Survival, differentiation, and migration of bioreactor-expanded human neural precursor cells in a model of Parkinson disease in rats

KARIM MUKHIDA, M.D.,1 BEHNAZ A. BAGHBADERANI, M.Sc.,1,2 MURRAY HONG, PH.D.,1 MATTHEW LEWINGTON, B.Sc.,1 TIMOTHY PHILLIPS, B.Sc.,1 MARCUS MCLEOD, PH.D.,1 ARINDOM SEN, PH.D.,2 LEO A. BEHIE, PH.D.,2 AND IVAR MENDEZ, M.D., PH.D.1

1Division of Neurosurgery, Department of Surgery, Cell Restoration Laboratory, Dalhousie Medical School; Departments of Anatomy and Neurobiology, Dalhousie University, Halifax, Nova Scotia; and 2Pharmaceutical Production Research Facility, Schulich School of Engineering, University of Calgary, Alberta, Canada

Object. Fetal tissue transplantation for Parkinson disease (PD) has demonstrated promising results in experimental and clinical studies. However, the widespread clinical application of this therapeutic approach is limited by a lack of fetal tissue. Human neural precursor cells (HNPCs) are attractive candidates for transplantation because of their long-term proliferation activity. Furthermore, these cells can be reproducibly expanded in a standardized fashion in suspension bioreactors. In this study the authors sought to determine whether the survival, differentiation, and migration of HNPCs after transplantation depended on the region of precursor cell origin, intracerebral site of transplantation, and duration of their expansion.

Methods. Human neural precursor cells were isolated from the telencephalon, brainstem, ventral mesencephalon, and spinal cord of human fetuses 8–10 weeks of gestational age, and their differentiation potential characterized in vitro. After expansion in suspension bioreactors, the HNPCs were transplanted into the striatum and substantia nigra of parkinsonian rats. Histological analyses were performed 7 weeks posttransplantation.

Results. The HNPCs isolated from various regions of the neuraxis demonstrated diverse propensities to differentiate into astrocytes and neurons and could all successfully expand under standardized conditions in suspension bioreactors. At 7 weeks posttransplantation, survival and migration were significantly greater for HNPCs obtained from the more rostral brain regions. The HNPCs differentiated predominantly into astrocytes after transplantation into the striatum or substantia nigra regions, and thus no behavioral improvement was observed.

Conclusions. Understanding the regional differences in HNPC properties is prerequisite to their application for PD cell restoration strategies. (DOI: 10.3171/FOC/2008/24/3-4/E7)

Key words • bioreactor expansion • differentiation • migration • neural precursor cell • Parkinson disease • transplantation
Human neural precursor cells perhaps could be used as an alternative to fetal tissue to improve the clinical efficacy of cell restoration strategies for PD. Precursor cells have been isolated from a variety of human CNS regions and propagated in culture in the presence of mitogens. Although the scaled-up production of HNPCs could be efficiently achieved in standard stationary culture systems, suspension bioreactors can be used to rapidly and reproducibly expand mammalian neural stem cells for extended periods of time and without adversely affecting characteristics such as cell growth rate, maximum cell density, viability, karyotype, and cell cycle dynamics to generate the large cell numbers required for clinical transplantation purposes. Expansion of HNPCs can therefore take place in a standardized manner in which optimal oxygen, osmolarity, and pH conditions are maintained.

The object in the present study was to characterize bioreactor-expanded HNPCs for potential use in a cell restoration strategy aimed at treating PD. First, HNPCs were isolated from different levels of the neuraxis, expanded in stationary cultures, and regional differences in their default differentiation characteristics studied. The ability of HNPCs to differentiate into a dopaminergic phenotype was investigated by supplementing cultures with exogenous factors known to enhance dopaminergic differentiation. Second, to generate quantities relevant for clinical application, HNPCs were expanded in suspension bioreactors and regional differences in their expansion properties were studied. Third, HNPCs were transplanted intracerebrally into hemiparkinsonian rodents to determine whether precursor cell origin influenced cell survival, differentiation, and migration in vivo. To determine whether these characteristics depended on the site of transplantation as well, HNPCs were transplanted into either the striatum or SN, 2 structures that have been targeted in clinical neural transplantation studies. To determine whether the amount of time in culture under bioreactor conditions could affect HNPC survival and differentiation posttransplantation, telencephalon-derived HNPCs were passaged for an extended period before transplantation. The characterization of bioreactor-expanded HNPC properties is prerequisite to HNPC application in clinical cell restoration strategies for PD.

**Materials and Methods**

**Stationary Cell Culture**

Human fetal tissues were harvested using protocols developed at our center. Fetal telencephalon (from 1 fetus of 10 weeks’ gestation), brainstem (pooled from 3 fetuses of 7.5, 7.5–8, and 10 weeks gestation), ventral mesencephalon (from 1 fetus of 9 weeks gestation), and spinal cord (from 1 fetus of 9 weeks gestation) were dissected in sterile conditions. The brainstem included tissue underneath the rhombencephalic flexure and inferior to the ventral mesencephalon. Single cell suspensions of each of these tissue types were prepared first by rinsing the tissues in 0.05% DNAse (Sigma-Aldrich)/PPRF-h2 medium, incubating them in 0.25% trypsin (Sigma-Aldrich)–EDTA at 37°C for 20 minutes, rinsing them again in 0.05% DNAse/PPRF-h2 medium, and mechanically dissociating them using 1-ml and 200-μl Eppendorf pipettes until uniform cell suspensions were achieved. Culture plates (25 cm²) containing PPRF-h2 medium were used to expand the primary HNPCs (passage level 0, or P0), with a 50% medium exchange performed every 4 days. After 2 weeks, the neurospheres were harvested and centrifuged at 1500 rpm for 5 minutes. The resulting pellets were incubated in 0.25% trypsin (HyClone) at 37°C for 20 minutes. After being rinsed with 0.05% DNAse/PPRF-h2 medium, the neurospheres were mechanically dissociated into single cell suspensions and placed in expanded medium (P1). The cells were passaged an additional 3 times in stationary culture.

At the end of P4, brainstem-, ventral mesencephalon-, and spinal cord–derived HNPCs were enzymatically dissociated into single cell suspensions. The differentiation patterns in vitro were determined by plating the cells on poly-L-lysine–coated 16-well glass chamber slides (Nunc, Fisher Scientific) at a density of 50,000 cells/ml in a medium containing 2% B27 (Stem Cell Technologies), 1% N2 (Gibco), and 1% penicillin-streptomycin in Dulbecco modified Eagle medium (HyClone) and then fixing the cells with 4% paraformaldehyde in 0.1M PB after 6 hours or 7 days, with the cultures fed every 3 days by replacing 50% of the spent medium with fresh medium. Additionally, the ability of brainstem-, ventral mesencephalon-, and spinal cord–derived HNPCs to be induced in vitro into a dopaminergic phenotype by using a variety of culture conditions was assessed immunocytochemically. Single cell suspensions of HNPCs were cultured for 7 days in medium that also contained BDNF (10 ng/ml, PeproTech), forskolin (10 μM, Sigma), and dopamine (10 μM, Sigma); BDNF, forskolin, dopamine, and GDNF (10 ng/ml, PeproTech); or BDNF, forskolin, dopamine, GDNF, and FGF8 (50 ng/ml, PeproTech). A schematic of the various culture conditions is presented in Fig. 1A.

**Suspension Bioreactor Expansion**

Aliquots of telencephalon, brainstem, ventral mesencephalon, and spinal cord cells initially expanded in stationary cultures were cultured separately in suspension bioreactors, 125-ml spinner flasks with a paddle impeller. Prior to use, the inner surface of these glass flasks and the outer surface of the impellers were siliconized using a 1:9 ratio of Sigmacote (Sigma) in hexane (Omnisolv). The bioreactors were then sterilized and filled with 100 ml of PPRF-h2 medium. The Thermolyne Cellgro system was used to provide suspension conditions at an agitation rate of 100 rpm in each bioreactor. Each bioreactor was inoculated at 100,000 cells/ml (single cell suspension) and then housed in a humidified incubator maintained at 37°C, 95% air saturation (20% O₂, and 5% CO₂). Every 5 days, 40% of the spent medium was replaced with fresh medium. All neurospheres formed during expansion were enzymatically dissociated into single cell suspensions by using 0.25% trypsin–EDTA. Cell density and viability following enzymatic dissociation were determined with trypan blue dye exclusion.

**Telencephalon-Derived HNPCs.** At the end of P1, telencephalon-derived neurospheres (~ 10⁶ cells) were transferred to a T-75 flask and cultured in PPRF-h2 medium.

Neurosurg. Focus / Volume 24 / March/April 2008
After 5 days, the neurospheres, which had increased in diameter to an average of ~500 μm, were harvested, enzymatically dissociated, and inoculated into T-25 flasks (P3). After another 14 days, the neurospheres were harvested, dissociated, and inoculated as single cells into duplicate 125-ml suspension bioreactors (P4). After 14 additional days, more than 2 × 10^8 cells were harvested and cryopreserved in PPRF-h2 medium with 10% DMSO.

For transplantation, cryopreserved bioreactor-expanded HNPCs were thawed and then passaged 2 additional times in stationary tissue culture flasks (P6). The neurospheres were rinsed in 0.05% DNAse/Dulbecco modified Eagle medium, incubated in 0.25% trypsin for 20 minutes at 37°C, and dissociated into a single cell suspension. The suspension was concentrated to 200,000 cells/μl (viability exceeded 95%) in preparation for transplantation.
Serial subculturing of telencephalon-derived HNPCs was continued in suspension bioreactors for 9 additional passages. Every 14 days, aggregates were harvested, enzymatically dissociated into single cells, and inoculated into duplicate 125-ml bioreactors. A cell bank was created by cryopreserving HNPC aggregates (10^6 cells) in PPRF-h2 medium with 10% DMSO at the end of each passage. In preparation for transplantation, these later-passage telencephalon-derived HNPCs were thawed and passaged an additional 5 times in stationary culture (P18). The neurospheres were prepared into a single cell suspension for transplantation at a concentration of 200,000 cells/μl (viability > 95%).

Ventral Mesencephalon–Derived HNPCs. At the end of P2, ~500,000 cells were transferred to a T-25 flask. After 24 hours, the cells were transferred into a T-25 flask containing 5 ml PPRF-h2 medium (P3) and left in culture for 7 days, after which the neurospheres were harvested and enzymatically dissociated into a single cell suspension. Cell counts revealed a cellfold expansion of 2.5 and viability of 91% (average viability of 87 ± 1%). The resulting single cell suspension was inoculated into T-25 flasks in which the cells adhered to the flask surface and did not form floating aggregates. To serially subculture the cells, 3 ml of 0.25% trypsin–EDTA was added to each T-25 flask, and the cultures were incubated at 37°C for 10 minutes. An equal volume of soybean trypsin inhibitor (0.25 g/L, Gibco) was then added to each culture to neutralize trypsin activity. The detached cells were transferred to a 15-ml centrifuge tube and spun at 1000 rpm for 10 minutes. After discarding the supernatant, 1.0 ml fresh PPRF-h2 medium was added to the pellet. A single cell suspension was generated, and cell counts revealed a cellfold expansion of 6.3 and viability of 91 ± 1%. The cells were serially subcultured under static conditions in the same manner for 2 additional passages. Counts revealed a cellfold expansion of 2.5 and viability of 93 ± 2% at the end of P5, and a cellfold expansion of 4.6 and viability of 92 ± 1% at the end of P6. The P6 HNPCs were inoculated into a 125-ml bioreactor (P7). After 14 days in suspension culture, the majority of the produced neurospheres were harvested and cryopreserved in PPRF-h2 medium with 10% DMSO. When required for transplantation, the cryopreserved vials were thawed, passaged twice in stationary culture, and then prepared into a single cell suspension at a concentration of 200,000 cells/μl (viability > 95%).

Brainstem-Derived HNPCs. At the end of P1, ~500,000 cells were transferred to a T-75 flask. After 3 days, the cells were harvested, enzymatically dissociated into a single cell suspension, and inoculated at 90,000 cells/ml into 2 T-25 flasks, each containing 5 ml of PPRF-h2 medium (P3). Although the cells initially adhered to the flask surface after inoculation, floating aggregates of cells formed over time in culture. After 14 days, cell counts revealed a cellfold expansion of 2.9 and viability of 88 ± 1.9%. After 2 additional serial subculture phases in static conditions (end of P4: cellfold expansion 12.2 and viability 92 ± 2%; end of P5: cellfold expansion 12.1 and viability of 96 ± 2%), the cells were expanded in 2 125-ml bioreactors (P6). After 14 days, most of the remaining neurospheres in culture were harvested and cryopreserved in PPRF-h2 medium with 10% DMSO. When required for transplantation, the cryopreserved vials were thawed, passaged twice in stationary culture, and then prepared into a single cell suspension at a concentration of 200,000 cells/μl (viability > 95%).

Spinal Cord–Derived HNPCs. At the end of the primary culture (P0), ~500,000 cells were transferred to a T-75 flask (P1). After 14 days, the neurospheres were harvested and enzymatically dissociated into a single cell suspension. Cell counts revealed a cellfold expansion of 5.8 and viability of 91 ± 1% at the end of P1. After being passaged once more in tissue culture flasks (end of P2: cellfold expansion 10.7 and viability 94 ± 1.7%), the cells were expanded in 2 125-ml bioreactors (P3). After 14 days in suspension culture, most of the neurospheres were harvested and cryopreserved in PPRF-h2 medium with 10% DMSO.

Surgical Procedures

Thirty-six female Wistar rats (Charles River) weighing 200-225 g were used for transplantation studies. The rats were housed in pairs in a room maintained at a constant temperature and humidity on a 12-hour light/dark cycle and allowed 7 days to acclimatize prior to surgery. Animals were allowed free access to food and water. The experiments were conducted in accordance with the standards and procedures of the Canadian Council on Animal Care and the University Committee on Laboratory Animals.

Rats received 2 stereotactic injections of 6-OHDA (Sigma) into the right nigrostriatal dopaminergic pathway via a metal cannula attached to a 10-μl Hamilton microsyringe while in a state of anesthesia induced by a 0.2 ml/100 g–body-weight dose of a ketamine-xylazine-acepromazine mixture (60 mg/kg ketamine hydrochloride [MTC Pharmaceuticals], 1.2 mg/kg xylazine [Bayer], and 1.6 mg/kg acepromazine maleate [Wyeth] in 0.9% saline). We used the following coordinates for the injections (in mm and with reference to the bregma and dura mater): 1) 2.5 μl of 6-OHDA (3.6 μg 6-OHDA hydrogen bromide [Sigma]/μl in 2.0 mg/ml L-ascorbate [Sigma] in 0.9% saline) injected at AP –4.4, ML –1.2, and DV –7.8, with the incisor bar set 2.0 mm above the IA. The injection rate was 1 μl/min, and the cannula was left in place for 5 minutes before its retraction. After a 3-week recovery period, the rats were given an amphetamine challenge (5 mg/kg, intraperitoneal), and their rotation scores were collected over a 70-minute period by using a computerized video activity monitoring system (Videomex, Columbus Instruments). Only animals that exhibited a mean ipsilateral rotation score of ≥ 8 complete body turns per minute were included in the study.

The hemiparkinsonian rats were randomly assigned to 1 of 7 treatment groups. Animals received injections of 400,000 bioreactor-expanded P6 HNPCs derived from the telencephalon into the striatum (Group 1, 6 rats) or SN (Group 2, 6 rats), from the brainstem into the striatum (Group 3, 6 rats) or SN (Group 4, 6 rats), or from the ventral mesencephalon into the striatum (Group 5, 4 rats) or SN (Group 6, 4 rats). An additional group (Group 7) of 4 rats received intranstriatal transplants of P18 telencephalon-derived HNPCs to determine whether the amount of time in culture in the suspension bioreactors affected the properties of the cells posttransplantation. A schematic of the various transplantation groups is presented in Fig. 1B and C.
Human neural precursor cell transplantation for Parkinson disease

The cells were stereotactically transplanted at a rate of 1 μl of cell suspension/minute in the striatum or SN ipsilateral to the 6-OHDA lesion by using a glass micropipette with an outer opening diameter of between 50 and 70 μm attached to a 2-μl Hamilton microsyringe. The capillary was left in place for an additional 5 minutes after each deposit before being retracted. The stereotactic coordinates for transplantation were as follows (in mm and with reference to the bregma and dura): 1) AP +1.3, ML −2.1, and DV −5.5 and −4.3; 2) AP +0.6, ML −2.9, and DV −5.5 and −4.3; and 3) AP +0.3, ML −3.7, and DV −5.5 and −4.3, with the incisor bar set 3.3 mm below the IA for all 3 sets of coordinates.

All animals received cyclosporine (10 mg/kg, intraperitoneal, Novartis) daily beginning 2 days before transplantation and until they were killed.

Histological Analysis

Seven weeks posttransplantation, rats were administered an overdose of an anesthetic agent and transected with a microsyringe and a 25-gauge needle. The total volume of the cell suspension was estimated using a microsyringe. The cell suspension was injected at a rate of 1 μl of cell suspension/minute in the striatum or SN ipsilateral to the 6-OHDA lesion by using a glass micropipette with an outer opening diameter of between 50 and 70 μm attached to a 2-μl Hamilton microsyringe. After each deposit, the capillary was left in place for an additional 5 minutes before being retracted. The stereotactic coordinates for transplantation were as follows (in mm and with reference to the bregma and dura): 1) AP +1.3, ML −2.1, and DV −5.5 and −4.3; 2) AP +0.6, ML −2.9, and DV −5.5 and −4.3; and 3) AP +0.3, ML −3.7, and DV −5.5 and −4.3, with the incisor bar set 3.3 mm below the IA for all 3 sets of coordinates.

All animals received cyclosporine (10 mg/kg, intraperitoneal, Novartis) daily beginning 2 days before transplantation and until they were killed.

Immunocytochemistry or immunohistochemistry was performed on cultured cells and brain sections, respectively. For fluorescence microscopy, cultured cells and selected brain sections were rinsed 3 times with 0.1M PB for 5 minutes each time, treated for 1 hour in 5% normal goat serum and 0.3% Triton X-100 in 0.1M PB, and incubated for 16 hours at room temperature in a solution of 5% normal goat serum/0.3% Triton X-100 in 0.1M PB that contained mouse anti–HuN (1:1000, Chemicon) and rabbit anti-GFAP (1:2000, Sigma), rabbit anti-nestin (1:1000, Chemicon), rabbit anti–TH (1:2500, Pel-Freeze), rabbit anti-GABA (1:1000, Sigma), rabbit anti–O4 (1:1000, Chemicon), rabbit anti–myelin basic protein (1:1000, Chemicon), rabbit anti–TUJ1 (1:1000, Chemicon), rabbit anti–microtubule-associated protein–2 (2:1000, Chemicon), or rabbit anti–glutamic acid decarboxylase 65/67 (1:1000, Chemicon). The cells and sections were rinsed with 0.1M PB and incubated for 1 hour in diluent containing secondary antibody (1:500, Vector Laboratories) or 0.3% Triton X-100 in 0.1M PB. After the sections were washed with 0.1M PB, they were incubated for 1 hour in avidin (1:200) and biotin (1:200, ABC kit, Vector Laboratories) in 0.1M PB. Peroxidase activity was developed using 3,3′-diaminobenzidine dissolved in 0.1M PB and 1% H2O2. Sections were rinsed with 0.1M PB before being mounted on gelatin-coated slides and covered with Permount.

Stereological Analysis of Stationary Cultured Cells

Each well of cultured cells was visualized at a magnification of 20 and then analyzed using a fractionator stereological probe (Stereo Investigator 6.01, Microbrightfield). Cells were counted at 86 randomly selected sites within each culture well. For each type of HNPC (brainstem, ventral mesencephalon, and spinal cord), the number of cells labeled for Hoechst, GFAP, or TUJ1 was determined for each of the 5 different culture treatments (6 wells were analyzed for each marker for each tissue type).

Stereological Analysis of Transplanted HNPCs

In an unbiased manner an investigator blinded to the animals’ treatments estimated the numbers of surviving transplanted telencephalon, brainstem, and ventral mesencephalon HNPCs in the striatum and SN by using stereological methods involving the optical fractionator formulae102 and stereological software (Stereo Investigator 6.01). The optical fractionator probe was used on every 4th HuN-immunostained section throughout the grafted areas and consisted of a 25 × 25-μm counting frame with a height of 15 μm. The sampling grid measured 125 × 125 μm. The counting grids were randomly placed in the grafted area. The section thickness was estimated for every 5th dissector measurement and then averaged for each section.

Migration Analysis of Transplanted HNPCs

Every 4th coronal section through the grafted areas was selected for cell plotting and counting in each animal. Plots were prepared using Neurolucida software (version 4.36, MicroBrightfield). The outlines of major landmarks (outer margin of the tissue section, corpus callosum, ventricles, and anterior commissures) were traced, and HuN cell profiles were plotted and counted. Nuclei that remained with the graft deposit were differentiated from those that migrated away from the deposit. Neurolucida files were exported to NeuroExplorer (MicroBrightfield). Stereological methods were not applied in this part of our analysis because the obtained cell counts were used not to estimate the total number of cells but instead to determine the proportion of transplanted cells that had migrated away from the graft deposit in relation to the total number of cells presents as well as to describe the migration pattern of the transplanted HNPCs.

Statistical Analyses

The number of surviving cultured brainstem, ventral mesencephalon, and spinal cord HNPCs and the proportion of surviving cells that differentiated into astrocytes and neurons in the various culture medium paradigms were determined.
assessed for within- and between-group differences at a probability level $< 0.05$ by using a one-way ANOVA followed by the Tukey post hoc test. Rotational behavior post–6-OHDA lesioning and 2, 4, and 6 weeks posttransplantation was assessed for within- and between-group differences at a probability level $< 0.05$ by using a two-way ANOVA followed by the Bonferroni post hoc test. The migration of HNPCs, expressed as the number of cells that migrated away from the graft core as a proportion of the number of surviving cells, and the survival of HNPCs were assessed for between-group differences at a probability value $< 0.05$ by using a one-way ANOVA followed by the Tukey post hoc test.

**Results**

*Expansion of HNPCs*

Regardless of the region of precursor cell origin, HNPCs derived from the fetal telencephalon, brainstem, ventral mesencephalon, and spinal cord formed neurospheres in both the stationary (Fig. 2) and suspension bioreactor cultures (Fig. 3). These spherical aggregates of HNPCs appeared similar when viewed with bright field microscopy or stained with Hoechst or antibodies to nestin. Telencephalon-derived neurospheres larger than 350 (Fig. 2C) and 600 $\mu$m (Fig. 3C) were observed after 14 days in stationary (P3) and suspension bioreactor cultures (P4), respectively. In static conditions in PPRF-h2 medium, telencephalon-derived HNPCs exhibited a cellfold expansion of $\sim 14$ per passage and a viability of 91 $\pm$ 1%. In the initial suspension culture (P4), the cellfold expansion increased to 32 and the viability was 94 $\pm$ 2%. At the end of P13 (after 9 consecutive serial subcultures in the bioreactors), the cells showed an average cellfold expansion of 17 and viability of 83 $\pm$ 1%.

On inoculation of single ventral mesencephalon–derived HNPCs into stationary cultures containing PPRF-h2 medium, the cells adhered to the surface of the T-flasks (Fig. 2D and E). Cells then formed a network of aggregates attached to the surface of the tissue culture flasks after Day 14 (Fig. 2F). Ventral mesencephalon–derived cells exhibited an average cellfold expansion of 4 per passage and a viability of 91 $\pm$ 1% in stationary culture. In suspension bioreactors, the HNPCs formed aggregates of cells that increased in size over time (Fig. 3D–F). After 7 days, some of the neurospheres agglomerated to each other in suspension bioreactors (data not shown), and they were removed from culture and excluded from the evaluation of HNPC expansion. After 14 days, neurospheres larger than 350 $\mu$m were observed in suspension bioreactors. The cells exhibited an average cellfold expansion of 2.3 and a viability of 87 $\pm$ 3% at the end of P7.

**Fig. 2.** Photomicrographs of HNPCs from different regions of the CNS and grown in stationary culture showing telencephalon-derived HNPCs immediately after inoculation (A) and 8 (B) and 14 days postinoculation (C); ventral mesencephalon–derived HNPCs immediately (D) and 8 (E) and 14 days postinoculation (F); brainstem-derived HNPCs immediately (G) and 8 (H) and 14 days postinoculation (I); and spinal cord–derived HNPCs immediately (J) and 8 (K) and 14 days postinoculation (L). Bars = 250 $\mu$m.
On inoculation of single brainstem-derived HNPCs into stationary cultures containing PPRF-h2 medium, the cells adhered to the surface of the T-flasks. After 8 days, the cells had formed aggregates, the majority of which remained adhered to the surface, although floating aggregates also could be observed in culture (Fig. 2H). After 14 days, the majority of the neurospheres were detached from the surface (Fig. 2I). Brainstem–derived HNPCs exhibited an average cellfold expansion of 9.1 per passage and a viability of 92 ± 1% in stationary culture. In the suspension bioreactors, brainstem–derived HNPCs grew in the form of neurospheres that increased in size over time in culture (Fig. 3G–I). After 14 days, aggregates with diameters of 500 μm were observed. The cells exhibited an average cellfold expansion of 4 and a viability of 86 ± 7% at the end of P6.

Single spinal cord–derived HNPCs adhered to the surface of the T-flasks following inoculation. After 8 days, some neurospheres adhered to the surface and some floated in culture (Fig. 2K). After 14 days, the majority of aggregates were detached from the surface (Fig. 2L). The cells exhibited an average cellfold expansion of 8.2 per passage and a viability of 92 ± 2% in stationary culture. In the suspension bioreactors, spinal cord–derived HNPCs grew in the form of neurospheres that increased in size over time (Fig. 3J–L). After 14 days, neurospheres with diameters > 500 μm were observed in suspension bioreactors. The cells exhibited an average cellfold expansion of 9.4 and a viability of 90 ± 2% at the end of P3 in the suspension bioreactors.

**Cultured HNPC Survival and Differentiation**

Immunocytochemistry demonstrated that HNPCs derived from the brainstem, ventral mesencephalon, and spinal cord differentiated into both astrocytic and neuronal phenotypes (Fig. 4A).

After only 6 hours of differentiation, HNPCs from all 3 levels of the neuraxis had few neuritic processes (Fig. 4A). After 7 days in culture, all HNPCs demonstrated extensive outgrowth of processes. Ventral mesencephalon–derived HNPCs demonstrated greater survival in culture compared with cells derived from the other tissues (p < 0.0001), and exposure to BDNF, dopamine, forskolin, GDNF, and FGF8 significantly enhanced their survival compared with ventral mesencephalon cells in the other treatment groups (p < 0.0001; Fig. 4B). Ventral mesencephalon HNPCs that were cultured in unsupplemented medium or medium supplemented with BDNF, dopamine, and forskolin demonstrated a significantly greater proportion of nestin-immunoreactive cells compared with other culture conditions (p < 0.0001) and other tissue types (Fig. 4C). The proportions of brainstem and spinal cord HNPCs that were immunopositive for...
nestin were not significantly affected by culture conditions. Culturing ventral mesencephalon HNPCs in the presence of dopamine and forskolin significantly increased the proportion of cells expressing the immature neuronal immunophenotype TUJ1 (p < 0.0001; Fig. 4D). Culturing brainstem HNPCs in supplemented medium decreased the...
proportion of cells that expressed TUJ1. The greatest proportion of TUJ1-immunoreactive spinal cord HNPCs was observed when the culture medium was supplemented with only BDNF, dopamine, and forskolin.

Typically, <$20\%$ of all cultures, regardless of tissue type and medium used, consisted of astrocytes (Fig. 4E). Whereas the proportion of astrocytes in the ventral mesencephalon and spinal cord HNPC cultures was similar regardless of the culture medium used, brainstem HNPCs cultured in unsupplemented medium or medium supplemented with BDNF, dopamine, forskolin, and GDNF demonstrated a significantly greater degree of astrocytic differentiation ($p < 0.0001$).

**Rotational Behavior**

Rats with 6-OHDA lesions of the right nigrostriatal dopaminergic pathway exhibited, in response to an amphetamine challenge, rotational asymmetry that was not attenuated by the transplantation of telencephalon-, brainstem-, or ventral mesencephalon–derived HNPCs, regardless of whether the cells were grafted to the striatum or SN (Fig. 5), as transplanted HNPCs differentiated predominantly into astrocytes.

**Transplanted HNPC Survival**

All hemiparkinsonian rats demonstrated surviving HNPC grafts in either the striatum or SN (Fig. 6). The greatest number of surviving transplanted HNPCs was observed in rats that had received telencephalon-derived grafts ($482,137 \pm 313,720$ cells and $321,498 \pm 85,420$ cells survived in the striatum and SN, respectively). Telencephalon-derived HNPC grafts appeared as dense clusters of closely packed cells within the striatum and SN (Fig. 6A–D). Significantly fewer ($p < 0.05$) brainstem-derived HNPCs survived posttransplantation ($62,902 \pm 13,993$) and appeared as less densely aggregated grafts (Fig. 6E–H). Ventral mesencephalon–derived HNPC grafts were the smallest in volume and, like the brainstem grafts, comprised clusters of HNPCs that were not as closely aggregated as those in the telencephalic transplants (Fig. 6I–L).
The number of surviving ventral mesencephalon HNPCs in the striatum and SN was 33,186 ± 16,743 and 86,926 ± 19,057, respectively, which was significantly less than the number of surviving telencephalon-derived HNPCs (p < 0.05) but not significantly different from the number of surviving transplanted brainstem derived HNPCs (p > 0.05).

For each tissue type, there was no significant difference in the number of surviving cells in the striatum compared with the SN (p > 0.05).

Transplanted HNPC Differentiation

Human neural precursor cells that demonstrated multipotentiality in vitro differentiated predominantly into astrocytes by 7 weeks after transplantation into the nonneurogenic striatum and SN (Fig. 7). Regardless of the region of precursor cell origin, HNPCs demonstrated immunoreactivity predominantly for GFAP and nestin. None of the grafts derived from any of the tissue types demonstrated immunoreactivity for neuronal (TUJ1 or microtubule-associated protein–2) or oligodendrocytic markers (O4 or myelin basic protein), and none of the cells exhibited neurochemical phenotypes (TH, glutamic acid decarboxylase 65/67, and GABA). Some cells, typically those at the margins of the graft deposits or HNPCs that had migrated away from the deposit, did not demonstrate immunoreactivity for any immunohistochemical markers, suggesting that they remained undifferentiated.

Transplanted HNPC Migration

Migration of transplanted HNPCs varied depending on the region of precursor cell origin and the site of transplantation. Telencephalon-derived HNPCs transplanted into the striatum migrated in a nondirected manner away from the graft deposits throughout the striatum (Fig. 8). In coronal sections through the striatum, some cells had migrated through this body and as far ventrally as the nucleus accumbens. Cells were also observed in the cortex and corpus callosum ipsilateral to the graft deposit, likely deposited as the transplantation capillary was withdrawn from the striatum. Cells migrated widely through the corpus callosum laterally toward the claustrum and dorsal endopiriform nucleus. Some cells migrated across the midline and into the corpus callosum of the contralateral hemisphere. The percentage of surviving HNPCs that migrated away from the striatum graft deposits was 51 ± 6%, and these cells migrated over a span of 2080 μm in the rostrocaudal axis. Significantly fewer of the telencephalon-derived HNPCs (29 ± 3%, p < 0.05) migrated away from the nigral graft deposit, although this movement occurred in an undirected manner. None was observed in the contralateral hemisphere. The span over which the HNPCs migrated was...
more restricted than that for the cells transplanted in the striatum and was 1600 μm in the rostrocaudal axis. Brainstem–derived HNPCs transplanted in the striatum also migrated away from the graft deposits over a span of 1480 μm in the rostrocaudal axis. The percentage of intrastriatally transplanted brainstem-derived HNPCs (44 ± 23%) was not significantly different (p > 0.05) from the percentage of intrastriatally and intranigrally transplanted telencephalic-derived HNPCs. In contrast, brainstem-derived HNPCs did not migrate extensively after transplantation into the SN and remained clustered at the site of transplantation. The HNPCs could be observed over a span of 1280 μm in the nigral brain sections. The ventral mesencephalon–derived cells did not migrate away from the site of transplantation in the striatum and SN, and the range over which they were observed spanned 800 and 960 μm in the rostrocaudal axis, respectively.

Later-Passage HNPCs

We also evaluated the survival and differentiation of intrastriatally transplanted telencephalon-derived HNPCs that had expanded in suspension bioreactors until they were at P13 and then had been passaged an additional 5 times in stationary culture. Hemiparkinsonian rats made 12 ± 2.2 amphetamine-induced rotations/minute and did not demonstrate significant recovery of amphetamine-induced rotational behavior at either 3 (11 ± 3 rotations/minute) or 6 weeks posttransplantation (10 ± 3 rotations/minute). Transplanted later-passage HNPCs demonstrated significantly reduced survival at 7 weeks posttransplantation compared with earlier-passage telencephalon-derived HNPCs, with 43,333 ± 2,834 cells surviving. The grafts derived from later-passage HNPCs appeared as dense clusters of cells but were much thinner in comparison to those derived from the earlier-passage cells (Fig. 9). Similar to the earlier-passage cells, however, the transplanted later-passage HNPCs differentiated predominantly into astrocytes with GFAP and nestin immunoreactivity (data not shown).

Discussion

The future of cell restoration strategies for the treatment of neurodegenerative disorders, such as PD, will depend on the availability of clinical-grade cells that can be expanded in a standardized manner to produce the large quantities necessary for widespread clinical application. Human neural precursor cells are attractive alternatives to primary fetal cells, given that they can proliferate in culture for extended periods of time and differentiate into a variety of phenotypes. In addition, there is growing evidence that characteristics of neural precursor cells, such as their differentiation potential, survival, and migration after transplantation into the CNS, can vary depending on their region of origin. The evaluation of the properties of HNPCs derived from different levels of the neuraxis, both in vitro before and in vivo after transplantation into the parkinsonian rodent, is therefore required to determine the optimal cell type for transplantation, which we did in this study.
Expansion and Survival of HNPCs

This study demonstrates the feasibility of expanding HNPCs derived from various levels of the neuraxis in a standardized manner in suspension bioreactors. The homogeneous conditions within the bioreactors resulted in significantly greater cell expansion compared with stationary culture. The rate of cell expansion as well as the structure of the neurospheres during expansion varied between tissue types. Telencephalon-derived HNPCs exhibited significantly higher cell fold expansion rates in culture and generated larger-diameter aggregates than cells derived from the ventral mesencephalon, for example. This result is consistent with other data indicating faster proliferation rates in neural precursor cells isolated from more rostral regions of the CNS. The propensity to migrate away from the site of graft placement into either the striatum or SN varied depending on the region of precursor cell origin. Telencephalon-derived HNPCs showed the greatest migration into parkinsonian rats varied depending on the region of precursor cell origin. Telencephalon–derived HNPCs demonstrated more restricted migration over a distance of 800 and 960 μm, respectively, in the rostrocaudal axis. Bar = 2000 μm for striatal tracings and 1000 μm for nigral tracings.

Differentiation of HNPCs

The differentiation of HNPCs is thought to be dependent on both intrinsic genetic programming and exogenous environmental factors and is supported by the results of in vitro experiments performed in the present study. Ventral mesencephalon-, brainstem-, and spinal cord–derived HNPCs demonstrated multipotentiality after culture in a variety of media, but the proportions of cells that differentiated into either neuronal or astrocytic immunophenotypes varied depending on the region of precursor cell origin and the manner in which the medium was supplemented. Among the factors that influence the neurogenic capacity of HNPCs are the region of precursor cell origin, which is greater for more rostrally derived cells, as well as the passage level of the cells, with a decrease in the proportion of neurons and an increase in the proportion of astrocytes over time. This finding may reflect preferential selection of HNPCs committed to glial phenotypes or a developmental program in which more astrocytes are generated as the brain matures.

In contrast, HNPCs, regardless of their region of origin, differentiated almost entirely into GFAP- and nestin-immunoreactive astrocytes after transplantation into the parkinsonian rat brain, suggesting that local in vivo microenvironmental factors, and not intrinsic cues, predominantly influence the ability of HNPCs to differentiate into neurons and determine HNPC phenotypic fate after implantation into the striatum or SN, 2 nonneurogenic regions of the brain. In this respect, the results presented...
here are consistent with the findings of other studies involving intracerebral transplantation of HNPCs in which only a small proportion of transplanted cells expressed neuronlike markers and even fewer expressed markers for dopaminergic neurons. The factors responsible for glial restriction of HNPC differentiation remain unknown, although in the telencephalon it has been associated with the presence of the leukocyte inhibitory factor, bone morphogenic protein 2, and FGF2. Additionally, astrocytes in nonneurogenic regions of the CNS are thought to inhibit neurogenesis and can affect the maturation and synapse formation by transplanted cells.

Consistent with observations in other studies, some precursor cells in the present study, typically those that had migrated away or were located on the periphery of the graft deposit, did not exhibit immunoreactivity for the phenotypic markers of either neurons or glial cells, suggesting that they remained in what Lundberg and colleagues referred to as a “quiescent state” similar to that demonstrated in adult rodent subventricular zone neural stem cells. It is possible that these cells retain the capacity to differentiate.

The assertion that environmental cues provided by the in vivo CNS play a critical role in determining transplanted precursor cell fate is supported by a number of observations. For example, precursor cells that exhibit glial restriction after transplantation into nonneurogenic regions of the CNS can differentiate into site-specific neuronal phenotypes when placed into neurogenic regions, such as the subventricular zone and dentate gyrus of the hippocampus. Additionally, neuronal differentiation of transplanted precursor cells may be dependent on the age of the host, given that precursor cells can differentiate into neurons after transplantation into postnatal rather than adult brains. Lastly, the phenotypic fate of transplanted precursor cells can vary depending on whether they are transplanted into normal or lesioned environments, which suggests that it may be necessary to predifferentiate HNPCs to desired phenotypes, such as a dopaminergic one for PD, prior to transplantation.

In this study, the ability to predifferentiate HNPCs from the various regions of the neuraxis into a dopaminergic phenotype was evaluated. In culture, HNPCs did not spontaneously differentiate into TH-immunoreactive cells and therefore were exposed to factors known to influence differentiation toward a dopaminergic phenotype, such as forskolin, GDNF, and FGF8. Nevertheless, dopaminergic differentiation still was not observed, even in cultures derived from the ventral mesencephalon. This finding may be related to the level of maturity of the tissue at the time of culture, given that mitogenic expansion has been shown to reduce the potential for fetal ventral mesencephalon–derived precursor cells to differentiate into dopaminergic neurons. Differentiation of HNPCs into dopaminergic cells may require exposure to additional exogenous factors in culture or genetic manipulation prior to transplantation. The differentiation of HNPCs into other neurochemical phenotypes, such as a GABAergic phenotype, already has been demonstrated.

Migration of HNPCs

The ability of transplanted HNPCs to migrate from their site of implantation may be a useful means of facilitating their widespread innervation of target structures, such as the striatum. In this study, the extent of the migration of HNPCs was dependent on both the region of precursor cell origin and the site of transplantation. Telencephalon-derived HNPCs demonstrated the most widespread migration, as did HNPCs targeted to the striatum. Migration appeared to be nondirected, with dispersion of HNPCs away from the graft core, which is consistent with other HNPC transplantation studies in which cells were observed to migrate, particularly in white matter structures. Transplanted precursor cells have been reported to migrate.
in a directed manner, toward sites of injury, for example;\textsuperscript{11,59,95} however, this type of movement was not the case in the present study, given that no HNPCs transplanted in the striatum were observed in the lesioned SN. Our findings also contrast with those indicating that HNPC migration was unrelated to the amount of time in culture.\textsuperscript{38} Here, telencephalon-derived HNPCs passed for extended periods in suspension bioreactors exhibited markedly less migration than cells passed fewer times, with a migration pattern that resembled that of the transplanted ventral mesencephalon–derived HNPCs.

**Influence of the Region of HNPC Origin**

Our finding that expanded populations of HNPCs displayed regional differences in their differentiation patterns in vitro and survival/proliferation and migration characteristics in vivo is consistent with data from studies demonstrating the regional specificity of neural precursor cells in the CNS. Regional identities of precursor cells are thought to develop early during embryogenesis,\textsuperscript{30,83} with graded levels of morphogens\textsuperscript{18,41,53} and the differential expression of regulatory genes influencing the inherent and site-specific properties of precursor cells along the neuraxis. Thus, neural precursor cells from different levels of the neuraxis express different molecular markers, some of which are retained even during mitogenic expansion and passing.\textsuperscript{2,14,45,75,76} Regional specification exists even within brain regions.\textsuperscript{24} For example, differentiation of the various cell types of the telencephalon occurs due to variations in the expression of transcription factors by subpopulations of precursor cells within this structure.\textsuperscript{32,42} Despite the regional differences that precursor cells exhibit, their phenotypic fate may be alterable and influenced by environmental cues,\textsuperscript{32,42} although this assertion remains controversial.\textsuperscript{45}

**Conclusions**

Data in this study demonstrate that HNPCs derived from different levels of the neuraxis can be expanded in a standardized fashion and in unlimited quantities in suspension bioreactors. When transplanted into a rat model of PD, these cells survive and migrate in a manner influenced by their region of origin and predominantly differentiate into astrocytes. These findings suggest the need to fully understand the factors that influence differentiation so that cell fate can be directed prior to transplantation.

**Acknowledgments**

Technical assistance was provided by Damaso Sadi, Rupert Ulalia, and Christopher Haughn.

**References [Au: see query #71]**

Human neural precursor cell transplantation for Parkinson disease


35. Kopyov OV, Jacques DS, Lieberman A, Duma CM, Rogers RL: Outcome following intrastriatal fetal mesencephalic grafts for Parkinson’s patients is directly related to the volume of grafted tissue. Exp Neurol 146:536–545, 1997


K. Mukhida et al.

Neurosurg. Focus / Volume 24 / March/April 2008
Human neural precursor cell transplantation for Parkinson disease


Accepted December 24, 2007.

This work was supported in part by fellowships from the Dalhousie Medical Research Foundation, Nova Scotia Health Research Foundation, and Parkinson Society Canada as well as a Killam Memorial Scholarship (K.M.), the Atlantic Innovation Fund (I.M.), the Stem Cell Network (I.M. and L.A.B.), and the Natural Sciences and Engineering Research Council of Canada (A.S. and L.A.B.). This work was presented in part at the Congress of Neurological Surgeons Annual Meeting, San Diego, California, in September 2007.

Address correspondence to: Ivar Mendez, M.D., Ph.D., Division of Neurosurgery, New Halifax Infirmary, Queen Elizabeth II Health Sciences Centre, 1796 Summer Street, Room 3806, Halifax, Nova Scotia, Canada B3H 4A7. email: mendez@dal.ca.