Stem Cell Reports, Volume *12*

Supplemental Information

Electroacupuncture Facilitates the Integration of Neural Stem Cell-Derived Neural Network with Transected Rat Spinal Cord

Hui Jin, Yu-Ting Zhang, Yang Yang, Lan-Yu Wen, Jun-Hua Wang, Hao-Yu Xu, Bi-Qin Lai, Bo Feng, Ming-Tian Che, Xue-Cheng Qiu, Zhi-Ling Li, Lai-Jian Wang, Jing-Wen Ruan, Bin Jiang, Xiang Zeng, Qing-Wen Deng, Ge Li, Ying Ding, and Yuan-Shan Zeng

SUPPLEMENTAL INFORMATION

Supplemental Experimental Procedures

Whole-cell Patch Clamp

NSC-derived cells in the neural network (NN) were transferred into a recording chamber. Artificial cerebrospinal fluid bubbled with 95% O_2 and 5% CO_2 was perfused into the recording chamber using a peristaltic pump (HEKA Inc., Germany) with a constant speed 3-4 ml/min. Cells were held in the current-clamp mode and their firing properties were assessed by delivering 600 ms depolarizing current steps. The internal solution for current-clamp recordings contained 130 mM K-gluconate, 10 mM KCl, 0.2 mM EGTA, 10 mM HEPES, 4 mM ATP, 0.5 mM GTP, and 10 mM Na-Phosphocreatine, pH 7.2-7.4, and the osmolarity was 270-290 mOsm. To record the miniature EPSC, cells were held on voltage-clamp mode and patch pipettes (2-4 MΩ) were filled with the internal solution consisting of the following: 120 mM Cs-methylsulfonate, 10 mM HEPES, 10 mM Na-phosphocreatine, 5 mM lidocaine N-ethyl bromide (QX-314), 4 mM ATP, 0.5 mM GTP, pH 7.2-7.3, and the osmolarity of the solution was 270-290 mOsm. mEPSCs were recorded in the presence of 1 μM TTX, 100 μM DL-APV and 50 μM picrotoxin. Cells were held at -70 mV to record mEPSCs. All electrophysiological recordings were performed at room temperature (22-24 ℃). All data were filtered at 3 kHz and digitized at 10 kHz using Igor Pro (Wave Metrics, Lake Oswego, OR).

Scanning Electron Microscopy

The surface morphology of NSC-derived neurons within the scaffolds cultured for 14 days was examined by scanning electron microscopy (SEM). For SEM examination, scaffolds were firstly washed 3 times with PBS, fixed in 2.5% glutaraldehyde in 0.1M phosphate buffer pH 7.4 overnight, dehydrated in a graded series of ethanol and then dried for 2 days. The dried samples were sputtered coated with gold and examined under a scanning electron microscope (Philips XL30 FEG, Holland).

Electron Microscopy

For electron microscopy (EM), the scaffolds were fixed with 2.5% glutaraldehyde in 0.1M phosphate buffer pH 7.4 at 4℃ for 1 h and post-fixed with 1% osmic acid for 1 h. The scaffolds were dehydrated through graded ethanol and embedded in Epon812 overnight, followed by polymerization at 60℃ for 48 h. Ultrathin sections were cut with an ultramicrotome (Reichert E, Co., Vienna, Austria) and examined under a transmission electron microscope (Philips CM10, Eindhoven, Holland).

Pseudorabies Virus Retrograde Tracing

Eight weeks after surgery, the rats were anesthetized with 1% pentobarbital sodium (40 mg/kg, i.p.). A longitudinal incision was made in the skin over the upper posterior part of the thigh and the gluteal region. The gluteus maximus muscle was then separated with a pair of iris scissors along the direction of its muscle fibres to expose the sciatic nerve as much as possible. To minimize animal suffering, 2% lidocaine was applied with a cotton-tipped stick. With the aid of a dissecting stereomicroscope (Leica Microsystems, Inc., Wetzlar, Germany), the needle tip of a 30g needle Hamilton syringe (Hamilton Co., Reno) was inserted into sciatic nerve along its longitude axis for 10 mm, and then withdrawn 2 mm to make a potential space for injection. Then, 1μl of pseudorabies virus (PRV-CMV-mRFP, red, 2.5×10^9 PFU/ml) was slowly injected and the needle was held in place for 5 min. The sciatic nerve was lightly crushed with a pair of blunt forceps on the top of the injection site to maximize PRV contact with nerve fibers. This was followed by a thorough rinsing of the injection site with sterile saline soaked cotton-tipped stick, and then the wound was sutured. Animals were sacrificed 5 days after injection.

Immunoelectron Microscopy

For immunoelectron microscopy (IEM), rats were perfused transcardially with 0.1 mol/L of sodium phosphate buffer containing 187.5 units/100 ml of heparin, followed by perfusion with 4% paraformaldehyde (PFA) containing 0.1% glutaraldehyde and 15% saturated picric

acid. The spinal cord segment containing injury/graft site was postfixed overnight at 4℃ in fresh 4% PFA and subsequently cut into 50 μm sagittal sections on a vibratome. To improve the penetration of antibodies, vibratome sections were transferred into cryprotectant solution containing 25% sucrose and 10% glycerol in 0.1 M PBS overnight at 4℃, followed by a quick freeze-thaw in liquid nitrogen three times. After washing with PBS, the sections were treated for 1 h with 20% goat serum (Tris buffer, pH 7.4) to block nonspecific binding of the antibody. Sections were first incubated with anti-GFP primary antibody in 2% normal goat serum solution at 4℃ for 24 h, then incubated with secondary antibodies overnight at 4℃, and postfixed in 1% glutaraldehyde for 10 min. The sections were incubated with ABC kit (Laboratories, CA, USA,) and detected by 3,3-diaminobenzidine (DAB, Sigma, USA), and then osmicated, dehydrated, and embedded in Epon812. The Epon blocks were sectioned and examined under the electron microscope (Philips CM 10, Eindhoven, Holland).

Western Blot

Eight weeks after surgery, 20 rats (n = 5 for each group) were sacrificed. The spinal cord was immediately removed and the injured spinal segments (0.5 cm) containing the injury/graft sites were dissected and readily homogenized on ice in Western lysis buffer (Beyotime Institute of Biotechnology, China) containing 1% protease inhibitor (Sigma, USA) and 1% phosphatase inhibitor (Sigma, USA) using homogenizers. Homogenates were centrifuged at 12,000 g for 15 min at 4℃, and the supernatant liquid was collected and stored at -80℃ for Western blot analysis. Equal amounts of the protein suspension were loaded on a 10% polyacrylamide gel, separated by gel electrophoresis, and transferred onto a polyvinylidene fluoride (PVDF) membrane. The membranes were incubated with the following antibodies (see Table 1): mouse anti-β-actin (1:1000), rabbit anti-NT-3 (1:500), rabbit anti-TRKC (1:1000), rabbit anti-AKT (1:1000), rabbit anti-Phospho-AKT (Ser473) (1:1000), rabbit anti-SYN (1:1000) and rabbit anti-PSD95 (1:1000) overnight at 4℃, respectively. After washing, the various biomarkers were revealed with a horseradish peroxidase (HRP)-conjugated secondary antibody (goat anti-mouse or goat anti-rabbit 1:5000, Jackson, USA) for 2 h at room temperature. The protein bands were visualized by using an ECL

(enhanced chemiluminescence) Western Blot Kit (Kangwei, Beijing, China).

Morphological Quantification

For *in vitro* quantification of immunopositive cells, 10 separate sections from each scaffold were selected (n = 5 for each group). After IFS with respective antibodies, five areas (four corners and one center) at 200× magnification for each of the sections were scrutinized. The percentage of immunopositive cells was obtained by the total number of immunopositive and Hoechst33342 double positive cells divided by the total number of all Hoechst33342 positive cells.

To observe the area of the injury/graft site of spinal cord, one in every nine of the whole series of longitudinal sections from each animal (a total of 10 sections per rats, n = 5 for each group) was stained with anti-GFP antibodies and imaged at 100× magnification, and the areas emitting detectable GFP positive signal at this magnification were outlined and measured using ImageJ software. Volume estimations were calculated using the formula: $V = \sum A \times T$, in which V = volume, A = area of GFP positive, T = distance between each sampled region (0.18 mm).

For the quantification of regenerating axons *in vivo*, NF or GAP43 positive axons were quantified. Briefly, two 0.32 mm × 0.32 mm (800 × 800 pixels) areas were selected respectively at three regions of the rostral site, graft site and caudal site to the injury site at 200× magnification for each section of spinal cord (a total of 10 sections per rats, n = 5 for each group). The area of GAP43 or NF positive axons signal in the unit area was automatically calculated by using Image J according to the procedure described earlier (Qiu et al., 2015). Following this, we calculated the ratio of GAP43 or NF positive signal area in unit area in three regions, respectively. Thresholding values on stained images were chosen to make sure that only immunolabeled axons were included. Weak non-specific background labeling was excluded.

To analyze the PRV labeled cells in the T9 (2 mm rostral to injury/graft site), T10 (injury/graft site), and T11 (2 mm caudal to injury/graft site) segments, one in every nine of the whole series of longitudinal sections from each animal (a total of 10 sections per rats, n =

3 for each group) was stained with anti-MAP2 antibodies and imaged at 100× magnification and counted using ImageJ software.

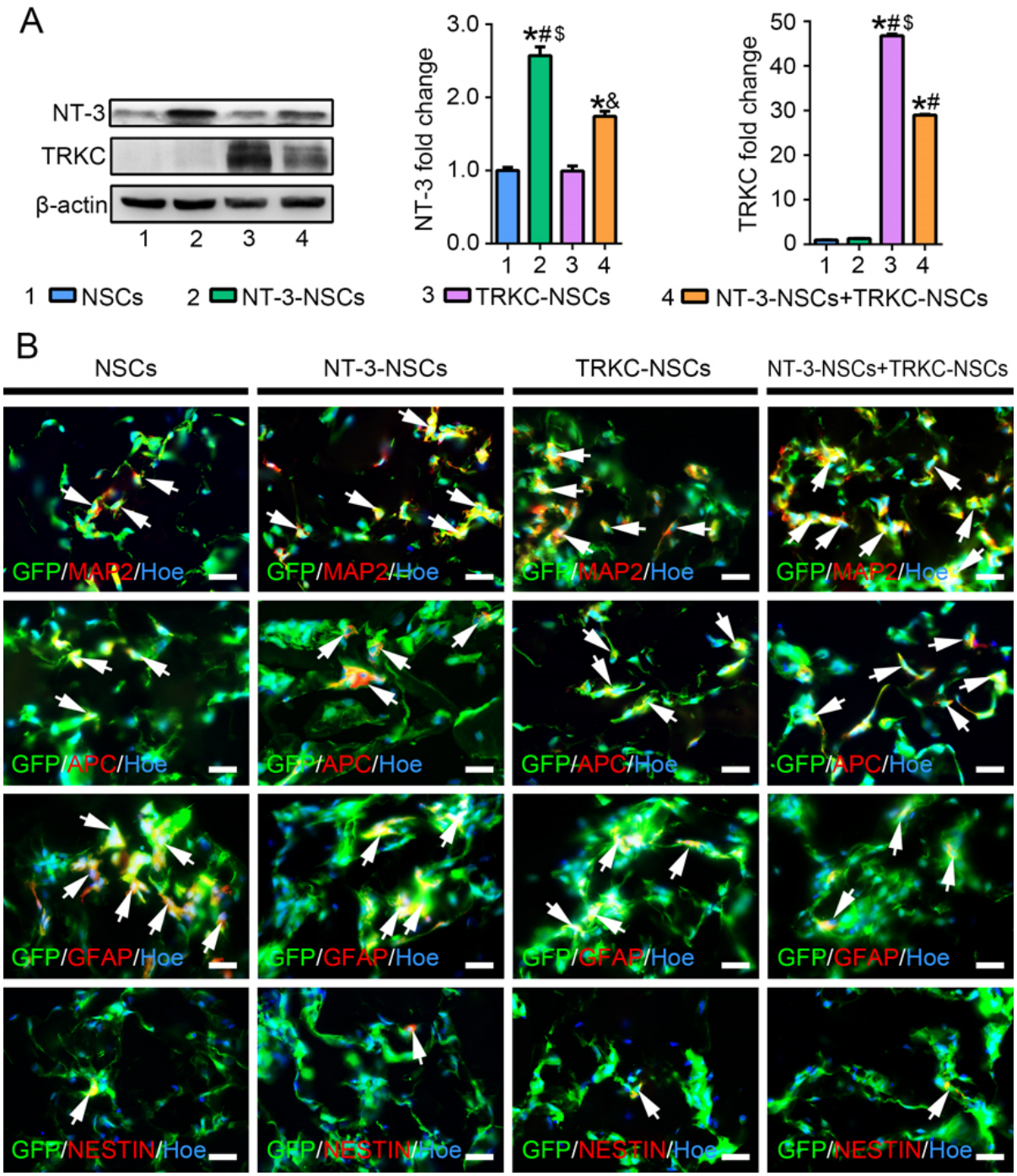

Figure S1. Analysis of Adenovirus (Ad) Vector-mediated Transgene Expression in NSC-derived Neural Network Scaffold and the differentiation of NSCs after 14-day co-culture *in vitro*.

(A) NT-3 and TRKC expression was detected by Western blot (1.the NSCs group, 2.the NT-3-NSCs group, 3.the TRKC-NSCs group, 4.the NT-3-NSCs+TRKC-NSCs group). Bar charts showing semi-quantitative comparison of NT-3 and TRKC in each group (n = 5 per

group; data presented as mean ± S.D.) * indicates significant difference from NSCs group (*p < 0.05); # indicates significant difference from the NT-3-NSCs (#p < 0.05); & indicates significant difference from the TRKC-NSCs group (&P < 0.05) by one-way ANOVA followed by LSD-t. (B) GFP^+ NSCs-derived cells (arrows) were respectively labeled by the neuron marker MAP2, oligodendrocyte marker APC, astrocyte marker GFAP and neural precursors marker NESTIN in the NSCs, NT-3-NSCs, TRKC-NSCs and NT-3-NSCs+TRKC-NSCs groups. The cell nuclei were labeled by Hoechst33342 (Hoe). Scale bars, 20 μm.

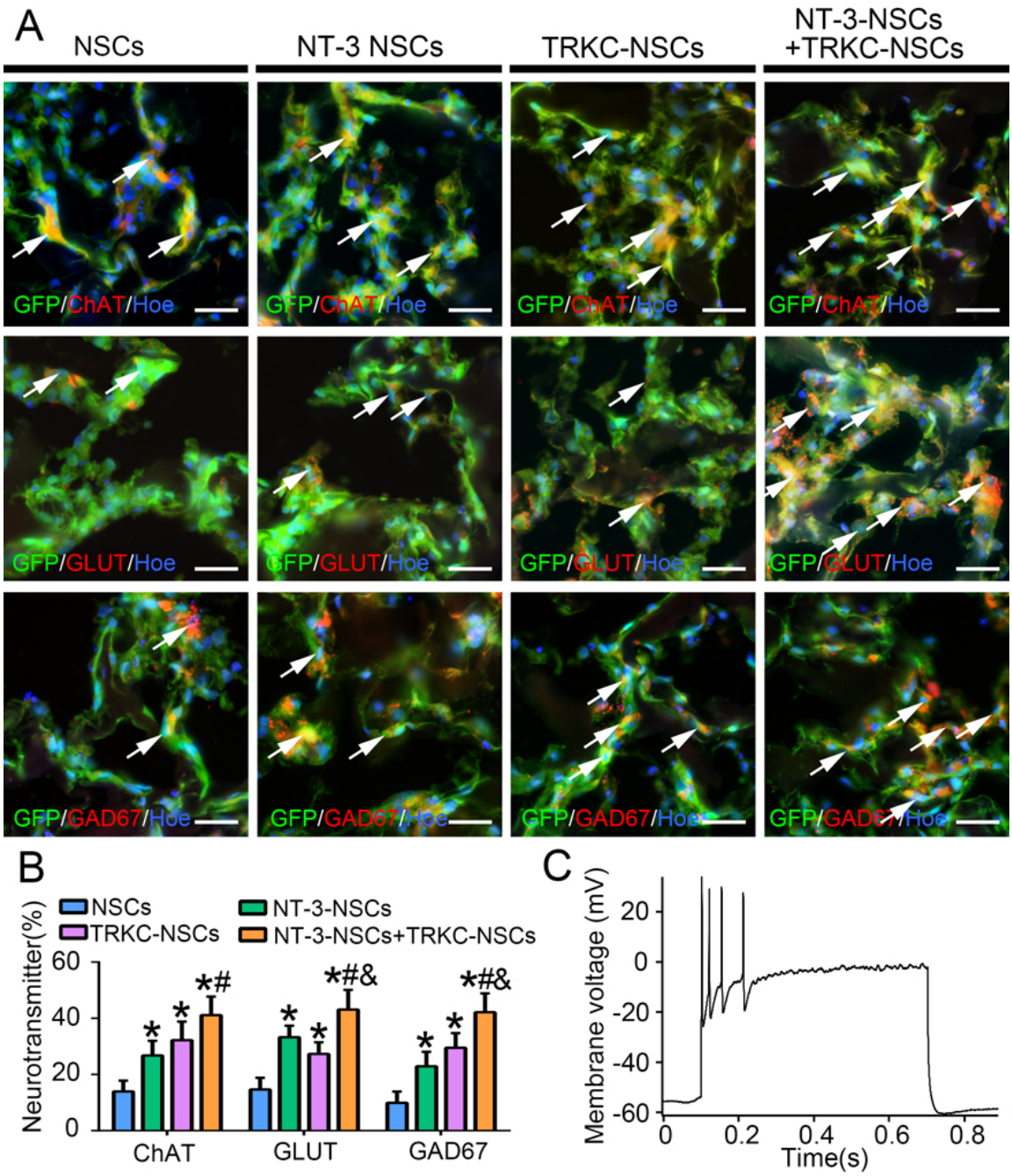

Figure S2. Neurotransmitters were expressed and Action Potentials were Recorded in the NSCs-derived Neurons in Neural Network Scaffold in 14 Day Culture *in vitro*. (A) GFP positive cells (arrows) were respectively labeled by ChAT, GLUT or GAD67 in the NSCs, NT3-NSCs, TRKC-NSCs and NT-3-NSCs+TRKC-NSCs groups. The cell nuclei were counterstained by Hoechst33342 (Hoe). Scale bars, 20 μm. (B) In the NT-3-NSCs+TRKC-NSCs group, consecutive action potentials (APs) were recorded in NSC-derived neurons.

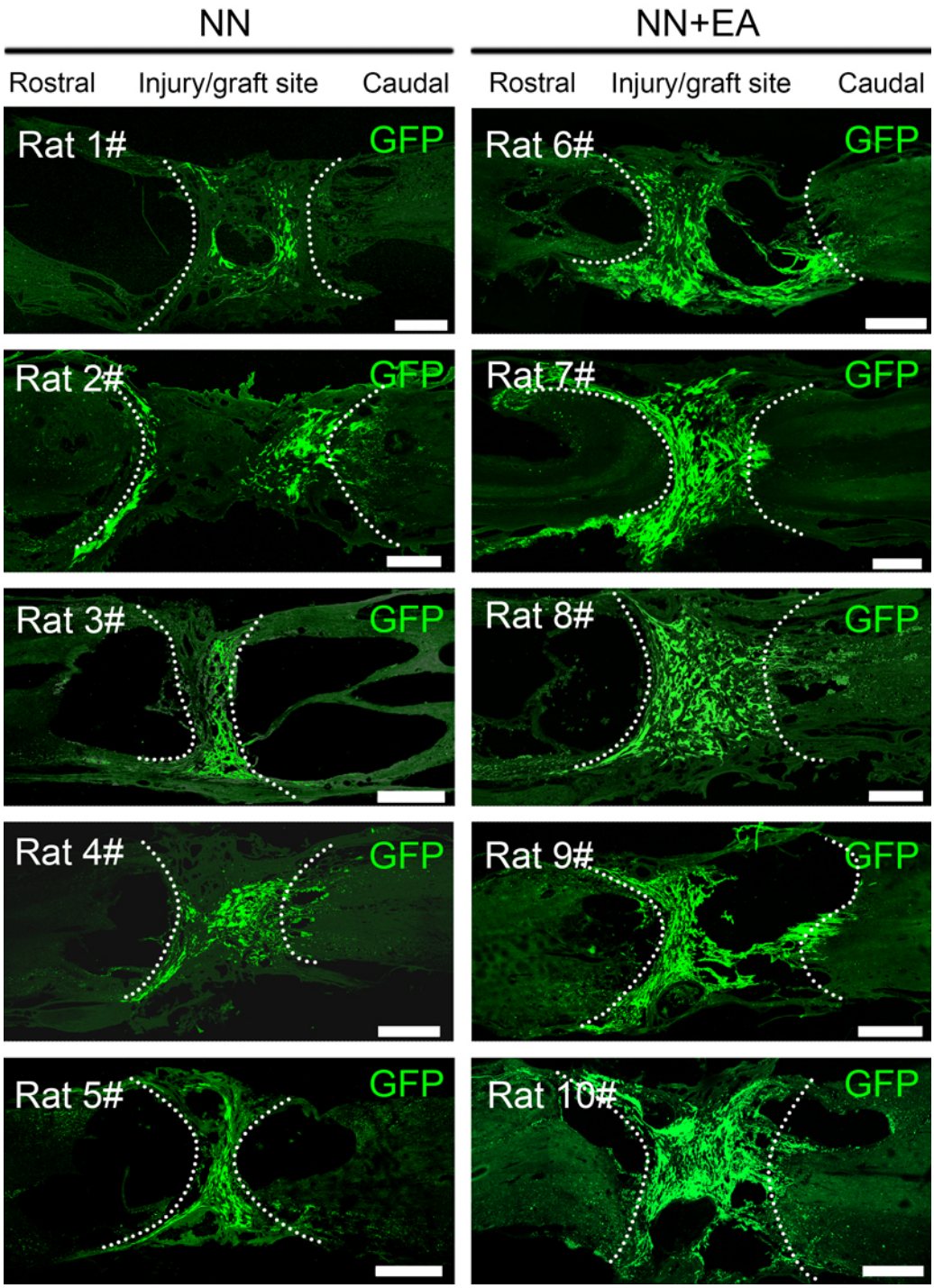

Figure S3. Electroacupuncture Promoted the Cell Survival of Transplanted Neural Network Scaffold in the Injury/graft Site of Spinal Cord.

Representative photomicrographs of horizontal longitudinal spinal cord sections showing the survival of grafted GFP-positive cells in the NN and NN+EA groups (n = 5 per group). Scale bars, 1 mm.

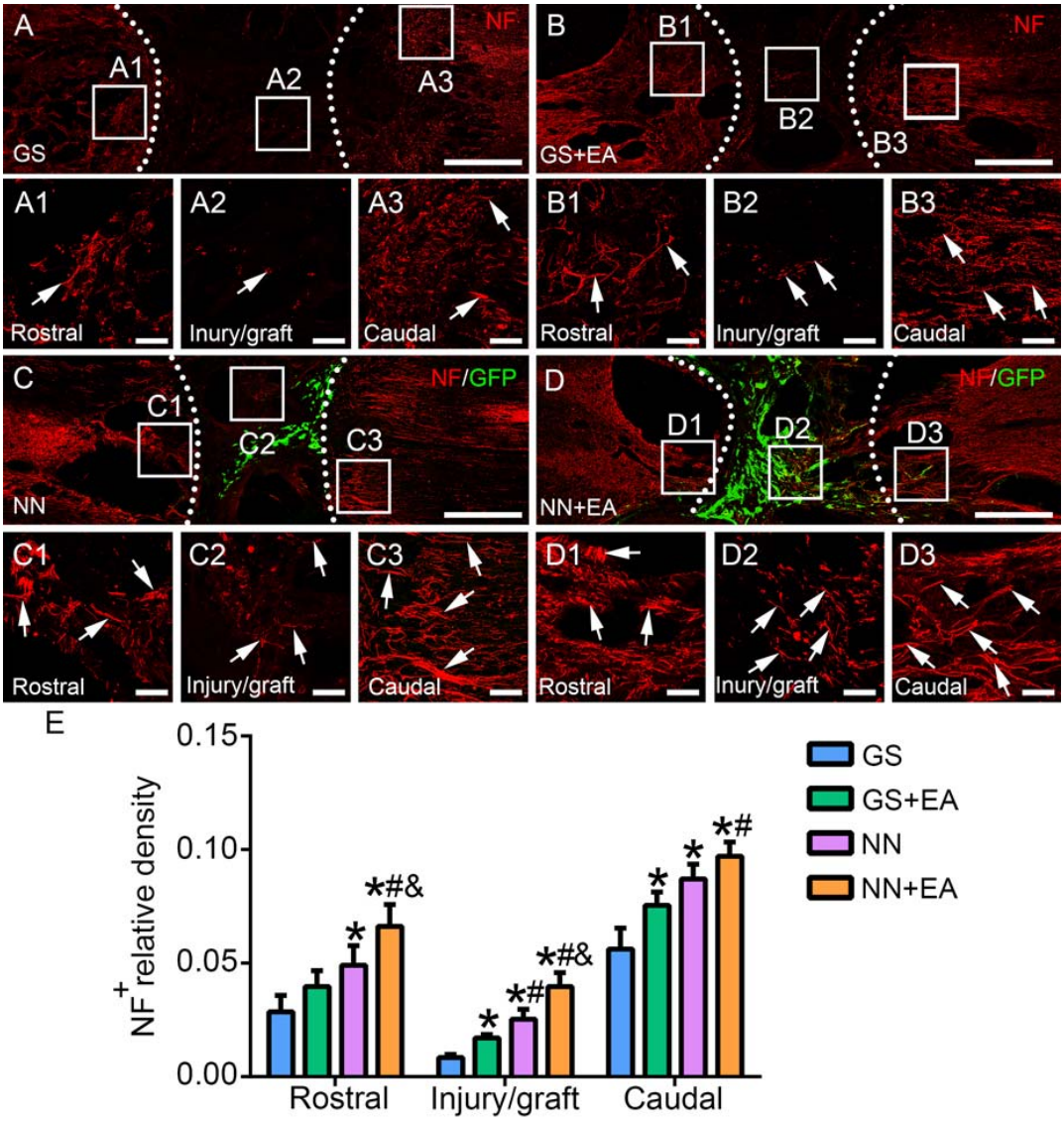

Figure S4. Assessment of Axonal Regeneration in the Injury/Graft Site of Spinal Cord.

(A-D) Representative images showing regenerating NF positive axons (arrows) in the GS (A), GS+EA (B), NN (C) and NN+EA (D) groups. The enlarged images from the rostral and caudal areas to/in the injury/graft site of spinal cord are showed in (A1-A3), (B1-B3), (C1-C3) and (D1-D3). (E) Bar chart showing NF positive axon relative density in the rostral and caudal areas to/in the injury/graft site in 4 groups (n = 5 per group; data presented as mean ± S.D.). * indicates significant difference from the GS group ($^{*}p < 0.05$); # indicates significant difference from the GS+EA ($^{\#}p < 0.05$); & indicates significant difference from the NN group ($^{\&}p < 0.05$) by one-way ANOVA followed by LSD-t. Scale bars, 1 mm in (A)-(D); 100 μm in (A1)-(A3), (B1)-(B3), (C1)-(C3) and (D1)-(D3).

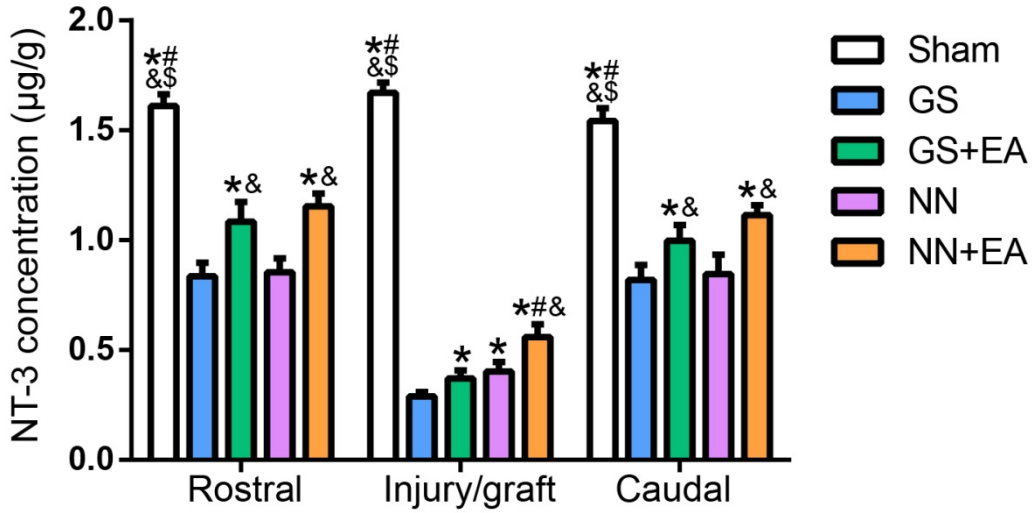

Figure S5. NT-3 Level of Three Segment Tissue of Injured Spinal Cord in Four Groups.

The concentration of NT-3 in the spinal cord tissue was measured by ELISA. The 5 mm long spinal cord tissue were collected from rostral and caudal areas to/in the injury/graft site of spinal cord at 8 weeks after injury, and were processed separately for ELISA analysis. * indicates significant difference from GS group (*$p < 0.05$); $^{\#}$ indicates significant difference from the GS+EA group ($^{\#}p < 0.05$); $^{\&}$ indicates significant difference from the NN group ($^{\&}p < 0.05$); $^{\$}$ indicates significant difference from the NN+EA group ($^{\$}p < 0.05$) by one-way ANOVA followed by LSD-t.

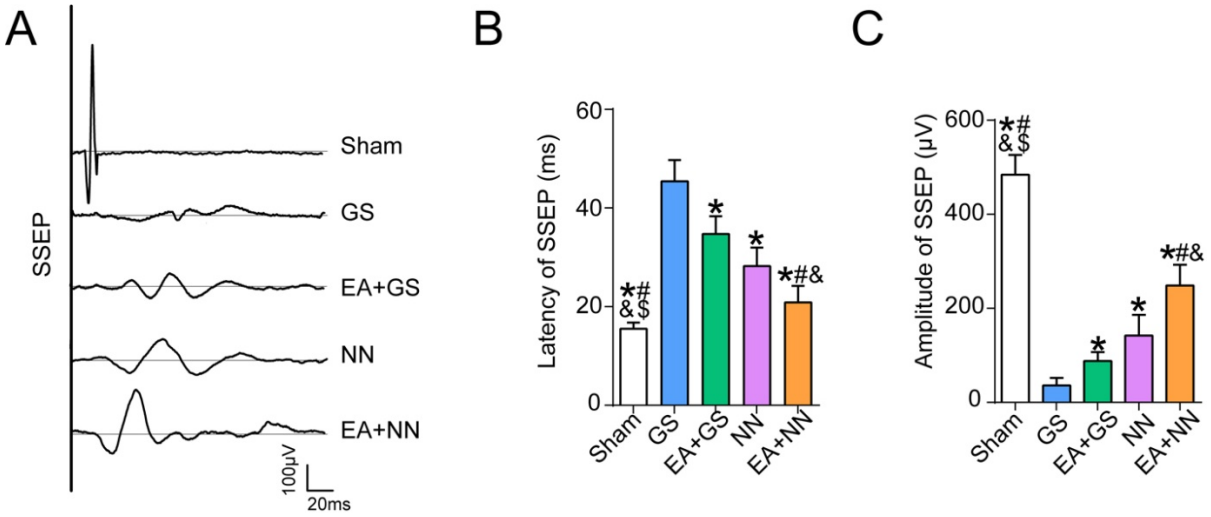

Figure S6. Latency and Amplitude of SSEPs.

(A) SSEPs were obtained by electrophysiological analysis in the GS, GS+EA, NN and NN+EA Groups. (B and C) Bar charts of the latency (B) and amplitude (C) of SSEP showing that shorter latency and higher amplitude of SSEPs were exhibited in the NN+EA group as compared with the GS ($^{*}p < 0.05$), GS+EA ($^{\#}p < 0.05$) and NN ($^{\&}p < 0.05$) groups. * indicates significant difference from the GS group (*$p < 0.05$); $^{\#}$ indicates significant difference from the GS+EA group ($^{\#}p < 0.05$); $^{\&}$ indicates significant difference from the NN group ($^{\&}p < 0.05$); $^{\$}$ indicates significant difference from the NN+EA group ($^{\$}p < 0.05$) by one-way ANOVA followed by LSD-t.

Table S1 Primary and secondary antibodies infromation

Antibodies	Species	Type	Dilution	Source(Catlog)
NESTIN	Rabbit	Polyclonal IgG	1:500	Sigma, St. Louis, USA(SAB5500150)
Neurotrophin-3 (NT-3)	Rabbit	Polyclonal IgG	1:200	Abcam, London, UK (ab53685)
TRKC	Mouse	Monoclonal IgG	1:400	R&D System, Minneapolis, USA(75219)
Microtubule-associated protein 2 (MAP2)	Rabbit	Polyclonal IgG	1:300	Sigma, St. Louis, USA(M4403)
Microtubule-associated protein 2 (MAP2)	Mouse	Monoclonal IgG	1:300	Sigma, St. Louis, USA(M3698)
APC (clone CC-1)	Mouse	Monoclonal IgG	1:200	Merck Millipore, Billerica, USA(OP80)
Glial fibrillary acidic protein (GFAP)	Rabbit	Polyclonal IgG	1:200	Abcam, London, UK(ab7260)
Choline acetyltransferase (ChAT)	Rabbit	Polyclonal IgG	1:200	Abcam, London, UK(ab6168)
Glutamate Decarboxylase 67(GAD67)	Rabbit	Polyclonal IgG	1:500	Abcam, London, UK(ab26116)
Glutaminase (GLUT)	Rabbit	Polyclonal IgG	1:300	Abcam, London, UK(ab93434)
Synapsin 1(SYN)	Rabbit	Polyclonal IgG	1:200	Merck Millipore, Billerica, USA(ab1543)
Postsynaptic density protein 95 (PSD95)	Rabbit	Polyclonal IgG	1:500	Abcam, London, UK (ab18258)
Neurofilament 200 (NF)	Rabbit	Polyclonal IgG	1:500	Sigma, St. Louis, USA(N4142)
GAP43	Rabbit	Polyclonal IgG	1:500	Abcam, London, UK (ab16053)
AKT	Rabbit	Polyclonal IgG	1:300	Cell Signaling Technology,USA(4685)
Phospho-AKT (Ser473)	Rabbit	Polyclonal IgG	1:300	Cell Signaling Technology,USA(4060)
Green fluorescent protein (GFP)	Chicken	Polyclonal IgY	1:1000	Thermo Fisher Scientific,USA(A10262)
Alexa 488 conjuncted anti chicken secondary antibody	Goat	Polyclonal IgG	1:1000	Thermo Fisher Scientific,USA(A11039)
Alexa 555 conjuncted anti mouse secondary antibody	Goat	Polyclonal IgG	1:1000	Thermo Fisher Scientific,USA(A21422)
Alexa 555 conjuncted anti rabbit secondary antibody	Goat	Polyclonal IgG	1:1000	Thermo Fisher Scientific,USA(A21428)
Alexa 647 conjuncted anti mouse secondary antibody	Goat	Polyclonal IgG	1:1000	Thermo Fisher Scientific,USA(A21236)
Alexa 647 conjuncted anti rabbit secondary antibody	Goat	Polyclonal IgG	1:1000	Thermo Fisher Scientific,USA(A21244)