Preconditioning crush increases the survival rate of motor neurons after spinal root avulsion

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Abstract

In a previous study, heat shock protein 27 was persistently upregulated in ventral motor neurons following nerve root avulsion or crush. Here, we examined whether the upregulation of heat shock protein 27 would increase the survival rate of motor neurons. Rats were divided into two groups: an avulsion-only group (avulsion of the L_4 lumbar nerve root only) and a crush-avulsion group (the L_4 lumbar nerve root was crushed 1 week prior to the avulsion). Immunofluorescent staining revealed that the survival rate of motor neurons was significantly greater in the crush-avulsion group than in the avulsion-only group, and this difference remained for at least 5 weeks after avulsion. The higher neuronal survival rate may be explained by the upregulation of heat shock protein 27 expression in motor neurons in the crush-avulsion group. Furthermore, preconditioning crush greatly attenuated the expression of nitric oxide synthase in the motor neurons. Our findings indicate that the neuroprotective action of preconditioning crush is mediated through the upregulation of heat shock protein 27 expression and the attenuation of neuronal nitric oxide synthase upregulation following avulsion.

Introduction

Spinal nerve root avulsion in adult rodents induces retrograde degeneration of motor neurons, leading to subacute death of motor neurons[1-6]. The molecular mechanisms of this degeneration and cell death are still not fully understood. One consensus is that neural cytotoxic molecules such as superoxide and glutamine accumulate[7-14] and neurotrophic factors, such as glial cell line-derived[15-16] and brain-derived[10, 17-19] neurotrophic factors, become depleted. Nitric oxide, mainly produced by neuronal nitric oxide synthase (nNOS) in the central nervous system, is considered a modulator of neurotransmission as well as a protector against neuronal death from several death stimuli[20]. However, besides this protective effect, nitric oxide is also cytotoxic and involved in neuronal degeneration or death after spinal nerve injury[2, 7, 20-23].

Recent evidence indicates that heat shock proteins are critically involved in protection against the cytotoxicity induced by reactive nitrogen species[23]. Among these proteins, special attention has been paid to a 27 kDa heat shock protein (HSP27) that is constitutively expressed in some populations of neurons. HSP27 plays an important role in cellular defense mechanisms[24-26]. A previous study showed that, 1 week after avulsion of the spinal nerve root, small motor neurons (< 500 μm^2) negative for HSP27 immunoreactiv-

ity died and only large (> 500 μm^2) HSP27-positive motor neurons survived in the spinal cord ventral horn[3]. This was followed by the induction of nNOS in the surviving large motoneurons, which showed a significant upregulation of HSP27. Furthermore, enhancement of HSP27 expression in motor neurons was observed after mild crush of the spinal nerve root, indicating that upregulation of HSP27 might be a protective response in neuronal cell bodies following the injury of peripheral axons[3]. But the effect on the survival rate of motor neurons has not been assessed after crushing before spinal root avulsion.

Here, we investigate whether preconditioning crush can increase the survival rate of motor neurons. We examined the effect of spinal nerve root crush on neuronal survival rate in spinal cord ventral horn at 2, 3 and 5 weeks after spinal nerve root avulsion. We also quantitatively compared the time courses of HSP27 and nNOS expression between the avulsion-only and crush-avulsion groups.

Results

Quantitative analysis of experimental animals

A total of 36 Wistar rats of both sexes, 4–6 weeks old, were randomly divided into an avulsion-only group and a crush-avulsion group (18 rats per group). The avulsion-only
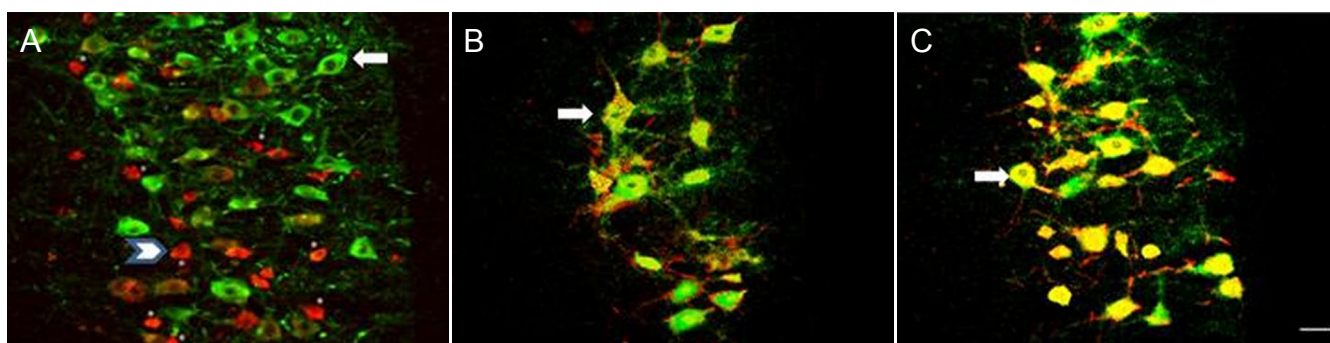
Figure 1 Effect of preconditioning crush on the expression of heat shock protein 27 (HSP27, green) and choline acetyltransferase (ChAT, red) in ventral motor neurons following L$_4$ nerve root avulsion.
Confocal images of horizontal sections through the L$_4$ segment of spinal cord 5 weeks after L$_4$ nerve root avulsion, double labeled using FITC (488 nm excitation wavelength) and Texas red (568 nm). Two types of the ventral motor neurons can be defined: large motor neurons with an angular or polygonal profile (some of which are indicated by arrows), and small motor neurons with an oval or ellipsoid profile (some of which are indicated by arrowhead). (A) Control side. The former is positive for both HSP27 and ChAT (yellow or yellowish green), whereas the latter is only positive for ChAT (red). (B) Lesion side, 5 weeks after L$_4$ nerve root avulsion only. (C) Lesion side, 5 weeks after L$_4$ nerve root avulsion with preconditioning crush 1 week prior to avulsion; number of surviving large motor neurons in the ventral horn is higher than that in the avulsion-only group, and the expression of HSP27 is stronger than that in the avulsion-only group. FITC: Fluorescein lsothiocyanate. Scale bar: 50 μm.

group underwent avulsion of the L$_4$ nerve root, while the crush-avulsion group was subjected to the same avulsion but 1 week prior to the avulsion also underwent crushing of the same nerve outside the vertebra. All 36 rats survived the surgery and were included in the final analysis.

Survival of HSP27- and choline acetyltransferase-immunoreactive ventral motor neurons
The number and size of motor neuron cell bodies was determined in the avulsion-only and crush-avulsion groups using double immunofluorescence labeling of HSP27 and choline acetyltransferase. On the intact control side of both groups, at all time points, small motor neurons (< 500 μm^2) were positive for choline acetyltransferase, while large motor neurons (> 500 μm^2) were double-positive for choline acetyltransferase and HSP27 (Figure 1A). Some ventral motor neurons, mainly of the large type, survived at all time points on both sides in the avulsion-only (Figure 1B) and crush-avulsion (Figure 1C) groups.

Preconditioning crush of the nerve root protected the spinal cord ventral motor neurons from death caused by nerve root avulsion
Nissl fluorescence staining and cell counts showed that there was no significant difference in the number of Nissl-positive ventral horn motor neurons on the intact control side of the L$_4$ segment between the two groups at 2, 3 or 5 weeks after avulsion (Figure 2A). However, at each time point, the number of Nissl-positive neurons in the injury side was significantly lower in the avulsion-only group than in the crush-avulsion group ($P < 0.05$; Figure 2B). The number of ventral horn motor neurons in the avulsion-only group was $56.5 \pm 3.2\%$, $53.7 \pm 3.1\%$ and $49.8 \pm 2.8\%$ at 2, 3 and 5 weeks, respectively, while that in the crush-avulsion group was $63.5 \pm 3.4\%$, $61.8 \pm 2.9\%$ and $57.9 \pm 3.2\%$. The large motor neurons were particularly abundant in the ventral horn on the injury side, although they seemed to be

undergoing light atrophy (Figure 2A).

Preconditioning crush upregulated HSP27 expression and attenuated upregulation of nNOS expression in the spinal cord ventral horn neurons following nerve root avulsion
Some motor neurons on the intact control side of the ventral horn expressed HSP27-immunofluorescent signals at all time points (Figure 3A), consistent with a previous report[27]. HSP27 expression was upregulated on the injury side in both groups (Figure 3A), but the degree of upregulation was significantly higher in the crush-avulsion group than in the avulsion-only group ($P < 0.01$), as shown by a higher number and density of HSP27-positive neurons (Figure 3B, C). The number of HSP27-immunoreactive neurons on the injury side of the crush-avulsion group at 2, 3 and 5 weeks was $63.7 \pm 3.4\%$, $58.9 \pm 3.0\%$ and $56.3 \pm 3.2\%$ respectively, while that in the avulsion-only group was $56.4 \pm 3.2\%$, $52.3 \pm 3.1\%$ and $49.8 \pm 2.9\%$ (Figure 3B).

In contrast, at all time points, immunoreactivity of nNOS was attenuated in the crush-avulsion group compared with the avulsion-only group (Figures 4, 5). Immunofluorescent staining of nNOS showed that ventral horn neurons in the intact control side of the two groups expressed only faint nNOS (Figures 4, 5), whereas in the injury side, nNOS-immunoreactive neurons were easily detected at the ventral horn in both groups. The preconditioning crush inhibited the upregulation of nNOS expression in the spinal cord ventral horn neurons (Figures 4, 5). Quantitative analysis revealed that the increase in the number of nNOS-immunoreactive neurons in the crush-avulsion group was lower than that in the avulsion-only group at 2, 3 and 5 weeks ($P < 0.05$).

Finally, among the surviving HSP27-immunoreactive motor neurons in the ventral horn at 2, 3 and 5 weeks, there were a greater number of large (> 500 μm^2) than small (< 500 μm^2) neurons in the two groups (Figure 6). An intrinsic relationship between HSP27 and nNOS expression was demonstrated by a negative correlation between the number

A

B
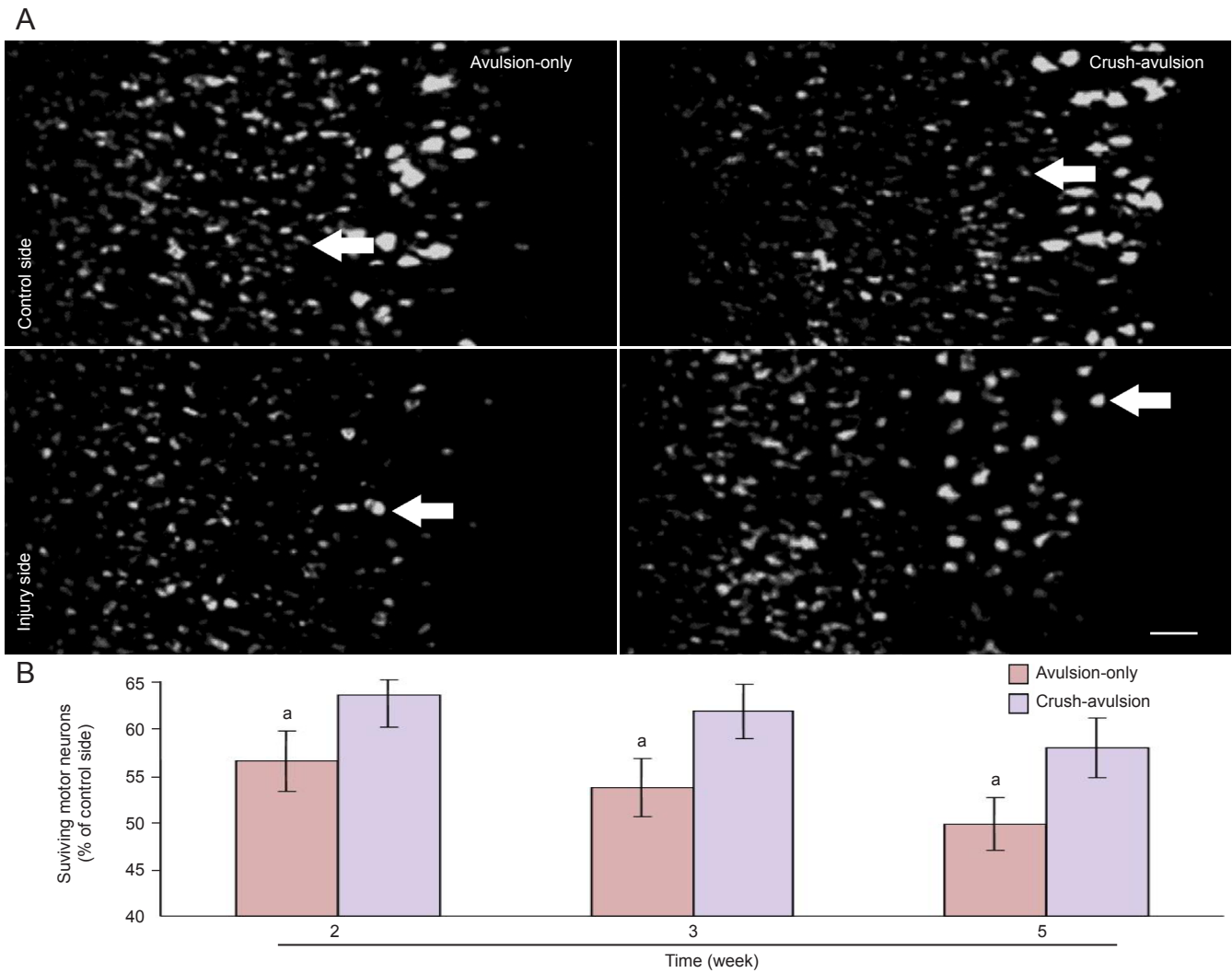
Figure 2 Effect of preconditioning crush on the survival of ventral motor neurons following L₄ nerve root avulsion.
(A) Nissl-stained horizontal sections through the ventral horn of the L_4 segment from the control side (upper panel) or the injury side (bottom panel) 5 weeks after L_4 nerve root avulsion in rats that underwent avulsion only (left panel) or preconditioning crush 1 week prior to avulsion (right panel). Motor neurons are indicated by arrows and observed under a fluorescence microscope. Scale bar: 100 μm.
(B) Number of surviving motor neurons in the spinal cord L_4 segment in the crush-avulsion and avulsion-only groups. Values represent percentages of the total Nissl-stained motor neurons in the contralateral side of the same spinal segment and data are expressed as mean ± SEM. $^aP < 0.05$, *vs.* crush-avulsion group. There were six rats per group per time point.

of HSP27-immunoreactive and nNOS-immunoreactive neurons ($r = 0.958$, $P < 0.001$; Table 1).

Discussion

We have shown that the time course of ventral motor neuron loss after nerve root avulsion is partially prevented by preconditioning crush. In accordance with previous quantitative analysis results[2-3], our statistical data indicated a progressive decline in the number of Nissl-stained motor neurons following avulsion. Severe loss of motor neurons was observed 2 weeks after avulsion, and neuronal loss progressed steadily and slowly. The extent of motor neuronal loss was significantly attenuated in the crush-avulsion group at each time point examined. Even 5 weeks after avulsion, 57.9% of the ventral motor neurons had survived. We therefore conclude that preconditioning crush has a persistent

effect on the maintenance of surviving motor neurons after nerve root avulsion.

We tentatively propose that the higher motor neuronal sur-

Table 1 Survival rate (%) of heat shock protein 27 (HSP27) and neuronal nitric oxide synthase (nNOS) immunoreactive motor neurons

Group	2 weeks		3 weeks		5 weeks	
	HSP27	nNOS	HSP27	nNOS	HSP27	nNOS
Avulsion-only (AO)	56.4	28.9	52.3	42.1	49.8	38.4
Crush-avulsion (CA)	63.7	23.5	58.9	35.2	56.3	29.1
(CA–AO)/AO%	12.9	−18.7	12.6	−16.4	13.1	−24.2

Values are expressed as percentages of the total motor neurons in the contralateral side of the same spinal segment. There is a negative correlation between (CA–AO)/AO% of HSP27 and nNOS using Pearson's correlation test ($r = 0.958$, $P < 0.001$).
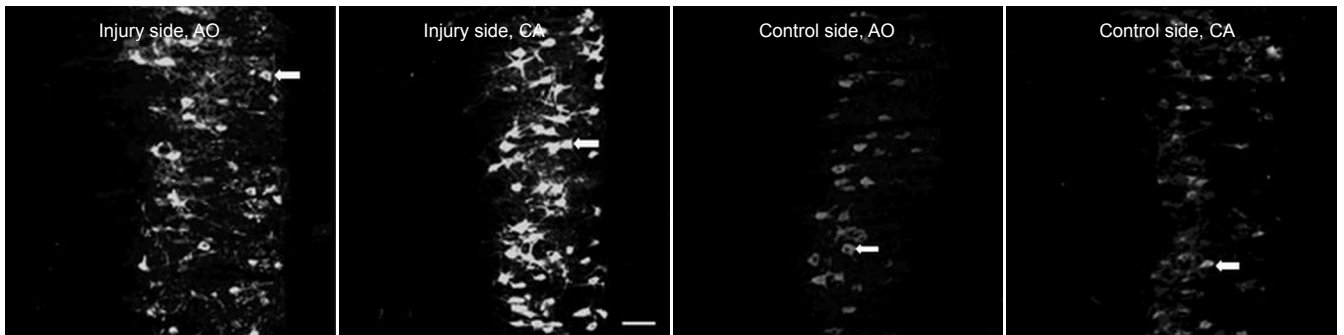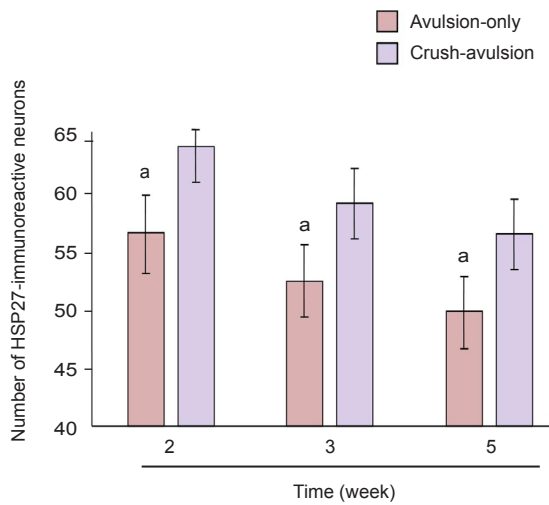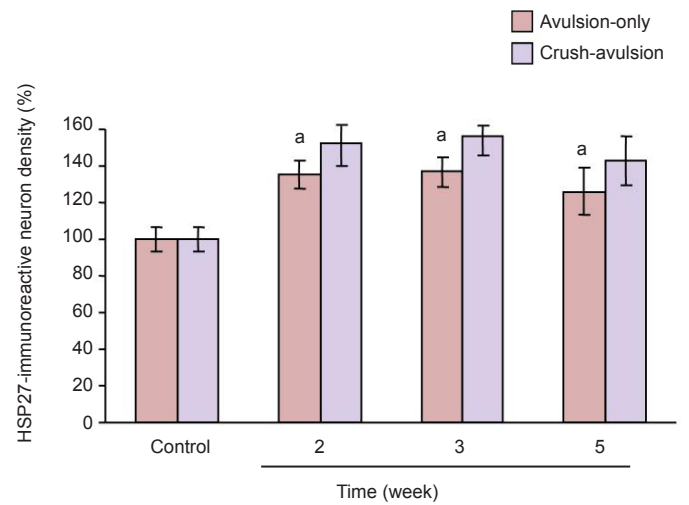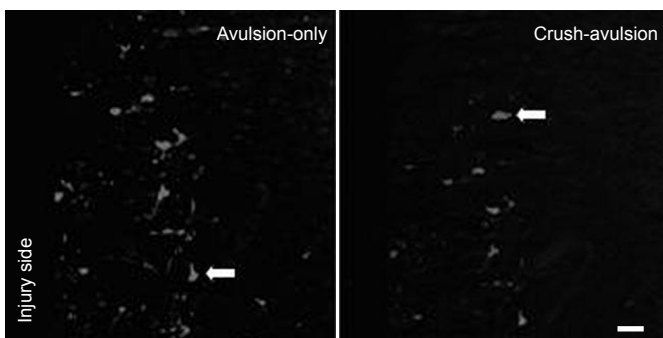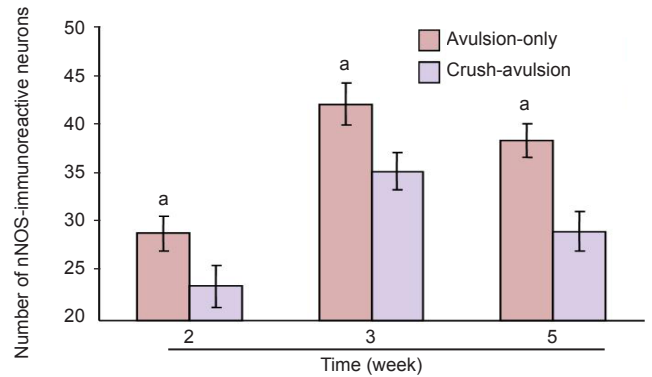
Figure 3 Effect of preconditioning crush on the expression of heat shock protein 27 (HSP27) in ventral motor neurons following L$_4$ nerve root avulsion.
(A) Confocal images of HSP27 immunofluorescence in horizontal sections through the ventral horn of the L$_4$ segment from the avulsion only or crush-avulsion groups 3 weeks after L$_4$ nerve root avulsion. Arrows: HSP27 positive motor neurons. Scale bar: 100 μm. AO: Avulsion only; CA: crush 1 week prior to avulsion. (B) Number of HSP27-immunoreactive neurons in the spinal cord ventral horn (injury side). (C) Time course of HSP27 immunoreactive motor neuron density in the avulsion-only group (black bars) and the crush-avulsion group (white bars). There were three animals per group per time point. A significant upregulation of HSP27-immunoreactive motoneurons can be seen in both groups after avulsion. Values are percentages (mean ± SEM) of the total number of motor neurons in the contralateral side of the same spinal segment. [a]$P < 0.01$, *vs.* crush-avulsion group (Student's *t*-test).

Figure 4 Effect of preconditioning crush on the expression of neuronal nitric oxide synthase (nNOS) in ventral motor neurons following the L$_4$ nerve root avulsion.
(A) Confocal images of immunofluorescence-labeled nNOS in horizontal sections through the ventral horn of the L$_4$ segment in the avulsion-only and crush-avulsion groups, 5 weeks after L$_4$ nerve root avulsion. Arrows: nNOS positive motor neurons. Scale bar: 100 μm. (B) Number of nNOS-immunoreactive neurons in the spinal cord ventral horn (injury side). [a]$P < 0.05$, *vs.* crush-avulsion group (Student's *t*-test). Data are presented as mean ± SEM.
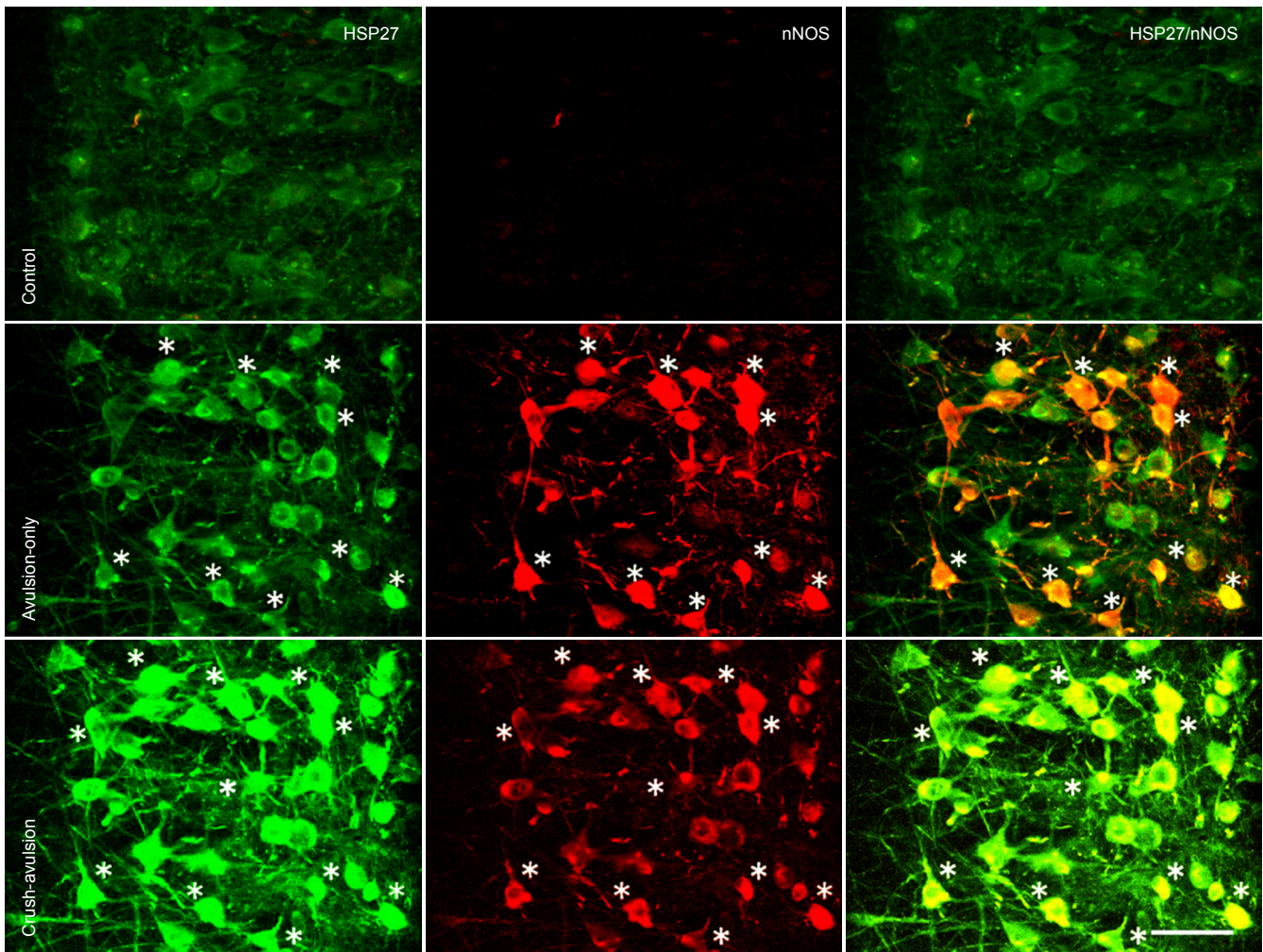
Figure 5 Effect of preconditioning crush on the expression of heat shock protein 27 (HSP27) and neuronal nitric oxide synthase (nNOS) in horizontal sections through the ventral horn of the L_4 segment following nerve root avulsion.
Confocal image of double immunofluorescent labeling with FITC (HSP27; green, 488 nm excitation wavelength) and Texas red (nNOS; 568 nm). Motor neurons in the control side (upper panel) of the two groups expressed low levels of nNOS, whereas in the injury side, nNOS-immunoreactive neurons were easily detected in the avulsion-only group (middle panel) and crush-avulsion group (lower panel). Asterisks indicate double nNOS- and HSP27-immunoreactive motor neurons. Scale bar: 50 μm.
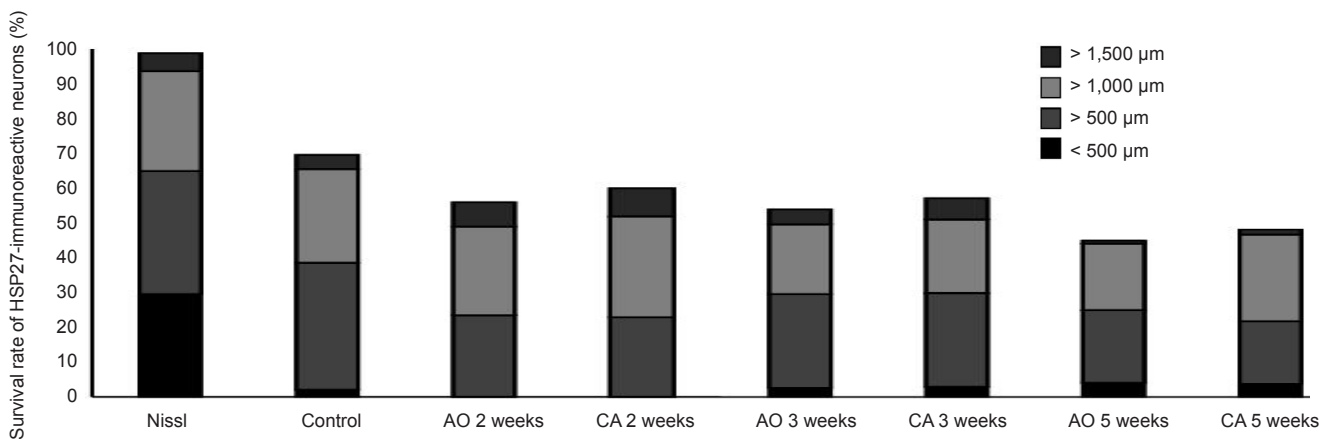
Figure 6 Time changes in cell body size in heat shock protein 27 (HSP27) immunoreactive motor neurons following nerve root crush prior to the avulsion of L_4 nerve root (CA) or avulsion alone (AO).
Most of the HSP27-immunoreactive motor neurons belonged to the large neuron group (≥ 500 μm^2 in size), while the HSP27-negative motor neurons belonged to the small neuron group (< 500 μm^2 in size). The small motor neurons disappeared 2 weeks after avulsion in both groups. There were three rats per time point. Values are expressed as percentages of the total Nissl-stained motor neurons in the contralateral side of the same spinal segment (considered to be 100%).

vival rate in the crush-avulsion group arises from a long-lasting and strong expression of HSP27. Previous studies have shown that HSP27 plays a role in promoting neuronal survival in the dorsal root ganglion after nerve axotomy[28-29] or nerve ligation[30]. Accumulating evidence[2-3] also demonstrates the persistently protective role of HSP27 in avulsed motor neurons, since all of the surviving large motor neurons continued to express HSP27 intensely over a long period. It is therefore conceivable that this protein might protect motor neurons from cell death. Neuronal protection was further demonstrated by a greater expression of HSP27, associated with a higher survival rate of motor neurons, in the crush-avulsion group compared with the avulsion-only group. Our previous study demonstrated an upregulation of HSP27 expression in ventral neurons by light nerve root crush[3]. Thus, the increase in HSP27 expression after mild nerve damage might protect neurons from degeneration and cell death caused by nerve root avulsion 1 week later. The induction of robust protection against noxious stimuli after a brief stress or toxic insult has been demonstrated in several models *in vitro* and *in vivo*[31-33]. For example, increasing endogenous HSP27 by heat shock preconditioning in cultured neonatal dorsal root ganglion neurons inhibits nerve growth factor withdrawal-induced apoptosis[33]. There are two temporally and mechanistically distinct types of protection afforded by preconditioning stimuli: acute and delayed preconditioning[34]. Although they share components of the signaling pathway, they differ in their requirements for new protein synthesis. The protective effect of acute preconditioning is short-lived protein synthesis mediated by post-translational protein modifications. Delayed preconditioning, however, requires new protein synthesis and the effects are sustained for days or weeks[34]. Based on the previously observed persistent upregulation of HSP27 expression following nerve root crush[3] and the long-lasting increase in HSP27 immunoreactivity in the crush-avulsion group in the present study, we hypothesize that the protection against crush lesions induced by preconditioning crush in motor neurons is mediated *via* the delayed preconditioning pathway. This long-term neuroprotective effect of HSP27 has also been demonstrated in another recent study[35].

We found that, following neonatal nerve crush, HSP27 overexpression *in vivo* provided substantial rescue of motor neurons 5–6 months after nerve injury. Furthermore, surviving motor neurons were able to regenerate and form motor units to preserve muscle function. These properties of HSP27 have considerable potential for improving long-term muscle function in motor neuron disorders.

How might endogenous HSP27 act to prevent or limit motor neuron degeneration after preconditioning crush? To address the underlying molecular mechanisms, we observed the time course of nNOS expression among the two groups and investigated the correlation between HSP27 and nNOS expression. Although nitric oxide has many functions following neurotrauma, it is generally accepted that the nitric oxide induced in motor neurons mediates motor neuron death, since the induction of nitric oxide occurs in avulsion models but not in other models of axotomy without cell death[3, 7, 36-37].

The motor neuronal toxicity of nitric oxide has been further demonstrated by the finding that nitroarginine, a specific inhibitor of nitric oxide synthase, significantly reduces the death of motor neurons following avulsion[7] and that nitric oxide mainly contributes to cultured motor neuron apoptosis induced by trophic factor deprivation *in vitro* [11].

In the present study, the number and staining density of nNOS-immunoreactive neurons was notably lower in the avulsion-only group than in the crush-avulsion group. There was a negative correlation between the number of neurons immunoreactive for HSP27 and for nitric oxide synthase after avulsion. Thus, it is reasonable to presume that the induction of HSP27 by preconditioning crush might attenuate upregulation of nNOS following crush to improve motor neuron survival.

Although we cannot provide direct evidence to elucidate their intrinsic relationship in the present study, we propose that persistent expression of HSP27 accompanied by nNOS may imply a keen competition in motor neuron survival between cytotoxic and cytoprotective systems. For example, Benn et al.[25] showed that the neuroprotective action of HSP27 is downstream of cytochrome c release from mitochondria and upstream of caspase-3 activation. On the other hand, Figueroa et al.[38] demonstrated that in cortical neurons nitric oxide toxicity is mediated by mitochondrial dysfunction. SNAP, a nitric oxide donor, induces apoptosis in these cells mainly by increasing p53 and inducing cytochrome C release, and activation of caspase-9 and caspase-3. Further study is necessary to elucidate how the molecular mechanism of HSP27 upregulation after preconditioning crush relates to the inhibition of nNOS following avulsion.

In summary, the present study is the first to demonstrate that loss of ventral motor neurons after nerve root avulsion is partially prevented by preconditioning crush. This injury tolerance might act through the induction of HSP27 by preconditioning crush, which inhibits the upregulation of nNOS to attenuate the cytotoxic effects of nitric oxide following crush. The present results open new avenues in the investigation of neuroprotective strategies for the treatment of motor neuron disorders in the future.

Materials and Methods

Design
A randomized, controlled animal experiment.

Time and setting
Experiments were performed from June 2011 to May 2012 at the Human Anatomy Laboratory, Nanjing Medical University, China.

Materials
A total of 36 Wistar rats of both sexes, aged 4–6 weeks, weighing 120–140 g, were purchased from the Experimental Animal Center of Jiangsu Province, China (license No. SYXK (Su) 2002-0013). The rats were housed at 20–26°C and 40–70% humidity in a 12 hour light/dark cycle, with free access

to water and food.

Experimental protocols were in strict accordance with the *Guidance Suggestions for the Care and Use of Laboratory Animals*, issued by the Ministry of Science and Technology of China[39].

Methods

Establishment of an animal model for lumbar nerve root avulsion

L_4 nerve root crush and avulsion were performed as described previously[2]. Briefly, rats were anesthetized with pentobarbital sodium salt (20 mg/kg). A left paramedial incision (about 2 cm) was made over the iliac crest. The left longissimus muscle was then split at the midline and the left transverse process of the L_5 vertebra was removed. In the avulsion-only group, the left L_4 nerve root (consisting of a motor and sensory root and dorsal root ganglion) was raised slightly and then pulled with steady and moderate traction. About 30 mm of the left L_4 mixed root was excised. The right nerve root was intact and used as the control.

In the crush-avulsion group, the left L_4 nerve root was crushed for 10 seconds at the exit point from the intervertebral foramen. One week later, the same nerve root was avulsed as described above. Crush and resection models resulted in little motor neuron loss and no expression of nNOS in motor neurons, but a significant increase of HSP27 was observed.

Perfusion and tissue processing

At 2, 3 and 5 weeks after surgery, six rats from each group were deeply anesthetized with ether and pentobarbital sodium salt (50 mg/kg), then perfused with 0.01 mol/L PBS (pH 7.4) and fixed by perfusion with 4% paraformaldehyde in 0.1 mol/L phosphate buffer (pH 7.4) through the ascending aorta. Under a stereomicroscope, the lumbar spinal cord with dorsal and ventral roots was carefully dissected. The avulsed L_4 dorsal and ventral nerve roots were confirmed, and the L_4 spinal cord segment was then dissected out using a pair of microscissors. The specimens were postfixed for 3 hours and subsequently stored in 30% sucrose solution at 4°C overnight.

The L_4 segment was cut horizontally from ventral to dorsal at 50 μm on a cryostat (Leica Microsystems, Wetzlar, Germany). Five serial sections from each animal were collected in 0.02 mol/L potassium phosphate-buffered saline (pH 7.4). The first section was used for fluorescent Nissl staining; the second and third sections were used for double-immunofluorescence staining of HSP27 with nNOS and with choline acetyltransferase, respectively. The protocol for each stain is described below.

Fluorescent Nissl staining

NeuroTrace fluorescent Nissl stains (Molecular Probes Inc., Eugene, Oregon, USA) were used to label the spinal cord ventral horn neurons. Sections were loaded on a slide, permeabilized in 0.1 mol/L PBS (pH 7.2) for at least 40 minutes and washed with PBS plus 0.1% Triton X-100 for 10 minutes followed by PBS twice for 5 minutes each. The NeuroTrace

stain, diluted 1:200 in PBS, was applied to the sections and incubated for 20 minutes at room temperature.

After washing once with PBS containing 0.1% Triton X-100, and twice with PBS, the sections were mounted onto glass slides and coverslipped with Gel Mount (Sigma, St. Louis, MO, USA). Staining was visualized with brightfield (cresyl violet) or epifluorescent (fluorescent staining) microscopy under an Olympus LSM-GB200 microscope (Olympus, Tokyo, Japan).

Immunofluorescent histochemical staining

Sections were stained for immunofluorescence of HSP27 or nNOS as described previously[2-3]. Briefly, non-specific binding sites were blocked overnight at 4°C by preincubation with 0.1% bovine serum albumin in potassium phosphate-buffered saline containing 0.5% Triton X-100. Primary antibodies were rabbit polyclonal antibody raised against murine HSP25/27 (1:1,500; Enzo Life Sciences Inc., Farmingdale, NY, USA) or sheep polyclonal antibody raised against murine nNOS (1:2,000; a generous gift from Dr. Emson (Kyushu University, Fukuoka, Japan) and were diluted in potassium phosphate buffered saline. Sections were incubated in primary antibody for 3 days at 4°C. As a negative control, preimmune serum was used in place of primary antibody. After washing, sections were incubated with FITC-conjugated swine anti-rabbit IgG (1:200; Chemicon, Temecula, CA, USA) or Texas red-conjugated donkey anti-sheep IgG (1:200; Chemicon) for 12 hours at 4°C. After washing with PBS, the sections were then mounted onto glass slides and coverslipped with Gel Mount.

For HSP27 and choline acetyltransferase double immunofluorescent labeling, a primary antibody mixture of rabbit polyclonal antibodies to HSP25/27 (1:1,500; StressGen, SPA-801) and rat monoclonal antibody to choline acetyltransferase (1:8; Boehringer Mannheim GMBH, Sandhofer Strasse, Mannheim, Germany) was used. Sections were then incubated in a secondary antibody mixture of FITC-conjugated swine anti-rabbit IgG (Dako, Glostrup, Denmark) and biotinylated goat anti-rat IgG (Chemicon). Texas red-conjugated streptavidin was used to visualize the biotin binding site. No difference in the morphology of any of the immunolabeled structures was observed between single and double labeling.

Counting of motor neurons

For each animal, motor neurons containing a clearly visible nucleus were counted in every fifth Nissl-stained, HSP27 and nNOS immunofluorescence-labeled section. The ratio of the number of motor neurons in the lesion side to that in the control side was expressed as a percentage of all surviving motor neurons. In immunofluorescence-labeled sections, the number of choline acetyltransferase- and HSP27-immunoreactive motor neurons on the ipsilateral side was counted separately in every fifth serial section.

The ratio of choline acetyltransferase- and HSP27-positive neurons to Nissl-stained motor neurons in the control side was expressed as a percentage of the surviving immunoreactive motor neurons.

Quantification of the size of HSP27-immunoreactive motor neurons

HSP27 was constitutively expressed in some motor neurons in the intact spinal cord, as indicated previously[27]. This labeling appeared to represent the complete figure of a motor neuron, so the size of HSP27-immunoreactive motor neurons on the lesion side was determined at 2, 3 and 5 weeks after nerve root avulsion using ImageJ (NIH software, Bethesda, Maryland, USA).

One of the criteria for including cells for measurement was that the nucleus be discernible. However, the fluorescence was bright and nuclei were difficult to visualize in some cases. In these cases, motor neurons were checked by scanning through different planes, and were included if they contained a major part of the cell body. Fifty to 100 spinal motor neurons were measured at each time point.

The upregulation of HSP27 expression on the lesion side and the density of HSP27 immunoreactivity was quantified by measuring the number of pixels per μm^2 in the area of the motor neuron displayed on the monitor of a computer running NIH Image version 1.62 software. The data from both groups were compared, and the density of HSP27 immunoreactivity in the control side was taken as 100%.

Data analysis

Immunofluorescent micrographs in each section were captured by confocal laser scanning microscopy (LSM-GB200 Olympus, Tokyo, Japan) as described previously[40]. Briefly, the optical section 2 μm from the upper surface was used as the lookup section, and sections 4–24 μm from the surface were reference sections after taking the lost caps bias into consideration[40].

The numbers of Nissl-positive, HSP27-immunoreactive and nNOS-immunoreactive motor neurons containing a clearly visible nucleus were counted in the spinal cord ventral horn of the L_4 segment.

The percentage of surviving motor neurons on the injured side was calculated by dividing the number of Nissl-positive neurons on the lesion side by that on the control side and multiplying by 100.

Statistical analysis

Excel 2007 (Microsoft Corporation, Redmond, Washington, USA) was used for statistical analysis. Data were presented as mean ± SEM. Statistical comparisons were made using Student's *t*-test, with $P < 0.01$ considered significant. The Pearson correlation test was used to investigate the relationship between HSP27 and nNOS expression, with $P < 0.05$ considered significant.

Author contributions: *Li L participated in the study concept and design, experimental implementation, data integrity and analysis, and manuscript writing. Zuo YZ participated in the study implementation. He JW was in charge of the study concept and design, served as principle investigator, and was responsible for manuscript authorization. All authors approved the final version of the manuscript.*

Conflicts of interest: *None declared.*

Peer review: *In this study, we avulsed spinal lumbar nerve roots in the rats, which caused a large number of neuronal degeneration and death in anterior horn, in an effort to compare the effect of avulsion-only versus crush-avulsion on the survival of motor neurons and the expression of heat shock protein 27. Experimental findings indicate that crush + avulsion of spinal nerve roots led to increased survival of motor neurons, and the underlying mechanism is mediated by the upregulation of heat shock protein 27 expression and the down-regulation of nitric oxide synthase expression.*

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