BIOCELL
2023 47(9): 2069-2080

Tech Science Press

Label-free quantitative proteomics analysis models *in vivo* and *in vitro* reveal key proteins and potential roles in sciatic nerve injury

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Key words: Oxygen glucose deprivation, Proteomics, Sciatic nerve injury, Peripheral nerve injury

Abstract: Background: The underlying mechanism of sciatic nerve injury (SNI) is a common motor functional disorder, necessitates further research. **Methods:** A rat model of SNI was established, with the injury group subjected to compressive injury of the right sciatic nerve exposed at the midpoint of the thigh and the sham surgery group undergoing the same surgical procedure. An oxygen-glucose deprivation model was employed to simulate *in vitro* SNI in PC12 cells. Following data acquisition and quality control, differentially expressed proteins (DEPs) in each model were identified through differential analysis, and enrichment analysis was used to explore the potential functions and pathways of the DEPs. Venn diagrams were drawn, and DEPs from both *in vivo* and *in vitro* SNI models were imported into the STRING database to construct a protein-protein interaction network and screen for hub proteins. **Results:** After the peptide segments obtained from rat nerve blockade and PC12 cells met quality requirements, 258 DEPs were identified in rat nerve samples, and 119 DEPs were screened in PC12 cells. Enrichment analysis revealed that DEPs in the rat model were predominantly concentrated in biological functions such as myogenic cell proliferation and signaling related to lipid and energy metabolism. DEPs in the *in vitro* model were mainly enriched in biological processes such as phagocytosis and were associated with lipid transport and metabolism. Two hub proteins, amyloid precursor protein (APP) and fibronectin 1 (FN1), were identified through MCC, MCODE, and Degree scoring. Both PC12 cells and external validation sets showed relatively higher expression of APP and FN1 in injured samples. Results of gene set enrichment analysis indicated that these two proteins were associated with metabolic pathways, such as biosynthesis of glycosaminoglycan chondroitin sulfate and biosynthesis of unsaturated fatty acids. **Conclusion:** APP and FN1 are potential key molecules involved in SNI and are associated with various metabolic pathways in nerve repair. These findings provide a theoretical basis for the development of therapeutic targets for SNI.

Introduction

Authors are encouraged to use the Microsoft Word template when preparing the final version of their manuscripts. In introduction, authors should provide a context or background for the study (that is, the nature of the problem and its significance). State the specific purpose or research objective of, or hypothesis tested by, the study or observation. Cite only directly pertinent references, and do not include data or conclusions from the work being reported.

Peripheral nerve injury (PNI) is a common clinical condition (Kamble *et al.*, 2019) that occurs due to the fragility of peripheral nerves, which are susceptible to sensory and motor nerve abnormalities resulting from acute trauma or chronic disease; in severe cases, it can lead to severe pain, severe disruption to life, and even permanent disability (Bray and Huggett, 2016; Hussain *et al.*, 2020). Due to the complexity of the extent and type of injury and the interaction between the microenvironment of the injury, nerve damage is often difficult to treat and the long healing period is often accompanied by complications that erode the

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#Yang Gu and Mingguang Bi made the equal contribution to this study and should be listed co-first authors
Received: 17 March 2023; Accepted: 27 April 2023;
Published: 28 September 2023

Doi: 10.32604/biocell.2023.029989

physical and mental health of the patient; besides, there is still a lack of perfect treatment to allow full recovery of nerve function (Arthur-Farraj and Coleman, 2021; Menorca *et al.*, 2013).

Among these, sciatic nerve injury (SNI) is a common type of PNI, and in the past, PNI models were often constructed by exposing and stimulating the sciatic nerve (Lee *et al.*, 2020; Yu *et al.*, 2023). SNI is usually accompanied by extensive soft tissue and skeletal injury, presenting significant nerve damage with slow recovery (Craig *et al.*, 2023; Ferdinand and Raduma, 2019; Jones *et al.*, 2018). Despite great progress in nerve repair research over the past few years, clinical outcomes are still unsatisfactory (Garg *et al.*, 2021; Stavrakakis *et al.*, 2022). If the damaged nerve is not repaired in time, it may even involve the muscle, leading to irreversible loss of muscle function (Sayad-Fathi *et al.*, 2019; Yue *et al.*, 2019). Therefore, uncovering the molecular mechanism of SNI is of crucial importance to facilitate the research and development of new drugs for nerve repair.

With the advancement of biotechnology, researchers have gained a deeper understanding of neural injuries. For example, microarray and RNA sequencing technologies have emerged in elucidating transcriptional changes in neural injury (Brosius Lutz *et al.*, 2022; Sun *et al.*, 2020a; Yao *et al.*, 2020). In this study, we used both *in vivo* and *in vitro* models to simulate SNI, constructing a rat SNI model through compressive injury to investigate the repair and functional recovery after neural injury and to explore the possibility that neural injury may deprive certain functions of neural innervation areas, leading to motor and sensory dysfunction. PC12 is a rat adrenal chromaffin cell line that is commonly used to study neural cell differentiation, regeneration, and neuron growth (Wiatrak *et al.*, 2020). One of the common neural cell injury models is the oxygen-glucose deprivation (OGD) model of PC12 cells (Bork *et al.*, 2015; Wang *et al.*, 2022b). Both *in vivo* and *in vitro* model samples were used. In this study, we used unlabeled quantitative proteomics to explore key proteins in sciatic nerve injury at the protein level. Through this approach, we hope to identify potential molecular targets and signaling pathways to promote neural injury repair and provide a basis for novel treatment strategies.

Materials and Methods

Establishment of nerve injury models of rats

All rats involved in experiments were acquired from the Institute of Zoology, Chinese Academy of Sciences (Shanghai, China), and all assays were done with permission from the Laboratory Animal Ethics Committee of Ningbo University with approval No. 11837. These adopted rats were eight-week-old male SD rats (200–220 g). Before the assay, the rats were raised at 23°C–25°C for at least 7 days under alternative day and night (12 h/12 h) and were allowed to have water and food freely. After a week of adaptive feeding, the 6 rats were assigned to an injury group (n = 3) or a sham operation group (n = 3). The rats were anesthetized through intraperitoneal injection of 10% chloral hydrate (3.5 mL/kg, aladdin, CellMark Biotech Ltd., Shenzhen, China). After skin preparation, the right sciatic nerve was horizontally exposed at the middle thigh through blunt dissection of the biceps femoris (Fig. 1A). At the proximal end (about 7 mm) of the trigeminal orifice of the sciatic nerve, the hemostatic forceps of the two blood vessels were placed about 2 mm apart to form moderate compression injury (Fig. 1B). After the suture of muscle and skin, cefazolin sodium (50 mg/kg, Meryer, Meryer Biochemical Technology, Ltd., Shanghai, China) was injected intravenously for seven days. The sham operation group received the same operation procedure, but the sciatic nerve was not compressed. After 7 days, the compressed nerve was acquired (Fig. 1C) and its pathological index was evaluated (Bennett and Xie, 1988).

Cell culture and establishment of oxygen-glucose deprivation models

PC12 cells were subjected to incubation (37°C, 5% CO_2) in RPMI-1640 complete culture medium (Solarbio, Solarbio Technology, Ltd., Beijing, China) with 5% fetal bovine serum (FBS), 10% equine serum (ES), and 1% double-antibody. The medium was refreshed every 2 days, and the passage was made every 3–4 days. Before the establishment of OGD models, first, 0.01% sterile poly-L-lysine (PLL) solution (Macklin, Trinity Biotechnology, Ltd., Guangzhou, China) was dripped onto the surface of the Petri dish until it completely covered the surface and then let to stand still at indoor temperature for 1 h. PLL solution was removed and recycled, and the Petri dish was placed in an incubator at 37°C for drying overnight. The plate was washed with sterile deionized water three times before planting. Then, PC12 cells was digested and removed, followed by counting. Then the PC12 cells were seeded in a 6-cm culture dish coated with PLL (1 × 106 cells per dish). After 24 h, the cells adhered to the wall. The medium was replaced with a complete culture medium having nerve growth factor (NGF) (Procell, Procell Life Sciences, Ltd., Wuhan, China) with a final concentration of 50 ng/mL, 10%FBS and 1% double-antibody. The medium was replaced every 2 days until the length of the nerve axon greatly increased on the 5th day, and the subsequent treatment was carried out at the end of induced differentiation. To build an OGD model, the air Inlet of the three-gas incubator (anaerobic workstation) was connected with the mixed gas containing 95% N_2, 4% CO_2, and 1% O_2, respectively, and the mixed gas was continuously ventilated to drain the air in the box for use. The culture medium of differentiated PC12 cells was absorbed, and the cells were cleaned gently through PBS 3 times, followed by the addition of a proper amount of glucose-free Hank's Balanced Salt Solutions (HBSS) medium (Gibco. Visions Biotechnology, Ltd., Guangzhou, China), and then the cells were transferred to the preset hypoxia three-gas incubator (Kang Heng, R&B Technology Development, Ltd., Beijing, China). The control group was subjected to incubation in the complete medium to induce differentiation under normal conditions. Cells were collected after 2 h of continuous hypoxia and sugar deficiency

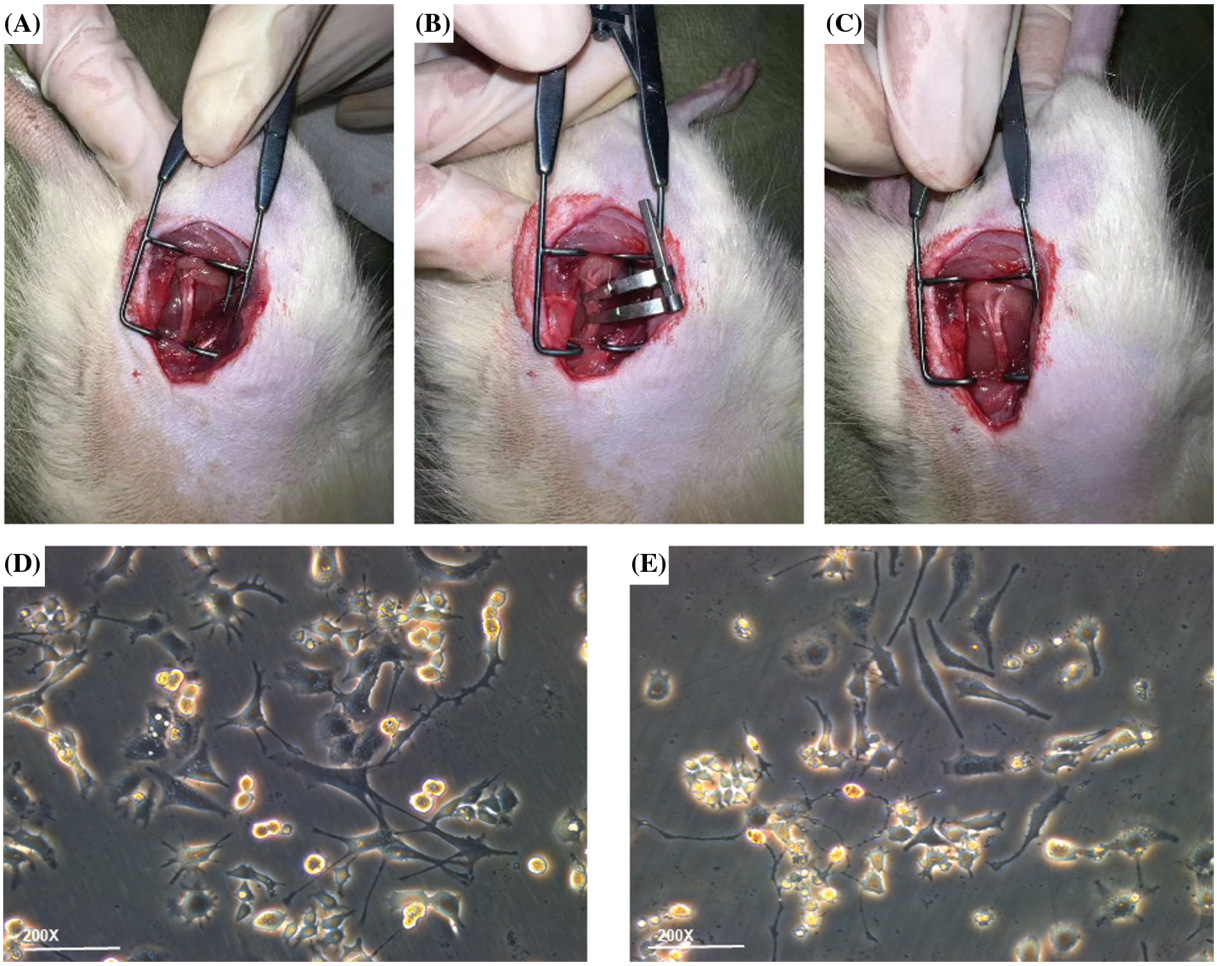

FIGURE 1. Establishment of *in vitro* and *in vivo* experimental models. (A) Sciatic nerve exposure, (B) sciatic nerve injury modeling, (C) edema of the sciatic nerve after being compressed. Compared to (D) untreated PC12 cell group, the (E) PC12 group after oxygen-glucose deprivation injury showed more swollen cells in the visual field; some cell protrusions appeared discontinuous and shortened in length, and there was less intertwining of intercellular protrusions and more cellular debris floating in the medium.

(Fig. 1E), respectively, for follow-up experiments. The control group consisted of the same batch of differentiated PC12 cells there were not treated for OGD modeling (Fig. 1D).

Label-free quantitative proteomics analysis

Extraction of proteins

The samples were taken out from −80°C refrigerators (AS-ONE, Myriad Biochemical Technology, Ltd., Shanghai, China), placed in one mortar precooled by liquid nitrogen, and ground to powder. Each group was added with lysis buffer with 4 times the volume of power (8 M urea, 1% protease inhibitor) and cracked ultrasonically, followed by 10-min centrifugation (4°C, 12000 g) to eliminate cell debris. The supernatant was then placed in one new centrifuge tube, followed by the determination of protein concentration by BCA kit (Beyotime, Beyotime Biotechnology, Shanghai, China). An equal amount of every sample protein was adopted for enzymolysis, and the volume was changed to be consistent with the lysate. The protein was added with TCA with a final concentration of 20%, followed by vortex mixing and 2-h precipitation (4°C), and then by 5-min centrifugation (4500 g) to remove the supernatant. The precipitate was cleaned through precooled acetone 2–3 times. After drying, the precipitate was added with TEAB (ThermoFisher, Sui-Ho Biotechnology Co., Ltd., Guangzhou, China) with the final concentration of 200 mM and then broken by ultrasonic waves (Ningbo Xinzhi,Gert Biotechnology Co., Zhuhai, China), followed by the addition of trypsin (Beyotime, Beyotime Biotechnology, Shanghai, China) at 1:50 (protease: protein, m/m) and enzymolysis all night long. Subsequently, it was put with dithiothreitol (DTT, Merck millipore, Sigma Aldrich (Shanghai) Trading Co., Shanghai, China) to change its final concentration to 5 mM, followed by a 30-min reduction (56°C). After that, iodoacetamide (IAA, Macklin, Trinity Biotechnology, Ltd., Guangzhou, China) was added to change its final concentration to 11 mM, followed by 15-min incubation (room temperature) with dark surroundings.

Liquid chromatography-mass spectrometry analysis

The peptide was dissolved by mobile phase A in liquid chromatography and then isolated by mobile phase system in EASY-nLC 1200 liquid chromatographer (ThermoFisher, Sui-Ho Biotechnology Co., Ltd., Guangzhou, China). The mobile phase A was an aqueous solution with 0.1% formic acid as well as 2% acetonitrile, and the mobile phase B was a solution with 0.1% formic acid as well as 100% acetonitrile. Liquid phase gradient setting: 0–68 min,

6%–23%B; 68–82 min, 23%–35%B; 82–86 min, 35%–80%B; 86–90 min, 80%B. The flow rate was kept at 550 nL/min. The peptides were isolated via ultra-high-performance liquid chromatography, followed by injection into the Capillary ion source for ionization and analysis through None mass spectrometry. The voltage of the ion source was set at 1.6 kV, and the parent ion of the peptide and its secondary fragments were determined and evaluated through high-resolution time-of-flight mass spectrometry. The scanning range of secondary mass spectrometry was set to 400–1200 m/z. The data acquisition mode was the parallel accumulation-serial fragmentation (PASEF) mode. One secondary spectrogram with the charge number of the parent ion within 0–5 was acquired through 10 times of PASEF mode after the first-order mass spectrometry was collected, and the dynamic exclusion time of tandem mass spectrum scanning was set to 20 s to avoid repeated scanning.

Selection of differentially expressed proteins (DEPs)

The reproducibility of protein quantification was assessed using principal component analysis (PCA) and relative standard deviation (RSD) methods. Fold Change (FC) was calculated between groups. After log2 transformation, the protein differences were calculated using the following formula:

$$P_{sample*protein} = T.test\left(\log_2 p_{sample*protein}, sample \in Group1\right), \log_2 p_{sample*protein}, sample \in Group$$

The screening criteria were set as $p \leq 0.05$, with FC > 1.5.

Enrichment analysis

The R package "clusterProfiler" was used to perform enrichment analysis of DEPs at three levels: Gene Ontology (GO), Kyoto Encyclopedia of Genes and Genomes (KEGG), and protein domain, to identify the enrichment trends of these proteins in terms of function, pathway, or structure. The R package ggplot2 was used for visualization. GSEA v4.3.2 software was used to perform single sample gene set enrichment analysis (ss GSEA) on hub molecules according to c2.cp.kegg.v2022.1.Hs.symbols.gmt.

Construction of protein-protein interaction (PPI) network

Venn diagrams were drawn based on DEPs, and the PPI network of DEPs was constructed using the STRING database. Cytoscape and Metascape were employed to analyze and visualize the PPI network. Utilizing the CytoHubba plugin, assign values to each node and calculate their attributes.

Dataset download and statistical analysis

Expression profiles from the SNI rat model (GSE24982, GSE63442) and the GSE150408 dataset of human SNI were selected and downloaded via Gene Expression Omnibus (GEO). GraphPad Prism 9.0 was used for data calculation and visualization. Quantitative data were analyzed using an independent samples *t*-test with a significance threshold set at $p < 0.05$.

Results

Sample collection and analysis of differentially expressed proteins

Peptide segments retrieved from the samples in the database (Figs. 2A and 2F) were analyzed for their length (Figs. 2B and 2G), quantity (Figs. 2C and 2H), protein coverage (Figs. 2D and 2I), and distribution of protein molecular weight (Figs. 2E and 2J) to ensure that the peptide segments obtained from both rat and PC12 cell samples met the quality standards.

Sciatic nerve samples of rats were collected from the injury group and the Sham group. PCA was performed to evaluate whether the biological repeatability within the group was consistent with the statistical consistency. The results revealed good repeatability of samples (Figs. 3A and 3B). Similarly, the PCA results of PC12 cells in the control and OGD groups also revealed good repeatability of samples (Figs. 2D and 2F).

Then, the DEPs of sciatic nerve samples of rats and PC12 cell samples were analyzed, and volcano plots and heat maps (orange represents proteins whose expression was up-regulated in the injury group/OGD group, and green represents proteins with down-regulated expression in the injury group/OGD group). Totally 258 DEPs were identified in sciatic nerve samples of rats, including 141 up-regulated proteins and 117 down-regulated ones (Figs. 3C and 3D). Totally 119 DEPs were identified in PC12 cells, including 29 up-regulated proteins and 90 down-regulated ones (Figs. 3G and 3H).

Function of differentially expressed proteins and pathway enrichment

The GO enrichment analysis showed the enrichment of DEPs in biological processes (BP), molecular functions (MF), and cellular components (CC) for both rat sciatic nerve samples and PC12 cells. In the edematous rat nerve samples after compression, DEPs were widely involved in BPs such as assembly of the spine apparatus, regulation of myogenic cell proliferation, and production of interleukin-8 (Fig. 4A), associated with CCs like endoplasmic reticulum lumen and MHC class I (Fig. 4B), and significantly enriched in MFs such as titin Z domain binding and retinol oxidase activity (Fig. 4C). In OGD-induced PC12 cells, DEPs repeatedly appeared in BPs like vitamin D metabolic processes, recognition, and response to BMP (Fig. 4F), CCs like layer-adhering protein-11 complex (Fig. 4G), and MFs like vitamin D metabolic processes and phagocytosis (Fig. 4H).

KEGG analysis revealed that DEPs in rat sciatic nerve samples were mainly enriched in pathways including graft-*vs*.-host disease, type I diabetes, autoimmune thyroid disease, allograft rejection, and IgA production in the intestinal immune network (Fig. 4D). DEPs in PC12 cells were mainly enriched in pathways like the biosynthesis of glycosphingolipids - globo and isoglobo series, extracellular matrix (ECM)-receptor interaction, other glycolysis, and biosynthesis of unsaturated fatty acids (Fig. 4I).

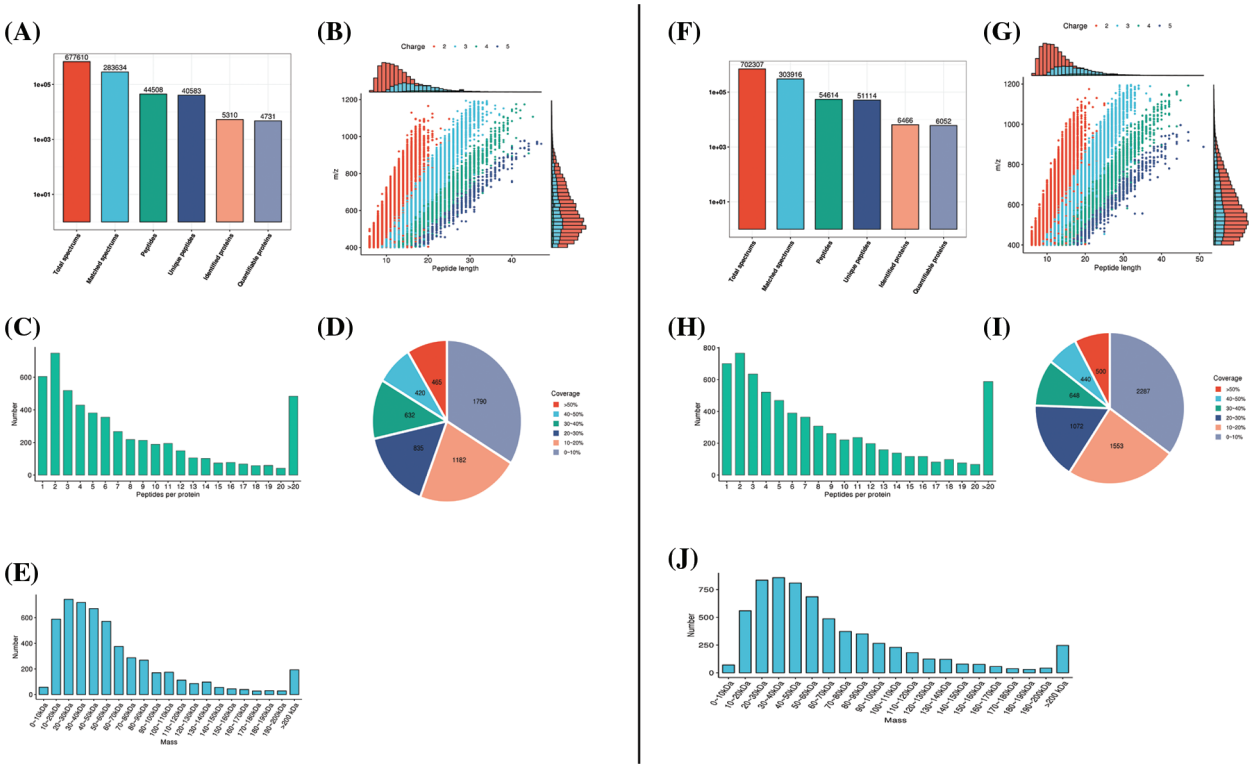

FIGURE 2. Mass spectrometry data were subjected to database retrieval and quality control analysis. In the rat sciatic nerve samples, (A) protein identification, (B) peptide length, (C) quantity analysis, (D) protein coverage, and (E) molecular weight distribution analysis was conducted. The right panels (F–J) represent the corresponding database and quality control analyses in PC12 cells.

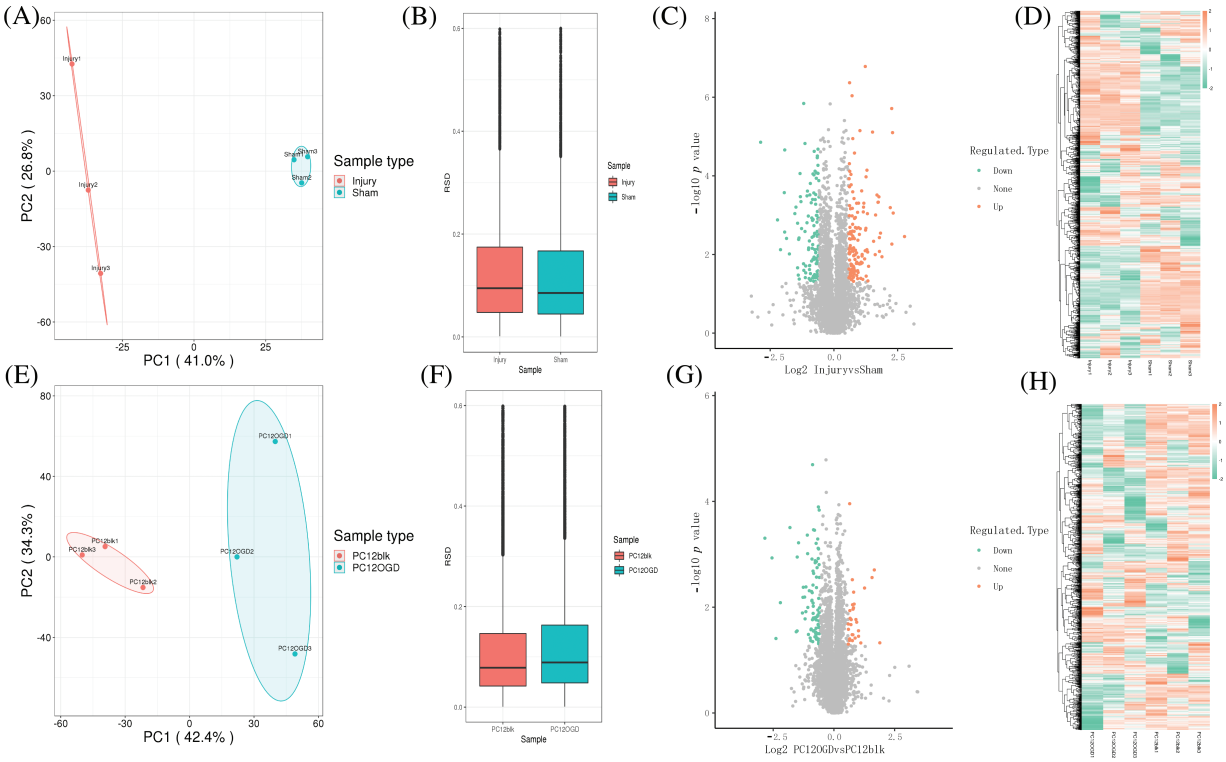

FIGURE 3. Sample collection and analysis of differentially expressed proteins (DEPs). (A) Principal component analysis (PCA) and (B) relative standard deviation (RSD) of rat sciatic nerve samples, volcano plot (C), and heat map (D) for differential expression analysis; (E) PCA, (F) RSD of PC12 cell samples, volcano plot (G), heat map, and (H) for differential expression analysis.

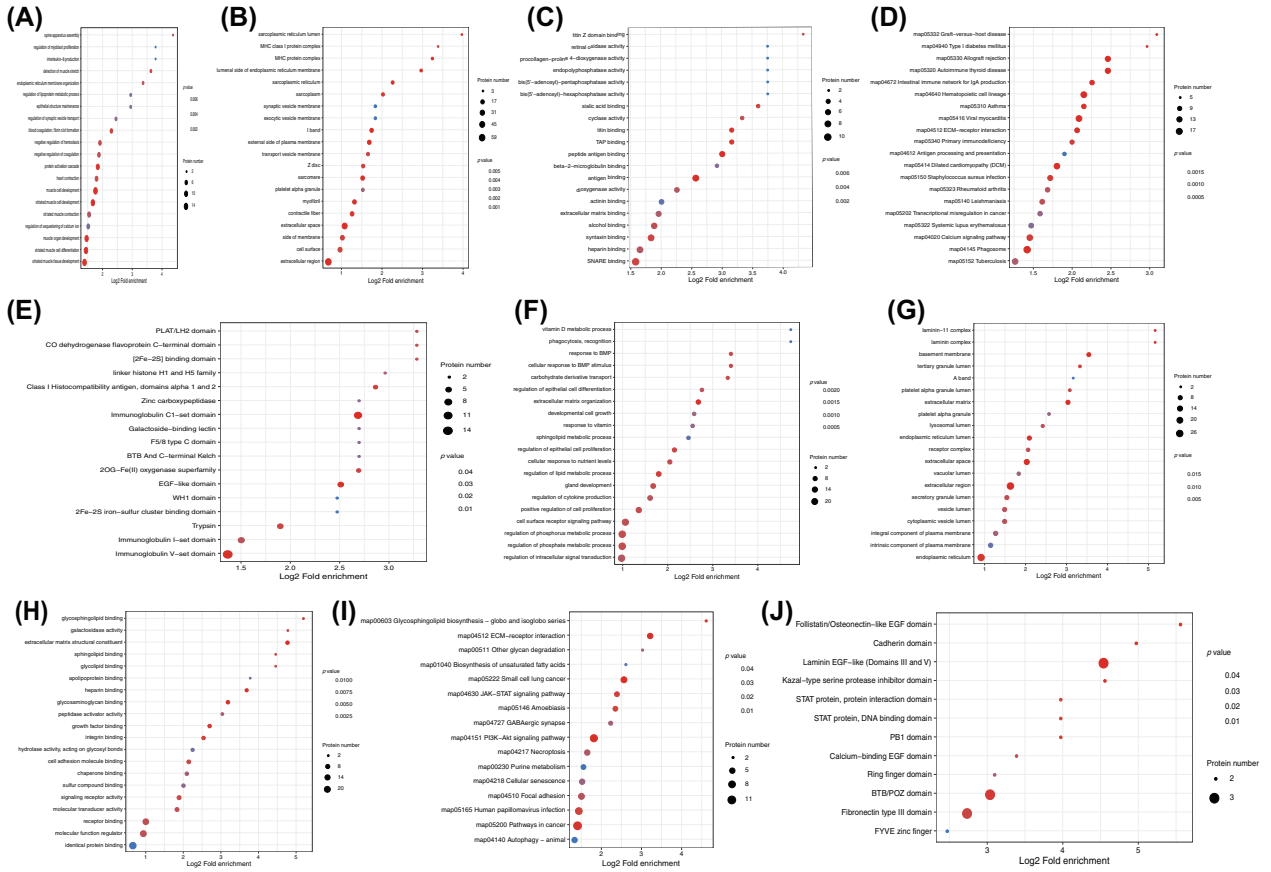

FIGURE 4. Functional and pathway enrichment of differentially expressed proteins (DEPs). Enrichment analysis of DEPs in rat sciatic nerve samples for (A) biological processes (BP), (B) cellular components (CC), (C) molecular functions (MF), as well as (D) Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis, and (E) protein-protein domain analysis. Enrichment analysis results for PC12 cells are shown in (F) BP, (G) CC, (H) MF, (I) KEGG, and (J) domain analysis.

DEPs in rat sciatic nerve samples were repeatedly enriched in domains such as PLAT/LH2, CO dehydrogenase flavoprotein C-terminal domain, and [2F–2S] binding domain (Fig. 4E). In PC12 cells, DEPs were mainly significantly enriched in domains like PLAT/LH2, CO dehydrogenase flavoprotein C-terminal domain, and [2F–2S] binding domain (Fig. 4J).

Prediction of hub proteins based on protein-protein interaction network

With the Venn diagram, the DEPs in sciatic nerve samples of rats and PC12 cells were combined. After the removal of three duplicate proteins, 374 DEPs were obtained (Fig. 5A). Based on the STRING database, a PPI network consisting of 374 proteins is established and is visually displayed through Metascape (Fig. 5B). A total of 13 sub-modules are identified (Fig. 5C). The functions and pathways of the individual modules are detailed in Table 1 and are highly correlated with ECM receptor interactions in MCODE 1. Genes in MCODE1 are screened by calculating the attributes of each node, with the top ten hub proteins based on MCC and Degree rankings, leaving only two proteins: amyloid precursor protein (APP) and fibronectin 1 (FN1) (Fig. 5D). Further, the expression of APP and FN1 in external datasets was determined by comparing the expression of APP and FN1 in the online database of sciatic nerve-related datasets (Fig. 6).

The potential functions of hub proteins

To further explore the pathways involved with hub proteins, we conducted GSEA enrichment analysis on two hub proteins in the GSE150408 dataset. The results revealed that in the low expression group of APP, biosynthesis of glycosaminoglycan chondroitin sulfate, lysosomes, and Fc Gamma R mediated phagocytosis were significantly enriched ($p < 0.05$). In contrast, FN1 was associated with the biosynthesis of unsaturated fatty acids, glycosaminoglycan biosynthesis of keratan sulfate, and the pentose phosphate pathway ($p < 0.05$). For more details, see Fig. 7.

Discussion

Acute mechanical and physical injury, chronic nerve compression, or nerve degeneration can all result in injury to the peripheral nerve, leading to functional impairment or neuropathic pain (Wang *et al.*, 2022a; Wang *et al.*, 2023). In contrast to the central nerve, the axons of injured peripheral nerves already have a remarkable capacity for regeneration (Gaudet *et al.*, 2011), although nerve regeneration involves a large pathophysiological network that requires the coordination of damaged parts and distal neurons, and the interaction between various growth factors, cytokines, inflammatory factors, etc. (Vijayavenkataraman, 2020). The complex inflammatory response to injury and the proliferation of microglia often impede nerve regeneration

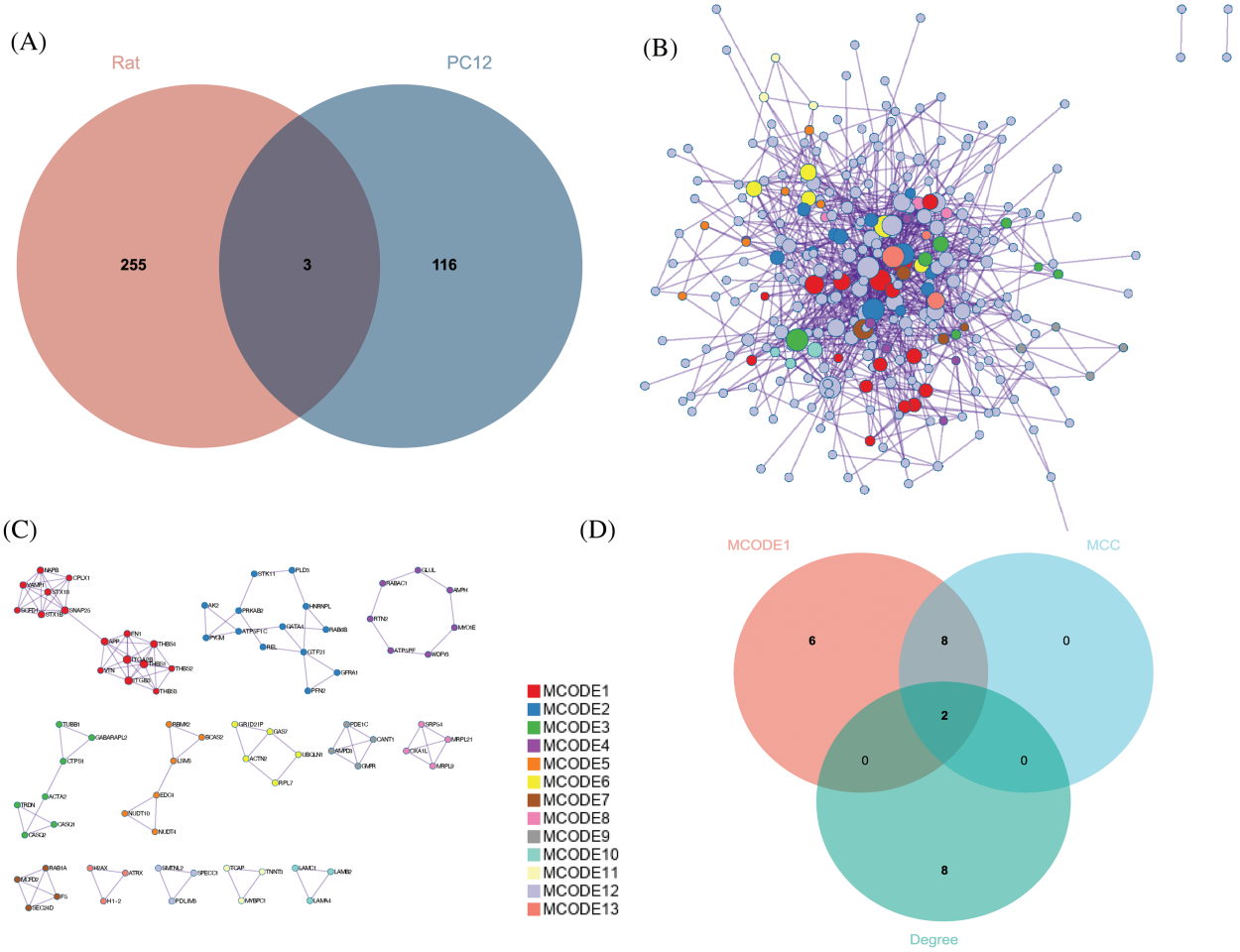

FIGURE 5. Identification of hub proteins. (A) Overlap of the two model differentially expressed proteins (DEPs), (B) Protein-protein interaction network (PPI) consisting of 347 DEPs, (C) 13 identified MCODE components, and (D) screening of hub proteins.

in SNIs (Molnár *et al.*, 2022), and the different trajectories followed by various cytokines after injury (Yuan *et al.*, 2021), make the outcome of nerve repair but the results are not encouraging. Repair of peripheral nerves takes months to years, and in some cases, cannot even be achieved (DeLeonibus *et al.*, 2021; Sulaiman and Gordon, 2013), especially in patients with SNI or shoulder brachial plexus injuries, where the prognosis is often poor. This is due to the long distances required for growth and repair, during which time the muscle atrophies and degenerates due to a prolonged lack of innervation, leading to irreversible loss of function. Accelerating nerve repair is an effective way to improve and prevent peripheral muscle atrophy (Gaudet *et al.*, 2011; Jiang *et al.*, 2020). However, the molecular mechanisms underlying nerve injury repair have not yet been explained, so uncovering the molecular mechanisms of SNI has become an important prerequisite for translational research and the development of new drugs to promote nerve recovery.

Analysis of the differences in translational and post-translational modifications in the nervous system that occur after injury by SNI can help identify key biological markers of SNI that can be used as major molecular targets for drug therapy and improve the efficacy of SNI treatment (Niederberger and Geisslinger, 2008). A number of studies have been conducted using different animal models to simulate the onset of SNI and for the triggers of SNI (e.g., viruses (Wang *et al.*, 2022c)), therapeutic agents (Chen *et al.*, 2022b; Xiong *et al.*, 2022), temporal changes (Ujcikova *et al.*, 2022; Vergara *et al.*, 2018). In contrast, the current study constructed *ex vivo* models of SNI using compressive injury and OGD induction and explored DEPs in SNI that suffered an injury based on label-free quantitative proteomics extraction and identification of proteins.

Enrichment analysis revealed the involvement of the DEPs from these rat models in the regulation of myogenic cell proliferation and interleukin-8 production, suggesting differences in muscle regeneration and inflammatory responses in rats after injury (Dort *et al.*, 2021; Jungen *et al.*, 2019) and that the search for molecular markers targeting myogenic cells and inflammatory factors may be able to promote muscle regeneration after SNI injury and ameliorate inflammation-induced sciatica. Vitamin D is known to have significant positive effects on nerve regeneration (El Soury *et al.*, 2021) and can significantly reduce neuropathic pain caused by sciatic nerve constriction in rats (Poisbeau *et al.*, 2019) and that DEPs in PC12 cells are significantly enriched in vitamin D metabolic processes and ECM-receptor interaction-related terms, suggesting that these DEPs may be key molecules in repairing nerve damage and reducing neuroinflammation (Hart and Karimi-Abdolrezaee, 2020; Kalinski *et al.*, 2020).

TABLE 1

For each MCODE component, the top three *p*-value scores are obtained following enrichment analysis of the respective terms

MCODE	GO	Description	Log_{10} (P)
MCODE_1	hsa04512	ECM-receptor interaction	−16.3
	WP306	Focal adhesion	−13.4
	hsa04510	Focal adhesion	−13.4
MCODE_2	hsa04530	Tight junction	−4.3
	GO:0048608	Reproductive structure development	−3.6
	GO:0061458	Reproductive system development	−3.6
MCODE_3	GO:0010880	Regulation of release of sequestered calcium ion into cytosol by sarcoplasmic reticulum	−7.5
	R-HSA-397014	Muscle contraction	−7.1
	R-HSA-5578775	Ion homeostasis	−6.7
MCODE_5	GO:0034655	Nucleobase-containing compound catabolic process	−7
	GO:0046700	Heterocycle catabolic process	−6.7
	GO:0044270	Cellular nitrogen compound catabolic process	−6.7
MCODE_7	R-HSA-204005	COPII-mediated vesicle transport	−10.6
	R-HSA-199977	ER to Golgi Anterograde Transport	−9.2
	R-HSA-948021	Transport to the Golgi and subsequent modification	−8.9
MCODE_8	R-HSA-72766	Translation	−8.1
	R-HSA-5389840	Mitochondrial translation elongation	−7
	R-HSA-5368286	Mitochondrial translation initiation	−7
MCODE_9	hsa00230	Purine metabolism	−9.5
	hsa01232	Nucleotide metabolism	−7.1
MCODE_10	R-HSA-8874081	MET activates PTK2 signaling	−9.1
	R-HSA-3000157	Laminin interactions	−9.1
	M5887	NABA BASEMENT MEMBRANES	−8.7
MCODE_11	R-HSA-390522	Striated Muscle Contraction	−8.8
	WP383	Striated muscle contraction pathway	−8.7
	R-HSA-397014	Muscle contraction	−6.5
MCODE_12	GO:0030036	Actin cytoskeleton organization	−5.3
	GO:0030029	Actin filament-based process	−5.1
MCODE_13	GO:0006334	Nucleosome assembly	−7.2
	GO:0034728	Nucleosome organization	−7
	GO:0065004	Protein-DNA complex assembly	−6.4

After further screening, APP and FN1 were identified as hub proteins, and both showed high expression in models of sciatic nerve injury. APP is a common transmembrane protein localized to chromosome 21 and is widely expressed in axons and dendrites, contributing to neurite growth and synaptogenesis (Hunter *et al.*, 2003; Kotulska *et al.*, 2010; Reinhard *et al.*, 2005). Also, APP is an important regulator in myelin formation and has now been identified as a key protein in neural development and neural repair processes after injury (Truong *et al.*, 2019). After PNI, APP expression was found to increase in the dorsal root ganglion and does not return to baseline levels until the nerve is reinnervated (Scott *et al.*, 1991); therefore, overexpressed APP is also considered one of the markers to identify nerve injury (Dzreyan *et al.*, 2021). This is consistent with our findings that APP can be used as a biological marker of SNI injury. In addition, low expression of APP seems to be associated with lysosomal activation. Lysosomal cytosolic action in Schwann cells contributes to axonal myelin regeneration and that regenerating sciatic nerve myelin regeneration is blocked in the presence of lysosomal dysfunction (Chen *et al.*, 2012). Most studies have also shown that lysosomal activation is an important target for the treatment of many inflammatory and neurological pathologies (Bonam *et al.*, 2019; Plotegher and Duchen, 2017). We speculate that APP inhibits lysosomal function and that targeted inhibition of APP may be able to mediate lysosomal action and promote myelin regeneration.

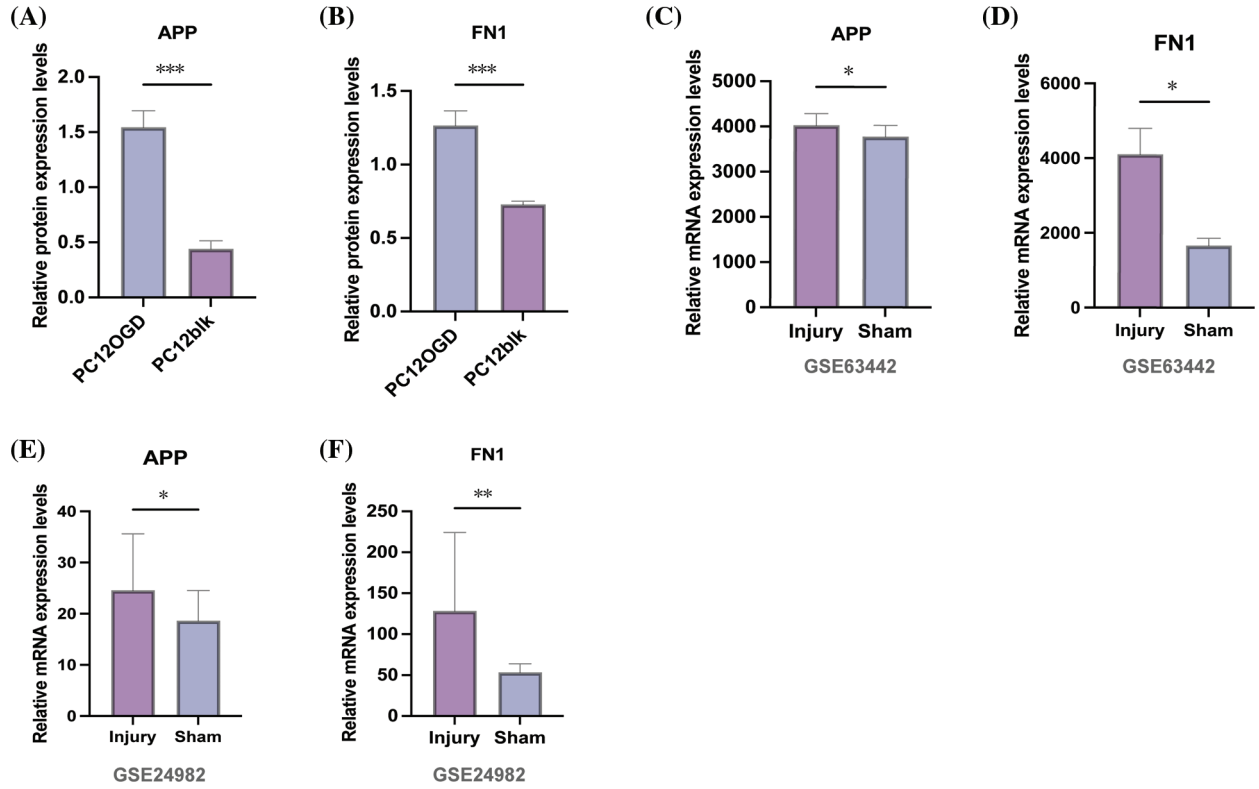

FIGURE 6. Amyloid precursor protein (APP) APP and fibronectin 1 (FN1) expression levels. (A–B) Relative protein expression of APP and FN1 in oxygen-glucose deprivation-induced and untreated PC12 cells, and APP and FN1 mRNA relative expression in (C–D) GSE63442 dataset and (E–F) GSE24982 dataset for the Injury group and the Sham group, respectively. Note: *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

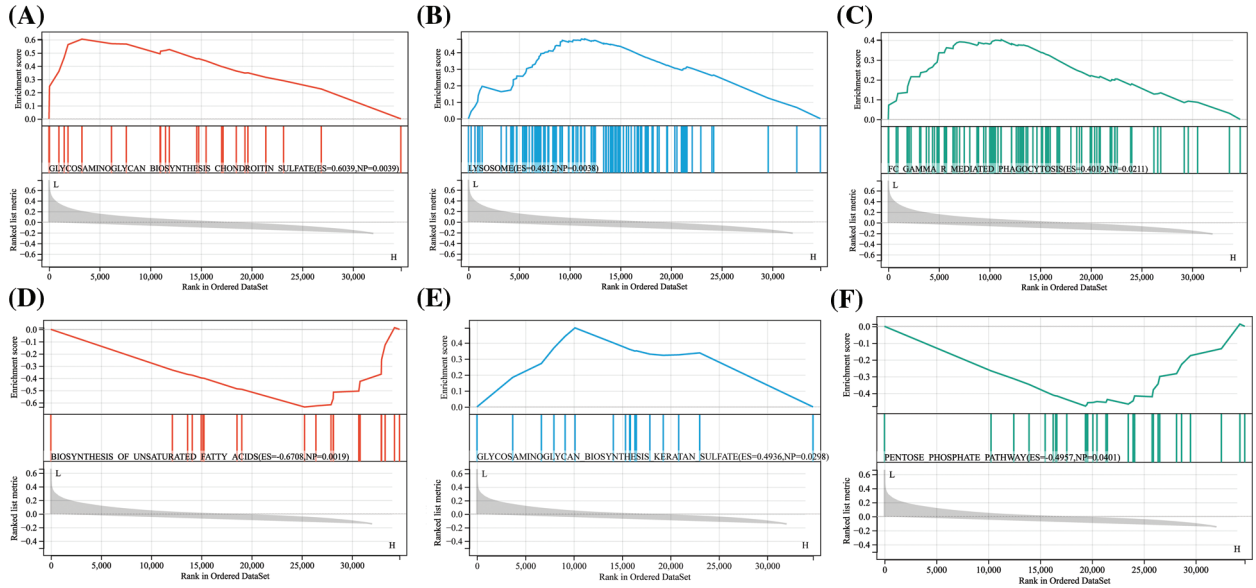

FIGURE 7. Single gene set enrichment analysis (GSEA) results for APP and FN1 APP was enriched in the GSE150408 dataset in (A) biosynthesis of glycosaminoglycan chondroitin sulfate, (B) lysosomes, and (C) Fc gamma R mediated Phagocytosis. fn1 was enriched in the GSE150408 dataset in (D) unsaturated fatty acid biosynthesis, (E) glycosaminoglycan biosynthesis keratin sulfate, and (F) pentose phosphate pathway.

FN1 is a member of the integrin receptor family of receptors that primarily mediates ECM interactions and is associated with cytoskeleton, adhesion, and growth (Li *et al.*, 2019; Pankov and Yamada, 2002; Sun *et al.*, 2020b). FN is also key to repairing nerve injury (Tsuda *et al.*, 2008), where fibronectin deposited in the sciatic nerve after injury alters the composition of the ECM and inhibits Schwann cell migration (Akassoglou *et al.*, 2003). In recent years, it has been shown that Sox2 and FN are co-expressed in a time-dependent manner in pro-regenerative Chevron cells *in vivo* (Torres-Mejía *et al.*, 2020). Even *in vitro*, FN1 expression is shown to increase through increased FN1 expression and

neural recovery through fibrillogenic fibril formation (Chen *et al*., 2022a). Besides, FN1 has been indicated as a key molecular target for axonal regeneration in DRG neurons after SNI (Mao *et al*., 2019). These studies also corroborate our findings that FN1 may be an important target in neural repair after SNI injury.

We acknowledge our study's limitations, which include the absence of western blotting validation for key protein expression and sciatic functional index (SFI) behavioral tests to confirm model construction success. In future research, we plan to address these limitations by conducting western blotting, SFI tests, and additional wet lab experiments to provide a more comprehensive validation of our results, ultimately strengthening the reliability of our findings.

In these studies, we have identified two hub proteins of SNI, namely, APP and FN1, through quantitative proteomics and bioinformatics analysis. Many studies have revealed their critical role in nerve-associated diseases, but the role they play in SNI and whether there is a regulatory relationship between them still need further experimental research.

Acknowledgement: None.

Funding Statement: This research was funded by the Project of 2022 Health Commission Technology Plan of Zhejiang Province (Project Number: 2022KY1169), and the 2022 Ningbo Natural Science Foundation Young Doctoral Project (Project Number: 2022J027).

Author Contributions: The authors confirm contribution to the paper as follows: study conception and design: Yang Gu, Mingguang Bi; data collection: Dehui Chen; analysis and interpretation of results: Ning Ni; draft manuscript preparation: Yang Gu, Mingguang Bi, Jianming Chen. All authors reviewed the results and approved the final version of the manuscript.

Availability of Data and Materials: The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics Approval: All assays were done with permission from the Laboratory Animal Ethics Committee of Ningbo University with approval No. 11837.

Conflicts of Interest: The authors declare that they have no conflict of interest.

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