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ORIGINAL RESEARCH

Repair of Peripheral Nerve Sensory Impairments via the Transplantation of Bone Marrow Neural Tissue-Committed Stem Cell-Derived Sensory Neurons

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Abstract
The present study aimed to investigate the efficacy of transplantation of bone marrow neural tissue-committed stem cell-derived sensory neuron-like cells for the repair of peripheral nerve sensory impairments in rats. Bone marrow was isolated and cultured to obtain the neural tissue-committed stem cells (NTCSCs), and the differentiation of these cells into sensory neuron-like cells was induced. Bone marrow mesenchymal stem cells (BMSCs), bone marrow NTCSCs, and bone marrow NTCSC-derived sensory neurons (NTCSC-SNs) were transplanted by microinjection into the L4 and L5 dorsal root ganglions (DRGs) in an animal model of sensory defect. On the 2nd, 4th, 8th, and 12th week after the transplantation, the effects of the three types of stem cells on the repair of the sensory functional defect were analyzed via behavioral observation, sensory function evaluation, electrophysiological examination of the sciatic nerve, and morphological observation of the DRGs. The results revealed that the transplanted BMSCs, NTCSCs, and NTCSC-SNs were all able to repair the sensory nerves. In addition, the effect of the NTCSC-SNs was significantly better than that of the other two types of stem cells. The general posture and gait of the animals in the sensory defect model exhibited evident improvement over time. Plantar temperature sensitivity and pain sensitivity gradually recovered, and the sensation latency was reduced, with faster sensory nerve conduction velocity. Transplantation of NTCSC-SNs can improve the repair of peripheral nerve sensory defects in rats.

Keywords Neural tissue-committed stem cells · Sensory neurons · Dorsal root ganglion · Transplantation · Neural repair

Introduction

Nerve injury can lead to impaired motor and sensory functions. Previous studies on nerve injury have mainly focused on the motor function recovery of the injured nerves, while attention to the recovery of sensory function was lacking. The main manifestations of sensory dysfunction after nerve injury include pain, temperature sensory impairment, and secondary sensory dysrhythmia. Repairing the sensory function of patients is an important aspect of the nerve repair and the patients' quality of life.

Neural stem cells (NSCs) represent a class of cells that is capable of self-renewal, proliferation, and differentiation, with the potential to produce neurons and induce the multidirectional differentiation of glial cells. NSCs have shown considerable promise for the treatment of nervous system injury, and NSC transplantation provides a new therapeutic approach for this disorder. However, the wide use of NSCs in clinical practice is difficult because of the difficulty in

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obtaining these cells and the ethical restrictions associated with their use. Therefore, appropriate methods for obtaining NSCs from adult non-nerve tissues have become a research focus in studies of cell resources in clinical transplantation. At present, NSCs can be obtained from the umbilical cord blood (Liao et al. 2015; Abraham and Verfaillie 2012), skin (Qin et al. 2015), hair follicles (Mistriotis and Andreadis 2013; Hoffman 2014; Najafzadeh et al. 2015), bone marrow, and other non-nerve tissues using a variety of approaches.

Bone marrow mesenchymal stem cells (BMSCs) are a type of fibroblast-like cells with self-renewal and immunoregulatory abilities. BMSCs can differentiate into mesoderm and other type of cells, such as nerve cells. They can be easily isolated, quickly proliferated, and autogenously transplanted with no ethical restrictions and have been widely used in scientific experiments and other applications (García-García et al. 2015; Zhang et al. 2014; Yang et al. 2014; Mezey 2011). Numerous studies (Zeng et al. 2015, 2011) have shown that BMSCs can be induced and can differentiate into neuron-like and glial cells under certain conditions to replace damaged tissue, and the neuron-like cells present some nerve electrophysiological characteristics. The expression of neurofilament protein in BMSCs is positive, indicating the local formation of neural circuits that can be integrated into the host neural network to restore nerve fiber conduction.

In 2004–2006, a study by Kucia confirmed the existence of precursor cells of the stem cell marker CXC chemokine receptor 4 (CXCR4) protein in bone marrow with directional tissue expression (Kucia et al. 2004, 2006). These precursor cells became known as neural tissue-committed stem cells (NTCSCs). NTCSCs are a subtype or component of BMSCs, which can naturally differentiate into neuronal cells. They are easy to obtain and exhibit good activity and induce to differentiate, making them the ideal seed cells for transplantation.

In 2005, Kondo found that, after BMSCs were pre-induced with Forskolin (an adenylate cyclase activator) and fibroblast growth factor-2 (FGF2) for 24 h, and then co-induced with sonic hedgehog and retinoic acid, they could differentiate into sensory neurons with specific expression of glutamatergic neuron markers, including VGluT1 (type I vesicular glutamate transporter), calretinin (calcium binding protein), and P_2X_3 (an ATP-regulated ion channel receptor) (Kondo et al. 2005).

Previously, our research group had successfully isolated and cultured NTCSCs from Sprague–Dawley (SD) rat bone marrow and induced their differentiation into neuron-like cells for the repair of peripheral nerve defects (Ren et al. 2007, 2008, 2009). These preliminary achievements provided a type of non-neural tissue-derived seed cells for use in the clinical repair of neurological diseases, which is widely applicable, can be derived from the autologous system, shows no immune rejection, and involves no ethical issues. However, the effect of this type of cells in repairing the sensory nerves of rats and whether they have a greater advantage over other NSCs, has never been reported.

In the present study, NTCSC-SNs were transplanted in the dorsal root ganglion (DRG) of an animal model with pyridoxine-induced sensory impairments. The effect of the transplanted cells in the repair of the sensory nerves in rats was observed. This study is expected to provide a theoretical and experimental basis for further clinical application of regenerative medicine with the transplantation of bone marrow-derived nerve cells to repair sensory nerve defects.

Materials and Methods

Extraction, Induced Differentiation and Identification of BMSCs and NTCSCs

Male SD rats (4–6 weeks old) were subjected to anesthesia with pentobarbital sodium (60 mg/kg, IP), and then, their femurs were aseptically harvested, and washed in a mixture of phosphate-buffered saline (PBS) and antibiotics for 5 min. Next, the soft tissue was dissected, transected at their epiphysis, and their marrow cavity was repeatedly rinsed with a mixture of heparin and Dulbecco's modified Eagle media (DMEM). The harvested cells were collected and centrifuged at 1500 rpm for 10 min. Cell pellets were resuspended with DMEM, and 2X Percoll separator liquid with a density of 1.073 g/ml was added to the tube. After centrifugation at 2500 rpm for 30 min, the single nucleated cell layer was separated, and the MSC layer was resuspended in DMEM and centrifuged at 1500 rpm for 10 min. Following washing, the cells were placed in DMEM culture medium supplemented with 10% fetal bovine serum, and the BMSCs were cultured to the third passage.

The mononuclear cell layer of the bone marrow whole blood cells from SD rats was obtained using NycoPrep™ lymphocyte separation medium (1.077A; Axis-Shield). DMEM/F12 (Invitrogen) serum-free medium [containing 20 ng/ml epidemical growth factor (EGF; Invitrogen), 20 ng/ml basic fibroblast growth factor (bFGF; Invitrogen), 1% N-2 supplement (Invitrogen), 2% B-27 supplement (Invitrogen), 100 µg/ml streptomycin, and 100 U/ml penicillin] was used to obtain the adherent cell clones. The cloning cells were selected and cultured using the serum-free medium suspension to obtain the NTCSC spheres.

Third-generation NTCSCs were seeded in a 6-well plate pre-coated with Poly-L-Ornithine solution (PLO, Sigma) and fibronectin (FN, Sigma) at a density of 0.5×10^6/ml. After culturing in serum-free medium for 24 h, the cells were cultured in the pre-induction medium [Dulbecco's modified Eagle's medium/F12 (DMEM/F12, Gibco) containing 20%

fetal bovine serum (FBS, Gibco), 10 ng/ml bFGF, 25 μM Forskolin (Sigma), 125 μM 3-isobutyl-1-methylxanthine (IBMX, Sigma), and 2% N2 SUPPLEMENT] for 24 h. After washing with PBS, the cells were continuously cultured with the induction medium [DMEM/F12 medium containing 10 ng/ml bFGF, 400 ng/ml SHH (Abcam), 5 μM Forskolin, 62.5 μM IBMX, 50 ng/ml brain-derived neurotrophic factor (BDNF, Invitrogen), 0.5 μM retinoic acid (RA, Sigma), 1% insulin-transferrin-selenium supplements (insulin, Invitrogen), and 2% N2] for 14 days. The cells obtained after induction were identified using the following antibodies: anti-microtubule-associated protein 2ab (MAP-2, Millipore, rabbit IgG, dilution 1:500), neuron-specific nuclear protein (NeuN, Abcam, rabbit IgG, dilution 1:200), and anti-glutamate receptor 4 (GluR-4, Millipore, dilution 1:500). Additionally, the nuclei were counterstained using 4′,6-Diamidino-2-phenylindole dihydrochloride (DAPI, Sigma).

Preparation of the Rat Model of Dorsal Root Ganglion Injury

Following the method described by Helgren (Helgren et al. 1997), adult female SD rats weighing 180–220 g were intraperitoneally injected with pyridoxine hydrochloride at a dosage of800 mg/kg twice a day for 8 days, to obtain the animal model of DRG injury. After the model was established successfully, the rats were allowed to recover for 12 days before the subsequent experiments (Yu et al. 2017).

Grouping of the Animals and the Method of Cell Transplantation

A total of 100 SD rats were purchased from the Experimental Animal Center of the Second Military Medical University, China. They were randomly assigned to the normal group ($n = 20$) and model group ($n = 80$). The rats in the normal group did not receive the experimental treatment, while the rats in the model group were treated according to the animal model method described above. The 80 rats in the model group were randomly assigned to four treatment groups using computer-generated random numbers. The five different levels of treatment were grouped as follows: Normal, blank control, BMSC, NTCSC and NTCSC-SN groups. Subsequent experiments on the rats of the model groups were performed 12 days after the model was established. Sensory function was examined at the end of the 2nd, 4th, 8th, and 12th week after the cell transplantation.

At 48 h before transplantation, the cells were labeled by incubating with 2 μg/ml chloromethyl-benzamidodialkylcarbocyanine (CM-Dil; Invitrogen, USA) for 30 min at 37 °C, followed by an additional 15 min at 4 °C, and then further incubation for 48 h. Before transplantation, cells were thoroughly washed by PBS. Labeled cells were injected into the left L4 and L5 DRG on the 13th day after the model was induced. Rats in the normal group were injected with PBS as the blank control. The cell density was adjusted to 5×10^4 cells/μl for the cell transplantation, which was performed using a glass micro-syringe with a tip diameter of 70–100 μm. The amount of cells injected into the L4 and L5 DRG of each rat was 4 μl, with an injection time of 60 s; the needle was retained for 5 min after the injection was completed to prevent overflow of the transplanted fluid. At the end of the 2nd, 4th, 8th, and 12th week after the transplantation, five rats were randomly selected from each group to undergo functional, electrophysiological and histomorphological tests.

Test Methods

The room temperature of the behavioral experiment laboratory was set to 24 °C, and fluorescent lighting was used. The wound recovery, gait, body weight, feces, and appetite of the rats after the cell transplantation were observed and recorded. Postoperative load-bearing, gait, and muscle atrophy of the posterior lower limb were observed.

Regarding the detection of the animal's sensory function, mirror-image pain caused by mechanical stimulation (performed using Stoelting's Von Frey Hairs fiber pain tester) was used to test the plantar sensory function of the rats (Chaplan et al. 1994). According to the modified method described by Hargreaves (Hargreaves et al. 1988), the change in plantar sensitivity to temperature was tested with a hot plate pain tester. Briefly, the rats were anesthetized by intraperitoneally injection of 3% pentobarbital sodium at the dose of 25 mg/kg. Next, the dorsal skin and fascia were cut open, the biceps femoris, semitendinosus, and semimembranosus muscles were separated, and the left sciatic nerve was exposed. The common peroneal nerve was cut off at the sciatic nerve branch in order to prevent the interference of positive conduction to the measurement results. The epineurium of the sciatic nerve was cleaned carefully and kept moist. Subsequently, the silver electrode was placed on the sciatic nerve, and the stimulation electrode was placed on the foot of the same side, about 0.5–1 cm near the foot center. The cathode was at the proximal end of the anode. The recording electrode was located at the distal end of the sciatic nerve, and the ground wire was fixed between the stimulation electrode and the recording electrode. Electrophysiological testing was performed using a MPA-2000M multi-channel biological signal analysis system, which was supplied by Shanghai Alcott Biotech Co., Ltd. The sampling rate of the instrument was adjusted to 20 KHz, with a filtration of 10 KHz, a time constant of 0.001 s, single stimulation, a synchronous trigger, a wave width of 0.45 ms, and an intensity of 1.06 V. The MPA-2000M was connected to measure the sensory nerve movement potential and sensory latency, and

the sensory nerve conduction velocity was calculated. The sensory nerve conduction velocity was detected using the antegrade method. In each experiment, the distance between the stimulating electrode and the recording electrode was measured using a vernier caliper, the sensory nerve latency and the action potential were recorded by the MPA-2000M analysis system, and the distance between the two electrodes was divided by the latency to obtain the sensory nerve conduction velocity.

All of the above experimental procedures involving the rats in this study were in accordance with the NIH guidelines (NIH. Pub. No. 85-23, revised 1996) and approved by the Medical Ethics Committee of Shanghai Second Military Medical University.

Statistical Methods

The data from the grouped experiment were analyzed using SPSS18.0 software. The experimental data are represented as the mean ± standard deviation (Means ± SD). A normal distribution test (Shapiro–Wilk test) and a variance homogeneity test (Levene test) were performed. Significant differences between groups were assessed by one-way ANOVA (LSD, least significance difference) when the data were normal distribution. Nonparametric test (Kruskal–Wallis test and Nemenyi test) was used when the data were non-normal distribution. Differences were considered significant at $P < 0.05$.

Results

Isolation, Induction and Identification of NTCSCs

The SD rat bone marrow mononuclear cells were isolated and cultured according to the method described earlier, and the cell spheres were obtained after screening with a single-cell cloning method and serum-free suspension culturing (Fig. 1a). After culturing to the third generation of NTCSCs, the neuronal cells were obtained using the induction method described earlier (Fig. 1b). The NTCSCs showed morphological characteristics of neuron-like cells, with the cytoplasm retracting to the nucleus to form round cells with a typical perikaryon shape that displayed significantly enhanced stereoscopic sensation and refraction. Several elongated protrusions from the cell body were similar to neuronal axons, and branches similar to the dendrites of neurons protruded from the ends of these axon-like protrusions. The protrusions intertwined with each other to form a network, making the cell body relatively larger and more round. The number of the protrusions ranged from 2 to 5.

For the cells obtained after the induction and differentiation of the NTCSCs, immunofluorescence staining was performed for the neuronal marker NeuN, the neuronal dendritic marker MAP-2, and the glutamatergic sensory neuron-specific receptor marker protein GluR4.

The results showed that the characteristic proteins NeuN, MAP-2, and GluR4 were expressed in the cells after induction (Fig. 2). The fluorescence of MAP-2 and NeuN was red, with MAP-2 staining observed mainly in the cytoplasm, and NeuN staining observed mainly in the nucleus and cytoplasm. The fluorescence of GluR4 was green, and GluR4 immunopositivity was found in the cytoplasm, with only a

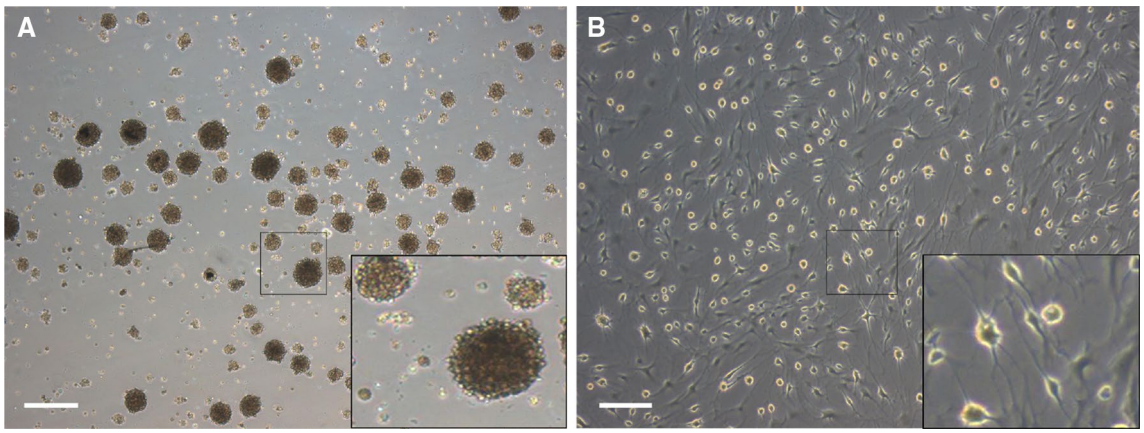

Fig. 1 Morphological characteristics of cell spheres and neuron-like cells. The bone marrow mononuclear cells were isolated and cultured, and the cell spheres were obtained after screening with a single-cell cloning method and serum-free suspension culturing (**a**). The third generation of NTCSCs were obtained using the induction method (**b**) bar = 100 μm

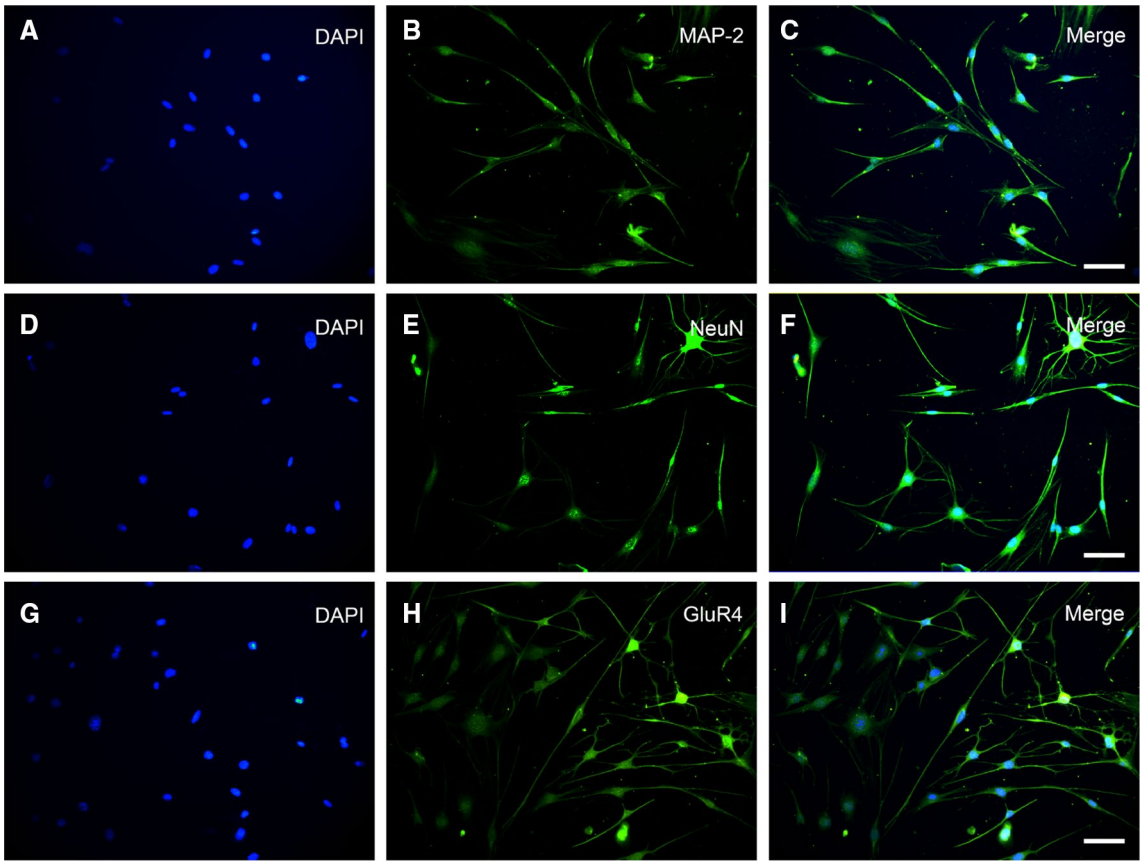

Fig. 2 Characteristic proteins of NeuN, MAP-2, and GluR4 were expressed in the cells. **a** DAPI dyeing nuclear, show blue fluorescence; **b** MAP-2 immunofluorescence staining, show green fluorescence; **c** merge figure of (**a**) and (**b**). **d** DAPI dyeing nuclear, show blue fluorescence; **e** NeuN immunofluorescence staining, show green fluorescence; **f** merge figure of (**d**)and (**e**). **g** DAPI dyeing nuclear, show blue fluorescence; **h** GluR4 immunofluorescence staining, show green fluorescence; **i** merge figure of (**g**) and (**h**)bar = 100 μm

small amount expressed on the cell membrane. DAPI staining of the nucleus was blue.

Repair Effects of Cell Transplantation on Sensory Function in the Animal Model

Survival of the Transplanted Cells in the DRG

At the 2nd week after cell transplantation, the distribution of CM-Dil-labeled NTCSC-SNs in the DRG was relatively aggregated. The morphology of the transplanted cells was relatively irregular, consisting of elliptical or polygonal shapes. The red fluorescence of the transplanted cells was very strong, and the cells were mainly distributed in the space between the nerve fibers of the DRG. At the same time, some CM-Dil-labeled NTCSC-SNs began to migrate to the space between the cells in the surrounding DRG tissue (Fig. 3).

At the 8th week after the transplantation of the NTCSC-SNs, the transplanted cells exhibiting red fluorescence in the DRG were no longer in an aggregated form, and the cells had migrated into the space between the internal cells of the DRG. The size of the labeled cells varied, and their shapes were mainly spherical or elliptical. At the 12th week after transplantation of the NTCSC-SNs, the CM-Dil-labeled red fluorescent cells were still stably expressed in the DRG and were interacting and intertwining with the DRG internal cells. The cell distribution was dispersed, and the cell bodies varied in size, with spherical or elliptical shapes observed.

Their distribution was in line with the distribution characteristics of DRG neurons, fully demonstrating that the NTCSC-SNs were already colonized in the DRG and had survived well.

Immunofluorescence staining was performed for the DRG sections using NeuN and GluR4 antibodies (Fig. 4). At the 12th week after cell transplantation, the CM-Dil-labeled NTCSC-SNs could survive in the DRG, and specific expression of NeuN and GluR4 proteins was detected in the living cells. The cell nuclei in the tissue were stained blue, while the CM-Dil-labeled NTCSC-SNs emitted red fluorescence. NeuN protein was expressed in the CM-Dil-labeled cells, indicating that the transplanted NTCSC-SNs

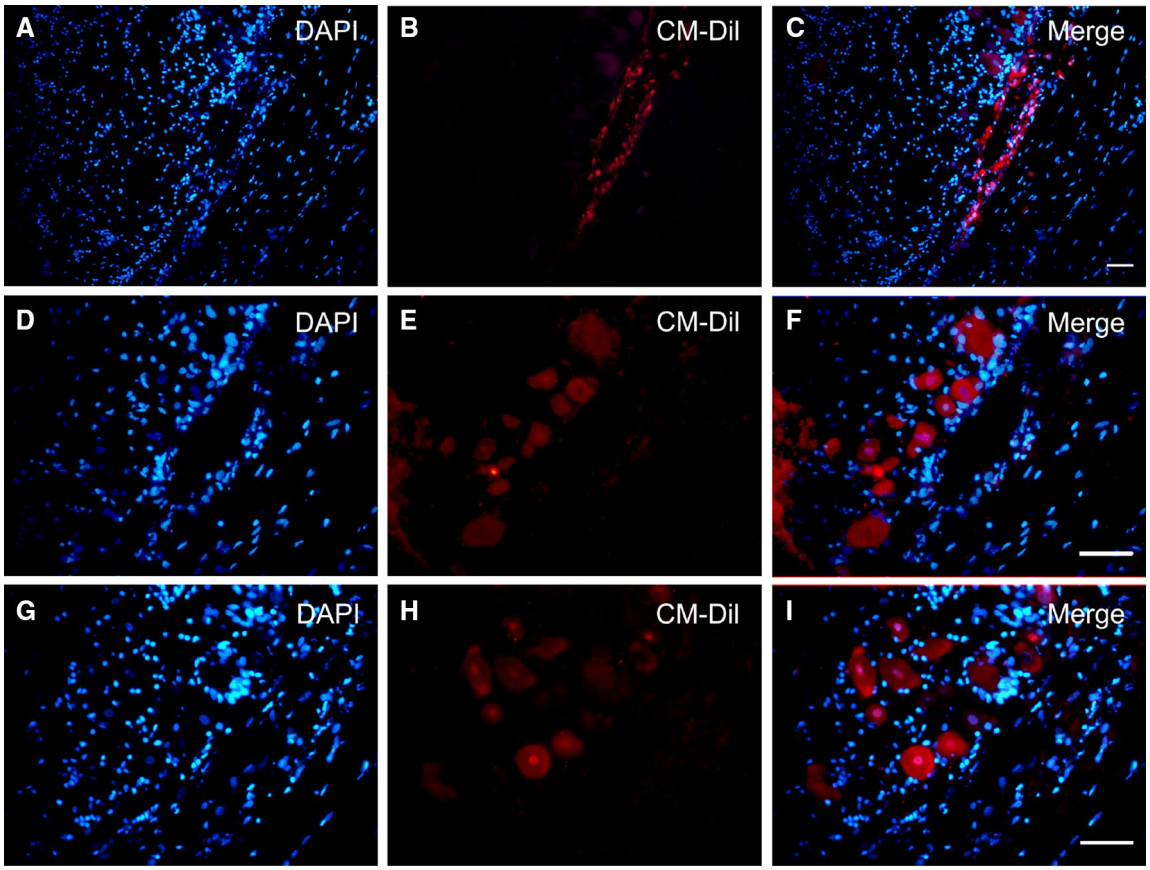

Fig. 3 Survival of the transplanted cells in the DRG. **a–c** NTCSCs source of CM-Dil marks the second week of sensory neurons transplantation. **a** DAPI dyeing nuclear, show blue fluorescence, **b** CM-Dil mark NTCSCs source sensory neurons; **c** merge figure of (**a**) and (**b**); **d–f** CM-Dil labeled NTCSCs source sensory neurons transplanted 8 weeks, **d** DAPI dyeing nuclear, show blue fluorescence, **e** CM-Dil mark NTCSCs source sensory neurons; **f** merge figure of (**d**)and (**e**); **g–i** CM-Dil labeled NTCSCs source sensory neurons transplant week 12, **g** DAPI dyeing nuclear, show blue fluorescence, **h** CM-Dil mark NTCSCs sensory neurons, **i** merge figure of (**g**) and (**h**) bar = 100 μm

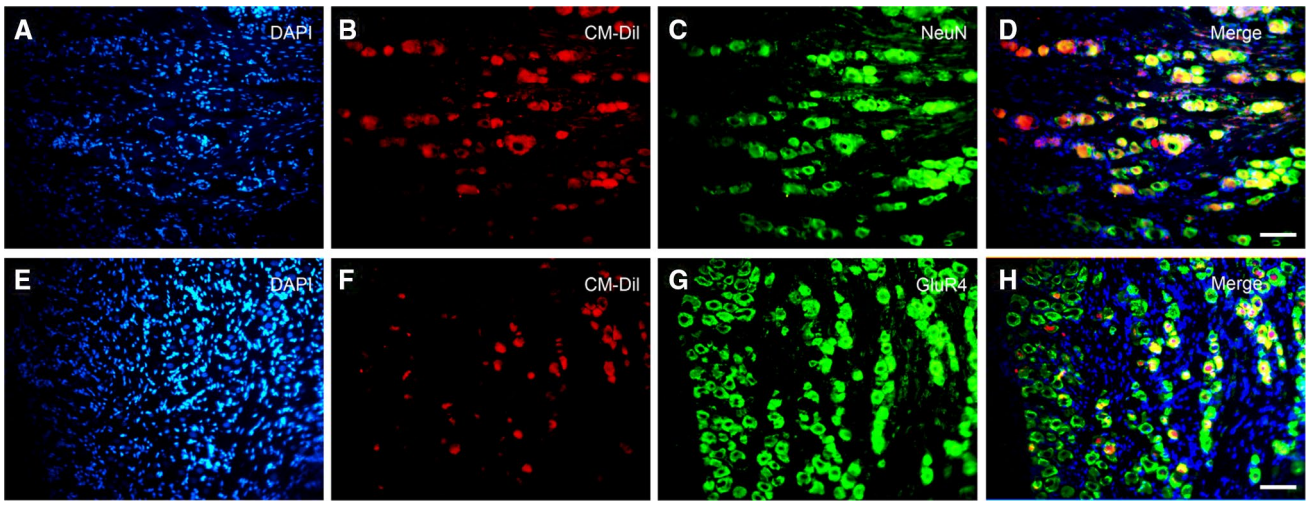

Fig. 4 Immunofluorescence staining for the DRG sections at week 12. The (**a–d**) DRG NeuN immunofluorescence test of the organization. **a** Dye DAPI nuclear; **b** CM-Dil labeled NTCSCs source sensory neurons; **c** visible NeuN dyed green fluorescence; **d** merge figure of (**a–c**). **e–h** DRG GluR4 immunofluorescence test of the organization; **e** dye DAPI nuclear; **f** CM-Dil labeled NTCSCs source sensory neurons; **g** GluR4 dyeing visible green fluorescence; **h** merge figure of (**e–g**) bar = 100 μm

had differentiated and matured, becoming neuronal cells. The CM-Dil-labeled transplanted cells could also express the glutamatergic sensory neuron marker GluR4, indicating that the transplanted cells had evolved into glutamatergic sensory-like neurons. Immunofluorescence staining revealed that NTCSC-SNs could survive and colonize in the damaged DRG of the model animals and could differentiate into sensory-like neurons.

Behavior and Sensory Function Tests

Behavioral Characteristics of the Animals The behavioral characteristics of the model rats were ataxic. Their movement was not coordinated, which manifested as unsteady gait and poor balance during walking. The locomotor function improved significantly over time. Mood, feeding, drinking, urination, and defecation of the rats were normal. By contrast, there was no significant change in the diet and behavior of the rats in the normal group, and their body weight stably increased during the experiment.

Detection of Sensory Function in the Model Animals Using Mirror Image Pain Behavior Evoked by Mechanical Stimulation At the different time points, the rats were subjected to the mirror-image pain behavior test, induced by mechanical stimulation (Table 1). The Von Frey value of the normal group was the lowest, which indicated that this group had the most sensitive plantar tactile function. The value of the blank control group also exhibited a statistically significant difference from the three treatment groups. Among groups that underwent treatment with the transplanted cells for sensory repair, the Von Frey value for the NTCSC-SN group at 12 weeks was the lowest, followed by the NTCSC group, and there were statistically significant differences among the three transplanted cell treatment groups. The Von Frey value of the NTCSC-SN group improved at 2 weeks, but there was no significant difference among the different time points (Fig. 6a).

Detection of Plantar Thermo-sensitivity in the Model Animals Using the Plantar Thermo-sensitivity Test The average thermal sensitivity value of the normal group was the lowest among all the groups at 2w, and there were statistically significant differences from the three transplanted cell treatment groups and the blank control group (Table 2). The NTCSC-SN group was statistically significantly different from the BMSCs group, the NTCSCs group, and the blank control group at different time points. The thermal sensitivity value of the NTCSC-SN group improved at 2w, and there was significant difference among the different time points (Fig. 6b).

Electrophysiological Detection of the Sciatic Nerve of the Model Animals: Detection of Sensory Nerve Latency and Sensory Nerve Conduction Velocity The amplitude of sensory nerve action potential in the model group was significantly reduced, and the latency of the sensory nerve was prolonged. The average latency of the sensory nerve of each experimental group was prolonged when compared with that of the normal group at 12 weeks. The wave amplitude of the sensory nerve action potential in the NTCSC-SN group was significantly increased at 12 weeks (Fig. 5).

The average latency of the sensory nerve in the normal group was the lowest(Table 3), and thus, the average sensory nerve conduction velocity of the normal group was the highest (Table 4). In the NTCSC-SN group, the average latency

Table 1 Test of mirror image pain caused by mechanical stimulation (g) ($\bar{x} \pm SD$, $n=5$)

Groups	Time points				X^2	P
	2w	4w	8w	12w		
Normal	5.20 ± 1.79	5.60 ± 1.67	6.40 ± 1.67	7.20 ± 1.10	4.269	0.234
Blank control	14.00 ± 2.24▲•	13.00 ± 2.74▲•	12.00 ± 2.74▲•□	12.00 ± 2.74▲•□	1.583	0.663
BMSCs	13.00 ± 2.74♦•♠	11.00 ± 2.24•♠	10.60 ± 2.61•♠	10.60 ± 2.61♦•♠	3.152	0.369
NTCSCs	14.00 ± 2.24■♠	13.00 ± 2.74■♠	9.60 ± 0.89♠□	9.20 ± 1.10■♠□	11.125	0.011
NTCSC-SN	12.60 ± 3.36▲■	10.60 ± 2.61▲■	10.20 ± 2.86▲	8.40 ± 1.67▲♦■	4.55	0.208
X^2	14.470	14.984	13.902	13.181		
P	0.006	0.005	0.008	0.010		

12W: Normal VS Other groups ($P<0.01$);•Blank control VS BMSCs ($P=0.003$);□Blank control VS NTCSCs ($P=0.000$);▲Blank control VS NTCSC-SN ($P=0.000$);♠BMSCs VS NTCSCs ($P=0.000$);♦BMSCs VS NTCSC-SN ($P=0.000$);■NTCSCs VS NTCSC-SN ($P=0.038$)

2W: Normal VS Other groups (P = 0.000);•Blank control VS BMSCs ($P=0.022$);▲Blank control VSNTCSC-SN (P = 0.024);♠BMSCs VS NTCSCs ($P=0.022$);■NTCSCs VS NTCSC-SN ($P=0.024$)

4W:Normal VS Other groups (P = 0.000);•Blank control VS BMSCs ($P=0.000$);▲Blank control VS NTCSC-SN ($P=0.000$);♠BMSCs VS NTCSCs ($P=0.000$);■NTCSCs VS NTCSC-SN ($P=0.000$)

8W: Normal VS Other groups ($P=0.000$);•Blank control VS BMSCs ($P=0.000$);□Blank control VS NTCSCs ($P=0.000$);▲Blank control VS NTCSC-SN ($P=0.000$);♠BMSCs VS NTCSCs ($P=0.007$)

Table 2 Thermal sensitivity value of plantar thermo-sensitivity test (s) ($\bar{x} \pm SD$, $n=5$)

Groups	Time points				*F*	*P*
	2w	4w	8w	12w		
Normal	11.31 ± 1.67	11.91 ± 1.22	12.15 ± 1.94	12.21 ± 1.14	0.358	0.784
Blank control	41.09 ± 7.08$^{▲}$	37.45 ± 3.31$^{▲}$	34.52 ± 4.05$^{▲}$	34.78 ± 5.81$^{▲}$	1.680	0.211
BMSCs	36.11 ± 3.43$^{◆}$	32.10 ± 2.71$^{◆}$	29.97 ± 3.89$^{◆}$	27.11 ± 3.37$^{◆}$	6.313	0.005
NTCSCs	34.60 ± 5.03$^{\#}$	29.64 ± 1.85$^{\#}$	27.11 ± 2.01$^{■}$	25.88 ± 3.72$^{■}$	6.406	0.005
NTCSC-SN	32.25 ± 4.97$^{▲◆\#}$	28.28 ± 3.53$^{▲◆\#}$	21.68 ± 2.95$^{▲◆■}$	17.50 ± 2.02$^{▲◆■}$	17.450	0.000
F	28.903	64.447	38.546	30.087		
P	0.000	0.000	0.000	0.000		

12W: Normal VS Other groups ($P<0.01$); $^{▲}$NTCSC-SN VS Blank control ($P=0.000$); $^{◆}$NTCSC-SN VS BMSCs ($P=0.000$); $^{■}$NTCSC-SN VS NTCSCs ($P=0.001$)

2W: Normal VS Other groups ($P=0.000$); $^{▲}$NTCSC-SN VS Blank control ($P=0.008$); $^{◆}$NTCSC-SN VS BMSCs ($P=0.216$); $^{\#}$NTCSC-SN VS NTCSCs ($P=0.446$);

4W:Normal VS Other groups ($P=0.000$); $^{▲}$NTCSC-SN VS Blank control ($P=0.000$); $^{◆}$NTCSC-SN VS BMSCs ($P=0.035$); $^{\#}$NTCSC-SN VS NTCSCs ($P=0.428$)

8W: Normal VS Other groups ($P=0.000$); $^{▲}$NTCSC-SN VS Blank control ($P=0.000$); $^{◆}$NTCSC-SN VS BMSCs ($P=0.000$); $^{■}$NTCSC-SN VS NTCSCs ($P=0.012$)

of the sensory nerve was significantly different from that in the other three groups since week 2. The average latency of the sensory nerve in the NTCSC-SN group decreased gradually with the extension of the time (Fig. 6c, d).

Discussion

The repair and regeneration of peripheral nerve injury is an important field of neuroscience research. The sensation and motor functions controlled by the injured nerve are abnormal after nerve injury. However, the understanding on the specificity of sensation and movement in the regenerated peripheral nerve is controversial (Allodi et al. 2012; He et al. 2012; Navarro et al. 2007). Clinical practice has primarily focused on the repair of motor function, while the importance of sensory repair is often ignored.

One of the core problems in regenerative medicine is the use of stem cells for transplantation. NSCs are primarily derived from embryonic or adult tissue. At present, the technology and methods to isolate and select the pluripotent stem cells derived from fetal central nervous tissue are mature (Gelati et al. 2013; Martens et al. 2000). However, because of complex issues related to the sources and clinical applications of the cells (such as ethical concerns, the possibility of immunological rejection, and the potential for self-tumorigenicity), wide clinical application of embryonic stem cells is greatly limited. Although NSCs (Andressen 2013; Wang et al. 2011; Ahmed 2009) derived from adult central nervous tissues can be obtained by isolating and culturing the adult hippocampus, striatum, dentate gyrus, olfactory bulb, and subventricular zone, these cells are difficult to obtain using this approach in clinical practice, since it poses a great risk of injury to the central nervous system, and the amount of obtained NSCs is extremely small. Therefore, this method has no clinical value.

In 2002, Kabos co-cultured unseparated bone marrow with EGF and bFGF, and successfully obtained cell spheres expressing neural nestin (a neural stem cell marker) and CD90 (a marker of mesenchymal stem cells) from adult rat bone marrow (Kabos et al. 2002). Additionally, the cells in the cell spheres could further differentiate and express neurogenin 1 (a neural differentiation-related transcription factor), as well as the neuron and glial cell markers NF-200, MAP-2, NSE, NeuN, CNPase, and GFAP proteins. Neural precursor cells obtained in this manner are similar to those obtained using NSCs from the central nervous system, while this method can effectively avoid the ethical issues and immunological rejection associated with the use of NSCs extracted from embryonic tissue.

Furthermore, BMSCs can be easily obtained and offer the potential for autologous transplantation, as they are free of immunogenicity and ethical restrictions. BMSCs have the functional characteristics of strong self-proliferative ability and a wide range of differentiation, along with the ability to repair injured tissue and provide immunoregulation, thus showing broad treatment prospects.

In recent years, experimental results (Amr et al. 2014; Zaminy et al. 2013; Nijhuis et al. 2013) have shown that the transplantation of BMSCs could indeed repair motor and sensory functions after spinal cord injury. However, the NSC transplantation results reported by Cao (Cao et al. 2001; Iwasaki et al. 2014; Hwang et al. 2009) revealed that, when undifferentiated NSCs and BMSCs were transplanted into the spinal cord tissue of normal adult rats, they could survive in the spinal cord tissue, but mainly differentiated into glial

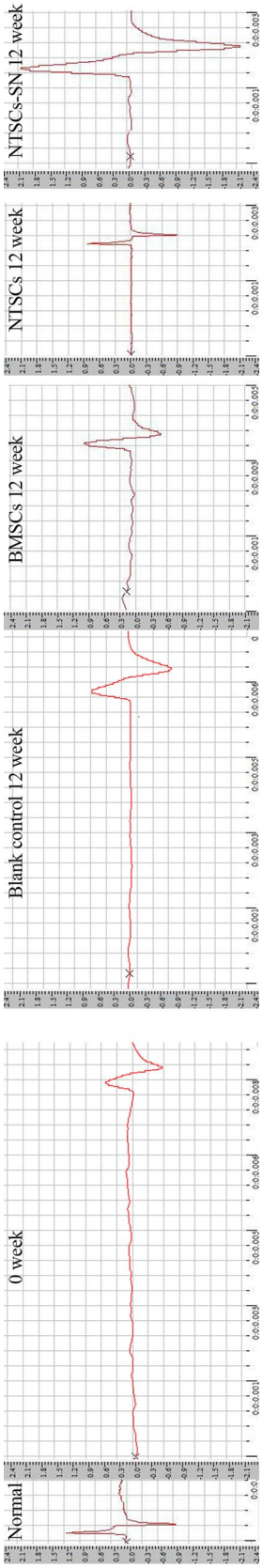

Fig. 5 Amplitude of the sensory nerve action potential. *Note* vertical axis: amplitude (mV); horizontal axis: time (ms)

cells. In addition, NSCs or BMSCs transplanted into injured spinal cord tissue could migrate to the lesion, but they also mainly differentiated into GFAP-positive astrocytes or remained in a nestin-positive stage with no differentiation. These findings suggested that, in order to allow the in vivo differentiation of transplanted seed cells into the expected nerve cells, the differentiation of seed cells to obtain the clinically desired nerve cells should be initially induced in vitro before cell transplantation, which should achieve a better repair result.

Some studies have suggested that BMSCs can affect the survival of DRG neurons; however, these studies have only examined in vitro cells (Xu et al. 2011; Kamishina et al. 2009; Gu et al. 2010). Our laboratory successfully isolated and purified NTCSCs from the bone marrow of SD rats, induced their differentiation into sensory neurons, and identified the cells obtained after the induction (Ren et al. 2007, 2008, 2009; Yu et al. 2015). The results showed that the cells obtained after the induction had the characteristics of glutamatergic sensory-like neurons, and these cells were identified as NTCSC-SNs. The NTCSC-SNs are developed from autologous bone marrow, which avoids ethical controversy and the possibility of rejection after transplantation, and are easy to obtain. Furthermore, these cells exhibit good stem cell characteristics and good survival ability, and their differentiation is easily induced.

The NTCSC-SNs were transplanted into the DRG of the model animals using regenerative medicine methods. An in vivo experimental study was conducted to determine whether the transplanted cells could survive and contribute to the repair and regeneration of the DRG neurons to ultimately restore the sensory deficits of the animals. Finally, the CM-Dil-labeled NTCSC-SNs were detected in the DRG of the model animals, indicating that they had been successfully colonized in the DRG, and the cells eventually survived in a good condition. The morphological results showed that the transplanted BMSCs or NTCSCs alone could also survive in the DRG of the model animals; however, they did not always take on an aggregated form and could not effectively differentiate into the sensory neurons required for the DRG tissue.

The functions of the fibers Aα, Aβ, Aδ and C vary in the sensory nerve. Aα fibers not only conduct the impulse of proprioception, but also transmit the somatic motor information; therefore, the measurement of potential latency and conduction velocity can reflect the conduct function of nerve fibers. Aβ fibers conduct the touch-pressure sensation, while they are unable to transmit the information of pain and temperature. Furthermore, Aδ and C fibers both conduct the pain, temperature and touch-pressure sensations. However, the diameter and conduction velocity of the fibers are significantly different. It has previously been proved that Aδ fibers mainly conduct the pain sensation evoked by mechanical

Table 3 Average latency of the sensory nerve (ms) ($\bar{x} \pm SD$, $n=5$)

Groups	Time points				*F*	*P*
	2w	4w	8w	12w		
Normal	0.62 ± 0.05	0.65 ± 0.05	0.64 ± 0.10	0.63 ± 0.06$^{\times}$	0.245	0.863
Blank control	4.05 ± 0.65$^{\blacktriangle}$	3.75 ± 0.33$^{\blacktriangle}$	3.75 ± 0.23$^{\blacktriangle}$	3.61 ± 0.49$^{\blacktriangle}$	0.846	0.489
BMSCs	2.81 ± 0.43$^{\blacklozenge}$	2.35 ± 0.36$^{\blacklozenge}$	1.99 ± 0.17$^{\blacklozenge}$	1.57 ± 0.16$^{\blacklozenge}$	14.963	0.000
NTCSCs	2.61 ± 0.20$^{\#}$	2.23 ± 0.15$^{\blacksquare}$	1.87 ± 0.25$^{\blacksquare}$	1.41 ± 0.06$^{\blacksquare}$	40.597	0.000
NTCSC-SN	2.21 ± 0.21$^{\blacktriangle\blacklozenge\#}$	1.77 ± 0.27$^{\blacktriangle\blacklozenge\blacksquare}$	1.38 ± 0.10$^{\blacktriangle\blacklozenge\blacksquare}$	0.92 ± 0.05$^{\blacktriangle\blacklozenge\blacksquare\times}$	47.013	0.000
F	54.600	93.027	200.914	126.644		
P	0.000	0.000	0.000	0.000		

12W: Normal VS Other 3 groups (Blank control, BMSCs and NTCSCs) ($P=0.000$); $^{\blacktriangle}$NTCSC-SN VS Blank control ($P=0.000$); $^{\blacklozenge}$NTCSC-SN VS BMSCs ($P=0.000$); $^{\blacksquare}$NTCSC-SN VS NTCSCs ($P=0.003$); $^{\times}$NTCSC-SN VS Normal ($P=0.066$)

2W: Normal VS Other groups ($P=0.000$); $^{\blacktriangle}$NTCSC-SN VS Blank control ($P=0.000$); $^{\blacklozenge}$NTCSC-SN VS BMSCs ($P=0.021$); $^{\#}$NTCSC-SN VS NTCSCs ($P=0.110$)

4W:Normal VS Other groups ($P=0.000$); $^{\blacktriangle}$NTCSC-SN VS Blank control ($P=0.000$);$^{\blacklozenge}$ NTCSC-SN VS BMSCs ($P=0.002$); $^{\blacksquare}$NTCSC-SN VS NTCSCs ($P=0.010$)

8W: Normal VS Other groups ($P=0.000$); $^{\blacktriangle}$NTCSC-SN VS Blank control ($P=0.000$); $^{\blacklozenge}$NTCSC-SN VS BMSCs ($P=0.000$); $^{\blacksquare}$NTCSC-SN VS NTCSCs ($P=0.000$)

Table 4 Average sensory nerve conduction velocity (m/s) ($\bar{x} \pm SD$, $n=5$)

Groups	Time points				*F*	*P*
	2w	4w	8w	12w		
Normal	44.03 ± 5.52	43.41 ± 3.56	45.04 ± 1.49	46.22 ± 2.93	0.558	0.65
Blank control	8.72 ± 1.90$^{\blacktriangle\square}$*	9.55 ± 0.85$^{\blacktriangle}$	9.20 ± 0.43$^{\blacktriangle}$	10.24 ± 1.50$^{\blacktriangle}$	1.219	0.335
BMSCs	12.78 ± 2.50*	15.49 ± 2.09$^{\blacklozenge}$	17.68 ± 1.17$^{\blacklozenge}$	21.95 ± 2.12$^{\blacklozenge}$	18.251	0.000
NTCSCs	13.25 ± 1.22$^{\square}$	15.73 ± 1.44$^{\#}$	18.77 ± 2.80$^{\blacksquare}$	25.01 ± 1.56$^{\blacksquare}$	37.320	0.000
NTCSC-SN	15.76 ± 1.76$^{\blacktriangle}$	19.75 ± 3.42$^{\blacktriangle\blacklozenge\#}$	25.22 ± 2.14$^{\blacktriangle\blacklozenge\blacksquare}$	38.47 ± 1.36$^{\blacktriangle\blacklozenge\blacksquare}$	92.546	0.000
F	113.163	137.343	281.381	256.154		
P	0.000	0.000	0.000	0.000		

12W: Normal VS Other groups ($P<0.05$); $^{\blacktriangle}$NTCSC-SN VS Blank control ($P=0.000$); $^{\blacklozenge}$NTCSC-SN VS BMSCs ($P=0.000$); $^{\blacksquare}$NTCSC-SN VS NTCSCs ($P=0.001$)

2W: Normal VS Other groups ($P=0.000$); $^{\blacktriangle}$Blank control VS NTCSC-SN ($P=0.001$); $^{\square}$Blank control VS NTCSCs ($P=0.027$); *Blank control VS BMSCs ($P=0.045$)

4W: Normal VS Other groups ($P=0.000$); $^{\blacktriangle}$NTCSC-SN VS Blank control ($P=0.000$); $^{\blacklozenge}$NTCSC-SN VS BMSCs ($P=0.035$); $^{\#}$NTCSC-SN VS NTCSCs ($P=0.428$)

8W: Normal VS Other groups ($P=0.000$); $^{\blacktriangle}$NTCSC-SN VS Blank control ($P=0.000$); $^{\blacklozenge}$NTCSC-SN VS BMSCs ($P=0.000$); $^{\blacksquare}$NTCSC-SN VS NTCSCs ($P=0.012$)

contact, which is measured by the Von Frey Hairs test. Additionally, Aδ fibers transmit the pain stimulus faster than C fibers. In the present study, the pain sensation induced by mechanical stimulation was reflected quickly, indicating that it should be conducted by the Aδ fibers. Once the pain sensation reached the threshold, the rats would lift their paws to avoid further pain. Therefore, the pain sensation evoked by mechanical stimulation was mainly transmitted through the Aδ fibers, which is consistent with the findings of previous studies. The data of the present study also demonstrated a prolonged potential latency and a slower conduction velocity of the sensory nerve after sensory defect was induced, while shorter potential latency and faster conduction velocity of sensory nerve was observed after cells transplantation.

The electrophysiological results of the present study showed that the transplantation of MSCs (Wang et al. 2010) and NSCs (Zhang et al. 2015) alone could also improve the electrophysiological parameters of the model animals, which is consistent with the results of the studies conducted by other scholars. However, in the present study, effects comparable to those of the NTCSC-SNs could not be achieved, suggesting that the transplantation of NTCSC-SNs has the best effect on sensory nerve latency and conduction velocity recovery in the model animals.

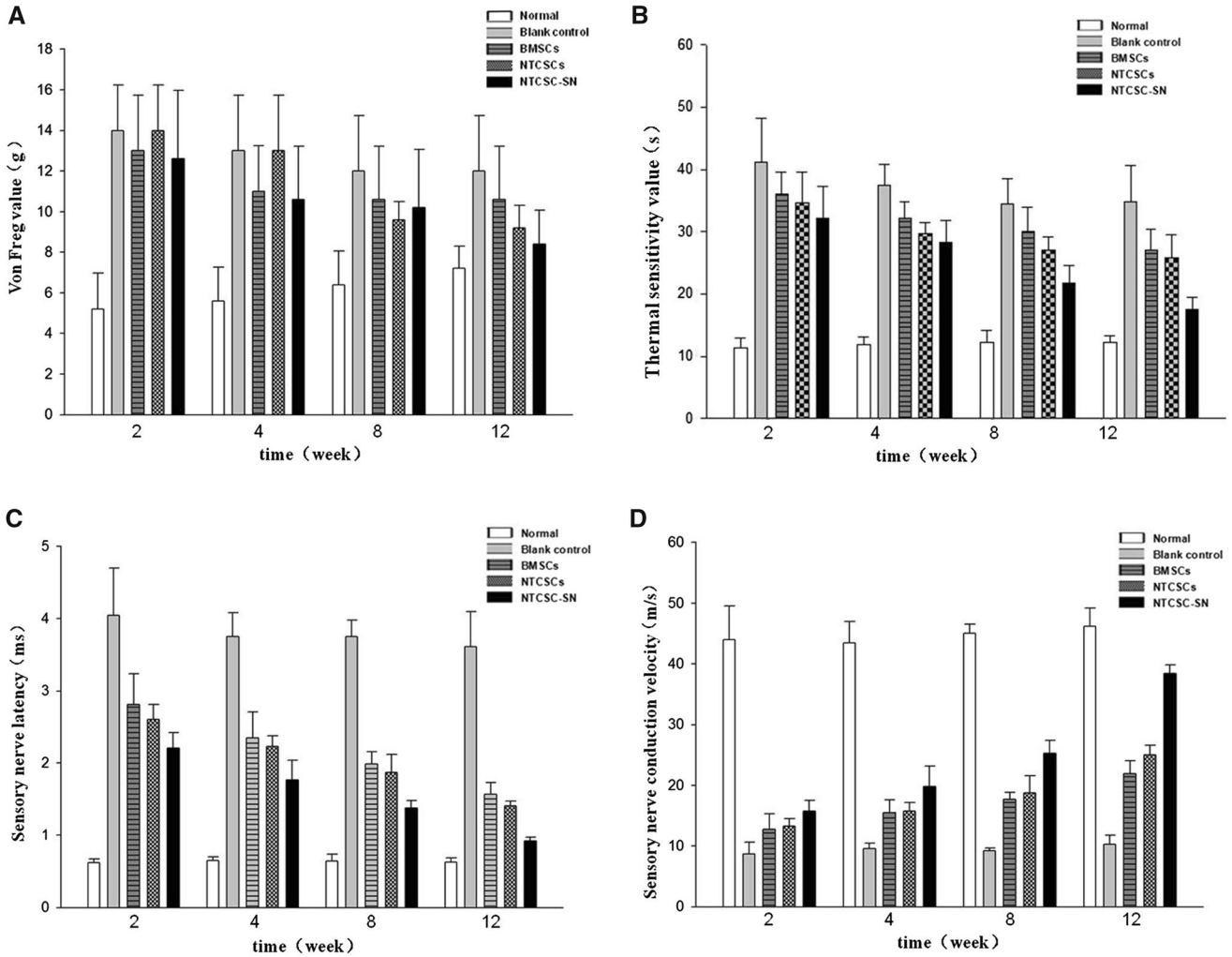

Fig. 6 Sensory function tests for the animals. **a** The value of mirror image pain results at different time points in different groups; **b** the thermal sensitivity value at different time points in different groups; **c** the sensory nerve latency at different time points in different groups; **d** the average sensory nerve conduction velocity at different time points in different groups

Furthermore, the results of the sensory function test indicated that with increasing time after cell transplantation, the plantar sensory function of the model rats was evidently restored. Previous studies have reported that BMSCs could repair sensory function (Ritfeld et al. 2012; Kakabadze et al. 2016; Sandner et al. 2016); however, the results of our experiments demonstrated that, among the variety of cells that were transplanted to treat sensory injury in the animal model, NTCSC-SNs showed the greatest efficacy.

Conclusion

In summary, the present study found that NTCSC-SNs could differentiate and develop into glutamatergic sensory-like neurons in the DRG of the model animals. The electrophysiological, behavioral and sensory function evaluations confirmed that the NTCSC-SNs had a good repair effect on the sensory deficits of the model animals. The specific signaling pathways and molecular mechanisms underlying the effect of the cells after transplantation into the DRG, as well as the interaction and relationship of the transplanted cells in the DRG with the surrounding environment and other cells, need to be elucidated in further in-depth studies.

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Author Contributions FH and CZ carried out the concepts. ZY, NX and NZ participated in the design of this study and performed the statistical analysis. These three authors contributed to this work equally and should be considered as co-first authors. YX, ZW, SL, DZ, carried out the study and collected important background information. ZY, NX, and NZ drafted the manuscript. All authors read and approved the final manuscript.

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Compliance with Ethical Standards

Conflict of interest The authors have declared no conflict of interest.

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