = 0.001$) (**a, d**). This decrease in MBP immunoreactivity (IR) was prevented in the short-term LPS-exposed animals that were preexposed to UP. At 7 days of LPS exposure no changes in MBP IR were found (2LPS vs. 7LPS, $p = 0.000$). Images were taken at $\times 100$ magnification (**insets** at $\times 400$ magnification). Scale bar, 200 μ m.

Table 2. Immune cells present in the choroid plexus

	SAL (n = 7)	42UP (n = 6)	2LPS (n = 5)	7LPS (n = 5)	UP&2LPS (n = 5)	UP&7LPS (n = 5)
MPO+ cells	3.31±2.72	6.80±10.29	8.58±2.84	14.66±8.96**	1.06±0.87	5.68±9.36
CD68+ cells	67.99±47.40	48.45±33.92	69.91±51.64	80.62±32.07	64.49±49.05	59.31±40.18
CD3+ cells	28.98±24.96	11.14±7.21	30.82±34.98	47.24±23.73	26.11±35.25	15.92±19.32

Values are expressed as means numbers of cells per square millimeter $\pm$ SD. One-way ANOVA with Dunnett's multiple comparisons testing was performed. ** $p < 0.1$ vs. control.

Fig. 6. Changes in the epigenetic markers 5-methylcytosine (5-mc) and 5-hydroxymethylcytosine (5-hmc) following intra-amniotic exposure to *Ureaplasma parvum* (UP) and short-term exposure to lipopolysaccharide (LPS) in the dentate gyrus of the hippocampus. A significant increase ($p < 0.05$) in the gene repression marker 5-mc IR was observed in short-term LPS-exposed animals and chronically UP-exposed animals compared to controls (SAL vs. 2LPS, $p = 0.008$; SAL vs. 7LPS, $p = 0.002$; and SAL vs. 42UP, $p = 0.008$) (a, c). The significant increase ($p < 0.05$) in transcription

activation marker 5-hmc IR was restricted to the group exposed to LPS for 2 days compared to controls (SAL vs. 2LPS, $p = 0.000$; 2LPS vs. 7LPS, $p = 0.000$), and it was prevented by preexposure to UP. 5-mc and 5-hmc are represented in the graphs as mean integrated density $\pm$ 95% CI. The integrated density was calculated by multiplying the area fraction and grey density measurements. Values were normalized to the data of the control group. Images were taken at $\times 200$ magnification (insets at $\times 400$ magnification). Scale bar, 200 μ m.

egrated density was only found in animals exposed to LPS for 2 days compared to controls and it was prevented by preexposure to UP (SAL vs. 2LPS, $p = 0.000$; 2LPS vs. 7LPS, $p = 0.000$; Fig. 6).

Accumulation of Lipids and Changes in the White and Grey Matter Lipid Profile following Acute LPS Exposure Are Prevented by Preexposure to UP

Using MALDI-MSI, we demonstrated unique regional differences in lipid composition in the preterm ovine brain between animals from the control, 42UP,

2LPS, and UP&LPS groups. Figure 7 shows the reconstructed image that represents the molecular differences in lipids between white (red area) and grey matter (green area). The lipid composition, characteristic of healthy white and grey matter of the preterm brain, was not altered following chronic UP exposure (Fig. 7a–c). In particular, in the white matter of control and 42UP animals, typical tentative assigned m/z values of different phosphocholine (PC) species such as m/z 782.5 PC 34:1+Na⁺, m/z 810.5 PC 36:1+Na⁺ or sphingomyelin m/z 725.5 SM 34:1+Na⁺ are found which are known to be representative for the white matter [29, 30] (Fig. 7c). m/z 756.5 PC 32:0+Na⁺, which is a characteristic grey matter lipid [31], was detected in the grey matter of control and 42UP animals, demonstrating that control and chronic UP-exposed animals had a similar and constitutive lipid profile.

Mosaic PCA images demonstrated that short-term LPS exposure resulted in lipid accumulation in the white matter and diffusion of white matter-specific molecular patterns into the grey matter and vice versa (Fig. 7d–i; principal components 4 and 6). These changes were reduced when LPS exposure was preceded by 42 days of UP infection (Fig. 7e, h). In particular, the abundance of the white matter-specific component m/z 725.5 in the grey matter of LPS-exposed animals was not present in the grey matter of control or UP animals (Fig. 7g–i; negative spectrum of principal component 6). m/z 734.5 PC 32:0+H⁺ was accumulated in the grey matter of LPS animals (negative spectrum of principal component 4), and increased in the white matter (positive spectrum of principal component 6). In addition, principal component 6 showed that other peaks such as m/z 760 PC 34:1+H⁺ accumulate in the white matter at 2 days of LPS exposure, which did not correspond with the pattern seen in our controls or those of other studies [29] in which these peaks were evenly distributed.

Other lipid species including phosphatidylinositols (PI) and sulfatide (SF) can be identified using the negative ion mode in MALDI-MSI. Regions of interest corresponding to the white matter were selected based on the results with the positive ion mode. DF1 (Fig. 8a) revealed that the highest differences were observed between the control group (negative scores) and the LPS group (positive scores). The UP&LPS group had negative scores and therefore possessed a molecular composition more similar to that of the control group. The DF1 spectrum (Fig. 8b) showed the lipid composition of the white matter of animals exposed to LPS for 2 days. This spectrum showed that LPS exposure reduced the amount of

tentative assigned SF species such as m/z 806.5 SF 18:0-H⁻, m/z 888.6 SF 24:1-H⁻, and m/z 890.6 SF 24:0-H⁻, which were described as lipids characteristic of white matter [28]. These species were mainly present in the white matter of the control, UP, and UP&LPS groups (spectrum not shown), whereas m/z 885.5 PI 38:4-H⁻ or m/z 718.6 PC 31:0-H⁻ was representative of the white matter in animals of the 2LPS group (Fig. 8b).

Discussion

Chronic intra-amniotic UP exposure decreased GFAP IR and increased Olig2-positive cells and 5-mc IR in the brain. These changes have potential clinical implications postnatally.

The observed decrease in GFAP IR and the number of astrocytes (GFAP+ cells) at 42 days of UP exposure is important because these cells possess several essential functions in brain development, including regulation of extracellular glutamate homeostasis, providing structural and metabolic support to surrounding cells (e.g., oligodendrocytes) and modulate neuronal connections [32]. Changes in astrocyte function or density result in altered neurological outcomes. In particular, altered astrocyte protein expression (GFAP) and disrupted astrocyte maturation have been implicated in the pathogenesis of neurodevelopmental disorders such as autism and cerebral palsy [33, 34]. Moreover, Sharma et al. [35] showed that LPS injection in the spinal cord of rodents decreased astrocytes, which was followed by hypomyelination. This suggests that white matter injury, a hallmark of preterm brain injury, can still occur in these fetuses considering the loss of GFAP IR at day 42 after UP exposure. Collectively, the astrocyte cell and protein loss in our study indicates that chronic UP exposure during the second trimester of gestation predisposes to brain pathologies that are often seen in newborns.

Second, the increase in oligodendrocyte lineage cells, as seen following 42 days of UP exposure, might indicate replenishment of oligodendrocytes upon an initial loss in the acute phase following UP exposure [36]. Importantly, UP was administered at 80 days of gestation, which is the premyelinating stage of brain development, with abundant vulnerable immature preoligodendrocytes sensitive to glutamate receptor-induced injury [37]. Interestingly, mature oligodendrocytes tended to be decreased following chronic UP exposure. Given these combined findings of increased Olig2+ cell numbers and reduced MBP+ IR, it is tempting to speculate that this indicates a maturation

a**b** **c** **d****e** **f** **g****h** **i**

arrest of oligodendrocyte progenitor cells, a key feature of white matter injury in preterms [38–40]. This oligodendrocyte maturation arrest can be linked to the decreased astrocytes that we found in this study. Astrocytes are essential contributors to extracellular glutamate homeostasis, which is disturbed by a loss of astrocytes [32]. Since immature oligodendrocytes are particularly vulnerable to glutamate receptor-induced injury, this can lead to oligo-

dendrocyte injury [37]. In addition, oligodendrocytes rely on astrocytes for their metabolic support via gap junctions [41]. Failure of metabolic support for oligodendrocytes following an astrocyte loss results in energy failure and eventually maturation arrest or death. Alternatively, it is tempting to speculate that oligodendrocyte maturation arrest may be connected to the apparent increase in the DNA methylation marker 5-mc at 42 days of UP exposure,

Fig. 8. Matrix-assisted laser desorption/ionization mass spectrometry imaging performed in negative ion mode followed by principal components analysis. DF1 (a) revealed that the greatest differences were observed between the control group (negative scores) and the lipopolysaccharide (LPS) group (positive scores). Both the 42UP and the UP&LPS groups had negative scores which were comparable to those of the control group. The DF1 spectrum of

the animals exposed to LPS for 2 days (b) showed that LPS infection reduced the amount of sulfatide (SF) species such as m/z 806.5 SF 18:0- H^- , m/z 888.6 SF 24:1- H^- , and m/z 890.6 SF 24:0- H^- . These species were mainly present in the white matter of the control, UP, and UP&LPS groups (spectrum not shown), whereas m/z 885.5 PI 38:4- H^- or m/z 718.6 PC 31:0- H^- was representative of the white matter in animals of the 2LPS group (b). UP, *Ureaplasma parvum*.

Fig. 7. Matrix-assisted laser desorption/ionization mass spectrometry imaging performed in positive ion mode followed by principal component analysis. a Reconstructed image that represents the molecular differences in lipids between the white (red area) and grey matter (green area) of control and *Ureaplasma parvum* (UP) animals. Principal component analysis 3 demonstrated similar lipid compositions of white and grey matter of control and UP animals (a–c). In particular, phosphocholine (PC) species m/z 782.5 PC 34:1+ Na^+ , m/z 810.5 PC 36:1+ Na^+ , or sphingomyelin m/z 725.5 SM 34:1+ Na^+ are present in white matter (positive spectrum principal component 3), whereas PC m/z 756.5 32:0+ Na^+ is present in the grey matter of control and 42UP animals (negative spectrum

principal component 3). Short-term LPS exposure results in lipid accumulation in the white matter as illustrated by an increased intensity of the white matter-related peaks, especially m/z 725.5 (d, g) (positive spectrum of principal component 4). In addition, a shift of white and grey matter-specific molecular patterns is observed at 2 days of LPS exposure, illustrated by more white matter-specific lipids present in the grey matter such as m/z 725.5 (d, negative spectrum of principal component 6), and grey matter-specific lipids into the white matter such as m/z 734.5 PC 32:0+ H^+ and m/z 760 PC 34:1+ H^+ (g, positive spectrum of principal component 6). These changes were prevented by preexposure to UP (e, h).

which is a very important repressor of gene transcription [42]. This theory is supported by several reports stating that changes in epigenetic regulatory mechanisms contribute to disturbed maturation and differentiation of immature oligodendrocytes [43–45]. Moreover, inflammation induced epigenetic changes during early development can cause substantial lasting neurodevelopmental impairments later in life [46, 47]. Altogether, these data offer a mechanistic insight into the association between intra-amniotic UP exposure and the increased incidence of adverse neurodevelopmental outcomes postnatally.

Interestingly, the cerebral phenotype following short-term LPS exposure was remarkably different compared to chronic UP exposure. In particular, we demonstrated that short-term LPS exposure induced a rapid and temporal increase in the number of microglia and decreased myelin IR, reflecting diffuse cerebral inflammation with hypomyelination. Microglia are important in inflammatory perinatal brain injury [43]. Aberrant or excessive microgliosis can be detrimental for the immature brain, resulting in white matter injury [14], which corresponds to the loss of myelin that we found in our study. The cerebral inflammatory response following LPS in this study seems to be temporal since no increase in microglial density was found following 7 days of LPS exposure. This dynamic response of activated microglia is consistent with distinct phases of cerebral inflammation [48] and can be explained by the presence of neutrophils in the choroid plexus, which are known to be important for the resolution of cerebral inflammation [26]. However, our immunohistochemical analysis does not rule out the possibility that phenotypic conversion of microglia might be induced following short-term LPS or chronic UP exposure, which might influence the cerebral immune response.

Although such short-term UP effects were not investigated in our model, in a study performed by Normann et al. [11] in rodents short-term UP exposure during early pregnancy resulted in an increased microglial density and a decreased MBP density in the fetal brain. This cerebral phenotype is consistent with our data following short-term LPS exposure, indicating that timing and not the inflammatory trigger is more important for the neurological outcome of the fetal brain.

Besides the DNA methylation marker 5-mc, the hydroxylated product and transcription activation marker 5-hmc was increased following 2 days of LPS exposure. 5-hmc is very important for proper neurodevelopment and it is altered in the umbilical cord of babies born after preeclampsia and gestational diabetes mellitus [49] and in the hippocampus of 7-week-old mice exposed to non-

infectious stress [50]. In addition, 5-hmc alterations are associated with severe neurodevelopmental disorders such as Rett syndrome, which is caused by mutations in the MeCP2 gene that encodes for proteins that directly bind to methylated DNA domains [51]. Therefore, the alterations we found in epigenetic markers following acute LPS exposure might explain, at least in part, the association between chorioamnionitis and the development of psychopathology later in life [52]. Since epigenetic changes are reversible, these findings provide new therapeutic targets to prevent long-lasting neurodevelopmental morbidities following prenatal stress [17].

In addition, our lipid data provide supporting evidence that short-term LPS exposure results in lipid accumulation and “diseased” lipid patterns in the preterm brain. Such lipid accumulation in the brain is associated with severe neurological damage and altered brain functions [20]. In addition, we noted a relative decrease in myelin-specific sphingolipids in the white matter of the LPS-exposed animals, which confirms and extends our findings concerning the loss of MBP in these animals. The abundance of PI following 2 days of LPS exposure was primarily seen within the region of increased IBA-1 IR. In line with this, the phosphorylated form of PI, phosphoinositide, is known to be upregulated in microglia and contributes to the activation of microglia following ischemia [53].

Third, we found that chronic intra-amniotic UP exposure prevented an increase in IBA-1 IR and IBA-1+ cells, 5-hmc IR, lipid profile changes, and a decrease in MBP IR upon a second inflammatory hit with LPS. This phenotype, also referred to as “preconditioning”, has been previously described in animal models in which preexposure to inflammation induced by LPS renders the brain less susceptible to a second hypoxic-ischemic insult, thereby resulting in less brain injury [54, 55]. This preconditioning effect of chronic UP exposure could be explained by the work of Cao et al. [56], which showed in pregnant sheep that microglia, once activated *in vivo* by intra-amniotic LPS exposure, display diminished inflammatory responses following reexposure to LPS. Moreover, they stated that the memory acquired by microglia upon the first exposure to inflammation might be mediated by epigenetic regulatory processes [56]. Although this hypothesis needs to be tested in future studies, it is noteworthy that changes in the global level of 5-hmc and 5-mc were observed in our study following acute LPS exposure that was prevented by chronic UP exposure. Clearly, long-term protection after inflammation-induced preconditioning needs to be confirmed in a longitudinal study, but it is considered to be

permanent since structural and functional protection for up to 8 weeks was established following hypoxic preconditioning in a neonatal rodent model [57].

One important limitation of a large animal study is the relative low number of animals per group. Given the relatively small animal numbers per group, we reported actual *p* values and tended to interpret *p* values between 0.05 and 0.1 as biologically relevant. This assumption decreases the chance of a false-negative finding but increases the chance that one of these differences is a false-positive result.

In this double-hit study, in which sequential different infectious hits were tested, we showed that microbial interactions, the moment of onset, and the duration of these potential injurious triggers determine the neurological outcome. These findings seem to be an important explanation for the diversity of neurological outcomes associated with intra-amniotic UP exposure. Altogether, these data emphasize that an accurate history of infections during pregnancy is essential to guide neonatal management, which warrants the need for biomarkers to diagnose antenatal infections.

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Disclosure Statement

The authors of this paper declare that there are no actual or potential conflicts of interest. The authors affirm that there are no financial, personal or other relationships with other people or organizations that have inappropriately influenced or biased this research.

References

- Hagberg H, Gressens P, Mallard C: Inflammation during fetal and neonatal life: implications for neurologic and neuropsychiatric disease in children and adults. *Ann Neurol* 2012;71:444–457.
- Strunk T, Inder T, Wang X, Burgner D, Mallard C, Levy O: Infection-induced inflammation and cerebral injury in preterm infants. *Lancet Infect Dis* 2014;14:751–762.
- Rovira N, Alarcon A, Iriando M, Ibanez M, Poo P, Cusi V, et al: Impact of histological chorioamnionitis, funisitis and clinical chorioamnionitis on neurodevelopmental outcome of preterm infants. *Early Hum Dev* 2011;87:253–257.
- Kuypers E, Ophelders D, Jellema RK, Kunzmann S, Gavilanes AW, Kramer BW: White matter injury following fetal inflammatory response syndrome induced by chorioamnionitis and fetal sepsis: lessons from experimental ovine models. *Early Hum Dev* 2012;88:931–936.
- Sweeney EL, Kallapur SG, Gisslen T, Lambers DS, Chougnet CA, Stephenson SA, et al: Placental infection with *Ureaplasma* species is associated with histologic chorioamnionitis and adverse outcomes in moderately preterm and late-preterm infants. *J Infect Dis* 2016;213:1340–1347.
- Viscardi RM: *Ureaplasma* species: role in diseases of prematurity. *Clin Perinatol* 2010;37:393–409.
- Viscardi RM: *Ureaplasma* species: role in neonatal morbidities and outcomes. *Arch Dis Child Fetal Neonatal Ed* 2014;99:F87–F92.
- Glaser K, Speer CP: Neonatal CNS infection and inflammation caused by *Ureaplasma* species: rare or relevant? *Expert Rev Anti Infect Ther* 2015;13:233–248.
- Kasper DC, Mechtler TP, Bohm J, Petricevic L, Gleiss A, Spengler J, et al: In utero exposure to *Ureaplasma* spp. is associated with increased rate of bronchopulmonary dysplasia and intraventricular hemorrhage in preterm infants. *J Perinat Med* 2011;39:331–336.
- Berger A, Witt A, Haiden N, Kaider A, Klebermasz K, Fuiko R, et al: Intrauterine infection with *Ureaplasma* species is associated with adverse neuromotor outcome at 1 and 2 years adjusted age in preterm infants. *J Perinat Med* 2009;37:72–78.
- Normann E, Lacaze-Masmonteil T, Eaton F, Schwendimann L, Gressens P, Thebaud B: A novel mouse model of *Ureaplasma*-induced perinatal inflammation: effects on lung and brain injury. *Pediatr Res* 2009;65:430–436.
- Goldenberg RL, Andrews WW, Goepfert AR, Faye-Petersen O, Cliver SP, Carlo WA, et al: The Alabama Preterm Birth Study: umbilical cord blood *Ureaplasma urealyticum* and *Mycoplasma hominis* cultures in very preterm newborn infants. *Am J Obstet Gynecol* 2008;198:43.e1–e5.
- Kirchner L, Helmer H, Heinze G, Wald M, Brunbauer M, Weninger M, et al: Amnionitis with *Ureaplasma urealyticum* or other microbes leads to increased morbidity and prolonged hospitalization in very low birth weight infants. *Eur J Obstet Gynecol Reprod Biol* 2007;134:44–50.
- Jin C, Londono I, Mallard C, Lodygensky GA: New means to assess neonatal inflammatory brain injury. *J Neuroinflamm* 2015;12:180.
- Hagberg H, Dammann O, Mallard C, Leviton A: Preconditioning and the developing brain. *Semin Perinatol* 2004;28:389–395.
- Fleiss B, Tann CJ, Degos V, Sigaut S, Van Steenwinckel J, Schang AL, et al: Inflammation-induced sensitization of the brain in term infants. *Dev Med Child Neurol* 2015;57(suppl 3):17–28.
- Gao Q, Tang J, Chen J, Jiang L, Zhu X, Xu Z: Epigenetic code and potential epigenetic-based therapies against chronic diseases in developmental origins. *Drug Discov Today* 2014;19:1744–1750.
- Liu Y, Hoyo C, Murphy S, Huang Z, Overcash F, Thompson J, et al: DNA methylation at imprint regulatory regions in preterm birth and infection. *Am J Obstet Gynecol* 2013;208:395.e1–e7.
- Farooqui AA, Horrocks LA, Farooqui T: Glycerophospholipids in brain: their metabolism, incorporation into membranes, functions, and involvement in neurological disorders. *Chem Phys Lipids* 2000;106:1–29.

- 20 Adibhatla RM, Hatcher JF: Role of lipids in brain injury and diseases. *Future Lipidol* 2007; 2:403–422.
- 21 Miura Y, Payne MS, Keelan JA, Noe A, Carter S, Watts R, et al: Maternal intravenous treatment with either azithromycin or solithromycin clears *Ureaplasma parvum* from the amniotic fluid in an ovine model of intrauterine infection. *Antimicrob Agents Chemother* 2014; 58:5413–5420.
- 22 Gavilanes AW, Strackx E, Kramer BW, Gantert M, Van den Hove D, Steinbusch H, et al: Chorioamnionitis induced by intraamniotic lipopolysaccharide resulted in an interval-dependent increase in central nervous system injury in the fetal sheep. *Am J Obstet Gynecol* 2009; 200:437.e1–e8.
- 23 Hankin JA, Farias SE, Barkley RM, Heidenreich K, Frey LC, Hamazaki K, et al: MALDI mass spectrometric imaging of lipids in rat brain injury models. *J Am Soc Mass Spectrom* 2011;22: 1014–1021.
- 24 Jellema RK, Lima Passos V, Zwanenburg A, Ophelders DR, De Munter S, Vanderlocht J, et al: Cerebral inflammation and mobilization of the peripheral immune system following global hypoxia-ischemia in preterm sheep. *J Neuroinflamm* 2013;10:13.
- 25 Lardenoije R, van den Hove DLA, Vaessen TSJ, Iatrou A, Meuwissen KPV, van Hagen BTJ, et al: Epigenetic modifications in mouse cerebellar Purkinje cells: effects of aging, caloric restriction, and overexpression of superoxide dismutase 1 on 5-methylcytosine and 5-hydroxymethylcytosine. *Neurobiol Aging* 2015;36: 3079–3089.
- 26 Schwartz M, Baruch K: The resolution of neuroinflammation in neurodegeneration: leukocyte recruitment via the choroid plexus. *EMBO J* 2014;33:7–22.
- 27 Chen Y, Ozturk NC, Zhou FC: DNA methylation program in developing hippocampus and its alteration by alcohol. *PLoS One* 2013; 8:e60503.
- 28 Wang Z, Tang B, He Y, Jin P: DNA methylation dynamics in neurogenesis. *Epigenomics* 2016; 8:401–414.
- 29 Veloso A, Astigarraga E, Barreda-Gomez G, Manuel I, Ferrer I, Giralt MT, et al: Anatomical distribution of lipids in human brain cortex by imaging mass spectrometry. *J Am Soc Mass Spectrom* 2011;22:329–338.
- 30 Carter CL, McLeod CW, Bunch J: Imaging of phospholipids in formalin fixed rat brain sections by matrix assisted laser desorption/ionization mass spectrometry. *J Am Soc Mass Spectrom* 2011;22:1991–1998.
- 31 Angerer TB, Dowlatshahi Pour M, Malmberg P, Fletcher JS: Improved molecular imaging in rodent brain with time-of-flight-secondary ion mass spectrometry using gas cluster ion beams and reactive vapor exposure. *Anal Chem* 2015; 87:4305–4313.
- 32 Carson MJ, Thrash JC, Walter B: The cellular response in neuroinflammation: the role of leukocytes, microglia and astrocytes in neuronal death and survival. *Clin Neurosci Res* 2006;6: 237–245.
- 33 Burd I, Brown A, Gonzalez JM, Chai J, Elovitz MA: A mouse model of term chorioamnionitis: unraveling causes of adverse neurological outcomes. *Reprod Sci* 2011;18:900–907.
- 34 Yang Y, Higashimori H, Morel L: Developmental maturation of astrocytes and pathogenesis of neurodevelopmental disorders. *J Neurodev Disord* 2013;5:22.
- 35 Sharma R, Fischer MT, Bauer J, Felts PA, Smith KJ, Misu T, et al: Inflammation induced by innate immunity in the central nervous system leads to primary astrocyte dysfunction followed by demyelination. *Acta Neuropathol* 2010;120: 223–236.
- 36 Bonestroo HJ, Heijnen CJ, Groenendaal F, van Bel F, Nijboer CH: Development of cerebral gray and white matter injury and cerebral inflammation over time after inflammatory perinatal asphyxia. *Dev Neurosci* 2015;37: 78–94.
- 37 Volpe JJ: Neurobiology of periventricular leukomalacia in the premature infant. *Pediatr Res* 2001;50:553–562.
- 38 Penn AA, Gressens P, Fleiss B, Back SA, Gallo V: Controversies in preterm brain injury. *Neurobiol Dis* 2016;92:90–101.
- 39 Segovia KN, McClure M, Moravec M, Luo NL, Wan Y, Gong X, et al: Arrested oligodendrocyte lineage maturation in chronic perinatal white matter injury. *Ann Neurol* 2008;63:520–530.
- 40 Back SA, Miller SP: Brain injury in premature neonates: a primary cerebral dysmaturation disorder? *Ann Neurol* 2014;75:469–486.
- 41 Kamasawa N, Sik A, Morita M, Yasumura T, Davidson KG, Nagy JI, et al: Connexin-47 and connexin-32 in gap junctions of oligodendrocyte somata, myelin sheaths, paranodal loops and Schmidt-Lanterman incisures: implications for ionic homeostasis and potassium siphoning. *Neuroscience* 2005;136:65–86.
- 42 Ficiz G, Branco MR, Seisenberger S, Santos F, Krueger F, Hore TA, et al: Dynamic regulation of 5-hydroxymethylcytosine in mouse ES cells and during differentiation. *Nature* 2011;473: 398–402.
- 43 Fleiss B, Gressens P: Tertiary mechanisms of brain damage: a new hope for treatment of cerebral palsy? *Lancet Neurol* 2012;11:556–566.
- 44 Favrais G, van de Looij Y, Fleiss B, Ramanantsoa N, Bonnin P, Stoltenburg-Didinger G, et al: Systemic inflammation disrupts the developmental program of white matter. *Ann Neurol* 2011;70:550–565.
- 45 van Tilborg E, Heijnen CJ, Benders MJ, van Bel F, Fleiss B, Gressens P, et al: Impaired oligodendrocyte maturation in preterm infants: potential therapeutic targets. *Prog Neurobiol* 2016; 136:28–49.
- 46 Jirtle RL, Skinner MK: Environmental epigenomics and disease susceptibility. *Nat Rev Genet* 2007;8:253–262.
- 47 Bilbo SD, Schwarz JM: Early-life programming of later-life brain and behavior: a critical role for the immune system. *Front Behav Neurosci* 2009;3:14.
- 48 Hagberg H, Mallard C, Ferriero DM, Vannucci SJ, Levison SW, Vexler ZS, et al: The role of inflammation in perinatal brain injury. *Nat Rev Neurol* 2015;11:192–208.
- 49 Sun M, Song MM, Wei B, Gao Q, Li L, Yao B, et al: 5-Hydroxymethylcytosine-mediated alteration of transposon activity associated with the exposure to adverse in utero environments in human. *Hum Mol Genet* 2016;25:2208–2219.
- 50 Li S, Papale LA, Kintner DB, Sabat G, Barrett-Wilt GA, Cengiz P, et al: Hippocampal increase of 5-hmC in the glucocorticoid receptor gene following acute stress. *Behav Brain Res* 2015; 286:236–240.
- 51 Szulwach KE, Li X, Li Y, Song CX, Wu H, Dai Q, et al: 5-hmC-mediated epigenetic dynamics during postnatal neurodevelopment and aging. *Nat Neurosci* 2011;14:1607–1616.
- 52 Meyer U: Prenatal poly(i:C) exposure and other developmental immune activation models in rodent systems. *Biol Psychiatry* 2014;75:307–315.
- 53 Jin R, Yu S, Song Z, Quillin JW, Deas DP, Penninger JM, et al: Phosphoinositide 3-kinase-gamma expression is upregulated in brain microglia and contributes to ischemia-induced microglial activation in acute experimental stroke. *Biochem Biophys Res Commun* 2010; 399:458–464.
- 54 Eklind S, Mallard C, Arvidsson P, Hagberg H: Lipopolysaccharide induces both a primary and a secondary phase of sensitization in the developing rat brain. *Pediatr Res* 2005;58:112–116.
- 55 Mallard C, Hagberg H: Inflammation-induced preconditioning in the immature brain. *Semin Fetal Neonatal Med* 2007;12:280–286.
- 56 Cao M, Cortes M, Moore CS, Leong SY, Durosier LD, Burns P, et al: Fetal microglial phenotype in vitro carries memory of prior in vivo exposure to inflammation. *Front Cell Neurosci* 2015;9:294.
- 57 Gustavsson M, Anderson MF, Mallard C, Hagberg H: Hypoxic preconditioning confers long-term reduction of brain injury and improvement of neurological ability in immature rats. *Pediatr Res* 2005;57:305–309.