

Supplemental Data

Neurons derived from transplanted neural stem cells restore disrupted neuronal circuits in the injured mouse spinal cord

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Supplemental Video Legends

Video 1

One week after injury, hindlimb movement has been completely lost.

Video 2

In the chronic phase (six weeks after injury), hindlimb movement in untreated mice has barely improved.

Video 3

NSC transplantation slightly improves functional recovery in hindlimbs (six weeks after injury).

Video 4

NSC transplantation and VPA administration results in dramatic functional recovery in hindlimbs (six weeks after injury).

Supplemental Methods

Cell culture.

To induce neuronal differentiation, NSCs were first plated onto O/F-coated chamber slides at a density of 5×10^4 cells/well in NS-A medium supplemented with bFGF (10 ng/ml). After 4 days, the medium was switched to NS-A supplemented with B27 (GIBCO) without any growth factors and the cells were cultured for 4 days. Half of the medium was exchanged every 2 days during this differentiation period. To induce oligodendrocyte differentiation, NSCs were plated onto 6-cm dishes at a density of 5×10^5 cells/dish in NS-A medium supplemented with bFGF (10 ng/ml), triiodothyronine (T3, 30 ng/ml) and thyroxine (T4, 40 ng/ml). After 4 days, cells were replated onto O/F-coated chamber slides at a density of 1×10^5 cells/well in NS-A medium with bFGF (5 ng/ml), T3 (30 ng/ml), and T4 (40 ng/ml), and further cultured for 4 days. Half of the medium was exchanged every 2 days during the differentiation period. To induce astrocyte differentiation, NSCs were plated onto O/F-coated chamber slides at a density of 5×10^4 cells/well in NS-A medium containing 1% fetal bovine serum (FBS) and cultured for 3 days. VPA (0.5 mM, Sigma), VPM (0.5 mM, Wako) or Trichostatin A (TSA, 25 nM, Upstate Biotechnology) was added to individual cultures during differentiation as appropriate.

Behavioral testing.

We evaluated hindlimb motor function for up to 14 weeks after injury. Two individuals, blinded to the treatment of the mice, examined motor function using the Basso-Beattie-Bresnahan (BBB) locomotor rating scale (Basso et al., 1995). Hindlimb movements of the mice were captured using a high-definition digital camcorder (Sony). We edited these movies and exported movie files using editing software (Apple).

Antibodies.

The following antibodies were used: rabbit anti-Sox2 (1:1000, Chemicon), mouse anti-nestin (1:250, Chemicon), mouse anti- β -tubulin isotype III (Tuj1; 1:500, Sigma), rabbit anti- β -tubulin isotype III (1:1000, Covance), goat anti-doublecortin (DCX) (1:200, Santa Cruz Biotechnology), rabbit anti-GFP (1:1000, Molecular Probes), chick anti-GFP (1:500, Aves Labs), guinea pig anti-glial fibrillary acidic protein (GFAP) (1:7500, Advanced Immunochemicals), rabbit anti-MAP2 (1:1000, Chemicon), mouse anti-MAP2ab (1:500, Sigma), rabbit anti-MBP (1:1000, Chemicon), rabbit anti-acetyl-histone H3 (1:2000, Upstate), rabbit anti-GAD65 (1:500, Millipore), and guinea pig anti-VGLUT2 (1:500, Chemicon).

Immunocytochemistry.

Immunocytochemistry experiments were performed as described previously (Setoguchi et al., 2004). Unless otherwise stated, all experimental manipulations were performed at room temperature. NSCs cultured on eight-well chamber slides were washed with PBS and fixed with

4% paraformaldehyde (PFA)/PBS for 10 min. Cells were washed with PBS and incubated for 1 h in blocking solution (PBS containing 10% FBS and 0.1% Triton X-100). Subsequently, cells were incubated overnight at 4 °C in an appropriate mixture of the primary antibodies described above. After three washes in PBS, cells were incubated for 1 h with the following secondary antibodies: FITC-conjugated donkey anti-chick IgG, FITC-conjugated donkey anti-rabbit IgG, Cy3-conjugated donkey anti-rabbit IgG, Cy5-conjugated donkey anti-mouse IgG, Cy5-conjugated donkey anti-guinea pig IgG (1:500, Jackson ImmunoResearch), Alexa Fluor 488-conjugated donkey anti-rabbit IgG, Alexa Fluor 594-conjugated donkey anti-rabbit IgG, Alexa Fluor 594-conjugated donkey anti-mouse IgG, Alexa Fluor 647-conjugated donkey anti-rabbit IgG, and Alexa Fluor 647-conjugated donkey anti-rabbit IgG (1:500, Molecular Probes). After three washes with PBS, nuclei were stained with Hoechst (bisbenzimidide H33258 fluorochrome trihydrochloride, Calbiochem). Samples were washed with PBS and mounted on glass slides with 70 µl of Immu-Mount (Thermo Scientific). The cells were examined using a fluorescence microscope (Axiovert 200M, Zeiss) equipped with the appropriate epifluorescence filters.

Immunohistochemistry.

We performed immunohistochemical analysis on tissue sections using three or more independent samples from the mice. Animals were anesthetized and perfused with PBS followed by 4% PFA in 0.1 M PBS, pH 7.4. The spinal cords were dissected, and postfixed overnight in

the same fixative at 4 °C. For cryosectioning, fixed tissues were cryoprotected in 10% sucrose in PBS overnight at 4 °C, then in 20% sucrose in PBS overnight at 4 °C, and embedded in OCT compound (Tissue Tek). Cryostat sections (20 µm) were cut and affixed to MAS-coated glass slides (Matsunami Glass). The sections were then permeabilized in PBS-T (PBS containing 0.1% Triton X-100) for 10 min and blocked with 10% FBS in PBS-T for 1 h. Subsequently, the sections were incubated overnight at 4 °C in an appropriate mixture of the primary antibodies described above. After three washes with PBS, they were incubated with the secondary antibodies described above for 1 h. After being rinsed with PBS, nuclei were stained using Hoechst. Sections were mounted and examined under a fluorescence microscope (Axiovert 200M, Zeiss) equipped with the appropriate epifluorescence filters. Sections were also viewed using a scanning laser confocal imaging system (LSM710, Zeiss).

Immunoelectron microscopy.

Five weeks after transplantation, animals were perfused intracardially with 0.9% saline followed by 50 ml of fixative consisting of 4% PFA and 0.08% glutaraldehyde in 0.1 M phosphate buffer (PB) at pH 7.4. The spinal cords (at the level Th4–L2) were removed, postfixed overnight at 4 °C in fresh fixative, and subsequently cut into 60-µm sagittal sections on a vibratome. To improve penetration of antibodies, vibratome sections were immersed in 20% sucrose in 0.1 M PB followed by quick freezing in liquid nitrogen for 15 sec. After washing with PBS, the sections were treated for 1 h with donkey serum diluted 1:10 to block nonspecific

binding of the antibody. The sections were immunostained with rabbit anti-GFP antibody (1:1000, Molecular Probes) for 48 h at 4 °C. Sections were then incubated with biotinylated donkey anti-rabbit IgG antibody for 4 h and further reacted with avidin-conjugated peroxidase (overnight at 4 °C). They were then processed in 0.02% diaminobenzidine tetrahydrochloride (DAB) and 0.005% hydrogen peroxide, yielding a black product in GFP-containing structures. The sections were then postfixated with 1% OsO₄ in 0.1 M PB, dehydrated in a graded series of ethanol concentrations and flat-embedded in Epon 812 (Shell Chemicals). After light microscopic examination, the tissue blocks were cut into ultrathin sections, collected on single-slot grids coated with formvar film, and observed under an electron microscope (H-7100, Hitachi).

Anterograde labeling of the CST.

Twelve weeks after injury, descending CST fibers were labeled with biotinylated dextran amine (BDA) (10% in saline, 2 µl per cortex, MW 10,000; Molecular Probes) (Bradbury et al., 2002; Hata et al., 2006; Kaneko et al., 2006) by injection into the left and right motor cortices (Pronichev and Lenkov, 1998) (for three uninjured mice, three mice that received SCI with no treatment, and three mice that received SCI and underwent combined treatment with NSCs and VPA). The skulls of anesthetized mice were tightly fixed to a stereotaxic apparatus (Narishige), and the body was supported on an elastic heating pad, which was maintained at 37 °C (FST). Scalping and craniotomy over the motor cortex area were carried out using a micromotor system

(Nakanishi). The injection site was 2.1 mm posterior to the bregma, 2 mm lateral to the bregma, and 0.7 mm in depth (Pronichev and Lenkov, 1998). For pressure injections with a 20- μ m outer diameter glass capillary attached to a microsyringe (Narishige), we used 0.1- μ l steps per minute until the desired amount (1 μ l per injection site) was injected. The region overlying the injection site was then sutured and the animals were returned to their normal housing for the duration of the survival period. Two weeks later, the animals were anesthetized and perfused with PBS followed by 4% PFA. The spinal cords were dissected, and postfixed overnight in the same fixative at 4 °C. For cryosectioning, fixed tissue was cryoprotected in 10% sucrose in PBS overnight, then in 20% sucrose in PBS overnight at 4 °C, and embedded in OCT compound (Sakura). Sections (30 μ m) were cut, collected on silane-coated slides, and air-dried overnight at 37 °C. To visualize the BDA, a tyramide signal amplification fluorescence system (Perkin Elmer) was used. Nuclei were stained using Hoechst.

Visualization of multisynaptic neural pathways.

To visualize selective and functional transsynaptic neural pathways, WGA-expressing recombinant adenoviruses were used (Kinoshita et al., 2002). In this system, injections of WGA-expressing adenovirus in well-mapped neural pathways result in labeling of first-, second-, and third-order neurons. WGA protein is efficiently transported in axons and dendrites in both anterograde and retrograde directions. Twelve weeks after injury, 1 μ l of saline containing WGA-expressing virus at a titer of 1×10^{11} pfu/ml was injected into the bilateral motor cortices

(0.5 μ l per injection site) (for three intact mice, three mice that received SCI with no treatment, and three mice that received SCI and underwent combined treatment with NSCs and VPA). The injection site was 2.1 mm posterior to the bregma, 2 mm lateral to the bregma, and 0.7 mm in depth. Two weeks later, animals were perfused and the spinal cords were fixed as described above. Sections (15 μ m) were cut and used for immunohistochemistry. Sections were incubated for 1 h with PBS-T and 10% FBS. Rabbit anti-WGA polyclonal antibody (3 μ g/ml, Sigma) was pre-absorbed with 1% acetone powder of mouse brains in blocking solution overnight at 4 °C. This pre-absorption procedure was necessary for clear detection of the WGA transgene product. Sections were then incubated overnight at 4 °C with pre-absorbed rabbit anti-WGA, chick anti-GFP, and mouse anti-MAP2ab antibodies in blocking solution. The sections were washed three times with PBS and then incubated with Alexa 594-conjugated anti-rabbit IgG, FITC-conjugated anti-chick IgG, and Cy5-conjugated anti-mouse IgG antibodies for 1 h. All images were analyzed and WGA signal intensity was measured using Axio Vision 4.6.3 software (Zeiss).

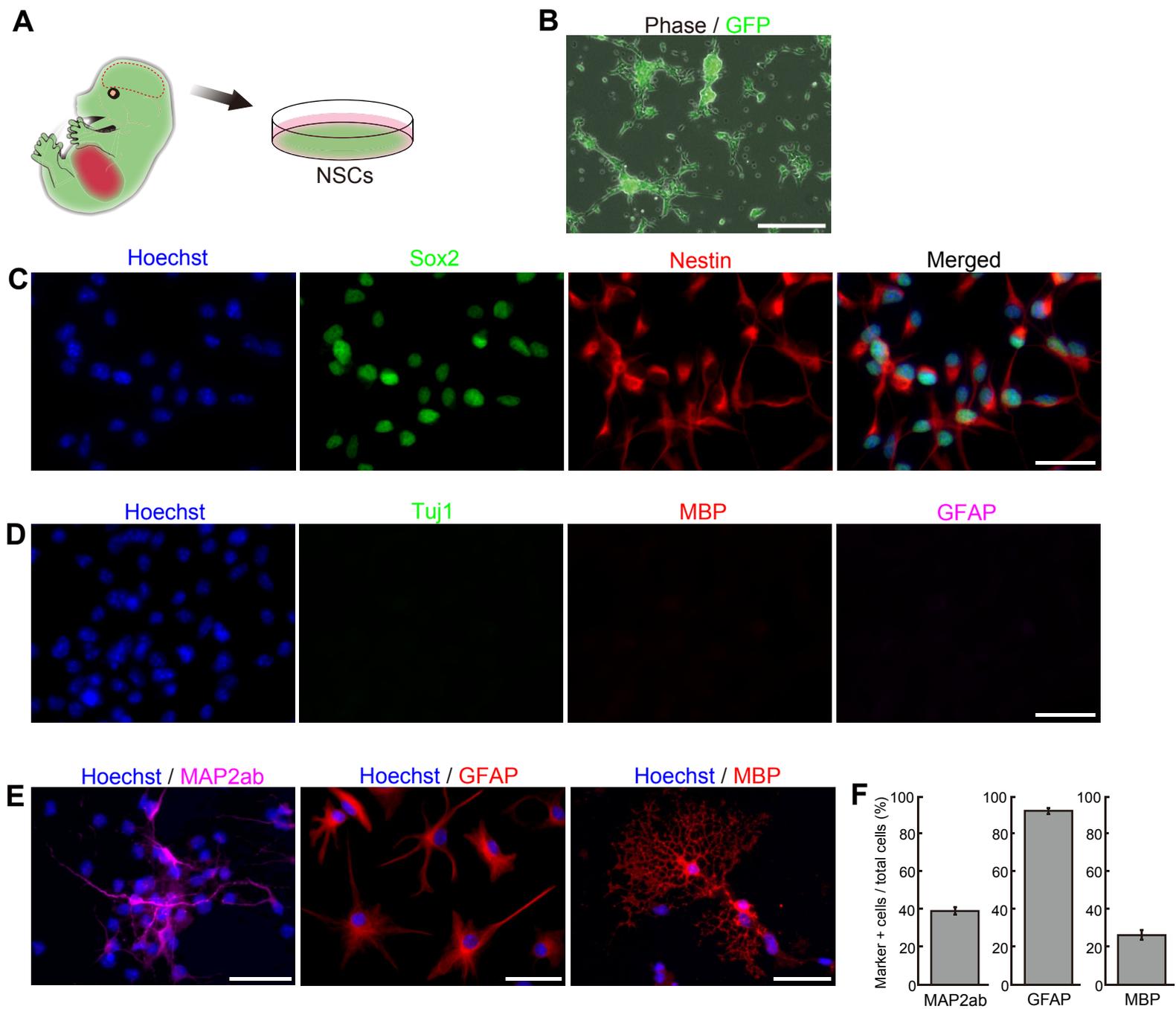
In vivo imaging of transplanted cells.

In vivo imaging experiments were performed as described previously (Okada et al., 2005). A Xenogen-IVIS 100 cooled CCD optical macroscopic imaging system (SC BioScience) was used for bioluminescence imaging. The signal intensity of NSCs was determined by plating cells at various densities and imaging immediately after D-luciferin (150 μ g/ml) was added. Signals are

reported as photons/cell/s. The integration time was fixed at 2 min duration for each image. For in vivo imaging, mice were given an intraperitoneal injection of D-luciferin (150 mg/kg body weight), and serial images were acquired from 20 min after administration until the maximum intensity was obtained with the field-of-view set at 10 cm. We found this time window to be optimal, because the signal intensity peaked at 15 min after administration and was followed by a plateau of 20 min (data not shown). All images were analyzed using Igor (WaveMetrics) and Living Image software (Xenogen), and optical signal intensity was expressed as photon flux, in units of photons/s/cm²/steradian. Each image was displayed as a false color photon count image superimposed on a grayscale anatomic image. To quantify the measured light, we defined regions of interest (ROIs) over the cell-implanted area and examined all values with the same ROI.

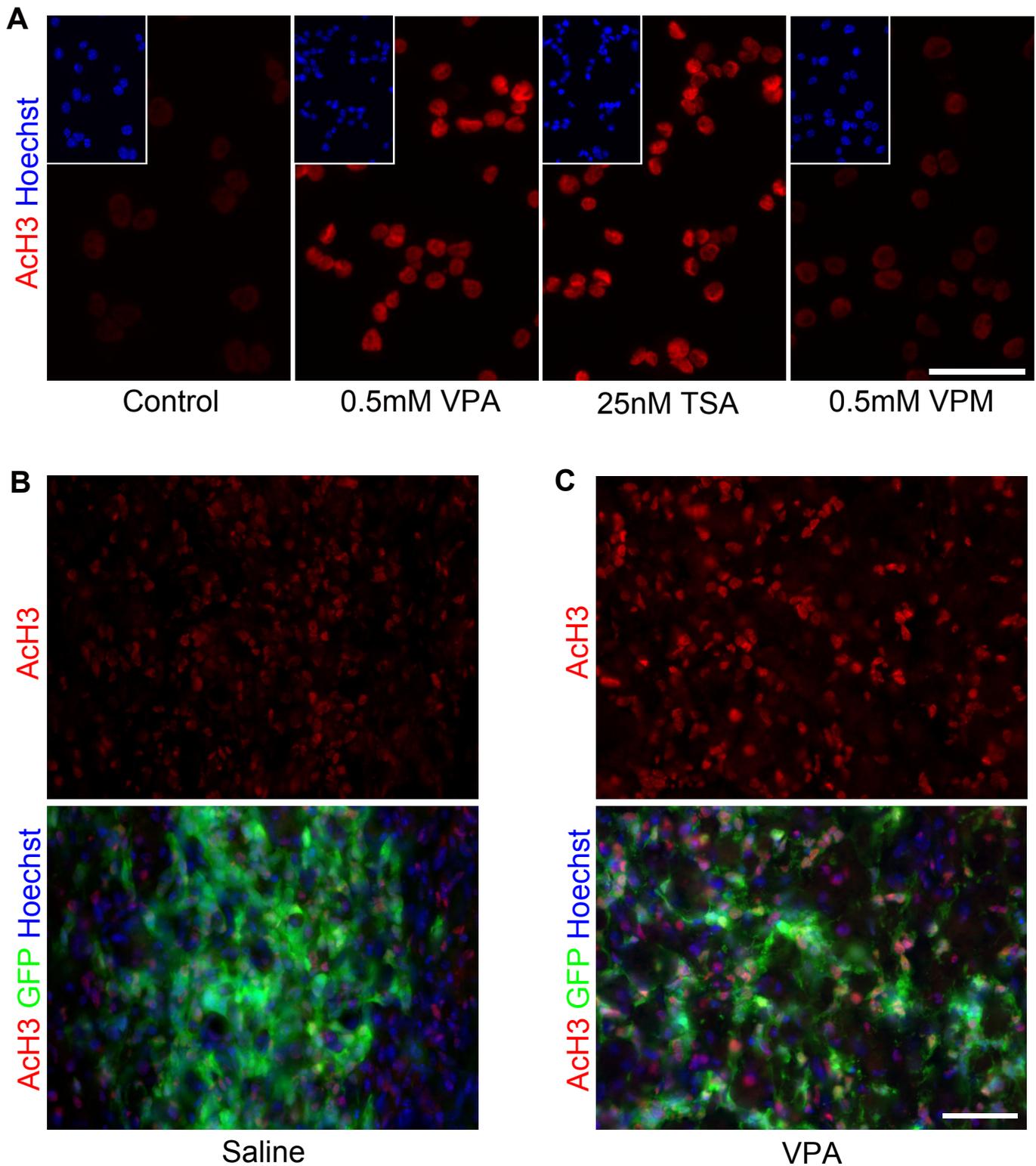
Ablation of transplant-derived cells.

Cell ablation experiments were performed as described previously (Furukawa et al., 2006; Saito et al., 2001). Diphtheria toxin was purified from conditioned medium of the PW8 strain of *Corynebacterium diphtheriae* by diethylaminoethyl Sepharose column chromatography and diluted to an appropriate concentration with saline. Six weeks after injury, DT solution (50 µg/kg/day × 2 days) was administered to mice by intraperitoneal injection (into nine GFP.Luc-NSC-transplanted and VPA-treated mice and six TR6.GFP.Luc-NSC-transplanted and VPA-treated mice).



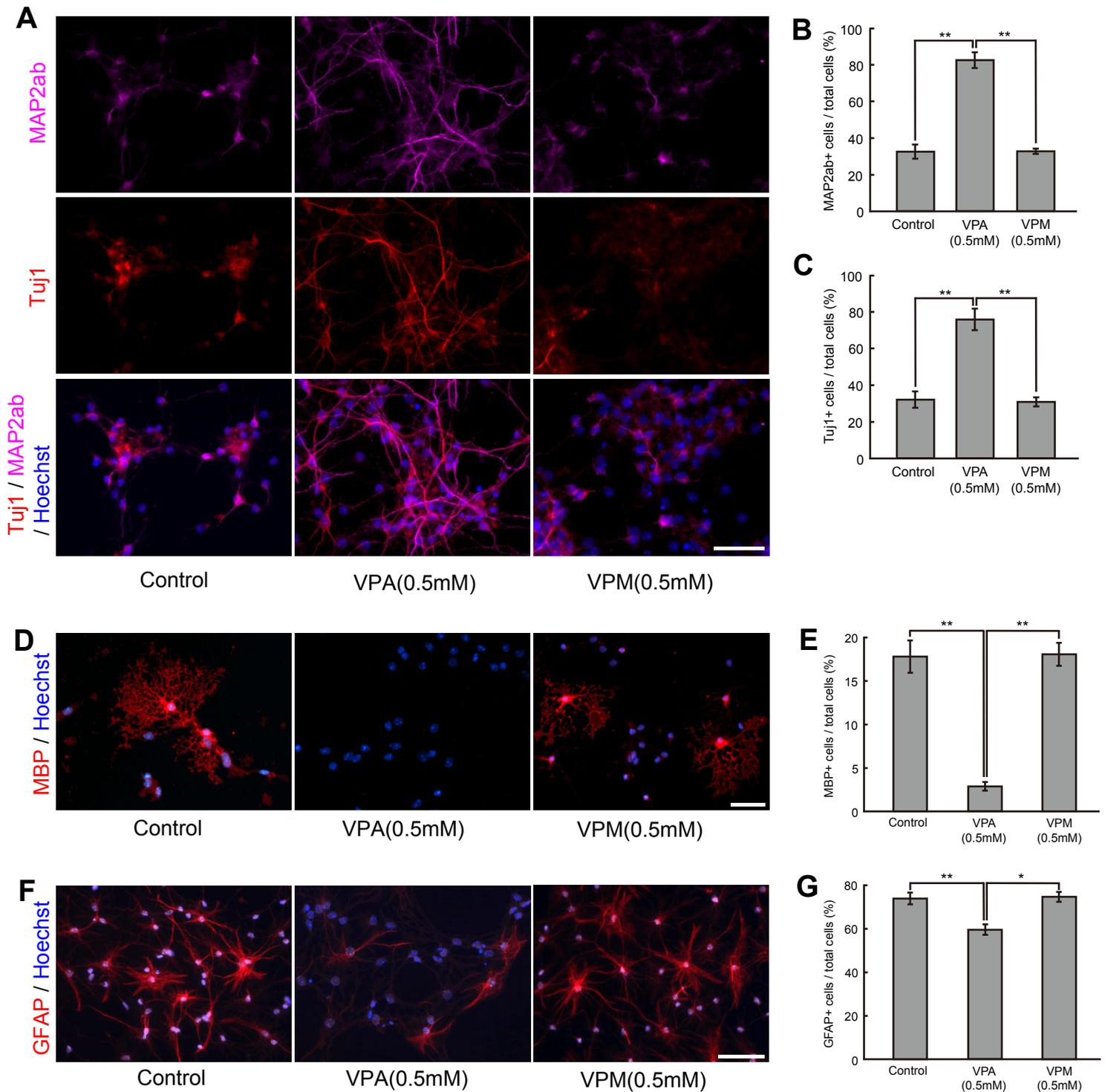
Supplemental Figure 1. Characterization of NSCs derived from embryonic forebrain.

Since we obtained almost the same results for GFP-NSCs, TR6.GFP.Luc-NSCs and GFP.Luc-NSCs, those for GFP-NSCs are shown here as representatives. (A) NSCs were prepared from the forebrains of E14.5 transgenic mice. (B) Phase and fluorescence images of GFP-expressing NSCs at passage five. Scale bar = 100 μ m. (C and D) NSCs were uniformly immunopositive for Sox2 and nestin, but negative for Tuj1, MBP, and GFAP. Scale bar = 50 μ m. (E) In neuronal, astrocytic and oligodendrocytic differentiation conditions, NSCs differentiate into MAP2ab-positive neurons (left), GFAP-positive astrocytes (middle) and MBP-positive oligodendrocytes (right), respectively. Scale bar = 50 μ m. (F) The percentages of cells that were immunopositive in each differentiation condition (E) were quantified. All data shown are from at least three experiments in parallel cultures, with error bars representing SD.



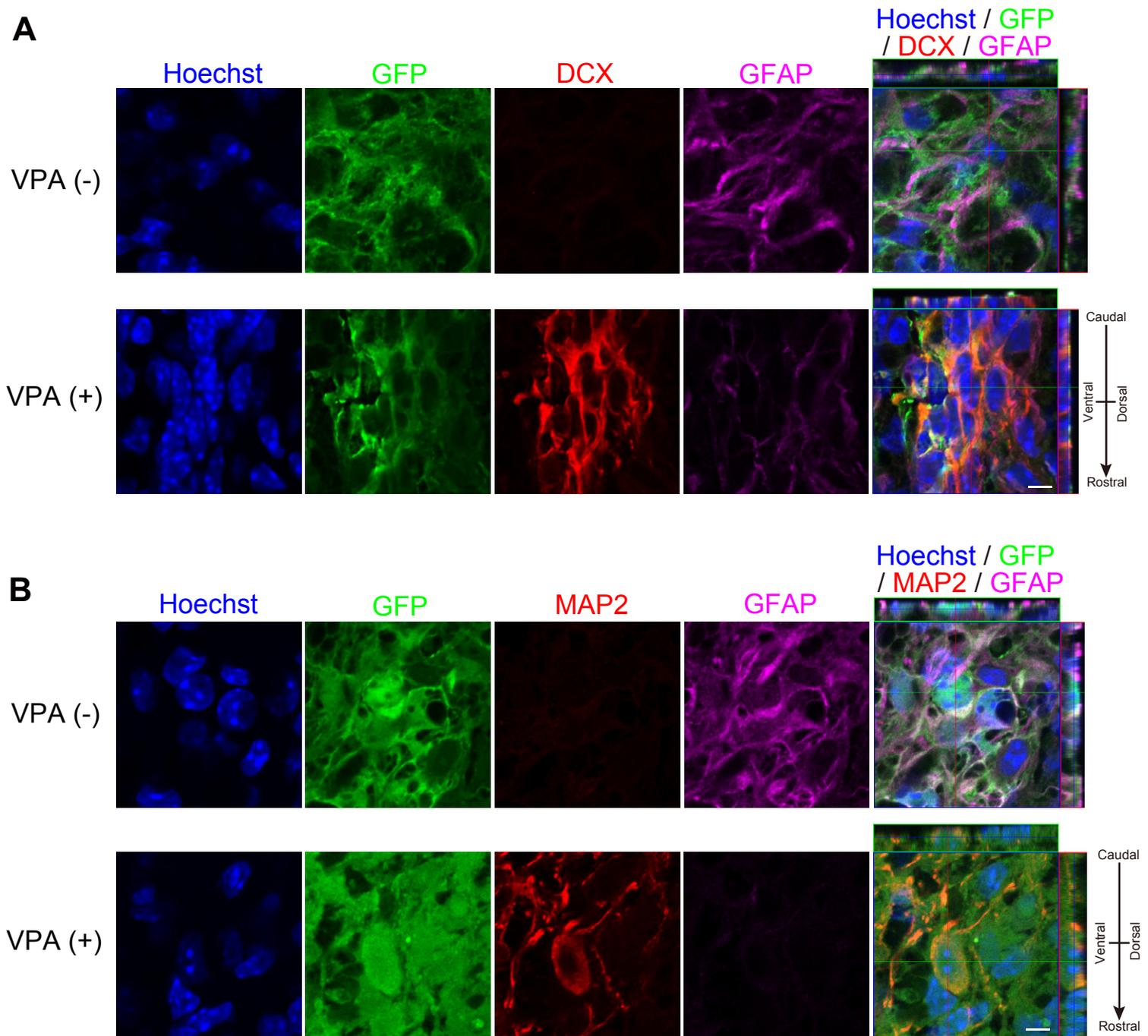
Supplemental Figure 2. VPA treatment enhances histone acetylation in vitro and in vivo.

(A) GFP-NSCs were treated with medium alone, 0.5 mM VPA, 25 nM TSA and 0.5 mM VPM for 3 h in the neuronal differentiation condition. Cells were then stained with anti-AcH3 antibody (red) and Hoechst (blue, insets in each field). Scale bar = 50 μ m. (B and C) Administration of VPA to SCI model mice led to an increase in histone H3 acetylation in transplanted and endogenous cells. GFP-NSC-transplanted spinal cords from mice administered with saline or VPA were fixed 6 h after the administration. Sections of the spinal cords were stained with anti-AcH3 (red) and -GFP (green) antibodies. Blue, Hoechst nuclear staining. Scale bar = 50 μ m.



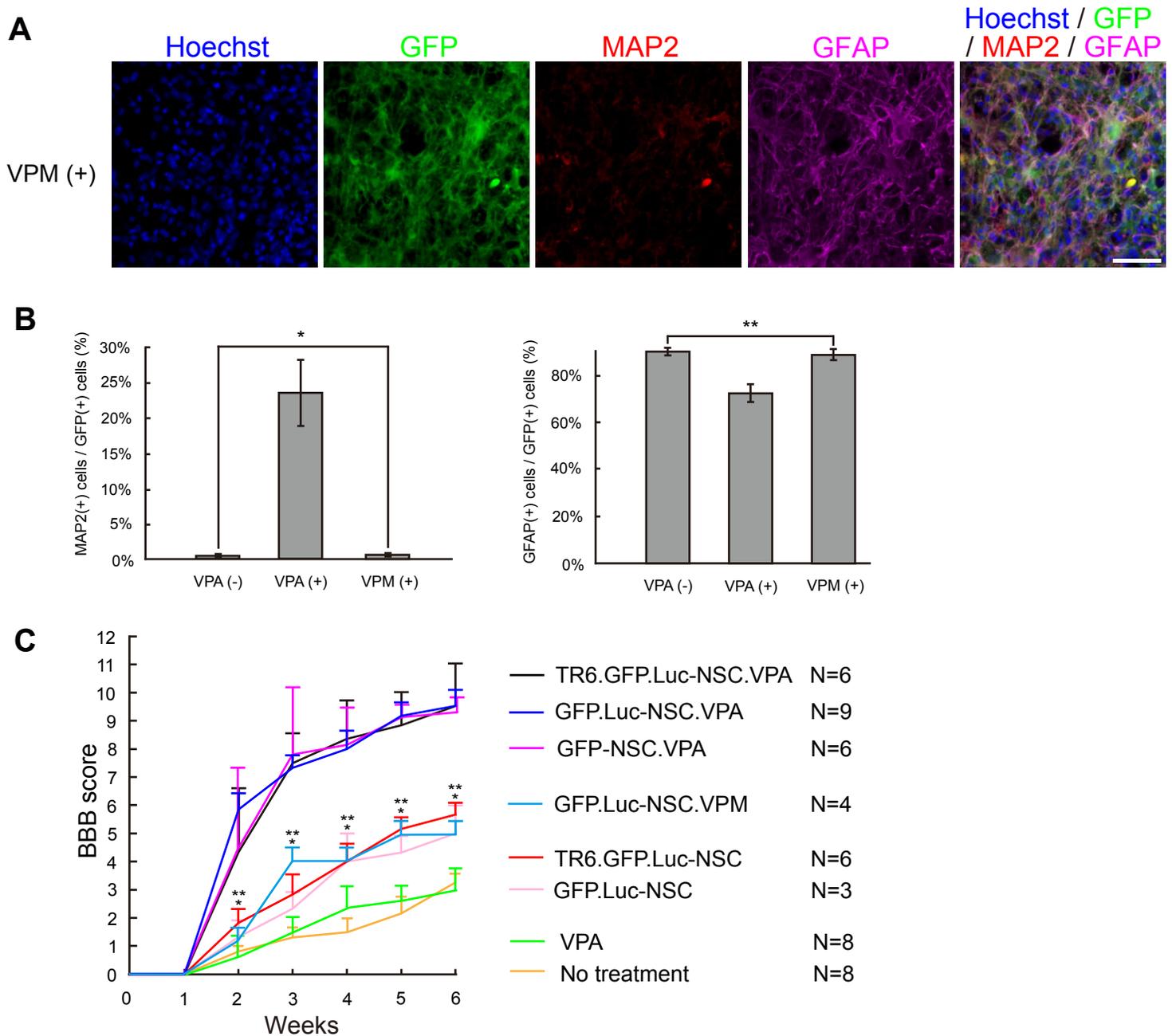
Supplemental Figure 3. Neuronal but not glial differentiation of NSCs is promoted by VPA in vitro.

(A) GFP-NSCs were treated with VPA (0.5 mM) or VPM (0.5 mM), or untreated, as indicated, for 8 days, in the neuronal differentiation condition. Cells were stained with antibodies for two different neuronal markers, MAP2ab (magenta) and Tuj1 (red). Blue, Hoechst nuclear staining. Scale bar = 50 μ m. (B and C) The percentages of MAP2ab+ and Tuj1+ cells in (A) were quantified. Means \pm SD. **, $P < 0.01$ compared with control or VPM-treated cells (t test). (D) NSCs were treated as in (A), but in the oligodendrocytic differentiation condition. Cells were stained with anti-myelin basic protein (MBP) antibody (oligodendrocyte marker, red) and Hoechst (blue). Scale bar = 50 μ m. (E) The percentages of MBP+ cells in (D) were quantified. Means \pm SD. **, $P < 0.01$ compared with control or VPM-treated cells (t test). (F) NSCs were treated as in (A), but in the astrocytic differentiation condition. Cells were stained with anti-glial fibrillary acidic protein (GFAP) antibody (astrocyte marker, red) and Hoechst (blue). Scale bar = 50 μ m. (G) The percentages of GFAP+ cells in (F) were quantified. Means \pm SD. *, $P < 0.05$ compared with controls; **, $P < 0.01$ compared with VPM-treated cells (t test). All data shown are from at least three experiments in parallel cultures.



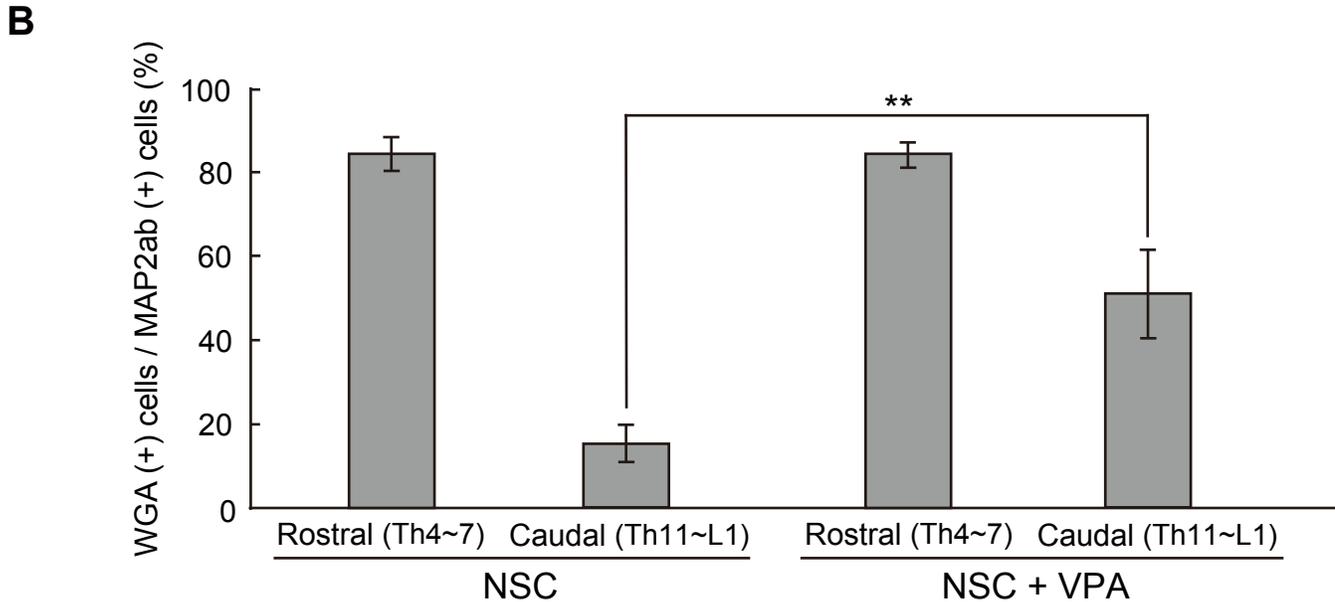
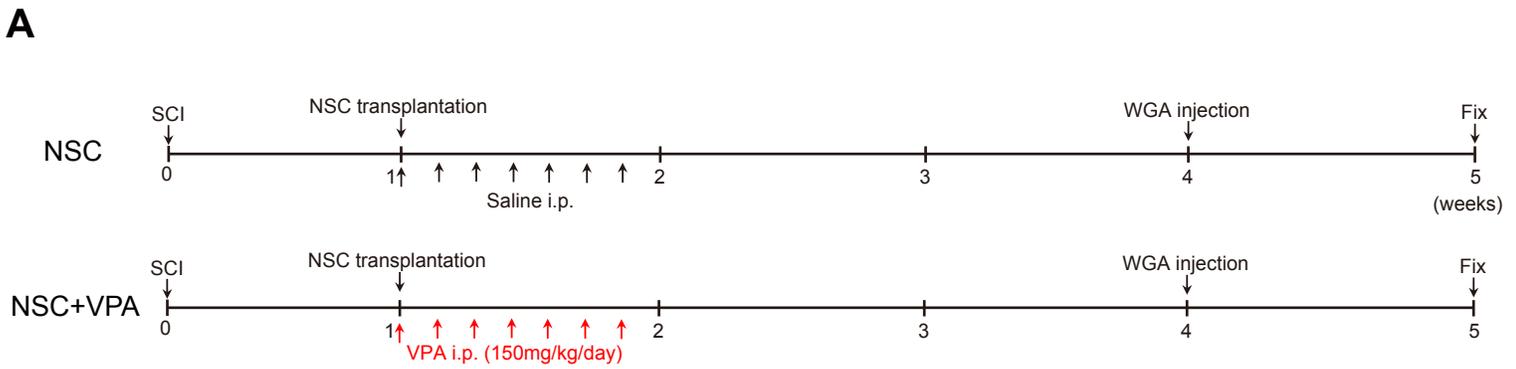
Supplemental Figure 4. VPA promotes neuronal differentiation of transplanted NSCs.

Representative high-magnification images of GFP-NSC-transplanted SCI model mice are shown. (A) Confocal images of transplanted cells 1 week after transplantation into the injured spinal cords. Spinal cord sections from VPA-treated (+) and untreated (-) mice were stained with anti-GFP (green), anti-doublecortin (DCX) (immature neuronal marker, red) and anti-GFAP (magenta) antibodies, and Hoechst (blue). VPA administration resulted in an increase in the number of DCX-positive neuronal precursors among transplanted cells (lower panel). Scale bar = 5 μ m. (B) Confocal images of NSCs 5 weeks after transplantation into injured spinal cords. Spinal cord sections from VPA-treated (+) and untreated (-) mice were stained with anti-GFP (green), anti-MAP2 (neuronal marker, red) and anti-GFAP (magenta) antibodies, and Hoechst (blue). VPA administration increased the numbers of MAP2-positive neurons (lower panel). Scale bar = 5 μ m.



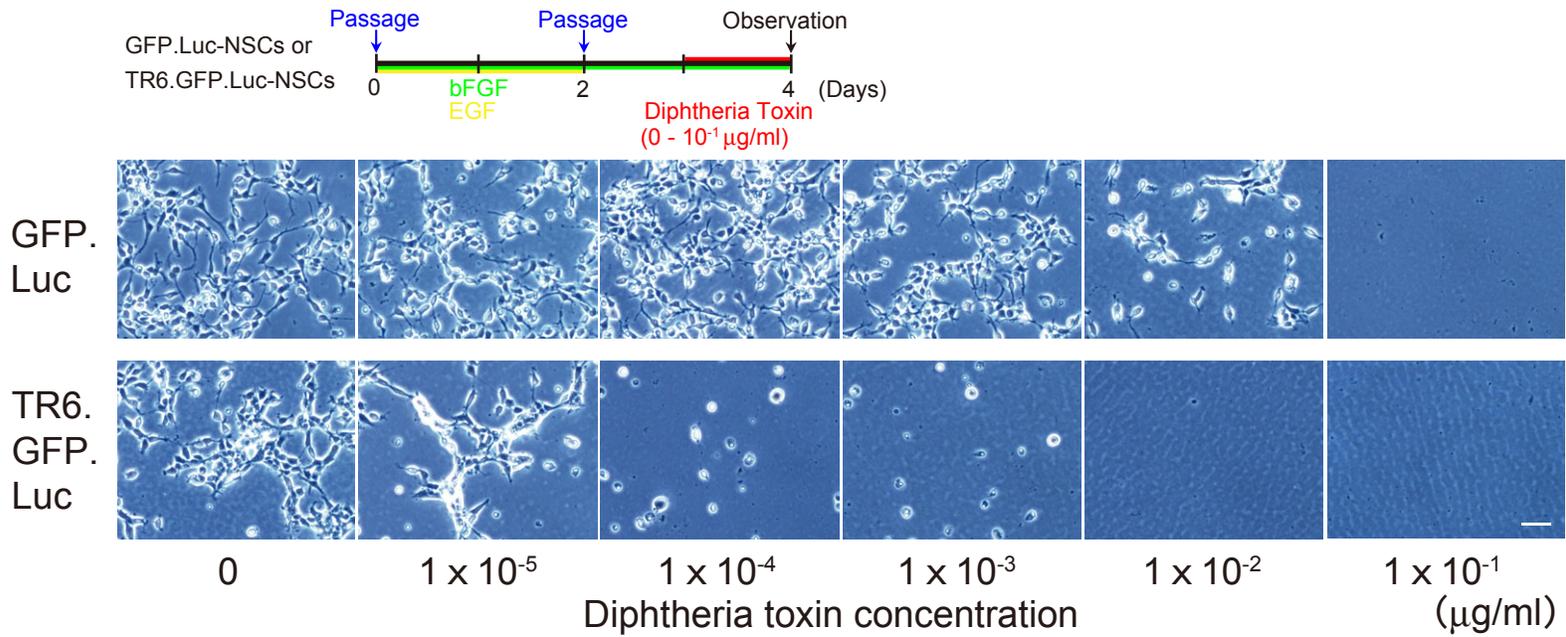
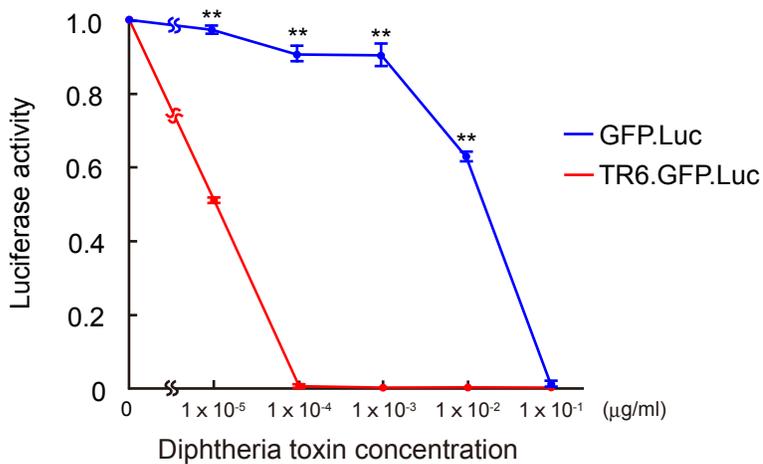
Supplemental Figure 5. VPM without HDAC-inhibition activity does not mimic the function of VPA.

(A) Images of sagittal sections of injured spinal cord 5 weeks after transplantation. SCI model mice received GFP.Luc-NSC transplantation and VPA or VPM administration as in Figure 1A. Spinal cord sections were stained with anti-GFP (green), anti-MAP2 (red), and anti-GFAP (magenta) antibodies, and Hoechst (blue). Scale bar = 50 μ m. (B) The percentages of MAP2- or GFAP-positive cells in GFP-positive transplanted cells were quantified. *: $P=0.805$, **: $P=0.642$. There is no significant difference in the percentage of MAP2- or GFAP-positive cells between VPM-treated and untreated mice. (C) Time course of the changes in BBB scores in SCI model mice. The VPM administration (sky blue line) had no influence on hindlimb motor function compared to that in mice which received the NSC transplantation only (red and pink lines). This result was superimposed on Figure 1b. *: $P=0.933$ compared with TR6.GFP.Luc-NSC-transplanted SCI model mice (red line). **: $P=0.704$ compared with GFP.Luc-NSC-transplanted SCI model mice (pink line) (repeated-measures ANOVA). Data are means \pm SEM.



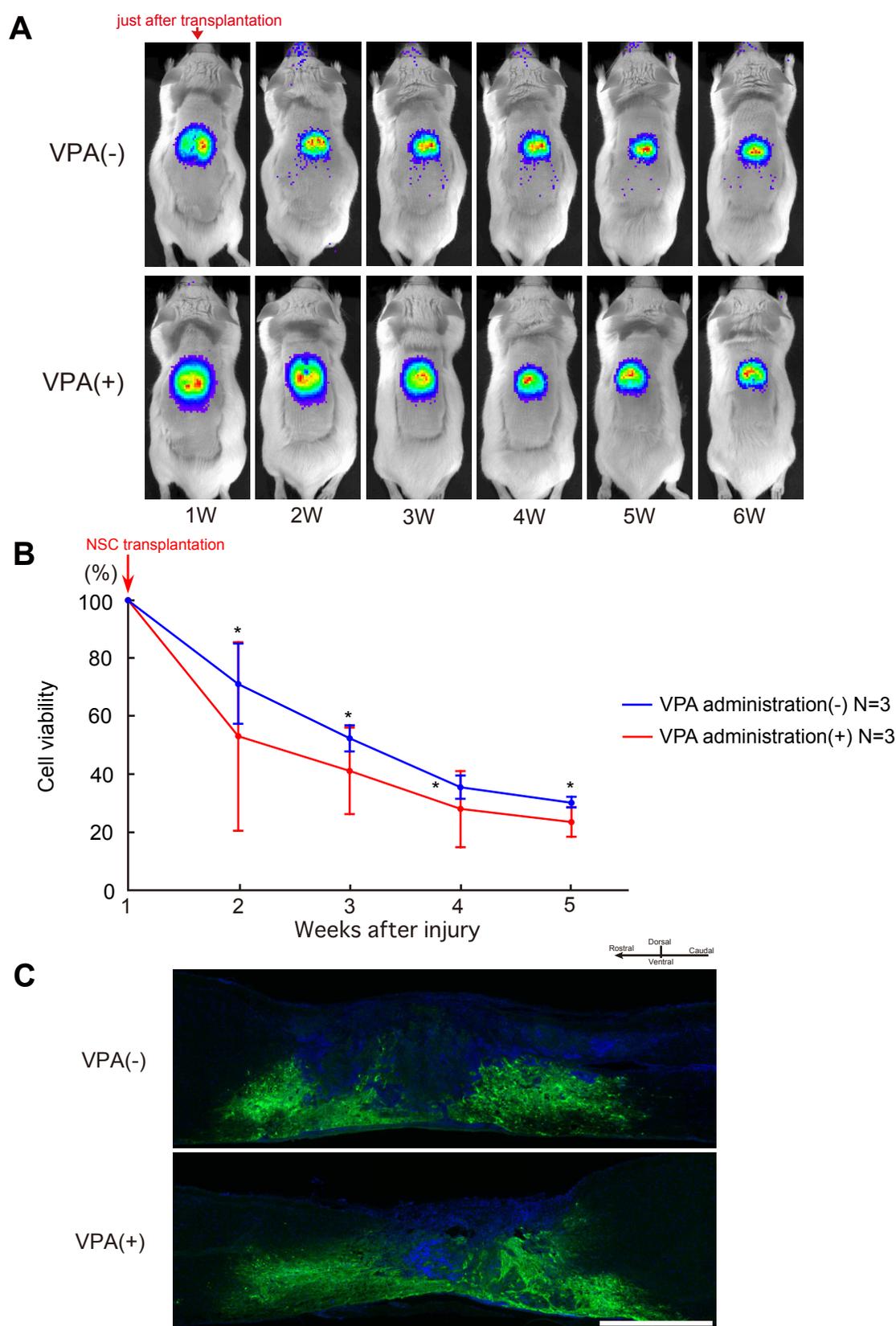
Supplemental Figure 6. Transplant-derived neurons reconstruct disrupted neuronal circuits in a relay manner.

(A) The time course of the WGA tracing experiment. WGA-expressing adenoviruses were injected into the motor cortices of mouse brain 4 weeks after SCI and spinal cord sections were analyzed after 1 week. Sections were stained with anti-WGA, anti-MAP2ab and anti-GFP antibodies, and Hoechst. (B) The percentage of WGA-positive cells per total MAP2ab-positive cells in the caudal region of spinal cord in mice that received NSC transplantation alone was $15.5 \pm 4.1\%$ ($n=3$). This is higher than that in mice untreated after SCI (see Figure 4C), but lower than that in mice treated with NSC transplantation and VPA administration ($51.2 \pm 10.6\%$ ($n=3$)), reflecting the degree of hindlimb functional recovery (Figure 1C). **, $P < 0.01$ (t-test). All data shown are from at least 30 images, containing more than 600 cells, from three individuals (five images per area) in parallel experiments, with error bars representing SD.

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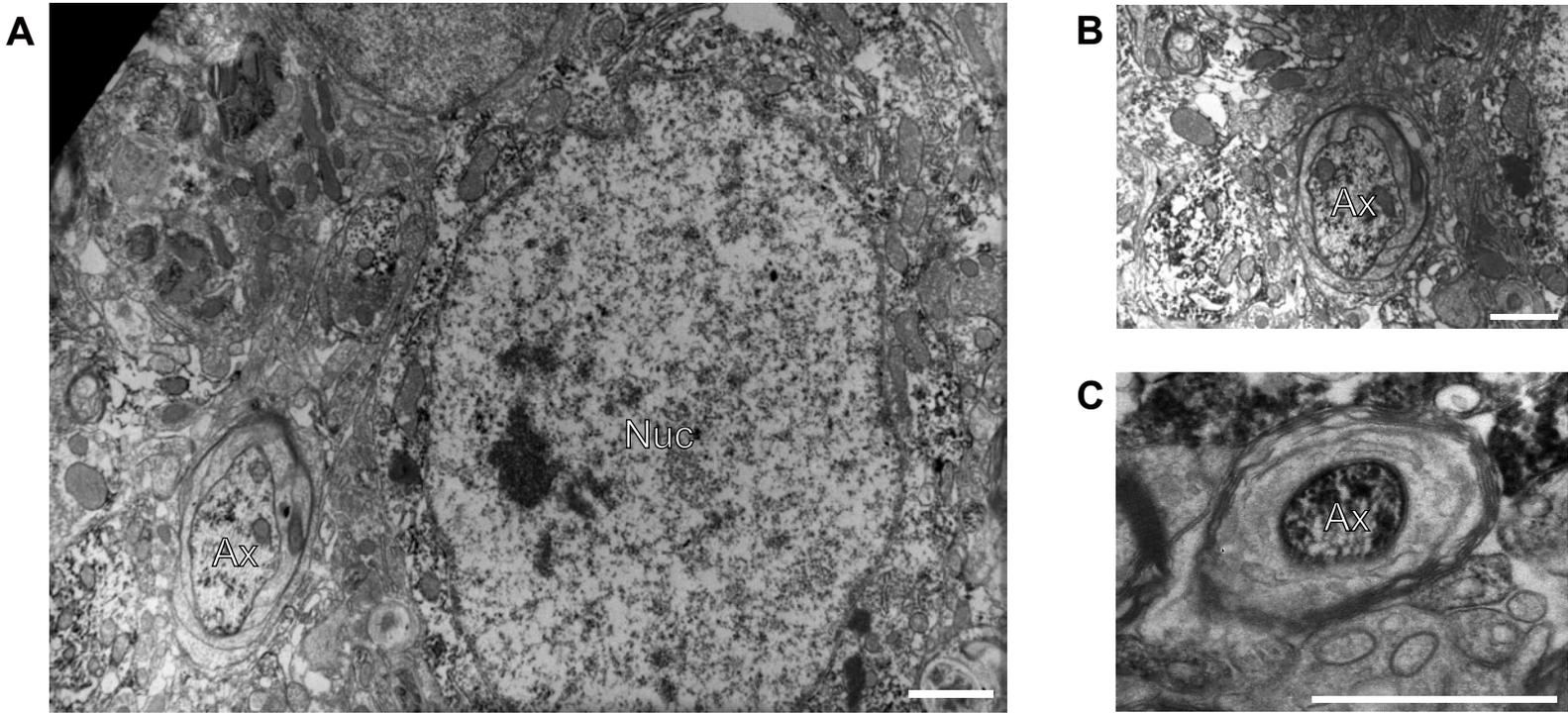
Supplemental Figure 7. Specific ablation of TR6.GFP.Luc-NSCs with DT.

(A) Phase contrast views of TG mouse-derived NSCs exposed to serial dilutions of DT. GFP.Luc- or TR6.GFP.Luc-NSCs were cultured with the indicated concentrations of DT for 24 h. TR6.GFP.Luc-NSCs were ~1000-fold more sensitive to DT than GFP.Luc-NSCs. Scale bar = 50 μm. (B) Luciferase activity of each NSC population cultured as in (A). A ~1000-fold difference in DT sensitivity was observed between the two types of NSCs. **, P<0.01 compared with TR6.GFP.Luc-derived cells (repeated-measures ANOVA). All data shown are from at least three experiments in parallel cultures, with error bars representing SD.



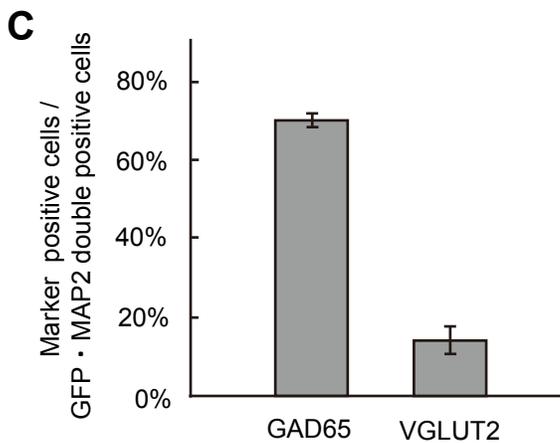
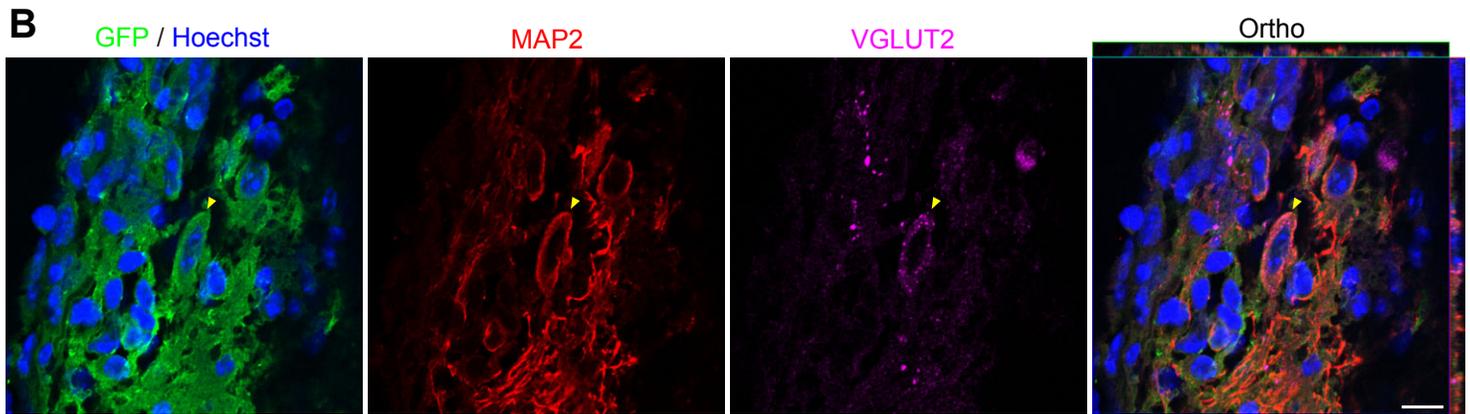
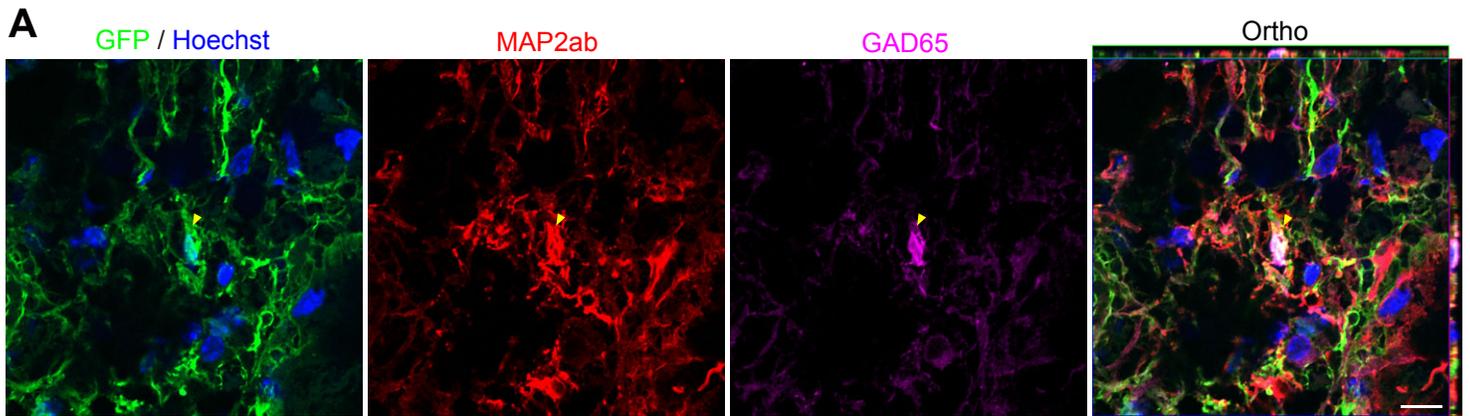
Supplemental Figure 8. Survival of the transplanted NSCs was not significantly influenced by VPA administration.

NSCs derived from GFP.Luc-tg mice were transplanted into SCI model mice 1 week after injury. VPA or Saline (VPA(-)) was intraperitoneally administered every day for 1 week. (A) Survival of transplanted cells was checked every week using a bioluminescence imaging system. (B) The time course of survival of transplanted NSCs in SCI model mice. The optical signal intensity was measured with IVIS (Details are available in the Supplementary methods). *, $P=0.1748$ compared with VPA-treated mice (repeated -measures ANOVA). Data are means \pm SEM. (C) Sagittal sections from SCI model mice treated with GFP.Luc-NSC transplantation and Saline or VPA administrations (5weeks after transplantation). Sections were stained with anti GFP (green) and Hoechst (blue). Scale bar = 1 mm.



Supplemental Figure 9. Transplant-derived neurons are myelinated by endogenous oligodendrocytes.

(A-C) Immunoelectron microscopy images of sequential sections of a GFP-positive neuron 5 weeks after transplantation. A GFP-positive axon (Ax) was remyelinated by GFP-negative endogenous oligodendrocytes. Nuc: Nucleus. Scale bar = 1 μ m.



Supplemental Figure 10. VPA induces GABAergic and glutamatergic neurons.

Representative images of GFP-NSC-transplanted and VPA-administered SCI model mice are shown. (A) Confocal images of GFP-positive cells 5 weeks after transplantation into the injured spinal cords. Spinal cord sections from VPA-treated mice were stained with anti-GFP (green), anti-MAP2ab (neuronal marker, red) and anti-GAD65 (GABAergic neuronal marker, magenta) antibodies, and Hoechst (blue). Scale bar = 10 μ m. (B) Confocal images of GFP-positive cells 5 weeks after transplantation into the injured spinal cords. Spinal cord sections from VPA-treated mice were stained with anti-GFP (green), anti-MAP2 (neuronal marker, red) and anti-VGLUT2 (glutamatergic neuronal marker, magenta) antibodies, and Hoechst (blue). Scale bar = 10 μ m. (C) The percentages of marker-positive cells in transplant-derived neurons. The data shown are from three experiments, with error bars representing SD.