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Original Article

Quantitative proteomic analysis of brain in rats with intrauterine infection

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Abstract: Intrauterine infection/inflammation can significantly affect perinatal brain development and result in significant alterations in brain structure and function. However, the pathophysiological mechanism of intrauterine infection remains elusive and therapy remains limited. Proteomics analysis might provide insight into potential mechanism and thus help to improve the clinical treatment. In this study, pregnant SD rats were divided into lipopolysaccharide (LPS) group and control group randomly, LPS or saline was injected for two days from gestation 18. Quantitative analysis of the brain tissue of 4 day-neonatal rat pups in two groups was performed using label-free shotgun analysis. Results showed that there were 283 proteins identified from control group and 260 proteins from intrauterine infection group. 11 proteins were 2 folds up-regulated and 11 proteins were 2 folds down-regulated in LPS group compared to control group. After analysis using DAVID database, we found that the differentially expressed proteins comprised mitochondrial component or proteins, cytoskeleton and organelle et al. Molecular function analysis revealed that the majority of proteins were involved in ion binding, nucleotide binding and enzyme binding et al. Biological process analysis showed that the majority of proteins were involved in regulation of nervous system development, regulation of ion transport, protein complex biogenesis and cytoskeleton organization et al. We then used STRING 9.0 software to analyze and explore the complex interactions existed among these proteins. Finally, we used Western blot to verify the fold changes of some proteins that we randomly chose and found that there existed the same trend compared to the results of proteomics. This novel study performed a full-scale screening of the proteomics research in intrauterine infection of immature rat.

Keywords: Intrauterine infection, proteomics, immature, rat

Introduction

Intrauterine infection/inflammation refers to that pregnant women infected during pregnancy and cause fetal intrauterine infection. It is a clinical syndrome which can damage various systems of the fetus and/or neonate. Intrauterine infection and inflammation are major reasons for preterm birth and may cause a lot of problems including development of cerebral palsy [1-3]. The pathophysiological mechanism of intrauterine infection is complex and remains elusive. Recently, it was demonstrated that inflammation has a modulating effect on adult neurogenesis. Studies found that many pathogenic microorganisms can be the reason for intrauterine infection, of which viruses are the most widely causes. Cytomegalovirus is one of the most common viruses which can cause more serious consequences [4-6]. Major Sequelae of cytomegalovirus induced infection include neurological disturbances such as mental retardation, hearing impairment language disabilities, learning difficulties, paralysis and so on. However, neonatal neuroprotection therapy for intrauterine infection induced nervous system damage remains elusive. Antivirus medicine is still the most effective therapy but there exist a lot of side effects after using antivirus treatment such as thrombocytopenia, leucopenia, abnormal liver function, et al. In recent years, people are trying new therapy in animal models and clinical research to intrauterine infection. But to date treatments can be used in clinical are still limited. Therefore, it is very necessary and important to full elucidate the physiopathology of intrauterine infection. However, the details of the mechanism leading to permanent nervous damage induced by intrauterine infection have

not yet been elucidated. Proteomics can analyze protein expression at the overall level and thus could provide insight into the potential mechanism of intrauterine infection. Furthermore, fully elucidated details in nervous damage after intrauterine infection/inflammation attack may help to develop protective therapies. We employed a label-free quantitative shotgun proteomic method [7] to make a global comparison of proteins of healthy brain tissue against those brain tissues from intrauterine infection insult in rats. Proteins down-regulated and up-regulated are considered to be important regulators during intrauterine infection injury and subsequent regenerative procedure, therefore, are also potential therapeutic target protein molecules.

Materials and methods

Animals and model

Pregnant rats (Sprague-Dawley rats) were bought from Beijing Vital River Laboratory Animal Technology Limited Company [SCXK (Beijing) 2011-0011]. Rearing conditions: exposure time: dark time=12 h:12 h, temperature: 24°C, Humidity: 40%. The experiment was approved by the University Animal Ethics Committee according to the local government legislation. The model of intrauterine infection of rats was made as Walker AK et al. [8]. Briefly, pregnant Sprague-Dawley rats were randomly divided into LPS group and control group, injected intraperitoneally (i.p) with LPS (0.2 mg/kg) or saline (0.5 ml) for two days from gestation day 18 (GD 18). On fourth day after delivery, the brain tissue of LPS group and control group were obtained. Brain tissue of each group were kept in -80°C refrigerator for further analysis (5 of each group for label-free quantitative shotgun proteomic analysis and 3 of each group for western blot verification).

Protein extraction and digestion

For the differential proteome study, brain tissues of P4 rats of two groups were investigated. Frozen brain samples were transferred to a cooled mortar and then liquid nitrogen were used to grind the samples to fine powder. Redissolved in 8 M UA buffer and determine the concentration of each protein.One hundred μg proteins were taken from each sample, 2 μg Lys-C were added and then incubated for 3 hours at room temperature. Added 25 mM NH_4HCO_3 to dilute the concentration of urea to 1.5 M and then added 2 μg trypsin, incubated in 37°C for 20 hours. Then proteins were prepared for examination of 2D-LC-MS/MS analysis.

2D-LC-MS/MS analysis of hydrolysates

According to the quantitative result, 5 μg hydrolysate of each sample of two groups were taken to conduct the 2D-LC-MS/MS analysis. Nanoflow liquid systemEASY-nLC1000 HPLC was used to separate the hydrosysate. Liquid phase solution A was 0.1% formic acid in acetonitrile (with 2% acetonitrile) and solution B was 0.1% formic acid in acetonitrile (with 84% acetonitrile). Thermo EASY column SC200 (RP-C18) was balanced by 100% solution A. Samples were loaded to the Thermo EASY column SC001 traps (RP-C18) by autosampler, and then separated by column chromatography, with a flow rate of 400 nL/min. Related phase gradients were as followed: linear gradient of solution B was increased from 0% to 45% during 0 minute to 100 minutes; linear gradient of solution B was increased from 45% to 100% during 100 minutes to 108 minutes and linear gradient of solution B was maintained at 100% during 108 minutes to 120 minutes. After separation by capillary liquid chromatography, hydrolyzate was identified by mass spectrum using Q-Exactive mass spectrometer (Thermo-Finnigan). The total time of analysis was 120 min, detection method was positive ion and precursor ion scan range was 300 m/z to 1800 m/z. The ratio of polypeptides and polypeptide fragments of the mass-charge was collected in accordance with the following methods: 10 pieces Atlas (MS2 scan, HCD) were collected after each full scan. The resolution of MS^1 at M/Z 200 is 70,000 and the resolution of MS^2 at M/Z 200 is 17,500.

Label-free analysis

The original 4 LC-MS/MS files were imported to Maxquant software for Label-free analysis, using the database of Swiss-Prot Rat (2010-04 edition). The peptides were constrained to be tryptic and up to 2 missed cleavages were allowed. Cysteine Carbamidomethylation was treated as a fixed modification, where as acetylation of protein N-term and oxi-

Table 1. Identification of down-regulated proteins (≥2-fold) in LPS group versus control group

ACC	DESC	Fold change	Molecular weight	PI
P11517	Hemoglobin subunit beta-2	-16.1	15851.24	8.91
P39069	Adenylate kinase isoenzyme 1	-5.5	21583.76	7.66
P51886	Lumican	-4.2	36485.83	6.01
P62959	Histidine triad nucleotide-binding protein 1	-3.7	13645.71	6.39
Q3T1J1	Eukaryotic translation initiation factor 5A-1	-3.3	16701.06	5.08
Q63362	NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 5	-3.1	13280.59	7.07
P61589	Transforming protein RhoA	-3.0	21442.68	5.83
Q4V8F9	Hydroxysteroid dehydrogenase-like protein 2	-2.9	58343.94	5.85
P70567	Tropomodulin-1	-2.4	40480.07	4.97
Q03626	Murinoglobulin-1	-2.2	162631.90	5.62
P11232	Thioredoxin	-2.2	11542.28	4.80

Table 2. Identification of up-regulated proteins (≥2-fold) in LPS group versus control group

ACC	DESC	Fold change	Molecular weight	PI
Q6P7Q4	Lactoylglutathionelyase	2.1	20688.42	5.12
P27605	Hypoxanthine-guanine phosphoribosyltransferase	2.1	24346.05	6.06
P07633	Propionyl-CoA carboxylase beta chain, mitochondrial	2.1	55658.59	6.25
P00762	Anionic trypsin-1	2.3	23604.71	5.02
P14668	Annexin A5	2.4	35613.32	4.91
P85834	Elongation factor Tu, mitochondrial	2.4	44984.90	6.20
P11661	NADH-ubiquinone oxidoreductase chain 5	2.4	68618.03	9.34
P07943	Aldose reductase	2.7	35666.08	6.28
Q63041	Alpha-1-macroglobulin	2.9	28267.83	6.10
P62138	Serine/threonine-protein phosphatase PP1-gamma catalytic subunit	3.0	37380.89	5.94
Q9JK11	Reticulon-4	3.0	126388.09	4.41

dation of methionine residues were considered to be variable modifications. Main search ppm was set to be 6 and MS/MS tolerance ppm was 20. Both of the false discovery rate (FDR) of peptide and protein were 0.05.

Statistical and bioinformatic analysis of perseus

Results from Maxquant were put to the software of Perseus for further analysis. The version number of Perseus software was 1.2.0.17. In this study, proteins more than 2-fold increased or decreased were identified to be dramatically changed. Data was showed as mean ± SEM. Comparisons of quantitative data were analyzed using the two-tail Student's t-test. Statistical significance was set as $P<0.05$. Analysis of cellular component, molecular function and biological process of differentially expressed proteins were performed using the tools on the database for annotation, visualization and integrated discovery (DAVID (http://david.abcc.ncifcrf.gov/). The association among the most dramatically changed proteins was analyzed through the STRING 9.0 software.

Western blot analysis

To verify the results of proteomics, Western Blot analysis was performed using brain tissue of LPS group (n=3) and control group (n=3). To be brief, added in protease inhibitor cocktail (Roche) to the precooled RIPA protein extraction reagent, and then used this reagent tothe brain tissue and homogenated. After incubating on ice for 20 min and centrifuging at 13000 rpm (4°C) for 20 min, we got the protein. Determined the protein concentration using

Table 3. Identification of proteins absent in LPS group versus control group

ACC	DESC	Molecular weight	PI
P02401	60S acidic ribosomal protein P2	11691.96	4.40
P02680	Fibrinogen gamma chain	47803.28	5.53
P02767	Transthyretin	13598.19	5.77
P04785	Protein disulfide-isomerase	54982.89	4.77
P12007	Isovaleryl-CoA dehydrogenase, mitochondrial	43097.43	6.12
P35738	2-oxoisovalerate dehydrogenase subunit beta, mitochondrial	37808.41	5.33
P38983	40S ribosomal protein SA	32692.86	4.80
P42930	Heat shock protein beta-1	22892.67	6.12
P48679	Lamin-A	73992.27	6.54
P61983	14-3-3 protein gamma	28302.59	4.80
P62963	Profilin-1	14826.02	8.50
P63102	14-3-3 protein zeta/delta	27771.14	4.73
P85125	Polymerase I and transcript release factor	43908.57	5.42
P08932	T-kininogen 2	45723.55	5.76
P50399	Rab GDP dissociation inhibitor beta	50537.13	5.93
P60711	Actin, cytoplasmic 2	41736.73	5.29
P69897	Tubulin beta-2B chain	49670.82	4.78
P05545	Serine protease inhibitor A3K	44601.02	5.31
Q5RKI0	WD repeat-containing protein 1	66050.26	6.15
P24090	Alpha-2-HS-glycoprotein	35929.80	5.95
P05506	NADH-ubiquinone oxidoreductase chain 3	13086.58	4.38
P15865	Histone H1.2	21856.16	11.1
P28480	T-complex protein 1 subunit alpha	60359.65	5.86
P13471	40S ribosomal protein S14	16127.49	10.08
P05544	Serine protease inhibitor A3L	43500.79	5.59
P09006	Serine protease inhibitor A3N	43574.12	5.63
P21396	Amine oxidase [flavin-containing] A	59507.83	8.12
P25113	Phosphoglyceratemutase 1	28700.79	6.75
P20761	Ig gamma-2B chain C region	Can not be computed	
P04639	Apolipoprotein A-I	28273.89	5.41
Q4G069	Regulator of microtubule dynamics protein 1	35400.55	7.6
P69527	Aminopeptidase O	92836.98	6.12
P02764	Alpha-1-acid glycoprotein	21630.66	5.7
O09175	Aminopeptidase B	72488.67	5.47
P06214	Delta-aminolevulinic acid dehydratase	36031.59	6.31
P13635	Ceruloplasmin	118667.02	5.30
P14669	Annexin A3	36363.20	5.96
P24473	Glutathione S-transferase kappa 1	25361.77	9.13
P30904	Macrophage migration inhibitory factor	12346.05	7.28
P62076	Mitochondrial import inner membrane translocase subunit Tim13	10457.94	8.42
P63029	Translationally-controlled tumor protein	19462.17	4.76
P85515	Alpha-centractin	42613.74	6.19
P97576	GrpE protein homolog 1, mitochondrial	21292.33	6.12
Q01129	Decorin	36363.99	9.06
Q5I0P2	Glycine cleavage system H protein, mitochondrial	13784.41	4.42
Q5XHZ0	Heat shock protein 75 kDa, mitochondrial	73943.52	5.91
Q62930	Complement component C9	60279.30	5.60

P85973	Purine nucleoside phosphorylase	32301.93	6.46
Q9QYE7	Integrin alpha-D	124731.98	5.72
P09456	cAMP-dependent protein kinase type I-alpha regulatory subunit	43094.98	5.27
Q62688	Inactive phospholipase C-like protein 1	122772.40	5.47
Q9WV75	Spondin-2	33273.41	5.59
P19332	Microtubule-associated protein tau	78432.81	5.95
P67999	Ribosomal protein S6 kinase beta-1	59131.52	6.35
Q9QW07	1-phosphatidylinositol-4,5-bisphosphate phosphodiesterase beta-4	134365.47	6.42
Q5PQS7	Uncharacterized protein C3orf19 homolog	54449.15	6.13
O54735	cGMP-specific 3',5'-cyclic phosphodiesterase	94556.18	5.74
O35878	Heat shock protein beta-2	20346.67	5.27
P06761	78 kDa glucose-regulated protein	70474.59	5.01
P85108	Tubulin beta-2B chain	49906.97	4.78
P0C2X9	Delta-1-pyrroline-5-carboxylate dehydrogenase, mitochondrial	59186.39	6.26
P05504	ATP synthase subunit a	25075.53	9.87
P41562	Isocitrate dehydrogenase [NADP] cytoplasmic	46603.23	6.53
Q5XIE6	3-hydroxyisobutyryl-CoA hydrolase, mitochondrial	39157.11	6.52
Q9QX79	Fetuin-B	39731.22	6.50
P11598	Protein disulfide-isomerase A3	54239.39	5.78
Q8VID1	Dehydrogenase/reductase SDR family member 4	29821.80	9.60
P17209	Myosin light chain 4	21282.18	4.96
P63251	G protein-activated inward rectifier potassium channel 1	56573.28	8.60
P24329	Thiosulfate sulfurtransferase	33275.55	7.82
P31211	Corticosteroid-binding globulin	42243.07	4.80
Q3T1K5	F-actin-capping protein subunit alpha-2	32835.87	5.58
Q62658	Peptidyl-prolylcis-trans isomerase FKBP1A	11791.44	8.08
Q6AY09	Heterogeneous nuclear ribonucleoprotein H	49293.60	5.89
Q8VIF7	Selenium-binding protein 1	52400.88	6.11
Q9QY17	Protein kinase C and casein kinase substrate in neurons 2 protein	55977.89	5.04
P18418	Calreticulin	46348.33	4.33
Q6AYL2	Germ cell-specific gene 1 protein	36057.13	5.59
Q9JM59	Kv channel-interacting protein 2	30932.76	4.93
Q9JJ79	Cytoplasmic dynein 2 heavy chain 1	492218.13	6.23
Q6IG02	Keratin, type II cytoskeletal 2 epidermal	69127.04	7.58
Q99PV3	Muskelin	84702.75	5.92
P0C0K7	Ephrin type-B receptor 6	107193.41	6.38
Q68FP1	Gelsolin	83510.51	5.65
P15146	Microtubule-associated protein 2	202410.75	4.77
P08009	Glutathione S-transferase Mu 2	25549.49	7.27
P14046	Alpha-1-inhibitor 3	161078.03	5.67
P07335	Creatine kinase B-type	42594.08	5.40
P14480	Fibrinogen beta chain	50671.00	7.94
Q6P6V1	Polypeptide N-acetylgalactosaminyltransferase 11	69039.10	8.58
P05708	Hexokinase-1	102408.01	6.29
P21263	Nestin	208797.47	4.30
O54728	PhospholipaseB1, membrane -associated	158727.08	6.21
Q9ERB4	Versican core protein (Fragments)	297749.90	4.48

Q704S8	Carnitine O-acetyltransferase	70800.73	8.73
Q6AYT9	Acyl-coenzyme A synthetase ACSM5, mitochondrial	61889.70	6.72
P51650	Succinate-semialdehyde dehydrogenase, mitochondrial	52188.67	6.40
P62919	60S ribosomal protein L8	27893.46	11.04
P70470	Acyl-protein thioesterase 1	24708.72	6.04
Q5XFX0	Transgelin-2	22262.22	8.45
Q5XIT9	Methylcrotonoyl-CoA carboxylase beta chain, mitochondrial	59037.20	7.27
Q66HB5	Radial spoke head 10 homolog B	101586.80	6.58
Q8R508	Protocadherin Fat 3	498700.77	4.70
Q9QYV8	DNA polymerase subunit gamma-1	136855.60	6.43
Q6LED0	Histone H3.1	15272.89	11.13
Q5QD51	A-kinase anchor protein 12	180979.01	4.34
Q9Z244	GMP reductase 1	37487.94	6.50
O88422	Polypeptide N-acetylgalactosaminyltransferase 5	105119.63	9.21
Q6UPR8	Endoplasmic reticulum metallopeptidase 1	99896.84	6.82
Q62671	E3 ubiquitin-protein ligase UBR5 (Fragment)	308026.95	5.72
P62828	GTP-binding nuclear protein Ran	24291.91	7.20
Q9JI51	Vesicle transport through interaction with t-SNAREs homolog 1A	26042.71	6.07
P41350	Caveolin-1	20421.41	5.30
Q497B0	Nitrilase homolog 2	30700.99	6.90
P70536	Oxytocin receptor	42868.60	9.56
P61016	Cardiac phospholamban	6094.51	9.15
Q9Z2A6	Mitogen-activated protein kinase 15	60723.56	9.83
Q710E6	Protein C1orf9 homolog	137121.91	4.94
Q535K8	GON-4-like protein	247902.63	4.82
Q8K3U6	Coagulation factor VII	17565.44	5.07
O88831	Calcium/calmodulin-dependent protein kinase 2	64315.07	5.64
P11608	ATP synthase protein 8	7629.91	9.30
Q99N02	Solute carrier organic anion transporter family member 3A1	76825.35	6.77
Q6RFZ7	Pleckstrin homology domain-containing family G member 5	115784.30	6.60
P51579	P2X purinoceptor 6	42450.59	6.45
Q9QZR8	PDZ domain-containing protein 2	293889.82	8.44
Q62667	Major vault protein	95667.05	5.43
Q9JHZ4	GRIP1-associated protein 1	95942.67	5.17
P19492	Glutamate receptor 3	98051.86	8.26
Q4V8H8	EH domain-containing protein 2	61237.48	6.12
Q62936	Disks large homolog 3	93539.44	6.32
Q5M965	Probable tRNA(His) guanylyltransferase	34849.81	8.44
Q07014	Tyrosine-protein kinase Lyn	58528.91	6.76
O54889	DNA-directed RNA polymerase I subunit RPA1	194192.16	6.43
Q62724	DNA replication licensing factor MCM6 (Fragment)	pI/Mw cannot be computed	
P35565	Calnexin	65129.16	4.48
P30427	Plectin-1	533540.00	5.71
P97526	Neurofibromin	316952.26	6.96
P52590	Nuclear pore complex protein Nup107	107208.89	5.34
Q3KRC5	tRNA-dihydrouridine synthase 3-like	71408.75	7.79
P15389	Sodium channel protein type 5 subunit alpha	227367.25	5.47
P23562	Band 3 anion transport protein	103172.71	5.28
Q5BK10	Calpain-13	76938.62	6.49

P30823	High affinity cationic amino acid transporter 1	67267.11	5.73
P16221	Cytochrome c oxidase polypeptide 8H, mitochondrial	4765.54	9.53
P20070	NADH-cytochrome b5 reductase 3	34043.44	8.57
P35467	Protein S100-A1	10428.65	4.73
P50398	Rab GDP dissociation inhibitor alpha	50536.64	5.00
P29995	Inositol 1,4,5-trisphosphate receptor type 2	307058.38	6.06
Q7TT49	Serine/threonine-protein kinase MRCK beta	194887.69	6.05
Q5XIA3	Leucine carboxyl methyltransferase 2	75532.60	6.54
Q3T1G7	Conserved oligomeric Golgi complex subunit 7	86211.84	5.24
P49621	Diacylglycerol kinase beta	90287.96	8.30
Q6IE52	Murinoglobulin-2	158868.77	6.12
P35365	5-hydroxytryptamine receptor 5B	41122.28	9.83
P50137	Transketolase	67643.64	7.22
Q5U300	Ubiquitin-like modifier-activating enzyme 1	117656.59	5.36
Q63618	Espin	90568.83	6.68
Q9JIR0	Peripheral-type benzodiazepine receptor-associated protein 1	200203.54	5.17
P05505	Cytochrome c oxidase subunit 3	29739.51	6.59
P05426	60S ribosomal protein L7	30329.26	10.87
O89040	1-phosphatidylinositol-4,5-bisphosphate phosphodiesterase beta-2	134882.94	5.81
P37199	Nuclear pore complex protein Nup155	155002.84	5.84
O54766	Zona pellucida sperm-binding protein 1	57997.92	6.23
Q8K4V4	Sorting nexin-27	61014.88	5.95
P19132	Ferritin heavy chain	21126.66	5.86
Q7TNK6	tRNA guanosine-2'-O-methyltransferase TRM11 homolog	52971.58	8.07
O35567	Bifunctional purine biosynthesis protein PURH	64208.42	6.69
Q9WV48	SH3 and multiple ankyrin repeat domains protein 1	226335.15	8.52
Q63148	Chordin	99459.77	7.26
Q62929	Interleukin-1 receptor-like 2	61820.83	7.03
P97571	Calpain-1 catalytic subunit	81988.04	5.46
Q5XI06	Probable histone acetyltransferase MYST1	52500.84	8.59
P11497	Acetyl-CoA carboxylase 1	265193.51	5.97
Q99PD6	Transforming growth factor beta-1-induced transcript 1 protein	50122.60	6.49
Q3SWT6	Serine/threonine-protein phosphatase with EF-hands 1	73966.47	6.65
P11950	Cytochrome c oxidase polypeptide VIc-2	8421.85	10.07
Q9R1J8	Prolyl 3-hydroxylase 1	81147.05	5.00
Q3T1I3	USH1C-binding protein 1	74611.76	5.41
O54975	Xaa-Pro aminopeptidase 1	69657.53	5.38
P68136	Actin, alpha skeletal muscle	41816.70	5.23
Q9EQT5	Tubulointerstitial nephritis antigen-like	50685.20	6.65
P54275	DNA mismatch repair protein Msh2	103896.82	5.77
P08050	Gap junction alpha-1 protein	42900.23	8.97
P85970	Actin-related protein 2/3 complex subunit 2	34391.10	6.84
Q9WUH4	Four and a half LIM domains protein 1	31772.57	8.76
O88280	Slit homolog 3 protein	164742.47	7.74
P97924	Kalirin	336586.63	5.67
Q66H98	Serum deprivation-response protein	46255.19	5.21
P15149	Cytochrome P450 2A2	56345.45	7.71
Q5U1Z0	Rab3 GTPase-activating protein non-catalytic subunit	154430.59	5.62

P11960	2-oxoisovalerate dehydrogenase subunit alpha, mitochondrial (Fragment)	45557.12	5.93
Q3ZBA0	UPF0584 protein KIAA1358 homolog P	130186.80	6.03
Q68FU4	CaiB/baiF CoA-transferase family protein C7orf10 homolog	46605.67	8.64
Q6RUV5	Ras-related C3 botulinum toxin substrate 1	21110.63	8.77
Q498S6	Zinc finger CCHC domain-containing protein 2	124731.61	6.37
P25809	Creatine kinase, ubiquitous mitochondrial	43144.17	7.78
P35525	Chloride channel protein 2	99328.40	9.01
P11915	Non-specific lipid-transfer protein	58813.30	6.62
Q4G064	Ubiquinone biosynthesis methyltransferase COQ5, mitochondrial	31865.48	7.28
Q8K430	Kelch-like protein 17	69732.04	7.64
B2RZ78	Vacuolar protein sorting-associated protein 29	20468.66	6.29
Q8K3K1	Usherin	164188.08	6.63
Q9ESP4	Probable G-protein coupled receptor 88	40200.00	9.55
Q63164	Dynein heavy chain 1, axonemal	515021.41	5.64
Q8CH84	ELAV-like protein 2	39505.84	9.14
P70478	Adenomatous polyposis coli protein	310401.48	7.07
P07687	Epoxide hydrolase 1	52581.58	8.59
Q4KM62	Palmdelphin	62418.82	5.43
Q9EPF2	Cell surface glycoprotein MUC18	68951.09	5.63
Q1XID0	Transcription factor LBX1	30433.11	6.47

BCA protein assay and adjusted the protein concentration of the sample to concentration of 3.5 μg/μL, added 5× protein sample buffer and incubated at 95°C water bath for 5 min.

We performed Western blot. In brief, protein samples were loaded and using the following lectrophoresis conditions: stacking gel 70 V/20 min; separating gel 120 V/40 min. Then the proteins were transferred to a pretreated PVDF membrane (use methanol to treat the membrane). The membranes were immersed in 5% non-fat milk and incubated 1 h (RT) to block nonspecific binding sites. After blocking, membranes were incubated with primary antibodies diluted in 5% non-fat milk overnight at 4°C. After three times washing using PBST, the membranes were incubated with secondary antibodies for 60 smin at room temperature. ECL was added to PVDF membrane and reacted for 2 min; film exposure: 10 s-5 min. Primary antibodies were used as follows: Tau (1:1000, Santa Cruz, Texas, USA), Neurofibromin (1:1000, Santa Cruz, Texas, USA), β-actin (1:1000, Guge Biotechnology company, Wuhan, China). We then used AlphaEase FC software to quantify the bands and t-test of SPSS software 16.0 to analyze the results.

Results

Label-free quantitative shotgun data and differentially expressed protein identification

To detect the differentially expressed proteins involved in the intrauterine infection procedure, we used a label-free quantitative shotgun proteomic method. Interestingly, 441 proteins were differentially expressed with 233 proteins displaying more than 2-fold difference when comparing the LPS group and the control group. Of these, 11 protein were more than 2-fold down-regulated (**Table 1**), 11 protein were more than 2-fold up-regulated (**Table 2**) in LPS group and 211 proteins were absent in LPS group (**Table 3**), determined by liquid chromatography-mass spectrometry/mass spectrometry. Classification of these proteins using Gene Ontology database showed that the majority of differentially expressed proteins comprised mitochondrial component or proteins, cytoskeleton and organelle et al. (**Figure 1**). DAVID classification of proteins by molecular function revealed that the majority of proteins were involved in ion binding, nucleotide binding and enzyme binding et al. (**Figure 2**). DAVID classification of proteins by biological process showed

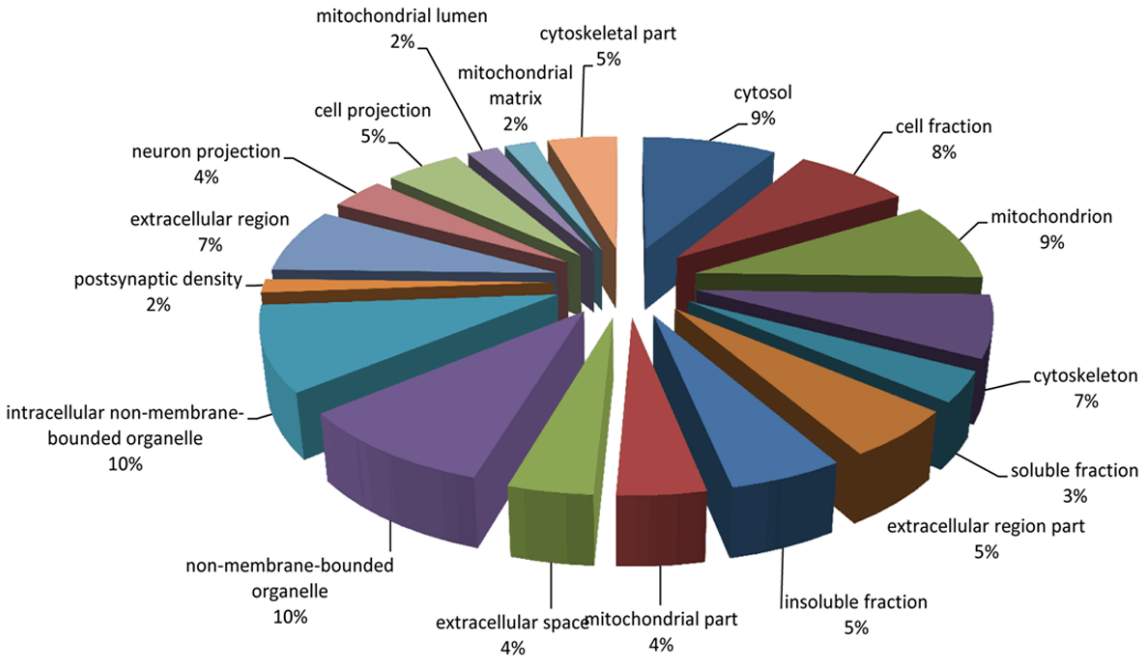

Figure 1. The cellular component categories according to bioinformatics analysis. Categorizations are based on information provided by the online resource Gene Ontology and DAVID Bio-informatics Resources.

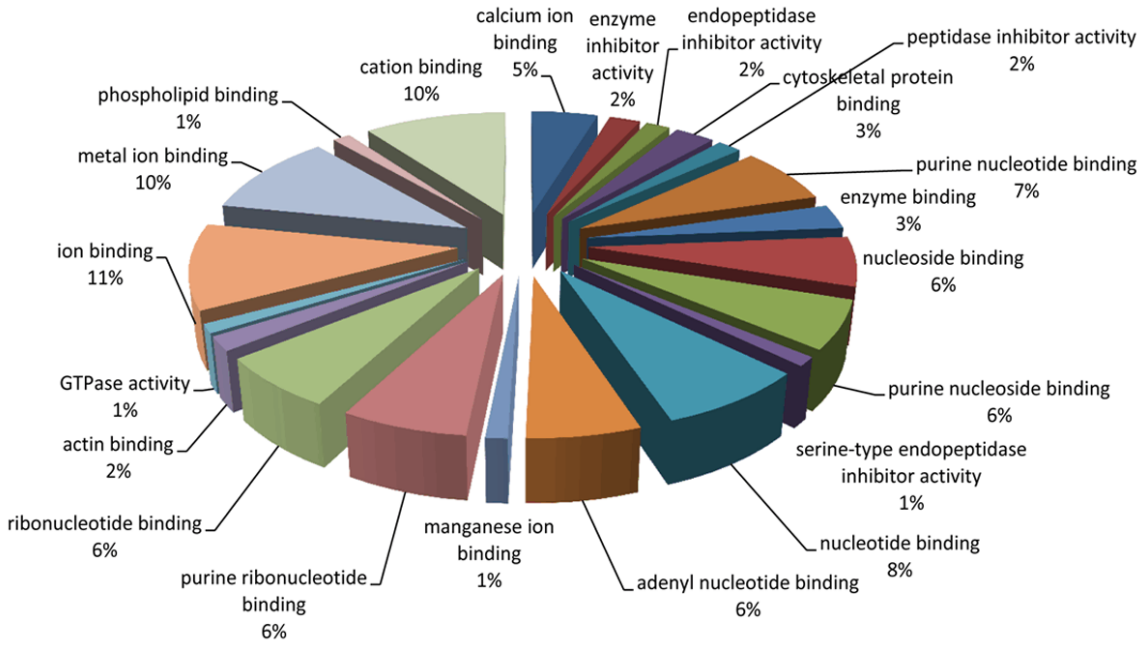

Figure 2. The molecular function categories by gene number according to bioinformatics analysis. Categorizations are based on information provided by the online resource Gene Ontology and DAVID Bio-informatics Resources.

that the majority of proteins were involved in regulation of nervous system development, regulation of ion transport, protein complex biogenesis and cytoskeleton organization et al. (**Figure 3**).

Network of the differential proteins change >2.0-fold

All the differentially expressed proteins with more than 2.0-foldchange were uploaded into

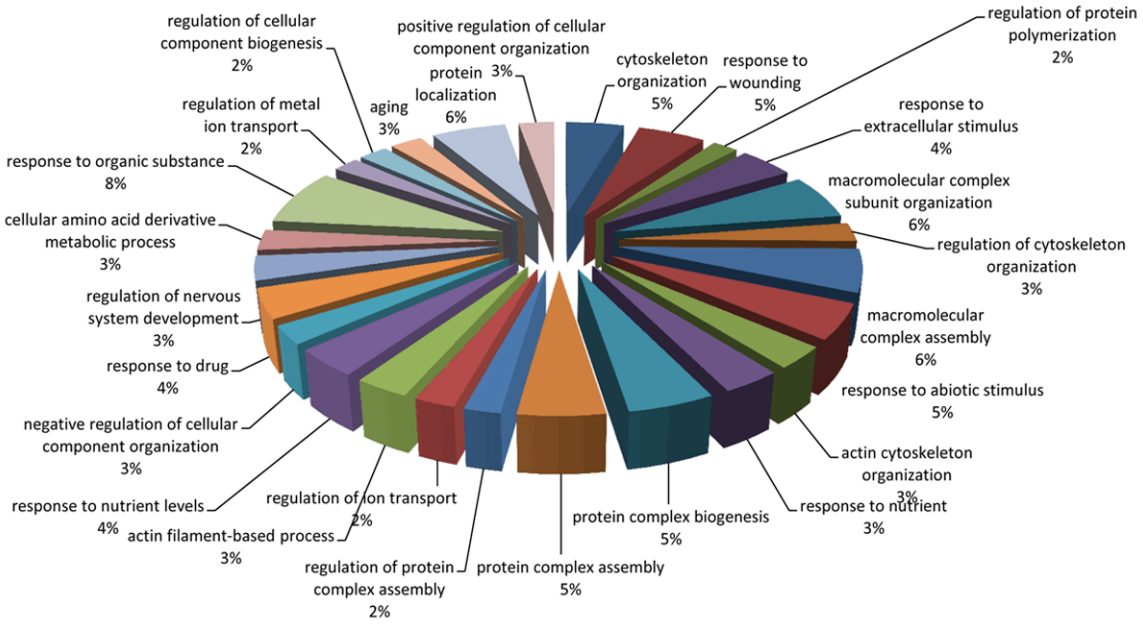

Figure 3. The biological process categories according to bioinformatics analysis. Categorizations are based on information provided by the online resource Gene Ontology and DAVID Bio-informatics Resources.

the STRING 9.0 software to analyze the interactions. As shown in **Figure 4**, interactions between proteins form a complex network. In the middle of this network, there existed several proteins with the most complicated interaction with other proteins. For example, there were several ribosomal related proteins such like Rpl30, Rps14 and Rps6kb1 et al. As we all known that ribosome was an important organelle which provide the location for protein synthesis. Rps6kb1 played a more critical role in protein synthesis which was a serine/threonine-protein kinase that acts downstream of mTOR signaling. In addition, there existed some mitochondrial heat shock proteins such as Trap1 and Hspe1 which could influence the ATPase cativity. Furthermore, there were also some glucose-regulated proteins (Hspa5) and some fatty acids related proteins (Acaca). Besides, some important proteins participated in neuron or axon related process including microtubule-associated protein tau (mapt) and neurofibromin 1 et al. were all interacted with several proteins detected in this study.

Increased expression of MAPT (Tau) and Neurofibromin 1 after hypoxic-ischemic brain damage

Among the differential expressed proteins, some important proteins participated in neuron or axon related process such as microtubule-associated protein tau (MAPT) and *Neurofibromin 1* et al. According to our study, both of these proteins are absent in LPS group. We used Western blot to verify the fold changes of MAPT and *Neurofibromin 1* mentioned above. Results of Western blot showed that a nearly 2.57-fold change was detected for Tau and 2.34-fold change was detected for *Neurofibromin 1* (**Figure 5**). The results were coincident with proteomic analysis.

Discussion

Intrauterine infection is one of the most important causes of white matter injury in preterm premature infants, can cause severe periventricular leukomalacia (PVL), then lead to spastic cerebral paralysis, cognitive and behavioral disorders and other sequelae [9-11]. Research [12] showed that intrauterine administration of TLR-4-specific LPS resulted in the activation of NF-kappaB in the maternal uterus and the fetal brain and up-regulation of proinflammatory proteins. Lee et al. [13] found that antenatal corticosteroid therapy can reduce infection associated with systemic immune response and reduce cytokines on developing oligodendrocytes damage, thereby reducing the incidence of PVL. But the mechanism and therapy of intrauterine infection remains elusive to date.

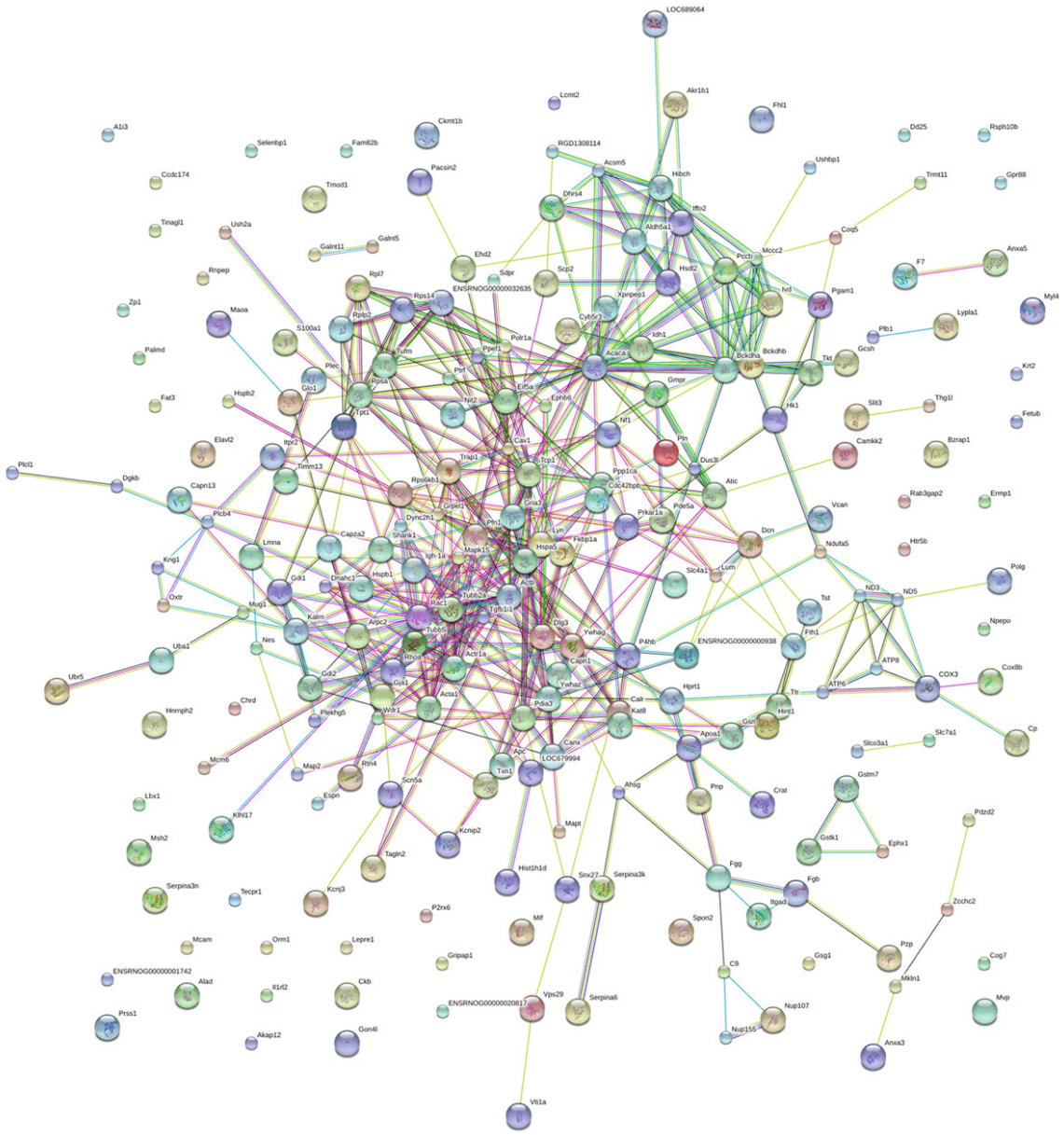

Figure 4. The network analysis of proteins differentially expressed using STRING 9. All the differentially expressed proteins with more than 2.0-fold change were uploaded into the STRING 9.0 software to analyze the interactions. Color ball: the changed protein; yellow line: text mining; purple line: experiments; blue line: databases; light blue: homology; black line: coexpression; green line: neighborhood; red line, gene fusion; deep blue: cooccurrence.

In this study, we established intrauterine infection animal model using pregnant SD rats. LPS was injected to pregnant rats for two days from gestation day 18 to mimics intrauterine infection. We used a label-free quantitative shotgun proteomic method to investigate the proteins expression profile of intrauterine infection. According to the results, 11 proteins were more than 2-fold up-regulated and 11 proteins were more than 2-fold down-regulated in LPS group, while 211 proteins were absent in LPS group. To identify what category they belonged to, we used Gene Ontology database to analyze the classification of these differentially expressed proteins. Results showed that the majority of these proteins comprised mitochondrial component or proteins, cytoskeleton and organelle et al. These results indicated that mitochondria, cytoskeleton and organelles reacted quickly and sensitively. And these also indicat-

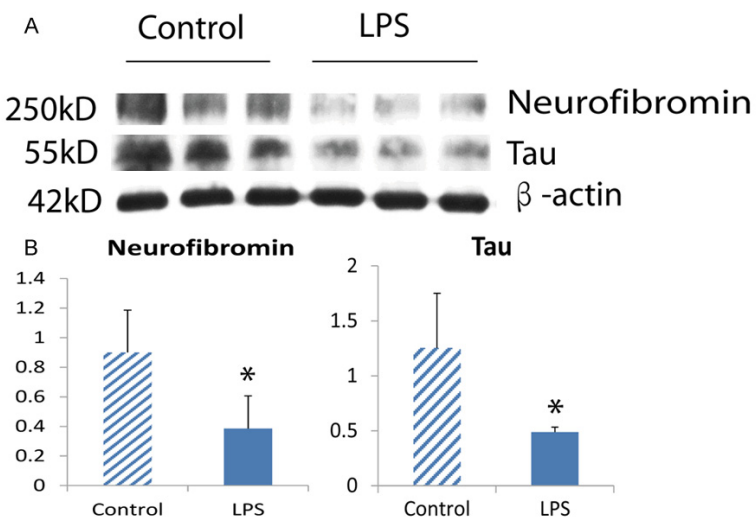

Figure 5. Changes in the protein expression of Neurofibromin and Tau determined by Western blot analysis. Expression of Neurofibromin was down-regulated in LPS group compared with control group (fold change=2.34). Expression of Tau was down-regulated in LPS group compared with control group (fold change=2.57). Expression of β-actin was used as control.

ed that proteins of mitochondria, cytoskeleton and organelle were more susceptible to intrauterine infection assault.

We used DAVID database to analyze the molecular function and biological process of these proteins. Molecular function analysis indicated that the majority of the differentially expressed proteins were involved in ion binding, nucleotide binding and enzyme binding et al. As we all know that the activation of energy metabolism and enzymes leads to a series of follow-up process. Then we want to know what biological process these proteins participate in. DAVID biological process analysis showed that the majority of proteins were involved in regulation of nervous system development, regulation of ion transport and cytoskeleton organization et al. This result can explain the most common sequelae of intrauterine infection such like mental retardation and so on.

STRING 9.0 software can be used to analyze and explore the complex interactions potentially existed among the differentially expressed proteins. We found that most differentially expressed proteins interacted with each other more or less (**Figure 4**). Within this complex network, the proteins with most relationship to others are almost enzymes. Besides, we chose two proteins to verify using Western blot-Tau and Neurofibromin 1 (NF1). Tau (also named (microtubule-associated protein tau) promotes microtubule assembly and stability, and also might be involved in the function of neuronal polarity. Neurofibromin 1 Stimulates the GTPase activity of Ras and might be a negative regulator of Ras signaling pathway.

According to the results of Western blot, we found that there existed the same trend with the results of label-free shotgun proteomics. This novel study performed a full-scale screening of the proteomics research in intrauterine infection of immature rat and may provide the potential targets to treatment in future.

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Disclosure of conflict of interest

None.

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