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2	Article Sub- Title		
3	Article Copyright - Year	**Springer Science+Business Media New York 2014 (This will be the copyright line in the final PDF)**	
4	Journal Name	Journal of Molecular Neuroscience	
5	Corresponding Author	Family Name	**Reglodi**
6		Particle	
7		Given Name	**D.**
8		Suffix	
9		Organization	University of Pecs
10		Division	Department of Anatomy
11		Address	Pecs, Hungary
12		e-mail	dora.reglodi@aok.pte.hu
13	Author	Family Name	**Maasz**
14		Particle	
15		Given Name	**G.**
16		Suffix	
17		Organization	University of Pecs
18		Division	Department of Analytical Biochemistry Institute of Biochemistry and Medical Chemistry
19		Address	Pecs, Hungary
20		Organization	University of Pecs
21		Division	Imaging Center for Life and Material Sciences
22		Address	Pecs, Hungary
23		Organization	University of Pecs
24		Division	Janos Szentagothai Research Center
25		Address	Pecs, Hungary
26		e-mail	
27	Author	Family Name	**Pirger**
28		Particle	
29		Given Name	**Z.**

30		Suffix	
31		Organization	University of Pecs
32		Division	Department of Anatomy
33		Address	Pecs, Hungary
34		Organization	Hungarian Academy of Sciences
35		Division	Chemical Ecology and Neurobiology, Department of Experimental Zoology, Balaton Limnological Institute, Centre for Ecological Research
36		Address	Tihany, Hungary
37		e-mail	
38	Author	Family Name	**Petrovics**
39		Particle	
40		Given Name	**D.**
41		Suffix	
42		Organization	University of Pecs
43		Division	Department of Analytical Biochemistry Institute of Biochemistry and Medical Chemistry
44		Address	Pecs, Hungary
45		e-mail	
46	Author	Family Name	**Schmidt**
47		Particle	
48		Given Name	**J.**
49		Suffix	
50		Organization	University of Pecs
51		Division	Department of Analytical Biochemistry Institute of Biochemistry and Medical Chemistry
52		Address	Pecs, Hungary
53		Organization	University of Pecs
54		Division	Imaging Center for Life and Material Sciences
55		Address	Pecs, Hungary
56		Organization	University of Pecs
57		Division	Janos Szentagothai Research Center
58		Address	Pecs, Hungary
59		e-mail	
60	Author	Family Name	**Kiss**
61		Particle	
62		Given Name	**P.**
63		Suffix	

64		Organization	University of Pecs
65		Division	Department of Anatomy
66		Address	Pecs, Hungary
67		e-mail	
68		Family Name	**Rivnyak**
69		Particle	
70		Given Name	**A.**
71		Suffix	
72	Author	Organization	Hungarian Academy of Sciences
73		Division	Chemical Ecology and Neurobiology, Department of Experimental Zoology, Balaton Limnological Institute, Centre for Ecological Research
74		Address	Tihany, Hungary
75		e-mail	
76		Family Name	**Hashimoto**
77		Particle	
78		Given Name	**H.**
79		Suffix	
80	Author	Organization	Osaka University
81		Division	Laboratory of Molecular Neuropharmacology, Graduate School of Pharmaceutical Sciences
82		Address	Osaka, Japan
83		e-mail	
84		Family Name	**Avar**
85		Particle	
86		Given Name	**P.**
87		Suffix	
88	Author	Organization	University of Pecs
89		Division	Department of Analytical Biochemistry Institute of Biochemistry and Medical Chemistry
90		Address	Pecs, Hungary
91		e-mail	
92		Family Name	**Jambor**
93		Particle	
94		Given Name	**E.**
95	Author	Suffix	
96		Organization	University of Pecs
97		Division	Department of Analytical Biochemistry Institute of Biochemistry and Medical Chemistry

98		Address	Pecs, Hungary
99		e-mail	
100	Author	Family Name	**Tamas**
101		Particle	
102		Given Name	**A.**
103		Suffix	
104		Organization	University of Pecs
105		Division	Department of Anatomy
106		Address	Pecs, Hungary
107		e-mail	
108	Author	Family Name	**Gaszner**
109		Particle	
110		Given Name	**B.**
111		Suffix	
112		Organization	University of Pecs
113		Division	Department of Anatomy
114		Address	Pecs, Hungary
115		e-mail	
116	Author	Family Name	**Mark**
117		Particle	
118		Given Name	**L.**
119		Suffix	
120		Organization	University of Pecs
121		Division	Department of Analytical Biochemistry Institute of Biochemistry and Medical Chemistry
122		Address	Pecs, Hungary
123		Organization	University of Pecs
124		Division	Imaging Center for Life and Material Sciences
125		Address	Pecs, Hungary
126		Organization	University of Pecs
127		Division	Janos Szentagothai Research Center
128		Address	Pecs, Hungary
129		e-mail	
130	Institutional Author	Institutional Author Name	**MTA-PTE Human Reproduction Research Group**
131		e-mail	
132	Institutional	Institutional Author Name	**PTE MTA Lendulet PACAP Research Team**

133	Author	e-mail	
134		Received	24 December 2013
135	Schedule	Revised	
136		Accepted	13 February 2014
137	Abstract	Pituitary adenylate cyclase-activating polypeptide (PACAP) is a widespread neuropeptide acting as a neurotransmitter, neuromodulator, or neurotrophic factor. The diverse biological actions provide the background for the variety of deficits observed in mice lacking endogenous PACAP. PACAP-deficient mice display several abnormalities, such as sudden infant death syndrome (SIDS)-like phenotype, decreased cell protective functions, and increased risk of Parkinson's disease but their molecular mechanisms and proteomic background are unclear in majority. Therefore, our aim was to investigate the differences in peptide and protein composition in the brains of PACAP-deficient and wild-type mice using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and mass spectrometric (MS)-based proteomic analysis. Brains from PACAP-deficient mice were removed, and different brain areas (cortex, hippocampus, diencephalon, mesencephalon, brainstem, and cerebellum) were separated. Brain pieces were weighed, homogenized, and further processed for electrophoretic analysis. Our results revealed several differences in diencephalon and mesencephalon. The protein bands of interest were cut from the gel, samples were digested with trypsin, and the tryptic peptides were measured by matrix-assisted laser desorption ionization time of flight (MALDI TOF) MS. Results were analyzed by MASCOT Search Engine. Among the altered proteins, several are involved in metabolic processes, energy homeostasis, and structural integrity. ATP-synthase and tubulin beta-2A were expressed more strongly in PACAP-knockout mice. In contrast, the expression of more peptides/proteins markedly decreased in knockout mice, like pyruvate kinase, fructose biphosphate aldolase-A, glutathione S-transferase, peptidyl propyl *cis*-trans isomerase-A, gamma enolase, and aspartate amino transferase. The altered expression of these enzymes might partially account for the decreased antioxidant and detoxifying capacity of PACAP-deficient mice accompanying the increased vulnerability of these animals. Our results provide novel insight into the altered biochemical processes in mice lacking endogenous PACAP.
138	Keywords separated by ' - '	PACAP - Knockout - MALDI - Proteomics	
139	Foot note information		

J Mol Neurosci
DOI 10.1007/s12031-014-0264-0

Comparative Protein Composition of the Brains of PACAP-Deficient Mice Using Mass Spectrometry-Based Proteomic Analysis

G. Maasz · Z. Pirger · D. Reglodi · D. Petrovics · J. Schmidt · P. Kiss · A. Rivnyak · H. Hashimoto · P. Avar · E. Jambor · A. Tamas · B. Gaszner · L. Mark · MTA-PTE Human Reproduction Research Group · PTE MTA Lendulet PACAP Research Team

Received: 24 December 2013 / Accepted: 13 February 2014

Abstract Pituitary adenylate cyclase-activating polypeptide (PACAP) is a widespread neuropeptide acting as a neurotransmitter, neuromodulator, or neurotrophic factor. The diverse biological actions provide the background for the variety of deficits observed in mice lacking endogenous PACAP. PACAP-deficient mice display several abnormalities, such as sudden infant death syndrome (SIDS)-like phenotype, decreased cell protective functions, and increased risk of Parkinson's disease but their molecular mechanisms and proteomic background are unclear in majority. Therefore, our aim was to investigate the differences in peptide and protein composition in the brains of PACAP-deficient and wild-type mice using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and mass spectrometric (MS)-based proteomic analysis. Brains from PACAP-deficient mice were removed, and different brain areas (cortex, hippocampus, diencephalon, mesencephalon, brainstem, and cerebellum) were separated. Brain pieces were weighed, homogenized, and further processed for electrophoretic analysis. Our results revealed several differences in diencephalon and mesencephalon. The protein bands of interest were cut from the gel, samples were digested with trypsin, and the tryptic peptides were measured by matrix-assisted laser desorption ionization time of flight (MALDI TOF) MS. Results were analyzed by MASCOT Search Engine. Among the altered proteins, several are involved in metabolic processes, energy homeostasis, and structural integrity. ATP-synthase and tubulin beta-2A were expressed more strongly in PACAP-knockout mice. In contrast, the expression of more peptides/proteins markedly decreased in knockout mice, like pyruvate kinase, fructose biphosphate aldolase-A, glutathione S-transferase, peptidyl propyl *cis*-trans isomerase-A, gamma enolase, and aspartate amino transferase. The altered expression of these enzymes might partially account for the decreased antioxidant and detoxifying capacity of PACAP-deficient mice accompanying the increased vulnerability of these animals. Our results provide novel insight into the altered biochemical processes in mice lacking endogenous PACAP.

Keywords PACAP · Knockout · MALDI · Proteomics

G. Maasz · D. Petrovics · J. Schmidt · P. Avar · E. Jambor · L. Mark
Department of Analytical Biochemistry Institute of Biochemistry and Medical Chemistry, University of Pecs, Pecs, Hungary

G. Maasz · J. Schmidt · L. Mark
Imaging Center for Life and Material Sciences, University of Pecs, Pecs, Hungary

G. Maasz · J. Schmidt · L. Mark
Janos Szentagothai Research Center, University of Pecs, Pecs, Hungary

Z. Pirger · D. Reglodi (✉) · P. Kiss · A. Tamas · B. Gaszner
Department of Anatomy, University of Pecs, Pecs, Hungary
e-mail: dora.reglodi@aok.pte.hu

Z. Pirger · A. Rivnyak
Chemical Ecology and Neurobiology, Department of Experimental Zoology, Balaton Limnological Institute, Centre for Ecological Research, Hungarian Academy of Sciences, Tihany, Hungary

H. Hashimoto
Laboratory of Molecular Neuropharmacology, Graduate School of Pharmaceutical Sciences, Osaka University, Osaka, Japan

Introduction

Pituitary adenylate cyclase activating polypeptide (PACAP) is a hypothalamic neuropeptide that was first isolated from ovine

hypothalamic (Miyata et al. 1989). PACAP occurs in the nervous system and almost all peripheral organs (Vaudry et al. 2009). PACAP has diverse biological effects, including behavioral actions, effects on biological rhythms, reproduction, and cardiovascular and gastrointestinal functions (Vaudry et al. 2009). It is also well-known that PACAP is part of the cellular protective machinery. The neuroprotective effects of both endogenous and exogenous PACAP are intensively investigated because PACAP is a promising neuroprotective peptide. Strong neuroprotective effects have been shown in numerous models of in vitro and in vivo injuries, including retinal and cerebral lesions (Fabian et al. 2012; Atlasz et al. 2007), neurodegenerative diseases (Brown et al. 2013), and toxic injuries (Wada et al. 2013). The protective effect of endogenous PACAP is mainly supported by observations in PACAP-deficient mice (Reglodi et al. 2012). The increased susceptibility of PACAP-deficient mice has been described in numerous models. The first descriptions come from cerebellar granule cells exposed to oxidative stress or ethanol. Vaudry and coworkers described that cultured granule cells from PACAP-deficient mice respond with increased cell death to the same injury (Vaudry et al. 2005). Subsequent studies have confirmed these original observations in other cultured cells (Horvath et al. 2010). In vivo, similar findings have been published. Knockout mice exposed to cerebral ischemia have increased brain infarct volume compared to wild-type mice (Reglodi et al. 2012; Ohtaki et al. 2006). For example, mice lacking endogenous PACAP have increased vulnerability to different stressors and toxic insults, and they also have accelerated aging. PACAP-deficient mice show increased infarct size in a stroke model (Ohtaki et al. 2008) and increased sensitivity to neuroinflammation accompanying a model of Parkinson's disease (Watson et al. 2013) and increased retinal injury in mice exposed to bilateral carotid artery occlusion (Szabadfi et al. 2012). Some biochemical alterations have been described in the background of these findings. However, very little is known about the proteomics of PACAP-deficient mice, and mass spectrometric analysis has not yet been done in these mice. Therefore, our aim was to map the proteomic profile in different brain regions of wild-type and knockout mice focusing on the present differences and quantitatively comparing the proteins found in both types.

Materials and Methods

Animals

The generation and maintenance of the knockout mice on the CD1 background have been previously described in detail (Hashimoto et al. 2001; Hashimoto et al. 2009); they were backcrossed for ten generations with the CD1 strain. Wild-type ($PACAP^{+/+}$, $n=5$) and homozygous PACAP-deficient mice ($PACAP^{-/-}$, $n=5$) were used. Animals were fed and watered ad libitum under light/dark cycles of 12/12 h. All procedures were performed in accordance with the ethical guidelines and under approved protocols (ethical permission number: University of Pecs BA02/2000-15024/2011).

Sample Preparation

Wild-type and homozygous PACAP-deficient mice were sacrificed under isoflurane anesthesia. Brains were removed and different brain areas (frontal cortex, temporal lobe–diencephalon complex, mesencephalon, rest of the brainstem (pons and medulla), and cerebellum) were dissected. Two hundred microliters of lysis buffer (2 mM EDTA, 10 mM EGTA, 20 mM HEPES, pH 7.5) was added to 50 mg brain tissue. LoRetention pipette tips (Eppendorf, Wien, Austria) and Lobind Eppendorf tubes (Eppendorf) were used during all steps of sample preparation to avoid protein loss. The tissue was homogenized, and cells were explored for 6×10 s with a high energy UIS250V ultrasonicator (Hielsher Ultrasound Technology, Teltow, Germany) applying ice cooling between the cycles. Samples were vortex mixed and centrifuged at 10,000 rpm for 10 min. Supernatant was transferred to new Eppendorf tubes, and 100 μl chloroform was added. Using the chloroformed precipitation, a high grade of purity was reached; a high percentage of the presented lipids could be removed, which resulted in a good degree of ionization. The mixture was gently shaken for several seconds and then immersed into an ultrasonic water bath for precipitation 3 min (Wang et al. 2012; Zhang and Lee 2012). Phase separation was performed by a centrifugation immediately at 4,000 rpm for 5 min. To get just the precipitated proteins, both the organic and the aqueous phases were removed. Residual chloroform was removed using Speed Vac Concentrator (Concentrator Plus, Eppendorf). The effectiveness of the protein precipitation was controlled by Autoflex II in both phases. It was used in linear mode, and 1 μl sample and 1 μl Sinapic acid (Bruker Daltonics, Bremen, Germany) matrix solution (the concentration was 10 mg/ml in acetonitrile/0.1 % TFA, 1/2 *v*/*v*%) was mixed. The samples were lyophilized and stored at −80 °C until further process.

Agilent-Automated Gel System

Following the instructions of the manual of Agilent 2200 TapeStation system (Agilent Technologies, Kromat KFT, Budapest, Hungary), P200 stain solution was prepared from the labeling dye and labeling buffer. Two microliters of buffer was mixed with 2 μl sample or ladder and heated for 7 min at 75 °C. The mixture was denatured with the P200 reducing sample buffer and heated for 5 min at 75 °C. Two microliters of P200 marker was added to each sample or ladder, mixed, centrifuged, and pipetted into the 2200 TapeStation system.

The experiments and semiquantitative data analysis were carried out using the software of the 2200 TapeStation system.

One-Dimensional SDS Gel Electrophoresis

All chemicals and solvents for gel electrophoresis were purchased from the BIO-RAD (Budapest, Hungary). The brain samples from wild-type and PACAP-deficient mice were homogenized in 1 M Tris/HCl buffer, pH 8, containing 0.5 M EDTA, 0.7 M beta-mercaptoethanol, and 10 % sodium dodecyl sulfate (SDS). After homogenization, the samples were boiled for 5 min and clarified by centrifugation (8,000 rpm for 10 min). SDS-polyacrylamide gel electrophoresis (PAGE) electrophoresis was carried out on 12 % polyacrylamide gel according to Laemmli. ProSieveTM QadColor™ protein marker, 4.6–300 kDa, was used for estimation of the molecular weight. Gels were stained with Coomassie Brilliant Blue R-250 and destined with a solution containing 5 % (*v*/*v*) acetic acid and 16.5 % (*v*/*v*) methanol. The gels were analyzed by Quantity One, BIO-RAD software.

The bands of interest were excised from the gel with a razor blade, placed in Eppendorf tubes, and destained by washing three times for 10 min in 200 μL of 50 % (*v*/*v*) acetonitrile solution containing 50 mM NH_4HCO_3 (pH: 8.3). Proteins were then reduced by 50 μL of 10 mM dithiothreitol in 50 mM NH_4HCO_3 for 1 h at 55 °C and alkylated in 50 μL of 55 mM iodoacetamide in 50 mM NH_4HCO_3 solution. The gel pieces were dehydrated at room temperature by a Speed Vac Concentrator and covered with 10 μL of modified trypsin (sequencing grade, Promega, Madison, WI, USA), 5 ng/μL in NH_4HCO_3 buffer (50 mM, pH: 8.3), and left at 37 °C overnight. The excised bands were crushed, and peptides were extracted by frequent vortexing with 50 μL aqueous solution of acetonitrile and formic acid (44/50/6 *v*/*v*/*v*). The samples were lyophilized and stored at −80 °C until further process.

Two-Dimensional SDS Gel Electrophoresis

The samples were homogenized in extraction buffer containing 8 M urea, 50 mM DTT, 4 % CHAPS, 0.2 % carrier ampholytes, and 0.0002 % bromophenol blue, which was also used for IPG strip rehydration. The solubilized samples were centrifuged at 10,000 rpm for 10 min to discard the insoluble materials. Isoelectro focusing of the supernatant was performed with rehydrated IPG strips (7 cm, pH 4–7 linear gradient) using PROTEAN IEF System (BIO-RAD) with the following parameters: 250 V for 20 min, 4,000 V for 1 h, and 4,000 V for 10,000 V for h. The current was 50 μA/strip. After the IEF, the strips were stored at −80 °C.

For the second dimension, the strips were incubated in equilibration buffer (6 M urea, 2 % SDS, 0.05 M Tris/HCl pH 8.8, and 20 % glycerol in ultrapure water) containing 2 % DTT for 10 min, and, after, in the same buffer containing 2.5 % iodoacetamide for 10 min. The strips were washed in SDS running buffer, placed on top of 12 % SDS-PAGE according to Laemmli. The gels were scanned using PharosFX™ (BIO-RAD) Image scanner and were analyzed using BIO-RAD's PDQuest™ 2-D analysis software. All samples were analyzed in triplicate. Gels were stained, and then proteins of interest were digested by the same method as described above.

MALDI TOF/TOF Mass Spectrometry-Based Identification

After SDS-PAGE, the peptide solutions were lyophilized and redissolved in 0.1 % trifluoroacetic acid (TFA). The aqueous solutions of the lyophilized protein digests were enriched on a Protein Anchor chip target plate (MTP AnchorChip™ 384 T F, Bruker Daltonics, Bremen, Germany) by using of 1 μL of sample solution; after that, 1 μl diluted matrix solution (the concentration was 0.7 mg/ml), prepared freshly before each measurement by dissolving α-cyano-4-hydroxycinnamic acid (CHCA) in acetonitrile/0.1 % TFA (1/2, *v*/*v*) was added. An Autoflex Speed TOF/TOF (Bruker Daltonics) mass spectrometer operated in reflector mode for peptide mass fingerprinting (PMF) or LIFT mode for laser-induced decay (LID), and collision-induced decay (CID) was used. The FlexControl 3.4 software was used to control the instrument. The accelerating voltage was set to 20.00 kV. The instrument uses a 1 kHz Smart beam II solid-state Nd:YAG UV laser (Lasertechnik Berlin GmbH., Berlin, Germany). External calibration was performed in each case using Bruker Peptide Calibration Standard (#206195 Peptide Calibration Standard, Bruker Daltonics). Peptide masses were acquired in the range of 500 to 5,000 *m*/*z*. Each spectrum was produced by accumulating data from 7,500 consecutive laser shots. Singly charged monoisotopic peptide masses were searched against Swiss-Prot and National Center for Biotechnology Information (NCBI) nr databases (last accessed 17 June 2013) by utilizing the MASCOT database search engine (version 2.3) (www.matrixscience.com, Matrix Science Ltd., London, UK) and Bruker ProteinScape server (Bruker Daltonics). Maximum two missed tryptic cleavage was considered, and the mass tolerance for monoisotopic peptide masses was set to a maximum of 150 ppm. The following possible modifications were included during data search: carbamidomethyl (C)-fixed, oxidation (M)-variable. Additionally, LID and CID fragmentation of three of the matched peptides were carried out with MALDI TOF/TOF to provide further evidence for the presence of the identified proteins.

Results

The peptide–protein profile of the different brain regions (frontal cortex, temporal lobe–diencephalon complex,

mesencephalon, cerebellum, and the rest of the brainstem, which consisted of the pons and the medulla) of wild-type- and PACAP-deficient mice was mapped on an Agilent 2200 TapeStation automated 1-D gel system (Fig. 1). Separated gel bands on the electropherogram show distribution of proteins with different molecular weights in orientation and intensity similar to detected peaks. Frontal cortex (a1 and a2 in Fig. 1) and the rest of the brainstem (pons and medulla) (b1 and b2 in Fig. 1) regions of wild-type and PACAP-knockout mice did not show marked differences in protein composition. In contrast, significant differences were found in the mesencephalon (c1 and c2 in Fig. 1) and temporal lobe–diencephalon complex regions (d1 and d2 in Fig. 1) using Agilent 2200 TapeStation system. Based on the electropherograms, different data from wild-type (a1 and b1 in Fig. 2) and PACAP-knockout mice (a2 and b2 in Fig. 2) were summarized in parallel. Figure 2(a2) shows several proteins (such as 50.8, 55.5, 61.1, 80.0, and 176.5 kDa) decreased in mesencephalon samples of PACAP-knockout (KO) mice. In contrast, 12.9 kDa protein concentrations increased in the same samples. Furthermore, Fig. 2(b2) indicates that 14.9, 35.8, and 52.8 kDa proteins markedly decreased in PACAP-knockout temporal lobe–diencephalon complex region in contrast to wild types in Fig. 2(b1).

The main advantages of Agilent 2200 TapeStation system are the simple sample preparation and the capability of quick

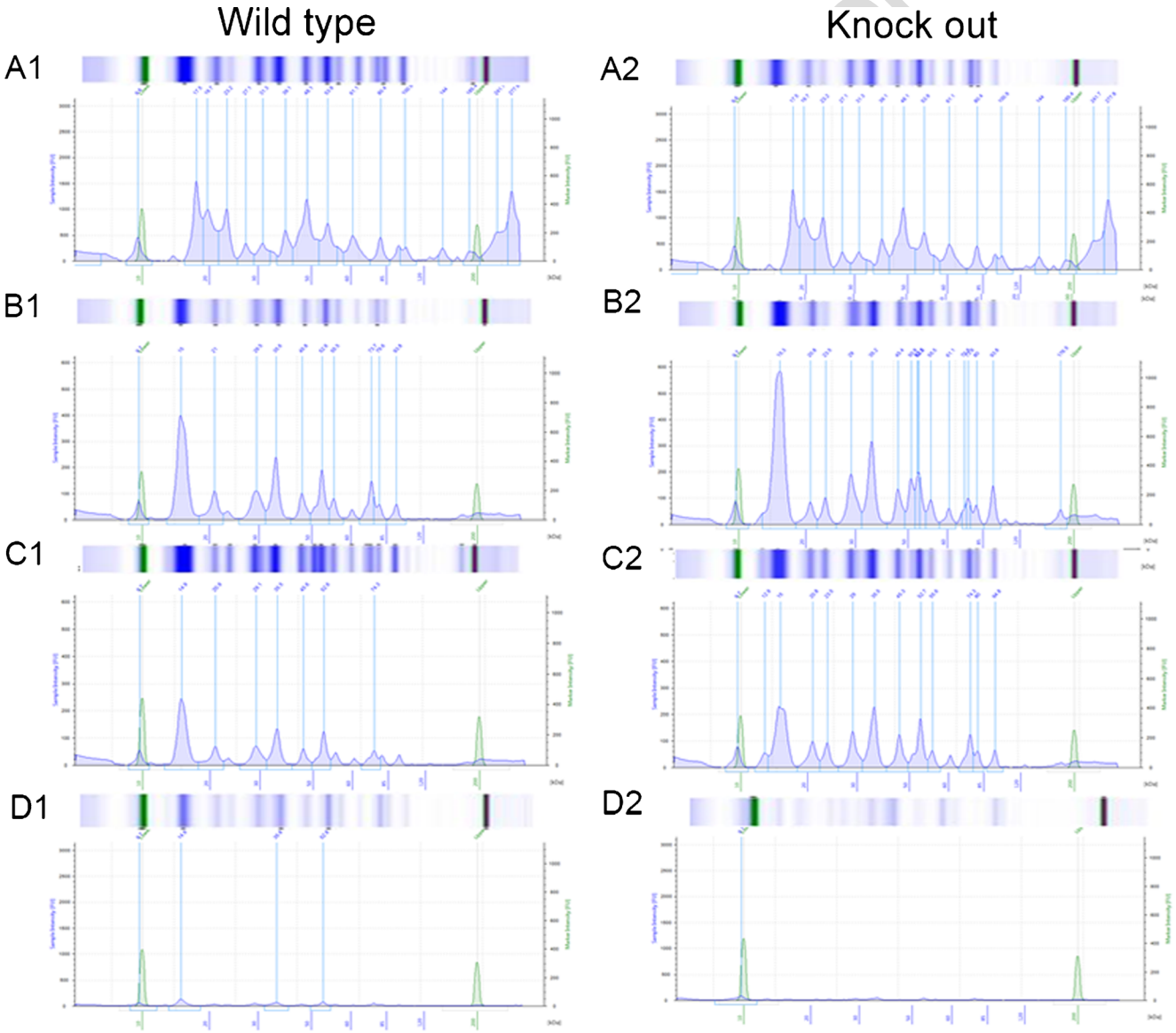

Fig. 1 Protein mapping of wild-type (*1*) and PACAP-knockout (*2*) mice brains were examined by Agilent 2200 TapeStation in the following regions: *A* frontal cortex, *B* rest of the brainstem (pons and medulla), *C* mesencephalon, and *D* temporal lobe–diencephalon complex. The *green* and the *brown* colors indicate the original P200 molecular weight markers and the *blue bands* represent the different proteins from samples. *Light blue peaks* are representing the relative intensities of different proteins Q4

measurements of a large number of samples, but its disadvantage is its limited resolution. Based on this, we were able to screen the different brain areas in a parallel fashion, but we could not reach a resolution that is high enough. Therefore, regions showing differences at first screening were further analyzed using SDS-PAGE. The differences from mesencephalon and temporal lobe–diencephalon complex, a commonly used SDS-PAGE method was applied (Fig. 3). The SDS-PAGE could separate the different protein bands with higher resolution. Figure 3(a) represents the different protein contents of mesencephalon in wild and PACAP-knockout samples. The insert shows the gel region with the most marked differences between wild-type and knockout mice in the range of ~40–70 kDa. In this region, eight protein bands (~39, 40, 45, 47, 50, 55, 60, and 70 kDa) with different intensity between two samples were identified. Figure 3(b) represents the different protein content of temporal lobe–diencephalon complex region in wild and PACAP-knockout samples. The insert shows the gel region with the most obvious differences between wild-type and knockout mice in the range of ~35–55 kDa. In this range, six protein bands (~35, 36, 37, 40, 47, and 50 kDa) with different intensity were identified. Since we could identify several proteins with MS analysis from the protein bands showing differences, 2-D electrophoresis was performed for more precise qualitative and quantitative analysis. Differences on 2-D map of brain homogenates are presented in Fig. 4a (mesencephalon) and b (temporal lobe–diencephalon complex). 2-D electrophoresis enabled the separation of proteins over the entire pH 3–10 range and comprised proteins between 4.6 and 300 kDa. The spot abundance values were highly comparable among all wild-type and knockout samples. The red arrows represent the main protein differences in wild-type animals, while the blue arrows show the same in knockout animals. Spots showing marked differences between the two animal groups were excised and were further processed for MS analysis.

We identified 22 proteins based on the sequences of the tryptic digests. From this protein pool, random representative

A1

Wavelength	MW [kDa]	Area	From [kDa]	Height	% of Total	% Integrated Area
Sample	9.7	14.044	8.8	85.696	2.49	2.79
-	-	-	-	-	-	-
Sample	15.3	177.356	12.8	584.34	31.44	35.28
Sample	20.8	17.222	18	84.092	3.05	3.43
Sample	23.5	17.029	21.9	100.655	3.02	3.39
Sample	29	45.725	25.6	192.658	8.11	9.09
Sample	35.2	61.78	31.9	317.694	10.95	12.29
Sample	45.4	23.544	40.6	134.689	4.17	4.68
Sample	50.8	29.596	48.2	175.831	5.25	5.89
Sample	52.2	19.187	51.7	186.902	3.4	3.82
Sample	52.5	14.319	52.8	200.041	2.54	2.85
Sample	55.5	15.396	54.3	93.553	2.73	3.06
Sample	61.1	9.771	57.9	60.14	1.73	1.94
Sample	70.8	6.604	69.3	57.486	1.17	1.31
Sample	73.6	9.041	72.4	99.965	1.6	1.8
Sample	80	12.718	75.3	71.374	2.25	2.53
Sample	93.8	19.821	85.7	148.691	3.51	3.94
Sample	176.5	9.604	152	56.075	1.7	1.91

A2

Wavelength	MW [kDa]	Area	From [kDa]	Height	% of Total	% Integrated Area
Sample	9.7	13.923	8.5	78.76	3.79	4.44
Sample	12.9	8.817	11.6	53.699	2.4	2.81
Sample	15	76.433	13.2	225.546	20.81	24.37
Sample	20.8	21.736	17.9	98.184	5.92	6.93
Sample	23.5	16.382	22.1	93.101	4.46	5.22
Sample	29	27.697	25.7	136.44	7.54	8.83
Sample	35.5	46.567	31.5	228.067	12.68	14.85
Sample	45.3	22.272	39.9	124.294	6.06	7.1
-	-	-	-	-	-	-
Sample	52.7	33.4	49.1	185.784	9.09	10.65
Sample	55.6	9.637	54.4	63.6	2.62	3.07
-	-	-	-	-	-	-
-	-	-	-	-	-	-
Sample	74.3	18.146	66.5	125.53	4.94	5.79
Sample	80	10.421	76.3	62.896	2.84	3.32
-	-	-	-	-	-	-
Sample	94.8	8.191	87.4	65.08	2.23	2.61
-	-	-	-	-	-	-

B1

Wavelength	MW [kDa]	Area	From [kDa]	Height	% of Total	% Integrated Area
Sample	9.7	12.899	8.8	59.62	8.9	17.91
Sample	14.9	29.825	13.2	129.038	20.58	41.42
Sample	35.8	16.229	31.8	66.942	11.2	22.54
Sample	52.8	13.06	49.4	76.236	9.01	18.14

B2

Wavelength	MW [kDa]	Area	From [kDa]	Height	% of Total	% Integrated Area
Sample	9.7	24.513	7.4	84.897	23.34	100
-	-	-	-	-	-	-
-	-	-	-	-	-	-
-	-	-	-	-	-	-

Fig. 2 Different parameters/data from Agilent 2200 TapeStation system; the *A1* and *B1* panels were summarized between mesencephalon and temporal lobe–diencephalon complex in wild-type samples. *A2* and *B2* panels show the measured results in the same regions in PACAP knockout mice. The proteins with similar molecular weight are presented in the same row of panel *a2* and *b2*, while the proteins under limit of detection are excluded and are presented with a *dash*

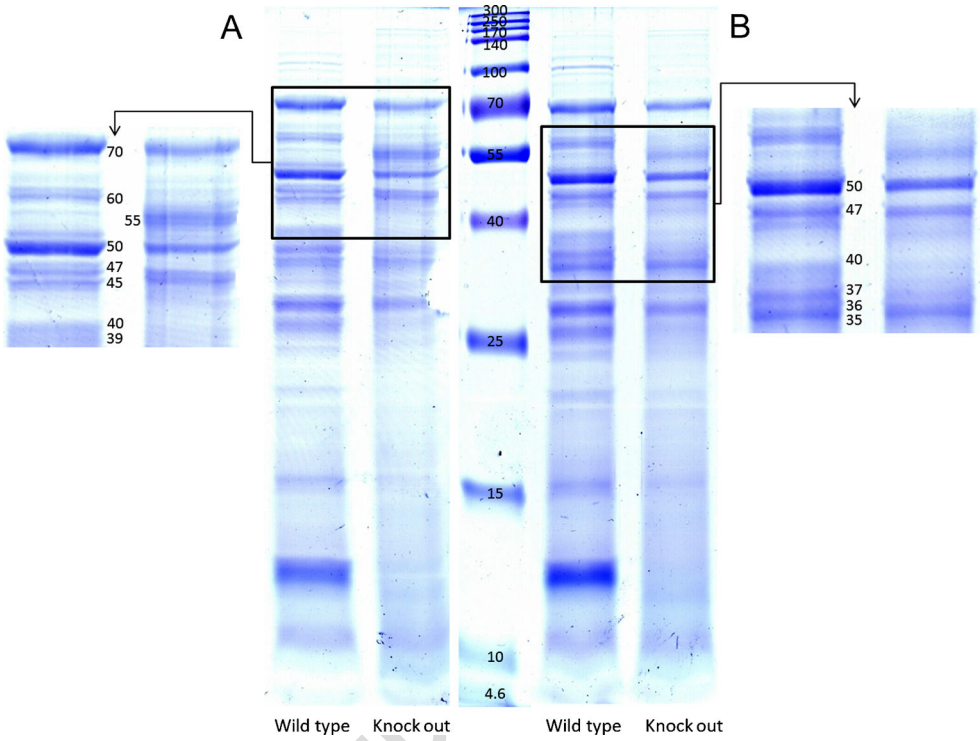

Fig. 3 SDS-PAGE of mesencephalon (*A*) and temporal lobe-diencephalon complex (*B*). The *inserts* show the gel regions with the biggest differences in both cases. The bands were analyzed with Quantity One BIO-RAD software

mass spectra are shown for a protein with no difference (Fig. 5a, glyceraldehyde-3-phosphate dehydrogenase), a protein being downregulated (Fig. 5b, glutathione S-transferase), and another one upregulated (Fig. 5c, ATP synthase) in PACAP-knockout samples. The identified 22 proteins are summarized in Table 1, with the NCBI codes, functions, Mascot score, and the sequence coverage. In PACAP-knockout mice, 14 proteins out of the 22 identified ones showed downregulation (peptidylprolyl isomerase A, glutathione S-transferase, malate dehydrogenase 1, enolase 2, aldolase 1, aspartate aminotransferase, leucine-rich repeat containing 9, phosphoglycerate mutase 1, pyruvate kinase, aconitase-2, hemoglobin subunit beta-1, albumin 1, histone (H1) domain, and secretin receptor), and in four proteins no difference was found (cytochrome c oxidase, glyceraldehyde-3-phosphate dehydrogenase, microphthalmia-associated transcription factor, and neurofascin). Four further proteins were identified that showed an upregulation in PACAP-knockout mice (ATP synthase, tubb2 protein–tubulin beta-2 chain, spectrin alpha chain, and vinculin).

Discussion

Our study revealed several proteins that were up- or downregulated in intact mice lacking endogenous PACAP. This is the first mass spectrometric analysis of PACAP-knockout mice using MS and Agilent 1-Dimension Automated 2200 TapeStation system.

Based on our current knowledge on mice lacking endogenous PACAP, it seems that there are no visible differences or only minor alterations are present in the brain morphology of

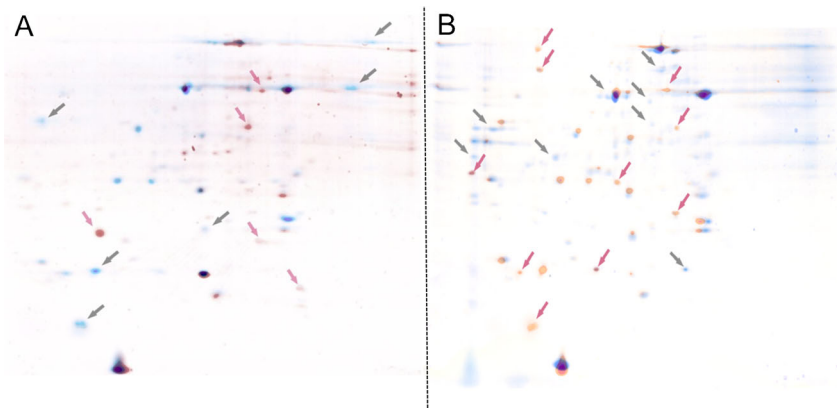

Fig. 4 Proteomic profiling by two-dimensional gel electrophoresis. **a** Mesencephalon, **b** temporal lobe–diencephalon complex. The results of wild-type and PACAP-knockout samples are merged. The *red arrows* represent the wild-type protein spots, and the *blue arrows* represent protein spots from the knockout samples

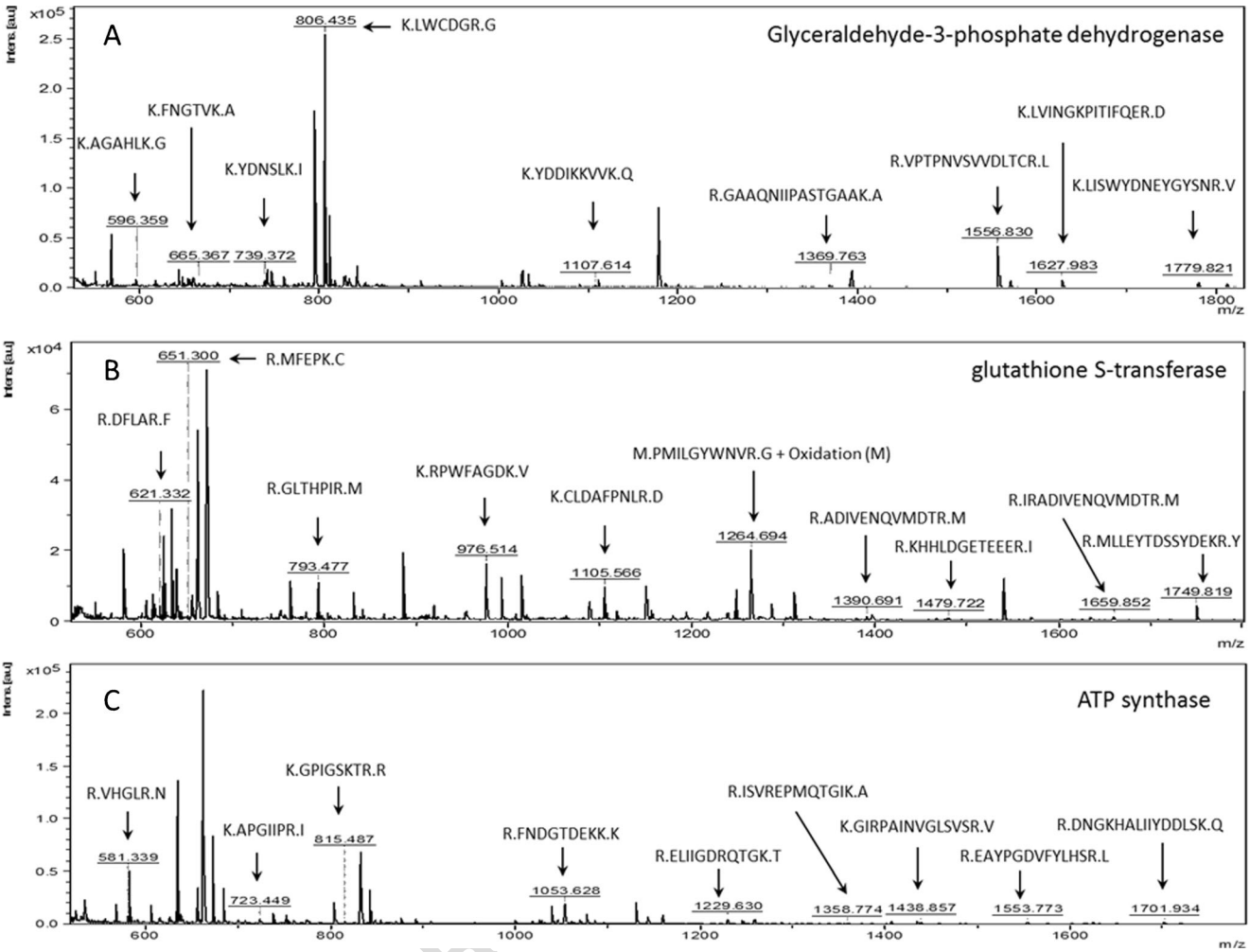

Fig. 5 Matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) representative mass spectral profiles of the tryptic peptides of different proteins: **a** glyceraldehyde-3-phosphate dehydrogenase, **b** glutathione S-transferase, and **c** ATP synthase. The identification of the resulted proteins was carried out by peptide mass fingerprinting workflow

PACAP-KO mice at macroscopic or light microscopic levels (Reglodi et al. 2012). However, when these mice are exposed to harmful challenges, including hypoxia/ischemia, trauma, and toxicity, PACAP-knockout mice respond with a significantly increased lesion. This has been proven in models of experimental autoimmune encephalomyelitis (Tan et al. 2009), brain ischemia (Ohtaki et al. 2006), retinal ischemia, and retinal excitotoxicity (Endo et al. 2011; Szabadfi et al. 2012). Furthermore, mice lacking endogenous PACAP have shown slower regeneration in spinal cord and peripheral nerve injury (Armstrong et al. 2008; Tsuchikawa et al. 2012). These results suggest that there must be biochemical alterations that can be compensated under unchallenged conditions in the absence of PACAP, but the compensatory mechanisms are not sufficient to overcome injuries and to provide cellular protection under challenged conditions. Our present results may shed further light on the increased vulnerability of PACAP-deficient mice against different challenges.

One group of proteins, where marked differences were found, was proteins related to oxidative stress and antioxidant defense. These proteins were found to be downregulated in PACAP-knockout mice. Peptidylprolyl isomerase A(PPIase), for example, plays a key role in heat shock protein-induced stress response. Glutathione S-transferase is important in detoxification and antioxidant defense. These results are in accordance with earlier observations showing that PACAP-knockout mice have increased oxidative stress levels, with increased malonedialdehyde, decreased glutathione, and decreased superoxide dismutase (Ferencz et al. 2010a, b). Furthermore, while no differences have been found in the antioxidant capacity and reactive oxygen species levels in the serum of knockout and wild-type mice at young ages, decreased antioxidant capacity accompanied by increased reactive oxygen species levels at older knockout mice has been found (Ohtaki et al. 2010). A recent study has investigated the PACAP-induced changes after cerebral ischemia in mice

t1.1 **Table 1** Identified proteins of wild-type and PACAP-knockout brain samples. The first column shows the identification number (ID), the second column represents the NCBI codes, the third column is the name of identified protein, the fourth and fifth columns show the change of amounts of proteins between wild-type and PACAP-knockout animals (+ the protein is present; − the protein is under limit of detection; +↓ the protein is present, but its level is decreased; +↑ the protein is present, but its level is increased). The sixth column shows the biological function of the named proteins. The last three columns represent the molecular weight (MW), Mascot Score and the sequence coverage (SC) in percentage

t1.2	ID	Accession	Name	Wild type	Knockout	Function	MW [kDa]	Mascot Score	SC [%]
t1.3	1	gi|498752597	Hemoglobin subunit beta-1	+	−	Oxygen transport	15.8	111.0	88.4
t1.4	2	gi|6679439	Peptidylprolyl isomerase A	+	+↓	Catalytic enzyme, interaction with HSPs	18.0	95.3	66.5
t1.5	3	gi|6754084	Glutathione S-transferase Mu 1	+	+↓	Detoxification	26.0	91.1	63.3
t1.6	4	gi|407261468	Cytochrome c oxidase subunit 1	+	+	Mitochondrial respiratory chain	28.9	67.5	50.0
t1.7	5	gi|74224797	Malate dehydrogenase 1, NAD isoform	+	+↓	Catalytic enzyme	36.5	70.5	43.7
t1.8	6	gi|55153885	Glyceraldehyde-3-phosphate dehydrogenase	+	+	Glycolytic enzyme	35.8	89.1	42.9
t1.9	7	gi|148677501	ATP synthase, isoform	−	+	ATP synthesis	54.6	96.4	39.2
t1.10	8	gi|7305027	Enolase 2, gamma neuronal	+	+↓	Glycolytic enzyme	47.3	105.0	38.9
t1.11	9	gi|6671539	Aldolase 1, A isoform	+	+↓	Glycolytic enzyme	39.3	74.3	31.6
t1.12	10	gi|387106	Aspartate aminotransferase	+	+↓	Enzyme in amino acid metabolism	46.2	81.7	31.0
t1.13	11	gi|148704587	Leucine-rich repeat containing 9, isoform	+	+↓	Protein structure motif	81.7	63.4	25.1
t1.14	12	gi|42561824	MITF protein	+	+	Transcription factor	38.6	83.7	33.8
t1.15	13	gi|114326546	Phosphoglycerate mutase 1	+	+↓	Glycolytic enzyme	28.8	61.0	43.7
t1.16	14	gi|13097483	Tubb2 protein	−	+	Structural protein	34.0	61.3	26.2
t1.17	15	gi|359807367	Pyruvate kinase, muscle isoform M1	+	+↓	Glycolytic enzyme	57.9	135.0	36.2
t1.18	16	gi|26340966	Albumin 1	+	+↓	Regulate the colloidal osmotic pressure of blood	68.7	106.0	34.5
t1.19	17	gi|914317	Neurofascin	+	+	Cell adhesion molecules	9.5	60.9	33.7
t1.20	18	gi|74189848	Spectrin alpha chain	−	+	Erythrocyte structural protein	97.8	76.8	25.5
t1.21	19	gi|51313	Histone (H1) domain	+	+↓	Histone protein	20.8	65.7	64.9
t1.22	20	gi|74188189	Aconitase 2	+	+↓	Catalytic enzyme	85.3	114.0	33.2
t1.23	21	gi|148669535	Vinculin, isoform	−	+	Cytoskeletal protein	123.8	68.0	18.7
t1.24	22	gi|81882894	Secretin receptor	+	+↓	G-protein coupled receptor	50.9	62.1	15.0

(Hori et al. 2012). The authors have found upregulation of antioxidant defense molecules after PACAP administration. In addition, an earlier study in PC12 cells found increased protective heat shock protein 27 levels while decreased neurotoxic heat shock protein expression after PACAP treatment (Lebon et al. 2006). All these results, in accordance with our present data, point to the importance of both endogenous and exogenous PACAP in protection against oxidative stress.

Another group of proteins, where we found major differences between the two groups, was the group of glycosylation enzymes. Malate dehydrogenase 1, enolase 2, aldolase 1, phosphoglycerate mutase 1 (PGM), and pyruvate kinase (PK) were downregulated, while ATP synthase was upregulated. Similarly to the oxidative stress markers, the changes in glycolytic enzymes in the present study are also in accordance with the findings of Hori et al. (2012) showing that exogenous PACAP influences the enzymes participating in glycosylation are altered after PACAP treatment in favor of a positive energy balance, supposedly providing protection in ischemic lesions. These results and our present observations suggest that endogenous PACAP is necessary for providing a favorable energy balance. In the lack of this regulatory mechanism, this energy balance is disturbed, making the organism more vulnerable to noxious stimuli (hypoxia, ischemia, aging, toxins, and neurodegenerative conditions). These results are also in accordance with studies showing that stimulating this enzymatic machinery provides neuroprotection in hypoxia (Zaman et al. 1999).

Our results also show that PACAP-knockout mice might compensate this disturbed energy balance by increasing ATP synthase levels under intact or unstressed conditions. This is in accordance with the observations of Ohtaki et al. (2010) showing that young knockout mice do not have increased oxidative stress in contrast to aging mice. We found several other differences in protein composition, including structural proteins, the functional significance of which is under further investigation. It is well-known that results obtained from knockout mice have to be handled carefully and no direct

functional consequences can be drawn regarding the exact endogenous actions of the molecule. The compensatory mechanisms in case of PACAP-knockout mice are not fully understood. Several attempts have been made to elucidate the compensatory changes in the lack of PACAP. However, the first studies found no differences in the monoaminergic neurotransmitter systems (Ogawa et al. 2005). Subsequent experiments hypothesized that there could be compensation by vasoactive intestinal peptide (VIP), the peptide with the closest structural homology to PACAP. In spite of this theoretical possibility, no compensatory changes were found in the expression of VIP in the brain (Girard et al. 2006). Therefore, it is still not known what mechanisms compensate the endogenous lack of PACAP, and it is possible that indirect compensatory effects exist, like the here-described differences in the proteins playing a role in the energy balance.

In summary, our present results open a novel direction to investigate alterations in PACAP-deficient mice that may explain their increased vulnerability to different harmful stimuli affecting the nervous system. Based on our present results, it seems that endogenous PACAP affects energy homeostasis and in lack of this neuropeptide, a disturbed energy balance exists which is cannot be compensated in case of an environmental challenge.

Acknowledgments This research was supported by the European Union and the State of Hungary, co-financed by the European Social Fund in the framework of TÁMOP-4.2.4.A/2-11/1-2012-0001 'National Excellence Programs' [A2-ACSJD-13-0302 (NKPR-2013-31609), A2-SZGYA-FOK-13-0003], OTKAK104984, PD109099, GVOP-3.2.1-2004-04-0172/3.0, TIOP 1.3.1-10/1-2010-0008, TIOP 1.3.1-07/1, TÁMOP-4.2.2.A-11/1/KONV-2012-0053, TÁMOP-4.2.2.A-11/1/KONV-2012-0024, NAP, PTE-MTA Lendület Program, and Arimura Foundation.

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