Adult Mammalian Central Nervous System Neural Precursors Undergo Rapid and Cathode-Directed Galvanotaxis

by

Robart Babona-Pilipos

A thesis submitted in conformity with the requirements for the degree of Doctoral of Philosophy
Institute of Biomaterials and Biomedical Engineering
University of Toronto

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2014

Abstract

Neural stem and progenitor cells (neural precursors) exist within the adult mammalian central nervous system, primarily within the brain’s neurogenic subependymal and subgranular zones, and in the lining of the spinal cord central canal. Neural precursors are ideal candidates for cell replacement strategies, due to their multipotency and neural stem cells’ inherent capacity for self-renewal. Indeed, following neural insult such as ischaemia, endogenous subependymal neural precursors undergo a proliferative and migratory response, whereby they migrate toward the lesion site and differentiate into neural cells. With the goal of enhancing this migratory response in order to facilitate improved neural repair, the work in this thesis has focused on utilizing electrical fields to direct and enhance neural precursor migration and understanding the mechanisms that underlie the effects. We have shown for the first time that primary undifferentiated neural precursors from the adult mammalian brain undergo rapid and cathode-directed migration in the presence of an electrical field, a phenomenon known as galvanotaxis. Strikingly, this response is entirely absent in neural precursors that are induced to differentiate into mature phenotypes. We utilized cross-perfusion techniques to demonstrate that the migratory response is not the result of a chemical gradient that may be electrophoretically generated within the culture media, establishing that the migration is a direct response of the applied electrical field. Second, we have provided evidence that neural precursor galvanotaxis is
mediated by epidermal growth factor receptor signalling – one of the mitogens that maintains neural precursors in an undifferentiated state – and by Ca$^{2+}$. Suppressing either epidermal growth factor or Ca$^{2+}$ signalling resulted in attenuation in the migration rate of galvanotaxis, without affecting the cells’ directedness. Importantly, we also demonstrate that neural precursor galvanotaxis can be induced via charge-balanced biphasic electrical pulses – a stimulation paradigm that is deemed safe for clinical neural stimulation applications. Overall, the work presented herein demonstrates the potential of incorporating galvanotaxis into the development of neural repair strategies as a mechanism for controlling the rate and direction of migration. This thesis contains supplementary content in the form of video files that are referenced throughout the text by the prefix ‘Movie S’ followed by the file number (for example Movie S1, Movie S2).
Acknowledgments

This thesis marks the end of a six-year journey. A journey that I was not entirely certain I was prepared to embark on at the outset, but ultimately I am proud and thrilled that I did. Six of the best, most fruitful, most inspiring, most educational – and simultaneously most difficult, most painstaking, most challenging - years of my life. I started and ended a business during these years. I fell in love with science, and also married the love of my life, during these years. I solved scientific problems, but also resolved the career path I wish to follow, during these years.

I am inexpressibly grateful to my co-supervisors Dr. Cindi Morshead and Dr. Milos Popovic for everything you have done for me along the way. My success would not have been possible without your support, your commitment, and your invaluable insight. I thank you both for providing me with the opportunity to develop and grow as a scientist and as an individual under your leadership. Your encouragement and your confidence in my potential have motivated me in innumerable ways. Working in your labs has been an absolute privilege. To my supervisory committee members, Dr. Molly Shoichet, Dr. Milton Charlton, and Dr. Taufik Valiante, thank you for your support, your stimulating discussions, and your insightful questions.

I extend my thanks to all members of the Morshead lab. As students of the sciences, we have grown together, stumbled together, succeeded together and failed together. We have worked late nights, we have worked early mornings, and sometimes we have worked through the nights into the mornings. I am fortunate to have learned from you and taught you. To former members Dave Piccin, Jessica Hunt, Shoeb Ahsan, and Fenggang Yu – thank you for your advice and training. To my long-standing colleagues Nadia Sachewsky, Wenjun Xu, and Amy Hoyles, thank you for your dedicated assistance, and for all the insightful discussions. Andrew Cheung, Parvati Dadwal, Neemat Mahmud, Labeeba Nusrat, Rebecca Ruddy, Ashkan Azimi, and Stephanie Iwasa - our time together has been shorter but I have watched each of you develop as students and researchers. I wish you all the best. To all of my former undergraduate and summer students: Ilya Droujinine, Mojdeh Shakiba, Alex Pritchard-Oh, Navid Javadi, Aleksandar Dikic, Kasra Tajdaran, and Alex Mok, I thank each of you for your assistance over the years, and I hope you learned as much from me as I benefitted from you. Each member of the lab uniquely contributed to my graduate experiences and the knowledge I have acquired. To the Rehabilitation Engineering Laboratory, although the nature of my project limited my time at the REL, I am
grateful for all the moments we have shared – at IBBME Scientific Day events, during IBBME seminar series talks, and during the lab outings I have been able to attend. The days that I have spent in your welcoming environment at Lyndhurst will not be forgotten.

Finally, to my family, words simply cannot express my gratitude for you all. To my parents, who sacrificed everything so that your children could have everything, I love you and thank you for all that you have done for Ivan and I. You gave up everything you were accustomed to and moved to a foreign country with next to nothing in your name, because you wanted to provide your children with the best lives possible. Dad, you have always been my rock. You have shaped and moulded me into the man I am today. As I sit here composing the closing words to my Ph.D. thesis on Fathers Day June 15, 2014, I cannot help but reflect on everything you have done for me, provided for me, and taught me. Your hard work and dedication to your family has instilled these same principles in me. Thank you, my loving, caring, compassionate mother, for teaching me how to love and be loved. Thank you for being our family chef, nurse, teacher, and home-keeper. You have dedicated your life to raising your children, and I have always aimed and hoped to make you proud. Thank you, mom and dad, for your relationship with each other – it is a shining example the relationship I aspire to achieve with Camille. To my brother, Ivan, you have been my role model since my toddler years. Constantly looking up to you and trying to be like you as a child has instilled within me a competitive spirit to which I attribute most of my life accomplishments. I am proud of you and I wish you nothing but success and happiness. To my indescribably beautiful, loving, caring, patient and brilliant wife, Camille, you are my driving force in life. Thank you for putting up with me during these last few months. I am certain I would not have made it this far without your love and support. I wake up everyday with a smile knowing that I get to spend the rest of my life with you.
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<th>Description</th>
<th>Abbreviation</th>
<th>Description</th>
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</thead>
<tbody>
<tr>
<td>Ach</td>
<td>Acetylcholine</td>
<td>K⁺</td>
<td>Potassium ion</td>
</tr>
<tr>
<td>Ara-C</td>
<td>Cytosine-β-D-arabinofuranoside</td>
<td>MAPK</td>
<td>Mitogen activated protein kinase</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AUC</td>
<td>Area under the curve</td>
<td>MCAO</td>
<td>Middle cerebral artery occlusion</td>
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<tr>
<td>BAPTA-AM</td>
<td>1,2-bis-(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid acetoxymethyl ester</td>
<td>Na⁺</td>
<td>Sodium ion</td>
</tr>
<tr>
<td>BDNF</td>
<td>Brain derived neurotrophic factor</td>
<td>NMDA</td>
<td>N-methyl-D-aspartate</td>
</tr>
<tr>
<td>bFGF</td>
<td>Basic fibroblast growth factor</td>
<td>NPC</td>
<td>Neural precursor cell</td>
</tr>
<tr>
<td>BMP</td>
<td>Bone morphogenic protein</td>
<td>NSC</td>
<td>Neural stem cell</td>
</tr>
<tr>
<td>BPBP</td>
<td>Biphasic bipolar</td>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>BPMP</td>
<td>Biphasic monopolar</td>
<td>PDK1</td>
<td>Phosphoinositide-dependent kinase 1</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>Calcium ion</td>
<td></td>
<td></td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
<td>PDEDF</td>
<td>Pigment epithelium-derived factor</td>
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<tr>
<td>CI-1</td>
<td>Calpain inhibitor 1</td>
<td></td>
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<tr>
<td>CNS</td>
<td>Central nervous system</td>
<td>PI3K</td>
<td>Phosphoinositide-3 kinase</td>
</tr>
<tr>
<td>CSF</td>
<td>Cerebrospinal fluid</td>
<td>PIP₂</td>
<td>Phosphatidylinositol-4,5-bisphosphate</td>
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<tr>
<td>DCEF</td>
<td>Direct current electrical field</td>
<td>PIP₃</td>
<td>Phosphatidylinositol-3,4,5-trisphosphate</td>
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<td>ECM</td>
<td>Extracellular matrix</td>
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<tr>
<td>EF</td>
<td>Electric field</td>
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<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
<td>PSA-NCAM</td>
<td>Polysialylated-neural cell adhesion molecule</td>
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<td>Epidermal growth factor receptor</td>
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<tr>
<td>EPO</td>
<td>Erythropoeitin</td>
<td>PTEN</td>
<td>Phosphatase and tensin homolog</td>
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<td>extracellular signal-regulated kinases 1/2</td>
<td>RMP</td>
<td>Resting membrane potential</td>
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<td>FBS</td>
<td>Fetal bovine serum</td>
<td>SDF-1</td>
<td>Stromal cell-derived factor 1</td>
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<td>FES</td>
<td>Functional electrical stimulation</td>
<td>SE</td>
<td>Subependyma</td>
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<td>GABA</td>
<td>γ-Aminobutyric acid</td>
<td>SEZ</td>
<td>Subependymal zone</td>
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<td>GFAP</td>
<td>Glial fibrillary acidic protein</td>
<td>SGZ</td>
<td>Subgranular zone</td>
</tr>
<tr>
<td>Acronym</td>
<td>Definition</td>
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<td>---------</td>
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<tr>
<td>SVZ</td>
<td>Subventricular zone</td>
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<tr>
<td>TAP</td>
<td>Transit amplifying progenitor</td>
<td></td>
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<tr>
<td>TEP</td>
<td>Transepithelial potential</td>
<td></td>
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<tr>
<td>TNF-α</td>
<td>Tumor necrosis factor α</td>
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<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
<td></td>
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<tr>
<td>VGCC</td>
<td>Voltage gated calcium channel</td>
<td></td>
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<tr>
<td>VZ</td>
<td>Ventricular zone</td>
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Chapter 1

1 Thesis Overview

Neural precursor cells exist primarily within two neurogenic regions in the adult mammalian brain: the subependymal zone within the walls of the lateral ventricles, and the subgranular zone of the hippocampal dentate gyrus. Their capacity to proliferate and differentiate into neural cell phenotypes has sparked great interest in utilizing neural precursors for repairing the injured or diseased central nervous system. Under baseline conditions, subependymal neural precursors normally give rise to neuroblasts (immature neurons) that migrate over long distances from the subependymal zone niche toward the olfactory bulb, where they differentiate into interneurons and integrate into the local neural circuitry. Upon neural insult, this process is perturbed by the recruitment and redirection of a subpopulation of neural precursors toward the affected region, even without any external manipulation. The introduction of mitogens and other soluble factors has been shown to enhance this process, but not to a capacity that is sufficient for significant repair. In addition, long-term safety concerns have limited the clinical potential of such approaches.

The overarching goal of the work in this thesis is to provide the basis for developing novel therapeutic tools for promoting central nervous system repair. In an effort to harness the therapeutic potential of neural precursors, we have explored the use of electrical fields to promote neural precursor migration. We have characterized their migratory properties – including rate, directedness, and tortuosity of migration – in the presence of an electrical field, and also discerned some of the cellular mechanisms that are involved in transducing the electrical signal into motility to better understand the behaviour of neural precursors. It stands to reason that by safely and effectively enhancing the migratory capacity of neural precursors toward injured or diseased areas within the central nervous system, it may be possible to augment their contribution toward reparative processes and to improve the recovery of lost function. Moreover, the electrical stimulation of neural tissue is not a novel concept – it has been used in clinical applications that include deep brain stimulation and transcranial direct current stimulation. In addition, electrical fields have been shown to be imperative for proper development and for wound repair processes. This makes electrical stimulation viable for
integration into neurorepair strategies as a method of promoting neural precursor migration *in vivo*.

Seven chapters comprise the body of this thesis. The contents of each chapter are as follows: 

**Chapter 2** provides a review of the literature, encompassing topics such as the origin of adult subependymal neural stem cells, a description of their niche *in vivo*, as well as their physiological behaviour under homeostatic and injury conditions. Also provided is a review of physiological electrical fields, including their roles in development and wound repair, and a discussion of the various cellular responses that they elicit. Lastly, a short summary of current clinical applications of electrical stimulation is provided.

**Chapter 3** summarizes the objectives and hypotheses of this thesis.

**Chapter 4** describes the characterization of neural precursor migration in the presence or absence of an electrical field, in their undifferentiated and differentiated states. It was shown that undifferentiated neural precursors undergo rapid and cathode-directed migration in the presence of an electrical field of strength 250 mV/mm, but that their migration is non-directed and less rapid in the absence of an electrical field. Additionally, it was shown that neural precursors induced to differentiate undergo almost no migration (indicated by translocation of the cell body), and that this behaviour was not attributable to prolonged adhesion to a substrate or to the lack of exogenous growth factors. Importantly, it was demonstrated that neural precursors retain their undifferentiated state following electrical stimulation. This chapter concludes by showing that undifferentiated neural precursor galvanotaxis is mediated in part by epidermal growth factor, since its exclusion or the inhibition of its receptor caused a reduction in the rate, but not the directedness, of migration.

**Chapter 5** delves further into the mechanisms that regulate undifferentiated neural precursor galvanotaxis. It was shown that undifferentiated neural precursors express L-type and T-type voltage gated Ca\(^{2+}\) channel subtypes. The remainder of this chapter demonstrates through various means – including chelation of intracellular Ca\(^{2+}\), placement of neural precursors in low-Ca\(^{2+}\) conditions, and the inhibition of L-type and T-type Ca\(^{2+}\) channels – that Ca\(^{2+}\) signalling, similar to epidermal growth factor signalling, is important in regulating the rate of neural precursor galvanotaxis, but does not seem to affect migration directedness.
Chapter 6 provides the first evidence that neural precursor galvanotaxis can be elicited via charge-balanced biphasic electrical pulses, a clinically safe method of electrically stimulating neural tissue. This represents the first time that the galvanotaxis of any mammalian cell type has been demonstrated using biphasic electrical fields. It was shown that undifferentiated neural precursors undergo rapid and cathode directed galvanotaxis using electrical pulses characterized by an initial cathodal phase that elicits the galvanotactic response, and a subsequent anodal phase that is one quarter the amplitude and four times the duration of the cathodal phase, that functions to balance the charge of the cathodal phase without eliciting a galvanotactic response. Importantly, it was shown that a galvanotactic response is not evoked when using electrical pulses in which the anodal and cathodal phases are equal in amplitude and duration but opposite in direction. Moreover, differentiated cells did not undergo galvanotaxis with either stimulation waveform. The differentiation state of neural precursors is shown to not be affected by the biphasic electrical stimulation.

Chapter 7 forms the conclusion of this thesis, and includes a discussion of the reported data as well as suggestions for future work.
Chapter 2

2 Literature Review

The aim of this review is to characterize the functions, behaviour, and neuroregenerative potential of adult neural stem cells (NSCs), and to describe the physiological importance of electric fields (EFs) in modifying this behaviour. This chapter is subdivided into two main sections. The first section will begin by summarizing current knowledge of the developmental origin of adult NSCs as well as their niche in the adult brain, followed by a review of the activity of NSCs and their progeny under normal and pathological conditions. The second portion will review the importance of EFs in regulating physiological processes such as development and wound repair, as well as highlight current clinical applications of electrical stimulation as a therapeutic tool.

2.1 Neural Stem Cells

The previous dogma in the literature was that neurogenesis in the mammalian brain ceases shortly after birth, and that neurons lost to injury or disease after this time, could not be replaced through de novo neurogenesis. In 1992, Brent Reynolds and Samuel Weiss demonstrated for the first time that a population of cells isolated from the adult mouse brain was capable of proliferating in response to epidermal growth factor (EGF) to form floating clonal colonies termed neurospheres [1]. A subpopulation of these neurosphere cells demonstrated the capacity for self-renewal by forming secondary neurospheres following mechanical dissociation and replating in the presence of EGF. Reynolds and Weiss further showed that individual neurospheres derived from single cells were capable of differentiating into neuron-specific enolase-expressing neurons and glial fibrillary acidic protein (GFAP)-expressing astrocytes. These observations established that a population of EGF-responsive cells in the adult mammalian brain displays the two cardinal properties of stem cells in vitro: i) self-renewal and ii) multipotency (the ability to generate progeny that give rise to cells with distinct differentiated phenotypes). These cells were designated as NSCs, and subsequent investigations have identified that the subependymal zone
(SEZ) lining the lateral ventricles in the adult forebrain is the true source of the NSCs [2] described in the work by Reynolds and Weiss.

The discovery that NSCs exist in the adult mammalian brain has sparked great interest in utilizing them as therapeutic agents, with the hope that their proliferative and differentiation potential can be harnessed to develop novel neurorepair strategies to treat the injured and diseased brain. Numerous studies, further described below, have explicated the developmental origin, functions, local microenvironment, and behavior of NSCs using in vivo and in vitro experimental models. The neurosphere assay, first developed by Reynolds and Weiss, has emerged as a powerful tool to investigate the proliferation, self-renewal and multipotency properties of NSCs [1].

To summarize the assay, the SEZ of the brain is dissected, enzymatically dissociated into single cells, and cultured in the presence of the mitogens EGF and basic fibroblast growth factor (bFGF) at 10 cells / µL for 7 days without movement of the culture to allow for clonal neurosphere formation [1,3,4]. Individual NSCs proliferate to form neurospheres consisting of a mixed population of self-renewing, multipotent NSCs (<1%) and to a much larger extent, actively proliferating neural progenitor cells (>99%) that are more restricted in their lineage potential and capacity for self-renewal [5]. Together, these populations of cells are pooled together and termed neural precursor cells (NPCs). The number of primary neurospheres that form reflects the number of NSCs that were present in the SEZ at the time of isolation [2], whereas the mean size (diameter) of the neurospheres reveals the size of the progenitor pool. To assay for the self-renewal properties of NSCs, primary neurospheres may be dissociated into single cells and plated individually in the same growth factor conditions to form secondary neurospheres. Since NSCs comprise the sphere-forming cells within a neurosphere, the number of secondary neurospheres that form reflects the self-renewing symmetric divisions undertaken by the NSCs. To assay for lineage potential, neurospheres may then be plated individually (either as whole spheres or following mechanical dissociation into single cells) onto culture surfaces coated with substrates such as Matrigel, laminin, or poly-l-ornithine, in the presence of serum or other differentiation factors [5-7]. The neurosphere assay is summarized in Figure 2-1. With a good understanding of the strengths and limitations of the assay, and when used with rigor, the
neurosphere assay is a powerful tool to investigate the properties of NSCs and their progeny [8,9].

Figure 2-1 – Overview of the neurosphere assay. The subependymal tissue is dissected from the mouse brain, enzymatically dissociated into single cells, and cultured for 7 days in the presence of EGF, bFGF, and heparin. Floating clonally derived colonies, termed primary
neurospheres, consisting entirely of undifferentiated NPCs, form after this period. Primary neurospheres can be dissociated into single cells and individually replated in identical mitogenic conditions for another 7 days to give rise to secondary neurospheres, in order to characterize the self-renewal properties of the NSCs. Neurospheres can be plated in differentiation conditions to give rise to neurons, astrocytes or oligodendrocytes, to characterize the multipotency of the NSCs.

2.1.1 Developmental Origin of Adult Neural Stem Cells

Development of the vertebrate central nervous system (CNS) during early embryogenesis occurs through a process known as neurulation. Neuroepithelial cells of ectodermal origin rapidly proliferate to form the neural plate, which subsequently invaginates and fuses to form the neural tube [10]. The neural tube ultimately develops into the brain and spinal cord, and as such, neuroepithelial cells are considered NSCs as they give rise to the neurons and glia that comprise the CNS. The single-cell-layered pseudostratified neuroepithelial cells that initially comprise the neural tube maintain contact with both the apical lumen of the neural tube and the basal pial surface [11]. In the early stages post-neural tube formation, neuroepithelial cells undergo symmetric divisions that exponentially expand the neuroepithelial population [12-15]. Later, an elevated proportion of neuroepithelial cells undergo asymmetric divisions such that one daughter cell remains a neuroepithelial cell, and the other daughter becomes a neuron or a basal progenitor cell that will asymmetrically generate neurons [16]. As newly generated neurons migrate toward the pial surface, the neural tube epithelium gradually thickens with the apical-most layer denoted as the ventricular zone (VZ) and its adjacent layer as the subventricular zone (SVZ). By the onset of neurogenesis (at embryonic day 11.5 in mice) neuroepithelial cells transform into radial glial cells, which are also identified as embryonic neural precursor cells [11]. Radial glia are similar to neuroepithelial cells in that they maintain apical-basal polarity, undergo division near the apical VZ, and exhibit interkinetic nuclear migration, but differ in their expression of astroglial markers such as GFAP and the astrocyte-specific glutamate transporter (GLAST) [16-18]. Fate-mapping studies using radial glia-specific promoters to drive Cre expression, as well as retroviral fluorescent labeling and time-lapse imaging of proliferating radial glia in slice cultures, have established that virtually all neurons in the brain derive from radial glial cells [19-21]. Similar to neuroepithelial cells, radial glia can divide symmetrically or asymmetrically. Asymmetric radial glia divisions produce radial glial cells and either post-mitotic neurons or intermediate progenitor
cells (basal progenitor cells) that occupy the SVZ and migrate basally along the processes of radial glia into progressively thickening cortical layers, ultimately differentiating into neuronal or glial lineages [11]. The lineage progression from neuroepithelial cells to cortical neurons is concisely illustrated in Figure 2-2.

Figure 2-2 – Illustration of the lineage progression from early embryonic neuroepithelial cells to adult SEZ NSCs. Neuroepithelial cells from the neuroectoderm initially undergo symmetric divisions to increase the neuroepithelial cell pool, and later divide asymmetrically to give rise to early neurons and another neuroepithelial cell. Neuroepithelial cells later give rise to radial glial cells that form the ventricular zone (VZ),
and which similarly either self-renew or give rise to neurons or intermediate progenitor cells that migrate along radial glial processes toward the pial surface. Radial glia-derived neurogenesis is followed by radial glia-derived gliogenesis. Late development radial glia give rise to ependymal cells that line the lateral ventricles, or transform into the definitive adult NSCs. MZ, marginal zone; CP, cortical plate; IZ, intermediate zone; SVZ, subventricular zone; VZ, ventricular zone; nIPC, neurogenic intermediate progenitor cell; RG, radial glia. (Source: adapted from Kriegstein and Alvarez-Buylla [22], with permission from Annual Reviews, Incorporated).

Later in development, neurogenic activity declines as cortical neurogenesis essentially completes and by embryonic day 14 (E14) to E16, a subpopulation of radial glia begin their transition to ependymal cells, and the VZ is reduced to a single-cell layer of ependymal cells [22]. Concomitantly, other radial glia detach from the apical VZ and migrate toward the pial surface, differentiating into astrocytes or astroglial progenitors [23,24]. Within the germinal zone, astrocytic derivatives of the radial glial cells become GFAP-expressing definitive NSCs that persist into adulthood [11,25,26]. These GFAP expressing, definitive NSCs give rise to neurospheres in the presence of EGF and bFGF. Further upstream in the NSC lineage, a leukemia inhibitory factor-dependent primitive NSC exists as early as E5.5 in mouse development [27]. These primitive NSCs give rise to definitive NSCs that are bFGF-responsive at E8.5, and EGF- and bFGF-responsive by E14.5, which ultimately develop into the GFAP expressing (first detectable at E15.5) NSCs in the adult brain [28,29]. Given that primitive NSCs exist as early as E5.5, and that gastrulation in the mouse initiates on E7-E7.5 [30,31], it is possible that the neuroepithelium and radial glia are descendants of the primitive NSC lineage, although further investigations are required to ascertain this notion. In mammals, adult neurogenesis in the brain is primarily restricted to two regions: the SEZ of the lateral ventricles, and the subgranular zone (SGZ) of the hippocampal dentate gyrus [32-34].

2.1.2 Adult Subependymal Zone Neural Stem Cell Niche Composition & Cytoarchitecture

The concept of the stem cell niche was first introduced by Schofield in 1978 [35]. Throughout the body, somatic tissue stem cells reside within specialized microenvironments called niches that regulate their behaviour including self-renewal, proliferation kinetics and differentiation activity [36]. A combination of more mature cells, signaling molecules, environmental factors
and extracellular matrices (ECMs) commonly comprises stem cell niches. The discovery of NSCs in the adult CNS generated considerable interest in characterizing the cellular composition, cytoarchitecture, and signaling profile of the neurogenic regions in the adult brain [6,37-40]. An understanding of the processes by which niches regulate stem cell behaviour may reveal novel mechanisms of harnessing the therapeutic potential of NSCs.

The adult SVZ neurogenic niche derives from the embryonic VZ/SVZ region. The VZ transforms into the ependymal layer but the SVZ (often referred to as the SEZ in adult brains) persists postnatally and into adulthood, albeit significantly reduced in size [11,26]. The SEZ is a four to five cell layer thick region adjacent to the ciliated ependymal cells, which separate the SEZ from the forebrain lateral ventricles [41]. A subset of embryonic radial glial cells retain their astroglial properties and develop into quiescent GFAP+/nestin+ astrocytes within the SEZ that are recognized as the bona fide adult NSCs [26,37,42], as evidenced through adenoviral lox-Cre-based fate mapping [25]. The conclusion that the definitive adult SEZ NSCs are astroglial cells largely derives from findings that demonstrate the ability of GFAP+ astrocytes to repopulate the SEZ following the complete ablation of their dividing progeny via cytosine-β-D-arabinofuranoside (Ara-C) treatment, as well as from retroviral lineage tracing [6,43].

The ependymal layer of the SEZ niche consists of multi- and bi-ciliated ependymal cells that largely separate the SEZ from the ventricular lumen and cerebrospinal fluid (CSF). CSF is primarily produced by the choroid plexus, and plays an important role in protecting the brain and transporting signals and nutrients. Factors found within the CSF are thought to play important roles in regulating the proliferation and differentiation of NSCs and their progeny. The beating of ependymal cilia causes the propulsion of CSF through the ventricular system. It was previously thought that some NSCs may be interspersed between ependymal cells and thus maintain contact with the lateral ventricle [42]. More recent evidence has revealed that NSCs extend apical processes that cluster together and contact the ventricular lumen, providing them with direct access to the soluble factors and signaling molecules within the CSF [44]. The apical clusters are surrounded by ependymal cells, and this organization resembles a pinwheel architecture with the apical NSC processes forming the central core of the pinwheel structure [44] (Figure 2-3). It is believed that ependymal cells help regulate NSC self-renewal through direct cell-cell contact via
gap junctions and by diffusible factors, such as pigment epithelium-derived factor (PEDF) and noggin, a modulator of bone morphogenic protein (BMP) signaling [45-48].

Figure 2-3 – The SEZ NSC niche is organized in a pinwheel structure. Top left panel: Lateral ventricle whole mount labelling of β-catenin (green) and γ-tubulin (red) to visualize ependymal cell membranes and basal bodies, respectively. GFAP⁺ NSCs extend a single apical process into the lateral ventricle wall that is surrounded by ependymal cells to form a pinwheel architecture. Top right panel: Colour-coded illustration of the top-left panel demonstrating that individual ependymal cells can occupy positions in multiple pinwheels. Blue regions represent the apical NSC processes that form the core of the pinwheel structures. Bottom: 3D reconstruction of the SEZ niche, including NSCs (blue) that extend basal processes toward underlying vasculature (orange) and a single apical process to form the center of a pinwheel structure in the ventricular wall. Also included are ependymal cells (light and dark brown) that surround the NSC apical process, TAPs (green) and neuroblasts (red). (Source: adapted from Mirzadeh et al. [45], with permission from Elsevier Science & Technology Journals).
NSCs and their progeny reside adjacent to the ependymal layer. Under normal conditions, NSCs divide slowly and asymmetrically to produce GFAP+/nestin+/Dlx2+ transit amplifying progenitors (TAPs) [6,37]. TAPs are rapidly dividing cells that give rise to GFAP+/Dcx+/Polysialylated-neural cell adhesion molecule (PSA-NCAM)+ neuroblasts. The neuroblasts form chains of migrating cells in the SEZ that are ensheathed within glial tubes formed by astrocytic processes [37,49] (Figure 2-4). Within the niche, cell-cell interactions, signaling factors, and neurotransmitters derived from local and distant inputs tightly regulate the proliferation of NSCs and their progeny.

Figure 2-4 – SEZ niche hierarchy and cytoarchitecture. Neural stem cells (Type B cells) located adjacent to ependymal cells (grey) divide slowly to give rise to highly proliferative transit amplifying progenitor cells (green) (Type C cells). TAPs produce neuroblasts (Type A cells) (red) that are arranged as chains of migrating cells ensheathed by the processes of astrocytes throughout the SEZ. (Source: adapted from Doetsch and Scharff [35], with permission from Karger AG).
The SEZ neurogenic niche is abundantly innervated by axons that extend from parenchymal regions such as the substantia nigra and striatum. Dopamine in the SEZ released from substantia nigra neurons positively regulates the proliferation of TAPs; depletion of dopamine signaling diminishes the number of dividing progenitors and subsequent neurogenesis [50,51]. In contrast, striatal nicotinamide adenine dinucleotide phosphate (NADPH) positive neurons are believed to inhibit subependymal NSC proliferation and neurogenesis through the secretion of nitric oxide [52,53]. SEZ neurogenesis is also regulated by intercellular interactions between niche cells. γ-Aminobutyric acid (GABA), which is locally produced within the SEZ by migrating neuroblasts, not only attenuates the proliferation of GFAP^+ NSCs [54], but also negatively regulates the speed of neuroblast migration in an autocrine fashion [55]. Wnt signaling has been shown to augment the proliferation and neuronal differentiation of NPCs [56], and elevated Wnt activity is observed when NSCs undergo symmetric divisions to enhance the stem cell pool size [57]. Strikingly, when frequently dividing populations (TAPs and neuroblasts) within the SEZ are eliminated via treatment with the antimotic agent Ara-C, NSCs activate and exit their state of quiescence to repopulate the SEZ niche within 4.5-6.5 days [43] suggesting that under homeostatic conditions NSCs are maintained in a quiescent state in part by cellular interactions with their progeny.

The SEZ hosts a broad vascular plexus that is enveloped by ECM-rich basal laminae. Basal laminae extend from the vasculature, make contact with various SEZ niche cells, and terminate just beneath ependymal cells in large bulb-shaped structures [49,58,59] (Figure 2-5). This organization provides the various niche cells with access to myriad growth factors, cytokines and signaling molecules available through the basal lamina that may regulate proliferation, differentiation and migration [60]. The vast majority of dividing NSCs and their progeny are found closely apposed (within 5-10 µm) to vasculature, and it is believed that SEZ NSC proliferation is in part mediated by binding to the vasculature through α6β1 integrin [59]. Consistent with this, disruption of NSC binding to the ECM causing detachment from their vascular niche increases their proliferation [59,61]. NSCs also extend long basal processes with end feet that terminate on the underlying vasculature [44]. These basal contacts, together with the ventricle-contacting apical processes, provide NSCs with direct access to soluble factors within the ventricular CSF and systemic circulation that may regulate the NSC niche.
Figure 2-5 – The SEZ niche contains a basal vascular plexus. Basal laminae (black) extend from the blood vessels (grey), contact the various niche cells (including Type B, Type A, and Tybe C cells), and terminate in bulb-shaped structures just beneath the ependyma. (Source: adapted from Doetsch [50], with permission from Elsevier Science & Technology Journals).

2.1.3 Neurogenic Activity Under Normal & Stroke Conditions

The identification of neurogenic regions that contain NSCs within the adult mammalian brain challenged the conventional belief that neurogenesis ceases shortly after birth, and that the brain was incapable of replacing neurons lost to injury or disease. In tandem, this discovery raised questions about the physiological roles of NSCs under normal and pathological conditions. What purpose do NSCs fulfill in the normal functioning of the brain? How do newborn neurons integrate into the existing neuronal circuitry? If NSCs are present in the adult brain, is endogenous repair of the injured brain possible, and why does the brain not fully heal itself following insult? Myriad studies have been conducted to answer these and other questions to characterize the function of resident NSCs in the brain.
2.1.3.1 NSC Behaviour Under Normal Conditions

Under homeostatic physiological conditions, quiescent SEZ NSCs divide asymmetrically with a cell cycle time of approximately 15 days, producing another NSC and a rapidly proliferating TAP [37,62]. It was previously believed that NSCs reside exclusively within the anterior portion of the SEZ of the lateral ventricular wall [63], but subsequent investigations have confirmed that NSCs are present along the entire rostrocaudal axis of the SEZ [64]. TAPs divide symmetrically with a cell cycle time of ~13 hours to exponentially expand the precursor population [65]. Ultimately, 60% of proliferating TAPs’ progeny undergo cell death, 25% give rise to migrating neuroblasts (or Type A cells), and 15% remain within the SEZ [62]. Neuroblasts undergo extensive tangential chain migration and continue to divide as they migrate [49,64]. The chains of migrating neuroblasts are enveloped within glial tubes formed by the processes of Type B astrocytes [37,49]. Proliferating TAPs can be found in clusters juxtaposed to the chains of migrating neuroblasts throughout the SEZ. The migratory chains of neuroblasts coalesce at the dorsolateral corner of the SEZ to form the rostral migratory stream (RMS), a well-defined path of migrating neuroblasts that terminates in the olfactory bulb where migrating cells differentiate into interneurons within the granular and periglomerular layers and contribute to normal neuronal turnover and olfaction [63,66-68] (Figure 2-6). This migratory process can take between 3-10 days in rodents [63,66,69]. Similar to the organization found within the SEZ, chains of migrating neuroblasts within the RMS are ensheathed by the glial processes of type B astrocytes [37,67].
Figure 2-6 – The adult mouse rostral migratory stream. SEZ NSCs give rise to transit amplifying progenitors, which in turn proliferate and differentiate into neuroblasts. Neuroblasts migrate and continue to proliferate along a defined path between the anterior SEZ and olfactory bulb, known as the rostral migratory stream. Neuroblasts give rise to olfactory granular layer or periglomerular layer interneurons. (Source: adapted from Guillemot and Parras [71], with permission from Nature Publishing Group).

Olfactory interneurons can be classified into distinct populations based on marker expression and localization. Periglomerular neurons are either calretinin-expressing, calbindin-expressing or tyrosine hydroxylase-expressing cells [70]. Granule layer neurons can be subdivided into groups of deep, superficial, or calretinin-expressing cells [71]. A recent study by Merkle et al. revealed the existence of an additional four previously unknown olfactory neuron subtypes: deep branching granule cells (type 1), shrub granule cells (type 2), perimitral cells (type 3), and satellite cells (type 4). The diversity of olfactory interneuron phenotypes raises the question of whether NSCs are homogeneous and behave in a multipotent capacity in vivo, capable of generating all cell fates, or if they are a population of fate-restricted heterogeneous progenitors that are committed to forming specific interneuron subtypes. It has long been held that SEZ NSCs are a homogeneous population of multipotent cells capable of generating neuronal and astroglial progeny in vitro, and all the olfactory interneurons in vivo. However, recent studies by Merkle et al. suggest the existence of regional fate-specificity within the SEZ, indicating that NSCs are indeed fate-restricted in vivo based on their location within the SEZ [71,72]. Merkle and colleagues utilized an adenoviral Cre recombinase model to permanently label postnatal
radial glia and their NSC progeny, tracking the lineage progression of labeled cells from NSC to differentiated interneuron [71]. They found by injecting minute volumes of adenovirus and thus labeling region-specific cells, that NSCs from different SEZ regions produce distinct populations of olfactory interneurons [71]. Calretinin-expressing granule and periglomerular cells originate largely from the anterior SEZ, whereas calbindin-expressing and tyrosine hydroxylase-expressing cells derive from ventrolateral and dorsolateral regions, respectively [71]. In addition, dorsal regions of the SEZ generate superficial granule cells, whereas ventral areas generate deep granule cells [71]. Similar findings of SEZ heterogeneity with respect to neuronal fate of NSCs were reported by Young et al. [73].

In the human brain, there is evidence of a hypocellular region that separates NSCs from the ependymal layer [37,74,75], in contrast to the rodent brain, in which NSCs are situated adjacent to ependymal cells. Although the presence of NSCs in the adult human brain has been established [76,77], the existence of a human RMS analogous to that of the rodent brain remains a subject of debate. A 2004 study by Sanai et al. concluded that an RMS (or RMS-like structure) was absent from the adult human brain, after failing to find any evidence of migratory Tuj1+/PSA-NCAM+ neuroblast chains in serial sections of autopsied and biopsied specimens [75]. However, a later study by Curtis et al., once again using autopsied human brain samples, demonstrated the existence of proliferating, PSA-NCAM+/Dcx+ cells that migrate from the SEZ and produce NeuN positive cells in the olfactory bulb [78]. Through extensive sagittal sectioning and immunostaining procedures, Curtis et al. revealed that the RMS structure in the human brain initially descends from the anterior horn of the lateral ventricle, takes a ventrocaudal direction beneath the caudate nucleus, then takes a rostral turn along the olfactory tract and into the olfactory bulb [78]. This latter study suggests that neurogenesis similar to that in rodent brains, in as much as producing new olfactory bulb neurons, does indeed occur in the adult human.

2.1.3.2 Neural Stem Cell Behaviour Under Neural Insult Conditions

The CNS is vulnerable to numerous disorders, encompassing both injury (such as traumatic brain injury) and disease (such as stroke and epilepsy). In adults, over 80% of strokes are ischemic, which are characterized by the obstruction of one or more blood vessels in the brain causing a transient or permanent reduction in blood flow [79]. Post-insult physiological responses within
the CNS are well established, and have been comprehensively reviewed [80-82]. The neurological damage occurs in two phases: primary and secondary injury. The primary injury immediately follows the insult. Cells become deprived of oxygen and glucose, both of which are critical for oxidative phosphorylation (the primary energy production process in the brain), causing depletion of the adenosine triphosphate (ATP) supply. Maintenance of the Na\(^+\)/K\(^+\) ionic gradient across neuronal membranes requires the ATP-dependent Na\(^+\)-K\(^+\) pump, and therefore reduced ATP production leads to disruption of ionic gradients, loss of neuronal membrane potential, and subsequent permanent neuronal depolarization [83,84]. Depolarization triggers the release of excitatory neurotransmitters and the activation of voltage-dependent Ca\(^{2+}\) channels, which together lead to excessive influx of Ca\(^{2+}\), Na\(^+\) and Cl\(^-\) [81]. Osmotic water influx and edema ensues, resulting in swelling and bursting of neuronal cell membranes. Elevated intracellular Ca\(^{2+}\) promotes excitotoxic processes such as the activation of proteases that degrade cytoskeletal and ECM components, and the generation of toxic free radicals that damage cellular components and induce caspase-mediated apoptosis, marking the onset of secondary injury [85,86]. Reactive oxygen species are then produced by the Ca\(^{2+}\)-dependent neuronal nitric oxide synthase pathway, and together with intracellular second messengers serve as signaling molecules to initiate inflammatory responses [81,86].

The events described in the previous paragraph occur primarily within the core of the insult region. Separating the core from healthy brain tissue is a region of reduced circulatory perfusion and semi-conserved bioenergetics known as the penumbra, which exhibits impaired function but mostly intact structure [87,88]. Insult conditions activate resident glial cells (astrocytes, NG2 cells, microglia) in a process known as gliosis [89-91]. Initially microglia become activated, infiltrate the injury site, retract their processes, and, together with reactive astrocyes, express pro-inflammatory cytokines and chemokines such as tumor necrosis factor α (TNF-α), interleukin 1β and macrophage inhibitory factor [92]. NG2 cells proliferate rapidly in response to insult, and astrocytes become activated to hypertrophy and proliferate in a process known as reactive astrogliosis [90,93]. Leukocytes (granulocytes, neutrophils, monocytes/macrophages) are subsequently recruited from the blood into the parenchyma and initiate post-insult inflammation (a major cause of secondary injury) within the penumbra. Reactive astrocytes and
microglia also play pro-survival and neuroprotective roles by releasing neurotrophic factors such as nerve growth factor, bFGF, and brain derived neurotrophic factor (BDNF), all of which are upregulated post-ischemia, and by protecting against infectious agents [89-91]. Another important function of reactive astrocytes is their formation of the so-called glial scar through their upregulation of GFAP expression and the interdigitation of their processes [94]. Glial scarring restricts the spread of neural damage, but as an unfortunate consequence, also inhibits axonal regeneration and elongation [95].

An interesting feature of NSCs – and one that motivates much of current neuroscience research – is their ability to detect pathological or traumatic damage to the brain and subsequently contribute to neuronal replacement and tissue repair efforts. Arvidsson et al. provided the first evidence that following stroke, neurogenesis from SEZ-derived cells is enhanced in the brain [96]. Using a middle cerebral artery occlusion (MCAO) model of stroke, they demonstrated a substantial increase in proliferating SEZ BrdU+/Dcx+ cells and a subsequent accumulation of BrdU+/NeuN+ cells within the ipsilateral striatum at 5 weeks post-stroke, concluding that stroke induced the proliferation and recruitment of SEZ neuroblasts into the injured striatum [96]. Similar findings of enhanced proliferation and recruitment of SEZ-derived cells were reported in models of cortical injury [97,98], status epilepticus [99], as well as in humans [78]. Elevated post-stroke neurogenesis is not solely due to increased proliferation of Type C and Type A progeny, but is also attributable to the enhanced recruitment of NSCs out of quiescence into a symmetric proliferative mode to expand the precursor pool [100,101]. Newly formed NPCs migrate to the site of injury in a chain-like fashion often closely apposed to blood vessels, and guided in part by the stromal cell-derived factor 1α/CXC chemokine receptor 4 signaling pathway [102-105].

Although newborn neurons migrate into parenchyma and integrate into the local circuitry, this post-insult neurogenic response falls short of producing any significant functional recovery. Arvidsson et al., for example, estimated that only 0.2% of the neurons lost due to injury were replaced by newborn neurons after 6 weeks [96]. It has been proposed that this limited cell-repair capacity may be due to the presence of inflammatory leukocytes, reactive glial cells, and inflammatory cytokines and chemokines within the infarct core that affect the proliferation and
differentiation activity of NPCs [106,107], in addition to the glial scarring process that physically impedes NPC recruitment. In support of this is the observation that post-insult (but not under baseline conditions) TNF-α expression by activated microglia is a negative regulator of NPC proliferation [108]. It follows that enhancing the endogenous neurogenic response may lead to improved neurorepair and functional recovery, and this is a subject of focus for much of current neuroscience research.

Numerous studies have shown that neurogenesis, and often functional recovery, can be enhanced post-insult via growth factors, immunomodulatory agents, environmental enrichment, and other factors. Infusion of EGF and bFGF enhanced neurogenesis and led to 40% recovery of lost hippocampal neurons following ischemia, as well as recovery of spatial-learning [109]. In a rat model of Parkinson’s disease, infusion of TGF-α induced the proliferation, migration, and differentiation of SEZ NPCs into striatal neurons and subsequent functional recovery [110]. Erythropoietin (EPO) is a cytokine believed to have neuroprotective, neurogenic and angiogenic effects. Wang et al. reported that i.p. injection of EPO in an MCAO model of stroke promoted angiogenesis, enhanced SEZ proliferation and neurogenesis, and promoted functional recovery [111]. Similarly, Kolb et al. demonstrated using a pial vessel disruption model of focal ischemia at the motor cortex that ventricular infusion of EGF followed by EPO – sequentially for one week each – after injury promoted the migration of SEZ-derived NPCs toward the injury site, regeneration of cortical tissue, and functional improvement [112]. Erlandsson et al. determined that immunosuppression – using both a genetically immunocompromised NOD/SCID animal model, and through administration of the immunosuppressant cyclosporine A – leads to enhanced recruitment of SEZ NPCs toward the pial vessel disruption infarct site, tissue regeneration, and functional recovery [113]. Interestingly, exercise and enriched environments (comprised of larger housing and enhanced stimulatory factors such as social interaction and physical activity) each enhanced neurogenic activity within the hippocampal germinal zone under baseline conditions, as well as improved functional recovery after injury [114-118]. However, the extent to which improved functional recovery is attributable to exercise- or environment-evoked neurogenesis is uncertain, since the effects of such activity are difficult to differentiate from brain plasticity and functional reorganization. Nevertheless, efforts remain
focused on developing mechanisms to enhance neurogenesis following injury or disease in order to improve the functional outcome of affected individuals.

### 2.1.4 Modes and Mechanisms of Neural Precursor Migration

The formation of the vertebrate brain’s intricate structure involves a complex pattern of cell migration during development and the early postnatal period. Similarly, adult SEZ NPCs exhibit elaborate migratory behaviour during baseline olfactory bulb neurogenesis, as well as in response to injury or disease. It may be possible to increase the potential of NPCs to contribute to neurorepair by enhancing their migratory capacity, but such an undertaking necessitates an understanding of the methods by which NPCs migrate and the mechanisms that regulate this behaviour. This section will review current knowledge on the migratory behaviour of neural precursors during development, as well as during homeostatic and pathological conditions in the adult.

As described above, radial glial cells comprise early embryonic NSCs from which virtually all neurons in the brain derive. During embryonic corticogenesis, radial glia are anchored in the VZ and extend a process toward the pial surface that is used as a scaffold by migrating newborn neurons destined toward the developing cortex [119,120]. This outward radial movement of neurons along glial processes is termed radial migration. The neurotrophins BDNF and NT4, as well as the neurotransmitters GABA and N-methyl-D-aspartate (NMDA) have been implicated in the modulation of radial migration, and interestingly neurotransmitter-regulated radial migration is mediated by intracellular Ca\(^{2+}\) levels [121-125]. EGFR also plays an important role in radial migration, as determined by the observations that embryonic neural precursors undergo reduced migration in EGFR null mice, and that overexpression of EGFR promotes enhanced neural precursor migration [126,127]. The interaction of migrating neurons with the glial processes that guide them is largely mediated by cell surface integrin receptors. Integrins, consisting of α and β subunits that combine to form distinct heterodimer structures, link ECM binding proteins (predominantly fibronectin and laminin in the developing CNS) with intracellular cytoskeletal components to facilitate cell motility [128,129]. Numerous studies have demonstrated that disruption of integrin receptors leads to the abnormal development of cortical
layers [130-133]. In addition, a few signaling effectors downstream from integrins have been identified, including Filamin A and Dab1, both of which bind to β1 integrins [134-136]. More recent evidence, however, suggests that β1 integrins specifically function to anchor radial glial processes at the pial surface, and thus the reduced neuronal migration observed in β1 integrin deficiency studies is a secondary effect. Importantly, once migrating embryonic neurons reach their cortical destination, they detach from their glial scaffold and occupy the developing cortical layer. This process occurs in an “inside-out” manner such that more superficial layers are occupied by more recently born neurons [137], and it is through successive waves of neuronal migration that the cortex develops, layer by layer.

In addition to radial migration, there exists another mode of neuronal locomotion known as tangential migration, whose name derives from the fact that the direction of movement is parallel to the ventricular surface and perpendicular to the orientation of radial glia [138]. Unlike radial migration, cells undergoing tangential migration do not require the support of radial glia. A prime example of tangential migration is that of adult brain neuroblasts that migrate in chains from the lateral ventricle along the RMS to the olfactory bulb [37,67]. Migrating neuroblasts utilize nearby neuroblasts as substrates for movement as they crawl along each other in a homotypic type of cell-cell interaction [138]. Upon arriving at the olfactory bulb, neuroblasts switch to a radial mode of migration in order to integrate into the local circuitry [66]. Although the guidance and regulatory mechanisms that govern neuroblast migration along the RMS have not been fully elucidated, several important factors have been identified. PSA-NCAM is highly expressed in neuroblasts and helps reduce cell adhesion thereby preventing cell aggregation and facilitating cell translocation [139,140]. In the absence of PSA-NCAM, neuroblasts exhibit greater binding with glial tube-forming astrocytes in the RMS [141], undergo early differentiation [142], and disperse from migrating chains as individual cells [143]. Dcx is a microtubule-associated protein that is transiently expressed in neuroblasts and is believed to be involved in the maintenance of neuroblasts’ bipolar morphology. Neuroblasts from Dcx knockout mutant mice exhibited increased branching and a multipolar (rather than bipolar) morphology, which reduced their velocity of migration [144]. Integrins have also been shown to play a role in RMS neuroblast migration. For example, laminin is abundant in the RMS and
functions as a ligand for α6β1 integrin, which is expressed on neuroblasts, and therefore acts as a chemoattractant for neuroblast migration in the RMS [145].

In the event of neural injury or disease, the brain possesses the ability to enhance NPC proliferation and redirect their migration from the SEZ toward the lesion site to promote neurogenesis, as described in greater detail above. The migration of NPCs through the parenchyma may occur along blood vessels and astrocytes [103,146]. The activation of local microglia and astrocytes as well as the infiltration of immune cells leads to the production and secretion of several cytokines, chemokines, and growth factors that appear to regulate the recruitment of NPCs toward the lesion site [147], possibly via chemotactic mechanisms. Stromal cell-derived factor 1 (SDF-1) and its receptor CXCR4 are the most commonly studied chemokine/receptor combination for adult brain NPC migration. SDF-1 is upregulated in the brain following injury and has been shown to direct NPC migration toward the site of injury [102,105,148]. CCL2 is another important chemokine that induces the chemotactic migration of NPCs, which express the receptor CCR2, following neural damage [149,150]. Amongst the various growth factors that impact post-damage neurogenesis and NPC migration are vascular endothelial growth factor (VEGF), bFGF, and EGF. These growth factors have all been shown to be overexpressed after neural damage and are believed to be involved in the directed migration of NPCs toward lesion sites [98,151-156]. The neurotrophin BDNF plays an important role in baseline NPC migration along the RMS, but has also been implicated in directing NPC migration toward the ischaemic striatum [157,158].

The inherent migratory properties of NPCs and their ability to sense and respond to neural damage makes them appealing candidate targets for the development of neurorepair strategies, but there is a clear need to enhance not only their migration, but also survival, differentiation and functional integration following neural damage to fully realize their therapeutic potential. The mobilization of NPCs from their SEZ niche to a lesion site is an early critical step in any such strategy. The limited endogenous response leaves room for improvement, and is a subject of focus for much of current neural stem cell research. One unique method of manipulating NPC motility is the use of EFs. Much like chemical gradients are able to direct the migration of cells through chemotaxis, a gradient in electrical potential has been shown to direct and enhance the
migration of certain cell populations through a phenomenon known as galvanotaxis. This phenomenon and its application to adult SEZ NPCs is the topic of my thesis and is reviewed in greater detail in the next section.

2.2 Physiological Electric Fields

2.2.1 History of Bioelectricity

Luigi Galvani, an anatomist and obstetrician by trade and regarded as the father of bioelectricity, performed several pioneering experiments in the late 18\textsuperscript{th} century that culminated in the definitive demonstration of the existence of what was termed “animal electricity” or bioelectricity. In his first experiment, Galvani noticed that the muscles in his frog dissection preparation (which was placed on the same table as a static electricity generator) would twitch each time his assistant placed a scalpel in contact with an exposed nerve [159,160]. Amusingly, he was able to replicate these observations using “atmospheric electricity” as an excitation source by placing his frog preparations outdoors during stormy weather and connecting them to a long insulated wire. The muscles twitched in conjunction with the appearance of lightning. In what is widely regarded as his second set of experiments, Galvani attempted to replicate these results in clear weather by affixing the frog preparations to iron railings outside his house using bronze hooks [159,160]. Frustrated by the lack of twitching in clear weather, Galvani pressed the bronze hooks against the iron railings and, to his surprise, the muscles contracted. He inferred from this reaction that inherent electricity must exist in the frog, since connecting the nerve to the leg muscle using bronze and iron metals elicited muscular contractions. He concluded that the electricity must flow from the interior of the muscle (where the nerves terminate) to the exterior of the muscle to induce contractions.
Figure 2-7 - Galvani’s third experiments. (A) Contacting the cut sciatic nerve of one frog leg with the muscles of the opposite leg elicits the contraction of the latter leg muscles. (B) Contacting the cut sciatic nerve of one frog leg with the intact sciatic nerve of the opposite leg elicits a muscular contraction in both legs, due to the potential difference between the cut and intact nerves, which evokes an action potential that propagates in both nerves. (Source: reproduced from Piccolino [162], with permission from Elsevier Science & Technology Journals).

Around this time, Alessandro Volta, another pioneer in the field of bioelectricity and who was initially convinced by Galvani’s work, began to perform his own experiments that challenged Galvani’s findings. Volta was able to generate frog leg contractions simply by connecting two points of the same nerve with two different types of metals, without needing to contact the muscles, seemingly contradicting Galvani’s conclusion [160]. From these results was born the principle of electropotential difference between dissimilar metals, which states that when two metals of different electrode potentials are placed within an electrolyte solution, an electromotive force develops between the metals. Volta argued that this external electromotive force, and not an internal “animal electricity” force, was responsible for the contractions produced in the frog leg muscles. Galvani responded by performing a third set of experiments that definitively demonstrated the existence of bioelectricity. He was able to generate muscular contractions by positioning the cut sciatic nerve of one leg in contact with the muscles of the opposite leg, without utilizing any metallic electrodes (Figure 2-7A). Even more convincingly, Galvani was
able to produce contractions in both frog legs by contacting the cut surface of one leg’s sciatic nerve to an intact region of the opposite leg’s sciatic nerve (Figure 2-7B).

Near this time, Volta had been furthering his own experiments by coupling together silver and zinc plates, piling them on top of each other, and interposing between them saline-soaked paper disks to effectively amplify the electromotive force. This invention became known as the battery and due to its massive success, Galvani’s experiments proving the existence of bioelectric potential went largely ignored by the scientific community. Owing to investigations performed by Leopoldo Nobili, we now know that the phenomena observed in Galvani’s third experiments were instigated by the potential difference between intact and damaged tissue, otherwise known as injury potentials [159]. Armed with knowledge of injury potentials, Carlo Matteucci elicited muscular contractions by inserting a section of a muscle’s nerve into a separate cut (injured) muscle, and ensuring a different section of the same nerve contacted an uninjured region of the cut muscle [159]. In another experiment, Matteucci demonstrated that by placing the nerve of one muscle onto the surface of another muscle, and contracting the latter, the nerve on the latter muscle’s surface would become stimulated and subsequently contract the former muscles. This was the discovery of another major bioelectric phenomenon – the action potential.

2.2.2 Endogenous Electric Fields

A brief summary of introductory principles related to electricity, voltage, and current that are pertinent to this thesis is provided below in the form of a simple circuit example (Figure 2-8). A voltage source (battery) connected to a resistor in series by means of conducting wires is a basic electric circuit commonly used to describe Ohm’s law. The voltage source drives current flow in the form of moving electrons through the wires and the resistor, causing a voltage drop across the resistor. The voltage drop across the resistor is proportional to the amount of current flowing through it, given by $V = IR$, where $V$ is the voltage drop across the resistor, $I$ is the amount of current flowing through it, and $R$ is the resistance of the resistor measured in Ohms.
Figure 2-8 – A simple electrical circuit. A voltage source, \( V \), applied in a closed circuit across a resistor with resistance, \( R \), will produce a current flow of magnitude \( I \). Since current is recognized as the flow of positive charge, the direction of current flow is from the voltage source’s cathode terminal (top), through the resistor, and into the voltage source’s anode terminal (bottom).

Unlike metallic conductive wires, physiological solutions lack free electrons, and therefore current flow is achieved by the movement of charged ions, such as Na\(^+\), K\(^+\), Cl\(^-\), and Ca\(^{2+}\). Another distinguishing feature of physiological electricity is that current flow occurs in a three-dimensional environment, in contrast to one-dimensional current flow in wires. Thus, the flow of charged ions in a physiological system is described as charge density (amps per square centimeter or \( \text{A/cm}^2 \)). The voltage difference per unit distance is called the electric field and the strength of the field, \( E \), is given by \( E = J \rho \) and is often measured in units of mV / mm, where \( J \) is the current density, and \( \rho \) is the bulk resistivity of the physiological solution (such as intracellular or extracellular fluid) – typically approximated as 100 \( \Omega \cdot \text{cm} \) [161]. Direct current electric fields (DCEFs) exist within all living organisms, and are characterized by a unidirectional flow of current.

2.2.2.1 Resting Membrane Potential

All living cells in multicellular organisms maintain a potential difference across their cell membranes of between 10 and 90 mV [162], with the inside negative relative to the outside. This resting membrane potential (RMP) arises due to concentration gradients of primarily K\(^+\) and to a lesser extent Na\(^+\) across cell membranes. Intracellular compartments typically possess high K\(^+\) and low Na\(^+\) concentrations, whereas extracellular spaces are high in Na\(^+\) and low in K\(^+\). Passive stochastic diffusion processes and active ion transporters that move ions against their
concentration gradients maintain the RMP. The importance of the RMP is often described in the context of excitable cells such as nervous system and cardiac cells. When an external stimulus such as neurotransmitter release in a synaptic cleft or mechanical stimulation [163] causes the rapid intracellular influx of Na\(^+\), the transmembrane potential becomes more positive and the cell is said to depolarize. If this depolarization surpasses a certain threshold, the excess local Na\(^+\) in a microdomain of the cytoplasm triggers the activation of nearby voltage-gated ion channels, thereby further increasing the influx of Na\(^+\) and generating a rapidly propagating electrical signal known as the action potential.

Membrane potential is also involved in various other cellular processes. There is a correlation between the RMP of a cell and its proliferative potential, a phenomenon first observed by Clarence Cone [164,165]. Proliferative and oncogenic cells tend to exhibit more depolarized (or more positive) RMPs [166,167], whereas fully differentiated cells commonly have hyperpolarized (or more negative) RMPs [166]. Cell migration is another important cellular process that is associated with modulation of the RMP [168,169]. Changes in the RMP can also modify gene expression and protein transcription levels [170,171]. In line with this, the RMP is implicated in cell maturation and differentiation processes. For example, human myoblasts experience membrane potential hyperpolarization prior to differentiation into myocytes. The production of K\(^+\) and Cl\(^-\) ion channels and their corresponding ionic currents across the plasma membrane, which hyperpolarize the RMP, are required for hematopoietic progenitor and fibroblast to myofibroblast differentiation, respectively.

2.2.2.2 Transepithelial Potential

Epithelial tissue lines the fluid-filled cavities and most organs of the body. Polarized epithelial cells featuring distinct apical and basal membrane domains directionally and selectively transport ions [172]. For example, in corneal epithelium, Na\(^+\) passively diffuses from the tear fluid into the apical side of the epithelium, and is then actively transported out through the basal region [173]. Concurrently, Cl\(^-\) uptake into the basal epithelium occurs through Na\(^+\)-K\(^+\)-Cl\(^-\) cotransporters, and Cl\(^-\) is released into the apical tear fluid through Cl\(^-\) channels [174]. The most apical region of the basolateral membrane contains tight junctions that provide an electrically resistive barrier between the apical and basal surfaces of the epithelium [175,176]. This separation of ions
together with the tight junction barrier creates a potential difference across the epithelium known as the transepithelial potential (TEP) (Figure 2-9). The TEP across corneal epithelium ranges between 25-40 mV (in the basal-to-apical direction) in mammals [161,173]. Similar TEPs exist across skin [177,178], kidney [179], gastrointestinal tract [180,181], urinary tract [182], respiratory tract [183-185], and prostate epithelium [186], among others. Endogenous TEPs play important physiological roles that include embryonic morphogenesis, limb formation and regeneration, and wound healing [161,172,187-190].

![Transepithelial potential across corneal epithelium](image)

Figure 2-9 - Transepithelial potential across corneal epithelium. Intact epithelial tissues separate ions such as Na$^+$ and Cl$^-$ through stochastic diffusion and active transport processes. Positive ions are transported basolaterally and negative ions are transported apically. This generates a potential difference across the epithelial tissue known as the transepithelial potential, which is maintained by the electrically resistive tight junction barrier near the apical domain of the epithelium. (Source: adapted from Zhao [174], with permission from Elsevier Science & Technology Journals).

2.2.2.3 Endogenous DCEFs During Development

DCEFs play crucial roles in proper embryonic development. Much of our understanding of the importance of developmental DCEFs is owed to the creation of the vibrating microelectrode [191]. Jaffé and Nuccitelli are credited with the invention of the vibrating microelectrode, which consists of a stainless steel sensing electrode with a platinum ball tip (20-30 µm diameter) and a reference electrode that is situated within the bathing medium that surrounds the embryo or tissue to be examined [191]. The sensing electrode is placed within close proximity of the
specimen, and vibrated laterally at up to 300 Hz such that the distance between the two extreme positions of the probe measures between 20-30 μm [191]. As the microelectrode vibrates, it continuously takes potential recordings, thereby providing the potential difference between the extreme positions of the probe. This potential difference, along with the resistivity of the bathing medium and distance between the probe’s two extreme positions (20-30 μm) permits the calculation of the current density flowing into or out of the sample.

The vibrating microelectrode technique has been used to establish the presence of transepithelial current flow and TEPs across the developing axolotl embryo [192]. Shi and Borgens determined that during the neurulation stage of axolotl embryonic development (stage 15), a TEP appears across the surface ectoderm and that a gradient of the TEP magnitude exists along the rostrocaudal and mediolateral axes [192]. Similar embryonic TEPs have been described in the developing Xenopus, chick, and mouse [187-189,193]. These endogenous embryonic fields are critical for proper embryonic development. The application of external DCEFs in the range of 25-100 mV/mm, depending on the orientation of the applied field, either hyperpolarized or depolarized the embryonic TEP causing severe disruption of proper morphological development [194]. Resulting deformities included incomplete neural plate folding and neural tube formation, missing or abnormal tail and gill structures, absence of body symmetry and improper body axis formation [194]. Notably, external DCEFs only produced developmental malformations when applied during neurulation – the stage when endogenous EFs are prominent; exposure to an external EF prior to or after neurulation had no effect on embryonic development [192,194]. Pharmacological blockade of developing axolotl neural tube Na⁺ channels via benzamil or amiloride attenuated the embryonic transneural tube potential and produced severe deformities in CNS structures [195]. Similar observations have been made in chick embryos by implanting conductive glass shunts to disrupt the endogenous EF at the posterior intestinal portal resulting in malformed tail, limb and cranial development [189]. Interestingly, epithelial tight junctions break down and the TEP collapses at precise locations in the skin several days prior to the emergence of limb buds, suggesting that TEPs play a role in proper limb development as well [193,196]. These studies clearly demonstrate the important roles that endogenous EFs play in proper
embryonic development, but equally important is their function in physiological wound healing processes.

2.2.2.4 Injury Potential

The separation of ionic charges that give rise to TEPs is maintained by the high-resistance electrical barrier formed by tight junctions between epithelial cells. When epithelium is wounded, this tight junction seal is disrupted and an electrical short-circuit is created across the epithelium at the wound site. The TEP at the wound site collapses to 0 mV and ions leak out through the wound region, creating an injury current [161]. Epithelial cells distal to the wound site continue to function normally, transporting and segregating ions across the epithelium to create a TEP. Thus, the TEP is 0 mV at the wound but maintains normal values at regions distal to the wound. This creates a lateral EF that is orthogonal to the TEP vector, and is known as the injury or wound potential [161,172,178,197] (Figure 2-10). The injury site is negative relative to distal regions, since the apical side of the epithelium and the wound site become roughly equipotential, and the basal side of the epithelium is positive relative to the apical side. Therefore, the wound site forms the cathode of the lateral EF, and the field vector points toward the wound [172]. The injury potential phenomenon has been well characterized in corneal and skin epithelium [178,197], but is applicable to all polarized epithelia, and has been detected elsewhere in amputated amphibian limbs and human fingertips [198-200], as well as injured bone [201] and spinal cord tissue [202].

The injury potential is spatially and temporally regulated. It is dependent on the existence of the TEP, and the magnitude of the TEP varies based on distance from the wound site. The injury potential is greatest near the wound site, and exponentially decreases at more distal regions. In Guinea pig, a skin wound generated a lateral EF of 140 mV/mm at the immediate wound edge, but this field strength exponentially diminished to 10 mV/mm between 500 to 1000 µm lateral to the wound [161,172,178]. Similar injury potential gradients have been measured in human skin wounds [203,204]. In addition, the injury current at the wound edge was found to be larger than the injury current at the center of the wound [205]. Injury potentials also exhibit a temporal profile. For example, immediately after wounding of rat cornea, an injury current of roughly 4 µA/cm² appeared, then gradually peaked at ~10 µA/cm² within the first hour, and subsequently
decreased to \(~5 \, \mu\text{A/cm}^2\) over the next several hours [205]. A comparable injury current temporal profile has been recorded in skin wounds [204,206]. Importantly, as discussed further below, these injury potentials play a critical role in the proper healing of wounded epithelium.

**Figure 2-10** – The generation of an injury potential. When epithelial tissue is damaged, the disruption of the tight junction barrier creates a local short circuit at the injury site, causing the TEP to drop to 0 at this region. At sites distal to the injured region, the TEP is maintained at normal levels, and therefore a lateral EF develops that is orthogonal to the TEP, and is directed toward the center of the wound. This lateral EF is called the injury potential. (Source: reproduced from McCaig [163], with permission from American Physiological Society).

### 2.2.3 Cellular Responses to Endogenous and Applied DCEFs

The significance of endogenous EFs is well established and was described above in the context of individual cells’ membrane potentials and cell excitability, as well as TEPs in developmental and wound healing processes. It is important to consider the specific effects of EFs on different cell populations, as well as the known cellular mechanisms that underlie the transduction of EFs into evoked responses. This section will review a few EF-induced cellular behaviours that have been characterized in the literature, including the growth and steering of nerves, cellular reorientation, wound closure/healing, and as an extension of the latter, the phenomenon of EF-induced whole cell body translocation, or galvanotaxis.
2.2.3.1 Nerve Growth and Steering

Ramon y Cajal first coined the term *growth cone* in 1890 when studying the morphology of 3-4 day old chick embryo spinal cord neurons, to describe a motile structure he observed at the end of developing axons [207]. Growth cones arise at the distal tips of neurons’ developing neurites (axons and dendrites), and are characterized by a central domain and a peripheral domain. The central domain is the enlarged region containing most of the cone’s protoplasm and consists primarily of microtubules [208]. The peripheral region consists of a meshwork of actin filaments that form flat webbed structures known as lamellipodia, and thin, finger-like protrusions known as filopodia [209] (Figure 2-11). Filopodia form when the cross-linked bundles of actin filaments project beyond the leading edge of the lamellipodium.

![Figure 2-11 – Differential interference contrast and fluorescent image of an Aplysia growth cone. Left: Differential interference contrast image showing the typical flat webbed lamellipodium in the peripheral region of the growth cone. Right: Fluorescent image of the same growth cone revealing the cross-linked bundles of actin filaments that comprise the filopodia (thin red protrusions) and the microtubules (green) that occupy the growth cone’s central domain. (Source: adapted from Munnamalai and Suter [210], with permission from Blackwell Science Ltd.).](image)

Growth cone activity is responsible for axon growth, steering, and synaptogenesis. Early evidence that the speed of neurite growth is enhanced in the presence of an externally applied EF came from work by Marsh and Beams, in which they demonstrated that when 7-10 day
embryonic chick medulla explants were exposed to a DCEF of minimum strength approximately 50 mV/mm, the cathode-facing neurites grew significantly faster than those facing the anode [211]. Below this threshold field strength, no preferential neurite growth was observed toward the cathode, and at even higher field strengths of approximately 90 mV/mm, neurites did not emerge at all from the anode-facing side of the explant [211]. Indeed, cathode-facing neurites not only undergo quicker outgrowth than anode-facing neurites, but anode-facing neurites also experience an attenuation of growth activity relative to control neurites from explants not exposed to a DCEF [212]. Jaffe and Poo demonstrated that the cathode-facing nerves from chick dorsal root ganglion explants grew up to five times faster than the anode-facing nerves when a 140 mV/mm DCEF was applied across the explant [213].

In addition to enhanced outgrowth speed, growth cones exposed to an external DCEF also exhibit turning or steering (generally) toward the cathode. To investigate the response of individual neurons rather than the aggregate response associated with explant models, two separate studies utilized a technique in which individual neurons were dissociated from embryonic *Xenopus* neural tube and subsequently placed in an external EF [214,215]. In field strengths ranging from 7 - 1000 mV/mm, cathode-facing neurites experienced accelerated outgrowth whereas anode-facing neurites displayed attenuated growth [214,215]. More interestingly, neurites that were roughly orthogonal to the EF vector underwent significant turning to curve toward the cathode [214,215]. While Hinkle et al. reported that DCEF stimulated cultures exhibited greater numbers of neurites emerging from somata without directional bias (similar numbers on both anode- and cathode-facing sides), Patel et al. determined that neurite outgrowth was preferential toward the cathode-facing side of the cell [214,215]. Importantly, these effects persisted when culture media was cross-perfused orthogonally in the neuronal culture to remove potential EF-induced chemical gradients from developing, indicating that the growth cone elongation and turning phenomena were a direct result of the applied EF [214,215]. Moreover, application of sinusoidal EFs did not alter growth cone activity, and removal of DCEF stimulation prompted the loss of growth cone turning toward the cathode [215]. Interestingly, the direction of neurite turning is dependent on the surface charge of the substratum on which the neurons are cultured; embryonic *Xenopus* neural
tube neurons turned toward the cathode when cultured on laminin (negatively charged), and toward the anode when cultured on lysine (positively charged) [216].

The mechanisms by which growth cones sense and respond to external EFs have been extensively studied, and although a complete understanding is yet to be achieved, a number of effectors have been implicated. It is believed that exposure of growth cones to an external DCEF induces the asymmetrical redistribution of membrane-bound acetylcholine (Ach) receptors toward the cathode [215], which, in concert with BDNF- and neurotrophin-3-mediated activation of trkB and trkC receptors, trigger a signaling cascade that ultimately functions to elevate intracellular Ca$^{2+}$ levels [161,217-219]. This is supported by observations that neurite growth cone turning is fully inhibited by blockade of Ach receptors, and partially inhibited by blockade of N-, T-, and P-type voltage-gated calcium channels [219,220]. However, a separate study by Palmer et al. reported that cathodal growth cone steering persisted when both extracellular and intracellular Ca$^{2+}$ was chelated, suggesting that growth cone steering activity may be calcium-independent [221]. A proposed model of this signaling cascade (Figure 2-12) postulates that activation of Ach, trkB and trkC receptors leads to elevated intracellular Ca$^{2+}$ both through extracellular Ca$^{2+}$ influx as well as Ca$^{2+}$ release from internal stores. Ach and trkB receptors also activate the phospholipase C and phosphoinositide-3 kinase (PI3K) pathways, further elevating cytoplasmic Ca$^{2+}$ levels, and as a result, stimulating the production of cyclic adenosine monophosphate (cAMP). Protein kinase A is activated by cAMP, and functions as a regulator of the Rho-GTPase signaling pathway, which is implicated in cytoskeletal reorganization processes [161,218,222,223]. In support of this model, the cAMP antagonist Rp-cAMPS and the Rho-GTPase family blocker Toxin B both inhibit cathodal turning of Xenopus spinal neuron growth cones [161,218] (Figure 2-13). Many details of the EF-to-steering transduction process remain to be uncovered, but a thorough understanding of the methods involved would prove beneficial to axon guidance and regeneration efforts.
Figure 2-12 – Model of EF-induced signalling cascade leading to increased intracellular Ca^{2+} levels. Ligand mediated activation of ACh receptors through spontaneous ACh release directly increases intracellular Ca^{2+} because ACh receptors are “leaky” to calcium. The binding of BDNF to TrkB receptors, and neurotrophin-3 to TrkC receptors stimulates the release of Ach. The PI3K and phospholipase C pathways are activated by Ach and TrkB receptors and result in further increased intracellular Ca^{2+} levels. Elevated intracellular Ca^{2+} is also accomplished through the activation of voltage gated calcium channels and Ca^{2+} release from internal stores. Increased intracellular Ca^{2+} induces the adenylate cyclase-mediated production of cAMP. cAMP in turn activates protein kinase A, an affector of Rho-GTPase signalling. The rac1, RhoA, and cdc42 family of small Rho-GTPases are known regulators of cytoskeletal dynamics. (Source: adapted from McCaig [163], with permission from American Physiological Society).
Figure 2-13 – Cathodal growth cone turning is mediated by cAMP and Rho-GTPase signalling. In the absence of an EF (A), growth cone steering occurs in all directions in a seemingly random fashion. In the presence of an applied EF (B), growth cones turn toward the cathode. The addition of the Rho-GTPase inhibitor Toxin B (C), or the cAMP antagonist Rp-cAMPS (D) into culture conditions eradicates the cathodal turning of growth cones in the presence of an applied EF. (Source: adapted from McCaig et al. [181] and Rajnicek et al. [226], with permission from Elsevier Science & Technology Journals and The Company of Biologists Ltd.).

2.2.3.2 Wound Healing

The concept of endogenous EF-guided wound healing was briefly introduced in section 2.2.2.4. Stochastic diffusion processes and active ion-transporters together facilitate the separation of ionic charges that generate a potential difference across the epithelial tissue that forms skin, lines fluid-filled body cavities, and surrounds most organs. Injury to the epithelium disrupts the integrity of the tight junctions that form the electrically resistive barrier between the epithelium’s (usually) negative apical side and positive basal side, causing a local short circuit at the injury site that results in the generation of a lateral EF orthogonal to the TEP known as the injury potential [161,172] (see Figure 2-10). Injury potentials arise immediately after disruption of the epithelium, and persist until integrity of the tight junction seal has been reestablished (the wound is closed) [172], and as such, it is not surprising that they function as important signals that regulate wound healing. The cornea and skin have been extensively utilized as models of epithelial wounding to investigate the involvement of injury potentials in the wound healing process.
The corneal epithelium normally maintains a TEP of roughly 40 mV (internally positive) [224,225]. Wounding of the corneal epithelium generates an injury potential that can be modulated by electrical and pharmacological means, and therefore presents an excellent model for investigating the role of endogenous EFs in wound healing. Song et. al investigated the rate of wound healing in a circular lesion model of rat cornea. They found that pharmacologically enhancing or attenuating the injury potential augmented or reduced the rate of wound closure, respectively [224,225]. Several pharmacological agents were topically applied to the wound, each of which achieved the same function of modifying the corneal TEP (and therefore the injury potential), but, importantly, acted through different mechanisms. Prostaglandin E2 increases the corneal TEP by enhancing Cl\(^{-}\) efflux, as do aminophylline and ascorbic acid through the inhibition of cAMP breakdown [225]. AgNO\(_3\) increases Na\(^{+}\) influx and Cl\(^{-}\) efflux, thereby also functioning to increase the TEP [225]. In contrast, ouabain and furosemide decrease the corneal TEP by inhibiting the Na\(^{+}\)-K\(^{+}\) pump and Cl\(^{-}\) efflux, respectively [225]. Prostaglandin E2, aminophylline, ascorbic acid, and AgNO\(_3\) each nearly doubled the rate of wound closure, whereas the wound healing rate significantly declined in the presence of ouabain and furosemide [225] (Figure 2-14). Similar reduction of bovine corneal TEP using the Na\(^{+}\)-channel blocker benzamil also reduced the rate of wound epithelialization, and interestingly, enhancing the TEP by direct injection of current into the wound enhanced the rate of epithelialization independent of the polarity of the applied EF [226].
Figure 2-14 – The rate of corneal epithelium wound healing is directly proportional to the TEP. (A) The effect of various pharmacological agents on the corneal TEP. Prostaglandin E2, aminophylline, AgNO3, and ascorbic acid each enhance the TEP above normal levels, each through independent mechanisms. Furosemide and ouabain, on the other hand, attenuate the corneal TEP. (B) The rate of wound healing at various times after injury is shown, in the absence of pharmacological modulation, and also in the presence of drugs that either enhance (aminophylline) or attenuate (ouabain) the TEP. When the TEP is enhanced, the corneal wound undergoes greater healing by 30h compared to control wounds. In contrast, when the TEP is attenuated, wound healing occurs slower than in control wounds. (Source: adapted from McCaig et al. [163], with permission from American Physiological Society).

Endogenous injury potentials also regulate the rate of epidermal epithelium wound healing. Chiang et al. developed an elegant experimental technique to determine the effect on wound healing of nullifying or enhancing the endogenous injury potential generated in newt skin wounds [227]. Identical wounds were made in the digits of opposite newt hindlimbs, and the injury potentials were subsequently modulated by injecting current through one hindlimb and out of the opposite, or by submerging the hindlimbs in benzamil to collapse the TEP (and therefore also the injury potential) [227]. The experimental setup was designed such that the injection of current reduced the injury potential of one hindlimb to approximately zero, and enhanced the injury potential of the opposite hindlimb. Consistent with the reports made in corneal epithelium, reducing or enhancing the injury potential decreased or increased the rate of wound healing, respectively [227]. Moreover, benzamil-induced reduction of the injury potential attenuated the
rate of wound healing, but this was rescued by injecting current into the hindlimb to restore the injury potential to normal levels [197]. Amputated limbs and digits also produce injury potentials [198-200]. The disruption of amputation-generated wound currents in amphibians either pharmacologically with the Na\(^+\) channel blocker amiloride, or by injecting opposing current into the animal significantly inhibited regeneration of the amputated limb [228,229].

Closure of epithelial wounds occurs through a number of cellular events including proliferation, reorientation, and migration, all of which are influenced by EFs of physiological strength. Corneal epithelial cells exposed to an EF orient their axis of cell division such that the cleavage plane is perpendicular to the EF’s direction. This has been shown using human corneal epithelial cells in vitro, but more importantly, in a rat corneal epithelial injury model in vivo in which the cleavage plane orientation was determined by the direction of the lateral injury potential [224,230]. Oriented cell division was only prominent in the vicinity of the wound edge; at regions distal to the wound (400 – 600 μm away) where the injury potential is effectively zero the axis of cell division was oriented randomly. Song and colleagues demonstrated that the cleavage orientation phenomenon could be enhanced or reduced by pharmacologically increasing or decreasing the strength of the injury potential, respectively, and enhancing the injury potential influenced the cleavage orientation even at distal regions [224]. Intriguingly, they also reported that modulating the injury potential could control the rate of epithelial cell proliferation; enhancing the injury potential with prostaglandin E2 or aminophylline increased the number of dividing cells whereas attenuating the injury potential with ouabain decreased dividing cell numbers. Cultured corneal epithelial cells also align the long axis of their cell bodies perpendicular to the direction of an applied EF [231,232]. This phenomenon has also been observed in neural crest cells [233], astrocytes [234], epidermal keratinocytes [235], osteoblasts and osteoclasts [236], muscle [237], and chondrocytes [238]. It is believed that this cellular response is made in an effort to minimize the voltage drop across the cell body [235].

A common feature of many of the aforementioned cell populations is that they undergo galvanotaxis in the presence of a DCEF. In the context of epithelial wound healing, the DCEF generated by the injury potential guides the migration of cells toward the center of the wound to facilitate wound closure. For example, the cathodal galvanotaxis of corneal epithelial cells has
been demonstrated \textit{in vitro} as well as \textit{in vivo} [226,231,232,239], and has been implicated as a
key regulator of the wound healing process, in such a capacity that it has been considered a
dominant signal that may override other wound repair cues [161,172]. Indeed, enhancement or
reduction of the endogenous injury potential has been shown to increase or decrease the rate of
wound healing as described above, but in addition to this, reversal of the direction of an applied
EF actually caused wounds to open up rather than heal in a corneal explant injury model [204].
This finding is particularly astonishing when one considers that all other cues that may regulate
the wound healing process – including chemical cues, and the removal of cell-contact inhibition
– persist even when the direction of the applied EF is reversed, suggesting that EFs act as an
overriding signal in wound healing. Thus endogenous injury potentials modulate the epithelial
wound healing process in part by regulating the orientation and frequency of cell division, as
well as promoting the directional migration of cells through galvanotaxis. Augmenting
endogenous TEPs (and therefore injury potentials) to enhance wound healing is an interesting
possibility that has been explored pharmacologically, and more recently electrically using a
novel technique called synchronization modulation in which a train of electrical pulses is used to
synchronize and enhance the activity of Na/K pumps to increase the TEP [205,240].

2.2.3.3 Galvanotaxis

It is worth delving into the phenomenon of galvanotaxis in greater detail. Cellular migration is
crucial to the development, regeneration and homeostasis of organisms. The phenomenology and
mechanisms underlying cellular migration have been investigated in a multitude of studies in
various cell populations. However, this work has predominantly focused on chemotactic (the
translocation of cells along a chemical gradient), haptotactic (the translocation of cells along a
gradient of substrate adhesion sites), and mechanotactic (the translocation of cells along a
gradient of substrate stiffness) migration. In comparison, galvanotaxis – or the translocation of
cells along a gradient of voltage – has been a subject of lesser focus in cell motility research.
Despite the discovery of galvanotaxis almost 125 years ago [241], it is only within the last few
decades that research into the phenomenon has intensified and it has begun to be appreciated as a
physiological behaviour that is imperative for proper development and maintenance of
 multicellular organisms as described in the sections above. There has also been renewed interest
in implementing galvanotaxis in regenerative medicine and wound healing paradigms. The galvanotaxis of adult mouse subependymal NPCs is the major focus of this thesis, and as such, this section will expand on and thoroughly review current knowledge of this cellular behaviour.

Over 15 cell populations from mammalian, amphibian, and fish species have been identified that undergo galvanotaxis [190]. These include neural crest cells [233], corneal epithelial cells [239,242,243], lens epithelial cells [244], retinal epithelial cells [245], vascular endothelial cells [246], Schwann cells [247], leukocytes [241], macrophages [248], keratinocytes [235,249], osteoblasts and osteoclasts [236], chondrocytes [238], fibroblasts [250], neuroblasts [248], NPCs [251,252], as well as human embryonic and induced pluripotent stem cells [253]. The range of DCEF field strengths reported in galvanotaxis studies ranges from as low as 3 mV/mm to greater than 1000 mV/mm [235,247]. The velocity and directedness of galvanotaxis can be a function of the strength of the applied DCEF [232,254]. The speed of cellular galvanotaxis generally ranges from 0.2–2 µm/min [246,255]. Most cells that undergo galvanotaxis do so cathodally, but some populations, such as osteoclasts, macrophages, and human vascular endothelial cells, migrate anodally [236,248,256]. Quite interestingly, the direction of galvanotactic migration may depend on the intensity of the applied DCEF; bovine lens epithelial cells exhibited anodal migration at 50 mV/mm, but between 150-200 mV/mm the migration was cathodal [244]. Also interesting is the fact that cells from the same tissue may experience opposite galvanotactic responses. For example, osteoblasts and osteoclasts migrate in opposite directions in the presence of a DCEF, as do corneal epithelial cells and corneal stromal fibroblasts [236,242]. These astonishing findings reveal the complexity of the sensing, transduction, and directional switching processes that must regulate this intricate cellular behaviour. Indeed, the mechanisms by which cells undergo galvanotaxis remain largely unknown. These details are just beginning to be elucidated.

All cellular migration occurs in five main steps [257,258]: i) actin polymerization and protrusion of the leading edge of the membrane toward the direction of migration; ii) binding of membrane integrins to extracellular matrix adhesion proteins; iii) proteolysis of extracellular membrane components (this primarily occurs in 3D migration such as in vivo); iv) binding of myosin to actin filaments to generate actomyosin contractions; and v) detachment of the trailing edge through a combination of actin filament breakdown, proteolysis of adhesion sites, and integrin
endocytosis. Persistent directional migration requires the coordinated polarization of these processes within cells. Potential methods by which applied EFs induce polarizing effects within cells are reviewed below, including known downstream signaling effectors that have been implicated in galvanotactic migration.

Early work in corneal epithelial cells established the importance of growth factor receptors in galvanotaxis. Corneal epithelial cells experienced cathodal galvanotaxis in serum containing media - a behaviour that was lost in serum free media but was rescued by the addition of EGF, bFGF, and transforming growth factor-β1 to serum free media [232]. It was postulated that an applied EF may polarize growth factor receptors toward one end of cells’ lipid membranes and thereby evoke a directionally-biased response. McCaig and Isseroff’s groups reported that this is indeed the case by demonstrating that fluorescently-tagged EGF receptors (EGFRs) polarized toward the cathode in keratinocytes and corneal epithelial cells [259,260] after as little as 5 minutes of EF exposure. In the same study, Zhao et al. demonstrated the cathodal redistribution of bFGF and TGF-β receptors as well, although these receptors required much longer EF exposure times (12-16h) [260]. EF-induced polarization of membrane proteins has also been shown with human lung and breast carcinoma cell EGFRs, as well as acetylcholine and concanavalin A receptors in muscle and fibroblast cells [261-265]. It is proposed that such polarized activation of growth factor receptors leads to asymmetric downstream signaling events that promote directed migration. Zhao et al. showed that the activation of cathodally-polarized EGFRs in corneal epithelial cells leads to the asymmetric activation of extracellular signal-regulated kinases 1/2 (ERK1/2) – downstream EGFR effectors that are members of the mitogen activated protein kinase (MAPK) family – which in turn induces cathodal accumulation and reorganization of F-actin microfilaments [266]. Polarized actin polymerization has also been observed in bovine vascular endothelial cells [246].

PI3K is another kinase that is redistributed toward the cathodal terminal of cell membranes in an applied EF, and has been implicated in galvanotaxis [204,267]. PI3K phosphorylates the inositol phospholipid phosphatidylinositol-4,5-bisphosphate (PIP₂) to form phosphatidylinositol-3,4,5-trisphosphate (PIP₃), a membrane phospholipid that is localized to the inner membrane leaflet [268]. Akt is a downstream PI3K effector kinase that is anchored to the inner membrane leaflet
by PIP3, where it is activated through phosphorylation by phosphoinositide-dependent kinase 1 (PDK1) [269]. An applied EF polarized the activation of the PI3K/Akt pathway toward the leading edge in keratinocytes undergoing cathodal galvanotaxis [204], suggesting that this pathway is important for transducing the applied EF signal into cellular motility. In support of this, both deletion of the PI3K gene and pharmacological block of PI3K with wortmannin inhibited the galvanotactic migration, and moreover, deletion of the phosphatase and tensin homolog (PTEN) – an antagonist of the PI3K/Akt pathway – potentiated galvanotaxis. The Rho family of small GTPases (including RhoA, Rac and cdc42) are regulators of actin cytoskeleton and their role in polarized chemotactic migration in various cell populations (often in crosstalk with the PI3K pathway) has been well-studied [270-274]. Direct evidence for the involvement of Rho GTPases in galvanotaxis was first provided by Yao et al. in hippocampal neurons [275]. Thus, membrane-bound growth factor receptors, PI3K, and their downstream signaling pathways are major regulators of galvanotaxis, but the observations that i) no single disruptive factor completely abrogated galvanotactic migration in the above-mentioned studies and ii) the velocity and directedness of migration may be separately modulated suggest that the phenomenon is most likely controlled by multiple redundant processes [266].

It is interesting to consider the mechanisms by which an applied EF may actually polarize membrane-bound receptors toward one end of the cell. Jaffe was the first to propose that applied EFs may asymmetrically reorganize membrane-bound receptors through electrophoresis [276]. The abundance of sialic acid on most membrane-bound glycoproteins confers on them a net negative charge, and therefore an applied EF would electrophoretically redistribute them toward the anode (Figure 2-15). While this may explain the mechanism by which anodally migrating cells initiate a polarized migratory response, it does not account for the cathodal aggregation of EGFR and phosphorylated Akt observed in cathodal galvanotaxis. An alternative explanation is that negatively charged membrane proteins are electroosmotically mobilized in the presence of an applied EF (Figure 2-15). Negatively charged surface residues attract mobile cations, such as Na$^+$ and K$^+$, from the extracellular fluid [161,277]. These cations form a thin layer a few angstroms from the surface of the cell membrane, and are surrounded by a hydration shell that arises due to hydrogen bonding of water molecules with the charged ions [161,277]. An applied
EF induces the electrophoresis of the cations, dragging their hydration shell along with them and thereby creating a hydrodynamic force that pulls the negatively charged surface glycoproteins toward the cathode by overpowering the electrophoretic force attempting to drag them toward the anode. In support of this hypothesis, when the negative surface charge was removed by cleavage of sialic acid via neuraminidase, membrane-bound proteins reorganized electrophoretically toward the anode [277]. Despite the multitude of evidence suggesting that the EF-induced polarization of membrane bound receptors such as EGFR triggers the asymmetric signaling cascades that induce galvanotaxis, there is one study worthy of mention that calls this hypothesis into question. Finkelstein et al. disrupted the net negative cell surface charge by conjugating the cationic protein avidin to surfaces of 3T3 fibroblast and HeLa cells, causing sialic acid-containing glycoproteins to reverse their polarization toward the anode of an applied EF. Surprisingly, there was not a significant change in the directedness of these cells’ cathodal migration, despite the loss of membrane protein polarization toward the cathode.
Another potential mechanism by which cells may sense externally applied EFs is the formation of asymmetric membrane potentials. An applied EF generates a voltage gradient on the exterior of a cell, resulting in the cell exterior’s anodal end having a greater potential than the cathodal end. As a result, the anode-facing membrane is slightly hyperpolarized and the cathode-facing membrane is slightly depolarized, and it is believed that such asymmetric membrane potentials differentially activate voltage gated ion channels on either side of the cell. Voltage gated calcium channels, in particular, are of central focus in this proposed mechanism [278]. Increased calcium

Figure 2-15 – Illustration of the potential mechanisms of EF-induced membrane receptor reorganization. The largely negatively charged membrane glycoproteins would experience an electrophoretic force toward the positive anode in the presence of an applied EF. However, the negatively charged glycoprotein residues attract mobile cations in solution, such as Na\(^+\) and K\(^+\). These ions are surrounded by a shell of hydration due to their hydrogen bonding with water molecules. An applied EF would electrophoretically redistribute these mobile cations along with their hydration shells toward the cathode, and the associated hydrodynamic forces facilitate the movement of membrane proteins toward the cathode by overcoming the opposing electrophoretic forces. (Source: adapted from Messerli and Graham [281], with permission from Marine Biological Laboratory).
influx at the depolarized cathodal end of the cell would establish an intracellular Ca\(^{2+}\) gradient (highest at the leading edge) that may direct migration toward the cathode. Contrary to this hypothesis, however, many motile cells exhibit an intracellular Ca\(^{2+}\) gradient that is highest at the rear trailing edge of the cell and lowest at the leading edge of the cell [279-281], and in the context of cathodal galvanotaxis this may be due to elevated passive influx of Ca\(^{2+}\) at the hyperpolarized anodal edge [233]. This contradiction may be reconciled by more recent findings made possible by advances in imaging technology that demonstrate the existence of gradients of calcium ‘flickers’ – highly localized and short-lived elevations in intracellular Ca\(^{2+}\) - that decrease in the front-to-rear direction, in opposition to cells’ rear-to-front decreasing global Ca\(^{2+}\) levels [282].

Still, researchers have been skeptical about the asymmetric membrane potential hypothesis. It has been argued that the minute difference in membrane potential is unlikely to significantly influence voltage gated calcium channel activity [278]. Consider a cell of 20 \(\mu\)m diameter undergoing galvanotaxis in the presence of a 100 mV/mm applied EF. This would produce a voltage drop of 2 mV across the entire cell body, but due to the cytoplasm’s much greater conductivity, the majority of the voltage drop would occur across the plasma membrane at the two ends of the cell facing the electrodes (approximately half of the voltage drop at each end) [190]. Therefore, the cathode-facing membrane would experience a \(\sim\)1 mV depolarization, and the anode-facing side would experience a \(\sim\)1mV hyperpolarization. In a cell with a -70 mV resting membrane potential, this would amount to a \(\sim\)1.4% perturbation in membrane potential. It is unlikely that such a perturbation would significantly affect the activity of calcium channels, which normally requires membrane potential changes an order of magnitude greater [283]. In addition, the role of Ca\(^{2+}\) in galvanotaxis has been controversial. While some studies have reported that galvanotaxis requires the influx of extracellular Ca\(^{2+}\) [284-287], other studies have demonstrated that galvanotaxis is calcium independent [288-290]. These discrepancies together with the ubiquitous nature of Ca\(^{2+}\) as a second messenger have made it difficult to elucidate the role of Ca\(^{2+}\) in galvanotaxis. It is not surprising that Ca\(^{2+}\) is suspected to be involved in galvanotaxis, since the role of Ca\(^{2+}\) in regulating cell motility is well known. The calcium-
dependent proteases, calpains, destabilize integrin-mediated cell adhesion to the ECM and induce detachment at the trailing edge of migrating cells, as well as modulate leading edge actin polymerization and membrane protrusion [291-294]. Ca$^{2+}$ also modulates actin filament destabilization and disassembly [295,296], and membrane ruffling in motile cells [297]. Actomyosin contraction is dependent on phosphorylated myosin ATPase, the phosphorylation of which is predominantly carried out by the calmodulin (and calcium)-dependent myosin light chain kinase [298]. Thus, the involvement of Ca$^{2+}$ in cell motility is not insignificant, but more finely controlled experiments are required to address whether or not this Ca$^{2+}$ activity is essential for galvanotaxis, for instance by separately modulating different mechanisms of intracellular Ca$^{2+}$ concentration, including passive transmembrane Ca$^{2+}$ influx, voltage gated calcium channel influx, and release from intracellular Ca$^{2+}$ stores.

To summarize, galvanotaxis has been studied in numerous cell populations across several species and has gained appreciation as a regulator of endogenous wound healing processes as well as a potential therapeutic tool for the treatment of wounds and tissue regeneration. Variability exists between cell populations in terms of the threshold strength EF required for galvanotaxis, as well as the speed and directedness of migration. Often, the speed and directedness of cellular galvanotaxis are proportional to the strength of the applied EF. Several candidate cellular mechanisms for sensing and transducing an EF, as well as activating motility machinery have been identified, but a complete understanding of the complex and redundant processes involved in galvanotaxis is yet to be achieved.

2.2.4 Clinical Applications of Applied Electric Fields

There is much interest in utilizing applied EFs in clinical settings for the treatment of injuries and pathological conditions. Potential applications include axon regeneration and guidance, directed precursor cell migration, chronic wound healing, and cancer intervention [161,299]. One of the most prominent uses of clinical electrical stimulation has been the treatment of chronic wounds. Continuous DCEFs, pulsatile DCEFs and even alternating current waveforms have been reported in clinical trials to enhance chronic skin wound healing [300-303]. Due to the variability of wound types, stimulation parameters and trial conditions, it has been difficult to establish an
optimized electrical stimulation protocol, or even to concur on the effectiveness of the treatment, despite its usage for several decades [190,304].

Deep brain stimulation involves the implantation of a neural pacemaker into the brain for the delivery of electrical impulses to modulate neural activity, and has experienced success in the treatment of movement and affective disorders including Parkinson’s disease [305-307], tremors [308], dystonia [309], chronic pain [310], and depression [311]. Another neural modulation application is the use of functional electrical stimulation (FES) therapy. FES therapy typically involves localized delivery of electrical pulses to peripheral nerves to activate paralyzed or paretic muscles in neurologically impaired individuals [312]. FES therapy has been used predominantly in spinal cord injured individuals to restore motor functions such as reaching, grasping, and gait [313-318], as well as bladder and bowel movements [319], and respiratory function [320]. Unfortunately, very little is known about the mechanisms underlying the effects of deep brain stimulation and FES therapy; elucidating the methods by which these interventions work could lead to significant developments in the effectiveness of the therapies.

There are still other clinical uses of electrical stimulation currently in existence, including transcranial direct current stimulation and transcranial magnetic stimulation for modulation of neural activity [321,322], anti-inflammatory stimulation [323], bone healing and spinal fusion [324,325], and cancer treatment. This section is not intended to comprehensively review clinical reports of these applications, but to convey the fact that electrical stimulation therapy is already widely used as a medical tool. With further research the utility of electrical stimulation may improve to enhance therapeutic outcomes of current treatments, or it may even expand into the realm of clinical tissue regeneration, for example to repair the injured brain and spinal cord.
Chapter 3

3 Thesis Objectives and Hypotheses

The overall goal of this thesis was to characterize the migratory behaviour of adult mammalian brain neural precursor cells in the presence of an electrical field, and to investigate the cellular mechanisms that regulate this behaviour. This work would form the basis of future projects that would investigate the utility of electrical field-induced migration in promoting neural precursor migration \textit{in vivo} for the purposes of enhancing neural repair. A secondary goal was to demonstrate to other researchers that properly designed electrical stimulation is a powerful tool for enhancing neural precursor migration, and should be considered in greater depth for integration into neural repair efforts.

3.1 Objectives

My specific objectives are described below.

1. To characterize the migratory behaviour of undifferentiated and differentiated neural precursors in the presence and absence of an electrical field
   
   i. Design a chamber suitable for application of an electrical field and for analyzing cell migration via time-lapse microscopy
   
   ii. Develop a protocol for plating and imaging neural precursors
   
   iii. Quantify parameters of migration, including magnitude of velocity, directedness, and tortuosity
   
   iv. Verify the differentiation state of each cell group analyzed

2. To investigate the cellular mechanisms involved in galvanotaxis

   i. Identify target pathways for investigation
ii. Develop a protocol to inhibit or suppress effectors within the signalling pathway being investigated

iii. Investigate the effects of suppressing the signalling pathway on neural precursor galvanotaxis

3. To develop a method of inducing undifferentiated neural precursor galvanotaxis using charge-balanced biphasic electrical fields

   i. Design charge-balanced biphasic waveforms capable of inducing galvanotaxis

   ii. Quantify parameters of migration, including magnitude of velocity, directedness, and tortuosity

   iii. Verify the differentiation state of each cell group analyzed

3.2 Hypotheses

My hypotheses are listed below:

1. Undifferentiated adult mouse SEZ NPCs are amenable to electrical field-induced locomotion (galvanotaxis).

2. In conditions that promote adult mouse NPC galvanotaxis, differentiated cells do not migrate.

3. SEZ NPCs migrate toward the cathode of an electrical field.

4. NPCs remain undifferentiated following EF exposure.

5. The magnitude of NPC galvanotaxis velocity is dependent on EGF signalling.

6. The magnitude of NPC galvanotaxis velocity is dependent on Ca\(^{2+}\) influx.

7. NPC galvanotaxis can be induced using charge-balanced biphasic EFs.
Chapter 4

4 Adult Subependymal Neural Precursors, but Not Differentiated Cells, Undergo Rapid Cathodal Migration in the Presence of Direct Current Electric Fields

4.1 Introduction

NPCs in the adult brain are promising candidates for the development of strategies to repair CNS tissue following injury or disease [326]. Adult NPCs (comprising both stem and progenitor cells) reside in a well-defined region lining the lateral ventricles known as the subependyma (SE). Under baseline conditions, adult NPCs migrate to the olfactory bulb where they differentiate into mature neurons. Following injury several studies have demonstrated the migration of SE-derived NPCs to the site of injury, both in the presence or absence of exogenous factors that activate the cells [96,100,112]. Factors such as EGF [112], the cytokine SDF-1α [104,105], and microglia derived inflammatory molecules [327] have been proposed as guidance cues that promote the migration of NPCs toward lesion sites. Post-injury NPC migration toward the site of injury has not been fully characterized and is relatively limited. The ability to enhance the migratory capacity and subsequent integration of NPCs into newly generated tissue would be beneficial for the development of neural repair strategies.

Physiological DCEF s play important roles during development and in tissue repair [189,195,224,225] and have been shown to cathodally direct the turning of growth cones during axon elongation [212,328]. In the context of wound repair, keratinocytes and corneal epithelial cells have been shown to migrate cathodally in the presence of DCEF s similar in strength to those that arise at the site of skin and cornea lesions during wound healing [232,235]. The

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1 The work presented in this chapter has been published in:
phenomenon by which cells migrate in a directed manner in the presence of an electric potential gradient is termed galvanotaxis. A previous study by Li et al. [329] demonstrated that a subpopulation of cells – primarily neuroblasts – within an explant of embryonic neural tissue undergoes cathode-directed galvanotaxis. A more recent study by Meng et al. showed that adult hippocampal cell line-derived NPCs (HCN-A94 cells) exhibit cathodal galvanotaxis in the presence of 250 mV/mm and 500 mV/mm DCEFs [252]. The existence of endogenous DCEFs in the mammalian brain [330] raises the possibility that wound-induced DCEFs (that may arise, for example, following stroke) may play a role in guiding endogenous NPCs to the site of injury.

Given our work demonstrating the significant contribution of endogenous SE-derived NPCs to tissue regeneration and functional recovery following stroke [112], we asked whether adult SE-derived NPCs could be induced to undergo cell body translocation in a rapid and directed fashion in the presence of a DCEF. Importantly, we examine the effects of DCEFs on differentiated neural cells as the ability to selectively target NPCs is an important consideration for developing neural repair strategies. Herein we have used live cell time-lapse imaging to perform an extensive kinematic analysis on pure populations of adult SE-derived NPCs and their differentiated progeny. We demonstrate rapid and directed cathodal migration of NPCs in vitro in the presence of a DCEF. The migration persists only for as long as the DCEF was applied, and removal of the DCEF results in the quick diminution of galvanotaxis. Moreover, we show that NPC cathodal galvanotaxis is unchanged in the presence of continuous media cross-perfusion demonstrating the phenomenon is a direct effect of the EF and not a secondary chemotactic effect. Most interesting, we show that the migration is specific to undifferentiated NPCs and is not observed in the differentiated progeny of NPCs. Finally, we demonstrate that EGF signaling plays a role in the speed of the migratory behaviour with little effect on the directedness. We suggest that harnessing the migratory potential of NPCs in the presence of an EF in vivo may provide means to enhance endogenous neurorepair and tissue regeneration elicited by SE-derived NPCs.
4.2 Materials and Methods

4.2.1 Ethics Statement

All animal work was approved by the University of Toronto Animal Care Committee in accordance with the institutional guidelines (protocol no. 20008754).

4.2.2 Cell Culture

NPCs were obtained as previously described [331]. Briefly, adult CD1 mice were sacrificed by cervical dislocation. Brains were dissected, and the periventricular region was enzymatically dissociated. Cells were plated at 10 cells/µL in T25 or T75 culture flasks (BD Falcon, Canada) in SFM (DMEM:F12 3:1) supplemented with EGF (20 ng/mL; Sigma-Aldrich, Canada), basic fibroblast growth factor (bFGF, 10 ng/mL; Sigma-Aldrich, Canada) and heparin (2 µg/mL; Sigma-Aldrich, Canada) [1,3]. After 7 days, primary neurospheres formed consisting purely of nestin-positive NPCs [1]. Primary neurospheres were either utilized for migration analysis or passaged and replated for another 7 days to form secondary neurospheres before being plated into galvanotaxis chambers.

4.2.3 Galvanotaxis chamber construction

Galvanotaxis chamber construction was adapted from Zhao et al. [260]. Briefly, galvanotaxis chambers were constructed by sealing acid-washed square no. 1 glass cover slides (22 mm x 22 mm x 0.17 mm) (VWR, Canada) to the base of 60x15 mm plastic Petri dishes with silicon vacuum grease (VWR, Canada). A pair of rectangular glass slide pieces (22 mm x 5 mm x 0.17 mm) were sealed to opposite edges of the square slide to yield a central chamber with dimensions 22 mm x 10 mm x 0.17 mm. The chambers were then UV sterilized for 15 minutes. The central troughs were coated with 100µg/mL poly-L-lysine (Sigma-Aldrich, Canada) for 2 hours at room temperature, rinsed 3 times with 1 mL of autoclaved water, and then incubated in 4% (v/v) Matrigel (BD Biosciences, Canada) in SFM for 1 hour at 37°C. The troughs were rinsed twice with SFM and covered with 300 µL of SFM supplemented with either EGF, bFGF and heparin, or fetal bovine serum (FBS, Invitrogen-Gibco, Canada) - depending on the experiment being performed - until ready to be plated with neurospheres. In experiments
investigating the galvanotactic properties of undifferentiated NPCs, primary or first passaged neurospheres were plated on the chamber for 17-20 hours in the presence of EGF, FGF, and heparin at 37°C, 5% CO2 and 100% humidity. In contrast, neurospheres were plated into chambers for 69-72 hours in the presence of 1% FBS in SFM for experiments investigating the galvanotactic properties of NPCs induced to differentiate into mature phenotypes.

4.2.4 Galvanotaxis Assay

Neurospheres plated into the chamber were covered with a square no. 1 glass cover slide to create a roof to the central trough, yielding a central chamber. A media reservoir was created on either side of the chamber and sealed with vacuum grease so as to permit electric current flow only through the chamber (Figure 4-1). Two 15 cm length PVC tubes (Fisher Scientific, Canada) (2.38 mm inner diameter, 3.97 mm outer diameter) were filled with 1.5% agarose gel. Silver wire (Alfa Aesar, USA) was cut into two 10 cm pieces, coiled and immersed in Javex bleach for 20 minutes to form Ag/AgCl electrodes. The galvanotaxis chamber to be analyzed was placed onto the stage of a Carl Zeiss Axiovert 200M microscope (Zeiss, Germany) that was situated within a temperature- and CO2-controlled, 100% humidity encasing. Each Ag/AgCl electrode was placed in a 60x15 mm Petri dish that was filled with 7.5 mL of SFM. These Petri dishes were placed on the microscope stage on either side of the Petri dish containing the galvanotaxis chamber. The agarose gel tubes were used to bridge the Petri dishes in order to establish electrical continuity between all three dishes (Figure 4-2). The Ag/AgCl electrodes were connected to an external constant-voltage power supply to establish a DCEF of strength 250 mV/mm [252,329] across the galvanotaxis chamber in the direction of the positive X-axis. Cell migration was recorded via time-lapse imaging microscopy using Zeiss Axiovision software, with images being captured at a frequency of one per minute for 2.5-8 hours. Cells were viewed at 5x for the largest field of view.

For cross-perfusion experiments, a microfluidic channel with two reservoirs (µ-Slide I, Ibidi, Germany) was pre-treated identically to the galvanotaxis chambers described above. Neurospheres were plated into the chambers in SFM + EGF, bFGF and heparin, and incubated for 17-20 hours as previously described. Each reservoir was filled with 1mL of SFM + EGF,
bFGF and heparin, PTFE thread sealant tape was wrapped around the rim of each of the channel’s reservoirs and the lids were replaced onto the reservoirs to create a tight seal. A 16G1½ stainless steel needle was inserted into each reservoir. The needles served two purposes: i) they were hollow and therefore permitted fresh media perfusion and ii) they were metallic and therefore electrically conductive. The chambers were secured to the microscope stage and a peristaltic pump (Ismatec, Switzerland) was connected to the inlet and outlet terminals of the chamber via the 16G1½ needles to perfuse fresh SFM + EGF, bFGF, and heparin at a flow rate of 0.83 mL/min. The electrodes of the external power supply were connected directly to the needles to form a DCEF of strength 250 mV/mm across the galvanotaxis chamber.

Figure 4-1 - Assembled galvanotaxis chamber. Strips of vacuum grease are used to create a pool of culture media on either side of the central trough.
4.2.5 Immunocytochemistry

Cells were fixed with 4% paraformaldehyde for 20 minutes at room temperature directly in the galvanotaxis chambers and then washed 3 times with phosphate buffered saline (PBS) for 5 minutes each. Cells were permeabilized with 0.3% Triton X-100 for 20 minutes at room
temperature, followed by a triple wash with PBS for 5 minutes each time. Blocking was performed with 10% normal goat serum (NGS, Jackson Immunoresearch Laboratories, Canada) in PBS for 1 hour at room temperature. Cells were incubated overnight in primary antibody at 4°C. The following day the chambers were washed three times with PBS for 5 minutes each time, and incubated at 37°C for 1 hour with secondary antibody. Primary and secondary antibody incubations were repeated for all antigens of interest. The following primary and secondary antibodies were used: primary: mouse monoclonal anti-nestin (1:400, Millipore, Canada), and rabbit polyclonal anti-GFAP (1:500, Sigma, Canada); secondary: goat-anti-mouse conjugated with Alexafluor 568 (1:400, Invitrogen-Gibco, Canada), and goat-anti-rabbit conjugated with Alexafluor 488 (1:400, Invitrogen-Gibco, Canada). Nuclear staining was performed with mounting medium containing DAPI (Vector Laboratories, Canada). Samples were stored at -20°C until they were imaged.

4.2.6 Quantification of Cell Migration

Cell migration was tracked via Zeiss Axiovision software’s automated tracking module. In order to ensure that cells could be followed for the duration of tracking, cells were selected for kinematic analysis if they were at least one cell body away from the nearest cell thereby decreasing the likelihood of cells overlapping each other during migration. For cells that were closer than one cell body to the surrounding cells manual tracking was performed using Zeiss Axiovision’s tracking module. Cell position was determined by cell centroid locations. A minimum of 45 cells from at least 3 separate experiments were analyzed for each experimental group. Four kinematic parameters were analyzed.

1. *Displacement* in the direction of the positive X-axis (which is parallel to the direction of the DCEF vector when DCEF is applied) was analyzed over 2.5 hours, because this was the maximum time common to all experimental groups that the DCEF state remained constant (i.e., the maximum time before the DCEF was either reversed or eliminated).
2. The magnitude of velocity (herein referred to as *velocity*) was defined as the total displacement between the initial and final positions of the cells divided by total experimental time.

3. *Directedness* was obtained by dividing the displacement along the DCEF vector by the total (x,y)-displacement between the initial and final positions of the cells. Directedness was taken as positive in the direction of the DCEF (positive X-axis) and negative in the direction opposite the DCEF (negative X-axis).

4. *Tortuosity* was defined as the total path length of the cells’ migration divided by the displacement between the initial and final cell positions.

The latter two parameters (3 and 4) characterize the extent to which the cells migrate in a straight line toward the cathode; a value of 1 for both directedness and tortuosity indicate a perfect straight-line migration parallel to, and in the direction of, the positive X-axis. In experiments where the direction of the DCEF was reversed, cells were deemed to have switched direction once their centroid exhibited a displacement in the direction of the new cathode for a minimum of two consecutive frames.

4.2.7 Statistical Analysis

All values are presented as group means ± S.E.M. Unless otherwise specified, differences between group means were determined using one-way ANOVA. Two-way ANOVA analysis with Bonferroni post-hoc tests were performed to determine if there was an interaction effect between the state of the DCEF and the differentiation state of the cells on the migratory behaviour of the cells. In all cases, statistical significance was set at \( p < 0.05 \).
4.3 Results

4.3.1 Undifferentiated NPCs Undergo Cathodally-Directed Galvanotaxis In The Presence Of A DCEF

We determined the pattern of migration that the NPCs exhibit in the presence or absence of an applied external DCEF. Neurospheres were plated into Matrigel-coated galvanotaxis chambers in growth factor conditions (EFG, FGF and heparin) to maintain the NPCs in an undifferentiated state for the duration of plating and imaging. Immunocytochemical analysis revealed the presence of nestin\(^+\) cells, verifying that the cells remained undifferentiated following 20 hours of culture on the chambers (Figure 4-3A). Neurosphere cells that adhered and dissociated in the chambers were analyzed for migration using time-lapse imaging for a period of 2.5-8 hours in either the absence or presence of a DCEF (250 mV/mm). Post-imaging immunostaining analysis revealed nestin expression was maintained in the NPCs regardless of the presence or absence of a DCEF when maintained under growth factor conditions (Figure 4-3A and 4-3B).

In the absence of a DCEF, mean cell body displacement over 2.5 hours in the direction of the cathode was 5.72 ± 1.63 µm vs. 157.35 ± 22.11 µm in the presence of a DCEF (Figure 4-3C and Figure A1). In the absence of a DCEF cells migrated in a random, non-directed manner at a mean velocity of 0.23 ± 0.12 µm/min, a mean directedness (defined as the total DCEF vector displacement divided by the straight-line distance between initial and final cell positions) of 0.12 ± 0.07, and mean tortuosity (the total path length of cell migration divided by the straight-line distance between initial and final cell positions) of 5.10 ± 0.69 away from the central core of the dissociated neurosphere (Figure 4-3D-G, Movie S1). Striking was the observation that when exposed to a DCEF, these undifferentiated NPCs underwent cathodal galvanotaxis at a rate of 1.09 ± 0.15 µm/min, >4 times that seen in the absence of a DCEF (p<0.05). The directedness increased to 0.96 ± 0.01 (a 9-fold increase, p<0.05), and tortuosity was reduced >3-fold to 1.56 ± 0.10 (p<0.05), compared to precursors not exposed to a DCEF (Figure 4-3D, 4-3E, 4-3H, and Movie S2). Hence, undifferentiated NPCs exposed to a DCEF undergo rapid and cathodally-directed galvanotaxis, whereas their migratory behaviour in the absence of a DCEF is slower and non-directed. During exposure to a DCEF the NPCs were observed extending filopodia toward the cathode (Movie S3).
Figure 4-3 – Undifferentiated NPCs undergo rapid and cathodally-directed migration in the presence of a DCEF. (A-B) NPCs are positive for nestin after 17h on matrigel in the presence of EGF, bFGF and heparin (A) as well as after 6h of DCEF exposure in the presence of EGF, bFGF and heparin (B). (C-F) NPCs exposed to a DCEF (n=4) exhibit a larger DCEF-vector displacement (C), larger velocity (D), larger directedness (E), and smaller tortuosity (F), compared to NPCs not exposed to a DCEF (n=3). (G-H) Typical cell migration paths for NPCs either not exposed (G), or exposed (H), to a DCEF of strength 250 mV/mm. Scale bars = 100um. Data are presented as means ± S.E.M., *=p<0.01, **=p<0.001.

To ensure that the rapid and directed migration observed in the presence of the DCEF was a property of the majority of NPCs, and not due to their relative position within the cell cluster, we
analyzed the migration of all cells within the field of view for a subset of three experiments using the manual tracking module of Zeiss Axiovision software. We found that 98.9% ± 0.4% of cells (1147 cells analyzed) had a positive overall displacement in the direction of the DCEF, indicating that galvanotactic migration is not a feature of a smaller sub-population of NPCs, but rather a phenomenon observed at the population level. Intraexperimental analyses between single cells that satisfied the cell selection criteria (cell situated near the outer edge of the dissociated neurosphere and at least one cell body away from the nearest cell) compared with cells that were situated near the cores of dissociated neurospheres revealed a significant difference in the velocity of migration (1.22 ± 0.10 μm/min vs. 0.56 ± 0.04 μm/min respectively, p < 0.05) and displacement along the DCEF vector (176.69 ± 15.16 μm vs. 77.71 ± 5.87 μm respectively, p < 0.05), but no change in tortuosity (1.37 ± 0.05 vs. 1.32 ± 0.03) or directedness (0.97 ± 0.01 vs. 0.92 ± 0.02). Hence, cells in higher density regions (at the centre of the clusters) migrated with a lower velocity than cells that were situated in lower density regions (near the perimeter of the cluster). Since cells can serve as physical obstructions to the migration of other cells, the regions of lower cell density may be more permissive to galvanotaxis than higher-density regions. For this reason, we focused the remainder of our analyses on cells residing near the outer edge of the dissociated neurosphere.

To determine whether exposure to the DCEF had long-term effects on the behaviour of the NPCs, we examined the migratory behaviour immediately following the removal of the DCEF. We found that within 20 minutes following the abrupt removal of the DCEF, the NPCs reverted to a non-directed, random migration similar to that observed when the cells had not been exposed to a DCEF at all (Figure 4-4A-D, Movie S4), and eventually their migratory behaviour became indistinguishable from NPCs that had never been exposed to a DCEF. Finally, we performed NPC galvanotaxis assays in which the direction of the DCEF was reversed after 2.5 hours so that it pointed in the direction of the negative X-axis instead of the positive X-axis. Strikingly, the majority of cells (80.0% ± 10.1%, 45 cells analyzed) reversed their direction of migration toward the new cathode within 15 minutes, although migration reversal was observed as early as 3 minutes (Figure 4-4E). Moreover, this reversal was observed at the population level as all cells within the field of view switched direction within 30 minutes. The cells maintained rapid and
directed migratory behaviour toward the new cathode (Movie S5). Our results indicate that the cellular machinery required for NPC galvanotaxis can be actuated to induce migration – as well as reorganized to reverse migration – within 15 minutes of the DCEF onset or reversal, suggesting that de novo protein synthesis is not necessary for the process.

Figure 4-4 – NPC galvanotaxis persists only for as long as the DCEF stimulus is present. (A-D) Analysis of cell migration for 20 minutes before and 20 minutes after the removal of the DCEF reveals a significant decrease in DCEF vector displacement (A), velocity (B), and directedness (C) of migration, as well as a significant increase in tortuosity (D). Following the reversal of the DCEF’s direction, 80.0% ± 10.1% of cells analyzed reverse their direction of migration to point toward the new cathode within 15 minutes (E). Data are presented as means ± S.E.M (n=3), *p<0.005, **p<0.05".
4.3.2 Cells With Differentiated Neural Phenotypes Do Not Exhibit Galvanotactic Migration

We next asked if galvanotaxis is specific to undifferentiated NPCs or also a property of differentiated phenotypes. Neurospheres were plated into galvanotaxis chambers as described in the presence of 1% FBS for 69-72 hours to induce cell differentiation into mature neural phenotypes. Immunocytochemical analysis demonstrated that the majority of the cells expressed GFAP after 69 hours (Figure 4-5A), confirming that the NPCs had differentiated into astrocytes. This phenotype was maintained in FBS-cultured cells after 6 hours of DCEF exposure (Figure 4-5B). A rare subpopulation of cells continued to express nestin (Figure 4-5A, arrowhead) representing undifferentiated precursor cells. Differentiated cells were maintained in 1% FBS and either exposed or not exposed to a DCEF. Pre-differentiated cells exposed to a DCEF exhibited a mean -8.83 ± 5.08 µm displacement in the direction of the DCEF–vector at a mean velocity of 0.14 ± 0.02 µm/min (Figure 4-5C-D, A2, and Movie S6) Their directedness of migration was -0.26 ± 0.16, with a mean tortuosity value of 2.92 ± 0.25 (Figure 4-5E and F). This migratory behaviour did not differ significantly from that of differentiated cells that were not exposed to a DCEF or from undifferentiated cells in the absence of a DCEF (Figure 3C-3H, and Movie S7). Notably, post-DCEF labeling revealed that the differentiated cells (primarily astrocytes) aligned their processes perpendicular to the direction of the DCEF (Figure 4-5B).
Figure 4-5 – Differentiated NPCs do not exhibit directed migration in response to a DCEF. (A-B) cells are positive for the astrocytic marker GFAP after 69 h on matrigel in the presence of 1% FBS (A), as well as after 6 h of DCEF exposure in the same conditions (B). (C-F) Differentiated cells (FBS, dark bars, n=4) exhibit no significant differences in DCEF-axis displacement (C), velocity (D), or directedness (E) when compared to differentiated cells in the absence of a DCEF (FBS, white bars, n=3) and undifferentiated NPCs (EFH, white bars, n=3) not exposed to a DCEF. However, undifferentiated NPCs in the absence of a DCEF exhibit greater tortuosity of migration than differentiated neural cells (F). (G-H) Typical cell migration paths for differentiated cells either not exposed (G), or exposed (H), to a DCEF of strength 250 mV/mm. Scale bars = 100 μm. Data are presented as means ± S.E.M, *p<0.05.

We considered that the lack of galvanotactic behaviour observed among differentiated cells could be due to the prolonged period of time that the cells are adhered to the Matrigel substrate in the galvanotaxis chambers prior to DCEF exposure. We asked if differentiated cells would undergo galvanotaxis if they adhered to the Matrigel substrate for only 17 hours, similar to the length of time that undifferentiated NPCs were maintained and exhibited galvanotaxis. Accordingly, NPCs were cultured for 52 hours in 1% FBS as free-floating neurospheres, and subsequently plated into Matrigel-coated galvanotaxis chambers for 17 hours in 1% FBS prior to application of the DCEF. We observed no significant difference in the migratory behaviour of differentiated cells after 17 hours versus ~70 hours of adhesion (Figure 4-6A-E) indicating that the lack of galvanotactic behaviour is due to their differentiated state, and not the prolonged binding period to Matrigel that is required to achieve FBS-induced maturation. Immunostaining post-DCEF application verified that the cells had differentiated (Figure 4-6F).

Studies have indicated that the galvanotactic response of corneal epithelial cells [266], keratinocytes [259], and hippocampal precursors [252] is dependent on EGFR signaling. We asked whether the lack of galvanotaxis exhibited by differentiated cells (in FBS conditions) was due to the lack of exogenous growth factors in the culture media during exposure to the DCEF. NPCs were first exposed to differentiation conditions (FBS) for 69-72 hours on matrigel-coated chambers. Following this, the culture medium was aspirated and replaced with growth factor-supplemented medium (as is used to maintain NPCs in a precursor state) and the pre-differentiated cells were then immediately exposed to a DCEF. We found that growth factor-supplemented media failed to rescue galvanotaxis in cells that had been pre-cultured in
differentiation conditions for 69-72 hours (Figure A3). Notably, differentiated cells transferred to growth factor conditions exhibited similar velocity and tortuosity compared to differentiated cells maintained in FBS conditions at all times. Interestingly, although differentiated cells consistently displayed low displacement in the direction of the DCEF and low directedness of migration, differentiated cells transferred to growth factor conditions showed a tendency to increased displacement towards the cathode relative to differentiated cells maintained in FBS at all times. Taken together, this suggests that the lack of rapid and cathodally-directed migration in differentiated cells is not due to the lack of EGF and bFGF signaling in the cells, and that growth factor signaling may impact the direction, but not the velocity, of these cells’ migration. Figure 4-7 summarizes and compares the migratory behaviour of both differentiated and undifferentiated cells in either the absence or presence of a DCEF using 2-way ANOVA analysis.
Figure 4-6 – Prolonged adherence to Matrigel is not responsible for the loss of galvanotactic behaviour in differentiated cells. (A-D) Differentiated cells plated on Matrigel for 70 h (white bar, n=4) or 17 h (dotted bars, n=3) exhibit similar DCEF-axis displacement (A), velocity (B), directedness (C), and tortuosity (D). Allowing the differentiated cells to adhere to Matrigel for an equal amount of time as the undifferentiated cells (hatched bar, n=4) does not prevent the loss of galvanotactic behaviour (A-D). (E) Typical cell migration paths for cells cultured in the presence of FBS for 70 hours and plated on Matrigel for 17 hours. (F) Immunostaining post DCEF-application reveals that the majority of cells are...
GFAP⁺ indicating that the NPCs have differentiated after 72 hours in FBS and 20 hours on Matrigel. Scale bar = 50 µm. Data are presented as means ± S.E.M, *p<0.001, **p<0.005.

Figure 4-7 – Summary of migratory properties of undifferentiated NPCs and differentiated cells in the absence and presence of a DCEF. (A-D) Undifferentiated NPCs exposed to a DCEF (n=4) exhibit larger DCEF-axis displacements (A), larger velocities (B), larger directedness (C) and smaller tortuosities (D), compared to NPCs in the absence of a DCEF (n=3), as well as compared to their differentiated counterparts in both the absence (n=3) and presence (n=4) of a DCEF. Data are presented as means ± S.E.M, *p<0.001, **p<0.05.
4.3.3 Electrically-Induced NPC Migration Is A Direct Effect Of The Electric Field

The conductivity of the culture media is imparted by its electrolyte constituents. As such, the existence of charged molecules within the media render the possibility of an EF-induced redistribution of the electrolytes to form a chemotactic gradient [172,332,333]. We asked whether the observed directed migration of the NPCs was a direct effect of the DCEF, or if the cells were responding to a DCEF-induced chemical gradient. To eliminate the possibility of a chemical gradient forming within the galvanotaxis chamber, we designed a novel chamber that permitted the continuous perfusion of fresh SFM + EGF, bFGF, and heparin. The DCEF was maintained in the direction of the positive X-axis as in previous experiments, while media was continuously perfused in the direction of the negative X-axis, opposing the electric current flow.

Remarkably, the NPCs migrated in a directed manner against the shear stress of the fluid flow toward the cathode, albeit with a higher tortuosity than that of NPCs in the presence of a DCEF without SFM cross-perfusion (2.16 ± 0.20 vs. 1.56 ± 0.10) (Figure 4-8A-C, Movie S8). There were no statistically significant differences in the velocity and directedness of migration between these two groups. Hence, these results demonstrate that the directed nature of the NPCs’ migration in the presence of a DCEF is indeed a galvanotactic effect, and not a chemotactic effect induced by the DCEF.
Figure 4-8 – The galvanotactic behaviour of undifferentiated NPCs is a direct effect of the applied DCEF. (A–C) NPCs exhibit no significant differences in the velocity (A), and directedness (B), of galvanotaxis when in either the presence (n=4) or absence (n=3) of a continuous cross-flow of media although they exhibit a higher tortuosity (C). Data are presented as means ± S.E.M, *=p<0.05.

4.3.4 The Galvanotactic Response Of Undifferentiated NPCs To An Externally Applied DCEF Involves EGFR Signalling

We demonstrated that the lack of migration of differentiated cells was not due to the lack of EGF since the addition of EGF could not rescue the galvanotactic response of differentiated cells. Next we asked if EGF signaling was important for the migratory behaviour of undifferentiated SE NPCs as previously described for other cell types [252]. We plated undifferentiated neurospheres into galvanotaxis chambers as before for 17 hours. Following this, the media was aspirated from the chambers, the troughs were gently washed and fresh SFM supplemented only with bFGF and heparin was immediately applied into the chamber and media reservoirs. The
bFGF was present in order to maintain the NPCs in an undifferentiated state. Time-lapse imaging revealed that in the absence of EGF, NPCs exhibited significantly reduced DCEF-axis displacement (62.4% ± 18.7% reduction), velocity (67.8% ± 17.7% reduction), and directedness (9.8% ± 3.2% reduction) of migration, as well as significantly increased tortuosity (112.9% ± 48.7% gain) (Figure 4-9A-D) compared to NPCs maintained in the presence of EGF at all times. We further demonstrated a role for EGF signaling in NPC galvanotaxis using the EGFR blocker, erlotinib which inhibits EGFR tyrosine kinase activity by preventing EGFR autophosphorylation via competitive binding to the ATP binding domain [334]. NPCs cultured in the presence of growth factors (EGF, FGF2 and heparin), with erlotinib (5 µg/mL in dimethyl sulfoxide, Santa Cruz Biotechnology, USA) migrated at a significantly decreased velocity, and increased tortuosity relative to vehicle controls and NPCs in growth factor conditions alone (without erlotinib) (Figure 4-10A-D, Movie S9). Immunostaining verified that the NPCs remained nestin-positive after 2.5 hours of DCEF exposure in the presence of erlotinib, suggesting that FGF2 is sufficient to maintain cells in an undifferentiated state within this time period (Figure A4). Notably, even in the absence of EGFR signaling (no EGF, or the presence of erlotinib) NPCs maintained their directional bias toward the cathode, with only a 14% reduction in directedness, relative to cells in the presence of EGFR signaling. This suggests that EGFR signaling predominantly impacts the velocity – and to a lesser extent the directedness – of SE NPC galvanotaxis. Further, the migratory behaviour of NPCs exposed to a DCEF in the absence of EGF was not significantly different from that of NPCs in the presence of erlotinib. Taken together, these data suggest that while EGF signaling plays a role in the galvanotactic response of NPCs, it is not responsible for all the cell behaviours observed.
Figure 4-9 – EGF is involved in regulating the rapid and directed NPC migration when exposed to a DCEF. (A-D) NPCs that are only exposed to bFGF and heparin (FH, n=3) during DCEF application undergo smaller DCEF-axis displacements (A), lower velocity (B), lower directedness (C), and higher tortuosity (D), of migration compared to NPCs maintained in the presence of EGF, bFGF and heparin (EFH) at all times (n=4). (E) NPCs remain positive for nestin following DCEF exposure in the presence of FH only. Scale bar = 100 µm. Data are presented as means ± S.E.M, *p<0.05.
4.4 Discussion

We have demonstrated that clonally-derived pure populations of adult SE-derived NPCs exhibit rapid and directed galvanotaxis toward the cathode of a DCEF. Moreover, we have shown this phenomenon to be unique to undifferentiated NPCs; inducing their maturation into differentiated phenotypes is associated with a loss of electrically-induced migratory capacity. Through continuous fresh media cross-perfusion experiments, we show that directed migration of NPCs in the presence of an applied DCEF is a direct effect of the field rather than an indirect chemotactic effect. We have provided evidence that NPC galvanotaxis is moderated by EGF signaling; both the removal of EGF from the culture medium as well as the blockade of EGFR via erlotinib significantly attenuate NPC galvanotaxis. Most interesting is the finding that loss of galvanotactic behaviour associated with FBS-induced maturation of NPCs could not be reversed by replacing the cells in the presence of EGF and bFGF. Thus our data indicate externally applied DCEF s can stimulate and guide the migration of undifferentiated SE NPCs, but not that of NPCs induced to differentiate into mature neural phenotypes.

The role of EFs in the central nervous system has been previously explored. The axons of embryonic rat hippocampal neurons align perpendicular to the direction of an applied DCEF in an EF strength-dependent manner after 24 hours of exposure [335], and interestingly, individual growth cones of dendrites, but not axons, undergo cathodal orientation [336]. Xenopus embryo neural tube cells have been shown to elicit EF strength-dependent cathodal turning of neurites, although the direction of neurite growth in response to an applied DCEF varies depending on the substrate adhesiveness and net surface charge; negatively charged substrates such as laminin promote cathodal outgrowth, whereas positively charged substrates such as lysine promote anodal outgrowth [214,216], reviewed in [337]. DCEF s also serve to modulate neuronal structure through differential neurite growth rate regulation (anode-facing neurites exhibit significantly slower outgrowth rates compared to cathode-facing neurites), and by enhancing neurite branching (predominantly cathodally) [212,337,338]. Interestingly, EF exposure has been reported to impact the differentiation profile of NPCs. In higher strength DCEF s (437 mV/mm) adult rat hippocampal NPCs exhibit a tendency to differentiate into neurons, whereas the differentiation profile of embryonic mouse NPCs encapsulated in alginate hydrogel beads and
exposed to lower-strength (1-16 mV/mm) alternating current EFs is dependent on the frequency and duration of stimulation [339,340]. While these studies investigated the neurite response or differentiation of relatively stationary somata in the presence of a DCEF, we were interested in the entire cell body translocation of NPCs.

Figure 4-10 – Epidermal growth factor signaling plays a role in the galvanotactic response of NPCs. (A-D) NPCs exposed to a DCEF in the presence of the EGFR blocker erlotinib (n=3) experience significantly reduced DCEF-axis displacement (A), velocity (B), and directedness (C), of migration, as well as significantly greater tortuosity (D), compared to NPCs in the absence of erlotinib (EFH, n=4, and EFH+vehicle, n=3). Data are presented as means ± S.E.M, * = p < 0.05, ** = p < 0.001
The findings reported here are similar to those of a recent study by Meng et al. [252], in which they showed that NPCs derived from an adult rat hippocampal cell line, as well as embryonic rat NPCs, undergo enhanced speed and cathodal directedness of migration in the presence of a DCEF. We have extended these findings to contrast and compare the galvanotactic capacity of undifferentiated SE NPCs with differentiated neural phenotypes. We have quantified total cellular displacement in the direction of the cathode as well as the tortuosity of migration (total path length divided by total displacement). The directedness measure of an individual cell’s migration can vary based on the initial and final time points chosen for analysis. The mean tortuosity, in combination with mean directedness, is a more informative indicator of how straight the cells migrate toward a particular direction than directedness alone. The SE-derived NPCs exhibited markedly higher velocity of migration, as well as increased directedness, compared to the hippocampal NPCs described by Meng et al. [252] in the presence of the same DCEF strength (250 mV/mm) and growth factor conditions. This may suggest that electrical stimulation of adult NPCs with a DCEF may yield differential migratory responses depending on the region of the brain from which the cells originate, although we cannot rule out the possibility that these observed differences are due to the differing substrates utilized in each study (poly-L-lysine/matrigel vs. polyornithine/laminin). A recent study demonstrated the galvanotaxis of postnatal rat hippocampal neurons [275] suggesting that maturing cell phenotypes can also respond to EFs during times of active neurogenesis. To our knowledge, the galvanotaxis of adult-derived mature neural cell types has not been shown. With the long-term goal of developing endogenous neurorepair paradigms, our findings that differentiated neural cells do not exhibit a galvanotactic response suggest that DCEF application may be a suitable approach to the development of such paradigms.

The cellular mechanisms involved in NPC migration have not yet been fully characterized. EGFR signaling has previously been shown to play a role in the galvanotaxis of several cell types including keratinocytes [259], breast cancer cells [341], corneal epithelial cells [266], and embryonic NPCs [252]. It has been suggested that EGFR polarization within the membrane leads to actin colocalization and polymerization, and these processes in turn trigger cathodal galvanotaxis [260]. Indeed the activation and polarization of EGFR toward the cathode-facing
side of NPCs was demonstrated in [252]. EGF has been extensively used to study NPC proliferation both in vitro and in vivo [1,331,342]. PI3K is a well-known downstream effector of the EGFR [343,344]. Rho GTPases (Rac1, Cdc42) are downstream targets of PI3K products and play key roles in the cytoskeleton remodeling process that is required for cell migration [345,346]. Meng et al. demonstrated that pharmacological and genetic inhibition of PI3K signaling significantly attenuated embryonic and hippocampal adult NPC migration [252]. Here we demonstrate that EGF also plays a role in the galvanotaxis of SE-derived NPCs. In the presence of the EGFR inhibitor erlotinib, undifferentiated NPCs experience significantly reduced migratory behaviour in the presence of a DCEF. However, their galvanotactic response is not completely eradicated in the presence of erlotinib, suggesting EGF is not exclusively responsible for NPC galvanotaxis. This is in line with the finding of Meng et al. [252] that FGF receptors are also involved in NPC galvanotaxis. In contrast to their findings, however, SE-derived NPCs in the presence of bFGF alone exhibited a significant decrease in the velocity and directedness of migration compared to NPCs in the presence of both EGF and bFGF. This suggests that the mechanisms by which growth factors mediate galvanotaxis may vary between hippocampal and SE-derived NPCs.

The identification of neural stem cells in the adult brain has led to the development of endogenous neural precursor activation paradigms to repair the injured CNS [112,113,347]. Critical to the success of such self-repair paradigms is the effective expansion and recruitment of NPCs to sites of injury or disease. Although SE NPC expansion occurs following injury alone, or in combination with exogenous factors, only a subpopulation of the newly formed NPCs migrate toward lesion sites in response to these stimulants [348]. Augmentation of neurorepair processes may be achieved by enhancing the numbers of SE-derived NPCs that are recruited to lesion sites, and our findings suggest that this may be accomplished with the application of external DCEFs as guidance cues for NPC migration.
Chapter 5

5  Adult Subependymal Neural Precursor Cell Galvanotaxis is Dependent on Calcium Influx

5.1  Introduction

Adult brain NSCs reside in the SEZ in a quiescent state where – under homeostatic conditions – they slowly divide and give rise to progeny that migrate along the rostral migratory stream toward the olfactory bulb and differentiate into interneurons [2,37,62-64,66,67]. Following neural insults such as stroke, SEZ NSCs are recruited into a proliferative mode to expand the neural stem and progenitor cell (NPC) pool [100,101]. NPCs migrate into the parenchyma and contribute to limited de novo neurogenesis in the damaged region [96]. The administration of soluble factors including mitogenic and immunomodulatory agents has been shown to enhance the endogenous neurogenic response and lead to functional recovery, by promoting increased survival, recruitment, and differentiation of NPCs [109,112,113,349].

We have previously shown that adult SEZ NPCs undergo rapid and cathode-directed galvanotaxis in the presence of DCEFs – a phenomenon that is not exhibited by their differentiated progeny [251,350]. Thus, galvanotaxis may be considered for integration into regenerative medicine strategies as a mechanism to selectively promote the directed migration of endogenous NPCs toward damaged regions in the brain. Such an undertaking would require a thorough understanding of the cellular mechanisms that are involved in transducing externally applied EFs into cell motility. To this end, we have previously reported that NPC galvanotaxis is mediated by EGF receptor signalling [251], a property shared by other galvanotactic cell populations including keratinocytes [259], breast cancer cells [341], and corneal epithelial cells [266].

Ca^{2+} is a well-known mediator of cell locomotion [280,351-354]. The formation of integrin-mediated cell-substratum adhesions, the generation of actomyosin contractile forces, and the regulation of actin cytoskeleton remodeling are all calcium-dependent processes [355-359]. Ca^{2+} has also been implicated in the galvanotaxis of several cell populations, including keratinocytes
[284,286], neural crest cells [285], human granulocytes [360], and osteoblast-like cells [361]. However, other studies have reported conflicting results about the involvement of Ca\(^{2+}\) in the galvanotaxis of fibroblasts. For example, Brown et al. determined that NIH 3T3 and SV101 fibroblast galvanotaxis is Ca\(^{2+}\)-independent, but Onuma and Hui described that the galvanotaxis of C3H/10T1/2 mouse embryonic fibroblasts requires Ca\(^{2+}\) influx [288,362]. The reason for this discrepancy is not certain, although proposed explanations include differences in culture conditions leading to distinct ion channel expression profiles, and different substrates eliciting variable cell responses [258].

With respect to NPC galvanotaxis, other cellular signalling effectors have been reported including bFGF, the PI3K/Akt pathway, and the NMDAR/Tiam1/Rac1/Pak1 pathway [329,363]. However, the role of Ca\(^{2+}\) in NPC galvanotaxis has not been investigated. Herein we report that adult mouse SEZ-derived undifferentiated NPC galvanotaxis is Ca\(^{2+}\)-dependent. We employed several Ca\(^{2+}\) modulation conditions in the presence of a DCEF of strength 250 mV/mm which is known to result in the rapid and directed migration of adult NPCs. Time-lapse imaging was performed and the kinematic properties of the cells’ migratory behaviour was analyzed. The chelation of intracellular Ca\(^{2+}\) with 1,2-bis-(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid acetoxymethyl ester (BAPTA-AM) – the cell permeable derivative of BAPTA – led to the abolition of migration. We used RT-PCR to show that adult derived NPCs express L- and T-type voltage gated calcium channels (VGCCs), the pharmacological blockade of which caused the retraction of cellular processes, cell rounding, and a dose-dependent reduction in the magnitude of the migration velocity (|velocity|). We demonstrated that when plated in low-Ca\(^{2+}\) SFM with 20 μM BAPTA, NPCs undergo a similar decrease in the |velocity| of migration and eventually cease migrating. Most important, restoring external Ca\(^{2+}\) concentrations completely rescues their migratory capacity. Cell viability analyses of NPCs in Ca\(^{2+}\) chelation and VGCC blockade conditions determined that the loss of migration was not due to cell death. We conclude that the galvanotactic migratory behaviour of NPCs is dependent on the influx of extracellular Ca\(^{2+}\).
5.2 Materials And Methods

5.2.1 Ethics Statement

Animal work was approved by the University of Toronto Animal Care Committee in accordance with the institutional guidelines (protocol no. 20009955).

5.2.2 Cell Culture

NPCs were isolated as previously described [331]. Briefly, adult male CD1 mouse brains were dissected and the periventricular regions were enzymatically dissociated. Cells were then plated at 10 cells per μL in serum free media (SFM) (DMEM:F12 1:1, Invitrogen, USA) containing 20 ng/mL EGF (Sigma-Aldrich, Canada), 10 ng/mL bFGF (Sigma-Aldrich, Canada) and 2 μg/mL heparin (Sigma-Aldrich, Canada) (herein referred to as Regular-SFM or R-SFM) and allowed to grow for 7d to form primary neurospheres (P0). Neurospheres were either utilized for galvanotaxis experiments after 7d in culture, or passaged by mechanical dissociation and replating to form secondary neurospheres (P1). Only P4 and younger neurospheres were used for experiments.

5.2.3 Galvanotaxis Chamber Construction

Galvanotaxis chambers were constructed exactly as previously described [251]. Briefly, square no.1 glass cover slides were placed in HCl for a minimum of 8 hours, and then individually adhered to the base of 60 mm Petri dishes using silicon vacuum grease (VWR, Canada). Two rectangular no.1 glass strips (22 mm x 5 mm) were cut using a diamond tipped glass cutter. Vacuum grease was used to attach the strips to opposite edges of the square slide within the Petri dish. These chambers were then UV-sterilized and coated with poly-L-lysine (100 μg/mL, Sigma, Canada) for 2h at room temperature, and subsequently with 4% (v/v) Matrigel (BD Biosciences, Canada) for 1h at 37°C. Following this, supplemented SFM was added to the chambers, and 5-6 neurospheres were transferred onto the central troughs of the chambers. Chambers were incubated overnight for 17-20h at 37°C, 5% CO₂ and 100% relative humidity.
5.2.4  Galvanotaxis Assay

The galvanotaxis assay was performed as previously described [350]. Chambers were selected for experimentation based on the level of NPC separation onto the Matrigel. Galvanotaxis chambers were transferred to the stage of an inverted Zeiss Observer Z1 microscope. Agarose gel bridges and pools of media in adjacent Petri dishes were used to connect the galvanotaxis chambers to Ag/AgCl electrodes that were coupled to an external power supply in order to provide DCEF stimulation. Images were captured at a frequency of 1 per minute with Zeiss Axiovision software.

5.2.5  Ca\(^{2+}\) Modulation

Intracellular Ca\(^{2+}\) was chelated via 20 µM BAPTA-AM (Invitrogen, USA) in R-SFM. Low extracellular Ca\(^{2+}\) conditions were produced by utilizing low-Ca\(^{2+}\) SFM (L-SFM) comprised of Ca\(^{2+}\)-free DMEM (Gibco, USA) and low-Ca\(^{2+}\) F12 (US Biological, USA) resulting in a 0.35 mM Ca\(^{2+}\) concentration, 30% of the concentration in R-SFM. L-SFM was coupled with 20 µM non-cell permeable BAPTA (Invitrogen, USA) to further reduce free extracellular Ca\(^{2+}\). Voltage gated Ca\(^{2+}\) channel inhibition was achieved with the L-type specific inhibitor Nifedipine (200 mM stock in DMSO) (Sigma, USA) and the T-type specific inhibitor ML218 (25 mM stock in H\(_2\)O) (Sigma, USA).

5.2.6  Quantification of Cell Migration

Cell migration was tracked using Zeiss Axiovision software. A minimum of 45 cells over 3 independent experiments was analyzed for each experimental group. Cells were tracked if they were a minimum of 1 cell diameter away from the nearest cell at the onset of the DCEF stimulation. The parameters of migration analyzed were the magnitude of velocity (|velocity|), the directedness, and the tortuosity. The |velocity| was determined by dividing the straight-line distance between the cells’ initial and final positions by the time taken to arrive at the final position. Directedness was given by the cosine of the angle between the positive X-axis (the direction of the EF vector) and the line segment formed by connecting the initial and final cell positions. Tortuosity was measured by dividing the total path length traversed by each cell by the length of the line segment connecting the cell’s initial and final positions.
5.2.7 Reverse Transcriptase Polymerase Chain Reaction

P0-P2 neurospheres were collected and lysed with a 25ga needle, and mRNA was isolated via the RNeasy extraction kit (Qiagen, USA). mRNA was reverse transcribed using the SuperScript II Reverse Transcriptase kit (Life Technologies, USA) according to the manufacturer’s protocol. PCR reactions were run using Taq polymerase (Invitrogen, USA), and PCR products were assessed via gel electrophoresis. PCR primer sequences were as follows: Cav1.1: forward – 5’-CTCCGCTATGATGTCACTCTTC-3’, reverse – 5’-GACGACATACCACACCTGATAC3’; Cav1.2: forward – 5’-ACAGCCAATAAAGCCCTCTTGGCCC-3’, reverse – 5’-GGAGGCAATGGAGCAGCAGCTGCTGTT-3’; Cav1.3: 5’-TTGCTGTGAGGACAGCTCTCCCA-3’, reverse: 5’-TAGGCTGCAACGGCCATGATCTGC-3’; Cav1.4: forward – 5’-CGCAATGGCTGGAAACCTGCTCGACT-3’, reverse – 5’-GTGTGACATGAAGCCAGGAGCCAGA-3’; Cav2.1: forward – 5’-CCAGCAGAGAACCAGAGGAG-3’, reverse – 5’-GCTCAGATCCTGCTCCCAAAC-3’; Cav2.2: forward – 5’-CGAATTGGCTCTGACCCTTA-3’, reverse – 5’-CCAGTGCTGAGTCCCAAAGT-3’; Cav2.3: forward – 5’-CAAGATGAGTGGAGAAGGAGG-3’, reverse – 5’-AGTAAGATGGAGATGCTGGTAG-3’; Cav3.1: forward – 5’-TGTCTCCGCACGGTCTGTAA-3’, reverse: 5’-AAGGCCGTCAATGCTCTCC-3’; Cav3.2: forward – 5’-CGAATGGCCAGCATGCTACAT-3’, reverse – 5’-TGAGGGTCTCGGAGTGCT-3’. Cav3.3: forward – 5’-TGAGGGTCTCGGAGTGCT-3’.

5.2.8 Statistical Analysis

All values are presented as means ± S.E.M. Significance was set at p<0.05. Differences between group means were analyzed via the Kruskal-Wallis nonparametric test. Post-hoc analyses were performed using the Mann-Whitney U test with Bonferroni correction for multiple pairwise comparisons.
5.3 Results

5.3.1 Chelation of Intracellular Cytosolic Ca\(^{2+}\) Rapidly Disrupts NPC Galvanotaxis.

We initially set out to investigate the role of intracellular cytosolic Ca\(^{2+}\) in NPC galvanotaxis. Neurospheres were plated in galvanotaxis chambers for 17-20h in regular SFM (R-SFM) containing 1.16 mM Ca\(^{2+}\). This period is sufficient to allow individual NPCs to adhere to the Matrigel substrate and separate from each other as they migrate radially. Following this period, the media was replaced with fresh R-SFM and NPCs were exposed to a 250 mV/mm DCEF and time-lapse imaged. Following 1-2h of imaging, the media was replaced with R-SFM containing 20 µM BAPTA-AM (the cell permeable derivative of the Ca\(^{2+}\) chelator BAPTA), and time-lapse imaging was resumed (Figure 5-1A). In its esterified form, BAPTA-AM does not chelate or otherwise affect extracellular [Ca\(^{2+}\)] due to the acetoxymethyl ester moiety localized at the Ca\(^{2+}\)-binding sites. Once transported across the cell membrane, the acetoxymethyl ester is hydrolyzed by nonspecific esterases to produce the active form of BAPTA. Within 25 minutes of the addition of BAPTA-AM, cells had begun exhibiting rounding of the cell body (Figure 5-1C), and by 75 minutes most cells had fully retracted their processes inwards, resulting in a globular morphology (Figure 5-1E, Movie S10). Considering that an incubation time of approximately 20 min is required for BAPTA-AM to be taken up by cells, hydrolyzed, and activated to chelate intracellular Ca\(^{2+}\) [364], this suggests that the observed loss of cellular processes and the abrogation of migration occurred rapidly after the perturbation of cytosolic [Ca\(^{2+}\)]. The rounding of cells following exposure to BAPTA-AM led to cells aggregating together, and this was not conducive to tracking cell migration or quantifying cell viability. To determine whether cells stopped migrating as a result of cell death, we performed a qualitative live/dead assay that demonstrated that the vast majority of cells remained viable 1h after they stopped migrating, with only a few non-viable cells (Figure 5-1F).
5.3.2 Low Extracellular Ca$^{2+}$ Conditions Decrease the $|\text{Velocity}|$ of NPC Galvanotaxis

Next we modulated the extracellular [Ca$^{2+}$] without directly manipulating intracellular levels. Neurospheres were once again plated in galvanotaxis chambers for 17-20h in R-SFM. Next, the
R-SFM was replaced with low-Ca\(^{2+}\) SFM (L-SFM) containing 20 \(\mu\)M non-cell permeable BAPTA (Figure 5-2A) and loaded with the cell viability reagents calcein-AM (2 \(\mu\)M) and ethidium homodimer-1 (4 \(\mu\)M). L-SFM contains 0.35 mM Ca\(^{2+}\), approximately 30\% of the concentration in R-SFM. NPCs were incubated in L-SFM + 20 \(\mu\)M BAPTA for 30 min and subsequently exposed to a 250 mV/mm DCEF and time-lapse imaged. In these conditions, NPCs migrated in a slow but directed manner, and eventually ceased migrating following the rounding of their cell bodies. Cells were imaged for an additional 60 min after >90\% of the population had ceased migrating and cell viability analyses were performed. Cells migrated for 121±17 min before >90\% of the population ceased migrating. In L-SFM+20 \(\mu\)M BAPTA, NPCs exhibited a reduced |velocity| of migration over time. The |velocity| was 0.49±0.04 \(\mu\)m/min during the first 60 min of DCEF exposure, and 0.17±0.02 \(\mu\)m/min during the 60 min prior to the cessation of migration (from 120 to 60 minutes prior to the end of the imaging period) (Figure 5-2B, Movie S11). Interestingly, the directedness and tortuosity of migration did not significantly change over these same time periods (Figure 5-2C-D). The abolition of migration was accompanied by rounding of cell bodies (Movie S11), similar to the response of NPCs in the presence of BAPTA-AM.

Cell viability was assayed at the beginning and end of DCEF exposure. Notably, the loss of migration |velocity| was not accompanied by significant cell death. 99.4±0.5\% of cells were viable at the onset of DCEF exposure, and 97.7±0.6\% had remained viable 1h following the abolition of migration (Figure 5-2E-F). It is important to note that the total number of cells in each experiment does not remain constant throughout the analysis. NPC proliferation, detachment from the substrate and electrophoresis of non-viable cells, and fusion of viable cells are all factors that potentially contribute to a difference in the total number of distinguishable cells between the initial and final analysis time points. The total number, and fraction of viable/non-viable cells at the onset and completion of each experiment is provided in Table B1.
Figure 5-2 – Low extracellular Ca\(^{2+}\) conditions cause a reduction in the |velocity| of galvanotaxis. (A) Experimental timeline demonstrating the replacement of R-SFM with L-SFM + BAPTA approximately 20h following plating of NPCs, as well as the first 60 min and last 60 min time points of migration analysis. ‘E’ and ‘F’ illustrate the time points at which cell viability was assessed in panels E and F. (B-D) NPCs undergo a reduction in |velocity| (B) in the presence of low extracellular Ca\(^{2+}\) conditions, but directedness (C) and tortuosity (D) remain unaffected. (E-F) The vast majority of NPCs are viable in low extracellular Ca\(^{2+}\) conditions at 0 min (E) and 150 min (F) of DCEF exposure. Scale bars = 100 µm. Data are presented as means ± S.E.M., * = p<0.05.

We asked if NPC galvanotaxis could be rescued if extracellular Ca\(^{2+}\) conditions were restored to normal levels by replacing L-SFM with R-SFM. Neurospheres were plated onto galvanotaxis chambers in R-SFM for 17-20h as before, and subsequently placed in L-SFM conditions and exposed to a DCEF of strength 250 mV/mm. 1h after >90% of cells had stopped migrating, L-SFM was aspirated from the galvanotaxis chamber and replaced with R-SFM (Figure 5-3A). Strikingly, following the addition of R-SFM, NPCs extended cellular processes and resumed
rapid and cathode-directed galvanotaxis (Movie S12). The \(|\text{velocity}|\) of migration increased >5-fold from 0.21±0.04 μm/min during the final 60 min in L-SFM to 1.12±0.11 μm/min within the first 60 min in R-SFM (Figure 5-3B), similar to what is seen in DCEF with R-SFM. Together, these data demonstrate that NPCs exhibit reduced galvanotaxis \(|\text{velocity}|\) in low extracellular Ca\(^{2+}\) conditions while maintaining highly cathode-directed and non-tortuous migratory paths. In addition, this reduction is not due to cell death and can be rescued by restoring the extracellular \([\text{Ca}^{2+}]\) to normal levels.

Figure 5-3 – NPC galvanotaxis can be rescued by restoring extracellular Ca\(^{2+}\) conditions. (A) Experimental timeline illustrating that NPCs are initially exposed to DCEF stimulation in the presence of L-SFM, and subsequently L-SFM is replaced with R-SFM. (B) NPCs experience a significant increase in their \(|\text{velocity}|\) of migration following replacement of L-SFM with R-SFM. (C-D) Representative images of NPCs immediately after replacement of L-SFM with R-SFM (C) or 60 min later (D). Cells in (C) possess a round morphology and generally lack membrane protrusions, whereas the extension of membrane processes is evident in (D) 60 min following restoration of extracellular Ca\(^{2+}\) conditions. Scale bars = 100 μm. Data are presented as means ± S.E.M., * = p<0.05.

5.3.3 Adult SEZ NPCs Express L- and T-type Voltage Gated Calcium Channels

We next investigated the expression profile of Ca\(^{2+}\)-channel subtypes in adult SEZ NPCs. We focused on VGCCs and assayed for the expression of the pore-forming subunit genes for L-, P/Q-, N-, R-, and T-type calcium channels. P1-P2 neurospheres were collected, and lysed. RNA
was extracted from the cells, followed by RT-PCR. Adult hippocampal explants served as positive controls. RT-PCR analysis revealed that adult SEZ NPCs express the Ca\textsubscript{v}1.2, Ca\textsubscript{v}3.2, and Ca\textsubscript{v}3.3 subunits (Figure 5-4). The Ca\textsubscript{v}1.2 subunit is the \( \alpha_1 \) pore-forming, voltage-sensing, and gating subunit of a L-type VGCC subtype that is localized in neurons throughout the central nervous system including the cortex, hippocampus, cerebellum, and spinal cord [365-367]. Similarly, Ca\textsubscript{v}3.2 and Ca\textsubscript{v}3.3 subunits are the \( \alpha_1 \) pore-forming subunits of T-type VGCC subtypes that are expressed in cortical, cerebellar, hippocampal, and olfactory bulb neurons [365]. Thus, adult SEZ NPCs express the \( \alpha_1 \) subunits of three VGCC subtypes, and therefore are amenable to manipulation in distinct Ca\textsuperscript{2+} modulation conditions.

![Figure 5-4](image)

**Figure 5-4** – RT-PCR analysis reveals that NPCs express Cav1.2 L-type and Cav3.2 and Cav3.3 T-type VGCC subtypes. Hippocampal explants represent positive primer controls.

### 5.3.4 Inhibition of Ca\textsubscript{v}1.2 or Ca\textsubscript{v}3.2-3.3 Channel Subtypes is Sufficient to Cease NPC Galvanotaxis

Given that NPCs express Ca\textsubscript{v}1.2 L-type and Ca\textsubscript{v}3.2 and Ca\textsubscript{v}3.3 T-type VGCC subtypes, and that the influx of Ca\textsuperscript{2+} is required for galvanotaxis, we sought to determine if the activity of individual VGCC subtypes is sufficient for inducing NPC galvanotaxis. Accordingly, NPCs were first plated onto galvanotaxis chambers for 17-20h in R-SFM. Next, The R-SFM was replaced with R-SFM containing either the L-type VGCC antagonist Nifedipine, or the T-type VGCC antagonist ML218 (Figure 5-5A), followed 30 min later by exposure to 250 mV/mm DCEF and time-lapse imaging. The |velocity|, directedness, and tortuosity of migration were analyzed for 0, 10, 100, and 200 \( \mu \text{M} \) concentrations of Nifedipine, and 0, 5, 10, and 25 \( \mu \text{M} \) concentrations of ML218. Most interestingly, the inhibition of L-type or T-type VGCCs individually was sufficient
to reduce the |velocity|, evoke cell rounding, and eventually abrogate migration, in a concentration-dependent manner, without affecting the directedness or tortuosity of migration (Figure 5-5B-G, Figure A5, Movies S13-14). Importantly, 1h after the cessation of migration, NPCs remained 96.7±0.8% and 95.5±1.4% viable in the presence of 200µM Nifedipine and 25 µM ML218, respectively, revealing that blocking individual VGCC subtypes did not disrupt NPC galvanotaxis through an apoptotic mechanism (Figure 5-5H-K).
Figure 5-5 – Inhibition of L-type and T-Type VGCCs individually causes a reduction in NPC galvanotaxis |velocity|.

(A) Experimental timeline illustrating the administration of ML218 or Nifedipine in R-SFM during DCEF stimulation approximately 20h following the plating of NPCs. ‘H,J’ and ‘I,K’ illustrate the time points at which cell viability was assessed in panels H-K. (B-G) NPCs experience a reduction in galvanotaxis |velocity| (B,E), but unaffected directedness (C,F) or tortuosity (D,G) in the presence of Nifedipine (B-D) or ML218 (E-G). (H-K) Cell viability analyses demonstrate that the majority of NPCs remain viable at the onset (H,J), and following 4h (I,K) of DCEF stimulation in the presence of 200 µM Nifedipine (H-I) or 25 µM ML218 (J-K). Scale bars = 100 µm. Data are presented as means ± S.E.M., * = p<0.05.
In the absence of electrical stimulation, NPCs maintain their migratory capacity, but undergo migration at a reduced velocity in a non-directed and tortuous manner [251,254,368]. Since inhibiting L-type and T-type VGCCs individually leads to reduced NPC galvanotaxis velocity, we reasoned that in the presence of Nifedipine, NPCs should exhibit a general reduction in their velocity of migration compared to vehicle controls even in the absence of an applied DCEF. Neurospheres were plated onto galvanotaxis chambers in R-SFM for 17-20h. The media was then replaced with fresh R-SFM containing either 200μM Nifedipine (Figure 5-6A), or vehicle, and NPC migration was time-lapse imaged in the absence of an applied DCEF for 2.5h-4h. In the presence of Nifedipine, NPCs shortened their processes and eventually ceased migrating as before, whereas NPCs in vehicle maintained their migratory capacity throughout the imaging period. The velocity of migration during the 60-minute period prior to the cessation of migration was assessed and compared to that of NPCs in vehicle-only conditions. We found that in the absence of an applied DCEF, the inhibition of L-type VGCC subtypes caused a reduction in the velocity of migration (Figure 5-6B). Notably, most NPCs exposed to 200 μM Nifedipine displayed rounded cell bodies after 3.5-4h of time-lapse imaging, but interestingly many cells still maintained their ability to migrate (Figure 5-6C-E).

Figure 5-6 – Inhibition of L-type VGCCs reduces the velocity of NPC migration in the absence of electrical stimulation. (A) Experimental timeline illustrating the administration of Nifedipine approximately 20h following cell plating. (B) The velocity of migration during the 60 min period prior to the cessation of migration in NPCs exposed to 200 μM
Nifedipine or vehicle in the absence of DCEF stimulation. (C-E) Representative images of NPCs in the presence of 200 μM Nifedipine in the absence of DCEF stimulation at various time points. Scale bars = 100 μm. Data are presented as means ± S.E.M.

5.3.5 L-Type Ca\(^{2+}\) Channel Inhibition Reduces the Length of NPC Membrane Processes

All three Ca\(^{2+}\) modulation methods described above elicited a cell body rounding response accompanied by the cessation of migration. The protrusion of membrane processes and substrate adhesion are critical steps in cell migration. We tested whether the cell rounding effect caused by attenuated Ca\(^{2+}\) signalling was due to a decrease in the number of membrane process extensions, a shortening of individual extensions, or both. NPCs were plated onto galvanotaxis chambers for 17-20h in R-SFM. The R-SFM was then replaced with R-SFM containing either 200 μM Nifedipine or vehicle as before. NPCs were exposed to a 250 mV/mm DCEF for 2h, and the number of processes as well as the length of each process was determined in 30 min increments. Only membrane protrusions > 5μm in length were counted. Interestingly, the inhibition of L-type VGCCs resulted in a reduction in the mean length of membrane processes over time (Figure 5-7A), but the mean number of processes per cell did not change (Figure 5-7B). This suggests that the inhibition of Ca\(^{2+}\) influx primarily causes cell rounding through the shortening of membrane processes, rather than through a reduction in the number of membrane protrusions. This process may be mediated by actin depolymerisation, but this remains to be determined.

Figure 5-7 – Inhibition of L-type VGCCs reduces the mean membrane process length. NPCs in the presence of 200 μM exhibit a reduction in the mean length of membrane
processes over time (A), but the mean number of processes (B) remains the same, relative to vehicle controls. Data are presented as means ± S.E.M., * = p<0.05.

5.4 Discussion

Our results demonstrate that the galvanotaxis of adult SEZ-derived NPCs is dependent on Ca\(^{2+}\). We determined via RT-PCR that NPCs express the Ca\(_{\alpha}1.2\) \(\alpha1\) subunit and the Ca\(_{\alpha}3.2\) and Ca\(_{\alpha}3.3\) \(\alpha1\) subunits of L-type and T-type VGCC subtypes, respectively. In addition, through the inhibition of L-type and T-type VGCCs and through the reduction of extracellular Ca\(^{2+}\) levels we have shown that galvanotaxis [velocity], but not directedness or tortuosity, is dependent on the influx of extracellular Ca\(^{2+}\). Moreover, if maintained in conditions of attenuated Ca\(^{2+}\) influx, NPCs eventually cease migrating. This behaviour is accompanied by morphological changes that include the shortening of cell membrane protrusions and the rounding of cell bodies, and is not attributed to cell death.

The abolition of NPC galvanotaxis occurred most rapidly with the application of the intracellular Ca\(^{2+}\) chelator BAPTA-AM. This may be due to the fact this method is more effective than VGCC inhibitors or extracellular Ca\(^{2+}\) chelators at reducing intracellular levels of free Ca\(^{2+}\). However, there is also evidence to suggest that in addition to Ca\(^{2+}\) chelation, BAPTA-AM may be directly responsible for the depolymerisation of actin and microtubule filaments in a Ca\(^{2+}\)-independent manner [369], offering an alternative explanation for the markedly rapid response. The chelation of intracellular Ca\(^{2+}\) inevitably affects other Ca\(^{2+}\)-dependent cellular processes and likely would lead to cell death over longer time periods, but importantly the vast majority of cells remained viable in the presence of BAPTA-AM 1h after cell migration had ceased, indicating that the abolition of cell migration is likely not due to apoptotic processes.

The role of Ca\(^{2+}\) in cell migration has been well-studied [280,370-373], and the role of Ca\(^{2+}\) in galvanotaxis has been previously described [168,285,286,360]. Interestingly, prior work in keratinocytes demonstrated that inhibition of VGCCs reduced the directedness of galvanotaxis without affecting their speed of migration, thus highlighting the possibility that these parameters of migration may be controlled by different mechanisms [286]. This is in line with our observations that VGCC inhibition or extracellular Ca\(^{2+}\) inhibition both predominantly impacted
the velocity of galvanotaxis but not the directedness or tortuosity. The explanation for the difference in the affected migration parameter is not clear. A simple explanation may be that the cellular mechanisms of galvanotaxis varies between the two cell types, in addition to the possibility that the different cell populations have different VGCC expression profiles. However, considering that the spatiotemporal characteristics of a change in intracellular [Ca$^{2+}$] are the determinants of the type of cellular response that is elicited by the change [374,375], an alternative or complementary explanation may be offered by noting that the study by Trollinger et al. employed general VGCC blockers instead of specifically targeting individual subtypes (inevitably resulting in different calcium influx dynamics), and a lower-strength DCEF of 100 mV/mm (compared to 250 mV/mm utilized in this study).

Cells that undergo persistent directional migration are known to possess a gradient in intracellular [Ca$^{2+}$] that is highest at the trailing edge and lowest at the leading edge [280]. In cathodal galvanotaxis, the trailing and leading edges correspond to the anodal and cathodal ends of the cell, respectively. This concentration gradient is enhanced by the application of a DCEF, which further increases the anodal intracellular [Ca$^{2+}$] via passive influx, although in cells containing VGCCs this may be balanced by the activation of the channels on the slightly depolarized cathodal side of the cell [258,376]. Elevated Ca$^{2+}$ at the rear of the cell is believed to be responsible for the retraction of the trailing edge [233,377] and the generation of actomyosin contractile forces for rear detachment and forward propulsion [373,378]. At the leading edge, there is evidence that Ca$^{2+}$ plays a role in F-actin polymerization mediated by the PI3K/Rac1 pathway, which is essential for membrane protrusion [297,379]. This is consistent with our findings that low extracellular Ca$^{2+}$ conditions or the inhibition of individual VGCC subtypes causes the shortening of cell processes, and rounding of the cell body. The abrogation of NPC migration in low Ca$^{2+}$-influx conditions is similar to previously described observations in human neutrophils and fish keratocytes [380,381]. However, other reports indicate that cells maintain their general ability to move [285].

Galvanotaxis is a powerful, but often under-appreciated, cellular behaviour that has significant implications for cell replacement therapies, particularly in stem cell populations. A thorough understanding of the cellular mechanisms that regulate galvanotaxis is necessary for the
realization of its clinical utility. While progress has been made on this front [258], a complete description is yet to be achieved. We conclude that Ca\(^{2+}\) plays a role in the |velocity| of galvanotaxis in undifferentiated SEZ NPCs. Further investigations may reveal mechanisms of augmenting NPC migration |velocity| that may benefit the development of neurorepair and cell replacement strategies.
Chapter 6

Biphasic Monopolar Electrical Stimulation Induces Rapid And Directed Galvanotaxis In Adult Subependymal Neural Precursors

6.1 Introduction

The discovery that neurogenesis persists into adulthood in the mammalian brain has altered our understanding of neuroplasticity and our outlook on repairing the injured brain following injury or disease. Adult NPCs reside in two neurogenic regions in the forebrain: the SEZ lining the lateral ventricles and the SGZ of the hippocampal dentate gyrus [2,382]. Under baseline conditions, SEZ NPCs give rise to neuroblasts that migrate along a well-defined pathway known as the RMS toward the olfactory bulb, where they differentiate into interneurons. The inherent proliferative, migratory and neurogenic properties of NPCs make them good candidates for contributing to neurorepair following neural insult, such as stroke. Indeed, SEZ derived NPCs have been shown to contribute to neurogenesis following injury [96]. Interestingly, neural insult alone results in the upregulation of multiple chemical and physical cues that enhance NPC proliferation and induce the redirection of their migration toward the lesion site, as comprehensively reviewed by Kahle et al. [383]. However, the neuroregenerative impact of endogenous NPC activity is limited. The introduction of exogenous factors can enhance this post-insult response and promote functional recovery [112,158,349], but long-term safety concerns have limited their clinical applicability. Targeting the recruitment of NPCs to appropriate areas remains a major challenge in neurorepair efforts, and the evolution of novel methods to direct their migration is instrumental to the development of successful neurorepair strategies.

Endogenous DCEFs play important roles in physiological processes that include development, wound healing, nerve growth, and angiogenesis [161,188-190,204]. On a cellular level, it has long been known that external application of DCEFs can induce the directed migration of certain cell types toward either the anode or the cathode of the EF in a process known as galvanotaxis [232,235,329,384]. It is interesting that stem cells, including neural stem cells [254,368], human induced pluripotent stem cells, and human embryonic stem cells [253], are included within these populations as this raises exciting possibilities of implementing EFs in regenerative medicine strategies to promote directional migration. We previously reported on the ability of DCEFs to induce rapid and directed cathodal galvanotaxis of adult SEZ-derived NPCs, but not in differentiated populations [251,350] making the application of EFs a viable approach to neuroregenerative strategies. However, direct current stimulation may not be suitable for clinical applications. Prolonged exposure to DCEFs results in charge accumulation at the electrode-tissue interface. Such charge build-up produces electrochemical reactions that may cause electrode corrosion, the formation of toxic chemical species and subsequent tissue damage [385,386]. Moreover, excessive charge accumulation at the electrodes could impede the flow of current from the stimulating electrodes [387]. These issues would be ameliorated by the use of balanced biphasic electrical stimulation [386], which is characterized by pulses that consist of both positive and negative phases. Importantly, with balanced biphasic stimulation the electrical charge delivered during one phase would be withdrawn during the opposite phase, resulting in no net charge accumulation and, in principle, the reversal of any electrochemical reactions that could produce toxic byproducts.

Successful galvanotaxis of mammalian cells using biphasic stimulation has not yet been reported. Hart et al. have demonstrated cathodal keratinocyte galvanotaxis using a combination of direct current and alternating current fields, but alternating fields alone resulted in non-directed, random migration [388]. Herein we report for the first time the ability of balanced biphasic EFs to induce rapid and directed galvanotaxis in clonally-derived pure populations of undifferentiated adult NPCs. Through live cell time-lapse imaging and cell-tracking techniques, we analyze the migratory properties of adult SEZ-derived NPCs and their differentiated progeny. Galvanotaxis was prominent in undifferentiated cells but absent in differentiated populations,
reminiscent of our previous work using direct current electric fields [251]. The present study demonstrates proof-of-principle evidence that the directed migration of endogenous NPCs through balanced biphasic stimulation is feasible and may provide a means for harnessing their potential to enhance endogenous neurorepair.

6.2 Materials And Methods

6.2.1 Ethics Statement

All animal work was approved by the University of Toronto Animal Care Committee in accordance with the institutional guidelines (protocol no. 20009955).

6.2.2 Cell culture

NPCs were isolated and cultured as previously described [331]. Briefly, adult male CD1 mice were sacrificed, the periventricular regions of the brain were excised and enzymatically dissociated. Cells were plated in SFM (DMEM:F12 3:1, Invitrogen) supplemented with EGF (20 ng/mL; Sigma-Aldrich, Canada), bFGF (10 ng/mL; Sigma-Aldrich, Canada) and heparin (2 µg/mL; Sigma-Aldrich, Canada) – herein referred to as SFM + EFH – at 10 cells/µL in T25 culture flasks (BD Falcon, Canada) [1,3]. After 7 days in culture primary neurospheres (P0) consisting purely of nestin expressing NPCs were collected and plated for galvanotaxis experiments or mechanically dissociated and re-plated in identical neurosphere forming conditions to form secondary neurospheres (P1). Neurospheres were passaged and plated in mitogenic conditions every 7 days. Neurospheres up to P4 were utilized for experiments.

6.2.3 Galvanotaxis Chamber Construction

Galvanotaxis chambers were constructed as previously described [350]. Briefly, square no.1 glass cover slides (22 mm x 22 mm x 0.17mm, VWR, Canada) were acid-washed in HCl overnight, then sealed to the base of 60 mm x 15 mm Petri dishes (VWR, Canada) using silicon vacuum grease (VWR, Canada). Other square no. 1 glass slides were then cut into rectangular strips (22 mm x 4mm x 0.17mm), washed with 70% ethanol followed by autoclaved water, and sealed with grease to opposite edges of the square slide in the Petri dish. The resulting central trough measured 22 mm x 12 mm x 0.5mm (l x w x h, and accounting for the thickness of the
grease). Chambers were UV sterilized, and the central troughs coated with poly-L-lysine (100 µg/mL) for 2 hours at room temperature, followed by 4% (v/v) Matrigel (BD Biosciences, Canada) in SFM for 1 hour at 37°C. Next, 350 µL of either SFM + EFH or SFM supplemented with 1% fetal bovine serum (FBS, Life Technologies, Canada) – herein referred to as SFM + FBS – were added to the central troughs of the chambers with 5-6 neurospheres that were 80-100 µm in diameter. Chambers were then incubated at 37°C, 5% CO₂ and 100% humidity. To investigate the galvanotaxis of undifferentiated NPCs, neurospheres were plated onto the chambers for 17-20 hours in SFM + EFH to allow the neurosphere cells to adhere to the Matrigel matrix while remaining undifferentiated. In contrast, to investigate the galvanotaxis of differentiated NPCs, neurospheres were plated onto the chambers for 72-96 hours in SFM + FBS to allow neurosphere cells to adhere and differentiate into mature neural phenotypes.

6.2.4 Galvanotaxis Assay

Two 15cm long pieces of PVC tubing (2.38mm i.d., 3.97 mm o.d., Fisher Scientific, Canada) were filled with 1.5% (w/v) agarose gel. Ag/AgCl electrodes were formed by coiling and immersing two 10cm pieces of silver wire (Alfa Aesar, USA) in bleach for 20 minutes. A square no. 1 glass cover slide was sealed with grease to the top of the rectangular strips on either side of the trough, creating a central chamber. Strips of grease were used to create pools of media on either side of the central chamber. The galvanotaxis chamber was transferred onto the stage of a temperature-, CO₂-, and humidity-controlled Zeiss Observer Z1 microscope (Zeiss, Germany) for time-lapse imaging. Two 60 mm x 15 mm Petri dishes were placed on the stage – one on either side of the galvanotaxis chamber – and filled with 7.5 mL of SFM. The Ag/AgCl electrodes were placed into the peripheral Petri dishes, and all three dishes were bridged with the agarose gel tubes to establish electrical continuity. The Compex Motion electrical stimulator (developed at the Rehabilitation Engineering Laboratory at the University of Toronto, and discussed further below) was connected to the Ag/AgCl electrodes for biphasic pulse application. Cells were electrically stimulated for 2.5-6 hours during which time-lapse imaging was performed via Zeiss Axiovision software to record cell migration at a capture rate of one image per minute.
The voltage across the galvanotaxis chamber was measured using a LabQuest 2 (Vernier, USA) data acquisition unit equipped with a Vernier differential voltage sensor. This apparatus was also used to obtain traces of the biphasic stimulator’s output waveforms. Output waveforms of 400 Hz frequency were captured for 0.5 seconds at a sampling rate of 20,000 samples per second. The peak cathodal potential difference across the galvanotaxis chamber was measured for each captured cycle to obtain the mean peak cathodal potential over the 0.5 second period. This value was divided by the length of the chamber (22 mm) to obtain the mean peak EF strength. The waveform traces were analyzed for the area under the curves AUC using Prism software (GraphPad Software, USA).

6.2.5 Kinematics of Cell Migration

Zeiss Axiovision software was used to track cell migration. At least 45 cells from 3 independent experiments were tracked for each experimental group. Cells were selected for tracking if they were at least one cell body away from the nearest cell at the beginning of the experiment (i.e. time=0). Cell centroid positions were used for tracking cell migration. The magnitude of velocity (or \(|\text{velocity}|\)), directedness, and tortuosity of migration were analyzed. The \(|\text{velocity}|\) of each cell was obtained by dividing the total displacement of a cell from its initial position (at time 0) to its final position, by the total time taken to arrive at this latter position. The directedness of a cell at was obtained by finding the cosine of the angle between the positive X-axis (the direction of the cathodal EF vector) and the straight-line displacement between the cell’s original position and its final position. The tortuosity was obtained by dividing the total path length traversed by the cell by the straight-line displacement between the cell’s initial position at time 0, and its final position.

6.2.6 Immunocytochemistry

Cells were fixed for 20 minutes at room temperature with 4% paraformaldehyde, then triple washed with PBS for 5 minutes each time. Cells were permeabilized for 20 minutes at room temperature with 0.3% Triton X-100, then blocked with 10% NGS (Jackson Immunoresearch Laboratories, Canada) in PBS for 1 hour at room temperature. Samples were processed with primary antibodies for NPCs (mouse monoclonal anti-Nestin 1:400, Millipore, Canada), neurons
(rabbit polyclonal anti-Class III β-Tubulin, Covance, USA), and astrocytes (rabbit polyclonal anti-GFAP 1:500, Sigma, Canada) overnight at 4°C. Samples were triple washed with PBS for 5 minutes, and incubated with appropriate secondary antibodies for 1 hour at 37°C. Secondary antibodies used were Alexafluor 568 (goat anti-mouse 1:400, Life Technologies, Canada), Alexafluor 488 (goat-anti-rabbit 1:400, Life Technologies, Canada), and Alexafluor 488 (goat anti-mouse 1:300, Life Technologies, Canada). Mounting medium containing DAPI (Vector Laboratories, Canada) was used for nuclear staining.

6.2.7 Statistical Analysis

Values are presented as group means ± S.E.M. Differences between group means were analyzed using the Kruskal-Wallis nonparametric test, followed by Mann-Whitney U test post-hoc analyses with Bonferroni correction for pairwise comparisons. Statistical significance was set at p<0.05.

6.3 Results

All investigations in this study utilized the Compex Motion electrical stimulator, developed by the Rehabilitation Engineering Laboratory at the University of Toronto, in collaboration with Compex SA (developer of electrostimulation products) and the Automatic Control Laboratory from the Swiss Federal Institute of Technology Zurich. The Compex Motion is a portable electrical stimulator used for FES therapy which has been shown in several studies and clinical trials to improve motor function in individuals with spinal cord injury [313-318,389]. The Compex Motion has four current regulated output channels, each of which are independently controlled. The device’s output waveforms are programmed and stored on chip cards, which can be easily exchanged to modify the stimulator’s output function.

The stimulator’s output functions can be classified under three operational modes: i) biphasic monopolar (BPMP): the negative phase of each pulse is balanced by a positive phase that is one-quarter the amplitude and four times the duration (Figure 6-1A); ii) biphasic bipolar (BPBP): the negative phase of each pulse is balanced by a positive phase that is equal in amplitude and duration but opposite in polarity (Figure 6-1B); iii) monophasic monopolar: each pulse consists
of a single negative phase, lacking a positive phase, and therefore this mode is not charge-balanced. The monophasic monopolar mode delivers unidirectional current, which is comparable to DCEF stimulation and therefore this mode was not investigated in the present study.

In order for cells to undergo directionally-biased galvanotaxis in the presence of balanced biphasic EFs, the electrical pulses must elicit a galvanotactic response in one phase, but not in the opposite balancing phase. We proceeded by assuming that there exists a threshold level of current, below which galvanotaxis would not be induced, consistent with our observations in earlier unpublished work. Our previous work demonstrated rapid and directed cathodal migration in a DCEF [251,350]. Accordingly, we designed a BPMP waveform such that the negative (cathodal) phase of the pulse would induce galvanotaxis, but the positive (anodal) balancing phase of the pulse (which is one-quarter the amplitude and four times the duration of the cathodal phase) would possess a current amplitude below this threshold, and would therefore not create a galvanotactic effect in the opposing direction. The Compex Motion stimulator has a current amplitude range between 0-100 mA in integer increments. However, when the cathodal amplitude of BPMP pulses is set to 2 mA and below, the anodal phase of the output waveform less accurately matches the parameters programmed onto the chip card, presumably because the device was designed to operate at higher current amplitudes suitable for clinical applications (15-30 mA). A cathodal amplitude of 4mA in BPMP mode would result in an amplitude of 1mA during the opposing anodal phase. Since our previous NPC galvanotaxis work with DCEFs utilized current amplitudes of 1-2 mA [251,350], we reasoned that a <1 mA amplitude during the anodal phase would be necessary to ensure that opposing anode galvanotaxis would not occur. Based on the above criteria, we utilized the following parameters to create the BPMP and BPBP waveforms used throughout the study. BPMP waveforms were characterized by a cathodal current amplitude of 3 mA and pulse width of 500 µs, followed by an anodal current amplitude of 0.75 mA and pulse width of 2000 µs, resulting in a total pulse duration of 2500 µs and duty cycle of 20%. In BPBP mode, a cathodal phase with 500 µs pulse width and 3 mA amplitude is followed by a balancing anodal phase of 500 µs pulse width and 3 mA amplitude, for a total pulse duration of 1000 µs and a duty cycle of 50%. The maximum frequency of each output channel is 100Hz. However, the outputs of the four channels are staggered (Figure 6-1), and
therefore the channels were connected in parallel to achieve maximum stimulation frequency, resulting in both BPMP and BPBP waveforms operating at a frequency of 400Hz. This allowed the cells to receive maximum exposure to the cathodal current without the overlapping of channel outputs. Other sets of stimulation parameters that failed to induce NPC galvanotaxis are listed in Table 6-1.

<table>
<thead>
<tr>
<th>Cathodal Amplitude (mA)</th>
<th>Anodal Amplitude (mA)</th>
<th>Cathodal Pulse Width (µs)</th>
<th>Anodal Pulse Width (µs)</th>
<th>Frequency (Hz)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
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<tr>
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<tr>
<td>3</td>
<td>0.75</td>
<td>500</td>
<td>2000</td>
<td>100</td>
</tr>
</tbody>
</table>

Table 6-1 – Table of biphasic monopolar pulse parameters that failed to induce NPC galvanotaxis.

The theoretical EF experienced by the cells within the chamber during the cathodal phase can be derived by Ohm’s law for conductive media: \( E = \frac{J}{\sigma} \), where \( E \) is the strength of the EF, \( J \) is the current density (total current through the chamber divided by the chamber’s cross-sectional area) and \( \sigma \) is the conductivity of the culture medium [390]. A current of 3 mA driven through chambers with cross-sectional area of ~7mm² (14 mm x ~0.5 mm) results in a current density of ~0.428 mA/mm². The conductivity of the culture medium at 37°C was measured as 14.90 mS/cm, or equivalently the resistivity was 671.14 Ωmm. By substituting these measured values of current density and conductivity into the above equation, a calculated value of ~288 mV/mm is obtained for the strength of the EF. This represents the field strength during the cathodal phase.
of the BPMP waveform, as well as during both phases of the BPBP waveform, and is close to the 250 mV/mm field strength utilized in previous work with DCEF’s by our group and others [251,350]. We measured the mean peak strength of the cathodal EF across the galvanotaxis chamber using a LabQuest 2 data acquisition unit equipped with a differential voltage sensor. The Compex stimulator’s output waveforms were captured at a sampling rate of 20,000 samples per second for a duration of 0.5 seconds. At a stimulation frequency of 400 Hz, this results in the tracing of approximately 200 cycles. The mean peak cathodal EF strength during BPMP and BPBP stimulation were measured as 234 mV/mm and 244 mV/mm, respectively (Table 6-2). We also measured the area under the curve (AUC) of the BPMP and BPBP waveform traces (with cathodal voltage measured as negative area and anodal voltage measured as positive area) to ensure that the stimulator’s output waveforms are indeed charge-balanced (Table 6-2). A perfectly charge-balanced system would produce a net AUC of zero.

<table>
<thead>
<tr>
<th>Stimulation mode</th>
<th>Cycles analyzed</th>
<th>Total positive AUC (V*s)</th>
<th>Total negative AUC (V*s)</th>
<th>Magnitude of net area</th>
<th>Peak cathodal field strength (mV/mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BPMP</td>
<td>160</td>
<td>0.4164</td>
<td>0.4130</td>
<td>0.0034</td>
<td>234</td>
</tr>
<tr>
<td>BPBP</td>
<td>201</td>
<td>0.5237</td>
<td>0.5270</td>
<td>0.0033</td>
<td>244</td>
</tr>
</tbody>
</table>

Table 6-2 - Analysis of the area under the curve and the mean peak cathodal EF strength for BPMP and BPBP pulses
Figure 6-1 - Oscilloscope traces of representative BPMP and BPBP pulses. *(A)* A typical BPMP pulse train with a negative cathodal phase that is four times the amplitude and one-quarter the duration of the balancing positive anodal phase. *(B)* A typical BPBP pulse train with a negative cathodal phase that is equal in amplitude and duration but opposite in direction to the opposing anodal phase.

### 6.3.1 BPMP pulses induce rapid and directed galvanotaxis in undifferentiated NPCs

We asked if BPMP pulses could elicit a galvanotactic response in undifferentiated NPCs. We plated neurospheres (P0-P4) onto galvanotaxis chambers for 17-20 hours in the presence of growth factors (SFM + EFH) to maintain the NPCs in an undifferentiated state. During this
period, neurospheres adhered to the Matrigel-coated base of the chambers and individual NPCs moved radially away from the plated neurosphere. The migration of undifferentiated NPCs was then analyzed using time-lapse imaging for a period of 2.5-6 hours in the presence of BPMP or BPBP stimulation, as well as without any applied EF.

In the absence of electrical stimulation, undifferentiated NPCs migrated at an overall mean $|\text{velocity}|$ of $0.20 \pm 0.02 \ \mu\text{m/min}$ (Figure 6-2A). Their migration was non-directed, with an overall mean directedness of $0.23 \pm 0.03$, and a mean tortuosity of $8.92 \pm 2.15$ (Figure 6-2B-C, F, Movie S15). Cells exposed to BPBP stimulation exhibited similarly non-directed and tortuous migratory behaviour (Figure 6-2B-C, E) but with a greater overall mean $|\text{velocity}|$ of $0.39 \pm 0.06 \ \mu\text{m/min}$ (Figure 6-2A, Movie S16). Interestingly, this suggests that although BPBP stimulation has no impact on the cells’ direction of migration, it still increases their rate of migration.

Strikingly, when undifferentiated NPCs were exposed to BPMP stimulation, they underwent rapid galvanotaxis at a rate of $0.78 \pm 0.07 \ \mu\text{m/min}$ (a significant 2-fold and 3-fold increase as compared to BPBP and non-stimulated groups, respectively, $p<0.05$) (Figure 6-2A). The migration of cells in the presence of BPMP stimulation was highly cathodally-directed, with mean overall directedness of $0.98 \pm 0.01$ ($>8$-fold and $>4$-fold increase compared to BPBP and non-stimulated groups, $p<0.05$) and mean overall tortuosity of $1.18 \pm 0.03$ ($>7$-fold decrease compared to non-stimulated groups, $p<0.05$) (Figure 6-2B-D, Movie S17). This analysis was also performed in 30-minute increments throughout the time-lapse (Figure A6). Notably, undifferentiated NPCs exposed to BPMP stimulation migrated at approximately 75% of the $|\text{velocity}|$ and 100% of the directedness of NPCs exposed to DCEF in our previous work [251], even though BPMP-stimulated cells had only 20% of the exposure to cathodal current as compared to DCEF stimulated cells. Out of 674 cells analyzed across 3 independent experiments, $95.9\% \pm 0.01\%$ of undifferentiated NPCs underwent cathodal galvanotaxis in the presence of BPMP stimulation. Hence, undifferentiated NPCs exposed to BPMP pulses undergo rapid and directed galvanotaxis toward the cathode, but their migration is slower and non-directed when either exposed to BPBP pulses or in the absence of electrical stimulation.
Pre- and post-imaging immunocytochemical analysis verified that the cells remained nestin$^+$ (a marker of undifferentiated NPCs) and did not express neuronal (β-III Tubulin) or glial (GFAP) markers when maintained in the presence of growth factors, regardless of whether or not they were exposed to biphasic stimulation (Figure 6-2G-K). This demonstrates that biphasic stimulation does not alter the differentiation state of the NPCs. A small subset of cells co-expressed GFAP and nestin (arrowhead in Figure 6-2I’ and Figure 6-2K’), and these may represent bona fide type-B neural stem cells [6].
Figure 6-2 - Balanced BPMP stimulation induces rapid and cathode-directed galvanotaxis in undifferentiated NPCs. (A-C) NPCs exposed to BPMP stimulation (n=3) have a greater velocity (A) and directedness (B), and a lower tortuosity (C) of migration compared to non-stimulated NPCs (n=3), and a greater velocity (A) and directedness (B) compared to BPBP stimulated groups (n=3). (D-F) Individual cell migration tracks localized to a common origin show that undifferentiated NPCs migrate toward the cathode terminal in the presence of BPMP pulses (D), but undergo non-directed migration in the presence of BPBP stimulation (E) or in the absence of stimulation (F). (G–K) NPCs maintained in the presence of EGF, bFGF and heparin express nestin, but not β-III Tubulin (G–H) or GFAP (I–J), prior to (G,J) and following (H,J) 6 hours of time-lapse imaging when exposed to biphasic electrical stimulation, or when not stimulated (K). (G’-K’) are higher magnification images of the regions within the dashed boxes in (G–K). Scale bars = 100µm. Data are presented as means ± S.E.M., *=p<0.05.
6.3.2 Differentiated neural cells do not undergo galvanotaxis

We previously demonstrated that when NPCs are induced to differentiate into neural phenotypes, they lose their ability to undergo galvanotactic migration in the presence of DCEFs [251]. We next asked if differentiated cells would undergo galvanotaxis when exposed to BPMP or BPBP pulses. Neurospheres (P0-P4) were plated onto Matrigel-coated galvanotaxis chambers in the presence of 1% FBS for 72-96 hours to induce cell differentiation. Differentiated cells were maintained in 1% FBS conditions and time-lapse imaged for 2.5-6 hours while either stimulated with BPMP or BPBP pulses, or not stimulated. Differentiated cells did not undergo galvanotaxis neither in the presence nor the absence of biphasic stimulation (both BPMP and BPBP modes), consistent with our previous observations with DCEFs. Their $|\text{velocity}|$ (BPBP: $0.07 \pm 0.01 \mu\text{m/min}$, BPMP: $0.06 \pm 0.01 \mu\text{m/min}$), directedness (BPBP: $0.07 \pm 0.17$, BPMP: $0.09 \pm 0.07$) and tortuosity (BPBP: $4.53 \pm 0.58$, BPMP: $2.85 \pm 0.68$) of migration did not significantly differ from that of non-stimulated differentiated cells ($|\text{velocity}|$: $0.06 \pm 0.01 \mu\text{m/min}$, directedness: $-0.04 \pm 0.04$, tortuosity: $2.39 \pm 1.01$) (Figure 6-3A-F, Figure A7, Movie S18). Similar to undifferentiated cells, this analysis was repeated throughout the entire time-lapse in 30-minute increments. Immunocytochemical analysis verified that the cells had differentiated into mature neuronal and glial phenotypes prior to time-lapse imaging, and their mature phenotypes were maintained after 6 hours of imaging in the presence or absence of biphasic stimulation (Figure 6-3G-L). We conclude that biphasic stimulation does not induce galvanotaxis in differentiated neural cells.
Figure 6-3 – Balanced biphasic stimulation does not elicit galvanotaxis in NPCs induced to differentiate. (A-C) Differentiated cells exhibit similar |velocity| (A), directedness (B), and tortuosity (C) of migration when exposed to BPMP stimulation (n=3), BPBP stimulation (n=3), or no stimulation (n=3). (D-F) Individual cell migration tracks localized to a common origin show that differentiated cells undergo little migration over 6 hours of time-lapse imaging in the presence of BPMP stimulation (D), BPBP stimulation (E) or in the absence of stimulation (F). (G-L) Differentiated cells express β-III Tubulin (G-H, arrowheads) or GFAP (I-J) prior to (G,I) and following (H,J) 6 hours of time-lapse imaging, when exposed to biphasic electrical stimulation, or not stimulated (K-L). (G’-L’) are higher magnification images of the regions within the dashed boxes in (G-L). Scale bars = 200µm. Data are presented as means ± S.E.M., *p<0.05.
6.4 Discussion

Effective endogenous neural repair strategies require efficient and controlled methods of mobilizing resident NPCs from their niche toward injured regions. The concept of using EFs to promote neuroregeneration has been proposed as a viable clinical approach [161]. To date, studies in the literature that investigate the effects of EF on cells utilize DCEF, hence their clinical applicability is limited due to the damaging effects of DCEF on neural tissue [386,391]. Electrochemical reactions that occur at the electrode-tissue interface produce by-products that elicit cytotoxic effects [392]. Balanced biphasic stimulation, in contrast, is not encumbered with this limitation and thus represents a more clinically-relevant approach to pursuing galvanotaxis-based therapies. Despite this, however, there have been no prior reports of successful galvanotaxis in any mammalian cell populations using balanced biphasic EFs.

Biphasic or alternating current fields have been previously investigated. Hart et al., demonstrated that high frequency sinusoidal alternating current fields in combination with direct current fields enhanced the speed of keratinocyte galvanotaxis, but they were unable to produce directed galvanotaxis using alternating current fields alone [388]. Pan et al., investigated the effects of asymmetric and symmetric alternating current EFs on the neurite outgrowth behaviour of chick sympathetic neurons [390], a phenomenon known as galvanotropism. However, here we report for the first time that the delivery of balanced biphasic monopolar pulses induces rapid and directed cathodal galvanotaxis (that is, whole cell body translocation in the presence of EFs) in undifferentiated adult SEZ-derived NPCs. To our knowledge, this is the first report of biphasic EFs inducing galvanotaxis in a mammalian cell population. We also demonstrate that galvanotaxis does not occur in NPCs exposed to biphasic bipolar pulses, in which the electrical pulses are completely symmetrical. We propose that the asymmetrical nature of BPMP pulses allows the cathodal phase of the pulses to elicit an effect that is not observed during the anodal phase, thus producing a cellular response that is biased to one phase of the EF. Conversely, symmetrical BPBP pulses would presumably elicit identical cellular responses during each phase but in opposing directions, yielding no net bias in migratory behaviour. Moreover we demonstrate that both BPBP and BPMP stimulation fail to produce a galvanotactic effect in NPCs that have been induced to differentiate into mature phenotypes, implying that balanced
biphasic stimulation can selectively target undifferentiated NPC populations. These findings are consistent with our previous work demonstrating that pure clonally-derived populations of adult NPCs undergo rapid cathodal galvanotaxis when exposed to DCEFs of strength 250 mV/mm [251]. Remarkably, the BPMP-stimulated cells in the present study migrate at 100% of the directedness and 71% of the |velocity| of cells that are exposed to DCEF stimulation, considering that BPMP-stimulated cells had only 20% of the exposure to cathodal current as compared to DCEF stimulated cells. There are infinite combinations of amplitude, pulse width, and frequency settings available for designing BPMP pulses, and it is conceivable that NPC migration velocity might be enhanced further by determining the optimal pulse parameter values. One must also consider that the cell behaviour observed in vitro may not accurately reflect their in vivo migratory capacity, which would be influenced by cell-cell and cell-ECM interactions, cell surface receptor expression, and gradients in various signaling molecules [384].

In addition to promoting galvanotaxis, balanced biphasic stimulation may exert other beneficial effects on neural stem and progenitor cells. Chang and colleagues demonstrated that balanced biphasic bipolar pulses promoted expansion and neuronal differentiation of fetal neural precursors [393]. A recent study reported that biphasic stimulation promotes cell survival and anti-apoptotic effects in growth factor-depleted conditions through BDNF and PI3K/Akt signalling [394]. The PI3K/Akt pathway has been implicated as a cellular mechanism by which external application of EFs is transduced into cellular motility through galvanotaxis [204,252]. Biphasic electrical stimulation is also a well-established clinical tool that has beneficial applications in neuromodulation, cardiac pacing, and deep brain stimulation [395,396]. Thus, balanced biphasic electrical stimulation may be a clinically relevant stimulant of neural precursor activity that can be integrated into therapeutic strategies for promoting neurorepair.

The discovery of neural stem cells in the adult brain has led to extensive investigation of their potential for promoting endogenous repair following neural injury or disease. Although neural insult alone is sufficient to expand resident NPC populations, and though this process can be augmented with exogenous factors, only a subpopulation of the expanded pool migrate to lesion sites in response to insult [348]. We propose that endogenous neurorepair processes may be enhanced by recruiting greater numbers of NPCs toward insult regions, and our data suggest that
balanced biphasic electrical stimulation may represent a clinically-relevant means to accomplish this by selectively targeting and controlling the directed migration of undifferentiated NPCs.
Chapter 7

7 General Discussion and Recommendations

7.1 General Discussion

The galvanotactic migration of SEZ NPCs was characterized in their undifferentiated and differentiated states using both direct current and biphasic EFs, and the cellular mechanisms involved in their galvanotaxis were investigated. Pure populations of NPCs were obtained by isolating subependymal tissue from the brains of adult male mice, enzymatically dissociating the tissue and subsequently culturing the cells in mitogenic conditions to yield clonally derived free-floating colonies termed neurospheres. The construction of galvanotaxis chambers and the method of electrical stimulation application were adapted from previously established protocols [397]. This configuration permits multi-hour image acquisition and electrical stimulation without affecting cell viability. Three main parameters of migration for each cell were analyzed following exposure or non-exposure to electrical stimulation: the \( |\text{velocity}| \) (magnitude of velocity) was determined by dividing the length of the line segment connecting the initial and final cell positions by the time taken to reach the final position; the directedness of migration was given by the cosine of the angle between the positive X-axis and the line segment connecting the initial and final cell positions; tortuosity was obtained by dividing the total length of the path traversed by a cell by the line segment joining the initial and final cell positions. The latter two parameters are measures of how “straight” the cells migrate. These kinematic properties of migration were analyzed in various conditions of culture that aimed to manipulate environmental and intracellular factors that may influence or regulate NPC galvanotaxis. These manipulations included the differentiation of NPCs into mature phenotypes prior to electrical stimulation, replacement of the migration substrate, as well as modulation of EGFR and \( \text{Ca}^{2+} \) signaling.

We demonstrated that NPCs undergo rapid and cathode-directed galvanotaxis while remaining in an undifferentiated state, confirming Hypotheses 1, 3, and 4. Among the most profound findings was that the phenomenon of galvanotaxis was limited to undifferentiated NPC populations only; when NPCs were induced to differentiate into mature phenotypes, they did not exhibit any
significant cell body translocation, confirming hypothesis 2. We demonstrated that this was neither due to the cells’ prolonged adherence to the Matrigel substrate, nor the result of a lack of exogenous mitogens in the culture media during electrical stimulation, suggesting that the absence of a galvanotactic response was inherent to mature phenotypes in neurosphere-derived cells and was not the result of external manipulations. Importantly, we also demonstrated that undifferentiated NPC galvanotaxis is not specific to Matrigel basement membrane, but rather is a robust phenomenon that occurs on other substrates such as laminin and MaxGel (a substrate derived entirely from human ECM constituents). The significance of these findings stems from the implication that NPCs as a cell population can be selectively targeted and induced to migrate across a variety of substrates using EFs, without eliciting a similar affect in mature cells – properties that are appealing for cell replacement and neurorepair strategies.

Galvanotaxis is a complex, multistep phenomenon. Fundamentally, it is a cellular response characterized by persistent directional migration that is elicited by the extracellular application of a voltage gradient. It is logical to postulate that this behaviour is evoked by the polarization of intracellular signals that lead to actin polymerization, membrane process protrusion and substrate attachment at the leading edge, and actin depolymerization, membrane process retraction and substrate detachment at the trailing edge. The cellular mechanisms that sense the applied voltage gradient and transduce it into polarized cell motility are not fully known, but two principal methods have been proposed: the redistribution of charged membrane surface receptors, and the biased activation of membrane ion channels. These methods were described in greater detail in Chapter 2.

In support of the idea that membrane receptor redistribution may mediate transduction of the EF signal, we have provided evidence that the EGF receptor is involved in regulating the |velocity| of NPC galvanotaxis, confirming Hypothesis 4. NPCs were either exposed to the EGFR antagonist erlotinib, or cultured in the absence of exogenous EGF for the duration of electrical stimulation. Under these conditions, NPCs maintained a cathode-directed trajectory of migration, but at a reduced |velocity| compared to vehicle controls and cells maintained in the presence of exogenous EGF (Figure 4-9, Figure 4-10). This is reminiscent of previous observations made in the galvanotaxis of corneal epithelial cells and keratinocytes, in which EGFRs were shown to
accumulate on the cathodal side [259,266]. Similar redistribution of EGFR in the presence of a DCEF occurred in embryonic NPCs [252]. In an earlier study by Zhao et al. the growth factors EGF, bFGF, and TGF-β1 significantly increased both the directedness and rate of migration in corneal epithelial cell galvanotaxis [232]. bFGF, TGF-β, and VEGF have also been implicated in the galvanotaxis of other cell populations [232,244,256]. Interestingly, the migration parameters (rate and directedness) seem to be controlled by different pathways downstream of growth factor activation, and may be cell-type specific. For example, the inhibition of EGFR tyrosine kinase predominantly affects the directedness of galvanotaxis in human keratinocytes, whereas the inhibition of the ERK1/2 MAP kinase (downstream of EGFR) primarily reduced the speed of galvanotaxis with a less pronounced effect on directedness in corneal epithelial cells [259,266]. Remarkably, TGF-α – another EGFR ligand – exerts a dose-dependent effect on corneal epithelial cell galvanotaxis; at lower concentrations TGF-α enhanced the speed and directedness of galvanotaxis, whereas the directedness was completely abrogated at higher concentrations without affecting the cells’ general motility [266], further highlighting the complexity and redundancy of growth factor signaling involvement.

The second proposed mechanism of cell polarization in an applied EF suggests that the extracellular voltage gradient leads to a slightly hyperpolarized anodal edge and a slightly depolarized cathodal edge. It is believed that this slight depolarization may be responsible for the biased activation of ion channels, particularly VGCCs, on the cathodal edge, and the anodal hyperpolarization would increase the passive influx of Ca$^{2+}$ on this side of the cell [233]. Ca$^{2+}$ is involved in a multitude of cellular processes that include neuronal excitation, muscle contraction, gene transcription, secretion, proliferation, differentiation, and apoptosis [398-400]. Ca$^{2+}$ has also been implicated in the regulation of cell motility [280,370-373,401], and specifically as a regulator of galvanotaxis in keratinocytes, neural crest cells, granulocytes, and osteoblast-like cells [168,285,286,360]. The intracellular Ca$^{2+}$ concentrations and spatiotemporal dynamics that arise due to passive influx and VGCC activation during electrical stimulation are thought to be involved in signaling cascades that promote cytoskeletal remodeling and the generation of motility forces that culminate in directional movement. Given that this model involves an elevation of intracellular Ca$^{2+}$ on both cathodal and anodal ends of the cell, Ca$^{2+}$ may play
multiple opposing roles in the regulation of galvanotaxis. This is evidenced by the involvement of Ca\(^{2+}\) in retraction of motile cells’ membrane processes at the trailing edge, as well as actin redistribution and polymerization at the leading edge [297,361,377]. This is in line with the data described in Chapter 5, in which NPCs were shown to experience a shortening in the length of membrane protrusions and a reduction in the |velocity| of migration (confirming Hypothesis 5) when placed in conditions of reduced extracellular Ca\(^{2+}\) or in the presence of VGCCs inhibitors, once again highlighting the decoupling of mechanisms that regulate the rate and directedness of galvanotaxis.

Given other groups’ reports of increased levels and cathodal polarization of F-actin during EF exposure, and our observations that the inhibition of Ca\(^{2+}\) influx leads to the retraction of membrane protrusions in NPCs, we propose a model in which the |velocity| of NPC galvanotaxis is regulated by the length and cathodal bias of membrane protrusions, which are dependent on the polarization and Ca\(^{2+}\)-mediated polymerization of actin (Figure 7-1). In the presence of an EF, F-actin would increase and accumulate toward the cathodal cytoplasm, and Ca\(^{2+}\) influx would promote actin polymerization and membrane protrusion (possibly via the PI3K/PIP\(_3\) signalling effectors) on this side (Figure 7-1A). The concentration of membrane processes on the cathodal side would permit overall greater substrate attachment and forward propulsion forces in this direction. When Ca\(^{2+}\) influx is inhibited, Ca\(^{2+}\)-mediated actin polymerization and membrane protrusion processes would be attenuated, resulting in shorter process lengths and reduced forward propulsion forces (Figure 7-1B), ultimately leading to a decrease in the |velocity| of migration. In the absence of an EF, F-actin localization would be ubiquitous and permissive of more evenly distributed membrane protrusions. This in turn would result in the generation of multiple push-pull forces on the cell body in various directions, and the net movement of the cell would be in the direction of the greatest pull (Figure 7-2A). Since the motility forces act in multiple directions, the |velocity| of migration in this case would be lower than that of cells exposed to an EF, in which the force-generating processes are biased toward the cathodal side. The inhibition of Ca\(^{2+}\) influx in NPCs not exposed to an EF would cause a similar reduction in membrane process length, accompanied by a decrease in the forces generated by the impartially distributed processes, and further reduced |velocity| of migration (Figure 7-2B).
Figure 7-1 - Model of F-actin- and Ca$^{2+}$-mediated regulation of NPC migration |velocity| in the presence of an EF. (A) EF-induced cathodal accumulation of F-actin, coupled with Ca$^{2+}$-mediated actin polymerization result in the biased protrusion of membrane processes on the cathodal side. This permits greater net forward propulsion forces in this direction and increased |velocity| of migration. (B) The inhibition of Ca$^{2+}$ influx causes the attenuation of Ca$^{2+}$-mediated actin polymerization and membrane protrusion, resulting in shorter process lengths, reduced propulsion forces, and decreased |velocity| of migration.
Figure 7-2 - Model of F-actin- and Ca$^{2+}$-mediated regulation of NPC migration |velocity| in the absence of an EF. (A) F-actin is ubiquitously distributed in the absence of an EF, and Ca$^{2+}$-mediated actin polymerization and membrane protrusion occurs around the cell. Multiple ‘push-pull’ forces are generated and the net movement of the cell is in the direction of the greatest pull. (B) The inhibition of Ca$^{2+}$ influx causes the attenuation of Ca$^{2+}$-mediated actin polymerization and membrane protrusion, resulting in shorter process lengths, reduced propulsion forces, and further decreased |velocity| of migration.

The work in Chapter 6 represents the first report of utilizing biphasic EFs to induce galvanotaxis in any mammalian cell type. Sinusoidal alternating current fields have been previously used to maintain *C elegans* worms in their current position within microfluidic channels, after inducing their migration with DCEFIs [402]. Alternating current fields failed to induce directional...
galvanotactic migration in keratinocytes. Remarkably, when alternating current fields were combined with DCEF stimulation, the effect on keratinocyte migration was dependent on the frequency of the alternating current waveforms; lower frequencies reduced the directedness of migration without affecting speed, whereas higher frequencies had the opposite effect of increasing the speed of migration without affecting the directedness [388]. Pan et al., utilized biphasic EFs to induce galvanotropic responses in chick embryonic neurons [390]. Our work shows for the first time in any mammalian cell population that charge-balanced biphasic monopolar waveforms can induce rapid and cathode-directed galvanotaxis, confirming Hypothesis 6. Interestingly, with our stimulation protocol NPCs maintained 100% of the directedness and ~71% of the |velocity| of galvanotaxis as compared to DCEF stimulated cells, despite only receiving 20% of the cathodal current. The need for such demonstrations, especially within the context of our work involving neural precursors, derives from early observations that direct current stimulation of neural tissue resulted in tissue damage [403,404]. Further investigations have revealed the cause of this to be unidirectional electrochemical reactions that generate toxic byproducts [386]. The integration of galvanotaxis into clinical treatment paradigms, particularly for neural applications, depends on the demonstration that this phenomenon can be safely and effectively achieved via biphasic stimulation, and the work presented in this thesis represents the first step in this direction.

7.2 Future Work

This section describes potential future investigations that may expand on the findings of each subproject in this thesis. As a general recommendation, it is advisable for in vitro investigations to pursue and validate a high-throughput microfluidic system to replace the Petri dish-based galvanotaxis chambers utilized in this thesis. The latter devices are taxing to construct, consume greater amounts of reagents, and only allow one type of culture condition to be investigated per chamber. Microfluidic chambers on the other hand may be manufactured or purchased in larger quantities, allow fine control of signal gradients (i.e. chemical or electrical gradients), and with today’s advanced imaging systems (such as the Mark&Find Zeiss Axiovision imaging module) allow multiple chambers with varying culture conditions to be imaged simultaneously. A more detailed description of the advantages of microfluidic systems maybe found in the review by Li
and Lin [405]. As may be expected, a current disadvantage of microfluidic systems is their increased costs relative to traditional Petri dish-based galvanotaxis chambers. Nonetheless, the implementation of microfluidic systems should be taken into consideration by future researchers.

### 7.2.1 Characterization of NPC galvanotaxis mechanisms

The work in Chapter 4 demonstrated that undifferentiated NPCs undergo cathodal galvanotaxis in a manner that is EGFR dependent and not influenced by an EF-generated chemotactic gradient. Much of the work presented was phenomenological in nature, with less emphasis placed on the mechanisms underpinning the phenomenology. A logical progression of this work would include further characterization of the cellular mechanisms involved in galvanotaxis. To expand on the results in Chapter 4, the role of other growth factors, including bFGF and TGF-α, and their downstream signaling pathways should be investigated. Given that currently known effectors of galvanotaxis (growth factor and Ca\(^{2+}\) signaling pathways) often have cell-type specific effects, it is not sufficient to draw conclusions of the involvement of these growth factors in NPC galvanotaxis from observations made in other cell populations. The PI3K signaling cascade has been implicated in the galvanotaxis of keratinocytes, hippocampal neurons, and embryonic and adult neural precursor cell lines [204,252,275], and is worth pursuing in primary cultures of NPCs. PI3K, which is downstream from EGFR activation, phosphorylates PIP\(_2\) into PIP\(_3\), a regulator of actin polymerization that is polarized toward the leading edge of galvanotactic cells [204,252]. Pharmacological and/or genetic inhibition of PI3K activity may provide an entry point for further elucidating the role of growth factors and their signaling pathways in adult brain derived NPCs. These methods may be combined with fluorescent tagging techniques to visualize the localization of effectors of interest, such as EGFR or PIP\(_3\), within migrating cells.

A second finding of the work in this chapter is that differentiated NPCs do not exhibit any galvanotactic migration. This has beneficial implications for the development of cell replacement and neurorepair strategies in which the selective targeting of undifferentiated NPCs without affecting mature phenotypes is desirable. However, this data conflicts with observations that neurons from postnatal hippocampal explants exhibit galvanotactic behaviour [275,406]. This may be due to the different origins of the cell populations, but the discrepancy may also be
explained by recent findings that the migration of the neuronal progeny of NPCs is impaired by their chemoattraction to VEGF and bFGF, factors that are released by nearby NPCs [407]. Such a chemotactic effect may offer an explanation for the lack of migration among any differentiated cells, including neurons, in our assay. To address this, NPCs could be derived from a transgenic mouse in which cells express a fluorescent marker (such as GFP) under the control of the promoter for a neuron specific gene (such as DCX or βIII Tubulin). The fluorescent neuronal progeny of NPCs induced to differentiate may then be isolated through fluorescence activated cell sorting in order to specifically analyze the galvanotaxis of neurons in the absence of other potentially inhibitory cell phenotypes.

Chapter 5 provided evidence that Ca$^{2+}$ signaling is involved in NPC galvanotaxis. This was demonstrated by various manipulations that affected the availability of free intracellular Ca$^{2+}$, including chelation of intracellular Ca$^{2+}$, placement of NPCs in low extracellular Ca$^{2+}$ conditions, and the inhibition of VGCCs. In response to these manipulations undifferentiated NPCs underwent a retraction or shortening of membrane processes and a reduction in their $|\text{velocity}|$ of galvanotaxis without affecting their directedness. These results raise several interesting questions and provide multiple avenues of further exploration. Ca$^{2+}$ is a ubiquitous second messenger with many cellular functions, as described above. The successful execution of these functions depends on the proper regulation of Ca$^{2+}$ concentrations both spatially and temporally within cells, which is achieved through binding with various Ca$^{2+}$-buffering and Ca$^{2+}$-transporting proteins. Therefore, of particular interest are the spatiotemporal dynamics of intracellular [Ca$^{2+}$] within NPCs in the presence and absence of an applied EF, and in the presence or absence of conditions that modulate Ca$^{2+}$ influx. A first step in this endeavor may be to measure the concentration levels of Ca$^{2+}$ within NPCs, which may be achieved with fluorescent ratiometric Ca$^{2+}$ indicator probes such as Fura-2. It would be interesting to determine using this approach if galvanotactic NPCs possess Ca$^{2+}$ gradients similar to those observed in chemotactic fibroblasts, namely a global Ca$^{2+}$ gradient in the rear-to-front decreasing direction accompanied by a Ca$^{2+}$ flicker gradient decreasing in the front-to-rear direction [282].

Similar to the additional work proposed above for growth factor signaling pathways, it would be prudent to investigate the Ca$^{2+}$-sensing effectors that translate Ca$^{2+}$ signals into cell motility.
Given that Ca\(^{2+}\) is most likely involved in cytoskeletal remodeling processes at both the leading and trailing edges of galvanotactic NPCs, several such signaling effectors are targets for investigation. Of particular interest is PI3K-mediated polymerization of F-actin at the leading edge, which has been shown to be Ca\(^{2+}\)-dependent in macrophages [297]. In addition, PIP\(_3\) (the lipid byproduct of PI3K) has been shown to trigger Ca\(^{2+}\) influx in neutrophils and basophils [408,409], further establishing a link between Ca\(^{2+}\) and PI3K activity. PI3K’s potential role in both growth factor- and Ca\(^{2+}\)-mediated galvanotactic signaling suggests that there may be crosstalk in these pathways, lending credence to the observation that both EGFR and VGCC inhibition primarily affected the |velocity| of galvanotaxis and not the directedness or tortuosity.

At the trailing edge, calpain has been implicated in the Ca\(^{2+}\)-mediated detachment of the rear cell membrane from the substrate. Calpain activity may be inhibited via Calpain Inhibitor I (CI-1), CI-2 or benzyloxy carbonyl-Leu-Leu-Tyr diazomethyl ketone [292], and we predict that this would reduce rear membrane detachment leading to decreased |velocity| and possibly reduced directedness of NPC galvanotaxis.

We have focused our efforts in Chapter 5 on VGCCs, specifically the Ca\(_{1.2}\) subunit of L-type, and the Ca\(_{3.2}\) and Ca\(_{3.3}\) subunits of T-type VGCC subtypes, since RT-PCR analysis revealed that these channels were expressed on undifferentiated NPCs. The role of ligand-gated Ca\(^{2+}\) channels in NPC galvanotaxis remains unexplored. Given that NMDA receptors (ligand gated Ca\(^{2+}\) channels) have been implicated in the chemotactic migration of embryonic neurons from the ventricular/subventricular zone toward the developing cortex [410], their involvement in NPC galvanotaxis may be a topic of interest. Other ion channels that may be of interest are stretch activated Ca\(^{2+}\) channels, which control cell steering and rear-edge detachment processes in migrating fibroblasts and keratocytes, respectively [282,351]. While the inhibition of VGCCs was sufficient to reduce the |velocity| of NPC galvanotaxis and eventually abolish migration, the directedness of galvanotaxis remained unchanged. Considering that Ca\(^{2+}\) has been shown to play a role in the directedness of keratinocyte galvanotaxis [286], it is plausible that Ca\(^{2+}\) influx through ligand-activated and stretch-activated channels that persists in the presence of VGCC inhibition is important for maintaining the directedness of migration. Specifically inhibiting different Ca\(^{2+}\) channels may provide insight into the mechanisms by which migration rate and
directedness are decoupled. Importantly, the role of other ion channels should not be neglected. For example, the galvanotaxis of metastatic rat prostate cells is dependent on voltage gated sodium channels [376].

Finally, an additional point for consideration would be to characterize the expression profiles of ion channels (including Ca\textsuperscript{2+} and Na\textsuperscript{+} channels) through microarray analysis in undifferentiated vs. differentiated NPC populations. This may provide insight into whether the lack of galvanotactic behaviour in differentiated neural progeny is attributable to a difference in ion channel expression patterns in this population. A similar analysis may be performed to investigate differences in growth factor receptor expression between undifferentiated and differentiated cells.

7.2.2 *In Vivo* Demonstration of NPC Galvanotaxis

Chapter 6 described a novel mechanism of selectively inducing the galvanotaxis of undifferentiated NPCs utilizing charge-balanced biphasic EFs. This work has significant implications for the development of neurorepair and cell replacement strategies that seek to incorporate galvanotaxis as a mechanism of promoting the directed migration of target cells. This is due to the fact that properly designed biphasic electrical pulses have been deemed safe for clinical applications of neural tissue, owing to their ability to avoid tissue damage, unlike direct current stimulation. To this end, future work in this aspect of the project should test the general hypothesis that NPCs can undergo galvanotaxis *in vivo*.

As a first step, to assess the safety of the BPMP pulses described in Chapter 6, electrodes may be implanted into mouse brains and biphasic pulses of varying pulse widths, amplitudes, and frequencies may be delivered over various time periods. Following stimulation, mouse brains should be excised, sectioned and analyzed for damage over several time points (to assess acute and chronic effects) using histopathological techniques and/or cell viability assays such as propidium iodide exclusion. Testing different pulse configurations is necessary because the parameter values that produce optimal galvanotaxis are not yet known.
In order to assess the ability of biphasic electrical stimulation to induce NPC galvanotaxis in vivo, two approaches may be considered: transplantation of exogenous cells or analysis of endogenous cells. Exogenous fluorescently tagged NPCs (derived, for example, from the brains of YFP transgenic mice) may be isolated and expanded in culture, and subsequently transplanted into the brains of host mice. Following this, electrodes may be implanted into the host mice brains, and electrical stimulation may either be applied or not applied, the latter condition serving as a control. Factors that would need to be considered in such an approach include: i) the location of transplantation within the host brain; ii) the number of cells transplanted; iii) whether NPCs are transplanted in the form of neurospheres or pre-dissociated into single cell suspensions; iv) the location of electrode implantation; v) the duration of stimulation (both the duration per session and the total number of sessions). Following stimulation, mice brains would be excised, sectioned, immunostained and analyzed over various time points for the extent and direction of NPC migration, as well as for their differentiation profile. This approach offers flexibility in terms of the numbers of cells that can be transplanted and assessed, as well as the starting location of the cells within the brain. In addition, this paradigm is not limited to only transplanted NPCs; the galvanotactic capacity of other transplanted cell populations, for example neuralized induced pluripotent stem cells, may also be investigated.

The second approach to assessing the in vivo efficacy of NPC galvanotaxis involves utilizing endogenous NPCs. This paradigm would require the specific and permanent labeling of endogenous NPCs in order to visualize their migratory response to electrical stimulation. This may be achieved by using a transgenic mice line, such as nestin-GFP mice, which express the fluorescent marker GFP under the control of the nestin promoter to label NPCs. Alternatively, lentiviral vectors encoding fluorescent or bioluminescent reporter genes can be injected into the lateral ventricle to transfet both dividing neural progenitors and slowly dividing neural stem cells. Electrode implantation, electrical stimulation and subsequent cell and tissue analysis would then be performed similar to the cell transplantation paradigm described above. Excitingly, it may also be possible to noninvasively visualize NPC galvanotaxis directly in vivo [411]. An endogenous labeling approach to demonstrating in vivo galvanotaxis is appealing because, if successful, it would demonstrate that the migration of the brain’s resident NPCs can be directed
and enhanced using EFs, thereby strengthening the notion that resident NPCs may be able to repair the injured or diseased brain without the need for exogenous delivery of cells.

Ultimately, in order to ascertain the utility of NPC galvanotaxis, it would be necessary to demonstrate that enhanced NPC migration toward a region of interest results in a measurable and quantifiable improvement. The indicator of improvement would vary depending on the application. For example, in the case of ischaemia, improvement would pertain to tissue repair through *de novo* neurogenesis, and recovery of lost motor or cognitive function. In the case of multiple sclerosis improvement may be manifested in the remyelination of neurons. In this regard, an injury or disease model that incorporates galvanotactic induction of NPC migration would need to be designed in order to analyze the benefits (if any) of NPC galvanotaxis *in vivo*. In addition to the electrical stimulation considerations outlined above, additional factors to be considered in such efforts include: i) the type of injury or disease to be investigated (for example ischaemia, multiple sclerosis, or spinal cord injury); ii) the injury model to be implemented (for example pial vessel disruption vs. endothelin-1 vasoconstriction in the case of ischaemia); iii) the source of NPCs to be investigated (exogenously transplanted vs. endogenously labeled); iv) the time between onset of injury and initiation of electrical stimulation; and v) whether or not galvanotactic stimulation should be coupled with other treatments. The results of such studies are imperative to furthering our understanding of how electrical stimulation may benefit strategies for repairing the injured or diseased CNS.

The importance of physiological EFs is incontestable. Their roles in development and wound healing are well established, and yet despite this, the phenomenology of galvanotaxis is generally underappreciated in the scientific community. This thesis has characterized the phenomenology of adult brain derived NPC galvanotaxis, uncovered some of the cellular mechanisms underlying the phenomenon, and demonstrated for the first time that it may be achieved via clinically-relevant biphasic electrical pulses. It is my hope that the scientific community continues to pursue a complete understanding of galvanotaxis, and that the phenomenon’s therapeutic potential be realized through widespread adoption and integration into regenerative medicine efforts.
References


73. Young KM, Fogarty M, Kessaris N, Richardson WD. Subventricular zone stem cells are heterogeneous with respect to their embryonic origins and neurogenic fates in the adult olfactory bulb. *J. Neurosci.* 27(31), 8286–8296 (2007).


79. Rosamond WD, Folsom AR, Chambless LE, *et al.* Stroke Incidence and Survival...


149 (2003).


119. Gadisseux J-F, Kadhim HJ, van den Bosch de Aguilar P, Caviness VS, Evrard P. Neuron migration within the radial glial fiber system of the developing murine cerebrum: an


144. Koizumi H, Higginbotham H, Poon T, Tanaka T, Brinkman BC, Gleeson JG.


156. Vergaño-Vera E, Méndez-Gómez HR, Hurtado-Chong A, Cigudosa JC, Vicario-Abejón C. Fibroblast growth factor-2 increases the expression of neurogenic genes and promotes the migration and differentiation of neurons derived from transplanted neural...


184. Al-Bazzaz FJ, Gailey C. Ion transport by sheep distal airways in a miniature chamber.


263. Stollberg J, Fraser SE. Acetylcholine receptors and concanavalin A-binding sites on...


266. Zhao M, Pu J, Forrester JV, McCaig CD. Membrane lipids, EGF receptors, and intracellular signals colocalize and are polarized in epithelial cells moving directionally in a physiological electric field. *FASEB J.* 16(8), 857–859 (2002).


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Appendix A – Supplemental Figures

Figure A1 – Undifferentiated NPCs undergo cathodal galvanotaxis. (A-D) Time-lapse images of undifferentiated NPCs in the presence (A,B) or absence (C,D) of a 250 mV/mm dcEF at 0 (A,C) and 300 (B,D) minutes.

Figure A2 – NPCs that are induced to differentiate into mature phenotypes do not undergo cathodal galvanotaxis. (A-D) Time-lapse images of differentiated neural cells in the presence (A,B) or absence (C,D) of a 250 mV/mm dcEF at 0 (A,C) and 300 (B,D) minutes.
Figure A3 – Growth factor conditions fail to rescue differentiated neural cell galvanotaxis. 
(A-D) NPCs induced to differentiate into mature phenotypes and then plated back into growth factor conditions exhibit low displacement in the direction of the dcEF (A), as well as low velocity (B), low directedness (C) and high tortuosity (D) of migration. This behaviour is similar to cells maintained in FBS conditions at all times, although cells transferred to growth factor conditions tend to display preferential overall displacement toward the cathode. * = p < 0.05.
Figure A4 – NPCs remain undifferentiated following 2.5 hours in the absence of EGFR signalling. (A-B) NPCs maintain nestin-expression after 2.5 hours of EGFR blockade with erlotinib, both in the absence (A), and presence (B) of a dcEF. Scale bar = 100 µm.
Figure A5 – Inhibition of L-type or T-type VGCCs reduces the velocity of NPC galvanotaxis, but not the directedness or tortuosity, in a dose-dependent fashion. NPCs were cultured in the presence of various concentrations of Nifedipine, or ML218, during DCEF exposure, and their migration was tracked over the entire time-lapse period. The velocity, directedness, and tortuosity of migration were analyzed over 30-minute intervals. For Nifedipine, *, #, †, ‡, and § indicate statistical significance between the groups 0 vs. 100 µM, 0 vs. 200 µM, 10 vs. 100 µM, 10 vs. 200 µM, and 100 vs. 200 µM, respectively. For ML218, *, #, †, ‡, and § indicate statistical significance between the groups 0 vs. 5 µM, 0 vs. 10 µM, 0 vs. 25 µM, 5 vs. 25 µM, and 10 vs. 25 µM, respectively.
Figure A6 - Undifferentiated NPCs undergo rapid and cathode-directed galvanotaxis in the presence of BPMP stimulation. (A-C) The |velocity| (A), directedness (B), and tortuosity (C) of migration of undifferentiated NPCs are plotted in 30 minute intervals over the course of time-lapse imaging. The average |velocity|, directedness, and tortuosity at each time point $t_x$, where $x$ is in increments of 30 minutes, were calculated based on $t=0$ as the initial time point. Tracking was performed for up to 210 minutes - the maximum amount of time that was common between all undifferentiated NPC groups. $n=3$ for each group, * $= p< 0.05$.

Figure A7 – Differentiated cells undergo negligible migration in the presence and absence of balanced biphasic stimulation. (A-C) The |velocity| (A), directedness (B), and tortuosity (C) of migration of differentiated cells are plotted in 30 minute intervals over the course of time-lapse imaging The average |velocity|, directedness, and tortuosity at each time point $t_x$, where $x$ is in increments of 30 minutes, were calculated based on $t=0$ as the initial time point. Tracking was performed for up to 150 minutes - the maximum amount of time that was common between all differentiated cell groups. $n=3$ for each group, * $= p< 0.05$. 
## Appendix B – Supplemental Tables

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<tr>
<th>Experiment</th>
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<th>No. of cells at completion of experiment</th>
<th>Mean viability at experiment completion</th>
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<td>V</td>
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Table B1 - Analysis of cell viability prior to and following DCEF stimulation in different Ca\(^{2+}\) modulation conditions. When NPCs are cultured either in L-SFM media with 20 µM BAPTA, or in the presence of 200 µM Nifedipine, or 25 µM ML218, the vast majority remain viable when assessed at least 1h after greater than 90% of cells have stopped migrating. Viability was determined by dividing the total number of viable cells by the total number of cells, per experiment, and mean viability was determined across three independent experiments. V, viable. NV, non-viable. Data are presented as mean ± S.E.M.
Appendix C – Full Protocol of *In Vitro* Neural Precursor Galvanotaxis Assay

1) **Isolation and Culture of Neural Precursors**

1.1) Anaesthetize a CD1 mouse (6-8 weeks old) with isofluorane and sacrifice via cervical dislocation.

1.2) Douse the head in 70% ethanol and decapitate the animal with sharp dissection scissors.

1.3) While holding the head with surgical forceps, remove the skin on the dorsal surface to expose the skull.

1.4) Using a scalpel and no. 11 blade, score the skull at the frontal sinus along the mediolateral axis, and also along the sagittal suture in the rostrocaudal direction.

1.5) Peel the parietal bones away from the head with no. 7 curved forceps, taking care not to pierce the brain tissue.

1.6) Insert a thin spatula underneath the brain starting from beneath the cerebellum and advancing toward the olfactory bulbs. While holding the skull in place with forceps, gently pull the brain from the skull and immediately place it in ice-cold artificial cerebrospinal fluid (see recipes below).

1.7) Under a dissection microscope, using sterile dissection scissors and forceps cut the brain in half along the midline. Rotate each hemisphere so that the medial (cut) surface faces upwards.

1.8) Select a hemisphere, and with the medial surface facing upward, locate the splenium of the corpus callosum (posterior region of the corpus callosum).

1.9) Make an incision from the surface of the cortex to the splenium of the corpus callosum along the dorsoventral axis.

1.10) Peel the incised cortex toward the olfactory bulb to expose the medial and lateral walls of the lateral ventricle.

1.11) Rotate the hemisphere so that the dorsal surface faces upwards, and use curved microscissors to cut out and collect the exposed medial and lateral walls, which contain the periventricular region where NPCs reside [331].

1.12) Repeat steps 1.8-1.11 for the other hemisphere.
1.13) Pipette the isolated tissue into 7 mL of trypsin solution (see recipe below) in a 15 cc tube, and place the tube on a rocker in a 37 °C incubator for 25 minutes.
1.14) Centrifuge the tube at 1500 RPM for 5 minutes, aspirate the supernatant, and resuspend the tissue in 2 mL of trypsin inhibitor solution (see recipe below).
1.15) Gently triturate the tissue with a small borehole Pasteur pipette 30-50 times carefully to avoid air bubbles.
1.16) Centrifuge the tube at 1500 RPM for 5 minutes, aspirate the supernatant, and resuspend in 1-2 mL of SFM (see recipe below) by triturating the pellet 3-5 times.
1.17) Centrifuge the tube at 1500 RPM for 3 minutes, aspirate the supernatant and resuspend in 1 mL of SFM + EFH.
1.18) Count live cell density with a haemocytometer and plate the cells in a T25 culture flask at a density of 10 cells per µL in SFM + EFH.
1.19) Allow the culture to grow for 7 days undisturbed to yield free-floating primary neurospheres comprised of NPCs.

2) **Galvanotaxis Chamber Preparation**

2.1) Place 3 square glass no. 1 cover slips (22 x 22 x 0.17 mm) in a bottle of 6N hydrochloric acid overnight.

2.2) The next day, use a diamond-tip glass-cutter to cut 6 rectangular strips (22 x 5 x 0.17 mm) of glass from square no. 1 cover slips.

2.3) Transfer the acid-washed square slips and rectangular slips into a laminar flow hood. Wash the rectangular and square strips first with 70% ethanol, then with tissue culture-grade autoclaved water, and allow to dry on a Kim Wipe (for added sterility, the glass may be allowed to air dry).

2.4) Apply vacuum grease to the perimeter of one surface of the square glass slides, and seal them to the base of 60 mm plastic Petri dishes.

2.5) Apply vacuum grease along the long axis of one surface of the rectangular glass strips, and seal them to opposite edges of the square glass slides (such that they are parallel to each other) in order to create a central trough.

2.6) UV-sterilize the chambers for at least 15 minutes in the laminar flow hood.
2.7) Pipette 250-300 µL of poly-L-lysine onto the central trough of the chambers and incubate at room temperature for 2 hours.

2.8) Approximately 15 minutes prior to the end of the incubation period, prepare the Matrigel solution (see recipe below).

2.9) Aspirate the poly-L-lysine, wash the central troughs with 1 mL of autoclaved water, and pipette 250-300 µL of Matrigel solution onto the central troughs.

2.10) Incubate the chambers at 37°C for 1 hour.

2.11) Aspirate the Matrigel solution and gently wash the central troughs with 1-2 mL of SFM.

2.12) Pipette 100 µL of EFH-SFM onto the central troughs and transfer the galvanotaxis chambers onto the stage of a counting microscope.

2.13) Pipette 3-4 mL of the neurosphere-containing culture into a 60 mm Petri dish and transfer the Petri dish to the stage of the counting microscope.

2.14) At a viewing objective of 5x, use a P10 pipette to transfer 5-8 whole neurospheres (up to four at a time) onto the central trough of each galvanotaxis chamber without dissociating them, and carefully spread the neurospheres around the central trough without disrupting the Matrigel substrate.

2.15) Add an additional 150-200 µL of EFH-SFM or FBS-SFM onto the central troughs.

2.16) Transfer the galvanotaxis chambers into a 37°C/5% CO2, 100% humidified incubator for 17-20 hours (if analyzing undifferentiated NPCs) to allow the neurospheres to adhere to the Matrigel substrate and dissociate into single cells as shown in Figure 1. If analyzing differentiated NPCs, the incubation period should be extended to 69-72 hours to allow the differentiation of the cells.

3) **Live Cell Time-Lapse Imaging**

3.1) Allow the live cell imaging system to equilibrate at 37°C/5% CO2 for a minimum of 30 minutes prior to initiation of the time-lapse recording.

3.2) Cut two 12 cm pieces of 1 mm diameter Silver wire, coil them from one end, and place them in Clorox bleach for 20 minutes to form Ag/AgCl electrodes.

3.3) Transfer the galvanotaxis chambers onto the stage of a counting microscope and select which chamber will be used for live-cell imaging migration analysis based on the
following criteria: i) the neurospheres should be almost completely dissociated into single cells and ii) the cells should possess round morphologies with little-to-no processes extending from the cell bodies.

3.4) Transfer the selected galvanotaxis chamber into a laminar flow hood, along with a separate square no. 1 glass cover slip and vacuum grease.

3.5) Wash the cover slip first with 70% ethanol, then with autoclaved water, and apply a strip of vacuum grease on two parallel edges of the cover slip.

3.6) Aspirate the culture media from the central trough of the chamber, then quickly place the cover slip (grease-side facing down) onto the chamber such that the grease strips rest on the two parallel rectangular glass strips, effectively creating a roof to the chamber.

3.7) Pipette 100 µL of fresh EFH-SFM or FBS-SFM into the central trough via capillary action.

3.8) Use vacuum grease to create borders for pools of culture media on each end of the central trough, as shown in Figure 2.

3.9) Cut two 15 cm pieces of PVC tubing, and use a 10 cc syringe with an 18 gauge needle to carefully inject agarose solution into the tubing, ensuring no bubbles form in the tubes, and allow the gel to solidify for 5 minutes.

3.10) Transfer the galvanotaxis chamber to the live cell imaging system, along with the agarose gel tubes, Ag/AgCl electrodes, and a pair of empty 60mm Petri dishes that will be used as culture media reservoirs and will contain the Ag/AgCl electrodes. Allow the galvanotaxis chamber to rest within the 37°C/5% CO₂ environment for 20-30 minutes.

3.11) During this time, prepare the lids of the 2 empty Petri dishes and the lid of the galvanotaxis chamber’s Petri dish by drilling holes into them with a Dremel or similar tool as shown in Figure 3.

3.12) Pipette 1-1.5 mL of EFH-SFM or FBS-SFM onto either side of the central trough, and 7-8 mL of SFM into each empty Petri dish. Place one Petri dish on each side of the galvanotaxis chamber’s central trough and place one Ag/AgCl electrode into each dish. Bridge the gap between the galvanotaxis chamber and the Petri dishes to establish electrical continuity with the agarose gel bridges, as shown in Figure 4.

3.13) Connect the Ag/AgCl electrodes to an external power supply, with an ammeter in series to measure electrical current, and turn on the power supply. Use a voltmeter to measure the
strength of the EF directly across the central trough, and adjust the output of the power supply until the desired EF strength is achieved (the assays performed in this lab utilize a dcEF strength of 250 mV/mm with electrical current between 1 and 1.5 mA).

3.14) Initiate the time-lapse module on the live cell imaging system, and allow the experiment to run for the desired amount of time. After completion of the assay, fix the cells in 4% paraformaldehyde for standard immunostaining analysis.