2015

Elucidating the Role of Endogenous Electric Fields in Regulating the Astrocytic Response to Injury in the Mammalian Central Nervous System

Matthew L. Baer
Virginia Commonwealth University

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Elucidating the Role of Endogenous Electric Fields in Regulating the Astrocytic Response to Injury in the Mammalian Central Nervous System

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University.

by

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July 2015
Acknowledgment

First and foremost, I would like to thank my wife Liz for her love and support, which has been a constant source of inspiration and motivation throughout this process. I am grateful for her begrudging acceptance of joint custody over my life that she has had to share with my research.

I would like to thank my parents and siblings for their encouragement and support, and for humoring my endless supply of scientific trivia at family gatherings. And I would like to thank my dear friends Michael, Caitlan, Wes, Colin, and Jackie, without whom I would have surely slipped beyond the realm of sanity long ago.

A special thank you goes to my mentor, Dr. Raymond Colello, who has been an insightful advisor, stalwart advocate, and friend. Over the years, Ray has allowed me the freedom to pursue research questions in which I took interest, and has simultaneously kept me focused on the larger project. His visionary approach to science has been an inspiration from the start, and his ability to communicate his ideas – both through presentations and in writing – has been invaluable to my training. His ability to balance his professional and personal lives is inspirational.

I would like to acknowledge, in chronological order, those people who have had a formative influence on my ability to think critically and on my passion for research: Meg Wastie for teaching me the importance of grammar and style; the University of Virginia School of Architecture for teaching me the importance of justifying every aspect of a new design; Dr. Daniel Willingham for his academic guidance and wisdom; and Dr. Michael Menaker for his mentorship and for teaching me the importance of humility in science: even the grandest and best planned experiments can end in utter catastrophe.

I would like to thank my graduate committee members, Drs. John Povlishock, Hadis Morkoç, Melissa McGinn Greer, and Dong Sun, for their guidance and support. I would also like to thank Dr. Scott Henderson, who acted as an unofficial committee member and has devoted an untold amount of time to training, counseling, advising, and supporting my work; and Dr. Roy Sabo, who has worked with me over 3 years to develop competence in the statistical analyses that I used.
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<th>Definition</th>
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<tbody>
<tr>
<td>2D</td>
<td>2 dimensional</td>
</tr>
<tr>
<td>°C</td>
<td>degrees Celsius</td>
</tr>
<tr>
<td>α</td>
<td>in statistics, probability of type 1 error</td>
</tr>
<tr>
<td>θ</td>
<td>direction (angle measure)</td>
</tr>
<tr>
<td>κ</td>
<td>concentration parameter (for circular statistics)</td>
</tr>
<tr>
<td>μ</td>
<td>mean (population parameter)</td>
</tr>
<tr>
<td>μA</td>
<td>microamperes</td>
</tr>
<tr>
<td>ρ</td>
<td>resistivity (i.e. electrical resistivity of the medium)</td>
</tr>
<tr>
<td>Ω</td>
<td>ohms (measure of resistance)</td>
</tr>
<tr>
<td>A</td>
<td>area</td>
</tr>
<tr>
<td>AC</td>
<td>alternating current</td>
</tr>
<tr>
<td>aCSF</td>
<td>artificial cerebrospinal fluid</td>
</tr>
<tr>
<td>AEC</td>
<td>apical ectodermal cap</td>
</tr>
<tr>
<td>Ag/AgCl</td>
<td>silver / silver chloride (type of electrode)</td>
</tr>
<tr>
<td>AMP</td>
<td>adenosine monophosphate</td>
</tr>
<tr>
<td>AMPK</td>
<td>adenosine monophosphate-activated protein kinase</td>
</tr>
<tr>
<td>AP</td>
<td>anteroposterior</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>BBB</td>
<td>blood brain barrier</td>
</tr>
<tr>
<td>BMP</td>
<td>bone morphogenic protein</td>
</tr>
<tr>
<td>BrdU</td>
<td>bromo-deoxyuridine</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>calcium ion</td>
</tr>
<tr>
<td>cm</td>
<td>centimeters</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>CO₂</td>
<td>carbon dioxide</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>CSPG</td>
<td>chondroitin sulfate proteoglycan</td>
</tr>
<tr>
<td>d</td>
<td>distance</td>
</tr>
<tr>
<td>DAPI</td>
<td>4',6-diamidino-2-phenylindole (DNA dye)</td>
</tr>
<tr>
<td>DC</td>
<td>direct current</td>
</tr>
<tr>
<td>dcEF</td>
<td>direct current electric field</td>
</tr>
<tr>
<td>dH₂O</td>
<td>deionized water</td>
</tr>
<tr>
<td>DIC</td>
<td>differential interference contrast</td>
</tr>
<tr>
<td>DIP</td>
<td>distal interphalangeal (joint)</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>DRG</td>
<td>dorsal root ganglion</td>
</tr>
<tr>
<td>DV</td>
<td>dorsoventral</td>
</tr>
<tr>
<td>ECM</td>
<td>extracellular matrix</td>
</tr>
<tr>
<td>EEG</td>
<td>electroencephalogram</td>
</tr>
<tr>
<td>EF</td>
<td>electric field</td>
</tr>
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<td>EGF</td>
<td>epidermal growth factor</td>
</tr>
<tr>
<td>EKG</td>
<td>electrocardiogram</td>
</tr>
<tr>
<td>FFT</td>
<td>fast fourier transform</td>
</tr>
<tr>
<td>FGF</td>
<td>fibroblast growth factor</td>
</tr>
<tr>
<td>GDP</td>
<td>gross domestic product</td>
</tr>
<tr>
<td>GFAP</td>
<td>glial fibrillary acidic protein</td>
</tr>
<tr>
<td>GTPase</td>
<td>guanosine triphosphatase</td>
</tr>
<tr>
<td>HCl</td>
<td>hydrochloric acid</td>
</tr>
<tr>
<td>HNO₃</td>
<td>nitric acid</td>
</tr>
<tr>
<td>hr</td>
<td>hour</td>
</tr>
<tr>
<td>Hz</td>
<td>hertz (cycles per second, unit of frequency)</td>
</tr>
<tr>
<td>I</td>
<td>current</td>
</tr>
<tr>
<td>IgG</td>
<td>immunoglobulin G</td>
</tr>
<tr>
<td>IgY</td>
<td>immunoglobulin Y</td>
</tr>
<tr>
<td>K⁺</td>
<td>potassium</td>
</tr>
<tr>
<td>ML</td>
<td>mediolateral</td>
</tr>
<tr>
<td>mL</td>
<td>milliliter</td>
</tr>
<tr>
<td>Acronym</td>
<td>Definition</td>
</tr>
<tr>
<td>---------</td>
<td>------------</td>
</tr>
<tr>
<td>MTOC</td>
<td>microtubule organizing center</td>
</tr>
<tr>
<td>mm</td>
<td>millimeters</td>
</tr>
<tr>
<td>mV</td>
<td>millivolts</td>
</tr>
<tr>
<td>nA</td>
<td>nanoamperes</td>
</tr>
<tr>
<td>Na⁺</td>
<td>sodium ion</td>
</tr>
<tr>
<td>Na⁺/K⁺-ATPase</td>
<td>sodium potassium ATPase</td>
</tr>
<tr>
<td>Naᵥ</td>
<td>voltage gated sodium channel</td>
</tr>
<tr>
<td>NCX</td>
<td>sodium-calcium exchanger</td>
</tr>
<tr>
<td>nF</td>
<td>nanofarads (measure of capacitance)</td>
</tr>
<tr>
<td>NPC</td>
<td>neural progenitor cell</td>
</tr>
<tr>
<td>OB</td>
<td>olfactory bulb</td>
</tr>
<tr>
<td>p</td>
<td>probability (for statistical evaluation)</td>
</tr>
<tr>
<td>P2</td>
<td>second day after birth</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>pH</td>
<td>measure of acidity (concentration of H⁺ ion)</td>
</tr>
<tr>
<td>PI3K</td>
<td>phosphoinositide-3-kinase</td>
</tr>
<tr>
<td>PKC</td>
<td>protein kinase c</td>
</tr>
<tr>
<td>PTEN</td>
<td>phosphatase and tensin homologue</td>
</tr>
<tr>
<td>RA</td>
<td>retinoic acid</td>
</tr>
<tr>
<td>RC</td>
<td>rostrocaudal</td>
</tr>
<tr>
<td>RMS</td>
<td>rostral migratory stream</td>
</tr>
<tr>
<td>SCI</td>
<td>spinal cord injury</td>
</tr>
<tr>
<td>SD</td>
<td>standard deviation</td>
</tr>
<tr>
<td>SEM</td>
<td>standard error of the mean</td>
</tr>
<tr>
<td>Shh</td>
<td>sonic hedgehog</td>
</tr>
<tr>
<td>SIK</td>
<td>salt-inducible kinase</td>
</tr>
<tr>
<td>SVET</td>
<td>scanning vibrating electrode technique</td>
</tr>
<tr>
<td>SVZ</td>
<td>subventricular zone</td>
</tr>
<tr>
<td>T75</td>
<td>cell culture plate with 75 cm² growing area</td>
</tr>
<tr>
<td>TBI</td>
<td>traumatic brain injury</td>
</tr>
<tr>
<td>TEP</td>
<td>transepithelial electrical potential</td>
</tr>
</tbody>
</table>
TGF-β  transforming growth factor β
TRPV1  transient receptor potential vanilloid 1
TNTP  trans neural tube potential
UV  ultraviolet
V_m  resting membrane potential
V  volts (i.e. electrical potential)
Wnt  wingless / int1 gene family
Abstract

Elucidating the Role of Endogenous Electric Fields in Regulating the Astrocytic Response to Injury in the Mammalian Central Nervous System

By Matthew Louis Baer, Ph.D.

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University.

Virginia Commonwealth University, 2015

Major Director: Raymond J. Colello, D. Phil.
Associate Professor, Department of Anatomy and Neurobiology

Endogenous bioelectric fields guide morphogenesis during embryonic development and regeneration by directly regulating the cellular functions responsible for these phenomena. Although this role has been extensively explored in many peripheral tissues, the ability of electric fields to regulate wound repair and stimulate regeneration in the mammalian central nervous system (CNS) has not been convincingly established. This dissertation explores the role of electric fields in regulating the injury response and controlling the regenerative potential of the mammalian CNS. We place particular emphasis on their influence on astrocytes, as specific differences in their injury-induced behaviors have been associated with differences in the regenerative potential demonstrated between mammalian and non-
mammalian vertebrates. For example, astrocytes in both mammalian and non-
mammalian vertebrates begin migrating towards the lesion within hours and begin to
proliferate after an initial delay of two days; subsequently, astrocytes in non-mammalian
vertebrates support neurogenesis and assume a bipolar radial glia-like morphology that
guides regenerating axons, whereas astrocytes in mammals do not demonstrate robust
neurogenesis and undergo a hypertrophic response that inhibits axon sprouting. To test
whether injury-induced electric fields drive the astrocytic response to injury, we exposed
separate populations of purified astrocytes from the rat cortex and cerebellum to electric
field intensities associated with intact and injured mammalian tissues, as well as to
those electric field intensities measured in regenerating non-mammalian vertebrate
tissues. Upon exposure to electric field intensities associated with uninjured tissue,
astrocytes showed little change in their cellular behavior. However, cortical astrocytes
responded to electric field intensities associated with injured mammalian tissues by
demonstrating dramatic increases in migration and proliferation, behaviors that are
associated with their formation of a glial scar in vivo; in contrast, cerebellar astrocytes,
which do not organize into a demarcated glial scar, did not respond to these electric
fields. At electric field intensities associated with regenerating tissues, both cerebellar
and cortical astrocytes demonstrated robust and sustained responses that included
morphological changes consistent with a regenerative phenotype. These results support
the hypothesis that physiologic electric fields drive the astrocytic response to injury, and
that elevated electric fields may induce a more regenerative response among
mammalian astrocytes.
Chapter 1: Physiologic electric fields regulate wound repair in the CNS

Dissertation Synopsis

This dissertation explores the hypothesis that physiologic electric fields (EF) regulate repair and regeneration in the mammal central nervous system, with a particular emphasis on their influence over astrocytes. This hypothesis is predicated upon three axioms: that the mammalian central nervous system (CNS) has a latent potential to regenerate that is largely a function of the astrocytic response to injury, that regeneration recapitulates the same physiologic mechanisms underlying embryogenesis and thus must be conserved in mammals, and that endogenous bioelectric fields regulate embryogenesis and regeneration by directly stimulating the underlying cellular behaviors. However, none of these axioms is taken for granted in this dissertation, and we thoroughly explore the existing literature underlying each supposition in chapter one. Through this discussion, it becomes clear that astrocytes determine the regenerative potential in the CNS, that EFs are well established in regulating embryogenesis and regeneration in peripheral tissues, and that EFs are also found in the CNS where they are similarly associated with embryogenesis. While this suggests that injury-induced EFs also regulate wound repair and regeneration in the CNS, it becomes equally clear that the role of physiologic EFs in the cellular response to CNS injury has not been explored.
Thus, the discussion in chapter one culminates in the overall hypothesis of this thesis: that physiologic EFs regulate tissue repair and the regenerative potential of the CNS by their influence on astrocytes. Chapters two and three detail studies investigating how electric fields regulate astrocyte behaviors associated with the injury response; we compare how EF effects differ between cortical and cerebellar astrocytes, and relate these differences to differences in regenerative potential and in phylogenetic origins between these two brain regions. In chapter four, we describe efforts to develop a protocol by which we can acquire longitudinal measurements of injury-induced EFs over the entire duration of wound healing in the mammalian CNS, which is a necessary prerequisite to designing an EF-based therapeutic strategy. In chapter five, we discuss the implications of our findings in the context of the larger hypothesis that endogenous bioelectric fields are a universal stimulus that regulates morphogenesis during both embryogenesis and regeneration in all metazoans. We discuss established criteria by which such a causal hypothesis is evaluated, and we explore how existing research and theory regarding regeneration, bioelectricity, and evolution supports this proposed causal relationship. Finally, we conclude by addressing the future experimental directions that are necessary to advance the therapeutic implications of these hypotheses.

**Historical context, epidemiology, and morbidity of CNS injury**

Injury to the CNS has fascinated human civilizations for millennia. People suffering from CNS disorders have often been treated as pariahs or have been presumed to suffer from demonic possession. These long-held beliefs often reflect a tragic misunderstanding of the brain and mind, and they have spawned countless
attempts to cure patients from their various afflictions. Many different cultures have embodied these fantasies of regeneration within their myths and folklore, as is evidenced by the Grecian myth of the hydra whose head regenerated two more upon amputation, by the Anglo-Celtic myth of Wayland the Smith who had the power to restore lost limbs that have undergone trauma\(^1\), by the Aztec legend of the god Xolotl who allegedly imbued the amphibian species Axolotl with the power to regenerate\(^2\), by Catholic biblical teachings of Jesus curing blindness and leprosy, by Old Norse poems about the “Mead of Poetry” that could revive the dead\(^3\), by the ancient Aryan drink called “Soma” that is described in poetry as a drink of immortality\(^4\), by the ancient Chinese goddess Woman Gua whose regenerative powers were so profound that her guts metamorphosed into ten different deities\(^5\), and in Nigerian Yoruba mythology by the regenerating serpent Oshunmare\(^6\). As early as eight millennia ago, cultures have taught that trephination – the practice of removing a portion of skull without damaging the underlying dura, meninges, or blood vessels – could treat epileptic seizures, migraines, and mental health disorders, with examples documented worldwide including among cultures from neolithic Europe (6500 BCE France)\(^7\), ancient China (5000 BCE)\(^8\), and Central America (900-1400 CE)\(^9\). Interest in disorders of the mind and behavior were also evident through a persistent obsession with epilepsy, which was presumed by the Babylonians, ancient Greeks, and Catholics to be caused by demonic possession. As science advanced through the Renaissance and into the modern era, there has been an increasing appreciation for the role of the brain as the seat of the mind and the source of behavior. The dualism between mind and body has gradually shifted from the realm of philosophy to that of science as our understanding of physiology has
progressed; the originally distinct fields of psychology and neurology have gradually
merged over the past 100 years as the neural circuitry and molecular signaling
underlying ever more complex behaviors continues to be elucidated. Yet in spite of the
persistent obsession with the role of the CNS in disorders of the mind and behavior, the
scientific understanding of how to cure them has been stubbornly intractable.

Each year, over 2.32 million Americans survive a CNS injury (an estimated 1.7
million patients suffer a traumatic brain injury (TBI)\textsuperscript{10-12}, 12,000 patients suffer a spinal
cord injury (SCI)\textsuperscript{13-17}, and 610,000 patients suffer a first-time stroke)\textsuperscript{18, 19}, which results
in direct costs for initial medical management of $45.0 - $73.4 billion annually (TBI:
$13.1\textsuperscript{11, 20, 21}$, SCI: $10.0 - $27.3 billion\textsuperscript{11, 22}$, and stroke: $21.9 - $33.0 billion\textsuperscript{11, 19}$).
Advances in acute management of these disorders have improved survival and initial
outcomes, but there is still no clear consensus on how to treat the resulting deficits and
recover lost function. Patients often develop significant deficits that require chronic care
and rehabilitation because the adult brain does not show a significant capacity to
regenerate itself after an injury. Consequently, up to 43% of patients discharged
following TBI hospitalization develop a long-term disability\textsuperscript{12}, and no more than 70% of
this population recovers sufficiently enough to return to work\textsuperscript{11, 23}; upwards of 81% of
patients surviving SCI are unemployed 1 year post injury, and 39.5% of patients remain
unemployed after 25 years’ recovery\textsuperscript{11}; and there is only a 50% 5-year survival rate for
patients suffering a stroke\textsuperscript{24}. There is a total prevalence of 10.78 million patients living
with the long-term consequences of these diseases (TBI: 3.2 – 5.3 million patients\textsuperscript{12};
SCI: 259,000 patients\textsuperscript{14}; and stroke: 6.27 million patients\textsuperscript{18, 19, 25}), which represents a
total of 3.38% of the estimated 319 million people living in the of the United States of
America in 2014\textsuperscript{26}; this enormous population of survivors results in indirect costs to American society through rehabilitation, supportive care, and lost productivity that are estimated at $84.1 - $94.9 billion annually (TBI: $63.9 billion\textsuperscript{12}; SCI: $3.7 billion\textsuperscript{14}; and stroke: $16.5-27.3 billion\textsuperscript{19})\textsuperscript{11}. As the total value of the US economy (GDP) is estimated at $16.768 trillion in 2013 as reported by the World Bank\textsuperscript{27}, CNS injuries represent 0.268\% - 0.438\% of GDP in direct costs, and 0.502\% - 0.566\% of GDP in indirect costs each year. (Note, all dollar values reported in this section are in inflation-adjusted 2013 values for the US dollar.) Thus, the gravity of this issue is enormous, and has led the United States Congress to pass the Traumatic Brain Injury Act in 2008\textsuperscript{28} that specifically authorizes research and public health activities related to TBI. As the impact of CNS injury is expected to grow substantially over the coming years, developing methods to promote functional recovery is imperative to improving the outcomes for these patients.

**Injury and regenerative potential in the CNS**

The CNS has evolved to perform extraordinarily complex functions that are of fundamental importance to the survival of the host organism – including regulating internal physiology, monitoring external stimuli, and generating complex motor programs – which requires the constant integration of diverse information. For instance, the visual system extracts information on colors, shapes, and locations to identify faces, avoid predators, or track prey; the auditory system extracts words from noise and can precisely localize sounds in 3-dimensions; the motor system plans and executes complicated behaviors and monitors the body’s position in space as these programs are executed. Neural processing for each component of a stimulus occurs in discrete nuclei throughout the CNS that are intricately interconnected through complex neural
networks. Any injury causing a disruption of these neural circuits, either by destroying cells within the nuclei where certain information is processed or by disrupting the axon tracts that connect discrete regions (Figure 1.1), can have profound functional consequences by interfering with the ability of the CNS to perform these fundamental activities. In mammals, plasticity in the remaining tissue often permits limited functional recovery, but these deficits are often permanent because the CNS does not demonstrate a robust ability to regenerate lost tissue.

In contrast to the absence of bona fide regeneration in the mammalian CNS, many non-mammalian vertebrates demonstrate robust regeneration that facilitates profound functional recovery after CNS injury. The difference in the regenerative potential between mammalian and non-mammalian vertebrates has been attributed to particular aspects of the injury-induced cellular response. In this section, we review the pathophysiology following injury to the vertebrate CNS, comparing the cellular responses inhibiting regeneration in mammals to those facilitating regeneration in non-mammalian vertebrates. Based on this understanding of the cellular behaviors necessary for successful CNS regeneration, we will explore the possibility that the mammalian CNS has a latent capacity to regenerate.

For the sake of the subsequent discussion, it is worth defining several terms involved in the injury response. As defined in one of the standard textbooks on medical pathology (page 100), Tissue “r]epair, sometimes called healing, refers to the restoration of tissue architecture and function after an injury. (By convention, the term repair is often used for parenchymal and connective tissues and healing for surface epithelia)… Repair of damaged tissues occurs by two types of reactions: regeneration
by proliferation of residual (uninjured) cells and maturation of tissue stem cells, and the deposition of connective tissue.” Organs are composed both of parenchyma, which refers to the functional tissue, and of stroma, which refers to the structural tissue; all organs are lined by an epithelium, which is a type of connective tissue that lines the surface of organs. Regeneration refers to the recovery of an organ’s function, which can refer to any of several pathways; for the scope of this dissertation, we are exclusively concerned with epimorphic regeneration, which is the recreation of the original structure and function of the damaged structure(s) through the proliferation of stem cells.

Pathophysiology of CNS injury inhibits regeneration in mammals

CNS injury induces a cellular response that protects the surrounding tissue from the damaging molecular milieu within the lesion site\textsuperscript{30-32}. The etiology of the primary injury initiating this secondary cellular response can be either hemodynamic or traumatic: hemodynamic instability from prolonged ischemia or infarction (i.e. a stroke) causes tissue necrosis through severe metabolic stress; mechanical impact from traumatic injury causes tissue necrosis and axon disconnection through physical damage to cell membranes, and also through traumatically-induced hemorrhage. Common to the pathophysiology of both hemodynamic and traumatic injury is a local disruption of the blood-brain barrier (BBB), the immunogenic response through which the necrotic tissue is removed, and the cellular response within the CNS that protects the surrounding healthy tissue by sequestering the immune response within the lesion site\textsuperscript{33, 34}. However, this initially protective cellular response to both hemodynamic and
traumatic injury ultimately resolves into the same maladaptive glial scar that becomes a physical and biochemical impediment to any spontaneous attempt at regeneration\textsuperscript{33, 35}.

Limited recovery can occur over time because adaptive plasticity allows the remaining neural circuitry in the adult CNS to assume some of the function of the tissue lost to injury, and communication between disconnected nuclei can be partially restored as it is rerouted through remaining fiber tracts\textsuperscript{36-39}. However, complete functional recovery requires restoring the original physiology of the damaged tissue, including both the local cytoarchitecture and the passing fiber tracts, through four basic functions (Figure 1.1)\textsuperscript{35, 40}: 1) each of the cell types present within the damaged tissue, including both neurons and glia, must be replaced and functionally incorporated into the remaining neural circuitry, 2) disconnected axons, including those projecting past the lesion from distant nuclei and those from newly-generated neurons within the lesion, must be capable of sprouting, 3) axons must sprout past the lesion and through the distal intact parenchyma, following local cues to their original target nuclei, and 4) axons must be able to reestablish their appropriate connections within their target nuclei. In the mammalian CNS, robust regeneration does not occur because these four fundamental functions are either absent or inhibited.

Although the mammalian CNS does not spontaneously regenerate, it does demonstrate a limited capacity for each of the cellular behaviors necessary for regeneration. The adult mammalian brain does contain neural stem cell populations that continue to divide throughout life, but they are only located in discrete regions and do not demonstrate the robust neurogenesis necessary for regeneration following an injury. Previous research has shown that axons have an inherent ability to sprout; however the
intact parenchyma of the mammalian CNS is not conducive to sprouting, as severed retinal ganglion cell axons cannot regenerate through the distal portion of the optic nerve but are readily able to sprout through a peripheral nerve graft\cite{41-44}. Furthermore, axons sprout towards the lesion after axotomy, but in vivo imaging demonstrates that they ultimately turn away and fail to regenerate after repeated efforts to pass the lesion\cite{45,46}. Nonetheless, axon terminals demonstrate local plasticity within their target nuclei that facilitates synaptic remodeling underlying both learning in the adult CNS and limited functional recovery after injury, which suggests that regenerating axons would be able to restore their original connections if they were able to regenerate past the lesion and reach their original targets\cite{36,47,48}. This abortive regenerative effort is due to a cellular response at the lesion site, which is initiated by BBB disruption and cellular injury, that is inhibitory to axon sprouting\cite{35,49-52}. However, the fact that each of the cellular behaviors necessary for regeneration is expressed following injury suggests that a latent and inducible capacity for regeneration may be conserved in the adult mammalian CNS.

BBB disruption at the lesion site allows an influx of neutrophils and macrophages that, while necessary to remove the necrotic tissue, release cytokines, enzymes, and reactive oxidative species that are actively toxic to the CNS parenchyma\cite{45,53}. Astrocytes contain this immune response by forming a physical barrier around the lesion site, which is necessary to reestablish the BBB (Figure 1.2); without this astrocytic response, these toxic metabolites produced by phagocytes would cause the lesion to expand, thereby exacerbating the effect of the initial injury\cite{32,54}. However, by sequestering the immune response within the lesion, this astrocytic response causes a
pseudocystic cavity to form as the necrotic tissue is removed (Figure 1.3). This acellular glial scar lacks a solid extracellular matrix (ECM) and thus does not have a physical substrate to support axon sprouting; moreover, damage to the microvasculature makes the lesion site avascular and the penumbra (the tissue immediately surrounding the lesion) ischemic, which represents a further barrier to the tendency of axotomized axons to spontaneously sprout. In addition to this physical barrier, astrocytes also molecularly modify the tissue around the glial scar, depositing chondroitin sulfate proteoglycans (CSPGs) in the ECM that further inhibit axon sprouting.49, 50, 55-57

Although the lesion site represents both a physical and biochemical barrier for regeneration, it is clearly not an insurmountable obstacle: the CNS does not form a glial scar after injury in embryonic and immature mammals, and instead demonstrates a substantial regenerative capacity.58, 59 Furthermore, focal lesions to the CNS of adult non-mammalian vertebrates are often healed through complete regeneration, even though the same variety of cells are present in these animals as are found in the injured mammalian CNS.60 By understanding why the lesion environment inhibits regeneration neither in young mammals nor in non-mammalian vertebrates, it may be possible to therapeutically modify the injured mammalian CNS to create a response more conducive to regeneration.

Physiology of CNS regeneration in non-mammalian vertebrates

In contrast to the mammalian CNS, the CNS in many non-mammalian vertebrates demonstrates a robust regenerative response following injury. Axons regenerate across the lesion site and reconnect with their original targets, facilitating
functional recovery after spinal cord transection in zebrafish\textsuperscript{61} (Figure 1.3) and in the freshwater turtle Trachemys dorbignyi\textsuperscript{62, 63}. Tail amputation in Urodele and Anuran amphibians includes loss of the caudal portion of the spinal cord, and the spinal cord completely regenerates new tissue that becomes functionally integrated into the remainder of the CNS\textsuperscript{64-66}. The brain also demonstrates regeneration following a stab wound in zebrafish\textsuperscript{67, 68}, and both Anuran and Urodele amphibians regenerate large portions of their brain after complete resection\textsuperscript{69, 70}. The injured non-mammalian vertebrate CNS also supports robust neurogenesis through which the injured tissue is replaced, and these new cells are able to restore the original neural circuitry by reconnecting with the same targets as the original cells had prior to the injury\textsuperscript{68-71}.

Radial glia, which are found throughout the adult CNS in non-mammalian vertebrates, are the predominant cell type that facilitates axon regeneration and neurogenesis underlying regeneration following injury. Interestingly, radial glia are functionally equivalent to, and express genetic markers associated with, both astrocytes and ependymal cells in the mature mammalian CNS\textsuperscript{72, 73}. Radial glia also express markers of mesenchymal cells (a description of which can be found on page 19) including smooth-muscle actin (Acta2), fibronectin, and several epithelial-mesenchymal transition transcription factors (slug, Zeb1, Zeb2); Zeb1 is necessary to maintain radial glia neurogenesis, while embryonic morphogens found in the spinal cord (Sonic hedgehog, Shh; and retinoic acid, RA) can cause radial glia to differentiate into subclasses of neurons\textsuperscript{72, 74}.

In addition to being orthologous to astrocytes and ependymal cells in the adult mammalian CNS, radial glia in the adult non-mammalian CNS also express markers
associated with radial glia in embryonic non-mammalian vertebrates, as well as with both radial glia and immature astrocytes in the embryonic mammalian CNS\textsuperscript{73, 75}, suggesting that they remain in an immature state throughout adulthood. Moreover, radial glia in the adult non-mammalian vertebrates CNS continue to demonstrate behaviors that are typically associated with neural development in the embryonic brain, which are also those that we have previously established are necessary for regeneration: neurogenesis, and facilitating axon outgrowth\textsuperscript{59, 72, 76}. Specifically, a subset of radial glia constitutively sustain neurogenesis throughout the adult non-mammalian vertebrate brain, and all radial glia retain a quiescent ability to function as pleuripotent neural progenitor cells (NPCs) that is regulated through the Notch signaling pathway\textsuperscript{67, 77, 78}; thus, radial glia near the lesion are the source of new neurons and glia that are necessary to regenerate the damaged parenchyma\textsuperscript{67}. Radial glia begin to migrate towards the lesion within hours of an injury\textsuperscript{76, 79} where they reestablish the BBB\textsuperscript{80} and, after an initial 48 hour delay, they begin to proliferate\textsuperscript{68}. Additionally, they extend their processes across the lesion to form a highly aligned cellular bridge\textsuperscript{79}, which facilitates regeneration by promoting neovascularization\textsuperscript{80} and by guiding axons as they sprout across the lesion towards their original target\textsuperscript{61}.

Throughout the injury response, radial glia demonstrate consistent changes in their genetic profile that emerge concurrently with their cellular behaviors that facilitate regeneration. Radial glia express Glial Fibrillary Acidic Protein (GFAP) in the adult Urodele and Teleost brain; upon spinal cord injury, GFAP expression decreases while vimentin and nestin, which are markers of immaturity and pleuripotent progenitors respectively, increase\textsuperscript{64}. As Nestin expression increases, an increasing proportion of the
radial glia daughter cells subsequently differentiate into neurons, with this neurogenesis being regulated through Notch signaling\textsuperscript{78}. Moreover, radial glia require activation of the signaling pathways associated with the morphogens transforming growth factor beta (TGF-β) and Wnt to control proliferation and progenitor cell fate during regeneration\textsuperscript{81, 82}. Thus, radial glia are sufficient to provide a source of new neurons in the regenerating non-mammalian vertebrate CNS, and they are sufficient to guide regeneration of damaged axons past the lesion site and to their original targets. Together, this shows that radial glia are the crucial cell type that facilitates CNS regeneration in non-mammalian vertebrates; as radial glia are orthologous to mammalian astrocytes, astrocytes may have a similarly important role in regulating regeneration in mammals.

*Regenerative physiology is conserved in the mammalian CNS*

CNS regeneration in non-mammalian vertebrates requires radial glia to facilitate the proper targeting of regenerating axons, and to replace the damaged parenchyma by producing new neurons and glia. The mammalian CNS also contains radial glia, but they are only present during development and they differentiate into astrocytes as the CNS matures. While there is debate about whether the radial glia in non-mammalian vertebrates are technically astrocytes, both of these cell types, which are characterized by robust GFAP expression, are genetically and functionally orthologous to each other. Given that radial glia facilitate regeneration in non-mammalian vertebrates, the astrocytic response to injury is likely to be similarly critical in determining the regenerative potential of the mammalian CNS.
Many of the astrocytic behaviors expressed throughout their injury response are strikingly similar to those through which radial glia facilitate regeneration, but the injury-induced response in mammalian astrocytes ultimately inhibits regeneration. Both astrocytes and radial glia begin to migrate towards the lesion within hours of the initial injury. Furthermore, both astrocytes and radial glia at the lesion border begin to proliferate after an initial delay, and the rate of proliferation peaks after 48 hours; astrocyte proliferation subsequently declines, while radial glia continue to proliferate at this elevated rate over at least seven days after the injury. The fate of their daughter cells is remarkably different between these cell types: astrocyte proliferation is predominantly gliogenic and these cells form a barrier that inhibits axon sprouting, while radial glia proliferation is neurogenic and provides both a source of neurons for regeneration and a substrate that axons use for sprouting. These cells also demonstrate divergent injury-induced morphologic changes: astrocyte processes hypertrophy and they up-regulate certain cytoskeletal elements associated with maturity; in contrast, radial glia assume a bipolar morphology resembling their shape during embryogenesis, and they down-regulate cytoskeletal elements associated with maturity and up-regulate those elements associated with immaturity. The fact that both radial glia and astrocytes demonstrate many of the same initial behaviors that develop along a similar timeline after injury suggests that both cell types respond to a similar signal that initiates their response to injury. Consequently, the divergent effect that astrocytes and radial glia have on facilitating regeneration is likely due to differences in the ways in which these individual cellular behaviors are subsequently regulated throughout the reparative response.
Although mammalian astrocytes do not facilitate overt CNS regeneration after injury, experimental evidence demonstrates that they retain a latent regenerative potential. While mammalian astrocytes do not facilitate axon sprouting after injury *in vivo*, multiple growth factors have been shown to make them more permissive to axon outgrowth *in vitro*. Astrocytes themselves are a source of some of these factors, including fibroblast growth factor (FGF) 2, which has an autocrine effect inducing astrocytes to promote neurite outgrowth and also acts directly on neurons to enhance outgrowth and branching *in vitro*. FGF-2 is also produced by reactive astrocytes around the lesion site *in vivo* and has been shown to promote axon sprouting following injury, suggesting that astrocytes can facilitate axon outgrowth in adult mammals given the appropriate molecular cues. Furthermore, while mitogenic and neurogenic growth factors are not robustly induced in the injured mammalian CNS, FGF-2 is induced upon injury in regenerating non-mammalian vertebrates where it drives proliferation and neurogenesis throughout regeneration. In addition to being able to promote neurite outgrowth, mammalian astrocytes – similarly to radial glia – have a latent neurogenic program. While astrocytic neurogenesis is repressed through Notch signaling *in vivo*, they can produce astrocytes, oligodendrocytes, and neurons *in vitro*, which suggests that the signaling environment within the CNS actively inhibits neurogenesis. The fact that astrocytes can be induced to promote axon sprouting and undergo neurogenesis strongly supports the notion that astrocytes retain a regenerative potential, and that they may be able to facilitate robust regeneration after CNS injury in mammals if this pathways regulating regeneration can be identified and appropriately targeted.
During embryonic development, immature astrocytes and radial glia facilitate neurogenesis and axon sprouting throughout the mammalian CNS. In embryonic vertebrates, radial glia expressing GFAP, Vimentin, and two astrocyte-specific glutamate transporters (GLAST and GLF-1) are the first cells to differentiate from neuroepithelial stem cells, and they function as the pleuripotent progenitors that give rise to astrocytes, neurons, and oligodendrocytes\textsuperscript{72, 107}. Radial glia help guide migrating neuroblasts to their appropriate brain regions\textsuperscript{108, 109} where their differentiation is specified by local inductive cues from master regulatory genes (e.g. Shh in the ventral floor plate of the spinal cord induces differentiation into dopaminergic lower motor neurons)\textsuperscript{72, 73, 110, 111}. Towards the end of neurogenesis, radial glia produce astrocytes, which facilitate embryonic axon outgrowth and targeting by modifying the ECM, including depositing some of the same CSPGs that they produce after injury\textsuperscript{112-115}. In contrast to their inhibitory role after injury, certain CSPGs produced by astrocytes promote axon outgrowth in the embryonic CNS\textsuperscript{116}, which suggests that CSPGs interact with other signals within the CNS to determine whether their effect on axon sprouting is inhibitory or excitatory. As astrocytes in the developing mammalian CNS demonstrate each of the same behaviors that radial glia express during both embryogenesis and regeneration in non-mammalian vertebrates, immature mammalian astrocytes may also be able to facilitate CNS regeneration in mammals.

Immature astrocytes lose the ability to facilitate axon outgrowth and function as NPCs as the CNS matures into adulthood. In the immature CNS, astrocytes and radial glia facilitate regeneration\textsuperscript{58, 59, 117}, concurrent with the ontogenetic decline in regenerative potential is the differentiation of radial glia into astrocytes at the end of
embryonic development, and the absence of robust regeneration in the adult mammalian CNS has been attributed to the inability to induce a signaling environment through which astrocytes can revert to an immature phenotype. Paralleling this ontogenetic decline in regeneration is a decrease in the magnitude of injury-induced proliferation and neurogenic fate of these cells, reintroducing growth hormones present in the juvenile brain enhances proliferation, drives neurogenesis, and promotes functional recovery following injury. The fact that both neurogenesis and axon outgrowth are enhanced by treating mature mammalian astrocytes with signaling molecules from the embryonic brain suggests that recreating the signaling environment within the immature CNS might be able to stimulate regeneration in adult mammals.

**Conclusion**

It is clear that astrocytes play a crucial role in determining the regenerative potential in the mammalian CNS: their response to injury inhibits regeneration in adults, but they facilitate regeneration in immature and embryonic animals. Moreover, regeneration in non-mammalian vertebrates is facilitated by radial glia, which are orthologous to mammalian astrocytes. Although mature astrocytes do not facilitate robust regeneration in the mammalian CNS, they retain a latent ability to demonstrate the same behaviors necessary for axon sprouting and neurogenesis during embryogenesis. Thus, recreating the signaling environment within the developing mammalian CNS may induce astrocytes to revert to an immature phenotype through which they could facilitate complete regeneration by recapitulating the same mechanisms by which the CNS originally developed.
Epimorphic regeneration recapitulates embryonic development

The ultimate goal of therapies and rehabilitation for CNS injury is complete functional recovery, which requires restoring the axon tracts, neurons and glia, and each of their connections that were destroyed by the lesion. In the previous section, we identified astrocytes as crucial determinants for regeneration in the CNS, and we established that they can be induced to express each of the behaviors through which their orthologous cell types facilitate regeneration in other vertebrate clades. In this section, we explore the physiology of spontaneous regeneration in non-mammalian vertebrates to try and understand why CNS regeneration is absent in mammals despite the presence of these physiologic mechanisms through which it might proceed. The facts that this neural circuitry develops spontaneously during embryogenesis and that many vertebrate species can completely regenerate this same circuitry following substantial injuries belie the notion that this circuitry is so extraordinarily complex as to be fundamentally incapable of regeneration. Indeed, regeneration in non-mammalian vertebrates is successful because it replaces the damaged tissue through recapitulating embryonic development, a process known as “Epimorphic Regeneration.” The physiology of embryogenesis is conserved among all vertebrates, so it may be possible to activate epimorphic regeneration in injured mammalian tissues if the stimulus through which these developmental pathways are reactivated upon injury in non-mammalian vertebrates can be identified.
Non-mammalian vertebrates demonstrate epimorphic regeneration

Non-mammalian vertebrates are able to completely regenerate injured or amputated tissues because the injury reactivates the same physiologic mechanisms through which these structures originally develop during embryogenesis (Figure 1.4). Just as embryogenesis begins with totipotent progenitors that gradually differentiate into increasingly mature cells as development progresses, a population of progenitors accumulates at sites of injury and supports regeneration in non-mammalian vertebrates\textsuperscript{122}. While progenitors are omnipresent in the embryo, they are not equally ubiquitous adult animals. Instead, in vertebrates that regenerate, terminally differentiated mesenchymal cells – those cells composing connective tissues such as the lymphatic system, circulatory system, and musculoskeletal system – dedifferentiate into pluripotent progenitors\textsuperscript{123, 124}. Upon injury, adjacent epithelial cells migrate into the lesion site where they form a wound epithelium that functions as the apical ectodermal cap (AEC) and induces underlying mesenchymal cells to dedifferentiate and form a mass called a regeneration blastema\textsuperscript{125}. New tissue develops as cells within the blastema proliferate, and gradients of morphogenic molecules guide their differentiation into the full complement cell types that were present in the original structure\textsuperscript{123, 125, 126}. As epimorphic regeneration progresses, these newly differentiated cells form tissues and organs that are anatomically and physiologically indistinguishable from the original tissue that was present prior to the injury.

Newly-produced progenitor cells require extracellular spatial signals to define the rostrocaudal (RC), mediolateral (ML), and anteroposterior (AP) body axes; these signals guide morphogenesis of new structures throughout regeneration\textsuperscript{127}. These morphogens,
which are conserved during embryogenesis and regeneration, provide spatial cues that
cells use to determine their 3-dimensional location within the developing embryo\textsuperscript{128-130}. For example, morphogen gradients determine whether the developing limb bud will
generate a forelimb or a hindlimb based on the relative location of the limb bud within
the RC axis; ectopic limb formation, either from a transplanted limb bud or from
induction of supernumerary limb buds, results in the formation of an additional limb that
assumes distinct forelimb or hindlimb anatomy based on the position of the limb bud
along the RC body axis of the embryo\textsuperscript{130-132}. Preservation of left-right symmetry and the
appropriate orientation of the new limb on the body indicate that the regeneration
blastema can detect the ML and AP axes of the limb, respectively\textsuperscript{125}. Thus, these
vertebrates demonstrate a profound capacity for spontaneous regeneration because
injury induces the morphogens that pattern all three body axes within the regenerating
structure.

Vertebrate regeneration is limited neither to a distinct clade nor to a particular
type of tissue. Beyond the Urodele limb, the widespread evidence of regeneration in
different tissues and across different clades illustrates the profound evolutionary
importance of regeneration\textsuperscript{133, 134}. Teleost fish, another common model of regeneration,
also demonstrate regeneration following amputation of their fins\textsuperscript{135-137}. Beyond the limb,
many vertebrates can regenerate their jaw\textsuperscript{138, 139}, eyes\textsuperscript{140}, skin\textsuperscript{141}, and tails\textsuperscript{64}. Even
injuries that would prove severely debilitating – if not fatal – in humans can often be
healed by regeneration in other vertebrate species. For example, zebrafish can
regenerate their hearts after more than 40% of the ventricular wall is removed by
amputation\textsuperscript{142, 143}. Urodeles also demonstrate profound cardiac regeneration, which was
incidentally discovered when animals whose hearts had been completely bisected for blood collection defied researchers' expectations that the animals would die and instead recovered completely within a week of the procedure\textsuperscript{144}; subsequent research demonstrated that the heart could regenerate up to 50\% of its structure following amputation, with formation of a clot within 30 minutes, initial regeneration of myocardial fibers within 2 hours, and restoration of circulation within 5 hours\textsuperscript{145}.

CNS regeneration is also widespread among vertebrates, demonstrating that the mechanisms to functionally restore injured tissue are retained in adult animals. Many vertebrates, including examples among such disparate clades as Teleosts, Urodeles, and Reptiles, functionally reconnect their spinal cord following a complete transection\textsuperscript{62, 79, 146}. Xenopus tadpoles and adult Urodeles are both able to regenerate portions of their spinal cord that are lost after tail amputation\textsuperscript{64, 147}. The retina, which is an extension of the CNS within the eye, also regenerates retinal pigment epithelium and ganglion cells following ablation in Teleost fish\textsuperscript{77, 148-150} and both Anuran\textsuperscript{151} and Urodele\textsuperscript{78, 152, 153} amphibians; moreover, retinal ganglion cell axons regenerate through the optic nerve to the optic tectum after either axotomy in the optic nerve or ablation of the retina\textsuperscript{154-158}. Furthermore, the neural circuitry within the adult CNS can be replaced, as Teleost fish regenerate following a telencephalic stab wound\textsuperscript{67}, and Anuran amphibians regenerate large portions of the cortex following resection\textsuperscript{69}. In each of these examples, regeneration is facilitated by radial glia, which recapitulate their neurogenic and axon guidance roles through which they facilitate embryogenesis\textsuperscript{71, 73, 77, 93, 150}. Mesenchymal cells in peripheral tissues facilitate epimorphic regeneration, and radial glia, which express mesenchymal cell markers\textsuperscript{72}, facilitate CNS regeneration through re-
development. Thus, CNS regeneration in non-mammalian vertebrates may be an example of epimorphic regeneration.

Among all of these examples, regeneration is a spontaneous process that begins immediately upon injury and forms of a new structure that is anatomically and physiologically indistinguishable from the original uninjured tissue. Moreover, while the specific cells that facilitate regeneration vary among each tissue, the cellular behaviors underlying regeneration are conserved among the disparate vertebrate species and across each of the tissues in which it has been studied. Many disparate vertebrates express epimorphic regeneration and fossil evidence demonstrates that amphibians were capable of limb regeneration over 300-million years ago, which suggests that epimorphic regeneration is a phylogenetically early adaptation that evolved in a common vertebrate ancestor\textsuperscript{133, 134, 159, 160}. Thus, the physiology underlying epimorphic regeneration is likely conserved among all vertebrates in which it is observed.

\textit{Epimorphic regeneration is conserved among all vertebrates}

During development, a small number of signaling molecules define the principal body axes and regulate tissue patterning in the embryo. These morphogens, which are called master regulatory genes, are conserved among diverse tissue types and across all vertebrates\textsuperscript{110, 127}. For example, Shh defines dorso-ventral (DV) patterning and regulates limb outgrowth\textsuperscript{161-164}; FGF regulates limb bud morphogenesis\textsuperscript{165, 166}; RA specifies the posterior-ventral-proximal portion of the embryo\textsuperscript{131, 167}; Wnt signaling is necessary for body axis extension in the mouse\textsuperscript{168} and both DV and AP axis formation in the Xenopus embryo\textsuperscript{167}; TGF-β contributes to longitudinal organization in the CNS\textsuperscript{110};
and both Notch and bone morphogenic protein (BMP) contribute to cellular
differentiation\textsuperscript{128, 169}. Gradients of these morphogens specify a Cartesian coordinate
system that defines bilateral body axes in all vertebrates, and the extraordinary diversity
in structures among different species is thought to be a function of differences in their
concentration, distribution of their gradients, and duration of expression\textsuperscript{127, 168, 170}.

The profound regenerative potential in non-mammalian vertebrates is facilitated
by the injury-induced expression of the same master regulatory genes that underlie
embryogenesis\textsuperscript{171}. Indeed, Shh regulates regeneration of the spinal cord and limb in
axolotl\textsuperscript{126, 163}, and of the limb in Xenopus tadpoles\textsuperscript{172}; FGF regulates regeneration of the
myocardium and fin in zebrafish\textsuperscript{143, 173-175}, and of the limb and tail in Xenopus\textsuperscript{176, 177}; RA
regulates regeneration of the limb in axolotl\textsuperscript{178}, and of the fin in zebrafish\textsuperscript{179}; Wnt
regulates regeneration of the limb and tail in Xenopus\textsuperscript{176, 177}; TGF-\beta is necessary to
initiate regeneration of the limb in axolotl\textsuperscript{180}; Notch regulates cellular differentiation
necessary for regeneration of the tail in Xenopus tadpoles\textsuperscript{181}, of the retina in axolotl\textsuperscript{178},
and of the heart in zebrafish\textsuperscript{182, 183}; and BMP regulates limb and tail regeneration in
Xenopus\textsuperscript{147, 176, 177}. The role of embryonic physiology in regeneration is further
underscored by certain vertebrate species in which immature animals are highly
regenerative but lose this ability once they mature and developmental physiology
becomes dormant. For example, Xenopus laevis regenerate their limbs and tail when
they are tadpoles, but they lose this regenerative ability after they progress through
metamorphosis and become adults; axolotl are closely related to Xenopus, but they
retain an immature phenotype similar to that of the Xenopus tadpole and they continue
to regenerate in adulthood\textsuperscript{125, 147, 159}. Together, these observations support the notion
that epimorphic regeneration is a recapitulation of embryogenesis, and that the injury-induced expression of master regulatory genes necessary for regeneration is widely conserved.

Given that ontogenetic physiology is conserved among all vertebrates, the basic physiologic mechanisms of regeneration must also be conserved regardless of whether the particular vertebrate species retains the ability to access these developmental programs and facilitate regeneration into adulthood. Indeed, although different cell types support regeneration in each tissue, the same set of master regulatory genes drives epimorphic regeneration by inducing a conserved series of cellular behaviors: migration, dedifferentiation, proliferation, and differentiation. Following limb amputation, TGF-β and RA are necessary to initiate regeneration in successfully regenerating animals. FGF and Wnt induction creates a gradient that stimulates epithelial cell migration towards the lesion site, where they form a wound epithelium within hours of the initial injury. Beneath the wound epithelium, FGF and Wnt induces mesenchymal cell dedifferentiation into pleuripotent progenitors, possibly by inhibiting Notch signaling. These progenitors form a regeneration blastema, where BMP and RA stimulate proliferation that causes the blastema to expand throughout regeneration. BMP, Shh, and RA gradients define a Cartesian coordinate system that signals the RC, AP, and ML axes of the regenerating structures. While the mechanisms of epimorphic regeneration are most thoroughly studied using models of the vertebrate limb (including the zebrafish fin), the cellular mechanisms of regeneration and the families of signaling molecules that orchestrate them are also conserved in other tissues. Regeneration of the zebrafish ventricular myocardium
requires the injury-induced expression of FGF, Notch, TGF-β, RA, and Shh: these master regulatory genes induce cardiomyocyte dedifferentiation into a regeneration blastema at the wound edge where these cells proliferate, migrate into the damaged tissue (or, in the case of amputation, into the clot that forms at the wound edge), and then transdifferentiate into new cardiomyocytes. Epimorphic regeneration of the vertebrate lens and jaw has also been shown to require the induction of FGF, RA, TGF-β, Wnt, and Shh. Among these regenerating species, induction of these master regulatory genes is necessary for regeneration, and interfering with these signaling pathways can block regeneration.

Differences in regenerative potential among vertebrates are associated with differences in the master regulatory genes and concomitant cellular behaviors induced upon injury. Manipulating these master regulatory genes restores regeneration by inducing these cellular behaviors in those tissues where regeneration does not spontaneously occur. Limb amputation in axolotl induces increased expression of FGF and stimulates directional epithelial cell migration towards the injury where a wound epithelium forms within hours, creating a structure resembling the AEC from which the limb develops during embryogenesis, and the axolotl limb completely regenerates. In contrast, wing amputation in the chick does not induce increased FGF expression, there is minimal epithelial cell migration towards the injury, a wound epithelium forms only after multiple days, and the wing does not regenerate; however, adding FGF to the injury site stimulates epithelial cell migration thus enhancing the rate of wound epithelium formation, the resulting wound epithelium develops characteristics of the AEC, and chick wing regenerates. Cardiac muscle necrosis and ventricular
amputation both induce Wnt and FGF expression in zebrafish, which is necessary for regeneration through stimulating cardiomyocyte dedifferentiation \(^{142, 143, 188}\). In contrast, cardiac regeneration induces neither Wnt nor FGF expression in mice, cardiomyocytes do not dedifferentiate, a regeneration blastema does not form, and the mouse heart does not regenerate after injury; however, Wnt expression stimulates cardiomyocyte dedifferentiation \textit{in vitro}, suggesting that this signaling pathway may be able to promote the formation of a regeneration blastema \textit{in vivo} \(^{191}\). Retinal regeneration in Urodeles and Teleosts requires the transdifferentiation of Müller glia (the resident radial glia population in the retina) into stem cells, and directly into retinal pigment epithelial cells, through a Notch-regulated pathway \(^{77, 78, 150}\); similarly, transdifferentiation of mature astrocytes and differentiation of NPCs after SCI in zebrafish requires the expression of Wnt through which neurogenesis is released from Notch-mediated inhibition, and cell fate is determined by expression of Shh and Wnt after injury \(^{126, 192}\). In contrast, SCI or tail amputation in mice or rats does not induce Shh or Notch expression, astrocytes do not dedifferentiate into NPCs, and the rodent spinal cord does not demonstrate robust regeneration \(^{33, 35}\); however, adult astrocytes express a latent Notch-regulated neurogenesis program in adults \(^{103}\), and adding FGF after injury induces astrocyte transdifferentiation and progenitor cell differentiation into neurons \(^{100, 193-195}\). Thus, master regulatory genes are sufficient to induce epimorphic regeneration in those tissues and species where regeneration does not spontaneously occur. The extraordinary degree of conservation among these master regulatory genes supports the notion that all vertebrates retain a latent ability to regenerate and suggests that a similarly robust regenerative potential is also conserved within mammals.
Latent epimorphic regenerative potential is conserved in mammals

Mammals retain a latent regenerative potential into adulthood because they express the same physiologic mechanisms during embryogenesis that facilitate both embryogenesis and regeneration in non-mammalian vertebrates. That mammals have conserved the physiology underlying regeneration is apparent in their ontogenetic decline in regenerative potential\textsuperscript{58, 196, 197}, which is similar to that in certain non-mammalian vertebrates, and in the sporadic instances of regeneration observed among diverse mammalian species where the same cellular behaviors and physiology are expressed. For example, spiny mice shed large portions of their skin through autotomy as a defense mechanism to escape predation, and this wound heals by regenerating fully functional skin\textsuperscript{198}. Following autotomy, epidermal cells migrate from the wound margin and form a wound epithelium and a regeneration blastema within 24 hours; Wnt and BMP expression both increase after injury, recreating the signaling environment during embryonic skin development, and sustaining the cellular proliferation and differentiation necessary to regenerate the underlying dermis, cartilage, sebaceous glands, and hair follicles throughout the new skin\textsuperscript{198}. In contrast, rat skin wounds take 5-7 days to re-epithelialize, which subsequently heals through the deposition of a collagenous scar rather than through regeneration\textsuperscript{199}. Deer antlers undergo an annual cycle of shedding and regrowth through the activation of stem cells in the pedicle periosteum, which is a form of epimorphic regeneration as these multipotent periosteal stem cells form a regeneration blastema that produce bone, vasculature, and an epidermis with functional sebaceous glands and hair follicles\textsuperscript{200-202}. Evidence from microarray studies suggests that the FGF signaling pathway is up-regulated in
regenerating antlers, and in vitro studies demonstrate that FGFs maintain a high rate of proliferation in cells cultured from the mesenchymal growth zone of the regenerating antler\textsuperscript{203, 204}. In certain young mammals, an amputation of the digit distal to the distal interphalangeal (DIP) joint can heal by complete regeneration. Digit regeneration in neonatal mice proceeds through endochondral ossification, which recapitulates embryonic development of the digit. BMP signaling regulates digit regeneration: regeneration can be induced in non-regenerating digits by applying exogenous BMP, while Noggin, which inactivates BMP by binding to it, inhibits regeneration\textsuperscript{197, 205}. Digit regeneration is also found in humans, where finger amputation distal to the DIP joint consistently heals by complete regeneration of the skin, muscle, vasculature, nerves, and bone\textsuperscript{196}. The expression of regeneration among different mammalian tissues and species demonstrates that the physiology of epimorphic regeneration is conserved in a latent state among all mammals. Thus, it may be possible to stimulate regeneration in non-regenerating tissues by manipulating these same physiologic pathways.

**Conclusion**

Ontogenetic physiology is conserved among all vertebrates. Epimorphic regeneration recapitulates embryogenesis, so all vertebrates – including mammals – must retain this physiology regardless of their ability to spontaneously regenerate. A small number of master regulatory genes regulate morphogenesis of each tissue throughout the embryo; these genes control the same set of behaviors – migration, proliferation, and differentiation – in each of the tissue-specific cell types responsible for development. Epimorphic regeneration also requires these same cellular behaviors,
which are regulated through the injury-induced expression of these master regulatory genes; the timing over which these behaviors emerge ultimately determines the regenerative potential.

In peripheral tissues, mesenchymal cells mediate regeneration in response to these morphogens. Radial glia express markers of mesenchymal cells, and they facilitate regeneration in the non-mammalian vertebrate CNS through the same cellular behaviors induced by the same master regulatory genes that induce peripheral regeneration. Astrocytes, the mammalian ortholog to radial glia, are crucial to wound repair; while they do not spontaneously facilitate regeneration, master regulatory genes can induce each of the behaviors necessary for regeneration. Thus, astrocytes retain a latent ability to regenerate, even though their obstreperous response to injury actively inhibits the spontaneous attempt of axons to regenerate.

Epimorphic regeneration emerges spontaneously within a variety of vertebrates, so there must be a signal associated with the injury in regenerating tissues that induces the expression of these master regulatory genes. Consequently, it is reasonable to postulate that the failure of certain vertebrates to regenerate is due to a lack of this injury-induced signal. Epimorphic regeneration relies on ontogenetic physiology, which is conserved among all vertebrates, so all vertebrates should be capable of epimorphic regeneration if the signal from the injury that induces these master regulatory genes can be identified.

**Physiologic electric fields regulate embryogenesis and regeneration**

Endogenous electric fields (EFs) are physiologically produced in all biological systems and influence the activity of many different cell types through electrostatic
interactions with individual cellular components ranging from small ions through biological macromolecules. Electricity was first demonstrated to have an effect on biologic activity in the 18th century when Luigi Galvani, with dramatic aplomb, made a dead frog leg jump by connecting its sciatic nerve to a source of electricity\textsuperscript{206}. In the early 19th century, Matteucci demonstrated that tissues produce their own bioelectricity by showing that a dead frog leg would contract if its sciatic nerve was placed over an incision made in the muscle from another frog\textsuperscript{207}. In 1855, Kollicker and Mueller found that, when the motor nerve to a frog’s leg was placed over a beating heart, the leg kicked with each heartbeat\textsuperscript{208}, demonstrating that the heart spontaneously produced bioelectricity. This electrical activity from the heart was first measured through the skin by Ludwig and Waller in the 1880s using a “capillary electrometer”\textsuperscript{209}. Hans Berger applied this same technique to the head, leading to the development of the electroencephalogram\textsuperscript{210}, which definitively demonstrated that the brain produces spontaneous electrical activity. Electrical activity has been subsequently demonstrated in all tissues, and multiple clinical tools, including the electrocardiogram (EKG) and the electroencephalogram (EEG), measure these bioelectric fields because their magnitude and polarity vary as a function of tissue physiology.

Endogenous bioelectric fields have been identified as a putative signal upstream of the master regulatory genes that regulates morphogenesis during development and regeneration. Elevated EFs are associated both with body patterning during embryogenesis, and EFs of sufficient magnitude are both necessary and sufficient for epimorphic regeneration in many non-mammalian vertebrates. As EFs have also been measured in the mammalian CNS, this suggests that EFs may also have a physiologic
effect on the cellular response to injury in the mammalian CNS, and that EF-based therapy may be able to promote regeneration.

Physics of electric fields

Two charges exhibit an electrostatic interaction between themselves because each charged particle has a region around it within which it is able to influence other charged particles or objects. Coulomb’s Law describes the magnitude of the force created by this interaction:

\[ F = \frac{kq_1q_2}{r^2} \]

where \( F \) is the electrostatic force, \( k \) is a constant, \( q_1 \) and \( q_2 \) are the two charges, and \( r \) is the distance between the two charges. Newton’s third law states that, for every action, there is an equal and opposite reaction: when a charge creates an electrostatic force on a second charge, that second charge creates an equal and opposite electrostatic force on the first charge. Therefore, a single charge in space cannot generate an electrostatic force: if \( q_2 = 0 \) Coulomb’s law predicts there would be no electrostatic force, and there is no second charge to satisfy the requirement of Newton’s third law that an equal and opposite charge be created. However, a single charge still has a region of electrical influence around it, which is defined as its electric field \( (E) \):

\[ E = \frac{kq_1}{r^2} \]

EFs for an individual charge can also be defined as

\[ E = \frac{F}{q_2} \]

which is a ratio of the electrostatic force between any two charges, and the second
charge involved in creating said force. It is worth noting that these charges can either be free electrons or ions; as the scope of this discussion covers biological systems, which are composed of aqueous environments, we are principally concerned with ions.

As a result of the force created by electrostatic interactions, EFs cause ions or molecules with a net charge move (a process called electrophoresis), while neutral molecules containing electrical dipoles (i.e. local separation of charges within the molecule but no overall charge) align their dipole in an EF. EFs are vectors and as such convey information about their magnitude and direction throughout their region of influence. EFs can either be static or dynamic: a static EF is one where charges are separated on either side of a physical barrier and do not travel between these poles; a dynamic EF is one where moving charges (i.e. an electrical current) induces an EF in the space around it. Electrostatic field strength is determined by the magnitude of the voltage gradient \( V \) and the distance \( d \) of separation: \( E = \Delta V / d \); electrodynamic field strength is determined by the resistivity of the medium \( \rho \), the ionic current \( I \), and the cross-sectional area \( A \): \( E = \rho I / A \). As Newton’s second law states, a particle that experiences a force \( F \) will undergo an acceleration \( a \) in proportion to its mass \( m \): \( F = ma \). Therefore, a charged particle will accelerate in an external EF, but the extent to which it will move depends on the ease of mobility of the charge in its external medium. At the extremes of the spectrum, the external medium can be a conductor, which allows completely free movement of a charge, or it can be an insulator, which restricts movement of a charge. Biological samples contain both conductors and resistors, which are created through the complex arrangements of cell membranes, the extracellular matrix, and the macromolecules holding each of these components together.
Cells physiologically produce and detect bioelectric signals

Physical properties of the tissue determine the magnitude of bioelectric fields that are produced by physiologic gradients in the transepithelial electrical potential (TEP) through the relationships of \( E = \Delta V/d \) and \( E = \rho I/A \). Voltage gradient (\( \Delta V \)) is a function of the TEP, which is determined by the metabolic activity in the cell (i.e. the number of ion transporters and their rate of activity) and by the resistivity of the medium; resistivity of the medium is the ability of the epithelium, or tissue parenchyma to resist the movement of ions; distance of charge separation is a function of the epithelial thickness, which often varies across the tissue; ionic current is affected by the magnitude of the voltage gradient, and also on the ability of the ions to diffuse; and cross sectional area is a function of the size of the tissue or, when the ionic current is across the epithelium, the portion of the epithelium supporting paracellular ion diffusion. These physical properties convert physiologic metabolic processes into EFs, which, because electrogenic ion transport is universal among cells, are omnipresent throughout all tissues.

All cells selectively segregate ions across their membranes, either by active transport up their concentration gradient or through ion channels that allow facilitated diffusion down their gradient. Ions, by definition, are charged molecules, so an electrical gradient is established when ion transport results in a net movement of charge across the membrane (Figure 1.5). Cellular activity that establishes electrical gradients is called electrogenic, and electrogenic activity is universal among cells because all cells use such electrochemical ion gradients for myriad metabolic processes. Electrogenic ion transport establishes a trans-membrane electrical potential (\( V_m \)) across the cell's plasma membrane, which is often characteristic for a given cell type\(^{212} \). \( V_m \) is generally
constant because these electrogenic transporters are often uniformly distributed across the cell membrane, but local variations in $V_m$ can arise over distances as short as 2 µm when they are segregated into domains by lipid rafts or anchored to the cytoskeleton; if opposite ends of the cell are organized into distinct regions, as can happen across epithelial cells which are organized into apical and basolateral domains, the resulting variation in $V_m$ can create a trans-cellular voltage spanning the entire cell.

Electrogenic activity is also a hallmark of tissues because all tissues are lined by cells containing a non-random distribution of membrane proteins that sustains a steady trans-cellular transport of metabolites and, thus, a trans-cellular ionic current (Figure 1.5F). These cells function as a barrier that regulate access of metabolites and molecules into and out of the tissue. In general, these cells are called epithelial cells, which can be derived from ectodermal, mesodermal, or endodermal tissues. Epithelial cells line most tissues, including the ventricular surface within the CNS; a notable exception is that the sub-pial surface of the CNS is lined by astrocytic end-feet that create the BBB which, while not a technical epithelium, regulate metabolite transfer into and out of the CNS similarly to how epithelia function in other tissues. In general, epithelial cells concentrate their sodium-potassium pumps ($Na^+/K^+$-ATPase) within their basolateral membranes, while sodium ($Na^+$) channels are concentrated in their apical membrane. The $Na^+/K^+$-ATPase is electrogenic because it pumps 3 $Na^+$ out of the cell while importing only 2 $K^+$, resulting in the net loss of 1 + charge for each adenosine triphosphate molecule (ATP) expended. As the intracellular $Na^+$ concentration decreases, a concentration gradient drives $Na^+$ diffusion from the tissue through the $Na^+$ channel and into the cell. Together, the basolateral $Na^+/K^+$-ATPase and apical $Na^+$
channels support a steady inward Na\(^+\)-ion current, which creates a large electrochemical Na\(^+\) gradient (Figure 1.6); tight junctions prevent paracellular ion diffusion, so the Na\(^+\) gradient is sustained\(^{214,215}\). Epithelial cells are oriented such that their individual trans-cellular voltage drops are aligned so they produce a large TEP. The magnitude of the TEP is determined by the metabolic rate of the Na\(^+\)/K\(^+\)-ATPases, and local variation in the TEP among epithelial cells creates EFs within the tissue. Although EFs originate at the periphery of the tissue, they spread throughout the entire parenchyma. Tissues are composed of ions and charged proteins, and EFs can influence biologic systems through electrophoresis and dipole alignment of charged ions and diffusible proteins. However, the influence of EFs throughout tissues is generally limited to the extracellular space because high plasma membrane capacitance causes intracellular EFs to decrease 1000x.

Extracellular EFs influence cellular activity both indirectly through effects on extracellular soluble molecules and directly through electrostatic interactions with membrane proteins. EFs cause electrophoresis of soluble molecules in the extracellular matrix, creating concentration gradients that, in turn, can serve as directional cues for cells to follow. EFs also cause electroosmosis\(^{216}\) of extracellular ions (Figure 1.8), with negatively-charged anions moving towards the positive pole of the EF and positively-charged cations moving towards the negative pole of the EF; those charged membrane proteins that are capable of lateral diffusion through the membrane (i.e. that are not anchored to the underlying cytoskeleton) passively diffuse along with these extracellular ions and become redistributed to either the positive or negative pole of the cell.
Cells can also respond to extracellular EFs directly because EFs induce a transcellular voltage drop, which affects the cell’s resting membrane potential and polarizes the cell by establishing depolarized and hyperpolarized domains at opposite ends of the cell such that the poles are parallel to the extracellular EF\textsuperscript{217-219}. These electrochemical effects allow cells to transduce the magnitude and direction of the applied EF through multiple distinct mechanisms. Cells transduce the EF magnitude by inducing a proportional depolarization of the cell membrane, causing a change in the opening probability of voltage-gated channels that activates intracellular second messengers. EFs also cause a polarized redistribution of membrane proteins, and the extent of this redistribution is proportional to the magnitude of the EF; as those membrane proteins with constitutive activity become increasingly concentrated at one area of the cell, the amount of this basal activity can become sufficient to cause a local activation of the downstream second messengers. EF-induced electroosmosis can transduce non-directional cues if the activated second messenger system is, for example, mitogenic\textsuperscript{218}; electroosmosis can also facilitate transduction of the directional component of the EF vector by causing excitatory membrane proteins to redistribute to one pole of the cell while inhibitory proteins redistribute to the opposite pole (Figure 1.8)\textsuperscript{220-222}. Another way cells can transduce the directional component of the EF is through depolarization and hyperpolarization of the membrane potential at opposite ends of the cell, which can result in activation and inhibition of certain voltage-gated channels. Together, each of these biophysical mechanisms allow cells to transduce extracellular EFs through multiple complex pathways, and the specific physiologic mechanisms depend on the particular extracellular proteins present in the tissue, the membrane proteins expressed
in the cell, basal activity of these membrane proteins, and the extent to which both the extracellular and the membrane proteins can diffuse. Thus, EFs may result in the same behavioral consequences on different cell types through completely independent physiologic pathways.

**Examples of established EF-transduction pathways**

Extracellular EFs, whose physiological effects have largely been studied using purified cell populations *in vitro*, influence the activity of both immature and terminally differentiated cells from all three embryonic germ layers. Many different pathways have been shown to contribute to cellular EF transduction for each cellular behavior, but the precise pathways responsible for specific behaviors have not been fully elucidated and are likely to vary among different cell populations. The best-studied example of EF transduction is electrotaxis (i.e. EF-induced directional migration), where electroosmosis of constitutively active membrane proteins to opposite ends of the cell causes increased activation of phosphoinositide-3-kinase (PI3K) at the leading end and phosphatase and tensin homologue (PTEN) at the lagging end of the cell, where they have excitatory and inhibitory effects on migration, respectively; interestingly, these second messengers have been implicated in electrotaxis in epithelial keratinocytes, hippocampal neurons, and neutrophils, but the specific receptors responsible for activating this common pathway vary\(^\text{221, 223-232}\).

A comprehensive study by Tseng and colleagues (2010) elucidated a pathway by which cells can respond to the magnitude of the EF independent of its orientation\(^\text{147}\). They demonstrated that EFs induce extracellular Na\(^+\) currents that open voltage-gated
sodium channels (Na\textsubscript{\textit{v}}), which activates the Salt Inducible Kinase (SIK) in direct proportion to its rising intracellular concentration\textsuperscript{147}. SIK is a Na\textsuperscript{+}-dependent member of the AMP-activated protein kinase (AMPK) family that modifies transcription and translation of downstream effector molecules, including Notch\textsuperscript{147}, to drive regeneration through re-development\textsuperscript{233, 234}. Interestingly, they also found that SIK is not involved in embryogenesis, suggesting that it may be part of a signaling pathway that reactivates developmental mechanisms to drive regeneration after injury. Furthermore, the SIK protein that they manipulated in Xenopus demonstrates sequence homology with a family of SIKs that was first identified in rats\textsuperscript{235} and has subsequently been demonstrated in mammalian astrocytes\textsuperscript{236}. Thus, SIK physiologically links bioelectric fields and scalar cellular responses through a second messenger system that is conserved among regenerating and non-regenerating vertebrates.

Another way calls can transduce EFs is through EF-induced depolarization and hyperpolarization of opposite ends of the cell. This is thought to contribute to directional neurite outgrowth that is towards either the anode or the cathode of the applied EF depending on the particular type of neuron being explored\textsuperscript{237-239}. Cellular morphology is also thought to be affected by the trans-cellular voltage drop induced by EFs, as astrocytes, Schwann cells, and fibroblasts align their processes perpendicularly to an applied EF, possibly to minimize the voltage drop across their membranes\textsuperscript{240-243}. Elevated EFs also affect differentiation in cardiomyocytes and neurons, likely through inducing changes in membrane potential\textsuperscript{244, 245}. Together, this demonstrates that bioelectric fields are produced throughout tissues as a consequence of physiologic epithelial cell activity, and that these EFs are able to regulate cellular activity.
Endogenous bioelectric fields vary in magnitude throughout life because changes in physiology affect the physical properties of the tissue. EF production begins early in development: fucoid eggs produce polarized electrical signaling shortly after fertilization when they are still unicellular, focal ionic currents are produced by the fertilized Xenopus egg during initial cleavage, and epithelial cells in vertebrate embryos begin producing directional trans-cellular ion currents that sustain EFs beginning early during development. EFs are typically elevated during embryogenesis because the epithelial cells sustain the same transcellular ion currents that they do in adult tissues, but the epithelium is thinner during development because the tissue is not fully formed; a smaller epithelium means the same voltage gradient occurs over a shorter distance, so the EF is higher. Additionally, the electrical resistivity of developing tissue is lower than it is during adulthood because there are fewer intercellular tight junctions between epithelial cells and the ECM in the tissue parenchyma is not fully formed, which further contributes to higher EFs within developing tissues (Figure 1.7C). During embryogenesis, a voltage drop of 90 mV is sustained across the developing axolotl neural tube; the presumptive neuroepithelium is initially 50 µm wide because it composed of only several cells, so this trans-neural-tube potential (TNTP) results in an electric field as high as 1800 mV/mm. As neurogenesis progresses, the EF within the neural tube decreases because the tissue thickens but the TNTP remains constant. EFs also increase during embryogenesis when epithelial tight junctions break down at sites of high cellular activity, decreasing resistance to paracellular ion diffusion, increasing the ionic currents, and inducing robust EFs; this happens at the AEC in
the developing embryo, and these large EFs have been shown to predict the site of limb bud formation in frog, chick, and mouse embryos\textsuperscript{248, 250, 252, 253}. Once development is complete, EFs become much lower because the adult tissues become electrically insulated as new epithelial tight junctions are formed that increase electrical resistance to ionic currents\textsuperscript{256-259}.

Physiologic EFs are a necessary signal that regulates cellular behaviors during embryogenesis. The developing embryo produces EF gradients in the RC, ML, and AP axes that contribute to embryonic morphogenesis by directing cellular behaviors\textsuperscript{215, 248, 255, 260}. High EFs are produced across the developing neural tube that are strongest in the sub-ependymal layer, which is the location of embryonic neurogenesis; inhibiting EFs either with pharmacologic antagonists or by using an implanted electrode to inject a counter-current causes gross abnormalities in neural tube development and can prevent closure of both the rostral and caudal neural pores\textsuperscript{261, 262}. Similarly, robust EFs are necessary for vertebrate limb development: high EFs precede initial outgrowth of the limb bud\textsuperscript{248, 263, 264}, and limbs develop abnormally if the EFs are inhibited either by pharmacological antagonists or by applying a countercurrent through an implanted electrode\textsuperscript{249, 250, 261, 262}. Thus, physiologic EFs within the vertebrate embryo are both necessary and sufficient to stimulate development.

EFs have also been measured in adult tissues in many vertebrate species. Ionic currents have been measured across the mammalian skin\textsuperscript{265}, respiratory epithelium\textsuperscript{266}, cornea\textsuperscript{267, 268}, and brain\textsuperscript{269}. Amphibians sustain an inward ionic current across the skin that varies in magnitude across different regions of the body\textsuperscript{270}. Similar ionic currents and electrical potentials have also been measured in human skin, revealing that human
skin produces a TEP that varies in magnitude across the body\textsuperscript{256, 257, 271, 272}. Although ionic currents and electrical potentials have been measured in a variety of adult vertebrate tissues, demonstrating that EFs have a role in regulating physiological activity in adults is more difficult than it is in developing animals. In adult tissues, cell populations typically do not demonstrate overt migration, proliferation, dynamic changes in protein expression, or marked changes in morphology. It is far more difficult to test the hypothesis that low EFs regulate cellular activity in adult tissues: the hypothesis is that the stimulus will not induce a response, but absence of evidence is not evidence of absence. However, one study has shown that a low dcEF (3-5 mV/mm) is present in the rostral migratory stream (RMS) in the adult CNS, which is a path through which nascent neuroblasts produced in the sub-ventricular zone (SVZ) migrate to the olfactory bulb. Low EFs have not been shown to elicit robust behavioral responses on other cell types in vitro, which makes intuitive sense: low EFs are found in adult tissues, so they should not be able to induce dramatic cellular behaviors in these mature cell populations. However, neuroblasts in vitro migrate cathodally upon exposure to a 3.5 mV/mm EF, which is the same direction in which neuroblasts are known to migrate in the adult CNS where EFs of this strength are present\textsuperscript{269}. This suggests that, similarly to their role in the embryonic CNS, physiologic EFs may regulate cellular activity in adult tissues.

\textit{Injury-induced electric fields regulate wound healing and regeneration}

Physiologic EFs are produced in embryonic and adult tissues, and the magnitude of these EFs reflects the physiologic properties of the developing tissues. Injury also induces a robust increase in EFs because epithelial damage creates an aqueous bridge
that short-circuits the trans-epithelial electrical resistance; this changes the physical properties of the tissue, instantly causing the TEP to collapse to 0 mV and consequently inducing large EFs between the lesion and the surrounding tissue (Figure 1.7). Kirchoff’s Current Law states that the current entering any point has to be the same as the current leaving that point, such that the algebraic sum of currents in a network being zero; the ionic current at the injury site reverses direction and establishes a current loop with the intact tissue at the lesion margin, so the total current flux through the lesion must be equal to the total current flux through the surrounding tissue. The intact tissue can be thought of as a concentric area surrounding the lesion site with a cross-sectional area of $2\pi r$ (assuming that there is only current parallel to the plane of the epithelium), where $r$ is the distance from the injury site; given that the total current $I$ is constant throughout the circuit, the current density ($\mu$) through any given part of the tissue decreases as the distance from the injury site increases. Moreover, Kirchoff’s Voltage Law states that the net voltage change through a circuit is 0, and Ohm’s Law states that the Resistance $= V/I$; as the surrounding intact tissue occupies a greater area than the injured tissue with a lower $I$ at any individual point, the $V$ within the healthy tissue must be smaller than that within the injury site. Thus, both the ionic current density and the voltage change within the tissue must vary spatially throughout the tissue, being low at the periphery and increasing substantially throughout the tissue approaching the lesion.

The injury-induced EF is a function of changing electrical resistance across the damaged epithelium, so it passively emerges instantly upon injury and is sustained throughout the entire repair process. As the TEP remains unchanged across the surrounding intact epithelium, the voltage gradient between the injury site and the
uninjured tissue must be larger in magnitude than those EFs typically found within intact tissues, and these EFs must be radially oriented between the lesion and the surround. Because these elevated EFs emerge automatically upon injury and are inherently directional with a vector directly towards the lesion, they are an ideal cue both to initiate the cellular response to injury and to direct surrounding cells to the lesion site. For tissues with an inward constitutive current, including the skin and cornea, the lesion site becomes the cathode of the injury-induced EF; epithelial keratinocytes, epidermal fibroblasts, and corneal fibroblasts, all of which are necessary for a regenerative healing response in their respective field, migrate towards the cathode upon exposure to an EF

\[\text{in vitro}^{223, 228, 230, 273}\], suggesting that these injury-induced EFs recruit these cells to the lesion site \textit{in vivo}.

The magnitude of injury-induced EFs is correlated with the regenerative potential of the injured tissue. EFs increase 50-100 fold in upon limb or tail amputation in axolotl, and this depolarization is sustained throughout the entire duration of regeneration\textsuperscript{270, 274, 275}. Tail amputation in Xenopus tadpoles induces robust ionic currents that are sustained throughout regeneration but, after tadpoles progress through metamorphosis, limb amputation induces a smaller current and the structure does not regenerate\textsuperscript{276-278}. Skin puncture in axolotl also induces strong EFs that are sustained throughout healing, during which time fully functional skin is regenerated\textsuperscript{141, 279}. In contrast, skin wounding in mice induces smaller ionic currents, and the skin heals by forming a collagenous scar rather than regenerating fully functional skin\textsuperscript{265}. In rats and cows, corneal injury induces an EF increase within the tissue surrounding the lesion; in rats, pharmacologically modifying the EF intensity causes a proportional change in the rate of wound healing.
that occurs\textsuperscript{267, 268}. Together, this shows that injury-induced EFs are more robust in those tissues that demonstrate epimorphic regeneration than they are in tissues that fail to regenerate. Moreover, these data suggest that the magnitude of these injury-induced EFs is closely associated with the regenerative potential of the tissue.

Elevated EFs are necessary and sufficient to stimulate epimorphic regeneration in injured tissues. Large ionic currents emerge from the amputated stump of the newt forelimb, and these currents persist throughout epimorphic limb regeneration\textsuperscript{270, 274}. Newt limb regeneration can be inhibited by attenuating these injury-induced EFs through either pharmacological or physical antagonists\textsuperscript{280, 281}, demonstrating that the EF itself, rather than any of the individual ions composing the associated injury current, is necessary for regeneration. In Xenopus tadpoles, a Na\textsuperscript{+} current induced by tail amputation is necessary for regeneration, and regeneration can be blocked by either injecting a countercurrent through an electrode or by genetically knocking out the sodium channel that allows the current’s creation\textsuperscript{147, 276}. In adult frogs, limb amputation produces relatively low EFs and the wound heals through a scar formation without robust regenerative outgrowth; enhancing the injury-induced EF through implanted electrodes promotes regenerative wound healing that is much more robust than the healing in animals without a functional stimulating electrode\textsuperscript{277, 278, 282}. Interestingly, although mammalian limbs do not regenerate after amputation and heal instead by scar formation, Robert Beck showed that electrical stimulation of the wound in rats promotes regenerative outgrowth of the amputation site with partial restoration of histologically normal tissue replete with vasculature, bone, and skin if the cathode of the applied EF is at the amputation site\textsuperscript{283, 284}. Together, this further supports the notion that endogenous
bioelectric fields are a main component of the signaling environment in injured tissue that regulates repair, and are both necessary and sufficient to induce regeneration.

*The CNS produces extracellular EFs*

Physiologic EFs have been measured throughout the mammalian CNS for over 100 years\(^\text{285}\). Richard Caton was the first person to measure EFs in the mammalian cerebral cortex when, in 1875, he demonstrated that the exposed cerebral hemispheres of rabbits and monkeys produce electrical phenomena\(^\text{286}\). Adolf Beck subsequently demonstrated that the spontaneous rhythmic electrical activity in the brain changed upon exposure to light, suggesting that this activity provides insight into the underlying function of the brain\(^\text{287}\). The first recording of these extracellular electric fields in living animals was made in 1912 by Ukrainian physiologist Vladimir Vladimirovich Pravdich-Neminsky, who measured evoked potentials in dogs\(^\text{288}\). Hans Berger invented the electroencephalogram (EEG) to measure this spontaneous electrical activity, and he used it to record the first human EEG in 1924\(^\text{210}\). The EEG measures extracellular EFs on the cortical surface using electrodes attached to the surface of the scalp with a conducting adhesive. By comparing the electric potential between two different electrodes, EFs can be calculated between different points of the brain. For clinical measurements, electrodes are typically applied in a standard configuration, and either the “absolute” EF is measured by comparing the individual electrode’s voltage to a ground electrode positioned far from the recording electrode, or relative EFs are measured between two locations within the cortex. These readings are often reported as voltages but, because they rely on differences in voltage at two electrodes separated
in space, there must be a voltage gradient (i.e. an EF) between the two locations. These voltages arise because the cytoarchitecture of the brain is highly organized, so synchronous activity among populations of cells results in large changes in the local electrical potentials. Even though the ions sustaining the electrical currents are relatively free to diffuse through the brain, the steady cellular activity and the highly organized cytoarchitecture result in local dcEFs that, cumulatively, are robust enough to be measured by the recording electrodes on the skin.

At the basic level, EEGs infer extracellular EFs within the brain parenchyma from recordings taken on the skin; while the 5 layers of tissue composing the scalp, the cranium, 3 layers of dura, and CSF separate the electrical activity in the brain parenchyma from the recording electrodes, the electrical signals produced by neural activity are so strong that these surface electrodes are able to filter out the ambient electrical noise and provide an accurate measurement of the surface cortical activity. Each pair of electrodes used to record an EEG measurement is called a lead, and only the change in electric potential in the vector parallel to the lead will be recorded; any component of electric activity perpendicular to the lead axis does not contribute to the difference in electric potential between the leads because the axis perpendicular to the lead vector falls along an isoelectric line for that lead and thus cannot be measured. EEG leads are arranged to measure electrical activity with an axis of measurement through the center of the head, which means that the EEG preferentially measures the portion of the cerebral cortex that is parallel to the skull (i.e. the outermost portion of the gyri), while the electric fields from cortical surface within the sulci cannot be measured because they are perpendicular to the electrodes. Moreover, the sulci are, necessarily,
anatomically arranged so that they are opposite each other, with the net result being that electrical activity in adjacent sulci cancels each other out. Nonetheless, EFs have been measured throughout the entire brain using electrocorticography, which uses a grid of electrodes applied directly to the surface of the brain to record electrical field potentials with much greater resolution than can be achieved with an EEG.

EEG and electrocorticography measurements are limited to the surface of the cerebral cortex, but subsequent research has demonstrated that extracellular EFs are present throughout the entire mammalian brain. Electrophysiological recording techniques for field potentials measure the voltage at an electrode implanted somewhere within the brain and compare it to a reference point either elsewhere within the brain, or in a bathing solution outside the brain; similarly to the EEG, these measurements produce a voltage recording that represents the net electrical activity of all cells within a certain distance (the recording sensitivity) of the electrode, and this field potential can be converted into an EF when compared to the recorded voltage at another recording electrode located at a known distance from the first. Multiunit extracellular electrodes are similarly implanted in the brain; they have a greater sensitivity than electrodes used for recording field potentials, so they are used to measure action potential activity from multiple axons within the region of the recording electrode. All of these recordings are traditionally used to measure action potential firings from increasingly small populations of cells, which result in alternating current (AC) signals of varying frequency. Nonetheless, these recording methodologies also consistently demonstrate a background dcEF in the CNS parenchyma, as the resting extracellular potential as compared to ground is not 0. (Of note, the EEG can also
measure dcEFs, although these measurements are not currently of clinical use and so are only typically recorded in experimental protocols.) However, experiments employing these techniques rarely report the extracellular dcEFs because they were developed to measure populations of axons firing action potentials; the measurement of interest for these types of recordings is the difference between electrical activity during the action potential and at rest, so the resting electric potential (i.e. the steady-state dcEF) is typically subtracted out of the measurement as the recordings are being made so that the baseline reading is 0 (using a direct current (DC) offset through a filter in the preamplifier that is attached to most electrophysiology set-ups).

Although dcEFs have been consistently disregarded as little more than background noise, a growing number of studies has specifically sought to measure these endogenous electrical signals. Physiologic EFs have been found in the vertebrate CNS during embryogenesis, in adulthood, and following injury. While it is clear physiologic EF strengths in the CNS vary in magnitude similarly to how EFs vary in peripheral tissues – high during developing and following injury, low in mature tissues – the precise magnitudes of these EFs have been less thoroughly explored because their measurements are more difficult to obtain. Shi and Borgens demonstrated that the axolotl neural tube maintains a voltage of 40-90 mV across itself, while Hotary and Robinson measured a voltage of 21 ± 2 mV across the Xenopus neural tube. A recent study by Cao and colleagues (2013) used an ex vivo preparation to demonstrate that a low EF of 2 – 5.7 mV/mm is present in the rostral migratory stream of the adult mouse brain, and that this EF is likely sustained due to a constitutive inward Na⁺ current of 1.5 ± 0.6 µA/cm² across the pial surface in the SVZ of the lateral ventricles and a
constitutive outward \( \text{Na}^+ \) current of 1.6 ± 0.4 \( \mu \text{A/cm}^2 \) across the surface of the olfactory bulb (OB)\(^{269} \). These ionic currents are produced by asymmetric distributions of the \( \text{Na}^+/\text{K}^-\text{-ATPase} \) in the lining of the brain, with the transporters located along the basolateral domain of ependymal cells in the SVZ and along the apical domain of astrocyte end feet in the glia limitans of the olfactory bulb\(^{269} \). Assuming that the outward ionic current over the intact olfactory cortex is representative of the direction of ionic current across the entire mammalian cortex, and knowing that the ionic current changes direction upon injury, this suggests that the orientation of an injury-induced EF in the mammalian brain would place the anode at the lesion site while the cathode would be in the surrounding intact tissue. A subsequent study used an \textit{ex vivo} preparation to demonstrate that the EF within adult mouse SVZ is 31.8 ± 4.5 mV/mm, but they measured this EF within a slice culture 300 \( \mu \text{m} \) thick so this reported EF seems to be more representative of a reading from an injured brain than one from an uninjured brain\(^{290} \). While the EF strength has not been measured upon injury in the mammalian brain, a group from the lab of Richard Borgens used an \textit{ex vivo} guinea pig SCI model to explore how extracellular EFs change in the injured CNS. By measuring the ionic current density around the spinal cord before and after a complete transection, they found that the injury induced an initial 100-fold increase in over 60 seconds, but that the EF decayed to a 10-fold increase that was sustained for at least the subsequent hour\(^{291} \). However, the Borgens lab’s experiment suffered from the same problem as did those experiments from by Cao: they used an \textit{ex vivo} model to measure electrical activity within the allegedly-intact spinal cord, neglecting the fact that removing the spinal cord from the animal is itself a severe injury that disconnects the tissue from its vascular
supply and disrupts its epithelia (for a further discussion of the problems of using ex vivo models and invasive recording techniques for measuring EFs, see Chapter 4: Considerations when measuring bioelectricity, page 174). Nonetheless, these biased studies suggest that the embryonic CNS produces EFs about 100-fold greater than those in the adult CNS, and that injury induces only a 10-fold increase in EFs over baseline. The reported change in EF intensity induced by injury in the mammalian CNS is similar to the magnitude of EFs induced by injury in other mammalian tissues. While neither the CNS nor peripheral tissues typically demonstrate robust regeneration in mammals, therapeutic EFs have been shown to promote regeneration in several peripheral tissues, suggesting that therapeutic EFs may similarly be able to promote a regenerative cellular injury response in the injured mammalian CNS.

*Extracellular EFs regulate cellular physiology in the CNS*

Physiologic dcEFs have been measured throughout the mammalian CNS, and EFs of these intensities influence the behavior of multiple neuronal and glial cell types *in vitro*\(^{269, 290, 291}\). Many cell types from the CNS respond to exogenous EFs, but the magnitude of EFs used in these studies often far exceeds the physiologic range reported in the CNS, which <10 mV/mm in the rostral migratory stream\(^{269}\), and up to 50 mV/mm in the hippocampus (sustained only for several minutes following an evoked potential)\(^{292}\). Upon exposure to extracellular EFs in culture ranging from 4-1,000 mV/mm for 3-20 hours, neurons from the Xenopus laevis neural tube preferentially grow towards the cathode and retract from the anode\(^{237, 239, 293-295}\). The rate of cathodal neurite outgrowth of Xenopus laevis neurons is dependent on Ca\(^{2+}\)\(^{296}\). The orientation of
EF-induced neurite outgrowth likely involves multiple mechanisms as some studies demonstrate that this directionality is Ca$^{2+}$ dependent$^{297}$ while other groups have found that neurite outgrowth remains directional even in Ca$^{2+}$-free media$^{298}$; spinal neurites have also been shown to mediate EF-induced neurite outgrowth through acetylcholine receptors in their growth cones, which are activated through autocrine acetylcholine release$^{299}$. Neurons from dorsal root ganglia (DRGs) in chick embryos also increase their rate of outgrowth$^{300}$ and preferentially grow towards the cathode of an EF of 15-140 mV/mm over 3.7-6.3 hours$^{294}$. Mammalian neurons similarly increase neurite outgrowth length parallel to an applied EF with a preference towards the cathode$^{293}$, and outgrowth is further improved by co-culture with Schwann cells$^{301}$ or astrocytes$^{240}$.

While the general conclusions from these studies are that neurons preferentially extend neurites towards the cathode and retract those facing the anode, the effects are often mixed, suggesting that there may be a heterogeneous response to EF exposure that depends on the sub-type of neuron tested. This is reinforced by observations that PC12 cells, which are a neuronal cell line derived from the rat adrenal medulla, extend neurites towards the anode over 48 hours’ exposure to 5-100 mV/mm EF$^{302}$, while neurite outgrowth from neurons derived from embryonic zebrafish is unaffected by a 100 mV/mm EF after 20 hours$^{303}$. EFs may also interact with the culture media and substrate to affect the orientation of Xenopus laevis neurite outgrowth$^{239}$, and chemoattractive and repulsive effects of CSPGs are modified by dcEFs in vitro$^{304}$. Moreover, the substrate through which neurites sprout interacts with EFs to determine the orientation of these sprouting neurites. DRG neurites project parallel to the polarity of a 50 mV/mm EF, and they will follow the orientation of their culture substrate when
grown without an EF; if the DRG is cultured on a substrate oriented perpendicularly to an EF, the substrate cue overrides the 50 mV/mm EF and the neurites will grow perpendicularly to the EF orientation\textsuperscript{305}. Interestingly, a 50 mV/mm EF increases the length of neurite outgrowth regardless of whether the DRGs are on a substrate growing with an orientation parallel or perpendicular to the EF orientation, showing that the EF effect on neurite outgrowth is independent of the orientation of the substrate\textsuperscript{305}.

Neuronal populations vary throughout the CNS and send projections to unique downstream targets through specific fiber tracts composed of different combinations of ECM molecules. The heterogeneity of EF effects on the rate and direction of neurite outgrowth, and the dependence of neurite outgrowth on the ECM substrate and on the morphology of glial cells within the parenchyma, may facilitate the development of the complex CNS cytoarchitecture: the same physiologic EFs may interact with the ECM to create precise signaling cues that guide different subpopulations of neurons to grow in different directions through different tracts within the CNS.

EFs also affect NPCs within the vertebrate CNS. NPCs from the mouse and rat migrate cathodally on exposure to EFs as low as 3 mV/mm, and the speed and directedness of this migration increases as EFs increase up to 400 mV/mm\textsuperscript{269, 290, 306-308}. EFs induce membrane asymmetry in NPCs through electroosmosis of membrane receptors, and they require the Wnt-GSK3β signaling pathway to transduce the EF signal into an electrotaxic response\textsuperscript{309}. Interestingly, a 3-5 mV/mm dcEF was recently been reported in the RMS, a pathway through which neuroblasts migrate from their place of origin in the SVZ to their destination in the OB, of adult rats oriented with the anode in the SVZ and the cathode in the olfactory bulb; as this is the same direction as
neuroblasts migrate on exposure to the same EF intensity in vitro, it suggests that this physiologic EF may be contributing to guiding neuroblast migration in vivo. EF intensity also affects NPC differentiation with 115 mV/mm and 437 mV/mm EFs promoting neurogenesis while 46 mV/mm does not alter the normal gliogenesis from hippocampal NPCs, and EFs have recently been shown to stimulate neuronal differentiation from human mesenchymal stem cells.

Astrocytes respond to EFs by aligning their processes perpendicularly to the EF vector at strengths above 100 mV/mm, and the extent of process alignment increases as the EF strength increases. EFs affect astrocyte glycolysis, decreasing metabolic rate at 50 mV/mm, having no effect at 100 mV/mm, and increasing the rate of glycolysis above 150 mV/mm. Only one study has explored EF effects on microglia, and it has demonstrated that EFs of 100 mV/mm increase the number of processes on the cells, and that these processes are oriented perpendicularly to the EF. Meanwhile, the effect of EFs on NG2 cells and on oligodendrocytes has not been explored. Thus, it is clear that multiple cells from the mammalian CNS respond to EFs, and that this response depends on the EF intensity and the extracellular signaling environment. However, these studies have not explored whether physiologic EFs contribute to the cellular response to injury and the regenerative potential in the CNS.

Although the ability of EFs to induce cellular behaviors associated with regeneration has not been explored in vivo, circumstantial observations suggest that EFs may be involved in certain cellular responses to injury. NPCs migrate physiologically from the SVZ to the OB, but they can be redirected to migrate towards sites of cortical injury, as the cortical EFs presumably demonstrate a similar
change in EF magnitude upon injury to that change observed in other tissues (see page 48), this injury-induced EF may direct NPC migration. In spite of the NPC migration towards the lesion site, there is not robust neurogenesis upon injury in the mammalian CNS\textsuperscript{74,121}. Elevated EFs are sufficient to drive neurogenesis from NPCs\textsuperscript{244,310}, but EFs produced by the non-regenerating tissues such as the mammalian cortex are known to be lower than those EFs found in regenerating tissues\textsuperscript{215,227,315}. As NPCs undergo electrotaxis at much lower EFs than are necessary to drive neurogenesis\textsuperscript{244}, injury-induced EFs in the mammalian cortex may be robust enough to drive NPC electrotaxis to the lesion site but insufficient to induce neurogenesis from NPCs once they arrive at the injured tissue. Assuming that physiologic EFs are involved in the NPC response to injury, this suggests that elevating the EFs at the lesion site would cause a more robust NPC migration and that it would also stimulate neurogenesis among NPCs at the lesion.

\textit{In vivo} evidence supports the hypothesis that therapeutic EFs may promote regeneration when used in the mammalian CNS. Fehlings & Tator (1992) used axon tracing to show that dcEFs promoted functional recovery of acutely injured spinal axons following clip compression injury in a rat model of SCI, and that the polarity of the applied EF was important to this effect\textsuperscript{316}. A series of studies by the Borgens lab showed that applied EFs promote recovery of the cutaneous trunci muscle (CTM) reflex in 25\% of guinea pigs following spinal cord hemisection (0\% of control guinea pigs demonstrated improvements)\textsuperscript{317}; qualitative observations showed that guinea pig sensory neurons projected axons towards a spinal cord hemisection and that EF application promoted their regeneration past the lesion and then through their original tract\textsuperscript{318}. In this study, the applied EF was oriented with the cathode rostral along the
spinal cord, and they emphasized sensory neurons because they previously found that spinal neurites preferentially projected towards the cathode. In a subsequent study, they reversed the orientation of the applied dcEF every 15 minutes following spinal cord transection in dogs under the assumption that all neurites project towards the cathode, so that the periodic EF reversal would promote outgrowth of all neurites (remember, according to the *in vitro* studies, EF-induced neurite cathodal outgrowth is faster than anodal retraction); while they found that dcEFs improved functional recovery after both 6 weeks and 6 months of treatment, the sample size was small and they did not histologically evaluate the impact of EFs on axon regeneration. Using rats, Borgens found that oscillating extracellular EFs after a spinal cord transection improved astrocyte alignment perpendicularly to the lesion site, and that the applied EF (technically a 40 µA current, with the EF neither measured nor calculated) decreased the number of GFAP+ astrocytes at the lesion site. However, these studies apply extraordinarily low EF intensities *in vivo* without measuring the magnitude of the EFs that are actually induced by this application at the lesion site, and these treatments are attempted in spite of the fact that neither the magnitude nor the physiologic role of injury-induced EFs in the cellular response to injury have been elucidated in the mammalian CNS. Moreover, the studies that reverse the orientation of the applied EF every 15 minutes do not apply EFs in a way that recreates the physiologic signaling environment that would otherwise allow regeneration to proceed spontaneously; instead, they use EFs to try and artificially promote axon regeneration by taking advantage of the fact that spinal axons preferentially grow towards the cathode faster than they retract from the anode. Although these studies have found that EFs promote axon regeneration, the EFs are
not applied in a way that recreates the physiologic lesion environment. Thus, these experiments do not actually test the hypothesis that physiologic EFs activate the otherwise latent physiology that would allow for spontaneous regeneration.

**Conclusion**

We have seen that bioelectric fields are physiologically produced in all tissues – including the CNS – in both mammalian and non-mammalian vertebrates, and that these endogenous EFs vary in intensity as a function of physiologic state. Evidence strongly suggests that elevated EFs are both necessary and sufficient to influence embryonic development and epimorphic regeneration in multiple vertebrate species. Moreover, EFs regulate a consistent series of behaviors – migration, proliferation, and differentiation – in a diverse variety of cell types from different tissues and germ cell layers. Physiologic EFs have been found in the mammalian CNS, and elevated EFs regulate these same behaviors in cells from the CNS; however, the EF intensities explored in these experiments have not generally been within the range previously demonstrated in the CNS, and effects of physiologically-relevant EF intensities have not been explored. Furthermore, therapeutically-applied EFs appear to promote histological and functional recovery after CNS injury, but these approaches did not attempt to recreate the physiologic EFs found in the CNS of regenerating animals. Thus, evidence suggests that endogenous EFs have the potential to serve as an important signal that regulates the cellular response to injury and determines the regenerative potential in the injured mammalian CNS, but the relevance of physiologic EF intensities on cellular activity needs to be explored.
Chapter Conclusions

Although mammals do not spontaneously regenerate, they retain a latent regenerative potential through which each of the cell types involved in wound repair can be stimulated to promote regeneration. Epimorphic regeneration proceeds through specific cellular behaviors – migration, proliferation, dedifferentiation, and differentiation – that are fundamentally important for regeneration. These behaviors are regulated by injury-induced expression of master regulatory genes: differences in the injury-induced expression of master regulatory genes, and the cellular behaviors that they stimulate, are associated with differences in regeneration; and master regulatory genes can induce regeneration in tissues where it is typically not expressed.

Endogenous electric fields are elevated at sites of injury and they have been shown to regulate each of the cellular behaviors necessary for epimorphic regeneration. Elevated EFs are necessary and sufficient to stimulate both embryogenesis and epimorphic regeneration in mammalian and non-mammalian vertebrates alike. This suggests that EFs may be the stimulus that initiates and regulates these processes, either by acting upstream of the master regulatory genes and inducing their expression, or by direct electrostatic interactions with the cellular receptors that transduce these genes. Physiologic EFs and their role in repair and regeneration have largely been studied in peripheral tissues; endogenous EFs have also been measured in the mammalian CNS, which suggests that physiological EFs similarly regulate wound repair and regeneration in the mammalian CNS. However, there is a remarkable dearth of evidence exploring the effect of physiologic EFs on each of the cellular behaviors necessary for the injury response and regeneration in the CNS.
The work presented in this dissertation was conducted with the overarching hypothesis is that the role of EFs in regulating wound repair and regeneration in peripheral tissues is conserved in the mammalian CNS. Specifically, we hypothesized that physiologic EFs induce a cellular response to injury characteristic of that observed \textit{in vivo}, and EF intensities associated with regenerating tissues would modify the cellular response towards one associated with regeneration. We chose to explore how EFs influence astrocytes, because the astrocytic response to injury is a key determinant in CNS regeneration, and because astrocytes facilitate regeneration in non-mammalian vertebrates through the same behaviors required for epimorphic regeneration in the periphery. We describe experiments in which we explore the role of EFs associated with intact, injured mammalian, and regenerating non-mammalian vertebrate tissues on regulating behaviors in cortical and cerebellar astrocytes in chapters two and three, respectively. In chapter four, we explore an approach to measure bioelectricity in the mammalian brain longitudinally after injury. While we were also interested in the mechanisms by which astrocytes transduced these behaviors, these studies are limited to an exploration of behaviors as the question of underlying physiology is ancillary to that of whether physiologic EFs induce the necessary astrocytic response: if EFs do not induce the behaviors necessary for regeneration, the mechanisms by which astrocytes transduce EFs are irrelevant. Instead, we discuss the physiologic implications of our findings together with the evolutionary origins of EFs and regeneration in chapter five, where we explore the concept of bioelectricity as a unifying force that regulates development and regeneration among all vertebrates.
Figure 1.1: Requirements for CNS regeneration

A simplified illustration of how injury to the CNS disrupts neural circuitry, using spinal cord injury as an example. (A) In the intact spinal cord, lower motor neurons (diamonds) receive descending projections from upper motor neurons through the corticospinal tract, as well as local connections between spinal interneurons (filled circles). (B) After a focal injury, the original neural circuitry is disrupted both because neurons within the lesion site have been damaged, and also because axons in the corticospinal tract passing through the lesion site have been disconnected from their original targets. Functional recovery through the complete regeneration of the original neural circuitry requires four basic steps: 1) each of the cell types present within the damaged tissue (in this example, the lower motor neurons and the interneurons) must be replaced, 2) disconnected axons, including those projecting past the lesion from distant nuclei and those from newly-generated neurons, must be capable of sprouting, 3) axons must sprout past the lesion and through the distal intact parenchyma, following local cues to their original target nuclei, and 4) axons must be able to reestablish their appropriate connections within their target nuclei. (This image was modified from Ben-Hur, 2010 and is reprinted here under the “fair use” limitation in title 107 of the U.S. copyright law.)
Figure 1.1: Requirements for CNS regeneration
Figure 1.2: Astrocytic response to CNS injury and the glial scar

An illustration and matching image GFAP-immunolabeled astrocytes, illustrating the morphological changes that astrocytes undergo as they become reactive; the images are aligned such that the illustrations are directly over their matching immunolabeled counterparts. (A) Healthy astrocytes in the intact parenchyma. (B) Reactive astrocytes become hypertrophic, developing thicker processes and increasing their expression of certain cytoskeletal elements, including GFAP (notice the brighter staining in the immunolabeled images). (C) Astrocytes in the glial scar border (white notched arrow) are hypertrophic and extend their processes circumferentially around the lesion; this response restricts immune cells (yellow cells in the illustration) to the lesion site, but it also prevents axons from sprouting past the lesion border. (The immunolabeled images are from unpublished observations in the Colello lab. The illustration in this image was modified from Sofroniew, 2009 and is reprinted here under the “fair use” limitation in title 107 of the U.S. copyright law.)
Figure 1.2: Astrocytic response to CNS injury and the glial scar
Figure 1.3: Comparison of astrocytes in wound repair among vertebrates

The astrocytic response to injury results in the formation of a chronic glial scar that inhibits axon regeneration in mammals, while astrocytes in non-mammalian vertebrates facilitate axon regeneration. (A, B) 3 weeks after a contusive spinal cord injury in the rat, astrocytes, which are immunolabeled for the intermediate filaments vimentin (A) and GFAP (B), surround the lesion site; these astrocytes form a barrier past which axons cannot sprout so, instead of regenerating, this cystic cavity resolves into a chronic glial scar. In contrast, 3 weeks after a complete spinal cord transection in zebrafish (C-F), astrocytes expressing both GFAP (C) and nestin (D) migrate into the injury site and extend elongated processes across the lesion cavity. (E, F) Astrocytes (immunolabeled for GFAP, red) function as a cellular bridge that facilitates axon regeneration (green) past the injury site. (The immunolabeled images in panels A and B are from unpublished observations in the Colello lab. The images in panels C-F are modified from Goldshmit et al, 2012\(^79\) and are reprinted here under the “fair use” limitation in title 107 of the U.S. copyright law.)
Figure 1.3: Comparison of astrocytes in wound repair among vertebrates
Figure 1.4: Mechanisms of epimorphic regeneration

Certain vertebrates, including Urodele amphibians, demonstrate robust epimorphic regeneration. (Top left) A newt is shown several months after tail amputation, demonstrating complete regeneration of a new tail past the amputation plain (indicated by the black line) with the new tail being anatomically and histologically indistinguishable from the original tail (gross anatomical images before amputation are shown at the top right). The basic cellular processes underlying epimorphic regeneration are conserved among all of the tissues and species in which these regenerative processes have been studied. These processes are illustrated for the Urodele amphibian limb, and matching gross anatomical images of the regenerating newt tail corresponding to these illustrations are shown. Immediately upon amputation, epidermal cells from the wound margin (indicated in yellow) migrate to the wound and cover it in a wound epithelium. The wound epithelium, which assumes the same function as the apical ectodermal cap during embryonic limb development, induces mesenchymal cells to dedifferentiate into pleuripotent progenitors (light blue). These progenitors form a cell mass called a Regeneration Blastema immediately beneath the wound epithelium and replace the amputated tissue through sustained proliferation. As the regenerating limb elongates, progenitor cells at the base of the blastema re-differentiate into cells from both ectodermal and mesodermal lineages, replacing the full complement of tissues and structures that were originally present. (The illustration was modified from Stewart et al, 2007, and the images of the newt tail were modified from McLean & Vickaryous, 2011; both sets of images are reprinted here under the “fair use” limitation in title 107 of the U.S. copyright law.)
Figure 1.4: Mechanisms of epimorphic regeneration
Figure 1.5: Electrogenic cellular ion transport produces bioelectricity

(A-C) An illustration of the physiologic mechanisms of electrogenic ion transport across cell membranes. Two Na\(^+\)/K\(^+\)-ATPases are shown in a portion of the cell membrane, as are only those cations (Na\(^+\) and K\(^+\)) together with their counter-ions (Cl\(^-\)) that are transported. (A) A hypothetical starting point where there is an equal number of anions and cations both on the intracellular and extracellular compartments. (B) The Na\(^+\)/K\(^+\)-ATPases transport 3 Na\(^+\) ions out of the cell and 2 K\(^+\) ions into the cell; this results in a net loss of 1 positive charge for every cycle of transporter (C), which produces a negative trans-membrane electrical potential (inside of the cell relative to the outside). If a Na\(^+\) channel present in the cell membrane opens, Na\(^+\) diffuses through the channel down its electrochemical gradient and across the cell membrane. (D) When the Na\(^+\)/K\(^+\)-ATPases are uniformly distributed around the cell, the entire cell develops a relatively uniform membrane potential. (E) If the cell is organized into distinct domains, the transmembrane potential and concentration gradients may not be uniform across the cell. For example, in epithelial cells (F), Na\(^+\)/K\(^+\)-ATPases are concentrated in the basolateral domain and produce an electrochemical gradient just across this portion of the membrane (F, left). (F, center) Na\(^+\) diffuses down the resulting concentration gradient into the cell through Na\(^+\) channels, which are concentrated in the apical domain, and the outside of tissue consequently develops a negative charge. (F, right) Continued transport of Na\(^+\) across the basolateral membrane sustains this net Na\(^+\) current across epithelial cells and into the tissue, and produces a tissue-positive trans-epithelial electrical potential \(V_{\text{TEP}}\).
Figure 1.5: Electrogenic cellular ion transport produces bioelectricity
Figure 1.6: Physiology of electric field generation by tissues

An illustration demonstrating how electrogenic ion transport across epithelial cells and physical properties of tissues result in the formation of endogenous electric fields. Three adjacent epithelial cells are shown bound together through tight junctions (indicated by the green ovals); consequently, distinct apical and basolateral domains form that segregate different membrane proteins and result in a net inward Na\(^+\) current (see Figure 1.5F for further details). (A) Across the intact epithelium, these tight junctions prevent paracellular ion diffusion, so the net inward current is sustained. Upon injury to the epithelium (B), damage to the cells decreases this resistance and allows Na\(^+\) to diffuse down its electrochemical gradient, resulting in a reversal of the current at the lesion site. (C) During embryogenesis, tight junctions disappear at sites of rapid tissue growth, so resistance to paracellular ion diffusion decreases and results in large currents being produced.
Figure 1.6: Physiology of electric field generation by tissues
Figure 1.7: Magnitude of electric fields in injured tissues

Epithelial damage short-circuits the trans-epithelial potential and allows Na⁺ to diffuse down its electrochemical gradient across the epithelium and out of the tissue. Within the tissue, large EFs develop between the intact tissue at the margin of the lesion and the lesion epicenter; the intact tissue becomes the anode of this injury-induced EF because it sustains its inward-positive TEP, while the lesion site becomes the cathode because the TEP collapses to 0 mV upon injury. The total charge must be conserved, so a current loop develops between the wound and the surrounding tissue such that the total current traveling through the lesion site ($I_W$) must be equal to the total current traveling through the surrounding healthy tissue ($I_T$) such that $\Sigma I_W - \Sigma I_T = 0$. Because the intact tissue has a greater surface area, less current travels through any individual point of intact tissue than through the wound. Consequently, the EFs are greatest at the wound, and decrease in magnitude with distance. The relationship between the relative magnitude of the injury-induced EF within the tissue and the distance from the lesion epicenter ($r$) is illustrated by the graph.
Figure 1.7: Magnitude of electric fields in injured tissues
Figure 1.8: Cellular transduction of electric fields by electroosmosis

(A) An illustration of a cell with membrane proteins randomly distributed throughout its membrane, and a uniform distribution of Na\(^+\) and Cl\(^-\) ions in the surrounding environment. The exemplar membrane proteins are ligand receptors responsible for chemotaxis, with receptors mediating a chemoattractive effect (green) having a relative positive charge while those receptors mediating a chemorepulsive effect (red) have a relative negative charge. (B) The cell is exposed to an external electric field, with the anode (+) towards the right side of the cell and the cathode (−) towards the left of the cell; the lines indicate the orientation of the EF vector, which is the direction that a freely-diffusible positive charge would move within the electric field. (C) The external EF interacts with the ions around the cell, creating electrostatic forces on each ion (the direction of which is indicated by the dashed arrows). As a result of the electrostatic force created by the external EF, Na\(^+\) and Cl\(^-\) ions redistribute towards the cathode and anode, respectively (D); this results in an accumulation of anions and cations at opposite ends of the cell, which, in turn, results in an electrostatic force acting on the charged membrane receptors (indicated by dashed lines). (E) As a result of the electrostatic forces between the ions and the membrane proteins, the membrane proteins undergo lateral diffusion through the membrane to opposite faces of the cell, resulting in a non-random distribution of these proteins such that chemoattractive receptors accumulate towards the anodal side of the cell while chemorepulsive receptors accumulate cathodally. (F) An illustration demonstrating how a stronger external EF results in a greater redistribution of these membrane proteins. Illustration by Matthew Baer, using ChemBioDraw v. 13.0.
Figure 1.8: Cellular transduction of electric fields by electroosmosis
Chapter 2: Cortical Astrocytes

Chapter overview

The previous chapter provides a review of CNS injury, establishing that the CNS in all vertebrates has a latent regenerative potential, and emphasizing the role of astrocytes and radial glia in determining whether tissue repair occurs through scar formation or regeneration. In summary, physiologic EFs regulate cellular physiology, and the magnitude of EFs induced by injury determines whether wound repair occurs through scar formation or epimorphic regeneration: a 50-100 fold increase is necessary to stimulate epimorphic regeneration, while a 10-fold increase is associated with scar formation. A 10-fold EF increase has been found in many mammalian tissues, including the CNS, and experimentally increasing these EFs has been shown to enhance regenerative outcomes in peripheral tissues. With this in mind, we hypothesized that the role of EFs in regulating wound repair and stimulating regeneration is conserved in the mammalian CNS. Consequently, physiologic EFs should induce a cellular response characteristic of that seen in vivo, and EFs elevated above those found physiologically in the mammalian CNS should alter this response to induce a more regenerative outcome. As the astrocytic response to CNS injury in mammals is crucial in determining the reparative outcome, we exposed cortical astrocytes to EF intensities associated with intact and injured mammalian tissues, as well as to those EF intensities measured in
regenerating non-mammalian vertebrate tissues, to determine whether physiologic EFs regulate astrocytic behaviors in the intact CNS, stimulate behaviors associated with their characteristic response to injury, and modify this injury response towards one associated with regeneration.

(Except for a portion of the methods, a portion of the results, Figure 2.3 and Figure 2.7, and table 2.1, this chapter is included in a manuscript in submission in: Baer ML, Henderson SC, Colello RJ. Elucidating the Role of Injury-Induced Electric Fields (EFs) in Regulating the Astrocytic Response to Injury in the Mammalian Central Nervous System. PLoS One; Manuscript submitted for publication.)

Introduction

The mammalian central nervous system (CNS) demonstrates limited functional recovery following traumatic injury, in large part because of the astrocytic response to the injury. In contrast, many non-mammalian vertebrates demonstrate a profound capacity to regenerate their tail and spinal cord after amputation, reconnect their spinal cord after complete transection, and even replace large regions of their brain lost to injury. Common among both groups of vertebrates, injury to the CNS induces an astrocytic response that has been well documented and is characterized by directional migration to the lesion site, by an enhanced rate of proliferation, and by changes in morphology. Each of these behaviors occurs in a similar temporal profile, relative to the onset of the injury, for both phylogenies. Unique among regenerating species, astrocytes also form a cellular bridge across the lesion consisting of highly-aligned bipolar processes that guide sprouting axons past the injury.
The absence of regeneration in mammals, despite the highly conserved astrocytic behaviors that occur following injury, suggests that all vertebrates use a conserved stimulus to induce the injury response and that this stimulus does not reach the threshold necessary to induce regeneration in mammals. If this is the case, this stimulus would be an ideal therapeutic target whose modification to recreate the environment present in regenerating tissues may enhance regeneration in the mammalian CNS.

One stimulus candidate found within the injury site that may direct astrocyte behavior is direct-current extracellular electric fields (EFs). EFs are produced by spatial variations in epithelial cell ion pump activity (see Chapter 1: Cells physiologically produce and detect bioelectric signals, page 33), which create voltage gradients within tissues. EFs have been measured in many different vertebrate tissues and have been shown to directly regulate multiple cellular behaviors. For example, ionic currents ranging from 1 to 1000 µA/cm² have been recorded in intact, injured, and developing tissues and have been shown to influence cellular migration, proliferation, differentiation, metabolism and process formation in a variety of ectodermally and mesodermally-derived cell types in vitro. These currents, which are low in mature tissues and are elevated during development and after injury at the site of growth or other cellular activity, generate corresponding electric fields that generally range from 1 to 200 mV/mm and have been reported to be as great as 1800 mV/mm. During embryogenesis, elevated EFs are necessary for limb development and neurulation. EFs also increase after injury, and a 50-100-fold increase is necessary for limb and tail regeneration in non-mammalian vertebrates.
Moreover, inhibiting EFs blocks regeneration\textsuperscript{275, 281, 330, 331}, whereas regeneration can be stimulated in non-regenerating amphibians by experimentally increasing EFs at the amputation site\textsuperscript{277, 283, 284, 319, 332-334}. Studies have also established that EF’s are present in the mammalian CNS\textsuperscript{269, 290, 291}. Although EFs have not been measured in the mammalian CNS \textit{in vivo}, ex vivo recordings of the mammalian brain demonstrate that low (3.5-5 mV/mm) EFs are present within the rostral migratory stream, and that EFs of this magnitude can direct neuroblast migration \textit{in vitro}\textsuperscript{269}. Furthermore, slice culture induces approximately a 10-fold increase of these EFs to 31.8 ± 4.5 mV/mm\textsuperscript{290}, and spinal cord injury has been shown to induce a rapid 10-fold increase in current density \textit{ex vivo}\textsuperscript{291}. As EFs and current density are proportional to the resistivity of the tissue\textsuperscript{335}, it is reasonable to assume that injury to the CNS induces a similar elevation in EF intensity. Together, these studies suggest that the physiologic EFs produced by the injured CNS may be capable of, and consequently responsible for, driving the astrocytic response to injury. Moreover, the EF intensities recorded in regenerating tissues in non-mammalian vertebrates may represent an intensity threshold that is necessary to induce astrocytic behaviors more favorable for regeneration in the mammalian CNS.

Previous work from our lab and others has shown that EFs elevated at levels associated with non-mammalian vertebrates cause mammalian astrocytes to assume a bipolar morphology and align their processes\textsuperscript{240, 241}, which is consistent with their demonstrated morphological changes during regeneration \textit{in vivo}. Furthermore, we have shown that the processes of these EF-exposed astrocytes are significantly more permissive to neurite outgrowth\textsuperscript{240}. Similarly, Schwann cells exposed to high EF’s
produce processes that are more conducive to neurite outgrowth. However, it is unclear whether EFs contribute to the astrocytic response in the injured mammalian CNS. In the current study, we tested the hypothesis that the EFs produced physiologically by the injured mammalian CNS are sufficient to induce behaviors associated with the astrocytic injury response. Furthermore, we hypothesized that increasing these EFs to levels found in regenerating non-mammalian vertebrates would stimulate a more robust behavioral response, and that only these elevated EFs would cause morphological changes that are closely associated with regeneration in vivo. Our findings in this study suggest that injury-induced EFs are an important stimulus for the astrocytic response to injury, and that EFs may represent a novel target to enhance the regenerative potential in the mammalian CNS.

Methods

Cell source and culture methods

Rat cortical astrocytes harvested from the cerebral cortex of animals at post-natal day 2 (P2) were purchased from ScienCell (cat # R1800). Cultures have greater than 99% purity as determined with GFAP immunolabeling by ScienCell; all of the astrocytes used for these experiments came from the first five passages after the initial thaw. Astrocyte cultures were maintained according to the protocol recommended by ScienCell. Briefly, astrocytes were thawed into poly-L-lysine (ScienCell # 0413) coated T75 culture flasks containing astrocyte media (pH 7.4; ScienCell AM-a 1831) supplemented with 2% fetal bovine serum (FBS; ScienCell # 0010) and 1%
penicillin/streptomycin (ScienCell # 0503). Cultures were maintained in a humidified 37 degree Celsius (°C) incubator with a 5% carbon dioxide (CO₂) atmosphere, and culture media was changed every 2-3 days. Once the cultures reached confluence, approximately 5,000 astrocytes were sub-cultured into each EF chamber (see description below) for migration, proliferation, and morphology experiments. For proliferation assays, the nucleotide anologue bromo-deoxyuridine (BrdU; Invitrogen, cat # 00-0103 diluted 1:100 in astrocyte media) was added to the culture for the last 6 hours of the electric field exposure. Each experiment was replicated at least three times using cortical astrocytes derived from different animals (different lot numbers of astrocytes were purchased from ScienCell), with all cells used in a given experiment being sister cultures derived from the same passage.

Electric field application and chamber design

Electric field chambers were constructed in a similar manner to those described by Babona-Filipos et al (2012) and Song et al (2007), with some modifications (Figure 2.1) as described below. EF chambers were coated with fibronectin (ScienCell # 8248) for 30 minutes, rinsed twice with deionized water, and allowed to dry. For EF experiments lasting longer than 24 hours, culture media and salt bridges were completely replaced every 24 hours. Constant-current electric fields were applied to the cells by connecting the EF chamber to a power supply; the anode and cathode determine the orientation of the electric field and are indicated in figures as either A/C or +/-, respectively. The magnitude of the electric fields were calculated according to the formula \[ E = \rho l / A, \] where \( E \) is the electric field strength (millivolts per millimeter;
mV/mm), the resistivity of the media (ρ) was measured as 700 Ohm-millimeters (Ωmm), and the cross-sectional area of the electric field chamber (A) is calculated in mm$^2$ (Figure 2.1B). EF strength is controlled by specifying the applied current and by changing the cross-sectional area of the cell culture chamber (by using coverslip spacers of different thicknesses, or by changing the width of the cell culture chamber). Two constant current power supplies were used, including Bio-Rad 1000 Power Pack, and Stoelting Precision Current Source 51413, in order to provide the full range of currents needed for these experiments. Ammeters in series with the electric field chambers were used to monitor the value of the applied current throughout the experiments. The power-packs were connected to Ag/AgCl electrodes (made by washing silver wire, Alfa Aesar 45852, in an HCl/HNO$_3$ solution for 15 seconds and then rinsing it in dH$_2$O), which were immersed in 50 mL flasks containing Steinberg Solution; these were connected to the electric field chambers through salt bridges made from a 2% agarose solution suspended in 2 mL plastic pipets that were bent into a U shape.

EF chambers were constructed using 50 x 7 mm glass-bottom petri dishes (Ted Pella, #14027) (Figure 2.1). Acid-washed 22 mm x 22 mm - 1.5 coverslips (average thickness 0.17 mm) were cut into two equal rectangles (11 mm x 22 mm) using a diamond knife. These coverslips were adhered to the bottom of the petri dish with hot dental wax to create a 10 mm x 22 mm x 0.17 mm central chamber and then sterilized under a UV light for at least 30 minutes. 5000 astrocytes were seeded onto these chambers and allowed to adhere to the dish overnight (at least 16 hours). At the start of the experiment, a 22x22-1.5 coverslip was used to create a roof for the EF chamber by using sterilized silicone vacuum grease (Dow corning # 1966898-0712) to seal it to the
cut coverslip spacers on either side of the cell culture lane. Double-sided tape placed over the coverslip roof on either end of the lane created wells for additional culture media; the junction between the double-sided tape and the edges of the petri dish were made water-tight by sealing the gaps with additional silicone vacuum grease. We applied an electric field of 0, 4, 40, or 400 mV/mm throughout the entire experiment by delivering a constant current of either 0, 10, 100, or 1000 µA. It is important that a good seal is maintained so that the only aqueous connection between the wells on either end of the culture dish is through the central trough containing the cells; otherwise, the applied current may leak around the area where the cells are, which would cause the actual applied electric field to be less than the calculated EF.

Time-lapse imaging

Electric field chambers were placed on a Zeiss AxioObserver Z1 inverted microscope (Carl Zeiss, Jena Germany) equipped with a fully automated and programmable Mährhäuser scanning stage, an Axiocam MRm camera, and a stage incubator system that regulates temperature, O₂, and CO₂ throughout the experiment. The cell culture chamber was placed on the stage, heated to 37°C, and the incubation chamber was maintained with a humidified 5% CO₂ environment at 37°C. A 20x 0.8 numerical aperture (NA) Plan-Apochromat objective lens was used to acquire images with differential interference contrast (DIC) optics every 3 minutes for the duration of the experiment, and these images were subsequently stitched together into time-lapse videos. Image acquisition was automated using the Zen Blue (2012, version 1.1.2.0) software package. We began imaging the first time-lapse video approximately 16 hours
after the cells were seeded into the EF chambers. Cells were imaged for at least 30 minutes prior to the start of the electric field to establish baseline cellular behavior, and then for at least 12 hours after electric field onset. As only one dish could be imaged at a time and each experiment ran for 15 hours, the last group that was imaged had been growing in the EF chamber for up to 36 hours longer than the first culture had been. To control for potential sequence effects (i.e. cells changing their responsiveness to EF exposure as a function of the length of time that has elapsed since they were sub-cultured into the EF chamber), the order in which the cells were exposed to each of the EF strengths was varied between experiments. We also directly tested whether the delay between sub-culturing the cells and beginning the EF exposure had any effect on the cellular response to the EF by exposing sister cultures to 40 mV/mm for 12 hours beginning either 16 or 48 hours after sub-culturing into the EF chamber. We found no evidence to suggest that this delay affected the response to the EF exposure, so data were pooled across experiments for the analysis.

Optimizing migration analysis

Note: this section was not included in the publication in which the rest of this chapter was first printed; it was added to the thesis to further explain the rationale for the experimental design.

We used pilot studies of astrocytes exposed to either 0 or 400 mV/mm to empirically determine the optimal frequency with which we would measure cell location to calculate velocity throughout these experiments. The time-lapse videos, which we described more fully above (page 82), revealed that astrocytes demonstrate a baseline
degree of movement with random direction *in vitro*, and that they responded to EF exposure within minutes (a detailed discussion of how EFs affect astrocyte migration can be found on page 97). In our initial attempt to measure astrocyte migration, we sampled cell position once every hour beginning at the time the EF was turned on; consequently, the first time point for which we could measure speed (as speed is calculated from a change in position over a certain period of time) was 1 hour after the start of the experiment and there was already a statistically significant difference between those astrocytes exposed to 0 mV/mm and those in the 400 mV/mm groups. We realized that we needed to modify our experimental design and methods of analysis so that we could demonstrate that the cell populations had the same baseline speed at the start of the experiment, and so that we could determine just how quickly the astrocytic response to EF exposure occurs. However, the optimal frequency for measuring cells is limited by the random measurement error caused by the imprecision in our tracking. Specifically, each cell is tracked by manually selecting the location corresponding to the center of its nucleus; this measurement is approximate and, thus, there is some variability in the location. As cell position is tracked with increasing frequency, the cells have less time to move so their displacement diminishes, but the amount of measurement error is constant so the proportion of the calculated cell speed that is represented by sampling error increases. However, at the other extreme of insufficient frequency, cells displaying randomly-directed migration may travel along a much greater path length than their net displacement over the tracking interval would indicate. Thus, an optimal measuring frequency would optimize the temporal resolution
of cell tracking while minimizing the proportion of the calculated speed represented by sampling error.

In order to empirically determine the optimal measurement frequency for cell tracking, we tracked each astrocytes used in this analysis every 3 minutes over the first 60 minutes of the experiment. We calculated the cell speed from these measured positions, using the location in every 1, 2, 5, 10, or 20 frames to calculate the cell speed with a frequency of 3, 6, 15, 30, or 60 minutes, respectively. (Each DIC image in the time-lapse videos was taken 3 minutes apart.) Because the astrocytes exposed to 400 mV/mm demonstrated an initial change in speed that plateaued within 30 minutes of EF onset (see Electric fields affect the speed of cortical astrocyte migration, page 97), we used the calculated cell speed over the last tracking interval (i.e. the last 3, 6, 15, 30, or 60 minutes of the first hour). As the same cell was used to calculate the cell speed for each of the tracking intervals, and as these cells were tracked over time, the data reflect a repeated measures design.

We tested the null hypothesis that there would be no significant measurement error at any of our measuring intervals against the alternative hypothesis that at least one of our sampling intervals would result in measurement error as measured either by an change in the standard deviation of the measured speeds or by an increase in the mean speed measured with a given interval. We used two different statistical approaches to test the overall hypothesis that certain tracking intervals would result in measuring error that would significantly affect the measured cell speed. We tested the null hypothesis that the sampling interval would have no effect on the mean cell speed against the alternative hypothesis that measurement frequency would cause sampling
error that would affect mean speed using a standard least squares analysis with a mixed-effects linear regression model to analyze whether the tracking interval influenced the measured velocity. The regression equation used for this analysis is:

\[ Y_{ij} = \beta_j + \mathcal{N}(0, \sigma_b^2) + \epsilon_{ij} \]

where \( Y_{ij} \) is the estimated cell speed for each observation \((i)\) at each tracking interval \((j)\); \( \beta_j \) is the coefficient parameter representing the mean value of cell speed at each tracking interval; \( \mathcal{N}(0, \sigma_b^2) \) represents the normal distribution of mean cell speeds for each cell, which we are using to estimate their variance \( (\sigma_b^2) \) only; and \( \epsilon_{ij} \) represents the error function of each observation \((i)\) around the mean value for each factor level \((j)\), which is synonymous with the within-groups variance and is also known as the normal distribution. We also tested the null hypothesis that measurement error does not vary among the different tracking intervals against the alternative hypothesis that more frequent measurements would cause an increase in measurement error; this error is reflected in the standard deviation of the observed cell speeds, and we used normal quantile plots with 95% confidence intervals to assess whether the studentized residuals of the model’s predicted values are normally distributed (i.e. the z-score of the difference between each actual and predicted measurement based on our regression model). We then compared these residuals between tracking intervals using a Levene test centered at the sample mean with a threshold of significance of \( \alpha = 0.05 \).

We ran separate regression models for cells exposed to 0 and 400 mV/mm because we were using this study to make inferences about differences in speed as a function of EF strength. In this model, we treated the tracking interval as an ordinal fixed variable and each cell was treated as a continuous random variable. The model
calculates an overall $F$-statistic for the fixed effect (i.e. the tracking interval) from which it calculates a $p$-value for the overall significance of the inferential statistic; the model also estimates both the cell speed for each tracking interval and the difference in cell speed between adjacent intervals (e.g. between 3 and 6 minutes, but not between 3 and 15 minutes), and then it calculates a t-score and $p$-value for each difference. The $F$-statistic and parameter estimates in this mixed-model approach correspond to the $F$-score and post-hoc tests that are traditionally associated with a 1-way ANOVA and we interpreted these statistics in the same way (the mixed-effect model has the added statistical benefit of accounting for the fact that the speed calculated at different tracking intervals from the same cell would exhibit some degree of dependence). We controlled for type 1 error using $\alpha = 0.05$ as our threshold of significance for the overall model, and for each parameter estimate (note, the $p$-value reported for each parameter estimate is adjusted for multiple comparisons because the regression model accounts for multiple comparisons when calculating the individual parameter estimates). We performed standard regression diagnostics to assess deviations from the model’s assumptions that would affect the predictive nature of the model (notably, we are using this model for inferential statistics, but we evaluated the predictive assumptions because we used one of these assumptions to evaluate effects of measurement error). We calculated the cell speeds that are predicted by the model (based on the tracking interval), and calculated the studentized residuals (i.e. the difference between the actual measured velocity and the measured velocity predicted by the model, normalized such that the predicted value $= 0$ and the standard deviation around the predicted value, which is equivalent to the error, is equal to 1; $t_i = \frac{\hat{e}}{\sigma \sqrt{1 - h_{ii}}}$ where $t_i$ is the studentized residual, $\sigma$ and $\epsilon$ represent
the estimated population parameters of the standard deviation and error function, respectively, and $h_i$ represents the leverage of each residual (the relative weight of each measurement on the entire model)). We assessed the assumption that the residuals are normally distributed by plotting the studentized residuals for each tracking interval with a Normal Quantile Plots (QQ-plots) with a 95% confidence interval (CI); the error function was determined to be normally distributed if the residuals fell within the 95% CI. We then tested the hypothesis that the tracking interval would affect the magnitude of the error by using the Levine test to assess heteroscedasticity (i.e. differences in standard deviation) among each of the tracking intervals using the actual residual values (not studentized); we used $\alpha = 0.05$ as our threshold of significance, so that a $p$-value < 0.05 indicates that the standard deviation is statistically different among at least one of the groups. As part of this analysis, we will report the overall $F$-statistic for the regression model, the estimated cell speed for each tracking interval (mean ± standard error of the mean), the parameter coefficients (mean ± standard error of the mean) with their t-score and $p$-value, and the $F$-ratio and $p$-value for the Levene test; the data are visually represented using box-plots. The data analysis and graphing were completed using the packages ggplot2\textsuperscript{336}, reshape\textsuperscript{337}, multcomp\textsuperscript{338}, car\textsuperscript{339}, and nlme\textsuperscript{340} with the statistical software R\textsuperscript{341} to calculate the regression model, evaluate the model assumptions, calculate the Levene test, and graph the data.

*Migration analysis*

To analyze astrocyte migration, time-lapse videos were imported into ImageJ and analyzed using the plugin MTrackJ\textsuperscript{342}. To track each cell, the point corresponding to the
center of each cell’s nucleus was manually selected in every 5th frame (15 minutes) throughout the 12-hour experiment; these positions were used to calculate the magnitude of the velocity (i.e. speed) and the direction of migration at each 15-minute interval (Figure 2.2). At least 8 fields of view were required for each experiment, and a minimum of 30 cells were tracked for each time point for each experiment, resulting in an overall n ≥ 90 cells for each EF strength at each time point once data for all 3 experiments were pooled. All data on cell tracking produced by MTrackJ for the migration analysis were compiled in Microsoft Excel 2011, saved as comma separated values files, and then imported into the statistical program R. All data analysis was performed using R (including the packages Circular, Ggplot2, Pastecs, Reshape, and Multcomp), with RStudio. The vector representing each cell’s velocity was broken down into the speed and direction components, and each component was analyzed individually. Mean cell speed was compared at each time point for statistical significance using a 1-way ANOVA with Tukey HSD post-hoc tests, with an overall threshold for significance at each time point of p = 0.05. The ability of each EF to induce directional cell movement was assessed at each time point using Rayleigh’s test, using a p-value = .05 with a Bonferroni correction for the number of comparisons (196 comparisons: 4 EF levels; 49 time points). For those EF strengths and time points where there was directional migration we measured the mean direction of alignment (μ ± SEM), the dispersion of direction about the mean angle with the concentration parameter (κ), and the circular standard deviation.
Analysis of EF effects on the orientation of the axis of cell division

To determine whether the EF exposure aligned the axis of cell division, each mitotic event in the time-lapse live cell videos was identified. The orientation of the axis of cell division was measured by drawing a line between the centers of each of the daughter nuclei in the first frame where the two daughter nuclei are distinctly identifiable. Image analysis was completed using the program Fiji\textsuperscript{346}. The angle of this line relative to the axis of the electric field was measured. Alignment of the mitotic axis was determined for each EF strength using a Rayleigh’s test, using $p < 0.01$ as the threshold for determining significance ($p < 0.01$ was chosen as a conservative adjustment for multiple comparisons based on a nominal $p < 0.05$ for 4 different groups). If the sample showed statistically significant alignment, the mean angle, concentration parameter ($\kappa$), and angular standard deviation are reported.

Immunocytochemistry

Cells from each independently-derived population were immunolabeled for GFAP, vimentin, and nestin to determine the purity and maturational state of the astrocyte population. For immuno-labeled astrocytes after EF exposure, cells were rinsed with ice-cold phosphate-buffered saline (PBS) (pH 7.4) and then fixed with 4% paraformaldehyde in 0.1M PBS for at least 12 hours. Cells were washed 3 times with PBS, blocked and permeabilized (4% normal goat serum, 0.5% bovine serum albumin, and 1% Triton X100 in 0.1M PBS) for 30 minutes at 25°C, and then incubated with the primary antibodies diluted in the blocking solution either for 2 hours at 25°C or overnight at 4°C. Cells were then washed 3 times with PBS, incubated with fluorescent-tagged
secondary antibodies (diluted in PBS) for 2 hours, counter-stained with DAPI (NucBlue Fixed Cell ReadyProbes kit, diluted per manufacturer's instruction; Molecular Probes # R37606), and mounted under glass coverslips with Vectashield (Vector Labs # H-1000). For cells stained for BrdU, an additional acid wash series was used to expose the BrdU for antibody binding prior to the start of the immunolabeling protocol: cell DNA was denatured for 10 minutes in 1N HCl on ice, 10 minutes in 2N HCl at 25°C, and 20 minutes in 2N HCl at 37°C, and then neutralized with 0.1M borate buffer for 10 minutes at 25°C. Primary antibodies used in the immunocytochemistry studies included the following: mouse IgG\textsubscript{1k} anti-BrdU (1:1,000; Dako # M0744), polyclonal rabbit anti-GFAP (1:5,000; Dako # Z0334), polyclonal chicken IgY anti-Vimentin (1:1,000; Millipore # AB5733), mouse IgG\textsubscript{1} anti-Nestin (1:1,000; clone rat-401, Millipore # MAB353). Secondary antibodies used were Goat IgG anti-rabbit Alexa-488 (Molecular Probes # A-11008), Goat IgG anti-chicken IgG Alexa-568 (compatible with chicken IgY primary antibody; Molecular Probes # A-11041), and Goat IgG anti-mouse Alexa-647 (Molecular Probes # A-21236). All secondary antibodies were diluted at 1:200. All cells except those stained for BrdU were counterstained with DAPI NucBlue Fixed Cell Stain (Molecular Probes, # R37606).

Confocal microscopy

Immuno-labeled cells were imaged by laser-scanning confocal microscopy (LSM-710, Zeiss, Jena, Germany) configured around an AxioObserver 21 (inverted) stand with a motorized XY stage. Image acquisition was performed using the Zen Black edition (Carl Zeiss, 2011; 64 bit, version 8.1.5.484) software package. 16 bit images
were acquired with a 20x/0.8 NA plan apochromat objective lens, with a pixel dwell time of 0.99 µsec and a pixel size of 0.13 µm². Images were acquired using 4x line averaging, with simultaneous scanning of the 405 Diode and 633 HeNe lasers, and a sequential scan for the 488 Argon and 561 DPSS lasers. The 488 laser line was also used to generate a transmitted light DIC image. At least 5 fields of view (424.84 µm²) were acquired for each condition (EF strength x time), and each experiment was repeated at least 3 times. Detector windows for each channel were adjusted to assure no cross talk between channels as follows: 405 nm (410 – 483 nm), 488 nm (492 – 560 nm), 561 nm (580 – 629 nm), and 633 nm (637 – 735 nm).

**Image analysis**

Images were imported into Fiji (an ImageJ distribution built for the Life Sciences; http://fiji.sc/Fiji) for quantifying cellular and nuclear morphology. To assess alignment of cell processes, gray-scale images of vimentin expression were analyzed using the 2D Fast Fourier Transform (FFT) algorithm and Oval Profile plugin (authored by Bill O’Connell, http://rsb.info.nih.gov/ij/plugins/oval-profile.html) as described previously. The 2D FFT produces an image that is the graphical representation of the spatial frequencies of the original images, which is related to directionality. With the Oval Profile Plugin, the radial summation of pixel intensities is used to determine whether these pixels are randomly distributed around the axis (i.e. are unaligned), or show clustering around a particular orientation (i.e. demonstrate alignment). The pixel intensities (in arbitrary greyscale units) are normalized for each image by dividing the value at each angle measure by the minimum radial pixel intensity sum for that image.
and then subtracting 1). Normalized pixel intensities for each angle measure in the oval profile are averaged across all images acquired from each group, and then those averaged values were normalized again. A graphical representation of orientation in the original image is obtained by plotting the summed pixel intensities between 0° and 180° (the directionality information is axial and does not distinguish between objects pointing in opposite directions; the data were plotted from 0° to 360° because double-plotting the data helped aid in visualizing directionality). It should be noted that the FFT image was first rotated 90° counterclockwise because the results of the FFT yields frequencies orthogonal to those in the original image. In our experiments, this rotation also defines the direction of the electric field application along the 0-180° axis (horizontal).

**Fluorescence microscopy and proliferation assay**

Digital images of BrdU-immunolabeled cells were acquired with a 25x/0.8 NA Plan-Neofluar objective lens using DIC optics and a GFP filter cube (filter set FS 38HE, Zeiss, Jena Germany) using a Zeiss Axiovert 200 inverted microscope (Zeiss, Jena Germany) equipped with a Hamamatsu ORCA ER CCD camera, Colibri LED illumination unit (blue, green, red), and a white light LED. Image acquisition was performed using the Zeiss Axiovision (version 4.8.2 sp1) software package. At least 20 fields of view were randomly acquired for each slide, allowing at least 1,000 cells to be counted for each group. The number of BrdU-positive or negative cells were counted using the Cell Counter plugin for Fiji (authored by Kurt De Vos, http://rsb.info.nih.gov/ij/plugins/cell-counter.html). For each time point within each experiment, we evaluated our hypothesis that EFs induce increased proliferation.
against the null hypothesis that EFs have no effect on proliferation using a test of homogeneity of proportions; if a significant effect was detected, we then performed individual $X^2$ tests between EF strengths to determine which specific groups were different. Results from individual experiments were used to develop a sense of trends of how EFs affect proliferation over time. To evaluate the effects that EF exposure has on proliferation at each time point among all of the experiments, we compiled the percentage of BrdU-positive cells from each of the individual experiments and compared these percentages between EF exposures using a Kruskal-Wallis test (using a threshold of significance of $p < 0.05$ at each time point) with nonparametric comparisons between each EF exposure and the 0 mV/mm control at each time point using the Dunn Method for Joint Ranking.

Statistical analysis

All data analysis and graphing were performed using R (including packages Ggplot2, Pastecs, Reshape, and Multcomp)\textsuperscript{336-338, 341, 344}, with RStudio\textsuperscript{345}. Directional data were evaluated using the Rayleigh test, which tests the research hypothesis of non-random directionality against a null hypothesis of random directionality based on the test statistic of the mean resultant vector (R). Circular statistics, including the circular mean direction ($\mu$), circular standard deviation, and concentration parameter ($\kappa$) were calculated using the R package Circular\textsuperscript{343}. For all experiments, the nominal threshold for significance was set at *$p < 0.05$, unless otherwise noted. Unless otherwise noted, data are reported as mean $\pm$ SEM. All figures were prepared using the ImageJ plugin FigureJ\textsuperscript{350}.
Results

Characterizing the cortical astrocytic population

The cortical astrocytes (rat primary cultures) used in these experiments were purified populations (>99%) as verified by the provider (ScienCell), with GFAP immunolabeling. Immunofluorescence labeling against GFAP, vimentin, and nestin, as well as morphological characteristics as visualized with DIC microscopy, were used to evaluate the purity and maturation of these astrocytes before each experiment (Figure 2.4). These cultured astrocytes expressed GFAP at varying levels of intensity, while vimentin and nestin were more consistently expressed in all cells. Morphologically, the astrocytes included both bipolar and lamellipodial morphologies. Together, this confirmed that >99% of the cells were astrocytes at varying degrees of maturation.

Optimizing the migration analysis protocol

Note: this section was not included in the publication in which the rest of this chapter was first printed; it was added to the thesis to further explain the rationale for the experimental design.

To determine the optimal interval to track astrocytes for migration measurements, we measured cell speed with different tracking frequencies (every 3, 6, 15, 30, or 60 minutes), and compared mean speeds measured among each group using a mixed-effects generalized linear regression model. We found that there was a statistically significant difference among the mean cell speeds measured with each tracking interval for astrocytes exposed to 0 mV/mm (n = 31 in each tracking interval, $F_4 = 24.7501$, $p =$
5.995 \times 10^{-15}), and the Tukey-HSD post hoc test demonstrated that there was a steady increase in the mean speed as the tracking interval increased (summarized in Figure 2.3 and Table 2.1); we found a similar trend towards increasing astrocyte speed measured as the tracking frequency increased for astrocytes exposed to 400 mV/mm \((n = 44, F_4 = 14.7062, p = 2.362 \times 10^{-10})\). Together, these results demonstrate that sampling error may affect the accuracy of the estimated mean cell speed. Each individual post-hoc comparison made with the Tukey-HSD test is reported in Table 2.1; the estimated differences for each comparison are reported, along with the t-score and adjusted \(p\)-values. The estimated group differences are also graphically demonstrated in Figure 2.3, where the measured migration speeds are plotted separately for 0 and 400 mV/mm. From this analysis of the means, there is no difference between tracking intervals of 30 or 60 minutes, but the mean speed begins to increase at intervals of 15 and 6 minutes, and becomes robustly larger in both groups at 3 minutes. In reviewing the model assumptions, we found that the residuals were normally distributed within each of the tracking intervals; however, the Levene test demonstrated heteroscedasticity among the different tracking intervals both with 0 mV/mm \((F_4 = 4.5879, p = 0.001597)\) and with 400 mV/mm \((F_4 = 2.8128, p = 0.02637)\), thus suggesting that measurement error significantly affects the precision of the measured cell speed. We calculated the standard deviation (SD) of the residuals for each tracking interval and found that there was a steady increase in SD with an interval of 3 minutes for astrocytes exposed to either 0 or 400 mV/mm, the SD for astrocytes tracked every 6 minutes in 400 mV/mm was slightly above the SDs for 15, 30 and 60 minutes (0 mV/mm: 3 min = 17.782; 6 min = 8.466; 15 min = 7.911; 30 min = 9.071; 60 min =
7.568. 400 mV/mm: 3 min = 15.570; 6 min = 10.788; 15 min = 10.000; 30 min = 9.109; 60 min = 10.049). Together, these results clearly demonstrate that a tracking interval of 3 minutes is prone to significant error; a 6 minute interval also appeared to have some error, although it was considerably less than 3 minutes; the lower measured mean and SD for cells tracked every 15 minutes indicated to us that the effect of measurement error was adequately controlled with this sampling interval. Thus, we determined that a tracking interval of every 15 minutes would provide us with the greatest temporal resolution without creating a significant component of measurement error and we used this interval for each of the cell migration experiments described hereafter.

*Electric fields affect the speed of cortical astrocyte migration*

To test our overall hypothesis that electric fields are capable of directing the astrocytic response to injury, we explored how EF exposure affects each of the behaviors that astrocytes characteristically display after injury in both mammalian and non-mammalian vertebrates. The first of these behaviors is migration, as astrocytes must move towards the lesion as they are recruited to restore BBB integrity and isolate the lesion environment from the surrounding healthy tissue. To assess the extent to which EFs influence astrocyte migration, time-lapse DIC live cell microscopy was used to record the astrocytic response to dcEF exposure over a 12-15 hour period. The migration of astrocytes following exposure to EF intensities associated with intact (4 mV/mm), injured mammalian (40 mV/mm), and injured non-mammalian vertebrate tissues (400 mV/mm) were compared to an untreated control (0 mV/mm). No evidence
of cell death was found during these experiments as a function of either EF exposure or
of phototoxicity from repeated exposure to light.

Time-lapse videos show that, in the absence of any EF, astrocytes displayed
heterogeneous morphologies (bipolar, stellate and lamellipodial) and displayed non-
directional movement. Cells exposed to 4 mV/mm showed similar morphologies but
demonstrated reduced speed as compared to astrocytes cultured in the absence of any
EF. In contrast, time-lapse videos showed that cells exposed to 40 mV/mm or 400
mV/mm responded rapidly to the EF exposure, with the entire cell population migrating
towards the anode of the EF within the first hour of the EF exposure.

To qualitatively evaluate the EF-induced effect on migration, the paths of
migration over the first six hours of EF exposure were plotted for individual astrocytes;
the starting position was normalized to the origin of the graph (0, 0), and the direction
was displayed relative to the orientation of the EF (Figure 2.5). An analysis of the mean
speeds for astrocytes exposed to each of the EF intensities showed that the mean
speed of cells in the control group (0 mV/mm) did not change over time (data not
shown), and that there were different effects on speed for each of the EF exposures
(Figure 2.6A). Differences in cell speeds were compared among all EF exposure groups
at each time point using a 1-factor ANOVA with a Tukey-HSD post-hoc test (Figure 2.6B
graphs this analysis for cells at the start of the experiment, and after 30 minutes and 4
hours of EF exposure). We found that the mean cell speed was equivalent among all
groups prior to the EF onset (0 mV/mm: 13.4 µm/hr; 4 mV/mm: 14.3 µm/hr; 40 mV/mm:
16.5 µm/hr; 400 mV/mm: 14.9 µm/hr; 1-factor ANOVA: p = 0.20) (Figure 2.6B, left
panel). However, astrocytes exposed to 40 and 400 mV/mm displayed a rapid increase
in migration speed within 30 minutes of EF exposure as compared to astrocytes exposed to 0 or 4 mV/mm (mean speed: 0 mV/mm: 12.7 µm/hr; 4 mV/mm: 14.9 µm/hr; 40 mV/mm: 22.3 µm/hr; 400 mV/mm: 21.5 µm/hr; 1-factor ANOVA: p = 1.1 x 10^{-6}; Figure 2.6B, middle panel). Interestingly, astrocytes exposed to 40 mV/mm sustained this increased speed for only one hour and returned to the baseline speed 1.75 hours after the EF onset. Similarly, astrocytes exposed to 400 mV/mm initially increased their migrational speed to that observed for astrocytes exposed to 40 mV/mm, but this initially robust increase in speed was sustained for over 4 hours, after which time it returned towards baseline while maintaining an increased speed that hovered between significant and a non-significant trend. Cells exposed to 4 mV/mm did not show an initial change in migration speed upon EF exposure, but the mean speed decreased relative to cells exposed to 0 mV/mm beginning 3.5 hours after the EF onset and persisting for the remainder of the experiment. Thus, EF strengths comparable to those present in intact tissue induced cortical astrocytes to decrease their speed, whereas EFs intensities comparable to those present in injured mammalian tissue initiated a rapid increased speed of migration by these cells. This migrational response was more pronounced and sustained in astrocytes exposed to EF intensities associated with regenerating tissues in non-mammalian vertebrates.

As the migrational assays were done sequentially, it was necessary to assess whether the time interval between when the cells were sub-cultured in the EF chamber and when EF exposure began had any effect on the cellular responsiveness to the electric field. Consequently, sister cultures were exposed to 40 mV/mm for 12 hours, beginning either 16 or 48 hours after the cells were sub-cultured into the EF chamber,
and migration speed was assessed. No difference in the mean migration speed between these groups was found (Figure 2.7), indicating that the sequence in which groups were exposed to each EF within an experiment does not serve as a confounding variable in these study.

*Electric fields are a directional cue for cortical astrocyte migration*

Having demonstrated that EF exposure alters cell speed in an intensity- and time-dependent manner, we assessed the extent to which EFs also serve as an orientational cue by causing directional migration. Directionality was assessed for each EF intensity at each time point using Rayleigh’s test (which tests the hypothesis of a non-random direction about a circle against the null hypothesis of a random direction), using an overall $p$-value = 0.05 with a Bonferroni correction for the total number of factor levels analyzed (196 comparisons: 4 EF levels at each of 49 time points). No directional migration was detected by astrocytes exposed to EF intensities of either 0 or 4 mV/mm. Interestingly, astrocytes displayed anodally-directed migration after 1.5 hours of exposure to 40 mV/mm, while this same anodally-directed migrational response only took 30 minutes to emerge for astrocytes exposed to 400 mV/mm (Figure 2.8). Once it emerged, directional migration continued throughout the remainder of the recording period. Moreover, cells exposed to 400 mV/mm moved with greater precision towards the anode as compared to astrocytes exposed to 40 mV/mm, which displayed a greater migrational dispersion. This was evident by a smaller circular standard deviation, and a larger concentration parameter ($\kappa$) of directional migration (data not shown).
As EFs rapidly induced directional migration by cortical astrocytes, we next set out to determine whether the cells remained sensitive to changes in the extracellular EF orientation. This was tested by exposing cells to 400 mV/mm for 6 hours, and then reversing the polarity of the EF exposure for another 6 hours (Figure 2.8, right panel). We found that, upon reversing the direction of the EF, cells stop moving towards the position that used to be the anode within 15 minutes, and reestablished directional migration towards the new anode position within 2 hours. This 2-hour loss of directionality occurred while the cells were reorienting to the new direction of the imposed EF, during which time half of the population turned left and the other half turned right (as indicated by the phase-shift in the directionality data, Figure 2.8).

Together, these results indicate that the cells are capable of detecting the external EF and move towards the anode, with the strength of the EF affecting the directionality and speed of migration. As the lesion site within the CNS becomes the anode of the injury-induced EF (see discussion in Chapter 1, page 48), EFs may be contributing towards astrocyte migration towards the lesion site in vivo.

Electric fields induce cortical astrocyte proliferation

After an injury, astrocytes around the lesion site proliferate with a well-described time course that begins within 24 hours of the injury, peaks after 48 hours, and begins to decline by 72 hours. This newly-proliferating population helps reestablish the damaged BBB, and serves to replenish some of the cells lost to injury. We tested the hypothesis that EFs associated with injured tissues (40, 400 mV/mm) may actually drive this proliferative response. Specifically, astrocytes were exposed to an EF of either 0, 4,
40, or 400 mV/mm for either 12, 24, 48, or 72 hours to determine whether EFs can induce astrocyte proliferation, and whether this effect mirrors the temporal profile of the proliferative response of astrocytes following injury in vivo. BrdU was added to the culture media for the last 6 hours of the EF exposure, and proliferating cells were identified using BrdU immunocytochemistry (Figure 2.9A-D). Cells were counted as either BrdU-positive or negative, and the percentage of BrdU-labeled cells was calculated for each group (16 groups: 4 EF strengths x 4 time points) within each experiment (Figure 2.9E). At all 4 time points, 5-10% of cells exposed to 0 mV/mm were BrdU-positive, and there was no statistically significant difference in BrdU labeling of these cells over time ($X^2_3 = 6.68, p = 0.828$). This was also the case for cells exposed to 4 mV/mm where, at each time point, 5-10% of cells were BrdU-positive. Likewise, astrocytes exposed to either 40 or 400 mV/mm did not display changes in proliferation after 12 hours of exposure. This persisted for 24 hours of exposure, when a non-significant trend towards increased proliferation began to emerge ($X^2_3 = 4.4643, p = 0.2155$). This trend towards increased proliferation for astrocytes exposed to 40 and 400 mV/mm is non-significant likely because there was a robust increase in proliferation only in a subset of the experimental groups exposed to 40 and 400 mV/mm for 24 hours and the non-parametric statistical test used for these comparisons was too conservative to detect a difference given the relatively small sample size that we had; however, this suggests that EFs-induced proliferation begins to emerge as early as 24 hours, but that the effect does not fully emerge until a slightly later time point. By 48 hours of exposure to either 40 or 400 mV/mm EF, there was a statistically significant effect on proliferation ($X^2_3 = 13.5526, p = 0.0036$), with a significant increase in astrocytes exposed to 40
mV/mm ($p = 0.0088$) and 400 mV/mm ($p = 0.0481$) relative to 0 mV/mm. This proliferative effect persisted through 72 hours of EF exposure, but only for cells exposed to 400 mV/mm ($p = 0.0386$); proliferation in cells exposed to 40 mV/mm had returned to baseline ($p = 0.1452$) within this period of time. Thus, EFs are capable of stimulating proliferation by astrocytes in an intensity- and time-dependent manner. The time-dependent manner in which astrocyte proliferation is affected mirrors that observed for astrocytes at sites of injury to the CNS. Moreover, only an EF intensity of 400 mV/mm maintained significant astrocyte proliferation through the 72-hour exposure period, suggesting that this EF intensity is most effective at facilitating cell replacement following injury.

One additional observation that was apparent in the time-lapse videos of astrocyte cultures exposed to 400 mV/mm EF is that the axis of division was related to the orientation of the electric field vector. As the orientation of division is known to influence cellular activity\textsuperscript{351}, we set out to determine the extent to which EF exposure influences the axis of division in mammalian astrocytes. Using the DIC time-lapse videos, mitotic cells were identified and the angle of the axis of division was measured relative to the EF vector by drawing a line between the two daughter nuclei in the first frame where they became distinctly visible (Figure 2.10A-D). The distribution of these axes relative to the EF vector are plotted for each EF strength (Figure 2.10E), with the axial data double-plotted on the x-axis relative to the anode (A) and cathode (C) to assist in visualizing the clustering of mitotic events perpendicularly to the EF vector. Using the Rayleigh test for alignment, we found that EF exposure aligned the axis of cell division for cells exposed to 400 mV/mm ($n = 124$, $R = 0.3740$, $p = 2.93 \times 10^{-8}$), with a
mean direction $\mu \pm \text{SEM} = 82.1 \pm 3.53^\circ$, $\kappa = 2.71$, and $\text{SD} = 39.7^\circ$ (the EF axis runs from $0^\circ$ to $180^\circ$; $90^\circ$ is perpendicular to the EF vector). We found that there was no significant alignment for cells exposed to 0 mV/mm ($n = 176, R = 0.1127, p = 0.1071$), 4 mV/mm ($n = 186, R = 0.1071, p = 0.1183$), or 40 mV/mm ($n = 260, R = 0.0437, p = 0.6092$).

Electric fields alter cortical astrocyte morphology

Having shown that EFs can control behaviors that are necessary for the initial astrocytic recruitment to the injury response, we tested the hypothesis that EFs can also regulate the hypertrophic and morphologic changes characteristic of the astrocytic response to injury in non-regenerating and regenerating animals, respectively. Following an injury, astrocytes in non-regenerating animals characteristically up-regulate the expression of the intermediate filament GFAP, relative to the intermediate filaments vimentin and nestin, while astrocytes in regenerating animals do not undergo this hypertrophic change. As this response generally emerges only after several days, we exposed astrocytes to 0, 4, 40, or 400 mV/mm for 72 hours, and then used immunofluorescence labeling for GFAP, vimentin, and nestin to determine whether EFs affect hypertrophy (Figure 2.11A-L). We found that astrocytes exposed to 4 mV/mm, which is an EF intensity associated with uninjured tissues, expressed low levels of GFAP, with greater intensity of both vimentin and nestin. However, astrocytes exposed to 40 mV/mm displayed elevated levels of both GFAP and vimentin, suggesting that EFs associated with injured mammalian tissues caused robust hypertrophy. Interestingly, we found that GFAP and vimentin expression in astrocytes exposed to 400 mV/mm were unchanged compared to that observed for astrocytes exposed to 4
mV/mm. Thus, EFs associated with injured mammalian tissues are sufficient to induce a hypertrophic response characteristic of reactive gliosis, while those associated with regeneration induce no such change.

Having found that EF strengths associated with non-regenerating tissues induce cytoskeletal hypertrophy of astrocytes, we next set out to determine whether EFs associated with regeneration induce morphological changes in astrocytes consistent with their regenerative phenotype 	extit{in vivo}. Our group and others have shown that astrocytes align their processes perpendicularly to a 500 mV/mm applied EF within 24 hours while 10 mV/mm has no effect, and we have previously shown that these EF-aligned astrocytes enhance the extent of neurite outgrowth compared to unaligned 0 mV/mm controls\textsuperscript{240, 241}. Our time-lapse videos from the migration studies indicate that EF exposure to 400 mV/mm induces astrocytes to transform into a bipolar morphology and to align their processes within the first 12 hours of EF exposure. In the current study, we exposed astrocytes to 0, 4, 40, or 400 mV/mm for either 12 or 72 hours and performed FFT analysis for alignment on vimentin immunolabeled images to determine how quickly alignment occurs, whether this alignment is consistent over time, and whether physiologic EFs found at the injury site in mammals affect process alignment (Figure 2.11M-O). Astrocytes exposed to either 4 or 40 mV/mm EF showed no change in their morphology at either 12 or 24 hours of exposure. However, astrocytes exposed to 400 mV/mm showed a robust alignment of their processes perpendicular to the EF orientation within 12 hours (not shown), and this alignment persisted through the 72 hour EF exposure. These results confirmed that only EF intensities associated with injury in regenerating vertebrates induce dramatic changes in astrocyte morphology that
mirror those demonstrated by astrocytes within the injury site of regenerating animals following trauma.

**Discussion**

Studies that have aimed to elucidate signals for regeneration in non-mammalian vertebrates have demonstrated that the intensity of injury-induced electric fields (EFs), which are 50-100 fold greater than in uninjured tissues, represent a potent signal to drive tissue repair\(^{215, 222, 227, 329, 331, 352, 353}\). In contrast, in many mammalian tissues where limited regeneration occurs, injury-induced EFs are much lower in their intensity. Interestingly, when an exogenous EF is applied to injured mammalian skin or cornea, a more robust reparative response occurs\(^{123, 326, 334}\). Studies have shown that the mammalian CNS also produces EFs\(^{269, 290}\) that increase in their intensity upon injury\(^{291}\), so it is reasonable to assume the EFs could present an important signal to drive CNS repair. As the astrocytic response to CNS injury is crucial to its reparative outcome, we evaluated how astrocytes respond to three different EF exposures within the ranges previously recorded in intact tissues (4 mV/mm\(^{256, 269}\)), injured mammalian tissues (40 mV/mm\(^{267, 326}\)), and highly regenerating non-mammalian vertebrate tissues (400 mV/mm\(^{270, 279, 280, 354, 355}\)). At the lowest EF strength, astrocytes displayed little change in their behavior. However, mammalian astrocytes rapidly responded to elevated EFs by displaying robust and sustained directional migration. The directionality and speed of this migration were dependent on the polarity and intensity of the EF, respectively. Astrocytes also displayed a robust proliferative response upon prolonged EF exposure. Interestingly, only EFs associated with regeneration induced morphological changes in astrocytes that mirror those that facilitate regeneration in non-mammalian vertebrates\(^{79}\).
Moreover, each of these astrocytic responses, which are necessary for CNS repair, developed along the same time line as has been reported following an injury in vivo. These novel findings suggest that injury-induced EFs are capable of, and may be responsible for, driving the astrocytic response at CNS injury sites, and that manipulating EF intensity may represent a therapeutic option to promote CNS regeneration.

The apparent role of EFs in regulating CNS regeneration is consistent with evidence that EFs regulate regeneration in a range of non-mammalian vertebrate tissues and structures, including skin, bone, cornea, lens, spinal cord, tail, and limb. Injury to these tissues produces a substantial increase in EF intensity, and these EFs remain elevated until regeneration is complete. Furthermore, studies manipulating only the injury-induced electric field have shown that EFs are both necessary and sufficient to drive regeneration. Specifically, reducing or blocking EFs can attenuate or completely inhibit regeneration, whereas increasing EF intensities can promote regeneration. Regeneration is not ubiquitous among non-mammalian vertebrates: Anuran amphibians regenerate their tails when they are tadpoles but they lose this ability as they progress through metamorphosis, during which time there is a concomitant decrease in the intensity of their injury-induced EFs. However, experimentally increasing EFs at amputation sites in the adult frog induces regeneration. Injury-induced EFs have also been measured in mammalian tissues, including skin, cornea, lens, and bone. In mammals, injury-induced EFs are consistently lower than that found in regenerating vertebrates, and experimentally increasing their intensity also
promotes regeneration in these tissues\textsuperscript{283, 284, 334, 367}. Recent studies have also established that EFs are present in the mammalian CNS. Although EFs have not been measured in the mammalian CNS \textit{in vivo}, ex vivo recordings of the mammalian brain demonstrate that low (3.5-5 mV/mm) EFs are present within the rostral migratory stream, and EFs of this magnitude can direct neuroblast migration \textit{in vitro}\textsuperscript{269}. Furthermore, slice culture induces a 10-fold increase in these EFs to 31.8 ± 4.5 mV/mm\textsuperscript{290}, and spinal cord injury has been shown to induce a rapid 10-fold increase in current density \textit{ex vivo}\textsuperscript{291}. As EFs and current density are proportional to the resistivity of the tissue, it is likely that injury to the CNS induces a similar elevation in EF intensity. Collectively, these studies suggest that, in a variety of tissues in both non-mammalian and mammalian vertebrates, the magnitude of injury-induced changes in electric fields represent a crucial determinant to that tissue’s regenerative potential.

Within the vertebrate CNS, the extent of regeneration is largely determined by the astrocytic response to injury\textsuperscript{60}. Common to all vertebrates studied, astrocytes quickly migrate towards the lesion site and begin to proliferate\textsuperscript{64, 79, 84, 321, 368}. This migration and expansion of the astrocytic population is necessary to re-establish the BBB and to prevent the lesion from enlarging into the surrounding healthy tissue\textsuperscript{32, 54, 83}. In mammals, astrocytes have been shown to hypertrophy\textsuperscript{91} following injury and release molecules that inhibit axon sprouting and limit regeneration\textsuperscript{35, 50}. In contrast, astrocytes facilitate axon regeneration in non-mammalian vertebrates by assuming a bipolar morphology and creating a cellular bridge that guides sprouting axons across the lesion site\textsuperscript{79}. These similarities and differences in astrocyte behavior among vertebrates suggests that a common signal may initiate these behaviors, but that the signal does
not reach the threshold in injured mammals to induce the astrocytic behaviors necessary for robust regeneration. Thus, modifying the mammalian astrocytic response towards that seen in successfully regenerating animals may improve regeneration in mammals.

As EFs have been shown to influence the repair of a variety of tissues through their action on cells within these tissues, it is reasonable to assume that EFs could also affect astrocytic behavior to induce a regenerative response in neural tissue. Our results are consistent with this notion: we found that EFs induce dramatic changes in astrocyte migration and proliferation, and that these effects are enhanced with increasing EF intensities. Indeed, astrocytes display rapid and sustained anodally-directed migration during EF exposure at both 40 and 400 mV/mm, and migration induced by a 400 mV/mm exposure has a significantly greater speed and a more precise directionality than that induced by 40 mV/mm. Previous literature suggests that the injury site in the mammalian cortex would become the anode of the injury-induced EF (see discussion in chapter one, page 48). Thus, our results that EFs induce anodal migration are consistent with the hypothesis that EFs contribute to astrocyte migration towards the lesion site in vivo. Interestingly, our observations that astrocytes exposed to 4 mV/mm decrease their speed relative to cells not exposed to an EF suggest that EFs associated with intact tissues may represent a signal that astrocytes use to maintain their stability in vivo, as astrocytes in intact tissues are thought to remain relatively stationary within defined domains.

Just as astrocytes proliferate beginning two days after injury in both mammalian and non-mammalian vertebrates in vivo, we found that both 40 mV/mm and 400
mV/mm induce astrocyte proliferation in vitro that begins to increase after 24 hours of exposure and peaks at 48 hours. While 40 mV/mm and 400 mV/mm induce a similar degree of proliferation, proliferation is sustained for at least 72 hours with 400 mV/mm exposure but decreases towards baseline during this time frame in the 40 mV/mm exposures. This mirrors observations showing that proliferation among mammalian astrocytes peaks two days after injury and then decreases, while proliferation among astrocytes in regenerating non-mammalian vertebrates increases two days after injury and remains elevated for many more days. Lastly, astrocytes maintain a more heterogeneous morphology at lower field intensity exposures and, consistent with previous work from our lab and others\textsuperscript{240, 241}, they uniformly transform to a bipolar morphology only when exposed to 400 mV/mm. Interestingly, we found that these morphological changes were independent of intermediate filament expression. Specifically, our immunolabeling suggested that 400 mV/mm did not induce changes in GFAP, vimentin, or nestin expression, while 40 mV/mm induced an increase in GFAP and vimentin expression that is consistent with a reactive astrocytic phenotype in mammals. These differences in astrocyte behavior and morphology induced by 40 mV/mm and 400 mV/mm mirror the differences in astrocyte behavior following injury in non-regenerating and regenerating vertebrates in vivo. This suggests that physiologic EFs may direct the astrocytic response to injury in mammals, and that therapeutically enhancing these EFs to levels found in non-mammalian vertebrates may induce an astrocytic response that is more favorable to regeneration. Indeed, we have previously shown that astrocytes exposed to high EF strengths generate processes that are more permissive to neurite growth than those generated at low field strength exposures\textsuperscript{240}. 

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Taken together, these findings suggest that EFs are capable of controlling a number of astrocytic behaviors that are necessary for the injury response in mammals and regeneration in non-mammalian vertebrates. Moreover, the degree of regenerative potential of astrocytes is a function of the EF strengths to which they are exposed.

The pronounced effects that EFs have on astrocytic behavior are likely a consequence of nonspecific interactions with charged membrane proteins in a variety of physiological pathways\textsuperscript{222, 369, 370}. The EF vector encodes both direction and intensity, each of which can be transduced independently. Although the mechanisms by which astrocytes transduce EFs have not been explored, research from other cell types suggests directional migration is a function of electroosmosis, through which external EFs drive non-anchored membrane receptors to accumulate at opposite ends of the cell (Figure 1.8)\textsuperscript{221, 223, 232, 309, 371-373}. The degree of membrane receptor clustering is proportional to the EF intensity, which is consistent with the increased precision of astrocyte migration we observed at the highest EFs tested. EF intensity may be transduced by astrocytes by creating a voltage drop across the cell that affects the cell membrane potential and causes ion channels to open\textsuperscript{335, 374}. Interestingly, changes in membrane potential are involved in inducing tail regeneration in the Xenopus tadpole, which requires the voltage-gated sodium channel Na\textsubscript{v}1.2\textsuperscript{147}. Regeneration is lost during metamorphosis when Na\textsubscript{v}1.2 expression declines, whereas transfecting human Na\textsubscript{v}1.5 into these maturing tadpoles reestablishes regeneration. In Xenopus, increased intracellular sodium through Na\textsubscript{v}’s induces regeneration through several pathways, including Notch. Mammalian astrocytes also express the voltage-gated sodium channel Na\textsubscript{v}1.5, which stimulates both migration and proliferation after injury by increasing
intracellular calcium (Ca^{2+}) through a Na^{+}/Ca^{2+} exchanger. Interestingly, in non-mammalian vertebrates, proliferating astrocytes are capable of producing neurons, thereby having an essential role in neurogenesis. Mammalian astrocytes also have a capacity for neurogenesis, which is regulated by Notch signaling. Higher EFs may promote regeneration by inducing a neurogenic program in astrocytes through regulating Notch signaling, while the lower EFs generated in the injured mammalian CNS may not sufficiently activate Na_{v}1.5 to regulate Notch-mediated neurogenesis in vivo. Taken together, these studies demonstrate that multiple physiologic pathways are involved in transducing the EF signal to drive the cellular response to injury through affecting migration, proliferation and morphology.

We have shown that electric fields within the physiologic ranges reported in injured mammalian tissues, and in regenerating non-mammalian vertebrate tissues, are able to elicit multiple behaviors in astrocytes that are necessary for their normal response to injury. Furthermore, the difference in responses induced by 40 mV/mm and 400 mV/mm closely correspond to the differences between astrocytic behaviors in the injured mammalian CNS and those in the regenerating non-mammalian vertebrate CNS. Assuming that our results reflect the signaling environment in vivo, this suggests that electric fields, which are induced immediately upon injury and remain elevated throughout wound healing, may represent an important astrocyte response signal to drive tissue repair. As specific astrocytic behaviors are induced by specific EF strengths, and as behaviors associated with regeneration are induced only at EF strengths greater than those reported in injured mammalian tissues, regeneration in the mammalian CNS may be improved by therapeutically supplementing the physiological
EFs produced at the injury site. Indeed, EF based therapy has been used successfully to enhance bone reunion following fractures in humans in clinical practice using an inexpensive, non-invasive application\textsuperscript{377-379}. Together, this suggests that therapeutically-applied EFs are a strong therapeutic candidate to promote regeneration in the mammalian CNS by inducing an astrocytic response more favorable to regeneration.
Figure 2.1: Electric field chamber

(A) An illustration of the electric field chamber, showing how it is connected to the circuit that creates the electric field. Wells for cell culture media are created using double-sided tape on either end of the cell culture chamber; vacuum grease is used to prevent any media from leaking around these barriers. Salt bridges (2% agarose in astrocyte media) connect the EF chamber to Ag/AgCl electrodes immersed in a Steinberg electrolyte solution. The power supply drives a redox reaction at each of the electrodes, converting the electrical current into an ionic current through the electric field chamber with cations moving towards the cathode (negatively-charged electrode) and anions moving towards the anode (positively-charged electrode). (B) Enlarged view of the electric field chamber, illustrating how specific EFs are calculated and applied. The EF chamber is made by sealing glass coverslip spacers to the bottom of the culture chamber with dental wax. The lane between the coverslip spacers is coated with fibronectin, and then astrocytes are seeded onto the lane. At the start of the experiment, a glass coverslip is sealed on top of the spacers with a thin bead of silicone vacuum grease to create a roof. EF magnitude is calculated with the formula $E = \rho I / A$, where $\rho$ is the resistivity of the media (700 Ωmm), $I$ is the applied current, and $A$ is the cross-sectional area of the EF chamber. The cross-sectional area (A) of the EF chamber is determined based on the thickness of the coverslip spacers (h), and the distance between the spacers (w). Varying the width between the coverslip spacers and the magnitude of the applied currents creates different EF strengths. (Note, Figure 2.1(A) was illustrated by Christina Delli Santi.)
Figure 2.1: Electric field chamber
Figure 2.2: Methods to analyze cell migration

(A) A point in the center of each cell’s nucleus (gold arrow) is manually selected every 5 frames (15 minutes). (B) The change in position is used to calculate the speed (magnitude, \( r \)) and direction (\( \theta \)) of migration relative to the anode and cathode at each time point. In the example in panel B, the velocity vector measured at the 6 hour time-point corresponds to the cell’s measured displacement and direction over the previous 15 minute interval. (C) The direction of cell migration relative to the anode and cathode is double-plotted over time. Starting at 0°, which is indicated by the gold line segment in (B) corresponding to the radius extending to \((1, 0)\) on the unit circle, angle measures increase counterclockwise such that 90° corresponds to the anode, 270° corresponds to the cathode, 450° corresponds to the anode, and 630° corresponds to the cathode. On the directionality double-plots (C, D), the direction relative to the anode and cathode is plotted along the x-axis, and time is plotted along the y-axis beginning at 0:00 hours (when the EF is turned on), and increasing in 15-minute increments over 12 hours. For the example of cell velocity measured in (B), the cell is traveling at approximately 120° 6 hours after the start of the EF. In the sample directionality double-plot (C), this data point is plotted along the line corresponding to the 6-hour mark both at 120° and at 480°. This double-plotting helps visualize the directionality of the data. (D) A directionality double-plot of the full data set for cells exposed to 0 mV/mm. The uniform distribution of cell directions visually indicates the lack of directional migration for these cells. (Panel D corresponds to part of the same data that is displayed in Figure 2.8.)
Figure 2.2: Methods to analyze cell migration
Figure 2.3: Optimizing the measuring interval for cell tracking

Astrocytes were exposed to either 0 or 400 mV/mm and a 20x DIC image was acquired once every 3 minutes for 1 hour. Individual cells were tracked by manually selecting the position corresponding to the center of their nucleus in every frame. By selecting sub-sets of these tracking positions, the cell speed was calculated every 3, 6, 15, 30, or 60 minutes throughout the first hour of EF exposure. We used a mixed-effects generalized linear regression model to test the hypothesis that the sampling interval used to track cells affected the measured cell speed by increasing the proportion of the measured cell displacement due to random error in manual tracking. Astrocytes exposed 0 and 400 mV/mm were analyzed separately, and box-plots summarizing the spread of data for cells tracked every 3, 6, 15, 30, or 60 minutes are shown. The box spans the middle two quartiles, and the central bar indicates the mean. We found that there was a statistically significant difference in the mean speed measured among the different tracking intervals (0 mV/mm: $F_4 = 4.5879, p = 0.001597$; 400 mV/mm: $F_4 = 2.8128, p = 0.02637$). We used a Tukey-HSD post-hoc test to make individual comparisons between groups, and the results are indicated above the graphs. The letters above each graph indicate the statistically significant differences between the groups; groups are statistically significant (i.e. $p < 0.05$) if they do not share a letter. Note: statistically significant differences exist between groups that do not share a common letter (for a table detailing these data, see Table 2.1).
Figure 2.3: Optimizing the measuring interval for cell tracking
**Figure 2.4: Characterizing the astrocyte population**

Representative confocal images used to characterize the astrocyte population based on immunolabeling. (A) GFAP, (B) vimentin, (C) nestin, and (D) an overlay show that the population is morphologically heterogeneous, consisting of both lamellipodial and process-bearing cells. The cells universally express all three markers, with the relative levels varying among the different cells, suggesting that our population includes astrocytes of varying degrees of maturation. Scale bar = 20 µm.
Figure 2.4: Characterizing the astrocyte population
Figure 2.5: Paths of astrocyte migration over the first 6 hours of EF exposure

To qualitatively evaluate the EF-induced effect on migration, the paths of migration over the first six hours of EF exposure were plotted for individual astrocytes; the starting position was normalized to the origin of the graph (0, 0), and the direction was displayed relative to the orientation of the EF with the cathode (+) at the top of the graph and the anode (-) at the bottom of the graph. 30 cells from each EF strength were randomly selected to be included in this plot (including more than 30 cells makes it difficult to discern individual tracks). X- and Y-units for the graph are in micrometers. These graphs demonstrate the different effects that each EF strength have on directional migration.
Figure 2.5: Paths of astrocyte migration over the first 6 hours of EF exposure
Figure 2.6: Electric field exposure affects cortical astrocyte migration speed

(A) Astrocyte migration speed is plotted every 15 minutes for 12 hours. Within 30 minutes of EF onset, cells exposed to both 40 and 400 mV/mm show a similarly rapid increase in mean speed. For cells exposed to 40 mV/mm, this is maintained for only the first 2 hours and then returns to the same speed as 0 mV/mm, while this is maintained in the group exposed to 400 mV/mm for over 4 hours. Initially, there is no effect on cells exposed to 4 mV/mm, but the mean speed begins to decrease after 3 hours. (B) Effects of EF exposure on speed were assessed at each time point. 3 representative time points are shown corresponding to the start of the experiment (0 hours), and 30 minutes and 4 hours after EF onset. Mean speed was compared between EF strengths at each time point with a 1-factor ANOVA followed by a Tukey-HSD post hoc test. There was no difference in mean speed between groups at the start of the experiment ($F_{(3, 471)} = 1.54$, $p = 0.20$). There was a significant effect of EF exposure at both 30 minutes ($F_{(3, 474)} = 10.5$, $p = 1.1 \times 10^{-6}$) and 4 hours ($F_{(3, 483)} = 11.8$, $p = 1.8 \times 10^{-7}$). The mean speed of cells exposed to 0 mV/mm did not significantly change over time. All data are expressed as mean ± SEM. # $p = 0.0509$; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. 
Figure 2.6: Electric field exposure affects cortical astrocyte migration speed
Figure 2.7: Assessing sequence effects on migration

Sister cultures of astrocytes were used to test whether the delay between sub-culturing astrocytes in the EF chamber and beginning the EF exposure affected the astrocytes’ migrational response to EFs. To test this, sister cultures were exposed to 40 mV/mm for 12 hours, with the EF exposure beginning either 16 or 48 hours after cells were sub-cultured (corresponding to (1) and (2) indicated in the legend, respectively). Migrational speed for astrocytes in these groups is plotted every 15 minutes as mean ± SEM. The same pattern of change in migrational speed over time was observed for both groups: speed increased transiently over the first hour and returned to baseline after the second hour. This demonstrates that there is not a robust sequence effect on these experiments.
Figure 2.7: Assessing sequence effects on migration
**Figure 2.8: Electric fields induce directional migration**

Astrocytes preferentially migrate towards the anode of an applied electric field of 40 or 400mV/mm. The direction of migration was measured for each cell every 15 minutes over 12 hours relative to the anode (A, indicated in red) and cathode (C, indicated in blue) of the applied EF and plotted, with each dot representing the direction of migration of a single cell at each time point. The x-axis is double-plotted for each field strength to help visualize the directionality (see Figure 2.2 for a description of how these plots are generated). The random direction of cell movement in 0 and 4 mV/mm is visually displayed by the even distribution of data points along the x-axis. Directional migration towards the anode emerges in cells exposed to 40mV/mm after 1.5 hours. 400 mV/mm induces anodally-directed migration after 30 minutes, which is more concentrated (greater concentration parameter, κ) towards the anode than it is for cells exposed to 40 mV/mm. If the polarity of the 400 mV/mm EF is reversed after 6 hours (panel labeled 400(R) mV/mm, time when current was reversed is indicated with the dashed gold line), cells reorient to the new EF vector over the following 2 hours.
Figure 2.8: Electric fields induce directional migration
Figure 2.9: Electric field exposure induces cortical astrocyte proliferation

Proliferation peaks 48 hours after onset of the EF. A-D: Representative BrdU immunolabeling overlaid on 20x DIC images of cortical astrocytes 48 hours after exposure to 0 (A), 4 (B), 40 (C), or 400 (D) mV/mm; scale bars: 20 μm. The orientation of the applied EF is indicated in each image, with the anode (+) to the right of the image and the cathode (-) to the left. (E) Quantification of change in proliferation over time as a function of EF strength by comparing the % BrdU-positive cells to the total population of cells present. For all field strengths, there was no change in proliferation at 12 hours ($X^2_3 = 0.7400, p = 0.8638$) or 24 hours ($X^2_3 = 4.4643, p = 0.2155$) after exposure. The proliferation at 24 hours in groups exposed to 40 or 400 mV/mm is not significantly different from 0 in spite of the visual trend indicating an increase because the non-parametric statistical test used for this evaluation is very conservative. By 48 hours after EF onset, there was a significant increase in proliferation ($X^2_3 = 13.5526, p = 0.0036$) in astrocytes exposed to 40 mV/mm ($p = 0.0088$) and 400 mV/mm ($p = 0.0481$) relative to 0 mV/mm, while 4 mV/mm had no effect on proliferation ($p = 1.0000$). After 72 hours, there was still an observed increase in proliferation ($X^2_3 = 13.0060, p = 0.0046$), but only cells exposed to 400 mV/mm remain elevated ($p = 0.0386$) relative to 0 mV/mm (4 mV/mm: $p = 1.0000$; 40 mV/mm: $p = 0.1452$). All data are expressed as mean ± SEM. Kruskal-Wallis test followed by individual nonparametric comparison post-hoc tests using the Dunn Method for Joint Ranking. *$p < 0.05$, **$p < 0.01$. 
Figure 2.9: Electric field exposure induces cortical astrocyte proliferation
Figure 2.10: Electric field exposure aligns the axis of cell division

(A-D) Mitotic cells (arrow, A) were identified in time-lapse DIC microscopy videos. The axis of division was defined for each cell by drawing a line through the center of each of the daughter nuclei (line, D) and measuring the angle of this axis relative to the applied EF (cathode (-) at the top of each image, anode (+) at the bottom of each image). The time (in minutes) of each image is provided to illustrate the duration of mitosis. Scale bar = 20 µm. (E) Frequency histograms representing the relative number of nuclei counted at each orientation relative to the anode (A) and cathode (C) (histogram bin width of 15°), with a density curve super-imposed on each graph. Nuclear alignment is double-plotted along the x-axis to aid in observing the alignment of these data. Each EF strength was evaluated for alignment with Rayleigh's test, and found that only 400 mV/mm induced alignment ($n = 124, R = 0.3740, p = 2.93 \times 10^{-8}$), with a mean direction $\mu \pm SEM = 82.1 \pm 3.53^\circ, \kappa = 2.71$, and SD = 39.7° (the EF axis runs from 0° to 180°, with 90° being perpendicular to the EF vector). There was no significant alignment for cells exposed to 0 mV/mm ($n = 176, R = 0.1127, p = 0.1071$), 4 mV/mm ($n = 186, R = 0.1071, p = 0.1183$), or 40 mV/mm ($n = 260, R = 0.0437, p = 0.6092$).
Figure 2.10: Electric field exposure aligns the axis of cell division
Figure 2.11: EF effects on intermediate filament expression and morphology

Representative confocal images of astrocytes after 72 hours’ exposure to 4, 40, or 400 mV/mm. (A-C) DAPI-labeled nuclei, with an overlay indicating the orientation of the electric field vector (+) and (−), corresponding to the anode and cathode, respectively. Immunolabeling for GFAP (D-F), vimentin (G-I), and nestin (J-L) show that only 40 mV/mm induces an up-regulation of vimentin and GFAP. (M-O) FFT analysis of normalized pixel intensity from vimentin-labeled images (averaged over 6-8 images) shows that astrocytes exposed to 4 and 40 mV/mm for 72 hours extend their processes in random directions, while only astrocytes exposed to 400 mV/mm display a strong preference for process alignment perpendicularly to the vector of the EF as indicated by the high peaks. All 4 graphs are plotted with the same scale on the vertical axis; the horizontal axis indicates directionality relative to the anode (+) and cathode (−). Scale bar: 50 µm.
Figure 2.11: EF effects on intermediate filament expression and morphology
Table 2.1: Optimizing the measurement interval for cell tracking

Coefficient parameters for the Tukey-HSD post hoc tests, indicating the estimated difference (μm/hour) between each group, the t-score of the difference, and the adjusted p-value for the comparison.

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(adjusted p values are reported, using the single-step method)
Chapter 3: Cerebellar Astrocytes

Chapter Overview

In the previous chapter, we found evidence to support our hypothesis that injury-induced EFs determine the regenerative potential in the CNS by affecting each of the astrocytic behaviors necessary for wound repair. However, our observations were limited to cortical astrocytes, but astrocytes demonstrate remarkable morphological and functional heterogeneity throughout different regions of the CNS. This heterogeneity among sub-populations of astrocytes suggests that the astrocytic response to injury may also vary among different regions within the mammalian CNS. Indeed, while reactive astrocytosis in the cortex is associated with the formation of a chronic glial scar, reactive astrocytes in the cerebellum do not reorganize into a similarly demarcated scar; moreover, there is evidence that certain neuronal cell types within the mammalian cerebellum regenerate. Similarly to how EFs drive behaviors associated with the astrocytic response to injury in the mammalian cortex, we hypothesized that EFs drive a reactive astrocytic phenotype in the cerebellum. Moreover, we hypothesized that the effect of EFs on cerebellar astrocytes would be different than that of EFs on cortical astrocytes, reflecting the difference in the injury response displayed by these two different populations of astrocytes.
Introduction

The mammalian CNS is characterized by minimal regeneration because the astrocytic response to parenchymal injury results in the formation of a chronic glial scar that inhibits axon regeneration\textsuperscript{33, 35}. However, astrocytes demonstrate remarkable heterogeneity in their response to injury across different regions of the brain\textsuperscript{380}, suggesting that glial scar formation and axon regeneration may be similarly variable. Indeed, the cerebellum remains conducive to axon outgrowth, as climbing fiber axons from olivocerebellar neurons are able to sprout through the intact parenchyma to their original targets after being individually axotomized using laser dissection (a procedure that does not induce tissue injury or a reactive response)\textsuperscript{47}, and these same intrinsic regenerative properties allow them to regenerate after injury past the lesion site\textsuperscript{381}; upon reaching their original targets, they functionally re-integrate into the remaining neural circuitry through local plasticity\textsuperscript{47, 381}. Moreover, cerebellar astrocytes promote migration of new cerebellar neurons throughout the life of the animal\textsuperscript{382}. The cerebellum, which is located in the hindbrain (Figure 3.1), is the phylogenetically oldest part of the mammalian brain, suggesting that its relatively greater inherent regenerative capacity may be a reflection of some vestigial phenotype that once facilitated regeneration in a common vertebrate ancestor.

Interestingly, the heterogeneous astrocytic population in the cerebellum reflects the phenotypic origins of the structure: astrocytes in the granular layer demonstrate a stellate morphology that is similar to cortical astrocytes, while astrocytes in the Purkinje-cell and molecular layers, which are eponymously known as Bergmann glia, more closely resemble immature astrocytes and radial glia (Figure 3.1B). Radial glia, which
are bipolar cells that span the entire width of the embryonic cerebellum, differentiate into Bergmann glia through as Shh and FGF-9 signaling\textsuperscript{382, 383}. As they differentiate, radial glia retain their basal branch, and Notch signaling induces additional processes to develop such that Bergmann glia ultimately have 3-6 branches that traverse the molecular layer\textsuperscript{75, 107, 382}. Bergmann glia express GFAP, but they are morphologically similar to radial glia\textsuperscript{384}, and they express the immature/stem-cell intermediate filament Nestin and the transcription factor Sox2\textsuperscript{382, 385}.

Following an injury, Bergmann glia and cerebellar astrocytes both develop hypertrophic processes within 10 days of a lesion, overexpress the intermediate filaments GFAP and vimentin, and deposit NG2 – a CSPG that inhibits axon outgrowth – into the ECM\textsuperscript{386, 387}. This response has been defined as characteristic reactive gliosis because of the strong similarities response to injury in cortical astrocytes. Nonetheless, cerebellar astrocytes and Bergmann glia also overexpress the intermediate filament nestin\textsuperscript{384} and a subset of cerebellar astrocytes express the embryonic neural cell adhesion molecule PSA-NCAM\textsuperscript{388}, which is a permissive substrate for neurite outgrowth, together suggesting that these cells also retain characteristics of immaturity associated with radial glia. Early studies that did not distinguish between sub-layers within the cerebellum reported a glial scar induced by injury\textsuperscript{389}, and biopsies from two case reports in human patients suggests evidence that implanted electrodes induce a gliotic response after 3 months\textsuperscript{390}. However, subsequent research suggests that Bergmann glia do not reorganize and form a glial scar in the molecular layer\textsuperscript{92, 384}, moreover, while cerebellar astrocytes do form a glial scar in the granular layer, their induction of PSA-NCAM expression beginning 3 months after axotomy corresponds to
the initiation of outgrowth from axotomized Purkinje cell axons, and up to 75% of axons sprouting past the glial scar are ensheathed by these reactive astrocytes\textsuperscript{388, 391}. Together, this suggests that cerebellar astrocytes and Bergmann glia may facilitate a regenerative response to injury in the cerebellum because they represent an intermediate astrocytic phenotype between that of regenerative radial glia in non-mammalian vertebrates and of non-regenerative cortical astrocytes in mammals.

In chapter one, we established that endogenous electric fields regulate wound repair and determine regenerative outcomes after injury by directly influencing the cellular response to injury. In chapter two, we showed that physiologic EFs regulate behaviors from cortical astrocytes associated with their injury response, and that elevated EFs modify this response towards a more regenerative phenotype. Based on these observations, we hypothesized that physiologic EFs also regulate the astrocytic response to injury in the mammalian cerebellum. Moreover, we hypothesized that the greater regenerative potential in the cerebellum is associated with a more robust regenerative response among cerebellar astrocytes to physiologic EFs. In the experiments described in the current chapter, we exposed rat cerebellar astrocytes to EFs following the same experimental design that we used in chapter two. Our findings from these studies suggest that cerebellar astrocytes demonstrate a robust response to EFs that is greater in its intensity and duration the response we have observed among cortical astrocytes. This suggests that cerebellar astrocytes maintain a greater regenerative potential because they are more intrinsically responsive to physiologic EFs present in the injury environment.
Methods

Cell source and culture methods

Rat cerebellar astrocytes harvested from P2 cerebellum were purchased from ScienCell (cat # R1800). Cultures have greater than 99% purity as determined with GFAP immunolabeling by ScienCell. The company makes no distinction among astrocytic sub-populations from the molecular and granular layers, and GFAP staining cannot differentiate between these cell types. Consequently, the astrocytes used in these experiments represent a heterogeneous population, similar to the mixed population present in an injury site in vivo. All of the astrocytes used for these experiments came from the first five passages after the initial thaw. Astrocyte cultures were maintained according to the protocol recommended by ScienCell. Briefly, astrocytes were thawed into poly-L-lysine (ScienCell # 0413) coated T75 culture flasks containing astrocyte media (pH 7.4; ScienCell AM-a 1831) supplemented with 2% fetal bovine serum (ScienCell # 0010) and 1% penicillin/streptomycin (ScienCell # 0503). Cultures were maintained in a humidified 37°C incubator with a 5% CO₂ atmosphere, and culture media was changed every 2-3 days. Once the cultures reached confluence, approximately 5,000 astrocytes were sub-cultured into each EF chamber (see description below) for experiments. Methods used to design the EF chamber were identical to the methods described in chapter two (page 80).
Experimental design and statistical analysis

For the experiments described in this chapter, we exposed cerebellar astrocytes to EFs of 0, 4, 40, or 400 mV/mm and repeated many of the analyses that we completed with cortical astrocytes in chapter two. We initially characterized the population of cerebellar astrocytes with immunolabeling for GFAP, vimentin, and nestin. We then assessed effects on migrational speed and direction, orientation of proliferation, and morphology. The methods and analyses used in these experiments are identical to those methods describe in chapter two (beginning on page 82), with the exceptions that cerebellar astrocytes used for immunocytochemistry studies were only exposed to EFs for 12 hours.

Results

Characterizing the cerebellar astrocyte population

We used cultured rat cerebellar astrocytes to test whether the EFs that are associated with intact and injured mammalian tissues may affect cerebellar astrocyte behaviors in vitro. The cerebellar astrocytes used in these experiments were purchased as purified populations from ScienCell (a company that specializes in preparing purified primary cell cultures), who verified population purity with GFAP staining. We also characterized the astrocytic population prior to conducting each experiment. We used immunofluorescence labeling against GFAP, vimentin, and nestin, as well as morphological characteristics in DIC, to evaluate the purity and maturation of these astrocytes (Figure 3.2). These astrocytes consistently expressed high levels of GFAP
and vimentin; in contrast, most of the cells did not express nestin, although certain sub-populations were strongly nestin-positive. Cell morphologies seemed to be consistent with the cytoskeletal elements that they expressed: nestin-negative cells maintained a lamellipodial morphology consistent with cerebellar astrocytes, while nestin-positive cells tended to have a more bipolar, process-bearing morphology similar to that characteristic of Bergmann glia. This suggests that this population contains a mixture of cerebellar astrocytes and Bergmann glia.

*Electric fields affect the speed of cerebellar astrocyte migration*

To test our overall hypothesis that EFs direct the astrocytic response to injury, we explored how EF exposure affects each of the behaviors that astrocytes characteristically display after injury in both mammalian and non-mammalian vertebrates. The first of these behaviors is migration, as astrocytes must move towards the lesion as they are recruited to restore BBB integrity and isolate the lesion environment from the surrounding healthy tissue. We tested the ability of EFs to influence astrocyte migration by using time-lapse DIC live cell microscopy to record how primary rat cerebellar astrocytes respond to dcEFs over a 12-15 hour period. We compared how EF intensities associated with intact (4 mV/mm), injured mammalian (40 mV/mm), and injured non-mammalian vertebrate tissues (400 mV/mm) affect migration relative to an untreated control (0 mV/mm). We observed no evidence of cell death during these experiments as a function of either EF exposure or of phototoxicity from repeated exposure to light. We measured migration by tracking cells every 15 minutes, which was the frequency that provided the greatest temporal resolution for velocity.
measurements without adding a significant component of error in selecting cell position (see page 95 for details). Each experiment was conducted at least 3 times using cells derived from different animals, and at least 30 cells were tracked at each time point from at least 5 fields of view for each experiment; results from each experiment were pooled and analyzed together (n ≥ 90 cells for each EF strength at each time point).

Time-lapse videos show that, in the absence of any EF, astrocytes have a heterogeneous morphology and display non-directional movement. Cells exposed to 4 mV/mm show similar morphologies and patterns of movement. In contrast to the cortical astrocyte response to 40 mV/mm, time-lapse videos show that 40 mV/mm does not induce directional migration or increased speed in cerebellar astrocytes. However, cerebellar astrocytes exposed to 400 mV/mm demonstrate a rapid response to the EF exposure, with the entire cell population migrating towards the anode of the EF within the first hour of the EF exposure.

To qualitatively evaluate the EF-induced effect on migration, the paths of migration over the first six hours of EF exposure were plotted for individual astrocytes; the starting position was normalized to the origin of the graph (0, 0), and the direction was displayed relative to the orientation of the EF (Figure 3.3). The mean cell speed is plotted for each EF-exposure at 15-minute intervals over the 12-hour experiment (Figure 3.4A). Using a 1-factor ANOVA, we found no significant differences in the mean cell speed among the EF-exposure groups prior to the EF onset (0 minutes: $F_{3, 360} = 2.13, p = 0.096$; mean speed ± SEM for 0 mV/mm: 16.64 ± 1.19 µm/hr; 4 mV/mm: 21.70 ± 2.62 µm/hr; 40 mV/mm: 17.89 ± 1.85 µm/hr; 400 mV/mm: 21.21 ± 1.52 µm/hr; Figure 3.4B, left panel). 15 minutes after EF onset, there is a statistically significant difference
in mean astrocyte speeds among the different EF-exposure groups \((F_{3, 357} = 17.1, p = 2.12 \times 10^{-10})\); mean speed ± SEM for 0 mV/mm: 16.26 ± 1.14 µm/hr; 4 mV/mm: 22.70 ± 1.69 µm/hr; 40 mV/mm: 17.42 ± 1.48 µm/hr; 400 mV/mm: 30.55 ± 2.00 µm/hr; Figure 3.4B, center panel). Using the Tukey-HSD post-hoc test, we found that cerebellar astrocytes exposed to 400 mV/mm have a greater cell speed than cells exposed to 0 mV/mm \((p = 1.761 \times 10^{-9})\); estimated difference: 14.28 µm/hr, 95% CI: 8.60, 19.96), 4 mV/mm \((p = 3.826 \times 10^{-3})\); estimated difference: 7.85 µm/hr, 95% CI: 1.93, 13.77), and 40 mV/mm \((p = 1.205 \times 10^{-7})\); estimated difference: 13.12 µm/hr, 95% CI: 7.22, 19.02); we also found that cells exposed to 4 mV/mm had an increased speed relative to 0 mV/mm \((p = 2.717 \times 10^{-2})\); estimated difference: 6.43.µm/hr, 95% CI: 0.51, 13.35), but that their speed was not different from astrocytes exposed to 40 mV/mm \((p = 0.1197)\).

Although this suggests that 4 mV/mm may transiently induce an increased migration speed, this more likely represents an aberration or artifact than a true effect as the difference between 0 and 4 mV/mm is relatively small, 4 mV/mm was not different from 40 mV/mm, and 0 mV/mm was not different from 40 mV/mm; moreover, 4 mV/mm was different from neither 0 nor 40 mV/mm at 30, 45, or 60 minutes after EF onset, while the increased speed for astrocytes exposed to 400 mV/mm was sustained at each of these time points and throughout the rest of the 12 hour experiment. Cerebellar astrocytes exposed to 400 mV/mm sustained this increased speed (relative to 0 mV/mm) throughout the entire 12 hours of the experiment, while there were no differences in mean cell speed among the cells exposed to 0, 4, or 40 mV/mm at any subsequent time point. (Differences between cell speeds were compared between EF exposure groups at each time point using a 1-factor ANOVA with a Tukey-HSD post-hoc test; see Figure
3.4B for analyses at the start of the experiment, and 0, 15 minutes and 2 hours after the EF onset.) Thus, only EFs elevated at levels associated with regenerating non-mammalian vertebrate tissues affect cerebellar astrocyte migration speed.

*Electric fields are a directional cue for cerebellar astrocyte migration*

Having demonstrated that EF exposure alters cell speed in an intensity- and time-dependent manner, we tested whether EFs also serve as an orientational cue by causing directional migration. Directionality was assessed for each EF intensity at each time point using Rayleigh’s test (which tests the hypothesis of a non-random direction about a circle against the null hypothesis of a random direction), using an overall $p$-value = 0.05 with a Bonferroni correction for the total number of factor levels analyzed (196 comparisons: 4 EF levels at each of 49 time points). We found that there was no directional migration in the 0 mV/mm control group, and that neither 4 mV/mm nor 40 mV/mm caused cerebellar astrocytes to move directionally. However, 400 mV/mm induced directional migration towards the anode within 15 minutes of EF exposure (i.e. the first time point measured after EF onset); the mean direction in which cells traveled was $93.29\pm 0.99^\circ$, $\kappa = 1.56 \pm 0.03$, where $90^\circ$ corresponds to the anode (circular mean direction ± circular standard deviation calculated for all cell directions at time points where there was statistically-significant directional migration) (Figure 3.5). As the lesion site within the CNS becomes the anode of the injury-induced EF (see discussion in Chapter 1, page 48), EFs may be contributing towards astrocyte migration towards the lesion site *in vivo*. Moreover, once directionality emerged, it continued throughout the entire recording period. We also found that, similarly to cortical astrocytes, cerebellar
astrocytes remain very sensitive to changes in the orientation in the extracellular EFs. We demonstrated this by exposing cerebellar astrocytes to 400 mV/mm for 6 hours and then reversing the polarity of the EF exposure for another 6 hours. We found that cerebellar astrocytes stop moving towards the original anode after 15 minutes of reversing the direction of the EF, and they orient themselves to the new anode after 30 minutes of non-directional movement. From the time-lapse videos and the directionality double-plot, this period of “non-directional” movement appears to reflect the cells actually turning around as they reorient themselves to the new direction of the imposed EF, during which time half of the population turned clockwise and the other half turned counter-clockwise (as indicated by the phase-shifts in the directionality data, Figure 3.5). This rate of reversal was much more rapid than the two-hours required for cortical astrocytes to reorient to a change in polarity of an applied EF, suggesting that cerebellar astrocytes may be more responsive to EFs than cortical astrocytes are.

*Electric fields align the axis of cell division*

While reviewing the time-lapse live cell videos, we observed that cells exposed to 400 mV/mm tended to divide with an axis of division perpendicular to the EF vector. The orientation of division is known to influence cellular activity during mammalian embryogenesis\(^{107}\), and regeneration in non-mammalian vertebrates is known to recapitulate development, so we were interested in determining whether EF exposure aligns the axis of division in cerebellar astrocytes. Using the DIC time-lapse videos, we identified mitotic cells and measured the angle of the axis of division relative to the EF vector by drawing a line between the two daughter nuclei in the first frame where they
were distinctly visible (Figure 3.6A-D). The distribution of these axes relative to the EF vector are plotted for each EF strength (Figure 3.6E), with the axial data double-plotted on the x-axis relative to the anode (A) and cathode (C) to assist in visualizing the clustering of mitotic events perpendicularly to the EF vector. Using the Rayleigh test for alignment, we found that EF exposure aligned the axis of cell division for cells exposed to 400 mV/mm (n = 123, R = 0.1721, p = 2.61 \times 10^{-2}), with a mean direction \( \mu \pm \text{SEM} = 98.21 \pm 5.97^\circ, \kappa = 1.35, \) and SD = 61.9° (the EF axis runs from 0° to 180°, with 90° being perpendicular to the EF vector). We found that there was no significant alignment for cells exposed to 0 mV/mm (n = 122, R = 0.1408, p = 0.089), 4 mV/mm (n = 99, R = 0.0727, p = 0.5925), or 40 mV/mm (n = 55, R = 0.0533, p = 0.8553). Thus, only EFs associated with regenerating tissues align the axis of division in cerebellar astrocytes.

*Electric fields align cerebellar astrocytes*

Cerebellar astrocytes are a heterogeneous population, with a sub-population morphologically similar to mammalian cortical astrocytes and another sub-population (Bergmann glia) with long processes spanning the molecular layer resembling radial glia in non-mammalian vertebrates. These aligned processes help certain classes of neurons to migrate through the adult cerebellum, and aligned astrocytic processes also promote a regenerative phenotype in the mammalian and non-mammalian vertebrate CNS. In chapter 2, we demonstrated that elevated EFs at levels associated with regeneration promote cortical astrocytes to align their processes perpendicularly to the polarity of the applied EF. Our time-lapse videos from the cerebellar astrocyte migration studies suggested that EFs induce morphological changes in cerebellar astrocytes.
similar those that we observed in cortical astrocytes and, given that these morphological changes are associated with a regenerative phenotype, we next set out to quantify these effects on morphology using FFTs on cells immunolabeled for DAPI, GFAP, vimentin, and nestin images (Figure 3.7). A qualitative review of these immunolabeled images showed that there was a relatively heterogeneous astrocytic population in the cells exposed to 0, 4, and 40 mV/mm, consisting of both lamellipodial and bipolar cells, while astrocytes exposed to 400 mV/mm appeared to assume a more process-bearing morphology. It also appears as though there is a relatively consistent expression of both GFAP and vimentin among these cells, with nestin-expressing cells intermittently present. We analyzed the vimentin-immunolabeled cells for alignment using an FFT analysis (Figure 3.8). These FFTs show that astrocytes exposed to 400 mV/mm align their processes perpendicularly to the applied EF; astrocytes exposed to 0, 4, and 40 mV/mm also demonstrate some periodicity in the alignment of their processes, but this is much smaller in magnitude than the alignment induced by 400 mV/mm, it is focused at an arbitrary orientation, and it is consistent among the 0, 4, and 40 mV/mm groups, and it may represent some sort of artifact rather than an actual alignment effect. These results summarize data from only one experiment, so we are unable to determine whether the alignment demonstrated by astrocytes exposed to 4 and 40 mV/mm represents something real about these cells or if it instead is an artifact.

Discussion

Increasing evidence supports the notion that bioelectric fields interact with all cells, and that injury-induced EFs regulate the cellular response to injury. This is evidenced by multiple studies demonstrating that elevated EFs induce the same
behaviors necessary for epimorphic regeneration among diverse cell types from many different tissues. We have previously described evidence that mammalian astrocytes, which are necessary for the cellular response to injury in the CNS, respond to elevated EFs through the same induced behaviors as are expressed by cells in other tissues upon EF exposure (chapter two). Our findings in the current study, that cerebellar astrocytes respond to elevated EFs, are consistent with the notion that EFs are a universal signal that regulates cellular activity and wound repair. We found that cerebellar astrocytes did not respond to EFs of 40 mV/mm; as 40 mV/mm induces behaviors in cortical astrocytes associated with glial scar formation, this suggests that cerebellar astrocytes may not form a glial scar because they do not respond to the injury-induced EFs within the cerebellum. In contrast, cerebellar astrocytes responded to EFs associated with regenerating tissues similarly to the response demonstrated by cortical astrocytes, demonstrating a rapid increase in cell speed and migrating towards the anode; we also found that only EFs associated with regeneration induced morphological changes in astrocytes that mirror those that facilitate regeneration in non-mammalian vertebrates. The difference between the EF intensities necessary to induce responses in cortical and cerebellar astrocytes also illustrates that the cellular response to EFs is an active one, whereby the cells are “choosing” to respond in certain nuanced ways; if instead cells were being forced to respond to gross electrochemical forces, both astrocytic sub-types should have had the same response. Assuming that the injury-induced EFs within the cerebellum are similar to those that are present in other injured mammalian tissues (and to the EFs within a hippocampal slice culture preparation), this would suggest that physiologic EFs within the injury site do not
induce a robust reactive astrocytic response but that therapeutically elevated EFs may still induce regenerative behaviors in cerebellar astrocytes.

This observed difference between the EF-induced response in cortical and cerebellar astrocytes is consistent with differences in their response to injury in vivo. Specifically, cortical astrocytes rapidly respond to an injury by restoring the BBB, but this response evolves into the formation of a glial scar; cerebellar astrocytes hypertrophy following an injury, but do not form a similarly robust glial scar. However, it does not explain why the same injury-induced EF would have different effects on these astrocytic behaviors. One possibility is that 40 mV/mm may induce only a subset of the behaviors necessary for regeneration in cortical astrocytes, so they form a glial scar once they are stimulated to migrate to and proliferate at the lesion site, but EF-intensity does not reach the threshold necessary to induce neurogenesis or to promote axon sprouting; in contrast, the EF-induced threshold for each of the regenerative behaviors in cerebellar astrocytes remains within a close range of each other so that these behaviors are induced together in an all-or-none regenerative effort, and 40 mV/mm is not sufficiently intense to reach this threshold. Alternatively, it is possible that injury-induced EFs within the cerebellum are higher than they are in the cortex, so the lack of response among cerebellar astrocytes to 40 mV/mm could reflect the fact that these EFs are not within the range necessary to induce injury-associated behaviors. In either case, the underlying axiom of these experiments is that injury-induced EFs determine the regenerative potential of the injured tissue. As the regenerative potential in the cerebellum is different from that in the cortex, and the astrocytic response to injury largely determines the regenerative potential of the injured
CNS, it is reasonable to assume either that the same injury-induced EFs are present in the cortex and cerebellum but that they have different effects on the resident astrocytes, or that different injury-induced EFs are present in these tissues which explains why the resident astrocytes respond differently to injury in each of these tissues. However, there is very little information as to the actual EF intensities within the intact and injured cerebral cortex, and there is no information about EFs within the cerebellum. Therefore, additional research is needed to explore the EFs throughout the mammalian CNS as we try to understand how physiologic EFs regulate the regenerative potential in the CNS.
Figure 3.1: Heterogeneity of cerebellar astrocytes

(A) Parasagittal section of the rat brain, Nissl stain. Astrocytes used in the experiments described in chapter two came from the cortex (black arrows), which is the phylogenetically newest region of the mammalian CNS. However, the resolution of CNS injury demonstrates regional variability throughout the CNS. The cerebellum (white arrowheads) is one of the phylogenetically oldest regions of the mammalian CNS and is thought to most closely resemble the CNS of non-mammalian vertebrates. (B) The rat cerebellum immunolabeled for GFAP, demonstrating the regional heterogeneity of the resident astrocyte populations. Bergmann glia have highly aligned processes spanning the molecular layer (white arrowhead) and resemble the radial glia found throughout the CNS of adult non-mammalian vertebrates; cerebellar astrocytes in the granular layer (white arrow with rounded base) have a stellate morphology more closely resembling that of cortical astrocytes in the adult mammalian CNS. (These images come from unpublished observations in the Colello lab.)
Figure 3.1: Heterogeneity of cerebellar astrocytes
Figure 3.2: Characterizing the cerebellar astrocyte population

Representative confocal images used to characterize the astrocyte population based on immunolabeling. (A) GFAP, (B) vimentin, (C) nestin, and (D) an overlay shows that the population is morphologically heterogeneous, consisting of both lamellipodial and process-bearing cells. These astrocytes consistently expressed high levels of GFAP and vimentin; in contrast, most of the cells did not express nestin, although certain sub-populations were strongly nestin-positive. Cell morphologies seemed to be consistent with the cytoskeletal elements that they expressed: nestin-negative cells maintained a lamellipodial morphology consistent with cerebellar astrocytes, while nestin-positive cells tended to have a more bipolar, process-bearing morphology similar to that characteristic of Bergmann glia. This suggests that this population contains a mixture of cerebellar astrocytes and Bergmann glia.
Figure 3.2: Characterizing the cerebellar astrocyte population
To qualitatively evaluate the EF-induced effect on migration, the paths of migration over the first six hours of EF exposure were plotted for individual astrocytes; the starting position was normalized to the origin of the graph (0, 0), and the direction was displayed relative to the orientation of the EF with the cathode (+) at the top of the graph and the anode (−) at the bottom of the graph. 30 cells from each EF strength were randomly selected to be included in this plot (including more than 30 cells makes it difficult to discern individual tracks). X- and Y-units for the graph are in micrometers. These graphs demonstrate the different effects that each EF strength have on directional migration.
Figure 3.3: Cerebellar astrocyte migration paths in an EF
Figure 3.4: EFs affect cerebellar astrocyte migration speed

(A) Astrocyte migration speed is plotted every 15 minutes for 12 hours. Within 15 minutes of EF onset, cells exposed to 400 mV/mm show a rapid increase in mean speed, which is sustained throughout the entire 12-hour observation period of the experiment. (B) Effects of EF exposure on speed were assessed at each time point and 3 representative time points are shown corresponding to the start of the experiment (0 Hours), and 15 minutes and 2 hours after EF onset. Mean speed was compared between EF strengths at each time point with a 1-factor ANOVA followed by a Tukey-HSD post hoc comparison. There was no difference in mean speed between groups at the start of the experiment ($F_{3, 360} = 2.13, p = 0.096$). There was a significant effect of EF exposure at both 15 minutes ($F_{3, 357} = 17.1, p = 2.12 \times 10^{-10}$) and 2 hours ($F_{3, 363} = 7.08, p = 2.30 \times 10^{-6}$). The mean speed of cells exposed to 4 or 40 mV/mm did not significantly change over time compared to 0 mV/mm. All data are expressed as mean ± SEM. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. 
Figure 3.4: EFs affect cerebellar astrocyte migration speed
Figure 3.5: EFs affect cerebellar astrocyte direction of migration

Astrocytes preferentially migrate towards the anode of an applied electric field of 400 mV/mm. The direction of migration was measured for each cell every 15 minutes over 12 hours relative to the anode (A, indicated in red) and cathode (C, indicated in blue) of the applied EF and plotted, with each dot representing the direction of migration of a single cell at each time point. The x-axis is double-plotted for each field strength to help visualize the directionality. The random direction of cell movement in 0, 4, and 40 mV/mm is visually displayed by the even distribution of data points along the x-axis. Directional migration towards the anode emerges in cells exposed to 400 mV/mm after 15 minutes. If the polarity of the 400 mV/mm EF is reversed after 6 hours (panel labeled 400(R) mV/mm, time when current was reversed is indicated with the dashed gold line), cells reorient to the new EF vector over the following 0.5 hours.
Figure 3.5: EFs affect cerebellar astrocyte direction of migration
Figure 3.6: EFs affect orientation of cerebellar astrocyte mitotic axis

Frequency histograms representing the relative number of nuclei counted at each orientation relative to the anode (A) and cathode (C) (histogram bin width of 15°), with a density curve super-imposed on each graph. Nuclear alignment is double-plotted along the x-axis to aid in observing the alignment of these data. Each EF strength was evaluated for alignment with Rayleigh’s test. Only 400 mV/mm induced alignment (n = 123, R = 0.1712, p = 2.61 x 10^{-2}), with a mean direction μ ± SEM = 98.21 ± 5.97°, κ = 1.35, and SD = 61.9° (the EF axis runs from 0° to 180°, with 90° being perpendicular to the EF vector). There was no significant alignment for cells exposed to 0 mV/mm (n = 122, R = 0.1408, p = 0.0890), 4 mV/mm (n = 99, R = 0.0727, p = 0.5925), or 40 mV/mm (n = 55, R = 0.0533, p = 0.8553). (Note, R is the test statistic for the Rayleigh test.)
Figure 3.6: EFs affect orientation of cerebellar astrocyte mitotic axis
**Figure 3.7: Cytoskeletal elements in cerebellar astrocytes after EF exposure**

Representative confocal images showing cerebellar astrocytes after 12 hours’ exposure to 0, 4, 40, or 400 mV/mm. Each EF-exposure is listed on its own row, with the EF intensity indicated in the left-most panel. Column (A): DAPI-labeled nuclei, with an overlay indicating the orientation of the electric field vector (+) and (-), corresponding to the anode and cathode, respectively. Immunolabeling for GFAP (column B), vimentin (column C), nestin (column D), and an overlay of all of these elements (column E); the same acquisition and display settings were used for each of these images. 400 mV/mm produced a dramatic effect on cell morphology and cytoskeletal element expression, with all of the astrocytes demonstrating elongated, bipolar morphologies with their processes perpendicular to the orientation of the applied EF. Furthermore, the merged images indicate that cells exposed to 400 mV/mm had a greater ratio of vimentin to GFAP expression relative to those cells exposed to 0, 4, or 40 mV/mm. These images also show that there is a heterogeneous population of cerebellar astrocytes upon exposure to 0, 4, and 40 mV/mm for 12 hours: the majority of these cells expressed GFAP and vimentin; while GFAP was seemingly constant, vimentin appears to vary with greater expression in bipolar cells and lower expression in lamellipodial cells. In contrast, nestin expression was sporadic. Scale bar: 50 µm.
Figure 3.7: Cytoskeletal elements in cerebellar astrocytes after EF exposure
Figure 3.8: EFs align cerebellar astrocyte processes

FFT analysis of normalized pixel intensity from vimentin-labeled images (averaged over 4-5 images); relative orientation is plotted in arbitrary units on the y-axis for each angle relative to the anode and cathode (in 1° increments; alignment is an axial unit, i.e. 0° = 180°, which is represented in the double-plotting of these graphs), and the same scale is used for the y-axis in each of the four graphs. These FFTs show that astrocytes exposed to 400 mV/mm align their processes perpendicularly to the applied EF; astrocytes exposed to 0, 4, and 40 mV/mm also demonstrate some periodicity in the alignment of their processes, but this is much smaller in magnitude than the alignment induced by 400 mV/mm, it is focused at an arbitrary orientation, it is consistent among the 0, 4, and 40 mV/mm groups, and it may represent some sort of artifact rather than an actual alignment effect.
Figure 3.8: EFs align cerebellar astrocyte processes
Chapter 4: In Vivo Experiments

Chapter Overview

In the first chapter, we discussed the physiologic EFs reported in biological systems. Intact tissues generally produce dcEFs ≤ 10 mV/mm, injured mammalian tissues (i.e. non-regenerating tissues) sustain an 8-15 fold EF increase, and regenerating tissues produce a 50-100 fold EF increase. This suggests that the magnitude of injury-induced EFs, which varies between regenerating and non-regenerating tissues, determines the regenerative outcome. In chapters two and three, we tested the hypotheses that physiologic EFs contribute to the astrocytic response to injury, and that a regenerative phenotype is favored by increasing the EF intensity towards levels found in regenerating tissues. The EFs used in these experiments were chosen based on previously published studies, but the existing literature also suggests the actual range of injury-induced EFs is broad. Moreover, certain types of equipment used to measure EFs is inherently biased, and the studies measuring EFs in the mammalian CNS are entirely based on ex vivo recordings. In previous chapters, we found that EFs induce intensity-dependent behaviors in mammalian astrocytes, and that the highest EF strength tested promoting a regenerative response. This suggests that physiologic injury-induced EFs may be therapeutically targeted to activate the physiologic mechanisms necessary for spontaneous regeneration. However, technical
challenges have prevented a thorough exploration of the strength, intensity, and duration of ionic currents produced by CNS injury. In this chapter, we discuss these limitations and our efforts to address them.

Introduction

Physiologic electric fields (EFs) drive the cellular response to injury and determine the regenerative potential in many vertebrate tissues. As we described in chapter one, EFs above a certain threshold are both necessary and sufficient to reactivate developmental physiology and induce complete regeneration of tissues and organs following injury or amputation. Injury-induced EFs are lower in tissues that do not regenerate, and regeneration can be stimulated by experimentally increasing these EFs towards levels found in highly regenerative tissues. Endogenous EFs have also been measured ex vivo in the mammalian CNS where they are similar in magnitude to the EFs that have been measured in other intact and injured mammalian tissues\textsuperscript{269}. Similarly to how injury-induced EFs drive the cellular response to injury in peripheral tissues, we found that EFs within the ranges reported in the injured mammalian CNS\textsuperscript{290} induce a series of behaviors in cortical and cerebellar rat astrocytes consistent with their physiologic response to injury (chapters two and three, respectively). Moreover, we found that EFs associated with highly regenerative tissues modify the astrocytic response, inducing changes that are associated with a more regenerative phenotype. Together, these observations suggest that the therapeutic manipulation of injury-induced EFs may be sufficient to stimulate regeneration in the mammalian CNS.

Endogenous EFs are sustained in intact tissues through an ionic current that is relatively constant in its magnitude and orientation over time, while injury-induced EFs
change in both magnitude and direction throughout the reparative process\textsuperscript{270}. In regenerating newts, EFs increase immediately upon injury, and they continue to rise until they peak several days after the injury\textsuperscript{270}. Moreover, the orientation of the injury current changes throughout limb regeneration in Urodele amphibians and tail regeneration in Anuran tadpoles: the initial outward current reverses direction after several days, and then gradually decreases in magnitude throughout regeneration, returning to its baseline value only once regeneration is complete\textsuperscript{270, 274, 276}. Although the differences between the injury-induced EFs produced in regenerating and non-regenerating tissues are often stated as function of the EF magnitude, this is a simplified version of reality. Following amputation, axolotl limbs regenerate while adult Xenopus do not, but the initial magnitude of the injury-induced current is very similar: 10-100 $\mu$A/cm$^2$ in salamanders\textsuperscript{270}, 20-40 $\mu$A/cm$^2$ in frogs\textsuperscript{278}. Instead, differences in the injury-induced currents emerge only after several days when the current reverses direction in salamanders but not in frogs\textsuperscript{270, 274, 278} (Figure 4.1). The importance of the injury current’s orientation is further illustrated through studies in which Xenopus limb regeneration was induced by an implanted electrode when the applied EF was oriented with the cathode at the lesion site, while anodal stimulation caused the amputated limb stump to degenerate proximal to the original plane of the injury\textsuperscript{277, 282}. These same studies have been replicated in rats\textsuperscript{283, 284}, suggesting that these EF-induced effects on regeneration are externally generalizable, and that they reflect neither an artifact nor a feature unique among amphibians. Together, these observations clearly demonstrate that the magnitude and orientation of the EFs within the injury site together play a nuanced and highly choreographed role in regulating tissue regeneration in vertebrates.

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Recordings of EFs that have been made in the mammalian CNS rely on an *ex vivo* model, which is prone to two obvious sources of bias. Endogenous EFs are a physiologic product of active ion transporters, which are highly metabolically demanding; removing the tissue from the body for an *ex vivo* preparation deprives it of vascular perfusion through which it receives the oxygen and glucose necessary to produce the ATP that drives the active transporters. While these nutrients are provided in artificial cerebrospinal fluid (aCSF) bathing the tissue, they rely on simple diffusion to permeate the tissue and the rate of diffusion is too low to satisfy the metabolic demands of cells. Thus, without vascular perfusion, the explanted tissue becomes ischemic, so the measured EFs may be less than their true value *in vivo*. Additionally, the *ex vivo* preparation involves dissecting the CNS out of the skull, a process that causes obvious trauma to the surrounding tissue and, if not performed correctly, to the CNS parenchyma itself. Moreover, the process of recording EFs within the CNS parenchyma using implanted electrodes causes damage to the tissue, which may further bias these recordings. As the preparation itself represents a considerable injury, any recording of current density or EF magnitude made from an *ex vivo* sample may actually reflect the bioelectric phenomena within the injured CNS or an artifact of the preparation and not a baseline recording from intact tissue. Interestingly, a previous study from the Borgens lab, which published recordings of injury-induced currents over the first hour after SCI *ex vivo*, demonstrated that the spinal cord is electrophysiologically “unstable” for the first hour after the *ex vivo* preparation, thus emphasizing the potential artifact in these *ex vivo* current density recordings\(^\text{394}\). This is most clearly illustrated in an experiment done by Cao and colleagues (2015), where they created a hippocampal slice culture
preparation that was 300-500 µm thick, measured an EF within the hippocampus of 31.8 ± 4.5 mV/mm, and declared this to be the EF within the “intact” parenchyma. However, current density measurements from *in vivo* cornea and skin, and from *ex vivo* spinal cord, suggest that there are injury-induced changes in bioelectric phenomena that extend at least up to 1 mm from the lesion site, so the entire thickness of this slice culture preparation more likely reflects an injured rather than an intact environment.

We found that 40 mV/mm EFs, which are comparable to the EF intensity measured in an “injured” hippocampal slice culture, induce behaviors in mammalian astrocytes *in vitro* that are characteristic of their injury response *in vivo*. However, our studies, and those from other labs, explore EF effects *in vitro* by applying a constant EF throughout the duration of the experiment. Instead, *in vivo* evidence from non-mammalian vertebrates suggests that injury-induced EFs that drive regeneration have a precise magnitude and direction, both of which change along a distinct temporal profile. Moreover, while EFs can stimulate robust regeneration in frogs and rats, applying them with incorrect polarity can actually exacerbate the wound. In the mammalian CNS, the only current observations measure injury-induced EFs over the first hour after an injury. This is far shorter than the multiple days after the initial injury over which EFs reach their apex and then reverse during axolotl limb regeneration.

While the injured mammalian CNS may indeed produce EFs that change upon injury, the temporal profile of the magnitude and orientation of endogenous EFs has yet to be characterized. Thus, while existing experimental data suggest that the injury-induced EFs drive the cellular response to injury and that therapeutic EFs might be able to
enhance regeneration, any such therapy aimed at enhancing regeneration through stimulating physiologic mechanisms of development would be purely speculative because the temporal and spatial dynamics of injury-induced EFs in the mammalian CNS are unknown. These parameters must be better understood before an EF-based therapy to promote regeneration can be developed, as certain elements of these bioelectric signals are fundamental to the success of any such approach.

Considerations when measuring bioelectricity

Electric fields strengths can be calculated either from the ratio of an electrostatic force to a known point charge \( E = F/q \) where \( E \) is the electric field, \( F \) is the force experienced by a charged particle in the presence of the electric field, and \( q \) is the charge of the second point charge, or from the difference in electrical potentials \( (V) \) measured at two electrodes that are a known distance apart \( E = dV/dx \) where \( E \) is the electric field, \( dV \) is the difference in voltage (i.e. \( dV = V_{source} - V_{reference} \)), and \( dx \) is the distance between the electrodes). (These equations are algebraically equivalent. Coulomb’s Law defines electrostatic force as \( F = kq_1q_2/r^2 \) where \( r \) represents the distance between the two charges, \( q_1 \) and \( q_2 \), and \( E = kq_1/r^2 \); electrical potential is calculated as \( V = kq_1/r \) so

\[
E = \frac{kq_1/r}{r} = \frac{\Delta V}{r} = \frac{dV}{dx}
\]

as \( r = dx \). Since it is impractical to measure an electrostatic force on a point charge in a biological system, EF strengths are instead calculated using electrodes to measure the electrical potential \( V \) at different points separated by a known distance. Although this system is more practical to calculate EF strengths in biological systems because the
distance between the two electrodes can be measured with great precision, it is still prone to inaccuracy. The electrodes detect the voltage in the sample through an electrochemical redox reaction at the interface between electrons in the metal electrode and ions in the surrounding solution, but this reaction, which causes ions to aggregate around the electrode, creates a hydration shell around the electrode that physically and chemically impedes the very reaction that the electrode is designed to measure\(^{395}\). This hydration shell, known as an “electric double layer,” insulates the recording electrode because the EF induces the water molecules to become aligned such that their molecular dipoles oppose the EF, causing the effective EF within the hydration shell – at the surface of the electrode – to be less than the actual EF within the tissue. This voltage artifact at the boundary between the electrode and the solution, which is termed the half-cell potential, can range from 1 mV to well over 200 mV\(^{395}\). The half-cell potential is dependent both upon the type of metal in the electrode and upon the ionic composition of the aqueous environment. As the electrolyte composition of the aqueous environment within each tissue is usually not well defined, the half-cell potential represents a significant source of measurement error that is very difficult to predict.

Technical challenges can prevent the accurate measurement of EFs by using electrodes. However, EFs can be calculated indirectly through measuring the ionic currents that they induce within the solution as a result of electrostatic force created between ions and the external EF. Jaffe and Nuccitelli first developed an ultra-sensitive probe that can accurately measure these ionic currents and endogenous electric potentials around biological specimens\(^{265, 396-398}\). Briefly, a highly-conductive platinum-plated metal electrode is vibrated over a known amplitude (typically 20-30 µm). The
electrode acts as a capacitor, so an external EF induces a voltage in the electrode as a function of the electrode’s capacitance and the EF intensity; this voltage is continuously measured as the probe vibrates, and the EF is calculated from the difference between the measured voltages and the distance that the probe travels as it oscillates (depending on the set-up of the system and the specific way in which the probe is calibrated, some vibrating probe set-ups measure the ionic current density instead of the EF). A half-cell potential cannot occur because the oscillating probe prevents a hydration shell from forming around the electrode, so EFs can be measured directly with minimal interference.

The vibrating probe method cannot be used to make recordings within a tissue because this technique requires the probe to move continuously. Instead, the vibrating probe technique is used to measure the ionic current density outside of the tissue. Although the extraparenchymal EF induced by the ionic current can be calculated \( E = \rho I / A \) where \( \rho \) is the resistivity of the conducting media, and \( I/A \) is the ionic current density, the EF within the tissue cannot be calculated from this measurement because the tissue resistivity is often unknown and the current density within the tissue is not uniformly distributed. Nonetheless, the total ionic current within the tissue is the same as the amount of current outside of the tissue (Kirchoff's Current Law states that the net current in the circuit is 0, see Chapter 1: Physics of electric fields, page 31), so the EF within the parenchyma is fundamentally proportional to the current density measured by the vibrating probe outside of the tissue and can thus be used as a measure of physiologic activity within the tissue.
Experimental goals

Although the vibrating probe can only measure current density outside of a tissue, these measurements are often used to make inferences about the physiologic activity and structural integrity within the tissue\textsuperscript{362}. Using an ion-sensitive vibrating probe, it was found that the physiologic ionic currents on the cornea and the limb are principally composed of Na\textsuperscript{+} ions; and experiments with different ion-channel and transporter blockers demonstrated that this current is sustained by constitutive activity of the Na\textsuperscript{+}/K\textsuperscript{+}-ATPase\textsuperscript{147, 268, 280}. Moreover, longitudinal current density measurements have also demonstrated that the current density is correlated to wound healing\textsuperscript{215, 274, 329}. Nonetheless, while measurements of external bioelectric phenomena provide insight into tissue physiology, they still cannot be used to calculate intraparenchymal EFs. One method to measure these intraparenchymal EFs is described by Cao and colleagues (2013): they implanted 4 electrodes into the brain, with the electrodes arranged in series throughout the fiber tract; they injected a known current through the outer two electrodes and measured the voltage drop between the inner two electrodes as a calibration, and then turned off their current source so that they could measure the endogenous EF\textsuperscript{269}. However, even if this method adequately controls for the half-cell reaction at the electrodes, it still does not account for the potential artifact caused by the fact that the electrodes themselves damage the parenchyma as evidenced by studies demonstrating glial scar formation around implanted electrodes \textit{in vivo}.

In order to develop a therapeutic strategy to promote regeneration in the mammalian CNS by manipulating EFs, we first need to establish how these endogenous bioelectric phenomena change in magnitude and orientation throughout
the duration of wound healing. This exploration requires that a method be developed to measure ionic currents and EFs in the CNS in vivo for two reasons: first, wound repair is a prolonged process and longitudinal observations will be necessary to monitor how physiologic EFs change over time; second, in vivo recordings will obviate measurement artifacts resulting either from ischemia or from injury occurring during the ex vivo preparation. In this chapter, we describe our initial efforts to develop a method to record current density in an in vivo model of CNS injury.

**Methods**

*Animals and surgical protocol*

Adult female Long-Evans rats (at least 6 months of age; n = 5) were used in these experiments (Harlan Laboratories). All animal protocols used in these studies were preapproved by the Virginia Commonwealth University IACUC. Rats were anesthetized with isoflurane and placed in a stereotaxic apparatus (see illustration, Figure 4.2); depth of anesthesia was monitored by respiratory rate and depth, and by regular assessment of reflexes. A 10 mm diameter area of skull was exposed with a midline scalp incision and the periosteum was removed. A plastic funnel (fabricated from the conical base of a 50 mL conical tube, Genesee Scientific #21-106; dimensions: approximately 15 mm tall, bottom diameter of 8-10 mm where the funnel was attached to the skull, upper diameter of 28 mm) was affixed to the skull with superglue to maintain a bathing solution of artificial cerebral spinal fluid (aCSF; 126.0 mM NaCl, 3.0 mM KCl, 1.25 mM NaH$_2$PO$_4$, 2.0 mM MgCl$_2$, 2.0 mM CaCl$_2$, 10.0 mM glucose, and 26.0
mM NaHCO₃) at least 7 mm deep over the recording area. The cortex was exposed by using a Dremmel tool to remove a portion of the parietal plate approximately 2 mm wide (ML) x 4 mm long (AP) in size; aCSF was frequently washed over the skull to prevent overheating of the bone and thermal injury to the underlying brain. The meninges were carefully removed to expose the intact cortex and surface vasculature was used to identify a landmark for the scan area and target for the injury.

**Measuring current with the Scanning Vibrating Electrode Technique**

Measurements of current density were obtained using the Scanning Vibrating Electrode Technique (SVET) apparatus (Applicable Electronics, New Haven, CT), based on the technique originally described by Jaffe. Briefly, Parylene-C insulated Platinum/Iridium electrodes attached to a gold connector and electrically arced to expose several micrometers of bare metal at the tip are used for these recordings (MicroProbes PI(2.5cm)0036.0A10). The electrode tips are platinum-coated by electroplating them in a solution of 1% Platinum Chloride, H₂PtCl₆ and 0.01% Lead II Acetate until they have at least a capacitance of 2.0 nanofarads (nF). The Electrode is inserted into a gold connector pin attached to the vibrator assembly, which is connected to a piezoelectric drive that vibrates in the X and Z planes. The probe vibration frequency is set at around 110 hertz (Hz) in the X plane and 70 Hz in the Z plane (optimal frequencies are at least ±10 Hz from 60 Hz to minimize electrical noise, and ±5 Hz from the resonance frequency of the probe), with an amplitude in each plane that is approximately twice the diameter of the electrode tip. The electrode position is controlled with a motorized 3-dimensional micromanipulator. An Optem Zoom 70 micro-
inspection videoscope lens system equipped with a 0.75x lens, a modular 1:7x zoom lens, and a 2x auxiliary lens system (total magnification range of 1.5 – 10.5x; Qioptiq), as well as a color uEyeLE camera (model UI-164xLE, CMOS sensor, 1280x1024 resolution, manufacturer: IDS) is used to visualize the probe and scan area. A computer running the ASET-LV4 software package (version 3.1.0.0; Science Wares, INC, Falmouth, MA) is connected to the SVET apparatus and is used to drive the micromanipulator and collect the data. The probe is calibrated per the protocol from the manufacturer, under 10.5x magnification using a 60 nanoampere (nA) current source delivered through a glass micropipette. Calibration is performed prior to the start of each experiment and every time a new probe is used. A region of interest is specified under 4x magnification, and the probe scans the current density approximately 50 µm above the surface of this region by moving in a grid-pattern to scan points every 40 µm in the x- and y-axes; a reference reading 5 mm above the brain surface is taken at the start and end of each scan to calibrate the measurements. An insect pin (approximately 150 µm diameter) attached to a 3D-micromanipulator is used to make a 2 mm deep stab wound in the center of the scan area. Current densities are measured immediately before and after the stab wound. At the end of the experiment, animals were euthanized with a single intraperitoneal injection of euthasol (350 mg/kg sodium pentobarbital, 45 mg/kg phenytoin sodium).

**Analysis**

Current density measurements and corresponding images were obtained for the z-axis (perpendicular to the surface of the tissue) over the surface of the cortex, both
before and after the injury. Data were analyzed and surface plots were generated using the JMP v. 11.2.1 statistical software package; bar-plots were generated using the statistical software R\(^{341}\). The dataset for each scan was reviewed for artifact from the vibrating probe contacting the surface of the brain by comparing readings from the Quadrature and In-phase channels of the recording (for a thorough discussion of how measurements are taken by the SVET apparatus, see Scheffey, 1986\(^{399}\)). Per the recommendation from the company from which the SVET apparatus was purchased, data points where the signal in the quadrature channel was >10% of the signal in the In-Phase channel were deemed suspect and excluded from analysis. Images of the scan area are automatically recorded before and after each scan, and the coordinates of each scan location are superimposed onto these images. Using these images, we labeled each scan location as being over intact cortex, surface vasculature (we were unable to differentiate between arterial and venous circulation), or the lesion site; we excluded scan locations for each animal that were on the border between two such classifications, defined as approximately 75-100 µm from the edge of a vessel or an injury site. We estimated the mean current density over the intact cortex and surface vasculatures separately, using a mixed-effects linear regression model

\[
Y_{ij} = \beta_j + \mathcal{N}(0, \sigma^2_b) + \epsilon_{ij}
\]

where \(Y_{ij}\) is the estimated mean current density at each scan location, \(\beta_j\) is the mean current density for each factor level (i.e. intact or injured cortex, or surface vasculature), and \(\sigma^2_b\) is the variance of the mean current density between animals. As our sample size for these pilot studies was relatively small and the only difference in mean current density with which we were interested was between the intact and injured cortex, we ran
separate models for the measurements over intact cortex and surface vasculature taken before the injury; we evaluated the hypothesis that there intact cortex and surface vasculature each sustain a current density across themselves against the null hypothesis that there was no such current density sustained by these tissues. We then ran a model with measurements over intact and injured cortex after the lesion (measurements over vasculature were excluded), and evaluated the estimated mean for each factor level as well as the difference between these groups. We used $\alpha = 0.05$ as an overall threshold for significance, and also for each of the post-hoc comparisons. We report the mean ± SEM for each of our parameter estimates, and we report the test-statistics with the degrees of freedom adjusted for the partial dependency of the observations made from each animal.

**Results**

**Current density measurements**

The goal of this pilot study is to develop a protocol by which we can make recordings of current density over a lesion site in the injured brain over time, with multiple measurements taken from the same animal at regular intervals (e.g. daily) throughout the recovery of the animal. To accurately identify the same scan area between measurements, especially at later time-points as the injury location becomes less visually distinct, we needed to identify a landmark that would be constant over time. Because the rodent brain is lissencephalic, we decided to use the surface vasculature, which forms distinct patterns that were visible at both low and high magnification (Figure
From pilot studies, we settled on a scan resolution (distance between adjacent scan positions) of 40-50 µm because we found that this allowed us to detect subtle variations in cortical current density and to distinguish between the current density measurements over the cortex and surface vasculature. We made grid-scans over regions of interest over the intact brain, estimating the scan to be approximately 50-75 µm above the cortical surface; however, the brain surface is not perfectly smooth so the exact distance from the cortical surface likely varies among the scan locations.

Consistent with previously-reported results\(^{269}\), we found that the intact rat cortex sustains an outward current, while the large blood vessels have an inward current over them. This pattern of current density over scan areas of both intact cortex and vasculature clearly reveals this pattern (Figure 4.3). We used images of the scan area to visually categorize each scan position as either surface vasculature or intact cortex; locations that appeared to be at the border between these two features were excluded from the analysis. We estimated the mean current density and standard error of the mean (SEM) over both the cortical surface and the vasculature using a mixed-effects linear regression model, with the location (i.e. cortex or vasculature) treated as a fixed effect and the animal treated as a random effect; we ran separate models for each location because we were not interested in estimating the difference between them (this is equivalent to running a 1-sample t-Test, controlling for the nested dataset). We found that the current density over the intact cortex was 33.04 ± 10.86 µA/cm\(^2\) (mean ± SEM), which was statistically different from 0 µA/cm\(^2\) (\(t_{4.005} = 3.04, p = 0.0383; N = 5\) animals, \(n = 591\)). We also found that there was an inward current over surface vasculature, which we estimated at 29.21 ± 20.24 µA/cm\(^2\); however, the model did not find that this was
significantly different from 0 μA/cm² ($t_{1.989} = 1.44$, $p = 0.2865$, $N = 3$ animals, $n = 100$ observations total), likely because there were far fewer points in the sample because the vasculature covers a small proportion of the scan area and we could not always find large enough vessels over which we could make distinct recordings.

We next set out to determine the how current density changes upon injury to the cortex. We used images of the scan area to categorize scan locations as either over intact or injured cortex, excluding locations on the border between the lesion and the surrounding intact tissue. We evaluated how the current density at the injury site changes compared to the surrounding healthy tissue using a mixed-effects linear regression model. Specifically, we estimated the fixed effect of the injury while controlling for the variability in average current density among the animals by treating the animal as a random effect. We found that there was an overall effect of injury on the current density ($F_{1, 667.8} = 479.22$, $p < 0.0001$). We found that the mean current density over the intact cortex was estimated at $13.35 \pm 4.394$ μA/cm², which was significantly different from 0 ($t_{4.414} = 3.04$, $p = 0.0339$); the mean current density over the injured cortex was estimated at $-34.85 \pm 4.500$ μA/cm² (the negative indicates that the current had the opposite direction as that over the intact cortex), and there was a statistically significant difference in current between the intact and injured cortex, which was estimated at $48.20 \pm 2.162$ μA/cm² ($t_{667.8} = 22.30$, $p < 0.0001$). (Note, JMP v. 11.2.1 was used for these analyses, and JMP does not provide specific $p$-values if they are smaller than 0.0001.) These results demonstrate that the rodent cortex sustains a physiologic ionic current across itself, and that the current density changes direction over sites of injury. Together, these observations are consistent with the hypothesis that the injured
mammalian brain produces physiologic EFs, and that these EFs change immediately upon injury. Moreover, our methodology suggests that the surface vasculature can provide an easily-identifiable landmark to make recordings from the same location at regular intervals over time.

Limitations of current density measurements: artifacts

While measuring the current density over both the intact and injured cortex, we noticed considerable variability in the current density estimates among different animals (i.e. the estimated current density over the intact cortex for each of the five animals from which we obtained good measurements were: 6.30 ± 1.655 µA/cm²; 39.44 ± 1.952 µA/cm²; 9.82 ± 0.560 µA/cm²; 47.66 ± 1.073 µA/cm²; and 61.97 ± 1.091 µA/cm²). While the pattern of current densities that we measured was consistent among each animal we used (e.g. outward current over intact cortex, inward current over intact vasculature and injured cortex), we were concerned about this variability. Specifically, as current density decays logarithmically as a function of the distance from the source, this variability could reflect differences in the distance between the probe and the brain surface due to topographical variations across the brain surface, small errors in estimating the height above the cortical surface, or the pulsatile nature of the brain due to the heart rate of the animal; however, it could just as easily reflect a methodological error. Moreover, the mean current densities that we estimated were over 10-fold greater than those over the intact cortex that have been previously published269, although they are within the range of current density measured upon limb amputation in both salamanders270,274 and frogs278. To try and determine whether artifact may have
affected the validity of our measurements, we set out to confirm that the SVET apparatus was measuring current density accurately by rigorously testing its calibration settings.

The protocol to calibrate the SVET apparatus specifies that the probe should be calibrated in the x- and z-axes separately. Specifically, the point-source of current for the calibration is defined as location (0, 0, 0) (x, y, z) in a 3-dimensional coordinate system; the probe is brought to (-150, 0, 0) to calibrate the x-axis, and to (0, 0, 150) to calibrate the z-axis (units are in µm). At each of these locations, the probe records a reference reading while vibrating to establish the background noise, and then it records while the point source passes a 60 nanoampere (nA) current. In physiologic saline, the real current density 150 µm from a 60 nA point current source is 21.2 µA/cm² (per the protocol provided by Applicable Electronics, which is the company that manufactured the SVET apparatus). This measurement is compared to the recordings made by the probe, and is used to calibrate the phase-offset and the resistivity of the media. We assessed the accuracy of the probe calibration by making line scans around the point current source while it was passing 60 nA. We assessed the x-axis using a line-scan from (-100, 100, 0) to (-100, -100, 0) (i.e. a straight line traveling only through the y-axis), and we assessed the accuracy of the z-axis by recording a line-scan from (0, 100, 100) to (0, -100, 100); we chose to have our line scan pass through the y-axis because the probe only vibrates in the x- and z- axes and thus cannot record current in the y-axis, so these line scans isolated the current recorded in the x- and z- axis channels, respectively. We also ran a line-scan through the y-axis to confirm that the SVET does not have cross-talk between detectors that would cause artifact. When we ran a line-
scan from (-100, 100, 0) to (100, 100, 0), we found that the SVET recorded a change in current in the z-axis channel. We were unable to determine whether this signal is a real measurement due to aberrant vibration in the y-axis, or if it represents an artifact due to cross-talk between the channels. Thus, the specific current density values that we estimated over the intact and injured cortex may be suspect and additional experiments are required to identify the source of the artifact in the SVET and to confirm the values of current density that we measured.

Considerations for improving experimental design

We assume that these potential artifacts can be addressed given sufficient resources and further discussion with the company producing the system, so it is worth discussing how this methodology might be optimized for these in vivo recordings. There are two principal considerations for further improving this approach. First, the current density produced over the cortex decreases as a function of the square of the distance from the surface; it is difficult to measure the exact distance of the probe above the surface, and the brain is highly contoured so this distance varies over space. Second, the grid scans we used in these studies take over 12 minutes to complete, which limits the temporal resolution over any individual point; these grid scans are very helpful in identifying changes in current density between areas of vasculature and cortex, and they help to identify the borders between intact and injured tissues. However, once these regions are identified, the 12 minute scan over the entire area does not afford sufficient temporal resolution to make consecutive current measurements after the injury to assess how these currents change over time immediately after the lesion.
Instead of one large grid scan at a single plane in the z-axis, we propose either smaller individual grid scans (e.g. 3x3 x, y) over regions of intact and injured cortex, or a single line scan covering the distance between the intact and injured tissue which can reveal how the current density changes over distance from the lesion margin. We also propose that future experiments employ scans with a step-back protocol whereby the same (x, y) scan pattern is repeated in multiple (at least 3) steps in the z-axis; this will allow the fall-off of the current density to be measured, which can be used to better determine distance between the measurement and the surface of the brain. (An additional technical note is that the surface vasculature is elevated above the level of the cortex; in several of our experiments, the vibrating probe hit a blood vessel during a scan, causing the vessel to rupture and making it impossible to take subsequent measurements from that scan area. By specifying smaller scans instead of relying on one large scan area, it would be much easier to avoid this type of hemorrhage.)

Discussion

These studies demonstrate that it is practical to take *in vivo* measurements of ionic current density with an SVET apparatus over the surface of the mammalian cortex, obviating concerns about bias in these measurements due to damage sustained during tissue dissection for *ex vivo* preparations or due to ischemia resulting from the fact that the *ex vivo* preparation does not have a functional vascular supply. We found that the intact rat cortex sustains a steady outward current while the surface vasculature sustains an inward current, which suggests that there may be a net ionic transport between the two tissues; we also found that the current over the cortex rapidly reverses direction upon injury. Importantly, we found that the current density over a cortical injury
reverses direction more rapidly than we were able to measure (less than 2-3 minutes), which is consistent with measurements from other studies showing that injury-induced changes in bioelectricity are immediate; as current density outside the tissue is fundamentally related to electric fields within the tissue, these results strongly support the notion that the mammalian cortex creates injury-induced electric fields within the lesion site and surrounding parenchyma. These results are consistent with the outwards orientation of current density that has been previously reported over mouse olfactory bulb\textsuperscript{269}, and with the rapid change in direction upon injury matches the rate of change reported over the spinal cord, skin, and cornea\textsuperscript{265, 268, 291}.

It is worth noting that the outward current density over the intact cortex that has been measured both by us and by other groups has an orientation that is opposite that reported in many other tissues (for a further discussion, see chapter 1, page 48). In the intact skin and cornea, which have been much more thoroughly studied than the CNS, there is a steady inward current that is sustained by an asymmetric distribution of Na\textsuperscript{+}/K\textsuperscript{+}-ATPases in the basolateral membrane of the epithelial cells lining the tissues\textsuperscript{147, 268, 270, 280, 281, 326}. In contrast, Cao and colleagues found that the astrocyte end feet in the glia limitans lining the cortical surface expressed Na\textsuperscript{+}/K\textsuperscript{+}-ATPase within the apical portion of their membranes; moreover, they measured an inward ionic current over the wall of the lateral ventricle, and they found that ependymal cells lining the lateral ventricles express Na\textsuperscript{+}/K\textsuperscript{+}-ATPases within their basolateral membranes\textsuperscript{269}. While this suggests that the discrepancies in the direction of ionic current between the intact mammalian cortex and other tissues may be a function of the localization of these ionic transporters, it does not address the reason for which these differences may arise.
Borgens and Shi found that the axolotl embryo produces ionic currents at least as early as embryonic stage 16, and they measured an inward ionic current across the entire ectodermal surface\textsuperscript{215, 249}. The ectoderm is still a laminar structure at this stage and the neural groove has not yet invaginated to form the neural tube, so the current across the neural groove has the same orientation as that over the rest of the ectoderm. The neural tube develops into the ventricular system and central canal of the spinal cord, and the lateral ectoderm develops into the skin and cornea. Thus, these two structures are topologically equivalent and ontogenetically derived from the same structure, so it makes sense that the distribution of ion transporters and the orientation of the ionic current would be conserved across the skin and cornea, and the ependymal cells lining the ventricular system. As the neural tube starts out only several cells thick, the inward current over its apical face would be matched by an outward current across its basolateral face; as the outer cortical surface develops from the basolateral face of the presumptive neural tube, it is reasonable to assume that the current density across it would have an opposite polarity.

The consistencies in our measurements in the context of previous literature, and the consistent variations we found in current density between the intact cortex and surface vasculature, strongly support the notion that our measurements reflect the physiologic activity of the tissue. However, the magnitude of the current densities that we measured over the intact cortex were approximately 10-fold greater than those previously reported\textsuperscript{269}; this could reflect the fact that our observations were made \textit{in vivo}, and the continued vascular perfusion allowed the brain to maintain a greater metabolic rate and, consequently, more activity in the ion transporters that drive the
ionic current. Alternatively, these differences could be methodological and reflect differences in the distance between the probe measurement position and the cortical surface. However, these measurements could also reflect an artifact due to a hitherto undetermined flaw in the setup of our SVET apparatus. Thus, while we are confident that the polarity of the current we measured reflects the true current density polarity, we are concerned about the potential contribution of artifact to our measurements and are therefore not confident about the accuracy of the magnitude of these measurements. Nonetheless, our results demonstrate that in vivo recordings of ionic current density are practical, and that this approach can be used to make repeated measurements from the same animal throughout the recovery period.
Figure 4.1: Injury currents in regenerating and non-regenerating vertebrates

A graph illustrating the evolution of the injury current at the site of an amputated limb in frogs and salamanders; these are summary data based on several studies. The initial injury currents measured within the tissues are very similar initially, but they begin to diverge after 2-3 days and remain different throughout the duration of regeneration.

(This image was modified from The Body Electric and is reprinted here under the “fair use” limitation in title 107 of the U.S. copyright law.)
Figure 4.1: Injury currents in regenerating and non-regenerating vertebrates
Figure 4.2: Scanning vibrating electrode technique

(A-D) Procedure for calibrating the vibrating probe, showing (A) the probe off, (B) the probe vibrating only in the x-axis, (C) the probe vibrating only in the z-axis, and (D) the probe vibrating in both the x- and z-axes. (Note, the probe cannot vibrate in the y-axis.) (E) Illustration showing where the skull was drilled, relative to bregma and lambda, to access the cortex for SVET recordings; a piece of bone approximately 3 mm wide and 5 mm long was removed. (F) Illustration showing the in vivo recording configuration (courtesy of Christina Delli Santi). (G) A low-magnification view of the exposed cortex, demonstrating the vascular landmarks that we used; the inset (H) demonstrates the size of the scan area relative to what we expose.
Figure 4.2: Scanning vibrating electrode technique
Figure 4.3: *In vivo* current density measurements

(A) An image showing the scan area prior to the injury, which was chosen because of the distinct landmarks made by the surface vasculature (indicated by the arrow and double-ended arrow), with the double-image of the vibrating probe visible (notched red arrowhead). (B) The same region, immediately after a 150 µm diameter insect pin was used to make a stab wound (arrowhead). Surface-plots of the intact (C) and injured (D) cortex, showing projections of the z-axis current density (µA/cm²) for each scan position in the x- and y-axes; the grid on each surface plot indicates 0 µA/cm² z-axis current, positive values correspond to outward current, and negative values correspond to inward current. Arrows in these surface plots correspond to the same areas indicated in the images of the scan area in (A, B). (Note, the surface plot in (C) was from a 5x5 (x, y) grid scan, while the plot in (D) was from a 20x20 (x, y) grid scan.) (E) Graphical depiction of the mean current density over the intact (13.35 ± 4.394 µA/cm²) and injured (-34.85 ± 4.50 µA/cm²) cortex; the random-effects model that we used for this analysis demonstrated that these two measurements were significantly different ($p < 0.0001$).
Figure 4.3: *In vivo* current density measurements
The research project described herein was undertaken to test the hypothesis that endogenous electric fields regulate the injury response in the mammalian CNS in an intensity-dependent manner. Specifically, we hypothesized that physiologic injury-induced EF intensities stimulate the characteristic cellular response to injury, while EF intensities associated with regenerating tissues modify this response towards one associated with regeneration. We were particularly interested in the effect of EFs on astrocytes because differences in particular injury-induced astrocytic behaviors have been attributed to the highly-regenerative response in non-mammalian vertebrates and the failure of regeneration in mammals. Through the experiments described in chapters two and three, we found evidence that EFs associated with injured mammalian tissues (including in the mammalian CNS) induce behaviors in cortical astrocytes associated with their response to injury (directional migration and increased proliferation), while no such responses were found in cerebellar astrocytes; these differences are consistent with the heterogeneous astrocytic response in vivo, where migration and proliferation of cortical astrocytes are associated with glial scar formation, whereas cerebellar astrocytes hypertrophy but do not form a demarcated glial scar. Most excitingly, EFs associated with regenerating tissues modified the response in cortical astrocytes and induced a robust response in cerebellar astrocytes, and the particular behaviors induced were consistent with a regenerative astrocytic phenotype. Our results indicate...
that physiologic EFs regulate wound repair in the mammalian CNS, and that these EFs may be a viable therapeutic target to promote regeneration after injury. Thus, the role of EFs in peripheral tissues is likely conserved in the CNS, suggesting that bioelectric fields may represent a single unifying force that regulates tissue morphogenesis during embryogenesis and epimorphic regeneration within all vertebrate tissues.

**Electric field: a unifying stimulus of embryogenesis and regeneration**

The results described throughout this dissertation fit into a broader argument that physiologic EFs regulate morphogenesis in embryonic and injured tissues. Evidence that EFs function as morphogens during embryogenesis and regeneration is obvious: EFs are necessary for both embryogenesis\(^{250,261}\) and regeneration\(^{275,280}\), and they are sufficient for regeneration\(^{277,284}\) (similarly thorough evidence that they are sufficient for embryogenesis is currently being explored\(^{400,401}\)). Moreover, EFs induce the same behavioral effects on diverse cell types *in vitro* as are necessary for both embryogenesis and regeneration *in vivo*. However, the notion that EFs have such a widely important role as the master regulator of both embryogenesis and regeneration in vertebrates – and, more broadly, all metazoans – is a hypothesis of causality and, while numerous experiments that we have described herein support this hypothesis, it is necessary to formally address this hypothesis.

Sir Austin Bradford Hill enumerated nine criteria to establish a causal relationship in a publication in 1965\(^{402}\), and these criteria have become the benchmark against which causality has been evaluated in medicine, epidemiology, and basic science research over the subsequent 50 years. His nine criteria are listed below, along with a description and interpretation of each criterion that we have paraphrased from this work:
1. **Strength**: the putative causal variable must have a robust effect.

2. **Consistency**: the association is replicated in studies in different settings using different methods.

3. **Specificity**: a single putative cause produces a specific effect; as Hill describes, this is the weakest of the criteria and is not necessary to establish causality (he cites as an example that the diseases attributed to cigarette smoking do not meet this criterion).

4. **Temporality**: the causal stimulus must necessarily always precede the observed effect; this is the only essential criterion.

5. **“Biological gradient” (i.e. dose-dependency)**: greater exposure to the putative causal agent should cause a greater effect.

6. **Plausibility**: the association should biologically plausible, although – as Hill points out – what is biological plausible depends on current scientific knowledge, which can change over time.

7. **Coherence**: the association should not conflict with the current scientific knowledge and theory, although occasional paradigm shifts serve as notable exceptions to this rule.

8. **Experiment**: altering the causal conditions can alter the effect.

9. **Analogy**: the proposed causal relationship is similar to other previously-established causal relationships, and alternative explanations have been considered and, if possible, ruled out.

Importantly, establishing causality is independent of establishing an underlying mechanism. Moreover, Hill states that there are no formal tests of significance that can
be used to quantitatively evaluate these criteria. Inferential statistical tests are quantitative tools that help the researcher evaluate a hypothesis objectively and answer the question of significance with a “yes” or a “no.” In contrast, Hill’s criteria are qualitative and are used to interpret the validity of the hypothesis in question.

Many groups have quantitatively demonstrated that EFs have a physiologic effect on tissues and on cells, during embryogenesis and during regeneration. However, the inference that the groups exposed to EFs are different in some characteristic from the groups without similar exposure does not necessarily imply that EFs are causing the effect in these experiments, nor that EFs are physiologically relevant stimuli in vivo. Thus, it is worth comparing the current understanding of electric fields in regulating development and regeneration against Hill’s criteria to establish whether existing knowledge is concordant with a causal relationship and, if not, to determine which aspects of the causal relationship are yet to be determined.

**Evaluation of EFs as a causal stimulus**

We have already provided evidence that EFs conform to a number of Hill’s criteria for causality. (We refer the reader to the section in chapter one entitled “Physiologic electric fields regulate embryogenesis and regeneration”, beginning on page 29, for a thorough discussion of this literature.) EFs are produced by all cells and in all tissues as a function of their normal physiologic activity, and all cells are affected by EFs through electrostatic interactions. EFs are increased during embryogenesis, and their elevation precedes limb bud outgrowth; EFs are also elevated upon injury and remain elevated throughout regeneration. The intensity of injury-induced EFs is
associated with the success of regeneration, such that tissues with lower EFs heal by scar formation while those with higher EFs regenerate. Multiple different groups have independently demonstrated this correlation between EF intensity and both embryogenesis and regeneration; moreover, multiple groups have shown that blocking EFs interferes with embryogenesis and with regeneration. *In vivo*, EFs have been manipulated physically by directly applying electrical current to the tissue, manipulated pharmacologically through multiple different ion transporters and channels, and manipulated chemically through changing ionic content of tissues; in all of these examples, similar effects of the manipulation have been reported. At a cellular level, EFs have similar effects on inducing the same set of behaviors in cells from multiple different tissues, and the behaviors that EFs induce are the same cellular behaviors that are necessary for both embryogenesis and for regeneration. Moreover, EFs have been shown to regulate cellular activity through the some of the same physiologic mechanisms by which cells have previously been shown to respond to chemotaxic, mitogenic, and fate-determining stimuli.

Cumulatively, this evidence is consistent with 7 of Hill’s criteria: strength, consistency, temporality, dose-dependency, plausibility, coherence, and experiment. It is worth noting that the two criteria not met, those of specificity and analogy, are actually consistent with the hypothesized role of EFs. EFs do not demonstrate specificity of effect on tissues or cells as master regulatory genes can stimulate the same cellular behaviors, developmental pathways, and regenerative effects; however, EFs are hypothesized to act upstream of the master regulatory genes, so this lack of specificity is necessary for the putative causal relationship. EFs also do not demonstrate a causal
relationship that is analogous to any other currently known relationship, but this lack of analogy is justified because EFs as a stimulus are hypothesized to be unique in their role as a trigger for morphogenesis. Of course, we could argue that EFs during embryogenesis are analogous to EFs during regeneration, but that is a mischaracterization of our hypothesis, which is that elevated EFs regulate the same set of cellular behaviors independent of the cell type, the tissue, or the species, in order to drive morphogenesis: in the embryo, morphogenesis is synonymous with embryonic development; after injury, morphogenesis is synonymous with epimorphic regeneration.

This qualitative review is consistent with our over-arching grand hypothesis: that EFs have a conserved effect on all cells in all tissues from all metazoans during both embryogenesis and regeneration. The plausibility of this hypothesis as described thus far is purely circumstantial: it is based on the fact that all cells produce electrogenic activity and can detect external electrical cues. However, to be truly plausible, there must be an evolutionary reason for which EFs would have developed a universal effect on morphogenesis. Furthermore, while causality does not require an understanding of the mechanisms underlying the effect, the plausibility of this hypothesis will be strongly supported if potential physiologic mechanisms can be identified. Thus, we will turn our attention now to exploring potential physiologic mechanisms and evolutionary origins of the hypothesized universal role of bioelectric fields.

**Injury-induced EFs stimulate regenerative physiology in astrocytes**

The plausibility of EFs as a stimulus for development and regeneration relies upon physiologic mechanisms through which EFs may be transduced, although it is not necessary for these mechanisms to be elucidated for the hypothesized causal
relationship to be valid. Nonetheless, we will turn our attention now to exploring potential transduction mechanisms, with an emphasis on how each of the EF-induced behaviors on astrocytes that we explored in chapters two and three may be regulated.

CNS regeneration in non-mammalian vertebrates is facilitated by radial glia, which restore the BBB, guide regenerating axons past the lesion site, and replace the damaged cell population by functioning as neural progenitor cells. These behaviors are mediated by a conserved set of cellular behaviors, including migration, proliferation, changes in differentiation, and morphological changes. Radial glia, which are the resident GFAP$^+$ cell in the non-mammalian vertebrate CNS, are orthologous to astrocytes in mammals; the absence of regeneration in the mammalian CNS has been attributed to the fact that, while astrocytes also migrate and proliferate upon injury, they do not undergo changes in differentiation or morphology that allow them to function as NPCs or to promote axon sprouting. Nonetheless, ample evidence demonstrates that astrocytes retain a latent regenerative potential as they can be induced both to function as NPCs and to promote axon outgrowth.

Although we found that EFs stimulate multiple astrocytic behaviors associated with wound repair and regeneration, we did not explore the mechanisms by which these behaviors are induced. Ultimately, these transduction pathways are an ancillary curiosity: the entire idea of exploring EF-based therapies is that EFs will activate regeneration by regulating each of the necessary physiologic mechanisms, thus obviating the need to individually target each of the contributing signal transduction pathways. Nonetheless, in order to establish the causal relationship between endogenous EFs and regeneration, they must have a plausible way of interacting with
the known mechanisms by which each of these cellular behaviors is transduced. For the purpose of exploring the biological significance of EF-mediated astrocytic regeneration, we will discuss pathways by which migration, proliferation, differentiation, and morphology are regulated in mammalian astrocytes, and explore potential ways by which EFs may be transduced by each of these pathways.

Migration

Astrocyte migration requires the directional extension of cellular processes, which is driven by the assembly of actin and microtubules beneath the leading ends of these protrusions, and the breakdown of these cytoskeletal elements at the trailing end of the cell. We observed this same type of membrane protrusion at the leading edge of migrating astrocytes upon EF exposure, suggesting that the cytoskeletal assembly drives astrocytes electrotaxis. Directional migration in astrocytes requires polarization of the Golgi apparatus and the microtubule organizing center (MTOC) within the cytosol; asymmetric activation of Rho-GTPases then drive microtubule assembly and the stabilization of polymerized microtubules towards the leading edge. Consequently, anything causing MTOC polarization can drive directional migration. One such pathway is driven by the Ca$^{2+}$-mediated activation of Protein Kinase C zeta (PKCζ) at the leading edge of the cell, which can be induced by the activation of either integrins (through the GTPase Cdc42) or transient receptor potential vaninoid-1 (TRPV1) channels, both of which are expressed in astrocytes. Integrins are heterodimeric membrane proteins that exist in an equilibrium between an active and inactive state and, when in their active state, an inside-out transduction pathway allows them to be
activated even without the presence of an external ligand; because these receptors demonstrate constitutive activity, the concentration of integrin receptors at the leading edge of the cell may create sufficient activation at the leading edge to drive directional migration. TRPV1 channels have a high Ca\textsuperscript{2+} permeability, and they have voltage-sensitive S1-S4 domains similar to those on Na\textsubscript{V}-channels that may become activated due to EF-induced depolarization of the cell membrane. As both integrins and TRPV1 channels are transmembrane proteins, an external EF may cause their redistribution to the leading edge of the cell through electroosmosis, thus allowing the cell to transduce the EF.

While the role of integrin- or TRPV1-mediated electrotaxis through PKC\zeta activation has not been explored, Ca\textsuperscript{2+}-sensing receptors and integrins have each been shown to modulate migration and differentiation in cerebellar granule cell precursors. Moreover, a different second messenger system has been shown to contribute to the transduction of EFs in hippocampal neurons: upon exposure to external EFs, phosphoinositide-3-kinase (PI3K) becomes activated at the leading edge of the cell and its negative regulator, phosphatase and tensin homologue (PTEN), becomes activated at the opposite side of the cell (Figure 1.8 uses these receptors as an example of cellular EF transduction through electroosmosis); this activation of antagonistic second messengers at opposite ends of the cell causes the MTOC and Golgi apparatus to become polarized, which establishes directional neuronal migration. Together, this demonstrates that the polarized organization of cellular organelles necessary to stimulate directional migration in astrocytes can be induced by external EFs.
Proliferation

Resting membrane potential ($V_m$) varies throughout the cell cycle, with a relative depolarization throughout G$_1$/S and G$_2$/M and a hyperpolarization in interphase$^{216}$. $V_m$ exerts a causal role in cell cycle regulation, as hyperpolarization induces a reversible mitotic block$^{217}$, while depolarization induces DNA synthesis and mitosis in mature neurons$^{218, 414}$. Cells transduce changes in $V_m$ through voltage-gated ion channels, whose opening-probability, and thus conductivity to ions, changes as a function of $V_m$. However, this relationship is not linear as many of the channels that determine $V_m$ are themselves affected by both pH and $V_m$, so small changes in $V_m$ can have large changes on ion channel conductance and intracellular ion concentration$^{392}$. Increased Na$^+$ conductance through Na$_v$1.5 channels has been shown to drive mammalian astrocyte proliferation following an in vitro scratch wound; subsequently, the passive Na$^+$/Ca$^{2+}$-exchanger (NCX) causes intracellular calcium levels to rise, which is necessary for astrocyte proliferation following injury$^{375}$. Another voltage-gated Na$^+$ channel, Na$_v$1.2, is necessary for the injury response in Xenopus tadpoles, where increased intracellular Na$^+$ stimulates Salt-Inducible Kinase (SIK), which induces regeneration following tail amputation in Xenopus tadpoles; regeneration can be rescued in those animals where Na$_v$1.2 is missing by transfecting human Na$_v$1.5 into these cells$^{147}$. SIK, which is a serine/threonine protein kinase – also known as SNF1LK – that belongs to a family of AMP-activated protein kinases, does not directly transduce Na$^+$; instead, increased Na$^+$ causes increased intracellular Ca$^{2+}$ through the NCX, which activates a calcium-calmodulin dependent kinase that, in turn, activates SIK$^{233, 234, 415, 416}$. The transcriptome of mammalian cortical astrocytes published in 2008
demonstrates that astrocytes express two isoforms of SIK\textsuperscript{236}, and SIK has also been found in Müller glia\textsuperscript{417} (the resident astrocytes of the retina), suggesting that this same pathway may allow astrocytes to transduce EF-induced changes in $V_m$ and regulate DNA synthesis and mitosis.

Extracellular EFs induce changes in $V_m$ that cause an increase in intracellular $Na^{+}\textsuperscript{418}$ that subsequently regulates $Ca^{2+}$ entry into the cell\textsuperscript{392, 419}, which suggests that EFs may be transduced through the SIK pathway. Assuming injury-induced EFs drive astrocyte proliferation after injury through a $Ca^{2+}$-regulated second messenger system (e.g. SIK), intracellular $Ca^{2+}$ and proliferation should be greatest at the lesion border where EFs are highest, and they should decline with distance to the lesion margin in parallel with the decay in EF-intensity over distance from through the penumbra (Figure 1.7). Indeed, intracellular $Ca^{2+}$ concentration increases in astrocytes upon injury, but this dissipates with distance from the lesion\textsuperscript{408}; and astrocyte proliferation following SCI is confined to the immediate penumbra\textsuperscript{33, 420}. Additionally, EFs should have a cell-type specific effect on proliferation as voltage-sensitive membrane protein expression varies among different sub-populations of cells, a supposition that is supported by observations that membrane depolarization in neurons stimulates DNA synthesis and mitosis\textsuperscript{218, 414} while 200 mV/mm EF application inhibits endothelial cell proliferation (50 and 100 mV/mm had no effect)\textsuperscript{421}. The hypothesis that EFs modify the $V_m$ to regulate cell cycle checkpoints that induce proliferation suggests that cells would only need to be exposed to EFs long enough to get past these cell cycle checkpoints. Consistent with this hypothetical mechanism, a single EF pulse stimulates the activation of members of the fos and jun gene families, as well as the persistent activation of multiple
transcription factors within one hour of exposure. Moreover, astrocyte proliferation 24 hours after a scratch wound requires a Na\(^+\) current through Nav1.5 only for the first 15 minutes after injury, and tail regeneration in Xenopus tadpoles requires only 1 hour of Na\(^+\) current to stimulate regeneration. Together, these observations demonstrate that EFs regulate a signal transduction pathway that is known to stimulate astrocyte proliferation, thus supporting the hypothesis that EFs have a causal effect in regulating proliferation.

**Differentiation and neurogenesis**

In peripheral tissues, injury-induced EFs stimulate terminally differentiated cells to revert to an immature progenitor phenotype, and these proliferating progenitors respond to master regulatory genes that guide morphogenesis throughout regeneration. In order for injury-induced EFs to stimulate regeneration in the mammalian CNS through astrocytes, elevated EFs must effect changes in astrocyte gene expression, and astrocytes must be able to respond to master regulatory genes. In the previous sections on migration and proliferation, we discussed possible mechanisms by which astrocytes may transduce EFs through second messenger systems associated with Ca\(^{2+}\), PI3K, PKC, and SIK; these same pathways can also contribute to transcriptional modification of genes associated with changes in differentiation, such as regulation of Notch1 and BMP by SIK-signaling during Xenopus tadpole tail regeneration. Moreover, we previously described how cells transduce EF-induced changes in V\(_m\) to regulate proliferation, and how V\(_m\) is also associated with differentiation: progenitor cells have relatively depolarized V\(_m\) that become increasingly hyperpolarized as they
differentiate⁴²³, changes in Vₘ have been shown to actively regulate differentiation of mesenchymal stem cells⁴²⁴, and elevated EFs increase neurogenesis from mammalian NPCs in vitro⁴²⁴. A comprehensive study of the transcriptome of murine astrocytes demonstrated that mature astrocytes express multiple receptors and downstream mediators necessary to transduce each of the master regulatory genes associated with epimorphic regeneration, including those for BMP, FGF, RA, Shh (the Ptch1 receptor), TGF-β, Wnt (the Fzd receptor family, especially Fzd2); and an ingenuity pathway analysis specifically identified Notch, Wnt/β-catenin, TGF-β, and Shh signaling pathways as being particularly enriched in astrocytes as compared to other cell types within the CNS²³⁶. Moreover, mature astrocytes actually express the transcripts for BMPs, FGFs, Shh, TGF-β, Wnt, suggesting that they may be a source of these morphogens in regenerating tissues²³⁶. It is well-established that astrocytes in the adult mammalian CNS have an ability to function as NPCs, but that this neurogenic potential is constitutively inhibited through Notch signaling¹⁰³,¹⁰⁴; as astrocytes express SIK, and SIK signaling promotes regeneration in other tissues through regulating Notch, EFs may be able to induce regeneration in astrocytes by inhibiting Notch-mediated repression of neurogenesis through SIK signaling.

**Biophysics of EF transduction**

Heretofore we have framed our discussion under the assumption that cells detect purely the vector components of the EF: their magnitude and their orientation. However, this is a gross oversimplification of the information encoded by EF vectors. We have already described the fundamental relationship between EFs and ionic currents, which
themselves may regulate cellular physiology through magnetic interactions induced by the moving charge and thus may have effects that are completely independent of EFs. (These potential magnetic interactions are beyond the scope of this already expansive disquisition and thus will not be further considered here.) Within the scope of EFs, we have already suggested that EFs may also respond to the magnitude of the change in EFs, and to the duration of EF exposure. In the context of our overarching hypothesis – that EFs have an intensity-dependent effect on wound repair – we must consider that cells are exposed to EFs both in the intact tissue prior to the injury, and in the injured tissue immediately thereafter. Thus, cells may respond to the absolute value of the injury-induced EF, the absolute value of the change in EF intensity induced by the injury, the relative increase in EF-intensity upon injury, or the rate of change in EF intensity upon injury. Moreover, as some EF-induced cellular responses only require the initial activation of certain second messenger cascades, the duration of EF exposure also may also be an important signal: cells may respond to the total duration of the applied EF, or they may only require a certain EF flux (e.g. a higher EF over a shorter duration is equivalent to a lower EF over a longer duration). Each of these signals may be relevant in certain circumstances, and multiple properties of EF signaling may be involved in regulating different behaviors within the same cell; for example, the initiation of a cardiac action potential requires the myocyte to depolarize to an absolute value (i.e. the threshold potential), but the rate of this electrical conduction through the myocardium is dependent on the rate of cell depolarization once the action potential is triggered. Thus, while our exploration of the relationship between EFs and regeneration may be facilitated by this simplification, the biophysics underlying this relationship is far
more complex. Moreover, this suggests that our experimental approach of manipulating the absolute value of EF intensities to assess EF-induced effects on cells *in vitro* that we employed in chapters two and three, and that have been employed by many other labs, may not detect the full effect that physiologic EFs have on cellular behavior if cells transduce components of the EF other than their absolute value.

**The evolutionary relevance of electric fields**

In the previous section, we established that EFs can plausibly interact with the physiologic mechanisms regulating each of the astrocytic behaviors necessary for epimorphic regeneration and, by extension, embryonic development. Now we turn our attention to exploring reasons for which cells would have evolved an ability to respond to EFs, and why EFs may play such an important role in regulating development and regeneration. The evolution of the important role that EFs play in development and regeneration presents a considerable conundrum given that EFs only encode bivariate information (i.e. magnitude and direction), while the complex tissues within vertebrates seem to require interactions among the myriad families of signaling pathways whose necessity for morphogenesis we have already established. However, this teleological understanding of EFs is not consistent with their evolutionary origins.

*Ubiquity of EFs explains their evolutionary significance*

As ontogeny recapitulates phylogeny, an understanding of when EFs begin to be involved in embryogenesis will inform our understanding of their evolutionary origins. The earliest endogenous bioelectric activity that has been measured in embryonic
vertebrates is immediately prior to limb bud formation\textsuperscript{248, 250, 252}, but this late measurement reflects technical difficulties in measuring EFs at earlier embryonic time points and is not an indication that they are absent at the start of development. Indeed, studies have found that asymmetric electrical activity precedes polarization of the fertilized egg from the alga Pelvetia, demonstrating that bioelectric signals are associated with the earliest stages of eukaryotic development. Moreover, the role that EFs play in establishing cell polarity in Pelvetia embryos\textsuperscript{246, 425} is similar to the role of EFs in defining body axes and guiding limb development in vertebrates\textsuperscript{252, 315, 400}, suggesting that EFs played a similar developmental role in the common ancestor to these two species – which existed before the division of the ancestral eukaryote into the Unikonta and Bikonta supergroups that is estimated at 1100 – 2300 million years ago\textsuperscript{160, 426}. As bioelectric signaling was already involved in developmental processes in the primordial eukaryote, it is likely that the interaction between EFs and physiology has evolutionary origins that are far older.

According to the theory of common descent, all extant life evolved through speciation from a universal protocellular ancestor. This protocell developed from the aggregation of a replicating genome containing hereditable information within a membranous vesicle that compartmentalized it from the surrounding environment\textsuperscript{427}. Consequently, these basic elements of cellular physiology – a semipermeable membrane and a self-replicating genome – would have had the opportunity to be conserved among all life. In turn, each of these elements originally developed from the spontaneous formation of organic molecules from inorganic ions, and the polymerization of these molecules into structures capable of catalyzing their own replication. These
chemical processes developed in an acellular environment and thus they would have been directly affected by the physical properties within the environment, including solute concentration, heat, and electrostatic forces. Evolution has had billions of years to shed the mechanisms by which life detects these fundamental properties of the environment, but the fact that there are species among extant archaea, bacteria, and eukarya that still respond to each of these cues underscores the importance of these physical properties to life.

Electric fields are the most important of the physical properties of the environment to molecular interactions because, while temperature and solute concentration may affect the rate of metabolic reactions (and may even affect the stability of the tertiary and quaternary structures of larger macromolecules), the actual interactions between different molecules are mediated through their electric fields. The arrangement of polar and charged residues on the surface of molecules creates EFs that propagate through the surrounding area; electrostatic interactions between the EFs from different molecules mediate their formation into molecular complexes, and EFs within the active site of enzymes are necessary for their catalytic activity. Moreover, certain electrostatic properties appear to be evolutionarily selected in proteins to maintain specific functions. Indeed, the earliest organic molecules may have reacted with each other exclusively through electrostatic interactions as more elaborate molecular signals and enzymes had yet to originate. These same charged residues mediating intermolecular interactions also allow biological macromolecules align their dipoles parallel to the orientation of environmental EFs. It follows that cells should also be able to detect and respond to environmental EFs, as cells are merely aggregates of
ions and biological macromolecules. As these basic molecules of heredity developed prior to the origin of the protocell, interactions between external EFs and biological molecules must predate the origin of cellular life.

Models of the membrane in primitive cells suggest that these original membranes were composed of simple fatty acids that allowed for the passage of polar solutes without the aid of specific transport machinery; such transport mechanisms gradually emerged as protocells developed nutrient requirements that exceeded those available through passive diffusion, and as the increasing complexity of membrane lipids caused membrane permeability to decrease. These transport mechanisms include selective ion channels and transporters whose very activity is electrogenic because they result in a net movement of specific ions. Consequently, not only did protocellular physiology develop in an environment in which they were constantly exposed to EFs, but cells also began to produce their own electrogenic signals early in evolutionary history. Moreover, EFs have a universal effect on charged molecules, so physiologic EFs would have allowed early cells to interact with each other prior to the evolution of specific signaling molecules.

EFs retained their physiologic relevance in intercellular communication throughout the evolution of multicellularity, which explains their integral role in morphogenesis during development and regeneration. In multicellular organisms, each tissue may have evolved a characteristic EF in parallel to the evolution of the tissue itself: each tissue has a characteristic structure that defines its electrical properties, and each tissue is composed of characteristic sub-populations of cells that each may have unique electrogenic activity. Epithelial cells lining the embryo sustaining the
same electrogenic activity as they do in adults but, because the undeveloped embryonic tissue is much thinner than the mature tissue, this electrogenic activity results in EFs that are elevated within the embryo (i.e. embryonic EFs are elevated in part because the same voltages created by epithelial cells are spread over a much shorter distance). Nascent tissues thicken throughout embryonic development due to EF-induced progenitor cell proliferation, which causes EFs to gradually diminish, which consequently drives the progressive differentiation of stem cells into increasingly-mature populations\textsuperscript{423, 424}. In the developing limb, epithelial cells in the apical ectodermal cap (AEC) produce a high EF that may sustain progenitor cell proliferation in the underlying blastema; these EFs dissipate with distance from the AEC, allowing more proximally located progenitors to differentiate, and this voltage gradient may contribute to planar cell polarity that helps pattern the proximodistal axis of the developing limb\textsuperscript{248, 252, 401}.

Characteristic physical properties of tissues may have also led to the evolution of EFs as a key signaling mechanism in the injury response. Injury changes the physical properties of tissues, causing a passive increase in the EF intensity that is immediate and is based purely on the physiologic electrogenic activity in the tissue prior to injury. In contrast to the immediacy of changes in bioelectric signaling following injury, molecular signals associated with the injury response are relatively slow as they are actively released only upon exposure to specific triggers, and certain signaling molecules first need to be synthesized. Moreover, molecular signaling is limited by the relatively slow process of diffusion through the lesion site, whereas bioelectricity propagates instantly through the tissue surrounding the lesion and dissipates only as a function of distance. Furthermore, these elevated EFs, which have been shown to drive
the dedifferentiation of cells into pleuripotent progenitors\textsuperscript{245, 424}, may drive epimorphic regeneration because they are the same signal that drives morphogenesis during development. Thus, the proposed importance of EFs in regulating molecular interactions within and between cells, and in tissue morphogenesis during development and regeneration, is compatible with the current understanding of the origins of life and subsequent metazoan evolution.

\textit{Evolutionary loss of EF-induced regeneration}

Given the ubiquity of EFs in biological systems and their conserved role in regulating both embryonic development and regeneration, it is particularly curious that mammals have generally lost the ability to spontaneously regenerate. Having described in the previous section multiple reasons for which EFs evolved to be integrally important to development and regeneration, we must now consider how mammals have evolved the loss of EF-induced regeneration. The form of regeneration with which we are concerned is that which recapitulates embryogenesis so, as we described in chapter one (page 18), these mechanisms must be conserved in mammals as they are identical to those underlying their original embryonic development. We have also described evidence that these pathways are expressed in mammals in a functional state, and the experiments that we described in chapters two and three demonstrated that EFs can induce each of the astrocytic behaviors necessary for regeneration. Furthermore, we have discussed evidence that injury induces an increase in EFs in multiple mammalian tissues, so the putative stimulus initiating regeneration is also clearly conserved. Therefore, the absence of regeneration must be due to a divergence between the
robustness of the injury-induced EFs in mammals and the sensitivity of the signal transduction pathway through which cellular regeneration is initiated. Injury-induced EFs may have declined in intensity due to evolutionary changes in the physical properties of mammalian tissues – either to a changing composition of the ECM altering the dielectric properties of the parenchyma\textsuperscript{431}, or to the development of the sub-dermal lymphatic system that electrically grounds the EF\textsuperscript{278} – or to an evolutionary diminution in the electrogenic capacity of epithelial cells. We have already described myriad pathways through which cells may transduce EFs; decreased efficiency of any of these pathways – due to a necessary molecule having reduced sensitivity, expression, or activity – could attenuate the cellular response to injury-induced EFs.

**Loss of regeneration as an evolutionary adaptation**

We have established that physiologic EFs regulate morphogenesis during embryonic development and regeneration, and that the physiology by which EFs are produced and transduced by cells is universally conserved. Therefore, the fact that regenerative potential exhibits variable expressivity among different vertebrate clades (Figure 5.1), and among different species within each clade, raises the question of why regeneration is not universal given that the underlying physiologic mechanisms are. This question is particularly pertinent in establishing the causality of endogenous electric fields as a universal regulator of embryogenesis and regeneration: in the previous section we posit that EFs evolved a loss-of-function mutation in certain vertebrates such that they continue to regulate embryogenesis but are insufficient to stimulate regeneration; for this hypothesis to be valid, we have to demonstrate that the loss of regeneration is evolutionarily plausible and coherent with preexisting theory.
Regeneration was a feature of the original metazoan

Two competing theories account for the observed variability in regenerative potential (Figure 5.2): regeneration may have evolved from a common non-regenerative ancestor independently in each lineage where it is expressed, or regeneration may have been evolutionarily lost from a common regenerative ancestor. Under the hypothesis that regeneration in extant vertebrates is expressed only in those species where it arose as a gain-of-function adaptation from a non-regenerative ancestor, the underlying physiology would be expected to vary widely among different species. Instead, the physiologic mechanisms underlying epimorphic regeneration involve the conserved role of endogenous EFs and master regulatory genes, which has been demonstrated in 10 extant vertebrate clades and absent only two – mammals and birds (Figure 5.1)\(^ {123, 125, 133, 136, 188}\). Thus, the principal of parsimony summarized eponymously as Occam’s Razor suggests that the common ancestor among vertebrates expressed regeneration and that loss-of-function adaptations independently arose in separate clades (theory posited in Figure 5.2A). However, this conclusion merely displaces the original quandary: mammals evolved the loss of regeneration from a highly-regenerative common ancestor, but it does not address the question of whether regeneration originated as a gain-of-function adaptation in this common ancestor or if regeneration is a more universal trait.

In addition to phylum Chordata\(^ {61, 124, 125, 133, 142, 143, 154, 432, 433}\), of which Vertebrata is a subphylum, epimorphic regeneration has also been demonstrated in phylum Arthropoda\(^ {434-436}\), and total body regeneration has been demonstrated in phyla Cnidaria\(^ {437-439}\), Platyhelminthes\(^ {440, 441}\), and Porifera\(^ {442}\). As regeneration has been
conserved across these diverse phyla, it is likely that regeneration is a feature present in the original metazoan – a multicellular organism resembling the modern sea-sponge – and that it was preserved through speciation to the common vertebrate ancestor after which it was subsequently lost within the mammalian clade subsequent to its evolutionary divergence from other vertebrates in which regeneration was retained.

It may be counter-intuitive to think that the loss of regeneration may confer an adaptive advantage to a species. However, evolution is not progressive: it is the result of cumulative selection pressures from immediate environmental factors acting on individuals, the culmination of which favors the emergence of a specific trait over multiple generations. Evolution requires that these traits are heritable characteristics with variable expression among different individuals within the population, and that environmental pressures acting on individuals at discrete points in time favor the spread of a subset of those characteristics over the expense of others. Not all traits are adaptive, and those traits that are beneficial in one generation may lead to a series of adaptations over subsequent generations that are ultimately maladaptive.\textsuperscript{443, 444}

It is posited that colonies of unicellular organisms resembling choanoflagellates gradually developed codependence, resulting in the original metazoan that is the progenitor of all multicellular life\textsuperscript{134, 445-448}; this organism likely resembled modern sea-sponges that are composed of individual choanocytes that are cytologically similar to the unicellular life forms from which they descended\textsuperscript{446}. These original metazoans represent the advent of regeneration and demonstrate its fundamental importance to multicellularity: codependence creates a situation in which the loss of an individual cell does not equate death of the entire organism, and thus those individual cells lost to
injury need to be replaced by the remaining cells within the animal. Similarly to colonies of unicellular organisms, these original multicellular organisms may survive after the death of a subset of their cellular composition, and new organisms may be regenerated from a small number of isolated individual cells. Sea-sponges – representing the origin of multicellularity and cellular codependence – are able to regenerate in much the same way: loss of a portion of the sponge is replaced by regeneration while isolating small numbers of dissociated cells results in the re-aggregation of these cells into a functional organism.  

Selection pressures favoring cellular codependence resulted in the development of a simple organism resembling the modern sea sponge and continued to encourage the speciation of this metazoan into increasingly diverse phyla. The original regenerative capacity of sea sponges is preserved in modern hydra and planaria as demonstrated by their capacity to regenerate complete organisms from only a small number of isolated cells, and this suggests that regeneration was an important feature worth preserving over the hundreds of millions of years of evolutionary history since the origin of multicellularity. Deuterostomes share a common ancestor with planaria and hydra but the totipotent regenerative phenomena expressed in planaria and hydra are lost in subset of deuterostomes from which vertebrates emerged. Within the phylum Chordata, Urodele amphibians and Teleost fish represent two examples of numerous non-mammalian vertebrates capable of completely regenerating their hearts, spinal cords, limbs/fins, and other organs after significant damage; mammals do not exhibit these robust regenerative properties in spite of the fact that all vertebrates share a common ancestor.
Because regeneration is a process found among most multicellular organisms, the principle of parsimony suggests that these regenerative mechanisms developed once and that its subsequent loss represents relatively unique evolutionary adaptations. Thus, the loss of regeneration in higher vertebrates in spite of the proficiency with which Urodeles and Teleosts recover from injury suggests a strong selection pressure against the ability to repair certain damaged tissues.

Mammals may have evolved an inability to regenerate, but many other organisms retained this feature over an equally long evolutionary opportunity to lose it. It is a fallacy to think that these early-branching organisms split from the vertebrate lineage and became evolutionarily stagnant; they have had as much time to evolve as have mammals. Although there has been an incredibly long time for the mechanisms underlying regeneration in these distantly-related organisms to evolve different mechanisms, the conservation of orthologous regenerative genes across multiple phyla indicates that there has been a constant selection pressure favoring the preservation of regenerative physiology. Mammals do not typically express epimorphic regeneration as adults, but the functional preservation of the underlying genes and pathways demonstrates that the potential to induce regeneration is retained.\textsuperscript{136, 437, 455–461}

Conservation of the pathways underlying regeneration throughout hundreds of millions of years of evolution indicates their fundamental importance. Given the extent to which regeneration can occur in other lineages, the loss of these phenomena in mammals is, when considered as an independent variable, likely a cause of increased mortality in these organisms following injury. That these pathways are present but unexpressed in mammals indicates that there must be a greater benefit afforded
elsewhere by suppressing regeneration than is gained by completely resolving organ
damage through regeneration after injury. Understanding the selection pressures
favoring the loss of regeneration is necessary to inform the development of a
therapeutic strategy to stimulate a regenerative phenotype while compensating for
those evolutionarily adaptive traits through which it is suppressed.

**Inclusive fitness: lost regeneration as an adaptive advantage**

Developing a multicellular lifestyle requires a shift in fitness strategy favoring
inclusive fitness over a direct reproductive approach. The metazoan concestor likely
resembled a simplistic sea sponge that, as already discussed, was capable of
regenerating its entire body plan from small aggregates of individual cells\(^{445-448}\). This
totipotent regenerative capacity was conserved past this point and is still expressed
among different modern phyla including Porifera\(^ {442}\), Cnidaria\(^ {437, 438}\), and
Platyhelminthes\(^ {441, 449}\). Organisms from each of these phyla show evidence of
immortality: Porifera re-aggregate upon being dissociated into individual cells\(^ {442}\); Hydra
reproduce when offspring bud from the parent while the parent remains capable of
surviving and reproducing over the course of at least four years (the longest published
example of hydra reproduction being studied)\(^ {462}\); Planaria reproduce by binary fission
producing two offspring from the original parent, and injury resulting in one Planaria
being torn in two results in the regeneration of two independent organisms\(^ {463, 464}\). This
same type of immortality is seen in many unicellular organisms\(^ {447, 465}\) that reproduce
symmetrically to form two identical and indistinguishable daughter cells and suggests
either that immortality results from convergent evolution that returned to metazoans

after the advent of multicellularity, or that the original metazoan concestor was a
conglomerate of immortal codependent cells. The preponderance of evolutionary
evidence and the simplicity required by Occam’s razor suggest that the totipotent
regenerative capacity in sea sponges, and the immortality of Porifera, Planaria, and
Hydra, are the result of immortality within the original metazoan.

Multicellular life most likely began with an immortal organism, so the
deuterostome clade in which modern mortal organisms must have evolved the trait of
mortality at some subsequent point in its speciation. Common ancestry between
mortal and immortal organisms implies that the concestor population from which the trait
of mortality arose contained coexisting mortal and immortal members. That this clade
survives and has undergone wildly successful speciation events over hundreds of
millions of years, and that examples of convergent evolution of mortality is seen in
examples of asymmetrically dividing prokarya in which the parent cell ages and dies is a clear indication that mortality must offer a significant benefit to the survival of a
species.

Mortality’s survival benefit is assumed because of the evolutionary success of
organisms in which this trait exists, but a net benefit is not required for its persistence in
a population. What is necessary for the continued existence of mortality is that its
development does not yield a net loss in fitness. While numerous other phyla
developed during this era of evolutionary history, widespread extinction of entire phyla
suggests that selection pressures are extremely effective at eliminating maladaptive
traits from the evolutionary gene pool. Modern computer models of the spread of
adaptive traits that benefit decreased individual reproduction support this and show that
novel phenotypic traits may spread rapidly through a population (within 500 generations) accompanied by the equally quick loss of the maladaptive version. Therefore, while the magnitude of the direct benefit offered by its evolution may be controversial, it is established that mortality is at least not maladaptive.

The development and success of mortality, together with the loss of totipotent regenerative potential in higher vertebrates, suggests that there must be an adaptive advantage to eliminating individual organisms for the benefit of the entire species. However, the adaptive benefit of death directly conflicts with the evolutionary axiom that the organism with the greatest fitness is the one producing the most offspring. Imagining the ancestor in which mortal and immortal organisms coexisted, it is intuitively obvious that the infinite reproductive capacity of immortal variants must have a fitness advantage over the mortal variant that eliminates itself from the reproductive population.

This hypothetical situation, while informative, is incomplete. Interpreting the best adaptation as the one leading to the greatest number of direct offspring is a conceptually-simple fallacy that obfuscates the reality that, although natural selection is based on unequal inheritance of phenotypes, the heritable unit of a phenotype is the genotype on which it is based. Thus, although inheritance of traits is easy to conceptualize, selection pressures favor the particular alleles that are best able to reach the next generation regardless of the method by which they are propagated. While individual reproduction creates immediate genetic transmission that increases the direct fitness of an organism, the ubiquitous evolutionary loss of directly-reproducing life within metazoans due to the distinction between somatic and germ cell lines indicates that selection pressures beyond direct fitness must also participate in evolution.
Following the evolution of mortality, further traits developed in which tissues lost their totipotent regenerative route of reproduction in favor of a distinct germ cell line from which all of that organism’s descendants arose. This development created a situation in which a subset of cells became completely incapable of directly reproducing, instead relegating reproduction to a specialized germ cell lineage. The remaining somatic cells sacrifice their direct inter-generational reproductive potential in favor of increasing the fitness of the specialized germ cells. Multicellular organisms represent an enormous conglomerate of clonal cells that, although differentiated into different phenotypes, are (for the most part) genetically identical. While non-germ cells forego direct reproduction in favor of assisting the germ cells, the genetic homogeneity means that each cell has the same reproductive fitness regardless of whether it is the direct progenitor of the reproducing germ cell. Thus, the somatic cell population can forego direct reproduction because it has the same fitness afforded to it by germ cell success that it would if it was directly responsible for reproduction.

More recent examples in evolution recapitulate the same axiom: that there are situations in which a greater reproductive benefit exists by foregoing opportunities to reproduce directly in favor of offering a benefit to other closely-related members of the population. Numerous examples within phylum Arthropoda illustrate the adaptive advantage of survival strategies that employ a helper caste whose primary fitness benefit is through assisting their close kin rather than by directly reproducing. Paper wasps employ an altruistic strategy by which fertile females forego direct reproduction in favor of assisting the reproductive efforts of their colony’s founding queen; gene expression changes when females become the foundress queen of a
new colony, which corresponds to a dramatic increase in reproductive behavior.\textsuperscript{473} Eusocial behavior is an alternative indirect fitness strategy exemplified by the honeybee in which a morphologically distinct worker caste of sterile female functions to maintain their closely-related hive: workers deter invaders with the kamikaze delivery of venom through a barbed stinger, which is left embedded in the victim along with the bee’s venom sac and abdominal viscera.\textsuperscript{474, 475}

Altruism and eusocial behavior pose an evolutionary paradox first solved by Hamilton in the 1960s. He predicted that indirect fitness would become a predominant fitness strategy in any species in which the allelic benefit from indirect fitness outweighed the cost of lost direct reproduction.\textsuperscript{469} Specifically, he predicted that indirect fitness strategies would emerge in a population whenever $rb - c > 0$ (where $c$ is the fitness cost to the altruist, $b$ is the benefit to the beneficiary, and $r$ is their genetic relatedness).\textsuperscript{468} The extent to which evolutionary altruism is advantageous is illustrated by multiple examples of convergent evolution towards this phenotype among different arthropod species, of which the altruistic paper wasp and the eusocial honeybee serve as two examples. The high degree of genetic relatedness among members of the paper wasp\textsuperscript{471, 473, 476} and honeybee hives\textsuperscript{472, 474, 475} and the prolific reproduction of the colony queens results in an incredibly high allelic benefit afforded by indirect fitness methods.

Kin selection also occurs within mammals, as is illustrated by the altruistic alarm call behavior seen in Belding’s ground squirrels.\textsuperscript{477} Ground squirrels selectively alert their close kin to the presence of predators so that they will have an increased opportunity to protect themselves, but this calling behavior significantly increases their risk of death through predation. The benefit of increased kin survival outweighs the
direct cost of increased predation. Similarly to the altruistic insects that forego direct fitness to benefit their hive, ground squirrels are much more likely to give alarm calls when they have closely related kin that stand to benefit from this behavior. However, squirrel populations are not as genetically homogenous as those of insects: while females stay close to their place of birth and are typically surrounded by kin, males travel to new territories and do remain to help raise their offspring. Females, therefore, are much more likely to have close kin that stand to benefit from altruistic behavior than males do, and they are also much more likely to emit alarm calls than are males. Although a less dramatic example than the suicidal behavior in insects, this still illustrates the benefit of foregoing individual reproductive opportunities to benefit close relatives.

Inclusive fitness helps resolve the paradox created by the evolutionary success of mortality and the potential benefit of an individual foregoing direct reproduction. Initially proposed by Hamilton, the overall benefit of an individual’s fitness – described as the sum of direct and indirect fitness – illustrates the idea that natural selection responds to the interaction between phenotype and environment by favoring the transmission of the responsible genes. An individual organism may increase its genetic impact on subsequent generations through direct reproduction, or through supporting other organisms that are genetically similar. In this context, the success of both mortality and altruism as adaptations implies that each trait must increase the overall inclusive fitness of the organism expressing it.

Complex organs evolved at the cost of direct fitness to the somatic tissue, which is evolutionarily understandable in the context that the cost of lost intergenerational
reproduction by the cells in each organ is outweighed by the benefit from a net fitness gain through inclusive fitness. To achieve this gain, each organ must have developed in a way that supported increasingly advantageous functions of the entire organism. Therefore, loss of organ function following injury is understood to be detrimental to the overall function of that organism. In non-mammalian vertebrates, and elsewhere throughout metazoans, totipotent regeneration permits complete recovery after tissue damage\textsuperscript{124, 125, 134, 438, 441, 449, 463}. Regeneration is a feature without which an organ cannot recover from injury, ergo the loss of regeneration in mammals must reflect an adaptation that is ultimately beneficial to the species’ fitness.

Establishing that the loss of regeneration is associated with a survival advantage in mammals implies that novel selection pressures, which would have arisen at the point when this clade diverged from a regenerative concestor, must have favored this development. It is likely that two distinct pressures drove this development in parallel to each other: one favoring the evolution of increasingly complex organs whose maintenance following injury required rapid recovery of function, and a second through which injured individuals somehow compromised the fitness of the entire group. Regeneration is energetically costly and time consuming, and the increasing importance of each organ likely favored a reparative response to rapidly restore functional integrity while inadvertently antagonizing the regenerative pathways. The decreased survival (and direct fitness) of the individual created by this failure to regenerate is insignificant when juxtaposed with the benefit to the surviving group members that no longer need to care for those infirm injured individuals that have decreased capacity to gather food, defend the group, or otherwise move.
Lost regeneration is not an epiphenomenon of evolution

The CNS and the heart are two examples of organs whose obstreperous response to injury does not support regeneration even though the pathways through which regeneration could be facilitated are preserved in a latent state. The benefit to inclusive fitness is only a partial explanation for why regeneration is not expressed: the loss of regeneration may not be directly advantageous to either the clade or the individual, but the fitness benefit resulting from regeneration was less than the fitness provided by the reparative processes of astrogliosis and cardiac fibrosis. As the physiology of regeneration and fibrosis directly conflict with each other, wound repair through fibrosis out-competed regeneration. Although not expressed, the preservation of latent regenerative physiology in mammalian tissues suggests that these mechanisms are still important for the normal function of the species and that there has been an active selection pressure to maintain it.

Traits may be eliminated by different mechanisms, each of which is informative about the reason for which it was lost. Convergent evolution of atrophied organs and genes illustrates the frequency with which obsolete functions accumulate loss-of-function mutations once the selection pressure to maintain them is removed: organs persist as vestigial remnants (e.g. the appendix and coccyx in humans\textsuperscript{478, 479}, and the recurrent convergent atrophy of eyes in blind cave fish\textsuperscript{480}), while genes become nonfunctional pseudogenes (e.g. hundreds of human odorant receptors became pseudogenes as they became increasingly visual animals, compared to the 1200-1500 functional receptors that remain in other olfactory vertebrates)\textsuperscript{481}. Obsolete genes and tissues persist in vestigial or pseudogene states because, while the pressure to
maintain them is removed, there is no selection favoring their loss. Alternatively, maladaptive phenotypes are actively selected against and, as in the case of the Irish Elk in which the initially adaptive enlargement of the male’s horns ultimately became too heavy for the males to lift their heads\textsuperscript{444}, may lead to the extinction of the entire species. Together, this supports the notion that regeneration is not expressed in mammals because it is subrogated by the gain-of-function of new physiology with which regeneration is mutually exclusive.

Although atrophy and negative selection are common mechanisms driving evolutionary change, neither of these mechanisms is responsible for the loss of regeneration in mammals. Instead of being selected against or being lost to evolutionary atrophy, regenerative physiology is maintained in a potentially functional state that may be actively suppressed during injury\textsuperscript{42-44, 450, 482}. Multiple mechanisms exist by which a trait may be lost as a species evolves, and each pathway reflects the emergence of a different selection pressure. While maladaptive traits often result in extinction and obsolete traits become vestigial, unexpressed pathways are preserved when the function they serve remains a strong benefit under specific circumstances. For regeneration, the functional preservation of the underlying physiology is likely due to the conserved use of these pathways during embryogenesis\textsuperscript{142, 437, 441}.

Mammals do not commonly regenerate following significant damage to their heart, CNS, or limbs, but the functional orthologous pathways promoting regeneration in other vertebrates are conserved in mammals. This implies that tissue regeneration in mammals was once adaptive and, while the preservation of these pathways may be due to their involvement in embryogenesis, that they persist in adulthood suggests that a
A therapeutic program has the potential to stimulate regeneration if the physiologic mechanism through which spontaneous regeneration is activated in other organisms can be identified in mammals. Given the health care burden of caring for patients suffering from long-term sequelae of CNS injury \(^{16, 21, 483, 484}\) and the inability to find a substantial therapeutic intervention to promote functional recovery \(^{485-487}\), a strategy to promote regeneration based on these physiologic mechanisms should be a research priority.

_Immune system and the effect on regeneration_

The complex immune system in mammals responds to tissue damage with an initial neutrophilic, and delayed macrophage, infiltrate to remove damaged tissue by phagocytosis; a concomitant proliferation of fibroblasts is responsible for generating scar tissue, which restores tensile strength to the injured tissue. (An exception to this is the CNS, which lacks fibroblasts; instead, astrocytes react to injury by isolating the lesion cavity \(^{488, 489}\), but there is no CNS equivalent of the fibroblast to fill the resulting cystic cavity with any cellular or collagen-based substrate \(^{35}\).) Immune cells are recruited by cytokines released from damaged tissue and they function to expedite recovery by rapidly clear damaged tissue to make way for proliferating fibroblasts \(^{490}\). These fibroblasts initially accumulate in the granulation tissue surrounding the necrotic injury focus and gradually deposit collagen into the area cleared by the immune response \(^{490}\).

Neutrophils and macrophages create free radicals and other molecules that can be directly toxic to healthy cells as part of their natural response to injury \(^{491, 492}\). In addition to serving as the source of fibroblasts, the granulation tissue surrounding the
injury site also functions to prevent the toxic compounds produced by the inflammatory process from spreading into the adjacent healthy tissue as shown by experimental paradigms in which attenuating the granulation tissue barrier results in a larger lesion cavity\textsuperscript{490}. Although lacking granulation tissue, the CNS exhibits a similar response to injury by which astrocytes isolate the lesion cavity from the adjacent healthy parenchyma to prevent the lesion from expanding due to secondary injury from toxic metabolites\textsuperscript{31, 32, 35, 493}.

Inflammation in response to injury is an incredibly important mechanism by which injured tissue is cleared, but it also appears to have maladaptive aspects in both the acute and chronic phases of recovery; the therapeutic efficacy of steroids in improving outcomes by attenuating inflammation is a clear indication of this\textsuperscript{491, 492}. Although methylprednisolone was the only approved pharmacologic intervention for CNS injury beginning on the 1990s, the therapeutic effect was small and the side effects were numerous\textsuperscript{485-487, 494}, thus, although inflammation is an important component of the injury response, it is clearly not the only factor in the associated pathophysiology.

Within the CNS, inflammatory mediators are toxic to neurons and are contained by a barrier of reactive astrocytes\textsuperscript{35, 50}. As previously mentioned, this barrier is incredibly important for preserving the integrity of the adjacent parenchyma and for restoring the blood brain barrier that is disrupted by the injury. The astrocytic barrier anatomically isolates the lesion site, allowing a cystic cavity of liquefactive necrosis to form as the immune system removes the necrotic tissue through phagocytosis. Reactive astrocytes also respond to injury by modifying the extracellular matrix with the addition of CSPGs, which prevent axons from accidentally sprouting into the lesion cavity by serving as a
repulsive cue to the growth cones\textsuperscript{49, 50}. Thus, the astrocytic reaction provides an immediate advantage by isolating a hostile lesion cavity\textsuperscript{35, 51, 495}, but ultimately creates an anatomical and functional barrier to axon regeneration.

\textit{Cardiac evolution illustrates the benefit of size over regeneration}

As evolution favored the emergence of increasingly large and complex organisms, passive diffusion ceased to be an efficient means of distributing nutrients throughout the body. Diffusion’s failure across significant distances is apparent in the extent to which cells expend energy on actively transporting substances through themselves using molecular motors such as kinesins and dyneins\textsuperscript{496-498}. Extracellular transport permits nutrient and waste exchange using a circulatory system that must be increasingly self contained and pressurized as organism size and complexity increases; the open circulatory system in arthropods is replaced by an increasingly efficient closed system in chordates\textsuperscript{134} that has developed the potential to generate high pressures that is necessary to support the larger body size within the vertebrate clade\textsuperscript{142, 441}. Vascular damage can compromise myocardial integrity, and physiologic pressures increase the risk of aneurysm formation and rupture\textsuperscript{499}.

Ischemic injury in the cardiovascular system results in coagulative necrosis\textsuperscript{29}. Disruption to the muscular layer reduces vessel integrity\textsuperscript{500, 501} and recruits the immune system to remove the necrotic tissue\textsuperscript{490-492}. Granulation tissue forming at the lesion border gradually replaces the necrotic vascular wall with fibrosis\textsuperscript{490}. Cardiac fibrosis begins within 48 hours after ischemic injury and continues over a period of 5-7 days; during this time, cardiac integrity is compromised and there is a significantly increased
risk of physiologic systolic pressure causing ventricular aneurysm rupture resulting in cardiac tamponade. Fibrosis quickly restores myocardial integrity, and risk of rupture decreases dramatically once it is completed. However, the scar tissue has decreased structural and functional integrity relative to the healthy myocardium. The fibrotic reparative response is necessary to obviate the morbidity associated with ventricular aneurysm formation and rupture, but it blocks endogenous stem cell proliferation and cardiac regeneration in the process. Because the scar tissue cannot be replaced with intact cardiomyocytes under physiologic conditions in mammals, the remaining myocardium undergoes compensatory hypertrophy to compensate for the decreased contractility of the fibrotic tissue. This beneficial compensatory response can become maladaptive and cause a progression to heart failure if it proceeds unchecked, which is reflected in the ~65% congestive heart failure complication rate over 5 years in patients recovering from a myocardial infarction.

Adult hearts in Urodele amphibians and Teleost fish are capable of complete regeneration following ventricular amputation by a combined proliferation of endogenous cardiac stem cells and by the dedifferentiation of mature cardiomyocytes into pluripotent stem cells; mature cardiomyocytes themselves may be capable of undergoing karyokinesis and cytokinesis without first dedifferentiating, even if they are multinucleated. Division requires a partial disassembly of the contractile apparatus within these cells from metaphase through cytokinesis that leaves the cell temporarily incapable of contracting. Thus, regeneration in these lower vertebrates results in a transient decrease in cardiac function while the cardiomyocytes return to the cell cycle, but this is ultimately permissive to the complete restoration of
cardiac function. Interestingly, a major model for cardiac regeneration involves amputation of the ventricular apex in newts\textsuperscript{142, 504} and zebrafish\textsuperscript{135, 142}, an injury that would be rapidly fatal in mammals\textsuperscript{142, 143, 450, 504}. Inducing ischemic injury in newt and zebrafish myocardium is extremely difficult because the thin tissue permits sufficient diffusion with ventricular blood such that coronary vasculature is unnecessary. The increased efficacy of nutrient transport by passive diffusion – rather than on active cardiovascular circulation – is recapitulated throughout their bodies such that the demand on the circulatory system for adequate perfusion is much less than that placed on the mammalian circulatory system. Smaller body size and decreased circulatory demand do not require the perfusion pressures seen in mammals, so the incision initiating clotting in a newt or zebrafish heart would cause rapid exsanguination and death in a mammal.

Within the hearts of lower vertebrates, cardiomyocytes and stem cells are vital components of regeneration\textsuperscript{142}. Although mammalian hearts contain both cell types, the granulation tissue is thought to inhibit or kill the cardiac stem cells, while the fibrotic response is thought to inhibit proliferation of cardiomyocytes\textsuperscript{490}. The fibrotic and regenerative responses are thought to be balanced such that the dominance of one process impedes the progress of the other\textsuperscript{482, 505}. The dramatically higher number of fibroblasts in mammalian hearts likely results in the fibrotic response easily out-competing the regenerative response, while the lower fibroblast content in non-mammalian vertebrate hearts is the reason for their successful regeneration. Studies endorsing this theory have shown that blocking regeneration in newts results in fibrosis and scarring instead of regeneration\textsuperscript{143}, while blocking fibroblast activation in mice has
resulted in essentially complete cardiac regeneration with function and structural capacity reaching that of uninjured animals over a six month recovery period\textsuperscript{482}. Significantly, mouse cardiac regeneration occurred over six months, during which time the lack of fibrosis likely compromised the cardiac integrity of these animals (although mortality rates in these mice were not reported); the accelerated rate of restoring structural cardiac integrity by fibrosis may result in a significant decreased human mortality following MI than the six-month regenerative period seen in mice would afford. Assuming a relatively constant rate of ventricular aneurysm formation and rupture throughout the six month regenerative process comparable to that seen during the fibrotic process in humans 0.2-0.3\%, increasing the period of compromised structural integrity to a six month window of regeneration would significantly increase the complication rate\textsuperscript{506, 507}.

In summary, the increased perfusion pressure that evolved in mammals to permit a larger body size created a selection pressure favoring the rapid restoration of structural integrity following an ischemic injury. Although the fibrotic process that developed in response to this stress is inhibitory to the endogenous regenerative physiology, blocking fibrosis can still stimulate this pathway. However, fibrosis is highly adaptive and any treatment aimed at blocking it must compensate for the substantially prolonged regenerative period and its concomitant decreased structural integrity.

**Conclusions and implications**

Throughout this dissertation we have put forth evidence supporting two interrelated hypotheses. First we explored the hypothesis that physiologic bioelectric fields regulate the injury response and determine regeneration in the mammalian CNS,
in chapters two, three, and four. The validity of this hypothesis is predicated upon, and nested within, the larger hypothesis that endogenous EFs are a universal stimulus that regulate morphogenesis underlying embryogenesis and epimorphic regeneration in all metazoans, the causal justification for which we explored throughout chapter five.

The implications for these findings are vast and important. First, EFs are a promising therapeutic option to promote CNS regeneration. Specifically, as EFs stimulate morphogenesis through endogenous physiologic pathways, therapeutic EFs could likely change the cellular response and facilitate regeneration by releasing these latent pathways from inhibition. Upon appropriate EF-based treatment, the tissue contains sufficient physiologic information to completely regenerate, obviating the need for supplemental drugs or combinatorial treatments. Beyond the CNS, the single unifying hypothesis of EFs as the stimulus underlying morphogenesis suggests that EF-based therapies may be used to promote regeneration following injury in any tissue or organ, and to treat structural abnormalities that arise as a consequence of congenital defects.

The successful application of EFs-based therapies relies upon a thorough understanding of three specific variables: the specific component of the EF that cells transduce, the parameters of physiologic EFs produced upon injury in the target tissue, and the parameters of EFs associated with tissues where successful regeneration occurs. Understanding these parameters is a necessary prerequisite to developing an EF-based therapy for stimulating regeneration. Before EFs should be therapeutically applied, it is necessary to fully elucidate how injury-induced EFs in non-regenerating tissues differ from those in regenerating tissues so that injury-induced EFs can
appropriately altered. Moreover, understanding how each physical component of the EF contributes to morphogenesis will inform the development of the most efficient application of therapeutic EFs. Multiple options for EF-based therapies have been explored in basic science, some of which have even been applied clinically; however, the optimal EF-based therapy cannot be designed and applied without fully understanding the underlying endogenous EFs.

We are left, then, with the conclusions that our research, especially when taken in the context of previous research by countless others, supports our hypotheses that EFs regulate wound repair in the mammalian CNS, and that this is an example of the universally conserved role of EFs in regulating morphogenesis throughout all metazoans. Based on these conclusions, further research is needed to identify differences in injury-induced EFs that correspond to differences in regenerative potential. Future projects are also needed to explore the physiologic mechanisms through which EFs are transduced, as this will provide insight into the specific physical attributes of bioelectricity to which cells respond. Once identified, differences in the expression of these physiologic pathways among species can be used to develop insight into the mechanisms underlying how certain species lost evolved the loss of spontaneous EF-induced regeneration. A more detailed understanding of the evolutionary role of EFs in morphogenesis is essential to the development of an optimal EF-based therapy to promote regeneration and thus would the translation of this approach to a clinical application.
Figure 5.1: Distribution of epimorphic regeneration throughout phylum Vertebrata

This figure illustrates the phylogenetic relationship among extant vertebrate clades. The expression of epimorphic regeneration among these groups based on published scientific literature is indicated by the colors to the left of each class: green indicates that there is at least one species within the group that demonstrates epimorphic regeneration, red indicates that epimorphic regeneration has been repeatedly shown to be absent, and black indicates an absence of evidence either way. (Note, mammals are listed as lacking epimorphic regeneration even though evidence discussed in chapter 1, page 22.) These data are based principally on a review by Bely and Nyberg, 2010\textsuperscript{133}. 
Figure 5.1: Distribution of epimorphic regeneration throughout phylum Vertebrata
Figure 5.2: Evolutionary origins of epimorphic regeneration among vertebrates

Phylogenetic tree illustrating two competing hypotheses about the origins of epimorphic regeneration in vertebrates based on the expression of epimorphic regeneration among extant vertebrate clades as illustrated in Figure 5.1. Green lines indicate a lineage capable of epimorphic regeneration, red lines indicates a lineage with evidence of absent epimorphic regeneration, and black lines indicate a lineage where no observations have been made. (A) Epimorphic regeneration was present within the common vertebrate ancestor and was retained throughout evolution except for two clades in which it was (mostly) lost. (B) Epimorphic regeneration was not present in the common vertebrate ancestor, so each vertebrate clade in which regeneration is observed evolved it independently. Assuming that epimorphic regeneration arose immediately upon the regenerative clade diverging from a common ancestor shared with either Mammalia or Aves, it would have had to have evolved convergently on at least seven separate occasions. The principle of parsimony as described by Occam’s razor suggests that the single evolutionary origin of epimorphic regeneration as posited in (A) is more probable than the convergent evolution of epimorphic regeneration through consistent molecular and cellular events that emerged through at least seven independent events as posited in (B). These data are based principally on a review by Bely and Nyberg, 2010\textsuperscript{133}. 

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Figure 5.2: Evolutionary origins of epimorphic regeneration among vertebrates
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Appendix 1: Miscellaneous Protocols

Solutions

General solutions procedure

Note: this protocol assumes 1L of final solution; adjust accordingly
1. Fill a 1L beaker to 900 mL with deionized water (for solutions with 1L final volume)
2. Add a stir bar and stir a 300 RPM (approximately)
3. Weigh out solids with accuracy of ± 1% and add to the beaker of water
4. Raise the fluid volume to 950-970 mL with deionized water and continue to stir until solution is clear
5. Adjust to specified pH
6. Transfer to a volumetric flask and fill to 1L line with deionized water
7. Clearly label container with contents, date made, initials of the person that made it, and pH (only once it has been measured)
Using the pH meter

Based on the instruction manual for the Accumet Basic AB15 pH meter and Accumet Gel-Filled Electrode

General protocol for using the pH Meter

- The pH meter should always be stored in electrolyte storage solution and must not dry out
- Never wipe the glass bulb within the electrode; wiping the electrode may scratch the glass tip, which will render it useless
- pH should be measured while the solution is being gently stirred on a stir plate

1. Press “std” on the Accument pH meter to turn on the display
2. Carefully remove the electrode from the electrolyte storage solution
3. Rinse the electrode with deionized water, blot (do not wipe!) the bottom with a Kim wipe, and wipe the sides of the pH meter
4. Immerse the electrode into the solution, being careful not to hit the electrode tip with the stir bar.
   - The pH meter will measure pH continuously. Measurements are complete when “STABLE” appears.
   - If calibrating the electrode, see instructions below for additional information at this point
5. Add HCl or NaOH drop-wise until the pH adjusts to the desired range
6. Rinse the electrode with deionized water (per step 3) after measuring each solution and before returning it to the electrolyte solution
7. When finished using the pH meter, press “stdby”

Calibration of the pH Meter

- For accurate pH measurements, it is recommended that calibration be done with every use.
- Calibration settings are saved between uses and must be cleared before beginning calibration

1. To clear the previously-stored standards:
   - Press setup to view the %slope of the standard curve
   - Press setup again to bring up the clear BUFFER icon and press enter to clear all existing buffers
2. Immerse the electrode into the buffer solution while stirring the solution moderately
3. Press “std” on the pH meter; the display will show a group of buffers.
• If the wrong buffer series is displayed, return to the main screen and press setup until the correct buffer series appears, then press enter to select it.

4. Press “std” a second time to initiate the standardization. The pH meter will recognize the buffer and return to the measure screen.

• Repeat the calibration with each of the buffered pH standards (pH = 4.01, 7, 10.01)

• At least two buffers must be used to establish the pH standard curve

5. The slope should be between 90-102% and a value in this range will return “Good Electrode”

• A slope reading outside of this range will return “Electrode Error”

• The electrode can still be used when “Electrode Error” is present

• Clear buffer data cache (per step 1) to reset the electrode error and re-calibrate the electrode

• The most common cause of an electrode error is old buffer that needs to be replaced
Phosphate-buffered saline

Note: this protocol was modified from Cold Spring Harbor Protocols

10x Phosphate-Buffered Saline (0.1M)

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
<th>pH = 7.4</th>
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<tbody>
<tr>
<td>pH = 7.4</td>
<td>1L</td>
<td>1L</td>
</tr>
<tr>
<td>Na₂HPO₄ (MW: 141.96)</td>
<td>6.78g</td>
<td>13.56g</td>
</tr>
<tr>
<td>NaCl</td>
<td>40g</td>
<td>80g</td>
</tr>
<tr>
<td>KCl</td>
<td>1g</td>
<td>2g</td>
</tr>
<tr>
<td>KH₂PO₄</td>
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<td>2g</td>
</tr>
</tbody>
</table>

Note: Sodium phosphate dibasic comes in many different hydrated forms; this recipe assumes that the salt is anhydrous; if hydrated salts are used, the amount of sodium phosphate added (by weight) will need to be increased in order to keep the resulting molarity the same.

Autoclave the 10x Phosphate-Buffered Saline Solution (40 minutes) to sterilize it. The mixture of salts will prevent them from crystallizing.

The following recipes are based on 10x Phosphate Buffer and 10x Normal Saline being prepared in separate containers. 10x phosphate buffer tends to crystallize out of solution, so the above recipe for 10x Phosphate Buffered Saline is preferable.

10x Phosphate Buffer (0.1 M)

<table>
<thead>
<tr>
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<th>Amount</th>
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<tbody>
<tr>
<td>pH = 7.4</td>
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</tr>
<tr>
<td>Sodium Phosphate</td>
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<tr>
<td>Sodium Chloride</td>
<td>90g</td>
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<tr>
<td>KCl</td>
<td>2g</td>
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<tr>
<td>KH₂PO₄</td>
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1x Phosphate-Buffered Saline (0.01 M)

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<tr>
<td>10x Phosphate Buffer</td>
<td>100 mL</td>
</tr>
<tr>
<td>10x Normal Saline</td>
<td>100 mL</td>
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</tbody>
</table>

10x Normal Saline (9%)

<table>
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<tr>
<td>Sodium Chloride</td>
<td>90g</td>
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1x PBS with 0.1% Triton X-100

<table>
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<th>Amount</th>
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<tbody>
<tr>
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<td>1L</td>
</tr>
<tr>
<td>10x Phosphate Buffer</td>
<td>100 mL</td>
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<tr>
<td>10x Normal Saline</td>
<td>100 mL</td>
</tr>
<tr>
<td>Triton X-100</td>
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</tbody>
</table>
4% paraformaldehyde

Note: Only use glassware and equipment that is labeled as “Para”; this equipment is stored on a cart to the right of the chemical fume hood. Keep these segregated from general purpose lab-ware as trace fixatives have been known to cross-contaminate and affect other experiments.

For 100 mL made from paraformaldehyde powder
1. Place a 250 mL beaker with a magnetic stir bar on the heater-mixer in the chemical fume hood. Add:
   a. 4 grams of paraformaldehyde (weighed in the hood)
   b. 90 mL deionized water
2. Mix while heating the solution (heat setting approximately 5.5); stir until dissolved
3. DO NOT heat above 60°C as this may result in the formation of formic acid
4. Add 1M NaOH drop-wise to the solution to help the paraformaldehyde dissolve
5. Add 10 mL 1.0M Phosphate Buffer while continuing to mix
6. Filter entire solution through a paper funnel into a flask
7. Adjust the pH to 7.4
8. Refrigerate or chill to room temperature

For 40 mL made from 16% paraformaldehyde ampules (specifically for fixation for electron microscopy)
1. Add 1 ampule (10 mL) of 16% paraformaldehyde to a marked container.
2. Rinse out the ampule with filtered 1x 0.1M PBS (to remove any crystals that may have formed) and add to the container with 16% paraformaldehyde. Add 30 mL total PBS for a total concentration of 4% paraformaldehyde.
3. Mix thoroughly before use.

- Paraformaldehyde solution is allegedly stable for a week once prepared and is ideally made fresh less than 24 hours prior to use.
- Paraformaldehyde is a regulated waste; collect it in a marked waste container. DO NOT DISPOSE DOWN THE DRAIN.
- Collect solid paraformaldehyde waste (e.g. weigh-boats, contaminated gloves) in a marked container that can be sealed (e.g. empty chemical bottle; sturdy plastic bag, but not one of the red biohazard bags)
Artificial cerebrospinal fluid

### 10x aCSF stock

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<thead>
<tr>
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<th>mM</th>
<th>Compound</th>
<th>grams in 1 Liter (for 10x)</th>
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<tbody>
<tr>
<td>58.44</td>
<td>126.0</td>
<td>NaCl</td>
<td>73.634</td>
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<tr>
<td>74.55</td>
<td>3.0</td>
<td>KCl</td>
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</tr>
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<td>137.99</td>
<td>1.25</td>
<td>NaH₂PO₄ • H₂O</td>
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<td>203.30</td>
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<td>MgCl₂ • 6H₂O</td>
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<tr>
<td>147.02</td>
<td>2.0</td>
<td>CaCl₂ • 2H₂O</td>
<td>2.94</td>
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<tr>
<td>180.20</td>
<td>10.0</td>
<td>Glucose</td>
<td>18.02</td>
</tr>
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### 10x Sodium Bicarbonate stock

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<th>grams in 1 Liter (for 10x)</th>
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<td>84.01</td>
<td>26.0</td>
<td>NaHCO₃</td>
<td>21.842</td>
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</table>

**Note:** The concentrations (mM) listed are those for the 1x working solution, **not** for the 10x stock.

To make a working solution, dilute equal volumes of the two stock solutions 1:10 (e.g. for 1 Liter of 1x solution, mix 100 mL 10x aCSF with 100 mL 10x sodium bicarbonate stock solution, and dilute with deionized water to a final volume of 1 Liter)

Note: the solution tends to form a precipitate after several hours, so make it fresh daily.
- The precipitate can be re-dissociated into solution by mixing the solution over gentle heat and bubbling oxygen

Note: this protocol was given to us from the Jacobs lab; it does not specify a pH for the solution.
**Borate buffer**

Used as part of the antigen retrieval protocol for BrdU immunostaining

**0.1M Borate Buffer:**

- 3.81 grams sodium tetraborate (Borax) per 100 mL deionized water

Mix solution until Borate dissolves
Adjust pH to 9.0

Note: Sodium Tetraborate is not acutely toxic, but it can cause respiratory and skin irritation in large quantities. Accordingly, measure it out and dissolve it in a chemical fume hood. Collect waste and dispose of it through OEHS.
Cell culture protocols

Coating culture flasks

Powdered Poly-L-Lysine Hydrobromide (Sigma P1524-25mg) Store at -20°C
• For astrocytes, recommended 2 µg / cm² (per ScienCell)

1. Dissolve 25 mg (entire contents of vial) in 41.6 mL of sterile de-ionized water to make 600 µg / mL (40x) stock solution.
2. Thoroughly-vortex, then use a 60 mL syringe to sterile-filter the solution through a Millex-GP filter unit (Ref # SLGP033RS) into a sterile 50 mL plastic centrifuge tube
3. Add 250 µL of 40x solution and 10 mL of sterile deionized water to a T-75 and gently tilt to evenly distribute
4. Store remaining solution in 250 µL aliquots in sterile microcentrifuge tubes at -20°C

Liquid Poly-L-Lysine (10 mg / mL) (ScienCell 0413) Store at -20°C
1. Add sterile water and poly-L-lysine to flask
2. Gently tilt to evenly distribute

<table>
<thead>
<tr>
<th>Water (mL)</th>
<th>Poly-L-Lysine (µL)</th>
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<tr>
<td>T-25</td>
<td>5</td>
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<tr>
<td>T-75</td>
<td>10</td>
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<tr>
<td>T-175</td>
<td>13</td>
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</tbody>
</table>

5 (10 mg / mL)
15 (10 mg / mL)
30 (10 mg / mL)

1x working concentration of poly-L-Lysine for astrocytes: 15 µg / mL (per ScienCell)
1. Once poly-L-Lysine has been added to the culture flask, return it to a 37°C incubator overnight (at least 1 hour)
2. Rinse the poly-L-lysine coated flask with sterile water twice and allow it to dry before adding culture media and cells.
Thawing astrocytes

- ScienCell estimates $7.5 \times 10^5$ cells in each vial, 90% ($6.75 \times 10^5$) survive the thaw

1. Remove cryogenic vial containing astrocytes from freezer, transfer it to a 37°C water bath to thaw
   - Gently mix vial as it thaws
   - Do not allow cap to be submerged in water; wrap in parafilm to protect cap if necessary

2. Transfer the cells directly to a poly-L-Lysine coated culture flask containing astrocyte media
   - 20 mL for a T-75, 7 mL for a T-25
   - Poly-L-lysine provides helps astrocytes adhere to the dish
   - Do not disturb the dish for 16 hours
   - Thawed cells are fragile; do not re-suspend, centrifuge, or otherwise manipulate cells before adding them to the culture flask, as these actions are more harmful to the cells than the effect of DMSO residue in the culture. Return them to the culture as quickly as possible with minimal handling.

3. Gently tilt flask to distribute cells evenly

4. Place cap on the flask and return to the incubator
Set up Initial Culture after Receiving the Order

**Prepare poly-L-lysine coated flask** (recommended 1 day before thawing cells)
1. 2 µg/cm², T-75 flask is recommended
2. Add 10 mL of sterile water to a T-75 flask
3. Add 15 µL of poly-L-lysine stock solution (10 mg/mL)
4. Leave the flask in the incubator overnight (minimum one hour at 37°C)

Prepare the Complete Medium
1. Decontaminate the external surfaces of all containers with 70% Ethanol
2. Transfer sterilized containers to sterile field
3. Aseptically add each supplement to the basal medium with a pipette.
4. Rinse each tube with medium to recover the entire volume

Take the poly-L-lysine coated flasks:
1. Rinse the poly-L-lysine coated flask with sterile water twice.
2. Add 20 mL of complete medium to the flask.

**Seeding frozen cells** (recommended density of 5,000 cells/cm²)
1. Place vial in 37°C waterbath, hold and rotate the vial gently.
2. Remove the vial from the waterbath as soon as they thaw completely.
3. Wipe vial down with 70% Ethanol and place in sterile field.
4. Remove cap, being careful not to touch the interior threads with fingers
5. Resuspend the vial contents carefully using a 1 mL eppendorf pipette
6. Dispense the contents into the equilibrated, poly-L-lysine coated flask
7. Replace the cap or cover, and gently rock the vessel to distribute cells evenly. Loosen cap if necessary to permit gas exchange.
8. Return the culture vessels to the incubator (for best results, do not disturb for at least 16 hours)

**Maintenance of the Culture**

Frequency of Changing the culture Medium
1. **1 day after seeding**, change the medium to fresh supplemented medium to remove residual DMSO and unattached cells
2. **Until 70% Confluent**: Change medium every 3 days
3. **Once 70% Confluent**: Change medium every 2 days
4. Once 90% Confluent: Split cells

Characteristics of Healthy Cells:
- Polygonally shaped sheets of contiguous cells
- Cell number doubles after 2-3 days in culture

Subculture (i.e. split cells) when 90% Confluent
Acid-washing slides & coverslips

**Purpose:** Acid washing cleans glass to prepare it for cell culture; do not assume that it is ready for cell culture
- Acid washing does not need to be completed in a sterile environment

**Protocol:**
1. Assemble glass on a rack that can hold
2. Soak glassware in alconox for 15 minutes
3. Rinse slides 3 times in dH$_2$O
4. Soak 20 minutes in 1N HCl
5. Rinse 3 times in dH$_2$O
6. Soak 20 minutes in 70% ethanol
7. Sterilize by either:
   - Place under UV light in sterile laminar flow hood until dry, at least 15 minutes
   - Autoclave

If glassware was previously contaminated with a hydrophobic substance, e.g. vacuum grease or wax:
- Clean slides by using a dehydration in ethanol (50%, 70%, 95%, 100%)
- Soak 2-3 times in xylene
- Rehydrate in ethanol (100%, 95%, 70%, 50%) then water
- Continue with acid wash starting at alconox above

Coating culture plates with fibronectin

ScienCell Catalogue # 8248 (1 mg / mL) Store at -20°C
1. Dilute Fibronectin in sterile 1x PBS
   - For astrocytes, 10 μL Fibronectin (1 mg / mL) per 1 mL PBS
2. Coat the culture surface with a minimal volume
3. Incubate at room temperature for 2 hours OR 2-8°C overnight.
4. Aspirate remaining fibronectin solution and rinse with deionized water. The culture vessels are now ready to use.
- We coat glass culture substrates in fibronectin for astrocytes
- Store in aliquots to minimize number of freeze-thaw cycles to which the Fibronectin is subjected
**Sub-culturing astrocytes**

Prepare Culture Dishes:
- Company recommends adding 2 µg / cm² poly-L-Lysine to culture flasks
- Company recommends warming all media to room temperature (not to 37°C with a water bath)

**Necessary components of media** (warmed to room temperature)
- Trypsin/EDTA solution
- HBSS (Hank’s Balanced Salt Solution)
- FBS (Fetal Bovine Serum)
- Trypsin Neutralization Solution (can be made from 10% FBS in HBSS)

Items to prepare prior to beginning:
- Coat new culture plates in appropriate substrate
- Clearly-labeled cryogenic vial (if freezing cells)
- Add 5 mL of fetal bovine serum (FBS) to a 50 mL conical tube (to receive the cells)
- Ensure 10 mL of trypsin neutralization solution is prepared at room temperature
  - 1 mL FBS
  - 9 mL HBSS
- Bring HBSS, Astrocyte media, FBS, Trypsin, and Trypsin-Neutralization Solution to room temperature
- Add astrocyte media to all new culture plates and place in the incubator

Dissociate astrocytes from a T-75 culture plate (adjust volumes for other plates)
1. Remove media from culture plate
2. Rinse cells in HBSS (remove serum-containing media)
3. Add **8 mL HBSS** to the flask, followed by **2 mL** of 0.25% Trypsin/EDTA solution
   a. Gently rock flask to ensure that cells are covered
   b. Incubate the flask in a **37°C** incubator for **2 minutes** (or until cells are completely rounded up)
4. Use 10 mL serological pipet to wash the plate with the Trypsin-containing media
   2-3 times to remove cells, then transfer the media to the 50 mL conical tube containing 5 mL FBS
5. Return the culture plate to the incubator (no solution in the flask). After **1-2 minutes**, gently tap the flask to detach remaining cells from the plate
6. Wash the plate twice with **5 mL** of Trypsin Neutralization Solution (TNS) to harvest the residual cells and transfer the solution to the 50 mL conical tube
7. Examine the flask under the inverted microscope to make sure that the cell harvesting was successful; if not, wash with additional TNS or add additional Trypsin as necessary; fewer than 5% of the cells should remain.

8. Centrifuge the 50 mL centrifuge tube at 1000 rpm for 5 minutes, remove the supernatant, and gently re-suspend the cells in 1 mL of astrocyte media.

9. Determine the concentration of cells using a hemocytometer. Then, either:
   a. Pipet the appropriate number of cells into each new culture plate
   b. Dilute the cells to a constant volume (e.g. 1x10^5 cells per mL)

**NOTE:**
- Estimated number of cells on a 100% confluent T-75: 2 million (26,500 cells per cm^2)
- 5 x 10^5 cells will produce an estimated 25% confluence
- Doubling time for astrocytes is estimated at 2-3 days (per ScienCell)

**Troubleshooting:** Cells not detaching from plate:
- Try new Trypsin; trypsin activity can decrease on repeated freeze-thaw cycles. Ideally, it should be stored in 1 or 2 use aliquots after initially thawed.
- Forcefully re-wash / pipet liquid over cells to dislodge them
- Pre-rinse cells with HBSS to remove serum-containing media prior to adding trypsin (serum competitively inhibits trypsin activity)
- Do NOT expose cells to trypsin for too much time; 2 minutes is sufficient.
Hemocytometer

Each square of a hemocytometer, with coverslip in place, represents a total volume of $0.1 \text{ mm}^3 (0.1 \mu\text{L})$

1. Seat coverslip onto the hemocytometer using a small amount of water along the edges to hold the coverslip firmly down
2. Make sure the cells are evenly distributed in the solution by gentle pipetting
3. Place 10 $\mu\text{L}$ of the cell suspension in the grooves of the hemocytometer (figure 1)
4. Place the hemocytometer on the stage of a microscope
5. Use a 10x objective with either phase contrast or DIC to focus on the central grid.
6. Count cells in the central 1 x 1 mm grid of the hemocytometer (figure 2); to avoid repeat counting, count cells touching the top or right lines of the hemocytometer, but not those touching the bottom or left lines.
7. Move backward and forward across the 5x5 grid of the hemocytometer to ensure that all cells are counted.
   - Depending on cell counts, either count the grids marked 1–5 (figure 2), or count all of the grids.
   - For accuracy, at least 100 cells should be counted.
8. To calculate the cells per mL:
   - If only a subset of squares in the grid were counted:
     \[
     \text{cell density} = \text{average cell count per square} \times 25 \times 10^4 \text{ cells per mL}
     \]
   - If all the squares in the grid were counted:
     \[
     \text{cell density} = \text{cell count} \times 10^4 \text{ (per mL)}
     \]

Hemocytometer layout

Example of cell counting with a hemocytometer
**Freezing astrocytes**

1. Suspend cells to be frozen in **800 µL of astrocyte media** and transfer to a labeled cryogenic vial
2. Add 100 µL FBS
3. Add **100 µL of sterile DMSO**
4. Close cryogenic vial and freeze in liquid nitrogen as quickly as possible
   - If possible, transfer to -80°C for 24 hours prior to transferring to liquid nitrogen; this theoretically limits damage to cells on freezing

   Note: DMSO damages cells; minimize time between adding it to the cells and freezing the vial

**Fixing adherent cell cultures**

**Preparation for Immunocytochemistry**

1. Wash culture 2-3 times with isotonic serum-free media
   - Washes should be gentle to minimize the risk of washing cells off of the culture plate
   - Goal of washes is to remove soluble proteins from the culture media, as these proteins can contribute to non-specific antibody binding.
2. Add 4% paraformaldehyde in 0.1M PBS (pH = 7.4) for 15-30 minutes
3. Wash off paraformaldehyde and replace with 0.1M PBS
   - Add 0.05% sodium azide to the PBS if cells will be stored for a prolonged period of time to minimize bacterial growth
4. Tightly wrap the culture dish with parafilm and store at 4°C until ready to use
Immunostaining

BrdU Cell Proliferation Assay

BrdU Preparation:
- From Solid BrdU (Sigma B5002): BrdU is difficult to dissolve; use 0.007N NaOH, place it into a heated sonicator, and vortex frequently.
- From Liquid BrdU stock (Invitrogen 000103): Dilute 1:100 in culture media
  Note: BrdU solution will need to be sterile-filtered before use

Protocol
5. Change culture media and add media containing BrdU; wash a second time to ensure the appropriate concentration of BrdU is achieved
6. Incubate cells for desired period of time
7. Wash off BrdU-containing media and replace with fresh media
8. Fix cells in 4% paraformaldehyde for at least 30 minutes
9. Immunolabel

Antigen retrieval for BrdU staining

This protocol is used for both tissue sections and cell staining

Solutions required:
2N HCl (Fisher SA-431)
1N HCl: Dilute 2N HCL in deionized water (1:1)
0.1M Borate Buffer: dissolve 3.81 g sodium tetraborate (Borax) per 100 mL deionized water, adjust pH to 9.0
- HCl and Borate Buffer are regulated wastes. Collect them in individual waste jars for disposal through OEHS.

Procedure
1. 1N HCl for 10 minutes on ice
2. 2N HCl for 10 minutes at room temperature
3. 2N HCl for 20 minutes at 37°C (place in an oven)
4. 0.1M borate buffer for 10 minutes at room temperature
5. Begin washes in PBs with 0.1% Triton X-100 per the beginning of normal immunostaining
Immunocytochemistry

Solutions Required:
- 0.1M PBS
- 0.1M PBS + 1% Triton X-100 (10 µL Triton per mL PBS)

**Blocking Solution**: 4% NGS, 0.5% BSA, 1% Triton-X100 in 0.1M PBS

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<tr>
<th>Ingredient</th>
<th>1 mL</th>
<th>5 mL</th>
<th>10 mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal Goat Serum (NGS)</td>
<td>40 µL</td>
<td>200 µL</td>
<td>400 µL</td>
</tr>
<tr>
<td>Bovine Serum Albumin (BSA)</td>
<td>5 mg</td>
<td>25 mg</td>
<td>50 mg</td>
</tr>
<tr>
<td>Triton X-100</td>
<td>10 µL</td>
<td>50 µL</td>
<td>100 µL</td>
</tr>
</tbody>
</table>

Primary Antibody Staining (Day 1)
1. 0.1M PBS: 3 washes for 5 minutes each
2. Blocking solution: 30 minutes
3. Primary antibody (diluted in blocking solution)
   • Incubate either for 2 hours at room temperature, or overnight in the refrigerator
   • To avoid cells drying out if using a small volume of antibody solution, cover in a piece of parafilm
   • If using parafilm, float it off with media to remove

Secondary Antibody Staining (Day 2)
1. 0.1M PBS: 3 washes for 5 minutes each
2. Secondary antibody (diluted 1:200 in PBS): 2 hours
3. 0.1M PBS: 3 washes for 5 minutes each

Nuclear Stain (if using)
• DAPI (1:10,000 diluted from 1 mg/mL stock): 15 minutes
• Bis-Benzimide (1:10,000 from 10 mg/mL stock): 1 minute
• DAPI NucBlue (2 drops/mL; from Molecular Probes R37606): 5 minutes
• 0.1M PBS: 3 washes for 5 minutes each after the nuclear stain

Coverslip
1. Deionized: 3 washes for 5 minutes each
   • This helps to remove the salt from the buffer and prevents the formation of salt crystals
2. Use vectashield as a mounting media
Appendix 2: Record keeping and Checklists

Cell culture experiments can generate extraordinary numbers of samples. Thorough records will facilitate identifying samples in the lab, confirm results, and locate information. Each sample should have a unique, unambiguous, understandable, and concise identifier associated with it that corresponds to a full set of records that is kept either in hard copy or, preferably, digitally where all of the details about the experimental conditions are recorded. For example, labeling specimens as E1, E2, E3, C1, C2, C3 for “experiment 1” (etc) is ambiguous: it only provides information about how the samples are related to each other without providing any other information. However, labeling each sample with its full set of records is absurdly onerous and unnecessary. A necessary compromise is a project name, an experiment number, the treatment group, the replicate number, the date, and the initials of the experimenter. This way, no two samples from a single experiment can be confused with each other, samples from each experiment can be readily identified, and there is sufficient information to look up the records associated with these experiments either by date or by project.

Considerations in devising a method for record-keeping should include the human elements involved. Hand-written records may be considered traditional, but digital records do not suffer from being incomprehensible due to hand-writing and they can easily re-printed in the event a hard copy version is damaged. Digital records are more readily backed-up, and they can also be easily accessed from remote locations.
Moreover, digital records simplify and expedite duplicating lengthy information about records, such as protocols with minor changes. And, most importantly, computers can search through digital records for keywords far more expeditiously than an experimenter can leaf through hard copy records.

Here, I will briefly describe my own record keeping system based on the previously-stated requirements. As my experiments were focused on cell culture, the following records are organized to reflect this. I devised standard forms for each level of record keeping, and arranged them hierarchically so that I could readily find the information I needed. Each cell line or genotype is referred to by a unique lot number, and the Cell Culture Record Sheet is used to keep track of each dish associated with that genotype; these records are used in real-time to help keep track of ongoing cell culture experiments, and they are also used as reference markers to try and recreate the history of certain cells if there are possible anomalies with analysis of experimental data.

Each project is given a name, and each experiment (full set of treatment groups) is assigned a unique number. A single Project Record Sheet provides an overview of ongoing progress with each experiment, tracks experimenter participation in these projects for subsequent attribution of credit (i.e. determining authorship on publications). Each experiment listed in this log has a corresponding Experiment Record Sheet, in which details of the treatment groups, the protocol, and the history of the experiment are kept. For experiments with multiple replicates, each replicate is listed on its own line and has a corresponding \textit{In Vitro} Staining Record (for ICC, other sheets can be composed for different types of studies); this staining record sheet includes the full staining
protocol and records of the procedure. Importantly, these record sheets include checklists for each experiments, allowing the experimenter to have access to the protocol in real time during the experiment and also providing a tangible record that each step of the procedure occurred; it also provides explicit instructions for each solution used within the experiment, minimizing experimental error due to mistakes in preparing solutions.

The hierarchical organization of these records can (and should) also be used for storing these records, both digitally and in hard copy. For example each Project Record Sheet should be assigned its own folder; each experiment within a project should be assigned a sub-folder in which the staining records for each replicate are stored. This organization ensures that all records for a single project are stored in one place and thus that they are readily found. Digital images can be stored in folders on a computer, with a hierarchical structure matching the organization of hard-copy records. (Another note on digital images: each folder and each image should be labeled according to the same strict guidelines established for samples. Do NOT label images as “image 1” because you will certainly forget everything about that particular image no matter how hard you try not to.)
### Cell Culture Record

<table>
<thead>
<tr>
<th>Date</th>
<th>Split #</th>
<th>Sub-Population</th>
<th>Plating Conditions</th>
<th>Media Changes</th>
<th>Split Date</th>
<th>Experiment</th>
<th>Notes</th>
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**Split #** Indicates the number of times cells were split since initial culture (i.e. 0 indicates initial thaw or culture, 1 = first split, etc.)

**Sub Population** (e.g. 1.3.2(01-12)): *numbers* represent sub-population, *decimal* indicate splits, *numbers in parentheses* indicate duplicates made for experiments.

- 1.3(01-12): 1*st* sub-population when initially thawed.
- 1.3(01-12): 3*rd* sub-population after the first split (first split indicated by the decimal), and these cells were plated into 12 equal conditions (e.g. 12 coverslips).
- 1.2-2: The dash-mark indicates that the second sub-population after the first split was frozen, and this is the second sub-population from when it was thawed.

**Plating Conditions:** Indicate both culture vessel and substrate (e.g. T75/PLL, Frozen, Glass-Bottom Dish/FN, etc.).

**Media Changes:** Include dates and the person that changed the media.

**Split Date:** Indicate the date that this population was split; there should be subsequent line(s) with corresponding dates for the sub-cultures made.

- If cells were frozen, split date indicates date of thaw.

**Experiment:** Indicate the experiment that the sub-population is intended for. If the cells are intended only to grow the population, leave blank.
## Project Record

**Project Name:**

**Goals:**

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<th>End Date</th>
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### Overview of Experiments

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<td>20.</td>
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</tbody>
</table>

**Notes:**

**Purpose:** Brief Description of experimental goals; include "rep. exp#" if it is to repeat a previous experiment

**Completed:** Check box only when experiment and analyses are finished

**Outcome:** e.g. "Analyzed" or "Failed" or "Must Repeat"
# In Vitro Experiment Record

**Project:**

<table>
<thead>
<tr>
<th>Investigator(s):</th>
<th>Experiment #:</th>
<th>Replicates:</th>
<th>Dates:</th>
<th>Project:</th>
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<tbody>
<tr>
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**Cell Type:**

<table>
<thead>
<tr>
<th>Cell Lot #:</th>
<th>Media:</th>
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**Cell Lot #:**

**Sub-ID #:**

**Time Lapse:**

<table>
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<th>Coating:</th>
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<td>Uncoated</td>
</tr>
</tbody>
</table>

**Purpose:**

**Groups:**

**Design:**

**Analysis:**

**Other Comments:**

## Culture Records

<table>
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<tr>
<th>Date</th>
<th>User</th>
<th>Manipulation</th>
<th>Comments</th>
</tr>
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<tbody>
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## Purpose for Each Replicate

<table>
<thead>
<tr>
<th>ICC / Western / Other</th>
<th>Purpose</th>
<th>Stained</th>
<th>Imaged</th>
<th>Analyzed</th>
<th>Outcome</th>
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<tr>
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</table>

Indicate dates of staining, imaging, and analysis

**Experiment Comments:**

**Overall Outcome:**

---

## In Vitro Experiment Record

**Results**

<table>
<thead>
<tr>
<th>Investigator(s):</th>
<th>Experiment #:</th>
<th>Replicate #:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1</td>
</tr>
</tbody>
</table>

For each result, indicate date(s) of analysis, methods used for analysis, outcome of any statistical tests, interpretation, and plan.
In Vitro Staining Record

Project: 
Investigator(s): 
Experiment #: 
Replicate #: 
Dates: -

Antibody Information

<table>
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<tr>
<th>Antigen</th>
<th>Host</th>
<th>Isotype</th>
<th>Dilution</th>
<th>Secondary</th>
<th>Label</th>
<th>Dilution</th>
<th>Host</th>
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</table>

Immunocytochemistry Protocol

<table>
<thead>
<tr>
<th>BrdU</th>
<th>Secondary</th>
<th>Nuclear Stain</th>
</tr>
</thead>
<tbody>
<tr>
<td>Y / N (If no, skip to Primary)</td>
<td>3x PBS washes (5 min)</td>
<td>None</td>
</tr>
<tr>
<td>1N HCl x 10 min (on ice)</td>
<td>1. 2. 3.</td>
<td>DAPI NucBlue (2 drops per mL, Molec. Probes R37606) 5 min</td>
</tr>
<tr>
<td>2N HCl x 10 min (25°C)</td>
<td>2. 3.</td>
<td>Bix-Benzimide (1:10,000 from 10 mg/mL) 1 min</td>
</tr>
<tr>
<td>2N HCl x 20 min (37°C)</td>
<td>2. 3.</td>
<td>DAPI (1:10,000 from 1 mg/mL) 15 min</td>
</tr>
<tr>
<td>0.1M Borate Buffer x 10 min (25°C)</td>
<td>2. 3.</td>
<td></td>
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</table>

<table>
<thead>
<tr>
<th>Primary</th>
<th>3x PBS washes (5 min)</th>
<th>Nuclear Stain (Y / N)</th>
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</thead>
<tbody>
<tr>
<td>3x PBS washes (5 min)</td>
<td>1. 2. 3.</td>
<td></td>
</tr>
<tr>
<td>1. 2. 3.</td>
<td>2. 3.</td>
<td></td>
</tr>
<tr>
<td>Block (30 min, 25°C)</td>
<td>3x PBS washes (5 min)</td>
<td></td>
</tr>
<tr>
<td>Per 1 mL 0.1M PBS:</td>
<td>1. 2. 3.</td>
<td></td>
</tr>
<tr>
<td>4% NGS (40 µL)</td>
<td>2. 3.</td>
<td></td>
</tr>
<tr>
<td>1% Triton (10 µL)</td>
<td>3x dH₂O (5 min)</td>
<td></td>
</tr>
<tr>
<td>0.5% BSA (0.05g)</td>
<td>1. 2. 3.</td>
<td></td>
</tr>
<tr>
<td>Primary (diluted in block)</td>
<td>Coverslip</td>
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</tr>
<tr>
<td>2 Hrs (25°C) / Overnight (4°C)</td>
<td>Mounting Media</td>
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</table>

Imaging

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<td>Label 3</td>
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<tr>
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General Imaging Comments:

Analysis

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<th>Results</th>
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<tbody>
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<td>Program &amp; Method</td>
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</table>

Outcome:

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Appendix 3: Data manipulation and analysis using R

Overview of using scripts in R

R is a powerful statistical language that is open source and widely supported. Data analysis in R involves writing scripts, which are algorithms involving step-by-step instructions that the software uses to analyze data and plot graphs. Accordingly, once a script is written to perform an analysis, new datasets can be analyzed (or old datasets can be re-analyzed) in exactly the same way by running the new data through the old script. This ensures that the same statistical tests are run in the same way on every dataset that you analyze. It also means that, after a script is initially developed, subsequent data analysis takes only as long as your computer takes to process the data.

R does require data to be stored in particular ways in order for it to read files correctly. All of the data files used in the scripts that I have developed should be .csv files, and the first row of the dataset should be the titles of each column. The easiest way to compile .csv files is with Microsoft Excel or any other spreadsheet-based program; enter the data, and then choose file -> Save As and choose “.csv” from the menu. The .csv files also need to be structured in the same way, with the same names for each column; names are case sensitive. A brief description of how to compile data to used by each script is found at the start of that section.
R Scripts for Chapter 2

Optimizing the measuring interval for tracking cell migration

Scripts are organized in the same fashion as those for the migration analysis.

See description found on page 305 for more information.

```r
library(ggplot2)
library(reshape)
library(multcomp)
library(car)
library(nlme)

### Load and sort data -----------------------------------------
# as of 6-26-15, use data files labeled
# Tracks.csv and Points(6-26-15).csv
points1 <- read.csv(file.choose(), header = TRUE)
points1 <- points1[!is.na(points1$vel),
   c("EF",
      "Position",
      "FrameInterval",
      "TrackID",
      "PointID",
      "hours",
      "vel",
      "dir"
   )]
points1$FrameInterval <- factor(points1$FrameInterval)
points1$EF <- factor(points1$EF)
levels(points1$EF) <- paste(levels(points1$EF),
   "mV/mm"
)

tracks1 <- read.csv(file.choose(),
   header = TRUE)

tracks2 <- tracks1
names(tracks2)[c(4:8)] <- c(3, 6, 15, 30, 60)

tracks2 <- melt(tracks2,
   id = c("EF",
      "Position",
      "TrackID"
   )
)```
variable_name = "interval"
)
tracks2$interval <- factor(tracks2$interval,
    levels(tracks2$interval),
    ordered = TRUE
)

tracks2$EF <- factor(tracks2$EF)
levels(tracks2$EF) <- paste(levels(tracks2$EF),
    "mV/mm"
)

# mixed effects model -----------------------------
t2 <- slh
t2$id <- paste(t2[,1],t2[,2],t2[,4], sep=".")

# random effects model and post-hoc test for 0 mV/mm
lr0 <- lme(vel ~ FrameInterval,
    random = ~ 1|id,
    data = t2[t2$EF == levels(t2$EF)[1],],
    method = "REML"
)
lr0
lrs0 <- summary(lr0)
lrs0
lr0.anova <- anova(lr0)
lr0.anova
tuk.lr0 <- glht(lr0,
    linfct = mcp(FrameInterval = "Tukey")
)
tuk.lrs0 <- summary(tuk.lr0)
tuk.lr0
tuk.lrs0

# random effects model and post-hoc test for 400 mV/mm
lr400 <- lme(vel ~ FrameInterval,
    random = ~1|id,
    data = t2[t2$EF == levels(t2$EF)[2],],
    method = "REML"
)

lr400
lrs400 <- summary(lr400)
lrs400
lr400.anova <- anova(lr400)
lr400.anova
tuk.lr400 <- glht(lr400,
    linfct = mcp(FrameInterval = "Tukey")
)
tuk.lr400
tuk.lrs400 <- summary(tuk.lr400)
tuk.lrs400

# evaluate constant variance using residuals
# i.e. Levene test for heteroscedasticity
# 0 mV/mm
leveneTest(y = residuals(lr0),
    group = lr0$data$FrameInterval,
    center = "mean"
)
# 400 mV/mm
leveneTest(y = residuals(lr400),
    group = lr400$data$FrameInterval,
    center = "mean"
)

# plots of 0 & 400 mV/mm
opar <- par(no.readonly = TRUE)
par(mfrow = c(1,2),
     mar = c(5, 4, 6, 2)
)
plot(
    cld(tuk.lr0),
    col = "lightgrey",
    xlab = "Tracking Intervals",
    ylab = expression(paste("Cell Speed (", mu, " m / hour)")),
    sub = levels(t2$EF)[1]
)
plot(
    cld(tuk.lr400),
    col = "lightgrey",
    xlab = "Tracking Intervals",
    ylab = expression(paste("Cell Speed (", mu, " m / hour)")),
    sub = levels(t2$EF)[2]
)
par <- opar
rm(opar)

**Migration analysis**

Migration analysis (including "Optimizing the measuring interval for tracking cell migration"): track cells in time-lapse videos using the ImageJ plugin MTrackJ. (Make sure that the time interval and distance unit in the image properties is appropriate.)

When tracking is finished, click "measure." Two datasets will appear: one labeled
“points” with data corresponding to each position that was clicked during tracking, and one labeled “tracks” that has summary statistic for each track (i.e. cell). The migration scripts use the “points” datasets from MTrackJ, including the same column labels. In addition, 3 columns labeled “Experiment” “Position” and “EF” must be added at the start of the dataset. (For these analyses, I made separate datasets for cells tracked before and after the onset of the EF.)

```r
### Load Packages  -------------------------------------------------------------
library(circular)
library(ggplot2)
library(pastecs)
library(reshape)
library(multcomp)
library(grid)

options(digits = 3)         # prints 3 significant figures

# Define Functions  ----------------------------------------------------------
data.subsample <- function(dataset, size, group) {
  data1 <- dataset
  data.subset <- data.frame()
  data1$factor <- as.factor(group)

  for (i in 1:length(names(table(data1$factor)))) {
    sub1 <- sample(x = names(table(data1$id[data1$factor ==
                      names(table(data1$factor))[[i]]])),
                   size = size
    data.subset <- rbind(data.subset,
                          data1[data1$id %in% sub1, -ncol(data1)]
    )
  }
  data.subset
}

id.create <- function(dataset) {
  paste(dataset[, "Experiment"],
        dataset[, "Position"],
        dataset[, "EF"],
        dataset[, "TID"],
        sep = "."
}
```

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# n = number of points selected per time, per group
# interval = how often the points are plotted
points.subset <- function(dataset, 
    n, 
    t.start = 0, 
    t.end = 12, 
    interval = 0.25, 
    group = "ef.factor"
) {
    # sub-samples individual data points within each group
    data1 <- dataset
    levels1 <- names(table(data1[, , group]))
    times <- seq(from = t.start, 
        to = t.end, 
        by = interval
    )
    output1 <- data.frame()
    for (i in 1:length(levels1)) {
        ef1 <- levels1[i]

        for (j in 1:length(times)) {
            t1 <- times[j]
            dsub <- data1[data1[, , group] == ef1 & data1$time == t1, ]
            id1 <- c(1:nrow(dsub))
            sub1 <- sample(x = id1,
                size = n,
                replace = FALSE
            )
            dsub <- dsub[sub1, ]
            output1 <- rbind(output1,
                dsub
            )
        }
    }
    output1
}

position.normalize <- function(dataset, 
    t.start, 
    t.end)
) {
    data1 <- dataset
    data1$id <- id.create(data1)
    data1 <- t.subset(dataset = data1, 
        t.start = t.start, 
        t.end = t.end
    )
    data1$t.norm <- data1$time - t.start
data1$x.norm <- NA
data1$y.norm <- NA

for (i in 1:length(names(table(data1$id)))) {
  cell.id <- names(table(data1$id))[i]

  x0 <- data1$x..micron.[data1$id == cell.id &
    data1$time == t.start]
  y0 <- data1$y..micron.[data1$id == cell.id &
    data1$time == t.start]

  data1$x.norm[data1$id == cell.id] <-
    data1$x..micron.[data1$id == cell.id] - x0
  data1$y.norm[data1$id == cell.id] <-
    y0 - data1$y..micron.[data1$id == cell.id]
}
data1


t.subset <- function(dataset, t.start, t.end) {
  data1 <- dataset[(dataset$time >= t.start) &
    (dataset$time <= t.end), ]
  cell.id <- data.frame("cell" = names(table(data1$id)))

  for (i in 1:nrow(cell.id)) {
    cell.id$t.min[i] <-
      min(data1$time[data1$id == cell.id$cell[i]]) == t.start
    cell.id$t.max[i] <-
      max(data1$time[data1$id == cell.id$cell[i]]) == t.end
  }
  cells.to.keep <- cell.id$cell[cell.id$t.min & cell.id$t.max ]
  data.new <- data1[data1$id %in% cells.to.keep &
    data1$time >= t.start & data1$time <= t.end, ]
}

vel.sub <- function(dataset, t.start = 0, t.end = 12,
  ef = "ef.factor",
  time = "time",
  speed = "speed",
  dir = "dir"
) {
  data1 <- dataset
  data1 <- data1[!is.na(data1[, speed]) & data1[, time] <=
    t.end & data1[, time] >= t.start & data1[, speed] != 0,
    c(ef,

time, speed, dir
}
data1[, dir][data1[, dir] < 0] <-
data1[, dir][data1[, dir] < 0] + 360
data1

# Load Data -----------------------------------------------
mig1 <- read.csv(file.choose(),
   header = TRUE
)
# mig2 is the data set that will be used to plot cell tracks
mig2 <- mig1
mig1$time[mig1$EF == 0] <- mig1$time[mig1$EF == 0] - 0.5
mig1.baseline <- read.csv(file.choose(),
   header = TRUE
)
mig1 <- rbind(mig1.baseline,
   mig1
)

mig1R <- read.csv(file.choose(),
   header = TRUE
)
mig1R.baseline <- read.csv(file.choose(),
   header = TRUE
)
mig1R$ef.factor <- "400(R)"
mig1R.baseline$ef.factor <- "400(R)"
mig1R <- rbind(mig1,
   mig1R,
   mig1R.baseline[mig1R.baseline$time == 0,]
)
rm(mig1.baseline, mig1R.baseline)

# create a variable for EF strengths that serves as a labeled factor
mig1$ef.factor <- as.factor(mig1$EF)
mig2$ef.factor <- as.factor(mig2$EF)
mig1R$ef.factor <- as.factor(mig1R$EF)

### Create dataframe for velocity analysis -----------------
# mig.vel stands for "migration data for analyzing velocity"
# excludes any time points greater than 12 hours
mig.vel <- vel.sub(mig1)

# make separate database for directionality double-plot so that dir
variable is not coerced into class circular
mig.vel1 <- vel.sub(mig1R)
levels(mig.vel1$ef.factor) <- paste(levels(mig.vel1$ef.factor),

"mV/mm"

# coerce directional data into circular
mig.vel$dir <- as.circular(mig.vel$dir, type = "angles", units = "degrees", template = "none", modulo = "2pi", zero = 0, rotation = "clock"

### aggregate mig.vel into dataframe for summary statistics
### aggregate mig.vel into dataframe for summary statistics
# create dataset of factor levels (EF level x time)
# create dataset of factor levels (EF level x time)
mig.vel.stats <- aggregate(mig.vel[, 3],
  by = list(ef.factor = mig.vel$ef.factor, time = mig.vel$time
    ),
  FUN = mean
)

# create dataset of summary statistics pertaining to migration
# create dataset of summary statistics pertaining to migration
mig.spd.stats <- as.data.frame(
  aggregate(mig.vel[, 3],
    by = list(ef.factor = mig.vel$ef.factor, time = mig.vel$time
      ),
    FUN = function(x) c(n = length(x),
      mean.speed = mean(x),
      sd.speed = stats::sd(x),
      sem.speed =
      as.numeric(stat.desc(x)["SE.mean"]),
      ci.95.speed =
      as.numeric(stat.desc(x)["CI.mean.0.95"]),
      med.speed = median(x),
      q25 = as.numeric(quantile(x, 0.25)),
      q75 = as.numeric(quantile(x, 0.75))
    )
  )[,3]
)

# create dataset of summary statistics pertaining to direction
# create dataset of summary statistics pertaining to direction
mig.dir.stats <- as.data.frame(
  aggregate(mig.vel[!is.na(mig.vel$dir), 4],
    by = list(ef.factor = mig.vel$ef.factor[!is.na(mig.vel$dir)]),
    FUN = function(x) c(rayleigh.p =
    )
  )
as.numeric(rayleigh.test(x)$p.value),
mean.dir =
as.numeric(mle.vonmises(x)$mu),
    # estimated mean
    # direction
sd.dir = deg(sd(x)),
se.mu.dir =
as.numeric(mle.vonmises(x)$se.mu),
    # standard error of the
    # mean direction estimate
kappa.dir =
as.numeric(mle.vonmises(x)$kappa),
    # kappa parameter
    # (concentration)
se.kappa.dir =
as.numeric(mle.vonmises(x)$se.kappa)
    # standard error of the kappa
    # parameter (concentration)
})[, 3]

# combine with statistics summary
mig.vel.stats <- cbind(mig.vel.stats,
    mig.spd.stats,
    mig.dir.stats
)

# remove unnecessary datasets
rm(mig.spd.stats,
    mig.dir.stats
)

# store this original mig.vel dataset
mig.vel.stats.original <- mig.vel.stats

# create dataset of rayleigh results
# include a variable indicating whether the test is significant
# TRUE/FALSE depending on significance of test
# Bonferroni correction built into analysis
# (i.e. threshold for significance is divided by
# the number of factor levels)
mig.vel.stats$r.is.signif <- mig.vel.stats$rayleigh <=
    .05/(length(levels(mig.vel.stats$ef.factor)) * 4  # number of EF
    # strengths
    length(table(mig.vel.stats$time))    # number of time points
)

# convert all circular statistical results to NA if
# the rayleigh test reveals that the time point is non-significant
mig.vel.stats[!mig.vel.stats$r.is.signif, c(12:16)] <- NA
# character string of statistical results
mig.vel.stats$r.text <- paste("p =", signif(mig.vel.stats$rayleigh, digits = 3))

### Display Results of Summary Stats ---------------------------------------
# display the results of the rayleigh tests
mig.vel.stats[, c(1, 2, 11, 17)]

# display the results of the rayleigh tests for specific EF strengths
mig.vel.stats[mig.vel.stats$ef.factor == 0, c(1, 2, 11, 17, 12)]

# tabular output of rayleigh results
xtabs(r.is.signif ~ ef.factor + time,
     data = mig.vel.stats)

# Plot mean speed by time with 95% CI ---------------------------------------
mspeed.plot <- ggplot(data = mig.vel.stats,
                       aes(x = time,
                            y = mean.speed,
                            fill = ef.factor,
                            shape = ef.factor
                       )
  ) +
geom_line(size = 1.5) +
geom_point(size = 7) +
scale_shape_manual(values = c(21, 19, 15, 22)) +
scale_fill_manual(values = c("#FFBA00",
                            "black",
                            "black",
                            "#FFBA00"
                            )
  ) +
coord_cartesian(xlim = c(0, 12.15),
                ylim = c(5, 30)
  ) +
scale_x_continuous(breaks = seq(0, 12,
                                  by = 1
                                  )
  ) +
scale_y_continuous(breaks = seq(0, 30,
                                  by = 5
                                  )
  ) +
theme_bw(base_size = 28) +
theme(legend.position = c(1, .95),
      legend.justification = c(1, 1),
      legend.direction = "horizontal",
      panel.border = element_blank(),
      panel.grid = element_blank(),
      plot.title = element_blank(),
axis.line = element_line(colour = "black",
    lineend = "square"
 )
) +
 labs(y = expression(paste("Cell Speed (", mu, " m / hour)")),
    x = "Duration of Electric Field Exposure (hours)",
    title = "Electric Fields Affect Cortical Astrocyte Migration Speed",
    shape = "EF (mV / mm)",
    fill = "EF (mV / mm)"
) +
 geom_errorbar(aes(ymin = mean.speed - sem.speed,
    ymax = mean.speed + sem.speed
 ),
    width = 0, # width of the bar-caps
    size = 1, # line thickness
    colour = "black"
 ) +
 geom_point(size = 7)

### Bar Plot of Mean Speed at Certain Times -------------------------------
# specify time and subset the data
time1 <- c(0, .5, 4)
mig.vel.stats.sub <- mig.vel.stats[mig.vel.stats$time %in% time1, ]
mig.vel.stats.sub$time <- paste(mig.vel.stats.sub$time,
    "Hours"
 )

# create bar plot
speed.bar.plot <- ggplot(data = mig.vel.stats.sub,
    aes(x = ef.factor,
    y = mean.speed,
    fill = ef.factor
 )

) +
geom_bar(stat = "identity") +
geom_errorbar(aes(ymin = mean.speed - sem.speed,
    ymax = mean.speed + sem.speed
 ),
    width = 0.25,
    size = 1
 ) +
 facet_grid(. ~ time) +
scale_y_continuous(breaks = seq(0, 50,
    by = 5
 )

) +
coord_cartesian(ylim = c(5, max(mig.vel.stats.sub$mean.speed +
mig.vel.stats.sub$sem.speed) + 6)) +
labs(y = expression(paste("Mean Speed (", mu, " m / hour)")),
    x = "Electric Field Strength (mV/mm)"
) +

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theme_bw(base_size = 28) +
    theme(legend.position = "none",
          strip.background = element_rect(size = 1.5,
                                            fill = "white",
                                            colour = "white"
          ),
          panel.grid = element_blank(),# Remove major & minor grid lines
          panel.border = element_rect(size = 1.5,
                                        colour = "black"
          ))
    ) +
    scale_fill_grey()

speed.bar.plot
rm(time1, mig.vel.stats.sub)

### Analysis of speed at a certain times ------------------------
# Statistical Analysis of differences between groups

time1 <- 4
mig.vel.sub <- mig.vel[mig.vel$time == time1, ]

means1 <- aggregate(mig.vel.sub$speed,
                     by = list(mig.vel.sub$ef.factor),
                     FUN = mean
                   )

means1
fit1 <- aov(mig.vel.sub$speed ~ mig.vel.sub$ef.factor)
summary(fit1)

plot(TukeyHSD(fit1))
TukeyHSD(fit1)
rm(mig.vel.sub, means1, fit1, time1)

# Analyze change in speed over time within a given EF

time1 <- c(0, .5, 4, 6)
mig.vel.sub <- mig.vel[mig.vel$time %in% time1 &
                       mig.vel$ef.factor == 400,]

aggregate(mig.vel.sub$speed,
          by = list(mig.vel.sub$time),
          FUN = mean
        )

fit <- aov(mig.vel.sub$speed ~ as.factor(mig.vel.sub$time))
summary(fit)

TukeyHSD(fit)
plot(TukeyHSD(fit))
rm(time1, mig.vel.sub, fit)

### Plot Direction by time (repeated x-axis) ------------------------
# if you want to plot all data points (not a subset)
mig.sub <- mig.vel1
# if you want to plot a subset of data points at each time
mig.sub <- points subset(dataset = mig.vel1,
   n = 45)

## Generate plot
dir2.plot <- {ggplot(data = mig.sub,
   aes(x = c(dir, dir+360),
       y = c(time, time))

   # add lines to indicate cathode and anode, respectively
   geom_vline(xintercept = c(90, 270, 90+360, 270+360),
       colour = rep(c("red", "blue"), 2),
       size = 1.5,
       alpha = 0.5)

   # Add points
   geom_point(size = 1.75,
               alpha = 0.5)

   # Black-White theme elements
   theme_bw(base_size = 28) +

   ## facets by EF strength
   facet_grid(. ~ ef.factor) +

   coord_cartesian(xlim = c(0, 720),
                   ylim = c(-.1, 12.1)) +

   scale_x_continuous(breaks = c(90, 270, 90+360, 270+360),
                      labels = rep(c("A", "C"), 2)) +

   scale_y_continuous(breaks = seq(0, 12, by = 1)) +

   ## Modify axis titles
   labs(x = "Direction of Astrocyte Migration Relative to the Electric Field",
        y = "Duration of EF Exposure (hours)",
        title = "Electric Field Effects on Direction of Cell Migration")

   # Remove minor grid lines}
theme(panel.border = element_rect(colour = "black", fill = NA, size = 2),
      panel.grid = element_blank(), panel.margin = unit(1, "lines"),
      strip.background = element_rect(fill = "white", colour = "white", size = 1.5),
      plot.title = element_blank(),
      axis.title = element_text(size = rel(1)),
      axis.text.x = element_text(colour = rep(c("red", "blue"), 2), size = rel(1), face = "bold"),
      strip.text = element_text(face = "bold", # modify facet header size = rel(1))
    ) +
# adds a horizontal line to the reverse-current plot to
# indicate when the current was changed
geom_segment(data = data.frame(xmin = 0, xmax = 720, ymin = 5.875, ymax = 5.875, ef.factor = levels(mig.sub$ef.factor)[length(levels(mig.sub$ef.factor))]),
             aes(x = xmin, xend = xmax, y = ymin, yend = ymax),
             show_guide = FALSE, colour = "#FFBA00", size = 1.5, linetype = 2)

dir2.plot

rm(mig.sub)

# Plot each track, normalized ----------------------------------------
t.start <- 0
t.end <- 6
n = 30

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mig2.sub <- position.normalize(dataset = mig2,
    t.start = t.start,
    t.end = t.end
)

mig2.sub2 <- data.subsample(dataset = mig2.sub,
    size = n,
    group = mig2.sub$ef.factor
)

levels(mig2.sub2$ef.factor) <-
    paste(levels(mig2.sub2$ef.factor), " mV/mm")
# change names of the levels
# so the facet labels include "mV/mm"

tracks.plot <- ggplot(data = mig2.sub2,
    aes(x = x.norm,
        y = y.norm,
        group = id
    )
)
    +
    geom_vline(xintercept = 0,
        colour = "darkgrey"
    ) +
    geom_hline(yintercept = 0,
        colour = "darkgrey"
    )+
    geom_path() +
    labs(y = expression(paste(mu, "m")),
        x = expression(paste(mu, "m"))
    ) +
    annotate("text",
        label = "+",
        size = 15,
        x = 0,
        y = -180,
        colour = "red"
    ) +
    annotate("text",
        label = ",",
        size = 15,
        x = 0,
        y = 180,
        colour = "blue"
    ) +
    facet_grid(. ~ ef.factor) +
    theme_bw(base_size = 28) +
    theme(panel.grid = element_blank(),
        panel.border = element_rect(colour = "black",
            size = 1.5
        ),
        panel.margin = unit(1, "lines"),
        strip.background = element_rect(colour = "white",
        )
)
tracks.plot

# Plot Total Displacement of each track
data = mig2.sub[mig2.sub$time == t.end, ],
aes(x = x.norm,
y = y.norm

) +
facet_grid(. ~ ef.factor) +
coord_cartesian(xlim = c(-200, 200),
ylim = c(-200, 200)
) +
theme_bw(base_size = 28) +
theme(panel.grid = element_blank(),
strip.background = element_rect(fill = "white",
colour = "white"
)
) +
geom_vline(xintercept = 0,
colour = "darkgrey"
) +
geom_hline(yintercept = 0,
colour = "darkgrey"
) +
labs(y = expression(paste(mu, "m")),
x = expression(paste(mu, "m"))

) +
annotate("text",
label = "+",
size = 15,
x = 0,
y = -180
) +
annotate("text",
label = "-",
size = 15,
x = 0,
y = 180
Migration following reversal of current

Scripts are organized in the same fashion as those for the migration analysis.

See description found on page 305 for more information.

```r
## Load Packages
library(circular)
library(ggplot2)
library(pastecs)
library(reshape)
library(multcomp)
library(grid)

options(digits = 3)  # prints 3 significant figures

# Define functions

t.subset <- function(dataset, t.start, t.end) {
  data1 <- dataset[(dataset$time >= t.start) & (dataset$time <= t.end),]
  cell.id <- data.frame("cell" = names(table(data1$id)))
  for (i in 1:nrow(cell.id)) {
    cell.id$t.min[i] <- min(data1$time[data1$id == cell.id$cell[i]]) == t.start
    cell.id$t.max[i] <- max(data1$time[data1$id == cell.id$cell[i]]) == t.end
  }
  cells.to.keep <- cell.id$cell[cell.id$t.min & cell.id$t.max]
  data.new <- data1[data1$id %in% cells.to.keep &
噪 data1$time >= t.start & data1$time <= t.end, ]
}
```
id.create <- function(dataset) {
  paste(dataset[, "Experiment"],
         dataset[, "Position"],
         dataset[, "EF"],
         dataset[, "TID"],
         sep = ".")
}

position.normalize <- function(dataset, t.start, t.end) {
  data1 <- dataset
  data1$id <- id.create(data1)
  data1 <- t.subset(dataset = data1, t.start = t.start, t.end = t.end)
  data1$t.norm <- data1$time - t.start
  data1$x.norm <- NA
  data1$y.norm <- NA
  for (i in 1:length(names(table(data1$id)))) {
    cell.id <- names(table(data1$id))[i]
    x0 <- data1$x..micron.[data1$id == cell.id &
                           data1$time == t.start]
    y0 <- data1$y..micron.[data1$id == cell.id &
                           data1$time == t.start]
    data1$x.norm[data1$id == cell.id] <-
        data1$x..micron.[data1$id == cell.id] - x0
    data1$y.norm[data1$id == cell.id] <-
        y0 - data1$y..micron.[data1$id == cell.id]
  }
  data1
}

data.subsample <- function(dataset, size, group) {
  data1 <- dataset
  data.subset <- data.frame()
  data1$factor <- as.factor(group)

  for (i in 1:length(names(table(data1$factor)))) {
    sub1 <- sample(x = names(table(data1$id[data1$factor ==
                      names(table(data1$factor))[i]])), size = size)
    data.subset <- rbind(data.subset,}
data1[data1$id %in% sub1, -ncol(data1)]
)
}
data.subset
)

# Load Data ---------------------------------------------

# data set for directionality double-plots
mig.rev <- read.csv(file.choose(), header = TRUE)

# data set for plotting individual tracks
mig.rev.tracks <- read.csv(file.choose, header = TRUE)

### Plot Direction by time (repeated x-axis) -------------------------

# only copies data if there is a value for speed (i.e. not NA)
mig.vel.rev1 <- mig.rev[!is.na(mig.rev$speed) & mig.rev$time <= 12 & mig.rev$time >= 0, c("EF", "time", "speed", "dir")]

# Convert range of angle measures for direction variable
# new range: 0-360 (degrees)
mig.vel.rev1$dir[mig.vel.rev1$dir < 0] <- mig.vel.rev1$dir[mig.vel.rev1$dir < 0] + 360

# if speed == 0, MTrackJ records direction as 0
# make dir == NA if(speed == 0)
mig.vel.rev1$dir[mig.vel.rev1$speed == 0] <- NA

## Generate plot
dir.rev.plot <-
ggplot(data = mig.vel.rev1[is.na(mig.vel.rev1$dir) == FALSE,],
aes(x = c(dir, dir+360),
y = c(time, time)
)
) +

# Add points
geom_point(size = 2,
alpha = 0.3
) +

# Black-White theme elements
theme_bw(base_size = 28) +
## facets by EF strength
## Plot Direction by time (400-Reverse only) (repeated x-axis) ------

# only copies data if there is a value for speed (i.e. not NA)
```
mig.vel.rev1 <- mig.rev[!is.na(mig.rev$speed) & mig.rev$time <= 12 & mig.rev$time >= 0, c("EF", "time", "speed", "dir")]
```

# Convert range of angle measures for direction variable
# new range: 0-360 (degrees)
mig.vel.rev1$dir[mig.vel.rev1$dir < 0] <- mig.vel.rev1$dir[mig.vel.rev1$dir < 0] + 360

# if speed == 0, MTrackJ records direction as 0
# make dir == NA if(speed == 0)
mig.vel.rev1$dir[mig.vel.rev1$speed == 0] <- NA

## Generate plot
```
dir.rev2.plot <- ggplot(data = mig.vel.rev1[is.na(mig.vel.rev1$dir) == FALSE & mig.vel.rev1$dir < 0] == "400-Reverse",), aes(x = c(dir, dir+360), y = c(time, time)) +
```

# Add points
```
geom_point(size = 2.5, alpha = 0.4)
```

# Black-White theme elements
```
theme_bw(base_size = 38) +
coord_cartesian(xlim = c(0, 720), ylim = c(-1, 12.1)) +
scale_x_continuous(breaks = c(0, 720, 90+360, 90+360), labels = rep(c("A", ...},

```
'C'
2

scale_y_continuous(breaks = seq(0,
12,
by = 1
)

## Modify axis titles
labs(x = "Direction Relative to the EF",
y = "Time (hours)"

# Remove minor grid lines
theme(panel.border = element_rect(colour = "black",
fill = NA,
size = 2
),
panel.grid = element_blank(),
axis.text.x = element_text(colour = rep(c("black", "black"),
2
),
size = rel(1),
face = "bold"
),
axis.text.y = element_text(colour = "black",
size = rel(1))

# remove legend
guides(colour = FALSE) +
# add colored lines to indicate cathode and anode, respectively
geom_vline(xintercept = c(90, 270, 90+360, 270+360),
colour = rep(c("black", "black"),
2
),
size = 1,
alpha = 0.5
) +

# add horizontal line to indicate where the current was reversed
geom_hline(yintercept = 5.875,
colour = "black",
size = 1,
linetype = 2
)
dir.rev2.plot
rm(mig.vel.rev1)

# Plot Tracks for 400 mV/mm only -------------------------------------
t.start <- 3
t.end <- 12
n = 15

mig.rev.norm <- position.normalize(dataset = mig.rev.tracks,
    t.start = t.start,
    t.end = t.end
)

n = 10
norm.sub <- data.subsample(dataset = mig.rev.norm,
    size = n,
    group = mig.rev.norm$EF
)

tracks.plot <- ggplot(data = norm.sub,
    aes(x = x.norm,
        y = y.norm,
        group = id
    )
    ) +
    geom_vline(xintercept = 0,
        colour = "darkgrey"
    ) +
    geom_hline(yintercept = 0,
        colour = "darkgrey"
    )+
    geom_path() +
    facet_grid(. ~ EF) +
    theme_bw(base_size = 28) +
    theme(panel.grid = element_blank(),
        panel.border = element_rect(colour = "black",
                size = 1.5
        ),
        panel.margin = unit(1, "lines"),
        strip.background = element_rect(colour = "white",
                fill = "white"
        ),
        axis.title = element_blank(),
        axis.text = element_text(size = rel(0.75))
    ) +
    coord_fixed(xlim = c(-201, 201),
        ylim = c(-201, 201)
    ) +
    scale_x_continuous(breaks = c(-200, -100, 0, 100, 200),
        labels = c("200", "100", "0", "100", "200")
    ) +
    scale_y_continuous(breaks = c(-200, -100, 0, 100, 200),
        labels = c(200, 100, 0, 100, 200)
    )

tracks.plot
**Orientation of proliferation**

Data are collected using ImageJ. Column names are: “Cell” [i.e. the cell type, e.g. cerebellar or cortical, in reference to astrocytes], “Experiment” “Position” “EF” “Measurement” “Angle” “Frame” and “Length”.

The second part of this script includes a graph that plots the orientation of proliferation over time. This graph was used for data exploration, but was not included in the analysis presented in this dissertation.

```r
### Load Packages .nextSibling
library(circular)  # For circular stats (seems more thorough than CircStats)
library(survival)
library(ggplot2)
library(grid)

### Load Data .nextSibling
prolif1 <- read.csv(file.choose(), header = TRUE)

## limit prolif1 data range to 12 hours (241 frames)
prolif1 <- prolif1[prolif1$Frame <= 241,]

### Data Management .nextSibling
# Create time variable (hours)
prolif1$Time <- (prolif1$Frame - 1) / 20

# Convert angle measurements into axial measurements
# Range from 0 - 180 degrees (original range -180 to +180)
prolif1$Axial[prolif1$Angle < 0] <- prolif1$Angle[prolif1$Angle < 0] + 180
prolif1$Axial[prolif1$Angle >= 0] <- prolif1$Angle[prolif1$Angle >= 0] - 180
prolif1$Axial[prolif1$Angle == 180] <- prolif1$Angle[prolif1$Angle == 180] - 180
head(prolif1)

# Determine whether alignment exists .nextSibling
# note: change the number for the ef1 variable to
# calculate the alignment for each EF strength
# factor levels: 0, 4, 40, 400
ef1 <- 4
a1 <- circular(prolif1$Axial[prolif1$EF == ef1], units = "degrees",
```
modulo = "pi"
)
rayleigh.test(2 * a1)[[2]]
rayleigh.test(sample(2 * a1,
    size = 30
) )
)[[2]]
length(a1)
mle.vonmises(a1)         # numbers in parentheses of results are the
standard error of those results
mean.circular(a1)
deg(sd.circular(a1))

# Distribution of Orientation of Mitotic Axis ------------------------
prolif1.dir <- ggplot(data = prolif,
    aes(x = c(Axial, Axial + 180),
        y=..density..)
    ) +
geom_histogram(binwidth = 15,
        size = .75,
        fill = "grey"
    ) +
geom_line(stat = "density") +
coord_cartesian(xlim = c(0, 360),
    ylim = c(0, .007)
    ) +
facet_grid(. ~ EF) +
theme_bw(base_size = 28) +
scale_x_continuous(breaks = seq(0, 360,
    by = 90
    ),
    labels = c("C", "", "A", "", "C")
    ) +
theme(panel.border = element_rect(colour = "black",
    size = 2
    ),
    panel.grid = element_blank(),
    panel.margin = unit(1.25,
        "lines"
    ),
    strip.background = element_rect(colour = "white",
        size = 1.5,
        fill = "white"
    ),
    axis.ticks.y = element_blank(),
    axis.text.y = element_blank()
    ) +
labs(x = "Orientation of the axis of cell division",
    y = "Relative number of cells"
    )
prolif1.dir
# ggplot2 Orientation of Division over Time -----------------------------
orient1 <- ggplot(data = prolif1,
  aes(x = c(Axial, Axial + 180),
       y = c(Time, Time))
  ) +
## add points for each speed value
  geom_point(data = prolif1,
     size = 2,
     alpha = 0.6
  ) +
scale_x_continuous(limits = c(0, 360),
     breaks = seq(0, 360, by = 90),
     labels = c("", "Cathode", "", "Anode", "")
  ) +
scale_y_continuous(limits = c(0, 12),
     breaks = seq(0, 12, by = 2)
  ) +
## Modify axis titles
  labs(x = "Orientation of Axis of Cell Division",
       y = "Duration of Electric Field Exposure\n(hours)",
       title = "Electric Field Effects on the Orientation of Cell Division over Time\n(horizontal axis repeated)"
  ) +
## facets by EF strength
  facet_grid(. ~ EF) +
# colour scheme is black and white
  theme_bw() +
# Remove minor grid lines
  theme(panel.grid.minor = element_blank()) +
# add colored lines to indicate cathode and anode, respectively
  geom_vline(xintercept = c(90, 270),
     colour = c("red", "blue")
  )
orient1
Proliferation – BrdU experiments

Only summary statistics are needed for this data file; these were calculated in the statistical software JMP v. 11.0.0. Column names are: “Time” “EF” “N Rows” “Mean(Percent)” “Median(Percent)” and “Std Err(Percent)”.

```r
library(ggplot2)

brdu1 <- read.csv(file.choose(),
  header = T)

brdu2 <- brdu1

brdu2$EF <- factor(brdu2$EF)
brdu2$Time <- factor(brdu2$Time)

levels(brdu2$Time) <- paste(levels(brdu2$Time),
  "Hours"
)

levels(brdu2$Time)

# Bar Plot -----------------------------------------------
brdu.plot <- ggplot(data = brdu2,
  aes(x = EF,
       y = Mean.Percent.,
       fill = EF
  )
  ) + 
  geom_bar(stat = "identity") + 
  scale_fill_grey() +
  facet_grid(. ~ Time) +
  labs(y = "BrdU-positive Cells (%)",
       x = "Electric Field Strength (mV/mm)"
  ) +
  theme_bw(base_size = 28) +
  theme(legend.position = "none",
        panel.border = element_rect(colour = "black",
                                      size = 1.25
                                 ),
        panel.grid = element_blank(),
        strip.background = element_rect(fill = "white",
                                          colour = "white"
                                 ),
        axis.ticks.x = element_blank()
  ) +
  coord_cartesian(ylim = c(0, max(brdu2$Mean.Percent. + brdu2$Std.Err.Percent.) + 8)) +
  geom_errorbar(aes(ymin = Mean.Percent. - Std.Err.Percent.,
                 ),
```
Morphology – FFT experiments

Only 3 columns are needed for this data file: “EF” “Angle” and “Value”.

```r
library(ggplot2)
library(grid)

# Load data
fft1 <- read.csv(file.choose(),
    header = TRUE)

# change the number in this variable to plot different EF intensities
ef.level1 <- 400

fft.plot <- ggplot(data = fft1[fft1$EF == ef.level1,],
    aes(x = Angle,
         y = Value,
    )
) +
geom_line(size = 2) +
coord_cartesian(xlim = c(0, 360),
    ylim = c(0, max(fft1$Value) + 1)
) +
scale_x_continuous(breaks = c(90, 180, 270, 360),
    labels = c("Anode", ",", "Cathode", ",")
) +
scale_y_continuous(breaks = NULL) +
labs(x = "Orientation (Relative to the EF)",
    y = "Relative Pixel Alignment"
) +
theme_bw(base_size = 24) +
theme(legend.position = "bottom",
    panel.border = element_blank(),
    panel.grid.major = element_blank(),
    axis.line = element_line(colour = "black",
        lineend = "square",
        size = 2
    ),
    axis.ticks.x = element_line(size = 2,
        lineend = "square"
    ),
    axis.ticks.length = unit(.4, "cm")
)
```
### Load Packages
--------------
library(circular)
library(ggplot2)

### Load Data
--------------
nuc1 <- read.csv(file.choose(),
                 header = TRUE)
head(nuc1)

t1 <- 12
boxplot(nuc1$AR[nuc1$time == t1] ~
        as.factor(nuc1$EF[nuc1$time == t1]),
        main = "Circularity Scores")
fit1 <- aov(nuc1$AR[nuc1$time == t1 & nuc1$Experiment == 2] ~
                    as.factor(nuc1$EF[nuc1$time == t1 & nuc1$Experiment == 2]))
summary(fit1)
TukeyHSD(fit1)
plot(TukeyHSD(fit1))
rm(t1, fit1)

# Determine whether nuclei are aligned
-----------------------
ef1 <- 0  # specify EF level for the test
t1 <- 12

# create a circular data set
a1 <- circular(nuc1$Angle[nuc1$EF == ef1 & nuc1$time == t1],
                units = "degrees",
                modulo = "pi")
rayleigh.test(sample(2 * a1,
                    size = 30)
             )[[2]]
length(a1)
mle.vonmises(a1)  # numbers in parentheses of results are the
mean.circular(a1)  # standard error of those results
deg(sd.circular(a1))

# compare kappa between groups
n1 <- 100
equal.kappa.test(circular(c(sample(nuc1$Angle[nuc1$EF == 400 &
nuc1$time == 12],
n1
),
sample(nuc1$Angle[nuc1$EF == 400 &
nuc1$time == 72],
n1
)
),
units = "degrees",
modulo = "pi"
),
c(rep(12, n1),
rep(72, n1)
)
)
rm(a1, ef1, n1, t1)

# Graph alignment distributions ----------------------------------------------
t1 <- 12
nuc1.dir <- ggplot(data = nuc1[nuc1$time == t1,],
aes(x = c(Angle, Angle + 180))
) +
geom_histogram(binwidth = 15,
size = .75,
fill = "grey"
) +
facet_grid(. ~ EF) +
coord_cartesian(xlim = c(-20, 380),
ylim = c(0, 280)
) +
theme_bw(base_size = 28) +
scale_x_continuous(breaks = seq(0, 360, by = 90
),
labels = c("C", "", "A", "", "C")
) +
theme(panel.border = element_rect(colour = "black",
size = 2
),
panel.grid = element_blank(),
strip.background = element_rect(colour = "white",
size = 1.5,
fill = "white"
)
) +
labs(x = "Orientation Relative to the Electric Field",
y = "Number of Nuclei"
)
nuc1.dir
rm(t1)
# Compare Nucleus Aspect Ratio

```r
t1 <- 72
ex1 <- c(6, 10)
nuc2 <- nuc1[nuc1$time == t1 & nuc1$Experiment %in% exp1,]

fit1 <- aov(nuc2$AR ~ as.factor(nuc2$EF))
summary(fit1)
plot(TukeyHSD(fit1))
TukeyHSD(fit1)
```

```r
rm(t1, exp1, nuc2)
```

# Graph Nucleus Aspect Ratio

```r
t1 <- 72
ex1 <- c(6, 10)
nuc2 <- nuc1[nuc1$time == t1 & nuc1$Experiment %in% exp1,]

ar1.plot <- ggplot(data = nuc2,
  aes(x = as.factor(EF),
       y = AR)
  ) +
  geom_boxplot() +
  theme_bw(base_size = 28) +
  theme(panel.grid = element_blank(),
        panel.border = element_blank(),
        axis.line = element_line(colour = "black",
                                  lineend = "square"
        )
  ) +
  labs(x = "Electric Field Strength (mV/mm)",
       y = "Aspect Ratio")

rm(t1, exp1, nuc2)
```

**R Scripts for Chapter 3**

For the cerebellar astrocyte analyses, the structure of the data files and the code for the analyses in R are identical for each of the analyses that were done for cortical astrocytes in chapter two. The only exception is a minor modification for the analysis of migration data. The migration analysis was identical, but the data were compiled slightly differently for the cortical astrocytes than they were for the cerebellar astrocytes. Specifically, the cortical astrocyte migration data for the current-reversal analysis were
stored in a separate .csv file, while all of the migration data for the cerebellar astrocytes were stored in the same .csv file. The changes in the script for data loading reflects the fact that only 1 .csv file had to be loaded, and a second data subset that did not contain the current reversal group was created. The following script is for loading the cerebellar astrocyte data only; the rest of the analysis (including the libraries, functions, statistical tests, and graphs) are identical.

Migration analysis modification for cerebellar astrocytes

Scripts are organized in the same fashion as those for the migration analysis.

See description found on page 305 for more information.

```r
# Load Data
----------------------------------------------------------
mig1R <- read.csv(file.choose(),
   header = TRUE
)

# mig2 is the data set that will be used to plot cell tracks
mig2 <- mig1R[mig1R$EF %in% c(0, 4, 40, 400) & mig1R$time <= 12, ]
mig2$ef.factor <- factor(mig2$EF,
   levels = c(0, 4, 40, 400)
)
mig1R$time[mig1R$EF == 0] <- mig1R$time[mig1R$EF == 0] - 0.5
mig1R.baseline <- read.csv(file.choose(),
   header = TRUE
)
mig1R <- rbind(mig1R.baseline,
   mig1R
)
mig1R <- mig1R[mig1R$time >= 0 & mig1R$time <= 12, ]
mig1R$ef.factor <- factor(mig1R$EF,
   levels = c(0, 4, 40, 400, "400-Reverse"),
   labels = c("0", "4", "40", "400", "400 (R)"
   )

mig1 <- mig1R[mig1R$ef.factor %in% levels(mig1R$ef.factor)[c(1:4)], ]
mig1$ef.factor <- factor(mig1$ef.factor,
   levels = levels(mig1$ef.factor)[c(1:4)]
)
### R Scripts for Chapter 4

*Plotting current density analysis*

```r
### Load Packages ------------------------------------------
library(ggplot2)

# These values were taken from the JMP analysis that I completed 
# on 7/6/15

g1 <- c("Intact", "Lesioned")
m1 <- c(13.43, -34.85)
s1 <- c(4.394, 4.500)

d1 <- data.frame(site = g1,
                  mean = m1,
                  sem = s1)

plot1 <- ggplot(data = d1,
                 aes(x = site,
                     y = mean)
                 ) +
geom_bar(stat = "identity",
         colour = "grey",
         fill = "grey",
         width = .8,
         position = "dodge" ) +
geom_errorbar(aes(ymin = mean - sem,
                 ymax = mean + sem
                  ),
               width = 0.25,
               size = 1
              ) +
geom_hline(yintercept = 0,
           colour = "black",
           size = 1.25
               ) +
coord_cartesian(ylim = c(-45, 30)) +
scale_y_continuous(breaks = c(seq(-40, 20, 10)),
                   labels = c(seq(-40, 20, 10))
                 ) +
```

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theme_bw(base_size = 28) +
theme(panel.grid = element_blank(),
  panel.border = element_rect(colour = "black",
    size = 1.25
  )
) +
labs(y = expression(paste("Current Density: \(\mu\ A/cm\) \(^2\)),
  x = element_blank()
) +
annotate("segment",
  x = 1,
  xend = 2,
  y = 23,
  yend = 23,
  colour = "black",
  size = 1.25
) +
annotate("text",
  x = 1.5,
  y = 25,
  size = rel(8),
  label = "*p < 0.0001",
  colour = "black"
)

plot1
Vita

Matthew Louis Baer was born on Friday, the 20th of September, 1985, in Brooklyn, New York. He graduated from Columbia High School, Maplewood, New Jersey in 2003. He earned his Bachelors of Arts in Psychology with a minor in Architecture from the University of Virginia in 2007. He matriculated into the MD-PhD program at Virginia Commonwealth University in 2009. During his doctoral training at Virginia Commonwealth University, he has served as a tutor for medical school courses and has served on the admission committee for both the MD and MD-PhD programs.