Int J Clin Exp Pathol 2015;8(10):12634-12645
www.ijcep.com /ISSN:1936-2625/IJCEP0015033

Original Article

Proteomics and bioinformatics analysis of mouse hypothalamic neurogenesis with or without *EPHX2* gene deletion

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Received August 24, 2015; Accepted September 25, 2015; Epub October 1, 2015; Published October 15, 2015

Abstract: The aim of this study was to identify differently expressed proteins in the presence and absence of *EPHX2* gene in mouse hypothalamus using proteomics profiling and bioinformatics analysis. This study was performed on 3 wild type (WT) and 3 *EPHX2* gene global knockout (KO) mice (*EPHX2*$^{-/-}$). Using the nano- electrospray ionization (ESI)-LC-MS/MS detector, we identified 31 over-expressed proteins in WT mouse hypothalamus compared to the KO counterparts. Gene Ontology (GO) annotation in terms of the protein-protein interaction network indicated that cellular metabolic process, protein metabolic process, signaling transduction and protein post-translation biological processes involved in *EPHX2*$^{-/-}$ regulatory network. In addition, signaling pathway enrichment analysis also highlighted chronic neurodegenerative diseases and some other signaling pathways, such as TGF-beta signaling pathway, T cell receptor signaling pathway, ErbB signaling pathway, Neurotrophin signaling pathway and MAPK signaling pathway, were strongly coupled with *EPHX2* gene knockout. Further studies into the molecular functions of *EPHX2* gene in hypothalamus will help to provide new perspective in neurogenesis.

Keywords: *EPHX2*, hypothalamic neurogenesis, proteomics profiling, protein network

Introduction

Soluble epoxide hydrolase (sEH), a ubiquitously expressed predominantly cytosolic enzyme that encoded by *EPHX2* gene, was found to be over expressed in liver, kidney, heart and ovary tissues [1]. Meanwhile, the epoxyeicosatrienoic acids or EETs have been reported to be important endogenous substrates for sEH which produced by cytochrome P-450 epoxygenases [2]. As a ubiquitously hydrolytic enzyme, sEH can catalyze EETs into dihydroxyeicosatrienoic acids (DHETs), which are biologically less active [3]. By far, a growing body of evidence indicates that EETs play a series of benignant roles in ischemia/reperfusion [4], inflammatory response [5], fibrinolysis [6], tube formation [7] *etc*.

In brain, EETs have been identified to promote vasodilatation, resulting in a protective effect against ischemia-induced tissue damage [8-10]. Besides, the stabilized EETs were also found to antagonize inflammation *via* negative regulation of nuclear factor-κB (NF-κB) [5]. Given the salutary effects of sEH deficiency, it global expression of proteins in tissues and fluids [11]. The proteins or peptides that are preferentially expressed and identified pathological state are well suited for diagnostic assays and medical treatment. In addition, with the rapid development of computational biology, rapid advances in network biology indicate that cellular is an attractive therapeutic target for several disorders [12-14]. Thus, insights into the physiological functions of sEH have emerged from studies in mice with global *EPHX2* gene deficiency or pharmacological inhibition of sEH, which in turn intrigues curiosities of neuroscientists.

Recent advances in analytic technique present a new opportunity to examine the networks are governed by universal laws and offer a new conceptual framework that could potentially revolu-

tionize our view of biology and disease pathologies in the twenty-first century [15]. Systematic mapping of protein-protein interactions networks was initiated in model organisms, starting with defined biological processes to proteome [16]. However, detailed network perspective associated with sEH in hypothalamic neurogenesis is still unclear. Given the role of sEH deficiency in neural systems and the lack of proteomics based network biological studies related to sEH, the aim of our present study is to extract the significantly expressed proteins with or without *EPHX2* gene knockout in mouse and explored the potential signaling pathways involved in *EPHX2* gene. Detailed elucidations are as follows.

Materials and methods

Ethics statement

This experiment was conducted according to the Guide for the Care and Use of Laboratory Animals of the US National Institutes of Health (NIH Publication, 8th Edition, 2011) and approved by the Peking University Committee on Animal Care and Use. All surgeries were performed under sodium pentobarbital anesthesia, and all efforts were made to ameliorate animal suffering and euthanasia.

Animal models

Male C57BL/6 background mice (8-10 weeks old, 20 ± 4 g body weight) with and without targeted disruption of *EPHX2* gene (*EPHX2*$^{-/-}$) were provided by Professor Yi Zhu at the department of physiology and pathophysiology in Peking University [17]. Mice were housed individually in air-conditioned facilities at room temperature with 55 ± 5% humidity under 12:12 h light/dark artificial cycle conditions, and supplied with food and water *ad libitum*.

Protein sample processing

Mouse hypothalamic tissues were washed with chilled phosphate buffered saline (PBS) and homogenized using a motor-driven glass-teflon homogenizer. After centrifuging and boiling at 100°C for 5 min, proteins from hypothalamus were extracted. The concentration of whole proteins was determined at 570 nm using the bicinchoninic acid (BCA, Pierce, Rockford, IL, USA) assay kit. Proteins from three tubes of *EPHX2*$^{-/-}$ and the control groups were pooled to minimize individual variation. Protein samples (200 μg) from each group were processed per the manufacturer's protocol for FASP (Filter Assisted Sample Preparation, Mann). Briefly, to Vivacon 500 filtrate tube (Cat No. VN01H02, Sartorius Stedim Biotech) containing protein concentrates, 100 μL of 8 M urea in 0.1 M Tris/HCL, pH 8.5 (UA) was added and samples were centrifuged at 14,000 g for 15 min at room temperature. Then 10 μL of 0.05 M TCEP in water was added to the filters and incubated at 37°C for 1 h. 10 μL of 0.1 M IAA in UA was added to the filters, and the samples were incubated for 30 min in darkness. Filters were washed twice with 200 μL of 50 mM NH_4HCO_3. Finally, 4 μg trypsin (Promega, Madison, WI) was added in 100 μL of 50 mM NH_4HCO_3 to each filter. The protein to enzyme ratio was 50:1. Samples were incubated over night at 37°C and released peptides were collected by centrifugation.

Protein separation

The digested peptide mixture was reconstituted with 600 μL buffer A (20 mM ammonium formate in water, pH = 10) and loaded onto a 2.1 × 150 mm Waters XBridge BEH130 C_{18} column containing 3.5 μm particles (Waters, Milford, MA). The peptides were eluted at a flow rate of 230 μL/min with a gradient of 5% buffer B (20 mM ammonium formate in 80% acetonitrile, pH = 10) for 5 min, 1-15% buffer B for 15 min, 15-25% buffer B for 10 min, 25-55% buffer B for 10 min, and finally 55-95% buffer B for 5 min. The system was then maintained in 95% buffer B for 5 min before equilibrating with 1% buffer B for 8 min prior to the next injection. Elution was monitored by measuring absorbance at 214 nm, and fractions were collected every 2 min. The eluted peptides were pooled as 15 fractions and vacuum-dried. Then samples were ready for nano-ESI-LC-MS/MS analysis.

Liquid chromatography tandem mass spectrometry (LC-MS/MS) analysis

The MS analysis experiments were performed on a nano-flow HPLC system (Easy-nLC II, Thermo Fisher Scientific, USA) connected to a LTQ-OrbitrapVelos Pro (Linear quadrupole ion trap-Orbitrap mass analyzer) mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA), equipped with a Nanospray Flex Ion Source (Thermo Fisher Scientific, USA). The

peptide mixtures were injected (5 μL) at a flow rate of 5 μL/min onto a pre-column (Easy-column C_{18}-A1, 100 μm I.D. × 20 mm, 5 μm, Thermo Fisher Scientific). The chromatographic separation was performed on a reversed phase C_{18} column (Easy-column C18-A2, 75 μm I.D. × 100 mm, 3 μm, Thermo Fisher Scientific) at a flow rate of 300 nL/min with a 60 min gradient of 2% to 40% acetonitrile in 0.1% formic acid. The electrospray voltage was maintained at 2.2 kV, and the capillary temperature was set at 250°C. The LTQ-Orbitrap was operated in the data dependent mode to simultaneously measure full scan MS spectra (m/z 350-2000) in the Orbitrap with a mass resolution of 60,000 at m/z 400. After the completion of the full-scan survey, the 15 most abundant ions detected in the full-MS scan were measured in the LTQ part by collision induced dissociation (CID), respectively.

Protein identification and quantization

As to the protein identification, data analysis was performed using MaxQuant software (version 1.4.1.2, http://www.maxquant.org/). Briefly, the raw MS/MS data were submitted to the Uniprot human protein database (http://www.uniprot.org/, release 3.43, 72,340 sequences) using the Andromeda search engine with the following settings: trypsin cleavage; fixed modification of carbamidomethylation of cysteine; variable modifications of oxidation of methionine; a maximum of two missed cleavages; the false discovery rate was calculated by decoy data base searching. Label-free quantization was also performed in MaxQuant. The Min. ratio count for LFQ was set 2, and the match-between-runs option was enabled. Other parameters were set as default.

Raw data analytical procedures

All spectra were exported as plain-text files and the *.txt files were read using Perseus software (version 1.5.0.3, http://www.perseus-framework.org/). Perseus, an architecture based on Windows. NET framework, was designed for the statistical analysis of omics data, such as mRNA microarray [18]. Briefly, raw data were firstly processed to remove random noises, subtract the low-frequency baseline, and detect and quantify individual sample peaks using the filter function button. Log 2 based normalized expression data was performed before differently expressed proteins identification.

Differentially expressed proteins identification

The differentially expressed proteins between the 3 couples of *EPHX2*$^{-/-}$ mice and the matched normal ones were assessed using two-sample *t* test method as previously described [18]. The parameter *p*-value was adjusted for multiple testing corrections with *p* value cutoff of 0.0001. No further false discovery rate was used for multiple testing correction in this experiment.

K-means clustering and principal component analysis

A nonsupervised analysis of global gene expression was performed using *k*-mean hierarchical clustering. Hierarchical clustering, also known as hierarchical cluster analysis or HCA, is a cluster analysis method which focuses on building a hierarchy of clusters using Agglomerative and Divisive strategies , such as Cluster 3.0 [19], NCSS statistical software, SPSS *etc*. In this study, cluster proteins with aberrant expression were agglomerated using *k*-mean average linkage hierarchical clustering rule and delineated based on the *Z* score method.

Besides, an orthogonal transformation to the linearly uncorrelated variables of differently expressed proteins was also visualized using principal component analysis (PCA). PCA, a variance-focused approach seeking to reproduce the total variable variance, creates variables that are linear combinations of the original variables and reflect both common and unique variance of the variable using correspondence analysis. To better merit the heterogeneity of protein candidate between *EPHX2* gene knock and the normal group, we performed a PCA analysis using SPSS for Windows (SPSS 18.0; SPSS, Chicago, IL, USA).

Protein-protein interaction network construction

The protein to protein interaction network associated with *EPHX2* was constructed as previously described [20]. To infer a reliable network from sEH to the possible proteins identified in our proteomics analysis, proteins found in proteomic analysis were used as seed to fish out other partners with direct interactions. Nodes relations were retrieved from Database of Interaction Proteins (DIP, http://dip.doe-mbi.

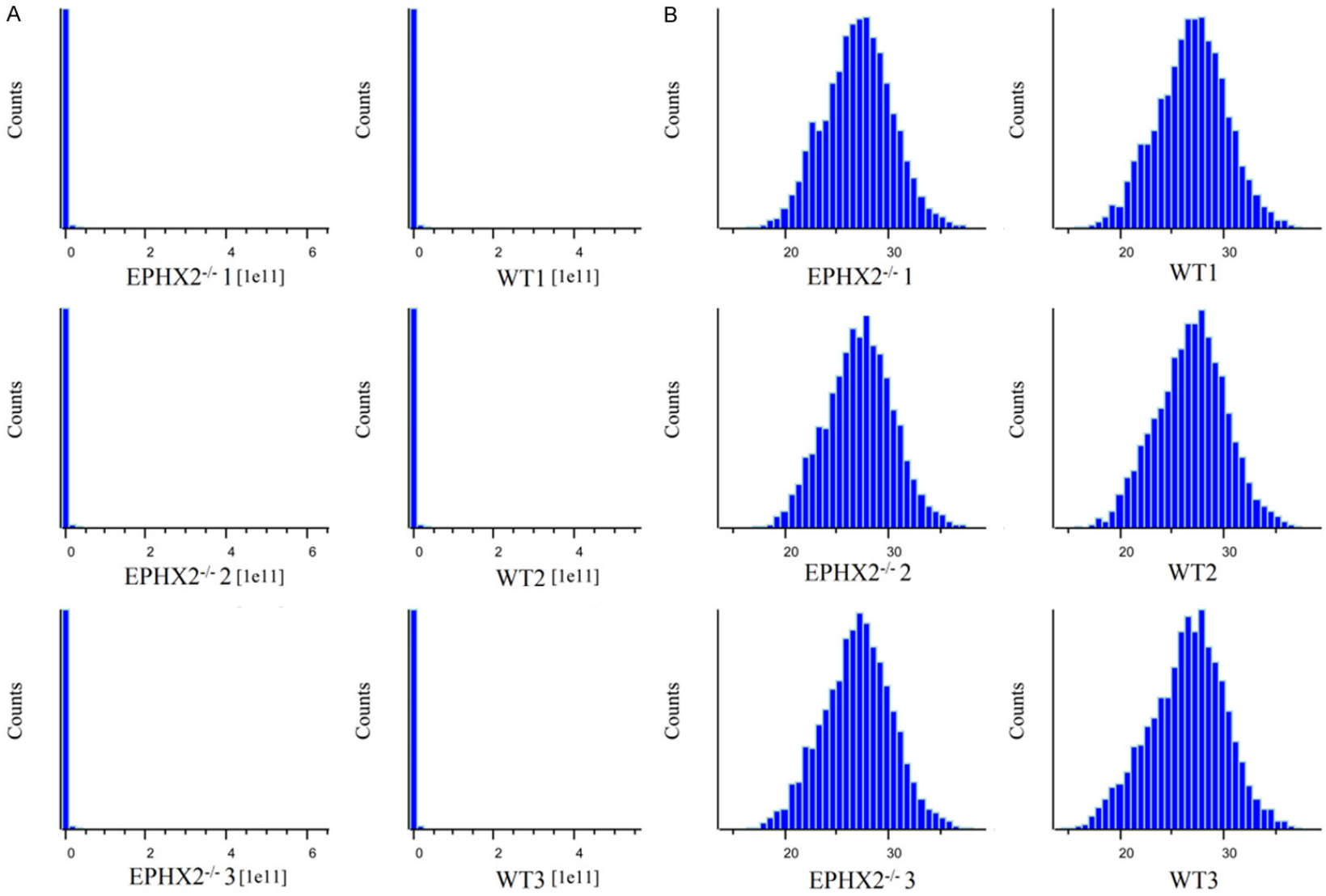

Figure 1. Histogram results of the raw abundance of proteins before and after logarithm transformation.

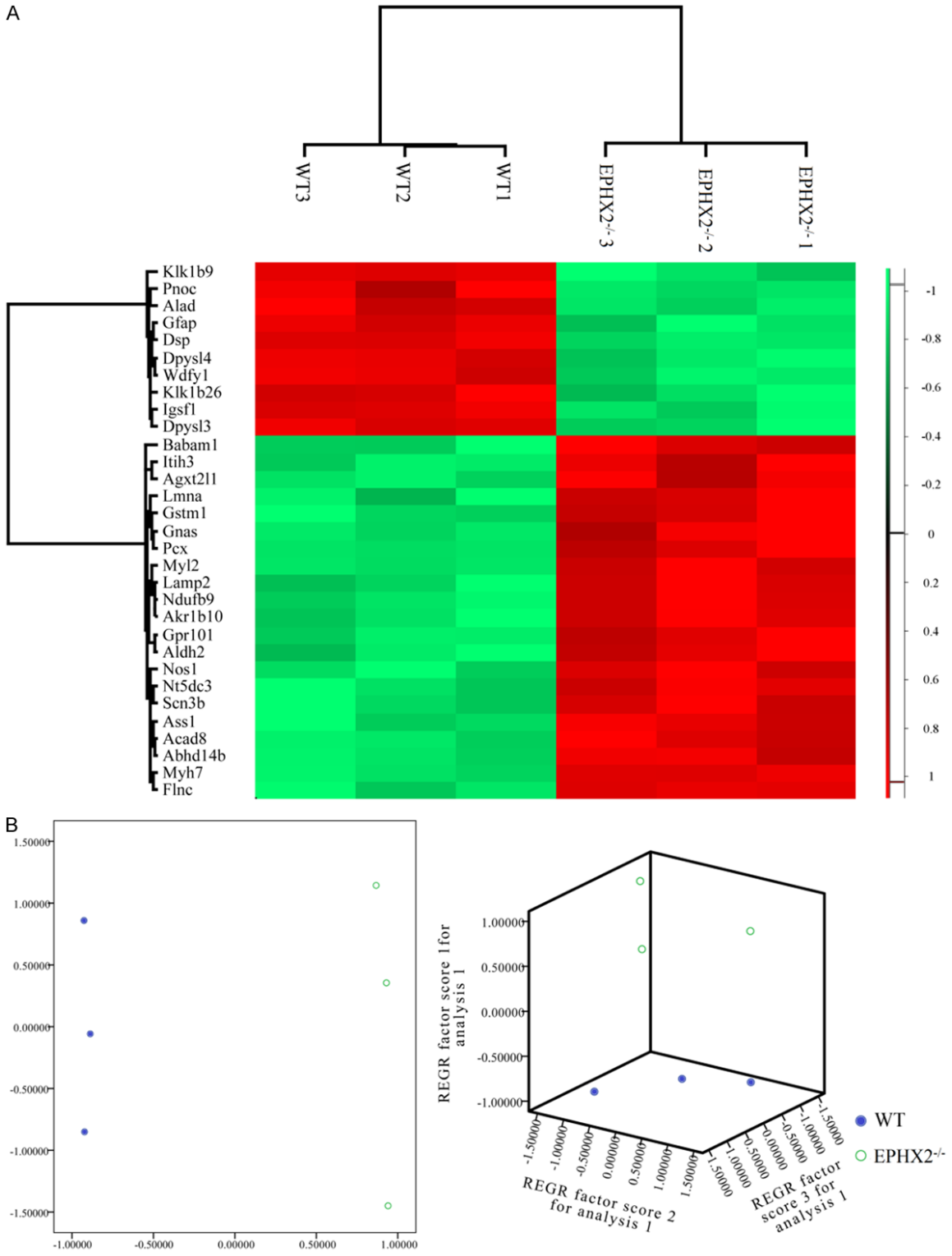

Figure 2. *K*-means clustering (A) and principal component analysis (B) of the 31 proteins with different expression in *EPHX2*$^{-/-}$ (n = 3) compared with the vehicle groups (n = 3). Color key in the hierarchical clustering represents the different expression levels. Red represents up-regulation, while green represents down-regulated proteins.

ucla.edu/dip/Main.cgi) [21], BIOGRID (Biological General Repository for Interaction Datasets, http://thebiogrid.org/) [22], HPRD (Human Protein Reference Database, http://www.

Table 1. Identification of proteins associated in *EPHX2*$^{(+/+)}$ and *EPHX2*$^{(-/-)}$ mouse hypothalamus using ESI-LC-MS/MS

Protein ID	Protein names	Official gene symbol	-Log t-test *p* value	t-test Difference
D3YW87	Filamin C, gamma	Flnc	5.09635	1.91472
E9PWE8	Dihydropyrimidinase-related protein 3	Dpysl3	4.61218	-1.14479
E9Q4P1	WD repeat and FYVE domain containing 1	Wdfy1	4.89347	-1.80857
E9Q557	desmoplakin	Dsp	5.72609	-1.37034
E9QN99	Abhd14b abhydrolase domain containing 14b	Abhd14b	4.75775	0.5864
E9QPD7	pyruvate carboxylase	Pcx	4.2864	0.408389
Q9Z0J4	nitric oxide synthase 1, neuronal	Nos1	4.51849	0.471614
G5E895	aldo-keto reductase family 1, member B10 (aldose reductase)	Akr1b10	4.43471	0.750383
H3BJR6	sodium channel, voltage-gated, type III, beta	Scn3b	4.11826	0.797281
Q3TMU8	dihydropyrimidinase-like 4	Dpysl4	4.79247	-0.763332
P03995	glial fibrillary acidic protein	Gfap	4.4619	-1.61872
P10518	aminolevulinate, delta-, dehydratase	Alad	4.28579	-0.703655
P10649	glutathione S-transferase, mu 1	Gstm1	4.17015	0.840785
P15949	kallikrein 1-related peptidase b9	Klk1b9	4.80132	-5.13527
P16460	argininosuccinate synthetase 1	Ass1	4.56585	0.339808
P17047	lysosomal-associated membrane protein 2	Lamp2	4.01747	2.33493
P36369	kallikrein 1-related petidase b26	Klk1b26	4.05746	-4.61297
P47738	aldehyde dehydrogenase 2, mitochondrial	Aldh2	4.10676	0.884048
P48678	lamin A	Lmna	4.01616	1.05971
P51667	myosin, light polypeptide 2, regulatory, cardiac, slow	Myl2	4.48729	3.80926
Q3UHB1	5'-nucleotidase domain containing 3	Nt5dc3	4.49016	1.8812
Q3UI43	BRISC and BRCA1 A complex member 1	Babam1	4.1279	1.24296
Q61704	inter-alpha trypsin inhibitor, heavy chain 3	Itih3	4.22979	0.659157
Q64387	prepronociceptin	Pnoc	4.184	-1.47249
Q6R0H7	GNAS (guanine nucleotide binding protein, alpha stimulating) complex locus	Gnas	4.16739	0.81181
Q7TQA1	immunoglobulin superfamily, member 1	Igsf1	5.0341	-0.607182
Q80T62	G protein-coupled receptor 101	Gpr101	4.27175	2.75136
Q8BWU8	ethanolamine phosphate phospholyase	Agxt2l1	4.35029	0.943425
Q91Z83	myosin, heavy polypeptide 7, cardiac muscle, beta	Myh7	5.75811	3.22376
Q9CQJ8	NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 9	Ndufb9	4.62392	0.991632
Q9D7B6	acyl-Coenzyme A dehydrogenase family, member 8	Acad8	4.66179	0.494231

hprd.org/) [23], BOND (Bimolecular Object Network Database, http://bind.ca), MINT (Molecular Interaction database, http://mint.bio.uniroma2.it/mint/Welcome.do) [24] and IntAct (http://www.ebi.ac.uk/intact/) [25]. The optimized network based on Steiner minimal tree algorithm was visualized in the Cytoscape environment [26]. Besides, nodes without connections were removed from the integrated network and only the largest component was regarded as the protein-protein interaction network associated with sEH.

Gene ontology and pathway enrichment analysis

The BiNGO plugin [27] in Cytoscape environment and DAVID web-server [28] (http://david.abcc.ncifcrf.gov/) were used to retrieve the Gene Ontology Consortium (GOC, http://geneontology.org/) [29] and Kyoto Encyclopedia of Genes and Genomes (KEGG) [30] annotations for the protein to protein interaction network as previously described [31].

Results

31 proteins were found to be differently expressed in mouse hypothalamic tissue with or without EPHX2 gene deletion

As a result, a total of 6,158 proteins were identified using shotgun method. A logarithm transformation (base 2) of the raw abundance of proteins was pre-performed before differently expressed proteins identification (**Figure 1**). As

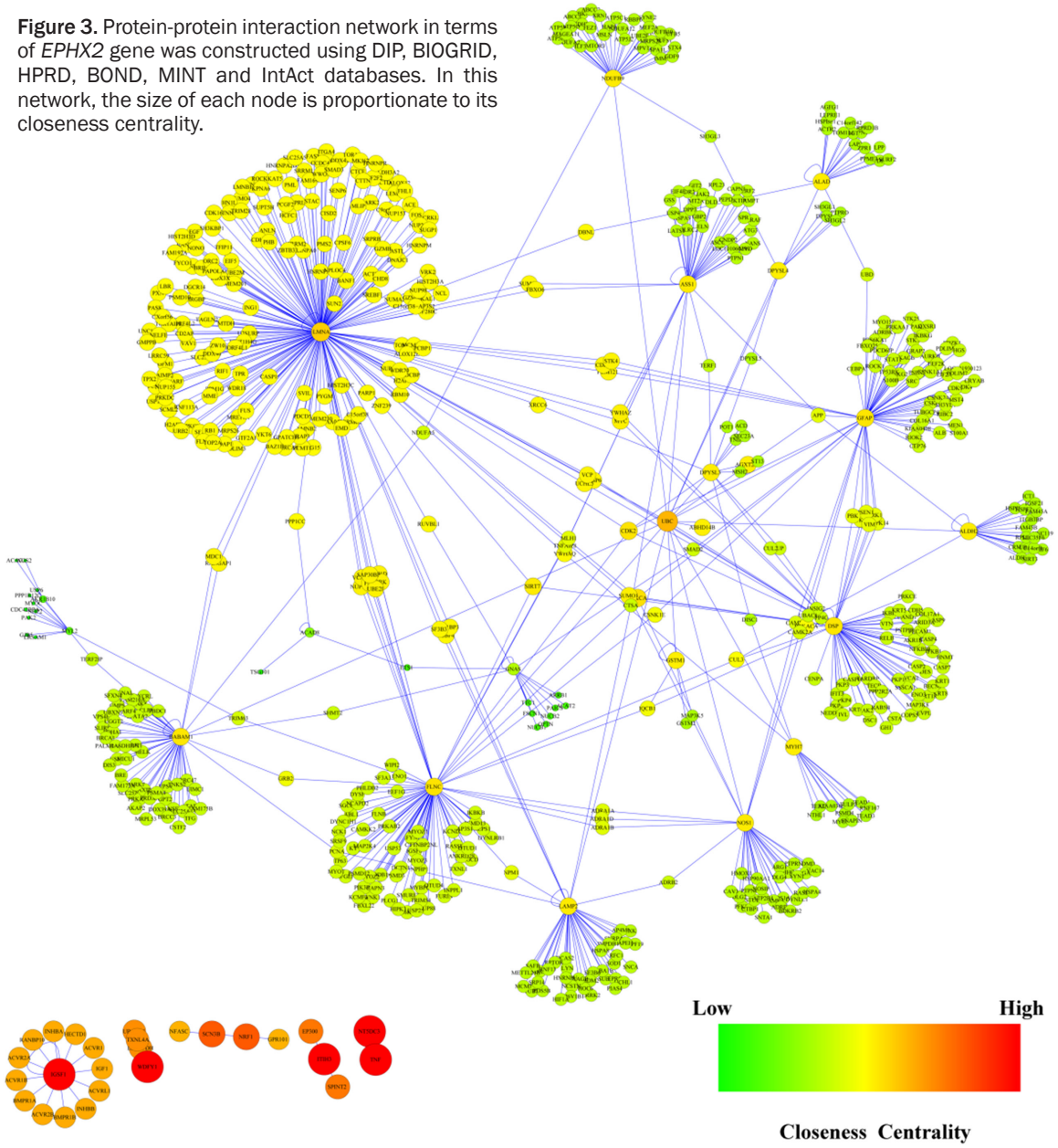

Figure 3. Protein-protein interaction network in terms of *EPHX2* gene was constructed using DIP, BIOGRID, HPRD, BOND, MINT and IntAct databases. In this network, the size of each node is proportionate to its closeness centrality.

shown in **Figure 2A** and **Table 1**, we found a total of 31 differently expressed proteins. 2D and 3D PCA (**Figure 2B**) also indicated that those 31 proteins had an excellent ability to discriminate mouse models with or without *EPHX2* gene deletion and could be used as phenotypic discriminators.

Protein-protein interaction regulatory network construction

To infer a protein-protein interaction network associated with sEH, we matched the 31 differently expressed genes with 6 public available warehouses to link the known regulatory data between transcriptional factors and the target genes. Totally, we obtained a cohort of 685 nodes and 768 relationships, and the integrated regulatory network was visualized using Cytoscape 2.8.3 (**Figure 3**).

Enrichment of the biological processes

To further extend our knowledge about the regulatory network associated with sEH, we enriched the large list of proteins for functional annotation using BiNGO plugin. As demonstrated in the **Figure 4**, the biological processes in

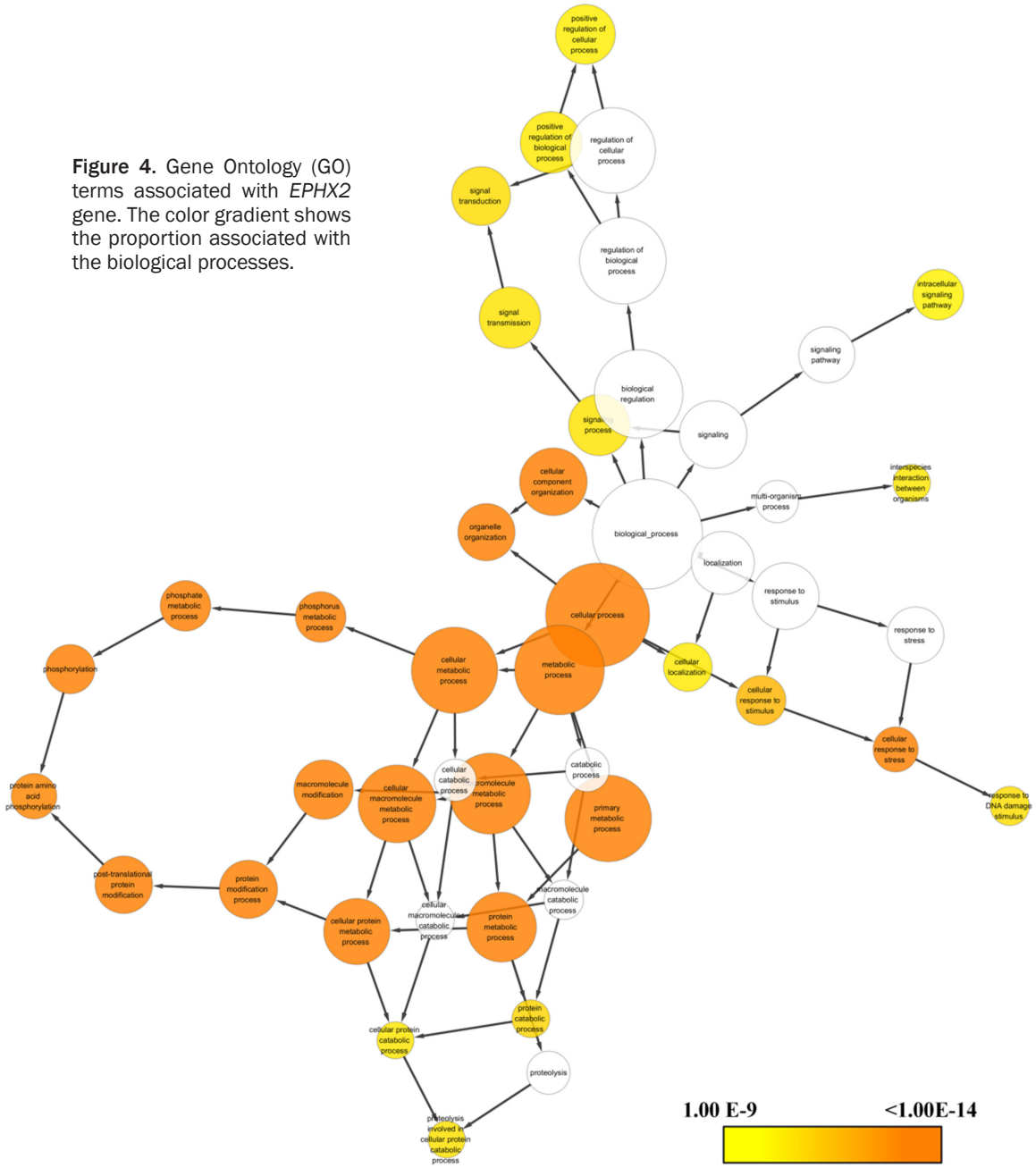

Figure 4. Gene Ontology (GO) terms associated with *EPHX2* gene. The color gradient shows the proportion associated with the biological processes.

term of Gene Ontology suggested that *EPHX2* gene was highly correlated with cellular metabolic process, protein metabolic process, signaling transduction and protein post-translation.

Signaling pathways enrichment analysis

To analyze the whole lists of proteins and better understand the functional annotation involved in *EPHX2*, differently expressed proteins were submitted to the DAVID Bioinformatics Resources 6.7 for signaling pathway enrichment analysis in terms of KEGG. In this study, we chose the *p*-value less than 0.001 as the cut-off criterion for canonical pathways. As a result, TGF-beta signaling pathway, T cell receptor signaling pathway, ErbB signaling pathway, Neurotrophin signaling pathway, MAPK signaling pathway, Parkinson's disease and Alzheimer's disease seem to be aberrant in *EPHX2*$^{-/-}$ mouse. All the detailed pathways were listed in **Table 2**.

Table 2. The enriched KEGG signaling pathways in DAVID Bioinformatics Resource ($P \leq 0.001$)

KEGG ID	Term	Count	%	*P* Value	Pop Hits	Fold Enrichment
hsa04350	TGF-beta signaling pathway	17	0.199157	3.73E-05	87	3.301065
hsa04660	T cell receptor signaling pathway	19	0.222587	4.88E-05	108	2.972038
hsa04012	ErbB signaling pathway	16	0.187441	1.41E-04	87	3.106885
hsa04722	Neurotrophin signaling pathway	19	0.222587	3.03E-04	124	2.588549
hsa04010	MAPK signaling pathway	31	0.363168	4.07E-04	267	1.961439
hsa05012	Parkinson's disease	19	0.222587	4.51E-04	128	2.507657
hsa05010	Alzheimer's disease	22	0.257732	5.20E-04	163	2.28013

Discussion

High throughput proteomics, in terms of vast amount of proteins, offers highly information towards biology [32]. However, owing to the complexity of biological system, it is difficult to identify and understand the entire proteins well. With the development of sophisticated separation techniques, mass spectrometry (MS)-based high throughput proteomics becomes a core instrumentation for proteins characterization due to its high sensitivity [33]. In the present study, shotgun proteomics method was introduced for protein analysis in a high-throughput way [34]. Interactome network basing on high throughput data, as well as its integration with disease phenotype, has been reported to be a conventional technology for the identification of disease-specific biomarkers, such as cancer [35]. Recently, Diederick *et al.* [36] discovered FASN, XPO1, ENO1 and PDCD61P were novel biomarkers for prostate cancer progression using a nanoLC and LTQ-Orbitrap-MS/MS mode. Similarly, Shen and colleagues [37] also revealed SSP411 was a band-new biomarker for cholangiocarcinoma, suggesting the high throughput proteomics profiling was a valuable tool for cancer biomarkers identification and diagnosis. Besides, proteomics analysis was also considered to be a powerful for neurodegeneration diagnosis [38]. In 2014, Liguori *et al.* detected the cerebrospinal fluid proteomic profiles in Multiple Sclerosis (MS) patients basing on the Matrix Assisted Laser Desorption Ionization Time of Flight (MALDI-TOP) mass spectrometer. They concluded Secretograin II and Protein 7B2 were highly expressed in clinically definite MS patients compared to the progressive ones. In addition, Tymosin β4 was also found to be aberrant in clinically isolated syndrome and relapsing remitting (RR) MS patients, suggesting the proteomic profiling technique in combination with the mass spectrometry evaluation provided useful and important information to improve our understanding of the complex pathogenesis of MS.

Previous animal studies have indicated that *EPHX2* gene deletion or treatment with sEH inhibitors results in increased levels of EETs and protection against stroke-induced brain damage [39]. Oxidative stress is hypothesized to play a major role in Alzheimer's disease. Epoxides are potentially reactive intermediates formed that contribute to cytotoxic damage mediated by oxidative stress [40]. In chronic neurodegenerative diseases, epoxide hydrolase were found to be significantly elevated in the hippocampus and associated cortex in Alzheimer's disease patients [41]. In agreement with the results of previous studies, KEGG based pathway enrichment analysis also confirmed that Neurotrophin signaling pathway, Parkinson's disease and Alzheimer's disease highly are strongly coupled with sEH. As an indispensable section of the brain, hypothalamus governed hormone production and regulated homeostasis. Hypothalamic hormones include thyrotropin-releasing, gonadotropin-releasing, growth hormone-releasing, corticotrophin-releasing, somatostatin, and dopamine hormones, which are released into the blood and link the nervous system to the endocrine system *via* hypophysis.

A number of documents proved that sEH inhibitors possessed protective effects in ischemic or cardiovascular disease. In 2010, Simpkins and colleagues discovered 12-(3-adamantan-1-yl-ure-ido)-dodecanoic acid, an inhibitor of sEH, could significantly improve the increment of inward remodeling. In addition, mice with *EPHX2* deletion enhanced inward vascular remodeling induced by carotid ligation [42]. Besides, using sEH-knockout mice, Xiaocui *et*

al. also discovered sEH deficiency and inhibition could decrease 1-methyl-4-phenyl-1,2,3,6-tetrahydro pyridine (MPTP)-treated mice *via* activating AKT signaling pathway to protect dopamine neurons (Xiaocui et al., 2014). In view of this, insights into the physiological functions of sEH have emerged from studies in mice with global *EPHX2* gene deficiency or pharmacological inhibition of sEH, which in turn provides beneficial effects in blood pressure [43], cardiovascular [44], renal [45] in murine models.

Many studies have shown that alternations of neuropeptides from hypothalamic pituitary axis resulted in neurodegenerative diseases, such as Huntington's disease [46]. Besides, postmortem studies pointed out that dopamine concentrations in the hypothalamus were involved in Parkinson's disease [47]. More recently, ^{18}F-dopa based positron emission tomography (PET) also discovered the hypothalamic monoamine storage capacity decreased in patients with idiopathic Parkinson's disease, which was in agreement with the postmortem observations [48].

Pathway enrichment analysis also indicated that TGF-beta signaling pathway, T cell receptor signaling pathway, ErbB signaling pathway and MAPK signaling pathway were engaged in *EPHX2* gene deficiency. However, detailed relations associated with *EPHX2* gene and these regulatory pathways still lacks and undefined.

In summary, we identified 31 significantly expressed proteins in mouse hippocampus using proteomics analysis and constructed a protein-protein interaction network associated with *EPHX2* gene knockout. Our study may shed new lights for sEH in the chronic neurodegenerative diseases, like Parkinson's disease and Alzheimer's disease.

Acknowledgements

We wish to thank Prof. Yi Zhu for the generous gift of the wild type and *EPHX2*$^{-/-}$ mouse.

Disclosure of conflict of interest

None.

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