Research Article

Ninjurin-1: a biomarker for reflecting the process of neuroinflammation after spinal cord injury

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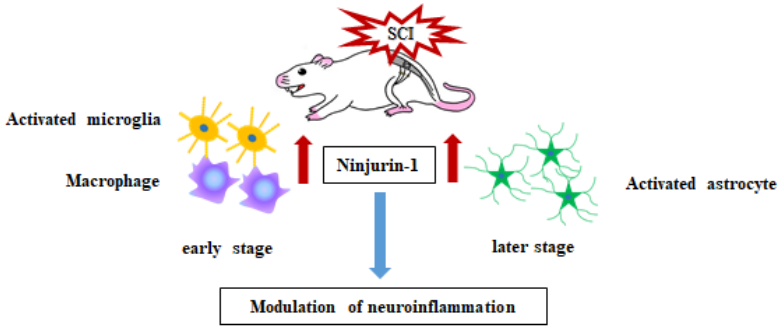
Graphical Abstract *Involvement of intensified early expression of Ninjurin-1 by macrophages and microglia and subsequently by reactive astrocytes in modulation of neuroinflammation of spinal cord after clip compression injury*



Abstract

Previous studies have shown that Ninjurin-1 participates in cell trafficking and axonal growth following central and peripheral nervous system neuroinflammation. But its precise roles in these processes and involvement in spinal cord injury pathophysiology remain unclear. Western blot assay revealed that Ninjurin-1 levels in rats with spinal cord injury exhibited an upregulation until day 4 post-injury and slightly decreased thereafter compared with sham controls. Immunohistochemistry analysis revealed that Ninjurin-1 immunoreactivity in rats with spinal cord injury sharply increased on days 1 and 4 post-injury and slightly decreased on days 7 and 21 post-injury compared with sham controls. Ninjurin-1 immunostaining was weak in vascular endothelial cells, ependymal cells, and some glial cells in sham controls while it was relatively strong in macrophages, microglia, and reactive astrocytes. These findings suggest that a variety of cells, including vascular endothelial cells, macrophages, and microglia, secrete Ninjurin-1 and they participate in the pathophysiology of compression-induced spinal cord injury. All experimental procedures were approved by the Care and Use of Laboratory Animals of Jeju National University (approval No. 2018-0029) on July 6, 2018.

Key Words: astrocytes; clip compression injury; macrophage; microglia; neuroinflammation; Ninjurin-1; rat; spinal cord

Chinese Library Classification No. R459.9; R364; R741

Introduction

Spinal cord injury (SCI) is caused by trauma to the spinal cord due to compression, contusion, or laceration (Mothe and Tator, 2013; Afshari et al., 2020) and is generally accompanied by secondary degeneration in conjunction with the infiltration of inflammatory cells (Jung et al., 2003; Shin et al., 2013; Ahn et al., 2015). The neuropathological outcomes after SCI often include local edema, axonal degeneration, hemorrhage, and the infiltration of inflammatory cells. Additionally, a secondary series of events result in exudation through the damaged blood-brain barrier, blood flow restriction, vasospasm, inflammation, and demyelination, which cause the formation of cystic cavitation as well as glial and connective tissue scarring (Ahn et al., 2012b; Mothe and Tator, 2013; Altinova et al., 2019; Wang et al., 2020).

Ninjurin-1 is a novel molecule in the mammalian Ninjurin group which is identified at low levels in the normal sciatic nerve but is upregulated in axons and Schwann cells following sciatic nerve injury (Araki and Milbrandt, 1996; Ekanayake et al., 2019; Jung et al., 2020). In the central nervous system (CNS) of rodents with experimental autoimmune encephalomyelitis (EAE), Ninjurin-1 is reported to be involved in the migration of T cells to the intraluminal surface of blood vessels (Ahn et al., 2009; Ifergan et al., 2011). Additionally, this protein is important in the migration of monocytes, macrophages, and dendritic cells to the inflammatory lesions of the CNS (Ahn et al., 2009; Ifergan et al., 2011) and contributes to the development and repair of the peripheral nervous system (Araki et al., 1997; Toma et al., 2020). *In vitro* experiments have demonstrated that Ninjurin-1 promotes neurite extension

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in dorsal root ganglion cultures (Araki and Milbrandt, 1996). Additionally, Ninjurin-1 is dynamically expressed in diverse immune cells at different time points following acute cerebral ischemia in rats, suggesting that it plays an essential role during rapid and delayed immune response modulation in the post-ischemic brain (Lee et al., 2016, 2018). Although these findings indicate that Ninjurin-1 is taking part in cell trafficking and axonal growth following neuroinflammation, its precise roles in these processes remain unclear. Thus, the present study investigated the changes in the expression levels and cell types expressing Ninjurin-1 that would be helpful to establish Ninjurin-1 as a reliable biomarker in assessing the neuroinflammatory course after acute SCI.

Materials and Methods

Animals

Thirty Sprague-Dawley male and female rats, weighing 180–220 g and aged 6–8 weeks, were obtained from Central Lab Inc. (Seocho-gu; Seoul, Korea). All rats were allowed to acclimate to the laboratory for 1 week prior to use in the experiment. They were housed in random groups of five rats/cage in a room maintained at 24 ± 2°C temperature and a relative humidity of 50 ± 5%, under 12-hour light/dark schedule. All rats were randomly divided into five groups: sham controls (day (D)21 post-injury (PI), n = 6) and SCI rats at the following time points: D1PI, D4PI, D7PI, and D21PI (n = 6 per time point). All experimental procedures were conducted with approval from the Care and Use of Laboratory Animals of Jeju National University (approval No. 2018-0029) on July 6, 2018 and all animal protocols conformed to current international laws and policies of the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, 1985, revised 1996). Additionally, all efforts were made to minimize animal pain and suffering and to reduce the number of animals used in this study.

Surgical procedure to induce SCI

SCI was induced by clip compression as described in our previous study (Ahn et al., 2012b). Briefly, the rats were anesthetized with Zoletil® 50 (Virbac, Carros, France) and subjected to laminectomy at the thoracic vertebrae (T9/T10). Next, the spinal cord was compressed at the thoracic level using a vascular clip (Stoelting; Wood Dale, IL, USA) at an occlusion pressure of 15–20 g for 1 minute. Immediately thereafter, the wounds, including the muscles and skin layers, were closed. Sham-operated control rats underwent only the laminectomy procedure. All rats with the exception of sham-operated controls had no hind limb locomotion soon after the SCI procedure. The behavioral function of SCI rats was evaluated using the Basso, Beattie, and Bresnahan (BBB) scoring system, which systematically evaluates the recovery of hind limb function from score 0, suggestive of no observed hind limb function, to score 21 (**Additional Table 1**), indicating a normal movement. Successful SCI models were confirmed with BBB score of 0 (Basso et al., 1996).

Tissue collection and histological examinations

Spinal cord tissues, including the thoracic lesion with the core injury (T8–11), were collected after being sacrificed using 95% CO_2 in the chamber, from rats in the five groups. After animals were sacrificed at the respective time points. The spinal cord tissues of 1 cm including the lesion core were removed and then fixed with 4% (v/v) paraformaldehyde in phosphate-buffered saline (PBS; pH 7.4) for 48 hours. Subsequently, 5-μm thick sagittal sections were stained with hematoxylin and eosin for immunohistochemical evaluation. The collected spinal cord tissues were stored at –80°C for western blot analysis.

Western blot analysis

Western blot analyses were conducted as described previously (Ahn et al., 2015). Briefly, the spinal cord samples, including the core region (length: 1 cm), were homogenized, electrophoresed, and immunoblotted onto nitrocellulose membranes (Schleicher and Schuell, Keene, NH, USA). The membranes were treated with mouse anti-Ninjurin-1 (1:1000, Cat# 610777, Lot No. 4108537, BD Transduction Laboratories, San Diego, CA, USA) and mouse anti-β-actin (1:10,000, Cat# A5441, Lot No. 028K4826, Sigma-Aldrich) antibodies and then with horse raddish peroxidase (HRP)-conjugated anti-mouse IgG antibody (dilution 1:1000, Lot No. Z1212, Vector Laboratories, Burlingame, CA, USA) for 1 hour at room temperature and visualized with BS-ECL Plus kits (W6002, Biosesang; Gyeonggi, Korea). Immunoblot signals were detected with the Fusion Solo 6X system (Vilber Lourmat; Collegien, France) and quantified using FUSION© software (Vilber Lourmat, Marne-la-Vallée cedex, France); the optical density of target samples was normalized to that of β-actin.

Immunohistochemistry

Immunohistochemistry was performed using a commercial avidin-biotin complex kit (Vetastain® Elite ABC Kit; PK-6102, Vector Laboratories; Burlingame, CA, USA) as previously described by our research group (Ahn et al., 2012a). In summary, deparaffinized sections were treated with 0.01 M citrate buffer (pH 6.0) for 2 minutes in a microwave and then with 0.3% hydrogen peroxide in methyl alcohol for 20 minutes to block endogenous peroxidase activity. Next, the sections were incubated with 10% (v/v) normal horse serum in PBS prior to incubation with the primary mouse anti-Ninjurin-1 antibody (1:1000, Cat# 610777, Lot No. 4108537, BD Transduction Laboratories) for 1 hour at room temperature. After three washes in PBS, sections were treated with biotinylated horse anti-mouse IgG prepared according to the manufacturer's protocol (1:100, PK-6101, Vetastain® Elite ABC Kit, Vector Laboratories) for 1 hour at room temperature. After washing, sections were incubated with an avidin-biotin peroxidase complex according to manufacturer's protocol and a peroxidase reaction was developed using a diaminobenzidine substrate kit (SK-4100, Vector Laboratories). Then, the sections were counterstained with hematoxylin prior to mounting. Presence of Ninjurin-1-immunoreactive cells in the spinal cord tissues of rats in each group was graded as follows: negative (–), < 20 positive cells (+), 20–50 positive cells (++), and > 50 positive cells (+++) under 20× magnification using an objective lens. Immunostaining was semi-quantified based on the Ninjurin-1 immunostained areas in the photographs using ImageJ software (NIH, Bethesda, MD, USA). Four different sections from each rat were analyzed and calculated as a percentage of positive area/total area per section selected (n = 3 per group). The results are expressed as the mean ± SEM.

Double-labeling immunofluorescence

For the double-labeling procedure, the sections were treated with 10% normal horse serum for 1 hour, with mouse monoclonal anti-Ninjurin-1 (1:500, Cat# 610777, Lot No. 4108537, BD Transduction Laboratories) antibody overnight at 4°C, and then with tetra-methyl rhodamine isothiocyanate-conjugated anti-mouse IgG (1:50, Cat# T5393, Lot No. 103K9165, Sigma-Aldrich) for 1 hour at room temperature. Next, the sections were washed and incubated with rabbit polyclonal anti-GFAP (1:1000, Cat# Z0334, Lot No. 00066092, Sigma-Aldrich) or anti-Iba-1 (1:1000, Cat# 019-19741, Lot No. LKH4161, Wako Pure Chemical Industries, Ltd., Osaka, Japan) overnight at 4°C. After washing, the sections were incubated with fluorescein isothiocyanate-conjugated anti-rabbit IgG (1:50, F0382, Lot No. 018K60572, Sigma-Aldrich) for 1 hour at room temperature. Next, the immunofluorescence-stained specimens were examined under a fluorescence microscope (BX-51; Olympus, Tokyo, Japan) and, following photography, the images were merged using Adobe Photoshop 7.0 software

(Adobe Systems, San Jose, CA, USA).

Statistical analysis

All data were analyzed with the GraphPad InStat software (version 3.1; San Diego, CA, USA). All variables were analyzed with one-way analysis of variance followed by *post hoc* Student-Newman-Keuls tests for multiple comparisons, and are expressed as the mean ± SE. *P*-values < 0.05 were considered statistically significant.

Results

Behavioral and histopathological examinations

Hind limb paralysis was not obvious in rats of the sham control group. However, all rats with SCI exhibited hind limb paralysis that improved on subsequent days (**Figure 1**). Similarly, the spinal cord tissues of sham-operated rats did not show pathological lesions (**Figure 2A** and **B**). By contrast, on D1PI, spinal cord tissues with clip compression injury exhibited a severe loss of cellularity in conjunction with edema and hemorrhage in the core region (**Figure 2C** and **D**); however, lesions were rarely observed in the penumbra region (**Figure 2C** and **D**). On D4PI, although hemorrhage and edema were less frequently observed, there was prominent and massive infiltration of inflammatory cells (**Figure 2E** and **F**) that was observable throughout D7PI (**Figure 2G** and **H**) and D21PI (**Figure 2I** and **J**). Further, these pathological outcomes have also been corroborated by other imaging methods in CNS traumatic injury (Gatto et al., 2015). Collectively, all pathological findings in the present study were comparable to those of previous reports (Kim et al., 2003; Shin, 2007; Moon et al., 2008; Ahn et al., 2012b).

Ninjurin-1 levels transiently increase after SCI

Western blot analyses were performed to evaluate variations in the expression levels of Ninjurin-1 in spinal cord tissues following clip compression injury (**Figure 3**). Ninjurin-1 was weakly expressed in the sham controls; and this value was arbitrarily set to "1". On D1PI and D4PI, the relative expression levels of Ninjurin-1 significantly increased by four- to five-fold (D1PI, $n = 6$, $P < 0.01$; D4PI, $n = 6$, $P < 0.001$, *vs*. sham control group), whereas these levels exhibited a marked decrease in D7PI compared to D4PI ($n = 6$, $P < 0.05$). On D21PI, Ninjurin-1 expression levels had decreased to control levels ($n = 6$, $P < 0.01$, *vs*. D4PI).

Immunohistochemical localization of Ninjurin-1 in SCI lesions

Figure 4 shows Ninjurin-1 expression levels in the neurons (**Figure 4A**) and vascular endothelial cells and glial cells (subsequently identified as astrocytes or microglia; **Figure 4A**) of the sham controls. On D1PI (**Figure 4B**), Ninjurin-1 was expressed in neurons, vascular endothelial cells, glial cells (subsequently identified as astrocytes or microglia; **Figure 4B**, arrow), and a few round cells (subsequently identified as Iba1-positive macrophages; **Figure 4B**, arrowhead). On D4PI (**Figure 4C**), some round cells that had infiltrated the core region were intensely stained for Ninjurin-1 (**Figure 4C**); some glial cells (**Figure 4C**) were also detected. On D7PI, Ninjurin-1 immunostaining was detected in neurons, glial cells (**Figure 4D**), and round cells (**Figure 4D**). On D21PI, Ninjurin-1 immunostaining was detected in neurons and glial cells (**Figure 4E**) with a higher intensity in glial cells. Furthermore, on D21PI, there was a relative lack of Ninjurin-1-immunopositive inflammatory cells (**Figure 4E**). In summary, Ninjurin-1 immunoreactivity increased sharply on D1PI and D4PI with respect to sham control and Ninjurin-1 immunoreactivity decreased on D7PI and D21PI (**Figure 4F**). **Table 1** summarizes the results of the Ninjurin-1 immunohistochemical analyses of spinal cord tissue samples from sham controls and at each SCI time point.

Table 1 | Ninjurin-1 immunoreactivity in the spinal cord tissues of sham controls and SCI rats[a]

Cell type	Sham control[b]	SCI[b]			
		D1PI	D4PI	D7PI	D21PI
Neurons	++	+	+	+	++
Microglia	+	++	++	+	+
Astrocytes	±	±	±	++	++
Macrophages	ND[c]	++	++	+	±
Vascular endothelial cells	–	++[d]	+	+	–

[a]Four different sections ($n = 4$) per group were examined by three observers blind to the conditions. [b]Spinal cord tissues of rats obtained from sham controls and day 1 post injury (D1PI), D4PI, D7PI, and D21PI rats. [c]ND, macrophages were not detected in the spinal cord tissues. [d]Intensity of Ninjurin-1 staining in the endothelial cells of vessels was classified as weak (+), moderate (++), or intense (+++) by three observers blind to the conditions. SCI: Spinal cord injury.

Phenotypes of Ninjurin-1-expressing cells in SCI lesions

To confirm the phenotypes of the cells that express Ninjurin-1, double-labeling immunofluorescence procedures for anti-Ninjurin-1 and anti-GFAP or anti-Iba-1, representative markers of astrocytes and microglia/macrophages, respectively (Velasco et al., 1980; Ahmed et al., 2007) were performed. On D7PI, Ninjurin-1 (**Figure 5A** and **D**) was co-localized with GFAP (**Figure 5B**) or Iba-1 (**Figure 5E**), demonstrating that astrocytes (**Figure 5C**) and macrophages/microglia (**Figure 5F**) expressed Ninjurin-1 post-SCI.

Discussion

The present study is the first to show that Ninjurin-1 levels are transiently upregulated in the lesioned areas of injured spinal cords using a rat model of SCI induced by clip compression. Although these findings suggest that Ninjurin-1 plays a role in the pathogenesis of early neuroinflammation, its precise role during the neuroinflammation process remains to be not fully elucidated.

Ninjurin-1 was originally identified as a molecule that interacts with neuron-glia which helps promote axonal sprouting and nerve repair in the rat sciatic nerve (Araki and Milbrandt, 1996; Araki et al., 1997). It is also upregulated in the neurons and Schwann cells of distal nerve segments following nerve transection or crush injury (Araki et al., 1997). Subsequently, it was revealed that Ninjurin-1 is also involved in cell adhesion, which is essential for the formation of early inflammatory reactions. As a cell adhesion molecule, Ninjurin-1 plays an important role in embryonic development, organogenesis, and even tissue regeneration after an injury (Araki et al., 1997; Lee et al., 2010). In case of ischemia in the CNS, Ninjurin-1 can be found in inflammatory cells as well as some glial cells (Lee et al., 2016). Moreover, Ninjurin-1 is upregulated in myeloid cells and inflamed endothelial cells in the spinal cord in mice with EAE (Ahn et al., 2014). The transient increases in Ninjurin-1 levels and myeloid cell expression in the early stage of SCI observed in the present study are consistent with the findings of previous studies, including those investigating brain ischemia and EAE (Ahn et al., 2014).

Although the role that Ninjurin-1 plays during CNS inflammation remains under debate, this protein is related to the induction of inflammatory processes in the CNS as well as in other tissues. For example, the functional repression of Ninjurin-1 reduces infiltration of leukocytes and clinical disease activity in mice with EAE (Ifergan et al., 2011) and repression of Ninjurin-1 reduces systemic inflammation in septic mice (Jennewein et al., 2015). Additionally, Ninjurin-1 is involved in the interplay between leukocytes and vascular endothelial cells during vascular remodeling and CNS inflammation (Lee et al., 2010; Marchetti and Engelhardt, 2020). In the present study, a significant increase in Ninjurin-1 expression was observed

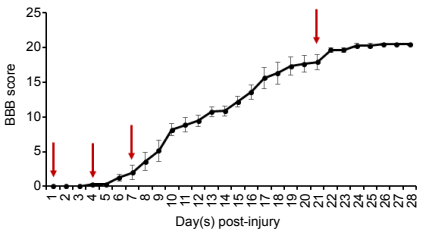

Figure 1 | BBB scores of locomotor function in SCI rats.
Following SCI, locomotor function was recorded and evaluated until D28PI. The BBB scores were low until D5PI and then gradually increased. Starting from D21PI, the BBB scores were maintained at the same higher levels until D28PI. Points of sampling are marked with arrows on post-injury days 1, 4, 7, and 21. BBB: Basso, Beattie, and Bresnahan locomotor scale; D5PI, D21PI, D28PI: days 5, 21 and 28 post-injury, respectively; SCI: spinal cord injury.

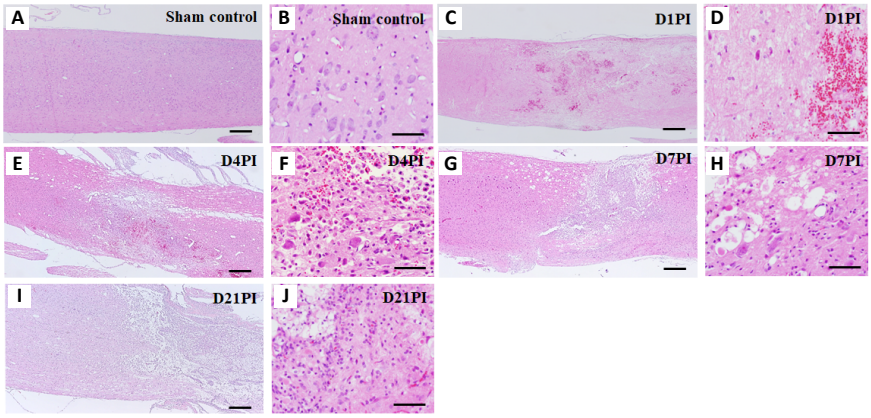

Figure 2 | Histopathological changes of the spinal cord after clip compression injury visualized with hematoxylin and eosin staining.
Longitudinal sections of spinal cord tissues from sham control (A), D1PI (C), D4PI (E), D7PI (G) and D21PI (I) showing both the core and the penumbra region. Higher magnification images of (B) sham controls and spinal cord injury rats on D1PI (D), D4PI (F), D7PI (H), and D21PI (J) illustrating the histopathological changes. Scale bars: 50 µm in A, C, E, G and I; 100 µm in B, D, F, H and J. D1PI, D4PI, D7PI, D21PI: days 1, 4, 7 and 21 post-injury, respectively.

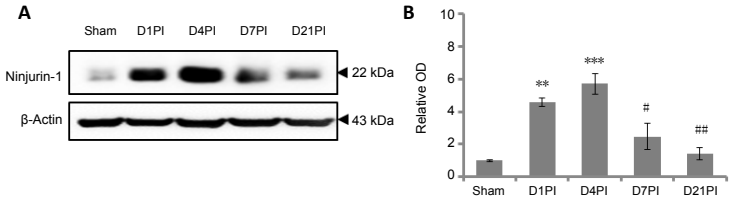

Figure 3 | Western blot analysis of Ninjurin-1 levels in the spinal cord tissues of sham controls and rats exposed to clip compression injury.
(A) Representative immunoblot images of Ninjurin-1 levels (approximately 22 kDa) and ß-actin (43 kDa) in the spinal cord tissues of sham controls and spinal cord injury rats on D1PI, D4PI, D7PI, and D21PI. (B) Bar graph showing the semi-quantitative analyses of the data (mean ± SE, *n* = 3/group). The relative expression levels of Ninjurin-1 were calculated after normalization to the β-actin band for each sample. Ninjurin-1 expression levels transiently increased on D1PI, peaked at D4PI, showed a tendency for a marked reduction on D7PI, and returned to control levels on D21PI. ***P* < 0.01, ****P* < 0.001, *vs*. sham controls (sham); #*P* < 0.05, ##*P* < 0.01, *vs*. D4PI. D1PI, D4PI, D7PI, D21PI: Days 1, 4, 7 and 21 post-injury, respectively; OD: optical density.

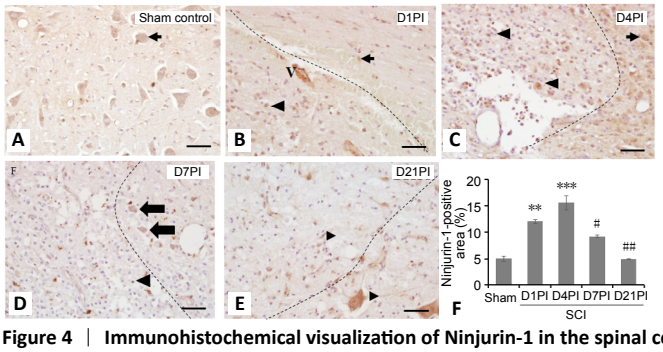

Figure 4 | Immunohistochemical visualization of Ninjurin-1 in the spinal cord tissues of sham control rats (sham, A) and SCI rats on D1PI (B), D4PI (C), D7PI (D), and D21PI (E).
Immunostaining of Ninjurin-1 with hematoxylin counterstaining in longitudinal sections of spinal cord tissues in sham (A) and SCI rats (B–E) on D1PI, D4PI, D7PI, and D21PI, respectively. In the post-SCI images (B–E), both the core and penumbra regions are in each figure; the dotted lines delineate the injury boundary. Neurons/glial cells are indicated by arrows while inflammatory cells are indicated by arrowheads. Scale bars: 20 µm. (F) The Ninjurin-1-positive area in the clip compression-injured spinal cord was sharply increased on D1PI and D4PI followed by a tendency to decrease on D7PI (mean ± SE, *n* = 3/group). ***P* < 0.01, ****P* < 0.001, *vs*. sham rats; #*P* < 0.05, ##*P* < 0.01, *vs*. D4PI. D1PI, D4PI, D7PI, D21PI: Days 1, 4, 7 and 21 post-injury, respectively; SCI: spinal cord injury.

on D1PI, and on D4PI, most of Ninjurin-1-immunopositive inflammatory cells were found. Oxidative and endoplasmic reticulum stress has been shown to increase the expression of Ninjurin-1. In the present study, disruption of blood supply after clip compression and subsequent outcomes may lead to oxidative stress induced upregulation of Ninjurin-1. Therefore, the present findings indicate that Ninjurin-1 is related to leukocyte recruitment and migration of inflammatory cells following SCI. Furthermore, these phenomena may be further confirmed with specific Ninjurin-1 knockout models in the future.

A full understanding of the expression of Ninjurin-1 in macrophages during the early stages of SCI will also require further investigation. In ischemic brains, Ninjurin-1 expression is induced in two specific populations of macrophages, microglia-derived macrophages and bone marrow-derived macrophages (Lee et al., 2016), which are both involved in the suppression of inflammation. Furthermore, *in vitro* studies where dorsal root ganglion cells were cultured with or without Ninjurin-1-positive environment, have shown to increase the neurofilament H-positive neurite extension when the environment is supported with Ninjurin-1 (Araki and Milbrandt, 1996). A decrease in myelinated axons with Ninjurin-1 deletion in nerve injury has also been reported (Tomita et al., 2019). In SCI rats in the present study, lesion periphery exhibited intense Ninjurin-1 immunoreactivity, where the prominent axon regrowth is involved, which indicates that Ninjurin-1 may involve in axonal regeneration. Moreover, this idea is further supported by a previous report through in situ hybridization in peripheral nerve injury (Araki and Milbrandt, 1996). By contrast, Ninjurin-1 derived from inflammatory cells supports adjacent axonal regeneration, at least in a rat model of SCI. Astrogliosis is another secondary consequence of CNS injury, including in EAE (Park et al., 2018), SCI (Moon et al., 2004), and brain ischemia (Park et al., 2018). It has been extensively suggested that astrogliosis is crucial in the CNS lesion recovery. Ninjurin-1 is constitutively expressed in astrocytes in spinal cord tissues, and reactive astrocytes with intense Ninjurin-1 immunoreactivity may involve in the resolution of CNS inflammation in the later stage of SCI.

Collectively, the present results suggest that transient increases in Ninjurin-1 expression levels are associated with macrophage activation and reactive astrogliosis and that these changes could activate inflammatory macrophages and/or modulate cell trafficking. The present study assessed the potential roles of

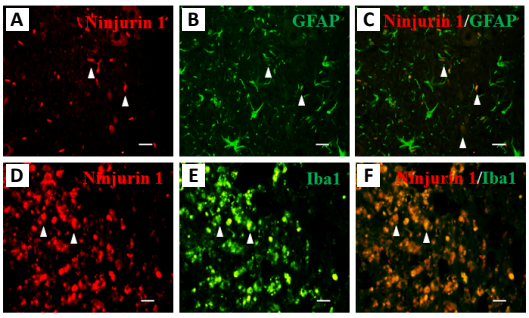

Figure 5 | Double immunofluorescence staining of Ninjurin-1 (A, D), GFAP (B), and Iba-1 (E) in spinal cord tissues following clip compression injury.
Double immunofluorescence staining of the sham control sections was performed to detect Ninjurin-1 (red, A) and GFAP (green, B) as well as Ninjurin-1 (red, D) and Iba-1 (green, E). Arrowheads indicate Ninjurin-1-expressing GFAP-positive astrocytes/Ninjurin-1-expressing Iba-1-positive macrophages/microglia. Scale bars: 20 μm. GFAP: Glial fibrillary acidic protein.

Ninjurin-1 following SCI and showed that the changes in these protein levels are associated with cell migration and neuroregeneration. However, further studies are recommended to evaluate the mechanisms involving Ninjurin-1 in SCI through genetical or molecular modifications in the future. With further advances in pharmacological interventions, Ninjurin-1 can also be considered to be developed as a biomarker for assessing neuropathogenesis of SCI. Furthermore, the possibility of regulating Ninjurin-1 expression to diminish neuroinflammation cannot be excluded.

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Data sharing statement: *Datasets analyzed during the current study are available from the corresponding author on reasonable request.*
Plagiarism check: *Checked twice by iThenticate.*
Peer review: *Externally peer reviewed.*

Additional file:
Additional Table 1: *Basso, Beattie, and Bresnahan locomotor rating scale.*

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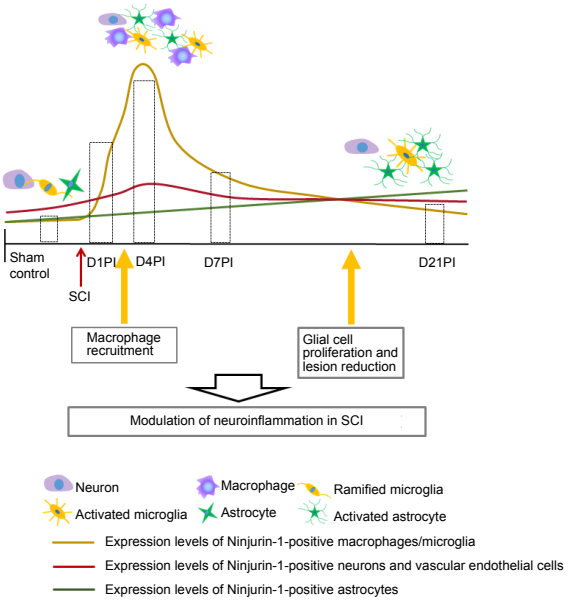

Figure 6 | Schematic illustration of spatiotemporal changes in Ninjurin-1-positive levels in rat spinal cord tissues following clip compression injury.
Ninjurin-1 was constitutively expressed in neurons and glial cells in the spinal cord tissues of sham controls. Clip compression-induced SCI of the spinal cord resulted in the expression of Ninjurin-1 within infiltrating macrophages other than neurons and glial cells. Soon after SCI, Ninjurin-1 was upregulated and expressed in infiltrating macrophages, reactive glia, and some neurons. However, subsequent Ninjurin-1 expression was primarily associated with reactive glia and some neurons. D1PI, D4PI, D7PI, D21PI: Days 1, 4, 7 and 21 post-injury, respectively; SCI: spinal cord injury.

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Additional Table 1 Basso, Beattie, and Bresnahan locomotor rating scale

0	No observable hindlimb movement
1	Slight movement of one or two joints, usually the hip and/or knee
2	Extensive movement of one joint or extensive movement of one joint and slight movement of the other joint
3	Extensive movement of two joints
4	Slight movement of all three joints of the hindlimb
5	Slight movement of two joints and slight movement of the third
6	Extensive movement of two joints and slight movement of the third
7	Extensive movement of all three joints of the hindlimb
8	Sweeping with no weight support or plantar placement of the paw with no weight support
9	Plantar placement of the paw with weight support in stance only (i.e., when stationary) or occasional, frequent, or consistent weight supported dorsal stepping and no plantar stepping
10	Occasional weight supported plantar steps: no forelimb-hindlimb coordination
11	Frequent to consistent weight supported plantar steps and no forelimb-hindlimb coordination
12	Frequent to consistent weight supported plantar steps and occasional forelimb-hindlimb coordination
13	Frequent to consistent weight-supported plantar steps and frequent forelimb-hindlimb coordination
14	Consistent weight supported plantar steps, consistent forelimb-hindlimb coordination, and predominant paw position during locomotion is rotated (internally or externally) when it makes initial contact with the surface as well as just before it is lifted off at the end of stance; or frequent plantar stepping, consistent forelimb-hindlimb coordination, and occasional dorsal stepping
15	Consistent plantar stepping and consistent forelimb-hindlimb coordination and no toe clearance or occasional toe clearance during forward limb advancement; predominant paw position is parallel to the body at initial contact
16	Consistent plantar stepping and consistent forelimb-hindlimb coordination during gait and toe clearance occurs frequently during forward limb advancement; predominant paw position is parallel to the body at initial contact
17	Consistent plantar stepping and consistent forelimb-hindlimb coordination during gait and toe clearance, predominant paw position is parallel at initial contact lift off
18	Consistent plantar stepping and consistent forelimb-hindlimb coordination during gait and toe clearance occurs consistently during forward limb advancement; predominant paw position is parallel at initial contact and rotated at lift off
19	Consistent plantar stepping and consistent forelimb-hindlimb coordinated gait, consistence toe clearance, predominant paw position is parallel at initial contact and lift off, and trunk instability; tail consistently up
20	Consistent plantar stepping and consistent coordinated gait, consistent toe clearance, predominant paw position is parallel at initial contact and lift off, and trunk instability; tail consistently up
21	Consistent planter stepping and coordinated gait, consistent toe clearance, predominant paw position is parallel throughout stance, and consistent trunk stability; tail consistently up