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Differences in Hippocampal Protein Expression at 3 Days, 3 Weeks, and 3 Months Following Induction of Perinatal Asphyxia in the Rat

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Perinatal asphyxia (PA) was induced in Sprague–Dawley rats; pups were sacrificed 3 days, 3 weeks, and 3 months following the asphyctic insult, and hippocampal protein levels were determined by a gel-based proteomic method. Levels of antioxidant, metabolic, cytoskeleton, signaling, channel, proteasomal, chaperone, splicing, and synaptosomal proteins were dysregulated depending on the age following induction of PA. These proteins are proposed to be potential markers or pharmacological targets for PA.

Keywords: Perinatal Asphyxia • Hippocampus • Rat • Protein Dysregulation

Introduction

A series of individual protein derangements have been reported in brain of animal models of perinatal asphyxia (PA), and a series of proteins from peripheral compartments were studied in Man and animals.[1–9] Only one systematic study at the nucleic acid level has been carried out, however, in order to search for transcripts differentially expressed in brain of rodents with PA.[10] Deficient protein synthetic machinery has been shown in brain of rats with PA in the early phase[11] lasting for at least 8 days following the asphyctic insult.[12] Deficient protein synthesis in terms of impaired RNA polymerase levels and activity would lead to expressional changes of brain proteins, thus, forming the rationale for designing a study to monitor protein levels at different time points following induction of PA. And indeed, brain protein derangement could be expected as a long-term study revealed longlasting effects of PA on brain protein levels in the rat still at the end of the life span.[13] As brain protein expression varies enormously between brain areas, we selected the hippocampus as a brain area that was permanently affected and accompanied by cognitive and behavioral changes.[14,15] The animal model of PA used herein was selected as this rat model is well-documented in terms of morphology, metabolism, and neurochemistry, and furthermore, asphyxiated pups can survive and be studied for the whole life span.[16–20]

The advent of high-throughput proteomics techniques allows generation of relatively large brain proteomes[21–23] that can be reliably compared between groups and are enabling concomitant and unambiguous identification and characterization of proteins. It was the aim of the study to search for differentially expressed hippocampal proteins that may be used as candidate markers for PA as representatives of individual pathways, cascades, and protein networks involved and representing pharmacological targets.

Although a handful of markers for PA including *S100*,[24] *CK-BB*,[25] *interleukin-6*, and *neuron-specific enolase*[26,27] have been tested in humans, novel and more specific candidate markers for the detection of neuronal damage have to be developed; *S100* is an astroglial marker, *interleukin-6* is not directly related to neuronal structures, and *neuron-specific enolase* can be observed in many tissues using proteomic techniques.[28,29] Likewise, many cascades have been incriminated to participate in pathomechanisms of PA[30] including signaling, cytoskeleton, antioxidant, nitric oxide synthetising proteins, and apoptosis-related proteins, but proteins of asphyxia-specific pathways remain to be identified. Finally, identification of tentative pharmaceutical targets has not been systematically envisaged.

With the use of stringent statistical evaluation, proteins from the protein synthetic, chaperoning, and degradation machinery; oxygen and intermediary metabolism; cytoskeleton; signaling; and synaptic apparatus were differentially expressed in PA at different time points.

Experimental Section

Animals. Female Sprague–Dawley rats (Institute of Animal Breeding, Medical University of Vienna, Himberg, Austria) were housed in groups of up to six per cage. Rats were maintained on 11/13 h light/dark cycle in a temperature (21 ± 1 °C) and humidity (50 ± 10%) controlled and well-ventilated room with access to food and drink ad libitum.

The animals were bred and kept under specific pathogen free (SPF) conditions, and all of the experiments were carried out in accordance with the rules of the American Physiology Society.[16] Experiments were approved by the Medical University of Vienna's Committee for Animal Experiments and the Ministry of Science (certificate GZ66.009/80-BrGT/2003).

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Induction of Perinatal Asphyxia. PA was induced as described elsewhere.[31,32]

Asphyxia was induced in pups delivered by Caesarian section on pregnant Sprague–Dawley rats. Within the last day of gestation as evaluated by estabularium protocols, animals were anesthetized with diethyl ether and hysterectomized. The uterus horns, still containing the fetuses, were extirpated and placed in a water bath at 37 °C for 20 min following removal of pups that served as normoxic controls.

Following the asphyctic period, that is, incubation at 37 °C, the uterus horns were rapidly opened and pups removed. Pups were cleaned, the umbilical cord was ligated, and the animals were allowed to recover on a heating pad. Pups were readily accepted and nourished by the foster mothers.

Only litters with pups weighing more than 4.5 g were used for the experiments. Animals from the offspring ($n = 10$ per group), normoxic and asphyctic, at 3 days, 3 weeks, and 3 months (the rationale to select these three age groups was that these are widely used for biochemical, genetic, and pharmacological studies) were sacrificed by decapitation, brains were rapidly removed, and complete hippocampal tissue was taken within 1 min, snap-frozen, and stored at −80 °C until chemical analysis, and the freezing chain was never interrupted.

Sample Preparation. Pooled left and right hippocampal tissue samples ($n = 10$ hippocampi per group), from 10 rats that were not littermates, were homogenized and suspended in 1.8 mL of sample buffer consisting of 8 M urea (Merck, Darmstadt, Germany), 2 M thiourea (Sigma, St. Louis, MO), 4% CHAPS (3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate) (Sigma, St. Louis, MO), 65 mM 1,4-dithioerythritol (Merck, Germany), 1 mM PMSF, and 0.5% carrier ampholytes Resolyte 3.5–10 (BDH Laboratory Supplies, Electran, England).

Samples were left at 21 °C for 1 h and then centrifuged at 14 000 × *g* for 60 min, and the supernatant was transferred into Ultrafree-4 centrifugal filter units (Millipore, Bedford, MA) for desalting and concentrating proteins.[33]

Protein content of the supernatant was quantified by the Bradford protein assay system.[34]

Two-Dimensional Gel Electrophoresis (2-DE). 2-DE was performed as reported.[35] Samples of 800 μg of protein were applied on immobilized pH 3–10 nonlinear gradient strips in sample cups at their basic and acidic ends. Focusing was started at 200 V, and the voltage was gradually increased to 8000 at 4 V/min and then kept constant for a further 3 h (approximately 150 000 Vh totally). After the first dimension, strips (18 cm) were equilibrated for 15 min in the buffer containing 6 M urea, 20% glycerol, 2% SDS, and 2% DTT, and then for 15 min in the same buffer containing 2.5% iodoacetamide instead of DTT. After equilibration, strips were loaded on 9–16% gradient sodium dodecyl sulfate-polyacrylamide gels for second-dimensional separation. The gels (180 × 200 × 1.5 mm) were run at 40 mA per gel. Immediately after the second-dimension run, gels were fixed for 12 h in 50% methanol, containing 10% acetic acid, and the gels were stained with Colloidal Coomassie Blue (Novex, San Diego, CA) for 12 h on a rocking shaker. Molecular masses were determined by running standard protein markers (Biorad Laboratories, Hercules, CA) covering the range 10–250 kDa. p*I* values were used as given by the supplier of the immobilized pH gradient strips (Amersham Bioscience, Uppsala, Sweden). Excess of dye was washed out from the gels with distilled water, and the gels were scanned with ImageScanner (Amersham Bioscience).

Electronic images of the gels were recorded using Adobe Photoshop.

Quantification of Protein Spots. Protein spots were outlined (first automatically and then manually) and quantified using the ImageMaster 2D Elite software (Amersham Biosciences, Uppsala, Sweden). The percentage of the volume of the spots representing a certain protein was determined in comparison with the total proteins present in the 2-DE gel.[36]

Matrix-Assisted Laser Desorption Ionization Mass Spectrometry. Spots were excised with a spot picker (PROTEINEER sp, Bruker Daltonics, Germany) and placed into 384-well microtiter plates, and in-gel digestion and sample preparation for MALDI analysis were performed by an automated procedure (PROTEINEER dp, Bruker Daltonics).[37,38] Briefly, spots were excised and washed with 10 mM ammonium bicarbonate and 50% acetonitrile in 10 mM ammonium bicarbonate. After washing, gel plugs were shrunk by the addition of acetonitrile and dried by blowing out the liquid through the pierced well bottom. The dried gel pieces were reswollen with 40 ng/L of trypsin (Promega) in enzyme buffer (consisting of 5 mM octyl-D-glucopyranoside (OGP) and 10 mM ammonium bicarbonate) and incubated for 4 h at 30 °C. Peptide extraction was performed with 10 μL of 1% TFA in 5 mM OGP. Extracted peptides were directly applied onto a target (AnchorChip, Bruker Daltonics) that was loaded with α-*cyano-4-hydroxy-cinnamic acid* (Bruker Daltonics) matrix thin layer. The mass spectrometer used in this work was an Ultraflex TOF/TOF (Bruker Daltonics) operated in the reflector mode for MALDI-TOF peptide mass fingerprint (PMF) or LIFT mode for MALDI-TOF/TOF fully automated using the FlexControl software. An accelerating voltage of 25 kV was used for PMF. Calibration of the instrument was performed externally with $[M + H]^+$ ions of angiotensin I, angiotensin II, substance P, bombesin, and adrenocorticotropic hormones (clip 1–17 and clip 18–39). Each spectrum was produced by accumulating data from 200 consecutive laser shots. Those samples that were analyzed by PMF from MALDI-TOF and were significantly different between groups were additionally analyzed using LIFT-TOF/TOF MS/MS from the same target. A maximum of three precursor ions per sample were chosen for MS/MS analysis. In the TOF1 stage, all ions were accelerated to 8 kV under conditions promoting metastable fragmentation. After selection of jointly migrating parent and fragment ions in a timed ion gate, ions were lifted by 19 kV to high potential energy in the LIFT cell. After further acceleration of the fragment ions in the second ion source, their masses could be simultaneously analyzed in the reflector with the Mascot software (Matrix Science Ltd., London, U.K.). Database searches, through Mascot, using combined PMF and MS/MS data sets, were performed via BioTools2.2 software (Bruker). A mass tolerance of 25 ppm and 1 missing cleavage site for PMF and MS/MS tolerance of 0.5 Da but no missing cleavage site for MS/MS search were allowed, and oxidation of methionine residues was considered. The probability score calculated by the software was used as criterion for correct identification. The algorithm used for determining the probability of a false-positive match with a given mass spectrum is described elsewhere.[39]

Statistical Evaluation. Values from densitometry of spots from 60 gels are expressed as means ± standard deviation (SD) of the percentage of the spot volume in each particular gel after subtraction of the background values. Between-group differences were analyzed using the Kruskal–Wallis test or, when appropriate, the Fisher's exact test was applied.

GraphPad InStat version (GraphPad Software, San Diego, CA) was used for all statistical analysis, and the level of significance

was set at $P < 0.001$ at all instances, thus, warranting stringent statistical criteria.

Results

A series of proteins, from several cascades and pathways has been identified (Tables 1 and 2; Supplemental Tables 3 and 4 in Supporting Information). A master map of proteins identified is shown in Figure 1.

In Table 1, means and standard deviation, as well as statistical level of significance (*P*-values < 0.001), are listed. With the use of stringent criteria, the level of significance was set at this reliable level of statistical significance correcting for multiple testing.[40]

As shown in Table 1, proteins with levels significantly different between groups represented antioxidant, chaperone, cytoskeleton, miscellaneous, neuron-associated, nucleid acid binding, proteasome, and signaling proteins.

Information on chemistry, biology, and medical relevance of the corresponding proteins is not given herein and can be tracked easily from the database (http://www.expasy.org/) as the Swiss-Prot numbers are provided.

As shown in Table 1, findings were highly specific for individual time points following the asphyctic insult.

Peroxiredoxin 6 was increased 3 months following the induction of PA and may indicate long-term sequelae and long-lasting effects on the antioxidant response.

Hsp90 co-chaperone *Cdc37* was undetectably low in control rat pups and became transiently detectable at 3 days following PA. No levels of other heat shock proteins or chaperones out of the many detected (Supplemental Table 3 in Supporting Information) were deranged by PA probably indicating the specificity of this co-chaperone for hypoxic/ischemic states in PA.

Only one of several fascin protein spots on 2-DE showed levels significantly dysregulated in PA, and this finding was observed in the early phase of PA. *Dynamin-1* levels were significantly dysregulated at 3 weeks following PA, but due to technical reasons, quantification was not carried out because of insufficient separation of some *dynamin-1* spots in the gel at the earlier period, and therefore, no conclusion can be drawn about the early time-point.

An about 6-fold difference between *3-mercaptopyruvate sulfurtransferase* levels between normoxic and asphyctic animals was shown at 3 days of PA probably indicating specific metabolic as well as redox derangement.[41]

Only one out of four detectable *synapsin IIb* expression forms, high-abundant proteins in hippocampus, showed levels different between normoxia and PA in the early phase of PA. The importance that the PA time point is studied is revealed by the long-term change of the *voltage-dependent anion-selective channel protein 1* expression form represented by spot no. 3.

During the early phase of PA, levels of a major component of the protein synthetic machinery were deranged proposing a role for *Heterogeneous nuclear ribonucleo-protein D-like* in the early asphyctic pathology.

An essential component of the proteasome, *subunit beta type 4*, presented with abnormal levels at 3 weeks following PA.

Aberrant levels of signaling proteins were either observed at the early phase of PA (*adenylyl cyclase-associated protein 1*) or in the late phase exclusively (*B-cell antigen receptor complex associated protein alpha-chain A*).

It must be noticed that Fisher's exact test was applied when numbers of present or absent spots were statistically analyzed (Table 1).

Table 2 lists the protein identification criteria for proteins with different levels between groups. The list of nonsignificantly different protein levels is shown in Supplemental Table 3 in Supporting Information. The identification of proteins with comparable levels between groups is provided in Supplemental Table 4 in Supporting Information.

Discussion

The major findings of this study reflect impairment of the brain's protein machinery, deranged signaling, and altered glia maturation. The observations at the protein chemical level are mainly in agreement with biological findings of PA in the rat and, in particular, with pathobiochemical and histological findings described in the identical animal model.

As shortly mentioned above, the protein machinery has been shown to be impaired in terms of protein levels and activity.[11] Detailed information of the impairment, however, remains to be elucidated, and no specific proteins or cascades have been addressed so far. The findings also clearly show that different cascades are shown at different time points, and indeed, early changes were showing most of the derangements in protein levels while only two proteins from cytoskeleton and a proteasomal component were affected at 3 weeks following PA. Antioxidant, ion channel, and two signaling proteins were specific for late changes of protein levels.

Heterogeneous nuclear ribonucleoproteins (*hnRNPs*) are key structures of the splicing apparatus, regulating mRNA-biogenesis and *hnRNP D-like* is a representative with increased levels in PA at the early (3d) phase following the asphyctic insult. Tsuchiya and co-workers reported a novel *hnRNP-like* protein and its expression in myeloid-leukemia cells,[42] and Kamei and co-workers showed the presence of two expression forms.[43] In the present study, one expression form was detected exclusively. Kamei and Yamada demonstrated domains with high-affinity binding sites that can be discriminated from *hnRNP-D*.[44] Kawamura et al. revealed that the dominant expression form shuttles between the nucleus and the cytoplasm and that the protein specifically accumulated upon polymerase transcription inhibition.[45]

It may well be that inhibition of polymerase I, as shown in the identical animal model of PA until at least day 8 following induction of PA,[11] led to the observed increase of *hnRNP D-like*. We may propose that this hnRNP is reflecting impaired nuclear export in PA.

Once proteins are synthetized, they are chaperoned by specific structures. *Hsp90 co-chaperone cdc37*(CDC) is a protein kinase-targeting molecular chaperone and is required in co-operation with *Hsp90* for various signaling kinases.[46] Harris and co-workers recently showed that CDC has a direct regulatory effect on endothelial NOS and may play an important role in mediating the eNOS protein complex formation as well as subsequent eNOS phosphorylation and activation.[47] The intriguing finding that CDC was expressed in hippocampi of rats with PA but not controls may indicate a role in the nitric oxide pathways: eNOS and other NOS systems are key mediators in hypoxic-ischemic states in early life.[48,49] In the applied rat model of PA, only nNOS was studied systematically,[18] and it is not known whether CDC is interacting with this expression form as well.

Table 1. Protein Levels Significantly Different between Groups [a]

accession number	name	3d CO mean ± SD (Fisher's exact)	3w CO mean ± SD (Fisher's exact)	3m CO mean ± SD (Fisher's exact)	3d PA mean ± SD (Fisher's exact)	3w PA mean ± SD (Fisher's exact)	3m PA mean ± SD (Fisher's exact)	3d CO vs PA	3w CO vs PA	3m CO vs PA
				Antioxidant						
O35244	Peroxiredoxin 6 (rat)	0.117 ± 0.037	0.397 ± 0.076	0.279 ± 0.081	0.125 ± 0.024	0.395 ± 0.110	0.670 ± 0.177	n.s.	n.s.	<0.0001
				Chaperone						
Q63692[1]	Hsp90 cochaperone Cdc37 (rat)	0.000 ± 0.000 (0/9)	x	x	0.033 ± 0.027 (6/7)	x	x	0.0009	x	x
				Cytoskeleton						
Q61553-spot 1[1]	Fascin (mouse)	0.239 ± 0.099 (9/9)	0.031 ± 0.053 (3/10)	0.069 ± 0.042 (7/8)	0.000 ± 0.000 (0/7)	0.000 ± 0.000 (0/8)	0.064 ± 0.058 (7/10)	<0.0001	n.s.	n.s.
Q61553-spot 2[2]		0.522 ± 0.136	0.334 ± 0.084	0.195 ± 0.058	0.571 ± 0.167	0.290 ± 0.118	0.192 ± 0.073	n.s.	n.s.	n.s.
Q61553-spot 3[2]		0.177 ± 0.072	0.085 ± 0.024	0.126 ± 0.027	0.115 ± 0.068	0.107 ± 0.074	0.080 ± 0.021	n.s.	n.s.	n.s.
Q61553-total[2]		0.938 ± 0.184	0.409 ± 0.169	0.395 ± 0.053	0.686 ± 0.168	0.370 ± 0.165	0.331 ± 0.052	n.s.	n.s.	n.s.
P39053 spot 1	Dynamin-1 (mouse)	x	0.112 ± 0.046	0.157 ± 0.116	x	0.020 ± 0.035	0.180 ± 0.121	x	0.0002	n.s.
P39053 spot 2[2]		x	0.186 ± 0.076	0.237 ± 0.151	x	0.124 ± 0.067	0.283 ± 0.176	x	n.s.	n.s.
P39053 total[2]		x	0.298 ± 0.111	0.377 ± 0.210	x	0.144 ± 0.091	0.463 ± 0.292	x	n.s.	n.s.
				Metabolic						
P97532	3-mercaptopyruvate sulfurtransferase (rat)	0.028 ± 0.055	0.135 ± 0.052	0.082 ± 0.043	0.173 ± 0.057	0.165 ± 0.103	0.057 ± 0.020	0.0008	n.s.	n.s.
				Miscellaneous						
P52555	Endoplasmic reticulum protein ERp29 [Precursor]	0.520 ± 0.093	0.443 ± 0.139	0.270 ± 0.075	0.729 ± 0.076	0.449 ± 0.103	0.288 ± 0.074	0.0002	n.s.	n.s.
Q63228	Glia maturation factor beta (rat)	1.770 ± 0.564	0.200 ± 0.035	0.162 ± 0.106	0.226 ± 0.178	0.192 ± 0.082	0.162 ± 0.055	<0.0001	n.s.	n.s.
				Neuron Associated						
Q 9QWV7 spot 1[2]	Synapsin IIb (mouse)	0.215 ± 0.083	0.219 ± 0.082	0.338 ± 0.168	0.170 ± 0.114	0.222 ± 0.073	0.466 ± 0.175	n.s.	n.s.	n.s.
Q 9QWV7 spot 2[2]		0.083 ± 0.042	0.310 ± 0.058	x	0.043 ± 0.083	0.265 ± 0.054	x	n.s.	n.s.	x
Q 9QWV7 spot 3[2]		0.161 ± 0.023	0.156 ± 0.084	0.293 ± 0.119	0.605 ± 0.232	0.310 ± 0.176	0.288 ± 0.126	0.0002	n.s.	n.s.
Q 9QWV7 total[2]		0.441 ± 0.078	0.654 ± 0.150	0.631 ± 0.204	0.751 ± 0.365	0.709 ± 0.186	0.710 ± 0.276	n.s.	n.s.	n.s.
Q 60932 spot 1[2]	Voltage-dependent anion-selective channel protein 1 (mouse)	0.214 ± 0.098	0.258 ± 0.082	0.244 ± 0.090	0.100 ± 0.038	0.281 ± 0.148	0.363 ± 0.136	n.s.	n.s.	n.s.
Q 60932 spot 2[2]		0.176 ± 0.066	0.360 ± 0.187	0.428 ± 0.181	0.147 ± 0.057	0.427 ± 0.126	0.578 ± 0.082	n.s.	n.s.	n.s.
Q 60932 spot 3[2]		0.213 ± 0.090	0.401 ± 0.181	0.154 ± 0.067	0.167 ± 0.135	0.302 ± 0.166	0.424 ± 0.101	n.s.	n.s.	0.0006
Q 60932 total[2]		0.536 ± 0.162	1.020 ± 0.369	0.717 ± 0.320	0.358 ± 0.203	0.974 ± 0.333	1.210 ± 0.438	n.s.	n.s.	n.s.
				Nucleic Acid Binding						
Q9CT01	JKTBP (syn: Heterogeneous nuclear ribonucleo-protein D-like)	0.377 ± 0.045	0.163 ± 0.051	0.148 ± 0.039	0.535 ± 0.061	0.168 ± 0.048	0.123 ± 0.012	0.0002	n.s.	n.s.
				Proteasome						
P99026[1]	Proteasome subunit beta type 4 [Precursor] (mouse)	0.000 ± 0.000 (0/9)	0.076 ± 0.015 (8/8)	0.084 ± 0.054 (7/7)	0.000 ± 0.000 (0/9)	0.000 ± 0.000 (0/8)	0.041 ± 0.027 (8/10)	n.s.	0.0002	n.s.
				Signaling						
P40124	Adenylyl cyclase-associated protein 1 (mouse)	0.163 ± 0.052	0.362 ± 0.094	0.203 ± 0.079	0.370 ± 0.128	0.466 ± 0.135	0.227 ± 0.082	0.0003	n.s.	n.s.
P11911[1]	B-cell antigen receptor complex associated protein alpha-chain [Precursor] A (mouse)	x	x	0.103 ± 0.064	x	x	0.000 ± 0.000	x	x	<0.0001
P31044	Phosphatidylethanolamine-binding protein (rat)	x	0.882 ± 0.163	0.448 ± 0.094	x	1.018 ± 0.234	1.062 ± 0.140	x	n.s.	<0.0001

[a] PA = perinatal asphyxia; CO = controls. Protein levels significantly different at the $P < 0.001$ level are indicated. In addition to comparison of groups by Kruskal–Wallis test the Fisher's exact test was performed when applicable: when spots were missing, the number of present spots/total amount of gels is indicated and listed in brackets.

Table 2. Identification of Proteins Significantly Different between Groups

accession number	protein name	matches	score	p*I*	(kDa)
	Antioxidant				
O35244	Peroxiredoxin 6 (rat)	25	198	5.65	24.68
	Chaperone				
Q63692	Hsp90 cochaperone Cdc37 (rat)	13	70	5.24	44.51
	Cytoskeleton				
Q61553	Fascin (mouse)	35	269	6.21	54.40
P39053	Dynamin-1 (mouse)	32	228	7.61	97.80
	Metabolic				
P97532	3-Mercaptopyruvate sulfurtransferase (rat)	11	68	5.88	32.80
	Miscellaneous				
P52555	Endoplasmic reticulum protein ERp29 [Precursor]	14	112	6.23	28.57
Q63228	Glia maturation factor beta (rat)	14	151	5.32	16.60
	Neuron Associated				
Q9QWV7	Synapsin IIb (mouse)	24	122	7.61	52.45
Q60932	Voltage-dependent anion-selective channel protein 1 (mouse)	14	128	8.35	32.35
	Nucleic Acid Binding				
Q9CT01	JKTBP (syn: Heterogeneous nuclear ribonucleoprotein D-like)	11	114	4.90	16.53
	Proteasome				
P99026	Proteasome subunit beta type 4 [Precursor] (mouse)	12	70	5.47	29.11
	Signaling				
P40124	Adenylyl cyclase-associated protein 1 (mouse)	19	74	7.30	51.44
P11911	B-cell antigen receptor complex associated protein alpha-chain [Precursor] A (mouse)	8	63	4.81	24.26
P31044	Phosphatidylethanolamine-binding protein (rat)	17	214	5.47	20.80

Endoplasmic reticulum protein ERp29 is a protein with chaperone activity and maybe involved in protein secretion. It is ubiquitously expressed in the ER, highly conserved in all mammalian species and may facilitate folding and/or export of secretory proteins in/from the ER. It is expressed by stress-like accumulation of unfolded proteins in the ER.[50]

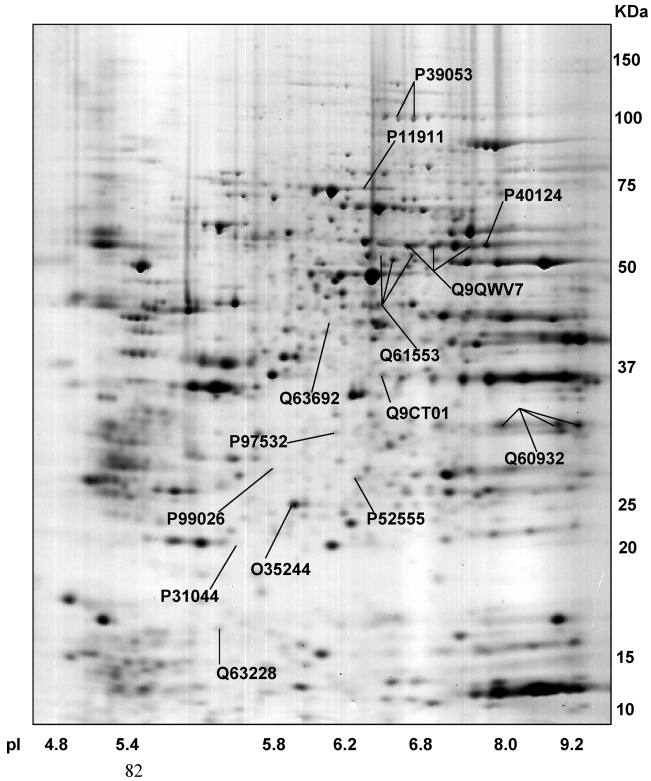

Figure 1. A Coomassie-blue stained master gel showing the identification of proteins with significantly different hippocampal levels between groups. (2-DE-gel of hippocampus at 3 months).

Hermann and co-workers proposed a distinctive type of folding activity of *ERp29* in the ER machinery probably mediated by the typical *Cys-125*.[51] Park and co-workers demonstrated cooperation of *ERp29* with other ER localized chaperones including *GRP94*, *BiP*, *ERp72*, and *calnexin* supporting the proposed chaperone activity.[52] Like *Hsp90 cochaperone cdc37*, an expression form (spot 1) and total *ERp29* were increased in the early phase of PA (at 3 days) probably due to ER stress and may help to cope with accumulation of misfolded/unfolded proteins. *ERp29* was never shown to be induced by hypoxic-ischemic states, and this is the first report probably providing a specific role for this protein.

The proteasome mediates nonlysosomal protein degradation in the cell, and the 26 S proteasome is a large eukaryotic protease complex involved in ubiquitin-mediated degradation of abnormal and many short-lived proteins.[53] This system is known to be involved in hypoxic-ischemic states and to control hypoxia-inducible factor 1 levels.[54–56] In this report, the absence or undetectably low levels of *proteasome subunit beta type 4* (PSBT4) was observed at 3 weeks following induction of PA. At this time point, any link to *hypoxia-inducible factor 1* are highly unlikely and would have been expected at very early time points of hours following the asphyctic insult that were not included in the present study. There is so far no explanation for this finding; that is, no biological role can be proposed. A possible clue for the interpretation comes from an observation that microglial activation by *interferon-gamma* and *lipopolysaccharide* leads to significantly altered proteasomal composition[57] and glial activation is a main finding in PA of rat and guinea pig.[58,59] Therefore, long-lasting microglial activation in PA includes the time point of 3 weeks following induction of PA and may be reflected by *PSBT4*. The result of undetectably low *PSBT4* in hippocampus of PA challenges and warrants further investigations into functions of proteasomes in PA and hypoxic-ischemic states.

Synapsins in the hippocampus are major mediators of cognitive functions, regulating synapse formation and neurotransmitter

release; thus, one could have expected derangement of synapsin levels as in the animal model used, in rats presenting with cognitive impairment. Oxygen and/or glucose deprivation was proposed to induce alterations of *synapsin I*:[60] in organotypic hippocampal slice cultures, *synapsin I* and *phosphosynapsin* were upregulated in areas CA1 and CA3. The model used herein leads to abnormal glucose transport[61] and hypoxia, and the observed increase of *synapsin IIb* may well be due to this mechanism. The biological meaning may be seen as altered neurotransmitter release in terms of altered exocytosis as well as reflecting compensative formation of new synapses following the asphyctic insult. Induction of formation of presynaptic terminals in neuroblastoma cells by *synapsin IIb* was shown,[62] and overexpression of known rat synapsins in NG108–15 neuronal cells enhanced functional synapse formation.[63] *Synapsin 1* levels were not altered in our assay system, and phosphorylation was not tested as in the proteomic technique for 2-DE used, no phosphatase-inhibitors were applied, and therefore, phosphorylation could not be reliably detected and quantified. As previously reported, *synapsin 1* phosphorylation was reduced in 7 day old rats with hypoxia-ischemia, however, at 21 h posthypoxia-ischemia, a time point not addressed in this study.[64] Out of three *synapsin IIb* expression forms, only one (spot 3) was increased pointing to hypoxia-specific regulation or at least hypoxia-ischemia-dependent regulation. The consequence of altered *synapsin IIb* levels may also lead to changes in levels and probably function of the many binding partners and interactions in particular with signaling proteins.[65,66]

One of the signaling proteins observed herein with increased levels at the early phase of PA is *adenylyl cyclase-associated protein 1*(CAP); it is an adenylyl cyclase activating protein cooperating with Ras, that binds to the cyclase at a different region. With another domain, CAP can bind monomeric actin and is therefore carrying out scaffolding functions.

CAP is clearly involved in Ras/cAMP-mediated signal transduction and most likely serves as adaptor protein translocating the adenylyl cyclase complex to the actin cytoskeleton. Involvement of cyclic AMP-dependent protein kinase in hypoxic-ischemic states[67] including PA of the rat[31] has been reported that may link the current findings of increased CAP to protein kinase A and probably to the cytoskeleton abnormalities observed in PA.

Phosphatidylethanolamine-binding protein (PEBP), a structure proposed to be of importance for neural development, has been demonstrated to bind to Raf-1 and *mitogen-activated protein kinase kinase*, components of the ERK signaling pathway[68,69] that inhibits cell proliferation. Its presence in the hippocampus has been documented by a proteomic technique,[70] and apart from its role for signaling, this multifunctional protein represents a novel family of serine protease inhibitors.[71] It has not been described so far in hypoxic-ischemic states (HIS), and we here show HIS-dependent protein levels in the hippocampus. A clue for a tentative role may come from the fact that PEBP contains a hippocampal cholinergic neurostimulating peptide.[72] This may be seen in context of cholinergic derangement that was observed at this time point in PA of the rat.[15] The consequence of the late increase at 3 months of PA is not clear, but long-term derangements of proteins in PA have been already reported.[13]

Another long-term effect observed 3 month following PA is reflected by the increased antioxidant protein *peroxiredoxin 6* in PA. *Peroxiredoxin 6* has not been linked to HIS yet to the best of our knowledge. Although there is some information relating peroxiredoxins to HIS, results cannot be extrapolated to PA or to an in vivo system. We therefore identify *peroxiredoxin 6* as a HIS-inducible antioxidant protein of the late phase.

At 3 weeks, *dynamin-1*, a protein involved in vesicular trafficking, was remarkably decreased in PA, and this may reflect impaired vesicular transport in the HIS. A significant decrease of *dynamin-1* was already reported in rats with PA at the end of the lifespan,[13] but at 3 months of PA, aberrant *dynamin-1* levels were no longer observed in the present study, due to the stringent statistical evaluation setting $P < 0.001$ as the threshold for significance.

Metabolic derangement of several pathways was already reported in PA, and we here add information on an upregulated high-abundance metabolic protein, *3-mercaptopyruvate sulfurtransferase* (3MPST), an antioxidant protein. Williams and co-workers showed that 3MPST is involved in antioxidant and thioredoxin metabolism of Leishmania.[73] Nagahara and Katayama revealed post-translational regulation of 3-*mercaptopyruvate sulfurtransferase* via a low redox potential cysteine-sulfenate in the maintenance of redox homeostasis in the rat.[74] The mercaptopyruvate pathway catalyzing transsulfuration from 3-mercaptopyruvate to pyruvate contributes to the cellular redox system.[75]

Most studies studying antioxidants were focusing on the well-known and documented antioxidant enzymes including SOD, glutathione, and other systems. Herein, we show for the very first time upregulation of 3MPST at the early phase of PA. We are therefore adding a so far hardly studied antioxidant protein to the list of antioxidants involved in PA and HIS, providing the basis for future studies including work on experimental therapies.

Finally, a major outcome of this study is decreased expression of *glial maturation facor beta* (GMFb). Lim and co-workers purified and characterized GMFb from bovine brain. It stimulated differentiation of normal neurons as well as glial cells.[76]

Molecular cloning and expression of biologically active human GMFb was carried out, and the recombinant protein showed activity identical to that of the native protein.[77]

Giulian et al. confirmed that GMFb stimulated myelin protein synthesis in mammalian brain,[78] and in GMF-knockout mice, impaired motor performance and learning was observed.[79] The rat model of PA studied shows myelination deficits, and the results of decreased GMFb at the early phase of PA may be associated if not causing these white matter changes.[80]

In conclusion, a series of proteins have been identified by a reliable and well-documented technology[23,38,81] and shown to be associated with PA in rat hippocampus that may help to explain neuropathology and mechanisms of PA at three different time points in postnatal life. These structures may be representing candidate markers for PA and are identified as hypoxia-ischemia-dependent. The outcome now challenges investigations of protein pathways and cascades, now that individual pathway members have been shown to be linked to the HIS. Moreover, transgenic animals for each of the aberrantly regulated proteins may be now tested for the outcome of PA, and pharmacological interventions may be proposed based upon the results shown herein. Our laboratory is now proceeding with characterization of post-translational modifications that may have occurred during the process of PA.

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Supporting Information Available: Tables listing the protein levels not significantly different between groups and nonsignificant proteins identified. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Costa, S.; Zecca, E.; De Rosa, G.; De Luca, D.; Barbato, G.; Pardeo, M.; Romagnoli, C. Is serum troponin T a useful marker of myocardial damage in newborn infants with perinatal asphyxia. *Acta Paediatr.* **2007**, *96*, 181–184.

(2) Rossiter, J. P.; Anderson, L. L.; Yang, F.; Cole, G. M. Caspase-3 activation and caspase-like proteolytic activity in human perinatal hypoxic-ischemic brain injury. *Acta Neuropathol.* **2002**, *103*, 66–73.

(3) Perrone, S.; Bracci, R.; Buonocore, G. New biomarkers of fetal-neonatal hypoxic stress. *Acta Paediatr. Suppl.* **2002**, *91*, 135–138.

(4) Fernández, A. P.; Alonso, D.; Lisazoaín, I.; Serrano, J.; Leza, J. C.; Bentura, M. L.; López, J. C.; Manuel Encinas, J.; Fernández-Vizarra, P.; Castro-Blanco, S.; Martínez, A.; Martinez-Murillo, R.; Lorenzo, P.; Pedrosa, J. A.; Peinado, M. A.; Rodrigo, J. Postnatal changes in the nitric oxide system of the rat cerebral cortex after hypoxia during delivery. *Brain Res. Dev. Brain Res.* **2003**, *142*, 177–192.

(5) Nagdyman, N.; Grimmer, I.; Scholz, T.; Muller, C.; Obladen, M. Predictive value of brain-specific proteins in serum for neurodevelopmental outcome after birth asphyxia. *Pediatr. Res.* **2003**, *54*, 270–275.

(6) Aly, H.; El Beshlawy, A.; Badrawi, N.; Mohsen, L.; Mansour, E.; Ramy, N.; Patel, K. Thrombopoietin level is increased in the serum of asphyxiated neonates: a prospective controlled study. *J. Perinatol.* **2005**, *25*, 320–324.

(7) Castillo-Meléndez, M.; Chow, J. A.; Walker, D. W. Lipid peroxidation, caspase-3 immunoreactivity, and pyknosis in late-gestation fetal sheep brain after umbilical cord occlusion. *Pediatr. Res.* **2004**, *55*, 864–871.

(8) Marchini, G.; Hagenäs, L.; Kocoska-Maras, L.; Berggren, V.; Hansson, L. O. Insulin-like growth factor binding protein-1 and interleukin-6 are markers of fetal stress during parturition at term gestation. *J. Pediatr. Endocrinol. Metab.* **2005**, *18*, 777–783.

(9) Florio, P.; Luisi, S.; Moataza, B.; Torricelli, M.; Iman, I.; Hala, M.; Hanna, A.; Petraglia, F.; Gazzolo, D. High urinary concentrations of activin A in asphyxiated full-term newborns with moderate or severe hypoxic ischemic encephalopathy. *Clin. Chem.* **2007**, *53*, 520–522.

(10) Labudova, O.; Schuller, E.; Yeghiazarjan, K.; Kitzmueller, E.; Hoeger, H.; Lubec, G.; Lubec, B. Genes involved in the pathophysiology of perinatal asphyxia. *Life Sci.* **1999**, *64*, 1831–1838.

(11) Kastner, P.; Mosgoeller, W.; Fang-Kircher, S.; Kitzmueller, E.; Kirchner, L.; Hoeger, H.; Seither, P.; Lubec, G.; Lubec, B. Deficient brain RNA polymerase and altered nucleolar structure persists until day 8 after perinatal asphyxia of the rat. *Pediatr. Res.* **2003**, *53*, 62–71.

(12) Mosgoeller, W.; Kastner, P.; Fang-Kircher, S.; Kitzmueller, E.; Hoeger, H.; Seither, P.; Labudova, O.; Lubec, G.; Lubec, B. Brain RNA polymerase and nucleolar structure in perinatal asphyxia of the rat. *Exp. Neurol.* **2000**, *161*, 174–182.

(13) Kitzmueller, E.; Krapfenbauer, K.; Hoeger, H.; Weitzdoerfer, R.; Lubec, G.; Lubec, B. Life-long effects of perinatal asphyxia on stress-induced proteins and dynamin 1 in rat brain. *Neurochem. Res.* **2004**, *29*, 1767–1777.

(14) Weitzdorfer, R.; Hoger, H.; Pollak, A.; Lubec, G. Changes of hippocampal protein levels during postnatal brain development. *J. Proteome Res.* **2006**, *5*, 3205–3212.

(15) Kohlhauser, C.; Mosgoeller, W.; Hoeger, H.; Lubec, G.; Lubec, B. Cholinergic, monoaminergic and glutamatergic changes following perinatal asphyxia in the rat. *Cell. Mol. Life Sci.* **1999**, *55*, 1491–1501.

(16) Hoeger, H.; Engidawork, E.; Stolzlechner, D.; Bubna-Littitz, H.; Lubec, B. Long-term effect of moderate and profound hypothermia on morphology, neurological, cognitive and behavioural functions in a rat model of perinatal asphyxia. *Amino Acids* **2006**, *31*, 385–396.

(17) Salchner, P.; Engidawork, E.; Hoeger, H.; Lubec, B.; Singewald, N. Perinatal asphyxia exerts lifelong effects on neuronal responsiveness to stress in specific brain regions in the rat. *J. Invest. Med.* **2003**, *51*, 288–294.

(18) Lubec, B.; Kozlov, A. V.; Krapfenbauer, K.; Berger, A.; Hoeger, H.; Herrera-Marschitz, M.; Nohl, H.; Koeck, T.; Lubec, G. Nitric oxide and nitric oxide synthase in the early phase of perinatal asphyxia of the rat. *Neuroscience* **1999**, *93*, 1017–1023.

(19) Lubec, B.; Marx, M.; Herrera-Marschitz, M.; Labudova, O.; Hoeger, H.; Gille, L.; Nohl, H.; Mosgoeller, W.; Lubec, G. Decrease of heart protein kinase C and cyclin-dependent kinase precedes death in perinatal asphyxia of the rat. *FASEB J.* **1997**, *11*, 482–492.

(20) Klawitter, V.; Morales, P.; Bustamante, D.; Goiny, M.; Herrera-Marschitz, M. Plasticity of the central nervous system (CNS) following perinatal asphyxia: Does nicotinamide provide neuroprotection. *Amino Acids* **2006**, *31*, 377–384.

(21) Lubec, G.; Krapfenbauer, K.; Fountoulakis, M. Proteomics in brain research: potentials and limitations. *Prog. Neurobiol.* **2003**, *69*, 193–211.

(22) Fountoulakis, M.; Juranville, J. F.; Dierssen, M.; Lubec, G. Proteomic analysis of the fetal brain. *Proteomics* **2002**, *2*, 1547–1576.

(23) Afjehi-Sadat, L.; Yang, J. W.; Pollak, A.; Kim, D. W.; Choi, S. Y.; Lubec, G. Structural and functional analysis of hypothetical proteins in mouse hippocampus from two-dimensional gel electrophoresis. *J. Proteome Res.* **2007**, *6*, 711–723.

(24) Thorngren-Jerneck, K.; Alling, C.; Herbst, A.; Amer-Wahlin, I.; Marsal, K. S100 protein in serum as a prognostic marker for cerebral injury in term newborn infants with hypoxic ischemic encephalopathy. *Pediatr. Res.* **2004**, *55*, 406–412.

(25) Nagdyman, N.; Komen, W.; Ko, H. K.; Muller, C.; Obladen, M. Early biochemical indicators of hypoxic-ischemic encephalopathy after birth asphyxia. *Pediatr. Res.* **2001**, *49*, 502–506.

(26) Martin-Ancel, A.; Garcia-Alix, A.; Pascual-Salcedo, D.; Cabanas, F.; Valcarce, M.; Quero, J. Interleukin-6 in the cerebrospinal fluid after perinatal asphyxia is related to early and late neurological manifestations. *Pediatrics* **1997**, *100*, 789–794.

(27) Tekgul, H.; Yalaz, M.; Kutukculer, N.; Ozbek, S.; Kose, T.; Akisu, M.; Kultursay, N.; Gokben, S. Value of biochemical markers for outcome in term infants with asphyxia. *Pediatr. Neurol.* **2004**, *31*, 326–332.

(28) Deloulme, J. C.; helies, A.; Ledig, M.; Lucas, M.; Sensenbrenner, M. A comparative study of the distribution of alpha- and gamma-enolase subunits in cultured rat neuronal cells and fibroblasts. *Int. J. Dev. Neurosci.* **1997**, *15*, 183–194.

(29) Bohnke, M.; Vogelberg, K.; Engelmann, K. Detection of neurone-specific enolase in long-term cultures of human corneal endothelium. *Graefe's. Arch. Clin. Exp. Ophthalmol.* **1998**, *236*, 522–526.

(30) Berger, R.; Garnier, Y. Perinatal brain injury. *J. Perinat. Med.* **2000**, *28*, 261–285.

(31) Lubec, B.; Dell'Anna, E.; Fang-Kircher, S.; Marx, M.; Herrera-Marschitz, M.; Lubec, G. Decrease of brain protein kinase C, protein kinase A, and cyclin-dependent kinase correlating with pH precedes neuronal death in neonatal asphyxia. *J. Invest. Med.* **1997**, *45*, 284–294.

(32) Klawitter, V.; Morales, P.; Johansson, S.; Bustamante, D.; Goiny, M.; Gross, J.; Luthman, J.; Herrera-Marschitz, M. Effects of perinatal asphyxia on cell survival, neuronal phenotype and neurite growth evaluated with organotypic triple cultures. *Amino Acids* **2005**, *28*, 149–155.

(33) Shin, J. H.; Gulesserian, T.; Verger, E.; Delabar, J. M.; Lubec, G. Protein dysregulation in mouse hippocampus polytransgenic for chromosome 21 structures in the Down Syndrome critical region. *J. Proteome Res.* **2006**, *5*, 44–53.

(34) Bradford, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **1976**, *72*, 248–254.

(35) Pollak, D. D.; John, J.; Schneider, A.; Hoeger, H.; Lubec, G. Strain-dependent expression of signaling proteins in the mouse hippocampus. *Neuroscience* **2006**, *138*, 149–158.

(36) Shin, J. H.; London, J.; Le Pecheur, M.; Hoger, H.; Pollak, D.; Lubec, G. Aberrant neuronal and mitochondrial proteins in hippocampus of transgenic mice overexpressing human Cu/Zn superoxide dismutase 1. *Free Radical Biol. Med.* **2004**, *37*, 643–653.

(37) Myung, J. K.; Lubec, G. Use of Solution-IEF-fractionation leads to separation of 2673 mouse brain proteins including 255 hydrophobic structures. *J. Proteome Res.* **2006**, *5*, 1267–1275.

(38) Chen, W. Q.; Kang, S. U.; Lubec, G. Protein profiling by the combination of two independent mass spectrometry techniques. *Nat. Protocols* **2006**, *1*, 1446–1452.

(39) Berndt, P.; Hobohm, U.; Langen, H. Reliable automatic protein identification from matrix-assisted laser desorption/ionization mass spectrometric peptide fingerprints. *Electrophoresis* **1999**, *20*, 3521–3526.

(40) Frischer, T.; Myung, J. K.; Maurer, G.; Eichler, I.; Szepfalusi, Z.; Lubec, G. Possible dysregulation of chaperon and metabolic proteins in cystic fibrosis bronchial tissue. *Proteomics* **2006**, *6*, 3381–3388.
(41) Nagahara, N.; Yoshii, T.; Abe, Y.; Matsumura, T. Thioredoxin-dependent enzymatic activation of mercaptopyruvate sulfurtransferase. An intersubunit disulfide bond serves as a redox switch for activation. *J. Biol. Chem.* **2007**, *282*, 1561–1569.
(42) Tsuchiya, N.; Kamei, D.; Takano, A.; Matsui, T.; Yamada, M. Cloning and characterization of a cDNA encoding a novel heterogeneous nuclear ribonucleoprotein-like protein and its expression in myeloid leukemia cells. *J. Biochem. (Tokyo)* **1998**, *123*, 499–507.
(43) Kamei, D.; Tsuchiya, N.; Yamazaki, M.; Meguro, H.; Yamada, M. Two forms of expression and genomic structure of the human heterogeneous nuclear ribonucleoprotein D-like JKTBP gene (HNRPDL). *Gene* **1999**, *228*, 13–22.
(44) Kamei, D.; Yamada, M. Interactions of heterogeneous nuclear ribonucleoprotein D-like protein JKTBP and its domains with high-affinity binding sites. *Gene* **2002**, *298*, 49–57.
(45) Kawamura, H.; Tomozoe, Y.; Akagi, T.; Kamei, D.; Ochiai, M.; Yamada, M. Identification of the nucleocytoplasmic shuttling sequence of heterogeneous nuclear ribonucleoprotein D-like protein JKTBP and its interaction with mRNA. *J. Biol. Chem.* **2002**, *277*, 2732–2739.
(46) Miyata, Y.; Nishida, E. CK2 binds, phosphorylates, and regulates its pivotal substrate Cdc37, and Hsp90-chaperone. *Mol. Cell. Biochem.* **2005**, 171–179.
(47) Harris, M. B.; Bartoli, M.; Sood, S. G.; Matts, R. L.; Venema, R. C. Direct interaction of the cell division cycle 37 homolog inhibits endothelial nitric oxide synthase activity. *Circ. Res.* **2006**, *98*, 335–341.
(48) Van den Tweel, E. R.; Nijboer, C.; Kavelaars, A.; Heijnen, C. J.; Groenendaal, F.; van Bel, F. Expression of nitric oxide synthase isoforms and nitrotyrosine formation after hypoxia-ischemia in the neonatal rat brain. *J. Neuroimmunol.* **2005**, *167*, 64–71.
(49) Kaur, C.; Sivakumar, V.; Ang, L. S.; Sundaresan, A. Hypoxic damage to the periventricular white matter in neonatal brain: role of vascular endothelial growth factor, nitric oxide and excitotoxicity. *J. Neurochem.* **2006**, *98*, 1200–1216.
(50) Sargsyan, E.; Baryshev, M.; Backlund, M.; Sharipo, A.; Mkrtchian, S. Genomic organization and promoter characterization of the gene encoding a putative endoplasmic reticulum chaperone, ERp29. *Gene* **2002**, *285*, 127–139.
(51) Hermann, V. M.; Cutfield, J. F.; Hubbard, M. J. Biophysical characterization of ERp29. Evidence for a key structural role of cysteine 125. *J. Biol. Chem.* **2005**, *280*, 13529–13537.
(52) Park, S.; You, K. H.; Shong, M.; Goo, T. W.; Yun, E. Y.; Kang, S. W.; Kwon, O. Y. Overexpression of ERp29 in the thyrocytes of FRTL-5 cells. *Mol. Biol. Rep.* **2005**, *32*, 7–13.
(53) Jager, S.; Groll, M.; Huber, R.; Wolf, D. H.; Heinemeyer, W. Proteasome beta-type subunits: unequal roles of propeptides in core particle maturation and a hierarchy of active site function. *J. Mol. Biol.* **1999**, *291*, 997–1013.
(54) Huang, L. E.; Gu, J.; Schau, M.; Bunn, H. F. Regulation of hypoxia-inducible factor 1alpha is mediated by an O2-dependent degradation domain via the ubiquitin-proteasome pathway. *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *95*, 7987–7992.
(55) Mottet, D.; Ruys, S. P.; Demazy, C.; Raes, M.; Michiels, C. Role for casein kinase 2 in the regulation of HIF-1 activity. *Int. J. Cancer.* **2005**, *117*, 764–774.
(56) Schnitzer, S. E.; Schmid, T.; Zjou, J.; Eisenbrand, G.; Brune, B. Inhibition of GSK3beta by indirubins restores HIF-1alpha accumulation under prolonged periods of hypoxia/anoxia. *FEBS Lett.* **2005**, *579*, 529–533.
(57) Stohwasser, R.; Giesebrecht, J.; Kraft, R.; Muller, E. C.; Hausler, K. G.; Kettenmann, H.; Hanisch, U. K.; Kloetzel, P. M. Biochemical analysis of proteasomes from mouse microglia: induction of immunoproteasomes by interferon-gamma and lipopolysaccharide. *Glia* **2000**, *29*, 355–365.
(58) Bernert, G.; Hoeger, H.; Mosgoeller, W.; Stolzlechner, D.; Lubec, B. Neurodegeneration, neuronal loss, and neurotransmitter changes in the adult guinea pig with perinatal asphyxia. *Pediatr. Res.* **2003**, *54*, 523–528.
(59) Kohlhauser, C.; Kaehler, S.; Mosgoeller, W.; Singewald, N.; Kouvelas, D.; Prast, H.; Hoeger, H.; Lubec, B. Histological changes and neurotransmitter levels three months following perinatal asphyxia in the rat. *Life Sci.* **1999**, *64*, 2109–2124.
(60) Jung, Y. J.; Park, S. J.; Park, J. S.; Lee, K. E. Glucose/oxygen deprivation induces the alteration of synapsin I and phosphosynapsin. *Brain Res.* **2004**, *996*, 47–54.
(61) Chiappe-Gutierrez, M.; Kitzmueller, E.; Labudova, O.; Fuerst, G.; Hoeger, H.; Hardmeier, R.; Nohl, H.; Gille, L.; Lubec, B. mRNA levels of the hypoxia inducible factor (HIF-1) and DNA repair genes in perinatal asphyxia of the rat. *Life Sci.* **1998**, *63*, 1157–1167.
(62) Han, H. Q.; Nichols, R. A.; Rubin, M. R.; Bahler, M.; Greengard, P. Induction of formation of presynaptic terminals in neuroblastoma cells by synapsin IIb. *Nature* **1991**, *349*, 697–700.
(63) Zhong, Z. G.; Noda, M.; Takahashi, H.; Higashida, H. Overexpression of rat synapsins in NG108–15 neuronal cells enhances functional synapse formation with myotubes. *Neurosci. Lett.* **1999**, *260*, 93–96.
(64) Moretto, M. B.; de Mattos-Dutra, A.; Arteni, N.; Meirelles, R.; de Freitas, M. S.; Netto, C. A.; Pessoa-Pureur, R. Effects of neonatal cerebral hypoxia-ischemia on the in vitro phosphorylation of synapsin 1 in rat synaptosomes. *Neurochem. Res.* **1999**, *24*, 1263–1269.
(65) Han, S. J.; Hong, S. H.; Kim, C. G.; Lee, J. B.; Choi, D. K.; Kim, K. R.; Kim, C. G. Synapsin IIb interacts with the C-terminal SH2 and SH3 domains of PLCgamma1 and inhibits its enzymatic activity. *Cell. Biol. Int.* **2004**, *28*, 943–948.
(66) Nicol, S.; Rahman, D.; Baines, A. J. Interaction of synapsin IIb with calmodulin: identification of a high affinity site. *Biochem. Soc. Trans.* **1998**, *26*, S109.
(67) Tanaka, K. Alteration of second messengers during acute cerebral ischemia-adenylate cyclase, cyclic AMP-dependent protein kinase, and cyclic AMP response element binding protein. *Progr. Neurobiol.* **2001**, 173–207.
(68) Yamazaki, T.; Nakano, H.; Hayakari, M.; Tanaka, M.; Mayama, J.; Tsuchida, S. Differentiation induction of human keratinocytes by phosphatidylethanolamine-binding proteins. *J. Biol. Chem.* **2004**, *279*, 32191–32195.
(69) Keller, E. T.; Fu, Z.; Brennan, M. The role of Raf kinase inhibitor protein (RKIP) in health and disease. *Biochem. Pharmacol.* **2004**, *68*, 1049–1053.
(70) Dahl, A.; Eriksson, P. S.; Persson, A. I.; Karlsson, G.; Davidsson, P.; Ekman, R.; Westman-Brinkmalm, A. Proteome analysis of conditioned medium from cultured adult hippocampal prgenitors. *Rapid Commun. Mass Spectrom.* **2003**, *17*, 2195–2202.
(71) Hengst, U.; Albrecht, H.; Hess, D.; Monard, D. The phosphatidylethanolamine-binding protein ist he prototype of a novel family of serine protease inhibitors. *J. Biol. Chem.* **2001**, *276*, 535–540.
(72) Ojika, K.; Kojima, S.; Ueki, Y.; Fukushima, N.; Hayashi, K.; Yamamoto, M. Purification and structural analysis of hippocampal cholinergic neurostimulating peptide. *Brain Res.* **1992**, *572*, 164–171.
(73) Williams, R. A.; Kelly, S. M.; Mottram, J. C.; Coombs, G. H. 3-Mercaptopyruvate sulfurtransferase of Leishmania contains an unusual C-terminal extension and is involved in thioredoxin and antioxidant metabolism. *J. Biol. Chem.* **2003**, *278*, 1480–1486.
(74) Nagahara, N.; Katayama, A. Post-translational regulation of mercaptopyruvate sulfurtransferase via a low redox potential cysteine-sulfenate in the maintenance of redox homeostasis. *J. Biol. Chem.* **2005**, *280*, 34569–34576.
(75) Nagahara, N.; Sawada, N. The mercaptopyruvate pathway in cysteine catabolism: a physiologic role and related disease of the multifunctional 3-mercaptopyruvate sulfurtransferase. *Curr. Med. Chem.* **2006**, *13*, 1219–1230.
(76) Lim, R.; Miller, J. F.; Zaheer, A. Purification and characterization of glia maturation factor beta: a growth regulator for neurons and glia. *Proc. Natl. Acad. Sci. U.S.A.* **1989**, *86*, 3901–3905.
(77) Kaplan, R.; Zaheer, A.; Jaye, M.; Lim, R. Molecular cloning and expression of biologically active human glia maturation factor-beta. *J. Neurochem.* **1991**, *57*, 483–490.
(78) Giulian, D.; Johnson, B.; Krebs, J. F.; Tapscott, M. J.; Honda, S. A growth factor from neuronal cell lines stimulates myelin protein synthesis in mammalian brain. *J. Neurosci.* **1991**, *11*, 327–336.
(79) Lim, R.; Zaheer, A.; Khosravi, H.; Freeman, J. H., Jr.; Halverson, H. E.; Wemmie, J. A.; Yang, B. Impaired motor performance and learning in glia maturation factor-knockout mice. *Brain Res.* **2004**, *1024*, 225–232.
(80) Kohlhauser, C.; Mosgoller, W.; Hoger, H.; Lubec, B. Myelination deficits in brain of rats following perinatal asphyxia. *Life Sci.* **2000**, *67*, 2355–2368.
(81) John, J. P.; Pintsov, O.; Petter-Puchner, A.; Redl, H.; Pollak, A.; Chen, W. Q.; Lubec, G. Nitric oxide and oxygen radical attack on GDP-dissociation inhibitor 2 (GDI-2) in spinal cord injury of the rat. *J. Proteome Res.* **2007**, *6*, 1500–1509.

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