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UNIVERSITY OF CALIFORNIA, SAN DIEGO

Neural Stem Cells in Models of Chronic Spinal Cord Injury

A Thesis submitted in partial satisfaction of the

requirements for the degree Master of Science

in

Biology

by

Kenneth Cong Nguyen

Committee in charge:

Mark Tuszynski, Chair
Andrew Huberman, Co-Chair
Kathy French

2013

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Co-Chair

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University of California, San Diego

2013

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ABSTRACT OF THE THESIS

Neural Stem Cells in Models of Chronic Spinal Cord Injury

by

Kenneth Cong Nguyen

Master of Science in Biology

University of California, San Diego, 2013

Professor Mark Tuszynski, Chair
Professor Andrew Huberman, Co-Chair

Recently, we reported that neural stem cells (NSCs) grafted into rat T3 complete transection sites at subacute stages of spinal cord injury (SCI) formed functional relays between transected segments and improved motor functions. In the current study, we explored the therapeutic effects of NSC grafts at chronic stages of SCI by delaying grafts until 6 months after injury. Grafted cells successfully filled lesion cavities with neurons and glia, but host derived collagenous tissue separated the entire graft, resulting in disruption of neuronal

relays. Compared to subacute grafts, delayed grafts showed similar neuron densities, but graft sizes were significantly smaller. Moreover, grafted neurons extended numerous axons into the chronically injured spinal cord for long distances: projections rostrally to C4 and caudally to T12. In addition, host raphespinal axons regenerated into delayed grafts as robustly as subacute grafts. Immunoelectron microscopic analysis confirmed new synapse formations between host and grafted neurons. Interestingly, the inhibitory scar that surrounds chronic lesion sites was significantly attenuated through delayed NSC grafts, suggesting a possible mechanism underlying great axonal growth from grafted and host neurons in chronic SCI. Despite of these findings, there was a lack of functional improvement, which is at least due to the presence of collagenous tissue separating the graft. Collectively, these findings indicate that NSC grafts have great potential to form functional relays at chronic stages of SCI. To achieve functional recovery, improvements in grafting methods and/or additional treatments targeting inhibitory mechanisms intrinsic to the chronically injured state will be required.

Introduction

The function of the spinal cord is to conduct information between the brain and the rest of the body. It is responsible for motor information descending from the brain, sensory information ascending to the brain, and coordinating various spinal reflexes. The spinal cord can be divided into 5 general regions: cervical, thoracic, lumbar, sacral, and coccygeal. Traumatic damage to the spinal cord is a spinal cord injury (SCI), with variable symptoms that depend on the injury type and location. Many SCI patients have loss of neurological function below the level of injury, and there are no known treatments to restore such deficits. Thus, it is imperative to understand the biology of SCI in order to establish a suitable therapy that will alleviate the sensorimotor deficits.

SCIs are classified by which spinal segment the injury is located, along with a description of the injury. Injuries at the cervical level are most frequently occurring, and often lead to tetraplegia or the loss of voluntary muscles required for respiration (McKinley et al., 2007; Sekhon and Fehlings, 2001). Injuries are described as incomplete or complete. Incomplete injuries damage only a portion of the spinal cord, leaving some spinal tracts intact while causing other tracts to become defunct. The result is a variable range of symptoms, from having no effect on the patient to allowing only a small degree of retained function. Complete injuries damage the entire spinal cord, leading to abolishment of all neurological function below the level of injury. The most devastating symptom of SCI is paralysis; with other symptoms including autonomic dysreflexia,

bladder/bowel dysfunction, sexual dysfunction, muscle spasms, and chronic pain (Hulsebosch et al., 2009; Karlsson, 2006; Khastgir et al., 2007; Monga et al., 1999; Rekand et al., 2012; Sipski and Arenas, 2006). SCI entails a myriad of complications that will continue to progress along with the injury and ultimately depreciate the quality of life.

SCI is described as a two-part process that involves primary and secondary damages. Primary damage is the initial mechanical disruption of the spinal cord and secondary damages are biochemical cascades and physiological changes that ensue; where secondary damage causes the majority of SCI pathology (Lipton and Rosenberg, 1994; Sekhon and Fehlings, 2001). Immediately following SCI involves vasculature changes, edema, hemorrhages, inflammation, neuronal damage and demyelination. SCI will progress with invading microglia, Wallerian degeneration, and hypertrophic astrocytes to finally be characterized by an astroglial scar surrounding a cystic lesion cavity in an atrophied spinal cord (Dumont et al., 2001; Fitch and Silver, 2008; Hagg and Oudega, 2006; Kakulas, 1999; Norenberg et al., 2004). The glial scar is a physical barrier mainly composed of reactive astrocytes and inhibitory extracellular matrices that express chondroitin sulfate proteoglycans (CSPGs) and it is thought to be the main hurdle to overcome for axonal regeneration in the chronically injured spinal cord (Faulkner et al., 2004; Jones et al., 2003a; Jones et al., 2003b; Silver and Miller, 2004). Effective approaches to SCI must take into consideration how far the injury has progressed.

One approach to SCI is cell replacement therapy, with various strategies involving modified fibroblasts, Schwann cells, peripheral nerve cells, olfactory ensheathing cells, bone marrow stromal cells, and neural stem cells (Boyd et al., 2005; Bregman et al., 1993; Faulkner and Keirstead, 2005; Liu et al., 1999; Lu et al., 2005; Lu et al., 2012; Richardson et al., 1980; Xu et al., 1995a; Xu et al., 1995b). Aims of cellular replacement therapy are to provide a permissive substrate for axonal regeneration, replace lost neural cells, and bridge cystic cavities (Thuret et al., 2006). Neural stem cells (NSCs) have the capability for self-renewal and differentiation into several neural lineages, thus a great potential for cellular replacement therapies (Barnabe-Heider and Frisen, 2008; Breunig et al., 2011). Numerous studies have reported the therapeutic effects of NSCs transplants at early phases of SCI (Cummings et al., 2005; Hill et al., 2004; McDonald et al., 1999), however there are only few studies of NSCs transplantation at chronic stages of SCI (Cusimano et al., 2012; Kumamaru et al., 2013; Nishimura et al., 2013) Despite recent advancements, there are no effective treatments for late stages of SCI, and inducing functional recovery through regenerating chronically injured axons remains a formidable challenge.

Failure of axon regeneration after SCI is caused by inhibitory mechanisms involving the refractive state of injured neurons and the formations of an inhospitable environment after SCI. Axotomized neurons form retraction bulbs at proximal stumps, which will not extend past the point of injury (Tom et al., 2004). Injured neuronal cell bodies undergo atrophy (Liu et al., 2011) and distal severed

ends of axons undergo Wallerian degeneration (Tsao et al., 1999), leaving myelin remnants that are inhibitory for axonal growth (Houle and Tessler, 2003). The chronically injured spinal cord is a biologically different environment when compared to the acutely or subacutely injured spinal cord, and presents additional obstacles. At chronic stages, the reactive glial scar is well developed, and expresses CSPGs that are inhibitory for axonal regeneration(Stichel and Muller, 1998). Degenerating neural tissue undergo phagocytosis, which develops into a cystic cavity that axons cannot traverse. (Tuszynski and Steward, 2012). In addition, collagen matrices may form to stabilize a severely injured spinal cord (Klapka and Muller, 2006). Finally, there is an absence of cytokines, growth factors, and inflammatory signals from the injury, leading to a quiescent environment with lack of stimulation (Fawcett, 2006; Houle and Tessler, 2003; Kwon et al., 2002; Norenberg et al., 2004; Pallini et al., 1988).

Recently, our lab showed the effectiveness of NSCs transplantation into spinal cord lesion sites at subacute stages of SCI (Lu et al., 2012). Engrafted NSCs embedded in fibrin matrices containing several growth factors were able to fill complete transection injury sites with glia and neurons, which extended long-distance axons and formed synapses in the host spinal cord. Reciprocal host supraspinal axon growth and communication with grafted NSCs led to functional relay formations that underlie functional recovery. To address whether the same treatment strategy is sufficient to overcome the inhibitory milieu of the long-term chronically injured spinal cord and elicit functional recovery, identical NSCs were grafted into lesion cavities of complete transection sites *six months* after injury.

Findings of this study show that NSCs were able to survive in chronically developed lesion cavities and attenuate the surrounding inhibitory chronic scar. Similar to subacute NSCs grafts, delayed grafts generated abundant neurons in lesion sites that were able to extend long-distance axons into the chronically injured spinal cord and form synapses with host motor neurons caudal to the injury site. In addition, delayed grafts were able to induce growth of raphespinal axons to allow for supraspinal input. However, delayed NSCs grafts were separated by reactive collagenous tissue that prevented formations of neuronal relays, leading to the lack of locomotor function improvement. These findings indicate that grafts of NSCs into chronically developed spinal cord lesion sites has great potential to form neuronal relays, but at a minimum, improvements in grafting methods will be required to induce functional recovery.

Materials and Methods

Animals:

Adult female Fischer 344 rats weighing 160-190g were used for this study. NIH guidelines for animal care and safety were followed. All survival surgeries were performed under anesthesia using a combination (2ml/kg) of ketamine (25mg/ml), xylazine (1.3g/ml), and acepromazine (0.25mg/ml).

Preparation of NSCs:

Grafted NSCs were prepared in the exact same manner as the previous study (Lu et al., 2012). Briefly, spinal cords at embryonic day 14 (E14) of transgenic Fischer 344 rat, ubiquitiously expressing GFP (Baska et al., 2008; Bryda et al., 2006) were dissociated using 0.2% trypsin (Harris et al., 2007) and resuspended to a concentration of 200,000 cells/µl in a fibrin matrix (fibrinogen at 25 mg/ml and thrombin at 25 U/ml). The following growth factors were added to the cell suspension: brain-derived neurotrophic factor at 50 mg/ml, neurotrophin-3 at 50 mg/ml, platelet-derived growth factor at 10 mg/ml, insulin-like growth factor-1 at 10 mg/ml, epidermal growth factor at 10 mg/ml, basic fibroblast growth factor at 10 mg/ml, acidic fibroblast growth factor at 10 mg/ml, glial-cell-line-derived neurotrophic factor at 10 mg/ml, hepatocyte growth factor at 10 mg/ml, and calpain inhibitor at 50 mM.

Surgical Procedures:

Total of 20 subjects underwent T3 complete spinal cord transection using fine micro scissors and aspiration described previously (Lu et al., 2012). 4 subjects received NSCs graft at 2 weeks after injury (subacute graft). 8 subjects received NSCs graft at 6 months after injury (chronically delayed graft). 8 subjects died from respiratory infections or urinary tract infections before sustaining injuries for 6 months. 4 subjects survived for 6 weeks after delayed grafts of NSCs. At the time of cell grafts, injury sites were re-exposed and 10μl of NSCs in the fibrin matrix was microinjected using a picosprizter into lesion cavities through a pulled micropipette. Subjects survived another six weeks to undergo functional testing. 6 subjects underwent C4 dorsal column lesions (DCL) to study chronic scar formation and electro-microscopic analysis for synapse confirmation. A tungsten wire knife (Kopf Instruments, Tunjunga, CA) was stereotaxically positioned at the dorsal midline of the exposed spinal cord, and then lowered 1mm into the spinal cord from the dorsal surface through a puncture in the dura. Knife blade was extruded to form an arc, which was than raised and met by a blunt glass rod to complete the transection. After injury, animals were sutured and allowed to recover. 6 months after injury, 2 subjects received grafts of 3 μl of NSCs in the fibrin matrix into lesion cavities using a picosprizter through a pulled micropipette.

Histology and Immunohistochemistry:

Six weeks after NSCs graft, animals were transcardially perfused using 4% PFA in 0.1M phosphate buffer, post-fixed overnight and then transferred to 30% sucrose for cryoprotection at 4 °C. After 72 hours submerged in 30% sucrose, spinal cords were dissected and sectioned on a cryostat at 30μm intervals. Spinal cords containing complete transections and NSCs grafts were sectioned horizontally, and transverse sections were taken from C4 and C8 rostral to the T3 injury site, and caudally at T6 and T12. Spinal cords receiving dorsal column lesions were sectioned sagittally. Free-floating sections were incubated with the following primary antibodies: GFP to label grafted cells (rabbit from Invitrogen at 1:1000 or chicken from AbCam at 1:1000); GFAP to label astrocytes (rabbit from Dako at 1:1500); NeuN to label mature neurons (mouse from Chemicon at 1:500); ChAT to label spinal cord motor neurons (goat from Chemicon at 1:500); 5-HT serotonin to label raphespinal axon system (from Immunostar at 1:5,000); synaptophysin to label presynaptic terminals (mouse from Chemicon at 1:1000); adenomatous polyposis coli, APC, to label oligodendrocytes (mouse from Oncogene at 1:400); IBA-1 to label macrophages/microglia (rabbit from Wako at 1:1500); βIII Tubulin to label neurons (mouse from Promega at 1:750); Collagen I to label type I collagen (rabbit from AbCam at 1:50); CS56 to label chondroitin sulfate proteoglycans (CSPGs) (mouse from Sigma at 1:200); Phosphacan, to label one of CSPGs (mouse from Developmental Studies Hybridoma Bank at 1:20); and Brevican, to label one of CSPGs (mouse from Developmental Studies Hybridoma Bank at

1:100). Duration of primary antibody incubation for fluorescence stains lasted overnight at 4 °C. Secondary antibodies used were Alexa conjugated 488, 594, or 647 fluorophores raised in goat or donkey (from Invitrogen at 1:200) and incubation period lasted 2.5 hours at room temperature. DAPI (200ng/ml) was used to stain nucleus and was added in the secondary incubation.

Light-level GFP immunolabeling was used to examine and quantify GFP-expressing axons. 0.6% hydrogen peroxide was used to block endogenous peroxidase activity for 30mins at room temperature. 5% donkey serum was used to block non-specific antigens for 1 hour at room temperature. Sections were incubated for 3 nights at 4 °C with a primary antibody for GFP (rabbit from Invitrogen at 1:1500). Secondary antibody was biotin-conjugated donkey anti-rabbit (from Jackson laboratory at 1:200), with an incubation time of 1 hour at room temperature. This was followed by 1-hour room temperature incubation of avidin-biotin peroxidase complex (Elite kit from Vector laboratories at 1:100 dilution). 0.05% diaminobenzidine with 0.04% nickel chloride were used as chromagens, and reacted for 15 minutes at room temperature.

Quantification of Graft Size and Host Spinal Cord:

GFP light level stained sections were used to quantify graft size and horizontal width of host spinal cord. 40x magnification photos were taken to generate a photomontage. 6 sections per subject underwent quantification to determine graft size. Photos were then converted to binary format and pixels were measured using ImageJ (Wayne Rasband, US NIH,

http://rsb.info.nih.gov/ij/). Pixel measurements were then converted to area measurement in mm^2. Two sections per subject were selected for quantification of width of host spinal cords. The outermost graft/host border was designated as the starting point. Graft / host border was measured, along with points at 1mm, 2mm, 3mm away from the graft, both rostrally and caudally.

Quantification of Neural Cell Phenotypes:

Triple and double fluorescence immunolabeled sections were used to quantify neural cell types. Serial boxed size images (mm^2) covering entire thickness of a section were taken by 400x magnification in the GFP-positive area, and then images were stacked by Image J software. Two areas per subject were taken. Individual DAPI positive cells co-labeled with NeuN, GFAP, APC, or IBA-1 were counted and divided by the total number of DAPI cells per image. Density of each cell type was obtained by dividing total number of each cell type by the size of image frame. Estimated number of neural cells in the graft was calculated through the sum of NeuN, GFAP, and APC densities multiplied by the size of graft. Estimated number of grafted neurons was calculated through NeuN densities multiplied by the graft size.

Quantification of Graft-Derived Axon Extension:

Emerging graft-derived axons were quantified using StereoInvestigator (MicroBrightField, http://www.mbfbioscience.com/) as previously described (Rosenzweig et al., 2010). Every sixth sections were labeled with light level GFP

staining and two widest sections per subject underwent quantification. Transverse lines were generated at 40x magnification at distances 1mm, 2mm, and 3mm rostral and caudal to the graft/host border and individual axons crossing the lines were counted under 400x magnification. Counted axon numbers at each distances were normalized to the number of axons in the subacute group at 1mm caudal point. Counted axon numbers were also normalized to the number of axons at 1mm point, under each respective condition.

Quantification of Host 5-HT Axon Regeneration:

Double fluorescent stained sections for GFP and 5-hydroxytryptamine (5-HT) were used to quantify the number of regenerating raphespinal axons, using StereoInvestigator as previously described above. Two sections per animal were subject to quantification. Rostral graft / host border was determined using GFP. Under 40x magnification, a transverse line was generated 1mm caudal to the pre-determined border. 5-HT positive fibers traversing the line were marked and counted under 400x magnification.

Electron Microscopy:

Immunoelectron microscopy was used to confirm synapse formation between host and grafted neurons. Subjects were perfused with 4% paraformaldehyde plus 0.1% glutaraldehyde and were post fixed overnight. Spinal cords were dissected and sagittally sectioned on a vibratome at 60μm

intervals. Sections underwent light level immunohistochemistry for GFP, with diaminobenzidine (DAB) and nickel chloride as chromagens. Immunolabeled sections underwent tissue processing for electron microscopy: 1% osmium tetroxide fix, dehydration, embed in resin, and 0.1% lead stain. Axon terminals were identified using electron microscope (FEI Tecnai Spirit G2 BioTWIN Transmission) at the UCSD Transmission Electron Microscopy Core Facility.

Quantification of Chronic Scar:

Fluorescently immunolabeled sections for GFAP, CS56, Brevican and Phosphacan were used to quantify the percentage of immunoreactivity around lesion cavity or grafted cells as previously described (Jones and Tuszynski, 2002). For lesion-only condition, sections from dorsal column lesion subjects were used. Delayed NSCs grafts into complete transection sites were used to assess the amount of chronic scar attenuation. Two images of every marker per subject were subject to quantification. A 100μm perimeter was used to assess the amount of immunoreactivity. Percentages were generated through the amount of immunoreactivity in pixels divided by pixel amount of the 100μm perimeter.

Functional Analysis:

The BBB 21-point locomotion scale (Basso et al., 1996) was used to assess functional recovery.

Statistical Analysis:

All quantification procedures underwent two-group comparison using Student's t-test. A significance criterion of *$p<0.05$, **$p<0.01$, ***$p<0.001$ were used. All data are presented with mean ± SEM

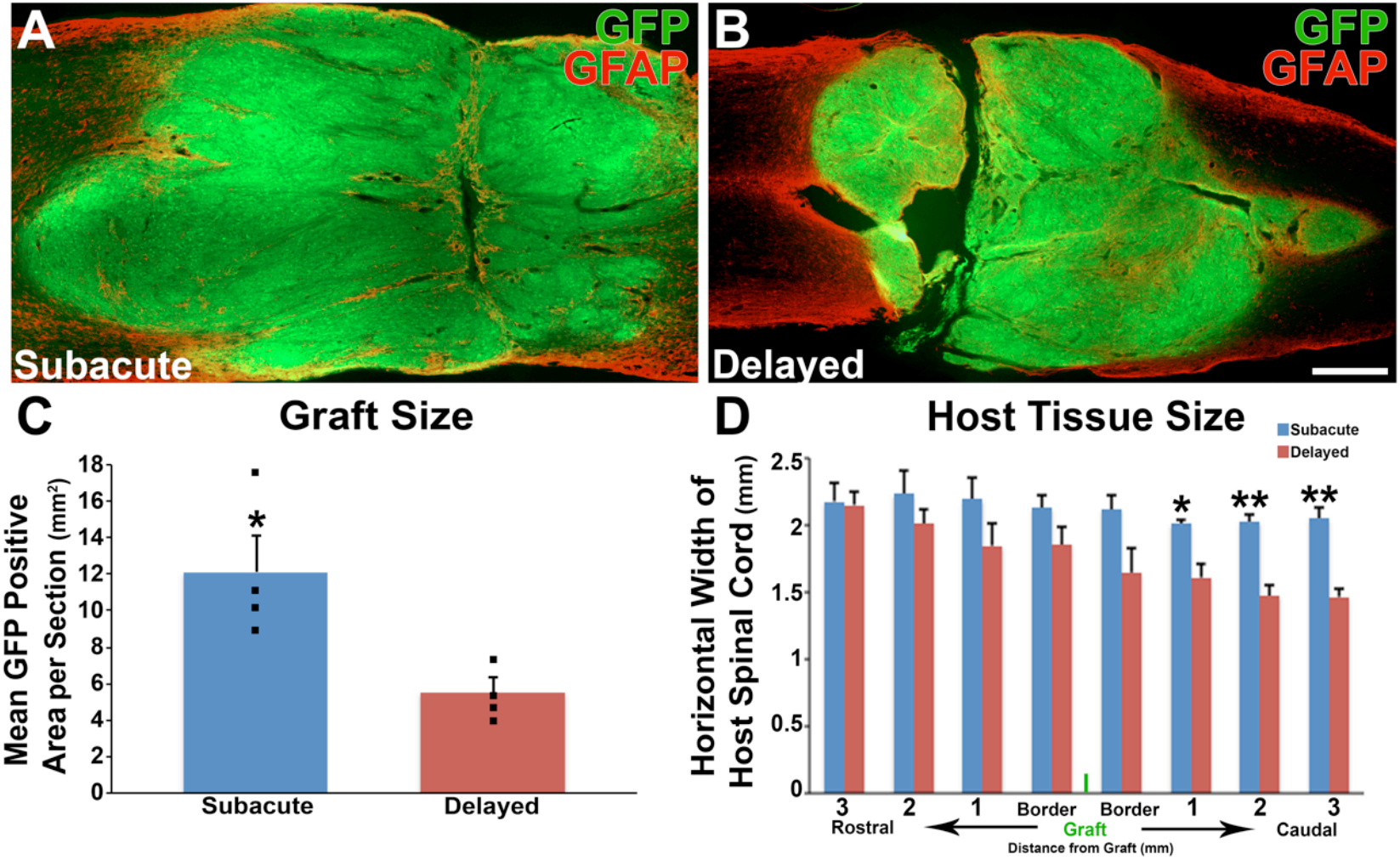
Results

Delayed NSCs Grafts Survive in Chronically Developed Lesion Cavities

To study the efficacy of using NSCs as cellular therapy for the chronically injured spinal cord, 16 adult female Fischer 344 rats received complete spinal cord transections at the thoracic T3 level. 8 subjects survived for 6 months after injury and received NSCs grafts. Importantly, when grafting NSCs into lesion cavities, no additional surgical manipulations of lesion sites (such as chronic scar resection or debris aspiration) were performed in order to avoid any further damage to the host spinal cord. 4 subjects survived for an additional 6 weeks after cell grafts and were used for the current analysis. As controls, 4 subjects received NSCs grafts 2 weeks after the same T3 injury (subacute graft) and all survived for an additional 6 weeks. Of note, the preparation methods of NSCs, total number of grafted cells, and injection methods were exactly same between these two groups, allowing us to perform a fair comparison. Grafted NSCs demonstrated the ability to survive in both subacutely and chronically developed lesion cavities of complete spinal cord transections (Figures 1A and B). However, graft size measurements showed that delayed grafts were significantly smaller in size (5.5 ± 0.8 mm^2) than subacute grafts (12.1 ± 2.0 mm^2, $p < 0.05$, Figure 1C). Horizontal width measurements of host spinal cords revealed that the spinal cord region caudal to the injury site was significantly narrower in subjects of delayed grafts than subjects of subacute grafts, indicating that significant atrophy of the region caudal to injuries will progress over time after complete transection injury.

(Figure 1D). This atrophied spinal cord appears to be, at least in part, attributing to the smaller size of delayed grafts.

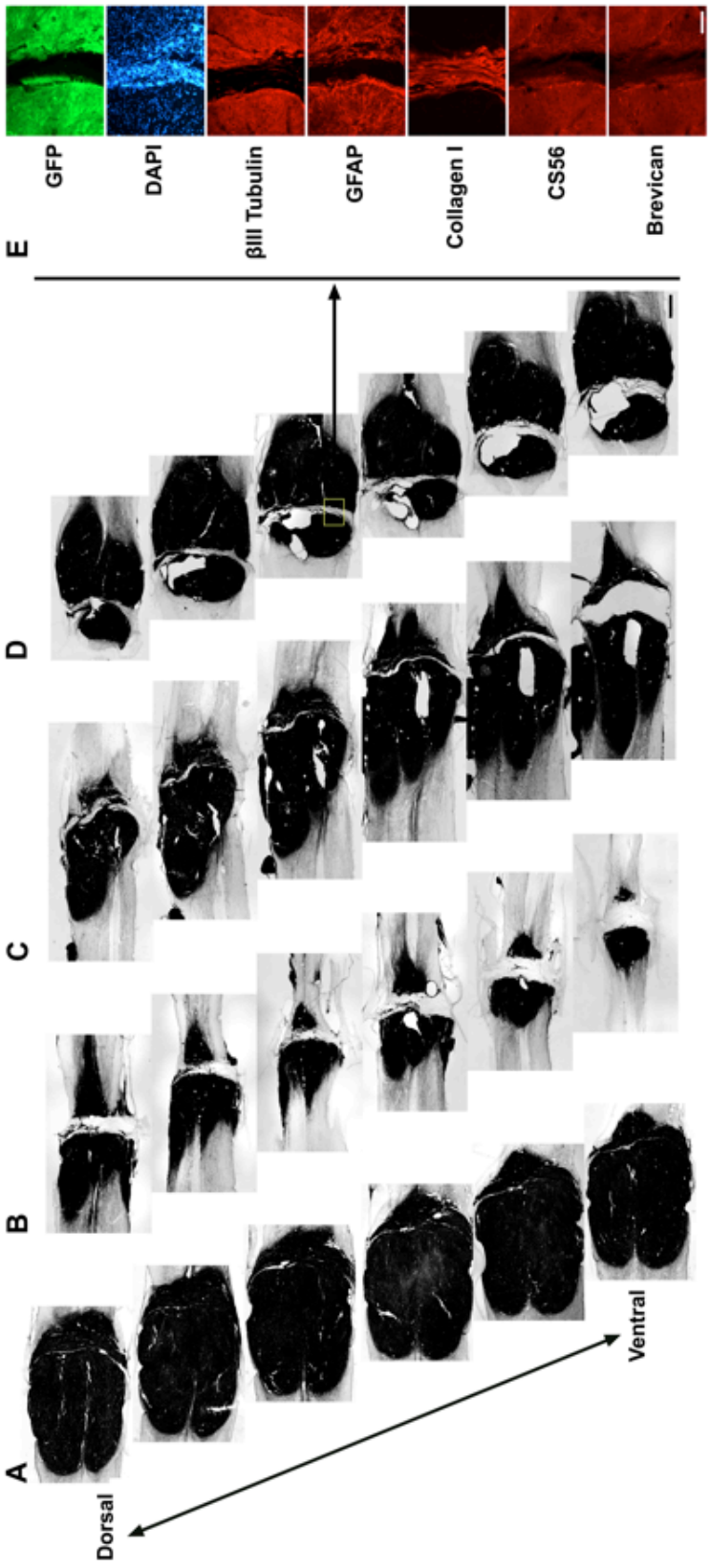
Figure 1. Six Months Delayed NSCs Grafts Fill Up T3 Complete Transection Lesion Sites.
(**A, B**) Overview of GFP and GFAP fluorescent immunolabeling in horizontal sections demonstrates that cells grafted at 2 weeks (subacute, **A**) or 6 months (delayed, **B**) after injury survived and filled up T3 complete transection site.
(**C**) Size of subacute grafts is significantly larger than delayed grafts. Individual measurements are represented by dots.
(**D**) Horizontal width measurement of host spinal cord showed that spinal cords caudal to graft/lesion were significantly narrower in subjects of delayed grafts than subacute grafts.
All data represented as mean ± SEM. (*$p<0.05$ and **$p<0.01$). Scale bar: **A** and **B,** 500μm.


A
GFP
GFAP
Subacute
B
GFP
GFAP
Delayed
C
Graft Size
Mean GFP Positive Area per Section (mm²)
18
16
14
12
10
8
6
4
2
0
*
Subacute
Delayed
D
Host Tissue Size
Subacute
Delayed
Horizontal Width of Host Spinal Cord (mm)
2.5
2
1.5
1
0.5
0
*
**
**
3
2
1
Border
Border
1
2
3
Rostral
Graft
Caudal
Distance from Graft (mm)

Reactive Collagenous Scar Separates Delayed NSCs Grafts

All delayed grafts showed presence of non-GFP tissue that completely separated the grafted area (Figure 2E). Serial images of every 6th section revealed that this separation occurred throughout the entire graft (Figures 2A – D). Because subjects of subacute NSCs grafts did not show separation by this non-GFP tissue, it is exclusive to cases of delayed grafts. This tissue layer was highly dense in DAPI positive cells, which were immunolabeled with βIII Tubulin negative, GFAP negative, type I collagen positive, and CSPG negative, indicating reactive collagenous scar tissue was formed through host non-CNS type cells. Notably, no βIII tubulin positive processes were able to traverse this collagenous scar, indicating a lack of neural connections across this barrier. Collectively, neural relay formations by delayed grafts of NSCs were blocked by the presence of this collagenous scar in lesion sites.

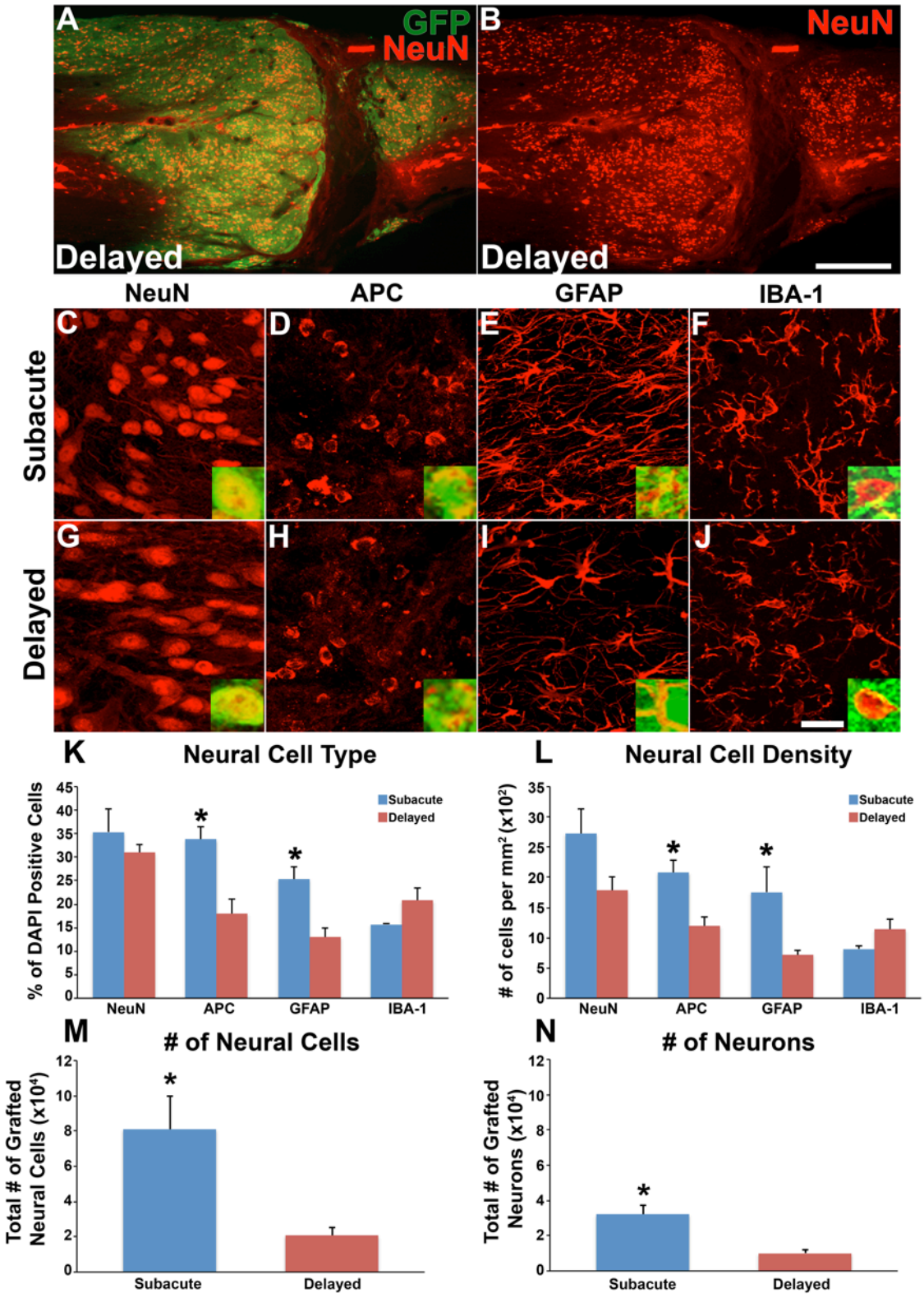
Figure 2. Collagenous Scar Separates Delayed NSCs Grafts.
(**A-D**) Every 6th serial horizontal section of all delayed grafted subjects was stained with light level GFP. Images are dorsal to ventral, from top to bottom. All of sections have a GFP negative tissue separating entire GFP graft.
(**E**) High magnification of the GFP negative tissue area is filled with DAPI positive cells and is negative for βIII Tubulin, GFAP, CS56 and Brevican, but positive for type 1 collagen.
Scale bars: **A**-**D**, 500μm; **E**, 100μm.


A
B
C
D
E
Dorsal
Ventral
GFP
DAPI
βIII Tubulin
GFAP
Collagen I
CS56
Brevican

Delayed NSCs Grafts Differentiate into Mature Neurons and Glia

Similar to subacute grafts (Figures 3C – E), delayed grafts of NSCs were able to mature into both neurons and glia (Figures 3 A – B, G – I). Proportions of total DAPI positive cells in delayed NSCs grafts were 31.0 ± 1.8% neurons, 18.0 ± 3.1% oligodendrocytes, 13.0 ± 2.0% astrocytes, and 20.8 ± 2.6% microglia/macrophages, while subacutely grafted cells consisted of 35.4 ± 4.8% neurons, 33.8 ± 2.8% oligodendrocytes, 25.3 ± 2.6% astrocytes, and 15.7 ± 0.1% microglia/macrophages, thus showing a similar proportion of neurons and a significant reduction in glia proportion in delayed grafts when compared to subacute grafts (Figure 3K). Host derived IBA-1 positive microglia/macrophages invaded grafted NSCs under both subacute and delayed grafting conditions, however a slightly larger number of microglia/macrophages were recruited into delayed NSCs grafts (Figures 3F and J). Actual cell density of neurons in delayed grafts was similar, but oligodendrocytes and astrocytes exhibited significantly decreased cell density in comparison (Figure 3L). Accordingly, estimated total number of neural cells in delayed grafts was a significantly lower than subacute grafts, partly due to the smaller size of delayed grafts (Figure 3M). Therefore, estimated total number of neurons in delayed grafts was also decreased, showing about 30% of neurons in subacute grafts (Figure 3N). Thus, delayed NSCs grafts into lesion sites could generate neuron density comparable to subacute grafts, but generated a lower glial density, resulting in decreased neural cells in delayed grafts.

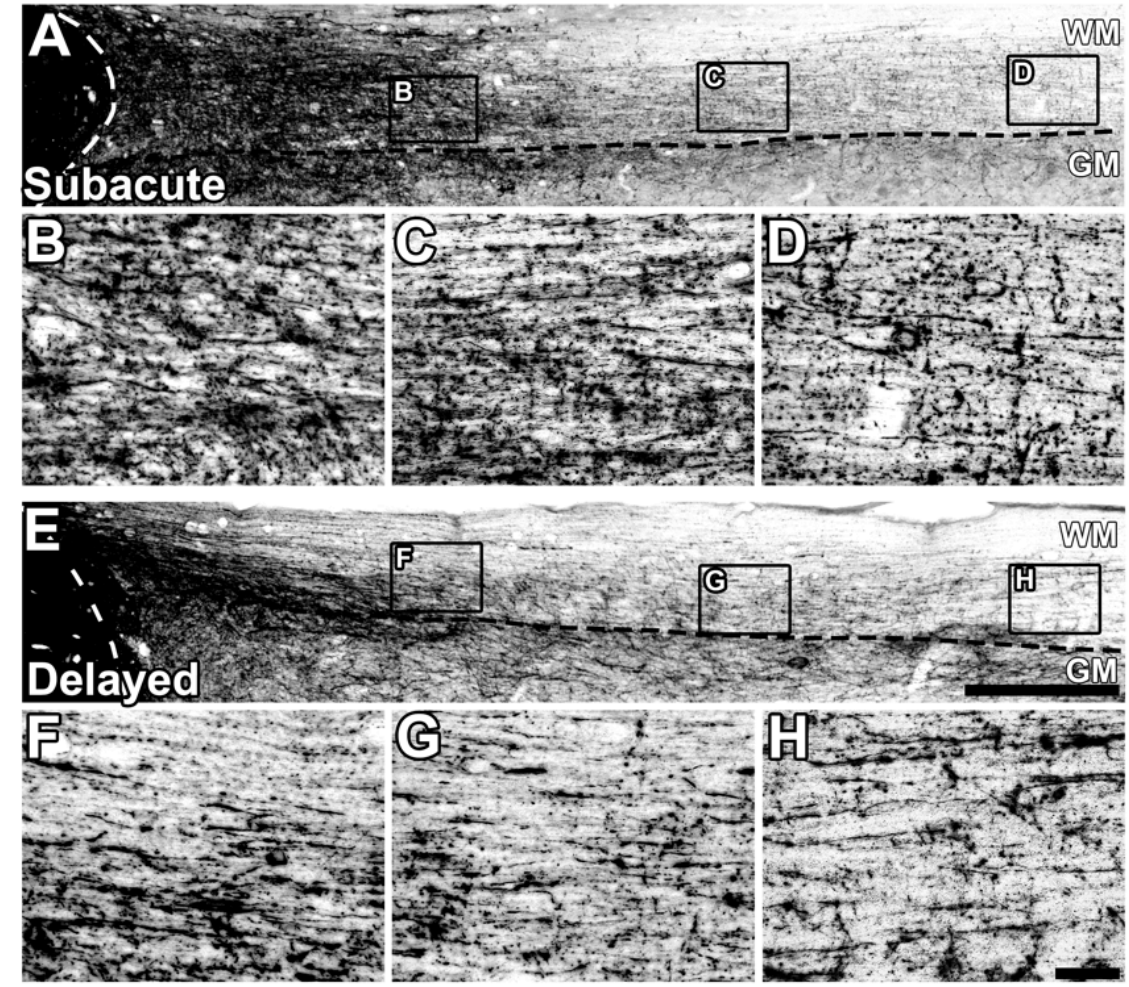
Figure 3. Delayed NSCs Grafts Differentiate into Mature Neurons and Glia.
(**A, B**) Overview of GFP and NeuN fluorescent immunolabeling in horizontal sections demonstrates that delayed grafts were able to fill lesion cavities with mature neurons.
(**C-J)** Subacute and delayed grafts were immunolabeled with NeuN, APC, GFAP, and IBA-1. Images were taken from GFP-positive area. Both grafting models were able to successfully differentiate into (**C, G**) neurons, (**D**, **H**) oligodendrocytes, (**E**, **I**) and astrocytes. (**F**, **J**) IBA-1 positive cells infiltrated grafted NSCs and did not express GFP.
(**K**) Proportions of each cell phenotypes per DAPI-positive cells.
(**L**) Cell densities of each cell phenotypes.
(**M**) Estimated total number of grafted neural cells.
(**N**) Estimated total number of grafted neurons.
All data represented as mean ± SEM. (*$p<0.05$). Scale bars: **A** and **B**, 500μm; **C-J,** 25μm.


A
GFP
NeuN
Delayed
B
NeuN
Delayed
NeuN
APC
GFAP
IBA-1
Subacute
Delayed
C
D
E
F
G
H
I
J
K
Neural Cell Type
% of DAPI Positive Cells
Subacute
Delayed
NeuN
APC
GFAP
IBA-1
L
Neural Cell Density
# of cells per mm² (x10²)
M
# of Neural Cells
Total # of Grafted Neural Cells (x10⁴)
N
# of Neurons
Total # of Grafted Neurons (x10⁴)

Delayed Grafts of NSCs Extend Long-Distance Axons into Host Spinal Cord

Next, we assessed axon extension from grafted neurons into chronically injured host spinal cord. Like subacutely grafted neurons (Figures 4A – D), neurons in delayed grafts extended large numbers of axons into host spinal cord (Figures 4E – H). These axons were detected rostrally at C4 (Figure 5E), and caudally at T12 (Figure 5H), indicating that delayed grafted NSCs conserve the ability to extend axons for long distances like subacute grafts (Figures 5A and D). Quantification of axon numbers at distances of 1, 2, and 3mm away from the host/graft interface, both in the rostral and caudal direction, showed that delayed grafted neurons extended significantly less axons than subacutely grafted neurons (Figure 4I). When comparing 1mm points, delayed grafts showed a 70% reduction of axon numbers extending rostrally and a 60% reduction of axon numbers extending caudally. This large reduction of protruding axons is at least partially due to the total number of neurons in the delayed grafts being only 30% in the subacute grafts (Figure 3L). Ratio calculations of axon numbers at 2mm or 3mm point to each respective 1mm point demonstrated that there were no significant differences between delayed and subacute grafts, suggesting that grafted NSCs can extend axons in chronically injured spinal cords as robustly as in subacutely injured spinal cords (Figure 4J). Collectively, delayed grafts of NSCs are able to extend numerous axons into the chronically injured spinal cord for long distances but the amount of growing axons is reduced when compared to subacute grafts of NSCs.

Figure 4. Axonal Outgrowth from Delayed NSCs Grafts.
(**A**, **E**) Low magnification of light level immunolabeling for GFP in (**A**) subacute and (**E**) delayed grafts. Left is rostral, right is caudal. White dashed lines indicate host/graft border. Black dashed lines separate white (WM) and gray (GM) matter of host spinal cord. Both of grafts robustly extend axons into host spinal cord caudal to the lesion.
(**B-D**, **F-H**) High magnification images of boxed areas in **A** and **E** show GFP axons growing at 1, 2, and 3mm caudal from the host/graft border.
(**I**) Quantified axon outgrowth from grafted NSCs. Average axon number at 1, 2, and 3mm both rostrally and caudally from the host/graft interface. Results are normalized to the average axon number of subacute grafts at 1mm caudal.
(**J**) Quantified axon outgrowth from grafted NSCs normalized to the average axon number at each respective 1mm point.
All data represented as mean ± SEM. (*$p<0.05$). Scale bars: **A** and **E**, 500μm; **B-D**, **F-H**, 50μm.



GFP Axon Growth

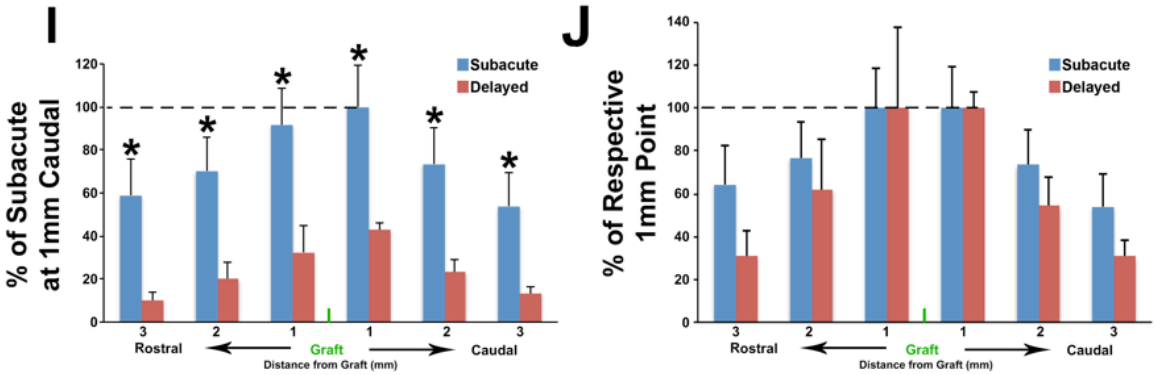
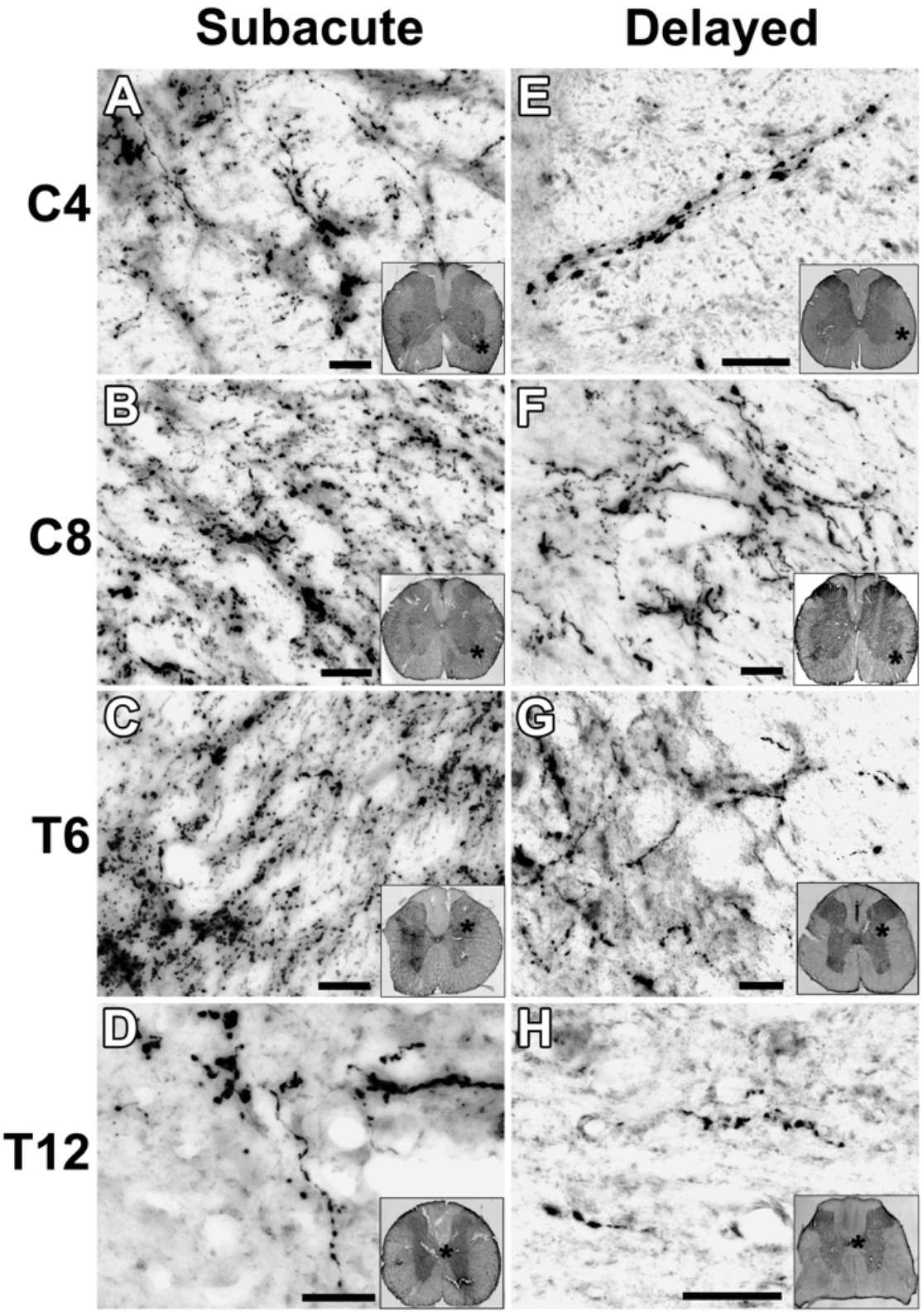


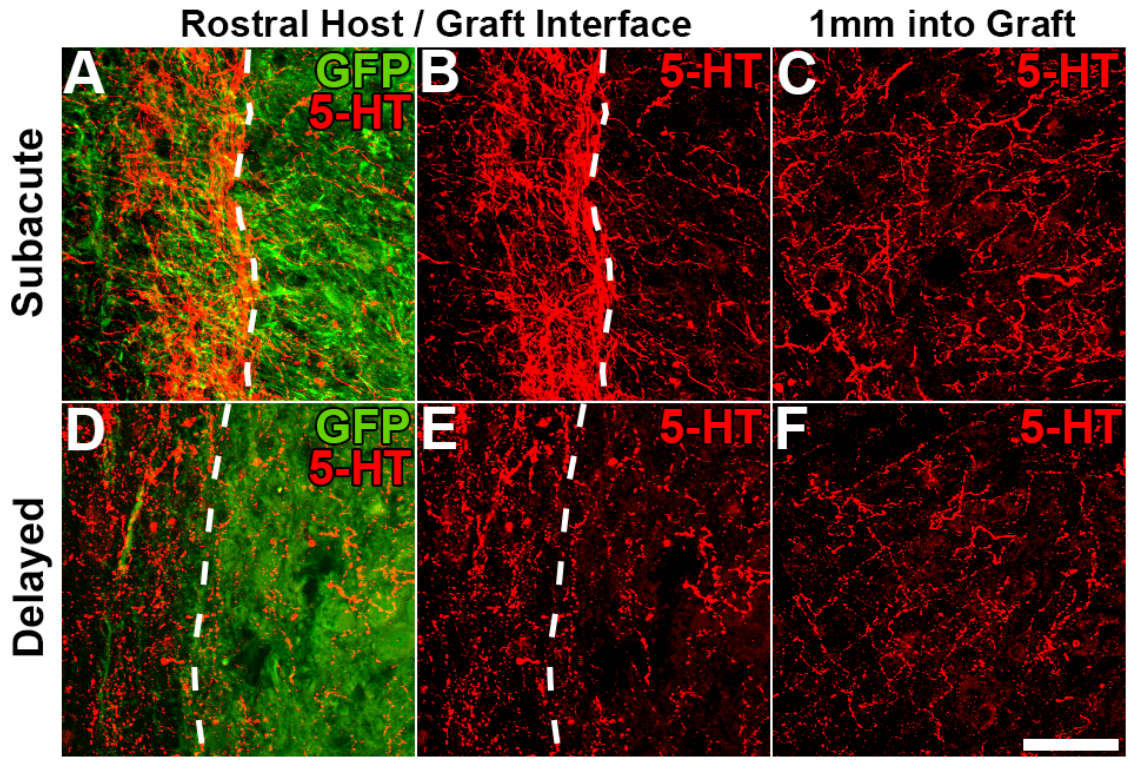
Figure 5. Long-Distance Axon Extension from Delayed NSCs Grafts.
(A-H) Transverse sections of different spinal segment levels in subacute (**A**-**D**) and delayed grafts (**E**-**H**) were immunolabeled for light level GFP. Low magnification insets depict origins of high magnification images, with asterisks indicating location. Delayed grafts of NSCs conserve the ability to extend long-distance axons like subacute grafts: projections rostrally to C4 and C8 (**E** and **F**) and caudally to T6 and T12 (**G** and **H**).
Scale bars: **A-H**, 20μm.

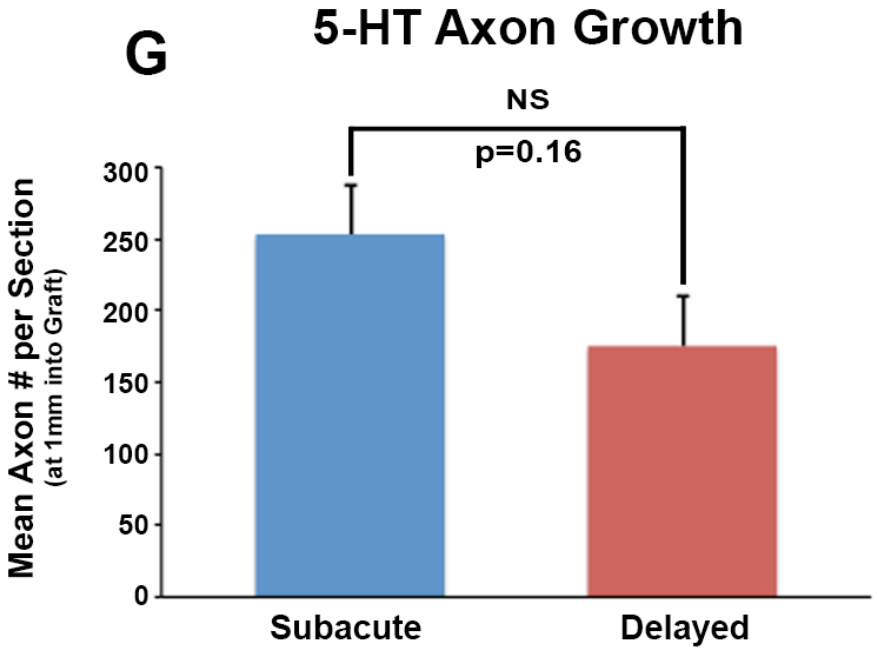

Subacute
Delayed
C4
C8
T6
T12
A
B
C
D
E
F
G
H

Host Supraspinal Axons Regenerate into Delayed NSCs Grafts

It is well known that inducing regeneration of chronically injured axons is much more difficult than acutely or subacutely injured axons. Next, we investigated host axonal growth into delayed NSCs grafts by labeling serotonergic (raphespinal) axons with 5-hydroxytryptamine (5-HT). 5-HT immunolabeling demonstrated that chronically injured 5-HT axons directly penetrated delayed grafts, similar to subacute grafts (Figures 6A – F). The current injury model transects all descending axons, and serotonergic axon trajectory in the graft is undirected, thus indicating that these are indeed regenerating axons. Quantification at 1mm into the graft from rostral host/graft border revealed no statistical difference of the number of regenerating 5-HT axons between subacute (235 ± 34 axons) and delayed (175 ± 34 axons) grafts (Figure 6G). Thus, delayed grafts of NSCs are able to induce regeneration of the chronically injured raphespinal axon system, at a comparable rate to subacute grafts of NSCs.

Figure 6. Host Raphespinal Axon Growth into Delayed NSCs Grafts.
(**A**-**F**) Host raphespinal axons, immunolabeled with 5-HT, regenerate into GFP-expressing (**A**-**C**) subacute and (**D**-**F**) delayed NSCs grafts.
(**A**-**B**, **D**-**E**) Rostral host/graft interface, indicated by white dashed lines. Host 5-HT axons penetrate through host/graft border.
(**C** and **F**) Images at 1mm from the rostral host/graft border.
(**G**) Quantified 5-HT axon number at 1mm from the rostral host/graft border.
Data represented as mean ± SEM. Scale bars: **A-F**, 50μm.


Rostral Host / Graft Interface
1mm into Graft
Subacute
Delayed
A
GFP
5-HT
B
5-HT
C
5-HT
D
GFP
5-HT
E
5-HT
F
5-HT


G
5-HT Axon Growth
NS
p=0.16
Mean Axon # per Section
(at 1mm into Graft)
300
250
200
150
100
50
0
Subacute
Delayed

Synapse Formations between Host and Grafted Neurons

Next, we asked whether delayed NSCs grafts have the ability to form synapses with host neurons like subacute NSCs grafts. Confocal image within the delayed grafted area demonstrated co-localization of 5-HT fibers and presynaptic marker synaptophysin, suggesting synapse formations between supraspinal host axons and delayed grafted neurons (Figure 7A). Immunoelectron microscopy confirmed unlabeled host axons formed synapses with delayed grafted neurons that were labeled with DAB (Figure 7C). Triple fluorescent immunolabeling of GFP, choline acetyltransferase (ChAT), and synaptophysin demonstrated that GFP positive fibers formed bouton-like structures surrounding host motor neurons and co-localized with synaptophysin, suggesting delayed graft-derived neurons were able to form synapses with host neurons (Figure 7B). Immunoelectron microscopy confirmed DAB labeled GFP axons formed synapses with host neurons at 1mm caudal to the graft (Figure 7D). These findings indicate that neurons generated from delayed grafts of NSCs can receive inputs from supraspinal systems and send outputs to host neurons below of the level of injury.

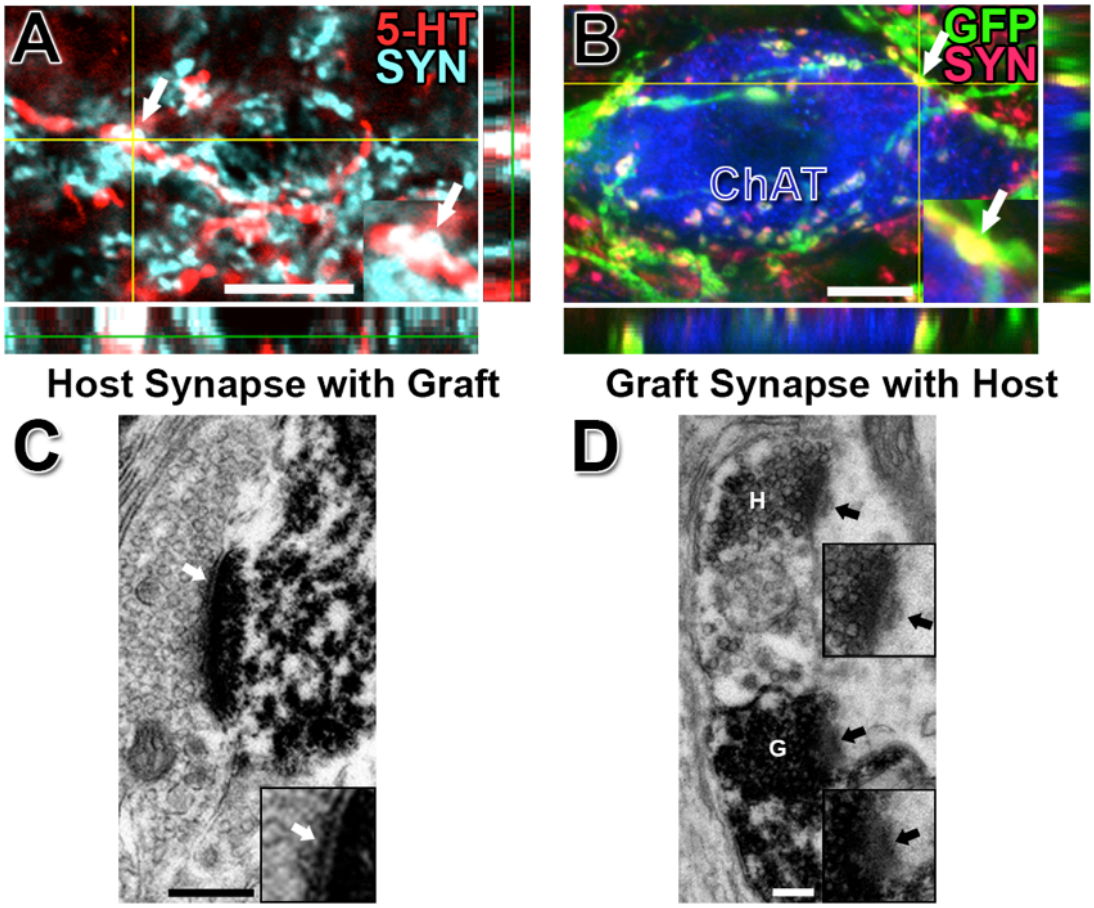


Figure 7. Synapse Formation between Host and Grafted Neurons.
(**A**) A confocal image double-labeled for 5-HT and presynaptic marker, synaptophysin, in the delayed graft shows bouton-like structures of host 5-HT axons are co-labeled with synaptophysin.
(**B**) A confocal image triple-labeled for GFP, synaptophysin, and choline acetyltransferase (ChAT) is taken in host tissue, 1mm caudal to grafted area. Graft-derived GFP axons are co-labeled with synaptophysin within proximity of ChAT positive motor neuron.
(**C**) Electron microscopy shows a host axon forms a synapse with a DAB-labeled grafted neuron. Arrows indicate synaptic cleft.
(**D**) Electron microscopy demonstrates an unlabeled host axon [H] and a DAB-labeled graft derived axon [G] form synapses with host neurons. Arrows indicate post-synaptic density.
Scale bars: **A** and **B**, 10μm; **C** and **D**, 300nm.

Delayed NSCs graft attenuates inhibitory chronic scar.

A key feature of the chronic stages of SCI is the deposition of an inhibitory scar surrounding lesion sites. It is composed of reactive astrocytes and chondroitin sulfate proteoglycans (CSPGs) and inhibits axonal growth as a physical and molecular barrier. As expected, dense immunoreactivities for GFAP, CS56 (broad marker for CSPGs), brevican (one of CSPGs), and phosphacan (one of CSPGs) were detected in the area surrounding lesion sites 6 months after injury (Figures 8A – D). Interestingly, subjects receiving NSCs grafts 6 months after injury demonstrated great attenuation of these chronic scar depositions (Figures 8E – L). Quantification showed that immunoreactivities of GFAP, CS56, brevican, and phophacan were respectively reduced by 65%, 94%, 84%, and 72% in subjects of delayed grafts, when compared to lesion alone controls. The ability of NSCs to modify and attenuate the chronic scar will be one of the mechanisms underlying great axonal growth from host and grafted neurons in chronic stages of SCI.

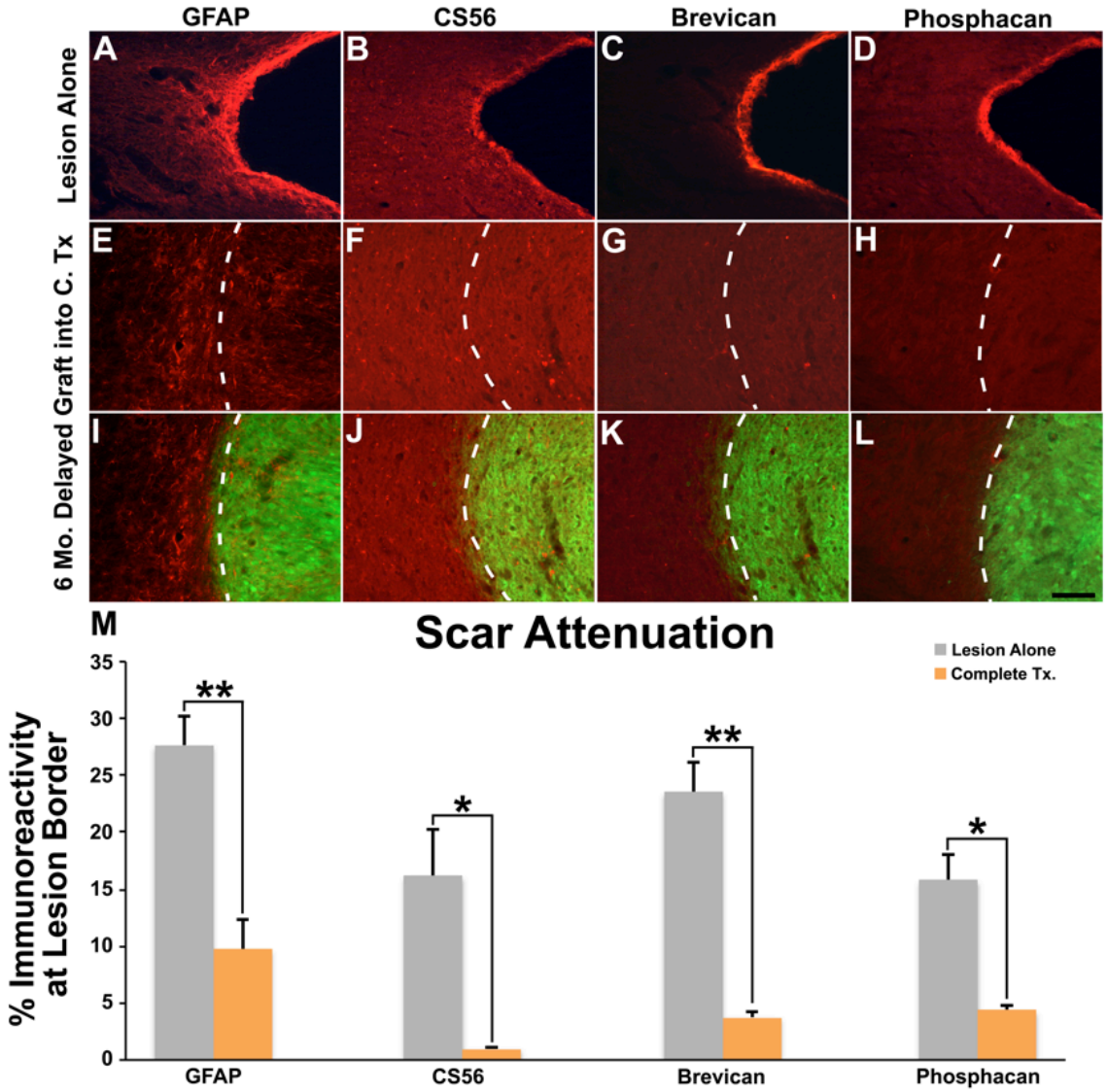


Figure 8. Glial Scar Attenuation through Delayed NSCs Grafts.
(**A**-**D**) Saggital sections showing lesion sites at 6 months after dorsal column lesion are immunolabeled for (**A**)GFAP, (**B**) CS56,(**C**) brevican, and (**D**) phosphacan. Chronic scar consisting of reactive astrocytes and CSPGs surrounds chronic lesion cavity.
(**E**-**L**) Horizontal sections showing GFP expressing NSCs were grafted into lesion sites 6 months after injury. White dashed line indicates host/graft interface. Immunoreactivities for (**E, I**) GFAP, (**F, J**) CS56, (**G, K**) brevican, and (**H, L**) phosphacan at host/graft interface are attenuated.
(**M**) Quantification displays significant attenuation of immunoreactivities of GFAP, CS56, brevican, and phosphacan in grafted subjects, when compared to lesion alone subjects.
Data represented as mean ± SEM. (*p<0.05 and **p<0.01). Scale bar: **A-L**, 100μm.

No locomotor improvement was acheived.

Hindlimb locomotion was measured weekly using the Basso, Beattie, and Bresnahan scale (BBB scale) (Basso et al., 1996). All subjects receiving T3 complete transection injuries exhibited severely impaired hindlimb functions at the time of cell grafts. Subjects of subacute NSCs grafts began to show significant locomotor improvement in two weeks, which plateaued at four weeks after grafting, while on the other hand, subjects of delayed NSCs grafts did not display any locomotor improvement. Thus, NSCs grafts are able to improve locomotor function, however only at subacute phases of SCI, and not chronic phases with the current method. This lack of locomotor improvement is partially due to the presence of collagenous tissue dividing delayed grafts, resulting in the disruption of neural relay formations at the lesion site.

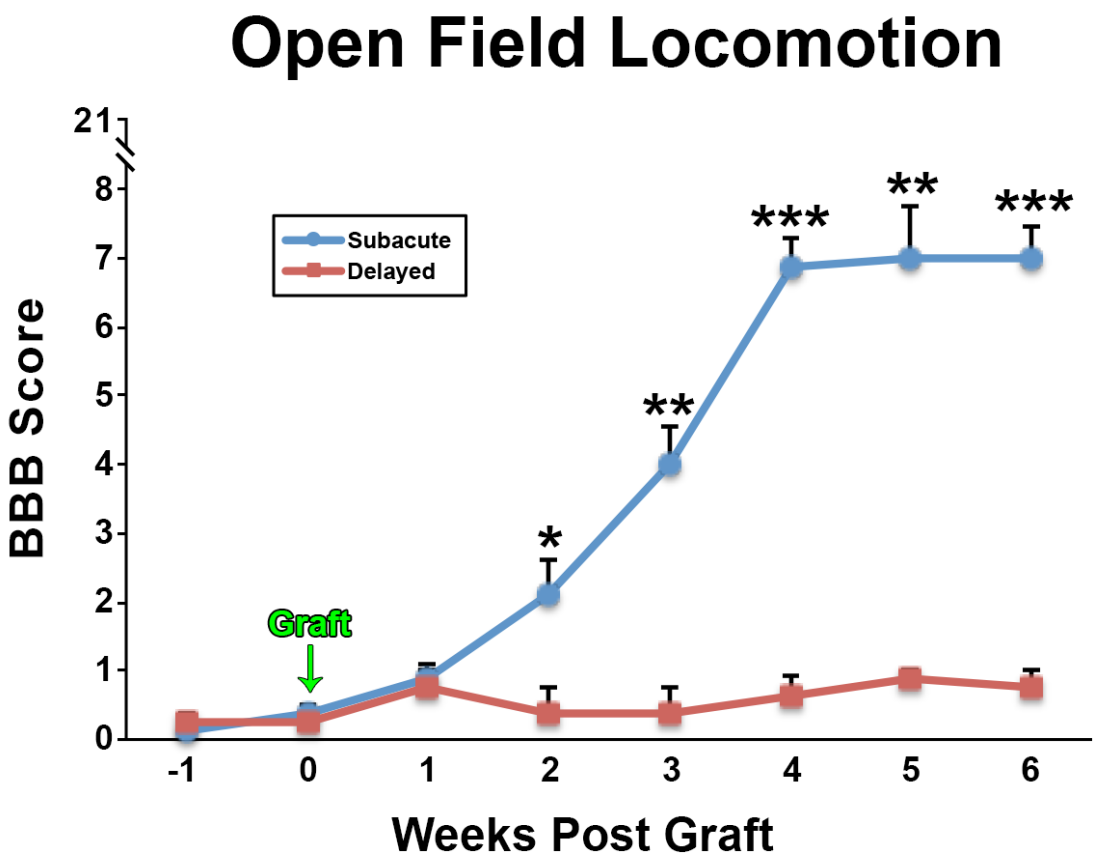


Figure 9. Locomotor Function after Delayed NSCs Grafts.
BBB scores were assessed weekly. Subacute NSCs grafts showed locomotor improvement, whereas delayed grafts did not elicit any improvement.
Data represented as mean ± SEM. (*p<0.05, **p<0.01, ***p<0.001)
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Discussion

The findings of the present study indicate that a 6 month delayed graft of NSCs can survive in the chronically developed lesion cavity of a completely transected spinal cord, and differentiate into mature neurons and glia. Graft-derived neurons extended long-distance axons into the chronically injured spinal cord to form connections with host neurons. Moreover, injured host raphespinal axons regenerated into delayed grafts of NSCs to allow supraspinal inputs. Finally, the inhibitory chronic scar formed around the lesion cavity is significantly attenuated after delayed grafts of NSCs. Despite these findings, delayed grafts of NSCs did not elicit any locomotor recovery of the hindlimbs. Absence of functional recovery is partially due to the presence of a collagenous scar tissue separating the graft, which prevents formation of neural relays. Collagen deposition at the site of SCI, will progress to form a thick fibrous tissue, an obstruction that is specific to chronic stages (Klapka and Muller, 2006; Shearer and Fawcett, 2001). In the current study, we emulated our successful grafting methods for subacute injures and did not perform any surgical manipulations of chronic lesion sites, which resulted in the presence of collagenous tissue separating grafts. Therefore, improvements in methods will include surgical procedures to open dura mater and resect collagenous tissue in lesion sites. Such modifications will also require optimization of fibrin matrix concentration and injection methods.

When compared to subacute grafts, delayed grafts were smaller in graft size, resulting in a reduced number of total neurons generated in the graft.

Reasons for the reduction in graft size include the atrophied host spinal cord in chronic stages of injury and the presence of collagenous tissue separating delayed grafts. Moreover, while the density of generated neurons in delayed grafts is comparable to subacute grafts, glial cell densities were significantly reduced, suggesting that the micro-environment of chronic lesion sites is inhibitory to glial differentiation and/or survival (Nishimura et al., 2013; Okano et al., 2003). Of note, the presence of a chronic scar at the time of grafting negatively impacts the survival of grafted NSCs (Karimi-Abdolrezaee et al., 2006). To increase glial cell densities after delayed NSCs grafts, optimization of growth factor combinations that are co-grafted with fibrin matrix and NSCs will be required.

The chronic scar surrounding lesion sites consists of reactive astrocytes and the extracellular matrix expressing various CSPGs and is known for physical and molecular impendence for axonal growth in chronic stages of SCI (Jones et al., 2003a; Morgenstern et al., 2002; Silver and Miller, 2004). In the current study, delayed NSCs grafts greatly attenuated chronic scars deposited around lesion sites, indicating that NSCs have the ability to modify lesion environment from inhibitory to permissive. This is particularly important, because previous attempts to make the lesion boundary more permissive for axonal growth through surgical resection of the chronic scar is not practical for clinical translation and has a high risk of further damaging the spinal cord (Tuszynski et al., 2003).

The amount of axon growth observed in this long-term chronic SCI model is unprecedented. Delayed graft-derived neurons were able to extend numerous

axons into the chronically injured spinal cord, although reduced when compared to the number of extending axons from subacute grafts. The large difference in grafted neurons between conditions may account for the observed reduction in axon numbers, however chronic stages of SCI present other hurdles that may impede axonal outgrowth from grafted NSCs that include the myelin debris left by degenerating axons (Schwab and Caroni, 1988) and the inhibitory chronic scar surrounding the lesion cavity (Silver and Miller, 2004). Finally, the chronically injured spinal cord is in a quiescent state in terms of injury and inflammation signals when compared to a more recent SCI, which may hamper the activated state of NSCs and lead to a decreased outgrowth.

Reciprocal host axon ingrowth into delayed grafts of NSCs allowed for supraspinal inputs. With attenuation of the chronic scar and the presence of a permissive substrate, host raphespinal axon system readily grew into grafted cells, which suggests delayed grafts of NSCs can induce growth of chronically injured supraspinal axon systems. Inducing growth of chronically injured axons is a marvelous feat, and particularly for the serotonergic axon system, which is involved in locomotor function (Thompson et al., 2011). NSCs have the capability to induce growth of chronically injured axons, in addition to extending axons and forming connections within the host spinal cord, thus being a very attractive substrate for SCI therapy.

Grafts of NSCs at the subacute phase of a severe spinal cord injury have been shown to promote functional recovery (Lu et al., 2012; McDonald et al., 1999; Ogawa et al., 2002), while no studies have reported improvement in

function by delayed NSCs grafts, even at early chronic stages of SCI (Cusimano et al., 2012; Nishimura et al., 2013). Besides the collagenous tissue separating the graft, there are several possibilities for the lack of functional recovery after treating chronic SCIs: continued cell death after injury (Beattie et al., 2002), reorganization of neural circuits that impede new inputs (Raineteau and Schwab, 2001), and/or atrophy of muscles and bone after SCI (Giangregorio and McCartney, 2006). Therefore, to maximize the effects of NSCs grafts for chronic stages of SCI, multiple treatments addressing these mechanisms may be needed.

Despite of lack of functional recovery, NSCs exhibited a great potential for making functional neural relays and restoring functions at prolonged time after SCI. In order to achieve functional recovery from the chronically injured state, improvements of both surgical procedures and preparation of NSCs, along with additional treatments addressing inhibitory mechanisms intrinsic to chronic stages of SCI may be required.

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