The Effect of Electrical Stimulation on

Endogenous Neural Stem and Progenitor Cell Behavior

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Abstract

Electrical stimulation to the central nervous system, such as deep brain stimulation, is a well-established neurosurgical technique used to treat a variety of neurological disorders. Despite its use as a promising therapy, the effects of electrical stimulation on different neural cells have not been fully explored. We examined the effects of electrical stimulation on the behavior of neural stem and progenitor cells (neural precursor cells; NPCs) found in the periventricular niche of the adult forebrain. We demonstrate that in vitro stimulation of primary periventricular cells at 250mv/mm gives more neural precursors. In vivo, an expansion in stem cells was concomitant with an increase in progenitor cell proliferation in vivo. The increase in the NPC pool can be accounted for by increasing the amount of symmetric divisions the stem cells undergo. These findings provide insight into the effects electrical stimulation on NPCs and its potential use in regenerative medicine strategies.
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Keywords

Neural stem cells, neural progenitor cells, electrical stimulation, regenerative medicine, endogenous repair, proliferation, quiescence, activation, EGFR, symmetric division, wnt3a, sFRP2
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Chapter 1

Introduction

Electrical stimulation in the brain is currently used as a non-pharmacological treatment for diseases such as essential tremor, Parkinson’s disease and dystonia where its application has seen success (Kinelbach et al, 2007). It has also been applied for pain and major depressive disorder, however its mechanism of action are not well understood. Deep brain stimulation (DBS) is thought to modify the balance of inhibitory and excitatory activation of neurons (Kringelbach et al, 2007; Chiken et al, 2016). It is further suggested that astrocytes could be activated to lead to changes in calcium and glutamate (Fenoy et al, 2013) which influence neuronal firing. DBS can lead to an increase in brain-derived neurotrophic factor and increased the number of immature and mature neurons (Cho et al, 2013). Increased proliferation has also been reported in the periventricular region of DBS treated Parkinson’s patients that died from unrelated illness (Vedam-Mai et al., 2014). Together, these findings highlight the potential role of neuroplasticity that underlies DBS, with the overarching goal to understand if electrical stimulation can regulate neural stem cell behavior in vivo. As stem cells provide a source of neuroplasticity and have promise in promoting neural repair following injury or disease, this study can help advance regenerative therapies using electrical stimulation.

Stem cells reside in restricted microenvironments where their development and behavior are controlled by a variety of cues. Signalling from the niche and microenvironment controls many aspects of stem cell behavior including regulation of quiescence versus proliferation, as well as regulating modes of division and migration. The environmental cues that activate signalling pathways can differ in uninjured versus injured conditions. We will ask specifically examine
how EFs regulate neural stem cell kinetics in vitro and in vivo with a view towards potential regenerative medicine application.

1.1 Neural Stem and Progenitor Cells

Neural precursor cells (defined as neural stem and progenitor cells, NPCs) are found in the periventricular regions surrounding the lateral ventricles in the forebrain and the central canal of the spinal cord. Stem cells are rigorously defined by two cardinal properties: unlimited self-renewal and the ability to give rise to cells that will differentiate into mature phenotypes (Siminovitch et al., 1963; Till and McCulloch, 1980). Firstly, neural stem cells have an unlimited self-renewal capacity and persist in the CNS throughout life (van der Kooy and Weiss, 2000). Second, neural stem cells are multipotent and can give rise to all cells within the neuro-ectodermal lineages of the CNS, including a variety of neuronal and glial sub-types (Gage et al., 1995; Weiss et al., 1996; McKay, 1997; Chiasson et al., 1999).

There are two approaches that have been examined to harness the potential of these cells in regenerative medicine strategies which include (1) the activation of resident NPCs (endogenous repair) and (2) the isolation of NPCs in vitro and transplantation in vivo (exogenous repair). Resident NPCs have been shown to migrate, proliferation and differentiate in the CNS following injury however their activation is not sufficient for brain repair and functional recovery (Jahanshahi et al, 2013). Thus strategies to promote the expansion, proliferation, and/or migration of these NPCs are a priority for regenerative medicine.

NPCs are neurogenic cells that express the intermediate filament protein Nestin and the transcription factors Sox2, and in vivo they give rise to neuroblasts that express doublecortin and migrate along the well defined rostral migratory stream towards the olfactory bulb where they
differentiation into mature interneurons (Sun et al, 2010) (figure 1). A study by Morshead et al revealed that the constitutively proliferating cells have an average life span of 15 days within the lateral ventricle subependyma. Examining the sizes and distributions of the clones under baseline conditions 6 days following retrovirus infection revealed that on average 60% of the cells undergo cell death (Morshead et al, 1998), one of the previously described fates of the constitutively proliferating cells (Smart, 1961; Morshead and van der Kooy, 1992). Of the 40% of the cells that survive, 15% are found in the lateral ventricle subependyma and the remaining 25% are within the olfactory bulb (including the rostral migratory stream; Lois and Alvarez-Buylla, 1994). The neural stem cells, which reside in the lateral ventricle walls, can repopulate the constitutively proliferating population following its depletion with high doses of [3H]thymidine (Morshead et al., 1994).

![Figure 1](image.png)

**Figure 1.** Sagital section showing the rostral migratory steam in adult mouse brain. New neurons born in the subventricular zone (SVZ) lining the lateral ventricle (LV) migrate to the olfactory bulb (OB) through the rostral migratory stream (RMS). In the RMS, migrating neuroblasts form chains and they are surrounded by a glial tube. Blood vessels running parallel to the RMS provide additional scaffolding for migrating neuroblasts. Abreviations: CC, corpus collosum; Str, Striatum; A, type A neuroblasts; G, glial tube; V, blood vessels (adapted from Sun et al, 2010).
NPC’s can be isolated in vitro using the simple and robust colony forming assay called the “neurosphere” assay. In the presence of growth factors EGF (epidermal growth factor) and bFGF (basic fibroblast growth factor), individual stem cells generate clonally derived colonies of cells (neurospheres) that consist of a small minority of stem cells and the vast majority of progenitors (Reynolds and Weiss, 1992). The numbers of neurospheres reflects the size of the neural stem cell pool. Neurospheres can be dissociated into single cells and re-plated to form secondary spheres in the presence of growth factors, reflecting their self-renewal capacity (Morshead et al, 1998). Finally, individual neurospheres plated in differentiation conditions can generate mature cells comprising all three lineages (neurons, astrocytes and oligodendrocytes) thereby displaying multipotentiality (Reynolds and Weiss, 1992).

1.2 Cell Cycle and Expanding the Neural Stem Cell Pool

The eukaryotic cell cycle consists of four distinct phases: G1 phase, S phase (synthesis), G2 phase (interphase), and M phase (mitosis and cytokinesis). M phase is itself composed of two tightly coupled processes: mitosis, in which the cell's nucleus divides, and cytokinesis, in which the cell's cytoplasm divides forming two daughter cells. Activation of each phase is dependent on the proper progression and completion of the previous one. Cells that have temporarily or reversibly stopped dividing are said to have entered a state of quiescence called G0 phase.

Heterogeneity exists within the NPC pools, whereby stem cells can be found in an “activated”/proliferative cell state or a “quiescent”/less-mitotic state. Quiescence is described as a reversible resting state in which a cell can re-enter the cell cycle and proliferate as needed to generate downstream progenitors (reviewed in Li and Clevers, 2010; Roccio et al., 2013), and stem cells can become quiescent by exiting the mitotic cell cycle and entering the G0 state (Li and Clevers, 2010). Small changes in cell cycle kinetics can significantly change the total number of cells
generated. Based on the total period of neurogenesis and the number of divisions of precursors during this period (based on cell cycle time,) it has been estimated that a stem cell undergoes a total of 10–12 divisions (Takahashi et al., 1995). Increasing or decreasing the number of precursor cell divisions by even one will double or halve neuronal number, a significant change but a level of variation not normally seen.

Given that not all stem cells may be in an activated state, their population can generally thought to be expanded in number through three main ways: (1) by activating quiescent stem cell; (2) by enhancing symmetric division of a stem cell: (3) or by enhancing stem cell survival (Obernier et al, 2018).

1.3 Stem Cell States

Quiescent and actively dividing (activated) stem cells coexist in adult stem cell niches (Li and Clevers, 2010). Stem cell quiescence and activation play an essential role in many organs, underlying tissue maintenance, regeneration, function, plasticity, aging, and disease. Quiescent stem cells dynamically integrate extrinsic and intrinsic signals to either actively maintain their dormant state or become activated to divide and give rise to differentiated progeny (Cheung and Rando, 2013). Recently, novel features of the anatomical organization of the SVZ stem cell niche have been uncovered. GFAP+ type B1 cells have a radial morphology and span different compartments of the stem cell niche (Silva-Vargas et al., 2013). Their apical processes contact the lateral ventricle at the center of pinwheel structures formed by ependymal cells, exhibit a primary cilium, and are exposed to signals in the cerebrospinal fluid (CSF) (Doetsch et al., 1999a, Kokovay et al., 2012). Their basal processes contact blood vessels, which are an important proliferative niche in the adult SVZ (Shen et al., 2008; Mirzadeh et al., 2008; Lacar et al., 2011, 2012).
Various molecular markers have been used for the *in vivo* identification of SVZ stem cells and their purification (reviewed in Pastrana et al, 2011). Nestin and Sox2 are widely used as NSC markers in both the embryonic and adult brain (Lendahl et al., 1990; Graham et al., 2003; Kazanis et al., 2010; Imayoshi et al., 2011; Marques-Torrejon et al., 2013). CD133 (Prominin), a transmembrane glycoprotein expressed on primary cilia of neural progenitors (Uchida et al, 2000; Marzesco et al, 2005; Pinto et al, 2009; Cesetti et al, 2011) has been used to distinguish GFAP+CD133+ stem cells from niche astrocytes (Mirzadeh et al. 2008, Beckervordersandforth et al, 2010). Combinations of markers are beginning to be identified that allow the purification of different subpopulations of SVZ cells, in particular of activated stem cells, including epidermal growth factor receptor (EGFR) (Doetsch et al, 2002; Pastrana et al, 2009), and brain lipid binding protein (BLBP) (Giachino et al, 2014). In 2014, Codega et al. revealed that CD133+ astrocytes comprise two functionally distinct populations, quiescent NSCs (qNSCs) and activated NSCs (aNSCs), which differ in their *in vivo* cell cycle status and lineage kinetics, their *in vitro* colony-forming efficiencies, and in their molecular signatures (Codega et al, 2014). Thus by using mice that are endogenously labelled GFP+ for GFAP and looking at the double positive CD133+EGFR+ cells, one can determine the number of activated NSCs present.

### 1.3.1 Changes in Stem Cell Kinetics

Several factors can influence the kinetics of stem cells in the mammalian brain. For example, aged animals (20-27 months of age) show a two-fold reduction of cell proliferation marker BrdU+ cells compared to young adults (2-5 months of ages) (Piccin et al, 2011). Neural stem cells in aged brains also have longer cell cycle times. A change in transforming growth factor-α (TGFα) was proposed to be responsible for the age related changes in precursor cell kinetics specific to progenitors (Craig et al., 1996; Tropepe et al., 1997). In addition, a reduction in the
number of proliferating cells, fewer migrating NPCs and decreased neurogenesis is observed in both the dentate gyrus (DG) of the hippocampus (~90% reduction) and OB (~50% reduction) (Tropepe et al., 1997; Sommer and Rao, 2002; Salomoni and Calegari, 2010). It has been argued that these changes are cell intrinsic and may result from the accumulation of DNA damage during aging (Sharpless and DePinho, 2004; He et al., 2009; Wei et al., 2018). Moreover, changes in DNA methylation have been implicated in NPC quiescence (Rhodes et al., 2018).

This age-related reduction in neurogenesis is concomitant with an inclination of NSCs to form astrocytes at the expense of neurons in both neurogenic regions (Bouab et al., 2011; Encinas et al., 2011). These changes ultimately lead to functional impairments affecting memory (Hattiangady and Shetty, 2008) and odour discrimination (DG and OB, respectively) with age (Hamilton et al., 2013; Tatar et al., 2013; Bouab et al., 2011; Jin et al., 2003; Enwere et al., 2004).

In addition, several groups have discovered that the numbers of in vitro stem cell-derived colonies is reduced from aged brains which is concomitant with an overall reduction in the numbers of NPCs that migrate to the OB with aging (Kuipers et al., 2015; Ahlenius et al., 2009; Enwere et al., 2004; Luo et al., 2006; Tropepe et al., 1997; Piccin et al., 2014; Bouab et al., 2011). This finding sets up the question of whether neural stem cells die through age or whether the lack of neurosphere formation is due to increase quiescence (changing the ratio of quiescent:activated stem cell states). Support for increased quiescence with aging is the increased gene expression of cell cycle regulators, such as p16INKA4a in vivo (Molofsky et al., 2006; Nityanandam et al., 2012). Further, in vitro experiments using conditioned media from old and young SVZ cultures reveal that factors from the young SVZ niche can completely restore the numbers of neurospheres that are generated from the aged SVZ (Piccin et al., 2014). Indeed, the number of SVZ derived neurospheres from an aged brain exposed to a young environment is
identical to the numbers generated from a young adult brain (Piccin et al., 2014). These findings provide convincing evidence that neural stem cells are not lost with aging, but rather, their activity is modified by the microenvironment and this has important implications for neural repair strategies aimed at harnessing the potential of endogenous NPCs.

1.4 Regulating Symmetry of Division

Stem cells can divide symmetrically to generate two stem cells, thereby expanding the stem cell pool, or asymmetrically to give rise to a stem cell and a more lineage restricted progenitor cell, thereby permitting maintenance of the stem cell pool and the generation of differentiated cell types (Potten et al, 1990). The canonical Wnt signaling pathway (Wnt/β-catenin pathway) has been linked to stem cell expansion in several systems, including the hematopoietic system (Reya et al, 2003), and the embryonic nervous system (Kalani et al, 2008). The mechanism that underlies the increase in stem cell numbers has been difficult to establish as a change in the symmetry of division, cell cycle time, or cell survival, could all account for the expansion.

Under baseline conditions, adult neural stem cells divide asymmetrically and give rise to progenitor cells that proliferate and migrate to the olfactory bulb, where they differentiate into interneurons (Lois & Alvarez-Buylla, 1994; Morshead at al, 1998; Doetsch et al, 2003). A number of signaling pathways that regulate the switch and balance between proliferation and differentiation of NPCs have been identified including the Notch, Sonic hedgehog, fibroblast growth factor, TGF-β/Smads, and Wnt pathways (Chenn and Walsh, 1999; Rowitch et al., 1999; Hirabayashi et al., 2004; Joksimovic et al., 2009; Aguirre et al., 2010; Menendez et al., 2011; Rash et al., 2011). Wnt signaling pathways play crucial roles in neurogenesis (Kuwabara et al., 2009; Durak et al., 2016). For example, the canonical Wnt/β-catenin pathway is required for NPC self-renewal and differentiation (Chenn and Walsh, 2003; Kalani et al., 2008; Bengoa-
Piccin et al demonstrate that under baseline conditions in vivo (when stem cells are dividing asymmetrically), active Wnt signaling is rarely observed within the SVZ stem cells. However, when stem cells are dividing symmetrically, that is during SVZ regeneration, following stroke, or during neurosphere formation, the proportion of stem cells with Wnt signaling is significantly increased (Piccin et al, 2014). Moreover, blocking this Wnt signaling significantly reduces the number of stem cells. They also showed that increased Wnt signaling both in vitro and in vivo increased the numbers of neural stem cell-derived colonies (neurospheres) with no concomitant change in the proliferation kinetics of the progenitor population (Piccin et al, 2014). However, when individual sFRP2-treated neurospheres (an inhibitor that works as an antagonist to wnt3a) were passaged into control media, a significant decrease in the numbers of secondary neurospheres, relative to controls, was seen. Together, these findings support the role for Wnt signaling regulating stem cell number by promoting symmetric divisions.

As Wnt signaling regulates the mode of division of neural stem cells by promoting symmetric divisions and expansion of the size of the neural stem cell pool, the signaling is significantly decreased in the SE with age (Piccin and Morshead, 2011; Piccin et al., 2014). Indeed, studies have shown upregulation of the Wnt antagonist Dkk1 through aging leads to decreased self-renewal and reduced neurogenesis in vivo (Hirota et al., 2016; Seib et al., 2013). Delivery of Wnt signaling molecules (SB216763, a GSK-3b inhibitor, and Wnt3) (Piccin et al., 2014; Okamoto et al., 2014) leads to an expansion of the size of the NPC pool in both in vitro and in vivo studies, in both the SVZ and the DG (Hirota et al., 2016; Sakakibara et al., 2001). Together, these findings support the idea that neural stem cell quiescence during aging is not intrinsic, but rather, is niche-dependent and can be reversed following the exposure to a factor from the young niche or
rescued upon transplantation into a young niche (Galan-Moya et al., 2014; Okamoto et al., 2014; Piccin et al., 2014). Of course, the question remains as to whether the changes in Wnt signaling are intrinsic to SE cells, or whether Wnt expression changes in response to environmental cues within the stem cell niche.

1.5 Neural precursor cell markers

1.5.1 Sox2

SRY (sex determining region Y)-box 2, also known as Sox2, is a transcription factor that is essential for maintaining self-renewal and pluripotency of undifferentiated embryonic stem cells. Sox2 also plays a critical role in maintenance of neural stem cells. The Sox family of transcription factors share highly conserved DNA binding domains known as HMG (High-mobility group) box domains containing approximately 80 amino acids. In neurogenesis, Sox2 is expressed in cells comprising the neural tube, and is downregulated during cell differentiation (Graham et al, 2003). Cells expressing Sox2 are capable of both producing cells identical to themselves and differentiated neural cell types, two hallmark features of stem cells (Suh et al, 2007). Hence, in the adult mammalian brain, Sox2 is expressed in neural stem cells and their progeny.

1.5.2 Nestin

Nestin is expressed by many types of cells during development and continues to be expressed into adulthood in NPCs in the neurogenic regions of the CNS: the periventricular region and the dentate gyrus of the hippocampus. Nestin is an intermediate filament protein in undifferentiated cells and upon differentiation, nestin becomes downregulated and is replaced by tissue-specific intermediate filament proteins such as neurofilaments and glial fibrillary acidic protein (GFAP).
Nestin expression is induced in astrocytes following injury and is prominent in the glial scar. Nestin expression has been extensively used as a marker for NPCs, however it is only present in those NPCs that are actively dividing (Codega et al, 2014).

1.5.3 GFAP

Glial fibrillary acidic protein is an intermediate filament (IF) protein that is expressed by astrocytes as well as in ependymal cells during development. Outside of the CNS is it expressed in glomeruli and peritubular fibroblasts taken from rat kidneys, Leydig cells of the testis in both hamsters and humans, human keratinocytes, human osteocytes and chondrocytes, and stellate cells of the pancreas and liver in rats. GFAP is closely related to its non-epithelial family members, vimentin, desmin, and peripherin, which are all involved in the structure and function of the cell’s cytoskeleton. GFAP is thought to help to maintain astrocyte mechanical strength as well as the shape of cells, but its exact function remains poorly understood. GFAP has also been shown to be expressed in stem cells, and thus combined with other markers has become a way to delineate stem cells from astrocytes (Doetsch et al., 1999a, 1999b; Raponi et al., 2007).

1.6 Additional Neural Types and Markers

1.6.1 Doublecortin

Neuronal migration protein doublecortin (DCX) is a microtubule-associated protein expressed by NPCs and neuroblasts in embryonic and adult cortical structures. NPCs begin to express DCX while actively dividing, and once they stopped migrating and differentiating DCX is downregulated. DCX is also found in oligodentrocyte precursor cells and is thought to be a a marker of migrating cells. DCX is used as a marker of neurogenesis and has been shown to increase in response to external stimuli including exercise (for example). DCX expression often
occurs in parallel with increased bromodeoxyuridine (BrdU) or 5-ethynyl-2’-deoxyuridin (EdU) labelling, which is currently a "gold standard" in measuring neurogenesis.

1.6.2 Microglia

Microglia are a collective type of neuroglia found throughout the CNS. There are currently thought to be up to 9 subtypes of microglia with different functions and morphology (Hammond, 2018). Microglia account for 10–15% of all cells found within the brain (Lawson et al, 1994). As the resident macrophage cells, they are actively involved in the immunological response of neural tissue following injury or disease. Moreover, microglia are key cells in overall brain maintenance—they are constantly scavenging the CNS for plaques, damaged or unnecessary neurons and synapses, and infectious agents. One common marker of microglia is allograft inflammatory factor 1 (AIF-1), also known as ionized calcium-binding adapter molecule 1 (IBA1). AIF1 is a protein that exists in the cytoplasm, and it is highly evolutionarily conserved. IBA1 is a 17-kDa EF hand protein that is specifically expressed in blood-borne macrophages and microglia and is upregulated during the activation of these cells following nerve injury, central nervous system ischemia, and other brain diseases (Ito et al, 1998).

1.6.3 Oligodentrocytes

Oligodendrocytes are myelinating cells within the central nervous system (CNS) that form the myelin sheath of axons to support rapid nerve conduction. The oligodendrocyte marker O4 is an antigen on the surface of immature oligodendrocytes and has been commonly used as an marker specific for the oligodendroglial lineage.
1.6.4 Neurons

Neurons are electrically excitable cells that communicate with other cells via specialized connections called synapses. A neuron is the main component of nervous tissue. The most common neuronal marker used is beta tubulin III, also known as Tuj-1, which is a member of the beta tubulin protein family. Beta tubulins are one of two structural components that form the microtubule network and play a role in a wide range of cellular processes (mitosis, motility, etc). Beta III tubulin’s expression correlates with the earliest phases of neuronal differentiation. For this reason, beta tubulin III is correlated with neurogenesis and other processes such as axon guidance and maintenance (Caccamo et al, 1989).

1.7 The Role of Electric Fields During Development and Injury

Electric fields (EFs) play a key role during development and in the mature body. During development, endogenous EFs play a key role in morphogenesis and tissue formation, with disruption of EFs causing abnormalities such as the loss of eye formation and misshapen head development (McCaig et al, 2005). EFs during development can range from 10-20mV/mm (found beneath the neural plate ectoderm), to larger EFs of 1000mV/mm across the neural tube (Shi and Borgens, 1994; Shi and Borgens, 1995). Endogenous EFs are hypothesized to be created by ion concentration differences between the intracellular and extracellular space – driven by different pumps being expressed on apical versus basal sides of tissue. In the brain, the extracellular space may compose 20% of total brain volume (Nicholson and Sykova, 1998). The concentration of ions (K⁺, Na⁺, Ca²⁺, and so on) in the extracellular space contributes to the strength of the extracellular fields (Cao et al, 2013) The field can provide a built-in driving force to move ion currents through cells and organs, and these currents are integrally involved in all of our sensory systems (Reid et al, 2007). Electrogenic pumps such as Na⁺/K⁺-ATPases are
expressed at high levels at the superficial layers of the OB (Khayari, et al, 1990) and are localized to apical region (Cao et al, 2013). This would drive electric currents (flow of positive charges) outwards, generating a voltage sink in the OB. At the ependyma of the lateral ventricles, a layer of multi-ciliated epithelial cells separates the SVZ from the ventricular lumen. The apical surfaces of these ependymal cells, including the microvilli, had little or no ATPase activity. Very strong ATPase reaction product was found in the slender processes terminating on the basal laminae of perivascular spaces (Cao et al, 2013).

The removal or reversal of these in vivo EFs causes significant developmental defects such as tail abnormalities and malformed limbs (Hotary and Robinson, 1992; Metcalf and Borgens, 1994; Shi and Borgens, 1994). For example, endogenous EF of around 20 mV/mm were measured in a 24 days old chick embryos and disruption of such field affected tail development structures. A similar EF was recorded in axolotl embryo, interference of which caused developmental abnormalities during neurulation but not gastrulation (McKasson et al, 2008). These examples demonstrate the importance of endogenous EF during different stages of embryo development. From the regeneration point of view, studies in cultured Xenopus embryonic neurons indicated striking orientational effects in such a way that applied EFs induced neurite sprouting and promoted the turning of the growth cones of extending neurons (Dineur et al, 1892). In addition, Hinkle et al. demonstrated that a low strength EF can induce amphibian neurite growth towards the negative pole in vitro and can control the bipolar orientation axis of developing muscle cells from spherical myoblasts (Hinkle et al, 1981).

Following development, EFs are maintained across epithelium through the transport of ions including sodium (Na⁺) and chloride (Cl⁻) (figure 2A). Tight junctions prevent leakage of ions and ions pass from the apical to the basolateral side and vice versa to create an EF. These EFs are
important to wound healing, as interfering with the EF can prevent wound closure (Zhao et al., 2006). EFs are created when charged particles are separated across cell membranes creating transmembrane electric potential differences, or across sheets of coupled cells in epithelium creating transepithelial potential differences (figure 2A) (Zhao et al., 2009). When an injury occurs, transepithelial potentials are disrupted. For example, a skin wound can disrupt the insulating cell barrier thus creating a net zero potential at the injury site. A new electric potential difference is then created between the injury site and the regions surrounding the wound (figure 2B). Depending on species, location of injury, distance from the wound edge, and time after injury, the strength of the EF can vary from around 0.6 to 200mV/mm (Barker et al., 1982; Nuccitelli et al., 2008; McGinnis et al., 1986). These injury-generated potentials are critical for directing the migration of cells at the wound margin towards the injury site and disrupting the wound generated EF prevents wound closure (Chiang et al., 1991; Zhao et al., 2006).

Figure 2. Creation of electric fields across tissue. (A) EFs are present across epithelium due to ions transported across tight junctions from the basolateral to the apical side and vice versa (Zhao et al., 2009). These EFs are disrupted when a wound occurs and another EF lateral to the epithelium is directed to the injury site (McCaig et al., 2005).

Before discussing more detailed examples of electrical fields and their control of development and regeneration, it is important to put the magnitude of these electrical signals into context. For example, the common technique of electroporation for drug or gene delivery into cells uses extremely large pulses of DC EF stimulation, with field strengths of roughly 100–500 V/mm.
(Mir et al, 1999). The fields strength of DC EFs that play physiological roles in development and regeneration are three or four orders of magnitude less than this (1–100 mV/mm).

### 1.8 Considerations for EF Application

Exogenous EFs are bioelectric fields generated from external power sources and typically applied to biological cells/tissues via electrodes. Currently, different kinds of electrical stimulation modalities are being applied in several experiments, *in vitro*. These include direct current (DC EF), pulsed (PEF), alternating current electric field (AC EF), oscillating magnetic flux induced electrical stimulation, and so on. Of them, DC EFs have gained significant attention among biomedical scientists and engineers, mainly for *in vitro* stimulation studies, as it can alter the migration and cell shape, apart from influencing the viability and proliferation of numerous cell types (Chao et al, 2007). Also, there are other ways of treating cells with EF such as capacitive coupling (CC) and inductive coupling (IC). Usually, in vitro DC EF setups are constructed from petri dishes or cell chambers with conductive electrodes (e.g. graphite, platinum wires or stainless steel) being placed directly in culture medium (Babona-Pilipos, 2011). Moreover, the electrodes can locally deliver the applied current between the anode and cathode accurately along a specified direction (Meng et al, 2011). Another experimental set up to apply EF to the cells or tissues is to custom-design an isolated chamber connected via agar salt bridges to external Ag/AgCl electrodes (figure 3).
Figure 3. Illustration of galvanotaxis chamber assembly for EF application and time-lapse imaging. The galvanotaxis chamber is the central Petri dish, in which the cells are plated. The media inside the central Petri dish housing the galvanotaxis chamber is filled with media and cells. The Petri dishes on either side of the galvanotaxis chamber are filled with SFM, and also contain the Ag/AgCl electrodes. These electrodes are connected to an external power supply, and bridging the three Petri dishes with agarose-gel bridges forms a complete circuit. (+) represents anode and (-) represents cathode (Babona-Pilipos et al, 2012).

Such a configuration can isolate the EF exposed cells from reactive faradaic products of electrolysis (hydrogen peroxide, hydroxyl and superoxide ions, or other free radical intermediates) generated from redox reactions occurring at the electrode-electrolyte interface (Banks et al, 2015). In addition, the adsorption of proteins on the electrodes leads to a reduction in the magnitude of the electrical stimulus. This occurs because the flow of electrons or current is impeded by the adsorbed proteins over time (Zhao et al, 2011).

Applied EFs have a number of properties that can be modified for application for tissue repair.

The optimal stimulation protocol depends on the application; however, the parameters of the current are important considerations. Unidirectional current has ions flowing in one direction and
the continued constant flow of these ions can cause chemical burns. Therefore, bidirectional currents which have a reversal of polarity, are a viable alternative. An alternating current has continuous flow with a reversal of polarity, while biphasic pulsed current has an interrupted reversal of polarity and flow of ions. As the exact mechanisms that facilitate wound healing are not well understood, utilizing these different stimulation protocols with additional research to harness the potential of the electric fields is essential (McCaig et al, 2005).

1.9 EF Induced Migration/Galvanotaxis

Adult-derived NPCs respond to EFs by migrating towards the cathode, a process called galvanotaxis. This EF-induced migration is specific to undifferentiated NPCs, and they respond within 15 minutes of application of a 250mV/mm EF (Babona-Pilipos et al, 2012). Differentiated progeny of NPCs (neurons, astrocytes, and oligodendrocytes derived from neurospheres in the presence of fetal bovine serum) do not migrate in the presence of an EF (Babona-Pilipos et al, 2011). Notably, the direction of migration varies between cell type and species, as human NPCs, mouse skin precursor cells, oligodendrocyte precursors, and astrocytes from other sources have been shown to migrate anodally, while at early stages of culture mouse bipolar hippocampal neurons with short processes and ESC-derived motor neurons can migrate to the cathode (Yao et al, 2008; Yao et al, 2009; Yongchao et al, 2014).

1.10 Electrosensitive Cells - Sensing the Electric Field

Ion channels and transport proteins are two traditional classes of ion-ophiric proteins. When cells are subjected to EF stimulation, both the ion channels and transport proteins are activated to contribute toward membrane polarization and a downstream cellular response. Specifically, calcium (Ca$^{2+}$) and sodium (Na$^+$) channels have been the most studied in terms of EF
application. Specifically, it has been shown that voltage-gated \( \text{Ca}^{2+} \) channels can be activated at the cathode-facing cell membrane to allow \( \text{Ca}^{2+} \) influx, which leads to the membrane polarization and cell migration (Mycielska and Djamgoz, 2004). The use of voltage-gated \( \text{Ca}^{2+} \) channel inhibitors reduces the cellular responses to EF stimulation (McCaig and Dover, 1989; Trollinger et al., 2002). Similarly, the EF-induced membrane polarization and ion channel/transport protein activation (\( \text{Na}^+ \), \( \text{Ca}^{2+} \)) alters the ion inflow/outflow (Mycielska and Djamgoz, 2004) and contributes to the disassembly of the cytoskeleton in response to EF stimulation (Borys, 2012; Rees et al., 1989; Mycielska and Djamgoz, 2004). Several other studies demonstrate that \( \text{Ca}^{2+} \) and \( \text{Na}^+ \) influx are detectable in EF stimulated cells (Shanley et al., 2006; Nesin and Pakhomov, 2012; Dube et al., 2012). It is evident however, that a single ion channel may not be sufficient to control the electrotactic response observed by some cells. For example, genetic abolishment of \( \text{Ca}^{2+} \) channels reduced electrotaxis, but could never fully inhibit the response, suggesting the existence of alternative signals as a compensating effect (Shanley et al., 2006). Previous studies argued that \( \text{Na}^+ \) may enter the depolarized cathodal pole of the cathodally moving cells, thereby pushing away the intracellular \( \text{Ca}^{2+} \) toward the anodal pole of the cells, which leads to actin depolymerization and myosin disassembly (Borys, 2012; Rees et al., 1989; Sheng and Kim, 1996).

1.10.1 EFs and Cell Polarization

Generally, EF stimulation is thought to polarize the signaling molecules at the cell membrane which induces an asymmetric activation of the signaling molecules and downstream cytoskeletal changes, resulting in the directed migration of cells which is termed “galvanotaxis”. (McCaig et al., 2005; Pullar et al., 2006; Li et al., 2008; Mycielska and Djamgoz, 2004). EF stimulation can also trigger the activation of EF sensitive receptors on the cell membrane and initiate the
signaling transduction to down-stream effectors, which ultimately determines cell behaviour including cell fate, survival, migration and/or proliferation. Membrane receptors such as epidermal growth factor receptor (EGFR) and acetylcholine receptor (AchR) are reported to be EF sensitive receptors (Wu et al., 2013; Zhang and Peng, 2011; Zhao et al., 2002a), whose activation by EF stimulation will initiate the intracellular pathways such as phosphatidylinositol-3-OH kinase (PI3K)/Akt, MAPK/ERKs, integrin, and Rho (Fukata et al., 2003; Etienne-Manneville and Hall, 2001; Li et al., 2012).

Cells undergoing cathodal galvanotaxis, such as corneal epithelial cells and keratinocytes, show asymmetric distribution of EGFR towards the cathodal membrane during migration (Zhao et al., 1999, 2002b; Fang et al., 1999). In contrast, cells that undergo electrotaxis towards the anode, including a human metastatic breast cancer cell line, reveal EGFR polarization to the anodal side of the electrotaxing cells (Wu et al., 2013). Although the mechanism of the differential redistribution of EGFR (cathodal vs. anodal) is still not clear, such EGFR repolarisation in different cell types may work as a switch to determine the cell cathodal or anodal migration in an applied EF. Previous studies also revealed that EGFR activation may be induced by an EF in a ligand-independent manner (Wolf-Goldberg et al., 2013).

Some of the chemical cues that direct neuronal migration are known, and these can differ according to the neuronal population (Hatten, 2002). In the tangential chain migration SVZ neurons to the OB via the rostral migratory stream, Slit proteins, ephrins and Eph receptors, and polysialylated NCAM have guidance roles, whereas neurons migrating from the germinal zones to the outer cortical layers along radial glial fibres initially use the extracellular-matrix molecule reelin as a guidance cue (Hatten, 2002; Ghashghaei et al., 2007). By contrast, in adult
neurogenesis, stromal-cell-derived factor 1α, which is transduced by the chemokine receptor CXCR4 on NPCs, directs neurons to sites of brain injury (Imitola et al., 2004).

Most cell migration in developing and damaged brain probably occur through tissues in which steady electrical signals exist. For example, there is a large voltage gradient across the wall of the *Xenopus* neural tube (between the central canal and the extracellular space) during early neurogenesis, when outward migration of neurons occurs (Hotary and Robinson, 1991). Epileptic seizure, stroke, ischemia, migraine and acute damage to the hippocampus, all induce extracellular electrical signals in the brain that persist for hours (Leiao, 1944; Jefferys, 1981; Hadjikhani et al., 2001; Strong et al., 2002; Reid et al., 2007). In addition to reading chemical gradients as molecular guidance cues, migrating neurons may also read the electrical 'language' in the confined extracellular spaces of brain. Newborn rat hippocampal neurons and progenitor neurons from the lateral ganglionic eminence both migrate cathodally and, for rat hippocampal neurons, this requires activation of both PI3K and Rho kinase (ROCK) as signalling elements. The receptor transduction mechanisms upstream of PI3K haven’t yet been explored. (Yao et al., 2008; Li et al., 2008).

Work have also been done looking at the cerebrospinal fluid (CSF) a source of guidance information. CSF flow may set up a chemical gradient of guidance molecules that directs the early emigration of neurons from the SVZ (Sawamoto et al., 2006). However, electrical signals might coexist in this area too. There are electrical gradients within the CSF, and the ependymal cells that line the CSF-filled ventricles of the brain and spinal cord also act as a selective ion-transporting epithelium with tight-junction-mediated electrical seals between the cells (Hornbein and Sorensen, 1972; Jarvis and Andrew, 1988; Lippoldt et al., 2000). It is speculated that electrical gradients along the ependymal lining layers might regulate the beating rate of
ependymal cilia and so control the establishment of gradients of guidance molecules within the flowing CSF (McCaig et al., 2009). In addition, an electrical gradient across the ventricular wall might act as a cue to regulate the axis of cell division or cell proliferation of neuroblasts (Song et al., 2002), or to initiate neuronal migrations from the germinal layers that line the ventricle. Again, the challenge is to outline respective roles for chemical and electrical cues and to determine their interactions.

Similar to neurons, astrocytes and Schwann cells undergo oriented growth in response to an EF, and Schwann cells migrate rapidly anodally in EFs as low as 3 mV/mm (Moriarty and Borgens, 2001; McKasson et al., 2008). Glial and neuronal cells are functionally integrated and the cells are connected by gap junctions. How the effects of an EF are integrated between neurons and glial cells is completely unexplored.

1.1 Membrane Potentials

1.1.1 Membrane Potentials and Cell Kinetics

Membrane potential (V_men) is the difference in electric potential between the interior and the exterior of a biological cell. With respect to the exterior of the cell, typical values of membrane potential, normally given in millivolts, range from –40 mV to –80 mV.

All animal cells are surrounded by a membrane composed of a lipid bilayer with proteins embedded in it. The membrane serves as both an insulator and a diffusion barrier to the movement of ions. Transmembrane proteins, also known as ion transporter or ion pump proteins, actively push ions across the membrane and establish concentration gradients across the membrane, and ion channels allow ions to move across the membrane down those concentration gradients. Ion pumps and ion channels are electrically equivalent to a set
of batteries and resistors inserted in the membrane, and therefore create a voltage between the two sides of the membrane.

Almost all plasma membranes have an electrical potential across them, with the inside usually negative with respect to the outside. The membrane potential has two basic functions. First, it allows a cell to function as a battery, providing power to operate a variety of "molecular devices" embedded in the membrane. Second, in electrically excitable cells such as neurons and muscle cells, it is used for transmitting signals between different parts of a cell. Signals are generated by opening or closing of ion channels at one point in the membrane, producing a local change in the membrane potential. This change in the electric field can be quickly affected by either adjacent or more distant ion channels in the membrane. Those ion channels can then open or close as a result of the potential change, reproducing the signal.

It has long been observed that Vmem levels are tightly correlated with cell proliferation-related events such as mitosis, DNA synthesis, and overall cell cycle progression. Resting potentials of various cell types fall within a wide range (generally −10 mV to −90 mV), and cells’ positions along such a Vmem scale generally correspond to their proliferative potential (Binggeli and Weinstein, 1996). Somatic cells that have a high degree of polarization (a hyperpolarized Vmem) tend to be quiescent and do not typically undergo mitosis (Sundelacruz et al, 2009).

1.11.2 Membrane Potentials and Stem Cell Kinetics

The ES effects on cell proliferation are widely reported and it includes both inhibitory and stimulating effects, depending on the cell type and exposure conditions (Sarimov et al, 2011). In several instances in vivo, cell divisions occur in the presence of DC physiological endogenous EF, such as, during morphogenesis of mammalian embryo, wound healing, or tumor formation. Similarly, a dose dependent increase in proliferation was observed in in vitro cultures, for
various cell types (HL-60 leukemia cells, Rat-1 fibroblasts and WI-38 diploid fibroblasts) exposed to low frequency EF (Wolf et al 2005). Under identical exposure conditions, unexpected DNA strand breaks in later hours were noticed - suggesting that the EF exposure caused a temporary mitogenic effect followed by a loss of DNA integrity. Hence, this study is an apt example validating that short-term exposures (<12 h) induce growth stimulation whereas prolonged exposures (>24 h) impede the advancement of the cell cycle by causing DNA damage (Wolf et al, 2005) Thus, exposing cells to EF for shorter duration is a viable option to harness its utility to enhance proliferation and differentiation. Another group came up with the inhibitory effects of intermediate frequency AC fields, termed as tumor treating fields (TT Fields) in preventing cancerous cell growth, in vitro and in vivo (Kirson et al, 2008). In a study aimed at promoting proliferation of adipose derived stem cells (ASCs), a 448 kHz EF stimulus, which is currently used in electrothermal capacitive-resistive electric transfer therapies, could activate proliferation without compromising the multipotentiality of ASCs to differentiate towards adipogenic, chondrogenic or osteogenic lineage (Hernandez-Bule et al, 2014). In the same way, studies have validated the effect of EF in prompting higher proliferation rates in adult stem/progenitor cells, however the exposure parameters and treatment protocols (intensity, type of EF, pulse duration, exposure time) were different in these experiments (Dubey et al, 2011; Liu et al, 2013; Griffin et al, 2005).

One possible explanation for the enhanced proliferation is the augmented convection of nutrients and soluble factors by electrokinetically driven flow (Yan et al, 2009) The applied EF can enhance the effective mass transport by either electrophoresis or electroosmosis. Under an applied EF, both these phenomena can aid in convection based transport of both charged and uncharged molecules. In addition EF causes a variety of proteins to re-distribute asymmetrically,
which in turn plays a critical role in determining the level of symmetry and orientation during cell division (Song et al, 2002). Unlike the randomly oriented cleavage plane of dividing cells in unstimulated cells, ES causes preferential alignment most often around 90 degrees to the field vector (Zhao et al, 1999).

1.12 Clinical Applications of Electrical Stimulation

A main type of electrical stimulation used clinically is Deep brain stimulation (DBS) – used as treatment for hyperkinetic diseases such as essential tremor, Parkinson’s disease and dystonia and has seen much success. It has also been applied for pain and major depressive disorder among other intractable disorders but its mechanisms of action are not well characterized. As previously mentioned, one hypothesis is that DBS can inhibit neurons and also activate axons to work at local and network levels. It is further suggested that astrocytes could be activated and increase cerebral spinal fluid or calcium and glutamate (Fenoy et al, 2013). Vedam-Mai et al. (2014) reported an increase in proliferating NPCs based on an increase in markers for proliferating NPCs in human patients that underwent DBS for Parkinson’s disease. However, although this hypothesis that DBS is thought to modify the balance of inhibitory and excitatory activation of neurons exists (Kringelbach et al, 2007; Chiken et al, 2016), its precise mechanisms have yet to be elucidated. In terms of cell-specific effects, it has been proposed that astrocytes play a key role in DBS therapies by regulating neuronal activity and contributing to long-term potentiation and depression (Rueger et al, 2012). Interestingly, Stone et al (2011) found an increase in proliferation in the neurogenic region of the hippocampus at short survival times following DBS. Together, these findings highlight the enhanced neuroplasticity that results from EF stimulation of the brain.
Chapter 2

Rationale, Hypothesis, and Objectives

2.1 Rationale

Despite its broad use as a promising therapy, the effects of electrical stimulation on neural cells has not been fully explored. Herein we propose to examine the effects of EF application on the behavior of neural stem and progenitor cells in the periventricular niche of the adult forebrain. Previous work has demonstrated that adult derived NPCs are electrosensitive cells that undergo rapid and directed migration in clinically relevant electric field strengths. To determine whether the proliferation kinetics of neural stem cells was affected by electric fields, we used in vitro and in vivo assays to examine the size of the neural stem cell pool.

2.2 Hypothesis

Electric field application will lead to an increase in the size of the adult NPC pool.

2.3 Objectives

1) Examine the effects of electrical stimulation on neural stem cell expansion in vitro.

We will use the in vitro neurosphere assay to examine the size of the neural precursor pool and differentiation profile of resulting colonies. The number of neurospheres will be assessed at various times post-stimulation, from primary SVZ cultures and passaged neurosphere derived cells.

(2) Examine the effects of in vivo electrical stimulation on the proliferation of endogenous NPCs and cells within their niche.
We will examine the numbers of neurospheres that are derived from the SVZ of adult brains following in vivo stimulation. The numbers of proliferating cells and the differentiation profile of cells will be examined in vivo using immunohistochemistry.

(3) Explore the mechanism of expansion of the NPC pool in response to electrical stimulation.

We will determine whether EF application leads to a change in the stem cell “state” (i.e. quiescent versus activated) or a change in the mode of division (symmetric versus asymmetric division) to effectively expand the size of the neural stem cell pool.

Chapter 3
Materials and Methods

3.1 Ethics Statement

Animal work was approved by the University of Toronto Animal Care Committee in accordance with institutional guidelines (protocol no. 20011279). Surgeries were performed on C57/BL6 mice aged 7-11 weeks (Charles River).

3.2 Electrode Construction Materials

Design and 3D Printing of Electrode Base (in collaboration with Taylor Morrisson and Dr. Hani Naguib)

The 3D printed electrode base was designed in SOLIDWORKS 2017. It consists of a 4.5 mm by 5 mm square ranging from 3 mm high at the four corners of the square base to 2.39 mm at the
bisector of each edge. The top face of the electrode base is level, while the bottom is concave as a result of each of the face edges having a slight inwards arc to better conform to the shape of the mouse skull. Two parallel cylindrical holes, each 2 mm in diameter, cut through the center of the printed part to create an open passage between two opposite side faces. The center axes of the cylinders are spaced 2 mm apart. Temporary features were added to the design to improve its printability. These parts can be easily removed after printing. The electrode base was oriented upside-down, with the level face facing downwards. Small columns (0.7 mm diameter) were placed on each corner of the part, extending downwards 2 mm to meet a 2 mm thick support layer. This support layer connects 4 electrode bases to form a 2 by 2 array within a single continuous piece.

3D printing was performed with the desktop stereolithographic 3D printer Form 2 by Formlabs Inc.. In a typical print, the 4-electrode base array was duplicated 8 times and arranged into a 3 by 3 array with 5 mm spacing between pieces, totalling 36 individual electrode bases per print job. The liquid photopolymer resin “Clear” was selected for its suitability for higher resolution prints. Specifically, “Clear” resin version 4 (FPGA04) was used. The resolution was set to the maximum allowable value of 0.025 mm. Under these settings, the print takes approximately 3 hours to complete.

Part Assembly

Approximately 5 mm of hard uncoated platinum wire 0.127 mm in diameter (AM Systems Inc.) was cut from the roll. The thinnest section at the end of a standard machine pin was removed with wire cutters. The cut machine pin and wire were soldered together by carefully transferring a small amount of solder from the end of a hot soldering iron to the machine pin tip as the wire was held in place against it extending straight out from the center axis of the pin. This process
was repeated a second time to produce 2 pin-platinum connectors per electrode base. The connectors were inserted into the electrode base by feeding them through the printed holes platinum end first. The machine pin end, which the electrode base has been designed to fit exactly, locks the connector in place when it is fed through the hole. The extruding platinum wires were bent 90 degrees towards the arched face of the electrode base to be perpendicular to the pins. Spare machine pins were inserted into the electrode pins and the electrode was balanced on these pins so that the platinum wires were horizontal. A small amount of super glue (LePage Super Glue – Ultra Liquid Control) was spread across the face of the electrode where the pins and wires meet to secure the wires in place and provide insulation on the conductive parts that will be exposed to the mouse after the electrode is surgically inserted. The glue was left to dry for at least 3 hours. Finally, the platinum wires were cut to be 2 mm long, as measured from the corners of the nearest electrode base edge rather than the center of the arc. The spare machine pins were removed.

3.3 Electrode Implant Surgery

Animals were placed in a stereotactic apparatus and an incision was made along the scalp’s midline. The skull surface was dried using a cue-tip to ensure the electrode would stick in place. Using a dental drill (#77, 0.018”, 8177, Kopf), two holes were drilled at (anterior +0.8, lateral -0.7, and -2.7, relative to Bregma). Just prior to being inserted, the electrode position was verified to ensure it was right above the two drilled holes. Insta-cure+ cyanoacrylate glue (Bob Smith Industries) was used to secure the electrode in place. The electrode was then positioned above the two drilled holes and lowered into the brain with forceps and pressed onto the electrode to apply pressure to secure the electrode to the skull. Once the electrodes were in place, the scalp was sutured closed with 4-0 sterile silk sutures.
3.4 Electrical Stimulation Paradigm

Beginning two days after electrode implantation and cell transplantation, mice received electrical stimulation. The implanted electrode was interfaced with a biphasic electrical stimulator for the duration of stimulation, and mice were anesthetized with 1.5-2.5% isoflurane during stimulation. Stimulation pulse parameters were under: 200 µA amplitude, 50-500 µs pulse width, 285 Hz pulse repetition frequency similar to our previous *in vitro* report (Babona-Pilipos et al., 2015). It has been shown that neuronal activity is suppressed at these high frequency settings (Durand et al, 2006). Following each stimulation session, mice were returned to their cages and sacrificed 24hr or 72hr following stimulation. For tissue analysis, mice were transcardially perfused with 4% ice cold paraformaldehyde or cervically dislocated for the neurosphere assay.

3.5 Neurosphere Assay

NPCs were isolated and cultured as described previously (Babona-Pilipos et al., 2012). Briefly, adult C57/BL6 mice aged 7-11 weeks were sacrificed, and the periventricular regions of the brain were excised and enzymatically dissociated. Cells were plated in serum-free media (SFM) (Dulbecco’s modified Eagle’s medium:F12, 3:1; Invitrogen, Burlington, ON, Canada) supplemented with epidermal growth factor (20 ng/ml; Sigma-Aldrich, Oakville, ON, Canada), basic fibroblast growth factor (10 ng/ml; Sigma-Aldrich) and heparin (2 µg/ml; Sigma-Aldrich) – herein referred to as SFM+ EFH – at 10 cells/µl in T25 culture flasks (BD Biosciences, Mississauga, ON, Canada) [35,36] for the neurosphere assay or right onto prepared plates for expansion assay (see below). After 7 days in culture, primary neurospheres (passage 0) were collected and plated for galvanotaxis experiments or for differentiation. Neurospheres up to passage 2 were utilized for experiments. Passaging involved collecting neurospheres from 7 day cultures in a 15mL falcon tube, centrifuging at 1500 rpm for 3 min at room temperature,
removing the supernatant, dissociating into a single cell suspension and replating cells in fresh serum free media supplemented with EFH at clonal density (10 cells/ul) as described above.

3.6 *In Vitro* Stimulation Assay

Galvanotaxis chambers were modified based on previously published designs (figure 3, Babona-Pilipos et al., 2012). Briefly, square no. 1 glass cover slides (22 mm× 22 mm× 0.17 mm; VWR, Mississauga, ON, Canada) and 60 mm× 15 mm Petri dishes were exposed to UV light overnight, then cover slides were sealed to the base of the Petri dishes (VWR) using silicone vacuum grease (VWR). Plates were then sterilized with 70% ethanol for five minutes, followed by 3x5 min washes with sterile ddH2O. Five min prior to cell seeding, a norborenene/tetrazine gel was prepared by mixing 100uL 75% norborene/ hyaluronic acid with 100uL 75% tetrazine/hyaluronic acid. The gel was mixed via pipetting and 100uL was added to the glass cover slip. Cells were then resuspended in 100uL to make a final cell density of about about 500,000 cells/100uL. 50uL of cell suspension was then added to each gel coated glass cover slip in the plate. Two 15 cm long pieces of PVC tubing (2.38mm i.d., 3.97mm o.d.; Fisher Scientific, Mississauga, ON, Canada) were filled with 1.5% (w/v) agarose gel. Ag/AgCl electrodes were formed by coiling and immersing two 10 cm pieces of silver wire (Alfa Aesar, Ward Hill, MA, 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 separate pools of media on either side of the central chamber. The galvanotaxis chamber was transferred onto the stage of a temperature-controlled, carbon dioxide-controlled and humidity-controlled Zeiss Observer Z1 microscope (Zeiss, Oberkochen, Germany) for time-lapse imaging. Two 60 mm× 15 mm Petri dishes were placed on the stage – one on either side of the galvanotaxis chamber – and filled with 7.5 ml 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, University of Toronto, ON, Canada; see below) was connected to the Ag/AgCl electrodes for biphasic pulse application. Cells were electrically stimulated for 3 hours with 1 plate sitting in the incubated microscope chamber as a control. Cells were collected following stimulation and plated at clonal density of 10 cells/μL in supplemented SFM as previously described. Spheres were counted 7 days later, and binned into spheres between 50um and 80um, and spheres greater then 80um. For conditioned media experiments, media and cells were collected and filtered through a 70um filter following stimulation, and the resulting media was diluted 5 times with supplemented SFM. Media was added to non-stimulated cells plated at clonal density. The numbers of neurospheres was compared between cell cultures plated in media from stimulated and unstimulated conditions.

For passaged cell stimulations, the same protocol as above was used to stimulate neurosphere derived cells (from 7 day colonies). Briefly, neurospheres were collected and plated in EFH conditions and stimulated in vitro.

3.7 Differentiation

On day 7 of neurosphere assay, 48 well plates were coated with 25uL of laminin dissolved in 5mL SFM for 4 hours. 250uL of 1% FBS in SFM was then added to each of the 24 wells in the middle of the 24 well plate, and spheres larger then 80um were picked via P20 pipette and added to the filled wells. Care was taken to ensure no bubbles were formed in the process. Seven days following differentiation induction, cells were fixed with 4% PFA for 10 min, following 3x5min PBS wash. Cells were then triple stained using a protocol adapted from Balbona, Pilipos et al, 2011. Briefly, cells were washed 3 times with 1x PBS, blocked for 1hr at room temperature with
10% normal goat serum in 1x PBS, and incubated with O4 mouse monoclonal IgM 1:1000 (R&D Systems MAB1326) in 10% goat serum in 1x PBS overnight at 4 degrees celcius. The following day cells were washed 3x5 min with 1xPBS, and incubated with goat anti-mouse IgM 568 1:500 (Invitrogen A110440) for 1hr. To stain for neurons and astrocytes, cells were then washed 3x with 1xPBS, permeabilized with 0.3% tritonx-100 for 20min, and then blocked with 10% goat serum in 1x PBS for 1hr at room temperature and BIII tubulin rabbit polyclonal IgG 1:1000 (Biolegend Poly18020), and GFAP mouse polyclonal IgG 1:500 (Sigma G3893) in 10% goat serum in 1xPBS overnight at 4 degrees. On day three cells were washed 3x5 min with 1x PBS and incubated with goat anti-rabbit IgG 647 (Invitrogen A21245), and goat anti-mouse IgG 488 (Invitrogen A11001) for 1hr at 1:500 in PBS (Balbona-Pilipos, 2011). Images were taken as for N=10 spheres/condition from 3 independent experiments, with each experiment combining 3 mice.

3.8 EdU Pulsing and Immunohistochemistry

To prelabel proliferating cells over 24hr, cell proliferation marker EdU (50mg/kg) in PBS was injected intraperitoneally at the time of stimulation. Animals were perfused 24hr later for day 1 timepoints with ice cold PBS and then 4% PFA. Four hours later brains were transferred to 30% sucrose for 48 hours before sectioning. Brains were sectioned frozen on a cryostat (ThermoScientific HM525 NX) for a thickness of 20µm/section with 5 sections/slide. EdU labeling was performed using the click-it kit with the 647 azide (Thermofisher C10419) as per the manufacturers instructions. Antibody staining was done prior to EdU labeling. Sections were permeabilized with 0.3% triton-x 100 for 20min at room temperature, blocked with either 10% normal goat serum or 5% normal donkey serum in 1x PBS for 1 hour at room temperature, stained with primary antibodies Sox2, mouse polyclonal Ab, 1:1000 (AB97579), Iba1, rabbit
polyclonal Ab, 1:500 (019-19741, Wako), and DCX mouse monoclonal 1:400 (Sigma SC271390) cocktail antibody with GFAP, mouse Ab, 1:1000 (Sigma G3893) overnight at 4 degrees in blocking solution. Secondaries used were goat anti-mouse IgG 488 (Invitrogen A11001) for Sox2, goat anti-rabbit IgG 568 (Invitrogen, A11036) for Iba1, and donkey anti mouse IgG 568 (Invitrogen 10037) for DCX, all at 1:500 in PBS. Dapi (Invitrogen D1306) was used for nuclear staining (1:1000) in PBS for 5 min.

3.9 Image Analysis

Fifteen sections from three mice were analyzed for each investigation (three slides/mouse with 5 20µm sections/slide). Imaging was performed with an Olympus FV1000 laser scanning microscope at 20x magnification or 40x magnification to generate 20 µm-thick z-stacks. Images of the dorsolateral corner of the lateral ventricle subependyma within a 350µm² region of interest (ROI). The total number of nuclei (DAPI+) was counted and the number of labeled cells (DCX, GFAP, Sox2, EdU) were counted and expressed as a percent of total DAPI+ cells. Images were analyzed using Fiji Imaging Software (Shindelin et al, 2012).

3.10 Fluorescent Activated Cell Sorting

GFAP::GFP mice age 7-11 weeks were implanted with electrodes as described and stimulated at 250mm/mV for 1hr. Five mice were used for each stimulated and control group, and protocol was used as cited in Codega et al, 2014. Briefly, the SVZs from 2-3 month old heterozygous GFAP::GFP mice (The Jackson Laboratory), which express GFP under the control of the human GFAP promoter (Zhuo et al., 1997), or wild-type C57/BL6 mice (Charles River Laboratories) were dissected, digested with papain (Worthington, 1,200 units per 5 mice, 10 min at 37°C) in PIPES solution (120 mM NaCl, 5 mM KCl, 50 mM PIPES (SIGMA), 0.6% glucose, 1x
Pen/Strep (Gibco) in water, pH adjusted to 7.6) and mechanically dissociated to single cells after adding ovomucoid (Worthington, 0.7 mg per 5 mice) and DNAse (Worthington, 1,000 units per 5 mice). Cells were centrifuged for 10 min at 4°C without brakes in 22% Percoll (SIGMA) to remove myelin and incubated for 15 min with A647-complexed EGF (1:300; Molecular Probes) and biotinylated rat anti-mCD133 (1:300, clone 13A4, eBioscience), washed by centrifugation and incubated for 15 min with PE-Cy7-conjugated streptavidin (1:1000; eBioscience). All stainings and washes were carried out on ice in 1% BSA, 0.1% Glucose HBSS solution. To check cell viability, 4’,6-diamidino-2-phenylindole (DAPI; 1:1000; SIGMA) was added to the cells before sorting. All cell populations were isolated in a single sort using a Becton Dickinson Influx or FACS Aria II using 13 psi pressure and 100-µm nozzle aperture. Cells were collected in SFM with EFH and plated at clonal density of 5-10 cells/uL. Gates were set manually by using control samples. Data were analyzed with FlowJo 9.3 data analysis software and displayed using biexponential scaling. Analysis was done using FlowJo and gating strategy was done similarly to Codega et al, 2014.

3.11 Blocking Symmetric Division

To determine if electrical stimulation causes an increase in symmetric divisions of neural stem cells, 0.2µg/mL of recombinant mouse sFRP2 (R and D Systems, 1169-FR-025) was added to cells grown from spheres during 3hrs of stimulation (set-up as previously described). Cells were then re-plated in supplemented SFM at clonal density and grown for seven days, with sphere numbers counted and compared to non-stimulated cells with sFRP2, and stimulated and non-stimulated cells without sFRP2.
3.12 Statistical Analysis

All data are reported as mean ± standard error of the mean unless otherwise indicated. Statistical analysis was performed using GraphPad Prism 6 (GraphPad, La Jolla, CA). For comparisons between multiple groups, an analysis of variance (ANOVA) followed by Tukey's post-hoc test or Bonferonni post-hoc test, as indicated. For comparisons between two groups, a two-tailed unpaired Student's t-test was used. A p-value of less than 0.05 (i.e., 95% confidence) was regarded as statistically significant (*p < 0.05, **p < 0.01, ***p < 0.001).

Chapter 4

Results

4.1 Electrical Stimulation In Vitro Increases the Size of the Neural Stem Cell Pool

To examine the effects of EF application on neural stem cells, we used the in vitro neurosphere assay. In a first series of experiment, primary dissected tissue from the adult mouse SVZ was plated in a galvanotaxis chamber, and exposed to (i) no EF (control) or (ii) an EF of ~250mV/mm, for 3 hours at 37 degrees celsius and 5% CO₂. Cells were collected from the chamber and re-plated at clonal density (figure 4A). After 7 days in vitro, the numbers of neurospheres >80um in diameter were counted. We observed 1.9 ± 0.1 fold increase in the total numbers of neurospheres following EF stimulation (figure 4B) compared to unstimulated cells.
**Figure 4. In vitro** electrical stimulation increases neurosphere numbers. (A) Experimental paradigm showing stimulation from tissue dissected from SVZ. (B) The numbers of neurospheres was counted in unstimulated cells (stim off) and unstimulated (stim on) from primary tissue (contains niche and stem cells) and a 1.9 ± 0.1-fold increase in sphere numbers was seen following stimulation (N=4, p<0.0001). (C) Sphere counts of spheres between 50 and 80 um diameter (progenitor spheres) remain equivalent following stimulation (N=4, p=0.8). (D) Experimental paradigm showing sphere numbers from stimulating dissociated spheres grown from culture. (E) We observed a 2.1 ± 0.3fold increase in sphere numbers following stimulation of pure NPC populations (N=3, p=0.03). Statistical analysis was done with a two-tailed unpaired t-test.

To address whether EF application resulted in increased proliferation of progenitor cells, the numbers of colonies that were less than 80um in diameter was also assessed. Smaller colonies are not multipotent and passagable and are derived from progenitor cells (Piccin et al, 2011). Interestingly, the numbers of spheres less than 80um did not change in the presence of EF (figure 4C). This suggests that the effects of the EF are not simply due to increased proliferation of NPCs, but instead, the EF expands the size of the neural stem cell pool.

We performed two experiments to confirm whether the EF is acting directly on the NSCs or indirectly affecting the behavior of the NSC through a niche cell that was present in the primary culture. First, we stimulated primary cells grown for seven days to obtain a pure NPC population
(figure 4D). As seen during stimulation of primary cultures, 3 hours of EF resulted in a significant two-fold increase (2.1 ± 0.3) in the total number of neurospheres following EF between control and stimulated groups (figure 3E). The second experiment utilized the conditioned media derived from primary dissected tissue exposed to 3 hours of EF that was collected and plated onto passaged spheres-cells (grown in the absence of an EF) (figure 5A). We predicted that if a factor from the stimulated niche was responsible for the expansion of the stem cell pool, then the conditioned media would increase the numbers of neurospheres after 7 days in culture. We observed no increase in the numbers of neurospheres in the presence of conditioned media compared to control media (figure 5B). We performed the same experiment using conditioned media from spheres grown for 7 days that were exposed to 3 hours of EF, then the media was collected and plated onto passaged spheres that were not exposed to an EF (figure 5C). Again, we saw no increase in neurospheres grown in media collected from stimulated spheres (figure 5D). These results reveal that secreted factors from both niche cells and pure NPC cells are not sufficient to cause an increase in neurosphere numbers. Thus, the EF’s are acting directly on the NSCs to expand the in number.
Figure 5. Media collected from in vitro electrical stimulations does not increase neurosphere numbers. (A) Co-culture experimental paradigm where the conditioned media from primary cultures exposed to 3 hours of stimulation was collected and plated onto passaged spheres that were not exposed to stimulation. (B) Cells plated at clonal density revealed no increase in the numbers of neurospheres in the presence of conditioned media compared to control media (N=3 independent experiments, p=0.1). (B) Experimental paradigm using conditioned media from dissociated sphere stimulations numbers. (D) The numbers of neurospheres plated with media from stimulated dissociated cells remains the same compared to spheres grown in unstimulated media (N=3 independent experiments, p=0.6). Statistical analysis was done with a two-tailed unpaired t-test.

4.2 In Vitro Electrical Stimulation Results in Increased Neurogenesis From Neurospheres

To determine whether electrical stimulation alters the differentiation profile of neurosphere derived cells in vitro, we performed immunohistochemistry on EF stimulated and non-stimulated neurosphere cells. We predicted that more neurons would be formed as a percentage of all cells following stimulation as EF application has been shown to enhance neurogenesis in vivo (Ariza et al, 2010). Neurospheres from stimulated and non-stimulated cultures were collected and plated in differentiation conditions for 7 days, all the while ensuring that neurospheres were of equivalent size in each condition. This was further ensured by comparing spheres of equivalent DAPI+ numbers. Neurospheres from EF conditions gave rise to significantly more BIII tubulin+ cells compared to unstimulated neurospheres (figure 6A,B). Unstimulated cells generated 20.8 ± 1.5% neurons, 35.9 ± 3.5% astrocytes, and 4.5 ± 0.7% oligodendrocytes, while stimulated cells generated 28.1 ± 0.8% neurons, 26.6 ± 1.4% astrocytes, and 7.3 ± 0.5% oligodendrocytes. This resulted in a 1.75 fold increase in neurons following stimulation and a 1.67 fold decline in astrocytes following stimulation, Hence, EF’s are able to modify the differentiated profile of NPCs by promoting neurogenesis at the expense of astrocytes.
4.3 In Vivo Stimulation Expands the Size of the Endogenous Neural Stem Cell Pool

To determine the effect of electrical stimulation on the endogenous neural stem and progenitor cells within their niche in vivo, stimulating electrodes were implanted into the cortex (figure 7A, Bi,ii) with the cathode near the midline and the anode placed laterally. Stimulation pulse parameters were 200 μA amplitude, 50-500 μs pulse width, 400 Hz pulse repetition frequency, similar to optimized parameters previously reported for NPC migration in vitro (Babona-Pilipos et al., 2015). Mice were implanted with electrodes on day 0 and stimulation was applied for 1 hour on day 2. This stimulation time was chosen as previous work had shown increased proliferation using this acute paradigm (Stone et al, 2011). The
neurosphere assay was performed 24 hours post stimulation, keeping the contralateral and ipsilateral hemispheres, relative to electrode implantation, separate. Similar to what was observed following in vitro stimulation, we observed a $2.3 \pm 0.2$ fold increase in neurosphere numbers in the ipsilateral hemisphere after stimulation compared to the contralateral hemisphere (no electrode). The brains that only had the electrode but received no stimulation had no significant difference in sphere numbers on the ipsilateral hemisphere compared to the contralateral hemisphere. This is not significantly different from the in vitro expansion following stimulation. Notably, since injury alone can affect the proliferation kinetics of NSCs in vivo (Sachewsky et al., 2014; Obernier et al, 2018) we compared the ipsilateral and contralateral hemispheres, in stimulated and unstimulated mice, and found that electrode implantation alone did not lead to a change in the size of the neural stem cell pool (figure 7C) compared to the contralateral hemisphere of both stimulated and unstimulated mice. This further supports the hypothesis that the EF is changing the kinetics of the neural stem cells in vivo.
Figure 7. *In vivo* electrical stimulation increases neurosphere numbers. (A) Experimental paradigm. (Bi) image of the 3D printed electrode. (Bii) schematic of the coronal section through the forebrain indicating the electrode implantation. (+) indicates anode while (-) indicates cathode in electrode. (C) There is a 2.3 ± 0.2 fold increase in the numbers of neurospheres between the control (stim off) and stimulated (stim on) ipsilateral hemispheres. (D) The increase in neurospheres is maintained from 24 hours to 72 hours post-stimulation. No significant difference is seen between the expansion one day and three days post stimulation p = 0.07 as determined by an unpaired two-way t-test. N=5 mice/24 hour and 3 mice/72 hour group, one-way ANOVA (α=0.05) with Tukey's post-hoc analysis.

We then asked if the expansion of the neural stem cell pool persisted in the absence of an EF. Using the identical stimulation paradigm in vivo, we performed the neurosphere assay 72 hours after stimulation. We observed a significant 1.5 ± 0.08 fold increase in neurosphere formation in the ipsilateral stimulated versus unstimulated hemispheres. Again, there was no significant difference between the contralateral hemispheres and the unstimulated ipsilateral hemisphere indicating that injury alone (electrode implantation) was not sufficient for the expansion (figure 7D). The size of the expansion between 24 hours post stimulation and 72 hours post stimulation were not significantly different with a 2.3 ± 0.2 - fold difference 24 hours post stimulation and 1.5 ± 0.1 fold difference at 72 hours post stimulation (figure 7E). These findings reveal that an initial expansion of the NPC pool is maintained for at least 72 hours post stimulation. The trend towards less of an increase over time leads to the hypothesis that waiting longer following stimulation would eventually show a return to baseline neurosphere levels.

Given that neural stem cells in vivo generate neurogenic proliferative progeny, we asked if the expansion of the NSC pool was coincident with increased proliferation *in vivo*. We injected mice with the proliferation marker, EdU, at the time of stimulation and perfused mice 24 hours later (figure 8A). The total numbers of EdU+ cells in the dorsolateral corner of the lateral ventricle SVZ revealed a significant 2.2 fold increase in the numbers of proliferating cells (31.5±1.5 vs
69.0±1.9 EdU positive cells/350um², control versus stimulated) (N=28 sections from 3 mice) (figure 8B,C). We also counted the number of DAPI+ cells to ensure that the EdU+ cells survived past the S phase or did not get stalled in S phase. We found a 2.0 fold increase in the number of DAPI+ cells in the stimulated brains compared to unstimulated brains (272.7 ± 10.1 DAPI+ cells in stimulated mice vs 135 ± 5.5 DAPI+ cells in unstimulated mice) (figure 8C). To determine the phenotype of the proliferating cells we used Sox2 for stem and progenitor cells and found a significant increase in stimulated brains, with 17.1 ± 0.9% Sox2+EdU+ cells in unstimulated group and 25.4±1.5% Sox2+EdU+ of total DAPI+ cells in the stimulated group (figure 9A,B). This resulted in a significant 1.48 fold increase in the number of stem cells that are proliferating (p=0.008).

Figure 8. In vivo electrical stimulation increases proliferation in the SVZ. (A) Experimental paradigm. (B) Sections showing the dorsolateral corner of the lateral ventricle ipsilateral to electrode/stimulation. Dotted line indicate the area counted with white arrows indicating examples of cells that are both DAPI+ and EdU+. SVZ = subventricular zone, LV = lateral ventricle, CC = corpus callosum. (C) Stimulation resulted in a 2.2-fold increase was seen in number of EdU+ cells compared to unstimulated brains. (D)
There are 2.0 fold more DAPI+ cells in stimulated mice in the ROI. Scale bar=100um. N=5 mice, two-tailed unpaired t-test, p<0.0001.

Figure 9. A subpopulation of EdU+ cells are Sox2+. (A) Sox2 (green) co-localizes with EdU (red), insert shows zoomed in image of yellow rectangle, white arrows show co-localization. (B) There is a significant increase in the numbers of Sox2+ cells in the stimulated brains. Scale bar=100um, N=3 mice/group, two-tailed t-test unpaired t-test.

We then looked at the number of migrating cells using the marker doublecortin (DCX). We found a significant increase in both the total number of DCX+ cells following stimulation (16.6 ± 2.7 cells/350um² vs 29.2 ± 3.9 cells/350um², unstimulated versus stimulated mice, respectively) (figure 10A,B). There was also a significant increase percentage of total DAPI+ cells that were also DCX+EdU+ positive (8 ± 1.095% in unstimulated mice vs 20.6 ± 2.205% in stimulated mice) (figure 10C). Thus the electrical stimulation increased the number of dividing cells that are also migrating (difference is 12.126 ± 2.426%). As doublecortin is a marker of newly formed...
neurons it is possible that electrical stimulation is increasing the propensity for neuroblast formation.

Figure 10. A subpopulation of proliferating cells are neuroblasts (A) DCX(green) co-localizes with EdU (red). White arrows indicate co-localized cells. (B) There is a 1.7 fold increase in DCX+ cells in stimulated mice per 350um² region. (C) The relative percentage of DCX+EDU+ cells is 12.126 ± 2.4 % increase in stimulated versus unstimulated mice, showing that stimulation resulted in a 2.6 fold increase DCX+EdU+ cells in stimulated mice (p = 0.0009). Scale bar=50um, N=3 mice/group, two-tailed unpaired t-test.

Importantly, since we were not able to account for all of the proliferating cells following stimulation, we investigated whether EF for 1 hour in vivo was causing an inflammatory
response and activating microglia and/or macrophages. We stained for Iba1 and found no difference in the numbers of Iba1+ cells in stimulated versus unstimulated, implanted mice (58.67±3.756 Iba1+ cells/350um² versus 51.67±5.239 Iba1+ cells/350um², unstimulated and stimulated respectively) (figure 11A,B. This corresponded to 11.3±1.7% of total DAPI+ cells in unstimulated mice, and 12.7±1.8% of total DAPI+ cells in stimulated mice (figure 11C). Together these findings suggest that the EF induced expansion of the stem cell pool leads to increased neural stem cell progeny but does not lead to increased inflammation with the short, 1 hour application.

Figure 11. No change in Iba1+ cells is seen after stimulation. (A) Images of Iba1 and EdU do not show any increase of Iba1 positive cells following stimulation. White arrows show EdU+Iba1+ cells as indicated by yellow from green and red overlap. (B,C) There is no change in numbers (B) or relative percentages (C) of Iba1+ cells between unstimulated and stimulated groups (p>0.05). N = 3 mice/group, two-tailed unpaired t-test.
4.4 EF Application Does Not Activate Quiescent Stem Cells

The EF induced expansion of the size of the stem cell pool could be due to (1); (2) (3); (1) causing quiescent neural stem cells to enter into the cell cycle; (2) increased symmetry of division of the stem cells; (3) enhanced survival of the stem cells or a combination of these mechanisms. In a next series of experiments we asked whether electrical stimulation changed the relative numbers of quiescent versus activated stem cells. We used FAC sorting for quiescent and activated cell populations from GFAP::GFP mice using CD133 to delineate GFAP+ stem cells from GFAP+ astrocytes, and the expression of EGFR to identify the activated stem cell pool (GFAP::GFP+CD133+EGFR+ versus GFAP::GFP+CD133+EGFR-; activated versus inactivated) (Codega et al, 2014). We performed the same electrical stimulation paradigm (1 hour of stimulation at 2 days post-electrode implantation) and sacrificed the mice 17-24 hours later. We observed no difference in the relative percentage of activated versus quiescent cell populations in the stimulated and non-stimulated groups (figure 12A,B). Hence, these data suggest that the stimulation is not activating quiescent cells.
Figure 12. Stimulation does not alter the ratio of quiescent to activated stem cells in vivo (A) Contour plots showing cell sorts from the unstimulated and stimulated groups. (B) The relative percentage of activated and quiescent cells. There was no significance in the percentage of quiescent vs activated cells in stimulated off versus stimulated on groups. N=4 independent experiments with five animals per experiment. Two-way ANOVA (α = 0.05) a with Sidak’s post-hoc multiple comparison test. Gating strategy shown in appendix I.
To rule out the possibility that activating quiescent cells could account for the expansion of the stem cell pool in vitro, we generated spheres from GFAP::GFP mice and dissociated and sorted cells for activated and quiescent cells. Interestingly, we found that 100% of the NSC’s within a neurosphere are GFAP::GFP+CD133+EGFR+ activated NSCs (figure 13-C). Thus, activating quiescent cells does not account for the presence of increased numbers of neurospheres after in vitro stimulation.

Figure 13. NSCs from 7-day old neurospheres are % activated. (A) Timeline of sorting cells day 1 after replating. (B) Representative histogram from sorting cells isolated from GFAP::GFP mice and grown as spheres in culture for 7 days. (C) 100% of these cells were had the activated phenotype (GFAP::GFP+ CD133+EGFR+ cells). N=3 independent experiments, two-tailed unpaired t-test, p<0.0001. Gating strategy performed as in figure 12.
4.5 Blocking Wnt Signalling Inhibits the EF Induced Expansion of the Stem Cell Pool

Wnt signalling has been shown to promote symmetric division of NSCs (Piccin et al, 2011). We hypothesized that if the electrical stimulation induced expansion of the NSC pool is due to symmetric division of the NSC, then blocking Wnt signaling during electrical stimulation would inhibit the 2-fold expansion observed following in vitro electrical stimulation. We exposed neurosphere derived cells to the Wnt3a antagonist, sFRP2 during electrical stimulation and compared the numbers of neurospheres between stimulated and unstimulated cells without sFRP2 (figure 14A). We observed a 17.36 fold decrease in neurospheres in the presence of sFRP2 during stimulation compared to stimulation in the absence of sFRP2 (figure 14B). This suggests that NSCs undergo symmetric division in response to electrical stimulation and that this can account for the increase in the size of the neural stem cell pool.

A

B

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Figure 14. Blocking Wnt signalling inhibits the expansion of the stem cell pool induced by EF. (A) Experimental paradigm. (B) Fold change of sphere numbers relative to unstimulated cells without sFRP2. N = 3 independent experiments, one-way ANOVA (α=0.05) with Tukey’s post hoc analysis.

Chapter 5

Discussion

In this study we have examined the response of neural stem cells and their progeny, using in vitro and in vivo assays, following electrical stimulation to the cortex. Building on our previous findings that NPCs are electro-sensitive cells that can undergo rapid and directed migration in response to an EF application, we have examined the proliferation kinetics and differentiation profiles of NPCs following short EF application. We investigated whether electrical stimulation had direct effects on the NPCs to induce their proliferation of whether it was mediated from indirect effects from factors released from neighboring cells. We show that the NSC pool was expanded with stimulation and this is concomitant with an increase in the number of proliferating cells. Finally, we show that this expansion may be cause by increasing the number of symmetric divisions the NSCs undergoes, as blocking in wnt signalling involved in symmetric divisions removed the two-fold increase in sphere numbers following stimulation in vitro.

Neurospheres increased following both in vitro and in vivo stimulation, demonstrating that 250mv/mm for 3 hr (in vitro) and 1 hr (in vivo), more stem cells from the SVZ can be made. If galvanotaxis was required in vivo, additional stimulation parameters might be required. However because only 1hr of stimulation is required in vivo to duplicate the number of stem cells from the SVZ, it is not unreasonable to hypothesize different stimulation paradigms added on to move the NSCs to their desired location.
Differentiating these cells from the \textit{in vitro} stimulation showed a shift from astrocyte fate to neuronal fate following the stimulation. This is consistent with other reports showing that electrical stimulation increases neuronal differentiation (Dong et al, 2017; Dong et al, 2019; Lee et al, 2019). During quantification we counted the total numbers of DAPI+ cells and these were found to be equivalent between conditions. This strengthens the interpretation that there is a change of fate rather than increased survival of neuronal phenotype.

\textit{In vivo} stimulation showed increased neurosphere formation 24 hours and 72 hours following stimulation. This indicates that not only are more NSCs formed following stimulation, but this increase persists for three days following stimulation. \textit{In vivo} stimulation also showed that an increase in EdU+ cells following stimulation, and that a subset of EdU+ cells are sox2+, and DCX+. while an even smaller subset are Iba1+. It is possible that the total numbers of sox2+ cells increased following stimulation, and counting the numbers of those sox2+ cells in stimulated and unstimulated conditions would help determine whether this occurred. An increase following stimulation would indicate that electrical stimulation increased the total numbers of stem cells and would be consistent with the other results here. The finding that a small number EdU+DCX+ cells were observed is an indication that electrical stimulation is increasing the propensity for neuroblast formation. The low numbers of DCX+EdU+ cells could be due to the fact that we looked early post-stimulation, and many new borne neuroblasts may not have had time to express DCX which is a marker of migrating cells. Waiting three days, with EdU injected every 12 hours, may help identify the formation of new neuroblasts, and to see if more neuroblasts are formed following stimulation. Another explanation is that the newly formed cells never fully mature once generated, and ultimately undergo cell death. Lineage tracing using an inducible, Sox2CreERT2, mouse model enabling prelabeling of NPCs and examination of their eventual phenotype. Further, the Sox2CreERT2 mouse will enable tracking of migrating cells in
vivo in the presence of EF application.

We found many proliferating cells in the brain parenchyma. The EdU+ cells were identified as Iba1+ microglia/macrophages, as well as Sox2+ NPCs. Infiltration of blood born cells following disruption of the blood brain barrier following electrode implant would lead to neutrophils infiltrating the brain being labeled by EdU. Importantly, we determined that electrical stimulation, a potential therapeutic tool for regenerative medicine strategies, did not increase the immune response over electrode implantation alone.

The idea that stem cells in the adult brain exist in different states (quiescent versus activated) has come to the forefront in stem cell biology. Based on a current understanding of the markers used to distinguish these populations (Codega et al., 2014) we found no significant shift in the relative proportions of activated versus quiescent NSC populations following stimulation. Thus stimulation does not activate quiescent cells, and can be ruled out as the mechanism by which more neurospheres are seen following stimulation. One limitation to the FAC sorting is that CD133 (marker of stem cells) is expressed at very low levels in NSCs, which can lead to an underestimation of the NSC pool. An alternative marker such as VCAM1, which is expressed at more abundant quantities in NSCs, may be a more robust marker for the analysis. It remains possible that the stimulation could be decreasing the cell cycle time of the already activated stem cells thereby causing them to undergo more cell divisions in a given period.

However, our studies to explore the mechanism revealed that electrical stimulation was expanding the NSC pool by enhancing symmetric divisions. Modifying the mode of division (symmetric versus asymmetric division) has been shown to occur during development (Obernier et al, 2018) and has been shown to change during aging (Piccin et al, 2011; Piccin et al., 2014). Herein we used inhibitors of symmetric divisions, sFRP2 which directly binds Wnt protein
thereby antagonizing the endogenous Wnt signaling (Piccin et al, 2010), to examine the potential that EF induced expansion is due to enhanced symmetry of division. No increase in sphere number was seen in the presence of both wnt3a inhibitor sFRP2 and electrical stimulation, supporting the hypothesis that the increase in sphere number following stimulation is due to enhanced symmetric divisions. Another way to further validate this result is to measure the amount of LEF transcription factor (lymphoid enhancer-binding factor) following electrical stimulation using qPCR, which would be increased should wnt signalling be involved. To test whether electrical stimulation increases symmetric division in vivo, the Wnt inhibitor sFRP2 can be infused by intraventricular pump into the ipsilateral hemisphere of mice immediately before stimulation, and the neurosphere assay can be performed 24 hours later. If the electrical stimulation was increasing neurosphere numbers by a mechanism other then increasing symmetric divisions, no decrease in neurospheres would be observed.

Sorting spheres grown for seven days for activated vs quiescent markers show that essentially 100% of clonally derived cells are activated, and thus in vitro stimulation is not activating quiescent cells because they are all activated already. This result is reasonable as EGFR is used to to delineate activated stem cells from quiescent stem cells, and EGF is given in culture to these cells – thus it is expected that all the cells should be EGFR+. Again, additional NSC markers such as LEX (Morizeur et al, 2018), along with transcript signatures of activated stem cells, will provide a more sensitive analysis of mechanisms induced during stimulation.

Finally, another potential mechanism to enhance the NSC numbers is promoting survival of NSCs that were activated following electrode implant. Looking at cell death markers would help determine if this was the case. However, the percentages of DAPI+ cells were unchanged after stimulation as indicated by FACS sorting. DAPI is used as a cell death marker during sorting,
and thus the percentage of DAPI+ cells give a crude estimate of how many cells were dying. More DAPI+ cells in unstimulated groups would have indicated that stimulation might be promoting more cell survival. However as both cells from both conditions undergo the same processing, it is unlikely that any subtle effects from promoting cell survival could be seen during FACS. Another crude way to look at cell survival following stimulation could be to use ethidium homodimer following in vitro stimulation. Ethidium homodimer is a membrane impermeable fluorescent dye which binds to DNA. After a cell sample has been stained with ethidium homodimer, the dead cells may be viewed and counted under a UV-light microscope. When cells die, the plasma membranes of those cells becomes disrupted. Because of this, ethidium homodimer may enter those cells and bind to DNA within those cells and since live cells don't have a compromised membrane, the ethidium homodimer cannot enter. As this is a crude way to measure cell death, a significant decrease in ethidium homodimer positive cells following stimulation compared to unstimulated cells would indicate that cell survival is enhanced.

Chapter 6

Future Directions

Future directions for this work can be divided into three categories: (1) Design strategies for additional electrodes; (2) Mechanism studies for understanding how electrical stimulation promotes NPC proliferation; and (3) Clinical relevance such as advancing treatments for Parkinson Disease and following stroke.
6.1 Design Strategies

6.1.1 Flexible Electrodes
To further optimize the stimulation parameters, flexible electrodes could be designed to lessen the amount of strain placed on the brain. If the device remains in the nervous tissue a chronic inflammation or other damage response will follow. A device can be secured onto the skull but micro-movements of a stiff electrode can affect the environment, causing blood brain leakage and be detrimental to neuronal regeneration while a flexible device would reduce the chronic damage (Du et al, 2017). However, the implantation of a flexible electrode would have a more complex implantation procedure as it would be hard to keep the implanted device in the right position in the brain tissue. Current designs for flexible electrode implantation include guide needles with dissolvable contacts to release the electrode once in place and microfluidic devices to prevent the electrode from causing damage upon insertion or during movement (Kozai et al, 2014; Gopalakrishnaiah et al, 2017; Vitale et al, 2018), and would give additional options for insertion location. Studies are moving forward to determine the best materials for electrical stimulation, with many of these studies look for materials for potential electrical recordings. Materials that have too high of an impedance for stimulation could be used for recording.

6.1.2 Neuroelectronic Interfaces for Measurements and Stimulation
To investigate endogenous and applied exogenous EFs in vivo careful design considerations must be made. These include but are not limited to chemical composition, topography, surface chemistry, electrode dimensions, stiffness and implantation procedure (Wellman et al, 2018). Current stimulation electrodes often use platinum, gold, palladium, iridium and stainless steel because of their general biocompatibility (Merrill et al, 2005). Immediately after implantations of microelectrodes an injury is created, the blood brain barrier is disrupted causing changes to the
vasculature, a glial scar is formed, and the microglia/macrophages react to the foreign body. Smaller electrode dimensions create smaller damage sites which is beneficial in minimizing damage. Finally, nanoelectrodes with a smaller surface area are an option that are currently being explored as these would also minimize tissue, however these bring the additional concern of the cell internalizing the device (Bussy, 2015).

6.2 Mechanistic Studies

6.2.1 Changes During Aging

A number of studies support the hypothesis that changes in the stem cell niche underlie differences in the NPC populations observed through aging (Piccin et al., 2014; Piccin and Morshead, 2010; Conboy et al., 2005; Villeda et al., 2011; Katsimpardi et al., 2014), so performing the electrical stimulations in older mice would be interesting to see if the EF induced cell NPC proliferation can enhance the NPC numbers in aged mice similarly to what is seen in mice that are two months old.

6.2.2 Optogenetic Electrical Stimulation

Several groups have described techniques for optically stimulating specific populations of excitatory neurons and inhibitory interneurons in vivo in combination with electrophysiology. Cell type selectivity is obtained using Cre-dependent expression of the light-activated channel Channelrhodopsin-2 (Debelke et al, 2017). These optogenetic techniques provide a spatially and temporally precise means of studying neural activity in the intact brain and allow a detailed examination of the effect of evoked activity on the surrounding local neural network. Injection of viral vectors requires 30–45 min, and in vivo electrophysiology with optogenetic stimulation requires 1–4 h (Debelke et al, 2017). These studies avoid the potential damage
caused by implantable electrodes, and thus untangle the confounding effects of damage caused by implants with the electrical stimulation induced effects.

6.2.3 Membrane Potentials and Cell Kinetics

Membrane potential (V\text{mem}) levels are tightly correlated with cell proliferation-related events such as mitosis, DNA synthesis, and overall cell cycle progression. Resting potentials of various cell types fall within a wide range (generally $-10$ mV to $-90$ mV), and cells’ positions along such a V\text{mem} scale generally correspond to their proliferative potential (Binggeli et al, 1986). Somatic cells that have a high degree of polarization (a hyperpolarized V\text{mem}) tend to be quiescent and do not typically undergo mitosis. Conversely, developing cells and cancerous cells tend to have a smaller degree of polarization (a depolarized V\text{mem}) and are mitotically active (Binggeli et al, 1986; Cone et al, 1971). In most systems, potassium (K\text{+}) flux changes resulting in depolarization favor proliferation, although there are cases where depolarization inhibits proliferation. However since K\text{+} flux can be very hard to measure, knocking out Kir (inward rectifier K\text{+} channel) or other membrane channels and stimulating NPCs could give evidence of mechanism. When K\text{+} currents were suppressed, resulting in V\text{mem} depolarization, ectopic induction of the neural crest regulator genes Sox10 and Slug was observed. A functional role was identified for the channel KCNQ1, in melanocytes whereby V\text{mem} regulated the mitotic and invasive activity of the melanocyte neural crest lineage. The channel KCNQ1 could be an additional target to modify to investigate the mechanism behind how electrical stimulation affects NPC kinetics.
6.2.4 Chemical Probes

Another approach to membrane potential measurements is the use of voltage-sensitive fluorescent dyes. Several of these dyes are thought to operate by an electrochromic effect, where the dye spectra are altered due to the coupling of molecular electronic states with the electric field present in the membrane, or an electrophoretic effect, where distribution of the dye across the membrane is voltage-sensitive (Millard et al, 2004; Plasek et al, 1996). These dyes typically respond to membrane potential with sensitivities of 10% per 100 mV (Loew, 1992; Millard et al, 2004). Advantages of optical detection of $V_{\text{mem}}$ changes include ease of use, simultaneous monitoring of many cells over many different regions, and the ability to resolve spatial differences over the surface of a single cell (Plasek et al, 1996; Gonzalez et al, 1997; Stuart et al, 2006). Voltage-sensitive dyes also facilitate $V_{\text{mem}}$ measurements in small cells or structures (such as the thin dendritic processes of neurons) that are traditionally difficult to impale or patch with electrodes (Stuart et al, 2006). Most data are reported as percentage changes in fluorescence over a basal fluorescence value and are sometimes converted into an estimated membrane potential value based on reported dye sensitivities (Stuart et al, 2006). Ratiometric imaging using fluorescence resonance energy transfer (FRET) between a mobile voltage-sensing dye and a membrane-bound fluorophore can improve voltage sensitivity, reduce experimental error, and provide information about the magnitude of the voltage change (Gonzalez et al, 1997) and thus add to mechanisms behind EF induced proliferation.
6.3 Clinical Applications

6.3.1 Applications for Parkinson’s Disease

After decades of DBS studies, some questions remain regarding its mechanisms. Several studies suggest that DBS can either excite or inhibit individual neurons, depending on whether electricity hits a particular cell’s body or its branches (McIntyre et al, 2004). Across a population of neurons, these effects can activate or suppress a brain area and influence activity in connected regions. There are also signs that DBS induces supporting brain cells to release neurochemicals (Ashkan et al, 2017) or alters the brain’s vasculature (Pienaar et al, 2015). How these various effects lead to improved health remains unclear and likely differs across disorders.

Although post-mortem studies have found increased neural precursors in the subventricular zones of patients with chronic PD (Vedam-Mai et al, 2014), human trials to date have failed to show a direct disease-modifying effect of STN-DBS. Given that striatal dopaminergic dysfunction and neural loss are already well-established at the time of diagnosis, and that DBS is typically considered after 4 years of symptoms, it is perhaps unsurprising that surgical intervention has failed to produce any detectable disease modification in clinical trials (Jakobs et al, 2019). Moreover, PD patients enrolled in DBS trials are typically at that late stages of the disease, where the purpose of electrical stimulation is largely symptomatic (Jakobs et al, 2019).

The design of future clinical trials should include more sensitive biomarkers and functional assessments of PD neuropathogenesis, and intervention at earlier stages where neurons with terminals in the striatum could be saved. (Fischer & Sortwell, 2018). Mouse models can be used to stage different times of disease progression, detect more sensitive biomarkers, and test new treatments with functional assessments. Building on the work presented in this thesis will help with designing experiments to test if cell proliferation correlates with either disease progression
or biomarker detection. Finally, this work would also help understanding if endogenous NPCs could play a role in PD treatment by using the electrical stimulation paradigm developed here to promote NPC expansion – these newly formed precursors could then be targeted to help repair the damage induced by PD pathology.

6.3.2 Calculating the Volume of Tissue Activation

A lot of work had gone into trying to calculating the volume of tissue activation during deep brain stimulation. The volume of tissue activated (VTA), defined as the spatial spread of direct neural activation in response to electrical stimulation, is a measure that allows for a computational assessment of the impact of electrical stimulation (Butson and McIntyre, 2005, Orozco-Gutiérrez et al, 2018). The gold standard for VTA estimation is to couple the electric potential generated by the DBS electrode with a model of multicompartment axons arranged in a field around the electrode shaft (Yousif et al, 2012). Axons that, as a result of the stimulation, fire action potentials in a one-to-one ratio with the DBS pulses are considered active, and their spatial distribution defines the VTA. Simplified methods exploit the relationship between the spatial location of the axons and the electrical stimulation, captured in the form of activation threshold curves that are then used to estimate the VTA (Astrom et al, 2015). However, these curves do not reproduce the results of multicompartment models accurately and cannot be applied successfully when multiple contacts of the DBS electrode are active (Chaturvedi et al, 2013). Chaturvedi et al. proposed a solution by training an artificial neuronal network, with the DBS stimulation parameters as inputs and the elliptic profiles defined by the active axons as outputs (Chaturvedi et al, 2013). Once trained, the neural network can estimate the VTA for any combination of stimulation parameters. This method can also be applied to mouse experiments and thus can help determine how much volume the stimulation in this and other stimulation
paradigms is being activated. Doing so will help in the translation of mouse work to human work in deep brain stimulation and help with translation of mouse work to clinical models.

6.3.3 Applications for Repair Following Stroke

Stroke is the 2
nd leading cause of death in the world with societal costs of over $100 billion annually in America alone (Collaborators CoD, 2017; Gooch et al., 2017). While acute ischemic therapies aimed at clot removal have rapidly advanced in recent years, methods to improve brain tissue recovery in the subacute and chronic windows remain at the level of preclinical and clinical research (Oh and George, 2019). Various non-conductive biomaterials and conductive have been used to interact with the nervous system to deliver drugs or optimize cellular therapies (Bible et al., 2009; George et al., 2018a; Li et al., 2017; Nih et al., 2018; Zhong et al., 2010). One example of a conductive polymer, Polypyrrole (PPy) has been widely utilized for neural tissue engineering due to its reasonable conductivity (1–75 S/m) under physiological conditions (George et al., 2017; Oh et al., 2018). In addition, it has shown great biocompatibility in both in vitro and in vivo systems as well as the ability to manipulate tissue interactions (George et al., 2005; Fahlgren et al., 2015; Wang et al., 2004). PEDOT has gained recent use given its ability to form highly stretchable, conductive polymers (Kayser and Lipomi, 2019). This provides the means to adapt the conductive polymer to the changing biological surface. PANI is another conductive polymer providing the flexibility to interact with the CNS electrically (Farkhondehnia et al., 2018).

Neural stem/progenitor cells can be also delivered using conductive polymer for stroke recovery. Previously, it has been reported that an electrically conductive scaffold made of PPy allows for in vitro electrical stimulation and subsequent implantation of human neural precursor cells (hNPCs) onto the peri-infarct cortex (the damaged part of the brain following stroke). (George et
A short period of electrical stimulation prior to implantation changed gene expression in the hNPCs to improve functional recovery by modulating vascular endothelial growth factor A (VEGF-A) production. A recent study also demonstrated that directional currents in the brain mobilizes migration of transplanted hNPCs (Feng et al., 2017). These studies revealed that electrical stimulation provides a potential strategy to manipulate, facilitate, and guide stem cell therapy for stroke.

Chapter 7

Conclusion and Significance

7.1 Conclusion

In conclusion, electric stimulation at clinically relevant doses (250mV/mm) activates endogenous neural precursors in vitro and in vivo, and causes more neurons to be made at the potential loss of astrocytes when stem cells are differentiated in vitro. Mechanisms such as activating quiescent stem cells have been explored, although the exact mechanism(s) by which the NSC increase is far from being defined. Given the high number of electrical stimulations performed via deep brain stimulation, and the lack of information on how these stimulations might affect the NSC pool, this study’s findings demonstrate that stem cells are regulated by EF application.

7.2 Significance

NPCs hold great promise for tissue repair following injury or disease due to their fundamental properties (Didbajnia et al, 2013). There are two approaches that have been examined to harness the potential of these cells in regenerative medicine strategies which include (1) the activation of
resident NPCs (endogenous repair) and (2) the isolation of NPCs in vitro and transplantation in vivo (exogenous repair). Resident NPCs have been shown to migrate, proliferation and differentiate in the CNS following injury however their activation is not sufficient for brain repair and functional recovery (Jahanshahi et al, 2013). Thus strategies to promote the expansion, proliferation, and/or migration of these NPCs are a priority for regenerative medicine. This work shows that NPC expansion occurs following just one hour of electrical stimulation.

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Appendix

I. Gating Strategy for Sorting GFAP::GFP+CD133+EGFR- and GFAP::GFP+CD133+EGFR+ populations.
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