Downregulation of Nck1 After Spinal Cord Injury in Adult Rats

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Background: Nck1 is an important molecule that participates in many cellular processes, including neurite outgrowth, synaptic plasticity, and apoptosis. However, the expression and function of Nck1 in the spinal cord and spinal cord injury remain unknown.

Aims: To investigate the role of Nck1 in spinal cord injury.

Study Design: Animal experimentation.

Methods: Adult Sprague–Dawley rats were used to establish an acute spinal cord injury model. Double immunofluorescence staining, Western blot, and quantitative reverse transcription polymerase chain reaction analysis were used to investigate the distribution, cellular localization, and expression of Nck1 in spinal cord injury processes. Short interfering RNA was used to silence Nck1 expression in VSC4.1 cells. The Shapiro–Wilk test was used for the normality distribution analysis; the Student's unpaired t-test, 1-way analysis of variance followed by post hoc Tukey's test were used for data analysis. Finally, RNA sequencing technology and gene ontology analysis were used to analyze the changes in Nck1-associated genes expression after spinal cord injury.

Results: Colabeled staining demonstrated that Nck1 was especially distributed in neurons. Western blot, quantitative reverse transcription polymerase chain reaction, and statistical analysis revealed that Nck1 expression reduced to the lowest levels at 1 day after nerve injury, and slowly increased to a stable level in 21 days ($P < .05$). Nck1-specific short interfering RNA transfection significantly reduced cell viability and neurite development in neurons. Bioinformatic analysis indicated that Nck1 participates in multiple pathological processes of spinal cord injury, and many Nck1-associated genes exhibited differential expression levels.

Conclusion: Nck1 is a vital protein in spinal cord injury processes and, therefore, further studies should be conducted to explore its potential functions and molecular mechanisms in spinal cord injury repair.

INTRODUCTION

Spinal cord injury (SCI) is a critical central nerve injury that greatly influences a patient's quality of life and has important socioeconomic effects.[1,2] In SCI, the primary injury causes distraction, squeezing, and shearing forces on the spinal cord,[3,4] which can harm both the central and peripheral nervous systems. Furthermore, secondary SCI effects include edema, ischemia, apoptosis, inflammation, and electrolyte imbalance, resulting in further damage in addition to that of the initial injury.[5-7] All of these changes lead to serious neurological deficits, such as cognitive and behavioral disorders.[8] A series of methods, including nerve growth factors and cell transplantation, can be considered to create a suitable environment for neuronal survival and axonal regeneration and to prevent any further development of SCI damage.[9] However, the identification of efficient molecular targets is a difficult task, and the underlying molecular mechanisms of SCI damage need to be further elucidated.

Nck1 includes 1 SH2 and 3 SH3 domains.[10] As a scaffold protein, Nck1 can bind surface receptors with the SH2 domain and then transfer signals to downstream molecules via the SH3 domains.[10-12] A great many of interacting partners have been reported to bind to Nck1.[13,14] Nck1 regulates various cellular processes, containing DNA synthesis, transcription, protein degradation, and cytoskeleton recombinant.[10-12] Many studies have shown that Nck1 is greatly expressed in cancer tissue and participates in extracellular signal-regulated kinase activation.[15] Nck1 can also regulate cell migration by linking phosphotyrosine signals.[16] Moreover, some studies have reported that Nck1 expresses in the developing nervous system and plays a role in neuronal growth cones.[17] Additionally, Nck1 recognizes specific extracellular signals and transmits them to neuronal bodies to induce axonal movement.[16,18] However, few researches have investigated the expression and functions of Nck1 in spinal cord tissue, or the potential molecular mechanisms of Nck1 after SCI.

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Hence, the main purpose of our study was to examine the localization and expression changes of Nck1 after SCI. We also aimed to observe its endogenous role in the pathological processes of SCI and to explore the potential molecular mechanisms. These data will facilitate a further understanding of the functions of Nck1 and the relative molecular mechanisms underlying SCI.

MATERIAL AND METHODS

SCI Model Construction

Twenty-five female SD rats weighing 200–220 g were randomly separated into the SCI group (n = 20) and sham group (n = 5). Under anesthesia, dorsal laminectomies were performed to expose the dorsal tissue between the T8 and T10 thoracic vertebrae. A contusive injury was made at the T9 region by dropping a 2.0 mm tip with 1.5 N force (Precision Systems & Instrumentation, Fairfax Station, Va, USA). The sham group animals were anesthetized and received laminectomy only, without the contusive injury. All laboratory animals' surgery and care were carried following the National Institutes of Health (USA) guidelines (NIH Publication No. 85-23). This research received ethical approval from the Laboratory Animal Ethics Committee of Nantong University (approval no. 2019-0306-010).

Behavioral Analysis

Recovery of rat function was checked using the Basso, Beattie, and Bresnahan (BBB) system, which is a rating scale from 0 to 21. Under blinded conditions, rats were handled in a round, open space and were allowed to walk freely. Each rat's trunk stability, knee coordination, and paw movement range were evaluated once each by 2 observers over 4 minutes. The mean scores were calculated and showed as the mean ± standard error.

Luxol Fast Blue Staining

The spinal cords were removed and cut into 10 μm sections after the rats had been anesthetized and perfused. Frozen slices were stained at 58°C in Luxol Fast Blue stain (LFB) mixture (0.1% LFB + 95% EtOH + 0.05% acetic acid). Next day, slices were washed in 95% EtOH and differentiated in 0.05% Li_2CO_3 solution, followed by a solution in 75% EtOH. The slices were then washed completely in distilled water and were kept in 0.05% Li2CO3 to stop differentiation. Finally, slices were washed in 75% EtOH, followed by 95% and 99% EtOH until the unmyelinated tissue appeared white.

Immunofluorescent Staining

The spinal cords were removed and cut into 10 μm sections after the rats had been anesthetized and perfused. All samples were blocked with 0.1% Triton X-100 in 3% bovine serum albumin (BSA) for 30 minutes at room temperature. First, samples were incubated in a solution of primary antibodies at 4°C overnight. Antibodies against Nck1 (rabbit, 1:1000, Abcam, Cambridge, UK) were simultaneously applied with either the neuronal markers Neu-N (1:500; Millipore, Burlington, Mass, USA), NF-200 (1:500; Millipore), or NF-M (1:400; Millipore) or the astrocytic marker

glial fibrillary acidic protein (GFAP) (1:400; Abcam). The next day, after 3 rinses in phosphate-buffered saline (PBS), the samples were incubated in secondary antibodies at room temperature for 2 hours. Three washes in PBS were performed, and samples were then mounted with 6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Burlingame, Calif, USA). All pictures were observed using a fluorescence microscope (Leica Microsystems, Wetzlar, Germany).

Cell Culture

VSC4.1 cells (ventral spinal cord 4.1 motor neuron line) were established by mixing rat ventral spinal cord neurons with mouse N18TG2 neuroblastoma cells. Cells were seeded on poly-l-lysine (PLL)-precoated culture flasks and were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal bovine serum (FBS). Cells were maintained in an incubator at 37°C with 95% O_2 and 5% CO_2. VSC4.1 cells were a gift from Dr Weidong Le (Baylor College of Medicine, Houston, Tex, USA).

Short Interfering RNA Transfection

To knock down Nck1, VSC4.1 cells were treated with Nck1 short interfering RNA (siRNA), which were purchased from Biomics Biotechnologies Co., Ltd. (Nantong, China). There were 3 siRNA sequences: si1 (forward 5'-GACCAUGUAGGUUCUCUGUDTDT-3', reverse 5'-ACAGAGAACCUACAUGGUCDTDT-3'), si2 (forward 5'-CAAAAAGGCA CCGAUCUUUDTDT-3', reverse 5'-AAAGAUCGGUGCCUUUUUGDTDT-3'), and si3 (forward 5'-GGA CACCUUAGGCAUUGGADTDT-3', reverse 5'-UCCAAU GCCUAAGGUGUCCDTDT-3'). Ineffective siRNA with low GC content was used as control. Each siRNA was transfected into cells using Lipofectamine 2000 (Invitrogen, Waltham, Mass, USA) following the instructions.

Western Blot

The spinal cord samples were excised and a 10-mm portion around the center of the damage was dissected out. The samples were manually minced, homogenized in radioimmunoprecipitation assay buffer (1% sodium dodecyl sulfate, 1% NP-40, 50 mM Tris base, and 1 mM phenylmethylsulfonyl fluoride) on ice for 60 minutes, and centrifuged at 1,2000 rpm for 15 minutes. The supernatants were harvested and kept in new microtubes, and the concentrations were then checked using bicinchoninic acid assay. The lysates were fractionated using 12% sodium dodecyl sulphate polyacrylamide gel (SDS-PAGE) before being transferred to polyvinylidene fluoride membranes (PVDF). The membranes then were blocked with 5% milk for 2 hours at room temperature and then incubated overnight with antibodies against Nck1 (rabbit, 1:2000; Abcam) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH; rabbit, 1 : 5000; Sigma-Aldrich, St Louis, Mo, USA). Next day, membranes were incubated for 2 hours with horseradish peroxidase secondary antibodies. Signal bands were then visualized and analyzed using a ChemiDoc™ XRS+ imaging system with Image Lab™ 5.1 software (Bio-Rad Laboratories Inc., Hercules, Calif, USA).

RNA Extraction and Quantitative Reverse Transcription Polymerase Chain Reaction

Total RNA was extracted from the spinal cord tissue or VSC4.1 cells using a QIAcube automated RNA extractor (QIAGEN, Chatsworth, Calif, USA) following the instructions. The following primers were used: GAPDH (forward 5'-GAGGTAGTTATGGCGTAGTGC-3', reverse 5'-CTGGTTTCTGGAGGATGG-3') and Nck1 (forward 5'-GCTCGGAAA GCATCTATT-3', reverse 5'-TACATGGTCACCCAAAGG-3'). GAPDH was used as an internal control.

Cell Counting Kit-8 Assay

VSC4.1 cells were treated with control or Nck1 siRNA, and the viability of the cells were then determined using a Cell Counting Kit-8 (CCK-8) assay (Beyotime Biotechnology, Shanghai, China) following the instructions. Cells were examined at 460 nm using a microplate reader (Infinite M200; Tecan, Männedorf, Switzerland). All cell viability assays were performed in triplicate.

RNA-Sequencing and Gene Ontology Analysis

Spinal cord tissue from 2 mm around the center of the damage was taken from each rat, including both the SCI and sham groups. Total RNA was extracted by RNA extractor kit (Qiagen) following the instructions. The RNA sample integrities were tested by using an Agilent 2200 (Agilent Technologies, Santa Clara, Calif, USA). Samples were used for the establishment of the RNA-Sequencing (RNA-seq) library. The library preparation and RNA-seq analysis were performed by RiboBio Corporation (Guangzhou, China).

The Gene Ontology (GO) analysis was performed using the GO website (http://geneontology.org), which indicates the biological function of target genes. The Chilibot database (http://www.chilibot.net/) was also used to search for SCI-associated genes. Finally, the genes related to Nck1 and SCI were screened for their biological properties.

Statistical Analysis

GraphPad Prism 8.0 (San Diego, Calif, USA) was used to perform data analyses. All data are shown as the mean ± standard deviation. The Shapiro–Wilk test was used as the numerical method for measuring data normality. The Student's unpaired t-test and 1-way analysis of variance with Tukey's post hoc test were chosen to check the significant interaction. Repeated-measurement analysis of variance with Tukey's post hoc test was used to check the significance for BBB score analysis. The statistical significance was $P < .05$.

RESULTS

Rat Behavioral Changes After SCI

To ensure the successful construction of SCI animal models, the BBB scoring system was used to assess spontaneous motor function recovery after SCI (Figure 1a). After injury, the SCI rats lost hind limb movement almost completely compared with the sham animals. The BBB scores were so low that slight recovery was seen until 7 days after the injury. Although spontaneous functional improvements occurred in the following days, the recovery of the SCI rats was much weaker than that of the sham group. These results indicate that the SCI animal model was successfully established.

Morphological Alterations of Spinal Cord After SCI

LFB staining was used to detect morphological alteration of spinal cord after SCI. The normal spinal cord exhibited a complete structure, with clear gray matter, white matter, and myelin. After SCI, however, the integrity of the spinal cord was disrupted and neural conduction tracts were blocked. There was abundant cell death and demyelination in the SCI rats. Around the lesion center, there were a large number of vacuoles and irregular spaces, axonal degradation, and cellular disorganization (Figure 1b).

Temporal Expression Analysis of Nck1 After SCI

To explore the role of Nck1 during the SCI process, we analyzed its expression at different time points using quantitative reverse transcription polymerase chain reaction (qRT-PCR) and western blot. The qRT-PCR results revealed that Nck1 expression levels were significantly decreased to the lowest level after SCI and then increased slowly over the following days (Figure 2a). The western blot assays demonstrated the same results (Figure 2b). Nck1 declined to a low level after SCI, and then slowly increased to a stable level at 35 days after SCI, although this level was still lower than that of the sham group (Figure 2c). Overall, the data mentioned earlier suggest that Nck1 might participate in the pathological processes of SCI in a time-dependent way.
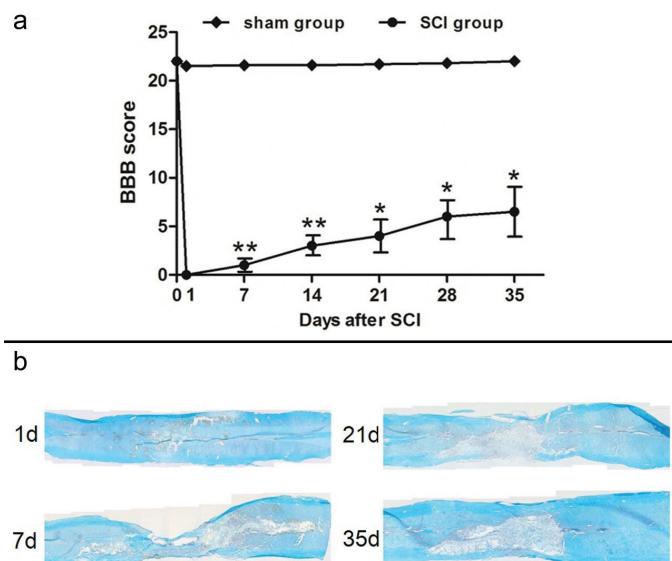

FIG. 1. a, b. Behavioral analysis of the rats after SCI and spinal cord LFB staining. (a) The time process and functional recovery levels of rats after SCI (n = 25). (b) LFB staining of the spinal cord at each time point (1, 7, 21, and 35 days).

LFB, Luxol Fast Blue stain; SCI, spinal cord injury.

Localization and Expression Alteration of Nck1 in the Spinal Cord After SCI
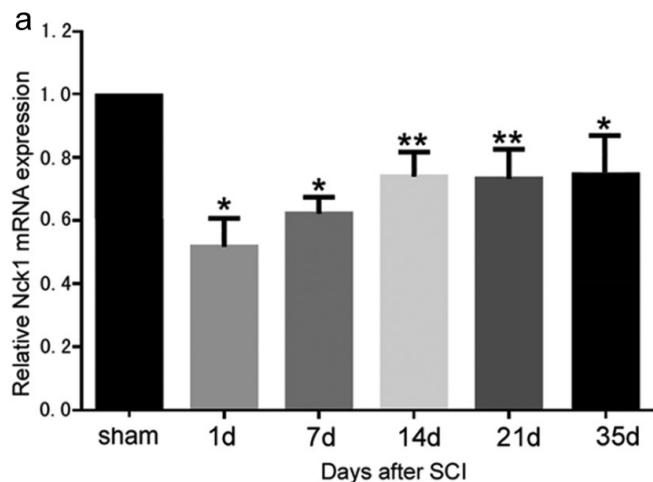
Immunocytochemistry was used to distinguish the cellular distribution of Nck1 in spinal cord of naïve rat. Nck1 was mainly expressed in cells with large bodies and nuclei, which shared similar morphological characteristics with neurons (Figure 3a). Notably, Nck1-positive signals were observed not only in the cytoplasm but also in the cell membrane (Figure 3b). To detect whether the Nck1-positive cells were neurons, we co-labelled sections with the neuron-specific markers Neu-N, NF-200, and NF-M (Figure 4). Indeed, Nck1 was selectively distributed in neurons in the gray matter, whereas no Nck1 staining was observed in astrocytes or any other cells (data not shown).

To further detect expression changes in Nck1 after injury, spinal cord samples from different time points of SCI model were chosen for immunofluorescence experiments. There were many Nck1-expressing neurons undergoing apoptosis, with shrunken morphology, at 1 day after SCI. However, Nck1 began to appear in the plasma and membranes of neurons with normal morphologies at 7 days compared with the sham group (Figure 5).

Changes in Neurite Growth in VSC4.1 Cells

With Reduced Nck1

Previous studies have reported that Nck1 can regulate neuronal differentiation and development.[19-21] To explore whether or not Nck1 is associated with neuronal development, we used Nck1 siRNA. To find the optimal silencing efficiency, 3 siRNAs (si1, si2, and si3) against Nck1 were used to transfect VSC4.1 cells along with a negative siRNA control. The qRT-PCR results revealed that of the 3 siRNAs, the si3-transfected cells had the greatest reduction in Nck1 compared with the negative control cells (Figure 6a). Thus, si3 was used for all further experiments.

To investigate whether Nck1 participated in neuronal proliferation, VSC4.1 cells were transfected with control- and Nck1-siRNA, and the CCK8 assay was then used to test cell proliferation and viability. The neurite lengths of Nck1-siRNA-transfected cells were markedly shorter than those of control cells. This result suggests that Nck1 is required for VSC4.1 cell proliferation (Figure 6b-c). In addition, the viability of si3-transfected cells decreased greatly compared with the control cells (Figure 6d). These results clearly indicate that Nck1 reduction specifically induces impairments in VSC4.1 cell proliferation and neurite growth.

GO Analysis of Differential Genes Expression After SCI

To investigate the role of Nck1 in SCI, we profiled the transcriptome of spinal cord tissue 14 days after injury using RNA-seq technology. We identified 1328 differentially expressed genes associated with Nck1 in the spinal cord tissue. There were 843 downregulated genes and 485 upregulated genes compared with the sham group. GO functional analysis was performed to check the potential functions of these differentially expressed genes. The downregulated genes were largely enriched in neuronal development, axon guidance, the immune response, cell surface receptor signaling, and cell migration. In contrast, the upregulated genes were closely associated with cell viability, apoptosis, tumor factor response, cell adhesion, and the MAPK signaling pathway (Figure 7a-b). Temporal expression analysis was used to screen for potential genes that were closely related to Nck1 and that had significant fold changes in their expressions (Table 1). Overall, the transcriptome analysis indicated that Nck1 might participate in multiple pathological processes in SCI, but its potential functions need to be further investigated.

DISCUSSION

Spinal cord injury could cause the impairment of sensory and motor functions.[1,6] Many molecular events occur during SCI,
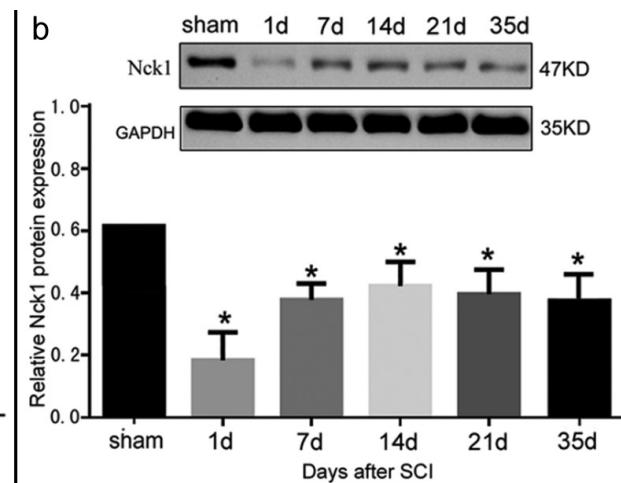

FIG. 2. a, b. Nck1 expression changes in spinal cord after SCI. (a) The expression of Nck1 after SCI as measured by qRT-PCR compared with the sham groups (b) Rat spinal cords at different times after the operation were used for western blot analysis. Representative bands labeled with Nck1 and GAPDH are displayed above, and the columns below display the ratio of Nck1 to GAPDH (**$P < .05$; **$P < .01$).

GAPDH, glyceraldehyde 3-phosphate dehydrogenase; qRT-PCR, quantitative reverse transcription polymerase chain reaction; SCI, spinal cord injury
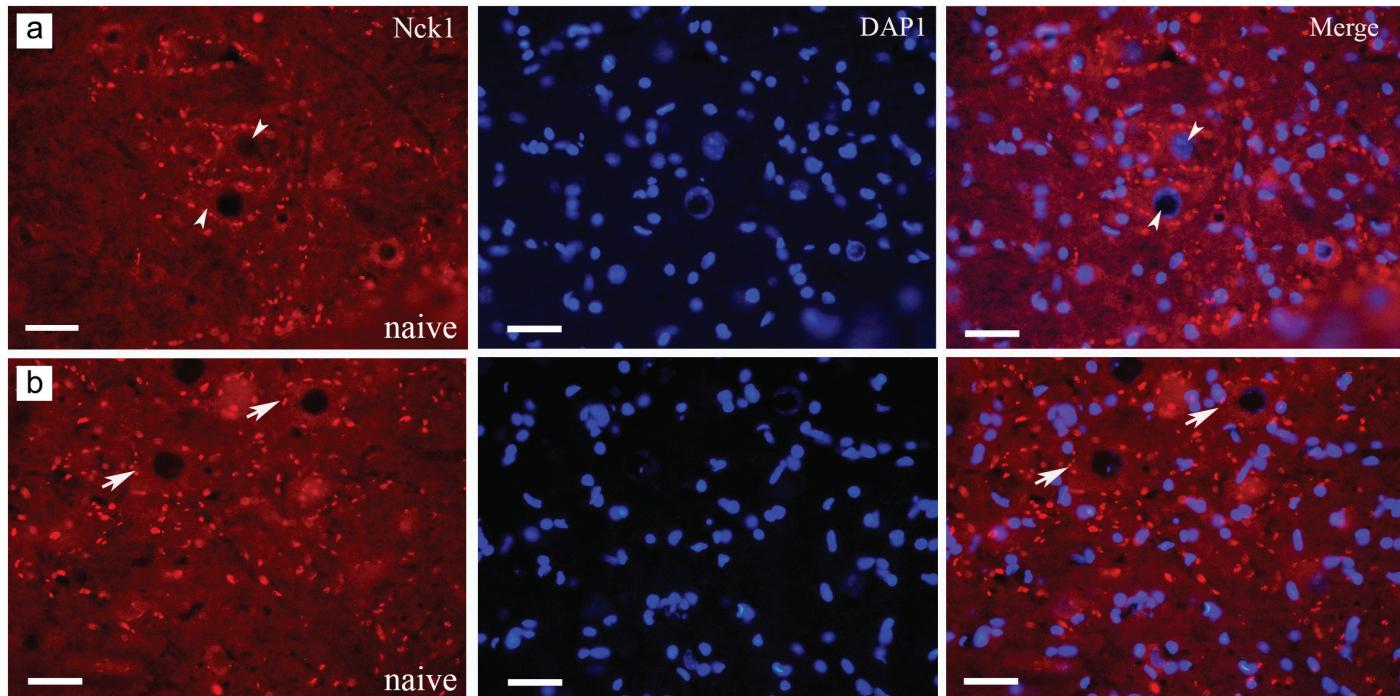
FIG. 3. a, b. Immunofluorescence staining of Nck1 in the spinal cord from naïve rat. (a) Nck1-positive cells had large bodies and big, round nuclei (DAPI was used to mark the nuclei). Note the big and round nuclei (arrows). (b) Nck1-positive signal was observed in both the cytoplasm and the membrane. Note the distribution of Nck1 in positive cells (arrowhead). Scale bars, 20 μm.
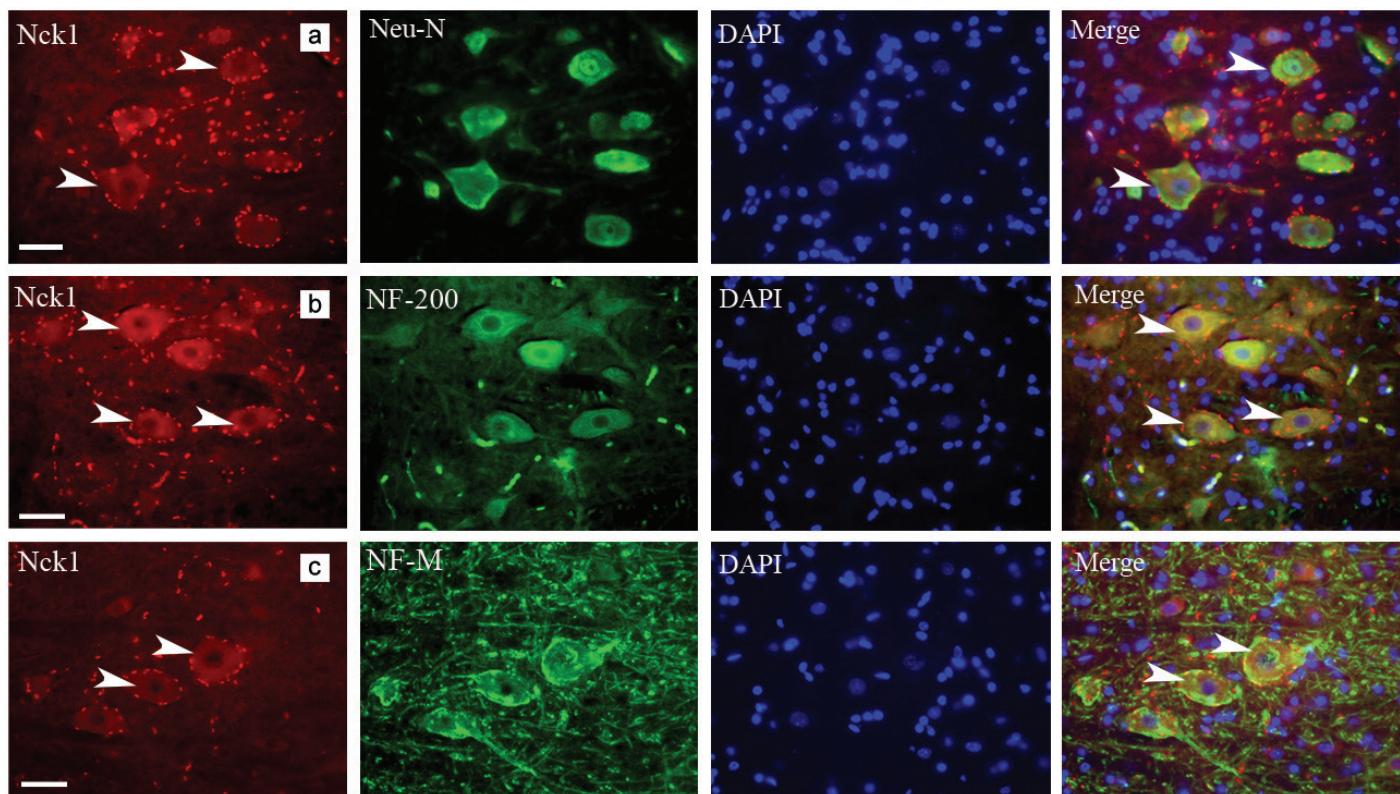

FIG. 4 (a-c). Immunofluorescence staining of Nck1 with various neuron-specific markers. (a) Nck1 are shown in red. (a-c) Neuron-specific markers are shown in green and include Neu-N, NF-200, and NF-M (a-c). Note the double staining of Nck-1 and neuronal markers (arrows).

including neuronal apoptosis, oxidative stress, inflammation, and astrocyte proliferation.[22-26] In recent years, research has studied the pathological processes and potential mechanisms of SCI, in an attempt to promote neuronal regeneration, but no effective therapeutic treatment.[6,9,23,27] In the present study, we constructed an SCI model and revealed the possible mechanisms during SCI progression. We constructed a spinal cord injury model in rats and revealed the potential molecular mechanisms during SCI progression. Nck1 is a well-known actin cytoskeleton regulator that can associate with multiple cell surface receptors.16 Nck1 could regulate a range of cellular processes, such as actin cytoskeleton, axon guidance, cell movement, and so on.[14,15,19,20,28] However, its function is still undetermined. In this study, we revealed the expression changes of Nck1 in SCI processes. Both the qRT-PCR and western blot results demonstrated that Nck1 expression was significantly decreased at 1 day after injury and then slowly

increased. The data suggest that Nck1 might participate in the pathological processes of SCI in a time-dependent way. Furthermore, immunofluorescence results revealed that Nck1 is mainly expressed in neurons, with positive signal detected in both the cytoplasm and cell membrane, suggesting that Nck1 might participate in the regulation of molecular mechanisms underlying SCI progression.

Previous studies have reported that Nck1 is essential for neuronal differentiation and neuritogenesis.[15,19,21,29,30] To further study the possible functions of Nck1, we obtained spinal cord tissue from different time points and used immunofluorescence to detect its distribution. We found that Nck1 was expressed at low levels in damaged neurons that were undergoing apoptosis, whereas it was expressed at relatively high levels in neurons with good morphology at 7 days after SCI. We also found that neurite outgrowth and length was significantly inhibited in Nck1-siRNA
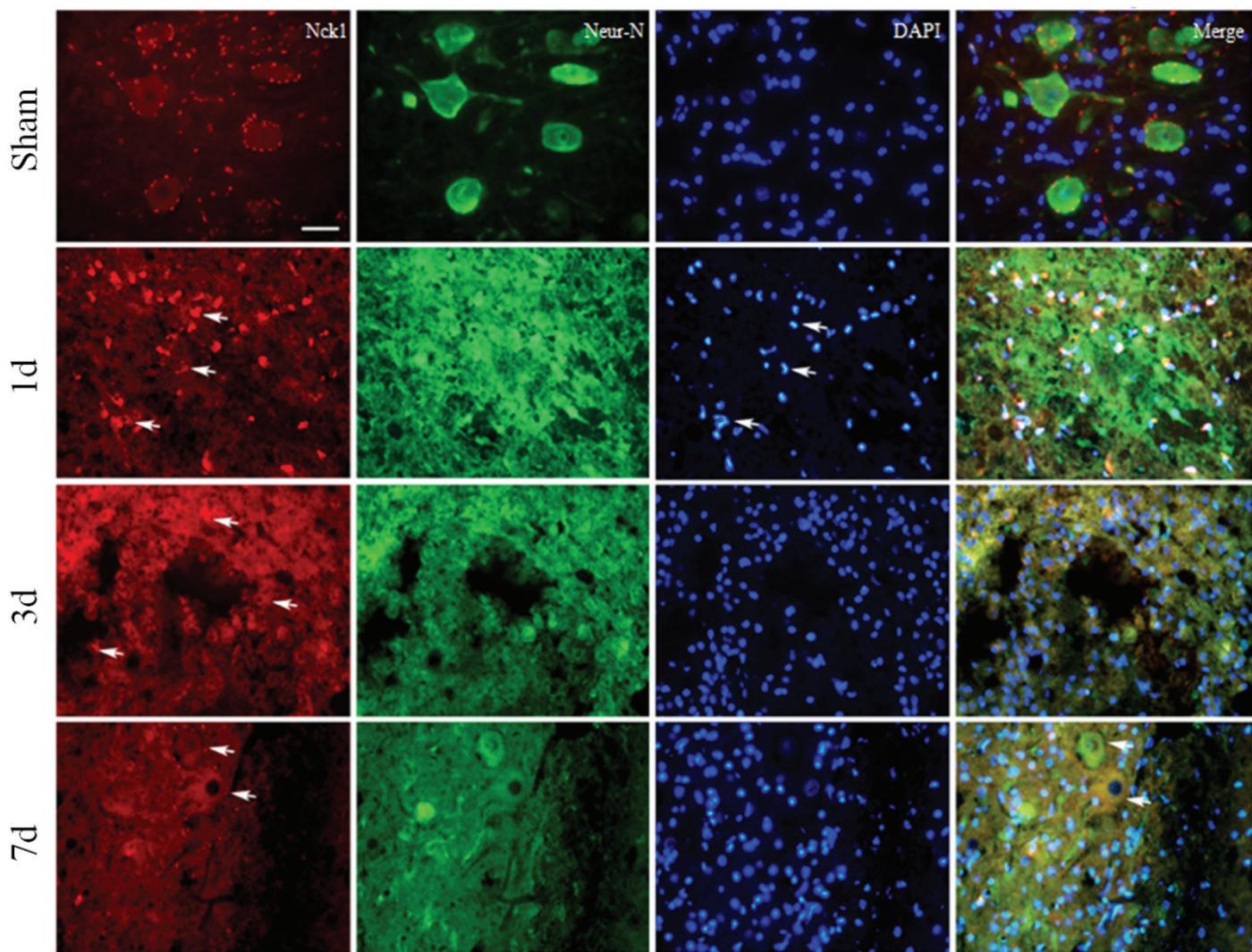

FIG. 5. Immunofluorescence staining of Nck1 at different time points after SCI. Rat spinal cord sections were probed with Nck1 (red), and neurons were labeled with Neu-N (green). Nck1 was observed in the plasma and membrane of neurons at 7 days. Nck1-positive neurons had normal morphology. Note the positive staining of Nck-1 in different days (arrowhead). Scale bars, 20 μm. SCI, spinal cord injury.

VSC4.1 cells compared with control groups. Additionally, knocking down Nck1 also significantly reduced the viability of VSC4.1 cells. Overall, these findings suggest that Nck1 might affect both the viability and morphological development of neurons in the progression of SCI.

We also used RNA-seq technology to investigate transcriptome-level differentiation of gene expression after SCI. There were 843 downregulated and 485 upregulated genes in the SCI model. Gene Ontology functional analysis revealed that multiple biological processes are closely associated with SCI repair, such as neuronal development, axon guidance, the immune response,

cell viability, and apoptosis. Temporal expression analysis was used to screen for potential genes that have previously been reported as closely related to Nck1, including TrkB, EphB, Grb2, LRFN4, Slit1, and EGF receptor.[11,16,17,19,31,32] The expression levels of these genes were detected, and they had significant fold changes in their expression.

In conclusion, we investigated the cellular localization and expression of Nck1 during SCI processes. Our data revealed that Nck1-specific siRNA transfection significantly reduced cell viability and neurite development in neurons. Additionally, our bioinformatic analysis indicated that Nck1 participates in multiple
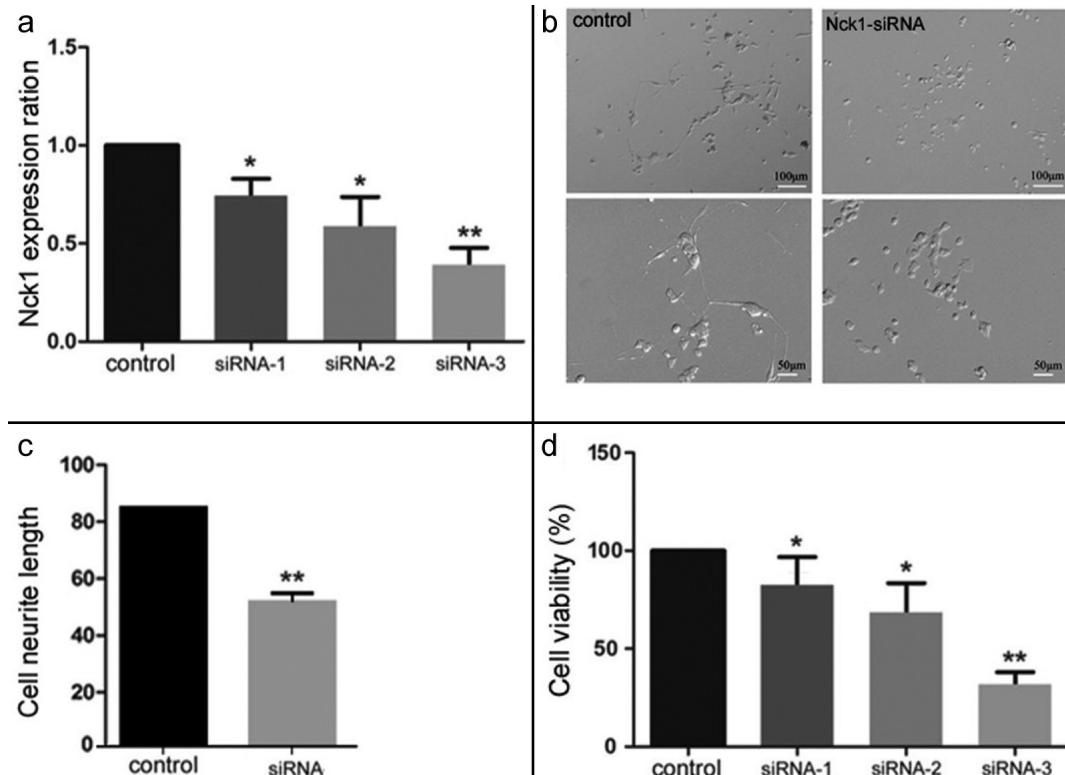

FIG. 6. a-d. VSC4.1 cells were treated with Nck1 siRNA. (a) Expression of Nck1 was decreased in Nck1-siRNA-treated VSC4.1 cells compared with controls. (b) Neurite length was markedly inhibited in VSC4.1 cells after Nck1-siRNA transfection. (c) The cell viability of Nck1-siRNA-treated VSC4.1 cells was tested and compared with that of controls (*P < .05). (d) The neurite length of Nck1-siRNA-treated VSC4.1 cells was calculated and compared with control cells (*P < .05; **P < .01).
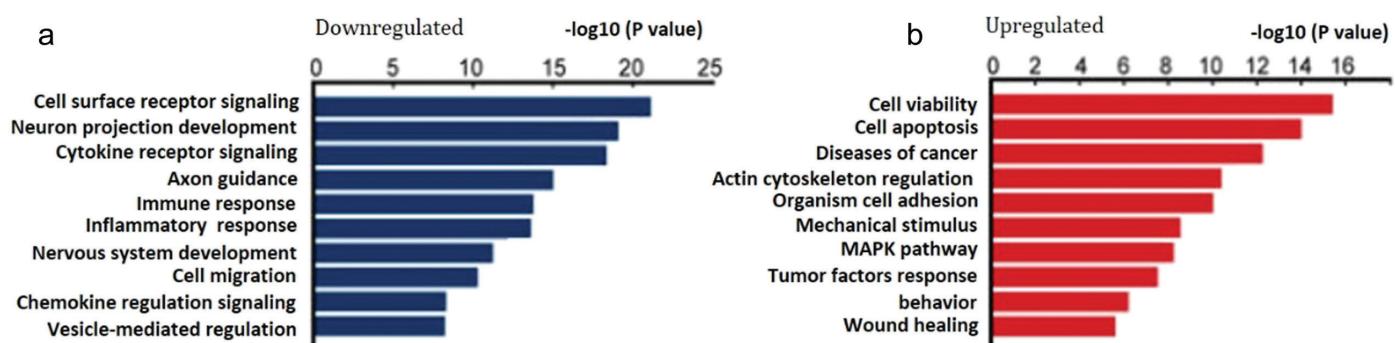

FIG. 7. a, b. GO terms associated with Nck1 after SCI. After SCI, the GO functional analysis showed expression changes of genes that are closely associated with Nck1. (a) Downregulated genes (blue). (b) Upregulated genes (red). GO, Gene Ontology; SCI, spinal cord injury.

TABLE 1. Nck1-Associated Genes that Showed Large Changes in Expression after SCI

Protein	Description	Gene expression fold change
TrkB	Neurotrophic receptor tyrosine kinase 2	823.345
EphB	Eph receptor B	765.542
Grb2	Growth factor receptor bound protein 2	684.582
LRNF4	Leucine-rich repeat and fibronectin type III domain-containing 4	18.Jul
Slit1	Slit guidance ligand 1	735.648
EGF receptor	Epidermal growth factor receptor	8.Dec
PDGF receptor	Platelet-derived growth factor receptor	435.864
Socs3	Suppressor of cytokine signaling 3	541.325
Ncf2	Neutrophil cytosolic factor 2	18.Apr
Dock8	Dedicator of cytokinesis 8	466.542

pathological processes of SCI, and many Nck1-associated genes exhibited differential expression levels. Overall, these findings suggest that Nck1 is a vital protein in SCI processes, and further studies should be conducted to explore its potential functions and molecular mechanisms.

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