A combination therapy of neural and glial restricted precursor cells and chronic quipazine treatment paired with passive cycling promotes quipazine-induced stepping in adult spinalized rats

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Introduction: In order to develop optimal treatments to promote recovery from complete spinal cord injury (SCI), we examined the combination of: (1) a cellular graft of neural and glial restricted precursor (NRP/GRP) cells, (2) passive exercise, and (3) chronic quipazine treatment on behavioral outcomes and compared them with the individual treatment elements. NRP/GRP cells were transplanted at the time of spinalization.

Methods: Daily passive exercise began 1 week after injury to give sufficient time for the animals to recover. Chronic quipazine administration began 2 weeks after spinalization to allow for sufficient receptor upregulation permitting the expression of its behavioral effects. Behavioral measures consisted of the Basso, Beattie, and Bresnahan (BBB) locomotor score and percent of weight-supported steps and hops on a treadmill.

Results: Rats displayed an increased response to quipazine (BBB ≥ 9) beginning at 8 weeks post-injury in all the animals that received the combination therapy. This increase in BBB score was persistent through the end of the study (12 weeks post-injury).

Conclusion: Unlike the individual treatment groups which never achieved weight support, the combination therapy animals were able to perform uncoordinated weight-supported stepping without a body weight support system while on a moving treadmill (6.5 m per minute) and were capable of supporting their own weight in stance during open field locomotion testing. No regeneration of descending serotonergic projections into and through the lesion cavity was observed. Furthermore, these results are a testament to the capacity of the lumbar spinal cord, when properly stimulated, to sustain functioning locomotor circuitry following complete SCI.

Keywords: Spinal cord injury, Chronic SCI, Embryonic stem cells, Transplantation, Neural restricted precursor cells, Glial restricted precursor cells, Rat, Locomotor training, Passive cycling, Quipazine, Locomotor recovery

Introduction

Complete spinal cord injury (SCI) disrupts both descending and ascending pathways within the spinal cord. The damage leads to a loss of locomotion and sensation that affects the body below the point of the SCI; damage to the cervical levels of the spinal cord results in tetraplegia, while damage to the thoracic or lumbosacral levels of the spinal cord results in paraplegia. Attempts to repair the spinal cord following a complete SCI have

produced many promising insights; however, actual reconnection has not been achieved. Despite the problems facing reconnection, several areas of SCI research, such as rehabilitative therapy, pharmacotherapy, and cellular transplantation, have demonstrated potential for anatomical plasticity and functional recovery.

In recent years, the use of combination therapies that incorporate elements from different therapeutic approaches, such as cellular transplantation, electrical stimulation, and pharmacotherapy have provided great insight into the recovery capacities of the damaged spinal cord. For example, spinalized rats are capable

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of performing weight-supported steps (WSS) when given an acute dose of quipazine and placed on a motorized treadmill with a body weight support (BWS) system that supports 85% of the body weight over the treadmill.^{1,2} Animals that were trained on a treadmill before testing had better stepping parameters than non-trained SCI animals^{1,3,4} and those placed in an upright training position compared to a horizontal position had improved plantar stepping.² Individually, transplantation of progenitor stem cells as well as cells modified to secrete-specific neurotrophic factors can increase spinal levels of neurotrophic factors and provide greater degrees of locomotor recovery following SCI (for a review, see Bregman *et al.*⁵ and Reier⁶). When incorporated into a combination therapy, spinalized animals that received cellular transplants and training also produced WSS when given an acute dose of quipazine and placed on a BWS motorized treadmill.^{3,4} Furthermore, the pairing of acute quipazine administration with epidural spinal cord stimulation also produced WSS in animals placed on the BWS motorized treadmill.^{7,8} These results suggest that the disconnected spinal cord can produce stepping motions even though the animals do not have the ability to support their lower body. This extends findings regarding activation of the central pattern generators and neuroplasticity within the spinal circuitry that have been mainly demonstrated in decerebrated cats^{9–12} that also required body support and exteroceptive stimulation to step on a motorized treadmill following complete SCI.

We examined a combination therapy for complete SCI that incorporated cellular transplants, passive cycling, and chronic quipazine administration. Cellular transplants of neural and glial restricted precursors (NRP/GRPs) were placed into the lesion cavity at the time of injury to fill the lesion cavity, provide neuroprotection, and integrate with the host tissue. Passive cycling was given 5 days a week to prevent muscle atrophy and stimulate locomotor circuitry below the level of the injury. Research in animal models of SCI has demonstrated that the use of passive exercise preserves muscle mass, prevents motor neuron degeneration, and prevents atrophy in limbs affected by immobility.^{13–16} Passive exercise also produces activation of specific muscle groups associated with the rhythmic motions of the cycling apparatus, thus providing central nervous system stimulation and release of neurotrophic factors that contribute to recovery.^{16–22} In order to avoid technical difficulties when pairing chronic quipazine administration with passive cycling, an acute dose that elicited locomotor effects and minimized hind limb side effects was determined. Chronic

quipazine was then paired with the passive cycling to further stimulate the denervated spinal system. We hypothesized that this combination therapy would provide increased locomotor recovery not achieved with the individual treatment elements alone by harnessing the neuroplasticity below the level of the lesion. Behaviorally, we examined locomotion improvements in the open field and on a motorized treadmill without body support. To determine the possibility of spinal reconnection through the lesion cavity, we examined serotonin (5-HT) and large diameter axons passing through the injury site. Since the vast majority of serotonin fibers originate from the raphe nuclei in the brain and descend throughout the spinal cord, the presence of 5-HT fibers below the level of the injury has been used to demonstrate connection through the lesion cavity.^{23–25}

Materials and methods

Subjects

Adult female Sprague-Dawley rats (Charles River Laboratory, Wilmington, MA, USA) were used for all experiments. Rats were housed under conditions of controlled temperature and humidity on a 12 hours light/dark cycle (lights on at 07:00). Animals ($n = 64$) were randomly divided into six groups. Animals received no treatment ($n = 12$) or were treated with either chronic quipazine ($n = 12$), daily cycling ($n = 12$), transplanted NRP/GRPs ($n = 6$) or a combination of chronic quipazine, daily cycling, and NRP/GRP transplants ($n = 12$). A separate group of animals were used for the acute quipazine dose response ($n = 10$). All procedures were performed under the guidelines of the National Institutes of Health and approved by the Institutional Animal Care and Use Committee (IACUC) of Drexel University College of Medicine.

Immunosuppression with cyclosporine A (CsA)

Subcutaneous administration of CsA injection solution (Sandoz Pharmaceuticals Co., East Hanover, NJ, USA) at a dose of 1 mg/100 g per 24 hours began 3 days before the transplantation procedures and continued for 2 weeks post-transplantation. After this, an oral CsA solution (50 µg/ml, Sandoz International, GmbH, Holzkirchen, Germany) was administered via the drinking water until sacrifice. Administration of CsA does not change the reaction of the spinal cord to injury and this is a standard practice for non-autologous stem cell transplantation protocols that is required or the stem cells will be destroyed by the innate immune system. This regimen has been well established to provide appropriate immunosuppression for

cellular transplants without affecting the spinal cord environment.^{26,27}

Surgery

All experiments were performed using female Sprague-Dawley rats (225–250 g) that received a complete spinal transection at T10 following our standard procedures.²⁸ Animals were anesthetized with 5% isoflurane and 2 l of oxygen and maintained at 2–3% isoflurane with 1-l oxygen for the duration of the surgery. Buprenex (0.02 mg/kg) and the antibiotic ampicillin (0.06 mg/kg) were given at the start of surgery. A laminectomy at the T9/T11 level exposed one spinal cord segment. A #10 scalpel blade was used to open the dura and pia mater and #11 scalpel blade was used to make the complete transection of the spinal cord. A fine-tipped microaspiration device was then used to remove 1–2 mm of spinal cord. Following the surgery, each animal was given 5 ml of lactated Ringer's solution (VWR, Radnor, PA, USA) subcutaneously. Animals recovered on heating pads and were closely observed until they achieved sternal recumbency, at the time of returning to their home cage. Bladder care was given three times daily for 2 weeks or until bladder control was regained. Buprenex was given every 12 hours for the first 48 hours after surgery for pain relief. Ampicillin was given for the first 6 days after surgery to stave-off infection. After that, at the first sign of infection, the rats were given subcutaneous injections of ampicillin (0.06 mg/kg) once a day for 7 days. Animals were housed two to three per cage (female rats weighing less than 350 g each may be housed up to three per cage. This is approved by IACUC for our facility) with highly absorbent Alpha-Dri bedding (Shepherd Specialty Papers Inc., Kalamazoo, MI, USA) and cages were kept on warm water pads throughout the study. All lesions were verified as complete through histochemistry, behavioral, and pharmacotherapy analysis. Animals were observed at 3 days after injury and were behaviorally indistinguishable from each other. Any incomplete lesions were removed from the study. Weight and general appearance (tumorigenesis, proper grooming behavior, vocalization, and coat color) were monitored though out the study. We did

not observe any measurable differences between the groups; however, animals that were exercised appeared to have a healthier coat compared to non-exercised animals. The experiment's timeline is detailed in Table 1.

Preparation of NRP and GRP for transplantation²⁷

NRPs and GRPs were isolated from embryonic day 14.0 transgenic Fischer 344 rats (Charles River Laboratory, Wilmington, MA, USA) that express the marker gene human placental alkaline phosphatase. Briefly, embryos were isolated in DMEM/F-12 (Invitrogen, Carlsbad, CA, USA). Trunk segments were incubated in collagenase type I (10 mg/ml; Worthington; Lakewood, NJ, USA)/dispase II (20 ng/ml; Roche Diagnostics, Indianapolis, IN, USA)/HBSS (Cellgro, Herndon, VA, USA) solution for 8 minutes at room temperature to remove meninges from the cords. Cords were dissociated using a 0.05% trypsin/EDTA (Invitrogen) solution for 20 minutes at 37°C. Cells were plated in complete medium ((DMEM/F-12, BSA (1 mg/ml; Sigma, St Louis, MO, USA), B27 (Invitrogen), basic FGF (bFGF) (20 ng/ml; Peprotech, Rocky Hill, NJ, USA), penicillin–streptomycin (100 IU/ml; Invitrogen), N2 (10 µl/ml; Invitrogen); bFGF (10 ng/ml) and neurotrophin-3 (NT-3) (10 µg/ml; Peprotech)) on poly-L-lysine-coated (13.3 µg/ml; Sigma) and laminin-coated (20 µg/ml; Invitrogen) dishes. After dissection, NRP and GRP were cocultured for 7 days in the complete medium to generate a mixed population for grafting. These cultures contained only precursors and were devoid of multipotent stem cells and mature cell types.²⁹ NRPs and GRPs were dissociated from culture flasks using 0.05% trypsin/EDTA, washed, and resuspended at a concentration of 100 000 cells/µl (in basal media) for transplantation. Cells were placed on ice throughout the grafting session and grafted in a 1:3 ratio of NRP to GRP.

After the completion of the grafting session, cell viability was assessed using the trypan blue assay. Viability was always found to be >90%. The composition of the NRP/GRP cultures, with respect to the absence of mature cells, was verified before grafting by staining for the mature neurons (neuronal-specific nuclear protein), astrocytes (glial fibrillary acidic protein), and

Table 1 Experimental Timeline

Day 0	5 days post SCI	2 wks post SCI	12 wks post SCI
SCI (all groups)+cellular transplant (combination and transplant only groups)	Begin passive cycling (combination and exercise only groups)	Begin chronic quipazine administration (combination and chronic quipazine only groups)	Sacrifice (all groups)

Figure 1 Image of passive cycling apparatus. Animals were placed in a custom-built apparatus consisting of four parallel mounted bicycles and entrained by a single sound attenuated electric motor with variable speed control. Rats were suspended above the pedals in an adjustable sling. The hindpaws were attached to the pedals with 3M micropore surgical tape (3M). Exercise began at 5 days post-surgery and continued for the duration of the experiment (12 weeks post-surgery).

oligodendrocytes (receptor interacting protein). The purity of the culture with respect to lineage-restricted precursors was verified by staining for the immature neural marker nestin, and the ratio of NRP to GRP was determined by their corresponding markers, embryonic neural cell adhesion molecule, and A2B5. Cellular transplants of NRP/GRPs were injected into the lesion cavity in an overall volume of 10 μ l (1 000 000 cells) at the time of injury. There were no other differences in the surgical procedure associated with these injections.

Exercise regimen

Animals were placed in a custom-built apparatus consisting of four parallel mounted bicycles and entrained by a single sound attenuated electric motor with variable speed control (Fig. 1). Rats were suspended above the pedals in an adjustable sling. The hindpaws were attached to the pedals with 3M micropore surgical tape (3M, St Paul, MN, USA). Exercise began at 5 days post-surgery and continued for the duration of the experiment (10 weeks post-surgery). Exercise consisted of two 30-minute sessions at 45 rpm with a 10-minute rest in between, 5 days a week. This protocol has been shown to reduce hind limb muscle atrophy following thoracic spinal transection.^{28,30}

Quipazine dose response

These data were obtained to determine a low dose of quipazine that maintained locomotor-like movements

(LM) but reduced the development of quipazine-induced side effects allowing us to place the animals in a passive cycling apparatus. At 3–6 weeks post-injury, the animals were challenged separately with the serotonin receptor agonist, quipazine. The day before serotonin challenge, baseline hind limb function was assessed by an intraperitoneal (IP) injection of saline followed 5 minutes later by BBB score, hind limb tremor, caudal serotonin syndrome, and then treadmill testing. Three doses of quipazine (0.075, 0.15, and 0.30 mg/kg) were administered intraperitoneally with a 2-day washout period between each drug administration. These doses for spinalized animals were selected based on those used in current SCI literature.^{1,31} Quipazine was purchased from Sigma (St Louis, MO, USA).

Chronic quipazine treatment

Starting at 2 weeks post-injury, animals received daily IP injections of 0.075 mg/kg quipazine dissolved in saline 5 minutes before being placed in their home cage (quipazine-only group) or on the exercise apparatus (combination group). This dose of quipazine was chosen based on our dose-response studies as the lowest dose of quipazine that produced LM with little to no negative hind limb side effects. Animals received drug treatments 5 days a week for 10 weeks post-surgery.

Behavioral outcomes

The effects of the drugs on hind limb LM³² and side effects in spinalized rats were assessed using three observational scales, the BBB score, hind limb tremors, and caudal serotonin syndrome. Each animal serves as its own control; therefore, preoperative behavioral data were collected. All testing was videotaped for confirmatory analysis. Once an optimal acute quipazine dose was determined in the acute dose–response study, it was used as the drug challenge dose to assess behavioral outcomes in the combination study.

BBB score

The open field locomotor score (BBB) measures hind limb function while the animals freely move in a 2.5 × 3 ft diameter enclosure. It is a widely used and replicable method of evaluating loss and recovery of over ground hind limb motor function after spinal injury. Rats were placed in an enclosure and scored by two individuals who were blind to the experimental conditions according to the BBB scale. This test is particularly valuable in detecting recovery in hind limb function after contusion, and has been adapted for other injury models including thoracic transection.^{28,33} The BBB scores range from 0 to 21. A score from 0 to 8 indicates the animal exhibits no, slight, or extensive hind limb LM up to coordinated hind limb sweeping. A score of 9 or above indicates some degree of hindquarter weight support with WSS.³³ BBB scoring was performed each subsequent week starting at week 1 post-injury to further insure completeness of the lesion (BBB < 1).

Hind limb tremors

Immediately following the BBB locomotor test, animals were placed for 3 minutes on a treadmill at a speed of 6.5 m per second. While adult spinalized rats do not regain the ability to perform WSS, low doses of 5-HT₂ receptor agonists reliably elicit hind limb tremors following stimulation on a treadmill.³⁴ Hind limb tremors were assessed by observation and palpation after the treadmill testing. Two observers, unaware of the drug treatment, rated hind limb tremors based on a four-point scale (0 = no tremors, 1 = mild (detectable by palpation), 2 = moderate (noticeable but does not interfere with locomotion), and 3 = intense (interferes with function)).

Caudal serotonin syndrome

When serotonin is depleted from lumbar spinal cord by thoracic transection, administration of serotonin or its agonists elicits a group of stereotypic behaviors referred to as the serotonin syndrome.³⁵ Typical behaviors include those expressed by musculature both rostral

(forepaw treading, border patrol, head bobbing, wet dog shakes, and oral dyskinesias) and caudal (hind limb activation and straub tail) to the mid thoracic injury site. Effects rostral to the thoracic injury are not observed in this animal model with low doses of 5-HT₂ receptor agonists, because 5-HT denervation does not occur above the injury level. Thus, the caudal serotonin syndrome score represents motor behavior expressed caudal to the injury and is comprised of the sum for the degree of hind limb activation, which consists of increased coordinated hind limb sweeping with alternating rhythmic movements, and for the degree of increased curvature of the tail. Animals were assessed in their home cage for 5 minutes following drug administration and rated on a four-point scale.³⁴ The rating scale measures the frequency and intensity of each behavior of the serotonin syndrome (0 = none, 1 = mild (about 1–2 bouts of short duration), 2 = moderate (1–2 bouts of long duration or 3–5 bouts of short duration), and 3 = severe (3 or more bouts of long duration)).

Treadmill

Rats that achieved weight support (BBB ≥ 9) were placed on a motorized treadmill (at a speed of 6.5 m per minute) and their unsupported locomotion was videotaped for 3 minutes. The percentage of weight-supported hind limb stepping and hopping during locomotion over total stepping was measured by an observer who was blind to the experimental conditions. A *WSS* is one in which the hindquarters are elevated above the surface of the treadmill, and only the plantar surface of the foot touches the treadmill surface. A *non-WSS* is one in which hind limb stepping movements are made, but the hindquarters, knee, toes, belly, or top of the paw (dorsal stepping) are in contact with the surface of the treadmill. Backwards movements during stance while on the treadmill were considered as a non-WSS. A *hop* was defined as both hindquarters being elevated-off of the treadmill at the same time.

Tissue processing for lesion verification

Animals were sacrificed by Euthasol (Virbac AH, Inc, Fort Worth, TX, USA) overdose (100 mg/kg IP) combined with cardiac perfusion. For lesion verification, spinal cords were removed following perfusion with 4% paraformaldehyde, placed in phosphate buffer (PB) containing 30% sucrose for 72 hours, frozen in OCT (Tissue Tek, Sakura Finetek Japan Co., Ltd., Tokyo, Japan) and sectioned on a freezing microtome at 20 μm. The lesion segment was sectioned parasagittally and alternate

sections were stained with Nissl-myelin to confirm that all lesions were complete. Briefly, sectioned tissue was dehydrated, placed in Citrisolve (VWR, Radnor, PA, USA) for 20 minutes, rehydrated then placed in the myelin stain (cyanine R, FeCl₃, and dH₂O) for 10 minutes followed by the Nissl stain (cresyl violet acetate stain and CV buffer) for 20 minutes. All transections were confirmed to be complete.

Tissue processing for imaging

Six animals per group were sacrificed by Euthasol overdose (100 mg/kg) followed by cardiac perfusion with physiological saline and then 4% paraformaldehyde in 0.1 M PB pH 7.4. Spinal cords were removed and washed with PB for 2 hours, then placed in PB containing 30% sucrose for 72 hours. Specimens were frozen in OCT compound (Tissue Tek, Sakura Inc.) and sectioned on a freezing microtome at 20 μ m. The lesion segment was sectioned parasagittally and alternate sections were stained to examine the cellular transplant survival, the migration of the transplant into the host spinal cord, and any transplant differentiation.

Neurofilament RT-97 and 5-HT immunocytochemistry

Following sacrifice by perfusion (see Materials and methods), 20 μ m sections were washed three times in phosphate buffered saline (PBS) for 5 minutes, incubated for 5 minutes in 0.2% triton PBS, and rinsed three times in PBS for 5 minutes. Non-specific binding was excluded by incubating sections in 10% normal goat serum (NGS) in PBS for 1 hour. The primary antibody RT-97 (1:500) or 5-HT (1:50 000; Eugene Tech., Ridgefield, NJ, USA) was applied overnight in 2% NGS PBS. The slides were rinsed three times in PBS for 5 minutes and incubated with a secondary antibody. For biotinylated secondary antibodies, the sections were incubated for 60 minutes in streptavidin (ABC, VectaStain Elite; Vector Laboratories, Burlingame, CA, USA), visualized with diaminobenzidine and coverslipped with DPX (Sigma, St Louis, MO, USA). For fluorescent protocols, the samples were incubated for 60 minutes with a fluorescent secondary antibody (secondary fluorescent IgG FITC, 1:200 (Sigma, St. Louis, MO, USA) and then coverslipped with Vectashield (Vector Laboratories, Burlingame, CA, USA). Sections used for quantification of immunocytochemistry were processed together in order to reduce background and variability.

Microscopy and imaging

All images were acquired with a DMER-B Leica microscope, through a three CCD chip Sensys Retiga EXi cooled 12-bit camera (PhotoMetrics Inc., Huntington

Beach, CA, USA). Images were processed and quantified through an Apple Powermac G4 computer, using the IPLab software (Scanalytics, Fairfax, VA, USA).

Quantification of axons in the graft

Area fraction of RT-97 or 5-HT immunostaining was quantified using the ImageJ software (National Institute of Health) from 20 μ m longitudinal sections through the center of the graft. The area fraction of immunoreactivity was obtained from micrographs taken at a magnification of 20 $\times$. We then created a template frame (550 $\times$ 822 pixels) that fit the center of the transplant and was applied to all samples in order to determine the area fraction of immunostaining (region of interest (ROI)). Threshold values were set by the observer in order to remove the background. If staining artifact was present, it was manually edited. The area fraction was subsequently calculated by ImageJ software for all sample frames. The mean area fraction of three sequential sections from each animal per group was obtained and used for comparison. The observer was blind to the nature of the experimental groups.

Statistical analyses

Data for BBB tests over time approximate normal distributions and are analyzed by two-way analysis of variance (ANOVA) between groups and times. However, *post-hoc* analysis between groups remains nonparametric and will be performed, where appropriate, using the Mann–Whitney test. Analysis of hind limb tremor expression is performed using the Mann–Whitney test. Catwalk and treadmill outcome measures are parametric and will be analyzed by ANOVA. Anatomical data were analyzed by ANOVA between groups with individual comparisons made by *post-hoc* test utilizing the Bonferroni correction where appropriate.

Results

Optimal dose ranges for quipazine

Activation of the 5-HT₂ receptors by quipazine at a dose of 0.30 mg/kg improves postural and locomotor functions in adult spinalized rats.^{28,36} As quipazine also induces hind limb tremors³⁷ and the serotonin syndrome caudal to the injury,^{34,38} our goal was to determine the dose of quipazine that maintains this established LM while minimizing the behavioral side effects in the adult spinalized rat. Our results (Fig. 2) show the optimal dose of quipazine to be 0.075 mg/kg where hind limb tremor intensity and caudal serotonin syndrome were almost completely eliminated (scores of <1) and the LM was maintained with a BBB score of 6.5 similar to the BBB score seen with the higher dose (0.3 mg/kg) currently used in the literature

Figure 2 Lower doses of quipazine reduce behavioral side effects (SE) while maintaining locomotor-like movements (LM). Higher doses (0.15 and 0.30 mg/kg) increased the expression of the behavioral SE of caudal serotonin syndrome and hind limb tremors. Only the lowest dose of quipazine tested (0.075 mg/kg) reduced the expression of the behavioral SE while maintaining the expression of LM in the hind limbs. BBB scores were similar throughout the remainder of the study (8–12 weeks post-injury). (* $P < 0.05$ compared to controls).

in both rats and mice.^{1,28,31,39,40} Our results are not in contradiction with this literature; however, the constraints imposed by motorized treadmill testing may not permit full expression of LM behavior and side effects seen in the open field.

Figure 3 The combination therapy promoted increased locomotor-like movements (LM). When tested at 4 weeks after spinalization, the average BBB score was 2 regardless of treatment intervention. At 8 weeks after spinalization, the highest BBB score was observed in the combination therapy animals with an average score of 3 compared to spinalized animals († $P = 0.06$ compared to controls. BBB scores were similar throughout the remainder of the study (12 weeks post-injury).

Under saline conditions, there were no differences between the treatment groups during open field locomotion (Fig. 3)

When tested under saline conditions at 4 weeks post-injury, the control animals had an average BBB score of 1.5. The injury, NRP/GRP, passive cycling, and chronic quipazine only groups were not different from control animals. At 8 weeks post-injury, a trend toward an increase in LM represented by an average BBB score of 3 was observed in the combination treatment group ($P = 0.06$). No differences were found between the rest of the treatment groups and injury only suggesting that long-term functional recovery was not achieved in the absence of drug challenge.

Under quipazine conditions, the combination treatment group exhibited weight-support during open field locomotion (Fig. 4)

When tested under acute quipazine conditions at 4 weeks after injury, control animals had an average BBB score of 6. The injury, passive cycling, chronic quipazine, and combination groups were not different than controls,

Figure 4 The combination therapy promoted quipazine-induced weight support. At 4 weeks after spinalization, all animals, when given an acute administration of quipazine, had an average BBB score between 3.5 and 6 indicating, at most, movement around all three joints of the hind limbs. The NRG/GRP only treatment group had a reduced response to acute quipazine administration with an average BBB score of 3.5 compared to controls. At 8 weeks after spinalization, the control animals and those treated with NRP/GRP cells only, exercise only, or quipazine only had an average BBB score of 6.5. The combination therapy group had an average BBB score of 9 indicating weight-support in stance. BBB scores at 8 weeks post-injury were similar throughout the remainder of the study (12 weeks post-injury). (* $P < 0.05$ compared to controls).

when tested under acute quipazine challenge, with an average BBB score of 5.5. The NRP/GRP only group exhibited an average BBB score of 3.5 suggesting a decrease in the expression of behavioral effects elicited by quipazine at this time point compared to controls. At 8 weeks post-injury, the control animals maintained an average BBB score of 7 under acute quipazine challenge indicating extensive reflexive movements of the hind limbs (see Supplementary video 1a). This was similar to the injury, NRP/GRP, passive cycling, and chronic quipazine only groups (BBB = 7.5). The combination treated group was the only group to reach weight support in the open field with an average BBB score of 9.5 with all animals in the group ($n = 12$) achieving weight support (see Supplementary video 1b). Furthermore, no hind limb tremor or caudal serotonin syndrome behaviors developed following chronic treatment with 0.075 mg/kg quipazine. This was confirmed at 12 weeks post-injury where animals remained at plateau performance on these outcome measures (data not shown). Although the other groups did not reach weight support, they were placed on the treadmill for locomotor analysis to confirm they could not take WSS when given cutaneous stimulation from the treadmill. None of these groups were capable of WSS on the treadmill at any time point though out the study.

The combination treatment group was capable of performing quipazine-induced WSS on the treadmill

When tested under saline conditions at 4 and 8 weeks post-injury, none of the animals were capable of performing WSS when placed on a moving (6.5 m per minute) treadmill. Under acute quipazine challenge, neither the control animals nor the injury, NRP/GRP, passive cycling, and chronic quipazine only groups could perform WSS when placed on the motorized treadmill. However, when the combination treated group was placed on the treadmill under acute quipazine challenge at 4 weeks post-injury, they were capable of performing a few WSS (Fig. 5; see Supplementary video 2).

The combination treatment group increased the percentage of WSS over time

Only animals in the triple combination group were capable of WSS. We analyzed the development of their degree of stepping over time. Of the 12 animals studied, 2 animals were capable of WSS at week 4, 7 animals at weeks 6–8, and all 12 animals at weeks 9–11. This was evident from the overall increase in the percentage of WSS for the entire group, which increased from 3% at week 4 to 6% at weeks 6–8, and 27% at weeks 9–11 (Fig. 5).

Figure 5 The combination therapy increased the percentage of quipazine weight-supported steps on the treadmill over time. At 4 weeks after spinalization, the percentage of weight supported steps (%WSS) that the combination therapy animals could perform was only 2%. Between 6 and 8 weeks after spinalization, the average %WSS was increased to 15% and between 9 and 11 weeks increased again to 27% (* $P < 0.05$ compared to controls).

Animals in the combination therapy group increased the percentage of hopping elicited by quipazine from 4 to 8 weeks post-injury

Hopping is an unusual locomotor behavior requiring bilateral hind limb hyperextension. At 4 weeks after

Figure 6 The combination therapy increased the percentage of hopping elicited by quipazine. At 4 weeks after spinalization, about 5% of the attempted stepping resulted in a hop. This percentage increased to over 15% between 6 and 8 weeks and slightly decreased to 11% between 9 and 11 weeks. Hopping was not observed in any of the other treatment groups throughout the study (12 weeks post-injury) (* $P < 0.05$ compared to controls).

injury, 3 of the 12 animals in the combination therapy group hopped 5% of the time they were attempting to walk on a motorized treadmill (Fig. 6). At 6–8 weeks, 7 of the 12 combination therapy animals hopped while on the motorized treadmill and the percentage of hopping increased to 17%. At the end of the study, all of the combination therapy animals exhibited hopping while on the motorized treadmill and the percentage of hopping was 11%. Hopping was not observed in any of the other groups.

Behavioral improvements in locomotor potential are not the result of spinal reconnection through the lesion site

To determine whether the combination therapy promoted reconnection of descending projections through the lesion cavity, we performed analysis on the degree

of 5-HT below the level of the injury as well as large diameter axons through the lesion site. Staining for 5-HT below the level of the injury showed no measureable 5-HT compared to the degree of 5-HT seen above the lesion site (Fig. 7A; ROI 0% versus ROI < 68%, respectively). RT-97 staining showed large diameter axons terminating at the edges of the rostral and caudal lesion cavity. They did not appear to traverse the lesion cavity (Fig. 7B; ROI 0% staining in 2 mm lesion cavity). We are unable to provide percentage of tissue spared in lesion region since these are complete transections with a 1–2 mm aspiration of the entire cord resulting in 0% tissue at the lesion cavity. Nissl-myelin staining was performed on all animals to confirm that there was no tissue sparing at the lesion epicenter (Fig. 7C; ROI 0% at lesion site). We are unable to provide percentage of spared white matter since these

Figure 7 (A) Serotonin staining was only observed rostral of the lesion site. There was an almost complete lack of 5-HT staining (ROI 0%) caudal to the lesion, represented by the black dotted line, when compared to the degree of staining rostral to the lesion (ROI < 68%). This image is representative of all animals within the study. (B) Large diameter axons are only in the host tissues and not in the lesion cavity. Staining for large diameter axons (RT-97) terminated at the rostral edge of the lesion cavity and did not continue through the lesion cavity (black arrows). The large diameter axons are then seen in the caudal portion of the spinal cord below the lesion cavity demonstrating the lack of their presence through the lesion epicenter. (C) Nissl-myelin staining verifies lesion completeness. All lesions were verified as complete through histochemistry, behavioral, and pharmacologic assessments. Apparent tissue sparing in the lateral borders of the lesion cavities show the presence of the NRP/GRP transplanted cells (unpublished data). Similar staining was seen in all other groups and lesion completeness was confirmed for all animals used in this study. These images are representative of all animals within each group.

are not incomplete lesions. All together, these data suggest that the behavioral improvement of quipazine-induced stepping was not the result of spinal reconnection through the lesion cavity.

Discussion

The lumbar spinal cord is capable of producing WSS without supraspinal input

When the lesion cavities of the combination animals were examined histologically, there was no measurable 5-HT staining below the lesion and there were no large diameter axons passing through the lesion cavity. Collectively, these results provide an overall negative picture of the level of reconnection through the lesion cavity resulting from our combination therapy. This lack of reconnection with the rostral portion of the spinal cord suggests that the ability of the combination animals to produce WSS in the open field as well as on a motorized treadmill occurred without supraspinal input. Therefore, although it has been suggested, and demonstrated in trained spinal cats and weight-supported spinalized rodents, that the spinal cord possessed the necessary circuitry at the segmental level to produce walking, this is the first time freely moving; unsupported rats spinalized as adults have demonstrated this ability. Further, the ability of 100% of the animals in the combination treated group to produce quipazine-induced stepping in the open field and on the treadmill is a testament to the capacity of the lumbar spinal cord, when properly stimulated, to sustain functioning locomotor circuitry following SCI.

Tolerance to chronic quipazine administration was not observed in treated animals by 8 weeks post-injury

Chronic administration of 5-HT₂ receptor agonists produces 5-HT₂ receptor downregulation in cortical areas of normal rats and 5-HT denervated rats.⁴¹ Therefore, it was likely that animals receiving chronic quipazine administration would develop a tolerance to the behavioral effects of the 5-HT₂ agonist. This effect has been shown to be due to 5-HT_{2A} receptor changes, but not 5-HT_{2C} receptors, which correlated with development of behavioral tolerance.⁴² Uncoupling between stimulation of second messenger systems and both 5-HT_{2A} and 5-HT_{2C} receptor downregulation has been reported following chronic 5-HT treatment in normal rats.⁴³ In our injury model, there is persistent 5-HT_{2A} and 5-HT_{2C} receptor upregulation caudal to the injury⁴⁴⁻⁴⁶ and no observed tolerance to its behavioral effects with chronic administration; therefore, it is our belief that these 5-HT₂ receptors are resistant to downregulation

from daily injections at the low dose we used in spinalized animals. Chronic IP administration with either quipazine (equally selective for 5-HT_{2A} and 5-HT_{2C}) or mCPP (100-fold more selective for 5-HT_{2C} over 5-HT_{2A}) to spinal animals did not produce tolerance to its motor enhancing effects.^{31,47} Therefore, the decrease in response to acute quipazine administration in the NRP/GRP only animals, which did not occur when NRP/GRP was combined with the other treatments, is unlikely due to a tolerance effect. Perhaps, the NRP/GRP secreted factors that delayed the receptor upregulation that was countered by the other treatments. Further, by 8 weeks post-injury these animals were pharmacologically responding similarly to the other individual treatment groups suggesting that the receptor upregulation of the 5-HT₂ receptors is maintained although extensive immuno-histochemistry would need to be performed at each time point to definitively support this hypothesis.

Quipazine-induced WSS and hopping began at 4 weeks after injury

Starting at 4 weeks after spinalization, animals treated with the combination therapy began to take a few WSS when placed on a motorized treadmill without any body weight support. Over the next several weeks, more animals were capable of performing WSS and those that had already been able to, increased the percentage of WSS taken. This increase in WSS ability over time suggests that the changes resulting from the combination therapy were a progressive process that improved throughout the last 8 weeks after injury. It is possible that given a longer window of recovery, the degree of WSS would increase or become more defined and precise and the emergence of long-term functional recovery might have been possible. Therefore, research focusing on complete thoracic SCI examining the beneficial effects of combination therapies over a much longer time frame than this investigation may be warranted.

There was also an increase in the percentage of hopping that the combination animals exhibited. Only animals that were capable of performing at least one WSS also produced hopping during the treadmill and open field testing under acute quipazine treatment. It appears that the production of hopping is a form of hyperextension. It often appeared between blocks of stepping suggesting that the spinal system may be at an over excited state leading to hyperextension of both hind limbs at the same time. There seemed to be an increase in the percentage of hopping between 4 and 8 weeks post-injury similar to the increase in the percentage of WSS. There was a trend for the percentage of

hopping to decrease between 9 and 11 weeks after injury, while the percentage of WSS continued to increase. This suggests that the hyperextension produced under acute quipazine administration may have begun to stabilize and that this may have helped in increasing the percentage of WSS at the end of the study.

Treadmill WSS preceded expression of WSS in the open field

Animals in the combination treated group tended to be able to take WSS on the motorized treadmill before they were capable of performing WSS in the open field. This is due to the increase in sensory input received by the disconnected spinal cord from the moving treadmill belt.^{10,48,49} There appeared to be a progressive increase in stepping ability that lead to a few single steps within the open field environment under acute quipazine administration. Open field testing was conducted before treadmill testing to insure that the emergence of WSS was not the result from increased sensory input from the treadmill belt.

Recent reports of quipazine-induced WSS

Acute administration of quipazine is often used in SCI animal research to examine the maximum locomotor potential of complete SCI animals. Increased LM (BBB score 3–7) are seen following acute administration due to activation of specific 5-HT₂ receptors located on motor neurons within the ventral horn of the spinal cord. Several recent studies^{7,8,50} have demonstrated that spinalized rats when given acute administration of quipazine and some other form of spinal stimulation such as electrical stimulation to the spinal cord itself can produce WSS on a motorized treadmill when placed in a harness that provides some degree of BWS. Our rats increased the percentage of WSS on the treadmill starting at 4 weeks after injury and were not given any form of BWS when attempting to walk. Furthermore, of the recent reports regarding quipazine-induced WSS, none have demonstrated the capacity to perform WSS in an open field unassisted, highlighting the scientific importance of our findings. It is possible that given more time, the spinalized animals described in several other studies would also have become able to perform WSS without the need of a body support.

Clinical implications, limitations, and future direction

Over the past 20 years, the percentage of patients with incomplete SCI has increased while patients with complete SCI has decreased, however, patients with complete SCI still encompass about 30% of all spinal cord

injuries.⁵¹ Unfortunately, complete spinal cord injuries have the lowest rate of spontaneous recovery highlighting the need for therapeutic interventions. Toward this goal, we have provided valuable information on the potential for the spinal cord to recover independent of descending connections which is invaluable for future comprehensive treatment options for patients with SCI.

In this study, the greatest functional recovery was observed only when the animals were stimulated by drug challenge, thus limiting the combination therapy's potential for immediate clinical translation. Our intention of this study was to explore the potential of these therapies to produce recovery, not to suggest an optimized treatment protocol. We do believe that the timing of the treatments will affect the potential for recovery. For example, since transplantation at the time of injury is not clinically relevant today, other timing options for this particular combination therapy will need to be explored to fully understand the most effective timing to improve locomotor recovery. In our rat model, we have established that 5-HT receptor upregulation occurs at 2 weeks post-SCI, and that quipazine administration beforehand can delay that receptor adaptation; however, this may not be the same in patients with SCI and is limited to complete spinal cord injuries. Furthermore, the use of passive cycling is also limited to complete SCI or very severe incomplete models due to possible injury associated with spasticity and hyperactive reflexes. However, future combination studies for patients with incomplete SCI may incorporate other exercise apparatuses, such as BWS treadmill systems or overground locomotor training.

Conclusion

We have shown that our combination of cellular transplants, passive cycling, and chronic quipazine administration resulted in substantial functional improvement in the hind limbs. We believe this is, in part, due to a cumulative interaction between the individual combination elements. We did not see significant locomotor improvements with the exercise, quipazine administration, or transplantation treatments alone. However, it is possible that the cellular transplantation promoted plasticity of the spinal environment that was harnessed by activation of spinal motor system (through quipazine administration), which could then be fully expressed by exercised hind limb muscles. Nevertheless, we have demonstrated that combining interventions in this manner can substantially modify the neuroplasticity of the spinal cord below the level of injury in ways consistent with the potential for recovery of function.

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Disclaimer statements

Contributors Only cited authors contributed to this paper.

Conflicts of interest None.

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