Experimental Neurology 287 (2017) 205–215

Contents lists available at ScienceDirect

Experimental Neurology

journal homepage: www.elsevier.com/locate/yexnr

Research Paper

# Intraspinal transplantation of subventricular zone-derived neural progenitor cells improves phrenic motor output after high cervical spinal cord injury

M.S. Sandhu [a], H.H. Ross [a], K.Z. Lee [a], B.K. Ormerod [c], P.J. Reier [b], D.D. Fuller [a,*]

[a] University of Florida, Department of Physical Therapy, P.O. Box 100154, Gainesville, FL 32610-0154, United States
[b] University of Florida, Department of Neuroscience, P.O. Box 100244, Gainesville, FL 32610-0244, United States
[c] University of Florida, Department of Biomedical Engineering, P.O. Box 116131, Gainesville, FL 32611-6131, United States

ARTICLE INFO

*Article history:*
Received 1 May 2016
Received in revised form 6 June 2016
Accepted 9 June 2016
Available online 11 June 2016

*Keywords:*
Phrenic
Neural precursor cells
Plasticity
Astrocyte

ABSTRACT

Following spinal cord injury (SCI), intraspinal transplantation of neural progenitor cells (NPCs) harvested from the forebrain sub-ventricular zone (SVZ) can improve locomotor outcomes. Cervical SCI often results in respiratory-related impairments, and here we used an established model cervical SCI (C2 hemisection, C2Hx) to confirm the feasibility of mid-cervical transplantation of SVZ-derived NPCs and the hypothesis that that this procedure would improve spontaneous respiratory motor recovery. NPCs were isolated from the SVZ of enhanced green fluorescent protein (GFP) expressing neonatal rats, and then intraspinally delivered immediately caudal to an acute C2Hx lesion in adult non-GFP rats. Whole body plethysmography conducted at 4 and 8 wks post-transplant demonstrated increased inspiratory tidal volume in SVZ *vs.* sham transplants during hypoxic ($P = 0.003$) or hypercapnic respiratory challenge ($P = 0.019$). Phrenic nerve output was assessed at 8 wks post-transplant; burst amplitude recorded ipsilateral to C2Hx was greater in SVZ *vs.* sham rats across a wide range of conditions (*e.g.*, quiet breathing through maximal chemoreceptor stimulation; $P < 0.001$). Stereological analyses at 8 wks post–injury indicated survival of ~50% of transplanted NPCs with ~90% of cells distributed in ipsilateral white matter at or near the injection site. Peak inspiratory phrenic bursting after NPC transplant was positively correlated with the total number of surviving cells ($P < 0.001$). Immunohistochemistry confirmed an astrocytic phenotype in a subset of the transplanted cells with no evidence for neuronal differentiation. We conclude that intraspinal transplantation of SVZ-derived NPCs can improve respiratory recovery following high cervical SCI.



## 1. Introduction

Multiple laboratories have confirmed that transplantation of neural precursor cells (NPCs) into the injured spinal cord can improve motor recovery (Bonner et al., 2011; Fischer, 2000; Mitsui et al., 2005; Reier, 2004; Snyder and Teng, 2012). While there are numerous potential NPC sources (reviewed in Reier, 2004), the current investigation focused on cells derived from the forebrain sub-ventricular zone (SVZ). The SVZ contains a robust NPC population that can be harvested and readily expanded *in vitro* (Alvarez-Buylla et al., 2002), and NPCs derived from the SVZ have been successfully transplanted into the injured spinal cord (Cao et al., 2001; Karimi-Abdolrezaee et al., 2006a; Mligiliche et al., 2005; Oka et al., 2004; Zhang et al., 2009). These spinal cord injury (SCI) studies have generally produced positive outcomes including enhanced myelination at or near lesions (Oka et al., 2004) and improved locomotor function (Karimi-Abdolrezaee et al., 2006b). The transplanted NPCs can develop into both astrocytes (Mligiliche et al., 2005) and oligodendrocytes (Karimi-Abdolrezaee et al., 2006b), but to our knowledge no prior studies show robust neuronal differentiation in the injured spinal cord. Thus, this NPC transplantation approach is not a "neuronal replacement" therapy, and mechanisms including neuroprotection (Llado et al., 2004), immunomodulation (Ziv et al., 2006) and remyelination (Xing et al., 2014) have been suggested to drive the observed motor recovery. Much of the aforementioned work was cited as the rationale for a recent study of NPC transplantation in humans with cervical SCI (Shin et al., 2015). In that trial, transplantation of human NPCs derived from the fetal telencephalon (a region containing the SVZ) was reported to be safe and well-tolerated by recipients.

Respiratory neuromotor output is always impacted by cervical SCI, and respiratory-related impairments are a primary cause of morbidity and mortality. Here, we used an established model of respiratory dysfunction after SCI (high cervical hemisection, C2Hx Goshgarian, 2003; Sandhu et al., 2009) to enable a quantitative evaluation of the impact of intraspinal transplantation of SVZ-derived NPCs on respiratory motor recovery. An important feature of the C2Hx model is that post-injury respiratory motor impairments can be readily quantified and interpreted both in anesthetized and unanesthetized animals (Sandhu

* Corresponding author.
*E-mail address:* ddf@phhp.ufl.edu (D.D. Fuller).

http://dx.doi.org/10.1016/j.expneurol.2016.06.007
0014-4886/

et al., 2009). In the current study, NPCs were isolated from the SVZ of embryonic green fluorescent protein (GFP) expressing neonatal rats, and then intraspinally delivered immediately caudal to an acute C2Hx lesion in adult non-GFP expressing rats that were otherwise syngeneic. This approach allowed us to test the hypothesis that high cervical transplantation of SVZ-derived NPCs would improve spontaneous respiratory motor recovery after cervical SCI. A secondary purpose was to histologically evaluate the local migration patterns of the transplanted NPCs, and to examine the differentiation patterns of the cells following transplantation.

## 2. Materials and methods

### 2.1. Animals

Experiments were conducted using adult female Sprague-Dawley rats (Harlan Inc., Indianapolis, IN, USA) and neonatal Sprague-Dawley rat pups carrying a germline enhanced green fluorescent protein (EGFP) transgene (bred under University of Florida in house breeding protocols). This colony was obtained *via* material transfer permission from the colony creators at the University of Missouri on behalf of the University of Missouri-Columbia. All procedures were approved by the University of Florida Institutional Animal Care and Use Committee.

### 2.2. NPC harvest and expansion

NPCs were derived from neonatal EGFP-expressing Sprague Dawley (SD-Tg(GFP)2BalRrrc) rats at postnatal day 4 as previously described (Ross et al., 2012). Brains were removed after rapid decapitation, and tissue blocks containing the SVZ were dissected, incubated for 5 mins in trypsin (0.05%), dissociated into single-cell slurry, and plated overnight in growth medium. Growth media consisted of Rat Neurocult base media (Stemcell Technologies Inc., Canada) containing a rat proliferation supplement (Stemcell Technologies Inc., Canada), basic fibroblast growth factor [10 ng/ml, bFGF], and epidermal growth factor [10 ng/ml, EGF]. To isolate neurosphere-forming cells, the non-adherent cell slurry was aspirated after 24 h, pelleted by centrifugation, and incubated in trypsin for 2 min. Cells were gently triturated, washed, resuspended and then plated in non-adherent flasks at clonal density (10,000 cells/cm$^2$) in growth medium. Cells were expanded in ultra-low attachment plates to promote floating sphere formation (Costar, #3471). The resultant neurospheres (NS) were passaged 2–3× in the same growth medium. To prepare a cell suspension for delivery into the injured adult spinal cord, NS were mechanically dissociated into single cells during passage, and cell number and viability was assessed using Trypan blue exclusion (Ross et al., 2012). An aliquot of the cells prepared for transplant were plated on poly-L-ornithine-coated coverslips and then fixed in 4% paraformaldehyde for 1 h at room temperature (RT) and blocked for 1 h at RT in PBS containing 0.01% Triton X-100 and 10% FBS. Primary antibody (rabbit anti-β-III tubulin 1:200; rabbit anti-glial fibrillary acidic protein (GFAP) 1:2000) was applied overnight at 4 °C. Coverslips were washed 2 × 10 min in wash buffer (PBS, 0.01% Triton X-100) and incubated with fluorescence-conjugated secondary antibody (goat anti-mouse, goat anti-rabbit) for 3 h at RT. Slips were washed 2 × 10 min in wash buffer, mounted on positively charged glass slides (Fisherbrand Superfrost/Plus, Fisher Scientific, Pittsburg, PA) and cover-slipped in Vectashield containing DAPI counterstain (Vector Laboratories, Burlingame, CA). Fluorescence micrographs were obtained with a Leica DMLB epifluorescence microscope equipped with a color Spot cooled CCD digital camera.

### 2.3. SCI and transplantation

Experiments were performed in separate cohorts of experimental animals, separated by approximately 6 months. All rats underwent C2Hx surgery as described below. In the first cohort, $N = 8$ rats had NPC transplant (body weight = 269 ± 3 g), $N = 4$ rats had intraspinal delivery of the growth medium (*i.e.*, sham transplant; body weight = 260 ± 3 g), and $N = 4$ rats received C2Hx only (body weight = 260 ± 7 g). In the second cohort, there were $N = 7$ NPC transplants (body weight = 262 ± 4 g) and $N = 3$ sham transplants (body weight = 261 ± 4 g). Qualitatively similar results were obtained in both cohorts of NPC transplants for all outcome variables, and the data were combined in the final presentation of the results. Similarly, no differences were detected in any of the neurophysiological or plethysmography measures (all $P$ values > 0.15) between the groups receiving sham transplant ($N = 7$) or C2Hx only ($N = 4$). Therefore, these animals were combined into a single control group for statistical comparison of functional outcomes with rats receiving NPC transplants. The final cohort of rats ($N = 6$, body weight = 284 ± 5 g) received NPC transplantation and were used only for spinal immunohistochemistry experiments.

Prior to SCI, rats were anesthetized with xylazine (10 mg/kg s.q.; Phoenix Pharmaceutical, Inc., St. Joseph, MO) and ketamine (120 mg/kg i.p.; Fort Dodge Animal Health, Fort Dodge, IA). The C2Hx lesion was induced as previously described (Doperalski and Fuller, 2006; Fuller et al., 2008; Lane et al., 2008). Following surgery, an analgesic (buprenorphine, 0.03 mg/kg, s.q.) was given every 12 h for 2 days, and lactated ringers solution (12 ml/day, s.q.) was provided as needed for 2–4 days. Seven days after C2Hx surgery, rats were anesthetized again, and the dissociated NPC suspension was delivered intraspinally after surgically exposing the C2–3 spinal cord. A total volume of 5 μl, containing approximately 500,000 live cells diluted in growth medium (described above), was injected using a 10 μl Hamilton glass syringe and a 31 gauge needle. The tip of the needle was positioned in the ventromedial white matter, approximately 1 mm caudal to the lesion site and 1.5 mm from the dorsal surface of the spinal cord. Sham injections consisted of an equal volume of growth medium. All animals received daily injections of cyclosporine A immunosuppressant (10 mg/kg, s.q., Sandimmune; Novartis, East Hanover, NJ) starting two days before transplantation and continuing until the end of the experiment.

### 2.4. Respiratory outcome measures

Ventilation was measured in unanesthetized, unrestrained rats using a whole-body plethysmography system (Buxco Inc., Wilmington, NC, USA) as previously described (Fuller et al., 2008). Baseline recordings lasted 1–1.5 h, and were made while the chamber was flushed with 21% $O_2$ (balance $N_2$). Rats were then exposed to two successive respiratory challenges separated by a 5-min normoxic recovery period. First, the chamber was flushed with 10% $O_2$ (balance $N_2$) for 5 min to provide an hypoxic respiratory stimulus. Second, the chamber was flushed with 7% $CO_2$, 21% $O_2$ mixture (balance $N_2$) for a hypercapnic stimulus. Data which were derived from the plethysmograph airflow recordings included inspiratory frequency (breaths per minute), inspiratory tidal volume (mls per breath), inspiratory minute ventilation (mls per minute), peak inspiratory airflow rate (mls/s), peak expiratory airflow rate (mls/s), inspiratory duration (sec), and expiratory duration (sec). These data were averaged over a 10-min period of stable breathing during the final 30 min of the baseline recording, and over the final minute of the hypoxic and hypercapnic challenges. Respiratory volume data were expressed in absolute units (*i.e.*, mls), per 100 g body mass, and also relative to the baseline value.

Phrenic nerve recordings were conducted as previously described (Sandhu et al., 2010). Rats were initially anesthetized with isoflurane (5% in 100% $O_2$) and then continued to breath an isoflurane mixture *via* a nose cone (2–3% isoflurane in 50% $O_2$, balance $N_2$) while the trachea was cannulated (PE-240 Tubing). After mechanical ventilation was initiated, a catheter (PE-50) was placed in the femoral vein, and isoflurane was withdrawn gradually in parallel with i.v. urethane delivery (1.6 g/kg; 0.12 g/ml distilled water). A femoral arterial catheter (PE-50) was inserted to measure blood pressure (Statham P-10EZ pressure transducer, CP122 AC/DC strain gauge amplifier, Grass Instruments,