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Research Article

Proteomic Analysis Provides Insights into the Molecular Regulatory Mechanism of Dengzhan Shengmai Capsule in a Rat Model of Vascular Dementia

Jing Zhang[1], Wei Liu[2], Dongfeng Wei[3], Zhanjun Zhang[4,5*]

[1]Department of BiochemisMtry and Molecular Biology, Capital Medical University, Beijing, China; [2]Department of Theoretical and Computational Photochemistry, Beijing Normal University, Beijing, China; [3]Department of Clinical Medicine, China Academy of Chinese Medical Sciences, Beijing, China; [4]Department of Cognitive Neuroscience and Learning, Beijing Normal University, Beijing, China; [5]Department of Regenerative Medicine, Beijing Normal University, Beijing, China

ABSTRACT

Dengzhan Shengmai (DZSM) capsule is a compound Chinese medicine that is widely used in the clinic for the treatment of ischemic cerebrovascular diseases along with symptoms of dementia and forgetfulness. DZSM can improve the cognitive function and quality of life of patients with Vascular Dementia (VD), but little is known about the therapeutic mechanisms. In this study, we found that DZSM rescued spatial memory impairment in VD rats. We next employed an isobaric Tag for Relative and Absolute Quantitation (iTRAQ) based quantitative proteomic approach to uncover the specific proteins and biological pathways underlying the exacerbation of cognitive deficits observed in VD and to investigate the molecular mechanism of the effect of DZSM against VD. We discovered that the proteome was broadly changed in VD rat brains; among the 222 identified proteins with altered expression after VD modeling, 136 were upregulated and 86 were downregulated. Gene ontology and ingenuity pathway analysis indicated that the altered proteins in VD brains strongly interacts with the TCA cycle, 14-3-3-mediated signaling, actin cytoskeleton signaling, the synaptogenic signaling pathway, etc. These top-ranked canonical pathways, key regulators of glucose metabolism, synaptic plasticity and synaptogenesis, were predicted to be downregulated in VD. We also examined the proteomic profile of DZSM-treated VD rat brains and identified 23 potential DZSM-targeting proteins. Our results suggest that DZSM may function by regulating key modulators, such as *RAP1A* and *H2AFX*, which are involved in signaling pathways important for neuronal functions. Our study offers resources to characterize the biological functions of DZSM regulating proteins and may aid in the identification of the molecular mechanism by which DZSM can treat VD.

Keywords: Vascular dementia; Gene ontology; Ingenuity pathway analysis; Molecular mechanism

INTRODUCTION

Vascular Dementia (VD) is one type of common dementia in elderly patients, second only to senile dementia [1]. VD refers to severe cognitive dysfunction syndrome caused by cerebrovascular attacks including ischemia or hemorrhage and hypoperfusion in brain regions related to memory, cognition and behavior [2]. The prevalence rate of VD in China is 1.1% ~ 3.0%. New cases of VD occur in approximately 5% ~ 9% of elderly people over 70 of age each year. Cognitive dysfunction in VD patients is progressively aggravated, which gradually affects patients' daily life and social competence. At present, there is no Food and Drug Administration (FDA) approved drugs for the prevention and treatment of VD.

Traditional Chinese Medicine (TCM) can induce multi-target effects to enhance vascular factors and cognition, thus offers unique advantages in prevention and treatment of VD. According to numerous studies, TCM showed promising clinical

Correspondence to: Zhanjun Zhang, Department of Cognitive Neuroscience and Learning, Beijing Normal University, Beijing, China, E-mail: zhangrzs@163.com

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prospects of VD [3-5]. Dengzhan Shengmai (DZSM) capsule, a type of TCM formula, was approved by authorities as a Chinese patent medicine. It has a wide application in the treatment of stroke sequelae in the clinic in China [6]. DZSM includes *Panax ginseng C.A.Mey*, *Schisandra chinensis* and *Ophiopogon japonicus* and its effective chemical constituents are dicaffenine acid, scutellarin, apigenin, hyperxanthin and other ketones. Recently, several clinical studies have shown that DZSM combined with nimodipine or other Western medicines can improve the cognitive function and quality of life of patients with VD and one recent study by our group confirmed the neuroprotective effect of DZSM against VD in a rat model [7-9]. However, the mechanism underlying the therapeutic effects of DZSM is almost unknown.

Considering that TCM have such characteristics as multi-ingredient, multi-target and multi-pathway to be effective, a comprehensive evaluation of proteomic changes in the rat brain in combination with bioinformatics studies is critical to further our understanding of the molecular mechanisms of DZSM for the treatment of VD. Since severe memory loss and learning deficits directly correlate with damage to the cerebral cortex and hippocampus caused by vascular pathology and since we previously found that DZSM ameliorates cognitive impairments in VD rats, we asked whether DZSM exerts its therapeutic effect by restoring neuronal functions in the cerebral cortex and hippocampus to some extent and hypothesized that this effect is largely attributed to modulation of protein expression profiles in the related regions of the VD rat brain [10,11].

Quantitative proteomics has been successfully used to detect proteomic changes in a variety of diseases and isobaric tags for relative and absolute quantitation (iTRAQ) is a widely used technique for quantitative proteome analysis [12-18]. Accordingly, in the present study, two-dimensional liquid chromatography combined with tandem mass spectrometry-based iTRAQ (2D LC-MS/MS-iTRAQ) was applied to quantitatively profile specific proteome changes in the cerebral cortex and hippocampus of the VD rat brain. Subsequently, specific proteins and pathways potentially involved in the development of VD were identified based on gene ontology and Ingenuity Pathway Analysis (IPA). Furthermore, the proteomic profile of the DZSM-treated VD rat brain was also investigated to identify potential DZSM-targeted proteins and their interactions to deepen our understanding of the effects of DZSM treatment on VD.

MATERIALS AND METHODS

Experimental animals

Male and female Sprague-Dawley rats (160 g-200 g, 2 months old) were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. The rats were kept in a temperature and humidity-controlled animal facility, and given free access to food and water. All procedures were approved by the Animal Care and Welfare Committee (ACWC) of Dongfang Hospital, Beijing University of Chinese Medicine, China.

Drug and reagent

Dengzhan Shengmai capsule was provided by Yunnan Biovalley Pharmaceutical Co., Ltd. 8-plex iTRAQ reagent was purchased from AB Sciex (Framingham, MA, United States). RIPA extraction buffer, phenylmethanesulfonyl fluoride and BCA kit were purchased from Beyotime Biotechnology (Haimen, China). Sequencing grade modified trypsin was purchased from Promega (Madison, WI, United States).

Animal modeling, grouping and intervention

The VD rat model was established by the *in vitro* injection of a microembolus into the internal carotid artery. The blood of the same kind of rat was dried into a blood clot in an oven under aseptic conditions. A suspension with a concentration of 30 mg/ml was prepared by dissolving the blood blot in sterile saline. After anesthesia, the median carotid artery was incised to expose the common carotid artery, which was temporarily clamped and retrograde intubation was performed at the left external carotid artery. After 0.3 ml of embolus saline was injected, the left external carotid artery was ligated immediately, and the common carotid artery was opened to cause the embolus to enter the cranium and brain from the internal carotid artery to induce multiple lumen infarction. The rats were divided into 3 groups randomly such as the sham control, VD model and DZSM treatment groups. DZSM (in saline) was intragastrically administered to rats at a dose of 112.5 mg/kg per day since the third day after surgery for 30 days. Rats in the sham control group and model group were administered with the same amount of saline instead.

Morris Water Maze (MWM) test

The rats were tested in a water maze (150 cm in diameter) filled with water and the temperature was maintained at 23°C. One day before the test, the rats were trained to find a platform, which was fixed 2 cm beneath the water surface in the second quadrant. Then, each rat was placed in any quadrant of the pool except the target quadrant. The rat was allowed to swim for up to 120 seconds to locate the submerged platform. If succeeded, the rat was allowed to stay for 30 seconds. Rat that failed to find the platform was guided to it and kept on it for 30 seconds. The rats were trained twice a day with a 4-hour interval for 5 consecutive days. On the 6th day, the platform was removed; the time and frequency that the rat took to cross the original platform within 2 min were measured.

Protein extraction and digestion

Rats (n=4 each group) were euthanized by decapitation and brain tissues from the hippocampus and cerebral cortex were obtained and frozen in liquid nitrogen. Cold RIPA buffer and 1 mm phenylmethanesulfonyl fluoride (Beyotime, Haimen, China) were added to the pulverized sample, the mixture was then sonicated. After centrifugation at 12,000 g for 10 min, the supernatant fraction was collected and the protein concentration was determined by a Bicinchoninic Acid (BCA) kit (Beyotime). Then, 100 μg of protein was digested at 37°C for 12 h with sequencing grade trypsin (Promega, Madison, WI, United States), with trypsin/protein ratio 1:50. After digestion, the obtained peptides were dried under vacuum freeze.

iTRAQ labeling and high pH Reverse Phase (RP) fractionation

According to the manufacturer's instructions, peptides were labeled using 8-plex iTRAQ reagent. High pH RP fractionation was then performed on an Agilent 1100 HPLC chromatography system (Agilent Technologies, Santa Clara, CA, United States). The peptides with iTRAQ tags were dissolved in 100 mL of buffer A (2%(v/v) acetonitrile, pH 10.0) and then loaded onto column (Agilent Zorbax Extend-C18, 2.1 mm × 150 mm, 5 μm). The column was eluted with buffer A at a flow rate of 300 μL/min under the gradient, 98% A for 8 min; 95% ~ 75% A for 30 min; 75% ~ 60% A for 12 min; 10% A for 10 min; 98% A for 5 min. The eluent was monitored by absorbance at 210 nm and 280 nm. The peptides were then dried by vacuum freeze.

Liquid Chromatography-tandem Mass Spectrometry (LC-MS/MS) analysis

The peptides were redissolved in buffer A, loaded onto a 3 μm, 150 Å, C18 column (100 μm × 3 cm, ChromXP Eksigent) and eluted on a 3 μm, 120 Å, C18 column (75 μm × 15 cm, ChromXP Eksigent) using the gradient, program 95% ~ 92% A for 30 s; 92% ~ 74% A for 47.5 min; 74% ~ 62% A for 13 min; 62% ~ 15% A for 6 s; 15% A for 5.9 min; 15% ~ 95% A for 6 s and 95% A for 2.9 min.

Samples were ionized by nano electrospray ionization using voltage of 2.4 kV. Pressure of the air curtain was 35 PSI, pressure of the atomization was 12 PSI, temperature of the heater was 150°C, the scanning mode of mass spectrometry was Information Dependent Analysis (IDA), the first full scan range was 400 m/z-1500 m/z, the scanning time was 250 milliseconds and 40 electricity was collected under each cycle of the IDA cycle. The second-order spectra, with a charge of +2 to +4 and a count of more than 260 per second, had a scanning range of 100 m/z-1500 m/z and a scanning time of 80 milliseconds. The energy of collision chamber was suitably set for Collision Induced Dissociation (CID) of all precursor ions, and the setting of dynamic exclusion was 16 seconds.

Protein identification and quantification

MS and MS/MS experiments were performed using TripleTOF 6600. The search parameters for peptide mass fingerprints were set as follows: sample type, iTRAQ 8-plex (peptide-labeled); cysteine alkylation, iodoacetamide; digestion enzyme, trypsin; database, Rattus norregicus. fasta; search effort, thorough. The experimental data were analyzed by Protein Pilot 5.0 (AB Sciex) using rat databases from UniProt. Protein expression between two groups was considered to have significant difference when the Fold Change (FC) was greater than 1.2 times or less than 0.83 times. P<0.05 was considered statistically significant.

Bioinformatics analysis of differentially expressed proteins

Genes that encode proteins were functionally classified based on biological process, protein class, and pathway using Gene Ontology (GO) database. Proteomics were analyzed using Ingenuity® Pathway Analysis (IPA) (Qiagen, Hilden, Germany) to investigate the biological significance of the data. The biologically relevant information extracted from the data analysis is shown as canonical signaling pathways and network connections.

Statistical analysis

All data were presented as mean ± SD. Group differences in MWM test were analyzed using one-way Analysis of Variance (ANOVA) with repeated measures, then by LSD post hoc test. P value less than 0.05 was considered statistically significant.

RESULTS

DZSM-treated VD rats exhibited better spatial learning memory ability

To examine the effect of DZSM treatment on spatial learning and memory in VD rats, MWM test was performed. In the place navigation experiment, their learning performance was assessed through measuring the latency to locate the submerged platform. The escape latency of three groups decreased over 5 days as shown in Figure 1A and on the 2nd, 4th and 5th days, the escape latency of the sham control group and DZSM group were significantly shorter than that of the VD model group (P<0.05) as shown in Figures 1B-1D. The spatial probe test on the 6th day showed that, in comparison with the time of model group needed to reach the target quadrant, the time of the sham control group was significantly shortened (P<0.01) and the number of times the rat cross the target quadrant was significantly increased (P<0.01). The time that the DZSM group needed to reach the original platform was significantly shorter than that in the model group (P<0.01) as shown in Figure 1E and the number of original platform location crosses was increased; however, the difference was not significant (P=0.068) as shown in Figure 1F. These results demonstrated that DZSM alleviates learning and memory impairment in VD rats (Figures 1A-1F).

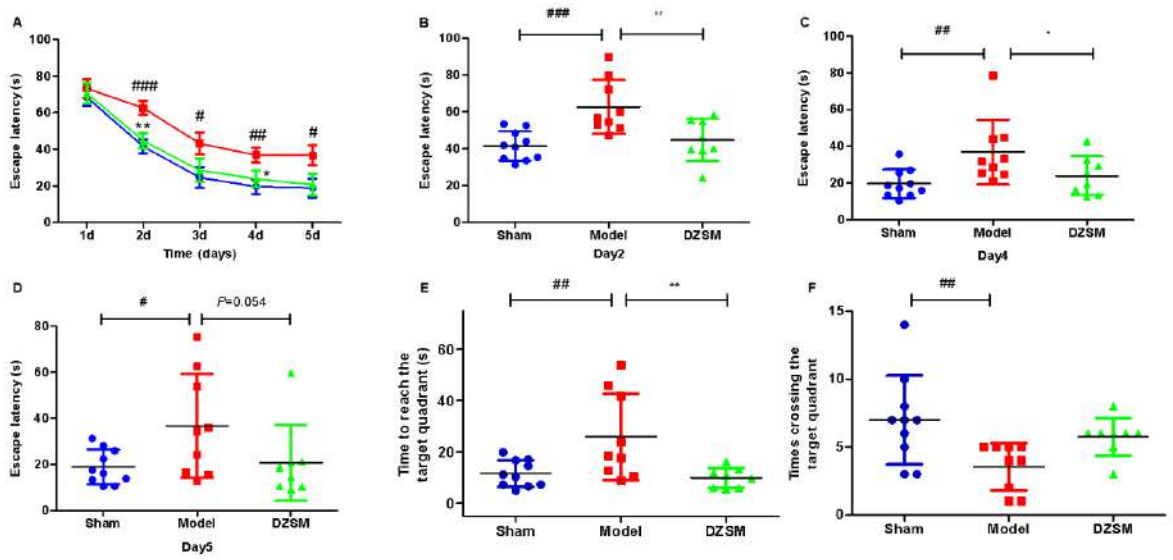


Figure 1: MWM test results. (A): Escape latency of five consecutive days; (B-D): Escape latency in each group during day 2,4 and 5; (E): The time to reach the target quadrant for the first time; (F): The number of times crossing the target quadrant in spatial probe tests. The data are presented as mean ± SD (Sham: n=10; Model: n=9; DZSM: n=8). **Note:** #: P<0.05; ##: P<0.01; ###: P<0.001; Sham vs. Model; *: P<0.05; **: P<0.01, Model *vs.* DZSM; (●): Sham; (■): Model; (▲): DZSM.

VD rats showed significant proteomic alterations in the hippocampus (Hp) and cerebral cortex (Cx) compared with the sham-operated control group

To identify critical proteins and their associated pathways that may lead to the behavioral and neuropathological changes observed in VD, we first investigated proteomic changes in the hippocampus and cerebral cortex in VD model rats *versus* normal rats. A total of 123 Hp proteins were differentially expressed between the VD model group and the control group; of these proteins, 64 were upregulated and 59 were downregulated. 99 Cx proteins were identified to be differentially expressed between the VD model group and the control group; of these proteins, 72 proteins were upregulated and 27 downregulation.

To clarify the biological relevance of these proteins, Gene Ontology (GO) analysis was performed by using the Panther database as shown in Figures 2A-2C. The identified proteins were classified into several sub-categories, including biological regulation (Hp: 11.6%; Cx: 10.1%), cellular process (Hp: 35.7%; Cx: 30.3%), localization (Hp: 17.0%; Cx: 13.1%), metabolic process (Hp: 17.0%; Cx: 21.2%), and multicellular organismal process (Hp: 11.6%; Cx: 13.1%). As part of these changes, cytoskeletal proteins (Hp: 11.1%; Cx: 9.2%), enzyme modulators (Hp: 15.3%; Cx: 9.2%), membrane traffic proteins (Hp: 11.1%; Cx: 7.7%), nucleic acid binding proteins (Hp: 12.5%; Cx: 9.2%), and oxidoreductases (Hp: 15.3%; Cx: 7.7%) were significantly altered (Figures 2A-2C).

To gain further insight into the potential biological networks and pathways mediating these changes, the identified proteins were uploaded to IPA as shown in Figures 3A and 3B. IPA mapped the differentially expressed proteins in the Hp to 250 canonical signaling pathways; among these pathways, the TCA cycle, 14-3-3 signaling, actin cytoskeleton signaling, the synaptogenesis signaling pathway, Rac signaling and glutathione-mediated detoxification were identified as top canonical pathways likely to be associated with the observed differences as shown in Figure 2C. IPA mapped the differentially expressed Cx proteins to 269 canonical signaling pathways, indicating changed activity in 3 important pathways such as GP6 signaling, Huntington's disease signaling and the synaptogenesis signaling pathway as shown in Figures 3A-3C.

Characterization of the differentially expressed proteins influenced by DZSM treatment

To characterize the molecular events involved in the neuroprotection of DZSM, we examined which differentially expressed proteins were further affected by DZSM treatment. Through two comparisons (the VD model group *vs.* the sham control group and the DZSM group *vs.* the VD model group), 14 Hp proteins and 9 Cx proteins were identified as shown in Figures 4A-4D. DZSM induced the elevated expression of 6 Hp proteins and 2 Cx proteins, including the important glycolytic enzyme GAPDH, the neuritogenesis modulator *RAP1A* and the genomic caretaker *H2AFX*. In contrast, the expression of 8 Hp proteins and 7 Cx proteins including the apoptosis-promoting protein PPM1F was down regulated by DZSM (Figure 4A).

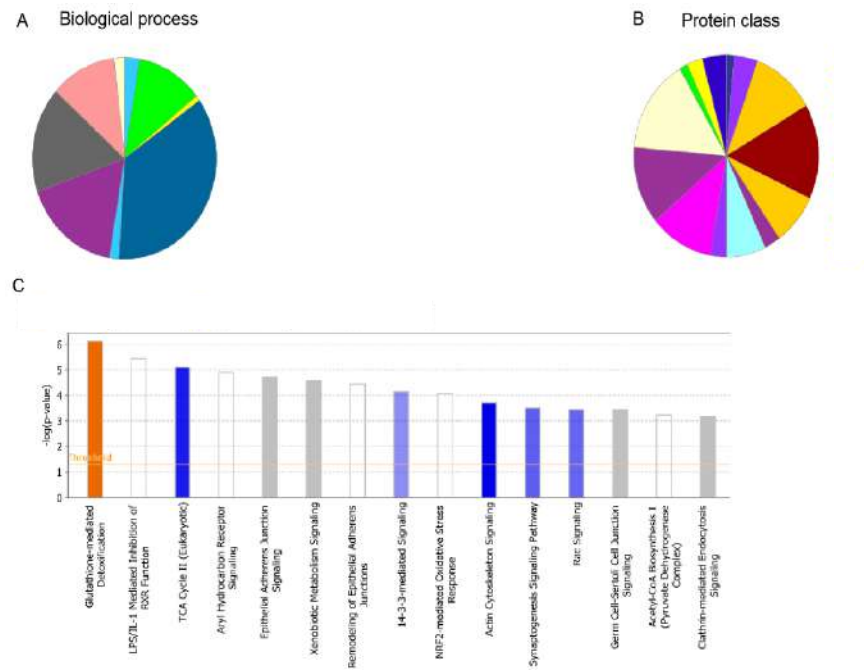


Figure 2: GO and pathway analysis of the hippocampal proteome of VD rats compared with the control group. (A): Biological process; (B): Protein class; (C): Top15 of IPA-annotated canonical signaling pathways. **Note:** (■): Biological adhesion (GO: 0022610); (■): Biological regulation (GO: 0065007); (■): Cellular component organization or biogenesis (GO: 0071840); (■): Cellular process (GO: 0009987); (■): Developmental process (GO: 0032502); (■): Localization (GO: 0051179); (■): Metabolic process (GO: 0008152); (■): Multicellular organismal process (GO: 0032501); (■): Response to stimulus (GO: 0050896); (■): Calcium-binding protein (PC00060); (■): Chaperone (PC00072); (■): Cytoskeletal protein (PC00085); (■): Enzyme modulator (PC00095); (■): Hydrolase (PC00121); (■): Isomerase (PC00135); (■): Ligase (PC00142); (■): Lyase (PC00144); (■): Membrane traffic protein (PC00150); (■): Nucleic acid binding (PC00171); (■): Oxidoreductase (PC00176); (■): Transfer/carrier protein (PC00219); (■): Transferase (PC00220); (■): Transporter (PC00227); (■): Positive z-score; (□): Z-score=0; (■): Negative z-score; (■): No activity pattern available.

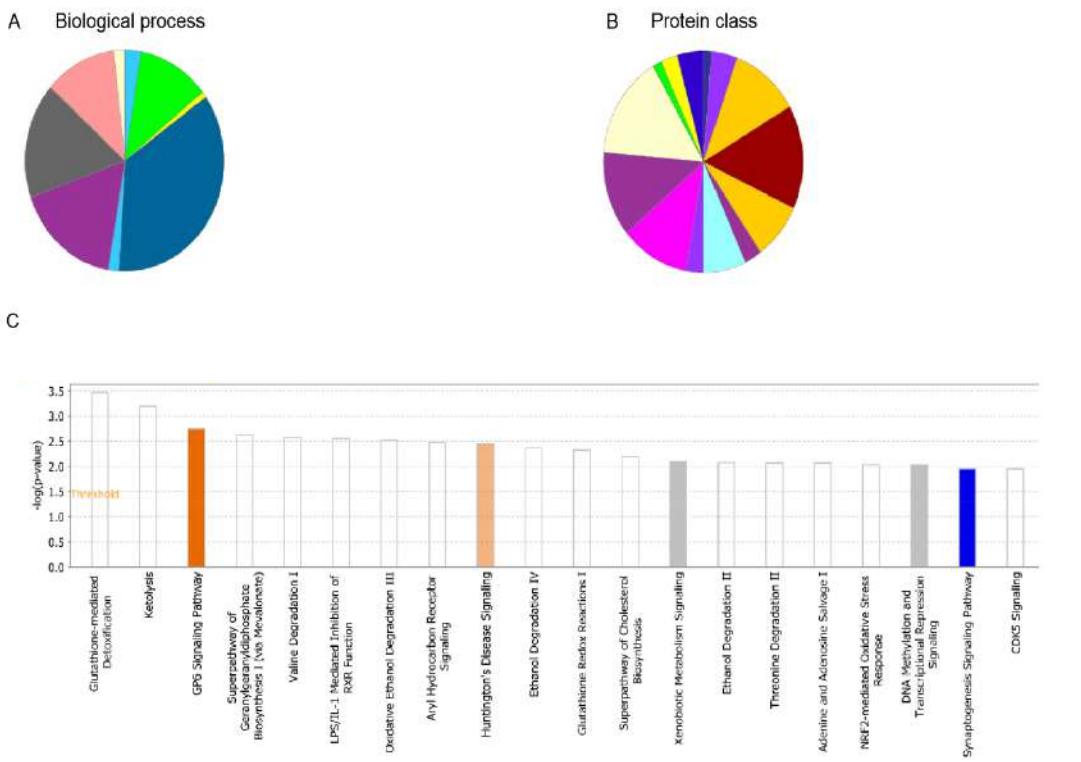


Figure 3: GO and pathway analysis of the cerebral cortex proteome of VD rats compared with the control group. (A): Biological process; (B): Protein class; (C): Top20 of IPA-annotated canonical signaling pathways. **Note:** (■): Biological adhesion; (■): Biological regulation; (■): Cellular component organization or biogenesis; (■): Cellular process; (■): Developmental process; (■): Localization; (■): Metabolic process; (■): Multicellular organismal process ; (■): Response to stimulus; (■): Calcium-binding protein; (■): Chaperone; (■): Cytoskeletal protein; (■): Enzyme modulator; (■): Extracellular matrix protein; (■): Hydrolase; (■): Isomerase; (■): Ligase; (■): Lyase; (■): Membrane traffic protein; (■): Nucleic acid binding; (■): Oxidoreductase; (■): Receptor; (■): Signaling molecule; (■): Transcription factor; (■): Transfer/carrier protein; (■): Transferase; (■): Transmembrane receptor regulatory/adapter protein; (■): Transporter; (■): Positive z-score; (■): Z-score=0; (■): Negative z-score; (■): No activity pattern available.

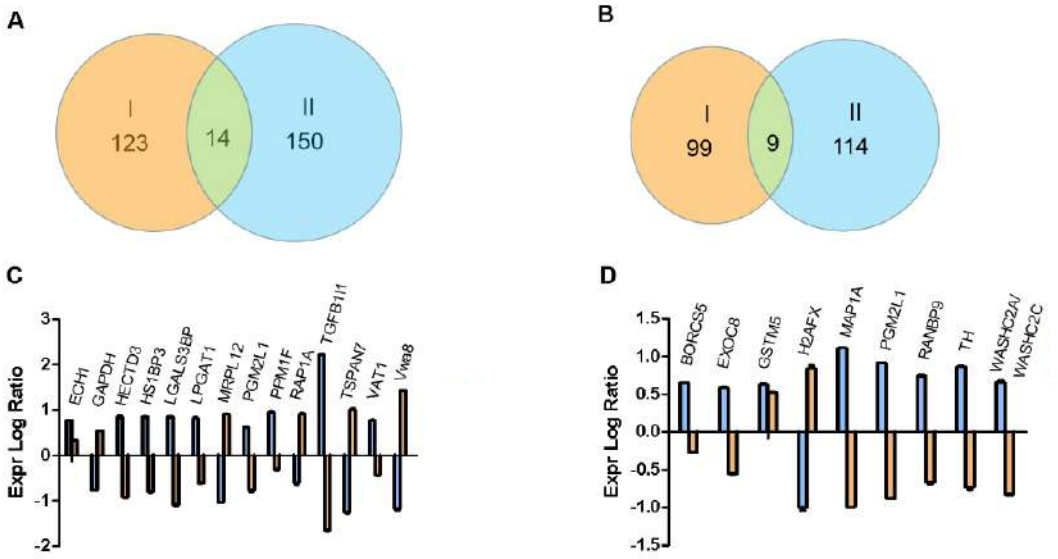
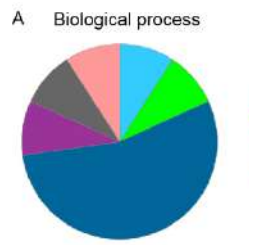
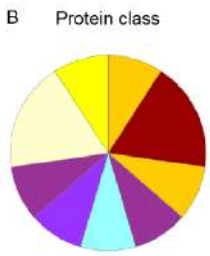


Figure 4: Proteomic analysis of the VD model group *vs.* sham control group and DZSM-treated group *vs.* VD model group in (A): Hippocampus; (B): Cerebral cortex; (I): Represented the differently expressed proteins between DZSM-treated group (VDZ) and VD model group (Vm); (II): Represented the differently expressed proteins between VD model group (Vm) and sham control group (Vc); (C): 14 DZSM-targeted hippocampal proteins identified by proteomic analysis; (D): 9 DZSM-targeted cortex proteins identified by proteomic analysis. **Note:** (■): Vm/Vc; (■): VDZ/Vm; (■): I; (■): II

We categorized the 23 differentially expressed proteins in the Hp and Cx in accordance with their biological functions as well as classes and pathways through the Panther database as shown in Figures 5A-5C and 6A-6C and further deciphered how these proteins interact with each other by IPA as shown in Figure 7A. The interaction networks identified by IPA highlighted that *H2AFX* works in synchrony with other proteins to combat potential DNA defects and oxidative damage in ischemia-induced VD as shown in Figure 7B. In both regions, cellular processes were the biological processes that were the most heavily interfered with. Specifically, the upregulated protein *RAP1A* was shown to be involved in several critical pathways, such as the integrin signaling pathway, which regulates neurite outgrowth and various synaptic functions by interacting with proteins such as GRIN2B or RHOA directly or indirectly (Figures 5A-5C-7A-7C).





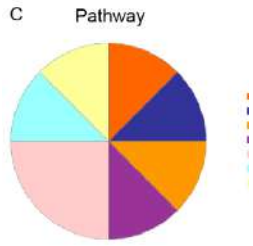
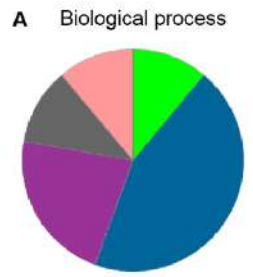
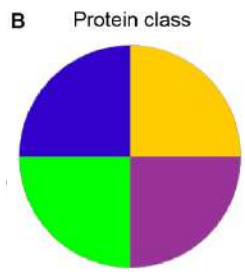


Figure 5: GO analysis of DZSM-targeted hippocampal proteins. (A): Biological process; (B): Protein class; (C): Pathway. **Note:** (■): Biological adhesion (GO: 0022610); (■): Biological regulation (GO: 0065007); (■): Cellular process (GO: 0009987); (■): Localization (GO: 0051179); (■): Metabolic process (GO: 0008152); (■): Multicellular organismal process (GO: 0032501); (■): Cytoskeletal protein (PC00085); (■): Enzyme modulator (PC00095); (■): Hydrolase (PC00121); (■): Isomerase (PC00135); (■): Ligase (PC00142); (■): Lyase (PC00144); (■): Nucleic acid binding (PC00171); (■): Oxidoreductase (PC00176); (■): Transferase (PC00220); (■): Angiogenesis (P00005); (■): Glycolysis (P00024); (■): Heterotrimeric G-protein signaling pathway-Gi alpha and Gs alpha mediated pathway (P00026); (■): Heterotrimeric G-protein signaling pathway-Gq alpha and Go alpha mediated pathway (P00027); (■): Huntington disease (P00029); (■): Integrin signalling pathway (P00034); (■): VEGF signalling pathway (P00056).





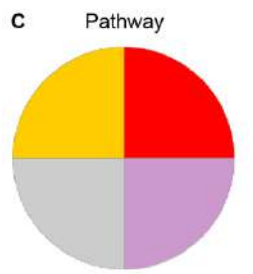


Figure 6: GO analysis of DZSM-targeted cortex proteins. (A): Biological process; (B): Protein class; (C): Pathway. **Note:** (■): Biological regulation (GO: 0065007); (■): Cellular process (GO: 0009987); (■): Localization (GO: 0051179); (■): Metabolic process (GO: 0008152); (■): Multicellular organismal process (GO: 0032501); (■): Cytoskeletal protein (PC00085); (■): Nucleic acid binding (PC00171); (■): Transfer/carrier protein (PC00219); (■): Transporter (PC00227); (■): Adrenaline and nor-adrenaline biosynthesis (P00001); (■): Dopamine receptor mediated signalling pathway (P05912); (■): Nicotine pharmacodynamics pathway (P06587); (■): Parkinson disease (P00049).

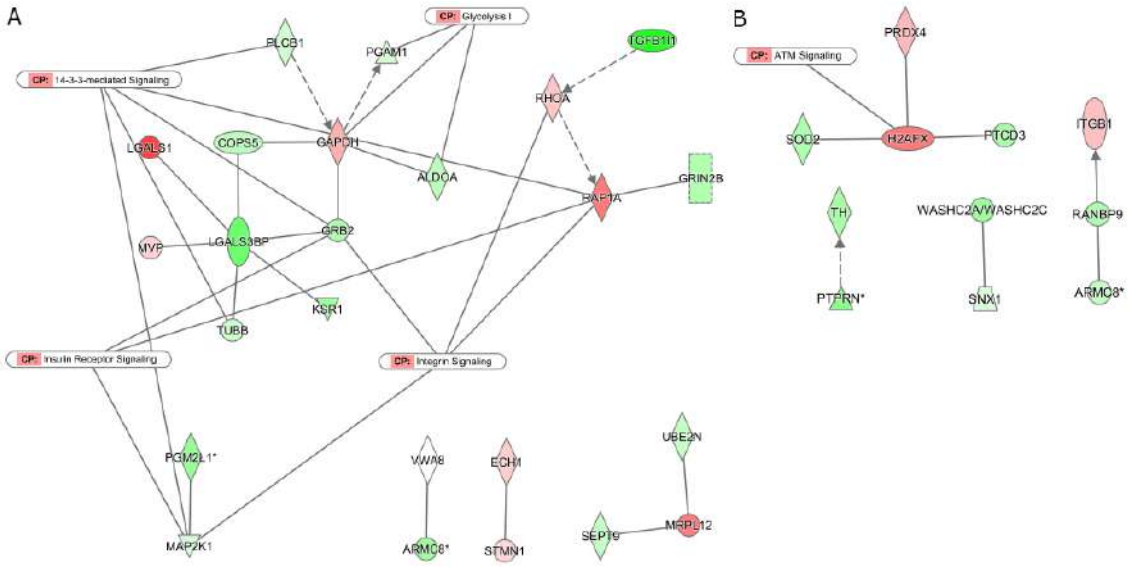


Figure 7: The interactions of (A): DZSM-regulated hippocampal proteins or (B): Cortex proteins. The interaction networks were predicted based on the ingenuity knowledge base. Upregulated or downregulated proteins are in red or green. Solid and dashed lines indicate direct and indirect interactions, respectively.

DISCUSSION

Vascular Dementia (VD), a syndrome with characteristics of gradual memory loss and cognitive impairment, is primarily caused by brain ischemia [19]. DZSM is a compound Chinese medicine that is widely used in the clinic for the treatment of ischemic cerebrovascular diseases along with symptoms of dementia and forgetfulness. One recent study confirmed the therapeutic effect of DZSM against VD in a rat model using neuroimaging methods. However, the underlying mechanism remains obscure. In this study, a clinically relevant VD rat model was established through the injection of microembolus into the internal carotid artery of rats [20]. By using iTRAQ-based quantitative proteomic analyses, we then investigated alterations in the proteome profile of the VD model group and the control group as well as DZSM-treated and non-treated VD rats, thereby providing further insights into the pathogenetic mechanisms of VD and the molecular mechanisms of the effects of DZSM against VD.

We explored the cognition-enhancing effect of DZSM treatment by using the MWM test. In the place navigation trials, escape latency declined as the training proceeded, and it was significantly shortened upon DZSM administration compared with that in the model group. The spatial probe test demonstrated that DZSM reduced the time needed to reach the original platform and increased the number of times the original platform was crossed. Overall, these data suggest that DZSM treatment rescued the spatial memory deterioration caused by the injection of microembolus into the internal carotid artery.

To reveal the specific proteins and pathways underlying the exacerbated cognitive deficits observed in VD, a label-free quantitative proteomic approach was applied, and bioinformatics analysis was conducted using the Panther database and IPA software. We found that compared with the control group, the VD model exhibited altered expression of 123 Hp proteins and 99 Cx proteins. Among these proteins, 64 Hp proteins and 72 Cx proteins were upregulated, and 59 Hp proteins and 27 Cx proteins were downregulated. Functional analysis identified multiple unique proteins, such as *CS, IDH3G, MDH2, RAP1A, SNCA, YWHAG, ACTR2, NCKAP1* and *GRIA2* that were primarily related to specific biological processes in the nervous system through their involvement in energy metabolism, synaptic functions, neuron morphogenesis, etc., [21-27]. Biocomputational protein classification analysis suggested that among all biological processes, cellular processes were most affected. Cytoskeletal proteins, enzyme modulators and oxidoreductases represented a significant proportion of regulated proteins in the VD brain.

Increasing evidence has suggested that the pathogenesis of VD is closely related to energy hypometabolism, the impairment of synaptic plasticity, and cytoskeletal malfunctions [28]. IPA indicated that the altered proteins in VD brain had strong interactions with the Tricarboxylic Acid (TCA) cycle, 14-3-3-mediated signaling, actin cytoskeleton signaling, the synaptogenesis signaling pathway, Rac signaling, etc. These top-ranked canonical pathways, key regulators of glucose metabolism, synaptic plasticity and synaptogenesis, were predicted to be downregulated in VD [29-32]. In particular, 14-3-3, one of the most abundant brain proteins in the brain, binds to a wide range of signaling proteins to mediate diverse cellular processes important for neuronal functions, including but not limited to synaptic plasticity, neurite outgrowth, and cell survival. Multiple studies have suggested a protective role for 14-3-3 proteins against neuronal damage and revealed downregulation of different 14-3-3 isoforms in neurological disorders such as Alzheimer's disease and Parkinson's disease [33,34]. In this study, we found that 6 vital proteins (*RAP1A, RAP1B, SNCA, TUBA8, TUBB2A*, and 14-3-3-gamma) involved in the 14-3-3-mediated signaling pathway all showed lower expression levels in VD compared with the

control and further analysis by IPA predicted decreased overall activity of this signaling pathway. Considering the crucial roles of 14-3-3 and its pathways in neuronal functions, the cognitive deficits observed in VD might be largely attributed to the downregulation of these critical signaling pathways.

To better resolve the specific mechanism of the effects of DZSM against VD, we looked for proteins potentially regulated by DZSM and their interactions. Through two comparisons of proteome changes, the VD model group *vs.* the sham control group and the DZSM group *vs.* the VD model group, 14 Hp proteins and 9 Cx proteins were identified to be susceptible to ischemic injury and DZSM simultaneously. Among these proteins, the glycolytic enzyme GAPDH, the neuritogenesis modulator *RAP1A* and the genomic caretaker *H2AFX* were upregulated, and the apoptosis-promoting protein PPM1F was downregulated. Specifically, Ras-related protein Rap-1A (*RAP1A*) is a Rap GTP-binding protein, a subfamily of the Ras superfamily (Ras, Rap1, and Rap2) [35]. Rap1 small G protein has been revealed by numerous studies to play critical roles in neurite outgrowth and various synaptic functions [36,37]. Our study revealed that *RAP1A* interacts with other proteins (e.g., RHOA and GRIN2B) to mediate multiple critical pathways, such as the integrin signaling pathway, Insulin Receptor (IR) mediated signaling, CREB signaling in neurons, synaptic long-term potentiation and synaptogenesis signaling [38-43]. Regarding histone H2A (*H2AFX*), evidence has suggested that this protein might serve as a docking platform for proteins involved in DNA damage/repair, function to promote double-strand break repair and preserve genome stability [44,45]. As shown in Figure 8, our results suggest that DZSM may function through regulating key modulators, such as *RAP1A* and *H2AFX*, which are involved in signaling pathways important for neuronal functions (Figure 8).

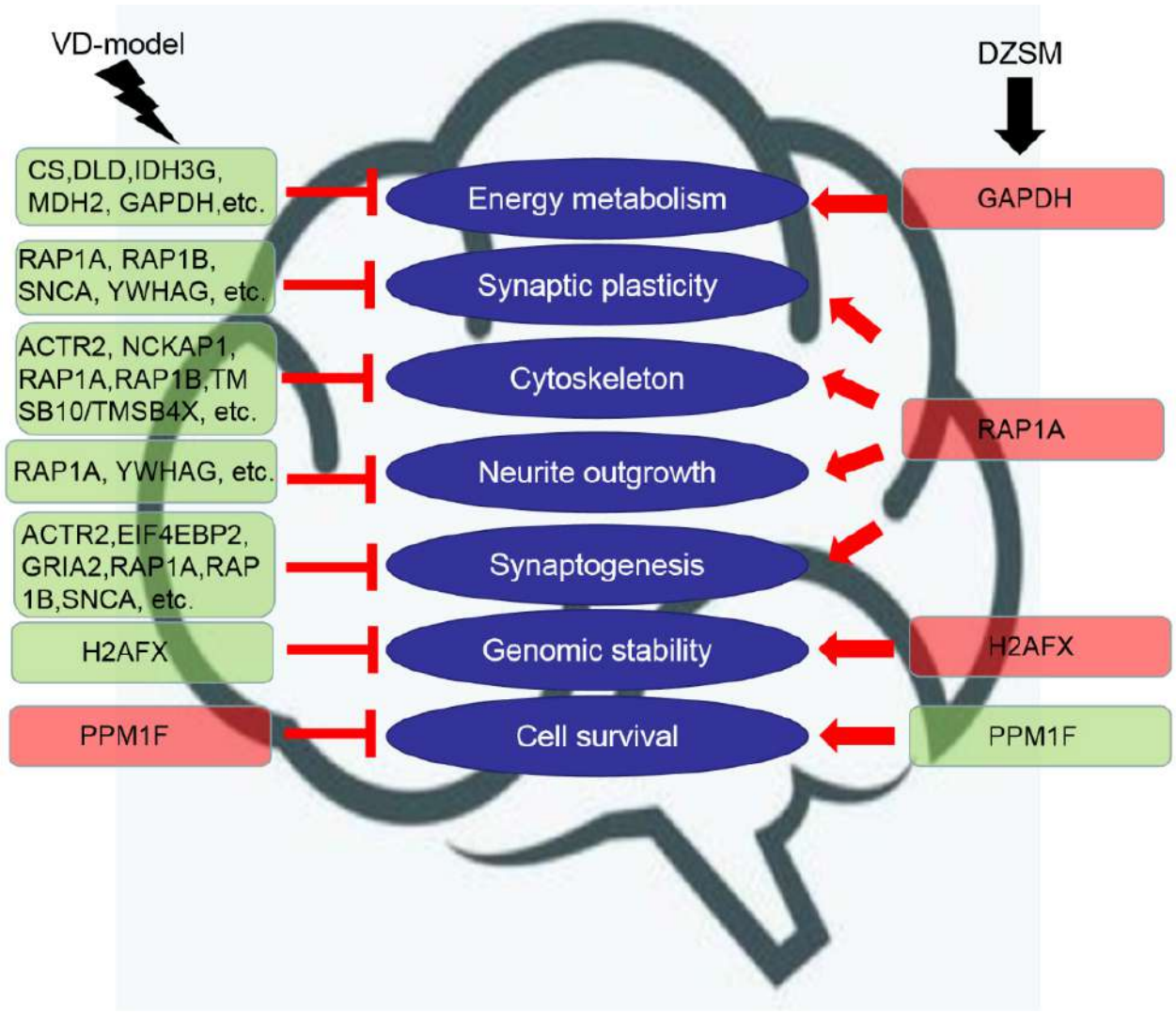


Figure 8: Summary of the overall findings of the iTRAQ-based proteomics analysis. Molecules in red or green rounded rectangular represent upregulated or downregulated proteins, respectively.

CONCLUSION

In summary, our study found that the proteome was broadly changed in the VD rat brain. With the aid of advanced bioinformatics analysis, we further revealed the dysregulation of multiple key proteins that are at the core of several crucial canonical pathways, including 14-3-3-mediated signaling, actin cytoskeleton signaling, and the synaptogenesis signaling pathway. We next examined the proteome profile of the DZSM-treated VD rat brain to identify potential DZSM-targeted proteins and their interactions. Since a diverse range of cellular activities are executed through proteins and their interactions, depicting the protein landscape of VD and the DZSM-treated VD brain will help us gain a deeper understanding of the underlying molecular mechanisms involved in the effects of DZSM treatment on VD.

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