**Clinical Progress**

**A First-in-Human, Phase I Study of Neural Stem Cell Transplantation for Chronic Spinal Cord Injury**

**Graphical Abstract**

**Preclinical Studies**
- Differentiation of grafted NSI-566:
  - neurons
  - oligodendrocytes
  - astrocytes
- Migration of grafted NSI-566 cells

**Intraspinal Injections**
- Phase 1 clinical trial
- n=4 adult subjects
- Thoracic ASIA A spinal cord injury
- 1-2 years after injury
- 6 bilateral injections
- 2 x 10^6 cells/injection via stereotactic floating cannula

**Clinical Follow-up**
- Presence of antibodies
- ISNCSCI Exam
- Neurophysiology
- Imaging
- Quality of Life
- Pain

**Highlights**
- NSI-566 grafted injured spines in rats with near complete cavity-filling
- The differentiation profile of grafted cells showed all three neural lineage cells
- High-density human axonal sprouting was seen throughout the NSI-566 grafted region
- NSI-566 transplanted in the spinal injury site of patients can be performed safely

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**In Brief**
After promising results were seen in a pre-clinical human-spinal-cord-derived neural stem cell NSI-566 transplantation study for spinal cord injury in rats, a phase I clinical trial for NSI-566 transplantation was initiated in patients with complete thoracic SCI.
SUMMARY

We tested the feasibility and safety of human-spinal-cord-derived neural stem cell (NSI-566) transplantation for the treatment of chronic spinal cord injury (SCI). In this clinical trial, four subjects with T2–T12 SCI received treatment consisting of removal of spinal instrumentation, laminectomy, and durotomy, followed by six midline bilateral stereotactic injections of NSI-566 cells. All subjects tolerated the procedure well and there have been no serious adverse events to date (18–27 months post-grafting). In two subjects, one to two levels of neurological improvement were detected using ISNCSCI motor and sensory scores. Our results support the safety of NSI-566 transplantation into the SCI site and early signs of potential efficacy in three of the subjects warrant further exploration of NSI-566 cells in dose escalation studies. Despite these encouraging secondary data, we emphasize that this safety trial lacks statistical power or a control group needed to evaluate functional changes resulting from cell grafting.

INTRODUCTION

Current pharmacological- or physical-rehabilitation-based therapies for chronic spinal cord injury (SCI) are limited and primarily focused on modulating symptoms associated with chronic SCI such as pain (Gwak et al., 2016; Saulino and Averna, 2016) and/or muscle spasticity (Koulousakis and Kuchta, 2007; McIntyre et al., 2014). With the exception of nerve transfer procedures aimed at optimizing motor control in dermatomes just below the injury (Emamhadi et al., 2016; Medina et al., 2017; Ray et al., 2016; Simcock et al., 2017), no therapy exists that would lead to a clinically relevant improvement of motor or sensory function in chronic spinal trauma patients.

RESULTS

Pre-clinical Efficacy and Safety Studies with a Spinally Grafted NSI-566 Cell Line

Extensive pre-clinical efficacy and safety studies were conducted using a spinally grafted NSI-566 cell line in small and large animal models of spinal injury or in naive immunodeficient or...
continuously immunosuppressed animals. A statistically significant improvement in motor and sensory neurological function, amelioration of muscle spasticity, and evidence of functional synaptic coupling between grafted NSI-566 cells with the host spinal neuronal circuitry was demonstrated in rat models of L3 spinal compression (van Gorp et al., 2013), complete spinal cord transection (Lu et al., 2012), or irreversible spinal ischemic injury (Cizkova et al., 2007). A comparable functional efficacy signal was recently reported using a non-human primate model of cervical hemisection (Rosenzweig et al., 2018). In addition, a cell dose escalation study to define the equivalent human cell dose was completed in adult pigs that have similar spinal cord anatomical dimensions to those of adult humans (Usvald et al., 2010).

To define the pre-clinical safety of spinally grafted NSI-566 cells, a long-term (9–10 months) study was conducted in adult spinally injured immunodeficient rats. A total of 90 athymic nude rats (mu-/mu-, NCI) were randomly assigned to injections of vehicle or NSI-566 (450,000 cells/45 μL of the vehicle) into the injury epicenter 7 days after T10 spinal segment impact injury (MASCIS 12.5 mm) (Figure 1A). After cell grafting, animals survived for an average of 9.7 months and were assessed for motor and sensory function as previously described (van Gorp et al., 2013). A sub-cohort of rats (n = 8) was also used to study the sprouting of GFP-tagged rubrospinal and corticospinal motor axons into NSI-566 grafts after bilateral injection of AAV9-UBI-GFP into the motor cortex and nucleus ruber. There were no significant differences between vehicle- and cell-injected animals with regard to neurological function deterioration. Qualitative confocal immunofluorescence microscopic analysis and quantitative analysis of graft survival, cell proliferation, and axonal outgrowth at 9 months post-grafting revealed

Figure 1. Survival and Long-Term Safety of Spinally Grafted NSI-566 in Th10 Spinal Segment-Injured Immunodeficient Rat
(A) Schematic diagram of the experimental design.
(B) Near complete injury-induced cavity-filling effect by injected NSI-566 cells (hNUMA+ cells) and continuing extensive syringomyelia in vehicle-injected animals (white asterisks).
(C–E) Differentiation of grafted NSI-566 to neurons (C) (hNSE), oligodendrocytes (D) (OLIG2), and astrocytes (E) (GFAP).
(F) Extensive migration of grafted NSI-566 cells (hNUMA+ cells) from cell-injected injury epicenter into host tissue.
Scale bars: (B), 500 μm; (C), 20 μm; (D), 100 μm; (E), 50 μm; (F), 200 μm.
Figure 2. Extensive Axonal Sprouting from Grafted NSI-566 and Innervation of Human Grafts by Descending Motor Axons of the Host in Immunodeficient Rat

(A) High density of human axons (rostral to the lesion site) (HO14; red) derived from grafted NSI-566 cells in lateral funiculi. Numerous GFP-tagged (green) descending motor axons of the host can also be seen.

(B–D) High density of descending GFP-tagged corticospinal and/or rubrospinal axons innervating hNSE + grafts.

(E) Human-specific synaptophysin terminals (hSYN; green) derived from grafted NSI-566 neurons. Numerous hSYN+ puncta residing on membrane of the host α-motoneuron (NeuN) distal to the injury epicenter can be identified.

Scale bars: (A), 100 μm; (B), 200 μm; (C), 50 μm; (D), 40 μm; (E), 10 μm.
increased axonal outgrowth in NSI-566 grafted animals (Figures 1 and 2; Table S6). Confocal images obtained following staining of sections with human-specific nuclear antibody showed near complete cavity-filling by grafted cells. This was in contrast to the extensive syringomyelia seen in vehicle-injected animals (Figure 1B). Examination of the differentiation profile of grafted cells showed all three neural lineage derivatives, including hNSE+ (human-specific neuronal enolase) neurons, OLG2+ (oligodendrocyte transcription factor) oligodendrocytes, and hGFAP+ (human-specific glial fibrillary acidic protein) astrocytes (Figures 1C–1E). Analysis of rostrocaudal migration of hNUMA+ cells showed a robust migration of injected cells through the thoracic spinal cord (Figure 1F). Analysis of axonal sprouting from grafted neurons showed a high density of human axons throughout the grafted region (rostral to the lesion site) and in lateral funiculi between GFP-tagged descending motor tracts of the host (Figure 2A). Endogenous GFP-tagged motor axons showed extensive innervation of human hNSE+ grafts (Figures 2B–2D). Moreover, human-specific synaptophysin+ punctae derived from grafted human neurons, on the membranes of the host interneurons and α-motoneurons above and below the level of injury, were seen (Figure 2E). In summary, previously published pre-clinical proof-of-concept and safety studies provided a scientific rationale and a large animal translational platform for designing the human clinical protocol for treatment of chronic SCI by spinal NSI-566 grafting.

A Phase I Neural Stem Cell Implantation Clinical Trial for Chronic Thoracic SCI

A total of four subjects have received NSI-566 spinal cord implantation (Video S1) with a post-procedure follow-up ranging from 18 to 27 months (Table 1). All subjects tolerated the procedure well with no serious adverse events in the post-procedure period. Prospective data have been collected including ISNCSCI scores, functional and pain surveys, SCIM scores, electromyography (EMG), Brain Motor Control Assessment (BMCA), and serial MRI. The presence of donor-specific HLA antibodies was also monitored periodically.

The first patient enrolled in the study is a 26-year-old female (subject 001) with a T8 neurological level of injury after a motor vehicle accident. After neural stem cell implantation, ISNCSCI neurological assessment showed two-level sensory and motor improvements (from T8 through T10) bilaterally at 6, 12, and 18 months, and one-level sensory and motor improvement at 27 months, compared to pre-treatment baseline. Moreover, BMCA showed newly developed muscle responsiveness in lower limbs to reinforcement maneuvers. This improvement developed between 18 to 27 months post-cell grafting visits with no evidence of circulating anti-donor HLA class I or class I antibodies at 3, 12, 18, or 27 months (Figures 3A–3D; Tables S1 and S5).

The second patient in the cohort is a 33-year-old male (subject 006) with a T7 neurological level of injury after a motorcycle accident. While no change in overall ISNCSCI neurological score was noted initially, the 12-month EMG analysis showed new voluntary activity in the right rectus abdominus and left T6 to T8 paraspinal muscles (Figures 3E and 3F) as well as the development of sensation during EMG needle insertion between T6 and T9 bilaterally at 18 months after grafting (Tables S2 and S5).

The third patient in the cohort of this phase I trial is a 35-year-old male (subject 008) who suffered a T2 neurological level of injury after a motor vehicle accident. No change in ISNCSCI neurological score (motor and sensory) was noted for up to 12 months after cell grafting (Tables S3 and S5).

Finally, the fourth patient in the cohort is a 24-year-old male (subject 010) with a T5 neurological level of injury after a motor vehicle accident. ISNCSCI neurological assessment showed one level of sensory and motor improvement (from T5 to T6) on the right side and two levels of sensory and motor improvement (from T5 to T7) on the left side at 6, 12, and 18 months visits compared to baseline. The functional response included gain in voluntary bilateral motor control of T6 to T7 abdominal wall musculature. Moreover, EMG analysis showed previously unrecorded activity in the right superficial paraspinal muscle at T7, one level lower at the 12 months visit than at the 6 months visit. This voluntary activity was reconfirmed at the 18 months visit (Figures 3G and 3H). Subject 010 was noted to have anti-HLA antibodies on post-implantation day 98. Measured anti-HLA antibodies of CW1, CW8, DRB1*04:04, and DR16 were not antibodies with specificity against the HLA alleles of the donor cells. The subject denied additional transfusions or blood products post-implantation. A bystander immune response was ruled out when months 12 and 18 revealed similar anti-HLA antibody results. Upon analysis of these assays and patient report, it was concluded that the immunoreactivity present in the patient was not related to the stem cell treatment (Tables S4 and S5).

For all patients, no significant change in quality of life scores were observed. MR imaging for all patients demonstrated varying degrees of focal spinal cord malacia, with one patient having bony fragments within the spinal canal at T11 level (subject 001). No patient demonstrated immediate or delayed complications after the stem cell injections. There were no new areas of cord or soft-tissue edema, enhancement, development of swelling, or fluid collections on immediate post-procedural or follow-up imaging. Evaluation of the area of cord malacia and cell injection site was limited in all patients due to susceptibility artifact either from metallic or bony fragments from the previous injury or from the sequelae of the previous fusion hardware. No visible morphologic change was observed in the area of spinal cord malacia on either the pure anatomic or diffusion tensor sequences. In all four patients, diffusion tensor imaging (DTI) revealed a stable appearance of spinal cord tracts both at the injury site and rostral/caudal to the injury site but did not show extensive evidence of remodeling or improvement of tractography (Figure 4).

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<th>Table 1. Patient Characteristics</th>
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<td><strong>Age (mean/range)</strong></td>
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<td><strong>Gender</strong></td>
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<td><strong>Number of injections (mean)</strong></td>
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<td><strong>Hospital length of stay (mean/range)</strong></td>
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<td><strong>Time since implantation (mean)</strong></td>
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DISCUSSION

SCI represents a devastating neurological condition. Depending on the segmental level and completeness of spinal injury, the neurological deficit can clinically be presented as paraparesis or quadripareisis or fully developed paraplegia or quadriplegia. Current pharmacological or physical rehabilitation-based therapies for SCI are limited and primarily focused on modulating symptoms associated with chronic SCI such as pain (Gwak et al., 2016; Saulino and Averna, 2016) and/or muscle spasticity (Koulousakis and Kuchta, 2007; McIntyre et al., 2014). With the exception of nerve transfer procedures aimed at optimizing motor control in dermatomes just below the injury (Emamhadi et al., 2016; Medina et al., 2017; Ray et al., 2016; Simcock et al., 2017), at present no therapy exists that would lead to a clinically relevant

Figure 3. Brain Motor Control Assessment (BMCA) and EMG Assessments to Identify Voluntary or Reinforcement Maneuver-Initiated EMG Activity

(A and B) Recording of voluntary or reinforcement maneuver-triggered BMCA activity showed no detectable BMCA responses at 18 months after cell transplantation in subject 001 (4.4.2016 tracers).

(C and D) Subsequent recording performed at 27 months after transplantation showed the presence of EMG response in triceps surae after a reinforcement maneuver (D). No activity is noted with volitional attempts at plantar flexion or dorsiflexion of the left ankle in either study (C). Red arrowheads show new activity in triceps surae with a reinforcement maneuver (neck flexion and deep breath) compared to prior recording; horizontal black bar indicates onset marker.

(E and F) EMG recording from right T6 paraspinal muscle in subject 006. In comparison to baseline recordings, multiple new motor units (red arrowheads) were recorded at 12 months after cell transplantation.

(G and H) EMG recording from T6 and T7 paraspinal muscles in subject 010. In comparison to baseline (12 weeks) recording, a new motor unit (red arrowheads) was recorded at 6 and 18 months after cell grafting from T7 paraspinal muscle.
improvement of motor or sensory function in chronic spinal trauma patients.

Over the past 3 decades, a substantial number of experimental studies have tested the treatment potency of spinally grafted cells with the aim of restoring trauma-induced loss of function. Based on known pathophysiology of SCI characterized by (1) loss of segmental neurons at the site of injury, (2) demyelination of descending axons and associated loss of signal conductivity, and (3) decreased synthesis of neurotrophic factors and increased and long-lasting inflammation, the cell replacement therapies and a specific cell source used for treatment can be considered in three principal categories. First are studies that aim to restore local axonal myelin by implanting oligodendrocyte-type cells (Brustle et al., 1999; Keirstead et al., 2005; Nistor et al., 2005). Second, the implantation of neurotrophic factor-producing cells was employed. In these studies, for example, implantation of BDNF or NGF-producing fibroblasts has been shown to promote axonal sprouting and facilitate functional recovery in rat and primate models of SCI (Grill et al., 1997; Liu et al., 1999; Tuszynski et al., 2002). Third, grafting of neuron-producing neural precursors (such as the NSI-566 line used in our current phase I study) was extensively used. The primary goals in these studies aim at reconstituting regional neuronal pools at the spinal injury site in order to (1) restore local segmental neuronal circuitry by newly established synapses between grafted neurons and the remaining neurons of the host, and (2) to provide additional neuronal targets for re-establishment of new synaptic contacts between sprouting trauma-injured descending motor axons.

As previously described, spinal grafting of NSI-566 in naive rat or rat and non-human primate L3 compression, complete or hemi-transected spinal cord, and spinal ischemic models has resulted in a significant improvement of neurological function and suppression of spasticity-characterized morphologically by extensive axonal sprouting and development of synaptic contacts with the host neurons (Cizkova et al., 2007; Lu et al., 2012; Rosenzweig et al., 2018; van Gorp et al., 2013).

Several phase I clinical trials to test the safety of spinally grafted human cells of different germ origin in peri-acute or chronic spinal trauma patients were previously reported. First, immuno-activated, autologous, peripheral blood macrophages have been transplanted intraspinally into the spinal cords of acute SCI patients using a hand-held microsyringe (Lammertse et al., 2012). Second, Ferón et al. investigated the effect of olfactory ensheathing cells implantation and postulated that these cells may promote axonal regeneration. Dissociated and cultured cells from autologous olfactory mucosal tissues were transplanted into 12 chronic ASIA-A patients. Neither functional improvements nor adverse effects due to the treatment were noted at 3-year follow-up (Feron et al., 2005; Mackay-Sim et al., 2008). Third, autologous bone-marrow-derived mononuclear cells were injected intraspinally into the injury area of nine chronic ASIA-A patients (Deda et al., 2008). No complications were seen. Collectively, these initial clinical studies have established the feasibility of spinal cell delivery procedures in acute or chronic spinal trauma patients. Until now no human spinal-cord-derived neural precursors were tested in the clinical setting for treatment of SCI. The NSI-566 line is the only spinal-cord-derived human cell line recently FDA-authorized for testing spinal grafting in human patients.

From a safety perspective, there are several key points to note from our current study. (1) No surgery-related complications such as laminectomy site infection or CSF leakage was seen in any of the subjects. (2) Analysis of pain scores showed that patients experienced no spontaneous or evoked pain up to 18–27 months post-procedure. (3) MRI analysis showed no detectable post-cell injection inflammatory changes, swelling,

![Figure 4. MRI Cervical-Thoracic T2 Sagittal and DTI Sagittal Sequences](image-url)
or fluid accumulation (indicative of syrinx formation) at or around the cell injection site.

Regarding secondary outcomes, analysis of neurological ISNCSCI level, EMG, and/or BMCA showed potentially positive changes in three subjects. Subject 001 reliably showed previously unrecorded activation of motor units in the triceps surae on BMCA, 27 months after injection, with reinforcement maneuvers (but not voluntarily). This change in the pattern of the EMG response is most consistent with an increased central state of excitability compared to the baseline recording and may be attributable to new activity in reticular circuitry, potentially via a functional reconnection of supraspinal motor centers within spinal circuitry. This transforms the patient status from a “complete” to an “incomplete” SCI status. Potential alternative explanations include a significant difference in patient effort between the studies or development of spasticity since the baseline recordings. However, patient effort is closely monitored by the examiner, and spasticity giving this result would be unusual given the chronicity of injury. Furthermore, the same subject showed a two-level sensory and motor improvement (ISNCSCI score) measured between 3 and 6 months post-procedure. Subject 010 demonstrated new voluntary muscle activity one level inferiorly on the right (T7) at 6 months post-cell grafting (compared to baseline or 4 weeks) which was re-confirmed at 18 months. Using the ISNCSCI score, a one to two level of motor and sensory improvement was seen 6 months after the procedure, supporting the EMG findings, but recognizing the caveats of interrater variability, sampling error, and multilevel innervation with paraspinal EMG. In subject 006, EMG showed perhaps the most robust change, recording previously unrecorded muscle activity in the left T7 and T8 paraspinal muscles at 12 months post-procedure as well as the development of new intolerance to needle EMG between T6 and T9 at 18 months post-procedure. However, no change in overall neurological ISNCSCI level was seen in this subject. The small changes seen in the level of injury on the left side of subject 006 and the small change seen at 27 months in subject 001 likely reflect the difficulty in assessing the exact motor and sensory level in the midthoracic spine and may indicate that one level of improvement may be within the margin of error of these assessments at the midthoracic level. Of note, it may be expected that subclinical reinnervation would be detected by EMG initially, prior to any manifestation of clinical improvement. This is due to EMG being capable of detecting motor units that are of insufficient power to be of clinical significance, as well as the fact that motor neurons newly under central control might be ineffective until maturation of central motor programming. The presence of new intolerance seen at T6–T9 is suggestive of alteration in spinal processing of afferent...
sensory signals and can be related to peripheral sprouting of sensory afferents. Electrophysiologically defined improvement seen in three of four of our subjects may reflect several mechanisms including improved myelination and/or development of new synaptic contacts with the host neurons and descending motor tracts (Cizkova et al., 2007; Lu et al., 2012; Rosenzweig et al., 2018; van Gorp et al., 2013).

DTI was performed for research purposes. DTI enables both qualitative and quantitative assessment of the spinal cord (by measurement of the fractional anisotropy and apparent diffusion coefficient parameters). After functional disruption of the spinal cord in SCI, these tracts could be improved after stem cell implantation. No apparent evidence of remodeling or remyelination was seen in the DTI imaging in this study. However, the stable appearance of DTI imaging suggests that no further disruption took place post-transplantation. Imaging was performed on 1.5T MRI machines in this study. The wider use of 3T scanners, as well as the development of improved acquisition and post-processing techniques, should result in an increased role of spinal cord DTI in both research and clinical SCI practice (Rajasekaran et al., 2012; Sgsiadek et al., 2012). Lastly, as described in the STAR Methods, the surgical procedure was a significant technical advance for this study (Figure 5). The use of a “floating cannula” allowed accuracy of delivery without suspension of respiration and other facets of homeostasis, including blood pressure and other vital signs (Video S1). This setup is different as compared to other injection devices that are fixed to the operating room bed and require suspension of ventilation to ensure proper injection.

Whether or not a higher degree of synapse formation and corresponding functional improvement would be achieved once higher cell doses are employed for spinal grafting is not defined at present. In addition to showing safety and tolerability, the dose of NSCs utilized was based on the safe and well-tolerated dose used for ALS and showed proof-of-concept results in this trial that are suggestive of functional improvement, supporting a further FDA-approved study in our cervical spine cohort.

Despite these potentially encouraging secondary data seen in our current clinical study, we emphasize that the study was designed as a safety trial without statistical power or a control group needed to evaluate any functional change resulting from cell grafting. Nonetheless, some of the clinical data at late time points after cell delivery are certainly intriguing and merit further investigation.

Conclusion and Future Directions
Our current clinical data demonstrate that spinal grafting of the human NSI-566 cell line in chronic spinal trauma patients is safe with no detectable side effects identified at 18–27 months after cell delivery. Published studies in this complex arena are usually small. Despite the small sample size, the key strengths of the study are the extensive follow-up period and the timeline of treatment, as everyone was treated after 1 year of injury when there is little to no chance of spontaneous recovery. Like other phase I studies, this study was intended to provide proof of safety and tolerability and proof-of-concept data that will justify the next cohort. This favorable human safety profile, in conjunction with signs of potential efficacy signal and promising prior animal studies, warrants future dose escalation studies in patients with chronic SCI.

STAR★METHODS
Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION
Supplemental Information includes six tables, further methods (inclusion/exclusion criteria and study visit protocols), and one video and can be found with this article online at https://doi.org/10.1016/j.stem.2018.05.014.

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AUTHOR CONTRIBUTIONS

DECLARATION OF INTERESTS
The authors declare no competing interests.

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REFERENCES


CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Joseph Ciacci (jciacci@ucsd.edu). Cell source requests should be directed to and will be fulfilled by Karl Johe, Ph.D. (Neuralstem Inc., kjohe@neuralstem.com).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Animals Safety Study in Immunodeficient Spinally Injured Rats

Animal welfare: The study design and animal usage were reviewed and approved by the Institutional Animal Care and Use Committee (MC 0071, UCSD, 9500 Gilman Dr., La Jolla, CA). Animal welfare for this study was in compliance with the U.S. Department of Agriculture’s (USDA) Animal Welfare Act (CFR 9 - Parts 1, 2, and 3), and the Guide for the Care and Use of Laboratory Animals. Animals: Rats, Athymic Nude (rnu-/rnu-) - Hsd: RH-Foxn1rnu (Harlan, Madison, WI, USA) were used. A total of 90 male rats were enrolled, of which 8 were used for descending motor tracts labeling (Figure 1A). Animal ages at injury ranged from 10 to 13 weeks, and body weights varied between 309 to 345 g.

Animal housing: Animals were single-housed in cages with irradiated bedding. Acclimation times prior to the experiment varied but were at least 8 days. Environmental controls were set to maintain temperatures from 64° to 79°F (18° to 26°C), with fluorescent lighting on a 12 hour/12 hour on/off inverted cycle. Animals were fed irradiated Harlan Teklad Global 18% Protein Rodent Diet T.2918.15 (Harlan Laboratories, Placentia, CA) ad libitum. In addition, animals that were selected for addition behavioral testing (see below) also received Fruit Crunchies (BioServ product # S05798-1) and HydroGel (ClearH2O®, ME 04101). Animals were provided with purified water (Mountain Dairy, Riverside, CA, USA) ad libitum.

Clinical Trial Design and Patient Selection

Inclusion and exclusion criteria are listed in Methods S1. This was a Phase I safety study of human spinal cord-derived neural stem cell transplantation for the treatment of chronic spinal cord injury, defined as at least one year but no more than 2 years after traumatic SCI. A sample of 4 subjects with chronic SCI classified as AISA-A, a motor and sensory complete SCI, levels T2-T12, who met eligibility criteria were enrolled. No control group was included. Inclusion and exclusion criteria are listed in Methods S1. All subjects received spinal cord injections of human spinal cord derived neural stem cells (NSI-566). The trial was registered in ClinicalTrials.gov as NCT 01772810. IRB approval granted by UCSD Health Center, Human Research Protections Program (HRPP), 9452 Medical Center Drive, La Jolla, CA 92037

NSI-566 Neural Stem Cell Line

NSI-566, is a human spinal cord-derived neural stem cell line. Neural stem cells are the precursor cells present in the neuro-epithelium along the neuraxis during mammalian fetal development. NSI-566 was derived from a single post-mortem spinal cord
of an 8-week gestational age fetus. This tissue was obtained in compliance with the National Institutes of Health (NIH) and Food and Drug Administration (FDA) Good Tissue Practice Guidelines, and under a protocol approved by an outside independent review board. Neural stem cells were isolated by dissociating a single piece of spinal cord tissue of lower cervical/upper thoracic region and expanding it as a single line. Three-tiered cell banks, consisting of a master cell bank (MCB), a working cell bank (WCB), and a clinical cell bank (CCB), were established and cryopreserved under cGMP. The current MCB is > 99% nestin-positive, adherent neural stem cells established at passage 6. A WCB at passage 9 was manufactured from this MCB, and a CCB at passage 12 was manufactured from the WCB. All cell doses used in this study were prepared from the passage 12 CCB. Karyology of the clinical lot cells showed normal 44, X, and Y chromosomes. Greater details of the cGMP generation of clinical material was previously described (Glass et al., 2012).

For cell administration, NSI-566 was provided as a live-cell suspension, ready-to-inject, formulation, requiring no further manipulation. Briefly, NSI-566 cell suspension was prepared one day prior to each scheduled surgery at a cGMP facility. One or more vials of the cryopreserved CCB were thawed at once, washed of the freezing medium by repeated centrifugation in a hibernation medium (HM), and concentrated to a final concentration of 2x10⁶ cells/mL of HM. This target concentration had been established for being safe and adequate for intraspinal injections by series of preclinical (Usvald et al., 2010; van Gorp et al., 2013) and clinical studies ((Glass et al., 2012; Glass et al., 2016). The HM is a sterile, buffer solution free of preservatives and antibiotics developed for safe and adequate for intraspinal injections by series of preclinical (Usvald et al., 2010; van Gorp et al., 2013) and clinical studies ((Glass et al., 2012; Glass et al., 2016). The HM is a sterile, buffer solution free of preservatives and antibiotics developed for being safe and adequate for intraspinal injections by series of preclinical (Usvald et al., 2010; van Gorp et al., 2013) and clinical studies ((Glass et al., 2012; Glass et al., 2016). The HM is a sterile, buffer solution free of preservatives and antibiotics developed for being safe and adequate for intraspinal injections by series of preclinical (Usvald et al., 2010; van Gorp et al., 2013) and clinical studies ((Glass et al., 2012; Glass et al., 2016). The HM is a sterile, buffer solution free of preservatives and antibiotics developed for being safe and adequate for intraspinal injections by series of preclinical (Usvald et al., 2010; van Gorp et al., 2013) and clinical studies ((Glass et al., 2012; Glass et al., 2016). The HM is a sterile, buffer solution free of preservatives and antibiotics developed for being safe and adequate for intraspinal injections by series of preclinical (Usvald et al., 2010; van Gorp et al., 2013) and clinical studies ((Glass et al., 2012; Glass et al., 2016). The HM is a sterile, buffer solution free of preservatives and antibiotics developed for being safe and adequate for intraspinal injections by series of preclinical (Usvald et al., 2010; van Gorp et al., 2013) and clinical studies ((Glass et al., 2012; Glass et al., 2016). The HM is a sterile, buffer solution free of preservatives and antibiotics developed for being safe and adequate for intraspinal injections by series of preclinical (Usvald et al., 2010; van Gorp et al., 2013) and clinical studies ((Glass et al., 2012; Glass et al., 2016). The HM is a sterile, buffer solution free of preservatives and antibiotics developed for being safe and adequate for intraspinal injections by series of preclinical (Usvald et al., 2010; van Gorp et al., 2013) and clinical studies ((Glass et al., 2012; Glass et al., 2016). The HM is a sterile, buffer solution free of preservatives and antibiotics developed for being safe and adequate for intraspinal injections by series of preclinical (Usvald et al., 2010; van Gorp et al., 2013) and clinical studies ((Glass et al., 2012; Glass et al., 2016). The HM is a sterile, buffer solution free of preservatives and antibiotics developed for being safe and adequate for intraspinal injections by series of preclinical (Usvald et al., 2010; van Gorp et al., 2013) and clinical studies ((Glass et al., 2012; Glass et al., 2016). The HM is a sterile, buffer solution free of preservatives and antibiotics developed for being safe and adequate for intras
(up to 30 days post injury). The day prior to grafting the animals were anesthetized to remove any remaining skin sutures and to shave the back of the animals. The spinal cord was re-exposed. The dorsal aspect of the vertebra immediately caudal to the existing laminectomy was removed using the dental drill. Special care was taken to keep the dura intact. For the current safety assessment, a maximum feasible cell dose was estimated by several preliminary dose-range finding studies using subcutaneous and intraspinal delivery. The cell suspension (Group A) or vehicle/hibernation buffer-only (Group B) was injected as follows:

1) 20 injections peripheral from the injury epicenter (1 µL each; 2 µL/min) were made bilaterally into about 2 segments above and/or below the injury epicenter at 1mm intervals.

2) 5 injections (5 µL each; 2 µL/min) were injected around the borders of injury epicenter.

A total of 25 injections were made in each spinal cord, resulting in a total of 45 µL (cells suspended at 10⁴ cells/µL, resulting in a total of ~4.5 x 10⁶ cells).

The required volume of dosing for completing all injections of one animal was drawn into a 100 µL Nanofil syringe (World Precision Instruments) with a 33-gauge needle (World Precision Instruments, Cat# NF33BV). The syringe was mounted onto a manually controlled syringe holder/injector (David Kopf, Model 5000+5001) attached to the stereotaxic frame. The needle tip was lowered to a depth of 1-1.5 mm from the pial surface at the Dorsal Root Entry Zone (DREZ) into the spinal parenchyma, and the injection was made. After a thirty second pause, the needle was gently drawn out of the spinal cord. The syringe/needle ensemble was cleaned to a depth of 1-1.5 mm from the pial surface at the DREZ into the spinal parenchyma, and the injection was completed.

**GFP labeling of descending motor tracts:** A small group of SCI animals (n = 8; 4 with cell treatment and 4 vehicle-injected), was used to study the innervation of grafted cells by descending motor axons of the host. To label descending motor tracts animals were anesthetized and received four brain injections of recombinant Adeno-Associated Virus (AAV) engineered to express Green Fluorescent Protein under the human synaptophysin promoter (AAV.synGFP; neuronal specific expression). Bilaterally injected vectors were targeted into motor cortex and nucleus ruber. Stereotaxic coordinates used for the injections were: motor cortex: bregma − 2.0mm, lateral 3.0mm, depth 1.5 mm, red nucleus: bregma −5.8, lateral 0.8mm, depth 7.1 mm. Each injection consisted of 5 µL of the virus suspension at 10¹⁴ gc/mL and was injected over 5 min period using 32G stainless steel needle. Vector was injected 4-6 weeks before sacrifice.

**Post-surgical care:** All animals received Sulfamethoxazole and Trimethoprim oral suspension, (USP 200 mg/40 mg per 5 mL) in the water (5 mL per 250 mL) for 1-2 days before, up to 30 days after initial contusion, and as needed in case of infections. Animals were checked for bladder retention at least two times daily for the duration of the study. If full, bladders were emptied by manual expression. Consequently, the surgical area was checked. Ketoprofen (4.0 mg/kg, s.c.) was given approximately one hour prior to surgery and approximately every 24 hours for up to 48 hours. Animals found ill were given up to 5 mL (s.c. ≤ 2 daily) Lactated Ringer’s solution until improvement was observed. Hexa-Caine Spray and/or Bicatrin/Neomycin/Polymyxin triple antibiotic ointment was used to treat scabs or wounds.

**Cells:** The cells, named “NSI-566RSC,” were produced by Neuralstem Inc. (Rockville, MD, USA), as described before (Johe et al., 1996). The grafted cells were a clinical lot (Lot No. CRL471925-3, passage 12), a human neural stem cell line derived from fetal spinal cord of 8 gestational weeks. They were suspended in hibernation buffer at 10⁴ cells/mL and was injected over 5 min period using 32G stainless steel needle. Vector was injected 4-6 weeks before sacrifice.

**Activity assessment in an open field:** One week prior to the beginning of the test trials, animals were adapted to the apparatus/arenas (76 x 152 x 50 cm; W x L x H) for five minutes on each day. After habituation, the animals were placed in the center of a clean arena and tracked by a video tracking system under dark conditions (night/active portion of their diurnal rhythm), over a period of three hours. The distance traveled and a number of various movement variables was assessed (EthoVision, Noldus Technology, the Netherlands). Three separate observation periods were evaluated. Each observation period was separated by at least 24 hours. During testing, animals had access to Hydrogel and food pellets.

**Beam walk:** Rats were trained to traverse elevated (1 m) narrow beams (3, 2.5, and 2 x 200 inches; W x L) toward a darkened goal box (10 x 10 x 10 inches). Only motor impaired animals would produce foot faults (slips from beam), which number than represent a
motor deficit. Bright light and white noise were produced near the start to promote beam crossing. Rats were allowed to fall from the beam (onto a container with padding). For training, each rat was placed in goal box for two minutes and afterward placed on the balance beam for 1 minute per trial. The rat was considered trained when it was able to remain on the beam for three consecutive trials. Mean scores out of two trials for each beam were used (i.e., right and left hind paw foot faults and falls). The animal was allowed to take ~5 min to complete a crossing. In between runs an animal was placed in their home cage for ≥ 15 s. If an animal was able to cross a beam for at least two thirds, it was also tested on the narrower beam (i.e., 2.5 or 2 inch).

**Catwalk gait analysis:** The CatWalk apparatus (CatWalk 7.1, Noldus Technology, the Netherlands) was used to quantify gait parameters by footprint analysis during walkway crossings. Animals walk down a horizontal glass walkway (109 × 15 × 0.6 cm; L × W × H), of which the glass is illuminated along the long edge. The light only enters the (side of the) glass and reflects merely internally (when the glass is bordered by air). As an animal crosses the walkway, light reflects off of the animal’s paws, producing a series of bright footprints when viewed through the glass, which are recorded by a video camera. Hence, testing was performed in a darkened room. Walkway crossing was stimulated by rewarding the animal with little Fruit Crunchies, placed at the end of the walkway. In conjunction, animals were further motivated by food restriction prior to training/testing. Hence, food was removed from their cages approximately 18 hours before testing (and returned after training/testing). In addition, the following criteria concerning walkway crossings needed to be met: (1) the animal needed to walk uninterrupted across the walkway, at a constant pace, and (2) a minimum of three such crossings per animal were required. Data from three proper crossings was averaged for statistical analysis.

**Sensory function assessment (mechanical and thermal):** In this test, pain thresholds for a supraspinal response (vocalization or escape behavior) to a below-level evoked mechanical or thermal stimulus were assessed. The animals were habituated to the set-up, test room, and investigator for one week (no responses were elicited), twice daily for five minutes, prior to testing. The investigator held the animal in an upright position, fixed the test hindpaw, and then the hindpaw was stimulated. When a response was elicited, the stimulus was stopped immediately and the maximal pressure elicited was recorded. Both hind paws were tested for four times while alternating between paws, with at least a 1-hour interval between trials. The first scores of each paw were removed.

The mechanical stimulus was created using a rigid tip mounted on a pressure transducer (IITC life sciences Electronic von Frey Anesthesiometer, Cat# 2393, Woodland Hills, CA), which was operated by a second investigator (SVG). The stimulus was applied on the dorsal side of a hind paw. Compressing the paw of the animal with the tip on the pressure transducer at the distal metatarsal or metacarpal area in a gradual incremental fashion (paw rests on table surface).

For eliciting the thermal stimulus an infrared beam apparatus (Ugo Basile, Cat# 37360, Collegeville, PA, USA) was used. For testing, the investigator held the animal’s plantar side of a hind paw over the infrared light beam mounted in the apparatus.

**Necropsy:** The post-grafting survival period varied between 272-274 days (~10 months). Animals were sacrificed by 2 mg pentobarbital and 0.25mg phenytoin (0.5mL of Beuthanasia-D, InterVet/Schering-Plough Animal Health Corp., Union, NJ, USA) followed by transcardiac perfusion of saline, trailed by 4% paraformaldehyde (phosphate buffered). The central nervous system tissue was dissected out and preserved in 4% paraformaldehyde. The following tissues were also taken out and placed in 10% formalin: skeletal muscle (thigh), skin, adrenals, aorta, rectum, cecum, colon, duodenum, esophagus, epididymides, eyes with optic nerves, femur, Harderian gland, heart, ileum, jejunum, stomach, kidneys, spleen, liver, lung with bronchi, mesenteric lymph node, mammary gland, pancreas, thyroid, thymus with parathyroid glands, trachea, pituitary, prostate, mandibular salivary glands, tongue, sciatic nerve, testes, seminal vesicles, and bladder.

Animals found clinically ill and moribund were euthanized, per protocol. On these animals, and all animals found dead, a gross necropsy was conducted (i.e., external surfaces and orifices, musculoskeletal system, cranial cavity (and brain), neck with associated tissues, and thoracic, abdominal and pelvic cavities (incl. associated organs and tissues).

At necropsy, any tissue with discoloration, lesion, necrosis, distension, malformation, and/or suspected tumor/mass were sent for external histopathological evaluations by a board-certified veterinary pathologist (J.E. Sagartz DVM PhD DACVP, Seventh Wave Laboratories LLC, Chesterfield, MO), as were the histological sections of the central nervous system. Evaluation was performed in a manner blinded to treatment nature. Severity grades for pathology diagnoses were based on a 4-point scale as follows: Grade 1 = minimal, Grade 2 = moderate, Grade 3 = marked and Grade 4 = severe. After completion of pathology analysis, the pathologist was unblinded and the final conclusion was made.

**Central nervous system histology:** Each cord was photographed against a ruler (cm) along with its identification number. The cords were bisected into approximately equal rostral and caudal pieces and embedded horizontally into a gelatin block (6.5 × 4.5 × 2.5 cm L × W × H). Spinal cords from 16 - 20 animals were embedded in 4 horizontal layers in a block 1. The rostral pieces were placed in the first and third layers and the caudal pieces were in the second and fourth layers, with the ventral side facing down. The layers were separated by ± 0.4 cm of gelatine. The cords (columns) were separated by ± 0.2 cm from each other. After solidification of the gelatin block, it was placed in 30% sucrose in 4% formaldehyde for 48 hours. Then, it was frozen in cold 2-methylbutane using dry ice, cryostat sectioned horizontally (40µm thickness), and mounted with intervals of 12 sections. Whole brains were also embedded into gelatin blocks, cryostat sectioned coronally at 40 µm thickness. All stained slides were sent to a Board-certified veterinary pathologist for evaluation (J.E. Sagartz DVM PhD DACVP, Seventh Wave Laboratories LLC, Chesterfield, MO). For histopathological stainings every 24th section was used. The spinal cords used to assess the neuronal integration (see below) were not gelatin embedded but were frozen directly (after 48h in 30% sucrose) using isopentane (~80°C), mounted in OCT compound (Tissue-Tek®) and cryostat sectioned at 30 µm in the horizontal, sagittal, or transverse plane, and collected for free-floating immunofluorescence staining.
Previously been used successfully in ALS trials with the same cell line (Glass et al., 2012; Tadesse et al., 2014). This included the use of
All 4 subjects were initiated and maintained for 12 weeks on a combination cocktail of immunosuppressive (IS) regimen that had pre-
Immunosuppression
post-anesthesia care unit, followed by recovering in an intermediate level care unit of the acute care hospital.

fashion, and the posterior spinal fascia and skin closed in meticulous layers. Subjects were then extubated and recovered in a
the injection procedure was repeated. At this time once all injections had been completed the dura was then closed in a watertight
was then injected using the syringe pump at flow rate of 5.0
were used. Tacrolimus and mycophenolate mofetil were withdrawn after 12 weeks post-transplantation. The dose of both medications was
Day 8 increased to 500mg in the morning and 1 g at night, and on post-transplant Day 15 increased to 1 g twice a day. In all 4 subjects
scheduled visits thereafter. Mycophenolate mofetil was started on post-transplant Day 1. It was initially dosed at 0.1 mg/kg/day divided every 12 hours by mouth (PO). Trough levels

Surgical and Cell-Grafting Procedure

The intervention included placing an anesthetized subject in the prone position and sterile processing of the associated surgical trial materials. An approximately 10 - 15 cm incision was performed in the dorsal midline and a bilateral laminectomy performed over the injured spinal cord segments. All prior fusion hardware was removed during the surgical procedure to allow for optimum serial magnetic resonance imaging (MRI).

Following laminectomy an incision of approximately 2-4 cm was made in the dura, which was then tackled up, allowing exposure of the spinal cord. The stereotaxic injection platform was then attached to 4 percutaneous posts through an approximately 1cm skin incision immediately above and below the laminectomy site (Figure 5E), (Tadesse et al., 2014). The injection device consisted of a Z-drive holding a 30-gauge beveled needle in perpendicular position over the exposed spinal cord. The top end of the needle was attached to tubing which was attached to a microprocessor-controlled syringe pump. The syringe is back-filled with mineral oil to eliminate air and to create an immiscible barrier against aqueous solution in the syringe. The syringe plunger is inserted into the syringe, pushed toward the end, and attached to the drive spindle of the injection pump. Separately, the injection cannula is manually filled with sterile injectable saline in order to eliminate air. The saline-filled cannula is attached to the Hamilton syringe. Using the injection pump in reverse, a small air space is created at the cannula tip to prevent mixing of the cell suspension with the saline. Using the injection pump in reverse, the cannula is loaded with the required volume of the cell suspension. The capacity of the cannula is at least 500 µL so that the injection volume never touches the tip of the syringe. Prior to initiating spinal cord injections, a 5 µL bolus (1 injection) is ejected under direct vision of the surgeon to ensure that the system is open and unobstructed. After the needle is inserted into the spinal cord, the guide sheath is retracted, converting the cannula into a “floating cannula.” This feature allows for accuracy of delivery without suspension of respiration and other facets of homeostasis, including blood pressure and other vital signs. This setup is different as compared to other injection devices that are fixed to the operating room bed and require suspension of ventilation to ensure proper injection. Bilateral injection positions were determined by preoperative MRI and target approximately 1mm lateral to the rim of the remaining tissue bordering the injury site. The needle was lowered into the spinal cord to the depth of approximately 4 mm from the pial surface (Figures 5D and 5F). The cell suspension was then injected using the syringe pump at flow rate of 5.0 µL per min for a period of 2 minutes. The needle was left in place for 1 min after injection and then slowly pulled out of the cord, advanced to the next position along the cord avoiding visible blood vessels and the injection procedure was repeated. At this time once all injections had been completed the dura was then closed in a watertight fashion, and the posterior spinal fascia and skin closed in meticulous layers. Subjects were then extubated and recovered in a post-anesthesia care unit, followed by recovering in an intermediate level care unit of the acute care hospital.

Immunosuppression

All 4 subjects were initiated and maintained for 12 weeks on a combination cocktail of immunosuppressive (IS) regimen that had previously been used successfully in ALS trials with the same cell line (Glass et al., 2012; Tadesse et al., 2014). This included the use of three separate medications. Basiliximab (Simulect) 20 mg intravenous (IV) administered within 2 hours prior to transplantation surgery. A second dose of 20 mg was given on postoperative Day 3 or 4. No additional doses of Basiliximab were used. Tacrolimus was started on post-transplant Day 1. It was initially dosed at 0.1 mg/kg/day divided every 12 hours by mouth (PO). Trough levels were measured while the subjects were hospitalized and the dose of tacrolimus was adjusted as necessary to ensure maintenance of a trough serum level of 4-8 ng/ml. Following discharge, trough levels were measured at the 2-week post-operative visit and at scheduled visits thereafter. Mycophenolate mofetil was started on post-transplant Day 1 at 500 mg twice a day, on post-transplant Day 8 increased to 500mg in the morning and 1 g at night, and on post-transplant Day 15 increased to 1 g twice a day. In all 4 subjects Tacrolimus and mycophenolate mofetil were withdrawn after 12 weeks post-transplantation. The dose of both medications was reduced by half at Week 13 and by another half at Week 14, followed by complete cessation at Week 15. Presence of antibodies against donor HLA’s were monitored during this period and at scheduled intervals thereafter. Because assessment of rejection is an efficacy metric rather than a safety metric, more comprehensive immunological workup thoroughly assessed in the Phase 2 study, including complement and interleukin levels. Changes in MRI intensity at the cell transplant area were also monitored before and after the IS withdrawal. All subjects tolerated the immunosuppressive regimen well. Subjects were monitored on a nearly weekly basis per our study protocol. See Methods S2.
Outcome Measures
Subjects were assessed for adverse events including pain and infection, motor function, and quality of life. Additional secondary outcome assessments were made to measure any postoperative changes from baseline in neurologic deficits, neurophysiology, imaging studies, bladder and bowel function, allodynia and neuropathic pain. ISNC-SCI (International Standards for the Neurological Classification of Spinal Cord Injury) examination was used to monitor neurologic deficits. Neurophysiological changes were monitored when feasible by needle electromyography (EMG) and/or surface poly-electromyography (Brain Motor Control Assessment (BMCA) (Lee et al., 2004; Sherwood et al., 1996). Imaging studies were done by standard 1.5T MRI for safety monitoring. Diffusion tensor imaging (DTI) imaging of the spinal cord was performed for longitudinal experimental studies (1.5T, TR 2500-5000ms, TE 64-95ms, slice thickness 3.5-4 mm, FA 90, DFOV 190-340, NSA 1-8). Bladder and Bowel Function and Pain and Allodynia questionnaires were administered. Quality of life was assessed by Functional Independence Measure (FIM) and Spinal Cord Independence Measure (SCIM) questionnaires. Subjects were followed postoperatively at 2 weeks, monthly for 6 months and at every 6 months thereafter in post-study safety are planned to be followed up for total 60 months post stem cell treatment. Patients did not receive any additional rehabilitation beyond their routine outpatient physical and occupational therapy.

Study Oversight
An independent Data Safety Monitoring Board (DSMB) was convened at approximately 4-week intervals to review the available safety data. The DSMB was charged with making specific recommendations regarding study continuation. It had not identified any safety issues which precluded continuation of the study.

Quantification and Statistical Analysis
For pre-clinical animal studies, results were analyzed using ANOVA (one-way, or two-way group x time repeated-measures, and using a fixed-effect model), with a Bonferroni post hoc test for multiple comparisons. If the unequal variances were observed (Bartlett’s test), the Kruskal-Wallis test with Dunn’s post hoc comparisons were used. To analyze differences between the two groups, we used Student’s t tests or Mann-Whitney test (Non-parametric), or a repeated-measures ANOVA (when appropriate). All statistical analyses were performed two-tailed, and a p value of 0.05 was considered significant. Between-group variations are reported as cell-injected versus vehicle-injected.

Additional Resources
The trial was registered in ClinicalTrials.gov as NCT 01772810.