

# Pannexin-1 Characterization after Spinal Cord Injury in Rats

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## Research article

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# Abstract

## Background

Pannexin-1 (Panx-1) is a homomeric membrane semi-channel mostly expressed in the central nervous system of mammals, including neurons and glial cells. Panx-1 channels are highly permeable to calcium and Adenosine Triphosphatase (ATP), it plays an important role in Hypoxic injury of cerebral ischemia through a variety of signal pathways, nerve cell apoptosis and inflammatory response. However, its specific role in spinal cord injury (SCI) is not clear. In the current study, we aimed to investigate the characterization of Panx-1 after SCI in rats, and further analysis were made on its effect and possible mechanism in SCI in order to provide the experimental evidence for potential interfering target spot on SCI therapy.

## Methods

A rat SCI model (Allen's model) was established by NYU Impactor-III and the hind limb motor function of rats was observed by BBB score. The expression of Panx-1 was detected by Real-time PCR and Western Blot. The correlation between Panx-1 expression and the BBB score in rats after SCI was analyzed to reveal the role of Panx-1 in SCI.

## Results

We found that the BBB score gradually recovered after SCI, but declined significantly at day 2 after SCI. Real-time PCR and Western Blot detection showed that compared with the normal control group and the sham operation group, the expression of Panx-1 increased significantly with time after SCI, and reached a peak at day 2 after SCI. Moreover, there was a significant negative correlation between the expression of Panx-1 protein and the BBB score of rat hind limb motor function at day 1, day 2, day 3 and day 5 after SCI.

## Conclusions

The characterization of of Panx-1 expression after SCI in rats suggests that Panx-1 had a significant effect on the motor function recovery after SCI, and it was one of the important mechanisms that aggravate the secondary injury after SCI in rats. This provided experimental basis for further exploring the potential intervention target of SCI.

## Introduction

The treatment of Spinal Cord Injury (SCI) has been one of the most difficult problems for clinicians. Until now, no significant breakthrough has been achieved, mainly because the lack of effective intervention

means for the pathophysiological changes after SCI. The pathological outcomes of SCI mainly include damage to the white and gray matter of the spinal cord, which is caused by primary injury and subsequent progressive secondary injury<sup>[1, 2]</sup>. Secondary injury is a complex cascade reaction amplification process of self-destruction caused by primary injury, including ischemia and hypoxia, immune inflammatory response, excitatory toxicity, free radical damage, lipid peroxidation, etc., which is the main cause of aggravating neurological dysfunction and has reversibility and regulation<sup>[3]</sup>. Therefore, prevention and intervention of secondary injury is the core content of early SCI treatment<sup>[4]</sup>.

In 2000, Panchin found a kind of coding sequence in vertebrates and named it Pannexin (Panx), which is homologous to the invertebrate connexin Innexin gene. Panx protein consists of three subtypes: Panx-1, Panx-2 and Panx-3<sup>[5]</sup>. Panx-1 is abundantly expressed in many organs including brain, spinal cord, bone, etc<sup>[6]</sup>. Panx-1 protein mainly functions by forming a homomer semi-channel<sup>[7]</sup>, which is open to allow charged ions, small molecules with molecular weight less than 1.5 kDa or diameter less than 1.5 nm to freely pass through, so as to realize rapid communication of information between cells and complete synchronous activities between cells. Studies have shown that the Pannexin1 semi-channel is in a closed state in a resting state, and the channel has various open states under different voltages. Mechanical stimulation, purine receptor activation, hypoxia, extracellular high potassium and other stimuli can open the Panx-1 semi-channel at the resting membrane potential level<sup>[8]</sup>. In the study of cerebral ischemia and anoxia injury, Thompson et al<sup>[9]</sup> also found that Panx-1 semi-channel plays an important role in cerebral ischemia and anoxia injury by regulating the transmission of small molecules such as intracellular and extracellular Ca<sup>2+</sup> and ATP, and inducing neuronal apoptosis and inflammatory response through various signal pathways. So far, the underlying mechanism of Panx-1 semi-channel in SCI is unknown.

In this study, a rat SCI animal model was established to observe the expression change of Panx-1 after SCI and to investigate its correlation with Basso Beattie Bresnahan (BBB) score<sup>[10]</sup>, which was a Sensitive and Reliable Locomotor Rating Scale for Open Field Testing in Rats after SCI, so as to explore the effect of Panx-1 on SCI and provide new ideas and target spot for further gene therapy of SCI.

## Materials And Methods

### Experimental animals and groups

A total of 65 male Sprague-Dawley rats with body mass of 250–270 g were purchased from SLAC Laboratory Animal Co., Ltd. (Shanghai, China). All laboratory procedures and animal use practices are conducted in accordance with the National Institutes of Health Guidelines for laboratory animals. The rats were randomly divided into the normal control group (NC group), Sham group (including 8 h, 12 h, day 1, day 2, day 3 and day 5) and the SCI group (including 8 h, 12 h, day 1, day 2, day 3 and day 5).

### Animal model of SCI

The rats were fasted for 12 h before operation, the surgical instruments and consumables were sterilized by high-pressure steam, and the room temperature in the operating room was suitable. After conventional disinfection, 3.6% chloral hydrate was injected into the abdominal cavity of the rats according to the anesthetic dose (3 ml/kg). Subsequently, the length of the spinal cord was exposed about 1.0 cm with T10 as the center (Figure. 1a, 1b). Spinal cord tissue was taken directly from the normal group (after perfusion). So much for the sham group, this procedure was performed without injury to the normal spinal cord. A thin aluminum sheet (about 3 × 8 mm, pre-bent into an arc consistent with the surface of the spinal cord) was placed on the surgical framework of NYU impact-III (W.M. Keck, USA) hammer in the SCI group, and the rat SCI model (Allen's model [11–13]) was made with a weight of 10 g and a height of 50 mm of free-fall hitting standard (up to 50 g cm of wound energy) [14] (Figure. 1c). The criteria to define the success of the model establishment were as follows: the spasmodic tail swing of the rat lasts for several seconds. After retraction and flapping for several seconds, both lower limbs and the body became paralyzed, with subdural hyperemia. After awakening, both lower limbs were completely paralyzed. At the same time, the correct parameters and curves are recorded by the NYU impact-III strike computer system (Fig. 1d).

## **BBB motor function score of rats after SCI**

The BBB motor function rating scale [10] was used to evaluate the posterior limb function recovery of rats in the NC group, Sham group and SCI group. According to the BBB scoring requirements, it was operated by 4 clinicians who were familiar with the experimental procedures and standards but did not know the specific group. Three clinicians performed manual scoring, and one person performed counting time, data collection and quality monitoring. Before the score, the rats were placed in the test area for a while to reduce their strangeness and fear of the unfamiliar environment. The score was completed within 5 minutes for each procedure, and each mouse was repeated for 3 times on each side. The average score was then used in the final result.

## **Real-time PCR detection**

About 100 mg of spinal cord tissue block at T10 of rats in the NC group, sham group and SCI group were collected, and total RNA of spinal cord tissue was extracted by Trizol (Invitrogen, USA). The PrimeScript™ RT reagent Kit with gDNA Eraser Kit (TaKaRa, Japan) was used to remove the genomic DNA from the total RNA and conduct a Reverse Transcription reaction to synthesize cDNA. The primers of panx-1 and  $\beta$ -actin (Sangon, China) were designed and synthesized, and the sequence was shown in Table 1. Real-time PCR reaction solution was prepared according to the instruction of SYBR® Premix Ex Taq™ (Tli RNaseH Plus) (TaKaRa, Japan), as shown in Table 2. Reaction conditions were set on the fluorescence quantitative PCR apparatus (LightCycler/LightCycler480 System (Roche Diagnostics)): denaturation at 95°C for 30 s. PCR at 95°C for 5 s, 60°C for 30 s, 40 cycles. Dissolution at 95°C for 5 s, 60°C for 1 min. The Ct value of the Panx-1 and  $\beta$ -actin of each groups was generated by the fluorescence quantitative PCR apparatus, and each gene expression of relative quantity is calculated by formula:  $F = 2^{-\Delta\Delta Ct}$ , and the NC group was used as the control sample in our study.

Table 1  
Primer of  $\beta$ -actin and Panx-1

Gene	Primer	bp
$\beta$ -actin	F: ACAGGATGCAGAAGGAGATTAC	117
	R: ACAGTGAGGCCAGGATAGA	
Panx-1	F: CTCCGACGAGTTTCTGTGTAG	136
	R: GGCGTACACTAGGAGGTTAATG	

Table 2  
Real-time PCR reaction solution

Component	Volume( $\mu$ l)
cDNA	2 $\mu$ l
Forward Primer	0.4 $\mu$ l
Reverse Primer	0.4 $\mu$ l
SYBR® Premix Ex Taq™(Tli RNaseH Plus)	10 $\mu$ l
Dye(II)	0.4 $\mu$ l
ddH <sub>2</sub> O	6.8 $\mu$ l

## Western blot analysis

About 100 mg of the spinal cord tissue blocks at T10 of each group were added into the lysis fluid (RIPA: PMSF = 100:1) 100  $\mu$ l. The electric tissue grinder was used to crush and dissolve on the ice, and the spinal tissue protein was obtained after centrifugation. After determining the protein concentration with a BCA protein assay kit (Beyotime, China), equivalent amounts of protein were separated by 10% SDS-PAGE. The proteins were transferred to PVDF membranes (Millipore, USA) using a transfer apparatus at 90–110 V for 1 h. And then the PVDF membrane was blocked with 5% nonfat milk at room temperature for 1 h and incubated with primary antibodies including anti Panx-1 (1:800, Abcam, UK), anti  $\beta$ -actin (1:1600, Abcam, UK) at 4 °C overnight. After washing by TBST, the membranes were incubated with secondary goat anti-rabbit IgG-HRP antibody (1:3000, ABclonal, USA) and goat anti-mouse IgG-HRP antibody (1:3000, ABclonal, USA) for 2 h at room temperature. Finally, the Supersensitive ECL Chemiluminescence Kit (Beyotime, China) was used to expose images by the gel imaging system (Tanon-2500, China). Semi-quantitative analysis was performed by Image J software.

## Statistical analysis

Since the BBB score of each group was not normally distributed, the non-parametric test was selected to compare the differences among three groups. Panx-1 expression measurement data were expressed as mean  $\pm$  standard deviation ( $\bar{x} \pm s$ ). The Image J software measured the gray value, took the average value, and used the One-Factor Analysis of Variance (ANOVA) T test for inter-group comparisons. Spearman

rank correlation was used to analyze the correlation between Panx-1 expression and BBB score. All the above statistics were conducted using SPSS 20.0 software, and a two-side  $P < 0.05$  was considered statistically significant.

## Ethical consideration

The current study was approved by the Experimental Animal Ethics Committee of The First Affiliated Hospital of Fujian Medical University.

## Result

### Temporal variation of BBB score of motor function of rats after SCI

BBB score was used to evaluate the changes of hind limb Function at 8 h, 12 h, day 1, day 2, day 3 and day 5 after SCI. Results were shown In Table 3 and Fig. 2. Although the BBB score of the SCI group gradually recovered to different degrees with the extension of injury time, it was still significantly lower than Sham group and NC group at the same time ( $P < 0.05$ ). We also observed that from the end of the attack to 8 h after SCI, the lower limbs were completely paralyzed. From 12 h to day 1 after SCI, the hind limb function recovered slightly but there was no significant differences ( $P > 0.05$ ). However, on day 2 after SCI, the score decreased, which was significantly different to the score of day 1, day 3 and day 5 ( $P < 0.05$ ). Until day 3 to day 5 after SCI, the posterior limb function gradually improved significantly, and the score was significantly different from that of 8 h, 12 h, day 1 and day 2 ( $P < 0.05$ ).

Table 3  
BBB score of rats in each group (n = 5, median (min, max))

Group	8 h	12 h	D 1	D 2	D 3	D 5
NC	21(21,21)	21(21,21)	21(21,21)	21(21,21)	21(21,21)	21(21,21)
Sham	21(21,21)	21(21,21)	21(21,21)	21(21,21)	21(21,21)	20.5(20,21)
SCI	0(0,0)	0.5(0,1.5)	1.5(1.5,2.5)	1(0.5,1)*	4.5(3,5)#	8(7,8.5)#
* $P < 0.05$ , VS. day 1, day 3, day 5 of SCI group						
# $P < 0.05$ , VS. 8 h, 12 h, day 1, day 2 of SCI group						

### Panx-1 mRNA expression in rats after SCI

The expression changes of Panx-1 at 8 h, day 1, day 2, day 3 and day 5 after SCI were detected by Real-time PCR. As shown in Table 4 and Fig. 3, the expression of Panx-1 mRNA in the SCI group increased significantly with the extension of injury time, which was significantly different from that in the NC group and Sham group at each time point ( $P < 0.05$ ). The expression of Panx-1 mRNA in SCI group reached a

peak at day 2 after injury, and then decreased slowly. Compared with the SCI group at 8 h, day 1, day 3 and day 5, the expression of Panx-1 mRNA in SCI group at day 2 was increased with statistically significant difference ( $P < 0.05$ ).

**Table 4.** Expression of Panx-1 mRNA in each treatment group (n = 5,  $x \pm s$ )

Group	-	8 h	D 1	D 2	D 3	D 5
NC	1	-	-	-	-	-
Sham	-	1.2652 ± 0.091	1.2794 ± 0.056	1.3228 ± 0.1	1.2298 ± 0.084	1.3087 ± 0.078
SCI	-	1.6029 ± 0.08	2.3667 ± 0.095	3.9846 ± 0.2	2.9885 ± 0.113	2.3762 ± 0.125

## Expression of Panx-1 protein in rats after SCI

Western Blot was used to detect the changes of Panx-1 protein expression at 8 h, day 1, day 2, day 3 and day 5 after SCI in rats. The results were shown in Fig. 4 and Fig. 5. The expression of Panx-1 protein in SCI group increased significantly with the extension of injury time, and there were significant differences compared with the NC group and Sham group at each time ( $P < 0.05$ ). The expression of Panx-1 protein in the SCI group reached a peak at day 2 after SCI, and then decreased slowly. Compared with the SCI group at 8 h, day 1, day 3 and day 5, the expression of Panx-1 protein in the SCI group at day 2 was significantly increased ( $P < 0.05$ ). Combined with the results of Real-time PCR, the changes of Panx-1 in protein level were consistent with the gene level at the above time points after SCI, and were consistent with the significant decline of rat hind limb function score at day 2 after SCI in BBB score.

## The correlation between Panx-1 expression and the BBB score in rats after SCI

After SCI, the expression of Panx-1 protein increased gradually with the extension of time, and it reached a peak at day 2, and then decreased slowly. BBB score of hind limb motor function of rats declined suddenly and significantly at day 2 after SCI, and then gradually increased. The above results suggested that there was a significant correlation between the expression of Panx-1 protein and the BBB score of the motor function of the hind limbs of rats at day 1, day 2, day 3 and day 5 after SCI. Spearman rank correlation analysis showed that at the above time points after SCI, the expression of Panx-1 protein was negatively correlated with the BBB score of hind limb motor function of rats, the specific statistical values were shown in Table 5.

Table 5  
Spearman rank correlation analysis between Panx-1  
and BBB score

Spearman	D 1	D 2	D 3	D 5
$r_s$	-0.973	-0.895	-0.889	-0.973
P	0.005	0.04	0.044	0.005

## Discussion

Panx-1 is a homomeric membrane semi-channel Located in the plasma membrane and endoplasmic reticulum<sup>[15]</sup>, mediating a variety of physiological functions such as intracellular ATP release, intracellular  $Ca^{2+}$  transfer, blood flow regulation and cellular immune response. ATP-mediated post-SCI inflammatory response is one of the important mechanisms of secondary injury. Studies have shown that after a few minutes of hypoxia in rats, the level of ATP in nerve cells significantly decreased, accompanied by the outflow of ATP into the intercellular space<sup>[16]</sup>. The low concentration of ATP can act as a chemokine of microglia<sup>[17]</sup>, guiding microglia to the damaged site, and the expression of ATP-specific P2 × 7 receptor on microglia is up-regulated<sup>[18]</sup>. These results suggested that ATP activation of P2 × 7 receptor caused the opening of P2 × 7 plasma membrane pores, released a large number of inflammatory mediators, induced inflammatory response after SCI<sup>[19]</sup>, and leads to secondary damage. At the same time, ATP can also activate MAPK or other signaling pathways through P2 × 7 receptor<sup>[20]</sup>, mediating cell inflammation and apoptosis, which is the important mechanism of ischemia-reperfusion injury.  $Ca^{2+}$  is closely related to neuronal cell apoptosis. The increased concentration of  $Ca^{2+}$  in neurons can trigger arachidonic acid metabolism cascade and activate the xanthine oxidation system through oxidative stress<sup>[21]</sup>, causing the formation of a large number of free radicals, forming a vicious cycle, and finally activating the  $Ca^{2+}$  dependent endonuclease, initiating the neuronal apoptosis process, leading to neuronal apoptosis<sup>[22]</sup>.

In this study, we used modified Allen's method and NYU impact-III hammer to establish the rat SCI model, and applied the BBB score of hind limb motor function to observe the degree of nerve injury and recovery of rats after SCI. The results showed that although the BBB score of SCI group gradually recovered to different degrees with the extension of injury time, it was still significantly lower than that of Sham group and NC group. In the process of BBB score gradually rising after SCI, BBB score significantly decreased at day 2 after injury, even lower than the level of day1 after injury. It was not until day 3-day 5 after the injury that the function of the hind limb of the rats began to recover significantly. This indicated that the rats after SCI can repair the damage spontaneously under the action of the central pattern generator (CPG) neural network<sup>[23]</sup>. However, BBB score of day 2 declined again after SCI, which should be considered as the peak of spinal cord edema caused by SCI secondary injury. Huang T<sup>[24]</sup> has observed that the BBB score of motor function basically fluctuated in a stable state and was much lower than the normal value in 14–28 days after SCI, which further explained the severity and irreversibility of SCI on nerve cell injury.

This phenomenon was closely related to the excessive proliferation of glial cells with scar formation, the excessive secretion of neurosuppressive factors, and the obvious inhibition of axonal regeneration.

After SCI, the expression of Panx-1 in rats significantly increased with the time extension compared with that in NC group and Sham group, reached a peak at day 2, and then decreased slowly. Moreover, the changes of Panx-1 in gene level and protein level were basically the same on day 1, day 2, day 3 and day 5 after SCI. This proved that the expression of Panx-1 in rat spinal cord had a certain regularity and adjustability after SCI, which makes it possible to regulate the expression of Panx-1 by genetic engineering technology.

After SCI, the expression of Panx-1 reached a peak at day 2, which was consistent with the significant decline of BBB score at the same time. In order to further study the correlation between Panx-1 protein expression changes and BBB score of hind limb motor function, we carried out correlation analysis. And the results showed that Panx-1 protein expression changed with time at day 1, day 2, day 3 and day 5, which had significant negative correlation with BBB score. This was consistent with the fact that secondary injuries of SCI such as ischemia, hypoxia, cell apoptosis, immune inflammatory response, excitatory toxicity, free radical damage and lipid peroxidation reached the peak of spinal cord edema 2–3 days after the injury, so the BBB score of day 2 after SCI declined significantly. The characterization of Panx-1 protein expression was negatively correlated with BBB score, which proved that Panx-1 has an important effect on the recovery of nerve function after SCI and Panx-1 might be one of the important mechanisms of secondary injury after SCI.

In conclusion, Panx-l proteins are distributed in the nervous system where they formed homomeric semi-channels, regulating the balance of  $\text{Ca}^{2+}$  and ATP in and out of the cell, and mediating cell apoptosis and inflammatory response in the activated state. The stimulation of SCI significantly up-regulated Panx-l expression in rats, and reached the peak at day 2 after SCI, while the motor function of hind limbs of rats significantly deteriorated, showing a significant negative correlation. Therefore, Panx-l might be one of the important mechanisms of the secondary injury after SCI. It is of great significance to further study how to block Panx-l semi-channel mediated cell apoptosis and inflammatory response for the treatment of the secondary injury of SCI, and it is a possible therapeutic target to improve the neurological function after SCI.

## Abbreviations

Not applicable

## Declarations

## Availability of data and materials

Data supporting the results reported in a published article can be found. Please contact author for data requests.

## Competing interests

The authors declare that they have no competing interests.

### Consent for publication

Not applicable

### Ethics approval and consent to participate

This study was approved by the ethics review board in the First Affiliated Hospital of Fujian Medical University, Fuzhou, China.

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## Authors' contributions

The authors' contributions to this study were as follow: study design, YH, JL, JHL, Data collection, YH, JL, Statistical analysis YH, JL, and XWC, Manuscript writing by all authors. None of authors had a personal or financial conflict of interest.

## Acknowledgments

Not applicable.

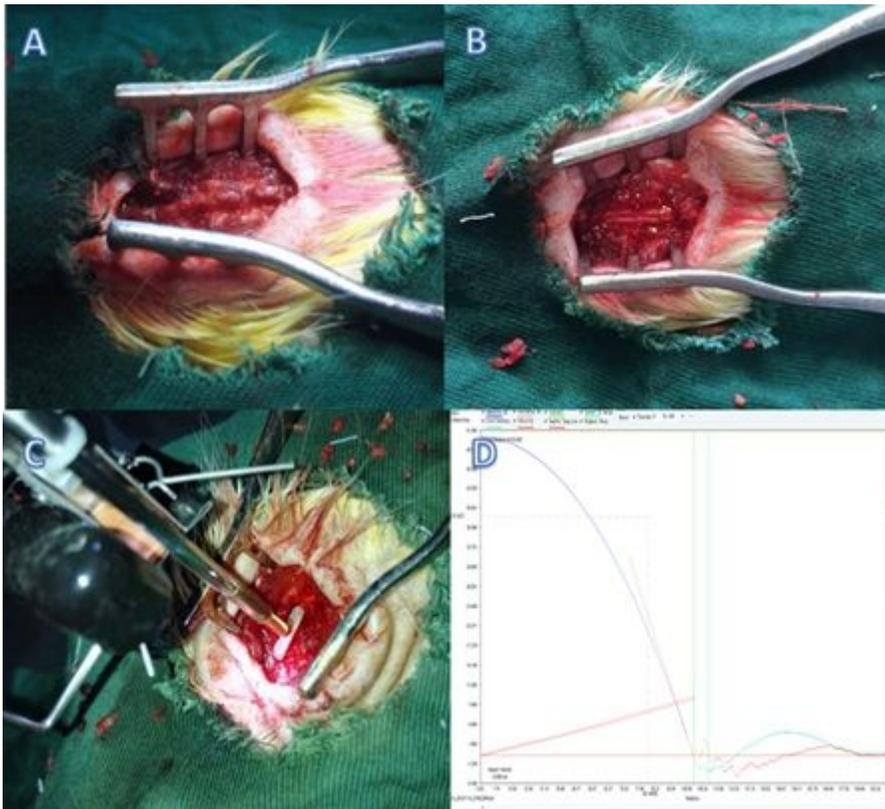
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## Figures



**Figure 1**

Animal model of SCI aA: Exposure of the T8-T12 lamina. bB: Exposure of the spinal cord. cC: Thin sheets of aluminum are placed on the surface of the spinal cord. dD: parameters and curves are recorded by the NYU impact-III strike computer system.

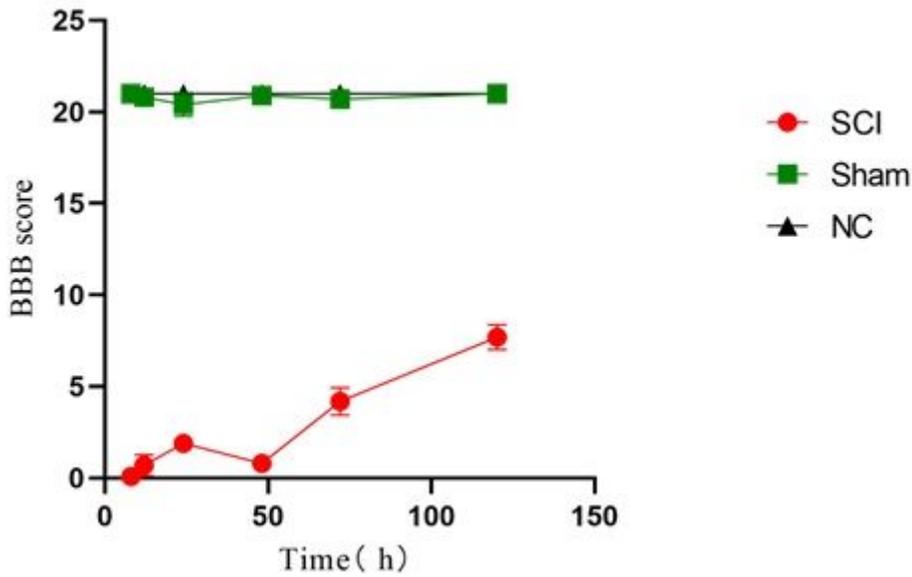


Figure 2

The BBB scores of each group varied with time

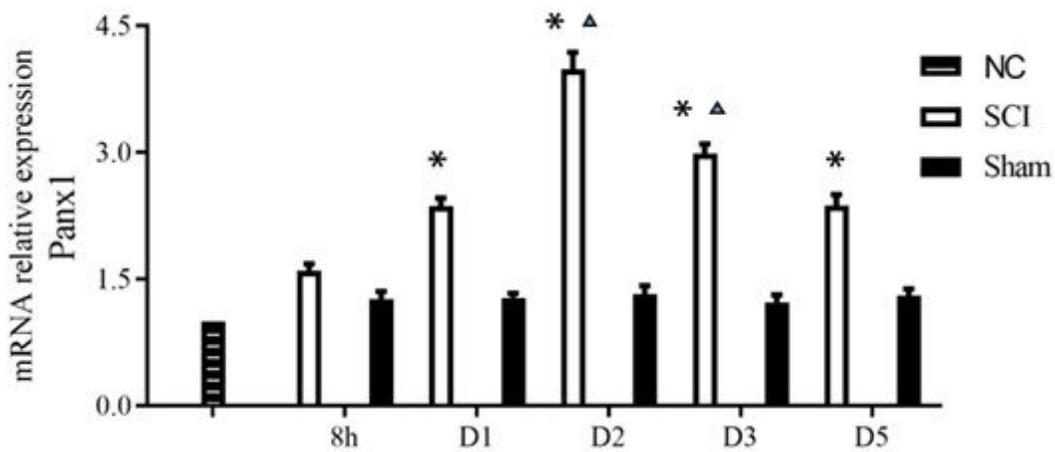
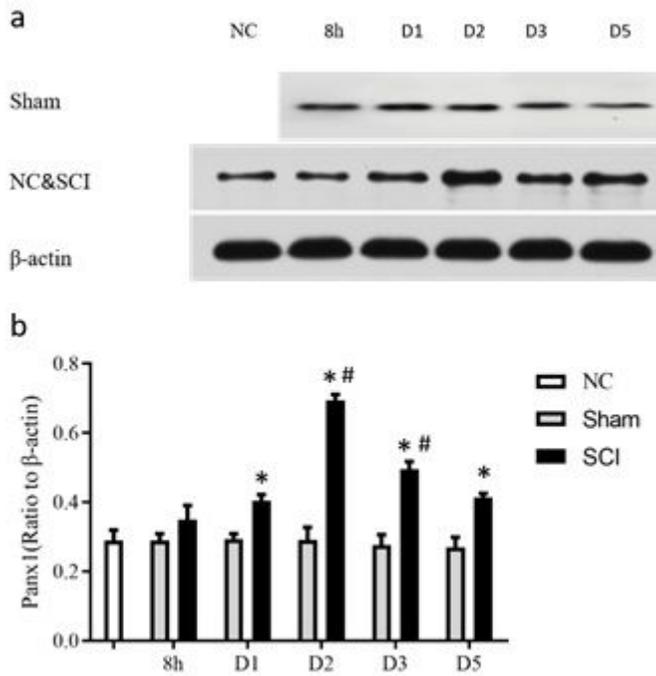


Figure 3

The expression of Panx-1 mRNA in each group changed with time (We used the normal group as the control, so the value of the NC group was 1, which was showed at the beginning.) \*P < 0.05, VS. NC group, Sham group at each time point P < 0.05, VS. 8 h-day1-day5 of SCI group P < 0.05, between



**Figure 4**

The expression of Panx-1 protein in each group changed with time after SCI (In the NC group of 5 rats, spinal cord was directly taken for detection, and there was no time change, so the value was showed at the beginning.) a: Representative Western Blot picture showing Panx-1 expression for each time point. b: Quantitative analysis of relative expression of Panx-1 protein for each time point. \* $P < 0.05$ , VS. NC group, Sham group at each time point # $P < 0.05$ , VS. 8 h, day 1, day 5 of SCI group # $P < 0.05$ , between