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Intraspinal transplantation of GABAergic neural progenitors attenuates neuropathic pain in rats: a pharmacologic and neurophysiological evaluation

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Abstract

Dysfunctional γ -aminobutyric acid (GABA)-ergic inhibitory neurotransmission is hypothesized to underlie chronic neuropathic pain. Intraspinal transplantation of GABAergic neural progenitor cells (NPCs) may reduce neuropathic pain by restoring dorsal horn inhibition. Rat NPCs pre-differentiated to a GABAergic phenotype were transplanted into the dorsal horn of rats with unilateral chronic constriction injury (CCI) of the sciatic nerve. GABA signaling in antinociceptive effects of NPC grafts was tested with the GABA_A receptor antagonist bicuculline (BIC), GABA_B receptor antagonist CGP35348 (CGP) and GABA reuptake inhibitor SKF 89976A (SKF). NPC-treated animals showed decreased hyperalgesia and allodynia 1-3 week post-transplantation; vehicle-injected CCI rats continued displaying pain behaviors. Intrathecal application of BIC or CGP attenuated the antinociceptive effects of the NPC transplants while SKF injection induced analgesia in control rats. Electrophysiological recordings in NPC treated rats showed reduced responses of wide dynamic range (WDR) neurons to peripheral stimulation compared to controls. A spinal application of BIC or CGP increased wind-up response and post-discharges of WDR neurons in NPC treated animals. Results suggest that transplantation of GABAergic NPCs attenuate pain behaviors and reduce exaggerated dorsal horn neuronal firing induced by CCI. The effects of GABA receptor inhibitors suggest participation of continuously released GABA in the grafted animals.

Keywords

neuropathic pain; GABAergic progenitors; GABA receptors; wind-up; post-discharges

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Introduction

Chronic pain often accompanies injury to the peripheral and central nervous systems. The mechanisms responsible for this type of pain are still not completely understood. Conventional therapies have low efficacy for neuropathic pain. Neither pharmacological treatment nor surgical intervention is optimal, since drug tolerance and addiction, untoward side effects and worsening pain emerge over time. Thus, there is a critical need to identify alternative approaches based on the pathophysiology of neurotraumatic pain syndromes.

Processing of somatosensory information in relation to pain occurring in the superficial laminae of the spinal dorsal horn is modulated by local and descending inhibitory circuits, in which γ -aminobutyric acid (GABA) and glycine play key roles as inhibitory neurotransmitters. The importance of GABA signaling has been shown by blockade of spinal GABAergic neurotransmission with intrathecally applied GABA receptor antagonists, which produce hypersensitivity to innocuous tactile stimuli (Gwak, et al., 2006, Hao, et al., 1994, Loomis, et al., 2001, Malan, et al., 2002, Sivilotti and Nistri, 1991). Also, transgenic mice that lack specific subunits of GABA receptors develop hyperalgesia and allodynia (Schuler, et al., 2001, Ugarte, et al., 2000). Decreases in GABA immunoreactivity (GABA-IR) and the GABA synthesizing enzyme GAD65/67 accompanied by the development of hyperalgesia and allodynia have been shown in rats after peripheral nerve injury (Castro-Lopes, et al., 1993, Eaton, et al., 1998, Gwak, et al., 2006, Ibuki, et al., 1997, Lee, et al., 2008). Correspondingly, administration of GABA into the spinal cord alleviates nerve injury-induced nociceptive behavior (Eaton, et al., 1999a). Intrathecal administration of GABA_A or GABA_B receptor agonists has been shown to produce a dose-dependent analgesia in animals with peripheral nerve injury, that is blocked by GABA receptor antagonists (Hwang and Yaksh, 1997, Malan, et al., 2002). One electrophysiological study on spinal cord slices from rats has confirmed a deficit in GABAergic inhibitory neurotransmission in spinal dorsal horn after peripheral nerve injury (Moore, et al., 2002). Such observations suggest that loss of inhibitory neural circuitry could play a role in allodynia and hyperalgesia developing after nerve injury (Eaton, et al., 1999a). Although there is controversy with regard to actual overt loss of GABAergic interneurons in the spinal cord (Polgar, et al., 2004), the above observations combine to suggest that there is a dysfunction of inhibitory processes in the spinal cord after peripheral nerve injury.

However, systemic pharmacological targeting of the GABAergic system has proven unsatisfactory for relieving such pain (Robert, et al., 2010, Slonimski, et al., 2004), perhaps because of the widespread distribution and multifunctional roles of GABA in the central nervous system (CNS). Novel ways to obviate the problems of ubiquitous GABA receptor distribution in the CNS and the temporary effects of pharmacologic treatment include direct delivery of GABA to spinal pain processing sites via cell-based or gene therapy. These are also better suited to the long-term management of chronic pain. Chronic pain behavior in rodent models has been previously reported to be improved by transplantation of GABAergic neurons or of cells bioengineered to secrete GABA (Eaton, et al., 1999a, Eaton, et al., 1999b, Eaton, et al., 2007, Stubbley, et al., 2001). In particular, pre-differentiated GABAergic neural progenitors (NPCs) have been shown to attenuate peripheral nerve injury-induced allodynia when transplanted intraspinally (Mukhida, et al., 2007b). Our laboratory has shown that primary neurospheres generated from E14 rat telencephalic NPCs, when intraspinally transplanted, attenuate spinal cord injury-induced neuropathic pain in rats. These neurospheres contain numerous GABAergic neurons when pre-differentiated by transient withdrawal of mitogenic factor FGF-2 (Furmanski, et al., 2009). In the present study, pre-differentiated GABAergic NPCs were intraspinally transplanted into rats with peripheral nerve injury and their effects on symptoms of neuropathic pain were assessed. To further characterize the effect of the NPCs, responses of dorsal horn neurons in close

vicinity of the graft to peripheral stimulation were evaluated by electrophysiology. Pharmacologic evaluations in both behavioral and electrophysiological outcomes were used to determine the involvement of GABAergic signaling in the effect of grafts. Preliminary accounts of these findings have been reported previously (Jergova, et al., 2009, Jergova, et al., 2010)

Materials and Methods

Animals

Male Sprague-Dawley rats (140-160g at the time of the first surgery) were used for peripheral nerve injury and intraspinal injections; 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 12h 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).

E14 cell isolation

Pregnant females were deeply anesthetized by intraperitoneal injection of ketamine (75mg/kg) and xylazine (5mg/kg). E14 embryos were harvested and placed in ice-cold Hank's Balanced Salt Solution (HBSS). The cortical lobes and underlying lateral ganglionic eminences were removed with fine forceps and collected in fresh ice-cold HBSS. Tissues were pooled, centrifuged at 4°C, resuspended in HBSS, dissociated by trituration and single cell suspensions were collected. Dissociated NPCs were pelleted and resuspended in growth medium DMEM/F12 with 1% N2 supplement and 10ng FGF-2/ml (R&D Systems). NPCs cultures were seeded in tissue culture flasks at 1×10^5 cells/cm². NPCs were allowed to grow and form neurospheres over 3 to 4 days *in vitro*. Withdrawal of FGF-2 one day prior transplantation was used to induce pre-differentiation of NPC to a GABAergic phenotype, as shown in our lab previously (Furmanski, et al., 2009). NPCs were then used for transplantation.

Surgical procedures

All surgical procedures were conducted under 2-3% Isoflurane/O₂ anesthesia. Chronic constriction of the sciatic nerve was used to induce peripheral neuropathic pain (Bennett and Xie, 1988). The common sciatic nerve was exposed on the right side at the mid-thigh level using aseptic surgical techniques. Four 4-0 chromic gut ligatures spaced about 1 mm apart were loosely tied around the sciatic nerve proximal to the trifurcation. The wound was closed in layers and animals (total n=72, utilized in various studies as described in relevant sections below) were left to recover in heated cages.

One week after CCI animals showing changes in the reaction to thermal or mechanical stimuli, (behavioral testing details below) were used for intraspinal injection of cells (n=38) or equal volume of saline vehicle (n=34). A T13-L1 laminectomy was done aseptically to expose L3-L4 lumbar spinal cord. Cells were loaded into a glass micropipette (tip diameter ~50 µm) attached to a Hamilton syringe, and injected into the right (ipsilateral) lumbar gray matter using a stereotaxic stage (Stoelting, Wood Dale, IL). The glass pipette was placed 0.5 mm right from the central vein and an injection was made at depth of 1 mm from the dorsal lumbar spinal surface. A volume of 3 µl (100,000 cells/µl) was injected at a rate of 1 µl/min; 3 µl of saline was injected in control CCI rats. The injection of saline vehicle was chosen as control to avoid any potential confounding effects that may result from release of trophic factors or other active agents by control cellular transplants. The use of vehicle or cell culture media as control has been previously employed in other transplantation studies

(Hendricks, et al., 2006, McDonald, et al., 1999, Mitsui, et al., 2003, Park, et al., 2010, Zhao, et al., 2004). After injection, the pipette was kept in place for additional 1 min. Muscles were sutured in layers, overlying skin was closed with wound clips 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.

Some of these animals at 1-2 weeks post intraspinal injection of either saline (n=10) or NPC (n=10) were used for pharmacologic evaluation of grafted cells by intrathecal injection of GABAergic drugs. An intrathecal catheter (7.5-8 cm; ReCathCo, PA) was threaded through a slit in the atlanto-occipital membrane down the intrathecal space and secured to the neck muscles with sutures under 2-3% isoflurane/O₂ anesthesia as described previously (Hama and Sagen, 2009, Yaksh and Rudy, 1976). This procedure brings the tip of catheter over the lumbar spinal segments. Rats were allowed to recover at least three days following intrathecal surgery prior to use in experiments.

Behavioral tests

Rats were tested weekly up to 5 weeks post injury. All behavioral tests were performed by a trained person blinded to the experimental treatment. The same person always performed a given test to reduce variability. On each testing day, animals were moved in the testing room and left to acclimate for 30 min before testing. When testing chambers were used, animals were placed in the chamber for another 20 min or until exploratory behavior ceased prior to sensory assessments. For each test the positive response was considered as the withdrawal of the paw during the stimulation. Such a response was usually a part of a more complex behavior such as licking and shaking the paw or vocalization.

Heat hyperalgesia—Sensitivity to noxious heat stimulation was evaluated according to the method of Hargreaves (Hargreaves, et al., 1988). Animals were placed in plastic chambers with the heat source underneath. A heat source was positioned under the middle of the plantar side of the hind paw and was controlled by a timer that was stopped by paw withdrawal and time latency was recorded. To avoid tissue damage in the absence of withdrawal, a cut-off time was set at 20 sec. Latency was tested for left and right hind paws with at least one minute interval between each paw. Three trials were conducted with 5 minute interval between each trial on the same paw. Difference scores were calculated by subtracting withdrawal latencies on the intact side from the injured side; thus negative difference scores are indicative of heat hyperalgesia.

Cold allodynia—To assess cold allodynia, 100 µl of acetone was applied to the plantar hind paw and reactions were observed for 1 minute. Lifting of the hind paw, licking and shaking were considered to be positive responses. Tests were repeated five times, with at least 2 min interval between successive trials; the percentage of positive responses was recorded.

Mechanical hyperalgesia—Mechanical hyperalgesia was evaluated using the Randall-Selitto method (Randall and Selitto, 1957). Briefly, an Ugo Basile pressure apparatus was used to assess the pressure threshold of hind paws. An increasing force was applied to the paw and the force inducing paw withdrawal was recorded. The test was repeated three times on each paw with at least 5 minute delay between tests on the same paw. Difference scores were calculated by subtracting withdrawal thresholds on the intact side from the injured side; thus negative difference scores are indicative of mechanical hyperalgesia

Drugs

For pharmacologic evaluation of grafted GABAergic cells, bicuculline methiodide (BIC, 0.3 $\mu\text{g}/5\text{ }\mu\text{l}$; a GABA_A antagonist), CGP35348 (CGP, 25 $\mu\text{g}/5\text{ }\mu\text{l}$; a GABA_B antagonist) and SKF89976A (SKF, 720 ng/5 μl ; a GABA transporter inhibitor) were used; all drugs were obtained from Sigma. Drugs were intrathecally injected in a volume of 5 μl followed by 5 μl of saline following determination of pre-drug responses. Rats were tested for heat hyperalgesia and cold allodynia as above at 30 min intervals up to 2 hours after drug injection. Only one test and one drug was used on the same day per one rat, and at least a 48 hr washout interval occurred between tests. As a control, 10 μl of intrathecal saline was injected. For electrophysiological evaluations, drugs were topically applied on the spinal cord in a volume of 5 μl .

Neurophysiology

The effect of grafted cells on the electrical activity of adjacent neurons was evaluated by extracellular recording of action potentials. Pre-injury activity was recorded from 3 rats. Post injury activity was recorded at weekly intervals up to 4 weeks from 3 NPC animals and 3 control saline animals per week (total $n=12/\text{group}$). Rats were initially anesthetized with 4-5% isoflurane in O₂ and maintained on 2-3% isoflurane/O₂. The body temperature was controlled by feedback and a heating blanket and kept at 37° C (Kent Scientific). The jugular vein and trachea were cannulated and the animal was positioned in a spinal frame. T11-L2 laminae were removed to enlarge the space for electrophysiological recording around the visually localized transplanted area. A single glass microelectrode filled with 3 M NaCl, with a tip diameter of 5-10 μm , was inserted into the spinal cord. Recordings were made 0.3-0.6 mm lateral to the midline at the side of transplant at a depth of 300-600 μm . Several recording sites 1-2mm rostral and caudal from transplant area were evaluated to obtain readings from 2-4 neurons per animal. Only neurons that responded to innocuous (touch) and noxious (pinch) stimuli were used for recording. To evoke responses, needle electrodes were inserted into the foot and a stimulus of intensity sufficient to excite C fibers (amplitude 10-40 V) was applied with frequency 0.1 Hz to 1 Hz. A frequency of 0.1 Hz was used to detect neurons. Only neurons responding to electrical stimulation and a light touch of the hind paw were further analyzed. The recording of neuronal responses started when stable responses of a neuron were observed for at least one minute. For baseline stimulation, a frequency of 0.1Hz was used for 20 seconds followed by 20 second stimulation at 1Hz frequency. Spikes were detected and sorted by software written in MatLab (MathWorks, Natick, MA) and counted in three time windows at poststimulus latencies of 0-20 ms (responses of A β fibers), 40-300 ms (responses of C fibers) and 300-500 ms (late responses of C fibers). For each window, responses were quantified as the average number of spikes per second during higher frequency stimulation (1Hz) divided by the average number of spikes per second during baseline stimulation (0.1Hz). To quantify wind-up, the number of spikes produced by the last five stimuli in each 20-stimuli train was divided by the number of spikes produced by the first five stimuli. For pharmacologic analysis, rats at 1-2 weeks post intraspinal cell implantation ($n=6$) or saline vehicle injection ($n=6$) were used to test the effect BIC or CGP ($n=3$ per each drug/group). GABAergic drugs were applied topically on the spinal cord in a volume of 5 μl and recordings were made at 30 min intervals up to 2 hours post drug application. Responses of 2-3 neurons per rat were obtained. To track the position of the electrode in the spinal cord, some electrodes were filled with 2% Fast Green dye which was manually injected into the spinal cord at the end of recording. At the end of electrophysiological experiment, rats were overdosed with anesthetics and perfused.

Immunohistochemistry and imaging

Animals were deeply anesthetized with ketamine (75 mg/kg) and xylazine (5 mg/kg) and intracardially perfused with 0.9% saline followed by 4% paraformaldehyde in 0.1 M PBS.

Spinal cords were removed and post-fixed for 4 hours in the same fixative and transferred to 25% sucrose for cryoprotection. Free floating cryostat sections were cut at 35 μ m and processed according to standard immunohistochemical protocols. Sections were incubated in 5% NGS for 2 hours followed by overnight incubation with primary antibodies rabbit anti-GABA (1:2000, Sigma) and mouse anti-NeuN (1:200, Chemicon) in 5% NGS. After washing, sections were incubated for 1 hour in anti-rabbit AlexaFluor 594 and anti-mouse AlexaFluor 594 secondary antibodies (1:250, Invitrogen), followed by DAPI (1:60, Invitrogen) incubation for 30min. Sections were then washed in PBS, slide-mounted and coverslipped. Image analysis was performed by fluorescent (Olympus BX 51) and confocal (Spectral Confocal Microscope Fluoview 1000) microscopy.

For the estimation of GABAergic profiles in the spinal cord of grafted or saline injected animals, every fourth immunostained section from lumbar spinal cord of three rats per group was used for analysis. Eight sections per animal within the graft/saline injection area were analyzed to cover the spread of the graft, total of 24 sections per group. Sections were digitalized under 20x magnification using Olympus BX51 fluorescent microscope and CCD camera and analyzed by ImageTool (UTHSCSA). The number of GABAergic profiles were counted in laminae I-III and IV-V according to Rexed (Rexed, 1952). The average number of profiles per section was compared between contralateral and ipsilateral sides in each group and between ipsilateral sides of control and experimental animals.

Statistics

Statistical analysis employed SigmaStat 3.1 software. Behavioral data were assessed by two-way ANOVA with repetitive measurements with group and time post-drug injection as variables followed by Holm-Sidak post-hoc analysis; level of significance was $p < 0.01$ and $p < 0.05$. Electrophysiological data were assessed by one-way ANOVA with level of significance $p < 0.05$ for comparison between groups and versus baseline responses. Estimation of GABAergic profiles was assessed by t-test with level of significance $p < 0.001$.

Results

Effects of NPC transplants on nociception following CCI

Behavioral tests for mechanical and heat hyperalgesia and cold allodynia were carried out weekly (Fig. 1). Since the total number of tested animals was reduced weekly as they were used in electrophysiological and pharmacologic experiments, the following results include the data from the 10 rats/group that completed the entire time course of the study.

Mechanical Hyperalgesia—Withdrawal thresholds of the ipsilateral paw compared to the contralateral paw decreased after CCI, resulting in negative difference scores by one week following the injury, which tended to worsen further during the 2-4 weeks post-CCI (Fig. 1A). Mechanical hyperalgesia persisted for at least 5 weeks post-CCI in both treatment groups. However, treatment with NPCs partially improved mechanical hypersensitivity. Differences between the NPC treated and control saline treated animals were significant on the second and third week post treatment (overall $F(df\ 1,16) = 4.944$; $p < 0.05$).

Heat Hyperalgesia—A decrease in paw withdrawal latency after CCI was also observed in the Hargreaves heat hyperalgesia test (Fig. 1B). By one week post-injury, significant heat hyperalgesia, as indicated by negative difference scores between ipsilateral and contralateral hindpaw withdrawal latencies, was observed in both groups of animals ($p < 0.01$ compared with pre-injury baselines). In control treated animals, difference scores fell to -2.3 ± 0.2 seconds following CCI, and animals continued to show significant heat hyperalgesia throughout the remainder of the follow-up ($p < 0.01$ compared with baseline). A partial

spontaneous improvement was observed 4-5 weeks after injury in the control group. In contrast with control animals, NPC treated rats showed significantly reduced heat hyperalgesia as indicated by nearly complete reversal of negative difference scores by one week post transplantation ($p > 0.05$ compared with baseline). Attenuated heat hyperalgesia by NPC grafts was sustained throughout the remainder of the study (overall F (df 1,16) = 4.964; $p < 0.05$ compared with saline control treated animals).

Cold Allodynia—Cold allodynia, measured by responses to application of acetone on the glabrous skin of the hind paws, was nearly absent at pre-surgical baseline, increasing to approximately 50% by one week following CCI (Fig. 1C; $p < 0.01$ compared with baselines). This increased sensitivity to cold continued to worsen in control treated animals with CCI in the ensuing weeks, to 60-70% through week 5. Cold allodynia in CCI rats that received NPCs did not worsen in the weeks following transplantation, and was significantly lower compared with control injected animals (overall F (df 1,16) = 3.925; $p < 0.05$).

Effects of NPC transplants on dorsal horn electrophysiology following CCI

Responses to peripheral electrical stimulation of 10-40 V were measured in laminae III-V neurons. A frequency of 0.1 Hz was used for baseline responses, which was changed to 1 Hz to examine wind-up (Schouenborg, 1984).

A β fiber responses—In pre-injury rats, A β fiber responses to 0.1 Hz baseline stimulation were not altered when 1 Hz stimulation was applied for 20 sec (Fig. 2A). In contrast, after CCI, a $30 \pm 13\%$ increase was seen 1 week post surgery in A β responses to 1 Hz stimulation compared to 0.1 Hz, but this difference disappeared in later weeks. NPC treatment did not significantly affect this potentiation of A β fiber responses.

Wind-up—Wind-up was measured as a ratio between the number of spikes during the last 5 stimuli and the number of spikes during the first 5 stimuli of a 20 sec 1-Hz train in the 40-300 ms window (C-fiber response window; Fig. 2B). Pre-injury wind-up levels showed a $41 \pm 13.5\%$ increase in neuronal firing. This was slightly increased, but not significantly, by CCI in the first week. Wind-up responses remained almost unchanged in subsequent weeks after ipsilateral spinal saline injection. Wind-up responses following transplantation of NPCs were significantly lower in NPC treated CCI animals, 2 weeks after transplantation ($p < 0.05$).

Post-discharges—CCI produced a strong effect on post-discharge responses (in the window 300-800ms; Fig. 2C). These increased significantly in both group of rats that underwent CCI by one week post-injury ($p < 0.05$ compared with pre-injury baselines). Nerve injury caused an increase in response to 1 Hz stimulation of up to $80 \pm 26\%$ at this time point. In the saline treated group, the post-discharge levels remained elevated in the following weeks and returned to baseline by four week post injury. In rats with NPC transplantation, post-discharges fell rapidly, returning to pre-injury baseline levels ($p > 0.05$ compared with baseline). Thus NPC treated animals showed significantly reduced post-discharges compared with saline treated rats at 3 weeks post injury ($p < 0.05$).

Effects of GABAergic agents on NPC transplants

In order to evaluate the potential contribution of GABAergic modulation in the observed behavioral and neurophysiological effects, intrathecal (for behavioral tests) or spinal (in neurophysiological experiments) application of BIC, CGP and SKF was examined. For control, treatment with saline was used. Rats were tested with GABAergic drugs at 1-2 weeks post NPC injections as at that time point differences in the behavior and electrophysiological responses were most robust.

Behavior

Heat Hyperalgesia: On test days prior to drug administration, baseline responses (BL) were obtained. Because of the effects of NPC transplants on attenuating heat hyperalgesia as shown above, pre-drug hindpaw difference scores (at BLs) were initially smaller than those of control injected animals with CCI (Fig. 3). Intrathecal saline injection (Fig. 3A) did not change difference scores in paw latency after heat stimulation in either group during the observation period. Bicuculline treatment (Fig. 3B) caused a significant enhancement in the ipsilateral hindpaw responses to noxious heat, as indicated by increased negative difference scores in NPC treated rats, which reached its peak effect at 60 min post BIC injection ($p < 0.05$ compared with pre-injection BL). At this time point, the difference scores reached levels similar to the control treated CCI animals, indicating a full reversal of the antihyperalgesic effects of the NPC transplants ($p > 0.05$ compared with control group). A small, but non-significant decrease in difference scores was noted in saline treated CCI rats. Following intrathecal injection of CGP, heat hyperalgesia was significantly increased in NPC treated rats, with peak exacerbation of difference scores at 30 min post injection (Fig. 3C; $p < 0.05$ compared with pre-injection BL). No changes in saline treated CCI rats were observed following CGP treatment. The injection of SKF (Fig. 3D) led to opposite results; an attenuation in the magnitude of difference scores was observed in both CCI groups by 30 min post injection. In particular in control injected animals, SKF produced a transient but nearly complete reversal of heat hyperalgesia during 60-90 min post injection ($p < 0.05$ compared with pre-injection BL).

Cold allodynia: Similar to heat responsiveness, at BL prior to drug treatment, cold allodynia was attenuated in animals with NPC transplants compared with saline injected CCI controls (Fig. 4). Intrathecal saline injection (Fig. 4A) did not cause any significant changes in responses to acetone application on the ipsilateral hind paw compared to pre-injection BL values in either group, and differences between saline-treated and NPC-treated groups remained significant during most of the observation period (overall F (df 1.8) = 11.322; $p < 0.05$). Intrathecal BIC appeared to exacerbate cold allodynia in both groups (Fig. 4B). However, the effect of BIC was more evident in NPC-treated rats, producing significantly increased responses to over 80% by 120 min post injection ($p < 0.05$ compared with pre-injection BL). CGP injection (Fig. 4C) did not change responses to stimulation dramatically, despite a slight non-significant increase in the NPC treated group 30 min post injection which resulted in comparable cold responses in both treatment groups. The injection of SKF (Fig. 4D) reduced responses to cold in both groups, with a significant anti-allodynic effect seen in saline treated CCI rats ($p < 0.05$ compared with pre-injection BL).

Since no significant effects of the GABAergic drugs were observed on responses to the mechanical hyperalgesia test, in either NPC or saline vehicle groups, these data are not shown.

Electrophysiology

Wind-up: Bicuculline (BIC) had a complex effect on wind-up (Fig. 5A-C). BIC gradually increased the firing of neurons during 1 Hz repetitive stimulation in both groups of animals (Fig. 5B, C), more so in the saline treated group, at 60 and 90 min post BIC application ($p < 0.05$ compared to baseline). However, wind-up itself, expressed as a difference between initial and final responses during each bout, was reduced in saline-treated rats at 60 and 90 min (Fig. 5A), because the mean number of action potentials at the beginning and at the end of stimulation was almost the same (Fig. 5B). The effect of CGP (Fig. 5D-F) was less marked. It increased wind-up in the NPC treated group at 90 min post injection (Fig. 5D; $p < 0.05$ compared with BL). However, the overall C-fiber response showed no differences at any time point following CGP (Fig. 5E, F).

Post-discharges: Post-discharges, the late responses of C fibers recorded in the 300-800 ms window, were compared following treatment with BIC or CGP (Fig. 6). Prior to drug administration, pre-treatment post-discharges were low in the NPC-transplanted animals, in contrast to the saline-injected control CCI animals. Bicuculline (Fig. 6A) facilitated post-discharges in both CCI groups, with peak effects at 60 min post-BIC ($p < 0.05$ compared with pre-drug BL for NPC group). By 120 min, the post-discharges in the NPC group dropped back down to baseline levels ($p > 0.05$ compared with pre-drug BL). Modestly enhanced post-discharges were observed 30 min post CGP injection in saline CCI rats, but this response was unstable and not statistically significant (Fig. 6B). No changes in post-discharges were observed in NPC transplanted rats following CGP treatment at any time point, which remained low during the recording period.

Histological analysis

The GABAergic phenotype of transplanted cells was identified in parallel *in vitro* cultures as described previously (Furmanski, et al., 2009) (data not shown). Histological examination of the spinal cords transplanted with NPCs revealed grafted cells located in the deep dorsal horn (Fig. 7A). The transplant area was identified as clusters of DAPI positive nuclei that were not present in this region on the contralateral side of the spinal cord (Fig. 7B). Some grafted cells were localized several millimeters caudal and rostral to the injection site. Using co-localization with neuronal marker NeuN, immunocytochemical examination showed the presence of GABA positive neuronal cells in the graft (Fig. 7C). The morphology of grafted cells was clearly distinct from endogenous GABAergic interneurons, as NPCs appeared as large and round cells in deeper dorsal horn in contrast to much smaller and elongated endogenous interneurons located mainly in superficial laminae. In saline injected animals, an injection track was identified based on the slightly changed morphology of the spinal dorsal horn, with a column of DAPI nuclei reaching from the spinal cord surface into the dorsal horn (Fig. 7D). No GABAergic profiles were observed in the saline injection area (Fig. 7E). The position of the recording electrode was traced by FastGreen dye (Fig. 7F). Recordings were performed in the close vicinity of the transplant area.

Evaluation of the GABAergic profiles in the spinal cord dorsal horn of saline injected rats showed reduced GABA-immunoreactivity in the dorsal horn ipsilateral to the constriction injury compared to contralateral side (Fig. 8; $p < 0.001$). GABA loss was observed in both superficial and deep dorsal horn regions. The intraspinal injection of GABAergic NPCs increased GABA-immunoreactivity in the ipsilateral dorsal horn of grafted rats. GABAergic profiles were increased in the dorsal horn ipsilateral to the CCI of NPC-grafted rats compared with the respective spinal cord areas of saline treated rats. ($p < 0.001$ vs saline; Fig. 8). The increased GABAergic immunoreactivity in grafted animals was primarily found in the deeper laminae (laminae IV-V), where the NPC transplants had been targeted, with significantly more GABAergic profiles in this area compared to saline-treated animals ($p < 0.001$ for laminae IV-V, $p > 0.05$ for laminae I-III). Although the GABAergic restoration in grafted animals appeared incomplete, total dorsal horn GABAergic immunoreactivity was not substantially reduced from the intact non-injured side ($p > 0.05$ compared with intact contralateral side).

Discussion

Dysfunctional GABAergic interneurons in the spinal cord are a likely contributing cause of chronic neuropathic pain. Thus transplantation of GABAergic cells is a promising strategy for relieving this pain, as it may provide a long-term renewable source of GABA rather than rely on repeated drug administration. The results of the present behavioral testing support this approach, by demonstrating reduced hyperalgesia and allodynia in injured rats that received pre-differentiated GABAergic NPCs. In fact, NPC treatment temporarily reversed

heat hyperalgesia back to the pre-injury values, and attenuated cold allodynia and mechanical hyperalgesia. Moreover, single neuron recordings showed an electrophysiological correlate in the decreased neuronal responsiveness to peripheral stimulation in rats with NPC transplants.

The relationship of dysfunctional GABAergic signaling with the development of pain has been shown previously in both pharmacological and electrophysiological experiments (Castro-Lopes, et al., 1993, Eaton, et al., 1998, Gwak, et al., 2006, Ibuki, et al., 1997, Moore, et al., 2002). Injury-induced neuropathic pain has been attributed to disinhibition of spinal nociceptive transmission caused by the loss of GABAergic interneurons. However, in spite of observations of apoptotic neurons in the spinal dorsal horn, detailed quantitative stereological studies have not revealed decreases in the number of GABAergic or glycinergic neurons in the spinal dorsal horn in rats with neuropathic pain after peripheral nerve injury (Polgar, et al., 2003, Polgar, et al., 2004, Polgar and Todd, 2008). Also, an electrophysiological study using a mouse model of CCI showed no major changes in membrane excitability and firing patterns of these spinal cord interneurons (Schoffnegger, et al., 2006). However, decreases in GABA synthesizing enzymes and transporters have been measured (Castro-Lopes, et al., 1993, Eaton, et al., 1998, Gwak, et al., 2006, Ibuki, et al., 1997, Lee, et al., 2008) so the question whether GABAergic interneurons are overtly lost or have become dysfunctional following nerve injury remains open.

In attempt to restore the inhibitory function in the spinal cord after peripheral nerve injury or spinal cord injury, several studies have explored the effects of intraspinal or intrathecal injection of GABAergic neuronal precursor cells or cells engineered to produce GABA. A reduction in tactile allodynia and thermal hyperalgesia induced by peripheral nerve injury has been reported after intrathecal injection of engineered rat neuronal cell lines releasing GABA (Eaton, et al., 1999b) and human GABAergic cell line NT2 (Vaysse, et al., 2011). The beneficial effect of such therapy was observed one week post-grafting and persisted up to 8 weeks. Intraspinal grafting of mouse striatum GABAergic cells or pre-differentiated human GABAergic neural precursor cells reduce tactile allodynia after L5/6 spinal nerve ligation (Mukhida, et al., 2007b). The enhancement of GABA production and pain alleviation has also been reported after intrathecal delivery of the human NT2 GABAergic cell line in the excitotoxic spinal cord injury model (Eaton and Wolfe, 2009, Wolfe, et al., 2007), and mouse embryonic stem cells can reduce spinal cord injury-induced pain behaviors in spinal cord hemisection (Kim, et al., 2010) and excitotoxic injury models (Hendricks, et al., 2006).

The attenuation of pain behaviors after transplantation in these and the current study could be due to secretion of GABA from grafted cells. GABA released from cells may act directly as to modulate signals from the periphery ascending to supraspinal sites (Stubley, et al., 2001). Indirect action of GABA may also be due to its neuroprotective effect on intrinsic interneurons. Neuroprotective actions of GABA have been suggested by studies where acute GABA therapy in ischemia models limited and reversed cell death (Hollrigel, et al., 1996, Jolkkonen, et al., 1996, Lyden and Lonzo, 1994). Alternatively, the transplanted cells could influence the pain transmission by releasing trophic factors. Several studies showed that grafted cells enhanced the survival of the neighboring cells and promote recovery after various kinds of injury by the release of trophic factors. In pain models, transplantation of neurotrophic factor-secreting cells has been shown to promote the recovery of GABA-IR neurons in the spinal cord (Cejas, et al., 2000, Eaton, et al., 1998, Ibuki, et al., 1997).

Possible migration of grafted cells to other parts of CNS could also influence the behavioral effect of such treatment observed in ours and in the other studies. Since GABAergic neurons could influence other physiological functions if misplaced or migrating to other regions,

safety concerns will need to be addressed further as transplantation therapies such as this proceed towards clinical application. However, no massive migration is reported in studies using neuronal progenitor grafts or bioengineered GABAergic cells. Grafted cells are usually found up to 1 cm around the injection site (McDonald, et al., 1999, Mitsui, et al., 2003, Mukhida, et al., 2007a, Su, et al., 2009, Wolfe, et al., 2007), and GABAergic cells identified in the current study also did not appear to migrate beyond the local graft site. Daily observations of the animals did not reveal any obvious physiological dysfunction or alterations in normal daily activities.

To test whether the antinociceptive effect of the grafts may involve enhanced GABAergic function, the effects of the GABA receptor antagonists BIC and CGP and of the GABA reuptake inhibitor SKF on pain-related behavior were evaluated 2 weeks post injury, when there were clear differences in pain-related behavior between grafted and control groups. Both BIC and CGP enhanced heat hyperalgesia and cold allodynia in NPC treated rats, but not in saline treated rats. SKF, on the other hand, attenuated pain-behavior in the saline treated group with minimal effect in the NPC treated rats. The enhanced responses to peripheral stimulation after injection of GABA receptor antagonists in rats receiving GABAergic grafts support the idea that the grafted cells elevated levels of GABA in the spinal cord. Since BIC showed a greater effectiveness than CGP, the GABA_A subtype of GABA receptor may have a more important role in the pain attenuation by NPC transplants.

The hypothesis that intraspinal grafts of NPCs can reduce peripheral neuropathic pain by reducing dorsal horn hyperexcitability was further tested by recording responses of cells near the transplant to peripheral stimulation, examining different times after transplantation. Some tests exploited the phenomenon of wind-up, which is a facilitation of responses in nociceptive spinal neurons that is seen when the frequency of electrical stimulation is changed from slow (0.1 Hz) to moderate (1 Hz) (Schouenborg, 1984). Wind-up is frequently used to study sensitization of dorsal horn neurons in electrophysiological and pharmacological studies (Vikman, et al., 2005), since it can be expressed as a dimension-free ratio. Wind-up is thought to be primarily caused by increased NMDA receptor-mediated postsynaptic currents, but a modulatory role for GABA receptors has also been shown (Sokal and Chapman, 2001, 2003, Vikman, et al., 2007). Several clinical studies have reported wind-up like phenomena in patients with neuropathic pain after spinal cord injury (Eide, et al., 1996, Felsby, et al., 1996, Price, et al., 1992). *In vivo* studies on wind-up in animal models report small changes after nerve injury (Chapman, et al., 1998, Laird and Bennett, 1993, Xu, et al., 1995). For the CCI model used in our study, disinhibition has been previously seen electrophysiologically (Reeve, et al., 1998), but there is no report of significant changes in the rate of wind-up. The current study corroborates this, as no significant changes in the intensity of wind-up were observed after injury compared to pre-injury values. However, our findings showed reduced neuronal excitability in NPC transplanted rats compared with control CCI animals. A significant reduction in neuronal hyperexcitability was observed two weeks post grafting, in concert with the observed reduction in thermal and mechanical hyperalgesia in the NPC transplanted rats. Since previous studies have suggested that wind-up is partially modulated by GABA signaling (Sokal and Chapman, 2001), the reduced wind-up response observed in GABAergic NPC treated rats could be partially due to release of GABA from grafted cells.

Post-discharges were also compared between the CCI rats with saline or NPC treatment. The post-discharges represent a late response of C fibers to peripheral stimulation, when firing of the cell occurs beyond the end of the stimulus. They are consistently present in a dorsal horn neuronal responses to mechanical stimulation after partial nerve injury (Laird and Bennett, 1993, Palecek, et al., 1992, Yakhnitsa, et al., 1999), although their cause remains unclear. The behavioral correlate of this phenomenon may be the prolonged paw withdrawal in

neuropathic animals following noxious stimulation (Hokfelt, et al., 2005). In the current study, changes in post-discharge responses paralleled behavioral outcomes, as increased post-discharge ratios were observed one week post injury when pain behavior was fully presented. In the saline treated CCI rats, the post-discharge rate remained elevated through the end of experiment. NPC treatment reduced this to levels comparable with naïve uninjured rats. A relationship between the rate of post-discharges and the disinhibition in the spinal cord was shown by Drew et al. (Drew, et al., 2004), who found that microiontophoretic application of BIC in normal rats increased the rate of late C fiber response firing. The reduced post-discharges in NPC treated rats in the current study could be due to enhanced GABA release from the graft.

The observed reduction in responses of deep dorsal horn neurons in the electrophysiological experiments correlate with the histological analyses of the graft position, showing increased numbers of GABAergic profiles particularly distributed in deeper dorsal horn laminae in grafted animals.

Electrophysiological recording was also used to evaluate the effects of spinal application of BIC and CGP on neuronal responses after peripheral stimulation in treated animals. In previous reports, spinal application of GABA or glycine antagonists was shown to enhance neuronal firing after low and high threshold stimulation (Sivilotti and Woolf, 1994), the changes being attributed to injury-induced disinhibition in allodynia-like states. In the current experiment, application of BIC or CGP facilitated wind-up responses in NPC treated rats. BIC also enhanced neuronal firing in control (saline treated) rats, although wind-up was paradoxically reduced, as also reported by others (Reeve, et al., 1998). This effect is due to an enhanced but stable increase in neuronal firing during repetitive stimulation, as opposed to progressively increased responses. Increased post-discharges as a result of disinhibition, as observed by Drew et al. (Drew, et al., 2004), was confirmed in the current study showing facilitated post-discharges after BIC application in the both groups of rats. CGP enhanced post-discharges in the saline treated CCI rats only.

Conclusions

The observation that GABAergic drugs, particularly the uptake blocker SKF elicited responses, albeit small, in CCI rats without graft suggests that dorsal horn GABAergic signaling is not totally abolished by peripheral nerve injury in these animals. The enhanced efficacy of GABAergic antagonist drugs in GABAergic transplanted rats support a role for increased GABA-mediated inhibitory tone generated in the spinal cord after NPC grafting. The effects of GABA_A and GABA_B antagonists may indicate altered levels of GABA receptor expression after the injury and GABAergic NPC grafting, and include a contribution of both receptor subtypes in these antinociceptive effects. Together these findings indicate the involvement of disrupted GABAergic inhibition in neuropathic pain behaviors and dorsal horn excitability, and support the beneficial effects of GABAergic NPC transplantation in partially attenuating these consequences of peripheral nerve injury.

However, considering the complexity of neuropathic pain mechanism, disrupted GABA signaling play only a partial role here. Therefore, to further enhance analgesic outcome using cell therapy and to promote recovery of spinal inhibitory tone, targeting of additional systems involved in pain processing has to be addressed.

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Highlights

Dysfunctional GABA signaling may contribute to neuropathic pain

Transplantation of GABAergic neuronal progenitors improved pain-related behavior

Grafted GABAergic cells reduced hyperexcitability of dorsal horn neurons

Antinociceptive effect of grafts is mediated through activation of GABA receptors

Figure 1.

Responses to mechanical (A), heat (B) and cold (C) stimulation after CCI. Negative difference scores for mechanical and heat stimulation indicate hyperalgesia. Increased responses to cold stimulation indicate cold allodynia. Animals with grafted cells (n=10) showed improvement of pain-related behavior compared to saline injected CCI animals (n=10). Inj= intraspinal injection of cells/saline. **p<0.01, *p<0.05 vs baseline; #p<0.05 vs saline group.M

Figure 2.

Electrophysiological recording of spinal neuronal responses (n 8 neurons) after hind paw stimulation. CCI induced enhanced firing of A fibers (A) and C fibers (B, C) compared to pre-injury values. Transplantation of GABAergic NPC reduced wind-up responses (B) and post-discharges of C fibers (C) compared to saline treated animals. Inj= intraspinal injection of cells/saline. * $p < 0.05$ vs baseline; # $p < 0.05$ vs saline group.

Figure 3.

The effect of intrathecal injection of drugs on heat hyperalgesia in CCI rats (n=10/group) at 1-2 weeks post grafting. Saline (A) did not show any effect of heat hyperalgesia. Bicuculline (B) and CGP (C) significantly reduced pain threshold in NPC grafted animals compared to baseline values. The opposite effect was observed after SKF (D) injection with enhancement of analgesia in control rats. BL= baseline. *p<0.05 vs baseline; #p<0.05 vs saline group.

Figure 4.

The effect of intrathecal injection of drugs on cold allodynia in CCI rats (n=10/group) at 1-2 weeks post grafting. Saline (A) did not change responses to cold stimulation. Bicuculline (B) enhanced cold sensitivity in NPC treated CCI rats. CGP (C) had only mild effect. Analgesic effect of SKF (D) was presented in both CCI groups with significant reduction of sensitivity in saline treated rats. * $p < 0.05$ vs baseline; # $p < 0.05$ vs saline group.

Figure 5.

The effect of spinal application of drugs on wind-up response of spinal neurons (n 8 neurons) after peripheral stimulation. Bicuculline (A-C) enhanced wind-up response in NPC treated group (A). The apparent decrease in wind-up potentiation in saline treated group (A) was caused by reduced ratio between initial and final firing of neurons although the overall response was significantly higher in this group (B) compared to NPC group (C). CGP (D-F) enhanced wind-up response in NPC treated animals while overall responses between NPC and saline group were comparable (E, F). * $p < 0.05$ vs baseline; # $p < 0.05$ vs saline group.

Figure 6.

The effect of spinal application of drugs on C-fibers post-discharges (n 8 neurons). Increased firing was observed in both groups up to 60 min post bicuculline application (A). Enhanced firing reappeared in saline treated group 120 min post drug application. The effect of CGP (B) was moderate. * $p < 0.05$ vs baseline; # $p < 0.05$ vs saline group.

Figure 7.

Photomicrograph showing GABAergic NPC graft in the spinal dorsal horn (A). The graft was identified morphologically based on DAPI (blue) and GABA (red) immunohistochemical staining revealing area with numerous cells nuclei that was not observed at the contralateral side of the spinal cord (B). Grafted cells were able to differentiate into mature GABAergic neurons as showed by overlapping of GABA (red) and NeuN (green) signals (C). The track of the needle in saline injected rats was identified by DAPI staining (D). No GABAergic profiles were found in the saline track (E). For electrophysiological experiment, the recording electrode was placed in the close vicinity of transplanted cells and its position was traced using FastGreen dye (F). Scale bar 50 μm (A,B,D,F), 10 μm (C, E).

Figure 8.

Estimation of GABAergic profiles in the spinal dorsal horn laminae of saline-injected or NPC-injected rats. As expected, reduced GABAergic profiles occurred in the ipsilateral spinal cord of saline injected rats compared to contralateral side (* $p < 0.001$, t-test). Intraplinal injection of GABAergic cells enhanced GABAergic immunoreactivity in the ipsilateral spinal cord of the grafted rats compared to saline group (# $p < 0.001$, t-test).