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Effect of Lipopolysaccharide on Global Gene Expression in the Immature Rat Brain

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ABSTRACT: To improve the understanding of the molecular mechanisms whereby lipopolysaccharide (LPS) affects the immature brain, global gene expression following LPS exposure was investigated in neonatal rats. Brains ($n = 5$/time point) were sampled 2, 6, and 72 h after LPS and compared with age-matched controls. The mRNA from each brain was analyzed separately on Affymextrix GeneChip Rat Expression Set 230. The number of genes regulated after LPS were 847 at 2 h, 1564 at 6 h, and 1546 genes at 72 h. Gene ontology analysis demonstrated that, at both 2 and 6 h after LPS, genes associated with protein metabolism, response to external stimuli and stress (immune and inflammatory response, chemotaxis) and cell death were overrepresented. At 72 h, the most strongly regulated genes belonged to secretion of neurotransmitters, transport, synaptic transmission, cell migration, and neurogenesis. Several pathways associated with cell death/survival were identified (caspase–tumor necrosis factor α [TNF-α]-, p53-, and Akt/phosphatidylinositol-3-kinase (PI3 K)–dependent mechanisms). Caspase-3 activity increased and phosphorylation of Akt decreased 8 h after peripheral LPS exposure. These results show a complex cerebral response to peripheral LPS exposure. In addition to the inflammatory response, a significant number of cell death–associated genes were identified, which may contribute to increased vulnerability of the immature brain to hypoxia-ischemia (HI) following LPS exposure. **(*Pediatr Res* 60: 161–168, 2006)**

Inflammatory processes are recognized key mediators of perinatal brain injury and intrauterine infection/inflammation is associated with an increased risk of cerebral white matter damage and subsequent cerebral palsy in the offspring. A number of experimental studies have shown that exposure to LPS during pregnancy induces cerebral white matter injury in the fetus or newborn animal (1–3). In addition, epidemiologic evidence suggests that a combination of adverse factors may have additive effects and further increase the risk of cerebral palsy in children. In particular, it was shown that infection during pregnancy in combination with potentially asphyxiating conditions at birth markedly increased the risk of spastic cerebral palsy in infants of normal birth weight (4). Studies in neonatal rats support these findings, and we and others have demonstrated that peripherally administered LPS aggravates HI brain injury (5,6). These studies suggest that LPS induces mediators in the brain that by themselves may be damaging or primes the brain to become more sensitive to later events. We have previously shown that the sensitizing effect of LPS on HI does not relate to alterations in cerebral blood flow or temperature (5), whereas LPS-induced tolerance is associated with the induction of glucocorticoids (7).

Systemically administered LPS induces complex biochemical alterations in the adult CNS; however, much less is known of the response in the immature brain, and evidence suggests that the immature brain may be more susceptible to inflammatory mediators than the adult brain (8). Maternally administered LPS induces increased expression of interleukin (IL)-1β and TNF-α mRNA in the fetal brain (1). We have demonstrated regulation of the LPS receptors toll-like receptor 4 (TLR-4) and CD14 in the neonatal brain after LPS exposure (5). However, very little is known of the global changes in the immature brain following a systemic endotoxin challenge.

A relatively new approach to examine global changes in gene expression is the use of microarrays, with analysis of several thousands of transcripts in a single sample. We have previously shown a very good correlation between this method and more conventional reverse transcriptase polymerase chain reaction (PCR) techniques (9,10). One study, using microarray technology, examined the short-term gene expression in the hypothalamus of adult mice after LPS exposure (11). However, there are no studies investigating the global gene expression in the immature brain following LPS expo-

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Abbreviations: FAIM2, fas apoptotic inhibitory molecule 2; **HI,** hypoxia-ischemia; **LPS,** lipopolysaccharide; **NF-κB,** nuclear factor-κB; **pAkt,** phosphorylated Akt; **PHLDA1,** pleckstrin homology-like domain, member 1; **PI3 K,** phosphatidylinositol-3-kinase; **PKB,** protein kinase B; **PND,** postnatal day; **RMA,** robust multiarray average

sure. The purpose of this study was therefore to characterize the global gene expression following exposure to LPS in a rat model approximately equivalent to a term baby. The specific aim was to identify genes that may be involved in inflammatory and cell death mechanisms in the brain because we hypothesized that these will be important and could contribute to increased sensitivity to further insults.

MATERIALS AND METHODS

Animal studies. Wistar rat pups from Mollegaard Breeding and Research Centre A/S, Skensved, Denmark were used in all experiments. The Animal Ethics Committee of the University of Göteborg approved all experiments. Animals from each litter were randomly assigned to the different groups below. LPS (0.3 mg/kg, Sigma Chemical Co. LPS O55:B5 in 0.9% NaCl, i.p.) was given to rat pups at postnatal day (PND) seven.

Gene analysis. LPS-treated pups ($n = 5$/time point) were killed 2, 6, and 72 h after the injection. Naïve control rats were killed at postnatal day (PND) 7 ($n = 5$) and PND 10 ($n = 5$). Animals were decapitated, and the brains, excluding the brainstem and cerebellum, were quickly removed and frozen on dry ice. Five micrograms of total RNA was extracted using Rneasy Mini Kit (Qiagen Inc.)

The mRNA from each brain was analyzed separately on Affymextrix GeneChip Rat Expression Set 230, which contains probe sets for detection of 15.923 transcripts (Swegene, Lund, Sweden). Data processing was performed on Affymetrix .CEL files using the robust multiarray average (RMA) algorithm (12), which performs three distinct operations: global background normalization, across-array normalization, and $\log_2$ transformation of perfect match values (http://stat-www.berkeley.edu/users/bolstad/RMAExpress/RMAExpress.html). All 25 arrays were normalized together as one experiment to reduce nonbiological variability. The RMA analysis, data management, statistical analysis, and gene ontology were performed using the Web-based GeneSifter software (http://login.genesifter.net/). In addition to PubMed (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?CMD = search&DB = pubmed), the database http://genome-www5.stanford.edu/cgi-bin/source/sourceSearch was used to obtain information about specific genes of interest.

Real-time PCR (RT-PCR). To confirm the microarray results, RT-PCR (Light Cycler, Roche) was performed on three selected genes 6 h after LPS administration. First-strand cDNA synthesis was performed using Superscript RNase H^- reverse transcriptase kit (Life Technologies, Inc.), random hexamer primers, and dNTP (dATP, dCTP, dGTP, and dTTP; Roche Molecular Biochemicals) from the same total RNA that had been used for microarray analysis.

Each PCR (20 μL) contained 1/80 cDNA, 2 or 3 μM $MgCl_2$ depending on the optimal concentration for each primer pair, 0.5 μM forward and reverse primers, and 2 μL of LightCycler–FastStart DNA Master SYBR Green I (Roche). Following primer pairs (CyberGene AB, Sweden), annealing temperatures and elongation times were used; metallothionein forward 5′-GCCTTCTTGTCGCTTACACC-3′ reverse 5′-CAGCAGCACTGTTCGTCACT-3′, 58°, 11s; CD14 forward 5′-CAGGAACTTTGGCTTTGCTC-3′ reverse 5′-ACCGATGGACAACTTTCAGG-3′, 58°, 10s and interferon induced transmembrane protein 2 forward 5′-CTCTACCTTTCGCTGCTGCT-3′ reverse 5′-GAACAGGGACCAGACCACAT-3′, 56°, 9s. Each sample from the individual animal was assayed in duplicate. Melting curves were performed to check that only one product had been produced. A standard curve was generated to every gene for quantification and estimating amplification efficiency using decreasing concentrations of cDNA. Transcripts were quantified with the relative standard curve and related against the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase.

Sample preparation for caspase-3–like analysis and Western blot. Pups were deeply anesthetized (thiopental, i.p.) and perfused intracardially with 0.9% NaCl at 2 h ($n = 7$), 8 h ($n = 7$), and 72 h ($n = 8$) after LPS and control animals at PND 7 ($n = 8$) and PND 10 ($n = 8$). The brains were rapidly dissected and quickly frozen. One hemisphere from each animal was sonicated in ice-cold homogenization buffer (50 mM Tris, pH 7.3, 5 mM ethylenediamine tetraacetic acid (EDTA), 1% protease inhibitor cocktail (P8340; Sigma Chemical Co.)). The protein concentration was determined according to Whitaker and Granum adapted for microplates (13).

Fluorometric assay of caspase-3–like activity. Samples, 25 μL, were added to a microplate and mixed with 75 μL extraction buffer (50 mM Tris, pH 7.3; 100 mM NaCl; 5 mM EDTA; 1 mM ethyleneglycol-bis-(β-aminoethylether)-N,N,N′,N′-tetraacetic acid (EGTA); 0.2% 3-[(3-cholamidoprophylene) dimethylamonio]propane sulfonic acid; 1% protease inhibitor cocktail (P8340; Sigma Chemical Co.), 1 mM phenylmethylsulfonylfluoride. After incubation for 15 min at room temperature, 100 μL peptide substrate, 25 μM Ac-Asp-Glu-Val-Asp-aminomethyl coumarin (Ac-DEVD-AMC; Enzyme Systems Products, Livermore, CA) in assay buffer (50 mM Tris-HCl, pH 7.3; 100 mM NaCl; 5 mM EDTA; 1 mM EGTA; 1 mM phenylmethylsulfonylfluoride, 10 mM dithiothreitol was added to the samples. Cleavage of the substrate was measured at 37°C using Spectramax Gemini microplate fluorometer (Molecular Devices, Sunnyvale, CA), with an excitation wavelength of 380 nm and emission wavelength of 460 nm. The degradation was followed at 2-min intervals for 2 h, and V_{max} was calculated from the entire linear part of the curve. Standard curves with AMC in the appropriate buffer were used to express the data in picomoles of AMC (7-amino-4-methyl-coumarin) formed per minute and per milligram of protein.

Western blot. Samples, 25 μg protein, were prepared as recommended by the manufacturer (Novex, San Diego, CA) and electrophoresed on NuPAGE Novex 4–12% Bis-Tris Gels (Novex, San Diego, CA). After electrophoresis, proteins were transferred to a nitrocellulose membrane (Optitran, 0.2 μm; Schleicher & Schuell, Inc., Dassel, Germany). Membranes were blocked in blocking buffer (30 mM Tris-HCl, pH 7.5; 100 mM NaCl; and 0.1% Tween 20 (TBS-T) containing 5% fat-free milk powder). After TBS-T washing, membranes were incubated with the following primary antibodies, diluted in TBS-T containing 3% bovine serum albumin and 9 mM NaN_3 overnight at 8°C: anti-phospho-Akt (Ser^{473}), #9271) rabbit polyclonal 1:2000 or anti-Akt (#9272) rabbit polyclonal 1:2000 (Cell Signaling Technology, Inc., Beverly, MA). Blots were washed three times with TBS-T and incubated with goat anti-rabbit peroxidase-conjugated secondary antibody (Vector Laboratories, Inc. Burlingame, CA) diluted in blocking buffer. Immunoreactive species were visualized using Super Signal Western Dura chemiluminescence substrates (Pierce Biotechnology, Inc., Rockford, IL) and a cooled CCD camera (LAS1000; Fuji Photo Film Co., Ltd., Tokyo, Japan). Immunoreactive bands were quantified using Image Gauge software (version 3.3, Fuji). To standardize quantification between the gels, five controls from PND 8 samples were run on every gel. Membranes were stripped for reprobing with new antibody by incubating them in stripping buffer (62.5 mM Tris-HCl, pH 6.7, 100 mM β-mercaptoethanol, and 2% SDS) at 50°C for 30 min.

Statistical analysis. Pairwise t test analysis with Benjamin Hochberg correction, the false detection rate set at 0.05, and a threshold of 1.2-fold change was applied to microarray data to determine significantly regulated genes. Time points 2 and 6 h were compared with PND 7 controls. The 72-h LPS animals were compared with age-matched PND 10 control pups. Gene ontology analysis was applied to genes that were found to be significantly regulated. Gene ontologies that included $\geq$10 genes are presented in the results. Caspase-3 and phosphorylated Akt (pAkt) measurements were compared using the Kruskal-Wallis test followed by Dunn's multiple comparison test and are presented as mean $\pm$ SEM.

RESULTS

Global gene changes. The number of genes regulated after LPS were 847 at 2 h, 1564 at 6 h, and 1546 at 72 h. At all time points, the majority of genes were down-regulated. At 2 h after LPS, 187 genes were up-regulated and 660 down-regulated, at 6 h 578 genes were up-regulated and 986 down-regulated and at 72h there were 656 genes up-regulated and 890 down-regulated.

The gene ontology analysis revealed that at 2 h after LPS administration, categories of protein modification and folding, immune and inflammatory response, response to chemical substance, chemotaxis, and programmed cell death were overrepresented (Table 1). There was also an overrepresentation of cellular processes, including cell-cell signaling, cell adhesion, and regulation of cell proliferation and differentiation. As shown in Table 2, at 6 h, gene ontologies associated with metabolism remained regulated. Furthermore, genes in the category of response to external stimuli and stress stayed highly overrepresented (immune, inflammatory, humoral immune response, cellular defense response, response to chemical substance and chemotaxis). Cell death continued to be overrepresented, as well as cell proliferation, cell organization

Table 1. *Gene ontologies regulated at 2 h after LPS compared to PND 7 control pups*

Ontology	No. of reg. genes	No. of up-reg.	No. of down-reg.	No. of genes on array	Z score up	Z score down
Physiological processes						
Metabolism						
Protein	125	28	97	1446	0.67	**4.92**
Modification	70	14	56	720	0.46	**4.71**
Folding	12	2	10	117	−0.02	**2.20**
Nucleobase, nucleotide, etc.	74	16	58	1186	−1.15	0.90
Biosynthesis	45	14	31	654	0.84	0.44
Phosphorous	35	8	27	452	0.06	1.69
Catabolism	29	7	22	525	−0.74	−0.26
Lipid	16	4	12	371	−1.00	−1.14
Organic acid	13	4	9	298	−0.53	−1.20
Response to external stimuli and stress						
Biotic	38	29	9	481	**7.53**	**−2.83**
Immune response	32	25	7	349	**8.01**	**−2.26**
Wounding	24	18	6	205	**7.86**	−1.05
Inflammatory response	17	15	2	126	**8.84**	−1.56
Abiotic	14	10	4	262	**2.64**	**−2.33**
Response to chemical substance	10	9	1	153	**3.98**	**−2.29**
Chemotaxis	10	9	1	74	**6.91**	−1.29
Death						
Programmed cell death	36	16	20	327	**4.50**	1.55
Cellular processes						
Cell growth and/or maintenance						
Transport	81	16	65	1308	−1.62	1.12
Cell proliferation	41	14	27	572	1.37	0.38
Cell organization and biogenesis	37	7	30	502	−0.61	1.79
Cell communication						
Signal transduction	91	30	61	1372	1.46	0.08
Cell-cell signaling	35	6	29	414	−0.46	**2.67**
Transmission of nerve impulse	24	0	24	258	**−2.18**	**3.92**
Cell adhesion	24	11	13	328	**2.31**	−0.41
Cell-cell adhesion	10	5	5	94	**2.68**	0.43
Regulation of cellular processes						
Regulation of cell proliferation	18	8	10	208	**2.37**	0.28
Cell differentiation	20	10	10	245	**2.87**	−0.26
Cell motility	16	7	9	247	1.35	−0.60
Development						
Morphogenesis						
Organogenesis	47	15	32	799	0.33	−0.60

For each ontology, the total number of regulated (reg.) genes, the number of up-regulated (up-reg.) and down-regulated (down-reg.) genes, the total number of genes on the array and the *Z* score are presented. *Z* scores that are >2.0 or >-2.0 are shown in bold. Analysis: pairwise analysis with Benjamin Hochberg correction set at 0.05 and fold change set at 1.2. Only regulated ontologies containing ≥ 10 genes are shown.

and biogenesis, cell growth, and signal transduction. Although several categories associated with metabolism were still regulated at 72 h after LPS administration, there was a smaller response in genes belonging to response to external stimuli, stress, and cell death (Table 3). The most highly regulated genes, 72 h after LPS, were found in the ontology secretions of neurotransmitter, cellular transport, ribosome biogenesis and assembly, synaptic transmission, cell migration, and neurogenesis, all which had *Z* scores >4.

Inflammation-related genes. There was a strong proinflammatory response in the brain 2 and 6 h after LPS, including up-regulation of α- and β- chemokines and pro- and anti-inflammatory cytokine genes (Table 4, Web based). Genes associated with immune cell activity were up-regulated including major histocompatibility complex (MCH) class I and II receptor-related genes, lymphocyte, macrophage, and cell adhesion protein-associated transcripts (Table 5, Web based).

Cell death–related genes. A significant number of regulated genes belonged to pro- and antiapoptotic pathways [caspase-3, transglutaminase 2, Nur77, inhibitor of apoptosis protein 1 (IAP 1), baculoviral IAP repeat-containing 2 (IAP 2), growth arrest and DNA damage–inducible gene beta, bcl2-associated athanogene 3, GTP cyclohydrolase 1, receptor-interacting serine-threonine kinase 3, myeloid cell leukemia sequence 1], including p53-dependent factors [IGF binding protein 3 (IGFBP3), Notch gene homology-like domain, member 1 and BH3 interacting domain death agonist] (Table 6, Web based). Several of the genes also belonged to Akt/phosphatidylinositol-3-kinase (Akt/PI3 K) cell survival mechanisms, such as serum/glucocorticoid-regulated kinase (SGRK), pleckstrin homology-like domain, member 1 (PHLDA1), phosphatase and tensin homolog (PTEN), guanine nucleotide binding protein (GNBP), fas apoptotic inhibitory molecule 2 (FAIM2), and tax 1 binding protein 1 (TAX1BP1).

Table 2. *Gene ontologies regulated at 6 h after LPS compared to PND 7 control rats*

Ontology	No. of reg. genes	No. of up-reg.	No. of down-reg.	No. of genes on array	*Z* score up	*Z* score down
Physiological processes						
Metabolism						
Protein	198	62	136	1446	−1.82	**5.04**
Modification	103	30	73	720	−1.34	**4.14**
Targeting	23	4	19	113	−0.81	**4.44**
Folding	19	6	13	117	−0.04	**2.01**
Complex assembly	11	3	8	47	0.36	**2.91**
Nucleobase, nucleotide, etc.	136	42	94	1186	−2.90	**2.13**
RNA metabolism	35	6	29	226	−1.76	**3.88**
Biosynthesis	66	27	39	654	−1.32	−0.66
Phosphorous	49	15	34	452	−1.88	0.86
Catabolism	63	33	30	525	1.17	−0.82
Lipid	35	21	14	371	0.41	**−2.24**
Organic acid	27	14	13	298	−0.41	−1.57
Amine	18	8	10	198	−0.75	−0.87
Amino acid	18	8	10	180	−0.47	−0.55
Electron	16	6	10	209	−1.55	−1.06
Regulation of metabolism	11	5	6	82	0.36	0.28
Alcohol	20	8	12	167	−0.25	0.33
Carbohydrate	19	10	9	235	−0.67	−1.73
Coenzyme and prosthetic group	10	6	4	112	0.07	−1.29
Response to external stimuli and stress						
Biotic	104	81	23	481	**11.99**	−1.65
Immune response	79	68	11	349	**12.38**	−2.65
Inflammatory response	25	24	1	126	**7.07**	−2.64
Humoral immune response	16	13	3	74	**4.82**	−0.88
Cellular defense response	10	8	2	51	**3.38**	−0.76
Abiotic	33	22	11	262	**2.38**	−1.58
Response to chemical substance	16	16	0	153	**2.96**	−3.32
Chemotaxis	11	11	0	74	**3.76**	−2.29
Response to DNA damage	13	4	9	111	−0.77	0.66
Death						
Cell death	59	32	27	346	**3.49**	0.96
Programmed cell death	55	29	26	327	**3.07**	1.04
Secretion						
Neurotransmitter secretion	10	5	5	50	1.53	0.99
Cellular processes						
Cell growth and/or maintenance						
Transport	155	63	92	1308	−0.73	0.78
Cell proliferation	73	24	49	572	−1.15	**2.04**
Cell cycle	39	5	34	278	**−2.62**	**3.91**
Cell organization and biogenesis	58	15	43	502	**−2.34**	1.90
Cell homeostasis	13	7	6	95	0.96	−0.10
Cell growth	14	11	3	101	**2.59**	−1.47
Cell communication						
Signal transduction	178	93	85	1372	**3.00**	−0.63
Receptor linked transduction	83	53	30	679	**3.24**	**−2.40**
Cell-cell signaling	57	30	27	414	1.94	−0.03
Cell adhesion	51	24	27	328	1.77	1.26
Regulation of cellular processes						
Regulation of cell proliferation	25	11	14	208	0.05	0.28
Regulation of cell growth	11	9	2	82	**2.37**	−1.52
Cell motility						
Cell migration	10	4	6	82	−0.14	0.28
Development						
Morphogenesis	101	48	53	862	0.52	−0.53
Neurogenesis	46	18	28	387	−0.51	0.55
Skeletal development	13	5	8	104	−0.19	0.47
Cell differentiation	35	21	14	245	**2.42**	−0.55

For each ontology, the total number of regulated (reg.) genes, the number of up-regulated (up-reg.) and down-regulated (down-reg.) genes, the total number of genes on the array, and the *Z* score is presented. *Z* scores that are >2.0 or >-2.0 are shown in bold. Analysis: pairwise analysis with Benjamin Hochberg correction set at 0.05 and fold change set at 1.2. Only regulated ontologies containing ≥ 10 genes are shown.

Caspase-3 activity and phosphorylation of Akt. To support the mRNA findings of the LPS effects on cell death/survival pathways, we examined the caspase-3 and Akt activation following LPS exposure. Activity measurements demonstrated an increase in caspase-3 activity and Western blot analysis showed a decrease in pAkt at 8 h after LPS (Fig. 1).

Table 3. *Gene ontologies regulated at 72 h after LPS compared to PND 10 control rat pups*

Ontology	No. of reg. genes	No. of up-reg.	No. of down-reg.	No. of genes on array	Z score up	Z score down
Physiological processes						
Metabolism						
Protein	201	93	108	1446	**3.80**	−1.19
Modification	115	48	67	720	**2.80**	1.14
Biosynthesis	36	21	15	288	**2.22**	−1.90
Targeting	20	11	9	113	**2.62**	−0.10
Nucleobase, nucleotide, etc.	132	45	87	1186	−1.52	−1.23
Phosphorous	67	25	42	452	0.96	0.87
Catabolism	51	22	29	525	−0.49	**-2.35**
Lipid	32	11	21	371	−1.57	−1.85
Organic acid	23	10	13	298	−1.07	**−2.49**
Carboxylic acid metabolism	23	10	13	296	−1.04	**−2.46**
Amine	16	5	11	198	−1.43	−1.39
Amino acid	15	5	10	180	−1.20	−1.32
Electron	17	8	9	209	−0.55	**−2.09**
Alcohol	15	4	11	167	−1.39	−0.78
Carbohydrate	21	3	18	235	**−2.49**	−0.31
Coenzyme and prosthetic group	10	5	5	112	−0.08	−1.46
Energy pathways	10	2	8	104	−1.32	−0.19
Response to external stimuli and stress						
Biotic	51	26	25	481	0.86	**−2.52**
Immune response	34	17	17	349	0.23	**−2.35**
Wounding	22	11	11	205	0.52	−1.51
Abiotic	37	7	30	262	−1.54	1.95
Perception of external stimuli	20	4	16	94	−0.17	**3.14**
Perception of sound	12	2	10	43	0.01	**3.61**
Response to pest/pathogen/parasite	24	12	12	243	0.24	−1.90
Response to DNA damage	16	8	8	111	1.31	−0.39
Death						
Cell death	55	21	34	346	1.33	1.13
Secretion						
Neurotransmitter secretion	18	2	16	50	−0.21	**6.15**
Homeostasis						
Cell homeostasis	13	5	8	95	0.30	0.07
Ion homeostasis	11	4	7	77	0.24	0.28
Cellular processes						
Cell growth and/or maintenance						
Transport	213	66	147	1308	0.84	**4.53**
Ion	81	26	55	482	0.85	**2.67**
Intracellular	64	28	36	366	**2.86**	1.17
Protein	54	24	30	314	**2.63**	0.89
Vesicle mediated	53	12	41	276	−0.22	**4.12**
Side chrome	35	6	29	169	−0.67	**4.30**
Organic acid	16	3	13	55	0.30	**4.19**
Neurotransmitter	14	2	12	41	0.08	**4.93**
Amine/polyamine	13	3	10	44	0.70	**3.52**
Cell proliferation	71	25	46	572	−0.30	−0.16
Cell organization and biogenesis	90	34	56	502	**2.41**	**2.51**
Organelle organization and biogenesis	56	19	37	303	1.41	**2.60**
Ribosome biogenesis and assembly	14	12	2	65	**5.35**	−1.52
Cell homeostasis	13	5	8	95	0.30	0.07
Cell growth	10	2	8	101	−1.27	−0.11
Cell communication						
Signal transduction	200	74	126	1372	1.57	1.50
Intracellular signaling cascade	99	36	63	553	**2.23**	**2.87**
Receptor linked signal transduction	95	32	63	679	0.13	1.08
Cell-cell signaling	75	16	59	414	0.76	**4.65**
Synaptic transmission	57	12	45	253	0.10	**5.67**
Cell adhesion	57	12	45	328	−0.85	**3.74**
Regulation of cellular processes						
Regulation of cell proliferation	21	7	14	208	−0.88	−0.79
Cell differentiation						
Regulation of cell differentiation	12	2	10	76	−0.83	1.58
Neuron differentiation	11	2	9	47	−0.12	**2.74**
Cell motility						
Cell migration	21	3	18	82	−0.42	**4.56**

Table 3. *Continued*

Ontology	No. of reg. genes	No. of up-reg.	No. of down-reg.	No. of genes on array	Z score up	Z score down
Muscle contraction	13	6	7	104	0.56	−0.56
Development						
Morphogenesis						
Organogenesis	108	22	86	799	**−2.71**	**2.83**
Neurogenesis	68	11	57	387	−1.72	**4.83**
Cellular morphogenesis	10	2	8	54	−0.32	1.78
Regulation of biological processes						
Regulation of enzyme activity	12	4	8	49	1.19	**2.08**

For each ontology, the total number of regulated (reg.) genes, the number of up-regulated (up-reg.) and down-regulated (down-reg.) genes, the total number of genes on the array and the *Z* score is presented. *Z* scores that are >2.0 or >−2.0 are shown in bold. Analysis: pairwise analysis with Benjamin Hochberg correction set at 0.05 and fold change set at 1.2. Only regulated ontologies containing ≥10 genes are shown.

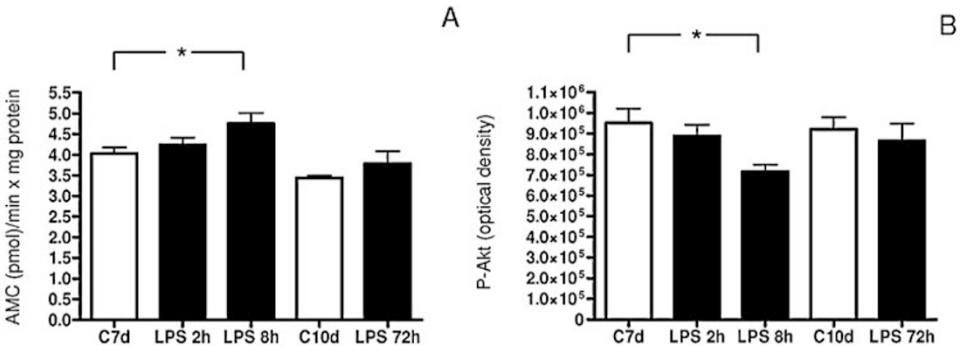

Figure 1. Caspase 3 and Akt activation. Cerebral caspase-3 like activity (DEVD cleavage) after LPS or vehicle treatment (*A*). There was increased caspase-3 like activity at 8 h after LPS compared with PND 7 rats. Phosphorylation of Akt (pAkt) in the brain after LPS or vehicle treatment (*B*). There was a decrease in pAkt at 8 h after LPS compared with PND 7 control. Kruskal-Wallis analysis with Dunn's multiple comparison test. *$p <$ 0.05 compared with PND 7 control.

RT-PCR. The RT-PCR results demonstrated a good agreement with the microarray data for the metallothionein, interferon-induced transmembrane protein 2 and CD14 genes (Fig. 2).

DISCUSSION

This is the first study investigating the global gene expression in the neonatal brain following peripheral LPS exposure. As we have previously shown that peripherally administered LPS at low doses aggravates HI brain damage both early (4 h) and at later (72 h) exposure in the neonatal rat (5,14), it was interesting to identify a large number of genes that are associated with deleterious processes in the brain, such as inflammation and apoptosis. Out of the nearly 16,000 transcripts on the array, >2000 genes were regulated during the first 3 d after LPS exposure. The majority of the most regulated genes belonged to the gene ontology categories of inflammatory responses, cell death, phosphorylation, and ion transport.

Several steps were taken to optimize the analysis; the mRNA from each individual brain was analyzed on a separate chip, which allowed for statistical comparisons including the Benjamin Hochberg correction for multiple comparisons, and all arrays were processed together using the RMA algorithm, which has been suggested to reduce nonbiological variability (12). Furthermore, we used age-matched control animals, which is particularly important when studying the immature brain because a large number of genes are altered due to development.

The early proinflammatory phase following infection, which involves the recruitment and activation of immune cells and their interactions with parenchymal cells, is believed to be detrimental to the brain (15). In support of this, within the first hours of LPS exposure, we found up-regulation of several proinflammatory genes that are known to be involved in the recruitment of immune cells, such as α- and β-chemokines. Cell adhesion is crucial in recruitment of immune cells and the array analysis revealed that a large proportion of the inflammatory genes were associated with cell adhesion. In addition, several genes associated with stimulated immune cells including macrophages, lymphocytes, and MHC class I and II cells, were up-regulated early after LPS. The inflammatory response in the brain was further evident by the expression of proinflammatory cytokine transcripts. Some of these, such as IL-1β, IL-6, and TNF-α, have previously been shown to be induced in the fetal rat brain after maternal LPS exposure (1).

Interestingly, several genes that are coupled to the transforming growth factor (TGF)-β pathway were down-regulated after LPS. TGF-β is considered an anti-inflammatory mediator and has, at least in the short term, also been shown to be neuroprotective after HI (16). A negative response in TGF-β associated these genes following LPS may therefore have contributed to the enhanced vulnerability of the brain after LPS.

LPS-induced microglial apoptosis has been demonstrated, which was associated with caspase-3 and 11 activation (17) and we also found regulation of caspase-3 and caspase-11 transcripts. In support of these findings we found that the caspase-3 activity was increased in the brain following peripheral LPS exposure. In addition, transglutaminase 2, a caspase substrate during apoptosis, which can also alter mitochondrial function and serve as an upstream effector of apoptosis (18) was regulated.

Several genes associated with the TNF-α pathway including Nur77, which prevents TNF-α induced death in macro-

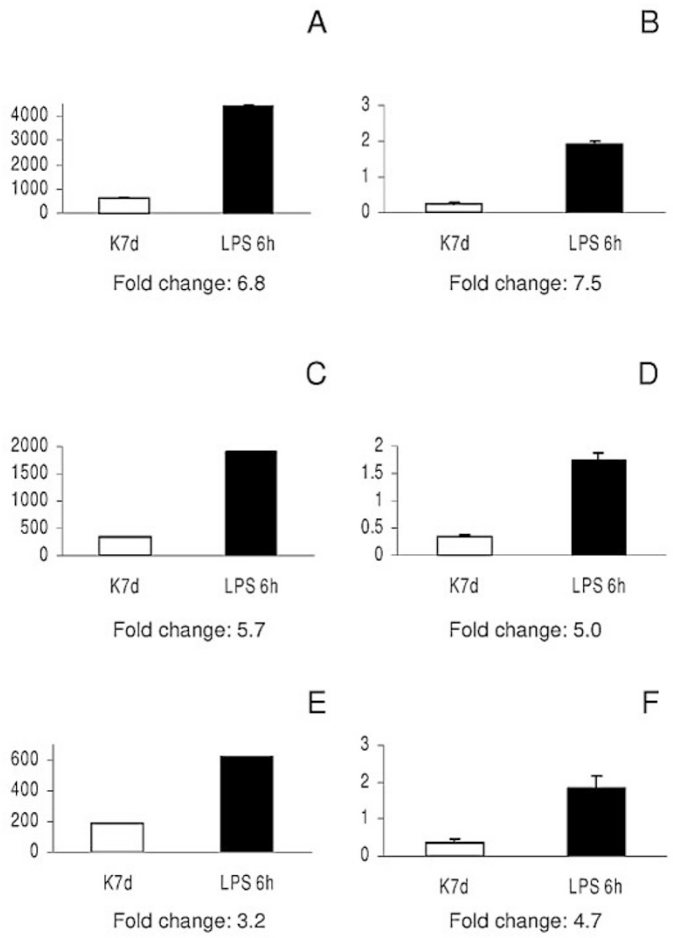

Figure 2. RT-PCR confirmation of microarray analysis. Three genes, metallothionein (*A*, *B*), interferon-induced transmembrane protein 2 (*C*, *D*), and CD14 (*E*, *F*), which were significantly changed between PND 7 control pups and 6 h after LPS injection according to the microarray analysis (*A*, *C*, *E*), were compared with RT-PCR (*B*, *D*, *F*) to confirm the accuracy of the microarray chip. When comparing nonlogged fold changes, there was an overall good agreement between RT-PCR and microarray results.

phages (19) and IAP 1 and 2, which inhibit apoptosis by binding to the TNF receptor associated factors 1 and 2. LPS has been shown to enhance the expression of IAP mRNA and protein in human monocyte-derived macrophages, which was associated with the inhibition of the caspase-3 activation, suggesting that LPS may inhibit apoptosis of macrophages through the induction of IAPs (20). The gene coding for the protein LPS-induced TNF-α factor (LITAF), which binds to a critical region of the TNF-α promoter and is believed to be involved in TNF-α expression following LPS induction, was regulated (21). TNF receptor 1 (TNFR1) initiates both the activation of NF-κB and caspase-8 signaling. When NF-κB is activated, it activates the caspase-8 inhibitor FLIP(L), which promotes cell survival. Thus, TNFR1-mediated signal transduction includes a checkpoint, resulting in apoptosis if FLIP fails to be activated by NF-κB (22). Several other p53-associated genes were also regulated, including IGFBP-3, which regulates IGF-1 bioactivity, but has also been shown to independently inhibit cell proliferation and induce apoptosis in a p53-dependent manner (23). Interestingly, concurrently the IGF-1 gene was down-regulated, which is in agreement, showing a negative regulation of IGFBP-3 on IGF-1 (24). Furthermore, IGF-1 is known to trigger Akt activation (25), and in support of these findings, we found a decrease in Akt phosphorylation. The mRNA for Notch 1, which selectively induces extensive apoptosis in progenitor cells, was up-regulated on the array. Notch activation in neural progenitor cells leads to elevated levels of nuclear p53 and transcriptional up-regulation of the target genes Bax and Noxa, and the promotion of apoptotic cell death by Notch activation is completely suppressed by p53 deficiency (26). Furthermore, the mRNA for BH3 interacting domain death agonist, which encodes a protein that is a member of the Bcl-2 family and is a mediator of mitochondrial damage *via* caspase-8 activation and cytochrome *c* release, was regulated (27). Taken together, these results indicate involvement of TNF-α and p53 dependent pathways in the immature brain following LPS exposure.

In support of previous studies, the mRNA expression of metallothionein was markedly increased in the brain following LPS (28), which is believed to be a protective response because metallothionein has been shown to reduce brain inflammation, caspase activation, and neurodegeneration (29). Other antiapoptotic genes were also regulated, particularly those associated with the Akt/PI3 K-dependent survival pathway. SGRK is a PI3 K-dependent kinase with structural homology to Akt, which provides cell survival responses to multiple types of stress stimuli (30). PHLDA1 may play an important role in the antiapoptotic effects of IGF-1/Akt pathway because the pleckstrin-homology domain of Akt/protein kinase B (Akt/PKB) is activated in response to growth factors (31). PHLDA1 negatively regulates intracellular levels of phosphatidylinositol-3,4,5-trisphosphate in cells and functions as a negative regulator of the PI3 K-Akt/PKB signaling pathway (32). Phosphorylation of PHLDA1 and Akt has also been demonstrated in astrocytes in the rat hippocampus following transient forebrain ischemia (33). The FAIM2 gene codes for a protein that can antagonize Fas-induced cell death (34) and exert neurite growth–promoting action in different neuronal systems (35). Furthermore, resistance of rat cerebellar granule neurons to FasL-induced apoptosis is mediated by PI3 K-AKT/PKB-dependent expression of FAIM2 (36). TAX1BP1, which modulates cell cycle progression and apoptosis (37), was also recently shown to be linked to Akt activation, NF-kB translocation, and p53 inhibition (38). These studies suggest that the Akt/PI3 K pathway is involved in the cerebral responses to LPS, which was further supported by our finding of a decrease in pAkt after LPS exposure.

Taken together, the microarray analysis suggests that both apoptosis-mediated mechanisms and the Akt pathway in the brain may be affected by peripheral LPS exposure. In support of the microarray data, we found evidence of increased caspase-3 activation and reduced phosphorylation of Akt. These results suggest that there may be an increase in cell death mechanisms at the same time as cell survival pathways are inhibited.

In conclusion, we have found a multiplicity of genes regulated in the immature brain, indicating a complex cerebral response to peripheral LPS exposure. The complexity is likely to be, at least partly, due to cell context and brain region,

which was not investigated in the present study. In addition to the expected inflammatory response, there was regulation of a significant number of cell death–associated genes, which could contribute to the increased vulnerability of the immature brain to HI following LPS exposure. Clearly, to further characterize the cerebral response to LPS, it will be important to study the cellular localization of the expressed genes and also the protein expression.

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