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Investigating the Effects of Applied Electric Fields on Microglial Cell Behaviour

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INVESTIGATING THE EFFECTS OF APPLIED ELECTRIC FIELDS ON MICROGLIAL CELL BEHAVIOUR

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Anatomy & Neurobiology at Virginia Commonwealth University

by

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Abstract

INVESTIGATING THE EFFECTS OF APPLIED ELECTRIC FIELDS ON MICROGLIAL CELL BEHAVIOUR

By Eman Bani, M.S.

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Anatomy & Neurobiology at Virginia Commonwealth University

Virginia Commonwealth University, 2014

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

As surveyors of the central nervous system (CNS), microglial cells play an integral part in the inflammatory response following traumatic injuries. Thus, they have been implicated in the limited capability of neurons to regenerate in the CNS. Additionally, the roles of endogenous electric fields in the regenerative process of neurons in the mammalian peripheral nervous system (PNS) or amphibian CNS have long been studied. Further, previous studies in our lab have shown that physiological electric fields are capable of directing behaviours in astrocytes and schwann cells. Therefore in this study, a BV-2 microglia cell line was utilized to investigate whether
microglial cells are capable of detecting electric fields. After determining whether microglia detected electric fields, the second aim was to investigate whether electric fields triggered microglial activation. This study showed that while BV-2 microglia were capable of detecting electric fields they did not become activated in response to them.
CHAPTER 1 Introduction

Traumatic Injuries of the Central Nervous System (CNS) cause numerous debilitating and long-term effects, ranging from memory loss to partial or complete paralysis in mammals (Xiong et al, 2013). These injuries include traumatic brain injuries (TBIs) and spinal cord injuries (SCIs). The etiology of these long-term effects of CNS injuries is predominantly attributed to the limited ability of neurons to regenerate after axotomy, or the severing of axons (Reier et al, 1983). Neurons in the mammalian peripheral nervous system (PNS) or in the CNS of non-mammalian vertebrate, on the other hand, are able to regenerate after the primary injury and axotomy. This difference is due to several mechanisms of secondary injury in mammalian CNS traumas that lead to an inhibitory environment to neuroregeneration and subsequent neurodegeneration (Fitch et al, 1999). Thus, glial cells and inflammatory cells, that release those inhibitory factors, have emerged as targets for therapies of traumatic injuries of the CNS.

As resident macrophages of the CNS, microglia, are the first responders upon traumatic injury. Additionally, they are integral players in the neuroinflammatory process during secondary injury (Fitch et al, 1999; Popovich et al, 1997). In healthy tissue, they survey conditions and are sensitive to any homeostatic changes. Upon exposure to signals of neuronal distress, microglia become activated, and migrate towards the site of injury. At
the injury site they release various factors and perform many roles that can either lead to the neuronal repair and regeneration or exacerbation of damage (Fitch et al, 1999; Prewitt et al, 1997). Thus, microglia have arisen as a therapeutic target in the topic of axonal regeneration in the CNS. Though many therapies targeting microglia and the inflammatory response have shown some promise, they have been limited in their complete resolution of traumatic injuries (Beattie, 2004).

An often forgotten player in the pathological processes of traumatic injuries of the CNS is electric fields. At rest, any cell within biological tissue has a set membrane potential due to ionic pumps maintaining a separation of charge across their cell membrane. Additionally, cells contain many charged proteins on their plasma membrane, which enables them to experience a set force in the presence of an electric field (Colello and Alexander, 2003). Damage or injury to tissue can induce electric fields or injury potential (McCaig et al, 2005). These injury currents have been implicated in directing regeneration seen in amphibians or embryonic development (Borgens et al, 1977; Hotary and Robinson, 1990). Additionally, in vitro studies have shown that electric fields are capable of directing migratory, proliferative, or morphological behaviours in several cell types that are conducive to wound repair or regeneration (Sheridan et al, 1996; Song et al, 2004; Zhao et al, 1996).

Thus far, there have not been any previous studies on the effects that exogenous electric fields exude on microglial cells. Therefore, this study aims to identify whether microglia are capable of detecting and responding to applied electric fields. Additionally,
this study attempts to identify whether electric fields can cause microglial cells to become reactive in response. We hypothesize that electric field intensities observed at injury sites will trigger the activation of microglial cells.

**Introduction to Microglia**

Franz Nissl was the first in the scientific field to identify microglial cells and suggest their identity as reactive neuroglia with the ability to migrate and phagocytose (Barron, 1995). In the 1930s, Del Rio Hortega furthered Nissl's observations and characterized microglia as a population of central nervous system (CNS) cells distinct from other glial cell types. Specifically, he hypothesized that they were of myeloid lineage unlike other cells of the CNS, which are of neuroectoderm lineage (Kettenmann et al, 2011). Though Del Rio Hortega's theories were a subject of debate, it is currently widely accepted that microglial cells are resident macrophages of the CNS within the scientific realm (Cuadros and Navascues, 1998; Alliot et al, 1999; Hickey and Kimura, 1988). Hortega's theory of the myeloid lineage of microglial cells were further supported, when it was shown that they share similar antigenic markers, F4/80 and CD11b, as other cells of monocyte lineage (Giulian and Baker, 1986; Perry et al, 1985).

Studies on embryonic development within the CNS have additionally reinforced the lineage of microglia. Research studies on carbon-tagged macrophage-like cells have shown that these cells migrated into the CNS parenchyma from the bloodstream during embryonic development, where they remained into adulthood (Ling, 1979; Ling 1980).
These cells participate in the early stages of the shaping of the brain through phagocytizing apoptotic cells and removing misdirected axons (Ajami et al, 2007; Leong and Ling, 1992; Alliot et al, 1999; Giulian and Baker, 1986). After the completion of brain development, they convert into a resting phenotype into adulthood, where they are distributed heterogeneously and comprise 20% of the glial cell population (Giulian and Baker, 1986, Lawson et al, 1990).

After embryonic development, microglial cells remain as highly plastic cells and are broadly classified into two different morphology types, ramified and amoeboid (Leong and Ling, 1992, See Figure 1.1). The differential morphologies observed predict the functionality of each microglial cell within the nervous system. Ramified cells are histologically characterized by their thin, extensive processes (Glenn et al, 1992). Though ramified cells are known as “resting cells”, they actively participate in the surveillance of the CNS (Nimmerjahn et al, 2005). Additionally, they are extremely sensitive to any homeostatic fluctuations as they constantly use their vast network of processes to screen the CNS environment for any signs of neuronal stress or presence of toxic substances (Nimmerjahn et al, 2005; Davalos et al, 2005). Beyond surveillance, there is additional evidence that ramified microglia can contribute a neuroprotective role in the support of neurons during excitotoxicity (Vinet et al, 2012).

As soon as ramified microglia are exposed to signals of neuronal death, pathogens or toxins, they become activated, retract their processes and transform into amoeboid
Figure 1.1: Activation of Microglia

Microglia are typically found in ramified morphology in healthy central nervous system tissue but upon exposure to signals of neuronal injury they retract their processes and become amoeboid. Their morphological transformation is coupled with several behavioural changes, such as an increased phagocytic rate and the release of pro-inflammatory cytokines as they migrate towards the site of neuronal injury. These behavioural and morphological changes are dependent on and proportional to the degree of microglial cell activation and the concentration of neuronal injury signals. Adapted from Kettenmann et al. (2007)
morphology (Davalos et al, 2005). Once amoeboïd in morphology, activated cells migrate towards the site of neuronal stress or injury and increase their proliferation rate (d’Avila et al, 2012, Honda et al, 2001). As these cells enter the site of neuronal distress, they exhibit several other changes in their behaviour as well. Microglial cells begin to express macrophage markers and release several pro-inflammatory cytokines, such as TNF-α, and reactive oxidative species (ROS) (d’Avila et al, 2012). Additionally, they increase their phagocytic activity to phagocytose toxins and cellular debris (Witting et al, 2000; Rogers and Lue, 2001). The magnitude of the morphological transformation and behaviour is dependent on the intensity of the signal of neuronal distress (Kettenmann et al, 2007). Details on the response of microglia to neuronal stress after activation will be detailed in the following section.

M1/M2 Polarization in Microglia

As previously stated, microglia survey CNS tissue during normal conditions and are capable of activating in response to neuronal stress signals. Thus, upon exposure to stress signals or pathogens, microglial cells activate and exhibit a wide range of behaviours that can either lead to neuronal degeneration or regeneration (Loane and Byrnes, 2010). Though microglia are broadly classified as resting versus activated cells, there are two phenotypes of activated microglia that have recently been identified, M1 and M2 (Crain et al, 2013; Durafourt et al, 2012; See Figure 1.2). M1 activation, or “classical activation”, typically occurs upon exposure to Interferon-γ (IFN-γ) or Lipopolysaccharide (LPS) and leads to the pro-inflammatory phase of the microglial
response (Ishizuka et al, 2012; Antonios et al, 2013). M1 microglia secrete an immense amount of nitric oxide (NO), pro-inflammatory cytokines [such as, Tumour Necrosis Factor-alpha (TNF-α) or Interleukin-6 (IL-6)], and matrix metalloproteinases (MMPs) (Block et al, 2007). While M1 activation can induce phagocytosis and recruitment of additional phagocytes to the injury site, excessive M1 activation leads to tissue damage and subsequent neurodegeneration (Loane et al, 2014).

In contrast, M2 activation, or “alternative activation”, typically occurs upon exposure to Interleukin-4 (IL-4) or Interleukin-13 (IL-13). It represents the anti-inflammatory phase of the microglial response and typically follows the pro-inflammatory phase (Kigerl et al, 2009; Gordon, 2003). M2 microglia produce a wide range of anti-inflammatory cytokines [such as, Interleukin-10 (IL-10) or Transforming Growth Factor-beta (TGF-β)] and growth factors [such as, Vascular Endothelial Growth Factor (VEGF) and Brain-Derived Neurotrophic Factor (BDNF)] (Miron et al, 2013). Therefore, M2 activation leads to the dampening of inflammation, tissue repair and subsequent neuroregeneration, even in the presence of inhibitory factors (Kigerl et al, 2009; Shechter et al, 2013). Studies have shown that M2 microglia additionally facilitate the differentiation of oligodendrocytes within remyelinating lesions (Miron et al, 2013).

Although M1 activation in microglia and inflammation is associated with tissue necrosis and neurodegeneration in the CNS, the regeneration of neurons in the PNS is attributed to the robust response by inflammatory cells (Perry and Brown, 1992). Additionally,
Figure 1.2: M1/M2 Polarization in Microglia

Activated microglial cells can further be categorized as either having an M1 or M2 phenotype. Exposure to Lipopolysaccharide (LPS) or Interferon-γ (IFN-γ) causes M1, or “classical”, activation in microglial cells. This leads to a series of behaviors including the release of pro-inflammatory cytokines [such as, Interleukin-6 (IL-6) or Tumour Necrosis Factor-α (TNF-α)], secretion of nitric oxide (NO), and phagocytosis. M1 activation leads to increased CNS tissue damage and subsequent neurodegeneration. However, exposure to Interleukin-4 (IL-4) or Interleukin-10 (IL-10) induces M2, or “alternative”, activation in microglial cells. Following M2 activation, microglial cells release anti-inflammatory cytokines [such as, IL-10], secrete growth factors [such as, Transforming Growth Factor-β (TGF-β) and Vascular Endothelial Growth Factor (VEGF)], and become phagocytic. This leads to a dampening of inflammation, tissue repair, and ultimately neuroregeneration. Following traumatic CNS injuries, microglial cells typically become M1 activated. A delay in M1-to-M2 switching of microglial phenotype contributes to limited nature of neuroregeneration in the CNS. Figure adapted from Laskin, 2009.
studies showed that mice that lack the ability to produce the pro-inflammatory cytokine TNF-α displayed expanded primary lesions (Klusman and Schwab, 1997). Therefore, M1 activation in microglial cells is essential for wound repair in the CNS. Thus, the balance and timing of M1/M2 microglial cell polarization determines the extent to which CNS tissue will be damaged or repaired after injury. Consequently, an adequate therapy targeting microglia would try to achieve the right balance of M1/M2 polarization in microglia, rather than blocking M1 activation.

The Etiology of CNS Trauma in Mammals

Traumatic injuries of the CNS represent both traumatic brain injuries (TBIs) and spinal cord injuries (SCIs) and are divided into two broad phases, primary and secondary. Primary injuries represent the initial damage that occurs following mechanical insult to CNS tissue (Cernack, 2005). Primary injuries include damage to the blood brain barrier (BBB), hemorrhage, axotomy, and localized cell death (Laplaca et al, 2007). Mechanical sheer or compression forces on the spinal cord are typically the cause of SCIs. In contrast, TBIs are typically the result of rapid acceleration or deceleration and rotation to the brain (Borgens and Liu-Snyder, 2012). Though TBI and SCI primary injuries result from slightly different methods of mechanical insult, both lead to a cascade of secondary injuries that exacerbate damage to CNS tissue and prevent the process of regeneration.
Secondary injuries contribute to the expansion of damage beyond the primary lesion and can start as early as minutes following mechanical insult (Gris et al, 2008; Sheerin, 2005). The cause of secondary injuries is attributed to a series of systemic responses to the primary injury, which include: edema and ischemia, inflammation, and formation of the gliotic scar. Following the primary injury and subsequent breakdown of the blood brain barrier (BBB), edema or swelling occurs due to increased permeability (Fishman, 1975). Another factor that induces edema is the release of cytotoxic factors by inflammatory cells (Barzo et al, 1997). Edema causes increased ischemia, or lack of blood flow, in CNS tissue leading to additional damage and cell death beyond the primary lesion (Borgens and Liu-Snyder, 2012).

The process of post-injury inflammation additionally contributes to secondary damage to CNS tissue (Kumar and Loane, 2012). As mentioned beforehand, microglia are sensitive to changes in their tissue environment and are the first responders to CNS injury. After activation, microglia produce several of pro-inflammatory cytokines, such as TNF-\(\alpha\) or IL-1, that leads to the recruitment of additional inflammatory cells and stimulation of astrocyte proliferation or hypertrophy (Klusman and Schwab, 1997; Gao et al, 2013). Examples of inflammatory cells recruited to the site of injury include: neutrophils, which release proteases and reactive oxidative species (ROS) that leads to further tissue damage and T-lymphocytes, which release pro-inflammatory cytokines that lead further to microglial activation (Saiwai et al, 2010; Brait et al, 2012; Pineau and Lacroix, 2007). Though the inflammatory process has protective effects in the PNS, its prolonged nature in the CNS leads to the amplification of damage to tissue (Loane et al,
Thus, the resolution of inflammation is important to the process of tissue repair in the CNS (Loane et al, 2013).

Pro-inflammatory cytokines released by microglia, such as TNF-\(\alpha\) or IL-6, stimulate the proliferation and hypertrophy of astrocytes leading to the formation of a gliotic scar, which is a direct obstacle to axonal sprouting (Sofroniew, 2009). Specifically, reactive astrocytes release chondroitin sulfate proteoglycans (CSPGs), collagen and tenascin into tissue creating an inhibitory environment to axonal regrowth (Gao et al, 2013). CSPGs contribute to a non-permissive nature of tissue post-injury through inhibiting the differentiation of neural stem cells (Barkho et al, 2006) and oligodendrocyte precursor cells (OPCs) (Siebert and Osterhout, 2011). Although gliotic scars form a direct barrier to axonal regrowth, they initially serve the neuroprotective role of preventing the spread of injury. This is seen in a study where the deletion of reactive astrocytes from transgenic mice led to the profound expansion of primary lesions of the spinal cord (Bush et al, 1999).

In brief, there are a wide range of systemic processes that occur following primary mechanical insult to CNS tissue. Though each of these processes discussed can have some protective roles in the process of repair following injury, they ultimately contribute to the limited regeneration of neurons in the CNS. See Table 1.1 (adapted from Burda and Sofroniew, 2014) on the next page to visualize a specific timeline of events that occur following traumatic injury to the central nervous system.
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**Table 1.1: CNS Traumatic Injury Timeline**

**Electric Fields in Biological Systems**

The concept of electric fields as an inherent property of biological systems has its foundations in the 1700s, when Luigi Galvani’s monumental experimentation on frog axons led to the discovery of a phenomenon termed “animal electricity”. His experimentation showed that a frog’s sciatic nerve’s cut end exhibited a long-standing direct current voltage gradient, or injury potential, due to it’s ability to stimulate contractions when touching muscles of the opposite leg. Emil Du Bois Reymond furthered Galvani’s work, when he used a galvanometer to show the existence of injury potential in a cut in his finger (McCaig et al, 2005). After centuries of the subject of steady electrical signals being ignored or explored in an erroneous manner, it reemerged as researchers began to explore the basis of embryonic development, wound healing in mammals, and regeneration of the limbs of non-mammalian vertebrates.
Before describing electric fields within biological systems, it is imperative to review the concept of electricity and circuits. Electricity can simply be described as a phenomenon that involves stationary or mobile charged particles. The movement of positively charged particles generates a current. Ohm’s Law states that current (I) is dependent on and proportional to potential difference or voltage (V) within an electrical circuit, while inversely proportional to the resistance (R) of the medium it flows through:

\[ V = IR \]

Electric field (E) is described as the force (F) imposed on a particle and the charge (q) that particle holds:

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

Both electric field and force are vector quantities and thus display direction and magnitude. Therefore, applying an electric field to charged particles causes them to move with magnitude and in a particular direction.

Electric field (E) can additionally be related to electric potential (V), measured in volts, through the equation shown below:

\[ E = \frac{-dV}{dx} \]

This equation shows that electric fields can be described as a measurement of the difference in potential energy and distance between two given points (Colello and Alexander, 2003). As shown in the above equations, electric fields are dependent on electric charges and voltage gradients. Furthermore, they can impose a force on charged particles causing them to move, thus creating an electrical current.
Consequently, electric fields must exist within electrical circuits as shown in Figure 1.3A. Additionally, as shown Figure 1.3B, electric fields lines pass through resistors within a circuit in parallel lines. For further information on the physical basis of electric fields review Colello and Alexander, 2003.

Within physiological fluids electricity is conducted as a result of the movement of ions, rather than free electrons (Colello and Alexander, 2003). This can be observed in epithelial cells that directionally move Na+ ions through their apical side and basolateral side into their extracellular space, through the action of ion pumps or channels, see Figure 1.4A. This creates a separation of charge and transepithelial potential (TEP) across the cell membranes of the epithelial cells. Additionally, tight junctions between these cells generate a high resistance barrier that neither current nor electric fields can cross, limiting the directionality of TEP to across a sheet of cells (McCaig et al, 2005). Transepithelial potential has been studied in intact epithelial cells of vertebrates and measured to be +10 mV to +40 mV, internally positive (Song et al, 2002).

A cut in the epithelium creates a local short circuit in transepithelial potential, dropping it to 0 mV in the area of the wound (Biber and Sanders, 1973). This is due to the formation of a hole through an area that previously exhibited a high resistance seal, through the action of tight junctions. Therefore, a gradient in injury potential, or an electric field, is established, progressively increasing from the wound edge. This creates an injury current towards the center of the wound that can serve as a galvanotactic signal for different cell types (Zhao et al, 2006). This can be visualized in Figure 1.4B.
A. The movement of electrons ($e^-$), or charges, within an electrical circuit causes the formation of a current ($I$). As shown by Ohm’s Law ($V=IR$), for current to flow through resistors ($R$) in an electrical circuit, a voltage ($V$) of some magnitude must be applied. Since electric fields ($E$) are both dependent on the movement of electric charges and voltage, then they must exist within electrical circuits. The directionally of an electric field, within an electrical circuit, shown in this figure is due to the presence of a positive charge. Due to laws of attraction, the electric field will move from the positive (+) to negative (-) terminals of this circuit. B. Resistors ($R$) contain a constant cross-sectional area. Thus, any point within the resistor will display the same electric resistance. Therefore, as currents ($I$) and electric fields pass through a resistor, each point within the resistor will experience an electric field identical in size. This means that the electric field lines passing through a resistor will have the same magnitude and directionality, and will be parallel to one another.
A. In normal conditions, directional ion transport of sodium inwards and chloride outwards form the buildup of positive charge beneath the epidermal layer and negative charge above the epidermal layer. This establishes a transepithelial potential (TEP) as large as 70 mV in mammalian skin. The TEP is further maintained due to tight junctions (red boxes), which cause areas between epithelial cells to be electrically resistant. B. Once mammalian skin is wounded, the TEP at the wound edge short-circuits and drops to 0 mV. This causes ions, mainly Na+, to leak into the wound forming an injury current (black arrows) and an electric field in the dermal layer (blue arrows). In regions distal to the wound edge, cells continue maintain a normal TEP of 70 mV, thus creating a gradient in electric potential towards the wound. Adapted from McCaig et al. (2005) & Vanhaesbroeck (2006).
The formation of an electric field can therefore lead to the re-epithelialization and re-innervation of the wound site and has been tested in literature (Sheridan et al, 1996; Song et al, 2004; Zhao et al, 2006).

Injury currents, measured to be between 10 to 100 $\mu$A/cm$^2$, have also be observed in regenerating amputated limbs of amphibians, such as newts and urodeles (Borgens et al, 1977; Borgens et al, 1979). These currents were found to be moving outwards from amputated, low-resistance, regions and decreased as the limb reformed, and resistance increased (Borgens et al, 1977). Finally, it was shown that these currents are an integral part of the regenerative process of amphibian limbs because the capacity of limb regeneration was enormously reduced when currents were blocked with the use of Amiloride (Borgens et al, 1979). Beyond guiding wound healing in mammals or the regenerative processes of amputated limbs in amphibians, electric fields and currents are similarly implicated as the signal for many aspects of embryonic development.

Intrinsic currents larger than 100 $\mu$A/cm$^2$ have been measured leaving the posterior region of chick embryos and are associated with the formation of its tail end (Hotary and Robinson, 1990). Disruption of these currents, using hollow capillaries, reduced outward currents from the posterior region and subsequently caused the formation deformities in the tail end of chick embryos (Hotary and Robinson, 1992). Currents as large as 100 $\mu$A/cm$^2$ have, also, been measured leaving low resistance areas of the Xenopus ectoderms. The addition of sodium-channel blockers, to disrupt those electrical currents, caused developmental abnormalities in the formation of head structures and neural tube
closure (Hotary and Robinson, 1994). These studies have shown the important role that intrinsic electric fields and currents have in the normal formation of the body, since disruption to these electrical signals causes detrimental developmental abnormalities.

In conclusion, endogenous electric fields are involved within many processes within biological systems. They naturally emerge in embryonic development to guide the formation of the body plan. Later into adulthood, they re-emerge as cues for wound healing in mammals and the regenerative processes in non-mammalian vertebrates. As past research has established the presence of naturally occurring electrical signals in physiological systems, much of current work is on exploring the effect of exogenous electric fields on different cell types. This will be discussed in depth in the next section on in vitro electric field studies.

**In Vitro Electric Field Studies**

All cells contain numerous charged proteins embedded in their plasma membrane, thus under the presence of applied electric field they experience a net force (Colello and Alexander, 2003). Therefore, the behavioral responses of different biological cells to applied direct current electric fields (dcEFs) can be monitored and tested through the use of galvanotaxis chambers. Galvanotaxis chambers are built to have a defined cross-sectional area, so that an electric field or current can be applied to a group of cells. Thus, these chambers allow researchers to apply an electric field in a controlled setting. Additionally, in vitro experimentations with electric fields are fueled by
electrolytic cells, which are redox reactions of a metal electrode driven by an external power source. More details on galvanotaxis chamber and electrolytic cell setup will be reviewed in the materials and methods chapter.

In vitro electric field studies have been investigated in several cell types of different biological systems. The typical observations upon application of an exogenous dcEF to various cell types include changes in migration, proliferation and morphology. In this section, I will start with reviewing studies that have been performed on cells of the central nervous system, since they are directly involved in the pathology of traumatic injuries in that region. Some of the earliest studies regarding the effects of electric fields on cell behaviour were on neurons, due to their ability to direct their growth based on differential signals. Studies by Jaffe and Poo (1979) on embryonic chick dorsal root ganglia showed that electric fields between 70 to 140 mV mm\(^{-1}\) caused neurites to grow faster towards the cathode than anode. Additionally, Gruler and Nuccitelli (1991) showed that applying electric field strengths ranging 7 to 390 mV mm\(^{-1}\) caused neural crest cells to migrate towards the cathode. Therefore, aspects of neuronal growth and migration can be directed by electric fields.

There are several studies on the effects of direct current electric fields (dcEFs) on glial cells as well. In 2008, McKasson et al, showed that embryonic chick schwann cells migrate towards the anode under exogenous electric fields as little as 3 mV mm\(^{-1}\). Additionally, experimentation on astrocytes showed that they align themselves perpendicularly to electric fields between 50 and 500 mV mm\(^{-1}\) (Borgens et al, 1994).
This is particularly important because neurite outgrowth is dependent on the spatial arrangement of astrocytes (Alexander et al, 2006). Later studies, by Huang et al (1997), showed that astrocytes increase their glycolysis rate under electric fields of 0.03 mV mm\(^{-1}\). Thus, electric fields are also capable of affecting the behaviour of glial cells as well.

Though the effects of electric fields on several cell types are well documented, to date there have not been such studies on microglial cell behaviour. However, the effects of exogenous electric fields have been studied on macrophages, which share a lineage with microglia. In 1982, Orida and Feldman showed that macrophages exhibit directed migration towards the anode under exogenous electric fields between 780 to 1170 mV mm\(^{-1}\). Additionally, they displayed increasing migrational speed with increasing electric field strengths. Thus, electric fields direct several different aspects of macrophage migrational patterns.

Based on the effects that electric fields have displayed on cells that share lineage or a tissue environment with microglia, it is plausible to hypothesize that they will also affect microglial cell behaviour in some manner. Thus, this study attempts to explore whether microglia sense physiological electric fields, 0-400 mV mm\(^{-1}\), through examining behaviours changes to cellular morphology. After establishing whether microglia detect physiological electric fields, the next aim of this study is to explore whether they become activated in response to electric fields, through looking at changes in migration, TNF-\(\alpha\) production, or phagocytic rates.
For a summary of cell behaviours seen in a variety of cell types in response to exogenous electric fields see Table 1.2, adapted from Colello and Alexander, 2003.

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>EF Strength (mV mm(^{-1}))</th>
<th>Cell Behaviour Observed</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neural Stem Cells</td>
<td>250</td>
<td>Migrate towards cathode</td>
<td>Li et al, 2008</td>
</tr>
<tr>
<td>Neurons</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Neural Crest</td>
<td>7-390</td>
<td>Migrate towards cathode</td>
<td>Gruler &amp; Nuccitelli, 1991</td>
</tr>
<tr>
<td>Dorsal Root Ganglion</td>
<td>70-140</td>
<td>Grow towards cathode</td>
<td>Jaffe &amp; Poo, 1979</td>
</tr>
<tr>
<td>Hipocampal</td>
<td>28-219</td>
<td>Orientation of growth</td>
<td>Rajnicek et al, 1992</td>
</tr>
<tr>
<td>PC12 Cells</td>
<td>1-5</td>
<td>Grow towards anode</td>
<td>Cork et al, 1994</td>
</tr>
<tr>
<td>Astrocytes</td>
<td>0.03</td>
<td>Enhanced glycolysis rate</td>
<td>Huang et al, 1997</td>
</tr>
<tr>
<td></td>
<td>50-500</td>
<td>Align Perpendicular to EF</td>
<td>Borgens et al, 1994</td>
</tr>
<tr>
<td>Schwann Cells</td>
<td>3-100</td>
<td>Migrate towards anode</td>
<td>McKasson et al, 2008</td>
</tr>
<tr>
<td>Macrophages</td>
<td>780-1170</td>
<td>Migrate towards anode</td>
<td>Orida &amp; Feldman, 1982</td>
</tr>
<tr>
<td>Leukocytes</td>
<td>150-200</td>
<td>Migrate towards cathode</td>
<td>Lin et al, 2008</td>
</tr>
<tr>
<td>Neutrophils</td>
<td>200</td>
<td>Migrate towards cathode</td>
<td>Zhao et al, 2006</td>
</tr>
<tr>
<td>Polymorphonuclear Cells (PMNs)</td>
<td>150-390</td>
<td>Migrate towards anode</td>
<td>Franke &amp; Gruler, 1990</td>
</tr>
<tr>
<td>Muscle Cells</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Myoblasts</td>
<td>36-170</td>
<td>Elongate perpendicular to EF</td>
<td>Hinkle et al, 1981</td>
</tr>
<tr>
<td>Cardiomyocytes</td>
<td>250-500</td>
<td>Elongate perpendicular to EF Cell Differentiation</td>
<td>Sauer et al, 1999</td>
</tr>
<tr>
<td>Osteoblasts</td>
<td>100-1000</td>
<td>Migrate towards cathode</td>
<td>Ferrier et al, 1986</td>
</tr>
<tr>
<td>Osteoclasts</td>
<td>100-1000</td>
<td>Migrate towards anode</td>
<td>Ferrier et al, 1986</td>
</tr>
<tr>
<td>Epithelial Cells</td>
<td>150</td>
<td>Orientation of cell division</td>
<td>Zhao et al, 1999</td>
</tr>
<tr>
<td></td>
<td>25-250</td>
<td>Migrate towards cathode</td>
<td>Zhao et al, 1997</td>
</tr>
<tr>
<td>Keratinocytes</td>
<td>100-400</td>
<td>Migrate towards cathode</td>
<td>Sheridan et al, 1996</td>
</tr>
<tr>
<td>Chondrocytes</td>
<td>80-1000</td>
<td>Migrate towards cathode</td>
<td>Chao et al, 2000</td>
</tr>
<tr>
<td>Fibroblasts</td>
<td>1-400</td>
<td>Cell Differentiation &amp; Elongate Perpendicular to EF</td>
<td>Erickson &amp; Nuccitelli, 1984</td>
</tr>
<tr>
<td>Endothelial Cells</td>
<td>150-400</td>
<td>Migrate towards anode &amp; Align Perpendicular to EF</td>
<td>Bai et al, 2004</td>
</tr>
</tbody>
</table>

Table 1.2: Cell Behaviours Observed Under Applied Electric Fields
CHAPTER 2 Materials and Methods

BV-2 Microglia Cell Culture

The immortalized murine BV-2 cell line was a generous gift from Dr. Block (Virginia Commonwealth University, Richmond, VA). BV-2 maintenance media was composed of Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (HI-FBS), and 1% penicillin/streptomycin (PS; 10,000 units/ml penicillin and 10,000 µg/ml streptomycin). BV-2 Treatment Media was composed of 2% HI-FBS and 1% PS. To seed cells, a 1 ml stock of frozen BV-2 Cells was thawed in a 37°C hot water bath and diluted to 10 ml using cold BV-2 maintenance media. The cells were then centrifuged at 1400 RPM (2100 g) for 4 minutes and the supernatant was discarded. The pellet formed was resuspended in cold BV-2 maintenance media and transferred to a T-75 flask in a 37°C / 5% CO₂ incubator. BV-2 cells were then allowed to attach to the flask bottom overnight and the BV-2 maintenance media was replaced after 24 hours to remove non-adherent cells or any DMSO residue. The cells were then allowed to grow to 80% to 90% confluence in a 37°C humidified atmosphere with 5% CO₂.

To subculture the BV-2 cells, adherent cells were detached from the T-75 flask through incubation with 0.25% Trypsin/EDTA (T/E) in a 37°C humidified atmosphere with 5%
CO₂ for four minutes. The cells that detached in 0.25% T/E solution were then centrifuged at 1400 RPM (2100 g). Afterwards, the supernatant was discarded and the formed pellet of cells was resuspended in pre-warmed BV-2 maintenance media. The cells were seeded at a density of $1 \times 10^6$ cells per T-75 flask with 15 ml of pre-warmed maintenance media and transferred to a 37°C / 5% CO₂ incubator. The cells were grown to 80% to 90% confluence before successive subculturing. Only cells that were in their third to tenth passage were used for experimentation.

A hemacytometer was used to determine the number of cells plated following subculturing or for experimentation. A cell suspension, obtained during passaging steps, was diluted by a factor of 10 in 0.5% Trypan Blue (Sigma-Aldrich) in 0.1 M Phosphate Buffered Saline (PBS). 10 µL of the cell suspension and 0.5% Trypan Blue mixture was then loaded into each hemocytometer well and allowed to spread underneath a coverslip. The number of viable cells within four 4x4 grid areas was counted. Cell number per milliliter was then calculated using the following equation:

$$\text{Cells per ml} = \frac{\text{Total Cells}}{4} \times \text{Dilution Factor} \times 10^4$$

**Galvanotaxis Chamber Setup**

A galvanotaxis chamber was constructed to evaluate the different aspects of microglial behavior that are affected by applied electric fields. The chamber was experimentally designed in a manner such that a constant electric field could be applied to a pre-measure area. Based upon this design, a constant current was calculated using a
known cross-sectional area, known resistivity of media, and desired field strength. The chamber was designed based similarly to descriptions shown in Orida and Feldman, 1982 and were altered to fit each specific experimental design.

To assess microglial migration and phagocytosis rates, a galvanotaxis chamber was built within 50 x 7 mm round glass-bottom dishes (Ted Pella, Inc; Redding, CA). Sterile 22 x 22 mm coverslips (1.5 thickness) were cut in half using a diamond knife. The two halves of the coverslip were then placed parallel to each other on the outer edges of the glass bottom dish and adhered using wax. This created a 10 mm distanced region, where the microglia cells were plated and allowed to attach overnight. Afterwards, a second 22 x 22 mm coverslip (1.5 mm thickness) was adhered on top of the two half coverslips using sterile vacuum grease, and served as the chamber’s roof. This created a chamber with a known cross sectional area of 10 mm (distance) x 0.175 mm (height). Double-sided scotch tape was placed on top of the top coverslip to allow for a larger reservoir of media and prevent media from leaking over the roof of the chamber. Furthermore, areas between the bottom coverslips, scotch tape and the glass bottom dish were sealed off with using hydrophobic, sterile vacuum grease. In all, the grease, coverslips and scotch tape create a sealed chamber with a known area, where a constant electric field of desired strength can be applied. Additionally, holes for the salt-bridges were drilled into the round glass-bottom dish covers to prevent media evaporation. To visualize this galvanotaxis chamber setup see Figure 2.1A.
Figure 2.1: Galvanotaxis Chamber Setup

A. This is a diagram of galvanotaxis setup within a 50 x 7 mm round glass-bottom dish. The area in the middle of the dish is where cells were seeded prior to experimentation.

B. This is a diagram of galvanotaxis setup within a 75 x 50 mm microscope slide. A silicone press-to-seal gasket was used to allow for a reservoir of media on top of the microscope slide.

C. This is a diagram showing a side view of the galvanotaxis chamber of the 50 x 7 mm round glass-bottom dish. The following are the different components of the diagram: (1) Base—composed of 22 x 22 mm half coverslips; (2) Roof—composed of 22 x 22 mm coverslips; (3) Cell Seeding Area.

D. This is a diagram showing how cross-sectional area was determined, where area (A) is calculated through multiplying distance (d) by height (h) of the cell seeding area.
To assess microglial cytokine production, a galvanotaxis chamber was designed on a 75 x 50 mm rectangular microscope slide (Ted Pella, Inc; Redding, Ca) within a 100 mm plastic petridish. A greater seeding area was used for the experiments that assessed cytokine production because larger amounts of samples are needed for ELISAs. The microscope slide was acid-washed using 1N HCl to strip it of any residues and prepare it for cell culture. A press-to-seal silicone gasket (dimensions: 75 (length) x 50 (width) x 0.5 (height) mm) was used to create a hydrophobic barrier and to keep media within a constrained area. Cells were plated within a 32.5 mm distanced region within the middle of the microscope slide. After the cells were allowed to attach to the microscope slide bottom overnight, a 35 x 35 mm microscope slide was added on top of the area where cells were seeded and used as the roof of the galvanotaxis chamber. This created a chamber with a known cross-sectional area, where a constant current can be applied. A petridish cover with holes for salt-bridges was used to prevent media evaporation. To visualize this galvanotaxis setup see Figure 2.1B.

**Electrolytic Cell Setup and Application of an Electric Field**

A constant electric field was applied using electrolytic cell setup. An electrolytic cell involves a redox reaction that occurs with an additional energy source, such as a power supply. The main components of an electrolytic cell include an electrolyte solution, electrodes at the cathode and anode, and salt-bridges. The electrolyte solution used was Steinberg’s Solution, which is composed of 60 mM NaCl, 0.7 mM KCl, 0.8 mM MgSO₄•7H₂O, 0.3 mM Ca(NO₃)₂•4H₂O, and 1.4 mM Tris. Silver wires (Alfa Aesar, Ward
Hill, MA) were gently sanded and chlorided using a solution of 15.8 N Nitric Acid: 6 N HCl: deionized water to generate the Silver Chloride (Ag/AgCl) electrodes. 2% agarose in 2% serum BV-2 media was used to make up salt-bridges, which separate the toxic Ag/AgCl redox reaction products from the galvanotaxis chamber. Finally, a Bio-Rad powersupply was used as an energy source to facilitate the spontaneous redox reaction of the Ag/AgCl electrodes at the anode and cathode. See the equations below for the redox reactions fueled at the cathode or anode:

\[
\text{Oxidation at Anode: } Ag(s) + Cl^- \rightleftharpoons AgCl + e^- \\
\text{Reduction at Cathode: } AgCl (s) + e^- \rightleftharpoons Ag (s) + Cl^-
\]

A Bio-Rad powersupply also supplied a constant current, which moved from the positive (anode) to the negative (cathode) electrode. The following equation was used to calculate the constant current (I) that should be used to achieve desired applied electric field strength (E) based on the cross sectional area of the BV-2 cells plated (A) and the resistivity of BV-2 treatment media (\(\rho\)):

\[E = \frac{I \times \rho}{A} = \frac{I \times \rho}{d \times h}\]

Note, that distance (d) multiplied by height (h) can alternatively used to calculate cross sectional area (A). The different physiological electric field strengths tested were 0 mV mm\(^{-1}\), 4 mV mm\(^{-1}\), 40 mV mm\(^{-1}\) and 400 mV mm\(^{-1}\). Where 0 mV mm\(^{-1}\) represented electric fields (EFs) seen in intact tissue, 40 mV mm\(^{-1}\) represented EFs seen in mammalian tissue injuries, and 400 mV mm\(^{-1}\) represented EFs seen in non-mammalian vertebrate regeneration. See Figure 2.2 to visualize the setup of an electrolytic cell and
Figure 2.2: Setup of an Electrolytic Cell and Application of an Electric Field

The direct current Electric Field (dcEF) is supplied using electrolytic cell setup, where a power supply is used to provide the energy required for a redox reaction of the Ag/AgCl electrodes. 2% agarose salt bridges were used to separate the galvanotaxis chamber, with the BV-2 cells, from toxic byproducts of the Ag/AgCl redox reaction. The Steinberg’s solution served as the electrolyte solution for the reaction. Once the power supply is turned on current flows from the positive to the negative electrode.
the circuit created to apply a constant current. See Table 2.1 for the different currents used to achieve our desired field strengths.

<table>
<thead>
<tr>
<th>$E$ (mV•mm$^{-1}$) desired</th>
<th>$\rho$ (Ω•mm)</th>
<th>$d$(mm)</th>
<th>$h$(mm)</th>
<th>$I$ (mA) used</th>
</tr>
</thead>
<tbody>
<tr>
<td>400</td>
<td>700</td>
<td>10</td>
<td>0.175</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>32.5</td>
<td>0.5</td>
<td>9</td>
</tr>
<tr>
<td>40</td>
<td>700</td>
<td>10</td>
<td>0.175</td>
<td>0.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>32.5</td>
<td>0.5</td>
<td>0.9</td>
</tr>
<tr>
<td>4</td>
<td>700</td>
<td>10</td>
<td>0.175</td>
<td>0.01</td>
</tr>
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<td></td>
<td></td>
<td>32.5</td>
<td>0.5</td>
<td>0.09</td>
</tr>
<tr>
<td>0</td>
<td>700</td>
<td>10</td>
<td>0.175</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>32.5</td>
<td>0.5</td>
<td>0</td>
</tr>
</tbody>
</table>

Table 2.1: Currents Used to Generate an Applied Electric Field

**LPS Positive Controls**

Lipopolysaccharide (LPS) treatment was used as a positive control for the electric field experiments. 5 mg of LPS (E. Coli serotype O111:B4, EMD Millipore, Billerica, MA) was diluted in 2 ml of sterile deionized water. The LPS mixture was then vortexed and incubated in a 37°C hot water bath for 15 minutes. This step was repeated three times to achieve full reconstitution. The mixture was then stored in 10 µl aliquots in -20 C. To obtain a working concentration of LPS, the 2.5 mg/ml LPS mixture (in deionized water) was diluted in BV-2 treatment media to 10 ng/ml. BV-2 cells were then treated with the 10 ng/ml LPS mixture for 3 hours and analyzed accordingly.
BV-2 Microglia Reactivity Test

A four-corner test and nitrite assay was performed to assess BV-2 cell reactivity through indirectly measuring Nitric Oxide (NO) produced. Due to the instability of NO released by microglia, it readily oxidizes into nitrite (NO$_2^-$), which can be measured using a nitrite assay. 0.125 x 10$^5$ BV-2 cells were plated with maintenance media into each well of a 24-well plate and allowed to attach overnight. The media was then changed to treatment media with the following concentrations of LPS: 0 ng/ml, 10 ng/ml, 100 ng/ml, and 1000 ng/ml. The table below represents the 24 well four-corner test setup:

<table>
<thead>
<tr>
<th></th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>0 ng/ml LPS</td>
<td></td>
<td></td>
<td>10 ng/ml LPS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B</td>
<td></td>
<td>100 ng/ml LPS</td>
<td></td>
<td></td>
<td>1000 ng/ml LPS</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 2.2: 24-well Plate Four-Corner Test Setup

BV-2 cells were then treated in a 37°C/5% CO$_2$ incubator for 24 hours. 50 µl duplicate media samples were collected per well and placed into a 96-well and subjected to a nitrite assay. 50 µl of Griess Reagent (1% Sulfanilamide, 0.1% Napthylethylenediamine dihydrochloride, and 2.5% phosphoric acid in deionized water) was added to each well and incubated for 10 minutes in dark conditions. The sulfanilamide in the Griess reagent forms a diazonium salt in the presence of nitrite. The Napthylethylenediamine
dihydrochloride in the Griess reagent is an azo dye agent, which forms a pink color in the presence of the diazonium salt. Absorbance was read at 540 nm using a Pherastar plate reader and measurements were relative to a standard curve. Standard curve r-values >0.98 was used to determine significance of sample measurements.

**BV-2 Timelapse Imaging**

To assess the effects of an applied electric field on the migrational behavior of microglia overtime, spinning disc confocal microscopy was performed at the VCU - Department of Neurobiology & Anatomy Microscopy Facility (supported with funding from NIH-NINDS Center core grant 5P30NS047463 and NIH-NCRR grant 1S10RR027957). BV-2 cells were plated at a density of $1 \times 10^4$ cells per galvanotaxis chamber within 50 mm x 7 mm round glass bottom dishes with BV-2 Treatment Media supplemented with 25 mM HEPES. Differential interference contrast (DIC) images (20x magnification) of migrating BV-2 cells were obtained every 10 minutes for 3-4 hours during electrotaxis under electric field strengths of 0 mV mm$^{-1}$ and 400 mV mm$^{-1}$. 10-13 relatively low cell density regions were chosen and were saved using a .czi format. Time-lapse imaging was analyzed for changes in migration or morphology using Image J (http://imagej.nih.gov/ij/).
Cell Migration Assay

To confirm observations from the time-lapse imaging, the BV-2 cells were also analyzed for changes in migration using a cell migration assay. Before exposure with or without a 400 mV mm\(^{-1}\) (1 mA) electric field, the cells were seeded within a circular area in the 50 x 7 mm galvanotaxis chamber. A circular ring cut from a 1.5 ml microcentrifuge tube was used as a barrier and defined area where the cells were seeded. The barrier was removed upon the start of experimentation the next day. Prior to the electric field exposure, images from five regions per electric field were taken at 10x magnification using Axioimager. After 24-hour electric field exposure, the same 5 regions were reimaged at 10x magnification. Images before and after electric field exposure were assessed to determine whether cells migrated. Additionally, images were analyzed for differences in the migration between cells exposed to different electric field strengths.

Cellular Morphometry

BV-2 cells were exposed to exogenous electric field strengths of 0 mV mm\(^{-1}\) (0 mA), 4 mV mm\(^{-1}\) (0.01 mA), 40 mV mm\(^{-1}\) (0.1 mA) and 400 mV mm\(^{-1}\) (1 mA) for 24 hours. The cells were then fixed in 4% paraformaldehyde. To label the membranes and nuclei of the cells, they were first washed with 0.1% Triton-X 100 in 0.1 M PBS solution for 20 minutes. They were then washed with 5 μl/ml of Vybrant DiO cell-labeling reagent (Invitrogen) and 2 drops/ml DAPI NucBlue (Invitrogen) for 5 minutes. The stained BV-2
cells were coverslipped in vectashield and imaged at 25x magnification using Axioimager. At least 10 fields of view per dish were imaged.

Images were analyzed for cellular and nuclear morphology using Image J. To assess cellular morphology, cells were first outlined, made binary, and measured for cell area and perimeter. Using cell area and perimeter, a parameter called form factor was calculated to assess the roundness or circularity of each cell. Perfectly circular objects have a form factor value of 1, while lower values represent irregularly shaped objects, such as rod-like structure. See below for the equation used to obtain form factor:

\[
\text{Form Factor} = \frac{4\pi \text{Cell Area}}{\text{Cell Perimeter}^2}
\]

The form factor parameter was used to analyze whether most cells were amoeboid versus displaying morphologies with cytoplasmic projections. At least 400 cells per field strength were measured from more than 10 fields of view per dish. For nuclear morphology, nuclei were outlined, made binary, and the area was measured. At least 400 nuclei per field strength were counted from more than 10 fields of view per dish.

**Phagocytosis Assays**

A phagocytosis assay was used to compare the percentage of BV-2 cells that were phagocytic when exposed to 0 mV mm\(^{-1}\) (0 mA) versus 400 mV mm\(^{-1}\) (1 mA) electric fields. Since BV-2 cells increase their phagocytic rate upon activation with LPS, a different group of cells was treated with 10 ng/ml of LPS as a positive control. The cells were seeded at a density of 2 x 10\(^4\) cells per galvanotaxis chamber (within 50 mm x 7
mm round glass bottom dishes) overnight in maintenance media. The following day, the cells were exposed to their respective applied electric field strength or LPS treatment for 3 hours. At the end of the 3-hour experiment, BV-2 cells were incubated with $1 \times 10^6$ carboxyl-coated Nile Red Latex beads (1.99 µm; Spherotech, Lakeforest, IL) in a dark 37°C humidified atmosphere with 5% CO$_2$ for one hour. Afterwards, the cells were washed five times with 37°C sterile 0.1 M PBS (Invitrogen) to remove non-phagocytosed beads. They were then fixed with 4% paraformaldehyde in 0.1 M PBS. 20x magnification DIC images were taken from at least eight randomized regions. The percentage of cells that had phagocytosed one or more beads was measured by using the equation below:

$$\text{% Phagocytic Cells} = \frac{\text{Cells that had phagocytosed } \geq 1 \text{ bead}}{\text{Total Cells Counted}}$$

The images were assessed using ImageJ. > 200 cells were counted per field strength using the Cell Counter plugin (http://fiji.sc/Cell_Counter).

**TNF-α ELISA**

BV-2 cells were exposed to an exogenous electric fields of the following strengths: 0 mV mm$^{-1}$ (0 mA), 4 mV mm$^{-1}$ (0.09 mA), 40 mV mm$^{-1}$ (0.9 mA), and 400 mV mm$^{-1}$ (9 mA). Media samples from the cells were then collected in a 96-well cell culture plate to perform a sandwich Enzyme linked Immunosorbent Assay (ELISA), to measure the amount of the pro-inflammatory cytokine Tumour Necrosis Factor-alpha (TNF-α) released. A mouse TNF-α Duoset kit (R&D Systems, Minneapolis, MN) was used to measure TNF-α produced by the BV-2 microglia. 100 µl of capture antibody diluted in
0.1 M PBS (0.8 µg/ml) was added to each well of a Nunc Maxisorp 96-well plate and incubated at room temperature overnight. The next day, each well was blocked using 300 µl of a 1% BSA and 5% sucrose in 0.1 M PBS solution for at least one hour. Samples were diluted by a factor of five in reagent diluent (1% BSA in 0.1 M PBS) and added to the Nunc 96-well plate. Each well was then incubated with 100 µl of detection antibody diluted in reagent diluent (150 ng/ml) for two hours. Afterwards, each well was incubated with 100 µl of Streptavidin-HRP diluted in reagent diluent (1:200) for 20 minutes in the dark. Wells were washed between each step using PBS-T (0.05% Tween-20 in 0.1 M PBS). 100 µl of 3,3’,5,5’-tetramethylbenzidine (TMB) liquid substrate system (Sigma-Aldrich Co. LLC) was added for 20 minutes. 50 µl of 2N H₂SO₄ stop solution was added to each well. Optical densities (OD) were then determined immediately at 450 nm using a Pherastar microplate reader. The OD of the samples were determined relative to a standard curve. A standard curve r-value >0.98 was used to determine significance of sample measurements.

**Statistical Analysis**

The data was analyzed for significance and graphed using JMP Pro 11 software (http://www.jmp.com/software/pro/) and Microsoft Excel. Specifically, ANOVA with Tukey-HSD post-hoc test was utilized with significance determined at p-values <0.05. Values in the results section represent Mean ± Standard Error Mean (SEM).
CHAPTER 3 Results

Identity of Cells Confirmed

Though electric field experimentation has been performed on other cell types of monocyte lineage (Orida and Feldman, 1982), none have specifically investigated the effect that they have on microglia. Thus, this project explores whether exogenous electric fields of different physiological strengths alter microglial cell behaviour. The cells obtained for this study were an immortalized (BV-2) murine microglial cell line. Therefore, the first step of this study was to affirm the identity of the cells obtained as BV-2 microglia. They were identified based upon the following features: morphology, Nitric Oxide (NO) secretion upon stimulation with lipopolysaccharide (LPS), and phagocytic activity.

The cells were first identified based upon morphological features. Imaging at 20x magnification showed a cell population that was heterogeneous in morphology (Figure 3.1A). Morphology ranged between small round cells to flat cells with cytoplasmic projections that varied in number. The varied morphologies detected were concurrent with previous studies that highlight morphologies found in BV-2 microglial cell culture.
A. The cells obtained for experimentation were first confirmed for their identity as BV-2 microglia through analysis of cell morphology. Cell cultures treated with (B) and without (A) 10 ng/ml of LPS both displayed a heterogeneous population of cells ranging from amoeboid cells (yellow arrows) to cells with cytoplasmic projections (red arrows). Unlike Primary microglia, BV-2 cells do not undergo morphological changes upon activation with LPS. Thus, confirming that the cells are not a primary cell culture. C. BV-2 microglia were also incubated with Nile Red carboxyl-coated latex beads. Images showed that the cells obtained are capable of phagocytizing beads (purple arrows). 25x magnification. Scale Bar= 50 µm
(Bocchini et al, 1992). BV-2 microglia were then exposed to 10 ng/ml of LPS for three hours. 20x magnification images showed that the cell culture displayed the same heterogeneity in cellular morphology observed for cells without LPS treatment (See Figure 3.1B). Unlike primary microglia, BV-2 cells do not display a change in cellular morphology upon activation with LPS stimulation (Stansley et al, 2012). Therefore, the lack of changes in cellular morphology after treatment with LPS is expected.

Since immortalized BV-2 cells are genetically modified, the next step was to affirm whether the cells obtained are able to perform the same behavioral functions as observed in BV-2 microglia or other microglial cells at baseline. This was first explored through assessing BV-2 phagocytic activity. At baseline, 20x imagining showed the cells obtained are capable of phagocytizing nile red carboxyl-coated fluorescent beads, as expected for BV-2 cells (Figure 3.1C).

Furthermore, BV-2 microglia are capable of displaying reactivity in response to stimulation with lipopolysaccharide (LPS), through releasing Nitric Oxide (NO). Since nitric oxide is unstable, once it is released by microglia it readily oxidizes into nitrite. Therefore, a nitrite assay was used to indirectly measure the amount of NO released by microglial cells. The assay is colorimetric therefore color changes occur after incubation with Griess Reagent when nitrite is detected (See Figure 3.2). The four-corner test and subsequent nitrite assay performed indirectly showed increased levels of NO production, through measurements of nitrite, upon 24-hour incubation with increasing levels of the following concentrations of LPS: 10 ng/ml, 100 ng/ml, and 1000 ng/ml.
Figure 3.2: Nitrite Assay Color Changes

This is an image of color changes observed within a 96-well plate after a nitrite assay on samples from a BV-2 microglia four-corner test. The LPS concentrations used in the four-corner test were: 0 ng/ml (A), 10 ng/ml (B), 100 ng/ml (C) and 1000 ng/ml (D). As more nitrite is detected with Griess Reagent the sample color becomes a darker shade of pink. Therefore, as expected samples from microglial cells exposed to 1000 ng/ml LPS were the darkest shade of pink, while samples from cells that were not exposed to any LPS were beige or clear. This assay is an indirect measure nitric oxide produced by microglial cells. Thus, the amount of nitrite detected is used as a relative measure of nitric oxide produced by the cells. The bottom two rows of the 96-well plate in the image were used to establish a standard curve, which was used to determine sample concentrations of nitrite in μM. Only sample measurements from assays with standard curve r-values>0.98 were considered significant.
Without LPS stimulation the cells obtained had a mean nitrite levels of 0.840 µM ± 0.24 measured (See Figure 3.3). However, BV-2 cells exposed 10 ng/ml LPS had a mean 16.420 µM ± 1.368 (p<0.001) nitrite measured, 100 ng/ml had a mean 29.401 µM ± 1.496 (p<0.001) nitrite measured, and 1000 ng/ml had a mean 34.719 µM ± 1.743 (p<0.01) nitrite measured (See Figure 3.3). Since the cells obtained secreted NO that was dependent on LPS concentration, it was confirmed that the cells are reactive. Additionally, it confirmed that LPS could be used as a positive control for studies on electric fields and the activation of BV-2 microglial cells.

**Timelapse Experimentation**

After confirming that the BV-2 cells obtained displayed similar behavioural characteristics to other microglia, timelapse experimentation was performed to examine whether 400 mV mm\(^{-1}\) applied electric fields affected the migratory behaviour or morphology of BV-2 microglia. Initial studies showed membrane blebbing and apoptosis of the BV-2 cells with or without an electric field in the timelapse environment within one to two hours of experimentation. This was attributed to the abrupt increase of the BV-2 media’s pH to >10.0 while in the time-lapse environment. Control cells that remained in the lab incubator were not apoptotic and their media’s pH remained between 7.0-7.5. Supplementation of 25 mM HEPES to BV-2 treatment media allowed for timelapse imaging experimentation for a maximum of four hours (See Figures 3.4 and 3.5).
Figure 3.3: Nitrite Levels Detected After a Four-Corner Test

BV-2 microglia were treated with the following concentrations of LPS: 0 ng/ml, 10 ng/ml, 100 ng/ml, and 1000 ng/ml. After 24 hours of treatment, media samples were measured for nitrite levels through the use of Griess Reagent. Since nitrite oxide (NO) is unstable and spontaneously oxidizes into nitrite, nitrite assays are used as an indirect measure of NO released by microglia. As expected, increasing levels of LPS increased NO production by BV-2 cells. Additionally, cells that were not treated with LPS released negligible amounts of NO. Measurements shown are mean ± SEM from 3 experiments. ANOVA with Tukey-HSD post-hoc test (**p<0.01, ***p<0.001).
Figure 3.4: Control BV-2 Cells in Timelapse

DIC images (20x magnification) for the control BV-2 cells, under an applied electric field of 0 mV mm$^{-1}$, were taken every 10 minutes for four hours using a Zeiss spinning-disk confocal microscope. Hourly images of the BV-2 cells showed no directional migration. Additionally, cells were heterogeneous morphology during the entire experiment, displaying amoeboid morphologies and morphologies with cytoplasmic projections. Note, that a subset of cells had undergone division (green arrows). Scale Bar= 50 µm
DIC images for experimental BV-2 cells, under an applied electric field of 400 mV mm\(^{-1}\), were taken every 10 minutes for four hours using a Zeiss spinning-disk confocal microscope. Similarly to BV-2 Cells that were not exposed to an electric field (Figure 3.2), cell exposed to a 400 mV mm\(^{-1}\) electric field did not display observable differences in cellular morphology. BV-2 cells were heterogeneous displaying amoeboid phenotypes or morphologies with cytoplasmic projections. Hourly images of the BV-2 cells showed the absence of directional migration (red arrows). Note that a subset of cells had undergone division during the experiment (green arrows). Additionally, membrane blebbing was observed in a subset of cells starting at hour 2 (white arrows; C, D, & E). Scale Bar= 50 µm
Four-hour exposure to 400 mV mm$^{-1}$ electric fields (See Figure 3.5) did not cause BV-2 cells to migrate. Cells that were not exposed to an electric field, 0 mV mm$^{-1}$, did not migrate as well (See Figure 3.3). Additionally, BV-2 cells remained heterogeneous in morphology with or without exposure to an exogenous electric field, 400 mV mm$^{-1}$.

Though changes in morphology or migrational pattern were not readily observed, membrane blebbing was observed in cells under an applied electric field at hour 2 of experimentation (Figure 3.5C). Additionally, the pH of media from both cell populations was between 8.0-8.5. This suggests that the BV-2 microglia did not migrate due to the pH changes in the timelapse environment. Thus, all of the following experimentation was not performed in the timelapse environment and were conducted in a 37°C/ 5% CO$_2$ incubator located in the laboratory.

**Migration Assay**

Due to the possibility that the pH fluctuations observed within the timelapse environment caused BV-2 cells to remain stationary, a migration assay was additionally performed. Cell clusters were assessed for changes in the migration front before and after 24-hour application of electric fields of the following strengths: 0 mV mm$^{-1}$ and 400 mV mm$^{-1}$.

Both cell clusters showed movement of the cell front after 24 hours of experimentation (Figure 3.6). However, there was not an apparent difference in the cell front movement of cells exposed to an electric field and those that were not (Figure 3.7). Although difference in cell front was observed after 24-hour experimentation, there were
Figure 3.6: Migration Assay of Control BV-2 Cells

Phase Images (10x magnification) for the control BV-2 cells, exposed to 0 mV mm$^{-1}$, were taken before (A) and after (B) 24-hours of experimentation. Note, that the cell front has shifted after 24 hours. Before experimentation a sharpie was used to outline the cell boundary, which was a reference point for movement of cell front (purple arrows). Scale bar= 200 $\mu$m
Figure 3.7: Migration Assay of BV-2 Cells Under 400 mV mm$^{-1}$ Electric Field

Phase Images (10x magnification) for BV-2 cells exposed to a 400 mV mm$^{-1}$ electric field were taken before (A) and after (B) 24-hours of experimentation. Note, that the cell front has shifted after 24 hours. However, it is difficult to determine whether there are any differences in cell front movement between cells that were or were not exposed to an electric field. Before experimentation a sharpie was used to outline the cell boundary, which was a reference point for movement of cell front (purple arrows). Scale bar= 200 µm
limitations in the method of analysis. BV-2 microglia are highly proliferative cells, thus changes to the changes in cell front observed could be due to an increase in cell number rather that the migration of cells.

**Morphological Changes**

The lack of difference in morphology between BV-2 cells that were or were not exposed to an electric field could be attributed to pH fluctuations and that timelapse imaging was limited to four hours. Thus, cells were assessed for changes in cell or nuclear area and form factor after 24-hour electric field exposure within a 37 °C/5% CO₂ incubator and a subsequent DiO and DAPI labeling. Our study showed that BV-2 cells exposed to exogenous electric fields displayed changes in cell area that correlated with electric field strength (See Figure 3.8). For example, cells that were not exposed to an electric field had a mean cell area of 186.66 µm² ± 2.94. However, cells exposed to electric fields of 4 mV mm⁻¹, 40 mV mm⁻¹, and 400 mV mm⁻¹ had mean cell areas of 285.56 µm² ± 4.80, 341.26 µm² ± 6.33, and 472.72 µm² ± 10.49 respectively (See Figure 3.9A). Therefore, BV-2 cells responded to higher field strengths through significantly increasing their cell area in a graded manner (p<0.001).

Changes to cellular morphology were also exhibited through changes to form factor, a dimensionless term that determines the circularity of an object (See Figure 3.8). Perfect circles have a form factor value of 1, while irregularly shaped objects display lower form
Following 24-hour exposure to electric fields of various magnitudes, > 2000 BV-2 cells were measured for cell area and form factor using ImageJ. This image shows outlines of the cells exposed to electric fields of the following strengths were obtained: 0 mV mm\(^{-1}\) (A) and 400 mV mm\(^{-1}\) (B). As shown in the figure, cells that were exposed to larger electric fields (B) were much larger and displayed more profound cytoplasmic projections than cells that were not exposed to an electric field (A).
Figure 3.9: Cellular Morphology Under Different Electric Field Strengths

After 24-hour exposure to electric fields of various magnitudes, > 2000 BV-2 cells were measured for cell area and form factor using ImageJ. **A.** The mean cell area of microglial cells increased linearly with increasing electric field strengths. **B.** Form factor is a dimensionless term used to describe cellular roundness. A form factor value of 1 describes perfect circles, while lower numbers describe irregularly shaped objects. In this study, form factor was related to electric field strength in an inverse manner. As electric field strengths were increased, BV-2 cells displayed lower form factors. Thus, under increasing field strengths cells were less circular and displayed cytoplasmic projections. **C.** Exposure to an electric field caused cells to increase their nuclear size but the effect seen was not dose dependent. All measurements graphed are mean ± SEM. ANOVA with Tukey-HSD post-hoc test (*p<0.05, **p<0.01, ***p<0.001).
factor values. Form factor was used to determine whether BV-2 cells displayed amoeboid, or circular, morphologies versus morphologies with cytoplasmic projections. Experimentation showed that BV-2 cells exposed to exogenous electric fields displayed changes in form factor that was dependent on electric field strength. For example, cells that were not exposed to an electric field had a mean form factor of 0.7136 ± 0.0063 (p<0.001). However, cells exposed to electric fields of 4 mV mm⁻¹, 40 mV mm⁻¹, and 400 mV mm⁻¹ had mean form factors of 0.6356 ± 0.0082 (p<0.001), 0.5955 ± 0.0084 (p<0.01), and 0.5572 ± 0.010 (p<0.05) respectively (See Figure 3.9B). The data obtained therefore suggests that as electric fields increase, BV-2 microglial cells were less circular and displayed cytoplasmic projections.

Measurements for nuclear morphology also showed changes that were dependent on exposure to an electric field (See Figure 3.9C). Control BV-2 cells, which were not exposed electric field, had a mean nuclear area of 68.49 μm² ± 0.82, which was significantly lower (p < 0.001) than for cells exposed to an electric field. However, cells exposed to electric fields of 4 mV mm⁻¹, 40 mV mm⁻¹, and 400 mV mm⁻¹ had mean nuclear areas of 80.81 μm² ± 0.89, 81.97 μm² ± 0.96, and 86.03 μm² ± 1.43 (p<0.01) respectively. Although the increase in nuclear area is not as linear as the changes in cell area, there was a significant increase in nuclear area of cells exposed to an electric field.
Phagocytosis

After investigating whether BV-2 microglia can sense electric fields, we explored whether electric fields can initiate activation. Phagocytosis was the first behaviour investigated to assess activation, since microglia are known to increase phagocytic activity upon exposure to stress signals. BV-2 microglia are capable of phagocytizing carboxyl-coated latex beads (Bocchini et al., 1992). Therefore, a phagocytosis assay was developed to assess changes to microglial phagocytic activity under an electric field. BV-2 cells that were treated with 10 ng/ml LPS were a positive control for phagocytic activity and cells that were not treated with LPS or an electric field were also assessed. Following 3-hour experimentation with each treatment or applied electric field and one hour phagocytosis assay, the number of cells that had phagocytosed and those that had not (See Figure 3.10 A-C) was determined. BV-2 cells exposed to an electric field of 400 mV mm\(^{-1}\) did not show a difference in the percent of cells that had phagocytosed latex beads when compared to control cells that were not exposed to an electric field. Cells under a 400 mV mm\(^{-1}\) electric field had 10.91% ± 0.95 phagocytic cells and cells that were not exposed to an electric field had 11.18% ± 1.01 phagocytic cells. However, cells that were treated with 10 ng/ml LPS had 16.23% ± 1.01 phagocytic cells, p < 0.05 (See Figure 3.11). Based on the data obtained, BV-2 cells that were exposed to a 400 mV mm\(^{-1}\) electric field and control cells had about the same percentage of phagocytic cells in culture. Additionally, cells that were treated with LPS did show a significant increase in the percentage of phagocytic cells.
**Figure 3.10: Phagocytosis of Latex Beads**

**A-B.** After 3 hours of experimentation, imaging (20x magnification) showed that relatively the same percentage of BV-2 cells were phagocytic (red arrows) with (A) and without (B) 400 mV mm\(^{-1}\) electric field exposure. **C.** BV-2 cells that were treated with 10 ng/ml LPS, as a positive control, however showed a slightly increased percentage of phagocytic (red arrows) cells. Scale Bar= 50 µm
Figure 3.11: Percent of Phagocytic Cells

After 3-hour electric field experimentation and a 1-hour phagocytosis assay, there was no difference in the percentage of phagocytic cells with and without 400 mV mm\(^{-1}\) electric field exposure. However, there was a slight increase in the percentage of phagocytic cells when BV-2 cells were treated with LPS. ANOVA with Tukey-HSD post-hoc test. (*p<0.05)
TNF-α Production

Release of the pro-inflammatory cytokine, TNF-α, is a common characteristic of M1, or classically activated, microglia. Thus, media samples from BV-2 cells exposed to electric field strengths of 0 mV mm$^{-1}$, 4 mV mm$^{-1}$, 40 mV mm$^{-1}$, and 400 mV mm$^{-1}$ following three and six hours of exposure were collected and tested for TNF-α production using an ELISA Duoset kit. Three and six hour time points were used because TNF-α release occurs as early as 3 hours after exposure to a stimulant. A subset BV-2 cells were also treated with 10 ng/ml LPS for three hours and used as a positive control. As expected the 0 mV mm$^{-1}$, did not release any TNF-α. Similarly, BV-2 cells exposed to an electric field did not release any TNF-α at all time-points tested (See Figure 3.12). However, the BV-2 cells treated with LPS secreted a mean 1201.56 pg/ml ± 41.29 (p<0.001) TNF-α. Since the positive control cells showed profound TNF-α release, it is affirmed that electric fields do not induce BV-2 microglia to release TNF-α.
Figure 3.12: TNF-α Release Under Different Electric Field Strengths

The BV-2 microglia were exposed to electric fields of the following strengths for 3 and 6 hours: 0 mV mm$^{-1}$, 4 mV mm$^{-1}$, 40 mV mm$^{-1}$ and 400 mV mm$^{-1}$. BV-2 cells were also treated with 10 ng/ml LPS for 3 hours as a positive control. Media samples were collected and TNF-α levels were measured using an ELISA kit. BV-2 cells that were exposed to electric fields did not release any TNF-α. However, cells that were treated with LPS released >1000 pg/ml TNF-α. A standard curve was used to determine sample concentrations of TNF-α in pg/ml. Only sample measurements from assays where standard curve r-values>0.98 were considered significant. Measurements shown are mean ± SEM from 3 experiments. All measurements graphed are mean ± SEM. ANOVA with Tukey-HSD post-hoc test (***p<0.001).
CHAPTER 4 Discussion

BV-2 Cells Can Detect Electric Fields

As mentioned beforehand, there are no previous research studies documenting the effects that exogenous electric fields have on directing microglial cell behaviour. Thus, the first aim of this study was to elucidate whether BV-2 microglia are capable of detecting exogenous electric fields through investigating changes to migration and cellular morphology. Morphometric studies on the BV-2 cells exposed to an electric field showed that they increased their cell area and decreased their form factor in a dose-dependent manner. This suggests that BV-2 cells are capable of detecting an electric field and respond by changing their cell area and forming cytoplasmic projections.

Although changes in cellular morphology have not been directly linked with the activation state or reactivity of BV-2 microglial cells (Henn et al, 2009), studies on the morphometry of microglia or other cell types can provide an explanation for the changes in cell area observed. One explanation for the increase in cellular area observed in BV-2 cells is that they could be adopting a large, multinucleated cell phenotype. Experimental studies on microglia and macrophages showed that a great number of large multinucleated cells were present after exposure to a pro-inflammatory signal.
(Leskovar et al, 2001; Hornik et al, 2014). Thus, the increase in BV-2 microglial cellular area could be in response to the electric field mimicking a stress signal.

Studies by McHugh et al, (2014) on BV-2 microglia revealed that the number of cells with cytoplasmic projections or branched morphology increased upon treatment with the neuronal signaling molecules Δ⁹-THC and N-arachidonoyl glycine (NAGly). The higher incidence of those morphologies was attributed to Δ⁹-THC and NAGly inducing “ramification” or branching to increase cell-cell contact between cells. Additional studies have shown that branched microglia can contact neuronal synapses and protect them against excitotoxicity (Fontainhas et al, 2011; Tremblay et al, 2010; Vinet et al, 2012). Thus, application of an electric field could be inducing cell branching and spreading to increase cell-to-cell contact by affecting their cytoskeletal structure. This is further supported by galvanotaxis studies on other cell types, which show that applied electric fields can directly affect the cytoskeletal structure of different cell types and induce changes to cell morphology (Onuma and Hui, 1988; Titushkin and Cho, 2009).

In brief, BV-2 cells changed their cellular morphology in a predictable manner following exposure to an electric field. Many research studies on cellular morphometry provide insight into why electric fields conferred those morphological changes in BV-2 microglia. In all, this study confirmed our hypothesis for our first aim that BV-2 cells are capable of detecting electric field.
Electric Fields Did Not Induce BV-2 Cell Migration

The timelapse studies and migration assays both showed that BV-2 microglia did not display a considerable difference in migration with and without electric field exposure. A factor that can explain the lack of migration observed during the microglial cell timelapse experimentation or migrational assays is the electric field strength used. Though directed migration towards the anode was observed in monocyte lineage cells, the electric field strengths tested were greater than 780 mV mm$^{-1}$ in strength (Orida and Feldman, 1982). Thus, their lowest experimental electric field strength tested was about twice the size of the electric field we tested (Marioty and Borgens, 1999). Additionally, in an in vivo study on spinal cord injuries, it was revealed that applied electric fields within physiological range, 400 µV mm$^{-1}$, did not affect the migration of macrophages into the injury site. The authors concluded that electric fields of physiological strength probably played a role in mediating macrophage behaviour during CNS injuries in a subtle manner.

Another reason why directed migration was not observed in BV-2 cells under an exogenous electric field can be attributed to other factors such as limitations of the experimental paradigm used. For example, at the end of the four-hour timelapse experiment, the cell media’s pH was measured at 8.0-8.5, suggesting that the lack of migration was probably cellular stress. Additionally, the before and after 10x magnification phase images from the migrational assay were difficult to assess for precise changes in migration. BV-2 cells display a very high proliferation rate and are
constantly changing their morphology through extending and retracting cytoplasmic projections. Thus, it was difficult to identify individual cells before and after experimentation, to use for measurements of migrational rate. It was additionally difficult to determine whether any changes in the cell front were due to increased proliferation versus migration.

Though there were some limitations in the experimental design, the BV-2 cells that were tested did not show an observable change in migrational rate upon exposure to an electric field. This suggests that the lack of migration could be due to the magnitudes of electric field strengths tested. Since the aim of this study was to determine whether microglia are capable of detecting electric field strengths that are within physiological range, it is plausible to deduce that endogenous electric fields of physiological strength do not direct BV-2 migration but do change their morphology.

**Electric Fields Could Be Modifiers of M1/M2 Polarization**

After showing that BV-2 cells are capable of detecting electric fields, the next aim was to explore whether electric fields can initiate behaviours associated with activation. Although cells did not release TNF-α upon exposure to electric fields as large as 400 mV mm⁻¹, they produced >1000 pg/ml TNF-α after treatment with LPS. Since LPS is a classic activator of M1 activity, this suggests that the applied electric fields did not activate BV-2 microglia. Additionally, the same effect was seen when measuring the percentage of phagocytic cells, where electric fields did not induce a change in the
number of phagocytic cells but treatment with LPS did. Therefore, it is possible that electric fields modulate, rather than initiate, microglial activation or that they affect other activation behaviours that have not been investigated.

As a reminder, activated microglia can further be classified into two different phenotypes after activation, M1 and M2. M1 cells are typically associated with neurodegeneration, since they release a wide range of pro-inflammatory cytokines and reactive oxidative species. However, M2 cells are associated with neuroregeneration, since they release a wide range of neurotrophic factors and anti-inflammatory cytokines. Many studies on the different factors that modulate M1/M2 polarization in microglia employ the use of LPS as a pre-treatment, since it is a known activator of the M1 response. For example, studies on minocycline showed that it induced anti-inflammatory effects through selectively blocking M1 polarization in LPS activated microglia (Kobayashi et al, 2013). Additionally, a study by Shen et al (2014) showed that exposure to electromagnetic fields reduced the release of the pro-inflammatory cytokine, IL-6, in BV-2 microglia after activation with LPS.

In conclusion, our study showed that electric fields did not activate microglia. This could be due to electric fields playing a role in modulating activated microglial behaviour. Therefore, it is possible that signals other than electric fields play a bigger role in activating microglia, while electric fields modulate microglial activity once they have become activated. Finally, it is possible that electric fields could be directing other
activation-related behaviours that were not investigated in this study, such as nitric oxide release.

**Limitations of Using a Cell Line**

When conducting in vitro research, there is an option of either using primary cells or immortalized cell lines. Primary cells are directly isolated from an animal and are the closest in behavior to cells in vivo (Stacey, 2005). However, primary cells are typically too time-consuming and expensive to maintain (Stansley, 2012). Thus, immortalized cell lines are used due to their ability to escape cell cycle control and grow indefinitely. In this study, an immortalized murine BV-2 microglia cell line was utilized to investigate the effects of applied electric fields on microglial cell behaviour. To develop the BV-2 microglia cell line, neonatal murine microglial cells were isolated and then purified through agitation and adhesion. Then, the cells were transformed through incubation with a recombinant J2 retrovirus with v-myc and v-raf oncogenes for three weeks to achieve immortalization (Blasi et al., 1990). Although the generation of this immortalized cell line combats issues associated with the harvest and maintenance of primary microglial cells, there are many limitations that emerge with the use transformed cells (Hovarth, 2008).

Through the process of immortalization, cells undergo numerous genomic alterations to their growth properties, which can subsequently affect their behaviour in comparison to primary cells. Though the observations of BV-2 cell line limitations were initially
anecdotal, several contemporary research studies have now documented their difference in behaviour in comparison to primary cells. In 2000, Stochwasser et al. showed that BV-2 cells are not as responsive to LPS stimulation as primary murine microglia. As stated previously, LPS is the most reliable model in achieving classic, or M1, activation in microglia. Thus, higher levels of LPS are needed to achieve the activation-based behaviors in BV-2 cells than in primary cells. Additionally, It was later shown that the up-regulation of activation-associated genes was less pronounced in BV-2 cells (Henn et al, 2009). Other research studies have shown that BV-2 cells displayed a decreased ability to release IL-12 and several other cytokines or chemokines (Hausler et al, 2002). Due to genetic modification, it should also be noted that BV-2 cells have an increased proliferative and adhesive capability in comparison to primary microglia (Hovath et al, 2008).

The use of immortalized cell lines can cause limitations on the behaviours that can be investigated when studying the effects of electric fields. For example, BV-2 cells have only been well-documented for studies on cytokine or nitric oxide release (Watters et al, 2002; Gresa-Arribas, 2012), phagocytosis (Shi et al, 2013), and chemotaxis or migration following activation (Zierler et al, 2008). Additionally, due to the genomic alterations to the growth properties of BV-2 cells, investigating proliferation rates under applied electric fields would be unreliable. Therefore, though BV-2 cells are useful for initial investigations on the effects of exogenous electric fields. The limitations and differences observed in this cell line warrants that this study should be repeated in primary microglia to confirm the observations.
Limitations of In Vitro Studies

Since there are not any documented studies on electric fields and microglia, the use of in vitro studies are invaluable to directly assess the effects they have on directing microglial behaviour. Although in vitro studies are essential in the beginning stages of studying the effect of exogenous electric fields on individual cell types, there are many limitations to keep in mind while conducting them. For example, it is difficult to maintain a stable environment when conducting in vitro studies, due to the presence of several external factors that need to be individually controlled. Although care was taken to ensure that the stability of the experimental environment, fluctuations in temperature, pH, and salt concentration can occur. Other than the environmental concerns of in vitro studies, there are also documented differences in microglial behaviour within in vitro conditions.

As surveyors of the CNS environment, microglia are particularly sensitive to signals from various cell types within their environment. Therefore, harvested microglia or microglia in cell culture can exhibit different behaviours when they are removed from CNS tissue. This is particularly highlighted in several studies on the electrophysiological properties of microglia in brain slices versus cell culture. Recordings from resting cells within cell culture showed that they displayed membranes with a much more reactive profile than microglia within brain slices (Boucsein et al, 2000; Kettenman et al, 1990; Norenberg and Gebicke-Haerter, 1994). Additionally, microglia that are typically used in
in vitro studies are isolated from neonatal brains, which can display different behaviours than matured cells within in vivo conditions.

Conclusion

In this study, BV-2 microglial cells changed their cellular morphology in response to applied electric fields. Though the exact reasoning behind the basis of those changes remains unknown, other studies on the microglial cell morphology suggest that the electric field could be inducing cytoplasmic projections to increase contact between cells. This is a neuroprotective activity seen in “resting” microglia during neurotoxicity. Additionally, it was shown that electric fields did not activate microglial cells nor increase TNF-α release or phagocytosis. This suggests that electric fields could play a role in modulating the behaviour of microglia rather than changing their activation state. In all, this study demonstrated that microglia are capable of sensing electric fields, but the extent to which they affect their behaviour as immune cells has yet to be explored.
Literature Cited


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