JRRD Volume 46, Number 1, 2009
Pages 145–166
Journal of Rehabilitation Research & Development

Clinical feasibility for cell therapy using human neuronal cell line to treat neuropathic behavioral hypersensitivity following spinal cord injury in rats

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Abstract—Management of neuropathic pain remains problematic; however, cell therapy to treat the effects of pain on the sensory system after spinal cord injury (SCI) could be a useful approach. Since many clinical trials ultimately do not succeed, use of cell therapy will require that safety and efficacy issues be addressed early in preclinical rat studies. We used the human neuronal cell line hNT2.17, which secretes the inhibitory neurotransmitters gamma-aminobutyric acid and glycine, in an excitotoxic SCI pain model after intraspinal injection of quisqualic acid into rats. One week after lumbar transplant of these cells, behavioral hypersensitivity was permanently reversed. Antinociceptive grafts displayed an optimal transplant time that included moderate effectiveness with chronic SCI and late graft placement and that required a minimal course of cyclosporine A 2 weeks after transplant for durable reversal of painlike behaviors. In addition, grafts did not need to be placed near the SCI level to be effective. These data suggest not only that these cells are safe and efficacious but also that they could be an effective clinical tool for treating SCI-associated neuropathic pain.

Key words: cell line, CsA, GABA, glycine, immunosuppression, inhibitory neurotransmitter, intrathecal, neuronal, preclinical, rehabilitation, NT2.

INTRODUCTION

Treatment of sensory neuropathies that result in chronic pain, whether inherited [1] or caused by trauma [2], the progress of diabetes [3], or other disease states [4], is one of the most difficult problems in modern clinical practice. The prevalence of sensory neuropathies has been conservatively estimated at 0.6 percent of the U.S. population [5]. To the extent that low-back pain is sometimes neuropathic, the actual figure might be far higher. Neuropathic pain might realistically affect 1.5 percent of

Abbreviations: BrdU = bromodeoxyuridine, CA = cutaneous tactile allodynia, CMF-HBSS = Ca^{2+}- and Mg^{2+}-free HBSS, CNS = central nervous system, CsA = cyclosporine A, CSF = cerebrospinal fluid, DAPI = 4′6-diamidino-2-phenylindol-2HCl, DMEM = Dulbecco's Modified Eagle Medium, DNA = deoxyribonucleic acid, DPBS = Dulbecco's Phosphate Buffered saline, EDTA = ethylenediaminetetra-acetic acid, FBS = fetal bovine serum, FDA = Food and Drug Administration, FGF = fibroblast growth factor, GABA = γ-aminobutyric acid, HBSS = Hank's Buffered Salt Solution, HCl = hydrochloric acid, HG = high-glucose, IND = investigational new device, IT = intrathecal, L = lumbar, NGS = normal goat serum, NSE = neuron-specific enolase, NT2 = NTera2cl.D/l, NuMA = nuclear matrix antigen, PBS = phosphate buffered saline, POC = proof-of-concept, PS = penicillin-streptomycin, QUIS = quisqualic acid, RA = retinoic acid, SCI = spinal cord injury, SEM = standard error of the mean, T = thoracic, TH = thermal hyperalgesia.

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DOI:10.1682/JRRD.2008.02.0018

the total population nationwide, and current pharmacological treatments have often proven ineffective or must be used at impractical dose levels, such as with morphine or its analogues [6]. Neuropathic pain is the most common type of chronic pain with spinal cord injury (SCI) and results from the abnormal processing of sensory input as a result of damage to the nervous system [7]. Identifying a specific stimulus or cause of neuropathic pain, which is notoriously unresponsive to conventional methods of pain treatment, is often difficult [8]. SCI pain (also called central, dysesthetic, or diffuse pain) is neuropathic pain at or below the level of injury and is often diffuse and poorly localized. The onset of SCI pain is usually weeks to months after injury [9–10]. Few research studies have examined treatments for SCI pain [11], and so far, few have shown any drug to be effective for a significant number of people. The most used, recently studied, and partially successful drug in animal [12–13] and human studies [14–15] is gabapentin, a calcium channel alpha2-delta ligand. But, a recent survey found that few patients continue to use this frequently prescribed agent [16]. Even so, individuals with SCI have found pain relief, sometimes from a combination of drugs and sometimes from drugs in combination with physical therapy or other treatment modalities. Cell therapy to release antinociceptive agents near the injured spinal cord to provide ongoing pain relief would be a logical next step in the development of treatment modalities [17–18]. But few clinical trials, especially for chronic pain, have tested the transplant of cells or a cell line to treat human disease [19].

Centrally induced excitotoxic SCI in an animal model has recently been developed as a model of neuropathic pain. Intraspinal injection of quisqualic acid (QUIS), a mixed AMPA (alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid)/metabotropic receptor agonist, produces injury with pathological characteristics similar to those associated with ischemic and traumatic SCI [20]. The pathological changes after QUIS injection include neuronal loss, demyelination, cavitation, glial responses, perivascular changes, breakdown of blood-brain barrier, and inflammation [21]. In addition, significant mechanical allodynia and thermal hyperalgesia, or behavioral hypersensitivity induced by the SCI, are important behavioral components. These behaviors indicate altered sensory function and/or pain similar to that reported after SCI. After spinal transplantation of primary adrenal tissue grafts following QUIS injections, behavioral hypersensitivity, including hypersensitivity to mechanical stimuli, was significantly reduced [22]. These results suggest that some sort of intrathecal (IT) cellular transplant would be a meaningful therapeutic strategy for the large proportion of SCI patients reporting intractable neuropathic pain [8].

Cell therapy to treat chronic pain, such as with adrenal chromaffin tissue, was conceived [23], demonstrated through multiple animal studies [24], and then tested clinically [25]. That therapy with adrenal chromaffin tissue was finally discontinued may illustrate the need for thorough preclinical testing before clinical trials with a cell therapy approach. Certain reports contend that this chromaffin cell therapy approach would have had problems clinically [26] with or without the clinically tested microencapsulation [27] that surrounded the grafts to prevent immune rejection [28]. These reports are in direct contrast to an extensive history of success with chromaffin cell therapy for pain in other animal studies [22,29]. These discrepant results suggest that translational research needs to be considered in order for such cell therapy to be successful [30].

The Food and Drug Administration (FDA) pathway for moving a basic research discovery toward testing with clinical trials includes a variety of steps. Beyond publication of the first peer-reviewed proof-of-concept (POC) study and initial discussions with the FDA to obtain investigational new device (IND) approval, many further POC studies can be implemented early in the process to support the feasibility of a clinical trial, such as (1) characterization of the cells in vitro and the in vivo graft phenotype; (2) safety of the graft transplanted in the naïve and injured animal; (3) correlation of a dose response curve with behavioral outcomes; (4) graft timing to duplicate efficacy acutely and chronically, including issues of graft survival over long periods; (5) examination of immunosuppression regimens to minimize the requirements for toxic immunosuppressive agents that support graft survival; and (6) examination of graft location to pinpoint the best placement of functional grafts. In addition to the POC studies, a second large animal study could be examined for safety (if not efficacy) of transplant. Unspoken here are topics such as appropriate control data, independent replication studies, reduction of possible investigator bias, and any procedural variations that should be tested to ensure reproducibility and integrity of data [30]. All are the minimum peer-reviewed evidence that should follow the scientific standards for

reliability of safety and efficacy before the considerable expense of second-level studies that adhere to current FDA good medical practice and current good laboratory practice regulations and before IND application and approval and clinical trials (phases I [safety], II [efficacy], and III [applicability]) for a final product license. Pre-IND discussions with the FDA determine appropriate POC data and guide the highly regulated second-level studies and initial clinical trial design and implementation.

Toward these ends, we have recently described the isolation, characterization, and preliminary use of a unique human neuronal inhibitory cell line, hNT2.17, a derivative of the human NTera2cl.D/l (NT2) cell line parentage, to treat neuropathic pain [31] in an animal SCI model. A phenotype abundantly present within the NT2 population synthesizes the inhibitory neurotransmitter γ-aminobutyric acid (GABA) [32]. From the variety of cell phenotypes expressed after differentiation from the NT2 cell line, we sought to subclone a human neuronal cell line from the NT2 heritage that was specific to the synthesis and secretion of GABA, characterize these cells in vitro, and test cell therapy with them in an SCI pain model. In this article, we describe the effects of IT grafted hNT2.17 cells in a model of excitotoxic SCI pain and test their optimal graft timing, immunosuppression regimen, and spinal IT location for attenuation of the behavioral hypersensitivity that accompanies SCI. Each study forms a subset of POC data that can be examined by the FDA and illustrates how such first-level studies can better ensure the clinical feasibility of treating SCI neuropathic pain with such a cell-based approach.

METHODS

Development of Human hNT2.17 Cell Line

We subcloned a human neuronal cell line from the parental NT2 cell line [33] by serial dilution and analyzed multiple subclonal cell lines by using a variety of immunohistochemical markers, including GABA, to determine the differentiated neurotransmitter phenotype of the various clones. We took advantage of a rapid aggregation method [34] for retinoic acid (RA) treatment and differentiation into the human neuronal phenotype to select cell lines. Although we derived a number of human NT2 neurotransmitter cell lines by these methods, we used the hNT2.17 cell line for further characterization and transplant in SCI pain.

The rapid aggregation method [34] for RA treatment and differentiation was also used to prepare cultures of differentiated hNT2.17 cells in vitro for characterization and transplant. Briefly, proliferating cultures of hNT2.17 cells were grown to near confluence at 37 °C in proliferation medium: Dulbecco's Modified Eagle Medium (DMEM)/Ham's F12 (GIBCO, Invitrogen Corporation; Carlsbad, California)/10 percent fetal bovine serum (FBS) (HyClone; Logan, Utah)/2 mM L-glutamine (GIBCO) freshly added/1 percent penicillin-streptomycin (PS) (GIBCO), with a media change every 3 days. When cells were near 100 percent confluent, they were replated to a 100 mm petri dish (VWR; West Chester, Pennsylvania) in DMEM/high-glucose (HG)/10 percent FBS/10 μM all-trans RA (Sigma; St. Louis, Missouri)/15 mM HEPES (N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid), pH 8.0/2 mM L-glutamine/1 percent PS, and continued for 2 weeks, with a media change every 2 days. After removal of the cells with 0.5 mM ethylenediaminetetra-acetic acid (EDTA), centrifugation, and resuspension, cells were replated to 100 mm tissue culture dishes (BD Falcon; San Jose, California) that had been coated with mouse laminin (Biomedical Technologies, Stoughton, Massachusetts; 20 μg/mL in Dulbecco's Phosphate Buffered Saline [DPBS]/poly-L-lysine, Sigma; 20 μg/mL in phosphate buffered saline [PBS]). They were then continued in DMEM/HG/5 percent FBS/1 percent PS/L-glutamine, 2 mM, at a pH of 7.4, for 9 to 24 h, before the addition of cytosine-D-arabinofuranoside (Sigma; 1 μM), plus uridine (Sigma; 10 μM) for nonneuronal growth inhibition. After 7 days, cells were briefly exposed to warmed trypsin/0.5 mM EDTA and adherent surface cells were removed with DMEM/HG/5 percent FBS/PS/L-glutamine, 2 mM, at a pH of 7.4. These cells were centrifuged, resuspended, and replated on 60 mm tissue culture dishes (BD Falcon) coated with mouse laminin (Biomedical Technologies, Inc; 20 μg/mL in DPBS/poly-L-lysine, Sigma; 20 μg/mL), and differentiated in DMEM/HG/5 percent FBS/PS/L-glutamine, 2 mM at a pH of 7.4 at 37 °C for 2 weeks before transplant, with a media change every 2 to 3 days.

Immunohistochemistry of hNT2.17 Cells In Vitro

Polyclonal antibody anti-fibroblast growth factor (FGF)-4 (dilution 1:20) was purchased from R&D Systems (Minneapolis, Minnesota). The polyclonal antibody anti-GABA (dilution 1:100) was purchased from Protos Biotech Corporation (New York, New York). The polyclonal antibody anti-glycine (dilution 1:200) was purchased

from Chemicon (Temecula, California). Monoclonal antibody anti-bromodeoxyuridine (BrdU) (dilution 1:10) was purchased from Becton-Dickson (San Jose, California). The hNT2-17 cells, after 2 weeks of RA treatment and mitotic inhibitors, were replated to eight-well laminin/poly-L-lysine-coated Permanox slides and differentiation continued for various times before immunostaining. The cells were then fixed for 10 min at 4 °C with cold 4 percent paraformaldehyde and 0.1 percent glutaraldehyde in 0.1M phosphate buffer, pH 7.4. For the BrdU immunostaining, after fixation and rinsing in PBS, pH 7.4 at room temperature, hNT2-17 cells were incubated with 2N hydrochloric acid (HCl) for 20 min at room temperature, rinsed three times with PBS, incubated with borate buffer (pH 8.5)/0.01M boric acid/0.5M Na borate (1:1) for 15 min at room temperature, rinsed three times with PBS, and then permeabilized for 30 min at room temperature with blocking buffer before incubation with the primary anti-BrdU antibody. For all in vitro immunostaining experiments, after fixation and rinsing in PBS, pH 7.4 at room temperature, fixed hNT2-17 cells were permeabilized for 30 min at room temperature with 0.5 percent Triton X-100 in PBS in the presence of 5 percent normal goat serum (NGS) (the blocking buffer) before the addition of the individual primary antibody, usually overnight at 4 °C. The staining was completed by incubation with the specific anti-species immunoglobulin G secondary conjugated to Alexa Fluor 488 Green (dilution 1:100; Molecular Probe; Eugene, Oregon) for 2 h at room temperature. After staining, slides were coverslipped with the use of Vectashield mounting medium with 4′6-diamidino-2-phenylindole-2HCl (DAPI) (Vector Laboratories; Burlingame, California). Photographic images were taken with a Zeiss microscope (Axioplan II Metamorphosis program, Carl Zeiss, Inc; Maple Grove, Minnesota). All immunohistochemistry studies included sister wells that were exposed to incubation in the absence of primary antibody to detect any autofluorescence and that served as negative controls for each experiment. All staining experiments were independently repeated at least three times to ensure that the micrographs were representative.

Animal Study Designs

The effect of graft timing, immunosuppression regimens, and location of intrathecal grafts of these hNT2.17 cells on behavioral hypersensitivity was studied. Adult male Wistar-Furth rats (approximately 200–250 g, Harlan Laboratories; Indianapolis, Indiana) were used for all behavioral experiments. The rats were housed two per cage with rat chow and water ad libitum on a 12/12 h light/dark cycle. They were acclimated and pretrained to two behavioral tests: cutaneous tactile allodynia (CA) (hindpaw withdrawal from normally innocuous mechanical stimuli) and thermal hyperalgesia (TH) (hindpaw withdrawal from a normally noxious heat source). These tests were performed before injury and weekly over the duration of the 60- to 90-day experiments. The animals underwent an excitotoxic SCI (QUIS injection, thoracic [T] 12 to lumbar [L] 1) to induce behavioral hypersensitivity to tactile and thermal stimuli, as confirmed by a vigorous response to behavioral testing. Two or six weeks after injury, rats randomly assigned to be transplanted received a lumbar or cervical IT cell graft with either viable hNT2.17 cells (either 2×10^6 or 10^6 cells/injection, which had been differentiated for 2 weeks in vitro before transplant) or negative-control nonviable hNT2.17 cells (10^6 cells/injection). Another rat group served as a control group and received the QUIS injection but no transplant. A third rat group received thoracic laminectomy, but neither QUIS injection nor transplant and served as the naïve controls. All animals received cyclosporine A (CsA) immunosuppression (10 mg/kg daily, intraperitoneal) for either 1 day before transplant and 14 days after transplant (2 weeks of CsA) or for only 1 day or 1 week. The animals were euthanized after 8 to 12 weeks of behavioral testing (after QUIS injection) and examined for surviving grafted hNT2.17 cells.

All surgical interventions, pre- and postsurgical animal care, and euthanasia were performed in accordance with the Laboratory Animal Welfare Act, Guide for the Care and Use of Laboratory Animals (National Institutes of Health; Department of Health, Education and Welfare, Pub. No. 78-23, Revised 1978) and the guidelines provided by the Animal Care and Use Committees of the Department of Veterans Affairs Medical Center and the University of Miami, both in Miami, Florida. All behavioral testing was performed under blinded conditions to eliminate experimental bias; the data were analyzed and unblinded by the statistician at the end of the experiment. Each specific intervention or test will be subsequently described in detail.

Excitotoxic Spinal Cord Injury

The spinal QUIS injury procedure with cell transplant has been previously described [21]. To produce this excitotoxic injury, we administered QUIS (nonsynthetic;

Sigma), a glutamate receptor agonist, in sufficient concentrations (125 mM) to cause neuronal spinal cell loss and demyelination.

The rats were anesthetized with a mixture of ketamine, xylazine, and acepromazine (0.65 mL/kg). A laminectomy was performed between T12 and L1. The rats were then placed in a stereotaxic frame and their dura and arachnoid were incised. Using a micropipette attached to a Hamilton syringe (Reno, Nevada), we unilaterally injected the QUIS into the dorsal horn 1,000 µm below the surface of the cord in three separate injections 500 µm apart. Each injection was 0.4 µL in volume for a total of 1.2 µL. Anatomically, the injection was located midway between the central vein and dorsal root entry zone, just lateral to the posterior columns. On pathological examination, these unilateral injections were centered in the gray matter between spinal laminae IV and VI. A small piece of sterile durafilm was placed over the dura (to protect the spinal cord and facilitate reopening of the dura for transplantation) and the fascia and skin were closed. Other than the anesthesia, no additional perioperative analgesics were given. The animals recovered at 37 °C for 12 h and then were returned to the animal care facility.

Cell Culture and Transplant of hNT2.17 Cells

Methods for the differentiation and transplant of hNT2.17 cells have been previously described [31]. The hNT2.17 GABA cells that had been predifferentiated for 2 weeks in vitro were prepared for transplant studies. Briefly, cells were rinsed with warmed Cellstripper (Voigt Global Dist; Lawrence, Kansas), media were replaced with another 3 mL of Cellstripper for 1 min, and then cells were rinsed with warmed Hank's Buffered Salt Solution (HBSS) for complete cell removal. Viability and cell counts were assessed by trypan blue exclusion, and the cells were suspended in 10^0 µL of Ca^{2+}- and Mg^{2+}-free HBSS (CMF-HBSS). Typically, an aliquot of about 1 million cells was prepared immediately before each transplant to assure near 100 percent viability at the beginning of the experiment; grafting occurred within 30 min of cell preparation. Nonviable hNT2.17 cells were prepared by initially resuspending 1 million cells in sterile water, centrifugating them, checking them for viability, and then resuspending them in CMF-HBSS for transplant.

One day after showing a vigorous behavioral hypersensitivity, the animals to be transplanted were anesthetized with a mixture of ketamine, xylazine, and acepromazine (0.65 mL/kg). For lumbar transplant, the previous laminectomy site (T12–L1) was exposed. A small dural and arachnoidal incision was made and a 2 to 3 mm segment of polyethylene tubing connected to a micropipette was inserted caudally through the durotomy. Either 2 or 6 weeks after QUIS injection, 1 to 2 million cells (viable or nonviable) were injected into the intrathecal space at spinal segment L1–L3 and then the fascia and skin were closed. The animals were allowed to recover at 37 °C for 12 h and then were returned to the animal care facility. All rats, including those not provided cell transplants, received immunosuppressive therapy with CsA injected intraperitoneally, typically beginning 1 day before cell transplant and then daily for 14 days. These rats were behaviorally tested for 56 to 90 days after the QUIS injection.

Excessive Grooming Evaluation

Evaluation of excessive grooming was based on the initial observations after QUIS injections to create behavioral hypersensitivity [35]. Animals that developed autophagia and excessive grooming over the hindlimb midthigh ipsilateral to the spinal injection were included in a weekly measure of lesion size after QUIS injection and transplant of hNT2.17 cells, as described previously [35]. Lesion size was measured from a grid drawing derived from digital photographs of the lesions, and grooming was categorized as either "improvement/resolution" or "worsening/no change" for each animal at the time of transplant and the end of each study.

Thermal Hyperalgesia Testing

Methods for testing TH with a Hargreaves' device have been described elsewhere [36]. Animals were placed in a clear Plexiglas box on an elevated Plexiglas floor where they were allowed to acclimate for approximately 5 min. A constant-intensity radiant heat source was aimed at the midplantar area of each hindpaw. The time in seconds from initial heat source activation until paw withdrawal was recorded. Five minutes were allowed between stimulations. Three to four latency measurements for each hindpaw were recorded and the mean and standard error of the mean (SEM) calculated for each hindpaw. Animals were tested weekly for the duration of the experiment.

Cutaneous Tactile Allodynia Testing

CA, the occurrence of foot withdrawal in response to normally innocuous mechanical stimuli, was tested with

an electronic von Frey anesthesiometer (IITC Life Science Inc; Woodland Hills, California) [37]. Each rat was placed in a Plexiglas box with an elevated mesh floor. After the rat was allowed to acclimate for 5 min, the device tip was applied perpendicular to the midplantar area of each hindpaw and depressed slowly until the animal withdrew the paw from pressure. The value, in grams, was recorded for each of three trials. A single trial of stimuli consisted of three to four applications of the instrument tip within 10 s to ensure that the response was constant. The scores for each hindpaw were averaged and the SEM calculated. These mean values were then used for further statistical analyses to determine the effects of the different treatments. The animals were tested weekly for the duration of the experiment.

Immunohistochemistry In Vivo

Fixation

Spinal cords were fixed for examination of cell graft survival and GABA and nuclear matrix antigen (NuMA) staining 8 to 12 weeks after QUIS injury. Transcardial perfusion with Lana's fixative (4% paraformaldehyde and 0.1% gluteraldehyde in PBS) was performed. Rats were euthanized for tissue fixation by a combination of pentobarbital overdose (interperitoneal injection, 12 mg/100 g) and exsanguination. Once the appropriate level of anesthesia was reached (i.e., no corneal or withdrawal reflexes), rats were transcardially perfused with aldehydes. After perfusion, the spinal cords, including transplant, were removed and histologically processed. After removal from the vertebral column, cords were stored in fix for 12 h at 4 °C. These cords were cryoprotected by equilibration in 30 percent sucrose and PBS overnight at 4 °C and then frozen and stored at –80 °C. Cords were embedded in Shandon-1 Embedding Matrix (Thermo Electron Corp; Waltham, Massachusetts) and sagittally or transversely cut in sequential 20 µm sections with a Cryostat (Leica CM3050 S Cryostat, Micro Optics of Florida Inc; Davie, Florida). They were collected on noncoated slides (micro Slides, Snowcoat X-tra, Surgipath; Richmond, Illinois). The slides were stored in a –20 °C freezer and removed for defrosting before the immunostaining procedures. Every second section was stained for the human marker NuMA or GABA and dehydrated, cleared, and mounted in Cytoseal 60 (Richard-Allan Scientific [Thermo Electron Corp]) after antibody staining. Processed slides were observed and photographed with a Zeiss Axioplan2 research microscope.

NuMA Staining

Methods for staining spinal cord sections for human-specific NuMA to identify NT2 neurons after grafting have previously been described [38]. The sections were washed with 0.1M PBS pH 7.4 and permeabilized with 0.4 percent Triton-X-100 in 0.1M PBS, 10 percent NGS, and 3 percent poly-D-lysine (Sigma) for 1 h. The sections were then incubated overnight at 4 °C in the primary anti-NuMA antibody (10 mg/mL DPBS; EMD Biosciences; San Diego, California) and the permeabilizing solution, followed by a 1 h incubation at room temperature with the secondary antibody solution, biotinylated mouse raised in goat (Vector Laboratories), a Peroxidase ABC reporter in 0.1M PBS (Vector Laboratories), and "VIP" substrate (Vector Laboratories). Some sections were stained in the absence of primary antibody and served as the negative controls.

GABA Staining

Methods for staining lumbar spinal cord sections for GABA were adapted from methods described elsewhere [39]. Sections were incubated with the primary antibody GABA (1:500; Protide Pharmaceuticals, Inc; Lake Zurich, Illinois) with 0.4 percent Triton-X-100 in 0.1M PBS and 10 percent NGS overnight at 4°C, followed by a 1 h incubation at room temperature with the secondary antibody solution, biotinylated guinea pig raised in goat (Vector Laboratories) in 0.4 percent Triton-X-100 in 0.1M PBS and 10 percent NGS, a Peroxidase ABC reporter in 0.1M PBS (Vector Laboratories), and "VIP" substrate (Vector Laboratories). Some sections were stained in the absence of primary antibody and served as the negative controls.

Statistical Analysis

Mean difference scores, based on three pairs of determinations, and the corresponding SEM were calculated for each sensory behavior session and each experimental animal. Evaluation of the SEM showed high stability of the within-animal daily measurements. Therefore, we performed further statistical analyses using the mean values, not the weighted mean values, obtained for each rat for each session. Repeated measures analysis of variance, with use of a General Estimating Equation approach (SAS Proc Mixed, SAS Institute Inc; Cary, North Carolina) was

used to assess overall treatment effects. We also performed a cross-sectional analysis, including pairwise comparisons of daily group mean values, using Tukey's method to adjust for the multiplicity of the comparisons. All analyses were performed with SAS software (SAS Institute) and *p*-values less than 0.05 are reported. After adjusting for multiple comparisons whenever possible, we considered all values statistically significant. The various data sets, including surgeries, behavioral outcomes, and statistical analysis, were all performed under double-blinded conditions, breaking the codes for the identity of rats and groups only with the statistician's analyses.

RESULTS

Differentiated hNT2.17 Neuronal Cells In Vitro

Critical to the eventual use of differentiated hNT2.17 cells as a transplant source are their morphological homogeneity and exclusive neuronal and inhibitory phenotype. To be scaled-up to a manufactured product, batches of differentiated cells should exhibit consistent physical properties. Once the hNT2.17 cells begin differentiation and after treatment with RA and mitotic inhibitors in vitro, they can easily be transferred to tissue culture flasks or slides for examination. Neuron-specific markers, such as human neuron-specific enolase (NSE) were present as soon as 4 days of differentiation, and NSE remained intense throughout at least 5 weeks of differentiation in vitro [31]. When examined by phase microscopy during differentiation, the cells immediately extend long processes at 1 day in vitro. Within 2 weeks, the cells continue to extend long fibers but aggregate as balls of cells, eventually forming dense fiber networks extending from the balled cells at 4 weeks of differentiation (**Figure 1(a)**). The hNT2.17 cells have been kept as long as 50 days of differentiation in culture, forming very dense fiber networks that cover the plate surface. Easily observable during early differentiation with an antibody stain for GABA, all the hNT2.17 cells stain for the inhibitory neurotransmitter. Both the cell soma and extending fibers contain a strong GABA signal (**Figure 1(b)**). As the fibers extend during differentiation, the fiber GABA signal becomes concentrated and punctate-like in bouton-like structures as early as 2 weeks in vitro. As the fibers become extensive, the punctate concentration of GABA becomes more pronounced in extending fibers [31].

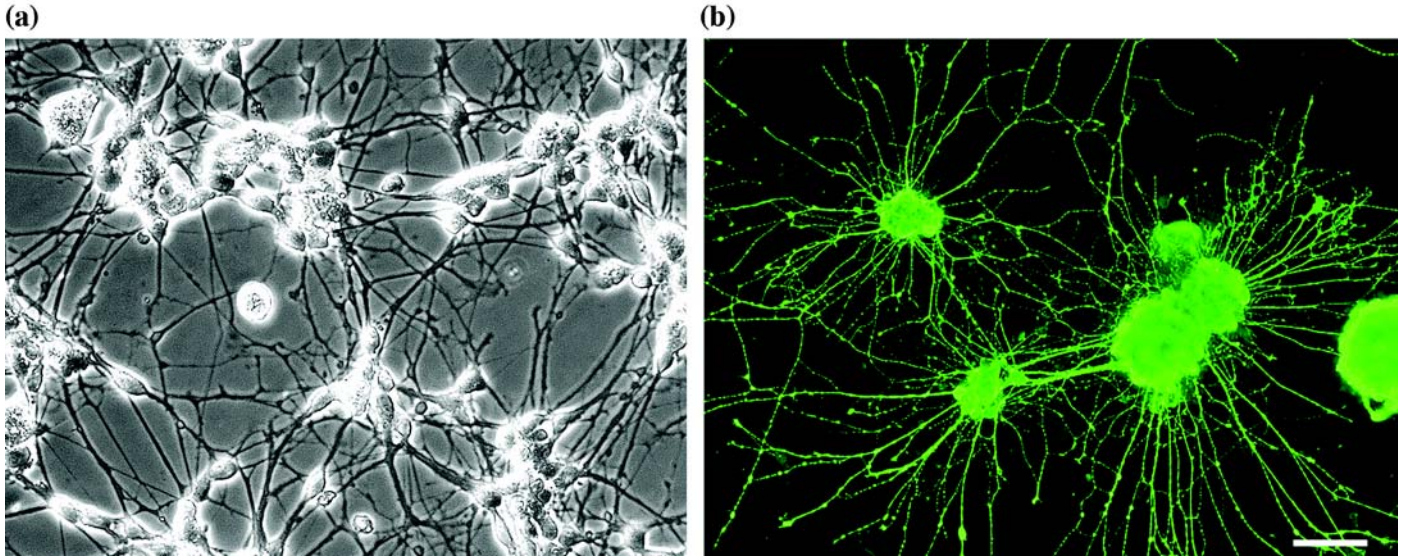


Figure 1.
Morphology of differentiation of hNT2.17 cell line in vitro. hNT2.17 cell line was treated for 2 weeks with retinoic acid and mitotic inhibitors and lifted to substrate-coated, eight-well plastic tissue culture slides for differentiation, phase microscopy, and immunostaining. **(a)** By 2 weeks in vitro, cells aggregate in balls and continue to extend multiple fibers during differentiation (4 weeks), which become a dense fiber network by 50 days of differentiation. **(b)** All hNT2.17 cells stain very brightly for inhibitory neurotransmitter γ-aminobutyric acid (GABA) (2 weeks). Both cell soma and extending fibers contain strong GABA signal. As fibers extend during differentiation, fiber GABA signal becomes concentrated and punctate-like in bouton-like structures as early as 2 weeks in vitro. Magnification bars = **(a)** 20 μm and **(b)** 30 μm.

hNT2.12 Cells are Nontumorigenic and Colocalize GABA and Glycine

The parental NT2 cell line has historically been demonstrated to change its phenotype to nontumorogenic after differentiation with RA in vitro [40], allowing it to be transplanted into the central nervous system (CNS) [41] and used safely in human studies [42]. Here, the hNT2.17 switches from a proliferating to a nonproliferating phenotype after RA exposure. BrdU immunostaining has been used as a marker for proliferating cells in vitro [43] and in vivo [44], since dividing cells incorporate BrdU-labeled uridine into newly made deoxyribonucleic acid (DNA). The hNT2.17 cells were exposed to 1 µM BrdU in vitro during either proliferation or differentiation before anti-BrdU immunostaining. Following 3 days of proliferation in the presence of BrDU, the BrdU signal was intense and found in all the dividing cells (**Figure 2(a)**). After 1 week of BrdU exposure during the first week of differentiation, hNT2.17 cells remained viable, as evidenced by DAPI staining (**Figure 2(b)**). The same field of differentiated hNT2.17 cells showed no BrdU signal (**Figure 2(c)**).

The inhibitory neurotransmitters GABA and glycine and their decrease in models of pain have been implicated as likely causes for the induction and maintenance of neuropathic pain. A cellular minipump that could supply both to the injured CNS might be the basis of a strategy for grafts that recover sensory modulation after SCI. In

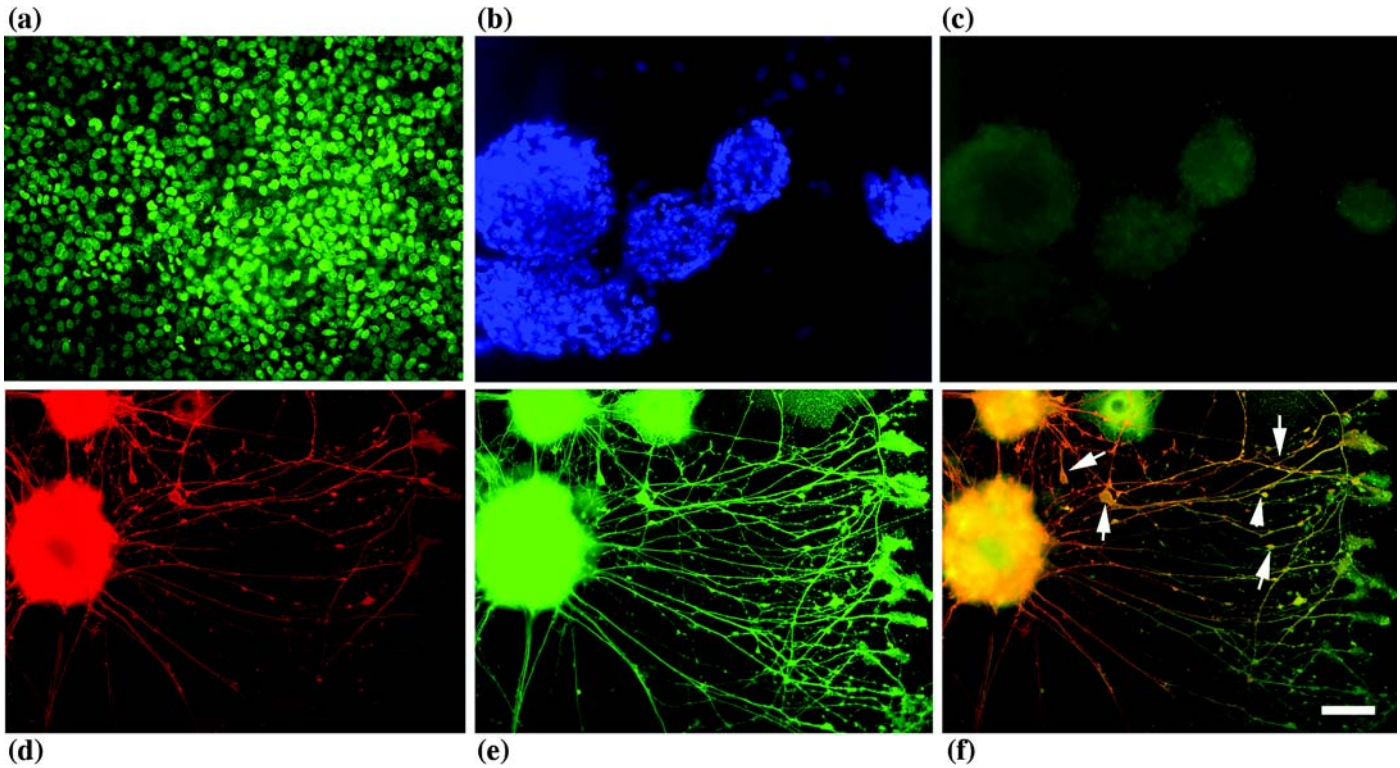


Figure 2.
hNT2.12 cells are nontumorigenic and colocalize γ-aminobutyric acid (GABA) and glycine. hNT2.17 cells were exposed to 1 µM bromodeoxyuridine (BrdU) **(a)** during 3 days of proliferation or **(b)–(c)** for 1 week during differentiation in vitro. **(a)** Proliferating cells incorporate abundant BrdU during proliferation. **(b)** Viable differentiated cells were labeled with DAPI (4′6-diamidino-2-phenylindal-2HCl) stain, while **(c)** the same field of differentiated cells did not incorporate any BrdU during differentiation. After 2 weeks of treatment with retinoic acid (RA) and mitotic inhibitors, hNT2.17 cells cease dividing and differentiation proceeds without further cell division. **(d)–(f)** hNT2.17 cell line was treated for 2 weeks with RA and mitotic inhibitors and lifted to substrate-coated, eight-well plastic tissue culture slides for differentiation and immunohistochemistry for glycine and GABA. **(d)** All hNT2.17 cells stain very brightly for inhibitory neurotransmitter GABA (red) at 4 weeks of differentiation. **(e)** These same cell bodies and extending fibers stain brightly for glycine (green). **(f)** When the two labels are merged with Metamorph imaging software, all cells colabel GABA and glycine (arrows). Magnification bar = 10 µm.

one example of hNT2.17 cells in vitro (**Figures 2(d)–(f)**), cells were differentiated for 4 weeks and stained for both GABA (**Figure 2(d)**, red) and glycine (**Figure 2(e)**, green). When these images are merged, the two neurotransmitters overlap 100 percent (**Figure 2(f)**, yellow/orange) and all the hNT2.17 cells synthesize both GABA and glycine with differentiation (**Figure 2(f),** arrows).

Localization and GABA Phenotype of Grafted hNT2.17 Cells After QUIS SCI

Important to safety considerations and prediction of the mechanisms of grafts on sensory behaviors after transplant is the ability to identify the grafts in vivo and determine the permanence of the neurotransmitter phenotype under those conditions. Transplanted spinal cord sections were collected 8 weeks after QUIS injection and visualized with specific human and neurotransmitter antibody markers GABA (**Figure 3(a)**, arrows) and NuMA (**Figure 3(b)**, arrows). Many of these grafted hNT2.17 cells survived (**Figures 3(a)–(b)**, arrows) on the pia near the lumbar cord for at least 6 weeks after QUIS injection and transplant, and apparently all cells retained their GABA expression after transplant, in a rat excitotoxic SCI pain model [31].

Sensory Behaviors After Transplant of hNT2.17 GABA Cell Line

Acute Versus Chronic Graft Times

Assessment of behavioral hypersensitivity (CA and TH) after transplant of hNT2.17 cells at 2 or 6 weeks after QUIS injection (**Figures 4(a)–(b)**), where cells had been predifferentiated in vitro for 2 weeks before grafting, showed no difference between QUIS alone (data not shown) and the QUIS plus nonviable hNT2.17-grafted animals. Baseline CA values for these groups ranged from 33.03 to 34.77 g (± 0.85 SEM). At 90 days after QUIS injection, the CA values ranged from 14.77 to 16.33 g (± 0.83 SEM). Baseline TH values for these groups ranged from 13.44 to 14.70 s (± 0.47 SEM). At 90 days after QUIS injection, the TH values ranged from 8.89 to 9.72 g (± 0.54 SEM). All animals undergoing excitotoxic QUIS injury demonstrated significant CA and TH in the hindpaw ipsilateral to the site of injury that persisted for the duration of the experiment. Animals undergoing

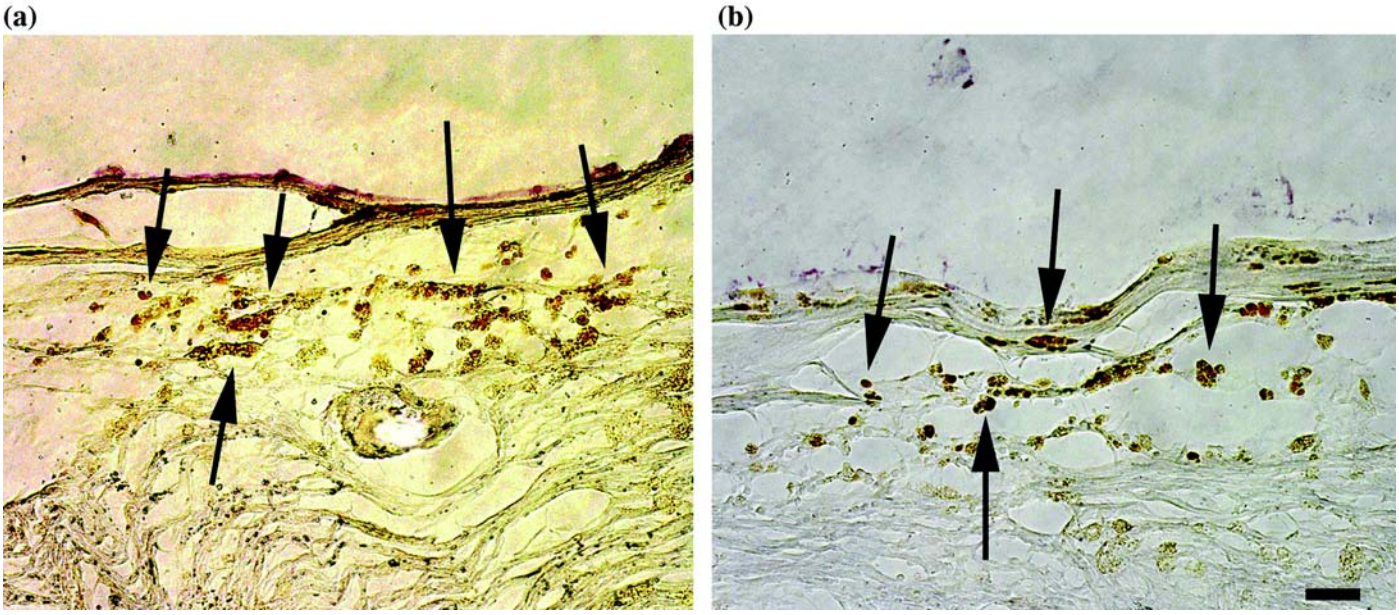


Figure 3.
γ-aminobutyric acid (GABA) and nuclear matrix antigen (NuMA) immunostain of quisqualic acid (QUIS) plus hNT2.17 transplanted rat spinal cord. **(a)** Sagittal section of anti-GABA immunostained QUIS + hNT2.17 transplanted lumbar spinal cord 6 weeks after grafting. Easily detectable hNT2.17 cells stain for GABA (arrows) on pial membranes. **(b)** Sagittal section of anti-NuMA immunostained QUIS + hNT2.17 transplanted lumbar spinal cord 6 weeks after grafting. Easily detectable hNT2.17 cells stain for NuMA (arrows) on pial membranes in adjacent sections. Magnification bar = 20 μm.

QUIS plus viable cell transplant (at 10^6 cells/injection) at 2 weeks after QUIS injection showed potent (near complete) reversal of CA and TH behaviors 1 week after transplantation that persisted for the duration of the experiment. For CA behaviors (**Figure 4(a)**), 2 weeks (28 days) after transplantation of 10^6 live hNT2.17 cells, withdrawal pressures had returned to 32.47 ± 0.65 g ($p < 0.001$). For TH behaviors (**Figure 4(b)**), 2 weeks (28 days) after transplantation of 10^6 live hNT2.17 cells, withdrawal latencies had returned to 12.66 ± 0.30 s ($p < 0.001$). At day 14 (immediately before cell grafting), no significant differences existed between the QUIS plus hNT2.17 transplant and QUIS alone or QUIS nonviable graft groups. From day 21 onward, only the QUIS plus hNT2.17 transplant animals had improved CA and TH scores; they showed significant differences compared with the QUIS alone or QUIS plus nonviable graft animals at 28 days onward. The IT hNT2.17 cell grafts completely and permanently reversed both CA and TH when transplanted 2 weeks after SCI, and these effects persisted throughout the 90 days. No significant difference was found in the results when the graft cell number was doubled to 2 million, rather than the 1 million, at the 2-week time point. Results when the 6-week graft time was used were mixed. When transplanted 6 weeks after SCI, the hNT2.17 grafts completely reversed TH. One week after the 6-week graft (49 days), a return to baseline values occurred (13.67 ± 0.34 s, $p < 0.001$). Immediately before the live hNT2.17 cell graft (42 days after QUIS injection), the values had been reduced to 10.92 ± 0.21 s, which was not significantly different from QUIS alone ($p < 0.001$). CA was only partially attenuated but was significantly improved over the nonviable cell transplant ($p < 0.01$). One week after the 6-week graft (49 days), the CA value was 24.71 ± 0.74 g ($p < 0.01$). The CA attenuation represents about 70 percent of that achieved when the grafts were placed 2 weeks after QUIS. These improvements in behavioral hypersensitivity lasted throughout the 3-month duration of the experiment.

Various Immunosuppression Regimens

The assessment of behavioral hypersensitivity (CA and TH) with various immunosuppression regimens (**Figures 5(a)**–**(b)**) after transplant of hNT2.17 cells,

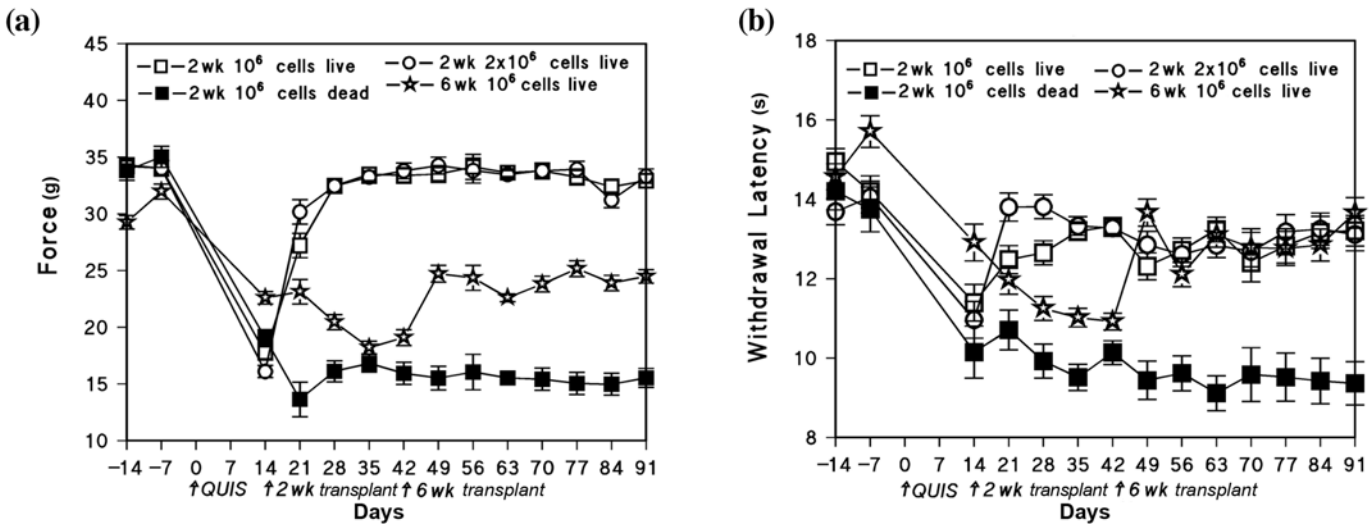


Figure 4.
Sensory behaviors after quisqualic acid (QUIS)-induced spinal cord injury (SCI) and acute versus chronic transplant of hNT2.17 cells in vivo. Rats were spinally injected with QUIS before cell transplant. All rats received cyclosporine A 1 day before and for 2 weeks after the 2- or 6-week time points at which some animals were injected with cells. Rats were either left untreated, injected with QUIS alone, or injected with QUIS plus viable (2×10^6 or 10^6 cells/injection) or nonviable hNT2.17 cells (10^6 cells/injection) into subarachnoid space. Rats were tested before SCI (baseline) and once a week following QUIS injection and treatments for hypersensitivity to **(a)** tactile or **(b)** thermal stimuli in hindpaws below SCI. QUIS injury negatively affected hindpaw responses bilaterally, but ipsilateral hindpaw was most affected by QUIS injection (shown here). Data represent mean ± standard error of the mean at each time point before and 91 days after QUIS injection. Only 2-week transplant time after SCI permanently and completely attenuated both cutaneous tactile allodynia and thermal hyperalgesia induced by QUIS injection. Six-week transplant time partially attenuated cutaneous tactile allodynia.

where cells had been predifferentiated in vitro for 2 weeks before grafting, showed no significant difference between the QUIS alone and the QUIS plus CsA animals. All animals undergoing excitotoxic QUIS injury demonstrated significant CA and TH in the hindpaw ipsilateral to the site of injury that persisted for the duration of the experiment. Baseline values for CA behaviors (**Figure 5(a)**) ranged from 25.20 to 33.47 g (± 0.93 SEM). At 56 days, the values for CA behaviors ranged from 14.33 to 22.22 g (± 0.91 SEM). Baseline values for TH behaviors (**Figure 5(b)**) ranged from 12.36 to 16.37 s (± 0.46 SEM). At 56 days, the values for the TH behaviors ranged from 9.56 to 12.50 s (± 0.38 SEM). Animals undergoing hNT2.17 cell transplantation accompanied by 2 weeks of CsA immunosuppression showed complete reversal of all behavioral hypersensitivity. The recovery occurred 1 week after transplantation and was maintained for the 56-day course of the experiment. One week of CsA administration following transplant resulted in permanent reversal of CA but only temporary reversal of TH. One day of CsA administration with transplant resulted in temporary reversal of both CA and TH. Regarding CA behaviors, when immunosuppression was provided for 2 weeks after transplantation (28 days) of 10^6 live hNT2.17 cells, withdrawal pressures returned to 31.13 ± 0.76 g ($p < 0.001$) (**Figure 5(a)**). Regarding TH behaviors, when immunosuppression was provided for 2 weeks after transplantation of 10^6 live hNT2.17 cells, withdrawal latencies returned to 14.09 ± 0.28 s ($p < 0.001$) (**Figure 5(b)**). Results were mixed when other CsA regimens were used. For CA behaviors, cell transplantation plus 1 week of CsA immunosuppression resulted in a significant ($p < 0.001$) and persistent return to baseline withdrawal latencies. Transplantation plus 1 day of immunosuppression showed an initial return to baseline levels by day 35 but a decrease in values starting at day 42 and a return to near-injury withdrawal levels by the end of the experiment at day 56. For TH behaviors, transplantation plus 1 week of CsA immunosuppression resulted in a significant ($p < 0.001$) but temporary return to baseline withdrawal latencies by day 35 that fell by day 56. Transplantation plus 1 day of immunosuppression showed an initial return to

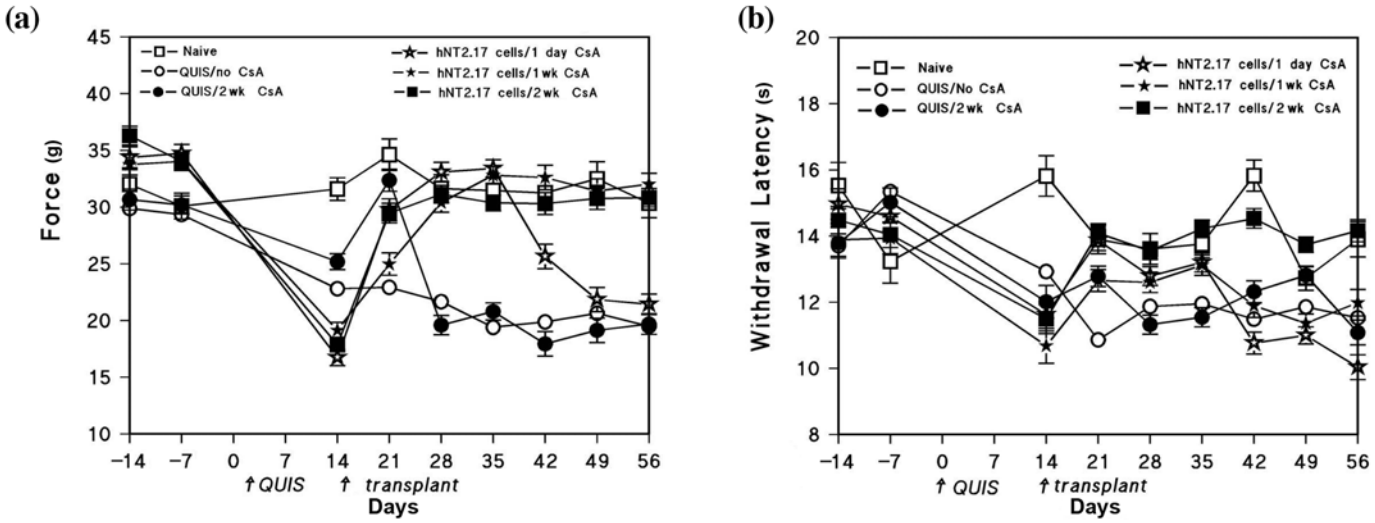


Figure 5.
Sensory behaviors after quisqualic acid (QUIS)-induced spinal cord injury (SCI) and transplant of hNT2.17 cells with various immunosuppression regimens in vivo. Rats were either left untreated, injected with QUIS alone, or injected with QUIS plus hNT2.17 cells (10^6 cells/injection) into subarachnoid space 2 weeks after QUIS injection. All rats received cyclosporine A (CsA) 1 day before or 1 day before and for either 1 or 2 weeks after the graft placement time point. Rats were tested before SCI (baseline) and once a week after QUIS injection and treatments for hypersensitivity to **(a)** tactile or **(b)** thermal stimuli in hindpaws below SCI. Both ipsilateral and contralateral hindpaws recovered at least partial degree of normal sensory responses to tactile and thermal stimuli after transplant of hNT2.17 at 2-week graft time point, compared with QUIS injury alone. QUIS injury negatively affected hindpaw responses bilaterally, but ipsilateral hindpaw was most affected by QUIS injection (shown here). Neither hindpaw recovered normal tactile or thermal responses after QUIS alone by 56 days after injection. Data represent mean ± standard error of the mean at each time point before and 56 days after QUIS injection. Only CsA injected 2 weeks perigraft permanently and completely attenuated tactile allodynia and thermal hyperalgesia induced by QUIS injection.

baseline levels by day 35 but a decrease in values starting at day 42 and a return to below injury withdrawal levels by day 56.

Grafts Placed at Two Different Spinal Levels

With grafts placed at either lumbar or cervical locations (**Figures 6(a)–(b)**), all animals undergoing excitotoxic QUIS injury demonstrated significant CA and TH in the hindpaw ipsilateral to the site of injury that persisted for the duration of the experiment compared with naïve animals ($p < 0.001$). Animals undergoing QUIS plus lumbar cell transplant (at 10^6 cells/injection) 2 weeks after QUIS injection showed potent (complete) reversal of CA and TH behaviors 1 week after transplantation that persisted for the duration of the experiment. For CA behaviors and lumbar transplant (**Figure 6(a)**), 2 weeks after grafting (28 days), values had returned to 30.48 ± 1.42 g ($p < 0.001$). For TH behaviors and lumbar transplant (**Figure 6(b)**), 2 weeks after grafting, values had returned to 14.22 ± 0.61 s ($p < 0.001$). Results were almost identical when the cervical transplant site was used. When cells were grafted cervically, the observed attenuation represented recovery of normal sensory TH and CA that was statistically indistinguishable from the recovery seen with a lumbar graft site for transplants. With CA behaviors and cervical transplant, 2 weeks after grafting (28 days), values had returned to 29.32 ± 1.12 g ($p < 0.001$). With cervical transplant and TH behaviors, at 2 weeks after grafting, values had returned to 13.37 ± 0.68 s ($p < 0.001$). Comparison of group mean values showed no significant differences between the two groups at days –14, –7, and 0. At day 14 (immediately before cell grafting), no significant differences were found between the QUIS plus hNT2.17 transplant and QUIS alone groups. From day 21 onward, both the lumbar and cervical QUIS plus hNT2.17 transplant animals had improved CA and TH scores, showing significant differences at 28 days onward compared with QUIS alone. Transplants collected 8 weeks after QUIS injection were visualized with the specific human and neurotransmitter antibody markers NuMA and GABA for grafts placed at cervical or lumbar locations (data not shown). Grafted cells expressing both NuMA and GABA were seen when the cells were grafted in the lumbar region. No grafts were visible when the cervical surface was examined after cervical placement of grafts. No surviving cells

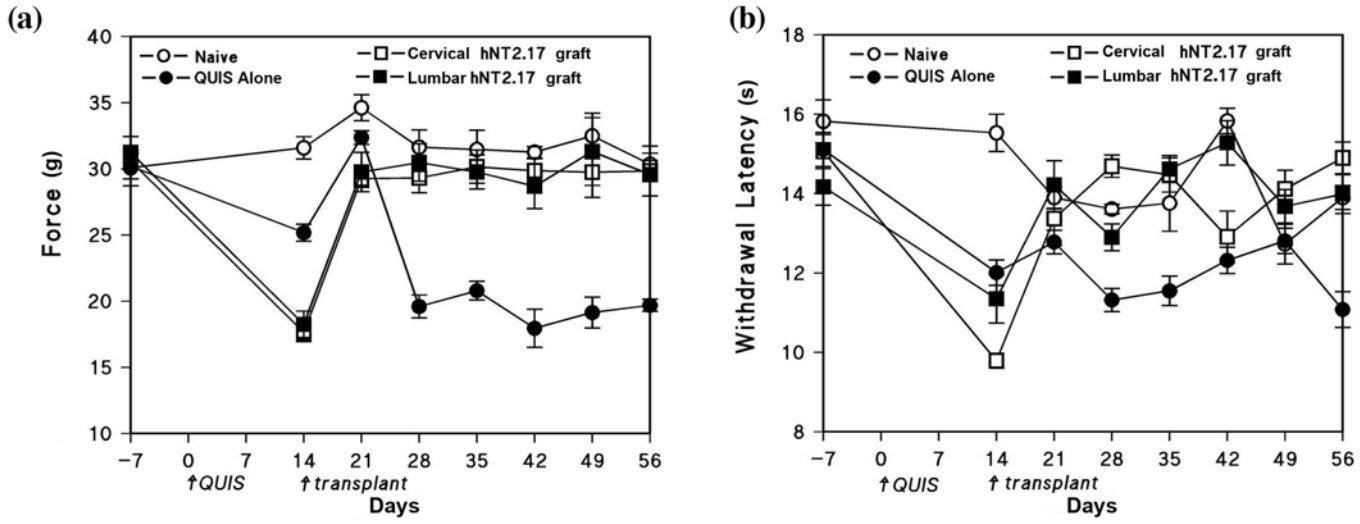


Figure 6.
Sensory behaviors after quisqualic acid (QUIS)-induced spinal cord injury (SCI) and transplant of hNT2.17 cells at two spinal levels in vivo. Rats were either left untreated, injected with QUIS alone, or injected with QUIS plus hNT2.17 cells (10^6 cells/injection) into lumbar or cervical subarachnoid space 2 weeks after QUIS injection. All rats received cyclosporine A (CsA) 1 day before and for 2 weeks after graft placement time point. Rats were tested before SCI (baseline) and once a week following QUIS injection and treatments for hypersensitivity to **(a)** tactile or **(b)** thermal stimuli in hindpaws below SCI. QUIS injury negatively affected hindpaw responses bilaterally, but ipsilateral hindpaw was most affected by QUIS injection (shown here). Neither hindpaw recovered normal tactile or thermal responses after QUIS alone 60 days after injection. Data represent mean ± standard error of the mean at each time point before and 56 days after QUIS injection. Both lumbar and cervical graft placement with SCI permanently and completely attenuated tactile allodynia and thermal hyperalgesia induced by QUIS injection.

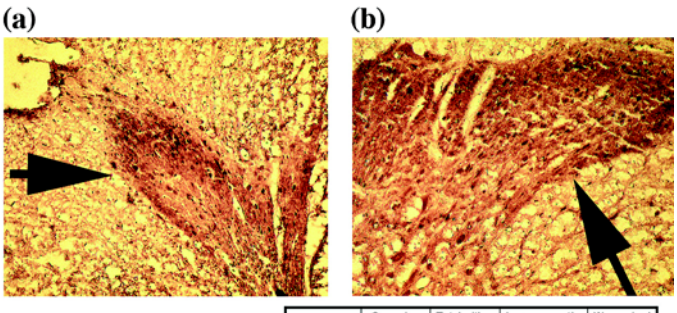
were detected at the cervical site with a lumbar graft or on the lumbar site with a cervical graft (data not shown).

Change of GABA Signal in Lumbar Spinal Cord with Injection of QUIS

When the sensory lumbar dorsal horn (**Figures 7(a)–(b)**, arrows) of the spinal cord is examined for the presence of a GABA signal 4 weeks after QUIS injection, the ipsilateral horn (**Figure 7(a)**) apparently contains few GABA-positive cells in the outer (laminae I–II) dorsal horn (arrow) compared with the contralateral side (**Figure 7(b)**) at the same lumbar level, but GABA positive dorsal horn cells are also seen (arrow).

Effects of hNT2.17 Cell Grafting on Excessive Grooming Related to QUIS Injection

Injection of QUIS causes ipsilateral excessive grooming behaviors and autophagia that does not resolve without intervention (**Figure 7(c)**, left). When excessively grooming rats that had been transplanted with either viable or nonviable hNT2.17 cells and exposed to different immunosuppression regimens were examined for development, resolution, worsening, or no change of excessive grooming, a trend toward improvement was associated with viable grafts and at least 1 week of accompanying CsA immunosuppression. When transplant was delayed to 6 weeks, no improvement in excessive grooming was seen (**Figure 7(c)**, right).



	Grooming at transplant	Total with grooming	Improvement/ Resolution	Worsening/ No Change
2 E6 cells viable	0	1	1	0
1 E6 cells viable	0	3	2	1
1 week Immuno	0	3	3	0
6 week Transplant	2	3	0	3
1 day Immuno	0	5	1	4
Dead cell transplant	0	5	0	5

Figure 7.
Change in lumbar spinal dorsal horn γ-aminobutyric acid (GABA) signal with injection of quisqualic acid (QUIS), and resolution of excessive grooming in rats with hNT2.17 cell graft. Transverse sections of lumbar spinal cord were collected 4 weeks after QUIS injection and stained for presence of GABA interneurons in dorsal horn sensory areas. **(a)** Ipsilateral to QUIS injection, GABA interneurons are barely detectable (arrow), with neck of dorsal horn greatly shrunk by SCI. **(b)** Contralateral side is much less damaged, and some GABA-stained cells are visible in outer laminae (arrow). Within 10 to 12 days after QUIS injection, rats begin to develop excessive grooming behaviors on ipsilateral dermatome associated with spinal injection level and **(c)** by 3 to 4 weeks, autophagia has progressed (left) to prominent lesion. Excessively grooming rats from different transplant groups (right; $n = 20$) were monitored for improvement or worsening after transplant of hNT2.17 cells (viable or dead), little or minimal CsA immunosuppression (1 day or 1 week immuno), and delayed transplant time (6 weeks) versus normal (2 weeks) of either 2×10^6 or 1×10^6 viable graft. At least 1 week of immunosuppression, 2-week graft time, and viable grafts of at least 1×10^6 hNT2.17 cells showed trend toward improved or resolved excessive grooming related to QUIS injection.

DISCUSSION AND CONCLUSIONS

More than two decades ago, it was discovered that when treated with RA, the human embryonal carcinoma cell line NT2 differentiates irreversibly into several morphologically and phenotypically distinct cell types, which include terminally differentiated postmitotic CNS neurons [45–46]. Successive replating of RA-treated NT2 cells in the presence of growth inhibitors results in the isolation of purified human neurons [45], which have been extensively characterized and tested in vivo in numerous animal models of traumatic injury and neurodegenerative disease [47–48]. This NT2 human neural cell line has been used for various studies that reveal regulation of an oncogenic phenotype by agents such as RA [49–50] and has been well characterized for the expression of a variety of neural phenotypic properties [51–53] and proteins [54–55] with differentiation of the cells in vitro and in vivo [56]. The potential application of NT2 neurons in cell transplantation therapy for CNS disorders and their use as vehicles for delivering exogenous proteins into the human brain for gene therapy have been envisioned [57]. Such NT2 neurons have been used in phase I clinical trials for the treatment of stroke [58–59], and this cell line or its derivatives can likely be used for further reparative transplant strategies. In our initial report, hNT2.17 cells, a unique and derivative cell line of

this NT2 parentage, have demonstrated the potential to treat the behavioral hypersensitivity associated with excitotoxic SCI [31].

Translational studies that describe basic research approaches to what is expected or hoped to be able to be moved to a clinical setting have seen a significant increase in recent peer-reviewed publications [60]. But even with the great interest in and funding directed toward translational research, this promise remains unfulfilled. Part of the problem, at least in the treatment of chronic and neuropathic pain, has been the difficulty with the most appropriate animal model [61] to use for basic research, as well as the disconnect between behavioral outcomes examined in animal studies and those reported in the presentation of pain in a clinical setting [62]. The animal model we used—excitotoxic SCI after spinal injection of the glutamate agonist QUIS—has many advantages over other commonly used SCI models, such as weight-drop contusion, clip-compression, focal-laser, or dorsal hemisection. These advantages include (1) the spinal injury (chemical lesion) has no effect on motor behaviors, including no apparent complications of paralysis or loss of bowel and bladder function [35], especially important when sensory evoked responses are used as an outcome; (2) the excitotoxic SCI pain model has been used to evaluate the effects of (other types of) cell transplantation to reverse the chronic behavioral allodynia and hyperalgesia [22] that this lesion of the dorsal horn pain-processing centers produces; (3) the model is reproducible for progressive syringomyelia and pain after SCI [63]; and (4) the spread of secondary injury over time to spinal segments rostral and caudal to the injury site is critical to the nature and distribution of SCI pain [64], and QUIS injection produces spontaneous pain that results in excessive grooming and autophagia behaviors in the dermatomes associated with the SCI level. In human hands, CA and TH are not only reproducible but also permanent. Only a severe SCI caused by spinal contusion or compression results in permanent and reproducible behavioral hypersensitivity [65–66], making long-term studies difficult and associated with poor animal survival and other complications. Only a few SCI models that induce behavioral hypersensitivity have shown good efficacy with cell transplant treatments, and with the exception of our own [31,67–72], few studies to date have extended the cell therapy concept for SCI beyond a single publication, no matter the animal model.

Such a history in translational work begs the question of investigator bias or reproducibility. These issues can be difficult to clarify, again using adrenal chromaffin graft therapy as a model, which was tested with encapsulation technology to limit rejection of xenografts [73]. Various laboratories were able to replicate the initial basic research in both peripheral [74–76] and central [29,77] models of pain. But the question of investigator bias has been suggested [26]. Certainly a good base of technical expertise is required to fairly replicate any scientific experiments. Most of the data presented in this article were gathered by an independent neurosurgeon at our facility but with shared technical staff. But more importantly, the various data sets, including surgeries, behavioral outcomes, and statistical analysis, were all performed under double-blinded conditions, breaking the codes with the statistician to attempt to limit possible bias. Indeed, some small differences exist in behavioral outcomes in the cell-graft dosing study [71] compared with the initial publication [31]. But the published conclusions, with replication by another investigator, are the same in all studies: (1) when the QUIS model of SCI-pain is used, hNT2.17 grafts provide 100 percent efficacy [72] and (2) 1×10^6 hNT2.17 cells/injection is the optimal dose for this efficacy [71].

A topic similar to optimal dose is the timing of the initiation of cell therapy, since cell grafts might not be the first treatment choice but rather used as an adjunct to a time course of pharmacotherapy with a drug such as gabapentin [14]. A recent study suggests that most neuropathic pain that accompanies SCI begins early after injury [15], but a variety of allopathic pharmacological treatments could be expected to be tried before cell therapy intervention, and the neuropathic pain would be chronic in these patients with nonoptimal pain relief. Indeed, our planned phase I trial design for the use of the hNT2.17 cells to treat SCI pain expects to use a population with stable SCI (2 years or longer). Although our data suggest that the earlier the initial grafting the better the outcome, even a graft provided 6 weeks after SCI in the rat provided ≥70 percent efficacy. The mechanism by which the human grafts provide such differences in efficacy is currently unknown, but reduced localization of pain perception in forebrain areas is one tempting theory [78]. If so, providing cell therapy in addition to a patient-based biofeedback method and the ability to modulate such perception is an exciting possibility [79]. But more studies on a possible spinal-level mechanism for the grafts are

needed and must examine changes to the animal host rather than have a singular focus on the precise phenotype of the hNT2.17 cells. Phenotype analysis could rather be the basis for establishing the unchanging potency of the cells once their preparation is scaled-up for clinical use, i.e., 2-week differentiated hNT2.17 cells synthesize and secrete a known range of GABA and glycine per million cells, as was measured by HPLC (high-performance liquid chromatography) in vitro [31]. Such information is expected to be part of the pharmacology profile for the cells but is not necessarily required for good pain control in the patient.

Additionally, the issues regarding any required immunosuppression regime for spinal intrathecal cell transplants are complex [80], even considering possible toxicity associated with CsA use and cell therapy [19]. But some trends are seen in the current animal work with the hNT2.17 grafts: (1) a minimal course of immunosuppression with CsA, about 1 to 2 weeks after transplants, is required; (2) this minimal CsA course ensures optimal efficacy in reversal of the behavioral hypersensitivity associated with SCI-pain; (3) less than minimal immunosuppression (1 day) only provides minimal efficacy; and, (4) longer than the optimal time course of CsA does not improve efficacy significantly. The differences in recovery of CA versus TH when less than 2 weeks of immunosuppression are provided is also interesting to note. A mixed CsA effect occurs after 1 week of treatment and seems to preserve the antiallodynic effects of the transplants but not the antihyperalgesic effects. Certainly the grafts do not survive as well as when 2 weeks of CsA is provided for immunosuppression. We examined immunostained sections at the end of the experiment (data not shown), and although reliable quantification of grafts is almost impossible, there were clearly fewer surviving grafts with less than 2 weeks of CsA. A “critical” number of functioning grafted cells could influence or permanently affect CA versus TH. Also, CA is likely modulated differently than TH; this difference is seen in the timing studies. We have seen similar differences in previous studies of the effects of timing with cell therapy and a peripheral model of neuropathic pain [81]. Precise answers as to possible mechanisms are difficult, but one value of preclinical studies is that they can reveal such differences in outcomes. All further studies using the hNT2.17 cell graft to attenuate pain will include experiments addressing behavioral testing of various immunosuppressive regimens, including models of diabetic neuropathic pain and peripheral nerve injury. One possibility would be to use other types of noxious and nonnoxious stimuli, such as cold and brush stimulation, to see whether behavioral outcomes remain differently modulated by different doses of CsA.

Another important clinical issue involves optimal location of grafts for pain relief. Lumbar intrathecal transplant is easiest in humans and was used in all the earlier adrenal chromaffin cell therapy for chronic pain in humans [82–83]. But neuropathies in upper limbs are common with SCI [84] and peripheral nerve injuries [85]. Our data suggest that a cervical graft completely reverses behavioral sensitivity measured in the hindlimbs about equally as well as a lumbar graft. Although these differing graft sites were examined some 6 weeks after transplant with no surviving cervical grafts visible, such cervical grafts were efficacious for the behavioral hypersensitivity from a low thoracic SCI. At this point, it is logical to speculate that upper-limb neuropathies could be attenuated by lumbar grafts of these same cells.

The absence of grafted cells after cervical transplant was surprising, but given our group’s expertise in finding grafted cells at late time points, such as 8 to 12 weeks after lumbar intrathecal transplant, this result is believable. Our own studies [31] and others indicate that NT2 grafted cells (hNT2.17 is a subclone of this parentage) do not migrate after placement in or near the spinal cord, so migration is not a likely explanation. Only when cells were placed in the (lumbar) IT space could grafts be found (within a few segments of L1–L4). One possible explanation comes from cerebrospinal fluid (CSF) flow studies [86]. Although the lumbar IT space is larger than the cervical space, it has relatively stagnant flow to compensate for intraspinal/intracranial pressure changes. CSF flow is greatest in the cervical space, perhaps a result of the “bottleneck” at the entrance to the brain. However long the grafts might survive on the cervical pia (and they must be there for awhile to produce identical effects as lumbar placement), by the end of the experiment, they will have likely eroded into the CSF flow.

The safety of use in humans is a final preclinical question for feasibility studies. The question of possible tumorigenicity in the eventual clinical use of any differentiated cells is relevant to their characterization in vitro and in vivo [40]. The parental NT2 cells are classified as embryonic carcinoma cells because they originate from testicular germ cells and express the same cell-surface antigens during proliferation. Exposure of NT2 (proliferating)

cells to RA results in postmitotic hNT2 cells, which do not form tumors or revert to a neoplastic state with transplantation [87]. When hNT2.17 cells are treated with RA and mitotic inhibitors and differentiated in vitro, they cease to express the tumor markers TGF-α (transforming growth factor) and FGF-4 [31]. Additionally, BrdU immunostaining has been used as a marker for proliferating cells in vitro [43] and in vivo [44], since dividing cells incorporate BrdU-labeled uridine into newly made DNA. We demonstrate here that differentiated hNT2.17 neurons cannot incorporate BrDU with differentiation, in contrast to the proliferating hNT2.17 cell line, suggesting that the differentiated hNT2.17 cells do not demonstrate any features of a tumor cell line, much like the NT2-N parent. Tumor proteins are abundant in the proliferating hNT2.17 cells, suggesting that only a differentiated hNT2.17 cell would be safe to transplant in vivo. Grafting well-differentiated hNT2.17 cells into the CNS does not form tumors in rats and supports such a contention.

The mechanism of action by which the grafts attenuate behavioral hypersensitivity in this SCI model is, admittedly, a weakness of our current understanding of hNT2.17 neurons. A role for GABA as an antinociceptive molecule to modulate excitatory neurotransmission both basally and temporarily after nerve damage or SCI is suggested by a number of studies with GABA receptor agonists, such as the GABA-B agonist baclofen. Baclofen has been shown to produce presynaptic inhibition of primary afferent terminals [88], and it inhibits the release of the excitatory neurotransmitters glutamate [89] and substance P [90] from the primary afferents. Baclofen has been shown to be antinociceptive in various behavioral studies in chronic pain models [91–94], probably due to degeneration or loss of dorsal horn GABA interneurons. Clinical studies of central pain following SCI and IT injection of GABA receptor agonist baclofen have had variable results [95–96]. Baclofen reduced central pain in one patient with SCI in a double-blind clinical trial [97]. An IT bolus injection of baclofen reduced continuous central pain and allodynia/hyperalgesia in three of six patients after SCI [98]. In another study using continuous IT baclofen infusion [99], benefits were seen only for musculoskeletal pain associated with spasticity. However, tolerance to IT baclofen is one of the many confounding problems associated with its long-term use for SCI-associated therapy [100]; additional complications are associated with the use of IT catheters for mechanical pump delivery of antinociceptive agents [101]. Alteration of the endogenous spinal GABA system after SCI plays a potentially major role in the induction and maintenance of chronic pain in humans. Here, the differentiated hNT2.17 cells can not only release the inhibitory neurotransmitters GABA and glycine but also secrete them basally without stimulation. We can argue that if, indeed, the grafts make and secrete a dose of GABA and glycine near the lumbar dorsal horn sensory areas or into the CSF, the balance might be tipped toward a recovered inhibitory influence, which seems to be lost in this SCI model. Future studies will include the use of IT GABA and glycine receptor antagonists during behavioral testing in the presence and absence of grafts so that we may understand whether GABA or glycine secreted from transplants is modulating these behaviors.

The question of the nature of the allodynia and hyperalgesia observed in animal studies remains a difficult one. One recent argument is that these behavioral hypersensitivities are evoked reflexive motor behaviors rather than spontaneous painful responses. Perhaps more appropriate is the inclusion of data from the nonreflexive excessive grooming associated with QUIS injections [35] and the aggressive grooming exhibited by Wistar Furth male rats [102]. Like these and other studies with the QUIS model, such behaviors are not consistent among all animals, with at least 30 percent of rats never developing this autophagia [35]. In the current study, a group of rats that developed significant excessive grooming were followed over time, and although the data can only be reported as a trend, early transplantation of viable hNT2.17 cells with adequate immunosuppression seemed to help resolve the autophagia behaviors and decrease lesion size. Whether allodynia and hyperalgesia actually represent clinical pain remains an open question, although both are widely used to report efficacy of various treatments for neuropathic pain of central or peripheral origin.

Data presented here suggest that grafts of the hNT2.17 inhibitory human neuronal cell line reverse the behavioral hypersensitivity induced by SCI. These grafts seem to improve the ability of the spinal cord sensory system to handle the aberrant spinal processing of both painful and nonpainful stimuli that might lead to chronic neuropathic pain. These grafts also at least partially ameliorate neuropathy even with chronic SCI. Whatever mechanism is induced by the presence of these transplants, they are able to be safely placed distant from spinal injury level, adding another desirable feature to their clinical feasibility. The differentiated hNT2.17 cells appear free

from the danger of tumor formation and possess a stable neuronal phenotype making them attractive candidates for further development as a clinical pain treatment.

In summary, many preclinical questions can be easily addressed by animal studies before the expense of second-tier FDA certification and phase I trials. Further development and transplant of the human hNT2.17 cell line, which is a stable human cell line that can be stored, expanded, analyzed for absence of human pathogens, and delivered to a surgical site for use in neuropathic pain, are reasonable first steps in moving cell therapy for pain closer to clinical application.

ACKNOWLEDGMENTS

This material was based on work supported by the Department of Veterans Affairs, Veterans Health Administration, Rehabilitation Research and Development Service (grant B4438I); the American Syringomyelia Alliance Project; the Column of Hope, Chiari and Syringomyelia Research Foundation; and the continuing support of The Miami Project to Cure Paralysis and Department of Neurosurgery, Miller School of Medicine, Miami, Florida.

The authors have declared that no competing interests exist.

REFERENCES

1. Keller MP, Chance PF. Inherited neuropathies: From gene to disease. Brain Pathol. 1999;9(2):327–41. [PMID: 10219749]
2. Schwartzman RJ, Maleki J. Postinjury neuropathic pain syndromes. Med Clin North Am. 1999;83(3):597–626. [PMID: 10386117] DOI:10.1016/S0025-7125(05)70126-7
3. Benbow SJ, Cossins L, MacFarlane IA. Painful diabetic neuropathy. Diabet Med. 1999;16(8):632–44. [PMID: 10477207] DOI:10.1046/j.1464-5491.1999.00028.x
4. Koltzenburg M. Painful neuropathies. Curr Opin Neurol. 1998;11:515–21. [PMID: 9848001] DOI:10.1097/00019052-199810000-00014
5. Bennett GJ. Neuropathic pain: New insights, new interventions. Hosp Pract. (Minneap). 1998;33(10):95–98, 1014, 107–10. [PMID: 9793544]
6. Bowsher D. Pain syndromes and their treatment. Curr Opin Neurol Neurosurg. 1993;6(2):257–63. [PMID: 8097640]
7. Dworkin RH. An overview of neuropathic pain: Syndromes, symptoms, signs, and several mechanisms. Clin J Pain. 2002;18(6):343–49. [PMID: 12441827] DOI:10.1097/00002508-200211000-00001
8. Yezierski RP. Pain following spinal cord injury: The clinical problem and experimental studies. Pain. 1996;68(2–3): 185–94. [PMID: 9121805] DOI:10.1016/S0304-3959(96)03178-8
9. Siddall PJ, Taylor DA, McClellan JM, Rutkowski SB, Cousins MJ. Pain report and the relationship of pain to physical factors in the first 6 months following spinal cord injury. Pain. 1999;81(1–2):187–97. [PMID: 10353507] DOI:10.1016/S0304-3959(99)00023-8
10. Siddall PJ, McClelland JM, Rutkowski SB, Cousins MJ. A longitudinal study of the prevalence and characteristics of pain in the first 5 years following spinal cord injury. Pain. 2003;103(3):249–57. [PMID: 12791431] DOI:10.1016/S0304-3959(02)00452-9
11. Widerström-Noga EG, Felipe-Cuervo E, Broton JG, Duncan RC, Yezierski RP. Perceived difficulty in dealing with consequences of spinal cord injury. Arch Phys Med Rehabil. 1999;80(5):580–86. [PMID: 10326925] DOI:10.1016/S0003-9993(99)90203-4
12. Hama A, Sagen J. Behavioral characterization and effect of clinical drugs in a rat model of pain following spinal cord compression. Brain Res. 2007;1185:117–28. [PMID: 17935699] DOI:10.1016/j.brainres.2007.09.013
13. Hulsebosch CE, Xu GY, Perez-Polo JR, Westlund KN, Taylor CP, McAdoo DJ. Rodent model of chronic central pain after spinal cord contusion injury and effects of gabapentin. J Neurotrauma. 2000;17(12):1205–17. [PMID: 11186233] DOI:10.1089/neu.2000.17.1205
14. Tai Q, Kirshblum S, Chen B, Millis S, Johnston M, DeLisa JA. Gabapentin in the treatment of neuropathic pain after spinal cord injury: A prospective, randomized, double-blind, crossover trial. J Spinal Cord Med. 2002; 25(2):100–105. [PMID: 12137213]
15. Siddall PJ, Cousins MJ, Otte A, Griesing T, Chambers R, Murphey TK. Pregabalin in central neuropathic pain associated with spinal cord injury: A placebo-controlled trial. Neurology. 2006;67(10):1792–1800. [PMID: 17130411] DOI:10.1212/01.wnl.0000244422.45278.ff
16. Cardenas DD; Jensen MP. Treatments for chronic pain in persons with spinal cord injury: A survey study. J Spinal Cord Med. 2006;29(2):109–117. [PMID: 16739554]
17. Eaton MJ. Cell therapy for neuropathic pain in spinal cord injuries. Expert Opin Biol Ther. 2004;4(12):1861–69. [PMID: 15571449] DOI:10.1517/14712598.4.12.1861
18. Eaton MJ. Cell and molecular approaches to the attenuation of pain after spinal cord injury. J Neurotrauma. 2006; 23(3–4):549–59. [PMID: 16629636] DOI:10.1089/neu.2006.23.549
19. Sagen J. Cellular therapies for spinal cord injury: What will the FDA need to approve moving from the laboratory

to the human? J Rehabil Res Dev. 2003;40(Suppl 1):71–79. [PMID: 15077651]

20. Liu S, Ruenes GL, Yezierski RP. NMDA and non-NMDA receptor antagonists protect against excitotoxic injury in the rat spinal cord. Brain Res. 1997;756(1–2):160–67. [PMID: 9187327] DOI:10.1016/S0006-8993(97)00137-6
21. Yezierski RP, Santana M, Park SH, Madsen PW. Neuronal degeneration and spinal cavitation following intraspinal injections of quisqualic acid in the rat. J Neurotrauma. 1993;10(4):445–56. [PMID: 8145267]
22. Brewer KL, Yezierski RP. Effects of adrenal medullary transplants on pain-related behaviors following excitotoxic spinal cord injury. Brain Res. 1998;798(1–2):83–92. [PMID: 9666085] DOI:10.1016/S0006-8993(98)00398-9
23. Sagen J, Pappas GD, Pollard HB. Analgesia induced by isolated bovine chromaffin cells implanted in rat spinal cord. Proc Nat Acad Sci U S A. 1986;83(19):7522–26. [PMID: 3463981] DOI:10.1073/pnas.83.19.7522
24. Ginzburg R, Seltzer Z. Subarachnoid spinal cord transplantation of adrenal medulla suppresses chronic neuropathic pain behavior in rats. Brain Res. 1990;523(1):147–50. [PMID: 2207684] DOI:10.1016/0006-8993(90)91649-2
25. Lazorthes Y, Sagen J, Sallerin B, Tkaczuk J, Duplan H, Sol JC, Tafani M, Bes JC. Human chromaffin cell graft into the CSF for cancer pain management: A prospective phase II clinical study. Pain. 2000;87(1):19–32. [PMID: 10863042] DOI:10.1016/S0304-3959(00)00263-3
26. Lindner MD, Frydel BR, Francis JM, Cain CK. Analagesic effects of adrenal chromaffin allografts: Contingent on special procedures or due to experimental bias? J Pain. 2003;4(2):64–73. [PMID: 14622717] DOI:10.1054/jpai.2003.6
27. Sagen J, Lewis-Cullinan C, Goddard M, Burgess FW. Encapsulated cell implants for pain surgery. In: Burchiel K, editor. Pain surgery. New York (NY): Thieme; 2002. p. 958.
28. Lindner MD, Francis JM, Saydoff JA. Intrathecal polymer-encapsulated bovine adrenal chromaffin cells fail to produce analgesic effects in the hotplate and formalin test. Exp Neurol. 2000;165(2):370–83. [PMID: 10993696] DOI:10.1006/exnr.2000.7472
29. Hains BC, Chastain KM, Everhart AW, McAdoo DJ, Hulsebosch CE. Transplants of adrenal medullary chromaffin cells reduce forelimb and hindlimb allodynia in a rodent model of chronic central pain after spinal hemisection injury. Exp Neurol. 2000;164(2):426–37. [PMID: 10915581] DOI:10.1006/exnr.2000.7439
30. Lindner MD. Clinical attrition due to biased preclinical assessments of potential efficacy. Pharmacol Ther. 2007; 115(1):148–75. [PMID: 17574680] DOI:10.1016/j.pharmthera.2007.05.002
31. Eaton MJ, Wolfe SQ, Martinez MA, Hernandez M, Furst C, Huang J, Frydel BR, Gomez-Marin O. Subarachnoid transplant of a human neuronal cell line attenuates chronic allodynia and hyperalgesia after excitotoxic SCI in the rat. J Pain. 2007;8(1):33–50. [PMID: 17207742] DOI:10.1016/j.jpain.2006.05.013
32. Yoshioka A, Yudkoff M, Pleasure D. Expression of glutamic acid decarboxylase during human neuronal differentiation: Studies using the NTera-2 culture system. Brain Res. 1997;767(2):333–39. [PMID: 9367265] DOI:10.1016/S0006-8993(97)00627-6
33. Andrews PW, Damjanov I, Simon D, Banting GS, Carlin C, Dracopoli NC, Fogh J. Pluripotent embryonal carcinoma clones derived from human teratocarcinoma cell line Tera-2. Lab Invest. 1984;50(2):147–62. [PMID: 6694356]
34. Cheung WM, Fu WY, Hui WS, Ip NY. Production of human CNS neurons from embryonal carcinoma cells using a cell aggregation method. Biotechniques. 1999; 26(5):946–54. [PMID: 10337488]
35. Yezierski RP, Liu S, Ruenes GL, Kajander KJ, Brewer KL. Excitotoxic spinal cord injury: Behavioral and morphological characteristics of a central pain model. Pain. 1998;75(1):141–55. [PMID: 9539683] DOI:10.1016/S0304-3959(97)00216-9
36. Hargreaves K, Dubner R, Brown F, Flores C, Joris J. A new and sensitive method for measuring thermal nociception in cutaneous hyperalgesia. Pain. 1988;32(1):77–88. [PMID: 3340425] DOI:10.1016/0304-3959(88)90026-7
37. Chaplan SR, Bach FW, Pogrel JW, Chung JM, Yaksh TL. Quantitative assessment of tactile allodynia in the rat paw. J Neurosci Meth. 1994;53(1):55–63. [PMID: 7990513] DOI:10.1016/0165-0270(94)90144-9
38. Saporta S, Willing AE, Colina LO, Zigova T, Milliken M, Daadi MM, Sanberg PR. In vitro and in vivo characterization of hNT neuron neurotransmitter phenotypes. Brain Res Bull. 2000;53(3):263–68. [PMID: 11113579] DOI:10.1016/S0361-9230(00)00329-4
39. Zhang A-L, Hao J-X, Seiger A, Xu X-J, Wiesenfeld-Hallin Z, Grant G, Aldskogius H. Decreased GABA immunoreactivity in spinal cord dorsal horn neurons after transient spinal cord ischemia in the rat. Brain Res. 1994; 656(1):187–90. [PMID: 7804836] DOI:10.1016/0006-8993(94)91383-8
40. Newman MB, Misiuta I, Willing AE, Zigova T, Karl RC, Borlongan CV, Sanberg PR. Tumorigenicity issues of embryonic carcinoma-derived stem cells: Relevance to surgical trials using NT2 and hNT neural cells. Stem Cells Devel. 2005;14(1):29–43. [PMID: 15725742] DOI:10.1089/scd.2005.14.29
41. Dmitrovsky E, Moy D, Miller WH, Li A, Masui H. Retinoic acid causes a decline in TGF-alpha expression, cloning efficiency, and tumorigenicity in a human embryonal cancer cell line. Oncogene Res. 1990;5(3):233–39. [PMID: 2320376]

42. Kondziolka D, Wechsler L, Goldstein S, Meltzer C, Thulborn KR, Gebel J, Jannetta P, DeCesare S, Elder EM, MocGrogan M, Reitman MA, Bynum L. Transplantation of cultured human neuronal cells for patients with stroke. Neurology. 2000;55(4):565–69. [PMID: 10953194]
43. Yong VW, Kim SU. A new double labelling immunofluorescence technique for the determination of proliferation of human astrocytes in culture. J Neurosci Meth. 1987; 21(1):9–16. [PMID: 3309485] DOI:10.1016/0165-0270(87)90098-7
44. Brown D, Stanfield B. The use of bromodeoxyuridine-immunohistochemistry to identify transplanted fetal brain tissue. J Neural Transplant. 1989;1(3–4):135–39. [PMID: 2519541]
45. Andrews PW. Retinoic acid induces neuronal differentiation of a cloned human embryonal carcinoma cell line in vitro. Dev Biol. 1984;103(2):285–93. [PMID: 6144603] DOI:10.1016/0012-1606(84)90316-6
46. Pleasure SJ, Lee VM. NTera 2 cells: A human cell line which displays characteristics expected of a human committed neuronal progenitor cell. J Neurosci Res. 1993;35(6): 585–602. [PMID: 8411264] DOI:10.1002/jnr.490350603
47. Borlongan CV, Tajima Y, Trojanowski JQ, Lee VMY, Sanberg PR. Transplantation of cryopreserved human embryonal carcinoma-derived (NT2N cells) promotes functional recovery in ischemic rats. Exp Neurol. 1998; 149(2):310–21. [PMID: 9500961] DOI:10.1006/exnr.1997.6730
48. Ferrare A, Ehler E, Nitsch RM, Gotz J. Immature human NT2 cells grafted into mouse brain differentiate into neuronal and glial cell types. FEBS Lett. 2000;486(2):121–25. [PMID: 11113451] DOI:10.1016/S0014-5793(00)02251-1
49. Cheung WM, Chu AH, Leung MF, Ip NY. Induction of trk receptors by retinoic acid in a human embryonal carcinoma cell line. Neuroreport. 1996;7(6):1204–8. [PMID: 8817533] DOI:10.1097/00001756-199604260-00022
50. Guillemain I, Fontes G, Privat A, Chaudieu I. Early programmed cell death in human NT2 cell cultures during differentiation induced by all-trans-retinoic acid. J Neurosci Res. 2003;71(1):38–45. [PMID: 12478612] DOI:10.1002/jnr.10458
51. Guillemain I, Alonso G, Patey G, Privat A, Chaudieu I. Human NT2 neurons express a large variety of neurotransmission phenotypes in vitro. J Comp Neurol. 2000; 422(3):380–95. [PMID: 10861514] DOI:10.1002/1096-9861(20000703)422:3<380::AID-CNE5>3.0.CO;2-C
52. Megiorni F, Mora B, Indovina P, Mazzilli MC. Expression of neuronal markers during NTera2/cloneD1 differentiation by cell aggregation method. Neurosci Lett. 2005;373(2): 105–9. [PMID: 15567562] DOI:10.1016/j.neulet.2004.09.070
53. Satoh J, Kuroda Y. Differential gene expression between human neurons and neuronal progenitor cells in culture: An analysis of arrayed cDNA clones in NTera2 human embryonal carcinoma cell line as a model system. J Neurosci Meth. 2000;94(2):155–64. [PMID: 106661835] DOI:10.1016/S0165-0270(99)00143-0
54. Lee VM, Andrews PW. Differentiation of NTERA-2 clonal human embryonal carcinoma cells into neurons involves the induction of all three neurofilament proteins. J Neurosci. 1986;6(2):514–21. [PMID: 2419526]
55. Daadi MM, Saporta S, Willing AE, Zigova T, McGrogan MP, Sanberg PR. In vitro induction and in vivo expression of bcl-2 in the hNT neurons. Brain Res Bull. 2001;56(2): 147–52. [PMID: 11704352] DOI:10.1016/S0361-9230(01)00621-9
56. Gortz P, Fleischer W, Rosenbaum C, Otto FR, Siebler M. Neuronal network properties of human teratocarcinoma cell line-derived neurons. Brain Res. 2004;1018(1):18–25. [PMID: 15262200] DOI:10.1016/j.brainres.2004.05.076
57. Trojanowski JQ, Kleppner SR, Hartley RS, Miyazono M, Fraser NW, Kesari S, Lee VM. Transfectable and transplantable postmitotic human neurons: Potential “platform” for gene therapy of nervous system diseases. Exp Neurol. 1997;144(1):92–97. [PMID: 9126157] DOI:10.1006/exnr.1996.6393
58. Nelson PT, Kondziolka D, Wechsler L, Goldstein S, Gebel J, DeCesare S, Elder EM, Zhang PJ, Jacobs A, McGrogan M, Lee VM, Trojanowski JQ. Clonal human (hNT) neuron grafts for stroke therapy: Neuropathology in a patient 27 months after implantation. Am J Pathol. 2002;160(4): 1201–6. [PMID: 11943704]
59. Meltzer CC, Kondziolka D, Villermagne VL, Wechsler L, Goldstein S, Thulborn KR, Gebel J, Elder EM, DeCesare S, Jacobs A. Serial [18F] fluorodeoxyglucose positron emission tomography after human neuronal implantation for stroke. Neurosurgery. 2001;49(3):586–92. [PMID: 11523668] DOI:10.1097/00006123-200109000-00011
60. Van Bekkum DW. Stem cell transplantation for autoimmune disorders. Preclinical experiments. Best Pract Res Clin Haematol. 2004;17(2):201–22. [PMID: 15302335] DOI:10.1016/j.beha.2004.04.003
61. Eaton MJ. Common animal models of spasticity and pain. J Rehabil Res Dev. 2003;40(4 Suppl 1):41–54. [PMID: 15077648]
62. Vierck CJ, Hansson PT, Yezierski RP. Clinical and preclinical pain assessment: Are we measuring the same thing? Pain. 2008;135(1–2):7–10. [PMID: 18215466] DOI:10.1016/j.pain.2007.12.008
63. Yang L, Jones NR, Stoodley MA, Blumbergs PC, Brown CJ. Excitotoxic model of post-traumatic syringomyelia in

the rat. Spine. 2001;26(17):1842–49. [PMID: 11568692] DOI:10.1097/00007632-200109010-00004

64. Yezierski RP. Pain following spinal cord injury: Pathophysiology and central mechanisms. Prog Brain Res. 2000; 129:429–48. [PMID: 11098709] DOI:10.1016/S0079-6123(00)29033-X
65. Lindsey AE, LoVerso RL, Tovar CA, Hill CE, Beattie MS, Bresnahan JC. An analysis of changes in sensory thresholds to mild tactile and cold stimuli after experimental spinal cord injury in the rat. Neurorehabil Neural Repair. 2000;14(4):287–300. [PMID: 11402879]
66. Bruce JC, Oatway MA, Weaver LC. Chronic pain after clip-compression injury of the rat spinal cord. Exp Neurol. 2002;178:33–48. [PMID: 12460606] DOI:10.1006/exnr.2002.8026
67. Hains BC, Johnson KM, McAdoo DJ, Eaton MJ, Hulsebosch CE. Engraftment of immortalized serotonergic neurons enhances locomotor function and attenuates pain-like behavior following spinal hemisection injury in the rat. Exp Neurol. 2001;171(2):361–78. DOI:10.1006/exnr.2001.7751
68. Hains BC, Fullwood SD, Eaton MJ, Hulsebosch CE. Subdural engraftment of serotonergic neurons following spinal hemisection restores spinal serotonin, downregulates the serotonin transporter, and increases BDNF tissue content in the rat. Brain Res. 2001;913(1):35–46. [PMID: 11532245] DOI:10.1016/S0006-8993(01)02749-4
69. Hains BC, Johnson KM, Eaton MJ, Willis WD, Hulsebosch CE. Serotonergic neural precursor cell grafts attenuate bilateral hyperexcitability of dorsal horn neurons after spinal hemisection in rat. Neuroscience. 2003;116(4):1097–1110. [PMID: 12617951] DOI:10.1016/S0306-4522(02)00729-7
70. Hains BC, Yucra JA, Eaton MJ, Hulsebosch CE. Intralesion transplantation of serotonergic precursors enhances locomotor recovery but has no effect on development of chronic central pain following hemisection injury in rats. Neurosci Lett. 2002;324(3):222–26. [PMID: 12009528] DOI:10.1016/S0304-3940(02)00194-5
71. Wolfe SQ, Garg M, Cumberbatch NM, Furst C, Martinez M, Hernandez M, Reimers R, Berrocal Y, Gómez-Marín O, Eaton MJ. Optimizing the transplant dose of a human neuronal cell line graft to treat SCI pain in the rat. Neurosci Lett. 2007;414(2):121–25. [PMID: 17306458] DOI:10.1016/j.neulet.2006.10.067
72. Wolfe SQ, Cumberbatch NM, Menendez I, Martinez M, Eaton MJ. Ronald Tasker Award: Intrathecal transplantation of a human neuronal cell line for the treatment of neuropathic pain in a spinal cord injury model. Clin Neurosurg. 2007;54:220–25. [PMID: 18504923] DOI:10.1227/01.neu.0000279957.81515.85
73. Buchser E, Goddard M, Heyd B, Joseph JM, Favre J, De Tribolet N, Lysaght M, Aebischer P. Immunoisolated xenogenic chromaffin cell therapy for chronic pain. Initial clinical experience. Anesthesiology. 1996;85(5):1005–12. [PMID: 8916816] DOI:10.1097/00000542-199611000-00007
74. Siegan JB, Sagen J. Attenuation of formalin pain responses in the rat by adrenal medullary transplants in the spinal subarachnoid space. Pain. 1997;70(2–3):279–85. [PMID: 9150303] DOI:10.1016/S0304-3959(97)03335-6
75. Eaton MJ, Herman JP, Jullien N, Lopez T, Martinez M, Huang J. Immortalized chromaffin cells disimmortalized with Cre/lox site-directed recombination for use in cell therapy for pain. Exp Neurol. 2002;175(1):49–60. [PMID: 12009759] DOI:10.1006/exnr.2002.7883
76. Lindner MD, Francis JM, Plone MA, McDermott PE, Frydel BR, Emerich DF, Saydoff JA. The analgesic potential of intraventicular polymer-encapsulated adrenal chromaffin cells in a rodent model of chronic neuropathic pain. Exp Clin Psychopharmacol. 2000;8(4):524–38. [PMID: 11127424] DOI:10.1037/1064-1297.8.4.524
77. Yu W, Hao JX, Xu X-J, Saydoff J, Sherman S, Eriksson A, Haegerstrand A, Wiesenfeld-Hallin Z. Immunoisolating encapsulation of intrathecally implanted bovine chromaffin cells prolongs their survival and produces antiallodynic effect in spinally injured rats. Eur J Pain. 1998; 2(2):143–51. [PMID: 10700310] DOI:10.1016/S1090-3801(98)90007-6
78. Schweinhardt P, Glynn C, Brooks J, McQuay H, Jack T, Chessell I, Bountra C, Tracey I. An fMRI study of cerebral processing of brush-evoked allodynia in neuropathic pain patients. Neuroimage. 2006;32(1):256–65. [PMID: 16679031] DOI:10.1016/j.neuroimage.2006.03.024
79. DeCharms RC, Maeda F, Glover GH, Ludlow D, Pauly JM, Soneji D, Gabrieli JDE, Mackey SC. Control over brain activation and pain learned by using real-time functional MRI. Proc Nat Acad. Sci U S A. 2006;102(51): 18626–31. [PMID: 16352728] DOI:10.1073/pnas.0505210102
80. Barker RA, Widner H. Immune problems in central nervous system cell therapy. NeuroRx. 2004;1(4):472–81. [PMID: 15717048] DOI:10.1602/neurorx.1.4.472
81. Stubley LA, Martinez MA, Karmally S, Lopez T, Cejas PJ, Eaton MJ. Only early intervention with GABA cell therapy is able to reverse neuropathic pain after partial nerve injury. J Neurotrauma. 2001;18(4):471–77. [PMID: 11336447] DOI:10.1089/089771501750171092
82. Bés JC, Tkaczuk J, Czech KA, Tafani M, Bastide R, Caratero C, Pappas GD, Lazorthes Y. One-year chromaffin cell allograft survival in cancer patients with chronic pain: Morphological and functional evidence. Cell Transplant.

1998;7(3):227–38. [PMID: 9647432] DOI:10.1016/S0963-6897(97)00161-9

83. Lazorthes Y, Sallerin B, Verdie JC, Sol JC, Duplan H, Tkaczuk J, Tafani M, Bastide R, Bes JC. [Management of intractable cancer pain: From intrathecal morphine to cell allograft.] Neurochirurgie. 2000;46(5):454–65. French. [PMID: 11084478]
84. Dalyan M, Cardenas DD, Gerard B. Upper extremity pain after spinal cord injury. Spinal Cord. 1999;37(3):191–95. [PMID: 10213328] DOI:10.1038/sj.sc.3100802
85. Bennett GJ. Chronic pain due to peripheral nerve damage: An overview. In: Fields HL, Liebeskind JC, editors. Pharmacological approaches to the treatment of chronic pain: New concepts and critical issues. Seattle (WA): IASP Press; 1994. p. 51.
86. Enzmann DR, Pelc NJ. Normal flow patterns of intracranial and spinal cerebrospinal fluid defined with phase-contrast cine MR imaging. Radiology. 1991;178(2):467–74. [PMID: 1987610]
87. Trojanowski JQ, Mantione JR, Lee JH, Seid DP, You T, Inge LJ, Lee VM. Neurons derived from a human tetratocarcinoma cell line establish molecular and structural polarity following transplantation into the rodent brain. Exp Neurol. 1993;122(2):283–94. [PMID: 8405265] DOI:10.1006/exnr.1993.1128
88. Henry JL. Pharmacological studies on the prolonged depressant effects of baclofen on lumbar dorsal horn units in the cat. Neuropharmacology. 1982;21(11):1085–93. [PMID: 7177339] DOI:10.1016/0028-3908(82)90165-4
89. Johnston GA, Hailstone MH, Freeman CG. Baclofen: Stereoselective inhibition of excitatory amino acid release. J Pharm Pharmacol. 1980;32(3):230–31. [PMID: 6103949]
90. Malcangio M, Bowery NG. Spinal cord SP release and hyperalgesia in monoarthritic rats: involvement of the GABA B receptor system. Brit J Pharmacol. 1994;113(4): 1561–66. [PMID: 7534191]
91. Aran S, Hammond DL. Antagonism of baclofen-induced antinociception by intrathecal administration of phaclofen or 2-hydroxy-saclofen, but not delta-aminovaleric acid in the rat. J Pharmacol Exp Ther. 1991;257(1):360–68.
92. Hammond DL, Washington JD. Antagonism of L-baclofen-induced antinociception by CGP 35348 in the spinal cord of the rat. Eur J Pharmacol. 1993;234(2–3): 255–62. [PMID: 8387011] DOI:10.1016/0014-2999(93)90961-G
93. Hao JX, Xu XJ, Yu YX, Seiger A, Wiesenfeld-Hallin Z. Baclofen reverses the hypersensitivity of dorsal horn wide dynamic range neurons to mechanical stimulation after transient spinal cord ischemia; Implications for a tonic GABAergic inhibitory control of myelinated fiber input. J Neurophysiol. 1992;68(2):392–96. [PMID: 1527566]
94. Smith DF. Stereoselectivity of spinal neurotransmission: Effects of baclofen enantiomer on tail-flick reflex in rats. J Neural Transm. 1984;60(1):63–67. [PMID: 6090584] DOI:10.1007/BF01254766
95. Fromm GH. Baclofen as an adjuvant analgesic. J Pain Symptom Manage. 1994;9(8):500–509. [PMID: 7852758] DOI:10.1016/0885-3924(94)90111-2
96. Slonimski M, Abram SE, Zuniga RE. Intrathecal baclofen in pain management. Reg Anesth Pain Med. 2004;29(3): 269–76. [PMID: 15138913]
97. Herman RM, D'Luzansky SC, Ippolito R. Intrathecal baclofen suppresses central pain in patients with spinal lesions. A pilot study. Clin J Pain. 1992;8(4):338–45. [PMID: 1493344] DOI:10.1097/00002508-199212000-00008
98. Taira T, Kawamura H, Tanikawa T, Iseki H, Kawabatake H, Takakura K. A new approach to control central deafferentation pain: Spinal intrathecal baclofen. Stereotact Funct Neurosurg. 1995;65(1–4):101–5. [PMID: 8916336] DOI:10.1159/000098905
99. Loubser PG, Akman NM. Effects of intrathecal baclofen on chronic spinal cord injury pain. J Pain Symptom Manage. 1996;12(4):241–47. [PMID: 8898508] DOI:10.1016/0885-3924(96)00152-2
100. Berg-Johnsen J, Røste G, Solgaard T, Lundar T. [Continuous intrathecal infusion of baclofen. A new therapeutic method for spasticity.] Tidsskr Nor Laegeforen. 1998;118(21): 3256–60. Norwegian. [PMID: 9772811]
101. Follet KA, Naumann CP. A prospective study of catheter-related complications of intrathecal drug delivery systems. J Pain Symptom Manage. 2000;19(3):209–15. [PMID: 10760626] DOI:10.1016/S0885-3924(99)00153-0
102. Gorman AL, Yu CG, Ruenes G, Daniels L, Yezierski RP. Conditions affecting the onset, severity, and progression of a spontaneous pain-like behavior after excitotoxic spinal cord injury. J Pain. 2001;2(4):229–40. [PMID: 14622821] DOI:10.1054/jpai.2001.22788

Submitted for publication February 6, 2008. Accepted in revised form May 9, 2008.