Comparative Phosphoproteomic Profiling in Prefrontal Cortex of Prenatal Stressed Offspring Rats

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Abstract

Background: Many investigations indicate that prenatal stress caused depressive-like disturbances in offspring rats. The underlying pathogenic mechanisms have not yet been fully unravelled. The PFC has been shown to play a role in susceptibility to stress during fetal, thus we focus our attention on differential protein phosphorylation in this region of PS-S(Susceptibility to PS) offspring rats.

Method: SPT was used to screen for susceptibility to PS. The validity of prenatally stressed model was verified by other common depression-like behaviors. We used MS-based TMT quantitative proteomics in combination with phosphopeptide enrichment method to compare phosphoproteomic profiling in prefrontal cortex of of PS-S and CON offspring rats.

Results: Totally, 3418 phosphoproteins, 8404 phosphopeptides and 12175 phosphosites were identified in this analysis. According to the screening criteria, 902 phosphopeptides increased and 609 decreased in the PFC of PS-S group compared to the control rats. GO enrichment analysis indicated that the main enriched terms in CC category were ‘synapse part’, ‘myelin sheath’, ‘synapse’, ‘neuron part’ and ‘axon’. The phosphorproteins enriched in MF and BP category were mainly related to cytoskeleton and projection morphogenesis associated proteins. KEGG pathway enrichment analyses identified 30 significant KEGG pathways, the top five pathways included salivary secretion, Endocrine and other factor-regulated calcium reabsorption, Pancreatic secretion and Insulin secretion. Motifs such as……_S_P...RR, ……_S_PE...., ……_S_PV...., ……_S_P.H...and ..S..._S_PT....were the top five motifs enriched in phosphorylated sites.

Conclusion: PS may induce depressive-like behaviors in offspring rats through regulating the phosphorylation of protein mainly related to synapse, myelin sheath, neuron and cytoskeleton. And the phosphorylation of related proteins may act as key pathogenic hits. Data are available via ProteomeXchange with identifier PXD026563.

Introduction

Depression is a common mental disorder characterized by depressed mood, loss of interest or pleasure, decreased energy, feelings of guilt or low self-worth, change in sleep or appetite, and poor concentration[1]. These problems can lead to substantial impairments in social functioning, defined as an individual’s ability to perform and fulfill normal social roles[2]. There have been few effective treatments developed for depressed patients. However, treatments currently available have limited in efficacy and can have serious side effects [3]. This multifactorial disorder involve in substantial molecular alterations and pathway dysfunction, therefore, it is significant to explore the underlying pathophysiology of depression.

The highest risk period for women to develop depression is during pregnancy which exposes fetus to prenatal stress (PS)[4]. PS is exposure of stress to an expectant mother which is caused by stressful life events or by environmental adversities resulting in altered brain development in fetus[5]. The fetus itself does not come into direct contact with the maternal nervous system[6, 7],but the placenta is in direct

contact with circulating maternal blood to mediate material exchange between the mother and fetus[8]. Throughout pregnancy, exposure to maternal depression or anxiety may have negative impact on the developing fetus [9]. The mechanism of the implication remains to be further investigated.

The prefrontal cortex (PFC), as a significant nerve center of thinking and behavior regulation in the brain, is associated with depression[10].The region is essential for top-down regulation of neuroendocrine and behavioral processes[11, 12].The PFC is highly sensitive to environmental stimuli (e.g. stress, sleep, diet and social experiences), in particular, stimuli present during fetal and early postnatal life[13]. PS changed global methylation in frontal cortex [14, 15]. Multiple genes in the frontal cortex were differentially expressed following PS[16]. In general, the PFC could be changed dramatically by events beginning prenatally.

In this study, we established the chronic restraint stress animal model of PS as in our previous study[17] to examine behavioral phenotypes of PS offspring rats firstly. As the PFC has been shown to play a role in susceptibility to stress during fetal[13], we focus our attention on this region. We identified differentially expressed phosphopeptides as PS-responsive phosphopeptides in the PFC of prenatal stressed offspring rats compared with the control group, which were carried out integrated analysis and exploration using a series of bioinformatics tools.

Results

Behavioral assessment of PS offspring rats

Experiments were carried out on forty-five stressed offspring male rats and eight control rats. Susceptibility(R), medium(M) or resistance(S)response to PS was assessed by SPT. 38.78% rats in stressed group exhibiting a significant decrease in preference for sucrose were regarded as susceptibility to PS and classified as PS-S offspring rats(PS-S group) (Fig. 1A),eight rats from this group were randomly selected for the subsequent experiments. The validity of prenatally stressed model and the filter criteria of the SPT were tested by common depression-like behaviors, including OFT and FST. Data were shown in Fig. 1the total distance of OFT in PS-S group compared to the control group is statistically significantly decreased using the One-Way ANOVA ($P < 0.05$Fig. 1B). Rats from PS-S group have a significant extended immobility time in FST compared with the control rats using the One-Way ANOVA ($P < 0.05$Fig. 1C). All above, these results provided evidences for the validity of the animal model and the screening approach, and the model may provide a tool to study the molecular and pathogenesis of offspring rats with depression-like behavior induced by prenatal restraint stress during pregnancy.

TMT-based quantitative phosphoproteomic analysis of the rat prefrontal cortex

MS-based TMT quantitative proteomics in combination with phosphopeptide enrichment method was applied to identify changes in phosphorylation status of proteome in the prefrontal cortex of prenatal stressed offspring rats. A flowchart of the steps used for the quantitative TMT-based phosphoproteomicanalysisis presented in Fig. 1D. Three rats per group were used for this study. Totally, this analysis identified 3418 phosphoproteins, 8404 phosphopeptides and 12175 phosphosites(Fig. 2A).The complete data set for all identified proteins can be found in supplementary Dataset 1. The phosphosites distribution was as follows: 2.06% phosphotyrosine site, 18.11% phosphothreonine site, and 79.83% phosphoserine site(Fig. 2B).We found that 60.4% proteins were detected with phosphorylation atmultiple (two or more) sites on the same one by the analysis of the distribution of all phosphosites(Fig. 2C), among which protein ENSRNOP00000023460 was identified the presence of phosphorylation sites up to 118. In all the modified proteins detected, the mean number of phosphosites every 100 amino acids was 3.51(Fig. 2D).

Screening and cluster analysis of differentially expressed phosphopeptides

Phosphopeptides were considered as differentially expressed according to the screening criteria of a fold change >1.2 (more than 1.2 or less than 0.83)and a p-value < 0.05. 1511 PS-responsive phosphopeptides were identified with these cut-offs. Compared with the control group, 902 phosphopeptides increased and 609 decreased in the prefrontal cortex of prenatal stressed offspring rats(Table 1).Quantitative results corresponding to differentially expressed phosphopeptides were displayed in the form of volcano plots(Fig. 3A), based on different biological repeats. The profiles of differentially expressed phosphopeptides between groups were then distinguished by applying a hierarchical cluster analysis. Data were presented in the form of heat-map(Fig. 3B). Heat map provided an important clue about the differentially expressed phosphopeptides obtained according to the screening criteria above can effectively distinguish between the groups, and comparison of duplicates per group showed similar the phosphorylation profile of all tested peptides.

Table 1

The quantitative statistical results forphosphopeptides

Comparisons	Significantly changing in abundance	
	Increased	Decreased
	902	609

Functional and pathway enrichment analysis/GO enrichment and KEGG pathway analysis

For high-throughput phosphoproteomic analysis, to understand which biological functions and pathways are significantly affected by PS treatments was the first priority. To infer the biological functions of proteins correspond to differentially expressed phosphopeptides, GO, KEGG analyses were used in this study to predict the function and pathway of these proteins in the pathogenesis of PS-S group.

All statistically significant phosphorylation proteins identified were subject by gene ontology (GO) annotation withBlast2GO (https://www.blast2go.com/)software, and then the GO category enrichment analysis was applied using Fisher's exact test with p value ≤0.05 as the cut-off. As shown in Fig. 4A, in the cellular component category, 'synapse part', 'myelin sheath', 'synapse', 'neuron part' and 'axon' were the main enriched terms. As for the molecular function category, 'structural constituent of cytoskeleton', 'calmodulin-Binding', 'cytoskeletal protein binding', 'actin binding' and 'protein binding' were most representative. While in the biological process category, 'cell projection morphogenesis', 'cell part morphogenesis', 'neuron projection morphogenesis', 'plasma membrane bounded cell projection morphogenesis', and 'cell morphogenesis involved in neuron differentiation' were dominant. Taken together, these results revealed the overall functional characteristics of all differentially phosphorylated proteins, and the significantly enriched GO terms were involved in biological functions we most concerned.

A similar to GO terms enrichment analysis was conducted for the KEGG pathways using Fisher's exact test. The most enriched KEGG pathway included salivary secretion, Endocrine and other factor-regulated calcium reabsorption, Pancreatic secretion and Insulin secretion. The top 20significantly enriched KEGG pathways in this analysis were shown in Fig. 4B.

Protein-protein interaction network

Proteins never exist singly in living organisms, the function of which was regulated or mediated by other associated proteins. The protein-protein interactions(PPI) network established by STRING online tool was used to investigate the interaction among all PS-responsive phosphopeptides. The PPI network graph were generated and visualized by CytoScape (Version 3.2.1) (Shown in Fig. 5).

Identification of phosphopeptide motif

Kinases phosphorylate substrates through recognizing substrate-specific motif sequence, the high throughput phosphoproteomic enabled us to identify consensus motif sequences reflecting the kinase-specific regulation of substrate phosphorylation and the identity of the corresponding kinases. All identified phosphorylation sites in differentially expressed phosphopeptides were analyzed using the motif discovery tool MEME (Multiple EM for Motif Elicitation) software. Motifs such as……_S_P...RR, ……_S_PE...., ……_S_PV...., ……_S_P.H...and ..S..._S_PT....were the top five motifs enriched in phosphorylated sites(Fig. 6A and Table S2). The distribution and enrichment of different conservative motif were showed in Fig. 6B and 6C, respectively.

We predicted the probable upstream kinases for motifs enriched in this study using PhosphoMotif Finder database. The five motifs above were identified to be potential substrates for the ERK1, ERK2 kinase, CDK1, 2, 4, 6 kinase, and growth associated histone HI kinase.

Discussion

In this study, prenatal restraint stress were used to establish offspring rats with depression-like behavior. Only the prenatal stress-susceptible male offspring rats (PS-S) assessed by SPT were selected for subsequent analyses in order to reduce heterogeneity.

Then we identified alterations in phosphorylation status of proteome in the PFC of PS-S offspring rats using TMT-based quantitative phosphoproteomic approach. Özgün Babur etc.[18]used phosphoproteomics approach coupled with the similar TMT labeling to reveal pathways in GPVI/ITAM-mediated platelet activation programs. In our study, TMT-based quantitative proteomics analysis indicated that a total of 6078 quantifiable proteins were identified, and 564 differentially expressed proteins (DEPs) were identified in PS-S group versus normal control group. The quality control experiment showed that the instrument analysis system of this experiment was stable and results reliable. Therefore, the experimentally obtained results reflect real biological differences between the two groups themselves. As a result, we observed that global changes of protein phosphorylation were exhibited in the PS-S model.

A GWAS-based pathway analysis reported significant associations of synaptic density with psychiatric disorders, including depression [19]. A study in the postmortem PFC revealed a reduced number of synapse in depressed subjects [20]. In this study, changed phosphorylated levels in synapse-associated proteins in the PFC of PS-S model appeared. Similar results also found in our previous study [17]. Cytoplasmic FMRP interacting protein 1(Cyfip1)，a candidate for a risk factor for psychiatric or neurodevelopmental disorders due to it's known relation with synaptic dysfunction [21, 22], was showed that it phosphorylation level was significantly increased in PS-S group. As far as a relationship with Fmr1 was concerned, Cyfip1 played antagonistic roles in regulating synaptic growth [23]. Either increases or decreases in Cyfip1 gene dosage was responsible for schizophrenia[24].A mechanism of Cyfip1 high phosphorylation in offspring rats with depression-like behavior induced by PS is still unclear. Investigation of whether consequences of Cyfip1 phosphorylation could be involved with intellectual disability, depression, and schizophrenia[25, 26] will be be an interesting point in future research.

Our results also revealed that the phosphorylation level of 17kDa, 18.5kDa and 21.5kDa myelin basic protein (Mbp) isoforms were all significantly increased in PS-S group. In particular, Mbp was thought to contribute to myelin structure and function by its post-translational modifications[27-29]. Mbp phosphorylation could disrupt of the Mbp interactions with lipid in the myelin sheath and alter the myelin sheath structure [29].It is reported that immunization with altered Mbp peptide produced antidepressant-like effects and reversed the CUS-induced depressive-like behaviors [30]. According to differential phosphorylation levels of myelin sheath related proteins, it was assumed that PS results in serious disturbance of myelination in offspring rat models. Further research is necessary to determine whether

and how those proteins related myelin sheath with significant differences in phosphorylation effect and play a role in PS induced depressive-like behavior in offspring.

Dihydropyrimidinase-like 3 (Dpysl3) was involved in neuronal differentiation, neuronal regeneration, neuronal polarity and axon elongation [31, 32]. The protein above is highly expressed in the adult brain, and its expression can be observed in new neurons during adult rat neurogenesis[33]. Treatment of depression with venlafaxine up-regulated expression of Dpysl3 [34]. The phosphorylation of Dpysl3 determined neuronal polarity and promotes axon elongation by regulating their binding to tubulin heterodimers in cultured hippocampal neurons[32]. In our study, the phosphorylation level of Dpysl3 decreased significantly in the PFC of prenatal stressed offspring rats. Combined with previous researches, the effect of exposure to PS on offspring rats might affect neuronal differentiation by the reduction of Dpysl3 phosphorylation.

Dual-specificity tyrosine-phosphorylation-regulated kinase 2 (Dyrk2), as an evolutionally conserved protein kinase [35-37], phosphorylates Dpysl3 [32, 38, 39] and reduced the growth and branching of axons and dendrites [40]. Dyrk2 has also been implicated in the regulation of the cytoskeleton [40]. Interestingly, we found here that phosphorylation of Dyrk2 were also reduced in PS-S group. The phosphorylation of Dpysl3 protein by Dyrk2 was presumably implicated in the regulation of cytoskeleton in axon guidance and dendrite branching. Altered phosphorylation of many other proteins related to cytoskeleton was observed in this study. Since the cytoskeleton forms the backbone of neuronal architecture[41], disruption of the synaptic cytoskeleton primarily interferes with the formation and maintenance of synapses[42, 43]. These abnormal phosphorylated proteins might regulate the morphology of synapse, neuron, axons and dendrites through remodeling of cytoskeleton during PS-induced depressive-like behavior.

Conclusions

The current study had some limitations. First of all, the relatively small sample size is a limitation of this study. In order to ensure the accuracy and stability of results, we experimentally explore the instrument detected with quality control samples. Accordingly, the differentially expressed phosphopeptides screened may reflect the group difference efficiently and objectively. Secondly, we did these experiments only with SD rats, which would limit the applicability of these findings to other species. Thirdly, protein phosphorylation has been observed only in PFC, the systematic analysis was required.

These results support the conclusion that PS may induce depressive-like behaviors in offspring rats through regulating the phosphorylation of protein mainly related to synapse, myelin sheath, neuron and cytoskeleton. And the phosphorylation of related proteins may act as key pathogenic hits. The mechanism of molecular mentioned above implicating in PS-induced depressive-like behaviors in offspring rats were, we suspected, just the tip of the iceberg. Further experimental analysis was clearly needed to verify the potential molecular mechanisms. The systematic analysis through the integration of various brain regions and different types of omics data should be applied in future to build a complete

and comprehensive information system for better elucidating the underlying associated pathway and molecular mechanisms.

Materials And Methods

Animal

Age matched Sprague-Dawley rat weighing approximately 230-300 g were housed under a 12-h light: 12-h dark schedule at 22°C with ad libitum access to food and water during experimental session. Following five-day-long habituation, 21 female rats were housed overnight with7 males by 3:1 during 20:00 - 22:00. Female rats were kept individually housed from gestational day (GD) 0when vaginas of female rats were sperm positive in the next morning (7:00~8:00). All experiments were approved by the Experimental Animal Care and Use Committee of Xi'an Jiaotong University. All animal treatments followed the rules of the National Institutes of Health guidelines (Clark et al., 1997).

Chronic restraint stress

The experimental procedure was referred to a previous article[44]. Briefly,during GD 14 - DG 20, pregnant dams in the stress group were exposed to the daily stress for 7 days. For every stress section,pregnant dams were placed in plastic bottles with adjustable lids to keep their heads still for 45 min and three optional times per day, and then they were allowed to return to their own cages. Correspondingly,control females were handled daily without stress stimulation.The pups selected in all experiments were from 6 to 12 rats in each litter, with no more than two offspring per litter. One month old postnatal male rats were used in subsequent experiments.

Behavioral tests

Sucrose preference test (SPT)

The SPT was employed to make a distinction ofmale offspring rats showed susceptibility(R)or resistance(S)response to PS, when they were one month old. SPT was conducted according to a previous article[45]. Briefly, rats were allowed to stay in their home cages with one bottle of 1% sucrose for at least 24 h before the experiments began. Then, following a 24 h period of food and water deprivation, rats were presented with two identical drinking bottles containing either tap water or 1% sucrose solution from 8: 00 to 9: 00.The sucrose preference was determined as the percentage of sucrose solution intake/total (water + sucrose liquid) intake. For the purpose of this experiment, only susceptible male offspring rats(PS-S) with a $\geq$ 30% reduction in sucrose intake compared to control rats were selected in the present experiment, the rest of male offspring ratsidentified as prenatal stress medium(PS-M)or prenatal stress resistance(PS-R) were excluded.

Open field test (OFT)

After screened by SPT, 8 rats in the PS-S group and 8 rats in the control group were randomly selected for OFT.The test apparatus was an open-field box measuring 80 cm length × 80 cm width × 40 cmheight with black side- walls, and the floor was divided into 25 squares of equal areas by black lines.Rats were gently placed at the center of the box and allowed to move freely,their behaviors were recorded using a video camera for 5 min.The total distancewas recorded for each trial.

Forced swimming test (FST)

FSTwas performed after the OFT. In brief,the animals were individually placed in a glass-cylinder (50 cm height × 20 cm diameter) with water at 30 °C and a depth of 30 cm in the test. Rats were adapted to swimming for 15 min on the first day and subsequentlydried by heater. Rats were placed individually in a vertical glass cylinder and video recorded (BW-DFS201; Shanghai Biowill Co., LTD. Shanghai, China) for 5 min to count and to analyze immobile time which was defined as a floating state in the water without struggling and making only those movements necessary to keep the head above water.

Protein extraction, protein enzymolysis, and peptide TMT labeling

After all behavioral tests, rats were sacrificed and the PFC was harvested. Tissue lysate was used to extract total protein from tissue (n =3) and the protein content was determined according to BCA Protein Measuring Kit instructions. Appropriate amount protein was taken from each sample and digested with trypsin according to FASP method[46]. The digest was desalted over a C18 cartridge and lyophilized, subsequently peptides were redissolved in 40uL dissolution Buffer and quantified by measuring absorbance at 280 nm (OD280).1μg peptides from each sample were labeled by TMT according to TMT-Labeling reagent kit (Thermo Scientific) instructions.

Phosphopeptides enrichment

The labeled peptide mixtures were pooled and vacuum-dried□then enriched by High-SelectTM Fe-NTA Phosphopeptides Enrichment Kit (Thermo Scientific). Phosphopeptides were concentrated using a vacuum and resuspended in 20 μl of 5% formic acid prior to mass spectrometry analysis.

LC-MS/MS

Samples were loaded and separated using an Easy nLCnanoflow HPLC liquid phase system.A total of 0.1% formic acid aqueous solution and a 0.1% formic acid-84% acetonitrile-water solution were used as

buffer A and buffer B, respectively. Following column equilibration 95% buffer A, the sample was injected via autosampler and loaded onto the column(Thermo Scientific Acclaim PepMap100,100μm*2cm, nanoViper C18), separation was carried out on the analytical column (Thermo Scientific EASY column, 10cm, ID75μm, 3μm,C18-A2) at a flow rate of 300 nl/min.After the separation by HPLC, the peptide samples were analyzed by a Q-Exactivemass spectrometer. Mass spectrometric analysis was performed in positive-ion mode, by a parent ion scan range of m/z 79, with the primary source with a resolution of 7000 at 200 m/z. AGC target was set to 1e6 with Maximum injection time of 50 ms and a dynamic exclusion of 60 s.The mass-to-charge ratios of polypeptides and polypeptide fragments are obtained by collecting 20 fragment patterns(MS2 scan) after each full scan at a resolution of 17,500 at 200 m/z, by high energy collision dissociation (HCD), with the normalized collision energy to 30eV, and isolation window of 2 m/z, and the underfill ratio as 0.1%.

Protein/phosphopeptides identification and quantitation

Original mass spectrometry data were in RAW files, MaxQuant software (MaxQuant, RRID:SCR_014485) was used to perform database searches and quantitative analysis. The relevant parameters were defined as Table S1.

Phosphopeptides were considered as differentially expressed according to the screening criteria of a fold change >1.2 (more than 1.2 or less than 0.83)and a p-value < 0.05.

Bioinformatics

Gene ontology and KEGG pathway

Gene ontology (GO) annotations of the set of target modified protein were performed using Blast2GO software (Blast2GO, RRID:SCR_005828). The process can roughly be summed up as sequence alignment, GO Mapping, GO Annotation, InterProScanAnnotation Augmentation.

The online service tool KAAS(KEGG Automatic Annotation Server) was used to annotate the KEGG database description of the set of target modified protein.

Fisher’s exact test was used to compare the differential distribution in the enrichment analysis of GO and KEGG annotations between target modified proteins and total modified proteins. GO annotations and KEGG pathway enrichment analysis of target modified proteins were carried out.

Cluster analysis of modified peptides

Firstly, the quantitative information of target proteins were normalized on the interval [-1,1].Then ComplexHeatmap (RRID:SCR_017270 Version 3.4) was employed in classifying the two dimensions of

the sample and protein expression (distance algorithm, Euclidean; connection, Average linkage), simultaneously. Further, heat map was generated using hierarchical clustering.

Network analysis of protein-protein interactions (PPI)

The IntAct (http://www.ebi.ac.uk/intact, RRID:AB_204334) was used to investigate the direct and indirect interactions among target proteins. CytoScape (http://cytoscape.org, RRID:SCR_003032 version 3.2.1) and String database (https://string-db.org/, RRID:SCR_005223) were used to generate the PPI network and analyze the network.

Prediction of Protein-Conserved Motifs

A (2*6 + 1)-mer on modified sites with 6 amino acids upstream and downstream were extracted to predict conserved motifs using MEME (MEME Suite - Motif-based sequence analysis tools, RRID:SCR_001783).

Statistical analysis

Data are presented as the means ± standard deviation (SD). Differences between groups were compared with one-way ANOVA followed by Dunnett's test.$p < 0.05$ was set as the significance level. Statistical analysis was accomplished by Prism version 5.0 software (GraphPad Prism, RRID:SCR_002798).

Abbreviations

PS: prenatal stress; PFC: prefrontal cortex ; SPT: Sucrose preference test ; OFT: Open field test ; FST: Forced swimming test ; TMT: tandem mass tags.

Declarations

Ethics approval and consent to participate

All experiments were approved by the Experimental Animal Care and Use Committee of Xi'an Jiaotong University. All animal treatments followed the rules of the National Institutes of Health guidelines.

Consent for publication

Not applicable.

Availability of data and materials

The datasets generated during the current study are available in ProteomeXchange with identifier PXD026563.

Competing interests

The authors declare that they have no competing interests.

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Author’s contributions

HS and WH were involved in study design. HH and HZ collected the data. WH analyzed the data, drafted the manuscript and performed statistical analysis. HS contributed to revising the manuscript and supervised the study. All authors read and approved the final manuscript.

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Figures

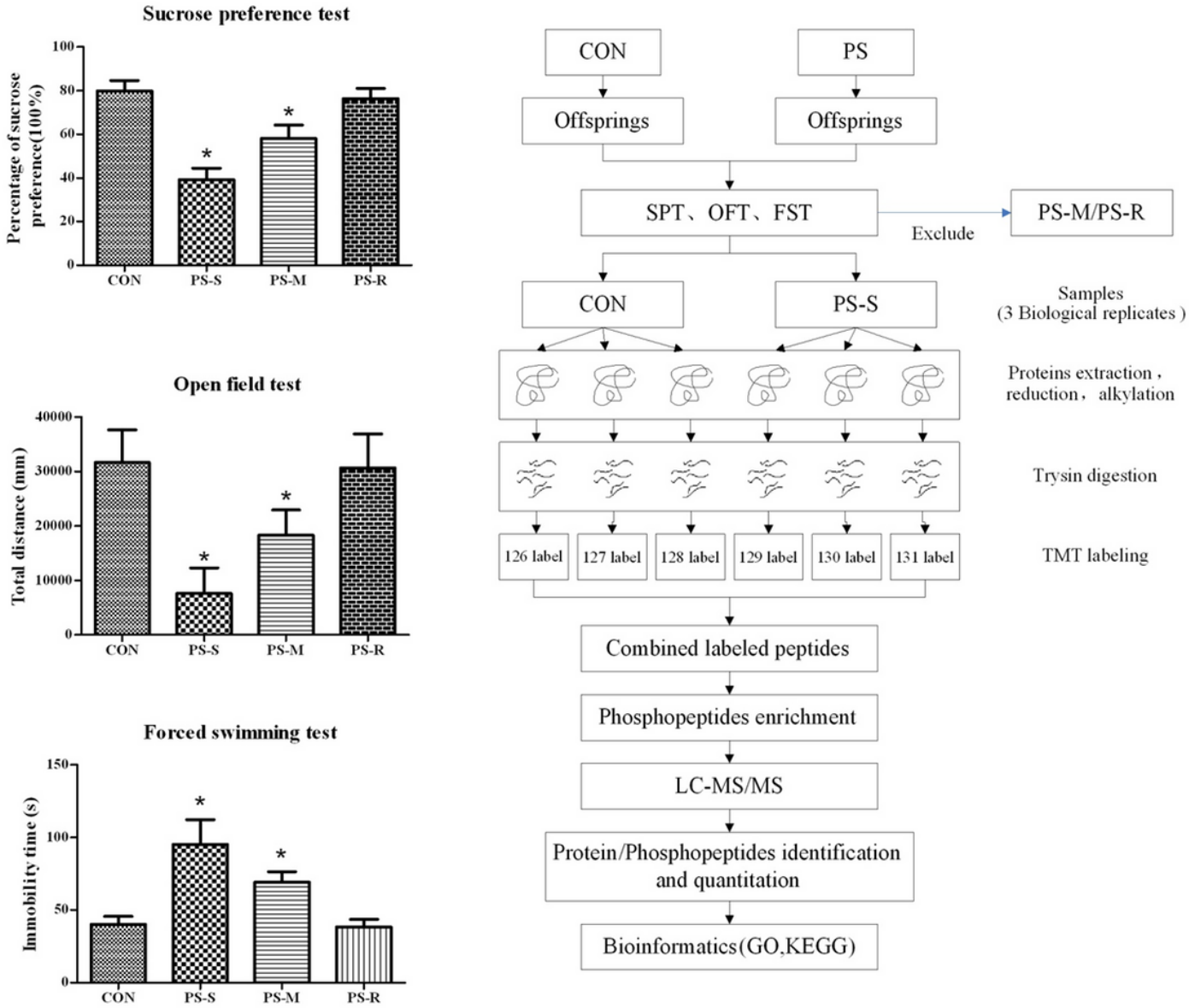

Figure 1

Behavioral assessment of the prenatal stress (PS) rat model and schematic workflow of the experimental design. (A) Percentage of sucrose preference in the sucrose preference test (SPT). (B) Total distance in the open field test (OFT). (C) Immobility time in the forced swimming test (FST). (D) Schematic workflow of the experimental design. Values represent means ± SD, n=8 per group. *p< 0.05 vs. CON.

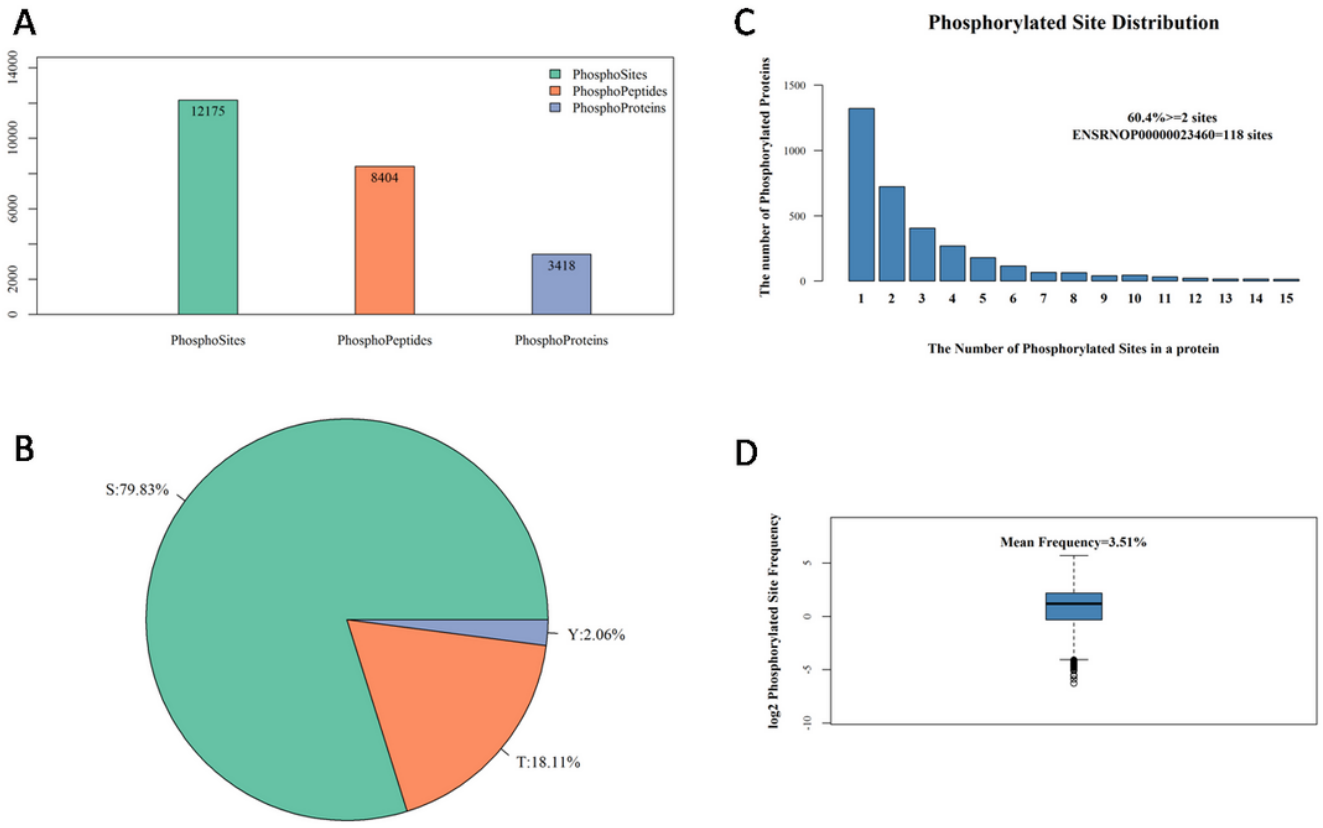

Figure 2

Description of phosphoproteome data. (A) Number of phosphorylation sites, phosphopeptides, and phosphoproteins identified. (B) Number of phosphorylation modifications for serine, threonine, and tyrosine. (C) Distribution of phosphorylated sites. (D) Frequency of phosphorylated sites.

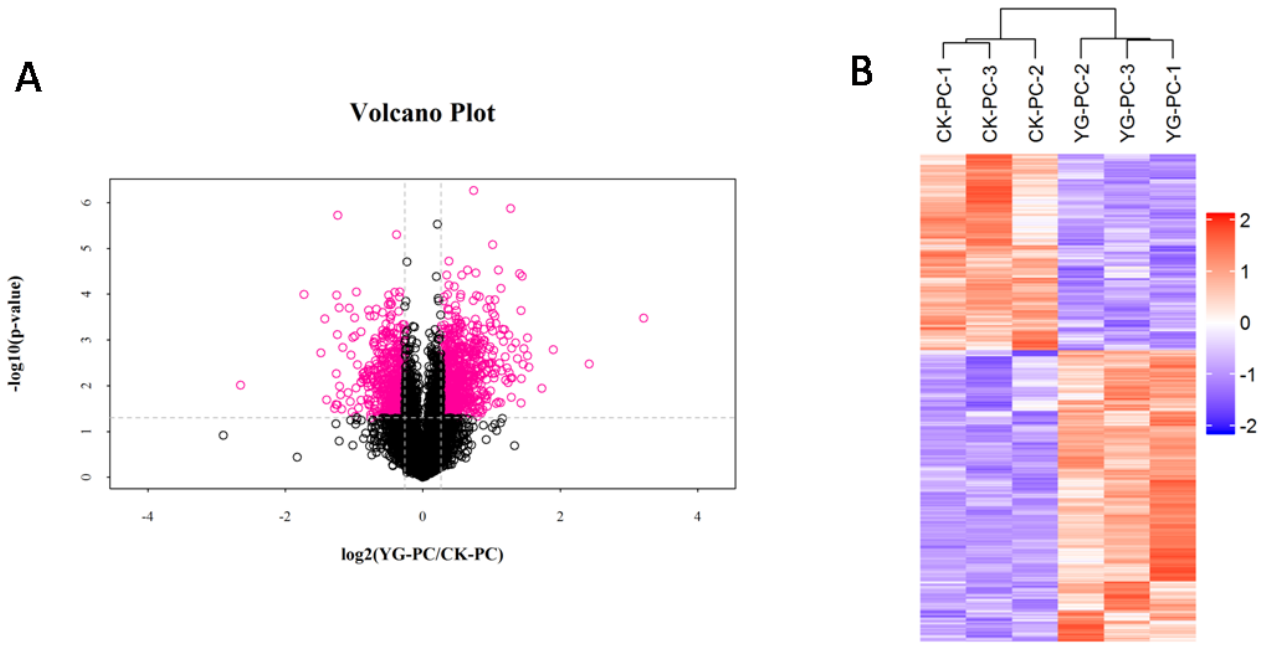

Figure 3

Analysis of the differentially expressed phosphopeptides from phosphoproteomic profiling.(A)Volcano plot of all phosphopeptides identified and quantified in the PS-S and control group. Phosphopeptides with significantly increased or decreased (±1.2(logarithm to base 2)-fold change(abscissa), p value(ordinate)< 0.05) abundance in prefrontal cortex of PS-S group compared to control are colored red.Black dots indicate no significant discrepancy. (B) Heat map generated by hierarchical clustering of differentially expressed phosphopeptides in PS-S and control group. Each column represents a sample; each row represents aphosphopeptide.The heat map shows the 2-logarithm value of the expression of significantly differentially expressed phosphopeptides in each samples. Blue color indicated significantly decrease of the phosphorylation status, red color indicated significantly increase of the phosphorylation status, and grey color depicted no quantitative information.

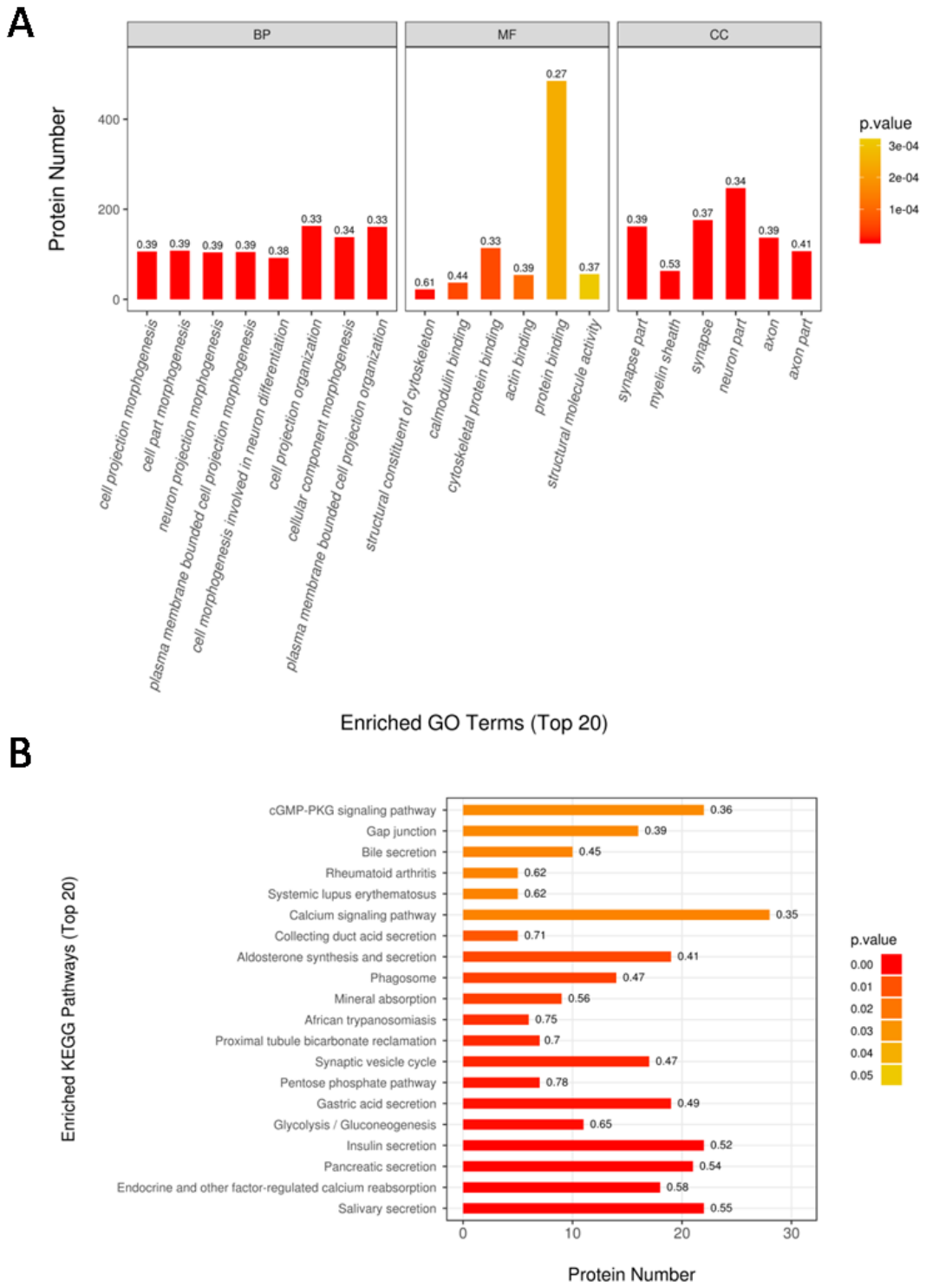

Figure 4

GO and KEGG enrichment analyses of the differentially expressed phosphoproteins. (A) Gene ontology (GO) classification of differentially phosphorylated proteins. The GO terms are classified into three main categories: biological process, cellular component, and molecular function. The ordinate represents the number of enriched differentially expressed phosphopeptides in each KEGG pathway. Colors of the bar graph represent the p-value (closer to red indicate lower p-values). The label above the bar graph shows

rich factor(rich factor<1).(B) Top 20 KEGG signaling pathways of differentially phosphorylated proteins. The abscissa represents significantly enriched KEGG pathway, and the ordinate represents the number of enriched differentially expressed phosphopeptides in each KEGG pathway.

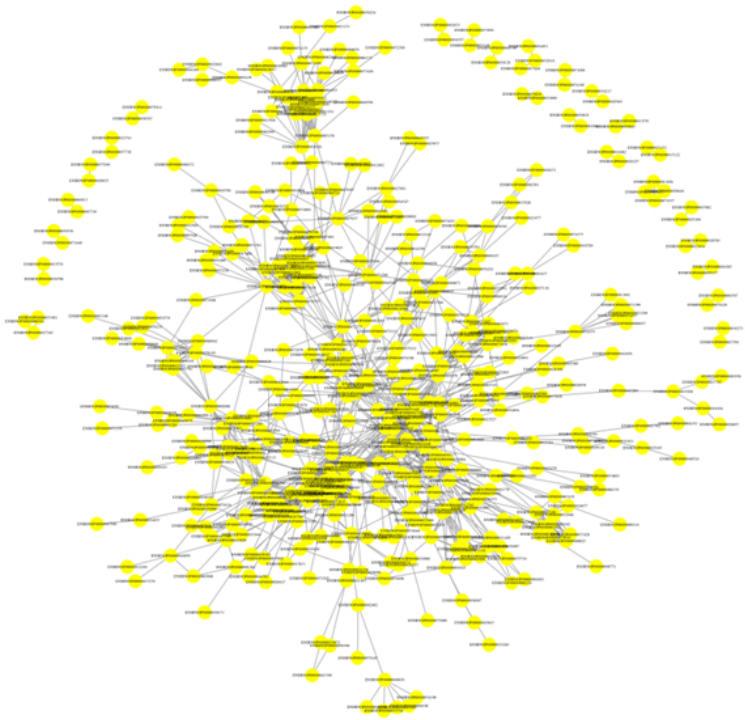

Figure 5

Protein-protein interaction network of differentially expressed phosphopeptides in PS-S and control group.

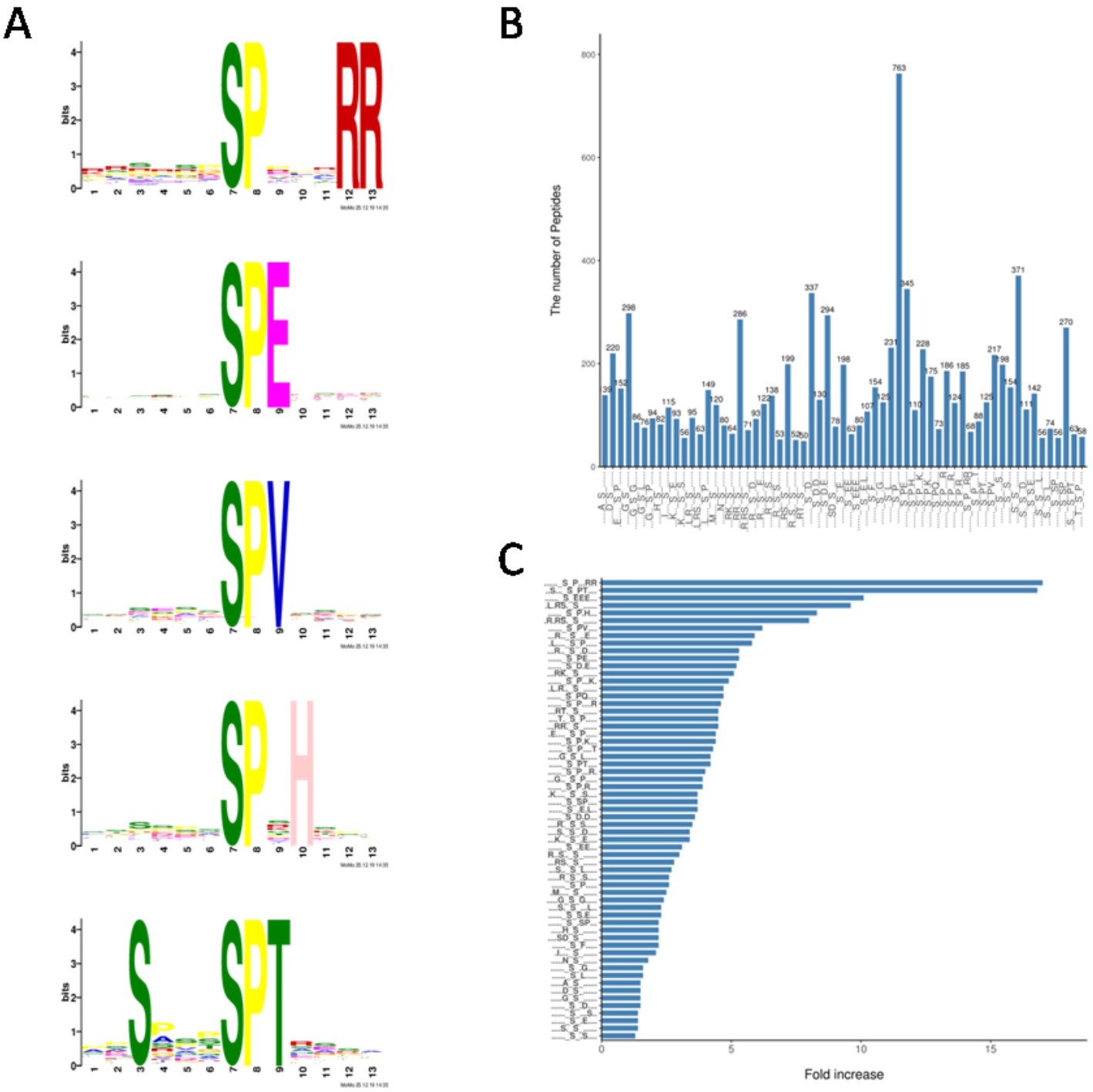

Figure 6

Phosphorylation consensus motif analysis of differentially expressed phosphopeptides in PS-S group. (A) Top five motifs identified from differentially expressed phosphopeptides. The x axis indicates sites, the seventh is the modification site, sites 1-6 and 8-13 represents6probable amino acids up- and downstream from the sites, respectively. Size of amino acid characters corresponds to the frequency of amino acid at that site. (B)The number of differentially expressed phosphopeptides containing specific motifs. Numbers above columns represent the number of specific phosphopeptides. (C)The enrichment analysis of conserved Motif contained in differentially expressed phosphopeptides. The x-axis represents

the ratio of the number of each motif corresponding to identifythe number of the same motif corresponding to theoretical peptides.

Supplementary Files

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