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Journal of Nutritional Biochemistry 22 (2011) 1003–1008

Journal of Nutritional Biochemistry

RESEARCH ARTICLES

Proteome alterations of cortex and hippocampus tissues in mice subjected to vitamin A depletion☆

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Received 13 October 2009; received in revised form 11 August 2010; accepted 23 August 2010

Abstract

Vitamin A regulates the development and maintenance of the central nervous system. Studies of vitamin A depletion (VAD) and mutations of retinoid receptors in rodents have revealed a dysfunction of motor and cognitive abilities. However, the molecular mechanisms underlying these behavioral changes are not well understood. In this study, VAD mice were examined and abnormal motor behavior related to psychosis symptoms was found. With the use of two-dimensional gel electrophoresis (2-DE), two-dimensional fluorescence difference gel electrophoresis (2D-DIGE) and mass spectrometric (MS) technologies, 44 and 23 altered protein spots were identified in the cortex and hippocampus, respectively, in VAD mice. By Western blot, the up-regulation of mitogen-activated protein kinase 1 (MAPK1) and proteasome subunit beta type 2 (PSMB2) in the cortex and that of dihydropyrimidinase-related protein 2 (DPYSL2) and PSMB2 in the hippocampus were observed in VAD mice. Bioinformatic analysis using DAVID revealed that altered proteins induced by VAD showed significant enrichment of (i) glycolysis, cytoskeleton, mitochondrion and glutamate metabolism in the cortex; and (ii) actin binding, dopamine receptor signaling and transmission of nerve impulse in the hippocampus. The up-regulations of DPYSL2, MAPK1 and PSMB2 may indicate the activated neuronal defensive mechanism in VAD brain regions, which may underlie the VAD-related psychosis behavior.

Keywords: Vitamin A depletion; Proteomics; Schizophrenia; Energy metabolism; Cytoskeleton

1. Introduction

Vitamin A, also known as retinol, is a pleiotropic micronutrient associated with a wide range of biological functions, including morphogenesis, vision, immune function and reproduction [1]. Vitamin A derivatives (retinoids) play an indispensable role in the development and maintenance of the central nervous system (CNS). During embryogenesis and early postnatal life, retinoic acid (RA) facilitates nervous system development *via* guiding patterning and neuronal differentiation [2]. By activating a developmental program in the adult CNS, RA could be an important regulator of regeneration [2]. Retinoids induce or repress gene transcription by interacting with distinct promotor sequence in target genes [3]. Two retinoid receptor families, namely, retinoic acid receptors (RARα, -β, -γ) and retinoid X receptors (RXRα, -β, -γ), have been identified in different brain regions, including the hippocampus and cortex [4], indicating the broad roles of retinoids in regulating nervous functions.

There is accumulating evidence suggesting that VAD contributes to motor and cognitive behavioral alterations in rodents. Vitamin A deprivation in adult rodents has been found to induce impairments in selective relational memory or spatial memory [5,6]. More recently, dysfunctions in locomotion and rotarod performance have been observed in VAD rats [3]. Compound null mutations in genes of RARβ and RXRβ and RXRγ would lead to locomotor defects in the adult mouse [7], whereas postnatal VAD produces no effect on working memory performance in rats [8]. Biochemical and electrophysiological methods have been applied to explain the impaired symptoms caused by VAD. Reduced expression of RARβ, RXRβ/γ, tTG mRNA and RC3 mRNA has been associated with impaired relational memory [5]. Dysfunctions of striatal cholinergic system and dopamine systems have been found related to impaired locomotion behavior [3,7]. In addition, damaged hippocampal long-term synaptic plasticity, which is generally thought to be the cellular mechanism for information storage and learning and memory, has been observed in VAD mice [9].

☆ The present study was supported by the S973 Program (2006CB910600), the National Natural Science Foundation of China (30700203) and the Shanghai Leading Academic Discipline Project (B205).

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doi:10.1016/j.jnutbio.2010.08.012

However, the detailed molecular basis underlying behavioral abnormality induced by VAD is still far from clear.

The dysfunction of vitamin A signaling was proposed to be related with neuropsychiatric diseases [schizophrenia and Alzheimer's disease (AD)]. In VAD rats, an accumulation of amyloid-β(Aβ) peptide in cerebral vessels, a down-regulation of RARα and a loss of choline acetyltransferase expression in forebrain cortical neurons have been observed, all of which are hallmarks of AD [10]. VAD also results in down-regulation of RARα, RARβ and components of the amyloid pathway [11]. The levels of these proteins are improved or restored by the subsequent administration of retinoic acid. In addition, retinoid signaling could be implicated in the etiology of schizophrenia at a number of levels [12]. Some loci possibly linked to schizophrenia are also the loci of genes associated with retinoid cascade. Numerous schizophrenia candidate genes (including dopamine D_2 receptor) are regulated by retinoid signaling. One microarray study found abnormal transport and synthesis of retinoic acid in schizophrenia patients [13]. Our previous study found alteration of retinoid transporter proteins (transthyretin and apolipoprotein E) in the plasma and cerebrospinal fluid (CSF) of schizophrenia patients [14]. Collectively, these clues suggest that VA signaling affects broad cerebral functions and a systemic study of proteome changes in VAD mice is necessary to illuminate the molecular mechanisms of symptoms implicated in neuropsychiatric diseases.

Previously, the motor behavior in VAD mice has been examined and enhanced locomotion in response to novel environment and increased stereotypy in response to the injection of MK-801 have been found [15], which are similar to some psychosis symptoms. To identify proteins related to VAD and to clarify the molecular mechanisms underlying VAD-induced behavioral alterations, two-dimensional gel electrophoresis (2-DE), two-dimensional fluorescence difference gel electrophoresis (2D-DIGE) and mass spectrometric (MS) technologies were used to explore the alteration of proteins in two brain regions (hippocampus and cortex). Western blot was used to validate altered proteins of interest. Bioinformatics tool was employed for data mining.

2. Materials and Methods

2.1. Animal preparation

C57BL6 Jico inbred strain mice (Shanghai Laboratory Animal Center, Chinese Academy of Science, Shanghai, China) were housed at a constant temperature of 25±2°C on a 12-h light–dark cycle with food and water available *ad libitum*. The experiments were conducted according to the People's Republic of China national standards for laboratory animal quality and the Chinese guidelines for care and use of laboratory animals. To produce VAD and control pups, pregnant C57BL/6J mice were fed a vitamin A-free diet (D03102201) or a vitamin A-enriched diet (D06051001) from the time of mating and the pups were weaned at the age of 3 weeks. Twenty-eight pups (male/female ratio=1:1) were selected randomly from litters generated by VAD mothers and were fed with vitamin A-free diet until they were sacrificed after the behavioral test. Another 28 pups (male/female ratio=1:1) were selected randomly from litters generated by control mothers and were fed with the diet containing vitamin A. In a separate group, animals were housed and receiving vitamin A-free food or normal food in the same manner as the previous group. Serums from 9-week-old VAD mice and the control group were extracted, and total retinol concentrations of serums were measured using the HPLC method. VAD mice had significantly lower serum retinol concentration than the control mice (data not shown). Behavioral measurements before and after MK-801 injection (0.6 mg/kg intraperitoneally) were conducted at the age of 9 weeks.

2.2. Tissue and protein preparation

After behavioral tests (at least 4 days after MK-801 injection), 12 VAD male mice and 12 control male mice were briefly maintained in a chamber filled with carbon dioxide until recumbent, immediately killed *via* decapitation and the brains were immediately put on ice. We focused on two brain regions of particular relevance to neuropsychiatric diseases. The entire hippocampus and the hemispheres (cerebral cortex) were separated and immediately stored in a −80° fridge. Resected cortex tissues were solubilized in 1-ml lysis buffer consisting of 7 M urea, 2 M thiourea, 4% CHAPS, 65 mM 1,4-dithiotrethiol, 40 mM Tris, 1 mM EDTA and anti-protease cocktail, and then centrifuged at 14 000×*g* at 20°C for 60 min. Desalting was carried out using an Ultrafree-4 centrifugal filter unit (Millipore, Bedford, MA, USA). Protein quantification was measured using the Bradford method.

2.3. Two-dimensional gel electrophoresis and image analysis

Proteins of cortex (control=12, VAD=11) were separated by 2-DE. Briefly, proteins were separated in the first dimension by isoelectric focusing (IEF) using pH 3–10, 24-cm immobilized pH gradient strips (GE Healthcare, Amersham, UK). A protein load of 150 μg for analytical gels and 1.2 mg for preparative gels was loaded onto the strips using in-gel rehydration. Samples were rehydrated and isoelectrically focused for a total of 90 kVh using the MultiPhor II (Amersham Pharmacia Biotech) and then stored at −70°C until further use.

Following IEF, strips were equilibrated and transferred to 12.5% homogeneous polyacrylamide gels. Electrophoresis was run for 5–6 h at 20 mA per gel at 15°C. Analytical gels of cortex samples were fixed overnight in a methanol/acetic acid solution and visualized with the PlusOne Silver Staining kit (Amersham Biosciences, Uppsala, Sweden). Preparative gels of cortex samples were stained with 0.1% Coomassie blue G-250.

All gels were scanned on a UMAX PowerLook III scanner (resolution 300 DPI). Protein spots were analyzed quantitatively using the ImageMaster Platinum 6.0 software (GE Healthcare, USA). Following automatic and subsequent manual editing, warping and matching procedures, the normalized volume of each protein, an integration of optical density and area, was measured objectively as a percentage of the total volume of all the detected spots and the data were exported for analysis. Differences in spot quantity between the VAD and control groups were analyzed using the independent *t* test from the Statistical Package for the Social Sciences (SPSS) 14.0, with the significance threshold set at $P<.05$. All results are shown as mean±S.D. Multivariate statistical analysis (PLS, SIMCA 11.0 software) was used to test how efficient these significantly altered spots were in generating the PLS model for distinguishing between the VAD and the control mice.

2.4. 2D-DIGE analysis

In the 2D-DIGE study, we took six hippocampus samples from VAD male mice and six from the control male mice. Hippocampus tissues were washed and suspended for 30 min on ice in 1-ml lysis buffer, consisting of 30 mM Tris-Cl, 2 M thiourea, 7 M urea, 4% CHAPS and 5 μl cocktail protease inhibitor. Samples were kept on ice and sonicated intermittently. They were then centrifuged at 4°C at 14 000×*g* for 30 min. Protein solutions were quantified by the Bradford method and stored at −70°C. Full details of the 2D-DIGE experimental procedures have been described in a previous study [16]. In summary, individual protein samples were firstly adjusted to moderate PH and minimally labeled with the fluorescent dye Cy2, Cy3 or Cy5 (GE Healthcare, Little Chalfont, UK). Half samples from the VAD group and control group were labeled with Cy3, and half were labeled with Cy5 to minimize potential dye artifacts. A protein pool consisting of all protein samples included in the study was used as an internal standard and was labeled with Cy2 fluorescent dye. Proteins labeled with Cy2 (pool), Cy3 and Cy5 were mixed and separated by IEF using pH 3–10, 24-cm nonlinear IPG DryStrips (GE Healthcare, Amersham, UK). Proteins were further separated using 12.5% homogeneous SDS-PAGE (Ettan DALT, GE Healthcare). Following electrophoresis, gels were scanned at appropriate wavelengths for Cy2, Cy3 and Cy5 fluorescence using a Typhoon 9400 scanner (GE Healthcare, Little Chalfont, UK). Gel images were cropped using ImageQuant V5.2 (GE Healthcare, Amersham, Uppsala, Sweden). Protein abundance was quantified using Image Master Platinum 6.0 software (GE Healthcare, USA), and the Cy2 internal standard included in all experiments for normalization purposes allowed both intra- and inter-gel analyses. After accurately matching the spots across six gels, independent *t* test was applied to identify significantly altered protein spots between the VAD and the control groups ($P<.05$). Multivariate statistical analysis (PLS) was also applied to screen protein spots which made the greatest contribution (VIP) to the PLS model for distinguishing between the VAD and control mice.

2.5. In-gel digestion and MALDI-TOF/TOF-TOF

Altered spots found by the *t* test analysis in cortex gels were directly excised from the Coomassie-stained gels. Protein spots showing significant changes and the top 30 VIP spots in the hippocampus tissues were selected from a colloidal Coomassie-stained gel for mass spectrometric identification purposes. All excised plugs were prepared as previously described [14]. Protein spots were destained, dried and digested by trypsin (sequencing grade, Ettan Chemicals, Amersham, Uppsala, Sweden) for 4 h. The resulting peptides were extracted using 10 μl of 50% ACN/0.1% TFA. The peptide solutions were dried in an Ettan Spot Handling Workstation (GE Healthcare, USA) and then resuspended with 3 μl of matrix solution, consisting of 50% acetonitrile, 0.03% TFA and semisaturated α-cyano-4-hydroxycinnamic acid. Samples were loaded to the MALDI target, and a time-of-flight mass spectrometer (Ettan MALDI-TOF/Pro, GE Healthcare, USA) was used to obtain PMF. ABI 4700 TOF/TOF ANALYZER (Applied Biosystems, USA) and AutoFlex TOF-TOF LIFT MS (Bruker Daltonics, Bremen, Germany) were used to obtain tandem MS data. The instrument's software was used to search for PMF in the NCBI 20080812 and Swissprot 56.1 mouse database. The MASCOT search engine was used to search for MS/MS data in the Swissprot 56.1 mouse database. Mono isotopic masses were used allowing a peptide tolerance of 100 ppm. MS/MS tolerance was set as 0.5 Da. The number of accepted missed cleavage sites was set to 1. Alkylation

of cysteine by carbamidomethylation was assumed as a fixed modification, and oxidation of methione was considered as a possible modification.

2.6. Western blotting

Four altered proteins (MAPK1, DPYSL2, PSMB2) were selected in VAD mice from the 2D or 2D-DIGE strategy for Western blotting. The protein samples (40–50 μg) were separated by 10% homogeneous SDS-PAGE before the proteins were transferred to NC membranes. After blocking with milk in PBS-Tween [0.1% (v/v)] buffer overnight at 4°C, the transferred membranes were incubated with four monoclonal antibodies: 20S proteasome β2 (MCP165) antibody: sc-58410 (Santa Cruz Biotechnology, California, USA); CRMP2 antibody [1B1]: ab62539 (Abcam, USA), and ERK2 antibody [1A8] (ab70532) (Abcam) for 2 h at 26°C; followed by horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology) for 2 h at 26°C. The blotting was developed by using ECL detection reagent (Amersham Pharmacia Biotech, Little Chalfont, UK) and visualized by autoradiography. HRP-conjugated monoclonal mouse anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was introduced as a reference. The intensities of each lane in Western blots were analyzed using Image Quant 5.2 (Molecular Dynamics). Four protein samples from VAD mice (hippocampus, cortex or liver) and four samples from control mice were run in the same gel. Independent *t* test was used to analyze the difference in bands' intensities between two groups.

2.7. Bioinformatics analysis

The DAVID2008 online platform (http://david.abcc.ncifcrf.gov/gene2gene.jsp) was used to conduct a functional enrichment analysis of significantly altered proteins [17]. Accession numbers of relevant proteins were input and "mouse" was set as a reference. DAVID covers multiple public databases (such as KEGG and GO). A conservative Fisher's Exact Test was used to calculate the EASE score of each pathway/function. $P<.05$ was set as significant, implying that the probability that these molecules are randomly associated with one pathway is relatively small.

Ingenuity pathway analysis (IPA) (http://www.ingenuity.com) was also conducted to explore enriched networks from altered proteins in the cortex and hippocampus as reported elsewhere [18].

3. Results

3.1. Proteome alteration in the cortex and hippocampus tissues induced by VAD

In total, 1000 well-matched spots from cortex tissues were analyzed for comparative proteomics study. Fig. 1 shows the representative 2-D gel of the cortex. The abundance of 51 spots was significantly altered in the VAD mice when compared to the control mice ($P<.05$, *t* test). Among them, 44 altered spots corresponding to 42 proteins were identified by MALDI-TOF-TOF (Supplementary Table 1). To validate these variations, 44 altered spots were selected to construct a PLS model. The PLS models based upon *t*-test results were able to distinguish between VAD and control mice with good calibration and validation scores (Supplementary Fig. 1).

For hippocampus, a total of 1842 spots were well matched across six VAD and six control DIGE gels. Normalized intensities of individual spots were generated for statistical analysis. *t* Test revealed seven altered spots with $P<.05$. From the total spots, we also used PLS to screen 30 spots with the highest VIP and found that the PLS model based upon these 30 spots had a good capability for distinguishing between VAD and control mice (Supplementary Fig. 2). The seven altered spots in the *t* test were all among the top 30 high VIP spots, and 21 of the remaining 23 high VIP spots had a *P* value of <.1. These 30 spots were selected as VAD-related spots and 23 of them were identified by MALDI-TOF-TOF. Fig. 2 shows the representative 2D gel of the hippocampus. There were 23 spots representing 22 proteins listed in Supplementary Table 2.

Some dysregulated proteins were found in duplicate on the 2-D gels, which may represent isoforms or various post-translational modification states of these proteins. Three proteins were found altered in both hippocampus and cortex tissues: DPYSL2, PSMB2 and GLUL.

3.2. Western blotting

The up-regulation of PSMB2 and MAPK1 protein in the cortex of VAD mice was confirmed by Western blot ($P=.022$, PSMB2; $P=.021$, MAPK1) (Fig. 3A and D). PSMB2 and DPYSL2 in the hippocampus of VAD mice were up-regulated significantly ($P=.005$, PSMB2; $P=.027$, DPYSL2) (Fig. 3B and D).

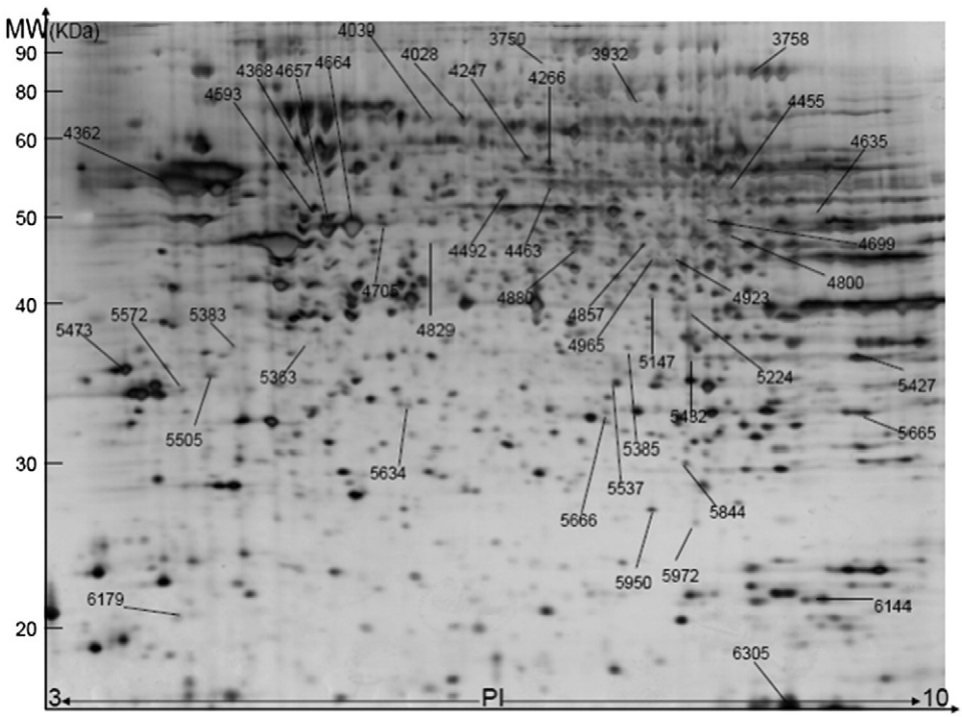

Fig. 1. Two-dimensional gel image of the cortex tissue of VAD mice. The 2D gel was silver stained. Marked spots are those significantly altered between VAD and control mice ($P<.05$) and identified by MALDI-TOF-TOF.

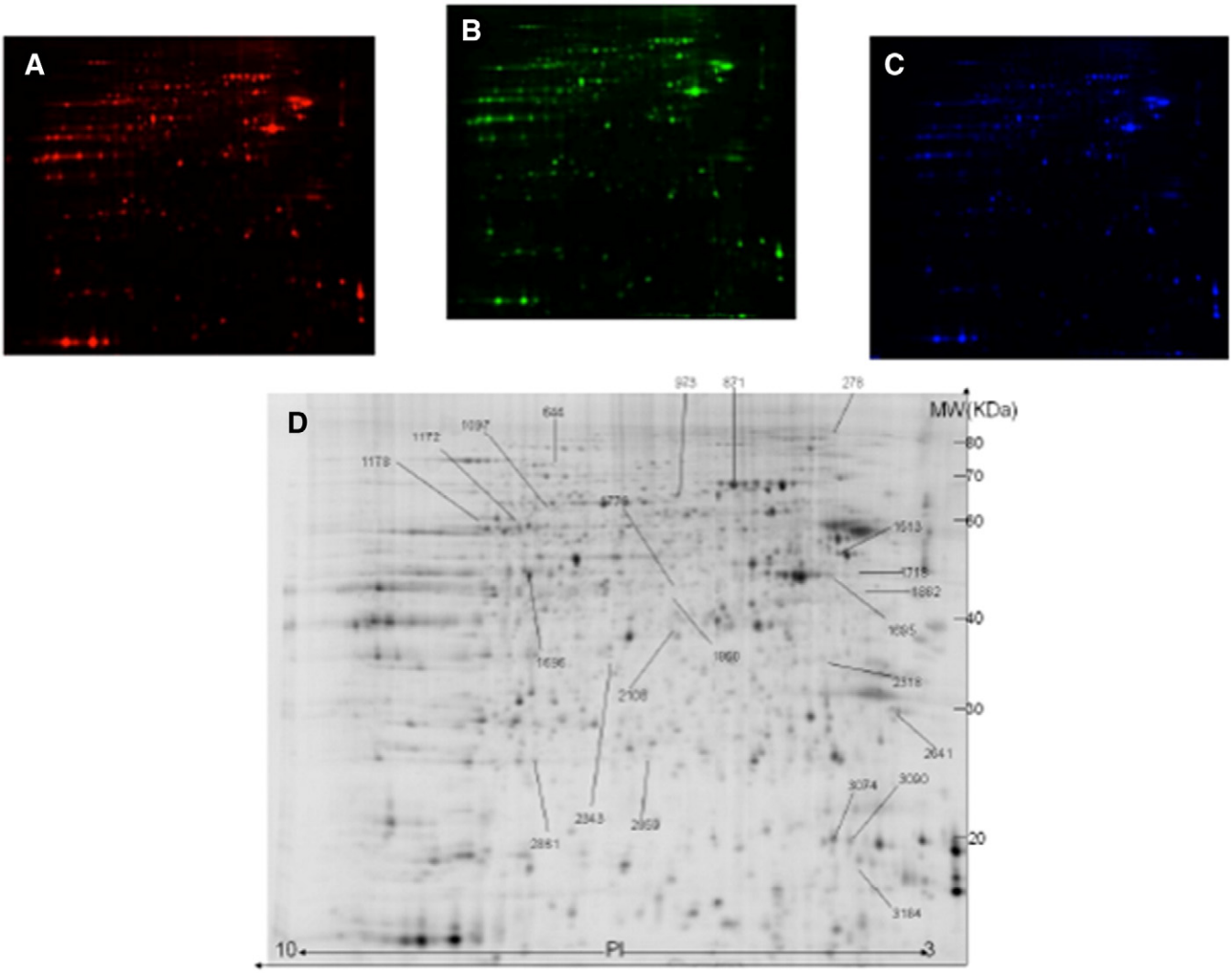

Fig. 2. Two-dimensional fluorescence difference gel electrophoresis of hippocampus proteins in VAD mice. (A) CY5-labelled, (B) CY3-labelled and (C) CY2-labelled gel images. (D) Marked spots are those significantly altered ($P<.05$) between VAD and control mice or top 30 VIP spots (PLS analysis) and identified by MALDI-TOF-TOF.

3.3. Bioinformatics analysis

Altered proteins in the cortex significantly clustered to the mitochondrion, glycolysis, cytoskeleton and the glutamine metabolic process ($P<.05$, conservative Fisher's Exact Test). Altered proteins in the hippocampus significantly clustered to actin binding, the dopamine receptor signalling pathway, mitochondrion and transmission of nerve impulse ($P<.05$, conservative Fisher's Exact Test) (Table 1).

IPA of altered proteins in the cortex and hippocampus indicated a hub molecule, GRIN2B, which has been reported to interact with six altered proteins (TPI1, ACO2, DPYSL2, BSN, MAPK1 and YWHAE) in the cortex and with four altered proteins (SNAP25, TUBB2C, MRLC2 and DPYSL2) in the hippocampus [19,20]. Seven altered proteins (ACTG1, ACTA1, IVD, TPI1, MAPK1, YWHAE and DPYSL2) are known to be regulated by retinoic acid [21–26]. Supplementary Fig. 3 shows the networks of altered proteins in cortex and hippocampus by IPA.

4. Discussion

The present results suggest that proteome alterations in VAD mice are involved in cytoskeleton and nervous system functions in both the cortex and hippocampus. These findings provide possible molecular mechanisms underlying altered motor abilities in VAD mice.

4.1. VAD induces protein changes in cytoskeleton and nervous system functions in the cortex

In the cortex, several cytoskeleton-related proteins were altered in VAD mice. The reduced level of RA may regulate the level of DPYSL2 [26], which is involved in cytoskeleton remodeling and axonal guidance. Other dysregulated proteins in VAD mice (ACTG1, ACTA1, MAPRE1, MAPRE2, CORO1A, ROCK1 and BSN) could also indicate their influence in cell migration and neurotransmitter release.

Dysregulated proteins in the cortex of VAD mice also suggest alteration in glutamate pathways. The altered level of GLUL and GMPS in the VAD mice may indicate reduced glutamine and increased glutamate. The up-regulation of PHGDH may point to the increased serine, which corresponds to our previous finding in astrocytes with retinoic acid deficiency [18]. Both glutamate and D-serine could influence NMDA receptors in the brain. Interestingly, six altered proteins in the VAD mice were found to interact with GRIN2B, suggesting dysfunction of glutamate-mediated pathways in the VAD cortex. The reduced levels of RA may up-regulate MAPK1 and YWHAE [24,25], which are important signalling molecules in regulating neuronal differentiation, neuronal migration, cortex development and other nervous system functions [27,28]. ERK pathway was previously reported to mediate the effect of mood stabilizers

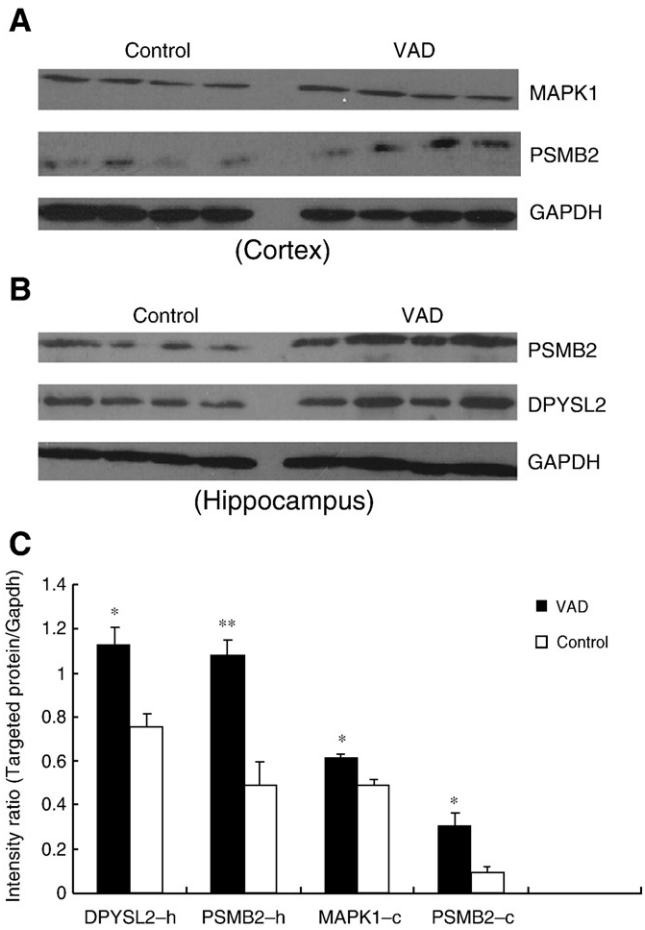

Fig. 3. Western blot of DPYSL2, PSMB2, DPYSL2 and PGM2 in the cortex (c) and hippocampus (h) tissues of VAD and control mice (A, B). The significance of mean difference between VAD and control groups was analyzed by using independent *t* test (*P<.05, **P<.01). DPYSL2-h, P=.027; PSMB2-h, P=.005; MAPK1-c, P=.021; PSMB2-c, P=.022 (C).

including locomotion in rats [29]. Therefore in VAD condition, the MAPK1/ERK pathway could be activated to promote neuronal growth and migration and influence the locomotion.

4.2. Proteome alterations in hippocampus points to actin binding, dopamine receptor signaling and glutamate metabolism

In the hippocampus, four VAD-related proteins (ACTR2, SPTAN1, GMFB and SYN1) were involved in actin binding, which may regulate neural regeneration and neurotransmitter release. The trend of down-regulation of G-proteins (GNO1 and GNA11) in VAD mice may provide the mechanisms of enhanced locomotion, as the disruption of GNO1 protein may increase the locomotor activity in the previous study [30].

Four VAD-related proteins have been reported as interacting with GRIN2B in the hippocampus, including the cytoskeleton (TUBB2C, MRLC) [19], synaptic vesicle (SNAP25) [19] and DPYSL2. In the present study, the up-regulation of DPYSL2 in the hippocampus of VAD mice was validated by Western blot. Therefore in the hippocampus, the reduced level of RA may up-regulate the level of DPYSL2 [26] and influence the guidance of axonal growth which could change the nervous circuits related to motor abilities.

4.3. VAD and neuropsychiatric diseases

With respect to neuropsychiatric diseases, several protein alterations in VAD mice correspond with findings in studies of postmortem brain tissues of patients. DPYSL2 was up-regulated in the hippocampus of VAD mice, which corresponds to a previous proteomics report of increased DPYSL2 in the hippocampus of schizophrenia patients [31]. The up-regulation of SNAP25 in CSF has been associated with schizophrenia [32], but our results showed a down-regulation of SNAP25 in the hippocampus of VAD mice. Interestingly, a systematic study of transcriptional, proteomics and metabolite perturbations in the schizophrenia prefrontal cortex [33] found significant dysfunctions of pathways related to glucoregulation and mitochondrial function. Multiple alterations of proteins in the schizophrenia prefrontal cortex have also been found in the VAD cortex (ACO2, TPI1, PGAM1, PDHA1, ACTG1, ACTC1, DPYSL2, PHGDH and CKB) and hippocampus (SPTAN1, DPYSL2, Crmp1, ALB and PKM2), indicating that schizophrenia and VAD produce similar effects on glycolysis, mitochondrion and cytoskeleton functions. Combined with the hyperactivity of locomotion and increased stereotypy in response to MK-801 in VAD mice, our proteomics findings support VAD as a risk factor for generating schizophrenia-related symptoms by regulating glycolysis, mitochondrial functions, cytoskeleton, dopamine receptor signalling and glutamate signalling.

Several VAD-related proteins have also been associated with other neuropsychiatric diseases. MPST and SYN1 have been found to be dysregulated in the cortex [34] and hippocampus [35] of bipolar patients. These alterations overlap with our findings of dysregulation of SYN1 and MPST in the cortex or hippocampus of VAD mice. Furthermore, two VAD-related proteins (PDHA1, MAPK1) in the cortex are associated with APP [36,37], indicating the potential involvement of VAD in the pathology of AD. CA2, CKB, PPI, SNAP25 and GNA11 have also been found to be altered in Huntington's disease [38,39]. These results suggest that broad molecular alterations induced by VAD could be associated with different neuropsychiatric diseases.

In summary, the present nutriproteomics study reported the effect of nutritional disruption (VAD) on proteome alterations in mice central nervous tissues and highlighted VAD-related pathways

Table 1
Enriched functional annotations in the cortex and hippocampus tissues of VAD mice

Category[a]	Tissue	Pathways	*P* Value[b]	Proteins
GO	Cortex	Mitochondrion	1.69E−04	O55126, Q04447, O08553, P15105, O88696, Q99KI0, P02088, Q8CAQ8, Q60931, P35486
GO	Cortex	Cytoskeleton	.01301	O89053, O88737, P68134, Q8R001, P70335, Q61166, P63260
GO	Cortex	Glutamine metabolic process	.036629	Q3THK7, P15105
GO	Hippocampus	Actin binding	.004277	P61161, Q9CQI3, P16546, O88935
GO	Hippocampus	Dopamine receptor signaling pathway	.024954	P18872, P21278
GO	Hippocampus	Mitochondrion	.030149	P52480, O08553, P15105, Q99J99, P56395
GO	Hippocampus	Transmission of nerve impulse	.036665	P60879, P21278, O88935
SP_PIR	Cortex	Glycolysis	.003549	P17751, P35486, Q9DBJ1

[a] Public databases used for functional enrichment analysis in the DAVID tool.
[b] *P* value was calculated by conservative Fisher's Exact Test. P<.05 was set as significance.

including cytoskeleton and nervous system functions in the cortex, and actin binding, dopamine receptor signalling and glutamate signaling in the hippocampus. The up-regulation of DPYSL2 in the hippocampus and MAPK1 in the cortex of VAD mice could indicate neuronal defensive mechanism involving active neuronal repair, regeneration and development, in response to possible inflammation and cell death in brain regions. PSMB2 was elevated in both the cortex and hippocampus of VAD mice, suggesting that broad protein damages may happen in both the cortex and hippocampus such that the protein degradation process was activated.

There is no doubt that further studies are required to verify the alterations of important signalling proteins and hub molecules (such as GRIN2B) identified in our study in the future. Furthermore, the developmental changes induced by VAD can be detected, including neuronal migration and differentiation, nervous architecture and neurotransmitter levels. The present study provides clues for understanding the role of nutritional factors (such as vitamin A) in the nervous system and sheds light on the pathological mechanisms underlying neuropsychiatric diseases.

Acknowledgments

We thank all persons participating in the experimental design, technological support and helpful discussions.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jnutbio.2010.08.012.

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