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Topography, extracellular matrix proteins, secreted molecules and endogenous electric fields: cues that influence the differentiation of neural progenitor cells

Carlos Atico Ariza

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Topography, extracellular matrix proteins, secreted molecules and endogenous electric fields: cues that influence the differentiation of neural progenitor cells

by

Carlos Atico Ariza

A dissertation submitted to the graduate faculty
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

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Iowa State University
Ames, Iowa
2009

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<td>3HT</td>
<td>Tritiated thymidine</td>
</tr>
<tr>
<td>AChR(s)</td>
<td>Acetylcholine receptor(s)</td>
</tr>
<tr>
<td>AHPC</td>
<td>Adult hippocampal progenitor cells</td>
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<tr>
<td>AMP</td>
<td>Adenosine monophosphate</td>
</tr>
<tr>
<td>BDNF</td>
<td>Brain derived neurotrophic factor</td>
</tr>
<tr>
<td>bFGF</td>
<td>Basic fibroblast growth factor</td>
</tr>
<tr>
<td>BMP</td>
<td>Bone morphogenic protein</td>
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<tr>
<td>CAM</td>
<td>Cell adhesion molecules</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic AMP</td>
</tr>
<tr>
<td>CCM</td>
<td>Co-culture media</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>CNTF</td>
<td>Ciliary neurotrophic factor</td>
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<tr>
<td>DIV</td>
<td>Days <em>in vitro</em></td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>EF(s)</td>
<td>Electric field(s)</td>
</tr>
<tr>
<td>EGF</td>
<td>Epithelial growth factor</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal growth factor receptor</td>
</tr>
<tr>
<td>GFAP</td>
<td>Glial fibrillary acidic protein</td>
</tr>
<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
</tr>
<tr>
<td>ICC</td>
<td>Immunocytochemistry or immunocytochemical</td>
</tr>
<tr>
<td>IL-6</td>
<td>Interleukin 6</td>
</tr>
<tr>
<td>iPS</td>
<td>Induced pluripotent stem cells</td>
</tr>
<tr>
<td>IR</td>
<td>Immunoreactive or immunoreactivity</td>
</tr>
<tr>
<td>LIF</td>
<td>Leukemia inhibitory factor</td>
</tr>
<tr>
<td>MAP2ab</td>
<td>Antibody against microtubule associated protein or the protein itself</td>
</tr>
<tr>
<td>NCCC</td>
<td>Non contact co-culture</td>
</tr>
<tr>
<td>NGF</td>
<td>Nerve growth factor</td>
</tr>
<tr>
<td>nHNPCs</td>
<td>Normal human neural progenitor cells</td>
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<td>NPC(s)</td>
<td>Neural progenitor cell(s)</td>
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<td>NSC(s)</td>
<td>Neural stem cell(s)</td>
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<td>NT-3</td>
<td>Neurotrophin 3</td>
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<td>PC12 cell</td>
<td>Adrenal tumor derived neuron precursor cell (not the same as a NPC)</td>
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<tr>
<td>PDGF</td>
<td>Platelet-derived growth factor</td>
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<tr>
<td>PEMF</td>
<td>Pulsed electromagnetic fields</td>
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<tr>
<td>PI-3K</td>
<td>Phosphatidylinositol 3-kinase</td>
</tr>
<tr>
<td>PKA</td>
<td>Protein kinase C-dependent kinase</td>
</tr>
<tr>
<td>PLC</td>
<td>Phospholipase C</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>PNS</td>
<td>Peripheral nervous system</td>
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<tr>
<td>RGD</td>
<td>Arginine, glycine, and aspartic acid common amino acid binding sequence</td>
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<tr>
<td>RIP</td>
<td>Oligodendrocyte antibody, recognizes 2',3'-cyclic nucleotide 3'-phosphodiesterase (CNP)</td>
</tr>
<tr>
<td>SCI</td>
<td>Spinal cord injury</td>
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<tr>
<td>SGZ</td>
<td>Subgranular zone</td>
</tr>
<tr>
<td>SHH</td>
<td>Sonic hedge hog</td>
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<tr>
<td>SVZ</td>
<td>Subventricular zone</td>
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<tr>
<td>TGF</td>
<td>Transforming growth factor</td>
</tr>
<tr>
<td>TUJ1</td>
<td>Antibody against type III β-tubulin</td>
</tr>
<tr>
<td>VGCC</td>
<td>Voltage gated calcium channels</td>
</tr>
<tr>
<td>θ</td>
<td>Angle between longest cell axis (furthest two points) and the EF vector</td>
</tr>
<tr>
<td>ζ</td>
<td>Zeta potential (~surface charge)</td>
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ABSTRACT

Neural progenitor cells (NPCs) have the potential to be used as a cell based therapy to treat Alzheimer disease, spinal cord injury and other significant damage to the central nervous system. In order to utilize the therapeutic potential of NPCs, we must first learn to control their migration, proliferation, differentiation and growth. An ideal methodology would entail directed stem cell migration to damaged tissue; proliferation until the target is reached; differentiation into the most beneficial phenotype; and integration of cells into the existing tissue. A myriad of stimulants that alter NPC behavior, exist in vivo. Characterizing the individual contributions of each stimulant or cue in vitro can be difficult to deconvolute, unless discerned in vitro. We investigate several of these cues individually and synergistically. We focus on the influence of extracellular matrix proteins, components secreted from surrounding cells in the central nervous system (i.e. - astrocytes, microglia) and the physical micro- and nano-architecture surrounding NPCs. One other stimulus that has not been explored much, but is investigated here extensively, is the influence of electric fields that mimic the electric gradient existent in the developing and adult nervous system. We investigate the contribution of electric fields as stimuli to NPCs and the possible use of electric fields to reap the therapeutic potential of NPCs. In summary, investigation of the influence of a synergistic combination of external cues on the behavior of neural progenitor cells provides insights into their behavior and enables potential control of stem cell differentiation.
CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW

1.1. Dissertation Organization

This dissertation is organized into five chapters. The first chapter is an extensive literature review which includes fundamental knowledge used in undertaking the research presented in following chapters. In part, the first chapter is modified from a recently published book chapter\(^1\). First, basic knowledge of stem cell research is provided. Second, insight into the relationship between soluble and insoluble proteins or electrical stimulation and the proliferation, growth and differentiation of NPCs is given. In brief, the importance of cell to cell, cell to substrate, and cell to extracellular matrix interactions are reviewed. Then, bioelectricity, the therapeutic use of electrical stimulation, and the complexity of investigating electrical stimulation of mammalian cells \textit{in vitro}, ends the first chapter. In general the first chapter conveys that physical, biological, chemical or electrical cues found extracellularly can alter cellular behavior.

The second, third and fourth chapters are original articles that have been or will be submitted to journals. In the first of these three, we rigorously investigated the use of multiple extracellular matrix proteins individually or combined with biological soluble factors and microscale substrate architecture on the behavior of NPCs. In the third chapter, the use of nanoporous or nanoscale topography as a means to control NPCs was investigated. Finally in the last journal article, the use of electric fields to control NPC behavior was investigated. The one common aspect of NPCs that was assayed was differentiation into the three possible phenotypes in the central nervous system. However, other aspects of NPC behavior were also investigated to determine the response to external cues. Overall conclusions from this research are presented in last chapter. Some possible future directions are presented as an important aspect of achieving further progress related to the control of stem cells \textit{in vitro} for cell based regenerative approaches.

1.2. Stem Cells

In 1960, stem cells were first acknowledged by Till, Becker and colleagues who observed single cells in the bone marrow of mice that gave rise to blood cells, \textit{in vivo}\(^2,3\). Broadly, stem cells have three properties that distinguish them from other cells: they are capable of dividing and renewing for long periods; are unspecialized (potential); and give rise (differentiate) to more committed or specialized cell types.

1.2.1. Stem Cell Potential

An uncommitted or unspecialized cell is one that does not perform any tissue-specific function. When stem cells differentiate they produce cells with lower potential or cells that are more committed, until a final functional cell fate is reached (Figure 1.3). A mammal is produced from the most potent or \textit{toti-}
potent stem cell: a fertilized egg. Thus, a totipotent stem cell is one that can create an entire organism. The progeny of the zygote are totipotent after cleavage, up to the formation of the morula (5 cell divisions of the zygote)\(^4\). Totipotent stem cells differentiate into pluripotent stem cells, which have the ability to produce tissues from the three embryonic germ layers (ectoderm, endoderm, and mesoderm). In other words, pluripotent stem cells eventually form tissues within the body but not those of the placenta. Embryonic stem cells are pluripotent stem cells. Further differentiation leads to an increase in committed cell fate, giving rise to multipotent stem cells. Multipotent stem cells differentiate into cells of a specific tissue or cell lineage (e.g. hematopoietic stem cells differentiate into red blood cells, white blood cells, platelets, etc.). Finally, unipotent stem cells only produce one cell type but can self-renew which separates them from non-stem cells.

1.2.2. Progenitor Cells

Further differentiation of multipotent or unipotent stem cells produces progenitor cells, which are similar to multipotent or unipotent stem cells except that progenitor cells have limited self-renewal and differentiation capacity. Progenitor cells can produce cells of a specific cell lineage but not indefinitely, instead progenitor cells go through several rounds of division before terminally differentiating into a cell fate. In other words, progenitor cells are more committed to a final cell fate than multipotent or unipotent stem cells.

1.2.3. Embryonic Stem Cells

Figure 1.1. Embryonic stem cells are derived from the inner mass cells in an embryo. Illustrated by

the University of Kansas Medical Center, Center for Reproductive Sciences Cell Imaging Core\(^5\)

About five days after an egg is fertilized, it develops into a ball of about 30 -150 cells called a blastocyst. The blastocyst can be divided into three structures: the trophectoderm, which is the layer of
cells (in this case called trophoblast) that surrounds the blastocyst and is responsible for negotiating implantation, developing into the placenta, and controlling the exchange of oxygen and metabolites between mother and embryo; the blastocoel, which is the hollow cavity inside the blastocyst; and the inner cell mass, which is a group of approximately 30 cells at one end of the blastocoel. Embryonic stem cells are obtained by removing the inner cell mass and proliferating these cells in culture (Figure 1.1). As mentioned, embryonic cells are pluripotent, producing germ layer tissues, but not those of the placenta, as the trophoblast develops into the placenta. Embryonic stem cells have been shown to proliferate for approximately a year or more in culture.

1.2.4. Sources of Embryonic Cells

One source of embryonic stem cells is in vitro fertilization, where a couple may decide to donate their unused fertilized eggs, for research purposes. Another potential source might be created by removing and replacing the nucleus of an unfertilized human egg cell with the nucleus of a cell from another person (therapeutic cloning). This process yields a blastocyst from which stem cells can be extracted. This may lead to embryonic stem cells that would be compatible with the person who needs treatment.

1.2.5. Concerns of Stem Cell Use

The use of embryonic stem cells is highly controversial because the embryo dies after the inner cell mass or stem cells are removed. Whether interfering with the development of a potential human life, is appropriate is a question of much debate. There are many profound viewpoints, but they will not be covered in this report. However, methods for keeping the embryo alive after embryonic stem cells are removed are being developed. The first successful trials were in rats and later with human embryos. Some embryos developed into normal rat pups, other embryos did not survive the procedure. Therefore, the removal of the inner cell mass is still detrimental to the embryo. The technique must allow all embryos to survive stem cell removal, and develop without abnormalities for the use of embryonic stem cells to be less controversial. However, this technique may not be developed fully because of the possibility of creating an unhealthy human.

Recently, exciting developments could potentially eliminate the need to derive stem cells from the embryo. Researchers at Kyoto, University have developed a promising technique which induces adult skin cells (dermal fibroblasts) to become pluripotent cells. The induced pluripotent stem cells (iPS) were found to differentiate and behave as embryonic stem cells. Additionally, iPS would greatly reduce the risk of patient rejection because iPS would be derived from the patient's skin and would contain a matching genetic code.

Even if the ethical controversy is resolved, the International Society for Stem Cell Research has stated that the use of embryonic stem cells can be dangerous. This is due to the rapidly dividing and
unspecialized nature of embryonic stem cells. If implanted, stem cells may proliferate into a tumor, instead of differentiating and integrating into the surrounding or damaged tissue. Thus, the tremendous therapeutic potential of stem cells will not be used effectively until they can be completely controlled.

1.2.6. Adult Stem Cells

Adult stem cells compared to embryonic stem cells, have decreased differentiation potential (multipotent or unipotent), and do not survive as long in vitro. Adult stem cells are often named after the tissue in which they are found or the cells that they produce. For example, some stem cells found in bone marrow are called hematopoietic (of the blood) stem cells because they produce all blood cell types. Another example are the stem cells found in central nervous system niches called neural stem/progenitor cells (NSCs or NPCs) as seen in Figure 1.3. NPCs differentiate into cells of the nervous system as their final cell fate. Other locations where adult stem cells have been found are hair follicles, skeletal muscle, the liver, the epidermis and the heart. Furthermore, tissues in which adult stem cells are found continue to increase. In short, embryonic stem cells are involved in the development of an organism and adult stem are involved in maintenance, repair and cellular replacement during normal cellular turnover.

1.2.7. Adult Stem Cells vs. Progenitor Cells

Progenitor cells also give rise to cell types belonging to specific lineages (i.e.–hematopoietic) and are similar to adult stem cells but have limited self-renewal. The distinction between adult and progenitor cells can be confusing because of the criteria used to define a stem cell.

Neural stem/progenitor cells found in the hippocampus are cells whose definition is somewhat unclear due to contradicting classifications. Some groups have classified these cells as progenitors and others say that they are stem cells. One recent study suggests that the cells found within the hippocampus are progenitor cells that arise from true neural stem cells found in a layer surrounding the hippocampus. Additionally, the ambiguous use of “adult stem cell”, “progenitor”, and “precursor” has led to skewed classifications.

To summarize, adult stem cell are proliferative, capable of long-term self-renewal (throughout the life of the animal) and multipotent; generating a large population of progeny that can terminally differentiated into the cell types of the tissue in which they are found. Thus, NSCs and NPCs are present in the brain throughout life and give rise to new neurons, astrocytes, and oligodendrocytes, just as in the prenatal or developing brain. However, NPCs do not self-renew indefinitely.

1.3. Cells in the Nervous System

The central nervous system (CNS) is comprised of two types of cells; neurons and glia (please refer to Figure 1.2 for the following). There exist 10 to 50 times more glial cells than neurons in the CNS of
vertebrates. Glial cells provide support and structure to the CNS; insulate axons with myelin; perform housekeeping duties regularly and during injury; guide migrating neurons and direct the outgrowth of axons in development; regulate presynaptic terminals at the nerve-muscle junction, and more. Not forgetting that neurons have subcategories, the glial cells within the nervous system are also further categorized into macroglia and microglia. Macroglia are further subcategorized in the CNS into oligodendrocytes and astrocytes, and in the peripheral nervous system (PNS) into Schwann cells.

First, microglia, as the name implies, are typically much smaller than the cells found in the CNS and are the smallest of the glial cells. Microglia are phagocytes produced outside the nervous system and are unrelated physiologically and developmentally to the other cells of the CNS. The brain owes its limited immunological response to the capability of microglia to remove apoptotic cells and debris. Phagocytosis by microglia (engulfing/eliminating waste or cell debris) is induced when mobilized or activated, because of injury, disease, or infection of the nervous system.

Second, oligodendrocytes in the CNS and Schwann cells in the PNS are responsible for making the transmission of neuronal signals more efficient. They do this by insulating axons with sheaths of a lipid-based substance called myelin. The sheaths are created by oligodendrocytes/Schwann cells densely wrapping their membranous processes around the axon repeatedly. A single oligodendro-
cyte myelinates an average of 15 internodes/axons, while a single Schwann cell, only envelops one internode/axon. The myelin produced by Schwann cells are similar, but chemically distinct, from that produced by oligodendrocytes.

Lastly, astrocytes derive their name from the Greek word astron which means of the stars. The reason for this name is easily understood when observing astrocytes through a microscope in cell culture, where astrocyte morphology resembles stars. Astrocytes are the most numerous cells in the CNS and were once consider solely supportive cells. Astrocytes filter nutrients to neurons, having end-feet at both capillaries and neurons. These end-feet encircle capillaries in the brain helping make an impermeable barrier that prevents blood from entering the brain, know as the blood-brain barrier. Neurotoxic substances and high concentrations of neurotransmitters such as norepinephrine and glutamate are prevented from entering or accumulation in the brain, by the blood-brain barrier. Astrocytic functions are indispensable for the maintenance and survival of neurons. Yet, astroglia are more than mere "support cells" for neurons, as once believed. Glial cells in the CNS are now known to serve a more profound role due to the discovery of neurogenesis 20. In neurogenic niches, NPCs are influenced by proteins that originate from astrocytes and other glia. Thus, glial cells create the proper environment for NPCs to proliferate, differentiate and integrate into the preexisting nervous system; making glial cells more than just neuronal "support cells".

1.4. Neural Progenitor Cells

NPCs are present in the adult nervous system throughout life, can self-renew, but not indefinitely, and give rise to (differentiate into) cells of the nervous system (neurons and glial cells) 8-11 (see Figure 1.3). In the adult CNS, differentiation occurs in specific niches. Some locations where NPCs are found within the adult nervous system are the olfactory bulb 11, 21, hippocampus 22, sub ventricular zone 23 and spinal cord 24-26. The research performed in our group characterizes the behavior of NPCs from the hippocampus of rats and humans in the presence of different cues or stimuli.

1.4.1. History of Postnatal Neurogenesis

Neurogenesis is the process of generating functional neurons from progenitor cells. This process includes proliferation and neuronal fate specification of neural progenitors, and maturation and functional integration of neuronal progeny into neuronal circuits. Originally it was thought that neurogenesis in mammals only occurred in early developmental stages 27. In 1913 Santiago Ramon y Cajal determined that neurons were generated only before birth 28. Limited laboratorial techniques prevented successful investigations into speculations of dividing cells in the postnatal CNS. Thus, Ramon y Cajal’s work became a dogma for about 90 years despite evidence that began to prove otherwise starting in 1961.
In 1959, a technique to label proliferating cells was developed using the radio labeled nucleoside, tritiated-thymidine (3HT). 3HT incorporates into replicating DNA during the S-phase of the cell cycle and is detected with radiography. So, 3HT positive cells in radiographs of tissue section would indicate that those cells were mitotic after the addition of 3HT. In 1961, Altman and colleagues detected 3HT in radiographs of three-day postnatal mice brains. Soon after, 3HT evidence in other regions of adult rats brains, such as, the neocortex, olfactory bulb and dentate gyrus of the hippocampus were published. Yet, adult neurogenesis was not accepted by the scientific community.

It is somewhat puzzling that these discoveries did not begin to dissolve the dogma against neurogenesis. There are several reasons for the dismissal of Altman’s discoveries. First, cells with 3HT positively labeled nuclei were not easily distinguishable as neurons; radiolabeled cells could be dividing glial cells. Second, 3HT also incorporates into DNA when it is repaired, therefore neurons could have been labeled during DNA repair. Third, the findings suggested migration from the ventricle to other parts of the brain such as the olfactory bulb which was considered very unlikely. Lastly, another possible reason was that Altman was a self-taught post doctoral fellow researching at MIT. Thus, neuroscientists would not go against Ramon y Cajal’s almost undisputed reputation without further proof.

From a spatial and mechanistic perspective, it seemed rather implausible for neurons to seamlessly integrate into the adult brain. How could each new neuron, integrate into the existing neuronal network without disrupting existing connections and accommodate itself in an already dense tissue (the brain)? Furthermore, the source of new neurons remained a mystery. It was unlikely that adult neu-
rons, some containing dendritic branches with many connections would retract all its projections to become mitotic. Therefore, skepticism remained toward neurogenesis.

Sixteen years after the initial discovery of neurogenesis, further proof was demonstrated. In 1977, Kaplan & Hinds found newborn neurons in the hippocampus were still alive after 30 days. Later, it was shown that new neurons received synaptic inputs and extended axons to targeted areas. These findings showed that new neurons served a long term purpose and could integrate into a pre-existing neural network without detrimentally disrupting that network.

Much later, a very important study of the telencephalon (brain region) in songbirds related neurogenesis to learning. Increases in postnatal neurogenesis along with the replacement of neurons coincided with increased knowledge or information gain. This was seen in canaries, which during the fall learn a new song and experience increased neurogenesis and replacement of dead neurons in the high vocal center of the brain. The song is used 8 months later during the mating season and the neurons acquired in the fall are not replaced. However, neurons created during the spring do not remain for so long and are replaced. Such evidence indicates a possible hierarchical selection process of neuronal replacement. In this manner the brain may balance limited space with the storage of important knowledge.

In 1982 an improved method to detect neurogenesis was successful in mice. The use of bromodeoxyuridine (5-bromo-2-deoxyuridine, BrdU), analogous to 3HT, as a S-phase indicator, eliminated the need for radiographs making the analysis faster. Additionally, with every cell division, the concentration of BrdU halves within the cell, leading to a lower intensity, which provides a quantifiable method for determining rate of cell division and the amount of cell divisions taken by a cell to obtain a non-mitotic or completely differentiated state. Furthermore, BrdU can be combined with immunocytochemistry to identify the final fates of differentiating stem cells. In humans, proof of neurogenesis was shown using BrdU combined with one of the neuronal markers, NeuN, calbindin or neuron specific enolase as the assay. This method is the most widely used and accepted technique to identify adult neurogenesis and isolate NPCs from the brain.

The mechanistic issue of accommodating and producing neurons was solved with the discovery of NPCs. The isolation of NPCs proved that in the CNS new neurons were being created from undifferentiated cells and not from pre-existing neurons. The first area of the brain where this was shown was the subventricular zone, followed by the dentate gyrus of the hippocampus.

Functional purpose to new neurons has been strongly shown in electrophysiological studies. Synaptic activity and integration into preexisting neural networks has been shown in the CNS, specifically in the olfactory bulb. Using combined retroviral-based lineage tracing in the developing nervous system with electrophysiological studies (i.e.- patch clamping), newborn neurons have been shown to functionally and synaptically incorporate into the adult CNS.
Now, the challenge lies in understanding why neurogenesis occurs only in certain areas of the brain, what functions this process serves, and how this processes can be exploited to develop a therapeutic approach to damage in the CNS.

1.4.2. NPCs in the CNS

Within the brain, there are two zones which are most proliferative or neurogenic: the subventricular zone (SVZ) of the lateral ventricles, and the subgranular zone (SGZ) of the dentate gyrus in the hippocampus. The SVZ and SGZ, as well as other areas with NPC activity, serve as models for the study of neurogenesis in vivo because of migration, differentiation and maturation of NPCs. These areas are interesting because the mature daughter progenitor cells that arise from asymmetric cell divisions, in both the SVZ and dentate gyrus of the hippocampus, migrate to distinct targeted areas as they mature. At the targets, the progenitor cells may completely differentiate or become quiescent. Cellular signaling occurs throughout this process. Thus, within these niches lie guidance, fate specific and proliferative cues that control NPC behavior. Suspected cues are studied by purifying and maintaining NPCs in vitro from these neurogenic niches, as demonstrated by Palmer et al. for hippocampal NPCs.

However, the SGZ and SVZ are not the only regions in the CNS where NPCs reside. Since, cells found in non-neurogenic regions, such as the spinal cord can produce neurons when dissected and transplanted into neurogenic regions, such as the dentate gyrus. It seems that quiescent NPCs are found throughout the CNS. These dormant NPCs differentiate only when given the appropriate signals or cues, in vivo or in vitro, as demonstrated by Jia and Chen. Some of these signals originate from neurogenic-niche astrocytes (neonatal brain, adult SGZ and SVZ) and include sonic hedgehog (SHH).

The exact phenotype of the adult neural stem cells (least differentiated with indefinite self-renewal) in the CNS, has not been clearly established. Some evidence indicates that a subpopulation of ependymal cells in the lining of the third ventricle are stem cells. More convincing evidence points to a subset of cells in the SVZ that are astrocyte-like (GFAP expressing) as being adult stem cells, as described in a recent review by Alvarez-Buylla and in a recent article by Jiao. The resolution of this issue is crucial in understanding neurogenesis and the development of cell based therapies for the nervous system.

1.4.3. NPC Fate Identification

NPCs studies usually require differentiated cells to be identified phenotypically and detected among cells that did not originate from NPCs. The phenotypes of differentiated can be identified with immunocytochemistry (ICC) and fluorescence microscopy. To distinguish NPCs and their progeny, retrovirus or transgenic mice can be used to produce NPCs that express fluorescent proteins, such a green fluorescent protein (GFP). The expression of the fluorescent proteins is tailored to be ubiqui-
tous in NPCs, making NPCs easily distinguishable from other cells, with a fluorescent microscope, when co-cultured with non-fluorescent cells.

In ICC, antibodies with specific antigenic targets are used to identify proteins that are unique to certain cell types. These antibodies are tagged with fluorescent probes that when exposed to a specific excitation wavelength(s) emit light allowing the probes to be visualized. Wherever the cell-type-specific protein, identified by the antibody, is found fluorescent light will be detected. This does not conclusively prove that the cells identified are of a specific type, unless their functionality is tested. However, this method is used most researchers to determine stem cells have differentiated into.

The proteins (antigens) or antibodies commonly used to identify cells that differentiate from NPCs in the research presented here are listed in Table 1.

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Antibody</th>
<th>Antibody Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Progenitor</td>
<td>Nestin</td>
<td>Intermediate filament protein expressed in both neuronal and glial progenitors</td>
</tr>
<tr>
<td>Neuron</td>
<td>TUJ1</td>
<td>Antibody against class III b-tubulin (in early neurons)</td>
</tr>
<tr>
<td></td>
<td>MAP2ab</td>
<td>Antibody against microtubule associated protein 2ab (in mature neurons)</td>
</tr>
<tr>
<td>Astrocyte</td>
<td>GFAP</td>
<td>Antibody against glial fibrillary acidic protein</td>
</tr>
<tr>
<td>Oligodendrocytes</td>
<td>RIP</td>
<td>Recognizes 2',3'-cyclic nucleotide 3'-phosphodiesterase (CNP), a known non-compact myelin protein</td>
</tr>
</tbody>
</table>

1.5. Control of Stem Cells and Therapeutic Applications

Since stem cells were first discovered in bone marrow, it is not surprising that these cells were the first used clinically. Bone-marrow transplants and peripheral blood transfusions (which contain stem cells) are still used to treat leukemia and inherited blood disorders. Stem cells have also been used to treat severe autoimmune disease and repair cartilage, cardiac muscle, liver tissue and other tissues. Thus, the discovery of stem cells in adults may provide a regenerative cell based therapy for the tissue in which they are found.

Currently, NPCs are one of the most investigated progenitor cells because of their ability to produce neurons and the demand for a bioengineered solution to neurodegenerative diseases and nervous system injury. For example, neurons lost due to Parkinson's or Alzheimer's diseases may potentially be replaced using NPCs. Additionally, the delivery of stem cells into the retina to treat retinal disease, such as Macular Degeneration, is being investigated. First, however, stem or progenitor cells must be completely understood to be used as an effective therapy.
One aspect of stem cell behavior that must be carefully characterized is the adoption of a final cell fate. Stem cell fate is dependent on both intrinsic (e.g. epigenetic factors) and extrinsic signals that alter the adoption of a cell fate. The dependence on the surrounding environment has been shown in many in vivo transplantations. For example, NPCs transplanted into the developing eye of a rat can migrate, integrate, and adopt similar morphology of adjoined but distinct cell layers in the retina (Figure 1.4). Thus, instructive signals reside within each tissue, that dictate where stem cells should migrate and what they should differentiate into.

Determining the cues that allow or prevent stem cell migration and integration into tissues is crucial for the advancement of cell-mediated therapies and cellular biology. If stem cells cannot be controlled in vivo, adverse effects, like the formation of teratomas, can arise when applied therapeutically. In clinical trials, fetal neural tissue implanted into patients with Parkinson’s disease alleviated the
symptoms of some patients \(^6\); yet, the inability to control stem cell behavior exacerbated the symptoms of others \(^6\). Thus, if we are to overcome our limited ability to treat damage to the CNS, using stem cells, we must first completely learn to control stem cells or NPCs \(^5\). Additionally, understanding the processes that occur in the diseased or injured CNS is necessary for cell-based therapies to overcome our limited therapeutic capability.

In order to control and apply stem cells therapeutically, we must consider all reasonable external interactions that occur in the stem cell niche and areas into which they are transplanted. This is quite a daunting task, considering the thousands of possible external interactions that any cell can have. In general, signaling proteins can originate from other cells, or in self-regulation are secreted by stem cells themselves. Furthermore, extracellular matrix molecules also interact with stem cells to alter their behavior.

1.6. The Extracellular Matrix

The ECM is a structural component of every tissue composed of molecules secreted by resident cells. The distinct physical properties of tissues are due to variations in the ECM composition (i.e.-bone compared to skin). Broadly, the ECM is composed of structural proteins (mainly collagen, elastin, or reticulin); glycoproteins (e.g. laminin and entactin); and proteoglycans. The ECM serves as a peptide scaffold or connective tissue that cells can anchor onto. However, it is not simply a scaffold, the ECM also influences the behavior of cells at all points in life: proliferation, differentiation, guidance and maturation \(^6\).

The ECM can be subdivided into the interstitial matrix and the basement membrane. The basement membrane is characteristically found under epithelial cells and is divided into the basal lamina and the lamina reticularis. The basal lamina is in contact with epithelial cells and is organized into sheet like layers of ECM proteins that form a mat 40-120nm thick. Compositionally, the basal lamina is mainly type IV collagen with specialized molecules on each face that help bind it to adjacent cells or ECM proteins. The basal lamina is divided into three layers: lamina rara interna, lamina densa and lamina rara externa. The lamina rara interna and externa are very similar; both contain the glycoproteins laminin and entactin. The lamina densa is 30–70nm thick, contains type IV collagen and is easily seen using electron microscopy because it is electron dense. Finally, the basement membrane is not complete without the underlying lamina reticularis, which is produced by fibroblast in connective tissue and is made of type VII collagen (fibrillary collagen) that tether it to the underlying connective tissue (typically contains fibronectin) \(^7\).

The ECM influences development and migration, and plays a very important role in healing. When cells are damaged and die the basal lamina remains, providing a scaffold for regenerating cells to migrate to the site of injury. In some cases, as in the skin or cornea, the basal lamina becomes chemically altered after injury, by the addition of fibronectin, which promotes cell migration towards the wound \(^7\).
1.7. Extracellular Interactions

In any cellular niche, cell to cell and cell to extracellular matrix interactions occur through cell adhesion molecules or CAMs. Cell-cell interactions occur via the cadherin and immunoglobulin protein superfamilies, and selectins (in bloodstream). Cells interact with the ECM through integrins and transmembrane proteoglycans. In order to function properly, the mentioned adhesion families, except for the immunoglobulin superfamily and transmembrane proteoglycans, require Ca\(^{2+}\) or Mg\(^{2+}\)\(^{72}\). Each family has a myriad of proteins with specific function(s), giving cells multiple methods to interact with each other and the ECM. Although there are two families of proteins that interact with the ECM, we shall focus on integrins, since the interactions we are studying depend on integrins.

1.7.1. Integrins

The principle receptors for binding ECM molecules (i.e. – collagen, laminin, fibronectin) in animal cells are the integrins. Integrins are a family of transmembrane protein receptors that bind to the ECM (also cell to cell adhesion in blood cells).

Integrins function through weak bonds or low affinity interactions and are very abundant in the cell membrane. When compared to other cell adhesion molecules integrins are 10 to 100 times more concentrated throughout the membrane. Though many integrins bind to the ECM, they do so with low affinity to facilitate cell movement while allowing the cell to adhere firmly to the ECM. If cell attachment to the ECM occurred via few integrins with strong affinity the cell would be incapable of migrating because it would be difficult for integrins to detach from the ECM. Therefore having many integrins with weak affinity for the ECM circumvents this problem.

Integrins are more than just cell adhesion molecules, they also communicate internally with the cell. All integrins do not cause the same internal change, allowing cells to respond to the surrounding ECM depending on its composition. One change that occurs is the re-organization of the cytoskeleton through the binding of actin filaments (to the \(\beta\) subunit) via molecules such as talin, \(\alpha\)-actinin, and filamin\(^{72,75}\). Integrins or ECM proteins can also change cellular migration\(^{76}\), as demonstrated in vitro, where NPCs migrated out of neurospheres faster if grown on ECM molecules than on poly-L-ornithine. Furthermore, results depend on the type of ECM molecule used, as demonstrated by NPCs migrating faster on laminin than on fibronectin\(^{77}\).

Cells can also change their specificity for ECM molecules in accordance to an up-regulation of a specific ECM molecule. For example, after post natal blood vessel formation and during blood vessel maturation in the adult CNS, brain endothelial cells switch from expressing \(\beta1\) integrins that bind fibronectin (\(\alpha4\beta1\) and \(\alpha5\beta1\)) to express more \(\beta1\) integrins that bind laminin (\(\alpha1\beta1\) and \(\alpha6\beta1\))\(^{78}\). In vitro studies demonstrate that fibronectin promotes brain capillary endothelial cell survival and proliferation\(^{79}\). Therefore, integrins not only bind to the ECM but can also modify cell behavior.
1.1.1.1. Integrin Structure and Function

Integrins are composed of two polypeptide subunits known as the α and β subunits. Each subunit is a glycoprotein which non-covalently associates to the other to make a heterodimer. In humans there are at least 18 α and 8 β subunit known. Every α and β combination has yet to be identified and it may not be possible for all α and β subunits to form an integrin. Currently, in humans, 24 α−β combinations have been found, these can be classified into three categories β1, β2, and αv because a majority of known integrins contain these subunits.

Across cell types the same integrin can bind to dissimilar proteins due to modifications. Cell-type-specific factors seem to modulate the binding activity of integrins. Also, divalent cations (Ca²⁺ or Mg²⁺) are crucial for the binding of integrins to their ligands; divalent-cation-binding domains are found on both α and β subunit. The integrin specificity and affinity is influenced by the bound divalent cation, therefore different cations allow the same integrin to bind to different ligands.

One way in which ECM proteins bind to cells is through a three amino acids sequence, Arg-Gly-Asp (RGD) found in binding domains. The RGD sequence is a common motif in extracellular adhesive proteins. Since the RGD sequence is common, so are the proteins that bind to it, which form a family of homologous with affinity to RGD. Peptides containing the RGD sequence compete for the binding sites on cells, so typically there are different types of adhesion proteins bound to the cells. Cell adhesion peptides can be coupled to a solid surface, causing cells to adhere to that surface. Common peptides used for this purpose are laminin, collagen, entactin and fibronectin. Even though the RGD sequence is competed for by receptors, each receptor recognizes its own set of adhesive proteins. Thus, the RGD sequence is not the only part of the adhesive proteins that is involved in receptor binding.

1.7.2. Fibronectin

The extracellular matrix contains a number of adhesive glycoproteins that bind to both cells and other matrix proteins. Fibronectin is the best characterized of the extracellular matrix proteins; it is a large fibril-forming glycoprotein found throughout the animal kingdom. Structurally, fibronectin is made of two similar but unique subunits (each almost 2500 amino acid long) which make a dimer. Fibronectin exists, as soluble dimers, and insoluble cell-surface oligomers or fibrils seen in the extracellular matrix. In the cell-surface and matrix aggregates, fibronectin dimmers are cross-linked to one another by disulfide bonds. Both fibronectin subunits have folds known as globular domains separated by lengths of unfolded polypeptide chain. These globular domains are the binding sites of specific proteins. Fibronectin can bind to collagen, heparin and specific receptors on the surface of various types of cells. The type III fibronectin repeat is among the most common of all protein binding domains, which binds to integrins, in vertebrates. There are two integrins that bind to fibronectin, α5β1 and α3β1. Integrin α5β1 is known to bind only to fibronectin and is suspected of being involved
in repair since fibronectin expression is increased around areas of injury, as seen in the CNS \(^\text{81}\). In the corpus callosum, fibronectin seems to be the principal ECM protein that astrocytes secret and that dorsal root ganglion cells depend on for axon growth, \textit{in vitro} \(^\text{82}\).

1.7.3. Laminin

Laminin is also a glycoprotein, made of three proteins chains (\(\alpha, \beta, \gamma\)) that interact to form an asymmetric cross heterodimer. The three subunits \(\alpha, \beta\) and \(\gamma\), have 5, 3, and 3 variations, respectively. Thus, a total of 45 isoforms of laminin are possible. Laminin-1 binds to collagen IV, entactin and heparan sulfate proteoglycan, in addition to binding to cells through integrin receptors and plasma membrane-associated molecules. The known integrin receptors that laminin binds to are \(\alpha_1\beta_1, \alpha_2\beta_1, \alpha_3\beta_1, \alpha_6\beta_1, \alpha_7\beta_1\) and \(\alpha_6\beta_4\) \(^\text{83}\).

The focus of this research is on NPCs, therefore let's consider how laminin is known to affect the behavior of neurons. \textit{In vivo} and \textit{in vitro}, laminin is required for neuronal migration \(^\text{84, 85}\) and adhesion, and neurite outgrowth \(^\text{86, 87}\). In development, the migratory path that neuroblasts follow contains laminin \(^\text{88}\). Furthermore, in rodents laminin is dispersed along radial glial fibers at higher concentrations in the prenatal cerebellum compared with the postnatal cerebellum \(^\text{89}\). In the prenatal cerebellum granule neurons elongate their processes along radial glial fibers suggesting that elongation is due to laminin found on the glial fibers. Supporting \textit{in vitro} work has shown that laminin alone is sufficient to provide neurite guidance \(^\text{90}\). As seen much attention has been given to migration. However, not much is known about the role that laminin plays in differentiation of NPCs.

1.7.4. Laminin vs. Fibronectin

Laminin and fibronectin differ structurally as well as functionally and the cellular responses that they evoke are different and sometimes opposing. However, there is some similitude, both laminin and fibronectin are expressed by immature cortical astrocytes \(^\text{91, 92}\); involved in migration \(^\text{90}\); and ligands to the \(\alpha_3\beta_1\) integrin.

Experiments \textit{in vitro} have compared NPCs from the brains of humans and rats on different ECM proteins in terms of migration, proliferation, morphology and differentiation \(^\text{77}\). The results show that laminin is better at promoting proliferation, influencing the cells to become neurons, facilitating migration and increasing the number of primary neurites extending from neurons, when compared to fibronectin. The differences in effects between fibronectin and laminin may be due to more \(\beta_1\) integrins binding to laminin than to fibronectin, five as apposed to two- \(\beta_1\) integrins, respectively. The availability for more interactions may allow laminin to have greater influence in NPC behavior.

1.8. Soluble Protein Interactions

Besides interactions that occur through CAMs and the ECM there are also transmembrane receptors specific for soluble factors that promote stem cell proliferation and differentiation. For NPCs, basic
fibroblast growth factor (bFGF) and epidermal growth factor (EGF) are known to maintain NPCs and promote their proliferation in vitro. Additionally, Wnt has also been shown to promote proliferation and is important in cell cycle regulation of NPCs.

The differentiation of NPCs into neurons, oligodendrocytes and astrocytes are also altered by soluble proteins which promote one of these phenotypes. First, the differentiation of NPCs into neurons is apparently promoted by platelet derived growth factor (PDGF), insulin like growth factor-I (IGF-I), interleukin-6 (IL-6; 95) brain-derived neurotrophic factor (BDNF), retinoic acid and forskolin. Second, the differentiation of NPCs into oligodendrocytes has been correlated with sonic hedge hog (SHH), PDGF, triiodothyronine, IGF-I and the inhibition of BMP signaling. Finally, astrocyte differentiation has been shown to be promoted through the IL-6 family of cytokines (e.g. LIF), BMP, and EGF (soluble factors were reviewed in 64).

Most soluble factors are secreted by cells surrounding NPCs in vivo. Thus, mature cells can assert their influence over NPCs without direct contact. Each cell type produces its own set of factors, therefore each cell type should have a different affect on NPC behavior (i.e.- proliferation, differentiation, migration, morphology).

1.9. Nano-scale Topography

In tissue engineering, the use of micron or submicron scale topography that mimics the surroundings of cells in vivo has been shown to elicit different cellular responses compared to planar surfaces. Highly ordered or random structures, such as, polymeric fibers 94, nanotubes 95, and ceramic materials 96 of nanometer dimensions are being investigated for biomedical applications. Including the developed of scaffolds for neural regeneration and bioelectrical interfaces 97. Carbon nanotubes, for example, are being researched for their use in recording and stimulating neural tissue to restore loss of function in disease or injury (reviewed by Cogan et al. 95).

Some key investigations demonstrate the applicability of nanoscale structure in neural tissue engineering {reviewed - 98, 99}. For example, twice as many NSC with elongated morphology were observed on 300 nm poly-L lactic acid nanofibers compared to smooth substrates 100. That difference was dependent on diameter of the nanofibers but not the order or arrangement. A cell that differentiates from a NSC into an elongated morphology is typically neuronal, and not oligodendroctic or astrocytic, which are highly branched or flat, respectively. Interestingly, the differentiation of embryonic stem cells into astrocytes decreased on uniaxially aligned poly (ε-caprolactone) nanofibers 101 and randomly ordered self assembled nanoscaffolds 102 compared to smooth substrates. Furthermore, mature astrocytes cultured on carbon nanotubes were not as adherent compared to those on planar surfaces 103. Thus, it is likely that on highly porous substrates such as nanofiber meshes or nanotubes arrays, a stem cell has trouble expanding into and establishing a flat astrocyte-like morphology. These discoveries are important for treatment of CNS injury where highly proliferative astrocytes can
potentially impede recovery. Thus, any control of differentiation into astrocytes would be beneficial in the development of methods to reestablish neural connectivity.

1.10. CNS Cells Interact with NPCs

1.10.1. Astrocytes and NPCs

Astrocytes, once thought to be merely support cells are now understood to serve vital roles. Astrocytes in the adult brain are in direct contact with NPCs as they proliferate, migrate and differentiate. Thus, astrocytes, the principle constituents in the surrounding environment of postnatal NPCs in the brain, contain or secrete cues that control and modify the existence of NPCs. Astrocytes are involved in routine but also critical neurogenesis where neurons must be replaced due to damage. When injury occurs, astrocyte activity increases, releasing growth factors and proteins that aid surviving neurons, promote neurogenesis, and affect synaptic organization.

Figure 1.5. Astrocytes from different brain regions and age co-cultured with AHPCs. From reprinted with permission from AAAS.
However, astrocytes throughout the CNS are not identical and do not secrete the same growth factors or promote neurogenesis; astrocytes differ according to location, function, and age. In a detailed study, astrocytes from newborn and adult rats from different areas in the brain, were co-cultured with NPCs. In this case, NPCs were derived from the hippocampus of adult rats (aka- Adult hippocampal progenitor cells (AHPCs)), and infected with a retrovirus to express GFP. AHPCs that differentiated into neurons expressed both MAP2ab (found in mature neurons) and GFP (a), and AHPCs that did not differentiate into neurons only expressed GFP (green cells with no red labeling, Figure 1.5, b). The percentage of AHPCs that differentiated into neurons (MAP2ab IR/GFP expressing) for each condition was represented as a bar graph (Figure 1.5, c).

The hippocampus was shown to contain neurogenic astrocytes in both adult and neonatal rats. However, neonatal-hippocampal astrocytes promote neurogenesis more than adult-hippocampal astrocytes (c), indicating that astrocytes in the hippocampus change as they mature. Since the nervous system is still developing a short time after birth, it is fitting of neonatal astrocytes to foster neural development.

If we compare astrocytes from different regions of the brain, from rats of the same age, we also find differences in neurogenic ability. Astrocytes from the hippocampus were most neurogenic compared to astrocytes from the spinal cord (Figure 1.5, c). These results demonstrate that astrocytes are not the same from different regions in the CNS and that astrocytes are integral in creating a neurogenic niche.

The mechanisms by which astrocytes limit or support neurogenesis, and the dependence on the place and age of the mammal that astrocytes reside in, are not well-understood. The answer possibly lies in the growth factors astrocytes secrete (soluble and surface adhesive), and the proteins associated to their membranes. Both surface-based and secreted proteins were investigated and seem to influence the adoption of the neural cell fate. The proteins that were neurogenic were Interleukins-1β and -6 (IL-1β and IL-6) at 20 ng/mL. A mixture of the proteins IL-1β, IL-6, VCAM-1, IP-10, cathepsin S and TGF-β2 caused the greatest increase on neuronal differentiation. Yet, individually all these proteins except IL-1β and IL-6 did not have an effect on AHPCs.

There is some controversy over the influence of IL-6, which has been shown both inhibit and promote neuronal differentiation. In the presence of retinoic acid, 50ng/mL of IL-6 proved to inhibit neurogenesis. Yet, IL-6 was found to be neurogenic in the absence of retinoic acid and 20 ng/mL. Yet, there are some differences between these in vitro studies that must be considered. First, the areas from which NPCs were derived are different. Therefore, the baseline differentiation profiles are dissimilar. Second, the concentrations at which IL-6 was added to the media are not equal (50 ng/mL vs. 20 ng/mL), which possibly indicates that the influence of IL-6 is concentration dependent. Additionally, other evidence suggests a dose depend response: microglial conditioned media (IL-6 is expressed by microglia), conditioned for 24h, fed to NPCs would cause cell death, but when NPCs
were fed media, conditioned by microglia for two hours, NPC health was not affected and the differenti- 
tiation prolife changed. The media that had been conditioned for a longer time most likely had 
higher concentrations of proteins secreted by the microglia which seem to be detrimental to cell sur-
vival. Third, retinoic acid combined with IL-6 maybe anti-neurogenic where as IL-6 alone is neuro-
genetic. Therefore, more studies are needed to understand how IL-6 plays a role in neurogenesis.

![Microglial response to injury in the brain](image1.png)

Figure 1.6. Microglial response to injury in the brain reprinted with permission from AAAS.

1.10.2. Activated Microglia and NPCs

Normally, IL-1β and IL-6 are secreted by activated microglia and macrophages in response to in-
jury or disease. These hormones trigger cascades that lead to the up-regulation of stress hormones 
that inhibit neurogenesis by causing inflammation (Figure 1.6). One of the downstream hormones 
produced has been shown to induce NPCs differentiation into astrocytes. Thus, one purpose of 
microglia may be to repopulate the brain of astrocytes since they are the most populous cell type in 
the brain and the most damaged when injury occurs.

![AHPC differentiation when fed microglial conditioned media](image2.png)

Figure 1.7. AHPC differentiation when fed microglial conditioned media. Reprinted with permis-
sion from AAAS.
In vitro, when AHPCs were fed activated-microglia conditioned media, neurogenesis decreased, but not gliogenesis. This is seen in Figure 1.7, where only the percentage of neuronal like cells (β Tubulin positive cells) decreased when fed media that was conditioned by activated microglia (+) as opposed to media condition by non activated microglia (-); the percentage of astrocytic (GFAP positive) and oligodendroctic (NG2 positive) cells did not significantly change in both conditions. When anti-inflammatory compounds were used, an increase in neurogenesis was shown 108. This is an important discovery because patients who receive radiative therapy experienced swelling in the brain accompanied by cognitive impairment. The cognitive impairment may be due to the cessation or decrease in neurogenesis. Therefore, restoring neurogenesis using anti-inflammatory drugs 108 may improve the overall life of a patient undergoing radiative therapy.

1.10.3. Neurons and NPCs

![Figure 1.8](image)

Figure 1.8. Differentiation of AHPC in neuron-AHPC co-culture after six days 107, reprinted with permission from AAAS

Besides astrocytes, there are also neurons, microglia, and oligodendrocytes in the neurogenic niche and these cells must also play a role in differentiation. In Figure, the percentage of differentiated AHPCs when co-cultured with primary astrocytes (red) or primary neurons (dark blue) is shown. Interestingly, when neurons are co-cultured with AHPCs, differentiation into oligodendrocytes is augmented 107 compared to mono-culture of AHPCs. Therefore, the niche where NPCs reside is also influenced by factors from pre-existing neurons.

1.11. Central Nervous System Damage

Generally, when injury occurs in the body, the immune system’s macrophages remove damaged cells and debris while releasing substances that promote healing or growth. However, the blood-spine barrier slows and limits macrophage entry only to the site of trauma where the blood-spine barrier is
When the spinal cord is injured, neurons and neural connections are destroyed and debris is created. Many glycoproteins found in the debris inhibit axonal growth. Since the immune system’s intervention is slow and restricted in the CNS (compared to the peripheral nervous system (PNS)), the injury is not properly “cleaned” and healing substances are not delivered. Furthermore, astrocyte proliferation is activated producing a physical and chemical (astrocyte secrete soluble factors) barrier, known as the “glial scar”, through which surviving neurons cannot extend their axons. Simply stated, an inhospitable environment which prevents axonal growth emerges and remains after CNS injury.

1.11.1. CNS Repair Strategies

Since the PNS can regenerate when damaged, early attempts to repair the CNS involved PNS tissue transplants. In 1980, PNS tissue grafted into damaged regions of the brain and spinal cord promoted axonal re-growth of CNS axons. Grafts composed of similar tissues, such as fetal spinal cord tissue, also showed beneficial results. Further research made nerve grafts the main approach when repairing transections of the PNS. However, additional surgery is necessary to remove donor tissue and after grafting total nerve function is not restored. Therefore, a bioengineered solution that does not depend on donor tissue that will restore total nerve function is needed in both the CNS and PNS.

Recent approaches in nerve repair investigate chemical, biological, physical and electrical stimuli to improve regenerative conditions. Chemical approaches use pharmacological agents that mimic the action of neurotransmitters and prevent cell death or interfere with inhibitors of axonal growth. Biological strategies utilize cells that secrete recuperative substances or replace damaged cells altogether (e.g. - stem cells). Physical techniques employ bridges or conduits that link together a transected nerve, and/or patterned surfaces that guide cells in the proper direction. Lastly, electrical, magnetic or electromagnetic stimulation has been applied to injured nerves in attempts to stimulate axonal growth and improve recovery time.

1.12. Electrical Stimulation

The use of electrical stimulation as an investigative or therapeutic tool was not taken very seriously 100 or so years ago. Most electro-therapeutic treatments were considered quackery, providing no beneficial outcome. In is an apparatus designed to immerse a patient in an electric field (EF) or "negative breeze" meant to cure a broad range of ailments including male pattern baldness. A metal receptor above the patient’s head and a metal plate placed beneath the patient, acted as the cathode and anode, respectively. The metal plate was connected to a static-electricity generator (inside a wooden cabinet in Figure 1.9). A patient might be occasional shocked due to faulty or incorrectly grounded wires, but no therapeutic benefit came from such a device.
Figure 1.9. 19th century “Electric Air Treatment” \cite{34,30}. Used with permission from The American Physiological Society

Such treatments were stopped by the next century, thanks to knowledge gained in human electrophysiology. However, the elimination of bogus treatments did not bring demise to electrotherapeutic treatment. Instead, the field advanced as evidence began to show correlations between electricity and anatomy. The role of electric fields (EFs) in tissues is still being investigated, but now the field of bioelectricity or electrical therapy is held with higher scientific regard.

1.12.1. Physiological Endogenous Electric Fields

The well-known action potential, one of the most significant discoveries in electrophysiology, is a momentary reversal in the potential difference across a plasma membrane (as in a nerve cell or muscle fiber) that occurs when a cell has been activated by a stimulus. The action potential is, in part, what makes neurons so unique from most other cells in the body. However, there is another electrical phenomenon discovered before the action potential, which is not usually mentioned in regular curriculum. That phenomenon is known as the injury potential and occurs when a nerve or other tissue is damaged. The injury potential is a voltage gradient established within the extracellular and intracellular space due to current flowing out of a wound.

In intact epithelium, a uniform potential is maintained perpendicular to the plane of the epithelium. Due to asymmetrically distributed membrane-bound ion channels and pumps on epithelial cells, dissimilar concentrations of ionic species are separated by the epithelium. Na⁺ channels are more numerous at the apical side of the epithelium while K⁺ channels and Na⁺/K⁺-ATPase (pump) are local-
ized at the basolateral membrane of epithelial cells. This causes the concentration of $K^+$ and $Na^+$ at the basolateral side of the epithelium to be larger than the apical side, establishing a potential difference across the epithelium. Thus, a concentration gradient drives ions to the apical side were possible, such as between the cells. Gap junctions unite epithelial cells creating a resistance to the flow of ionic species from the basolateral to the apical side of the epithelium 136.

When a break occurs across multiple cell layers, as occurs in injury, a low resistance pathway is created for ionic current flow between the basolateral and apical sides of the epithelium. Therefore, a potential gradient (or EF) with a positive vector pointing toward the wound is created. The EF that arises due to a wound is found in the subepithelium or at the basolateral side of the epithelium and runs parallel to the plane of the epithelium. The EF or injury potential guides wound healing by making cells migrate in the direction of the wound. Furthermore, the rate of mitosis increases, the mitotic spindle aligns perpendicularly to the EF and neurite outgrowth is directed in the direction of the EF vector 136.

For most cell types that respond to EFs or the injury potential, there seems to be a minimum EF strength that will elicit a cellular response and an EF strength that maximizes the response. In the case of the human corneal endothelial cells, alignment of their mitotic spindles perpendicularly to the vector of a DC EF is most prominent at 200mV/mm 137. There are two recent, illustrated, thorough and well written articles about the injury potential related to wound healing and the history of the injury potential 135, 136.

1.12.2. Electrical Stimulation of the Nervous System

Electrical stimulus has been applied therapeutically to improve the regeneration of nerve cells in the nervous system. The type of EFs used can be direct (DC), alternating current (AC), and electromagnetic. The sciatic nerve of rats is a common model used to study the regeneration of the PNS. The two types of trauma commonly studied are crush lesions and transections. After the animal is injured the electrical stimulation is applied and the results are evaluated.

1.12.3. Constant Electric Fields In Vivo

The use of constant EFs to treat nervous system injury is effective at stimulating re-growth of axons as long as the cathode is present distally, otherwise no benefits are observed 138. When an EF is created in air, electrons flows form the anode to the cathode. However, when an EF applied is applied through tissue or culture media, ions carry the charge from the anode to the cathode. The movement of ions from anode to cathode is the reason that constant EFs are generally effective only when the cathode is placed past the injury in the direction of desired growth. Conversely, if the cathode is placed proximally to the injury, the EF vector will be against the direction of desired growth, which seems to hinder regeneration 139.
Constant EFs show similar types of improvements in the regeneration of nerves. For example, electrodes placed intraluminally in transected rat sciatic nerves were used to apply a weak-DC EF. After treatment, the regenerated-axonal distance in the rats sciatic nerve was 69% longer than that of untreated rats \(^{132}\). However, the age of the treated rat has an effect on the improved rate of recovery due to constant EF treatment. A constant EF in 10-month old rats showed an increase in recovery rate (measured by a behavioral test) of 21% compared to controls \(^{140}\). However, younger rats (3 months) did not have significant improvement in recovery. The unaltered recovery rate in younger rats is thought to be due to younger rats having a better healing ability than older rats: two- to three-month rats heal 24% faster than 9 to 10 month old rats \(^{141}\).

1.12.4. Electromagnetic Stimulation In Vivo

P. Jagadeesh and D. Wilson \(^{142}\) were one of the first to explore the effects of electromagnetic fields on nerve regeneration by stimulating the median-ulnar nerves with a radio frequency signal (5-120mV/cm\(^2\)) \(^{142}\). Transected median-ulnar nerves were stimulated for 15 minutes each day for up to 60 days. From observations seen thirty days from the start of treatment, the animals treated with the pulsed electromagnetic field (PEMF) showed significant restoration of nerve conduction activity and larger diameter nerve fibers as compared to untreated rats \(^{142}\). Similarly, rats with transected sciatic nerves were completely subjected to PEMF. Treated rats regained motor function in four weeks instead of untreated rats, which regained motor function in eight weeks \(^{143}\). Additionally, sinusoidal electromagnetic fields were applied to a crushed sciatic nerve model with similar results. However, regeneration was attributed to earlier stages in re-growth of the transected nerve \(^{132}\).

1.12.5. Therapeutic Electrical Stimulation in the CNS

Jagadeesh and Wilson extended their studies of electrical stimulation from the PNS to the CNS using cats. Cats were treated with PEMF stimulation after transversely cutting half-way into the spinal cord (hemicordotomy). The PEMF treatment consisted of 50mW/cm\(^2\) at 400 pulses/second applied 30 minutes each day for one month. After three months, the spinal cord was dissected, sectioned and histologically analyzed. In treated cats, extent of scarring was reduced and neurons traversed the region of the hemicordotomy \(^{142}\). Therefore, electrical stimulation has shown potential in achieving axonal growth across the glial scar, which is one of the greatest challenges in spinal cord repair.

More recent use of electrical stimulation to repair spinal cord injury (SCI) has also been researched in guinea pigs and dogs. The research of Borgens et. al. in guinea pigs, first demonstrated that axons could grow into the glial scar and in some cases around the glial scar \(^{144, 145}\). However, axons were not shown to grow through the glial scar. Despite this, functional recovery occurred in guinea pigs with SCI treated with a 200μV/mm voltage gradient was demonstrated \(^{146}\). Behavioral recovery was studied using the cutaneous trunci muscle reflex, a useful reflex when studying SCI recovery \(^{147}\). This led to trials in dogs \(^{148}\) using implantable electrical stimulators. The trials in dogs used an oscillating
electrical field switching polarity every 15 min as opposed to the previous studies in guinea pigs where the field did not oscillate. The use of an oscillating EF on dogs with SCI showed improvement in every category of functional evaluation at 6-weeks and 6-months, with no reverse trend. The stimulators used in dogs were designed for future use in human clinical trials in humans. Recently, human phase 1 clinical trials of SCI with the oscillating field stimulation were shown to be safe and neurologically beneficial to patients. No severe adverse effects were observed after 1 year of treatment in 10 patients and there was an improvement in somatosensory tests. These trials are evidence that electrical stimulation may achieve significant therapeutic use. Yet, these EF treatments seem to be beneficial only if applied during the initial recovery period post SCI.

Other studies, that use electrical stimulation in combination with locomotive training to treat chronic SCI, have shown to help rehabilitate walking. In either case, electrical stimulation was shown to have a promising future in the regeneration of the spinal cord and possibly the entire nervous system.

1.12.6. Extracellular Electrical Stimulation In Vitro

When undertaking extracellular stimulation, one encounters the complexity of merging three fields of study into one; electrical engineering, electrochemistry and cellular biology. In engineering a system to stimulate cells in vitro knowledge must be used. The electrical stimulation apparatuses and the characteristics of the stimuli that may be applied gives researchers a myriad of conditions or stimuli to choose from. This can be frustrating when determining the appropriate variable combination that will produce a desired result. The probability of discerning those values can require many repetitions and thorough statistical analysis. Since experiments are done on live cells/tissue, one must consider possible electrochemical reactions that occur when placing electrodes in cell culture media/tissue. Electrode material must be chosen wisely so that the vitality of the cells is not affected or the electrodes are not rendered useless. Most cells are sensitive to minute changes in the media and thus any change in the media will usually negatively affect the cells. Finally, the assays used to determine the effect of electrical/electromagnetic/magnetic stimulation have to be carefully thought of and incorporated into the design. For example, if immunocytochemistry is used as an assay, multiple sets of statistically significant cell populations need to be electrically stimulated. So, when designing such an apparatus, many stipulations must be considered.

1.12.7. Stimulation Methodology

First, there are many ways to stimulate cells with a form of electricity. One thing that most devices have in common is the way in which stimulation is applied to the cells. Usually an EF is created around growing or proliferating cells. Ironically, this is essentially a scaled down version of the electric air bath that served no therapeutic purpose (Figure 1.9). EFs can be applied in two fashions, with electrodes submerged in the media and electrodes that surround the chamber where the cell are grown. In the first case an electric current can be delivered to the cells with micro-wires placed
around or on the cells, semiconductor-based multi-electrode arrays, electrically conducting polymers, agar saline bridges, or graphite rods.

Furthermore, cells can be grown on a surface that is electrically conductive and serves as one of the electrodes (working electrode). For this, most groups use a potentiostat: an instrument that holds a constant voltage, across the growth media, by varying the current in response to changes in resistance. Two electrodes are placed in the media, one is for the potentiostat to measure the voltage change (reference electrode) and the other is to deliver the electrical stimulus (counter electrode) to the cells via the media. The counter electrode acting as the opposing terminal that completes the circuit with the electrically conductive surface or reference electrode. Of course, the working electrode must allow the cells to grow without any abnormality.

Others have stimulated cells without electrode to media contact, reducing changes in pH, chances for contamination, and electrode by-products from electrochemical reactions. The caveat being that more power is needed to supply the same dose compared to electrodes placed directly in the growth media where the cells are maintained. One of the simplest ways to achieve this is to use parallel metal plates (i.e.-stainless steal) to create a capacitive EF that surrounds petri dishes. Similarly, electromagnetic stimulation through a large solenoid or with other arrangements, and magnetic fields have been used.

1.12.8. Signal Selection

Second, the electrical signal that is chosen for stimulation has many variables. The stimulus can be direct current (DC - constant voltage), alternating current, biphasic, or monophasic (pulses with only either positive or negative components). Furthermore, the signal can be modified in terms of frequency, amplitude, impulse duration, impulse delay and waveform. Typical waveforms used are square or rectangular, triangular and sinusoidal. Most function generators now allow unique waveform to be created so the signal possibilities are endless.

1.12.9. Electrode Material

When considering the material to be used as electrodes, one must consider cost, reusability, reversibility, and how the material may affect the cells. Some common electrode materials used in electrophysiology are Ag/AgCl, Pt, Au and graphite. These electrodes are used to establish an EF directly in the media, converting the flow of electrons into the flow of ions and vice versa, allowing current to be passed in a consistent manner.

Ag/AgCl electrodes are economic and reversible, yet they are exhaustible and brittle. They can also become imbalanced when using two half-cells to drive create an EF. In other words, differences in the concentration of AgCl can build up on the electrodes (Figure 1.10). This decreases or increases the amount of current that is being applied (depending on direction of applied current) from one electrode to the other. Electrodes pairs can be equilibrated by connecting and placing them in same
saline solution. To increase the lifetime of the electrodes, when using a constant current or EF, electrodes should be alternated as cathode and anode, so that the AgCl that is removed (at the negative pole) can then be regenerated (at the positive pole) and vice versa. In other words the amount of current and time that passes through the electrode as a cathode should be approximately the same when using that same electrode as an anode to limit electrode exhaustion and keep electrodes balanced.

**Figure 1.10.** Electrochemical Reactions of Ag/AgCl and Pt in Aqueous Solution modified from 181

Graphite electrodes are not easily exhaustible and do not accumulate ionic species. Graphite is a good electrode material because of high electrical conductivity; acceptable corrosion resistance; high purity; inertness with ionic species; low cost; and ease of fabrication into composite structures. However, graphite is brittle and will easily crumble when handled. Platinum is inexhaustible and does not accumulate ionic species, but is expensive. Some studies indicate that Pt dissolution occurs and electrochemical byproducts are produced when Pt is used as an electrode 182,183, but this was ameliorated when proteins were added to the media 183. Also, the pH of the media can change due to electrolysis of water (H₂ and O₂ gas release). To minimize electrolysis low currents should be used (1mA was suggested by 184). Yet, compared to other electrode materials Pt is a good choice.

1.12.10. Changes in Hydrogen Concentration

With the complexity of choosing a signal, it is logical to attempt a constant EF signal that eliminates variables (e.g. – frequency, waveform, etc) and allows any effect to be correlated with the strength
and time of stimulation. The caveat is that the pH of the media will be changed when using a constant current, so one must design a system that prevents cells from experiencing change in pH to use DC. Solving this problem can often be difficult. Most often media perfusion is a good solution, but this increases the risk of contamination.

One other solution is the use of agar salt bridges to prevent byproducts and changes in pH caused by the electrodes to enter the media. However, the saline in the salt bridges will diffuse out and media will diffuse into the salt bridge when stimulating cells for an extended period and temperatures close to the agar gelation temperature. The concentration difference between the media and agar-saline bridge drives the diffusion of salt into the media and of media into the salt bridge. Diffusion will be slower at room temperature than at incubator temperature which is 37°C. At temperatures close to 37°C agar is close or at its gelation temperature allowing diffusion in and out of the agar. Additionally, agar has been shown to cause changes in the genetic material of cells when an electromagnetic field is applied this may not be related to DC stimulation but should be taken as a precaution 185. Therefore, using agar salt bridges is an appropriate solution for experiments on cold blooded animal cells, but for mammalian cells media perfusion may be better.

1.12.11. Cellular Response to Electric Fields In Vitro

The effect of any electrical stimulation is not predictable or the same for all cell types. There is no individual signal that results in the same cellular response in all cell types. Albeit a strong electric signal that passes high enough current through the cell to cause cell death. A thorough review, written by Nuccitelli et. al, where the effects of in vitro electrical stimulation on several cells types are listed 136, shows that no single response to an identical stimulus is the same for all cell types. The cells mentioned by Nuccitelli et. al. were stimulated with DC EFs. Cells types that have been stimulated with other methodologies are: PC12 cells 159, 163, astroglial cells 186, HeLa cancer cells 187, and epithelial cells161, again, with no identical response to a particular stimulus.

The focus of this chapter is on electrical stimulation related to the nervous system; therefore studies with PC12 and astroglial cells are considered here in greater detail. PC12 cells were stimulated with rectangular impulses of 200mV and 400mV, peak-to-peak, with frequencies of 50Hz, 100Hz, 500Hz and 1 kHz for 96h. The result was that PC12 cells matured and extended neurites without the use of NGF. In other words electrical stimulation caused the cells to differentiate into more mature neurons, which normally only occurs by incorporating NGF into the media 163.

In the latter study, astroglial cells were shown to secrete NGF when an electrical stimulation was applied 186. A 10Hz, sine wave with a potential difference of +0.3V was shown to maximize the amount of NGF secreted into the media. These two studies are related since they both show that electrical stimulation affects the dependency on or the quantity of secreted NGF. These relationships help determine the mechanisms or pathways through which electrical stimulation alters cell behavior.
1.13. Neurons in Electric Fields

Despite the different cell types investigated using electrical stimulation; neurons are probably the most thoroughly researched in this context, with myocardial cells following the lead. The relationship between neurons and EFs has been investigated mostly using neurons derived from cold blooded animal, which eliminates the need for equipment such as temperature and CO₂ controlled incubators. The work of McCaig et. al. on spinal cord neurons from embryonic frogs (*Xenopus laevis*) using an agar bridge setup is a great example. McCaig et. al., investigated the effects of EFs on axon guidance or turning, axonal growth rate, growth cone receptors, secondary messengers and cytoskeletal proteins.

1.13.1. Axon Guidance

Galvanotropism occurs at different thresholds for different cells types. The same can be said about the different types of neurons and the strength of the EF needed to initiation neurites turning. Threshold as low as 7mV/mm have been reported to initiate neurite grow cone turning. If neurite turning were dependent only on EF strength, then determining an underlying mechanism would not be such a daunting task.

Surface adhesion molecules or extracellular matrix components affect the influence an EF has on cells. Neurons examined by Rajnicek et. al. turned cathodally in the presence of an EF on culture plastic (control). Neurites on laminin or on PLL with laminin remained cathodal, although not as prominent as on culture plastic. However, on PLL, neurite growth under an EF changed direction (compared to control) toward the anode. PLL is strongly cationic, these experiments demonstrated that surface charge affected neurite guidance, but does not cause a complete change in growth in an EF.

A misconception can be that neurites turn towards the cathode, because the overwhelming majority of experiments were carried out using embryonic *Xenopus* neurons to demonstrate neurites are directed towards the cathode in an EF (Figure 1.11). For motor neurons from cold blooded animals like the *Xenopus*, cathodal turning is usually true. However, neurite turning varies for other neuronal cell types and species. For example, processes from sensory neurons did not turn at all, neurites from mammalian PC12 cells (adrenal tumor cells that produce cells with neuronal properties) turned towards the anode, and rat hippocampal neurons aligned their process perpendicularly to the EF vector.
Thus, the influence an EF may have on neurites was dependent on EF strength, time of exposure, neuronal cell type and species, the ECM proteins used or the charge on the substratum (on which the cells are cultured), and if the neuronal projection is axonal or dendritic (for axonal turning). These variables create a complicated “puzzle to piece together” when determining the cellular mechanics that produce responses to EFs.

1.13.2. Cellular Level Changes in EFs

It has been proposed that neural growth cone guidance or galvanotaxis in an EF is due to an accumulation or concentration of receptor and voltage gated channels in the membrane facing the direction of movement or turning (Figure 1.12, A). The receptors and channels involved are similar to those involved in chemotropic guidance. For example, in neurons, poly-saccharide-binging plant lectins receptors, such as, concanavalin A receptor and acetylcholine receptors (AChRs) were asymmetrically distributed in an EF. Similarly, in corneal epithelial cells and fibroblasts, epidermal growth factor receptor (EGFR) became unequally distributed in an EF. These receptors were shown to accumulate on areas of the cell membrane facing the cathode.
Since more than one type of AChR exists, the dependency of neurite guidance on nicotinic and muscarinic AChR in an EF was tested. When nicotinic AChRs were blocked with D-tubocurare, cathodal turning did not occur, but when muscarinic AChRs were blocked with the antagonist atropine and/or suramin, cathodal turning was enhanced. Atropine and suramin are also P2-purinoceptor and bFGF receptor antagonists. Therefore, the interaction of P2-purinoceptors and bFGF receptors with an EF cannot be ruled out.

The release of ACh can be enhanced using neurotrophin 3 (NT-3) and BDNF as demonstrated in embryonic Xenopus neuromuscular synapses. The addition of either NT-3 or BDNF to the media of cells grown in an EF, enhanced growth cone attraction 3-fold at 150mV/mm and also reduced the threshold required for cathodal guidance. This effect was shown to be dependent on trkB and trkC (both bind NT-3, but trkC binds with greater affinity) receptors by blocking NT-3-trk receptor interac-
tion using antagonist K252a. Not all growth factors tested enhanced growth cone turning. For example, nerve growth factor (NGF) and ciliary neurotrophic factor (CNTF) had no affect on growth cone guidance. These results implicate three receptors in the regulation of EF induced growth cone turning or neurite guidance: P2-purinoceptor, bFGF receptor and AChR. Therefore, there does not seem to be a single receptor responsible for galvanotaxis or growth cone guidance by an EF. Instead, combinations of signals/receptors dictate whether cells are guided by an EF.

1.13.3. Asymmetric Accumulation of Membrane Bound Proteins

As demonstrated, some receptors accumulate at the cathode facing side of the cell membrane, in an EF. Proteins can be forced to migrate in an EF due to the charge of the protein and the charge on the cell membrane. Typically membrane bound proteins (i.e. – integrins, neurotrophic receptors) are negatively charged and so is the cell membrane. In the presence of an electric field, membrane bound proteins are acted on by: (1) electrophoretic force, as observed in Western Blot (electrophoresis in gel) and (2) electro-osmotic fluid flow due to the movement of counter ions. The electrophoretic and electro-osmotic forces are opposing in direction (for a typical negatively charged protein), attracting the protein towards the anode (negative in electrolytic cell) and cathode, respectively. The balance of these counteracting forces dictates the direction in which membrane bound proteins move. The magnitude of these forces depends on the surface charge of the protein; the relative viscosity of the membrane, to that of the aqueous phase; and the size of the membrane bound protein, the extracellular portion and the portion embedded in the cell membrane.

Surface charge can explain why sometimes negatively charged proteins don’t accumulate at one side of the cell in an EF. If the zeta potential (~surface charge) for the membrane bound protein, \(\zeta_1\), is less negative than the zeta potential for the cell surface, \(\zeta_2\), the protein will be pushed by electro-osmotic flow toward the cathode (-). Conversely, if \(\zeta_2\) is less negative than \(\zeta_1\) the membrane bound protein will move toward the anode (+) facing side of the cell by a stronger electrophoretic force. Thus, if \(\zeta_1=\zeta_2\) the protein should not move because the forces are equally balanced. This has been demonstrated experimentally with the concanavalin A receptor.

When receptors bind their ligands, a conformational change usually occurs, which can change how the receptor migrates in an EF. Therefore, cells sometimes do not respond to an applied EF if ligands are bound to their receptors. This was demonstrated using concanavalin A (Con A), which recognizes a commonly occurring sugar structure, \(\alpha\)-linked mannose, found in many membrane bound glycoproteins. The use of Con A prevented 88% of neurites from responding to a small EF; where without Con A, 75% of neurites turned towards the cathode.

Receptor migration and accumulation can affect the behavior of a cell partly because of autoregulation. A good example of this is the AChR (yellow in Figure 1.12). As mentioned previously, AChRs
accumulate at the cathode-facing side of the growth cone. Also at the growth cone, acetylcholine (ACH; green in Figure 1.12) is spontaneously released, regulating cone growth by activating nearby AChR. Since AChR accumulate on the cathode facing side of the growth cone in an EF, AChR signaling cascades initiate inside the growth cone closer to the cathode promoting preferential growth toward the cathode (Figure 1.12, A).

Additionally, the cell should receive or detect more signals from receptors and integrins where membrane bound proteins have accumulated. Thus, there is a greater propensity for the cell to respond to signals asymmetrically toward the face of the cell with the greater concentration of receptors. As demonstrated by increased growth rates of neurites facing the cathode and directed migration of epithelial cells towards the cathode.

1.13.4. Calcium

Calcium plays a very important role in mechanism of growth cone turning; substantial research by McCaig et al. has led to such a hypothesis (illustrated in Figure 1.12, B): “Cathodal turning requires influx of Ca\(^{2+}\) via voltage-gated Ca\(^{2+}\) channels (VGCC) and Ca\(^{2+}\) release from ryanodine and thapsigargin-sensitive intracellular stores. Activation of AChRs (yellow) by spontaneous release of Ach (green) induces cytoplasmic Ca\(^{2+}\) elevation further, since the receptors are “leaky” to Ca\(^{2+}\). Activation of the trkC and trkB receptors is also required for cathodal turning. Addition of NT-3, the ligand for the trkC receptor (blue) or BDNF, the ligand for the trkB receptor (magenta) to the culture medium enhances the cathodal response. This implicates the AChR further because NT-3 and BDNF stimulate release of ACh from the growth cone, therefore enhancing the asymmetric signaling via AChRs at the cathodal side of the growth cone. trkB receptors and AChRs activate the phospholipase C (PLC), phosphatidylinositol 3-kinase (PI-3K) pathway, elevating intracellular Ca\(^{2+}\) even further. Ca\(^{2+}\) elevation stimulates cAMP production via adenylate cyclase. cAMP activates the protein kinase C-dependent kinase (PKA), which affects signaling by the rho family of small GTPases (rac1, rhoA, and cdc42). Activation of rac1 and cdc42 by PKA stimulate lamellipodial and filopodial formation, respectively. This is hypothesized to underlie the EF-stimulated orientation of filopodia and lamellipodia on the cathode-facing sides of growth cones, which are essential for cathodal orientation. Inhibition of rhoA by PKA activation cathodally prevents cathodal growth cone collapse, but relatively low levels of PKA signaling anodally permit rho-mediated growth cone collapse, further enhancing growth cone asymmetry. This leads to asymmetric tension within the growth cone and turning toward the cathode. (pg 963 in 135).” Support for this theory can be visualized using fluorescent Ca\(^{2+}\) labeling within growth cones (Figure 1.12, C and D). When exposed to an EF of 120mV/mm the Ca\(^{2+}\) present in the growth cone of a neurite increased (Figure 1.12, D), compared to Ca\(^{2+}\) prior to EF exposure (Figure 1.12, C).
1.14. Neural Progenitor Cells and Electric Fields

NPCs share some characteristics with neurons; some of the same receptors and ion channels may be affected by an EF. One example is the trkB signaling cascade, which is known for promoting differentiation of NPCs. Changes in these or other membrane bound proteins may influence the trkB cascade or other signaling cascades, causing changes in differentiation, such as, the preferential adoption of a specific cell fate when NPCs are exposed to an EF. Another example is bFGF, which is known to control the proliferation of NPCs, and may also be involved in growth cone guidance as mentioned above. Furthermore, corneal epithelial cells did not migrate or orient in EF strengths below 250mV/mm unless growth factors such as EGF, bFGF and TGF-β were used. Finally, calcium channels also seem to be involved in the differentiation of NPCs into neurons. When L-type Ca^2+ channels are blocked the rate of differentiation into neurons (TUJ1/MAP2ab IR) decreases. Speculation provides possible routes for EFs to influence NPC differentiation. Once these routes are investigated, a mechanism for the differentiation of NPCs in an EF will be even more rigorous to establish than that of mature neurons, because differentiation is also complex and not well understood.

1.15. Final Thoughts

If the differentiation of NPCs were completely controllable and the use of electrical stimulation to treat CNS damage continues to improve, then cell-based and electrical-stimulatory therapies should be developed in the near future. The use of electrical stimulation as a therapeutic tool is already showing promising results in clinical trials by improving recovery in patients with SCI. NPC based therapies are also being investigated, but much progress is needed in determining what extracellular and intracellular cues may be used to effectively control and exploit the therapeutic potential of NPC. Electrical stimulation is an addition cue that needs to be further investigated in the quest to control stem cells behavior. The use of NPCs as a cell-based therapy has enormous potential to treat cases where neural cell damage is present. Despite the complexity in understanding how electrical stimulation affects CNS cells, understanding how NPCs may be used in combination with electrical stimulation is worthwhile. Combing these two fields of research could produce powerful techniques that treat injury or disease in the CNS synergistically by aiding surviving neurons and guiding new neural cells that differentiate from NPCs.
CHAPTER 2: EXTRACELLULAR MATRIX PROTEINS AND ASTROCYTE-DERIVED SOLUBLE FACTORS INFLUENCE THE DIFFERENTIATION AND PROLIFERATION OF ADULT NEURAL PROGENITOR CELLS

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2.1. Abstract

To control the differentiation of neural progenitor cells (NPCs), the synergistic influence of topography, extracellular matrix (ECM) proteins and soluble factors were investigated. Previously, in our laboratory, astrocyte-derived soluble factors were found to promote differentiation of adult hippocampal progenitor cells (AHPCs) into neurons when grown on a laminin substrate. Here we determined that the ECM protein on which AHPCs are cultured does not seem to alter this neurogenic effect or the differentiation of AHPCs when grown alone. However, AHPCs cultured on ECL (a combination of entactin, collagen and laminin) in the presence of soluble factors from hippocampal astrocytes, differentiated into a significantly greater percentage of oligodendrocytes (~34% on ECL vs. ~19% on laminin). Furthermore, a concomitant decrease in the percentage of proliferating cells was observed on the ECL (~38% on ECL vs. ~55% on laminin). Additionally, the increase in AHPC differentiation into oligodendrocytes on ECL occurred only in the presence of soluble factors from astrocytes, and...
not when AHPCs were cultured alone. Finally, we demonstrated that microscale topography did not influence the phenotypic differentiation in all conditions tested. These results show that a combination of astrocyte-derived soluble factors and ECM can dramatically affect the differentiation and proliferation of NPCs.

2.2. Introduction

The stem cell microenvironment is a complex milieu containing extracellular matrix (ECM) components, chemical factors, cell-cell interactions and distinct topography which can regulate proliferation, differentiation, guidance, adhesion, and migration of stem cells. The regulation of stem cells occurs spatially at the microscopic level where changes in the microenvironment create differences in adjacent stem cells. Determining the stimuli influencing stem cells in vivo that alter or control differentiation is crucial in harnessing the potential of stem cells to regenerate tissue with limited autonomous repair, such as the central nervous system (CNS). Here we investigate the use of the aforementioned stimuli as methods to control the proliferation and differentiation of neural progenitor cells (NPCs) for the possible use of NPCs as a cell source for rescue and repair of the diseased or damaged nervous system. Previous studies indicated that the differentiation of AHPCs cultured on poly-L-lysine (PLL) and laminin (LAM) were not influenced by micropatterning, even though AHPC processes aligned with the microscale topography. Here we determined if the null effect on differentiation due to microscale patterning was dependent on the ECM protein used to culture AHPCs. Furthermore we explored the effect of combining micropatterning, ECM proteins and soluble factors from hippocampal astrocytes. AHPCs were co-cultured with hippocampal astrocytes without any physical contact using a Transwell® culture insert system (non-contact co-culture (NCCC)). In NCCC, AHPCs are only influenced by astrocyte secreted factors, and ECM proteins were used to coat the polystyrene (PS) microscale patterned films for culturing the AHPCs. Previously, astrocytes in NCCC with AHPCs cultured on LAM, created a neurogenic environment where 64% of AHPCs differentiated into early neurons compared to approximately 14% when AHPCs were cultured alone. Thus, astrocytes created a neurogenic environment in vitro, through indirect interaction with AHPCs via secreted soluble factors. Besides LAM, no other ECM proteins were used in these previous studies, and therefore it was not determined if the neurogenic effect was dependent on the ECM protein used and on potential interactions between the ECM protein and soluble factors. Astrocyte-secreted soluble factors known to promote neurogenesis, such as IL-6 or IL-1β, may be dependent on NPCs growing on a specific ECM molecule. The binding of specific integrins to the ECM are known to alter cellular activity in NPCs, and therefore changes in integrin binding may alter the effect of soluble factors on NPCs. In the work presented here, the effects of different ECM proteins (LAM, fibronectin (FN) and ECL) on AHPCs alone or in NCCC with hippocampal astrocytes, with or without microscale topography, were explored. In other words, the role that ECM proteins can have independently and synergistically with soluble factors and/or topography were ex-
explored to increase our knowledge in the quest for spatial control of NPC differentiation on biomaterial substrates.

2.3. Materials and Methods

2.3.1. Astrocytes

Astrocytes were dissected from postnatal day two (PN2) rat pups as described by Recknor et al. All procedures performed on rat pups were in accordance with and approved by the Iowa State University Committee on Animal Care. Briefly, hippocampal astrocytes were obtained by dissection of the hippocampus followed by mechanical shear, and the enzymatic treatment with papain (20 IU/mL; Sigma-Aldrich Corp., St. Louis, MO). After subsequent treatment with trypsin inhibitor solution (10 mg/mL; Sigma), the tissue was mechanically dissociated in modified minimal essential culture medium (MMEM: Invitrogen, Carlsbad, CA). The hippocampal astrocytes were grown to confluence in 25 cm² tissue culture flasks (T-25; Falcon) in a culture incubator. The culture medium, MMEM, consisted of minimum essential medium (MEM; Invitrogen, Carlsbad, CA) supplemented with 40 mM glucose, 2 mM L-glutamine, 1 mM sodium pyruvate and 14 mM sodium bicarbonate, penicillin (100 IU/mL) and streptomycin (100 µg/mL) with 10% v/v fetal bovine serum (FBS; HyClone, Logan, UT), pH 7.35.

Enriched type-1 astrocyte cultures were prepared and maintained as previously described. To purify astrocyte cultures, less adhesive cells such as microglia and neurons were removed with rinses of cold culture media (5°C), and continuous stirring on a rotating table for 18 hours. Astrocyte cultures containing 95% or more immunoreactive (IR) cells for anti-GFAP, (Glial fibrillary acidic protein, found in astrocytes) and no IR oligodendrocytes (RIP) or neuronal cells (TUJ1) were observed (determined via immunocytochemical (ICC) analysis). No more than 5 passages were allowed for the astrocyte cultures.

2.3.2. Adult Hippocampal Progenitor Cells

Adult hippocampal progenitor cells (AHPCs) were a generous gift from Dr. F. Gage (Salk Institute for Biological Studies, La Jolla, CA). The AHPCs were isolated from the dentate gyrus of the hippocampus of adult Fischer 344 rats as reported by Palmer and colleagues. The AHPCs were maintained in 75 cm² tissue culture flasks (T-75; Fisher Scientific, Pittsburgh, PA) coated with poly-L-ornithine (10 µg/mL; Sigma) and mouse-derived laminin-1 (5 µg/mL; BD Biosciences, Bedford, MA) in phosphate-buffered saline (PBS; Invitrogen, Carlsbad, CA). The AHPCs were maintained under proliferation conditions by culturing in “complete medium” containing Dulbecco’s modified Eagle’s medium/Ham’s F-12 (DMEM/F-12, 1:1; Omega Scientific, Tarzana, CA) supplemented with N2 (Gibco BRL, Gaithersburg, MD), 20 ng/mL basic fibroblast growth factor (human recombinant bFGF; Promega Corporation, Madison, WI), and 2.5 mM L-glutamine (Gibco BRL, Gaithersburg, MD). For in vitro analysis, AHPCs were detached from a T-75 flask using 0.05% Trypsin-EDTA (Gibco BRL,
Gaithersburg, MD), harvested and collected by centrifugation at 1000g for 5 minutes. The pellets were re-suspended in complete medium without bFGF (referred to as “differentiation medium”) or co-culture medium (CCM, described below) and triturated gently. The AHPCs were seeded initially at ~94 cells/mm² on micropatterned PS substrates coated with ECM proteins. Cells were maintained at 37°C in a 5% CO₂/95% humidified air atmosphere for 6 days in culture medium. Culture media was replenished every two days.

2.3.3. Polystyrene Micropatterned Films

Polystyrene (PS) films were made from a solution of PS dissolved in toluene (0.08 g/mL), and cast or poured over wafers that were micropatterned. The pattern was fabricated on wafers using UV lithography and reactive ion etching as described by Recknor et al. The PS solution was created by dissolving PS beads (MW = 125,000-250,000, Polysciences, Inc, Warrington, PA) in toluene. After casting the solution, the wafer was covered with a large glass petri dish to prevent dust from settling on the pattern. The toluene from this solution evaporated, leaving a film of PS that is approximately 50-70 µm thick, with the shape of the micropattern cast onto the film. The micropattern consisted of parallel ridges with height, thickness and separation of 4 µm, 14 µm and 16 µm, respectively (for scanning electron microscopy images and diagram, please see supplemental materials).

2.3.4. Polystyrene Patterned Film Setup

A reservoir was created around each PS film to minimize use of ECM proteins and AHPCs. The reservoirs were made from 22mm² glass cover slips and 9/16 inner diameter PTFE (Teflon®) O-rings (Small Parts, Inc., Miami Lakes, FL) were used. O-rings were adhered to 22 mm², 0.15 mm thick glass coverslips (Fisher Scientific, Pittsburgh, PA) with Sylgard® (Dow Chemical, Midland, Michigan) a silicone adhesive, and allowed to dry for one day, and autoclaved the following day. Patterned PS films were dipped in 70% ethanol and rinsed in sterile H₂O, 1 cm² pieces were cut out using a square hole puncher (EK Success Ltd., Clifton, NJ), attached to the center of the O-ring with a small amount of Sylastic® medical adhesive (Dow Chemical, Midland, MI), and allowed to dry for a day. The next day, 0.5 mL of sterile water was added to the middle of the o-ring chamber and allowed to soak for one day to remove residual medical adhesive solvent. The coverslips (each with an O-ring and PS film adhered) were then placed in a 6-well plate and exposed to UV light for sterilization.

2.3.5. ECM Protein Coating

PS films were coated with poly-L-lysine (PLL, Sigma, St. Louis, MO) at 100 µg/mL prior to coating with laminin-1 (LAM; R & D Systems, Minneapolis, MN) or fibronectin (FN; BD Biosciences Inc, Franklin Lakes, NJ) at 10 µg/mL in Earle’s Balanced Salt Solution (EBSS; Invitrogen, Carlsbad, CA) free of divalent cations (e.g. Mg²⁺, Ca²⁺). PS films were coated with the Entactin-Collagen-Laminin cell attachment matrix (ECL; Millipore, Billerica, MA) at 10 µg/mL in DMEM/F-12 but were not pre-
coated with PLL. Patterned PS films were submerged in PLL for one day, aspirated, rinsed with sterile water, and aspirated again. LAM, FN or ECL were incubated on the PS films for approximately 1 to 3 days at 37°C, aspirated and rinsed with sterile water or cell culture media prior to AHPC seeding.

2.3.6. Non-Contact Co-Culture

In the non-contact co-cultures (NCCC), astrocytes and AHPCs were cultured in the same media, but not in direct contact. Astrocytes were seeded at approximate 100 cells/mm² on 0.4 μm pore diameter polyester membrane Transwell® inserts (Corning, Inc., Corning, NY) and allowed to grow for two days in astrocyte growth media with 10% Fetal Bovine Serum (FBS) (MMEM). Two days later, AHPCs were seeded at approximately 94 cells/mm² on 1 cm² PS films (0.5 cm² patterned with parallel grooves) within a 9/16 inch ID O-ring, in 0.5 mL of AHPC differentiation media. The cells were allowed to settle and attach to the PS film for two hours. A combination of astrocyte growth media without FBS (MMEM - FBS), and AHPC differentiation media were added to the area outside the O-rings in the 6-well petri dishes, so that when the O-rings were removed, the medias mixed and a 1:1 ratio of AHPC-Astrocyte co-culture media (CCM) was created. The O-rings were carefully removed, using sterile forceps, from each 22 mm² #1 glass cover slips that both the PS film and O-ring were adhered to. Prior to seeding AHPCs onto the PS films, the MMEM (containing FBS) in the astrocyte containing semipermeable Transwell® inserts, and the corresponding 6-well petri dishes was aspirated; the remaining MMEM was rinsed twice with EBSS, and then replaced by CCM. The astrocytes were then incubated for 4 hrs while the AHPCs were seeded and allowed to adhere to the PS films. Two hours after seeding the AHPCs, the Transwell® membrane inserts containing astrocytes were placed in the 6-well petri dishes above the AHPCs (Illustrated in Figure 2.1). The semipermeable membrane inserts allow soluble factors to diffuse across the membrane. After 6 days in vitro (DIV), the cultures were prepared for immunocytochemistry.

2.3.7. Immunocytochemical Procedures

After 6 DIV, cells were immunochemically processed. Briefly, the media was removed with two dilutions of 0.1 M PO₄ buffer, then cells on the PS micropattern were fixed using 4% paraformaldehyde in 0.1 M PO₄ buffer, and rinsed in filtered PBS 7 times with 5 minutes rinses. Cells were then incubated in blocking solution {5% normal donkey serum, 0.4% bovine serum albumin (BSA; Sigma), and 0.2% Triton X-100 (Fisher Scientific)} for 90 minutes. Cell type specific antibody markers were used to identify differentiated AHPCs. Neurons were identified using anti-class III β tubulin (TUJ1, mouse monoclonal IgG; R&D systems, Inc., Minneapolis, MN) and microtubule-associated protein antibody (MAP2ab, mouse monoclonal IgG; Sigma); oligodendrocytes, anti-RIP (mouse monoclonal IgG; Developmental Studies Hybridoma Bank); and astrocytes, anti-glial fibrillary acidic protein (GFAP, mouse monoclonal IgG; ICN, Costa Mesa, CA) were used. Primary antibodies, TUJ1, MAP2ab, RIP and GFAP were diluted with blocking solution to 1:500, 1:750, 1:1500, and 1:1000, respectively.
Additionally, bromodeoxyuridine (BrdU), a thymidine analogue that incorporates into DNA during the S phase of mitosis, was added to at least two PS substrates in each condition during the last 14hrs of the experiment. The cells which had incorporated BrdU were identified with a primary antibody against BrdU (anti-BrdU, rat monoclonal IgG; Abcam Inc., Cambridge, MA) diluted with blocking solution 1:200.

One phenotypic antibody was used per substrate; anti-BrdU was typically used in combination with the phenotypic primary antibody. Cells on 1 cm² PS substrates were incubated in primary antibodies overnight at 4°C in a humid chamber. Then they were washed in PBS with 0.1% Triton X-100, incubated in the appropriate biotinylated secondary antibodies for 1.5 hours and rinsed. Finally cells were incubated in the dark for 30 minutes with streptavidin-conjugated Cy3 (Jackson ImmunoResearch, West Grove, PA) and 4', 6-diamidino-2-phenylindole, dilactate (DAPI), at a dilution of 1:15,000 and 1:1000 in PBS, respectively. Cy3 and DAPI were used to label the primary antibodies and cell nuclei, respectively. Additionally, Cy5 (Jackson ImmunoResearch, West Grove, PA) was used to indicate anti-BrdU immunoreactivity. The PS patterned films containing cells were then rinsed with PBS, 7 times for 5 minutes and mounted onto microscope slides using a mounting medium (Gel Mount; Biomeda Corp., Foster City, CA).

Cells were observed using light microscopy (Nikon Diaphot-TMD bright field/phase contrast microscope) during culture. Images were captured with a charge-coupled device camera (Megaplus; Model 1.4; Kodak Corp., San Diego, CA) connected to a frame grabber (Megagrabber; Perceptics, Knoxville, TN) using NIH Image 1.58VDM software (Wayne Rasband, National Institutes of Health, Bethesda, MD).

Images were taken after immunocytochemical labeling using a Nikon Eclipse (Nikon Corp.) inverted microscope equipped with standard epifluorescence illumination and differential interference contrast (DIC) optics equipped with a cooled digital camera (ORCA, Hamamatsu) controlled by MetaMorph software (Universal Imaging Corporation, West Chester, PA). For cells with Cy5 epifluorescence, a Leica DM5000B (Leica Microsystems Inc., Bannockburn, IL) upright microscope equipped with a Retiga 1300i (QImaging Corporation, Surrey, BC, Canada) digital camera controlled by QCapture Pro software (QImaging Corporation, Surrey, BC, Canada) was used.

2.3.8. Quantitative Analysis of Immunocytochemical Procedures

Six to ten areas were analyzed on the patterned and the non-patterned side of each film. Two to three images were taken for each field, corresponding to fluorescent emissions of Cy3 or DAPI. The third image captured was of Cy5 fluorescent cells labeled with anti-BrdU. Nuclei and immunoreactive (IR) cells were counted semi-automatically using Image J. The sum of IR cells was divided by the sum of nuclei (DAPI-labeled) for all images per treatment to determine the percentage of immunoreactive cells for each primary antibody. In other words, the percentage of AHPCs that differentiated into neurons, oligodendrocytes and astrocytes after 6 DIV and those that were proliferating 14hrs
before the end of the experiment, were determined for each experimental condition. The background or non-specific immunoreactivity intensity was determined from cells that were exposed to secondary antibodies and CY3/CY5 fluorophore, but not primary antibodies (ICC controls). Only immunoreactive cells with emission intensity above the non-specific intensity threshold were counted.

2.3.9. Image-J Semiautomatic Quantitative Analysis

In an attempt to make cell counting automatic, a routine or macro was programmed on free imaging software, Image J\textsuperscript{214}. Images were processed in batches, and for each image the background fluorescence was subtracted, DAPI/anti-BrdU immunoreactive nuclei were encircled, and counted automatically if approximately 15 to 315\(\mu\)m\(^2\) in area. The procedure was semi-automatic because user intervention was required to include nuclei with low fluorescent intensity and/or areas not within the specified range (Figure 2.1). A log of DAPI/anti-BrdU immunoreactive counts for each image was generated and cells that were not counted were added to create a total cell count per image. For each experimental condition, all nuclei counted were summed to determine the number of cells examined. Analogously, BrdU containing nuclei were semi-automatically counted using the macro created for ImageJ.

2.3.10. Statistical Analysis

For each antibody marker (e.g. –TUJ1, MAP2ab, RIP, GFAP and BrdU) a split-plot analysis was performed. The main plot had a randomized complete block design with a 3 x 2 factorial structure. Day was treated as a random block effect. The whole plot factors were the extracellular matrix proteins (LAM, FN, or ECL) and the cells in culture (AHPCs only or AHPCs in NCCC with astrocytes). The split plot factor was the topography on which the AHPCs were cultured; half of the PS film had a micropatterned topography while the other half did not, providing a built-in control. Pairwise comparisons among levels of a factor were made for each treatment combination of the other two factors. For example, AHPCs cultured alone (1 level for astrocyte-secreted components as a factor) on LAM, FN and ECL (3 levels of ECM factors) were compared amongst each other for the patterned side (1 level for topography as a factor) of the PS film. In the previous example, there are 3 comparisons. Analogously for the smooth side, there would be three more pairwise comparisons. In total 24 pairwise comparisons were made among the conditions (or nine when pooling data from patterned and smooth surfaces). Multiple comparison adjustments for the p-values were made for these pairs of treatment combinations using Bonferroni’s Method. JMP version 6.02 (Copyright © 2006 SAS Institute) was used to analyze the data collected for all experiments.

2.4. Results
2.4.1. Differentiation

When AHPCs were cultured alone on LAM and phenotypic data were pooled across the PS substrate, ~16% of the AHPCs were TUJ1-IR, 17% MAP2ab-IR, 18% RIP-IR, and 3% GFAP-IR which are in agreement with previous results. Similarly for AHPCs cultured alone on FN, ~12% of the AHPCs were TUJ1-IR, 17% MAP2ab-IR, 14% RIP-IR, and 3% GFAP-IR; and for ECL, ~22% of the AHPCs were TUJ1-IR, 12% MAP2ab-IR, 16% RIP-IR, and 8% GFAP-IR. Comparisons between different ECM proteins, when AHPCs were cultured alone, revealed no statistically significant differences in phenotypic differentiation (Figure 2.2). When AHPCs were cultured on LAM in NCCC with hippocampal astrocytes, ~49% of the AHPCs were TUJ1-IR, 19% MAP2ab-IR, 19% RIP-IR, and 6% GFAP-IR averaging across the entire substrate. Likewise, for AHPCs in NCCC FN, ~49% of the AHPCs were TUJ1-IR, 20% MAP2ab-IR, 24% RIP-IR, and 6% GFAP-IR; and for NCCC ECL ~43% of the AHPCs were TUJ1-IR, 12% MAP2ab-IR, 34% RIP-IR, and 5% GFAP-IR (Figure 2.2). Furthermore, differences in phenotypic differentiation of AHPCs between patterned and the smooth sides of the PS films for each condition were not statistically significant (Figure 2.3).

Comparing phenotypic results for AHPCs in NCCC with hippocampal astrocytes, the largest increases can be observed for TUJ1-IR and RIP-IR cells. The phenotypic differentiation into early neurons (TUJ1-IR) on LAM increased when AHPCs were in NCCC with hippocampal astrocytes compared to AHPCs alone (AHPC alone LAM: 17% versus NCCC LAM: 49%, Figure 2.2 and Figure 2.3), which is consistent with our previous results for AHPCs on LAM in NCCC with cortical astrocytes. Yet here, the increase in early neuronal differentiation due to soluble factors from astrocytes observed on LAM prevailed on FN and ECL (AHPCs alone FN: 12% versus NCCC FN: 49%; AHPCs alone ECL: 21% versus NCCC ECL: 44%). When comparing differentiation into the oligodendrocyte fate, a significant increase was induced when AHPCs were cultured on ECL in NCCC (AHPCs alone ECL: 16% versus NCCC ECL: 34%), yet no other ECM tested produced a significant increase in oligodendrocyte differentiation when AHPCs were in the NCCC condition. However, for the astroglia and mature neuronal phenotypes (GFAP and MAP2ab, respectively), significant differences in differentiation were not observed between AHPCs cultured alone and in NCCC conditions (Figure 2.2, and Figure 2.3). These results demonstrated that the ECM protein, on which AHPCs were cultured, altered the differentiation into a specific phenotype (i.e. - oligodendrocytes) in the presence of hippocampal-astrocyte derived soluble factors. Surprisingly, increased oligodendrocyte differentiation occurred without a significant decrease in AHPC differentiation into any of the other phenotypes tested, and despite a majority of AHPCs differentiating into early neurons (TUJ1) in NCCC. Thus, the total percentage of AHPCs that differentiated in NCCC ECL compared to other conditions was increased.

To support this result, the percent of proliferative cells during the last 16 hours of the experiment was assayed using BrdU (Figure 2.4, BrdU-IR). The data gathered indicated no significant differences in
AHPC proliferation between the micro-patterned and smooth side of PS films for all conditions; or between all ECM components used to culture AHPCs alone (AHPCs alone LAM: 59%, FN: 60% and ECL: 63%). However, soluble factors from hippocampal astrocytes, in synergy with ECL, significantly reduced the percent of proliferative cells compared to soluble factors and other ECM proteins, or compared to ECL alone (NCCC ECL: 38% versus NCCC LAM: ~55% or NCCC FN: 58%, or compared to AHPCs alone on ECL: 63%, respectively).

The significant differences mentioned were visually apparent before performing a quantitative analysis. NCCC conditions displayed more TUJ1-IR (leftmost column, Figure 2.5) cells compared to AHPCs cultured alone, where NCCC conditions had more cells with fluorescent processes axially aligned, reminiscent of neuronal morphology. Stunning differences were observed in NCCC ECL conditions, where oligodendrocyte-like cells (RIP-IR, center column, Figure 2.5) were more numerous and highly branched, covering a large portion or all of the field of view. The processes of oligodendrocytes and early neurons were usually found confined to the corridors or grooves of the microscale pattern (not quantitated). In NCCC ECL, fewer BrdU immunoreactive nuclei (green in the rightmost column of Figure 2.5) were observed compared to all other conditions. Finally, fewer cells were typically observed for AHPCs alone, cultured on FN, where the mean cells/mm² for FN was lower than all other conditions (data not shown).

2.5. Discussion

A dramatic shift in differentiation of AHPCs toward any of the assayed phenotypes could not be attributed to the use of LAM or FN, when AHPCs were cultured alone. However, a combination of ECM proteins (ECL) and soluble factors from the astrocytes in NCCC conditions increased differentiation into oligodendrocytes significantly compared to other conditions, which indicates cellular changes are induced with combinations of ECM proteins not pure ECM substrates. This may be due to increased signaling received from different ECM proteins, causing a greater variety of integrin receptors to interact with the substrate (e.g.- cells on ECL can interact with laminin, collagen type IV and entactin, not laminin only). It is also possible that ECL interacts with soluble factors to form a complex(s) or may accumulate soluble factors increasing substrate concentration of soluble factors that promote oligodendrogenesis and decreased proliferation. The accompanying decrease in proliferation (BrdU-IR) observed for AHPCs in NCCC on ECL agrees with the notion that the ECL substrate and astrocyte factors combined, stimulate AHPC differentiation towards oligodendrocytes decreasing the percentage of cells that are mitotic. Especially since the differentiation into other cell types did not drastically change in NCCC on ECL, the data indicate that the decrease in proliferation was due to an increase in oligodendrocyte differentiation.

ECL contains traces of growth factors that may have influenced the differentiation of AHPCs in NCCC toward the oligodendrocyte fate. To determine this, individual growth factors must be tested in combination with NCCC with hippocampal astrocytes. The growth factors present in ECL are similar to
those found in Matrigel™, IGF-I, PDGF, NGF, and TGF-β, and research indicates that some of these growth factors can increase the differentiation of NPCs into oligodendrocytes. Specifically, PDGF is known to promote bipotent O2-A progenitor proliferation and differentiation into oligodendrocytes at 5 ng/mL or 10 ng/mL (for a review on factors affecting gliogenesis see Lee, et. al.). Furthermore, IGF-I is known to promote the differentiation of AHPCs into oligodendroglia, at comparable percentages to our results, when applied at 20 - 500 ng/mL. However, PDGF and IGF-I are present at lower concentrations in Matrigel™: 12 pg/mL and 15 ng/mL, respectively (Millipore Inc.). Furthermore, the ECL solution used to coat our PS substrates was diluted 1:100 from the manufacturer stock solution, and then aspirated after coating. Thus, the concentrations of PDGF and IGF-I should have been greatly reduced once the culture well was filled with media and cells were cultured on the PS substrates. It is likely that an insignificant increase in RIP-IR would be attributed to traces of PDGF and IGF-I found when AHPCs were cultured on ECL in NCCC with hippocampal astrocytes. However, this increased differentiation into oligodendrocytes was not observed when AHPCs were cultured alone on ECL. Therefore, soluble factors from hippocampal astrocytes were not considered to magnify the effects of trace PDGF and IGF-I in NCCC ECL; instead ECL most likely altered the effect that soluble factors had on AHPCs, increasing the differentiation into oligodendrocytes. The majority of Matrigel™ is laminin, 58% (Millipore Inc.), but LAM alone did not produce the same effects on AHPCs in NCCC. Therefore, to determine if the increase in RIP-IR and decrease in proliferation in NCCC ECL can be attributed to a single component in ECL, collagen and entactin (nidogen-1) should be investigated. Collagen for example, decreased the proliferation of Schwann cells, in similar research. Nidogen is a ubiquitous glycoprotein that bonds non-covalently with laminin and collagen type IV to create a structural network/framework of the basal lamina. Both, nidogen and collagen type-I (not collagen type IV as used here) has been shown to promote the survival and proliferation of Schwann cells, which are analogous to oligodendrocytes in the CNS. The ECL combination is closer to what NPCs may encounter in vivo since neurogenic niches are not composed of individual ECM components. The possible number and type of integrin receptors that interact with ECL should be greater than those that interact with FN or LAM. Finally, the maturation of neurons, as indicated from the MAP2ab IR (Figure 2.3) was not conclusively influenced by the use of different ECM proteins. The mean for each column in Figure 2.3 shows that FN accelerates the establishment of the neuronal phenotype when AHPCs are grown alone and in NCCC, compared to ECL. However, this difference is not statistically significant. In similar research, human and mouse NPCs grown on FN did not produce as many neurons (MAP2ab immunoreactive cells) as NPCs cultured on LAM, 6% vs. 8% (human), and 1% vs. 10% (mouse), respectively; additionally, migration and cellular process growth were slightly less proficient on FN as on LAM. Here however, an increase in MAP2-IR cells was not observed when comparing any substrate. We believe
that extending our experiment for longer durations would allow for NPCs to become mature neurons (MAP2ab immunoreactive) and allow differences between ECM substrates to become more apparent. Purified ECM components may not influence the differentiation profile of AHPCs grown alone or in NCCC, and combinations of ECM proteins may be more effective at overcoming or synergistically working with potent soluble growth factors. Generally, it is possible that soluble factors have greater influence over cell behavior than the ECM, as is the case with bFGF which controls proliferation or IL-6 and IL-1β which are powerful neurogenic factors secreted from astrocytes. The data presented here, demonstrated that FN and LAM influenced the differentiation of AHPCs in a similar way, and that the ECM proteins alone were not as influential as soluble factors, from hippocampal astrocytes, at altering the differentiation of AHPCs (i.e. - differentiation into neurons). These findings can benefit the development of cell based tissue engineering strategies for nervous system repair. Biodegradable conduits could be selectively coated with a combination of ECM proteins such as ECL to increase the number of oligodendrocytes in select spatial locations in the conduits for the treatment of de-myelinating diseases like multiple sclerosis. A successful cell based strategy will likely be a combination of multiple approaches: micro or nano topography to provide guidance to cellular processes; soluble factors released slowly by incorporating these into biodegradable polymers to further promote differentiation into a desired cell type (e.g. - IGF-1 for oligodendroglial differentiation); and ECM proteins to further control cell fate and proliferation. Therefore the knowledge presented here should help understand how NPCs can be controlled and bring us closer to developing a cell based strategy for nerve repair.

2.6. Acknowledgments

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Figure 2.1. Illustration of one well in a 6 well culture plate containing an astrocyte and AHPC non-contact co-culture. A semipermeable membrane insert, suspends the astrocytes above AHPCs cultured on 1cm² polystyrene film (half micro-patterned). Astrocyte-secreted molecules permeate through the membrane and affect AHPCs. In “AHPC alone” conditions, AHPCs were cultured on the PS films without a hovering suspension of astrocytes. SEM images of AHPCs on PS films were modified from Recknor et al. 2006.
Figure 2.2. Florescent nuclei semi-automatically counted with a software script we developed for Image J to process multiple images rapidly. Nuclei labeled with DAPI or immunocyto-chemically with anti-BrdU were, automatically counted and circled when they were above an automatically determined background intensity. Nuclei (i.e.-white arrows) not counted or over-counted by the program were manually accounted for to corrected cell counts.
Figure 2.3. Percentage of AHPCs that were immunoreactive for neuronal (TUJ1 and MAP2ab), oligodendroglial (RIP) and astroglial (GFAP) markers, after 6 days in vitro. Significant differences were not observed between smooth and micro-patterened side of the PS films, therefore the data were averaged for each ECM substrate when AHPCs were cultured alone (LAM, FN, ECL) or in the presence of hippocampal astrocyte soluble factors (NCCC LAM, NCCC FN, NCCC ECL). Significant contrasts were AHPCs cultured alone versus NCCC conditions, and AHPCs cultured alone versus NCCC ECL, where approximately 30% and 18% more AHPCs differentiated into early neurons and oligodendrocytes, respectively. N (number of experiments) = 3 to 7. For each phenotypic marker, conditions not connected by the same letter or character were significantly different, p<0.006. Values are mean ± SEM.
Figure 2.4. Percentage of AHPCs that were IR with phenotypic antibodies for neurons (a; TUJ1 IR cells, N (number of experiment) = 4 to 7); mature neurons (b; MAP2ab IR, N= 3 to 5); oligodendrocytes (c; RIP-IR cells, N = 4 or 5); and astrocytes (d; GFAP-IR cells, N = 3 or 4) after 6DIV on the pattern and smooth sides of the PS film. Conditions not connected by the same letter are significantly different, p<0.0021. Values are mean ± SEM.
Figure 2.5. Percent of cells that went through the S phase of mitosis during the last 16 hours of the experiments as indicated by the percent of BrdU-IR cells. Data presented are for AHPCs on the smooth and patterned sides of the PS films separately and pooled together. N (number of experiment) = 3 to 5. Conditions not connected by the same letter are significantly different, p<0.0021. Values are mean ± SEM.
Figure 2.6. Epi-fluorescent images of antibody immunoreactivity in AHPCs that had significant differences between conditions (within each antibody). Each column represent a different immunocytochemical labeled and each row represent the different condition to which the entire PS films were exposed. Images here are for the patterned side of the PS films. Alignment of cellular processes can be observed in TUJ1 and RIP IR cells. The major notable differences are the increase in NPC differentiation of oligodendrocytes (RIP IR) and early neurons (TUJ1), in NCCC, ECL vs. all NCCC conditions, and all NCCC conditions vs. culture of only NPCs. Furthermore, fewer proliferative (BrdU containing cells) were seen for NCCC ECL conditions. Similar results were observed for NPCs cultured on the non-patterned side of the PS films (not shown). All nuclei were labeled with DAPI. Scale bar = 50um.
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CHAPTER 3: TITANIUM DIOXIDE NANOTUBE ARRAY AFFECTS NEURAL STEM CELL DIFFERENTIATION

Modified from an original manuscript to be submitted to Nanoletters

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3.1. Abstract

This study provides the first investigation of how highly ordered nanotube arrays can be used to control the differentiation of neural stem/progenitor cells (NSCs). Decreased NSC differentiation into astrocytes was observed on highly ordered TiO\textsubscript{2} nanotube arrays compared to planar substrates such as glass, while differentiation into neurons or oligodendrocytes was not altered. Furthermore, adhesion may have been slightly improved on nanotubes. This demonstrates that it is possible to control NSCs differentiation through nanoporous/nanotubular structures.

3.2. Manuscript Text

The development of a methodology to control stem cell differentiation into specific cell types is crucial in implementing a cell based approach to potentially ameliorate central nervous system (CNS) diseases, disorders or damage. Cell based therapies for the CNS may eventually be based on the control of adult neural stem/progenitor cells (NSCs)\textsuperscript{223-226}. Methodologies to control NSC proliferation and differentiation include, but are not limited to, use of soluble molecules such as leukemia inhibitory factor and bone derived neurotropic factor\textsuperscript{64}; extracellular matrix proteins that interact with integrin receptors\textsuperscript{77, 209-211, 227}; electric\textsuperscript{228} or electromagnetic\textsuperscript{229} fields; and substrate architecture\textsuperscript{102, 230}. 
Nanoscale substrate architecture and topography, especially, has shown the most interesting effects on NSCs. Mats of electrospun polymeric nano-fibers have demonstrated to be useful in the developing field of tissue engineering\textsuperscript{231}. For example, laminin-coated electrospun polyethersulfone fiber meshes increased NSC differentiation into neurons and oligodendrocytes, and that increase was found to be dependent on nano-fiber diameter\textsuperscript{230}. Furthermore, processes aligned\textsuperscript{100} and grew longest\textsuperscript{100, 232} on highly ordered fibers compared to random nanofibers.

Here we investigate another important nano-structure: nanotubes. Previously, multilayer single walled carbon nanotube (SWNT) films were demonstrated to be biocompatible with embryonic NSC\textsuperscript{233}. However, SWNT films are not highly ordered, and therefore the “tactile” stimulus that NSCs receive is probably not equivalent for each cell. To our knowledge we are the first to culture NSCs and investigate their differentiation on highly ordered nanotube arrays. The physical stimulation that NSCs received here is not random for each cell, and changes to NSC behavior can potentially be related to a measurable dimension, such as nanotube diameter. Our aim was to determine if highly ordered tubular nanoscale arrays can be used to control NSCs.

Nano-scale topography is potentially beneficial for the culture of cells due to improved adhesion and control of differentiation compared to smooth substrates. Essentially, the nanotubes provide greater surface to area for the cells to adhere on. Better cell adhesion leads to better integration of biomedical devices in the body. For example bone formation around TiO\textsubscript{2} oxide implants has been improved by changing the diameter of nanotubes on the surface\textsuperscript{234}. Implants that integrate well into the bone are very desirable and have shown promising results even in dentistry\textsuperscript{235}. Furthermore, increased healing rates have been attributed to increased differentiation into the appropriate phenotype\textsuperscript{236} due to surface modified materials. TiO\textsubscript{2} is used extensively in bone repair since it is generally known to be biologically inert. Inevitably MSC contained in bone marrow come into contact with TiO\textsubscript{2} biomedical implants. Therein lies the reasoning for many investigations into the effects of nano-structure on MSC\textsuperscript{96, 237, 238}. However, the response of NSCs to these surfaces had not been studied previously, even though MSC have the ability to differentiate into neurons and glia\textsuperscript{239}.

Highly ordered TiO\textsubscript{2} nanotube (60 nm) arrays were fabricated according to our previously reported procedure\textsuperscript{240-242}. Briefly, Ti foil (99.7% purity, 0.25 mm thick, Sigma-Aldrich) was cut into pieces with dimension of 1 inch x 0.5 inch, followed by degreasing in acetone, isopropanol, and methanol sequentially in an ultrasonication bath. TiO\textsubscript{2} 60 nm nanotube arrays were grown on the Ti foil by potential-static electrochemical anodization in fluorine containing electrolytes. TiO\textsubscript{2} 60 nm arrays with pore diameter of 60 nm were formed by anodizing Ti foil in 0.5 wt% hydrofluoric acid in an ice bath with anodization potential of 20 V for 30 minutes. After anodization, the Ti foil with nanotube arrays grown on it was immediately rinsed with large amount of DI water, followed by drying with N\textsubscript{2} gas. The as-prepared TiO\textsubscript{2} nanotube arrays were amorphous, and anatase crystalline phase was induced by thermal annealing at 500°C for 3 hours, which was confirmed by Raman scattering\textsuperscript{240-242}. The nanos-
tructure of TiO2 nanotube arrays was characterized with scanning electron microscopy (SEM, JEOL 5800 LV).

The NSCs were a generous gift from Dr. F. Gage (Salk Institute for Biological Studies, La Jolla, CA). NSCs were isolated from the dentate gyrus of the hippocampus of adult Fischer 344 rats as reported by Palmer and colleagues \(^{23}\). They were then maintained in 75 cm\(^2\) tissue culture flasks (T-75; Fisher Scientific, Pittsburgh, PA) coated with poly-L-ornithine (10 \(\mu\)g/mL; Sigma) and mouse-derived laminin-1 (5 \(\mu\)g/mL; BD Biosciences, Bedford, MA) in phosphate-buffered saline (PBS; Invitrogen, Carlsbad, CA). NSCs were propagated in "complete medium", which consisted of Dulbecco's modified Eagle's medium/Ham's F-12 (DMEM/F-12, 1:1; Omega Scientific, Tarzana, CA) supplemented with N2 (Gibco BRL, Gaithersburg, MD), 20 ng/mL basic fibroblast growth factor (human recombinant bFGF; Promega Corporation, Madison, WI), and 2.5 mM L-glutamine (Gibco BRL, Gaithersburg, MD). NSCs were harvested from a T-75 flask using 0.05% Trypsin-EDTA (Gibco BRL, Gaithersburg, MD) and collected by centrifugation at 1000g for 5 minutes. The pellets were re-suspended and triturated gently in complete medium without bFGF and with 5% FBS. NSCs were seeded at 100 cells/mm\(^2\) in 35mm petri dishes (Fisher Scientific) containing one 12mm glass coverslip and a TiO\(_2\) nanotube array. TiO\(_2\) arrays and 12mm coverslips were cleaned with boiling RBS-35™ concentrate (Chemical Products R. Borghgraef S.A., Fisher Scientific) detergent (1:50 in DI water) and rinsed repeatedly in 18MΩ water prior to cell culture. Substrates were then coated with poly-L-lysine (100 \(\mu\)g/mL) and laminin-1 (10 \(\mu\)g/mL). Culture conditions were maintained at 37\(^\circ\)C in a 5% CO\(_2\) / 95% humidified air atmosphere for 6 days in culture medium. Cell culture media was replenished every two days.

After 6 days in vitro (DIV), the cells were immunohistochemically processed. Briefly, the media was removed with two dilutions of 0.1 M PO\(_4\) buffer. Then the NSCs on 12mm glass coverslips or TiO\(_2\) nanotube arrays were fixed using 4% paraformaldehyde in 0.1 M PO\(_4\) buffer, and rinsed in filtered PBS 7 times with 5 minutes rinses. Cells were then incubated in blocking solution {5% normal donkey serum, 0.4% bovine serum albumin (BSA; Sigma), and 0.2% Triton X-100 (Fisher Scientific)} for 120 minutes. Cell type specific antibody markers were used to identify differentiated NSCs. Early neurons were identified using anti-class III β tubulin (TUJ1, mouse monoclonal IgG; R&D systems, Inc., Minneapolis, MN); oligodendrocytes were identified using anti-RIP (mouse monoclonal IgG; Developmental Studies Hybridoma Bank); and astrocytes using anti-glial fibrillary acidic protein (GFAP, mouse monoclonal IgG; ICN, Costa Mesa, CA) were used. Primary antibodies, TUJ1, RIP and GFAP were diluted with blocking solution at 1:500, 1:1500, and 1:600, respectively.

One phenotypic antibody was used per substrate. Cells were incubated in primary antibodies overnight at 4\(^\circ\)C in a humid chamber and repeatedly rinsed in PBS. Next, cells were incubated in the appropriate biotinylated secondary antibodies and 4', 6-diamidino-2-phenylindole, dilactate (DAPI), at 1:60 in blocking solution for 90 minutes and rinsed. Finally, cells were incubated in the dark with streptavidin-conjugated Cy3 (Jackson ImmunoResearch, West Grove, PA) for 30 minutes, at
1:15,000 in PBS. The substrates were then rinsed repeatedly with PBS and mounted onto microscope slides using a mounting medium (Gel Mount; Biomeda Corp., Foster City, CA).

To observe and photograph immunocytochemical labeling, a Nikon TE2000 (Nikon Corp., Melville, NY) inverted microscope equipped with standard epifluorescence illumination and equipped with a cooled digital camera (Cascade® 512B or Coolsnap® EZ; Photometrics, Tucson, AZ) controlled by MetaMorph software (Universal Imaging Corporation, West Chester, PA) was used.

Ten to twelve areas were imaged under fluorescent illumination. Two images were taken for each field analyzed, corresponding to fluorescent emissions of DAPI and CY3, and superimposed (Figure 3.2). Nuclei and immunoreactive (IR) cells were counted semi-automatically using Image J (see supporting information). The percentage of NSCs that differentiated into neurons, oligodendrocytes and astrocytes after 6 DIV, were determined for each experimental condition. The background or non-specific immunoreactive intensity was determined from cells that were exposed to secondary antibodies and CY3 fluorophore, but not to primary antibodies (ICC controls). Only immunoreactive cells with emission intensities above the non-specific intensity threshold were considered positively labeled. ANOVA was performed on data of antibody marker counts (e.g. – TUJ1, RIP, and GFAP) using JMP version 8.0.1 (Copyright © 2006 SAS Institute) to statistically analyze the data collected for all experiments.

After 6 day of culture, the density of neural progenitor cells seemed greater on the nanotubes as indicated by DAPI staining (Figure 3.2). Cell counts of fluorescent micrographs images were used to quantify the cell density after immunocytochemical procedures (Figure 3.3). The mean values indicated fewer cells on the glass coverslips than on the nanotubes (163 ± 42 vs. 219 ± 42, p = 0.19). However, statistical analysis indicated no difference between the NSCs density on 60 nm nanotubes arrays compared to glass coverslips.

The percentage of NSCs that differentiated into each of the three neural phenotypes (Figure 3.2 and Figure 3.4) was determined. Out of four experiments, the least squared mean percentage of TUJ1 immunoreactive NSCs on glass and 60 nm TiO2 nanotubes was found to be 41.1 ± 4.7% and 35.9 ± 4.4%. Similarly for RIP immunoreactive cells, the percentage of RIP positive cells for the control and 60 nm TiO2 nanotubes was 36.3 ± 6.7% and 29.7 ± 5.2%, respectively. The least mean square percentage of TUJ1 and RIP immunoreactive cells were statistically consistent over the three conditions. Most importantly, the percentages of GFAP immunoreactive cells in the control and 60 nm TiO2 nanotubes were 16.5 ± 2.6% and 6.7 ± 2.5%. Thus, there were significantly fewer GFAP positive cells on the 60 nm TiO2 compared to glass (n = 4, p < 0.034).

The only other investigation of growth and differentiation of NSC on nanotubes by Jan and Kotov is very different from our study. In our study, the nanotubes here were highly ordered and aligned vertically in an array, as opposed to being randomly dispersed in a film. Second, NSCs here were derived from an adult rat, and not an embryonic mouse. Third, TiO2 and not carbon was used to cre-
ate the nanotubes. We demonstrate a decrease in the differentiation into astrocytes (GFAP\(^+\)) without changes to the differentiation into neurons or oligodendrocytes (TUJ1\(^+\) and RIP\(^+\), respectively) on highly ordered TiO\(_2\) nanotube arrays. In contrast, embryonic NSCs differentiated mostly into astrocyte-like cells (~50\%) and their differentiation was not altered by carbon SWNT \(^233\). At the site of spinal cord injury, where active gliosis leads to the glial scar \(^243-245\), it would not be desirable for transplanted NSCs to differentiate into astrocytes. Instead the reestablishment of neuronal connections and remyelination of those connections by new neurons and oligodendrocytes, respectively, is necessary. Thus, modifying the surface of grafts with highly ordered nano-porous topography using TiO\(_2\) nanotube arrays may increase the regeneration efficacy of transplanted NSCs. The nano-topography would minimize NSC differentiation into astrocytes, while maintaining the differentiation of NSC into neurons and oligodendrocytes.

Also, a possible method of drug delivery via nanotubes has been demonstrated chemically \(^246\). Therefore, besides the topographical influence that nanotubes provide, pharmacological agents or growth factors could also be released at a specific time to induce differentiation before or after transplantation. For example insulin-like growth factor-1 could be used and released to cause differentiation into oligodendrocytes. Similarly, “nano-patterned” surfaces may eventually be tailored with polymer coatings \(^247\) to further biologically interact with its surroundings.

We have previously investigated the use of microscale substrate architecture to control NSC \(^129,248\). The major advantage of nanoscale over microscale is that it tends to be more biologically interactive altering growth, survival, and differentiation \(^249\). Hence, some nanoscale architecture is considered more biologically active and more capable of integrating with cellular function. Use of free nanoscale particles or nanotubes however, may negatively affect cell health and viability. Free titanium dioxide nanoparticles have been demonstrated to be cytotoxic to human neural crest cells and fibroblasts \(^250\). Furthermore, nanowires and nanotubes can become incorporated into the cytoplasm and cause cytotoxic effects \(^251\). In the lungs, TiO\(_2\) nanofibers and other ultrafine-TiO\(_2\) particles produced pulmonary fibrosis regardless of particle size \(^252,253\). Therefore, the use of nanotube arrays as opposed to free nanotubes circumvents these potential issues.

Studies of NSCs on electrospun nanofibers have shown that NSCs differentiate preferentially into neurons or oligodendrocytes. However, they do not demonstrate a decrease in GFAP expression as presented here for NSCs on nanotubes. This is important for therapeutic applications because prevention of the “glial scar” would allow neurons to reestablish connections after spinal cord injury. This may lead to applications were the glial scar is prevented or ameliorated through the use of a nanoporous material to treat of spinal chord injury. The size of the TiO\(_2\) nanotube in the arrays can be potentially controlled \(^240-242\) to investigate the effect of nanotube diameter on NSC differentiation. With progress, a highly ordered nanoporous topography may improve the propagation of NSC colonies used in transplants, or induce NSC differentiation into specific phenotypes in transplanted tissues.
3.3. Acknowledgments

We thank Christian Tormos for diligently taking a multitude of microscope images. Many thanks to Jisun Oh for maintaining and managing a well kept colony of NPCs. Finally, we appreciate the help of Dr. Curtis Mosher in microscopy. Financial support granted from The National Science Foundation’s Alliance for Graduate Education and the Professoriate (NSF-AGEP), and The National Institutes of Health (NIGMS 1 RO1 GM072005).

3.4. Supporting Information Paragraph

Semi-automatic Image Analysis- In an attempt to make cell counting automatic, a routine or macro was programmed on free imaging software, Image J \(^{214}\). Images were processed in batches, and for each image, the background fluorescence was subtracted. DAPI immunoreactive nuclei that were approximately 15 to 315 \(\mu m^2\) were encircled, and counted automatically. The procedure was semi-automatic, as user intervention was required to determine the total cell count by manually counting nuclei with low fluorescent intensity, clumped nuclei, and/or areas out of the specified range. For each experimental condition, all nuclei counted were summed to determine the number of cells examined. The total DAPI stained nuclei divided by the total actual area photographed yielded the cell density (cells per mm\(^2\)) for each treatment.
Figure 3.1. Scanning electron microscopy (SEM) characterization of TiO$_2$ nanotube arrays. Nanotubes used for cell NSC culture had an average pore diameter of 60 nm (Scale bar = 1 µm).
Figure 3.2. Neural Stem/Progenitor Cells immunocytochemically labeled with antibodies to determine phenotypes of differentiated cells. DAPI was used to label DNA in nuclei. No drastic changes in morphology or differentiation were apparent. However, there appeared to be fewer NSCs on the glass substrates compared to the nanotube arrays. (Scale bar = 100 µm)
Figure 3.3. Cell density was statistically similar between glass coverslips and nanotube array substrates.
Figure 3.4. The differentiation of NSC on 60 nm Nanotubes compared to glass coverslips. Statistically similar percentages of TUJ1 and RIP positive cells were observed. However, fewer astrocytes were observed on the nanotube arrays (p = 0.0151; n = 4).
CHAPTER 4: ALTERED NEUROGENESIS, ALIGNMENT, AND CELL VIABILITY: THE INFLUENCE OF ELECTRIC FIELDS ON HIPPOCAMPAL NEURAL PROGENITOR CELLS

Modified from an original manuscript to be submitted to The Journal of Stem Cells

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4.1. Abstract

The differentiation and proliferation of neural progenitor cells (NPCs) are dependent on various in vivo environmental factors or cues, which may include an endogenous electrical field (EF), as observed during nervous system development and repair. In this study, we investigate the morphologic, phenotypic, and mitotic alterations of adult hippocampal NPCs that occur when exposed to two EFs of endogenous strength. NPCs treated with a 437 mV/mm continuous EF aligned perpendicularly to the EF vector and had a greater tendency to differentiate into neurons, but not into oligodendrocytes or astrocytes, compared to controls. Yet fewer cells were observed in the continuous EF, which in part was due to a decrease in cell viability. A 46 mV/mm alternating EF was applied to determine if an alternating EF could cause changes in NPC differentiation. However, the alternating EF of this magnitude showed no major differences, compared to control conditions. Similarly, the percent of mitotic cells during the last 14 hours of the experiment was not statistically different between all treatments. To our knowledge, this is the first evidence demonstrating the effects of EFs on the differentiation of NPCs. Further investigation and application of EFs on stem cells is warranted to elucidate the utility of EFs to control phenotypic behavior. With progress, the use of EFs may be engineered to control differentiation and target the growth of transplanted cells in a stem cell-based therapy to treat nervous system disorders.
4.2. Introduction

Endogenous EFs occur during development of the central nervous system (CNS)\textsuperscript{254, 255}, in wound healing\textsuperscript{136} and in the healthy\textsuperscript{256} or highly active epileptic adult brain\textsuperscript{257, 258}. So, both in development and in the adult endogenous electric fields can be found. In the adult, evidence of EFs has been demonstrated from recording of extracellular potential gradients of the hippocampus. In a isolated intact or “injured” hippocampus an outward and inward current were recorded, respectively\textsuperscript{259}. In vivo hippocampal recording of steady potential gradients of 4 mV/mm have been recorded under physiological conditions\textsuperscript{256}, from 0.5 to 4 mV/mm during normal slow wave activity\textsuperscript{260, 261}, and up to 50 mV/mm after induced synchronous activity of granule cells\textsuperscript{262}. During epileptic seizure, steady potential gradients of 10 mV/mm\textsuperscript{257} and EFs up to 20 mV/mm\textsuperscript{258} were recorded. These EF are very important, as disruption of endogenous EF causes developmental abnormalities demonstrated in chick and Xenopus embryos\textsuperscript{255, 263}, and reduces the rate of wound healing\textsuperscript{136}. Also, drastically important for proper generation, repair or maintenance of the nervous system are neural progenitor or stem cells (NSCs)\textsuperscript{46} which proliferate and differentiate into neurons and glia\textsuperscript{8-11}. In the adult brain, one of the areas known to contain neural progenitor cells (NPCs) is the hippocampus\textsuperscript{46, 264}. These adult NSCs may be influenced by the endogenous EFs. For example, rat models with varying degrees of status epilepticus (continuous seizure) all had increased neurogenesis in the adult dentate gyrus in the hippocampus, and the survival of these neurons depended on the degree of seizure severity or activity\textsuperscript{131}. Similarly, after stroke\textsuperscript{265} or in hypoxic ischemia\textsuperscript{266}, NPCs have been shown to migrate, differentiate and integrate in areas of the brain where they otherwise would not. Could occurrences of endogenous EF be another cue that alters the residing population of adult NPCs; for example to induce differentiation, migration and integration in the developed nervous system in learning, or to ameliorate nervous system damage?

To our knowledge, the analysis of adult NPC differentiation, alignment, proliferation, and viability in an in vitro culture system within an EF have not been investigated. The application of EFs on nervous system cells has been predominantly on amphibian cells\textsuperscript{135, 197, 198, 200, 255, 267, 268}, but response of cells to EF exposure is not the same for all cell types or species\textsuperscript{136}. Culture of mammalian CNS cells in EFs has been previously explored. Embryonic hippocampal neurons were subjected to EFs of 28 to 219 mV/mm\textsuperscript{193} and astrocytes with 500 mV/mm\textsuperscript{269}, in both cases cellular alignment became perpendicular to the EF vector. Recently, EFs of 50, 100 and 250 mV/mm directed migration of NSPCs (emerging out of neurospheres) towards the cathode, in a dose dependent manner and that migration was dependent on N-methyl-D-aspartate receptors\textsuperscript{270}. However, the role of EFs on the differentiation of multipotent stem cells is unknown. Here, the extent of NPC exposure to EFs surpasses that of the sole study performed on NSPCs\textsuperscript{270} by three days. Our objective was to investigate and characterize the effects of EFs on NPCs, and to assess our ability to use EFs to control their differentiation and proliferation. Furthermore, NPC migration occurs after injury in the brain\textsuperscript{265}, thus increasing the
amount of NPCs that reach the site of injury using an EF would be beneficial. The application of an EF may amplify the therapeutic ability that NPCs exercise on the CNS. The utilization of EF treatment combined with stem cell therapy may become a rational strategy to facilitate neuro-regeneration and repair in order to treat a variety of neurodegenerative diseases and CNS injury (Alzheimer’s or Parkinson’s disease, spinal cord injury and other previously non-treatable damages to the CNS 1, 63, 135, 223, 225, 226).

4.3. Materials and Methods

4.3.1. Neural Progenitor Cell Cultures

 Neural progenitor cells (NPCs) were a generous gift from Dr. F. Gage (Salk Institute for Biological Studies, La Jolla, CA). The NPCs were isolated from the dentate gyrus of the hippocampus of adult Fischer 344 rats as reported by Palmer and colleagues 23. The NPCs were maintained in 75 cm² tissue culture flasks (T-75; Fisher Scientific, Pittsburgh, PA) coated with poly-L-ornithine (10 µg/mL; Sigma) and mouse-derived laminin-1 (5 µg/mL; BD Biosciences, Bedford, MA) in phosphate-buffered saline (PBS; Invitrogen, Carlsbad, CA). The NPCs were maintained under proliferating conditions by culturing in “complete medium” containing Dulbecco’s modified Eagle’s medium/Ham’s F-12 (DMEM/F-12, 1:1; Omega Scientific, Tarzana, CA) supplemented with N2 (Gibco BRL, Gaithersburg, MD), 20 ng/mL basic fibroblast growth factor (human recombinant bFGF; Promega Corporation, Madison, WI), and 2.5 mM L-glutamine (Gibco BRL, Gaithersburg, MD). For in vitro analysis, NPCs were detached from a T-75 flask using 0.05% Trypsin-EDTA (Gibco BRL, Gaithersburg, MD), harvested and collected by centrifugation at 1000g for 5 minutes. The pellets were re-suspended and triturated gently in complete medium without bFGF and with 5% FBS. NPCs were then seeded at 100 cells/mm² within stimulation chambers for experimental observation. Culture conditions maintained at 37°C in a 5% CO₂ / 95% humidified air atmosphere for 6 days in culture medium. Culture media was replenished every two days.

4.3.2. Stimulation Apparatus

Electric field exposure chambers were constructed based on work described by McCaig et al. and Alexander et. al. 189, 269, but modified for sterile mammalian cell culture over an extended period. Stimulation chambers were created by adhering machined Lexan® polycarbonate pieces with two 0.3 mm x 14.5 mm x 40 mm recesses (Figure 4.1, A and B) to 50 mm x 70 mm glass slides using Sylgard. Ag/AgCl electrodes were placed in 25 mL bottles containing Dulbecco’s modified Eagle’s medium/Ham’s F-12 (DMEM/F-12, 1:1; Omega Scientific, Tarzana, CA) with penicillin (100 IU/mL), and streptomycin (100 µg/mL) on both sides of the chamber. The electrodes were provided a constant electrical current from an electrophoresis system power supply (FB 600; Fisher Scientific, Pittsburg, PA). Glass tubes containing 1% agarose (42°C gelation temp; Sigma-Aldrich, St. Louis, MO) gels made in Dulbecco’s modified Eagle’s medium/Ham’s F-12 with 5% Fetal Bovine Serum
(FBS, Atlanta Biologicals Inc., Laurenceville, GA), penicillin (100 IU/mL) and streptomycin (100 µg/mL) connected the electrodes to wells containing media that supplied the NPCs in the apparatus (Figure 4.1, C). The electrodes convert the electron current to a Cl⁻ ion flow from the cathode to the anode through the agarose gel bridge. The ion flow generated a consistent EF through the media. The strength of the EF (E) produced was calculated by the formula: E = Ip/A, using the intensity of the electric current (I), the resistivity of the media (ρ = 655 ± 6 Ω•mm), and the cross sectional area of the chamber (A= 4.35 mm²). The resistivity of the media at 37°C was measured using a conductance meter (YSI 35; YSI, Inc., Yellow Springs, OH).

The machined polycarbonate pieces and glass slides were cleaned by boiling in diluted RBS-35 detergent concentrate (Pierce, Rockford, IL). They were then rinsed repeatedly with purified, filtered water (18 MΩ Millipore, Billerica, MA) and sterilized by exposure to UV light for 30 minutes. Each glass slide was aligned to the longest side of its corresponding machined polycarbonate part before adhering the two parts. After allowing the adhesive to dry for two days, 70% ethanol was flushed through the chambers and the apparatus was rinsed with distilled water (Gibco BRL, Gaithersburg, MD). Chambers were then coated with poly-L-lysine (100 mg/mL) and laminin-1 (10 mg/mL). NPCs (with culture media) were seeded at a concentration of 100 cells/mm² on the glass slides within chambers.

4.3.3. Electric Field Treatment

NPCs were treated with two different EFs of physiological strength: a 46 mV/mm alternating EF (0.338 mAmps) with a 30 minute period and square bi-polar waveform, and 437 mV/mm continuous EF (3.383 mAmps). The magnitude of EFs in embryos are typically less than 100 mV/mm but approximately 1800 mV/mm across the neural tube. In vitro, the effects on mammalian brain cells have been investigated for EFs between 1 and 500 mV/mm, thus we chose to stay within this range. The alternating EF frequency was chosen based on EF treatment of spinal chord injury in clinical trials. The alternating and continuous EF treatments were applied to the NPCs using a 15 MHz waveform generator (Hewlett-Packard Company, model 3321A; Palo Alto, CA) electrophoresis power supply (FB600 and for time-lapse FB 452; Fisher Scientific Pittsburg, PA), respectively. EF treatment began 4 to 6 hours after NPCs were seeded in the chambers. The continuous EF treatment was applied for 16 to 24 hrs a day for the first 3 days, and on the final day of each experiment. To avoid electrode exhaustion in the continuous EF treatment, electrodes were alternated as the positive and negative electrode (while maintaining the EF vector direction by rotating the chambers 180 degrees) after each day of stimulation. Media in the electrode-containing bottles was replaced after pH changes were noticeable, due to extended stimulation. The alternating EF was applied for the duration of each experiment. NPCs in control conditions did not receive EF treatment but were cultured in the custom culture apparatus. Culture media in the chambers (where the NPCs resided)
was replenished every two days. NPCs were maintained at 37°C in 5% \( \text{CO}_2 \) / 95% humidified air atmosphere for the entire 6 days \textit{in vitro} (DIV).

4.3.4. Cell Viability

Lactate dehydrogenase (LDH) assay kit from Sigma-Aldrich (St. Louis, MO) was used to determine the viability of cells after one day of treatment. LDH content in the media of each experimental condition was determined colorimetrically. Colorimetric readings were normalized between 0 and 1, corresponding to positive and negative controls, respectively. Positive controls consisted of killed cells using 0.2% triton-X-100 in media. Negative controls were NPCs in the apparatus that received no EF treatment. Cell density was determined along with immunocytochemical quantification (see below).

4.3.5. Immunocytochemistry

After 6 DIV, cells were immunochemically processed. Briefly, the media was removed with two dilutions of 0.1 M \( \text{PO}_4 \) buffer, and NPCs were fixed on the 50 x 70 mm\(^2\) glass slides using 4% paraformaldehyde in 0.1 M \( \text{PO}_4 \) buffer, and rinsed in filtered PBS 7 times with 5 minutes rinses. Cells were then incubated in blocking solution {5% normal donkey serum, 0.4% bovine serum albumin (BSA; Sigma), and 0.2% Triton X-100 (Fisher Scientific)} for 120 minutes. Cell type specific antibody markers were used to identify differentiated NPCs. Early neurons were identified using anti-class III β tubulin (TUJ1, mouse monoclonal IgG; R&D systems, Inc., Minneapolis, MN); oligodendrocytes, anti-RIP (mouse monoclonal IgG; Developmental Studies Hybridoma Bank); and astrocytes, anti-glial fibrillary acidic protein (GFAP, mouse monoclonal IgG; ICN, Costa Mesa, CA) were used. Primary antibodies, TUJ1, RIP and GFAP were diluted with blocking solution at 1:500, 1:1500, and 1:600, respectively. Additionally, bromodeoxyuridine (BrdU), a thymidine analogue that incorporates into DNA during the S phase of mitosis, was added to all experimental conditions 14hrs before the end of experiments. The cells which had incorporated BrdU were identified with a primary antibody against BrdU (anti-BrdU, rat monoclonal IgG; Abcam Inc., Cambridge, MA) diluted with blocking solution to 1:200.

The area of each glass slide containing cells within the chambers was divided using a PAP Pen (Electron Microscope Sciences, Hatfield, PA) to apply the different phenotypic antibodies aforementioned; anti-BrdU was typically used in combination with the phenotypic primary antibody. Cells on 50 x 70 mm\(^2\) glass slides or coverslips were incubated in primary antibodies overnight at 4°C in a humid chamber and repeatedly rinsed in PBS. Next, cells were incubated in the appropriate biotinylated secondary antibodies and 4', 6-diamidino-2-phenylindole, dilactate (DAPI), at 1:60 in blocking solution for 90 minutes and rinsed. Finally, cells were incubated in the dark with streptavidin-conjugated Cy3 (Jackson ImmunoResearch, West Grove, PA) for 30 minutes, at 1:15,000 in PBS. Additionally, Cy5 (Jackson ImmunoResearch, West Grove, PA) was used to indicate anti-BrdU immunoreactivity. The
50 x 70 mm² glass slides containing cells were then rinsed repeatedly with PBS and mounted onto microscope slides using Gel Mount (Biomeda Corp., Foster City, CA).

4.3.6. Microscopy

Cells were observed using light microscopy (Nikon Diaphot-TMD bright field/phase contrast microscope) during culture. Images were captured with a charge-coupled device camera (Megaplus; Model 1.4; Kodak Corp., San Diego, CA) connected to a frame grabber (Megagrabber; Perceptics, Knoxville, TN, in a Macintosh computer; Apple Computer, Cupertino, CA) using NIH Image 1.58VDM software (Wayne Rasband, National Institutes of Health, Bethesda, MD). For immunocytochemical labeling, a Nikon TE2000 (Nikon Corp., Melville, NY) inverted microscope equipped with standard epifluorescence illumination and a cooled digital camera (Cascade® 512B or Coolsnap® EZ; Photometrics, Tucson, AZ) controlled by MetaMorph software (Universal Imaging Corporation, West Chester, PA) was used. Time-lapse videos were captured with a cooled mono-chrome 12-bit digital camera (Retiga; QImaging, Surrey, Canada), mounted on a Leica DMIRE2 (Leica Microsystems, Wetzlar, Germany) controlled through OpenLab v. 3.5.1.

4.3.7. Quantitative Analysis of Immunocytochemistry

Images of NPCs in the chambers were captured where the EF would be uniform in the EF treated experimental conditions (as indicated Figure 4.2, A). Ten to twelve areas were analyzed per primary antibody. Two to three images were taken for each field analyzed, corresponding to fluorescent emissions of DAPI, CY3 and/or CY5. Nuclei and immunoreactive (IR) cells were counted semi-automatically using Image J 214. The sum of IR cells was divided by the sum of nuclei (DAPI-labeled) for all images per treatment to determine the percentage of immunoreactive cells for each primary antibody. In other words, the percentage of NPCs that differentiated into neurons, oligodendrocytes and astrocytes after 6 DIV and those that were proliferating 14hrs before the end of the experiment, were determined for each experimental condition. The background or non-specific immunoreactive intensity was determined from cells that were exposed to fluorescent secondary antibodies (CY3/CY5) only. Only immunoreactive cells with emission intensities above the non-specific intensity threshold were counted. JMP version 8.0.1 (Copyright © 2006 SAS Institute) was used to analyze the data collected for all experiments.

4.3.8. Image-J Semiautomatic Quantitative Analysis

In an attempt to automate cell counting, a macro was programmed using ImageJ 214. Images were processed in batches, and for each image the background fluorescence was subtracted, DAPI/anti-BrdU immunoreactive nuclei that were approximately 15 to 315 µm² were encircled, and counted automatically. The procedure was semi-automatic, as user intervention was required to determine the total cell count by manually counting nuclei with low fluorescent intensity, clumped nuclei, and/or
areas out of the specified range. For each experimental condition, all nuclei counted were summed to determine the number of cells examined. Likewise, BrdU-immunoreactive (IR) nuclei were semi-automatically counted using the macro created for ImageJ. The total DAPI stained nuclei divided by the total actual area photographed yielded the cell density (cells per mm²) for each treatment.

4.3.9. Quantitative Analysis of Cell Morphology

To determine cellular alignment, images were processed using ImageJ (NIH). Briefly, images were converted to 8-bit after thresholding image brightness to make the cellular processes of each cell visible. The Particles8 plug-in for ImageJ was used to determine the angle between the longest axis of each cell with the horizontal of the image. Cell angles were then adjusted relative to the EF vector, absolute from 0° to 90°, with 0° and 90° indicating cellular alignment parallel and perpendicular to the EF vector, respectively. For clarity, the outline of a cell at 6 DIV and the angle, θ, made by a line between its furthest points with the EF is shown (Figure 4.2, A). Furthermore, primary processes emanating from cells were counted and separated into quadrants as shown in Figure 4.3, similar to Cork et al. Alignment and process count data were pooled from all experiments and the distribution for each experimental treatment was determined.

4.4. Time-lapse Digital Imaging and Quantification of Migration

Similarly, the migration rate was determined from time-lapse images using the Manual Tracking plug-in for ImageJ. Time-lapse recordings were initiated 6-12 hours after NPCs were plated into chambers, with images captured every 5 minutes for 12-36 hours. Cells were maintained in the custom chamber, on a heated stage at 37°C. All media were supplemented with 10 mM HEPES. A thin film of mineral oil was used to prevent evaporation and changes in pH due to gas exchange at the media-air interface, similar to Cell migration was quantified as shown by Yao et al. Briefly, the distance of cell displacement was measured as the length between starting point and the final cell position of the migrating cell (in μm). It was calculated from pixel coordinate data using the Pythagorean theorem. The rate of cell displacement was calculated based on the distance of cell displacement in a given time. The cell track velocity was calculated from the full distance of cell migration in a given time. Finally, the mean directedness index of total cell movement was calculated from \( \sum \cos \Phi \), where \( \Phi \) is the angle between the EF vector and the cellular translocation direction, and N is the total number of cells from at least three experiments. Thus, the mean directedness index would equal 1 or -1 if all cells migrated directly toward or away from the cathode (-), respectively.

4.4.1. Statistical Analysis

ANOVA was performed on data of antibody marker counts (e.g. – TUJ1, RIP, GFAP and BrdU), cellular alignment angles, and all quantities derived from time-lapse digital imaging. The EF treatment and the date of each experiment were set as fixed and random effects, respectively. To determine statis-
tical significance, a student’s t test was used with adjustments for the p-values using the Bonferroni’s Method. A Chi-Squared test was performed to determine if the angle of cellular alignment was evenly distributed for each EF treatment. JMP (v. 8.0.1) or SAS (v. 9.1) (Copyright © 2006 SAS Institute), were used to analyze the data collected for all experiments.

4.5. Results

4.5.1. Cell Alignment

The cellular alignment of NPCs relative to the EF vector is displayed in Figure 4.2. The alignment of each NPC’s longest axis was evaluated for 1648, 910, and 880 NPCs cultured in a 437 mV/mm continuous EF, 46 mV/mm alternating EF or no EF, respectively (n = 4). The data revealed an uneven distribution (chi-squared test for equal proportions, p < 0.0001) with the majority of NPCs aligned perpendicularly to the EF vector (49.1% between 70° and 90°, compared to only 5.3% of NPCs that aligned between 0° and 20°). In contrast the distributions of the alternating and non-stimulated conditions were approximately even (Chi-squared test for equal proportions, p = 0.1614 and 0.7891). Comparisons between the control and alternating conditions revealed no statistically significant differences in alignment. The cellular alignment in the continuous EF was significantly greater compared to non-stimulated and alternating EF conditions (p < 0.001). The trend towards perpendicular alignment of NPCs in the continuous EF and the contrast between this and the other conditions is clearly evident in Figure 4.2.

4.5.2. Process Growth Inhibited Anodally and Promoted Perpendicularly

We quantified the number of processes emanating out of the NPC somas and grouped these counts into quadrants. The quadrants corresponded to the anode (+), cathode (-), left and right facing sides, as shown in Figure 4.3, A. Since the control and 40 mV/mm alternating EF were similar in alignment, only cells cultured without EF and in the 437 mV/mm EF treatment were analyzed. From the analysis (Figure 4.3, B) it was determined that significantly fewer primary processes emanated out toward the anode facing side (+) compared to the cathode (-) and the mock anode on NPCs in control conditions (0.63 ± 0.09 vs. 0.97 ± 0.03 and 1.12 ± 0.11, respectively; p<0.0001, n=4). Furthermore, significantly fewer processes emanated from sides of the soma perpendicular to the EF vector, compared to control cells (left side – 1.38 ± 0.10 vs. 1.13 ± 0.12, right side - 1.28 ± 0.13 vs. 1.08 ± 0.14 , respectively; p<0.0001, n=4). Results were similar when comparing the left and right sides to the cathode and anode sides for cells treated with the 437 mV/mm EF (p<0.0001). Thus, in an EF, process growth is promoted at sides that are perpendicular to the EF, and inhibited at the anode (+) facing side.
4.5.3. EF Influences NPC Differentiation

For each condition, fluorescent microscope images were analyzed for nuclear labeling with DAPI and primary antibody (TUJ1, RIP, GFAP, and BrdU) immunoreactivities (Figure 4.4). Cells immunoreactive for the primary antibody and the total DAPI and BrdU were counted. The percentage of NPCs that differentiated into each of the three neural phenotypes was determined (immunoreactive cells for each phenotypic primary antibody) and divided by total DAPI labeled nuclei (Figure 4.5). The least squared mean percentages of TUJ1 immunoreactive cells for the control, 46 mV/mm alternating and 437 mV/mm continuous EF conditions were found to be 24.1 ± 1.7% (± S.E.M.), 23.8 ± 1.9% and 41.7 ± 2.2%. The percentage of immunoreactive TUJ1 cells is significantly greater in the continuous EF conditions when compared to control and alternating EF conditions (n = 6, p < 0.001). The percentage of RIP immunoreactive cells for the control, 46 mV/mm alternating and 437 mV/mm continuous EF conditions were 24.5 ± 3.6%, 22.3 ± 3.1% and 18.6 ± 3.5%, respectively. Finally, the percentages of GFAP immunoreactive cells in the control, 46 mV/mm alternating and 437 mV/mm continuous EF groups were 13.7 ± 2.3%, 9.6% ± 3.7% and 14.9% ± 4.4%. The least mean square percentage of RIP and GFAP immunoreactive cells was statistically consistent over the three conditions.

4.5.4. Bromodeoxyuridine (BrdU) Cell Birth-Dating Analysis

Bromodeoxyuridine was used to label newly generated cells during the final 14 hours of cell culture. The percentages of cells immunoreactive for BrdU was 19.1 ± 6.7%, 29.7 ± 6.7%, and 31.7 ± 7.6% (n = 3) for the control, alternating EF, and continuous EF treatments, respectively. Statistical analysis revealed no significant difference in proliferation between the cells with and without EF treatment (Figure 4.6, B).

4.5.5. Cell Density

After 6 DIV, cell density was assessed by determining the number of DAPI labeled nuclei in the area of each image (Figure 4.6, A). The continuous EF treatment had a significantly lower number of cells per area, 136 ± 45 cells/mm² compared to the control, 268 ± 45 cells/mm² (n=6, p = 0.017). However, no significant differences were observed between the control and alternating EF (209 ± 41 cells/mm²).

4.5.6. Lactate Dehydrogenase Assay

To determine if NPC viability was being negatively affected by the EF treatment, the amount of lactate dehydrogenase (LDH) was assayed, after 1DIV. The data revealed significantly greater LDH content in the 437mV/mm EF treatment compared to the control (17.8 ± 4.0 vs. 0.0 + 4.0 %, respectively; n=3, p=0.001). In contrast no statistical difference was observed between cultures of NPCs not treated with an EF and treated with a 46 mV/mm alternating EF (3.2 ± 5.2%).
4.5.7. EF Influences NPC Migration

We examined NPC migration in control and continuous EF treatments for 8 hours via time-lapse digital imaging. The alternating EF was not analyzed because of its similarity to control conditions. Clearly the superimposed paths of migrating NPCs (Figure 4.7, A) indicate that EF influenced and directed the migration of adult NPCs. NPCs were displaced an average of $114.2 \pm 15.4 \, \mu m$ from the origin in the $437 \, mV/mm$ EF, which was significantly further than in control conditions ($32.7 \pm 19.0 \, \mu m$ (p = 0.0242; Figure 4.7, B). The direction of migration (Figure 4.7, C) was significantly greater towards the cathode (-) in the continuous EF than in controls (Directedness index- $0.43 \pm 0.3$ vs. $0.01 \pm 0.04$, respectively; p = 0.001). At 8 hours, the mean track velocity in the $437 \, mV/mm$ continuous EF was, surprisingly, not significantly different than in controls ($26.1 \pm 1.6 \, \mu m/h$, respectively; Figure 4.7, D). Similarly the displacement rate during each 5 min interval averaged over 8 hours, was also not significantly different between conditions ($28.9 \pm 3.4 \, \mu m/h$, respectively, p =0.18; Figure 4.7, E).

4.5.8. Changes in Migration Depend on Exposure Duration

To determine if changes in migration parameters were dependent on the duration of EF treatment, each time interval was analyzed during the first 85 min. Time dependence analysis revealed that characteristics of migration were dependent on EF exposure duration and became asymptotic after approximately 30 min (Figure 4.7, F and G). Differences in directedness between EF and the control conditions increased with time and were significant from 5 min (p=0.0025, 408 cells; Figure 4.7, F) onwards. Similarly, for displacement, significant differences were observed starting at 25 min (p =0.010, 405 cells; Figure 4.7, G). In both cases, directedness and displacement differences became very significant with an additional 5 min (p<0.0001, n=3, student t-test). However, significant differences were not observed between NPC track velocity in the continuous EF compared controls (data not shown).

4.6. Discussion

We have investigated and observed how EFs affect mammalian NPCs. To our knowledge, this is the first study to examine the effects of EF exposure on the differentiation into CNS phenotypes (neurons, oligodendrocytes, and astrocytes), proliferation and viability of adult mammalian NPCs. Only one other study explored changes in cellular viability due to EFs treatment in vitro. Our alignment results are in agreement with the majority of prior investigations of mammalian nervous system cells treated with EFs, which aligned perpendicular to the EF vector in a continuous EF.

4.6.1. Electric Field Induced Neurogenesis

The preferential differentiation of EF-treated NPCs into neurons agrees with similar published results for PC12 cells. Yet PC12 cells, are derived from an adrenal tumor, and when exposed to nerve
growth factor (NGF) develop into cells with the capacity to sprout neurites, generate action potentials, and form synapses. In an EF, PC12 cells developed into neurons without the use of NGF. However, PC12 cells do not have the potential to become oligodendrocytes or astrocytes like multipotent NPCs. EFs may promote the differentiation into neurons by depolarizing transmembrane potential; in similitude to culturing NPCs in media with an elevated K⁺ concentration, which depolarizes NPCs and increases the differentiation into neurons.

Indirect influence on the differentiation of NPCs could potentially be attributed to EF effects on differentiated astrocytes and oligodendrocytes. Astrocytes have been shown to release greater quantities of NGF when stimulated with an EF. We can speculate that endogenous EF may also indirectly influence NPCs to differentiate into early neurons or mature into neurons through increased astrocytic production and secretion of the potent neurogenic growth factor, NGF. Thus, the endogenous EF found in development or after injury to the nervous system may favor the production of new neurons by influencing NPCs synergistically via membrane potential depolarization and increased neurogenic receptor stimulation (increased neurotrophic factor secretion by mature nervous system cells influenced by the endogenous EF). However, there are a multitude of receptors potentially involved in galvanotaxis and galvanotropism, which are also related to NPC differentiation. For example, N-methyl D-aspartate (NMDA) receptors were recently demonstrated to be involved in the directed migration of micro-explants containing NSPCs, and augmented stimulation of NMDA receptors increased proliferation and neuronal differentiation in the adult hippocampus. Thus, NPC migration and neuronal differentiation seem to be linked together at least through the NMDA receptor. Our work creates a stronger link between the electrical activity in the adult mammalian brain (normal or injured) and the resident populations of NPCs. The increase we see in neuronal differentiation agrees with increased neurogenesis in the hippocampus of epileptic models, where elevated firing of neurons produces an extracellular electric potential. What has not been previously alluded to is that, in the hippocampus, the differentiation of NPCs into oligodendrocytes or astrocytes may not be altered by extracellular EF activity (applied or created by heightened neuronal activity), as indicated by our results. Thus, it seems that neurogenesis is favored in an endogenous EF in the adult CNS. Indeed, transplanted neural human stem cells into the brains of epileptic rat models produced inhibitory interneurons that reduced seizure reoccurrence. Furthermore, it may be that injury induced EFs promote the production of new neurons, partially compensating for an environment conducive to active gliosis and glial scar formation.

4.6.2. Aligned Morphology

The overall alignment of NPCs was visibly perpendicular to the continuous EF vector as shown in light microscopy images taken throughout the 6 days in vitro (DIV) and in the fluorescent microscope images obtained after immunostaining procedures at 6DIV (Figure 4.4). Moreover, a greater number of processes were observed emanating out of NPCs on the sides perpendicular/lateral to the EF...
compared to other sides in the 437 mV/mm EF and controls. In agreement, EF treated cultures of
disassociated rat brain cells, such as astrocytes and neurons and neurons grew perpendicular to the EF
vector. The perpendicular alignment of neonatal rat cortical astrocytes was proportional to the EF
strength or time of exposure, with a maximum response at 500 mV/mm for 10 hours. Similarly,
Alexander et al. demonstrated that astrocytes aligned perpendicularly to the EF vector when ex-
posed to an EF of 500 mV/mm, for a duration of 24hrs. Furthermore, the presumptive axons of rat
hippocampal neurons derived from 18-day-old fetus aligned perpendicularly to the EF vector in EF of
29-290 mV/mm, but were not affected by EFs of less than 9 mV/mm. Thus, the results found in
literature agree that mammalian brain cells align perpendicularly in an EF as is seen in this study. It
seems that the ionic distribution inside the cell is changed the least at the sides of the cell that are
perpendicular to the EF. Therefore, if changes in the trans-membrane potential negatively affect
process growth, then process growth would be least likely to change perpendicular to the EF vector.
In addition to the longest axis aligning perpendicularly, we qualitatively (Figure 4.2, C) and quantita-
tively (Figure 4.3, B) determined that fewer primary processes emanated out of the anode facing side
(+) compared to all other sides of NPCs in the 437 mV/mm EF, and the mock anode facing portion of
NPCs in control conditions. Thus, process growth is inhibited at the anode-facing side of the cell in
an EF. Growth was definitely not promoted at the cathode (-) facing side, as the number of proc-
esses were the same on the side of NPCs that faced the mock cathode in controls. Furthermore,
cathodally directed processes were not qualitatively more robust or longer than processes emanating
from other areas. Without a proper simultaneous morphologic analysis of EF treated and non-treated
cells, claims of promoted cathodal growth are not as conclusive. Thus, we are in disagreement
that an EF would make process growth faster at the cathode (-) facing side. Some controversy sur-
rounds the use of EFs and the claim that neural processes (neurites) grow faster in an EF. Similar effects have been observed for neurite growth of PC12 cells, where alignment was
described to be both perpendicular and toward the anode (+) when exposed to a 30 mV/mm EF for
48hrs. Decreased cytoskeletal architecture (decrease in actin polymerization) in human umbilical
vein epithelial cells and in human mesenchymal stem cells and osteoblasts demonstrate one
reason for inhibited growth on the anode (+) facing side of cells. Changes in intracellular Ca²⁺
concentration indicate that the Ca²⁺ channels are mechanistically involved in directed growth and
migration of cells in EFs. Recently, neonatal rat hippocampal neurons (dissociated from micro ex-
plants) exposed to EFs up to 300 mV/mm for 1 - 1.5 hours directed their golgi, nucleosomes,
neurites, and migration asymmetrically toward the cathode. Besides these morphological results,
mild turning of cathodal processes was qualitatively observed, not as drastic as observed for
Xenopus neurons. The mechanism for alignment, migration and differentiation most likely involves membrane receptor
movement due to electrophoresis and electro-osmosis (competing forces). These two forces are
considered to cause the migration of different receptors towards the electric poles depending on the size and charge of the receptor. Plus, an asymmetric charge distribution of the transmembrane potential may slightly inhibit or facilitate the opening of ion channels, depending on which electrode the membrane is facing. There are potentially many membrane components involved in induced neuronal differentiation and directed migration of NPCs, such as Ca\textsuperscript{2+} channels and NMDA, epidermal, basic fibroblast and transforming (\(\beta\)-1) growth factor, and acetylcholine receptors. Furthermore, EF induced directionality has been shown to be dependent on substrate, serum and cell type.

4.6.3. Small Alternating EF has No Effect

The frequency of the 46 mV/mm alternating EF was chosen based on clinical trials to treat spinal cord injury with an alternating EF applied in vivo in an attempt to guide the growth of motor neurons and restore function and sensation to patients. The idea of switching the polarity every 15 min was derived from the finding that “a small population of cultured Xenopus neurites was stimulated to grow cathodally upon switching EF polarity, while anodal inhibitory effects were delayed.” However, that observation was made upon a single reversal of polarity and has not been extended to long term exposure of Xenopus neurites to switched EF. To our knowledge this is the first study to apply a slowly alternating EF to mammalian cells (where the polarity was switched every 15 min) and investigate effects during 6 DIV. Even though we used an alternating EF greater in amplitude than in clinical trials (46 mV/mm vs. 0.5–0.6 mV/mm, respectively), it was surprising that there were no observed changes in NPCs in terms of alignment, differentiation, proliferation or viability in vitro. However, in the clinical trials, an alternating EF of lower strength was shown to have a slight regenerative effect.

On the other hand, the majority of in vitro studies of mammalian nervous system cells cultured in EFs (ours included) dictate that cellular process growth will be perpendicular to the EF. One might argue that an alternating EF may have been chosen by Shapiro et. al. in an attempt to align the growth of mammalian neurites parallel to the EF. Whatever the reasoning, NPC did not align to an alternating EF after treatment. Therefore, our results are in agreement with the argument that there is no in vitro basis for the therapeutic effects of an alternating EF used to treat spinal cord injury, when considering that the current used in the clinical trials was much lower than that of EF experiments in vitro described in literature.

We must however, consider that in the clinical trials, the stimulation was spread over two years, which is much longer compared to this study, where the cells are maintained for only 6 DIV. Thus, with time, a smaller EF may cause cellular changes in the surrounding tissue that are not noticeable or cannot be described by our experiments.
4.6.4. Directed Migration

The 437 mV/mm treatment directed migration of NPCs cathodally (-), which agrees with the migration of embryonic derived NSPCs, neonatal neurons, and nineteen out of twenty-six reviewed cell types, cultured in EFs. Furthermore, the directedness and net displacement of migrating neonatal rat hippocampal cells, like that of our NPCs, was proportional to EF strength and treatment time. Here, NPCs were about 35 μm/h slower than neonatal rat hippocampal cells, but closely agreed with the velocity of rat embryonic NSPC in EFs. Compared to controls, the velocity of EF treated NPCs was not significantly different at 8 hours, similar to that of neonatal rat hippocampal cells. However, the average track velocity became more similar between control and EF treated NPCs with time. Early on, significant differences were observed 45 min after EF exposure commenced (analyzed with student t-test). Although time-lapse micrographs were analyzed for only 8 hours, the response of NPCs during the 6 DIV is not expected to change drastically. If anything, migration rate would probably slightly decrease due to process growth and increased adhesion as time progressed.

Some cell types migrate toward the anode (+) while others are capable of dual-directional migration. For example, peripheral bovine lens epithelial cells migrated toward the cathode at 50 mV/mm and towards the anode at 150 - 250 mV/mm. EF induced directionality has been shown to be dependent on substrate, serum and cell type. For example, Xenopus spinal cord neurons are known to migrate parallel to the EF or have neurites turn toward the EF vector, while Xenopus neural crest cells aligned perpendicularly to the EF. Although, the mechanisms responsible for regulating the directionality were not investigated in this work, this study demonstrates for the first time that EFs can be used to direct the migration of adult NPCs.

4.6.5. Decreased Viability

When treated with 437 mV/mm continuous EF, a noticeable decrease in cell density was observed as EF treatment progressed, leading to a significantly lower cell density (Figure 4.6, A) at the end of the experiment compared to chambers not treated with an EF. Lower cell density has been attributed to a decrease in proliferation for human umbilical vein endothelial cells in a 200 mV/mm continuous EF applied for 72hr (3 DIV), but not for EFs of 50 or 100 mV/mm. Therefore, one might anticipate a decrease in proliferation of NPCs in a stronger (437 mV/mm) EF applied for a similar duration. We cannot conclude that there is a decrease in proliferation during the last 14 hours of the experiment (Figure 4.6, B). It may be that during the first day of experimentation there is a decreased in proliferation, in addition to a decrease in cell viability as determined via LDH assay (Figure 4.6, C). The accumulation of ions or electrode by-products in the bottles may begin to negatively affect the cells within 1 day of stimulation. Since the media in the bottles containing the Ag/AgCl electrodes was not replaced before the pH changes reached the cells in the 437 mV/mm EF treatment, the methodology used to apply an EF may be less than ideal for extended stimulation and may require active monitor-
ing of changes in pH. Since, the 40 mV/mm alternating EF treatment did not show a significant decrease in cell density, it may be best to decrease the EF strength for the continuous EF treatment. The alternating EF strength could also be increased to elicit a response and eliminate the accumulation of cation/ions at the negative/positive electrodes. It should be noted that cellular density/viability in 437 mV/mm was too low to quantify any observations without the use of FBS.

4.7. Conclusions

This study supports the hypothesis that adult NPCs are affected both morphologically and phenotypically by an EF gradient. Thus, endogenous EFs should be considered as a potential cue in the myriad of chemical, biological and physical cues present in the neurogenic niche. As shown, adult NPCs in a continuous EF favored neuronal differentiation, but had no apparent effect on the differentiation into other neural phenotypes. This demonstrates that EFs may be useful when attempting to increase the number of neurons in neuron deficient tissue. Our work shows the potential of using EF to control migration, differentiation and alignment of stem cells transplanted to treat nervous system disorders. Surely, other electrical signal parameters should be investigated to determine if an electric signal can be tailored to produce a desired neural phenotype. The tremendous therapeutic potential of stem cells may increase the efficacy of EFs used in treatments of nervous system disorders. A broad range of work must be done to integrate transplanted adult NPCs and the application of an EF, but such a combination may potentially develop into an indispensable therapeutic approach for the treatment of the CNS.

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Figure 4.1. Apparatus used to treat NPC with EFs. Modified computer aided drawing (courtesy of D. Jones, Ames National Lab, Iowa) of designed Lexan® polycarbonate (General Electric; Schenectady, New York) pieces with three machined holes and two recesses (B). Once adhered to 50 mm x 70 mm glass slides, the recesses become chambers of 0.3 mm x 14.5 mm x 40 mm dimension with the holes serving as media containing wells. Glass tubes containing Agarose gelled media connected Ag/AgCl electrodes in culture media in 25mL bottle to each 28 mm x 13.5mm well that maintain the cells (C). The culturing/stimulating chamber was housed in a 100 mm glass petri dish with modified lid for attachment of the stimulating electrodes. Electrodes were supplied via a function generator or a constant current source to create an EF.
Figure 4.2. The angle ($\theta$) of the longest axis with respect to the continuous EF vector (A) was determined, at 6 DIV, for NPC cultures without EF treatment, a 46 mV/mm EF (alternating every 15 minutes) and a 437 mV/mm continuous EF, and distributed into bins of 10 degrees from 0 to 90 relative to the EF vector (B). A chi-square test for equal proportions indicated that cellular alignment was evenly distributed in the control and alternating EF groups, but not in the continuous EF group ($p < 0.0001, n = 4$). A significant difference in alignment was seen between the control and 437 mV/mm continuous EF ($p < 0.0001$), and 46 mV/mm alternating EF and the 437 mV/mm continuous EF ($p < 0.0001$). Similarly, superimposition of tracings of major cell processes (40 cells per condition) after 6 DIV revealed that the majority of processes in the continuous EF treatment demonstrate perpendicular alignment to the EF vector (C). Cell processes in the alternating EF at 40 mV/mm did not align in any direction with the EF vector, and were very similar to those without EF treatment (controls).
Figure 4.3. Process growth is inhibited at the anode in an EF. Phase images of a control NPC (A1) and a NPC after 14 hours in a 437 mV/mm EF (A2). Cells and their process were separated into quadrants using cross-hairs as shown in A. The primary processes in each quadrant were counted and analyzed statistically for images taken at 3 DIV (B). There were significantly fewer processes toward the anode (+) compared to the cathode (-) in the 437 mV/mm EF treatment* and controls*. Furthermore, a significantly greater number of processes were counted emanating from sides of the cell that were perpendicular to the EF vector (left and right), compared to cathode and anode facing sides* and the corresponding sides in controls* (* and ♣ indicate significance, p<0.0001, n=4, 225 cells per treatment; Scale bar = 25 μm).
Figure 4.4. Fluorescent microscopy images (20 X magnification) of immunoreactive cells labeled with the primary antibodies (TUJ1, RIP, GFAP, or BrdU) and nuclei stained with DAPI in three conditions: control (no electrical stimulation), alternating EF (46 mV/mm with oscillation of 15 minutes), and continuous EF (437 mV/mm). Each image was created by merging the three fluorescent channels: DAPI (blue), phenotypic antibody immunoreactivity (red) and BrdU immunoreactivity (green).
Figure 4.5. Differentiation of NPCs cultured in two externally applied EFs, after 6DIV. Phenotypic antibodies TUJ1, RIP, and GFAP were used to determine differentiation into early neurons, oligodendrocytes and astrocytes, respectively. Differentiation into early neurons increased significantly when NPCs were exposed to an EF of 437 mV/mm compared to the 46 mV/mm alternating and control conditions (n = 6, *p < 0.001).
Figure 4.6. Analysis of the effects of EFs on cell density, cell proliferation and cell death. (A) Exposure to a 437 mV/mm continuous EF resulted in a significant decrease in cell density when compared to the control conditions (n=6, *p = 0.017). (B) Percent of proliferative NPCs (positively labeled with BrdU) in the two types of externally applied EFs during the last 14 hours of the experiment. No significant differences in the average number of BrdU immunoreactive cells was observed when comparing the control to the 46 mV/mm alternating EF or the 437 mV/mm continuous EF (p = 0.103 and 0.086, respectively, n=3). (C) Lactate dehydrogenase assay after 16-24 hours of EF treatment. Cell death is represented as a relative percentage between 0 and 100%, where 0 indicates the amount of cell death in the control group and 100% indicates complete cell death via culture media containing 0.02% Triton X. Analysis reveals a significant difference in NPC viability between the 437 mV/mm continuous EF treated conditions and the control (n=3, p = 0.001).
Figure 4.7. Time-lapse digital image analysis. (A) The migration paths of 191 individual cells during the first 8 hours of EF exposure graphed to initiate at the origin and superimposed. Migration was clearly directed towards the cathode (-) in the 437 mV/mm EF. In contrast control cells migrated randomly. (B) After 8 hours EF, exposed NPCs were significantly further away from their origin compared to NPCs that did not receive EF treatment. (C) The average direction of NPC migration was significantly more in the direction of the EF vector than in controls. (D-E) Neither the displacement rate nor the mean track velocity of NPCs in a 437 mV/mm EF were different from control cells at 8 hours. (F) Directedness was dependent on the time of EF exposure and peaked at approximately 30 min. Differences between EF and control conditions increased with time and were significant from 5 min (p=0.0025, 408 cells) onwards (p <0.0001). (G) The net displacement of NPCs away from the origin was dependent on time. Significant differences between the displacement of control and EF treated NPCs commenced at 25 min (p =0.010, 405 cells) and increased with time. (*p< 0.0001, n=3).
CHAPTER 5: GENERAL CONCLUSIONS

Overall, we have shown that control of neural progenitor cells (NPCs) can be achieved through manipulation of their surrounding environment with biological, physical and electrical cues. We have demonstrated that NPC migration, alignment, cell viability, proliferation and differentiation can all be controlled externally. The external cues used here provide spatial control over NPCs cultured on substrates, as opposed to soluble cues added to the media that do not.

Control of NPC differentiation is necessary to use stem cells in a regenerative framework such as a conduit loaded with NPCs. The different methodologies presented here altered NPC differentiation into all neural phenotypes possible. Nano-scale topography decreased the differentiation into astrocytes. A combination of microscale topography, ECM proteins, and soluble factors increased differentiation into oligodendrocytes. Finally, a continuous EF increased the differentiation into early neurons. This creates opportunities to improve and combine these different cues to obtain greater fractions of the desired cell types.

Nano-scale, unlike microscale topography, altered differentiation of NPCs. A smaller percentage of NPCs differentiated into astrocytes on 60 nm nanotubes compared to planar substrates. Although the decrease was not drastic, it indicates that ordered nanotubes can be used to control NPC differentiation. Culturing NPCs on a range of nanotube diameters would determine if NPC changes are dependent on pore size like they were for MSCs. After determining the range of useful pore sizes, a flexible and biodegradable material with controllable pore dimensions could potentially be created and incorporated into conduits for nerve repair. If successful, NPC differentiation into a desirable phenotype would be controlled by pore size.

We have shown for the first time that continuous EFs in vitro alter NPC differentiation. A continuous 437 mV/mm EF caused NSCs to directionally align and increase their differentiation into early neurons. If the EF strength were decreased, the magnitude of changes observed would likely decrease, as demonstrated for hippocampal neurons by Yao et. al. EFs of 100, 200 and 300 mV/mm should be investigated for both the alternating and continuous EF treatment of NPCs to determine dependence on EF strength. Nevertheless, the control of NPC alignment and differentiation prior to or after transplantation would be one application of EF treatment. EF application would be useful to control migration over small distances (less than 4 mm) and to cause alignment once NPCs are implanted. The use of stem cells combined with EF treatment during transplantation however has not been attempted. Thus, the development of EF uses and bioelectrical interactions in neural tissue engineering should continue.

Culturing with a single cue does not necessarily cause changes in NPCs. For example, NPCs alone on micropatterned substrates did not influence differentiation or proliferation, but when combined with different extracellular matrix proteins (e.g. - ECL) and soluble molecules, the differentiation into oli-
godendrocytes was increased significantly. Thus, a combination of physical, biological and electric cues may prove to be a valuable methodology to control stem cells in the creation of nerves \textit{in vitro}. Combinations of nanotubes and different extracellular matrix proteins are possible\textsuperscript{100, 102, 232}. Also, polymers that are electrically conductive have been developed and used in culture of PC12 cells\textsuperscript{159}. Such a combination could be used to make a conduit with concentric or overlapping layers, spaced apart, to allow for stem cell plating. Each layer could be tailored to provide cues to augment differentiation into a specific cell type. For example cues that promote differentiation into neurons and oligodendrocytes could be alternated by layer. Oligodendrocytes would then be available to ensheath neurons with myelin. Alignment in the appropriate orientation within the conduit would be controlled by nano or micro scale structure. Finally, the conduit could be placed onto a culture system that would allow for EF stimulation to mimic endogenous EFs during development, which are necessary for proper nerve formation. The alignment observed in the continuous EF was perpendicular to the EF vector. Thus, the conduit would have to be placed perpendicularly to the EF for the processes of NPCs to grow along the length of the conduit. In this way a conduit with cultured nerve tissue could be used to repair the peripheral or central nervous system, instead of having to use nerve grafts from donor locations within the patient.

However, despite all these advances, controlling stem cell behavior is still challenging. Vast optimization of individual and combined cues to achieve total control of stem cells will be very tedious but necessary. Otherwise the use of stem cells could exacerbate the problems that cell based therapies are meant to treat. Using the appropriate cues and developing implantable, three dimensional scaffolds on which stem cells will behave as desired, will pose a further challenge. Once \textit{in vivo} it will be difficult to control stem cell behavior because of the external stimuli from surrounding tissue. Conduits could be used to isolate and provide the desired cues to stem cells. Despite all the challenges, the investigation of external cues is a necessary, but first step, to develop the therapeutic approaches of the future.

If the mechanism involved in the external control of stem cells were elucidated, optimizing of cues or combinations of those would be greatly facilitated. Understanding the mechanistic response to external cues would facilitate the creation of materials or procedures that imitate conditions in vivo. Most external stimuli interact with cells via membrane receptors and channels on cells. These channels or receptors can lead to changes in the internal ionic concentration. Fluorescent dyes used to investigate the ionic changes within cells (i.e. – Fluo-4 A.M, used in Ca\textsuperscript{2+} imaging) should be investigated, especially during EF treatment. Since, the ions are affected by the EF, using fluorescent dyes specific for certain ions, the type of ionic flow most affected by EF could be determined. Additionally, blockage of specific channels or receptor pharmaceutically combined with fluorescent ionic imaging would further elucidate the specificity of the EF effects on ionic channels. Visualizing the ionic flow in and out of the NPCs and determining changes in differentiation in different EFs would allow the ob-
ervation of a link between increased differentiation into a phenotype and changes in ionic activity. In the EF parameters that would maximize the differentiation (e.g. – into neurons as observed here, or to another phenotype) due to an EF could be tuned.

The use of EF to treat an entire population of NPCs has been used here, but selective local and spatially controlled stimulation can be delivered using micro electrode arrays. Flexible microelectrode arrays such as those used in retinotopic activation of the visual cortex\textsuperscript{294}, could be developed to house and stimulate NPCs for use in nerve repair. Furthermore, nerve activity and regeneration could be monitored with such devices\textsuperscript{295}. Applying an electrical stimulus would potentially control the differentiation of transplanted stem cells to help improve the mild regenerative effect of electrical stimulation applied to the nervous system\textsuperscript{133}.

Responses to individual or combined external stimuli were determined, here, for NPCs from adult rats. One ponders if the responses would be the same for human NPCs. Furthermore, would the changes be similar for stem cells that are less committed to a specific lineage, such as pluripotent stem cell? The possibility of testing human pluripotent stem cells derived autologously instead of from embryos now exists\textsuperscript{7}. Thus, testing these approaches using human autologous pluripotent stem or adult stem cells would be most applicable to clinically relevant situations.
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