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# Transcriptomic regulations in oligodendroglial and microglial cells related to brain damage following fetal growth restriction

Abbreviated title: Fetal growth restriction and the developing brain

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## Abstract

Fetal growth restriction is a major complication of human pregnancy, frequently resulting from placental vascular diseases and prenatal malnutrition, and is associated with adverse neurocognitive outcomes throughout life. However, the mechanisms linking poor fetal growth and neurocognitive impairment are unclear. Here, we aimed to correlate changes in gene expression induced by fetal growth restriction in rats and abnormal cerebral white matter maturation, brain microstructure, and cortical connectivity in vivo. We investigated a model of fetal growth restriction induced by low-protein-diet malnutrition between embryonic day 0 and birth using an interdisciplinary approach combining advanced brain imaging, in vivo connectivity, microarray analysis of sorted oligodendroglial and microglial cells and histology.

We show that myelination and brain function are both significantly altered in our model of fetal growth restriction. These alterations, detected first in the white matter on magnetic resonance imaging significantly reduced cortical connectivity as assessed by ultrafast ultrasound imaging. Fetal growth retardation was found associated with white matter dysmaturation as shown by the immunohistochemical profiles and microarrays analyses. Strikingly, transcriptomic and gene network analyses reveal not only a myelination deficit in growth-restricted pups, but also the extensive deregulation of genes controlling neuroinflammation and the cell cycle in both oligodendrocytes and microglia.

Our findings shed new light on the cellular and gene regulatory mechanisms mediating brain structural and functional defects in malnutrition-induced fetal growth restriction, and suggest, for the first time, a neuroinflammatory basis for the poor neurocognitive outcome observed in growth-restricted human infants.

## Main points

Fetal growth restriction induced by low-protein-diet in rat significantly alters myelination, and brain microstructure and function.

Transcriptomic analyses reveal deregulation of genes controlling neuroinflammation in oligodendrocytes and microglia.

## Introduction

Fetal growth restriction (FGR), which has affected in 2010 more than 32 million newborns and 27% of all births in low and middle income countries, is one of the most common causes of disabilities in children and adults (Black et al., 2013). The rate of FGR is likely to rise further due to the increasing rates of multiple pregnancies and exposure to several FGR-inducing factors, including stress, nicotine and maternal malnutrition (Ergaz et al., 2005). Infants exposed to such adverse prenatal conditions are at high risk for neonatal death, neurological morbidities including cerebral palsy, learning disabilities, attention and executive deficits, behavioral disorders and autism spectrum disorders (Hack et al., 2002; Wiles et al., 2006; Jarvis et al., 2003; Abel et al., 2013). Given that the incidence of maternal malnutrition is higher than 10% in developing countries and accounts for a significant proportion of FGR and small-for-gestational-age infants at birth, it is crucial to better delineate this impact on brain maturation.

Besides infectious, genetic and immunological factors, FGR is most frequently associated with disorders of the placental vasculature, in which the placenta does not provide sufficient nutrients to the fetus to sustain normal growth. During pregnancy, maternal malnutrition or undernutrition could have a deleterious effect, mimicking placental insufficiency and inducing FGR (O'Keeffe et al., 2003; Hoyert et al., 2006; Guellec et al., 2011). Animal studies of FGR induced by protein deficiency or caloric restriction during gestation have revealed several effects on brain development and function, including structural and biochemical abnormalities in different brain regions such as the neocortex and the hippocampus (Garcia-Ruiz et al., 1993; Cintra et al., 1997; Lister et al., 2005; Tolcos et al., 2011; Leonhardt et al., 2002; Salas et al., 2012; Diaz-Cintra et al., 2007) leading to altered cognitive function and abnormal behavior (Lukoyanov and Andrade, 2000; Delcour et al., 2012). However, none of

these studies has pinpointed the transcriptomic or neuroanatomical mechanisms linking prenatal malnutrition to the subsequent brain phenotype, limiting effective neuroprotective strategies.

We asked the question whether transcriptomic deregulations of specific gene networks involved in brain development could account for abnormal brain structure and function related to FGR. Here, we demonstrate that FGR induced by a gestational low-protein diet (LPD) in rats has a severe impact on cerebral white matter maturation, brain microstructure and brain function/connectivity *in vivo*, and that these changes are correlated by the deregulation of gene expression, including that of mediators of neuroinflammation, in oligodendroglial and microglial cells.

## Materials and Methods

### Animals and diets

All experiments were carried out in compliance with INSERM ethical rules and approved by the institutional review board (Bichat-Robert Debré ethics committee, Paris, France, approval number 2010-13/676-0010).

Sprague-Dawley rats (Janvier SAS, Le Genest-St-Isle, France) were randomly divided into two groups according to their diet: a normal 23% protein diet or an isocaloric 9% protein diet (LPD), as previously described (Buffat et al., 2007; Zana-Taieb et al., 2015). Dams were fed the different diets from the day of conception until postnatal day (P) 0. Prenatal LPD-induced malnutrition resulted in a significant but transient restriction in body weight, although no postnatal mortality was observed (Supplemental Figure 1). All aspects of the study design and tools used for assessing brain structure and function were summarized in the Supplemental Figure 2.

### MRI assessment

*Ex-vivo* MRI experiments were performed at P10 and P21 in LPD and control groups (N=8 in each group from two litters) on an actively-shielded 9.4T/31cm magnet (Agilent) equipped with 12-cm gradient coils (400mT/m, 120µs) with a 2.5 mm diameter birdcage coil. A multi-b-value shell protocol was acquired using a spin-echo sequence with the following parameters: FOV ($mm^2$)/matrix size/number of slices/thickness (mm)=20×20/96×64/12/0.6 at P10 and 27×27/128×64/14/0.6 at P21, axial slices, 3 averages with TE/TR=45/2000ms. A total of 96 diffusion weighted images were acquired, 15 of them as $b_0$ reference images. The

remaining 81 were separated among 3 shells with the following distribution (# of directions/b-value in s/mm$^2$): 21/1750, 30/3400 and 30/5100. All 81 directions were non-collinear and were uniformly distributed in each shell. Acquired data were fitted using DTI-TK and the NODDI toolbox for DTI and NODDI, respectively (Zhang et al., 2006, 2012). Principal eigenvectors were tracked in a voxel zoomed into the primary somatosensory. Parameters derived from DTI were axial diffusivity (AD), radial diffusivity (RD), mean diffusivity (MD) also called apparent coefficient diffusion (ADC) and fractional anisotropy (FA). Parameters derived from NODDI were intracellular volume fraction (*ficvf*), isotropic volume fraction (*fiso*) and orientation dispersion index (ODI). The diffusion tensor (DT) was spatially normalized to the study-specific DT template using DTI-TK (Zhang et al., 2006). Six regions of interest (ROI) were drawn on the DT study-specific template and were then transformed back to the subject space in order to compute ROI-averaged estimates of DTI and NODDI maps. Six different brain regions were identified on the DT-template: primary motor cortex, primary and secondary somatosensory cortices, corpus callosum, external capsule and basal ganglia (Supplemental Figure 3).

**Functional ultrasound imaging of intrinsic brain connectivity in living rats**

Resting state fUS imaging was performed at P28 on 6 rats from two litters in each group. Animals were anesthetized and placed in a stereotaxic frame under constant body temperature (Kopf, Tujunga, California, USA). Ultrasound coupling gel was placed on the skull of the rat and the linear ultrasound probe (15MHz central frequency, 160 elements; Vermon, Tours, France) was positioned directly above the cranial surface. We used the central 128 elements of the transducer, which was connected to an ultrafast ultrasound scanner (Aixplorer, SuperSonic Imagine, Aix-en-Provence, France). The software-based

architecture of the scanner enables Matlab programming (MathWorks, Natick, Massachusetts, USA) of custom transmit/receive ultrasound sequences. Ultrafast Doppler relies on compounded plane-wave transmission (Montaldo et al., 2009), meaning that the brain is insonified using a succession of ultrasonic plane waves with different tilt angles. This enables high-quality images of the entire brain to be acquired at a frame-rate of 500Hz. The ultrasound sequence consisted of the transmission of five different tilted plane waves (-4°, -2°, 0°, +2°, +4° tilt angle) with a 2,500-Hz pulse repetition frequency (PRF). Backscattered echoes were added to produce enhanced ultrasound images at a 500-Hz frame rate over 400ms (200 images). This is enough to capture a few cardiac cycles, and consequently to obtain a mean estimation of the blood volume in every pixel during these 400ms. This ultrafast cineloop of 200 images is filtered using a dedicated spatiotemporal SVD-based filter to remove the tissue signal and keep only the blood signal. Then the average intensity (L1 norm) of this blood signal is computed over the cineloop, yielding an intensity image called a Power Doppler image, representative of the blood volume level in the brain section. This cycle was then repeated every 2 seconds during 10 minutes, yielding a stack of 300 Power Doppler images, which were subdivided into ROIs corresponding to known functional areas using Paxinos and Watson atlas (1998). For each ROI, the Power Doppler signal was averaged in space to give a 300-point time signal for each functional region: this signal exhibit fluctuations in the 0.05-0.5Hz range that are related to the local cerebral blood volume variations due to spontaneous neuronal activity (Osmanski et al., 2014). To study the intra- and inter-hemispheric functional connectivity, correlations between spontaneous activities in the different functional areas were measured. Consequently, the Pearson product-moment correlation coefficient was computed between all the ROI signals and the results gathered in a "correlation matrix". This connectivity matrix can be thought of a “fingerprint”

of the functional connectivity pattern of an animal under a given physiological condition. To test for statistical differences, the matrix Pearson coefficients were transformed using a Fisher transformation before running Student's T-tests.

## Immunohistochemistry

In each experimental group, we studied 8 to 10 pups from two litters on postnatal days P4, P10 and P21 depending on experiments.

Paraffin embedded or frozen sections were immunolabeled with primary antibodies listed in Table 1 and labeling visualized using either the streptavidin-biotin-peroxidase method and the chromogen diaminobenzidine, or secondary antibodies coupled to the green fluorescence marker Alexa488 (Lifetechnology) or the red fluorescent marker Cyanine 3 (Jackson Immunoresearch Laboratories, USA), as previously described (Baud et al., 2004).

## Neural tissue dissociation and magnetic-activated cell sorting

Brains were collected for cell dissociation (N=9-12 brains from 3 separate litters), and pre-oligodendrocyte/microglial cell enrichment using a magnetic-bead-coupled antibody extraction technique (MACS), as previously described and according to the manufacturer's protocol (Jungblut et al., 2012) (Miltenyi Biotec, Bergisch Gladbach, Germany). Microglial cells were sorted at P1, P2 and P4, and oligodendroglial cells were sorted at P4 based on cell-surface expression of CD11b and O4 respectively. In brief, after removing the cerebellum and olfactory bulbs, the brains for each age were pooled (N=3 brains for each point) and dissociated using the Neural Tissue Dissociation Kit containing papain. From the resulting brain homogenates, pre-oligodendrocyte/microglial cells were enriched by MACS using anti-

O4/CD11b microbeads, and after elution, isolated cells were centrifuged for 5 min at 600g and conserved at -80°C. The purity of the eluted O4-positive or CD11b-positive fraction was verified using qRT-PCR for glial fibrillary acid protein (GFAP) and neuronal nuclear antigen (NeuN), and revealed gene expression levels 95% lower than found in the primary cultures of astrocytes and neurons respectively.

**RNA preparation, cDNA synthesis, microarray hybridization and bioinformatics analysis**

Total RNA was extracted from purified cells of each animal (FGR or control) on P1, P2 and P4 for microglia and on P4 for oligodendrocytes, using RNeasy Mini RNA Extraction Kit (Qiagen, Chatsworth, CA, USA). Purified RNA was quantified using NanoDrop™ Spectrophotometer technology (Thermo Fischer Scientific, Wilmington, DE, USA). For microarray hybridization, labeled cRNA was generated using classical protocols for Affymetrix array hybridization. The labeled cRNAs were then hybridized to the RaGene-2_0-st microarray, according to Affymetrix protocols. 36685 probes are examined in this array. Three points per condition (oligodendroglial and microglial cells), each point pooled from 3 animals, were analyzed using Affymetrix array hybridization. Image analysis was performed using classic Affymetrix software to generate .cel files that were directly analyzed using GSEA developed by the Broad Institute (http://software.broadinstitute.org/gsea/index.jsp) (Subramanian et al., 2005), to which the reference RaGene-2_0-st, was downloaded. GSEA principle, developed by the Broad Institute, depends on the analysis of a complete list of ranked genes from a microarray, which are compared to predefined reference gene sets. Then the distribution of genes in a given gene set is analyzed and compared to a random distribution. An increased,

i.e. non-homogeneous, density of genes belonging to a given gene set at the right or left of the distribution indicates their non-random enrichment (quantified by an Enrichment Score, ES, itself normalized according to the number of genes in the gene set to yield a Normalized Enrichment Score or NES). For instance a NES of 2.5 indicates that genes of the gene set are 2.5 fold more frequently detected than expected by chance alone. The statistics are based upon a Monte-Carlo simulation, fixed here at 1000 iterations. Among the genes belonging to a given gene set, some are responsible for the enrichment (the curve reaching its extremity); these are the so-called core-genes. In our study, GSEA was first used to identify pathways against “hallmarks”, a collection of 50 massive gene sets that provide a clear and simplified vision of clustered deregulated genes in a given microarray. Following this preliminary analysis, the dataset was reanalyzed against the CGP database (Chemical and Genetic Perturbations), a rich database of the GSEA containing almost 3400 datasets, mainly from microarray experiments, thus allowing the analysis to be refined.

The microarrays were also analyzed as networks built using string (http://string-db.org/). First, the list of names was entered in the multiple names option, and a network was generated using medium confidence (0.40). The numerical data from the network were then exported as a tab delimited table to Cytoscape, which was used for further analysis and the search for the hub genes, meaning genes having more than a threshold number of connections with other genes. For this, the "Network Analysis" tool of Cytoscape was used to highlight.

To validate the microarray observations, qRT-PCR has been performed on ten genes in sorted oligodendrocytes (N=8-10 samples from two litters).

**RNA purification for real-time PCR**

For qRT-PCR quantification, brains were immediately snap-frozen in liquid nitrogen and stored at -80°C from 8 to 10 pups from two litters. Total RNA from the samples was extracted with the RNeasy mini kit according to the manufacturer's instructions (Qiagen, Courtaboeuf, France). RNA quality and concentration were assessed by spectrophotometry using a NanoDrop™ apparatus (Thermoscientific, Wilmington, DE, USA). One µg of total RNA was subjected to reverse transcription using the Iscript™ cDNA synthesis kit (Bio-Rad, Marnes-la-Coquette, France). qRT-PCR was performed in duplicate for each sample on a CFX384 Real Time System (Bio-Rad), using SYBR Green Supermix (Bio-Rad) for 40 cycles (Pham et al., 2015). Amplification specificity was assessed by melting curve analysis. Primers were designed using Primer3 software and manufactured by Eurofins Genomics (Ebersberg, Germany). Primer sequences are summarized in Table 2. The expression of genes of interest was calculated relative to the expression of the reference gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Analyses were performed using Bio-Rad CFX Manager 3.0.

## Statistical analysis

All data are reported as means±S.E.M or S.D. Statistical analysis of all data was performed using GraphPad PRISM version 5.0 (GraphPad Software, San Diego, CA). The normal distribution of all results was assessed with the D'Agostino and Pearson omnibus normality test. To compare two groups with a normal distribution, a t-test was performed whereas in the absence of a normal distribution, a Mann–Whitney U test was used. For more than 2 groups, a one-way ANOVA followed by a Dunnett's post hoc test was performed due to non-Gaussian distributions.

## Results

### Changes in postnatal brain microstructure associated with LPD-induced FGR

To assess changes in brain structure associated with FGR, we acquired advanced MRI sequences *ex vivo* from the brains from control and LPD-exposed animals at P10 and P21 (Fig. 1A). Axial diffusivity (AD), radial diffusivity (RD), mean diffusivity (MD) and fractional anisotropy (FA) were measured using Diffusion Tensor Imaging (DTI). The principal eigenvectors in a voxel zoomed into the primary somatosensory cortex, corresponding to the principal diffusion direction of water in the cortex, are shown. Derived from Neurite Orientation Dispersion and Density Imaging (NODDI), the intraneurite volume fraction (*ficvf*), isotropic volume fraction (*fiso*) and orientation dispersion index (ODI) were also computed.

At P10, compared to control animals, white matter fibers in the corpus callosum and external capsule of LPD animals presented a significantly decreased AD and increased ODI (Fig. 1B). The FA was also significantly decreased ($p<0.001$). The *ficvf* was significantly increased in the external capsule. No significant change was observed in the motor or somatosensory cortex, or in the basal ganglia.

At P21, significant changes were no longer observed in corpus callosum and the *ficvf* was lower in the external capsule of LPD animals as compared to controls (Fig. 1C). Significant changes mainly occurred in the grey matter (both motor and somatosensory cortices), with increased AD, FA and *fiso* as well as decreased ODI in LPD animals.

In order to determine whether these changes in MRI parameters, in particular of the white matter, were reflected by changes in brain histology, we next focused on the myelination process. While there was no detectable difference in the gross morphological aspect of the brain in the two groups, immunohistochemistry for myelin basic protein (MBP) at P10 and

P21 revealed a significant decrease in the myelin content of the external capsule in LPD-exposed animals compared to controls (Figs. 2A, 2B). In accordance with these results, the density of mature myelinating oligodendrocytes (APC-positive cells; Fig. 2C) was decreased in the external capsule and cingulate white matter in the LPD-exposed group both at P10 and P21. The density of immature oligodendrocytes (NG2+) was unaffected between the two groups at P10 (Figs. 2D, 2E). As a result, the total oligodendroglial population (olig2-positive cells; Figs. 2D, 2E) was significantly decreased within the lateral white matter.

**Effect of LPD-induced FGR on intrinsic cortical connectivity in the living rat brain**

In order to determine whether the changes in brain microstructure observed above had functional consequences, we next decided to examine brain connectivity in animals exposed to LPD during development. Ultrafast Doppler allows functional ultrasound (fUS) imaging through neurovascular coupling, as cerebral blood volume variations can be mapped with very high sensitivity (Mace et al., 2011). The degree of correlation between spontaneous hemodynamic fluctuations of cerebral blood flow in different brain regions was measured here to assess connectivity between brain regions in a reproducible manner *in vivo* (Fig. 3A). We compared the pattern of resting state cerebral connectivity between live LPD and control animals at P28. Interhemispheric somatosensory connectivity was significantly depressed in LPD animals in the medial and lateral parietal association cortices. Moreover, intrahemispheric connectivity was found severely altered in several areas within the somatosensory cortex in LPD-exposed animals (Figs. 3B-D). Investigating the molecular basis of the brain functional changes described above by analyzing MACS-sorted oligodendroglial cells at the earlier time-point P4, the expression of several genes involved in axonal guidance was significantly deregulated, as shown in the volcano plot presented in Supplemental

Figure 4. In particular, an imbalance in the expression of several semaphorin genes (Sema3e, Sema4d, Sema4c, Sema3b, and Sema3a) and their plexin receptors (Plxna3, Plxnc1 and Plxna2) could account for the impaired cortical microstructure and connectivity observed above.

## Impact of prenatal LPD on gene expression changes in oligodendrocytes and microglial cells

Considering the key developmental role of oligodendrocytes and microglia in myelination as well as axonal connectivity and synaptogenesis, we used cell sorting followed by microarray analysis at the early developmental stage (P4) in order to analyze gene expression changes specifically in these two cell types (Figs. 4A, 4B). This cell-specific analysis revealed significant changes in 501 genes for oligodendrocytes and 677 genes for microglia. Gene Set Enrichment Analysis (GSEA; http://software.broadinstitute.org/gsea/index.jsp) was used to match these genes to gene sets and then to the CGP (Chemical and Genetic Perturbations) database. Using a 0.25 False Discovery Rate (FDR) as the threshold, the analysis of genes whose expression was altered above revealed an enrichment of several "hallmark" gene sets (Figs. 4C, 4D). Gene sets related to cell differentiation, cell proliferation, apoptosis and the inflammatory response were among the most deregulated. In the CGP database, GSEA revealed 1022 gene sets deregulated in oligodendrocytes, with a huge predominance of gene sets composed of up-regulated genes (1010 up-regulated *vs* 12 down-regulated), and 1143 pathways deregulated in microglial cells (607 up- and 536 down-regulated). To validate the microarray observations, qRT-PCR has been performed on ten genes in sorted oligodendrocytes. The graph presents the correlation between the two methods of gene

expression analysis with a $r^2$=0.9554, suggesting a high level of reliability of the microarray data (Figs. 4E, 4F).

**Genomic basis of deregulation of oligodendroglial lineage maturation induced by prenatal LPD exposure**

We next explored oligodendroglial lineage differentiation/maturation, neuroinflammation and cell death, all mechanisms known to play a role in abnormal myelination in the developing brain. Among the gene sets down-regulated in oligodendrocytes from LPD-exposed animals, GSEA analysis revealed a highly significant enrichment of the "LEIN_OLIGODENDROCYTE_MARKERS" gene set (http://software.broadinstitute.org/gsea/msigdb/geneset_page.jsp?geneSetName=LEIN_OLIGODENDROCYTE_MARKERS; Fig. 5A), including a number of genes encoding myelin-related proteins such as MBP, MAG, MOBP and CNP (Fig. 5B), in accordance with the immunohistological and MRI results. Indeed, MBP immunoreactivity (as shown in Figure 2A) and CNP staining both confirmed microarrays analysis and genes downregulation. Among the top-ranking gene sets whose expression changed significantly in oligodendrocytes sorted from LPD animals at P4, GSEA also revealed a highly significant enrichment of the "GOBERT_CORE_OLIGODENDROCYTE_DIFFERENTIATION" gene set (http://software.broadinstitute.org/gsea/msigdb/cards/GOBERT_CORE_OLIGODENDROCYTE_DIFFERENTIATION), defined by their deregulation in the Oli-neo oligodendrocyte precursor cell line after treatment with factors known to promote oligodendrocyte maturation (Fig. 5C). We noted a correlation with both the up-regulated and the down-regulated gene groups identified in the original GOBERT study (179 genes were in the 'Gobert down' group and 219 genes were in the 'Gobert up' group). Surprisingly, the expression of genes of this set

appears to be up-regulated in LPD (Fig. 5D), suggesting that there may be a compensatory triggering of the oligodendrocyte differentiation/maturation process when the deleterious environment (prenatal LPD) is finally interrupted at birth.

Next, we analyzed the transcriptome data for these genes using the network analysis tool Cytoscape (http://www.cytoscape.org/), after generating a network using String (http://string-db.org/). The network obtained was then simplified by selecting “hub” genes, defined as genes known from the literature to be connected to several others. Among "GOBERT_CORE_OLIGODENDROCYTE_DIFFERENTIATION" hub genes (Figs. 5E, 5F), connectivity was much higher in the Gobert-up than in the Gobert-down gene set, with more than 30 connections per hub gene for the up-regulated group and more than 10 connections for the down-regulated group. Interestingly, the genes with the highest number of connections were those involved in the cell cycle, i.e. the DNA topoisomerase TOP2A and CDK1, involved in G1/S and G2/M phase transitions of the eukaryotic cell cycle, in oligodendroglial sorted cells from animals subjected to prenatal LPD.

**Up-regulation of inflammation-related pathways following prenatal LPD exposure in sorted oligodendrocytes and microglial cells**

Some key factors involved in the balance between proliferation and differentiation in pre-oligodendrocytes (including PDGFRα (induction ratio LPD/Ctl=1.78, p=0.001) and Olig1 (induction ratio LPD/Ctl=1.47, p=0.003)) suggest an arrest of the developmental program of the oligodendroglial lineage in response to prenatal LPD. PDGFRα gene expression has been assessed in oligodendroglial cells sorted using O4 markers, at a later stage compared to NG2 or OPCs that could explain why NG2-positive cells density has been found consistently increased.

Because neuroinflammation has been recognized as a major risk factor associated with the disruption of oligodendroglial maturation (Favrais et al., 2011), we then assessed if prenatal LPD induced significant changes in inflammation-related pathways. Although the density of Tomatolectin- and Mrc1-positive microglial cells in the white matter did not differ between groups at P4 and P10 (Fig. 6A), we further studied *in vivo* transcriptional changes in both sorted oligodendrocytes and microglial cells.

We first assessed the expression of genes related to microglial phenotypes (cytotoxic, repair/regeneration, immunomodulation) in microglial cells sorted at P1, P2 and P4. Microglia were activated at P1 in a non-specific manner, with an increase in all markers in LPD animals compared to controls (Fig. 6B). This activated state was transient, as microglia displayed a similar phenotype profile between groups at P2 and P4. Next, to better delineate the genomic basis of this inflammatory activation in animals subjected to LPD before birth, we performed microarray analysis of deregulated inflammatory pathways. In microglial cells, 4 gene sets with highly significant enrichment were observed: 1) SEKI_INFLAMMATORY_RESPONSE_LPS_UP; 2) PHONG_TNF_TARGET_UP; 3) CROONQUIST_IL6_DEPRIVATION_DN and LEE_EARLY_T_LYMPHOCYTE_UP (http://software.broadinstitute.org/gsea/index.jsp). Microarray analysis of two major inflammatory pathways ("inflammation" and "TNF-alpha") revealed 55 genes up-regulated in oligodendroglial cells and 50 in microglial cells sorted at P4. Among them, we found 8 common genes that were significantly up-regulated both in pre-oligodendrocytes and microglial cells and in both pathways, all involved in the induction or control of brain inflammation (Figs. 6C, 6D).

Findings regarding oligodendroglial lineage reported in Figure 2D suggested an imbalance between cell proliferation and cell death of oligodendroglial precursors following prenatal

LPD exposure. As mentioned above, microarray analysis also revealed an enrichment of the "APOPTOSIS" hallmark gene set. This was confirmed by a highly significantly increase in interactions between genes involved in apoptosis in oligodendrocytes at P4 (Fig. 7A). Using a network based on core apoptosis genes that contribute to the normalized enrichment score (NES), an indicator of the interrelatedness of genes that are co-regulated in the same direction, we found almost three times more interactions as expected due to chance alone, which is highly significant (p=0, Fig. 7B).

Finally, network analysis, based on the connectivity of the genes, also emphasized the central role of c-Jun, Cdkn1a and Ccnd1, genes essential for cell division and proliferation, in modulating apoptosis. Additionally, other factors involved in regulating oxidative stress (Sod1, Hmox1, Gpx3, Gpx4) and inflammation (TNF, Rela, IL1a) as well as chromatin remodeling (Gadd45b, Top2A, Dnaja1) (Fig. 7C), could be involved in oligodendroglial cell death or dysmaturation, and subsequent deficits in connectivity observed following prenatal LPD exposure.

## Discussion

In this study, we demonstrate a substantial impact of maternal protein malnutrition on brain structure and function using a rodent model of gestational low protein diet (LPD). The main phenotypic effects of LPD reported here are a deficit of myelination and a disturbance of oligodendroglial maturation. Impaired myelination is a common feature of growth restriction in different animal models of placental vascular disease induced by unilateral or bilateral ligation of the uterine arteries (Olivier et al., 2005; Reid et al., 2012), oxidative stress induced by chronic gestational hypoxia (Pham et al., 2015), maternal undernutrition (Piorkowska et al., 2014) and prenatal exposure to toxins such as alcohol (Alfonso-Loeches and Guerri, 2011). Three main mechanisms have been shown to lead to white matter injury: arrested maturation of the oligodendroglial lineage, increased apoptosis of immature oligodendrocytes, and decreased proliferation of oligodendrocyte progenitors. Other rodent models of FGR based on hypoxia or uterine artery ligation demonstrate delayed oligodendrocyte differentiation, astrogliosis and microglial activation (Baud et al., 2004; Olivier et al., 2005; Reid et al., 2012). In the present FGR model of protein restriction, disrupted maturation occurred in combination with a pro-inflammatory state and activated apoptotic genes networks.

Neuroinflammation is commonly associated with delayed or arrested maturation of the developing white matter (Favrais et al., 2011). In our model, maternal protein restriction induced transient microglial activation following birth. The transcriptomic study performed in sorted oligodendrocytes and microglia demonstrated an up-regulation of pro-inflammatory pathways.

MRI scans evidenced microstructural changes at earlier time points in the white matter and later in the grey matter. At P10, the decreased AD and FA, and increased ODI within the

developing white matter indicate axonal degeneration and subsequent defects in myelination or fiber compaction (Lodygensky et al., 2010). At this stage, myelination has just begun, meaning that changes in DTI-derived parameters depicted diffuse structural defects rather than specific myelination defects (van de Looij et al., 2012). Later, at P21, more significant changes occurred in the grey matter (both motor and somatosensory cortices), with a more organized cortical structure that could correspond to a simplification and/or retardation of dendritic arborization (van Velthoven et al., 2012). This is very consistent with previous MRI studies performed in humans. Growth-restricted neonates without significant brain lesions *in utero* display delayed myelination and decreased grey matter volume (Mace et al., 2011; Padilla et al., 2011). In coherence with these observations, genes involved in axonal guidance were among the most deregulated in oligodendrocytes from LPD animals. The expression of the soluble Sema3a, which acts as a repulsive factor on neuronal cells, was strikingly induced in oligodendrocytes after LPD while Sema3E, known to promote axonal growth in the developing brain, was severely repressed (Koncina et al., 2007; Bernard et al., 2012).

These microstructural changes in LPD brains were associated with impaired cerebral connectivity at P28, as revealed by ultrafast Doppler. While the lack of evidence linking this new tool with neurobehavioral testing batteries is a limitation of the study, the correlation between functional brain connectivity and behavior and cognition has been already reported (Gonzalez-Castillo et al., 2015; Smith, 2015). Altered patterns of brain connectivity have been correlated with motor, cognitive and behavioral outcomes in children born small for gestational age as compared to children showing age-appropriate growth, using EEG and MRI diffusion tractography techniques (Batalle et al., 2012; Eixarch et al., 2015). To date, only one study has assessed regional brain connectivity in an experimental model of FGR (Illa

et al., 2013). Using MRI diffusion eigenvectors, the authors revealed microstructural changes in grey matter structure, decreased connectivity and a higher degree of anxiety, attention and memory problems in a vascular model of FGR in rabbits. In contrast to functional MRI of brain connectivity *in vivo,* neurovascular coupling imaged by ultrafast Doppler allows reproducible comparisons between experimental groups (Osmanski et al., 2014). Microstructural changes in the grey matter rather than the white matter may be the major factor underlying impaired cerebral connectivity, as suggested by the expression profiles of gene sets involved in synaptogenesis and axonogenesis.

One strength of this study is that we could correlate gene expression regulation in response to LPD exposure during fetal life with phenotypic findings in the developing brain. We analyzed these parameters in sorted oligodendrocytes and microglial cells, two key players in perinatal brain injury. The intrauterine environment could influence fetal and neonatal brain development through a wider transcriptomic impact and epigenetic changes, thought to interfere with normal brain development and maturation and to account for the fetal programming of metabolic diseases (Hochberg et al., 2011; Kalhan and Marczewski, 2012; Lee, 2015; Cottrell et al., 2012; Moisiadis and Matthews, 2014). To our knowledge, our study is the first to do systematic network analysis and investigate gene expression alterations induced by prenatal malnutrition in the developing brain.

In conclusion, this study underscores the deleterious impact of maternal protein restriction during gestation on the developing brain, even though this restriction is transient and followed by a normal diet after birth. Our data creates new prospects for neuroprotective strategies based on gene networks deregulated in oligodendrocytes and microglia.

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## Figure legends

**Figure 1: Microstructural changes following LPD exposure assessed using advanced MRI brain imaging.**

(A) Average DTI (AD: Axial diffusivity, RD: radial diffusivity MD: mean diffusivity, FA: fractional anisotropy, E. Vec: principal eigenvectors in a voxel zoomed into the primary somatosensory cortex and direction encoded color (DEC)-derived maps as well as NODDI (*ficvf*: intracellular volume fraction, *fiso*: isotropic volume fraction and ODI: orientation dispersion index)-derived maps of Control (Ctl) and LPD pups at P10 and P21 (N=8 in each group).

(B, C) Mean values (SD) of the corresponding DTI and NODDI derived parameters at P10 (B) and P21 (C) in the main ROIs.

AD, RD and MD are expressed in $\times 10^{-4} mm^2.s^{-1}$, FA, *ficvf*, *fiso* and ODI are indices between zero and one. *: p<0.05; **: p<0.01; ***: p< 0.001, using a Mann–Whitney U test.

**Figure 2**: **Effect of LPD exposure on *in vivo* myelination**

(A) Representative photomicrographs of MBP-positive fibers in the lateral white matter in P10 and P21 rat pups subjected to LPD before birth (right), compared to controls (left). Bars = 300 µm. Co: cortex; LV: lateral ventricle; LWM: lateral white matter; Str: striatum.

(B, C) Quantitative analysis of the density of MBP-positive fibers (B) and APC-positive cells (C) in the cingulate (CWM) and lateral white matter (LWM) in P10 and P21 rat pups from LPD and the Control (Ctl) groups. LWM areas analyzed are indicated by boxes in A.

(D) Quantitative analysis of the density of Olig2-, NG2- and APC-positive cells within the lateral white matter in LPD-exposed P10 animals compared to controls. Data show that the

density of both total (Olig2) and mature (APC) oligodendroglial cells was decreased in the white matter of animals exposed to LPD, while the density of NG2-positive cells was found to be unaffected, suggesting that oligodendroglial cell death could be increased following prenatal LPD exposure.

(E) Photomicrographs show representative immunohistochemical labeling for Olig2-, NG2- and APC-positive cells within the lateral white matter. Arrows indicate NG2-positive immature oligodendrocytes and Olig2-positive nuclei, and asterisks indicate mature APC-positive oligodendrocytes. Bars = 20 µm.

*: $p<0.05$, **: $p<0.01$, for comparisons between the Ctl and LPD groups, using a one-tailed t-test.

**Figure 3: Effect of LPD-induced FGR on the functional connectivity of the brain, measured by fUS imaging during the resting state.**

(A) Setup containing a stereotactic frame and a dedicated 15 MHz ultrasonic probe mounted on 3D translation motors. The acquisition sequence consists of 10-minute fUS recordings.

(B) Image of cerebral blood volume assessed in a 2D slice by fUS imaging and superimposed on the corresponding functional regions extracted from the Paxinos Atlas.

(C) Average functional connectivity map in the control and LPD group (N=6, each).

(D) Loss of interhemispheric functional connectivity in the MPtA cortex and in several intra-hemispheric areas in LPD rats compared to controls. 1, 10 = S1BF, primary somatosensory cortex Barrel field; 2, 9 = LPtA, lateral parietal association cortex; 3, 8 = MPtA, medial parietal association cortex; 4, 7 = RSD, retrosplenial dysgranular cortex; 5, 6 = RSGc, retrosplenial granular cortex, c region. For statistical analyses, matrix Pearson coefficients were transformed using a Fisher transformation before Student's T-tests.

**Figure 4**: **Alterations to the transcriptome in sorted glial cells in response to prenatal LPD exposure.**

A, B: Microarray analysis of pre-oligodendrocytes (A) and microglial cells (B) sorted using magnetic-bead-coupled antibodies (MACS) at P4. Threshold for significance was at least 1.5-fold changes. Among genes with significant changes (in green), the graphs represent the number of genes induced (red) or repressed (blue). The intensity of gene deregulation is shown as the degree of induction/repression 1.5-fold or above relative to control rats: > 3-fold (/3, x3), from 3-fold to 2-fold (/2, x2); and from 2-fold to 1.5-fold (/1.5, x1.5).

C, D: Graphical representation of the significant hallmark gene sets uncovered by GSEA in purified oligodendrocytes (C) and microglial cells (D). The y-axis represents the absolute value of the Normalized Enrichment Score (NES). While most hallmark gene sets are composed of up-regulated genes in oligodendrocytes, the deregulation ratio is much more balanced in microglial cells. In both cell types, several hallmark gene sets related to inflammation are composed principally of up-regulated genes.

E, F: qRT–PCR analysis of cDNA prepared from rat pre-oligodendrocytes sorted at P4 for external validation of microarray analysis. A comparison of induction ratios between qRT–PCR and microarray data was performed for ten induced or repressed genes listed in (E), with a $r^2$ estimated at 0.9554, indicating that the two methods are in good agreement (F).

**Figure 5: Microarray analysis of oligodendroglial maturation in MACS-sorted oligodendrocytes.**

A, B: Gene Set Enrichment Analysis demonstrating a highly significant enrichment of the gene set «LEIN_OLIGODENDROCYTE_MARKERS» (A) in oligodendrocytes from P4 rat pups

prenatally subjected to LPD. Many genes included in this gene set and significantly down-regulated encoded crucial myelin-related proteins including notably MBP, MAG, MOBP and CNP (B). Each column in (B) represents one microarray analysis including 3 sorted cell samples each.

C, D: Significant enrichment of this gene set in oligodendrocyte sorted at P4 (C) and list of genes most up-regulated in LPD-exposed animals compared to controls (D). Each column in (D) represents one microarray analysis including 3 sorted cell samples each.

E, F: Gobert oligodendrocyte gene networks: 179 down-regulated genes and "hub" genes with > 10 connections (E) and 219 up-regulated genes and "hub" genes with > 30 connections (F). These “hub” networks were obtained using the Network Analysis tool of Cytoscape (http://www.cytoscape.org/), after generating a network using String (http://string-db.org/).

**Figure 6**: **Microglial cell activation and neuroinflammation following LPD exposure in the neonatal brain and MACS-sorted microglia and oligodendrocytes.**

(A) Quantitative analysis of the density of Tomatolectin- and Mrc1-positive microglial cells in the cingulate white matter in P4 and P10 rat pups from the LPD and Control (Ctl) groups, demonstrating no significant difference.

(B) Gene expression levels of various microglial phenotypic marker in microglial cells sorted at P1, P2 and P4 from rat pups subjected to LPD before birth, compared to controls. IL6, TNF$\alpha$ and Cox2 are cytotoxic markers, IL1RN and IL4RA are immunomodulatory markers, and Mrc1 is a repair/regeneration marker. **: $p<0.01$ and ***: $p<0.001$ for comparisons between the two groups indicated, using a one-tailed t-test.

(C, D) Microarray analysis for two inflammatory pathways (“inflammation” and “TNF-alpha”) deregulated in microglial and oligodendroglial cells sorted at P4. Eight genes, listed in (D), all involved in the induction or control of brain inflammation, were found to be significantly up-regulated in both cell types.

**Figure 7: Apoptotic oligodendroglial cell death associated with prenatal LPD prenatal exposure.**

A: GSEA analysis of genes modified in oligodendrocytes analyzed against the 50 hallmark gene sets. The apoptosis hallmark set appears to be a prominent one among the significant hallmark sets detected by this non-supervised approach. The Normalized Enrichment Score was estimated at 1.86, meaning that up-regulated genes were present 1.86 times more in the oligodendroglial dataset than expected by chance. The corrected False Discovery Rate (FWER) was highly significant (p=0.003). The highly dense genes on the left contributed to the increase of the NES.

B: The apoptosis genes that contributed to the NES were used to build a network using the String database, with a confidence threshold fixed at 0.4 (medium). There are almost 3 times more relations than expected by chance, with a p value close to 0. The mathematical parameters of this network were collected as a text file.

C: The network of (B) was analyzed using Cytoscape, and this basic analysis was followed by a Network analysis. In this simplified network, the color is linked to the connectivity (the effect that a given node gene will have on the network if withdrawn) of the genes. The size of the dots is proportional to the number of connections.

**Supplemental figure legends**

**Supplemental Figure 1:** Time course of body weight gain in animals subjected to LPD compared to controls during the first 3 weeks of postnatal life.

**Supplemental Figure 2:** Overall study design and methods used for brain assessment and molecular analyses.

**Supplemental Figure 3: Anatomical location of regions of interest for quantitative analysis of MRI brain imaging**

Region of interests (ROIs) displayed on the averaged control P10 diffusion-encoded map (Cx_M1: primary motor cortex Cx_S1 and Cx_S2: primary and secondary somatosensory cortices, EC: external capsule, CC: corpus callosum and BG: basal ganglia). ROIs in the P21 diffusion encoded map were similarly located at several rostrocaudal levels.

**Supplemental Figure 4: Effect of LPD-induced FGR on the regulation of genes involved in axon guidance and synaptogenesis.**

Effect of LPD-induced FGR on the regulation in gene sets involved in axon guidance and synaptogenesis in P4 oligodendrocytes. Volcano Plot with the x-axis indicating the level of gene induction/repression, versus the p-value on the y-axis. Significant induction ratios (LPD/Ctl) of genes encoding axon-guidance- and synaptogenesis-related proteins were listed in the table.