UNIVERSITY OF CALIFORNIA, SAN DIEGO

HUMAN NEURAL STEM CELL THERAPY IN A PRIMATE MODEL FOR PARKINSON'S DISEASE

A dissertation submitted in partial satisfaction of the requirements

of the degree Doctor of Philosophy

in

Biomedical Sciences

by

Dustin Robert Wakeman

Committee in Charge:

Professor Evan Y. Snyder, Chair
Professor Laurence L. Brunton, Co-Chair
Professor Joseph G. Gleeson
Professor Mark H. Tuszynski
Asst. Professor Binhai Zheng

2010

ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346

The dissertation of Dustin Robert Wakeman is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

_____

_____

_____

_____

_____

_____
Chair

University of California, San Diego

2010

DEDICATION

To my family, who have always been my biggest supporters, especially my grandparents, Marjorie V. Wakeman, Harrison "Bud" Wakeman, and Wilma Stookey who taught me honesty and integrity in life.

EPIGRAPH

"There is a road, no simple highway
Between the dawn and the dark of night
And if you go, no one may follow
That path is for your steps alone"

- Robert Hunter -

"Who can unlearn all the facts that I've learned
As I sat in their chairs and my synapses burned
The torture of chalk dust collects on my tongue
Thoughts follow my vision and dance in the sun
All my vasoconstrictors they come slowly undone
Can't this wait till I'm old? Can't I live while I'm young?"

- Tom Marshall -

"Once in awhile you get shown the light
In the strangest of places if you look at it right"

- Jerry Garcia -

"Sometimes you eat the bar and sometimes the bar eats you"

- Sam Elliott -

"The Dude Abides"

- Jeffrey "Duder" Lebowski -

"It is a paradox of our time that, seduced by the similarities, we often neglect the importance of distinctions. Indeed it should be expected that millions of years of evolution which selected enhanced mental capacity for survival would leave a significant mark on the organization of the cerebral cortex"

- Pasko Rakic -

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

bFGF	basic fibroblast growth factor
BLBP	brain lipid binding protein
BrdU	5-Bromo-2'-Deoxyuridine
BSA	bovine serum albumin
CAPIT	Core Assessment Program for Intracerebral Transplantation
CC	corpus callosum
CDB	cell dissociation buffer
Ch	Chemicon
CM	conditioned media
CNS	central nervous system
CPu	caudate putamen
DA	dopamine; Dopaminergic
DAT	dopamine transporter
DCX	doublecortin
DPBS	Dulbecco's modified phosphate buffered saline
EAE	experimental allergic encephalomyelitis
ECM	extracellular matrix
EM,	Erlenmeyer
EGF	epidermal growth factor
eGFP	enhanced green fluorescent protein
FB	Fast Blue
FBS	fetal bovine serum
FG	fluorogold
GDM	glial defined medium
GDNF	glial cell line-derived neurotrophic factor
GFAP	glial fibrillar acidic protein
GFP	green fluorescent protein
HBSS	Hank's balanced salt solution
HD	Huntington's disease
huNuc	human nuclei,
(h)ESC	(human) embryonic stem cell
(h)NPC	(human) neural precursor cell
(h)NSC	(human) neural stem cell
(h)fNPC	human fetal neural precursor cell
(h)fNSC	human fetal neural stem cell
IGF-1	insulin-like growth factor-1
ir	immunoreactive
iPS	induced pluripotent stem (cell)
LIF	leukemia inhibitory factor
MAN	multilayer adherent network
MAPK	mitogen activated protein kinase
mCherry	monomeric cherry
MCI	mitochondrial complex I
MFB	medial forebrain bundle
MHC	major histocompatibility complex
MMS	MAN membrane system
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine

MS	Multiple Sclerosis
NB-B27	Neurobasal-B-27 Medium
Neurobasal-N	Neurobasal-neuronal medium
NFM	neurofilament
NGF	nerve growth factor
NS	nigrostriatal
NSA	neurosphere assay
NuMa	nuclear mitotic apparatus
OB	olfactory bulb
OL	oligodendrocyte
OLP	oligodendrocyte progenitor
OSM	oligodendrocyte specification medium
PBS	phosphate buffered saline
PD	Parkinson's disease
PDGF	platelet derived growth factor
PFA	paraformaldehyde
PFS	Parkinson's factor score
PSA-NCAM	polysialylated neural cell adhesion molecule
RG	radial glia
RMS	rostral migratory stream
PS	protamine sulfate
SC	Santa Cruz
SCI	spinal cord injury
SPIO	super-paramagnetic iron oxide
SN	substantia nigra
SNpc	substantia nigra pars compacta
SVZ	subventricular zone
TBS	Tris-buffered saline.
TC	tissue culture
TH	tyrosine hydroxylase
VM	ventral mesencephalic
2D	two dimensional
3D	three dimensional
6-OHDA	6-hyhroxydopamine

LIST OF FIGURES, TABLES, AND DIAGRAMS

ACKNOWLEDGEMENTS

Many people helped me accomplish the research presented in this thesis. First of all, I would like to thank my thesis advisor, Evan Y Snyder, and mentor Eugene Redmond for their continual support and guidance throughout my graduate career, and for creating an environment in which I could be successful. I also thank my thesis committee members, Larry Brunton, Mark Tuszynski, Joseph Gleeson, and Binhai Zheng for their helpful feedback and advice throughout this process. I am very grateful to past and present members of the Snyder lab, especially Ilyas Singec, Martin Hofmann, Seung Kim, Kook-In Park, Marcel Daadi, Michael Marconi, Andrew Crain, Jean-Pyo Lee, Brian Tobe, Tsaiwei Olee, Franz-Josef Mueller, and Chris Stubban, for their collaborative help. Many others assisted in my research, specifically the entire primate transplantation team: John Elsworth, Csaba Leranth, Jeremy Bober, John Sladek, Barbara Blanchard, SKBRF staff (Rodrigo Valles, Zyka, Wenti, Danny), Kim Bjugstad, as well as those at Sanford-Burnham Institute for Medical Research: Ed Monosov, Joseph Russo, Yoav Altman, Robbin Newlin, Jeanne Loring, Roy Williams. I would also like to specially thank the following professors for always sharing critical comments in a positive manner: Phillip Schwartz, Maurizio Zanetti, Don Cleveland, Martin Marsala, and Alysson Muotri. Finally, Id like to acknowledge the training from David Song, Stephanie Lessig, Mark Kamps, Jess Mandel and the HHMI Med-Into-Grad program for reinvigorating my research and reconnecting with the patient.

Chapter 1B, is a reprint in part the material as it appears in *Regenerative Med*. (2006) 1(4), 405–413 with co-authors Andrew C. Crain and Evan Y Snyder. The dissertation author was the primary investigator and author of this paper. DRW wrote and compiled figure, ACC helped print set figure, and EYS supervised research. Together with his coauthors, he thanks DE Redmond and JH Kordower for helpful suggestions. DRW is supported by the NIH/NIGMS funded UCSD Genetics Training Program (T32 GM08666) and is a recipient of

an American Society for Neural Therapy and Repair Travel Fellowship; DRW and AMC are supported by an NIH Stem Cell Center Grant (1 P20 GM075059-01) as well as a grant from the American Parkinson's Disease Association (APDA).

Chapter 2, is a reprint in part of material as it appears in *Curr. Protoc. Stem Cell Biol.* Chapter 2:Unit2D.3, May 2009 with co-authors Martin R Hofmann, D. Eugene Redmond Jr., Ted Teng, and Evan Y Snyder with the addition of a Table and Diagram from Derivation, Expansion, and Characterization of Human Fetal Forebrain Neural Stem Cells. In: Masters JR, Palsson BO (eds). Human Cell Culture: Adult Stem Cells. Volume 7. Dordrecht: Springer, 2009 with co-authors Martin R. Hofmann, Ted Teng, and Evan Y Snyder. The dissertation author was the primary investigator and author of both papers. DRW wrote, designed, and performed all experiments. MRH helped with figure production and cell culture. EYS and YDT supervised DRW. DER wrote methods for preparation of monkeys for transplantation. Together with his coauthors, he thanks Steven A. Wakeman and Pamela S. Burnett for constructive comments and support, as well as Ilyas Singec, Scott R. McKercher, Michael Marconi, Phillip H. Schwartz, Jeanne F. Loring, Franz-Josef Mueller, Jean-Pyo Lee, Seung U. Kim, and Kook I. Park for technical advice and procedural training. Funding for D.R.W. comes from (NIH/NIGMS T32 GM008666) UCSD Institutional Training Fellowship in Basic and Clinical Genetics, HHMI Med-Into-Grad Training Fellowship, American Society for Neural Therapy and Repair, and the American Parkinson's Disease Association. Additional support was provided by the Stem Cell Center at the Burnham Institute for Medical Research (NIH P20 GM075059-03).

Chapter 3, is a reprint in part of material as it appears in *Proc Natl Acad Sci U S A*. 2007 Jul 17;104(29):12175-80 with co-authors Redmond DE Jr, Bjugstad KB, Teng YD, Ourednik V, Ourednik J, Parsons XH, Gonzalez R, Blanchard BC, Kim SU, Gu Z, Lipton SA, Markakis EA, Roth RH, Elsworth JD, Sladek JR Jr, Sidman RL, Snyder EY. The dissertation

author was a co-author of this paper. DRW performed transplantations, immunohistochemistry/ cytochemistry, analyzed data, co-prepared manuscript, co-assembled figures, and edited text. DER was principal investigator, performed transplantations and oversaw all animal operations, co-prepared entire manuscript, and managed the project. KBB performed stereotactic counting. BCB, EAM and ZG performed IHC. JRS prepared histological figures. YDT, VO, JO, RG, XHP cultured cells. RHR, JDE, RLS, EYS, SAL, SUK supervised. Together with his coauthors, we thank the staff of St. Kitts Biomedical Research Foundation for their contributions to the *in vivo* primate studies, Csaba Leranth and Robert Makuch for histological and statistical advice, and Marcel Daadi for advice and studies differentiating hNSCs into DA neurons. This work was supported by National Institute of Neurological Disorders and Stroke Grants RO1NS40822, PO1NS44281 (to D.E.R.), and R21NS053935; Veterans Affairs Biomedical Laboratory Research and Development Grant 121F (to Y.D.T.); the National Institutes of Health/National Institute of General Medical Sciences Grant T32GM08666 (to D.R.W.); the Axion Research Foundation; Project ALS; the American Parkinson's Disease Association; the Michael J. Fox Foundation; the International Organization of Glutaric Acidemia; the A-T Children's Project; and an anonymous donor to the Combined Jewish Philanthropies.

Work presented in Chapter 4 is being prepared for publication to *PLOS: Biology* with co-authors Jeremy Bober, John R. Sladek Jr., Csaba Leranth, R. Jude Samulski, Evan Y. Snyder, and D. Eugene Redmond Jr. DRW designed and performed all experiments, produced all data, and oversaw the entire project. DER designed experiments, performed transplantations, and edited text. JB co-performed IHC. CL supervised JB and analyzed DAB IHC. RJS produced AAV-5 virus. EYS supervised DRW. The dissertation author is the primary author of this work and along with co-authors would like to thank Joseph Russo, Robbin Newlin, and Yoav Altman for CORE services, as well as Jeff H. Kordower, Ole

Isacson, John Elsworth, Lars Olson, Barbara Blanchard, and Ilyas Singec for critical historical insight in transplantation of neuronal tissue and general knowledge about neurogenesis. We also thank Walter Niles, Brian Tobe, Andrew Crain, and Rachel Krupa for procedural help and putting up with our incessant video-conferences.

Appendix A, is a reprint in part of the material as it appears in *Curr. Protoc. Stem Cell Biol.* Chapter 2:Unit 2D.4., Sep 2009 with co-authors Araceli Espinosa-Jeffrey, Seung U. Kim, Evan Y. Snyder, and Jean de Vellis.. The dissertation author was the co-primary investigator and co-author of this paper. DRW and AEJ performed all experiments , co-wrote, and co-prepared the manuscript. EYS, SUK, and JdV supervised. Together with his coauthors, he thanks the MRRC Media Core for preparation of figures and Dr. D. Birt for photograph of the dissection set up. This work (J. de V. and A. E.) was supported in part by PPGHD065-76 and by a Pilot grant from the National Multiple Sclerosis Society PP1498. DRW thanks M. Hudson for critical review and comments. DRW is supported in part by the American Parkinson's Disease Association, HHMI Med-Into-Grad Training Fellowship, and the UCSD-NIH Training Fellowship in Clinical Genetics.

Last, but not least, I would like to thank my parents, Pamela and Gene Burnett and Steve and Kathy Wakeman, as well as my brother, Dr. Derek S. Wakeman for their unwavering support with life in general.

CURRICULUM VITA

2003 Bachelor of Science in Biology:
University of Illinois at Urbana- Champaign (UIUC)

Honors/Awards:
- Senior Distinction Thesis Award, Life Sciences
- Magna Cum Laude
- Phi Beta Kappa, Phi Kappa Phi, Golden Key International Honor Society
- Dean's List
- Environmental Fellows Scholar

2010 Doctor of Philosophy, Biomedical Sciences
University of California, San Diego

Honors/Awards:
- 2005: Outstanding Merit Qualifying Thesis (UCSD)
- 2006: Travel Award: American Society for Neural Therapy and Repair-NIH
- 2006-2008: Training Fellowship in Basic and Clinical Genetics
- 2007-2008: Howard Hughes Medical Institute Med-Into-Grad Training Fellowship
- 2009: Travel Award: American Society for Neural Therapy and Repair-NIH
- 2009: Best Student Seminar Presentation: UCSD-Biomedical Sciences Retreat

PUBLICATIONS

Abstracts:

1. **D.R. Wakeman**, J.F. Loring, E.Y., D.E. Redmond Jr.' E.Y. Snyder, F.J. Mueller. Genome wide expression analysis of human neural stem cells in vitro. *Experimental Neurology,* 198(2), p593, 2006.

2. **D. R. Wakeman**, D. E. Redmond, Jr., J. R. Sladek, Jr., and E. Y. Snyder. Human neural stem cells engraft and survive concomitantly with exogenous AAV-5 GDNF expression in the MPTP-lesioned non-human primate brain. *Cell Transplantation*, 18, p239, 2009.

Articles:

1. Franz-Josef Mueller, Naira Serobyan, Ingrid U. Schraufstatter, Richard DiScipio, **Dustin Wakeman**, Jeanne F. Loring, Evan Y. Snyder, Sophia K. Khaldoyanidi. Adhesive interactions between human neural stem cells and inflamed human vascular endothelium are mediated by integrins. *Stem Cells*, 2006;24;2367-2372.

2. **Wakeman Dustin R,** Crain Andrew C, Snyder Evan Y. Large animal models are critical for rationally advancing regenerative therapies. *Regenerative Med*. (2006) 1(4), 405–413. *Editorial*

3. Redmond DE Jr, Bjugstad KB, Teng YD, Ourednik V, Ourednik J, **Wakeman DR**, Parsons XH, Gonzalez R, Blanchard BC, Kim SU, Gu Z, Lipton SA, Markakis EA, Roth RH, Elsworth JD, Sladek JR Jr, Sidman RL, Snyder EY. Behavioral improvement in a primate Parkinson's model is associated with multiple homeostatic effects of human neural stem cells. *Proc Natl Acad Sci U S A*. 2007 Jul 17;104(29):12175-80.

4. **Wakeman DR**, Hofmann MR, Teng YD, Snyder EY. Derivation, Expansion, and Characterization of Human Fetal Forebrain Neural Stem Cells. In: Masters JR, Palsson BO (eds). Human Cell Culture: Adult Stem Cells. Volume 7. Dordrecht: Springer, 2009. *Book Chapter*

5. **Wakeman DR**, Hofmann MR, Redmond Jr. DE, Teng YD, Snyder EY. Long-Term Multilayer Adherent Network (MAN) Expansion, Maintenance, and Characterization, Chemical and Genetic Manipulation, and Transplantation of Human Fetal Forebrain Neural Stem Cells. *Curr Protoc Stem Cell Biol*. Chapter 2:Unit2D.3, May 2009.

6. Araceli Espinosa-Jeffrey, **Dustin R. Wakeman**, Seung U. Kim, Evan Y. Snyder, and Jean de Vellis. Culture system for rodent and human oligodendrocyte specification, lineage progression and maturation. *Curr Protoc Stem Cell Biol*. Chapter 2:Unit2D.X (In press).*Curr Protoc Stem Cell Biol.* Chapter 2:Unit 2D.4., Sep 2009.

7. Teng YD, Kabatas S, Li J, **Wakeman DR**, Snyder EY, and Sidman RL. Functional Multipotency of Neural Stem Cells and Its Therapeutic Implications. In: Stem cells: From tools for studying mechanism of neuronal differentiation towards therapy. Henning Ulrich (ed.), Springer-Verlag: San Diego, 2009. (In press). *Book Chapter*

8. Photo Contribution in: Jandial R, Snyder EY. A safer stem cell: on guard against cancer. *Nat Med.* 2009 Sep;15(9):999-1001. *Editorial*

9. D.E. Redmond Jr, S. Weiss, J.D. Elsworth, R. H. Roth, **D.R. Wakeman**, E.Y. Snyder, K. B. Bjugstad, T.J. Collier, B. C. Blanchard, Y. D. Teng, and J.R. Sladek Jr. Cellular Repair in the parkinsonian non-human primate brain. *Rejuvenation Research*. 2009. *Review* IN PRESS

ABSTRACT OF THE DISSERTATION

HUMAN NEURAL STEM CELL THERAPY IN A PRIMATE MODEL FOR PARKINSON'S DISEASE

by

Dustin Robert Wakeman

Doctor of Philosophy in Biomedical Sciences

University of California, San Diego, 2010

Professor Evan Y. Snyder, Chair

Professor Laurence L. Brunton, Co-Chair

Neural stem cells derived from various sources have shown considerable promise for the treatment of parkinsonian symptoms in a variety of animal models; however, the long-term potential of human neural stem cells to engraft, differentiate into dopamine neurons, and restore function in the dopamine-depleted non-human primate brain remains unknown. This dissertation describes a clinically relevant paradigm for transplantation of undifferentiated human fetal subventricular-zone derived NSC in the parkinsonian primate, including gene therapy enhancement with GDNF. Chapter 1 reviews literature regarding functional properties of NSC that make them a candidate for cellular transplantation in neurodegenerative disorders, the potent neurotrophic effects of GDNF, as well as the rationale for utilizing large animals to adequately test stem cell therapeutics. Chapter 2 describes a novel culturing system to overcome cellular senescence and efficiently expand large-scale human fetal NSC long-term

without aberration. Included are clinically pertinent techniques to produce and deliver hfNSC into both the rodent and primate brain, as well as new advances in radiological tracking and imaging utilizing FDA-approved supra-magnetic iron-oxide particles. Chapter 3 demonstrates the first reported evidence that undifferentiated hfNSC differentiate into some TH-ir neurons and restore functional deficits in parkinsonian in non-human primates. Normalization of dopamine-levels and nigrostriatal circuitry argue for neuroprotective effects of endogenous cells rather than direct cell replacement. To allow for morphological analysis of donor grafts, reporter hfNSC were created (Ch.2) for long-term studies. Chapter 4 presents a new paradigm in which hfNSC are homotopically transplanted concomitantly with striatal AAV-GDNF to enhance graft survival and promote axonal outgrowth. Transplanted cells engrafted for up to 11-months and differentiated extensively congruent with host circuitry, demonstrating for the first time that undifferentiated hfNSC retain developmentally relevant programs of differentiation and respond to host signals in the dopamine-depleted primate brain. Further, evidence supports the standing argument that fetal subventricular-zone derived NSC do not significantly differentiate into mature A9-subtype midbrain dopamine neurons in-vivo, even in a GDNF-rich environment. Chapter 5 discusses the significance of these findings to cellular transplantation in the adult CNS and the future application of stem cell transplantation with regards to Parkinson's disease.

CHAPTER 1A:

INTRODUCTION-
THERAPEUTIC RATIONALE FOR HUMAN NEURAL STEM CELL TRANSPLANTATION IN PARKINSON'S DISEASE

1A.1 Background

Parkinson's disease (PD) presently affects 1 to 1.5 million Americans, and this number is expected to increase with the aging of the population. Present treatments are palliative and some possibly contribute to disease progression. The proposed research, as next steps for developing a biologically restorative treatment method from "replacement" cells[1], could lead to a treatment for patients with early stage as well as untreatable late-stage Parkinson's disease.

Loss of DA neurons in the substantia nigra (SN) and the resulting deficit in DA release in the striatum and other areas appear to be responsible for the characteristic motor and cognitive manifestations of PD[2]. Substantial improvements result from the systemic administration of the DA precursor, L-Dopa, or DA agonists[3], supporting the idea that DA release provided by transplanted replacement neurons might have therapeutic effects. This seems now to have been verified in numerous studies in rodents[4-8] and non-human primates[9-17] which have shown that grafts of fetal DA neurons can lead to improvements in biochemical and behavioral indices of DA deficiency. However, in clinical studies, the improvements in parkinsonism have been rather modest and variable[18-30]. The main reasons appear to be insufficient restoration of striatal DA tone which, in turn, stems from the large volume of striatum that has to be reinnervated in man, together with the low rate of survival of transplanted fetal DA neurons, (reviewed by Redmond[31] and Wakeman[32]).

It is the goal of this dissertation to test the potential of human neural stem cells (hNSC) to reverse dysfunction in the neurotoxic model of parkinsonism produced by MPTP in monkeys. We have chosen to focus on how best to achieve permanent dopamine (DA) replacement and functional recovery from human stem cells in the most physiologic and functional manner without side effects or cell over-growth. The following dissertation chapters address fundamental unknown questions relevant to stem cell therapeutics and introduce novel methodological advances critical for advancing stem cell transplantation therapies into clinically relevant paradigms. Specifically, the development of new defined culturing systems for long-term mass-production of human fetal neural stem cells (hfNSC), as well as a developmentally defined system to systematically produce oligodendrocyte precursor cells in-vitro. In addition, we developed methods for tracking transplanted hNSC with iron-particles and magnetic resonance imaging and refined neurosurgical techniques to mimic the clinical surgical setting in the most relevant non-human primate model of parkinsonism. With the development of these tools, we sought to determine whether:

(1) Undifferentiated hfNSCs will engraft, survive, differentiate, and induce significant recovery of function in the dopamine-depleted striatum and substantia nigra (SN) (Chapters 3 and 4).

(2) HfNSCs will promote a return to homeostasis through multiple compensatory mechanisms.

- **i.** Provide neuroprotective support to host DA neurons and circuitry and preserve or increase DA concentrations, DA cell survival, and the endogenous connections that already exist (Chapter 3).
- **ii.** Differentiate into other relevant support cells (astrocytes, oligodendrocytes)

and secrete neurotrophic factors (Chapters 2A, 3, 4, Appendix A)

(3) AAV-GDNF overexpression in the host will increase survival and distribution of endogenous and hfNSC derived cells, specifically the induction of the A9-nigral midbrain phenotype, and favorably direct their neuritic outgrowth toward striatal target regions (Chapters 3 and 4).

1A.2 Beneficial Characteristics of hNSC

Stem cells may provide a solution to many of the known and suspected problems of neural transplantation with fetal DA precursor cells. Multiple classes of human "stem cells," have been defined, including hNSCs, the most primordial, uncommitted and multipotent cells of the nervous system, which give rise to the vast array of more specialized cells of the CNS[33-53]. HfNSCs have been most effectively obtained from neuroectodermal-derived germinal zones, e.g., the ventricular zone of the fetal telencephalon[54] and are operationally defined by their ability to differentiate into most (if not all) neuronal and glial lineages in multiple anatomical and developmental contexts, and to populate developing and/or degenerating CNS regions. They can be readily isolated from the brain and *efficiently expanded* in vitro by multiple safe and effective means, propagated, characterized, manipulated, and tested for efficacy and lack of pathogenicity. In addition, NSCs have been reported to have a number of properties that might make them useful for brain repair, including (1) Facile engraftability (particularly into germinal zones), (2) Permeability within the blood brain barrier, (3) Genetic manipulability (easily transduced ex-vivo by most viral and non-viral gene transfer methods), (4) Migratory behavior enabling replacement of cells and genes at disseminated lesion sites, [55] (5) Plasticity, (6) Tropism and trophism within regions of active CNS degeneration[56-58], and (7) Low immunogenicity despite high MHC expression[59] (Reviewed by Park[60,61]). Prior data in rodents (by many investigators) and progress with hNSCs obtained from human fetal brain

(reported by our group) suggest that stem cells may provide both cellular replacement and neurorestorative effects in the host that may be applicable to the treatment of PD.

1A.3 Non-Human Primates are Critical for Pre-clinical Evaluation

There are numerous dissimilarities between the brains of rodents and primates that limit the clinical predictive power and experimental utility of rodent experiments alone. The most relevant differences begin with genomics but are manifested as differences in (a) biochemistry, (b) pharmacological responsiveness, (c) development, (d) brain size and organization, and (e) behavior. In all of these areas, Old World non-human primates are far more like humans. In addition, recent genetic studies showing that an alpha-synuclein substitution/mutation at P53 (an alanine for a threonine), which is associated with neurodegenerative diseases, including PD, occurred about 38 million years ago at the divergence of Old World from New World primates[62]. This difference may help to explain the unique vulnerabilities of Old World primates to neurotoxins such as MPTP and, perhaps, with additional genetic alterations, the unique vulnerability of humans to PD. Significant other biochemical and pharmacological differences exist between rodent and primate brains in aspects of the monoamine systems relevant to PD[63]; there are fewer differences between monkey and human. The existence *only* in primates of the excellent symptomatic model of human parkinsonism after systemic MPTP exposure raises powerful ethical arguments in *favor* of using it to develop the most effective clinical interventions possible. Misinformation from rat studies has led to erroneous but tenacious interpretations of human clinical trials. For example, despite the large number of rodent PD "model" studies using fetal tissue, they failed to anticipate the dyskinetic side effects ultimately observed following the use of such graft material in actual PD patients and overestimated its chances for success. Work with primates, while having the advantage of being much more directly connected to the human condition, is

extremely labor-intensive and requires longer periods of time to prepare animals, carry out experiments, and analyze post mortem specimens. However, we believe that the value of the data ultimately acquired provides information that cannot be obtained or predicted from studies in rodents or even from post mortem outcomes in human patients (See also: Chapter 1B for a detailed description)

1A.4 MPTP Model of PD in Non-Human Primates

For functional studies to have relevance to PD, it is important to study the best available models. MPTP was inadvertently discovered to induce Parkinson's disease in humans as the toxic by-product of a synthetic street drug[64,65]. MPTP is a potent, irreversible inhibitor of mitochondrial complex I (MCI) when converted by astrocytes to MPP+ and taken into DA neurons by the dopamine transporter (DAT)[66-71], causing selective degeneration of A9 DA neurons within the midbrain structure, substantia nigra pars compacta (SNpc), thus mimicking human PD. The range of variability, course, and duration of the systemic MPTP syndrome in the African green monkey has been studied for periods up to 14 years and shown that this species shows most of the characteristics of PD. The use of highly symptomatic parkinsonian animals in order to improve the parallels with PD and to assure the stability (or deterioration) of the condition over time, also has drawbacks due to the care required to maintain life and to minimize stress and discomfort. Unfortunately, no other model of PD (not unilateral intra-carotid MPTP infusion or unilateral or bilateral 6-OHDA administration) in non-human primates mimics as closely the full behavioral syndrome or neuropathology[66-68]. Fortuitously, the St. Kitts green monkey appears to show a more complete model of parkinsonism than any other species of monkey[69]. In addition, our behavioral assessment method for parkinsonism correlates extremely well with measures of DA transporters *in vivo* and DA concentrations post mortem. Our studies of DA transporters in vivo using SPECT

imaging with [^{123}I]β-CIT in the striatum of MPTP monkeys have included determination of the reproducibility of the imaging measures, optimization of the SPECT outcome measure, and longitudinal assessment of MPTP animals with SPECT. Mean DA punch biopsy measures were significantly correlated with SPECT striatal uptake ($r= 0.80$, $n=18$, $p <0.015$). DA concentrations in post-mortem biochemical punches highly correlate with both the Parkinson Score and Healthy Behavior Score. This finding is similar to the finding that UPDRS correlates with SPECT beta-CIT in patients[70,71].

1A.5 HNSCs Effect on the Parkinsonian Brain

A major aim of the proposed studies will be to determine the adequacy of hNSC to (1) provide properly distributed, functionally integrated dopamine neurons to restore normal function and (2) to provide neuroprotective support to host DA neurons and circuitry in order to preserve the connections that already exist. Though multiple studies have now demonstrated that implanted neural progenitors, when transplanted in *non*-neurogenic regions of the intact adult brain do not yield neurons[41,72,73], more recent evidence suggests that, within neurodegenerative environments, neurogenic signals are recapitulated (at least transiently) such that new, donor NSC-derived neurons now emerge[37,74,75] successfully engraft and assume legitimate neural phenotypes. It appears that such "self-repair" signals to which NSCs are responsive may be elaborated constitutively in the adult DA-depleted primate brain and now confirmed by our studies in monkeys. However, they may not be of sufficient magnitude to yield optimal functional recovery. Hence, an additional strategy that must be compared with unmodified hNSCs would be to *increase* the proportion of hNSCs that differentiate into DA neurons. The most obvious method for increasing these numbers is to inject more hNSCs. But based upon some on-going and published studies in the St. Kitts green monkey, we failed to increase the number of surviving TH+ cells by doubling the amount of primary fetal tissue

injected, suggesting that the host environment may limit this approach for stem cells also. In our recent studies, there also appeared to be no clear relationship between the number of hNSC injected and the number that were found post-mortem.

1A.6 GDNF enhances survival and outgrowth of immature DA neurons:

A recognized problem with conventional primary fetal neural tissue transplantation is the high percentage of transplanted cells that die. It appears that most DA neurons are, in fact, apoptotic within the first 24 hours of grafting[76]. Thus, early loss of DA neurons probably accounts in large part for the observation that 90-99% of transplanted neurons are lost by the end of a study, typically several weeks after grafting occurred[77-81]. The relatively impoverished neurotrophic environment of the adult brain is one factor that may trigger death of grafted DA neurons[82-87]. There have been attempts to counter the effects of an experimentally induced DA loss with transplants to the striatum of primate stem cells converted ex vivo to a DA phenotype. Success has been mixed with regard to survival of transplanted cells or loss of phenotype and hence functional impact[95].

GDNF has been found to be a potent neurotrophic factor necessary for DA neuron survival in the developmental and adult stages[88], and its actions on the VM are rather selective for DA neurons[89, 90]. When administered exogenously to rodents and humans with Parkinsonism, it appears to protect endangered DA neurons[91-94]. Indeed, GDNF supplied via viral vectors in non-transplant paradigms to parkinsonian animal models has been shown to be a potent neuroprotective agent. Overexpression of GDNF induced by a lentiviral vector provides an additional means of trophic support during possibly critical periods[91]. An added benefit of GDNF may not only be the autocrine/paracrine effect such a neurotrophin may have on donor-derived cells, but the protective effect this factor may have on *host* cells that may be in the process of undergoing apoptotic degeneration[95]. Recent work from our collaborators[96]

demonstrated that injection of AAV2-GDNF into the monkey striatum lead to stable and long-lasting (12 month) overexpression of GDNF in that region of the brain. We have also shown that AAV2-mediated gene transfer of GDNF to the striatum of MPTP-treated monkeys results in a marked increase in survival of primary fetal VM DA-precursor cells by several fold and elicits directional outgrowth of axons[97-99]**.** Furthermore, recent data indicates that GDNF can act as a chemoattractant in stem cell migration in the brain[100-102]. These data provide solid support for the hypothesis that injections of AAV-GDNF in combination with hNSC transplantation may also enhance the survival and outgrowth of DA neurons derived from stem cells.

1A.7 EGFP labeling improves viability of donor cells

Traditional methods for pre-labeling NSCs prior to transplantation require harsh DNA intercalating thymidine analogs such as BrdU and CldU, which have been shown to greatly underestimate overall engraftment success and create a variety of false positives when administered for extensive periods of time. Generation of an independently labeled fluorescent hNSC line eliminates the need for these toxic compounds, while increasing both cell viability and engraftment efficiency. In addition, fluorescently labeled donor cells can be easily identified among their host counterparts allowing for enhanced visualization of axonal processes and their dendritic counterparts. These optical properties allow us to easily assess the overall multi-potentiality of each subline. The stem cell community now demands extensive validation of donor cell origin to effectively prove that exogenous benefits were directly derived from transplanted donor hNSCs, therefore, extensive characterization of these reporter hNSC sublines is pertinent to the progression of NSC therapy in neurodegenerative disease.

1A.8 Significance

Considerable data derived from studies of rodents and now our initial progress in primates indicate that stem cells can be transplanted, survive, migrate, differentiate into TH+ cells, have other normalizing effects on the host, and perform at least some of the functions for which they are specialized. There is a clear possibility that the functional changes and motor deficits produced by MPTP, similar to those in Parkinson's disease, could be reversed, and this possibility is supported by our studies in primates to date. It is important also that the desirable and undesirable consequences of stem cell engraftment be determined in animals which can be studied over long periods of time and whose brains can be examined directly to determine morphological, biochemical, and behavioral consequences of grafting. Such studies may lead to new understanding of the disease processes affecting DA systems, as well as adding to our understanding of brain function and plasticity.

1A.9 References

1. **Bjorklund A, Dunnett SB, Brundin P, et al. Neural transplantation for the treatment of Parkinson's disease. Lancet Neurol. 2003 Jul;2(7):437-45.**

2. **Bernheimer H, Birkmayer W, Hornykiewicz O, Jellinger K, Seitelberger F. Brain dopamine and the syndromes of Parkinson and Huntington: clinical, morphological and neurochemical correlations. Journal of Neuroscience. 1973;20:415-55.**

3. **Rinne UK. Dopamine Agonists as Primary Treatment in Parkinson's Disease. In: Yahr MD, Bergmann KJ (eds). Parkinson's Disease, 1986:519-23.**

4. **Perlow MJ, Freed WJ, Hoffer BJ, Seiger A, Olson L, Wyatt RJ. Brain grafts reduce motor abnormalities produced by destruction of nigrostriatal dopamine system. Science. 1979;204:643-53.**

5. **Björklund A, Stenevi U. Reconstruction of the nigrostriatal dopamine pathway by intracerebral nigral transplants. Brain Research. 1979;177:555-60.**

6. **Björklund A, Stenevi U. Neural Grafting in the Mammalian CNS. Amsterdam: Elsevier Science Publishers; 1985.**

7. **Björklund A, Stenevi U. Intracerebral neural grafting: a historical perspective. In: Björklund A, Stenevi U (eds). Neural Grafting in the Mammalian CNS. Amsterdam: Elsevier Science Publishers, 1985:3-14.**

8. **Brundin P, Duan WM, Sauer H. Functional effects of mesencephalic dopamine neurons and adrenal chromaffin cells grafted to the rat striatum. In: Dunnett SB, Bjorklund A (eds). Functional Neural Transplantation. Volume 2. New York: Raven Press, 1994:9-46.**

9. **Redmond DE, Jr., Sladek JR, Jr., Roth RH, et al. Fetal neuronal grafts in monkeys given methylphenyltetrahydropyridine. Lancet. 1986;1(8490):1125-7.**

10. **Redmond DE, Jr., Sladek JR, Jr., Roth RH, et al. Transplants of primate neurons [letter]. Lancet. 1986;2(8514):1046.**

11. **Sladek JR, Jr., Collier TJ, Haber SN, Roth RH, Redmond DE, Jr. Survival and growth of fetal catecholamine neurons transplanted into primate brain. Brain Research Bulletin. 1986;17(6):809-18.**

12. **Annett LE, Martel FL, Rogers DC, Ridley RM, Baker HF, Dunnett SB. Behavioral assessment of the effects of embryonic nigral grafts in marmosets with unilateral 6-OHDA lesions of the nigrostriatal pathway. Experimental Neurology. 1994;125(2):228-46.**

13. **Elsworth JD, Sladek JR, Jr., Taylor JR, Collier TJ, Redmond DE, Jr., Roth RH. Early gestational mesencephalon grafts, but not later gestational mesencephalon, cerebellum or sham grafts, increase dopamine in caudate nucleus of MPTP-treated monkeys. Neuroscience. 1996;72(2):477-84.**

14. **Taylor JR, Elsworth JD, Roth RH, Sladek JR, Jr., Collier TJ, Redmond DE, Jr. Grafting of fetal substantia nigra to striatum reverses behavioral deficits induced by MPTP in primates: a comparison with other types of grafts as controls. Experimental Brain Research. 1991;85(2):335-48.**

15. **Bankiewicz K, Mandel RJ, Sofroniew MV. Trophism, transplantation, and animal models of Parkinson's disease. Experimental Neurology. 1993;124(1):140-9.**

16. **Fine A, Hunt SP, Oertel WH, et al. Transplantation of embryonic marmoset dopaminer-gic neurons to the corpus striatum of marmosets rendered parkinsonian by 1-methyl-4-phenyl-1,2-3,6-tetrahydropyridine. Prog Brain Res. 1988;78:479-89.**

17. **Bakay RAE, Barrow DL, Fiandaca MS, Iuvone PM, Schiff A, Collins DC. Biochemical and behavioral correction of MPTP Parkinson-like syndrome by fetal cell transplantation. Annuals of the New York Academy of Science. 1987;495:623-40.**

18. Freed CR, Breeze RE, Rosenberg NL, et al. Transplantation of human fetal dopamine cells for Parkinson's disease: results at 1 year. Archives of Neurology. 1990;47:505-12.

19. Hitchcock ER, Kenny BG, Clough CG, Hughes RC, Henderson BT, Detta A. Stereotactic implantation of fetal mesencephalon (STIM): the UK experience. Progress In Brain Research. 1990;82:723-8.

20. Henderson BT, Clough CG, Hughes RC, Hitchcock ER, Kenny BG. Implantation of human fetal ventral mesencephalon to the right caudate nucleus in advanced Parkinson's disease. Archives of Neurology. 1991;48(8):822-7.

21. Lindvall O, Rehncrona S, Brundin P, et al. Neural transplantation in Parkinson's disease: the Swedish experience. Progress In Brain Research. 1990;82:729-34.

22. Lindvall O, Sawle G, Widner H, et al. Evidence for long-term survival and function of dopaminergic grafts in progressive Parkinson's disease. Annals of Neurology. 1994;35:172-80.

23. Freed CR, Breeze RE, Rosenberg NL, et al. Survival of implanted dopamine cells and neurologic improvement 12 to 46 months after transplantation for Parkinson's disease. New England Journal of Medicine. 1992;327:1549-55.

24. Spencer DD, Robbins RJ, Naftolin F, et al. Unilateral transplantation of human fetal mesencephalic tissue into the caudate nucleus of Parkinsonian patients: functional effects for 18 months. New England Journal of Medicine. 1992;327(22):1541-8.

25. Widner H, Tetrud J, Rehncrona S, et al. Bilateral fetal mesencephalic grafting in two patients with parkinsonism induced by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) [see comments]. N Engl J Med. 1992;327(22):1556-63.

26. Kordower J, Freeman T, Snow B, et al. Neuropathological evidence of graft survival and striatal reinnervation after the transplantation of fetal mesencephalic tissue in a patient with Parkinson's disease. New England Journal of Medicine. 1995;332:1118-24.

27. Kordower J, Rosenstein J, Collier T, et al. Functional fetal nigral grafts in a patient with Parkinson's disease: Chemoanatomic, ulturastructural, and metabolic studies. Journal of Comperative Neurology. 1996;370:203-30.

28. Redmond DE, Jr., Roth RH, Spencer DD, et al. Neural transplantation for neurodegenerative diseases: past, present, and future. Ann N Y Acad Sci. 1993;695:258-66.

29. Olanow CW, Kordower JH, Freeman TB. Fetal nigral transplantation as a therapy for Parkinson's disease. Trends Neurosci. 1996;19(3):102-9.

30. **Freed CR, Greene PE, Breeze RE, et al. Transplantation of embryonic dopamine neurons for severe Parkinson's disease.[see comment]. New England Journal of Medicine. 2001;344(10):710-9.**

31. **Redmond DE, Jr. Cellular replacement therapy for Parkinson's disease--where we are today? Neuroscientist. 2002 Oct;8(5):457-88.**

32. **Wakeman DR. Large animal models are critical for rationally advancing regenerative therapies. Regenerative Medicine. 2006 July 2006;1(4):405-13.**

33. **Martinez-Serrano A, Snyder E. Neural stem cell lines for CNS repair. In: Tuszynski M, Kordower J (eds). CNS Regeneration: Basic Science & Clinical Applications. San Diego: Academic Press, 1998:203-50.**

34. **Snyder E, Wolfe J. CNS cell transplantation: a novel therapy for storage diseases? Current Opinion in Neurology. 1996;9(2):126-36.**

35. **Snyder EY, Yoon C, Flax JD, Macklis JD. Multipotent neural precursors can differentiate toward replacement of neurons undergoing targeted apoptotic degeneration in adult mouse neocortex. Proc Natl Acad Sci U S A. 1997;94(21):11663-8.**

36. **Fisher L. Neural precursor cells: application for the study & repair of the central nervous system. Neurobiol Dis 1997 4:1-22.**

37. **Snyder E, Park K, Liu S, et al. Transplantation of multipotent neural progenitors or "stem-like cells" for CNS gene therapy and repair. Adv Neurol 1997;72:121-32.**

38. **Snyder E. Neural stem-like cells: Developmental lessons with therapeutic potential. The Neuroscientist 1998 4(6):408-25.**

39. **Gage F, Ray J, Fisher L. Isolation, Characterization & Use of Stem Cells from the CNS. Ann Rev Neurosci. 1995;18:159-62.**

40. **Alvarez-Buylla A, Temple S. Stem cells in the developing and adult nervous system. J Neurobiology. 1998;36:105-10.**

41. **McKay R. Stem cells in the central nervous system. Science. 1997;276:66-71.**

42. **Gage F, Christen Y. Research & Perspectives in Neurosciences: Isolation, Characterization, & Utilization of CNS Stem Cells. Heidelberg, Berlin: Springer-Verlag, 1997**

43. **Stemple D, Mahanthappa N. Neural stem cells are blasting off. Neuron. 1997;18:1-4.**

44. **Weiss S, Reynolds B, Vescovi A, Morshead C, Craig C, van der Kooy D. Is there a neural stem cell in the mammalian forebrain. Trends Neurosci. 1996;19:387-93.**

45. **Vescovi A, Reynolds B, Fraser D, Weiss S. bFGF regulates the proliferative fate of unipotent (neuronal) & bipotent (neuronal/astroglial EGF-generated CNS progenitor cells. Neuron. 1993 11:951-66.**

46. **Reynolds B, Tetzlaff A, Weiss S. A multipotent EGF-responsive striatal embryonic progenitor cell produces neurons & astrocytes. J Neurosci. 1992;12:4565-74.**

47. **Reynolds B, Weiss S. Generation of neurons & astrocytes from isolated cells of the adult mammalian central nervous system. Science. 1999;27:1707-10.**

48. **Reynolds B, Weiss S. Clonal & population anayses demonstrate that an EGF-repsonsive mammalian embryonic CNS precursor is a stem cell. Develop Biol. 1996 175:1-13.**

49. **Morshead C, Reynolds B, Craig C, et al. Neural stem cell in the adult mammalian forebrain: a relatively quiescent subpopulation of subependymal cells. Neuron. 1994 13:1071-82.**

50. **Craig C, Tropepe V, Morshead C, Reynolds B, Weiss S, van der Kooy D. In vivo growth factor expansion of endogenous subependymal neural precursor cell populations in the adult mouse brain. J Neurosci. 1996 16(8):2649-58.**

51. **Vescovi A, Snyder E. Establishment & properties of genetically & epigenetically propagated neural stem cell clones: plasticity in vitro & in vivo. Brain Pathology. 1999;9:569-98.**

52. **Weissman IL. Stem cells: Units of development, units of regeneration, and units of evolution. Cell. 2000;100:157-68.**

53. **Fuchs E, Segre JA. Stem cells: A new lease on life. Cell. 2000;100:143-55.**

54. **Flax JD, Aurora S, Yang C, et al. Engraftable human neural stem cells respond to developmental cues, replace neurons, and express foreign genes. Nature Biotechnology. 1998;16(11):1033-9.**

55. **Imitola J, Raddassi K, Park KI, et al. Directed migration of neural stem cells to sites of CNS injury by the stromal cell-derived factor 1alpha/CXC chemokine receptor 4 pathway. Proc Natl Acad Sci U S A. 2004 Dec 28;101(52):18117-22.**

56. **Ourednik V, Ourednik J, Park KI, Snyder EY. Neural stem cells -- a versatile tool for cell replacement and gene therapy in the central nervous system. Clin Genet. 1999 Oct;56(4):267-78.**

57. **Ourednik V, Ourednik J, Park KI, et al. Neural stem cells are uniquely suited for cell replacement and gene therapy in the CNS. Novartis Found Symp. 2000;231:242-62; discussion 62-9, 302-6.**

58. Ourednik J, Ourednik V, Lynch WP, Schachner M, Snyder EY. Neural stem cells display an inherent mechanism for rescuing dysfunctional neurons. Nat Biotechnol. 2002 Nov;20(11):1103-10.

59. Odeberg J, Piao JH, Samuelsson EB, Falci S, Akesson E. Low immunogenicity of in vitro-expanded human neural cells despite high MHC expression. J Neuroimmunol. 2005 Apr;161(1-2):1-11.

60. Park KI, Lachyankar M, Nissim S, Snyder EY. Neural stem cells for CNS repair: state of the art and future directions. Adv Exp Med Biol. 2002;506(Pt B):1291-6.

61. Park KI, Ourednik J, Ourednik V, et al. Global gene and cell replacement strategies via stem cells. Gene Ther. 2002 May;9(10):613-24.

62. Hamilton BA. alpha-Synuclein A53T substitution associated with Parkinson disease also marks the divergence of Old World and New World primates. Genomics. 2004 Apr;83(4):739-42.

63. Roth RH, Elsworth JD. Biochemical pharmacology of midbrain dopamine neurons. In: Bloom FE (ed). Psychopharmacology, Fourth Generation of Progress. New York: Raven, 1995:227-44.

64. Langston JW, Ballard P, Tetrud JW, Irwin I. Chronic Parkinsonism in humans due to a product of meperidine-analog synthesis. Science. 1983 Feb 25;219(4587):979-80.

65. Langston JW, Ballard PA, Jr. Parkinson's disease in a chemist working with 1-methyl-4-phenyl-1,2,5,6-tetrahydropyridine. N Engl J Med. 1983 Aug 4;309(5):310.

66. Jenner P, Marsden CD. 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), an update on its relevance to the cause and treatment of Parkinson's disease. In: Franks AJ, Ironside JW, Mindham RHS, Smith RJ, Spokes EGS, Winlow W (eds). Function and dysfunction of the basal ganglia. Manchester: Manchester University Press, 1990:140-60.

67. Bankiewicz KS, Plunkett RJ, Mefford I, Kopin IJ, Oldfield EH. Behavioral recovery from MPTP-induced parkinsonism in monkeys after intracerebral tissue implants is not related to CSF concentrations of dopamine metabolites. Progress In Brain Research. 1990;82:561-71.

68. Guttman M, Fibiger HC, Jakubovic A, Calne DB. Intracarotid 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine administration: Biochemical and behavioral observations in a primate model of hemiparkinsonism. J of Neurochemistry. 1990;54:1329-34.

69. Emborg ME. Nonhuman primate models of Parkinson's disease. ILAR journal / National Research Council, Institute of Laboratory Animal Resources. 2007;48(4):339-55.

70. Marek K, Innis R, van Dyck C, et al. [123I]beta-CIT SPECT imaging assessment of the rate of Parkinson's disease progression. Neurology. 2001 Dec 11;57(11):2089-94.

71. Seibyl JP, Marek KL, Quinlan D, et al. Decreased single-photon emission computed tomographic [123I]beta-CIT striatal uptake correlates with symptom severity in Parkinson's disease. Ann Neurol. 1995 Oct;38(4):589-98.

72. Martinez-Serrano A, Bjorklund A. Immortalized neural progenitor cells for CNS gene transfer & repair. Trends Neurosci. 1997;20:530-8.

73. Snyder E, Yoon C, Flax J, Macklis J. Multipotent neural progenitors can differentiate toward replacement of neurons undergoing targeted apoptotic degeneration in adult mouse neocortex. Proc Natl Acad Sci USA. 1997 94:11645-50.

74. Snyder EY, Macklis JD. Multipotent neural progenitor or stem-like cells may be uniquely suited for therapy for some neurodegenerative conditions. Clinical Neuroscience. 1995;3(5):310-6.

75. Park K, Liu S, Flax J, Nissim S, Stieg P, Snyder E. Transplantation of neural progenitor & stem-like cells: developmental insights may suggest new therapies for spinal cord and other CNS dysfunction. Journal of Neurotrauma. 1999;16/8::675-87.

76. Zawada W, Zastrow D, Clarkson E, Adams F, Bell K, Freed C. Growth factors improve immediate survival of embryonic dopamine neurons after transplantation into rats. Brain Research. 1998;786:96-103.

77. Bjorklund A, Stenevi U, Schmidt RH, Dunnett SB, Gage FH. Intracerebral grafting of neuronal cell suspensions. II. Survival and growth of nigral cell suspensions implanted in different brain sites. Acta Physiol Scand Suppl. 1983;522:9-18.

78. Boonman Z, Isacson O. Apoptosis in neuronal development and transplantation: role of caspases and trophic factors. Exp Neurol. 1999;156(1):1-15.

79. Brundin P, Barbin G, Isacson O, et al. Survival of intracerebrally grafted rat dopamine neurons previously cultured in vitro. Neurosci Lett. 1985;61(1-2):79-84.

80. Brundin P, Isacson O, Bjorklund A. Monitoring of cell viability in suspensions of embryonic CNS tissue and its use as a criterion for intracerebral graft survival. Brain Res. 1985;331(2):251-9.

81. Rosenstein JM. Why do neural transplants survive? An examination of some metabolic and pathophysiological considerations in neural transplantation. Exp Neurol. 1995;133(1):1-6.

82. **Choi-Lundberg D, Bohn M. Ontogeny and distribution of glial cell line-derived neurotrophic factor (GDNF) mRNA in rat. Devel Brain Research. 1995;85:80-8.**

83. **Mehta V, Hong M, Spears J, Mendez I. Enhancement of graft survival and sensorimotor behavioral recovery in rats undergoing transplantation with dopaminergic cells exposed to glial cell line-derived neurotrophic factor. J Neurosurg. 1998;88(6):1088-95.**

84. **Nosrat CA, Tomac A, Hoffer BJ, Olson L. Cellular and developmental patterns of expression of Ret and glial cell line-derived neurotrophic factor receptor alpha mRNAs. Exp Brain Res. 1997;115(3):410-22.**

85. **Schaar D, Sieber B, Dreyfus C, Black I. Regional and cell-specific expression of GDNF in rat brain. Experimental Neurology. 1993;124:368-71.**

86. **Stromberg I, Bjorklund L, Johansson M, et al. Glial cell line-derived neurotrophic factor is expressed in the developing but not adult striatum and stimulates developing dopamine neurons in vivo. Exp Neurol. 1993;124(2):401-12.**

87. **Widenfalk J, Parvinen M, Lindqvist E, Olson L. Neurturin, RET, GFRalpha-1 and GFRalpha-2, but not GFRalpha-3, mRNA are expressed in mice gonads. Cell Tissue Res. 2000;299(3):409-15.**

88. **Pascual A, Hildalgo-Figueroa M, Piruat JI, Pintado CO, Gomez-Diaz R, Lopez-Barneo L. Absolute requirement of GDNF for adult catecholaminergic neuron survival. Nature neuroscience. 2008;11:755-61.**

89. **Akerud P, Canals JM, Snyder EY, Arenas E. Neuroprotection through delivery of glial cell line-derived neurotrophic factor by neural stem cells in a mouse model of Parkinson's disease. J Neurosci. 2001 Oct 15;21(20):8108-18.**

90. **Lin L, Doherty D, Lile J, Bektesh S, Collins F. GDNF: a glial cell line-derived neurotrophic factor for midbrain dopaminergic neurons. Science. 1993 260:1130-2.**

91. **Kordower JH, Emborg ME, Bloch J, et al. Neurodegeneration prevented by lentiviral vector delivery of GDNF in primate models of Parkinson's disease. Science. 2000;290(5492):767-73.**

92. **Gash DM, Zhang Z, Ovadia A, et al. Functional recovery in parkinsonian monkeys treated with GDNF. Nature. 1996 Mar 21;380(6571):252-5.**

93. **Choi-Lundberg DL, Lin Q, Chang YN, et al. Dopaminergic neurons protected from degeneration by GDNF gene therapy. Science. 1997 Feb 7;275(5301):838-41.**

94. **Bjorklund A. Cell replacement strategies for neurodegenerative disorders. Novartis Found Symp. 2000;231:7-15.**

95. **Liu WG, Wang XJ, Lu GQ, Li B, Wang G, Chen SD. Dopaminergic regeneration by neurturin overexpressing c17.2 neural stem cells in a rat model of Parkinson's disease. Mol Neurodegener. 2007 Oct 1;2(1):19.**

96. **Elsworth JD, Redmond DE, Jr., Leranth C, et al. AAV2-mediated gene transfer of GDNF to the striatum of MPTP monkeys enhances the survival and outgrowth of co-implanted fetal dopamine neurons. Experimental neurology. 2008 May;211(1):252-8.**

97. **Bloch J, Redmond DE, Jr, Leranth C, et al. GDNF released from encapsulated cells increases fetal dopaminergic cell survival in a primate Model of Parkinson's disease. European Society for Stereotactic and Functional Neurosurgery. Toulouse, 2002.**

98. **Redmond DE, Jr.,, Elsworth JD, Leranth C, et al. Co-implantation of AAV2-GDNF viral vector with fetal ventral mesencephalon markedly increases long-term survival of grafted dopamine neurons in the MPTP monkey striatum. Soc Neurosci Abstr. 2006;470.13.**

99. **Elsworth JD, Redmond DE, Jr.,, Leranth C, et al. AAV2-mediated gene transfer of GDNF to the striatum of MPTP monkeys enhances the survival and outgrowth of co-implanted fetal dopamine neurons. submitted. 2007.**

100. **Brederlau A, Correia AS, Anisimov SV, et al. Transplantation of human embryonic stem cell-derived cells to a rat model of Parkinson's disease: effect of in vitro differentiation on graft survival and teratoma formation. Stem cells (Dayton, Ohio). 2006 Jun;24(6):1433-40.**

101. **Paratcha G, Ibanez CF, Ledda F. GDNF is a chemoattractant factor for neuronal precursor cells in the rostral migratory stream. Molecular and cellular neurosciences. 2006 Mar;31(3):505-14.**

102. **Shi H, Patschan D, Dietz GP, Bahr M, Plotkin M, Goligorsky MS. Glial cell line-derived neurotrophic growth factor increases motility and survival of cultured mesenchymal stem cells and ameliorates acute kidney injury. American journal of physiology. 2008 Jan;294(1):F229-35.**

103. **Patel NK, Bunnage M, Plaha P, Svendsen CN, Heywood P, Gill SS. Intraputamenal infusion of glial cell line-derived neurotrophic factor in PD: a two-year outcome study. Ann Neurol. 2005 Feb;57(2):298-302.**

CHAPTER 1B:

INTRODUCTION-

LARGE ANIMAL MODELS ARE CRITICAL FOR RATIONALLY ADVANCING REGENERATIVE THERAPIES

1B.1 Introduction

Enthusiasm for therapies based on the transplantation of exogenous cells or the transfer of genes by viral vectors has burgeoned over the past thirty years, accompanied by a predictable exhortation to launch clinical trials as soon as possible. The majority of data regarding safety, efficacy, and mechanisms of these therapies have been derived from studies in rodents alone. While such "small animal" systems offer invaluable insights into fundamental biological questions, it is often misleading and perilous to unquestionably equate the higher order motor, sensory, and cognitive processes that characterize human disease with that gleaned from a mouse or rat. Indeed, the literature is littered with clinical trials that failed and, in some cases, led to unforeseen adverse outcomes because the field had leap-frogged over the requisite large animal model. Large animals often provide an essential bridge between insights into fundamental biology and pathophysiology gleaned from simple systems and the realities of treating a human disease. This is often especially true for neurological disorders where not only do differences in size and scale pertain, but also in neuroanatomic connections and organization, cognitive capacities, signaling pathways, genetic redundancy, or the etiology of the disease.

While the gene therapy field has increased their use of non-human primates prior to the application of viral vectors in clinical trials, the cellular therapy field – represented most conspicuously of late by the stem cell field – has only recently begun to properly address this

need. Monkeys and the mini-pig may prove to be excellent preclinical models because of their similar comparative anatomy, pharmacokinetics, and physiologic and metabolic interactions. These models have proven to be extremely useful for studying endocrinological diseases such as type-1 diabetes and neurological disorders including Parkinson's disease (PD), spinal cord injury (SCI), and multiple sclerosis (MS). Prudence would argue that clinical trials for diseases in which differences between rodent and human transcend size and scale should require clear and definitive proof-of-concept in at least one relevant large animal model, in order to safeguard patients. This need, of course is counterbalanced by considerations of the substantial cost, time, and ethical circumspection that typically accompanies such research. In this editorial, we will attempt to help researchers reason through the potential need and advisability of using a large animal model for their particular biological question.

There are numerous differences between rodents and humans that make the unqualified translation of rodent data inadequate. The most relevant differences start at the genomic level and are manifested as differences in pharmacological, biochemical, developmental, behavioral, and functional responses to perturbations and interventions (1). Anatomical and cytoarchitectural differences are particularly profound in the brain, where innervation of specific regions is critical to addressing a neurological deficit (Figure 1A-1). In PD for example, it has been estimated that for each volume of tissue innervated by a rat dopaminergic (DA) neuron, a monkey neuron must innervate 20 times that volume, while humans would need a staggering volume of 200 units to mimic similar reinnervation of nigra-striatal projections (2).

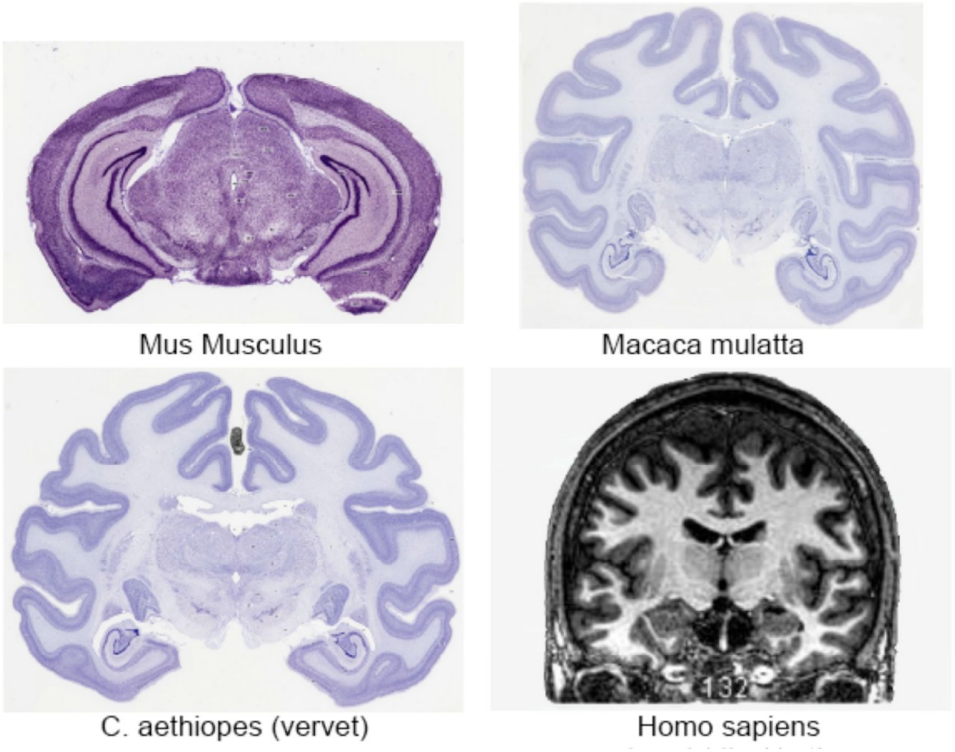


Figure 1B-1. Coronal images of Nissl stained sections from Mus musculus, Chlorocebus aethiops, Macaca mulatta, and MRI of human cortex (135).

In addition, differences in monoamine biochemistry (3-7) and developmental life span make extrapolating rodent data to human therapies risky and insufficient. Behavioral differences between rodents and humans are also of great significance (8-10). It is likely that only a large animal model can adequately mirror the complexities of studying and restoring bipedal gait, balance, tremor, hand preference, fine motor coordination, spontaneous blink rate and cognitive deficits (11-16).

1B.2 Lesson's from Parkinson's Disease

PD is characterized by degeneration of dopaminergic (DA) neurons in the substantia nigra pars compacta (SNpc) and subsequent loss of striatal DA release. Cell based strategies

for replacement of damaged DA neurons have been a promising strategy since the early 1970's, when Olsen, Stenevi, Bjorkland, and colleagues performed their initial transplantation experiments in rat brains (59-63). Several subsequent studies in rats provided extended evidence for fetal cell transplants repairing DA deficiency (64, 65), however, despite numerous attempts by many groups, these results could never be recapitulated in primates (1). Coupled with mounting ethical considerations, researchers turned to adrenal allografts as a new source of tissue, but these also failed, and subsequent clinical trials were abandoned (66-68). New hope came again after 1985 when human fetal neural cells were derived and successfully implanted into the rat and monkey brain (69-76), however ethical opponents argued that the large amount of fetal tissue required made these procedures for routine regenerative therapy unfeasible and, therefore, unfundable.

Based on the varying methods published in the mid 1970's, several groups began preliminary efficacy and safety clinical studies of fetal ventral mesencephalic grafts into PD patients. Initial studies in Sweden (77), England (78), and Mexico (79) produced variable results, elevating the ethical debate and political controversy, eventually leading to a funding moratorium in the United States (80, 81). Subsequent studies seemed promising but the overall consensus was that the results were inconclusive due to variability within small studies, limited short-term functional improvements, and overall difficulty in comparing independent procedures (1). To investigate this problem and improve the ability to compare studies, a panel of experts published a recommended protocol for future investigations termed, Core Assessment Program for Intracerebral Transplantation (CAPIT) (82).

In the same year, the first randomized controlled clinical trial, headed by Curt Freed and Stanley Fahn, was initiated studying 40 PD patients over seven years, half of which underwent bilateral stereotactic transplantation of human embryonic mesencephalic tissue into the putamen (83). Although initial results appeared promising, it was determined that no

benefit in primary outcome measure had been attained after 12 months post-op. However, a small subset of patients < 60 years old did show small double blind improvements on a validated PD rating scale. More importantly, predictions made from animal models failed to predict the relatively high occurrence of dystonia and dyskinesia associated with long-term survival (84). These gross abnormalities occurred in 5 of 33 patients that lived 3 years post-transplant, of whom all five were less than 60 years of age and had displayed improvements in PD score after 12 months post surgery.

The human trials appeared to have a solid rationale based on a decade of rodent studies. Hundreds of investigators had reported survival and functional recovery from fetal ventral mesencephalic derived neural tissue transplanted into the striatum of rats (85). The grafts were shown to express tyrosine hydroxylase (TH), increase DA concentrations, project long outgrowths, form synaptic connections, and respond to afferent stimulation (86-90). However, the rat models of PD overestimated the likely success of fetal precursor transplants and failed to anticipate the dyskinetic side effects in human trials (84). And, although large animal models were tested, it is possible that they were not followed for a long enough time, simply because the vast difference between rodents and primates (of which human is an example) was not appreciated. Furthermore, while the Freed et al. study made extremely valuable contributions to our understanding of PD, small design flaws such as method of tissue preparation, limited target area, and lack of immunosuppression may have inherently impeded the overall success of the study (1). Many of these details may have been worked out in large animals before reaching the clinic, potentially predicting adverse effects. Unfortunately, cell replacement therapy received negative media coverage (91-94), creating an impression of defeat in the public's mind and even creating dissention among PD investigators (84, 92, 95-102). Much of this may have been avoided had a higher priority been placed on more extensive primate studies with larger numbers and longer follow-up.

1B.3 Going Forward in PD- A Case-in-Point for Choosing the Correct Model

Having detailed above the pitfalls into which the PD field slipped by relying upon non-representative data for its first cell-based clinical trial, how might it recover its momentum – and can this serve as a lesson for future trials for other diseases?

As noted above, most of the early transplantation studies in PD were performed in rodents that had received a unilateral injection of the dopaminergic toxin 6-hydroxydopamine (6-OHDA) into the medial forebrain bundle or nearby (103, 104). Although transplantation appeared to promote recovery from the resultant PD-like symptoms, these behaviors actually bore little resemblance to human PD. If therapies were to be tested solely on such a rodent model, the results would likely have poor predictive value for actual patients. On the other hand, through serendipity in the 60's and 70's, it was discovered that the complex I inhibitor, 1-methyl-4-phenyl-1, 2,3,6-tetrahydropyridine (MPTP), created pathology quite similar to human idiopathic PD in both humans (105-107) and monkeys (70, 108-112), especially the vervet (113, 114). MPTP depletes SN TH-ir and striatal DA to levels similar to PD, as well as producing characteristic Parkinsonian symptomatology including akinesia, rigidity, postural alterations, and tremor (9, 10). Overexpressing alpha-synuclein in conjunction with MPTP may create Lewy bodies (115, 116). Hence, systemically administered MPTP in African green monkeys has become acknowledged as the most authentic model for actual human idiopathic PD and the model in which the safety and efficacy of viral vector- and cell-based therapies in humans can most reliably be judged (expert reviews- 1, 8-10, 117). Indeed, the potential use of lentiviral and AAV-based vectors for delivering neurotrophic factors in PD patients has been and is currently being tested in monkey first (118-120). Such studies, in turn, did set the stage for some phase-1 clinical trials (121-131). It is disheartening that very few investigators to date in the cell therapy field (including the stem cell field) have taken advantage of this model

for work in PD despite widespread recognition of its power. Most likely this reticence is due to the huge expense entailed in performing such complex studies with a sufficiently large sample size and with suitably objective and predictive readouts over a long enough period of observation. Nevertheless, that expense is vastly smaller than the cost to the health care system, to science, and to patient well being when an ineffective or injurious clinical intervention becomes misguidedly launched. Funding agencies and scientists must begin to change their thinking and view adequately powered large animal studies for some diseases not as a luxury, but as a necessity.

Another source of hesitancy in the use of large animals, particularly monkeys, is not solely financial but also ethical. There is no question that experimentation on "higher order" animals provoke, more than any other type of scientific study, circumspection regarding the appropriateness of animal experimentation. Although we have outlined above its scientific justification, there is no question that investigators must be acutely sensitive (as they should be for all animal work) to using the minimal number of monkeys required to statistically overcome type 1 and type 2 error rates (1), to treating the animals humanely, and to using animals only in the most judicious manner, for only exquisitely well-conceived and rigorously designed experiments.

Another level of ethical consideration that has recently emerged as a consequence of the burgeoning human stem cell field is whether integrating human cells into the brains of non-human primates (particularly prenatally) somehow "humanizes" them. In other words, might a human-primate chimeric brain develop consciousness and hence merit consideration beyond that of just an experimental laboratory animal (131-134). While an interesting metaphysical consideration, chimerism in our hands, even under the most optimal transplantation conditions, yields only a relatively small human cell contribution to an

overwhelmingly primate CNS structure. Furthermore, we have never observed anything but typical primate behaviors, emotions, and skills in our animals.

1B.4 Summary and Conclusions

Appropriately assessing the safety and efficacy of many future interventions in regenerative medicine will require large animal pre-clinical testing in order to avoid the failures of previous studies in patients. Such recognition requires scientists, investors, funding agencies, and the public to recalibrate their thinking. Simpler rodent models, while useful for unraveling certain biological conundrums often fall short in their predictive value for human disease or their appropriateness for devising effective delivery methods. A greater number of multi center collaborations may be required to insure adequate funding and expertise for large animal studies. Such an approach requires a less egocentric approach to scientific accomplishment. The ultimate cost of *not* including the rationale use of large animal models in preclinical studies – the price of unsuccessful trials, injury to patients, and failure to bring potentially groundbreaking therapies to the bedside – far outweighs the short-term expense of the studies themselves.

1B.5 Acknowledgements

Chapter 1B, is a reprint in part of material as it appears in *Regenerative Med*. (2006) 1(4), 405–413 with co-authors Andrew C. Crain and Evan Y Snyder. The dissertation author was the primary investigator and author of this paper. DRW wrote and compiled figure, ACC helped print set figure, and EYS supervised research. Together with his coauthors, he thanks DE Redmond and JH Kordower for helpful suggestions. DRW is supported by the NIH/NIGMS funded UCSD Genetics Training Program (T32 GM08666) and is a recipient of

an American Society for Neural Therapy and Repair Travel Fellowship; DRW and AMC are supported by an NIH Stem Cell Center Grant (1 P20 GM075059-01) as well as a grant from the American Parkinson's Disease Association (APDA).

1B.7 References

1. Redmond DE Jr: Cellular replacement therapy for Parkinson's disease--where we are today? Neuroscienist 8(5): 457-88 (2002).

2. Lindvall O, Rehncrona S, Brundin P et al.: Human fetal dopamine neurons grafted into the striatum in two patients with severe Parkinson's disease: a detailed account of methodology and a 6-month follow-up. Arch Neurol 46: 615–31 (1989).

3. Bacopoulos NG, Maas JW, Hattox SE, Roth RH: Regional distribution of dopamine metabolites in human and primate brain. Comm Psychopharmacol 2:281–6. (1978).

4. Felten DL, Sladek JRJ: Monoamine distribution in primate brain: V. monoamine nuclei: anatomy, pathways and local organization. Brain Res Bull 10:171–284 (1983).

5. Waddington JL, O'Boyle KM: Drugs acting on brain dopamine receptors: a conceptual reevaluation five years after the first selective D1 antagonist. Pharmacol Ther 43:1–52 (1989).

6. Haycock JW: Four forms of tyrosine hydroxylase are present in human adrenal medulla. J Neurochem 56:2139–42 (1991).

7. Nagatsu T: Genes for human catecholamine-synthesizing enzymes. Neurosci Res 12:315–45 (1991).

8. Steece-Collier K, Maries E, Kordower JH.: Etiology of Parkinson's disease: Genetics and environment revisited. Proc Natl Acad Sci U S A. Oct 29;99(22):13972-4. Review (2002).

9. Collier TJ, Steece-Collier K, Kordower JH.: Primate models of Parkinson's disease. Exp Neurol. Oct;183(2):258-62 Review (2003).

10. Emborg ME.: Evaluation of animal models of Parkinson's disease for neuroprotective strategies: J Neurosci Methods. Oct 30;139(2):121-43. Review (2004).

11. Taylor JR, Roth RH, Sladek JR Jr, Redmond DE Jr.: Cognitive and motor deficits in the performance of an object retrieval task with a barrier-detour in

monkeys (Cercopithecus aethiops sabaeus) treated with MPTP: long-term performance and effect of transparency of the barrier. Behav Neurosci Aug;104(4):564-76 (1990).

12. Taylor JR, Elsworth JD, Roth RH, Sladek JR Jr, Redmond DE Jr.: Cognitive and motor deficits in the acquisition of an object retrieval/detour task in MPTP-treated monkeys. Brain Jun;113 (Pt 3):617-37 (1990).

13. Taylor JR, Elsworth JD, Roth RH, Collier TJ, Sladek JR, Redmond DE Jr.: Improvements in MPTP-induced object retrieval deficits and behavioral deficits after fetal nigral grafting in monkeys. In: Richards SJ, Dunnett SB, editors. Progress in brain research. Amsterdam: Elsevier. p 543–59 (1990).

14. Elsworth JD, Lawrence MS, Roth RH et al.: D1 and D2 dopamine receptors independently regulate spontaneous blink rate in the vervet monkey. J Pharmacol Exp Ther 259:595–600 (1991).

15. Taylor JR, Elsworth JD, Roth RH, Sladek JR Jr, Collier TJ, Redmond DE Jr.: Grafting of fetal substantia nigra to striatum reverses behavioral deficits induced by MPTP in primates: a comparison with other types of grafts as controls. Exp Brain Res 85(2):335–48 (1991).

16. Kordower JH, Liu YT, Winn S, Emerich DF.: Encapsulated PC12 cell transplants into hemiparkinsonian monkeys: a behavioral, neuroanatomical, and neurochemical analysis. Cell Transplant Mar-Apr;4(2):155-71 (1995).

17. Vodicka P, Smetana K Jr, Dvorankova B et al.: The miniature pig as an animal model in biomedical research. Ann N Y Acad Sci. May;1049:161-71 (2005).

18. Bollen P, Ellegaard L: Developments in breeding Go¨ttingen minipigs. In: Tumbleson ME, Schook LB, eds. Advances in Swine in Biomedical Research. New York: Plenum Press. p 59-66 (1996).

19. Bollen P, Ellegaard L: The Go¨ttingen minipig in pharmacology and toxicology. Pharmacol Toxicol 80:3-4 (1997).

20. Brown D, Terris J: Swine in physiological and pathophysiological research. In: Tumbleson ME, Schook LB, eds. Advances in Swine in Biomedical Research. New York: Plenum Press. p 5-6 (1996).

21. Murakami T, Hitomi S, Ohtsuka A, Taguchi T, Fujita T: Pancreatic insulo-acinar portal systems in humans, rats, and some other mammals: Scanning electron microscopy of vascular casts. Microsc Res Tech 37:478-488 (1997).

22. Larsen MO, Rolin B.: Use of the Gottingen minipig as a model of diabetes, with special focus on type 1 diabetes research. ILAR J. ;45(3):303-13. Review (2004).

23. Markussen J, Havelund S, Kurtzhals P et al.: Soluble, fatty acid acylated insulins bind to albumin and show protracted action in pigs. Diabetologia 39:281-288 (1996).

24. Pieber TR, Plank J, Go¨rzer E et al.: Duration of action, pharmacodynamic profile and between-subject variability of insulin determine subjects with type 1 diabetes. Diabetologia 45:A257 (2002).

25. Miller ER, Ullrey DE.: The pig as a model for human nutrition. Annu Rev Nutr 7:361-82. Review (1987).

26. Broughton DL, Taylor R: Review: Deterioration of glucose tolerance with age: The role of insulin resistance. Age Ageing 20:221-225 (1991).

27. Rosenthal M, Doberne L, Greenfield M, Widstrom A, Reaven GM: Effect of age on glucose tolerance, insulin secretion, and in vivo insulin action. J Am Geriatr Soc 30:562-567 (1982).

28. Marshall M, Sprandel U, Zollner N: Streptozotocin diabetes in a miniature pig. Res Exp Med (Berl) 165:61-65 (1975).

29. Marshall M: Induction of chronic diabetes by streptozotocin in the miniature pig. Res Exp Med 175:187-196 (1979).

30. Gabel H, Bitter-Suermann H, Henriksson C, Save-Soderbergh J, Lundholm K, Brynger H:Streptozotocin diabetes in juvenile pigs. Evaluation of an experimental model. Horm Metab Res 17:275-280 (1985).

31. Wilson JD, Dhall DP, Simeonovic CJ, Lafferty KJ: Induction and management of diabetes mellitus in the pig. Aust J Exp Biol Med Sci 64:489-500 (1986).

32. Kjems LL, Kirby BM, Welsh EM et al.: Decrease in beta-cell mass leads to impaired pulsatile insulin secretion, reduced postprandial hepatic insulin clearance, and relative hyperglucagonemia in the minipig Diabetes 50:2001-2012 (2001).

33. Mellert J, Hopt UT, Hering BJ, Bretzel RG, Federlin K: Influence of islet mass and purity on reversibility of diabetes in pancreatectomized pigs. Transplant Proc 23:1687-1689 (1991).

34. Morsiani E, Fogli L, Lanza G, Lebow LT, Demetriou AA, Rozga J: Long-term insulin independence following repeated islet transplantation in totally pancreatectomized diabetic pigs. Cell Transplant 11:55-66 (2001).

35. Kin T, Iwata H, Aomatsu Y,et al.: Xenotransplantation of pig islets in diabetic dogs with use of a microcapsule composed of agarose and polystyrene sulfonic acid mixed gel. Pancreas 25:94-100 (2002).

36. Buhler L, Deng S, O'Neil J et al.: Adult porcine islet transplantation in baboons treated with conventional immunosuppression or a nonmyeloablative regimen and CD154 blockade. Xenotransplantation 9: 3-13. (2002).

37. Cantarovich D, Blancho G, Potiron N, et al.: Rapid failure of pig islet transplantation in non human primates. Xenotransplantation 9:25-35 (2002).

38. Groth CG, Korsgren O, Tibell A, et al.: Transplantation of porcine fetal pancreas to diabetic patients. Lancet 344:1402-1404 (1994).

39. Elliott RB, Escobar L, Garkavenko O et al. No evidence of infection with porcine endogenous retrovirus in recipients of encapsulated porcine islet xenografts. Cell Transplant 9:895-901 (2000).

40. Groth CG, Tibell A, Wennberg L et al.: Clinical aspects and perspectives in islet xenotransplantation. J Hepatobil Pancreat Surg 7:364-369 (2000).

41. Shapiro AMJ, Lakey JRT, Ryan EA, et al.: Islet transplantation in seven patients with type 1 diabetes mellitus using a glucocorticoid-free immunosuppressive regimen. N Engl J Med 343:230-238 (2000).

42. Sachs D, Leight HG, Cone J, Schwarz S, Stuart L, Rosenberg S: Transplantation in miniature swine. I. Fixation of the major histocompatability complex. Transplantation 22:559-567 (1976).

43. Sachs DH: MHC-homozygous miniature swine. In Swindel, M.M, moody, D.C., Philips, L.D. (Eds.), Swine as Models in Biomedical Research, Iowa State University Press, Ames, pp 3-15 (1992).

44. Mezrich JD, Haller GW, Arn JS, Houser SL, Madsen JC, Sachs DH. Histocompatible miniature swine: an inbred large-animal model. Transplantation 2003 Mar 27;75(6):904-7.

45. Allan JS, Wain JC, Schwarze ML, Houser SL, Benjamin LC, Madsen JC, Sachs DH.: Modeling chronic lung allograft rejection in miniature swine. Transplantation Feb 15;73(3):447-53 (2002).

46. Gollackner B, Dor FJ, Knosalla C et al.: Spleen transplantation in miniature swine: surgical technique and results in major histocompatibility complex-matched donor and recipient pairs. Transplantation. Jun 15;75(11):1799-806 (2003).

47. Kenmochi T, Mullen Y, Miyamoto M, Stein E: Swine as an allotransplantation model. Vet Immunol Immunopathol. Oct;43(1-3):177-83. Review (1994).

48. Emery DW, Sachs DH, LeGuern C.: Culture and characterization of hematopoietic progenitor cells from miniature swine. Exp Hematol Jul;24(8):927-35 (1996).

49. Tumbleson ME.: Brain weight, as a function of age, in miniature swine. Growth Mar;37(1):13-7 (1973).

50. Holloway RL, Heilbroner P: Corpus callosum in sexually dimorphic and nondimorphic primates. Am J Phys Anthropol Mar;87(3):349-57 (1992).

51. Singer BA, Tresser NJ, Frank JA, McFarland HF, Biddison WE: Induction of experimental allergic encephalomyelitis in the NIH minipig. J Ieuroimmunol Jun 1;105(1):7-19 (2000).

52. Matsuyama N, Hadano S, Onoe K et al.: Identification and characterization of the miniature pig Huntington's disease gene homolog: evidence for conservation and polymorphism in the CAG triplet repeat. Genomics Oct 1;69(1):72-85 (2000).

53. Mikkelsen M, Moller A, Jensen LH, Pedersen A, Harajehi JB, Pakkenberg H: MPTP-induced Parkinsonism in minipigs: A behavioral, biochemical, and histological study. Neurotoxicol Teratol Mar-Apr;21(2):169-75 (1999).

54. Fink JS, Schumacher JM, Ellias SL et al.: Porcine xenografts in Parkinson's disease and Huntington's disease patients: preliminary results. Cell Transplant Mar-Apr;9(2):273-8 (2000).

55. Schumacher JM, Ellias SA, Palmer EP et al.: Transplantation of embryonic porcine mesencephalic tissue in patients with PD. Neurology Mar 14;54(5):1042-50 (2000).

56. Valdes-Gonzalez RA, Dorantes LM, Garibay GN, et al.: Xenotransplantation of porcine neonatal islets of Langerhans and Sertoli cells: a 4-year study. Eur J Endocrinol Sep;153(3):419-27 (2005).

57. Savitz SI, Dinsmore J, Wu J, Henderson GV, Stieg P, Caplan LR: Neurotransplantation of fetal porcine cells in patients with basal ganglia infarcts: a preliminary safety and feasibility study. Cerebrovasc Dis 20(2):101-7 (2005).

58. Iwanami A, Kaneko S, Nakamura M et al.: Transplantation of human neural stem cells for spinal cord injury in primates. J Neurosci Res Apr 15;80(2):182-90 (2005).

59. Olson L, Seiger A: Brain tissue transplanted to the anterior chamber of the eye: 1. Fluorescence histochemistry of immature catecholamine and 5-hydroxytryptamine neurons reinnervating the rat iris. Z Zellforsch Mikrosh Anat 135:175–94 (1972).

60. Olson L, Seiger A: Locus coeruleus: fiber growth regulation in oculo. Med Biol 54:142–5 1976.

61. Bjorklund A, Stenevi U, Svendgaard N: Growth of transplanted monoaminergic neurones into the adult hippocampus along the perforant path. Nature 262(5571):787–90 (1976).

62. Stenevi U, Bjorklund A, Svendgaard N: Transplantation of central and peripheral monoamine neurons to the adult rat brain: techniques and conditions for survival. Brain Res 114(1):1–20 (1976).

63. Hoffer B, Seiger A, Freedman R, Olson L, Taylor D: Electrophysiology and cytology of hippocampal formation transplants in anterior chamber of the eye: II. Cholinergic mechanisms. Brain Res 119:107–32 (1977).

64. Bjorklund A, Stenevi U: Reconstruction of the nigrostriatal dopamine pathway by intracerebral nigral transplants. Brain Res 177(3):555–60 (1979).

65. Perlow MJ, Freed WJ, Hoffer BJ, Seiger A, Olson L, Wyatt RJ: Brain grafts reduce motor abnormalities produced by destruction of nigrostriatal dopamine system. Science 204:643–53 (1979).

66. Backlund EO, Granberg B, Hamberger E et al.: Transplantation of adrenal medullary tissue to striatum in parkinsonism: first clinical trials. J Neurosurg 62:169–73 (1985).

67. Rehncrona S: A critical review of the current status and possible developments in brain transplantation. Adv Tech Stand Neurosurg 23:3–46 (1997).

68. Hallett M, Litvan I.: Scientific position paper of the Movement Disorder Society evaluation of surgery for Parkinson's disease. Task Force on Surgery for Parkinson's Disease of the American Academy of Neurology Therapeutic and Technology Assessment Committee. Mov Disord 15(3):436–8 (2000).

69. Redmond DE Jr, Roth RH, Sladek JR: MPTP produces classic parkinsonian syndrome in African green monkeys. Neurosci Abstr 11(1):166 (1985).

70. Redmond DE Jr, Sladek JR Jr, Roth RH, et al.: Fetal neuronal grafts in monkeys given methylphenyltetrahydropyridine. Lancet 1(8490):1125–7 (1986).

71. Redmond DE Jr, Sladek JR Jr, Roth RH et al.: Transplants of primate neurons [letter]. Lancet 2(8514):1046 (1986).

72. Redmond DE Jr, Naftolin F, Collier TJet al.: Cryopreservation, culture, and transplantation of human fetal mesencephalic tissue into monkeys. Science 242(4879):768–71 (1988).

73. Bakay RAE, Barrow DL, Fiandaca MS, Iuvone PM, Schiff A, Collins DC: Biochemical and behavioral correction of MPTP Parkinson-like syndrome by fetal cell transplantation. Ann N Y Acad Sci 495:623–40 (1987).

74. Sladek JR Jr, Redmond DE Jr, Collier TJ et al.: Transplantation of fetal dopamine neurons in primate brain reverses MPTP induced parkinsonism. Prog Brain Res 71:309–23 (1987).

75. Sladek JR Jr, Redmond DE Jr, Collier TJ et al.: Fetal dopamine neural grafts: extended reversal of methylphenyltetrahydropyridine-induced parkinsonism in monkeys. Prog Brain Res 78:497–506 (1988).

76. Brundin P, Strecker RE, Widner H et al.: Human fetal dopamine neurons grafted in a rat model of Parkinson's disease: immunological aspects, spontaneous and drug-induced behaviour, and dopamine release. Exp Brain Res 70:192–208 (1988).

77. Lindvall O, Rehncrona S, Gustavii B et al.: Fetal dopamine-rich mesencephalic grafts in Parkinson's disease. Lancet 2:1483–4 (1988).

78. Hitchcock ER, Kenny BG, Henderson BT, Clough CG, Hughes RC, Detta A: A series of experimental surgery for advanced Parkinson's disease by fetal mesencephalic transplantation. Acta Neurochirurgica Suppl 52:54–7 (1991).

79. Madrazo I, León V, Torres C et al.: Transplantation of fetal substantia nigra and adrenal medulla to the caudate nucleus in two patients with Parkinson's disease. N Engl J Med 318:51 (1988).

80. Mahowald MB, Areen J, Hoffer BJ et al.: Transplantation of neural tissue from fetuses. Science 235:1307–8 (1987).

81. Hoffer BJ, Olson L: Ethicalissues in brain-cell transplantation. Trends Neurosci 14:384–8 (1991).

82. Langston JW, Widner H, Goetz CG et al.: Core Assessment Program for Intracerebral Transplantations (CAPIT). Mov Disord 7:2–13 (1992).

83. Freed CR, Greene PE, Breeze RE et al.: Transplantation of embryonic dopamine neurons for severe Parkinson's disease. N Engl J Med 344(10):710–9 (2001).

84. Hagell P, Piccini P, Bjorklund A et al.: Dyskinesias following neural transplantation in Parkinson's disease. Nat Neurosci 5(7):627–8 (2002).

85. Björklund A, Dunnet SB, Stenevi U, Lewis ME, Iverson SD: Reinnervation of the denervated striatum by substantia nigra transplants. Functional consequences as revealed by pharmacological and sensorimotor testing. Brain Res 199:307–33 (1980).

86. Kordower J, Freeman T, Snow B et al.: Neuropathological evidence of graft survival and striatal reinnervation after the transplantation of fetal mesencephalic tissue in a patient with Parkinson's disease. N Engl J Med 332:1118–24 (1995).

87. **Freund TF, Bolam JP, Björklund A et al.: Efferent synaptic connections of grafted dopaminergic neurons reinnervating the host striatum: a tyrosine hydroxlase immuncytochemical study. J Neurosci 5:603–16 (1985).**

88. **Fisher LJ, Young SJ, Tepper JM, Groves PM, Gage FH: Electrophysiological characteristics of cells within mesencephalon suspension grafts. Neuroscience 40(1):109–22 (1991).**

89. **Kordower J, Rosenstein J, Collier T et al.: Functional fetal nigral grafts in a patient with Parkinson's disease: chemoanatomic, ulturastructural, and metabolic studies. J Comp Neurol 370:203–30 (1996).**

90. **Kordower JH, Styren S, Clarke M, DeKosky ST, Olanow CW, Freeman TB: Fetal grafting for Parkinson's disease: expression of immune markers in two patients with functional fetal nigral implants. Cell Transplant 6(3):213–9 (1997).**

91. **Craven R: Disappointment for Parkinson's disease patients. Trends Pharmacol Sci 22(5):221 (2001).**

92. **Kolata G: Parkinson's research is set back by failure of fetal cell implants. The New York Times, A1(2001).**

93. **Vogel G: Parkinson's research. Fetal cell transplant trial draws fire. Science 291(5511):2060–1(2001).**

94. **Williams N: Setback spurs Parkinson's disease research. Curr Biol 11(8):R285–6 (2001).**

95. **Abbott A: Trials offer way forward for Parkinson's. Nature 410(6827):401(2001).**

96. **Bakay RA: Is transplantation to treat Parkinson's disease dead? Neurosurgery 49(3):576–80 (2001).**

97. **Brundin P, Dunnett S, Bjorklund A, Nikkhah G: Transplanted dopaminergic neurons: more or less? Nat Med 7(5):512–3 (2001).**

98. **Dunnett SB, Bjorklund A, Lindvall O: Cell therapy in Parkinson's disease—stop or go? Nat Rev Neurosci 2(5):365–9 (2001).**

99. **Fischbach GD, McKhann GM: Cell therapy for Parkinson's disease. N Engl J Med 344(10):763–5 (2001).**

100. **Isacson O, Bjorklund L, Pernaute RS: Parkinson's disease: interpretations of transplantation study are erroneous. Nat Neurosci 4(6):553 (2001).**

101. **Olanow CW, Freeman T, Kordower J: Transplantation of embryonic dopamine neurons for severe Parkinson's disease. N Engl J Med 345(2):146; discussion 147 (2001).**

102. Redmond DE Jr, Sladek JR, Spencer DD: Transplantation of embryonic dopamine neurons for severe Parkinson's disease. N Engl J Med 345(2):146–7 (2001).

103. Ungerstedt U, Arbuthnott GW: Quantitative recording of rotational behavior in rats after 6-hydroxy- dopamine lesions of the nigrostriatal dopamine system. Brain Res 24(3):485–93 (1970).

104. Cenci, M.A., Whishaw, I.Q., Schallert, T: Animals models of neurological deficits: how relevant is the rat. Nat. Rev. Neurosci. 3, 574–579 (2002).

105. Langston J, Irwin I, Langston E, Forno L: 1-Methyl-4- phenylpyridinium ion (MPP+): identification of a metabolite of MPTP, a toxin selective to the substantia nigra. Neurosci Lett 48:87–92 (1984).

106. Burns RS, LeWitt PA, Ebert MH, Pakkenberg H, Kopin IJ: The clinical syndrome of striatal dopamine deficiency: parkinsonism induced by 1-methyl-4-phenyl- 1,2,3,6-tetrahydropyridine (MPTP). N Engl J Med 312(22):1418–21 (1985).

107. Langston JW, Forno LS, Tetrud J, Reeves AG, Kaplan JA, Karluk D: Evidence of active nerve cell degeneration in the substantia nigra of humans years after 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine exposure. Ann Neurol 46(4):598–605 (1999).

108. Burns RS, Chiueh CC, Markey SP, Ebert MH, Jacobowitz DM, Kopin IJ: A primate model of parkinsonism: selective destruction of dopaminergic neurons in the pars compacta of the substantia nigra by N-methyl-4-phenyl- 1,2,3,6-tetrahydropyridine. Proc Natl Acad Sci USA 80(14): 4546–50 (1983).

109. Forno LS, DeLanney LE, Irwin I, Langston JW: Neuropathology of MPTP-treated monkeys: comparison with the neuropathology of human idiopathic Parkinson's disease. In: Markey SP, editor. MPTP: a neurotoxin producing a parkinsonian syndrome. Orlando (FL): Academic Press. p 119–40 (1986).

110. Deutch AY, Elsworth JD, Goldstein Met al.: Preferential vulnerability of A8 dopamine neurons in the primate to the neurotoxin 1-methyl-4-phenyl- 1,2,3,6-tetrahydropyridine. Neurosci Lett 68(1):51–6 (1986).

111. Elsworth JD, Deutch AY, Redmond DE Jr, Sladek JR Jr, Roth RH: Differential responsiveness to 1-methyl-4-phenyl- 1,2,3,6- tetrahydropyridine toxicity in sub-regions of the primate substantia nigra and striatum. Life Sci 40(2):193–202 (1987).

112. Elsworth JD, Deutch AY, Redmond DE Jr, Sladek JR Jr, Roth RH: Effects of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) on catecholamine and metabolites in primate brain and CSF. Brain Res 415:293–9 (1987).

113. Taylor JR, Elsworth JD, Sladek JR Jr, Roth RH, Redmond DE Jr.: Behavioral effects of MPTP administration in the vervet monkey: a primate model of Parkinson's disease. In: Woodruff ML, Nonneman AJ, editors. Toxin-induced models of neurological disorders. New York: Plenum Press. p 139–74 (1994).

114. Taylor, J.R., Elsworth, J.D., Roth, R.H., Sladek Jr., J.R., Redmond Jr., D.E.: Severe long-term 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine induced parkinsonism in the vervet monkey (Cercopithecus aethiops sabaeus). Neuroscience 81, 745–755 (1997).

115. Kirik D, Rosenblad C, Burger C et al.: Parkinson-like neurodegeneration induced bytargeted overexpression of α−synuclein in the nigrostriatal dopamine system. J Neurosci 22(7):2780–91 (2001).

116. Kirik D, Annett LE, Burger C, Muzyczka N, Mandel RJ, Bjorklund A.: Nigrostriatal alpha-synucleinopathy induced by viral vector-mediated overexpression of human alpha-synuclein: a new primate model of Parkinson's disease. Proc Natl Acad Sci U S A Mar 4;100(5):2884-9 (2003).

117. Dass B, Olanow CW, Kordower JH: Gene transfer of trophic factors and stem cell grafting as treatments for Parkinson's disease. Neurology May 23;66(10 Suppl 4):S89-103 (2006).

118. Kordower JH, Emborg ME, Bloch J et al.: Neurodegeneration prevented by lentiviral vector delivery of GDNF in primate models of Parkinson's disease. Science 290(5492):767–73 (2000).

119. Palfi S, Leventhal L, Chu Y et al.: Lentivirally delivered glial cell line-derived neurotrophic factor increases the number of striatal dopaminergic neurons in primate models of nigrostriatal degeneration. J Neurosci Jun 15;22(12):4942-54 (2002).

120. Soderstrom K, O'Malley J, Steece-Collier K, Kordower JH: Neural repair strategies for Parkinson's disease: insights from primate models. Cell Transplant 15(3):251-65 (2006).

121. Slevin JT, Gash DM, Smith CD et al.: Unilateral intraputaminal glial cell line-derived neurotrophic factor in patients with Parkinson disease: response to 1 year each of treatment and withdrawal. Neurosurg Focus May 15;20(5):E12 (2006).

122. Barker RA: Continuing trials of GDNF in Parkinson's disease. Lancet Neurol Apr;5(4):285-6 (2006).

123. Chebrolu H, Slevin JT, Gash DA et al.: MRI volumetric and intensity analysis of the cerebellum in Parkinson's disease patients infused with glial-derived neurotrophic factor (GDNF). Exp Neurol Apr;198(2):450-6 (2006).

124. Lang AE, Gill S, Patel NK et al.: Randomized controlled trial of intraputamenal glial cell line-derived neurotrophic factor infusion in Parkinson disease. Ann Neurol Mar;59(3):459-66 (2006).

125. Patel NK, Bunnage M, Plaha P, Svendsen CN, Heywood P, Gill SS: Intraputamenal infusion of glial cell line-derived neurotrophic factor in PD: a two-year outcome study. Ann Neurol Feb;57(2):298-302 (2005).

126. Nutt JG, Burchiel KJ, Comella CL et al.: Implanted intracerebroventricular. Glial cell line-derived neurotrophic factor. Randomized, double-blind trial of glial cell line-derived neurotrophic factor (GDNF) in PD. Neurology, Jan 14;60(1):69-73 (2003).

127. Gill SS, Patel NK, Hotton GR et al.: Direct brain infusion of glial cell line-derived neurotrophic factor in Parkinson disease. Nat Med. May;9(5):589-95 (2003).

128. Kordower JH: In vivo gene delivery of glial cell line--derived neurotrophic factor for Parkinson's disease. Ann Neurol 53 Suppl 3:S120-32; discussion S132-4. Review (2003).

129. McBride JL, Kordower JH: Neuroprotection for Parkinson's disease using viral vector-mediated delivery of GDNF. Prog Brain Res 138:421-32. Review (2002).

130. During MJ, Kaplitt MG, Stern MB, Eidelberg D: Subthalamic GAD gene transfer in Parkinson disease patients who are candidates for deep brain stimulation. Hum Gene Ther. Aug 10;12(12):1589-91 (2001).

131. During MJ, Kaplitt MG, Stern MB et al.: A Dose-Ranging Study of AAV-hAADC Therapy in Parkinsonian Monkeys. Mol Ther Jun 15 (2006).

132. Shreeve J: The other stem-cell debate: to test the potential curative powers of human embryonic stem cells, biologists want to inject them into lab animals. Creating such chimeras makes perfect sense, to a point: a sheep with a human liver? O.K. A mouse brain made up of human cells? Maybe. But a chimp that sobs? N Y Times Mag 42-7 (2005).

133. Robert JS: The science and ethics of making part-human animals in stem cell biology. FASEB J 20(7):838-45 (2006).

134. Greene M, Schill K, Takahashi S et al.: Ethics: Moral issues of human-non-human primate neural grafting. Science Jul 15;309(5733):385-6 (2005).

135. Laird AR, Lancaster JL, Fox PT: BrainMap: The social evolution of a functional neuroimaging database. Neuroinformatics (3) 65-78 (2005).

CHAPTER 2:

LONG-TERM MULTILAYER ADHERENT NETWORK (MAN) EXPANSION, MAINTENANCE, AND CHARACTERIZATION, CHEMICAL AND GENETIC, MANIPULATION, AND TRANSPLANTATION OF HUMAN FETAL FORBERAIN NEURAL STEM CELLS

2.1 Summary:

Human neural stem/precursor cells (hNSC/hNPC) have been targeted for application in a variety of research models and as prospective candidates for cell-based therapeutic modalities in central nervous system (CNS) disorders. To this end, the successful derivation, expansion, and sustained maintenance of undifferentiated hNSC/hNPC in vitro, as artificial expandable neurogenic micro-niches, promises a diversity of applications as well as future potential for a variety of experimental paradigms modeling early human neurogenesis, neuronal migration, and neurogenetic disorders, and could also serve as a platform for small-molecule drug screening in the CNS. Furthermore, hNPC transplants provide an alternative substrate for cellular regeneration and restoration of damaged tissue in neurodegenerative disorders such as Parkinson's disease and Alzheimer's disease. Human somatic neural stem/progenitor cells (NSC/NPC) have been derived from a variety of cadaveric sources and proven engraftable in a cytoarchitecturally appropriate manner into the developing and adult rodent and monkey brain while maintaining both functional and migratory capabilities in pathological models of disease. In the following unit, we describe a new procedure that we have successfully employed to maintain operationally defined human somatic NSC/NPC from developing fetal, pre-term postnatal, and adult cadaveric forebrain. Specifically, we outline the

detailed methodology for in vitro expansion, long-term maintenance, manipulation, and transplantation of these multipotent precursors.

2.2 Introduction

A number of techniques have been devised to attempt to identify and isolate rodent and human neural stem/precursor cells (NSCs/NPCs). Some have relied on the aggregation of cells in suspension cultures—termed "neurospheres" and giving rise to the "neurosphere forming assay" (NSA; Reynolds and Weiss, 1992; Reynolds et al., 1992; Rietze and Reynolds, 2006)—for artificially expanding nonclonal NSC/NPC populations in vitro (Singec et al., 2006) in serum-free medium. However, other techniques have been employed prior to (Ryder et al., 1990; Redies et al., 1991; Renfranz et al., 1991; Snyder et al., 1992) and since (Flax et al., 1998; Shihabuddin et al., 1996; Rubio et al., 2000) popularization of the NSA which, in fact, have been found to have beneficial properties compared to the NSA. It is these techniques that our group has long employed to great advantage and success—particularly when interested in using NSCs/NPCs for transplantation, genetic manipulation, rigorous clonal analyses, and developmental studies—and which will be described in this unit. Human embryonic, fetal, newborn, and adult cadaveric CNS precursors have been shown to thrive when derived and maintained as two-dimensional (2-D) adherent cultures. This technique offers many growth and culture advantages over the NSA and, in fact, has come to supplant the NSA in many neurobiological laboratories.

Over the past two decades, numerous techniques have been described for the derivation and expansion of suspension of human neural precursors either in suspension or as adherent monolayers (Ray et al., 1995; Svendsen et al., 1999; Wu et al., 2002; Walsh et al., 2005; Rajan and Snyder, 2006; Ray and Gage, 2006; Pollard et al., 2006a,b), utilizing an assortment of growth factors (Buc-Caron, 1995; Chalmers-Redman et al., 1997; Moyer et al.,

1997; Sah et al.,1997; Svendsen et al., 1998, 1999; Carpenter et al., 1999; Kukekov et al., 1999; Vescovi et al., 1999a,b; Roy et al., 2000; Uchida et al., 2000; Villa et al., 2000; Piper et al., 2000, 2001; Arsenijevic et al., 2001a,b; Keyoung et al., 2001; Palmer et al., 2001; Cai et al., 2002; Laywell et al., 2002; Nunes et al., 2003; Schwartz et al., 2003; Zhang et al., 2005; Conti et al., 2005; Li et al., 2005; Pollard et al., 2006a,b; Yin et al., 2006; Ray, 2008).

In this unit, we outline methodology for the expansion, long-term maintenance, manipulation, and transplantation of human fetal (10- to 25-week) neural precursor cells (hNPC). Specifically, we describe a new method for long-term expansion of karyotypically stable hNPC, termed the Multilayer Adherent Network (MAN), to generate large scale self-renewing multipotent hNPC populations, amenable to in vitro manipulation and transplantation in vivo. We describe in detail the methods we have successfully utilized to prepare and transplant hNPC into the neonatal mouse and adult nonhuman primate. In addition, we provide basic procedures for characterization of undifferentiated and differentiated hNPC, as well as the processing of engrafted brains. Furthermore, we illustrate techniques for the efficient labeling of hNPC, including lentivirus infection and noninvasive super-paramagnetic iron oxide (SPIO) particle transfection. For simplicity's sake, we will refrain from the operational NSC debate and simply refer to both neural stem and progenitor cells as NPCs from here forward.

2.3 STRATEGIC PLANNING

2.3.1Growth Factor Signaling

Long-term expansion and maintenance of self-renewing NPC in serum-free media (Reynolds et al., 1992; Reynolds and Weiss, 1992; Svendsen et al., 1996; Rosser et al., 1997) requires mitogenic support from either epidermal growth factor (EGF) or basic fibroblast

growth factor (bFGF) to activate mitogen-activated-protein-kinase (MAPK) signaling and support hNPC division (Gensburger et al., 1987;Walicke, 1988; Kornblum et al., 1990; Murphy et al., 1990; Drago et al., 1991a,b; Ray et al., 1993; Vescovi et al., 1993a,b; Bartlett et al., 1994; Kitchens et al., 1994; Ray and Gage, 1994; Ghosh and Greenberg, 1995; Kilpatrick and Bartlett, 1993, 1995; Kilpatrick et al., 1995; Palmer et al., 1995; Vicario-Abejon et al., 1995; Gritti et al., 1996; Kuhn et al., 1997; Qian et al., 1997; Shihabuddin et al., 1997; Caldwell and Svendsen, 1998; Ciccolini and Svendsen, 1998; Gritti et al., 1999; Palmer et al., 1999; Arsenijevic et al., 2001a,b; Caldwell et al., 2001; Temple, 2001; Ostenfeld and Svendsen, 2004; Tarasenko et al., 2004; Kelly et al., 2005; Ray and Gage, 2006). In addition, the neurotrophic leukemia inhibitory factor (LIF) has been shown to enhance telomerase expression, improve viability, and extend the time until terminal senescence of hNPC when used in combination with bFGF and/or EGF (Galli et al., 2000; Molne et al., 2000; Shimazaki et al., 2001; Wright et al., 2003; Bonaguidi et al., 2005; Gregg and Weiss, 2005). Although LIF signaling appears to induce gliogenesis in rodent NPC, in our experience, LIF not only enhances survival and doubling time of human NPC but is absolutely essential for the sustained maintenance of symmetric cell divisions in long-term multilayer adherent network cultures. Direct comparisons of NPC derived from different species or by alternate techniques have shown that NPC characteristics are drastically altered by their environmental inputs and retain these intrinsic cellular properties in direct relation to how they are manipulated in vitro (Ray and Gage, 2006). We have empirically determined the specific regimen of growth factors that best supports growth of human fetal forebrain NPC. As a result, we have adopted a strategy for sustained proliferative expansion of karyotypically normal undifferentiated hNPC in basal growth medium consisting of bFGF and LIF (without EGF).

2.3.2 Media Formulations

Although traditional serum-free rodent NPC culture has generally utilized DMEM/F12 supplemented with N2, we have adjusted the recipe to accommodate hNPC by utilizing Neurobasal medium (Invitrogen) with B-27 supplement (without vitamin A) to support long-term proliferation of hNPC in vitro (Brewer et al., 1993; Brewer, 1995, 1997; Svendsen et al., 1995; Brewer and Price, 1996; Brewer and Torricelli, 2007). In addition, heparin is added to stabilize the binding of the bFGF heparin-sulfate proteoglycan to its FGFR-1 receptor (Balaci et al., 1994; Caldwell et al., 2004), potentiating cell-cell attachments that favor adherent monolayer hNPC growth (Richard et al., 1995, 2000). On the day of use, prepare fresh hNPC growth medium plus 20 ng/ml bFGF plus 10 ng/ml LIF (see Reagents and Solutions). Growth factors are added fresh on the day of use due to their relative instability (Kanemura et al., 2005). Contamination is possible and thus Normocin (InvivoGen) is supplemented regularly (48-hr half-life) as an antipathogenic agent (replaces penicillin/streptomycin/amphotericin B to deter mycoplasma, Gram-positive and -negative bacteria, and fungal contamination). Normocin and any other antibiotics employed may be gradually withdrawn from the culture after an adequate period of time as desired. Due to the relatively large amount of time and resources involved in hNPC culture, we highly recommend the use of pathogen-control agents. Normocin has remained the most gentle yet potent and comprehensive single treatment application we have tested thus far.

2.4 LONG-TERM EXPANSION AND MAINTENANCE OF hNPC

Throughout the expansion process, cryopreservation and functional testing of hNPC lines is necessary for the continued long-term maintenance of healthy proliferative progenitors. Cultures are monitored superficially under the light microscope for morphological aberrations that may occur in artificial culture. Once sufficient cell numbers have been established, a more intensive battery of screens for in vitro and in vivo multipotency should be

employed, particularly when hNPC reach high passage number or whenever a new vial of early passage progenitors are thawed from cryopreservation for mass expansion, to ensure hNPC cultures do not change phenotypically or become lineage restricted with time. To test functionality, several vials are reconstituted to assess the overall freeze/thaw success, cell viability, and sustained multipotency. Throughout culture, the genetic stability of hNPC should be confirmed periodically through spectral karyotyping, microarray fingerprinting, and transcriptome and proteomic analysis to demonstrate a normal chromosomal complement and sustained expression profile of all classical stemness-associated genes (Cai et al., 2006; Chang et al., 2006; Luo et al., 2006a,b; Maurer and Kuscinsky, 2006; Shin and Rao, 2006; Anisimov et al., 2007; Shin et al., 2007). In an effort to reduce time and costly resources, hNPC lines should be regularly tested for these attributes before proceeding with any large animal transplantation studies.

NOTE: All incubations are performed in a 37◦C, 5% CO2 humidified incubator, unless otherwise noted.

NOTE: All reagents and equipment coming into contact with living cells must be sterile, and aseptic technique should be used accordingly.

NOTE: Numerous different types and sizes of tissue culture vessels are described in this unit; the plating volumes for common tissue culture petri dishes, multiwell plates, slides, and flasks are listed in Table 2-1.

Table 2-1. Plating Volumes for Different Culture Vessels

Vessel	Area (cm^2/well)	Volume
Petri dishes		
20 mm	3	1 ml
25 mm	8	2.5 ml
60 mm	25	6 ml
100 mm	78.5	18 ml
Multiwell plates		
6 well	9.6	3.5 ml
12 well	3.8	2 ml
24 well	2	1 ml
48 well	0.75	500 µl
96 well	0.32	250 µl
Slides		
1 well	9.4	3 ml
2 well	4.2	2 ml
4 well	1.8	1 ml
8 well	0.8	250 µl
Flask		
25-cm^2	25	6-8 ml
75-cm^2	75	16-20 ml
225-cm^2	225	40-50 ml

2.4.1 ESTABLISHING AND MAINTAINING MULTILAYER ADHERENT NETWORK (MAN) CULTURES

Traditionally, we have thawed and grown hNSC as small, slightly adherent aggregates for the first 2 to 3 weeks of culture post-thaw. More recently, however, we have developed a new method for expansion of newly thawed or freshly dissociated undifferentiated hNPC on noncoated flasks free of extracellular matrix (ECM). Establishment of these high-density multilayer adherent networks (MAN) is founded on the basic theory of aggregate formation, but is adapted into a novel adherent system that offers many growth advantages for both the progenitor population and the researcher. As a whole, the MAN assay relies on a combination of the inherent hNPC property of forming fusion aggregates at high density, coupled with the

intrinsic behavior of resting hNPC aggregates to attach and migrate over time. The end result is a highly dynamic, proliferative population of undifferentiated hNPC displaying a variety of advantageous growth parameters. In general, we find that mature MAN hNPC cultures proliferate and expand at an elevated doubling rate (3 to 5 days) compared to their neurosphere counterparts (4 to 7 days; Kanemura et al., 2002, 2005; Mori et al., 2006). In addition, feeding MAN cultures fresh medium can easily be achieved by simply tilting the flask, aspirating or collecting CM, and refilling the flask with new medium. This fast and easy process allows the researcher to replace all or portions of the medium as often as necessary without the harsh mechanical stresses involved in centrifugation.

The key to transitioning traditional aggregate cultures into MAN cultures is the overall density of the hNPC initially plated. Simply stated, the greater the density of hNPC initially plated, the larger the aggregate units, the more quickly they attach, and, thus, the more quickly subsequent mature multilayer adherent networks are established. It should be noted that replating hNPC at densities greater than 4 °— 106 cells per 25-cm2 flask will result in overcrowding and subsequent formation of large spheroid cellular masses, negating the entire premise for the initial dissociation. For the most part, high density passaging is only recommended for preparing small cellular clusters prior to cryopreservation, or to quickly establish mature MAN cultures for short-term study. A brief history of the early stages of MAN formation is:

a. 0 to 24 hr: Cells equilibrate and settle to bottom of flask following an even distribution pattern.

b. 24 to 48 hr: Cells begin to lightly attach and spread (as evidenced by small microspikes and several small projections; Fig. 2-1A,B).

c. 48 to 72 hr: Aggregated cell clusters continue to spread, elongate, and begin to proliferate and extend into adjacent neighboring clusters, becoming adherent three-dimensional clusters, creating the first evidence of an interlinked network (Fig. 2-1C).

d. 72 to 96 hr: Cell clusters continue to migrate into each other at the periphery and become anchored strongly enough to change medium. These cultures consist mainly of slightly adherent clusters and a small proportion of nonadherent floating aggregates. The cultures can be carefully removed from the incubator to change medium without disrupting the newly formed MAN (Fig. 2-1 D-F).

Materials

Human NPC (Support Protocol 1): frozen (Support Protocol 2) and freshly thawed (Support Protocol 3) or freshly dissociated as described in Support Protocol 1

25% (v/v) conditioned medium (CM; Support Protocol 4)/75% (v/v) NB-B-27 complete medium (see recipe) containing 40 ng/ml bFGF and 10 ng/ml LIF (bFGF and LIF concentrations based on total volume of medium)

NB-B-27 complete medium (see recipe)

Leukemia inhibitor factor (LIF; Millipore, cat. no. LIF1010)

Basic fibroblast growth factor (bFGF; Millipore, cat. no. GF003)

Normocin (InvivoGEN, cat. no. ant-nr-1)

Dulbecco's PBS with Ca2+ andMg2+ (DPBS; Mediatech, cat. no. 21-030-CM)

Dulbecco's PBS without Ca2+ or Mg2+ (CMF-DPBS; Mediatech, cat. no. 21-031-CM)

Accutase (Millipore, cat. no. SCR005) *or* Cell Dissociation Buffer (Invitrogen, cat. no. 13150-

016)

Conditioned medium (CM; Support Protocol 4)

15-ml conical tubes

25-cm2 and 75-cm2 tissue culture flasks

Battery-powered pipetting aid (e.g., Drummond Pipet-Aid XP)

1000-µl extended-length pipet tip and 1000-µl automatic pipettor

Centrifuge

Additional reagents and equipment for counting viable cells by trypan blue exclusion

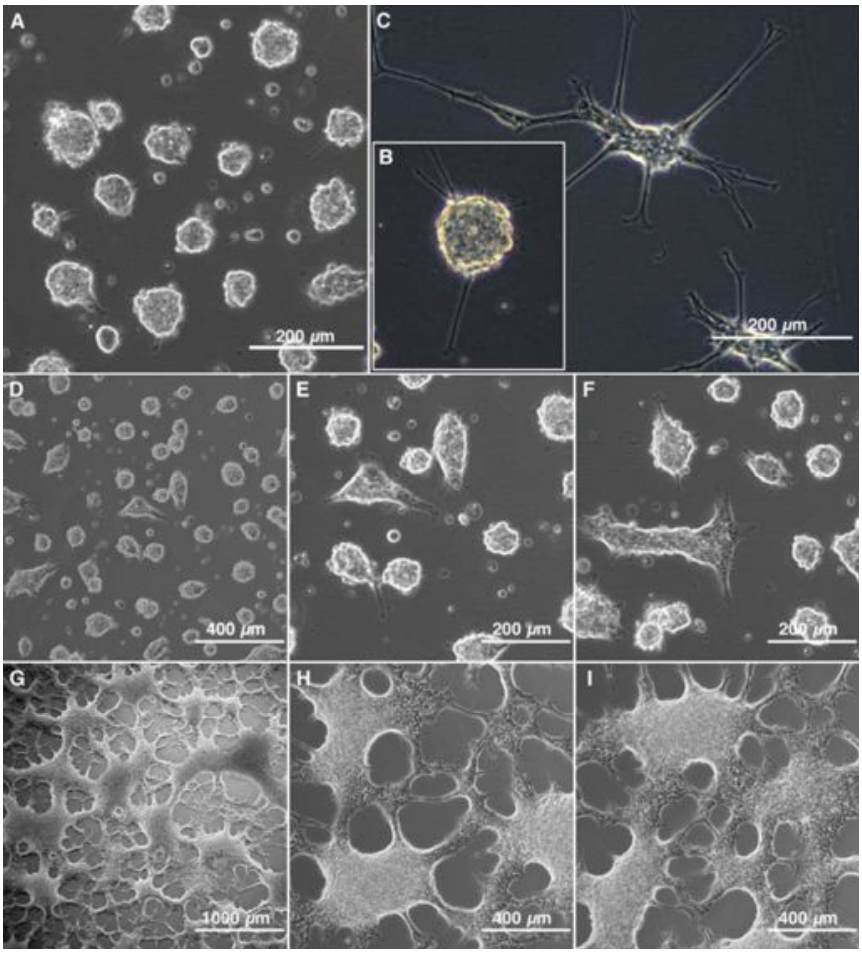


Figure 2-1. Establishment of multilayer adherent network (MAN). (**A**) 24 to 48 hr after plating, hNSC (HFB-2050) readily form evenly spaced, proliferative aggregated cell clusters. Small hNPC clusters initially attach to the culturing surface and sample the local microenvironment with meandering growth-cone like protrusions (**B**), and eventually flatten and spread out (**C**). Taking advantage of higher plating densities, the MAN culturing technique creates optimal spacing between colonies, allowing each aggregate cluster close access to neighboring signaling molecules. (**D-F**) After 72 hr, hNSC aggregates are lightly attached to the surface and begin to actively proliferate. Over the next 3 to 4 weeks, hNSC rapidly expand and form extensive honeycomb-shaped, mature multilayer adherent networks (**G-I**).

2.4.1.1Establish MAN cultures

1. To establish MAN cultures from freshly thawed cells or freshly dissociated cells, resuspend hNPC 2:1 (i.e., at 2–3 °— 106 cells/flask) in 25% (v/v) CM/75% (v/v) NB-B-27 complete medium (containing 40 ng/ml bFGF, 10 ng/ml LIF, and 2 µl/ml Normocin) in a 15-ml conical

tube. Transfer hNPC to an uncoated 25-cm2 flask (Fig. 2-2).

Ratios such as 2:1 refer to the surface area used—i.e., if starting with one 25-cm2 flask, when expanding cells, one would use a 1:2 split, meaning that one should start with one 25-cm2 flask and resuspend the dissociated cells into two 25-cm2 flasks—increasing the surface area from 25 to 50 or 1:2. However, if referring to establishment of a culture with frozen cells, the ratio is 2:1, i.e., the number of frozen cells that were originally in two 25-cm2 flasks would need to be thawed into one 25-cm2 flask. Similarly, when dealing with freshly dissociated cells, the ratio is 2:1. In this step, 2 million fresh cells or 3 million frozen cells are diluted into 8 ml media into one 25-cm2 flask.

Plating a higher density of hNPC leads to the quicker (24- to 72-hr) formation of small (2–3 °— 106 cells) to medium size (3–4 °— 106 cells) clusters, respectively, initiating close cell-cell contacts critical for enhanced paracrine and autocrine support. This means that if you plate 2–3 °— 106 cells (dissociated) into one 25-cm2 flask, it will give you small clusters within 24 to 72 hr, whereas 3–4 °— 106 will give medium-size clusters in this same period of time.

Interestingly, we have found that leukemia inhibitory factor (LIF) is absolutely necessary and essential for the long-term maintenance of MAN cultures. Removal of LIF from the basal growth medium results in the rapid breakdown of elongated projections into ropelike, flexible, spindly, nonadherent protrusions that eventually disappear, ultimately resulting in the loss of proliferation capacity, increased senescence, and eventual cellular crisis.

2. Place the flask into a humidified 5% CO2 incubator at 37◦C and shake horizontally in both planes to evenly disperse cells throughout the flask without sloshing medium into the neck of the flask.

It is imperative that small (4- to 16-cell) to medium size (16- to 64-cell) clusters (from thawed sample; Support Protocol 3) or single cells (from dissociation) are dispersed uniformly onto the surface to avoid clumping and uneven coating of the flask.

3. Once the cells have been fully distributed, allow the flask to incubate and equilibrate for 3 days without moving the flask for any reason from its original resting position.
It is equally important that the incubator remain motionless and not be bumped or shaken in any way, or else the even hNPC coating will be disrupted and the organization of the MAN system will become disordered.

During the 72-hr hands-free period, the individual evenly spaced cellular clusters settle to the bottom of the flask, lightly attach, and migrate out over the surface, proliferating into each other, creating an interlinked lattice of three-dimensional adherent clusters displaying elongated processes that extend and connect each cellular island into a global multilayer adherent network (MAN). Perhaps the most important aspects of successful MAN culture setup are the initial plating conditions coupled with diligent patience and a steady hand during the initial week after hNPC thaw.

Throughout the process, adherent clusters can easily become detached by simply moving the flask; therefore, it is of utmost importance for the integrity of the culture system to absolutely avoid any movement of the flask or its content during the crucial aggregate-to-MAN transition process. Once hNPC clusters have detached, they will immediately merge with any other suspension aggregates they come into contact with (via integrins and secreted ECM proteins), thereby perturbing the essential spacing component of adherent growth. Even removal of the flask to view under the microscope disrupts the culture setup and should be avoided. For the same reasons, it is not prudent to supplement growth factors during this time; therefore, MAN cultures are started in 40 ng/ml bFGF to account for rapid degradation

and resultant mitogen loss over the first 48 hr.

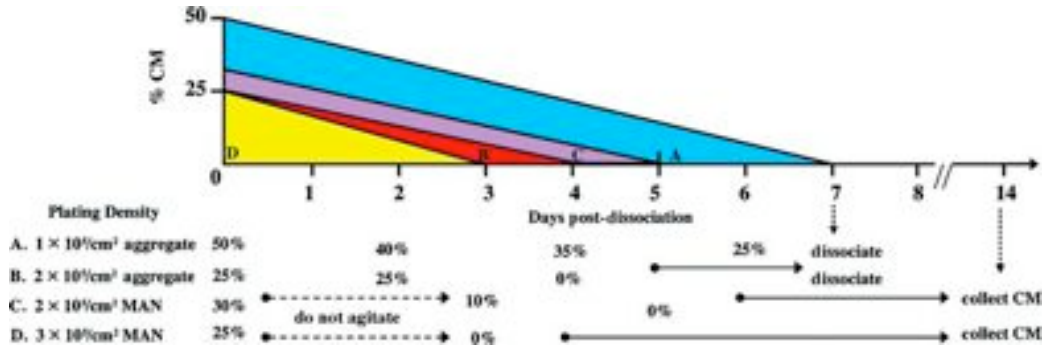


Figure 2-2. Human neural precursor cell basic culture schedule. Human NPC are grown as either lightly adherent aggregates or as multilayer adherent networks in 25-cm2 flasks. Conditioned medium (CM) is gradually reduced from cultures as they progress and can be collected after MAN cultures reach ~75% confluence. Aggregate cultures are dissociated once a week or as growth parameters dictate, whereas MAN cultures can be cultured for up to 1 month before passaging.

4. After 3 to 4 days of untouched growth, gently move the flask from the incubator to the sterile hood.

5. Slowly tilt the flask up to 90◦, then slowly rock backwards so that the flask is now upside-down, the CM is now facing downward on the top of the flask, and the cellular plane is facing upwards.

You should be able to visibly identify exposed adherent clusters attached to the flask.

6. Aspirate all of the medium from the flask and, quickly but gently, add 8 ml fresh NB-B-27 complete medium containing 20 ng/ml bFGF, 10 ng/ml LIF, and 2 µl/ml Normocin to the downward (noncellular) plane, being careful not to slosh medium onto the upper (cellular) plane, which would dislodge the lightly adherent cells.

Do not allow the flask to dry out after the medium has been aspirated, as hNPC may begin to detach upon reintroduction of fresh medium to the culture.

7. In a reverse motion, rock the flask back slowly to its original position, paying careful attention as the medium re-covers the adherent cells.

During this process, it is absolutely imperative to reintroduce the fresh medium in a slow fluid motion to minimize waves as the medium spreads across the flask. Any major fluctuations or tapping of the flask can easily dislodge the clusters from their equally spaced positions, threatening the overall integrity of the MAN. No matter how careful you may be, there will always be a small percentage of cells that either did not attach or have detached during the feeding process. These floating cells will either reattach or can be removed from the culture at the time of the next feeding.

8. After the medium has been changed, place the flask back into the incubator and repeat the process every 2 to 3 days as necessary to replenish growth factors (48-hr half-life) or replace metabolized medium (indicated by an orange acidic appearance).

The literature and product datasheets support a general half-life for most of the growth factors used in this unit at 24 to 72 hr at 37oC in these medium formulations. The cells also utilize a large proportion, so we generally assume that the majority of the growth factors need to be replenished; therefore, we supplement according to the volume in the flask and adjust the concentration to the full concentration on the assumption that there is no growth factor remaining.

As the cultures expand, it will become necessary to alter the percentage of CM exchanged. During the first week, 75% to 100% of the medium should be exchanged to account for metabolized nutrients while maintaining adequate paracrine conditioning.

As cultures develop from week one onward, it will become essential to exchange 100% fresh NB-B-27 growth medium every 1 to 2 days to replenish the highly metabolized

nutrient stores and remove toxic metabolic by products. CM does not need to be added back in this case, as the high density-to-volume ratio leads to quick paracrine conditioning, adequate for immediate sustained survival. Furthermore, these fully developed MAN cultures can be utilized for the collection of high-quality CM (Support Protocol 4).

Over the next 2 to 3 weeks, MAN hNPC continue to proliferate and spread into a webbed culture, whereby adherent cellular islands will not only expand into each other but also proliferate in the vertical z dimension, creating the characteristic multilayer three dimensional appearance (Fig. 2-1 G-I).

As the MAN matures, it will develop into a highly mitotic (75% to 85%) confluent culture. Although clusters will continue to merge, there will always be demarcated areas on the flask surface where no hNPC grow; therefore, these cultures never attain the classic two-dimensional monolayer morphology.

2.4.1.2 Feed Multilayer Adherent Network (MAN)

MAN cultures offer many time and growth advantages over classic aggregate or suspension sphere assays. Care should be taken to minimize sloshing of medium or excessive vibration that will detach the fragile network of cells. The basic rule for ease of use with this system is to minimize mechanical stress, especially at the edges of the flask, which can easily loosen the outer edges of the MAN, exposing the undersurface and resulting in uplifting of the entire sheet of adherent progenitor cells. Although these adherent networks of cells appear to be stably anchored to the flask, it takes relatively little force to disrupt their fragile connections. Furthermore, once detached, the cells will remain adherent in their networks and organize into large clumps, floating or partially attached to the remaining sheet of cells, which may become necrotic if not dissociated in ample time. Any cellular debris and insoluble salt residues that may develop from prolonged culture are removed by the methods described

below.

9. Slowly tilt the flask up to 90◦ and rock backwards so that the CM is facing downward on the top of the flask and the cellular plane is facing upwards. Carefully aspirate or collect conditioned medium

See Support Protocol 4 for treatment of the conditioned medium.

10. Gently rinse the flask once with 8 ml DPBS (for 25-cm2 flask) or 12 ml DPBS (for 75-cm2 flask) by expelling DPBS onto the downward (noncellular) plane at low speed, being careful not to slosh liquid onto the upper (cellular) plane, which would dislodge lightly adherent hNPC.

Do not allow the flask to dry out after DPBS has been aspirated, as hNPC will begin to detach upon reintroduction of fresh media to the culture.

11. In a reverse motion, rock the flask back slowly to its original culture position, paying careful attention as the DPBS re-covers the adherent cells.

During this process, it is absolutely imperative to reintroduce the fresh DPBS in a slow fluid motion to minimize mechanical fluctuations as it spreads across the flask

12. Repeat steps 9 to 11, transferring 8 to 10 ml (for 25-cm2 flask) or 15 to 20 ml (for 75-cm2 flask) fresh NB-B27 complete medium (containing 20 ng/ml bFGF, 10 ng/ml LIF, and 2 µl/ml Normocin) to each flask. Slowly move the flask to a humidified incubator at 37◦C, 5% CO2.

2.4.1.3 Dissociate Multilayer Adherent Network (MAN)

13. When cultures are ready for passaging (see Critical Parameters and Troubleshooting), slowly tilt the flask upwards to 90◦, then rock backwards so that the CM is facing downward on the top of the flask and the cellular plane is facing upwards. Aspirate or collect conditioned medium.

14. Gently rinse the flask once with 8 ml CMF-DPBS (for 25-cm2 flask) or 15 ml CMFDPBS (for 75-cm2 flask) by expelling CMF-DPBS onto the downward (noncellular) plane, being careful not to slosh CMF-DPBS onto the upper (cellular) plane, which would dislodge lightly adherent cells.
Do not allow the flask to dry out after medium has been aspirated, as hNPC will begin to detach upon reintroduction of fresh liquids to the culture.

15. In a reverse motion, rock the flask back slowly to its original position, paying careful attention as the CMF-DPBS re-covers the adherent cells. Repeat step 13 and aspirate.
During this process, it is absolutely imperative to reintroduce the CMF-DPBS in a slow fluid motion to minimize mechanical fluctuations as it spreads across the flask.

16. Gently add 3 to 5 ml (for 25-cm2 flask) or 7 to 10 ml (for 75-cm2 flask) of Accutase (prewarmed to 37◦C, 10 min before use) to flask without disrupting the integrity of the cellular sheet (as described for CMF-DPBS rinse in steps 13 to 15).

17. Carefully transfer the flask into a 37◦C, 5% CO2 humidified incubator for 3 to 5 min (depending on density), minimizing any significant motion that will release the multilayer adherent network prematurely.
The key to the successful dissociation of a MAN culture relies on learning to recognize the

following properties throughout the incubation in dissociation agent.

a. As the enzyme initially begins to break down cell-cell contacts, the adherent culture releases from the plastic dish from the outside in. Generally speaking, the outermost edges of the network will flap up and off of the dish, generating an organized sheet that eventually releases from the plastic dish below. If the dish is prematurely interrupted during this incubation process by moving the flask or sloshing the Accutase solution, the precise coordinated lifting of the multilayer adherent network is disturbed and subsequently leads to breakdown of the intact sheet of cells. Inadvertent disruption of the intact sheet can lead to gross clumping and compromise the integrity of cells as they dissociate.

b. In addition, prolonged exposure to enzymes can puncture the cell membrane and render hNPC extremely vulnerable to mechanical shearing, resulting in lysis and release of DNA into the cell suspension. The results of enzyme overexposure are visibly apparent, as evidenced by increased viscosity of the cell suspension accompanied by discernibly large floating aggregates. These aggregates have a propensity to float to the top of the cell suspension and are characterized by their sticky, slimy properties that render them problematic in culture as they accrue and amass live cells on the surface. As the aggregates continue to bind live hNPC, they become heavier and eventually fall by gravity from the top of suspension to the bottom, thus allowing for removal from the remaining population. The overall result of enzyme overexposure is decreased hNPC recovery; therefore, it is imperative to time the enzymatic process and visually inspect the flask after 3 to 3.5 min, to monitor the dissociation progress closely.

c. During the 3- to 5-min incubation process, the MAN layer will gradually detach completely

from the underlying flask, effectively shrinking into an intact rectangular sheet, resembling a miniature compacted version of the original MAN. The exact timing for completion of this process is variable, but should be minimized to account for overexposure. In general, the entire sheet should be detached and shrunken into the center of the flask for at least 1 to 3 min before the desired stage of dissociation is attained. Note that this is an extremely time-sensitive process. Lesser incubation times will result in incomplete dissociation of larger hNPC clusters, requiring additional cycles, ultimately leading to increased clumping and subsequent cell death.

18. After 3.5 to 5 min, when the MAN displays the above characteristics, gently transfer the flask to a sterile hood, paying special care to retain the free-floating cellular sheet in its intact form for easy removal.

The intact sheet is extremely fragile and will most likely begin to dissociate as the flask is moved. Try to retain the sheet in as many large pieces as possible. Furthermore, lower density cultures will not retain the structural integrity that their mature MAN counterparts display.

19. Carefully tilt the flask so that the sheet of cells aggregates to the bottom corner of the flask with gravity. With a 5-ml pipet, carefully suck up the concentrated network of cells in 1 to 3 ml of the Accutase solution and transfer to a 15-ml conical tube.

It should be possible to reclaim the cells into a small volume without extensive single cell dissociation or disruption of the cellular sheet. The remaining Accutase should appear clear and may contain a few smaller cell clusters.

20. Gently triturate contents of the conical tube with a 5-ml pipet attached to a pipetting aid (e.g., Drummond) on medium speed (five to seven times) to break the cell suspension into

smaller floating cellular aggregates.

Be very careful not to over-triturate, as the cell suspension is extremely fragile at this stage.

21. Using the same 5-ml pipet, immediately triturate the remaining contents of the flask to break up remaining clusters, gently but thoroughly, paying extra attention to the removal of adherent hNPC at the edges of the flask where they tend to attach preferentially and with increased strength. Transfer the contents of the flask to the previous conical tube.

22. Continue trituration of hNPC inside the conical tube to break the cells up into smaller clusters by gently expelling the cell suspension at a 45◦ angle against the wall of the conical tube at medium speed (8 to 10 times).

23. If necessary, recap the conical tube and incubate in a 37◦C water bath for 1 to 2 min more with constant swirling to avoid clumping of aggregates at the bottom of the tube and reduce accumulation of sticky DNA from lysed cells.

It is very important to ensure the hNPC do not aggregate and begin clumping during the dissociation process; therefore, care should always be taken to continuously swirl or triturate the cells during steps 20 to 23.

24. Using a 1000-µl extended-length pipet tip with a standard automatic pipettor set to 750 µl, slowly triturate hNPC suspension at a 45◦ angle against the wall of the conical tube at a consistent rate.

Excessive or high-rate trituration against the plastic wall is not well tolerated at this stage. We recommend slow to medium trituration at a position near, but not touching directly against the wall of the conical tube (five to ten times or until large clumps are no longer

visible and the dissociated solution has a homogenous milky and sandy appearance). Ideally passaged cultures will be fully dissociated into single cells, >95% viable, and free of floating aggregates if the time of initial Accutase exposure was within the correct window (step 17), cells are not allowed to aggregate, and trituration remains moderate and minimal. Cell clusters will readily stick to the meniscus (~750-µl line) of the pipet tip.

25. To recover cells that have stuck to the meniscus, reset the plunger from 750 µl to 1000 µl (with tip remaining intact). Rinse the 1000-µl tip once with 1000 µl NB-B-27 complete medium to dislodge residual clusters, and transfer the contents to a new 15-ml conical tube containing 10 ml fresh NB-B-27 complete medium containing 20 ng/ml bFGF, 10 ng/ml LIF, and 2 µl/ml Normocin (prewarmed to 37◦C) to inactivate the reaction.

26. Inactivate fully dissociated preparation from step 24 by adding it to the 10 ml medium in the conical tube from step 25.

Variability in hNPC culture densities and morphology will dictate the specific timing and rate of dissociation for each culture. As a result, it is often the case that a small percentage of undissociated cell clusters remain and require a second round of enzymatic treatment, while the majority of cells are fully dissociated and ready to be inactivated and released from enzymatic shock.

27. To process partially dissociated cell suspensions, place the conical tube vertically for 1 to 2 min until the visible cellular clusters have settled by gravity to the bottom. Carefully transfer the top portion of supernatant containing dissociated cells to the previously inactivated cell suspension. To the remainder of undissociated hNPC, add 1 ml fresh prewarmed Accutase, triturate twice, and repeat steps 24 to 26.

28. Transfer the appropriately dissociated cell suspension to the previously inactivated 10 ml hNPC suspension from step 26.

In rare cases, some clusters may remain after the second round of dissociation (often seen in necrosis) and are considered behaviorally abnormal and subsequently discarded.

CAUTION: *Overexposure to any dissociating agent will cause significant cell death and deter growth from lysed hNPC. The solution will become more viscous when this occurs. Thus, the procedure should be optimized to break up the cell clusters, while minimizing the amount of time in the dissociation agent. Generally, the larger the flask, the more dissociation agent that will be needed, which means more cell death and greater difficulty in controlling the timing of the process. We recommend 25-cm2 or 75-cm2 flasks for optimal conditions.*

29. Centrifuge the cell suspension for 4 min at 400 °— *g*, room temperature. Carefully aspirate the supernatant.

Adherent cultures exhibit a highly branched, polarized cellular morphology, and unfortunately many of these delicate processes are cleaved by dissociating agents and mechanical stress, resulting in a greater amount of cellular debris. As a result, an additional rinse and centrifugation with 10 ml of either CMF-DPBS or Neurobasal medium (Invitrogen) is recommended to remove any problematic residual debris.

30. Resuspend the hNPC pellet in the conical tube with 1 ml fresh NB-B-27 complete medium using an extended-length 1000-µl pipettor and tip, gently triturating five to seven times to thoroughly liberate the cell pellet.

31. Count viable cells using a hemacytometer and trypan blue (*UNIT 1C.3*) for correct

replating density.

32. After counting, add 8 ml CM (for a 1:2 dilution) to the conical tube, adjust for the desired final volume of fresh NB-B-27 complete medium to CM ratio accordingly (i.e., 8 ml fresh NB-B27 medium for 50% CM final), bring cells to desired density, and replate into new 25-cm2 flasks.

In general, more concentrated splits survive and proliferate more effectively than their diluted counterparts. As a guideline, a 25-cm2 flask containing 1–3 °— 106 cells is fed 25% to 50% CM, and 4 °— 106 cells do not require CM as they quickly condition the medium due to high density.

33. Add bFGF and LIF to achieve a final concentration of 20 ng/ml and 10 ng/ml, respectively. Gently swirl contents of flask horizontally to evenly disperse hNPC and place in a humidified incubator at 37◦C, 5% CO2.

Subsequent culturing methods will depend on the density of cells plated and method for further expansion.

34. Passage MAN cultures.

Typically, the growth parameters of hNPC MAN cultures dictate passaging once every 1 to 2 months depending on the original plating density and desired confluency. We typically split MAN cultures at a 1:2 dilution for 3–4°—106 cells/25-cm2 flask of mature 65% to 75% confluent culture, or 1:4 for 5–10 °— 106 cells/25-cm2 flask of very mature 80% to 90% confluent extremely high-density 2-month-old cultures, as they contain many more cells per flask than a typical aggregate culture where high density cannot be achieved at the cost of fusion, large globular aggregate formation, and ensuing necrosis.

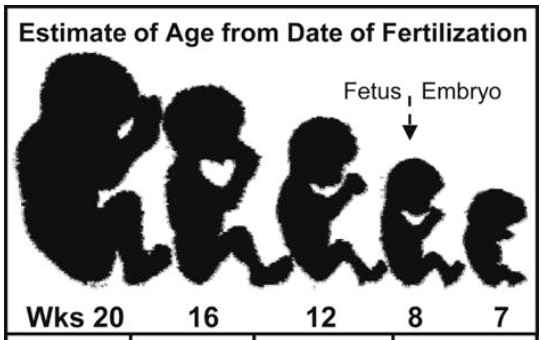
We consider the above modifications of the enzymatic process, specifically the precisely timed controlled release of the entire MAN as an intact sheet, to be one of the key components of successful passaging and subsequent expansion of hNPC using this assay. Consistent high viability and overall health of the resultant hNPC preparations coupled with the intrinsic quantitative qualities of the assay (i.e., increased population doubling rate, apparent increase in proliferation capacity for>100 passages without senescence or decease in rate of replication, and decreased cost in consumables and personal time) all mark the overall utility and advantages for employing the MAN assay to obtain long-term expansion of large quantities of undifferentiated hNPC.

MAN cultures can also be processed by traditional methods used for aggregate cultures. Simply triturate adherent cells thoroughly from the flask and proceed as described for aggregate cultures (Alternate Protocol 1). It should be noted that enzymatic dissociation times will be greatly enhanced, requiring multiple rounds of gravity-based cluster separation, enzymatic treatment, and subsequent centrifugation cycles. Unfortunately, this procedure results in significant cell death (60% to 70% viability) in even the most skilled hands, and should only be employed when cells are accidentally detached by mechanical force. In these cases, a second rinse and centrifugation step should be added prior to final plating.

2.4.2 DERIVATION OF HUMAN FETAL NEURAL STEM/PRECURSOR CELLS

Fetal spatial features and their specific neuroanatomical coordinates are used to determine the cadaver's specific stage of CNS development and dictate the exact location for tissue dissection. Proficiency in fetal neuroanatomy is essential for efficient assessment and subsequent resection of specified CNS regions. We, along with others, have described various methods for the derivation of hNPC. Here, we detail the methodology we have successfully employed to isolate and expand fetal forebrain periventricular zone human NPC.

The investigator typically comes into possession of fetal tissue post-mortem as cadaveric material (Prior to 23 weeks of gestation, a fetus is non-viable outside of the womb). Between the eighth and ninth week of development, the embryo undergoes several distinct developmental transitions. Both the feet and hands lose their webbing and become separated into distinct digits, and the stubby tail disappears. At 9 weeks post-fertilization, by convention, the embryo is called a fetus. Estimation of fetal age can be extremely tricky without prior knowledge of the last normal menstrual period or day of fertilization (which is rarely known by clinicians); however there are a few decent methods to gauge estimated fetal age and developmental stage. Fetal entities are most accurately staged using pre-term ultrasound measurements, however, these vital records are rarely granted to the investigator, therefore, we must rely on post-mortem methods including crown to rump length (CRL), foot length, head to trunk, cheek to cheek, and fetal weight for quantitative analysis [Figure 2-3]. When utilized individually, these methods are often inaccurate, as fetal specimens are affected by stretching. A combination of strategies, however, can be used to accurately determine fetal age within several days of fertilization.



Age	CRL	Foot Length	Fetal weight
week	mm	mm	gm
9	45	5	7
10	55	10	15
12	85	15	45
14	120	20	110
16	140	25	200
18	160	35	310
20	190	40	450
22	210	45	620
24	230	50	800
26	250	55	1000

Figure 2-3. Fetal developmental staging. Determining the relative fetal developmental age is crucial for proper anatomical assessment prior to dissection of the brain. Specifically, Crown Rump Length (CRL) is used as an appropriate measure for staging post-mortem fetuses. All measurements are averages based on deviations from Streeter's original tables from fixed fetuses based on the *time from fertilization* and intended as general reference points to gauze developmental staging of the brain. Gestational age begins 2 weeks after fertilization age. Fetuses born prematurely after 22 weeks may survive with artificial support but have limited capacity due to underdevelopment of both the respiratory and nervous systems.

The most common and useful parameter for aging and developmental analysis has remained CRL due to its high correlation with definitive gestational changes. Starting at week 9 (from date of fertilization), the head contributes to almost half of the CRL, which more than doubles by the end of week 12. From weeks 12-16, fetal growth is rapid, albeit the head remains relatively stable, contributing less to the total CRL than during weeks 9-12.

Importantly, skull and long bone ossification begins to set in and should be considered when deriving hNPC from fetuses of this developmental period. By the end of the 16th week, the eyes are repositioned anteriorly from anterolateral and overall fetal growth slows down considerably.

As development proceeds over the next four weeks, the CRL continues to increase by about 50 mm, and the lower limbs reach their final proportions. By twenty weeks, hair and genitalia begin to develop and the mother will start to experience fetal movements, or *quickening*. It is at this time that the fetal entity begins to fully develop and take on substantial weight. Furthermore, between weeks 21 and 25, the fetus develops blood filled capillaries, fingernails, and rapid eye movements reminiscent of newborns. A thorough and accurate assessment should be made to properly identify the age of the fetus before dissection. All procedures must be carried out in strict accordance with federal regulations. For example, in the United States, federal law mandates that the latest time a woman may legally terminate pregnancy to be roughly 24 weeks. Typically, cadavers become available to the investigator at 9-15 weeks of age, a time at which landmarks are admittedly hard to identify.

NOTE: Use of human fetal cadaveric CNS must follow all safety and bioethical guidelines, including but not limited to full informed consent, IRB approval, and strict adherence to all state and federally mandated laws and guidelines for the ethical use and treatment of patients or specimens derived thereof.

NOTE: Perform all procedures aseptically in a sterile Biosafety Level 2 hood. Sterilize all surgical tools in a hot bead sterilizer or autoclave (121◦C, 2 hr), or by gas sterilization. During the procedure, place all of the tools in fresh 70% ethanol when not in use. Immediately following removal from ethanol, briefly rinse twice in fresh sterile DPBS (Mediatech, cat. no.

21-031-CM).

Materials

Fetal tissue

10% (v/v) formalin (optional)

Enzymes for tissue dissociation (optional): e.g., Accutase, trypsin-EDTA, PPD (papain-protease-DNase I)

Fetal bovine serum (FBS; optional)

NB-B-27 complete medium (see recipe)

Basic fibroblast growth factor (bFGF; Millipore, cat. no. GF003)

Leukemia inhibitory factor (LIF; Millipore, cat. no. LIF1010)

Normocin (InvivoGEN, cat. no. ant-nr-1)

Epidermal growth factor (EGF; Millipore, cat. no. 01-107)

Surgical equipment, including scalpel, sterile

15-ml conical tubes

Battery-powered pipetting aid (e.g., Drummond Pipet-Aid XP)

Additional reagents and equipment for counting viable cells by trypan blue exclusion (*UNIT 1C.3*)

2.4.2.1 Isolate and digest human fetal periventricular zone

1. Stage the fetus using neuroanatomical coordinates, open the head cavity, and remove the brain.

2. Cut sagittally across the midline to separate the cerebral hemispheres then cut again coronally from frontal to occipital poles (Diagram 2-1).

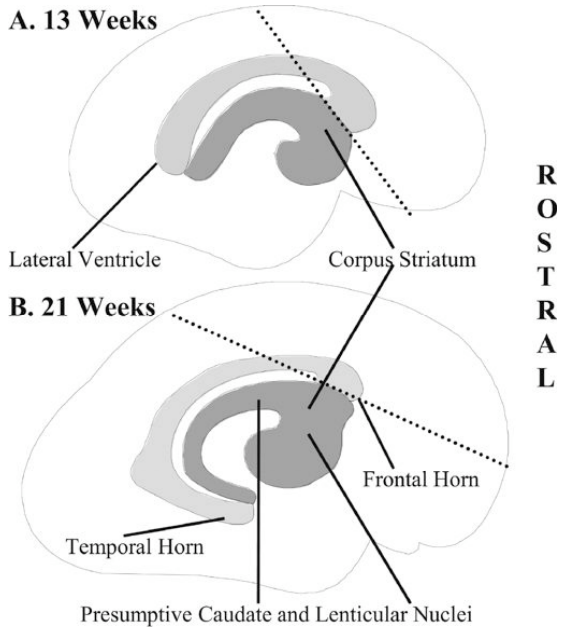


Diagram 2-1. Fetal cerebral hemispheres and ventricular zone development
Sagittal anatomical rendering of the medial surface of the fetal brain at 13 and 21 weeks after fertilization. As the brain develops, the cerebral hemispheres expand and meet at the midline, giving rise to the corpus striatum and shaping the cerebrospinal fluid filled, C-shaped cavities known as the lateral ventricles (**A, B**). As the cerebral cortex begins to differentiate, the corpus striatum eventually divides into the horseshoe shaped caudate nucleus and the bulbous lentiform nucleus, which houses the putamen and globus pallidus (**B**). At 13 weeks, the surface of the cerebral hemispheres are smooth and underdeveloped, highly resembling lower order mammals, however by 21 weeks, patterned convolutions (gyri) and grooves or furrows (sulci) form complex folds increasing the total surface area with little change in cranial parameters. In addition, the lateral sulcus begins to narrow as the insula becomes buried (not pictured).We generally dissect along the *dashed line* and remove the periventricular zone adjacent to the head of the caudate nucleus.

3. Select the brain slice containing the region of interest for dissociation.

Optional: Fix the remaining tissue in 10% (v/v) formalin for a more extensive neuropathological examination.

4. Carefully scrape the ventricular wall and adjacent subventricular zone region from the

forebrain section with a surgical scalpel. Delicately mince the dissected tissue into small pieces with the scalpel blade.

5. Transfer the tissue pieces into a 15-ml sterile conical tube that contains 6 ml cold NB-B-27 medium, 20 ng/ml bFGF, 20 ng/ml EGF, and 4 µl/ml Normocin.

6. Place the conical tube vertically and allow the tissue to pellet by gravity (1 to 2 min), aspirate supernatant carefully, and rinse three times, each time with 8 ml cold medium.
7. After final rinse, resuspend the tissue in 8 ml cold medium.

8. Gently triturate the fetal tissue suspension (10 to 15 times) with a 5-ml pipet attached to a pipetting aid (e.g., Drummond Pipet-Aid XP) at medium speed against the wall of the 15-ml conical tube to further dissociate the tissue into a homogenous milky solution.
The cell suspension will contain both single cells and a few small cellular clumps. Try to avoid introducing air bubbles during the trituration process. It is important that the primary tissue not be overzealously digested into a single-cell suspension, due to the subsequent damage incurred by mechanical stress on the progenitor fraction. CNS tissue from young fetal brains is softer than that from fully developed myelinated adult brains; therefore, later-stage CNS preparations include the addition of an enzymatic agent such as Accutase, trypsin-EDTA, papain-protease-DNase I (PPD), dispase, or any commercially available reagent, according to the manufacturer's instructions, to efficiently dissociate primary cultures before their initial plating. In general, enzymatic fetal tissue dissociation averages ~5 to 10 min, while adult tissue can take upwards of 45 to 90 min to generate the desired breakdown of brain tissue.

9. Remove large undissociated tissue bits remaining after the initial trituration by allowing

them to settle by gravity (2 to 3 min), then collect the suspension of cells in the upper supernatant. Dissociate remaining undissociated cell clumps again as in the steps above and pool together with the originally dissociated cell suspension.

10. Inactivate enzymatic preparations by diluting them 1:5 in fresh prewarmed NB-B27 medium and centrifuge for 5 min at 400 °— *g*, room temperature. Remove supernatant and retain pellet.

2.4.2.2 Establish primary hNPC cultures

11. Following primary dissociation, bring the cell suspension to working volume in 8 ml pre-warmed NB-B-27 medium with 20 ng/ml bFGF, 20 ng/ml EGF, and 4 µl/ml Normocin at a final density of 1 °— 105 cells/cm2 in one 25-cm2 flask and place in a humidified 5% CO2 incubator at 37◦C.

Primary cultures plated onto tissue culture treated flasks will generally produce mixed aggregate and adherent cultures. Primary cell suspensions may also be plated onto fibronectin-coated tissue culture–treated flasks for monolayer-like (two-dimensional) adherent cultures.

12. Determine cell viability using either the propidium iodide or trypan blue exclusion assay and a hemacytometer.

Sticky cellular debris and small undissociated neural clumps may make this process difficult initially.

13. *Optional*: Add 0.1% to 1% (v/v) fetal bovine serum (FBS) at the time of initial derivation to enhance initial NPC expansion efficiency, promote adhesion, and decrease overall cell

death with a relatively low risk of differentiation.

CAUTION: *Using FBS may introduce unwanted variability. Serum components are removed after a short period of time and replaced with a defined, serum-free medium so as not to potentiate long-term side effects on primary hNPC cultures. In some cases, it is desired that newly derived stem cell lines be established utilizing serum-free protocols so as not to introduce animal proteins into culture.*

14. Incubate cells. At a time point 12 to 48 hr after plating, rinse any serum-containing cultures twice with 10 ml DPBS and transfer cultures to serum-free conditions in NB-B-27 medium containing 20 ng/ml EGF, 20 ng/ml bFGF, 10 ng/ml LIF, and 4 µl/ml Normocin. Continue incubation.

15. At a time point 3 to 4 days after the primary plating, supplement cultures by carefully removing the top half of medium from each flask, termed conditioned medium (CM), and replace with fresh NB-B-27 complete medium containing 40 ng/ml EGF, 40 ng/ml bFGF, 20 ng/ml LIF, and 8 µl/ml Normocin for the final working concentration of 20 ng/ml EGF, 20 ng/ml bFGF, 10 ng/ml LIF, and 4 µl/ml Normocin.

These final concentrations are based on the assumption that the growth factors have been completely deleted by this point.

16. For more efficient recovery, remove the CM containing free-floating aggregates and small clumps of primary tissue and transfer the contents to a new flask. Triturate the cell suspension thoroughly to redissociate the remaining clumps, and supplement with fresh growth factors and antibiotics by the above procedure.

Alternatively, centrifuge suspension aggregates and debris for 3.5 min at 400°—g, aspirate,

and either add the cells back to the original parent culture flask for further expansion or replate the primary cultures into 8 ml fresh pre-warmed NB-B27 complete medium containing 20 ng/ml EGF, 20 ng/ml bFGF, 10 ng/ml LIF, and 4 µl/ml Normocin.

17. Repeat steps 15 and 16 throughout the first few weeks of primary culture.
Initially, hNPC will proliferate throughout the flask as a mixture of adherent and free floating aggregates and can be detached from the culturing vessel through repeated trituration. We stress the inclusion of adherent monolayer-like hNPC within primary cultures during the initial hNPC expansion stage. As cultures mature, adherent hNPC cultures may also spontaneously give rise to a few spherical balls. These aggregates detach from the initial colony and continue to expand and self-renew as free-floating suspension cultures as well.

18. After several weeks, select the hNPC cultures that proliferate in a morphologically relevant manner and dissociate into single-cell suspensions or small clumps (3 to 8 cells/clump) with Accutase or cell dissociation buffer (CDB)/cellstripper. Dissociate when cellular aggregates are larger than 12 to 15 cells in diameter and can no longer be mechanically separated by simple trituration or when adherent cultures become greater than 75% confluent. Pool both adherent and free-floating cells and discard any remaining large clumps that do not readily dissociate.

19. Replate hNPC at a 1:1 or 1:2 ratio as either multilayer adherent aggregates or as suspension aggregates in NB-B-27 complete medium, 20 ng/ml EGF, 20 ng/ml bFGF, 10 ng/mL LIF, and 4 µg/ml Normocin for 2 more weeks.

20. Exchange one-half of the culture medium as described in step 15 every 2 to 3 days to

replenish growth factors and antibiotics. Dissociate and replate cultures (1:1 or 1:2) once per week or as necessary. After 2 weeks, exclude LIF and EGF for mitogen selection.

2.4.2.3 Mitogen-select primary hNPC cultures

After 2 to 4 weeks of primary expansion, undifferentiated hNPC colonies will proliferate and establish a healthy culture of precursors. At this point, successful cultures are subjected to a 10-week sequential growth factor selection process utilizing parameters of growth rather than markers alone to select for the proliferative EGF/FGF responsive population of cells.

21. Expand hNPC as a mixed population of both adherent clusters and free-floating aggregates in NB-B-27 complete medium containing 20 ng/ml bFGF alone (and 2 µl/ml Normocin) for 2 weeks with (1:1 or 1:2) dissociation once per week throughout the selection process as dictated by size exclusion and morphological parameters described above in step 18.

22. After 2 weeks, omit bFGF and supplement the medium with 20 ng/ml EGF alone (and 2 µl/ml Normocin) for 2 weeks.

23. Maintain the bFGF/EGF 2-week rotation schedule for two to three sequential rounds (10 weeks) and complete after the final bFGF-alone cycle.

24. After the final selection process, a few primary hNSC/hNPC cultures will continue to proliferate and display appropriate morphology; dissociate these cultures and pool together into NB-B27 complete medium containing 10 ng/ml LIF, for a final hNPC complete basal maintenance medium composed of NB-B-27 growth medium containing 20 ng/ml bFGF, 10

ng/ml LIF, and 2 μl/ml Normocin for secondary hNPC expansion.

2.4.3 Replating Dissociated hNSC on Extracellular Matrix (ECM) as Adherent Two-Dimensional Monolayer Cultures.

In addition to the MAN assay described in detail here, hNPC can also be replated onto a variety of extracellular matrix (ECM) components at 1–2 °— 106 cells/25-cm2 flask (maximum of 2–3 °— 106 cells/25-cm2 flask) to induce attachment for more traditional two-dimensional, adherent monolayer growth parameters (Fig. 2-4). As with MAN cultures, ECM attachment should not be utilized for low-density cultures where very few cell-cell contacts are present. The resulting cultures will likely become post-mitotic and differentiate prematurely. We prefer to expand primary hNPC lines without additional biological components, but we also recognize the utility and beneficial growth parameters that many ECM components confer in hNPC culture, especially when assaying and analyzing cells for migration and immunocytochemistry. That being said, not all ECM components are created equal, and each hNPC line will have its own particular characteristic adhesion properties. In our hands, hNPC tend to adhere to a variety of ECM proteins displaying a continuum for strength of adhesion—in order from weakest to strongest adhesion, fibronectin (human or mouse), laminin (human or mouse), Matrigel, collagen, and vitronectin. We recommend trying Millipore's ECM cell culture optimization assay to determine the optimal ECM protein and concentration desired for the specific growth parameters chosen. In addition, a number of commercially available cell-binding enhancement solutions (Cell Bind) or specially scaffolded substrates (Cell Web, Corning) are also available, with a variety of binding properties to circumvent the use of biological attachment substrates. Furthermore, pre-coating flasks with electrostatically charged molecules such as poly-D-lysine or poly-L-ornithine in combination with extracellular matrix proteins provide a secondary level of support, often conferring an

additional degree of adhesion. One warning is that poly-D-lysine should not be used for experiments involving electrophysiology, as it may interfere with ion-channel function.

In our hands, prolonged enhanced adhesion and exposure to matrix signaling molecules can have significant effects on hNPC phenotypic variation and related changes in cellular differentiation profile. For example, fibronectin supports a similar lightly adherent mode of growth to freshly dissociated MAN cultures on non-coated tissue culture–treated flasks, with the added benefit of slightly enhanced adhesion, quicker attachment, and higher rates of attachment. Laminin, likewise, retains many of the essential properties of the undifferentiated MAN, with the caveat that the initial adhesion is stronger, resulting in more flattened, monolayer-like, two-dimensional, multi-polar progenitor colonies. In slight contrast, Matrigel, a soluble basement membrane extract of the Engelbreth-Holm-Swarm tumor, which is composed mainly of laminin as well as collagen IV, heparin sulfate proteoglycans, and entactin, but contains trace amounts of the platelet-derived growth factor (PDGF), nerve growth factor (NGF), insulin-like growth factor-1 (IGF-1), and TGF-β, supports exuberant growth of highly mitotic, extremely adherent, bipolar and multipolar neural precursors that will self-assemble into a highly dynamic neural niche (Watt and Hogan, 2000; Palmer, 2002; Wurmser et al., 2004; Lathia et al., 2007) composed of a heterogeneous population resembling type A, B, and C cells of the subventricular (SVZ) niche (D.R. Wakeman, unpub. observ.). Furthermore, substrates such as collagen IV and vitronectin bind hNPC, conferring an exceptional propensity for attachment, but typically at the cost of mass cellular differentiation. These findings introduce a secondary criticism of ECM components, in that ECM molecules naturally guide neuronal migration (Thomas et al., 1996; Murase and Horwitz, 2002, 2004; Labat-Robert and Robert, 2005; Flanagan et al., 2006; Hall et al., 2008) and are thought to play a critical role in differentiation of hNPC in vivo. As a result, culturing hNPC in the presence of these molecules in vitro may actually trigger primary differentiation of hNPC and

an irreversible exit from the cell cycle. It is important, therefore, to choose an ECM accordingly and with respect to the specific assay of interest, as long-term cultures will adapt to their environment and may not continue to behave as true undifferentiated hNPC. We are comfortable with prolonged undifferentiated culture and expansion on either human fibronectin or human laminin (Ray et al., 1993; Vicario-Abejon et al., 1995; Walsh et al., 2005; Flanagan et al., 2006; Ray and Gage, 2006; Hall et al., 2008) and temporary undifferentiated growth on Matrigel for 1 to 2 weeks More adherent substrates such as vitronectin and collagen type IV are best utilized for differentiation assays. Specific brands and lots of ECM vary; therefore, proper testing is essential to determine individual growth parameters. It is worth noting that enzymatic lifting and dissociation of hNPC grown on strongly adherent ECM components generally require longer incubation times and often generate 10% to 20% cell death accordingly, due to the increased prevalence of fragile projections.

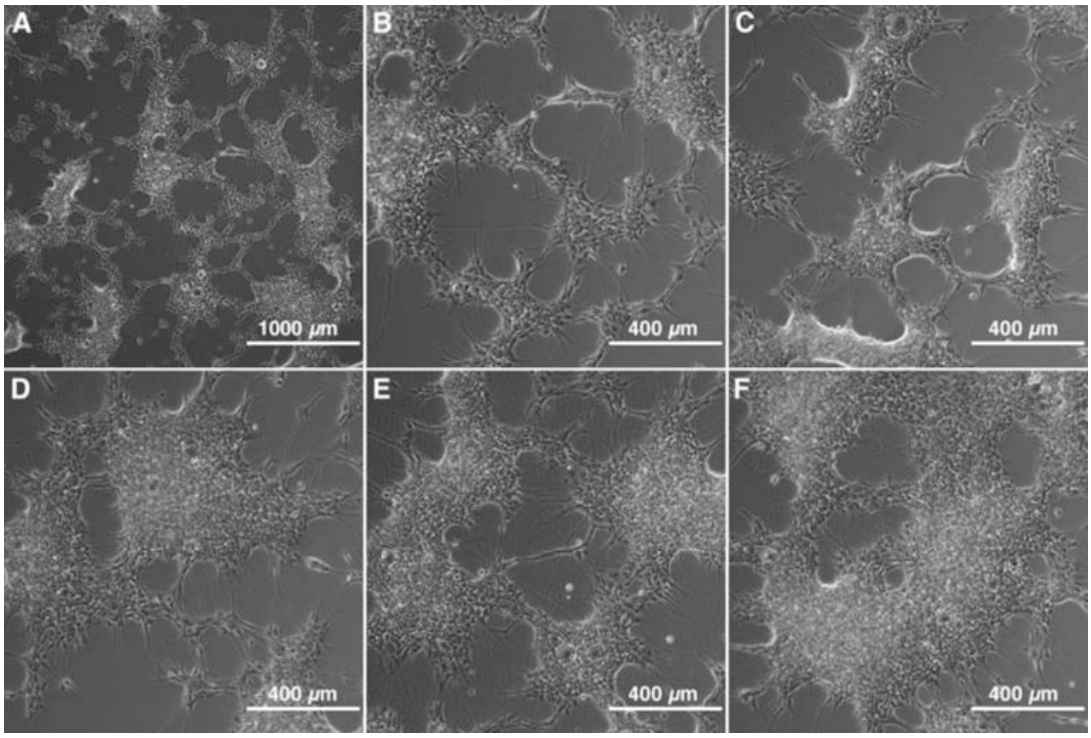


Figure 2-4. Extracellular matrix confers two-dimensional monolayer phenotype in hNSC cultures. Human NSC (HFB-2050) were plated onto tissue culture–treated flasks previously coated with a combination of poly-D-lysine and the extracellular matrix protein fibronectin. After 7 days in vitro, hNSC attain a similar composition and phenotype as MAN cultures, although they flatten and proliferate in a more two-dimensional manner in contrast to the three-dimensional architecture of MAN cultures (**A-C**). After 2 weeks, individual aggregate clusters are indistinguishable from each other, and begin to merge into a confluent layer of hNSC (**D,E**). In contrast to their MAN counterparts, these cultures will form a classic monolayer and lose their honeycomb appearance (**F**).

2.5 LABELING hNPC PRE-TRANSPLANTATION

In order to identify transplanted donor cells, hNPC must be pre-labeled chemically or genetically with a definitive nontransferable marker. One can also utilize human-specific antibodies, such as huNuc, after transplantation to recognize donor-derived cells. For proper validation, hNPC should be identified with at least two of these markers and preferably three to ensure that results are not simply false-positive artifacts. We generally prefer colocalization of BrdU and a reporter gene with at least one human-specific epitope to locate successful donor grafts. Upon implantation into the mammalian CNS, HNPC may undergo one to three

rounds of division before becoming post-mitotic; therefore, non-integrating labels will not become too diluted for later detection. Theoretically, thymidine analogs, iron particles, and lipophilic dyes become diluted by a factor of ½ for every symmetrical cell division; therefore, these markers can become diluted below standard detection levels within five to six cell divisions. Careful selection of labeling method should be based on the specific assay of interest.

2.5.1 Labeling hNPC with BrdU

Human NPC that have not been genetically labeled are preincubated with the S phase, DNA-intercalating thymidine analog, bromodeoxyuridine (BrdU) for proper postmortem graft identification. Both monoclonal (Gratzner, 1982) and polyclonal antibodies have been raised to detect BrdU using immunofluorescence and multiphoton confocal microscopy for graft analysis. As a result, BrdU was reinforced as a popular prelabeling technique for grafting proliferative cellular substrates. Although BrdU only labels at best 42% to 50% (neurospheres) to 74% to 82% (MAN cultures; Fig. 2-5) of donor hNPC nuclei (in our experience), presents a variety of false-positive artifacts (Rakic, 2002a,b; Burns et al., 2006), and can be highly toxic when administered for extended periods of time (Caldwell et al., 2005), it has remained one of the most highly used pre-transplantation labeling methods to verify donor cell origin (Dolbeare, 1995, 1996; Carbajo et al., 1995; Carbajo-Perez et al., 1995).

Materials

NB-B-27 complete medium (see recipe)

Normocin (InvivoGEN, cat. no. ant-nr-1)

Leukemia inhibitor factor (LIF; Millipore, cat. no. LIF1010)

Basic fibroblast growth factor (bFGF; Millipore, cat. no. GF003)

5-bromo-2_-deoxyuridine (BrdU; Sigma, cat. no. 59-14-3; see recipe)

25-cm2 tissue culture flasks

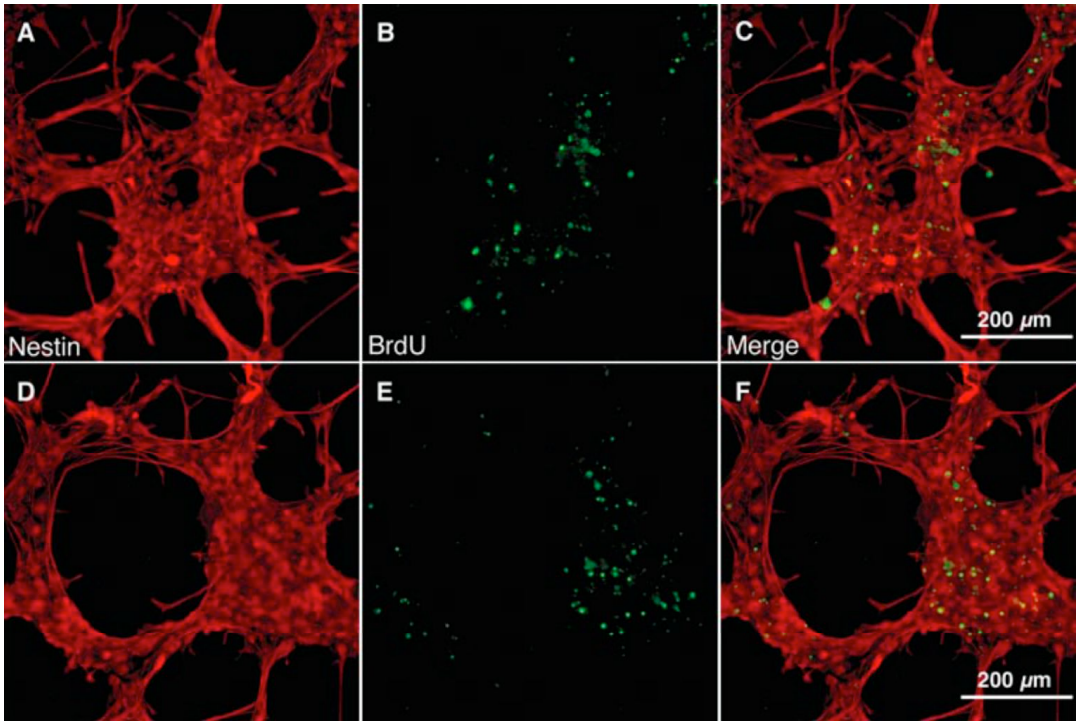


Figure 2-5. hNSC MAN cultures incorporate BrdU. Human HSC (HFB-2050) grown as multilayer adherent networks express nestin (**A,D**) extensively and readily incorporate BrdU (**B,E**), indicating that they remain in a highly proliferative, immature state throughout MAN culture. Of interest, cells within the clusters appear to proliferate preferentially in comparison to their peripheral counterparts (**C,F**), indicating a possible niche component within each adherent cluster.

1. To efficiently label cells, dissociate hNPC 48 to 72 hr prior to transplantation and replate as described in Basic Protocol 1 or Alternate Protocol 1, as applicable, using fresh NB-B-27 complete medium containing 2 μl/ml Normocin, 10 ng/ml LIF, 20 ng/ml bFGF, and 10 to 20 μM BrdU (added from 1000°— stock).

BrdU is highly toxic to low-density hNPC cultures; therefore, we recommend plating cells at no less than 2 °— 106 cells/25 cm2.

CAUTION: *BrdU acts by incorporation in the place of thymidine during DNA synthesis, and thus may cause birth defects or heritable genetic effects. Be extremely careful when handing BrdU.*

2. Allow cultures to equilibrate and return to homeostasis in a 37◦C,5%CO2 humidified incubator.

Low-density cultures fail to equilibrate properly and display extremely slow division rates, impeding efficient labeling during S-phase.

3. Every 24 to 36 hr, replenish the medium by adding fresh 1000°— BrdU to 1°— final, LIF to 10 ng/ml final, and bFGF to 20 ng/ml final. Lightly triturate.

Gentle trituration of cells is absolutely essential to reduce merging of cell clusters and formation of premature aggregates before transplantation. Cells are extremely fragile when incubating in BrdU; therefore, slow trituration is recommended to avoid shearing.

4. After a total of 48 to 72 hr, prepare hNPC for transplantation as described (see Basic Protocols 6 and 7).

In our hands, 40% to 50% (neurospheres) and 70% to 80% (MAN) of the total hNPC population will be labeled after 48 to 72 hr. It should be noted that longer incubation times are notorious for introducing false positives into nondividing cells. Furthermore, hNPC do not proliferate well past 3 to 4 days in media that include BrdU (D.R.Wakeman, unpub. observ.), indicating a time threshold for toxicity.

2.5.2.1 Lentiviral Infection of hNPC

Traditional methods for prelabeling NPC prior to transplantation require harsh DNA intercalating thymidine analogs such as BrdU and CldU, which have been shown to result in great underestimation of overall engraftment success and create a variety of false positives when administered for extensive periods of time. Generation of independently labeled fluorescent reporters, animals, and cell lines eliminates the need for these toxic compounds while increasing both cell viability and engraftment efficiency (Shimomura et al., 1962; Chalfie et al., 1994, 1995;Ward et al., 1998; Zhang et al., 2002; Tsien, 2003; Vintersten et al., 2004; Shaner et al., 2005, 2008; Shimomura, 2005; Giepmans et al., 2006). In addition, fluorescently labeled donor cells can be easily identified among their host counterparts, allowing for enhanced visualization of axonal and dendritic processes. These optical properties also allow us to perform classic electrophysiological assays to test the synaptic potential of differentiated cells and access the overall multi-potentiality of each subline.

Lentiviral infection has proven a reliable way to stably express genes of interest (~8 kb) into slowly dividing hNPC with little to no long-term effects on behavior or morphological phenotype (Consiglio et al., 2004; Capowski et al., 2007; also see *UNIT 2D.2*). We have used the following protocols to generate hNPC engineered to constitutively express second and third-generation lentiviruses carrying either a cytosolic CAG-eGFP (kind gift of Mark H. Tuszynski) or PGK-mCherry (kind gift of Mark Mercola) fluorescent marker protein for greater than 5 months with 75% mCherry and 85% eGFP (aggregate) and 89% mCherry and 98% eGFP (MAN) efficiency for aggregate and MAN cultures respectively.

All procedures involving live infection-competent lentivirus are performed in a Level 2 or better biosafety hood in accordance with your institution's specific safety standards. We recommend full disposable safety coat, sleeves, glasses, and double nitrile and latex gloves for adequate personal protection. Any materials (pipets, tips, flasks, conical tubes) that come into

contact with virus should be properly sanitized by soaking for at least 20 min in 10% to 20% (v/v) bleach, followed by 15 min in 70% ethanol, and properly disposed of according to safety regulations. Medium is prepared fresh prior to infection. Conditioned medium can be utilized, but metabolic components may influence the overall efficiency in cell lines (D.R. Wakeman, unpub. observ.) and subsequent gene expression (McCarthy et al., 1995).

Aggregate cultures can be infected as either single cells at high density or as small size clusters (8 to 16 cells/cluster) to medium-size clusters (16 to 32 cells/cluster) (Fig. 2-6). Utilizing cellular aggregates has the added benefit of essential cell-cell contacts, whereas single cells must be plated at high density to induce cell-cell contacts quickly following infection. In addition, larger aggregates contain internal cells that may not be exposed to the viral particles, resulting in decreased infection efficiency. Although most of these cells are migratory within each cluster and eventually become labeled with increased incubation times, we recommend infecting hNPC as either small cellular aggregates or high-density single cells (3–4 °— 104 cells/cm2), so that all cells are accessible to viral particles for optimal infection efficiency.

Materials

Human NPC, single cells or lightly adherent aggregates (Basic Protocol 1 or Alternate Protocol 1)

NB-B-27 complete medium (see recipe)

Normocin (InvivoGEN, cat. no. ant-nr-1)

Leukemia inhibitor factor (LIF; Millipore, cat. no. LIF1010)

Basic fibroblast growth factor (bFGF; Millipore, cat. no. GF003)

10 mg/ml polybrene (Chemicon)

Lentivirus (see protocol introduction for information)

Dulbecco's PBS with Ca2+ and Mg2+ (DPBS; Mediatech, cat. no. 21-030-CM)

25-cm2 tissue culture flasks

15-ml conical tubes

1. Dissociate hNPC as described in Basic Protocol 1 or Alternate Protocol 1, as applicable, immediately prior to infection, or utilize small clusters (dissociated 48 to 72 hr before infection, similar to cryopreservation or transplantation).

2. Replate hNPC in 6 ml fresh NB-B-27 complete medium containing 2 µl/ml Normocin, 10 ng/ml LIF, 20 ng/ml bFGF, and 6 µl of 10 mg/ml polybrene in a 25-cm2 flask.
Polybrene enhances the infection efficiency but can be omitted if desired.

3. Carefully add lentivirus at a concentration of 100 ng p24 particles for every 1 °— 106 cells.
CAUTION: *Properly sanitize any virus-exposed waste with bleach and ethanol.*

4. Incubate in a 37◦C, 5% CO2 humidified incubator for 12 to 48 hr.
Longer incubation times will result in higher efficiency rates and expression levels, but at the cost of multiple integration sites.

5. To remove any remaining live virus, transfer hNPC to a 15-ml conical tube, rinse the flask once with 10 ml DPBS, combine with the cells in the tube, and allow the cells to settle by gravity. Aspirate the supernatant and repeat 10-ml DPBS rinse three times.
Alternatively, centrifuge contents for 4 min at 400 °— g in a lentivirus-approved containment centrifuge.

6. After final rinse, add 8 ml fresh NB-B-27 complete medium containing 2 µl/ml Normocin, 10 ng/ml LIF, and 20 ng/ml bFGF. Incubate in a at 37◦C, 5% CO2 humidified incubator.

7. Repeat DPBS rinse every 24 hr for the first 7 days to remove any residual viral particles.

Excessive trituration and centrifugation can be detrimental to hNPC survival during this crucial time period. We recommend replating single cells as high-density aggregate or MAN cultures to enhance paracrine signaling required for enhanced expansion. Extensive expression of both transgenes is seen after 48 to 72 hr at an efficiency of 82% to 85% for eGFP and 75% to 79% for mCherry single cells and small clusters, respectively. Larger aggregates are much more variable and range between 45% to 85% in labeling efficiency.

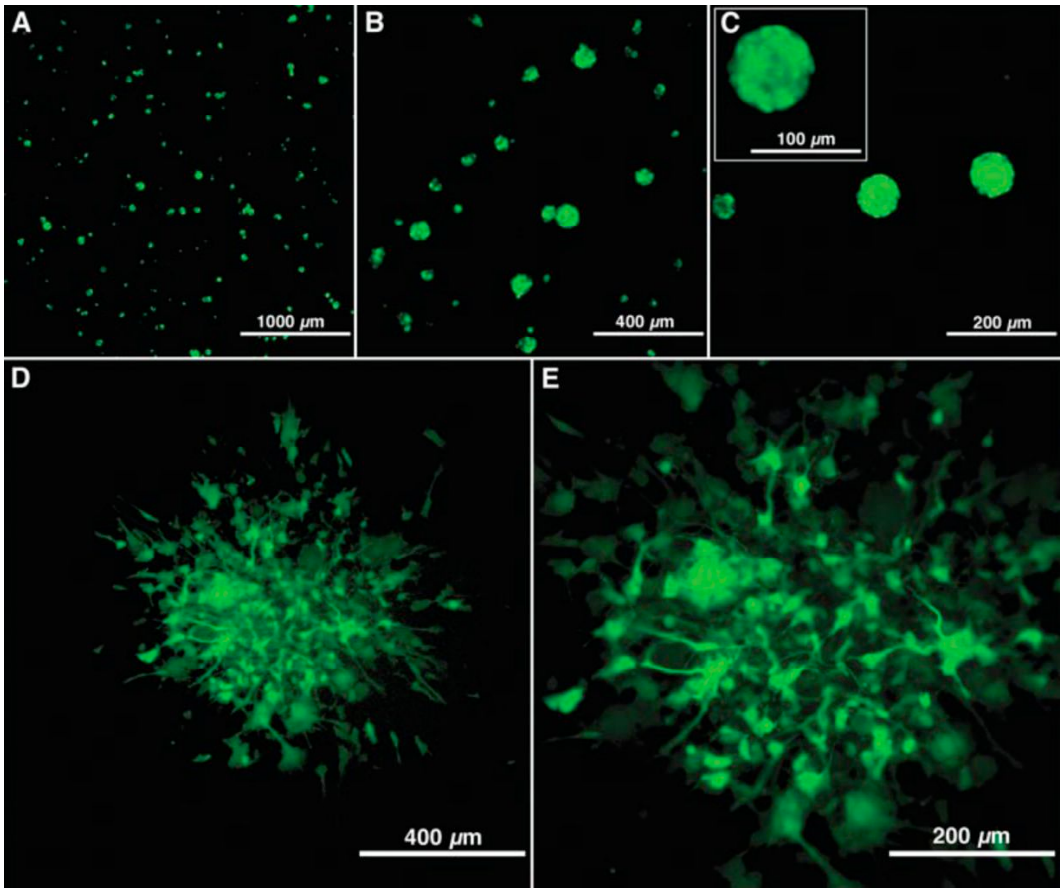


Figure 2-6. Aggregate hNSC cultures express lentiviral eGFP. Human NSC (HFB-2050) were dissociated into single cells at medium density (1°—106 cells/25 cm2) and cultured as lightly adherent aggregates (with trituration every 12 hr) for 48 hr before transfection with a CAG-eGFP lentivirus. 72 hr after exposure (5 days in vitro), free-floating spherical aggregates readily expressed the transgene (**A-C**). Inset in (C), a close-up of the aggregate in the middle of the frame. When plated onto poly-D-lysine-coated tissue culture–treated flasks, hNPC-eGFP aggregates rapidly attached and flattened, confirming 80% to 90% efficiency, and sustained eGFP expression in vitro (**D,E**). Green = live eGFP expression.

2.5.2.2 Lentiviral Infection of Multilayer Adherent Network (MAN)

The MAN assay allows for temporary modification and optimization during lentivirus infections, due to the ease of changing medium; therefore, we reduce the volumes of culture medium to 5 ml fresh hNPC medium in each 25-cm2 flask to concentrate virus and decrease waste. We have applied the following procedure to new and mature (60% to 80% confluent) MAN hNPC cultures (3–5 °— 106 cells/flask), infecting with lentivirus containing either a

PKC or CAG promoter–driven cytosolic eGFP (Matz et al., 2002; Tao et al., 2007) or mCherry (Merzlyak et al., 2007) fluorescent protein (Figures. 2-7 and 2-8).

Additional Materials

60% to 80% confluent (2 to 3 weeks in vitro) MAN hNPC culture in 25-cm2 flask (Basic Protocol 1) or in transmembrane basket

6-well tissue culture plates

Hanging Basket Cell Culture Insert, 1.0 μm (Millipore, cat. no. PIRP30R48)

25-cm2 tissue culture flasks

1. Replace 100% of the medium in 60% to 80% confluent MAN hNPC culture (in 25- cm2 flask) with 5ml fresh NB-B-27 complete medium containing 2 μl/ml Normocin, 10 ng/ml LIF, 20 ng/ml bFGF, and 5 μl of 10 mg/ml polybrene.

Polybrene enhances the infection efficiency but can be omitted if desired.

2. Carefully add the lentivirus at a concentration of 100 ng p24 particles/1 °— 106 cells.

CAUTION: *Properly sanitize any virus-exposed waste with bleach and ethanol. For a 60% to 80% confluent culture, there will be 3–4 °— 106 cells per 25-cm2 flask, or 1–2 °— 106 cells/ hanging basket cell culture insert.*

3. Incubate 12 to 48 hr in a humidified 37◦C, 5% CO2 incubator.

Longer incubation times will result in higher efficiency rates and expression levels but at the cost of multiple integration sites.

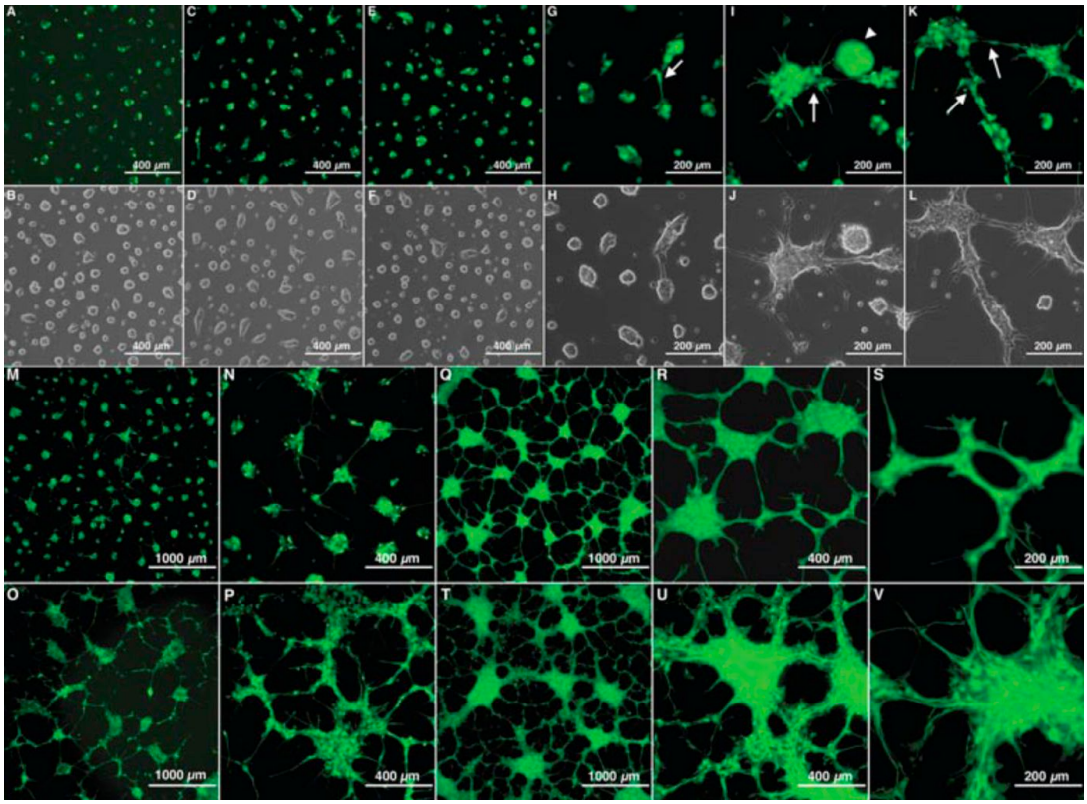


Figure 2-7. MAN cultures efficiently express lentiviral eGFP. Human neural stem cells (HFB-2050) were dissociated into single cells and exposed to a CAG-eGFP lentivirus (as described in text) upon replating (2.5 °— 106 cells/25-cm2 flask). hNSC were allowed to attach without agitation to induce formation of multilayer adherent networks (MAN). (**A,B**) 48 hr post-exposure, hNSC formed small, evenly spaced, adherent aggregate clusters and express the eGFP transgene. (**C-H**). By 72 hr, transgene expression markedly increased to nearly 90% of hNSC constitutively expressing eGFP throughout the cytoplasm. In addition, adherent clusters continued to proliferate and spread, making initial contacts with neighboring colonies (arrow). (**I-L**) Over the next week, adherent hNSC aggregates rapidly proliferated and actively migrated between adherent three-dimensional aggregates, creating the initial foundation for the multilayer adherent network. (**I-J**) Many peripheral anchor cells displayed long protruding feet resembling growth cones that sampled the local microenvironment and rapidly reorganized in response to local guidance cues (arrow). In addition, some clusters detached and continued to proliferate as floating suspension aggregates (arrowhead). (**K-N**) After 10 days, multilayer adherent clusters began to coalesce, established the classic honeycomb architecture, and actively exchanged migratory proliferative cells between colonies. Individual colonies became unrecognizable as the meandering protrusions of neighboring clusters (arrows) met and rapidly joined into one two-way hNSC highway. (**O, P**) At 15 days, MAN cultures continued to proliferate and expand across the culturing surface, covering nearly 40% to 60% of the flask by 20 days (**Q-S**). After 30 days, hNSC (HFB-2050- eGFP) assumed mature form (**T-V**), covering nearly 70% of the culturing surface and were ready to be dissociated. MAN cultures can be maintained for up to 6 weeks; however, overcrowding generally leads to a reduced proliferation rate, and should be avoided if possible. Green = live eGFP expression.

4. Remove any remaining virus by rinsing five times with 5 ml DPBS.

One of the greatest benefits of utilizing the MAN assay is the ease of changing medium and rinsing live virus from the culture without extensive manipulation or harsh mechanical stress. Using PBS without salts often leads to detachment of cells from the flask; therefore MAN cultures are always rinsed with DPBS containing both Mg2+ and Ca2+.

5. Add 8 ml fresh NB-B-27 complete medium containing 2 µl/ml Normocin, 10 ng/ml LIF, and 20 ng/ml bFGF. Incubate in a humidified 37◦C, 5% CO2 incubator. 6. Repeat steps 4 and 5 every 12 hr for the first 7 days to remove any residual viral particles.

Theoretically, all live virus will be removed with the first few rinses; however, we err on the side of safety to ensure adequate removal of all viral particles over time. In fact, we treat lentiviral-infected cultures as if they contain live virus until the cultures have been fully dissociated and passaged at least three times.

7. After 1 to 2 weeks in culture, dissociate (1:3) and expand the newly labeled hNPC population.

Extensive expression of both transgenes was seen after 48 to 72 hr at an efficiency of 98% eGFP and 89% mCherry. After three to five additional population doublings, fluorescently labeled hNPC populations are pooled together and sorted by FACS into three polyclonal populations based on relative fluorescence intensity, labeled low-, medium-, and high intensity expression. These cells are then expanded in 25-cm2 or 75-cm2 tissue-culture treated flasks (Falcon) as MAN or lightly adherent aggregate cultures and frozen for future expansion. Polyclonal subpopulations can then be individually subcloned and transplanted intraventricularly into neonatal (P0) mice to assay both hNPC migration and differentiation capacity.

8. As an example, perform MMS lentiviral infection of hNSC (HFB-2050) as follows.
The human NPC line HFB-2050 was engineered to constitutively express either a cytosolic eGFP or mCherry fluorescent marker protein by the following methods. After 20 days of culture, hNPC MMS-multilayer adherent networks were infected with lentivirus containing either a cytosolic eGFP or mCherry protein driven by the CAG or PKC promoters respectively (Fig. 2-8).

a. Because the MMS allows for temporary modification and optimization during lentivirus infections due to the basket's inherent mobility, reduce the volume of culture medium to 1.5 ml fresh NB-B-27 complete medium containing 2 µl/ml Normocin, 10 ng/ml LIF, 20 ng/ml bFGF in each well of a 6-well dish, and insert a transmembrane basket with attached hNSC into each well.
For a 60% to 80% confluent culture, there will be 1–2 °— 106 cells per hanging basket cell

b. To each basket insert, add 10 mg/ml polybrene and lentivirus at a concentration of 100 ng p24 particles/1 °— 106 cells. Incubate in humidified 37◦C, 5% CO2 incubator.
CAUTION: *Properly sanitize any virus-exposed waste with bleach and ethanol.*

c. At a time point 48 hr later, aspirate viral waste and wash hNPC five times for 30 sec each in 5 ml DPBS. Transfer the basket insert to a new 6-well plate and return to standard culture medium volumes.
This procedure is repeated every day for 7 days to remove any residual viral particles.
Extensive expression of both transgenes was seen in HFB-2050 after 72 hr at an efficiency of 98% eGFP and 89% mCherry (quantified by FACS).

d. After 7 days, dissociate hNPC networks into single-cell suspension by adding 1.5 ml per well of Accutase and seed one well into one 25-cm2 flask with 8 ml NB-B-27 medium containing 2 µl/ml Normocin, 10 ng/ml LIF, and 20 ng/ml bFGF per flask, to induce MAN or lightly adherent aggregate cultures.

In the case of HFB-2050, each well was dissociated into a 25-cm2 flask, cultured, and expanded as a webbed MAN for 20 days before passaging again into one 75-cm2. After three total passages (90 days), all hNSCs were pooled together and sorted by FACS into three polyclonal populations based on relative fluorescence intensity, labeled low-, medium-, and high-intensity expression. These cells were then expanded in 25-cm2 or 75-cm2 tissue culture–treated flasks (Falcon) in modified adherent networks or as suspension aggregates, frozen for future expansion, or cloned and transplanted into (P0) neonatal mice intraventricularly (Basic Protocol 8) to assay both migration and differentiation capacity.

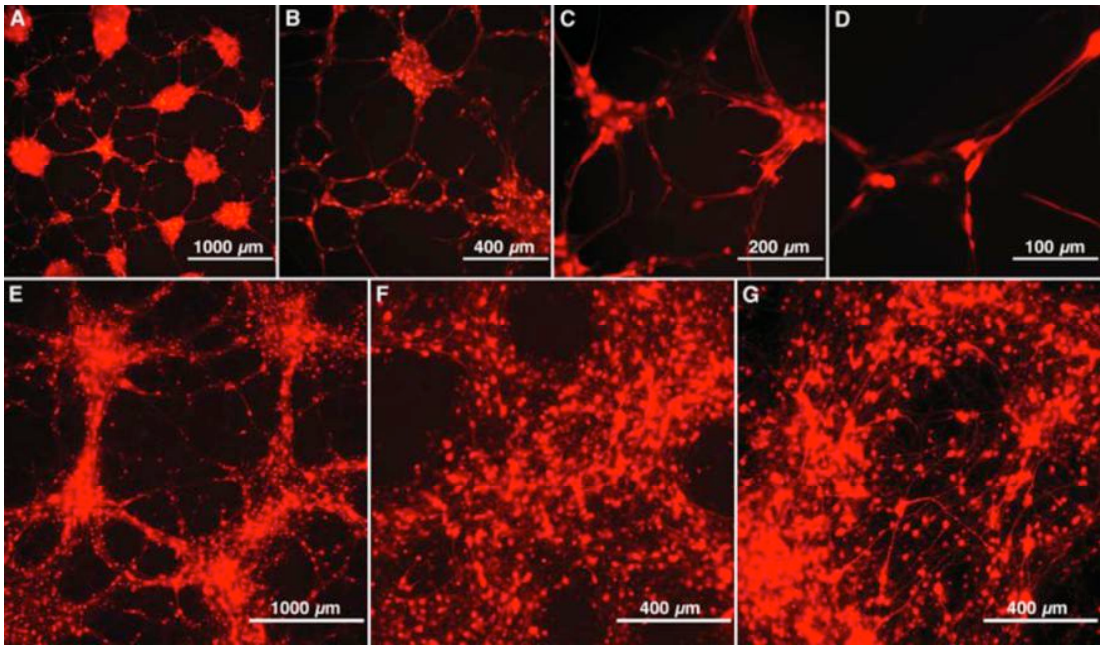


Figure 2-8. MAN and MMS mCherry lentivirus expression. Human NSC (HFB-2050) grown as multilayer adherent aggregate (MAN) cultures maintain expression of the PGK-mCherry transgene 10 days post transfection (**A-D**). In addition, the MAN culturing technique can be adapted to a semi-porous membrane culturing surface and grown using the MMS technique, highly resembling MAN cultures in phenotype and composition. MMS cultures are then easily transfected with viral vectors (as described in the text) to produce fluorescent reporter cultures that are easy to manipulate (**E-G**).

2.5.3 Labeling hNPC with Super-Paramagnetic Iron Oxide (SPIO)

To follow survival and migration of transplanted donor hNPC in vivo, it has become increasingly important to develop noninvasive techniques (Manganas et al., 2007; Gilad et al., 2008; Ruiz-Cabello et al., 2008) for monitoring and imaging engrafted donor cells (Rogers et al., 2006; Slotkin et al., 2007; Schroeder, 2008; Sumner et al., 2007). Common post-mortem immunohistological staining techniques do not afford the opportunity to trace migration within the same animal over time; however, hNPC transfected with super-paramagnetic iron oxide (SPIO) particles have been used with MRI in rodents to successfully track migration of stem cells after engraftment in the normal and diseased animal (Lewin et al., 2000; Bulte et al., 2002; Frank et al., 2003; Hinds et al., 2003; Arbab et al., 2003a,b, 2004a,b; Jendelova et

al., 2004; Miyoshi et al., 2005; Magnitsky et al., 2005; Sykova and Jendelova, 2005, 2006, 2007a,b; Lepore et al., 2006a,b; Shapiro et al., 2006; Guzman et al., 2007, 2008; Politi et al., 2007; Walczak and Bulte, 2007; Neri et al., 2008; Walczak et al., 2008). Successfully labeled cells can be detected and followed weeks to months after implantation as they migrate contralaterally through predominantly white matter to sites of pathological insult. In addition, MRI findings can then be verified post-mortem utilizing immunohistochemistry by costaining for human specific markers and iron particles (Prussian blue or dextran staining) utilizing the MRI guided coordinates.

Additional Materials

Human NPC

Feridex (Bayer Healthcare Pharmaceuticals, cat. no. NDC-59338-7035-5)

Protamine sulfate injection, USP, 50 mg/5 ml (Bayer Healthcare Pharmaceuticals,

cat no. NDC-63323-229-05)

25-cm2 tissue culture flasks

15-ml conical tubes

Centrifuge

1. Dissociate hNPC into single cells as applicable. Replate 2–3 °— 106 cells in a 25-cm2 flask containing 5 ml of 25% CM/75% fresh NB-B-27 complete medium /10 ng/ml LIF/20 ng/ml bFGF/2 µl/ml Normocin. Incubate for 6 hr at in a 37◦C, 5% CO2 humidified incubator. *Alternatively, skip the incubation and proceed directly to step 2.We prefer to allow freshly dissociated hNPC to recover for 6 to 12 hr after dissociation to increase viability before SPIO incubation.*

2. Prepare 2°— SPIO mixture, 45 to 60 min before labeling, by adding 10 to 20 μg/ml Feridex and 5 μg/ml protamine sulfate to 5 ml fresh NB-B-27 growth medium containing 10 ng/ml LIF and 20 ng/ml bFGF. Incubate in a 37◦C water bath for 45 to 60 min with mixing.
We have had the best short-term in vitro results utilizing a final working concentration of 10 μg/ml Feridex.

3. Add 5 ml of 2°— SPIO solution to the medium over the plated cells from step 1 and triturate gently for a final working concentration of 5 to 10 μg/ml Feridex/2.5 μg/ml protamine sulfate. Incubate for 20 to 24 hr in a 37◦C, 5% CO2 humidified incubator to allow iron particles to enter the cell by endosomal transport.
Increasing the Feridex concentration will enhance the overall signal but can result in intracellular clumping and aggregation of iron particles that may be detrimental to the long-term viability, stability, and behavioral phenotype of the cell.

4. After 20 to 24 hr, gently triturate hNPC and transfer the contents to a 15-ml conical tube. Rinse the flask once with 4 ml DPBS and add to the conical tube. Centrifuge for 3 to 4 min at 400 °— *g*, room temperature.
Increasing the incubation time will enhance the number of iron particles that enter the cell by only a small percentage and possibly at the cost of cellular differentiation or decreased post-incubation survival. In addition, the total number of particles per cell will vary within the individual culture. 24 hr is sufficient for nearly 95% labeling efficiency (Figure 2-9).

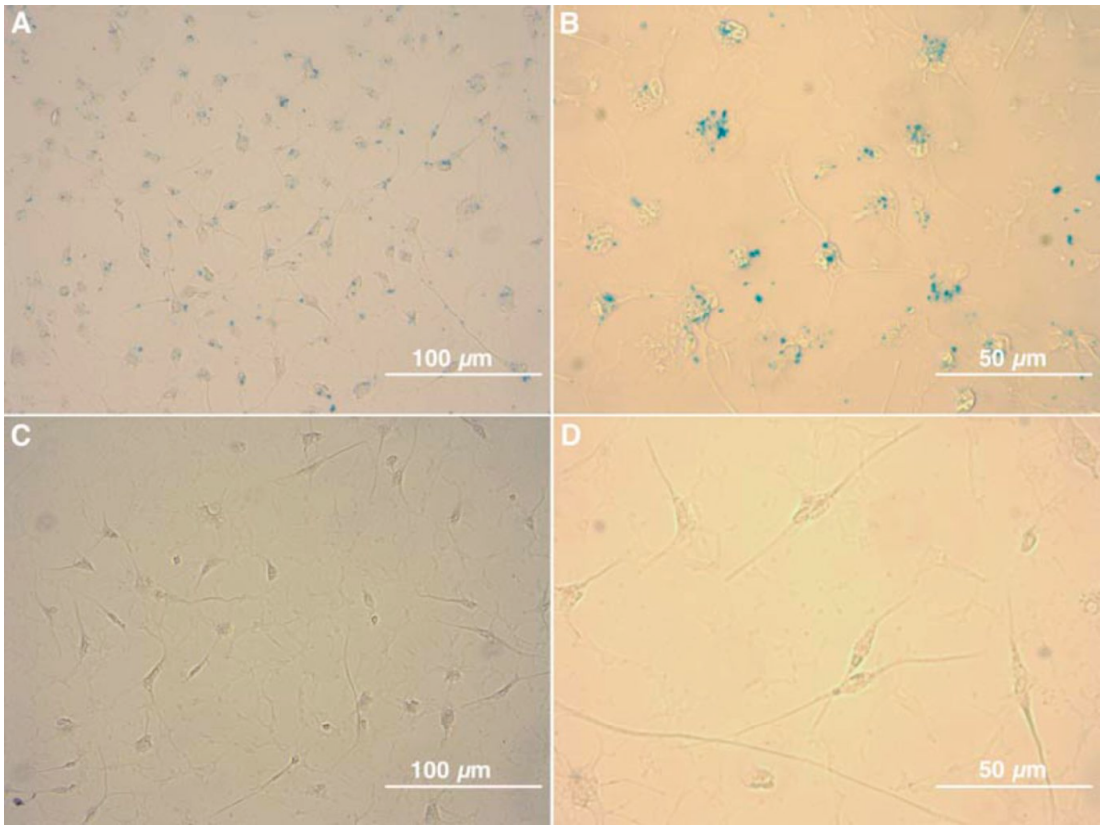


Figure 2-9. hNPC readily engulf SPIO particles in vitro. Human NSC (HFB-2050) were plated onto Matrigel with SPIO particles for 20 hr and processed with Perl's Prussian blue to detect labeled cells. Most hNSC readily took in the Feridex particles (blue particles) (**A,B**), whereas unlabeled control cells (**C,D**) were completely devoid of blue staining.

5. Vacuum aspirate the supernatant and rinse by resuspending in 8mlDPBS. Centrifuge for 3 to 4 min at 400 °— *g*, room temperature. Repeat DPBS rinse twice.

6. Resuspend the SPIO-labeled hNPC pellet in 8 ml fresh prewarmed NB-B-27 complete medium/10 ng/ml LIF/20 ng/ml bFGF/2 µl/ml Normocin. Replate in a 25-cm2 tissue culture flask and incubate in a 37◦C, 5% CO2 humidified incubator for 6 to 12 hr, or proceed immediately to transplantation procedures.

Alternatively, replate SPIO-labeled hNPC onto Matrigel-coated 24-well plates for in vitro characterization and differentiation assays. Recommended plating densities are 50,000

cells/well for a 24-well plate, 100,000 cells/well for a 12-well plate, or 250,000 cells/well for a 6-well plate. The hNPC may also be labeled 24 hr after plating onto Matrigel by simply adding the SPIO mixture to the newly established adherent monolayer culture.

2.6 PREPARING hNPC FOR TRANSPLANTATION

Human NPC injections are performed with cells in a uniformly undifferentiated state, displaying log-phase growth, and able to incorporate BrdU during DNA synthesis. To enhance synchronization, undifferentiated hNPC are dissociated 2 days prior to transplantation and disaggregated into a single-cell suspension immediately prior to surgery. Maintaining hNPC in vitro for a longer period of time prior to injection promotes clumping due to spontaneous elaboration of extracellular matrix (ECM) or subsequent premature spontaneous cellular differentiation, both of which may deter efficient engraftment. Careful dissociation of hNPC is essential for sustained viability and long term post-transplantation survival.

Many global neurodegenerative disorders affect multiple CNS cell types; therefore, undifferentiated hNPC provide the plasticity needed for the host microenvironment to naturally direct differentiation into multiple phenotypes that may be necessary to ameliorate and restore the host cytoarchitectural milieu. On the other hand, focal CNS disorders may benefit more from lineage-directed pre-differentiation strategies, as a priori ex vivo manipulation of hNPC along specific neuronal fate pathways may be more suitable for alleviating lineage-specific CNS deficits. These cells are presumably lineage-restricted and committed, effectively eliminating nonspecific, regionally inappropriate differentiation that could lead to potentially detrimental off-target side effects. In contrast to their undifferentiated hNPC counterparts, pre-differentiated precursors and mature post-mitotic neurons to do not have the same capacity for long-distance migration or the multipotency beneficial to global neurodegenerative diseases (Le Belle et al., 2004). The decision to implant a semi-

homogenous population of undifferentiated hNPC or lineage-defined progenitors is ultimately determined by the specific cellular properties that best suit the experimental paradigm (Svendsen et al., 1997a; Armstrong et al., 2000; Teng et al., 2002; Pluchino et al., 2003; Burnstein et al., 2004;Kelly et al., 2004; McBride et al., 2004;Yasuhara et al., 2006; Lee, H.J. et al., 2007; Lee, J.P. et al., 2007; Tarasenko et al., 2007). Both strategies have their merits, allowing the natural host tissue to effectively direct maturation and terminal differentiation in accordance with local signaling cues (Fricker et al., 1999).

Materials

Human NPC (hNPC): small to medium aggregate clusters (Basic Protocol 1 or Alternate Protocol 1)

Dulbecco's PBS with Ca2+ and Mg2+ (DPBS; Mediatech, cat. no. 21-030-CM)

0.4% (v/v) trypan blue

Hemacytometer (*UNIT 1C.3*)

Additional reagents and equipment for counting viable cells using a hemacytometer and trypan blue (*UNIT 1C.3*)

1. Triturate hNPC lightly against the bottom of the flask to detach any cellular aggregates that may have attached, and transfer the contents to a 15-ml conical tube. Centrifuge for 3 to 4 min at 400 °— *g*, room temperature.

Utilize small- to medium-size aggregates plated 48 hr before transplantation.

2. Dissociate hNPC by the methods previously described (Basic Protocol 1 or Alternate Protocol 1, as applicable) and resuspend in 10 ml cold DPBS.

Dissociation should not take longer than 1 to 3 min to fully break apart small aggregates.

3. Centrifuge the 15-ml conical tube for 4 min at 400 °— *g*, room temperature. Remove supernatant. Repeat steps 2 to 3.

4. With a pipettor and 100-µl pipet tip, carefully resuspend the hNPC pellet in a small volume of cold DPBS (20 to 40 µl), approximately equal to the volume of the cell pellet, and transfer into a 1.5-ml microcentrifuge tube.

Resuspend hNPC in half the volume of DPBS first, then wash the 15-ml tube with the second half volume and transfer all cells into the 1.5-ml microcentrifuge tube. Be very careful not to triturate aggressively, as hNPC will easily shear when resuspended at such high density.

5. Remove 2 µl of cell solution and transfer to a microcentrifuge tube containing 17 µl DPBS, add 1 µl of 0.4% trypan blue to the tube, and triturate well.

6. Count the cells with a hemacytometer, making sure to factor in the 1:10 dilution (*UNIT 1C.3*).

The purpose of this dilution is to use the fewest cells possible for counting, while maintaining accuracy.

7. Adjust the amount of DPBS in the microcentrifuge tube as needed to achieve the desired cell concentration.

We aim for ~50,000 cells/µl as an optimal concentration for transplantation. Cells should not be suspended at higher than 100,000 cells/µl for long periods of time, as excessive cell death will likely occur, resulting in a sticky DNA precipitate that will easily clog the needle.

8. Immediately following resuspension in DPBS, place the vial of hNPC onto wet ice (4◦C) and gently flick the tube every minute to deter clumping of cells that may clog the needle during stereotactic injection. Proceed without delay to Basic Protocol 7.

Cells should be utilized as quickly as possible to reduce cell death and increase overall viability and engraftment success. For multiple animal studies, it is beneficial to prepare separate biological replicate batches as the procedures continue, preparing enough cells for use no longer than 1 hr post-dissociation.

2.7 LOADING AND INJECTION OF hNPC FOR TRANSPLANTATION INTO ST. KITTS AFRICAN GREEN MONKEY

The following methods have been refined over a number of years for stereotaxic injection of cells and tissue into the nigrostriatal system of monkeys. There are some variations in the methods which other investigators have used, but this procedure has evolved to work successfully in our hands (Redmond et al., 1986, 1988; Taylor et al., 1995; Sladek et al., 1995, 2008; Bjugstad et al., 2005, 2008; Wakeman et al., 2006; Redmond et al., 2007, 2008).

NOTE: All protocols involving live animals must be reviewed and approved by an Institutional Committee for Ethical Animal Care and Use (IACUC) and must conform to government regulations for the care and use of laboratory animals.

Materials

Experimental animals: St. Kitts African Green Monkey (MPTP-treated or PBS sham control)

Ketamine hydrochloride injection, USP (Ketaset, Fort Dodge Animal Health)

Atropine for i.m. or s.c. administration

Pentobarbital for i.v. administration

Lubrivet (optional; Butler Animal Health; *http://www.accessbutler.com/*)

Dulbecco's PBS with Ca2+ and Mg2+ (DPBS; Mediatech, cat. no. 21-030-CM)

70% ethanol

Human NPC (hNPC; Basic Protocol 6), labeled according to any of the pretransplantation labeling procedures described in this unit

NB-B-27 complete medium (see recipe) without Normocin

Phosphate-buffered saline (PBS)

100-µl syringe (Hamilton) for stereotactic injection

22-G, 2-in. needle (Hamilton) for stereotactic injection

Single syringe microinjectors for Hamilton syringes (Stoelting)

Primate or large animal size stereotaxic head holder and bars (David Kopf Instruments)

Ear bars (David Kopf Instruments)

Animal clippers

Endotracheal tube

Sterile surgical supplies including:

Stereotactic bone drill with 0.5-mm drill burrs

Drapes

Forceps

Hemostats

Needle holders

Periostial elevators

Scalpel with no. 10 surgical blade (Bard–Parker)

Self-retaining retractor

Towel clips

Standard neurosurgical supplies—sutures, bone wax, Gelfoam, sterile NaCl irrigation solution

3-in. sterile gauze

EKG machine with monitoring leads

Leads for temperature and O2 saturation

Additional reagents and equipment for counting viable cells with a hemocytometer and trypan blue (*UNIT 1C.3*)

1. Mount the syringe into the stereotactic apparatus, calibrate according to ear bar zero, and calculate target sites from atlas or prior studies.

For injecting monkeys, we steam-autoclave all of the equipment in the surgical field, including the stereotaxic frame with the carriers. The syringe pumps, syringes, and controllers are sterilized by ethylene oxide gas.

2. Anesthetize monkeys initially with 7 to 15 mg/kg ketamine by i.m. injection and administer 0.02 to 0.05 mg/kg atropine by i.m. or s.c. injection. Shave and prepare the surgical area. Place an intravenous line into the saphenous or other peripheral vein and induce the animal to light anesthesia with 20 to 30 mg/kg pentobarbital for placement of an endotracheal tube to ensure adequate airway and for supplemental ventilation with room air or O2, if necessary.

3. Place the anesthetized and monitored monkey into the stereotaxic frame with the ear bars (it helps to lubricate and clean the ear canals with Lubrivet) and check the centering. Repeat if necessary.

For accuracy of targeting, this is the most critical step of the procedure, if it is performed without MRI targeting.

4. Place EKG monitoring leads and a lead for temperature and O2 saturation.

5. Scrub and prep the already shaved head from the eyebrows to the back of the neck and from ear to ear. Remove the drape which protects the sterile cover over the ear bar on the stereotaxic frame, and then drape the head with sterile drapes and an incise drape so that nothing remains exposed except the sterile ear bar, which remains accessible for the syringe holder/carrier.

6. Recheck the zero coordinates for the drill and for the syringe carriers against a calibrated zero bar.

7. Compare the midline of the skull with the calculated coordinates for the drill. Adjust if necessary and then recalculate all targets for the drill and the carriers if the midline was adjusted. After the coordinates have been verified, slowly drill holes, lubricating with sterile DPBS. Replace the drill holder with the needle carrier and repeat steps 6 to 7.

8. Clear the Hamilton syringe by drawing and expelling 60 µl sterile DPBS two to three times. *The DPBS rinse removes residual ethylene oxide, helps lubricate the needle, and ensures proper working condition of the syringe*

9. To fill the needle, wipe the hNPC suspension tube (optimally containing ~50,000 cells/µl) thoroughly with 70% ethanol and carefully uncap the tube inside the surgery suite. *Gently flick or triturate the hNPC microcentrifuge tube immediately prior to filling the needle.*

10. Grasp the microcentrifuge tube with hemostats and carefully move the tube up to the needle from underneath.

The bevel of the needle should be located at the middle of the cell suspension.

11. Slowly move the tube vertically to mix cells and begin drawing 10 to 20 µl hNPC into the needle with the controlled-rate syringe pump.

Cells should be drawn into the syringe immediately before the injection. Do not touch the sides or bottom of the tube, introduce air bubbles, or bend the needle. Prolonged time within the syringe will result in clumping and subsequent clogging of the needle. Always draw 2 to 3 µl excess cell suspension into the syringe for post-injection analysis.

12. Immediately before insertion of needle, slowly expel 1 µl cell suspension from needle into a clean sterile microcentrifuge tube. Add 1 ml fresh NB-B-27 complete growth medium without Normocin to the microcentrifuge tube.

13. Lower the needle into target area of interest at slow speed (10 to 15 sec) to the proper depth for the given stereotactic location.

At the appropriate vertical depth, wait 2 min to allow the brain tissue to adjust to the proper level at the needle tip.

14. Inject the prepared cell suspension using the syringe pump at a maximum constant rate of (1 µl/min).

15. During autoinjection, count viable hNPC in the microcentrifuge tube with a hemocytometer and trypan blue (*UNIT 1C.3*) to determine viability at time of injection and replate the 1 ml of hNPC into one well of a 24-well dish. Incubate at 37◦C for 48 to 72 hr and examine under the microscope to determine if any contamination was introduced during the

procedure.

16. After the cells are fully expelled from the syringe, allow the needle to remain in place for 2 min before withdrawing.

The extra settling time will prevent significant backflow and leakage of hNPC through the needle track during retraction of the needle.

17. Retract needle slowly, at a maximum of 1 μm/min. If another injection is planned, flush out the syringe with sterile PBS. Refill and repeat step 8 above.

18. After the last injection, make sure that any bleeding is controlled, suture animal, and proceed to post-operative care.

2.8 INTRAVENTRICULAR INJECTION OF hNPC INTO NEONATAL MICE

Neonatal mice (post-natal day 0 to 3; P0 to P3) are relatively effortless to handle and can easily be injected in the lateral ventricles with hNPC, offering several advantages over adult mice for transplantation. The undeveloped newborn skull is soft and translucent, obviating the need for drilling or cutting into the head while facilitating penetration of the needle or drawn glass micropipet. In addition, hNPC survive, migrate, and integrate within the developing neonatal CNS at a higher efficiency than when transplanted into the mature adult brain. It should be noted that engraftment, migration, synaptic maturation, and development of fully competent neuronal subtypes often take longer with human-derived NPC than with rodent-derived NPC; therefore, we highly recommend the use of immunodeficient rodent models such as the SCID genetic background to increase overall success and eliminate the need for expensive immunosuppressants (Chidgey et al., 2008). Other strains with variations

in T, B, and NK cell deficiencies such as BALB/c and Rag2 backgrounds may also be used, but may display some leakiness with age. We prefer the NIHS-beige-nude-xid (NIHBNX) or C.B-17 scid beige (CBSCBG) mice available from Taconic or Harlan. The extra cost for maintenance of these animals is far outweighed by time and resource expenditures involved in continually testing and rederiving new hNPC lines, which could be avoided by eliminating the false negatives involved in using non-immunopriveleged strains.

Materials

Neonatal mice (P0 to P3) of appropriate strain

70% ethanol

Dulbecco's PBS with Ca2+ and Mg2+ (DPBS; Mediatech, cat. no. 21-030-CM)

Human NPC (hNPC; Basic Protocol 6; 50,000 cells/μl) in microcentrifuge tube

Borosilicate glass (Sutter Instrument Co., cat. no. B100–75-15)

Micropipet puller (Sutter Instrument Co., Model P-87)

Aspirator tube assemblies for calibrated microcapillary pipets (Sigma Aldrich, cat. no.A5177–5EA)

Fiber optic light source for transillumination (Dolan-Jenner Industries)

Warm-water glove balloon

Additional reagents and equipment for preparing injection micropipet (Lee et al., 2008) and processing mouse brains

1. Prepare calibrated drawn borosilicate glass micropipet using borosilicate glass and a micropipet puller (Lee et al., 2008).

2. Anesthetize the neonatal mouse by placing the pup on wet ice for ~1.5 to 3 min until the

animal no longer retains locomotion or responds to gentle toe and tail pinch.
Carefully monitor the pup to minimize time on ice, as overexposure to low temperatures can lead to death of newly born mice. Immediately proceed to transplantation

3. Insert a calibrated drawn borosilicate glass micropipet into an aspirator tube assembly. Just prior to drawing up hNPC, rinse the micropipet by drawing up and then expelling 5 µl of 70% ethanol ten times, followed by sterile DPBS ten times, to sterilize and lubricate the glass.

4. Gently flick or triturate the microcentrifuge tube containing the hNPC suspension immediately prior to filling the needle. Wipe the tube thoroughly with 70% ethanol and carefully uncap the tube.

5. Slowly move the tube vertically to mix cells and begin drawing 4 to 5 µl hNPC into the micropipet by mouth suction.
Do not touch the sides or bottom of the tube, introduce air bubbles, or break the glass needle. Prolonged time within the micropipet will result in clumping, subsequently clogging the needle. Always draw 2 to 3 µl excess cell suspension into the syringe for post-injection analysis.

6. Loosely secure the skull by hand at the cranium and place directly over a non heat conducting (fiber-optic) light source to visualize the bregma and lateral ventricles by transillumination.
Gentle handling of the pup throughout the procedure is important to avoid trauma.

7. Carefully insert glass needle ~0.5 to 1 mm deep into the head at the midline between

bregma and eye and slowly inject 1 to 2 µl hNPC suspension at 5 °— 104 cells/µl into the lateral ventricle of either the left or right hemisphere.

Correct location and accurate dispersion within the ventricles can be confirmed by addition of an inert dye to the cell suspension. A correctly placed glass needle will deliver cells without resistance by mouth-pressure delivery from a micropipet aspirator tube assembly. We recommend practicing on nonexperimental animals with trypan blue, to become acquainted with the correct pressure and distance necessary for accurate intraventricular injection of experimental animals with hNPC cell suspensions. P0 mice generally will tolerate 3 µl total divided between both ventricles, while the later stages

P2 to P3 can tolerate 4 to 5 µl total.

8. Slowly remove the needle and check for leakage through the needle track. Repeat step 7 in the contralateral hemisphere.

The entire procedure starting from grasping the pup off of ice to injection should not take longer than 40 to 60 sec per animal to avoid having the anesthetic wear off.

9. Immediately following injection, warm the lower extremities under tepid flowing water, gently pat dry with a cotton swab or piece of gauze, and place on a warm-water glove balloon on top of a heating pad to adequately increase the body temperature of each pup before returning the pup to its mother, to avoid post-operation parental rejection.

Monitor pups after transplantation for several hours to ensure adequate post-operational recovery. It is very important to increase core body temperature quickly, for best results. The brain is not an immunopriveleged organ; therefore immunosuppression regimes are recommended to avoid rejection in applicable genetic backgrounds (e.g., wild-type strains).

10. Process brains for differentiation potential at the following time points (see Basic Protocol 9):

a. Injection location confirmation (2 to 12 hr).

Location and structures of interest, lateral ventricles and luminal cavity: Trypan blue tracer can easily be seen within the lateral ventricles at this time, confirming correct needle position at time of injection. In addition, the overall integrity of the brain and amount of damage incurred by the glass needle track can be assessed to validate technical procedure for optimal hNPC transplants. Cells will begin to engraft in the first 8 to 12 hr following transplantation; however, accurate assessment for cell survival can not be attained this early.

b. Engraftment (12 to 24 hr).

Location and structures of interest, lateral ventricular walls, including SVZ and choroids plexus: Cells will initially incorporate within the ventricular walls and preferentially accumulate in the SVZ as bulbous nodules where they eventually incorporate into the host SVZ and rostral migratory stream (RMS). Dead cells are often cleared and become trapped within the choroid plexus, the natural "kidney" of the brain that actively filters CSF. In general, there is a give and take between the moderate amount of cell death that occurs during the transplantation process and the subsequent cell divisions that take place post-transplantation.

c. Integration and migration within SVZ-RMS niche (1 to 3 days).

Location and structures of interest, SVZ, RMS, olfactory bulbs (OB): Successfully engrafted hNPC incorporate within the developing ventricular walls, forming nodules reminiscent of SVZ protrusions induced by intraventricular growth factor infusion (Kuhn et al., 1997; de Chevigny et al., 2008). The bulbous cluster eventually flattens back out into a normal

ventricular wall, and the hNPC integrate within the host neurogenic niche. Within the endogenous SVZ niche, a small population of donor cells will continue to proliferate (Ostenfeld et al., 2000) for at most two to four cell divisions (in our experience) before exiting the cell cycle as they mature and coordinately migrate tangentially and by chain migration through the RMS. The number of cells that die during and following transplantation far outweighs the additional proliferative load; therefore, unchecked tumor-like growth does not occur.

d. Migration, differentiation, and synaptic integration (3 to 14 days).

Location and structures of interest, RMS and olfactory bulbs (OB), neuraxis: As hNPC preferentially migrate through the RMS to the OB, they receive signaling and guidance inputs that eventually direct them to defer from tangential or chain migration and turn radially where they continue to migrate and differentiate into synaptically integrated OB interneurons. In addition, many cells will migrate through predominantly white matter and integrate appropriately into the developing striatum and to a lesser extent contribute to the cortex as well (Fig. 2-10). Utilizing non-immunopriveleged animals that do not exhibit pathological deficit often leads to a significant decrease in overall graft survival, integration, and long-term synaptic connectivity. When testing hNPC differentiation potential, we recommend always using immunodeficient mouse models (SCID, BALB/c, or Rag2) to decrease time-consuming false negatives. Assaying donor cell survival after 2 to 3 weeks in vivo in nondiseased animals can often be misleading, as many of the cells are discarded, become quiescently integrated within the ventricular wall and SVZ, or simply undergo apoptosis in response to local microenvironment niche signaling cues. It seems that once the initial process of fetal development has concluded, only a small portion of transplanted hNPC remain quiescently undifferentiated within the neurogenic niches, while the remainder of donor cells

continue to migrate, differentiate, and eventually be replaced by the natural host neurogenic process. In non-immunopriveleged animals, we speculate that these donor cells undergo additional selectional pressure as the host immune system develops, and this may eventually deter the long-term survival and maturation of OB interneurons. One way or another, very few transplanted hNPC will be found after 1 to 3 months in vivo in nondiseased, non-immunopriveleged animals; therefore, careful consideration of genetic background and terminal end point is essential for transplantation success [Figure 2-10].

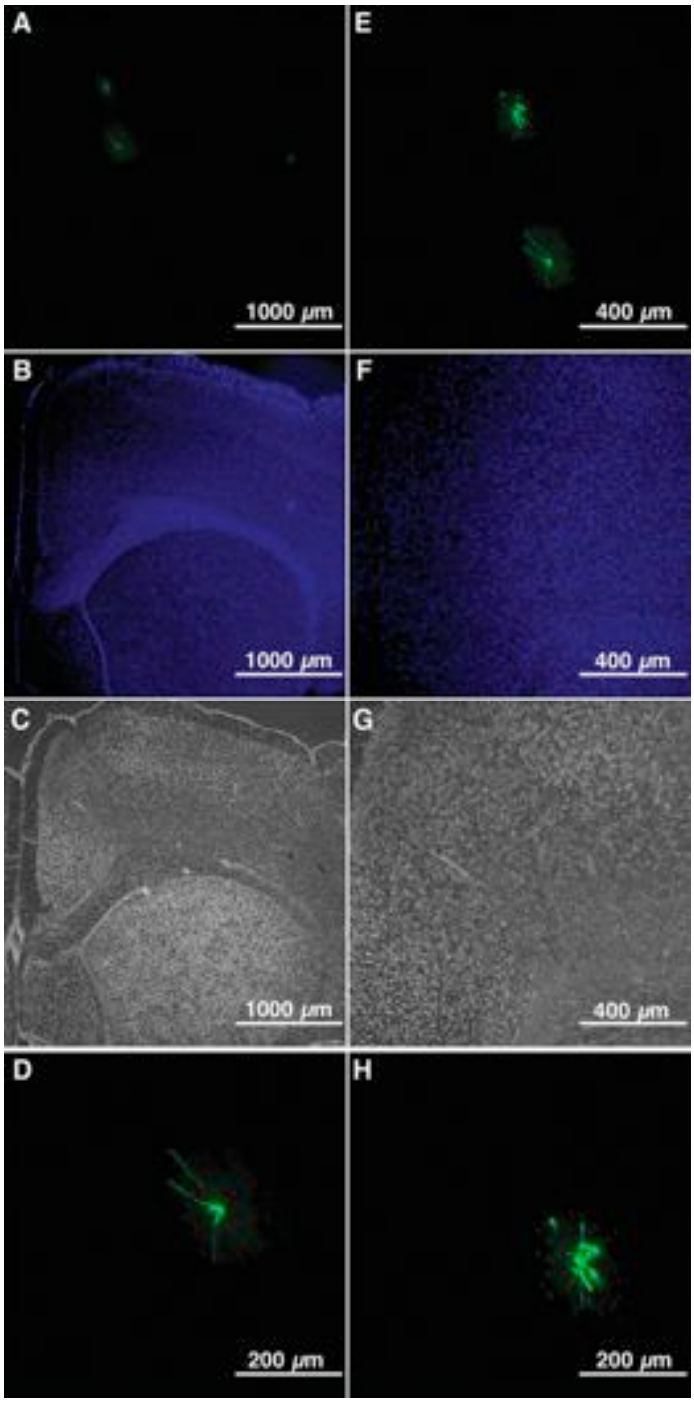


Figure 2-10. Engrafted hNSC survive, mature, and express the eGFP transgene in vivo. Human NSC (HFB-2050) engineered to express eEGP under the CAG promoter were established and transplanted at low density into both lateral ventricles of P0 neonatal mice (as described in the text). Mature eGFP+, donor-derived neurons were found in clusters throughout the forebrain up to 10 weeks post-transplantation (**A-H**); DAPI (**B,F**), phase (**C,G**), GFP (**A,D,E,H**).

2.9 CHARACTERIZING hNPC

Throughout expansion and long-term maintenance, hNPC are periodically assayed for in vitro expression of known "stemness" markers. Human NPC should be analyzed by standard immunoassays to demonstrate sustained undifferentiated characteristics and cellular morphology. Prior to transplantation, a subset of hNPC are set aside and assayed for the following markers (see below) in multiple combinations to confirm morphologically relevant expression profiles exist.

The fate of donor cells in the CNS can be assessed in vivo by dissecting the brains and processing by standard immunohistochemical methods for lineage-specific antigens such as nestin/vimentin for hNSCs; GFAP and s-100-Beta for astrocytes; and NeuN, NFM70/200, and synaptophysin for mature neurons. Antibodies to the human nuclear antigen (HuNuc) can be used to co-label hNSCs for donor confirmation. The functionality of donor cells in the brain can also be assessed by classic electrophysiological methods ex vivo.

2.9.1 Undifferentiated hNPC

Healthy proliferative Ki-67+ (Scholzen and Gerdes, 2000; Ab available from Abcam, use at 1:200 dilution) or PCNA+ (Hall and Levison, 1990; Hall et al., 1990; Ab available from Santa Cruz Biotechnology, use at 1:100 dilution) undifferentiated hNPC express the nuclear transcription factor Sox-2 (D'Amour and Gage, 2003; Komitova and Erickson, 2004; Baer et al., 2007; Ab available from Santa Cruz Biotechnology, use at 1:100 dilution), musashi-1/2 (Chan et al., 2006; Ab available from Abcam, use at 1:100 dilution), the filamentous cytoplasmic proteins Nestin (Ab available from Chemicon, use at 1:400 dilution; Fig. 2-11A,B,E-J) and Vimentin (Ab available from Chemicon, use at 1:400 dilution), and the surface protein LexA/SSEA-1 (Ab available from Chemicon, use at 1:250 dilution) in cellularly appropriate locations (Temple, 2001; Pixley and de Vellis,1984; Pixley et al.,

1984a,b; Hockfield and McKay, 1985; Lendahl et al., 1990; Dahlstrand et al., 1992; Zimmerman et al., 1994; Garcia et al., 2004). In addition, fetal hNPC also express glial fibrillary associated protein (GFAP; Ab available from DAKO, use at 1:400 dilution) in morphologically appropriate (nonprotoplasmic) locations, highly correlating with Nestin expression (Fig. 2-11. E,G-J), consistent with rodent data (Doetsch et al., 1999; Laywell et al., 2000; Imura et al., 2003), suggesting that GFAP also labels human fetal NPC or that astrocytes may be hNPC at specific times during early development. It is critical that these markers be utilized in combination to confirm the stem/precursor phenotype of donor cells before transplantation. In addition to the classic stem cell markers, we have also confirmed the expression of radial glia (RG) associated brain lipid binding protein (BLBP; Feng et al., 1994; Feng and Heinz, 1995; Ab available from Chemicon, use at 1:350 dilution), which has been linked to fetal NSC in vivo (Fig. 2-12; Garcia et al., 2004; Malatesta et al., 2000; Hartfuss et al., 2001; Alvarez-Buylla et al., 2001, 2002; Miyata et al., 2001; Noctor et al., 2001, 2002; Gotz et al., 2002; Gregg et al., 2002; Doetsch, 2003; Malatesta et al., 2003; Goldman, 2003; Gotz, 2003; Gregg and Weiss, 2003; Noctor et al., 2004, 2008; Merkle et al., 2004; Gotz and Bard, 2005; Merkle and Alvarez-Buylla, 2006; Merkle et al., 2007).

In addition, a small population of bipolar migratory cells express the microtubule associated protein doublecortin (DCX; Fig. 2-12E,F; Gleeson et al., 1999; Francis et al., 1999; Friocourt et al., 2003; Ab available from Santa Cruz, use at 1:400 dilution) and the membrane-bound polysialylated neural cell adhesion molecule (PSA-NCAM; Hu et al., 1996; Curtis et al., 2007; El Marouf and Rutishauser, 2008; Rutishauser, 2008; Burgess et al., 2008; Ab available from Chemicon, use at 1:250 dilution). Furthermore, some later-passage hNPC populations also express the intermediate filament protein, beta-3-tubulin (Tuj-1; Fig. 2-11 F; Caccamo et al., 1989; Geisert and Frankfurter, 1989; Lee et al., 1990; Menezes and Luskin, 1994; Menezes et al., 1995; Ab available from Covance Research, use at 1:400 dilution) and

colocalizes with GFAP (Rakic, 1972; Sidman and Rakic, 1973; Levitt and Rakic, 1980; Levitt et al., 1981, 1983). Although beta-3-tubulin has been described as an early marker for immature neuron ally–restricted NPCs in rodents, these hNPC continue to self-renew and maintain multipotency in vitro and in vivo. Furthermore, they are phenotypically and behaviorally indistinguishable from early-passage predecessors. As a result, assuming the same stemness profile for human cells may not be entirely appropriate, and this should be taken into account when assessing hNPC fate. For example, expression of GFAP or beta-3-tubulin alone may not be entirely sufficient to assume loss of multipotency or regional differentiation into neurons and astrocytes, respectively. Instead, we favor a mode of characterization based on both morphology and multiple marker comparison to appropriately assess stem cell fate both before and after transplantation.

It is well known that cell lines accumulate in vitro artifacts in response to long-term artificial cell culture environments (Doetsch et al., 2002; Pollard et al., 2008). Whether the trends we see in vitro appropriately mimic the in vivo nature of hNPC remains to be determined. Certainly, it stands to reason that hNPC share many developmental markers in common with their rodent counterparts; however, it is not beyond the scope of reason to assert that perhaps some of the key players involved in human neural developmental processes may be differently regulated spatiotemporally from the corresponding processes in lower-order mammals. We have verified these findings by both standard immunocytochemistry and western blotting in three hNPC lines ranging from 10 to 22 weeks (from fertilization date), further suggesting that hNPC are not homogenous populations of identical stem cells, but rather highly dynamic heterogeneous populations (Kukekov et al., 1999; Laywell et al., 2002; Suslov et al., 2002; Steindler et al., 2003; Chen et al., 2006) of neural progenitors. Clonal analysis assays and in-depth time-lapse fluorescence video microscopy are currently being used to determine the specific lineage relationships involved.

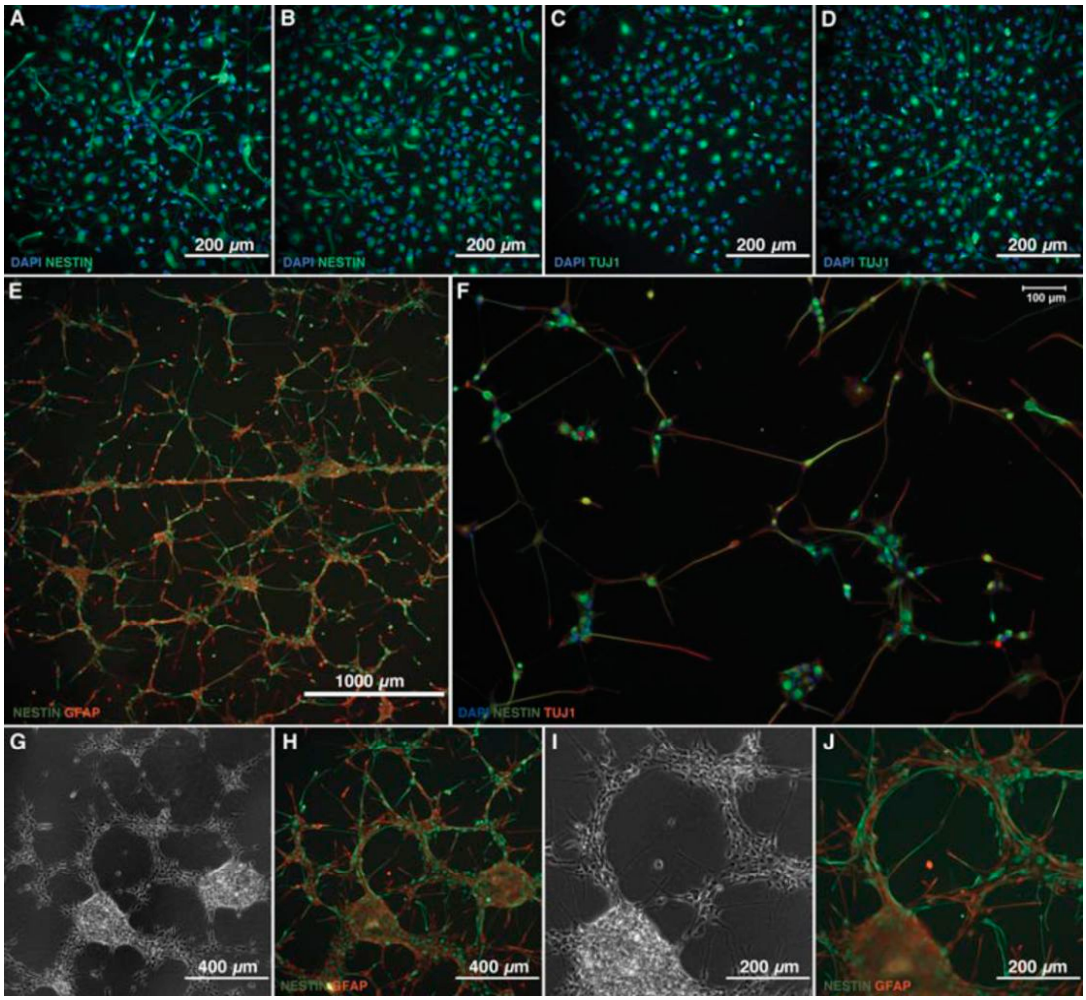


Figure 2-11. hNSC express classic neural stemness genes. Human NSC (HFB-2050) express nestin (**A,B**) and Tuj-1 (**C,D**) uniformly when plated as aggregates on poly-D-lysine-coated tissue culture–treated flasks (blue = DAPI). Cells rapidly attach and begin to elongate, sending processes throughout the culture. (**A**) and (**D**) were processed 24 hr after plating, while (**B**) and (**C**) represent the more immature morphology seen after 12 hr of culture. DAPI+ nuclear morphology indicates active mitosis and sustained proliferation at both time points. In addition, nestin+ hNSC (green) also express GFAP (red) (**E,H,J**), typically in inverse proportion to each other. For example, a cell with high GFAP expression will also express nestin, but at a much lower level, whereas a highly nestin+ cell will express GFAP at a lower level. Interestingly, both cell types are intimately interwoven within each other, forming a meshwork of migratory cells (pictured here on poly- L-ornithine/fibronectin-coated tissue culture–treated flasks). Furthermore, nestin+ hNSC also express Tuj-1 (**F**) throughout most of the cytoplasm. Typically, nestin expression is highest surrounding the nuclear box, whereas Tuj-1 expression is greatest at the feet of meandering processes. Expression patterns are typically opposite of each other, and tend to colocalize in the middle of the cell's architecture, similar to nestin and GFAP coexpression. These filamentous proteins may play distinct roles at their specific positions within the stem cell, conferring or coordinating cell polarity within the in vitro microniche (**G,I** = phase contrast).

Whether these distinct phenotypic outliers represent bona fide stem cells or restricted progenitors that have been reprogrammed in response to mitogenic signaling cues remains to be determined. Certainly, the new wave of research dedicated to studying induced pluripotent stem cells (iPS) suggests that this process is much easier to induce than previously appreciated (Takahashi and Yamanaka, 2006; Takahashi et al., 2007 a,b; Maherali et al., 2007; Wernig et al., 2007; Okita et al., 2007; Meissner et al., 2007; Yu et al., 2007; Nakagawa et al., 2008; Park et al., 2008; Brambrink et al., 2008; Wernig et al., 2008; Stadtfeld et al., 2008; Shi et al., 2008; Kim et al., 2008; Maherali et al., 2008; Maherali and Hochedlinger, 2008a,b). Therefore, it is only through careful phenotypic characterization that we can begin to understand the nature of hNPC in vitro. Until better methods and markers are discovered to accurately access cellular identity, we are forced to apply the borrowed phrase, "It's hard to define, but I know it when I see it" (Morrison et al., 1997).

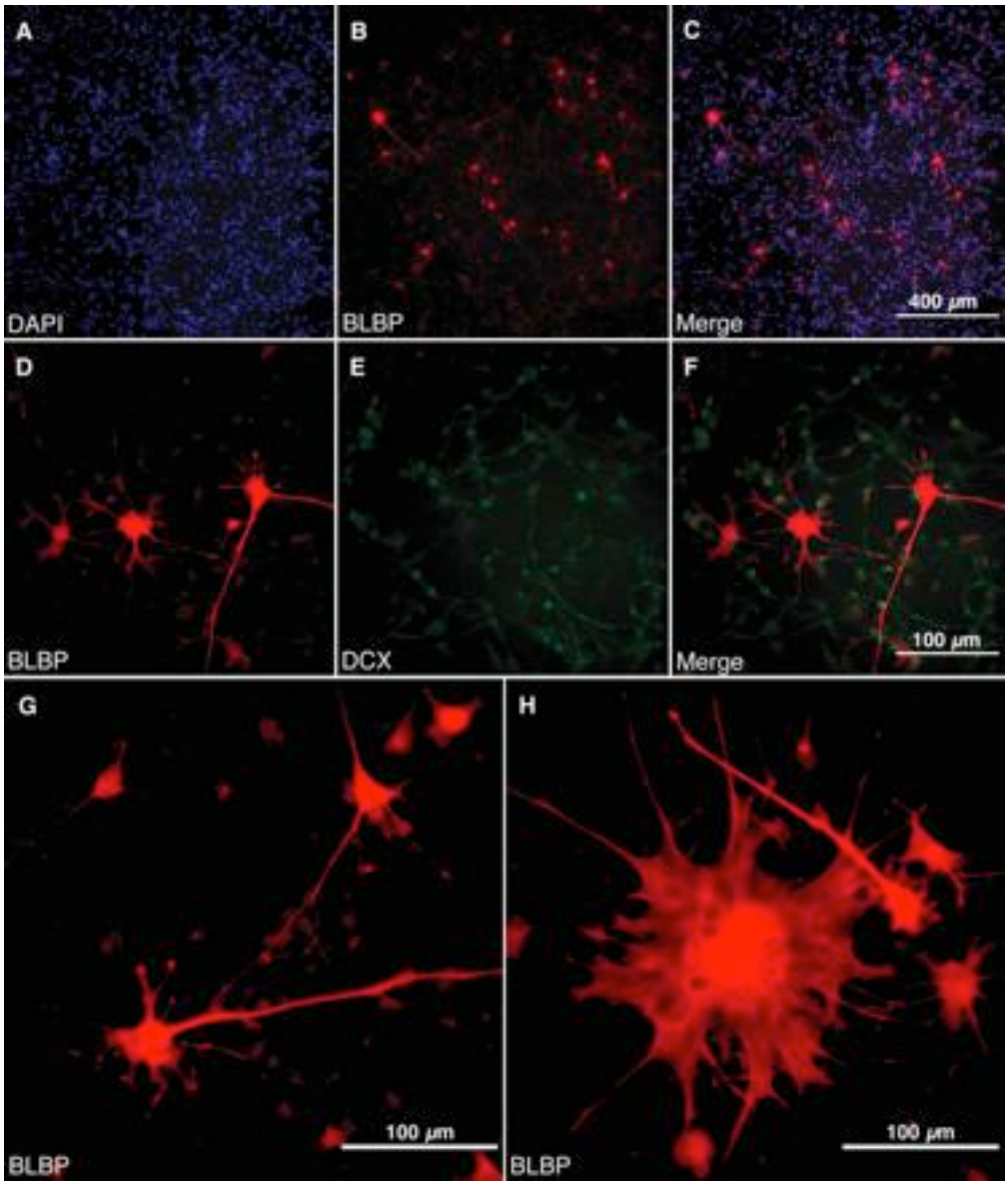


Figure 2-12. hNPC exist as heterogeneous populations resembling the SVZ niche. In addition to the classic NSC marker proteins, some cells also highly express brain lipid binding protein (BLBP) (**A-H**) or doublecortin (DCX) (**E,F**). Cells that highly express BLBP typically assume an astrocyte-like star morphology and have one or two long meandering processes with a highly arborized cell body, resembling radial glia. In contrast, most cells that do not express BLBP highly or have radial glia morphology express DCX and highly resemble the migratory transit amplifying cells found in vivo within the subventricular zone NSC niche. Images were taken from hNSC (HFB-2050) plated on Matrigel-coated tissue culture plates and cultured for 3 days in vitro to induce attachment and spreading. Of note, extracellular matrix components and growth factors found in Matrigel have a profound impact on cell morphology and may affect the differentiation profile of cultures over time. It is likely that these cultures have started an initial differentiation process and may not retain all stemness properties. Nonetheless, the heterogeneity of hNSC cultures is evident as 72 hr is probably not sufficient time to considerably differentiate hNSC.

2.9.2 Differentiated hNPC

In addition to the classical stemness markers, cells may be differentiated into neurons, astrocytes, or oligodendrocytes by a variety of methods and assayed for lineage-specific differentiation markers. Specific methods for in vitro differentiation are detailed elsewhere (Wakeman et al., 2009; Johe et al., 1996; Hsieh and Gage, 2004; Androutselis- Theotokis et al., 2008). Differentiated cells are fixed in 4% cold PFA, stained with the appropriate antibodies by standard protocols, and analyzed by indirect immunofluorescence for the expression of pro-neuro/gliogenic markers. To determine whether hNPC are capable of giving rise to neurons (after allowing at least 3 weeks of in vitro maturation), the cells are stained for an extensive panel of pro-neuronal markers—first (immature): doublecortin (Santa Cruz, dilute 1:500), b-III-tubulin (Chemicon, dilute 1:200), Pax6 (Covance, dilute 1:400), Ptx3 (Abcam, dilute 1:500, or R&D Systems, dilute 1:400), Lmx1a/b (Santa Cruz, dilute 1:200), Gbx1/2 (Santa Cruz, dilute 1:250), Ngn1/2/3 (Santa Cruz, dilute 1:250), and then more mature neuronal phenotypes: PSA-NCAM (Chemicon, dilute 1:100), high-molecular-weight neurofilament (Boehringer, dilute 1:150), tau (Sigma, dilute 1:400), NeuN (Chemicon, dilute 1:50), MAP-2 (Sigma, dilute 1:200), synaptophysin (Sigma, dilute 1:500), calbindin (Sigma, dilute 1:400), calretinin (Chemicon, dilute 1:500), Mash1 (BD Biosciences, dilute 1:100), Msx1 (Abcam, dilute 1:500), En1 (Iowa Developmental Hybridoma Bank, dilute 1:50), Girk2 (Alomone Laboratories, dilute 1:500), Nurr-1 (Santa Cruz, dilute 1:500), TH (PelFreeze Biologicals, dilute 1:500, or primary rabbit antiserum, Eugene Tech, dilute 1:3000), DAT (rabbit, Affinity Bioreagents, dilute 1:500), VMAT2 (PelFreeze, dilute 1:500), AADC (Chemicon, dilute 1:1000; dopaminergic), GABA (Sigma, dilute 1:5000 or rabbit polyclonal antibody for GAD65/67, Chemicon, dilute 1:1000), ChAT (Chemicon, dilute 1:400; cholinergic), 5-HT (Sigma, dilute 1:50 or rabbit polyclonal, Calbiochem;, dilute 1:1200), as well as the radial glial, BLBP (Chemicon, dilute 1:1000), A2B5 (R&D Systems, dilute 1:400),

the mature astroglial, GFAP (polyclonal rabbit, Dako-Patts, dilute 1:1000, Sternberger Monoclonals, dilute 1:200, or Chemicon, dilute 1:200), S100B (Abcam, dilute 1:500), glutamate transporters Glast/GluT-1/EAAT1 (Chemicon, dilute 1:400, or Santa Cruz, dilute 1:500) and GLT1/EAAT2 (Chemicon, dilute 1:400, or Santa Cruz, dilute 1:500), and oligodendrocyte fate, MBP (Chemicon, dilute 1:200), O4/O1 (Chemicon, dilute 1:200), CNPase (Chemicon, dilute 1:200), otx2 (Santa Cruz, dilute 1:100), and RIP (Chemicon, dilute 1:400).

2.10 COMMENTARY

The great neuroanatomist Ram´on y Cajal wrote, "In the adult centers the nerve paths are something fixed, ended and immutable. Everything must die, nothing may be regenerated" (Ram´on y Cajal, 1928; Ram´on y Cajal and May, 1959). This observation, based on the primitive methods of the time, held up as neurodevelopmental dogma for centuries. It wasn't until Joseph Altman and Michael Kaplan's classic autoradiographic experiments using tritiated thymidine (Altman, 1962a,b, 1963; Altman and Chorover, 1963; Altman and Das, 1965a,b, 1966) that neurobiologists even considered rethinking the notion of adult neurogenesis (Allen, 1912; Messier et al., 1958; Messier and Leblond, 1960; Smart, 1961; Smart and LeBlond, 1961; Kaplan and Hinds, 1977), let alone embraced it as an intrinsic process active throughout adulthood until death.

In the past quarter century, it has become undeniably clear that neural stem/progenitor cells reside within the developing embryonic, neonatal, and adult songbird (Goldman and Nottebohm, 1983), rodent (Alvarez-Buylla et al., 2002; Snyder et al., 1992; Morshead et al., 1994;Weiss et al., 1996; Johanson et al., 1999), monkey (Gould et al., 1998, 1999a,b; Kornack and Rakic, 2001a,b), and human forebrain (Merkle et al., 2007; Curtis et al., 2007; Sanai et al., 2004; Howard et al., 2006; Quinones-Hinojosa et al., 2006, 2007; Sanai et al., 2007), primarily

lining the posterior to anterior subventricular zone (SVZ; Doetsch et al., 1999; Merkle et al., 2004; Menezes et al., 1995; Lois and Alvarez-Buylla, 1993; Luskin, 1993; Lois and Alvarez-Buylla, 1994; Rousselot et al., 1995; Luskin et al., 1997) of the lateral ventricular walls and within the subgranular zone of the hippocampal dentate gyrus (Ray and Gage, 2006; Ray et al., 1993; Gage et al., 1995a). These cells can be derived from various regions of the brain, with limited capacity, where they exist naturally as relatively quiescent populations of stem and progenitor cells (Palmer et al., 1995) within the complex microenvironment of a highly dynamic, tightly junctioned, neurogenic niche (Sanai et al., 2007; Alvarez-Buylla and Lim, 2004;Verdugo and Alvarez-Buylla, 2006; Lim et al., 2007).

During mammalian CNS development, hNSC undergo an initial expansion phase of symmetric divisions followed by nonsymmetric divisions and extensive migration in accordance with electrical stimulation (Deisseroth et al., 2004; Spitzer, 2006) and chemical (Ghashghaei et al., 2007) guidance cues. Differentiation is characterized by stages of neurogenesis followed by gliogenesis (Qian et al., 1997, 1998, 2000; Namihira et al., 2009), differentiating in temporal waves of first neurons, then astrocytes and oligodendrocytes, to shape and form the mature human brain (Levison and Goldman, 1993; Levison et al., 1993; Menn et al., 2006). In the adult, NPC continue to proliferate and migrate by a combination of tangential and chain migration from the SVZ through the rostral migratory stream (RMS) into the olfactory bulbs (OB; Lois et al., 1996; Doetsch and Alvarez-Buylla, 1996), generating new neurons and glia (Kuhn et al., 1996; Goldman et al., 1997; Petreanu and Alvarez- Buylla, 2002; Carleton et al., 2003; Lledo et al., 2006) as an active pool to replace or restore homeostasis to the aging or injured brain (de Chevigny et al., 2008; Lim et al., 2007; Nait-Oumesmar et al., 1999; Picard- Riera et al., 2002; Lie et al., 2004; Parent et al., 2006; Leung et al., 2007; Hellstrom et al., 2008).

In addition, cultured NSC/NPC can be directed in vitro and in vivo to give rise to all

three neuroectodermal lineages (Arsenijevic et al., 2001a; Johe et al., 1996; Gage et al., 1995b; Kirschenbaum et al., 1994; McKay, 1997; Levison and Goldman, 1997; Murray and Dubois-Dalcq, 1997; Pincus et al., 1998;Takahashi et al., 1999; Rao, 1999; Brannen and Sugaya, 2000; Dietrich et al., 2002; Riaz et al., 2004; Scheffler et al., 2005; Christophersen et al., 2006; Pistollato et al., 2007; Rao et al., 2008), as well as a variety of intermediate cellular phenotypes (Markakis et al., 2004) when presented with the appropriate signaling cues. These cells may act by a variety of mechanisms, either by providing potential new raw material for regenerating the damaged CNS or by rescuing the endogenous nervous system through secondary neuroprotection mechanisms (Pluchino et al., 2005a), thereby modulating the host microenvironment (Madhavan et al., 2005, 2006, 2008), conferring a return to baseline nonpathological stasis (Bjugstad et al., 2005). In this manner, neurological function may be restored through transplantation therapies by either directly integrating and replacing host neural circuitry, or, secondarily, by rescuing and restoring the endogenous host milieu through growth factor or neurotransmitter paracrine signaling (Brustle and McKay, 1996; Park et al., 2002a; Svendsen and Langston, 2004; Tai and Svendsen, 2004; Emsley et al., 2005; Pluchino et al., 2005a,b; Schwartz, 2006). Moreover, multipotent hNSC have been shown to display low immunogenicity (Mason et al., 1986; McLaren et al., 2001; Odeberg et al., 2005), readily express foreign transgenes (Flax et al., 1998; Ostenfeld et al., 2002a; Wu, P. et al., 2002; Park et al., 2003; Behrstock and Svendsen, 2004; Kim, 2004; Klein et al., 2005; Behrstock et al., 2006; Capowski et al., 2007; Roy et al., 2007; Suzuki et al., 2007; Ebert et al., 2008), and inherently home to sites of pathological insult (Aboody et al., 2000; Park et al., 2002b; Ourednik et al., 2002; Imitola et al., 2004; Park et al., 2006).

Traditionally, human neural stem cells (hNSC) have been operationally defined (Weissman et al., 2001; Anderson, 2001; Seaberg and van der Kooy, 2003; Parker et al., 2005; Navarro-Galve and Martinez-Serrano, 2006) by two cardinal criteria, first, the ability to *self-*

renew indefinitely by division into two identical (symmetric) or nonidentical (asymmetric) daughter cells, and, second, to maintain the *multi-potential* capacity to differentiate into all three neuroectodermal CNS lineages: neurons, astroglia, and oligodendrocytes in vivo (Gage et al., 1995a; Brustle et al., 1997; Lundberg et al., 1997; Winkler et al., 1998; Zigova et al., 1998; van der Kooy and Weiss, 2000;Weissman et al., 2001; Buchet et al., 2002). Asymmetric daughter cells that are not identical to the parental hNSC have a reduced stemness and are therefore referred to as intermediate or transit amplifying neural progenitor cells (Doetsch et al., 1999; Merkle and Alvarez-Buylla, 2006; Verdugo and Alvarez-Buylla, 2006; Doetsch et al., 1997; Garcia-Verdugo et al., 1998; Lim et al., 2008; for reviews seeKemperman, 2006; Slack, 2008). In vertebrates, NSC self-renewal can be viewed as a property of the entire population as opposed to just the single cellular entity, in that self-renewal is thought of more globally as the capacity to maintain the absolute number of neural stem cells in a given area at a steady level throughout time (Gritti et al., 2003). This more relaxed definition allows for fluctuations in absolute NSC population size, evenly balancing symmetric stem cell divisions with asymmetric differentiated progenitors as physiological conditions dictate.

Although there may be no true NSC capable of self-renewing indefinitely throughout adulthood in vivo, NPCs seem to maintain prolonged self-renewal (Ravin et al., 2008) and regain their multipotentiality when exposed to mitogenic growth factors in vitro (Doetsch et al., 2002; Gabay et al., 2003). In addition, transplantation of SVZ-derived NPC suggests that they retain both migratory and differentiation capabilities when homotopically reintroduced into appropriate anatomical locations, but may only differentiate and fail to migrate when heterotopically positioned (Betarbet et al., 1996; Zigova et al., 1996; Hererra et al., 1999; Yang et al., 2000; Ourednik et al., 2001; Tamaki et al., 2002; Seidenfaden et al., 2006). The results demonstrate that there may be an intrinsic spatiotemporal program that determines the developmental potential of NPC. This apparent positional restriction suggests heterogeneity of

hNPC as restricted populations of bipotent or unipotent glial and neural precursor cells in vivo; however, these finding do not exclude the possibility that precursor cells may reconfer multipotentiality in vivo (Ostenfeld et al.,
2002b; Hitoshi et al., 2002; Parmar et al., 2002, 2003; Lepore et al., 2004; Kim et al., 2006; Kallur et al., 2006) upon long-term exposure to the local cellular milieu or complementary signaling of mitogenic growth factors from secondary expansion in vitro.

Recent evidence from Cre-lox and retroviral lineage mapping studies has challenged the past notion that NPC become fate restricted before adulthood (Garcia et al., 2004; Doetsch et al., 1999; Malatesta et al., 2000; Hartfuss et al., 2001; Miyata et al., 2001; Noctor et al., 2001; Gotz et al., 2002; Doetsch, 2003; Malatesta et al., 2003; Goldman, 2003; Gotz, 2003; Noctor et al., 2004; Merkle et al., 2004; Gotz and Barde, 2005; Noctor et al., 2008; Weissman et al., 2003; Martinez- Cerdeno et al., 2006). These data have led to three major changes in the tenets of developmental neurobiology (Merkle and Alvarez-Buylla, 2006; Malatesta et al., 2008). First, adult NSC are of glial origin, but are not fate restricted and can therefore give rise to all three central nervous system (CNS) cell subtypes. Second, embryonic, fetal, and adult NSC are uniformly lineage related through radial glia (RG), and, third, fetal NPC actually divide asymmetrically to increase the number of progeny they generate via symmetrically dividing intermediate NPCs (Alvarez- Buylla et al., 2001; Tramontin et al., 2003; Rakic, 2007).

The revised unified model demonstrates the lineal transition from neuroepithelium to radial glia and eventually adult astrocyte-like NPC and may help explain the relative heterogeneity that NPC display throughout embryonic and fetal development into adulthood. Furthermore, the unified theory proposes that functional CNS stem cells will display heterogeneous phenotypes throughout neural development depending on temporal and spatial cues, suggesting that the nature of in vivo NSC/NPC is highly dynamic. Moreover, the relative

heterogeneity and plasticity of the in vivo neurogenic niche suggests a similar component may exist within artificial in vitro dissociated stem cell culture preparations.

Although it has been published that passaging cells with enzymes results in "high risk of high rates of cell death, lack of adherence, or differentiation" (Nethercott et al., 2007) as well as induction of karyotypic abnormalities, utilizing the procedures described here, we have been able to maintain behaviorally normal, karyotypically stable, undifferentiated forebrain hNPC (Villa et al., 2004; Foroni et al., 2007) as highly proliferative, multilayer adherent networks for >100 passages without marked senescence or phenotypic adaptation by means of enzymatic (Accutase) single-cell dissociation. It is our opinion that overall expansion rates and possibly time to senescence (Carpenter et al., 1999; Goyns and Lavery, 2000; Wright et al., 2006) can be greatly increased by simply improving the overall condition of hNPC during and after dissociation, regardless of the technique employed. The repetitive combination of mechanical shear stress from trituration, centrifugation, and osmotic shock simply provides more opportunities to destroy the fragile neural progenitors and ultimately results in a gradual decline in hNPC numbers. Furthermore, we speculate that as the gross number of actively mitotic progenitors decreases, the subsequent loss of paracrine signaling (Taupin et al., 2000; Toda et al., 2003; Agasse et al., 2004, 2006) between hNPC eventually falls below a threshold concentration, whereby the delimited hNPC culture no longer maintains the capacity to properly condition its own basal substrate and subsequently becomes quiescently static, undergoing an irreversible halt in paracrine/autocrine regulatory signaling. The ultimate result of such events is a small population of nonproliferative hNPC in severe crisis; these cells are not suitable for study and should be distinguished from their proliferative counterparts and discarded.

We propose a model, whereby hNPC end-term senescence and proliferative potential is influenced by population density through "conditioned signaling" and can be controlled by

manipulating various combinations of these factors. Moreover, in vitro human manipulation can play a huge impact on the overall health and success of cultures, impacting the combined intrinsic signaling cascades that govern the phenotype of hNPC. On a global scale, the ultimate capacity for longterm self-renewal and ability to generate extremely large quantities of undifferentiated neural precursors (Svendsen and Smith, 1999) may be vastly improved with minimal adaptation to currently employed procedures. We therefore posit that the potential for somatic hNPC therapy and diagnostics would best benefit by a paradigm shift in culturing techniques from low- to high-density adherent populations, paying special attention to the importance of re-establishing essential cell-cell contacts. Investigating these properties may restructure the current theory of in vitro populations of somatic hNPC as limited-capacity progenitors (Hayflick, 1968; Temple and Raff, 1986; Durand et al., 1998; Svendsen et al., 1998; Quinn et al., 1999; Palmer et al., 2001; van Heyningen et al., 2001) incapable of amassing the relatively large quantities of cells (like their embryonic counterparts) necessary for regenerative therapies (Gottlieb, 2002).

2.11 Acknowledgements

Chapter 2, is a reprint in part of material as it appears in Curr Protoc Stem Cell Biol. Chapter 2:Unit2D.3, May 2009 with co-authors Martin R Hofmann, D. Eugene Redmond Jr., Ted Teng, and Evan Y Snyder with the addition of a Table and Diagram from, Derivation, Expansion, and Characterization of Human Fetal Forebrain Neural Stem Cells. In: Masters JR, Palsson BO (eds). Human Cell Culture: Adult Stem Cells. Volume 7. Dordrecht: Springer, 2009 with co-authors Martin R. Hofmann, Ted Teng, and Evan Y Snyder. The dissertation author was the primary investigator and author of both papers. DRW wrote, designed, and performed all experiments. MRH helped with figure production and cell culture. EYS and YDT supervised DRW, DER wrote methods for preparation of monkeys for transplantation.

Together with his coauthors, he thanks Steven A. Wakeman and Pamela S. Burnett for constructive comments and support, as well as Ilyas Singec, Scott R. McKercher, Michael Marconi, Phillip H. Schwartz, Jeanne F. Loring, Franz-Josef Mueller, Jean-Pyo Lee, Seung U. Kim, and Kook I. Park for technical advice and procedural training. Funding for D.R.W. comes from (NIH/NIGMS T32 GM008666) UCSD Institutional Training Fellowship in Basic and Clinical Genetics, HHMI Med-Into-Grad Training Fellowship, American Society for Neural Therapy and Repair, and the American Parkinson's Disease Association. Additional support was provided by the Stem Cell Center at the Burnham Institute for Medical Research (NIH P20 GM075059-03).

2.12 References

1. Aboody, K.S., Brown, A., Rainov, N.G., Bower, K.A., Liu, S., Yang, W., Small, J.E., Herrlinger, U.,Ourednik,V.,Black, P.M., Breakefield, X.O., and Snyder, E.Y. 2000. Neural stem cells display extensive tropism for pathology in adult brain: Evidence from intracranial gliomas. ***Proc. Natl. Acad. Sci. U.S.A.*** **97:12846-12851.**

2. Agasse, F., Roger, M., and Coronas, V. 2004. Neurogenic and intact or apoptotic non-neurogenic areas of adult brain release diffusible molecules that differentially modulate the development of subventricular zone cell cultures. ***Eur. J. Neurosci.*** **19:1459-1468.**

3. Agasse, F., Benzakour, O., Berjeaud, J.M., Roger, M., and Coronas, V. 2006. Endogenous factors derived from embryonic cortex regulate proliferation and neuronal differentiation of postnatal subventricular zone cell cultures. ***Eur. J. Neurosci.*** **23:1970-1976.**

4. Allen, E. 1912. The cessation of mitosis in the central nervous system of the albino rat. ***J. Comp. Neurol.*** **22:547 568.**

5. Altman, J. 1962a. Are new neurons formed in the brains of adult mammals? ***Science*** **135:1127- 1128.**

6. Altman, J. 1962b. Autoradiographic study of degenerative and regenerative proliferation of neuroglia cells with tritiated thymidine. ***Exp. Neurol.*** **5:302-318.**

7. Altman, J. 1963. Autoradiographic investigation of cell proliferation in the brains of rats and cats. ***Anat. Rec.*** **145:573-591.**

8. Altman, J. and Chorover, S.L. 1963. Autoradiographic investigation of the distribution and utilization of intraventricularly injected adenine- 3H, uracil-3H and thymidine-3H in the brains of cats. *J. Physiol.* 169:770-779.

9. Altman, J. and Das, G.D. 1965a. Autoradiographic and histological evidence of postnatal hippocampal neurogenesis in rats. *J. Comp. Neurol.* 124:319-335.

10. Altman, J. and Das,G.D. 1965b. Post-natal origin of microneurones in the rat brain. *Nature* 207:953-956.

11. Altman, J. and Das, G.D. 1966. Autoradiographic and histological studies of postnatal neurogenesis. I. A longitudinal investigation of the kinetics, migration and transformation of cells incorporating tritiated thymidine in neonate rats, with special reference to postnatal neurogenesis in some brain regions. *J. Comp. Neurol.* 126:337-389.

12. Alvarez-Buylla, A. and Lim, D.A. 2004. For the long run: Maintaining germinal niches in the adult brain. *Neuron* 41:683-686.

13. Alvarez-Buylla, A., Garcia-Verdugo, J.M., and Tramontin, A.D. 2001. A unified hypothesis on the lineage of neural stem cells. *Nat. Rev. Neurosci.* 2:287-293.

14. Alvarez-Buylla, A., Seri, B., and Doetsch, F. 2002. Identification of neural stem cells in the adult vertebrate brain. *Brain Res. Bull.* 57:751-758.

15. Anderson, D.J. 2001. Stem cells and pattern formation in the nervous system: The possible versus the actual. *Neuron* 30:19-35.

16. Anderson, L., Burnstein, R.M., He, X., Luce, R., Furlong, R., Foltynie, T., Sykacek, P., Menon, D.K., and Caldwell, M.A. 2007. Gene expression changes in long term expanded human neural progenitor cells passaged by chopping lead to loss of neurogenic potential in vivo. *Exp. Neurol.* 204:512-524.

17. Androutsellis-Theotokis, A., Murase, S., Boyd, J.D., Park, D.M., Hoeppner, D.J., Ravin, R., and McKay, R.D. 2008. Generating neurons from stem cells. *Methods Mol. Biol.* 438:31-38.

18. Anisimov, S.V., Christophersen, N.S., Correia, A.S., Li, J.Y., and Brundin, P. 2007. "NeuroStem Chip": A novel highly specialized tool to study neural differentiation pathways in human stem cells. *BMC Genomics.* 8:46.

19. Arbab, A.S., Bashaw, L.A., Miller, B.R., Jordan, E.K., Lewis, B.K., Kalish, H., and Frank, J.A. 2003a. Characterization of biophysical and metabolic properties of cells labeled with superparamagnetic iron oxide nanoparticles and transfection agent for cellular MR imaging. *Radiology* 229:838-846.

20. Arbab, A.S., Bashaw, L.A., Miller, B.R., Jordan, E.K., Bulte, J.W., Frank, J.A. 2003b. Intracytoplasmic tagging of cells with ferumoxides and transfection agent

for cellular magnetic resonance imaging after cell transplantation: Methods and techniques. *Transplantation* 76:1123- 1130.

21. Arbab, A.S., Yocum, G.T., Kalish, H., Jordan, E.K., Anderson, S.A., Khakoo, A.Y., Read, E.J., and Frank, J.A. 2004a. Efficient magnetic cell labeling with protamine sulfate complexed to ferumoxides for cellular MRI. *Blood* 104:1217-1223.

22. Arbab, A.S., Yocum, G.T., Wilson, L.B., Parwana, A., Jordan, E.K., Kalish, H., and Frank, J.A. 2004b. Comparison of transfection agents in forming complexes with ferumoxides, cell labeling efficiency, and cellular viability. *Mol. Imaging* 3:24-32.

23. Armstrong, R.J., Watts, C., Svendsen, C.N., Dunnett, S.B., and Rosser, A.E. 2000. Survival, neuronal differentiation, and fiber outgrowth of propagated human neural precursor grafts in an animal model of Huntington's disease. *Cell Transplant.* 9:55-64.

24. Arsenijevic,Y.,Villemure, J.G., Brunet, J.F., Bloch, J.J., D´eglon, N., Kostic, C., Zurn, A., and Aebischer, P. 2001a. Isolation of multipotent neural precursors residing in the cortex of the adult human brain. *Exp. Neurol.* 170:48-62.

25. Arsenijevic, Y., Weiss, S., Schneider, B., and Aebischer, P. 2001b. Insulin-like growth factor- I is necessary for neural stem cell proliferation and demonstrates distinct actions of epidermal growth factor and fibroblast growth factor-2. *J. Neurosci.* 21:7194-7202.

26. Baer, K., Eriksson, P.S., Faull, R.L., Rees,M.I., and Curtis, M.A. 2007. Sox-2 is expressed by glial and progenitor cells and Pax-6 is expressed by neuroblasts in the human subventricular zone. *Exp. Neurol.* 204:828-831.

27. Balaci, L., Presta, M., Ennas, M.G., Dell'Era, P., Sogos, V., Lauro, G., and Gremo, F. 1994. Differential expression of fibroblast growth factor receptors by human neurones, astrocytes andmicroglia. *Neuroreport* 6:197-200. Bartlett, P.F., Kilpatrick, T.J., Richards, L.J., Talman, P.S., and Murphy, M. 1994. Regulation of the early development of the nervous system by growth factors. *Pharmacol. Ther.* 64:371-393.

28. Bartlett, P.F., Kilpatrick, T.J., Richards, L.J., Talman, P.S., and Murphy, M. 1994. Regulation of the early development of the nervous system by growth factors. *Pharmacol. Ther.* 64:371-393.

29. Behrstock, S. and Svendsen, C.N. 2004. Combining growth factors, stem cells, and gene therapy for the aging brain. *Ann. N.Y. Acad. Sci.* 1019:5- 14.

30. Behrstock, S., Ebert, A., McHugh, J., Vosberg, S., Moore, J., Schneider, B., Capowski, E., Hei, D., Kordower, J., Aebischer, P., and Svendsen, C.N. 2006. Human neural progenitors deliver glial cell line-derived neurotrophic factor to parkinsonian rodents and aged primates. *Gene Ther.* 13:379-388.

31. Betarbet, R., Zigova, T., Bakay, R.A., and Luskin, M.B. 1996. Migration patterns of neonatal subventricular zone progenitor cells transplanted into the neonatal striatum. *Cell Transplant.* 5:165-178.

32. Bjugstad, K.B., Redmond, D.E. Jr., Teng, Y.D., Elsworth, J.D., Roth, R.H., Blanchard, B.C., Snyder, E.Y., and Sladek, J.R. Jr. 2005. Neural stem cells implanted into MPTP-treated monkeys increase the size of endogenous tyrosine hydroxylase-positive cells found in the striatum: A return to control measures. *Cell Transplant.* 14:183-192.

33. Bjugstad, K.B., Teng, Y.D., Redmond, D.E. Jr., Elsworth, J.D., Roth, R.H., Cornelius, S.K., Snyder, E.Y., and Sladek, J.R. Jr. 2008. Human neural stem cells migrate along the nigrostriatal pathway in a primate model of Parkinson's disease. *Exp. Neurol.* 211:362-369.

34. Bonaguidi, M.A., McGuire, T., Hu, M., Kan, L., Samanta, J., and Kessler, J.A. 2005. LIF and BMP signaling generate separate and discrete types of GFAP-expressing cells. *Development* 132:5503-5514.

35. Brambrink, T., Foreman, R., Welstead, G.G., Lengner, C.J., Wernig, M., Suh, H., and Jaenisch, R. 2008. Sequential expression of pluripotency markers during direct reprogramming of mouse somatic cells. *Cell Stem Cell* 2:151-159.

36. Brannen, C.L. and Sugaya, K. 2000. in vitro differentiation of multipotent human neural progenitors in serum-free medium. *Neuroreport* 11:1123-1128.

37. Brewer, G.J. 1995. Serum-free B27/Neurobasal medium supports differentiated growth of neurons from the striatum, substantia nigra, septum, cerebral cortex, cerebellum, and dentate gyrus. *J. Neurosci. Res.* 42:674-683.

38. Brewer, G.J. 1997. Isolation and culture of adult rat hippocampal neurons. *J. Neurosci. Methods* 71:143-155.

39. Brewer, G.J. and Price, P.J. 1996. Viable cultured neurons in ambient carbon dioxide and hibernation storage for a month. *Neuroreport* 7:1509- 1512.

40. Brewer, G.J. and Torricelli, J.R. 2007. Isolation and culture of adult neurons and neurospheres. *Nat. Protoc.* 2:1490-1498.

41. Brewer, G.J., Torricelli, J.R., Evege, E.K., and Price, P.J. 1993. Optimized survival of hippocampal neurons in B27-supplemented Neurobasal, a new serum-freemedium combination. *J. Neurosci. Res.* 35:567-576.

42. Brustle, O. and McKay, R.D. 1996. Neuronal progenitors as tools for cell replacement in the nervous system. *Curr. Opin. Neurobiol.* 6:688-695.

43. Brustle, O., Spiro, A.C., Karram, K., Choudhary, K., Okabe, S., and McKay, R.D. 1997. in vitro–generated neural precursors participate in mammalian brain

development. ***Proc.Natl. Acad. Sci. U.S.A.*** **94:14809-14814.**

44. Buc-Caron, M.H. 1995. Neuroepithelial progenitor cells explanted from human fetal brain proliferate and differentiate in vitro. ***Neurobiol Dis.*** **2:37-47.**

45. Buchet, D., Buc-Caron, M.H., Sabate, O., Lachapelle, F., and Mallet, J. 2002. Long-term fate of human telencephalic progenitor cells grafted into the adult mouse brain: Effects of previous amplification in vitro. ***J. Neurosci. Res.*** **68:276-283.**

46. Bulte, J.W., Duncan, I.D., and Frank, J.A. 2002. In vivo magnetic resonance tracking of magnetically labeled cells after transplantation. ***J. Cereb. Blood Flow Metab.*** **22:899-907.**

47. Burgess, A., Wainwright, S.R., Shihabuddin, L.S., Rutishauser, U., Seki, T., and Aubert, I. 2008. Polysialic acid regulates the clustering, migration, and neuronal differentiation of progenitor cells in the adult hippocampus. ***Dev Neurobiol.*** **68:1580-1590.**

48. Burns,T.C.,Ortiz-Gonzalez, X.R.,Gutierrez-Perez, M., Keene, C.D., Sharda, R., Demorest, Z.L., Jiang, Y., Nelson-Holte, M., Soriano, M., Nakagawa, Y., Luquin, M.R., Garcia-Verdugo, J.M., Pr´osper, F., Low, W.C., and Verfaillie, C.M. 2006. Thymidine analogs are transferred from prelabeled donor to host cells in the central nervous system after transplantation: A word of caution. ***Stem Cells*** **24:1121-1127.**

49. Burnstein, R.M., Foltynie, T., He, X.,Menon, D.K., Svendsen, C.N., and Caldwell, M.A. 2004. Differentiation and migration of long term expanded human neural progenitors in a partial lesion model of Parkinson's disease. ***Int. J. Biochem. Cell Biol.*** **36:702-713.**

50. Caccamo, D., Katsetos, C.D., Herman, M.M., Frankfurter, A., Collins, V.P., and Rubinstein, L.J. 1989. Immunohistochemistry of a spontaneous murine ovarian teratoma with neuroepithelial differentiation: Neuron-associated beta-tubulin as a marker for primitive neuroepithelium. ***Lab. Invest.*** **60:390-398.**

51. Cai, J.,Wu,Y.,Mirua, T., Pierce, J.L., Lucero, M.T., Albertine, K.H., Spangrude, G.J., and Rao,M.S. 2002. Properties of a fetal multipotent neural stem cell (NEP cell). ***Dev Biol.*** **251:221-240.**

52. Cai, J., Shin, S.,Wright, L., Liu, Y., Zhou, D., Xue, H., Khrebtukova, I., Mattson, M.P., Svendsen, C.N., and Rao, M.S. 2006. Massively parallel signature sequencing profiling of fetal human neural precursor cells. ***Stem Cells Dev.*** **15:232- 244.**

53. Caldwell, M.A. and Svendsen, C.N. 1998. Heparin, but not other proteoglycans potentiates the mitogenic effects of FGF-2 on mesencephalic precursor cells. ***Exp. Neurol.*** **152:1-10.**

54. Caldwell, M.A., He, X., Wilkie, N., Pollack, S., Marshall, G., Wafford, K.A., and Svendsen, C.N. 2001. Growth factors regulate the survival and fate of cells derived from human neurospheres. ***Nature Biotechnol.*** **19:475-479.**

55. Caldwell, M.A., Garcion, E., terBorg, M.G., He, X., and Svendsen, C.N. 2004. Heparin stabilizes FGF-2 and modulates striatal precursor cell behavior in response to EGF. ***Exp. Neurol.*** **188:408-420.**

56. Caldwell, M.A., He, X., and Svendsen, C.N. 2005. 5-Bromo-2_-deoxyuridine is selectively toxic to neuronal precursors in vitro. ***Eur. J. Neurosci.*** **22:2965-2970.**

57. Campos, L.S., Leone, D.P., Relvas, J.B., Brakebusch, C., F¨assler, R., Suter, U., and Ffrench-Constant, C. 2004. Beta 1 integrins activate a MAPK signalling pathway in neural stem cells that contributes to their maintenance. ***Development*** **131:3433-3444.**

58. Capowski, E.E., Schneider, B.L., Ebert, A.D., Seehus, C.R., Szulc, J., Zufferey, R., Aebischer, P., and Svendsen, C.N. 2007. Lentiviral vector mediated genetic modification of human neural progenitor cells for ex vivo gene therapy. ***J. Neurosci. Methods.*** **163:338-349.**

59. Carbajo, S., Orfao, A., Alberca, V., Ciudad, J., L´opez, A., Hern´andez, L.C., and Carbajo-P´erez, E. 1995. in vivo bromodeoxyuridine (BrdU)- labelling index of rat thymus: Influence of different BrdU doses and exposure times as analyzed both in tissue sections and in single cell suspensions. ***Anal. Cell Pathol.*** **8:15-25.**

60. Carbajo-Perez, E., Carbajo, S., Ciudad, J., Lopez, A., Dolbeare, F., and Orfao, A. 1995. in vitro bromodeoxyuridine-labelling of single cell suspensions: Effects of time and temperature of sample storage. ***Cell Prolif.*** **28:609-615.**

61. Carleton, A., Petreanu, L.T., Lansford, R., Alvarez-Buylla, A., and Lledo, P.M. 2003. Becoming a new neuron in the adult olfactory bulb. ***Nat. Neurosci.*** **6:507-518.**

62. Carpenter, M.K., Cui, X., Hu, Z.Y., Jackson, J., Sherman, S., Seiger, A., and Wahlberg, L.U. 1999. in vitro expansion of a multipotent population of human neural progenitor cells. ***Exp. Neurol.*** **158:265-278.**

63. Chalfie, M. 1995. Green fluorescent protein. ***Photochem. Photobiol.*** **62:651-656.**

64. Chalfie, M., Tu, Y., Euskirchen, G., Ward, W.W., and Prasher, D.C. 1994. Green fluorescent protein as a marker for gene expression. ***Science*** **263:802-805.**

65. Chalmers-Redman, R.M., Priestley, T., Kemp, J.A., and Fine, A. 1997. in vitro propagation and inducible differentiation of multipotential progenitor cells from human fetal brain. ***Neuroscience*** **76:1121-1128.**

66. Chan, C., Moore, B.E., Cotman, C.W., Okano, H., Tavares, R., Hovanesian, V., Pinar, H., Johanson, C.E., Svendsen,C.N., and Stopa, E.G. 2006. Musashi1

antigen expression in human fetal germinal matrix development. *Exp. Neurol.* 201:515-518.

67. Chang, H.Y., Thomson, J.A., and Chen, X. 2006. Microarray analysis of stem cells and differentiation. *Methods Enzymol.* 420:225-254.

68. Chen, K.A., Laywell, E.D., Marshall, G., Walton, N., Zheng, T., and Steindler, D.A. 2006. Fusion of neural stem cells in culture. *Exp. Neurol.* 198:129-135.

69. Chidgey, A.P., Layton, D., Trounson, A., and Boyd, R.L. 2008. Tolerance strategies for stem-cell based therapies. *Nature* 453:330-337.

70. Christophersen, N.S., Meijer, X., Jorgensen, J.R., Englund, U.,Grønborg, M., Seiger, A., Brundin, P., and Wahlberg, L.U. 2006. Induction of dopaminergic neurons from growth factor expanded neural stem/progenitor cell cultures derived from human first trimester forebrain. *Brain Res. Bull.* 70:457-466.

71. Ciccolini, F. and Svendsen, C.N. 1998. Fibroblast growth factor 2 (FGF-2) promotes acquisition of epidermal growth factor (EGF) responsiveness in mouse striatal precursor cells: Identification of neural precursors responding to both EGF and FGF-2. *J. Neurosci.* 18:7869-7880.

72. Consiglio, A., Gritti, A., Dolcetta, D., Follenzi, A., Bordignon, C., Gage, F.H., Vescovi, A.L., and Naldini, L. 2004. Robust in vivo gene transfer into adult mammalian neural stem cells by lentiviral vectors. *Proc. Natl. Acad. Sci. U.S.A.* 101:14835-14840.

73. Conti, L., Pollard, S.M., Gorba, T., Reitano, E.,Toselli, M., Biella, G., Sun, Y., Sanzone, S., Ying, Q.L., Cattaneo, E., and Smith, A. 2005. Niche-independent symmetrical self-renewal of a mammalian tissue stem cell. *PLoS Biol.* 3:e283.

74. Curtis, M.A., Kam, M., Nannmark, U., Anderson, M.F., Axell, M.Z., Wikkelso, C., Holt°as, S., van Roon-Mom, W.M., Bj¨ork-Eriksson, T., Nordborg, C., Fris´en, J., Dragunow, M., Faull, R.L., and Eriksson, P.S. 2007. Human neuroblasts migrate to the olfactory bulb via a lateral ventricular extension. *Science* 315:1243-1249.

75. Dahlstrand, J., Zimmerman, L.B., McKay, R.D., and Lendahl, U. 1992. Characterization of the human nestin gene reveals a close evolutionary relationship to neurofilaments. *J. Cell Sci.* 103:589-597.

76. D'Amour, K.A. and Gage, F.H. 2003. Genetic and functional differences between multipotent neural and pluripotent embryonic stem cells. *Proc. Natl. Acad. Sci. U.S.A.* 100:11866-11872.

77. de Chevigny, A., Cooper, O., Vinuela, A., Reske-Nielsen, C., Lagace, D.C., Eisch, A.J., and Isacson, O. 2008. Fate mapping and lineage analyses demonstrate the production of a large number of striatal neuroblasts after transforming growth factor alpha and noggin striatal infusions into the dopamine-depleted striatum.

Stem Cells **26:2349-2360.**

78. Deisseroth, K., Singla, S., Toda, H., Monje, M., Palmer, T.D., and Malenka, R.C. 2004. Excitation-neurogenesis coupling in adult neural stem/progenitor cells. *Neuron* 42:535-552.

79. Dietrich, J., Noble, M., and Mayer-Proschel, M. 2002. Characterization of A2B5+ glial precursor cells from cryopreserved human fetal brain progenitor cells. *Glia* 40:65-77.

80. Doetsch, F. 2003. The glial identity of neural stem cells. *Nat. Neurosci.* 6:1127-1134.

81. Doetsch, F. and Alvarez-Buylla, A. 1996. Network of tangential pathways for neuronalmigration in adult mammalian brain. *Proc. Natl. Acad. Sci. U.S.A.* 93:14895-14900.

82. Doetsch, F., Garcia-Verdugo, J.M., and Alvarez- Buylla,A. 1997. Cellular composition and threedimensional organization of the subventricular germinal zone in the adult mammalian brain. *J. Neurosci.* 17:5046-5061.

83. Doetsch, F., Caille, I., Lim, D.A., Garcia-Verdugo, J.M., and Alvarez-Buylla, A. 1999. Subventricular zone astrocytes are neural stem cells in the adult mammalian brain. *Cell.* 97:703- 716.

84. Doetsch, F., Petreanu, L., Caille, I., Garcia- Verdugo, J.M., Alvarez- and Buylla, A. 2002. EGF converts transit-amplifying neurogenic precursors in the adult brain into multipotent stem cells. *Neuron* 36:1021-1034.

85. Dolbeare, F. 1995. Bromodeoxyuridine: A diagnostic tool in biology and medicine. Part I. Historical perspectives, histochemical methods and cell kinetics. *Histochem. J.* 27:339-369.

86. Dolbeare, F. 1996. Bromodeoxyuridine: A diagnostic tool in biology and medicine. Part III. Proliferation in normal, injured and diseased tissue, growth factors, differentiation, DNA replication sites and in situ hybridization. *Histochem.J.* 28:531-575.

87. Drago, J., Murphy, M., Carroll, S.M., Harvey, R.P., and Bartlett, P.F. 1991a. Fibroblast growth factor-mediated proliferation of central nervous system precursors depends on endogenous production of insulin-like growth factor I. *Proc. Natl. Acad. Sci. U.S.A.* 88:2199-2203.

88. Drago, J., Nurcombe, V., Pearse, M.J., Murphy, M., and Bartlett, P.F. 1991b. Basic fibroblast growth factor upregulates steady-state levels of laminin B1 and B2 chain mRNA in cultured neuroepithelial cells. *Exp. Cell Res.* 196:246- 254.

89. Durand, B., Fero, M.L., Roberts, J.M., and Raff, M.C. 1998. p27Kip1 alters the response of cells to mitogen and is part of a cell-intrinsic timer that arrests the

cell cycle and initiates differentiation. *Curr. Biol.* 8:431-440.

90. Ebert, A.D., Beres, A.J., Barber, A.E., and Svendsen, C.N. 2008. Human neural progenitor cells over-expressing IGF-1 protect dopamine neurons and restore function in a rat model of Parkinson's disease. *Exp. Neurol.* 209:213-223.

91. El Maarouf, A. and Rutishauser, U. 2008. Use of PSA-NCAM in repair of the central nervous system. *Neurochem. Res.* In press.

92. Emsley, J.G., Mitchell, B.D., Kempermann, G., andMacklis, J.D. 2005. Adult neurogenesis and repair of the adult CNS with neural progenitors, precursors, and stem cells. *Prog.Neurobiol.* 75:321-341.

93. Feng, L. and Heintz, N. 1995. Differentiating neurons activate transcription of the brain lipidbinding protein gene in radial glia through a novel regulatory element. *Development* 121:1719-1730.

94. Feng, L., Hatten, M.E., and Heintz, N. 1994. Brain lipid-binding protein (BLBP): A novel signaling system in the developing mammalian CNS. *Neuron.* 12:895-908.

95. Flanagan, L.A., Rebaza, L.M., Derzic, S., Schwartz, P.H., and Monuki, E.S. 2006. Regulation of human neural precursor cells by laminin and integrins. *J Neurosci. Res.* 83:845-856.

96. Flax, J.D., Aurora, S., Yang, C., Simonin, C., Wills, A.M., Billinghurst, L.L., Jendoubi, M., Sidman, R.L., Wolfe, J.H., Kim, S.U., and Snyder, E.Y. 1998. Engraftable human neural stem cells respond to developmental cues, replace neurons, and express foreign genes. *Nat. Biotechnol.* 16:1033-1039.

97. Foroni, C., Galli, R., Cipelletti, B., Caumo, A., Alberti, S., Fiocco,R., and Vescovi,A. 2007. Resilience to transformation and inherent genetic and functional stability of adult neural stem cells ex vivo. *Cancer Res.* 67:3725-3733.

98. Francis, F., Koulakoff, A., Boucher, D., Chafey, P., Schaar, B., Vinet, M.C., Friocourt, G., McDonnell, N., Reiner, O., Kahn, A., McConnell, S.K., Berwald-Netter, Y., Denoulet, P., and Chelly, J. 1999. Doublecortin is a developmentally regulated, microtubule-associated protein expressed in migrating and differentiating neurons. *Neuron* 23:247-256.

99. Frank, J.A., Kalish, H., Jordan, E.K., Anderson, S.A., Pawelczyk, E., and Arbab, A.S. 2007. Color transformation and fluorescence of Prussian blue–positive cells: Implications for histologic verification of cells labeled with superparamagnetic iron oxide nanoparticles. *Mol. Imaging.* 6:212-218.

100. Frank, J.A., Miller, B.R., Arbab, A.S., Zywicke, H.A., Jordan, E.K., Lewis, B.K., Bryant, L.H. Jr., and Bulte, J.W. 2003. Clinically applicable labeling of mammalian and stem cells by combining superparamagnetic iron oxides and transfection agents. *Radiology* 228:480-487.

101. Fricker, R.A., Carpenter,M.K.,Winkler, C., Greco, C., Gates, M.A., and Bjorklund, A. 1999. Sitespecific migration and neuronal differentiation of human neural progenitor cells after transplantation in the adult rat brain. *J. Neurosci.* 19:5990- 6005.

102. Friocourt, G., Koulakoff, A., Chafey, P., Boucher, D., Fauchereau, F., Chelly, J., and Francis, F. 2003. Doublecortin functions at the extremities of growing neuronal processes. *Cereb. Cortex* 13:620-626.

103. Gabay, L., Lowell, S., Rubin, L.L., and Anderson, D.J. 2003. Deregulation of dorsoventral patterning by FGF confers trilineage differentiation capacity on CNS stem cells in vitro. *Neuron* 40:485-499.

104. Gage, F.H., Ray, J., and Fisher, L.J. 1995a. Isolation, characterization, and use of stem cells from the CNS. *Annu. Rev. Neurosci.* 18:159-192.

105. Gage, F.H., Coates, P.W., Palmer, T.D., Kuhn, H.G., Fisher, L.J., Suhonen, J.O., Peterson, D.A., Suhr, S.T., and Ray, J. 1995b. Survival and differentiation of adult neuronal progenitor cells transplanted to the adult brain. *Proc. Natl. Acad. Sci. U.S.A.* 92:11879-11883.

106. Galli, R., Pagano, S.F., Gritti, A., and Vescovi, A.L. 2000. Regulation of neuronal differentiation in human CNS stem cell progeny by leukemia inhibitory factor. *Dev. Neurosci.* 22:86-95.

107. Garcia, A.D., Doan, N.B., Imura, T., Bush, T.G., and Sofroniew, M.V. 2004. GFAP-expressing progenitors are the principal source of constitutive neurogenesis in adult mouse forebrain. *Nat. Neurosci.* 7:1233-1241.

108. Garcia-Verdugo, J.M., Doetsch, F., Wichterle, H., Lim, D.A., and Alvarez-Buylla, A. 1998. Architecture and cell types of the adult subventricular zone: In search of the stem cells. *J. Neurobiol.* 36:234-248.

109. Geisert, E.E. Jr. and Frankfurter, A. 1989. The neuronal response to injury as visualized by immunostaining of class III beta-tubulin in the rat. *Neurosci. Lett.* 102:137-141.

110. Gensburger, C., Labourdette, G., and Sensenbrenner, M. 1987. Brain basic fibroblast growth factor stimulates the proliferation of rat neuronal precursor cells in vitro. *FEBS Lett.* 217:1-5.

111. Ghashghaei, H.T., Lai, C., and Anton, E.S. 2007. Neuronal migration in the adult brain: Are we there yet? *Nat. Rev. Neurosci.* 8:141-51.

112. Ghosh, A. and Greenberg,M.E. 1995. Distinct roles for bFGF and NT-3 in the regulation of cortical neurogenesis. *Neuron* 15(1):89-103.

113. Giepmans, B.N., Adams, S.R., Ellisman, M.H., and Tsien, R.Y. 2006. The fluorescent toolbox for assessing protein location and function. *Science* 312:217-

224.

114. Gilad, A.A., Walczak, P., McMahon, M.T., Na, H.B., Lee, J.H., An, K., Hyeon, T., van Zijl, P.C., and Bulte, J.W. 2008.MRtracking of transplanted cells with "positive contrast" using manganese oxide nanoparticles. *Magn. Reson. Med.* 60:1-7.

115. Gleeson, J.G., Lin, P.T., Flanagan, L.A., and Walsh, C.A. 1999. Doublecortin is a microtubule associated protein and is expressed widely by migrating neurons. *Neuron* 23:257-271.

116. Goldman, S.A. 2003. Glia as neural progenitor cells. *Trends Neurosci.* 26:590-596.

117. Goldman, S.A. and Nottebohm, F. 1983. Neuronal production, migration, and differentiation in a vocal control nucleus of the adult female canary brain. *Proc. Natl. Acad. Sci. U.S.A.* 80:2390- 2394.

118. Goldman, S.A., Kirschenbaum, B., Harrison- Restelli, C., and Thaler, H.T. 1997. Neuronal precursors of the adult rat subependymal zone persist into senescence, with no decline in spatial extent or response to BDNF. *J. Neurobiol.* 2:554-566.

119. Gottlieb, D.I. 2002. Large-scale sources of neural stem cells. *Annu. Rev. Neurosci.* 25:381-407.

120. Gotz, M. 2003. Glial cells generate neurons—master control within CNS regions: Developmental perspectives on neural stem cells. *Neuroscientist* 9:379-397.

121. Gotz, M. and Barde, Y.A. 2005. Radial glial cells defined and major intermediates between embryonic stem cells and CNS neurons. *Neuron* 2005 46:369-372.

122. Gotz, M., Hartfuss, E., and Malatesta, P. 2002. Radial glial cells as neuronal precursors: A new perspective on the correlation of morphology and lineage restriction in the developing cerebral cortex of mice. *Brain Res. Bull.* 57:777-788.

123. Gould, E., Tanapat, P., McEwen, B.S., Flugge, G., and Fuchs, E. 1998. Proliferation of granule cell precursors in the dentate gyrus of adult monkeys is diminished by stress. *Proc. Natl. Acad. Sci. U.S.A.* 95:3168-3171.

124. Gould, E., Reeves, A.J., Fallah, M., Tanapat, P., Gross, C.G., and Fuchs, E. 1999a. Hippocampal neurogenesis in adult OldWorld primates. *Proc. Natl. Acad. Sci. U.S.A.* 96:5263-5267.

125. Gould, E., Reeves, A.J., Graziano, M.S., and Gross, C.G. 1999b. Neurogenesis in the neocortex of adult primates. *Science* 286:548-552.

126. Goyns, M.H. and Lavery, W.L. 2000. Telomerase and mammalian ageing: A critical appraisal. *Mech. Ageing Dev.* 114:69-77.

127. Gratzner, H.G. 1982. Monoclonal antibody to 5- bromo- and 5-iododeoxyuridine: A new reagent for detection of DNA replication. *Science* 218:474-475.

128. Gregg, C. and Weiss, S. 2003. Generation of functional radial glial cells by embryonic and adult forebrain neural stem cells. *J. Neurosci.* 23:11587-11601.

129. Gregg, C. and Weiss, S. 2005. CNTF/LIF/gp130 receptor complex signaling maintains a VZ precursor differentiation gradient in the developing ventral forebrain. *Development* 132:565-578.

130. Gregg, C.T., Chojnacki, A.K., and Weiss, S. 2002. Radial glial cells as neuronal precursors: The next generation? *J. Neurosci. Res.* 69:708-713.

131. Gritti, A., Parati, E.A., Cova, L., Frolichsthal, P., Galli, R., Wanke, E., Faravelli, L., Morassutti, D.J., Roisen, F., Nickel, D.D., and Vescovi, A.L. 1996. Multipotential stem cells from the adult mouse brain proliferate and self-renew in response to basic fibroblast growth factor. *J. Neurosci.* 16:1091-1100.

132. Gritti, A., Frolichsthal-Schoeller, P., Galli, R., Parati, E.A., Cova, L., Pagano, S.F., Bjornson, C.R., and Vescovi, A.L. 1999. Epidermal and fibroblast growth factors behave as mitogenic regulators for a single multipotent stem celllike population from the subventricular region of the adult mouse forebrain. *J. Neurosci.* 19:3287-3297.

133. Gritti, A., Vescovi, A., and Galli, R. 2003. Neural stem cells and their plasticity. *In* Neural Stem Cells for Brain and Spinal Cord Repair (T. Zigova., E.Y. Snyder, and P.R. Sanberg, eds.) pp. 45-68. Humana Press, Totowa, N.J.

134. Gritti, A., Galli, R., and Vescovi, A.L. 2008. Clonal analyses and cryopreservation of neural stem cell cultures. *Methods Mol. Biol.* 438:173-184.

135. Guzman, R., Uchida, N., Bliss, T.M., He, D., Christopherson, K.K., Stellwagen, D., Capela, A., Greve, J., Malenka, R.C., Moseley, M.E., Palmer, T.D., and Steinberg, G.K. 2007. Longterm monitoring of transplanted human neural stem cells in developmental and pathological contexts with MRI. *Proc. Natl. Acad. Sci. U.S.A.* 104:10211-10216.

136. Guzman,R., Bliss,T.,DeLos Angeles, A.,Moseley, M., Palmer, T., and Steinberg, G. 2008. Neural progenitor cells transplanted into the uninjured brain undergo targetedmigration after stroke onset. *J. Neurosci. Res.* 86:873 882.

137. Hall, P.A. and Levison, D.A. 1990. Review: Assessment of cell proliferation in histological material. *J. Clin. Pathol.* 43:184-192.

138. Hall, P.A., Levison, D.A., Woods, A.L., Yu, C.C., Kellock, D.B., Watkins, J.A., Barnes, D.M., Gillett, C.E., Camplejohn, R., Dover, R., Waseem, N.H., and Lane, D.P. 1990. Proliferating cell nuclear antigen (PCNA) immunolocalization in paraffin sections: An index of cell proliferation with evidence of deregulated expression in some neoplasms. *J. Pathol.* 162:285- 294.

139. Hall, P.E., Lathia, J.D., Caldwell, M.A., and Ffrench-Constant, C. 2008. Laminin enhances the growth of human neural stem cells in defined culture media. *BMC Neurosci.* 9:71.

140. Hartfuss, E., Galli, R., Heins, N., and Gotz, M. 2001. Characterization of CNS precursor subtypes and radial glia. *Dev. Biol.* 229:15-30.

141. Hayflick, L. 1968. Human cells and aging. *Sci. Am.* 218:32-37.

142. Hellstrom, N.A., Bjork-Eriksson, T., Blomgren, K., and Kuhn, H.G. 2008. Differential recovery of neural stem cells in the subventricular zone and dentate gyrus after ionizing radiation. *Stem Cells* In press.

143. Herrera, D.G., Garcia-Verdugo, J.M., and Alvarez- Buylla, A. 1999. Adult-derived neural precursors transplanted into multiple regions in the adult brain. *Ann Neurol.* 46:867-877.

144. Hinds, K.A., Hill, J.M., Shapiro, E.M., Laukkanen, M.O., Silva, A.C., Combs, C.A., Varney, T.R., Balaban, R.S., Koretsky, A.P., and Dunbar, C.E. 2003. Highly efficient endosomal labeling of progenitor and stem cells with large magnetic particles allows magnetic resonance imaging of single cells. *Blood* 102:867-872.

145. Hitoshi, S., Tropepe, V., Ekker, M., and van der Kooy, D. 2002. Neural stem cell lineages are regionally specified, but not committed, within distinct compartments of the developing brain. *Development* 129:233-244.

146. Hockfield, S. and McKay, R.D. 1985. Identification of major cell classes in the developing mammalian nervous system. *J. Neurosci.* 5:3310- 3328.

147. Howard, B., Chen, Y., and Zecevic, N. 2006. Cortical progenitor cells in the developing human telencephalon. *Glia* 53:57-66.

148. Hsieh, J. and Gage, F.H. 2004. Epigenetic control of neural stem cell fate. *Curr. Opin. Genet. Dev.* 14:461-469.

149. Hu, H., Tomasiewicz, H., Magnuson, T., and Rutishauser, U. 1996. The role of polysialic acid in migration of olfactory bulb interneuron precursors in the subventricular zone. *Neuron* 16:735-743.

150. Hynes, R.O. 2002. Integrins: Bidirectional, allosteric signaling machines. *Cell* 110:673-687.

151. Imitola, J., Raddassi, K., Park, K.I., Mueller, F.J., Nieto, M., Teng, Y.D., Frenkel, D., Li, J., Sidman, R.L., Walsh, C.A., Snyder, E.Y., and Khoury, S.J. 2004. Directed migration of neural stem cells to sites of CNS injury by the stromal cell-derived factor 1alpha/CXC chemokine receptor 4 pathway. *Proc. Natl. Acad. Sci. U.S.A.* 101:18117-18122.

152. Imura, T., Kornblum, H.I., and Sofroniew, M.V. 2003. The predominant neural stem cell isolated from postnatal and adult forebrain but not early embryonic forebrain expresses GFAP. *J. Neurosci.* 23:2824-2832.

153. Jacques, T.S., Relvas, J.B., Nishimura, S., Edwards, G.M., Streuli, C.H., and Ffrench-Constant, C. 1998. Neural precursor cell chain migration and division are regulated through different beta1 integrins. *Development* 125:3167-3177.

154. Jendelova, P., Herynek, V., Urdzikova, L., Glogarov´a, K., Kroupov´a, J., Andersson, B., Bryja, V., Burian, M., H´ajek, M., and Sykov´a, E. 2004. Magnetic resonance tracking of transplanted bone marrow and embryonic stem cells labeled by iron oxide nanoparticles in rat brain and spinal cord. *J. Neurosci. Res.* 76:232-243.

155. Jessberger, S., Clemenson, G.D. Jr., and Gage, F.H. 2007. Spontaneous fusion and nonclonal growth of adult neural stem cells. *Stem Cells* 25:871-874.

156. Johansson, C.B.,Momma, S., Clarke,D.L., Risling, M., Lendahl, U., and Frisen, J. 1999. Identification of a neural stem cell in the adultmammalian central nervous system. *Cell* 96:25-34.

157. Johe, K.K., Hazel, T.G., Muller, T., Dugich- Djordjevic, M.M., and McKay, R.D. 1996. Single factors direct the differentiation of stem cells from the fetal and adult central nervous system. *Genes Dev.* 10:3129-3140.

158. Kallur, T., Darsalia, V., Lindvall, O., and Kokaia, Z. 2006. Human fetal cortical and striatal neural stem cells generate region-specific neurons in vitro and differentiate extensively to neurons after intrastriatal transplantation in neonatal rats. *J. Neurosci. Res.* 84:1630-1644.

159. Kanemura, Y., Mori, H., Kobayashi, S., Islam, O., Kodama, E., Yamamoto, A., Nakanishi, Y., Arita, N., Yamasaki, M., Okano, H., Hara, M., and Miyake, J. 2002. Evaluation of in vitro proliferative activity of human fetal neural stem/progenitor cells using indirect measurements of viable cells based on cellular metabolic activity. *J. Neurosci. Res.* 69:869-879.

160. Kanemura, Y., Mori, H., Nakagawa, A., Islam, M.O., Kodama, E., Yamamoto, A., Shofuda, T., Kobayashi, S.,Miyake, J.,Yamazaki, T.,Hirano, S., Yamasaki, M., and Okano, H. 2005. in vitro screening of exogenous factors for human neural stem/progenitor cell proliferation using measurement of total ATP content in viable cells. *Cell Transplant.* 14:673-682.

161. Kaplan, M.S. and Hinds, J.W. 1977. Neurogenesis in the adult rat: Electron microscopic analysis of light radioautographs. *Science* 197:1092-1094.

162. Kelly, S., Bliss, T.M., Shah, A.K., Sun, G.H., Ma, M., Foo, W.C., Masel, J., Yenari, M.A., Weissman, I.L., Uchida, N., Palmer, T., and Steinberg, G.K. 2004. Transplanted human fetal neural stem cells survive, migrate, and differentiate in

ischemic rat cerebral cortex. *Proc. Natl. Acad. Sci. U.S.A.* 101:11839-11844.

163. Kelly, C.M., Tyers, P., Borg, M.T., Svendsen, C.N., Dunnett, S.B., and Rosser, A.E. 2005. EGF and FGF-2 responsiveness of rat and mouse neural precursors derived from the embryonic CNS. *Brain Res. Bull.* 68:83-94.

164. Kempermann, G. 2006. Neural Stem Cells. Adult Neurogenesis: Stem Cells and Neuronal Development in the Adult Brain. pp. 50-95. Oxford University Press, New York.

165. Keyoung, H.M., Roy, N.S., Benraiss, A., Louissaint, A. Jr., Suzuki, A., Hashimoto, M., Rashbaum, W.K., Okano, H., and Goldman, S.A. 2001. High-yield selection and extraction of two promoter-defined phenotypes of neural stem cells from the fetal human brain. *Nat. Biotechnol.* 19:843-850.

166. Kilpatrick, T.J. and Bartlett, P.F. 1993. Cloning and growth of multipotential neural precursors: Requirements for proliferation and differentiation. *Neuron* 10:255-265.

167. Kilpatrick, T.J. and Bartlett, P.F. 1995. Cloned multipotential precursors from the mouse cerebrum require FGF-2, whereas glial restricted precursors are stimulated with either FGF-2 or EGF. *J. Neurosci.* 15:3653-3661.

168. Kilpatrick, T.J., Richards, L.J., and Bartlett, P.F. 1995. The regulation of neural precursor cells within the mammalian brain. *Mol. Cell. Neurosci.* 6:2-15.

169. Kim, H.T., Kim, I.S., Lee, I.S., Lee, J.P., Snyder, E.Y., and Park, K.I. 2006. Human neurospheres derived from the fetal central nervous system are regionally and temporally specified but are not committed. *Exp. Neurol.* 199:222-235.

170. Kim, J.B., Zaehres, H.,Wu, G., Gentile, L., Ko, K., Sebastiano, V., Ara´uzo-Bravo, M.J., Ruau, D., Han, D.W., Zenke, M., and Sch¨oler, H.R. 2008. Pluripotent stem cells induced from adult neural stem cells by reprogramming with two factors. *Nature* 454:646-650.

171. Kim, S.U. 2004. Human neural stem cells genetically modified for brain repair in neurological disorders. *Neuropathology* 24:159-171.

172. Kim, M. and Morshead, C.M. 2003. Distinct populations of forebrain neural stem and progenitor cells can be isolated using side-population analysis. *J. Neurosci.* 23:10703-10709.

173. Kirschenbaum, B., Nedergaard, M., Preuss, A., Barami, K., Fraser, R.A., and Goldman, S.A. 1994. in vitro neuronal production and differentiation by precursor cells derived from the adult human forebrain. *Cereb Cortex* 4:576-589.

174. Kitchens, D.L., Snyder, E.Y., and Gottlieb, D.I. 1994. FGF and EGF are mitogens for immortalized neural progenitors. *J. Neurobiol.* 25:797-807.

175. Klein, S.M., Behrstock, S., McHugh, J., Hoffmann, K., Wallace, K., Suzuki, M., Aebischer, P., and Svendsen, C.N. 2005. GDNF delivery using human neural progenitor cells in a rat model of ALS. *Hum. Gene Ther.* 16:509-521.

176. Komitova, M. and Eriksson, P.S. 2004. Sox-2 is expressed by neural progenitors and astroglia in the adult rat brain. *Neurosci. Lett.* 369:24-27.

177. Kornack, D.R. and Rakic, P. 2001a. The generation, migration, and differentiation of olfactory neurons in the adult primate brain. *Proc. Natl. Acad. Sci. U.S.A.* 98:4752-4757.

178. Kornack, D.R. and Rakic, P. 2001b. Cell proliferation without neurogenesis in adult primate neocortex. *Science* 294:2127-2130.

179. Kornblum, H.I., Raymon, H.K., Morrison, R.S., Cavanaugh, K.P., Bradshaw, R.A., and Leslie, F.M. 1990. Epidermal growth factor and basic fibroblast growth factor: Effects on an overlapping population of neocortical neurons in vitro. *Brain Res.* 535:255-263.

180. Kuhn, H.G., Dickinson-Anson, H., and Gage, F.H. 1996. Neurogenesis in the dentate gyrus of the adult rat: Age-related decrease of neuronal progenitor proliferation. *J. Neurosci.* 16:2027-2033.

181. Kuhn, H.G., Winkler, J., Kempermann, G., Thal, L.J., and Gage, F.H. 1997. Epidermal growth factor and fibroblast growth factor-2 have different effects on neural progenitors in the adult rat brain. *J. Neurosci.* 17:5820-5829.

182. Kukekov, V.G., Laywell, E.D., Suslov, O., Davies, K., Scheffler, B., Thomas, L.B., O'Brien, T.F., Kusakabe, M., and Steindler, D.A. 1999. Multipotent stem/progenitor cells with similar properties arise from two neurogenic regions of adult human brain. *Exp. Neurol.* 156:333-344.

183. Labat-Robert, J. and Robert, L. 2005. The extracellular matrix during normal development and neoplastic growth. *Prog. Mol. Subcell. Biol.* 40:79-106.

184. Lathia, J.D., Rao, M.S.,Mattson, M.P., and Ffrench-Constant, C. 2007. Themicroenvironment of the embryonic neural stem cell: Lessons from adult niches? *Dev. Dyn.* 236:3267-3282.

185. Laywell, E.D., Rakic, P., Kukekov, V.G., Holland, E.C., and Steindler, D.A. 2000. Identification of a multipotent astrocytic stem cell in the immature and adult mouse brain. *Proc. Natl. Acad. Sci. U.S.A.* 97:13883-13888.

186. Laywell, E.D., Kukekov, V.G., Suslov, O., Zheng, T., and Steindler, D.A. 2002. Production and analysis of neurospheres from acutely dissociated and postmortem CNS specimens. *MethodsMol. Biol.* 198:15-27.

187. Le Belle, J.E., Caldwell, M.A., and Svendsen, C.N. 2004. Improving the survival

of human CNS precursor-derived neurons after transplantation. *J. Neurosci. Res.* 76:174-183.

188. Lee, H.J., Kim, K.S., Kim, E.J., Choi, H.B., Lee, K.H., Park, I.H., Ko, Y., Jeong, S.W., and Kim, S.U. 2007. Brain transplantation of immortalized human neural stem cells promotes functional recovery in mouse intracerebral hemorrhage stroke model. *Stem Cells (Dayton)* 25:1204-1212.

189. Lee, J.P., Jeyakumar, M., Gonzalez, R., Takahashi, H., Lee, P.J., Baek, R.C., Clark, D., Rose, H., Fu, G., Clarke, J., McKercher, S., Meerloo, J., Muller, F.J., Park, K.I., Butters, T.D., Dwek, R.A., Schwartz, P., Tong, G., Wenger, D., Lipton, S.A., Seyfried, T.N., Platt, F.M., and Snyder, E.Y. 2007. Stem cells act through multiple mechanisms to benefit mice with neurodegenerative metabolic disease. *Nat. Med.* 13:439-447.

190. Lee, J.-P., Muller, F.-J., and Snyder, E.Y. 2008.Neural stem cell transplantation in mouse brain. *Curr. Protoc. Neurosci.* 42:3.10.1-3.10.23.

191. Lee, M.K., Tuttle, J.B., Rebhun, L.I., Cleveland, D.W., and Frankfurter, A. 1990. The expression and posttranslational modification of a neuronspecific beta-tubulin isotype during chick embryogenesis. *Cell Motil. Cytoskeleton* 17:118-132.

192. Lendahl, U., Zimmerman, L.B., and McKay, R.D. 1990. CNS stem cells express a new class of intermediate filament protein. *Cell* 60:585-595.

193. Leone,D.P., Relvas, J.B., Campos, L.S., Hemmi, S., Brakebusch, C., F¨assler, R., Ffrench-Constant, C., and Suter, U. 2005. Regulation of neural progenitor proliferation and survival by ! 1 integrins. *J. Cell Sci.* 118:2589-2599.

194. Lepore, A.C., Han, S.S., Tyler-Polsz, C.J., Cai, J., Rao, M.S., and Fischer, I. 2004. Differential fate of multipotent and lineage-restricted neural precursors following transplantation into the adult CNS. *Neuron Glia Biol.* 1:113-126.

195. Lepore, A.C., Neuhuber, B., Connors, T.M., Han, S.S., Liu, Y., Daniels, M.P., Rao, M.S., and Fischer, I. 2006a. Long-term fate of neural precursor cells following transplantation into developing and adult CNS. *Neuroscience* 142:287-304.

196. Lepore, A.C., Walczak, P., Rao, M.S., Fischer, I., and Bulte, J.W. 2006b. MR imaging of lineage-restricted neural precursors following transplantation into the adult spinal cord. *Exp. Neurol.* 201:49-59.

197. Leung, C.T., Coulombe, P.A., and Reed, R.R. 2007. Contribution of olfactory neural stem cells to tissue maintenance and regeneration. *Nat. Neurosci.* 10:720-726.

198. Levison, S.W. and Goldman, J.E. 1993. Both oligodendrocytes and astrocytes develop from progenitors in the subventricular zone of postnatal rat forebrain. *Neuron* 10:201-212.

199. Levison, S.W. and Goldman, J.E. 1997. Multipotential and lineage restricted precursors coexist in themammalian perinatal subventricular zone. *J. Neurosci. Res.* 48:83-94.

200. Levison, S.W., Chuang, C., Abramson, B.J., and Goldman, J.E. 1993. The migrational patterns and developmental fates of glial precursors in the rat subventricular zone are temporally regulated. *Development* 119:611-622.

201. Levitt, P. and Rakic, P. 1980. Immunoperoxidase localization of glial fibrillary acidic protein in radial glial cells and astrocytes of the developing rhesus monkey brain. *J. Comp. Neurol.* 193:815-840.

202. Levitt, P.,Cooper, M.L., and Rakic, P. 1981. Coexistence of neuronal and glial precursor cells in the cerebral ventricular zone of the fetal monkey: An ultrastructural immunoperoxidase analysis. *J. Neurosci.* 1:27-39.

203. Levitt, P., Cooper, M.L., and Rakic, P. 1983. Early divergence and changing proportions of neuronal and glial precursor cells in the primate cerebral ventricular zone. *Dev. Biol.* 96:472-484.

204. Lewin, M., Carlesso, N., Tung, C.H., Tang, X.W., Cory, D., Scadden, D.T., and Weissleder, R. 2000. Tat peptide-derivatized magnetic nanoparticles allow in vivo tracking and recovery of progenitor cells. *Nat. Biotechnol.* 18:410-414.

205. Li, X., Xu, J., Bai, Y., Dai, X., Liu, Y., Zhang, J., Zou, J., Shen, L., and Li, L. 2005. Isolation and characterization of neural stem cells from human fetal striatum. *Biochem. Biophys. Res. Commun.* 326:425-434.

206. Lie, D.C., Song, H., Colamarino, S.A., Ming, G.L., and Gage, F.H. 2004. Neurogenesis in the adult brain: New strategies for central nervous system diseases. *Annu. Rev. Pharmacol. Toxicol.* 44:399-421.

207. Lim, D.A., Huang, Y.C., and Alvarez-Buylla, A. 2007. The adult neural stem cell niche: Lessons for future neural cell replacement strategies. *Neurosurg. Clin. N. Am.* 18:81-92.

208. Lim, D.A., Huang, Y.C., and Alvarez-Buylla, A. 2008. Adult subventricular zone and olfactory bulb neurogenesis. *In* Adult Neurogenesis, 52nd Edition (F.H. Gage, G. Kempermann, and H. Song, eds.) pp. 159-174. Cold Spring Harbor Press, New York.

209. Lledo, P.M., Alonso, M., and Grubb, M.S. 2006. Adult neurogenesis and functional plasticity in neuronal circuits. *Nat. Rev. Neurosci.* 7:179-193.

210. Lois, C. and Alvarez-Buylla, A. 1993. Proliferating subventricular zone cells in the adult mammalian forebrain can differentiate into neurons and glia. *Proc. Natl. Acad. Sci. U.S.A.* 90:2074-2077.

211. Lois, C. and Alvarez-Buylla, A. 1994. Longdistance neuronal migration in the adult mammalian brain. *Science* 264:1145-1148.

212. Lois, C., Garcia-Verdugo, J.M., and Alvarez-Buylla, A. 1996. Chain migration of neuronal precursors. *Science* 271:978-981.

213. Lundberg, C., Martinez-Serrano, A., Cattaneo, E., McKay, R.D., and Bjorklund, A. 1997. Survival, integration, and differentiation of neural stem cell lines after transplantation to the adult rat striatum. *Exp. Neurol.* 145:342-360.

214. Luo, Y., Bhattacharya, B., Yang, A.X., Puri, R.K., and Rao, M.S. 2006a. Designing, testing, and validating a microarray for stem cell characterization.*Methods Mol. Biol.* 331:241-266.

215. Luo, Y., Schwartz, C., Shin, S., Zeng, X., Chen,N., Wang, Y., Yu, X., and Rao, M.S. 2006b. A focused microarray to assess dopaminergic and glial cell differentiation from fetal tissue or embryonic stem cells. *Stem Cells* 24:865-875.

216. Luskin,M.B. 1993. Restricted proliferation and migration of postnatally generated neurons derived from the forebrain subventricular zone. *Neuron* 11:173-189.

217. Luskin,M.B., Zigova, T., Soteres, B.J., and Stewart, R.R. 1997. Neuronal progenitor cells derived from the anterior subventricular zone of the neonatal rat forebrain continue to proliferate in vitro and express a neuronal phenotype. *Mol. Cell Neurosci.* 8:351-366.

218. Madhavan, L., Ourednik, V., and Ourednik, J. 2005. Grafted neural stem cells shield the host environment from oxidative stress. *Ann N.Y. Acad. Sci.* 1049:185-188.

219. Madhavan, L., Ourednik, V., and Ourednik, J. 2006. Increased "vigilance" of antioxidant mechanisms in neural stem cells potentiates their capability to resist oxidative stress. *Stem Cells* 24:2110-2119.

220. Madhavan, L., Ourednik, V., and Ourednik, J. 2008. Neural stem/progenitor cells initiate the formation of cellular networks that provide neuroprotection by growth factor-modulated antioxidant expression. *Stem Cells* 26:254-265.

221. Magnitsky, S.,Watson, D.J.,Walton, R.M., Pickup, S., Bulte, J.W., Wolfe, J.H., and Poptani, H. 2005. in vivo and ex vivo MRI detection of localized and disseminated neural stem cell grafts in the mouse brain. *Neuroimage* 26:744-754.

222. Maherali, N. and Hochedlinger, K. 2008a. Guidelines and techniques for the generation of induced pluripotent stem cells. *Cell Stem Cell* 3:595-605.

223. Maherali, N. and Hochedlinger, K. 2008b. Induced pluripotency of mouse and human somatic cells. *Cold Spring Harb. Symp. Quant. Biol.* 2008 Nov 6. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

224. Maherali, N., Sridharan, R., Xie, W., Utikal, J., Eminli, S., Arnold, K., Stadtfeld, M., Yachechko, R., Tchieu, J., Jaenisch, R., Plath, K., and Hochedlinger, K. 2007. Directly reprogrammed fibroblasts show global epigenetic remodeling and widespread tissue contribution. *Cell Stem Cell* 1:55-70.

225. Maherali, N., Ahfeldt, T., Rigamonti, A., Utikal, J., Cowan, C., and Hochedlinger, K. 2008. A highefficiency system for the generation and study of human induced pluripotent stem cells. *Cell Stem Cell* 3:340-345.

226. Malatesta, P., Hartfuss, E., and Gotz, M. 2000. Isolation of radial glial cells by fluorescent activated cell sorting reveals a neuronal lineage. *Development* 127:5253-5263.

227. Malatesta, P., Hack, M.A., Hartfuss, E., Kettenmann, H., Klinkert, W., Kirchhoff, F., and G¨otz, M. 2003. Neuronal or glial progeny: Regional differences in radial glia fate. *Neuron* 37:751-764.

228. Malatesta, P., Appolloni, I., and Calzolari, F. 2008. Radial glia and neural stem cells. *Cell Tissue Res.* 331:165-178.

229. Manganas, L.N., Zhang, X., Li, Y., Hazel, R.D., Smith, S.D., Wagshul, M.E., Henn, F., Benveniste, H., Djuric, P.M., Enikolopov, G., and Maletic-Savatic, M. 2007. Magnetic resonance spectroscopy identifies neural progenitor cells in the live human brain. *Science* 318:980-985.

230. Markakis, E.A., Palmer, T.D., Randolph-Moore, L., Rakic, P., and Gage, F.H. 2004. Novel neuronal phenotypes from neural progenitor cells. *J. Neurosci.* 24:2886-2897.

231. Marshall, G.P. II, Reynolds, B.A., and Laywell, E.D. 2007. Using the neurosphere assay to quantify neural stem cells in vivo. *Curr. Pharm. Biotechnol.* 8:141-145.

232. Martinez-Cerdeno,V.,Noctor, S.C., and Kriegstein, A.R. 2006. The role of intermediate progenitor cells in the evolutionary expansion of the cerebral cortex. *Cereb. Cortex* 1:i152-i161.

233. Mason, D.W., Charlton, H.M., Jones, A.J., Lavy, C.B., Puklavec, M., and Simmonds, S.J. 1986. The fate of allogeneic and xenogeneic neuronal tissue transplanted into the third ventricle of rodents.

234. ***Neuroscience*** **19:685-694. Matz, M.V., Lukyanov, K.A., and Lukyanov, S.A. 2002. Family of the green fluorescent protein: Journey to the end of the rainbow. *Bioessays* 24:953-959.**

235. Maurer,M.H. and Kuschinsky,W. 2006. Screening the brain: Molecular fingerprints of neural stem cells. *Curr. Stem Cell Res. Ther.* 1:65-77.

236. McBride, J.L., Behrstock, S.P., Chen, E.Y., Jakel, R.J., Siegel, I., Svendsen, C.N.,

and Kordower, J.H. 2004. Human neural stem cell transplants improve motor function in a rat model of Huntington's disease. *J. Comp. Neurol.* 475:211-219.

237. McCarthy, M., Wood, C., Fedoseyeva, L., and Whittemore, S.R. 1995. Media components influence viral gene expression assays in human fetal astrocyte cultures. *J. Neurovirol.* 1:275-285.

238. McKay, R. 1997. Stem cells in the central nervous system. *Science* 276:66-71.

239. McLaren, F.H., Svendsen, C.N., Van der Meide, P., and Joly, E. 2001. Analysis of neural stem cells by flow cytometry: Cellular differentiation modifies patterns of MHC expression. *J. Neuroimmunol.* 112:35-46.

240. Meissner, A., Wernig, M., and Jaenisch, R. 2007. Direct reprogramming of genetically unmodified fibroblasts into pluripotent stem cells. *Nat. Biotechnol.* 25:1177-1181.

241. Menezes, J.R. and Luskin, M.B. 1994. Expression of neuron-specific tubulin defines a novel population in the proliferative layers of the developing telencephalon. *J. Neurosci.* 14:5399-5416.

242. Menezes, J.R., Smith, C.M., Nelson, K.C., and Luskin, M.B. 1995. The division of neuronal progenitor cells during migration in the neonatal mammalian forebrain. *Mol. Cell Neurosci.* 6:496-508.

243. Menn, B., Garcia-Verdugo, J.M., Yaschine, C., Gonzalez-Perez, O., Rowitch, D., and Alvarez- Buylla, A. 2006. Origin of oligodendrocytes in the subventricular zone of the adult brain. *J. Neurosci.* 26:7907-7918.

244. Merkle, F.T. and Alvarez-Buylla, A. 2006. Neural stem cells in mammalian development. *Curr. Opin. Cell Biol.* 18:704-709.

245. Merkle, F.T., Tramontin, A.D., Garcia-Verdugo, J.M., and Alvarez-Buylla, A. 2004. Radial glia give rise to adult neural stem cells in the subventricular zone. *Proc. Natl. Acad. Sci. U.S.A.* 101:17528-17532.

246. Merkle, F.T., Mirzadeh, Z., and Alvarez-Buylla, A. 2007. Mosaic organization of neural stem cells in the adult brain. *Science* 317:381-384.

247. Merzlyak, E.M., Goedhart, J., Shcherbo, D., Bulina, M.E., Shcheglov, A.S., Fradkov, A.F., Gaintzeva, A., Lukyanov, K.A., Lukyanov, S., Gadella, T.W., and Chudakov, D.M. 2007. Bright monomeric red fluorescent protein with an extended fluorescence lifetime. *Nat. Methods* 4:555-557.

248. Messier, B. and Leblond, C.P. 1960. Cell proliferation and migration as revealed by radioautography after injection of thymidine-H3 into male rats and mice. *Am. J. Anat.* 106:247-285.

249. Messier, B., Leblond, C.P., and Smart, I. 1958. Presence of DNA synthesis and

mitosis in the brain of young adult mice. *Exp. Cell Res.* 14:224-226.

250. Miyata, T., Kawaguchi, A., Okano, H., and Ogawa, M. 2001. Asymmetric inheritance of radial glial fibers by cortical neurons. *Neuron* 31:727-741.

251. Miyoshi, S., Flexman, J.A., Cross, D.J., Maravilla, K.R., Kim, Y., Anzai, Y., Oshima, J., and Minoshima, S. 2005. Transfection of neuroprogenitor cells with iron nanoparticles for magnetic resonance imaging tracking: Cell viability, differentiation, and intracellular localization. *Mol. Imaging Biol.* 7:286-295.

252. Molne, M., Studer, L., Tabar, V., Ting, Y.T., Eiden, M.V., and McKay,R.D. 2000. Early cortical precursors do not undergo LIF-mediated astrocytic differentiation. *J. Neurosci. Res.* 59:301-311.

253. Mori, H., Ninomiya, K., Kino-oka,M., Shofuda, T., Islam, M.O., Yamasaki, M., Okano, H., Taya, M., and Kanemura, Y. 2006. Effect of neurosphere size on the growth rate of human neural stem/progenitor cells. *J. Neurosci. Res.* 84:1682-1691.

254. Morrison, S.J., Shah, N.M., and Anderson, D.J. 1997. Regulatory mechanisms in stem cell biology. *Cell* 88:287-298.

255. Morshead, C.M., Reynolds, B.A., Craig, C.G., McBurney,M.W., Staines,W.A., Morassutti,D., Weiss, S., and van der Kooy, D. 1994. Neural stem cells in the adult mammalian forebrain: A relatively quiescent subpopulation of subependymal cells. *Neuron* 13:1071-1082.

256. Moyer, M.P., Johnson, R.A., Zompa, E.A., Cain, L., Morshed, T., and Hulsebosch, C.E. 1997. Culture, expansion, and transplantation of human fetal neural progenitor cells. *Transplant. Proc.* 29:2040-2041.

257. Mueller, F.-J., Serobyan, N., Schraufstatter, I.U., DiScipio,R.,Wakeman, D., Loring, J.F., Snyder, E.Y., and Khaldoyanidi, S.K. 2006. Adhesive interactions between human neural stem cells and inflamed human vascular endothelium are mediated by integrins. *Stem Cells* 24:2367-2372.

258. Murase, S. and Horwitz, A.F. 2002. Deleted in colorectal carcinoma and differentially expressed integrins mediate the directional migration of neural precursors in the rostralmigratory stream. *J. Neurosci.* 22:3568-3579.

259. Murase, S. and Horwitz, A.F. 2004. Directions in cell migration along the rostral migratory stream: The pathway for migration in the brain. *Curr. Top. Dev. Biol.* 61:135-152.

260. Murphy, M., Drago, J., and Bartlett, P.F. 1990. Fibroblast growth factor stimulates the proliferation and differentiation of neural precursor cells in vitro. *J. Neurosci. Res.* 25:463-475.

261. Murray, K. andDubois-Dalcq,M. 1997. Emergence of oligodendrocytes fromn

human neural spheres. *J. Neurosci. Res.* 50:146-156.

262. Nait-Oumesmar, B., Decker, L., Lachapelle, F., Avellana-Adalid, V., Bachelin, C., and Van Evercooren, A.B. 1999. Progenitor cells of the adult mouse subventricular zone proliferate, migrate and differentiate into oligodendrocytes after demyelination. *Eur. J. Neurosci.* 11:4357-4366.

263. Nakagawa, M., Koyanagi, M., Tanabe, K., Takahashi, K., Ichisaka, T., Aoi, T., Okita, K., Mochiduki, Y., Takizawa, N., and Yamanaka, S. 2008. Generation of induced pluripotent stem cells without Myc from mouse and human fibroblasts. *Nat. Biotechnol.* 26:101-106.

264. Namihira, M., Kohyama, J., Semi, K., Sanosaka, T., Deneen, B., Taga, T., and Nakashima, K. 2009. Committed neuronal precursors confer astrocytic potential on residual neural precursor cells. *Dev. Cell* 16:245-255.

265. Navarro-Galve, B. and Martinez-Serrano, A. 2006. "Is there any need to argue..." about the nature and genetic signature of in vitro neural stem cells? *Exp. Neurol.* 199:20-25.

266. Neri, M., Maderna, C., Cavazzin, C., Deidda- Vigoriti, V., Politi, L.S., Scotti, G., Marzola, P., Sbarbati, A., Vescovi, A.L., and Gritti, A. 2008.

267. Efficient in vitro labeling of human neural precursor cells with superparamagnetic iron oxide particles: Relevance for in vivo cell tracking. *Stem Cells* 26:505-516.

268. Nethercott, H., Maxwell, H., and Schwartz, P.H. 2007. Neural progenitor cell culture. *In* Human Stem Cell Manual: A Laboratory Guide. First Edition (J.F. Loring, R.L. Wesselschmidt, and P.H. Schwartz, eds.) pp. 309-331. Elsevier Academic Press, New York.

269. Noctor, S.C., Flint, A.C., Weissman, T.A., Dammerman, R.S., and Kriegstein, A.R. 2001. Neurons derived from radial glial cells establish radial units in neocortex. *Nature* 409:714-720.

270. Noctor, S.C., Flint, A.C., Weissman, T.A., Wong, W.S., Clinton, B.K., and Kriegstein, A.R. 2002. Dividing precursor cells of the embryonic cortical ventricular zone have morphological and molecular characteristics of radial glia. *J. Neurosci.* 22:3161-3173.

271. Noctor, S.C., Martinez-Cerdeno, V., Ivic, L., and Kriegstein, A.R. 2004. Cortical neurons arise in symmetric and asymmetric division zones and migrate through specific phases. *Nat. Neurosci.* 7:136-144.

272. Noctor, S.C., Martinez-Cerdeno,V., and Kriegstein, A.R. 2008. Distinct behaviors of neural stem and progenitor cells underlie cortical neurogenesis. *J. Comp. Neurol.* 508:28-44.

273. Nunes, M.C., Roy, N.S., Keyoung, H.M., Goodman, R.R., McKhann, G. 2nd, Jiang, L., Kang, J., Nedergaard, M., and Goldman, S.A. 2003. Identification and isolation of multipotential neural progenitor cells from the subcortical white matter of the adult human brain. *Nat. Med.* 9:439-447.

273. Odeberg, J., Piao, J.H., Samuelsson, E.B., Falci, S., and Akesson, E. 2005. Low immunogenicity of in vitro-expanded human neural cells despite high MHC expression. *J. Neuroimmunol.* 161:1-11.

274. Okita, K., Ichisaka, T., and Yamanaka, S. 2007. Generation of germline-competent induced pluripotent stem cells. *Nature* 448:313-317.

275. Ostenfeld, T. and Svendsen, C.N. 2004. Requirement for neurogenesis to proceed through the division of neuronal progenitors following differentiation of epidermal growth factor and fibroblast growth factor-2-responsive human neural stem cells. *Stem Cells* 22:798-811.

276. Ostenfeld, T., Caldwell, M.A., Prowse, K.R., Linskens, M.H., Jauniaux, E., and Svendsen, C.N. 2000. Human neural precursor cells express low levels of telomerase in vitro and show diminishing cell proliferation with extensive axonal outgrowth following transplantation. *Exp. Neurol.* 164:215-226.

277. Ostenfeld, T., Tai, Y.T., Martin, P., Deglon, N., Aebischer, P., and Svendsen, C.N. 2002a. Neurospheres modified to produce glial cell linederived neurotrophic factor increase the survival of transplanted dopamine neurons. *J. Neurosci. Res.* 69:955-965.

278. Ostenfeld, T., Joly, E., Tai, Y.T., Peters, A., Caldwell, M., Jauniaux, E., and Svendsen, C.N. 2002b. Regional specification of rodent and human neurospheres. *Brain Res. Dev. Brain Res.* 134:43-55.

279. Ourednik, V., Ourednik, J., Flax, J.D., Zawada, W.M., Hutt, C., Yang, C., Park, K.I., Kim, S.U., Sidman, R.L., Freed, C.R., and Snyder, E.Y. 2001. Segregation of human neural stem cells in the developing primate forebrain. *Science* 293:1820-1824.

280. Ourednik, J., Ourednik, V., Lynch,W.P., Schachner, M., and Snyder, E.Y. 2002. Neural stem cells display an inherent mechanism for rescuing dysfunctional neurons. *Nat. Biotechnol.* 20:1103-1110.

281. Palmer, T.D. 2002. Adult neurogenesis and the vascular Nietzsche. *Neuron* 34:856-858.

282. Palmer, T.D., Ray, J., and Gage, F.H. 1995. FGF-2- responsive neuronal progenitors reside in proliferative and quiescent regions of the adult rodent brain. *Mol. Cell. Neurosci.* 6:474-476.

283. Palmer, T.D., Markakis, E.A., Willhoite, A.R., Safar, F., and Gage, F.H. 1999. Fibroblast growth factor-2 activates a latent neurogenic program in neural stem

cells from diverse regions of the adult CNS. *J. Neurosci.* 19:8487-8497.

284. Palmer, T.D., Schwartz, P.H., Taupin, P., Kaspar, B., Stein, S.A., and Gage, F.H. 2001. Cell culture: Progenitor cells from human brain after death. *Nature* 411:42-43.

285. Parent, J.M., von dem Bussche,N., and Lowenstein, D.H. 2006. Prolonged seizures recruit caudal subventricular zone glial progenitors into the injured hippocampus. *Hippocampus* 16:321-328.

286. Park, I.H., Zhao, R.,West, J.A., Yabuuchi, A., Huo, H., Ince, T.A., Lerou, P.H., Lensch, M.W., and Daley,G.Q. 2008. Reprogramming of human somatic cells to pluripotency with defined factors. *Nature* 451:141-146.

287. Park, K.I., Ourednik, J., Ourednik, V., Taylor, R.M., Aboody, K.S.,Auguste,K.I., Lachyankar, M.B., Redmond, D.E., and Snyder, E.Y. 2002a. Global gene and cell replacement strategies via stem cells. *Gene Ther.* 9:613-624.

288. Park, K.I., Teng, Y.D., and Snyder, E.Y. 2002b. The injured brain interacts reciprocally with neural stem cells supported by scaffolds to reconstitute lost tissue. *Nat. Biotechnol.* 20:1111-1117.

289. Park, K.I., Palacino, J.J., Taylor, R., Aboody, K.A., Tate, B.A., Ourednik, V., Ourednik, J., Lachyankar, M., and Snyder, E.Y. 2003. Global gene and cell replacement strategies via stem cells. *In* Neural Stem Cells for Brain and Spinal Cord Repair (T. Zigiva, E.Y. Snyder, and P.R. Sanberg, eds), pp. 289-332. Humana Press Inc., Totowa, N.J.

290. Park, K.I., Hack, M.A., Ourednik, J., Yandava, B., Flax, J.D., Stieg, P.E., Gullans, S., Jensen, F.E., Sidman, R.L., Ourednik, V., and Snyder, E.Y. 2006. Acute injury directs the migration, proliferation, and differentiation of solid organ stem cells: Evidence from the effect of hypoxiaischemia in the CNS on clonal "reporter" neural stem cells. *Exp. Neurol.* 199:156-178.

291. Parker, M.A., Anderson, J.K., Corliss, D.A., Abraria, V.E., Sidman, R.L., Park, K.I., Teng, Y.D., Cotanche, D.A., and Snyder, E.Y. 2005. Expression profile of an operationally-defined neural stem cell clone. *Exp. Neurol.* 194:320-332.

292. Parmar, M., Skogh, C., Bjorklund, A., and Campbell, K. 2002. Regional specification of neurosphere cultures derived from subregions of the embryonic telencephalon. *Mol. Cell. Neurosci.* 21:645-656.

293. Parmar, M., Skogh, C., and Englund, U. 2003. A transplantation study of expanded human embryonic forebrain precursors: Evidence for selection of a specific progenitor population. *Mol. Cell. Neurosci.* 23:531-543.

294. Petreanu, L. and Alvarez-Buylla, A. 2002. Maturation and death of adult-born olfactory bulb granule neurons: Role of olfaction. *J. Neurosci.* 22:6106-6113.

295. Picard-Riera, N., Decker, L., Delarasse, C., Goude, K., Nait-Oumesmar, B., Liblau, R., Pham-Dinh, D., and Evercooren, A.B. 2002. Experimental autoimmune encephalomyelitis mobilizes neural progenitors from the subventricular zone to undergo oligodendrogenesis in adultmice. *Proc. Natl. Acad. Sci. U.S.A.* 99:13211-13216.

296. Pincus, D.W., Keyoung, H.M., Harrison-Restelli, C., Goodman, R.R., Fraser, R.A., Edgar, M., Sakakibara, S., Okano, H., Nedergaard, M., and Goldman, S.A. 1998. Fibroblast growth factor-2/brain-derived neurotrophic factor-associated maturation of new neurons generated from adult human subependymal cells. *Ann. Neurol.* 43:576-585.

297. Piper, D.R., Mujtaba, T., Rao, M.S., and Lucero, M.T. 2000. Immunocytochemical and physiological characterization of a population of cultured human neural precursors. *J. Neurophysiol.* 84:534-548.

298. Piper, D.R., Mujtaba, T., Keyoung, H., Roy, N.S., Goldman, S.A., Rao, M.S., and Lucero, M.T. 2001. Identification and characterization of neuronal precursors and their progeny from human fetal tissue. *J. Neurosci. Res.* 66:356-368.

299. Pistollato, F., Chen, H.L., Schwartz, P.H., Basso, G., and Panchision, D.M. 2007. Oxygen tension controls the expansion of human CNS precursors and the generation of astrocytes and oligodendrocytes. *Mol. Cell. Neurosci.* 35:424-435.

300. Pixley, S.K. and de Vellis, J. 1984. Transition between immature radial glia and mature astrocytes studied with a monoclonal antibody to vimentin. *Brain Res.* 317:201-209.

301. Pixley, S.K., Kobayashi, Y., and de Vellis, J. 1984a. A monoclonal antibody against vimentin: Characterization. *Brain Res.* 317:185-199.

302. Pixley, S.K., Kobayashi, Y., and de Vellis, J. 1984b. Monoclonal antibody to intermediate filament proteins in astrocytes. *J. Neurosci. Res.* 12:525-541.

303. Pluchino, S., Quattrini, A., Brambilla, E., Gritti, A., Salani, G., Dina, G., Galli, R., Del Carro, U., Amadio, S., Bergami, A., Furlan, R., Comi, G., Vescovi, A.L., and Martino, G. 2003. Injection of adult neurospheres induces recovery in a chronic model of multiple sclerosis. *Nature* 422:688-694.

304. Pluchino, S., Zanotti, L., Rossi, B., Brambilla, E., Ottoboni, L., Salani, G., Martinello, M., Cattalini, A., Bergami, A., Furlan, R., Comi, G., Constantin, G., and Martino, G. 2005a. Neurosphere-derived multipotent precursors promote neuroprotection by an immunomodulatory mechanism. *Nature* 436:266-271.

305. Pluchino, S., Zanotti, L., Deleidi, M., and Martino, G. 2005b. Neural stem cells and their use as therapeutic tool in neurological disorders. *Brain Res. Rev.* 48:211-219.

306. Politi, L.S.,Bacigaluppi,M., Brambilla, E., Cadioli, M., Falini, A., Comi, G.,

Scotti, G., Martino, G., and Pluchino, S. 2007. Magnetic-resonancebased tracking and quantification of intravenously injected neural stem cell accumulation in the brains of mice with experimental multiple sclerosis. *Stem Cells* 25:2583-2592.

307. Pollard, S.M., Benchoua, A., and Lowell, S. 2006a. Neural stem cells, neurons, and glia. *Methods Enzymol.* 418:151-169.

308. Pollard, S.M., Conti, L., Sun, Y., Goffredo, D., and Smith, A. 2006b. Adherent neural stem (NS) cells from fetal and adult forebrain. *Cereb Cortex* 16:i112-i120.

309. Pollard, S.M., Wallbank, R., Tomlinson, S., Grotewold, L., and Smith, A. 2008. Fibroblast growth factor induces a neural stem cell phenotype in foetal forebrain progenitors and during embryonic stem cell differentiation. *Mol. Cell. Neurosci.* 38:393-403.

310. Qian, X., Davis, A.A., Goderie, S.K., and Temple, S. 1997. FGF2 concentration regulates the generation of neurons and glia from multipotent cortical stem cells. *Neuron* 18:81-93.

311. Qian, X., Goderie, S.K., Shen, Q., Stern, J.H., and Temple, S. 1998. Intrinsic programs of patterned cell lineages in isolated vertebrate CNS ventricular zone cells. *Development* 125:3143-3152.

312. Qian, X., Shen, Q., Goderie, S.K., He, W., Capela, A., Davis, A.A., and Temple, S. 2000. Timing of CNS cell generation: A programmed sequence of neuron and glial cell production from isolated murine cortical stem cells. *Neuron* 28:69-80.

313. Quinn, S.M., Walters, W.M., Vescovi, A.L., and Whittemore, S.R. 1999. Lineage restriction of neuroepithelial precursor cells from fetal human spinal cord. *J. Neurosci. Res.* 57:590-602.

314. Quinones-Hinojosa, A., Sanai, N., Soriano-Navarro, M., Gonzalez-Perez, O., Mirzadeh, Z., Gil-Perotin, S., Romero-Rodriguez, R., Berger, M.S., Garcia-Verdugo, J.M., and Alvarez- Buylla, A. 2006. Cellular composition and cytoarchitecture of the adult human subventricular zone: A niche of neural stem cells. *J. Comp. Neurol.* 494:415-434.

315. Quinones-Hinojosa, A., Sanai, N., Gonzalez-Perez, O., and Garcia-Verdugo, J.M. 2007. The human brain subventricular zone: Stem cells in this niche and its organization. *Neurosurg. Clin. N. Am.* 18:15-20.

316. Rajan, P. and Snyder, E. 2006. Neural stem cells and their manipulation. *Methods Enzymol.* 419:23-52.

317. Rakic, P. 1972. Mode of cell migration to the superficial layers of fetal monkey neocortex. *J. Comp. Neurol.* 145:61-83.

318. Rakic, P. 2002a. Neurogenesis in adult primate neocortex: An evaluation of the evidence. *Nat. Rev. Neurosci.* 3:65-71.

319. Rakic, P. 2002b. Neurogenesis in adult primates. *Prog. Brain Res.* 138:3-14.

320. Rakic, P. 2007. The radial edifice of cortical architecture: From neuronal silhouettes to genetic engineering. *Brain Res. Rev.* 55:204-219.

321. Ram´on y Cajal, S. 1928. Degeneration and Regeneration of the Central Nervous System. Hafner, New York.

322. Ram´on y Cajal, S. and May, R.T. 1959. Degeneration and Regeneration of the Nervous System. Volume 2. Hafner, New York.

323. Rao, M.S. 1999. Multipotent and restricted precursors in the central nervous system. *Anat. Rec.* 257:137-148.

324. Rao, R.C., Boyd, J., Padmanabhan, R., Chenoweth, J.G., and McKay, R.D. 2008. Efficient serum-free derivation of oligodendrocyte precursors from neural stem cell-enriched cultures. *Stem Cells* In press.

325. Ravin, R., Hoeppner, D.J., Munno, D.M., Carmel, L., Sullivan, J., Levitt, D.L., Miller, J.L., Athaide, C., Panchision, D.M., and McKay, R.D. 2008. Potency and fate specification in CNS stem cell populations in vitro. *Cell Stem Cell* 3:670-680.

326. Ray, J. 2008. Monolayer cultures of neural stem/progenitor cells. *In* Adult Neurogenesis (F.H. Gage, G.Kempermann, andH. Song, eds.), pp. 135-157. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

327. Ray, J. and Gage, F.H. 2006. Differential properties of adult rat and mouse brain-derived neural stem/progenitor cells. *Mol. Cell. Neurosci.* 31:560-573.

328. Ray, J. and Gage, F.H. 1994. Spinal cord neuroblasts proliferate in response to basic fibroblast growth factor. *J. Neurosci.* 14:3548-3564.

329. Ray, J., Peterson, D.A., Schinstine, M., and Gage, F.H. 1993. Proliferation, differentiation, and long-term culture of primary hippocampal neurons. *Proc. Natl. Acad. Sci. U.S.A.* 90:3602-3606.

330. Ray, J., Raymon, H.K., and Gage, F.H. 1995. Generation and culturing of precursor cells and neuroblasts from embryonic and adult central nervous system. *Methods Enzymol.* 254:20-37.

331. Redies, C., Lendahl, U., and McKay, R.D. 1991. Differentiation and heterogeneity in T-antigen immortalized precursor cell lines from mouse cerebellum. *J. Neurosci. Res.* 30:601-615.

332. Redmond, D.E. Jr., Sladek, J.R. Jr., Roth, R.H., Collier, T.J., Elsworth, J.D., Deutch, A.Y., and Haber, S. 1986. Fetal neuronal grafts in monkeys given methylphenyltetrahydropyridine. *Lancet* 1:1125-1127.

333. Redmond, D.E. Jr., Naftolin, F., Collier, T.J., Leranth, C., Robbins, R.J., Sladek, C.D., Roth, R.H., and Sladek, J.R. Jr. 1988. Cryopreservation, culture, and transplantation of human fetal mesencephalic tissue into monkeys. *Science* 242:768-771.

334. Redmond, D.E. Jr., Bjugstad, K.B., Teng, Y.D., Ourednik, V., Ourednik, J., Wakeman, D.R., Parsons, X.H., Gonzalez, R., Blanchard, B.C., Kim, S.U., Gu, Z., Lipton, S.A., Markakis, E.A., Roth, R.H., Elsworth, J.D., Sladek, J.R. Jr., Sidman, R.L., and Snyder, E.Y. 2007. Behavioral improvement in a primate Parkinson's model is associated with multiple homeostatic effects of human neural stem cells. *Proc. Natl. Acad. Sci. U.S.A.* 104:12175-12180.

335. Redmond, D.E. Jr., Vinuela,A.,Kordower, J.H., and Isacson, O. 2008. Influence of cell preparation and target location on the behavioral recovery after striatal transplantation of fetal dopaminergic neurons in a primate model of Parkinson's disease. *Neurobiol. Dis.* 29:103-116.

336. Ren, P., Guan, Y.Q., Zhang, Y. 2007. Comparisons among different methods of culturing neural stem cells isolated from human fetal cortex. *Fen Zi Xi Bao Sheng Wu Xue Bao* 40:79-83.

337. Renfranz, P.J., Cunningham, M.G., and McKay, R.D. 1991. Region-specific differentiation of the hippocampal stem cell lineHiB5 upon implantation into the developing mammalian brain. *Cell* 66:713-729.

338. Reynolds, B.A. and Rietze, R.L. 2005. Neural stem cells and neurospheres–re-evaluating the relationship. *Nat. Methods.* 2:333-336.

339. Reynolds, B.A. and Weiss, S. 1992. Generation of neurons and astrocytes from isolated cells of the adult mammalian central nervous system. *Science* 255:1707-1710.

340. Reynolds, B.A., Tetzlaff,W., andWeiss, S. 1992. A multipotent EGF-responsive striatal embryonic progenitor cell produces neurons and astrocytes. *J. Neurosci.* 12:4565-4574.

341. Riaz, S.S., Theofilopoulos, S., Jauniaux, E., Stern, G.M., and Bradford, H.F. 2004. The differentiation potential of human foetal neuronal progenitor cells in vitro. *Brain Res. Dev. Brain Res.* 153:39-51.

342. Richard, C., Liuzzo, J.P., and Moscatelli, D. 1995. Fibroblast growth factor-2 can mediate cell attachment by linking receptors and heparan sulfate proteoglycans on neighboring cells. *J. Biol. Chem.* 270:24188-24196.

343. Richard, C., Roghani, M., and Moscatelli, D. 2000. Fibroblast growth factor (FGF)-2 mediates cell attachment through interactions with two FGF receptor-1 isoforms and extracellular matrix or cell-associated heparan sulfate proteoglycans. *Biochem. Biophys. Res. Commun.* 276:399-405.

344. Rietze, R.L. and Reynolds, B.A. 2006. Neural stem cell isolation and characterization. *Methods Enzymol.* 419:3-23.

345. Robinson, J.P., Darzynkiewicz, Z., Hoffman, R., Nolan, J.P., Orfao, A., Rabinovitch, P.S., and Watkins, S. (eds.) 2009. Current Protocols in Cytometry. JohnWiley & Sons, Hoboken, N.J. Rogers,W.J., Meyer, C.H., and Kramer, C.M. 2006. Technology insight: In vivo cell tracking by use of MRI. *Nat. Clin. Pract. Cardiovasc. Med.* 3:554-562.

346. Rosser, A.E., Tyers, P., ter Borg, M., Dunnett, S.B., and Svendsen, C.N. 1997. Co-expression of MAP-2 and GFAP in cells developing from rat EGF responsive precursor cells. *Brain Res. Dev. Brain Res.* 98:291-295.

347. Rousselot, P., Lois, C., and Alvarez-Buylla, A. 1995. Embryonic (PSA) N-CAM reveals chains of migrating neuroblasts between the lateral ventricle and the olfactory bulb of adult mice. *J. Comp. Neurol.* 351:51-61.

348. Roy, N.S., Benraiss, A., Wang, S., Fraser, R.A., Goodman, R.,Couldwell,W.T., Nedergaard, M., Kawaguchi, A., Okano, H., and Goldman, S.A. 2000. Promoter-targeted selection and isolation of neural progenitor cells from the adult human ventricular zone. *J. Neurosci. Res.* 59:321-331.

349. Roy, N.S., Chandler-Militello,D., Lu,G.,Wang, S., and Goldman, S.A. 2007. Retrovirally mediated telomerase immortalization of neural progenitor cells. *Nat. Protoc.* 2:2815-2825.

350. Rubio, F.J., Bueno, C., Villa, A., Navarro, B., and Mart´ınez-Serrano, A. 2000. Genetically perpetuated human neural stem cells engraft and differentiate into the adult mammalian brain. *Mol. Cell Neurosci.* 16:1-13.

351. Ruiz-Cabello, J., Walczak, P., Kedziorek, D.A., Chacko, V.P., Schmieder, A.H., Wickline, S.A., Lanza, G.M., and Bulte, J.W. 2008. In vivo "hot spot" MR imaging of neural stem cells using fluorinated nanoparticles. *Magn. Reson. Med.* 60:1506-1511.

352. Rutishauser, U. 2008. Polysialic acid in the plasticity of the developing and adult vertebrate nervous system. *Nat. Rev. Neurosci.* 9:26-35.

353. Ryder, E.F., Snyder, E.Y., and Cepko, C.L. 1990. Establishment and characterization of multipotent neural cell lines using retrovirus vector-mediated oncogene transfer. *J Neurobiol.* 21:356-375.

354. Sah, D.W., Ray, J., and Gage, F.H. 1997. Bipotent progenitor cell lines from the human CNS. *Nat. Biotechnol.* 15:574-580.

355. Sanai, N., Tramontin, A.D., Quinones-Hinojosa, A., Barbaro, N.M., Gupta, N., Kunwar, S., Lawton, M.T., McDermott, M.W., Parsa, A.T., Manuel-Garc´ıa Verdugo, J., Berger, M.S., and Alvarez-Buylla, A. 2004. Unique astrocyte ribbon in adult human brain contains neural stem cells but lacks chain migration.

Nature **427:740-744.**

356. Sanai, N., Berger, M.S., Garcia-Verdugo, J.M., and Alvarez-Buylla, A. 2007. Comment on "Human neuroblasts migrate to the olfactory bulb via a lateral ventricular extension". *Science* 318:393.

357. Scheffler, B.,Walton, N.M., Lin,D.D., Goetz, A.K., Enikolopov, G.,Roper, S.N., and Steindler,D.A. 2005. Phenotypic and functional characterization of adult brain neuropoiesis. *Proc. Natl. Acad. Sci. U.S.A.* 102:9353-9358.

358. Scholzen, T. and Gerdes, J. 2000. The Ki-67 protein: From the known and the unknown. *J. Cell. Physiol.* 182:311-322.

359. Schroeder, T. 2008. Imaging stem-cell-driven regeneration in mammals. *Nature* 453:345-351.

360. Schwartz, P.H. 2006. The potential of stem cell therapies for neurological diseases. *Expert Rev. Neurotherapeutics* 6:153-161.

361. Schwartz, P.H., Bryant, P.J., Fuja, T.J., Su, H., O'Dowd, D.K., and Klassen, H. 2003. Isolation and characterization of neural progenitor cells from post-mortem human cortex. *J. Neurosci. Res.* 74:838-851.

362. Seaberg, R.M. and van der Kooy, D. 2003. Stem and progenitor cells: The premature desertion of rigorous definitions. *Trends Neurosci.* 26:125-131.

363. Seidenfaden, R., Desoeuvre, A., Bosio, A., Virard, I., and Cremer, H. 2006. Glial conversion of SVZ-derived committed neuronal precursors after ectopic grafting into the adult brain. *Mol. Cell. Neurosci.* 32:187-198.

364. Shaner,N.C., Steinbach, P.A., and Tsien,R.Y. 2005. A guide to choosing fluorescent proteins. *Nat. Methods* 2:905-909.

365. Shaner, N.C., Lin, M.Z., McKeown, M.R., Steinbach, P.A., Hazelwood, K.L., Davidson, M.W., and Tsien, R.Y. 2008. Improving the photostability of bright monomeric orange and red fluorescent proteins. *Nat. Methods* 5:545-551.

366. Shapiro, E.M.,Gonzalez-Perez, O.,Manuel Garcia-Verdugo, J., Alvarez-Buylla, A., and Koretsky, A.P. 2006. Magnetic resonance imaging of the migration of neuronal precursors generated in the adult rodent brain. *Neuroimage* 32:1150-1157.

367. Shi,Y.,Do, J.T., Desponts, C., Hahm, H.S., Scholer, H.R., Ding, S. 2008. A combined chemical and genetic approach for the generation of induced pluripotent stem cells. *Cell Stem Cell* 2:525-528.

368. Shihabuddin, L.S., Brunschwig, J.P., Holets, V.R., Bunge, M.B., and Whittemore, S.R. 1996. Induction of mature neuronal properties in immortalized neuronal precursor cells following grafting into the neonatal CNS. *J. Neurocytol.*

25:101-111.

369. Shihabuddin, L.S., Ray, J., and Gage, F.H. 1997. FGF-2 is sufficient to isolate progenitors found in the adultmammalian spinal cord. *Exp. Neurol.* 148:577-586.

370. Shimazaki, T., Shingo, T., and Weiss, S. 2001. The ciliary neurotrophic factor/leukemia inhibitory factor/gp130 receptor complex operates in the maintenance of mammalian forebrain neural stem cells. *J. Neurosci.* 21:7642 7653.

371. Shimomura, O. 2005. The discovery of aequorin and green fluorescent protein. *J. Microsc.* 217:1-15.

372. Shimomura, O., Johnson, F.H., and Saiga, Y. 1962. Extraction, purification and properties of aequorin, a bioluminescent protein from the luminous hydromedusan, Aequorea. *J. Cell Comp. Physiol.* 59:223-239.

373. Shin, S. and Rao, M.S. 2006. Large-scale analysis of neural stem cells and progenitor cells. *Neurodegener. Dis.* 3:106-111.

374. Shin, S., Sun, Y., Liu, Y., Khaner, H., Svant, S., Cai, J., Xu, Q.X., Davidson, B.P., Stice, S.L., Smith, A.K., Goldman, S.A., Reubinoff, B.E., Zhan, M., Rao, M.S., and Chesnut, J.D. 2007. Whole genome analysis of human neural stem cells derived from embryonic stem cells and stem and progenitor cells isolated from fetal tissue. *Stem Cells* 25:1298-1306.

375. Sidman, R.L. and Rakic, P. 1973. Neuronal migration, with special reference to developing human brain: A review. *Brain Res.* 62:1-35.

376. Singec, I., Knoth, R., Meyer, R.P., Maciaczyk, J., Volk, B., Nikkhah, G., Frotscher, M., and Snyder, E.Y. 2006. Defining the actual sensitivity and specificity of the neurosphere assay in stem cell biology. *Nat. Methods.* 3:801-806.

377. Singec, I. and Quinones-Hinojosa, A. 2008. Neurospheres. *In* Adult Neurogenesis (F.H. Gage, G. Kempermann, and H. Song, eds) pp. 119-134. Cold Spring Harbor Press, New York.

378. Slack, J.M. 2008. Origin of stem cells in organogenesis. *Science* 322:1498-1501.

379. Sladek, J.R. Jr., Elsworth, J., Taylor, J., Roth, R., and Redmond, D. Jr. 1995. Techniques for neural transplantation in non-human primates. *In* Methods in Cell Transplantation (C. Ricordi, ed.) pp. 391-408. R.G. Landes, Austin, Tx.

380. Sladek, J.R. Jr., Bjugstad, K.B., Collier, T.J., Bundock, E.A., Blanchard, B.C., Elsworth, J.D., Roth, R.H., and Redmond, D.E. Jr. 2008. Embryonic substantia nigra grafts show directional outgrowth to cografted striatal grafts and potential for pathway reconstruction in nonhuman primate. *Cell Transplant.* 17:427-444.

381. Slotkin, J.R., Cahill, K.S., Tharin, S.A., and Shapiro, E.M. 2007. Cellular

magnetic resonance imaging: Nanometer and micrometer size particles for noninvasive cell localization. ***Neurotherapeutics*** **4:428-433.**

382. Smart, I. 1961. The subependymal layer of the mouse brain and its cell production as shown by radiography after thymidine-H3 injection. ***Comp. Neurol.*** **116:325-347.**

383. Smart, I. and Leblond, C.P. 1961. Evidence for division and transformation of neuroglia cells in mouse brain as derived from radioautography after injection of thymidine-H3. ***J. Comp. Neurol.*** **116:349-367.**

384. Snyder, E.Y., Deitcher, D.L., Walsh, C., Arnold-Aldea, S., Hartwieg, E.A., and Cepko, C.L.1992. Multipotent neural cell lines can engraft and participate in development of mouse cerebellum. ***Cell*** **68:33-51.**

385. Spitzer, N.C. 2006. Electrical activity in early neuronal development. ***Nature*** **444:707-712.**

386. Stadtfeld, M., Maherali, N., Breault, D.T., and Hochedlinger, K. 2008. Defining molecular cornerstones during fibroblast to iPS cell reprogramming in mouse. ***Cell Stem Cell*** **2:230-240.**

387. Steindler,D.A., Scheffler,B., Laywell, E.D.,Zheng, T., Reiniger, T., and Kukekov, V.G. 2003. Neural stem/progenitor cell clones or "neurospheres": A model for understanding neuromorphogenesis. ***In*** **Neural Stem Cells for Brain and Spinal Cord Repair (T. Zigova, E.Y. Snyder, and P.R. Sanberg, eds) pp. 183-202. Humana Press, Totowa, N.J.**

388. Sumner, J.P., Conroy, R., Shapiro, E.M.,Moreland, J., and Koretsky, A.P. 2007. Delivery of fluorescent probes using iron oxide particles as carriers enables in-vivo labeling of migrating neural precursors for magnetic resonance imaging and optical imaging. ***J. Biomed. Opt.*** **12:051504.**

389. Suslov, O.N., Kukekov, V.G., Ignatova, T.N., and Steindler, D.A. 2002. Neural stem cell heterogeneity demonstrated by molecular phenotyping of clonal neurospheres. ***Proc. Natl. Acad. Sci. U.S.A.*** **99:14506-14511.**

390. Suzuki, M., McHugh, J., Tork, C., Shelley, B., Klein, S.M., Aebischer, P., and Svendsen, C.N. 2007. GDNF-secreting human neural progenitor cells protect dying motor neurons, but not their projection to muscle, in a rat model of familial ALS. ***PLoS ONE*** **2:e689.**

391. Svendsen,C.N. and Langston, J.W. 2004. Stem cells for Parkinson disease and ALS: Replacement or protection? ***Nat. Med.*** **10:224-225.**

392. Svendsen, C.N. and Smith, A.G. 1999. New prospects for human stem-cell therapy in the nervous system. ***Trends Neurosci.*** **22:357-364.**

392. Svendsen, C.N., Fawcett, J.W., Bentlage, C., and Dunnett, S.B. 1995. Increased

survival of rat EGF-generated CNS precursor cells using B27 supplemented medium. *Exp. Brain Res.* 102:407-414.

393. Svendsen, C.N., Clarke, D.J., Rosser, A.E., and Dunnett, S.B. 1996. Survival and differentiation of rat and human epidermal growth factorresponsive precursor cells following grafting into the lesioned adult central nervous system. *Exp. Neurol.* 137:376-388.

394. Svendsen, C.N., Caldwell, M.A., Shen, J., ter Borg, M.G., Rosser, A.E., Tyers, P., Karmiol, S., and Dunnett, S.B. 1997a. Long-term survival of human central nervous system progenitor cells transplanted into a rat model of Parkinson's disease. *Exp. Neurol.* 148:135-146

395. Svendsen, C.N., Skepper, J., Rosser, A.E., ter Borg, M.G., Tyres, P., and Ryken, T. 1997b. Restricted growth potential of rat neural precursors as compared to mouse. *Brain Res. Dev. Brain Res.* 99:253-258.

396. Svendsen, C.N., ter Borg, M.G., Armstrong, R.J., Rosser, A.E., Chandran, S., Ostenfeld, T., and Caldwell, M.A. 1998. A new method for the rapid and long term growth of human neural precursor cells. *J. Neurosci. Methods.* 85:141-152.

397. Svendsen, C.N., Caldwell, M.A., and Ostenfeld, T. 1999. Human neural stem cells: Isolation, expansion and transplantation. *Brain Pathol.* 9:499-513.

398. Sykova, E. and Jendelova, P. 2005. Magnetic resonance tracking of implanted adult and embryonic stem cells in injured brain and spinal cord. *Ann. N.Y. Acad. Sci.* 1049:146-160.

399. Sykova, E. and Jendelova, P. 2006. Magnetic resonance tracking of transplanted stem cells in rat brain and spinal cord. *Neurodegener. Dis.* 3:62-67.

400. Sykova, E. and Jendelova, P. 2007a. In vivo tracking of stem cells in brain and spinal cord injury. *Prog. Brain Res.* 161:367-383.

401. Sykova, E. and Jendelova, P. 2007b. Migration, fate and In vivo imaging of adult stem cells in the CNS. *Cell Death Differ.* 14:1336-1342.

402. Tai, Y.T. and Svendsen, C.N. 2004. Stem cells as a potential treatment of neurological disorders. *Curr. Opin. Pharmacol.* 4:98-104.

403. Takahashi, K. and Yamanaka, S. 2006. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell* 126:663-676.

404. Takahashi, J., Palmer, T.D., and Gage, F.H. 1999. Retinoic acid and neurotrophins collaborate to regulate neurogenesis in adult-derived neural stem cell cultures. *J. Neurobiol.* 38:65-81.

405. Takahashi, K., Okita, K., Nakagawa, M., and Yamanaka, S. 2007a. Induction of

pluripotent stem cells from fibroblast cultures. *Nat. Protoc.* 2:3081-3089.

406. Takahashi, K., Tanabe, K., Ohnuki, M., Narita, M., Ichisaka, T., Tomoda, K., and Yamanaka, S. 2007b. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell* 131:861-872.

407. Tamaki, S., Eckert, K., He, D., Sutton, R., Doshe, M., Jain, G., Tushinski, R., Reitsma, M., Harris, B., Tsukamoto, A., Gage, F., Weissman, I., and Uchida, N. 2002. Engraftment of sorted/expanded human central nervous systemstem cells from fetal brain. *J. Neurosci. Res.* 69:976-986.

408. Tao,W., Evans, B.G., Yao, J., Cooper, S., Cornetta, K., Ballas, C.B., Hangoc, G., and Broxmeyer, H.E. 2007. Enhanced green fluorescent protein is a nearly ideal long-term expression tracer for hematopoietic stem cells, whereas DsRedexpress fluorescent protein is not. *Stem Cells* 25:670-678.

409. Tarasenko, Y.I., Yu, Y., Jordan, P.M., Bottenstein, J., and Wu, P. 2004. Effect of growth factors on proliferation and phenotypic differentiation of human fetal neural stem cells. *J. Neurosci. Res.* 78:625-636.

410. Tarasenko, Y.I., Gao, J., Nie, L., Johnson, K.M., Grady, J.J., Hulsebosch, C.E., McAdoo, D.J., and Wu, P. 2007. Human fetal neural stem cells grafted into contusion-injured rat spinal cords improve behavior. *J. Neurosci. Res.* 85:47-57.

411. Taupin, P., Ray, J., Fischer, W.H., Suhr, S.T., Hakansson, K., Grubb, A., and Gage, F.H. 2000. FGF-2-responsive neural stem cell proliferation requires CCg, a novel autocrine/paracrine cofactor. *Neuron* 28:385-397.

412. Taylor, J.R., Elsworth, J.D., Sladek, J.R. Jr., Collier, T.J., Roth, R.H., and Redmond, D.E. Jr. 1995. Sham surgery does not ameliorate MPTPinduced behavioral deficits in monkeys. *Cell Transplant.* 4:13-26.

413. Temple, S. 2001. The development of neural stem cells. *Nature* 414:112-117.

414. Temple, S. and Raff, M.C. 1986. Clonal analysis of oligodendrocyte development in culture: Evidence for a developmental clock that counts cell divisions. *Cell* 44:773-779.

415. Teng, Y.D., Lavik, E.B., Qu, X., Park, K.I., Ourednik, J., Zurakowski, D., Langer, R., and Snyder, E.Y. 2002. Functional recovery following traumatic spinal cord injury mediated by a unique polymer scaffold seeded with neural stem cells. *Proc. Natl. Acad. Sci. U.S.A.* 99:3024-3029.

416. Thomas, L.B., Gates, M.A., and Steindler, D.A. 1996. Young neurons from the adult subependymal zone proliferate and migrate along an astrocyte, extracellular matrix-rich pathway. *Glia* 17:1-14.

417. Toda, H., Tsuji, M., Nakano, I., Kobuke, K., Hayashi, T., Kasahara, H., Takahashi, J., Mizoguchi, A., Houtani, T., Sugimoto, T., Hashimoto, N., Palmer,

T.D., Honjo, T., and Tashiro, K. 2003. Stem cell-derived neural stem/progenitor cell supporting factor is an autocrine/ paracrine survival factor for adult neural stem/progenitor cells. *J. Biol.Chem.* 278:35491-35500.

418. Tramontin, A.D., Garcia-Verdugo, J.M., Lim, D.A., and Alvarez-Buylla, A. 2003. Postnatal development of radial glia and the ventricular zone (VZ): A continuum of the neural stem cell compartment. *Cereb. Cortex* 13:580-587.

419. Tsien, R.Y. 2003. Imagining imaging's future. *Nat. Rev. Mol. Cell. Biol.* Suppl:SS16-SS21.

420. Uchida, N., Buck, D.W., He, D., Reitsma, M.J., Masek, M., Phan, T.V., Tsukamoto, A.S., Gage, F.H., and Weissman, I.L. 2000. Direct isolation of human central nervous system stem cells. *Proc. Natl. Acad. Sci. U.S.A.* 97:14720-14725.

421. van der Kooy, D. and Weiss, S. 2000. Why stem cells? *Science* 287:1439-1441.

422. van Heyningen, P., Calver, A.R., and Richardson, W.D. 2001. Control of progenitor cell number by mitogen supply and demand. *Curr. Biol.* 11:232-241.

423. Verdugo, J.M. and Alvarez-Buylla, A. 2006. Cellular composition and cytoarchitecture of the adult human subventricular zone: A niche of neural stem cells. *J. Comp. Neurol.* 494:415-434.

424. Vescovi, A. and Snyder, E. 1999. Establishment and properties of genetically and epigenetically propagated neural stem cell clones: Plasticity in vitro and in vivo. *Brain Pathol.* 9:569-598.

425. Vescovi, A., Reynolds, B., Fraser, D., and Weiss, S. 1993a. bFGF regulates the proliferative fate of unipotent (neuronal) & bipotent (neuronal/ astroglial EGF-generated CNS progenitor cells. *Neuron* 1:951-956.

426. Vescovi, A.L., Reynolds, B.A., Fraser, D.D., and Weiss, S. 1993b. bFGF regulates the proliferative fate of unipotent (neuronal) and bipotent (neuronal/astroglial) EGF-generated CNS progenitor cells. *Neuron* 11:951-966.

427. Vescovi, A.L., Gritti, A., Galli, R., and Parati, E.A. 1999a. Isolation and intracerebral grafting of nontransformed multipotential embryonic human CNS stem cells. *J. Neurotrauma.* 16:689-693.

428. Vescovi, A.L., Parati, E.A., Gritti, A., Poulin, P., Ferrario, M.,Wanke, E., Fr¨olichsthal-Schoeller, P., Cova, L., Arcellana-Panlilio, M., Colombo, A., and Galli, R. 1999b. Isolation and cloning of multipotential stem cells from the embryonic human CNS and establishment of transplantable human neural stem cell lines by epigenetic stimulation. *Exp. Neurol.* 156:71-83.

429. Vicario-Abejon, C., Johe, K.K., Hazel, T.G., Collazo, D., and McKay, R.D.G. 1995. Functions of basic fibroblast growth factor and neurotrophins in the differentiation of hippocampal neurons. *Neuron* 15:105-114.

430. Villa, A., Snyder, E.Y., Vescovi, A., and Martinez-Serrano, A. 2000. Establishment and properties of a growth factor-dependent, perpetual neural stem cell line from the humanCNS. *Exp. Neurol.* 161:67-84.

431. Villa, A., Navarro-Galve, B., Bueno, C., Franco, S., Blasco, M.A., and Martinez-Serrano, A. 2004. Long-term molecular and cellular stability of human neural stem cell lines. *Exp. Cell Res.* 294:559-570.

432. Vintersten, K., Monetti, C., Gertsenstein, M., Zhang, P., Laszlo, L., Biechele, S., and Nagy, A. 2004. Mouse in red: Red fluorescent protein expression in mouse ES cells, embryos, and adult animals. *Genesis* 40:241-246.

433. Wakeman, D.R., Crain, A.M., and Snyder, E.Y. 2006. Large animal models are critical for rationally advancing regenerative therapies. *Regen Med.* 1:405-413.

434. Wakeman, D.R., Hofmann, M.R., Teng, Y.D., and Snyder, E.Y. 2009. Derivation, expansion, and characterization of human fetal forebrain neural stem cells. *In* Human Cell Culture: Adult Stem Cells. Volume 7. (J.R. Masters, ed.) Springer, Dordrecht, The Netherlands.

435. Walczak, P. and Bulte, J.W. 2007. The role of noninvasive cellular imaging in developing cell-based therapies for neurodegenerative disorders. *Neurodegenerative Dis.* 4:306-313.

436. Walczak, P., Zhang, J., Gilad, A.A., Kedziorek, D.A., Ruiz-Cabello, J., Young, R.G., Pittenger, M.F., van Zijl, P.C., Huang, J., and Bulte, J.W. 2008. Dual-modality monitoring of targeted intraarterial delivery of mesenchymal stem cells after transient ischemia. *Stroke* 39:1569-1574.

437. Walicke, P.A. 1988. Basic and acidic fibroblast growth factors have trophic effects on neurons from multiple CNS regions. *J. Neurosci.* 8:2618-2627.

438. Walsh, K., Megyesi, J., and Hammond, R. 2005. Human central nervous system tissue culture: A historical review and examination of recent advances. *Neurobiol. Dis.* 18:2-18.

439. Ward, W.W., Chalfie, M., and Kain, S. 1998. *In* Green Fluorescent Protein: Properties, Applications, and Protocols (M. Chalfie and S. Kain, eds.) Wiley–Liss, New York.

440. Watt, F.M. and Hogan, BL. 2000. Out of Eden: Stem cells and their niches. *Science* 287:1427-1430.

441. Weiss, S., Dunne, C., Hewson, J., Wohl, C., Wheatley, M., Peterson, A.C., and Reynolds, B.A. 1996. Multipotent CNS stem cells are present in the adult mammalian spinal cord and ventricular neuroaxis. *J. Neurosci.* 16:7599-7609.

442. Weissman, I.L., Anderson, D.J., and Gage, F. 2001. Stem and progenitor cells:

Origins, phenotypes, lineage commitments, and transdifferentiations. ***Annu. Rev. Cell Dev. Biol.*** **17:387-403.**

443. Weissman, T., Noctor, S.C., Clinton, B.K., Honig, L.S., and Kriegstein, A.R. 2003. Neurogenic radial glial cells in reptile, rodent and human: From mitosis to migration. ***Cereb. Cortex.*** **13:550-559.**

444. Wernig,M., Meissner, A., Foreman, R., Brambrink, T., Ku, M., Hochedlinger, K., Bernstein, B.E., and Jaenisch, R. 2007. in vitro reprogramming of fibroblasts into a pluripotent ES-cell-like state. ***Nature*** **448:318-324.**

445. Wernig, M., Meissner, A., Cassady, J.P., and Jaenisch, R. 2008. c-Myc is dispensable for direct reprogramming of mouse fibroblasts. ***Cell Stem Cell*** **2:10-12.**

446. Winkler, C., Fricker, R.A., Gates, M.A., Olsson, M., Hammang, J.P., Carpenter, M.K., and Bj¨orklund, A. 1998. Incorporation and glial differentiation of mouse EGF-responsive neural progenitor cells after transplantation into the embryonic rat brain. ***Mol. Cell Neurosci.*** **11:99-116.**

447. Wright, L.S., Li, J., Caldwell, M.A., Wallace, K., Johnson, J.A., and Svendsen, C.N. 2003. Gene expression in human neural stem cells: Effects of leukemia inhibitory factor. ***J. Neurochem.*** **86:179-195.**

448. Wright, L.S., Prowse, K.R., Wallace, K., Linskens, M.H., and Svendsen, C.N. 2006. Human progenitor cells isolated from the developing cortex undergo decreased neurogenesis and eventual senescence following expansion in vitro. ***Exp. Cell Res.*** **312:2107-2120.**

449. Wu, P., Ye, Y., and Svendsen, C.N. 2002. Transduction of human neural progenitor cells using recombinant adeno-associated viral vectors. ***Gene Ther.*** **9:245-255.**

450. Wu, Y.Y., Mujtaba, T., and Rao, M.S. 2002. Isolation of stem and precursor cells from fetal tissue. ***Methods Mol. Biol.*** **198:29-40.**

451. Wurmser, A.E., Palmer, T.D., and Gage, F.H. 2004. Neuroscience: Cellular interactions in the stem cell niche. ***Science*** **304:1253-1255.**

452. Yang, H., Mujtaba, T., Venkatraman, G., Wu, Y.Y., Rao, M.S., and Luskin, M.B. 2000. Region specific differentiation of neural tube-derived neuronal restricted progenitor cells after heterotopic transplantation. ***Proc. Natl. Acad. Sci. U.S.A.*** **97:13366-13371.**

453. Yasuhara, T., Matsukawa, N., Hara, K., Yu, G., Xu, L., Maki, M., Kim, S.U., and Borlongan, C.V. 2006. Transplantation of human neural stem cells exerts neuroprotection in a rat model of Parkinson's disease. ***J. Neurosci.*** **26:12497-12511.**

454. Yin, X.J., Ju, R., and Feng, Z.C. 2006. Experimental study on growth, proliferation and differentiation of neural stem cell from subventricular zone of human fetal brain at different gestational age. *Zhonghua Er Ke Za Zhi* 44:500-504.

455. Yu, J., Vodyanik, M.A., Smuga-Otto, K., Antosiewicz-Bourget, J., Frane, J.L., Tian, S., Nie, J., Jonsdottir, G.A., Ruotti, V., Stewart, R., Slukvin, I.I., and Thomson, J.A. 2007. Induced pluripotent stem cell lines derived from human somatic cells. *Science* 318:1917-1920.

456. Zhang, J., Campbell, R.E., Ting, A.Y., and Tsien, R.Y. 2002. Creating new fluorescent probes for cell biology. *Nat. Rev. Mol. Cell. Biol.* 3:906-918.

457. Zhang, H., Zhao, Y., Zhao, C., Yu, S., Duan, D., and Xu, Q. 2005. Long-term expansion of human neural progenitor cells by epigenetic stimulation in vitro. *Neurosci. Res.* 51:157-165.

458. Zigova, T., Betarbet, R., Soteres, B.J., Brock, S., Bakay, R.A., and Luskin, M.B. 1996. A comparison of the patterns of migration and the destinations of homotopically transplanted neonatal subventricular zone cells and heterotopically transplanted telencephalic ventricular zone cells. *Dev. Biol.* 173:459-474.

459. Zigova, T., Pencea, V., Betarbet, R., Wiegand, S.J., Alexander, C., Bakay, R.A., and Luskin, M.B. 1998. Neuronal progenitor cells of the neonatal subventricular zone differentiate and disperse following transplantation into the adult rat striatum. *Cell Transplant.* 7:137-156.

460. Zimmerman, L., Parr, B., Lendahl, U., Cunningham, M., McKay, R., Gavin, B., Mann, J., Vassileva, G., and McMahon, A. 1994. Independent regulatory elements in the nestin gene direct transgene expression to neural stem cells or muscle precursors. *Neuron* 12:11-24.

CHAPTER 3:

BEHAVIORAL IMPROVEMENT IN A PRIMATE PARKINSON'S MODEL IS ASSOCAITED WITH MUTIPLE HOMEOSTATIC EFFECTS OF HUMAN NEURAL STEM CELLS

3.1 Summary

Stem cells have been widely assumed to be capable of replacing lost or damaged cells in a number of diseases, including Parkinson's disease (PD), in which neurons of the substantia nigra (SN) die and fail to provide the neurotransmitter, dopamine (DA), to the striatum. We report that undifferentiated human neural stem cells (hNSCs) implanted into 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine- treated Parkinsonian primates survived, migrated, and had a functional impact as assessed quantitatively by behavioral improvement in this DA-deficit model, in which Parkinsonian signs directly correlate to reduced DA levels. A small number of HNSC progeny differentiated into tyrosine hydroxylase (TH) and/or dopamine transporter (DAT) immunopositive cells, suggesting that the microenvironment within and around the lesioned adult host SN still permits development of a DA phenotype by responsive progenitor cells. A much larger number of hNSC-derived cells that did not express neuronal or DA markers were found arrayed along the persisting nigrostriatal path, juxtaposed with host cells. These hNSCs, which express DA-protective factors, were therefore well positioned to influence host TH-ir cells and mediate other homeostatic adjustments, as reflected in a return to baseline endogenous neuronal number-to-size ratios, preservation of extant host nigrostriatal circuitry, and a normalizing effect on alpha-synuclein aggregation. We propose that multiple modes of reciprocal interaction between exogenous hNSCs and the pathological host milieu underlie the functional improvement observed in this model of PD.

3.2 Introduction

Degeneration of dopamine (DA) neurons in the substantia nigra (SN) and the consequent deficit of DA release in the striatum and other target areas appear to be responsible for the characteristic manifestations of Parkinson's disease (PD). Although substantial improvements result from the systemic administration of the DA precursor L-DOPA or DA agonists, such pharmacological replacement does not address the etiology of the disease, provide a permanent redress of the pathophysiology, or forestall progression of the degenerative process. It does, however, support the idea that DA provided by exogenous replacement cells might be therapeutic, a notion verified in rodents (1–3) and monkeys (4–6), where grafts of fetal DA neurons led to improvements in biochemical and behavioral indices of DA deficiency. However, in graft studies, the improvements in Parkinsonism have been limited and variable (see review in ref. 7). Therefore, we hypothesized that, in addition to DA replenishment, PD treatment should also restore functional equilibrium in the host SN-striatal system. A clinically relevant strategy might be to implant human neural stem cells (hNSCs) and progenitor cells constitutively capable of multiple actions, including neural differentiation and cytokine secretion, and allow them to develop within the PD affected brains of nonhuman primates to yield cells whose types, numbers, locations, and regulation are determined by the interplay of donor elements and the local host milieu. Outcomes derived from such donor–host interactions may result in a new level of bio-equilibrium among the DA-related neuro-structures (i.e., homeostasis), which could benefit behavioral recovery. hNSCs, either isolated directly from the developing normal brain (8–11) or derived from embryonic stem cells (12, 13), appear to be well suited for testing implementation of such a hypothesis. As the CNS' most primordial cell, the hNSC has attributes that appear to promote anatomical and functional preservation and/or restoration in neurodegenerative diseases. These properties include the potential for yielding appropriate ratios of cell types that constitute a normal

anatomical structure (i.e., both neurons and glia, plus other ‘‘chaperone-like’’ cells) (14–16). In addition, large numbers of hNSCs can be grown in culture as homogeneous, well-defined populations. For this study, we used hNSCs directly isolated from a neuroectoderm-derived structure, the telencephalic ventricular zone of normal, early second-trimester human cadavers (8). We selected two identically derived, non-immortalized hNSC lines (maintained *in vitro* as monolayers in serum-free, mitogen-supplemented medium) for their ability to engraft and migrate *in vivo* (8). One of these cell lines was known to pursue a ventral mesencephalic lineage when presented with appropriate cues *in vitro* (17) and to express a number of markers associated with a mesencephalic cell lineage [supporting information (SI) Figure 3-6]. We have reasoned that our hNSC-based comprehensive approach might better alleviate some of the limitations of previous strategies that placed partially differentiated cells, apparently without natural regulatory mechanisms, in ectopic locations such as the striatum (7).

3.3 Results

We studied 29 adult male African green monkeys of St. Kitts origin (*Chlorocebus sabaeus*). Four were untreated normal control monkeys, and the remainder were injected systemically with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). This neurotoxin causes selective permanent bilateral destruction of mesencephalic DA neurons and their striatal projections, depletion of DA concentrations, and the full signs of Parkinsonism (4, 18, 19). hNSCs were injected stereotaxically into the right SN and bilaterally into caudate nuclei. The approach of implanting undifferentiated (as opposed to precommitted) hNSCs allowed us to investigate whether cues might be present in the host milieu that could permit, or even direct and sustain, an appropriate anatomical and physiological restoration. The animals were studied over periods of ≤8 months and categorized according to MPTP treatment,

immunosuppression, numbers of cells injected, and other treatment variables (Groups 1–5; see SI Table 3-1). To ensure reproducibility, hNSCs were obtained from two separate lines (designated as ‘‘H1’’ and ‘‘HFB2050’’) that were initially derived by the same method: mitogen selection and expansion without immortalization (8). Numerous aliquots of early passaged cells were banked, thawed, and expanded as needed for new studies, hence minimizing cell variability from experiment to experiment over time.

To assess the possible impact of exogenous hNSCs on DA function, we studied a group of severely Parkinsonian monkeys (Group 1). Severity was determined with a well - validated and reliable behavioral scoring method consisting of time-sampled, quantitative behaviors and qualitatively rated items that reflect manifestations of Parkinsonism as well as normal behaviors in this primate species. A Parkinson's factor score (PFS), derived from these observations, correlates inversely with postmortem striatal DA concentrations (r = -0.72; n=18; P<0.01) (18, 19). Monkeys in this ‘‘most severe’’ category do not spontaneously or significantly improve over periods of greater than 1 year (18–20). Furthermore, the PFS in monkeys closely matches the 5-point Hoehn–Yahr scale, which is used clinically to categorize PD patients; the ‘‘most severe’’ category in monkeys corresponds to Stage 5 in humans.

Based on the PFS, eight monkeys that met the ‘‘Stage 5–Severely Parkinsonian’’ criteria were selected for study after their behavioral abnormalities were stable. Stage 5 monkeys show severe difficulty in ambulation, poverty of movement, delayed initiation of movement, lack of responses to food, difficulty eating, periods of ‘‘freezing’’ (remaining motionless for 5 sec), as well as head and limb tremors. The monkeys were randomly assigned to receive hNSC infusions or sham operations. Five hNSC-injected monkeys (10^6 cells X3 sites per monkey) were compared to three monkeys that received sham surgical injections, with observations starting 4 months before and continuing to 4 months after surgery. The hNSCs, maintained and prepared to optimize engraftment, were injected into the SN and

caudate. These severely affected hNSC injected monkeys improved progressively and were significantly different from controls for the entire post-treatment period (Figure 3-1). These differences were highly significant functionally as well as statistically, and they included ‘‘activities of daily living’’ (such as ability to sit, walk, and self-feed) compared to the sham-injected monkeys, which were unable to do so. Although the hNSC-engrafted monkeys were less improved in the final 60-day period, at the end of the experiment, they remained significantly improved compared to their pre-implantation levels and compared to sham-operated monkeys, which remained severely Parkinsonian (Figure 3-1).

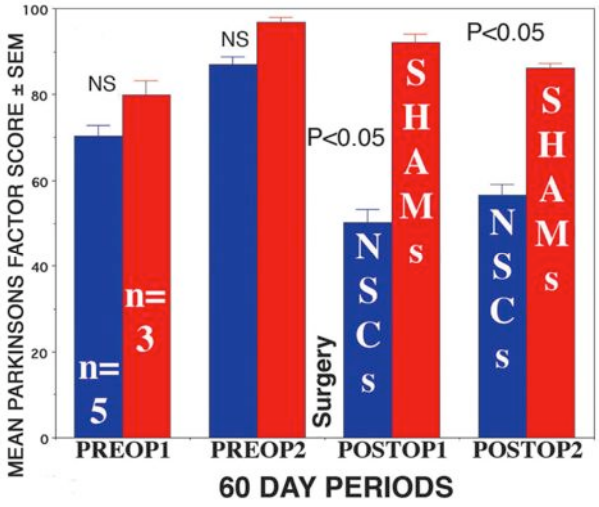


Figure 3-1. Behavioral recovery in severely Parkinsonian monkeys after hNSC injections. Severely Parkinsonian monkeys engrafted with hNSC (blue bars) demonstrated a significant decrease in a quantitative PFS, compared to sham operated monkeys (red bars), which remained severely Parkinsonian. Mean values ±SEM are divided into 60-day periods (PREOP, before injections; POSTOP, after injections). After treatment, the hNSC group improved dramatically and significantly. ANOVA revealed a significant interaction among treatment group (hNSC vs. sham), treatment (before or after), and day of observation (F=65.87, df=1,1096, P<0.0001). Tests of main effects showed that differences between the treatment groups were not significant before surgery (F =1.06, df = 1,6, P =NS), but became significantly different after (F = 6.16, df = 1,6, P < 0.05).

During formal (as well as extended periods of informal) observation of monkeys with ‘‘chimeric’’ human neural cell-bearing brain regions, there were no indications of any qualitative or quantitative behaviors that were not typical of the species, nor were any Parkinsonian dyskinesias noted. The duration and magnitude of functional recovery, compared to the controls, convinced us to terminate the behavioral experiment and begin a more extensive search for biochemical and histological correlates of improvement that might justify longer duration experiments and the investigation of immune and other factors and side effects that might help inform future long-term treatment of human PD.

To understand the basis for this functional recovery, histological sections from brains of these Group 1 monkeys and additional hNSC-injected monkeys were processed to assess the fate of donor and host cells [4 months after hNSC injections, designated as Group 2 (4-

month monkeys)]. Another group of MPTP-treated and hNSC-injected monkeys (Group 3) were studied and killed after 7 months (designated as 7-month monkeys). Four monkeys that were sham-operated but not MPTP-treated were controls (Group 4). In Group 1, although hNSCs were injected unilaterally immediately dorsal to the right SN (Figure 3-2*A*), we noted that donor-derived cells [identified by BrdU prelabeling (Figure 3-2*C*) and Beta-gal expression (Figure 3-2*D*)] were distributed bilaterally throughout the DA pathway, suggesting migration to the contralateral SN (Figure 3-2*B* and *C*) and/or migration from the engrafted ipsilateral caudate. Small numbers of donor-derived cells expressed tyrosine hydroxylase (TH) in the ventral mesencephalic region of Group 1 hNSC-injected monkeys (Figure 3-3). Such double-labeled TH-ir cells (identified by multiple independent markers) were not seen in non-lesioned hNSC-injected adult monkeys, although there was robust survival of hNSCs in all monkeys, whether normal or MPTP-lesioned. (In no monkeys were neoplasms, tumors, deformation, or overgrowth noted.)

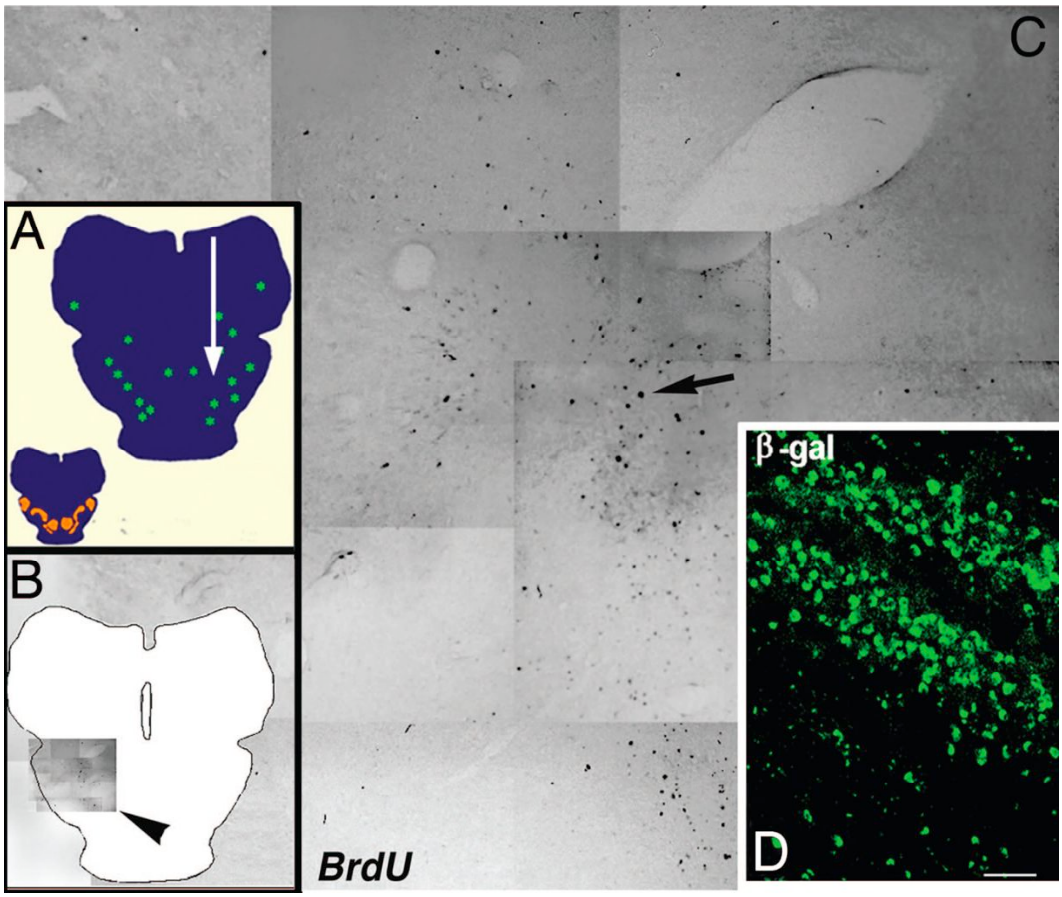


Figure. 3-2. Survival and migration of engrafted hNSCs associated with functional improvement after transplantation into the nigrostriatal system. (*A*) hNSCs were injected unilaterally, dorsal to the right SN (white arrow) in the monkeys studied behaviorally (Figure. 3-1, Group 1). Donor-derived cells were mapped (green stars) and detected throughout the area where DA nuclei are located (DA neuron distribution in this region is delineated in orange in the lower left corner, as recorded with a camera lucida). (*B–C*) Donor-derived cells labeled with BrdU and ß-gal were also detected on the side contralateral to the implant, as shown by widely distributed BrdU-ir donor-derived hNSCs (black nuclei, black arrow) in the region (arrowhead in *B* and enlarged in *C*). (*D*) ß –gal-ir cells (green) were present in substantial numbers in the ventral mesencephalon.

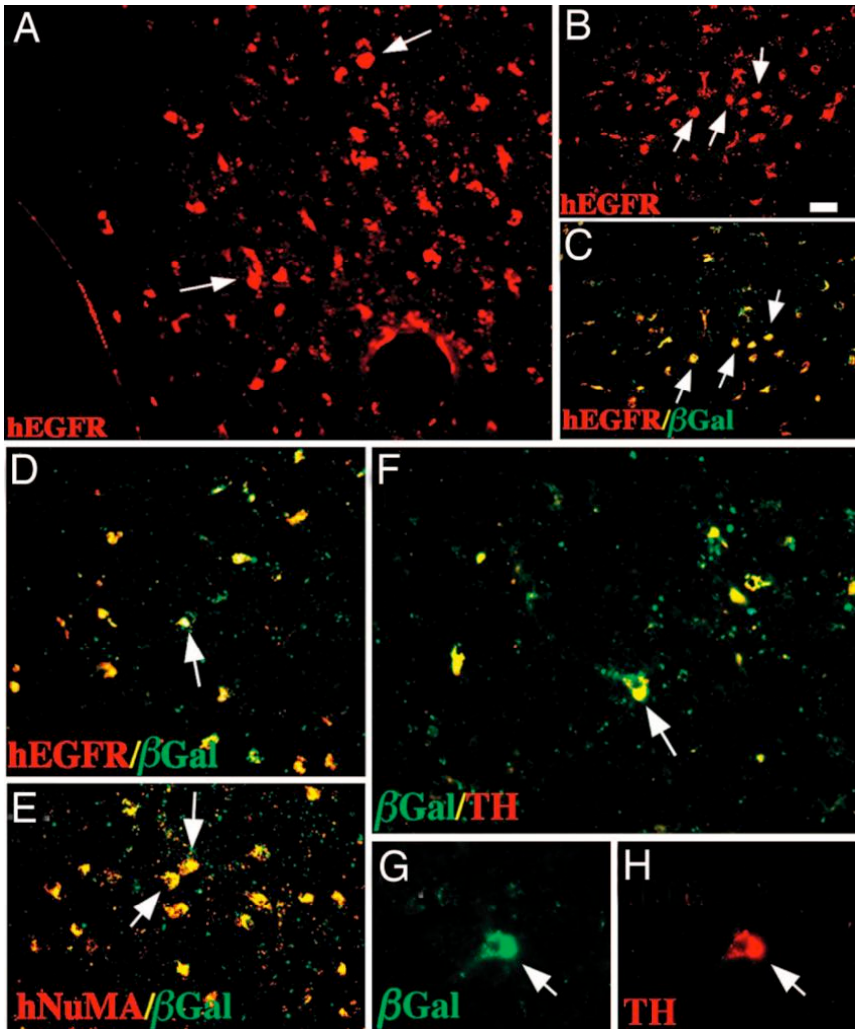


Figure 3-3. Additional independent markers identify engraftment and survival of donor-derived hNSCs within structures relevant to Parkinsonism and differentiation of some of them into TH-ir cells. Donor-derived cells in the ventral tegmentum are identified by antibodies to the human EGF receptor (hEGFR), nuclear mitotic apparatus (NuMA), or b-gal (arrows). EGFR-ir and NuMA-ir (*A* and *E*) co-localized with *lacZ*-expressing b-gal-ir (*C–E*) donor hNSCs. Furthermore, some donor-derived ß -gal-ir cells (*G*) co-labeled with TH-ir cells (*H*) in the DA-deficient nigra (*F*) (merged). Confocal microscopic analysis of such cells (with optical dissection and *z*-stacks) is shown in Figure 3-4*D* and SI Figure 3-7.

To confirm the presence and numbers of hNSC-derived neurons expressing markers consistent with a DA phenotype, we injected hNSCs into six additional MPTP-lesioned monkeys and performed additional histological studies on them after >7 months (SI Table 3-1, Group 5). Although in the earlier studies no differences were noted between cyclosporine-

and noncyclosporine-treated animals, azathiaprine and prednisolone were added to cyclosporine in this later group of monkeys to increase immunosuppression. These animals showed extensive survival of hNSCs, yielding a variety of neural cell types, including significant numbers of TH-ir and DAT-ir expressing cells in the disabled SN (Figure 3-4 and SI Figure 3-7). Although such cells constituted ≤1% of donor-derived cells in the SN, they represented 4–7% of the total TH-ir cellular population in that region. Further, because of migration of the unilaterally injected hNSCs to the contralateral equally impaired SN, the percentage of TH-ir cells that were donor-derived was not significantly different between the two sides [implanted, 6.74 ± 1.75% vs. unimplanted, 5.99 ± 1.74%; $F(1,8) = 0.15$, P = NS]. Accordingly, the actual concentrations of DA measured biochemically in punches from these regions were also statistically not different [$t(3) = 0.087$, P value not significant]. The number of BrdU-ir cells that were also TH-ir was not significantly different from those that were also DAT-ir [TH-ir, 6.37 ± 1.23% vs. DAT-ir, 4.69 ± 1.03%; $F(1, 8) = 0.70$, P value not significant]. (Because not all hNSCs become pre-labeled *ex vivo* with BrdU, a larger number of TH-ir and/or DAT-ir cells in the SN may have been derived from donor hNSCs.) There was no significant difference between the total number of counted BrdU-labeled cells between the implanted and unimplanted sides (5,931 ± 312 vs. 4,672 ± 988, respectively) [$t(3) = 1.86$, P value not significant].

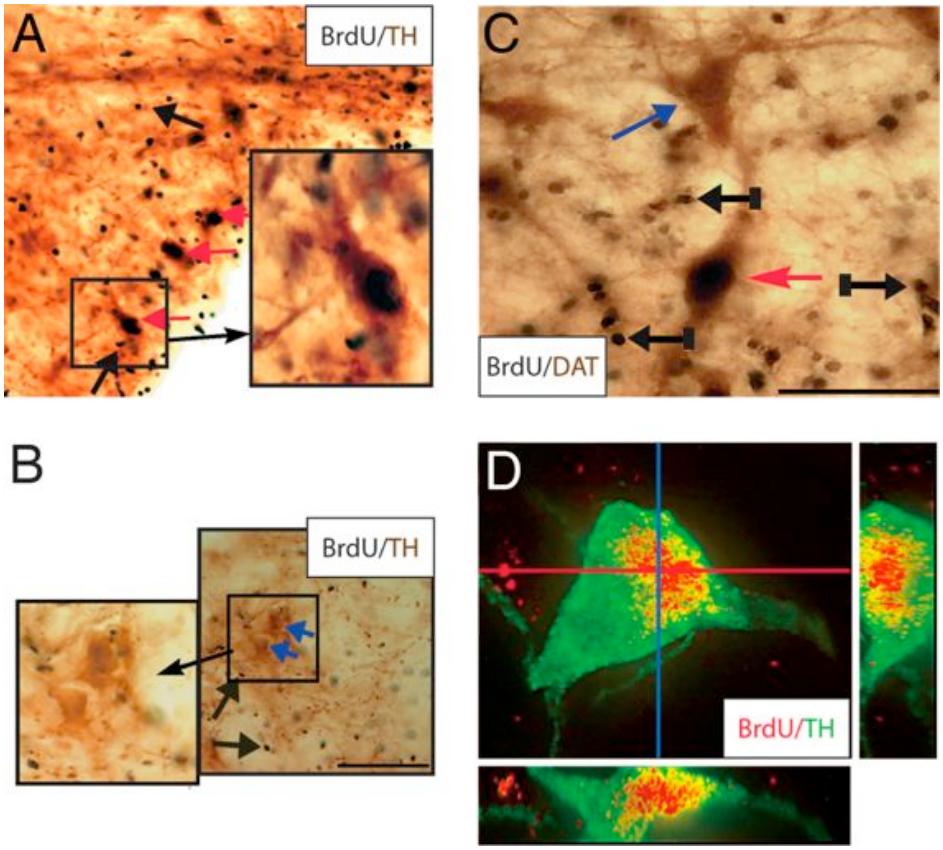


Figure 3-4. Some hNSCs transplanted into the SN of MPTP-lesioned monkeys showed key markers of DA neurons. (*A*) A black BrdU-ir nucleus indicates a donor-derived cell, with a small proportion also containing brown cytoplasmic TH-ir (6.75±1.28% of all TH-ir cells, red arrows). (*Inset*) Magnification of blocked cells. (*B*) Most BrdU-ir cells are not TH-ir (black arrows). Compared with host BrdU-negative TH-ir cells in *B* (blue arrows), cells in *A* are most likely donor-derived. (*Inset*) Magnification of blocked host BrdU-negative TH-ir cells. (*C*) Some donor-derived BrdU-ir cells in this region were also immunoreactive for the DAT (red arrow with tail); 3.91± 1.04% of DAT-ir cells were also labeled with BrdU. A DAT-ir neuron with a nucleus void of BrdU (blue arrow), presumably an endogenous host cell, is seen above the hNSC-derived neuron (red arrow), as well as many DAT-negative BrdU-ir (black nucleus) hNSCs (black arrows with tails) juxtaposed to DAT-ir fibers. (*D*) Double-label immunofluorescence of an hNSC cell expressing TH viewed by confocal microscopy with *z*-stacks; a BrdU-ir nucleus (red) is surrounded by a TH-ir cytoplasm (green). Red and blue lines indicate corresponding points in the orthogonal planes, confirming localization of the label within the pictured cell after the summation of serial optical sections. See also SI Figure 3-7. (Scale bars: *B*, 100 um; *C*, 50 um.)

Besides bilateral distribution of hNSCs in the SN after a unilateral injection, the hNSCs and their progeny appeared to migrate from the depleted striatum toward the SN along the nigrostriatal pathway (Figure 3-5 *A* and *B*). Most donor-derived cells were found between

SN and the striatum and ventral to the SN. Donor hNSC-derived TH-ir cells were closely associated with host-derived cell bodies and TH-ir fibers in the SN and nigrostriatal pathway (Figure 3-5*B*). Indeed the close physical association suggested that stimulus–response intercellular relationships might be in process between donor-derived non-neuronal cells and host DA neurons and their fibers (Figure 3-5*B*). This nonrandom distribution pattern was reminiscent of one of the proposed routes followed by progenitors during embryonic emergence of the nigrostriatal functional unit and might suggest that this pathway can still be used by progenitors, with possible behavioral consequences in the adult primate brain with PD pathology. No cells in the striatum of any of the monkeys were double-labeled for TH-ir and B-gal, nuclear mitotic apparatus (NuMA), or other human-specific markers studied. (Because of the extensive migration of hNSCs and their progeny, it was not feasible to count the proportion of implanted cells that survived. A more detailed study of cell migration in these monkeys is in progress.)

We also noted significant increases in the size of host TH-ir neurons in the SN by 7 months after hNSC injections (Figure 3-5*C* and SI Figure 3-8 *A* and *B*), associated with the presence of donor hNSC that were not TH-ir or DAT-ir. Thus, hNSCs appeared to exert homeostatic effects on host circuitry, increasing the size of abnormally small endogenous TH-ir neurons of the SN toward normal values. MPTP-induced changes in the size and number of TH-ir host cells in the striatum were also normalized after hNSC injections (SI Figure 3-9). Although the molecules mediating the impact of hNSCs on host DA systems are unknown, some BrdU-ir cells expressed a marker associated with an astrocytic lineage (Figure 3-5*G*) and expressed glial cell line-derived neurotrophic factor (GDNF) (Figure 3-5*H*), a growth factor known to augment and/or protect DA systems (21–24). Also, increased aggregation of _-synuclein has been reported after MPTP treatment in rodents (25) and primates (26). We found by immunohistochemical analysis of the nigrostriatal system in eight animals that _-

synuclein aggregation was present in approximately 80% of cells in monkeys that were MPTP-exposed only, but aggregation was found in less than 20% of cells after hNSC implantation had followed MPTP exposure (Figure 3-5*I* and SI Figure 3-10). No aggregates were seen in non-MPTP-lesioned monkeys regardless of whether they were transplanted with hNSCs. In summary, hNSC implantation appeared to return a number of abnormalities after MPTP lesioning to the parameters seen in normal animals.

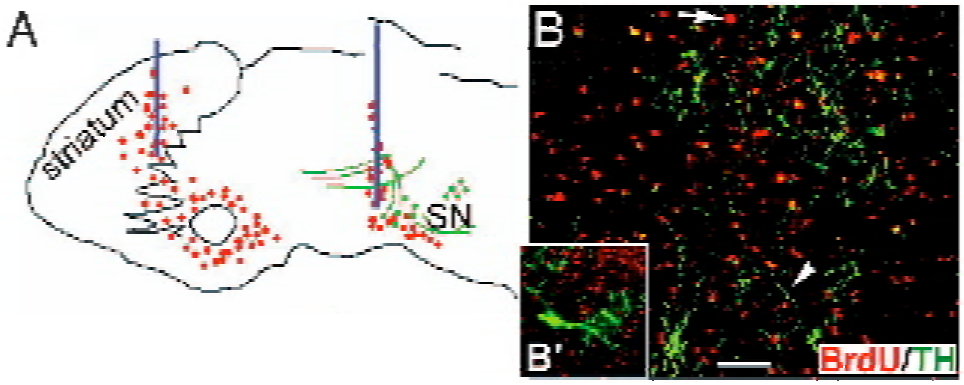


Figure 3-5. hNSC engraftment is associated with multiple influences on the host DA nigrostriatal system that might contribute to the observed functional improvement. Migration of hNSCs, normalization of pathological numbers and sizes of host TH-ir cells, and effects on alpha-synuclein aggregation are shown, with evidence of secretion of a growth factor known to preserve fibers in the host nigrostriatal system. (*A*) Four and 7 months after hNSCs were placed in the SN and caudate, the majority of donor-derived BrdU-ir cells had migrated to the nigrostriatal pathway as illustrated in a composite of serially sampled sections from an entire brain. Red dots represent an approximate density and locations where the majority of BrdU-ir cells were found. Green dots and lines indicate host TH-ir cells and their fibers. Blue lines indicate the locations where hNSCs had been implanted. (*B*) Many non-neuronal hNSC-derived cells (BrdU-ir in red, marked by white arrow) were found in the SN and closely associated with host TH_ cell bodies and their neurites (green, marked by white arrowhead). (*Inset*) Robust, healthy host DA neuronal soma with extensive processes (see also SI Figure 3-8 *A* and *B*). (*C*) In the SN, MPTP reduced the size of host TH-ir cells, which were then significantly increased 7 months after hNSC injections, compared to sham-operated MPTP-lesioned monkeys [ANOVA post hoc group differences; *, smaller than corresponding control group only; **, smaller than all other treatment groups ($P < 0.05$)]. (*D*) Endogenous TH-ir cells are also found in small numbers in the primate striatum. The arrow points to the most prominent type of striatal TH-ir neuron, which is small and bipolar (see SI Figure 3-9 *A–D*). (*E* and *F*) Their size-to-number ratios become disordered after MPTP lesioning. After MPTP lesioning, striatal TH-ir neurons increase in number (*E*) and decrease in size (*F*), a compensatory but abnormal change. They do not restore DA function and, in fact, are at their peak in animals that show the greatest signs of DA deficiency. In monkeys receiving hNSC implants, the aberrant size-to-number relationships of striatal DA neurons return to near normal control parameters (see SI Figure 3-9). (*G* and *H*) Some hNSCs (BrdU-ir cells, black nuclei) along the nigrostriatal pathway were also immunoperoxidase-positive (brown cytoplasm) for glial fibrillary acidic protein (*G*) and GDNF (*H*), suggesting that they had differentiated into astrocytes spontaneously and constitutively produced this trophic factor as a potential mechanism for hNSCs' effects on host neurons. (*I*) hNSCs transplanted into MPTP-lesioned monkeys appeared to diminish the alpha-synuclein-ir aggregation pattern (arrows) in the host striatum, approximating a more normal profile (as seen in non-lesioned monkeys with and without hNSCs). (Scale bars: *B*, 100 um; *D*, 20 um; *H* and *I*, 50 um.)

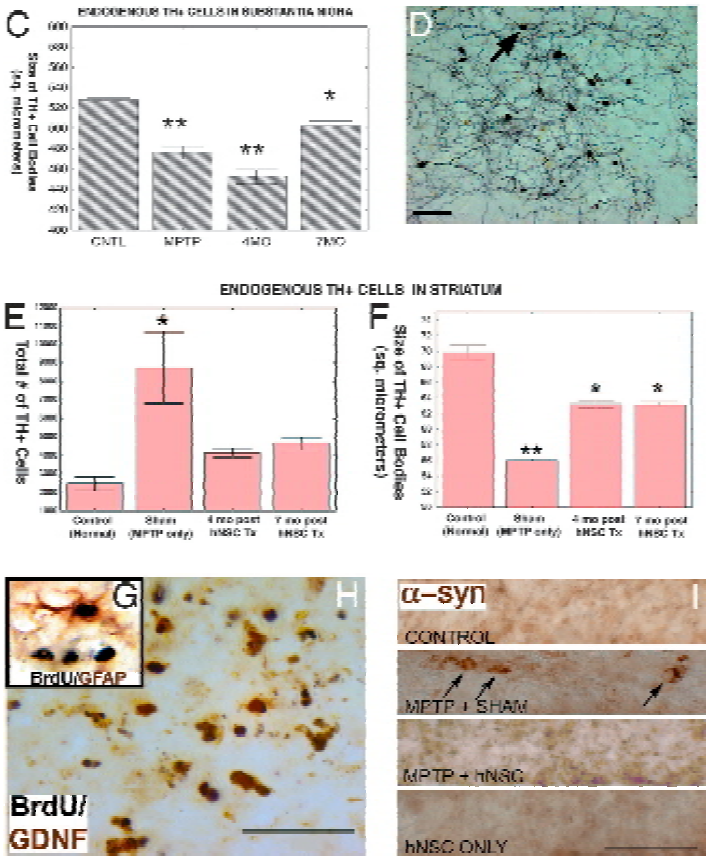


Figure 3-5. Continued

3.4 Discussion

Our studies demonstrate that the MPTP-lesioned adult monkey brain retains intrinsic microenvironmental signals that may direct differentiation of an uncommitted human stem cell toward a DA phenotype and suggest that hNSCs have the capacity to respond to a DA deficiency (27) even without pre-induction by factors or transgenes. However, the predominant functional action of hNSCs in the presence of damage to DA systems was most likely one of promoting homeostatic adjustment of host nigral DA neurons and their nigrostriatal projections. Some of the hNSCs, particularly those juxtaposed to host cells and fibers along the nigrostriatal trajectory, pursued an astrocytic lineage, which included expression of the neurotrophic factor GDNF. This observation is consistent with previous findings that epigenetic signals promoting the differentiation of Nurr1-expressing precursors may emanate from neighboring astrocytes (28). In fact, the role of the astrocyte in directing neurogenesis (15), mediating rescue (14), and potentiating the function of other neural cells is becoming increasingly appreciated (29). In particular, GDNF (one of possibly several natural products of the astrocytic progeny of these hNSCs) shows developmental, trophic, and protective support of DA neurons and promotes effective processing and release of DA (21–23). The normalization of _-synuclein aggregation in the presence of injected hNSCs in this study illustrates another potentially beneficial effect of hNSCs, but is probably independent of GDNF (30).

Most prior studies have focused on the concept that the host environment, as it changes during the course of development and aging, or after injury or cell degeneration, influences the transplanted stem cell, as exemplified here by the homeostatic emergence of some donor-derived TH-ir and DAT-ir cells. Based on past reports that small reversals of DA depletion can underlie large functional improvements, even a small elevation in DA might be

behaviorally relevant, whether from stem cell differentiation into DA neurons or from preservation and even augmentation of host DA pathways via hNSC-derived trophic/neuroprotective effects. This study also reveals that improvement in function might result from significant reversal of abnormalities in sizes and distributions of endogenous TH-ir cells in the nigrostriatal system as well as an interesting normalization of alpha-synuclein aggregation. These effects are consistent with other studies that have shown NSCs yielding multiple interacting cell types, not only key effector neurons but also undifferentiated progenitor cells that mediate neuroprotection and neuroplasticity (31) and glia that nurture, detoxify, myelinate, or direct the differentiation of neurons (32, 33). NSCs have also been suggested to restore equipoise to a disequilibrated milieu by fueling cell turnover (8, 34) and regulating gene expression and signaling pathways (35, 36). We believe that our data suggest, therefore, that the Parkinsonian primate CNS may benefit from such homeostatic effects, including (*i*) replacement of degenerating DA neurons by differentiated human stem cells, and (*ii*) the trophic, protective, and guidance effects of non-neuronal stem cell-derived progeny. These latter actions may manifest themselves by promoting recovery through the variety of effects described here, as well as by others that remain to be elucidated by additional experiments and controls. Although long-term studies of these effects and potential side effects, such as dyskinesia (although not observed in the present study) and possible immunorejection of exogenous stem cells, are needed before attempting clinical application, this report provides evidence that permissive signals are present in the milieu, and that stem cells respond with multiple homeostatic actions to restore functionality to an adult primate brain that presents with severe Parkinsonian pathology.

3.5 Materials and Methods

3.5.1 Source and Maintenance of hNSCs

Cells were obtained from stable, self-maintaining populations of hNSCs dissected from the ventricular germinal zone of a 13-week-old human fetal cadaver (8, 37) and maintained in neurobasal (Gibco/ Invitrogen, Grand Island, NY) medium supplemented with N2 or B-27 (Gibco) plus bFGF (20 ng/ml) (Chemicon International, Temecula, CA), heparin (8 mcg/ml), and LIF (5 ng/ml) (Chemicon International). Both adherent cells and floating clusters were chemically dissociated with Accutase (Chemicon International), triturated, and passaged every 3 to 10 days. hNSC lines (8, 37) were propagated with mitogens alone. More details are provided in *SI Materials and Methods*.

3.5.2 MPTP Lesioning of Monkeys and Treatment Groups.

Five groups of monkeys were studied with or without injections with MPTP-HCl (RBI/Sigma–Aldrich, Natick, MA). Seventeen monkeys received cumulative doses of 2.25 mg/kg over a 5-day period to induce degeneration of the nigrostriatal pathway, and six monkeys received 1.5 mg/kg aimed to produce DA depletion but without functional impairments. Seven monkeys were sham-injected, and 20 received hNSCs injections. Four monkeys, which were not treated with MPTP, were studied as controls (see SI Table 3-1 for individual details of cell numbers, immunosuppression, cell types, and numbers). The animal experiments were approved by the relevant institutional animal care and use committees of the collaborating institutions.

3.5.3 Behavioral Scoring and Statistical Analysis.

Blinded observers scored the MPTP-treated monkeys by using a published and

validated quantitative time-sampling method (4, 18) two periods per day, 5 days per week, a regime that has been shown empirically to sample Parkinsonian behaviors efficiently and accurately. Statistical analysis of behavioral changes used a multifactor ANOVA of the daily PFS of each monkey. All 1,304 individual observations were analyzed in 60-day blocks from 120 days before to 120 days after hNSC implantation, when monkeys were killed; >95% concordance was recorded among five blinded observers for all behaviors tested.

3.5.4 Preparation and Transplantation of hNSCs.

hNSCs were injected into the right SN and the right and left caudate nuclei by using stereotaxic procedures. Donor-derived cells were identified by multiple independent techniques. Dissociated hNSCs were preincubated *ex vivo* with BrdU for 48 to 72 h *in vitro* before transplant. Some hNSCs were subcloned to stably express the *lacZ* transgene and produce B-gal. Control experiments confirmed that donor cell-specific markers were never transferred to host cells after cell destruction; donor cells never produced recombinant replication-competent helper virus. hNSCs were dissociated and passaged 24 to 48 h before transplantation to help synchronize and make as uniform as possible their state of differentiation and stage in the cell cycle (8, 37). The number of hNSCs injected was 1,000,000 cells in most animals, although it ranged from 1 to 8.75 E 06 in Group 5. Additional monkeys were MPTP-treated only and received injections of vehicle or needle passage alone (‘‘sham-transplanted’’) (see SI Table 3-1).

3.5.5 Histological Analyses.

Donor-derived and host cells were distinguished in postmortem fixed tissue by immunocytochemistry with antibodies against multiple independent markers, including BrdU, B-gal, and human-specific epitopes (8, 37, 38). Unbiased stereology was used for counting

labeled cells. The number and size of TH-ir immunoreactive (ir) cells in the caudate and SN were quantified, and BrdU-ir, TH-ir and DAT-ir cells were calculated as a percentage of total cells of each type. Immunocytochemistry for alpha-synuclein was performed with standard methods and primary antibody (Cell Signaling Technology, Danvers, MA) at 1:500 dilution and second antibody (Jackson ImmunoResearch, West Grove, PA) at 1:500 dilution and second antibody (Jackson ImmunoResearch, West Grove, PA) at 1:200.

3.6 Acknowledgements

Chapter 3, is a reprint in part of material as it appears in *Proc Natl Acad Sci U S A*. 2007 Jul 17;104(29):12175-80 with co-authors Redmond DE Jr, Bjugstad KB, Teng YD, Ourednik V, Ourednik J, Parsons XH, Gonzalez R, Blanchard BC, Kim SU, Gu Z, Lipton SA, Markakis EA, Roth RH, Elsworth JD, Sladek JR Jr, Sidman RL, Snyder EY. The dissertation author was a co-author of this paper. DRW performed transplantations, immunohistochemistry/ cytochemistry, analyzed data, co-prepared manuscript, co-assembled figures, and edited all text. DER was principal investigator, performed transplantations and oversaw all animal operations, co-prepared entire manuscript, and managed the project. KBB performed stereotactic counting. BCB, EAM and ZG performed IHC. JRS prepared histological figures. YDT, VO, JO, RG, XHP cultured cells. RHR, JDE, RLS, EYS, SAL, SUK supervised. Together with his coauthors, we thank the staff of St. Kitts Biomedical Research Foundation for their contributions to the *in vivo* primate studies, Csaba Leranth and Robert Makuch for histological and statistical advice, and Marcel Daadi for advice and studies differentiating hNSCs into DA neurons. This work was supported by National Institute of Neurological Disorders and Stroke Grants RO1NS40822, PO1NS44281 (to D.E.R.), and R21NS053935; Veterans Affairs Biomedical Laboratory Research and Development Grant 121F (to Y.D.T.); the National Institutes of Health/National Institute of General Medical

Sciences Grant T32GM08666 (to D.R.W.); the Axion Research Foundation; Project ALS; the American Parkinson's Disease Association; the Michael J. Fox Foundation; the International Organization of Glutaric Acidemia; the A-T Children's Project; and an anonymous donor to the Combined Jewish Philanthropies.

3.7 References

1. **Perlow MJ, Freed WJ, Hoffer BJ, Seiger A, Olson L, Wyatt RJ (1979)** ***Science*** **204:643–653.**

2. **Bjorklund A, Stenevi U (1979)** ***Brain Res*** **177:555–560.**

3. **Brundin P, Strecker RE, Widner H, Clarke DJ, Nilsson OG, Åstedt B, Lindvall O (1988)** ***Exp Brain Res*** **70:192–208.**

4. **Redmond DE, Jr, Sladek JR, Jr, Roth RH, Collier TJ, Elsworth JD, Deutch AY, Haber S (1986)** ***Lancet*** **1:1125–1127.**

5. **Taylor JR, Elsworth JD, Roth RH, Sladek JR, Jr, Collier TJ, Redmond DE, Jr (1991)** ***Exp Brain Res*** **85:335–348.**

6. **Bankiewicz K, Mandel RJ, Sofroniew MV (1993)** ***Experimental Neurology*** **124:140–149.**

7. **Redmond DE, Jr (2002)** ***Neuroscientist*** **8:457–488.**

8. **Flax JD, Aurora S, Yang C, Simonin C, Wills AM, Billinghurst LL, Jendoubi M, Sidman RL, Wolfe JH, Kim SU,** ***et al.*** **(1998)** ***Nat Biotechnol*** **16:1033– 1039.**

9. **Uchida N, Buck DW, He D, Reitsma MJ, Masek M, Phan TV, Tsukamoto AS, Gage FH, Weissman IL (2000)** ***Proc Natl Acad Sci USA*** **97:14720–14725.**

10. **Vescovi A, Snyder E (1999)** ***Brain Pathol*** **9:569–598.**

11. **Villa A, Snyder EY, Vescovi A, Martinez-SerranoA(2000)** ***Exp Neurol*** **161:67–84.**

12. **Zhang SC, Wernig M, Duncan ID, Brustle O, Thomson JA (2001)** ***Nat Biotechnol*** **19:1129–1133.**

13. **Kim JH, Auerbach JM, Rodriguez-Gomez JA, Velasco I, Gavin D, Lumelsky N, Lee SH, Nguyen J, Sanchez-Pernaute R, Bankiewicz K,** ***et al.*** **(2002)** ***Nature*****418:50–56.**

14. **Ourednik J, Ourednik V, Lynch WP, Schachner M, Snyder EY (2002)** ***Nat Biotechnol*** **20:1103–1110.**

15. **Song HJ, Stevens CF, Gage FH (2002)** ***Nat Neurosci*** **5:438–445.**

16. **McKay IJ, Lewis J, Lumsden A (1997)** ***Eur J Neurosci*** **9:1499–1506.**

17. **Daadi MM, Weiss S (1999)** ***J Neurosci*** **19:4484–4497.**

18. **Taylor JR, Elsworth JD, Sladek JR, Jr, Roth RH, Redmond DE, Jr (1994) in** ***Toxin-Induced Models of Neurological Disorders*****, eds Woodruff ML, Nonneman**

AJ (Plenum, New York), pp 139–174.

19. Elsworth JD, Taylor JR, Sladek JR, Jr, Collier TJ, Redmond DE, Jr, Roth RH (2000) *Neuroscience* 95:399–408.

20. Taylor JR, Elsworth JD, Roth RH, Sladek JR, Jr, Redmond DE, Jr (1997) *Neuroscience* 81:745–755.

21. Gash DM, Zhang Z, Ovadia A, Cass WA, Yi A, Simmerman L, Russell D, Martin D, Lapchak PA, Collins F, *et al*. (1996) *Nature* 380:252–255.

22. Choi-Lundberg DL, Lin Q, Chang YN, Chiang YL, Hay CM, Mohajeri H, Davidson BL, Bohn MC (1997) *Science* 275:838–841.

23. Kordower JH, Emborg ME, Bloch J, Ma SY, Chu Y, Leventhal L, McBride J, Chen EY, Palfi S, Roitberg BZ, *et al*. (2000) *Science* 290:767–773.

24. Patel NK, Bunnage M, Plaha P, Svendsen CN, Heywood P, Gill SS (2005) *Ann Neurol* 57:298–302.

25. Vila M, Vukosavic S, Jackson-Lewis V, Neystat M, Jakowec M, Przedborski S (2000) *J Neurochem* 74:721–729.

26. Kowall NW, Hantraye P, Brouillet E, Beal MF, McKee AC, Ferrante RJ (2000) *NeuroReport* 11:211–213.

27. Bjorklund LM, Sanchez-Pernaute R, Chung S, Andersson T, Chen IY, McNaught KS, Brownell AL, Jenkins BG, Wahlestedt C, Kim KS, *et al*. (2002) *Proc Natl Acad Sci USA* 99:2344–2349.

28. Wagner J, Akerud P, Castro D, Holm P, Snyder E, Perlmann N, Arenas E (1999) *Nature Biotechnol* 17:653–659.

29. Talbott JF, Loy DN, Liu Y, Qiu MS, Bunge MB, Rao MS, Whittemore SR (2005) *Exp Neurol* 192:11–24.

30. Lo Bianco C, Deglon N, Pralong W, Aebischer P (2004) *Neurobiol Dis* 17:283–289.

31. Teng YD, Lavik EB, Qu X, Park KI, Ourednik J, Zurakowski D, Langer R, Snyder EY (2002) *Proc Natl Acad Sci USA* 99:3024–3029.

32. Song H, Stevens CF, Gage FH (2002) *Nature* 417:39–44.

33. Christopherson KS, Ullian EM, Stokes CC, Mullowney CE, Hell JW, Agah A, Lawler J, Mosher DF, Bornstein P, Barres BA (2005) *Cell* 120:421–433.

34. Rosario CM, Yandava BD, Kosaras B, Zurakowski D, Sidman RL, Snyder EY (1997) *Development (Cambridge, UK)* 124:4213–4224.

35. Mehler MF, Gokhan S (2001) *Prog Neurobiol* 63:337–363.

36. Li J, Imitola J, Snyder EY, Sidman RL (2006) *J Neurosci* 26:7839–7848.

37. Imitola J, Raddassi K, Park KI, Mueller FJ, Nieto M, Teng YD, Frenkel D, Li J, Sidman RL, Walsh CA, *et al*. (2004) *Proc Natl Acad Sci USA* 101:18117–18122.

38. Ourednik V, Ourednik J, Flax JD, Zawada WM, Hutt C, Yang C, Park KI, Kim SU, Sidman RL, Freed CR, *et al*. (2001) *Science* 293:1820–1824

3.8 Supplemental Figures and Methods:

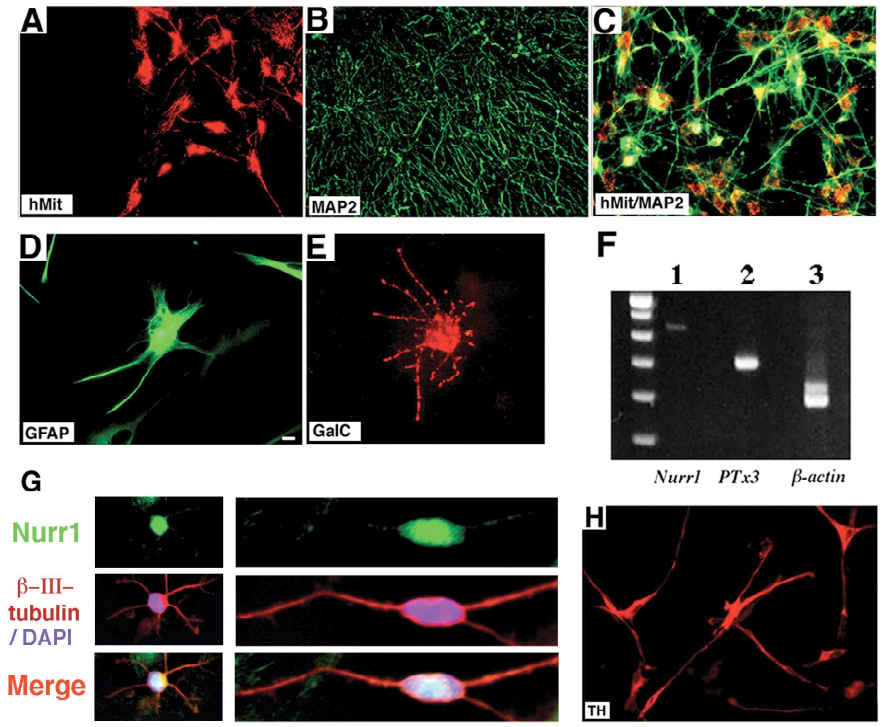


Figure. 3-S6. Human neural stem cells used in these studies show a number of markers associated with potential development into a DA phenotype when presented with appropriate cues in vitro. (A) Human NSCs were identified in vitro by human-specific markers as illustrated by their immunoreactivity to a monoclonal antibody against human mitochondria (hMit). (B-E) The hNSCs differentiated into mature neurons [MAP-2 (B), dual-labeled with hMit in (C)], astrocytes [GFAP (D)], and oligodendrocytes [GalC (E)]. (F) When maintained under appropriate culture conditions (27), the hNSCs expressed midbrain-specific molecular markers, Nurr1 and Ptx3 (the RT-PCR amplification product shown). (G) Prolonged culture produced neurons that co-labeled for b-III-tubulin (red), Nurr1 (green nuclear/perinuclear-ir product), or TH (H).

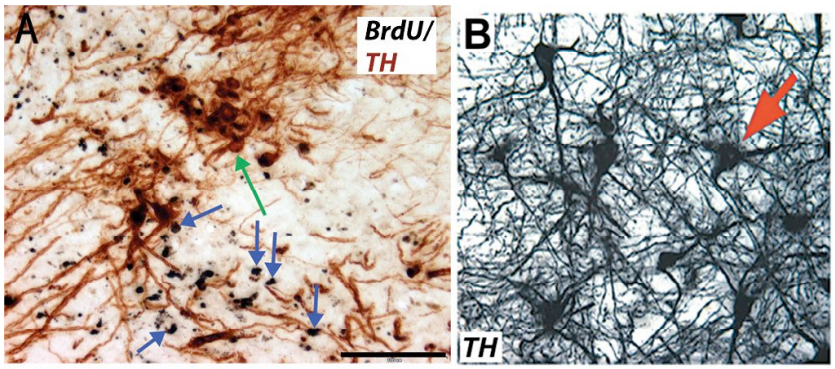


Figure 3-S7. hNSC engraftment normalizes some MPTP-induced changes in host DA neurons in the SN of the recipient Parkinsonian monkey. (A) Many undifferentiated hNSC (BrdU+, black nuclei, blue arrows) were located within and just ventral to the SN 7 months after transplantation. Furthermore, they were often closely juxtaposed to large BrdU-negative, endogenous TH+ (brown cytoplasm) cell bodies and their (brown) fibers (green arrows) (see also Figure 5 A-C).Typically, MPTP decreases the size of surviving nigral DA neurons, as well as their numbers. In MPTP-lesioned monkeys transplanted with hNSCs, however, as shown in B, host SN neurons were significantly increased in size 7 months after grafting. Note large exuberantly TH+ host nigral DA neurons (arrow). These sizes are quantified in Figure 5.C.

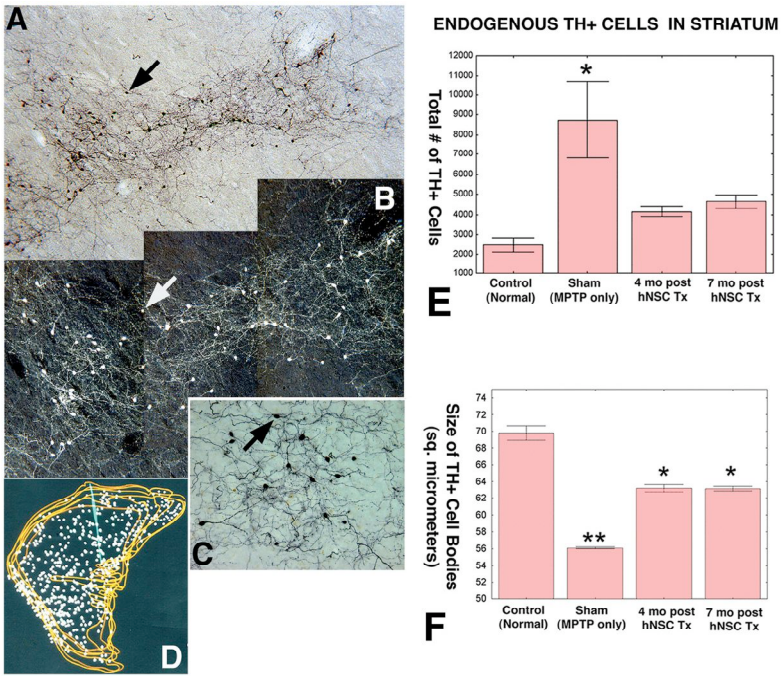


Figure 3-S8. hNSC engraftment also normalizes MPTP-induced changes in the Parkinsonian primate host striatum (i.e., aberrant size-to-number ratios). (A-D) Endogenous TH+ cells are also found in the primate striatum. A representative overview of TH+ cells in the caudate post-transplantation (in a representative monkey whose behavior was analyzed in Figure 3-1) is presented in brightfield (A) and in darkfield (B). Note the exuberant network of extensively branching TH+ processes. At higher magnification (C), arrows indicate a typical TH+ caudate neuron, characteristic of >95% of neurons in the caudate: small (60 mm), TH-immunoreactive, bipolar with smooth or varicose processes. It is this cell, meeting these criteria (similarly indicated by arrows in A and B), that was analyzed to yield the quantitative data (E and F) (see also Figure 3-5 D-F). The computer reconstruction (D) demonstrates the dense yet uniform and extensive distribution of endogenous dopaminergic neurons throughout the caudate of this monkey (as culled from multiple serial parasagittal 50mm histological sections) (needle track is indicated as a blue line). (E and F) The size-to-number ratios of these endogenous striatal TH+ cells become disordered following MPTP-lesioning: they increase in number (E) and decrease in size (F), a compensatory but abnormal change with unclear functional consequences. They do not restore DA function and, in fact, are at their peak in animals that show the greatest signs of DA deficiency. In monkeys receiving hNSC implants, the size and number of striatal DA neurons return to normal control parameters (E) and (F) (repeated from Figure 3-5 E and F). Details of specific identifiable TH cell subtypes and their normalization after hNSC implantation are reported elsewhere (34).

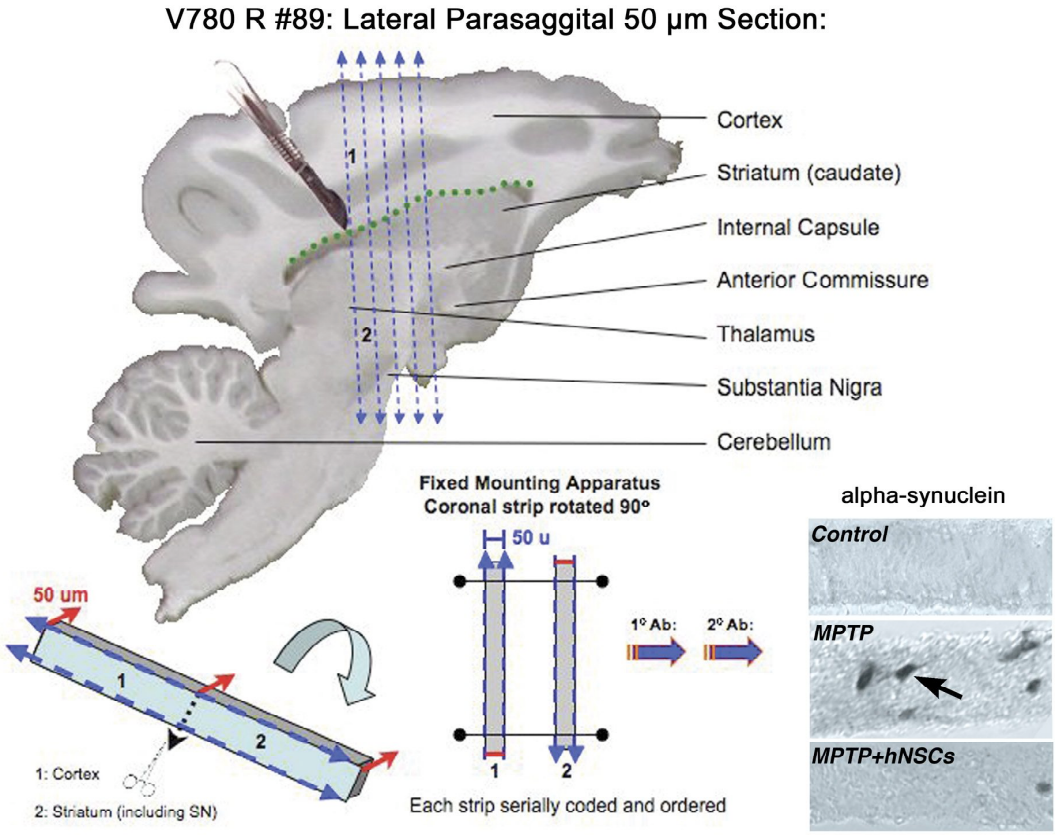


Figure 3-S9. Processing of monkey brain to provide tissue for a-synuclein immunohistochemistry. Three serial sections each ~200-300 mm wide were cut from lateral to medial surfaces from an existing 50-mm parasagittal tissue slice in an orientation indicated by the blue dotted lines. Individual strips were then divided at the green dotted line into region 1, consisting principally of cerebral cortex, and region 2, including principally the striatum and extending ventral to the nigra (below the green dotted line). These regions were numbered for record keeping and sectioned for a-synuclein Multiple samples from two or three monkeys in each of the following four experimental conditions were analyzed by two observers for extent, distribution, and pattern of a-synuclein immunoreactivity in both cortex and nigrostriatum (see SI Table 1). Group 1: Not MPTP lesioned, not transplanted with hNSCs (n = 14 samples); Group 2: Not MPTP lesioned, transplanted with hNSCs (n = 7 samples); Group 3: MPTP lesioned, sham-treated but not transplanted with hNSCs (n = 37 samples); and Group 4: MPTP lesioned, transplanted with hNSCs (n = 27 samples). Representative photomicrographs of a-synuclein-immunostained sections are shown in Figure 3-5 and here (from Groups 1, 3, and 4). The arrow indicates a cell immunopositive for a-synuclein.

SI Materials and Methods

Parkinsonian and Healthy Behavior Score and Scoring Methods.

Blinded observers who are trained and maintain a coefficient-of-concordance (Kendall's) >0.95 in their ratings of all behaviors scored the monkeys using a time-sampling method for the presence or absence of 20 normal and abnormal behaviors, in addition to ratings of a number of Parkinsonian signs (1-3). Monkeys were observed and scored two periods per day, 5 days per week, a regime that has been shown empirically to sample Parkinsonian behaviors efficiently and accurately. Monkeys were selected into treatment groups based on quantitative scores 1 month after MPTP exposure. A Parkinsonian factor score (PFS) was derived from a prior large study involving a principal component factor analysis on 15,000 individual behavioral observations from 77 MPTP or control monkeys, and the score was averaged per week of observations. For the purposes of predicting outcome and assigning monkeys to treatment groups, the monkeys were classified into five severity categories based on their Parkinsonian scores. These were quintiles from the initial study. Monkeys with the highest severity category, which, like Parkinson's patients, have previously been shown not to recover spontaneously, were assigned to hNSC treatment or sham surgery for the studies of function (2). Validity of the score has been tested by a number of methods. The score is highly responsive to pharmacological changes in DA function and correlates highly (inversely) with striatal DA concentrations postmortem (4). These categories at 1 month predict the extent of postmortem DA damage. Categorizing monkeys in this fashion at 1 month allows the selection of treatment groups that have similar extent of DA depletion and are known to remain significantly parkinsonian over long periods. Although the categories were derived empirically and mathematically, the five categories correlate well with the five levels of the Hoehn and Yahr scale, which is used clinically to categorize PD patients.

Eight monkeys that matched criteria for "severe Parkinsonism" (corresponding to Hoehn and Yahr category 5) were randomly assigned to receive hNSC injections or sham surgery. A total of 1,304 observations of the monkeys were made from a period from 120 before to 120 days after hNSC implantation. Five monkeys implanted bilaterally into the caudate nucleus and unilaterally dorsal to the SN with »1 million hNSCs per site were compared with three monkeys that received sham surgery using a multifactor analysis of variance. All monkeys were observed using the above-described standard behavioral scoring system (2-10).

Induction of Markers of Mesencephalic Precursors by hNSCs in Vitro.

This procedure is derived from that reported by Daadi and Weiss (27). hNSCs are induced to differentiate by first omitting growth factors from the medium. During the first week of differentiation, 7 days in vitro (DIV), ~5% of the total population express the neuronal marker b-tubulin class III. After 15 days in vitro, ~60% of the differentiated progeny express b-tubulin and MAP2. Of the remainder, 38% are astrocytes as identified by their immunoreactivity to GFAP and <2% are oligodendrocyte that express the cell surface antigen galactocerebrocide (SI Figure 3-6). To induce the dopaminergic phenotype, hNSCs are single cell dissociated at 0.5×10^6 cell per ml and plated at a density of 2.5×10^5 cell per cm2 on PLO-coated glass coverslips in 24-well culture dishes containing the baseline differentiation medium. After a 2-h culture period, the baseline differentiation media is replaced by the TH-inducing media containing 20 ng/ml of bFGF and 75% (vol/vol) of glial conditioned media (CM).

CM is prepared from cultures of postnatal striatal astrocytes and from the B49 rat glial cell line. B49 cells are cultured in DMEM/10% FBS until confluency. Confluent astrocyte or

B49 glial cell cultures are rinsed once with PBS and twice with serum-free DMEM/F12 (1:1) with hormone mix and replaced in the incubator with 20 ml of the same medium. The CM is collected after 24, 48, or 72 h and centrifuged at 1,000 ´g and 2,000 ´ g to remove cellular debris. The CM is carefully removed, filtered, aliquoted, and stored at -80°C until used. The CM likely fulfills a role similar to that reported by Wagner et al. (28).

The cultures are incubated and investigated for the TH induction within a period of 7 DIV. In each culture, the neuronal and dopaminergic lineage species are determined using immunocytochemistry for b-III-tubulin and TH, respectively. These cultures demonstrate a stable and steady increase of the TH-IR cells in cultures. After 24 h in culture, 1% of the total cells express TH, which represents ~30% of the total number of b-III-tubulin-IR neurons. The number of TH-ir cells steadily increases to 3% of the total DAPI positive live cells after 7 DIV. This proportion of TH-ir cells represents ~76% of the total b-III-tubulin-ir neuronal population (SI Figure 3-6). In vivo, the specification and maintenance of the midbrain DA fate during development is controlled by the nuclear receptor transcription factor Nurr1 and the homeobox transcription factors engrailed and Ptx-3. Semi-quantitative RT-PCR analysis confirms that DA-induced cultures (i.e., FGF2+CM-treated hNSCs after 7 DIV) markedly up-regulate expression of Nurr1 and Ptx-3, supporting the acquisition by these forebrain-derived hNSCs of a molecular profile consistent with mesencephalic dopamine precursors (SI Figure 3-6). The expression of Nurr1 and Ptx-3 without gene transduction in these cultures, a technique that has been previously used (12), suggests that they would be able to develop into dopaminergic neurons in vivo if exposed to the appropriate conditions.

Immunosuppressive Drug Administration.

Approximately half of the monkeys in each experimental and control group were

immunosuppressed with cyclosporine (0.6 mg/kg) in the behavioral and initial histological studies. In the final triple "immunosuppression" study, all monkeys also received prednisolone (0.3 mg/kg) and azathioprine (0.5 mg/kg) twice daily starting 3 days before implantation and continuing until they were killed, a dose that was tolerated by the monkeys.

Quantitative Morphology.

Unbiased stereology was used for counting all labeled cells. The number and size of TH cells in the caudate and SN were quantified, and BrdU+, TH+, and DAT+ cells were calculated as a percentage of total cells of each type. For cell size, a digital camera was used to take 4 to 10 pictures of TH-positive cells found at three different levels of the caudate and corresponding SN (Olympus MicroSuite software; Olympus, Tokyo, Japan). Each picture contained 1 to 15 different cells. Scion Image for Windows (Scion Corp., based on NIH Image by Wayne Rashan, NIH, Bethesda, MD) was used to measure the diameter and area of each cell pictured. ANOVA based on weighted means with posthoc Fisher's LSD was used to determine significant differences in cell soma size. Total cell counts were computed from the mean number of cells in five slides taken from a 2-mm lateral expanse and multiplied by the number of sections in 2 mm. For total cell counts, a standard ANOVA, with post hoc tests, was used to determine significance between groups of animals at $P < 0.05$ (two-tailed). In the Group 4 monkeys, this procedure was adjusted to compensate for a postmortem punch to obtain tissue for analysis of monoamines. Paired t tests of the adjusted cell counts ($P < 0.05$, two tailed) compared the injected versus the uninjected sides. Double-labeled TH/BrdU, TH (endogenous), DAT/BrdU, DAT (endogenous), and BrdU alone (NSC-derived) were statistically analyzed (five tests). None was significant.

Statistical Analyses of Behavioral Effects.

For the behavioral studies, sample size was estimated from the numbers required to show significant effects in previous studies of fetal tissue transplantation in MPTP-treated monkeys. All of the monkeys at a Stage 5 severity level were studied behaviorally because, as confirmed by our prior studies (2, 10), this level of severity does not spontaneously recover even after observation periods of >1 year. Monkeys were randomly assigned to receive hNSC's or sham surgery. Because we anticipated the possibility that actual cell engraftment could have adverse consequences (either perioperatively or afterward), the group sizes were skewed to have more hNSC animals than sham-operated animals. Data analyses were done using methods we have tested repeatedly to construct a PFS and Healthy Behavior score (similar to the clinical rating subscore, "activities of daily living." Significance was determined by ANOVA using the multifactor repeated measures model described by Winer (33) and carried out using the Statistical Analysis System. The two-tailed significance level was set at $P < 0.05$. As described in Figure 3-1, ANOVA revealed a significant interaction between treatment group (hNSC vs. sham) and treatment (before or after) and day of observation ($F = 65.87$, $df = 1,1096$, $P < 0.0001$). Tests of main effects showed that differences between the treatment groups were not significant before surgery ($F = 1.06$, $df = 1,6$, $p = NS$), but became significantly different afterward ($F = 6.16$, $df = 1,6$, $P < 0.05$). In summary, hNSC transplantation had a statistically significant and sustained impact on improving behavior (i.e., diminishing the PFS while improving the Healthy Behavior Score). These differences were functionally significant and included the ability to perform critical activities of daily living, compared to the controls, which could not.

We also performed linear regressions on the PFS data to determine whether there were significant trends toward improvement before hNSC injection in either the active hNSC or the

sham-injected group and whether any slopes were significantly different between the groups (Statistical Analysis System). This analysis examined the possibility that the Stage 5/severely Parkinsonian monkeys were already spontaneously improving before hNSC transplantation. There were no differences in the highly significant increases in Parkinsonism in both groups during the first 60 days of observation, but both groups were stable (had no significant slopes) and were not different from each other in the 2 months before surgery. After surgery, the hNSC group showed a highly significantly slope of improvement ($P < 0.001$). The sham-injected animals also showed a statistically significant improvement ($P < 0.01$), but this slope was tiny and was different from the hNSC animals. In the final 60-day period, the shams had no significant slope and remained highly symptomatic, whereas the hNSC's appeared to worsen slightly, consistent with the interpretation that differentiated TH+ and possibly other hNSC cells were being rejected over this time period. They remained dramatically better, however, than before the hNSC injections. The significance of linear regression slopes were determined using Statistical Analysis System, and predicted slopes and 95% confidence intervals were calculated and plotted to compare the two treatment groups.

Supplemental References:

1. **Baulu J, Redmond, DE, Jr (1978) Primates 19:391-399.**

2. **Taylor JR, Elsworth JD, Sladek JR, Jr, Roth RH, Redmond DE, Jr (1994) in Toxin-Induced Models of Neurological Disorders, eds Woodruff ML, Nonneman AJ (Plenum Press, New York), pp 139-174.**

3. **Redmond DE, Jr, Sladek JR, Jr, Roth RH, Collier TJ, Elsworth JD, Deutch AY, Haber S (1986) Lancet 1:1125-1127.**

4. **Elsworth JD, Taylor JR, Sladek JR, Jr, Collier TJ, Redmond DE, Jr, Roth RH (2000) Neuroscience 95:399-408.**

5. **Elsworth J, Collier T, Redmond D, Jr, Roth R, Sladek J, Jr, Taylor J (1996)**

Neuroscience 72:477-484.

6. **Elsworth JD, Brittan MS, Taylor JR, Sladek JR, Jr, al-Tikriti MS, Zea-Ponce Y, Innis RB, Redmond DE, Jr, Roth RH (1996) Cell Transplant 5:315-325.**

7. **Elsworth JD, Deutch AY, Redmond DE, Jr, Sladek JR, Jr, Roth RH (1987) Brain Research 415:293-299.**

8. **Elsworth JD, Deutch AY, Redmond DE, Jr, Taylor JR, Sladek JR, Jr, Roth RH (1989) Neuroscience 33:323-331.**

9. **Taylor JR, Elsworth JD, Roth RH, Sladek JR, Jr, Collier TJ, Redmond, DE, Jr (1991) Exp Brain Res 85:335-348.**

10. **Taylor JR, Elsworth JD, Roth RH, Sladek JR, Jr, Redmond DE, Jr (1997) Neuroscience 81:745-755.**

11. **Fisher L (1997) Neurobiol Dis 4:1-22.**

12. **Vescovi AL, Reynolds BA, Fraser DD, Weiss S (1993) Neuron 11:951-966.**

13. **Gritti A, Cova L, Parati EA, Galli R, Vescovi AL (1995) Neurosci Lett 185:151-154.**

14. **Flax JD, Aurora S, Yang C, Simonin C, Wills AM, Billinghurst LL, Jendoubi M, Sidman RL, Wolfe JH, Kim SU, et al. (1998) Nat Biotechnol 16:1033-1039.**

15. **Caldwell MA, He X, Wilkie N, Pollack S, Marshall G, Wafford KA, Svendsen CN (2001) Nat Biotechnol 19:475-479.**

16. **Ciccolini F, Svendsen CN (1998) J Neurosci 18:7869-7880.**

17. **Gritti A, Frolichsthal-Schoeller P, Galli R, Parati EA, Cova L, Pagano SF, Bjornson CR, Vescovi AL (1999) J Neurosci 19:3287-3297.**

18. **Gritti A, Parati EA, Cova L, Frolichsthal P, Galli R, Wanke E, Faravelli L, Morassutti DJ, Roisen F, Nickel DD, et al. (1996) J Neurosci 16:1091-1100.**

19. **Reynolds BA, Tetzlaff W, Weiss S (1992) J Neurosci 12:4565-4574.**

20. **Murphy M, Drago J, Bartlett PF (1990) J Neurosci Res 25:463-475.**

21. **Kilpatrick TJ, Bartlett PF (1993) Neuron 10:255-265.**

22. **Wright LS, Li J, Caldwell MA, Wallace K, Johnson JA, Svendsen CN (2003) J Neurochem 86:179-195.**

23. Schwartz PH, Bryant PJ, Fuja TJ, Su H, O'Dowd DK, Klassen H (2003) J Neurosci Res 74:838-851.

24. Palmer TD, Schwartz PH, Taupin P, Kaspar B, Stein SA, Gage FH (2001) Nature 411:42-43.

25. Svendsen CN, Fawcett JW, Bentlage C, Dunnett SB (1995) Exp Brain Res 102:407-414.

26. Caldwell MA, Svendsen CN (1998) Exp Neurol 152:1-10.

27. Daadi MM, Weiss S (1999) J Neurosci 19:4484-4497.

28. Wagner J, Akerud P, Castro D, Holm P, Snyder E, Perlmann N, Arenas E (1999) Nature Biotech 17:653-659.

29. Ourednik V, Ourednik J, Flax JD, Zawada WM, Hutt C, Yang C, Park KI, Kim SU, Sidman RL, Freed CR, et al. (2001) Science 293:1820-1824.

30. Sladek JR, Jr, Elsworth JD, Taylor JR, Roth RH, Redmond DE, Jr (1995) in Methods in Cell Transplantation, ed Ricordi, C (Landes RG, Austin, TX), pp 391-408.

31. Burns TC, Ortiz-Gonzalez XR, Gutierrez-Perez M, Keene CD, Sharda R, Demorest ZL, Jiang Y, Nelson-Holte M, Soriano M, Nakagawa Y, et al. (2006) Stem Cells 24:1121-1127.

32. Ourednik J, Ourednik W, Mitchell DE (1998) J Comp Neurol 395:91-111.

33. Winer BJ (1974) Statistical Principles in Experimental Design (McGraw-Hill, New York).

34. Bjugstad KB, Redmond DE, Jr, Teng YD, Elsworth JD, Roth RH, Blanchard BC, Snyder EY, Sladek, JR, Jr (2005) Cell Transplant 14:183-192.

CHAPTER 4:

HUMAN NEURAL PROGENITOR CELL GRAFTS PROJECT NEURITIC PROCESSES ALONG HOST CIRCUITRY CONCOMITANTLY WITH AAV5-GDNF CHEMOTAXIS IN THE MPTP-LESIONED PRIMATE BRAIN

4.1 Summary

Human fetal and embryonic stem cell (hESC) derived neural precursor cells (hNPC) have been suggested as a renewable alternative substrate to fetal dopaminergic (DA) neurons/progenitors for transplantation in Parkinson's disease; however, little is known about the long-term potential of these grafts to differentiate and integrate into anatomically correct loci in the adult non-human primate brain. We transplanted undifferentiated hfNPC that were derived from human fetal forebrain subventricular zone homotopically into the ventral midbrain of MPTP-lesioned monkeys. In the same animals, we concomitantly delivered striatal, glial-derived neurotrophic factor (GDNF) by an AAV serotype5 vector. The fate of grafted cells was assessed after 11-months in vivo. Donor soma remained predominantly within the ventral mesencephalon in the area of injection and extended numerous, morphologically-relevant, monoamine fiber types including both smooth and beaded varicose profiles. While these neurofilament positive neurites appeared to project in multiple directions, they also coursed in trajectories, often circuitous, to adjacent disease relevant targets, such as the substantia nigra. Donor processes paralleled tyrosine hydroxylase positive fibers of the host nigrostriatal tract, but did not differentiate substantially into fully mature, striatally-integrated, A9 dopaminergic neurons. This work demonstrates that human fetal NPC are capable of generating neuronal phenotypes long-term, retain the capacity to direct axonal projections with trajectories comparable to the intrinsic nigrostriatal pathway, and respond to

specific local endogenous signaling cues in the adult dopamine-depleted primate brain; suggesting that the adult primate brain retains specific axonal guidance cues and maintains a permissive environment for xenotransplantation of hNPC and possible pathway construction.

4.2 Introduction

Human stem/precursor cells derived from multiple cellular origins[1] have been touted for their potential as regenerative substrate in a broad range of human disorders. Experimental evidence supports the functional benefit of cell based therapies for diseases of the blood, bone, pancreas, and central nervous system (CNS)[2-5], specifically neurodegenerative disorders such as Parkinson's disease (PD)[6-15] Degeneration of midbrain A9 DA neurons in the substantia nigra pars compacta (SNpc) and the resulting deficit in dopaminergic (DA) innervation in the striatum and other areas appear to be responsible for the majority of the characteristic motor and cognitive dysfunction of PD[16-18].

Significant motor improvements result from systemic administration of the dopamine precursor, L-Dopa, or dopamine agonists[19], supporting the rationale that dopamine release provided by transplanted replacement grafts might provide therapeutic effects. Supporting evidence from numerous studies in rodents[3,20-23] and non-human primates[24-31] have shown that grafts of fetal DA neurons can lead to biochemical and functional behavioral improvements[31-36]. However, in clinical studies, the improvements in parkinsonism have been rather modest and variable[37-49].

GDNF is essential for nervous system development and survival of ventral midbrain dopaminergic (DA) neurons in-vivo[50-52] and has been shown to act as a potent chemo-attractant in stem cell migration and neuritic maturation in the brain[53-56]. Exogenous delivery of GDNF by numerous modalities including, protein pump, cell secretion, and viral vectors[52,57-71] have yielded promising functional improvement in several PD models[72-85] at the

pre-clinical level, but present a more complicated picture in the clinic thus far[86].

To further aid in these attempts, we and others have shown that exogenous overexpression of AAV-driven GDNF in the host striatum can increase the survival of ectopically placed fetal VM grafts by several fold as well as elicit highly directional axonal outgrowth[87,88]. In addition, we demonstrated by fluorogold tract tracing, that VM grafts placed in the substantia nigra (SN) harbor the capacity to project TH-ir neuritic processes across substantial distances and innervate the host striatum when exposed to exogenous GDNF[89]. Furthermore, dopamine neuroblasts implanted into the SN of 6-Hydroxydopamine (6-OHDA) lesioned mice are capable of regenerating a new anatomically aligned, functionally relevant nigrostriatal pathway[90]. This target directed growth was further enhanced by striatal GDNF overexpression and supports the rationale for cellular transplantation and gene co-therapy as a dual modality therapeutic approach for nigrostriatal (NS) reconstruction in animal models of PD.

Until recently[89,90], attempts to establish new anatomically appropriate nigrostriatal axonal projections from fetal DA neurons placed within the VM had been met with relatively moderate success [91-95]. As a result, exogenous growth factors, such as GDNF, were utilized to help overcome what was suspected to be a relatively non-permissive growth environment[84,96,97]. In addition, studies utilizing immature neuroblasts or early post-mitotic neurons transplanted into other adult central nervous system locations (homotopic and heterotopic) *clearly demonstrate* the capacity to extend long distance, target specific axonal projections[21,23,26,98-119] While these results are extremely promising, it is still relatively unknown whether undifferentiated hNPC also retain the capacity to differentiate [120] and respond to relevant signaling cues in the adult dopamine depleted primate brain.

While clinical trials have demonstrated *promising* results[37-42,45,46,48,49,121-133], acquiring sufficient high-quality donor tissue remains a definitive obstacle for fetal VM cell

transplantation. To circumvent this issue, undifferentiated hNPC can be readily expanded in-vitro to provide sufficient cellular substrate for multiple patients from a single donor source. These cells retain an inherent developmental plasticity and respond to local excitatory and inhibitory axon guidance molecules[134], differentiating in relation to the changing microenvironment in-vivo.

Previously, we demonstrated that human fetal forebrain neural stem/progenitor cells (hfNPC) engraft, migrate, and promote functional improvements in behavioral deficits when transplanted into the MPTP-lesioned, African green monkey[11,135,136]. Graft analysis revealed few donor-derived DA cells (BrdU+/TH+), suggesting that the reduction in parkinsonian behaviors and subsequent normalization of endogenous TH-ir cell numbers/area were most likely a result of indirect, secondary support mechanisms. Specifically, we hypothesized the homeostatic effect to be a result of alternative mechanisms: release of neuroprotective growth factors, astrocytic differentiation, and recruitment of inflammatory cytokine signaling molecules.

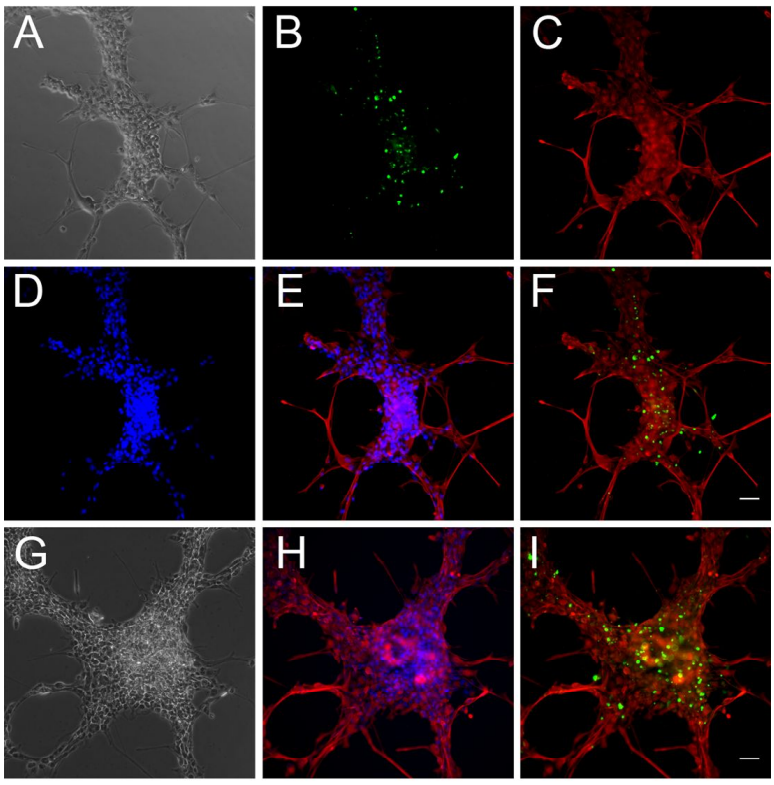
Given strong evidence for a permissive nigrostriatal growth environment to newly generated neuritic processes in rodents[90,101,102] and primates[89], it is feasible that hNPC might also respond to similar trophic stimuli in-vivo. Immature fetal VM DA neuroblasts and other post-mitotic VM cell types have been shown to respond to environmental stimuli[137,138] and develop into mature DA neurons in-vitro[139,140]and in vivo[105,106,141], however, it is still relatively unclear whether undifferentiated SVZ-derived hfNPC retain the intrinsic capacity to significantly differentiate into mature disease relevant A9-DA neurons and innervate appropriate target regions in the adult degenerative brain. Therefore, we sought to determine whether overexpression of AAV5-GDNF, delivered into the primate striatum could enhance graft integration and DA differentiation of donor hfNSC injected into the SN, as well as elicit directional neuritic outgrowth from the SN to the caudate nucleus. After eleven months,

healthy donor-derived cells with multiple morphologically relevant neuronal phenotypes successfully engrafted and extended axonal projections parallel to endogenous fiber tracts, indicating that hfNPC retain the capacity to respond to endogenous signaling molecules and differentiate in accordance with a permissive growth environment in the adult dopamine-depleted primate brain.

4.3 Results

4.3.1 Pre-Labeling hfNSC for Transplantation

To our knowledge, no single marker exists to adequately distinguish human from non-human primate cells in the CNS; therefore, donor hNPC require pre-labeling in-vitro to ensure accurate graft identification post-mortem. While harsh DNA-intercalating thymidine analogs like BrdU have traditionally been used to label proliferative donor hNSC, we found that exposure beyond 4-DIV was toxic and resulted in only ≈ 48-57% labeling efficiency when grown as suspension aggregates. In contrast, when hNPC were grown as multilayer adherent networks (MAN)[142], cell density appeared to overcome cellular toxicity up to 7-DIV, allowing for a larger percentage (≈ 83%) of cells to pass through the cell-cycle and acquire a BrdU label [Figure 4-1].



J

HfNPC In-Vitro BrdU Incorporation

	DIV	0	1	2	3	4	5	6	7	7, No-Ab
FACS	% BrdU-ir MAN	0.8	20.7	35.3	57.4	68.4	77.5	81.1	83.5	1.4
	% BrdU-ir NS	0.9	17.3	29.5	46.4	56.7	32.4	19.3	17.4	0.9
ICC-IF	% BrdU-ir MAN	0.5	19.5	33	59	62.5	73.5	83.5	82	2
	% BrdU-ir NS	1	18	27.5	49	48.5	34.5	22.5	20	2.5

Figure 4-1. HfNPC readily incorporate BrdU and are efficiently transduced with eGFP when cultured as multilayer adherent networks (MAN).
HFB-2050 hfNPC were cultured utilizing the MAN technique or as aggregate "neurospheres" in the presence of BrdU for up to seven days in-vitro (DIV) and analyzed by immunocytochemistry (ICC) with both FACS and immunofluorescence (IF) to determine labeling efficiency. MAN culturing conditions improved labeling efficiency and allowed for incubation without toxicity beyond 4DIV. HFB-2050 hfHNPC grown as MAN cultures were labeled for Nestin (red) and BrdU (green) at 4-DIV (A-F) and 7-DIV (G-I). Proliferative BrdU-ir cells were typically located within the center of the three-dimensional cellular clusters, opposed to the highly arborized webbed end-feet protruding from and connecting each larger cluster. [J], The efficiency of BrdU incorporation was determined for hfNPC grown as either neurospheres (NS) or multilayer adherent networks (MAN) for up to 7 days in-vitro (DIV) and analyzed by fluorescence microscopy (ICC-IF) or fluorescent activated cell sorting (FACS). Cells grown for 7-DIV in the presence of BrdU but not stained with fluorescent antibody are shown as a control. Data for 7-DIV without BrdU but stained with antibody were similar (not shown).

In previous studies, nigrostriatal tract tracing and donor-derived neuritic profiles could not be assessed due to histological limitations with BrdU and coronal sectioning. Generation of a stably-integrated fluorescent hNPC line eliminates the need for these toxic compounds[143], eliminates false-positive artifacts[144], while increasing both cell viability and graft detection efficiency. Therefore, in addition to the nuclear BrdU tracer, donor hfNPC were also pre-labeled with a stable, constitutively active, cytosolic, eGFP lentivirus [HFB-2050-eGFP] to capture donor cell morphology and accurately trace neuritic branching and cellular differentiation profiles post-mortem [Figure 4-2]. After two months of expansion, stable polyclonal populations were analyzed by flow cytometry (average,90.5% eGFP+ n=5) and fluorescence microscopy (average, 87.5% eGFP+, n=3) to ensure efficient stable transgene expression [Figure 4-2 A-D]. Upon addition of 10% fetal bovine serum for seven months, HFB-2050-eGFP became extremely adherent and elongated into a morphologically differentiated profile with cells resembling astrocytes and neurons similar to non-transduced counterparts [Figure 4-2 E-G].

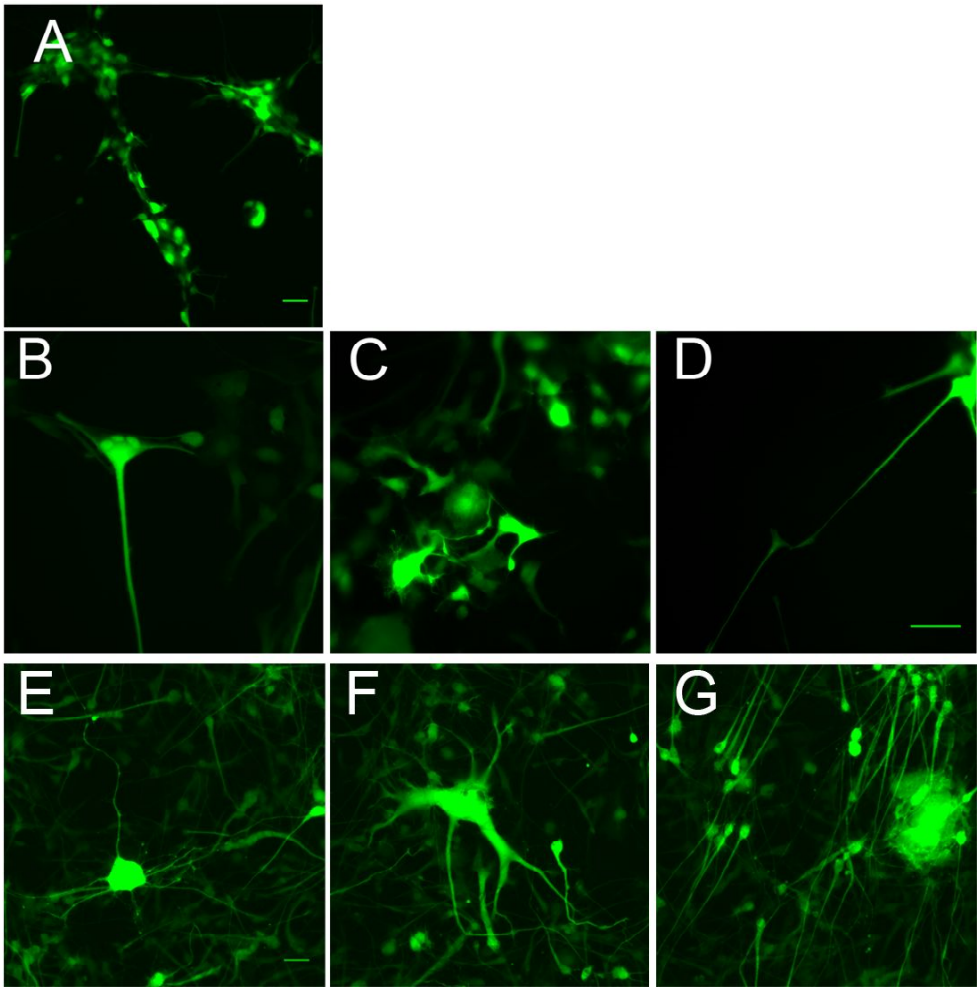


Figure 4-2. Undifferentiated hfNPC were stably transduced with a cytosolic-eGFP lentivirus and expanded for several months in-vitro. While often found in multi-cellular clusters [A], examination of individual migratory cells at low density typically revealed a bifurcated leading edge growth cone adorned with meandering micro-cilia [B-D]. Cells appear to constantly survey and sample the local microenvironment, protruding and retracting, often at great distances, until they come into contact with an established "colony," whereby they ascend and migrate toward the leading edge, eventually joining into aggregate units. In addition, HFB-2050-GFP assumed multiple differentiated cell morphologies after addition of 10%FBS for seven months (E-G) confirming that lentiviral transduction did not affect multipotency.

4.3.2 Multilayer Adherent Network hfNPC Culturing System

Adherent culturing conditions[142,145-152] have several advantageous characteristics compared to their suspension "neurosphere" counterparts and have gained attention for their utility in high-throughput applications like chemical screening[153]. In order to produce

sufficient cellular substrate for multiple transplants, we developed a novel culturing method for expanding hfNPC as multilayer adherent networks (MAN) [Diagram 4-1], with enhanced growth parameters compared to aggregated suspension spheres[142]. Utilizing these conditions, primary hfNPC, HFB-2050, were maintained for over 120 population doublings without significant chromosomal aberration or apparent senescence as previously reported utilizing suspension aggregate methods[154,155]. MAN cultures demonstrate an increased population doubling time as well as a greater percentage of actively dividing cells [Figure 4-1 J], while retaining a gene expression profile signature (mRNA and miRNA) similar to hNPC derived from several other central nervous system tissue sources. When compared to human embryonic stem cells (hESCs) and neural stem cells derived using alternative in-vitro manipulation techniques, HFB-2050 hfNPC mRNA and miRNA gene expression profiles clustered most closely within other human fetal CNS NPC preparations[156,157]. In fact, they were extremely similar to a primary human fetal telencephalon NPC line derived utilizing identical techniques, termed HFT-13[158], that were recently shown to be fully reprogrammable to induced pluripotent stem (iPS) cells utilizing only the transcription factor Oct-4[159].

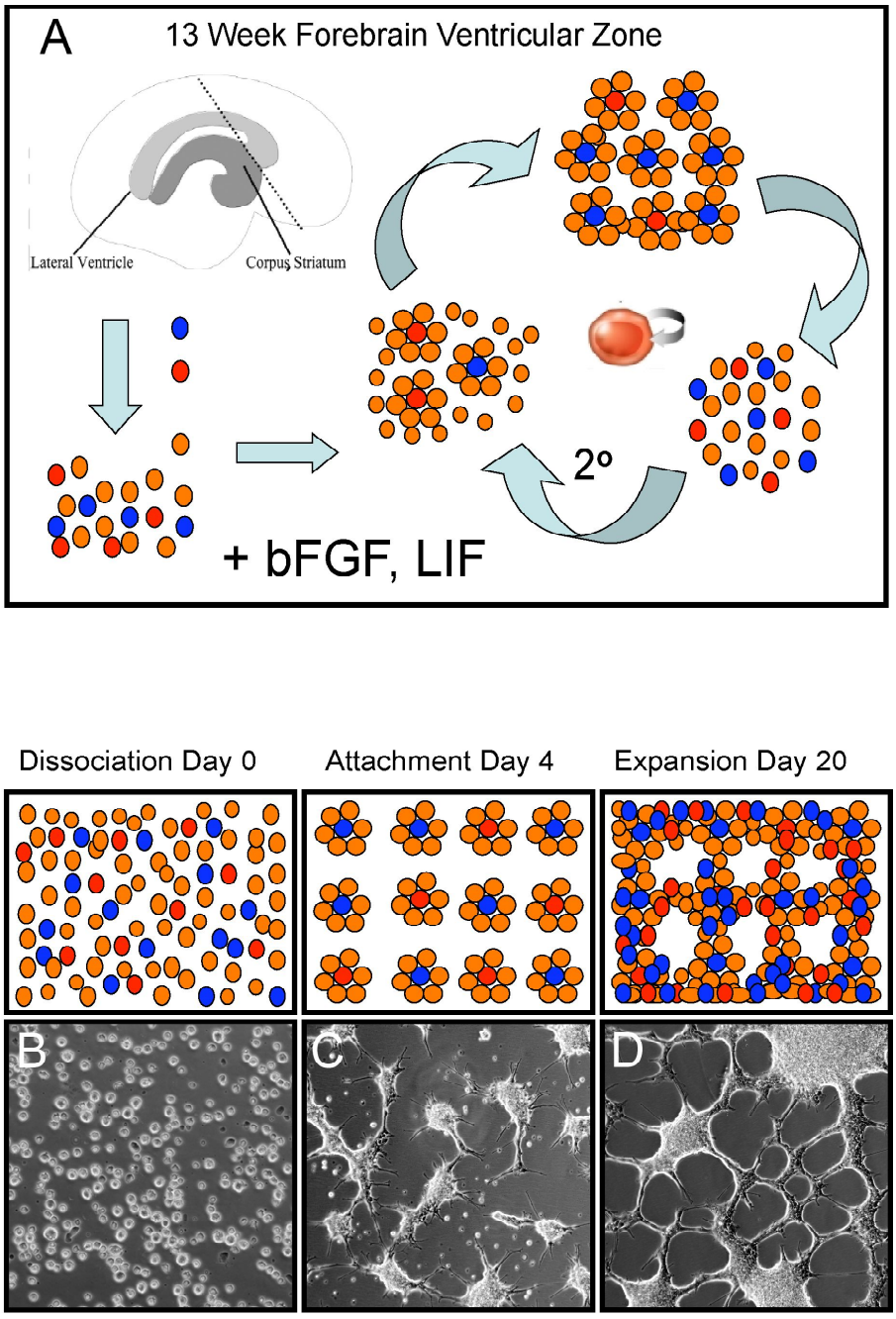


Diagram 4-1. Derivation and multilayer-adherent-network (MAN) expansion of hfNPC, HFB-2050. Human fetal forebrain periventricular-zone from a 13-wk cadaver was roughly dissected and dissociated in the presence of mitogens. After growth-factor selection for proliferative stem-like cells, primary cultures were further cultured in bFGF and LIF [A]. Expansion of hfNPC was enhanced utilizing the MAN culturing method [B-D]. Dissociated cells are re-plated at a greater density [B] compared to "neurosphere" suspension culture techniques and allowed to grow for 3-4 days without disruption inducing attachment of small aggregate clusters [C] that eventually proliferate and expand into densely organized adherent 3D-networks [D].

4.3.3 HfNPC HFB-2050 Gene Expression

QRT-PCR confirmed a neural stem/progenitor cell lineage identity for HFB-2050 when compared to undifferentiated WA-H9 hESCs. HFB-2050 had a marked increase in expression of the NSC associated SoxB transcription factors, sox1 and sox2[160,161], as well as similar levels of Myc and significantly less Klf4 compared to H9 hESC [Table 4-1]. These genes are essential in maintaining pluripotency in hESC as well, acting in a regulatory network with Oct-4, Nanog and several other factors[162-174]. In agreement, HFB-2050 displayed significantly lower mRNA levels for hESC regulatory genes Oct-3/4 (Pou5F1), Nanog, and Lin28, as well as similar expression of the stem cell associated intermediate filament Nestin, excluding the possibility that these cells were partially undifferentiated pluripotent progenitors [Table 4-1]. Furthermore, compared to hESC, HFB-2050 hfNPC expressed moderately elevated levels of Pax6, an essential regulator of NSC self-renewal, neurogenesis, and fate specification in vivo [175-178], as well as low levels of Ptx3, an important factor in DA maturation in the mouse brain. Pax6 and SoxB family members have been shown to regulate the transition from neuroepithelial precursors to radial glia in-vivo and in hESC derived cultures in-vitro[179-181], while Ptx3 appears to play a role in downstream signaling events during DA differentiation. Moreover, undifferentiated HFB-2050 hfNPC retained little to no gene expression for multiple pro-neurogenic genes, specifically those associated with signaling and maturation of the DA lineage including Wnt1, Lmx1a, Lmx1b, EN1, FoxA2, Nurr1 (NR4A2), and TH[182-191] [Table 4-1]. These combined expression data strongly support HFB-2050 lineage as a bona-fide fetal neuroectodermal hNPC population.

Table 4-1. Quantitative PCR profile of HFB-2050 hfNPC and hESC-WAH9 for known regulators of pluripotency and neural differentiation. Gene expression levels are consistent with a primitive neuroectodermal fate (SoxB family members, Nestin, pax6) and argue against a pluripotent ESC-like (Oct-4, Nanog) or neuronally restricted (Lmx1, EN1, TH, etc.) progenitor. NS = Not Significant; - denotes a significant decrease in expression; + denotes a significant increase in expression; NE = Not Expressed

GENE	WA-H9 Δ CT	hfNPC Δ CT	Δ
NANOG	4	11.5	-
POU5F1	0.4	8.2	-
LIN28	1.1	14.4	-
KLF4	9.4	13.4	-
MYC	7.6	6.3	NS
SOX1	10.6	5.8	+
SOX2	4.4	2.3	NS
NES	6.5	5.8	NS
PAX6	11.9	9.8	NS
WNT1	12	17.9	-
FOXA2	11.1	N/E	-
EN1	15.1	19	-
LMX1A	13.9	20.4	-
LMX1B	13.6	19.3	-
PTX3	17	13.1	+
NR4A2	12.4	13.1	NS
TH	15.4	14.4	NS

ΔCT= (CTgene of interest) –(CTGAPDH);
A lower ΔCT denotes higher gene expression

4.3.4 HfNSC HFB-2050 Express Neurogenic Proteins:

We next examined expression of NSC-niche related proteins in-vitro by ICC-IF to characterize their morphological distribution in hfNPC. Highly proliferative (Ki-67-ir) MAN cultures of HFB-2050 hfNPC [Figure 4-3 C] displayed robust cytoplasmic co-expression of the NSC-lineage related filamentous proteins Nestin [Figure 4-3 D, H] and GFAP [Figure 4-3 I] as well as transcription factors sox2 [Figure 4-3 K] and sox-3 [Figure 4-3 L] and membrane

bound Tie-2 [Figure 4-3 N]. Surprisingly, hfNPC also markedly expressed the "immature" neuronal markers, cytoplasmic filament beta-III-tubulin/Tuj1[192,193] [Figure 4-3 M] and extracellular polysialic-neural cell adhesion molecule (PSA-NCAM) [Figure 4-3 O] attributed to migratory type-A neural progenitors[194-202]. In addition, HFB-2050 hfNPC express cytoplasmic brain lipid binding protein (BLBP) [Figure 4-3 P], a marker for radial glia[203,204], the NE derived NSC of the fetal brain[205-213] suggesting HFB-2050 express developmentally appropriate forebrain hfNPC lineage markers[194,204,214-229]

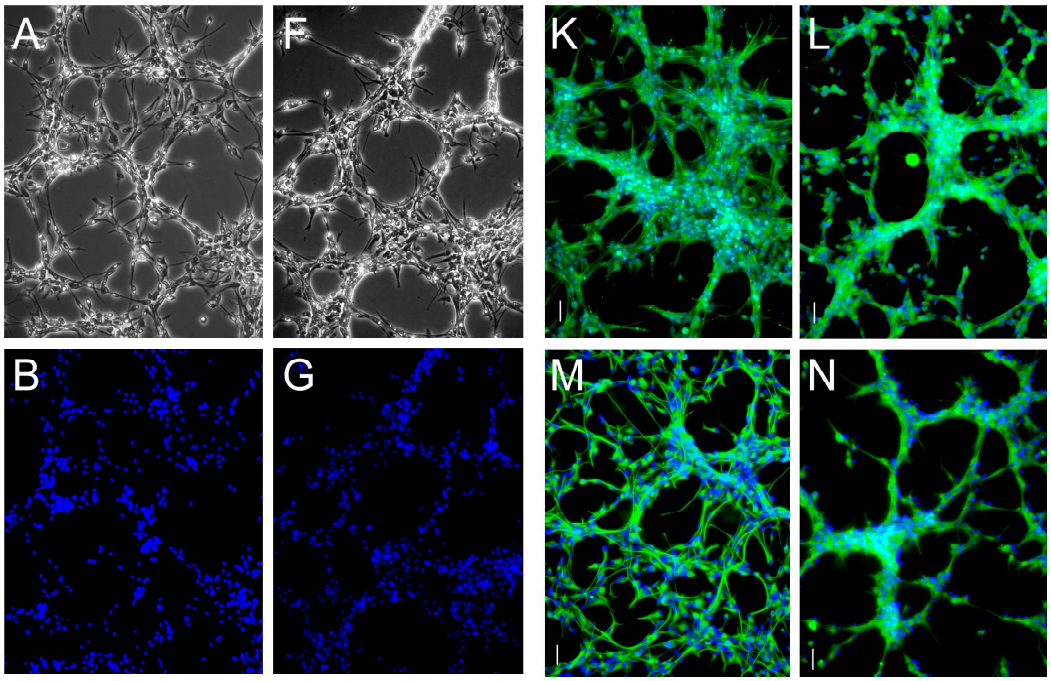


Figure 4-3. Human fetal NPC express proteins characteristic of stem/precursor cell in-vitro. Proliferative, Ki-67-ir HFB-2050 cultures [A-E] expanded and maintained as multilayer adherent networks (MAN) expressed the neural stem cell related filaments Nestin, [A-J] and GFAP [F-J], as well as the NSC transcriptional regulators sox2 [K] and sox3 [L] and membrane associated Tie-2 [N]. Of interest, other neurogenesis related proteins such as Tuj-1 [M] and PSA-NCAM [O] (markers for immature neuroblasts in-vivo), along with BLBP [P] (marker for radial glia) were also highly expressed in-vitro. (A,F = phase contrast, B, G = DAPI, G = Ki-67, H, D = Nestin, I = GFAP, E, J = Merged images. K-P are merged with DAPI. Western blotting confirmed expression of neurogenic proteins in undifferentiated hfNPC [Q]. When compared to "neurosphere" (NS) suspension cultures, MAN cultures express increased levels of high molecular weight Nestin, multiple isoforms of GFAP, and similar levels of BLBP and Beta-3-tubulin/Tuj1, while NS cultures had a marked increase in DCX protein levels. N2A neuroblastoma cells were used as a +/- controls where applicable. Lanes labeled 1-10, Running order as follows. GFAP, ladder = 1, 10; 2,5,8 = MAN, 3,6,9 = NS; 4,7 = N2A neuroblastoma negative control. TUBB3/TuJ1/Beta-3-Tubuln: ladder = 1,8; MAN = 2,5,9; NS = 3,6,10; N2A = 3,6 as a positive control. Lanes 9, 10 probed with antibody from Chemicon vs. Covance in lanes 1-7 demonstrating specificity to only the Covance antibody. DCX: ladder = 6,10; Lanes 1,2 = BSA – loading control, lanes 3-5 stained without primary DCX antibody as a control for background from secondary antibody alone. Ladder = 6, 10; MAN = 4, 8; NS =5,9 N2A = 3,7 as a negative control. Nestin and BLBP: Ladder = lane 1; NS = lanes 2-4; MAN = 5-7; N2A = 8-10. Nestin and BLBP lanes represent serial 1/10 dilutions (100X, 10X, 1X)

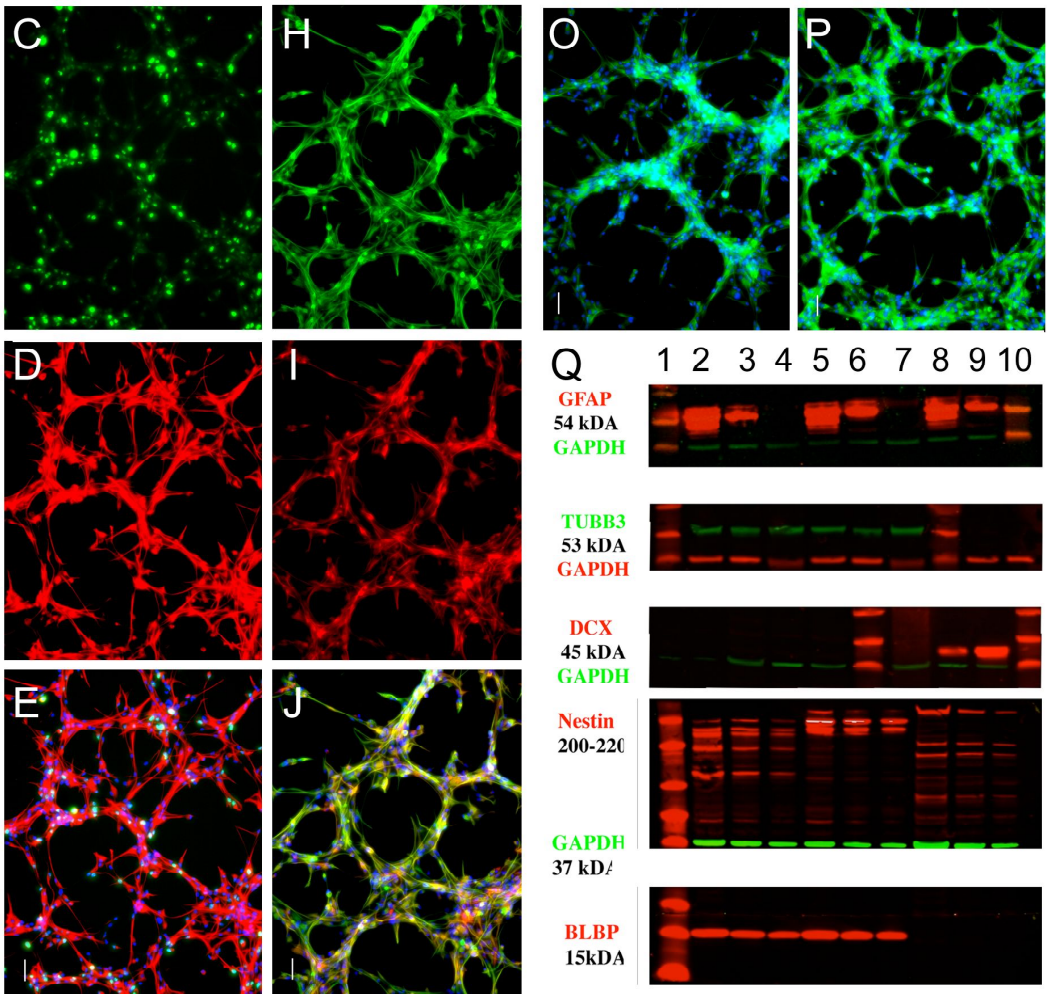


Figure 4-3. Continued

Biochemical analysis of hfNPC grown as either MAN or suspension "neurospheres" confirmed the expression of NSC niche associated proteins. Western blotting revealed elevated expression of high molecular weight, human-specific Nestin as well as multiple isoforms of GFAP[224], when grown under MAN conditions [Figure 4-3 Q]. In addition, BLBP and Tuj1/beta-3-tubulin were expressed at similar levels, while the migratory neuroblast associated protein doublecortin (DCX)[230-232] was elevated under suspension "neurosphere" conditions [Figure 4-3 Q]. The combined results suggest that hfNPC exist as proliferative,

highly plastic stem/precursor cells, "primed" for neurogenesis. In addition, biochemical analysis indicates that in-vitro culturing conditions dramatically alter NSC lineage related protein expression, and neurosphere growth parameters may actually artificially select for slightly differentiated neuroblast like neural precursor cells (NPC). These observations may help explain previous reports of early senescence and loss of neurogenic potential in hfNPC (<60 passages) utilizing suspension aggregate culturing methods[154,155,233].

4.3.5 HFB-2050 hfNSC Recapitulate the Subventricular Zone Niche

To further investigate the developmental plasticity of hfNPC, HFB-2050-WT cells were cultured in an extracellular matrix (ECM) protein rich environment. Matrigel contains laminin, collagen-IV, heparin sulfate proteoglycans, and entactin, as well as trace amounts of platelet-derived growth factor (PDGF), nerve growth factor (NGF), insulin-like growth factor-1 (IGF-1), and TGF-B; proteins with functional roles in maintenance of the NSC niche, as well as differentiation and migration during normal CNS development[145,234-244]. We therefore sought to emulate the early neurogenic microenvironment, utilizing Matrigel to model early neurogenesis and induce differentiation in-vitro.

HfNPC were cultured on Matrigel to induce intermediate cell phenotypes and analyzed by ICC-IF for lineage specific NSC niche proteins. Immunostaining revealed a phenotypically *heterogeneous* population of precursor cells comprised of at least two specific replicating sub-populations of cells; supporting radial glial like ($BLBP^{high}$/DCX-) and migratory ($BLBP^{low}$/DCX+) precursor cells [Figure 4-4]. $BLBP^{high}$/DCX- cells phenotypically resemble type-B astrocyte like NSCs of the adult subventricular zone (SVZ) niche[194,207,208,214-217,219]. Many $BLBP^{low}$/DCX+ cells displayed distinct β-3-tubulin+(Tuj-1) leading and lagging processes [Figure 4-5], highly reminiscent of the in-vivo neuroblast found in the murine rostral migratory stream. These cells appear to migrate along the $BLBP^{high}$/DCX- radial glial

scaffolding forming a multifaceted, in-vitro *microniche* resembling the SVZ neurogenic niche, in-vivo [207,216,245]. Overall, these data suggest that HFB-2050 populations retain characteristic properties of multipotent forebrain hfNSC, in-vitro[246]. Specifically, when introduced to ECM instructive cues, they readily differentiate into early precursor phenotypes and maintain critical components reminiscent of the ventricular zone neurogenic niche.

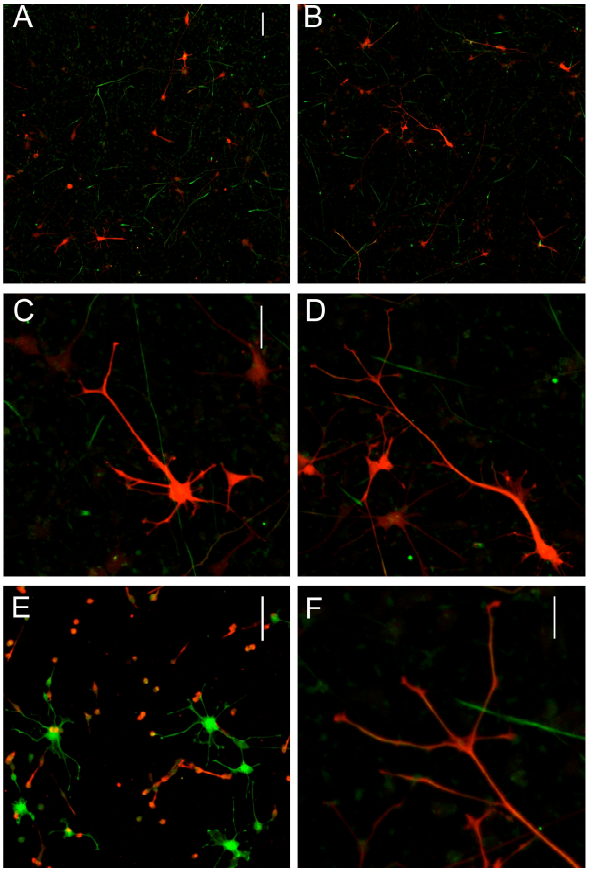


Figure 4-4. Modeling early subventricular-zone (SVZ) neurogenesis in-vitro.
HFB-2050 hfNPC were cultured on Matrigel to induce developmental programs involved in early neurogenesis. Stem/precursor cell phenotype were analyzed by immunocytochemistry revealing a relatively heterogeneous population comprised of two morphologically distinct phenotypes. Nearly all cells expressed some degree of Nestin, Vimentin, and GFAP (not shown); however, there were marked differences in structural characteristics for cells that highly expressed either BLBP (green) or DCX (red) independently [E]. Morphologically, $BLBP^{high}$/DCX- cells were characterized by a large stellate cell body and nuclei [A-D, red = BLBP, green = NFM], as well as extensive outgrowth of multipolar arborizations [C, D], often with one or two elongated extensions protruding up to several mm in length, resembling radial glia or astrocyte-like type-B cells in-vivo [D, F]. The branched processes appear to function as neural scaffolding or "highways" upon which the $BLBP^{low}$/DCX+ sub-population migrates [E]. In contrast, $BLBP^{low}$/DCX+ cells [E] are distinguished by their highly migratory behavior, smaller cell body and nuclei, bipolar morphology, and leading edge protrusion, resembling both type-C transit amplifying cells and type-A neuroblasts. After 3DIV [E], cells are still beginning to develop in accordance with ECM cues (red= DCX, green = BLBP). By 7DIV [A-D, F], cultures mature significantly revealing extensive arborization and outgrowth from BLBP-ir (red) radial-glia like cells as well as the emergence of neuronal-related NFM-ir (green) fibers.

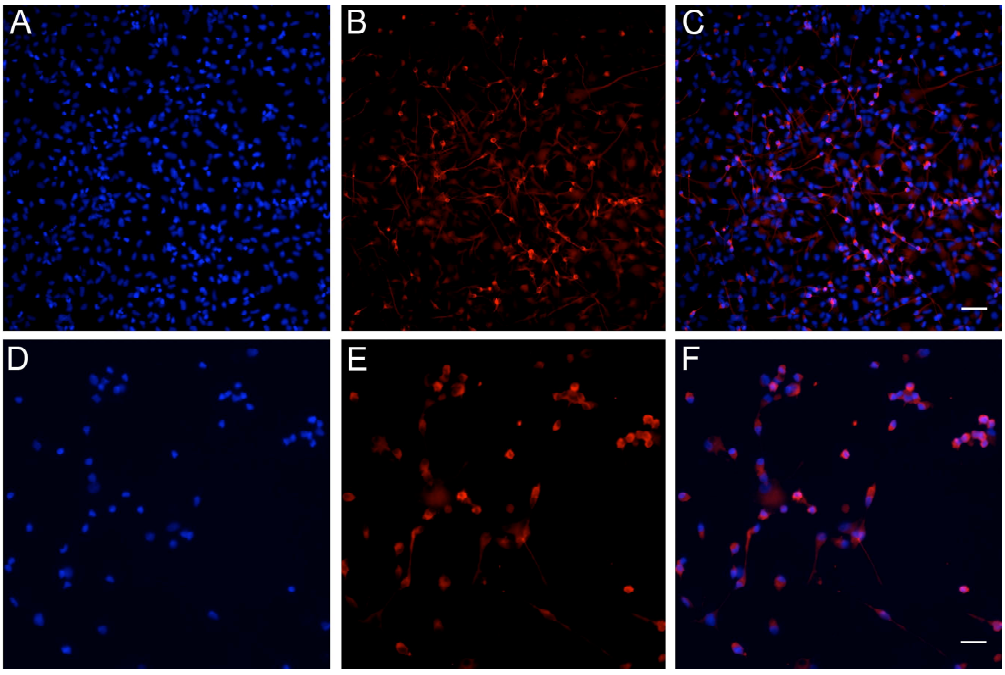


Figure 4-5. Extracellular matrix induction of immature-neuron associated Beta-3-tubulin/Tuj-1. Undifferentiated cells cultured on Matrigel extracellular matrix proteins expressed Tuj1 in immature bipolar, migratory cells after 3-DIV [D-F]. Development of more mature Tuj1-ir neuronal morphologies were seen after 7-DIV [A-C] indicating HFB-2050 hfNPC retain developmentally appropriate programs relevant to early neurogenesis in-vitro.

4.3.6 HfNSC HFB-2050 Are Multipotent In-Vitro and In-Vivo

Previously, we demonstrated that undifferentiated HFB-2050 hfNPC retain the capacity to express proteins associated with the VM lineage in-vitro, expressing Nurr1, Ptx3, and TH[11], when cultured in the presence of glial conditioned media and growth factors[247-249]. In addition, we have shown that these cells can be selectively directed into oligodendrocytes in-vitro[250]. Furthermore, upon transplantation into the lateral ventricles of P0 neonatal mice, HFB-2050-eGFP hfNPC participate in normal CNS development, including migration from germinal zones through the RMS to olfactory bulbs, into the corpus callosum, and along vascular networks throughout the brain[142]. These data suggest HFB-2050 hfNPC retain a responsiveness to regional and temporal developmental cues to become multiple cell types in these regions as well as integrating long-term and non-disruptively within the host SVZ niche.

Collectively, our data support HFB-2050 hfNPC as long-term self-renewing, undifferentiated, multipotent (in-vitro and in-vivo) progenitors suitable for in-vivo transplantation.

4.3.7 Nigral HfNPC Engraft Long-Term Concomitantly with Striatal AAV5-GDNF:

To determine whether undifferentiated hfNPC grafts retain the capacity to differentiate and respond to GDNF chemotaxis in-vivo, ten MPTP-treated adult male St. Kitts green monkeys received injections of AAV-5 GDNF unilaterally into both the rostral caudate and post-commissural putamen, followed by a hfNPC graft unilaterally into the ipsilateral hemisphere, immediately rostral to the SN [Diagram 4-2; Supplementary Table 4-1]. Animals were sacrificed at 1.5 months or 11 months and analyzed for GDNF expression and hfNPC-derived graft distribution. Parasagittal sections were utilized in order to capture the complex neuronal circuitry of the entire NS unit.

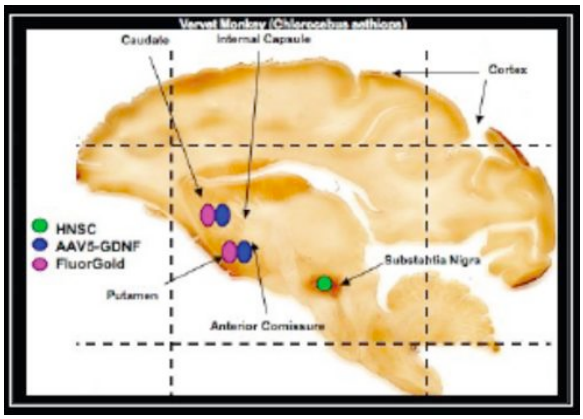


Diagram 4-2. Schematic representation of injection targets in vervet brain. HfNSC (green oval) were homotopically transplanted into the rostral SN, and AAV5-GDNF (blue oval) was administered into both the caudate and putamen. After 11-months, Fluorogold (Purple oval) was injected into the identical striatal coordinates previously used for AAV5-GDNF. The dotted lines demarcates where the tissue was cut for histological analysis.

4.3.7 AAV5-GDNF Distribution

Extensive long-term overexpression of AAV-5-GDNF at 1.5 months [Supplementary Figure 1] and 11 months [Figure 4-6] was confirmed unilaterally throughout the injected striatum, emanating and diffusing outward from the injection sites throughout both the caudate and putamen. GDNF-ir cells were distributed from the most dorsal-rostral regions of the far-lateral extent of the striatum extending ventrally throughout the caudate and into the most caudal regions of the putamen [Figure 4-5 A,B,C]. Transduced host striatal cells highly expressing GDNF had the characteristic morphology and molecular profile of predominantly medium spiny neurons and to a lesser extent, stellate astrocytes [Figure 4-6 G,H]. Immunostaining for GDNF, NeuN and GFAP revealed the majority of transduced cells had a NeuN-ir neuronal phenotype and morphology consistent with medial spiny neurons throughout most of the striatum and a small percentage of astrocytes in the most dorsal-rostral regions of the far-lateral extent of the caudate (data not shown).

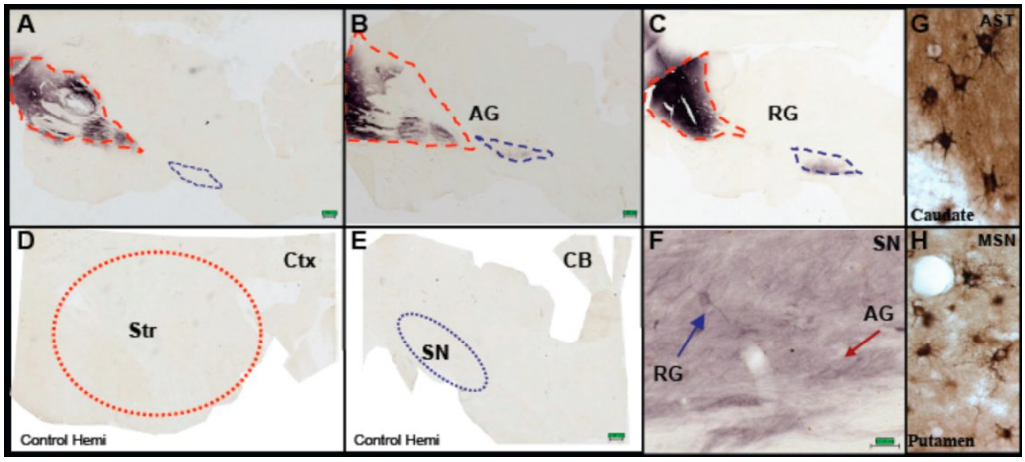


Figure 4-6. Exogenous AAV5-GDNF expression after 11-months. Extensive diffusion and expression of GDNF (black staining) from AAV-serotype-5 was verified throughout the striatum (striatum = red dotted lines) [A, B], emanating from the injection sites [C] into adjacent tissue. Exogenous GDNF was not found in contralateral hemispheres [D, E]; however GDNF-ir cells bodies and fibers were also seen within the ipsilateral SN (SN = blue dashed lines), suggesting retrograde (RG, blue arrow) and anterograde transport (AG, red arrow) [F], respectively, from striatal injection sites. [G, H] Most GDNF-transduced cells (brown staining) had the characteristic morphology of striatal medium spiny neurons throughout the striatum as well as astrocytes in some regions of the caudate.

GDNF-ir was also abundant within the ipsilateral SN of the injected hemisphere [Figure 4-6 F] providing evidence for transport of exogenous GDNF from the striatum to the SN. Specifically nigral neuronal soma with DAergic morphology and processes located within the nigra were densely immunoreactive, suggesting either retrograde transport from nigrostriatal terminals or anterograde transport downstream along the striatonigral pathway to relevant targets including the SN. In addition, exogenous AAV-GDNF was never seen in the contralateral hemisphere within the striatum or SN [Figure 4-6 D,E], indicating localized delivery. Furthermore, no adverse behaviors or changes in feeding or body mass previously reported using AAV2 serotypes[58] were observed at any point throughout the experiments.

4.3.9 Endogenous TH-IR within the Nigrostriatum:

Analysis of TH-ir cell bodies located within the SN and their axonal projections to the striatum revealed a characteristic reduction in the number of endogenous TH-ir cell bodies (asymptomatic monkey) compared to control non-MPTP injected animals (data not shown). Interestingly, by 11 months, host TH-ir cells appeared to expand and fill the subsequently degenerated interstitial spaces with extensively arborized neuritic processes, indicative of endogenous regeneration. In addition, a moderate level of intact host TH-ir nigrostriatal fibers[251] were spared or rescued from degeneration. Host outgrowth was likely a result of predominantly GDNF neurotrophic support within the SN, as well, to a lesser extent, hfNPC graft derived factors in concert with local signaling cues.

4.3.10 HfNPC Graft Distribution and Morphology:

Robust donor-derived grafts were typically located immediately dorsal and adjacent to the most dorsal-rostral region of the host SN, with some variability toward more dorsal-caudal extents of the SN in one animal. After 1.5 months, donor cells generally remained within the

target site, and did not migrate to the hemisphere contralateral from the injection site in any animals [Figure 4-7 A-C]. Grafted BrdU-ir cells were typically clustered within and immediately adjacent to TH-ir dense regions of the SN, located in an adjacent plane to endogenous TH-ir cell bodies [Figure 4-7 D-I, Supplementary Video 1] and directly juxtaposed to some endogenous TH-ir neurite tracts. After 1.5 months, donor cells retained a relatively undifferentiated phenotype, typically with little to no arborization.

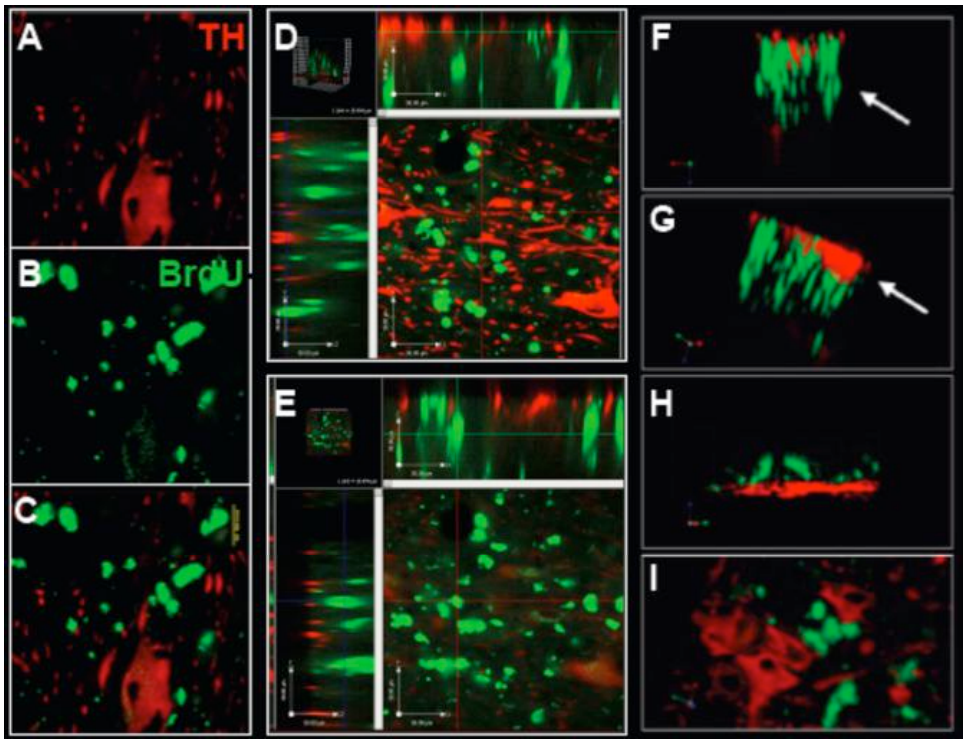


Figure 4-7. Robust engraftment of hfNPC adjacent to host neurons after 1.5 months. BrdU-ir donor cells (green) [A-I] were located at the more dorsal and rostral regions of the SN and typically were found to engraft near host TH-ir cells (red). No TH-ir/BrdU-ir cells were found at 1.5 months [A-C]; however several BrdU-ir nuclei had morphological features consistent with mitotic-figures (top left in panel B) but were not Ki-67 or PCNA-ir. [D-I], Confocal z-stack reconstruction. Different z-planes 30um apart at the identical (x, y) plane clearly indicated that BrdU-ir cells had integrated immediately adjacent to, but typically not within, the endogenous layer of TH-ir (red) DAergic cells [D, E]. 3D-reconstruction of z-plane images further demonstrate that donor cells occupy space near but not generally in the same plane as endogenous TH-ir cell bodies [F-I].

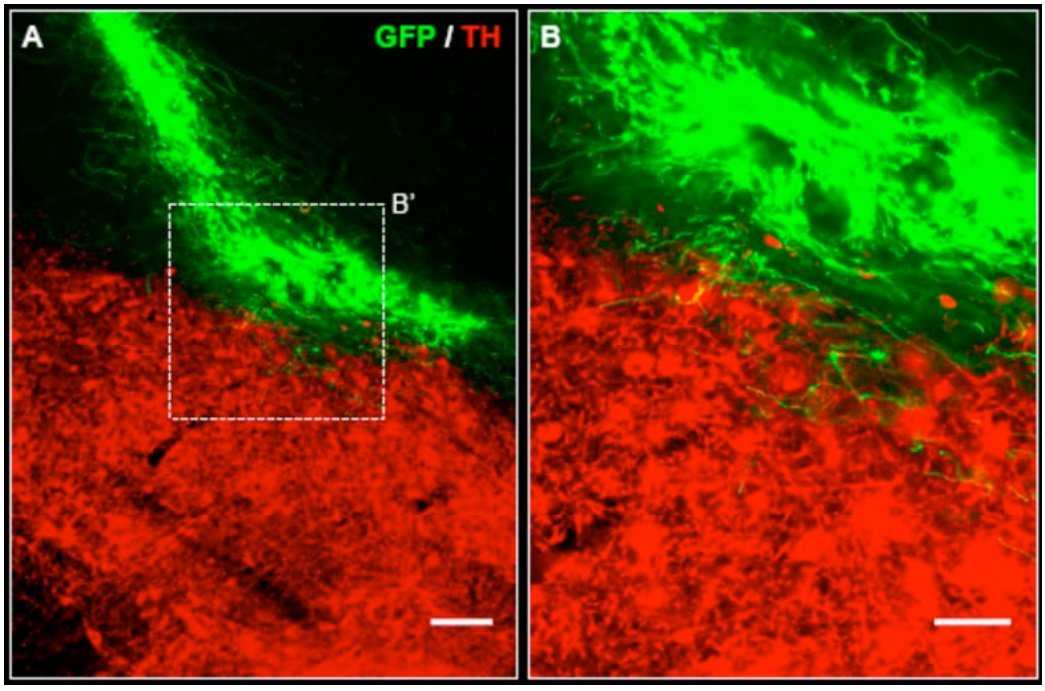


Figure 4-8. Long-term engraftment of GFP-ir donor cells. Fluorescence immunohistochemistry confirmed significant GFP-ir graft survival (green) at the dorsal extents of the SN, as indicated by TH-ir dopamine cells (red) [A, B (inset of dashed box in A]. Another GFP-ir graft was located in an equivalent location, while two other grafts were located at the most dorso-rostral extent of the SN. In all four cases, donor-derived fibers projected within the host SN.

Analysis of hfNPC grafts at 11-months post-transplantation confirmed long-term survival of dense GFP-ir grafts at the dorsal region of the SN [Figure 4-8 A, B], bearing extensive neuritic processes in 4 out of 5 animals [Figure 4-9 A-C]. Hemispheres were analyzed and scored arbitrarily from (0-5) for overall engraftment success [Supplementary Table 4-2] and GFP-ir serial sections were analyzed volumetrically to determine overall graft density [Supplementary Table 4-2]. The one animal that did not show markers for graft survival and the two animals transplanted with dead cells (control) displayed no aberrant host pathology in the SN or apparent long-term injury from the transplantation procedure. In addition, GFP-ir grafts retracted into the needle tract in three out of four animals [Figure 4-9

A, C; Figure 4-10 A], where many dorsally oriented GFP-ir cells displayed a highly branched stellate morphology, but lacked GFAP-ir. Interestingly, the largest grafts, such as the ones in [Figure 4-9 A, C, Figure 4-10 A], bear a striking morphological resemblance to grafts reported previously utilizing human dopaminergic mesencephalic neuroblast implanted into the rostral mesencephalon or internal capsule[106].

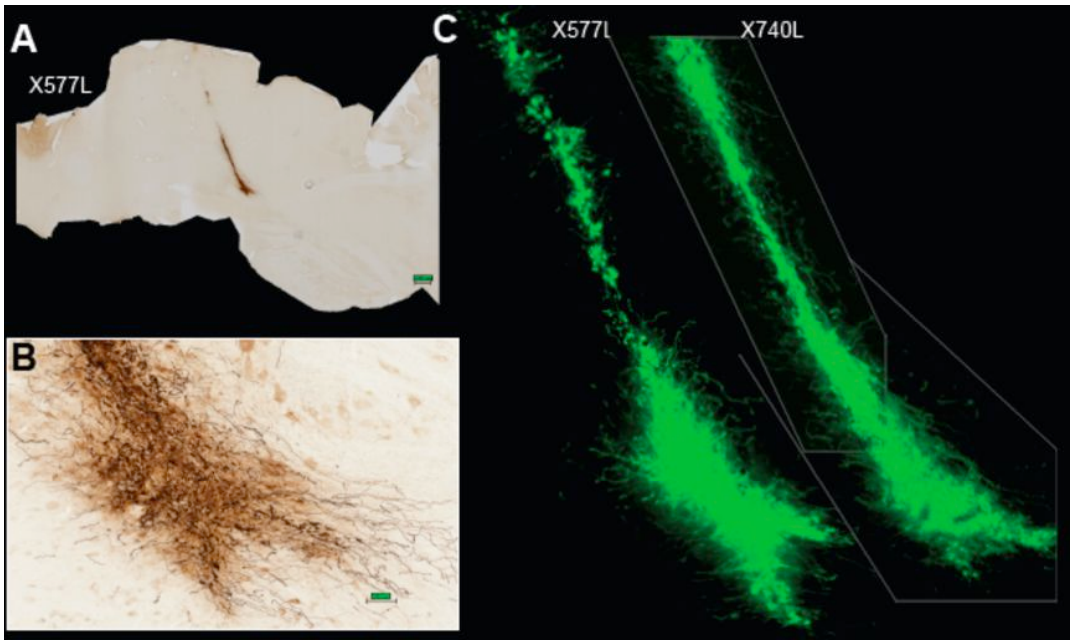


Figure 4-9. Long-term engraftment of donor cells and extensive neuritic profile 11-months post-transplantation. After 11-months, robust GFP-ir donor-derived grafts [A-C] were found to project extensive fibritic output into peripheral tissue [B], including SN. Cells were also found filling the injection cavity created from needle insertion-extraction in 3 of 4 animal successfully transplanted with GFP-ir [A, C]. The most robust grafts analyzed at 11-months are remarkably comparable to grafts reported previously utilizing human DA-neuroblasts implanted into the NS, however lack extensively long (> 3mm) neuritic projections growing toward striatal target regions. Migration of cell bodies from the graft into peripheral regions was never seen [C], including to the contralateral hemisphere.

4.3.11 Characterization of Neuritic Outgrowth

Morphologically, donor GFP-ir cells generally bore a soma located within the core of the graft site [Figure 4-10 B, E] and one or more processes extending up to greater than 1mm out into host tissue from the periphery of the graft zone [Figure 4-10 C, D]. Numerous neurites

were found coursing in multiple trajectories, with no definitive directional preference of growth; however, GFP-ir cells located at the dorsal-rostral region of the graft core [Figure 4-10 D] displayed a more rostral pattern of growth up through the zona inserta ascending past the subthalamic nuclei toward the internal capsule and fields of Forel, while more caudal cellular protrusions generally remained within the graft or projected ventrally into the SN and further toward the cerebral peduncle and medial lemniscus of the lower brainstem. Interestingly, many GFP-ir fibers coursed immediately adjacent and/or through endogenous myelinated white matter tracts.

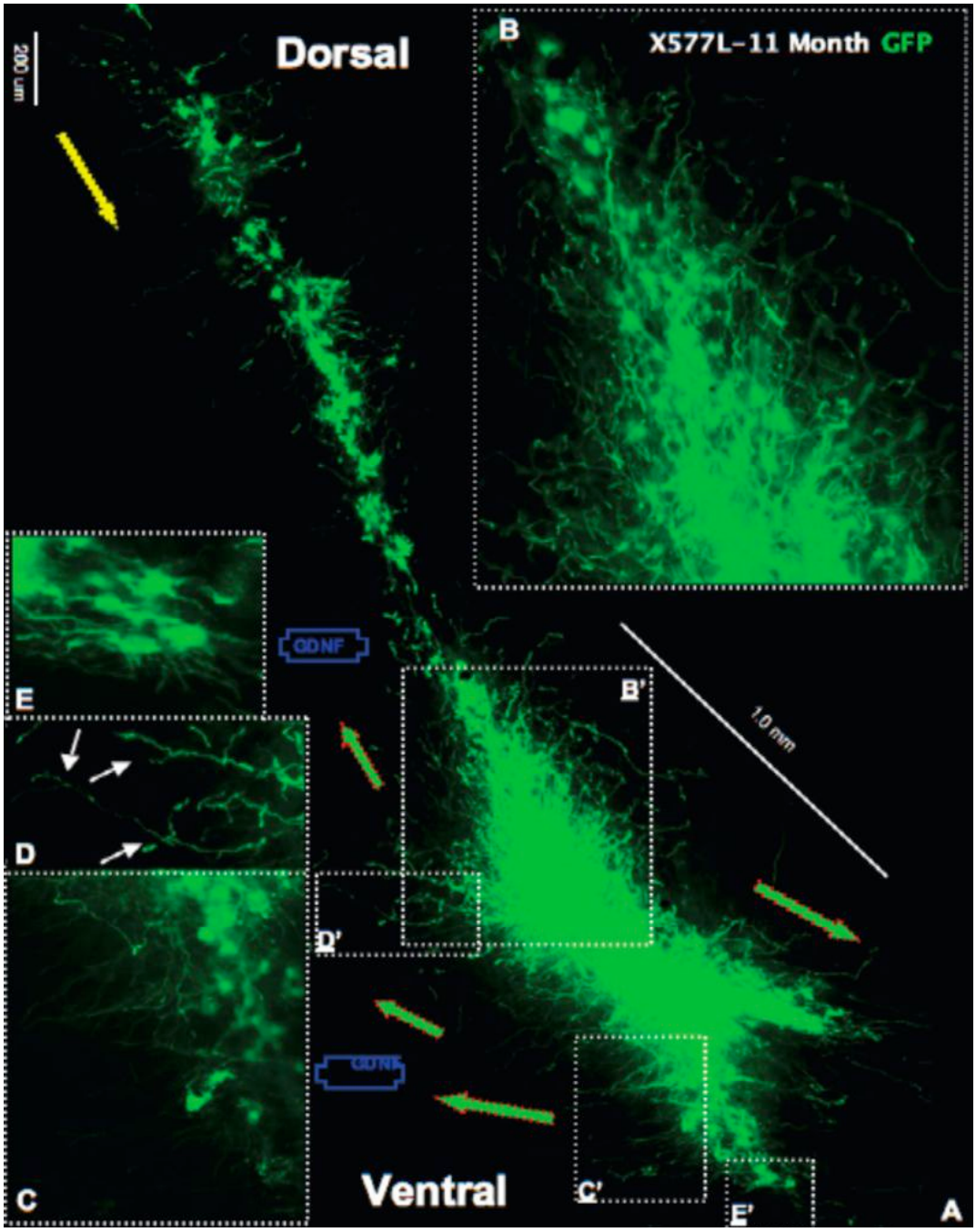


Figure 4-10. HfNPC develop an elaborate neuronal profile after 11-months in-vivo. A typical GFP-ir section (same section from Fig. 4-3C) from within the core of a hfNPC-derived graft after 11-months demonstrating a variety of morphological characteristics within the graft, including concentration of nuclei within the immediate graft zone [B, E], as well as extensive neuritic branching resembling developing axons [C, D] into peripheral tissue.

Analysis of GFP-ir neurites revealed patterned growth along a trajectory congruent with host fiber tracts [Figure 4-11]. Long protruding GFP-ir neurites coursed the SN and the needle tract, extending rostrally with a striatal trajectory in parallel with host-TH-ir fibers of the medial forebrain bundle (MFB) and NS pathway [Figure 4-11 G, H]. GFP-ir processes located at the dorsal-rostral region of the graft, as well as those located where the needle tract overlapped with the NS tract, often terminated in bifurcate forked end-feet, morphologically resembling growth-cones [Figure 4-11 H]. Throughout the graft, intricate rostro-caudal branching of thin GFP-ir fibers could be seen at the ends of thick axons, with highly ramified terminals located at both short and long distances from the transplantation zone.

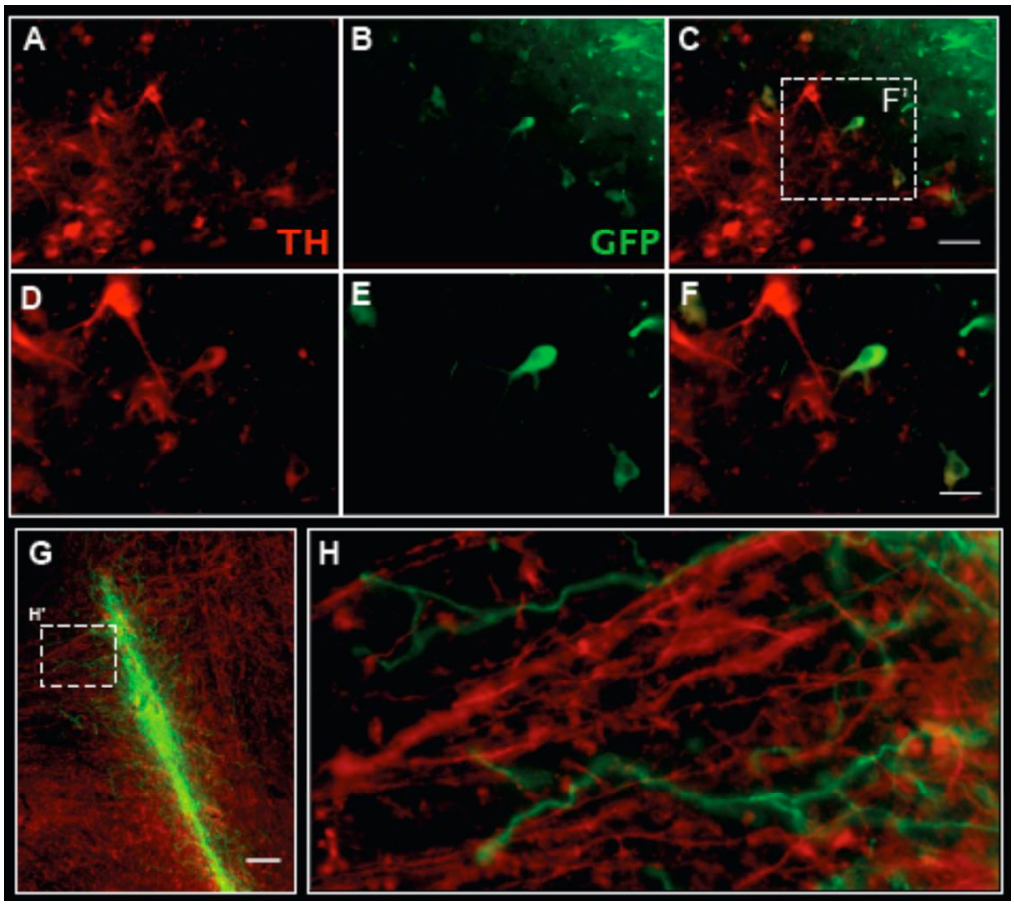


Figure 4-11. HfNPC graft development is influenced by host-environment. Donor derived GFP-ir cells (green) co-express TH (red) [A-F] and project growth cone-like processes parallel to spared endogenous TH-ir nigrostriatal circuitry G, H]. Some GFP-ir soma were also TH-ir but typically lacked significant arborization in areas where grafted cells overlapped with spared endogenous TH-ir dopamine cells in the SN [C, F]. Regions where grafted cells overlapped with host fiber tracts displayed a clear preference of growth congruently alongside these pre-existing networks [G, H], arguing for a permissive, instructive environment with the adult primate brain.

In addition, some GFP-ir/pan-NF-ir fibers projected rostrally into host tissue then coursed back caudally towards the direction of the SN, resembling a circuitous loop [Figure 4-12 A-F]. It is possible that these projections are responding to local signaling molecules and coursing back towards a nigral target trajectory, in the same manner as young striatonigral neurons would correctly home to their target and innervate the developing brain in-vivo. Directional outgrowth, therefore, appeared to also be significantly altered by a permissive

local microenvironment and relevant endogenous excitatory and inhibitory guidance molecules in addition to direct chemotropic guidance from AAV5-GDNF.

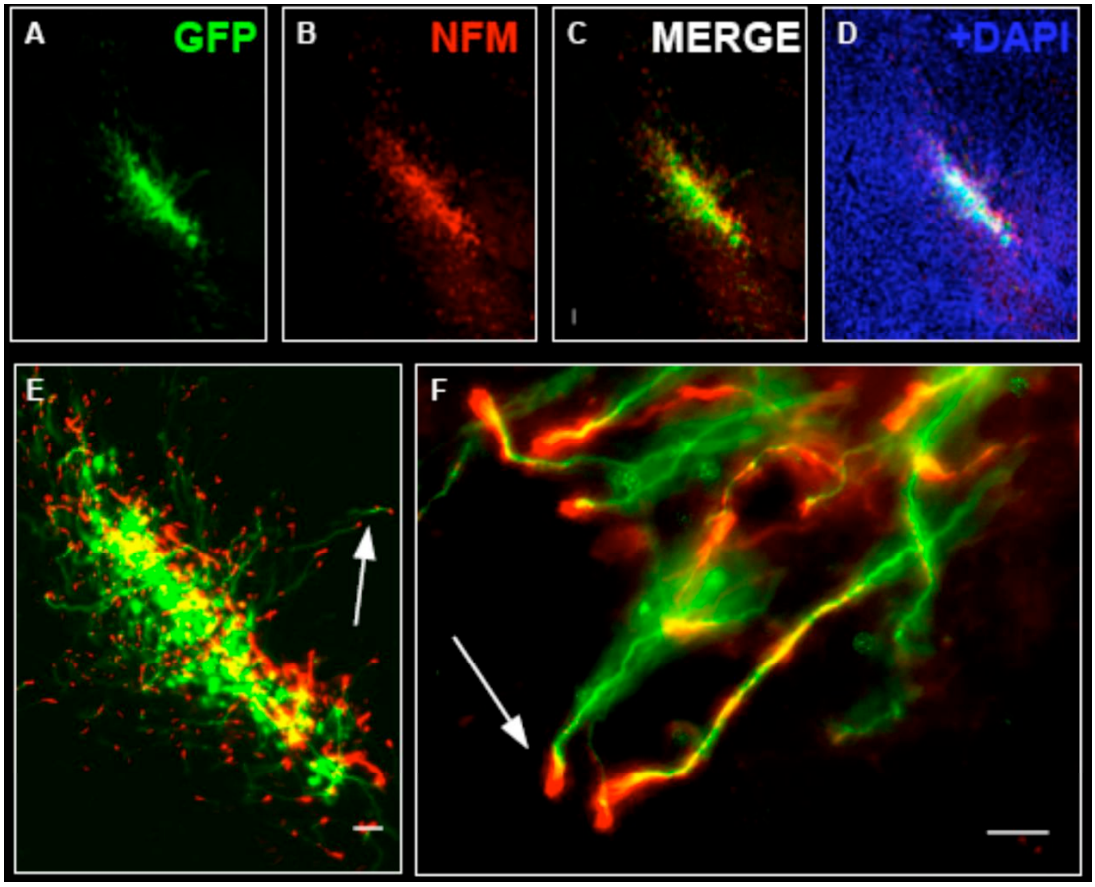


Figure 4-12. Neuritic processes pursue multiple trajectories along host circuitry. In some instances, GFP-ir/pan-NF-ir fibers (green/red) were found exiting rostrally into host tissue and coursing back caudally towards the SN, resembling a circuitous loop [Figure 4-6 A-F]. NF-ir was most abundant at the distal tips of GFP-ir projections (arrows) and became spotty towards the soma. Interestingly, NF staining revealed that the direction of GFP-ir fibers often favored that of host fiber-tracts and suggests local signaling molecules may override GDNF chemotaxis. DAPI staining [D] demonstrates no morphological aberrations to host cytoarchitectural arrangements were induced by hfNPC grafts.

4.3.12 Graft Lineage Specification:

The lineage fate of grafted hfNPC was analyzed by IHC-IF for neuronal and glial lineage-related markers. After 11-months, 12-17% of grafted GFP-ir neurites co-localized with the undifferentiated lineage filament Nestin [Figure 4-13 E-H], with a lesser proportion

(3-9%) also expressing Vimentin (data not shown). Interestingly, 67-87% of GFP-ir cells co-localized with pan-neurofilament protein (NFM) [Figure 4-12 A-F] however they did not express significant levels of the mature neuronal protein, MAP-2, (~1% of GFP-ir/NFM-ir cells). In addition, few (≅ .1-1.0%) donor-derived GFP-ir cells co-expressed the DA marker, tyrosine hydroxylase (TH) [Figure 4-11 A-F]. Some GFP-ir/TH-ir cells developed larger soma, but without marked arborization of processes, suggesting that these cells were of an immature DA phenotype. Interestingly, grafted cells no longer expressed GFAP or Tuj1/beta-3-tubulin, indicating downregulation of these proteins post-transplantation, even in astrocyte rich regions of the brain [Figure 4-13 A-C].

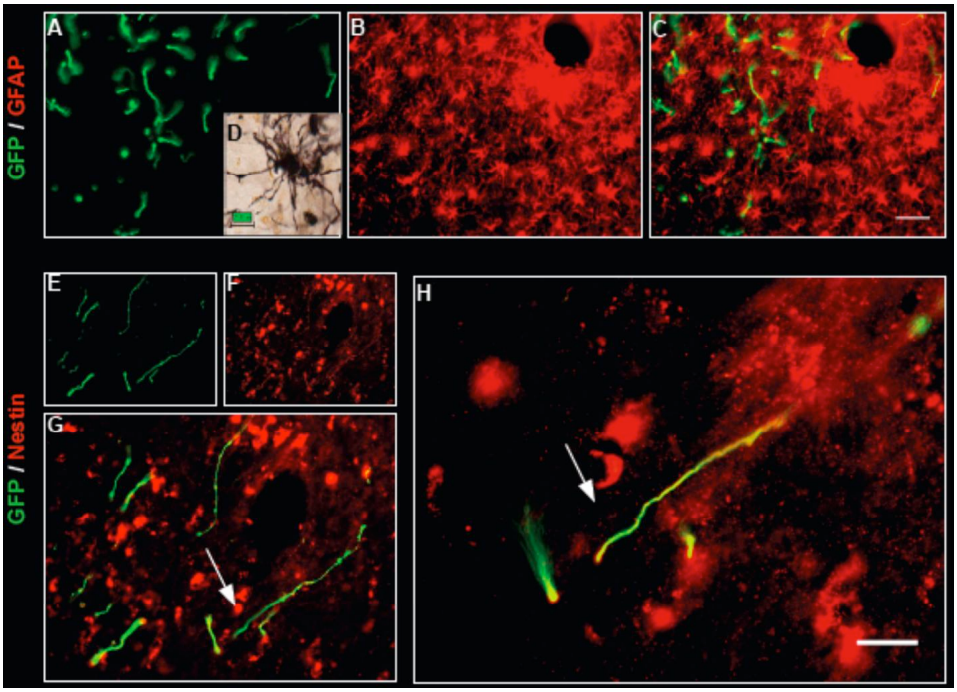


Figure 4-13. Engrafted GFP-ir cells downregulate immature filamentous proteins in-vivo. Eleven month's post-transplantation, some GFP-ir donor cells (green) had the characteristic morphological appearance of astrocytes [D]; however, surprisingly, grafted cells did not co-localize with GFAP (red), even in regions where many endogenous astrocytes were present [A-C] and despite high expression in-vitro. Analysis of the "immature" neural marker filament Nestin (red), revealed co-localization with GFP-ir donor cells (green) only at the distal ends of some fibers [E-H], further indicating a switch from expression of stem cell to neuronal related proteins.

In one animal, GFP-ir cells were also found within the host ventricular lumen and SVZ at the dorsal region of the needle tract [Supplementary Figure 4-2]. Cells remaining at the ventricular surface retained a type-B/C cell phenotype, displaying either elongated radial processes resembling radial glia or bipolar migratory cells. Some of these cells were also Vimentin-ir (data not shown), indicating that HFB-2050 hfNSC were capable of integrating into the adult primate SVZ and likely incorporated into the host SVZ niche, thus further supporting HFB-2050 as a bonafide forebrain SVZ hfNPC. Furthermore, some GFP-ir neurites were also found lining branched vascular walls and expressed complimentary markers in-vitro.

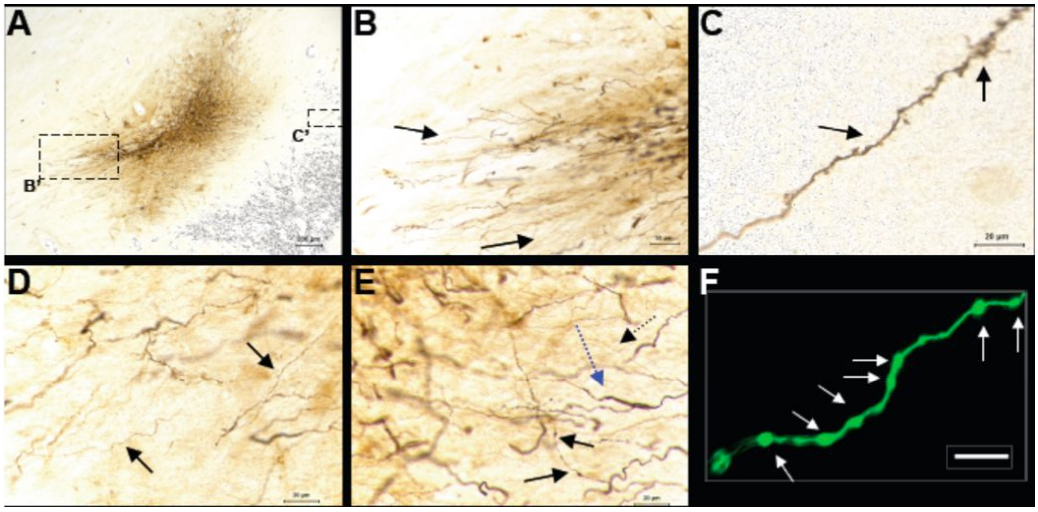


Figure 4-14. Morphological characteristics of 11-month grafts suggest differentiation into multiple neuronal phenotypes. Histological enhancement of GFP-ir fibers revealed extensive fibritic growth reminiscent of mature monoamine neurons [A, B, D-F]. Extremely fine GFP-ir fibers (arrows in [B] and [D]; dashed-black arrow in [E]) adorned with varicosities (solid-black arrows in [E], white-arrows in [F]) projected within the SN and were extremely dense at the ventro-caudal region [B] and dorsal-rostral of the graft [B]. Some GFP-ir processes were thicker and/or smooth (blue arrow) [E] and may reflect non-terminal ends of axons, while other longer processes terminated in, "spine-like" structures (black arrows, [C]).

Many longer (>800um) GFP-ir neurites exhibited morphological features of a variety of more mature, monoamine neuronal phenotypes[252]. Very fine, densely packed, small (0.3-.06 um) beaded varicose GFP-ir fibers, characteristic of DA neuron terminals[253-256] were found as

diffuse spotted masses within the SN and protruding from the dorsal-rostral and rostro-caudal regions of the graft [Figure 4-14 A, B, D, E]. In addition, many GFP-ir cells bore both thick and thin smooth processes [Figure 4-14 D, E] similar to non-terminal ends of monoamine axons, as well as those bearing both small and large beaded processes typical of the catecholamine (DA and norepinephrine (NE)) and 5-HT neurons, in-vivo[257-281]. Many processes of variable thickness had abundant round or fusiform enlargements, "varicosities", spaced at variable distances and number per unit length [Figure 4-14 F]. These structures are morpholocially consistent with pre-synaptic sites for amine synthesis, storage, and release, analogous to synaptic boutons found at axo-dendritic junctions. Furthermore, many longer processes terminated in highly branched, "spine-like" structures [Figure 4-14 C].

4.3.13 Fluorogold Tract Tracing:

To determine if GFP-ir neuritic processes innervated striatal targets, we analyzed retrograde transport from striatal terminals to the SN using Fluorogold (FG). Densely labeled FG-ir endogenous cells were found extensively throughout the injected striatum [Supplementary Figure 4-3 A], however relatively few (1-3%) endogenous TH-ir cell bodies in the SN displayed marked FG-ir [Supplementary Figure 4-3 B]. No GFP-ir/FG-ir donor cells were found in the SN, suggesting a lack of striatal innervation after 11 months in-vivo. In addition, GFP-ir processes were never found near the most caudal striatal regions arguing against striatal innervation or NS reconstruction. These data also suggest that FG may not be an efficient retrograde tracer in the primate or that endogenous TH+ nigral cells were deinnervated by MPTP exposure at their striatal terminals, the latter of which can be negated as we saw many spared endogenous TH+ projections throughout the NS.

4.3.14 Safety Concerns and Immunological Response:

In order to access safety concerns involving cellular transplantation of fetal CNS tissue[282], we analyzed brains for signs of aberrant pathology and immune response. We found no evidence for gross anatomical differences within grafted tissue by Cresyl Violet [Supplementary Figure 4-3 C] or DAPI staining. In addition, Ki-67 and PCNA immunostaining revealed no evidence for sustained proliferation within grafts at 1.5 or 11 months, suggesting that "bonafide" hfNPC do not continue to replicate long-term, initiate glioma-like tumors, or induce aberrant host pathology. Furthermore, cd11b staining indicated no major immune response activation of microglia around the grafted region at 11 months in hfNPC grafted animals or in the two animals that received killed-cell control transplants. Together, these results indicate no major anatomical changes or inflammatory reaction in animals with successful grafts, unsuccessful graft (one of five animals injected with HFB-2050-GFP), or controls that emulate an unsuccessful graft (dead cells). Occasionally, red blood cells were found within the injection site, indicating that a small amount of blood likely entered through the needle tract and into the VM during surgical injection. In addition, FG markedly damaged striatal tissue directly adjacent to the injection and was often accompanied by a small striatal hemorrhage.

All subjects were video monitored extensively both pre-MPTP injection and throughout the entire experimental timeline to ensure good health. Although animals selected for this study were deemed behaviorally asymptomatic for both PARK Score and Healthy normal behaviors, we continued to monitor them for negative side-effects. No animals regressed statistically for the entire post-surgical intervention period for both parameters; however, two hfNPC transplanted animals displayed improved scores for signs of healthy behaviors that neared statistical significance. Interestingly, these animals also displayed the highest survival and most extensive neuritic output, suggesting that these grafts may be

functionally active. At no point did any participants show adverse behaviors or signs of distress. These data support the safety of hfNPC grafts in the primate brain.

4.4 Discussion

The results demonstrate that human fetal NPC xenografted into the primate VM are able to generate new immature neuronal phenotypes and project neuritic processes along the host NS in the dopamine-depleted midbrain. Previous studies have demonstrated axonal outgrowth along the nigrostriatal pathway and other brain regions in rodents using human or pig neuroblasts[90,100-102,105,106,283-285] indicating the adult NS as a permissive environment for regenerative growth. In addition, exogenous GDNF has been shown to enhance survival and direct neuritic outgrowth from grafted cells in both the rodent and primate dopamine-depleted brains[69,71,84,87-90,96,286,287]. Furthermore, striatal over-expression of GDNF has been shown to enhance endogenous axonal regeneration in the lesioned NS as well[75,287]

In a series of studies, we demonstrated that hfNPC, derived from the forebrain germinal zone, retain the capacity to engraft in the primate CNS, migrate to disease loci, and promote functional improvements in parkinsonian monkeys[11,135,136]. Histological analysis indicated only a small percentage of grafted cells differentiated into phenotypically DA cells (BrdU+/TH+ or DAT+), suggesting that the behavioral rescue and normalization of endogenous TH-ir cell numbers/area were related to indirect, secondary support of spared host cells. Similarly, others have shown lesion induced-migration[288], differentiation into NFM-70-ir/Map2-ir neurons and GFAP-ir astrocytes, as well as extensive long-term axonal outgrowth[289-292] from undifferentiated hfNPC grafted into rodents. In addition, when differentiated in-vitro or transplanted into dopamine-depleted, 6-OHDA lesioned rats, differentiated cells with neuronal phenotype also expressed TH, albeit sometimes transiently[289,290,292,293], suggesting that hfNPC retain the plasticity to produce disease relevant

DA neurons in-vivo; however, the extent to which these cells might fully differentiate and mature into disease-specific DA neurons, long-term, has never been fully or adequately accessed. Interestingly, hfNPC engineered to overexpress GDNF appear to remain predominantly undifferentiated (90%nestin, 5-8% GFAP, O% and <1% NeuN discrepancy) when transplanted into rodents and one primate and do not differentiate into DA neurons[294,295]. While these results appear promising, it remains relatively unclear to what extent SVZ-derived hfNPC can differentiate into mature striatally integrated, A9-(Girk2-ir) DA neurons and respond to local axon guidance molecules when transplanted into the lesioned adult primate brain.

The use of sagittal plane sectioning and hfNPC engineered to express an eGFP reporter allowed us to unambiguously identify graft-derived cells and their neuritic output from endogenous cells long-term and evaluate their morphological and histological profile in relation to host architecture. Robust long-term graft survival and extensive neuritic outgrowth was evident at 11-months post-transplantation. Large, densely clustered GFP-ir grafts projected neurites in many directions from the core of the graft out into the surrounding tissue; however, the directional output was clearly polarized at anatomical loci where grafted cells overlapped with host neural circuitry. Many GFP-ir processes at the dorsal end of the graft exited at the rostral pole and positioned themselves rostrally in a parallel trajectory to host TH-ir NS or MFB fibers. Some of the processes resembled developing/regenerating neurons with bifurcate endfeet, similar to growth cones. While there were several clearly polarized areas of growth, neurites were found projecting three-dimensionally from the periphery of the graft in nearly all directions. Therefore, the grafts generally had an ovular, 3D shape (much like a watermelon) with axonal projections extending out from the periphery (rind of the melon). It appears that wherever the graft happened to overlap with endogenous circuitry, the donor axons aligned and positioned themselves in a similar orientation to the host neuronal fibers.

The specificity for host fiber trajectories suggests that local axon guidance molecules persist in a permissive environment and may directly influence hfNPC neuritic output in the dopamine-depleted primate brain. Netrins, Semaphorins, Ephrins and Slits play critical roles during the development of midbrain DA neurons[296-310], but it is still relatively unclear to what extent these axonal guidance cues persist into adulthood in the primate brain and if they retain their instructive patterning cues long-term. Supporting previous studies utilizing more mature neural substrates, hfNPC also display directionally polarized outgrowth along host NS trajectories, specifically when the position of the graft was such that it intersected along or adjacent to endogenous fiber tracts. Importantly, the results demonstrate that a partially intact endogenous neural circuitry is likely to facilitate (and may be necessary for) NS reconstruction utilizing homotopic neural grafts. In addition to maintaining instructive guidance cues, endogenous DA fibers likely secrete neurotrophic growth promoting factors like BDNF as well. In this study, animals with partial lesions (loss of approximately 50% TH-ir) were specifically chosen to allow spared nigrostriatal fibers to facilitate directional outgrowth through the NS pathway, as seen in the 6-OHDA-lesioned mouse[90]. Fully lesioned animals with little to no DA nigrostriatal fibers remaining likely would not retain the axonal guidance molecules or produce trophic stimuli necessary for directed outgrowth. Therefore, as a whole, it appears that the partially lesioned primate brain retains a permissive environment to harbor neuronal differentiation and outgrowth of undifferentiated hfNPC.

The most plausible explanation for this targeted axonal outgrowth is that hfNPC retain their developmental plasticity and are able to utilize preserved trophic signaling cues from positions surrounding the source of their target in the adult brain. Newly grafted pioneer-like axons[311-316] may use existing spared adult axons as tracts, utilizing endogenous TH-ir fibers as nutrient and matrix rich scaffolding for generating new tissue. Interestingly DARPP32-ir medial spiny striatonigral projection neurons extend long fibers along congruent trajectories to

TH-ir nigrostriatal fibers[317-321]. These neurons are a potent source of GDNF for NS neurons and thus may facilitate a permissive growth environment for new graft-derived axonal processes. Grafts of DA neuroblasts have been shown to project axons that course in direct apposition to striatonigral DARPP-32-ir fiber bundles in the adult 6-OHDA lesioned adult mouse brain[90]. Furthermore, in the study, overall graft survival, efficiency of NS reconstruction, and degree of neuritic branching were enhanced by the addition of exogenous striatal AAV2/5-GDNF. Limited tissue constraints did not allow for co-analysis of GFP-ir neurites with DARPP-32 in the present study; however it is possible that a similar pattern might be found for hfNPC as well.

Congruent with studies in rodents and primates[90,322], we found that AAV5-GDNF most efficiently transduced striatal projection neurons, providing GDNF neurotrophic support to surrounding striatal tissue, as well as being anterogradely transported downstream along the striatonigral pathway to relevant targets including the SN. Others have shown a similar pattern of anterograde transport from the caudate to the globus pallidus, internal globus pallidus, and substantia nigra pars reticulata after 3 months using AAV5-GFP in Cynomolgus monkeys[322]. In addition, the percentage of transduced GFAP-ir glial cells in the present study ranged from at most 10% to less that 5% compared to NeuN-ir neuron structures, in agreement with Cynomolgus monkeys transduced with AAV5-GFP[322]. Interestingly, far fewer GFAP-ir cells were found transduced with AAV5-GDNF than previously reported using AAV5-GFP in vervet monkeys, where nearly half of the transduced cells in the striatum were GFAP-ir[323]. Large host cell bodies within the SN were also transduced, indicating some degree of retrograde transport as well. Thus, donor and endogenous cells are simultaneously exposed to GDNF at multiple levels: within the SN enhancing donor survival within the GFP-ir graft site, along GFP-ir neurites adjacent to GDNF transduced striatonigral projections, as well as locally within the striatal target to enhance target innervation. Therefore, GDNF acts to

enhance endogenous striatonigral projections that deliver GDNF downstream, preserving spared host TH-ir fibers and promoting graft survival and possibly a permissive growth environment to establish new NS projections.

Interestingly, some NFM-ir fibers appeared to project with circuitous trajectories. These cells generally coursed dorsal-rostral along or adjacent to NS fibers, then appeared to turn back caudally, coursing with a ventral trajectory in a similar manner as developing striatonigral projections. Newly developed pioneer-like axons and existing NS and striatonigral axons may be playing dual roles in these circumstances. Donor axons may be receiving multiple signaling cues, first locally in the SN to project in accordance with NS axonal signaling cues then once reaching and interacting with existing striatonigral projection neurons, turning back under the influence of their opposing guidance molecules.

Multiple neuronal phenotypes with morphological parameters relevant to monoamine neurons were present, displaying ramifications that closely resembled that of host circuitry, specifically those of the intrinsic DA pathways. Fine, densely packed, GFP-ir processes with small beaded varicosities were found at the ventral region of the graft coursing in a ventral-caudal trajectory within the host SN. These thin fibers were often bundled together with a diffuse or dotted staining pattern, reminiscent of DAT bearing terminals, in-vivo[259-268,270,271,273-275]. Morphologically, DA, NE, and 5-HT monoamine neuron terminals are generally indistinguishable without the aid of histochemical enhancement, bearing abundant varicosities spaced somewhat irregularly throughout the length of the fiber[259-263,266-269]. We found many GFP-ir fibers coursing from the periphery of the graft with both small and large oval to round varicose enlargements. In general, varicosities varied in length, and width, and were not spaced with any marked regularity. These structures highly resemble pre-synaptic boutons and are the site for amine synthesis, storage, and release of amines in-vivo. In contrast, many processes were long and generally smooth at non-terminal ends (closer to soma), but often

terminated in highly branched structures, also characteristic of monoamine axons in-vivo[259,261,266,267]. Other fibers terminated in highly branched spine-like structures, indicative of inhibitory complexes.

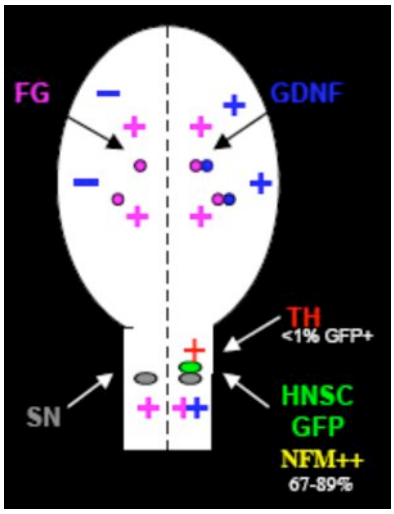


Diagram 4-3. Schematic representation of 11-month histological data. After 11-months in-vivo, robust expression of AAV5-GDNF (blue +) was confirmed throughout most of the striatum, as well as within the ipsilateral SN, but never expressed in the contralateral hemisphere. HfNPC-eGFP (green oval) differentiated into a predominantly NFM-ir phenotype, but with a very small number of GFP-ir/TH-ir cell bodies (red +). Fluorogold (pink +) was found surrounding the injection site but rarely noticeably transported into the SN (gray oval) and never found within grafts themselves.

Immunohistochemically, the majority of grafted cells appeared to be transitioning from immature (Nestin+) precursors and preferentially differentiating into highly arborized, NFM-ir neuronal phenotypes within the dopamine depleted VM [Diagram 4-3]. While many GFP-ir cells morphologically resembled monoamine neuronal phenotypes, most donor-derived cells lacked expression of several characteristic neuronal markers, including TUBB3 and MAP-2(a+b). However, a few donor derived GFP-ir cells possessed characteristics of immature DA cells, including TH-ir soma, providing evidence that hfNPC may develop into lineage-appropriate DA cells when placed homotopically into the SN. Interestingly, a small percentage of larger cells located at the periphery of the caudal region of graft were GFP-ir to

a lesser extent and had a morphology reminiscent of host nigral DA neurons. While these cells were excluded from analysis, it is very possible that these are an example of donor cells that have turned down expression of the eGFP transgene upon differentiation and maturation in-vivo. Previous reports have demonstrated similar results utilizing the CAG-promoter in rodents and primates; therefore, future studies utilizing a secondary reporter under the control of the TH-promoter similar to those used in the mouse[90] will be necessary to further eliminate these apparent discrepancies and determine whether SVZ-derived hfNPC can truly recapitulate a relevant A9-DA neuron in-vivo. Limited tissue did not allow for analysis of DA subtype, specifically to determine if any TH-ir donor cells had matured into A9 (Girk2-ir) or A10 (Calbindin-ir) regionally specific DA subtypes. Given the substantial 11-month period of time between transplantation and histological analysis, as well as the facilitated support of exogenous GDNF along disease relevant tracts (NS), it is reasonable to suggest that transplanted SVZ derived hfNPC do not generate *significant numbers of new mature* DA neurons, even in the presence of neurotrophic chemotaxis. Furthermore, there was no conclusive histological evidence that GFP-ir/TH-ir co-labeled cells retained morphological parameters of size or axonal profile consistent with mature A9-DA neurons.

A distinct possibility remains that grafted human cells still need longer to mature and at 11 months are just now becoming receptive to chemotaxic guidance cues. At first glance, this scenario appears unlikely, given such a long period of time. Undifferentiated hfNPC should have fully matured by eleven months in-vivo; however, it may be true that human cells simply need a far greater period of time to fully develop and only once upon reaching a certain level of maturation, will they activate necessary signaling programs to correctly respond and project processes in accordance with permissive endogenous cues. In previous rodent and primate studies, hfNPC generally retained Nestin-ir and/or GFAP-ir up to 20 weeks post-transplantation[288,290,324], suggesting that they had limited capacity to differentiate into mature

neuronal phenotypes; however, given that grafted cells retained a relatively immature but pro-neuronal immunohistochemical profile after 11-months in the current study, it is certainly plausible that these cells require a more extensive period of time to fully develop.

In theory, as cells continue to mature and begin utilizing axonal guidance molecules from existing host circuitry, they may actually be capable of integrating striatal targets, similar to results seen utilizing fetal VM neurons[89], where outgrowth was found coursing to the most caudal portion of the caudate, roughly 5-7mm. In this theoretical model, regeneration and NS reconstruction would occur simultaneously utilizing two distinct complementary mechanisms. In the first phase, exogenous GDNF promotes regeneration and protection of spared endogenous DA neurons and their striatal projections via mechanisms discussed above while also promoting a survival advantage to grafted hfNPC through a permissive neurotrophic environment. During this extended period of time, host neurocircuitry begins to regenerate as well as produce potent stores of local trophic factors that may act on developing grafts, preferentially exerting DA signaling cascades and enacting parallel receptor programs, thus priming grafted cells to now respond to axonal guidance molecules being displayed by locally restored circuitry. In the second phase, newly committed neuronal processes would then utilize rejuvenated host circuitry and local guidance cues to properly course the NS pathway and reinnervate the GDNF-rich striatum.

Surprisingly, GFP-ir cells did not express GFAP, although many had a morphological profile consistent with that of astrocytes. In previous studies, where hfNPC were placed into the striatum, we found that grafted cells migrated extensively and differentiated into a small percentage of astrocytes[136]. Similarly, hfNPC grafted into normal or 6-OHDA lesioned rats differentiate into astrocytes[7,288,289,291,292] and migrate extensively when implanted ectopically into the striatum of fully lesioned rats[292] or transplanted homotopically into the SN of striatal partial-lesion rats[288]. Interestingly, homotopic grafts of both undifferentiated and

predifferentiated (TUBB3-ir) hfNPC retained expression of both Nestin and GFAP 12-weeks post-transplantation in 6-OHDA partial-lesion rats. The data suggest that the signaling cues at the local transplantation zone as well as lesion zone may profoundly impact the fate of grafted cells. In this case, hfNPC were placed directly adjacent to the lesion zone (SN) of partial-lesion primates, and we saw no migration or astrocytic differentiation. Therefore, the striatal environment likely maintains astrocytic differentiation cues, whereas the VM appears to preference towards neuronal phenotypes. These studies highlight significant differences in the fate of undifferentiated hfNPC in relation to their site of transplantation as well as the species into which they are delivered. Secondarily, the degree of lesioning and persistence of existing circuitry are likely to have a profound impact on donor cell fate. Fully lesioned, behaviorally parkinsonian animals have extensive degeneration of both DA circuitry as well as a general breakdown of the cytoarchitectural milieu, whereas asymptomatic animals retain enough DA transmission to maintain most healthy daily behaviors. With only partial degeneration, asymptomatic animals may not require secondary glial support or enact the same signaling mechanisms employed by a fully lesioned brain.

Safety considerations and immunological response to grafted cells are important obstacles to consider for any cell-based therapy. Host tissue within the transplantation zone displayed no aberrant pathology by Cresyl Violet or DAPI stain. In addition, grafted cells were negative for the proliferative markers, Ki-67 and PCNA, confirming previous findings that grafted hfNPC exit the cell-cycle shortly after transplantation[292]. Interestingly, after 1.5 months, we found a few cells within the core of the graft with morphological characteristics of late stage symmetric division; however, these cells did not stain positive for PCNA or Ki-67 as expected. These results are consistent with those seen in the rat[292], where some hfNPC replicated several times before exiting the cell-cycle, but never maintained long-term sustained proliferation. Staining for inflammatory activation revealed little to no infiltration of

CD-11b-ir microglia or apparent long-term immune response of grafted cells. Overall, the results demonstrate that "bonafide" hfNPC do not proliferate long-term, initiate tumor like growth, or induce aberrant host pathology. Furthermore, hfNPC appear to be well-tolerated by the endogenous environment without major immunological rejection long-term or adverse effects derived from the transplantation procedure itself.

It is still relatively unclear whether undifferentiated NSC or lineage specific post-mitotic DA precursors/neurons represent the ideal substrate for neural regeneration in PD. It is important to examine each individually defined resource and test its potential separately from the others. Careful and meticulous side-by-side characterization of NSCs or DA progenitors derived from fetal cadavers, hESC, and induced pluripotent stem cells (iPSC) may ultimately lead to successful treatments. Biochemical and gene expression arrays indicate that HFB-2050 *SVZ-derived* hfNPC retain multiple characteristic consistent with a neural stem/precursor phenotype. Immunocytochemical analysis indicated that these cells exist as a heterogeneous population of neural stem and progenitor cells, highly resembling the neurogenic niche in-vivo. Interestingly, almost all hfNPC are Vimentin-ir, Nestin-ir, GFAP-ir, Sox-2-ir, Ki-67ir replication competent, and readily incorporate the thymidine analogue BrdU; however, upon exposure to an extracellular matrix-rich environment, a greater percentage of type-B, radial glial like cells were multi-nucleated and aligned symmetrically, suggesting they may be the true hfNSC that give rise to and support their slightly differentiated $BLBP^{low}$/DCX+, type-C and type-A hfNPC counterparts. Moreover, there is no evidence to suggest these cells are not still fully plastic and can revert to either lineage in response to local signaling molecules that may govern the overall percentages of each cellular sub-population. In fact, it has been shown that secondary type-C transit amplifying cells can function as multipotent NSCs, in-vitro, when exposed to appropriate mitogenic stimuli[148,325], suggesting the cell fate determination transition from type-B to type-C cell may not be a unilinear event in-vitro, but rather a plastic

bi-directional spectrum of differentiation, poised to respond to local signaling molecules[326,327]. It is therefore likely that mature, high density, HFB-2050 MAN cultures are intrinsically interconnected networks comprised of both populations co-existing as one functional unit.

HfNPC (SC-23: derived from a premature infant of about 23 weeks gestation)[150] were sorted by immuno-flow cytometry for CD9, a marker associated with pluripotence in hESCs, revealed both positive and negative-staining cells in standard cultures (Phillip H Schwartz, CHOC; personal communication). A few days after sorting and separately replating each population, the morphology of the CD9+ cells differed from the CD9- population; a promising result in hopes of separating the stem from progenitor and differentiated cells. After two weeks in culture; however, the cultures appeared morphologically indistinguishable by phase microscopy. In addition, flow cytometry showed an equal distribution of CD9+ and CD9- cells in both cultures, suggesting that they had both "re-set" to the original distribution found in the initial parent culture. Over several weeks, daughter cultures inherently repopulated as a heterogeneous mixture; therefore, cells appeared to reorganize and adjust accordingly, demonstrating the inherent plasticity hfNPC possess. Interestingly, these cells were extremely similar to HFB-2050 in both mRNA and miRNA expression[156,157] and have similar morphological features in-vitro.

4.5 Conclusion

These results provide evidence that hfNPC can engraft and survive long-term in the MPTP lesioned primate SN while their distant striatal target is releasing an elevated concentration of GDNF. We demonstrate for the first time, extensive neuritic outgrowth from undifferentiated hfNPC in the primate dopamine-depleted midbrain. Specifically, smooth and fine varicose GFP-ir processes were found to course along trajectories congruent to endogenous neural circuitry suggesting that grafted hfNPC, much like their VM counterparts,

can be placed into appropriate homotopic loci and develop and respond appropriately to axonal guidance signaling molecules in the degenerative adult primate brain. The results also confirm previous reports in other regions of the CNS, indicating that the adult brain retains permissive instructive cues for repair and reconstruction[90,100-103,106,110,328]. In these studies, fate committed DA-neuroblasts were capable of innervating multiple highly specific short and long-distance target regions in the lesioned adult CNS.

Similarly, undifferentiated hfNPC demonstrate the capacity to differentiate and utilize local signaling cues retained within spared endogenous nigrostriatal circuitry. Interestingly, after 11-months, grafted cells remained somewhat immature and still appeared to be developing and growing in relation to host axon guidance cues, indicating that human stem cell derived neurons likely need a much longer time to fully develop and mature than rodent or primate fate committed DA-neuroblasts within the primate brain compared to rodent brain. Human cells have a much longer developmental timescale compared to rodents in-vivo; therefore, it is likely that they also remain receptive to growth factors and guidance cues throughout this longer process as well. Furthermore, undifferentiated stem cell derived grafts are likely to give rise to multiple alternative cell types, which may explain why outgrowth was somewhat promiscuous and did not appear to be quite as target-restricted compared to DA-neuroblasts, which have been shown to direct circuit-specific projections toward dennervated targets along relevant nigrostriatal fiber tracts (medial forebrain bundle and internal capsule). It is still relatively unclear how long it would take in-vivo and to what degree SVZ-derived hfNPC can differentiate into fully differentiated A9-specific DA neurons and integrate distant targets. In the present study, we found ~.01-1% of GFP-ir donor cells also co-expressed TH, confirming our previous findings where AAV5-GDNF was not used combinatorially. GDNF, therefore, did not appear to exert a significant effect on DA differentiation, but rather improved graft survival and rescue of endogenous NS fibers. The experiments further support

the rationale that early initial functional improvements in parkinsonian primates reported previously[11] were likely due to trophic support and restoration of spared endogenous host-neurons not from direct cell replacement. Cell replacement strategies utilizing undifferentiated NPCs, therefore, may likely require several years to fully mature when directed by host tissue. In contrast, fate-committed DA-neuroblasts readily differentiate into TH-ir neurons and have been shown to accurately and efficiently reconstitute new nigrostriatal projections along existing host circuitry. It is likely that hESC and iPSC derived NSC and their differentiated DA precursors will also retain many of these valuable characteristics.

Great strides have been made in neural induction and maturation of A9-DA neurons from hESC and iPSC, in-vitro[12-14,182,188,329-338]. Additionally, the identification of new extracellular markers for FAC sorting specific populations of progenitors[339,340] will be crucial to predictably establish and reliably expand transplantable populations in a clinical setting. With the advent and present onslaught of iPSC biology, as well as the initiation of new fetal DA cell clinical trials in Europe, cellular transplantation appears to have come full circle. It may be time to test each specific cellular substrate side-by-side, to determine if we can make a more therapeutically relevant, renewable cell in large quantities, in-vitro and determine which stage of differentiation provides the most functionally relevant substrate for treatment of Parkinson's disease.

4.6 Methods

4.6.1 Derivation of hfNSC/hfNPC HFB-2050: Primary neural stem/progenitor cell line (HFB-2050) was derived from the neuroectoderm-derived forebrain ventricular zone of a 13-week human fetal cadaver as previously described[11,142,341-343]. Cultures with normal karyotype and sustained proliferative capacity for greater than 20 population doublings were maintained as multilayer adherent networks (MAN) in *NB-B27 complete media* containing: Neurobasal

medium (Gibco) supplemented with B-27/without Vitamin A (Gibco), Glutamax (Gibco), heparin [8μg/ml] (Sigma), Normocin (InvivoGen), FGF-2 [20ng/ml] (Peprotech), and LIF [10ng/ml] (Millipore). Stable populations of hfNPCs were frozen as aggregates in 1 ml aliquots, approximately 10-15 cells in diameter as previously described[142] and stored as large batches for future use as a library of early passage biological replicates.

4.6.2 Establishment and Maintenance of hfNSC/NPC Multilayer Adherent Network (MAN): Frozen HfNPC aggregates were "quick thawed" at 37°C from identical previously frozen aliquots containing 2-3x10^6 cells, washed immediately in NB-B27 complete media, centrifuged for 3.5 min at 300 rcf, resuspended in 8-ml fresh NB-B27 complete media, and plated at *uniform* density into vented Falcon T-25 cm2 flasks. Flasks were incubated at 37°C, 5% CO^2, *undisturbed*, to allow cellular clusters to form homogenous, "low affinity" adherence, replication competent MAN *colonies* as described previously[142,344]. After 72 hours, metabolized media was gently aspirated and replaced with 8-ml fresh NB-B27 complete media to expand hfNSCs colonies and allow individual cell clusters to extend processes and begin migrating into adherent *networks*. This process was repeated every 3 days to refresh mitogens and remove toxic metabolites. Within 7 DIV, cellular clusters were "moderately" adherent, highly migratory, and came into direct contact with adjacent neighboring clusters forming the "webbed" structural foundation for the multilayer network. After 2-weeks in culture, these "monolayer-like" colonies continued to expand into contact with each other forming a uniform, densely webbed, interconnected, migratory, *multilayer adherent network* of hfNPC.

4.6.3 FACS Analysis: HFB-2050-eGFP hfNPC were enzymaticaly dissociated into single cells with Accutase (Millipore), allowed to equilibrate for two hours and resuspended at $1x10^6$

cells/ml as previously described[142]. For lentiviral labeling efficiency, eGFP epifluorescence was analyzed in live cells without antibody enhancement. BrdU labeled cells were fixed in 4%PFA, incubated in 2N HCL @ 45^{o}C for 30min, and stained by methods described below with AlexaFluor mouse-anti-BrdU-488 (1:250, Molecular Probes, Eugene OR). GFP+ or BrdU:488-ir cells were analyzed on a fluorescent activated cell sorter (FACS). For detection of BrdU, data was acquired with a FACSCanto (BD Biosciences, San Jose) flow cytometer and analyzed with FACSDiVa version-4 software. For GFP sorting, cells were sorted on a FACSVantageSE DiVa sorter with FACSDiVa version-4 software at 15psi with a 100-um ceramic nozzle and a drive frequency of 31,000 drops per second. BrdU-488 was excited at 488nm and detected with a 530/30 bandpass filter and PI was detected with a 670 longpass filter. 30,000 cell events were collected in each data file. Events were gated in forward and side scatter to eliminate debris and dead cells. Aggregates were gated out using pulse shape parameters. Fluorescence was determined and gated in comparison to appropriate controls: (unlabeled GFP-negative cells for GFP labeling efficiency) or (labeled + unstained, unlabeled alone, unlabeled + antibody; for BrdU labeling efficiency). All comparisons were carried out on cells at an identical stage of maturity and prepared in parallel. To assure specificity, sorted cells were verified visually by immunofluorescence microscopy (see below) and by FACS reanalysis. All sorts were repeated at least three times.

4.6.4 Microscopic Confirmation of eGFP & BrdU Labeling: Flow sorted cells were collected and reanalyzed with standard immunofluorescence microscopy. Briefly, cell suspensions were gently triturated and plated onto standard microscope slides or hemacytometer, coverslipped, and visualized with an Olympus IX-71 fluorescent microscope. Cells in ten random fields of ten coverslips per sample were counted in three experimental replicate samples.

4.6.5 Immunocytochemistry (ICC)-Immunofluorescence (IF): Routine IF techniques were applied to dissociated cells as well as cells grown on coverslips or TC-treated plastic chambers/wells as previously described[142]. Briefly, cells were fixed in cold 4% PFA (Electron Microscopy Sciences, Hatfield, PA USA) for 15-20min at 4C, then permeablized (non-surface antigens) in 0.1%TritonX-100 (Sigma-Aldrich, St Louis, MO USA) for 20min, and blocked in 3-5% Normal Donkey Serum (Jackson ImmunoResearch Laboratories, Inc., West Grove PA, USA) + 1-3% BSA (Sigma-Aldrich, St Louis, MO USA) in 0.2% TritonX-100 for 1hr at RT. Primary antibodies were diluted in 1:1 (blocking solution: dPBS + 0.1% TritonX-100) and incubated on cells overnight at 4C. Subsequently, cells were washed thoroughly, followed by secondary antibody incubation with species appropriate fluorescence-labeled AlexaFlour conjugates; Donkey anti-mouse, rabbit, or goat 488 (green), 555 or 568 (near red), 647 (far-ir) (Molecular Probes, Eugene OR, 1:500). Where applicable, nuclei were visualized with Hoechst 33342 (Molecular Probes, 6 ug/ml) (blue). Primary antibody was omitted from specified controls. Coverslipped samples were mounted on permafrost-coated slides in Vectashield (Vector Labs).

4.6.6 Q-PCR: RNA was extracted from cell pellets using the SV Total RNA Isolation System (Promega). Following purification, the extracted RNA was treated with RQ1 RNase-Free DNase (Promega) to ensure complete removal of any contaminating genomic DNA. In preparation for real-time PCR amplification, first-strand cDNA was synthesized from 100ng of each RNA template, using the ImProm-II(tm) Reverse Transcription System (Promega). Following thermal inactivation of the reverse transcriptase, the cDNA was used as the template in quantitative PCR amplifications. Amplification of pluripotency-associated transcripts was performed as two-color duplex reactions, with each pluripotency-associated

gene (labeled in FAM) duplexed with a reference transcript (GAPDH, labeled in JOE). Amplification of neural-associated transcripts was performed as monoplex reactions, with the reference transcript (GAPDH) also amplified as a monoplex. Quantitative PCR was performed using the StemElite(tm) Gene Expression System (Promega) and a LightCycler 480 instrument (Roche). Analysis of qPCR data was performed using the Plexor(r) Analysis Software (Promega). Primer sequences were developed and provided by Promega. Additional information or specific sequence inquiries available by request to Promega. During the amplification, the point where the decrease in fluorescence crosses the detection threshold is the CT; samples are compared using this number. A smaller CT value denotes a higher level of gene expression. CT was determined and normalized to the housekeeping gene, GAPDH; average CT=19.5. ΔCT= (CT-gene of interest) –(CT-GAPDH). Significant expression differences between samples are defined as a difference in ΔCT > 2.

4.6.7 Immunoblotting: Undifferentiated HFB-2050 MAN cultures were rinsed five times in cold dPBS and immediately lysed in RIPA buffer (Pierce #89900, Rockford, IL) + fresh HALT phosphatase and protease inhibitor cocktails (Pierce 78410, 78420) for 5-7 min with shaking. When lysis was complete, homogenate was pulled through an insulin syringe 3-5 times, sonicated for 5sec, and centrifuged for 10 min to clear debris. A small aliquot was removed and placed on ice for protein quantification (BCA Assay, Pierce) and the remaining cleared lysate aliquoted and stored at -20C. 5-30ug of solubilized protein from each sample were mixed 1:4 with sample buffer and NuPAGE reducing agent and heated to 70C for 10 min according to manufacturers instructions (Invitrogen, NuPAGE electrophoresis system). Samples and molecular weight standards (New England Biolabs, Ipswich, MA) were loaded onto precast NuPAGE Novex 4-12% Bis-Tris gels (Invitrogen) and electrophoresed in NuPAGE MES SDS Running Buffer + Antioxidant at 200V, 35min. After electrophoresis, the

proteins were electrically transferred to Immobilon-FL membranes at 30V for 1hr in NuPAGE Transfer Buffer + Antioxidant. After transfer, blots were incubated in PBS with 0.1% Tween-20 containing 5% Odyssey Blocking Buffer (LI-COR) or 5% nonfat dry milk. Blots were then incubated with the appropriate primary antibody (Nestin-human Ms 1:2000; Chemicon; GFAP Rb Dako 1:3000; BLBP Rb Chemicon 1:2000; DCX Gt SantaCruz 1:1000; Tuj1 Ms 1:2000; Covance) plus 0.1% Tween-20 and 3% Odyssey Blocking Buffer at 4°C overnight. Subsequently, fluorescently-conjugated secondary antibodies (AlexaFluor-680, Rockland-IR800, Ms, Rb, Gt; 1:10,000) were applied in 0.1% Tween-20 and 0.01% SDS, and immunoreactive bands were visualized using the Odyssey infrared imaging system (LiCorr Biosciences) with GAPDH (Chemicon 1:1000) serving as a loading control.

4.6.8 Stereotactic Surgeries, Transplantation of hfNSC, and GDNF Vector Delivery: St. Kitts (African) green monkeys (*Chlorocebus sabaeus*) were anesthetized with ketamine (10 mg/kg IM) and sodium pentobarbital (15-25 mg/kg IV), as previously described[142]. Briefly, animals were given atropine, intubated and the head was prepped, shaved, and mounted into a stereotactic frame using sterile technique. The animal was monitored for vital signs, EKG, pO2, and temperature and given a 30-50 ml/hour intravenous infusion of Lactated Ringer's solution. Each animal received 300,000 Units of Flocillin (penicillin G benzathine and penicillin G procaine) before surgery. After the animal was anesthetized to a level that showed no deep pain responses, the scalp was incised. Small 1-2 mm holes in the cranium were drilled at the desired coordinates (David Kopf, Tujunga, CA). A 22-gauge needle connected to a Hamilton (Reno, NV) syringe was lowered through drilled holes to the desired depth, and left *in situ* for 2 minutes pre- and post-injection, and in cases where the depth of the needle was varied, the needle was left *in situ* for 2 minutes before each withdrawal in an upwards direction.

Two days prior to transplantation, HFB-2050-eGFP MAN cultures were enzymatically dissociated into single cells with Accutase and replated at low sphere forming density to deter mass aggregation. Ten minutes prior to transplantation, donor cells were gently re-dissociated into a single-cell suspension at [50,000 cells/ul] in dPBS and aseptically loaded into a 22-gz needle attached to a 20 ul Hamilton syringe (Reno, NV) for stereotactic injection as previous. Six animals received undifferentiated HFB-2050-eGFP hfNSC, two received unmodified wild-type HFB-2050 hfNSC, and two received killed hNSC-eGFP (controls for transgene fusion and host immune response). HfNPC were grafted into the rostral mesencephalon immediately dorsal to or within the host SN using Kopf Instruments stereotaxic devices (Tujunga, CA) to target empirical stereotactic coordinates determined based on previous studies[89] [AP 11.1, Lateral 3.5, and Vertical 9.6 mm from actual ear bar zero] and driven by a microperfusion pump (Stoelting Instruments, Wood Dale IL) at a rate of 1 ul/min.

For vector delivery, 100 µl Hamilton syringe was driven by a perfusion pump delivering vector at 1 µl per minute (Stoelting Instruments, Wood Dale IL). The vector dose was 1×10^{12} viral genomes per milliliter. Two targets were injected within the hemisphere ipsilateral to the hfNPC injection; the rostral caudate nucleus (CD) and post commissural putamen (PM). After injection of vector was completed for each target, the needle was slowly withdrawn (1 mm/min) over a period of five minutes. The measurements used for the target regions of the brain were as follows: Caudate: AP: 21.1, Lateral: 4, Vertical relative to ear bar zero: 19; Putamen: AP: 21.1, Lateral: 10, Vertical relative to ear bar zero: 18.9. All animals were immunosuppressed for the duration of the experiments.

4.6.9 GDNF Viral Vector Synthesis: The methods for vector production have been published elsewhere [345] and are briefly, as follows: A single-stranded rAAV vector was constructed as

follows: Digest the plasmid pSub201 with XbaI and HindIII restriction endonucleases to remove the rep and cap fragments, and gel purify the 4000-bp plasmid backbone containing the AAV2 wt-ITRs. The desired transgene expression cassette was inserted between the XbaI sites to construct the single-stranded rAAV vector plasmid. For pSub201, HindIII was used in the digest to cut the rep and cap fragment in half for easy isolation of the plasmid backbone.

Self-complementary rAAV vectors were constructed as follows: The plasmid pHpa-trs-SK was digested with Acc65I and SalI to remove the CMV-GFP-poly(A) gene expression cassette and gel purify the 4166-bp plasmid backbone containing the mutated AAV2 ITR (left ITR) and wt-ITR (right ITR). Replace it with the desired transgene expression cassette between Acc65I and SalI. The size of the resulting rAAV construct (including two wild-type ITRs, which are each 145 bases for single-stranded rAAV genome, or two wild-type ITR and a mutated ITR, which is 120 bp, for self-complementary rAAV genome) should be between 3.4 and 4.8 kb, with the optimum size being that of the wild-type AAV genome of 4681 bp. Therefore, the size of the transgene cassette in the cloning plasmid, which includes the gene of interest and the promoter sequences necessary for its expression, spans between 3400 and 4400 bp (for single-stranded rAAV) or 1500 and 1950 bp (for self-complementary rAAV). Filler sequence was included if the transgene cassette is <3000 bp. The AAV ITRs are unstable in E. coli.

A large-scale plasmid preparation (at least 1 mg) of the rAAV vector and the suitable AAV helper plasmid and pXX6 plasmids was purified by double CsCl gradient fractionation. AAV serotype 5 was then generated by using different AAV helper plasmids of the pXR series (e.g., pXR1 for generating AAV serotype 1 capsids). For pXR plasmids, restriction digests tested plasmids upon arrival prior to transfer into bacteria (e.g., DH10b) and large-scale plasmid preparation. The restriction digest pattern was: pXR1: BamH1 and SphI digest yielded 2949-bp and 4516-bp fragments; pXR2: BamH1 and NcoI digest yielded 420-bp,

2722-bp and 4320-bp fragments; pXR3: BamH1 digest yielded 1528-bp and 5937-bp fragments; pXR4: BamH1 and SphI digest yielded 2051-bp and 5411-bp fragments; pXR5bam: BamH1 digest yielded 212-bp, 1443-bp and 5761-bp fragments. When growing the rAAV vector plasmid, the culture was not allowed to remain in stationary phase too long, as ITR deletions occur more rapidly after the culture has proceeded past log phase. The pXX6 plasmid was also unstable in bacteria. After receiving the plasmid, pSub201, pHpa-trs-SK, and XX6 was transformed into SURE bacteria (Stratagene) and the integrity of the miniprepped plasmid was checked by restriction digest, and the integrity of AAV ITRs by SmaI restriction digest, followed by electrophoresis. After selection of an intact clone, individual glycerol stocks were prepared. The plasmid preparations were analyzed to be free of contaminants (ethidium bromide, CsCl, and RNA) before use.

4.6.10 General Methods Used for Animal Care and Systematic MPTP Treatment: All animal work complied with National Research Council guidelines and was approved by Axion Research Foundation's animal care and use committee. The study was performed in accordance with U.S. federal guidelines and with the approval of the BIMR Institutional Animal Care and Use Committees (IACUC). Mature adult male African green monkeys (Chlorocebus sabaeus, St. Kitts) were singly housed with natural daylight light/dark cycle at 17° N. Latitude. Monkeys were systemically treated with MPTP HCL (RBI, Natick, MA) for a five-day period (total dose 2 mg/kg of body weight)[346-348] at least five weeks before surgical injections with a rAAV-hu-GDNF serotype5 vector and hfNPC as described below. Four doses were administered with a spacing of approximately twelve hours between each dosing for the first three days, with the fifth dose being administered on the morning of the fifth day. MPTP was handled using published guidelines[349]. Behavioral scoring before and after MPTP injection confirmed a moderately low PFS (non-parkinsonian) for all animals selected

indicative of low to moderate pathological deficit. These animals were selected based on their likelihood to harbor a relatively intact NS unit, while having been previously exposed to pathological insult and related toxic microenvironment.

4.6.11 Fluorogold Injection: Ten to twelve days prior to sacrifice, previous striatal targets used for GDNF delivery were injected bilaterally with 10 ul of the retrograde transport tracer, Fluorogold to interpret the extent of donor-derived striatal integration in long-term animals. Methods used to deliver viral vector were identical for fluorogold.

4.6.12 Sacrifice & Histological Preparation: Animals were sacrificed according to IACUC guidelines and regulations after 1.5 months (short-term) [1 hNSC-WT, 1 hNSCeGFP] or 11 months (long-term) after grafting [1 hNSC-WT, 5 hNSC-eGFP, 2 hNSC-Kill]. Animals were deeply anesthetized with ketamine (10mg/kg, i.m.) and sodium pentobarbital (50mg/kg i.v.) until complete loss of corneal reflexes. Whole brains were collected following cardiac perfusion with cold heparinized saline and cold 4%PFA, then suspended in 4%PFA at 4^{o}C for deep fixation. Following fixation, brains were allowed to "sink" in 30% sucrose preservative then transected at the mid-sagittal plane into two hemispheres and sectioned serially on a vibratome at 50um in the parasagittal plane to include the entire striatum and SN up through the extent of the lateral ventricle into the midline. Free-floating sections comprising every sixth section were stored in buffer at 4^{o}C.

4.6.13 Immunohistochemistry: Free-floating sections were treated with standard IF techniques. Briefly, sections were incubated (non-surface, intracellular antigens) in 0.3%TritonX-100 (Sigma-Aldrich, St Louis, MO USA) for 20min, and blocked in 5% Normal Donkey, Horse, or Goat Serum (Jackson ImmunoResearch Laboratories or Vector Labs

Burlingame, CA) + 1% BSA (Sigma-Aldrich) in 0.3% TritonX-100 for 1hr at RT. Primary antibodies (see below) were diluted 1:1 (blocking solution: dPBS + 0.3% TritonX-100) and incubated for 24-72hrs at 4C. Primary antibody was omitted from specified controls. Sections were then washed thoroughly, followed by incubation with species appropriate secondary antibodies. For IF: fluorescence-labeled AlexaFlour conjugates; Donkey anti-mouse, rabbit, or goat 488 (green), 555 or 568 (near red), 647 (far-ir) (Molecular Probes, Eugene OR, 1:500). Where applicable, nuclei were visualized with Hoechst 33342 (Molecular Probes, 6 ug/ml) (blue). For immunoperoxidase staining: sections were treated by standard protocols using the Vectastain ABC-Elite kit with Nickel-DAB chromagen substrate enhancement. Secondary antibodies used were biotinylated horse anti-mouse IgG (1:500) (Vector, BA2000) and biotinylated horse anti-goat IgG (1:250) (Vector, BA9500). Sections were rinsed, mounted onto gel coated slides, and coverslipped in PVA DABCO mountant for Nickel-DAB or mounted on permafrost-coated slides and mounted in Vectashield (Vector Labs) for fluorescence.

4.6.14 GFP-ir Graft Quantification: One series (every sixth section) was immunolabeled for GFP with a mouse monoclonal or rabbit polyclonal antibody against GFP (Molecular Probes; 1:250-1:500) and treated and enhanced with the ABC+DAB method described above. We quantified the volume of tissue occupied by GFP immunoreactivity using the Aperio Scancope XT (Aperio) at 40X magnification and analyzed for total GFP-ir area. Every 6^{th} section was analyzed using the optical fractionator probe. The area containing concentrated GFP immunoreactivity was outlined at 20x magnification and GFP-ir cells were analyzed at 20-40x. Non-specific staining was extracted from the analysis by removing the highlighted area from the total area per image. The volume of spread of concentrated GFP immunoreactivity

was determined by multiplying the area of the outline by the individual section thickness and the sampling interval.

4.6.15 Imaging and Processing: Tissue sections and in-vitro samples were examined under either a standard light microscope or one of several fluorescence microscopes (Olympus IX71, Leica DMI400B) and images captured with a Hamamatsu-ORCA-ER C47/42 camera using ImagePro 6.2 or Metamorph 7.5.6. All confocal data was taken from a Radiance 2100/AGR-3Q BioRad Multiphoton Laser Point Scanning Confocal Microscope and acquired with Lasersharp 2000 V6.0. Image J v1.38 was used to process original images in the following order: application of look-up-table (red or green), scale bar applied, smoothed with the 2-D or 3-D hybrid median filter, segmented with multi-thresholder (maximum entropy or mixture modeling methods), and then combined into a RGB image. Confocal z-stacks were arranged into 3D reconstructions and analyzed using Volocity 5 software. Figures were arranged in Adobe Photoshop or Illustrator (Adobe Systems, San Jose, CA).

Primary Antibodies

Rabbit-anti-Ki-67	(1:500)	(AB15580-100 AbCam)
Goat anti-PCNA	(1:200)	(SC-9857 Santa Cruz)
Mouse anti-Sox2	(1:200)	(MAB2018 R&D)
Goat anti-Vimentin	(1:200)	(AB1620 Chemicon)
Rabbit anti-GFAP	(1:500)	(Dako)
Rabbit anti-Nestin	(1:200)	(MAB5922 Chemicon)
Mouse anti-Nestin Human Specific	(1:200)	(MAB5326 Chemicon)
Mouse anti-Tuj-1	(1:500)	(MMS-435P Covance)
Mouse anti-Beta-III-tubulin	(1:250)	(MAB1637 Chemicon)
Mouse anti-NeuN	(1:100)	(MAB377 Millipore)
Goat anti-Doublecortin	(1:100)	(SC8066 SantaCruz)
Mouse anti-PSA-NCAM	(1:100)	(MAB5324 Chemicon)
Rabbit anti-BLBP	(1:300)	(AB9558 Chemicon)
Mouse anti-MAP2	(1:250)	(M4403 Sigma)
Mouse anti-Neurofilament (PAN)	(1:250)	(18-017 Invitrogen)
Mouse anti-TH	(1:5,000)	(MAB 318 Chemicon)
Rabbit anti-TH	(1:500)	(PRB-515P Covance)
Goat anti-GDNF Biotinylated	(1:500)	(BAF 212 / R&D)
Rabbit anti-GFP	(1:500)	(A11122 Molecular Probes)
Mouse anti-BrdU-488	(1:500)	(A21303 Molecular Probes)
Goat anti-Ang-1	(1:300)	(SC-6319 Santa Cruz)
Rabbit anti-Tie-2	(1:300)	(SC-324 Santa Cruz)
Rabbit anti-p-Tie-2	(1:300)	(SC-130607 Santa Cruz)
Mouse anti-Cd11b	(1:100)	(CD11b00 CALTAG Labs)

Microscopes		Excitation	Dichroic	Emission
Leica DMI4000B	DAPI	320-410	400	430-500
	GFP	460-500	505	512-542
	RFP	534-558	565	560-640
Olympus IX71	DAPI	360-370	400	435-485
	GFP	470-490	500	510-560
	RFP	540-580	595	600-660

Radiance 2100 Confocal

Collected wavelengths: 520-560
520 Long Pass = Block filter
560 Short Pass = Emission filter

Collected wavelengths: 580-700
580 Long Pass = Block filter

Open = Emission filter

Photomultiplier Tubes
488 channel, 100%
568 channel, 100%
No mixing between PMTs

4.7 Acknowledgements

Work presented in Chapter 4 is being prepared for publication to *PLOS: Biology* with co-authors Jeremy Bober, John R. Sladek Jr., Csaba Leranth, R. Jude Samulski, Evan Y. Snyder, and D. Eugene Redmond Jr. DRW designed and performed all experiments, produced all data, and oversaw the entire project. DER designed experiments, performed transplantations, and edited text. JB co-performed IHC. CL supervised JB and analyzed DAB IHC. RJS produced AAV-5 virus. EYS supervised DRW. The dissertation author is the primary author of this work and along with co-authors would like to thank Joseph Russo, Robbin Newlin, and Yoav Altman for CORE services, as well as Jeff H. Kordower, Ole Isacson, John Elsworth, Lars Olson, Barbara Blanchard, and Ilyas Singec for critical historical insight in transplantation of neuronal tissue and general knowledge about neurogenesis. We also thank Walter L. Niles, Brian Tobe, Andrew M. Crain, and Rachel Krupa for procedural help and putting up with our incessant video-conferences.

4.8 References

1. Arsenijevic Y, Villemure JG, Brunet JF, et al. Isolation of multipotent neural precursors residing in the cortex of the adult human brain. Exp Neurol. 2001 Jul;170(1):48-62.

2. Ahmad I, Hunter RE, Flax JD, Snyder EY, Erickson RP. Neural stem cell implantation extends life in Niemann-Pick C1 mice. J Appl Genet. 2007;48(3):269-72.

3. Björklund A, Stenevi U. Intracerebral neural grafting: a historical perspective. In: Björklund A, Stenevi U (eds). Neural Grafting in the Mammalian CNS. Amsterdam: Elsevier Science Publishers, 1985:3-14.

4. Blunt SB. Fetal neural graft survival. Lancet. 1990 Nov 3;336(8723):1131-2.

5. Pluchino S, Zanotti L, Deleidi M, Martino G. Neural stem cells and their use as therapeutic tool in neurological disorders. Brain researchBrain research reviews. 2005 Apr;48(2):211-9.

6. Brundin P, Bjorklund A, Lindvall O. Practical aspects of the use of human fetal brain tissue for intracerebral grafting. Prog Brain Res. 1990;82:707-14.

7. Anderson L, Caldwell MA. Human neural progenitor cell transplants into the subthalamic nucleus lead to functional recovery in a rat model of Parkinson's disease. Neurobiol Dis. 2007 Aug;27(2):133-40.

8. Armstrong RJ, Watts C, Svendsen CN, Dunnett SB, Rosser AE. Survival, neuronal differentiation, and fiber outgrowth of propagated human neural precursor grafts in an animal model of Huntington's disease. Cell transplantation. 2000 Jan-Feb;9(1):55-64.

9. Bjorklund A, Dunnett SB, Brundin P, et al. Neural transplantation for the treatment of Parkinson's disease. Lancet Neurol. 2003 Jul;2(7):437-45.

10. Bjorklund A, Stenevi U, Dunnett SB, Gage FH. Cross-species neural grafting in a rat model of Parkinson's disease. Nature. 1982 Aug 12;298(5875):652-4.

11. Redmond DE, Jr., Bjugstad KB, Teng YD, et al. Behavioral improvement in a primate Parkinson's model is associated with multiple homeostatic effects of human neural stem cells. Proceedings of the National Academy of Sciences of the United States of America. 2007 Jul 17;104(29):12175-80.

12. Sanchez-Pernaute R, Lee H, Patterson M, et al. Parthenogenetic dopamine neurons from primate embryonic stem cells restore function in experimental Parkinson's disease. Brain. 2008 Aug;131(Pt 8):2127-39.

13. Soldner F, Hockemeyer D, Beard C, et al. Parkinson's disease patient-derived induced pluripotent stem cells free of viral reprogramming factors. Cell. 2009 Mar 6;136(5):964-77.

14. Wernig M, Zhao JP, Pruszak J, et al. Neurons derived from reprogrammed fibroblasts functionally integrate into the fetal brain and improve symptoms of rats with Parkinson's disease. Proc Natl Acad Sci U S A. 2008 Apr 15;105(15):5856-61.

15. Brundin P, Nilsson OG, Strecker RE, Lindvall O, Astedt B, Bjorklund A. Behavioural effects of human fetal dopamine neurons grafted in a rat model of Parkinson's disease. Exp Brain Res. 1986;65(1):235-40.

16. Abdo WF, van de Warrenburg BP, Burn DJ, Quinn NP, Bloem BR. The clinical approach to movement disorders. Nat Rev Neurol. Jan;6(1):29-37.

17. Bernheimer H, Birkmayer W, Hornykiewicz O, Jellinger K, Seitelberger F. Brain dopamine and the syndromes of Parkinson and Huntington: clinical,

morphological and neurochemical correlations. Journal of Neuroscience. 1973;20:415-55.

18. Bjorklund A, Dunnett SB. Dopamine neuron systems in the brain: an update. Trends Neurosci. 2007 May;30(5):194-202.

19. Rinne UK. Dopamine Agonists as Primary Treatment in Parkinson's Disease. In: Yahr MD, Bergmann KJ (eds). Parkinson's Disease, 1986:519-23.

20. Perlow MJ, Freed WJ, Hoffer BJ, Seiger A, Olson L, Wyatt RJ. Brain grafts reduce motor abnormalities produced by destruction of nigrostriatal dopamine system. Science. 1979;204:643-53.

21. Björklund A, Stenevi U. Reconstruction of the nigrostriatal dopamine pathway by intracerebral nigral transplants. Brain Research. 1979;177:555-60.

22. Björklund A, Stenevi U. Neural Grafting in the Mammalian CNS. Amsterdam: Elsevier Science Publishers; 1985.

23. Brundin P, Duan WM, Sauer H. Functional effects of mesencephalic dopamine neurons and adrenal chromaffin cells grafted to the rat striatum. In: Dunnett SB, Bjorklund A (eds). Functional Neural Transplantation. Volume 2. New York: Raven Press, 1994:9-46.

24. Redmond DE, Jr., Sladek JR, Jr., Roth RH, et al. Fetal neuronal grafts in monkeys given methylphenyltetrahydropyridine. Lancet. 1986;1(8490):1125-7.

25. Redmond DE, Jr., Sladek JR, Jr., Roth RH, et al. Transplants of primate neurons. Lancet. 1986 Nov 1;2(8514):1046.

26. Annett LE, Martel FL, Rogers DC, Ridley RM, Baker HF, Dunnett SB. Behavioral assessment of the effects of embryonic nigral grafts in marmosets with unilateral 6-OHDA lesions of the nigrostriatal pathway. Experimental Neurology. 1994;125(2):228-46.

27. Elsworth J, Collier T, Redmond D, Jr., Roth R, Sladek J, Jr., Taylor J. Early gestational mesencephalon grafts, but not later gestational mesencephalon, cerebellum, or sham grafts, increase dopamine in caudate nucleus of MPTP-treated monkeys. Neuroscience. 1996;72:477-84.

28. Taylor JR, Elsworth JD, Roth RH, Sladek JR, Jr., Collier TJ, Redmond DE, Jr. Grafting of fetal substantia nigra to striatum reverses behavioral deficits induced by MPTP in primates: a comparison with other types of grafts as controls. Experimental Brain Research. 1991;85(2):335-48.

29. Bankiewicz K, Mandel RJ, Sofroniew MV. Trophism, transplantation, and animal models of Parkinson's disease. Experimental Neurology. 1993;124(1):140-9.

30. Fine A, Hunt SP, Oertel WH, et al. Transplantation of embryonic marmoset dopaminer-gic neurons to the corpus striatum of marmosets rendered parkinsonian by 1-methyl-4-phenyl-1,2-3,6-tetrahydropyridine. Prog Brain Res. 1988;78:479-89.

31. Bakay RAE, Barrow DL, Fiandaca MS, Iuvone PM, Schiff A, Collins DC. Biochemical and behavioral correction of MPTP Parkinson-like syndrome by fetal cell transplantation. Annuals of the New York Academy of Science. 1987;495:623-40.

32. Bankiewicz KS, Plunkett RJ, Mefford I, Kopin IJ, Oldfield EH. Behavioral recovery from MPTP-induced parkinsonism in monkeys after intracerebral tissue implants is not related to CSF concentrations of dopamine metabolites. Progress In Brain Research. 1990;82:561-71.

33. Bolam JP, Freund TF, Bjorklund A, Dunnett SB, Smith AD. Synaptic input and local output of dopaminergic neurons in grafts that functionally reinnervate the host neostriatum. Exp Brain Res. 1987;68(1):131-46.

34. Brundin P, Strecker RE, Londos E, Bjorklund A. Dopamine neurons grafted unilaterally to the nucleus accumbens affect drug-induced circling and locomotion. Exp Brain Res. 1987;69(1):183-94.

35. Brundin P, Strecker RE, Widner H, et al. Human fetal dopamine neurons grafted in a rat model of Parkinson's disease: immunological aspects, spontaneous and drug-induced behaviour, and dopamine release. Exp Brain Res. 1988;70(1):192-208.

36. Brundin P, Strecker RE, Lindvall O, et al. Intracerebral grafting of dopamine neurons. Experimental basis for clinical trials in patients with Parkinson's disease. Ann N Y Acad Sci. 1987;495:473-96.

37. Freed CR, Breeze RE, Rosenberg NL, et al. Transplantation of human fetal dopamine cells for Parkinson's disease: results at 1 year. Archives of Neurology. 1990;47:505-12.

38. Hitchcock ER, Kenny BG, Clough CG, Hughes RC, Henderson BT, Detta A. Stereotactic implantation of fetal mesencephalon (STIM): the UK experience. Progress In Brain Research. 1990;82:723-8.

39. Henderson BT, Clough CG, Hughes RC, Hitchcock ER, Kenny BG. Implantation of human fetal ventral mesencephalon to the right caudate nucleus in advanced Parkinson's disease. Archives of Neurology. 1991;48(8):822-7.

40. Lindvall O, Brundin P, Widner H, et al. Grafts of fetal dopamine neurons survive and improve motor function in Parkinson's disease. Science. 1990 Feb 2;247(4942):574-7.

41. Lindvall O, Sawle G, Widner H, et al. Evidence for long-term survival and function of dopaminergic grafts in progressive Parkinson's disease. Annals of Neurology. 1994;35:172-80.

42. Freed CR, Breeze RE, Rosenberg NL, et al. Survival of implanted dopamine cells and neurologic improvement 12 to 46 months after transplantation for Parkinson's disease. New England Journal of Medicine. 1992;327:1549-55.

43. Spencer DD, Robbins RJ, Naftolin F, et al. Unilateral transplantation of human fetal mesencephalic tissue into the caudate nucleus of Parkinsonian patients: functional effects for 18 months. New England Journal of Medicine. 1992;327(22):1541-8.

44. Widner H, Tetrud J, Rehncrona S, et al. Bilateral fetal mesencephalic grafting in two patients with parkinsonism induced by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). N Engl J Med. 1992 Nov 26;327(22):1556-63.

45. Kordower J, Freeman T, Snow B, et al. Neuropathological evidence of graft survival and striatal reinnervation after the transplantation of fetal mesencephalic tissue in a patient with Parkinson's disease. New England Journal of Medicine. 1995;332:1118-24.

46. Kordower J, Rosenstein J, Collier T, et al. Functional fetal nigral grafts in a patient with Parkinson's disease: Chemoanatomic, ulturastructural, and metabolic studies. Journal of Comperative Neurology. 1996;370:203-30.

47. Redmond DE, Jr., Robbins RJ, Naftolin F, et al. Cellular replacement of dopamine deficit in Parkinson's disease using human fetal mesencephalic tissue: preliminary results in four patients. Res Publ Assoc Res Nerv Ment Dis. 1993;71:325-59.

48. Olanow CW, Kordower JH, Freeman TB. Fetal nigral transplantation as a therapy for Parkinson's disease. Trends Neurosci. 1996;19(3):102-9.

49. Freed CR, Greene PE, Breeze RE, et al. Transplantation of embryonic dopamine neurons for severe Parkinson's disease. N Engl J Med. 2001 Mar 8;344(10):710-9.

50. Andressoo JO, Saarma M. Signalling mechanisms underlying development and maintenance of dopamine neurons. Curr Opin Neurobiol. 2008 Jun;18(3):297-306.

51. Bartlett PF, Kilpatrick TJ, Richards LJ, Talman PS, Murphy M. Regulation of the early development of the nervous system by growth factors. Pharmacology & therapeutics. 1994;64(3):371-93.

52. Barroso-Chinea P, Cruz-Muros I, Aymerich MS, et al. Striatal expression of GDNF and differential vulnerability of midbrain dopaminergic cells. Eur J Neurosci. 2005 Apr;21(7):1815-27.

53. Pahnke J, Mix E, Knoblich R, et al. Overexpression of glial cell line-derived neurotrophic factor induces genes regulating migration and differentiation of neuronal progenitor cells. Exp Cell Res. 2004 Jul 15;297(2):484-94.

54. Paratcha G, Ledda F. GDNF and GFRalpha: a versatile molecular complex for developing neurons. Trends Neurosci. 2008 Aug;31(8):384-91.

55. Pozas E, Ibanez CF. GDNF and GFRalpha1 promote differentiation and tangential migration of cortical GABAergic neurons. Neuron. 2005 Mar 3;45(5):701-13.

56. Trupp M, Belluardo N, Funakoshi H, Ibanez CF. Complementary and overlapping expression of glial cell line-derived neurotrophic factor (GDNF), c-ret proto-oncogene, and GDNF receptor-alpha indicates multiple mechanisms of trophic actions in the adult rat CNS. J Neurosci. 1997 May 15;17(10):3554-67.

57. Bohn MC, Choi-Lundberg DL, Davidson BL, et al. Adenovirus-mediated transgene expression in nonhuman primate brain. Hum Gene Ther. 1999 May 1;10(7):1175-84.

58. Eberling JL, Kells AP, Pivirotto P, et al. Functional effects of AAV2-GDNF on the dopaminergic nigrostriatal pathway in parkinsonian rhesus monkeys. Hum Gene Ther. 2009 May;20(5):511-8.

59. Emborg ME, Moirano J, Raschke J, et al. Response of aged parkinsonian monkeys to in vivo gene transfer of GDNF. Neurobiol Dis. 2009 Nov;36(2):303-11.

60. Bloch J, Redmond DE, Jr, Leranth C, et al. GDNF released from encapsulated cells increases fetal dopaminergic cell survival in a primate Model of Parkinson's disease. European Society for Stereotactic and Functional Neurosurgery. Toulouse, 2002.

61. Georgievska B, Carlsson T, Lacar B, Winkler C, Kirik D. Dissociation between short-term increased graft survival and long-term functional improvements in Parkinsonian rats overexpressing glial cell line-derived neurotrophic factor. Eur J Neurosci. 2004 Dec;20(11):3121-30.

62. Georgievska B, Jakobsson J, Persson E, Ericson C, Kirik D, Lundberg C. Regulated delivery of glial cell line-derived neurotrophic factor into rat striatum, using a tetracycline-dependent lentiviral vector. Hum Gene Ther. 2004 Oct;15(10):934-44.

63. Georgievska B, Kirik D, Bjorklund A. Aberrant sprouting and downregulation of tyrosine hydroxylase in lesioned nigrostriatal dopamine neurons induced by long-lasting overexpression of glial cell line derived neurotrophic factor in the striatum by lentiviral gene transfer. Exp Neurol. 2002 Oct;177(2):461-74.

64. Georgievska B, Kirik D, Bjorklund A. Overexpression of glial cell line-derived neurotrophic factor using a lentiviral vector induces time- and dose-dependent

downregulation of tyrosine hydroxylase in the intact nigrostriatal dopamine system. J Neurosci. 2004 Jul 21;24(29):6437-45.

65. Georgievska B, Kirik D, Rosenblad C, Lundberg C, Bjorklund A. Neuroprotection in the rat Parkinson model by intrastriatal GDNF gene transfer using a lentiviral vector. Neuroreport. 2002 Jan 21;13(1):75-82.

66. Kirik D, Georgievska B, Rosenblad C, Bjorklund A. Delayed infusion of GDNF promotes recovery of motor function in the partial lesion model of Parkinson's disease. Eur J Neurosci. 2001 Apr;13(8):1589-99.

67. Kozlowski DA, Bremer E, Redmond DE, Jr., George D, Larson B, Bohn MC. Quantitative analysis of transgene protein, mRNA, and vector DNA following injection of an adenoviral vector harboring glial cell line-derived neurotrophic factor into the primate caudate nucleus. Mol Ther. 2001 Feb;3(2):256-61.

68. Lapchak PA, Araujo DM, Hilt DC, Sheng J, Jiao S. Adenoviral vector-mediated GDNF gene therapy in a rodent lesion model of late stage Parkinson's disease. Brain Res. 1997 Nov 28;777(1-2):153-60.

69. Rosenblad C, Kirik D, Devaux B, Moffat B, Phillips HS, Bjorklund A. Protection and regeneration of nigral dopaminergic neurons by neurturin or GDNF in a partial lesion model of Parkinson's disease after administration into the striatum or the lateral ventricle. Eur J Neurosci. 1999 May;11(5):1554-66.

70. Rosenblad C, Georgievska B, Kirik D. Long-term striatal overexpression of GDNF selectively downregulates tyrosine hydroxylase in the intact nigrostriatal dopamine system. Eur J Neurosci. 2003 Jan;17(2):260-70.

71. Rosenblad C, Kirik D, Bjorklund A. Sequential administration of GDNF into the substantia nigra and striatum promotes dopamine neuron survival and axonal sprouting but not striatal reinnervation or functional recovery in the partial 6-OHDA lesion model. Exp Neurol. 2000 Feb;161(2):503-16.

72. Akerud P, Canals JM, Snyder EY, Arenas E. Neuroprotection through delivery of glial cell line-derived neurotrophic factor by neural stem cells in a mouse model of Parkinson's disease. J Neurosci. 2001 Oct 15;21(20):8108-18.

73. Bilang-Bleuel A, Revah F, Colin P, et al. Intrastriatal injection of an adenoviral vector expressing glial-cell-line-derived neurotrophic factor prevents dopaminergic neuron degeneration and behavioral impairment in a rat model of Parkinson disease. Proc Natl Acad Sci U S A. 1997 Aug 5;94(16):8818-23.

74. Bjorklund A, Rosenblad C, Winkler C, Kirik D. Studies on neuroprotective and regenerative effects of GDNF in a partial lesion model of Parkinson's disease. Neurobiol Dis. 1997;4(3-4):186-200.

75. Brizard M, Carcenac C, Bemelmans AP, Feuerstein C, Mallet J, Savasta M. Functional reinnervation from remaining DA terminals induced by GDNF

lentivirus in a rat model of early Parkinson's disease. Neurobiol Dis. 2006 Jan;21(1):90-101.

76. Connor B, Kozlowski DA, Unnerstall JR, et al. Glial cell line-derived neurotrophic factor (GDNF) gene delivery protects dopaminergic terminals from degeneration. Exp Neurol. 2001 May;169(1):83-95.

77. Eslamboli A, Georgievska B, Ridley RM, et al. Continuous low-level glial cell line-derived neurotrophic factor delivery using recombinant adeno-associated viral vectors provides neuroprotection and induces behavioral recovery in a primate model of Parkinson's disease. J Neurosci. 2005 Jan 26;25(4):769-77.

78. Kirik D, Rosenblad C, Bjorklund A. Preservation of a functional nigrostriatal dopamine pathway by GDNF in the intrastriatal 6-OHDA lesion model depends on the site of administration of the trophic factor. Eur J Neurosci. 2000 Nov;12(11):3871-82.

79. Kirik D, Rosenblad C, Bjorklund A, Mandel RJ. Long-term rAAV-mediated gene transfer of GDNF in the rat Parkinson's model: intrastriatal but not intranigral transduction promotes functional regeneration in the lesioned nigrostriatal system. J Neurosci. 2000 Jun 15;20(12):4686-700.

80. Mandel RJ, Snyder RO, Leff SE. Recombinant adeno-associated viral vector-mediated glial cell line-derived neurotrophic factor gene transfer protects nigral dopamine neurons after onset of progressive degeneration in a rat model of Parkinson's disease. Exp Neurol. 1999 Nov;160(1):205-14.

81. Mandel RJ, Spratt SK, Snyder RO, Leff SE. Midbrain injection of recombinant adeno-associated virus encoding rat glial cell line-derived neurotrophic factor protects nigral neurons in a progressive 6-hydroxydopamine-induced degeneration model of Parkinson's disease in rats. Proc Natl Acad Sci U S A. 1997 Dec 9;94(25):14083-8.

82. Ramaswamy S, Soderstrom KE, Kordower JH. Trophic factors therapy in Parkinson's disease. Prog Brain Res. 2009;175:201-16.

83. Sauer H, Rosenblad C, Bjorklund A. Glial cell line-derived neurotrophic factor but not transforming growth factor beta 3 prevents delayed degeneration of nigral dopaminergic neurons following striatal 6-hydroxydopamine lesion. Proc Natl Acad Sci U S A. 1995 Sep 12;92(19):8935-9.

84. Wang Y, Tien LT, Lapchak PA, Hoffer BJ. GDNF triggers fiber outgrowth of fetal ventral mesencephalic grafts from nigra to striatum in 6-OHDA-lesioned rats. Cell Tissue Res. 1996 Nov;286(2):225-33.

85. Winkler C, Sauer H, Lee CS, Bjorklund A. Short-term GDNF treatment provides long-term rescue of lesioned nigral dopaminergic neurons in a rat model of Parkinson's disease. J Neurosci. 1996 Nov 15;16(22):7206-15.

86. Gill SS, Patel NK, Hotton GR, et al. Direct brain infusion of glial cell line-derived neurotrophic factor in Parkinson disease. Nat Med. 2003 May;9(5):589-95.

87. Elsworth JD, Redmond DE, Jr., Leranth C, et al. AAV2-mediated gene transfer of GDNF to the striatum of MPTP monkeys enhances the survival and outgrowth of co-implanted fetal dopamine neurons. Exp Neurol. 2008 May;211(1):252-8.

88. Redmond DE, Jr.,, Elsworth JD, Leranth C, et al. Co-implantation of AAV2-GDNF viral vector with fetal ventral mesencephalon markedly increases long-term survival of grafted dopamine neurons in the MPTP monkey striatum. Soc Neurosci Abstr. 2006;470.13.

89. Redmond DE, Jr., Elsworth JD, Roth RH, et al. Embryonic substantia nigra grafts in the mesencephalon send neurites to the host striatum in non-human primate after overexpression of GDNF. J Comp Neurol. 2009 Jul 1;515(1):31-40.

90. Thompson LH, Grealish S, Kirik D, Bjorklund A. Reconstruction of the nigrostriatal dopamine pathway in the adult mouse brain. Eur J Neurosci. 2009 Aug;30(4):625-38.

91. Nikkhah G, Bentlage C, Cunningham MG, Bjorklund A. Intranigral fetal dopamine grafts induce behavioral compensation in the rat Parkinson model. J Neurosci. 1994 Jun;14(6):3449-61.

92. Nikkhah G, Cunningham MG, Jodicke A, Knappe U, Bjorklund A. Improved graft survival and striatal reinnervation by microtransplantation of fetal nigral cell suspensions in the rat Parkinson model. Brain Res. 1994 Jan 7;633(1-2):133-43.

93. Mendez I, Sadi D, Hong M. Reconstruction of the nigrostriatal pathway by simultaneous intrastriatal and intranigral dopaminergic transplants. J Neurosci. 1996 Nov 15;16(22):7216-27.

94. Mukhida K, Baker KA, Sadi D, Mendez I. Enhancement of sensorimotor behavioral recovery in hemiparkinsonian rats with intrastriatal, intranigral, and intrasubthalamic nucleus dopaminergic transplants. J Neurosci. 2001 May 15;21(10):3521-30.

95. Bentlage C, Nikkhah G, Cunningham MG, Bjorklund A. Reformation of the nigrostriatal pathway by fetal dopaminergic micrografts into the substantia nigra is critically dependent on the age of the host. Exp Neurol. 1999 Sep;159(1):177-90.

96. Wilby MJ, Sinclair SR, Muir EM, et al. A glial cell line-derived neurotrophic factor-secreting clone of the Schwann cell line SCTM41 enhances survival and fiber outgrowth from embryonic nigral neurons grafted to the striatum and to the lesioned substantia nigra. J Neurosci. 1999 Mar 15;19(6):2301-12.

97. Ahn YH, Bensadoun JC, Aebischer P, et al. Increased fiber outgrowth from xeno-transplanted human embryonic dopaminergic neurons with co-implants of

polymer-encapsulated genetically modified cells releasing glial cell line-derived neurotrophic factor. Brain Res Bull. 2005 Jul 30;66(2):135-42.

98. Abrous N, Guy J, Vigny A, Calas A, Le Moal M, Herman JP. Development of intracerebral dopaminergic grafts: a combined immunohistochemical and autoradiographic study of its time course and environmental influences. J Comp Neurol. 1988 Jul 1;273(1):26-41.

99. Annett LE, Dunnett SB, Martel FL, et al. A functional assessment of embryonic dopaminergic grafts in the marmoset. Prog Brain Res. 1990;82:535-42.

100. Galpern WR, Burns LH, Deacon TW, Dinsmore J, Isacson O. Xenotransplantation of porcine fetal ventral mesencephalon in a rat model of Parkinson's disease: functional recovery and graft morphology. Exp Neurol. 1996 Jul;140(1):1-13.

101. Isacson O, Deacon TW. Specific axon guidance factors persist in the adult brain as demonstrated by pig neuroblasts transplanted to the rat. Neuroscience. 1996 Dec;75(3):827-37.

102. Isacson O, Deacon TW, Pakzaban P, Galpern WR, Dinsmore J, Burns LH. Transplanted xenogeneic neural cells in neurodegenerative disease models exhibit remarkable axonal target specificity and distinct growth patterns of glial and axonal fibres. Nat Med. 1995 Nov;1(11):1189-94.

103. Li Y, Raisman G. Long axon growth from embryonic neurons transplanted into myelinated tracts of the adult rat spinal cord. Brain Res. 1993 Nov 26;629(1):115-27.

104. Wictorin K. Anatomy and connectivity of intrastriatal striatal transplants. Prog Neurobiol. 1992 Jun;38(6):611-39.

105. Wictorin K, Brundin P, Gustavii B, Lindvall O, Bjorklund A. Reformation of long axon pathways in adult rat central nervous system by human forebrain neuroblasts. Nature. 1990 Oct 11;347(6293):556-8.

106. Wictorin K, Brundin P, Sauer H, Lindvall O, Bjorklund A. Long distance directed axonal growth from human dopaminergic mesencephalic neuroblasts implanted along the nigrostriatal pathway in 6-hydroxydopamine lesioned adult rats. J Comp Neurol. 1992 Sep 22;323(4):475-94.

107. Wictorin K, Clarke DJ, Bolam JP, Bjorklund A. Host Corticostriatal Fibres Establish Synaptic Connections with Grafted Striatal Neurons in the Ibotenic Acid Lesioned Striatum. Eur J Neurosci. 1989 May;1(3):189-95.

108. Wictorin K, Clarke DJ, Bolam JP, Bjorklund A. Fetal striatal neurons grafted into the ibotenate lesioned adult striatum: efferent projections and synaptic contacts in the host globus pallidus. Neuroscience. 1990;37(2):301-15.

109. Wictorin K, Clarke DJ, Bolam JP, et al. Extensive efferent projections of intra-striatally transplanted striatal neurons as revealed by a species-specific neurofilament marker and anterograde axonal tracing. Prog Brain Res. 1990;82:391-9.

110. Wictorin K, Lagenaur CF, Lund RD, Bjorklund A. Efferent Projections to the Host Brain from Intrastriatal Striatal Mouse-to-rat Grafts: Time Course and Tissue-type Specificity as Revealed by a Mouse Specific Neuronal Marker. Eur J Neurosci. 1991 Oct;3(1):86-101.

111. Wictorin K, Ouimet CC, Bjorklund A. Intrinsic Organization and Connectivity of Intrastriatal Striatal Transplants in Rats as Revealed by DARPP-32 Immunohistochemistry: Specificity of Connections with the Lesioned Host Brain. Eur J Neurosci. 1989 Jan;1(6):690-701.

112. Wictorin K, Simerly RB, Isacson O, Swanson LW, Bjorklund A. Connectivity of striatal grafts implanted into the ibotenic acid-lesioned striatum--III. Efferent projecting graft neurons and their relation to host afferents within the grafts. Neuroscience. 1989;30(2):313-30.

113. Aguayo AJ, Bjorklund A, Stenevi U, Carlstedt T. Fetal mesencephalic neurons survive and extend long axons across peripheral nervous system grafts inserted into the adult rat striatum. Neurosci Lett. 1984 Mar 9;45(1):53-8.

114. Benfey M, Aguayo AJ. Extensive elongation of axons from rat brain into peripheral nerve grafts. Nature. 1982 Mar 11;296(5853):150-2.

115. Bjorklund A, Dunnett SB, Stenevi U, Lewis ME, Iversen SD. Reinnervation of the denervated striatum by substantia nigra transplants: functional consequences as revealed by pharmacological and sensorimotor testing. Brain Res. 1980 Oct 20;199(2):307-33.

116. Bjorklund A, Schmidt RH, Stenevi U. Functional reinnervation of the neostriatum in the adult rat by use of intraparenchymal grafting of dissociated cell suspensions from the substantia nigra. Cell Tissue Res. 1980;212(1):39-45.

117. Bjorklund A, Stenevi U, Dunnett SB, Iversen SD. Functional reactivation of the deafferented neostriatum by nigral transplants. Nature. 1981 Feb 5;289(5797):497-9.

118. Bjorklund A, Stenevi U, Schmidt RH, Dunnett SB, Gage FH. Intracerebral grafting of neuronal cell suspensions. II. Survival and growth of nigral cell suspensions implanted in different brain sites. Acta Physiol Scand Suppl. 1983;522:9-18.

119. Holden C. Neuroscience. Fetal cells again? Science. 2009 Oct 16;326(5951):358-9.

120. Gaietta GM, Giepmans BN, Deerinck TJ, et al. Golgi twins in late mitosis revealed by genetically encoded tags for live cell imaging and correlated electron microscopy. Proc Natl Acad Sci U S A. 2006 Nov 21;103(47):17777-82.

121. Lindvall O. Update on fetal transplantation: the Swedish experience. Mov Disord. 1998;13 Suppl 1:83-7.

122. Lindvall O, Brundin P, Widner H, et al. In Reply: Fetal Brain Grafts and Parkinson's Disease. Science. 1990 Dec 7;250(4986):1435.

123. Lindvall O, Hagell P. Clinical observations after neural transplantation in Parkinson's disease. Prog Brain Res. 2000;127:299-320.

124. Lindvall O, Rehncrona S, Brundin P, et al. Human fetal dopamine neurons grafted into the striatum in two patients with severe Parkinson's disease. A detailed account of methodology and a 6-month follow-up. Arch Neurol. 1989 Jun;46(6):615-31.

125. Lindvall O, Rehncrona S, Brundin P, et al. Neural transplantation in Parkinson's disease: the Swedish experience. Prog Brain Res. 1990;82:729-34.

126. Lindvall O, Widner H, Rehncrona S, et al. Transplantation of fetal dopamine neurons in Parkinson's disease: one-year clinical and neurophysiological observations in two patients with putaminal implants. Ann Neurol. 1992 Feb;31(2):155-65.

127. Kordower JH, Freeman TB, Chen EY, et al. Fetal nigral grafts survive and mediate clinical benefit in a patient with Parkinson's disease. Mov Disord. 1998 May;13(3):383-93.

128. Hauser RA, Freeman TB, Snow BJ, et al. Long-term evaluation of bilateral fetal nigral transplantation in Parkinson disease. Arch Neurol. 1999 Feb;56(2):179-87.

129. Hagell P, Brundin P. Cell survival and clinical outcome following intrastriatal transplantation in Parkinson disease. J Neuropathol Exp Neurol. 2001 Aug;60(8):741-52.

130. Hagell P, Schrag A, Piccini P, et al. Sequential bilateral transplantation in Parkinson's disease: effects of the second graft. Brain. 1999 Jun;122 (Pt 6):1121-32.

131. Freeman TB, Olanow CW, Hauser RA, et al. Bilateral fetal nigral transplantation into the postcommissural putamen in Parkinson's disease. Ann Neurol. 1995 Sep;38(3):379-88.

132. Olanow CW, Freeman T, Kordower J. Transplantation of embryonic dopamine neurons for severe Parkinson's disease. N Engl J Med. 2001 Jul 12;345(2):146; author reply 7.

133. Redmond DE, Jr. Cellular replacement therapy for Parkinson's disease--where we are today? Neuroscientist. 2002 Oct;8(5):457-88.

134. Baier H, Bonhoeffer F. Attractive axon guidance molecules. Science. 1994 Sep 9;265(5178):1541-2.

135. Bjugstad KB, Redmond DE, Jr., Teng YD, et al. Neural stem cells implanted into MPTP-treated monkeys increase the size of endogenous tyrosine hydroxylase-positive cells found in the striatum: a return to control measures. Cell Transplant. 2005;14(4):183-92.

136. Bjugstad KB, Teng YD, Redmond DE, Jr., et al. Human neural stem cells migrate along the nigrostriatal pathway in a primate model of Parkinson's disease. Experimental neurology. 2008 Jun;211(2):362-9.

137. Agasse F, Benzakour O, Berjeaud JM, Roger M, Coronas V. Endogenous factors derived from embryonic cortex regulate proliferation and neuronal differentiation of postnatal subventricular zone cell cultures. The European journal of neuroscience. 2006 Apr;23(8):1970-6.

138. Agasse F, Roger M, Coronas V. Neurogenic and intact or apoptotic non-neurogenic areas of adult brain release diffusible molecules that differentially modulate the development of subventricular zone cell cultures. The European journal of neuroscience. 2004 Mar;19(6):1459-68.

139. Maciaczyk J, Singec I, Maciaczyk D, Nikkhah G. Combined use of BDNF, ascorbic acid, low oxygen, and prolonged differentiation time generates tyrosine hydroxylase-expressing neurons after long-term in vitro expansion of human fetal midbrain precursor cells. Exp Neurol. 2008 Oct;213(2):354-62.

140. Jin G, Tan X, Tian M, et al. The controlled differentiation of human neural stem cells into TH-immunoreactive (ir) neurons in vitro. Neurosci Lett. 2005 Sep 30;386(2):105-10.

141. O'Keeffe FE, Scott SA, Tyers P, et al. Induction of A9 dopaminergic neurons from neural stem cells improves motor function in an animal model of Parkinson's disease. Brain. 2008 Mar;131(Pt 3):630-41.

142. Wakeman DR, Hofmann MR, Redmond Jr. DE, Teng YD, Snyder EY. Long-Term Multilayer Adherent Network (MAN) Expansion, Maintenance, and Characterization, Chemical and Genetic Manipulation, and Transplantation of Human Fetal Forebrain Neural Stem Cells (Unit 2.D) Current Protocols in Stem Cell Biology. Hoboken: Wiley, 2009.

143. Caldwell MA, He X, Svendsen CN. 5-Bromo-2'-deoxyuridine is selectively toxic to neuronal precursors in vitro. Eur J Neurosci. 2005 Dec;22(11):2965-70.

144. Burns TC, Ortiz-Gonzalez XR, Gutierrez-Perez M, et al. Thymidine analogs are transferred from prelabeled donor to host cells in the central nervous system after transplantation: a word of caution. Stem Cells. 2006 Apr;24(4):1121-7.

145. Glaser T, Pollard SM, Smith A, Brustle O. Tripotential differentiation of adherently expandable neural stem (NS) cells. PLoS One. 2007;2(3):e298.

146. Pollard S, Conti L, Smith A. Exploitation of adherent neural stem cells in basic and applied neurobiology. Regen Med. 2006 Jan;1(1):111-8.

147. Pollard SM, Conti L, Sun Y, Goffredo D, Smith A. Adherent neural stem (NS) cells from fetal and adult forebrain. Cereb Cortex. 2006 Jul;16 Suppl 1:i112-20.

148. Pollard SM, Wallbank R, Tomlinson S, Grotewold L, Smith A. Fibroblast growth factor induces a neural stem cell phenotype in foetal forebrain progenitors and during embryonic stem cell differentiation. Mol Cell Neurosci. 2008 Jul;38(3):393-403.

149. Conti L, Pollard SM, Gorba T, et al. Niche-independent symmetrical self-renewal of a mammalian tissue stem cell. PLoS Biol. 2005 Sep;3(9):e283.

150. Schwartz PH, Bryant PJ, Fuja TJ, Su H, O'Dowd DK, Klassen H. Isolation and characterization of neural progenitor cells from post-mortem human cortex. J Neurosci Res. 2003 Dec 15;74(6):838-51.

151. Nethercott H, Maxwell H, Schwartz PH. Neural Progenitor Cell Culture. In: Loring JF, Wesselschmidt RL, Schwartz PH (eds). Human Stem Cell Manual: A Laboratory Guide. 1 ed. NY, NY: Elsevier Academic Press, 2007:309-31.

152. Palmer TD, Schwartz PH, Taupin P, Kaspar B, Stein SA, Gage FH. Cell culture. Progenitor cells from human brain after death. Nature. 2001 May 3;411(6833):42-3.

153. Pollard SM, Yoshikawa K, Clarke ID, et al. Glioma stem cell lines expanded in adherent culture have tumor-specific phenotypes and are suitable for chemical and genetic screens. Cell Stem Cell. 2009 Jun 5;4(6):568-80.

154. Wright LS, Li J, Caldwell MA, Wallace K, Johnson JA, Svendsen CN. Gene expression in human neural stem cells: effects of leukemia inhibitory factor. Journal of Neurochemistry. 2003 Jul;86(1):179-95.

155. Wright LS, Prowse KR, Wallace K, Linskens MH, Svendsen CN. Human progenitor cells isolated from the developing cortex undergo decreased neurogenesis and eventual senescence following expansion in vitro. Exp Cell Res. 2006 Jul 1;312(11):2107-20.

156. Laurent LC, Chen J, Ulitsky I, et al. Comprehensive microRNA profiling reveals a unique human embryonic stem cell signature dominated by a single seed sequence. Stem Cells. 2008 Jun;26(6):1506-16.

157. Muller FJ, Laurent LC, Kostka D, et al. Regulatory networks define phenotypic classes of human stem cell lines. Nature. 2008 Sep 18;455(7211):401-5.

158. Kim HT, Kim IS, Lee IS, Lee JP, Snyder EY, Park KI. Human neurospheres derived from the fetal central nervous system are regionally and temporally specified but are not committed. Exp Neurol. 2006 May;199(1):222-35.

159. Kim JB, Greber B, Arauzo-Bravo MJ, et al. Direct reprogramming of human neural stem cells by OCT4. Nature. 2009 Oct 1;461(7264):649-3.

160. Kondoh H, Kamachi Y. SOX-partner code for cell specification: Regulatory target selection and underlying molecular mechanisms. Int J Biochem Cell Biol. 2009 Sep 9.

161. Genethliou N, Panayiotou E, Panayi H, et al. SOX1 links the function of neural patterning and Notch signalling in the ventral spinal cord during the neuron-glial fate switch. Biochem Biophys Res Commun. 2009 Dec 25;390(4):1114-20.

162. Babaie Y, Herwig R, Greber B, et al. Analysis of Oct4-dependent transcriptional networks regulating self-renewal and pluripotency in human embryonic stem cells. Stem Cells. 2007 Feb;25(2):500-10.

163. Baltus GA, Kowalski MP, Tutter AV, Kadam S. A positive regulatory role for the mSin3A-HDAC complex in pluripotency through Nanog and Sox2. J Biol Chem. 2009 Mar 13;284(11):6998-7006.

164. Campbell PA, Perez-Iratxeta C, Andrade-Navarro MA, Rudnicki MA. Oct4 targets regulatory nodes to modulate stem cell function. PLoS One. 2007;2(6):e553.

165. Chavez L, Bais AS, Vingron M, Lehrach H, Adjaye J, Herwig R. In silico identification of a core regulatory network of OCT4 in human embryonic stem cells using an integrated approach. BMC Genomics. 2009;10:314.

166. Chen X, Vega VB, Ng HH. Transcriptional regulatory networks in embryonic stem cells. Cold Spring Harb Symp Quant Biol. 2008;73:203-9.

167. Chen X, Xu H, Yuan P, et al. Integration of external signaling pathways with the core transcriptional network in embryonic stem cells. Cell. 2008 Jun 13;133(6):1106-17.

168. Kashyap V, Rezende NC, Scotland KB, et al. Regulation of stem cell pluripotency and differentiation involves a mutual regulatory circuit of the NANOG, OCT4, and SOX2 pluripotency transcription factors with polycomb repressive complexes and stem cell microRNAs. Stem Cells Dev. 2009 Sep;18(7):1093-108.

169. Kim J, Chu J, Shen X, Wang J, Orkin SH. An extended transcriptional network for pluripotency of embryonic stem cells. Cell. 2008 Mar 21;132(6):1049-61.

170. Orkin SH, Wang J, Kim J, et al. The transcriptional network controlling pluripotency in ES cells. Cold Spring Harb Symp Quant Biol. 2008;73:195-202.

171. Pan G, Thomson JA. Nanog and transcriptional networks in embryonic stem cell pluripotency. Cell Res. 2007 Jan;17(1):42-9.

172. Suzuki A, Raya A, Kawakami Y, et al. Nanog binds to Smad1 and blocks bone morphogenetic protein-induced differentiation of embryonic stem cells. Proc Natl Acad Sci U S A. 2006 Jul 5;103(27):10294-9.

173. Tay Y, Zhang J, Thomson AM, Lim B, Rigoutsos I. MicroRNAs to Nanog, Oct4 and Sox2 coding regions modulate embryonic stem cell differentiation. Nature. 2008 Oct 23;455(7216):1124-8.

174. D'Amour KA, Gage FH. Genetic and functional differences between multipotent neural and pluripotent embryonic stem cells. Proceedings of the National Academy of Sciences of the United States of America. 2003 Sep 30;100 Suppl 1:11866-72.

175. Tonchev AB, Yamashima T, Sawamoto K, Okano H. Transcription factor protein expression patterns by neural or neuronal progenitor cells of adult monkey subventricular zone. Neuroscience. 2006;139(4):1355-67.

176. Osumi N, Shinohara H, Numayama-Tsuruta K, Maekawa M. Concise review: Pax6 transcription factor contributes to both embryonic and adult neurogenesis as a multifunctional regulator. Stem Cells. 2008 Jul;26(7):1663-72.

177. Mo Z, Zecevic N. Is Pax6 critical for neurogenesis in the human fetal brain? Cereb Cortex. 2008 Jun;18(6):1455-65.

178. Kallur T, Gisler R, Lindvall O, Kokaia Z. Pax6 promotes neurogenesis in human neural stem cells. Mol Cell Neurosci. 2008 Aug;38(4):616-28.

179. Baer K, Eriksson PS, Faull RL, Rees MI, Curtis MA. Sox-2 is expressed by glial and progenitor cells and Pax-6 is expressed by neuroblasts in the human subventricular zone. Exp Neurol. 2007 Apr;204(2):828-31.

180. Barraud P, Thompson L, Kirik D, Bjorklund A, Parmar M. Isolation and characterization of neural precursor cells from the Sox1-GFP reporter mouse. Eur J Neurosci. 2005 Oct;22(7):1555-69.

181. Suter DM, Tirefort D, Julien S, Krause KH. A Sox1 to Pax6 switch drives neuroectoderm to radial glia progression during differentiation of mouse embryonic stem cells. Stem Cells. 2009 Jan;27(1):49-58.

182. Chung S, Leung A, Han BS, et al. Wnt1-lmx1a forms a novel autoregulatory loop and controls midbrain dopaminergic differentiation synergistically with the SHH-FoxA2 pathway. Cell Stem Cell. 2009 Dec 4;5(6):646-58.

183. Lee HS, Bae EJ, Yi SH, et al. Foxa2 and Nurr1 Synergistically Yield A9 Nigral Dopamine Neurons Exhibiting Improved Differentiation, Function and Cell Survival. Stem Cells. 2010 Jan 4.

184. Inestrosa NC, Arenas E. Emerging roles of Wnts in the adult nervous system. Nat Rev Neurosci. Feb;11(2):77-86.

185. Smits SM, Burbach JP, Smidt MP. Developmental origin and fate of meso-diencephalic dopamine neurons. Prog Neurobiol. 2006 Jan;78(1):1-16.

186. Toledo EM, Colombres M, Inestrosa NC. Wnt signaling in neuroprotection and stem cell differentiation. Prog Neurobiol. 2008 Nov;86(3):281-96.

187. Friling S, Andersson E, Thompson LH, et al. Efficient production of mesencephalic dopamine neurons by Lmx1a expression in embryonic stem cells. Proc Natl Acad Sci U S A. 2009 May 5;106(18):7613-8.

188. Hedlund E, Pruszak J, Lardaro T, et al. Embryonic stem cell-derived Pitx3-enhanced green fluorescent protein midbrain dopamine neurons survive enrichment by fluorescence-activated cell sorting and function in an animal model of Parkinson's disease. Stem Cells. 2008 Jun;26(6):1526-36.

189. Jonsson ME, Ono Y, Bjorklund A, Thompson LH. Identification of transplantable dopamine neuron precursors at different stages of midbrain neurogenesis. Exp Neurol. 2009 Sep;219(1):341-54.

190. Kadkhodaei B, Ito T, Joodmardi E, et al. Nurr1 is required for maintenance of maturing and adult midbrain dopamine neurons. J Neurosci. 2009 Dec 16;29(50):15923-32.

191. Kim HJ, Sugimori M, Nakafuku M, Svendsen CN. Control of neurogenesis and tyrosine hydroxylase expression in neural progenitor cells through bHLH proteins and Nurr1. Exp Neurol. 2007 Feb;203(2):394-405.

192. Martinez-Cerdeno V, Noctor SC, Kriegstein AR. The role of intermediate progenitor cells in the evolutionary expansion of the cerebral cortex. Cereb Cortex. 2006 Jul;16 Suppl 1:i152-61.

193. Menezes JR, Luskin MB. Expression of neuron-specific tubulin defines a novel population in the proliferative layers of the developing telencephalon. The Journal of neuroscience : the official journal of the Society for Neuroscience. 1994 Sep;14(9):5399-416.

194. Doetsch F, Garcia-Verdugo JM, Alvarez-Buylla A. Cellular composition and three-dimensional organization of the subventricular germinal zone in the adult mammalian brain. J Neurosci. 1997 Jul 1;17(13):5046-61.

195. Lois C, Alvarez-Buylla A. Proliferating subventricular zone cells in the adult mammalian forebrain can differentiate into neurons and glia. Proc Natl Acad Sci U S A. 1993 Mar 1;90(5):2074-7.

196. Lois C, Alvarez-Buylla A. Long-distance neuronal migration in the adult mammalian brain. Science. 1994 May 20;264(5162):1145-8.

197. Lois C, Garcia-Verdugo JM, Alvarez-Buylla A. Chain migration of neuronal precursors. Science. 1996 Feb 16;271(5251):978-81.

198. Rousselot P, Lois C, Alvarez-Buylla A. Embryonic (PSA) N-CAM reveals chains of migrating neuroblasts between the lateral ventricle and the olfactory bulb of adult mice. J Comp Neurol. 1995 Jan 2;351(1):51-61.

199. Rutishauser U. Polysialic acid in the plasticity of the developing and adult vertebrate nervous system. Nat Rev Neurosci. 2008 Jan;9(1):26-35.

200. Angata K, Huckaby V, Ranscht B, Terskikh A, Marth JD, Fukuda M. Polysialic acid-directed migration and differentiation of neural precursors are essential for mouse brain development. Mol Cell Biol. 2007 Oct;27(19):6659-68.

201. Burgess A, Wainwright SR, Shihabuddin LS, Rutishauser U, Seki T, Aubert I. Polysialic acid regulates the clustering, migration, and neuronal differentiation of progenitor cells in the adult hippocampus. Dev Neurobiol. 2008 Dec;68(14):1580-90.

202. Hu H, Tomasiewicz H, Magnuson T, Rutishauser U. The role of polysialic acid in migration of olfactory bulb interneuron precursors in the subventricular zone. Neuron. 1996 Apr;16(4):735-43.

203. Feng L, Hatten ME, Heintz N. Brain lipid-binding protein (BLBP): a novel signaling system in the developing mammalian CNS. Neuron. 1994 Apr;12(4):895-908.

204. Hartfuss E, Galli R, Heins N, Gotz M. Characterization of CNS precursor subtypes and radial glia. Developmental biology. 2001 Jan 1;229(1):15-30.

205. Tramontin AD, Garcia-Verdugo JM, Lim DA, Alvarez-Buylla A. Postnatal development of radial glia and the ventricular zone (VZ): a continuum of the neural stem cell compartment. Cerebral cortex (New York, NY: 1991). 2003 Jun;13(6):580-7.

206. Weissman T, Noctor SC, Clinton BK, Honig LS, Kriegstein AR. Neurogenic radial glial cells in reptile, rodent and human: from mitosis to migration. Cereb Cortex. 2003 Jun;13(6):550-9.

207. Alvarez-Buylla A, Garcia-Verdugo JM, Tramontin AD. A unified hypothesis on the lineage of neural stem cells. Nature reviewsNeuroscience. 2001 Apr;2(4):287-93.

208. Alvarez-Buylla A, Lim DA. For the long run: maintaining germinal niches in the adult brain. Neuron. 2004 Mar 4;41(5):683-6.

209. Pollard SM, Conti L. Investigating radial glia in vitro. Progress in neurobiology. 2007 Sep;83(1):53-67.

210. Malatesta P, Appolloni I, Calzolari F. Radial glia and neural stem cells. Cell Tissue Res. 2008 Jan;331(1):165-78.

211. Merkle FT, Alvarez-Buylla A. Neural stem cells in mammalian development. Current opinion in cell biology. 2006 Dec;18(6):704-9.

212. Merkle FT, Tramontin AD, Garcia-Verdugo JM, Alvarez-Buylla A. Radial glia give rise to adult neural stem cells in the subventricular zone. Proceedings of the

National Academy of Sciences of the United States of America. 2004 Dec 14;101(50):17528-32.

213. Noctor SC, Flint AC, Weissman TA, Wong WS, Clinton BK, Kriegstein AR. Dividing precursor cells of the embryonic cortical ventricular zone have morphological and molecular characteristics of radial glia. The Journal of neuroscience : the official journal of the Society for Neuroscience. 2002 Apr 15;22(8):3161-73.

214. Alvarez-Buylla A, Temple S. Stem cells in the developing and adult nervous system. J Neurobiology. 1998;36:105-10.

215. Alvarez-Buylla A, Seri B, Doetsch F. Identification of neural stem cells in the adult vertebrate brain. Brain Res Bull. 2002 Apr;57(6):751-8.

216. Doetsch F. The glial identity of neural stem cells. Nature neuroscience. 2003 Nov;6(11):1127-34.

217. Doetsch F. A niche for adult neural stem cells. Curr Opin Genet Dev. 2003 Oct;13(5):543-50.

218. Doetsch F, Alvarez-Buylla A. Network of tangential pathways for neuronal migration in adult mammalian brain. Proc Natl Acad Sci U S A. 1996 Dec 10;93(25):14895-900.

219. Doetsch F, Caille I, Lim DA, Garcia-Verdugo JM, Alvarez-Buylla A. Subventricular zone astrocytes are neural stem cells in the adult mammalian brain. Cell. 1999 Jun 11;97(6):703-16.

220. Garcia-Verdugo JM, Doetsch F, Wichterle H, Lim DA, Alvarez-Buylla A. Architecture and cell types of the adult subventricular zone: in search of the stem cells. J Neurobiol. 1998 Aug;36(2):234-48.

221. Tavazoie M, Van der Veken L, Silva-Vargas V, et al. A specialized vascular niche for adult neural stem cells. Cell Stem Cell. 2008 Sep 11;3(3):279-88.

222. Garcia AD, Doan NB, Imura T, Bush TG, Sofroniew MV. GFAP-expressing progenitors are the principal source of constitutive neurogenesis in adult mouse forebrain. Nature neuroscience. 2004 Nov;7(11):1233-41.

223. Imura T, Kornblum HI, Sofroniew MV. The predominant neural stem cell isolated from postnatal and adult forebrain but not early embryonic forebrain expresses GFAP. The Journal of neuroscience : the official journal of the Society for Neuroscience. 2003 Apr 1;23(7):2824-32.

224. Middeldorp J, Boer K, Sluijs JA, et al. GFAPdelta in radial glia and subventricular zone progenitors in the developing human cortex. Development. Jan;137(2):313-21.

225. Quinones-Hinojosa A, Sanai N, Soriano-Navarro M, et al. Cellular composition and cytoarchitecture of the adult human subventricular zone: a niche of neural stem cells. J Comp Neurol. 2006 Jan 20;494(3):415-34.

226. Quinones-Hinojosa A, Sanai N, Gonzalez-Perez O, Garcia-Verdugo JM. The human brain subventricular zone: stem cells in this niche and its organization. Neurosurg Clin N Am. 2007 Jan;18(1):15-20, vii.

227. Gotz M. Glial cells generate neurons--master control within CNS regions: developmental perspectives on neural stem cells. The Neuroscientist : a review journal bringing neurobiology, neurology and psychiatry. 2003 Oct;9(5):379-97.

228. Gotz M, Barde YA. Radial glial cells defined and major intermediates between embryonic stem cells and CNS neurons. Neuron. 2005 May 5;46(3):369-72.

229. Gotz M, Hartfuss E, Malatesta P. Radial glial cells as neuronal precursors: a new perspective on the correlation of morphology and lineage restriction in the developing cerebral cortex of mice. Brain research bulletin. 2002 Apr;57(6):777-88.

230. Gleeson JG, Lin PT, Flanagan LA, Walsh CA. Doublecortin is a microtubule-associated protein and is expressed widely by migrating neurons. Neuron. 1999 Jun;23(2):257-71.

231. Francis F, Koulakoff A, Boucher D, et al. Doublecortin is a developmentally regulated, microtubule-associated protein expressed in migrating and differentiating neurons. Neuron. 1999 Jun;23(2):247-56.

232. Friocourt G, Koulakoff A, Chafey P, et al. Doublecortin functions at the extremities of growing neuronal processes. Cerebral cortex (New York, NY: 1991). 2003 Jun;13(6):620-6.

233. Anderson L, Burnstein RM, He X, et al. Gene expression changes in long term expanded human neural progenitor cells passaged by chopping lead to loss of neurogenic potential in vivo. Experimental neurology. 2007 Apr;204(2):512-24.

234. Aberg MA, Aberg ND, Palmer TD, et al. IGF-I has a direct proliferative effect in adult hippocampal progenitor cells. Mol Cell Neurosci. 2003 Sep;24(1):23-40.

235. Arsenijevic Y, Weiss S, Schneider B, Aebischer P. Insulin-Like Growth Factor-I Is Necessary for Neural Stem Cell Proliferation and Demonstrates Distinct Actions of Epidermal Growth Factor and Fibroblast Growth Factor-2. Journal of Neuroscience. 2001 September 15;21(18):7194-202.

236. Bogler O, Wren D, Barnett SC, Land H, Noble M. Cooperation between two growth factors promotes extended self-renewal and inhibits differentiation of oligodendrocyte-type-2 astrocyte (O-2A) progenitor cells. Proceedings of the National Academy of Sciences of the United States of America. 1990 Aug;87(16):6368-72.

237. Cai J, Wu Y, Mirua T, et al. Properties of a fetal multipotent neural stem cell (NEP cell). Dev Biol. 2002 Nov 15;251(2):221-40.

238. Caldwell MA, He X, Wilkie N, et al. Growth factors regulate the survival and fate of cells derived from human neurospheres. Nature biotechnology. 2001 May;19(5):475-9.

239. Chojnacki A, Weiss S. Isolation of a Novel Platelet-Derived Growth Factor-Responsive Precursor from the Embryonic Ventral Forebrain. Journal of Neuroscience. 2004 December 1;24(48):10888-99.

240. Galli R, Pagano SF, Gritti A, Vescovi AL. Regulation of neuronal differentiation in human CNS stem cell progeny by leukemia inhibitory factor. Developmental neuroscience. 2000;22(1-2):86-95.

241. Johe KK, Hazel TG, Muller T, Dugich-Djordjevic MM, McKay RD. Single factors direct the differentiation of stem cells from the fetal and adult central nervous system. Genes Dev. 1996 Dec 15;10(24):3129-40.

242. van Heyningen P, Calver AR, Richardson WD. Control of progenitor cell number by mitogen supply and demand. Current biology : CB. 2001 Feb 20;11(4):232-41.

243. Ciccolini F, Svendsen CN. Neurotrophin responsiveness is differentially regulated in neurons and precursors isolated from the developing striatum. J Mol Neurosci. 2001 Aug;17(1):25-33.

244. Takahashi J, Palmer TD, Gage FH. Retinoic acid and neurotrophins collaborate to regulate neurogenesis in adult-derived neural stem cell cultures. J Neurobiol. 1999 Jan;38(1):65-81.

245. Goldman S. Glia as neural progenitor cells. Trends in neurosciences. 2003 Nov;26(11):590-6.

246. Anderson DJ. Stem cells and pattern formation in the nervous system: the possible versus the actual. Neuron. 2001 Apr;30(1):19-35.

247. Daadi M, Arcellana-Panlilio M, Weiss S. Activin co-operates with fibroblast growth factor 2 to regulate tyrosine hydroxylase expression in the basal forebrain ventricular zone progenitors. Neuroscience. 1998;86:867-80.

248. Daadi MM. In vitro assays for neural stem cell differentiation: induction of dopaminergic phenotype. Methods Mol Biol. 2008;438:205-12.

249. Daadi MM, Weiss S. Generation of tyrosine hydroxylase-producing neurons from precursors of the embryonic and adult forebrain. J Neurosci. 1999 Jun 1;19(11):4484-97.

250. Espinosa-Jeffrey A, Wakeman DR, Kim SU, Snyder EY, de Vellis J. Culture system for rodent and human oligodendrocyte specification, lineage progression, and maturation. Curr Protoc Stem Cell Biol. 2009 Sep;Chapter 2:Unit 2D 4.

251. Bedard P, Larochelle L, Parent A, Poirier LJ. The nigrostriatal pathway: a correlative study based on neuroanatomical and neurochemical criteria in the cat and the monkey. Exp Neurol. 1969 Nov;25(3):365-77.

252. Anden NE, Dahlstrom A, Fuxe K, et al. Ascending monoamine neurons to the telencephalon and diencephalon. . Acta Physiol Scand. 1966; 67:313–26.

253. Anden NE, Carlsson A, Dahlstroem A, Fuxe K, Hillarp NA, Larsson K. DEMONSTRATION AND MAPPING OUT OF NIGRO-NEOSTRIATAL DOPAMINE NEURONS. Life Sci. 1964 Jun;3:523-30.

254. Anden NE, Dahlstroem A, Fuxe K, Larsson K. FURTHER EVIDENCE FOR THE PRESENCE OF NIGRO-NEOSTRIATAL DOPAMINE NEURONS IN THE RAT. Am J Anat. 1965 Jan;116:329-33.

255. Anden NE, Dahlstrom A, Fuxe K, Larsson K. Functional role of the nigro-neostriatal dopamine neurons. Acta Pharmacol Toxicol (Copenh). 1966;24(2):263-74.

256. Anden NE, Fuxe K, Hamberger B, Hokfelt T. A quantitative study on the nigrostriatal DA neuron system in the rat. . Actaphysiol Scand 1966;67:306–12.

257. Anden NE, Dahlstrom A, Fuxe K, Larsson K. Mapping out of catecholamine and 5-hydroxytryptamine neurons innervating the telencephalon and diencephalon. Life Sci. 1965 Jul;4(13):1275-9.

258. Anden NE, Dahlstrom A, Fuxe K, Olson L, Ungerstedt U. Ascending noradrenaline neurons from the pons and the medulla oblongata. Experientia. 1966 Jan 15;22(1):44-5.

259. Dahlstroem A, Fuxe K. EVIDENCE FOR THE EXISTENCE OF MONOAMINE-CONTAINING NEURONS IN THE CENTRAL NERVOUS SYSTEM. I. DEMONSTRATION OF MONOAMINES IN THE CELL BODIES OF BRAIN STEM NEURONS. Acta Physiol Scand Suppl. 1964:SUPPL 232:1-55.

260. Dahlstroem A, Fuxe K. A METHOD FOR THE DEMONSTRATION OF MONOAMINE-CONTAINING NERVE FIBRES IN THE CENTRAL NERVOUS SYSTEM. Acta Physiol Scand. 1964 Mar;60:293-4.

261. Dahlstroem A, Fuxe K. EVIDENCE FOR THE EXISTENCE OF MONOAMINE NEURONS IN THE CENTRAL NERVOUS SYSTEM. II. EXPERIMENTALLY INDUCED CHANGES IN THE INTRANEURONAL AMINE LEVELS OF BULBOSPINAL NEURON SYSTEMS. Acta Physiol Scand Suppl. 1965:SUPPL 247:1-36.

262. Dahlstroem A, Fuxe K, Olson L, Ungerstedt U. ASCENDING SYSTEMS OF CATECHOLAMINE NEURONS FROM THE LOWER BRAIN STEM. Acta Physiol Scand. 1964 Dec;62:485-6.

263. Dahlstrom A, Fuxe K. Localization of monoamines in the lower brain stem. Experientia. 1964 Jul 15;20(7):398-9.

264. Fuxe K. CELLULAR LOCALIZATION OF MONOAMINES IN THE MEDIAN EMINENCE AND IN THE INFUNDIBULAR STEM OF SOME MAMMALS. Acta Physiol Scand. 1963 Aug;58:383-4.

265. Fuxe K. CELLULAR LOCALIZATION OF MONOAMINES IN THE MEDIAN EMINENCE AND THE INFUNDIBULAR STEM OF SOME MAMMALS. Z Zellforsch Mikrosk Anat. 1964;61:710-24.

266. Fuxe K. EVIDENCE FOR THE EXISTENCE OF MONOAMINE NEURONS IN THE CENTRAL NERVOUS SYSTEM. 3. THE MONOAMINE NERVE TERMINAL. Z Zellforsch Mikrosk Anat. 1965 Feb 9;65:573-96.

267. Fuxe K. EVIDENCE FOR THE EXISTENCE OF MONOAMINE NEURONS IN THE CENTRAL NERVOUS SYSTEM. IV. DISTRIBUTION OF MONOAMINE NERVE TERMINALS IN THE CENTRAL NERVOUS SYSTEM. Acta Physiol Scand Suppl. 1965:SUPPL 247:37+.

268. Fuxe K, Dahlstrom A, Hoistad M, et al. From the Golgi-Cajal mapping to the transmitter-based characterization of the neuronal networks leading to two modes of brain communication: wiring and volume transmission. Brain Res Rev. 2007 Aug;55(1):17-54.

269. Fuxe K, Dahlstrom A, Jonsson G, et al. The discovery of central monoamine neurons gave volume transmission to the wired brain. Prog Neurobiol. 2009 Oct 21.

270. Fuxe K, Hoekfelt T, Nilsson O. OBSERVATIONS ON THE CELLULAR LOCALIZATION OF DOPAMINE IN THE CAUDATE NUCLEUS OF THE RAT. Z Zellforsch Mikrosk Anat. 1964 Aug 18;63:701-6.

271. Fuxe K, Hokfelt T, Nilsson O, Reinius S. A fluorescence and electron microscopic study on central monoamine nerve cells. Anat Rec. 1966 May;155(1):33-40.

272. Fuxe K, Hokfelt T, O. N. A fluorescence microscopic and electronmicroscopic study on certain areas in the central nervous system, rich in monoamine terminals. J Amer Anat. 1964;117:33-46.

273. Fuxe K, Hokfelt T, Ungerstedt U. Morphological and functional aspects of central monoamine neurons. . Int Rev Neurobiol 1970;13:93–126.

274. Fuxe K, Jonsson G. The histochemical fluorescence method for the demonstration of catecholamines. Theory, practice and application. J Histochem Cytochem. 1973 Apr;21(4):293-311.

275. Fuxe K, Manger P, Genedani S, Agnati L. The nigrostriatal DA pathway and Parkinson's disease. J Neural Transm Suppl. 2006(70):71-83.

276. Fuxe K, Ungerstedt U. Histochemical, biochemical and functional studies on central monoamine neurons after acute and chronic amphetamine administration. . In: Costa E, Garattini S (eds). Amphetamines and Related Compounds. New York: Raven Press, 1970:257–88.

277. Hillarp NA, Fuxe K, Dahlstrom A. Demonstration and mapping of central neurons containing dopamine, noradrenaline, and 5-hydroxytryptamine and their reactions to psychopharmaca. Pharmacol Rev. 1966 Mar;18(1):727-41.

278. Olson L, Seiger A, Fuxe K. Heterogeneity of striatal and limbic dopamine innervation: highly fluorescent islands in developing and adult rats. Brain Res. 1972 Sep 15;44(1):283-8.

279. Olson L, Seiger A. Early prenatal ontogeny of central monoamine neurons in the rat: fluorescence histochemical observations. Z Anat Entwicklungsgesch. 1972;137(3):301-16.

280. Bjorklund A, Falck B, Stenevi U. Classification of monoamine neurones in the rat mesencephalon: distribution of a new monoamine neurone system. Brain Res. 1971 Sep 24;32(2):269-85.

281. Bjorklund A, Nobin A. Fluorescence histochemical and microspectrofluorometric mapping of dopamine and noradrenaline cell groups in the rat diencephalon. Brain Res. 1973 Mar 15;51:193-205.

282. Amariglio N, Hirshberg A, Scheithauer BW, et al. Donor-derived brain tumor following neural stem cell transplantation in an ataxia telangiectasia patient. PLoS Med. 2009 Feb 17;6(2):e1000029.

283. Englund U, Bjorklund A, Wictorin K. Migration patterns and phenotypic differentiation of long-term expanded human neural progenitor cells after transplantation into the adult rat brain. Brain Res Dev Brain Res. 2002 Mar 31;134(1-2):123-41.

284. Englund U, Fricker-Gates RA, Lundberg C, Bjorklund A, Wictorin K. Transplantation of human neural progenitor cells into the neonatal rat brain: extensive migration and differentiation with long-distance axonal projections. Exp Neurol. 2002 Jan;173(1):1-21.

285. Eriksson C, Bjorklund A, Wictorin K. Neuronal differentiation following transplantation of expanded mouse neurosphere cultures derived from different embryonic forebrain regions. Exp Neurol. 2003 Dec;184(2):615-35.

286. Rosenblad C, Martinez-Serrano A, Bjorklund A. Glial cell line-derived neurotrophic factor increases survival, growth and function of intrastriatal fetal nigral dopaminergic grafts. Neuroscience. 1996;75(4):979-85.

287. Rosenblad C, Martinez-Serrano A, Bjorklund A. Intrastriatal glial cell line-derived neurotrophic factor promotes sprouting of spared nigrostriatal dopaminergic afferents and induces recovery of function in a rat model of Parkinson's disease. Neuroscience. 1998 Jan;82(1):129-37.

288. Burnstein RM, Foltynie T, He X, Menon DK, Svendsen CN, Caldwell MA. Differentiation and migration of long term expanded human neural progenitors in a partial lesion model of Parkinson's disease. The international journal of biochemistry & cell biology. 2004 Apr;36(4):702-13.

289. Svendsen CN, Caldwell MA, Shen J, et al. Long-term survival of human central nervous system progenitor cells transplanted into a rat model of Parkinson's disease. Experimental neurology. 1997 Nov;148(1):135-46.

290. Svendsen CN, Clarke DJ, Rosser AE, Dunnett SB. Survival and differentiation of rat and human epidermal growth factor-responsive precursor cells following grafting into the lesioned adult central nervous system. Experimental neurology. 1996 Feb;137(2):376-88.

291. Fricker RA, Carpenter MK, Winkler C, Greco C, Gates MA, Bjorklund A. Site-specific migration and neuronal differentiation of human neural progenitor cells after transplantation in the adult rat brain. The Journal of neuroscience : the official journal of the Society for Neuroscience. 1999 Jul 15;19(14):5990-6005.

292. Ostenfeld T, Caldwell MA, Prowse KR, Linskens MH, Jauniaux E, Svendsen CN. Human neural precursor cells express low levels of telomerase in vitro and show diminishing cell proliferation with extensive axonal outgrowth following transplantation. Exp Neurol. 2000 Jul;164(1):215-26.

293. Svendsen CN, ter Borg MG, Armstrong RJ, et al. A new method for the rapid and long term growth of human neural precursor cells. Journal of neuroscience methods. 1998 Dec 1;85(2):141-52.

294. Behrstock S, Ebert A, McHugh J, et al. Human neural progenitors deliver glial cell line-derived neurotrophic factor to parkinsonian rodents and aged primates. Gene therapy. 2006 Mar;13(5):379-88.

295. Behrstock S, Ebert AD, Klein S, Schmitt M, Moore JM, Svendsen CN. Lesion-induced increase in survival and migration of human neural progenitor cells releasing GDNF. Cell Transplant. 2008;17(7):753-62.

296. Yue Y, Widmer DA, Halladay AK, et al. Specification of distinct dopaminergic neural pathways: roles of the Eph family receptor EphB1 and ligand ephrin-B2. J Neurosci. 1999 Mar 15;19(6):2090-101.

297. Lin L, Isacson O. Axonal growth regulation of fetal and embryonic stem cell-derived dopaminergic neurons by Netrin-1 and Slits. Stem Cells. 2006 Nov;24(11):2504-13.

298. Lin L, Rao Y, Isacson O. Netrin-1 and slit-2 regulate and direct neurite growth of ventral midbrain dopaminergic neurons. Mol Cell Neurosci. 2005 Mar;28(3):547-55.

299. Culotti JG, Kolodkin AL. Functions of netrins and semaphorins in axon guidance. Curr Opin Neurobiol. 1996 Feb;6(1):81-8.

300. Goodman CS. Mechanisms and molecules that control growth cone guidance. Annu Rev Neurosci. 1996;19:341-77.

301. Kennedy TE, Serafini T, de la Torre JR, Tessier-Lavigne M. Netrins are diffusible chemotropic factors for commissural axons in the embryonic spinal cord. Cell. 1994 Aug 12;78(3):425-35.

302. Kennedy TE, Tessier-Lavigne M. Guidance and induction of branch formation in developing axons by target-derived diffusible factors. Curr Opin Neurobiol. 1995 Feb;5(1):83-90.

303. Round J, Stein E. Netrin signaling leading to directed growth cone steering. Curr Opin Neurobiol. 2007 Feb;17(1):15-21.

304. Serafini T, Colamarino SA, Leonardo ED, et al. Netrin-1 is required for commissural axon guidance in the developing vertebrate nervous system. Cell. 1996 Dec 13;87(6):1001-14.

305. Huber AB, Kolodkin AL, Ginty DD, Cloutier JF. Signaling at the growth cone: ligand-receptor complexes and the control of axon growth and guidance. Annu Rev Neurosci. 2003;26:509-63.

306. Matthes DJ, Sink H, Kolodkin AL, Goodman CS. Semaphorin II can function as a selective inhibitor of specific synaptic arborizations. Cell. 1995 May 19;81(4):631-9.

307. Puschel AW. Semaphorins: repulsive guidance molecules show their attractive side. Nat Neurosci. 1999 Sep;2(9):777-8.

308. Tran TS, Rubio ME, Clem RL, et al. Secreted semaphorins control spine distribution and morphogenesis in the postnatal CNS. Nature. 2009 Dec 24;462(7276):1065-9.

309. Plachez C, Richards LJ. Mechanisms of axon guidance in the developing nervous system. Curr Top Dev Biol. 2005;69:267-346.

310. Placzek M, Tessier-Lavigne M, Yamada T, Dodd J, Jessell TM. Guidance of developing axons by diffusible chemoattractants. Cold Spring Harb Symp Quant Biol. 1990;55:279-89.

311. Hidalgo A, Brand AH. Targeted neuronal ablation: the role of pioneer neurons in guidance and fasciculation in the CNS of Drosophila. Development. 1997 Sep;124(17):3253-62.

312. Klose M, Bentley D. Transient pioneer neurons are essential for formation of an embryonic peripheral nerve. Science. 1989 Sep 1;245(4921):982-4.

313. Lin DM, Auld VJ, Goodman CS. Targeted neuronal cell ablation in the Drosophila embryo: pathfinding by follower growth cones in the absence of pioneers. Neuron. 1995 Apr;14(4):707-15.

314. McConnell SK, Ghosh A, Shatz CJ. Subplate neurons pioneer the first axon pathway from the cerebral cortex. Science. 1989 Sep 1;245(4921):978-82.

315. McConnell SK, Ghosh A, Shatz CJ. Subplate pioneers and the formation of descending connections from cerebral cortex. J Neurosci. 1994 Apr;14(4):1892-907.

316. Pittman AJ, Law MY, Chien CB. Pathfinding in a large vertebrate axon tract: isotypic interactions guide retinotectal axons at multiple choice points. Development. 2008 Sep;135(17):2865-71.

317. Fienberg AA, Hiroi N, Mermelstein PG, et al. DARPP-32: regulator of the efficacy of dopaminergic neurotransmission. Science. 1998 Aug 7;281(5378):838-42.

318. Foster GA, Schultzberg M, Hokfelt T, et al. Development of a dopamine- and cyclic adenosine 3':5'-monophosphate-regulated phosphoprotein (DARPP-32) in the prenatal rat central nervous system, and its relationship to the arrival of presumptive dopaminergic innervation. J Neurosci. 1987 Jul;7(7):1994-2018.

319. Foster GA, Schultzberg M, Kokfelt T, et al. Ontogeny of the dopamine and cyclic adenosine-3':5'-monophosphate-regulated phosphoprotein (DARPP-32) in the pre- and postnatal mouse central nervous system. Int J Dev Neurosci. 1988;6(4):367-86.

320. Ouimet CC, Miller PE, Hemmings HC, Jr., Walaas SI, Greengard P. DARPP-32, a dopamine- and adenosine 3':5'-monophosphate-regulated phosphoprotein enriched in dopamine-innervated brain regions. III. Immunocytochemical localization. J Neurosci. 1984 Jan;4(1):111-24.

321. Walaas SI, Greengard P. DARPP-32, a dopamine- and adenosine 3':5'-monophosphate-regulated phosphoprotein enriched in dopamine-innervated brain regions. I. Regional and cellular distribution in the rat brain. J Neurosci. 1984 Jan;4(1):84-98.

322. Dodiya HB, Bjorklund T, Stansell Iii J, Mandel RJ, Kirik D, Kordower JH. Differential Transduction Following Basal Ganglia Administration of Distinct Pseudotyped AAV Capsid Serotypes in Nonhuman Primates. Mol Ther. 2009 Sep 22.

323. Markakis EA, Vives KP, Bober J, et al. Comparative Transduction Efficiency of AAV Vector Serotypes 1-6 in the Substantia Nigra and Striatum of the Primate Brain. Mol Ther. 2009 Dec 15.

324. Emborg ME, Ebert AD, Moirano J, et al. GDNF-secreting human neural progenitor cells increase tyrosine hydroxylase and VMAT2 expression in MPTP-treated cynomolgus monkeys. Cell Transplant. 2008;17(4):383-95.

325. Doetsch F, Petreanu L, Caille I, Garcia-Verdugo JM, Alvarez-Buylla A. EGF converts transit-amplifying neurogenic precursors in the adult brain into multipotent stem cells. Neuron. 2002 Dec 19;36(6):1021-34.

326. Santa-Olalla J, Baizabal JM, Fregoso M, del Carmen Cardenas M, Covarrubias L. The in vivo positional identity gene expression code is not preserved in neural stem cells grown in culture. Eur J Neurosci. 2003 Sep;18(5):1073-84.

327. Hunter KE, Hatten ME. Radial glial cell transformation to astrocytes is bidirectional: regulation by a diffusible factor in embryonic forebrain. Proc Natl Acad Sci U S A. 1995 Mar 14;92(6):2061-5.

328. Gaillard A, Prestoz L, Dumartin B, et al. Reestablishment of damaged adult motor pathways by grafted embryonic cortical neurons. Nat Neurosci. 2007 Oct;10(10):1294-9.

329. Lee SH, Lumelsky N, Studer L, Auerbach JM, McKay RD. Efficient generation of midbrain and hindbrain neurons from mouse embryonic stem cells. Nat Biotechnol. 2000 Jun;18(6):675-9.

330. Ben-Hur T, Idelson M, Khaner H, et al. Transplantation of human embryonic stem cell-derived neural progenitors improves behavioral deficit in Parkinsonian rats. Stem Cells. 2004;22(7):1246-55.

331. Hedlund E, Pruszak J, Ferree A, et al. Selection of embryonic stem cell-derived enhanced green fluorescent protein-positive dopamine neurons using the tyrosine hydroxylase promoter is confounded by reporter gene expression in immature cell populations. Stem Cells. 2007 May;25(5):1126-35.

332. Karki S, Pruszak J, Isacson O, Sonntag KC. ES cell-derived neuroepithelial cell cultures. J Vis Exp. 2006 Dec(1):118.

333. Pruszak J, Isacson O. Molecular and cellular determinants for generating ES-cell derived dopamine neurons for cell therapy. Adv Exp Med Biol. 2009;651:112-23.

334. Sanchez-Pernaute R, Studer L, Ferrari D, et al. Long-term survival of dopamine neurons derived from parthenogenetic primate embryonic stem cells (cyno-1) after transplantation. Stem Cells. 2005 Aug;23(7):914-22.

335. Pankratz MT, Li XJ, Lavaute TM, Lyons EA, Chen X, Zhang SC. Directed neural differentiation of human embryonic stem cells via an obligated primitive anterior stage. Stem Cells. 2007 Jun;25(6):1511-20.

336. Roy NS, Cleren C, Singh SK, Yang L, Beal MF, Goldman SA. Functional engraftment of human ES cell-derived dopaminergic neurons enriched by coculture with telomerase-immortalized midbrain astrocytes.[see

comment][erratum appears in Nat Med. 2007 Mar;13(3):385]. Nature Medicine. 2006;12(11):1259-68.

337. Do JT, Joo JY, Han DW, et al. Generation of parthenogenetic induced pluripotent stem cells from parthenogenetic neural stem cells. Stem Cells. 2009 Dec;27(12):2962-8.

338. Morizane A, Li JY, Brundin P. From bench to bed: the potential of stem cells for the treatment of Parkinson's disease. Cell Tissue Res. 2008 Jan;331(1):323-36.

339. Pruszak J, Ludwig W, Blak A, Alavian K, Isacson O. CD15, CD24, and CD29 define a surface biomarker code for neural lineage differentiation of stem cells. Stem Cells. 2009 Dec;27(12):2928-40.

340. Pruszak J, Sonntag KC, Aung MH, Sanchez-Pernaute R, Isacson O. Markers and methods for cell sorting of human embryonic stem cell-derived neural cell populations. Stem Cells. 2007 Sep;25(9):2257-68.

341. Flax JD, Aurora S, Yang C, et al. Engraftable human neural stem cells respond to developmental cues, replace neurons, and express foreign genes. Nature biotechnology. 1998 Nov;16(11):1033-9.

342. Ourednik J, Ourednik V, Lynch WP, Schachner M, Snyder EY. Neural stem cells display an inherent mechanism for rescuing dysfunctional neurons. Nat Biotechnol. 2002 Nov;20(11):1103-10.

343. Ourednik V, Ourednik J, Flax JD, et al. Segregation of human neural stem cells in the developing primate forebrain. Science (New York, NY). 2001 Sep 7;293(5536):1820-4.

344. Wakeman DR, Hofmann MR, Teng YD, Snyder EY. Derivation, Expansion, and Characterization of Human Fetal Forebrain Neural Stem Cells. In: Masters JR, Palsson BO (eds). Human Cell Culture: Adult Stem Cells. Volume 7. Dordrecht: Springer, 2009.

345. Haberman R, Kroner-Lux G, McCown T, Samulski RJ. Production of recombinant adeno-associated viral vectors and use in in vitro and in vivo administration. In: Crawley J, Gerfen C, Rogawski M, Sibley D, Skolnick P, Wray S (eds). Current protocols in neuroscience, vol 1. Volume 1. New York: John Wiley & Sons, Inc., 1999:p. 4.17.1-4..25.

346. Elsworth JD, Taylor JR, Sladek JR, Jr., Collier TJ, Redmond DE, Jr., Roth RH. Striatal dopaminergic correlates of stable parkinsonism and degree of recovery in old-world primates one year after MPTP treatment. Neuroscience. 2000;95(2):399-408.

347. Taylor JR, Elsworth JD, Sladek JR, Jr., Roth RH, Redmond DE, Jr. Behavioral effects of MPTP administration in the vervet monkey: a primate model of Parkinson's disease. In: Woodruff ML, Nonneman AJ (eds). Toxin-Induced Models of Neurological Disorders. New York: Plenum Press, 1994:139-74.

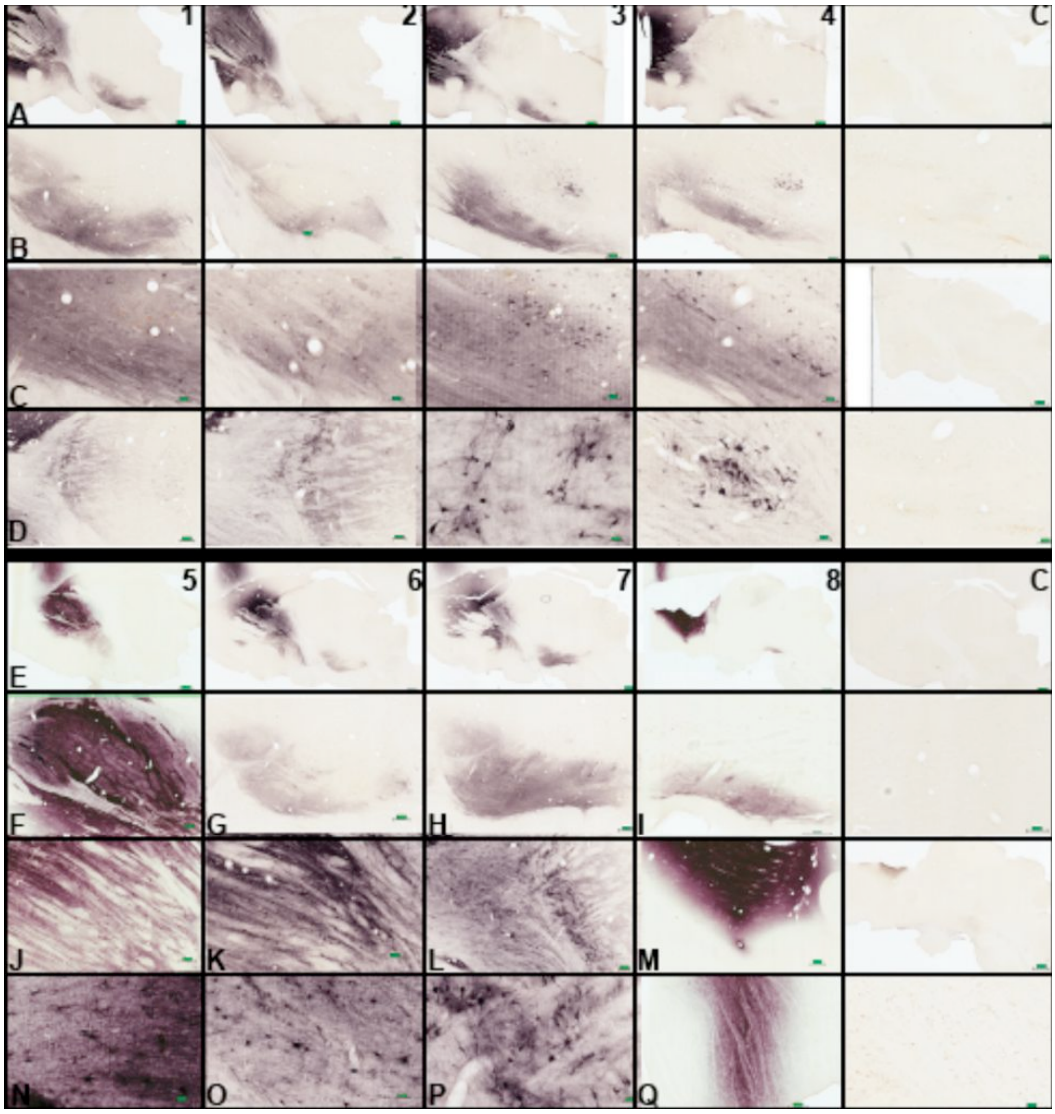
348. Taylor JR, Elsworth JD, Roth RH, Sladek JR, Jr., Redmond DE, Jr. Severe long-term 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-induced parkinsonism in the vervet monkey (Cercopithecus aethiops sabaeus). Neuroscience. 1997;81(3):745-55.

349. Przedborski S, Jackson-Lewis V, Yokoyama R, Shibata T, Dawson VL, Dawson TM. Role of neuronal nitric oxide in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced dopaminergic neurotoxicity. Proc Natl Acad Sci U S A. 1996 May 14;93(10):4565-71.

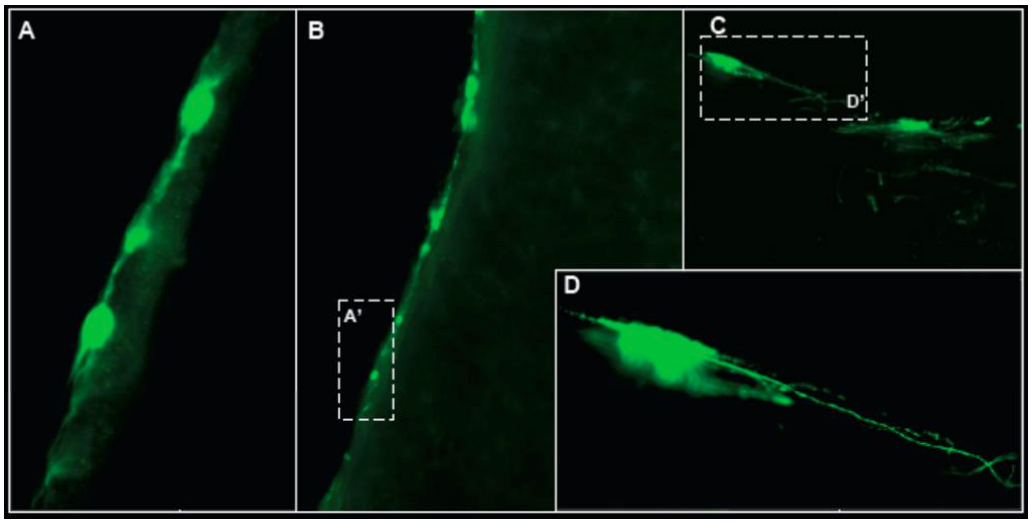
4.9 Supplementary Information:

Supplementary Table 4-S1. Experimental subjects and treatment guide.

Monkey	hfNSC subtype	Reporter	FG	Sacrifice
W843	2050-NS-eGFP	GFP	NO FG	1.5 Months
W820	2050-WT-3BIC-3.3	NO GFP	NO FG	1.5 Months
W883	2050-WT-3BIC-3.3	NO GFP	NO FG	11 Months
X955	HFB-2050-WT-DEAD	NO GFP	FG	11 Months
X850	HFB-2050-eGFP-DEAD	GFP	FG	11 Months
W841	2050-p32-eGFP	GFP	FG	11 Months
X896	2050-3BIC-3.2-GFP	GFP	FG	11 Months
Y092	2050-3BIC-3.2-GFP	GFP	FG	11 Months
X740	2050-NS-eGFP	GFP	FG	11 Months
X577	2050-p32-eGFP	GFP	FG	11 Months



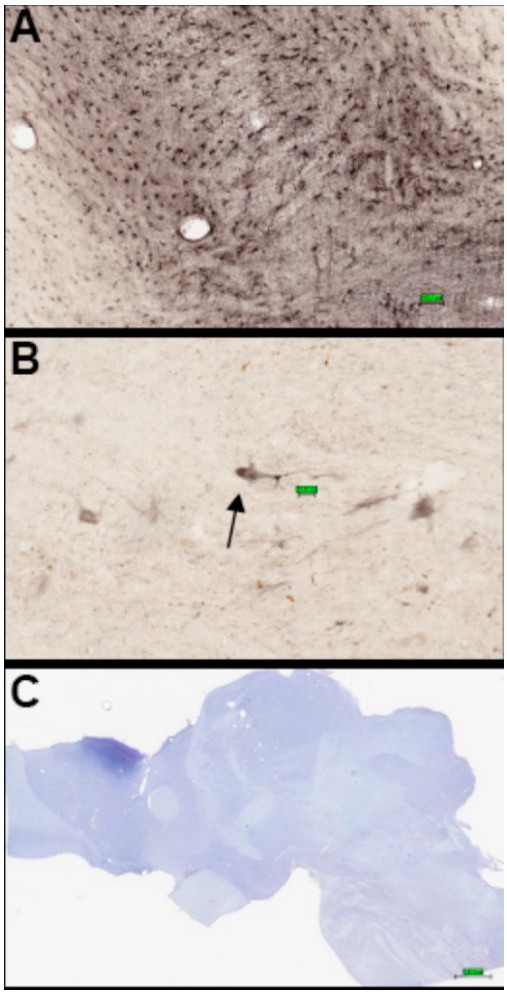
Supplementary Figure 4-S1. GDNF expression at multiple anatomical levels 1.5 months post-injection. Robust exogenous GDNF was detected throughout the striatum [Columns 1-8 A,E; Columns 1,2, row D; J, K, M, and Q] and ipsilateral SN (Columns 1-4, rows B and C; Column 3, row D, Columns 6-8, rows G-I] throughout multiple anatomical planes within the same animal [Columns1-8], but never on the contralateral side [Column C]. Robust GDNF-ir cells were also found near the locus coeruleus [Column 4, row D] and dorsal extents of the internal capsule. [L, P]. Most GDNF-transduced striatal cells had a morphology indicative of medium spiny projection neurons [N, O]



Supplementary Figure 4-S2. Incorporation of GFP-ir hfNPC into host ventricular wall. In one animal, residual donor cells (green) retracted dorsally through the needle tract into the ventricle. Some of the cells which engrafted near the ventricle incorporated into the lumen [A-D] and appeared to retain morphological characteristics consistent with the SVZ-NSC niche.

Supplementary Table 4-S2. GFP-ir whole-graft quantification in the four animals that received successful hfNPC transplants (0-5, with 5 being the highest rank).

	X577L	X740L	Y092R	X896R
GFP-ir Rank	5	5	4	2
SN (mm2)	9.834	13.341	8.674	7.108
Needle(mm2)	22.704	28.186	21.254	0
Area (mm2)	32.538	41.527	29.928	7.108
Volume (mm3)	1.6269	2.07635	1.4964	0.3554



Supplementary Figure 4-S3. Fluorogold (FG) and Nissl at 11-months post-transplantation. Striatal tissue adjacent to injection sites displayed robust FG-ir cells [A]; however, few FG-ir were found in the corresponding SN [B], labeling at best 5% of the spared endogenous TH-ir population. No FG-ir/GFP-ir cells were detected after 11-months suggesting non-innervation of the striatum at this time-point. [C] A representative example of Nissl staining demonstrates that hfNPC grafts do not induce aberrant pathology or alter host neuronal organization.

CHAPTER 5:

CONCLUSIONS AND FUTURE DIRECTIONS

5.1 Summary

This dissertation describes a clinically relevant paradigm for hNSC transplantation in the dopamine-depleted non-human primate brain. Several conclusions can me ascertained from this data. Contrary to previous reports utilizing suspension culturing conditions, human fetal subventricular-zone derived NSC can be maintained as multilayer-adherent-networks and expanded long-term (at least three years) without senescence or karyotypic aberration, in sufficient numbers to treat multiple patients over time. Previous groups have reported mass senescence in fetal cortical derived neural stem cells after just sixty passages, indicating that these cells would not have the capacity to be expanded indefinitely to treat multiple patients long-term. Furthermore, Clive Svendsen's group has shown that these cortical progenitors also accumulate chromosomal abnormalities, specifically chromosomes in 7 and 19, when cultured long-term. I have managed to overcome both of these obstacles utilizing the novel multilayer adherent network culturing system, thus providing a clinically relevant neuroectoderm derived undifferentiated fetal NSC population.

Transplantation of these undifferentiated fetal neural stem cells into rodent and non-human primates indicates they may retain developmentally appropriate cues and respond to local trophic and guidance molecules in vivo. Specifically, I have shown recovery in behavioral indices of parkinsonism in MPTP-lesioned vervet monkeys as well as long-term survival and axonal outgrowth up to eleven months post-injection when transplanted concomitantly with exogenous GDNF. Prior to these studies, it was unknown whether

undifferentiated human fetal NSC would even engraft into the CNS, let alone incorporate within the dopamine-depleted primate brain and provide functional improvement in severely lesioned animals. Previous reports by Svendsen's group indicated that fetal NSC do not migrate or significantly differentiate when transplanted striatally into parkinsonian rodent models. In stark contrast, we found extensive migration of fetal NSC derived from the subventricular zone of the lateral ventricles when transplanted into the striatum. Interestingly, migration was never found when cells were transplanted into the substantia nigra, indicating regionally specific cues are present within the striatum to induce not only migration but also glial differentiation of donor cells into astrocytes.

Previous reports have suggested that hfNSC do not differentiate in the primate brain, specifically when induced to express GDNF. In contrast, development of GFP-labeled hfNSC allowed for the unequivocal determination of "monoamine-like" differentiated morphologies with extensive varicose projections. I found morphological evidence consistent with neuronal differentiation including branching dendrites with spine-like structures as well as elaborately ramified fine-beaded structures. Unfortunately, standard biochemical analysis for mature neuronal marker proteins did not confirm the histological results that indicated extensive differentiation in vivo. It is possible that these grafted human cells simply need more time or more instruction in vitro or in vivo to assume appropriate biochemical indices of mature neuronal differentiation. Additional staining with 5-HT and GABA were inconclusive and unfortunately limited tissue did not permit for further testing.

Anatomically, the data were quite impressive, as historically, the biology of the NS unit is heavily rooted in morphological anatomical analysis (immunohistochemistry isn't always perfect and often depends on specific fixation techniques). Unfortunately, there appears to be a relative disconnect between what is found morphologically compared to biochemical readouts. Most classic neuropathologists would stop at this point and declare

these grafts as neuronally mature, however there appears to be a disconnect between anatomy and true molecular maturation, therefore, extreme caution should warranted when making assumptions about the maturity of grafted cells in relation to their overall anatomical appearance. Either way, this is the first report to our knowledge of human fetal neural stem cells engrafting long-term and projecting extensive neuritic fibers within the normal or lesioned adult primate brain, and provides validation that human NSC preparations retain the capacity to undergo programs of early neuronal differentiation in vivo.

To determine if these morphologically relevant fibers are truly neuronal in function, a more thorough analysis of grafted cells is absolutely necessary. Specifically, electron microscopy would be ideal to determine if the "bouton-like" enlarged fusiform structures that highly resemble sites of synaptic neurotransmission are truly integrated and appear functionally active. If so, we would expect to find evidence for pre- and post-synaptic contact structures as well as vesicles carrying neurotransmitters at the pre-synaptic sites. In addition, determining neuronal functionality relies heavily on electrophysiological techniques to measure pre- and post-synaptic transmission. As neuronal precursors begin to mature, they develop the potential to carry "mini-spikes" of electrical activity and will eventually fire full-on action potentials as they mature into neurons. A combination of specific dopamine agonists/antagonists could be used to selectively inhibit or excite grafted cells and determine if they respond in a similar manner as dopamine neurons in vivo. Utilizing these techniques, we could determine what specific types of neuronal properties grafted cells retain, specifically, whether they are of an excitatory or inhibitory nature and if so, which specific types of neurotransmitters they have the capacity to respond to. Moreover, dopamine neurons, like many other mature neuronal sub-types, typically retain a characteristic set of electrophysiological properties that may distinguish them from other cells thus allowing for a more unambiguous determination of cellular properties within grafted cells (Reviewed by

Grace and Bunney in Electrophysiological Properties of Midbrain Dopaminergic Neurons, in *Neuropsychopharmacology* Volume 5).

Combined results appear to confirm previous studies indicating that SVZ-derived human fetal NSC lack the inherent potential to significantly differentiate into mature A9-DA neurons in vivo. In both transplantation paradigms, only 1-3% of the total grafted cells expressed tyrosine-hydroxylase after several months in vivo, indicating that even in the presence of neurotrophic stimuli known to induce dopaminergic neurogenesis (i.e., GDNF), SVZ-derived fetal NSC cannot be induced to differentiate preferentially into A9 dopamine neuron phenotypes, further indicating regional specificity within CNS derived stem cell preparations. These data are highly congruent with those reported by Ole Isacson's group, clearly showing that SVZ-derived neural progenitor cells do not retain the capacity for midbrain-specific neurogenesis, whereas ventral mesencephalic precursors readily express tyrosine hydroxylase and secrete dopamine in vitro and in vivo. One possibility is that these cells simply need more time to mature in the primate brain compared to the rodent brain, as described by Lars Olsen (personal communication) who has found congruent morphological data when transplanting similar cells into rodent substantia nigra. Another equally plausible explanation is that these cells are only capable of expressing midbrain-associated proteins, specifically tyrosine hydroxylase, transiently. The only way to accurately assess this question is through multiple time points to determine at what time grafted cells begin to express tyrosine hydroxylase and to what extent they maintain expression past 11-months in-vivo. In addition, it would be extremely beneficial to determine whether grafted cells that appear dopaminergic and co-express tyrosine hydroxylase actually make and secrete dopamine. While technical limitations do not allow for such biochemical analysis, future studies would benefit by by techniques that identify the presence and/or absence of specific enzymes involved in chatecholamine synthesis such as DOPA decarboxylase, dopamine-B-

hydroxylase, and phenylethanolamine N-methyl transferase to determine specifically which types of monoamine neurons may be present.

Interestingly, many NFM-ir neurites had morphological parameters consistent with developing axons and appeared polarized parallel to endogenous neural circuitry when grafted cells were in close proximity or overlapped, demonstrating for the first time that undifferentiated hfNSC retain developmentally relevant programs of early differentiation and may respond to host signals in the dopamine-depleted primate brain. As previously indicated, a full time-course with multiple short and long-term time-points is necessary to determine if eGFP-ir graft derived fibers are still elongating and extending into host tissue. Interestingly, staining for the growth cone associated protein (GAP-43) expressed at the distal tips of developing neurons was negative, once again indicating that biochemical and morphological differentiation status do not necessarily overlap.

In addition to severely Parkinsonian monkeys, I also examined a large number of hNSC-transplanted monkeys treated with lower doses of MPTP to induce significant (50-80%) depletion in the DA system, but few functional effects. (If functional impairments are not essential, we attempt to minimize incapacitating the monkeys and having to provide special care). These animals were observed and scored on the same behavioral rating system that also produces a sum factor "healthy behavior" analogous to the "activities of daily living" part of the UPDRS scale. It includes such items as walking about the cage, eating, drinking, looking out of the cage, threatening other monkeys, self-grooming, picking at the cage, etc. Monkeys decrease the amount of healthy behavior in response to many different types of disturbance, drugs, or illness, including MPTP-induced Parkinsonism.

Data from 42 monkeys were analyzed which had received hNSCs injected into the striatum and/or SN (not including the stage 4 animals reported in Chapter 4). Normal and healthy behaviors were not reduced by the surgery or the hNSC implantations suggesting that

the cells were exerting no behaviorally apparent toxicity. Rather, normal behaviors appeared to trend toward and increase in these animals and no abnormal behaviors such as dyskinesia or dystonia were seen. Although, with these monkeys, we cannot infer any functional improvement associated with the NSCs, at least they appear to do no harm or cause any apparent behavioral toxicity. The pathological results of the monkeys reported here demonstrate that no tumors or overgrowth has ever been seen under any stem cell transplantation paradigm we have implemented with these cells. These results are paramount for safety and efficacy of future clinical trials.

One of the most difficult obstacles to overcome with any research involving large animals, specifically in this case, non-human primates, lies in attaining a large enough experimental group of animals to attain significance and control for all experimental variables. In this case several historical controls were necessary to compare GDNF-injected animals. Ideally, each experimental group would be assigned the same number of replicate animals, however, in this case, expenses and ethical considerations did not allow for a complete set of experimental controls. Specifically, no comparisons could be made between animals injected with neural stem cells alone and those co-injected with GDNF as the original hfNSC were not engineered to express the GFP-reporter. As a result, we have no way to accurately compare the effects of exogenous striatal GDNF on total graft survival or the degree to which GDNF enhanced neuritic fiber outgrowth in comparison to transplanted cells alone without exogenous growth factor support.

5.2 Conclusions and Future Directions

Significant progress has been made towards testing the hypotheses of the specific aims, showing survival, migration, and conversion to the DAergic phenotype in the nigrostriatal system of normal and diseased monkeys, normalizing effects on host brain

morphology, as well as reversal of parkinsonism induced by MPTP through multiple homeostatic mechanisms. Specifically, we have shown survival of hfNSCs after implantation into normal and DA-depleted monkey striatum, including differentiation into TH-expressing cells and other neural cell types using multiple methods for identifying hNSCs and their progeny. We found functional improvements in severely affected MPTP-parkinsonian monkeys compared with sham-operated monkeys. We also found that hNSCs "normalized" host systems by returning them toward normal cell numbers, cytoarchitecture, size, and function.

Experiments during this dissertation have supported the original hypothesis that human neural stem cells can reverse dysfunction in the neurotoxic model of parkinsonism produced by MPTP in monkeys and suggested that a combination of endogenous effects and cellular replacement may be responsible for functional improvements in severely parkinsonian monkeys. The effects we have observed appear to fall into 2 broad categories (1) the impact the abnormal milieu has upon the stem cell (e.g., shifting its differentiation fate and neuritic output along the nigrostriatal pathway) & (2) the stem cell's impact upon the host (e.g., providing neurotrophic & neuroprotective support to endogenous dopaminergic (DA) neurons & their projections, restoring homeostasis to the cytoarchitecture including the size-to-number ratios of endogenous cells, preserving compromised neural circuits, and possibly "circuit restoration" achieved by a small percentage of stem cells that acquire characteristics of dopamine neurons. However, significant questions remain as to how these functional effects occur and, more importantly, can be enhanced.

While the recovery is very impressive and without apparent toxicity or dyskinesias, it is not yet complete and few hNSC derived dopaminergic cells survived, even after GDNF enhancement for eleven months. We believe that the multiple effects of stem cells on the nigrostriatal system are interesting and should be further investigated, specifically the

relationship between transplanted cells and the specific axonal guidance molecules along spared endogenous circuitry. Additional studies confirming these results should also include a TH-promoter driven reporter construct integrated into donor hfNSC to verify the potential of these cells to express tyrosine hydroxylase and determine to what extent the TH-ir donor-derived cells express A9-DA neuron specific Girk2 at time points beyond 1-year. Importantly, there was evidence for transgene silencing of eGFP in larger TH-ir cells that retained morphological features consistent with endogenous mature dopamine neurons (large cell body and axonal arborization). The data indicate that we still may not be appropriately accessing the actual dopaminergic potential of grafted cells and further argue for a more detailed analysis before a definitive conclusion can be made. In addition, a side-by-side comparison with fetal DA-neuroblasts as well as embryonic stem cell derived and induced pluripotent stem (iPS) derived NSC and their DAergic derivatives to determine which tissue source provides the most therapeutic cellular substrate is essential to the advancement of cellular transplantation based therapies.

Most prior studies have focused on the concept that the *host* environment –- as it changes during the course of development and aging, or after injury or degeneration — influences the transplanted stem cell, as exemplified here by the emergence of some donor-derived TH+ and DAT+ cells. Based upon past reports of the small difference in DA depletion that appears to underlie large functional differences, even a 5% elevation in DA may be sufficient to produce significant functional improvement, either from stem cell-derived DA neurons or from augmentation of host DA pathways via trophic/neuroprotective effects. We believe our data suggest that the primate CNS may benefit (1) from human stem cell-derived replacement of missing cell types (including neurons) and (2) from the supportive effects of other stem cell-derived progeny that may be necessary for promoting optimal recovery via the

variety of mechanisms described in this dissertation (trophic, homeostatic and guidance) as well as others that remain to be elucidated.

Given the analysis we have performed utilizing undifferentiated human fetal NSC in the non-human primate brain, and the extensive knowledge our colleagues and others have attained transplanting fetal DAergic tissue, it is still relatively unclear which neural substrate may provide the most therapeutic substrate to restore DAergic tone in Parkinson's disease. While NSC have the advantage of being expandable nearly indefinitely, DAergic neuroblasts appear to functionally integrate the striatum and developmentally reconstruct the nigrostriatal pathway in response to GDNF chemotaxis in the rodent and monkey. Unfortunately, fetal grafts may also induce dyskinesias in patients over time given their apparent vigor and sustained uncontrolled production of dopamine, therefore, a more slowly developing substrate may, with time, eventually be a more therapeutic option clinically.

The degree of degeneration within the patient may also have a dramatic impact on how neural grafts survive and respond in vivo. Currently, neurologist are only able to make a correct diagnosis for Parkinson's disease based on motor deficits (Tremor, Rigidity, Akinesia, Postural Instability). These characteristic motor behaviors do not become clinically diagnosable until greater than 80% of the dopaminergic tone has been lost, therefore a typical Parkinson's disease patient may not have enough time to wait for a NSC graft to mature and develop into relevant cell types in-vivo. When diagnostic tools have improved and biomarkers are available to diagnose and treat Parkinson's disease earlier, before massive degeneration has already taken place, perhaps NSC therapies aimed at neuroprotection can provide therapeutic value for endogenous dopamine neurons. Therefore, under current clinical circumstances, the data described here argue against the use of undifferentiated hfNSC as a therapeutic option for cell replacement in Parkinson's disease. Specifically, simple neuralization (in this case by natural development in vivo) of stem cells does not appear to be

sufficient for in-vivo differentiation into dopaminergic cells. Rather, neural stem cells will most likely require further developmental cues, maturation, and specification into DA precursors in vitro to attain neural replacement. An intriguing question remains as to whether all undifferentiated neural stem cell preparations maintain similar characteristics in vivo.

Future studies will be necessary to determine exactly where the differentiation "sweet-spot" lies for creating safe, transplantable cells with high survival and sustained controlled release of dopamine in-vivo. A useful approach might be to systematically determine the phenotypic characteristics of ventral mesencephalic cells that have previously been shown to reduce behavioral deficits in parkinsonian primates pre-clinically. It is known that only a small portion of these cells are responsible for motor recovery, whereas others are expected to be involved in deleterious mismatch synapses inducing dyskinetic behaviors. Developing techniques to mimic phenotypic characteristics of the beneficial cellular components of these grafts may ultimately reshape how we prepare and differentiate cells in vitro for further optimization of a transplantable therapeutically relevant neural substrate capable of restoring dopaminergic tone in Parkinson's disease patients. With the advent of human embryonic stem cells, iPS derived neural cells, and most recently induced neural (iN) cells, we are now equipped with more tools than ever before to create and test neural transplantation therapies for PD, however it should be noted that extreme caution is warranted for any therapy utilizing undifferentiated, non-midbrain specified neural stem cells.

APPENDIX A:

CULTURE SYSTEM FOR RODENT AND HUMAN OLIGODENDROCYTE SPECIFICATION, LINEAGE PROGRESSION, AND MATURATION

Summary:

Here we document protocols for the production, isolation, and maintenance of the oligodendrocyte phenotype from rodent and human neural stem cells. Our unique method relies on a series of chemically defined media, specifically designed and carefully characterized for each developmental stage of oligodendrocytes as they advance from oligodendrocyte progenitors to mature, myelinating oligodendrocytes

Introduction:

Here we document a protocol for the production, isolation and maintenance of the oligodendrocyte (OL) phenotype from rodent and human neural stem cells (NSC). Our unique method relies on a *series of chemically defined media,* specifically designed, and carefully *characterized for each developmental stage* of OL, as they advance from OL progenitors (OLP) to mature myelinating OL [Figure A-1] (Neman and de Vellis, 2008). Providing hNSC with the nutrients specifically required at a particular moment in OL development, our system allows for *the propagation of OL at a desired stage* from OLP to mature premyelinating OL. Therefore, lineage progression can be manipulated by controlling the duration of a given developmental stage as needed, in a more "natural" manner, and without using gene transfer (Park et al., 2002b; Kim, 2004; Müller et al., 2006; Ahn et al., 2008), co-cultures, or undefined substrates such as, cell line-derived conditioned medium or animal serum.

Preparation of embryonic neural stem cells (NSC)

The following methodology can be used to isolate and derive NSC lines from various species. Specific methods for the derivation of human NSC are detailed elsewhere (Svendsen et al., 1999; Villa et al., 2000; Palmer et al., 2001; Schwartz et al., 2003; Kim et al., 2006; De Filippis et al., 2007; Ebert et al., 2008; Kim et al., 2008; Wakeman et al., 2009a.b).

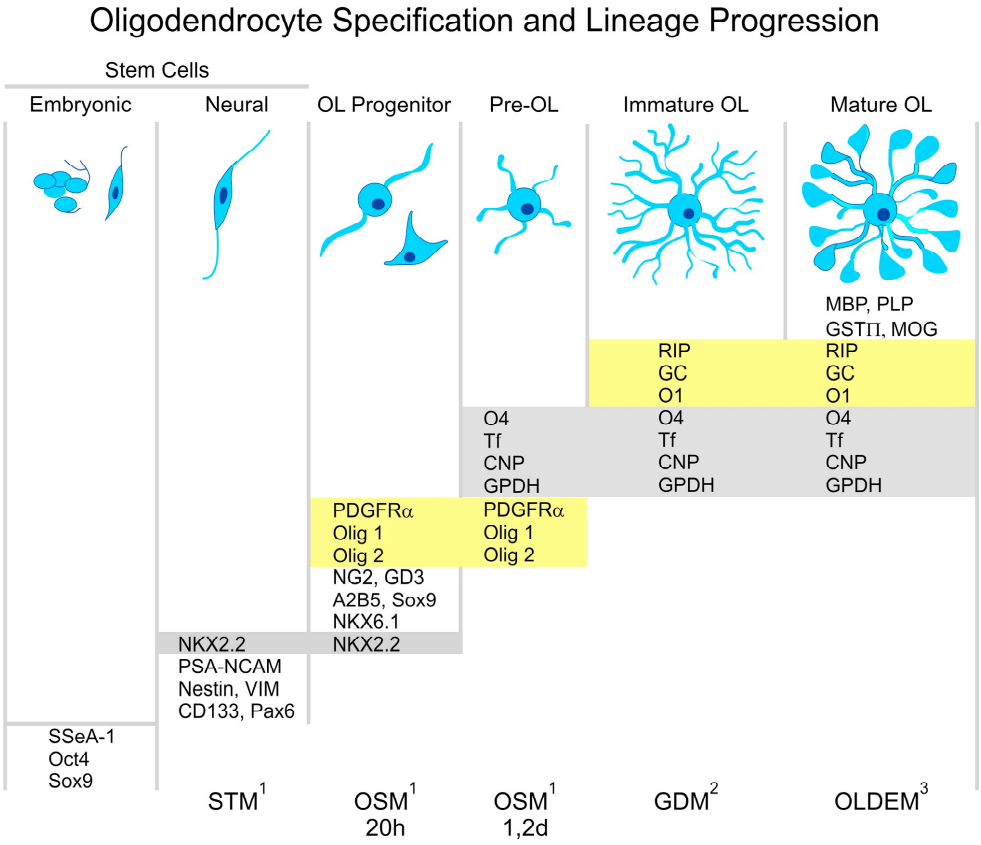


Figure A-1. **Oligodendrocyte Specification and Lineage Progression.** Oligodendrocytes undergo sequential morphological changes as they develop from uncommitted NSC to a committed OLP and acquire characteristics inherent to a functional OL. The list of OL markers below each developmental stage is not exhaustive but represents frequently used markers to identify OLs and their developmental stage.

BASIC PROTOCOL 1

I. RODENT NSC ISOLATION AND MAINTENANCE

Materials:

Remarks: All dissection instruments, plastic, and glassware must be sterile.

Animals: Time-pregnant embryonic day 14-16 (ED14-16) Sprague-Dawley rats.

(Charles- River, Wilmington, MA)

Dissection Instruments: Refer to numbers in [Diagram 1].

1) Mayo Scissors 14010-17 FST
2) Lister Scissors 14131-14 FST
3) Blunt-Pointed Forceps 08-887 Fisher
4) Iris Scissors 14060-09 FST
5) Moma Iris Forceps 11373-12 FST
6) Dumont #7 Forceps 11297-10 FST
7) 140 μm and 230 μm sieves Cellector, E-C Apparatus Corp.
8) Syringe 520673 Jone Kendall

Bovine Serum Albumin (BSA)

Conical tubes

Flasks (T-12.5 cm^2, T-75 cm^2)

Hank's Balanced Salt Solution (HBSS)

Hemacytometer

Isoflurane

Petri Dish (Bacterial grade, non TC-treated)

PSA-NCAM Coated Dishes (See Support Protocol 1)

Sterile surgical gauze

STMc media

14-gauze Penetration needle, 14-825-15 Fisher

Step 1. Isolation of Neural Stem Cells from Newborn Rat Brain

Procedure:

1. Prepare the work area and sterile tools in a biosafety hood [Diagram 1].
2. Euthanize the rat by isofluorane inhalation.
3. Extract the placenta containing embryos and place in STM Basal + 1% BSA.
4. Remove the cerebellum.
5. Dissect the brain of each embryo and place in STM complete (STMc) medium.
6. Separate the cortex from the rest of the brain and remove the meninges with forceps.
7. *Combine the tissue of all the brains without meninges and mechanically dissociate with the needle by gently aspirating the brain pieces (10 times) and releasing the suspension slowly with the needle attached to the wall of the tube (try to minimize foaming).
8. *Recover the supernatant with the cells in suspension and transfer to a 15ml tube.
9. *Add 2-4ml of complete medium to the chunks left over in the dissociation tube and dissociate again 5 to 8 times.
10. Filter the suspension of dissociated cells through the 230 μm and 140 μm sieves to remove cell clusters.
11. Rinse the sieves sequentially with basal STM + 1% BSA at room temperature, and add this medium to the tubes containing the cells.

12. Collect the cells by centrifugation at 45 RCF for 8 min.
13. Gently discard the supernatant.
14. Resuspend the cell pellets in 4 ml of STM complete (freshly prepared), and gently dissociate the pellets.
15. Assess cell viability (see next section), count cells using hemacytometer, and plate onto fresh PSA/NCAM coated dishes (2×10^6 cells/100 mm dish).
16. Incubate plated cells overnight at 37°C with 4.5% CO_2 and 95% humidity. *Note:* Younger cells do not yet express PSA-NCAM and will remain floating as small clusters, whereas the older cells will attach overnight.
17. **On the next day, recover the non-attached cells, pellet as previously described, and remove and save the conditioned media (CM).
18. Resuspend the pellet and dissociate in 4 ml of fresh STMc medium by passing through a 14-gauge needle eight times. Complete the volume to 8ml with conditioned medium and plate on additional anti-PSA-NCAM coated plates. *Alternatively:* Place 1.0 ml of the cell suspension in a 75ml Erlenmeyer [EM] flask in 25ml of STMc, and incubate with shaking at 37˚C [Figure A-2].
19. Feed cells every other day by removing 1/3 of the culture medium and adding the same volume of fresh STMc.
20. Switch the cells from 4.5% to 5.0% CO_2 only if your cells are growing slowly. Leave them at 4.5% if they maintain the color of the medium red/orange.

Notes: A chart of the STM cell isolation method is illustrated in [Figure A-2].

*Steps 7-9 can be substituted by dissociating the cells 2.5 min using a Stomacher 80 (Seward, London, UK).

**Collect, filter, and save the conditioned medium at 4˚C for immediate use (or frozen for later use). CM is an excellent supplement to start NSC cultures from frozen stocks. Allow cells to grow to 70-90% confluency.

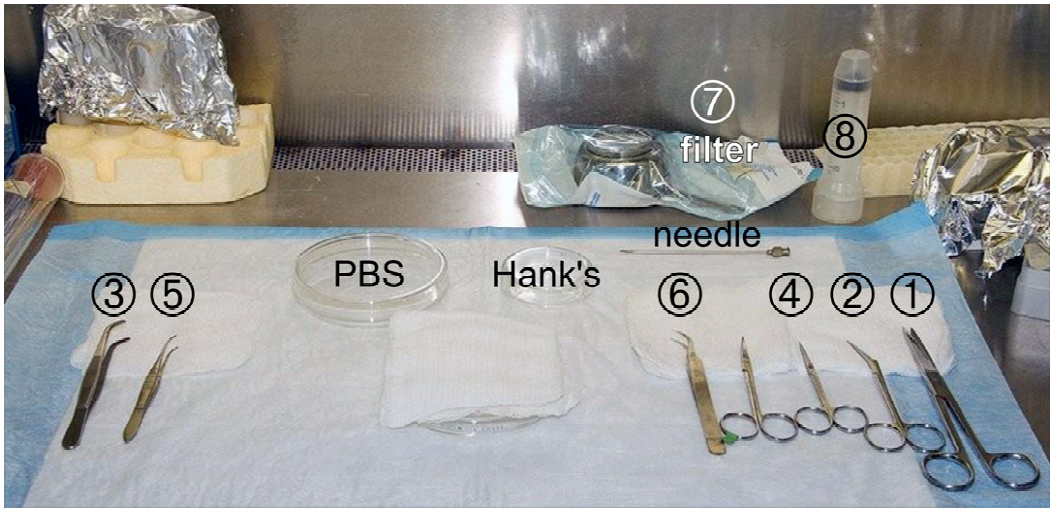


Diagram A-1. Instruments required for dissection. #1, Scissors for decapitation; #2,scissors to cut the head skin to expose the skull; #3, forceps to hold the head in place as you cut the skin and cut the skull cartilage with scissors, #4 to expose the brain. Some users prefer the curved scissors, #2 instead of #4. Use the same scissors to transfer the brain to the Petri dish containing PBS. #5 & #6, Forceps to hold the brain in place and remove the meninges (respectively). After removal of the meninges place the brains in Hanks solution while dissecting the rest of the brains. #7, filter mesh to filter the cell suspension after dissociation. #8, 20ml sterile syringe and sterile dissociation needle.

Cell Viability Assay

Cell viability can be determined with the SYTOX blue nucleic acid stain (Molecular Probes, Eugene, OR). Cells with compromised plasma membranes are labeled by SYTOX binding to nucleic acids and detected by fluorometry.

Materials:

PBS

SYTOX blue nucleic acid stain

Tris-Buffered Saline (TBS)

Procedure:

1. Harvest and wash cells with 1× TBS
2. Incubate for 12 min in 1 μM SYTOX in PBS
3. Remove the solution and wash the cells with 1× TBS, five times
4. Determine the *number of positive cells per random field* and record as a percentage of the total number of cells.

Step 2. NSC Propagation and Maintenance

Propagation of Rodent NSCs as 2D-cultures

NSCs can be propagated in bi-dimensional (2D) or three-dimensional (3D) cultures. When attached, NSCs (2D cultures) tend to grow faster and are therefore ideal to create a large cell stock quickly before starting specific studies. In addition, we have developed a new method for expansion and maintenance of human NSC in NB-B27 (see Media Preparation) as multilayer adherent network (MAN) cultures, with increased proliferation rates compared to standard sphere forming assays (Wakeman et al., 2009b). In order to accommodate for the difference in basal media, human NSC can either be initially derived in STMc media (replacing NB-B27), or previously established cultures may be slowly transitioned away from

the basal NB-B27. Simply substitute 25% STMc for 1 week, followed by successive weeks at 50%, 75%, and finally 100% STMc after one month.

Materials:

Cell Scraper

Conical tubes

Flasks (T-12.5 cm^2, T-75 cm^2)

Hank's Balanced Salt Solution (HBSS)

Petri Dish (Bacterial grade, non TC-treated)

PSA-NCAM Coated Dishes (See Support Protocol 1)

STMc media

Procedure:

1. When confluency has been reached, remove the supernatant conditioned media (CM), add 5ml of Hank's Buffered Salt Solution (HBSS) without Ca^{2+} or Mg^{2+}, detach the cells with a cell scraper, and transfer to a 15ml conical tube (1 to 3 Petri dishes).
2. Rinse the Petri dish once with 2ml HBSS and centrifuge at 45 RCF for 8min.
3. Resuspend the cell pellet in 3 ml STMc, dissociate gently using a 14-gauge needle, and centrifuge at 45 RCF for 5min.
4. Save the CM (as above), resuspend cells in fresh STMc + CM (2:1), and re-plate. If you have repeated this process several times and the cell pellet is 0.5ml volume or larger, divide the cell suspension into two parts.

5. One part of the suspension will be used to start a frozen stock (see next section for cryopreservation). The second half of the cell suspension is further dissociated using a needle (as described above, however, the sieves are not necessary).
6. Count the number of cells/ml and adjust the volume (to 15ml) with freshly prepared STM containing 1/3 self-conditioned-STM + 2/3 of fresh STMc.
7. Plate the cells in five T-12.5 cm^2 cell culture flasks coated with anti-PSA/NCAM as described.
8. Feed cells with 1/3 of self-conditioned-STM + 2/3 of fresh STMc every other day until they reach 80 to 90% confluency, and repeat the process to increase the number of cells.
9. When four or more T-12.5 cm^2 flasks reach confluency, harvest the cells as described above, and seed the equivalent content of cells from three T-12.5 cm^2 flasks into one T-75 cm^2 flask (coated with anti-PSA/NCAM)**.** Feed the cells with 1/3 self-conditioned-STM + 2/3 of fresh STMc in a total volume of 10ml/ flask.
10. *After 1-2 days, the culture medium should be red. If the medium is turning orange, add 3ml of STMc and repeat this step as needed. Add 3ml of STMc every day only if the medium changes color.
11. **When cells reach confluency, you may elect to freeze the contents of one T-75 cm^2 flask (see cryopreservation section).
12. After accumulating at least 10 to 15 vials of cryopreserved NSC in a frozen stock, NSCs may be grown as neurospheres (3D-cultures) for slower growth, allowing more time to devote to experiments.

Notes:

***** If cells seem not to grow but look healthy, or if the culture medium is not red but rather purple, you will need to remove 1/2 of the plating medium and complete to 10ml volume of the mixture (1/3 self-conditioned-STM + 2/3 of fresh STMc freshly prepared). If the opposite is true and the culture medium turns orange overnight, the cells have proliferated heavily, and you will need to replace the culture medium and seed more T-75 cm^2 flasks (per three T-12.5 cm^2).

****** When propagating cells to create frozen stocks, we strongly recommend maintaining a "mother flask/dish" by scraping most, but not all of the cells attached to the flask. After removing the detached cells, feed the mother flask with fresh medium and CM (1:1) to ensure continuity of these cultures (in case re-plated cells do not look healthy, grow slowly or die).

Formation, propagation and maintenance of Neurospheres *(3D cultures)*

Suspension aggregate, or "neurosphere", 3D cultures are an alternate strategy to propagate NSCs at a slower pace than attached cells, while preserving most of the standard characteristics of a proper NSC. NSC suspension cultures are started from freshly dissociated NSC (2D cultures) and grown in Erlenmeyer flasks (EM) to prevent attachment and encourage free-floating spherical growth.

Materials:

Conical tubes

Flasks (T-12.5, 75 cm^2)

Glass Erlenmeyer flasks (EM) 25 ml or 50 ml with cap.

Hank's Balanced Salt Solution (HBSS)

Petri Dish (Bacterial grade, non TC-treated)

PSA-NCAM Coated Dishes (See Support Protocol 1)

STMc media

Procedure:

1. Place 15ml of fresh STM + 3ml of self-conditioned medium (CM) into the EM.
2. Add 2ml of the cell suspension (freshly harvested from 2D cultures), and close the top of the flask partially to allow for O_2/CO_2 exchange.
3. Place the EM, continuously shaking, at 90 rpm in the incubator. If placing a shaker in the incubator is not an option due to safety regulations, the cell suspension may be placed directly into two Petri dishes (bacterial grade) to prevent cell attachment.
4. Add 1.5ml fresh STMc, every other day, and dissociate routinely (3 times gently) with a syringe and needle (as described for pellet dissociation in the previous section) to keep the spheres at a small size. This process allows for increased sphere formation without the negative potential for spontaneous differentiation. It also permits cells more exposure to the fresh nutrients in the culture medium, allowing for preservation of "stemness" in all cells.
5. When the culture medium turns orange overnight, it is time to renew the culture medium and split the cells. Collect the contents of the EM flask and place into one 50ml conical tube. Centrifuge for 6 min at 45 RCF.
6. Slowly collect the CM, filter (0.22 μm), and save for replating cells.

7. Resuspend the pellet in 4ml of STM to dissociate larger spheres, *(at this point they should all be easily dissociated). Add freshly prepared STMc + 1/3 self-conditioned medium.
8. Seed cells on desired containers for experiments, or continue to propagate NSCs as 2D or 3D cultures.

Notes: A diagram for the propagation of NSC in 2D & 3D cultures is shown in [Fig. A-2].

*If some spheres remain large in spite of repeated dissociation, use the sieves to eliminate the clumps instead of drastically dissociating them. This step will prevent significant cell death at the time of re-plating.

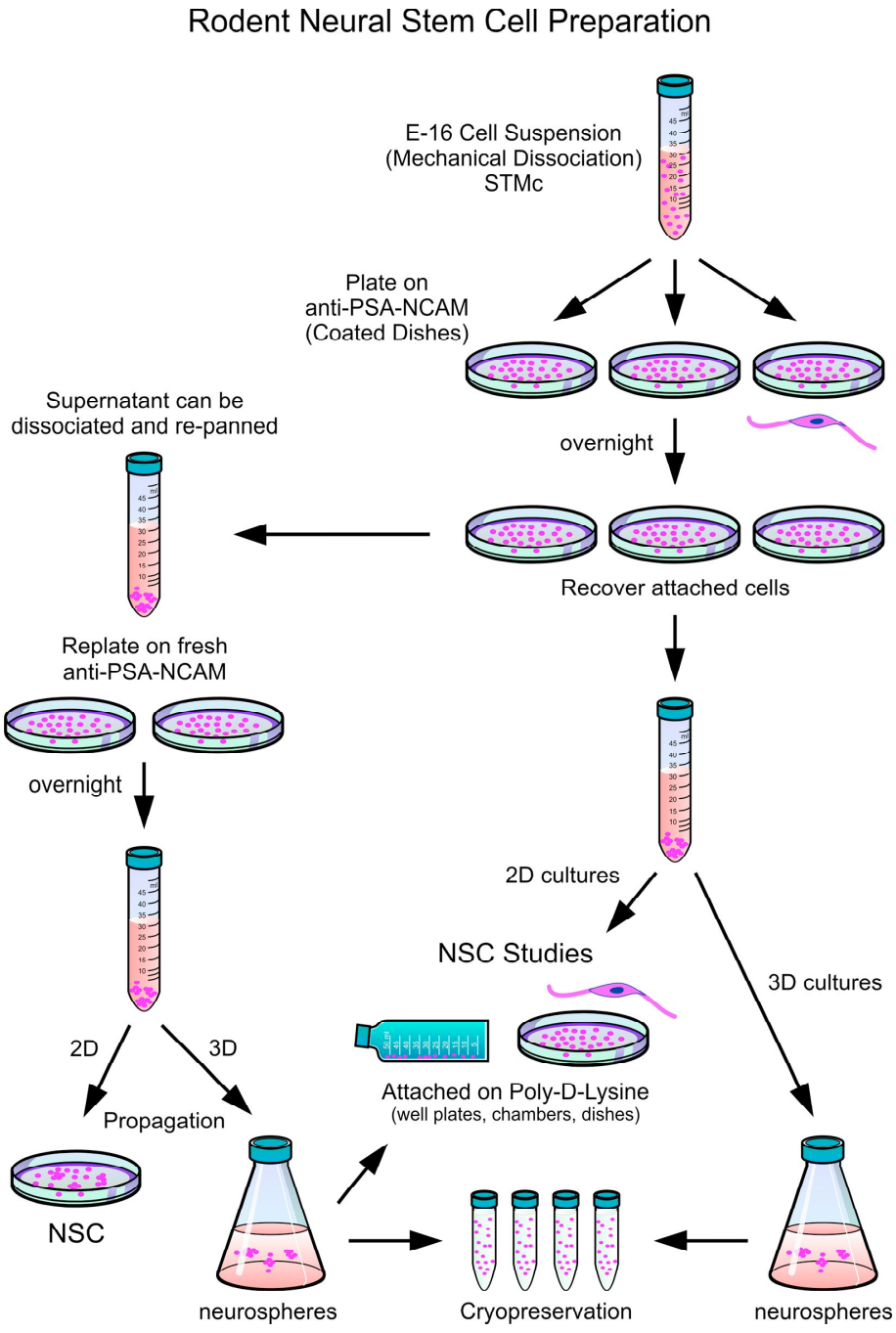


Figure A-2. Rodent Neural Stem Cell Preparation. Following dissection, the cell suspension is plated on anti-PSA/NCAM antibody coated dishes and allowed to adhere. The process can be performed repeatedly to increase the numbers of neural stem cells, as 2D or 3D "sphere" cultures. (shown in the left side of the diagram). Alternatively, cells can be propagated and immediately used for cell culture experiments (as shown on the right side of the diagram). While we prefer to use committed OL-progenitors for cell transplants, other investigators also use uncommitted progenitors for grafting.

Support Protocol 1:

Immunopanning

The following method was developed based on published work (Wysocki and Sato, 1978 and Williams and Gard, 1997) to isolate the rodent NSC population from the other cell populations in the brain during initial plating. We also use anti-PSA/NCAM coated dishes to propagate rodent NSC in 2D cultures (Espinosa et al., 2002). Please refer elsewhere for specific methods on the selection of human NSC during primary derivation (Wakeman et al., 2009a).

Materials:

Anti-PSA-NCAM

Bovine Serum Albumin (BSA)

Flasks (T-12.5 cm^2, T-75 cm^2)

PBS

Petri Dish (Bacterial grade, non TC-treated)

Tris (pH 9.5)

Procedure:

1. Prepare the immunopanning cocktail by mixing: Tris (pH 9.5) +1% BSA + 50 μg/ml anti-PSA/NCAM.
2. Coat the bottom surface of non tissue-culture grade Petri dishes (351029) with the anti-PSA/NCAM mixture (4 to 5ml/per dish).
3. Incubate for 30 min at 37°C.
4. Wash Petri dishes 3 times with PBS and once with PBS + 1% BSA just before using. Do not allow the plates to dry.
5. Extra plates may be covered with foil and stored at 4˚C for up to 10 days.

Support Protocol 2

Cryopreservation / Thawing of NSC stocks.

We recommend collecting cells for frozen stocks at low passage number. Human NSC are cryopreserved using modified methods found elsewhere (Wakeman et al., 2009a). In addition, the method formerly described for rat and mouse NSC (Espinosa et al., 2002) can also be used to stock human NSC.

A) *Freezing NSCs*

Materials:

Cryogenic slow-freezing chamber

Cryogenic freezing vials

HBSS

STM media

14-gauze needle

Procedure:

1. Allow NSCs to grow to 70-90% confluency. Remove all of the cell culture medium, add 5ml of HBSS to each Petri dish or 10ml to T-75 cm^2 flasks, and detach cells by gently scraping the culturing surface.
2. Centrifuge the cells at 45 RCF for 8 min and resuspend in 3 ml STM media.
3. Gently dissociate cells using a 14-gauge needle, pellet at 45 RCF for 8 min with a centrifuge, and discard the supernatant.
4. Gently resuspend the pellet from one (100mm Petri dish or T-75 flask) in 1ml of serum-free freezing medium.
5. Transfer the contents to a 1.2ml cryovial, and place the vial(s) in a cryogenic freezer container overnight for slow freezing.
6. Next day, place the vials in liquid nitrogen for long-term storage.

B) *Thawing NSCs*

Materials:

Anti-PSA-NCAM coated plates

CM and STM media

NSC Cryovial

Procedure:

1. To "reanimate" NSCs, defrost cryovials quickly in a 37°C water bath, and transfer the contents of the vial to a 2ml tube containing 1ml of STMc + CM at 37°C.
2. Centrifuge gently at 35 RCF for 5-7 min.
3. Remove the supernatant, add fresh plating medium (STMc + CM) and remove a small aliquot to test the initial cell viability (as described above).
4. Count the number of viable cells in the tube (approx. 1 $x10^6$ cells expected).
5. Plate cells onto anti-PSA/NCAM coated surface (Petri dishes or T-75 cm^2 flasks, plate the equivalent of 1 vial/T-75 cm^2 flask). If the yield is lower, utilize T-25 cm^2 flasks to increase the cell density necessary for healthy growth. Seeding low-density cultures in large containers decreases the proliferation rate and might be detrimental to the culture.
6. To propagate NSCs after replating, proceed as described in step 2 for "propagation and maintenance of NSCs".

Basic Protocol 2:

OL commitment in 2D and 3D cultures

During development, the nutritional and environmental needs of cells change as they lose multipotency and become lineage restricted. The present system is based on the modification of nutrients contained in the cell culture medium and the percentage of CO_2 needed to optimize and direct lineage restriction towards the OL phenotype. Like NSCs, OLPs can be propagated in 2D and 3D cultures. When attached (2D cultures), OLPs grow

faster, and thus ideal to create an OLP cell stock quickly before starting specific *in vitro* cell culture or *in vivo* transplantation studies. A diagram of the following steps can be found in [Figure A-3].

Materials:

Media: STM-CM, OSM, GDM, OLDEM

Anti-IgM coated plates

Anti-PSA-NCAM coated plates

BFGF

Cell Scraper

Erlenmeyer (EM) flask

HBSS without Ca^{2+} or Mg^{2+}

Petri dishes

14-gauge needle

15 μm sieve

24-well plate

4.5% CO_2 incubator

Procedure:

1. When NSCs reach confluency, remove the supernatant (CM), and add 5ml of Hank's Buffered Salt Solution (HBSS) without Ca^{2+} or Mg^{2+}.
2. Detach the cells with a cell scraper, transfer into a 15ml tube (1 to 3 Petri dishes), rinse once with 2ml of HBSS, and centrifuge at 45 RCF for 8min.

3. Resuspend the cell pellet in 3 ml OSM media and gently dissociate (3X) using a 14-gauge needle. Centrifuge at 45 RCF to pellet the cells.
4. Resuspend the cells in fresh OSM + STM-CM and seed cells on anti-IgM coated dishes or flasks (use the same procedure as for anti-PSA/NCAM), and maintain the cells as described in *Basic Protocol 1* but using OSM/self-CM (2:1) respectively. From this point on, the *CO_2 concentration in the incubator should remain at 4.5%.
5. Feed the cells with 1/3 self-conditioned-OSM + 2/3 fresh OSM every other day until they reach 80-90% confluency. This process can be repeated several times to attain a large number of cells for freezing (if desired).
6. Alternatively, to grow OL spheres to create/enrich a frozen stock of OLP, place the equivalent of $2mm^2$ (pellet size after dissociated and in suspension) in a 25ml Erlenmeyer flask with 15ml of OSM + self-CM (2:1). If the pellet is $4mm^2$, use a 50ml Erlenmeyer flask. Prepare the cell suspension and place in a total volume of 25ml of OSM + self-CM (2:1). Feed OL-spheres with fresh OSM every other day by adding 3 ml of freshly prepared OSM (no CM). When spheres start to become larger than 2mm, gently dissociate 1-2 times in the same flask with the 14-gauge needle using a 12ml syringe (sterile).
7. When the culture medium starts to turn orange, recover and centrifuge the spheres, and split into more Erlenmeyer flasks. These may be used for experiments or cryopreserved as previously described (cryopreservation section).

*We recommended pre-calibrating the percentage of CO_2 one day before plating the cells. If the incubator is shared with other people or needed at 5% for NSC propagation and maintenance, we recommend using T-flasks for 2D cultures instead of Petri dishes. Close the cap completely and then open it ¼ of a turn before placing in the incubator at 5% CO_2. For

propagation and maintenance of OL-spheres, the Erlenmeyer flask should also be kept open just enough to ensure O_2 / CO_2 exchange. When using 4.5% CO_2, loosen the caps of the flasks until half-way open.

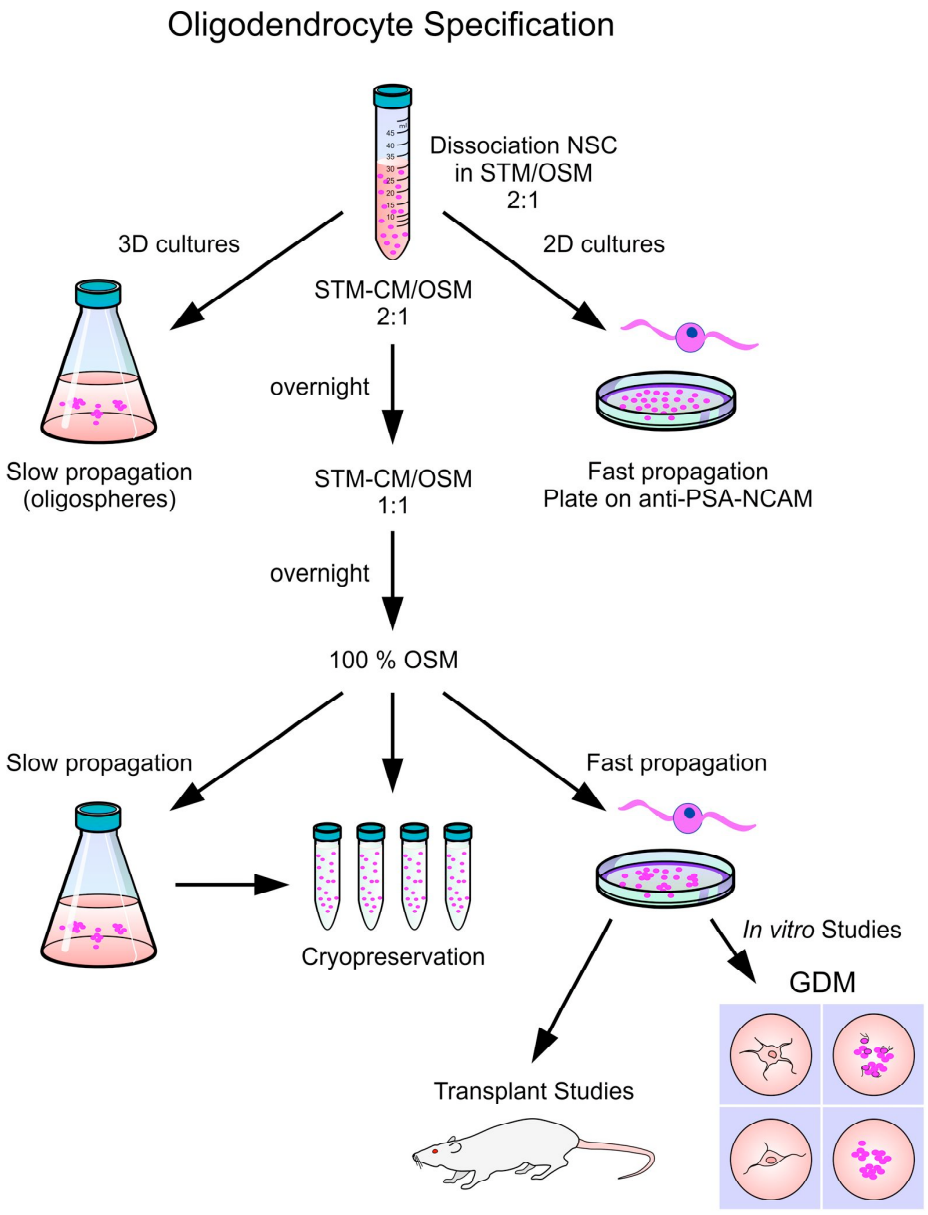


Figure A-3. Oligodendrocyte Specification. The transition of NSC to commit to the OL lineage is brief but sequential, rather than abrupt. In order for cells to survive, they must acclimate to their new environment. OLP can be propagated to create frozen stocks as 3D "oligosphere" cultures (shown on the left of the diagram) or frozen without propagation (as shown in the sequence in the center of the diagram). OLP can also be propagated in 2D cultures for cryopreservation, for specific cell culture experiments, or for cell replacement therapies (as shown in the right side of the diagram).

Support Protocol 3

Oligodendrocyte lineage progression and maturation

The nutritional needs for a committed cell within the OL lineage differ considerably as they progress and mature to the next developmental stage. These cells need to start synthesizing enzymes and proteins related to myelination, therefore, the energy demand is enormous compared to their earlier stage where migration and proliferation are the basic functions. The culture medium "GDM" (glial defined medium) was first designed to maintain 04+, GC+/-, CNP+/- cells (for details see Pre-OL in [Figure A-1]). Later, we realized that GDM also induced the transition of OLP to pre-OL (Espinosa et al., 1997).

Materials:

Media: OSM, GDM, OLDEM

bFGF

Petri dishes with cells

Poly-d-lysine coated wells/plates

24-well plate

Procedure:

1. In order to obtain “pre-OL” (along the OL lineage), plate OLPs using OSM (as in Basic Protocol 2).
2. Next day, remove ½ of the volume of the plating medium (OSM) and add the same volume of GDM. As in previous steps, they may be propagated as OL-spheres (3D) or as 2D cultures on anti-IgM coated flasks, Petri dishes, or directly on cell culture grade plastic.
3. To obtain more OLP/pre-OL, cells are grown as 2D or 3D cultures in the presence of bFGF [Figure A-4]. Cells will remain at the same stage as the parent cells by adding fresh GDM + [20ng/ml] bFGF. For cell replacement therapies, we suggest using cells at this stage (1-2 days after plating without bFGF) as cells are still highly motile and readily migrate within the host post-natal and/or adult rodent brain and/or spinal cord.
4. To enhance maturation of cells into the next developmental stage, OL are cultured as 2D cultures in GDM for at least 2 days (if plated in GDM without bFGF), or 4 days (if plated in GDM + bFGF) without further bFGF supplementation [Figure A-5].
5. After exposure to GDM, cells express myelin enzymes and proteins, and they display multipolar, branched cell processes, but not a myelin-like membrane. In addition, OL maintained in GDM for at least 4d (without bFGF or any other factors) can be further induced to a fully mature myelinating stage.
6. To fully mature OL, plate as 2D cultures onto poly-d-lysine coated wells/plates or Petri dishes in GDM/OLDEM (OL maturation medium) 1:1 for 1-5 days, followed by 100% OLDEM thereafter [Figure A-4].

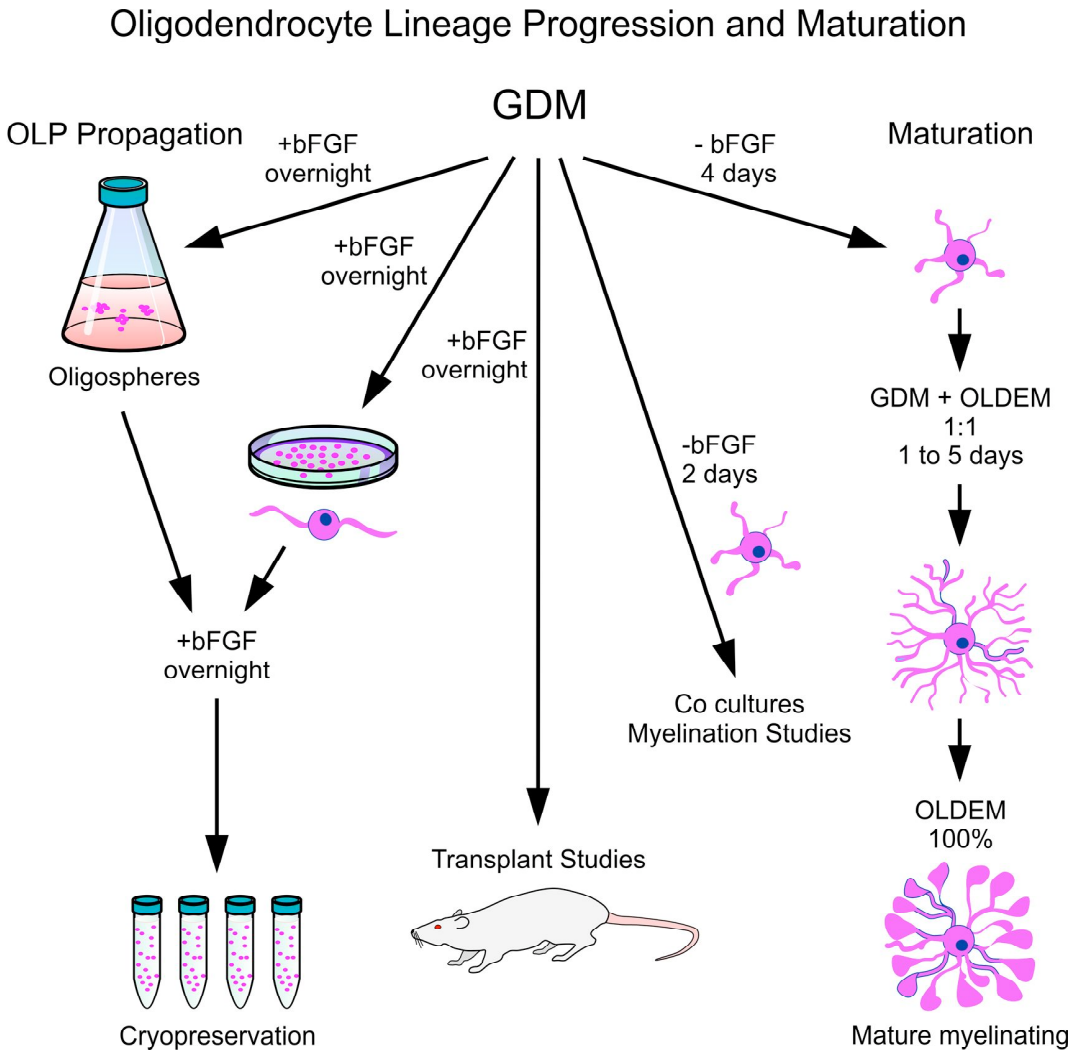


Figure A-4. Oligodendrocyte Lineage Progression and Maturation. After commitment of NSC to the OL lineage, cells are propagated at the OLP stage to create a frozen stock (steps indicated on the left portion of the flow chart), or processed further for transplantation studies (as shown by the middle arrow on the diagram). To allow OLP to further mature along the OL lineage and become myelinated, cells are transitioned into OLDEM for at least 48h. Once OL have reached this stage of maturation, they are excellent for cell culture studies but are not recommended for cell grafting as detachment from the substrate can damage the numerous delicate cell processes.

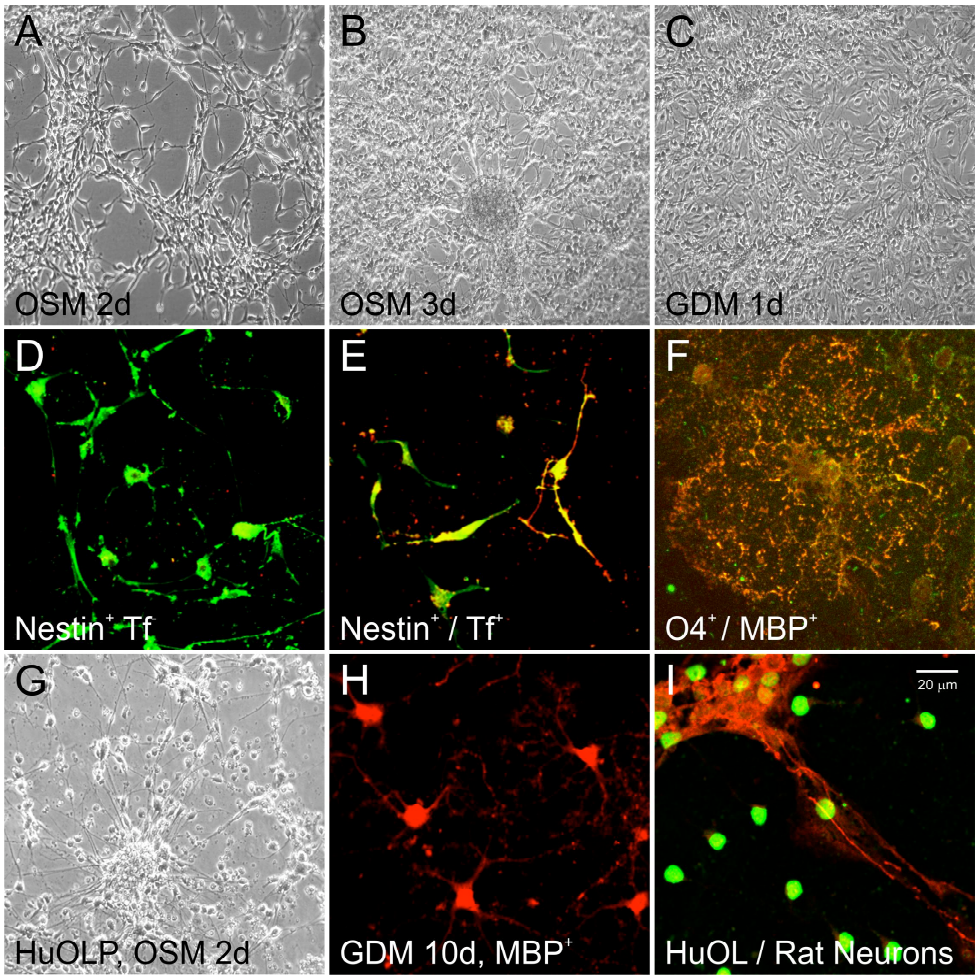


Figure A-5. Oligodendrocyte specification of NSC's derived from E16 rat. Phase contrast view of NSCs derived from E16 rat brain at passage number two (P2), [A-C]. NSC were plated and maintained in OSM for 2 days [A], 3 days [B], or 3 days in OSM then switched to GDM for 1 day, [C]. Cells in OSM still proliferate while in OSM. When cells from [A or B] are plated and maintained in OSM on poly-d-lysine coated coverslips for one day, they start to display a bipolar or multipolar morphology, [D] and most express the immature precursor marker, nestin (green) but not Tf (red), an early marker for OL. After two days in OSM, bipolar nestin+ cells co-express Tf, [E]. After four days in OSM, cells were switched to GDM for one day, developed numerous cell processes and co-expressed sulfatides (recognized by the anti-O4 antibody, green) and MBP (red), [F]. (Panels G to I are human cells) [G], Phase contrast view of human NSCs (HFB-2050) acclimated and expanded in STM, then re-plated and maintained in OSM for 2 days. [H], OL derived from human NSCs (HFB-2050) were specified to the OL lineage with OSM and maintained in GDM for 10 days. OL matured and started to express MBP (red). [I], Rat cortical neurons (NFM-200-red) were cultured for 10 days, then human OLP derived from NSC (HFB-2050) were added in co-culture for 24 hours. Human Nuclei marker (HuNu green).

7. Every 4 days, feed the cells by replacing all of the culture medium with fresh OLDEM*. The medium should look red, not orange. If it turns orange, add more medium while feeding the cells.

* These cells will express myelin enzyme levels comparable to those found in pure myelin within 5 days after having been introduced to 100% OLDEM. As they mature, cells will synthesize myelin-like membranes *in vitro* even in the absence of neurons. They can be maintained for various weeks if they are sub-confluent, however, if the culture becomes overcrowded, cells will deteriorate and die.

Support Protocol 4

Propagation for *In Vitro* Myelination Assays

To perform myelination studies *in vitro,* it is recommended to start with OL plated on plastic alone (rather than poly-d-lysine) and maintained in GDM for 2 days.

Materials:

Media: GDM and OLDEM,

Cell Scraper

Neuronal Co-culture (See Support Protocol 5 below)

25 µm sieve

24-well plates

Procedure:

1. Detach cells with cell scraper and centrifuge at 45 RCF for 8 minutes in the same culture medium.
2. Remove the supernatant and resuspend the cells in GDM-CM + fresh OLDEM (1:2).
3. A single cell suspension preparation is necessary for this step. Remove any cell clusters with a 25 μm sieve (as described previously).
4. Count the cells and adjust the cell suspension to approximately [200,000 cells/ml] of OLDEM medium.
5. Remove half the volume of culture medium from the neuronal cultures without disturbing the cells (cortical neurons or dorsal root ganglion cells).
6. Slowly add the OL suspension to one 24-well plate containing the neuronal cultures to complete the original total volume in each well.
7. Follow the co-cultures for at least 10 days. To feed, replace ½ CM with fresh OLDEM. If the cultures are not overcrowded, they can be kept for at least 4 weeks.

Support Protocol 5

Preparation of Cortical Neurons

Materials:

Instruments, animals and materials are the same as described in Basic Protocol 1.

Animals: Time-pregnant (ED14-16) Sprague-Dawley rats (Charles-River).

Bovine Serum Albumin (BSA)

Conical tubes

Culture tubes with cap, Sterile. Fisher 17 x 100mm

Hank's Balanced Salt Solution (HBSS)

Hemacytometer

Isoflurane

Needle

Poly-d-lysine

Scissors

Sterile Surgical Gauze

230 μm and 140 μm sieves

24 or 12 well plates

Procedure:

1. Prepare the work area and sterile tools in a biosafety hood [Diagram 1].
2. Euthanize the rats by isofluorane inhalation.
3. Extract the placenta containing embryos and place into STM Basal + 1% BSA.
4. Remove the cerebellum.
5. Dissect the brain of each embryo and place into Neurobasal-N complete (see Media Preparation).
6. Separate the cortex from the brain and remove the meninges with forceps.

7. *Combine the cortical tissue of all of the brains without meninges. Mechanically dissociate with the needle by gently aspirating the brain pieces and releasing the suspension slowly against the wall of the tube ten times (try to minimize foaming).
8. *Recover the supernatant with the cells in suspension and transfer to a 15ml tube.
9. Add 2 to 4 ml of Neurobasal-N medium to the chunks left over in the dissociation tube and dissociate again 5 to 8 times.
10. Filter the suspension of dissociated cells through 230 μm and 140 μm sieves to remove cell clusters.
11. Rinse the sieves sequentially with basal Neurobasal-N + 1% BSA at room temperature and add this medium to the tubes containing the cells.
12. Collect the cells by centrifugation in the culture tubes at 40 RCF for 8 min.
13. Discard the supernatant very gently as the pellet is very loose.
14. Resuspend the pellet in 4 ml of fresh Neurobasal-N medium with a 5ml pipette by gently triturating 2 or 3 times. Complete the volume to 12ml (or the equivalent of 1 embryo/ml) with 2 parts of fresh medium and 1 part of conditioned medium.
15. Assess *cell viability, count cells using a hemacytometer, and plate onto poly-d-lysine coated well plates.
16. Incubate plated cells at 37°C with 4.5% CO_2/95% humidity and monitor with a Combustion Test Kit (Bacharach # 10-500) (most electronic panels do not give an accurate reading).

Note: Cortical neurons maintained in Neurobasal-N do not need to be fed as frequently as other cell types. Simply add 500μl of Neurobasal-Nc every third day. Six days after plating, remove 1/4 of the culture medium and add the same volume of fresh Neurobasal-Nc.

*****Cell viability can be determined with SYTOX as described in Cell Viability Assay in Basic Protocol 1.

Support Protocol 6

Transplantation of OL Progenitors into Neonatal Rats

Neural progenitor cells and their differentiated OL counterparts can be stereotaxically transplanted into the newborn developing rat brain relatively non-invasively as previously described (Snyder et al., 1997; Flax et al., 1998; Espinosa et al., 2002). Similar results can be obtained with variations on the transplant method that are more suitable depending on the needs of the host brain and the type of study (Yandava et al., 1999; Ourednik et al., 2001, 2002; Park et al., 2002a; Teng et al., 2002; Wakeman et al., 2006; Lee et al., 2007; Redmond et al., 2007). A selection of detailed protocols for neonatal and adult mouse transplantation are described elseware (Espinosa et al., 1992; 1993a,b; Lee et al., 2008; Wakeman et al., 2009a,b; Yan et al., 2004). Upon implantation into the lateral ventricles, donor cells engraft and migrate from the subventricular zone into the host RMS in much the same manner as host NSC.

Materials: (may vary depending on the grafting method of choice).

Aspirator tube assembly (Sigma, A5177–5EA)

Borosilicate glass tube (Sutter Instrument, B100–75-15)

DPBS

Transillumination light source

Microfuge tube with cell sample

Micropipette puller (Sutter Instrument Co., Model P-87)

Neonatal rat (P0-P5)

Warming pad

Warm Water Glove Balloon

Wet Ice

Procedure:

1. Anesthetize the rat pup until the animal no longer retains locomotion or responds to gentle toe and tail pinch. Carefully monitor the pup and proceed to transplantation.
2. Insert a calibrated, drawn borosilicate glass micropipette into the aspirator tube assembly, and rinse the micropipette by drawing and expelling 70% Et-OH five times followed by sterile dPBS ten times to clean the needle.
3. Gently flick sample in microcentrifuge tube prior to filling the needle, wipe the tube with 70% Et-OH, and uncap the tube.
4. Slowly draw 4-5μl cell suspension into the micropipette.
5. Loosely secure the head of the anesthetized pup and place directly over the light source to visualize the eyes and bregma.
6. Carefully insert the glass needle into the head at the midline between eye and bregma and slowly inject 2-5 μl cell suspension at [$5x10^4$ cells/μl] into both lateral ventricles Slowly remove the needle and check for leakage through needle tract. Repeat step 6 into the contralateral hemisphere.
7. After the injection, warm the pup by placing on a warm water balloon glove or heating pad to increase the body temperature before returning to the mother.

Note: In addition to the lateral ventricles, NSC can also be transplanted into the striatum, SN, and corpus callosum (CC) (Bjugstad et al., 2005, Redmond et al., 2007, and Bjugstad et al., 2008). Upon implantation into the CC of the host, HFB-2050 donor cells recognized by the fluorescent Fast Blue (FB) label migrated along the CC and into the CPu. [Figure A-6]. Pre-committed-OL can also be placed locally within focal sites of injury to decrease the need for extensive migration.

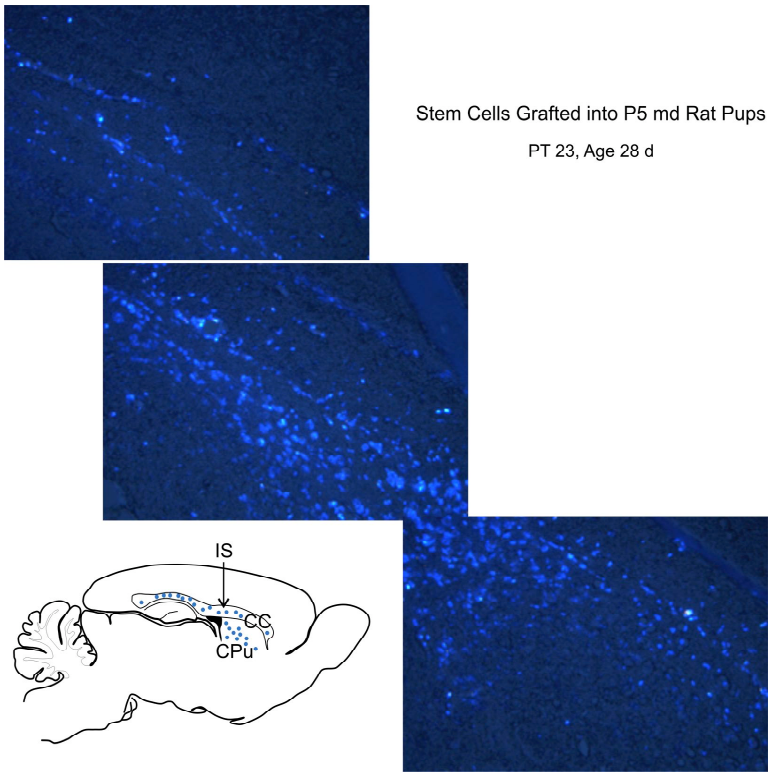


Figure A-6. Stem cells engrafted into P5 rat pup. Human OL derived from human fetal NSC were labeled with fluorescent fast blue (FB). A total of 60,000 cells were grafted into the corpus callosum (CC) of P(5) rat pups born to a md carrier mother. Twenty-three days after grafting, samples were harvested and examined. Grafted NSC survived and migrated extensively within the host brain parenchyma extending along the corpus callosum (CC) and caudate putamen (CPu). In the sketch, dots represent the location where FB+ cells were found. The sketch represents a sagittal view of the transplanted rat brain at 28 days of age, Injection Site (IS) indicates where cells were originally implanted.

Commentary:

Background Information:

The described culturing system allows for the production of relatively homogeneous primary OL cultures in adequate numbers for cryopreservation. These cell stocks can be used for basic research in further *in vitro* studies. Moreover, these cells are never exposed to animal or human sera, and therefore remain as suitable candidates for cell replacement therapies in

developmental disorders of the central nervous system (CNS) as well as neurodegenerative diseases.

Numerous methods and culture media described in the literature (even before, the times of NSC), were the basis for the optimization of the culture media formulations described here (some examples are, Botenstein and Sato, 1979; Saneto and de Vellis, 1985; Espinosa et al., 1988; Yang et al., 2005; Larsen et al., 2008., etc). Unndoubtedly, all previous reports on how to obtain and culture OL derived from NSC have also been instrumental in designing the present protocol. For example, the group of Lachapelle, and Baron-Van Evercooren described floating oligospheres derived from newborn rat brain (1996). This concept has been applied to NSC to generate OLs by Zhang (et al., 1998, Espinosa et al., 2002) and in the present protocol. Zhang and coworkers (1998) described the use of B104 neuroblastoma cell-conditioned medium (B104CM) to induce the oligodendrocyte phenotype on neurospheres and induce proliferation. This approach provides OL for many kinds of studies, but they are unsuitable as donor cells for cell replacement therapies to be used in translational studies, having used uncharacterized conditioned medium from B104 cells that have been grown in the presence of fetal bovine serum (as originally described by Louis et al., 1992). An example on the use of the protocols described can be found in Chattopadhyay et al., 2008.

Critical Parameters:

We want to emphasize that fate restriction towards commitment from NSC to OLP (as defined in *"Basic Protocol 2"*) becomes *irreversible* after NSCs have been in OSM for at least 20 hours (2D and 3D cultures). Therefore, the progeny of these cells will define a homogeneous OLP population, ideal for biochemical, toxicological and pharmacological studies, as well as an appropriate and reproducible source of committed cells to be used in cell

therapy studies. Phenotype reversal of induced OLPs may be possible with genetic manipulation but we have not attempted such studies to date.

Always monitor the concentration of CO_2 with a Combustion Test Kit (Bacharach # 10-500), as most electronic panels do not provide an accurate reading. The proper lineage progression relies on precise control of CO_2 to maintain a pH that should remain accurate and controlled.

Troubleshooting:

Human NSC are more fragile than their rodent counterparts, therefore, we recommend dissociation protocols that favor as little mechanical stress as possible. In our hands, enzymatic dissociation with 2-4 ml Accutase (Millipore) @ 37^{o}C for 3-5 minutes or light mechanical trituration through a 14-gauze needle (3-5 times) is sufficient to dissociate hNSC into single cells and small 2-6 cell clusters. Detailed methodology can be found elseware (Wakeman et al., 2009a,b).

Anticipated Results:

OLPs obtained utilizing this system are plated on anti-PSA/NCAM plates and will attain a bipolar morphology if maintained in freshly supplemented OSM. Cells can also be plated directly onto plastic (tissue culture grade). The morphology may look more flattened or fibroblastic, but if maintained in fresh OSM, the early markers such as Olig2, Tf, PDGF-R and NG2 will be expressed. At this stage, cells are still highly motile but will migrate less if plated onto poly-d-lysine. During this time, cells attain a more mature phenotype that truly represents their *in vivo* counterparts.

Our culture media formulation includes the minimum and sufficient nutrients to support a given developmental stage, thus, cells can not be kept indefinitely in these

conditions because the substratum dictates the organization of the molecules on the cell membrane and poly-d-lysine confers a more permanent adhesion to the cells. Consequently, they would have the tendency to mature based on the signals coming from the cell membrane-substrate interaction (rev. Linnemann and Bock, 1989; Mauro et al., 1994). Unfortunately, cells will not survive or remain healthy if maintained in OSM due to a lack of nutrients to support their transition to the next developmental stage. The same concept applies to the transition to more mature OL stages. The nutrients and substrate together contribute to support cell signaling that will result in the formation of multiple cell processes followed by the synthesis of myelin components and their organization for membrane formation.

Time Considerations:

The initial dissection and preparation of the primary cell suspension takes approximately 2h. From the moment cells are plated on anti-PSA-NCAM (if fed regularly with fresh humoral factors), 100mm dishes can be confluent within 3-4 days. Thus, generating 20 vials of rat NSCs for cryostorage would take approximately 16 days. The generation of OLP from rNSC takes approximately 24h; yet, generating OLP in high numbers (15 vials) for storage would take 4-6 weeks. Lineage progression of rat OL towards more mature phenotypes takes approximately 48h in the specific culture medium (GDM or OLDEM). In addition, OL will still proliferate in GDM but at a much slower rate. Both, GDM and OLDEM media are favorable to protein synthesis but less favorable for cell proliferation.

Previously isolated ES cells and their NSC derivatives will need a longer period of time to provide high numbers of NSC for frozen stocks. This time will vary depending on the origin of the sample. We have had similar success directing NSC from several species, utilizing the same chemically defined media; however, incubation times may need to be increased for full maturation in higher order mammals, such as primates. Induced cells loose

NSC characteristics and acquire OLP features within 72h, yet their cell cycle is much slower and therefore, it would be necessary to propagate these cells 8 to 10 weeks to be able to create a healthy stock (6 to 8 vials) of human OLP. Previously established NSC lines (Snyder et al., 1992) can also be propagated and specified into the OL phenotype using the system described here

Acknowledgements:

Appendix A, is a reprint in part of material as it appears in *Curr Protoc Stem Cell Biol.* Chapter 2:Unit 2D.4., Sep 2009 with co-authors Araceli Espinosa-Jeffrey, Seung U. Kim, Evan Y. Snyder, and Jean de Vellis.. The dissertation author was the co-primary investigator and co-author of this paper. . DRW and AEJ performed all experiments , co-wrote, and co-prepared the manuscript. EYS, SUK, and JdV supervised. Together with his coauthors, he thanks the MRRC Media Core for preparation of figures and Dr. D. Birt for photograph of the dissection set up. This work (J. de V. and A. E.) was supported in part by PPGHD065-76 and by a Pilot grant from the National Multiple Sclerosis Society PP1498. DRW thanks M. Hudson for critical review and comments. DRW is supported in part by the American Parkinson's Disease Association, HHMI Med-Into-Grad Training Fellowship, and the UCSD-NIH Training Fellowship in Clinical Genetics.

References:

1. **Ahn, S.M., Byun ,K., Kim, D., Lee, K., Yoo, J.S., Kim, S.U., Jho, E.H., Simpson, R.J., Lee, B. 2008. Olig2-induced neural stem cell differentiation involves downregulation of Wnt signaling and induction of Dickkopf-1 expression. *PLoS ONE*. 3(12): e3917.**

2. **Arenander, A., De Vellis, J. 1995. Early-response gene expression in glial cells. In: Neuroglia (Kettenmann H, Ransom BR, eds), pp 510-522. New York: Oxford UP.**

3. **Avellana-Adalid, V., Nait-Oumesmar, B., Lachapelle, F., Baron-Van Evercooren, A. 1996. Expansion of rat oligodendrocyte progenitors into proliferative "oligospheres" that retain differentiation potential. *J. Neurosci. Res.* 45(5): 558-70.**

4. **Bjugstad, K.B., Redmond, D.E. Jr, Teng, Y.D., Elsworth, J.D., Roth, R.H., Blanchard, B.C., Snyder, E.Y., Sladek, J.R. Jr. 2005. Neural stem cells implanted into MPTP-treated monkeys increase the size of endogenous tyrosine hydroxylase-positive cells found in the striatum: a return to control measures. *Cell Transplant.* 14(4): 183-92.**

5. **Bjugstad, K.B., Teng, Y.D., Redmond, D.E. Jr, Elsworth, J.D., Roth, R.H., Cornelius, S.K., Snyder, E.Y., Sladek, J.R. Jr. 2008. Human neural stem cells migrate along the nigrostriatal pathway in a primate model of Parkinson's disease. *Exp. Neurol*. 211(2): 362-9.**

6. **Bottenstein, J.E., Sato, G.H. 1979. Growth of a rat neuroblastoma cell line in serum-free supplemented medium. *Proc. Natl. Acad. Sci*. 76(1): 514-7.**

7. **Chattopadhyay, N., Espinosa-Jeffrey, A., Tfelt-Hansen, J., Yano, S., Bandyopadhyay, S., Brown, E.M., de Vellis, J. 2008. Calcium receptor expression and function in oligodendrocyte commitment and lineage progression: potential impact on reduced myelin basic protein in CaR-null mice. *J. Neurosci. Res.* 86(10): 2159-67.**

8. **De Filippis, L., Lamorte, G., Snyder, E.Y., Malgaroli, A., Vescovi, A.L. 2007. A novel, immortal, and multipotent human neural stem cell line generating functional neurons and oligodendrocytes. *Stem Cells*. 25(9): 2312-21.**

9. **Ebert, A.D., McMillan, E.L., and Svendsen, C.N. 2008. Isolating, expanding, and infecting human and rodent fetal neural progenitor cells. *Curr. Protoc. Stem Cell Biol.* Chapter 2: Unit 2D.**

10. **Espinosa de los Monteros, A., Roussel, G, Neskovic, N.M. and Nussbaum, J.L. 1988. A chemically defined medium for the culture of mature oligodendrocytes. *J. Neurosci. Res*. 19(2): 202-211.**

11. **Espinosa de los Monteros, A., Zhang, M., Gordon, M., Aymie, M, de Vellis, J. 1992. Transplantation of Cultured Premyelinating Oligodendrocytes into Normal and Myelin-Deficient Rat Brain. *Dev. Neurosci.* 14(2): 98-104.**

12. **Espinosa de los Monteros, A., Zhang, M.-S., and de Vellis, J. 1993a. O2A Progenitor Cells Transplanted into the Neonatal Rat Brain Develop Into Oligodendrocytes but not Astrocytes. *Proc. Natl. Acad. Sci.* 90(1): 50-54.**

13. Espinosa de los Monteros, A., Bernard, R., Tiller, B., Rouget, P., and de Vellis, J. 1993b. Grafting of fast blue labeled glial cells into neonatal rat brain: differential survival and migration among cell types. *Int. J. of Dev. Neurosci.* 11(5): 625-639.

14. Espinosa de los Monteros A., Yuan, J., McCartney, D., Madrid, B.R., Cole, R., Kanfer, J.N., and de Vellis, J. 1997. Acceleration of the maturation of oligodendroblasts into oligodendrocytes and enhancement of their myelinogenic properties by a chemically defined medium. *Dev. Neurosci.* 19(4): 297-311.

15. Espinosa-Jeffrey, A., Becker-Catania, S., Zhao, P.M., Cole, R., and de Vellis, J. 2002. Phenotype Specification and Development of Oligodendrocytes and Neurons from Rat Stem Cell Cultures Using two Chemically Defined Media. Special Issue on Stem cells, *J. Neurosci. Res.* 69: 810-825.

16. Flax, J.D., Aurora, S., Yang, C., Simonin, C., Wills, A.M., Billinghurst, L.L., Jendoubi, M., Sidman, R.L., Wolfe, J.H., Kim, S.U., Snyder EY. 1998. Engraftable human neural stem cells respond to developmental cues, replace neurons, and express foreign genes. *Nat. Biotechnol.*16(11):1033-9.

17. Kim, H.T., Kim, I.S., Lee, I.S., Lee, J.P., Snyder, E.Y., Park, K.I. 2006. Human neurospheres derived from the fetal central nervous system are regionally and temporally specified but are not committed. *Exp. Neurol.* 199(1): 222-35.

18. Kim S.U. 2004. Human neural stem cells genetically modified for brain repair in neurological disorders. *Neuropathology.* 24(3): 159-71. Review.

19. Kim, S.U., Nagai, A., Nakagawa, E., Choi, H.B., Bang, J.H., Lee, H.J., Lee, M.A., Lee, Y.B., and Park, I.H. 2008. Production and Characterization of Immortal Human Neural Stem Cell Line with Multipotent Differentiation Property. From: Methods in Molecular Biology, vol. 438: Neural Stem Cells 2nd Edition. Ed. L. P. Weiner. Humana Press, Totowa, NJ 103-121.

20. Larsen, E.C., Kondo, Y., Fahrenholtz, C.D., Duncan, I.D. 2008. Generation of cultured oligodendrocyte progenitor cells from rat neonatal brains. *Curr. Protoc. Stem Cell Biol.* Chapter 2:Unit 2D.1.1-2D.1.13.

21. Lee, H.J., Kim, K.S., Kim, E.J., Choi, H.B., Lee, K.H., Park, I.H., Ko, Y., Jeong, S.W., Kim, S.U. 2007. Brain transplantation of immortalized human neural stem cells promotes functional recovery in mouse intracerebral hemorrhage stroke model. *Stem Cells*. 25(5): 1204-12.

22. Lee, J.P., McKercher, S., Muller, F.J., and Snyder, E.Y. 2008. Neural stem cell transplantation in mouse brain. *Curr. Protoc. Neurosci.* Chapter 3: Unit 3.10.

23. Linnemann, D., Bock, E. 1989. Cell Adhesion Molecules in Neural Development. *Dev. Neurosci.* 11(3): 149-173.

24. Louis, J.C., Magal, E., Muir, D., Manthorpe, M., Varon S. 1992. CG4, a new bipotential glial cell line from rat brain, is capable of differentiating in vitro

either mature oligodendrocytes or type-2 astrocytes. *J. Neurosci. Res.* 31(1): 193–204.

25. Mauro, V.P., Wood, I.C., Krushel, C., Crossin, K.L., and Edelman, G.M. 1994. Cell adhesion alters gene transcription in chicken embryo brain cells and mouse embryonal carcinoma cells. *Proc. Natl. Acad. Sci.*.91(7): 2868-2872.

26. Müller, F.J., Snyder, E.Y., Loring, J.F. 2006. Gene therapy: can neural stem cells deliver? *Nat. Rev. Neurosci.* 7(1): 75-84. Review.

27. Neman, J., and De Vellis, J., Eds. 2008 Handbook of Neurochemistry and Molecular Neurobiology: Myelinating Cells in the Central Nervous System-Development, Aging, and Disease. Springer US.

28. Ourednik, V., Ourednik, J., Flax, J.D., Zawada, W.M., Hutt, C., Yang, C., Park, K.I., Kim, S.U., Sidman R.L., Freed, C.R., Snyder, E.Y. 2001 Segregation of human neural stem cells in the developing primate forebrain. *Science*. 293(5536): 1820-4.

29. Ourednik, J., Ourednik, V., Lynch, W.P., Schachner, M., Snyder, E.Y. 2002. Neural stem cells display an inherent mechanism for rescuing dysfunctional neurons. *Nat. Biotechnol*. 20(11): 1103-10.

30. Palmer, T.D., Schwartz, P.H., Taupin, P., Kaspar, B., Stein, S.A., and Gage, F.H. 2001. Cell culture. Progenitor cells from human brain after death. *Nature* 411(6833): 42-3.

31. Park, K.I., Teng, Y.D., Snyder, E.Y. 2002a. The injured brain interacts reciprocally with neural stem cells supported by scaffolds to reconstitute lost tissue. *Nat. Biotechnol.* 20(11): 1111-7.

32. Park, K.I., Ourednik, J., Ourednik, V., Taylor, R.M., Aboody, K.S., Auguste, K.I., Lachyankar, M.B., Redmond, D.E., Snyder, E.Y. 2002b. Global gene and cell replacement strategies via stem cells. *Gene Ther.* 9(10): 613-24. Review.

33. Redmond, D.E. Jr, Bjugstad, K.B., Teng, Y.D., Ourednik, V., Ourednik, J., Wakeman, D.R., Parsons, X.H., Gonzalez, R., Blanchard, B.C., Kim, S.U., Gu, Z., Lipton, S.A., Markakis, E.A., Roth, R.H., Elsworth, J.D., Sladek, J.R. Jr, Sidman, R.L., Snyder, E.Y. 2007. Behavioral improvement in a primate Parkinson's model is associated with multiple homeostatic effects of human neural stem cells. *Proc. Natl. Acad. Sci.* 104(29): 12175-80.

34. Saneto, R.P., de Vellis, J. 1985. Characterization of cultured rat oligodendrocytes proliferating in a serum-free chemically defined medium. *Proc. Natl. Acad. Sci.* 82(10): 3509-3513.

35. Schwartz, P.H., Bryant, P.J., Fuja, T.J., Su, H., O'Dowd, D.K., and Klassen, H. 2003. Isolation and characterization of neural progenitor cells from post-mortem human cortex. *J Neurosci. Res*. 74(6): 838-51.

36. **Snyder, E.Y., Deitcher, D.L., Walsh, C., Arnold-Aldea, S., Hartwieg, E.A., Cepko, C.L. 1992. Multipotent neural cell lines can engraft and participate in development of mouse cerebellum. *Cell.* 68(1): 33-51.**

37. **Snyder, E.Y., Yoon, C., Flax,J.D., Macklis, J.D. 1997. Multipotent neural precursors can differentiate toward replacement of neurons undergoing targeted apoptotic degeneration in adult mouse neocortex. Proc. Natl. Acad. Sci. 94(21): 11663-8.**

38. **Teng, Y.D., Lavik, E.B., Qu, X., Park, K.I., Ourednik, J., Zurakowski, D., Langer, R., Snyder, E.Y. 2002. Functional recovery following traumatic spinal cord injury mediated by a unique polymer scaffold seeded with neural stem cells. *Proc. Natl. Acad. Sci.* 99(5): 3024-9.**

39. **Svendsen, C.N., Caldwell, M.A., and Ostenfeld, T. 1999. Human neural stem cells: isolation, expansion and transplantation. *Brain Pathol*. 9(3): 499-513.**

40. **Villa, A., Snyder, E.Y., Vescovi, A., Martínez-Serrano, A. 2000. Establishment and properties of a growth factor-dependent, perpetual neural stem cell line from the human CNS. Exp Neurol. 161(1): 67-84.**

41. **Wakeman D.R., Crain, A.C., Snyder, E.Y. 2006. Large animal models are critical for rationally advancing regenerative therapies. *Regenerative Med*. 1(4): 405-413.**

42. **Wakeman D.R., Hofmann M.R., Teng Y.D., Snyder E.Y. Derivation, Expansion, and Characterization of Human Fetal Forebrain Neural Stem Cells. 2009a. *In Human Cell Culture: Adult Stem Cells: Vol. 7.* (J.R. Masters & B.O. Palsson,eds). Springer Dordrecht.**

43. **Wakeman, D.R., Hofmann, M.R., Redmond Jr., D.E., Teng, Y.D., Snyder, E.Y. 2009b. Long-Term Multilayer Adherent Network (MAN) Expansion, Maintenance, and Characterization, Chemical and Genetic Manipulation, and Transplantation of Human Fetal Forebrain Neural Stem Cells *Current Protocols in Stem Cell Biology* Hoboken: Wiley. (In press)**

44. **Williams, W.C. 2nd, Gard, A.L. 1997. In vitro death of jimpy oligodendrocytes: correlation with onset of DM-20/PLP expression and resistance to oligodendrogliotrophic factors. *J. Neurosci. Res.* 50(2): 177-89.**

45. **Wysocki, L.J., and Sato V.L. 1978. "Panning" for lymphocytes: a method for cell selection. *Proc. Natl. Acad. Sci.* 75(6): 2844-8.**

46. **Yan, J., Welsh, A.M., Bora, S.H., Snyder, E.Y., Koliatsos, V.E. 2004. Differentiation and tropic/trophic effects of exogenous neural precursors in the adult spinal cord. *J. Comp. Neurol.* 480(1): 101-14.**

47. Yandava, B.D., Billinghurst, L.L., Snyder, E.Y. 1999. "Global" cell replacement is feasible via neural stem cell transplantation: evidence from the dysmyelinated shiverer mouse brain. ***Proc Natl Acad Sci*** **96(12): 7029-34.**

48. Yang, Z., Watanabe, M., and Nishiyama, A. 2005. Optimization of Oligodendrocyte progenitor cell culture method for enhanced survival ***J. Neurosci. Methods*****. 149(1): 50-56.**

49. Zhang, S. Ch, Lundberg, C, Lundberg, Lipitz, D. O'connor, L. T. Duncan I. D. 1998. Generation of oligodendroglial progenitors from neural stem cells. ***Journal of Neurocytol.*** **27(7): 475-489.**