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Cervical Spinal Cord Therapeutics Delivery: Preclinical Safety Validation of a Stabilized Microinjection Platform

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Abstract

Objectives—The current series represents a preclinical safety validation study for direct parenchymal microinjection of cellular grafts into the ventral horn of the porcine cervical spinal cord.

Methods—Twenty-four 30–40 kg female Yorkshire pigs immunosuppressed with cyclosporine underwent cervical laminectomy and ventral horn human neural progenitor cell (hNPC) injection. Groups 1–3 (n=6 pigs each) were undertaken with the intent of assessing the safety of varied injection volumes: 10 µl, 25 µl, and 50 µl injected at 1 µl/min, 2.5 µl/min, and 5 µl/min, respectively. Groups 4 & 5 (n=3 pigs each) received prolonged immunosuppressant pre-treatment in an attempt to demonstrate graft viability. The latter was undertaken in an alternate species (mini-pig vs. Yorkshire farm pig).

Results—Neurologic morbidity was observed in one animal and was attributable to the presence of a resolving epidural hematoma noted at necropsy. While instances of ventral horn targeting were achieved in all injection groups with a coordinate-based approach, opportunities exist for improvement in accuracy and precision. A relationship between injection volume and graft site cross sectional area suggested limited reflux. Only animals from Group 5 achieved graft survival at a (t=1 wk) survival endpoint.

Conclusions—This series demonstrated the functional safety of targeted ventral horn microinjection despite evidence for graft site immune rejection. Improvements in graft delivery may be augmented with an adapter to improve control of the cannula entry angle, intra-operative imaging, or larger graft volumes. Finally, demonstration of long-term graft viability in future preclinical toxicity studies may require tailored immunosuppressive therapies, an allograft construct, or tailored choice of host species.

Keywords

Microinjection; Neural Progenitor Cells; Cervical Spinal Cord; Cell Therapy; Pigs

INTRODUCTION

In light of the improving understanding of neural stem cell (NSC) biology, insight into neurodegenerative disease pathogenesis, and research findings indicating effectiveness of cell-based therapeutics in small animal disease models, we have previously described a targeting and delivery approach for transplant of a cellular graft into the parenchyma of the porcine conus medullaris(10). We developed a lumbar microinjection platform capable of delivering a defined volume of cells to a specific location within the spinal cord through a microinjection cannula. Precise volume injection and depth control were obtained through use of a microinfusion pump and a microdrive, respectively. Targeting to the ventral horn was achieved through the use of microelectrode recording (MER) and subsequent stimulation. Though targeting proof-of-principle was demonstrated, peri-operative morbidity in this acute survival series required the implementation of procedural alterations designed to maximize the safety of direct parenchymal injection prior to further consideration of this method as a viable therapeutics delivery approach.

The current study extends the proof-of-principle concept for direct parenchymal injection from the lumbar to the cervical spinal cord. An initial emphasis has been placed on characterization of the safety of intraspinal microinjection over a range of infusion parameters. In order to accomplish this, a unique cervical microinjection platform was constructed capable of attachment to porcine and human spines with firm rostral fixation at the occiput and caudal attachment to the C7 spinous process. Second, a coordinate-based targeting approach has been adopted in lieu of an MER-based strategy, addressing the elevated peri-operative morbidity reported previously. Third, multiple groups comprising unique injection volumes and rates were followed for one week post-transplant to ensure safety and to observe the histological effects of different infusion parameters on the cell graft and local tissue architecture. Multiple immunostaining markers were used to assess cell graft viability at endpoint.

The findings presented herein demonstrate the safety of direct intraparenchymal cellular graft delivery to the ventral horn across a range of infusion parameters, provides insight into strategies to further improve the accuracy of delivery to ventral horn, and underscores the challenges of preclinical validation in the context of a xenograft-based cellular transplant.

METHODS

Cells

All infused pigs were injected with human neural progenitor cells (hNPCs), prepared as neurospheres in a similar manner to that described previously(7,12). Neurospheres were dissociated using Accutase (Innovative Cell Technologies, San Diego, CA) and suspended in transplant medium (49% Leibovitz L-15 / 49% PBS-glucose / 2% B-27 supplement) prior to transplantation. Cells were prepared for injection at a concentration of 200,000 cells/ μ l. Prior to injection, cell viability was confirmed with Trypan Blue dye and through cell counting with use of a hemacytometer.

Study Design

Twenty-one 30-40kg female Yorkshire pigs were utilized for cervical intraspinal cell transplantation in four groups and three female minipigs comprised a fifth group. Cell transplants in Groups 1-3 (n=6 pigs each) were undertaken with the primary intent of assessing the safety of cervical intraspinal therapeutics delivery. Each group was characterized by a separate cell suspension volume: 10 μ l, 25 μ l, and 50 μ l. The injection rate was controlled with a programmable Harvard p99 microinfusion pump (Harvard, Inc) connected to a Hamilton syringe by silastic tubing and was set at 1 μ l/min, 2.5 μ l/min, and 5 μ l/min, respectively. This

ensured an equal time of intraspinal residence for the microinjection cannula, regardless of the injection volume. Group 4 and Group 5 (n=3 pigs each) were undertaken with the intent of demonstrating cell transplant viability following prolonged immunosuppressant pre-treatment and followed the same volume and infusion rates as Group 2. In a further attempt to demonstrate graft viability, Group 5 was further characterized by use of an alternate species. As opposed to the Yorkshire farm pig used in all other groups, the mini-pig was used. Procedures on the first three cohorts were approved by the Cleveland Clinic Institutional Animal Care and Use Committee (IACUC). Procedures on the fourth and fifth cohort were approved by the Emory University IACUC.

Pre- and Post-operative Immunosuppression

For Groups 1-3, cyclosporine (Neoral®, Novartis Pharmaceuticals Corporation) administration began one day prior to cell grafting and was maintained throughout the study survival period. Cyclosporine was started at 10mg/kg PO BID. For Group 4 and Group 5, the immunosuppression regimen was begun at pre-operative Day 10 and serum trough levels were determined twice weekly using a MS/MS Tandem Mass Spectrophotometry C assay. Dosage was adjusted to maintain serological levels of 300-400ng/ml.

Surgical Technique

Animal sedation and anesthesia induction were performed with ketamine 20mg/kg, xylazine 1mg/kg, atropine 0.02mg/kg, and acepromazine 0.1mg/kg. Isoflurane 1-3% was used to maintain intra-operative general anesthesia. Both during electrocautery-aided exposure of the cervical spine and during microinjection a short-acting non-depolarizing paralytic, Pavulon® (pancuronium) was used. This pharmacologic aid helped to prevent undesired movement of the animal during critical elements of the procedure.

Two-level cervical laminectomies were performed at the position of the third and fourth cervical vertebrae. The pig was placed in the prone position, and the operative field was aseptically prepared. After appropriate marking, a 22cm incision was created over the cervical spine and a multi-level laminectomy performed. A combination of Surgicel® and Gelfoam® were used as hemostatic aids when necessary. A 2.5cm incision was made through the dura, exposing the cervical spinal cord. The dura was reflected away from the pial layer using 4-0 Nurulon® suture and secured to the deep paraspinal musculature. The rostral end of the stabilized microinjection platform was attached to the base of the porcine occiput using two occipital screws with bicorticate purchase. The caudal attachment point was at C7 using a screw tightened clamp on the spinous process providing rigid fixation of the microinjection platform. The platform itself consists of two rigid bars spaced to provide easy visualization of the dura and spinal cord. A separate stage was positioned along these rods and locked over target allowing for coronal trajectory adjustment. This approach provided secure fixation and access to the cervical spinal cord, as well as easy manipulation of the hydraulic microdrive.

The microinjection procedure represents a modification of the technique we have previously published for lumbar intraspinal microinjection(10). A hydraulic microdrive (Narishige MO-97) was rigidly attached to the stabilization platform for controlled advancement of the microinjection cannula. The dorsal root entry zone (DREZ) was identified under loupe magnification. The pial surface was opened with a microscalpel under loupe magnification with use of microbipolar coagulation as required to provide hemostasis while minimizing damage to dorsal cord structures. The spinal cord was penetrated to the left of midline on an orthogonal trajectory to the cord surface at a point 2mm medial to the dorsal root entry zone (DREZ). The cell suspension was infused at different volume/rates at a depth of 3.5mm from contact at the pial surface following initial cannula introduction to a depth of 4mm. Mediolateral coordinates for cord penetration and infusion depth coordinates were chosen on

the basis of histologic examination of porcine cervical spinal cord at the C3 level. Following infusion, the cannula was left in place for five minutes to minimize the potential for cell suspension reflux along the cannula track.

Following withdrawal of the injection cannula, the entire apparatus was removed and the incision was closed in four layers. Upon removal of the microdrive and prior to removal of the stabilization platform, a single 6-0 blue prolene dural stitch was placed to mark the site of injection. Skin closure was achieved with skin stapling.

Microinjection Platform and Hydraulic Microdrive

The prototype cervical microinjection platform was fabricated in the Department of Biomedical Engineering - Prototype Laboratory in the Lerner Research Institute at The Cleveland Clinic. As described above, the device is optimized for attachment rostrally to the occiput and caudally to the C7 human or porcine spinous process. The distance between spinous process clamps can be adjusted to accommodate a variety of laminectomy lengths. A hydraulic microdrive (Narishige model MO-97) permits additional rostrocaudal and mediolateral electrode or cannula position adjustment.

Behavioral Assessments

Both pre-operative and post-operative (POD1, POD3, and at POD7) behavioral assessments were gathered to assess neurological morbidity associated with the cell infusion procedure. Neurological examination of the animals was performed using a modified Tarlov score. The score is as follows: (0) no voluntary movements; (1) perceptible movements at joints; (2) good movements at joints but inability to stand; (3) ability to get up and stand with assistance <1 min; (4) ability to get up with assistance and stand unassisted <1 min; (5) ability to get up with assistance and stand unassisted >1 min; (6) ability to get up and stand unassisted >1 min; (7) ability to walk <1 min; (8) ability to walk >1 min; (9) full recovery and normal walking. After assessment of the Tarlov scores on postoperative day 7, the animals were euthanized.

Histology

For Groups 1-3, following euthanasia, spinal cords segments were dissected at the levels of C2 and C5 and subsequently fixed in a 4% Paraformaldehyde (PFA) (Sigma-Aldrich Co, St Louis, MO) solution in phosphate buffered saline (PBS), pH 7.4 for 10 days. Dura was left on specimens with the 6-0 prolene stitch to ensure localization of the injection site. The following day, they were transferred to 30% sucrose in PBS for 24-48 hrs. The excised cord segments were frozen and cut into 30 μ m sections with a cryostat (Jung Frigocut 2800N, Leica Microsystems, Nussloch, Germany). For Group 4 & Group 5, transcardiac perfusion was performed. This technique was employed to better preserve local tissue cytoarchitecture given the increased quantity of immunostaining performed to detect either evidence for graft viability or an immune-mediated rejection response. In this case, just prior to euthanasia, pigs were sedated and given a 10,000 USP Units intravenous dose of Heparin Sodium. The animals were then euthanized and transcardially perfused with 0.9% NaCl solution, followed by 4% PFA.

Anatomical Analysis of Transplanted hNPCs

The area and anatomic location of the transplanted cell grafts were quantitatively compared between pigs that received 10, 25 and 50 μ l injections of hNPCs. Nissl staining was performed in order to visualize transplanted hNPCs. Sections were photographed with a SPOT Insight digital camera (Model 3.2.0, Diagnostic Instruments, Sterling Heights, MI) using SPOT Software (Version 4.6, Diagnostic Instruments) and analyzed for area and anatomic location of the transplants using Axiovision LE software (Version 4.3, Carl Zeiss, Thornwood, NY). The area measurement was taken from the section corresponding to the epicenter of the graft

site. The x and y-coordinates of the graft site epicenter were measured relative to the center of the ventral horn grey matter, and by arbitrary convention positive values were assigned for ventral and medial displacements.

Statistical Analysis

Mean values ($\pm$ standard error of the mean; SEM) are shown throughout. One-way ANOVA was used to make comparisons of the transplant areas and their relative x,y-displacements. Tukey's Honestly Significant Difference (HSD) Post Hoc test was used to determine where the significant differences occurred if the F-value exceeded F-critical ($p < 0.05$).

Immunostaining and Y probe in situ hybridization (FISH)

The sections were processed for Nissl staining or were immunostained for human nuclei (hNUC, mouse monoclonal, 1: 100, Millipore, Billerica, MA), human glial fibrillary acidic protein (GFAP, rabbit polyclonal, 1:500, DAKO, Carpinteria, CA), human nestin (rabbit polyclonal, 1:200, Millipore), choline acetyl transferase (ChAT, goat polyclonal, 1:200, Chemicon), or CD8b (mouse monoclonal, 1:100, BD Biosciences, San Jose, CA). Primary antibodies were followed by secondary antibodies conjugated to Cy3 or Alexa Fluor 488 (anti-IgG, 1:1000, Jackson ImmunoResearch Laboratories Inc., West Grove, PA) or biotinylated secondary antibody (1:200, Jackson ImmunoResearch Laboratories) followed by DAB with avidin-biotin method (Vector Laboratories, Burlingame, CA). Y Probe in situ hybridization was performed using a CEP XY DNA FISH probe (Vysis, Downers Grove, IL) as described previously(2).

RESULTS

Stabilized Cervical Spine Microinjection Platform

The stabilization platform is demonstrated in both *in situ* and *in vivo* environments in Figure 1. This platform has been specifically designed for attachment to the cervical spine in contrast to the platform we have previously described for attachment to the lumbar spine(10). As opposed to the lumbar platform which uses pressure clamps for both rostral and caudal spinous process fixation, the cervical microinjection platform achieves rostral fixation through use of a hinged plate with four holes designed to accommodate bone screws. As with the lumbar microinjection platform, the caudal end of the microinjection platform firmly attaches to spinous processes through use of a pressure clamp. The initial design not employed in the present study provides for additional "outrigger" laminar screws to prevent roll in the apparatus. In the present study, no significant roll was encountered. As such, outriggers were not employed.

Post-operative Behavioral Outcomes

Table 1 demonstrates both pre- and post-operative neurological outcomes for Groups 1-3, following intraspinal microinjection of hNPC cell grafts at different volumes. In seventeen of eighteen pigs assessed in Groups 1-3, baseline motor function was regained by sacrifice at POD7. One animal in Group 1 represented the only animal that failed to return to neurologic baseline; however, a trend towards improvement was noted between POD1 and sacrifice at POD7. Evaluation at necropsy revealed the presence of an epidural hematoma overlying the laminectomy defect. Despite varying microinjection volumes and rates, no difference was observed between groups regarding post-operative return to baseline neurological motor function at POD 1, POD3, or POD7. Figure 2 illustrates findings of similar behavioral outcomes and rates of post-operative recovery between Groups 1-3. This indicates that neither increases in microinjection volumes nor infusion rate were associated with development of post-operative neurologic sequelae.

hNPC Intraspinal Cell Graft Targeting

Targeting data from Groups 1-3 is presented in Figure 3. Figure 3A is a Nissl stained series of images that demonstrates the volumetric distribution of the implanted cells from the approximate epicenter with the cannula infusion track evident in the third image. This series of images was taken from Group 2, corresponding to a total injection volume of 25ul. In some specimens, there were intense regions of Nissl staining that could represent either reactive inflammation of the host spinal cord, or surviving transplants. However, following extensive attempts to locate human specific cells using an antibody against human nuclear protein no cells could be detected(1). Neither remote rostral, nor caudal control segments, nor the contralateral cord showed any regions of Nissl reactivity. Figure 3B provides an illustration of the approximate microinjection epicenter as calculated by analysis of the specimen section with the maximum area of Nissl stain. Deviation of the microinjection epicenter relative to the center of the ventral horn was compared between groups along both mediolateral and dorsoventral axes. No difference in mediolateral deviation was detected ($p=0.88$); however, dorsoventral deviation was observed ($p=0.05$). Subsequent analysis indicated a difference only between Group 2 & 3 at an alpha level of ($\alpha=0.05$). Figure 3C demonstrates an approximately equivalent percentage of each injection observed within the ventral horn ($p=0.96$). This result was determined by calculating the injection volume area present in the ventral horn expressed as a fraction of the overall cord injection volume. Figure 3D illustrates a trend towards an increase in the mean cord injection area with respect to increasing injection volume ($p=0.04$). Further testing indicated a significant difference between the means of Group 1 & 3 at an alpha level of ($\alpha=0.05$). These findings provide evidence that reflux was neither present to a significant degree nor associated with an increasing cell graft volume or infusion rate. Finally, an association of increased cross-sectional Nissl staining with increasing microinjection volume indicates that Nissl staining is not likely a result of cannulation-associated tissue trauma. Nissl staining secondary to trauma from cannulation, if observed, would be expected to be consistent between microinjection groups.

Attempts to Obtain Intraspinal Cellular Graft Survival

We next asked whether surgical manipulation, mechanical trauma, or an ongoing immune rejection response related to the cell microinjection process affected motor neuron viability. To address this concern, spinal cord sections adjacent to the injection core were immunostained to observe for the presence of choline acetyltransferase (ChAT) positive cells. Multiple ChAT-positive cells were present both ipsilateral and contralateral to the injection site in the cervical spinal cord (Figure 4A, B), indicating that neither the surgical procedure nor the injection process nor an ongoing rejection response, discussed below, induced gross changes in motor neuron viability.

As evidenced by the results in Figure 3, Nissl staining consistently demonstrated apparent inflammation at the graft site in the absence of hNUC staining. Together, these findings strongly suggest that an immune reaction resulted in rejection of the xenograft. To further explore this possibility, specimens that demonstrated a positive reaction to Nissl stain were assessed for the presence of other graft-specific markers including human nestin and human GFAP. Since the original fetal tissue from which the hNPC's were derived was male, we attempted to perform in situ analysis using a Y chromosome probe, a technique we have used previously in primate xenograft studies(2). In Groups 1-3, attempts to detect markers of cell viability using these methods were unsuccessful.

To address the inability to isolate a viable cell graft at the study endpoint, we attempted to increase the survival of the transplants through prolonged immunosuppressive pre-treatment and to maximize the quality of tissue specimens through transcardiac perfusion with PFA immediately prior to sacrifice. Despite these additional steps, Group 4 histology was similar

to that obtained for Groups 1-3 with observation of positive Nissl stain but negative results for graft-specific markers. The lack of evidence for viable cells prompted further evaluation for the cause of cell graft death. Tissue sections at and adjacent to the C3 injection site were immunostained for the presence of T-cell recruitment (CD8b). Although we used immunosuppressive cyclosporine pre-treatment, significant T-cell infiltration was detected in the injection core of the cervical spinal cord (Figure 4C-E) but not in contralateral or remote ipsilateral specimens. However, equivalent survival studies since undertaken in an alternate species, the mini-pig, have demonstrated cell graft survival at a (t=1wk) survival endpoint as shown in Figure 5. The ability to demonstrate graft survival on a species-dependent basis may have important implications for further studies devoted towards clinical translation of a molecular therapeutics-based delivery strategy.

DISCUSSION

Safety: Microinjection Across A Range of Infusion Parameters

These findings extend prior work from our laboratory that has focused on delivery of therapeutics to the ventral horn of the large animal spinal cord. Previously, we developed the technology and approach to support direct parenchymal microinjection of a cellular graft to the ventral horn of the lumbar spinal cord(10). In that acute survival series (t=3hrs), targeting of the ventral horn was undertaken with the aid of microelectrode recording and confirmed with stimulation. While that study was successful in demonstrating accurate microelectrode recording, an attendant peri-operative morbidity of approximately 40% (n=7 pigs) raised concern regarding the overall safety of the approach. In the present series, the targeting methodology was modified to a coordinate-based approach, ensuring that the same site would only undergo one cannulation, as opposed to the previous approach that required at minimum two but often three penetrations. Modification of the approach resulted in one episode of neurologic morbidity at endpoint (t=1wk) in the larger current series, with an epidural hematoma as the likely cause of a persistent but resolving motor deficit. No adverse behavioral effect was present between microinjection volume groups, demonstrating that the spinal cord is capable of tolerating variable graft volumes infused over a range of rates. The observation of increasing positive Nissl area with increasing injection volumes argues that appreciable cannula reflux did not occur at the combination of chosen injection volumes and rates, as shown in Figure 3D.

Accuracy & Platform Improvements: Coordinate-Based Ventral Horn Targeting

The data presented herein supports the safety of the proposed approach with an attendant sacrifice in accuracy when employing the coordinate-based targeting approach as opposed to the MER-based ventral horn targeting strategy. The image series in Figure 3A demonstrates the potential to achieve both safety and accuracy using a coordinate-based targeting approach, while Figure 3B illustrates the range of Nissl reactive epicenters obtained from microinjection site specimens. Opportunities exist for pursuit of improvements in the accuracy and precision of graft targeting as well as in platform design.

First, conceivable improvement in both targeting accuracy and precision may be achieved with attempts to more rigorously ensure a constant entry angle of the cannula with respect to the spinal cord. A modified adapter (Figure 6, **Left**) will allow articulation of the microdrive and injection cannula relative to the platform and, therefore, alteration of the spinal cord entry angle without requiring modification of either the occipital or C7 spinous process attachment points. Second, (Figure 6, **Right**) a modified occipital attachment will help to ensure firm skull fixation despite minor variations in cranial anatomy. Third, we have observed spinal cord pulsations that are comprised by both observable cardiac and respiratory components. Experimentation to compensate for respiratory-associated cord movement will explore expedited microinjection

protocols undertaken during periods of oxygenation without ventilation. This is achieved through a pause in the ventilator with high flow 100% O₂ delivered through the endotracheal tube. Fourth, we anticipate the use of intraoperative imaging to augment accuracy in placement and definition of grey/white interfaces in the spinal cord. Optical coherence tomography, intraoperative MRI, and ultrasound may serve this purpose(4,5,8,9,11). Both the utility of intra-operative imaging for improvement in accuracy and in long term follow-up for graft viability are enhanced through the recent development of imaging compatible tracers that will allow tagging of viable graft for both immediate and delayed follow-up(3,6). Fifth, observations from this study indicate that simple intra-operative manipulations can improve targeting accuracy. Ensuring that the cannula entry angle retains a trajectory medial to the DREZ helps to ensure accuracy along the mediolateral plane. Further, split infusions at different depths can help to ensure achievement of gray matter targeting along dorsoventral axis. This is demonstrated in Figure 5 (**Left, Top Middle, Bottom Middle**) where a 25µl hNPC payload delivered to female mini-pigs was split over a depth of 6mm and 4mm. Finally, because safety data from this series did not demonstrate evidence of infusion volume or rate dependent sequelae, a larger infusion volume may be more likely to achieve deposition of a cellular graft in the ventral horn even if the injection epicenter is not within the ventral horn.

Graft Viability: Cell Rejection & Transplant Survival

An inability to observe evidence of viable hNPC graft at sacrifice, despite rigorous and successful attempts to maintain therapeutic cyclosporine levels, led to further series, Groups 4 & 5, which received prolonged pre-operative immunosuppression. Histological findings of evidence for inflammation shown in Figure 4, including indication of CD8⁺ T cell infiltrate localized at the injection site, are likely related to characteristic features of xenograft rejection. Importantly, neither motor neuron viability nor behavioral outcomes appeared to be affected by this rejection response. Subsequent studies within our laboratory have used an equivalent microinjection protocol and immunosuppression regimen in the minipig as opposed to the farm pig. Figure 5 demonstrates initial data from these (t=1 wk) survival series in the mini-pig, which have consistently demonstrated hNPC graft survival.

Clinical Translation: Future Challenges Toward Human Application

To our knowledge, this study represents the first reported attempt to vertically target and cannulate a specific intraspinal target within the cervical cord of a large animal for cell-based therapeutic application. Demonstration of microinjection safety across a range of infusion parameters, an ability to target the ventral horn, and recommendations for further targeting improvements represent the first necessary steps towards validation of this novel therapeutics delivery approach. However, unique developmental challenges remain in this emerging field of study prior to validation of a hybrid therapeutic paradigm that consists of a biologic payload but requires neurosurgical intervention for delivery. Unlike traditional pharmacological therapies, assessment of treatment toxicity must simultaneously consider potential roles for the agent in question, the device used for delivery, and the employed surgical technique. Moreover, cross-species validation testing of human cells in animals complicates interpretation of survival data given the use of a xenograft rather than an allograft transplant. This immunologic confounding may require generation of parallel xenograft and allograft toxicity data in the preparation of investigational new drug applications to the Food and Drug Administration (FDA). Finally, while a variety of safe and effective immunosuppressive agents have been used for human solid organ transplants, limited information has been reported on neural transplantation approaches. Hence, although efficient in rodents, further characterization of available immunosuppressants for application to the CNS may also be required with the aim of developing an updated graft-specific immunosuppressive protocol.

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Figure 1. Stabilized Cervical Intraspinous Microinjection Platform, *in situ* and *in vivo*
A) *In situ* demonstration of the platform while attached to a model of the human occiput rostrally and to the spinous process of C7 caudally. The spinous processes and lamina of C3-C4 have been removed, as occurs intra-operatively. **B)** An *in vivo* demonstration of the platform with microdrive and microinjection cannula attached. This picture was taken during microinjection following opening of the dura, incision of the pial layer, and targeting of the ventral horn. Due to anatomic variation between the human and porcine occiput, the rostral fixation plate is angled downwards during porcine application of the platform.

Figure 2. Neurologic Outcome Behavioral Data

This is a graphical illustration of the Tarlov scores demonstrated in Table 1. **Top Panel)** Post-Operative Day 1 Tarlov Scores. **Middle Panel)** Post-Operative Day 3 Tarlov Scores. **Bottom Panel)** Post-Operative Day 7 Tarlov Scores.

Figure 3. Targeting the Ventral Horn, Accuracy Observed with Coordinate-Based Mapping

A) This Nissl-stained series approximates the volume of the cell graft at the time of sacrifice (t=1wk) in one animal in the 25µl microinjection group. Despite rigorous attempts to achieve and document therapeutic cyclosporine immunosuppression levels, immunostaining was unable to detect viable cells at a timepoint of t=1wk. **B)** This scaled schematic demonstrates the epicenter of the microinjection site as determined by histological assessments at the slide with maximal area. **C)** No significant change between groups was observed regarding the percentage of cell graft volume within the ventral horn ($p=0.96$). **D)** Between the three groups, a trend was present demonstrating increased injection area with increases in injection volume. This was significant at the 0.05 level ($p=0.04$).

Figure 4. hNPC Cellular Graft Rejection

(A, B) Photomicrographs of choline acetyltransferase (ChAT) immunostaining in the section in the group 1 pig. Healthy ChAT positive motor neurons were found in the adjacent section of the injection site, indicating that the surgery procedure did not induce overt motor neuron damage. (C-E) Immunostaining for T cell marker CD8b shows an active cellular immune response in the core of the transplanted site. Animals in Group 4 received immunosuppression beginning 10 days prior to surgery with subsequent serial documentation of therapeutic CysA levels. While Nissl staining (C) showed the volumetric distribution of the cannula infusion track, significant T cell infiltration (designated by arrows in E) was detected in the injection core of the cervical spinal cord. Scale bars: 500 μm in A, C; 100 μm in B, D, E.

Figure 5. hNPC Cellular Graft Survival

Demonstration of hNPC cell graft survival at postoperative Day 7 using cyclosporine and a minipig as opposed to farm pig. **(Left)** hNUC-positive cells present in the ventral horn and subjacent white matter. **(Top Middle)** hNUC-positive cells in the ventral horn. **(Bottom Middle)** hNUC-positive cells in white matter below ventral horn. **(Right)** BrdU positive staining in a separate animal is observed in the ventral horn, midline grey, and midline white matter. No appreciable reflux is observed. Scale bars: 500 µm in **Left, Right**; 100 µm in **Top Middle, Bottom Middle**.

Figure 6. Design Alteration for Improved Ventral Horn Targeting

(Left) This figure demonstrates an attachment for the microinjection platform rail system designed to serve as an alternative microdrive fixation mount. The attachment is unique in its capability of pivoting in the axial plane. This will allow more consistent angulation of the cannula and greater control of the intraspinal entry trajectory. **(Right)** Demonstrated is an alternative mount designed to accommodate variations in occipital anatomy.

Table 1

Neurologic Outcome Behavioral Data

Pre-Operative		POD1	POD3	POD7
Group 1	1	9	6	9
	2	9	6	9
	3	9	7	9
	4	9	8	9
	5	9	9	9
	6	9	2	5
Group 2	1	9	9	9
	2	9	6	7
	3	9	8	9
	4	9	7	9
	5	9	7	9
	6	9	8	9
Group 3	1	9	8	9
	2	9	9	9
	3	9	6	7
	4	9	8	9
	5	9	7	8
	6	9	8	9
ANOVA p value		p=0.33p=0.68p=0.39		