

Ronald Tasker Award: Intrathecal Transplantation of a Human Neuronal Cell Line for the Treatment of Neuropathic Pain in a Spinal Cord Injury Model

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Neuropathic pain is prevalent after spinal cord injury (SCI), affecting up to 40% of this population.¹¹ Although difficult to define, neuropathic pain is generally accepted to be pain of neuronal origin, resulting in unpleasant burning, stabbing, and dysesthetic sensations unrelated to mechanical pain. The mechanism is incompletely understood, but an imbalance between excitatory and inhibitory spinal systems seems to play a significant role. Melzack and Walls⁸ proposed the gate theory in 1965, postulating that the interneurons of the dorsal horn function as modulators of afferent excitatory impulses of the spinothalamic tract and that the larger fibers of the dorsal column system contribute to pre-synaptic inhibition. The presence and function of these inhibitory γ -aminobutyric acid (GABA)-ergic interneurons, located in Rexed laminae II to V, are markedly diminished after injury,⁶ causing unimpeded ascending excitatory signals to reach the brain. Without descending or local inhibition, excitatory pathways, such as A- δ and C pain fibers predominate, leading to hypersensitivity and neuropathic pain.

It follows that replacement of inhibitory neurotransmitters in the region of the dorsal horn may prevent and alleviate hypersensitivity, which is supported by the reversal of neuropathic pain after peripheral nerve injury with an intrathecal GABA injection.³ Because intrathecal pumps are not feasible because of the labile nature of bioactive neurotransmitters, we sought to develop a cell line that could function as a biological “minipump,” synthesizing and secreting inhibitory neurotransmitters. This cell line needed to be a stable, readily available, and reproducible source of human cells that could be developed for clinical use.

More than 20 years ago, it was discovered that a human embryonal carcinoma cell line, NTera2cl.D/1 (NT2), differentiates irreversibly into several morphologically and phenotypically distinct cell types when treated with retinoic acid (RA).⁹ One of these cell lines includes terminally differenti-

ated postmitotic central nervous system (CNS) neurons, and has been extensively characterized both in vivo and in vitro.⁹ This neuronal cell line has previously been used clinically for cerebral transplantation in stroke patients by Kondziolka et al.⁷ The senior author (ME) subcloned, isolated, and characterized a daughter cell line, called hNT2.17, which permanently differentiates into a human neuronal phenotype that secretes inhibitory neurotransmitters, such as GABA and glycine.⁴

The hNT2.17 cell line was used in a pain model of excitotoxic SCI previously described by Yeziarski.¹³ Using an intraspinal injection of the glutamate receptor agonist, quisqualic acid (QUIS), this model results in neuronal loss, cavitation, and demyelination, similar to that found in post-traumatic syringomyelia. This injury results in spontaneous and evoked hypersensitivity behaviors associated with neuropathic pain. When transplanted into the subarachnoid space of SCI rats, the hNT2.17 cell line results in complete recovery from behavioral hypersensitivity after QUIS injury. This novel treatment is efficacious, durable, and without the adverse side effects of current pharmacotherapy for neuropathic pain.

MATERIALS AND METHODS

Development and Characterization of the hNT2.17 Cell Line

A human neuronal cell line was subcloned from the parental NTera2cl.D/1 (NT2)⁹ cell line by serial dilution, using a rapid aggregation method² for RA treatment and differentiation into a human neuronal phenotype. From several derived NT2 neuronal cell lines, the hNT2.17 cell line, which produced the inhibitory neurotransmitters GABA and glycine, was chosen for further characterization and transplantation into a SCI pain model.

As we have previously described the procedure of hNT2.17 cell growth and proliferation in detail,⁴ we will present here a brief summary. Cultures of hNT2.17 cells were grown at 37°C in proliferation medium (Dulbecco’s Modified

Eagle Medium/Ham's F12/10% fetal bovine serum/2 mmol/L l-glutamine/1% Pen-Strep [Invitrogen Corp., Carlsbad, CA]). When cells were near 100% confluence, they were replated into 10 $\mu\text{mol/L}$ all-trans RA media (Sigma Chemical Co., St. Louis, MO) for 2 weeks. After removal with 0.5 mmol/L ethylenediaminetetraacetic acid (EDTA), centrifugation, and resuspension, the cells were replated into culture dishes coated with mouse laminin and poly-L-lysine. Cultures were continued in proliferation media at a pH of 7.4 for 9 to 24 hours before the addition of cytosine-d-arabino-furanoside (araC) (Sigma Chemical Co.; 1 $\mu\text{mol/L}$) and uridine (Sigma Chemical Co.; 10 $\mu\text{mol/L}$) for nonneuronal growth inhibition. After 7 days, cells were briefly exposed to warmed trypsin/0.5 mmol/L EDTA, and adherent surface cells removed. The cells were again centrifuged, resuspended, and replated on mouse laminin and poly-L-lysine in proliferation media for 2 weeks before transplantation.

To examine the neurotransmitter content and release in differentiated hNT2.17 cells, high-performance liquid chromatography (HPLC) analysis was performed in vitro for both GABA and glycine, as described previously.⁴ Immunohistochemistry was performed in vitro for GABA, vesicular inhibitory amino acid transporter (VIAAT), glycine, nuclear matrix antigen (NuMA), neurofilaments (NF), human neuron-specific enolase (NSE), and β -tubulin III to characterize the neuronal nature of the differentiated cells, as previously described.⁴ Immunohistochemistry was performed in vitro for tumor growth factor (TGF)- α , fibroblast growth factor (FGF)-4 and bromodeoxyuridine (BrdU) to show nontumorigenicity of the hNT2.17 cell line, as previously described.⁴ Negative antibody controls were used for all immunohistochemistry to ensure the validity of the stains.

Animal Studies

All animal care, interventions, and euthanasia were in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23) revised 1996 and guidelines provided by the Animal Care and Use Committees of the Veteran's Association Medical Center and the University of Miami. All behavioral testing was performed under blinded conditions to eliminate experimental bias. Data was analyzed and unblinded by the statistician at the end of the experiment.

Forty-eight adult male Wistar-Furth rats weighing 250 to 300 g were randomized into four groups ($n = 12$): naïve; QUIS alone; QUIS plus viable cell transplant; and QUIS plus nonviable cell transplant. The rats were housed two per cage with rat chow and water ad libitum on a 12/12 hour light/dark cycle. All animals were acclimated to cutaneous allodynia (CA) and thermal hyperalgesia (TH) behavioral testing, which was performed weekly for the duration of the 60-day experiment. CA, the occurrence of foot withdrawal in response to normally innocuous mechanical stimuli, was tested

using an electronic von Frey anesthesiometer.¹ Animals were placed in a Plexiglas box with an elevated mesh floor and acclimated for 5 minutes. The von Frey tip was applied perpendicularly to the midplantar area of each hind paw and depressed slowly until the animal withdrew from the pressure (in grams), which was recorded for each of three trials. These scores were averaged and the standard errors of the mean (SEM) was calculated. TH, or hind paw withdrawal from a normally innocuous heat source, was tested with a Hargreave's heat source.⁵ Animals were placed in a clear Plexiglas box on an elevated floor and allowed to acclimate for 5 minutes. A radiant heat source with constant intensity was aimed at the midplantar area of the hind paw. The time, in seconds, from initial heat source activation to paw withdrawal was recorded. Five minutes were allowed between the first and second stimulations, and 8 minutes between the second and third. Three latency measurements for each paw were recorded and the mean and SEM calculated for each hind paw.

An excitotoxic SCI, as described by Yeziarski,¹³ was performed on all animals in the QUIS injury groups on Day 0. Animals were anesthetized with a mixture of ketamine, xylazine, and acepromazine (0.65 mL/kg) and absence of blink and withdrawal reflexes were ensured. A laminectomy was performed between T12-L1, and the rat was placed in a stereotaxic frame. After a dural incision, a Hamilton syringe was used to inject 125 mmol/L of QUIS into the right dorsal horn. The injection was performed 1000 μm below the surface of the cord in three injections measuring 500 μm apart. Each injection was 0.4 μl , 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 the spinal laminae IV to VI. A small piece of sterile durafilm was then placed over the dura (to protect the spinal cord and facilitate reopening the dura for transplantation), and the fascia and skin were closed. In accordance with the guidelines provided by the Animal Care and Use Committees of the Veteran's Association Medical Center and the University of Miami, no postoperative analgesics were used to prevent interference with the cellular treatment for pain.

Two weeks after injury (Day 14), the animals to be transplanted received a lumbar intrathecal cell graft with either viable hNT2.17 cells (a dose of 10^6 cells/injection, differentiated for 2 weeks in vitro before transplant) or nonviable hNT2.17 cells (10^3 cells/injection). On the day of transplant, cells were rinsed with warmed Cellstripper (Voigt Global Distribution, Kansas City, MO), the media replaced with another 3 ml of Cellstripper for 1 minute, and then 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 μl of $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free (CMF) HBSS. Nonviable hNT2.17 cells were prepared by centrifuging the cells in sterile water, assuring nonviability, and resuspending them in CMF-HBSS for transplant. The appropriate cell dosage was prepared immediately before each transplant to assure nearly 100% viability at the beginning of the experiment, and transplantation was within 30 minutes of cell preparation.

The animals were anesthetized as previously described, the previous incision at T12–L1 was reopened, the dura incised, and a 2 to 3 mm segment of polyethylene tubing (PE-10) passed caudally into the intrathecal sac, through which the cells were injected. All groups received cyclosporine immunosuppression (intraperitoneal, 100 mg/kg daily) for 1 day before transplant and 14 days after transplant.

Sixty days after QUIS injury, the animals were killed to examine cell graft survival. Rats were euthanized for tissue fixation by a combination of pentobarbital overdose and exsanguination. Transcardial perfusion with 4% paraformaldehyde and 0.1% gluteraldehyde was performed and the spinal cord was removed and stored in fixative for 12 hours at 4°C. The cords were then cryoprotected by equilibration in 30% sucrose and phosphate-buffered saline for several days at 4°C. Cords were embedded in Shandon-1 Embedding Matrix (Thermo Electron Corp., Waltham, MA) and sagittally cut in sequential 20- μm sections with a Cryostat (Leica 1900; Leica). Every second section was stained for the human marker, NuMA, or GABA to identify the grafted human cell line, as previously described.⁴

RESULTS

hNT2.17 Characterization

After 2 weeks of differentiation in RA, the hNT2.17 cell line exclusively demonstrates the morphology of mature CNS neurons. The cells develop long processes as quickly as after 1 day in vitro and, within 2 weeks, aggregate and form dense networks of dendritic fibers (Fig. 36.1). The hNT2.17 cells have been kept for as long as 50 days of differentiation in culture, forming very dense fiber networks that cover the plate surface. In addition to adopting a neuronal morphology after retinoic differentiation, the hNT2.17 cells express human neural markers. As soon as after 4 days of differentiation, β -tubulin, human NSE, NF light chain, and NF medium chain were present and stained intensely until at least 6 weeks of differentiation in vitro. Immunoreactivity for NF heavy chain, a marker for mature NF, appeared at 28 days of differentiation. The human-specific NuMA, commonly used to identify NT2 neurons in vitro and in vivo,⁷ was abundant in all differentiating hNT2.17 cells. Immunostaining for glial cells with glial fibrillary acidic protein or vimentin antibodies showed no uptake of signal.

All hNT2.17 cells stain for the inhibitory neurotransmitters GABA and glycine, with 100% colocalization in

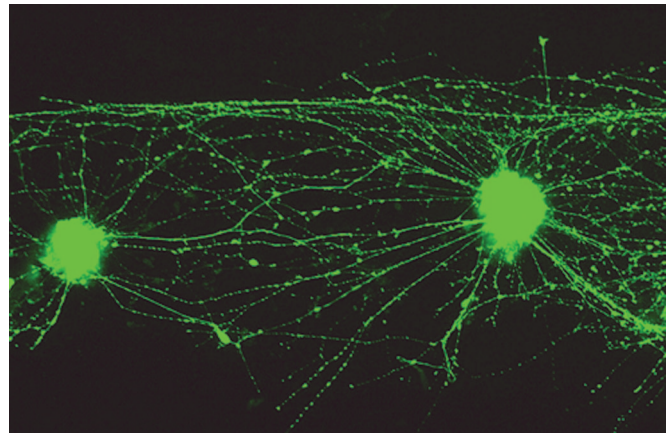


FIGURE 36.1. GABA immunofluorescence showing the mature neuronal phenotype, cellular aggregation, and a dense network of dendritic fibers in a differentiated hNT2.17 cell culture at 5 weeks.

vitro. Both the cell soma and dendritic fibers show strong immunofluorescence, and bouton-like structures, or vesiculations, with concentrated GABA and glycine signal, are seen (Fig. 36.2). Other elements of the GABA phenotype appear early during differentiation. Both the GAT3 GABA transporter protein and the VIAAT are abundant at 2 weeks of differentiation. With continued differentiation, the antibody signal for the vesicular GABA transporter VIAAT becomes concentrated in bouton-like structures, similar to the GABA signal in late differentiated cells.

HPLC analysis of GABA and glycine (Fig. 36.3) content, basal secretion in the presence of physiological K^+ (2.95 mmol/L) and stimulated release in the presence of high K^+ (100 mmol/L) in the media was performed at 2 weeks differentiation, and showed significant in vitro neurotransmitter secretion.⁴ The mean \pm SEM GABA content was

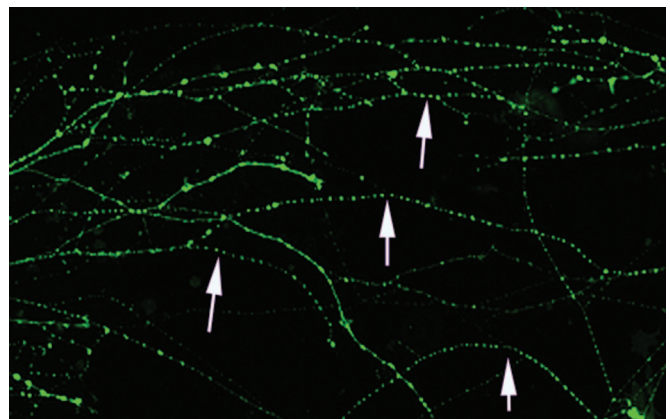


FIGURE 36.2. GABA immunofluorescence showing dendritic fibers with numerous densely fluorescent secretory vesicles, containing concentrations of GABA.

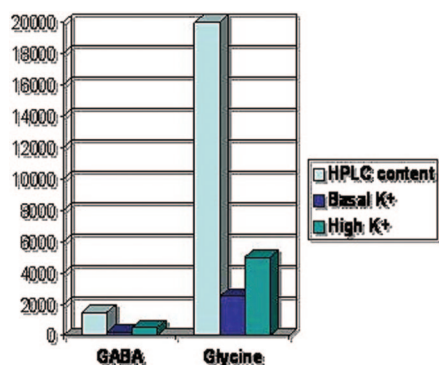


FIGURE 36.3. In vitro HPLC showing the GABA and glycine content, basal secretion, and stimulated secretion per 1 million hNT2.17 cells.

1554.7 ± 186.6 pmoles per 10⁷ cells, with basal release of 303.5 ± 29.7 pmoles per 10⁷ cells and stimulated release of 497.5 ± 81.8 pmoles per 10⁷ cells during 15 minutes. Glycine content (2026.4 ± 159.8 pmoles per 10⁵ cells), secretion (278.3 ± 15.5 per 10⁵ cells) and stimulated release (584.3 ± 24.4 per 10⁵ cells) in the presence of basal and high concentrations of K⁺ was approximately 10 times higher than that for GABA 100,000 cells for glycine.

The hNT2.17 cells also synthesize low to moderate levels of vasopressin and oxytocin, as well as low, but apparent, signals for the inhibitory opioid peptides met-enkephalin and neuropeptide Y, at 6 weeks of differentiation (Table 36.1). No other significant neurotransmitter markers have been found by immunohistochemical methods in the hNT2.17 cell line, including catecholamine enzymes (tyrosine hydroxylase, dopamine β hydroxylase, acetylcholine, substance P, galanin, or serotonin).⁴

Safety is paramount to the usefulness of the hNT2.17 cell line in human studies. Exposure of proliferating NT2 cells to RA results in postmitotic hNT2 cells, which do not form tumors or revert to a neoplastic state with transplantation.¹⁰ The hNT2.17 cells do not stain for BrdU, a marker for proliferating cells because of its incorporation into newly made deoxyribonucleic acid (DNA), after BrdU exposure during the first week of differentiation.⁴ In addition, differentiated hNT2.17 cells cease to express markers of tumorigenicity. Differentiated hNT2.17 cells stop producing the protein TGF-α, involved in stimulation of cell proliferation, and FGF-4, abundant in a subset of germ cell cancers for malignant growth promotion.⁴

Behavioral Results

Analysis of sensory behavior (CA and TH) was determined using the combined mean and SEM for ipsilateral hind paw withdrawal in each experimental group. A parametric one-way analysis of variance (ANOVA) was used to determine statistical significance (*P* < 0.05).

TABLE 36.1. Neurotransmitters synthesized by the hNT2.17 cell line

γ-Aminobutyric acid
Glycine
Met-enkephalin
Oxytocin
Neuropeptide Y
Vasopressin

Animals undergoing viable hNT2.17 cell transplantation demonstrated complete reversal of all hypersensitivity (*P* < 0.001). The recovery occurred 1 week after transplantation and was maintained for the 60-day course of the experiment.

The assessment of CA (Fig. 36.4) showed baseline withdrawal values of 30.36 ± 0.80 g to 35.57 ± 0.76 g. The naïve control group maintained these values throughout the 60-day duration of the experiment. All animals undergoing excitotoxic QUIS injury demonstrated significant behavioral hypersensitivity ipsilateral to the site of injury that persisted for the duration of the experiment, with a drop in withdrawal values to 17.30 ± 0.70 g to 23.29 ± 1.00 g by 14 days after injury. One week after transplantation of 10⁶ live hNT2.17 cells, there was a return to withdrawal pressures of 32.23 ± 1.14 g at a significance level of *P* < 0.001. This return to baseline remained statistically significant throughout the duration of the experiment. Transplantation of the nonviable cells never showed statistical difference from the injury-alone group.

Baseline withdrawal values for TH (Fig. 36.5) ranged from 13.9 ± 0.44 seconds to 14.4 ± 0.34 seconds. The naïve

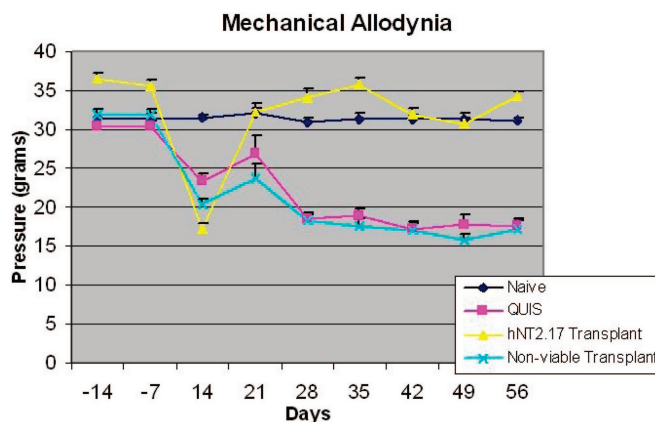


FIGURE 36.4. CA in the hind paw ipsilateral to the side of QUIS injury. QUIS injury was performed at Day 0 and transplant at Day 14, with complete and durable recovery in the viable hNT2.17 transplant group (1 million cells) and no recovery in the injury alone or nonviable cell transplant group.

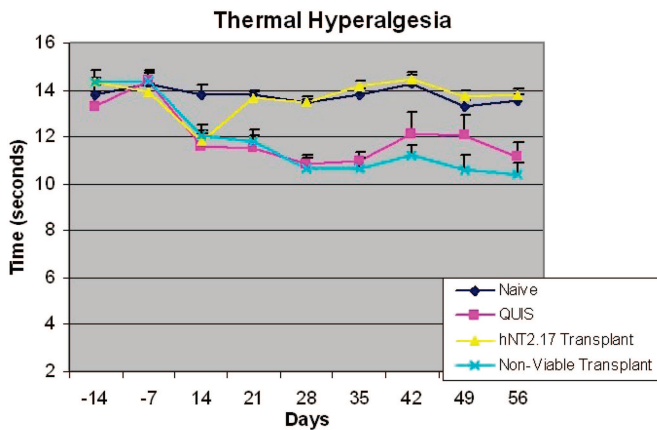


FIGURE 36.5. TH in the hind paw ipsilateral to the side of QUIS injury. QUIS injury was performed at Day 0 and transplant at Day 14, with complete and durable recovery in the viable hNT2.17 transplant group (1 million cells) and no recovery in the injury alone or nonviable cell transplant group.

control group maintained these values throughout the 60-day duration of the experiment. All animals undergoing QUIS injury demonstrated significant behavioral hypersensitivity ipsilateral to the site of injury ($P < 0.001$) by 14 days after injury 11.6 ± 0.57 seconds to 12.1 ± 0.44 seconds. At 2 weeks after transplantation, the viable transplant group showed a statistically significant return to baseline (13.6 ± 0.26 s; $P < 0.001$), which persevered throughout the duration of the experiment. Transplantation of the nonviable cell grafts never showed a statistically significant improvement in TH values.

Perfused spinal cords collected 8 weeks after QUIS were visualized with the specific human and neurotransmitter antibody markers, GABA (Fig. 36.6A) and NuMA (Fig. 36.6B), to view the surviving hNT2.17 cell grafts. These grafts reliably clustered on the dorsal pial surface of the lumbar cord near the site of injury and colocalized both GABA and NuMA immunoreactivity. Immunohistochemis-

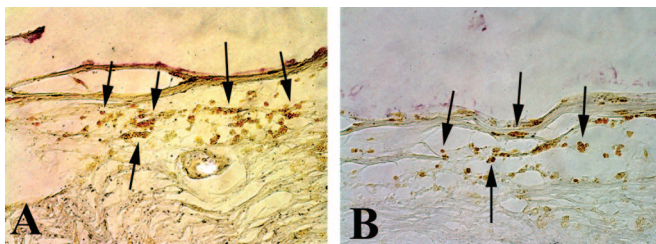


FIGURE 36.6. GABA (A) and NuMA (B) immunoreactivity showing sagittal sections of thoracolumbar spinal cord 8 weeks after QUIS injury (6 wk after transplantation of 10^6 hNT2.17 cells). The cell grafts appear as round cells clustered on the dorsal pial surface (arrows).

try confirmed that grafted cells were present and synthesizing GABA.

DISCUSSION

Chronic neuropathic pain in those with SCI is a common, often underreported clinical problem that has proven difficult to treat. Few clinical studies have examined treatments for this type of pain and, to date, no pharmacological approach has been shown to be effective over time in a significant number of individuals. The possibility of transplanting cultured cells that release pain-relieving agents into the subarachnoid space offers a new approach to the treatment of chronic neuropathic pain in a syringomyelia model of SCI.

Our laboratory has pioneered the development of cell lines bioengineered to express specific antinociceptive agents, such as the hNT2.17 human neuronal cell line, which produces the inhibitory neurotransmitters GABA and glycine, endogenously present in the dorsal horn and spinal pain-processing pathways. After SCI, levels of GABA in the dorsal horn are low below the region of the injury,⁶ likely causing a sensory signal imbalance that induces chronic neuropathic pain. We hypothesized that the release of inhibitory neurotransmitters by hNT2.17 neurons grafted into the subarachnoid space near the spinal cord will reduce behavioral symptoms of chronic neuropathic pain after excitotoxic SCI.

Current understanding of central and supraspinal¹⁴ mechanisms for the induction and maintenance of chronic pain after SCI suggests a major role for decreased function of GABAergic inhibitory systems.¹² Because we have seen no change in the spinal cytoarchitecture after hNT2.17 cell transplantation, these cells probably alter the neurotransmitter environment rather than changing the spinal cord structure, with resultant improvement in neuropathic pain. All hNT2.17 cells retain their GABA phenotype in vitro and all surviving grafts are GABA immunoreactive. Although these mechanisms continue to be incompletely understood, a recognized fault of this study, we can infer that the hNT2.17 cells function in the reestablishment of normal neurotransmitter balance.

Our overall purpose was to create a nonmitotic, GABA-secreting, human neural cell line that can be used as a transplant source for the therapeutic treatment of the consequences of SCI and syringomyelia, including chronic pain. The human Ntera/D1 (NT2) precursor cell line, known to express a neuronal phenotype in vitro and in vivo after treatment with RA, has been used as the cell source for subcloning an individual GABA and glycinergic cell line. Ordinarily, NT2 precursors require 6 to 8 weeks of differentiation with RA to provide stable neuronal cultures, which are no longer proliferative and can be transplanted into the CNS without danger of tumor formation. Here, we used a new

shortened cell-aggregation method for RA-mediated differentiation and serial dilution to subclone the hNT2.17 cell line. This cell line expresses an exclusively neuronal phenotype, does not incorporate BrdU, and does not express the tumor-related proteins FGF-4 and TGF- α during differentiation after 2 weeks of treatment with RA and mitotic inhibitors. In addition, the cell line synthesizes, secretes, and releases measurable amounts of GABA and glycine, measured by HPLC at 2 weeks of differentiation in culture.

When hNT2.17 cells are transplanted into a SCI model with confirmed initiation of pain-like behaviors, both CA and TH induced by the excitotoxic injury are potently and durably reversed. Control cell transplants of hNT2.17 cells that had been rendered nonviable before grafting had no effect on the hypersensitivity induced by QUIS injury. The hNT2.17 cell grafts reverted pain behaviors to before injury levels, with maximum effect 1 week after transplantation and lasting the entire 60-day course of the experiment. Immunohistochemistry to identify the grafts was used to ensure that the grafted cells were present and synthesizing GABA at time of perfusion, 8 weeks after QUIS injection. This data suggests that the initiation of chronic pain after SCI is sensitive to spinal levels of inhibitory neurotransmitters, and human neuronal GABA and glycinergic cells can be used as biological minipumps for antinociception in models of SCI and chronic neuropathic pain.

CONCLUSIONS

The inhibitory neuronal cell line, hNT2.17, is a safe, readily available source of human cells that potently and durably reverse neuropathic pain after intrathecal transplantation. This cell line, acting as a biological minipump, holds great clinical promise for treating neuropathic pain of central and peripheral origin.

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