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Optimizing the transplant dose of a human neuronal cell line graft to treat SCI pain in the rat

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Abstract

Neuropathic pain is a prevalent and difficult problem in the setting of spinal cord injury (SCI). The use of cellular transplant therapy to treat this pain has been successful with the use of a human neuronal cell line, hNT2.17 [M.J. Eaton, S.Q. Wolfe, M.A. Martinez, M. Hernandez, C. Furst, J. Huang, B.R. Frydel, O. Gomez-Marin, Subarachnoid transplant of a human neuronal cell line attenuates chronic allodynia and hyperalgesia after excitotoxic SCI in the rat, J. Pain 8 (2007) 33–50]. Intrathecal transplant of these cells potently reverses behavioral hypersensitivity after excitotoxic spinal cord injury in the rat model. This study focuses on delineating the optimal dose of these cell grafts in the same model. Two weeks after intraspinal injection of quisqualic acid (QUIS) with subsequent behavioral hypersensitivity, terminally differentiated hNT2.17 cells were transplanted into 300 g Wistar-Furth rats in a logarithmic variation of doses: 10^6, 10^5 and 10^3 cells. Behavioral hypersensitivity testing was performed weekly for 6 weeks following transplant. The dose of 10^6 cells (or approximately 3 million/kg) potently and permanently reversed both cutaneous allodynia (CA) and thermal hyperalgesia (TH). Reduced transplant doses of the hNT2.17 cell line did not permanently reverse behavioral hypersensitivity, suggesting that there is an optimal dose that can be used as a clinical tool to treat SCI-associated neuropathic pain.

Keywords: hNT2.17; Neuronal cell line; Cellular transplant; Intrathecal dose; Neuropathic pain; Spinal cord injury

Neuropathic pain is prevalent after spinal cord injury (SCI) population, affecting up to 40% of this population [12]. The mechanism is incompletely understood, but an imbalance between excitatory and inhibitory spinal systems seems to play a significant role. In 1965 Melzack and Wall proposed the gate theory which states that the interneurons of the dorsal horn functions as modulators of afferent excitatory impulses of the spinothalamic tract and that the larger fibers of the dorsal column system contribute to presynaptic inhibition [10]. The presence and function of these inhibitory GABAergic interneurons, located in Rexed laminae II–V, are markedly diminished after injury [7], causing unimpaired ascending excitatory signals to reach the brain. Replacement of sensory inhibition can be accomplished in the form of cellular "minipumps", human neurons which tonically produce inhibitory neurotransmitters [4]. This novel treatment appears to be efficacious, durable and without the adverse side effects of current pharmacotherapy for neuropathic pain.

This study investigates the optimal dosing of intrathecal transplantation of the human neuronal cell line, hNT2.17, required to permanently reverse behavioral hypersensitivity after excitotoxic spinal cord injury. Varying doses were used to investigate the lowest possible dose that proves efficacy, to minimize the necessary volume of transplant grafts and to formulate dose-response.

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)

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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 were analyzed and unblinded by the statistician at the end of the experiment.

Adult male Wistar-Furth rats weighing 250–300 g were used for all experiments. The rats were housed two per cage with rat chow and water ad lib on a 12-h light:12-h dark cycle. At the beginning of the experiment, animals were randomly assigned to one of seven groups: naïve controls ($n = 12$), injury-alone controls ($n = 12$), 10^6 live cell naïve intrathecal transplant group ($n = 4$), 10^6 live cell injured intrathecal transplant group ($n = 12$), 10^5 live cell injured transplant group ($n = 6$), 10^3 live cell injured transplant group ($n = 6$) and 10^6 dead cell injured transplant controls ($n = 6$).

All animals were then acclimated to cutaneous allodynia (CA) and thermal hyperalgesia (TH) behavioral testing, which were performed weekly for the duration of the 60-day experiment. Cutaneous allodynia, the occurrence of foot withdrawal in response to normally innocuous mechanical stimuli, was tested using an electronic von Frey anesthesiometer [2]. Animals were placed in a Plexiglas box with an elevated mesh floor and acclimated for 5 min. The von Frey tip was applied perpendicularly to the midplantar area of each hindpaw and depressed slowly until the animal withdrew from the pressure (grams), which was recorded for each of three trials. The mean of these scores was used for subsequent analyses. Thermal hyperalgesia, hindpaw withdrawal from a normally innocuous heat source, was tested with a Hargreave's heat source [5] (3 A) with a Halogen Photo Optic Lamp (15 V, 150 W). The average temperature (at the animal's hindpaw surface) was 36.2 °C at 10 s, 39.2 °C at 14 s and 41.3 °C at 16 s. Animals were placed in a clear Plexiglas box on an elevated floor and allowed to acclimate for 5 min. A radiant heat source with constant intensity was aimed at the midplantar area of the hindpaw. 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 min between the second and third. Three latency measurements for each hindpaw were recorded and their mean used for subsequent analyses.

An excitotoxic spinal cord injury, as described by Yezierski et al. [14], was performed on the SCI groups. Animals were anesthetized with a mixture of ketamine, xylazine, and acepromazine (0.65 ml/kg) and absence of blink and withdrawal reflexes were ensured before surgery. 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 mM of quisqualic acid (QUIS), a glutamate receptor agonist, 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 pathologic examination, these unilateral injections were centered in the gray matter between the spinal laminae IV–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 post-operative analgesics were used to prevent interference with the cellular treatment for pain.

Two weeks after injury, the animals to be transplanted received a lumbar intrathecal cell graft with either viable hNT2.17 cells (doses of 10^6, 10^5 or 10^3 cells/injection, differentiated for 2 weeks in vitro before transplant) or non-viable hNT2.17 cells (a dose of 10^6 cells/injection). Doses of 10^6 live cells were transplanted into naïve animals at this same time to act as a control group. The hNT2.17 cell line was subcloned from the parental NT2 [1] cell line, as previously described in detail [4]. Briefly, proliferating cultures of hNT2.17 cells were grown to near confluence at 37 °C before exposure to 2 weeks of all-trans retinoic acid with fresh media changes every 2 days. After removal with 0.5 mM EDTA, centrifugation and resuspension, cells were replated to 100 mm tissue culture dishes (Falcon) which had been coated with mouse laminin [(Biomedical Technologies, Stoughton, MA; 20 μg/ml in DPBS)/poly-L-lysine (Sigma; 20 μg/ml in PBS)]. They were continued in DMEM/HG/5% FBS/1% Pen-Strep (PS)/L-glutamine (2 mM) at a pH of 7.4 for 9–24 h, before the addition of cytosine-D-arabinofuranoside (araC) (Sigma; 1 μM) plus uridine (Sigma; 10 μM), for non-neuronal growth inhibition. After 7 days, cells were briefly exposed to warmed trypsin/0.5 mM EDTA and adherent surface cells removed with DMEM-HG/5% FBS/PS/L-glutamine (2 mM). The cells were centrifuged, resuspended, replated on 60 mm tissue culture dishes (Falcon), coated with mouse laminin [(Biomedical Technologies, Inc; 20 μg/ml in DPBS)/poly-L-lysine (Sigma; 20 μg/ml)] and continued in DMEM-HG/5% FBS/PS/L-glutamine, 2 mM at a pH of 7.4 at 37 °C for 2 weeks before transplant, with media change every 2–3 days. On the day of transplant, cells were rinsed with warmed Cellstripper (Voigt Global Dist.), the media replaced with another 3 ml of Cellstripper for 1 min, 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 Ca^{2+}-Mg^{2+}-free (CMF) HBSS. Non-viable hNT2.17 cells were prepared by centrifuging the cells in sterile water, assuring non-viability and resuspending them in CMF-HBSS for transplant. The appropriate cell dosage was prepared immediately prior to each transplant to assure near 100% viability at the beginning of the experiment and transplantation was within 30 min of cell preparation.

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

Sixty days after QUIS injury, the animals were sacrificed to examine cell graft survival. Rats were euthanized for tissue fixation by a combination of pentobarbital overdose and exsanguination. Transcardial perfusion with 4% paraformaldehyde

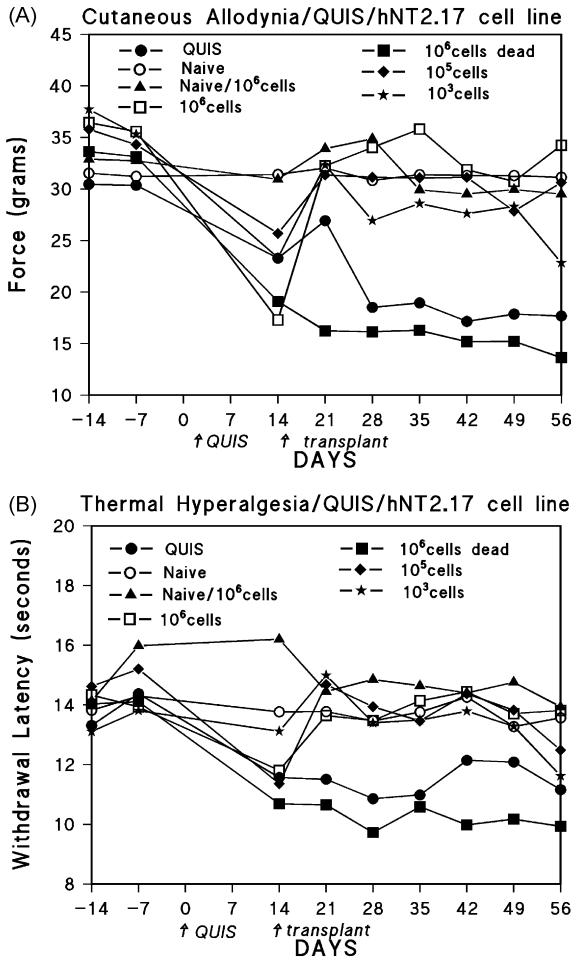

Fig. 1. Cutaneous allodynia (A) and thermal hyperalgesia (B) behaviors showing significant hypersensitivity 14 days after SCI with complete and persistent recovery after transplant of the 10^6 cells/dose group ($p < 0.02$) vs. the incomplete and unsustained recovery of the 10^5 and 10^3 cell dose groups. Data shown represents the mean value (±S.E.M.) of the ipsilateral hindpaw for each group.

and 0.1% gluteraldehyde was performed and the spinal cord was removed and stored in fixative for 12 h at 4 °C. The cords were then cryoprotected by equilibration in 30% sucrose and PBS for several days at 4 °C. Cords were embedded in Shandon-1 Embedding Matrix (Thermo Electron Corp.) and sagittally cut in sequential 20 μm sections with a Cryostat (Leica 1900). Every other section was stained for the human marker Nuclear Matrix Antigen (NuMA) alternating with GABA to identify the grafted human cell line, as previously described [4].

As stated before, statistical analysis of sensory behaviors (CA and TH) were performed using, for each animal, the average of three measurements for ipsilateral hindpaw withdrawal. Both, one-way ANOVA and its non-parametric counterpart, the Kruskal–Wallis test, were used for cross-sectional comparisons of group means at each point time. These cross-sectional overall analyses were followed by pairwise comparisons of daily group means, using the Tukey's method to adjust for the multiplicity of the comparisons. All analyses were performed in SAS (SAS Institute, North Carolina) and results of tests with p-values less than 0.05 were considered statistically significant.

The results for tactile allodynia can be seen in Fig. 1A. As seen in this figure, baseline withdrawal values (mean ± S.E.M.) ranged from 30.36 ± 0.86 to 35.32 ± 1.21 g. The naïve control group maintained its values throughout the 60 days duration of the experiment. Live cell intrathecal transplants into naïve animals showed the same withdrawal values as the naïve animals, with no effect on sensory behaviors. All animals undergoing excitotoxic QUIS injury showed significant behavioral hypersensitivity ipsilateral to the site of injury that persisted for the duration of the experiment, with a drop in withdrawal values to 18.25 ± 1.05 to 25.68 ± 0.75 g by 14 days post-injury.

One week after transplantation of hNT2.17 cells, the 10^6 live cell group showed a significant return ($p = 0.019$) to withdrawal pressures of 30.0 ± 1.55 g. This return to baseline level remained throughout the duration of the experiment.

The assessment of cutaneous allodynia showed baseline withdrawal values of 30.36 ± 0.86 to 35.32 ± 1.21 g. The naïve control group maintained these values throughout the 60 days duration of the experiment. Live cell intrathecal transplants into naïve animals showed the same withdrawal values as the naïve animals, with no affect on sensory behaviors. 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 18.25 ± 1.05 to 25.68 ± 0.75 g by 14 days post-injury.

One week after transplantation of hNT2.17 cells, the 10^6 live cell group showed a return to withdrawal pressures of 30.0 g ± 1.55 at a significance level of $p = 0.0192$. This return to baseline remained statistically significant throughout the duration of the experiment. Transplantation of the 10^5 live cell dose showed initial return to baseline after 1 week (31.35 g ± 2.2, $p = 0.019$), but declined 3 weeks after transplant. Transplantation of the 10^3 live cell and 10^6 dead cell doses never showed statistically significant difference from the injury-alone group.

The results for thermal hyperalgesia can be seen in Fig. 1B. Baseline withdrawal values for thermal hyperalgesia testing ranged from 13.9 ± 0.39 to 15.20 ± 0.55 s. The naïve control group maintained its values throughout the 60 days duration of the experiment. The naïve live cell intrathecal transplants group showed no significant variation from naïve animals. All animals undergoing QUIS injury showed significant behavioral hypersensitivity ipsilateral to the site of injury with a p-value of 0.019 by 14 days post-injury. At 2 weeks post-transplantation, the 10^6 live cell group showed a statistically significant return to baseline (13.48 ± 0.39, $p < 0.001$), which persevered throughout the duration of the experiment. Transplantation of the 10^6 dead cell dose never showed a statistically significant improvement in TH values. Animals undergoing transplantation of the 10^3 live cell dose briefly showed return to baseline at 2 weeks post-transplant but declined for the remainder of the study. These results for thermal hyperalgesia can be seen in Fig. 1B.

Spinal cords collected 8 weeks after QUIS were visualized with specific human and neurotransmitter antibody markers GABA and NuMA (Fig. 2) 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. As all NuMA positive

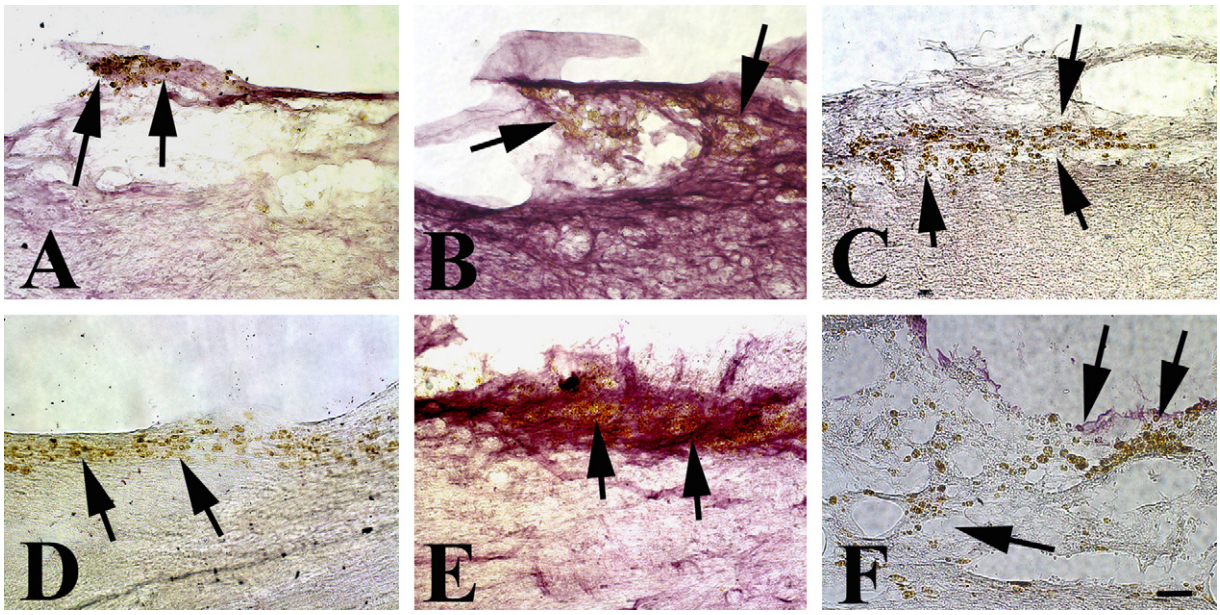

Fig. 2. Transplant of hNT2.17 cell line in the QUIS model of SCI and chronic pain: NuMA and GABA immunohistochemistry. GABA (A–C) and NuMA (D–F) immunostaining of the 10^3 (A and D), 10^5 (B and E) and 10^6 (C and F) hNT2.17 cell grafts (arrows) placed 2 weeks after QUIS injury and examined 6 weeks after transplant. It is important to note that these slides represent a single cluster of cells, but the most significant difference between dosing is the number of clusters found over the surface of the cord. Magnification bar = 40 μm.

cells retain their GABA expression after transplant in this pain model [4], only NuMA was used to identify cell grafts in a dorsal view of the lumbar cord (Fig. 3).

In cutaneous allodynia, all doses of grafted cells were able to initially recover normal sensory functioning below the level of injury, but this effect quickly became suboptimal after 2 weeks and continued to decline in both the 10^3 cells/dose and 10^5 cells/dose groups. Thermal hyperalgesia showed the same trend toward recovery at 2 weeks in both groups, but only the 10^3 live cell group showed significance. Neither group retained any significant recovery over the duration of the experiment. This small discrepancy between the TH and CA results is probably due to the smaller scale of the TH withdrawal (an average of 4 s difference between naïve and uninjured animals in TH versus a 15 g difference in withdrawal in CA). These results lead us to believe that there is both an early effect of transplantation of these inhibitory cells but there is a critical dose below which behavioral recovery is not sustained. A graft of at least 10^3 hNT2·17 cells may act in a similar way to a single bolus of GABA [3] or baclofen [11], producing presynaptic inhibition of primary afferent terminals [6] and inhibiting the release of excitatory neurotransmitters glutamate [8] and substance P [9] from the primary afferents. While histologic pictures of 10^3, 10^5 and 10^6 cells/dose appear similar, this is deceiving (Fig. 2). These cells have a tendency to cluster, making any individual picture unrepresentative of the total.

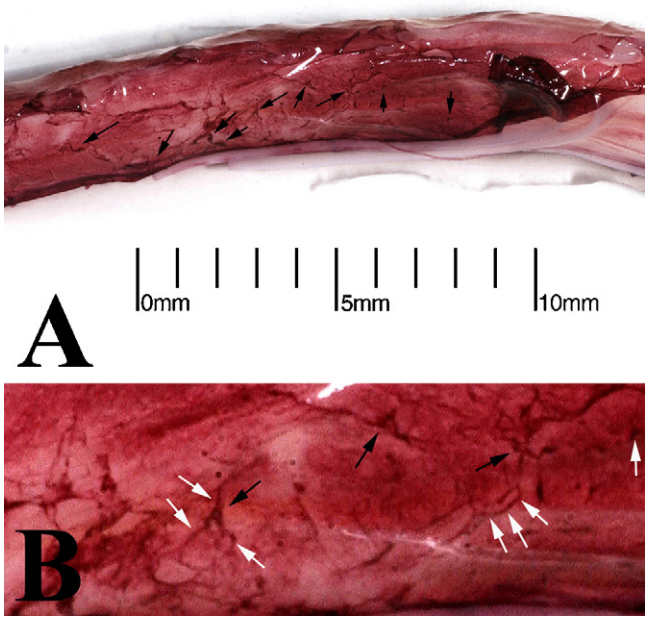

Fig. 3. Pial surface of lumbar spinal cords with grafts of hNT2.17 cells. Low (A) and high magnification (B) images of hNT2.17 grafts on the pial surface of lumbar spinal cord placed 2 weeks after QUIS injury and examined 10 weeks after transplant. Grafts were stained with the human marker anti-NuMA. Individual graft cell bodies (small black arrows) extend extensive neurite arbors (small white arrows). Magnification scale = 10 mm (A).

Current understanding of central and supraspinal [15] mechanisms for the induction and maintenance of chronic pain after SCI suggests a major role for decreased function of GABAergic inhibitory systems [13]. In vitro, the hNT2.17 cell line synthesizes significant amounts of the GABA neurotransmitter (1554.700 (mean) ± 186.631 (S.E.M.) pmoles/10 million cells) and secretes it under basal or stimulated K^+ conditions [4]. All hNT2.17 cells retain their GABA phenotype in vitro and all surviving grafts are GABA positive. The inhibitory neurotransmitter, glycine, is also synthesized and secreted in large amounts; also, small amounts of met-enkephlin, neuropeptide Y, vaso-

pressin, oxytocin are also synthesized by the hNT2.17 cell line [4]. As 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. Without completed studies to evaluate the precise location or mechanism of action of the hNT2.17 cell grafts, we can only hypothesize that they may function in inhibitory restoration of the inhibitory dorsal horn interneurons as they mediate incoming excitatory responses. We cannot rule out dorsal column transmission and/or spinal roots as possible sites of action. Whether it is the GABA or a combination of other bioactive substances secreted by the hNT2.17 cells into the intrathecal environment, there is a temporary effect even at the lowest dose, suggesting that it is these grafts that cause recovery of sensory function (compared to injury-alone or non-viable cells grafts). The incomplete understanding of the mechanisms of this treatment is a recognized weakness of this study, which we will continue to address with ongoing investigations.

Transplants of live 10^6 cells in naïve animals under the same conditions and behavioral testing show that hNT2.17 grafts at the highest dose do not affect motor behaviors. We are examining BBB motor scores after grafts of hNT2.17 cells in a severe contusion SCI model with no effect of grafts on motor behaviors, while behavioral hypersensitivity is ameliorated (unpublished observations).

These results suggest that graft dose determines the duration and degree of reversal of behavioral hypersensitivity in a SCI pain model.

This study helps to better define the early effect of inhibitory neuronal cellular transplantation as well as a critical cell number, below which suboptimal effect is seen. The use of cellular therapy, where grafts function as cellular minipumps in the subarachnoid space, is a promising clinical tool for the treatment of neuropathic pain syndromes.

Acknowledgments

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