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Supplemental Information

Activation of Intrinsic Growth State Enhances Host Axonal Regeneration into Neural Progenitor Cell Grafts

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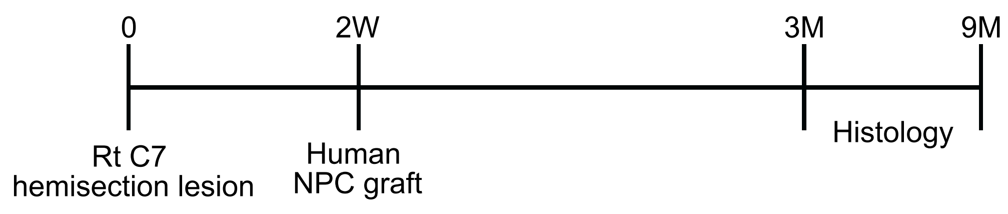
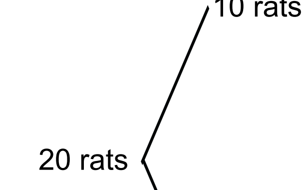
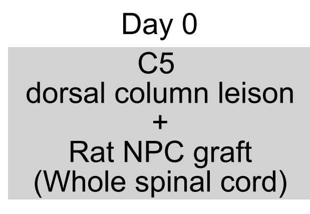
A

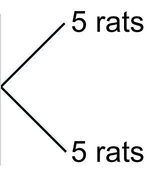
Primate study (Fig.1)

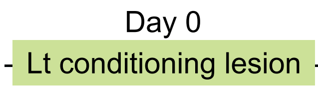


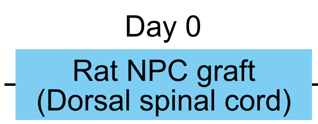
B

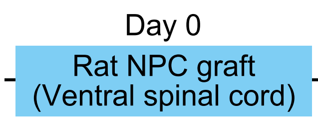












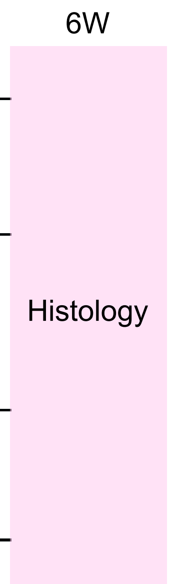


Figure S1. Study Schematic
(**A**) Primate study
(**B**) Rat study

Supplemental Experimental Procedures

Animals

Primate Studies: We studied five male rhesus macaques (*Macaca mulatta*, 7-9 -year-old, 8.5-11 kg). *S*ubjects were housed at the California National Primate Research Center (Davis, CA). All surgery was done under deep anesthesia with 1.5–2.5% isoflurane. Tissue processing and analysis was performed at the Center for Neural Repair (University of California, San Diego; La Jolla, CA).

Rat Studies: A total of 23 adult female Fischer 344 rats (150-250g, The Jackson Laboratory, Bar Harbor, ME) were used in this study. Animals had free access to food and water throughout the study. All surgery was done under anesthesia using a combination (2 ml/kg) of ketamine (25 mg/ml), xylazine (1.3 g/ml), and acepromazine (0.25 mg/ml). All surgeries were approved by the Institutional Animal Care and Use Committee of the Department of Veterans Affairs (VA) San Diego Healthcare System. National Institutes of Health guidelines for laboratory animal care and safety were strictly followed.

Primate Study

The monkeys described here were reported previously (Rosenzweig et al., 2018). GFP-expressing human fetal spinal cord neural stems (NSI-566RSC-GFP) were a gift of NeuralStem, Inc. Culture methods were reported previously (Lu et al., 2012). Briefly, the cervical and upper thoracic human fetal spinal cord from a single week 8 gastrulation male donor was dissociated and suspended in serum-free DMEM/F12 media containing N2 supplement, 100 mg/l human plasma apo-transferrin, 25 mg/l recombinant human insulin, 1.56 g/l glucose, 20 nM progesterone, 100 mM putrescine, and 30 nM sodium selenite. Cells were plated on 100 mg/ml poly-D-lysine coated flasks and expanded. 10 ng/ml FGF2 was added every other day. Cells were replated when they reached 75% confluence. At each passage, cells were harvested and stored in liquid nitrogen. For shipping, cells were thawed 1 day prior to grafting, washed, and shipped overnight at 2-8°C. Surgical procedures for placement of right C7 hemisection lesions were described previously (Rosenzweig et al., 2018). Briefly, after deep anesthesia, a C5 laminectomy was performed and a right lateral hemisection was created in the underlying C7 spinal cord. Two weeks after the C7 hemisection, twenty million human fetal spinal cord neural progenitor cells were suspended in a fibrin matrix containing a cocktail of growth factors, including brain-derived neurotrophic factor (BDNF; 50 µg/mL, Peprotech, Rocky Hill, NJ), neurotrophin-3 (NT-3; 50 µg/mL, Peprotech), glial cell-derived neurotrophic factor (GDNF; 10 µg/mL, Sigma, St. Louis, MO), epidermal growth factor (EGF; 10 µg/mL, Sigma), basic fibroblast growth factor (bFGF; 10 µg/mL, Sigma), acidic FGF (aFGF; Sigma, 10 µg/mL), hepatocyte growth factor (HGF; 10 µg/mL, Sigma), insulin-like growth factor 1 (IGF-1; 10 µg/mL, Sigma), platelet-derived growth factor (PDGF-AA; 10 µg/mL, Peprotech), vascular endothelial growth

factor (VEGF; 10 µg/mL, Peprotech), and a calpain inhibitor (MDL28170, 50 µM, Sigma) (Lu et al., 2012). The cell / growth factor mix were injected into the lesion site. After grafting, animals received a 3-drug immunosuppressive regimen consisting of mycophenolate modafeniul (MMF; CellSept, the initial dose, 50 mg/kg twice a day), tacrolimus (FK-506; ProGraf, the initial dose, 0.5 mg/kg twice a day), and prednisone (The initial dose, 2 mg/kg/day; the maintenance dose1 mg/kg/day). Dosages of MMF and FK-506 were adjusted based on their blood concentration. After three to nine months, subjects were transcardially perfused with 4% paraformaldehyde (PFA), and the spinal cords were removed and sectioned into 30-µm-thick horizontal sections. Two sections per animal were labeled for calcitonin gene-related peptide (CGRP) regeneration using Green fluorescence protein (GFP, chicken, Aves Labs GFP-1020, 1:2000), CGRP (mouse, GeneTex GTX82726, 1:500), and NeuN (mouse, Millipore MAB377, 1:500) antibodies. For quantification of primate CGRP axonal regeneration into the human graft, whole graft images were sampled using the BZ-9000 digital microscope system (N = 5, 2 sections per monkey). GFP + area was outlined and inner contour lines were drawn at 100 µm intervals from the surface of the graft. CGRP intensity was thresholded and total CGRP density in the total GFP + area and each area (0-100um, 101-200um, 201-300um, 301-400um, 401-500um, 501- um from the surface) was calculated using ImageJ. The phenotype of grafted neurons penetrated by regenerating host sensory axons was identified by immunolabeling for TLX3 (generous gift from T. Müller and C. Birchmeier, Max-Delbrück-Center for Molecular Medicine, Berlin, Germany), a transcription factor that is specifically expressed by spinal sensory neurons. For quantification of CGRP density in TLX3 + domains, images were sampled using the confocal microscope (200x magnification, N = 5, 2 fields, 2 sections per monkey). TLX3 + domains were outlined using ImageJ. CGRP intensity was thresholded and their density was calculated in each region of interest (TLX3+) and remaining region (TLX3-). Statistical analysis was performed using the Student's T-test. P <0.05 was considered statistically significant. Data are presented as mean ± standard error of the mean (SEM).

Rodent Study

In **Study 1** (**Fig. 2**), we examined the effects of peripheral conditioning lesions on the regeneration of sensory axons into neural progenitor cell grafts placed into sites of SCI. A total of ten F344 rats underwent C5 bilateral dorsal spinal cord lesions; two weeks later, these animals received grafts of E14 spinal cord-derived multipotent neural progenitor cells into the lesion site. Half of these animals (N = 5) also received peripheral nerve conditioning lesions at the time of grafting; conditioning lesions were created by transecting the left musculocutaneous, median, ulnar, and radial nerves, ensuring that all axons projecting into the C5 spinal cord segment were conditioned (Wang et al., 2008). In **Study 2** (**Fig. 4**), we studied the effects of enriching grafts with more targets for regenerating host sensory axons. Ten F344 rats underwent C5 bilateral dorsal column spinal cord lesions, with neural progenitor cell grafts and

conditioning lesions, as described above. In this study, the neural progenitor cell grafts were either enriched or depleted of appropriate targets for regenerating sensory neurons by grafting either the *dorsal* half of the developing E14 spinal cord (enriched in sensory neuronal targets, N = 5 animals), or the *ventral* half of the developing E14 spinal cord (depleted in sensory neuronal targets, N = 5 animals). In all studies, animals were sacrificed six weeks after grafting.

Peripheral nerve surgery, Spinal cord lesions, and Rat NPC preparation

Peripheral nerve transection was performed as described previously, with a slight modification (Wang et al., 2008). The musculocutaneous, median, and ulnar nerves in the left forelimb were exposed through a medial longitudinal incision above the elbow. The ulnar and median nerves were transected at the level of the medial epicondyle and the musculocutaneous nerve was transected above the elbow. After transection of these three nerves, the radial nerve was exposed and transected after developing the interval between the biceps muscles and the humeral shaft. We placed the conditioning lesions on the same day as the spinal cord injury (Blesch et al., 2012). Conditioning lesions were placed on only on one side (left) because subjects require one functional forelimb to eat and groom. The functional forelimb without a conditioning lesion served as a control for analysis of growth-associate gene expression analysis after conditioning lesions in affected forelimb. Due to the peripheral nerve transections, we could not examine functional outcomes in this study.

Lesions: Rat C5 bilateral dorsal column wire knife lesions were made as described previously (Kadoya et al., 2016). Briefly, the spinal cord dorsal surface was exposed by C5 laminectomy. The tungsten wire knife was inserted 1 mm from the dorsal surface and raised; the most dorsal aspect of the dorsal column was left uncut to support graft retention without the need for growth factor supplementation (Kadoya et al., 2016).

Grafts: Multipotent neural progenitor cell grafts were prepared from E14 F344 developing rat spinal cords as described previously (Lu et al., 2012). Briefly, E14 spinal cords were carefully dissected out to avoid any dorsal root ganglia (DRG) tissue contamination. Dissected spinal cords were treated with 0.125% trypsin were mechanically dissociated into single cells and then filtered with a 40 um cell strainer. These multipotent neural progenitor cell grafts were re-suspended to a concentration of 5.0×10^5 cells/µl in phosphate buffered saline. For dorsal and ventral spinal grafts, the spinal cords were opened in the middle of dorsal plane and then separated into dorsal and ventral halves. Cell viability was measured with trypan blue exclusion (Thermo fisher scientific, Waltham, MA): in all cases, graft viability exceeded 90%. In all experiments, 1.0×10^6 viable cells were grafted into the lesion cavity, through the dura, using a pulled glass micropipette and a PicoSpritzer II (General Valve, Inc., Fairfield, NJ). After six-week survival period, animals were transcardially perfused with 4% PFA and post-fixed in 4% PFA overnight.

Immunohistochemistry: Fixed tissues were immersed in 30% sucrose for two days. Spinal cord tissues were cut into 30-μm-thick transverse free-floating sections. DRGs were sectioned at 10-um-thick intervals and directly mounted onto gelatin-coated slides. Sections were incubated with primary antibodies against GFP, CGRP, NeuN, TLX3, c-Jun (mouse, Santa Cruz Biotechnology sc-74543, 1:200), phospho- signal transducer and activator of transcription 3 (pSTAT3: rabbit, Cell Signaling 9145S, 1:200), growth associated protein 43 (GAP-43, rabbit, Abcam ab75810, 1;200), or beta-III Tubulin (TUJ1, mouse, BioLegend 801202, 1:200) overnight and then incubated with Alexa 488, 568, or 647 conjugated donkey secondary antibodies (1:500, Invitrogen, Carlsbad, CA) and DAPI (1:1000, Invitrogen) for 1 hr. For DRG labeling, heat-induced antigen retrieval was performed in the sodium citrate buffer (10 mM sodium citrate (Sigma), 0.05% Tween 20 (Thermo fisher scientific), pH 6.0 at 90**°C** for 30 min. For CGRP labeling of spinal cord sections, the CGRP signal was amplified by the tyramide signal amplification method. Briefly, after incubation of primary antibody, sections were incubated with biotinylated donkey secondary antibodies (Jackson Immunoresearch, West Grove, PA) for 1 hr, ABC solution (Vector Laboratories, Burlingame, CA) for 30 min, biotinyl tyramide in tris-buffered saline (TBS) solution containing 0.1% H_2O_2 for 30 min, and then Alexa Fluor-conjugated streptavidin (Invitrogen) for 1 hr. Sections were washed with TBS three times between each step. Sections were mounted on glass slides and cover slipped with Mowiol mounting medium (Sigma). Images were captured using an Olympus AX-70 fluorescence microscope (Olympus, Tokyo, Japan) equipped with an Optronics Microfire A/R digital camera Microfire A/R, (Optronics, Goleta, CA), a confocal microscope (FV-1000, Olympus, Tokyo, Japan), or the BZ-9000 digital microscope system (Keyence, Osaka, Japan).

Quantification and Statistical Analysis

For quantification of sensory regeneration into the graft, whole graft images were sampled using the BZ-9000 digital microscope system (N = 5, 2-3 sections per rat). GFP + area was outlined, CGRP intensity was thresholded, and CGRP + area in the GFP + area (%) was calculated using ImageJ. For quantification of sensory density in TLX3 + regions, images were sampled using the confocal microscope (200x magnification, N = 5, 3 sections per animal). TLX3 + regions were outlined using ImageJ. CGRP intensity was thresholded and calculated in each region of interest (TLX3+) and remaining region (TLX3-).

For quantification of c-Jun, pSTAT3, GAP-43, and TUJ1 in DRGs, whole DRG images were sampled using the Olympus AX-70 fluorescence microscope (100x magnification, N = 3, 4 sections per animal). c-Jun, pSTAT3, GAP-43, and TUJ1 signal intensity was thresholded using ImageJ. Total c-Jun, pSTAT3, and TUJ1 expressing cells were counted (**Fig.3B and C**) and GAP-43 intensity was normalized by TUJ1+ area (**Fig. 3A**).

Statistical analysis was performed using the Student's T-test. $P < 0.05$ was considered statistically

significant. Data are presented as mean ± standard error of the mean (SEM).