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Recombinant neural progenitor transplants in the spinal dorsal horn alleviate chronic central neuropathic pain

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Abstract

Neuropathic pain induced by spinal cord injury (SCI) is clinically challenging with inadequate long-term treatment options. Partial pain relief offered by pharmacologic treatment is often counterbalanced by adverse effects after prolonged use in chronic pain patients. Cell-based therapy for neuropathic pain using GABAergic neuronal progenitor cells (NPC) has the potential to overcome untoward effects of systemic pharmacotherapy while enhancing analgesic potency due to local activation of GABAergic signaling in the spinal cord. However, multifactorial anomalies underlying chronic pain will likely require simultaneous targeting of multiple mechanisms. Here we explore the analgesic potential of genetically modified rat embryonic GABAergic NPCs releasing a peptidergic NMDA receptor antagonist, Serine¹-histogranin (SHG), thus targeting both spinal hyperexcitability and reduced inhibitory processes. Recombinant NPCs were designed using either lentiviral or adeno-associated-viral vectors (AAV2/8) encoding single and multimeric (6 copies of SHG) cDNA. Intraspinal injection of recombinant cells elicited enhanced analgesic effects compared to nonrecombinant NPCs in spinal cord injury-induced pain in rats. Moreover, potent and sustained antinociception was achieved, even following a 5 weeks post-injury delay, using recombinant multimeric NPCs. Intrathecal injection of SHG antibody attenuated analgesic effects of the recombinant grafts suggesting active participation of SHG in these antinociceptive effects. Immunoblots and immunocytochemical assays indicated ongoing recombinant peptide production and secretion in the grafted host spinal cords. These results support the potential for engineered NPCs grafted into the spinal dorsal horn to alleviate chronic neuropathic pain.

Introduction

The long-term management of chronic neuropathic pain presents significant challenges in current clinical practice owing in part to difficulties in maintaining pharmacologic effectiveness and minimizing untoward side effects over extended periods of time. Alternative approaches intended to produce long-term or permanent alterations in CNS pain transmission, such as gene therapy or cell transplantation, could overcome limitations of traditional pharmacotherapy and may be particularly indicated for patients suffering from

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chronic disabling pain. Cell-based delivery for the management of pain has been explored in our laboratory and others over the past 25 years ((65) for review). Key advantages include the ability to deliver therapeutic agents locally, reducing detrimental systemic side effects, capacity for sustained renewable delivery of therapeutic molecules by living cells, and ability to engineer cells to produce additional potent antinociceptive peptides which otherwise have limited CNS access. Stem cells of various origins have emerged as promising candidates for therapeutic intervention, and are currently being evaluated in early phase clinical trials. In contrast to cells transplanted in the spinal subarachnoid space, which may act as local minipumps to provide analgesic agents, a potential advantage of stem cell sources for grafting into the CNS is the possibility of neuronal differentiation and integration within host neural circuitry.

Dysfunctional GABA signaling is thought to contribute to chronic pain, and GABAergic neural progenitor cells (NPCs) have shown promise in reducing neuropathic pain behavior following transplantation in rat and mouse pain models (7,38,46,52,54). Previous studies in our laboratory showed amelioration of neuropathic pain in rats induced by either peripheral nerve injury or spinal cord injury by intraspinal GABAergic NPC grafts (42,43,48,49). Potential integration with host dorsal horn neurocircuitry is supported by both electrophysiological (43) and neuroanatomical (7) findings. Despite the promise of cellular transplantation therapies to replace lost neuronal populations, current technologies appear limited in their restorative capabilities, and reversal of neuropathic pain symptoms incomplete. Only a small percentage of grafted cells may remain at 1 month (7). An approach in overcoming this apparent ceiling and improving beneficial outcomes of the grafts is to enhance the antinociceptive capabilities of the cells that do survive and integrate. Towards this end, the goal of this study was to enhance the pharmacologic potency of the grafted cells by genetically engineering them to produce complementary antinociceptive molecules. Although there are a number of possible targets, our lab has identified a particularly promising peptide as an initial candidate and proof of concept, Serine¹-histogranin (SHG), which has NMDA antagonist activity. Since neuropathic pain is thought to be mediated in part by activation of dorsal horn NMDA receptors and consequent neuronal hyperexcitability, SHG may be an ideal complementary candidate for reducing abnormal hyperexcitability and restoring inhibitory balance in conjunction with the GABAergic NPC transplants.

Materials & Methods

Animals

Male Sprague-Dawley rats were used for the spinal cord injury, implantation of intrathecal catheter and intraspinal injections (140–160g at the time of the first surgery); pregnant female Sprague-Dawley rats were used for E14 embryo harvesting (Harlan Lab, IN). Animals were housed two per cage with free access to food and water in 12 h light/dark cycle. Experimental procedures were reviewed and approved by the University of Miami Animal Care and Use Committee and followed the recommendations of the “Guide for the Care and Use of Laboratory Animals” (National Research Council). All surgical procedures were conducted under 2.5% Isoflurane/O₂ anesthesia using aseptic conditions.

Spinal cord injury pain model

Spinal cord clip injury (SCI) was used to induce central neuropathic pain (34). Spinal cord segments T6-T8 were exposed by laminectomy and an aneurism clip 1 mm wide (20 g compression force; Harvard Apparatus) was placed in the vertical orientation over the exposed thoracic spinal cord without disturbing dura or dorsal roots for 60 seconds. The clip was then removed and the wound closed with silk sutures and clips. Animals were treated with gentamicin (2mg/kg, i.p) to prevent infection of the urinary tract. Bladders were expressed daily until voiding was regained (total n=62; n's for individual experiments and treatment groups appear in figure legends).

Intrathecal catheter implantation

For pharmacological experiments, drugs were delivered via intrathecal catheter. Pharmacologic reversal studies were conducted at 2–4 weeks post-transplantation, when anti-allodynic effects were maximum and stable. Indwelling catheters (7.5–8 cm; ReCathCo, PA) were inserted through a slit in the atlanto-occipital membrane down the intrathecal space and secured to the neck muscles with sutures similar to previously described methods (35,75). Rats were allowed to recover at least three days following intrathecal surgery prior to use in experiments. A 48 hr washout period was included between drug dosing. The following drugs were used for intrathecal injections: SHG antibody (0.5 µg/ml, custom synthesized by 21st Century Biochemicals), bicuculline (0.3µg; Sigma), and albumin (bovine serum albumin, 1 µg, Sigma). Drugs were dissolved in saline and injected in 5 µl volumes, followed by a 5µl flush with saline. For mixed injections (bicuculline and SHG antibody) 5 µl of each drug was injected.

Preparation of engineered NPCs

Viral vectors: The Miami Project based Viral Vector Core generated the required lenti- and adeno-associated viruses (AAV) for this study. Our laboratory previously generated lenti-SHG constructs which resulted in successful production of the SHG peptide in a variety of cell types (26). Briefly, double stranded DNA flanked by BglII and XbaI was produced based on the SHG protein sequence. The ppNGF-β signal sequence that mediates stimulus-independent release of mature peptide (19) was added to achieve production of a secretable peptide. The resulting ppNGF-β-SHG cDNA was fused with monomeric Red Fluorescent Protein (mRFP) cDNA and the fragment was subcloned into the lentiviral vector pRRL.

To further increase antinociceptive potential of the construct, we engineered SHG multimers to generate multiple copies of the SHG peptide. In order to accomplish this, Bgl II restriction sites were added at the ends of the SHG cDNA using PCR. The Bgl II flanked SHG sequence was confirmed, excised with Bgl II, and annealed to itself in a ligation buffer. This generated multimers with varying copy numbers of SHG. The SHG multimers (mSHG) were subcloned in a vector containing a single copy of SHG linearized with Bgl II. The resulting clones were screened for increases in insert size and verified for the correct orientation (Genewiz). We were able to purify clones with up to 6 copies of SHG (6(SHG)). Lentiviral vectors were used for subcloning in initial studies; however, due to low yields of DNA, we switched to AAV vectors in later studies for subcloning the 6(SHG) construct and subsequent NPC transduction. Our screenings of different serotypes of AAV vector for

transduction of neuronal cells showed the hybrid AAV2/8 as the most efficient. This vector has also been shown by other studies to have 5–100 fold higher efficiency for transgene delivery as compared to AAV2 serotypes in cardiac muscle (6), skeletal muscle (72), brain (8), lungs (51) and liver (27).

Neural progenitor cells: E14 fetal neocortical tissue (cortical lobes and underlying lateral ganglionic eminences) from Sprague-Dawley rats was microdissected in Hank's balanced salt solution (HBSS, Gibco) and mechanically triturated to obtain single cell suspensions. Cells were plated at a concentration of 5×10^5 cells/ml Stemline NSC medium (Sigma) containing 10 ng/ml of human recombinant basic fibroblast growth factor (FGF-2, Sigma), incubated for 5–7 days with media change after 2–3 days. Twenty-four hours prior to transplantation, FGF was withdrawn from media in order to initiate pre-differentiation to GABAergic progenitors, as we have found this environment to be favorable for GABAergic differentiation (25). For transduction with viral plasmids, NPCs were harvested and prepared as above. Twenty-four hours after harvesting, cells were pelleted at 1000 rpm for 5 min and 10 μ l of purified virus (1×10^6 TU/ml for lentivirus, 1×10^9 TU/ml for AAV) prepared by the Miami Project Viral Vector Core was added to a minimal volume of culture media for 4–5 hours at 37° C. After washing the pellet to remove unbound virus, cells were plated with an appropriate amount of culture media. Production and secretion of recombinant peptide were verified by immunocytochemistry and dot blot analysis.

Immunocytochemical evaluations

For phenotypic characterization of cultured NPCs, standard immunocytochemical protocols were used. Cells were plated in poly-L-ornithine/fibronectin coated Lab-Tek (Nunc) chambers at concentration 5×10^5 /well and incubated at 37°C for 2–3 days. After that, cells were fixed with 4% paraformaldehyde, washed and incubated in 5% normal goat serum for 2 hours. Primary antibodies (anti-GABA (1:200, Millipore); TuJ1 (1:1000, Neuromics), anti-SHG (1:50, 21st Century Biochemicals) were added for 24–72h incubation, followed by wash and incubation with appropriate secondary antibodies (anti-mouse and anti-rabbit and anti-guinea pig AlexaFluor 488 and 597, 1:250, Invitrogen) and DAPI for nuclear staining. After a final wash the upper structure of the chamber was carefully removed, slides were air-dried and coverslipped (Vectashield, VectorLabs). To estimate the transduction rate and to compare differentiation of NPCs into GABAergic proneuronal phenotype between non-recombinant NPC, 1(SHG) NPC and 6(SHG) NPC, at least 2 images of areas with clearly identified single cells per well labeled with GABA, TuJ1, SHG and DAPI were captured by confocal microscope (Spectral Confocal Microscope Fluoview1000) and analyzed by ImageTool software by manual counting based on the overlapping of single-colored images and colocalization of markers. Experiment was designed to have at least 2 chambers stained with the same combination of antibodies, thus having at least 4 images per count. DAPI labeled objects with diameter under 8 μ m were not included in counting as they may present artifacts of staining or apoptotic nuclei.

Cell stimulation

Recombinant and non-recombinant cells were pelleted by centrifugation, washed and treated with Krebs solution. To compare the relative amount of SHG present in samples, we

collected cell supernatant samples from approximately the same number of cells ($\sim 2 \times 10^7$) in NPC cultures. For basal release of peptides, cells were incubated for 15 min in normal $[K^+]$ Krebs (124.3mM NaCl; 2.95mM KCl; 1.3mM $MgSO_4 \cdot 7H_2O$; 2.41mM $CaCl_2 \cdot H_2O$; 26mM $NaHCO_3$; 1.25mM KH_2PO_4 ; 10.4mM D-Glucose). Supernatant was harvested and filtered through a 22 μm syringe filter. Cells were then washed, incubated for 30 min in high $[K^+]$ Krebs (87mM NaCl; 100mM KCl; 1.3mM $MgSO_4 \cdot 7H_2O$; 2.41mM $CaCl_2 \cdot H_2O$; 26mM $NaHCO_3$; 1.25mM KH_2PO_4 ; 10.4mM D-Glucose) for induced release and supernatant was harvested and filtered (13). The protein concentration in each sample was determined by a Pierce BCA Protein Assay Kit (Thermo Scientific) according to the manufacturer protocol. The method is based on the colorimetric reaction of bicinchoninic acid (BCA) with cuprous cations (Cu^{1+}), reduced by protein. Briefly, a known dilution of standard peptide (albumin) and two dilutions of samples were loaded into 96 well plates in triplicates. BCA working reagent was added to all wells and incubated for 30 min in 37°C. The colorimetric reaction was evaluated by spectrophotometry at 562nm absorbance and the concentration of protein in samples was determined based on standard curve.

The FLISA method was used to quantify the amount of SHG in cell supernatants. Based on BCA results, samples were prepared to contain the same amount of peptide in 100 μl . This volume was loaded into 96 well plates in capture buffer according to the manufacturer protocol (Odyssey FLISA, Li-Cor), incubated overnight at 4°C, followed by incubation in the blocking buffer for 3 hours at room temperature and anti-SHG primary antibody (1:100, 21st Century Biochemicals) for 18 hours at 4°C. Plates were then washed and incubated in secondary antibody (IRDye goat anti-rabbit) for 2 hours, washed and absorbance measured by Odyssey Infrared Imager (Li-Cor). SHG peptide at different concentrations was used to calculate the standard curve and the relative amount of SHG in samples. Data were analyzed by Graph Prism polynomial analysis.

Intraspinal NPC transplantation

At 5 weeks post SCI, animals received intraspinal injection of either NPCs or equal volumes of saline. NPC transplantation was done at the lumbar enlargement, despite the thoracic location of the SCI. The rationale for this was to target a primary clinical concern for SCI patients, which is below-level neuropathic pain, frequently reported as the most severe and bothersome by these patients (14,66–68,73). In addition, several studies have shown that there are significant changes in the spinal dorsal horn at levels far removed from the actual injury site, suggesting that aberrant signaling in those regions may underlie or contribute to below-level SCI pain (16,28,31,64). Further, this target transplant site allows us to utilize standard hind-paw tests for allodynia and hyperalgesia, mediated at the L3–L5 level (1). Prior to transplantation, cells were pelleted at concentration of 50,000/ μl . Under anesthesia, rats were placed in a stereotaxic holder with spinal clamps (Kopf, CA) and a laminectomy was performed to expose the L3–L4 spinal cord level. Intraspinal injection was done at L3–L4 using a pulled glass microneedle (diameter $\sim 50 \mu m$) attached to a Hamilton syringe. The needle was placed 0.5 mm from the central vein and 2 μl of cell suspension or saline were injected bilaterally at 0.5 $\mu l/min$ with a digital microinjector (Stoelting, Wood Dale, IL) at a depth 0.5 mm from the dorsal lumbar spinal surface. The needle was left on place for another 3 min to prevent backflow. Muscles were sutured in layers, overlying skin was

closed with staples, and animals were transferred to heated cages for recovery. All transplanted rats received cyclosporine A (IP, 10 mg/kg; Bedford Labs, OH) from –1 day until sacrifice.

Behavioral evaluations in SCI animals

Rats were tested weekly for up to 11 weeks post-SCI using a battery of innocuous and noxious stimuli. Tests for tactile and cold allodynia were performed on the same day. For drug evaluation experiments rats were tested every 30 min up to 120 min following intrathecal injections with 48h wash-out between drugs/tests. Behavioral responses were recorded by observers blinded to experimental treatments.

Tactile Allodynia—The threshold response to an innocuous mechanical stimulus was measured with calibrated von Frey hairs ranging from 0.25 to 15 g. Animals were placed beneath an inverted clear plastic cage on an elevated wire mesh floor and filaments were applied to the plantar skin of the hind paw sequentially with increasing or decreasing force based on negative or positive responses respectively (17). A brisk hind paw withdrawal together with at least one other pain-related behavior, such as turning the head towards the stimulus, licking or shaking the paw was considered as a positive response, in order to incorporate involvement of nociceptive processing at the supraspinal level (3).

Cold Allodynia—Sensitivity to a non-noxious cooling stimulus was evaluated using acetone. 100 μ l of acetone was dropped onto the plantar hind paws and responses were recorded. Acetone was applied 5 times, with about 1–2 min between applications. Hind paw lifting following acetone application was usually accompanied by supraspinal responses (head turning and licking of the affected paw or shaking of the paw). The total number of positive lifting responses out of five was converted to a percent response frequency.

Neurochemical and immunohistochemical evaluations of NPC transplants

To confirm transplant survival and expression of transgene, some spinal cord samples were prepared for neurochemical or immunohistochemical evaluations. For neurochemical analyses, spinal cord samples were dissected from SCI animals grafted with either NPC only, 1(SHG) or 6(SHG) NPCs at 5–6 weeks post grafting (n=3/group). The spinal lumbar region around the grafting site was dissected and frozen on dry ice. Samples were homogenized in RIPA lysis buffer (Santa Cruz) and protein concentration was estimated by the BCA method as stated above. The FLISA method was used to quantify the amount of SHG in spinal cord samples. 100 μ l of sample homogenates were loaded into 96 well plates in capture buffer according to the manufacturer protocol (Odyssey FLISA, Li-Cor), incubated overnight at 4°C, followed by incubation in the blocking buffer for 3 hours at room temperature and anti-SHG primary antibody (1:100, 21st Century Biochemicals) for 18 hours at 4°C. Plates were then washed and incubated in secondary antibody (IRDye goat anti-rabbit) for 2 hours, washed and absorbance measured by Odyssey Infrared Imager (Li-Cor). SHG peptide at different concentrations was used to calculate the standard curve and the relative amount of SHG in samples. GAPDH was used as loading control. Data were analyzed by Graph Prism polynomial analysis.

For immunohistochemical analyses, sham animals (n=2), SCI animals (5 weeks post SCI, n=2) and SCI animals with graft (5–6 weeks post grafting, n=10) were deeply anesthetized and intracardially perfused with 0.9% saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer. Spinal cords were removed and post-fixed for 12 hours in the same fixative and transferred to 25% sucrose for cryoprotection. Cryostat sections, either free floating or slide mounted, were prepared and processed according standard immunohistochemical protocols. Sections were incubated in 5% normal goat serum for 2 hours followed by overnight or 48h incubation with primary antibodies as above. After washing, sections were incubated in the appropriate secondary antibodies, washed and coverslipped. The following antibodies were used in immunocytochemical and histochemical methods: *Primary*: rabbit anti-GABA (1:200, Sigma), guinea pig anti-GABA (1:200, Millipore), mouse anti-NeuN (1:200, Chemicon), and rabbit anti-SHG (1:1000, 21st century Biochemicals); *Secondary*: goat anti-guinea pig and goat anti-mouse AlexaFluor 488 (1:250, Invitrogen), goat anti-rabbit (1:250, Invitrogen), and DAPI (Invitrogen). Images were analyzed by confocal microscope (Spectral Confocal Microscope Fluoview 1000).

To compare the relative survival rate and differentiation of transplanted cells between non-recombinant and recombinant groups, we performed quantitative analyses in the subset of samples. mRFP and/or SHG were used as immunohistochemical markers to identify grafted cells. All images were evaluated by StereoInvestigator (MBF Bioscience). Five to seven 40 μ m sections (at least 160 μ m apart) of spinal cords with visible grafts from NPC (n=4), 1(SHG) NPC (n=3) and 6(SHG) NPC (n=3) treated SCI animals have been evaluated. The area with grafted cells was outlined at low magnification, and mRFP, SHG, GABA and NeuN positive cells were manually counted in each slide at 20 $\times$ magnification. From these, the estimated number of cells per animal labeled by certain markers and colocalization of markers were calculated for each group.

Statistical analysis

Data are expressed as mean $\pm$ S.E.M. For statistical evaluation of behavior, all animals were included in the NPC groups if at least one GABA or DAPI cell was found in the graft site within the lumbar dorsal horn, as non-detectable grafts may be the result of misplacement or non-viability(7). Using these criteria, the transplant success rate was approximately 80%. Comparisons between treatment groups were done using t-test and ANOVA. To compare the effect of treatments over time, two-way ANOVA for repeated measures was done, followed by the Student-Newman-Keuls *post-hoc* test. Relative optical density was evaluated by t-test and one-way ANOVA. Statistical analyses were performed using SigmaStat. Statistical significance was taken at $p < 0.05$ and 0.01.

Results

Generation of SHG-producing neural progenitor cells

Since previous findings suggested that supplementing NPC transplants with intrathecal injection of SHG can increase antinociception, we therefore embarked on generating NPCs engineered to produce this peptide. Initial studies utilized the single SHG construct in a lentiviral vector backbone, as previously described by our group (Gajavelli et al., 2008).

However, in order to increase antinociceptive potency, multimeric constructs were developed for later studies using the SCI model. To accomplish this, SHG oligonucleotides were annealed and subcloned into a pGEMt vector with a single SHG to obtain multi-SHG constructs (mSHG). Several mSHG constructs were successfully designed and purified, containing up to 6 copies of SHG (6(SHG)). The 6(SHG) constructs were used to engineer recombinant GABA NPCs, since the most potent effect was expected from this construct compared to those with fewer SHG copies.

The SHG signal was detected in the majority of cultured cells for both 1(SHG) NPC and 6(SHG) NPC groups (Fig. 1). Image analysis estimated a transduction rate of $73.5 \pm 4.8\%$ for 1(SHG) NPC (Fig. 1A–D) and $82.62 \pm 16.2\%$ for 6(SHG) NPC (Fig. 1E–H) of all DAPI positive objects ($>8\mu\text{m}$). Co-staining with TujI showed a similar rate for 6(SHG) NPCs and 1(SHG)NPC expressing this neuronal marker, ($38.6 \pm 7.3\%$ of 1(SHG) NPC and $40.2 \pm 10.3\%$ for 6(SHG) NPC). Quantification of TujI colocalization with the SHG signal showed a similar ratio of TujI cells expressing SHG in 1(SHG)NPC ($85.6 \pm 12.6\%$) and 6(SHG)NPC ($78.3 \pm 15.4\%$). These results suggest that the transduction rate and differentiation of transduced cells were not affected by the length of cDNA used in the recombinant viral vector. Colocalization of GABA and SHG signals was detected in $73.5 \pm 10.3\%$ and $58.6 \pm 5.6\%$ of the GABA population in 1(SHG)NPC and 6(SHG)NPC (Fig. 1I–L) respectively. No SHG signal was detected in the nontransduced cells, confirming the specificity of SHG antibody binding (Fig. 2M–T). Although the majority of cultured cells express the proneuronal phenotype, there were also glial cells in the culture as well, expressing GFAP and O4 (data not shown). These likely contributed to the observation of DAPI cells which do not co-localize with either neuronal marker. Differentiation into the GABAergic phenotype was not markedly affected by transduction; $51.2 \pm 3.6\%$ were GABA positive cells in 1(SHG) NPC, $45.6 \pm 12.1\%$ in 6(SHG) NPC and $62.3 \pm 8.9\%$ in NPC cultures respectively. Although transduction with recombinant genes does not seem to have detrimental effects on the morphology or phenotype of surviving cells, it is necessary to mention that the survival of the cells was reduced early after transduction. When replating into fresh media 48 hours post transduction, survival and differentiation of recombinant cell cultures was similar to non-recombinant cells. The NPC culture composition and transduction rates are summarized in Supplementary Table I.

The ability of transduced cells to release SHG was evaluated by FLISA (Fig. 2). Both transduced and non-transduced NPCs were stimulated with 100mM KCl Krebs solution following collection of basal release samples, and the levels of SHG released into the media was evaluated. No detectable SHG signal was observed in either basal or high K^+ conditions from non-recombinant control NPCs (Fig. 2A). A positive SHG signal was detected in the media collected from transduced cells. Cells transduced with the 6(SHG) showed higher basal levels of released SHG in the supernatant than 1(SHG). High K^+ stimulation increased the SHG signal in 6(SHG) transduced cells (Fig. 2B, $*p=0.001$ vs NPC; $\#p=0.01$ vs Baseline; $+p=0.001$ 1(SHG) vs 6(SHG)).

Effects of recombinant NPC grafts on spinal cord injury neuropathic pain

To demonstrate that injury at thoracic levels of the spinal cord can result in pathophysiological changes at sites well below the injury level, in lumbar spinal cord level, spinal cord sections from sham (n=2) and SCI animals (n=2) were immunostained for GABA and NeuN markers. Fig. 3 indicates a reduction in GABA immunoreactivity in neuronal cell bodies after SCI, similar to reports in other neuropathic pain models (7,18,29,32,36,48). The reduced immunoreactivity is observed mainly at lateral and medial side of the dorsal horn in laminae I-III. As it has been reported, reduced inhibitory GABA signaling and subsequent development of chronic pain is not likely due to the actual loss of neurons but rather due to the disruption of signaling at the level of GABA receptors (57–59). In our samples we have not observed any marked loss of NeuN immunoreactivity and the slides from sham and SCI animals were undistinguishable using NeuN staining (Fig. 3C, G). Differences however were observed in the intensity and the amount of GABA positive bodies and processes (Fig. 3B, F). Reduced inhibitory GABA signaling may underlie neuronal hyperactivity and contribute to the below-level pain reported by SCI patients (3,9,28,40,76,77).

As the *in vitro* results indicated successful transduction of NPCs and generation of SHG peptide, the antinociceptive properties of recombinant NPCs were evaluated in the SCI-induced pain model. Viral vectors encoding single and multiple copies of SHG were used in these evaluations. GABAergic cells harvested from E14 rat embryos were transduced by recombinant lentiviral (single SHG) or AAV vector (multiSHG) and maintained in culture for 5–7 days prior to transplantation. Non-transduced cells served as controls. Since the current study was based on previous findings in our laboratory using untransduced NPCs, it was designed to directly compare these natural NPCs in transplants. However, in a small pilot study, natural untransduced NPCs were compared with NPCs transduced with AAV-GFP. Evaluation of tactile and cold allodynia did not show any significant differences between these and untransduced NPCs (Supplementary Fig. 1). Recombinant and non-recombinant NPCs were intraspinally injected at 5 weeks post SCI when pain related behavior was fully developed. The clip compression model has been utilized for evaluation of below-level neuropathic pain following SCI as it produces pain behavioral symptoms sustained for at least 12 weeks post-injury (34). This time frame allows the opportunity to evaluate transplant interventions at more clinically relevant stages post-injury.

The antinociceptive effects of recombinant 1(SHG) NPC, 6(SHG) NPC and non-recombinant NPCs are shown in Fig. 4. Both tactile allodynia (Fig. 4A) and cold allodynia (Fig. 4B) develop within 2–3 weeks following clip compression spinal cord injury, and stably plateau in severity by approximately 3 weeks following injury. The intraspinal injection of recombinant or non-recombinant GABAergic NPCs at 5 weeks post-SCI reduced symptoms of both tactile and cold allodynia in SCI rats, in contrast to the sustained allodynia observed in control saline injected animals for the remaining duration of the study. All three groups receiving NPC grafts, non-recombinant NPCs, 1(SHG) NPCs and 6(SHG) NPCs, showed significant improvement in tactile allodynia compared with saline grafted rats (overall $F(df3,11)=3.71$, $*p=0.001$). Recombinant NPCs producing SHG showed enhanced effects over non-recombinant cells, with both longer lasting and more robust antinociceptive

effects. The recombinant cells 1(SHG) and 6(SHG) showed significantly stronger antinociceptive effect than non-recombinant NPCs ($p < 0.05$) starting at 2 weeks following transplantation. In particular, recombinant cells with 6(SHG) copies induced the most stable and consistent antinociceptive effects on both tactile and cold allodynia. Stronger antinociceptive effects were observed in animals receiving 6(SHG) vs 1(SHG) ($p < 0.05$ at 1 and 2 weeks post grafting). Robust, nearly complete reversal of tactile allodynia was maintained for the duration of the study following transplantation of 6(SHG) NPCs.

A reduction in cold allodynia was also observed in animals grafted with NPC, 1(SHG) and 6(SHG) cells beginning on week 2 post transplantation compared with the saline group (overall $F(df3,11) = 2.82$; $p < 0.05$). Animals grafted with non-recombinant cells showed reduced sensitivity to cold stimuli that appeared to gradually increase over time (by week 6 post-transplantation, responses were not different from saline control animals). The recombinant grafts produced more robust and prolonged effects, with significant differences from saline controls detected through the end of the study. Significant differences between rats grafted with non-recombinant and recombinant cells were observed between 4 to 6 weeks post-injection ($p < 0.05$). The 6(SHG) grafts in particular appeared to nearly completely abolish cold allodynia and maintain effectiveness throughout the time course of the study.

To evaluate the contribution of SHG released from the grafts to the observed antinociceptive effects, SHG antibody was intrathecally injected in some animals with 6(SHG) and 1(SHG) grafts (Fig. 5). Since the reversal studies were conducted at 4 weeks post-transplantation, the pre-injection baselines for both the 1(SHG) and 6(SHG) were both at time points near maximum anti-allodynic effects; thus at this point, pre-injection responses, although slightly higher in the 6(SHG) animals, were similar in both groups. In animals grafted with 6(SHG)NPCs the intrathecal injection of anti-SHG significantly attenuated the antinociceptive effects in both behavioral tests ($p = 0.02$ compared with pre-injection responses). In animals grafted with 1(SHG) NPC, the antinociceptive effects of the graft on either tactile or cold allodynia were slightly reduced, but not significantly reversed ($p > 0.05$ compared with pre-injection responses) after anti-SHG injection. Injection of GABA-A receptor antagonist bicuculline methiodide (Bic) significantly reversed the antinociceptive effects of the grafts in both groups and in both tests ($p < 0.05$ vs baseline). Reversal of the effect of the grafts was also observed after combined injections of the SHG antibody and bicuculline ($p < 0.05$ vs baseline). In all cases, there appeared to be enhanced reversal of the engineered NPC effects with combined anti-SHG and Bic administration compared with either agent alone; however this was only statistically significant for tactile allodynia in the 1(SHG)NPC group. ($p = 0.045$ vs baseline, $p = 0.03$ vs anti-SHG). Intrathecal injection of an irrelevant control protein (albumin) did not significantly alter the anti-allodynic effects of the engineered NPCs ($p > 0.05$ vs baseline).

To evaluate the possible nonspecific effects of anti-SHG, it was injected into animals grafted with non-recombinant NPC. No differences in either tactile or cold allodynia were observed compared to pre-injection (baseline) values.

Immunohistochemical analysis and quantification of the graft

Grafts were localized bilaterally in the dorsal horn of the lumbar spinal cord (Fig. 6A). In graft regions, DAPI positive cells were densely packed and circumscribed compared with the host dorsal horn cellular distribution. Transplanted recombinant cells were partially identified in the spinal dorsal horn based on the red fluorescent signal of mRFP and/or positive staining for the SHG peptide. Some grafted cells were detected in the deeper dorsal horn areas where they may have migrated. The rostrocaudal extent of the graft was approximately 0.5 mm from the injection site in all cases. Although the signal of the mRFP and SHG markers using immunohistochemical staining was weak for precise identification and quantification of the graft, probably due to cryoprocessing, biochemical detection of SHG in the grafted tissue by the sensitive FLISA method (below) yielded consistent results confirming the presence of SHG in the tissue.

Quantification of mRFP signaling in the grafted area of the SCI group (bilateral injection) showed an average of 423.8 ± 23.5 mRFP positive cells in the 6(SHG) NPC group (Fig. 6B) and 235.1 ± 12.5 in the 1(SHG) NPC group. Colocalization with GABA was detected in $19.2 \pm 4.1\%$ and $13.6 \pm 6.3\%$ of SHG cells in the 6(SHG) (Fig. 6C, E–G) and 1(SHG) group, respectively. Quantification of SHG/NeuN colocalization showed $13.6 \pm 8.4\%$ of SHG objects associated with the NeuN marker (Fig. 6D, H–J). Orthogonal analysis further confirmed colocalization of these markers in some of the cells. Figure 7 depicts the analysis of cells labeled by stars in Fig. 6G and 6J. The peaks of optical densities for red (SHG), green (NeuN/GABA), and blue (DAPI) color along the vertical and horizontal lines show the presence of the respective marker labeled by immunofluorescent antibody. Overlapping or juxtaposition of peaks suggests colocalization of respective markers (Fig. 7 Ga–b, Ja–b). Single-labeled cells displayed peaks for DAPI and red (Fig. 7 Gc, SHG and DAPI) or green (Fig. 7 Jc, NeuN) signals only.

Using the total amount of injected cells (100,000/side) and ratio of SHG transduction in the cell culture (~70% of neuronal cells), and the number of mRFP objects identified in the spinal cord, we estimate about 0.5% remaining recombinant NPCs at 6 weeks following transplantation. However, these numbers may be underestimated due to technical issues including the low signal of mRFP and SHG staining in the spinal cord, and possible loss of NPCs along the injection barrel during transplantation. To overcome the technical difficulties using immunocytochemical identification of recombinant cells, we measured SHG expression in the spinal cords of grafted animals using biochemical assays.

Biochemical analysis of grafted tissue

The presence of SHG in the spinal cord tissue of animals grafted with recombinant cells was evaluated by FLISA (Fig. 8). Since SHG is a synthetic peptide not normally produced in the mammalian spinal cord, its presence is indicative of successful production by engineered transplanted cells. Loading the SHG peptide in different concentrations allowed us to quantify the relative amount of SHG in the spinal tissue. Analysis of spinal cord samples from SCI animals grafted with 1(SHG) and 6(SHG) NPCs confirmed the presence of recombinant SHG in the graft region of these animals (Fig. 8A). There was significantly enhanced SHG signal in 6(SHG) samples compared to 1(SHG); no detectable signal

observed in nonrecombinant NPC samples. Analysis showed 0.04 ± 0.02 mg/ml and 0.18 ± 0.03 mg/ml of SHG in 1(SHG) NPC and 6(SHG) NPC samples respectively (Fig. 8B; $p < 0.01$, t-test). The average ratio of the SHG concentration for 1(SHG) and 6(SHG) NPC samples of 1:4.5 is close to the expected 1:6 (with some samples at the 1:6 ratio) and further supports the hypothesis of SHG expression by recombinant cells transduced by 1(SHG) or 6(SHG) cDNAs.

Discussion

The findings of this study suggest that strong and sustained attenuation of persistent neuropathic pain can be achieved using neural progenitor grafts enhanced by genetic engineering to produce additional antinociceptive molecules. In animal models of neuropathic pain there are qualitative changes in the processing of nociceptive and non-nociceptive signals due to injury-induced hyperexcitability of the dorsal horn neurons. Those changes may lead to development of increased sensitivity to noxious and non-noxious stimuli that are observed after peripheral nerve injury or spinal cord injury. While several mechanisms likely contribute to dorsal horn hyperexcitability after injury, disrupted GABA signaling and enhanced NMDA-receptor activity seem to be common in persistent neuropathic pain of both etiologies (2,18,20,21,29,30,32,45,46,49,70).

Regardless whether or not overt GABAergic cellular death occurs, it is clear that loss of spinal inhibitory tone and consequent abnormal hyperexcitability contribute to the maintenance of neuropathic pain, and is a promising target for intervention. Although the reasons for reduced GABA signaling after peripheral or central nerve injury are still matter of debate (57,58,60), several studies showed that transplantation of GABA-releasing cells is an effective way to reduce neuropathic pain symptoms in experimental models of pain (7,22,23,38,43,54,71,74). The findings in our lab suggest that the positive effects of neural progenitor grafts are likely mediated, at least in part, by releasing GABA in the dorsal horn, as both antinociceptive effects and attenuated dorsal horn hyperexcitability by NPC grafts were reduced by GABAergic antagonists (42,43).

Nevertheless, current technologies appear limited in their restorative capabilities, as reversal of symptoms has been incomplete in both peripheral and central models of neuropathic pain in our lab (42,43,48,49). Since it is thought that only a small percentage of grafted cells (perhaps as low as 1–3% in peripheral nerve injured animals (7,45), ~0.5% in our study) remain at 1 month, an approach to improving beneficial outcomes of the grafts is to enhance the antinociceptive capabilities of the cells that do survive and integrate. This and other studies demonstrated that even a fraction of surviving grafted GABAergic cells is sufficient to reduce pain related behavior and influence neuronal responses to peripheral stimulation (7,43,45), probably as a results of direct release of GABA from grafted cells and enhancing of survival of endogenous GABAergic cells (11,21,22,41). Similarly, studies focused on neurodegenerative diseases report therapeutic effect of transplanted NPCs with less than 10% of cells producing desired therapeutic substances (4,63).

Here we attempted to augment this approach by concurrent attenuation of excitatory signaling via glutamate NMDA receptors. The activation of NMDA receptors initiates

hyperexcitability of spinal neurons that cause abnormal responses to peripheral stimuli (5,10,24,56). NMDA receptor antagonists have been shown to attenuate injury-induced hyperalgesia in animal models; however, their clinical use is compromised by their narrow therapeutic window and adverse side effect such as motor weakness and hallucinations (44). Previous studies in our lab showed that the novel peptide NMDA antagonist SHG can reduce pain symptoms without motor deficits and may be a promising adjunct in pain therapies (33,37,39,55,69). SHG is a synthetic analog of histogranin, a natural peptide with NMDA antagonist activity produced by chromaffin cells (50,61). Since neuropathic pain is thought to be mediated in part by activation of dorsal horn NMDA receptors and consequent neuronal hyperexcitability, SHG may be an ideal complementary candidate for reducing abnormal hyperexcitability and restoring inhibitory balance, in conjunction with the GABAergic NPC transplants. Therefore we have designed recombinant GABAergic progenitor cells producing NMDA antagonist peptide SHG (SHG-NPCs) for intraspinal grafting in animals with spinal cord injury-induced neuropathic pain.

The current results demonstrated enhanced antinociceptive effects of the SHG-engineered NPCs, particularly using multimeric SHG. A role for the engineered SHG transgene in reducing chronic SCI pain is suggested by partial reversal using intrathecal SHG antibodies in the current study. In addition, sustained SHG transgene expression in the grafted host spinal cord can be achieved using this approach as indicated by neurochemical and immunocytochemical assays. Results showing the ability of both the SHG antibody and GABA_A antagonist bicuculline to partially block the anti-allodynic effects of SHG-NPCs suggest a contribution of both NMDA-antagonist and GABAergic mechanisms of the grafted cells. The differing efficiencies of anti-SHG in reversing the analgesic effects of the 1(SHG) and 6(SHG) grafts may suggest different dominant mechanisms. It is possible that SHG expression reduces allodynia via both an acute antagonism of NMDA receptor-mediated events and by longer term effects on graft survival and/or host-graft integration (e.g. trophic effects). If the latter effects are predominant, they are likely not readily reversible by acute anti-SHG administration in the 1 (SHG) grafts, in contrast to the 6(SHG) grafts which produce higher SHG levels and may include both acute and trophic effects on dorsal horn pain neurocircuitry. Attenuation of anti-allodynia by bicuculline also suggests an important role of GABA in the effects of engineered NPCs. A likely source of GABA is from the transplanted NPCs since GABAergic differentiation is a default neuronal differentiation for NPCs, and this can be further enhanced by pre-differentiation in vitro by FGF withdrawal. Previous findings in our lab have shown that these pre-differentiated NPCs express GABA following transplantation into the spinal dorsal horn (25,48) and a role for NPC-derived GABA was suggested in electrophysiological and behavioral evaluations of NPC grafts in a model of peripheral neuropathy (43).

Cells transplanted into the CNS can provide therapeutic molecules by acting as a local minipump or by integration within the host neurocircuitry. NPC grafts are particularly promising because of their potential to differentiate to appropriate neuronal phenotypes and restore damaged neural circuitry. As dysfunction of vulnerable GABAergic interneurons is considered a leading pathological candidate in chronic pain models, the transplantation of GABAergic NPCs may contribute to the restoration of dorsal horn inhibition by integration within the host inhibitory neurocircuitry (7,43). The functional integration of GABAergic

NPC may also overcome the excitatory action of GABA reported after injury (7,12,15,62). Although viral vectors can be utilized to deliver analgesic peptides, the use of GABAergic cells as delivery vehicles may thus additionally contribute to the restoration of disrupted endogenous GABAergic signaling induced by the injury. Recombinant cells also may provide neurotrophic support for endogenous host cells thus enhancing restorative capacities of damaged tissue. Further beneficial anti-nociceptive effects may be achievable by using multi-segmental cell transplantation.

Chronic SCI neuropathic pain is a notoriously challenging and difficult to treat clinical target. Thus, the robust antinociceptive effects of SHG-producing NPC grafts are particularly encouraging since cell transplantation was delayed following the onset of SCI pain symptoms (5 weeks post-injury) as is likely to be encountered in the clinical setting. In spite of the low number of grafted cells detected we were able to demonstrate production of SHG in concentration-related levels corresponding with the use of 1(SHG) or 6(SHG) gene constructs. These data support our hypothesis that even a small number of grafted cells producing analgesic peptides are sufficient to induce behavioral changes and overcome limitations of low cell survival.

In conclusion, findings from this study support the potential for engineered neural progenitor cell transplants to alleviate neuropathic pain resulting from spinal cord injury. Stem cells of various origins have emerged as promising candidates for therapeutic intervention, and are currently being initiated and evaluated in early phase clinical trials. NPC transplantation is a promising means for accomplishing the goal of long-term pain relief by both replacing lost or dysfunctional spinal inhibitory neurons, and by providing a constant source of pain-reducing agents. Our findings suggest targeting spinal NMDA-mediated hyperexcitability in conjunction with inhibitory GABAergic restoration using engineered NPC grafts may be particularly beneficial in the management of chronic SCI pain.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Immunocytochemical identification of serine histogranin (SHG) produced by 1(SHG) and 6(SHG) transduced NPCs. Colocalization of the SHG signal with TuJ1 marker suggests transduction of proneuronal cells (A–H). Colocalization with GABA confirms transduction of GABAergic cells (I–L). The cell morphology and the expression rate of TuJ1 and GABA markers were similar between recombinant and nonrecombinant cells, suggesting that transduction did not have effects on cellular phenotypes of NPC cultures. No SHG signal

was detected in nonrecombinant cells (M–T). DAPI was used as nuclear staining to identify cells in cultures.

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Figure 2.

FLISA analysis of cell culture supernatant for detection of SHG peptide. A) The fluorescent signal of SHG is significantly enhanced in 6(SHG)NPC compared to 1(SHG)NPC in the baseline level and after the cell stimulation. No signal was detected for non-recombinant NPCs. B) Estimated amount of SHG in samples * $p=0.001$ vs NPC; # $p=0.01$ vs Baseline; + $p=0.001$ 1(SHG) vs 6(SHG).

Figure 3. Immunohistochemical analysis of sham (A–D) and SCI (E–H) spinal cord tissue (5 weeks post injury) for the presence of GABA positive neurons in the lumbar spinal cord. Reduction of GABA immunoreactivity in SCI samples indicate remote changes in the spinal cord after thoracic level of SCI. Reduction of GABA immunoreactivity was mostly observed in the cytoplasm of neurons.

Figure 4.

Intraspinal injection of non-recombinant NPC, 1(SHG)NPC, 6(SHG)NPC, or saline into SCI animals at 5 weeks post injury. Both recombinant cell groups demonstrated enhanced and prolonged reduction of tactile (A) and cold (B) allodynia. * $p < 0.05$ vs saline, # $p < 0.05$ vs nonrecombinant NPC, + $p < 0.05$ vs 1(SHG)NPC. $n = 6/\text{saline}$; $n = 10/\text{transplant}$

Tactile Allodynia

Cold Allodynia

Figure 5.

Intrathecal injection of SHG antibody (antiSHG), bicuculline (Bic), and antiSHG + bicuculline into SCI rats grafted with 1(SHG)NPC or 6(SHG)NPC. As controls for antiSHG effect, nonspecific peptide albumin (BSA) was injected to 6(SHG) group and antiSHG was injected to animals with nonrecombinant graft (NPCs only). Animals were tested 30 min post drug injection. * $p < 0.01$ compared with pre-injection responses; # $p < 0.05$ vs anti-SHG. $n = 6-8$ /group (except $n = 4$ for albumin).

Figure 6.

Immunofluorescent detection of grafted cells in the spinal cord. Grafts were localized bilaterally in the dorsal horn within the laminae L II-L IV (A). For graft quantification, the grafted area was overlaid with grid, DAPI and mRFP positive profiles were estimated in each grid square and used to calculate total of mRFP profiles per treatment group (B). Colocalization of SHG signal with GABA immunostaining (C, E-G, orange arrows) demonstrated differentiation and survival of grafted progenitors in the host tissue in SCI animals. Differentiation of grafted SHG cells into neuronal phenotype was observed as well (D, H-J, orange arrows). Some of the single stained cells are labeled by white arrows.

Figure 7. Orthogonal profiles of cells labeled in by stars in Fig. 6G and Fig. 6J shows colocalization of markers (Ga-b, Ja-b) or a presence of single marker only (Gc, Jc). DAPI was used as nuclear staining.

Figure 8.

FLISA analysis of spinal cord tissue homogenates of SCI animals treated with nonrecombinant and recombinant NPCs. A) Differences in the SHG signal demonstrate the production of SHG in the grafted area. Authentic peptide SHG is shown in the first column, GADPH was used to normalize data. B) Estimated amount of SHG detected in the tissue. Differences were detected between 1(SHG) and 6(SHG) NPCs (* $p < 0.01$ vs NPC; # $p < 0.01$ vs 1SHG) grafted tissue in SCI animals.