Quantitative proteomics reveals EVA1A-related proteins involved in neuronal differentiation

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EVA1A is an autophagy-related protein, which plays an important role in embryonic neurogenesis. In this study, we found that loss of EVA1A could decrease neural differentiation in the brain of adult Eva1a−/− mice. To determine the mechanism underlying this phenotype, we performed label-free quantitative proteomics and bioinformatics analysis using the brains of Eva1a−/− and wild-type mice. We identified 11 proteins that were up-regulated and 17 that were down-regulated in the brains of the knockout mice compared to the wild-type counterparts. Bioinformatics analysis indicated that biological processes, including ATP synthesis, oxidative phosphorylation, and the TCA cycle, are involved in the EVA1A regulatory network. In addition, gene set enrichment analysis showed that neurodegenerative diseases, such as Alzheimer’s disease and Huntington’s disease, were strongly associated with Eva1a knockout. Western blot experiments showed changes in the expression of nicotinamide nucleotide transhydrogenase, an important mitochondrial enzyme involved in the TCA cycle, in the brains of Eva1a knockout mice. Our study provides valuable information on the molecular functions and regulatory network of the Eva1a gene, as well as new perspectives on the relationship between autophagy-related proteins and neural differentiation.

Keywords:
Bioinformatics / Eva1a / Neuronal differentiation / Quantitative proteomics

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1 Introduction

EVA1A, also known as FAM176A or TMEM166, was first identified as a lysosomal and endoplasmic reticulum-associated protein involved in autophagy and apoptosis [1]. The Eva1a gene encodes 152 amino acids with a molecular weight of 17.5 kDa and isoelectric point of 6.5. EVA1A is conserved in human, chimpanzee, rat, mouse, and dog, and shows no obvious homology to any known genes or proteins.

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Significance of the study

The mechanisms that mediate neural differentiation are of interest to researchers in the context of developmental processes as well as neurodegenerative disease pathogenesis. In this study, we used quantitative proteomics and bioinformatics analysis to show that the loss of EVA1A in the brain could disrupt neural differentiation. Our results provide valuable information about the regulatory network associated with EVA1A and the influence of EVA1A on the neural system. Further, we showed that the protein nicotinamide nucleotide transhydrogenase (NNT) has important roles in EVA1A regulation and EVA1A-mediated neural differentiation. Our work is among the first to show the involvement of EVA1A in the processes of neuronal differentiation and neuron loss, and could form a basis for future studies on novel therapeutic targets for neurodegenerative diseases, such as Parkinson’s disease and Alzheimer’s disease.

Overexpression of EVA1A induces cell death in 293T and HeLa cells [1]. Results of studies in rat suggest that EVA1A plays a significant role in cell death following middle cerebral artery occlusion injury; blocking the activity of EVA1A by using siRNA prevented cell loss following cerebral ischemia injury [2]. Decreased expression of EVA1A has been observed in several types of human tumors, including lung cancers [3]. Our previous study using Eva1a brain knock out (KO) mouse showed that EVA1A can influence embryonic neurogenesis by negatively activating the PIK3CA-AKT axis, which ultimately leads to the inhibition of autophagy [4].

Autophagy is important for the degradation of bulk cytoplasm, long-lived proteins, and entire organelles [5]. There are three main forms of autophagy: microautophagy, macroautophagy, and chaperone-mediated autophagy [6]. Komatsu et al. found that the loss of autophagy in the central nervous system causes neurodegeneration in mice [7], and many neurodegenerative conditions in humans, such as Huntington’s disease, Parkinson’s disease, amyotrophic lateral sclerosis, and Alzheimer’s disease [8]. Thus, in organism growth and development, autophagy and autophagy-related genes likely play important roles in cellular reorganization and renovation rather than cell death.

Mass spectrometry (MS)-based proteomics has improved significantly, owing to advances in high-resolution instrumentation, in recent years. The combination of a linear ion trap with the Orbitrap analyzer is a popular instrument configuration [9]. Liquid chromatography coupled with mass spectrometry (LC-MS) enables the simultaneous analysis of thousands of proteins from complex biological samples [10], providing information on the abundance of proteins or peptides under different biological conditions, interplay of proteins, protein complexes, signaling pathways, and network modules. Although isotopic labeling quantification has slightly better quantitative precision than label-free quantification does, label-free quantitative proteomics can achieve a good balance between quantitative precision and number of quantified features [11], thereby providing a cost-effective alternative to labeled quantification.

EVA1A could regulate the progress of microautophagy [1, 12], and the association of EVA1A with autophagy suggests that EVA1A could play a critical role in the process of neural differentiation in the nervous system. The aim of this study was to combine quantitative proteomic workflows with bioinformatics analysis, and identify proteins that were differentially expressed with or without Eva1a gene knockout in mouse brains, and to determine the potential signaling pathways by which the Eva1a gene regulates neural differentiation.

2 Materials and methods

2.1 Materials

Ammonium bicarbonate, sodium deoxycholate, iodoacetamide, and dithiothreitol were purchased from Sigma (St. Louis, MO, USA). Tris-(2-carboxyethyl) phosphine (TECP) was acquired from Thermo Scientific (Rockford, IL, USA). Modified sequencing-grade trypsin was obtained from Promega (Madison, WI, USA). All mobile phases and solutions were prepared with HPLC grade solvents (i.e. water, acetonitrile, methanol, and formic acid) from Sigma Aldrich. All other reagents were from commercial suppliers and of standard biochemical quality.

2.2 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.

2.3 Animal models

Male C57BL/6 background mice (8 weeks old, 20 ± 4 g body weight) with and without targeted disruption of Eva1a gene (Eva1a+/+ and Eva1a−/−) were housed individually in...
2.4 Hematoxylin and eosin staining and Nissl staining

For hematoxylin and eosin staining, mice were perfused through the heart with saline and 4% paraformaldehyde, and after post-fixing overnight the brains were embedded in paraffin. Brain sections (5 μm thick) were prepared, deparaffinized and then stained with hematoxylin and eosin. For Nissl staining, mice were anesthetized and perfused with 4% cold paraformaldehyde in 4% paraformaldehyde saline (PBS, 10 mM, pH 7.4). The brains were dissected and fixed in 4% paraformaldehyde at 4°C overnight, processed for embedding in paraffin, and cut into 4-μm-thick serial sections. The sections were hydrated in 1% toluidine blue at 37°C for 1 h. After rinsing with double distilled water, they were dehydrated and mounted with permount. After Nissl staining, staining cells were identified under a light microscope.

2.5 RT-PCR

Total RNA was extracted from mice tissues or cells using the Trizol reagent (Invitrogen, 15596-026). RT-PCR was performed using 0.1 μg of cDNA with a 2 × Taq mastermix (CWBio, cw0682) and 10 pmol/μL of primers. Reactions were run on a Bio-Rad MyCycler Thermal Cycler, and PCR products were analyzed by agarose gel electrophoresis. The RNA level of β-tubulin III (a neuron marker) was used to determine the number of neurons in different brain regions (cortex, hippocampus, and cerebellum), and the RNA level of GFAP (an astrocyte marker) was used to determine the number of astrocytes in these regions.

2.6 Real-time PCR

Real-time PCR was carried out using the SYBR green real time PCR kit from Qiagen on an Eppendorf mastercycler, eprealplex (Eppendorf, Germany). Relative mRNA expression was determined by normalization to the expression of a housekeeping gene—GAPDH. The following primers were used: 5′-TGTGTCCGTGCTGGATCTGA-3′ and 5′-TTGCTGT-TGAAGTCGCGAGGAG-3′ for GAPDH.

2.7 Immunofluorescence

Brain frozen sections from both Eva1a−/− and Eva1a+/+ mice at 8 weeks were fixed with 4% formaldehyde, washed with PBS, and blocked with 5% BSA (in PBS) and 0.25% Triton-X100 at room temperature for 1 h. The sections were incubated with the indicated primary antibody at 4°C overnight, washed with PBS three times and incubated with FITC or rhodamine-labeled secondary antibodies at 37°C for 1 h. Nuclei were stained with Hoechst33342 for 5 min. Finally, immunofluorescence was detected under a confocal fluorescence microscope (LSM 510 Meta plus Axiovert zoom, Carl Zeiss) with a 63×1.40 NA oil immersion objective lens (PlanApochromat; Carl Zeiss) and a camera (AxioCam HRm, Carl Zeiss). Images were processed and viewed using the LSM Image Browser software. The protein level of β-tubulin III (a neuron marker) was used to determine the number of neurons in different brain regions (cortex, hippocampus, and cerebellum), and the protein level of GFAP (an astrocyte marker) was used to determine the number of astrocytes in these regions.

2.8 Shotgun proteomic analysis

2.8.1 Shotgun analysis sample preparation

Proteins extraction and digestion were conducted. In this study, mouse brain 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 were extracted. The concentration of whole proteins was determined using the bicinchoninic acid (BCA, Pierce, Rockford, IL, USA) assay kit. Protein samples (200 μg) from each group were processed according to the manufacturer’s protocol for filter-aided sample preparation (FASP). Protein concentrates in Vivacon 500 filter tube (Cat No. VNO1HO2, Sartorius Stedim Biotech) were mixed with 100 μL of 8 M urea in 0.1 M Tris/HCl (pH 8.5) and samples were centrifuged at 14 000 g for 15 min. This step was performed twice, after which 10 μL of 0.05 M Tris-(2-carboxyethyl) phosphate (TCEP) in water was added to the filters, and samples were incubated at 37°C for 1 h. Then, 10 μL of 0.1 M iodoacetamide (IAA) was added to the filters, and samples were incubated in darkness for 30 min. Filters were washed twice with 200 μL of 50 mM NH4HCO3. Finally, 4 μg of trypsin (Promega, Madison, WI) in 100 μL of 50 mM NH4HCO3 was added to each filter. The protein to enzyme ratio was 50:1. Samples were incubated overnight at 37°C and released peptides were collected by centrifugation. The experiment was performed in triplicate.

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High pH reverse phase chromatography was performed using the Dionex Ultimate 3000 Micro Binary HPLC Pump system. 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 mm × 150 mm Waters BEH130 C-18 column with 3.5 μm particles. 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, 5 to 15% buffer B for 15 min, 15 to 25% buffer B for 10 min, 25 to 55% buffer B for 10 min, and 55 to 95% buffer B for 5 min. The system was then maintained in 95% buffer B for 5 min before equilibrating with 5% buffer B for 10 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.

2.8.2 LC-MS/MS analyses

The MS analysis experiments were performed on a nano-flow HPLC system (Easy-nLC II, Thermo Fisher Scientific, USA) connected to a LTQ-Orbitrap Velos Pro (Linear quadrupole ion trap-Orbitrap mass analyser) mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA), equipped with a Nanospray Flex Ion Source (Thermo Fisher Scientific, USA). The peptide mixtures (5 μL) were injected at a flow rate of 5 μL/min onto a pre-column (Easy-column C18-A1,100 μm I.D. ×20 mm, 5 μm, Thermo Fisher Scientific). The chromatographic separation was performed on a reversed phase C18 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 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 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. Each sample was analyzed in triplicate.

2.8.3 Protein identification and quantification

The data analysis was performed with MaxQuant software (version 1.4.1.2, http://www.maxquant.org/). For protein identification, the MS/MS data were submitted to the Uniprot human protein database (release 3.43; 72 340 sequences) using the Andromeda search engine with the following settings: trypsin cleavage; fixed modification of carboxymethylation of cysteine; variable modifications of methionine oxidation; a maximum of two missed cleavages; and false discovery rate was calculated by decoy database searching. Other parameters were set as default. The up-regulated or down-regulated proteins were defined as those with significantly changed protein ratio (p-values <0.05). The significance p-values were calculated by Perseus software (version 1.4.1.3, http://www.perseus-framework.org/). A twofold change and p-value of 0.01 were used as combined thresholds to define biologically regulated proteins.

2.9 Bioinformatic analyses

Protein to protein interaction network without Eva1a gene was expanded using BisoGenet plugin in Cytoscape environment. The 28 significantly dysregulated proteins revealed from the proteomic analysis were used as queries firstly. Then the experimentally supported hyperlinks were connected from DIP, BIOGRID, HPRD, BIND, MINT and INTACT to retrieve known protein to protein interaction relationships. Finally the constructed protein interaction network was visualized in Cytoscape. The BiNGO plugin in a Cytoscape environment was used to retrieve the Gene Ontology Consortium (GOC, http://geneontology.org/).

2.9.1 Gene set enrichment analysis (GSEA) and network construction

We used the GSEA method, a running-sum statistic method for the whole gene set on rank list of all the available expression values instead of selecting the most differentially expressed proteins for functional enrichment analysis. Proteins with more than two unique peptides from LFQ intensity were defined as qualified proteins for GSEA analysis. The GSEA was performed using java GSEA. The phenotypes of analyzed data were given to two classes, A (Eva1a+/-) and B (Eva1a+/-). All curated canonical pathways (C2) in curated molecular signature database (MSigDB, v.4.0) were selected as the gene sets. The permutation type was set to gene set, and other settings were set as default. A normal p-value <0.05 and FDR q-value <0.05 was considered as a significantly enriched pathway according to GSEA documentation. The significantly enriched pathways, expression data, and all curated canonical pathways were subsequently subjected to Cytoscape (version 3.2.1) and interpreted by the Enrichment Map plugin according to user manual. The representative pathways were obtained using an overlap coefficient cutoff > 0.5.

2.10 Western blot

After the addition of a sample loading buffer, protein samples were electrophoresed on a 12% SDS-PAGE and subsequently
transferred to a nitrocellulose membrane (Amersham PharmaciaBiotech, UK). The membrane was incubated in fresh blocking buffer (0.1% Tween 20 in Tris-buffered saline, pH 7.4, containing 5% nonfat dried milk) at room temperature for 30 min and then probed with the corresponding antibody in blocking buffer at 4°C overnight. The membrane was washed thrice for 5 min each using PBST (PBS and0.1% Tween 20). Subsequently, the membrane was incubated in the appropriate horseradish peroxidase (HRP)-conjugated secondary antibody at room temperature for another 1 h and washed again thrice in PBST buffer. The membrane was incubated with ECL substrate solution (Shanghai Pufei Biotechnology Co., Ltd, China) for 5 min according to the manufacturer’s instructions and visualized with autoradiographic film. The following antibodies were used: monoclonal mouse anti-EVA1A antibody (Abcam, Cambridge, UK), mouse anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody (Shanghai Kangchen Biotechnology Co., Ltd, China), anti-β-TUBULIN III (TuJ1, Covance, MRB-435P, Princeton, New Jersey, US), anti-GFAP (Abcam, ab68428, Cambridge, MA, US), anti-ATG12 (CST, 4180, Danvers, MA, US), anti-β-ACTIN/ACTB (Proteintech, 60008–1-Ig, Campbell Park, Chicago, USA). Secondary antibodies were anti-rabbit (sc-2004, Santa Cruz, US) and anti-mouse antibody (sc-2005, Santa Cruz, USA).

2.11 Statistical analysis

Results were expressed as mean ±S.E.M. Statistical evaluation was performed with the Student’s t-test when two value sets were compared. \( P < 0.05 \) was considered to be significant \((^{*}P < 0.05, {**}P < 0.01)\).

3 Results

3.1 Evaluation of the degree of neural differentiation in Eva1a\(^{-/-}\) mice

In order to elucidate the role of EVA1A in the adult mouse nervous system, we first explored the effect of EVA1A in Eva1a\(^{-/-}\) mice and wild type mice (8 weeks old). Hematoxylin and eosin (H&E) and Nissl staining results showed a lower number of neurons in the brains of Eva1a\(^{-/-}\) mice (Fig. 1A–H). The neuron marker β-tubulin III was markedly down-regulated in Eva1a\(^{-/-}\) mice at the mRNA and protein levels (Fig. 1I–P), suggesting damage to the new neuron population. No significant difference in the expression of GFAP (the marker for astrocytes in the brain) was observed in the Western blot and immunofluorescence assay results, indicating no difference in astrocyte number between the two groups. The mRNA and protein levels of the neuron marker MAP2 were also significantly lower in Eva1a\(^{-/-}\) mice brain (Supporting Information Fig. S1). Thus, neural differentiation was significantly disrupted in the Eva1a\(^{-/-}\) mice but not in the WT mice. We also measured markers for other types of cells, including neural stem cells (NESTIN), oligodendrocyte (OLIG2), type I astocytes (FGFR3), and type II astrocytes (MUSASHI1) in the brain (Supporting Information Fig. S2). No significant differences were observed in the levels of these markers in WT and Eva1a KO mouse brains. We therefore concluded that, apart from neurons, other types of cells are not altered in the Eva1a KO mouse brain.

3.2 Proteomic study of the Eva1a\(^{-/-}\) mice model

A total of 5438 proteins were identified using the shotgun proteomic method and 4462 proteins were quantified using the LFQ algorithm (proteins with at least two unique peptides identified in all samples). A logarithmic transformation (base 2) of the raw abundance of proteins was pre-performed to obtain a normal distribution before differentially expressed protein identification (Supporting Information Fig. S3). As shown in Table 1, we found 28 significantly dysregulated proteins, involved in a variety of pathways such as nicotine and nicotinamide metabolism (Nnt), amino sugar and nucleotide sugar metabolism (Ugp2), oxidative phosphorylation (Uqcrq), and neurodegenerative disease-relevant pathways (Plcb3, Uqcrq). Figure 2A shows the number of proteins identified in each of the three biological replicates. The expression levels of the 28 proteins in all samples are shown in the heat map generated by hierarchical cluster analysis, and show a clear difference in mouse models with or without Eva1a (Fig. 2B).

3.3 Protein–protein interaction regulatory network construction and gene ontology analysis

To investigate the protein-protein interaction network associated with EVA1A, we matched the 28 significantly dysregulated proteins with 6 public sources to link known regulatory data between transcriptional factors and the target genes by using the Biogenten plugin of Cytoscape 3.0. We obtained a cohort of 139 nodes and 1574 relationships (Supporting Information Fig. S4). To extend our knowledge about the regulatory network associated with EVA1A, we matched the entire gene set of the constructed network for functional annotation using the BiNGO plugin. As shown in Fig. 3A, the molecular function in terms of Gene Ontology suggested that the Eva1a gene is highly correlated with functions such as protein binding, transcriptional factor binding, and nucleic acid binding. Further, Eva1a and its network are mainly associated with membrane-bound organelles, the nucleus, membrane-enclosed lumen and so on (Fig. 3B). To analyze the regulatory pathways associated with EVA1A, differentially expressed proteins were submitted to the Pathway mapping.

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Figure 1. Evaluation of the degree of neurodegeneration in Eva1a^{-/-} mice. (A, B, C, and D) Frozen sections obtained from the brain samples of Eva1a^{+/+} and Eva1a^{-/-} mice at 8 weeks. Representative light micrograph showing H&E staining in the hippocampus (Scale bar = 100 μm). The right panel shows higher magnifications (Scale bar = 10 μm). (E, F, G, and H) Nissl staining of neurons in the hippocampal dentate gyrus (Scale bar = 100 μm). The right panel shows higher magnifications (Scale bar = 20 μm). (I, J, K, and L) Immunofluorescence assay results showing β-TUBULIN III expression in the cortex. (Scale bar = 100 μm). The right panel shows higher magnifications (Scale bar = 10 μm). (M, N, O, and P) Immunofluorescence assay results showing GFAP expression in the cortex (Scale bar = 100 μm). The right panel shows higher magnifications (Scale bar = 10 μm). (Q) RT-PCR analysis of EVA1A, β-TUBULIN III, and GFAP mRNA in whole-brain tissue from Eva1a^{+/+} and Eva1a^{-/-} mice at 8 weeks. (R and S) Quantification of the ratio of β-TUBULIN III/GAPDH and GFAP/GAPDH. The average value in the Eva1a^{+/+} group was normalized as 1. Data are shown as mean ± SD of the results from three independent experiments. **P < 0.001. (T) Immunoblot analysis of β-TUBULIN III and GFAP expression in whole-brain tissue from Eva1a^{+/+} and Eva1a^{-/-} mice at 8 weeks. (U and V) Histogram shows the ratio of β-TUBULIN III/ACTB and GFAP/ACTB. The average value in the Eva1a^{+/+} group was normalized as 1. Data are means ± SD of the results from three independent experiments. *P < 0.05.
## Table 1. Differentially expressed proteins revealed by LC-MS

| Majority protein IDs | Protein names                               | Gene names      | Ratio (HOMO/WT) | -Log t-test p-value |
|---------------------|---------------------------------------------|-----------------|-----------------|---------------------|
| P06880              | Somatotropin                                | Gh1             | 0.01            | 4.82                |
| P35455              | Vasopressin-neurophysin 2-copeptin          | Avp             | 0.12            | 3.89                |
| Q91ZJ5-2            | UTP-glucose-1-phosphate uridylytransferase  | Ugp2            | 0.38            | 1.37                |
| H7BWX9              | Small ubiquitin-related modifier 2          | Sumo2           | 0.43            | 2.00                |
| P07759              | Serine protease inhibitor A3K;Serine protease inhibitor A3M | Serpina3k | 0.31            | 3.55                |
| P61924              | Coatomer subunit zeta-1                    | Copz1           | 0.41            | 2.34                |
| Q61941              | NAD(P) transhydrogenase, mitochondrial      | Nnt             | 0.42            | 3.11                |
| Q8GBK5              | Solute carrier family 35 member F1          | SLC35F1         | 0.43            | 1.45                |
| P51432              | 1-phosphatidylinositol 4,5-bisphosphate phosphodiesterase beta-3 | Plcb3      | 0.43            | 2.00                |
| Q9Z2M6              | Ubiquitin-like protein 3                    | Ubi3            | 0.45            | 1.37                |
| Q64288              | Olfactory marker protein                    | Omp             | 0.46            | 2.58                |
| Q99JF5              | Diposphoehemalate decarboxylase             | Mvd             | 0.46            | 1.67                |
| Q520L0              | Trophoblast glycoprotein                    | Tpbg            | 0.47            | 2.15                |
| Q80XQ2              | TBC1 domain family member 5                | Tbc1ds          | 0.48            | 1.54                |
| P97384              | Annexin A11/Annexin A11                    | Anxa11          | 0.48            | 2.83                |
| Q9C69               | Cytochrome b-c1 complex subunit 8           | Uqcrq           | 0.49            | 3.16                |
| Q9JIX0              | Enhancer of yellow 2 transcription factor homolog | Eny2         | 0.50            | 1.34                |
| Q00623              | Apolipoprotein A-I;Truncated apolipoprotein A-I | Apoa1         | 2.26            | 2.23                |
| Q7M750              | Opalin                                      | Opalin          | 2.29            | 1.31                |
| Q8CIZ8              | von Willebrand factor;von Willebrand antigen | Vwf            | 2.33            | 1.50                |
| Q8VCN6              | CD99 antigen                                | Cd99            | 2.34            | 1.57                |
| O5S028              | [3-methyl-2-oxobutanoate dehydrogenase [lipoamide]] kinase, mitochondrial | Bckdk         | 2.38            | 2.94                |
| Q9JM63              | ATP-sensitive inward rectifier potassium channel 10 | Kcnj10        | 2.43            | 1.72                |
| Q90P4               | Small acidic protein                        | Smap            | 2.81            | 2.27                |
| E9QMF4              | Probable tRNA N6-adenosine threonylcarbamoyltransferase | Osgep         | 2.89            | 1.32                |
| P04919              | Band 3 anion transport protein              | SLC4A1          | 3.02            | 2.36                |
| P29555              | NEDD8                                       | Nedd8           | 3.83            | 2.38                |
| Q8R2R3              | Alpha- and gamma-adaptin-binding protein p34 | Aagab           | 2.76            | 1.68                |

Figure 2. Proteomic study of the Eva1a<sup>−/−</sup> mouse model. (A) Venn diagram of the identified protein numbers in each of the three biological replicates. (B) Heat map of the 28 differently expressed proteins; red color represents high abundance and green represents low abundance.
tools of KEGG (http://www.kegg.jp/kegg/). The results indicated that several pathways, including those associated with Alzheimer’s disease, Huntington’s disease, and PI3K-Akt signaling could be altered in the Eva1a+/− mouse. All the altered pathways are listed in Supporting Information Table S1.

3.4 Gene set enrichment analysis

To improve our understanding of the gene expression changes in the Eva1a+/− mouse, we applied gene set enrichment analysis to detect more biology-driven gene sets without biases toward significantly differentially expressed genes. The results showed that two gene sets in phenotype A (Eva1a+/− group) and 20 gene sets in phenotype B (WT group) were significantly enriched at p-value <0.01 and FDR <25%. Gene sets that were significantly down-regulated in the Eva1a+/− group at both NOM p-value <0.01 and FDR <5% included genes encoding respiratory electron transport, oxidative phosphorylation, and neuroactive ligand receptor interaction (Fig. 4, Table 2). A network of gene sets was constructed using the Enrichment map plugin to visualize the significantly enriched gene sets and their relation with each other. The details of the significantly enriched gene sets, including name, size, NES, NOM p-value, and FDR q-value, are listed in Table 2. The Enrichment plot (profile of the running ES Score & positions of gene set members on the rank ordered list) of the top ten significantly enriched gene sets are shown in Supporting Information Fig. S5.

3.5 Western blot verification of the differentially expressed proteins NNT and UGP2

Nicotinamide nucleotide transhydrogenase (NNT) is a mitochondrial enzyme that transfers reducing equivalents from NADH to NADPH, thereby playing an important role in neuronal energy metabolism. Considering that the loss of EVA1A may influence pathways relevant to respiratory electron transport and oxidative phosphorylation (Table 2), which is closely related to the molecular function of NNT, we used Western blot to validate the protein level of NNT in the brain. Further, another significantly dysregulated protein, UGP2, was randomly chosen for Western blot analysis to validate the results of the proteomic study. We observed significant differences in NNT and UGP2 expression levels between the two groups (Fig. 5A). When we calculated the intensity of the bands, the Eva1a+/− group showed significant down-regulation of NNT and UGP2 compared with the WT group, consistent with the proteomics result (Fig. 5B). To ensure that the test is reliable, a positive control (ATG5-ATG12, a direct interactor of Eva1a, which is down-regulated in the Eva1a+/− mouse brain [12]) and a negative control (ACTB) were included in the test.

4 Discussion

The relationship and interaction between genotype and phenotypes is one of the fundamental avenues of biological research. Protein and mRNA levels often do not correlate [13]. Considering that proteins are essential to the progression of
Figure 4. Gene set enrichment analysis (GSEA) of the EVA1A regulation network. Functional enrichment analysis was obtained from GSEA results and interpreted using Cytoscape installed with the enrichment map plugin. A node represents each enriched gene set of canonical pathways from the molecular signature database (MSigDB), obtained using an overlap cutoff >0.5. The node color represents the pathways enriched in the Eva1a+/− (red, up-regulated functions) or Eva1a+/+ mouse brains (blue, up-regulated functions). The colors of node borders represent the FDR q-value and the thickness of the line represents the similarity coefficient of overlapping proteins between nodes.

Table 2. Details of the gene sets enriched by GSEA

| Enriched group | Gene set name | Size | NES     | NOM p-value | FDR q-value |
|---------------|--------------|------|---------|-------------|-------------|
| Eva1a+/+      | REACTOME_RESPIRATORY_ELECTRON_TRANSPORT | 53   | 2.47    | 0           | 0           |
| Eva1a+/+      | REACTOME_RESPIRATORY_ELECTRON_TRANSPORT_ATP_SYNTHESIS_BY_CHEMIOSMOTIC_COUPLING_AND_HEAT_PRODUCTION_BY_UNCOUPLING_PROTEINS_ | 63   | 2.44    | 0           | 0           |
| Eva1a+/+      | KEGG_OXIDATIVE_PHOSPHORYLATION | 73   | 2.36    | 0           | 0           |
| Eva1a+/+      | KEGG_HUNTINGTONS_DISEASE | 93   | 2.22    | 0           | 0           |
| Eva1a+/+      | MOOTHA_VOXPHOS | 55   | 2.31    | 0           | 0           |
| Eva1a+/+      | KEGG_NEUROACTIVE_LIGAND_RECEPROR_INTERACTION | 28   | 2.3     | 0           | 0           |
| Eva1a+/+      | KEGG_PARKINSONS_DISEASE | 72   | 2.28    | 0.001       | 0.002       |
| Eva1a+/+      | REACTOME_TCA_CYCLE_AND_RESPIRATORY_ELECTRON_TRANSPORT | 93   | 2.21    | 0           | 0.002       |
| Eva1a+/+      | KEGG_ALZHEIMERS_DISEASE | 86   | 2.13    | 0           | 0.006       |
| Eva1a+/+      | REACTOME_G_ALPHA_S_SIGNALLINGEVENTS | 30   | 1.9     | 0.005       | 0.134       |
| Eva1a+/+      | NABA_ECM_AFFILIATED | 28   | 1.89    | 0.002       | 0.133       |
| Eva1a+/+      | STEIN_ESR1_TARGETS | 19   | 1.87    | 0           | 0.158       |
| Eva1a+/+      | REACTOME_ACTIVATION_OF_KAINATE_RECEPTORS_UPON_GLUTAMATE_BINDING | 18   | 1.86    | 0           | 0.152       |
| Eva1a+/+      | KEGG_CARDIAC_MUSCLE_CONTRACTION | 38   | 1.84    | 0           | 0.183       |
| Eva1a+/+      | REACTOME_NEUROTRANSMITTER_RECEPTOR_BINDING_AND_DOWNSTREAM_TRANSMISSION_IN_THE_POSTSYNAPTIC_CELL | 74   | 1.8     | 0           | 0.238       |
| Eva1a+/+      | WOO_LIVER_CANCER_RECURRENTENCE_UP | 21   | 1.79    | 0.002       | 0.248       |
| Eva1a+/+      | REACTOME_TRANSMISSION_ACROSS CHEMICAL_SYNAPSES | 109  | 1.78    | 0           | 0.244       |
| Eva1a+/−      | KEGG_LYSOSOME | 47   | 2       | 0           | 0.124       |
| Eva1a+/−      | REACTOME_RESPONSE_TO_ELEVATED_PLATELET_CYTOSOLIC_CA2 | 30   | 1.93    | 0           | 0.189       |
significant down-regulation of NNT and UGP2 compared to the
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between mouse models with or without the
differences in protein expression patterns
Fig. 2B) showed marked differences in protein expression patterns
EVA1A, in the regulation of neuronal differen-
tation. Furthermore, hierarchical cluster analysis (Fig. 2B)

network of EVA1A, in the regulation of neuronal differen-
tation. Furthermore, hierarchical cluster analysis (Fig. 2B)

the nervous and endocrine systems [20]. All these differen-
cade, with olfactory canonical signaling components between
OMP is involved in the OR-mediated signal transduction cas-
dependent biosynthetic enzymes, including NNT [23]. From
The recent improvement of
biological processes, study of the proteome is important for
revealing the function of genes. The recent improvement of
the performance and sensitivity of MS promises increased
sampling depth and enhance capabilities for more effective
label-free quantification [14]. Bioinformatics combines com-
puter science, statistics, mathematics, and engineering to an-
alyze and interpret biological data, and has proven a use-
ful tool for analyzing data from genomics and proteomics
[15]. Data retrieved from high-throughput experiments and
literature are available in several databases, such as DIP,
BIND, and Intact, which represent the major repositories
of protein-protein interactions from multiple organisms. On
the other hand, databases such as KEGG [16] and Reactome
can provide information on metabolic and signaling path-
ways [17]. Proteomic profiling techniques, in combination with bioinformatics, are a useful and important source of
information to improve our understanding of complex patho-
geneses in mouse models [17, 18]. In our protein profiling
experiment, we identified 28 significantly differentially ex-
pressed proteins. Some of the corresponding genes have been
linked to development and energy generation. For example,
GH1 is a member of the somatotropin/prolactin family of
hormones, which plays an important role in growth con-
trol. The presence of GH has been reported in several ex-
traptituary sites, such as neural, ocular, reproductive, im-
mune, cardiovascular, muscular, dermal, and skeletal tis-
sue [18]. An endocrine disorder whose symptoms include
severe proportionate growth failure can be caused by het-
erozygous mutations of Gh1 [19]. Another differentially ex-
pressed protein, OMP (olfactory marker protein), an olfa-
tory receptor (OR)-associated protein, has also recently been
observed in non-olfactory tissues, Kang et al. reported that
OMP is involved in the OR-mediated signal transduction cas-
cade, with olfactory canonical signaling components between
the nervous and endocrine systems [20]. All these differenti-
tally expressed proteins may be involved in the regulatory
network of EVA1A, in the regulation of neuronal differen-
tiation. Furthermore, hierarchical cluster analysis (Fig. 2B)
showed marked differences in protein expression patterns
between mouse models with or without the Eva1a gene. The

phenotypes associated with the differentially expressed pro-
teins, and the exact mechanism by which they regulate neu-
ronal differentiation are promising avenues for future study.

In this study, a regulation network was constructed to show the
known interactions between the 28 differentially expressed proteins and other genes (Supporting Information Fig. S4).
Further Gene Ontology analysis using the BINGO plugin also provided information about the molecular func-
tions and cellular components associated with Eva1a, such as
protein binding, and transcriptional factor binding. Further-
more, a previous study of the Eva1a−/− mouse showed that
the Pik3ca-Akt-Mtor axis is involved in the EVA1A regulation
network [4], which also confirmed our KEGG-based pathway
enrichment analysis: the PI3K-Akt signaling pathway, Parkin-
son’s disease, and Alzheimer’s disease are all associated with
the Eva1a regulation network.

In addition to analysis using the 28 significantly dysregu-
lated proteins, we also performed gene set enrichment analy-
sis (GSEA) using all the quantified proteins, in order to fully
extract information from our protein profiling data. GSEA
could be used to distinguish between the two mouse groups at
the level of gene sets (groups of genes that share common bi-
ological function) by using gene expression profiles instead of
the significantly dysregulated genes only. Thus, GSEA could
help overcome some analytical challenges and limitations as-
associated with common analysis approaches, which cannot
cannot fully extract useful information from profiling results [21].
Figure 4 shows the GSEA results, indicating that EVA1A de-
ciciency can influence gene sets related to neurodegenerative
diseases such as Parkinson’s disease and Alzheimer’s dis-
case. These neurodegenerative disease-relevant gene sets cor-
responded with the phenotype of the Eva1a−/− mouse model
(Loss of neurons due to the decreased efficiency of neuronal
differentiation). Further, EVA1A may influence the neuronal
differentiation process by regulating energy generation path-
ways, such as ATP synthesis, oxidative phosphorylation, and
the TCA cycle (Fig. 4). To validate the role of EVA1A in en-
ergy generation, we examined the expression level of NNT,
an important component of the TCA cycle, by using Western
blotting.

NNT is a mitochondrial enzyme that transfers reducing equivalents from NADH to NADPH. Gameiro et al. found
that NNT produces NADPH for reductive carboxylation and
modulates glucose catabolism [22]. Ghosh et al. reported that
the redox buffers GSH and NAD(P)H are important in age-
related neurodegeneration of mice neurons. They also ob-
served an age-dependent loss of gene expression of key redox-
dependent biosynthetic enzymes, including NNT [23]. From
the results of previous studies and our Western blot results of
NNT expression (Fig. 5), we may infer that NNT plays an im-
portant role in the EVA1A network, mediating the progress of
neuronal differentiation regulated by EVA1A. Because Eva1a
is an autophagy-related gene, its loss may result in the dis-
rupption of neurogenesis during nervous system development
[4], and may further reduce of neuronal differentiation in the
mature brain. Therefore, Eva1a, NNT, and other related genes

Figure 5. Western blot verification of differential expression of NNT and UGP2. (A) Western blot results showing expression lev-
els of NNT, UGP2, and ATG5-ATG12 in the two groups (Eva1a+/−
and Eva1a−/−). The direct interactor of EVA1A (ATG5-ATG12) was
used as a positive control. (B) Statistical analysis of the intensity
of the bands after triplicate analysis; the Eva1a−/− group showed
significant down-regulation of NNT and UGP2 compared to the
Eva1a+/− group.
may be promising targets for drug discovery and further study of regulatory mechanisms.

In conclusion, our results indicated disrupted neuronal differentiation in the nervous system of Eva1a−/− mice. We identified 28 significantly dysregulated proteins in the brains of Eva1a−/− mice by using proteomics analysis, and constructed a protein-protein interaction network associated with Eva1a gene knockout. Bioinformatics analysis revealed several associated pathways, including ATP synthesis, oxidative phosphorylation, and the TCA cycle. We therefore elucidated the function of EVA1A in the processes of neuronal differentiation and neuron loss. Our results could be relevant in the context of neurodegenerative diseases such as Parkinson’s disease and Alzheimer’s disease.

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The authors declare no conflict of interest.

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