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Application of the sodium hyaluronate-CNTF scaffolds in repairing adult rat spinal cord injury and facilitating neural network formation

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The present study aimed to explore the potential of the sodium hyaluronate-CNTF (ciliary neurotrophic factor) scaffold in activating endogenous neurogenesis and facilitating neural network re-formation after the adult rat spinal cord injury (SCI). After completely cutting and removing a 5-mm adult rat T8 segment, a sodium hyaluronate-CNTF scaffold was implanted into the lesion area. Dil tracing and immunofluorescence staining were used to observe the proliferation, differentiation and integration of neural stem cells (NSCs) after SCI. A planar multielectrode dish system (MED64) was used to test the electrophysiological characteristics of the regenerated neural network in the lesioned area. Electrophysiology and behavior evaluation were used to evaluate functional recovery of paraplegic rat hindlimbs. The Dil tracing and immunofluorescence results suggest that the sodium hyaluronate-CNTF scaffold could activate the NSCs originating from the spinal cord ependymal, and facilitate their migration to the lesion area and differentiation into mature neurons, which were capable of forming synaptic contact and receiving glutamatergic excitatory synaptic input. The MED64 results suggest that functional synapsis could be established among regenerated neurons as well as between regenerated neurons and the host tissue, which has been evidenced to be glutamatergic excitatory synapsis. The electrophysiology and behavior evaluation results indicate that the paraplegic rats' sensory and motor functions were recovered in some degree. Collectively, this study may shed light on paraplegia treatment in clinics.

spinal cord injury, endogenous neural stem cells, neural network reconstruction, CNTF, new born neuron

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INTRODUCTION

The spinal cord, as an important part of the central nervous system (CNS), participates in multiple sensory and motor functions that control the body (Fink et al., 2014). The ascending and descending paths of the spinal cord form the main part of the spinal cord neural network, and serve as a critical link to connect the peripheral nervous system and different areas of the brain as well as neural nuclei (Dickson and Gilestro, 2006; Liang et al., 2012). Spinal cord injury (SCI) may cause the patients to lose sensory and motor functions beneath the lesion interface, i.e. paraplegia, because of the destruction of the neural network that structured by the ascending and descending paths in the spinal cord (Maegele et al, 2005). Various methods have been attempted for

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adult mammals SCI treatment, including cell transplantation (Sharp et al., 2010; Lowry and Temple, 2007), delivery of exogenous neurotrophic factors (Tuinstra et al., 2012; Alto et al., 2009), and biomaterial scaffolds (Horn et al., 2007; Yang et al., 2015; Wang et al., 2014). Cell transplantation is usually restricted by such issues as immunologic rejection, tumor formation and cell sources. Delivery of neurotrophic factors is constantly circumscribed by the relatively short half-life under the physiological condition and the limited administration methods (Martin Bauknight et al., 2012). In the present study, we integrated the ciliary neurotrophic factor (CNTF) into sodium hyaluronate to prepare a sodium hyaluronate-CNTF scaffold slow-releasing system, which was capable of slowly releasing CNTF at 37°C for up to 105 days (Wang et al., 2014). Next, we implanted this scaffold into the lesioned area of the adult rat spinal cord to observe its repair effect on the completely sectioned thoracic spinal cord of adult rats as well as its potential in facilitating the rebuilding neural network after SCI.

RESULTS

The sodium hyaluronate-CNTF scaffold facilitated the generation and maturation of new born neurons after SCI

Thirty days after operation, it was observed under an anatomical microscope that, in the sodium hyaluronate-CNTF scaffold group, the semi-transparent and smooth-surfaced regenerated tissue had bridged the gap (Figure 1A and B); in the lesion-control group, the lesioned area was filled with scar-like tissue (Figure 1C and D), which was non-transparent, with a rough and uneven surface.

Thirty days after operation, in the sodium hyaluronate-CNTF scaffold group, Nestin$^+$ and BrdU$^+$ cells were observed in the lesioned area, indicating that endogenous NSCs had been activated and entered the lesioned area (Figure 2). We also observed the new born Tuj1$^+$ & BrdU$^+$ immature neurons (Figure S1 in Supporting Information) and new born NeuN$^+$ & BrdU$^+$ mature neurons (Figure 3) in the lesioned area. While in the lesion-alone group, only a small amount of Nestin$^+$ & BrdU$^+$ cells were observed in the lesion area, with few new born Tuj1$^+$ & BrdU$^+$ immature neurons and NeuN$^+$ & BrdU$^+$ mature neurons (Figures S2–S4 in Supporting Information). Thirty days after operation, synapsin1-positive presynaptic components that were distributed in dots on these β-tubulin3$^+$ & BrdU$^+$ neurons fibers were observed in the lesion area of the sodium hyaluronate-CNTF scaffold group (Figure S5 in Supporting Information). The synapses formed between new born neurons included vGluT1$^+$ glutamatergic excitatory synapses (Figure S6 in Supporting Information), indicating that the sodium hyaluronate-CNTF scaffold could facilitate the activation of endogenous neural stem cells after SCI, and induce them to migrate to the lesion area and further differentiate into mature neurons.

The origin of new born neurons

As previously reported, ependymal cells in the adult spinal cord central canal are neural stem cells in the spinal cord. They can renew themselves *in vitro* and differentiate into neurons, astrocytes and oligodendrocytes (Barnabé-Heider et al., 2010; Shihabuddin et al., 2000). To clarify the origin of endogenous neural stem cells activated by the sodium hyaluronate-CNTF scaffold, we injected the Dil (a lipid membrane dye) into the lateral ventricle 3 days after operation, to label the intrinsic ependymal cells in the spinal cord. Next, we observed the migration and differentiation of Dil$^+$ cells at different time points after operation.

The coronal spinal cord section results show that, in adult rats, Dil$^+$ cells confined in the central canal and did not migrate to surrounding spinal cord tissue (Figure S7A and A1 in Supporting Information). After SCI, only a small number of Dil$^+$ cells migrated into the spinal cord parenchyma (Figure S7E and E1 in Supporting Information). On the contrary, in the sodium hyaluronate-CNTF scaffold group, a large number of Dil$^+$ migrating cells were observed in the spinal cord parenchyma outside the central canal. By comparing the numbers of Dil$^+$ cells at different sites from the rostral end among groups, we found that there existed a large number of Dil$^+$ cells at 5 mm from the rostral end; the farther away from

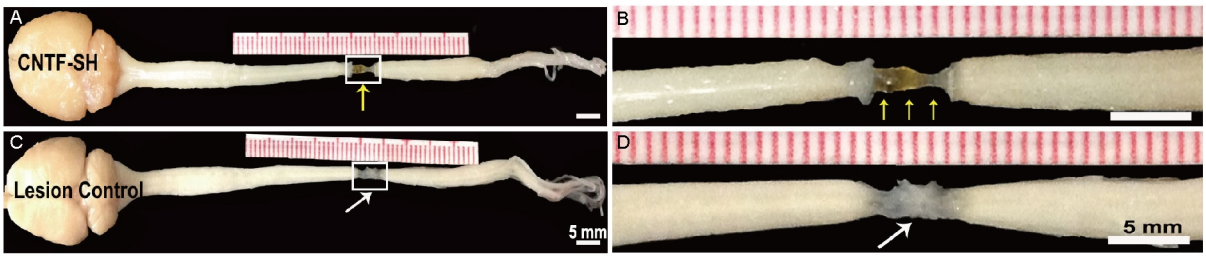

Figure 1 Dorsal view (under an anatomical microscope) of the rat brain and spinal cord in the sodium hyaluronate-CNTF scaffold group and lesion-control group. A, 30 days after implantation of the sodium hyaluronate-CNTF scaffold, new born spinal cord (indicated by the arrow) bridged the gap. B, Amplification of the boxed area in A. The new born spinal cord tissue is semi-transparent and smooth-surfaced. C, The spinal cord tissue 30 days after SCI in the lesion-control group. The arrow indicates scar tissue. D, Amplification of the boxed area in C. The scar tissue in the lesioned area is non-transparent and rough-surfaced.

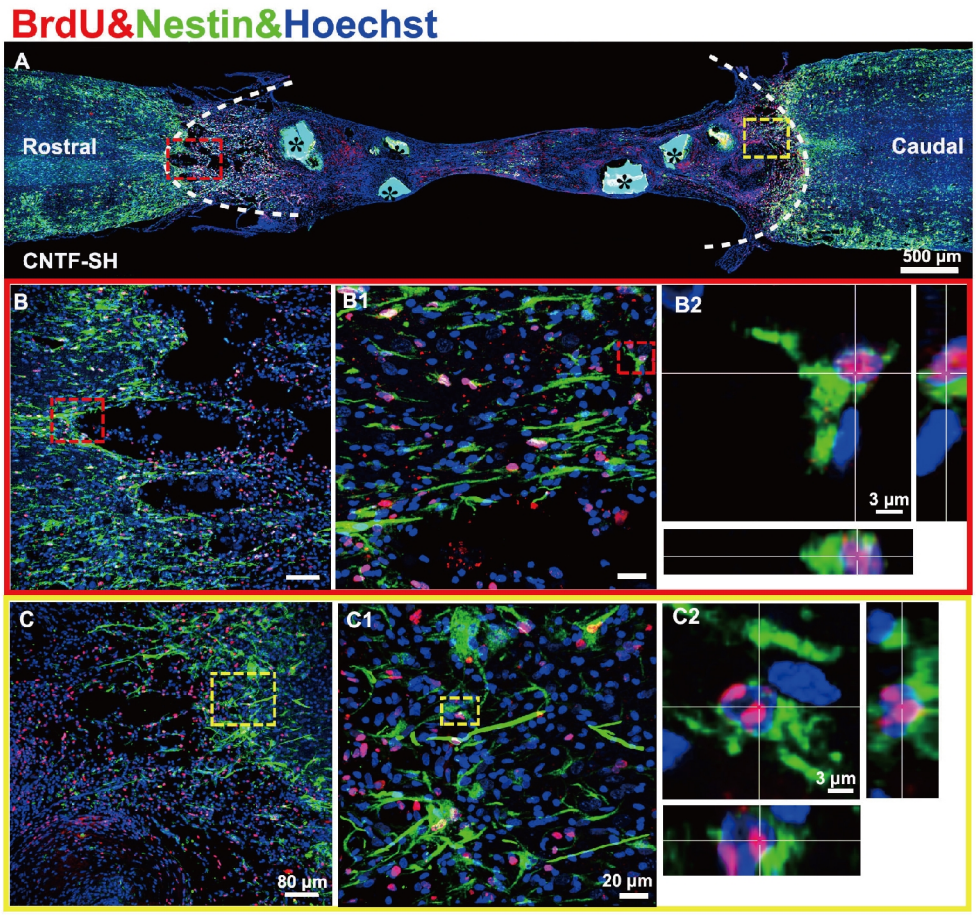

Figure 2 Thirty days after operation, activated NSCs were observed in the lesioned area of the sodium hyaluronate-CNTF scaffold group. A, Longitudinal spinal cord sections including the lesioned area, which were Nestin, BrdU and Hoechst immunofluorescence triple-labelled. B–B2, Continuous amplification of the red boxed area at the R (rostral) end of the lesioned area in A. C–C2, Continuous amplification of the yellow boxed area at the C (caudal) end of the lesioned area in A. Nestin$^+$ & BrdU$^+$ cells were observed, indicating that the implanted sodium hyaluronate-CNTF scaffold had activated endogenous NSCs, which then migrated to the lesioned area. * indicates the undegraded scaffold, and the white dotted line indicates the boundary between the lesioned area and host tissue.

the rostral end, the less the Dil$^+$ cells; at 30 mm from the rostral end, Dil$^+$ cells significantly reduced (Figure S7B–D in Supporting Information). Taken together, the implanted sodium hyaluronate-CNTF scaffold stimulated the activation and migration of Dil$^+$ NSCs originating from the ependymal. In the lesion-control group, only a small number of Dil$^+$ migrating cells could be detected, which had significant difference from that in the sodium hyaluronate-CNTF scaffold group (Figure S7G in Supporting Information).

Using immunofluorescence staining, we detected the differentiation status of Dil$^+$ cells that migrated to the lesioned area. In the sodium hyaluronate-CNTF scaffold group, 28 days after operation, a large number of Dil$^+$ & Nestin$^+$ cells were observed in the lesioned area (Figure 4); 56 days after operation, Dil$^+$ & Tuj1$^+$ immature neurons and Dil$^+$ & NeuN$^+$ mature neurons were observed at the middle and caudal end of the lesioned area, implicating that the activated endogenous NSCs could differentiate into mature neurons. In the lesion-control group, at different time points after operation, only a small number of Dil$^+$ & Nestin$^+$ could be observed, but almost no Dil$^+$ & Tuj1$^+$ immature neurons and Dil$^+$ & NeuN$^+$ mature neurons (data not shown). Collectively, the sodium hyaluronate-CNTF scaffold could significantly activate endogenous NSCs (i.e., ependymal cells), and recruit them to migrate to the lesioned area and differentiate into mature neurons.

The functional neural network formed by new born cells and its developmental characteristics

To determine the electrophysiological activity of these morphologically synapse-like structures detected at 8 weeks after operation, we used a planar multielectrode dish system (MED64; Alpha MED Scientific) to test the characteristics of the regenerated neuronal network formed in the sodium hyaluronate-CNTF scaffold group. Using pharmacologic compounds, we also determined whether the field potential recorded by the MED64 system in the lesioned area was transmitted by the synapses formed among regenerated neurons. When stimulating the caudal part of the regenerated spinal cord tissue in the lesioned area, multisite neural

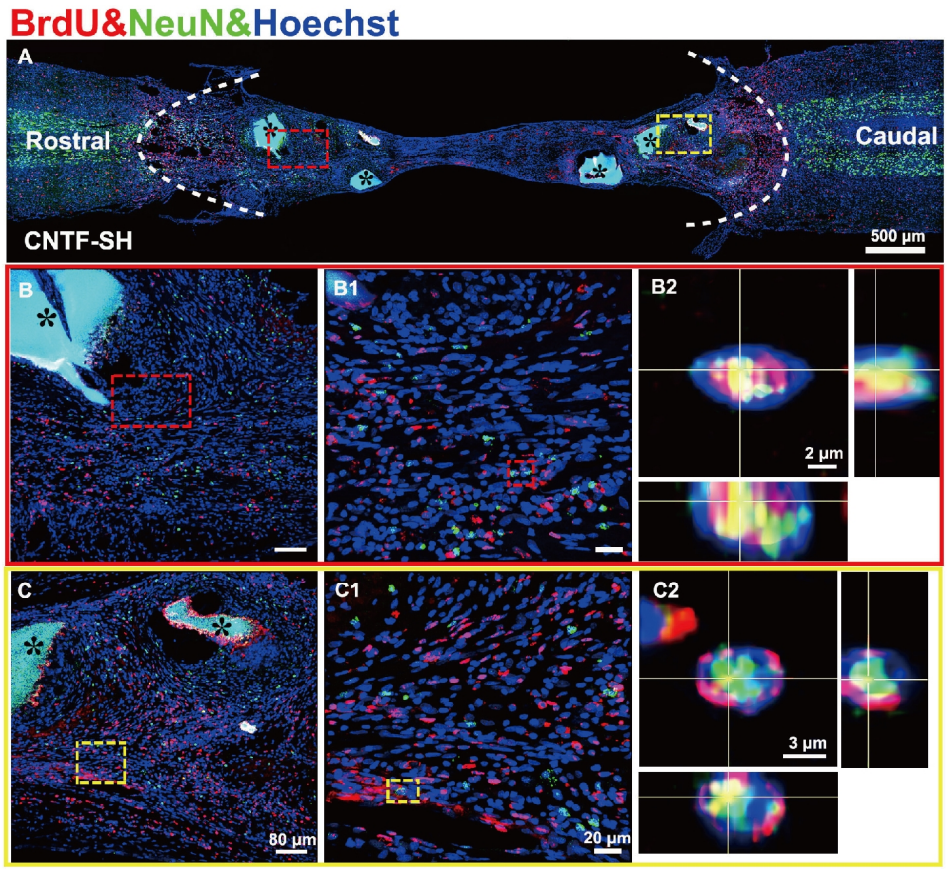

Figure 3 Thirty days after operation, new born mature neurons were observed in the lesioned area of the sodium hyaluronate-CNTF scaffold group. A, Longitudinal spinal cord sections including the lesioned area, which were Nestin, BrdU and Hoechst immunofluorescence triple-labelled. B–B2, Continuous amplification of the red boxed area at the R end of the lesioned area in A. C–C2, Continuous amplification of the yellow boxed area at the C end of the lesioned area in A. NeuN$^+$ & BrdU$^+$ new born mature neurons were observable. * indicates the undegraded scaffold, and the white dotted line indicates the boundary between the lesioned area and host tissue.

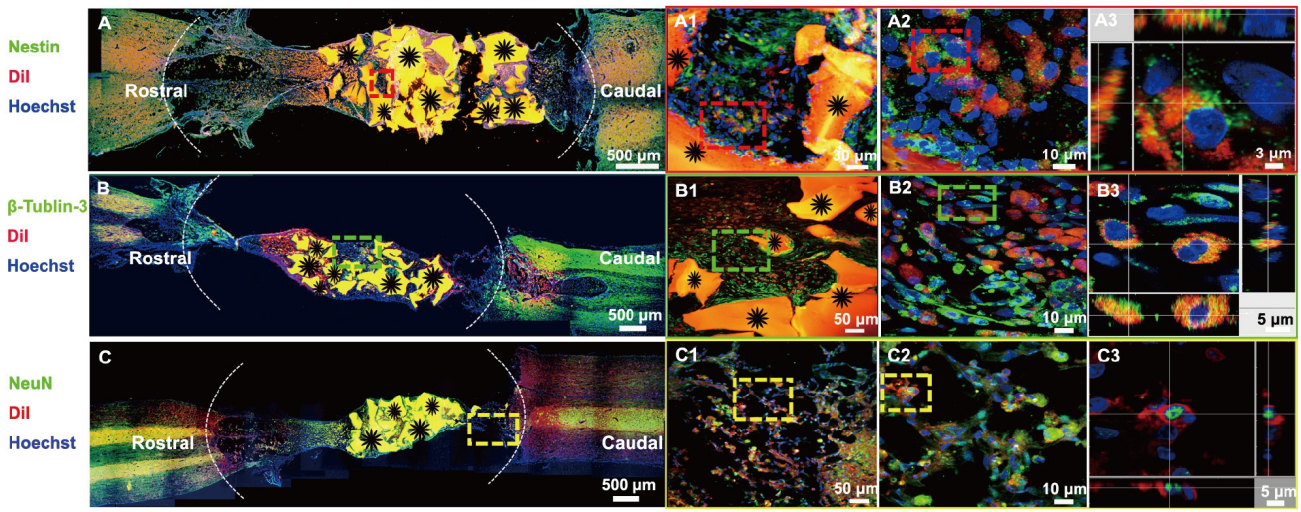

Figure 4 Immunofluorescence staining was used to test differentiation status of Dil$^+$ cells that had migrated to the lesioned area. A, Immunofluorescence images of the sodium hyaluronate-CNTF scaffold group 28 days after operation. A1–A3, Amplification of the red boxed area in A, where a large amount of Dil$^+$ & Nestin$^+$ cells were observed in the lesioned area. B, Immunofluorescence images of the sodium hyaluronate-CNTF scaffold group 56 days after operation. B1–B3, Amplification of the green boxed area in B, where Dil$^+$ & Tuj1$^+$ immature neurons were observed in the lesioned area. C, Immunofluorescence images of the sodium hyaluronate-CNTF scaffold group 90 days after operation. C1–C3, Amplification of the yellow boxed area in C, where Dil$^+$ & NeuN$^+$ mature neurons were observed at the caudal end. * indicates the undegraded sodium hyaluronate-CNTF scaffold, and the white dotted line indicates the boundary between the lesioned area and host tissue.

responses were produced within the lesioned area as well as in the host spinal cord caudal to the lesion site (Figure 5A and B), where the amplitude of field excitatory postsynaptic potentials (fEPSP) could be significantly suppressed by 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX; 10 μmol L^{-1}), suggesting that major excitatory neurotransmissions were glutamatergic. All effects of these pharmacologic agents on fEPSP were reversible on reperfusion (i.e., washout of CNQX), as expected. Taken together, functional synaptic connections could be established among regenerated neurons in the lesioned area as well as in the host spinal cord, and that these new synaptic connections likely participated in the functional recovery.

The sodium hyaluronate-CNTF scaffold facilitated the functional recovery of bilateral hindlimbs of SCI rats

To further confirm sensory and motor functional recovery, we performed electrophysiological analysis. The somatosensory evoked potentials (SEP) and motor evoked potentials (MEP) of normal rats were taken as normal control in all groups. In the lesion-control group, at 1, 7, 14, and 21 day after the first operation, under gradually increasing electric stimulation, no SEP could be evoked from the cerebral sensory cortex, and no MEP could be evoked by stimulating the cerebral motor cortex. By contrast, in the sodium hyaluronate-CNTF scaffold group, SEP and MEP started to recover from week 4 after operation; at week 12 after operation, the latency and amplitude of both SEP and MEP were partially restored, but not to the normal level before SCI (Figure 6A and B); when re-transecting the regenerated spinal cord, neither SEP nor MEP could be induced (data not shown). These results evidence the partial recovery of nerve function in the sodium hyaluronate-CNTF scaffold group.

To determine whether the regenerated neural tissues enabled functional recovery in terms of behavior, we used the BBB open-field walking scale to measure hindlimb locomotor activity of SCI rats. As expected, hindlimb locomotion score reduced to zero immediately after operation for all experimental rats. In the lesion-control group, the right and left hindlimb BBB scores remained no higher than 1.5 over 12 weeks after operation (Figure 6C). In the sodium hyaluronate-CNTF scaffold group, the mean BBB scores of both right and left hindlimbs had a significant increase at week 4, and kept rising steadily till week 12 (Figure 6C). To find out the reason for this increase, a compensatory effect or spinal cord regeneration, we then re-transected the regenerated cable and placed a plastic diaphragm at the lesion area. As a result, the mean hindlimb BBB scores dropped sharply from 7.5 to

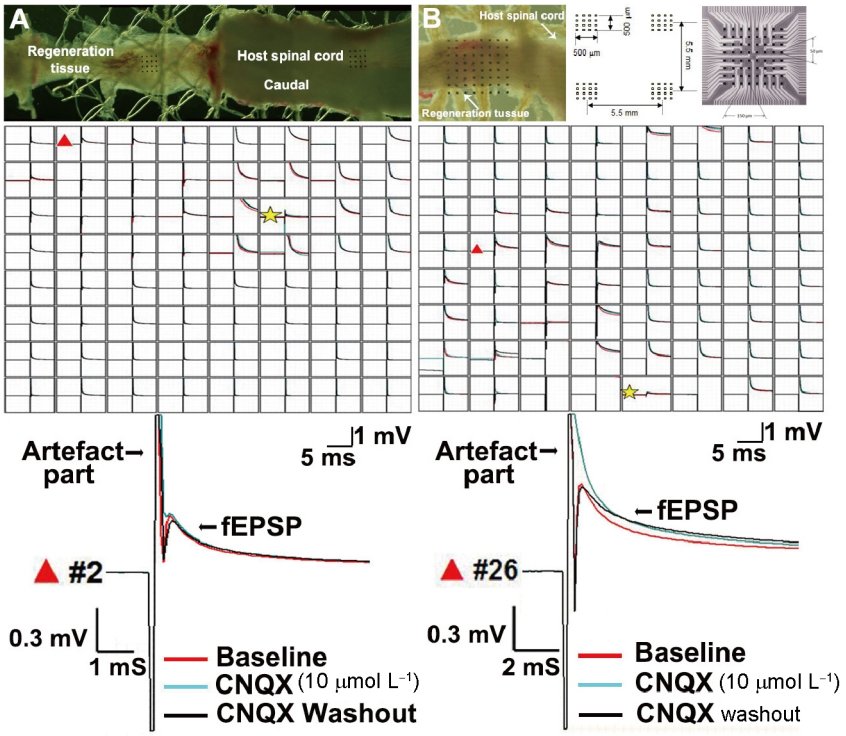

Figure 5 Functional synaptic connection was established among the new born neurons in the lesioned area or between the new born neurons and host spinal cord tissue of the sodium hyaluronate-CNTF scaffold group. The rat spinal cord tissue (including the lesioned area) of the sodium hyaluronate-CNTF scaffold group was longitudinally sectioned, then the sections were placed on the MED64 planar multielectrode dish system. The narrow part represents the regenerated tissue. When stimulating a point of the regenerated tissue, fEPSPs could be recorded at other points of the host tissue (A) or regenerated tissue (B). These fEPSPs could be inhibited by CNQX (10 μmol L^{-1}), suggesting that these synaptic transmissions were glutamatergic. The yellow star indicates the stimulating point, and the red arrow indicates fEPSP recorded at point 2 and 26.

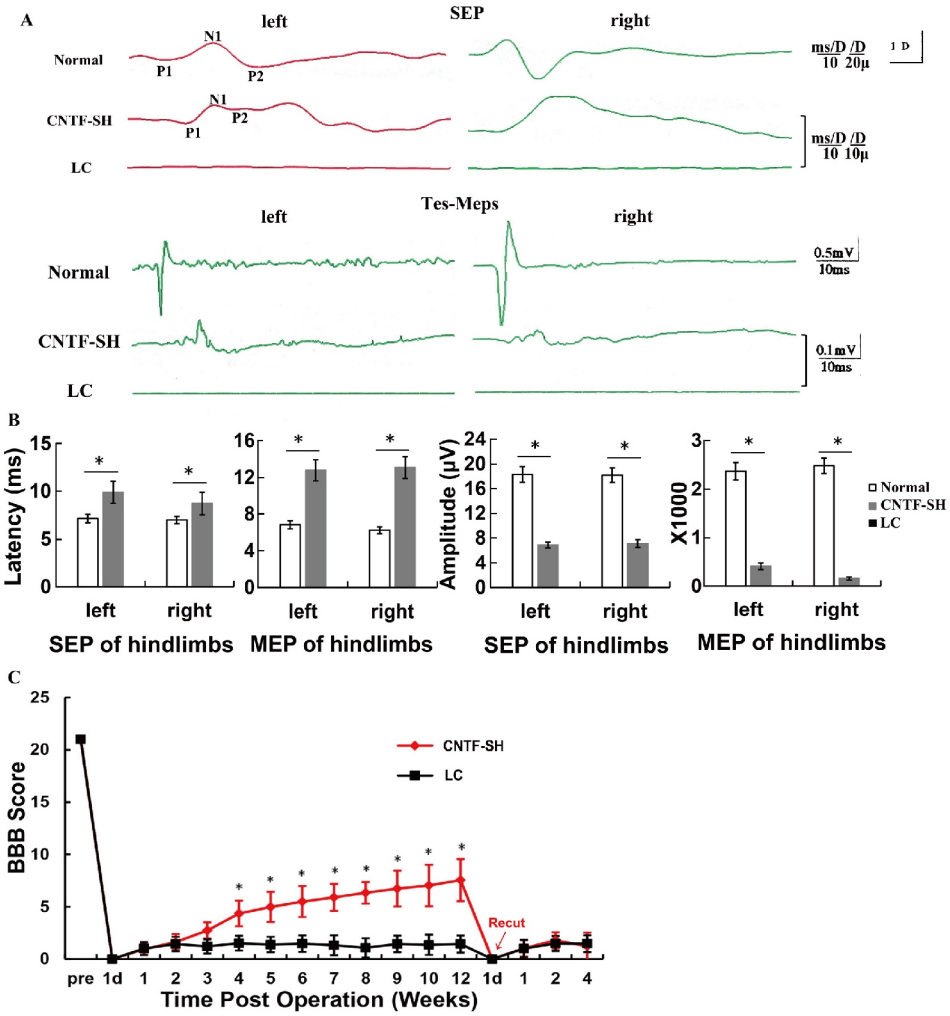

Figure 6 Electrophysiological and behavioral evaluation of functional recovery of the SCI rat hindlimbs. A, SEP and MEP data for each group 4 weeks after operation. Only the sodium hyaluronate-CNTF scaffold group showed partial functional recovery. B, Quantitative analysis of the amplitude and latency of SEP and MEP of bilateral hindlimbs for each group. Compared with the normal group, the sodium hyaluronate-CNTF scaffold group showed significantly prolonged latency of SEP and MEP as well as remarkably reduced amplitude of SEP and MEP (mean±SD; n=6; P<0.05), indicative of partial recovery of sensory and motor function. No signal was detected in the lesion-control group. C, Basso-Beattie-Bresnahan (BBB)-open field scoring for each group. From week 4 after operation, compared with the lesion-control group, the sodium hyaluronate-CNTF scaffold group showed significantly increased BBB scores (mean±SE; n=6; P<0.05, one-way ANOVA (analysis of variance) with Bonferroni post hoc test). 12 weeks after the first operation, the regenerated cable was re-transected, and a plastic diaphragm was placed at the re-lesion site (n=5). According to the BBB scoring for successively 4 weeks, all rats again lost hindlimb motor function (mean±SE; n=5; P<0.05, paired Student t test comparing before and after recutting).

0–1 (not significantly different from that in the lesion-control groups), and remained at this low level for the following 4 weeks (Figure 6C). This suggests that the locomotor function recovery observed in the sodium hyaluronate-CNTF scaffold group was most likely attributed to the re-establishment of synaptic transmission from motor axons, rather than the compensatory response beneath the lesion interface.

DISCUSSION

Adult neurogenesis is a complicated process of neuron development under the CNS environment. Starting with the proliferation and fate-decision of stem/progenitor cells, this process includes in turn new born neurons' differentiation, survival, migration and synaptic integration with the host neural circuit (Ming and Song, 2005). At present, it is generally accepted that adult mammal neurogenesis constantly exists in the subventricular zone (SVZ) and subgranular zone (SGZ), as well as in the central spinal canal (Barnabé-Heider et al., 2010; Gage, 2000; Luo et al., 2015). In this study, we completely cut and removed the 5 mm length T8 segment of adult rats, and then implanted a sodium hyaluronate-CNTF scaffold into the lesioned area. Via BrdU injection, Dil tracing and immunofluorescence multi-label staining, $BrdU^+$ & $NeuN^+$ cells in the lesioned area were observed, which were also Dil positive. These results suggest that new born neurons might originate from the neural stem cells in the spinal cord ependymal.

CNTF is a part of cytokine family. Studies have shown that CNTF could promote the survival and axonal growth of neurons (de Almeida, et al., 2001; Wang et al., 2014), induce the promyelinating effect and increase the proliferation or survival of oligodendrocyte precursor cells (Stankoff et al., 2002). Under the physiological environment, the half-life of CNTF is very short (about 3 min) (Poduslo and Curran, 1996). Thus, local using of soluble CNTF can result in rapid diffusion and lose efficacy. In the previously published study, we had attempted to load CNTF into degradable sodium hyaluronate gelatinous particles to achieve the sustained and local CNTF releasing. We implanted the CNTF-SH into the lesion area of spinal cord which could modify the harsh micro-environment in the lesion area and make the greatest CNTF concentration in the lesion area and lower concentrations in the adjacent tissue. The gradient may facilitate axonal growth toward the lesion area. We suggested that the CNTF-SH might create a favorable microenvironment for the differentiation of neural stem/progenitor cells to the neuronal cell line.

Being neurons, these new born cells had to possess the cellular morphological structure and protein expression characteristics similar to those of neurons, enable production and transmission of electronic signals (Magloire and Streit, 2009), and be capable of forming a neural circuit or integrating with the existing neural circuit. The neurons of different characteristics and functions in the CNS connect with each other in various patterns, form neural circuits and networks at different levels, and take actions in multiple ways, such as series connection, parallel connection, feedforward, feedback, positive feedback, and negative feedback.

In previous research, morphological experiments were usually conducted to demonstrate neural circuit formation. In the present study, when the experimental rats got significantly high BBB scores 4 weeks after SCI operation, the MEP and SEP experiment was conducted to prove that the sodium hyaluronate-CNTF scaffold did facilitate the partial recovery of sensory and motor functions of the paraplegic rat hindlimbs. Moreover, the MED64 planar multielectrode dish system (Alpha MED Scientific, Japan) was used to record neural network activities in different regions of *in vitro* spinal cord sections. The results suggest that functional synaptic connection had been established between new born neurons as well as between new born neurons and the host spinal cord, and this connection was mediated by glutamatergic receptors.

In this study, sodium hyaluronate was stably combined with CNTF to realize a long-term release for up to 105 days (Wang et al., 2014). This not only provided a scaffold for neural cell migration and processes extension, but also improved the micro-environment in the lesioned area, thus benefiting seed (i.e., endogenous neural stem cells) activation, further cell recruitment and migration to the lesioned area, where these cells ultimately differentiated into mature neurons. This research may shed light on clinical SCI treatment.

MATERIALS AND METHODS

CNTF scaffold preparation

The sodium hyaluronate-CNTF scaffold was prepared as previously reported (Wang et al., 2014; Li et al., 2009). In brief, under sterile conditions, 200 Da sodium hyaluronate was dissolved in 0.5% sodium hydroxide aqueous solution, and then vinylcyclohexane (5 wt %) was added. After stirring for 6–8 h at 25°C, the gelatin block was added to deionized water and allowed to swell for 6 h at 25°C. The swollen gelatin block was washed with phosphate-buffered saline totally for 12 h. After 100-mesh sieve filtration, the sodium hyaluronate gelatinous particles were prepared with CNTF protein. 1 mg sodium hyaluronate gelatinous particles and 15 μg CNTF were dissolved in 100 mL phosphate-buffered saline under 4°C environment. Next, the sodium hyaluronate gelatinous particles were vacuum cooled, dried and stored at 4°C for use.

Preparation of the rat SCI model

The detailed preparation process has been reported in refs. (Wang et al., 2014; Li et al., 2009). In brief, adult female Wistar rats, each weighing 220–250 g, were anesthetized by intraperitoneal injection of Equithesin (3 mL kg^{-1} body weight). A dorsal laminectomy was performed at T8 to expose the spinal cord. A segment of spinal cord tissue was excised completely to form a 5-mm-long gap, and any residual fiber at the lesioned area was removed by aspiration. All the rats were divided into two groups, the sodium hyaluronate-CNTF scaffold implantation group (n=36, the sodium hyaluronate-CNTF scaffold were implanted into the lesion cavities), lesion-control group (n=36, nothing was implanted into the lesion cavities), respectively. Penicillin was injected intraperitoneally (100 thousand units $time^{-1}$) for a period of 7 days. The bladder was massaged twice daily until bladder function recovered.

The above experimental procedures were approved by the Experimental Animal Center and Animal Care Committee, Capital University of Medical Sciences (Beijing).

Labeling of endogenous neural stem cells

The detailed labelling process has been reported in ref. (Yang et al., 2015). In brief, BrdU (50 mg kg^{-1} body weight; Sigma-Aldrich) was injected i.p. every 12 h for 1 week after the operation. The injected BrdU was used to label the dividing cells, to allow observation of the dynamic process of cell division. The animals were sacrificed at different time points to examine the cell fate of BrdU-labelled cells.

The detailed preparation process has been reported in ref. (Nakatomi et al., 2002). In brief, four rats respectively

from the sodium hyaluronate-CNTF scaffold group and lesion-control group were randomly selected to label cells in myelocoele by injecting 20 μL of DiI (0.2% in dimethylsulfoxide, Molecular Probe, USA) into the lateral ventricle.

Tissue handling and immunofluorescence staining

The detailed process has been reported in reference (Yang et al., 2015). In brief, the primary antibodies included polyclonal rabbit anti-Nestin (diluted 1:1000, Cell Signaling Technology, USA) used to label NPCs, chicken polyclonal anti-β-tubulin 3 (diluted 1:500, Abcam, USA) to label immature neurons, polyclonal rabbit anti-GFAP (diluted 1:300, Zymed, USA) to label astrocytes, monoclonal rabbit anti-NeuN (diluted 1:200, Chemicon, USA) to label mature neurons, monoclonal rabbit anti-synapsin1 (diluted 1:200, Cell Signaling Technology) to label synapse, and mouse anti-BrdU to label proliferative cells.

At each time point after the operation, four or five rats were selected at random from each group and sacrificed by an overdose of anesthesia. After transcardial perfusion with 4% paraformaldehyde, the brain and spinal cord were excised and fixed at 4°C in fixing solution for 6–8 h. The lesion area was examined under a dissecting microscope. The spinal cord tissue including the lesion area was embedded and sliced longitudinally with a freezing microtome to produce 10-μm sections.

The sections were washed three times with 0.01 mol L^{-1} PBS and then incubated with the primary antibodies at 4°C overnight. After that the sections were incubated with appropriate secondary antibodies conjugated to various fluorescent labels, such as Texas red-conjugated Affinipure goat anti-mouse IgG and CyTm2-conjugated Affinipure goat anti-rabbit IgG (diluted 1:200, Jackson Laboratory, USA), at room temperature for 6 h in the dark. The sections were covered with coverslips and Vectashield-mounting medium containing Hoechst (Vector Laboratories, USA), and examined under a laser scanning confocal microscope (SP-8; Leica, Germany).

MED64 planar multielectrode dish system recording

The detailed operational process has been reported in ref. (Yang et al., 2015). In brief, the multielectrode dish system (Alpha MED Scientific, Japan) was prepared basically as described previously (Yang et al., 2015; Oka et al., 1999). The device had an array of 64 planar microelectrodes, each 50 μm×50 μm, arranged in an 8×8 pattern (interelectrode distance, 150 μm). Preparation of acute spinal cord slices including the lesioned area 8 weeks after operation as well as the electrophysiological recordings protocol were almost the same as described previously (Yang et al., 2015; Oka et al., 1999; Mladinic et al., 2013) with slight modifications; i.e., the spinal cord tissue including the lesion area was sliced longitudinally.

Electrophysiological studies

The detailed operational process has been reported in ref. (Yang et al., 2015). In brief, electrophysiological assays were performed for each group (n=6) with a Keypoint-II bichannel evoked potential/electromyograph before the operation and at 1 month after the operation. The electrophysiological data obtained before the operation served as a normal control (n=6). The indices measured included SEP and MEP, which could comprehensively reflect the sensory and locomotor function in the healthy state, after SCI, and during the recovery process (Yang et al., 2015; Arunkumar et al., 2001).

The rats were anesthetized by an i.m. injection of ketamine (50 mg kg^{-1} body weight), and their limbs were abducted and fixed on a board by cloth bands. The room temperature was kept at 25–28°C. For MEP, the stimulating positive electrode (a 2-mm ball) was placed on the skull surface at the midline of the motor area of cerebral cortex, 2.5 mm behind the anterior fontanel and 2 mm on the left or right side of the midline. The stimulating negative electrode (a 4-mm disk) was placed on the skull surface of the hard palate. The recording electrodes (i.e., the needle electrodes) were inserted into the tibialis anterior muscle of the bilateral hindlimbs at the depth of 1.5 mm. The reference electrode was placed 2 cm away from the distant end of the recording electrode, and the ground line was placed between the stimulating and recording electrodes. A single square wave was used to stimulate the motor area of the cerebral cortex through the skull, at an intensity of 5–12 mA (Schlag et al., 2001), duration of 0.2 ms, stimulating frequency of 1 Hz, a bandpass filter from 2 to 10 kHz, and an amplifier sensitivity of 0.1 mV D^{-1}. MEP was recorded at the tibialis anterior muscle of the bilateral hindlimbs, i.e., the latency and the amplitude from the negative peak to the adjacent positive peak was measured. The distance between the stimulating electrode and the recording electrode was measured as well. Before the MEP measurements, the motor threshold intensity was set as described previously (Schlag et al., 2001).

SEP measurements were also done with a Keypoint-II bichannel evoked potential/electromyograph. The positive electrodes (i.e., needle electrodes) were inserted into the tibialis anterior muscle of the bilateral hindlimbs. This muscle was successively stimulated with an average of 200 pulses at a stimulating intensity of 3–5 mA (to make the toes of the hindlimbs move slightly) (Schlag et al., 2001), a duration of 0.2 ms, an amplifier sensitivity of 10 μV D^{-1}, a bandpass filter from 20 to 3000 Hz, and a sweep length of 80 ms. SEP were recorded on the skull surface of the sensory area of cerebral cortex, including P1 and P1-N1. The distance between the stimulating electrode and the recording electrode was measured as well.

Behavioral assessment

The detailed operational process has been reported in ref.

(Yang et al., 2015). In brief, once prior to the operation, 1 day after, and then once every week after the operation, two observers blind to the treatment carried out the BBB test on experimental rats in an open field to evaluate the degree of recovery of locomotor function after SCI in accordance with the 21-point BBB scale (Li et al., 2009). The normal rats act as the normal control. Repeated measures, ANOVA, and Bonferroni post hoc analysis were adopted to determine the statistical significance of differences for multiple group comparisons (Basso et al., 1995). 12 weeks after the operation, when the BBB behavioral scores tended to be stable, five rats were chosen from each group, and anesthetized by intraperitoneally injecting Equithesin (3 mL kg^{-1} body weight). Next, skin and muscles were incised, and the original surgical site was exposed. Under an operating microscope, the regenerated cable/scar tissue in the lesioned area was transected. After that, the muscles and skin were closed with suture. BBB test was performed successively again for the above-mentioned rats within one month after the transection.

Statistical analysis

All data are presented as mean±SE. The Shapiro-Wilk method is used for data normality analysis, and the Levene test is used to test for homogeneity of variance. Data are analyzed by one- or two-way ANOVA, then by Fisher's least significant difference (LSD) test. Values are considered significantly different at $P<0.05$.

Compliance and ethics *The author(s) declare that they have no conflict of interest. All authors conformed to the Helsinki Declaration of 1975 (as revised in 2008) concerning Human and Animal Rights, and we followed out policy concerning Informed Consent as shown on Springer.com.*

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SUPPORTING INFORMATION

Figure S1 Thirty days after operation, new born immature neurons were observed in the lesioned area of the sodium hyaluronate-CNTF scaffold group.

Figure S2 Thirty days after operation, a small amount of activated NSCs were observed in the lesioned area of the lesion-control group.

Figure S3 Thirty days after operation, no new born immature neuron was observed in the lesioned area of the lesion-control group.

Figure S4 Thirty days after operation, no new born mature neurons were observed in the lesioned area of the lesion-control group.

Figure S5 Thirty days after operation, new synapses formed in the lesioned area of the sodium hyaluronate-CNTF scaffold group.

Figure S6 Thirty days after operation, glutamatergic synapses formed in the lesioned area of the sodium hyaluronate-CNTF scaffold group.

Figure S7 Coronal spinal sections to show ependymal cells traced by DiI.

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