In Vitro Modeling of the Central Nervous System:
Towards Optimized Cell-based Therapies

by
Yu-Ting Liu Dingle
M.S., Yale University, 2009
Sc.M., Brown University, 2007

Submitted in partial fulfillment of the requirements for the degree of
Doctor of Philosophy in Biomedical Engineering, a joint program in the
Division of Biology and Medicine and the School of Engineering at Brown University

Providence, Rhode Island
May, 2015
This dissertation by Yu-Ting Liu Dingle is accepted in its present form by the Biomedical Engineering program, a joint program in the Division of Biology and Medicine and the School of Engineering as satisfying the dissertation requirement for the degree of Doctor of Philosophy.

Date ________________
Diane Hoffman-Kim, Ph.D., Advisor

Recommended to the Graduate Council

Date ________________
Jeffrey Morgan, Ph.D., Reader

Date ________________
Eric Darling, Ph.D., Reader

Date ________________
Kareen Coulombe, Ph.D., Reader

Date ________________
David Kaplan, Ph.D., External Reader

Approved by the Graduate Council

Date ________________
Peter Weber, Dean of the Graduate School
Curriculum Vitae

Yu-Ting Liu Dingle

Education

Doctor of Philosophy, Biomedical Engineering, May 2015
Brown University, Providence, RI

Master of Science, Biomedical Engineering, May 2009
Yale University, New Haven, CT

Master of Science, Medical Science, May 2007
Master’s Thesis – Mechanisms of Polystyrene Microspheres Uptake in Rat Ileum
Brown University, Providence, RI

Bachelor of Science with Honors, Biomedical Engineering, May 2006
Bachelor of Arts, Economics, May 2006
Thesis – Comparative Study of Gastrointestinal Polystyrene Microspheres Uptake and Biodistribution: Site and Size
Brown University, Providence, RI

Publications


Conference Podium Presentations


- **Liu (Dingle) Y**, Morgan J, and Hoffman-Kim D, Neural, astroglial and endothelial heterotypic cell-cell interactions in 3D. Biomedical Engineering Society Annual Meeting, Austin, Texas, 2010


Poster Presentations

- Evans E, **Dingle Y**, and Hoffman-Kim D, Simulated microgravity can alter cell viability, density, and transport in 3d microtissues. Biomedical Engineering Society Annual Meeting, San Antonio, TX, October 2014


• Reineke J, Liu (Dingle) Y, and Mathiowitz E, Quantitative resident time analysis of microsphere drug delivery systems. Undergraduate Student Poster Session, American Association for the Advancement of Science Annual Meeting, St. Louis, Missouri, USA, 2006

Awards

• NASA Rhode Island Space Grant Fellowship 2013 – 2014
• Brown Institute for Brain Science Graduate Research Award 2012
• Keystone Symposia Scholarship 2012
• U.S. Department of Education GAANN Fellowship 2011 – 2012
• National Science Foundation GK-12 Fellowship 2009 – 2011
• Faculty of Engineering Fellowship, Yale University 2007
• Incumbent Workers Masters Training Program Award 2007

Teaching Experience

• Adobe Photoshop Workshop, 2015
  Five one-hr workshops on how to use Adobe Photoshop to adjust scientific images to graduate students

• Lectures, Tissue Engineering (BIOL 1140), 2013 and 2014
  One-hr lecture "Design Considerations: Mechanical Forces in the Human Body" and a one-hr lecture "How to Make Slides for Scientific Presentations" to undergraduate students

• Lecture, Analysis of Development (BIOL 1310), 2013
  One-hr lecture "Vascularization in Tissue Engineering" to undergraduate and graduate students

• Adobe Illustrator Workshop, 2013
  Three one-hr workshops on how to use Adobe Illustrator to make scientific figures and posters to graduate students

• Lecture, Howard Hughes Medical Institute (HHMI) Summer Scholars Program, 2011
  Two-hr lecture on neural stem cell to undergraduate students
• **Lecture, Summer@Brown – Engineering Biomedical Systems, 2011**
  Two-hr lecture on stem cell engineering to high school students

• **"How to Make a Scientific Poster" Workshop, 2011**
  One-hr workshop to teach undergraduates in Research Experience for Undergraduates Program (REU) and in Undergraduate Teaching and Research Awards (UTRA) on how to make a scientific poster for their summer research.

• **Second and Third Grade Science, Vartan Gregorian Elementary School, 2009 – 2011**
  Weekly science classes to second and third graders at this local elementary school. Each class was an hour and fifteen minutes. I designed my own classes, handouts, and lab activities.

• **Brown Summer High School, 2009**
  I collaborated with four other graduate students to teach a 4-week long science course with labs for high school students.

• **Mentoring, 2011-Present**
  I have trained graduate and undergraduate students, and research assistants various lab techniques and provided guidance on their projects.

**Professional Development**

• **Brown University Sheridan Center Teaching Certificate III Program: The Professional Developmental Seminar, 2012 – 2013**
  Throughout the course, I developed my teaching portfolio, including my teaching philosophy, CV, and sample syllabus. I also worked on other important aspects of job search such as cover letters and interview skills.

• **Brown University Sheridan Center Teaching Certificate I Program: Reflective Teaching, 2011 – 2012**
  I learned several teaching modules from this program through lectures and workshops: 1. Reflections on Teaching and Learning, 2. Developing Student Learning Goals, 3. How Students Learn, 4. Grading and Evaluation, and 5. Rhetorical Practice. I practiced using these learning modules by designing a course syllabus, drafting evaluations, and giving lectures. One of my lectures was video-taped and received detailed feedback from the teaching consultants.

• **Neural Stem Cell Training Course, Stem Cell Technology, Vancouver, BC, Canada, 2012**
  In this two-day training course, I gained hands-on experience on various techniques of isolating and culturing neural stem cells from rodent brains.

**Service**

• **Brown Graduate Biomedical Engineering Society**
  2009 – Present

• **Founding President, Brown Graduate Biomedical Engineering Society**
  2009 – 2010

• **Graduate Affiliate – Trumbull College, Yale University**
  2007 – 2008

• **Judge, Rhode Island Science and Engineering Fair**
Selected Technical and Specialized Skills

- Photolithography: mask designing, clean room operation
- Soft lithography: protein micropatterning, biomimetic substrate fabrication
- Small animal surgery: bowel resection, carotid artery repair, nephrectomy, sciatic nerve guidance channel placement, subcutaneous cell transplantation, vascular graft placement
- Basic animal handling
- Tissue culture: 3D spheroid culture, mouse embryonic stem cell culture, neural stem cell culture, primary neuron isolation and culture
- Processes and techniques: cryosectioning, ELISA, immunocytochemistry,
- Imaging: confocal microscopy, epifluorescence microscopy, scanning electron microscopy (SEM), time-lapse microscopy
- Imaging software: AxioVision, ImageJ, Volocity, ZEN
- Graphing and statistics software: Excel, Prism, PowerPoint, SigmaPlot, SPSS
- Scientific illustration: Adobe Illustrator and Photoshop

Language Skills

Fluent in English and Chinese (Mandarin)
2 years of Japanese study

Other Interests

Photography (digital), illustration and drawing (digital and traditional media)
Acknowledgements

First, I would like to thank my advisor Prof. Diane Hoffman-Kim for being an amazing mentor. I was very lucky to be your student, and I have grown so much as a scientist and as a person under your guidance. You trusted me and allowed me the freedom to explore areas that were new to our lab. I have become an independent researcher working with you. You have taught me important people skills that will take me a long way in my career. The support you gave me when I had my son Watson showed me that it is possible for me to be a good mom and a great scientist at the same time. You have been a great role model and I hope I will be a good mentor like you in the future.

Thank you to my thesis committee members, Prof. Jeffrey Morgan, Prof. Eric Darling, Prof. Kareen Coulombe, Prof. Mark Zervas, and Prof. David Kaplan. You have challenged me and provided me with insightful knowledge to make me a better scientist. I would especially like to thank Jeff. Our interactions have made me a confident scientist and taught me how to advocate for myself. I would also like to acknowledge my former mentors, Prof. Michael Lysaght, Prof. Edith Mathiowitz, and Prof. Joshua Renieke, who inspired me to pursue a Ph.D. degree in biomedical research.

It has been a wonderful time working in the Hoffman-Kim Lab. Liane Livi, thank you for always being there to help me with my cultures and experiments and to make sure the lab is running smoothly. You have also helped me so much outside of the lab. Molly Boutin, thank you for your help and support with my science and writing. You are a great scientist, and I look forward to seeing your future work. Liz Evans, it has been fun to
have you in the lab, and I always appreciate your new perspectives and ideas. To the colleagues who graduated before me — Celinda Kofron, Julie Morabito, Jen Mitchel, and Cristina López-Fagundo, it was great working with you and I look forward to joining the other side with you. Talisha Ramchal, Cameron Remeter, Sam Brady, Payal Patel, Kari Truong, Cindy Oh, Ryan Din, Michael Kader, Renan Ribeiro e Ribeiro, Danielle Chau, Emily Hsieh, Melissa Tsang, and Jennifer Pallay — thank you for making working in the DHK lab a great experience.

To the people on the third floor of BioMed Center — Jackie Schell, Toni-Marie Achilli, Andrew Blakely, Beth Leary, Mike Susienka, Sean Curran, Ben Wilks, Claire Rhee, Nick Labriola, Hetal Marble, Olivia Beane, Manisha Kanthilal, Jess Sadick, Bryan Sutermaster, Rafael Gonzalez Cruz, Vera Fonseca, Vé Léandre, Chris Baker, Stacia Furtado, and Elaina Atherton — thank you for your support and friendship (and the occasional homemade baked goods) throughout the years. Thank you for making the third floor such a supportive and interactive environment where people share ideas and help out each other. Special thanks to Jackie, you have always helped me think new ideas. I will miss the time that we chat about both science and non-science topics at the lunch table, and I wish the best for all of you.

Thank you to all the administrative staff — Jess Bello, Carol Folan, Cheryl Pariseau, Elly Peimer, Brian Quinn, Kris McCutcheon, Crystal Miller, and Tim Durning. I was able to focus on research because you took care of everything else.

I would especially like to thank my family. Mom and Dad, you have provided me with a tremendous amount of support throughout my entire education year after year.
Now as a mom myself, I understand how hard it was for you to send me away to attend high school in the U.S. so I could get a better education. I would not have achieved what I am today without you. I really appreciate all that you have done for me, and I hope you are proud of me. Andy, Dicky, and Flora, best wishes for your medical school and graduate studies.

To my son Watson, your arrival has changed my life in so many ways. Most importantly, it has motivated me to be a good role model for you as a good person and a good scientist. You have taught me to be patient and to put things in perspective. I would still appreciate it if you would let me get some extra sleep once in a while.

Finally, I would like to thank my husband Patrick for your continuous support and encouragement. I am glad that I found someone who is nice and smart and shares the same passion for science. You have provided me with brilliant ideas and Matlab code to solve questions that I encountered. You have always had my back. You took care of our son when I needed the extra hours in the lab, and you have been there to encourage me when research doesn't go well. My Ph.D. years have been so much more enjoyable with you in the picture, and I look forward to the next stage of our lives together.
Table of Contents

Overview of Thesis Chapters ........................................................................................................... 1

Chapter 1  Introduction.................................................................................................................... 3

1.1 Relevance ..................................................................................................................................... 3
1.2 Background and significance ......................................................................................................... 5
1.3 Overview of CNS diseases and cell-based therapy strategies ....................................................... 7
1.4 Overview of CNS injuries and cell-based therapy strategies ......................................................... 9
1.5 Choice of parameters in cell-based therapy .................................................................................. 11
  1.5.1 Cell source .......................................................................................................................... 11
  1.5.2 Pre-transplantation culture condition .................................................................................... 13
  1.5.3 Delivery method, location, and timing .................................................................................. 14
  1.5.4 Co-delivery of soluble factors ............................................................................................. 16
1.6 In vitro Models of the CNS.......................................................................................................... 16
  1.6.1 Design criteria ....................................................................................................................... 16
  1.6.2 2D monolayer cultures .......................................................................................................... 19
  1.6.3 Organotypic slice cultures ..................................................................................................... 20
  1.6.4 3D scaffold culture ................................................................................................................ 21
  1.6.5 Aggregates ............................................................................................................................ 24
1.7 Closing remarks ........................................................................................................................... 27
1.8 References .................................................................................................................................... 27

Chapter 2  Quantitative analysis of dopamine neuron subtypes generated from mouse embryonic stem cells .............................................................................................................. 41

2.1 Abstract ...................................................................................................................................... 42
2.2 Introduction ................................................................................................................................. 42
2.3 Materials and methods ................................................................................................................ 45
  2.3.1 Mouse embryonic stem cell culture ....................................................................................... 45
  2.3.2 DA neuron differentiation ...................................................................................................... 46
  2.3.3 Immunocytochemistry .......................................................................................................... 49
  2.3.4 Quantification ....................................................................................................................... 50
  2.3.5 Sampling size selection by Monte Carlo simulation .............................................................. 51
  2.3.6 Quantitative real time PCR (qRT-PCR) ................................................................................ 52
  2.3.7 Statistical method .................................................................................................................. 52
2.4 Results ......................................................................................................................................... 54
  2.4.1 Selection of quantification approach using Monte Carlo simulation .................................... 54
  2.4.2 5-stage method generated more CALB+ than CALR+ subtype in D3 ESC-derived DA neurons .......................................................... 57
2.4.3 The role of exogenous SHH and FGF8 during NP expansion (Stage IV) on DA neurons subtype generation from D3 ESCs ................................................................. 59
2.4.4 Exogenous SHH and FGF8 during NP expansion (Stage IV) did not influence total DA neuron production ................................................................. 60
2.4.5 The role of exogenous SHH and FGF8 on developmental signaling pathways .......... 62
2.4.6 Comparative analysis with R1 ESCs: DA neuron production and subtype generation . 65
2.4.7 Exogenous SHH and FGF8 during NP selection stage (Stage III) did not substantially change the proportions of subtypes in D3-derived DA neurons ......................... 67
2.4.8 Treatment with SHH- and FGFR3-neutralizing antibodies did not alter CALB^2 or CALR^2 subtype generation ................................................................. 68
2.5 Discussion .................................................................................................................... 70
  2.5.1 The role of SHH and FGF8 in ESC-derived DA neurons ........................................ 71
  2.5.2 DA neuron subtypes derived from mouse ESCs .................................................. 75
2.6 Conclusion .................................................................................................................. 77
2.7 Acknowledgements .................................................................................................... 78
2.8 References ................................................................................................................ 78
2.9 Supplementary materials .......................................................................................... 84

Chapter 3  3D neural spheroid culture: an in vitro model for the central nervous system .................................................................................................................. 93

3.1 Abstract ....................................................................................................................... 94
3.2 Introduction ................................................................................................................ 94
3.3 Materials and methods ............................................................................................ 97
  3.3.1 Cell isolation and culture ..................................................................................... 97
  3.3.2 3D self-assembled cortical spheroid fabrication ................................................... 98
  3.3.3 Viability assay .................................................................................................. 98
  3.3.4 Immunostaining and optical clearing ................................................................. 98
  3.3.5 Imaging and image analysis ............................................................................. 99
  3.3.6 Electrophysiology ........................................................................................... 100
  3.3.7 Mechanical characterization ............................................................................ 101
3.4 Results ....................................................................................................................... 102
  3.4.1 Cortical cells self-assembled into 3D spheroids ............................................... 102
  3.4.2 Cortical spheroids remained viable for at least three weeks in culture .......... 103
  3.4.3 Neurons and astrocytes formed complex 3D structures in the spheroids .......... 104
  3.4.4 Neurons in spheroids were electrically active and formed synaptic connections.. 106
  3.4.5 Cortical spheroids had similar elastic moduli to cortical tissue ......................... 110
3.5 Discussion ................................................................................................................ 111
  3.5.1 Versatile culture .............................................................................................. 111
  3.5.2 In vivo-like cell morphology and structure ....................................................... 112
  3.5.3 ECM production ............................................................................................. 113
  3.5.4 Active circuitry in the spheroid ...................................................................... 114
  3.5.5 Brain-like mechanical properties ................................................................... 115
3.6 Conclusion ............................................................................................................... 116

xiii
List of Tables

Table 1.1 Nature and synthetic materials used for neural cell culture ........................................... 22
Table 2.1 Quantification and statistical comparisons of CALB<sup>-</sup> and CALB<sup>+</sup> subtypes ........... 59
Table 2.2 Quantification and statistical comparisons of total neurons and DA neurons ............ 62
Table S2.1 Sequence of primers used for qRT-PCR ................................................................. 91
## List of Figures

### Chapter 2  Quantitative analysis of dopamine neuron subtypes generated from mouse embryonic stem cells

<table>
<thead>
<tr>
<th>Figure</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1</td>
<td>Summary of culture conditions.</td>
<td>48</td>
</tr>
<tr>
<td>2.2</td>
<td>Sampling and quantification approach.</td>
<td>56</td>
</tr>
<tr>
<td>2.3</td>
<td>Quantification of CALB(^+) and CALR(^+) DA neuron subtypes in S0F0(<em>{IV}) vs S500F100(</em>{IV}).</td>
<td>58</td>
</tr>
<tr>
<td>2.4</td>
<td>Exogenous SHH and FGF8 during NP expansion did not enhance DA neuron production.</td>
<td>61</td>
</tr>
<tr>
<td>2.5</td>
<td>Endogenous SHH, FGF8, and WNT1 signaling components in ESCs and NPs.</td>
<td>64</td>
</tr>
<tr>
<td>2.6</td>
<td>CALB(^+) and CALR(^+) subtypes of DA neurons were generated from R1 ESCs.</td>
<td>66</td>
</tr>
<tr>
<td>2.7</td>
<td>SHH and FGF8 at Stage III did not govern CALB(^+) DA neuron subtype generation.</td>
<td>68</td>
</tr>
<tr>
<td>2.8</td>
<td>Blocking endogenous SHH and FGF8 signaling did not alter DA neuron subtypes.</td>
<td>70</td>
</tr>
<tr>
<td>S2.1</td>
<td>Cell morphology in the 5-stage culture.</td>
<td>84</td>
</tr>
<tr>
<td>S2.2</td>
<td>Heterogeneous distribution of TH(^+) neurons.</td>
<td>85</td>
</tr>
<tr>
<td>S2.3</td>
<td>GIRK2(^+) subtype identity was acquired later.</td>
<td>86</td>
</tr>
<tr>
<td>S2.4</td>
<td>LMX1a expression in Stage III NPs.</td>
<td>87</td>
</tr>
<tr>
<td>S2.5</td>
<td>Exogenous SHH/FGF8 or anti-SHH/FGFR3 neutralizing antibodies did not alter DA neuron production.</td>
<td>88</td>
</tr>
<tr>
<td>S2.6</td>
<td>Relationship between TH(^+) neuron clustering and subtype differentiation.</td>
<td>89</td>
</tr>
<tr>
<td>S2.7</td>
<td>In vitro CALB(^+) and CALR(^+) subtype specification did not correlate with SHH and FGF8 concentrations.</td>
<td>90</td>
</tr>
</tbody>
</table>

### Chapter 3  3D neural spheroid culture: an in vitro model for the central nervous system

<table>
<thead>
<tr>
<th>Figure</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.1</td>
<td>Size-controlled 3D cortical spheroids.</td>
<td>103</td>
</tr>
<tr>
<td>3.2</td>
<td>Neurons and astrocytes formed laminin-containing 3D networks.</td>
<td>105</td>
</tr>
<tr>
<td>3.3</td>
<td>7 DIV spheroids contained neurons with immature properties.</td>
<td>108</td>
</tr>
<tr>
<td>3.4</td>
<td>14 DIV spheroids contained mature neurons with synapses.</td>
<td>109</td>
</tr>
<tr>
<td>3.5</td>
<td>Mechanical properties of 3D spheroids.</td>
<td>110</td>
</tr>
<tr>
<td>S3.1</td>
<td>21 DIV spheroids contained mature neurons with functional synapses.</td>
<td>122</td>
</tr>
<tr>
<td>S3.2</td>
<td>3D hippocampal spheroids.</td>
<td>123</td>
</tr>
</tbody>
</table>

### Chapter 4  3D in vitro stem cell transplantation model for the central nervous system

<table>
<thead>
<tr>
<th>Figure</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.1</td>
<td>The in vitro transplantation model consists of a cortical trampoline microtissue and a NSC spheroid.</td>
<td>134</td>
</tr>
</tbody>
</table>
4.2 Utility of the *in vitro* model - study of the effect of FGF2 on NSC migration. ................. 136
S4.1 Critical seeding density for cortical trampoline............................................................ 142
S4.2 Migration assay in 2D models. .................................................................................. 143

**Appendix A**  
Endothelial cell and neural cell spheroid co-culture for *in vitro* modeling of the neurovascular unit

A.1 Self-assembly of ECs ........................................................................................................ 156
A.2 Spheroid co-cultures of neural cells and ECs ................................................................. 158

**Appendix B**  
Scientific illustrations

B.1. Methods of fabrication of topographical platforms......................................................... 160
B.2. Topographies presented to neurons *in vitro*. ................................................................. 161
B.3. Schwann cells perform a range of functions in the body ................................................ 162
B.4. Bioengineered model of stem cell manipulation and cell transplantation for neurological disorders. .................................................................................................................. 163
B.5. Investigating the effects of simulated microgravity on 3D neural stem cell Microtissue .... 164
B.6. Axon guidance by critical cues – Engineering nerve growth *in vitro* and observing from afar ........................................................................................................................................ 165
B.7. *In vitro* neuroma model .................................................................................................. 166
B.8. Biomimetic synthetic feeder layer .................................................................................... 167
B.9. Cell origami ...................................................................................................................... 168
Overview of Thesis Chapters

The work presented in this thesis focuses on two areas of *in vitro* central nervous system (CNS) modeling related to cell-based therapy: Part 1. The generation of neurons from stem cells (Chapter 2). Part 2. The development of *in vitro* CNS models for testing graft-host interactions (Chapters 3 and 4). Chapter 1 provides broad background and motivation of the thesis work, with emphasis on the clinical demand for improved cell-based therapies, current problems, and the need to fine-tune the therapy approach for better clinical outcomes. Following the motivation, I provide a review of the existing *in vitro* culture platforms for the study of CNS function, disease and injury mechanisms, and cell-based therapies.

Chapters 2 - 4 are presented in the style of scientific journal articles including Introduction, Materials and Methods, Results, Discussion, References, and Supplemental Materials. Complementary experiments and results not reported in the submitted journal article manuscript are included in the end of each chapter.

Chapter 2 - Quantitative Analysis of Dopamine Neuron Subtypes Generated from Mouse Embryonic Stem Cells - I describe a study assessing the heterogeneity of dopamine neurons differentiated from mouse embryonic stem cells. I report the percentages of dopamine neuron subtypes generated using an *in vitro* differentiation protocol and show that dopamine neuron subtype specification *in vitro* is not governed by exogenous soluble factors sonic hedgehog and fibroblast growth factor 8. In addition, I describe an optimized quantitative approach developed to address the inherent
variability of embryonic stem cell cultures and the uneven spatial distribution of the differentiated cells.

Chapter 3 - 3D Neural Spheroid Culture: An In Vitro Model for the Central Nervous System - I describe an accessible and reproducible method without the use of scaffolds to fabricate 3D neural spheroids using primary rat CNS cells. Characterization of the spheroids showed that the spheroids present key features of the in vivo microenvironment, including cell type, structure, extracellular matrix type, mechanical properties, and electrophysiological properties. This study demonstrates that 3D neural spheroid culture is a feasible in vitro model for the investigation of CNS. The work has been submitted as a manuscript to the following journal: Tissue Engineering Part C.

Chapter 4 - 3D In Vitro Stem Cell Transplantation Model for the Central Nervous System - This chapter expands on the scaffold-less culture presented in Chapter 2. I describe a 3D co-culture consisting of "graft" neural stem cell (NSC) spheroids and "host" cortical neural microtissues to evaluate interactions between graft neural stem cells and host brain tissues, and to screen stem cell transplantation parameters in vitro. This chapter is formatted in the style of a Short Communication.

Chapter 5 summarizes overall the key findings and proposes future experiments to build upon the work presented here.

Appendix A describes a pilot study using spheroid co-cultures for in vitro modeling of neurovascular unit.

Appendix B is a portfolio of scientific illustrations I have done for publications, grant proposals, and fellowship applications.
Chapter 1

Introduction

1.1 Relevance

The central nervous system (CNS), which consists of the brain and the spinal cord, has limited self-restorative ability after disease or injury. Cell-based therapy is an emerging treatment option that facilitates the healing of the CNS via two mechanisms of action: 1) cell replacement - replacing damaged cells using stem cell-derived neurons and 2) neuroprotection - delivering cell-secreted soluble factors produced by the transplanted cells. A variety of stem cells have been proposed and tested for cell-based therapies. For a cell-based therapy to be successful, stem cells first need to differentiate to the cell type of interest and survive after transplantation. Following transplantation, the cells need to migrate to the damaged area, and reconnect the neuronal circuitry or secrete the neuroprotective factors. Current technologies have yet to achieve full control of cell fate and behavior before and after transplantation, and safety and efficacy still need to be carefully addressed.

A cell's intrinsic genetic makeup and the microenvironmental cues together determine the cell's fate and behavior. These microenvironmental cues include neighboring cells, extracellular matrix (ECM), soluble factors, substrate-bound factors, topography, and mechanical properties. During development, the intrinsic and microenvironment cues work in a precise spatial and temporal fashion to generate all the
tissues and organs in an organism. However, we do not fully understand the exact role of each factor to use them to control cell fate and behavior. There is a need for understanding how the cues within a cell's microenvironment affect biological activities in order to fine-tune cell behavior and achieve better clinical outcomes. Broadly speaking, this thesis focuses on two aspects of cell-based therapy: *in vitro* differentiation of stem cells into dopamine (DA) neurons, and the interaction of transplanted neural stem cells (NSCs) within the host tissue.

The use of animal models to study CNS therapies is necessary because animal models present the full picture of complex host microenvironments that transplanted cells would be surrounded by and are the most translatable in terms of evaluating functional outcome before a therapy moves to clinical trials. In contrast, *in vitro* platforms allow for deconstruction of this complexity to enable researchers to closely examine cell behavior and manipulate microenvironmental cues. In the earlier part of this thesis (Chapter 2), we used a traditional 2D culture to investigate cell fate specification of DA neurons derived from ESCs, and further evaluated the effect of soluble factors on ESC-derived DA neurons. The methodology and information provided by this chapter of the thesis contribute to the field of neurobiology that studies DA neuron-associated diseases and to the field of regenerative medicine that uses stem cell-derived DA neurons for cell replacement therapy.

While simplified 2D cultures let us study individual input variables, many microenvironmental cues are missing in the tissue culture plastic dish. Therefore, the second part of this thesis (Chapter 3 and 4) aims to develop a 3D *in vitro* culture platform with *in vivo*-like features to model the CNS and to study graft-host interactions. The
reported 3D spheroid culture resembles many characteristics of the CNS tissue and can potentially recapitulate native cell behavior better than simplified 2D cultures. Further, the 3D culture can be used to model many CNS diseases and injuries. During the design of various cell-based therapies, there are many variables that can impact treatment outcome, such as cell source, developmental stage, delivery method, timing, and co-delivery of trophic factors. We showed that the 3D neural culture described in this thesis can facilitate more rapid testing of these transplantation parameters. Knowledge of how these variables impact cell fate and behavior can advance the design of a cell-based therapy procedure and potentially improve cell-based therapy outcome.

1.2 Background and significance

Neurodegenerative diseases and injuries are often incurable, can leave the patient with permanent damage, and create heavy financial burden on the patients and society. For example, more than five million Americans live with Alzheimer's disease (AD), and more than $200 billion is spent per year for the care of AD patients (www.alz.org). Parkinson's disease (PD) affects one million Americans, with estimated $25 million per year spent on the patient care (Parkinson's Disease Foundation, http://www.pdf.org/). In the US, stroke affects 800,000 people per year and is the fourth leading cause of death. The cost of care for stroke survivors combined with lost in productivity due to premature mortality adds up to more than $34 billion per year (CDC, http://www.cdc.gov/stroke/). The number of people living with spinal cord injury is estimated to be 270,000 in the U.S, with the direct and indirect cost combined assumed to be over $20 billion (The National SCI Statistical Center, https://www.nscisc.uab.edu/).
Disease and injury in the CNS are especially debilitating because mature CNS neurons do not typically regenerate. The death of neurons leads to lifelong impairment of motor or cognitive functions. However, stem cells have the ability to differentiate into multiple types of cells, including neurons. The classical model of cell-based therapy is to replace lost cells and reconnect the neuronal circuitry using stem cell-derived neurons. It has become evident that paracrine effects are beneficial to functional restoration in the CNS, including secreting of neuroprotective factors, modulating inflammatory responses, providing substrates for axon growth and remyelination, and promoting endogenous neurogenesis (Dunnett and Rosser, 2014; Joyce et al., 2010). Mesenchymal stem cells (MSC), which secrete a variety of these paracrine factors, have also been used for cell-based therapy for CNS.

Although some functional recovery has been reported in animal models, early clinical trials of cell-based therapy for Parkinson's disease had mixed results (Olanow et al., 2003, 2009). Low survival, lack of migration to the lesion site, uncontrolled differentiation, deficient integration with host tissue, and tumor formation remain the major issues with cell-based therapies (Lindvall and Kokaia, 2010). Better understanding and optimization of the parameters involved in the design of cell-based therapy procedures can aid in improved functional recovery.
1.3 Overview of CNS diseases and cell-based therapy strategies

Parkinson's disease (PD)

The midbrain consists of the ventral tegmental area (VTA) and the substantia nigra (SN). DA neurons in the VTA project to limbic and cortical areas, giving rise to the mesocortical and mesolimbic pathways that are involved in cognition, emotional balance, reward-associated behavior, and addiction. DA neurons in the SN project to the dorsal striatum. This nigrostriatal pathway is essential for motor functions, and the hallmark of PD is the degeneration of these SN DA neurons (Björklund and Dunnett, 2007). Patients with PD experience rigidity, hypokinesia, tremor, and lack of postural control. Despite the impact on the patient's motor functions, life expectancy is not affected. Current standard treatment is the use of a DA agonist L-3,4-dihydroxyphenylalanine (L-DOPA) to temporarily alleviate the symptoms, but drug treatment does not stop disease progression (Lindvall, 2003).

A cell-based therapy approach involves the generation of stem cell-derived DA neurons to restore lost DA neurons (Lindvall and Bjorklund, 2009). DA neurons have been generated from ESCs and induced pluripotent stem cells (iPSCs) of mouse, non-human primate, and human origins, although the differentiated culture is generally "contaminated" with other cell types (Lee et al., 2000; Maria et al., 2013). Enrichment of DA neurons can be achieved by forced expression of transcription factors associated with DA neuron progenitors, such as Nurr1 and Lmx1a (Chung et al., 2002; Friling et al., 2009). Transplantation of mouse and human stem cell-derived DA neurons in animal
models of PD has shown improvement of motor functions (Kim et al., 2002; Kriks et al., 2011; Moon et al., 2013). However, some clinical trials transplanting fetal tissue-derived cells failed to show significant improvement (Olanow et al., 2003).

**Motor neuron diseases - amyotrophic lateral sclerosis (ALS) and spinal muscular atrophy (SMA)**

There are two major neurodegenerative diseases involving the function of motor neurons - ALS and SMA. ALS is an adult-onset neurodegenerative disease which involves the death of cortical and spinal motor neurons. The cause is unknown in the majority (90%) of the cases. Patients experience rapid progression of muscle weakness and eventually have difficulty swallowing and speaking. There is currently no cure for ALS, and most patients do not survive due to respiratory failure within three to five years (Kiernan et al., 2011). SMA is a childhood autosomal recessive genetic disorder that results in the death of spinal motor neurons. Children with the severe types of SMA do not survive beyond age four (Davis-Dusenbery et al., 2014).

Motor neurons have been generated from both mouse and human stem cells (Wichterle et al., 2002) and transplantation has shown some improvement in the lifespan in ALS mouse models (Kondo et al., 2014). The current clinical goal of cell-based therapies for motor neuron diseases is to promote survival of existing motor neurons in the hostile microenvironment (Lindvall and Kokaia, 2010). Transplantation of stem cells overexpressing neuroprotective trophic factors such as vascular endothelial growth factor (VEGF) and glial derived neurotrophic factor (GDNF), have been shown to have positive
effects on motor neuron survival in animal models (Hwang et al., 2009; Suzuki et al., 2007).

**Alzheimer's disease (AD)**

AD involves multiple cell types in multiple brain regions. Neurofibrillary tangles and amyloid-β plaques cause degeneration of neurons and synapses in the basal forebrain cholinergic system, amygdala, hippocampus, and cortical areas (Lindvall and Kokaia, 2010). Symptoms include memory loss, disorientation, mood swing, dementia, and inability of self-care. Antipsychotics are used to treat dementia-related symptoms, however, there is no permanent treatment. Cell replacement therapy for AD is challenging because of the involvement with multiple cell types in wide-spread regions. Controlled generation of multiple cell types from stem cells has not yet been accomplished (Dunnett and Rosser, 2014). Current focus with cell-based therapy is to deliver neuroprotective factors and to promote neurogenesis from endogenous neural stem cells (NSCs).

**1.4 Overview of CNS injuries and cell-based therapy strategies**

**Stroke**

Ischemic stroke is caused by the blockage of blood flow in the brain, and hemorrhagic stroke is caused by rupture of the blood vessels. Both types of stroke result in acute regional death of multiple cell types. Although there has been evidence of endogenous neurogenesis (Ohab et al., 2006), patients generally do not achieve full recovery. Transplanting mouse and human ESCs and ESC-derived neural precursor cells
in animal models have shown graft cell survival, migration, differentiation, as well as improvement in motor function (Darsalia et al., 2007; Kelly et al., 2004). Other studies focus on using cells to deliver neurotrophic factors, such as VEGF and GDNF to enhance survival, protect surviving cells, promote angiogenesis, modulate the inflammatory environment, and recruit endogenous NSCs (Lee et al., 2007; Nomura et al., 2005).

**Traumatic brain injury**

Physical trauma to the brain causes cell and structural damage via multiple mechanisms. Mechanical stress and shear force on the cells and tissues create acute necrosis. Ischemia, hematoma formation, glutamate release, and hyper-inflammatory response lead to prolonged apoptosis. Axonal swelling due to membrane perforations causes dispersed lesions beyond the focal injury (Aertker et al., 2015). Cell-based therapies have been shown to improve motor functions in animal models through modulation of local and systemic inflammatory responses and local production of neurotrophic factors. Low survival of transplanted cells in the highly inflammatory environment has been a major challenge (Coyne et al., 2006; Riess et al., 2002).

**Spinal cord injury**

During spinal cord injury, the axons are severed and the lesion at the site of injury is filled with glial scars that inhibit axon regeneration. Patient symptoms include loss of sensory and motor control below the lesion. Human stem cells transplanted in rat spinal cords have shown differentiation into neurons and oligodendrocytes. These neurons reinnervated existing neurons, and oligodendrocytes promoted remyelination support (Cummings et al., 2005; Yan et al., 2007). However, there has been no evidence of long
distance precise point-to-point reinnervation (Dunnett and Rosser, 2014). The neuroprotective mechanisms of stem cell-therapy has been shown to be beneficial, and several clinical trials are ongoing.

1.5 Choice of parameters in cell-based therapy

1.5.1 Cell source

Fetal tissues

Region-specific neural precursors can be obtained from fetal tissues. These cells continue their region-specific differentiation *in vitro* (Darsalia et al., 2007). Transplanting fate-committed cells that match the affected host cell type has better potential to restore the native neuronal circuitry. However, due to the ethical concerns and the limited sources, the use of human fetal tissues for stem cell therapy is unlikely to meet the clinical demand. Clinical trials using cells derived from aborted human fetal mesencephalon to treat PD also showed variability in clinical improvement, as well as some side effects of dyskinesia (Kordower et al., 1998; Olanow et al., 2003). Nevertheless, fetal neural precursors still remain valuable for research in order to understand the effects of cell types and cell-fate commitment on engraftment success.

ESC and ESC-derived neural stem/progenitor cells

ESCs can be expanded indefinitely and are not genetically modified. They can differentiate into multiple CNS cell types both *in vitro* and *in vivo*. Transplantation of ESCs or ESC-derived cells have shown motor function recovery in animal models of PD
and spinal cord injury (Kim et al., 2002; Liu et al., 2000; Nishimura et al., 2003). However, ethical concerns and regulations limit the use of human ESCs. In addition, teratoma formation from ESCs after transplantation remains a problem (Brederlau et al., 2006).

**iPSCs and iPSC-derived neural stem/progenitor cells**

Reprogramming adult somatic cells into iPSCs that have ESC-like pluripotency was a groundbreaking success in the field of stem cell and regenerative medicine (Takahashi et al., 2007). iPSC-derived neural cells enable the modeling of human CNS disease and injury, bridging the gap created by the limited access to human CNS tissues (Ouyang et al., 2013; Soldner and Jaenisch, 2012). Patient-derived iPSCs opened the possibility of autologous cell transplantation (Soldner et al., 2009; Zhang et al., 2013) and circumvent the ethical concerns with ESCs.

**Mesenchymal stem cells (MSCs)**

MSCs are of stromal origin and can be derived from cord blood, adult peripheral blood, and adipose tissue in large quantities. MSCs can shelter themselves from the host immune system and their safety has been demonstrated in multiple clinical trials (Le Blanc, 2006). The usefulness of MSC for the treatment of CNS diseases and injury is due to their secretion of neurotrophic factors, such as BDNF and GDNF, as well as their production of ECM to promote endogenous repair (Aizman et al., 2009). Although MSCs have been shown to transdifferentiate to express neuronal markers in vitro, their function and capacity to replace neurons is unknown (Engler et al., 2006; Joyce et al., 2010).
**Endogenous adult neural stem cells**

Evidence of adult neurogenesis in the subventricular zone (SVZ) of the lateral ventricles and the subgranular zone (SGZ) of the dentate gyrus in the hippocampus was first reported in the 90's (Eriksson et al., 1998; Gage, 2000; Reynolds and Weiss, 1996). It is now widely accepted that the mature brain retains a limited ability to regenerate (Zhao et al., 2008). NSCs from these regions are capable of differentiating into neurons, astrocytes, and oligodendrocytes. In rodents, NSCs in the SVZ replenish olfactory neurons, and NSCs in the SGZ are believed to be involved in memory formation. Live human NSCs *in vivo*, which would require invasive and unnecessary procedures to isolate, are not a feasible source for transplantation. However, there is great interest in promoting endogenous NSC proliferation, differentiation, and migration toward the lesion for self-repair as a mean of regeneration.

### 1.5.2 Pre-transplantation culture condition

The disease or injury determine the types of cells needed for cell-based therapy. Not only is there a lack of resources of fully differentiated CNS cells, mature neurons have poor survival after cell isolation procedures. However, stem cells and progenitor cells are more robust for transplantation than mature neurons, but controlling their terminal fate in the host is not a trivial issue. Therefore, the developmental stage of the cells need to be optimized and understood for better survival and controlled differentiation.

Hahn et al. showed lineage-restricted DA progenitors isolated from embryonic day 13 (E13) rats resulted in better graft cell survival and functional recovery in rat PD
models than progenitors from E15 rats (Hahn et al., 2009). In another transplantation study using human ESC-derived DA neurons, pre-differentiated *in vitro* for 16-23 days, results showed no functional recovery in rat PD models. Teratoma formation was observed from graft cells that were pre-differentiated for only 16 days (Brederlau et al., 2006). *In vitro* pre-treatment of the graft cells with neurotrophic factors, such as BDNF and GDNF can also improve survival and functional recovery following transplantation (Rosenblum et al., 2014; Wang et al., 2011).

*In vitro* differentiation of stem cells rarely generate a homogenous population of a target cell type. More than 70% of the cells in the culture do not become DA neurons from an established *in vitro* ESC differentiation protocol (Chung et al., 2002; Lee et al., 2000). Genetic modification can enhance the specification of certain neuronal type but can also sometimes create side effects. Overexpression of the transcription factor Nurr1 enhanced *in vitro* DA neuron differentiation, but when these cells were transplanted, PD model animals exhibited incorrect overcorrection motor behavior (Kim et al., 2002). In order to optimize the outcome of cell-based therapy, the effects of *in vitro* culture condition and treatment on graft cell behavior need to be carefully addressed pre-clinically.

### 1.5.3 Delivery method, location, and timing

Cells can be delivered either intravenously or locally to or away from the injury. Intravenous injection is less invasive and is usually used for systemic immunomodulation, whereas local engraftment in the brain or the spinal cord allows better control of the location of the graft cells. For local engraftment, cells can be delivered as suspended cells,
as aggregates, or be embedded in scaffolds. The highly inflammatory injury environment can lead to poor graft cell survival (Kelly et al., 2004). Encapsulating cells in scaffolds or transplanting cells as aggregates may offer protection from inflammatory response immediately following transplantation (Li et al., 2012; Tate et al., 2009). Laminin and fibronectin scaffolds have been shown to enhance graft NSC survival in injured rat brain (Tate et al., 2009). Biodegradable scaffolds further allow later integration of the graft cells with the host tissue (Choi et al., 2013; Mahoney and Anseth, 2006; Perale et al., 2011).

The location in the host tissue for engraftment greatly affects outcome. Kelly et al. showed poor cell survival of human NSCs transplanted at the lesion site of an animal stroke model, likely due to the hostile inflammatory environment. Cells transplanted laterally away from the lesion had better health and were able to migrate toward the lesion (Kelly et al., 2004). For PD, most of the studies have been done by transplanting cells in the striatum (the target region of the DA neurons) instead of the SN of the midbrain (the original location of DA neurons) to circumvent the challenge of axon guidance. However, the transplanted DA neurons in the striatum do not receive upstream communications. One such study has showed that an adult rat brain still retained axon guidance ability for DA neurons transplanted in the SN (Thompson et al., 2009), opening the discussion on the optimal location for transplantation.

After injury, astrocytes and fibroblasts remodel the ECM to form glial and fibrotic scars. Inflammatory responses lead to continued cell death and spreading of the lesion (Aertker et al., 2015). If patient-derived iPSCs are used, it can take weeks to generate iPSCs and further neural lineage-restricted cells. Therefore, there must be a balance
between targeting the effective therapeutic, time window, and the differentiation stage of the graft cells.

1.5.4 Co-delivery of soluble factors

Families of neurotrophic factors - GDNF, brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF), are important for the survival, maintenance, and regeneration of specific neural cells in the adult CNS (Allen et al., 2013; Allodi et al., 2011). Other soluble factors such as VEGF and FGF2 have also been shown to enhance stem cell and neural progenitor cell survival and migration (Mani et al., 2010). Co-delivery of trophic factors in soluble form or by overexpression in the graft cells, has been suggested to promote translatable outcomes in cell-based therapy. (Deng et al., 2013; Hwang et al., 2009; Lee et al., 2007; Thompson et al., 2009). Besides trophic factors, delivery of enzymes that remodel the glial scar in rat spinal cord injury models has also been shown to enhance stem cell migration toward the lesion and neurite outgrowth (Ikegami et al., 2005).

1.6 In vitro Models of the CNS

1.6.1 Design criteria

In vitro CNS models allow faster pre-animal screening of the parameters mentioned previously for the optimization of cell-based therapy. With a variety of CNS modeling approaches, it is important to consider the appropriate design criteria to fit the problem statement and purpose.
Cell types

The main cell types in the mature CNS are neurons, astrocytes, oligodendrocytes, microglia, endothelial cells, and pericytes (Hopkins et al., 2014). A small number of NSCs also are present in the SVZ and SGZ. Different types of neurons are involved in distinct neurological diseases. Ideally, cell types used for the model should match the cells affected within the disease or injury. Region-specific neurons can be isolated from the CNS, but mature neurons are post-mitotic and cannot be expanded to obtain in large numbers. Access to human primary CNS neurons is very limited. Thus human neural cell line and human ESC- and iPSC-derived neurons are the main source to model the human CNS in vitro.

ECM

ECM accounts for 20% of the adult brain mass, and within the 20% of ECM in the brain, there are three major ECM components: the basement membrane, perineuronal nets, and neural interstitial matrix (Lau et al., 2013). The basement membrane is composed of laminin (LN), fibronectin (FN), type IV collagen (Col IV), and heparan sulfate proteoglycans (HSPG). Perineuronal nets are composed of hyaluronan (HA), chondroitin sulfate proteoglycans (CSPG), and tenascin R. The neural interstitial matrix is composed of FN, HA, CSPG, and a small amount of LN, FN, and Col IV. ECM regulates neural stem cell survival, migration, and differentiation, axon guidance, and synapse formation. ECM is remodeled throughout development. Changes in ECM are often hallmarks of CNS disease and injury (Lau et al., 2013).
Dimensionality

Neural cells in vivo communicate with other cells and the ECM in all directions but are forced to a planar morphology and can only interact with other neurons laterally in 2D culture. In 2D, cell-cell and cell-ECM communication is altered, and the 3D topography in the tissue is lost (Bruder et al., 2007; Hoffman-Kim et al., 2010). Difference in biological activities, including survival, proliferation, differentiation, protein and gene expression, response to toxicity, and synapse formation have been shown in 2D vs 3D (Baraniak and McDevitt, 2012; Kofron et al., 2009; Li et al., 2007; Xu et al., 2009).

Mechanical properties

The CNS is soft (~ 0.5 kPa) in comparison to other tissues and organs in the body (ex. 12 kPa for skeletal muscle) (Elkin et al., 2007; Levental et al., 2007). Cells sense and respond to the mechanical properties of their microenvironment via various mechanotransduction mechanisms. The impact of substrate stiffness in neural stem cell differentiation, migration, neurite outgrowth, and response to toxins has been well-documented (Franze et al., 2013). For example, neurite branching was reduced when the substrate stiffness changed from brain-like stiffness to fibrotic tissue-like stiffness (Flanagan et al., 2002). Enhanced neuronal differentiation over glial differentiation was observed on softer substrate (Georges et al., 2006). Therefore, substrates with brain-like mechanical properties are more likely to produce more translatable results.
Compatibility with analytical methods

Having measureable outputs is essential for assessing the effect of input variables on cell behavior. Many of the analytical techniques (i.e. viability, migration, phenotypic characterization, electrophysiology) are optimized for one platform but may either be incompatible or have lower resolution in other platforms. For example, materials used for 3D cultures can have obstacles for light microscopy and single cell patch-clamping electrophysiology characterization.

1.6.2 2D monolayer cultures

Culturing cells as a monolayer in a tissue culture dish is the gold standard and most widely used in vitro method. This type of culture is reproducible and requires less training and technical resources than the other in vitro models. There is a high degree of freedom when manipulating the culture conditions. Related cell manipulation, analytical, and characterization techniques have been optimized for 2D cultures. Generating DA neurons or motor neurons from stem cells in vitro was first developed using 2D cultures (Kawasaki et al., 2000; Lee et al., 2000; Wichterle et al., 2002). For modeling injury environments, glial and fibrosis scar models that are capable of inhibiting neuronal growth have been developed in 2D cultures (Kimura-Kuroda et al., 2010; Wanner et al., 2008). 2D cultures are compatible with many high-resolution imaging techniques and allow for the close examination of cellular mechanisms. The phenomenon of microglia-mediated cell fusion between stem cells and neurons was also first observed in 2D and following confirmation in vivo (Cusulin et al., 2012).
Despite access to powerful analytical techniques, many of the \textit{in vivo} microenvironmental cues are absent in 2D cultures (Page et al., 2013; Pampaloni et al., 2007). ECM are presented as discrete fibrils \textit{in vivo} but as a continuous 2D layer (Baker and Chen, 2012). The mechanical properties of the tissue culture plastic - in gigapascal - are many orders of magnitude stiffer than the mechanical properties of the CNS. Soluble molecules diffuse into the media and do not establish gradients (Choi et al., 2014). Due to the striking differences between 2D cultures and \textit{in vivo} 3D microenvironment, there is an increasing demand for more physiologically relevant cultures for translatable medicine (Baker and Chen, 2012).

\subsection*{1.6.3 Organotypic slice cultures}

Organotypic slice cultures are ex vivo models prepared from thin sections (typically hundreds of micrometers in thickness) of the brain and spinal cord. The slices preserve native cell types, ECM, cellular organization, and parts of the circuitry and function, which therefore gives a better indication to \textit{in vivo} responses than other \textit{in vitro} models (Sundstrom et al., 2005). Slice cultures are often prepared from postnatal (P0-P10) animals and some from adult animals (Daviaud et al., 2013). Disease and injury can be modeled through oxygen glucose deprivation, chemical insult, or mechanical loading (Kearns et al., 2006; Morrison et al., 2011). Graft cells can be added to the culture media in suspension, injected directly to the slice, or grafted as spheroid microtissues (Kress and Reynolds, 2005). The visual inspection of tagged graft cell survival, migration, differentiation, and integration is easier than animal models (Tanvig et al., 2009;
Tønnesen et al., 2011). Functional integration between graft cells and host via gap junction formation was first observed using slice culture (Jäderstad et al., 2010).

Organotypic cultures also have their own disadvantages. The slices cannot be kept for more than one month in culture. Confounding factors are difficult to remove due to the presence of multiple cell types and factors in the slice cultures. Most importantly, access to human CNS organotypic slices is extremely limited.

1.6.4 3D scaffold cultures

Scaffolds facilitate cells to form 3D morphology and organization, as well as present the cells with micro- and nanoscale 3D structures and topography that better mimic the in vivo microenvironment (Drury and Mooney, 2003; Lee et al., 2008). The types of scaffolds can be loosely grouped into hydrogels, solid materials, and decellularized tissues. Both natural and synthetic materials have been successfully used for 3D neural cultures. Examples of common scaffold materials are listed in Table 1.1. Many natural materials contain integrin-binding peptides for cell adhesion, and synthetic materials that require additional conjugation of peptide or protein for the cells to anchor (Frampton et al., 2011; Hopkins et al., 2014). 3D scaffolds offer more control of cell types, substrate materials, cell organization, and soluble molecules in the system than slice cultures. Cullen et al. modeled CNS injury by using neuron-astrocyte co-culture embedded in 3D Matrigel treated with mechanical loading. Using this injury model, they showed that delivery of NSCs in a LN-containing scaffold (vs. suspended) can reduce graft cell apoptosis (Cullen et al., 2007).
Scaffolds can be further designed to incorporate sophisticated architectures, such as layered neuronal and glial cells as observed in the six-layered cerebral cortex, or to compartmentalize soma and axons, mimicking the grey and white matter in the brain (Frampton et al., 2011; Odawara et al., 2013; Tang-Schomer et al., 2014). Tang-Schomer et al. demonstrated a cortex model using layered silk and collagen scaffolds. The neurons responded to mechanical insult similarly to cortical neurons in vivo (Tang-Schomer et al., 2014). These scaffold designs that incorporate histotypical structures can better reflect the complex organization and interconnectivity in the CNS in vivo compared to 2D culture and bulk scaffolds.

<table>
<thead>
<tr>
<th>Natural materials</th>
<th>Synthetic materials</th>
</tr>
</thead>
<tbody>
<tr>
<td>Agarose</td>
<td>Poly (ethylene glycol) (PEG)</td>
</tr>
<tr>
<td>Alginate</td>
<td>Poly(2-hydroxyethyl methacrylate) (pHEMA)</td>
</tr>
<tr>
<td>Collagen I</td>
<td>Poly((β-hydroxybutyrate) (PHB)</td>
</tr>
<tr>
<td>Hyaluronic acid</td>
<td>Poly(ε-caprolactone) (PCL)</td>
</tr>
<tr>
<td>Chitosan</td>
<td>Poly(lactic-co-glycolic acid) (PLGA)</td>
</tr>
<tr>
<td>Methyl-cellulose</td>
<td></td>
</tr>
<tr>
<td>Silk fibroin</td>
<td></td>
</tr>
</tbody>
</table>

**Table 1.1 Nature and synthetic materials used for neural cell culture.** Reviewed in (Hopkins et al., 2014; Subramanian et al., 2009)

Hydrogels are crosslinked hydrophilic polymers swollen in fluids. This material is widely used because of their tunable stiffness, efficient diffusion properties, and transparency (Annabi et al., 2010). Polymers in the hydrogels are generally randomly oriented, but hydrogels containing aligned fibrils have also been fabricated to direct neurite extension (Odawara et al., 2013). The health of neurons has been shown to be well-supported by hydrogels (O’Connor et al., 2001; Xu et al., 2009). Hydrogels with
brain-like mechanical properties make them an excellent candidate to study neural cells in a biomimetic microenvironment. In addition, hydrogel cultures are able to establish a gradient and accumulation of soluble factors secreted by cells, whereas these factors disperse into media in a 2D culture. Because accumulation of amyloid-β is an important pathological features of AD, recent studies have demonstrated that 3D hydrogel cultures are more capable at recapitulating AD pathology than traditional 2D cultures (Choi et al., 2014; Seidel et al., 2012; Zhang et al., 2014).

Solid material scaffolds presenting engineered topography are good for directing and aligning cells and cell extensions (Hoffman-Kim et al., 2010). Solid materials include electrospun fibers, porous sponges, layered colloids, pillared structures, and grooved substrates (Bakhru et al., 2011; Frega et al., 2014; Hayman et al., 2004; Kofron et al., 2009; Pautot et al., 2008; Puschmann et al., 2014). The micro- and nano-structures of solid materials can be designed via material fabrication processes. The mechanical properties of solid materials are typically stiffer than hydrogels - ranging from kilo- to megapascals (Palchesko et al., 2012). In addition, many solid material scaffolds are anisotropic and support less z-direction growth than in x and y directions compared to hydrogel scaffolds (Limongi et al., 2013; Pautot et al., 2008; Puschmann et al., 2014).

The shared disadvantage between hydrogel and solid material-based scaffolds is the introduction of foreign materials. Because cell-ECM signaling directs many aspects of normal and pathological cell function, the presence of foreign materials can potentially mask or alter native cell behavior. Neural cells in vivo remodel their ECM as they migrate, differentiate, mature, and form synapses. Nevertheless, many of the scaffold materials cannot be enzymatically remodeled by the cells to reflect the dynamic change of the
microenvironment (Laperle et al., 2014). ECM-to-cell ratio is often much higher in scaffold cultures than \textit{in vivo}. The resulting lower cell density, lower cell-cell communications, and higher cell-ECM interactions can also impact cell behavior.

Decellularized tissue scaffolds retain native ECM types, ECM organization, and substrate-bound factors. Thus, decellularized tissue scaffolds present a more \textit{in vivo}-like microenvironment than the other types of scaffolds. Brain tissues have been decellularized and repopulated with cells (Baiguera et al., 2014; DeQuach et al., 2011). However, intact tissues need to be first collected from animals, making decellularized tissue scaffolds less feasible for high throughput tests.

### 1.6.5 Aggregates

Culturing cells as 3D multicellular aggregates, also known as spheroids, initially gained great interest in cancer biology and has recently been adopted to the neurobiology field. Free-floating cells cultured in various scaffold-free methods adhere to each other and self-assemble into aggregates. Techniques for self-assembly include hanging-drops, non-adhesive dishes/molds, conical tubes on roller drums, or spinning bioreactors (Birenboim et al., 2013; Layer et al., 2002; Napolitano et al., 2007). The term organoids are special aggregates of organ-specific cells that self-organize in a manner similar to \textit{in vivo} (Lancaster and Knoblich, 2014; McCracken et al., 2014).

In the aggregates, cells produce their own ECM, and thus the native ECM type is preserved (Fennema et al., 2013). Tumor cells grown as aggregates have been shown to reflect the clinical gene and protein expression pattern and mass transport properties, likely due to the appropriate cell-cell and cell-ECM interactions (Achilli et al., 2014;
Friedrich et al., 2009). iPSC-derived neuron aggregates showed higher neuronal markers compared to 2D cultures (Pamies et al., 2014). Aggregate cultures allow for higher cell density and cell-cell communication. Normal physiological functions such as intercellular signal transduction via synapses highly correlates with the amount of cell-cell interactions and intercellular distances (Cullen et al., 2010). During development, newly-born neurons migrate along radial glial cell guidance fibers to their final locations in the cortex (Anton et al., 1999). Pathological features like amyloid-β-induced apoptosis have also been reported to relate to intercellular distance. Therefore, there is an increasing trend to use aggregate 3D culture for AD and other CNS disease modeling (Choi et al., 2013; Seidel et al., 2012).

The *in vivo*-like microenvironment in the aggregates can foster organogenesis-like processes. Cells in the aggregates have been shown to retain their intrinsic self-organization ability. (Eiraku et al., 2011; Sato et al., 2009). Lancaster et al. modeled human brain development in an organoids culture (Lancaster et al., 2013). Neural progenitor cells derived from human ESCs and iPSCs formed organoids, these organoids spontaneously developed ventricle-like cavities, and cells had proper polarized adhesion protein expression. Markers of different brain regions showed layered organization within the organoids. They successfully modeled microcephaly using microcephaly patient-derived iPSCs and RNA interference, a developmental disorder that has been difficult to recapitulate in mouse models. The resulting organoids were smaller with very few progenitor regions. Their results demonstrated that CNS disease modeling can be effectively achieved by the use of human cells in aggregate culture.
Although organoid studies have demonstrated cell's self-organization capability within an aggregate, random-oriented cellular structures are typically observed in 3D neural aggregates (Choi et al., 2013). An approach to gain better spatial control of cell positions is to use the spheroids as individual building units to create larger microtissues. Kato-Negishi et al. reported a platform using cortical neuron spheroids and hippocampal neuron spheroids as building units. They allowed the cortical spheroids and hippocampal spheroids to fuse to form larger microtissue constructs for modeling the cortex-hippocampus interface (Kato-Negishi et al., 2013). A recent study published by our collaborator's lab developed an instrument to pick up the microtissues and place them at precise locations to fabricate larger complex microtissues (Blakely et al., 2014).

The high cell density in aggregates has its own caveat. Light scattering in the aggregates can limit the depth for image acquisition in light microscopy, even with confocal or two-photon microscopy. Recent advances in tissue clearing techniques have improved optical properties of otherwise opaque tissues, organs, or whole animals (Hama et al., 2011; Ke et al., 2013; Kuwajima et al., 2013; Zhu et al., 2013). Our lab has previously optimized the ClearT2 technique for spheroids of neural cells and showed that we could image 100µm into the spheroid (Boutin and Hoffman-Kim, 2014). Clearing has enabled the visualization and phenotypic characterization within whole aggregates.

Analysis that rely on diffusion and cellular uptake of reagents can also be difficult to carry out due to the limited diffusion beyond the outer layers of the aggregates (Achilli et al., 2014). Destruction of the aggregates by lysing or physical sectioning is sometimes required, but the process disrupts fine structures and can also be labor-intensive. (Ju et al., 2006). Analytical methods and technologies have started to adapt to the emerging field of
3D cultures. We can expect to see more techniques become available for the functional evaluation of 3D neural spheroids (Hopkins et al., 2014).

1.7 Closing remarks

Stem cell biology and regenerative medicine have opened many opportunities for treatment of the human body. A successful cell-based therapy requires finding the right combination of cells and the presentation of microenvironmental cues to direct cell fate and behavior. A systematic approach is to understand the relationship between the input parameters and the cells' biological activities and use this collective information to determine an optimized therapy regimen. By improving the ways to recreate the in vivo microenvironment in the cell culture, we can extract the information from a cellular level and translate into clinical applications. Interdisciplinary collaborations are essential for the complete understanding of cell behavior. Our tissue engineering strategies contribute to the field by providing new tools to explore the complex interactions between cell and their surroundings, with the goal of advancing cell-based therapies for the CNS diseases and injuries.

1.8 References


Maria, S., Helle, B., Tristan, L., Gaynor, S., Arnar, A., Michele, M., Teresia, O., Oliver, C., Roger, S., Penelope, H., et al. (2013). Improved Cell Therapy Protocol for Parkinson’s Disease Based on Differentiation Efficiency and Safety of Hesc-, Hipsc and Non-Human Primate iPSC-Derived DA Neurons. Stem Cells N/A – N/A.


Chapter 2

Quantitative analysis of dopamine neuron subtypes generated from mouse embryonic stem cells

Manuscript in preparation

Yu-Ting L. Dingle\textsuperscript{1,2¶}, Katherine B. Xiong\textsuperscript{3¶}, Jason T. Machan\textsuperscript{4}, Kimberly A. Seymour\textsuperscript{3}, Debra Ellisor\textsuperscript{3}, Diane Hoffman-Kim\textsuperscript{1,2,5,6}, and Mark Zervas\textsuperscript{3,5}

\textsuperscript{1}Department of Molecular Pharmacology, Physiology and Biotechnology, Providence, RI, USA
\textsuperscript{2}Center for Biomedical Engineering, Brown University, Providence, RI
\textsuperscript{3}Department of Molecular Biology, Cell Biology, and Biochemistry, Division of Biology and Medicine, Brown University, Providence, RI
\textsuperscript{4}Departments of Orthopedics and Surgery at Rhode Island Hospital and The Warren Alpert Medical School at Brown University, Providence, RI
\textsuperscript{5}Brown Institute for Brain Science, Providence, RI, USA
\textsuperscript{6}School of Engineering, Brown University, Providence, RI

\textsuperscript{¶}These authors contributed equally to this work.

Author contributions

YLD, DHK, and MZ conceived of the project and all experiments therein. YLD and MZ designed the dopamine neuron differentiation experiments. YLD conducted initial experiments, developed the quantification method, performed preliminary quantification, and oversaw all data analysis. KBX conducted and completed dopamine neuron differentiation experiments and quantification. JTM conducted statistical analysis with YLD, MZ, and DHK. KAS conducted mRNA analysis. DE contributed to the initial design of experiments. DHK and MZ oversaw all experiments and analysis.
2.1 Abstract

The diverse subtypes of dopamine (DA) neurons modulate specific physiological functions and are involved in distinct neurological disorders. Embryonic stem cell (ESC)-derived DA neurons have the potential to aid in the study of disease mechanisms, drug discovery, and future cell replacement therapies. DA neurons can be generated from ESCs in vitro, but despite the diversity of DA neurons, the subtypes of ESC-derived DA have not been investigated in detail. Due to cell culture heterogeneity, sampling methods applied to ESC-derived cultures can be ambiguous and potentially biased. Therefore, we developed a quantification method to capture the depth of DA neuron production in vitro by estimating the error associated with systematic random sampling. Using this method, we quantified calbindin\(^+\) and calretinin\(^+\) subtypes of DA neurons generated from mouse ESCs and found a higher production of calbindin\(^+\) subtype (11-27\%) than calretinin\(^+\) subtype (2-13\%) of DA neurons; in addition, DA neurons expressing neither subtype marker were also generated. We then examined whether exogenous sonic hedgehog (SHH) and fibroblast growth factor 8 (FGF8) affected subtype generation. Our results demonstrated that exogenous SHH and FGF8 did not alter DA neuron subtype generation in vitro. This suggests that a deeper understanding of other mechanisms that govern in vitro subtype differentiation of ESC-derived DA neurons is required.

2.2 Introduction

Dopamine (DA) neurons in the midbrain are a diverse population and consist of subtypes with distinct anatomical and molecular identities (Grimm et al., 2004). When
defined by anatomical region, the major groups are the substantia nigra (SN) or the A9 
group and the ventral tegmental area (VTA) or the A10 group (Smidt and Burbach, 
2007). SN DA neurons, which innervate the dorsal striatum, are essential for initiating 
movement and controlling posture reflexes (Björklund and Dunnett, 2007; Grealish et al., 
2010; Grimm et al., 2004; Thompson et al., 2009). VTA DA neurons, which project to 
the ventral striatum, limbic, and cortical areas, are involved in cognition, reward, and 
emotional behavior (Ikemoto, 2007; Prakash and Wurst, 2006; Smidt and Burbach, 
2007). The degeneration of SN DA neurons leads to the loss of motor function in 
Parkinson's disease (Olanow et al., 2009), while abnormal activity of VTA DA neurons 
has been suggested to underlie schizophrenia, addiction, and attention deficit 
hyperactivity disorder (Björklund and Dunnett, 2007; Grimm et al., 2004; Ikemoto, 2007; 
Nestler, 2001; Viggiano et al., 2003).

The heterogeneous molecular identity of DA neurons adds further complexity to 
the subtypes. Calbindin (CALB), a calcium-binding protein, is enriched in the majority of 
VTA DA neurons and in a subset of SN DA neurons (Brichta and Greengard, 2014; 
Brown et al., 2011; Fu et al., 2012; Liang et al., 1996; Nemoto et al., 1999; Reyes et al., 
2012). A recent study suggested that CALB buffers DA vesicle release in healthy VTA 
neurons (Pan and Ryan, 2012). The expression of CALB may protect DA neurons from 
calcium cytotoxicity-induced degeneration as VTA DA neurons are less affected than SN 
DA neurons in Parkinson's disease (Brichta and Greengard, 2014; Chung et al., 2005; 
Neuhoft et al., 2002). Calretinin (CALR), another calcium-binding protein, is expressed 
in DA neurons scattered in the VTA and SN (Brown et al., 2011; Liang et al., 1996; 
Nemoto et al., 1999). The physiological function of CALR is not well understood, but it
has been suggested that CALR may have similar neuroprotective properties to CALB (Kim et al., 2000; Mouatt-Prigent et al., 1994). A third protein, G-protein-regulated inward-rectifier potassium channel 2 (GIRK2), is expressed in nearly all SN DA neurons, which undergo the greatest degree of degeneration in Parkinson's disease (Mendez et al., 2005; Reyes et al., 2012; Thompson et al., 2005). The expression of CALB, CALR, and GIRK2 is not mutually exclusive and gives rise to diverse subtypes of DA neurons (Alfahel-Kakunda and Silverman, 1997; Brown et al., 2011; Liang et al., 1996; McRitchie et al., 1996; Nemoto et al., 1999).

Because of their clinical relevance the generation of DA neurons in vitro offers opportunities to study disease mechanism, progression and therapeutics and holds potential for cell replacement therapy (Hargus et al., 2010; Kirkeby et al., 2012; Lindvall et al., 2004; Soldner et al., 2009). An established method of deriving DA neurons from embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) in vitro is a 5-stage differentiation method first described by Lee et al. (Lee et al., 2000). Each stage utilizes a defined culture substrate and media and cells become lineage-restricted as they pass through each stage. Differentiated neurons are observed at the end of the protocol with DA neurons accounting for a small percentage of all neurons. The DA neuron population generated using this 5-stage method is not homogeneous (Chung et al., 2002; Friling et al., 2009; Sánchez-Danés et al., 2012), but the subtype profile has not been quantitatively analyzed.

The efficiency of DA neuron production with the 5-stage method may be enhanced by exposing neural progenitors at the fourth stage to exogenous sonic hedgehog (SHH) and fibroblast growth factor 8 (FGF8). SHH and FGF8 are two morphogens
required for inducing DA neuron fate in the midbrain during embryonic development (Ye et al., 1998). At embryonic day 8.5 (E8.5) SHH is expressed along the ventral midline and FGF8 in the isthmus at the mid/hindbrain boundary (Zervas et al., 2005); the intersection of these two morphogens is where DA neuron progenitors appear during embryogenesis (Ye et al., 1998). A SHH gradient has been shown to be involved with motor neuron subtype specification in the spinal cord (Ericson et al., 1997), but it is unknown whether soluble morphogens play a role in subtype specification in the midbrain.

In this study, we established a sampling approach to quantify DA neuron subtypes in heterogeneous cultures. We then evaluated the generation of DA neuron subtypes from a baseline 5-stage culture, in which no exogenous SHH and FGF8 were added. Among the DA neurons, we detected the expression of CALB and CALR, but not GIRK2. We then conducted quantitative analysis of the CALB+ subtype and CALR+ subtype of DA neurons. We further investigated whether the distribution of CALB+ and CALR+ subtypes could be manipulated by the addition of SHH and FGF8 at different stages. We discuss these findings and the relative roles of endogenous and exogenous factors in subtype specification of DA neurons.

2.3 Materials and methods

2.3.1 Mouse embryonic stem cell culture

Mitomycin C-inactivated mouse embryonic fibroblasts (iMEFs), obtained from Brown University's Transgenic Facility, were cultured for 24 hrs in MEF media [Dulbecco's
Modified Eagle Medium (DMEM, Invitrogen, 11965), 10% fetal bovine serum (FBS, Gemini, 100-106) and 1% Penicillin-Streptomycin-Glutamine (Pen/Strep/Glut, Invitrogen, 10378) in 10 cm tissue culture dishes (BD, 353003) coated with 0.1% gelatin (Sigma, G2500). D3 ESCs (ATCC, CRL-11632) and R1 ESCs (ATCC, SCRC-1011) were each cultured on confluent iMEF monolayers at 2.5-3x10⁶ cells/dish in ES media [Knock-out DMEM (Invitrogen, 10829), 15% FBS, 100 μM MEM nonessential amino acids (Invitrogen, 11140), 1% Pen/Strep/Glut, and 55 μM 2-mercaptoethanol (Invitrogen, 21985)] supplemented with 1400 U/mL ESGRO leukemia inhibitory factor (LIF, Millipore, ESG1107). ESCs were cultured for 1-2 days before colonies contacted each other and were dissociated using 0.05% trypsin (Invitrogen, 25300). The ESCs were passaged onto a new iMEF monolayer, cryopreserved in ES media with an additional 10% FBS and 10% DMSO (Sigma, D2650), or used for DA neuron differentiation (see below). Passage number of D3 ESCs was not provided by ATCC, and therefore we designated passage 1 (P1) as the purchased vial.

2.3.2 DA neuron differentiation

We used a 5-stage protocol, with a baseline culture that was slightly modified from the 5-stage method first described by Lee et al. in 2000 (Lee et al., 2000) and commercialized by R&D Systems (Human/Mouse DA Neuron Differentiation Kit, R&D Systems, SC001b). As illustrated in Figure 2.1 and Supplementary Figure S2.1 the five stages of the baseline culture were: Stage I, ESC expansion - ESCs were cultured on gelatin-coated tissue culture dish in ESC media with LIF for 1-2 days before colonies contacted each other. Stage II, EB formation - ESC colonies were dissociated with 0.05%
trypsin. Monodispersed ESCs were cultured in non-adherent bacterial culture dishes (BD, 351029) at 2x10^6 cells/dish for 3-4 days in ES media without ESGRO to form EBs. Stage III, Neural precursor (NP) selection - The EBs were transferred to tissue culture dishes in ES media. After 24 hrs, the media was replaced with ITSFn media [DMEM/F12 (Invitrogen, 11320), 1X insulin-transferrin-selenium supplement (ITS, Invitrogen, ITS-G), 50 µg/mL fibronectin (Fn, R&D Systems, 1030-FN), and 1% Pen/Strep (Invitrogen, 15140)]. The cells were cultured in ITSFn media for 6 days and then dissociated with 0.05% trypsin. Stage IV, NP expansion - monodispersed Nestin^+ NPs were cultured at 3x10^5 cells/well on 12-mm glass coverslips coated with 15 µg/mL poly-L-ornithine (PLO, Sigma, P4957) and 1 µg/mL Fn in 24-well plates in N2aa media [DMEM/F12, N-2 Max supplement (R&D, AR009), 1% Pen/Strep, and 200 µM ascorbic acid (Sigma, A4403)] supplemented with 10 ng/mL basic fibroblast factor (bFGF, R&D, 233-FB-025/CF). Cells were cultured for 4 days. Stage V, Differentiation - Media was replaced with N2aa media without bFGF. Cells were cultured in this differentiation media for either 10 days for D3 or 15 days for R1.
Figure 2.1. Summary of culture conditions.

A modified version of the 5-stage protocol was used in this study. Baseline control conditions (S0F0\textsubscript{IV}, S0F0\textsubscript{III}, and S0F0\textsubscript{α}): Undifferentiated ESCs were expanded in ESC media with LIF (Stage I, ESC Expansion). ESCs were cultured as EBs in suspension in ESC media without LIF (Stage II, EB Formation). NPs (blue) were selected in ITSFn media (Stage III, NP Selection). NPs were expanded in N2aa media with bFGF (10 ng/mL) (Stage IV, NP Expansion). Cells differentiated after growth factor withdrawal (Stage V, Differentiation). Experimental conditions: (a) SHH (500 ng/mL) and FGF8 (100 ng/mL) were added during Stage IV (S500F100\textsubscript{IV}). The paired control (S0F0\textsubscript{IV}) was conducted in parallel. (b) SHH (500 ng/mL) and FGF8 (100 ng/mL) were added during Stage III (S500F100\textsubscript{III}). The paired control (S0F0\textsubscript{III}) was conducted in parallel. (c) Anti-SHH (10 μg/mL) and anti-FGFR3 (10 μg/mL) antibodies were added at the start of Stage III until the end of the experiment (αSaFr). The paired control (S0F0\textsubscript{α}) was conducted in parallel. Differentiated cells at the end of Stage V were immunolabeled for TH (DA neurons), NeuN (neurons), and subtype markers CALB, CALR, and GIRK2. NPs at the end of Stage III were immunolabeled for Nestin (NP) and LMX1a (transcription factor associated with DA neuron progenitors). Undifferentiated ESCs and cells at the end of Stage IV were analyzed for the mRNA expression of SHH and FGF8 signaling components, namely Shh, Gli1, Fgf8, Fgfr3, Wnt1, Tcf4, Nfat, and Tcf4.

In the first experiment (S500F100\textsubscript{IV}, Figure 2.1a), P3 of D3 ESCs and P21 of R1 ESCs were used. SHH (500 ng/mL, R&D, 461-SH-025/CF) and FGF8 (100 ng/mL, R&D, 423-F8-025/CF) were added to the N2aa media and refreshed upon media exchange during NP expansion (Stage IV). In the second experiment (S500F100\textsubscript{III}, Figure
2.1b), P5 of D3 ESCs was used, and SHH and FGF8 were added to the ITSFn media during NP selection (Stage III) and refreshed upon media exchange. In the third experiment (αSαFr, Figure 2.1c), P5 of D3 ESCs was used. Anti-SHH antibody (10 µg/mL, R&D, MAB4641) and anti-FGFR3 (10 µg/mL, R&D, MAB710) were added from the beginning of Stage III to the end of Stage V. Antibodies were refreshed upon media change.

A common problem with ESC culture is the variation among subclones (Martinez et al., 2012). To remove confounding variables, all experiments described in this study were conducted in parallel with a paired control group (S0F0 IV, S0F0 III, and S0F0 α) using the baseline culture protocol. The experimental group and the control group started with the same passage and subclone of ESCs. To evaluate the expression of tyrosine hydroxylase (TH), NeuN, CALB, CALR, and GIRK2 in the differentiated culture, cells were fixed with 4% paraformaldehyde at the end of Stage V. To evaluate the expression of Nestin and LMX1a in NPs, cells at the end of Stage III were trypsinized and re-plated on PLO/Fn-coated glass coverslips in ITSFn media for 24 hrs and then fixed.

2.3.3 Immunocytochemistry

Immunocytochemistry was carried out using standard protocols (Brown et al., 2011). Primary antibodies were diluted in working solution [phosphate buffered saline (PBS), 10% normal donkey serum and 0.2% Triton X-100] with the following dilutions: mouse anti-nestin (Millipore, MAB353, 1:200), rabbit anti-LMX1a (Millipore, AB10533, 1:200), mouse anti-Tuj-1 (R&D, MAB1195, 1:1000), rabbit anti-TH (Millipore, AB152, 1:1000), sheep anti-TH (Millipore, AB1542, 1:1000), rabbit anti-calbindin (SWANT,
1:5000), goat anti-calretinin (Millipore, AB1550, 1:5000). Mouse anti-NeuN antibody (Millipore, MAB377, 1:100) was diluted in PBS. Primary antibody incubation was done at 4°C overnight, followed by three washes with PBT [0.2% Triton-X in PBS]. Appropriately matched secondary antibodies were diluted in working solution (all at 1:500): Alexa 488 donkey anti-sheep (Invitrogen, A11015), Alexa 488 donkey anti-rabbit (Invitrogen, A21206), Alexa 555 donkey anti-mouse (Invitrogen, A31570), Alexa 555 donkey anti-rabbit (Invitrogen, A31572), and Alexa 555 donkey anti-goat (Invitrogen, A21432). Secondary antibody incubation was done at room temperature for 1 hr, followed by three PBT washes. Nuclei were counter-stained with Hoechst or DAPI. All experiments were performed with n ≥ 3 coverslips. 20X z-stack images with 2-µm z-steps were acquired with Volocity 5.2 software and a Leica fluorescence microscope (DM6000B) or a Nikon Eclipse (TS2000) fluorescence microscope. All images were pseudo-colored. Raw images were used for quantification. Images shown in the figures are z-stacks (typical thickness 20-40 µm) reconstructed using the Extended Focus function in Volocity to show cells in focus from multiple z-planes, and brightness and contrast were adjusted using the level function in Photoshop for better visualization of markers.

2.3.4 Quantification

To quantify neurons that expressed markers of interest, z-stack raw images were opened in Volocity with one single z-plane shown at a time. The green channel with TH⁺ labeling was uncloaked while the other channels were cloaked. TH⁺ cells in each field of view (FOV) were tagged using the point tool. TH⁺ cells were examined through all the z-
planes to ensure that each object was counted only once. The red channel (CALB, CALR, or NeuN labeling) was then uncloaked to determine double-positive cells in each FOV. The percent of DA neurons was calculated as the number of TH$^+$ cells divided by NeuN$^+$ cells. We used NeuN because it is restricted to neuronal nuclei and facilitates more accurate counting than using TUJ-1, which labels neuronal soma and processes. The percent of CALB$^+$ DA neurons was calculated as the number of TH$^+$CALB$^+$ cells divided by TH$^+$ cells, and the percent of CALR$^+$ DA neurons was calculated as the number of TH$^+$CALR$^+$ cells divided by TH$^+$ cells.

2.3.5 Sampling size selection by Monte Carlo simulation

The Monte Carlo error model was used to estimate error and help determine our sub-sampling method. The model was based on large coverage sampling of DA neuron-containing cultures by imaging eighty non-overlapping fields of view (operationally, we define multiple fields as FOVs) with a 20X objective; this was applied to two separate culture samples. The number of TH$^+$ neurons in each FOV was manually counted as described above. A Matlab simulation was developed to randomly select subsets of N numbers of FOVs and calculate the average number of TH$^+$ neurons based on the actual counts from the subset of FOVs. The simulation was executed 1000 times for all subset sizes. The standard errors were calculated from the standard deviations of the 1000 averages for each sampling size.
2.3.6 Quantitative real time PCR (qRT-PCR)

Three experimental groups including undifferentiated ESCs and NPs derived from S0F0IV and S500F100IV conditions were harvested for RNA extraction. For each of the groups, multiple wells (n > 2) were collected to generate biological replicates. RNA was extracted using Trizol (Invitrogen, 15596-026) and purified using a RNeasy Mini Kit (Qiagen, 74104) according to manufacturers' instructions. RNA was quantified using Nanodrop ND-1000. A 0.5 µg RNA template was used to synthesize cDNA using iScript cDNA Synthesis Kit (BioRad, 170-8890) according to manufacturer's instruction. The cDNA from each biological replicate was run in three separate qRT-PCR reactions (in triplicate) for each primer set. Quantitative RT-PCR (qRT-PCR) was performed with SYBR Green (Applied Biosystems, 4367650) using ABI 7900 Fast Sequence Detection Instrument. Expression of *Shh*, *Gli1*, *Fgf8*, *Fgfr3*, *Wnt1*, *Tcf4*, and *Nfat* were analyzed and normalized to 18S expression. Results are reported as mean cycle threshold, standard deviation, and significance determined by two-tailed p value. Cycle times were graphed using a log(2) scale to match cycle times. See S1 Table for primer sequences.

2.3.7 Statistical method

Thirty FOVs were analyzed for each marker combination in each culture sample. Statistical analyses of *TH*+/mm², *NeuN*+/mm², *TH*/NeuN+, *TH*+CALB+/TH+, and *TH*+CALR+/TH+ neurons were performed in SAS software and Generalized Estimating Equations were used to test all hypotheses. FOVs within each coverslip were modeled to have correlated residual error. For analyses comparing the percent of one type of cell within a larger pool of cells (e.g. *TH*+CALB+/TH+, *TH*+CALR+/TH+, and *TH*/NeuN+) a
binomial distribution was used, which describes the probability distribution of the number of successes (e.g. TH\(^+\)CALB\(^+\)) in a sequence of trials (e.g. TH\(^+\)). Binomial distribution appropriately constrains values between 0-100\%, models the way the variability of percentages is skewed above and below 50\%, and scales the variability by the size of the denominator. For analyses comparing the number of TH\(^+\) neurons per area (e.g. TH\(^+\)/mm\(^2\)) a Poisson distribution was used, which describes the number of events (e.g. TH\(^+\)) one would observe in a fixed event rate (events per mm\(^2\)). Unlike binomial distribution, Poisson distribution appropriately constrains values only to ≥ 0.

Once the appropriate distribution models were fit for the dependent variable (TH\(^+\)/mm\(^2\), TH\(^+\)/NeuN\(^+\), TH\(^+\)CALB\(^+\)/TH\(^+\), and TH\(^+\)CALR\(^+\)/TH\(^+\)), individual orthogonal linear estimates were constructed within the models to test specified hypotheses. Families were defined within a dependent variable as comparisons between experimental conditions and their paired controls. Familywise alpha was maintained at 0.05 using the Holm test to create adjusted p-values, and statistical significance was defined as adjusted p < 0.05. Comparisons among controls were also conducted to assess the variability among ESC lines and passages. Finally, classical sandwich estimation was used to adjust the final model parameters based on how closely the parameters fit the distribution of the data. Means and 95\% confidence intervals (CI) are reported.
2.4 Results

2.4.1 Selection of quantification approach using Monte Carlo simulation

We generated DA neurons from ESCs using a modified version of the 5-stage method (See Methods, Figure 2.1). Immunolabeling for TH revealed that the *in vitro* spatial distribution of TH\(^+\) (DA) neurons generated from ESCs was highly heterogeneous (Figure 2.2a and Supplementary Figure S2.2). To quantitatively evaluate our cultures in an unbiased manner we selected sampling size to ensure accuracy while taking into account sampling error and efficiency. As illustrated in Figure 2.2b, we sampled 80 evenly spaced FOVs positioned in the center of the coverslip with a 1 FOV-space between sampled FOVs. We made the assumption that the 80 FOVs yielded sufficient coverage to represent the entire culture. We manually counted the number of TH\(^+\) neurons in each FOV and calculated the average from the 80 FOV counts (Average TH\(^+\)\(_{80}\)) (Figure 2.2c). We used a Monte Carlo error model in Matlab to assess the error associated with randomly sampling smaller subsets from the 80 FOVs (Figure 2.2e). Specifically, we defined sample size N, where 0 < N < 80. A set of N numbers of FOVs was randomly selected from the 80 FOVs, and the average TH\(^+\) counts from this subset was calculated (Average TH\(^+\)\(_N\)). This process was executed 1000 times for each sample size N and as illustrated in Figure 2.2f the 1000 Averages TH\(^+\)\(_N\) deviated from Average TH\(^+\)\(_{80}\) to varying extents. The standard deviation of the 1000 averages was calculated, which by definition is the standard error of the sample (Figure 2.2h). As expected, it was found that with larger sampling size there was a smaller resulting error. For example, the
errors were ±27.43% for 10 FOV, ±17.04% for 20 FOV, ±13.05% for 30 FOV, and ±9.75% for 40 FOV. Taking into consideration the error and the efficiency of performing the counts, we concluded that 30 FOVs per sample provided sufficient representation of the cultures and an acceptable amount of error. We repeated this process with TH⁺CALB⁺ numbers (Figure 2.2d, g, h) and reached a similar conclusion. Subsequently, all cultures described in this study were analyzed by imaging 30 randomly selected FOVs.
Figure 2.2. Sampling and quantification approach.

The spatial distribution of differentiated cells was highly heterogeneous. To determine a sufficiently accurate quantitative estimation method, we evaluated the error associated with sampling size. (a) Stitched fluorescence image shows the distribution of TH+ (green) and CALB+ (red) neurons. Box represents a 20X FOV. Scale bar, 100 µm. (b) A high coverage of the culture sample was obtained with 80 FOVs. The illustration shows the size and spacing of the 80 FOVs relative to the coverslip (diameter = 10 mm). Each FOV was 1 FOV-distance apart from the next
The numbers of TH⁺ neurons (c) and TH⁺CALB⁺ neurons (d) in each FOV were manually counted. The numbers show the actual counts in the corresponding FOVs. The averages from all 80 FOVs were calculated (Average TH⁺, Average TH⁺CALB⁺). (e) Monte Carlo simulation in Matlab was used to randomly choose a subset of FOVs with a defined sampling size (N number of FOVs) among the 80 and calculate the average from the subset (Average TH⁺_N, Average TH⁺CALB⁺_N). This process was repeated to generate 1000 averages for each sampling size N. (f, g) Representative box plots of the 1000 Average TH⁺_N (f) and Average TH⁺CALB⁺_N (g) from a simulation run of each sampling size N. Red dashed lines show the medium value for each simulation, box boundaries show the 25th and 75th percentile. Red crosses indicate outliers. Average TH⁺_N and Average TH⁺CALB⁺_N are shown as the gray lines. (h) Representative graph of the standard error vs. sampling size from a simulation. Dashed lines indicate the chosen 30 FOVs for our study and the corresponding errors for TH⁺ and TH⁺CALB⁺.

2.4.2 5-stage method generated more CALB⁺ than CALR⁺ subtype in D3 ESC-derived DA neurons

We assessed the generation of DA neuron subtypes by immunolabeling for TH and CALB, CALR, or GIRK2 following a 5-stage culture paradigm in the absence of exogenous SHH and FGF8. We observed qualitatively more TH⁺CALB⁺ DA neurons versus TH⁺CALR⁺ DA neurons (Figure 3a,c). We also observed many TH⁺CALB⁺ neurons and a smaller number of TH⁺CALR⁺ neurons, presumably interneurons, which were not included in our study. We did not detect TH⁺GIRK2⁺ neurons at the end of the 10-day differentiation period. GIRK2⁺ expression was observed when we extended differentiation to 25 days (Supplementary Figure S2.3), but for the purpose of this study, we restricted our quantitative analysis to the standard differentiation duration typically cited in the literature (Lee et al., 2000). As shown in Figure 2.3e, the percentage of TH⁺ DA neuron subtypes generated with the S0F0IV baseline culture condition was 26.24% for
the CALB\(^+\) subtype, which was significantly higher than 13.14\% for the TH\(^+\)CALR\(^+\) subtype (p < 0.0001). See Table 2.1 for the details of quantitatively analyzed parameters.

Figure 2.3. Quantification of CALB\(^+\) and CALR\(^+\) DA neuron subtypes in S0F0\(_{IV}\) vs S500F100\(_{IV}\).

D3 ESCs cultured in S0F0\(_{IV}\) baseline or S500F100\(_{IV}\) conditions in which exogenous SHH (500 ng/ml) and FGF8 (100 ng/ml) were added during NP expansion (Stage IV). (a, b) Images of TH (green) and CALB (red) immunofluorescent labeling in S0F0\(_{IV}\) (a) and S500F100\(_{IV}\) (b). (c, d) Images of TH (green) and CALR (red) immunofluorescent labeling in S0F0\(_{IV}\) (c) and S500F100\(_{IV}\) (d). Arrowheads indicate examples of neurons that are TH\(^+\)CALB\(^+\) or TH\(^+\)CALR\(^+\); high magnification images of the same regions are shown in the insets. (e) Quantification of CALB\(^+\) and CALR\(^+\) DA neuron subtypes revealed a higher percent of CALB\(^+\) than CALR\(^+\) subtype in both S0F0\(_{IV}\) and S500F100\(_{IV}\) conditions. The addition of SHH and FGF8 resulted in a small, but statistically significant reduction in CALR\(^+\) subtype. Means and 95\% CI are shown with values provided in Table 2.1. * p = 0.010, *** p < 0.0001. Scale bar (a - d), 50 μm; insets, 20 μm.
The percentage of CALB⁺ and CALR⁺ subtypes of DA neurons and adjusted p values. CI, confidence interval (95%).

2.4.3 The role of exogenous SHH and FGF8 during NP expansion (Stage IV) on DA neurons subtype generation from D3 ESCs

The addition of SHH and FGF8 during NP expansion (Stage IV), but not prior or after, has been reported to enhance DA neuron production (Lee et al., 2000). To study if exogenous SHH and FGF8 shape DA neuron subtype generation, we supplemented culture media with exogenous SHH (500 ng/mL) and FGF8 (100 ng/mL) during Stage IV (denoted as S500F100IV, Figure 2.3b,d) in parallel to the S0F0IV baseline culture. As shown in Figure 2.3 and Table 2.1, the S500F100IV conditions yielded 27.25% CALB⁺ DA neurons, which was significantly higher than 10.95% of the CALR⁺ DA neuron subtype (p < 0.0001). These findings were similar between the S0F0IV and S500F100IV groups. The comparison of CALB⁺ subtype across the S0F0IV and S500F100IV groups revealed no difference (p = 1.000) while the comparison of CALR⁺ subtype across the S0F0IV and S500F100IV groups revealed a 2.19% difference (p = 0.010).
2.4.4 Exogenous SHH and FGF8 during NP expansion (Stage IV) did not influence total DA neuron production.

To validate the effect of exogenous morphogens on total DA neuron production we asked whether exogenous SHH and FGF8 in S500F100 IV altered the production of TH+ neurons from D3 ESCs compared to S0F0 IV. As shown in Figure 2.4 and Table 2.2, total TH+ neuron production in S0F0 IV (123.12 TH+/mm²) was not significantly different from S500F100 IV (98.34 TH+/mm², p = 0.406). Total neuron production in S0F0 IV (2,110 NeuN+/mm²) was not significantly different from S500F100 IV (1,867 NeuN+/mm², p = 0.877). The percent of TH+ neurons within the total NeuN+ neuronal population in S0F0 IV (6.18%) was not significantly different from S500F100 IV (6.49%, p = 1.000). The data shows that exogenous SHH and FGF8 did not affect total neuron or DA neuron production derived from D3 ESCs.
Figure 2.4. Exogenous SHH and FGF8 during NP expansion did not enhance DA neuron production.

Neurons and DA neurons in S0F0<sub>IV</sub> and in S500F100<sub>IV</sub> were immunolabeled for NeuN and TH, respectively. (a, b) Images of TH (green) and NeuN (red) immunofluorescent labeling in S0F0<sub>IV</sub> (a) and S500F100<sub>IV</sub> (b). Arrowheads show examples of neurons that are double-positive for TH<sup>+</sup>NeuN<sup>+</sup>; insets show higher magnification images of DA neurons. (c, d) Corresponding phase contrast images. (e-g) Quantification of TH<sup>+</sup> neurons (e) and NeuN<sup>+</sup> neurons (f) per unit area and percent TH<sup>+</sup> neurons compared to NeuN<sup>+</sup> neurons (g) showed no difference between S0F0<sub>IV</sub>, and S500F100<sub>IV</sub> cultures. Means and 95% CI are shown with values provided in Table 2.2. Scale bar (a - d), 50 μm; insets, 20 μm.
Table 2.2 Quantification and statistical comparisons of total neurons and DA

The numbers of DA neurons (TH⁺) and total neurons (NeuN⁺), the percentage DA neurons versus total neurons, and adjusted p values. CI, confidence interval (95%).

2.4.5 The role of exogenous SHH and FGF8 on developmental signaling pathways

SHH, FGF8, and WNTs are morphogens maintained by interdependent and hierarchical genetic loops during embryonic patterning that continue to function during dopamine neuron development in vivo (reviewed in (Joyner et al., 2000; Prakash and Wurst, 2006; Zervas et al., 2005)). Therefore, we determined whether endogenous SHH, FGF8, and WNT signaling pathway components in ESCs programmed to become DA neurons were affected by exogenous SHH and FGF8. We performed quantitative RT-PCR analysis on D3 ESCs and D3 ESC-derived neuronal precursors at the end of Stage IV in S0F0ΙV and S500F100ΙV conditions. We analyzed the mRNA expression of Shh and Gli1 (a downstream transcription factor in the SHH signaling pathway) (Blaess et al., 2006; Hayes et al., 2013) as well as Fgf8 and Fgfr3, which encodes a receptor required
for FGF8 signaling in DA neurons (Chi, 2003; Ellisor et al., 2012; Olsen et al., 2006; Saarimäki-Vire et al., 2007; Yang et al., 2013). As shown in Figure 2.5a, Shh expression was nearly undetectable in Stage I ESCs but was expressed at Stage IV in both S0F0IV and S500F100IV. However, exogenous SHH and FGF8 in S500F100IV did not increase Shh expression (6296.52 ± 2014.11, cycle threshold) compared to S0F0IV baseline (6184.75 ± 551.23, p = 0.9306). Gli1 was detected in Stage I ESCs and was upregulated at the end of Stage IV. There was no difference in Gli1 in the presence of exogenous morphogens (5.90 ± 2.39) compared to the S0F0IV baseline (5.89 ± 1.13, p = 0.9961, Figure 2.5b). Fgf8 and Fgfr3 were also expressed in Stage I ESCs and upregulated at Stage IV. Treatment with exogenous morphogens did not alter Fgf8 expression (3.68 ± 0.27) compared to the S0F0IV (3.38 ± 0.53, Figure 2.5c, p = 0.4253). Fgf3r levels in S500F100IV (12.59 ± 3.40) were lower compared to the S0F0IV (23.87 ± 4.00, Figure 2.5d, p = 0.0205). Finally, Wnt1 as well as Nfat and Tcf4 (downstream components in WNT signaling) were expressed at low levels in Stage I ESCs and up-regulated at Stage IV (Figure 2.5e,f). Again, exogenous morphogens failed to alter the expression of these genes: Wnt1 level in the presence of morphogens was 175.80 ± 37.10 and without was 140.37 ± 48.24, p = 0.3703, Figure 5e); Tcf4 in the presence of morphogens was 5.29 ± 1.17 and without was 6.49 ± 0.86 (Figure 5f, p = 0.2268); Nfat in the presence of morphogens was 2.87 ± 0.01 and without was 2.38 ± 0.70 (p = 0.2268). The expression levels of these transcripts in ESC-derived NPs are in good agreement with qRT-PCR analysis of DA neuron progenitors isolated in vivo (Yang et al., 2013).
Figure 2.5. Endogenous SHH, FGF8, and WNT1 signaling components in ESCs and NPs.

RNA was harvested from NPs at the end of Stage IV and compared to ESCs by qRT-PCR analysis. Expression of *Shh* (a), *Gli1* (b), which is a high fidelity readout of SHH signaling, and *Fgf8* (c) increased during the transition from ESC to NP, but these genes were not affected by the exogenous morphogens SHH and FGF8. In contrast, *Fgfr3* (d), which encodes a receptor for FGF8 was decreased in the presence of exogenous SHH and FGF8. *Wnt1* (e) and *Tcf4* (f), which
is activated by canonical WNT signaling were increased in NPs compared to ESCs but unaffected by exogenous morphogens. The Y-axis is in log scale 2 to match cycle fractions. Three biological replicates are indicated by diamonds (and sometimes obscured by close values of the replicates); the bar indicates the mean. The values and standard deviations are indicated in the results section.

2.4.6 Comparative analysis with R1 ESCs: DA neuron production and subtype generation

To investigate whether the results we observed were specific to the D3 ESCs, we performed the same experiment using R1 mouse ESCs. R1 ESCs were cultured using the same baseline control and experimental conditions (S0F0Ⅷ vs. S500F100Ⅷ). TH⁺ neuron production in S0F0Ⅷ (186.65 TH⁺/mm²) was not significantly different from S500F100Ⅷ (202.12 TH⁺/mm², p = 0.508, Figure 2.6 and Table 2.2). These results show that similar to D3 ESCs, the production of TH⁺ neurons in R1 ESCs was not enhanced by the presence of exogenous SHH and FGF8 during the NP expansion stage.

R1 ESCs also produced both CALB⁺ and CALR⁺ subtypes of DA neurons. As shown in Figure 2.6f and detailed in Table 2.1, the CALB⁺ subtype (10.92%) was significantly higher than the CALR⁺ subtype (2.41%, p < 0.0001) in S0F0Ⅷ. In the presence of exogenous SHH and FGF8 in the S500F100Ⅷ experimental condition, the CALB⁺ subtype (10.87%) was again significantly higher than the CALR⁺ subtype (3.49%, p < 0.0001). The absence or presence of SHH and FGF8 resulted in no difference in the specification of CALB⁺ subtype (p = 1.000) or CALR⁺ subtype (p = 0.144). These results showed that R1 ESCs yielded more of the CALB⁺ subtype of DA neurons over the CALR⁺ subtype and that exogenous SHH and FGF8 did not shift this distribution - similar to findings with D3 ESCs.
Figure 2.6. CALB$^+$ and CALR$^+$ subtypes of DA neurons were generated from R1 ESCs. The S0F0$_{IV}$ and the S500F100$_{IV}$ conditions were repeated using R1 ESCs. (a, b) Images of TH (green) and CALB (red) immunofluorescent labeling in S0F0$_{IV}$ (a) and S500F100$_{IV}$ (b). (c, d) Images of TH (green) and CALR (red) immunofluorescent labeling in S0F0$_{IV}$ (c) and S500F100$_{IV}$ (d). Arrowheads indicate examples of neurons that are double-positive for TH$^+$CALB$^+$ or TH$^+$CALR$^+$, and high magnification images of the same region are shown in the insets. (e) Quantification of TH$^+$ neurons showed no difference in DA neuron production with the addition of SHH and FGF8. (f) Quantification of CALB$^+$ subtypes and CALR$^+$ subtypes of DA neurons revealed a higher percent of CALB$^+$ than CALR$^+$ subtype in both S0F0$_{IV}$ and S500F100$_{IV}$ cultures, but no difference in CALB$^+$ or CALR$^+$ subtype when compared across culture conditions. Means and 95% CI are shown with values provided in Tables 1 and 2. *** $p < 0.0001$. Scale bar (a - d), 50 μm; insets, 20 μm.
2.4.7 Exogenous SHH and FGF8 during NP selection stage (Stage III) did not substantially change the proportions of subtypes in D3-derived DA neurons.

Previous studies suggested that SHH and FGF8 did not affect DA neuron production when added before Stage IV (Lee et al., 2000). Immunolabeling of ESC-derived NPs at Stage III revealed that LMX1a, a transcription factor and determinant of DA neurons, was distributed amongst Nestin+ NPs (Supplementary Figure S2.4). We therefore tested whether exposure to exogenous SHH and FGF8 at this earlier stage impacted DA neuron subtype generation. As illustrated in Figure 2.7a-d, SHH (500 ng/ml) and FGF8 (100 ng/mL) were added to the culture only during Stage III, denoted as S500F100III (Figure 2.1b). The paired control was cultured using the baseline culture condition, denoted as S0F0III. As shown in Figure 2.7e and detailed in Table 2.1, the CALB+ subtype (12.31%) was significantly higher than CALR+ subtype (8.53%, p < 0.001) in S0F0III. The CALB+ subtype (11.01%) was also significantly higher than CALR+ subtype (8.81%, p = 0.0142) in S500F100III. Comparison of CALB+ subtype across the two culture conditions revealed a 1.30% difference (p = 0.025). There was no difference in CALR+ subtype between S0F0III and S500F100III (p = 1.000). There was no increase in neuron or TH+ neuron production with the presence of SHH and FGF8 during NP selection. As shown in Supplementary Figure S2.5 and Table 2.2, we did not observe differences in DA neuron production, neuron production, or the percentage of DA neurons to total neurons between S0F0III and S500F100III.
Figure 2.7. SHH and FGF8 at Stage III did not govern CALB\(^+\) DA neuron subtype generation.

D3 ESCs were cultured in parallel in the S0F0\(_{III}\) baseline condition and in the S500F100\(_{III}\) condition, in which exogenous SHH (500 ng/ml) and FGF8 (100 ng/ml) were added during NP selection (Stage III). (a, b) Images of TH (green) and CALB (red) immunofluorescent labeling in S0F0\(_{III}\) (a) and S500F100\(_{III}\) (b). (c, d) Images of TH (green) and CALR (red) immunofluorescent labeling in S0F0\(_{III}\) (c) and S500F100\(_{III}\) (d). Arrowheads indicate examples of neurons that were double-positive for TH\(^-\)CALB\(^+\) or TH\(^-\)CALR\(^+\), and high magnification images of the same region are shown in the insets. (e) Quantification of CALB\(^+\) subtypes and CALR\(^+\) subtypes of DA neurons revealed higher percent of CALB\(^+\) than CALR\(^+\) subtype in both S0F0\(_{III}\) and S500F100\(_{III}\) cultures. The addition of SHH and FGF8 resulted in a small but statistically significant reduction in CALB\(^+\) subtype. Means and 95% CI are shown with values provided in Table 2.1. \(\ast\) \(p = 0.025\), \(\ast\ast\) \(p = 0.0142\), \(\ast\ast\ast\) \(p = 0.0003\). Scale bar (a - d), 50 \(\mu\)m; insets, 20 \(\mu\)m.

2.4.8 Treatment with SHH- and FGFR3-neutralizing antibodies did not alter CALB\(^+\) or CALR\(^+\) subtype generation.

Because \(Shh\) and \(Fgf8\) expression were detected at Stage IV regardless of exogenous SHH and FGF8 (Figure 2.5), we used neutralizing antibodies to block
endogenous SHH and FGFR3, which is the receptor for FGF8 (Ye et al., 1998). Cells were incubated with anti-SHH (10 µg/mL) and anti-FGFR3 (10 µg/mL) antibodies from the beginning of Stage III through the end of Stage V (Figure 2.1c; denoted as αSαFr). Under these conditions, the cultures did not receive exogenous SHH and FGF8. The paired control group received neither exogenous SHH and FGF8, nor anti-SHH and anti-FGFR3, denoted as S0F0. As shown in Figure 2.8 and Table 2.1, in S0F0, the CALB+ subtype (12.49%) was significantly higher than CALR+ subtype (9.04%, p < 0.0001), in agreement with the previous baseline control groups. In αSαFr, CALB+ subtype (12.43%) was also higher than CALR+ subtype (8.21%, p = 0.0084). There was no significant difference in either CALB+ subtype (p = 1.000) or CALR+ subtype (p = 0.467) between S0F0 and αSαFr. As shown in Supplementary Figure S2.5 and Table 2.2, we did not observe differences in DA neuron production, neuron production, or the percentage of DA neurons to total neurons between S0F0 and αSαFr.
Figure 2.8. Blocking endogenous SHH and FGF8 signaling did not alter DA neuron subtypes.

D3 ESCs were cultured in parallel in the S0F0’a baseline condition or in the αSαFr condition, in which anti-SHH (10 µg/mL) and anti-FGFR3 (10 µg/mL) neutralizing antibodies were added from Stage III through Stage V. (a, b) Images of TH (green) and CALB (red) immunofluorescent labeling in S0F0’a (a) and αSαFr (b). (c, d) Images of TH (green) and CALR (red) immunofluorescent labeling in S0F0’a (c) and αSαFr (d). Arrowheads indicate examples of neurons that were double-positive for TH+CALB+ or TH+CALR+, and high magnification images of the same region are shown in the insets. (e) Quantification of CALB+ subtypes and CALR+ subtypes of DA neurons revealed higher percent CALB+ than CALR+ subtype in both S0F0’a and αSαFr culturues. The addition of anti-SHH and anti-FGFR3 did not affect CALB+ and CALR+ subtype generation. Means and 95% CI are shown with values provided in Table 2.1. ** p = 0.0084, *** p < 0.0001. Scale bar (a - d), 50 μm; insets, 20 μm.

2.5 Discussion

DA neurons in vivo consist of several subtypes with unique molecular identities and are involved in distinct neurological disorders (Björklund and Dunnett, 2007; Brichta and Greengard, 2014; Ikemoto, 2007). An understanding of how specific subtypes of DA
neurons are generated in vitro is important for developing appropriate research tools for studying disease mechanisms, investigating potential therapeutics, and adopting stem cells for clinical applications (Olanow et al., 2009). In this study, we differentiated DA neurons from ESCs using a 5-stage method and evaluated DA neuron subtypes by immunolabeling for CALB or CALR and TH and tested whether subtype generation in vitro was influenced by exogenous SHH and FGF8. Our initial observation of the differentiated cultures revealed a high degree of heterogeneity in the spatial distribution of all neurons and DA neurons. A brief survey of the literature reveals a limitation in the application and/or description of determining quantification methods for analyzing ESC-derived neurons. We developed a method to approximate errors associated with different sizes of random sampling, which was used to select the number of FOVs in our quantitative analysis. Our sampling and quantitative approaches can readily be applied to a variety of in vitro experiments as demonstrated here for assessing DA neuron diversity.

2.5.1 The role of SHH and FGF8 in ESC-derived DA neurons

SHH and FGF8 are morphogens that function in embryonic patterning and play a role in the induction of DA neurons in the embryonic brain (Blaess et al., 2006; Ellisor et al., 2012; Hayes et al., 2013; Ye et al., 1998). These two morphogens have been suggested to enhance DA neuron production in vitro (Chung et al., 2002; Lee et al., 2000; Tang et al., 2009). However, we did not observe an increase in the production of DA neurons in the presence of exogenous SHH and FGF8 from two independent ESC lines. There are several potential explanations for these findings including the following: 1)
variations in ESCs and experimental procedures, 2) saturating levels of endogenous SHH and FGF8, and 3) context-dependent functions of SHH and FGF8.

The production of DA neurons from ESCs can be influenced by variations in cell line and experimental procedures. With regard to cell lines, we chose D3 and R1 ESCs for our experiments because these commercially available lines have been used previously in DA neuron differentiation experiments with the 5-stage protocol (Chung et al., 2002; Kim et al., 2002; Lee et al., 2000). It is noteworthy that the descriptions of DA neuron production in the literature have indicated a range in the yield of DA neurons derived from ESCs treated with SHH and FGF8 (7% → 33% of total neurons reported by Lee et al.; 3 →11% reported by Chung et al.; 6% in this study) (Chung et al., 2002; Lee et al., 2000). One experimental difference between these studies and ours is that we counted TH⁺ neurons versus NeuN which is restricted to neuronal nuclei and more amenable to cell counting than TUJ-1 that is expressed in neuronal soma and processes. However, it is also possible that our ESCs were at a passage that did not maximally respond to exogenous SHH and FGF8. Studies have reported variations in proliferation, differentiation, and genetic abnormalities with ESC subclones and passages (Martinez et al., 2012; Park et al., 2008). Because passage numbers of ESCs are often unknown or not reported in the literature it is difficult to directly compare across studies, which necessitates the need to run an untreated control group. To eliminate reagent variability as a potential influence on cell response, we compared reagents from a commercial DA neuron differentiation kit (R&D, sc001b) and reagents that were individually purchased (listed in Methods) and we observed no differences in DA neuron production.
With regard to endogenous morphogen levels, our qRT-PCR analysis showed that mRNA levels of SHH and FGF8 signaling components were upregulated in cells at the NP expansion stage as compared to undifferentiated ESCs indicating that some cells in vitro acquire robust expression of endogenous Shh and Fgf8. Along with the upregulation of the Wnt1, these results are consistent with previous in vitro studies and are in alignment with in vivo development (Lee et al., 2000; Yang et al., 2013). The addition of exogenous SHH and FGF8 did not increase the expression levels of endogenous pathway components, but interestingly, there was a decrease in the expression of Fgfr3 in the presence of exogenous FGF8, consistent with a negative feedback loop of FGF8 signaling (Mott et al., 2010). The dynamic change in intrinsic gene expression from ESCs to NPs and the lack of a robust functional response to exogenous SHH and FGF8 suggest that NPs produce sufficient and/or saturated levels endogenously and may be a self-sufficient system for limited DA neuron production. We directly tested the role of endogenous SHH and FGF8 signaling with neutralizing antibodies (Ye et al., 1998) and saw no decrease in the number of TH+ neurons. These results suggest that a low level of endogenous morphogen persisted in the presence of the antibodies or that a short pulse of morphogen activity occurred prior to blocking antibody disruption, which would be consistent with the dynamic temporal requirement for morphogen induction of DA neurons in vivo (Brown et al., 2011; Ellisor et al., 2012; Ye et al., 1998).

We confirmed D3 ESCs' pluripotency with OCT4 expression, which was further supported by the ability to generate DA neurons, CALB+ and CALR+ neurons, and a substantial number of neurons. In addition, we observed the acquisition of LMX1a expression in DA progenitors at an appropriate stage. However, our findings and previous
studies collectively indicate that the majority (67 - 94%) of NPs do not differentiate into DA neurons in response to exogenous SHH or FGF8 (Chung et al., 2002; Lee et al., 2000). SHH and FGF8 have been observed to fail to transdifferentiate striatal neurons to express TH \textit{in vitro} (Stull and Iacovitti, 2001). While SHH and FGF8 are potent morphogens for DA neuron induction \textit{in vivo}, it is important to note that they act on specific precursor cells suggesting context-dependent functions of these morphogens. SHH is involved in the induction of not only DA neurons but also serotonergic neurons, motor neurons, and GABAergic interneurons, showing that the same factor can have different effects depending on the recipient cells (Ericson et al., 1997; Gulacsi and Lillien, 2003; Hayes et al., 2011; Tanabe et al., 1995). Further, it is only within a critical window \textit{in vivo} that SHH and FGF8 can induce ectopic DA neuron production (Ye et al., 1998). It is interesting to note that when ESC-derived NPs overexpress key transcription factors such as \textit{Nurr1} and \textit{Lmx1a}, these NPs produced 60-80% DA neurons when exposed to SHH and FGF8 (Chung et al., 2002; Friling et al., 2009; Kim et al., 2002). Taken together, these studies suggest that many of the microenvironmental cues that can influence cell fate and behavior may be missing \textit{in vitro}. Alternatively, there may be an unidentified mechanism(s) that ensures only a limited number of progenitors are poised to acquire a DA neuron identity. Other mechanisms are likely to be involved with subtype specification, such as other soluble morphogens, progenitors niche, cell-cell communication, migration and refinement of progenitors, and intrinsic gene expression (Xiong et al., 2013). It is likely that additional intrinsic and extrinsic factors and mechanisms are required to work synergistically with exogenous SHH and FGF8 to enhance DA neuron production.
### 2.5.2 DA neuron subtypes derived from mouse ESCs

After ten days of differentiation, a small percentage of DA neurons in our culture expressed CALB (11 - 27%) or CALR (2 - 13%). Remarkably, our culture conditions recapitulated the relative distribution of these subtypes of DA neurons in the mouse brain, which also has a higher percentage of CALB$^+$ versus CALR$^+$ DA neurons in both the VTA and SN (Gerfen et al., 1987; Liang et al., 1996; Nemoto et al., 1999). The differences in percentages between DA neuron subtypes produced *in vitro* and established *in vivo* may reflect differences in the time, context, or embryonic niche where DA progenitors are located compared to culture conditions. CALB and CALR expression in DA neurons are not mutually exclusive *in vivo* (Nemoto et al., 1999). Although we did not perform triple-immunolabeling of TH, CALB, and CALR due to antibody compatibility complications, it will be interesting in the future to quantify the co-expression of CALB and CALR in DA neurons. Because cognitive function and reward-associated behaviors involve DA neurons located in the VTA, which is high in CALB$^+$ and CALR$^+$ DA neuron subtypes, our findings regarding the *in vitro* production of these subtypes is potentially applicable to aid in the study of complex disorders such as schizophrenia, addiction, reward, and arousal. The GIRK2$^+$ subtype of DA neurons in the SN is vulnerable to degeneration in Parkinson's disease (Brichta and Greengard, 2014) and transplanting an enriched population of SN DA neurons has led to greater motor function recovery in a rat Parkinson's disease model (Grealish et al., 2010; Reyes et al., 2012). In this study, we did not detect GIRK2 expression at the end of fairly standard differentiation duration (10-15 days) in the 5-stage paradigm. However, previous studies have detected GIRK2 expression in DA neurons derived from a similar paradigm after
being engrafted in animal models with DA neuron lesions (Friling et al., 2009; Geeta et al., 2008; Moon et al., 2013). Thus, DA neurons derived from ESCs have the ability to become the GIRK2$^+$ subtype of DA neurons. The absence of GIRK2 expression may be explained by the temporal dependency, intrinsic mechanisms, or microenvironment (Blaess et al., 2006). The assertion of temporal dependency is supported by our observation that a small number of GIRK2 expressing DA neurons was observed after 25 days in vitro.

Finally, we hypothesized that the generation of DA neuron subtypes is influenced by exogenous SHH and FGF8. Subtype specification through a SHH gradient has been demonstrated in spinal cord motor neurons (Ericson et al., 1997) while SHH and FGF8 gradients also control cell fate specification in limb bud development (Lewandoski et al., 2000; Riddle et al., 1993). The rationale for testing our hypothesis is that in the developing midbrain it is likely that precursor cells are exposed to different concentrations of SHH and FGF8 secreted by the ventral midline and isthmus, respectively (Ye et al., 1998). Surprisingly, the addition of SHH and FGF8 at the NP expansion stage did not alter the production of the CALB$^+$ subtype of DA neuron and resulted in a minor decrease of CALR$^+$ subtype generation. Even though the decrease was statistically significant, the change was very small and in our opinion unlikely to be an efficient method to manipulate DA neuron subtype generation in vitro. Similarly, neutralizing antibodies did not alter the number of CALB$^+$ or CALR$^+$ DA neuron subtypes. These findings suggest that subtype generation in vitro is not governed by exogenous SHH and FGF8, but may be controlled by intrinsic mechanisms. We did note a significant difference in the percentages of both CALB$^+$ and CALR$^+$ DA neuron
subtypes derived from earlier versus later ESC passage, although the pattern of more CALB$^+$ than CALR$^+$ DA neurons remained. These results suggest that DA subtype specification may be sensitive to intrinsic factors and starting ESC passage, which agrees with literature that reported variability in the yield of differentiated cells among subclones of the same ESCs (Martinez et al., 2012).

2.6 Conclusion

We established a rigorous sampling analytical approach to quantify DA neuron subtypes generated from ESCs using a 5-stage method. Higher percentages of CALB$^+$ than CALR$^+$ subtype of DA neurons were produced with this method. Manipulating SHH and FGF8 signaling through exogenous SHH and FGF8 or SHH- and FGFR3-neutralizing antibodies was not effective in altering subtype generation in vitro. Taken together, our findings point toward endogenous mechanisms that govern DA neuron production and subtype differentiation of CALB$^+$ and CALR$^+$ DA neurons from mouse ESCs in the 5-stage method. Subtype specification likely involves multiple complex mechanisms that were not identified in this study and not controlled solely by SHH and FGF8. A deeper understanding of the intrinsic gene expression patterns, both spatial and temporal, and their relation to allocating DA progenitors and subtype specification will be particularly useful for the field of ESC and DA neuron research.
2.7 Acknowledgements

The authors thank Patrick Dingle for the Monte Carlo simulation Matlab script, Samantha Brady for assistance in quantification of R1 ESC studies, and Kirsten Sigrist and Marissa Furey at the Brown Transgenic Facility for sharing their knowledge of ESC techniques. This research was funded by the National Institutes of Health National Institute of General Medical Sciences (NIH/NIGMS) [Grant #8P20GM103468-04] (MZ) and a Brown Institute for Brain Science Pilot Grant 4-63662 (MZ, DHK).

2.8 References


Hargus, G., Cooper, O., Deleidi, M., Levy, A., Lee, K., Marlow, E., Yow, A., Soldner, F., Hockemeyer, D., Hallett, P.J., et al. (2010). Differentiated Parkinson patient-


## 2.9 Supplementary materials

<table>
<thead>
<tr>
<th>Stage I</th>
<th>Stage II</th>
<th>Stage III</th>
<th>Stage IV</th>
<th>Stage V</th>
</tr>
</thead>
<tbody>
<tr>
<td>ESC Expansion</td>
<td>EB Formation</td>
<td>NP Selection</td>
<td>NP Expansion</td>
<td>Differentiation</td>
</tr>
</tbody>
</table>

**Supplementary Figure S2.1 Cell morphology in the 5-stage culture.**

Cells had distinct morphologies at each stage and qualitative assessment revealed good health of cells. R1 cells are shown. Stage I. Undifferentiated R1 ESC formed colonies on gelatin. Some MEFs were carried over from ESC maintenance culture. Stage II. Free-floating EB were formed in non-adherent dishes. Stage III. EBs were transferred to adherent dishes in ITSFn media to select for NPs. Monolayers of NPs emerged from the EBs. Stage IV. NPs were re-plated on PLO/LN and expanded in N2aa media supplemented with bFGF. Stage V. Cells differentiated after growth factor withdrawal and became highly confluent. Both cluster and monolayer morphologies were observed. Scale bar, 50µm.
Supplementary Figure S2.2 Heterogeneous distribution of TH\(^+\) neurons.

Histogram of relative frequency of the number of TH\(^+\) neurons per FOV. The data includes TH\(^+\) counts from all the control and experimental conditions in this study (total 1,980 FOVs). The number of TH\(^+\) neuron per FOV ranged from 0 to 152. 5\(^{th}\) percentile = 1, 25\(^{th}\) = 5, 50\(^{th}\) = 14, 75\(^{th}\) = 28, and 95\(^{th}\) = 51.
Supplementary Figure S2.3 GIRK2⁺ subtype identity was acquired later. GIRK2⁺ expression (red) in TH⁺ neurons (green) was detected when differentiation (Stage V) was extended to 25 days. Neurons were immunolabeled with TUJ-1 (blue). Scale bar, 20 µm.
Supplementary Figure S2.4 LMX1a expression in Stage III NPs.
Corresponding phase contrast (left) and fluorescence (right) images of D3 cells at the end of Stage III, harvested and re-plated as monolayers. The majority of the cells expressed Nestin (red), and cohorts of cells expressed LMX1a (green), a transcription factor associated with DA neuron progenitors. Arrowheads indicate examples of neurons that are double-positive for Nestin$^+$LMX1a$^+$, and high magnification images of the same region are shown in the insets. Scale bar, 100 µm; inset, 50 µm.
Supplementary Figure S2.5 Exogenous SHH/FGF8 or anti-SHH/FGFR3 neutralizing antibodies did not alter DA neuron production.

(a-c) D3 ESCs were cultured in parallel in the S0F0<sub>III</sub> baseline condition and in the S500F100<sub>III</sub> condition, in which exogenous SHH and FGF8 were added during NP selection (Stage III). Quantification of TH<sup>+</sup> neurons (a), NeuN<sup>+</sup> neurons (b), and percentage of TH<sup>+</sup> neurons to all (NeuN<sup>+</sup>) neurons (c) revealed that exogenous SHH and FGF8 did not affect neuron or TH neuron production. (d-f) D3 ESCs were cultured in parallel in the S0F0<sub>α</sub> baseline condition and in the αSaFr condition, in which anti-SHH and anti-FGFR3 blocking antibodies were added from the start of Stage III to the end of the experiment. Quantification of TH<sup>+</sup> neurons (d), NeuN<sup>+</sup> neurons (e), and TH<sup>+</sup> neurons normalized to NeuN<sup>+</sup> neurons (f) revealed that attenuating SHH and FGF8 signaling pathways did not affect neuron or TH neuron production. Means and 95% CI are shown with values provided in Table 2.2. No statistical significance was detected between each pair.
Supplementary Figure S2.6 Relationship between TH⁺ neuron clustering and subtype differentiation.

Plots of (a) percent CALB⁺ in individual FOVs against the densities of TH⁺ neurons in the same FOVs, and (b) percent CALR⁺ in individual FOVs against the densities of TH⁺ neurons in the same FOVs of the paired controls and experimental conditions tested for D3 and R1 ESCs. Trendlines based on generalized linear models. Shaded areas show the confidence intervals of the trendlines. * indicates slope of the trendline significantly different from zero. No significant difference in between paired-control and experimental conditions was detected. *p < 0.05.
Supplementary Figure S2.7 *In vitro* CALB$^+$ and CALR$^+$ subtype specification did not correlate with SHH and FGF8 concentrations.

We tested a dose-response of CALB$^+$ and CALR$^+$ subtype specification in a pilot study using D3 ESCs. SHH and FGF8 of the indicated concentrations were added during Stage IV. Across culture conditions, percent CALB$^+$ subtype was higher than percent CALR$^+$ subtype. However, neither CALB$^+$ subtype nor CALR$^+$ subtype showed a correlation with SHH/FGF8 concentrations. n = 1.
<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fgf8</td>
<td>F: CATGTGAGGGACCAGACCC</td>
<td>R: GTAGTTGTCTCCAGCAGGATC</td>
</tr>
<tr>
<td>Fgfr3</td>
<td>F: ATCCTCGGGAGATGACGAAGAC</td>
<td>R: GGATGCTGCCAAACTTTGTTCCTC</td>
</tr>
<tr>
<td>Shh</td>
<td>F: GGAAGATCACAAGAAACTCCGAAC</td>
<td>R: GGATGCGAGCTTTGGATTCATAG</td>
</tr>
<tr>
<td>Gli1</td>
<td>F: TCCACAGGCATAACAGGATCA</td>
<td>R: TGCAACCTTTCTGCTCACAC</td>
</tr>
<tr>
<td>Wnt1</td>
<td>F: ATGAACCCTTCACAACACGAG</td>
<td>R: GGTTGCTGCCCTCGGTTG</td>
</tr>
<tr>
<td>Tcf4</td>
<td>F: CACCCCAAGACCCTTACAGA</td>
<td>R: TGCTTTGAGCTCTCATCG</td>
</tr>
<tr>
<td>Nfat</td>
<td>F: ACCCTCCGGTACAGAGGACT</td>
<td>R: TAGGGCAGGGTCTGAAGA</td>
</tr>
<tr>
<td>18s</td>
<td>F: CCRCGCTCTTCTATTTGGTGG</td>
<td>R: GGCCTCCCTCTAAATCATG</td>
</tr>
</tbody>
</table>

Supplementary Table S2.1  Sequence of primers used for qRT-PCR
Chapter 3

3D neural spheroid culture: an in vitro model for the central nervous system

Manuscript in revisions to Tissue Engineering Part A

Yu-Ting L. Dingle\textsuperscript{1,2}, Anda M. Chirila\textsuperscript{1*}, Molly E. Boutin\textsuperscript{1,2*}, Liane L. Livi\textsuperscript{1}, Nicholas R. Labriola\textsuperscript{1,2}, Lorin M. Jakubek\textsuperscript{1,2}, Jeffrey R. Morgan\textsuperscript{1,2,3}, Eric M. Darling\textsuperscript{1,2,3,4}, Julie A. Kauer\textsuperscript{1,5,6}, and Diane Hoffman-Kim\textsuperscript{1,2,3,6}

\textsuperscript{1}Department of Molecular Pharmacology, Physiology, and Biotechnology, Brown University, Providence, Rhode Island, USA
\textsuperscript{2}Center for Biomedical Engineering, Brown University, Providence, Rhode Island, USA
\textsuperscript{3}School of Engineering, Brown University, Providence, Rhode Island, USA
\textsuperscript{4}Department of Orthopedics, Brown University, Providence, Rhode Island, USA
\textsuperscript{5}Department of Neuroscience, Brown University, Providence, Rhode Island, USA
\textsuperscript{6}Brown Institute for Brain Sciences, Brown University, Providence, Rhode Island, USA
\textsuperscript{*}Equal contributions to this work

Author Contributions

YLD and DHK conceived of the project. YLD designed and conducted initial spheroid culture, immunocytochemistry, and confocal microscopy, and oversaw data analysis. AMC conducted and analyzed whole-cell electrophysiological characterization. MEB conducted immunocytochemistry, confocal microscopy, and sample preparation for mechanical and electrophysiological characterization. LLL conducted spheroid culture and sample preparation for mechanical characterization. NRL conducted mechanical characterization. LMJ conducted hippocampal spheroid culture. JAK consulted on electrophysiology experimental design and analysis. EMD consulted on mechanical characterization experimental design and analysis. JRM consulted on spheroid culture procedure. DHK oversaw all experiments and analysis.
3.1 Abstract

There is a high demand for in vitro models of the central nervous system to study neurological disorders, injuries, toxicity, and drug-efficacy. Three-dimensional (3D) in vitro models can bridge the gap between traditional 2D culture and animal models because they present an in vivo-like microenvironment in a tailorable experimental platform. Within the expanding assortment of sophisticated 3D cultures, scaffold-free, self-assembled spheroid culture avoids the introduction of foreign materials and preserves the native cell populations and extracellular matrix types. In this study, we generated 3D spheroids with primary postnatal rat cortical cells using an accessible, size-controlled, reproducible, and cost-effective method. These cortical spheroids remained viable for at least 3 weeks. Neurons and astrocytes formed laminin-containing 3D networks within the spheroids. The neurons were electrically active and formed circuitry via both excitatory and inhibitory synapses. The mechanical properties of the spheroids were in the range of brain tissue. These in vivo-like features of the 3D cortical spheroids provide the potential for relevant and translatable investigations of the central nervous system in vitro.

3.2 Introduction

Disease and injury in the central nervous system (CNS) have tremendous impacts on quality of life, and there is a high demand for models to study CNS pathologies and therapeutic strategies. Neurons and glial cells in the brain form a complex three-dimensional (3D), intertwined system, and therefore, engineering the microenvironment for in vitro investigations contains many inherent challenges (Hopkins et al., 2014).
Microenvironmental cues, such as cellular, biochemical, mechanical, and topographical cues, can influence cell behaviors including viability, proliferation, differentiation, migration, and protein and gene expression (Baker and Chen, 2012; Balgude et al., 2001; Bruder et al., 2007; Hoffman-Kim et al., 2010; Li et al., 2007). Notably, many native microenvironmental cues are lost in traditional two-dimensional (2D) cultures, in which cells spread on substrates that are often several orders of magnitudes stiffer than brain tissue and lack the brain's extracellular matrix (ECM) organization (Georges et al., 2006). Cells in 2D are forced to adapt a planar morphology and can form intercellular connections only in the lateral direction. These deficiencies have led to the development of 3D in vitro models of the nervous system.

3D in vitro neural models have exciting potential to bridge the gap between traditional 2D cultures and in vivo models (Hopkins et al., 2014; Pampaloni et al., 2007). For example, 3D culture of human induced pluripotent stem cells (iPSCs) has enabled the study of microcephaly (Lancaster et al., 2012), and the hallmarks of Alzheimer's disease, amyloid-β plaques and neurofibrillary tangles, have been more effectively modeled in 3D cultures than in 2D (Choi et al., 2014). Historically, 3D neural cultures were developed using material scaffolds (Cullen et al., 2007; Irons et al., 2008; Li et al., 2007; O’Shaughnessy et al., 2003; Xu et al., 2009). and complex cell compartmentalization and organization have been achieved via scaffold design (Frampton et al., 2011; Odawara et al., 2013; Tang-Schomer et al., 2014). Recently, neural cells have been cultured through self-assembly into spheroid structures in which they can produce their own matrix (Choi et al., 2013; Kato-Negishi et al., 2013); these spheroids have been suggested to
approximate *in vivo*-like cell behavior better than scaffold-based cultures (Fennema et al., 2013; Lin et al., 2008).

Several studies of nervous system function or disease have incorporated high levels of technological requirements in their cell cultures (Lancaster et al., 2012; Odawara et al., 2013; Pautot et al., 2008; Puschmann et al., 2014; Tang-Schomer et al., 2014). Combinations of advances including multi-component scaffold design, progenitor cells, and long-term culture have given rise to sophisticated *in vitro* models of the nervous system. However, complex neural cultures may be unattainable to many labs due to the resources and expertise needed to assemble and maintain them. Here we have characterized a relatively simple 3D neural culture approach with accessibility for laboratory groups that wish to address the myriad of interesting questions in neurobiology and neuroengineering.

With this reproducible method to generate 3D neural spheroid cultures, the culture materials are commercially available, or alternatively can be fabricated by a laboratory via standard biomaterial techniques. This approach requires no progenitor or embryonic cell isolation; only postnatal cultures are needed. We show that in the relatively short time frame of two weeks, the 3D postnatal cortical neural spheroid contains neurons, glia, and cell-synthesized matrix, is mechanically similar to *in vivo* cortex, and is electrically active. This approach can provide relatively large numbers of 3D microtissues for the study of CNS function, disease, and therapeutics.
3.3 Materials and methods

3.3.1 Cell isolation and culture

Primary cortical tissues were collected from postnatal day 1-2 CD rats (Charles River), and primary rat hippocampus tissues (embryonic day 18) were purchased from BrainBits, LLC. Cell isolation protocol was modified from BrainBits, LLC. The following buffer solutions and media were used: Hibernate A buffer solution - Hibernate A (BrainBits) supplemented with 1X B27 supplement (Invitrogen) and 0.5 mM Gluta-Max (Invitrogen); Papain solution - 2 mg/mL papain dissolved in Hibernate A without Calcium (BrainBits); Neurobasal A/B27 medium - Neurobasal A medium (Invitrogen) supplemented with 1X B27, 0.5 mM Gluta-Max, and 1X Penicillin-Streptomycin (Invitrogen). Briefly, the tissues were cut into small pieces and placed in papain solution for 30 min at 30°C. Papain solution was removed, and the tissues were triturated with fire-polished Pasteur pipettes 20 times in Hibernate A buffer solution. The cell solution was centrifuged at 150 xG for 5 min, and the supernatant was removed. The cell pellet was resuspended in Neurobasal A/B27 medium, and debris were removed by passing the solution through a 40 µm cell strainer. The cell solution was washed once more by centrifuging at 150 xG for 5 min, resuspending in Neurobasal A/B27 medium, and filtering with a cell strainer. Cell viability at time of isolation was determined by a Trypan Blue exclusion assay (Invitrogen). Cortical cells were seeded at densities of 1000, 2000, 4000, and 8000 (1k, 2k, 4k, 8k) cells/spheroid (see below). Hippocampal cells were seeded at densities of 125, 1000, and 3300 cells/spheroid.
3.3.2 3D self-assembled cortical spheroid fabrication

Molten 2% agarose (Invitrogen) solution was poured onto the spheroid micromold (#24-96-Small, Microtissues, Inc) to obtain agarose hydrogels with 400 µm diameter round-bottom recesses, termed "microwells." Agarose gels were equilibrated in culture medium with three medium exchanges over a 48-hr period. Cell solution containing the appropriate number of cells was centrifuged and resuspended in Neurobasal A/B27 medium. Medium was aspirated from the gels, and the cell solution (75 µL/gel) was seeded in the agarose gels. Cells were allowed to settle into the microwells for 30 min, and 1 mL Neurobasal A/B27 medium was added. Cell medium was exchanged 48 hrs after seeding and subsequently every 3-4 days.

3.3.3 Viability assay

Medium was removed from the hydrogel, and the hydrogel was rinsed once with phosphate buffered saline (PBS). Spheroids were incubated in 2 µM Calcein-AM and 4 µM Ethidium homodimer-1 (EthD, Live/Dead Viability Assay Kit, Invitrogen) for 45 min at 37°C. Hydrogels were rinsed once with PBS before imaging.

3.3.4 Immunostaining and optical clearing

Whole spheroid immunostaining and Clear\textsuperscript{T2} clearing protocols were adopted (Boutin and Hoffman-Kim, 2014; Kuwajima et al., 2013). Briefly, spheroids were fixed in 4% v/v paraformaldehyde and 8% w/v sucrose in PBS overnight, followed by three 1-hr PBS washes. All of the following steps were performed on a shaker at room
temperature. The following antibodies were used: mouse anti-β-III-tubulin (Covance, 1:50), rabbit anti-glial fibrillary acidic protein (GFAP, DAKO, 1:200), rabbit anti-laminin (BTI, 1:100), Cy3 goat anti-mouse (Jackson, 1:500), and Alexa488 goat anti-rabbit (Jackson, 1:500). Spheroids were permeabilized and blocked with 1% Triton X-100, 10% normal goat serum, and 4% bovine serum albumin in PBS (B-PBT) for 2 hrs, and subsequently incubated in primary antibodies diluted in B-PBT overnight. Spheroids underwent two 2-hr washes with 0.2% TritonX-100 in PBS (PBT), followed by one 2-hr B-PBT wash. Spheroids were incubated with secondary antibodies in B-PBT overnight. Spheroids underwent two 2-hr PBT washes and incubated in 1 µg/mL mL 4',6-diamidino-2-phenylindole (DAPI) in PBT for 1 hr and returned to PBS.

ClearT2 20% and 40% w/v poly-ethylene glycol (PEG) solutions were made in water and 50% v/v formamide (Sigma) solution was made in PBS. The following incubation steps were used: 1) 25% formamide/10% PEG for 10 min, 2) 50% formamide/20% PEG for 5 min, and 3) 50% formamide/20% PEG for 60 min. Spheroids were kept in final clearing solution and transferred to glass-bottom confocal dishes for imaging.

3.3.5 Imaging and image analysis

For spheroid top-view diameter measurement, phase contrast images were taken by a Nikon CoolPix 995 camera connected to a Nikon TS200 microscope with a 10X objective. Spheroid diameters were measured in ImageJ software (n ≥ 12 spheroids per time point). Cell densities at 1 day-in-vitro (DIV) were calculated by dividing the number of cells seeded by the spheroid volumes at 1 DIV.
Cell densities at 14 and 21 DIV were calculated by dividing the number of cells in a confocal slice by the slice volume (Equation 3.1). Cell number was found by normalizing DAPI-stained nuclei counts (N) to take into account the potential for a nucleus (diameter, d = 10.23 µm) to cross multiple slices (thickness, t = 2.01 µm). Volume was calculated by measuring the spheroid diameter (D) within the confocal slice (t = 2.01 µm).

\[
\text{Cell density (cells/mm}^3) = \frac{N * (t/d)}{\pi * (D/2)^2 * t} = 124.46 * \frac{N}{D^2} \quad \text{Equation 3.1}
\]

For Live/Dead analysis, fluorescent images were taken on a Nikon Eclipse TE2000-S microscope with Volocity software with 4X and 10X objectives. Exposure times for live and for dead signal were kept constant across time points.

For immunofluorescence, confocal imaging was performed on a Zeiss LSM 510 Meta Confocal Laser Scanning Microscope built on an Axiovert 200M inverted microscope with ZEN 2009 software. 10X, 20X, and 40X objectives were used. For z-stack images, laser power was kept constant, and gain was adjusted using the AutoZ Brightness Correction function. 3D projections were reconstructed in ZEN.

### 3.3.6 Electrophysiology

Cortical spheroids were transferred from the hydrogels and immobilized by allowing the bottom of the spheroids to adhere onto poly-D-lysine (50 µg/mL)-coated coverslips for two days prior to clamp recordings. Spheroids were perfused at ~1 mL/min at room temperature with oxygenated artificial cerebrospinal fluid (aCSF) containing 2.5 mM CaCl₂, 1 mM NaH₂PO₄, 119 mM NaCl, 2.5 mM KCl, 1.3 mM MgSO₄; 26 mM...
NaHCO₃, 25 mM dextrose, and 1.3 mM Na ascorbate. Whole-cell voltage clamp recordings at -70 mV were made from neurons near the surface (< 3 cell layers) on the spheroid using patch pipettes filled with a KCl-based internal solution containing 125 mM KCl, 2.8 mM NaCl, 2 mM MgCl₂, 2 mM ATP-Mg²⁺, 0.3 mM GTP-Na⁺, 0.6 mM EGTA and 10mM HEPES. Current clamp recordings were made with no added holding current. For spontaneous excitatory postsynaptic currents (sEPSCs), aCSF contained bicuculline (10 μM) and strychnine (10 μM), to block GABAₐ and glycine receptors; for spontaneous inhibitory postsynaptic currents (sIPSCs), 6,7-dinitroquinoxaline-2,3-dione (DNQX; 10 μM) was added to block AMPA receptors.

3.3.7 Mechanical characterization

Mechanical properties were tested with an MFP-3D-BIO atomic force microscope (AFM, Asylum Research) connected to a Nikon Eclipse Ti-U epifluorescence microscope. Spheroids were harvested and immobilized (see previous section). Neonatal cortex was manually sectioned and immobilized on a glass coverslip with low-melt SeaPrep Agarose (Cambrex). A 40x40 μm² measurement area was selected on the top surface of each spheroid, and 4x4 measurement points were evenly placed in the area. Indentation curves were measured using a spherically tipped cantilever (5 μm diameter, average k ~ 0.027 N/m, Novascan Technologies, Inc.) with an approach velocity of 10 μm/s, and force trigger of 1 nN, following previously established procedures.(Darling et al., 2010) Between 11-19 spheroids were tested at each time point from 2-3 independent experiments. Average indentation depth was less than 2 μm. Elastic modulus was calculated from force (F) vs indentation curves (δ) using a Hertz model (Equation 3.2)
previously described. (Darling et al., 2006, 2007; Harding and Sneddon, 1945) In the equation, R is the effective radius of the cantilever tip, and \(\nu\) is the Poisson's ratio, assumed to be 0.5 for incompressible materials. Outliers, defined as less than 10 Pa and greater than 2.5X standard deviation, were removed. Geometric means of the moduli were calculated. One-way ANOVA with post hoc multiple comparisons using Bonferroni test was used to perform statistical analysis.

\[
F(\delta) = \frac{4R^{1/2}E_{\text{elastic}}}{3(1-\nu^2)} \delta^{3/2} \quad \text{Equation 3.2}
\]

3.4 Results

3.4.1 Cortical cells self-assembled into 3D spheroids.

Monodispersed cortical cells were seeded in the micromolded hydrogels at seeding densities of 1k, 2k, 4k, and 8k per microwell. The hydrogel microwells (400 µm diameter) facilitated the assembly of spheroids within 24 hrs. Spheroid diameters at 1 DIV ranged from 100-230 µm, with size dependent on the initial seeding density (Figure 3.1a,b). Cell density across spheroids at 1 DIV was estimated to be 2x10^6 cells/mm^3. Spheroid diameter increased from 1 DIV to 7 DIV, and between 7-21 DIV, less growth was observed (Figure 3.1b). Cell densities in 14 and 21 DIV spheroids (seeded at 8k cells/spheroid) were estimated to be 2-4x10^5 cells/mm^3.
3.4.2 Cortical spheroids remained viable for at least three weeks in culture.

The viability of cortical cells at the time of isolation was 84-96%. Therefore, a small population of dead cells was expected to be present in the spheroids. Epifluorescent images showed strong calcein (live) signal and low EthD (dead) signal in spheroids both immediately after self-assembly (1 DIV) and at 21 DIV, suggesting high viability after three-week culture (Figure 3.1c).

![Figure 3.1 Size-controlled 3D cortical spheroids.](image)

Monodispersed cortical cells self-assembled into spheroids in the micromolded hydrogels and remained viable for 21 days. (a) Phase contrast images of cortical spheroids at 1 DIV and 7 DIV with initial seeding densities of 1, 2, 4, and 8k per spheroid. (b) Graph of spheroid diameter over time. Spheroid sizes initially increased, then plateaued. (c) Epifluorescence images of Live/Dead
3.4.3 Neurons and astrocytes formed complex 3D structures in the spheroids.

Neurons and astrocytes were present in the cortical spheroids (Figure 3.2). Neurons accounted for the majority of cells with a small percentage of astrocytes. At 1 DIV, 8k spheroids exhibited a globular pattern of β-III-tubulin neuronal staining and short GFAP⁺ astrocyte extensions. Cells were highly packed at this initial stage. From 1-7 DIV, the distance between nuclei increased (Figure 3.2a, DAPI). Neurites and longer astrocyte processes were visible by 7 DIV. From 7-21 DIV, neurites elongated, formed bundles, and formed a complex network, and astrocytes continued extending processes. Neurites and astrocyte processes extended in all x, y, z directions (Figure 3.2b). To demonstrate that cortical spheroids formed ECM, 8k spheroids were immunostained for laminin at 1, 3, 7, 14, and 21 DIV. As shown in Figure 3.2c, laminin expression was detected in spheroids at all time points. Laminin expression was generally diffuse, with some areas of localized brighter staining.
Figure 3.2 Neurons and astrocytes formed laminin-containing 3D networks. Neurites and astrocyte processes extended within the spheroids over time. (a) Confocal projections of 1, 3, 7, 14, and 21 DIV 8k spheroids revealed the increasing distance between nuclei (DAPI, white) and the morphology of neurons (β-III-tubulin, red) and astrocytes (GFAP,
green). (b) Confocal projections with z-axis depth-code-pseudocoloring of a 14 DIV 8k spheroid show β-III-tubulin (left) and GFAP (right) structures in 3D. Color represents the z-depth from top surface. (c) Confocal projections of 1, 3, 7, 14, and 21 DIV 8k spheroids showed laminin expression in the spheroids. Scale bars, (a,c) 100 µm, (b) 70 µm.

### 3.4.4 Neurons in spheroids were electrically active and formed synaptic connections.

Whole cell patch-clamp recordings were used to characterize the neurons' electrophysiological properties, including resting membrane potential, evoked action potentials, spontaneous action potentials, and spontaneous postsynaptic currents (PSCs). Average resting membrane potential for 7 DIV spheroids was -42.7 ± 6.8 mV (n = 8 cells). In response to a current injection, neurons in 7 DIV spheroids usually failed to fire an action potential or fired rapidly failing, broad small action potentials (Figure 3.3a). Although sEPSCs (Figure 3.3b, +bicuculline +strychnine panel) and sIPSCs (Figure 3.3c, +DNQX panel) could be detected, they were of low frequency and low amplitude.

In 14 DIV spheroids, average resting membrane potential was -56 ± 5.7 mV (n = 7). Neurons fired both evoked action potentials upon current injection (Figure 3.4a) and spontaneous action potentials (Figure 3.4b), suggesting the neurons in 14 DIV spheroids were more mature than in 7 DIV spheroids. Among the neurons recorded, we detected repetitive spike, single spike, and delayed evoked action potential firing patterns (repetitive firing is illustrated in Figure 3.4a). sEPSCs (Figure 3.4c, +bicuculline +strychnine panel) and sIPSCs (Figure 3.4d, +DNQX panel) of both large and small amplitudes were isolated using AMPA and GABA<sub>A</sub> receptor antagonists, respectively. Inhibiting sodium channels with TTX blocks action potential-triggered PSCs. Quantal
events (miniature PSCs) caused by spontaneous fusion of neurotransmitter vesicles with the presynaptic membrane were detectable under these conditions (Figure 3.4d, +TTX panel). These results demonstrate that both glutamatergic and GABAergic neurons are present in the spheroids, and the spontaneous PSCs demonstrate that both types of neurons make active synaptic connections onto the recorded neuron. Similarly to 14 DIV spheroids, evoked and spontaneous action potentials, as well as sEPSCs and sIPSCs, were detected in 21 DIV spheroids (Supplementary Figure S3.1), showing that the neurons remained active. The average resting membrane potential for 21 DIV spheroids was $-59 \pm 5.2$ mV (n=8).
Figure 3.3  7 DIV spheroids contained neurons with immature properties.

Neurons in 7 DIV 8k spheroid either failed to fire action potential upon current injection or fired low amplitude, failing action potentials. (a) Example of a recorded neuron responding to somatic current injections by firing short, rapidly failing action potentials (current clamp recording). Lower panel: current injection protocol. (b) Example traces of spontaneous synaptic activity (voltage clamp recording holding the neuron at -70 mV). Left panel: spontaneous PSCs recorded in aCSF. Right panel: sEPSCs were isolated with GABA_A receptor antagonist bicuculline and the glycine receptor antagonist strychnine. (c) Left panel: traces of spontaneous PSCs recorded in aCSF. Right panel: sIPSCs were isolated with the AMPA receptor antagonist DNQX.
**Figure 3.4** 14 DIV spheroids contained mature neurons with synapses.

Neurons in 14 DIV 8k spheroids fired both evoked and spontaneous action potentials. Both sEPSCs and sIPSCs were detected. (a) Evoked action potential. Lower panel: current injection step. (b) Example traces showing evoked action potentials followed by a spontaneous action potential. (c) Left panel: traces of spontaneous PSCs recorded in aCSF (voltage clamp recording at -70 mV). Middle panel: sEPSCs were isolated with GABA_A receptor antagonist bicuculline and glycine receptor antagonist strychnine. Right panel: spontaneous PSCs were completely blocked with glutamate receptor antagonist DNQX, confirming that they were mediated by AMPA-type glutamate receptors. (d) Left panel: spontaneous PSCs recorded in aCSF (voltage clamp recording at -70 mV). Middle panel: sIPSCs were then isolated using DNQX. Right panel: traces of miniature IPSCs remaining after addition of the sodium channel blocker TTX. These are individual quantal events.
3.4.5 Cortical spheroids had similar elastic moduli to cortical tissue.

Elastic moduli of spheroids at 1, 7, 14, and 21 DIV and neonatal rat (P0-2) cortex tissues were assessed using AFM. As shown in Figure 3.5, there was a trend of increasing elastic moduli over time. The modulus for 7 DIV spheroids (160 ± 80 Pa) was twice that of 1 DIV spheroids (80 ± 30 Pa, p < 0.05), and 14 DIV spheroids (190 ± 70 Pa) and 21 DIV spheroids (240 ± 60 Pa) were 2.4-3 fold stiffer than 1 DIV spheroids (p < 0.001). The elastic modulus of neonatal cortex tissue (110 ± 70 Pa) was similar to the moduli of 1 DIV and 7 DIV spheroids but lower than the moduli of 14 and 21 DIV spheroids (p < 0.05).

![Figure 3.5 Mechanical properties of 3D spheroids.](image)

Elastic moduli of 8k spheroids on 1, 7, 14, and 21 DIV and of neonatal rat cortex tissues (P0-P2) were measured by AFM. There was a general trend of increasing elastic moduli over time in culture. Elastic moduli for 1 DIV and 7 DIV spheroids were not significantly different from those of neonatal rat cortex tissue while late-stage spheroids (14 and 21 DIV) had greater elastic moduli than neonatal rat cortex tissue. *p < 0.05, **p < 0.001.
3.5 Discussion

In scaffold-free, self-assembled spheroid culture, cells form microtissues that contain a physiologically relevant microenvironment. In this study, we report a versatile and controllable 3D spheroid culture using postnatal, region-specific neural cells. These spheroids present many key features of the CNS microenvironment, including neuronal subtypes, astrocytes, 3D structure, electrical activity, synapse formation, ECM protein production, and in vivo-like stiffness.

3.5.1 Versatile culture

The method we described herein provides several advantages. It uses commercially available materials, is cost-effective, and is reproducible. It generates on the order of 1000 spheroids per neonate rat cortex, depending on the requisite spheroid cell density, thus demonstrating its potential for high throughput assays and for reducing animal use. Spheroid sizes were controlled by initial seeding densities, with the largest spheroids (8k cells/spheroid) having a final radius less than 160 μm, within the typical oxygen and nutrient diffusion limit of 200 μm (Lovett et al., 2009). Spheroids of all four seeding densities in this study reached a stable size by 14 DIV. It is possible that the 8k spheroids' growth was constrained by the hydrogel microwells. The spheroids in the micromolded hydrogels are easy to maintain, are viable for at least 3 weeks, and are not allowed to fuse due to the micromold's physical barrier, thus providing researchers with a stable culture tool.
This culture method can be applied to a wide range of neural cell types (Boutin and Hoffman-Kim, 2014). Here we have described rat postnatal cortical spheroids, and additionally, to demonstrate that this 3D spheroid culture method can be adapted to other types of neural cells, we fabricated spheroids using rat hippocampal cells. Hippocampal cells from a commercially available CNS tissue source formed spheroids, and the spheroid sizes were dependent on the seeding densities (Supplementary Figure S3.2). We have previously fabricated spheroids from rat dorsal root ganglion neurons, rat adult hippocampal neural stem cells, glioma cells (Boutin and Hoffman-Kim, 2014), neuroblastoma cells, and human iPSC-derived neurons (data not shown). This method also allows for co-culture to study cell-cell interactions (Achilli et al., 2012), such as brain endothelial cells with astrocytes (data not shown). Of note, the seeding protocol allows for independent treatment of cell groups before combining them in the spheroid (Youssef et al., 2012).

3.5.2 *In vivo*-like cell morphology and structure

Cell density in the brain (~1x10^5/mm^3 in mouse) is higher than in most other organs (Herculano-Houzel and Lent, 2005; Pautot et al., 2008). The spheroid culture allows for high density, which can mimic *in vivo* intercellular distance and communication better than scaffold cultures (Hopkins et al., 2014). Cell density in spheroids at 1 DIV (2x10^6/mm^3) was approximately one magnitude higher than *in vivo* density. Cell aggregation in the spheroid can be described as close packing of spheres (Curcio et al., 2007), thus the initial cell density within the spheroid is dependent on cell size rather than the number of cells per spheroid. Densities in the spheroids decreased as
cells spread and extended processes over time, so that densities in 14-21 DIV spheroids (2-4x10⁵/mm³) approached the magnitude of in vivo density.

Within the spheroids, neurites and astrocyte processes extended in all directions. Previous research showed that neurites in spheroids can extend to millimeters in length (Kato-Negishi et al., 2013). The time period of increasing spheroid diameter correlated with the internal structural changes of growing extensions and increasing distance between nuclei.

Self-sorting of cell types has been seen in many heterotypic spheroid cultures (Dean and Morgan, 2008; Napolitano et al., 2007; Rago et al., 2009). In our cortical spheroids, astrocytes were dispersed among the neurons, suggesting neuron-astrocyte interactions, which are important in vivo for neuron homeostasis (Allen and Barres, 2009). Qualitative assessment showed high neuron-to-astrocyte ratios in the spheroids, consistent with the cellular composition of neonatal brain (70-90% neurons), when neurogenesis comes to an end but gliogenesis is just beginning (Bandeira et al., 2009). In future experiments, to approximate other cellular composition such as that of adult brain, defined cell media could be used to promote proliferation of other neural cells within the spheroid.

3.5.3 ECM production

ECM accounts for 10-20% of the adult brain tissue mass in vivo, and its importance is highlighted by the fact that many neurological diseases are hallmarked by a change in ECM composition (Lau et al., 2013). One important ECM protein in the CNS is laminin, which is involved in neurogenesis, cell migration, axon guidance,
oligodendrocyte maturation, and axon myelination (Barros et al., 2011; Relucio et al., 2012.) Laminin expression has been shown to be altered in injury and disease (Liesi et al., 1983; Morgan and Inestrosa, 2001). In scaffold-based cultures, foreign ECM is introduced to the culture, and ECM-to-cell mass ratios are often much higher than in vivo. These changes can potentially modify cell behavior and mask disease-related changes in the culture model. In the present study, we showed that laminin was produced in the cortical spheroids, demonstrating the culture's advantage of inherent production of physiologically relevant ECM, as well as the absence of introduced foreign materials.

3.5.4 Active circuitry in the spheroid

Electrical activity and network formation via synapses are key functional features of neurons (Dichter, 1978; Frega et al., 2014; O’Shaughnessy et al., 2003; Tang-Schomer et al., 2014; Zhang et al., 2013). Whole-cell patch clamp recording, used to assess electrophysiological properties of single neurons, has been difficult in 3D because scaffold materials break or occlude the ultrathin glass pipette tips. Thus, 3D cultures have typically been limited to extracellular field potential recordings of far lower resolution and generally do not identify neurotransmitter type. (Hopkins et al., 2014; Odawara et al., 2013) Here, spheroids can be removed from the hydrogel mold and immobilized on coverslips, making this 3D culture compatible with patch-clamp recording. Whole-cell recordings at 14 and 21 DIV showed that the neurons in the spheroids had matured and were able to fire spontaneous and evoked action potentials associated with mature neurons. The difference in firing patterns among the neurons recorded may result from different neuronal subtypes within the spheroids. We note that resting membrane
potentials of neurons in spheroids were less negative than \textit{in vivo} (-64 mV) (Jia et al., 2010). One possible explanation is the greater synaptic connectivity in the spheroids. Spontaneous PSCs revealed functional synapse formation and robust communication among cells in the culture. Using specific pharmacological tools to block AMPA receptors and GABA\textsubscript{A} receptors, respectively, allowed us to characterize both excitatory and inhibitory synapses in the circuitry.

3.5.5 Brain-like mechanical properties

Neural cell functions such as differentiation, migration, neurite outgrowth, and response to toxins are affected by substrate stiffness (Balgude et al., 2001; Georges et al., 2006; Man et al., 2011; Ramamoorthi et al., 2014; Saha et al., 2008). In contrast to typical tissue culture materials – i.e. polystyrene with an elastic modulus on the order of gigapascals – these cortical spheroids had elastic moduli in the range of neonatal brain tissue (110 Pa, this study) and of adult brain tissue (< 1000 Pa) (Elkin et al., 2007). The increase in spheroid elastic modulus over time correlated with changing cell morphology within the microtissues, as cells in spheroids were round when spheroids first self-assembled and spread in late-stage spheroids. These changes may reflect a previously demonstrated correlation between internal tension and actin bundle formation within spread cells (Darling et al., 2007).

Although the commonly used cell culture hydrogels, collagen I gels (0.5 - 12 kPa) (Raub et al., 2010) and Matrigel (450 Pa) (Soofi et al., 2009), also have elastic moduli in the range of brain tissue, they contain proteins that are not normally present in the brain. In the present study, spheroids contained only cell-secreted proteins. Taken together,
spheroids' physiological mechanical properties may influence spheroid cellular behavior in beneficial ways.

### 3.6 Conclusion

Studies of the formation, health, pathology, and repair of the CNS have tremendous potential for advancement with the use of 3D *in vitro* approaches. We have reported an efficient and effective method to culture primary neural cells in 3D spheroids. The spheroids recapitulate several key features of the *in vivo* CNS tissue, including cell types, cell morphology, circuitry formation, ECM production, and mechanical properties. This 3D culture method can serve as an attractive tool to study neurological disease and injury, to screen therapeutic agents, and to gain insights into the organization and function of the CNS.

### 3.7 Acknowledgements

The authors thank Geoffrey Williams of the Leduc Bioimaging Facility for excellent technical support with confocal microscopy. Funding was provided by NSF GK-12 Fellowship, Brown Institute for Brain Science Graduate Research Award, and Department of Education through GAANN Award P200A090076 administered by the Institute for Molecular and Nanoscale Innovation at Brown University to Y.L.D.; NSF GRFP to M.E.B.; NIH NINDS SBIR 1R43NS073195 to J.R.M; NIAMSD R01 AR063642 to E.M.D.; NINDS R01 NS088453 and NIDA R01 DA011289 to J.A.K.; NSF
CBET 1134166, NIH 5R01HL110791 subcontract, NIH R21HL113918 subcontract, and Brown Institute for Brain Science Pilot Research Award to D.H.K.

Disclosure Statement

J.R.M. has an equity interest in Microtissues, Inc. This relationship has been reviewed and managed by Brown University in accordance with its conflict of interest policies. No other competing financial interests exist.

3.8 References


3.9 Supplementary materials

Supplementary Figure S3.1 21 DIV spheroids contained mature neurons with functional synapses.

Neurons in 21 DIV spheroids fired both evoked and spontaneous action potentials. Both sEPSCs and sIPSCs were detected. (a) Trace of evoked action potentials. Lower panel: current injection protocol. (b) Example of evoked action potentials followed by a spontaneous action potential. (c) Left panel: sEPSCs were isolated by addition of GABA<sub>A</sub> receptor antagonist bicuculline and glycine receptor antagonist strychnine (holding potential, -70 mV). Right panel: after addition of TTX, remaining miniature sEPSCs are apparent. (d) Left panel: sIPSCs were isolated using DNQX. Right panel: miniature IPSCs remaining in the presence of sodium channel blocker TTX.
Supplementary Figure S3.2 3D hippocampal spheroids.

Monodispersed rat hippocampal cells self-assembled into spheroids in the micromolded hydrogels. Phase contrast images of hippocampal spheroids at 1 DIV with initial seeding densities of 125, 1k, and 3k cells per spheroid. Scale bar, 100 µm.
Chapter 4

3D *in vitro* stem cell transplantation model for the central nervous system

Yu-Ting L. Dingle

*Formatted in the style of Short Communication*

4.1 Abstract

Neural stem cell transplantation is a potential treatment option for central nervous system diseases and injuries. Optimization of transplantation parameters to improve neural stem cell engraftment has been conducted mainly in animal models, which are expensive and labor-intensive. Although *in vitro* models can offer a platform for cheaper and faster preliminary evaluation of prospective parameters, information from traditional 2D cultures does not always correlate well with *in vivo* results because the culture microenvironment is oversimplified. We describe a novel 3D *in vitro* transplantation model, which presents a physiologically relevant microenvironment, to investigate host-graft interactions. This model consists of a "host" trampoline-shaped microtissue of primary rat cortical neural cells and a "graft" spheroid microtissue of neural stem cells. The graft neural stem cell spheroid is "transplanted" onto the host trampoline, and cell behavior can be monitored in the construct. The proposed model can potentially provide
more translatable results and better insights than traditional 2D culture. In a proof-of-concept experiment, we show that exposing the transplant model construct to fibroblast growth factor 2 enhanced NSC migration and tissue fusion.

### 4.2 Introduction

The central nervous system (CNS) has limited self-repair capability, and thus prognosis is often poor for CNS diseases and injuries. Stem cell transplantation is a promising treatment to restore CNS functions through multiple mechanisms, including replenishment of lost neurons, restoration of circuitry, modulation of inflammatory response, protection of existing neurons, and reversal of disease state (Lindvall and Kokaia, 2010). Neural stem cells (NSCs) are commonly used because they are lineage-restricted. For the therapy to be successful, the grafted NSCs must survive, migrate to the injury site, differentiate into the appropriate cell type, and reconnect the neuronal network. Survival, migration, differentiation, and integration are influenced by numerous variables, such as origin of graft cells (Darsalia et al., 2007), cells' developmental stage (Brederlau et al., 2006; Hahn et al., 2009), delivery method (Tate et al., 2009), timing of transplantation, number of cells, additional soluble factors administered for promoting successful engraftment (Ikegami et al., 2005), and microenvironmental cues in the host tissue (Kelly et al., 2004).

Current issues with NSC transplantation include low viability, lack of migration due to scar or absence of guidance cues, differentiation into undesired cell types, tumor formation, and lack of functional integration (Brederlau et al., 2006; Ikegami et al., 2005). These problems demonstrate that optimization of prospective variables is still required.
for a successful outcome. *In vivo* models have been widely used to test these parameters; NSCs are injected into the animals, and the tissues are collected at a targeted time point to study graft cells and host response. These *in vivo* studies can be expensive, labor-intensive, and time-consuming. Moreover, it can be difficult to monitor graft cells and study cellular-level mechanisms in the timeframe between transplantation and tissue collection (Tønnesen et al., 2011).

*In vitro* cultures are a powerful tool to study cellular interactions and are cheaper and faster testing platforms than animal models. Researchers have utilized *in vitro* cultures for the optimization of transplantation parameters (Alfaro et al., 2009; Cullen et al., 2007; Pillekamp et al., 2007; Tønnesen et al., 2011). However, because the microenvironment has profound influence on the survival, migration, and differentiation of the graft cells, discrepancies can occur between *in vivo* results and results generated from oversimplified, hard tissue culture polystyrene cultures. NSCs are highly viable *in vitro*, but survival is low after transplantation (Kelly et al., 2004). NSCs *in vitro* and in healthy brain differentiate into both neurons and glia, but neuronal differentiation of the graft NSCs was predominant in stroke-damaged brain (Darsalia et al., 2007). The number of proliferating graft cells are also different *in vivo* (10%) from *in vitro* (40%) (Darsalia et al., 2007). Therefore, an *in vitro* model that recapitulates the *in vivo* microenvironment will provide more translatable data than simple 2D cultures (Cullen et al., 2007; Stabenfeldt et al., 2010).

We previously described a scaffold-free, self-assembled 3D cortical neural culture and showed that the cortical microtissue presents several key features of the brain microenvironment, including cell types, extracellular matrix, synaptic connectivity, and
mechanical properties (Chapter 3). In the current study, we expanded our culture method to generate a 3D CNS in vitro transplantation model, composed of a host microtissue and a graft microtissue. The host microtissue is a trampoline-shaped microtissue with primary rat cortical neural cells. The graft microtissue is a NSC spheroid, which is placed onto the host microtissue. We monitored radial migration of the graft NSCs in this transplantation model construct as well as the fusion of the two microtissues (Rago et al., 2009). We demonstrated that the described in vitro transplant model has the potential to serve as a pre-animal screening platform for transplantation parameters.

4.3 Materials and methods

4.3.1 Cell isolation and culture

Primary rat cortical cells

Primary cortical cells were prepared as described in (Chapter 3, Section 3.2.1). Briefly, cerebral cortices from postnatal rat (P0-P2) were isolated and dissected into small pieces in Hibernate A buffer solution [Hibernate A (BrainBits), 1X B27 supplement (Invitrogen), and 0.5 mM Gluta-Max (Invitrogen)]. The tissues were enzymatically-dissociated with papain solution [2 mg/mL papain (BrainBits) in Hibernate A without Calcium (BrainBits)] for 30 min at 30°C. Tissues were then mechanically-dissociated with trituration with fire-polished Pasteur pipettes 20 times in Hibernate A buffer solution. Cells were centrifuged and resuspended in neuronal media [Neurobasal A (Invitrogen), 1X B27, 0.5 mM Gluta-Max, and 1X Penicillin-Streptomycin (Invitrogen)]
and filtered through a 40 µm cell strainer. Cells were washed once more with Neurobasal A/B27 media. All centrifugations were performed at 150 xG for 5 min.

**Adult hippocampal NSCs**

Rat adult hippocampal NSCs (NSCs, Millipore) were cultured as previously described (Boutin and Hoffman-Kim, 2014). NSCs were expanded on tissue culture flasks coated with poly-L-ornithine (PLO, 10 µg/mL, Sigma) and laminin (LN, 5 µg/mL, Life Technologies) in NSC media [Neurobasal A, 1X B27, 2 mM Gluta-Max, 1X Penicillin-Streptomycin, and 20 ng/mL fibroblast growth factor 2 (FGF2, Millipore)]. Cells were passaged with Accutase (Millipore). All centrifugations were performed at 300 xG for 3 min. NSCs were labeled with Vybrant DiI cell-labeling solution (Invitrogen) according to the manufacturer's instructions.

**4.3.2 Microtissue fabrication**

**Cortical trampoline microtissues**

Trampoline molds were designed using computer-assisted design (CAD) on SolidWorks software. Key features are shown in Figure 4.1a. Wax molds were printed using 3D systems Thermojet Solid Object Printer (3D Systems). Negative molds were made by casting silicone elastomer (Oomoo 30 Silicone Rubber, Smooth-On) on the wax molds. After curing, silicone negative molds were removed and thoroughly cleaned using alconox, 70% ethanol, and water. The silicone molds were autoclave-sterilized each time before use. Molten agarose solution (2% w/v) was poured on the negative molds to produce agarose hydrogels containing the original design features. Agarose hydrogels
were equilibrated in neuronal media over 48 hrs with several media exchanges. Media were aspirated prior to cell-seeding.

Cortical cells were resuspended in neuronal media at densities of 0.5 - 2 x 10^6 cells per trampoline. 10 µL cell solution was added to the seeding chamber of the trampoline hydrogel. Cells were allowed to settle to the bottom by gravity for 30 min and an additional 3 mL of media was added to each well. Media was exchanged every 2-3 days. Cortical trampolines were allowed to mature for 6 days-in-vitro (DIV) prior to in vitro transplantation.

**NSC spheroids**

3D Petri-Dish silicone spheroid negative molds were purchased from Microtissues, Inc (Providence, RI). Agarose hydrogels were made as described above and were equilibrated in NSC media. The resulting hydrogels contained 96 round bottom microwells, 400 µm in diameter. NSCs were resuspended at densities of 500 cells per spheroid. 75 µL cell solution was added to the seeding chamber. Cells were allowed to settle for 30 min, and an additional 1 mL media was added. NSC spheroids were fabricated one-day prior to in vitro transplantation.

**4.3.3 In vitro transplantation**

On the day of transplantation, media in the cortical trampoline culture was refreshed. In the migration experiment, FGF2 (20 ng/mL) was added to the neuronal media. NSC spheroids were harvested by tilting the gel and gently rinsing the seeding chamber with a P1000 pipet. A single spheroid was transferred to the top surface of the
trampoline hydrogel using a P1000 pipet, and the spheroid was maneuvered with forceps to the center area of the trampoline. The two microtissues were allowed to adhere for 1 hr at room temperature without physical disturbance before transferring to a 37°C incubator.

### 4.3.4 2D migration assay

Tissue culture polystyrene (TCPS) multiwell plates were coated with PLO (10 µg/mL) and LN (50 µg/mL). For migration on 2D cortical cells, cortical cells (210k/cm²) were pre-cultured on PLO/LN-coated TCPS for 6 days. 1 DIV DiI-NSC spheroids were placed on PLO/LN-coated TCPS or on 2D cortical cells. Samples were imaged daily for 3 days.

### 4.3.5 Immunocytochemistry and optical clearing

Microtissue immunostaining and optical clearing protocols were adopted (Chapter 1, Boutin 2014). Briefly, microtissues were fixed overnight [4% v/v paraformaldehyde and 8% w/v sucrose in phosphate buffered saline (PBS)], followed by three 1-hr PBS washes. The following steps were performed on a shaker at room temperature. Microtissues were permeabilized and blocked with B-PBT [1% Triton X-100, 10% normal goat serum, and 4% bovine serum albumin in PBS] for 2 hrs. Spheroids were incubated overnight in primary antibodies, mouse anti-β-III-tubulin (Covance, 1:50) and rabbit anti-glial fibrillary acidic protein (GFAP, DAKO, 1:200), diluted in B-PBT. Spheroids underwent two 2-hr washes with PBT [0.2% Triton X-100 in PBS], followed by one 2-hr B-PBT wash. Spheroids were incubated overnight with secondary antibodies, Cy 3 goat anti-mouse (Jackson, 1:500), and Alexa488 goat anti-rabbit (Jackson, 1:500),
diluted in B-PBT. Spheroids then underwent two 2-hr PBT washes and incubated in 1 µg/mL mL 4',6-diamidino-2-phenylindole (DAPI) in PBT for 1 hour and were then returned to PBS.

For Clear\textsuperscript{T2} optical clearing, poly-ethylene glycol (PEG) was diluted in water and formamide (Sigma) was dissolved in PBS. The following incubation steps were used: 1) 25% formamide/10% PEG, 10 min, 2) 50% formamide/20% PEG, 5 min, and 3) 50% formamide/20% PEG, 60 min.

4.3.6 Imaging and image analysis

Epifluorescence imaging was performed on a Nikon Eclipse TE2000-S microscope with Volocity software. 4X and 10X objectives were used. Images were stitched in Photoshop software. Confocal imaging was performed on a Zeiss LSM 510 Meta Confocal Laser Scanning Microscope built on an Axiovert 200M inverted microscope with ZEN 2009 software. 5X and 20X air objectives were used. Microtissue dimensions and fusion angle were measured on Volocity and ImageJ software.

4.4 Results

4.4.1 In vitro transplantation model

The 3D in vitro transplant model is illustrated in Figure 4.1b. A trampoline-shaped microtissue composed of cells from the CNS is the model host microtissue. A spheroid of graft NSCs is placed on the host tissue. The cell types and pre-transplantation
culture conditions of the host and graft cells can be selected to fit the objectives of the experiment. In this study, primary rat cortical neural cells were used to form the host trampoline, and rat adult hippocampal NSCs were the model NSC.

We determined that at least $1.5 \times 10^6$ cells were required to form consistent, symmetrical, and stable trampolines (Figure 4.1c). At lower densities, cells frequently formed incomplete trampolines, formed asymmetrical trampolines, or failed to form trampolines (Supplementary Figure S4.1). The complete trampolines were able to maintain their shape for the duration of the study (9 days). The diameter of the center area was approximately 900 µm, providing room to place the graft. Live/Dead viability assay confirmed that the trampolines were viable (data not shown). Side profile images showed that the trampoline started relatively flat with approximately 150 µm in thickness at 1 DIV, and became thicker and uneven up to approximately 250-350 µm in thickness at 6 DIV (Figure 4.1d). Thus, distance from the surface of the trampoline to the core still fell within the oxygen and nutrient diffusion limit of 200 µm, suggesting that tissue necrosis is mostly avoided. Immunostaining showed neuronal and astroglial network formation in the trampoline (Figure 4.1e).
Figure 4.1 The *in vitro* transplantation model consists of a cortical trampoline microtissue and a NSC spheroid.

(a) Design of trampoline mold. Key dimensions: seeding chamber, 3.5 mm in depth, 3.4 mm in diameter; posts, 600 µm in diameter, 400 µm edge-to-edge distance from chamber wall, and 400 µm edge-to-edge distance from adjacent post. (b) Schematic of the *in vitro* transplantation model. A NSC spheroid is formed to model the graft. A trampoline microtissue composed of cortical cells is fabricated and serves as the host microtissue. The NSC spheroid is placed or "transplanted" onto the trampoline. Cell behavior is monitored on the transplant model. (c) Phase contrast image of a 7 DIV cortical trampoline microtissue. Dashed line shows the approximate cut plane for images in (d). (d) Phase contrast images of cortical trampolines dissected across the center on 1 DIV (left) and 6 DIV (right) and imaged from the side. Dashed line outlines the profile of the microtissue in the plane of focus. (e) Confocal images show extensions of neurons (β-III-tubulin, left) and astrocytes (GFAP, right) in the center region of a 6 DIV cortical trampoline. (f) Phase contrast images of 1 DIV NSC spheroids. Scale bars, (c,d) 300 µm, (e) 100 µm, (f) 200 µm.
NSCs were pre-labeled with DiI prior to spheroid formation in order to be tracked in the transplantation model. NSCs self-assembled into spheroids in round-bottom microwells of the agarose hydrogels, with diameters dependent on initial seeding densities (data not shown). We chose a seeding density of 500 cells/spheroid, which yielded spheroids large enough (194 ± 17 µm in diameter, Figure 4.1f) to be visible under a bench-top dissection microscope for manual manipulation.

We were able to place a single spheroid in the center of the trampoline using simple manipulation with a pipette and forceps. The spheroids adhered to the trampoline in under 1 hr. In 24 hrs, the two tissues could not be delineated with phase contrast microscopy. DiI-labeled NSCs could be identified using epifluorescence and confocal microscopy (Figure 4.2 and Supplementary Figure S4.2).

4.4.2 NSC migration in the transplantation model

We performed a proof-of-concept experiment by assessing NSC migration in the presence of FGF2. FGF2 is a multipotent growth factor that maintains NSC stemness and promotes proliferation, survival, and migration of NSCs in vitro. Injection of FGF2 promotes migration of endogenous NSCs in vivo (Azim et al., 2012). A 1 DIV spheroid was placed on a 6 DIV trampoline in the presence or absence of FGF2. As shown in Figure 4.2, confocal microscopy allowed the visualization of individual DiI-NSCs, and enhanced radial migration of NSCs from the spheroid was observed in the presence of FGF2. Migration enhancement was correlated with an increase in fusion angle, defined as the angle between the host and graft microtissues as seen on side views of confocal images.
Figure 4.2 Utility of the in vitro model - study of the effect of FGF2 on NSC migration.

A 1 DIV NSC spheroid, pre-labeled with DiI, was grafted on a 6 DIV cortical trampoline in the presence and the absence of FGF2. Nuclei were stained with DAPI. Confocal projections showed enhanced NSC migration in the presence of FGF2. Orthogonal XZ plane view of confocal images showed an increased fusion angle between the two microtissues in the presence of FGF2. Scale bar, 200 µm.
4.5 Discussion

*In vitro* cultures have enabled the evaluation of candidate factors to enhance transplantation success. For example, Ikegami et al. first demonstrated that the addition of chondroitinase can promote NSC migration *in vitro* and then translated the results to a rat spinal cord injury model (Ikegami et al., 2005). The ability to closely examine the NSCs *in vitro* also allows researchers to elucidate mechanisms through which NSCs interact with host cells. Jäderstad et al. reported that gap junction coupling was an early functional integration between graft and host cells in culture (Jäderstad et al., 2010). Cusulin et al. demonstrated a cell fusion phenomenon between NSCs, microglia, and cortical neurons first *in vitro* and then confirmed *in vivo* (Cusulin et al., 2012).

In this study, we described a novel 3D *in vitro* transplantation model for the CNS. This model offers several advantages over traditional 2D cultures. Here, NSCs are in contact with a host microtissue that presents physiologically relevant features, including cell types, ECM, soluble factors, and mechanical properties (Chapter 3). Using the scaffold-free culture method, we avoid the introduction of scaffold materials that are not normally present in the CNS. We observed differences in NSC migration and tissue fusion in the presence of FGF2. Because of the *in vivo*-like microenvironment of the model, we hypothesize that results obtained are more applicable to *in vivo* than traditional 2D cultures. Future experiments include quantitative analysis of migration and fusion, as well as the assessment of NSC viability and differentiation after transplantation.

The host microtissue in our platform is tailorable to appropriately model the diseased or injured microenvironment where the NSCs will be grafted. NSC behavior is
highly impacted by the host microenvironment. Neural cells can be isolated from different regions of the CNS for the fabrication of the host microtissue. Pre-treatment with defined culture conditions can be used to simulate disease and injury states, such as ischemic culture for stroke model, mechanical loading for traumatic injury, and exposure to inflammatory cytokines and mediators (Cullen et al., 2007; Kelly et al., 2004; Tønnesen et al., 2011). This model also opens up the possibility of using neurons derived from human induced pluripotent stem cells (iPSCs), including patient-specific iPSCs. Our group has cultured human iPSCs as self-assembled spheroids (data not shown). The ability to use human cells can fill in the gap created by the limited availability of human brain samples. The incorporation of human cells will be more relevant and will have a higher chance of translating than animal models.

The geometry of the trampoline provides its own benefits. The near-centimeter size of the trampoline can accommodate delivery methods other than spheroids, including suspended cells, scaffold-encapsulated cells, and whole tissues. While many scaffold-less microtissues contract and change shape in a short period of time (Dean et al., 2007; Livoti and Morgan, 2010), the stable shape of the trampoline enables tracking of NSC migration. We measured the fusion angle between the two microtissues, which can be an indication of the integration between the graft and host microtissues (Rago et al., 2009).

Importantly, our in vitro transplantation model provides a fast and economical platform to systematically test the scores of factors that may influence the outcome of transplantation. A study using DA neuron progenitors from embryonic rats showed that transplantation of less differentiated DA neuron progenitors from embryonic day 13 (E13) rats resulted in better functional recovery in a rat Parkinson's disease model than of
progenitors from E15 rats (Hahn et al., 2009). However, another study using human embryonic stem cell-derived DA progenitors showed that if the in vitro differentiation was too short, there was inadequate DA neuron production and adverse teratoma formation after transplantation (Brederlau et al., 2006). These examples show that transplantation strategies require fine-tuning to achieve a favorable clinical outcome. In addition, several soluble factors besides the aforementioned FGF2 have profound influence on NSC behavior, such as sonic hedgehog (SHH), brain-derived neurotrophic factor (BDNF), glial-derived neurotrophic factor (GDNF), and vascular-endothelial growth factor (VEGF) (Martino et al., 2006). We can generate approximately six host trampoline microtissues per rat neonate, which can reduce animal use. Our model can therefore allow investigators to pre-screen a larger number of factors to narrow down the higher-impact parameters to perform in vivo studies.

In conclusion, we describe a 3D in vitro transplantation model for the CNS that presents a physiologically relevant microenvironment, is tailorable and versatile, and can be used to perform preliminary screening of NSC transplantation parameters.

## 4.6 Acknowledgements

The author thanks Jacquelyn Schell for the design and technical support of the trampoline mold, Molly Boutin for confocal imaging, and Liane Livi for the help with culture and imaging. Funding was provided by Brown Institute of Brain Science Graduate Research Award to Y.L.D.
4.7 References


4.8 Supplementary materials

Supplementary Figure S4.1 Critical seeding density for cortical trampoline.
Phase contrast images at 1 DIV. 0.5 x 10^6 cells/gel failed to form a complete trampoline. 1 x 10^6 cells/gel formed trampolines, but some trampolines were incomplete or asymmetrical. 2 x 10^6 cells/gel formed complete trampolines. A minimum seeding density of 1.5 x 10^6 cortical cells was determined to be required for consistent, complete trampoline microtissue formation.
Supplementary Figure S4.2 Migration assay in 2D models.
FGF2 enhanced radial migration from NSC spheroids on PLO/LN-coated TCPS (a) and on 2D cortical cells pre-cultured on PLO/LN-coated TCPS (b). Spheroids were fabricated with DiI-labeled NSCs (red) at an initial seeding density of 500 cells/spheroid and allowed to mature for 1 DIV. The substrates were PLO/LN-coated (a) or cortical cells (210k/cm²) on PLO/LN-coated TCPS (b) pre-cultured for 6 DIV prior to NSC transplantation. Epifluorescence images and corresponding phase contrast images on 2 days post-transplantation. Scale bar, 200 µm.
Chapter 5

Conclusions and future directions

Cell-based therapies hold great promise for patients who suffer CNS diseases and injuries, yet, significant and reliable clinical improvements have not been achieved. We are motivated by the need for better in vitro culture platforms to aid in the study of CNS disease, injury, and stem cell differentiation and transplantation. This thesis is divided into two main parts to address different aspects of cell based-therapy: 1) the study of in vitro neuronal differentiation of stem cells (Chapter 2), and 2) the development of 3D in vitro microtissue cultures to model the CNS (Chapters 3 and 4).

The first goal of this thesis was to address the heterogeneity of ESC-derived DA neurons. ESC-derived DA neurons are proposed for cell transplantation for PD and for studying DA neuron disease mechanisms. Because DA neuron subtypes are involved in distinct physiological functions and disorders, the ability to generate appropriate subtypes of DA neurons can lead to more relevant findings and better transplantation outcomes. In Chapter 2, we reported the presence of CALB$^+$ and CALR$^+$ DA neuron subtypes with quantitative analysis, and the absence of the GIRK2-expressing subtype. Since cells are not typically sorted prior to transplantation, this information on ESC-derived DA neurons' heterogeneity should be taken into consideration when analyzing transplantation results. The generation of CALB$^+$ and CALR$^+$ DA neuron subtypes is also useful for investigators who focus on VTA DA neurons that are associated with addiction and psychiatric disorders. The quantitative analysis approach presented in this chapter, which
involves the assessment of sampling errors in cultures with high degree of spatial distribution variation, can also be adapted by research groups that encounter similar problems.

The fields of stem cell biology and regenerative medicine have been searching for the right combination of factors to control stem cell differentiation. We demonstrated that DA neuron yield using the 5-stage differentiation protocol in 2D culture was low, and SHH and FGF8, potent morphogens in the induction of DA neuron differentiation in vivo, did not enhance DA neuron generation in vitro. Possible explanations include that the recipient cells were not the precursor cell types responsive to SHH and FGF8, and/or that these cells were in a microenvironment without other critical cues. In vitro, SHH and FGF8 did not govern DA neuron subtype generation, and attenuation of SHH and FGF8 signaling using neutralizing antibodies neither resulted in changes of DA neuron production or subtype generation. The rationale for selecting SHH and FGF8 was the spatial distribution of DA neuron subtypes in the VTA (medial) and in the SN (lateral) in relation to the locations of SHH (ventral midline) and FGF8 (isthmus) sources during development. The uncommitted precursors are potentially exposed to gradients of SHH/or and FGF8, and this differential exposure may be one of the mechanisms behind subtype specification. In the developing spinal cord, a SHH gradient sets up the pattern for spinal cord motor neuron and interneuron subtype specification. The absence of subtype specification by SHH and FGF8 in vitro should not rule out the morphogens' potential role in vivo. Another approach to evaluate whether SHH and/or FGF8 play a role in DA neuron subtype specification is to expose precursor cells isolated from the region of early embryonic midbrain (approximately E8.5) to an array of concentrations
and combinations of SHH and FGF8. An alternative way to test this hypothesis is to use a microfluidic approach to set up gradients of SHH and FGF8 in the same and opposing directions and observe the spatial distribution of CALB⁺, CALR⁺, and GIRK2⁺ subtypes of DA neurons.

Cell fate and cell behavior are determined by both intrinsic gene expression and extrinsic cues in the microenvironment. The spatial and temporal patterns of these factors are important for proper development of an organism. However, the traditional 2D plastic culture is very different from the in vivo embryonic niche where developing DA neuron progenitors reside. The results from Chapter 2 highlighted the differences between in vitro and in vivo, the impact of these differences on cells, and the demand for culture platforms that better mimic the in vivo microenvironment.

Developing novel 3D in vitro platforms for neural culture to bridge the gap between traditional 2D culture and in vivo models is the second goal of this thesis. As described in Chapters 3 and 4, we used a scaffold-free, self-assembled microtissue culture approach. Such an approach has several advantages: cell types can be tailored, the introduction of foreign materials is avoided, and cells are allowed to engineer their own microenvironment. In Chapter 3, we showed that 3D spheroids of primary rat cortical neural cells present several key features of the in vivo microenvironment, including 3D cell morphology, network formation, ECM production, electrical activity, synapse formation, and mechanical properties. The results demonstrate that spheroid culture of primary CNS neural cells is an effective in vitro platform to model the CNS.
We see great potential for applying this 3D neural spheroid culture to model CNS disease and injury. The accessible fabrication method allows easy adaptation of the technique by other research groups. An example field that can benefit from this 3D culture model is AD, a disease hallmarked by amyloid-β plaque, neurofibrillary tangles, and eventually synapse loss and cell death. Recent studies have shown that 3D cultures (hydrogel scaffold) were able to recreate the disease features better than traditional 2D culture. This was partially due to the fact that secreted amyloid-β accumulates in 3D cultures to produce toxic effects, whereas in 2D cultures the protein diffuses freely into the media. AD is a disease of synaptic dysfunctions resulting in memory loss and dementia. However, culturing neurons in 2D has been shown to alter neurons' electrophysiology. For example, long-term potentiation and depression are important features to evaluate synaptic functions and plasticity in vivo and slice cultures, but these features often disappear in 2D cultures. We believe that our 3D spheroid culture, which supports an in vivo-like microenvironment, is able to better recapitulate in vivo synaptic functions. Another advantage of 3D spheroid culture over scaffold 3D culture is that spheroid culture is more compatible with the whole-cell patch-clamping technique, which generates high resolution and specific electrical activity recordings. Future directions include the characterization of synaptic plasticity in spheroids, which will provide insights on the feasibility of using spheroids to study synaptic functions to model diseases that affect synapses. From there, we can model AD with cortical spheroids treated with amyloid-β or in spheroids fabricated using AD patient iPSC-derived neurons and evaluate the change in synaptic functions. Such a model will be valuable for understanding the disease pathology of AD and for testing drugs to reverse the pathology.
With immunocytochemistry and confocal microscopy, we showed elaborate 3D neuronal networks in the spheroids. However, immunocytochemistry was unable to visualize individual neurites due to the high neurite density. Single cell labeling by viral transfection or microinjection can enable the visualization of neurites from a single neuron and the measurement of their length and branching. The ability to characterize neurites is useful to study diseases that affect neurite extension and arborization.

We showed the presence of laminin in the spheroids, demonstrating that the spheroids contain cell-secreted, physiologically relevant ECM. ECM accounts for approximately 20% of the tissue weight in the adult brain. Since ECM composition is important for healthy cell functions in the CNS and alterations in ECM are associated with many CNS pathologies, future directions will involve in-depth characterization of the types and amount of ECM. One interesting ECM component is the perineuronal nets (PNN) with the constituents hyaluronan, chondroitin sulfate proteoglycan, and tenascin R, which stabilizes newly formed synapses. Changes in PNN affect synaptic plasticity and function. Since we have electrophysiological evidence of synapse formation in cortical spheroids during the period of 7-14 DIV, it will be interesting to see if the formation of PNN correlates with this time frame.

Because spheroid culture allows the researchers to tailor cell types and ratios, this culture can be used to assess cell-cell interactions that would not be possible in brain slice culture. For example, increasing amount of evidence has shown that neuronal functions are regulated by astrocytes through ion and neurotransmitter buffering. However, artificially changing the numbers of astrocytes and neurons in vivo to perform gain of function or loss of function experiments can be difficult. One future direction is to
investigate synaptic functions under different astrocyte-to-neuron ratios. Chapter 3 showed that our 3D cortical spheroids had a low astrocyte-to-neuron ratio, likely due to the neonatal stage of the dissected brain. *In vivo*, the astrocyte-to-neuron ratio increases as the brain matures. Enrichment of astrocytes can be achieved by culturing primary neural cells that contain mixed cell types in defined media promoting astrocyte proliferation, and we can then add these astrocytes to newly isolated primary neural cells. Changes in electrophysiology under the influence of higher numbers of astrocytes will provide information on the functions of astrocytes. Alternatively, we could investigate the effects of reactive astrocytes in an injury environment on neurons. One method is to treat the astrocytes with inflammatory cytokines that are released after injury (e.g. TGFβ), co-culture these reactive astrocytes with healthy neurons in the spheroids, and conduct electrophysiology characterization on the neurons. Therefore, we are examining the isolated effects of reactive astrocytes on the neurons rather than the combination effects of reactive astrocytes and cytokines.

We showed that cortical neural spheroids present *in vivo*-like features, but there still remains many differences between 3D spheroid culture and native tissue. For example, compartmentalized *in vivo* structure are absent in the spheroids. Spheroids have higher cell densities than *in vivo*, but they are within the same magnitude. Many other 3D *in vitro* cultures have cell densities many magnitudes lower than *in vivo*. Depending on the specific questions, it may be a reasonable to trade off perfect reflection of the native tissue and simplicity of the culture. Genomics, proteomics, and electrophysiology comparisons among CNS tissues, spheroids, scaffold cultures, and 2D monolayer cultures can offer valuable information on similarities and differences of cells in their native niche.
versus cells cultured in the variety of *in vitro* platforms and provide insights on the impact of microenvironmental cues on cells' biological activities. The results will aid researchers to choose the *in vitro* culture method that is appropriate for their specific scientific questions.

In addition to the healthy cortical and hippocampal neural cells we reported in this study, the spheroid culture has the potential to use iPSC-derived neural cells or genetically modified neural cells. Because access to live human brain slices is very limited, using patient iPSCs cultured as 3D microtissues can be a practical alternative.

Chapter 4 builds on the results from Chapter 3 to develop a 3D co-culture platform to model stem cell transplantation in the CNS. This study aims to facilitate the screening of input variables prior to animal studies in order to make the optimization process for cell-based therapies faster and more cost-effective. Although animal brain slice cultures are frequently used for this purpose, the microtissue approach is especially useful when brain slices are not feasible, such as for long term cultures or when animal models of human disease are unavailable. The reported platform consists of a large surface "host" microtissue of CNS cells and a "graft" stem cell spheroid. Because we showed in Chapter 3 that CNS neural cells cultured as scaffold-free microtissues recapitulate many key *in vivo* features and can be used for long term culture, the host microtissue in this chapter can be a good alternative to brain slices. In a proof-of-concept experiment, we were able to evaluate the enhanced NSC graft migration and fusion with the host cortical microtissue in the presence of FGF2. Quantification of NSC migration and fusion of the NSC spheroid with the host trampoline will provide comprehensive analysis for this project.
Other outputs that can potentially be obtained from this model include viability, proliferation, differentiation, and circuitry formation. Viability can be assessed using a TUNEL assay on tissue sections, proliferation can be addressed using BrdU labeling, differentiation can be evaluated using immunostaining on the sections, and circuitry formation can be examined by electrically stimulating the host tissue and recording downstream electrical responses in the spheroids.

Differences in viability, proliferation, and differentiation profile of NSCs in different microenvironments have been reported in the literature. Based on these studies, investigating NSC behavior in a 3D CNS model holds great potential to provide more translatable results than traditional 2D culture. Future directions include the assessment of graft cell behavior using a host microtissue that is fabricated and pre-conditioned for a specific disease or injury model. For example, an ischemic stroke model can be created by culturing the host microtissue using oxygen and glucose deprivation. NSCs will be placed on the stroke model microtissue, and viability, migration, and differentiation will be examined. Comparison of these results to *in vivo* data will help validate our 3D *in vitro* model.

Next, this transplantation model can be used to test the various parameters associated with cell-based therapy procedures, such as cell types, developmental stages, delivery methods, and co-delivery of soluble factors. An example is to compare NSC migration and differentiation of cells that are pre-differentiated (by growth factor withdrawal) versus undifferentiated NSCs. Another example is to treat the transplant constructs with additional soluble factors such as BDNF, GDNF, and VEGF to test whether these factors affect migration, tissue fusion, and differentiation.
In conclusion, this thesis contributes to a deeper understanding of *in vitro* generation of neurons from stem cells and provides novel engineered 3D *in vitro* models to evaluate CNS disease and injury. Our 3D platforms can complement traditional 2D culture and animal models to gain translatable information and to efficiently and effectively screen variables associated with cell-based therapy. Together, this thesis provides new information and tools to the field to optimize treatment strategies for cell-based CNS therapies.
Appendix A

Endothelial cell and neural cell spheroid
co-culture for *in vitro* modeling of the
neurovascular unit

A.1 Introduction

Endothelial cells (ECs) play an important role in the CNS including the formation of the blood-brain-barrier (BBB) and regulation of neural stem cell (NSC) differentiation. In the adult NSC niche, active neurogenesis occurs in NSCs that are in close proximity with the ECs (Goldberg and Hirschi, 2009; Tavazoie et al., 2008). In a normal neurovascular unit, ECs form a continuous BBB that are covered and regulated by pericytes and astrocytes (Urich et al., 2013). In contrast, the NSC niches have distinct neurovascular unit architecture with regions of incomplete tight junctions. There is a significant decrease of pericyte and astrocyte endfoot coverage of the EC with NSCs often in direct contact with ECs (Tavazoie et al., 2008). NSCs co-cultured and co-transplanted with ECs has lead to higher survival and neuronal differentiation after transplantation in animal models (Nakagomi et al., 2009).

Heterotypic cell interactions are often studied in non-contacting co-culture method such as the Transwell assay. Non-contacting methods allow soluble factors to
reach to the other groups of cells. Contacting co-culture method provides the advantage of studying cell surface-bound factors and cell-cell interactions in addition to secreted factors. In this preliminary study, we used the scaffold-free self-assembled spheroid method to co-culture 1) EC and astrocyte for a BBB model and 2) EC and neuronal cell for a neurovascular unit model.

**A.2 Materials and methods**

**A.1.1 Cell types**

Mouse endothelioma line bEnd.3 and transformed rat endothelial cells RBE4 were used as the model ECs. A7, a transformed astrocyte cell line were used as the model astrocyte. B104, a neuroblastoma cell line was used as model neuronal cells. Cells were cultured and expanded according to the suppliers' instructions.

**A.1.2 Spheroid culture**

Cells were pre-labeled with 5µM CellTracker Red or CellTracker Green according to the manufacturer's instructions. Spheroid culture was conducted as described in Section 3.2. For heterotypic spheroid cultures, cells were either pre-mixed or sequentially seeded, and media was mixed at a 1:1 ratio.
A.3 Results and discussion

As shown in Figure A.1, RBE4 (50-300 cells/microwell) formed loose aggregates with many round individual cells seen on the surface of the aggregates. bEnd.3 (25-500 cell/microwell) self-assembled into compact aggregates with smooth surface. B104 (150 cells/microwell) also self-assembled into compact aggregates with a smooth surface. A7 (150 cells/microwell) self-assembled into aggregates, and most of the aggregates did not have a smooth surface (Figure A.2a).

![Figure A.1 Self-assembly of ECs](image)

(a) RBE4, seeded at 50, 150, 300 cells/microwell, formed loose aggregates. bEnd.3, seeded at 25, 50, 100, 200, and 500 cells/microwell self-assembled into aggregates. Images were taken at 24 hrs.

When A7 and B104 were co-cultured with RBE4 or bEnd.3, the ECs sorted to the outside of the aggregates (Figure A.2a). Additionally, bEnd.3/B104 were co-cultured at different ratios from 8:1-1:4 (Figure A.2b). We found that bEnd.3 sorted to the outside of the heterotypic spheroid in all seeding ratios.
In sequential seeding experiments, A7 and B104 spheroids were seeded alone in the hydrogels (150 cells/microwell) or pre-mixed with bEnd.3 (150 cells/microwell). Dispersed bEnd.3 (1:1 ratio) were added on day 1, 3, or 5. In the absence of bEnd.3, A7 spheroid continued to contract (Figure A.2c, blue line). When bEnd.3 and A7 were pre-mixed prior to spheroid formation, the A7s showed less compactability (Figure A.2c, red line). When A7 aggregates were first formed on day 0 and bEnd.3s were added sequentially on day 1 (orange line), the contractibility of A7 was slowed. Similar effects were not observed when bEnd.3s were added on day 3 (yellow line) or day 5 (green line).

In comparison, when B104s were seeded alone, the aggregates continued to expand (Figure A.2d, blue line). When B104 were pre-mixed with bEnd.3, the expansion of B104 was slowed. When B104 aggregates were initially formed and bEnd.3 were added sequentially on day 1 (orange line), day 3 (yellow line), or day 5 (green line), the rate of expansion also decreased.

The results showed that the spheroid culture can be used to co-culture EC and neural cells and potentially be used to model BBB, NSC niche, and neurovascular unit. We observed interesting results in A7's compaction and B104's expansion when bEnd.3 was introduced. Analytical techniques still need to be optimized for heterotypic spheroid culture to characterize cellular functions in order to appropriately model EC and neural cell interactions.
Figure A.2 Spheroid co-cultures of neural cells and ECs

(a) A7 and B104 (both at 150 cells/microwell) formed small aggregates within 24 hrs. When pre-mixed with ECs (bEnd.4 or RBE4, 150 cells/microwell) in a heterotypic co-culture, ECs generally self-sort to the outside. (b) In a bEnd/B104 heterotypic co-culture with different seeding ratio (1=150 cells/microwell), bEnd.3 continued to sort to the outside and B104 to the core. (c) Graph of A7 spheroid top-view size, measured using red fluorescence images. When A7 were seeded alone, the spheroid continued to contract. The presence of bEnd.3 on day 0 or day 1 reversed the compaction. (d) Graph of B104 spheroid top-view size, measured using red fluorescence images. When B104 were seeded alone, the spheroid continued to expand. The presence of bEnd.3 slowed the expansion.
A.4 References


Appendix B

Scientific illustrations

Figure B.1 Methods of fabrication of topographical platforms.
Figure B.2 Topographies presented to neurons *in vitro*.
Figure B.3 Schwann cells perform a range of functions in the body.
Figure B.4 A bioengineered model of stem cell manipulation and cell transplantation for neurological disorders.

Specific aims for an awarded Pilot Research Grant proposal to Brown Institute for Brain Science.
Figure B.5 Investigating the effects of simulated microgravity on 3D neural stem cell microtissue.

Objectives for an awarded fellowship application to NASA Rhode Island Space Grant Consortium.
Figure B.6 Axon guidance by critical cues – Engineering nerve growth *in vitro* and observing from afar.

Illustrations of methods used in an awarded NSF grant proposal.
**Figure B.7 In vitro neuroma model**

Illustrations of methods (a) and specific aims (b) for an awarded grant proposal to the Emerging Areas of New Science (DEANS) Awards, Brown University.
Figure B.8 Biomimetic Synthetic Feeder Layer (BSFL).
Specific aims for a grant proposal in progress.
Figure B.9 Cell origami.

Methods for a grant proposal in progress.