2013

Characterizing the Effect of Substrate Stiffness on Neural Stem Cell Differentiation

Colleen Curley
Lehigh University

Characterizing the Effect of Substrate Stiffness on Neural Stem Cell Differentiation

By
Colleen T. Curley

A Thesis
Presented to the Graduate and Research Committee
of Lehigh University
in Candidacy for the Degree of
Master of Science
in
Bioengineering

Lehigh University
January 2013

This thesis is accepted and approved in partial fulfillment of the requirements for the Master of Science.

_____

Date

_____

Thesis Advisor: Sabrina Jedlicka

_____

Chairperson of Department: Anand Jagota

Acknowledgements

This research was supported by NSF CBET Grant #1014957 and the Lehigh University 2012 Faculty Innovation grant. I would also like to acknowledge Lehigh University for the Presidential Scholarship.

Table of Contents

List of Figures

Abstract

Differentiated neurons (dorsal root ganglia and cortical neurons) have been shown to develop longer neurite extensions on softer materials than stiffer ones, but previous studies do not address the ability of neural stem cells to undergo differentiation as a result of material elasticity. In this study, I investigate neuronal differentiation of C17.2 neural stem cells due to growth on polyacrylamide gels of variable elastic moduli. Neurite growth, synapse formation, and mode of division (asymmetric vs. symmetric) are all assessed to characterize differentiation. Data indicates that C17.2 differentiation (as dictated by number and type of division events) is dependent upon substrate stiffness, with softer polyacrylamide surfaces (140 Pa) leading to increased populations of neurons and increased neurite length. Additionally, this study is the first to verify synapse formation of C17.2 neurons *in vitro* and show that soft gel substrates support synaptogenesis, with increased synapse numbers seen after high potassium stimulation. Results illustrate the importance of substrate stiffness in directing fate of neural stem cells and bring the field one step closer to mechanically tunable scaffolds for neuroregeneration.

Chapter 1

Review of Current Literature

Stem cell therapies have the potential to treat and cure a number of diseases within the next 10-15 years**.** One specific area of use will be for the treatment of neurodegenerative disorders such as Parkinson's and Alzheimer's disease. Since the brain itself has limited capacity for self-repair, these treatments would include implantation of stem cells to replace damaged cells populations [1, 2]. Although the brain is a complex organ, results from animal studies show that implanted cells do have the capacity to integrate into the existing circuitry [3-5]. To effectively use stem cells to treat these diseases, it is essential to have control over the final fate of the implanted cell population. Factors contributing to cell fate *in vivo* consist of both chemical and mechanical cues from the cell microenvironment [6]. In recent studies, the impact of the mechanical properties of the extracellular environment has been shown to play a large role in determining stem cell fate [7-9]. Specifically, more research is needed to determine the ideal mechanical properties of a substrate to direct neural stem cells toward clinically relevant neuronal fates.

Certain chemical stimuli are involved in activation and progression of stem cell differentiation pathways. For example, chemical signals, like growth factors and cytokines activate specific members of the mitogen-activated protein kinase (MAPk) signal transduction pathway. Studies have illustrated that this pathway regulates differentiation of muscle cells, mesenchymal stem cells, and neuronal cells [10-12]. *In*

vivo, factors such as basic fibroblast growth factor (bFGF) and transforming growth factor beta (TGF-β) are involved in neuronal differentiation in the neural tube, leading to development of mature spinal cord. For stem cells of the central nervous system, cilliary neurotrophic factor (CNTF) is a driver of astrocytic differentiation, while thyroid hormone (T3) yields lineage-restricted progenitors for oligodendrocytes [13].

Much of the work regarding mechanical properties of the extracellular environment dictating cell fate focuses on substrate elasticity. The property of elasticity is a material's resistance to deformation [14]. Many cellular processes, including cell proliferation, migration, and differentiation are regulated by matrix elasticity [7-9, 15-18]. Previous studies indicate that response to the elasticity of the extracellular environment is cell-type specific and seems to correlate to the elasticity of the cell's native tissue [8, 19]. Increased spreading of fibroblasts occurs on stiffer surfaces [20], whereas primary neuronal cells form more neurite branches on softer substrates [21]. It is believed that cells can sense the stiffness of the matrix through a feed-back mechanism of the actin-myosin cytoskeleton.

Cells are able to sense extracellular mechanical force in various ways, including stress-sensitive ion channels, caveolae, integrins, and cadherins [22-25]. For this the purposes of this study, I am particularly interested in cell-extracellular matrix connections that allow for direct sampling of the mechanical properties of the extracellular environment. Integrins are extracellular matrix (ECM) receptors that essentially link the intracellular (cytoskeleton) and extracellular (ECM) environment. Yeung *et al*. 2005, correlates cells spreading on different stiffnesses with the expression of the α-5 integrin.

This work showed that on stiffer substrates, fibroblasts exhibit a more well-spread morphology that correlates to a five-fold increase in α5 integrin expression, whereas these cells on softer substrates exhibit a more compact, circular morphology [19].

Although integrins serve as a physical link between the external and intracellular environments, they are part of a larger signaling complex involved in mechanotransduction, known as focal adhesions [26]. Focal adhesions are protein complexes that link the cytoskeleton of cells to the extracellular matrix at the sites of integrin binding. These complexes are thought to possibly act as a signaling hub in the translation of mechanical cues from the extracellular environment into biochemical signals inside the cell [27]. A 1997 study by Pelham and Wang looked at the effect of substrate stiffness on focal adhesion complexes of normal rat kidney epithelial and 3T3 fibroblastic cells. They observed differences in morphology and also in motility of the cells cultured on soft and stiff substrates. The authors hypothesized that the cells were able to sense the differences in extracellular elasticity through the sites of adhesion. To test this, they looked at fluorescently labeled vinculin on the different substrates. They found that on stiff substrates the cells had formed arrays of stable focal adhesion, whereas cells on softer substrates the adhesion sites were unstable and irregular punctate structures. They also showed that the extent of tyrosine phosyphorylation matched results for focal adhesions, implicating this pathway in the mechanism for mechanosensing [20].

The 2006 study by Engler *et al*. illustrates the importance of substrate stiffness in directing stem cell fate. Mesenchymal stem cells are adult multipotent stem cells with a

variety of potential fates. In this study, mesenchymal stem cells were cultured on polyacrylamide gel substrates of various stiffnesses. The investigators found that MSCs grown on gels with comparable stiffness to brain tissue exhibit cellular morphology similar to neurons, while cells grown on substrates with stiffnesses similar to muscle and bone tissue exhibit morphologies similar to that of myoblasts and osteoblasts, respectively. PCR analysis confirmed morphological observations, showing that cells grown on the softest gels had 5-fold greater expression of neurogenic transcripts, cells grown on gels with elasticity comparable to muscle show 6-fold greater expression of myogenic markers and cells grown on gels with elasticity comparable to bone show 4-fold greater expression of osteogenic markers with respect to early passage MSCs. This study also implicates myosin II in the mechanical sensing pathway, illustrating that inhibition of myosin II with blebbistatin blocked expression of all differentiation markers on all stiffnesses [8]. While many studies have looked at the effect of substrate stiffness on stem cell differentiation, the subject of neural stem cell differentiation in response to external mechanical cues remains relatively unexplored.

In this study, I investigate the effect of substrate stiffness on specific aspects of neural stem cell differentiation, including neurite growth, synapse formation, and mode of division (symmetric or asymmetric). Formation of functional nervous tissue requires neurons to guide axons along an appropriate route to a suitable synaptic partner, and type of division during differentiation influences the final fate of the cell, and ultimately the makeup of the adult tissue [28,29]. Therefore, these aspects of neuronal differentiation are crucial for cells intended to integrate and replace damaged cell populations in the

nervous system. The cytoskeleton plays a large role in the guidance of the axonal growth cone and formation of synapses [30]. It also is largely involved in spindle orientation during cell division [31]. Since the proposed pathway for mechanotransduction involves feedback mechanisms of the acto-myosin cytoskeleton, it is possible that all aspects of differentiation studied here are affected by the stiffness of the extracellular environment through cytoskeletal sensing and response to external elasticity.

Many previous studies, discussed below, use primary neuronal cultures. In this research, I am using neural stem cells, which are self-renewing multipotent cells, meaning that they have the ability to ultimately become one of several neural cell types. While primary neurons serve as an established means for studying neurons and the CNS, they are not the best option for therapeutic use. Primary neurons are restricted to a single developmental fate and cannot be expanded in culture, yielding a limited supply. Neural stem cells, however, are more amenable for regeneration of nervous tissue, and in this research, I aim to guide neural stem cell fate. This is fundamentally different from the studies performed with primary neuronal cultures, which only look at the effect of substrate mechanics on physical properties of cells already committed to a specific neuronal phenotype.

The first aspect of neural stem cell differentiation that I will explore in response to substrate stiffness is neurite growth. Neurite outgrowth is crucial for the formation of proper connections between cells, allowing for signal transduction, a process essential to proper brain function. Several studies, detailed below, have explored the effect of substrate stiffness on neurite growth and formation using primary neuronal cultures. A

2001 study performed with chick dorsal root ganglion cells in 3D agarose gels shows that the rate of neurite extension rate decreases with increasing gel stiffness [32]. Flanagan *et al*. 2002 looks at neurite extension of mouse spinal cord primary neuronal cells on polyacrylamide gels of varying stiffnesses, finding that cells grown on softer substrates had significantly more branches than cells grown on stiffer substrates [21]. Georges *et al*. 2006 reported that growing mixed cultures of glial cells and neurons on soft substrates generates populations that are mostly neurons [33]. Leach, *et al*. 2007 performed neurite growth studies with PC12 cells, a rat adrenal pheochromocytoma cell line that can be induced into a neuronal phenotype with neurite growth factor. This study found more an increase in number and length of neurite branching on the stiffer substrates tested (190 Pa, 2 kPa, and 19 kPa), as opposed to the softest substrate (7 Pa) in which they saw few short, unbranched neurites. No correlation was found between increasing modulus in the stiffer substrates [34]. This threshold result varies from those found in other neurite growth studies and could be attributed to differences in cell type used and ranges of stiffnesses that were tested.

To gain a better understanding of the effect of substrate stiffness on neural stem cell differentiation, additional studies are needed. One study that has been performed with neural stem cells is Saha, *et al.* 2008, in which researchers were able to vary the stiffness of interpenetrating polymer networks and characterize growth of adult rat neural stem cells. Results indicated that the softest substrates promoted neuronal growth using differentiation media that promotes a mixed culture of glial cells and neurons. It was found that under media conditions that promote neuronal differentiation, a peak for this

differentiation occurred on substrates with a stiffness of around 500 Pa. Under conditions promoting astrocytic differentiation, cells did not survive as well on softer substrates. This led to the conclusion that chemical and mechanical factors can be combined to obtain populations with desired compositions of glial and neuronal cells [35]. Studies in this area suggest that neural stem cells, like primary neuronal cultures, develop longer neurite extensions on softer substrates. Therefore, differentiation of neural stem cells into functional neurons for therapeutic use may be best achieved on substrates of a specific stiffness, particularly on the scale of hundreds of Pascals. I hope to confirm and quantify this notion in these studies.

Synapses are critical connections between neurons that allows for communication between cells. Synapses are composed of a pre-synaptic specialization on the cell that is sending the signal, a synaptic cleft between the two neurons, and a post-synaptic specialization on the cell that receives the signal. When a signal is transmitted across a synapse, synaptic vesicles fuse with the membrane in the active zone of the pre-synaptic cell. The vesicles release neurotransmitters into the synaptic cleft, containing a matrix of cell adhesion molecules and extracellular matrix proteins. Neurotransmitters diffuse to receptors on the post-synaptic cell, triggering the appropriate post-synaptic response. Both pre-synaptic and post-synaptic specializations consist of a dense network of proteins that ensure proper signal transmission. Current knowledge of synaptogenesis is limited, but factors such as co-culture with glial cells, neurotrophins, and cell-adhesion molecules have been shown to enhance synapse formation [36, 37]. Synaptogenesis occurs during development, but also during adulthood, playing a role in learning and memory. During

development, the process or synapse formation is coupled with neuronal differentiation. Initial and often transient synapse formation occurs shortly after the cells differentiate into neurons and begin to extend neurites [38]. The extracellular matrix has been implicated in directing the axonal growth cone [29], and previous studies have found that substrate stiffness affects neurite growth and branching of primary neurons [21, 32, 33]; therefore, it is logical to assume that this may also have an impact on synapse formation. In these studies, I examine the role of substrate stiffness on synapse formation, a subject that has scarcely been explored to this point.

Multicellular organisms are made up of a diverse range of cell types that have all come from the fertilized egg. For normal development, there must be a proper balance of symmetric and asymmetric division events [39-42]. Symmetric cell division results in two daughter cells with the same developmental fate, and serves primarily to expand the pool of progenitor cells. Asymmetric division events, however, result in two daughter cells with different developmental fates, and therefore give rise to much of the cellular diversity in multicellular organisms [43]. Balance of these processes is also important in fully developed organisms with respect to adult stem cell populations [44]. Whether these cells undergo proliferation or differentiation processes is crucial to the proper function and maintenance of adult tissues. The occurrence of one of these two division types over the other depends on the position of the mitotic spindle and is thought to be regulated by both extrinsic and intrinsic factors. Although exact mechanisms of regulation are unknown, intrinsic factors implicated in this process include segregation of certain proteins and transcription factors and proteins such as numb and prospero in

Drosophila [45, 46]. Extrinsic factors in the stem cell niche, such as the extracellular environment have also proven to guide the orientation of the cell division axis [47]. Specifically, properties of the extracellular environment are sensed by the cytoskeleton in a feedback manner and translated into signals within the cell, contributing to spindle position and ultimately mode of division. This phenomenon remains relatively unexplored and is an important factor that must be taken into consideration when designing scaffolds to control stem cell differentiation.

To investigate the effects of substrate stiffness on neural stem cell differentiation, I will be utilizing a version of a widely used protocol first developed by Pelham and Wang [20]. This method utilizes thin polyacrylamide gels, which are coated with collagen to allow for cell adhesion, to control mechanical properties. In using polyacrylamide, investigators are able to alter the physical properties of the substrates by varying the ratios of acrylamide to bis-acrylamide while keeping the chemical properties constant. Differing ratios of acrylamide to bis-acrylamide results in more or less cross-linking, ultimately allowing for controlled variation in the elastic modulus of the material. This material has also proven useful for cell growth since polyacrylamide is porous.

This project will utilize C17.2 neural stem cells, a gift of Evan Snyder at the Burnham Institute. This cell line was generated via retro-virus-mediated v-myc transfer into murine cerebellar progenitor cells [48]. These cells are a relevant model because of their therapeutic potential in the treatment of neurodegenerative diseases. Snyder *et al.* found that C17.2 cells that were transplanted into the adult mouse neocortex can differentiate into neurons within regions of targeted apoptotic neuronal degeneration [49].

Chapter 2

Effect of Substrate Stiffness on Neurite Length and Synapse Formation

Introduction

Neurodegenerative diseases are conditions that target specific groups of neurons for degradation. The brain has a limited capacity for self-repair, and therefore one possible treatment for these disorders involves the implantation of neural stem cells to integrate into existing circuitry and replace damaged cell populations [2,3]. For these treatments to reach the clinic, it is crucial to understand the factors affecting neural stem cell differentiation and to use this knowledge to direct cells into therapeutically useful fates [1]. Factors contributing to cell fate consist of both chemical and mechanical cues from the cell microenvironment [6], and recent studies show that mechanical properties of the extracellular matrix are involved in the regulation of many cellular processes, including cell proliferation, migration, and differentiation [7-9, 15-18].

Response to the elasticity of the extracellular environment is cell-type specific and correlates to the elasticity of the cell's native tissue [7-9, 19]. Increased spreading of fibroblasts occurs on stiffer surfaces [20], whereas primary neuronal cells form more neurite branches on softer substrates [21]. Research has shown that mesenchymal stem cells grown on gels with comparable stiffness to brain tissue exhibit cellular morphology similar to neurons, while cells grown on substrates with stiffnesses similar to muscle and bone tissue exhibit morphologies similar to that of myoblasts and osteoblasts, respectively. PCR analysis confirms morphological observations, showing increased

mRNA expression of transcripts associated with cell-type correlating to matrix elasticity [8].

Most studies regarding neural cells and substrate stiffness focus on neurite extensions of primary neuronal cells, indicating that rate of extension, neurite length, and neurite branching of these cells decrease with increased matrix stiffness [21, 32-34]. Neural stem cells are a more desirable source for implantable neurons for disease treatment; however, the effect of matrix elasticity on the differentiation of neural stem cells is relatively unexplored. Studies in this area suggest that neural stem cells, like primary neuronal cultures, are sensitive to matrix elasticity, with softer substrates favoring neuronal differentiation [35]. Therefore, differentiation of neural stem cells into functional neurons for therapeutic use may be best achieved on substrates of a specific stiffness, particularly on substrates of comparable stiffness to brain tissue.

Construction of functional nervous tissue requires formation of neurons that guide axons along an appropriate route to a suitable synaptic partner [28]. Therefore, both neurite outgrowth and formation of synaptic connections are crucial for cells intended to integrate and replace damaged cell populations in the nervous system. The extracellular matrix has been implicated in directing the axonal growth cone [29], and previous studies have found that substrate stiffness affects neurite extension [21, 32-34]. For these reasons, I explore the role of substrate stiffness on two aspects of neural stem cell differentiation, neurite growth and synaptic protein expression. To my knowledge, these specific aspects of differentiation have not been explored and quantified using neural stem cells in response to substrate elasticity

I utilize thin polyacrylamide substrates with different ratios of acrylamide to bis-acrylamide, resulting in controlled variation in the elastic modulus of the material while keeping consistent chemical properties [20]. This study uses C17.2 neural stem cells, a gift of Evan Snyder at the Burnham Institute, a neural stem cell line generated via retro-virus-mediated v-myc transfer into murine cerebellar progenitor cells [48]. These cells are capable of differentiation into neurons within regions of targeted apoptotic degradation in the adult mouse neocortex [49]. My aim is to find the optimal material elasticity to guide neural stem cells to neuronal fates most beneficial for treatment of neurodegenerative disorders, so these cells provide a useful model. Ultimately, the results of this study will bring us one step closer to neural stem cell scaffolds with precise control over cell fate and successful integration into existing cell circuitry via cues from mechanical properties of the material.

Materials and Methods

Polyacrylamide Substrate Fabrication: Polyacrylamide gels were fabricated on 22mmx22mm cover glass (VWR) as described previously in Pelham and Wang 2007, [20, 50, 51] with slight modification. Briefly, coverslips were flamed, coated with 0.1N-NaOH and air dried. A small amount of 3-aminopropyltrimethoxysilane (Acros Organics) was spread evenly across the cover glass and allowed to dry for 5-10 minutes. Coverslips were washed thoroughly with ddH_2O and then incubated in 70% glutaraldehyde (Alfa Aesar) in PBS for 30 minutes. The coverslips were washed again with ddH_2O and allowed to air dry. Next, polyacrylamide gels of various stiffnesses were fabricated on the surface of the activated coverslips. Differences in stiffness were

achieved by varying the amounts of acrylamide and bis-acrylamide in the gel solution according to the Table III in Johnson *et al.* 2007. Twenty microliters of a degassed gel solution, containing acrylamide concentrations ranging from 3% to 10% and bis-acrylamide concentrations ranging from 0.04% to 0.5%, were placed on the coverslips. Rain-X coated 18 mm circular cover glass were then placed on top of the gel solution, and the polyacrylamide was allowed to polymerize for 25-60 minutes. After polymerization, the circular cover glass was removed and the gels were then treated to allow for cell adhesion. Treatment consisted of applying Sulfo-SANPAH (Thermo Scientific) cross-linker solutions and incubating under ultraviolet light for 15 minutes. After several rinses with 50 mM HEPES pH 8, a 0.2 mg/ml collagen I solution was placed on the gels. Substrates were incubated overnight to sterilize before cell seeding.

Cell Culture: C17.2 neural stem cells, a gift of Evan Snyder from the Burnham Institute were cultured according to accepted protocol. NSCs were cultured in DMEM high glucose with 10% Fetal Bovine Serum, 5% Horse Serum, and 1% L-Glutamine. Cells were split at less than 1:10 at least once a week. All experiments were performed with cells at passage number 20 or below. Cells were fed 3 times per week by removing half of the old culture media and replacing with an equal amount of fresh media. For the serum withdrawal procedure, cells were fed with serum-free culture media, DMEM high glucose with 1% L-Glutamine. Cells were seeded onto the polyacrylamide gel substrates at a density of 10,000 cells/cm^2 and allowed to grow to about 80% confluency, at which point the serum withdrawal process began. Cells were fixed 14 days after the start of serum withdrawal. A number of synapse samples were cultured for an additional period

after this point, during which they were subjected to stimulation in high potassium Locke's buffer (95mM NaCl, 50mM KCl, 2.3mM $CaCl_2$, 1mM $MgCl_2$, 3.6mM $NaHCO_3$, 5mM HEPES, 20mM Glucose), either for 15 minutes or 5 minutes, or placed in low potassium Locke's buffer (154mM NaCl, 5.6mM KCl, 2.3mM $CaCl_2$, 1mM $MgCl_2$, 3.6mM $NaHCO_3$, 5mM HEPES, 20mM Glucose) for 15 minutes. Samples were subjected to this stimulation procedure every 12 hours for a total of 5 days.

Immunocytochemistry: For the neurite growth studies, cells were fixed with 10% formalin in PBS for 10 minutes. They were then rinsed with PBS and treated with methanol for 7 minutes. After three more PBS rinses, cells were permeablilized in 0.1% Triton X-100 in PBS for 15 minutes. Three additional rinses were performed, and cells were then blocked with 1% BSA for 15 minutes. The primary antibody solution contained 1:1000 β-tubulin III-AF488 (Covance) and rhodamine phalloidin (Cytoskeleton, Inc.) at a concentration of 7 µl per ml in 0.1% BSA in PBS. Samples were incubated in the primary antibody solution at 37°C for 2 hours, and overnight at room temperature. Samples were then rinsed with PBS and counterstained with Hoechst dye (Invitrogen) at 0.002 mg/ml in ddH_2O for 5 minutes. Additional rinses were performed and samples were left in PBS for imaging.

For synapse studies, cells were fixed either as stated previously, with 10% formalin (synaptotagmin/synaptophysin), or with 4% paraformaldehyde (synaptophsyin/homer/psd-95) in PBS for 15 minutes. Samples were then rinsed with PBS permeablilized in 0.1% Triton X-100 in PBS for 15 minutes. Three additional rinses were performed, and cells were then blocked with 1% BSA with 0.01% Triton X-100 in

PBS for 15 minutes. The primary antibody solutions contained 1:500 synaptophysin (Millipore), 1:500 synaptotagmin (mAB 30, DSHB), 1:500 homer (Synaptic Systems), and 1:500 PSD-95 (Santa Cruz Biotechnology) in 0.1% BSA solution, with 0.001% Triton X-100, in PBS. The synaptotagmin antibody developed by Louis Reichardt was obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by The University of Iowa, Department of Biology, Iowa City, IA 52242. Samples were incubated in the primary antibody solution at 37°C for 2 hours, and overnight at room temperature. Secondary antibodies were all obtained from Invitrogen and were as follows: Alexa Fluor 546 goat anti-mouse IgG1, Alexa Fluor 488 goat anti-mouse IgG2a, Alexa-Fluor 488 goat anti-rabbit IgG, and Alexa Fluor 488 goat anti-mouse IgG2a, respectively. Samples were incubated in the secondary antibody solutions at 37°C for 1 hour, rinsed with PBS, and counterstained with Hoechst dye (Invitrogen) at 0.002 mg/ml in ddH_2O for 5 minutes. Additional rinses were performed and samples were left in PBS for imaging. Images were taken with a Zeiss Observer Z1 inverted fluorescence microscope.

Data Analysis: Neurite growth quantification was performed using the NeuronJ plug-in for ImageJ [52, 53]. Tracings were performed on each visible neurite from the nucleus until the edge of the extension. All images included at least 10 neurite tracings.

Statistical Analysis: Statistical analysis of the data was performed using a student's t-test.

Results and Discussion

After 14 days of serum withdrawal, cells were fixed and immunocytochemically analyzed for proteins of interest. Substrate samples of each stiffness (140 Pa, 1050 Pa, and 60,000 Pa), and control samples (tissue-culture treated glass) were stained for actin, nuclei, and β-tubulin III, a neuron specific tubulin, to assess cell fate. Cells expressing β-tubulin III were present on all samples, indicating differentiation of the C17.2 neural stem cells into post-mitotic neurons, as seen in **Figure 2.1**. Throughout serum withdrawal, cells would often sheet off of the control samples, which could indicate differences in proliferation and differentiation of the stem cells on these chemically and mechanically different substrates.

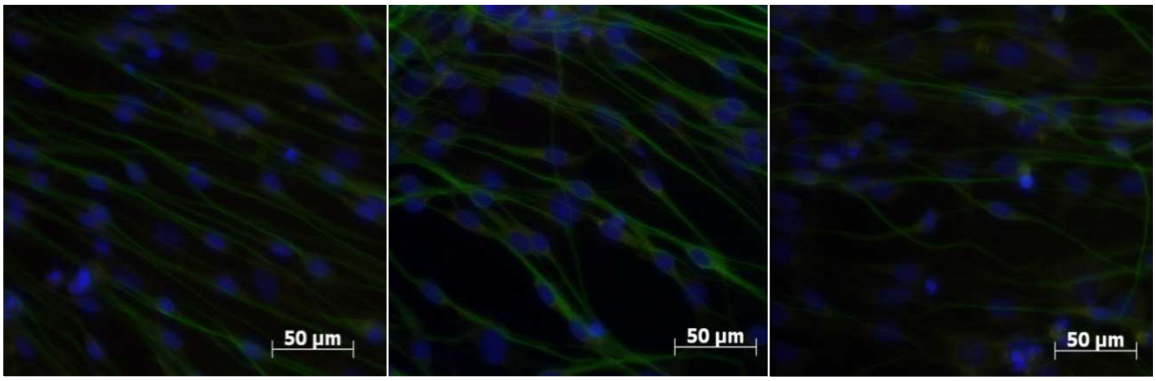

Figure 2.1. Neurite pictures β- tubulin III (green), actin (red), nuclei (blue). (a.)140 Pa, (b.) 1050 Pa, (c.) 60000 Pa

Neurite lengths of neuronal populations on each of the tested substrates were quantified using NeuronJ plug-in for ImageJ [52, 53]. I found that the neurite length decreases with increasing substrate stiffness, shown in **Figure 2.2**. Specifically, the softest substrates (140 Pa) facilitated formation of the longest neurite extensions, possibly indicating a more mature population of neurons. For all tested substrates, differences in

neurite length were statistically significant, as determine using a student's t-test. This data is consistent with previous studies performed with primary neuronal cultures [21, 32-34], indicating that soft substrates with mechanical properties similar to the brain support neuronal differentiation, and promote neurite growth.

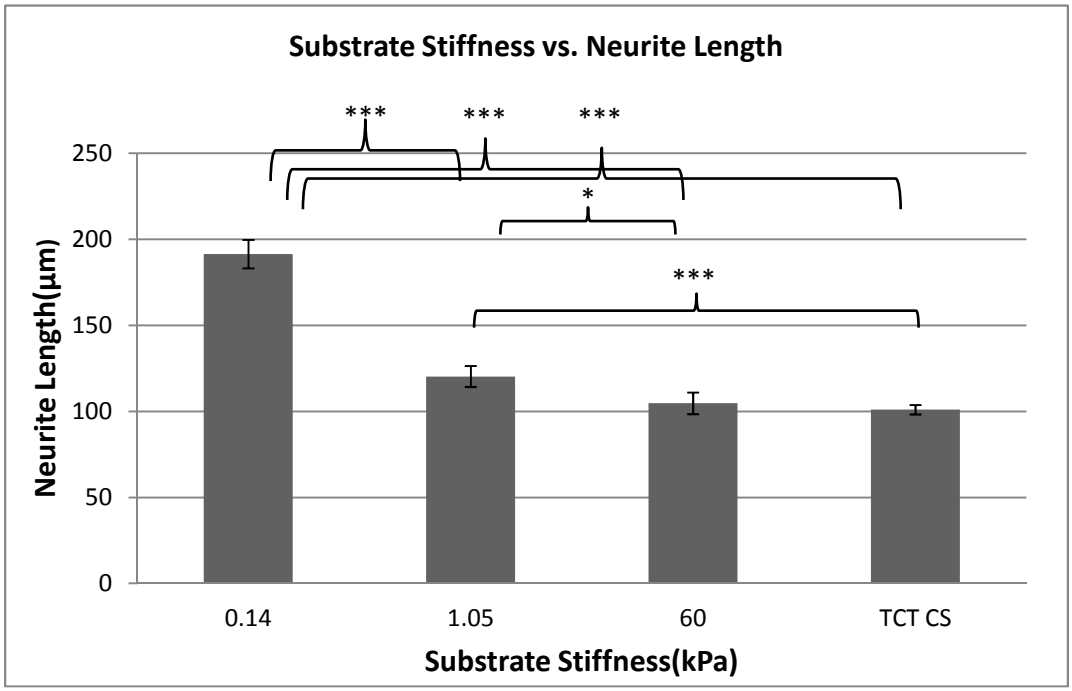

Figure 2.2. Graph of Substrate Stiffness vs. Neurite Length. Data is a combination of results from 3 separate trials of the experiment. Statistical significance is represented by asterisks with * = $p < 0.1$, ** = $p < 0.05$, and *** = $p < 0.01$.

I also tested for expression of synaptic proteins and synapse formation after completing the serum withdrawal procedure. This testing was only performed on the softest substrates, as neurite length data indicates this as the best substrate for neuronal differentiation. These samples were subjected to additional treatments in High Potassium Locke's buffer for either 15 minutes or 5 minutes or in Low Potassium Locke's buffer for 15 minutes. This procedure was repeated every 12 hours for a period of 5 days after the

completion of serum withdrawal. Incubation in the high potassium buffer creates high extracellular potassium levels, depolarizing the neurons in the sample and causing them to fire. Incubation in the low potassium buffer serves as a control to allow for comparison of stimulated and unstimulated samples.

Synapses consist of pre-synaptic specializations on the cell that is sending the signal, a synaptic cleft between the two neurons, and a post-synaptic specialization on the cell that receives the signal. I stained for several pre-synaptic and post-synapatic proteins to visualize synapses, an area of colocalization between the two. Synaptotagmin (pre) and PSD-95 (post) were not expressed in C17.2 neurons, but synaptophysin (pre) and homer (post) were expressed in my samples. **Figure 2.3** shows expression and colocalization of these synaptic proteins on samples that underwent the various stimulation treatments. This indicates formation of synapses with all three treatments, with the 15 minute high potassium stimulation yielding more colocalization, and therefore a greater abundance of synapses. Stimulation may have induced neuronal firing, yielding a more mature population of neurons and facilitating the formation of stable synapses.

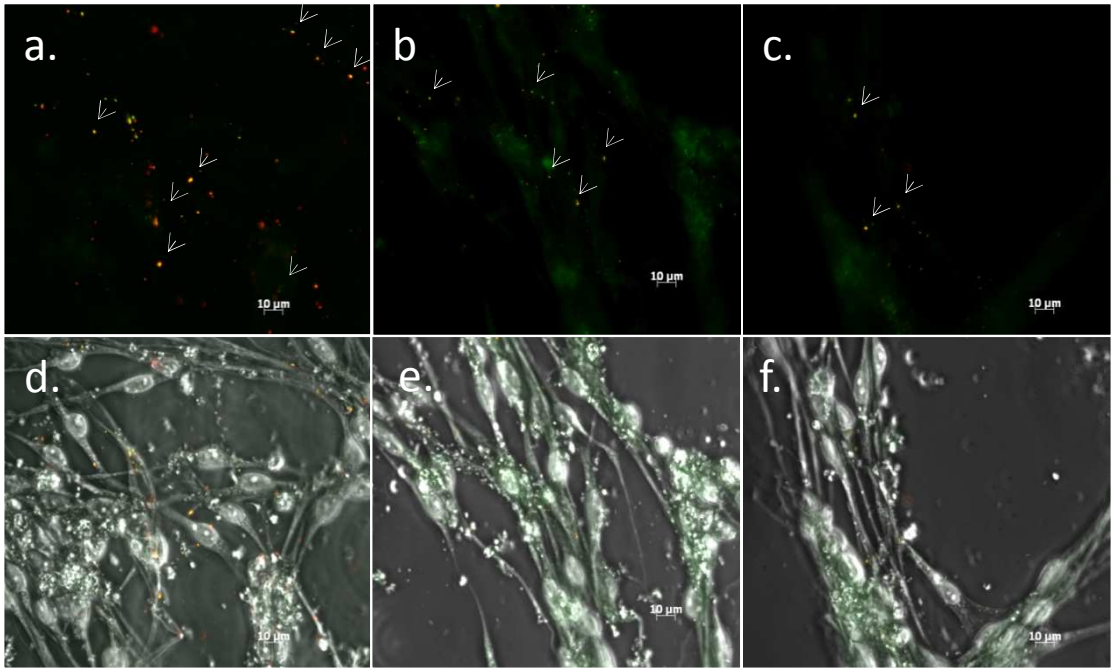

Figure 2.3. 140 Pa gel samples stained for synaptic proteins, synaptophysin (red) and homer (green). Arrows indicate synapses (yellow). Stimulation Treatments: (a.)/(d.) 15 min. high K+ Locke's buffer, (b.)/(e.) 5 min. high K+ Locke's buffer, (c.)/(f.) 15 min. low K+ Locke's buffer.

Conclusion

In this study I have shown that soft gel substrates, with stiffness comparable to that of brain tissue [21], provide an environment conducive for neural stem cell differentiation into neurons. These substrates facilitate differentiation of NSCs into neuronal populations with the longest neurite extensions and support the formation of synapses. To my knowledge, this is the first report of *in vitro* synapse formation of differentiated C17.2 NSCs, illustrating a greater potential for use of similar NSC models for neural stem cell therapy.

These results further current knowledge of the role of mechanical properties of the extracellular environment on neural stem cell differentiation. Future work will include

analysis of the neuronal subtypes that form on the various stiffnesses. This will allow for greater control of NSC fate, and will be useful for treatments of diseases such as Parkinson's, which is hallmarked by degradation of dopaminergic neurons and would require replacement of this very specific neuronal population [5]. Results from this study will help with design of biomaterials scaffold for directing fate of implanted cell populations, inching one step closer to the use of stem cells for treatment of neurodegenerative diseases.

Chapter 3

Effect of Substrate Stiffness on Mode of Division throughout Differentiation

Introduction

One potential therapy for the treatment of neurodegenerative diseases involves the implantation of neural stem cells to replace damaged cell populations in the brain. For this technique to progress into clinical use, the ability to control the fate and compositions of the final implantable cell population is crucial [1-3]. During differentiation of neural stem cells, two modes of division have been documented, asymmetric and symmetric. The balance of these processes affects the composition of the cell population, and therefore, I am interested in assessing this aspect of differentiation [39-41]. Both mechanical and chemical cues contribute to produce the final fate of a cell, and I will explore the role of mechanical properties of the microenvironment in mode of cell division throughout differentiation [6].

Multicellular organisms are made up of a diverse range of cell types that have all come from the fertilized egg. For normal development, there must be a proper balance of symmetric and asymmetric division events [42]. Symmetric cell division results in two daughter cells with the same developmental fate, and serves primarily to expand the pool of progenitor cells. Asymmetric division events, however, result in two daughter cells with different developmental fates, and therefore give rise to much of the cellular diversity in multicellular organisms [43].

Balance of these processes is also important in fully developed organisms with respect to adult stem cell populations [44]. Whether these cells undergo proliferation or differentiation processes is crucial to the proper function and maintenance of adult tissues. The occurrence of one of these two division types over the other depends on the position of the mitotic spindle and is thought to be regulated by both extrinsic signals from the stem cell niche and intrinsic factors inside the cells, such as segregation of transcription factors [45, 46]. The physical properties of the cell niche play a role in guiding the orientation of the cell division axis [47]. Specifically, the mechanical properties of the extracellular environment may contribute to spindle position and ultimately mode of division. Further investigation into this phenomenon will aide in future design of scaffolds for controlling stem cell differentiation.

To investigate the effects of substrate stiffness on frequency of symmetric division during neural stem cell differentiation, I will be utilizing a version of a widely used protocol first developed by Pelham and Wang [20]. This method utilizes thin polyacrylamide gels that are coated with collagen to allow for cell adhesion. Polyacrylamide allows for tailoring of the physical properties of the substrates by varying the ratios of acrylamide to bis-acrylamide, while keeping the chemical properties constant. Differing ratios of acrylamide to bis-acrylamide results in more or less cross-linking, yielding controlled variation in the elastic modulus of the material.

I will use C17.2 neural stem cells, a gift of Evan Snyder at the Burnham Institute. This cell line was generated via retro-virus-mediated v-myc transfer into murine cerebellar progenitor cells [48]. These cells are a relevant model because of their

therapeutic potential in the treatment of neurodegenerative diseases. Snyder *et al*. found that C17.2 cells that were transplanted into the adult mouse neocortex can differentiate into neurons within regions of targeted apoptotic neuronal degeneration [49].

Materials and Methods

Cell Culture: C17.2 neural stem cells, a gift of Evan Snyder from the Burnham Institute, were cultured according to accepted protocol. NSCs were cultured in DMEM high glucose with 10% Fetal Bovine Serum, 5% Horse Serum, and 1% L-Glutamine. Cells were split at less than 1:10 at least once a week. All experiments were performed with cells at passage number 20 or below. Cells were fed 3 times per week by removing half of the old culture media and replacing with an equal amount of fresh media. For the serum withdrawal procedure, cells were fed with serum-free culture media, DMEM high glucose with 1% L-Glutamine.

Biostation: Time-lapse imaging was performed with the Nikon Biostation IM over extended periods of time, with pictures captured every 5 minutes. Mattek dishes (35 mm) were used for cell observation on the biostation, with cells seeded at a density of 3500 cells/cm^2.

Data Analysis: Daughter cell size quantification was performed using ImageJ software [53]. The size of each daughter cell was measured after each captured division. The ratio of the area of the smaller daughter to the larger daughter was calculated, and asymmetric divisions were defined as those in which this ratio was less than 0.7.

Transfections: Addgene plasmid 26740 [54], Addgene plasmid 26739 [54], Addgene plasmid 28310 [55], Addgene plasmid 13777 [56], mCherry-UtrCH, mRFP-UtrCH, CALNL-DCX-eGFP, pCAG-ERT2CreERT2, were obtained from Addgene. Plasmids were isolated using the alkaline lysis method. Cationic lipid transfections were performed using the Lipofectamine LTX and PLUS Reagents (Invitrogen) according to the suggested protocol. Triple transfections were performed with 2500 ng each of the pCAG-ERT2CreERT2 and CALNL-DCX-eGFP plasmids, and 5000 ng of either mCherry-UtrCH or mRFP-UtrCH and 10 μl of Lipofectamine LTX and 10 μl of plus reagent. Transfections were performed on cells that had been seeded the previous day with 20,000 cells in each 35 mm Fluorodish. For the pCAG-ERT2CreERT2 and CALNL-DCX-eGFP double transfection, doublecortin expression was induced with 4-OHT (Sigma/Enzo). Electroporation was also performed and optimized to transfect cells with these plasmids.

Results and Discussion

Daughter cell size measurements were performed on time-lapse images captured at full serum and at various serum concentrations throughout serum withdrawal on glass, collagen-coated glass, and 140 Pa gel substrates. For each data point presented, measurements images were captured at 4 different areas in a single dish. **Figure 3.1** shows data from a 48 hour biostation run on each tested substrate, with images captured at 40x. Very few divisions were captured, about 12 total for each substrate, resulting in large standard errors. For these reasons, additional data is needed to draw conclusions

about frequency of asymmetric division events on the various substrates at full serum.

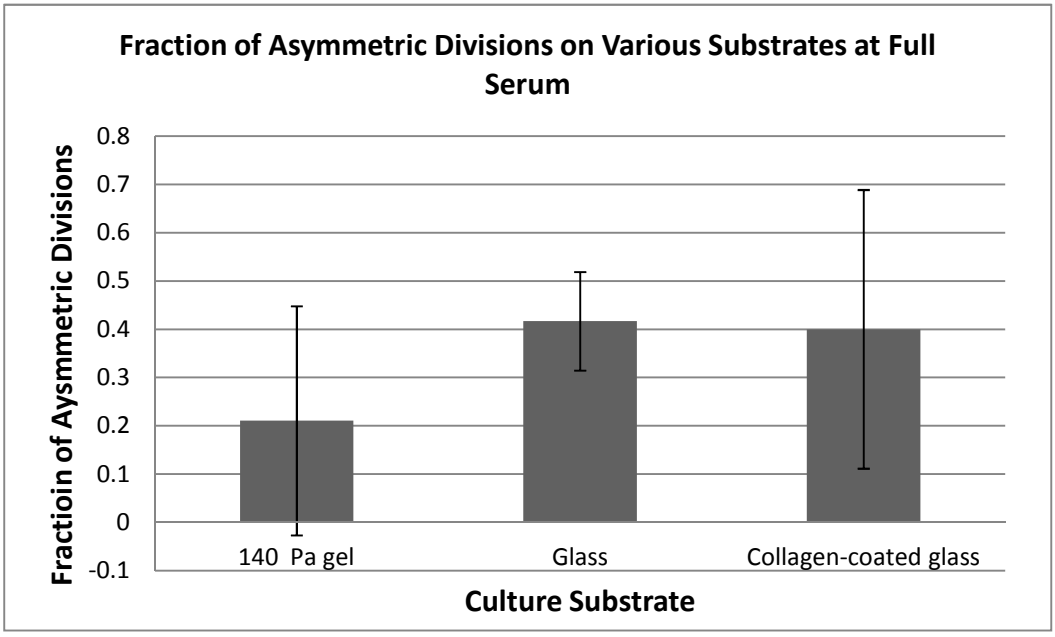

Figure 3.1. Fraction of asymmetric division events at full serum. Asymmetric events are divisions in which the area ratio of the smaller daughter to the larger daughter is less than 0.7.

Data from measurements performed throughout the serum withdrawal procedure, at 7.5% serum, 3.75% serum, 1.875% serum, and 0.94% serum, can be seen in **Figure 3.2**. All serum points consist of a 48 hour biostation run, with images captured with the 20x objective every 5 minutes, except for the 3.75% serum point. This data is from a 72 hour biostation run with images captured every 5 minutes, due to the observed protocol for serum withdrawal. Large standard errors are observed for the 7.5% serum data point. Cells were initially seeded at a low density, so large errors can be attributed to a small amount of observed divisions due to a low cell number. Slightly larger errors for the last serum point can also be attributed to low division numbers, since less cells are dividing at this point in serum withdrawal. Data indicates that NSCs on the softest substrate, 140 Pa

gel, have a higher frequency of divisions earlier on in the serum withdrawal procedure, while cells on the glass and collagen-coated glass substrates seem have lower frequencies of asymmetric divisions earlier in serum withdrawal. This may indicate a delayed start of differentiation on the stiffer glass substrates. Earlier entry into differentiation on the softer gel substrate is consistent with observations from my previous studies. Previously, I have seen cells sheeting off of tissue-culture-treated glass substrates during serum withdrawal, which was not seen on the softer polyacrylamide gel samples. If cells experience delayed differentiation on the stiffer substrates, they would continue to proliferate for a longer period of time, yielding samples with much more cells than the gel counterparts.

Measurements from every observed division were not necessary included in the data for **Figure 3.2**. For some divisions, one daughter cell would spread out on the surface well before the other, or one or both would never spread out at all. Daughter cell size measurements for these types of divisions were not included; however, these instances were recorded and the frequency of such events on each substrate can be seen in **Figure 3.3**. Cell death events were also observed and recorded along with the total number of divisions for each serum concentration on all tested substrates. This data is shown in **Figure 3.4** and **Figure 3.5**, respectively. Each bar on the charts represents an average of data from 4 different points on a single dish, allowing for a more accurate representation of what is occuring throughout the dish. All substrates seem to follow the same pattern for frequency of cell death, peaking at 1.875% serum. It is logical that there would be an increase in cell death with reduction of serum. As differentiation proceeds,

cells that cannot survive in the low serum conditions will die. Lower frequency of death on the gel could indicate more direct and complete differentiationof the NSCs into neurons. For the number of measured divisions, all substrates once again follow a similar trend. Division peaks at 3.75% serum, and then drops off with the progression of serum withdrawal, and the presumed differentiation of the cells into their final, postmitotic state. The data indicates that by the final observed point of serum withdrawal, there are very few divisions on the gel substrate. While the number of divisions on the other two substrates do decrease at the last two serum concentrations, there are higher numbers of divisions than on the gels, possibly meaning that there is still a significant amount of cells that have not differentiated and remain NSCs, as opposed to the mostly differentiated, posmitotic neurons on the gels.

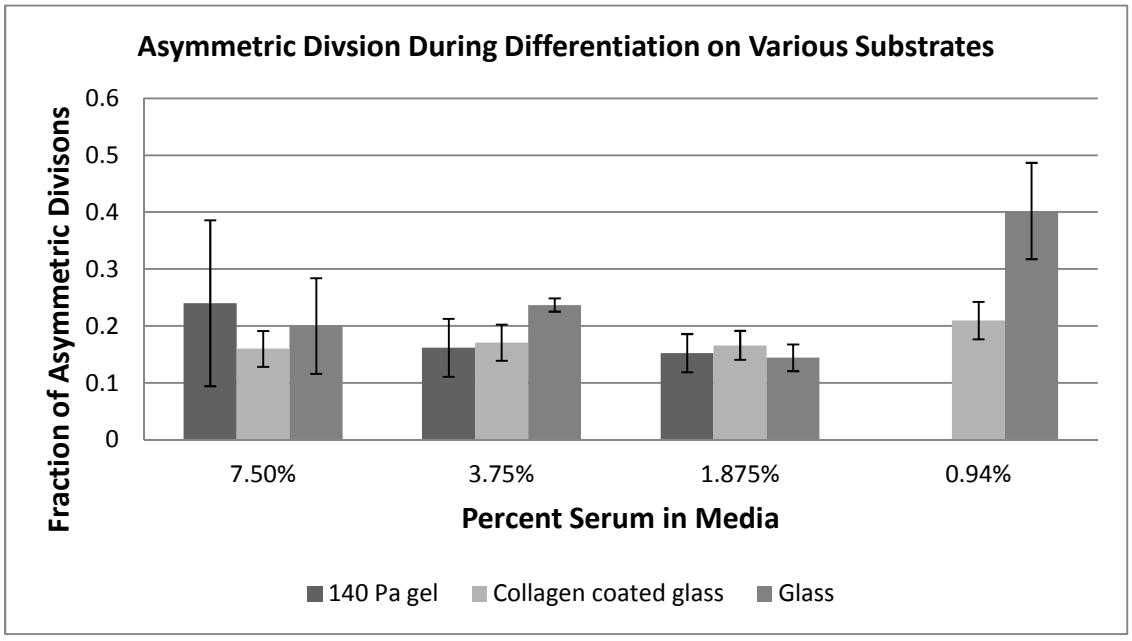

Figure 3.2. Fraction of asymmetric division events throughout serum withdrawal. Asymmetric events are divisions in which the area ratio of the smaller daughter to the larger daughter is less than 0.7.

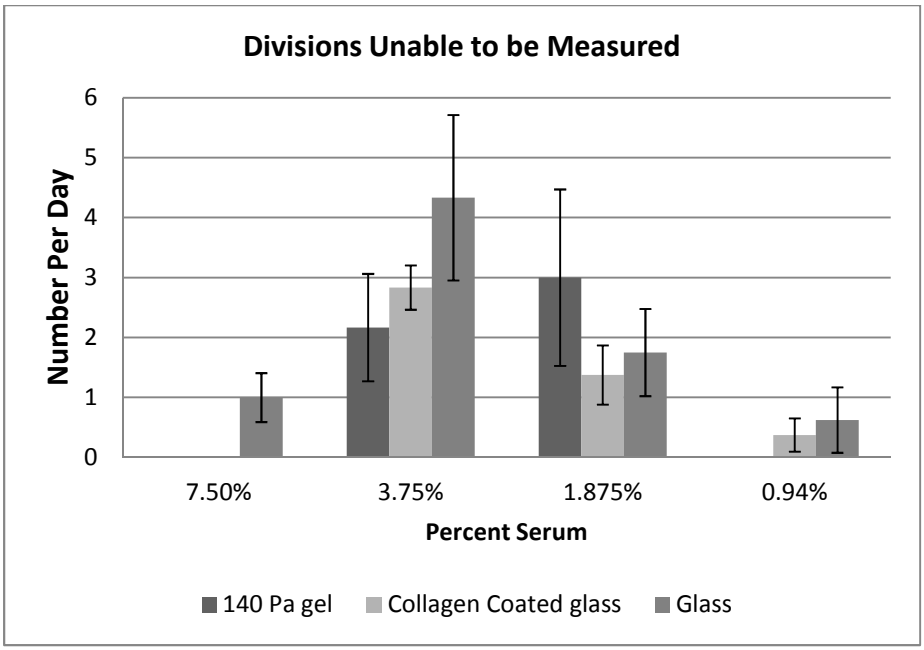

Figure 3.3. Number of division events per day in which daughter cell sizes could not be measured throughout serum withdrawal on various substrates. This was usually due to daughter cells not spreading on the material.

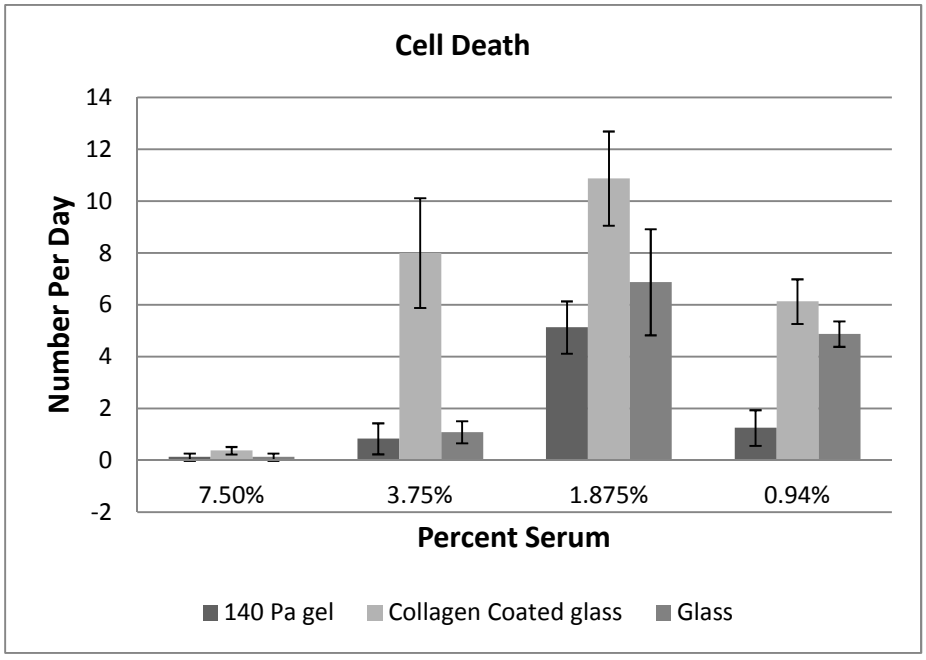

Figure 3.4. Number of cell deaths per day observed throughout serum withdrawal on various substrates.

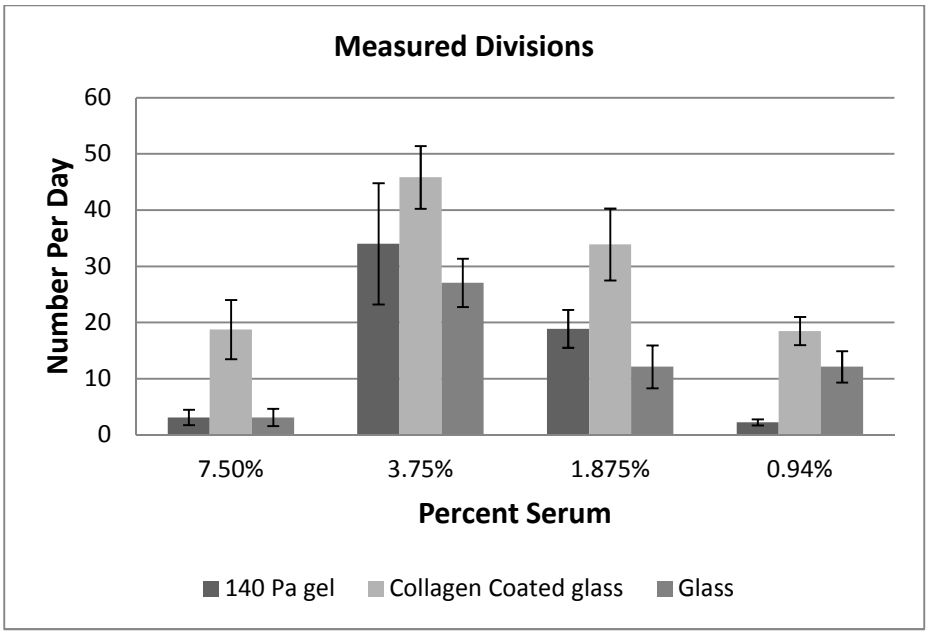

Figure 3.5. Number of division events per day that were measured throughout serum withdrawal on various substrates.

I successfully transfected C17.2 neural stem cells with the plasmids listed previously. Images of the transfected cells can be seen in **Figure 3.6**. Fluorescently labeled utrophin, an actin binding protein, is shown in **Figure 3.6a**, and fluorescently labeled doublecortin, a microtubule binding protein is shown in **Figure 3.6b**. I was successful in both transfection methods utilized, cationic lipid and electroporation, with slightly higher efficiency obtained via electroporation. These cells will be used in additional studies to visualize suspected perturbation in the dynamics of the cytoskeleton, yielding differing frequencies of asymmetric division as a result of substrate properties.

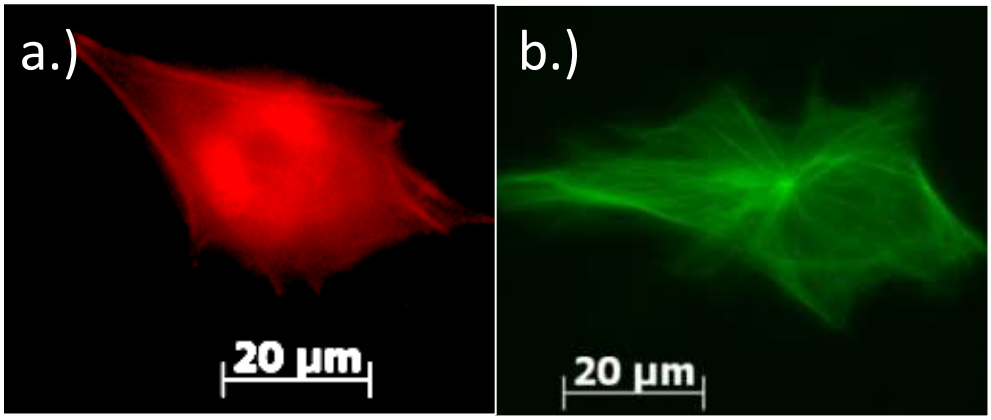

Figure 3.6. C17.2 neural stem cells expressing fluorescent cytoskeletal proteins: a.) mRFP-utrophin, b.) CALNL-DCX-eGFP/ pCAG-ERT2CreERT2

Conclusion

In this study, I show that substrate properties have an effect on the frequency of asymmetric and symmetric division events during the differentiation process of C17.2 neural stem cells. Asymmetric, or differentiative, division events have been reported as a normal occurrence throughout differentiation and yield much of the cellular diversity in the adult brain [39-41]. To obtain a desired cell population for therapeutic implantation, it is important to control the frequency of each type of division. Therefore, it is vital to address the effect that scaffolding material properties will have on mode of division. While the extracellular matrix has been noted to play some role in orientation of the division plane [47], to my knowledge, this is the first study addressing the impact of substrate material properties on division type, *in vitro*. This is an aspect of neural stem cell differentiation that will need to be addressed in the design of biomaterial scaffolds for use in treating neurodegenerative diseases with implanted stem cell populations.

Future work will more closely examine the mechanism of asymmetric division by visualizing the cytoskeleton of these cells throughout differentiation.

Acknowledgements

I would like to acknowledge William Bement, Joseph Loturco, and Connie Cepko for the Addgene plasmids utilized in this study.

Chapter 4

Conclusion

In this work, I have shown that substrate stiffness affects several aspects of neural stem cell differentiation. Specifically, the softest gel substrates, with stiffness comparable to brain tissue, support differentiation of C17.2 neural stem cells into neurons with the longest neurite extensions. This stiffness also supports formation of synapses, the first report of synapses using this cell type. Lastly, substrate properties impact the frequency of asymmetric division events during differentiation. The frequencies of asymmetric and symmetric division events, as classified by daughter cell size after division, vary on soft gel substrates, glass, and collagen coated glass. This affects the composition of the resulting cell population after differentiation. In studying these aspects of neural stem cell differentiation, I now have a greater understanding of how substrate stiffness impacts differentiation into neurons, yielding insight into the design of biomaterials scaffolds for use in treatment of neurodegenerative diseases.

In the future, I would like to further characterize the differentiated cell populations that form on each of the tested stiffnesses. I have verified formation of neurons on each of the stiffnesses, but I do not know the types of neurons that are present. Exploring the subtypes of neurons that are forming on specific stiffness would introduce a greater degree of control over final cell fate. This is important for therapeutic use in treatment of diseases such as Parkinson's Disease, in which dopaminergic neurons are targeted for degradation [5].

Future work would also include additional research into the role of matrix elasticity in mode of division, asymmetric or symmetric. Greater knowledge in this area can be obtained by testing a wider range of gel stiffnesses. Also, I have engineered C17.2 neural stem cells to express fluorescently labeled utrophin, an actin-binding protein, and doublecortin, a microtubule-binding protein, to allow for visualization of the cytoskeleton. I will perform time-lapse imaging of these cells on various substrates to observe the actin and microtubule cytoskeleton throughout the division process, capturing both symmetric and asymmetric events. I hypothesize that mode of division is regulated through the cytoskeleton, which can sense elasticity of the extracellular matrix. I hope to compare and study distribution of actin and microtubules in both modes of division.

List of References

1. Bjorklund, A., and O. Lindvall. "Cell Replacement Therapies for Central Nervous System Disorders." Nature neuroscience 3.6 (2000): 537-44.
2. Rossi, F., and E. Cattaneo. "Opinion - Neural Stem Cell Therapy for Neurological Diseases: Dreams and Reality." Nature Reviews Neuroscience 3.5 (2002): 401-9.
3. Wernig, M., Benninger, F., Schmandt, T., Rade, M., Tucker, K.L., Büssow, H., Beck, H., and Brüstle, O. "Functional Integration of Embryonic Stem Cell-Derived Neurons in Vivo." Journal of Neuroscience 24.22 (2004): 5258-68.
4. Zhang, S.C., Wernig, M., Duncan, I. D., Brüstle, O., and Thomson, J. A. "In Vitro Differentiation of Transplantable Neural Precursors from Human Embryonic Stem Cells." Nature biotechnology 19.12 (2001): 1129-33.
5. Kim, J.H., Auerbach, J. M., Rodríguez-Gómez, J. A., Velasco, I., Gavin, D., Lumelsky, N., Lee, S. H., Nguyen, J., Sánchez-Pernaute, R., Bankiewicz, K., and McKay, R. "Dopamine Neurons Derived from Embryonic Stem Cells Function in an Animal Model of Parkinson's Disease." Nature 418.6893 (2002): 50-6.
6. Discher, D. E., Mooney, D. J., and Zandstra, P. W. "Growth Factors, Matrices, and Forces Combine and Control Stem Cells." Science 324.5935 (2009): 1673-7.
7. Engler, A. J., Griffin, M. A., Sen, S., Bönnetnann, C. G., Sweeney, H. L. and Discher, D. E. "Myotubes Differentiate Optimally on Substrates with Tissue-Like Stiffness: Pathological Implications for Soft Or Stiff Microenvironments." Journal of Cell Biology 166.6 (2004): 877-87.
8. Engler, Adam J., Sen, S., Sweeney, H.L, and Discher, D.E. "Matrix Elasticity Directs Stem Cell Lineage Specification." Cell 126.4 (2006): 677-89.
9. Guilak F., Cohen D. M., Estes B.T., Gimble J. M., Liedtke W., Chen C. S. "Control of Stem Cell Fate by Physical Interactions with the Extracellular Matrix." Cell Stem Cell 5.1 (2009): 17-26.
10. Bennett, A. M., and N. K. Tonks. "Regulation of Distinct Stages of Skeletal Muscle Differentiation by Mitogen-Activated Protein Kinases." Science 278.5341 (1997): 1288-91.
11. Jaiswal, R. K., Jaiswal, N., Bruder, S. P., Mbalaviele, G., Marshak, D. R., and Pittenger, M. F. "Adult Human Mesenchymal Stem Cell Differentiation to the Osteogenic Or Adipogenic Lineage is Regulated by Mitogen-Activated Protein Kinase." Journal of Biological Chemistry 275.13 (2000): 9645-52.
12. Traverse, S., Gomez, N., Paterson, H., Marshall, C., and Cohen, P. "Sustained Activation of the Mitogen-Activated Protein (Map) Kinase Cascade may be Required for Differentiation of Pc12 Cells - Comparison of the Effects of Nerve Growth-Factor and Epidermal Growth-Factor." Biochemical Journal 288 (1992): 351-5.
13. McKay, R. "Stem Cells in the Central Nervous System." Science 276.5309 (1997): 66-71.

14. Buxboim, A., I. L. Ivanovska, and D. E. Discher. "Matrix Elasticity, Cytoskeletal Forces and Physics of the Nucleus: How Deeply do Cells 'Feel' Outside and in?" Journal of cell science 123.3 (2010): 297-308.
15. Hadjipanayi, E., V. Mudera, and R. A. Brown. "Guiding Cell Migration in 3D: A Collagen Matrix with Graded Directional Stiffness." Cell motility and the cytoskeleton 66.3 (2009): 121-8.
16. Lo, C. M., Wang, H. B., Dembo, M. and Wang, Y. L. "Cell Movement is Guided by the Rigidity of the Substrate." Biophysical journal 79.1 (2000): 144-52.
17. Hadjipanayi, E., V. Mudera, and R. A. Brown. "Close Dependence of Fibroblast Proliferation on Collagen Scaffold Matrix Stiffness." Journal of Tissue Engineering and Regenerative Medicine 3.2 (2009): 77-84.
18. Winer, J. P., Janmey, P. A., McCormick, M. E. and Funaki, M. "Bone Marrow-Derived Human Mesenchymal Stem Cells Become Quiescent on Soft Substrates but Remain Responsive to Chemical Or Mechanical Stimuli." Tissue Engineering Part a 15.1 (2009): 147-54.
19. Yeung, T., Georges, P. C., Flanagan, L. A., Marg, B., Ortiz, M., Funaki, M., Zahir, N., Ming, W., Weaver, W., Janmey, P. A. "Effects of Substrate Stiffness on Cell Morphology, Cytoskeletal Structure, and Adhesion." Cell motility and the cytoskeleton 60.1 (2005): 24-34.
20. Pelham, R. J., and Y.L. Wang. "Cell Locomotion and Focal Adhesions are Regulated by Substrate Flexibility." Proceedings of the National Academy of Sciences of the United States of America 94.25 (1997): 13661-5.
21. Flanagan, L. A., Ju, Y. E., Marg, B., Osterfield, M., Janmey, P. A. "Neurite Branching on Deformable Substrates." Neuroreport 13.18 (2002): 2411-5.
22. Sukharev, S., and D. P. Corey. "Mechanosensitive channels: multiplicity of families and gating paradigms." Sci. STKE 2004 (2004): re4.
23. Rizzo, V., Sung, A., Oh, P., Schnitzer, J. E. "Rapid Mechanotransduction in Situ at the Luminal Cell Surface of Vascular Endothelium and its Caveolae." Journal of Biological Chemistry 273.41 (1998): 26323-9.
24. Geiger, B., Bershadsky, A., Pankov, R., Yamada, K. M. "Transmembrane Extracellular Matrix-Cytoskeleton Crosstalk." Nature Reviews Molecular Cell Biology 2.11 (2001): 793-805.
25. Ralphs, J. R., A. D. Waggett, and M. Benjamin. "Actin Stress Fibres and Cell-Cell Adhesion Molecules in Tendons: Organisation in Vivo and Response to Mechanical Loading of Tendon Cells in Vitro." Matrix Biology 21.1 (2002): 67-74.
26. Geiger, B., and A. Bershadsky. "Assembly and Mechanosensory Function of Focal Contacts." Current opinion in cell biology 13.5 (2001): 584-92.
27. Ingber, D. E. "Cellular Mechanotransduction: Putting all the Pieces Together again." Faseb Journal 20.7 (2006): 811-27.
28. Geraldo, S., and P. R. Gordon-Weeks. "Cytoskeletal Dynamics in Growth-Cone Steering." Journal of cell science 122.20 (2009): 3595-604.
29. Tessier-Lavigne, M., and C. S. Goodman. "The Molecular Biology of Axon Guidance." Science 274.5290 (1996): 1123-33.

30. Dent, E. W., and K. Kalil. "Axon Branching Requires Interactions between Dynamic Microtubules and Actin Filaments." Journal of Neuroscience 21.24 (2001): 9757-69.
31. Palmer, R. E., Sullivan, D. S., Huffaker, T., and Koshland, D. "Role of Astral Microtubules and Actin in Spindle Orientation and Migration in the Budding Yeast, Saccharomyces-Cerevisiae." Journal of Cell Biology 119.3 (1992): 583-93.
32. Balgude, AP, Yu, X., Szymanski, A., and Bellamkonda, R. V. "Agarose Gel Stiffness Determines Rate of DRG Neurite Extension in 3D Cultures." Biomaterials 22.10 (2001): 1077-84.
33. Georges, P. C., Miller, W. J., Meaney, D. F., Sawyer, E. S., and Janmey, P. A. "Matrices with Compliance Comparable to that of Brain Tissue Select Neuronal Over Glial Growth in Mixed Cortical Cultures." Biophysical journal 90.8 (2006): 3012-8.
34. Leach, J. B., Brown, X. Q., Jacot, J. G., Dimilla, P. A., Wong, J. Y. "Neurite Outgrowth and Branching of PC12 Cells on very Soft Substrates Sharply Decreases Below a Threshold of Substrate Rigidity." Journal of Neural Engineering 4.2 (2007): 26-34.
35. Saha, K., Keung, A. J., Irwin, E. F., Li, Y., Little, L., Schaffer, D. V., Healy, K. E. "Substrate Modulus Directs Neural Stem Cell Behavior." Biophysical journal 95.9 (2008): 4426-38.
36. Dodla, M. C., J. Mumaw, and S. L. Stice. "Role of Astrocytes, Soluble Factors, Cells Adhesion Molecules and Neurotrophins in Functional Synapse Formation: Implications for Human Embryonic Stem Cell Derived Neurons." Current Stem Cell Research & Therapy 5.3 (2010): 251-60.
37. Ullian E. M., Sapperstein S. K., Christopherson K. S., Barres B. A. "Control of Synapse Number by Glia." Science 291.5504 (2001): 657-61.
38. Waites, C. L., A. M. Craig, and C. C. Garner. "Mechanisms of Vertebrate Synaptogenesis." Annual Review of Neuroscience 28 (2005): 251-74.
39. Horvit, H. R., and I. Herskowitz. "Mechanisms of Asymmetric Cell-Division - 2 Bs Or Not 2 Bs, that is the Question." Cell 68.2 (1992): 237-55.
40. Shapiro, L., and R. Losick. "Protein Localization and Cell Fate in Bacteria." Science 276.5313 (1997): 712-8.
41. Jan, Y. N., and L. Y. Jan. "Asymmetric Cell Division." Nature 392.6678 (1998): 775-8.
42. Siller, K. H., and C. Q. Doe. "Spindle Orientation during Asymmetric Cell Division." Nature cell biology 11.4 (2009): 365-74.
43. Lu, B. W., L. Jan, and Y. N. Jan. "Control of Cell Divisions in the Nervous System: Symmetry and Asymmetry." Annual Review of Neuroscience 23 (2000): 531-56.
44. Yamashita, Y. M. "The Centrosome and Asymmetric Cell Division." Prion 3.2 (2009): 84-8
45. Rhyu, M. S., L. Y. Jan, and Y. N. Jan. "Asymmetric Distribution of Numb Protein during Division of the Sensory Organ Precursor Cell Confers Distinct Fates to Daughter Cells." Cell 76.3 (1994): 477-91.

46. Betschinger, J., and J. A. Knoblich. "Dare to be Different: Asymmetric Cell Division in Drosophila, C-Elegans and Vertebrates." Current Biology 14.16 (2004): R674-85.
47. Théry, M., Racine, V., Pépin, A., Piel, M., Chen, Y., Sibarita, J. B., and Bornens, M. "The Extracellular Matrix Guides the Orientation of the Cell Division Axis. Nature cell biology 7.10 (2005): 947-U29.
48. Snyder, E. Y., Deitcher, D. L., Walsh, C., Arnold-Aldea, S., Hartwieg, E. A., and Cepko, C. L. "Multipotent Neural Cell-Lines can Engraft and Participate in Development of Mouse Cerebellum." Cell 68.1 (1992): 33-51.
49. Snyder, E. Y., Yoon, C., Flax, J. D., and Macklis, J. D. "Multipotent Neural Precursors can Differentiate Toward Replacement of Neurons Undergoing Targeted Apoptotic Degeneration in Adult Mouse Neocortex." Proceedings of the National Academy of Sciences of the United States of America 94.21 (1997): 11663-8.
50. Aplin, J. D., and R. C. Hughes. "Cell-Adhesion on Model Substrata - Threshold Effects and Receptor Modulation." Journal of cell science 50.AUG (1981): 89-103.
51. Johnson, K. R., J. L. Leight, and V. M. Weaver. "Demystifying the Effects of a Three-Dimensional Microenvironment in Tissue Morphogenesis." Cell Mechanics 83 (2007): 547-83.
52. Meijering, E., Jacob, M., Sarria, J. C., Steiner, P., Hirling, H., Unser, M. "Design and Validation of a Tool for Neurite Tracing and Analysis in Fluorescence Microscopy Images." Cytometry Part a 58A.2 (2004): 167-76.
53. Schneider, C. A., W. S. Rasband, and K. W. Eliceiri. "NIH Image to ImageJ: 25 Years of Image Analysis." Nature Methods 9.7 (2012): 671-5.
54. Burkel, B. M., G. von Dassow, and W. M. Bement. "Versatile Fluorescent Probes for Actin Filaments Based on the Actin-Binding Domain of Utrophin." Cell motility and the cytoskeleton 64.11 (2007): 822-32.
55. Manent, J.B., Wang Y., Chang, Y., Paramasivam, M, and LoTurco, J. J. "Dcx Reexpression Reduces Subcortical Band Heterotopia and Seizure Threshold in an Animal Model of Neuronal Migration Disorder." Nature medicine 15.1 (2009): 84-90.
56. Matsuda, T., and C. L. Cepko. "Controlled Expression of Transgenes Introduced by in Vivo Electroporation." Proceedings of the National Academy of Sciences of the United States of America 104.3 (2007): 1027-32.

Vita

Colleen Curley was born on June 11, 1989 in Philadelphia, PA to Richard and Theresa Curley. She earned a B.S. in Biology from Lehigh University in May 2011. She was awarded the Lehigh University Presidential Scholarship to fund the tuition of her M.S. degree.