THE MITOCHONDRIAL MEMBRANE POTENTIAL AS A SCREENING TOOL FOR IMMUNOSTIMULATION.

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Abstract

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The rise of neuroinflammatory disorders highlights the importance of early detection and intervention for more effective treatment options. Neuroinflammation is associated with the pathogenesis of many neurological disorders, including Major Depressive Disorder, Alzheimer's disease, and Multiple Sclerosis. There has been a focus on neurons to advance our understanding of the underlying mechanisms of neuroinflammation and its role in neurodegeneration. However, recent studies have highlighted the pivotal role of glial cells, particularly microglia, in neuroinflammation due to their active participation in the immune response. This study investigates glial-specific indicators of morphology, metabolic changes, and drug efficacy in neuroinflammatory conditions. By analyzing the glial-specific activity of microglia at various levels, we uncover distinct patterns that correlate with disease progression. These findings offer insights into the early stages of neurological diseases and provide potential biomarkers for early detection. Moreover, this study explores the modulation of microglial metabolic activity as a screen for therapeutic approaches. We examine the impact of pharmacological interventions on

glial cells and their subsequent effects on disease outcomes. In summary, this dissertation expands the current understanding of the dynamic interplay between glial cells, neuroinflammation, and disease progression, ultimately contributing to the development of a novel drug screening tool. Identifying glial-specific metabolic indicators and evaluating drug efficacy offer promising avenues for early detection and targeted therapeutic interventions in neurological diseases.

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Dedication

To my beloved mother, my dear sisters, and my supportive boyfriend,

This dissertation is not just a reflection of my academic journey but a testament to the unwavering love, support, and encouragement you have all provided me throughout this journey. To my mother, whose strength and resilience have been my guiding light; to my sisters, who have always believed in me and stood by my side; and to my boyfriend, whose love and understanding have been a constant source of comfort and motivation. Each of you has played an integral role in shaping my path and inspiring me to pursue my dreams. This accomplishment is as much yours as it is mine.

With all my love and gratitude,

Kendra

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List of Abbreviations

5-HT	Serotonin
AMPK	AMP-Activated Protein Kinase
ATP	Adenosine Triphosphate
CNS	Central Nervous System
COX-2	Cyclooxygenase-2
CSF	cerebrospinal fluid
DAMPs	Damage-Associated Molecular Patterns
DC	Dendritic Cells
FA	Fatty Acid
FADH2	Flavin Adenine Dinucleotide
HPA	Hypothalamic-Pituitary-Adrenal
MDD	Major Depressive Disorder
MMP ΔΨm	Mitochondrial Membrane Potential
NADH	Nicotinamide Adenine Dinucleotide
NE	Norepinephrine
NF-κB	Nuclear Factor kappa-light-chain-enhancer of activated B cells
NK	Natural Killer
NLR	NOD-like receptors
NSAIDs	Nonsteroidal Anti-inflammatory Drugs
ox-mtDNA	oxidized mitochondrial DNA

OXPHOS	Oxidative Phosphorylation
OXPHOS	oxidative phosphorylation
PAMPs	Pathogen-Associated Molecular Patterns
PFK	Phosphofructokinase
PPARγ	Peroxisome Proliferator-Activated Receptor Gamma
PRRs	pattern recognition receptors
PTSD	Post Traumatic Stress Disorder
ROS	Reactive Oxygen Species
SSRIs	Selective Serotonin Reuptake Inhibitors
TCA	Tricarboxylic Cycle
TLR	Toll-Like Receptors
TNF-α	Tumor Necrosis Factor-Alpha
TSPO	Translocator Protein
TST	Tail Suspension Task
TZDs	Thiazolidinediones

Chapter One: The Proposed Study

Targeting Microglial Metabolism as a Screen for Therapeutic Approach

Neuroinflammatory disorders, including Major Depressive Disorder (MDD) and Post Traumatic Stress Disorder (PTSD), represent some of the most significant healthcare challenges today (Jun Du et al. 2022). These conditions bring substantial emotional, societal, and economic burdens, affecting millions worldwide (Feigin et al. 2020; GBD 2016 Neurology Collaborators 2019). Despite the severity of these disorders, relatively limited progress has been made in current treatment options (M.-N. Liu et al. 2023; Sachdeva et al. 2023). These neuroinflammatory disorders vary in how they appear clinically and affect individuals of different ages and backgrounds (Price and van Stolk-Cooke 2015). However, they all share a common characteristic: the gradual damage and loss of neurons, the cells responsible for transmitting signals from the central nervous system to other parts of the body (Whitaker, Gilpin, and Edwards 2014). As these diseases progress, cumulative dysfunction in these neurons leads to severe impairment, with patients experiencing persistent sadness, hyperarousal, or potentially losing their ability to walk.

The impact on those affected and their families can be profound, highlighting the urgent need for improved healthcare approaches. However, the complexity of studying and directly targeting the progressive nature of these disorders, given the challenges in accessing specific cells, presents a significant hurdle. Within the extensive realm of these disorders, a common theme emerges—the intricate interplay between neuroinflammation, the metabolism of glial cells, and the underlying energy dynamics governing these processes (Niranjan 2018). Inflammation, a natural and adaptive

response to injury and illness, becomes a target of exploration within these diseases, offering a new direction for research. While neurological research and therapeutic efforts have historically prioritized neurons as central players in brain function, mounting evidence highlights the pivotal contributions of glial cells, particularly microglia, in the onset and progression of these conditions (Stevenson et al. 2020; Karthikeyan et al. 2016; Hanslik, Marino, and Ulland 2021).

Microglia, as the resident immune cells of the central nervous system (CNS), play a dynamic role in orchestrating immune responses within the brain. Their ability to swiftly respond to and modulate immune reactions relies on their capacity to engage various metabolic processes intricately linked with energy dynamics (Chausse, Kakimoto, and Kann 2021). These metabolic processes not only impact the brain's immunological balance but also serve as integral components of microglia's diverse functions, encompassing immune surveillance, synaptic maintenance, tissue repair, and more. Understanding the finely tuned interplay between microglial dynamics and metabolism provides insights into their multifaceted roles in brain health and neuroinflammatory disorders. Despite the growing recognition of microglia's significance, the development of targeted therapeutic interventions centered on these dynamic cells has been impeded, partly due to limited investigative tools. A critical knowledge gap exists in harnessing microglial-centered approaches to address the neuroinflammatory components of these disorders. Consequently, potential therapeutic pathways rooted in microglial metabolic dynamics have remained largely unexplored.

This proposed study endeavors to bridge this knowledge gap by illuminating the intricate relationship between neuroinflammation, microglial metabolic activities, and

their influence on the progression of neuroinflammatory disorders. Through innovative exploration of microglial-centered tools and their potential therapeutic applications, we aim to unveil novel strategies for managing these challenging conditions. By deciphering the complex network of interactions within the central nervous system, this study seeks to provide fresh insights and innovative approaches to combat the global health challenge posed by neuroinflammatory disorders.

Experimental Hypotheses and Implications

The experimental hypothesis proposed for this study is that the mitochondrial membrane dynamics of microglia are critical for their rapid response to immune stimuli. We predict that changes in microglial metabolic states, as reflected in the mitochondrial membrane potential ($\Delta\Psi m$), occur in a sequential pattern originating in the soma and progressing outward to the branches and endfeet during immune activation. Furthermore, we anticipate that Emapunil, as an inverse agonist, will modulate this process, leading to a domain-specific regulation of microglial metabolic activity.

These findings hold significant implications for understanding the underlying mechanisms of microglial responses to immune stimuli. If our hypothesis is supported, it suggests that microglial metabolic processes and mitochondrial dynamics are tightly linked to their ability to react rapidly to inflammatory conditions in the brain. This could open doors to novel therapeutic interventions aimed at regulating microglial metabolic activity during neuroinflammatory disorders. Additionally, the domain-specific effects of Emapunil may provide a valuable tool for targeting specific aspects of microglial function, potentially minimizing unwanted side effects in therapeutic applications. Overall, this research may contribute to a better understanding of the intricate role of

microglia in neuroinflammation and pave the way for developing more effective treatments for inflammatory brain conditions.

Chapter Two: Literature Review

The Role of Inflammation in Neurological Disorders

Neuroinflammation, a common hallmark of various neurological disorders, plays a pivotal role in shaping the trajectory of conditions ranging from infectious or autoimmune diseases to mental health disorders (Gorji 2022). The intersection of neuroinflammation and mental health disorders provides a critical vantage point for understanding the underlying pathophysiology. Conditions like Major Depressive Disorder (MDD) and Post Traumatic Stress Disorder (PTSD) have been increasingly recognized for their association with dysregulated immune responses within the CNS (Drevets et al. 2022).

At its core, neuroinflammation serves as an initial defense mechanism, safeguarding the brain by removing pathogens and promoting tissue repair. However, sustained inflammatory responses, triggered by factors such as genetic mutations, protein aggregation, infections, trauma, or drugs, can lead to detrimental effects, inhibiting regeneration and exacerbating various disorders (Zhao et al. 2021; Gao et al. 2023; Tavares, Teixeira, and Garcia 2017; Huber-Lang, Lambris, and Ward 2018). While acute and chronic forms of inflammation are well-characterized, the transition between these states remains a less-explored terrain, necessitating a deeper understanding. Unpacking the nuances of neuroinflammation is crucial for developing innovative interventions. This involves not only delineating the acute and chronic phases but also delving into the dynamic transition between them. Refining the tools and approaches currently used to study and treat neuroinflammation is critical in enhancing our ability to alleviate the burden of associated neurological disorders. In this context, a more detailed exploration of the current research and treatments for conditions like MDD and

PTSD adds a layer of understanding to the dynamic relationship between neuroinflammation and mental health.

Major Depressive Disorder

The pathogenesis of MDD is intricate and not yet completely comprehended. The monoamine transmitter hypothesis, considered a cornerstone of depression research, suggests that insufficient levels of monoamine transmitters like serotonin (5-HT), norepinephrine (NE), and dopamine in the brain contribute to depression (B. Liu et al. 2017). Studies indicate that these neurotransmitters regulate energy metabolism in the central nervous system and can induce glycogen breakdown in a concentration-dependent manner (Hirschfeld 2000; Mulinari 2012). Antidepressants, such as selective serotonin reuptake inhibitor paroxetine, can significantly alter metabolite levels within energy-related pathways when combined with phosphofructokinase (PFK), strongly associated with PFK activity (Park et al. 2020) . Another widely accepted theory is the neuroendocrine hypothesis. Research reveals elevated adrenocorticotropic hormone and corticosterone expression in the serum of MDD-afflicted rats, highlighting neuroendocrine involvement in depression (Q. Wang et al. 2017). Dysregulation of the hypothalamic-pituitary-adrenal (HPA) axis, which interfaces with brain monoamine neurotransmitters, is a recurring feature in depressive patients. Hyperactivity of the HPA axis can lead to glucocorticoid secretion, which plays a significant role in overall energy balance (X. Du and Pang 2015). The presence of corticotropin-releasing hormone receptors and the majority of 5-HT and NE cells within the extrahypothalamic region underscores their role in depression (Waters et al. 2015). Depression is often linked to varying levels of inflammation, with increased expression of proinflammatory cytokines,

such as interleukin-6 and tumor necrosis factor-alpha (TNF-α), observed in affected individuals (Osimo et al. 2020). Acute systemic inflammation can disrupt brain energy metabolism, as exemplified by lipopolysaccharide (LPS)-induced inflammation and interleukin-1β, leading to hypoglycemia and decreased cerebral glucose levels (Kealy et al. 2020).

Stress significantly impacts emotions, cognition, and energy metabolism, and imbalances in energy metabolism are commonplace in neurological diseases. Oxidative phosphorylation, a principal means of energy generation, is often disrupted in these disorders, resulting in oxidative stress and contributing to neurodegenerative conditions like Alzheimer's, Parkinson's, and amyotrophic lateral sclerosis (Singh et al. 2019). An emerging body of research underscores the association between depression and energy metabolism (Sharma and Akundi 2019; Bansal and Kuhad 2016; Khan, Baussan, and Hebert-Chatelain 2023; Giménez-Palomo et al. 2021; Rezin et al. 2009), suggesting that irregular energy metabolism may play a pivotal role in its etiology and pathogenesis, thus presenting a potential target for therapeutic interventions.

Post-Traumatic Stress Disorder

MDD and PTSD share commonalities, particularly in the context of stress-induced inflammation, suggesting interconnected mechanisms. The impact of stress on energy metabolism becomes a critical focal point in understanding these disorders. PTSD is a neuropsychiatric disorder characterized by the manifestation of psychological symptoms in a subset of individuals, estimated to be around 20 to 30%, following exposure to highly distressing and traumatic events, including but not limited to combat situations, incidents of sexual assault, or life-threatening accidents (Moye et al. 2022; Gong,

Kamboj, and Curran 2019; Bown et al. 2019). Although a substantial proportion of individuals experience a noteworthy traumatic event during their lifespan, the documented lifetime prevalence of PTSD stands at approximately 6.8% in the United States and 8% worldwide (Brier et al. 2023) . The probability of developing PTSD, as opposed to experiencing a transient acute stress response lasting for a duration of 1-3 weeks, is modulated by a multitude of factors, encompassing biological sex and sociocultural components (Cahill and Pontoski 2005; Marin et al. 2019). According to a recent meta-analysis, it has been observed that unintentional traumas have a higher likelihood of leading to the development of acute PTSD when compared to intentional events during the acute phase (Diamond et al., n.d.; Y. Chen et al. 2020). The percentage of individuals experiencing acute PTSD following unintentional traumas was found to be 30.1%, whereas for intentional events, it was 11.8% (Santiago et al. 2013; Cyr et al. 2021). Nevertheless, it is noteworthy that individuals who have been subjected to deliberate traumas exhibit a comparatively elevated prevalence of chronic PTSD after a period of 12 months, with rates of 14% and 23.3% observed, respectively (Santiago et al. 2013). This empirical finding implies the presence of a cognitive component involved in the patient's response to traumatic experiences. The magnitude of the traumatic event also modulates the probability of developing PTSD, preexisting stressors encountered by an individual, cumulative exposure to traumatic events, and an individual's personal resilience and risk factors.

The field of PTSD demands innovative therapeutic interventions and biomarkers. A comprehensive understanding of advanced imaging and spectroscopy techniques is crucial to progress in diagnostic and treatment approaches for this condition. According

to the DSM-V, stress disorders from trauma are initially categorized as acute stress disorder within the first month. The official diagnosis of PTSD requires symptoms to persist for at least one month. Differentiating between acute stress disorder and PTSD during this observational period is critical for initiating intensive therapeutic interventions promptly.

Current treatment options for PTSD have limitations. Conventional research methods struggle to provide precise diagnoses, assess treatment efficacy, and track disorder progression. Established diagnostic biomarkers for psychiatric disorders, including PTSD, are lacking. The emerging technologies in neuroscience are not fully leveraged within suitable experimental frameworks, revealing a discernible need for improved research methodologies in PTSD investigation. The existing dearth of established diagnostic biomarkers for psychiatric disorders, including PTSD, underscores the pressing need for advancements in research methodologies within this realm.

Current Treatments and Limitations

Effective treatments for neuroinflammatory disorders like MDD and PTSD remain a substantial challenge. Current therapeutic approaches primarily involve psychotherapy, antidepressant medications, and cognitive-behavioral interventions. Antidepressants like selective serotonin reuptake inhibitors (SSRIs) are commonly prescribed for MDD but often yield limited efficacy and side effects (A. Chu and Wadhwa 2024). Similarly, exposure-based therapies and pharmacological interventions, such as serotonin-norepinephrine reuptake inhibitors (SNRIs), are employed for managing PTSD, yet treatment resistance remains a significant concern (Santarsieri and Schwartz 2015).

One overarching limitation in treating neuroinflammatory disorders is the absence of therapies directly targeting the underlying neuroinflammatory processes. Traditional treatment strategies primarily address symptoms rather than the root causes of these conditions. This unmet need underscores the urgency to develop novel interventions that modulate neuroinflammation, aiming to provide more effective and enduring relief for individuals with MDD and PTSD. Understanding the intricate relationship between neuroinflammation and these disorders is critical for advancing treatment approaches. While current therapies provide some relief, their limitations highlight the pressing need for novel interventions directly targeting neuroinflammatory mechanisms.

Prominent Pharmaceutical Screening Techniques

Screening techniques are essential in biomedical research, aiding in discovering novel drugs, understanding cellular responses, and unraveling complex biological mechanisms. Among the prominent screening techniques, cell culture, electrophysiological, calcium imaging, and behavioral assays have garnered significant attention due to their versatility and applicability in various research domains. However, each of these techniques possesses distinct limitations that must be considered when designing experiments and interpreting results.

Cell Culture

Cell culture, an integral technique in biomedical research, facilitates the cultivation and study of cells in a carefully controlled environment, deviating from their natural surroundings. This method serves as a controlled setting to explore intricate cellular processes, assess the efficacy of potential drug candidates, and unravel the nuances of

cell behavior. Researchers widely employ cell culture as a versatile platform, contributing significantly to our understanding of fundamental biological mechanisms.

Cell culture has inherent limitations that necessitate careful consideration despite its utility. One prominent concern is the potential disparity between cell lines and in vivo systems, as the simplified environment of cell culture may not faithfully replicate the complexity of living organisms (Corrò, Novellasdemunt, and Li 2020). This discrepancy can lead to variations in results obtained in vitro and those observed in actual biological contexts. Another drawback is the cost and time associated with maintaining cell lines. The continuous effort required for their upkeep and expense poses challenges for researchers, particularly in resource-intensive projects (Geraghty et al. 2014). Moreover, prolonged passages of cell lines may lead to genetic drift, introducing unintended alterations in cellular behavior and compromising the reliability of experimental outcomes (Torsvik et al. 2014). Despite these limitations, studies have leveraged cell culture as a pharmaceutical screening technique, utilizing its controlled environment to assess the effects of potential drug candidates on cellular processes and identify promising therapeutic interventions.

Electrophysiological Assays

Electrophysiological assays, such as patch-clamp recordings and field potential measurements, directly measure cellular electrical activity. These techniques have played a pivotal role in unraveling neuronal function's intricacies, elucidating ion channels' properties, and documenting changes in membrane potential. The advent of electrophysiological assays has significantly advanced our understanding of the

fundamental processes governing neural communication and has been instrumental in shaping contemporary neuroscientific research.

Despite their undeniable contributions, electrophysiological assays come with inherent challenges that warrant careful consideration. These techniques are technically demanding and necessitate skilled operators who are proficient in their execution (Annecchino and Schultz 2018). The intricacies involved in achieving accurate recordings and measurements require a level of expertise that may pose a barrier for researchers unfamiliar with the methodology. Furthermore, electrophysiological assays often involve invasive procedures, introducing the potential for cellular disruption. This inherent invasiveness can make it challenging to conduct long-term experiments, limiting the duration and scope of investigations (Papaioannou and Medini 2022).

Another significant limitation of electrophysiological assays lies in their high equipment costs, which can be prohibitive for many research laboratories. The specialized instruments and apparatus required for precise recordings come with a substantial financial burden, potentially restricting access to these techniques for researchers with limited resources (Roth and Ding, n.d.). As a result, despite their wealth of information, the practical constraints associated with electrophysiological assays underscore the importance of considering alternative approaches and complementary methods.

Calcium Imaging Calcium imaging is a powerful technique also commonly utilized in pharmacological screening, enabling real-time monitoring of intracellular calcium ion concentrations ([Ca2+]). This method has proven invaluable for studying cellular signaling pathways, deciphering neuronal activity, and unveiling dynamic cellular responses to various stimuli (Russell 2011). The ability to visualize and quantify

changes in intracellular calcium levels provides researchers with critical insights into the intricacies of cellular function.

However, calcium imaging is not without its limitations. One significant constraint is the requirement for specialized equipment and fluorescent indicators (Suzuki, Kanemaru, and Iino 2016). Obtaining and maintaining the necessary tools for calcium imaging can pose logistical challenges and financial constraints for research laboratories. Additionally, the spatial and temporal resolution of calcium imaging may prove insufficient to capture rapid or localized calcium dynamics accurately (Nietz et al. 2022). This limitation is particularly relevant when studying highly dynamic cellular processes, and it underscores the importance of considering alternative methods with higher resolution capabilities.

Traditionally, calcium imaging has predominantly focused on neurons, reflecting its historical roots in neuroscientific research (Grienberger and Konnerth 2012; Geng et al., n.d.). While this focus has yielded valuable information about neuronal function and communication, it also poses a limitation in the broader context of cellular signaling. The emphasis on neurons may overlook crucial aspects of cellular activity in non-neuronal cells, limiting the technique's ability to provide a comprehensive view of complex cellular processes. Exploring the multifaceted nature of cellular signaling of diverse cell types will become imperative for a more holistic understanding of cellular function and drug efficacy.

Behavioral Assays

The Tail Suspension Task (TST) is a well-established preclinical behavioral assay used to study depressive-like behaviors in rodents. It was first introduced by Steru and

colleagues in 1985 as a simple and reliable method for assessing depression-like phenotypes in mice and rats (Steru et al. 1985). Since then, the TST has become a widely employed tool in the field of neurobehavioral research to evaluate the efficacy of potential antidepressant compounds and investigate the underlying mechanisms of depression (Cryan, Mombereau, and Vassout 2005). In the TST, a rodent is suspended by its tail, typically using adhesive tape, in an inescapable situation. The immobility time, defined as the period when the animal ceases active escape-oriented behaviors and hangs passively, is considered a measure of depressive-like behavior (Can et al. 2012). Shorter immobility times are interpreted as a reduced depressive-like state.

However, despite its widespread use, the TST is not without its limitations. One major concern is the lack of specificity in interpreting immobility time as solely indicative of depressive-like behavior (Mayorga and Lucki 2001). Immobility in the TST can also result from factors unrelated to depression, such as motor impairments, fatigue, or adaptation to stress. Additionally, the TST primarily measures acute responses to stressors rather than chronic depressive states, potentially overlooking the dynamic nature of depressive disorders. Furthermore, the TST does not capture the complexity of depressive symptoms, such as anhedonia or changes in social behavior, which are crucial for a comprehensive understanding of depression. These limitations highlight the importance of complementing TST results with other behavioral assays and considering contextual factors to accurately interpret depressive-like behaviors in preclinical models.

While cell culture, electrophysiological, and behavioral assays are indispensable for scientific inquiry, their limitations underscore the importance of a multidisciplinary approach to research. Combining these techniques with complementary methods, such

as in vivo studies, molecular biology, and computational modeling, allows researchers to mitigate the inherent constraints of each technique. Collaborative efforts across scientific disciplines can provide a more comprehensive and nuanced understanding of cellular and physiological processes, ultimately advancing our knowledge and improving the accuracy of drug discovery and disease understanding.

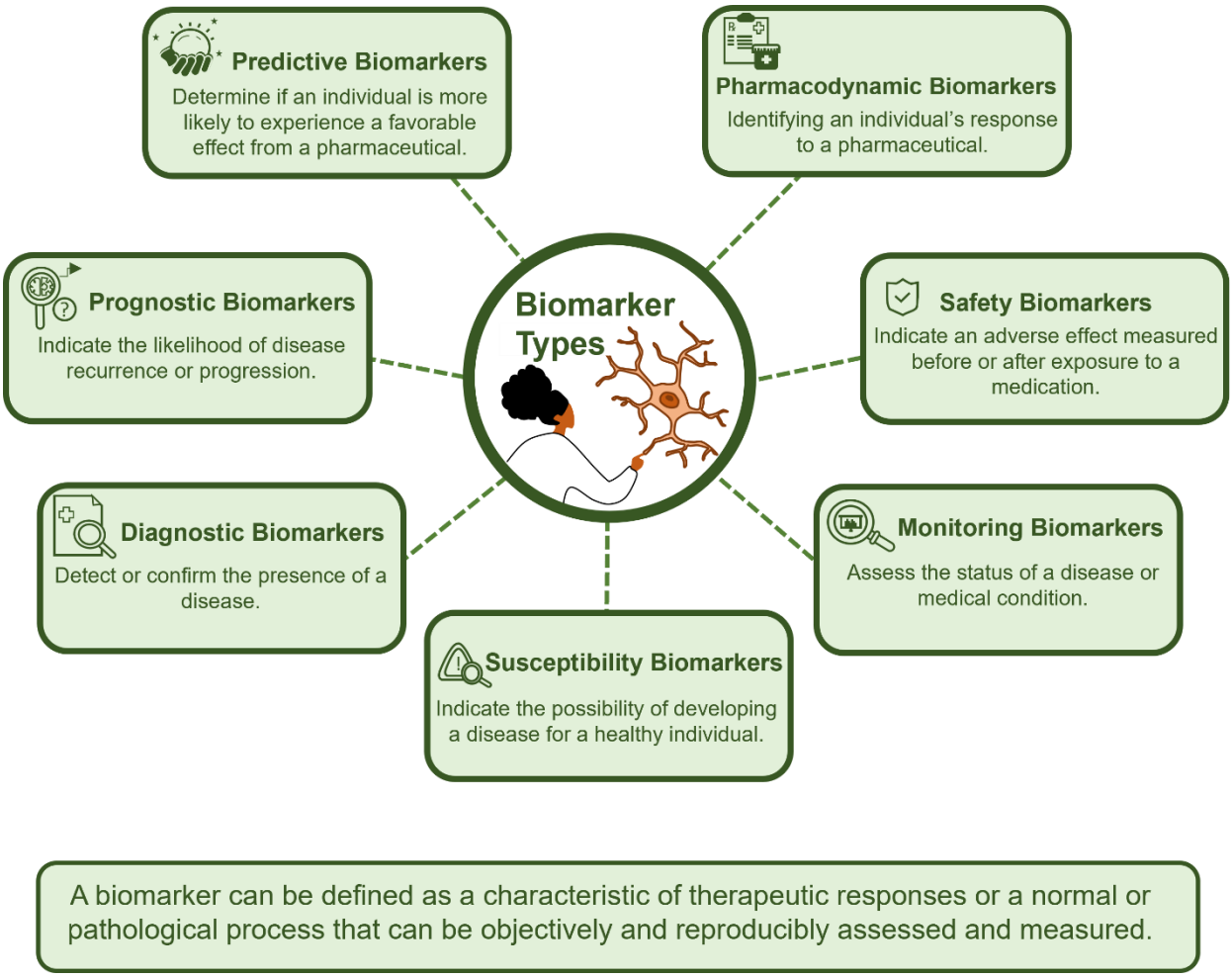


Figure 1: Diagram of Biomarker Types.

This comprehensive diagram delineates various biomarker categories crucial in biomedical research and clinical settings, illustrating their diverse roles in advancing our understanding of neuroinflammatory conditions such as Major Depressive Disorder (MDD) and Post Traumatic Stress Disorder (PTSD). Biomarkers, characteristics of therapeutic responses or normal pathological processes that can be objectively and reproducibly assessed and measured, offer a promising avenue for research in these neuroinflammatory conditions. The highlighted biomarker types include Predictive, Pharmacodynamic, Safety, Monitoring, Susceptibility, Diagnostic, and Prognostic, each contributing to more accurate diagnoses, improved assessment of treatment effectiveness, and enhanced tracking of disorder progression.

Metabolic Demands of Cells

Brain cell metabolism directly impacts the overall health of the brain. Proper energy production, nutrient utilization, and waste removal are essential for maintaining the structural and functional integrity of brain cells. Imbalances in brain metabolism can lead to cellular damage, oxidative stress, and inflammation, all of which contribute to various neurological disorders (Uttara et al. 2009; Singh et al. 2019). Disruptions in brain metabolism can contribute to the development or exacerbation of symptoms in individuals with PTSD or MDD (Sherin and Nemeroff 2011; aan het Rot, Mathew, and Charney 2009). Examining these metabolic disruptions provides valuable insights into the mechanisms underlying these conditions and opens the door to innovative treatment strategies. Targeting metabolic pathways to slow disease progression or even prevent damage will transform the landscape of neurological disease treatment. Such treatments go beyond symptom management, addressing the root causes of these conditions. Different cell types exhibit unique metabolic characteristics that underscore the exceptional energy demands of the human brain (Neves, Costalat, and Pellerin 2012). Metabolism is the intricate network of chemical reactions that occur within a cell to maintain life, including the processes of anabolism (building molecules) and catabolism (breaking down molecules). Cellular respiration is a central component of metabolism, as it specifically deals with the catabolic pathways responsible for extracting energy from nutrient molecules and generating adenosine triphosphate (ATP), the cell's primary energy currency (Alberts et al. 2002).

Glucose and Alternative Energy Sources

Cellular respiration involves the breakdown of various organic molecules, including glucose, fatty acids, and amino acids, through metabolic pathways like glycolysis, the tricarboxylic cycle (TCA), and oxidative phosphorylation (Arnold and Finley 2022). Cellular respiration is the process by which cells extract energy from nutrient molecules, primarily glucose, to generate ATP. Glycolysis is a metabolic pathway that exists in two main forms: aerobic and anaerobic respiration. Aerobic respiration is the more efficient and prevalent form of energy production, occurring in the presence of oxygen (Alberts et al. 2002). This process takes place within the mitochondria, the cell's energy powerhouses. Aerobic respiration revolves around the glycolytic pathway's metabolic activities, which involve the conversion of glucose into pyruvate, a metabolic intermediary required for cellular energy production.

Glycolysis takes place in the cytoplasm and involves a series of enzymatic reactions that ultimately break down one molecule of glucose into two molecules of pyruvate, generating a modest amount of ATP and reduced nicotinamide adenine dinucleotide (NADH) along the way (Chaudhry and Varacallo 2024). The significance of glycolysis extends beyond ATP production as it provides intermediates for numerous biosynthetic pathways, including the synthesis of amino acids, nucleotides, and fatty acids. Furthermore, the product of pyruvate acts as a central hub in cellular metabolism by serving as a link between glycolysis and the TCA cycle, allowing the efficient oxidation of glucose-derived carbon to generate more ATP through oxidative phosphorylation.

The TCA cycle, also known as the Krebs cycle or citric acid cycle, is a pivotal metabolic pathway that occurs within an organelle known as the mitochondria of eukaryotic cells

and serves as a crucial intermediary step in cellular respiration. Pyruvate is transported into the mitochondrial matrix, where it undergoes a series of enzymatic reactions to enter the TCA cycle (McCommis and Finck 2015). The initial steps of the TCA cycle begin with the condensation of pyruvate to form acetyl-CoA, which then enters the cycle. During its progression, the cycle generates reduced coenzymes such as NADH and FADH2, which carry high-energy electrons. These coenzymes are vital contributors to the subsequent electron transport chain, the final step in oxidative phosphorylation. As the TCA cycle proceeds, it releases carbon dioxide and regenerates oxaloacetate, a compound that combines with acetyl-CoA to continue the cycle. Pyruvate can be shunted into alternative metabolic pathways under specific conditions, such as lactate production in anaerobic glycolysis or conversion to acetyl-CoA for fatty acid synthesis (Cater et al. 2003; Xiaolu Li et al. 2022).

Anaerobic respiration occurs in the absence of oxygen or under conditions where oxygen is scarce. This metabolic pathway involves pyruvate being converted into alternative end products such as lactic acid or ethanol, which allows glycolysis to continue in the absence of oxygen (Melkonian and Schury 2024). While anaerobic respiration is less efficient in terms of ATP production (generally producing only 2 ATP molecules per glucose molecule), it serves as a temporary energy source when oxygen is limited. Both aerobic and anaerobic respiration are essential for meeting the energy needs of cells in various physiological conditions.

Neurons for instance, are nerve cells that specialize in transmitting information within the nervous system to carry out various functions (X. Zheng et al., n.d.). Unlike many other cells in the body, neurons rely predominantly on aerobic respiration as their

primary energy source. This heavy reliance on the production of glucose reflects the brain's extraordinary energy requirements, considering that the brain constitutes only about 2% of the body's weight but consumes approximately 20% of its total energy (*Basic Neurochemistry* 1999). The metabolism of glucose has been shown to support intricate neural processes that underpin cognition, learning, memory, and a myriad of other higher-order functions (Messier and Gagnon 1996).

Neurons and various glial cell types work collaboratively to ensure the energy demands of the brain are met and to maintain brain homeostasis. Neurons, as the primary signaling units, require a substantial amount of energy to sustain their activities (Vergara et al. 2019). Action potentials, neurotransmitter release, and the maintenance of ion gradients across the cell membrane primarily drive neuronal energy demands. These processes necessitate a continuous supply of glucose, which is catabolized through glycolysis and oxidative phosphorylation in mitochondria to meet the ATP requirements.

Glial cells, which include astrocytes, oligodendrocytes, ependymal cells, and microglia, play pivotal roles in supporting the energy needs of neurons and maintaining brain homeostasis. Astrocytes are especially renowned for their metabolic support, as they actively participate in the uptake and storage of glucose, thus ensuring a steady supply for neighboring neurons (Z. Chen et al. 2022). Furthermore, astrocytes contribute to the regulation of ion concentrations and neurotransmitter recycling, all of which have profound impacts on neuronal energy metabolism (Beard et al. 2022). Oligodendrocytes, responsible for myelinating axons, play an indirect yet crucial role in neuronal energy conservation (Bradl and Lassmann 2010). By providing electrical

insulation, myelin enables faster saltatory conduction of action potentials along axons, reducing the energy expenditure associated with repeated depolarization of unmyelinated segments. Ependymal cells, residing in the brain's ventricles, are involved in cerebrospinal fluid (CSF) production and circulation (Deng et al. 2023). CSF acts as a nutrient source and waste removal system for neurons, contributing to their metabolic requirements. Microglia, the CNS's resident immune cells, participate in immune surveillance and inflammation. While their primary role is not in energy support, they contribute indirectly to homeostasis by defending against pathogens and assisting in the removal of cellular debris, which requires high amounts of energy to maintain (Lannes et al. 2017).

Recent research has unveiled the multifaceted roles of glial cells in the CNS and highlighted their capacity for metabolic flexibility. The metabolic flexibility of a cell represents the ability to vary the nutrients being metabolized when changes occur in the brain parenchyma. This adaptability is often critical to maintain cellular function. Microglial cells have been shown to rely on glycolysis, glutaminolysis, or FA oxidation (FAO) in the face of fuel source availability (Lepiarz-Raba et al. 2023). Microglia, for instance, predominantly rely on glycolysis under normal conditions, similar to neurons. However, recent studies have revealed the remarkable metabolic flexibility of microglia in response to various challenges (Bernier et al. 2020). In response to inflammatory signals or specific environmental cues, microglia can undergo a metabolic shift. They switch to alternative energy sources, such as fatty acids, to meet their energy needs, a process known as metabolic reprogramming. This metabolic plasticity extends beyond microglia's energy requirements. It has been shown that microglial metabolic

reprogramming is intimately linked to their functions in immune responses, phagocytosis, and the production of cytokines and other signaling molecules (Towriss, MacVicar, and Ciernia 2023). In this context, the ability of microglia to adapt their metabolism serves as a critical component of their role in maintaining the CNS's homeostasis, responding to neural injury, and contributing to the resolution of inflammation. This capacity for metabolic reprogramming is not merely a reflection of microglial adaptability; it is intricately linked to their functional roles. For example, during neuroinflammation, microglia can transition to a more pro-inflammatory state, accompanied by a shift towards glycolysis to support their heightened immune response (Yang et al. 2021). During the resolution of inflammation, a more anti-inflammatory phenotype may prevail, with microglia returning to oxidative phosphorylation to maintain homeostasis and mitigate excessive immune activation.

Studies have reported abnormalities in glucose metabolism, mitochondrial function, and inflammation markers in individuals with MDD (Gebara et al. 2021; Cataldo et al. 2010; Wu et al. 2019). Altered glucose metabolism has been observed in brain regions associated with mood regulation, potentially affecting the availability of energy for neuronal processes and neurotransmitter synthesis (S. Zhang et al. 2023). Dysfunctional mitochondria, which play a central role in energy production, are implicated in both the etiology and progression of depression (Allen et al. 2018). Additionally, heightened neuroinflammatory markers, such as cytokines and oxidative stress, are commonly associated with MDD (Bakunina, Pariante, and Zunszain 2015). These metabolic disturbances can lead to neuronal damage, synaptic dysfunction, and

impairments in neuroplasticity, all of which contribute to the neurobiological underpinnings of depression (Miller et al., 2013; Belmaker & Agam, 2008).

Similarly, PTSD exhibits metabolic alterations that profoundly impact brain function. Neuroinflammation and oxidative stress have been linked to the development and maintenance of PTSD, often stemming from traumatic experiences (Miller et al. 2018). Elevated levels of pro-inflammatory cytokines can disrupt normal neuronal function, affecting mood, cognition, and behavior. The interaction between stress hormones, such as cortisol, and metabolic pathways further compounds these disturbances (B. Chu et al. 2024). Abnormalities in glucose metabolism and mitochondrial dysfunction have been reported in individuals with PTSD, potentially influencing the brain's response to stress and emotional regulation. These metabolic changes can contribute to the long-term structural and functional alterations observed in PTSD, affecting memory processing and the development of symptoms like intrusive thoughts and hypervigilance (O'Donovan et al. 2015; Lindqvist et al. 2017). Metabolic modulators, which target key metabolic pathways in the brain, have shown potential in ameliorating the symptoms of these disorders and improving overall neurological function.

In the case of MDD, the use of metabolic modulators has gained attention due to the observed metabolic abnormalities in individuals with depression. One such approach involves targeting mitochondrial function, as dysfunctional mitochondria have been implicated in the pathophysiology of MDD. Research has shown that interventions with compounds like coenzyme Q10, which support mitochondrial function and reduce oxidative stress, can lead to improvements in mood and cognitive symptoms in individuals with MDD (Giménez-Palomo et al. 2021). Similarly, dietary interventions

involving omega-3 fatty acids, which can influence mitochondrial function and reduce inflammation, have demonstrated potential benefits for individuals with depression (Jing Du et al. 2016; Ortega et al. 2022).

In the context of PTSD, metabolic modulators offer a unique perspective on managing the neuroinflammatory and oxidative stress aspects of the disorder. Emerging research suggests that nutritional interventions targeting the kynurenine pathway, which is linked to inflammation and oxidative stress, can potentially mitigate PTSD symptoms (Pathak et al. 2024). Compounds like kynurenine aminotransferase inhibitors, which modulate this pathway, have shown promise in preclinical studies for reducing PTSD-like behaviors. Furthermore, dietary interventions that enhance the availability of antioxidants, such as polyphenols and vitamins, may help counteract oxidative stress and neuroinflammation in individuals with PTSD (Rudrapal et al. 2022; Naomi et al. 2023). Strategies aimed at modulating brain metabolism through metabolic modulators represent a novel approach to address the underlying metabolic disturbances associated with these disorders.

Immunometabolism

Intimate connections between the functions of immune cells and their metabolic states are rapidly being recognized and expanding the field of immunometabolism (Bernier, York, and MacVicar 2020). This emerging discipline is dedicated to unraveling how the reconfiguration of intracellular metabolic pathways can fundamentally alter the behavior of immune cells. Specific cell types like T cells and macrophages demonstrate remarkable metabolic adaptability that enables them to respond effectively to changing

inflammatory conditions and support coordinated immune reactions (Domblides, Lartigue, and Faustin 2018).

The capacity of immune cells to shift their energy production between glycolysis, oxidative phosphorylation (OXPHOS), amino acid, and fatty acid (FA) metabolism is a testament to their versatility. This metabolic flexibility equips them to address challenges stemming from altered nutrient availability or metabolic constraints encountered within inflammatory microenvironments. Moreover, the functions of immune cells can evolve over the course of an immune response, necessitating corresponding shifts in their metabolic strategies (Artyomov, Sergushichev, and Schilling 2016). Processes such as phagocytosis, migration, proliferation, and cytokine release all demand a recalibration of the balance between glycolysis and OXPHOS.

Within the innate immune system, macrophages and dendritic cells provide illustrative examples of how metabolic shifts coincide with immune activation. When exposed to LPS, macrophages elevate their reliance on glycolysis as an essential step in their proinflammatory activation (Gauthier and Chen 2022). Dendritic cells, when engaging with pathogens, also exhibit an increased glycolytic flux as they assume their immunogenic roles (Pearce and Everts 2015). In contrast, immature dendritic cells lean more towards OXPHOS.

The adaptive immune system mirrors this metabolic plasticity. Developing T cells, upon activation and differentiation into effector T cells, undergo a transition towards a glycolytic profile (Rangel Rivera et al. 2021). A similar glycolytic shift accompanies the stimulation of natural killer (NK) cells (Z. Wang et al. 2020). Intriguingly, a recurring theme in these studies is the role of intracellular metabolites as signaling molecules that

influence the phenotype of immune cells and the immune response itself. Consequently, research into the peripheral immune system has illuminated the transient metabolic signatures of immune cells as pivotal regulators with profound implications for immunity.

Mitochondrial Function

Mitochondria, often referred to as the "powerhouses of the cell," are essential organelles with a diverse array of functions critical for the overall health and vitality of eukaryotic cells. Mitochondria are double-membraned organelles with a distinctive inner membrane that folds into structures known as cristae. The mitochondria are equipped with their own genetic material, separate from the nuclear genome, and they contain machinery for protein synthesis. This intricate structure facilitates the execution of multiple functions, including energy production, calcium regulation, and reactive oxygen species (ROS) management.

One of the most renowned functions of mitochondria is their role in energy production through oxidative phosphorylation. This process, carried out within the inner mitochondrial membrane, involves the electron transport chain and ATP synthase. Mitochondria generate adenosine triphosphate (ATP), the primary energy currency of the cell. The research of Mitchell (1961) greatly contributed to our understanding of the chemiosmotic theory, elucidating how proton gradients drive ATP synthesis (Mitchell 1966).

Mitochondria contribute significantly to the maintenance of metabolic homeostasis in brain cells by facilitating the electron transport chain, an assembly of protein complexes embedded in the inner mitochondrial membrane (Osellame, Blacker, and Duchen 2012). These complexes are highly involved in the transfer of electrons that are

extracted from molecules derived from nutrients, such as glucose or fatty acids. As electrons journey along the chain, they release energy. This energy is harnessed to pump protons (hydrogen ions) from the mitochondrial matrix into the intermembrane space, creating an electrochemical gradient. The build-up of protons in the intermembrane space acts as a form of stored energy. To restore equilibrium, protons flow back into the mitochondrial matrix through ATP synthase, an enzyme that functions as a molecular turbine. As protons rush through ATP synthase, they induce a conformational change that drives the phosphorylation of adenosine diphosphate (ADP) into ATP. This crucial step is what provides the cell with a continuous and ample supply of ATP for various energy-requiring processes.

The TCA cycle is a major source of electrons for the electron transport chain, which ultimately leads to the synthesis of ATP. For every turn of the cycle, one molecule of ATP is produced directly, and the coenzymes NADH and FADH2 generated in the process participate in oxidative phosphorylation, facilitating the production of numerous ATP molecules. These electron carriers shuttle high-energy electrons to the electron transport chain yielding a significantly greater amount of ATP compared to glycolysis alone. Aerobic respiration is highly efficient, producing up to 36 ATP molecules per glucose molecule.

The pivotal role of mitochondria in brain cell metabolism extends beyond ATP production, as they are intimately involved in calcium ion (Ca2+) homeostasis, regulating the production of reactive oxygen species (ROS), and regulating the metabolism of neurotransmitters and lipids (Matuz-Mares et al. 2022; Starkov 2008; Casanova et al. 2023). Dysregulation of mitochondrial function can have far-reaching

consequences, impacting not only energy production but also the overall well-being of brain cells and potentially leading to neurodegenerative diseases and other neurological disorders. As such, the profound involvement of mitochondria in brain cell metabolism underscores their significance in maintaining the delicate balance of energy and homeostasis in the brain.

Mitochondria also partake in cellular signaling by releasing signaling molecules and participating in calcium buffering. The release of mitochondrial-derived molecules like cytochrome c and apoptosis-inducing factor plays a pivotal role in apoptosis, a programmed cell death process (C. Wang and Youle 2009). Moreover, calcium signaling and buffering by mitochondria impact various cellular functions, including muscle contraction and neurotransmitter release.

Impaired mitochondrial function is closely associated with numerous diseases. For instance, in neurodegenerative disorders like Parkinson's and Alzheimer's, mitochondrial dysfunction and oxidative stress are implicated. The work of Schapira et al. (1989) contributed to understanding the role of mitochondria in Parkinson's disease (Schapira et al. 1989). Mitochondrial diseases, often caused by mutations in mitochondrial DNA or nuclear genes, can result in a broad spectrum of clinical presentations, from muscle weakness to organ failure. The exploration of mitochondrial dysfunction in cancer by Hanahan and Weinberg (2011) revealed its role in sustaining the proliferative and survival capabilities of cancer cells (Hanahan and Weinberg 2011).

The process of mitochondrial biogenesis is essential for maintaining a functional mitochondrial population. The PGC-1α signaling pathway, discussed in the work of Scarpulla (2011), orchestrates this vital aspect of mitochondrial function (Scarpulla

2011). Additionally, quality control mechanisms, such as mitophagy and the mitochondrial unfolded protein response (UPRmt), safeguard mitochondrial health (Svaguša et al. 2020). Recent research has unveiled novel facets of mitochondrial function. Mitochondrial dynamics, including fission and fusion events, are under scrutiny for their roles in regulating mitochondrial morphology and function. The link between mitochondria and cellular metabolism, particularly in the context of metabolic diseases, is a rapidly evolving field. Understanding the contribution of mitochondrial dysfunction in aging and age-related diseases is another area of active investigation.

Mitochondrial Membrane Potential

The mitochondrial membrane potential serves as a critical indicator of cellular health and function, reflecting the dynamic interplay between cellular metabolism and inflammatory responses. Under basal conditions, a stable mitochondrial membrane potential is maintained, indicating efficient energy production and cellular homeostasis. However, upon encountering inflammatory stimuli, such as pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs), mitochondria undergo profound alterations in their membrane potential (Wilkins et al. 2017). This is often characterized by depolarization, a decrease in the electrochemical gradient across the mitochondrial inner membrane. This depolarization event signifies a shift in mitochondrial function, transitioning from energy production to initiating signaling pathways associated with inflammation (Silva Santos Ribeiro, Willemen, and Eijkelkamp 2022). As a consequence, the mitochondrial membrane potential serves as an early biomarker, reflecting the impending onset of an inflammatory response.

Moreover, alterations in the mitochondrial membrane potential can modulate immune cell function and influence the magnitude and duration of inflammatory responses. Studies have demonstrated that changes in mitochondrial membrane potential can regulate the activation and polarization of immune cells, such as macrophages and microglia, towards pro-inflammatory or anti-inflammatory phenotypes. Depolarization of the mitochondrial membrane can trigger the release of pro-inflammatory mediators, such as ROS and cytokines, amplifying the inflammatory cascade. Conversely, maintaining a stable or hyperpolarized mitochondrial membrane potential may promote an anti-inflammatory phenotype, dampening excessive inflammation and promoting tissue repair processes. Thus, the mitochondrial membrane potential serves as a crucial nexus linking cellular metabolism to immune responses, highlighting its significance as a biomarker and potential therapeutic target in inflammatory diseases.

Microglia

Origin and Development of Microglia

Microglia research has deep historical roots, with pivotal contributions from Franz Nissl, Santiago Ramón y Cajal, and Pío del Río Hortega. Río Hortega, who studied under Ramón y Cajal, holds a significant place as the Father of Microglia. It was he who first identified and christened these cells, making notable observations of their tree-like processes and foreseeing their phagocytic role. His meticulous histological investigations form the cornerstone of our current knowledge about microglial biology. In the years following Río Hortega's discoveries, the microglia research field has grown substantially. Soon after the identification of microglia by Río Hortega, discussions regarding their lineage and origin emerged. The phenotypical resemblances microglia

spurred these discussions shared with peripheral monocytes/macrophages and dendritic cells (DCs), leading to suggestions that microglia might have hematopoietic origins. However, Cuadros et al.'s pioneering research in the 1990s demonstrated that microglia have a unique developmental path (Cuadros et al. 1993). Microglia are derived from primitive myeloid precursors, originating in the yolk sac before migrating into the CNS during early embryogenesis. These yolk sac progenitors give rise to tissue-resident macrophages throughout the body, including microglia in the brain.

The recent addition of fate mapping studies and transplantation approaches have shed new light on the longstanding debate surrounding microglial identity and plasticity. This interplay between research techniques has yielded insights into microglial cells' origin and developmental processes, particularly in the context of different model organisms. In mice, a widely used model for neurobiological research, there is mounting evidence to support that microglia originate from a pool of macrophages that emerge during primitive hematopoiesis within the yolk sac. These macrophages subsequently infiltrate the neuroepithelium around embryonic day 8.5 (E8.5), marking a crucial stage in the development of these resident immune cells within the central nervous system. This emerging consensus among researchers highlights the importance of understanding the early origins of microglia in mouse models to elucidate their functions and contributions to brain health (Alliot, Godin, and Pessac 1999; Ginhoux et al. 2010). In contrast, the timeline for microglial precursor invasion in humans presents a distinct pattern. Research in human embryonic development suggests that microglial precursors infiltrate the brain primordium at a significantly earlier gestational stage, around 4.5 to 5.5 weeks.

Microglial development is a multistep process that includes colonization, maturation, and maintenance phases. A key signaling pathways that governs their development and maintenance is the colony stimulating factor receptor (CSF1R). The signaling cascades initiated by CSF1R activation are crucial for the regulation of microglial populations within the central nervous system (CNS). This pathway is modulated by specific ligands, including two cytokines, IL-34 and CSF1, both of which exhibit distinct origins and primary sequences but share similar tridimensional structures and an affinity for binding to CSF1R. Interestingly, the sources of these ligands vary significantly. IL-34, crucial for microglial sustenance, is primarily produced by neurons, while CSF1, with its distinct cellular origin, is predominantly secreted by oligodendrocytes and astrocytes. These differential sources underscore the non-overlapping functions of IL-34 and CSF1 in the establishment and maintenance of microglial populations within both gray and white matter regions of the CNS (Greter & Merad, 2013; Wang & Colonna, 2014).

Microglia's remarkable capacity for self-renewal under specific conditions is an intriguing facet of their biology. This phenomenon, often termed "microglial repopulation" or "microglial self-renewal," allows microglia to repopulate the CNS rapidly, typically within a week, even when more than 99% of the microglial population is ablated using CSF1R antagonists or diphtheria toxin-based approaches. This endogenous replenishment mechanism serves as an integral component of microglial maintenance within the CNS, ensuring a continuous presence of these resident immune cells. It should be noted that "microglial self-renewal" is distinct from "microglia replacement," a process where endogenous microglia are replaced by exogenous cells, which can include bone marrow-derived myeloid cells, peripheral blood cells, and stem-cell- or induced-

pluripotent-stem-cell (iPSC)-derived peripheral blood cells. This replacement process can occur under various experimental or pathological conditions, further emphasizing the intricate dynamics of microglial populations in the CNS (Prinz & Priller, 2014; Askew et al., 2017).

Upon entering the CNS, they undergo maturation processes, transforming from a round amoeboid morphology to a ramified, surveilling state. This transformation includes the acquisition of surface markers, including CX3CR1 and CD11b, and the downregulation of CD45. Maturing microglia also establish contacts with neurons, astrocytes, and other glial cells. Once microglia reach their ramified state, they become long-lived and self-renewing. In the adult brain, microglia continuously monitor the neural microenvironment, surveying for signs of damage or infection. They play critical roles in synaptic pruning, synaptic plasticity, and immune surveillance. Research suggests that microglial phenotype and functions are tightly regulated by their microenvironment, and any perturbation can lead to neuroinflammation and neurodegeneration.

Microglial Morphology and Phenotypes

Recent advancements in our understanding of neuroimmunology have departed from the traditional dualistic classifications of microglial activation (Paolicelli et al. 2022). In the past microglial activation was categorized as either resting or activated, failing to account for the intricate and multifaceted nature of microglial responses.

Microglia have long been recognized for their role in maintaining brain homeostasis and responding to insults. Over the years, research has unveiled the remarkable plasticity of microglia, highlighting the intricate relationship between their morphology and functional phenotypes.

The first aspect of microglia that researchers encounter is their morphology, which reflects their state of activation and functional phenotype. In their resting state, microglia appear as highly ramified cells with numerous fine processes continuously surveying the microenvironment. However, when challenged by injury, infection, or disease, microglia transform into an activated state. The morphology of activated microglia shifts dramatically, as these cells adopt an amoeboid or hypertrophic form characterized by retraction of processes. These morphological changes are indicative of the switch to a pro-inflammatory phenotype, essential for mounting an immune response.

Microglia possess diverse functional phenotypes depending on the microenvironment and the nature of the stimulus. These phenotypes are broadly categorized into M1 (pro-inflammatory) and M2 (anti-inflammatory) states. Activated M1 microglia are associated with the release of pro-inflammatory cytokines and reactive oxygen species, contributing to neuroinflammation and damage. In contrast, M2 microglia are considered neuroprotective, contributing to tissue repair, immunosuppression, and phagocytosis of debris.

The dichotomous M1/M2 classification has proven overly simplistic. Microglial activation exists along a spectrum, reflecting a continuum of phenotypes. The unique microenvironment and nature of the stimulus determine where microglia fall within this spectrum. Recent studies have identified a broad spectrum of microglial activation states, from classical pro-inflammatory states to alternative anti-inflammatory and immunoregulatory states. These states can coexist within the same tissue or even within a single microglial cell.

Various factors modulate microglial phenotypes. These include cytokines, chemokines, damage-associated molecular patterns (DAMPs), and pathogen-associated molecular patterns (PAMPs). Additionally, genetics, epigenetics, and neural signaling profoundly influence microglial responses. The role of microglia in various neurological conditions, including neurodegenerative diseases, neuroinflammation, and psychiatric disorders, underscores the importance of understanding the factors that dictate their phenotypic states.

The functional consequences of microglial activation and the resulting phenotypes are substantial. While pro-inflammatory states contribute to tissue damage, immunosuppressive and reparative phenotypes are integral for tissue repair and resolution of inflammation. Microglial phenotype shifts in response to neural plasticity, development, and various neurological disorders. The ongoing challenge lies in harnessing this knowledge for therapeutic purposes, as efforts are directed toward modulating microglial phenotypes to mitigate damage and enhance recovery in the CNS.

Homeostatic Functions

The term "homeostatic" has traditionally been applied to microglia in physiological conditions, yet its interpretation can vary when describing microglia in the context of health and disease. While "homeostatic" is often linked to the "physiological" state of microglia within specific temporal and spatial boundaries, it does not necessarily correspond to a unique and static molecular profile. Microglia exhibit a remarkable degree of morphological and functional diversity even in the absence of external perturbations, as they continuously adapt to signals from the central nervous system

(CNS) microenvironment. This constant microglial sensing results in a multitude of transcriptional signatures, reflecting the dynamic nature of microglial function across various developmental stages, from infancy to aging (Lavin et al., 2014; Masuda et al., 2019).

Intriguingly, microglia can transition into states that may be considered more "homeostatic" in certain contexts, characterized by reduced responsiveness to challenges. However, this reduced responsiveness may hinder their ability to effectively respond to damage or pathological cues, particularly in aging and disease contexts. For example, in the context of aging and neurodegenerative diseases, microglia may exhibit diminished capacity to swiftly respond to brain challenges, such as the removal of toxic amyloid or the clearance of infected, damaged, or degenerating neurons. This impaired responsiveness can contribute to central nervous system dysfunction and the progression of neurodegenerative diseases. Studies involving TREM2 knockout mice have described microglia as being "locked in a homeostatic state" because they are less responsive to challenges, like amyloid accumulation, and do not adopt the typical transcriptional signature associated with disease-associated microglia (DAM) in contexts of neurodegenerative disease. This example underscores the necessity of clearly defining and contextualizing the term "homeostatic" with regard to microglial function to facilitate a comprehensive understanding of their roles in health and disease (Keren-Shaul et al., 2017; Jay et al., 2015).

Microglia- Neuron Interactions

Microglia, the resident immune cells of the central nervous system, exhibit remarkable chemotactic and migratory capabilities crucial for their surveillance and response to

neuronal signals. Recent studies have delved into the molecular mechanisms governing microglial movement and chemotaxis. Chemokines, such as CX3CL1 and CCL2, have emerged as key players in guiding microglial migration towards sites of injury or synaptic activity. The CX3CR1 receptor, expressed on microglia, interacts with neuronal CX3CL1, orchestrating directed migration toward neurons. Additionally, purinergic signaling, involving ATP release from neurons, has been implicated in microglial chemotaxis. These findings underscore the intricate molecular dialogue that directs microglia towards specific locations within the brain, emphasizing the dynamic nature of microglia-neuron interactions.

The crosstalk between microglia and neurons extends beyond mere physical proximity, influencing synaptic regulation and plasticity. Research has elucidated the role of microglia in shaping synaptic connections and modulating neuronal activity. Microglia actively participate in synaptic pruning, a process crucial for refining neural circuits during development. Molecular mechanisms involved in this regulation include the complement system, with C1q and C3 playing pivotal roles in tagging synapses for elimination (Gomez-Arboledas, Acharya, and Tenner 2021). Furthermore, the recognition of neuronal activity by microglial receptors, such as P2Y12 and CX3CR1, suggests a nuanced interplay between microglia and neurons in the maintenance of synaptic homeostasis. These findings highlight the active contribution of microglia to synaptic plasticity and the fine-tuning of neuronal networks.

Microglia in Neuroinflammation

Neuroinflammation, often instigated by microglial activation, represents a critical facet of microglia-neuron communication. Recent investigations have unveiled intricate signaling

pathways that underlie the neuroinflammatory responses mediated by microglia. Toll-like receptors (TLRs), crucial components of the innate immune system, are expressed on microglia and recognize various danger-associated molecular patterns (DAMPs) released during neuronal stress or injury. Activation of TLRs triggers downstream signaling cascades, involving nuclear factor-kappa B (NF-κB) and mitogen-activated protein kinases (MAPKs), ultimately leading to the production and release of pro-inflammatory cytokines. The delicate balance between neuroinflammatory and neuroprotective responses in microglia underscores the complexity of their signaling pathways and their pivotal role in shaping the neural microenvironment. Understanding these molecular mechanisms opens avenues for targeted therapeutic interventions in neuroinflammatory conditions.

Signaling Pathways Involved in Microglial Activation

Microglial cells, often referred to as the vigilant custodians of the central nervous system, play a pivotal role in maintaining brain homeostasis. These resident immune cells, with their dynamic and multifaceted functions, respond to various stimuli by undergoing a process known as microglial activation. The intricacies of microglial activation are underpinned by a complex network of signaling pathways that regulate the extent and nature of their responses. Understanding this phenomenon is crucial, as microglial activation is not only an essential component of normal brain function but also a key player in the pathogenesis of various neurological disorders.

At its core, microglial activation represents a finely tuned response to changes in the brain's microenvironment. These changes can arise from diverse sources, including infection, injury, or neurodegenerative processes. Microglia, equipped with an array of

receptors on their surfaces, serve as the first responders to these challenges. These receptors, such as Toll-like receptors (TLRs) and purinergic receptors, recognize molecular patterns associated with pathogens or cellular damage. Upon activation, microglia transition from a resting state to an activated state, triggering a cascade of events that involve the release of various signaling molecules.

The significance of microglial activation lies in its role as a double-edged sword. On one hand, activated microglia are essential for combating pathogens, clearing cellular debris, and promoting tissue repair. On the other hand, dysregulated or chronic microglial activation has been implicated in the pathogenesis of neuroinflammatory conditions and neurodegenerative diseases. Thus, unraveling the molecular mechanisms behind microglial activation is essential for comprehending both the physiological and pathological aspects of brain function.

Signaling pathways serve as the conductors orchestrating the symphony of microglial activation. These pathways, which include but are not limited to MAPK, NF-κB, and JAK-STAT, act as molecular switches that regulate gene expression, cytokine release, and the overall inflammatory profile of activated microglia. The intricate interplay between these signaling cascades determines the phenotype of activated microglia, ranging from a pro-inflammatory M1 phenotype to an anti-inflammatory M2 phenotype. Striking a balance between these phenotypes is crucial for maintaining a healthy brain microenvironment.

The delicate equilibrium between the beneficial and detrimental aspects of microglial activation underscores the importance of understanding the signaling pathways that govern these processes. As we delve deeper into the molecular intricacies, we gain

insights not only into the fundamental mechanisms of microglial biology but also into potential therapeutic targets for modulating microglial responses in the context of neurological disorders.

Key Signaling Molecules in Microglial Activation: Unraveling the Molecular Orchestra

The activation of microglia is a finely orchestrated symphony, with key signaling molecules serving as the instrumental players that dictate the tempo and intensity of the response. In this exploration of microglial activation, we delve into the intricate world of signaling molecules, specifically focusing on cytokines, chemokines, and growth factors. These molecular entities form a dynamic network that initiates and modulates microglial responses, shaping the intricate landscape of neuroinflammation.

Cytokines, small proteins crucial in cell signaling, emerge as central players in the microglial activation saga. Pro-inflammatory cytokines such as interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α), and interleukin-6 (IL-6) are released during microglial activation, contributing to the inflammatory milieu. These molecules not only recruit immune cells to the site of injury or infection but also stimulate microglia to amplify their responses. Conversely, anti-inflammatory cytokines like interleukin-10 (IL-10) act as regulators, dampening the inflammatory cascade and promoting resolution. The delicate balance between pro- and anti-inflammatory cytokines dictates the nature of microglial activation and its impact on the surrounding neural environment.

Chemokines, another class of signaling molecules, choreograph the movement of immune cells, including microglia, within the central nervous system. Chemokines such

as CCL2 (chemokine ligand 2) and CXCL10 (C-X-C motif chemokine 10) are released by activated microglia, guiding the migration of immune cells to sites of injury or infection. This chemotactic response is a crucial aspect of the neuroinflammatory process, facilitating the recruitment and coordination of immune cells to effectively address the underlying challenge.

Growth factors add another layer of complexity to the microglial activation narrative. Insulin-like growth factor 1 (IGF-1), for instance, has been identified as a potent modulator of microglial activation, influencing their morphology, proliferation, and cytokine release. Moreover, transforming growth factor-β (TGF-β) has dual roles, acting both as an anti-inflammatory agent and as a mediator of tissue repair. The interplay between these growth factors and microglial responses highlights the multifaceted nature of signaling molecules in shaping the outcomes of microglial activation.

As we navigate through the realm of these key signaling molecules, it becomes evident that their interactions form a complex regulatory network, finely tuning the microglial response to ensure an appropriate reaction to diverse challenges. Dissecting the roles of cytokines, chemokines, and growth factors in microglial activation not only expands our understanding of the molecular mechanisms at play but also provides potential avenues for therapeutic interventions in neuroinflammatory and neurodegenerative conditions. In the continued exploration of microglial activation, unraveling the nuances of these signaling molecules promises to uncover novel insights into the dynamic interplay between immune responses and neural health.

Intracellular Signaling Cascades in Microglial Activation: Unveiling the Molecular Choreography

The activation of microglial cells represents a remarkable dance of intracellular signaling cascades, where molecular pathways choreograph a nuanced and context-dependent response to various stimuli. This exploration delves into the intricacies of these intracellular signaling events, with a focus on two key players: Mitogen-Activated Protein Kinase (MAPK) and Nuclear Factor-kappa B (NF-κB). These pathways serve as integral components in the orchestration of microglial activation, unraveling a complex molecular ballet within the confines of these immune cells.

MAPK, a multifaceted signaling pathway, stands at the forefront of the microglial activation repertoire. Comprising three major branches — extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38 MAPK — this cascade integrates diverse extracellular signals into precise cellular responses. Upon microglial activation, MAPK pathways are activated, triggering a series of phosphorylation events that culminate in the modulation of gene expression. ERK, for instance, has been implicated in promoting anti-inflammatory responses, while JNK and p38 MAPK often drive pro-inflammatory cytokine production. The intricate balance and crosstalk between these MAPK branches dictate the nuanced phenotype adopted by microglia, influencing their roles in immune surveillance, inflammation, and tissue repair.

NF-κB emerges as another central protagonist in the drama of microglial activation. This transcription factor, residing in the cytoplasm in its inactive state, undergoes activation in response to various stimuli. Upon activation, NF-κB translocates to the nucleus, where it orchestrates the expression of genes involved in immune and inflammatory

responses. In microglia, NF-κB activation is a pivotal event in the pro-inflammatory cascade, driving the expression of cytokines, chemokines, and other mediators that amplify the neuroinflammatory milieu. The tight regulation of NF-κB activity is crucial, as dysregulation can lead to chronic inflammation and contribute to the pathogenesis of neurodegenerative diseases.

The interplay between MAPK and NF-κB pathways adds layers of complexity to the microglial activation narrative. These signaling cascades do not operate in isolation but rather crosstalk and influence each other, shaping the overall response of microglia to diverse stimuli. While MAPK pathways regulate the immediate early events in microglial activation, NF-κB serves as a master regulator, steering the sustained inflammatory response. Understanding the intricacies of these intracellular signaling cascades provides a roadmap for unraveling the molecular events that underlie microglial activation and its impact on neural health.

In conclusion, the exploration of MAPK and NF-κB pathways within microglial activation unveils the molecular choreography governing these immune cells' responses. The delicate balance and intricate crosstalk between these signaling cascades illuminate the dynamic nature of microglial activation, offering insights into potential therapeutic targets for modulating neuroinflammation in the context of neurological disorders. As we navigate the molecular intricacies, the story of microglial activation continues to unfold, promising deeper understanding and innovative approaches to harness the therapeutic potential within these intracellular pathways.

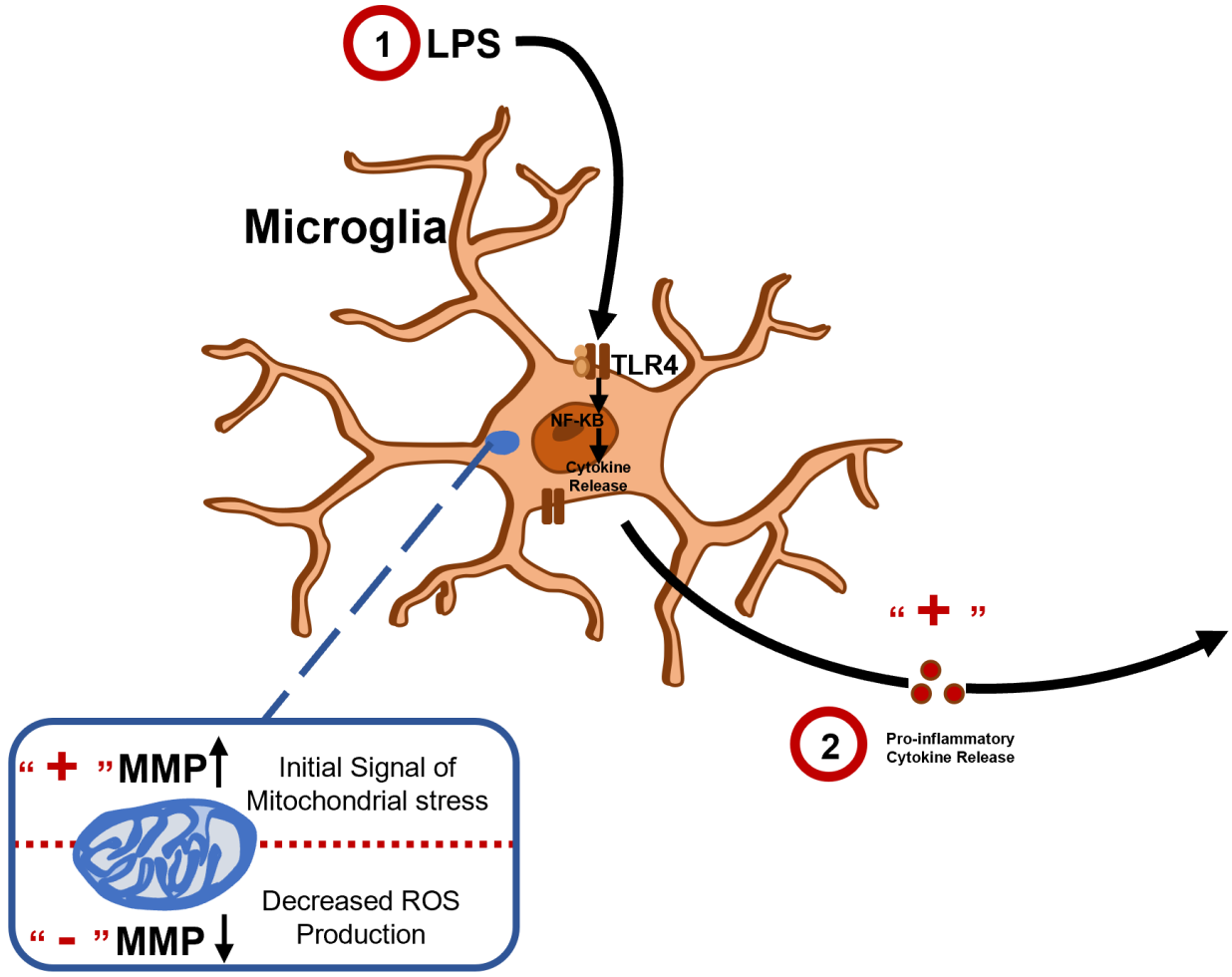


Figure 2: Intracellular Signaling Cascades in Microglial Activation.

Visual representation of the molecular events underlying the inflammatory response in microglia following exposure to LPS. The process begins with LPS binding to Toll-Like Receptor 4 (TLR4) on microglial cells, triggering the activation of the NF-κB pathway. The subsequent signaling cascade leads to alterations in the mitochondrial membrane potential, ultimately culminating in the release of pro-inflammatory cytokines including tumor necrosis factor-alpha (TNF-α), interleukin-1 beta (IL-1β), and interleukin-6 (IL-6).

Reactive Oxygen Species Signaling in Microglial Activation: Unveiling the Dual Nature of Oxidative Stress

Reactive Oxygen Species (ROS) emerge as enigmatic players in the intricate signaling pathways orchestrating microglial activation. This exploration delves into the pivotal role of ROS in shaping the inflammatory responses of microglia, shedding light on the dual nature of oxidative stress as both a signaling mediator and a potential driver of neuroinflammation. As we unravel the impact of ROS on microglial activation, a nuanced understanding of the delicate balance between physiological signaling and pathological consequences begins to emerge.

ROS, encompassing free radicals like superoxide anion ($O_2^{\bullet-}$) and non-radical species like hydrogen peroxide (H_2O_2), serve as essential signaling molecules in cellular processes. In the context of microglial activation, ROS generation is a hallmark response to various stimuli. While low levels of ROS are involved in physiological signaling, acting as secondary messengers in intracellular cascades, excessive ROS production can tip the balance towards oxidative stress. This dual nature of ROS highlights their Janus-faced role in microglial activation.

Oxidative stress in microglial activation is intricately linked to the activation of NADPH oxidase, a major source of ROS in these immune cells. Upon stimulation, NADPH oxidase is recruited to the cell membrane, where it catalyzes the production of superoxide anion. This initial burst of ROS acts as a signaling mediator, activating redox-sensitive pathways, including those involving MAPK and NF-κB. The activation of these pathways shapes the inflammatory responses of microglia, influencing the expression of pro-inflammatory cytokines, chemokines, and other mediators.

However, the fine line between physiological ROS signaling and pathological oxidative stress can be easily disrupted. Prolonged or excessive ROS production can lead to cellular damage, lipid peroxidation, and DNA oxidation, contributing to a state of chronic inflammation. The oxidative stress-induced damage becomes a self-perpetuating cycle as damaged cells release signals that further stimulate microglial activation, creating a feedforward loop that exacerbates neuroinflammation.

The impact of ROS on microglial activation extends beyond the realm of inflammation. ROS have been implicated in the modulation of microglial phagocytosis and their interactions with neurons. Additionally, the influence of oxidative stress on the blood-brain barrier integrity further underscores the multifaceted role of ROS in the neuroinflammatory milieu.

In conclusion, the exploration of ROS signaling in microglial activation unravels a complex interplay between physiological signaling and oxidative stress. The Janus-faced nature of ROS highlights the delicate balance that must be maintained to preserve the physiological functions of microglia while preventing the detrimental consequences of chronic inflammation. As we delve deeper into the molecular intricacies of ROS in microglial activation, potential therapeutic strategies targeting this signaling axis emerge, holding promise for mitigating neuroinflammatory processes in various neurological disorders.

Neuroinflammatory Implications of Microglial Activation Signaling Pathways

In the complex landscape of neurobiology, microglial activation signaling pathways unfold as critical determinants of both health and pathology. As we conclude our exploration, we delve into the broader neuroinflammatory implications of these

pathways, examining how their dysregulation can intricately contribute to the onset and progression of neurological disorders. Moreover, we scrutinize the emerging therapeutic targets that hold promise in modulating microglial responses, offering a potential beacon of hope in the realm of neurodegenerative diseases.

In its physiological state, microglial activation is a defensive mechanism aimed at maintaining neural homeostasis. However, when these responses become dysregulated, the consequences can be profound and contribute to the pathogenesis of various neurological disorders. Alzheimer's disease, Parkinson's disease, multiple sclerosis, and amyotrophic lateral sclerosis are among the conditions where aberrant microglial activation is a common denominator. In Alzheimer's disease, for instance, activated microglia surround amyloid plaques, releasing pro-inflammatory mediators that exacerbate neuronal damage and cognitive decline. Similarly, in Parkinson's disease, microglial activation is implicated in the inflammatory processes that contribute to dopaminergic neuronal degeneration.

The dysregulation of microglial activation signaling pathways, particularly MAPK and NF-κB cascades, plays a central role in these neuroinflammatory scenarios. Uncontrolled activation of these pathways can lead to an excess of pro-inflammatory cytokines, chemokines, and reactive oxygen species, creating a neurotoxic environment that contributes to neuronal damage and death. Understanding the specific molecular events that drive these neuroinflammatory responses provides a foundation for identifying targeted interventions aimed at restoring balance.

Therapeutic strategies targeting microglial activation signaling pathways are actively being explored as a means to mitigate neuroinflammation in various disorders. Small

molecules and biologics that modulate MAPK and NF-κB pathways are among the leading contenders(Kim et al. 2019). In preclinical studies, inhibitors of these pathways have shown promise in reducing neuroinflammation and ameliorating disease progression(Y. Zheng et al. 2023). Additionally, targeting specific receptors involved in microglial activation, such as Toll-like receptors, has emerged as a potential avenue for therapeutic intervention.

The evolving landscape of therapeutic targets extends beyond traditional pharmacological approaches. Immunomodulatory strategies, including the use of anti-inflammatory agents and immunotherapies, are being investigated for their potential to modulate microglial responses. Harnessing the potential of regenerative medicine, such as stem cell therapies, also holds promise in restoring balance to the neuroinflammatory milieu. The neuroinflammatory implications of microglial activation signaling pathways provide a critical lens through which we can understand and potentially intervene in the pathogenesis of neurological disorders. The dysregulation of these pathways contributes to the chronic inflammation observed in various conditions, underscoring the need for targeted therapeutic approaches. As research progresses, unraveling the complexities of microglial activation signaling pathways holds the key to developing innovative strategies that may one day alter the trajectory of neurodegenerative diseases, offering renewed hope for improved outcomes and quality of life for affected individuals.

Potential Therapeutic Targets for Modulating Microglial Responses

Potential therapeutic targets for modulating microglial responses are a subject of growing interest in neuroinflammation and neurodegenerative diseases. Microglia, the

primary immune cells in the central nervous system, play a crucial role in maintaining homeostasis and responding to various insults. Dysregulation of microglial responses is implicated in the pathogenesis of neuroinflammatory conditions, making the identification of therapeutic targets a critical pursuit.

Among the potential targets, inhibitors of MAPK (Mitogen-Activated Protein Kinase) signaling pathways have emerged as promising candidates. These pathways are integral to the intracellular signaling cascades that regulate microglial activation. By targeting specific components of MAPK pathways, researchers aim to modulate the molecular landscape associated with microglial activation. The intricate interplay between MAPK signaling and microglial functions presents a nuanced area for therapeutic intervention.

Inhibitors of MAPK Signaling Pathways: Navigating the Molecular Landscape in Microglial Activation Modulation

The intricate world of Mitogen-Activated Protein Kinase (MAPK) signaling pathways stands at the forefront of potential therapeutic interventions aimed at modulating microglial responses in neuroinflammatory conditions. This exploration focuses on small molecules and biologics designed to target the MAPK cascade, unraveling their potential to fine-tune the phosphorylation events that underlie microglial activation. By seeking to mitigate the excessive pro-inflammatory responses associated with neuroinflammation, these inhibitors represent a promising avenue for therapeutic advancement in the realm of neurological disorders.

The MAPK signaling pathways, comprised of the extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38 MAPK, play a pivotal role in transducing extracellular signals into intracellular responses within microglial cells (Farkhondeh et al. 2020). In the context of neuroinflammation, dysregulation of these pathways contributes to an exaggerated release of pro-inflammatory mediators, exacerbating neuronal damage. Small molecules and biologics designed as inhibitors within the MAPK cascade aim to disrupt this dysregulation, offering a potential brake on the inflammatory cascade.

One prominent class of inhibitors targets the phosphorylation events within the MAPK cascade. These small molecules are engineered to interfere with the enzymatic activity of kinases involved in the phosphorylation of MAPKs, disrupting the signaling cascade at critical junctures. By selectively inhibiting specific kinases, these molecules aim to modulate the intensity and duration of the MAPK response, preventing the escalation of neuroinflammatory processes (Cargnello and Roux 2011).

Furthermore, biologics, including monoclonal antibodies and kinase inhibitors, provide a targeted and sophisticated approach to intervene in the MAPK pathways. Monoclonal antibodies can specifically bind to critical components of the MAPK cascade, blocking their interactions and hindering the transmission of signaling events (Zahavi and Weiner 2020). On the other hand, Kinase inhibitors directly target the enzymatic activity of kinases within the MAPK pathways, serving as precision tools to disrupt phosphorylation events and downstream signaling.

Several compounds have shown promise in preclinical studies and early-phase clinical trials. For instance, inhibitors targeting p38 MAPK have demonstrated anti-inflammatory

effects in models of neurodegenerative diseases, showcasing their potential for mitigating microglial activation-induced neuroinflammation. Similarly, compounds modulating ERK and JNK pathways are under investigation for their ability to regulate microglial responses and influence the inflammatory milieu in neurological disorders.

Despite these promising strides, challenges remain. Achieving the delicate balance between inhibiting excessive inflammation and preserving essential immune functions poses a complex task. Moreover, these inhibitors' specificity and potential side effects require meticulous consideration. The journey toward harnessing the therapeutic potential of MAPK inhibitors in neuroinflammation necessitates further research, with ongoing efforts aimed at refining these compounds for optimal efficacy and safety.

In conclusion, inhibitors of MAPK signaling pathways stand as beacons of hope in the pursuit of modulating microglial responses and attenuating neuroinflammation. As researchers unravel the molecular intricacies of these pathways, the promise of targeted interventions grows, offering potential avenues for therapeutic advancement in the challenging landscape of neurological disorders.

As we delve into the intricate landscape of neuroinflammation, the spotlight turns to Toll-Like Receptors (TLRs), the sentinels positioned on the cell surface of microglia. This exploration focuses on agents designed to modulate TLRs, unraveling their potential as therapeutic targets for regulating microglial responses and alleviating the neuroinflammatory consequences that underlie various neurological disorders. Positioned at the frontline of pathogen recognition, TLRs emerge as promising points of intervention in the quest to mitigate neuroinflammation.

TLRs are integral components of the innate immune system, serving as molecular sentinels that recognize conserved patterns associated with pathogens, such as bacteria, viruses, and damaged cells. Microglia, as the resident immune cells of the central nervous system, express a repertoire of TLRs, enabling them to detect and respond to potential threats. However, dysregulation of TLR signaling in microglia has been implicated in the perpetuation of neuroinflammation, contributing to the pathogenesis of various neurological disorders.

Agents targeting Toll-Like Receptors present a multifaceted approach to modulating microglial responses. These agents can be categorized into various classes, including small molecules, antibodies, and synthetic ligands, each designed to interact with specific components of the TLR signaling cascade. By doing so, these agents aim to fine-tune the microglial response, preventing the disproportionate release of pro-inflammatory mediators associated with TLR activation.

Small molecules, such as TLR inhibitors and antagonists, represent a key class of TLR-targeting agents. These compounds are designed to interfere with the binding of ligands to TLRs or inhibit downstream signaling events, mitigating the cascade of inflammatory responses. For instance, small molecules targeting TLR4, a receptor implicated in neuroinflammation, have shown promise in preclinical studies for their ability to modulate microglial activation and reduce inflammation in the central nervous system.

Monoclonal antibodies provide another avenue for TLR modulation. These antibodies can specifically bind to TLRs, preventing ligand-receptor interactions and inhibiting downstream signaling. Antibodies targeting TLR2 and TLR4, in particular, have been explored for their potential to attenuate neuroinflammatory responses in various

neurological disorders. The precision offered by monoclonal antibodies allows for a targeted approach, minimizing off-target effects.

Synthetic ligands, including agonists and antagonists, offer a nuanced strategy to manipulate TLR signaling. Agonists can stimulate TLRs, potentially promoting anti-inflammatory responses and enhancing neuroprotective effects. Conversely, antagonists can block TLR activation, preventing the initiation of pro-inflammatory cascades. The design of synthetic ligands provides researchers with a toolkit to modulate TLR responses selectively, tailoring interventions to the specific needs of the microglial environment.

While the exploration of TLR-targeting agents is a burgeoning field, challenges persist. Achieving the delicate balance between dampening excessive inflammation and preserving essential immune functions remains a critical consideration. Additionally, the potential off-target effects and unintended consequences of manipulating TLR signaling necessitate careful evaluation and refinement of these therapeutic approaches.

In conclusion, Toll-Like Receptor targeting agents represent a dynamic frontier in the pursuit of modulating microglial responses and alleviating neuroinflammation. As researchers unravel the intricate dance of TLR signaling in neurological disorders, the potential for targeted interventions grows, offering a promising avenue for therapeutic advancement in the challenging landscape of neuroinflammatory conditions.

Immunomodulatory strategies have also emerged as versatile tools aimed at recalibrating the immune response. These strategies go beyond the direct modulation of microglial behavior, extending their influence to the broader immune milieu. By doing

so, they aspire to restore balance in neuroinflammatory conditions, offering a comprehensive and nuanced approach to tackling the complexities of immune dysregulation in various neurological disorders.

One pillar of immunomodulatory strategies in neuroinflammation involves the use of anti-inflammatory agents. These compounds are designed to dampen excessive immune responses and mitigate the release of pro-inflammatory mediators, thereby reducing the neurotoxic environment associated with microglial activation. Non-steroidal anti-inflammatory drugs (NSAIDs), corticosteroids, and specific cytokine inhibitors represent key players in this category. NSAIDs, such as ibuprofen, act by inhibiting enzymes involved in the synthesis of pro-inflammatory mediators, while corticosteroids, like prednisone, exert broad anti-inflammatory effects by modulating immune cell activity. Specific cytokine inhibitors, such as monoclonal antibodies targeting tumor necrosis factor-alpha (TNF-α), offer a targeted approach to mitigating neuroinflammation by neutralizing key inflammatory mediators.

Beyond anti-inflammatory agents, immunotherapies harness the body's own immune system to modulate the neuroinflammatory response. This category encompasses a diverse array of strategies, including monoclonal antibodies, adoptive cell therapies, and vaccines. Monoclonal antibodies, designed to target specific components involved in neuroinflammation, offer precision in neutralizing key players without broad immunosuppression. Adoptive cell therapies involve the infusion of immune cells, such as regulatory T cells, with the aim of restoring immune balance. Vaccines, particularly those targeting specific pathogens associated with neuroinflammatory conditions, seek to induce a controlled immune response, preventing excessive inflammation.

Glucocorticoids, a class of corticosteroids, represent a nuanced facet of immunomodulation. These agents not only possess potent anti-inflammatory properties but also exert immunosuppressive effects by modulating the function of various immune cells. While their broad-spectrum impact raises concerns about potential side effects, including immunosuppression, their judicious use under appropriate clinical supervision can yield therapeutic benefits in certain neuroinflammatory conditions.

Modulating Microglial Phenotype: In addition to systemic immunomodulatory approaches, there is a growing focus on strategies that directly modulate microglial phenotype. This involves promoting a shift from a pro-inflammatory M1 phenotype to an anti-inflammatory M2 phenotype, fostering a more reparative and neuroprotective microglial state. Compounds targeting specific receptors on microglia, such as PPAR-γ agonists, have shown promise in preclinical studies for their ability to promote an M2 phenotype, potentially offering a more targeted and nuanced approach to immunomodulation.

Challenges in immunomodulatory strategies include the need for precise temporal and spatial control to avoid unwanted immune suppression and potential side effects. Moreover, the heterogeneity of neuroinflammatory conditions requires tailored approaches, emphasizing the importance of personalized medicine in the field of immunotherapy. The realm of immunomodulatory strategies offers a multifaceted approach to navigating neuroinflammation. By encompassing both anti-inflammatory agents and immunotherapies, these strategies aim to recalibrate the immune response and restore balance in the complex milieu of neurological disorders. As research continues to unravel the intricacies of immune dysregulation, immunomodulatory

strategies hold promises as innovative and targeted interventions in the evolving landscape of neuroinflammatory conditions.

Chapter Three: Methodology

Description/Justification of Subjects

All experimental protocols were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Nevada Las Vegas. The University of Nevada Las Vegas vivarium is maintained at 70 degrees Fahrenheit, and the colony lights are on a 12-h light/dark cycle. Mice are provided access to food and water ad libitum. Slices were prepared from CX3CR1-EGFP transgenic mice aged 15-25 days postnatal. Pups were group housed with the dam until the time of experimentation.

$CX_3CR\text{-}1^{GFP}$

CX3CR-1GFP mice represent a valuable tool in the field of neuroscience and immunology, offering a unique means to investigate the dynamics of specific cell populations within the central nervous system, particularly microglia. This transgenic mouse strain has been intentionally designed to express green fluorescent protein (GFP), a naturally occurring protein found in certain jellyfish species. The genetic modification introduces the GFP gene under the control of the CX3CR-1 promoter, which is a specific genetic sequence that regulates gene expression in cells, such as microglia, where CX3CR-1 is active. Consequently, when CX3CR-1 is expressed in microglia or other cells, it induces the production of GFP concurrently, leading to the emission of green fluorescence by those cells.

GFP, as a fluorescent marker, is an indispensable asset for scientists working with CX3CR-1GFP mice. It emits a vivid green light when illuminated with specific wavelengths of light falling within the range of 395-470 nanometers (nm). This property allows for non-invasive and real-time visualization of cells expressing CX3CR-1 in living

tissues. We have leveraged this green fluorescence to track the movements, interactions, and activation states of microglia within the central nervous system. The capacity to monitor microglia behavior in situ provides insights into their role in neuroinflammation, neuronal support, and various neurological diseases. Overall, CX3CR-1GFP mice, by combining genetics and fluorescence, offer a dynamic platform for investigating the intricate workings of microglia and other CX3CR-1-expressing cells in the context of neurobiology and immunology.

General Experimental Procedures

Acute Slicing and Imaging

Slice Preparation and Solutions

A Vibratome was used to prepare acute hippocampus brain slices, which were 400µm thick. Before imaging, slices were allowed to acclimate to the room temperature (68–74 °F) for 30 minutes. The oxygenated artificial cerebrospinal fluid (aCSF) contained the following amounts (in mM): NaCl 126, KCl 2.5 or 4.2, $NaHCO_3$ 26, glucose 10, $MgCl_2$ 2, NaH_2PO_4 1.25 and $CaCl_2$ 2. After being moved to a recording chamber, slices were maintained by being continuously infused with oxygenated aCSF at a flow rate of 1-3 mL/min.

Live Imaging

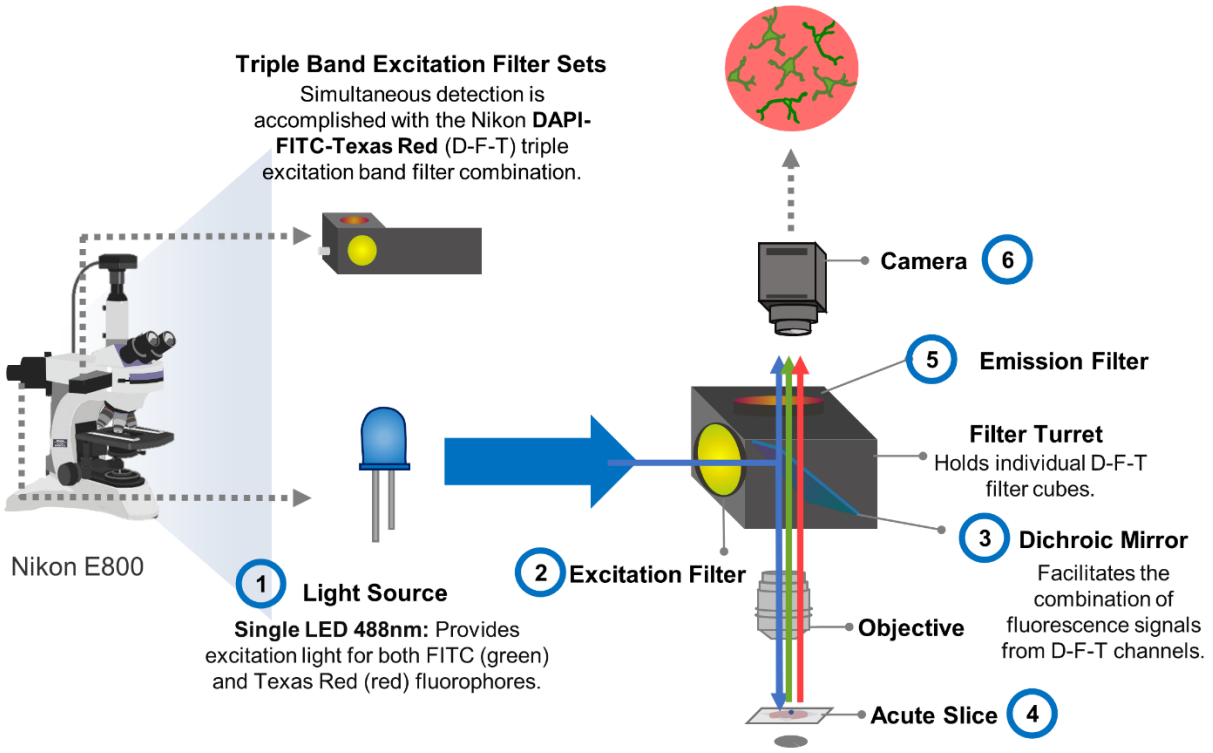
Fluorescence microscopy has revolutionized our ability to investigate biological specimens, both fixed and alive. This particular microscopy technique possesses the capacity to selectively observe fluorescent entities with exceptional precision and sensitivity, even when applying low quantities of fluorophores. The historical roots of fluorescence microscopy can be identified in the nineteenth century. The initial

investigations with naturally occurring fluorescent chemicals, such as quinine, established the fundamental basis for the advancement of this imaging methodology. The significant progress achieved by August Köhler and Carl Zeiss during the early 20th century was instrumental in the advancement and refinement of fluorescence microscopy, which ultimately enabled its widespread application.

Fluorescence microscopy leverages the inherent ability of certain molecules, referred to as fluorophores, to absorb light at a particular wavelength and then emit it at a different wavelength. The utilization of particular excitation wavelengths induces the stimulation of fluorophores, hence causing the emission of light. The ability to discriminate between excitation and emission wavelengths is key to the exceptional selectivity of this method. This enables the visualization of biological structures or molecules, even in complex environments, with an exceptional signal-to-background ratio. Fluorescence microscopy has permeated virtually all facets of biological research. In cell biology, it facilitates the tracking of cellular processes and the observation of subcellular structures. Immunofluorescence and live-cell imaging are pivotal for studying molecular interactions and dynamic events. Furthermore, fluorescence microscopy is instrumental in neuroscience, genetics, microbiology, and pharmacology.

Imaging was conducted utilizing a Nikon Eclipse e800 microscope equipped with a Hamamatsu digital CMOS camera that was directly coupled to a 40X-W/0.80 numerical aperture objective lens. EGFP was typically excited at 488 nM and TMRE was excited at 552 nM. For acquiring images, the LED-driver LEDD1B did not exceed 1000mA. A single LED at 488nm serves as the light source for both FITC (green) and Texas Red

(red) fluorophores. The excitation filter selectively allows wavelengths for DAPI, FITC, and Texas Red fluorophores to pass through, initiating excitation (Figure 3). Neither photobleaching nor cellular injury was detected during the acquisition of time-lapse images. In every instance, the LED current limit was meticulously monitored and maintained consistently across all experiments. Imaging was conducted at a rate of 100ms exposure, 200ms delay, and a gain of 200.



Nikon Triple Band Excitation Filter Combination Specifications

Filter Set	Excitation Filter (nm)	Dichroic Mirror (nm)	Emission Filter (nm)	Profiles
DAPI	395-410	445	450-470	Violet EX/ Blue
FITC	490-505	510	515-545	Blue EX/ Green EM
TEXAS Red	560-580	590	600-650	Green Ex/ Red EM

Figure 3: Multichannel Microscopy Imaging.

Diagramed illustration of the imaging process for EGFP and TMRE using a Nikon Triple Band excitation filter combination. A single LED at 488nM serves as the light source for both FITC (green) and Texas Red (red) fluorophores. The excitation filter selectively allows wavelengths for DAPI, FITC, and Texas Red fluorophores to pass through, initiating excitation. The dichroic mirror then combines the fluorescence signals from D-F-T channels, providing simultaneous detection. After passing through an acute slice, emitted fluorescence is filtered by emission filters specific to DAPI, FITC, and Texas Red, directing the signals to the camera for image capture.

Fluorescent Dye Loading

Fluorescent dye loading, a key technique in fluorescence microscopy, has significantly enhanced the ability of researchers to visualize and analyze biological specimens with great precision. The diverse range of available fluorophores, from classic dyes like fluorescein and rhodamine to advanced quantum dots and green fluorescent protein (GFP), offers researchers the flexibility to tailor their experiments according to the unique requirements of their biological specimens. This flexibility is not just about choosing the right dye; it extends to the methods of introducing these dyes into cells or tissues for detailed observation and analysis.

One key technique in this realm involves loading individual cells with fluorescent probes through patch pipettes. This method allows for the exact and targeted introduction of dyes into cells, enhancing the specificity and accuracy of fluorescence microscopy. Its versatility accommodates a broad spectrum of dyes, suitable for various research needs. Markers such as Lucifer yellow facilitate detailed morphological reconstructions, while ion-sensitive indicator dyes like fura-2 are crucial for studying cellular signaling processes. Additionally, dye-labeled proteins are essential for advanced fluorescence techniques like FRET, FCS, and FRAP, which investigate protein interactions and dynamics within cells (Eilers and Konnerth 2009). This adaptability makes the method a vital tool in modern biological research.

A prominent application of this technique is in calcium (Ca2+) imaging, integral to understanding cellular signaling. Combining dye loading with electrophysiological methods like whole-cell patch-clamp recordings provide a comprehensive approach to studying cellular processes. Researchers can simultaneously perform

electrophysiological measurements and optical imaging, offering a multifaceted view of cellular behavior. This method's ability to quantitatively assess electrophysiological properties while visually tracking cellular activities is a significant advancement in biological research.

In a similar vein, this study utilized an approach that combined recording electrophysiological properties while visualizing specific cells - in this case, microglia. Microglia, known for their role in brain immunity and neuroinflammation, are crucial to understanding various neurological conditions. By applying this dual technique, the study was able to observe microglial behavior and properties in real time, shedding light on their functions and interactions within the brain.

Loading and Treatment

The cell permeant dye Tetramethylrhodamine ethyl ester (TMRE) was applied to acute slices at a concentration of 1 nM, prepared in 4mLs of artificial cerebral spinal fluid, for a duration of 5 minutes. LPS was dissolved in saline at a concentration of 10µg/mL and bath applied at a rate of 1-3 mL/ min during acute slicing experiments. Emapunil was dissolved in saline to a concentration of 50µg/mL. In experiments where both LPS and emapunil were applied, the pretreatment of emapunil preceded LPS by 10 minutes.

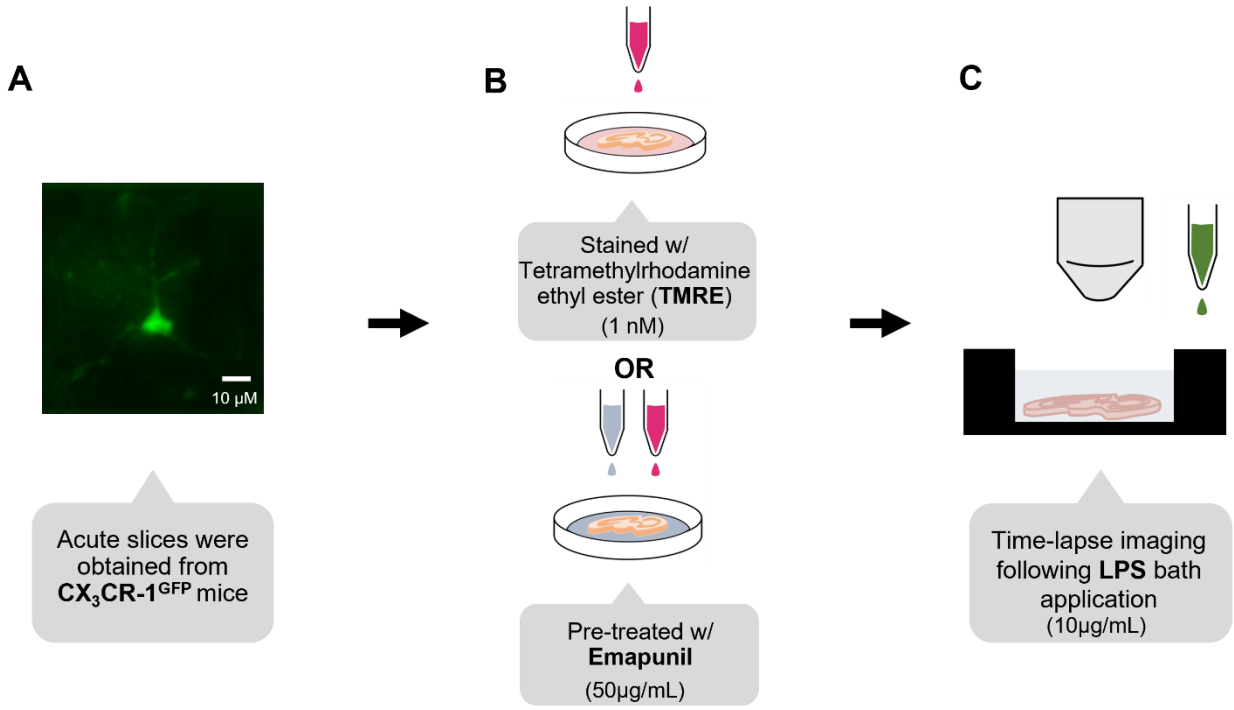


Figure 4: Dye loading and Treatment Protocol.

A. Representative image of acute slices obtained from fractalkine mice with a targeted deletion of the microglial CX3CR1 receptor and the insertion of EGFP. **B.** TMRE was dye-loaded onto acute slices at a concentration of 1 nM, prepared in 4 mLs of artificial cerebral spinal fluid. **C.** In groups treated with both emapunil and LPS, emapunil was pretreated. **D.** Tetramethylrhodamine ethyl ester (TMRE) labeling was followed by the bath administration of LPS to observe changes in mitochondrial membrane potential. This protocol strategically exploits mitochondrial membrane potential fluctuations as an indicator of microglial inflammatory states, offering a screening tool for potential pharmaceutical interventions.

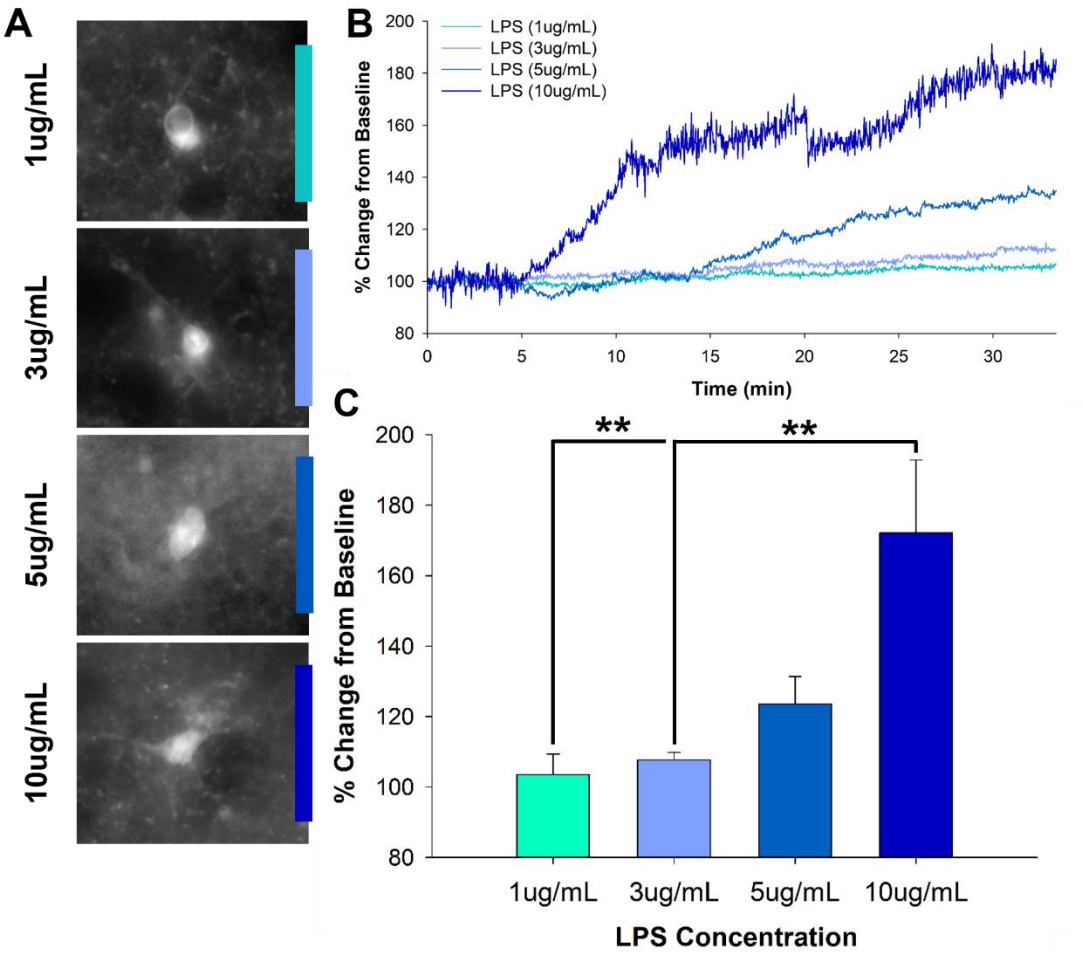


Figure 5: Microglial Mitochondrial Membrane Potential Modulation in Response to Varying LPS Doses.

A. Representative images of microglia bath applied with concentrations of 1, 3, 5, or 10 µg/mL lipopolysaccharide (LPS) (n=3). **B.** Representative traces of the percentage change from baseline activity of microglial mitochondrial membrane potential (MMP) over time. **C.** Cumulative percent change from baseline, indicating a positive correlation between increased LPS concentrations and enhanced microglial MMP. These results highlight the dose-dependent relationship and suggest that higher LPS concentrations lead to increased microglial MMP (p= 0.017; p=0.021).

Two-Photon Induced Micro-Lesioning

A Zeiss LSM510-Axioskop-2 two-photon laser scanning microscope with a 40X-W/0.80 numerical aperture objective lens was used for imaging, and it was directly connected to a 10 W Chameleon ultrafast laser (Coherent). Large two-photon absorption cross-sections of lipofuscin and EGFP were usually stimulated around 820 nm, and epifluorescence was seen using external detectors equipped with 510 nm (40 nm bandpass) and 605 nm (55 nm bandpass) filters. When obtaining time-lapse photos, prolonged scanning revealed no signs of cellular damage or photobleaching, and laser intensities exiting the objective during image acquisition were less than 25 mW. utilizing the same wavelength used for picture acquisition, a circular area with a diameter of 5 μm was briefly lit (total cumulative illumination time of 110 msec at 10× intensities) in order to produce discrete micro-lesions utilizing two-photon stimulation. Every time, the laser's intensity was closely observed and maintained constant throughout the experiment. Brain slices were imaged at depths of up to 100 μm and greater than 50 μm. For imaging lesions, the average depth was 75 μm. Z-stacks covering a field of 64.5 x 64.5 μm were collected in 1.5 μm increments. For the z-stack, the average scan duration was around one minute and eighteen seconds.

The images were thresholded to remove the background, excluding pixels with a value equal to or less than the threshold that was not part of the lesion. Image J was used to calculate the number of microglial processes following the introduction of 3 separate lesions to determine changes in morphology. The laser lesion appears orange due to the broad fluorescence emission of lipofuscin-like degradation products in green and red channels. Micro-lesions are detected by fluorescence emission at the 610-640nm

range. Lipofuscin has a broad fluorescence emission spectrum from 460 to 630 nm; therefore, when simultaneously acquiring EGFP fluorescence at 510-550 nm and lipofuscin fluorescence. Sholl analysis was conducted with ImageJ to measure changes in microglial morphology over increasing cycles of stress.

Data and Statistical Analysis

ImageJ and Clampfit 10.7 were utilized to trace ROIs and measure the percent change from baseline in stack images. Statistical significance of differences in mean values were assessed by conducting a t-test or one-way analysis of variance (ANOVA) as appropriate. Differences between means were considered significant at values of * $p \leq .05$, **: $p \leq .01$, ***: $p \leq .001$.

Chapter Four: Results

Introduction

In the intricate landscape of neuroinflammation, microglia stand as sentinels, orchestrating complex responses vital for maintaining central nervous system (CNS) homeostasis. This duality is the focus of two innovative studies that converge on understanding the nuanced transitions between acute and chronic inflammatory states in the brain. The first study delves into the predictive modeling of microglial stress, drawing parallels with principles used to forecast metal fatigue. By hypothesizing that increased cycles of stress induce irreversible microglial deformations, compromising their ability to withstand future stressors, the study aims to unravel the delicate balance microglia maintain in either promoting healing or exacerbating CNS conditions. This novel approach offers valuable insights for advancing therapeutic interventions in neuroinflammatory diseases, emphasizing the pivotal role of microglial mitochondrial dynamics.

Complementing this endeavor, the second study focuses on the dynamic role of microglia in acute states of inflammation, emphasizing their responsiveness to immune stimuli. Leveraging live imaging techniques and fluorescent reporting of mitochondrial membrane potential ($\Delta\Psi m$) changes in response to lipopolysaccharide (LPS), a known immune stimulant, the research sheds light on distinct mitochondrial membrane dynamics driving microglial responses. The convergence of these studies underscores the essentiality of microglia in the context of CNS health and disease, offering a comprehensive exploration of their role in both acute and chronic inflammatory scenarios. Together, these investigations contribute to a more holistic understanding of

microglial dynamics, providing a foundation for targeted therapeutic strategies aimed at modulating neuroinflammatory responses across diverse states.

Results for Aim #1: Modeling Cycles of Microglial Stress

Considering the essential role of microglia in neuroinflammation, developing a predictive model of microglial stress becomes a useful tool. This model parallels the principles used in predicting metal fatigue, adapting them to understand the transitions from acute to chronic inflammatory states in the brain. By hypothesizing that increased cycles of stress lead to irreversible microglial deformations, diminishing their capacity to withstand future stress, this study explores the delicate balance microglia maintain in either healing or exacerbating CNS conditions. The predictive model applied in this research offers a novel approach to understanding the nuanced transition between acute and chronic neuroinflammation, providing invaluable insights for advancing therapeutic interventions in neuroinflammatory diseases. Such an innovative approach underscores the importance of microglial mitochondrial dynamics in the broader context of CNS health and disease.

In predictive modeling studies of metal fatigue, stress and strain measures are fundamental for predicting the endurance limit of metal components (Serjouei and Afazov 2022). Young's Modulus, a measure of a material's stiffness, is utilized to assess stress and strain in metal components. In an experiment, stress is incrementally applied to a metal component, and the corresponding strain is measured. By plotting stress against strain, engineers can determine the Young's Modulus of the material. This information, along with data on stress amplitudes and cycle numbers, is then used to

predict the endurance limit of the metal component (Bannantine, n.d.), beyond which it will fail.

Similarly, in the investigation of microglial stress in brain tissue, the concept of stress and strain is applied, albeit in a different context. Instead of testing tensile strength directly, changes in microglial formations and morphology serve as indicators of the tissue's endurance limit. Through cyclic stress induced by micro-lesions in the brain tissue, alterations in microglial branches are observed, reflecting the strain experienced by these cells. By correlating stress amplitudes and cycle numbers with microglial changes, akin to plotting stress against strain in materials testing, the study predicts the tissue's endurance limit, crucial for understanding the transition from acute to chronic neuroinflammation. This approach bridges principles from material science and neuroscience, offering a unique perspective on assessing the resilience of brain tissue under pathological conditions.

To empirically validate our predictive model of acute to chronic inflammation, we first examined the dynamic nature of microglia in acutely prepared brain slices with EGFP-expressing microglia (Figure 17A; T_0). The morphology of microglia localized in the healthy interior regions of brain slices was observed using two-photon laser scanning microscopy. These regions of microglia cells appeared to have typical surveilling phenotypes through their ramified processes. Filopodia extension and retraction were observed from microglia, indicating active sensing of the local environment by the microglial processes. Delicately handling the prepared tissue ensured that the only cells affected by the slicing process were located on the surface of the slice (<10um). At the

depth at which we conducted the present study, microglia did not appear to be activated.

Acute fractalkine slices were introduced to brief high-intensity two-photon laser bleaching within a discrete region of tissue. Additional focal lesions were made to continue increasing cycles of stress. Over the course of three micro-lesions performed at an interval of 5 minutes, autofluorescence was detected at each lesioned region due to lipofuscin. Previous studies have shown lipofuscin is created through the breakdown of membranes and the peroxidation of lipids and can be detected on a broad fluorescence emission spectrum ranging from 460 to 630nm (Eichhoff et al., 2008; Seehafer and Pearce,2006). When imaging both EGFP and lipofuscin fluorescence at 520-550nm and 610-640nm, respectively, we could reliably discern between the microglial processes and the lipofuscin (Figure 17A; T_{final}). The swift response of microglia to the stress of the lesion resulted in the initiation of outgrowing processes towards the damage. Processes of the neighboring microglia also oriented themselves towards the damage (Figure 17A; T_1) until another cycle of stress (Figure 17A; T_2) was introduced.

Predictions were made based on microglia located near the site of injury to determine if the cells would be reliable or reach their failure point following increased cumulative damage (Figure 17B). As the cumulative damage continued to increase, microglia cells closer in proximity showed variations in their morphology. Decreases in the branch number of microglia near the lesion highlight differences in the cyclic applied stress. This indicated that both the mean stress amplitude and the number of times the microglial cells experienced a cycle of applied stress determined how many more cycles

could be endured (Figure 3B). A higher mean stress amplitude is reflected in morphology changes, such as transitioning from a ramified state to an activated state. The estimation of an endurance limit or the number of times microglia could cycle through these morphological changes was based on stress amplitude and cycle number (Figure 3C).

The range of stress microglia can endure is dependent on what state the cell has begun at. For instance, the microglial cells closest to the damaged region during the first cycle of stress (Figure 17A; T_1) did not have any additional resources to go toward their neighboring cells by the time the third cycle of stress (Figure 17B) took place. The breakdown in the formation of previously tiled microglia cells is similar to the elastic deformation that occurs during micro-fracture cumulation (Figure 16). Applying cumulative stress in the form of micro-lesions induced cycles of microglial stress and resulted in decreases in the number of microglial branches proportional to cyclic applied stress (Figures 18).

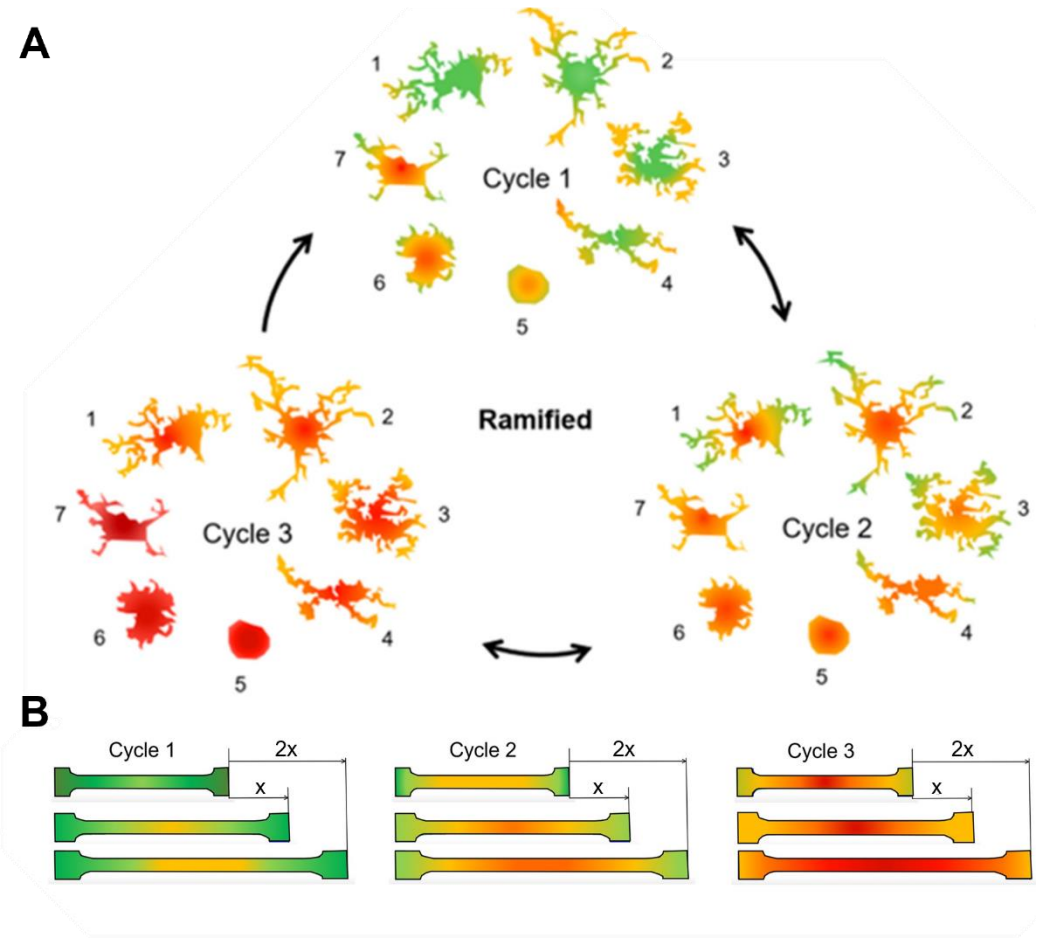


Figure 6: Linear Relationship Between Strain and Stress Responses in Microglia.

A. schematic diagram illustrates the dynamic transitions of microglia through increasing inflammatory states. The depiction captures the ramified microglial cycles, showcasing their morphological alterations and responses during the progression of inflammatory stimuli. **B.** Hooke's law was employed to quantify the strain and stress experienced by ramified microglia at each cycle. This analysis establishes a linear relationship between the strain exerted on microglia, indicative of their deformation, and the corresponding stress responses, providing insights into the mechanical aspects of microglial responses to inflammatory challenges.

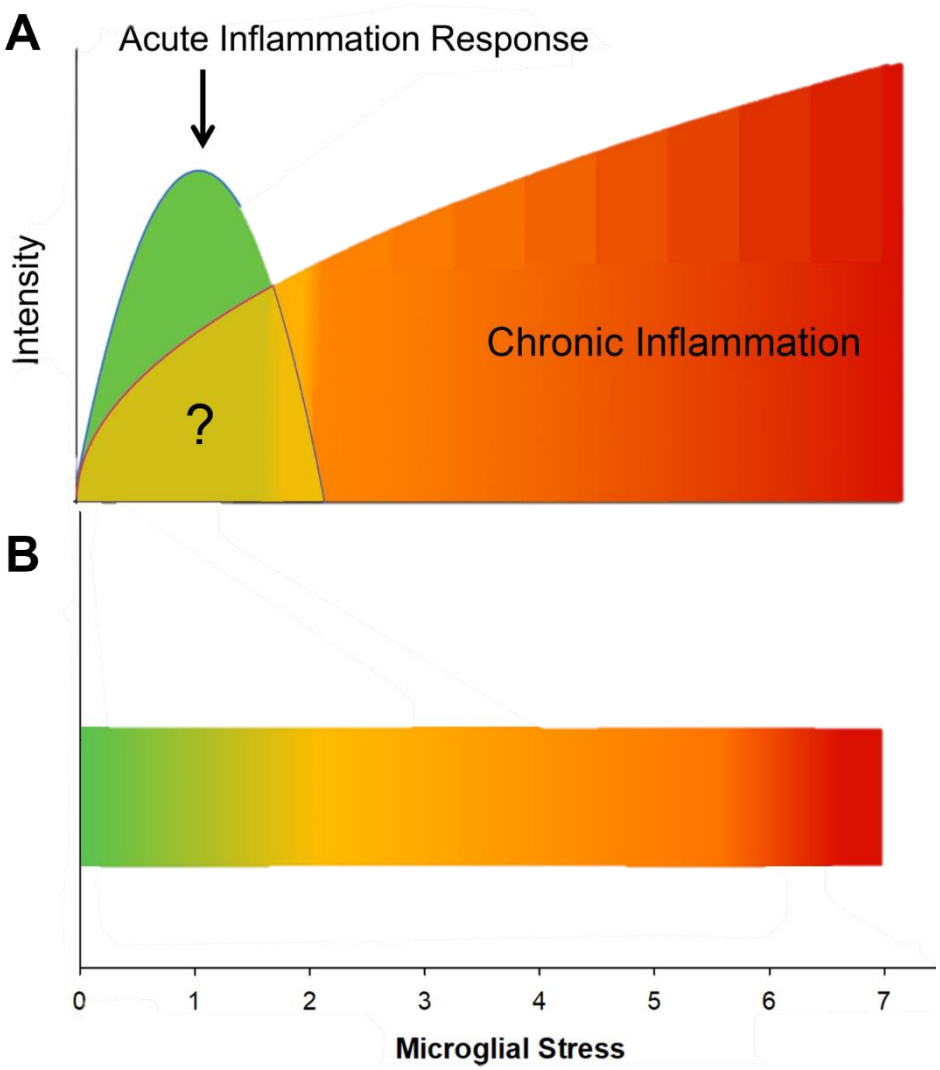


Figure 7: Distribution of Acute to Chronic States of Microglial Inflammatory Responses.

A. Distribution of microglial inflammatory responses is delineated concerning the intensity of stress over time. The diagram visually portrays the instances of both acute and chronic cases, offering insights into the temporal dynamics of microglial responses to varying stress levels. **B.** Schematic illustrating the proportional relationship between escalating microglial inflammatory states and systemic stress, providing insights into their interdependence. The diagram contributes to our understanding of the intricate dynamics between microglial responses and overarching stress levels.

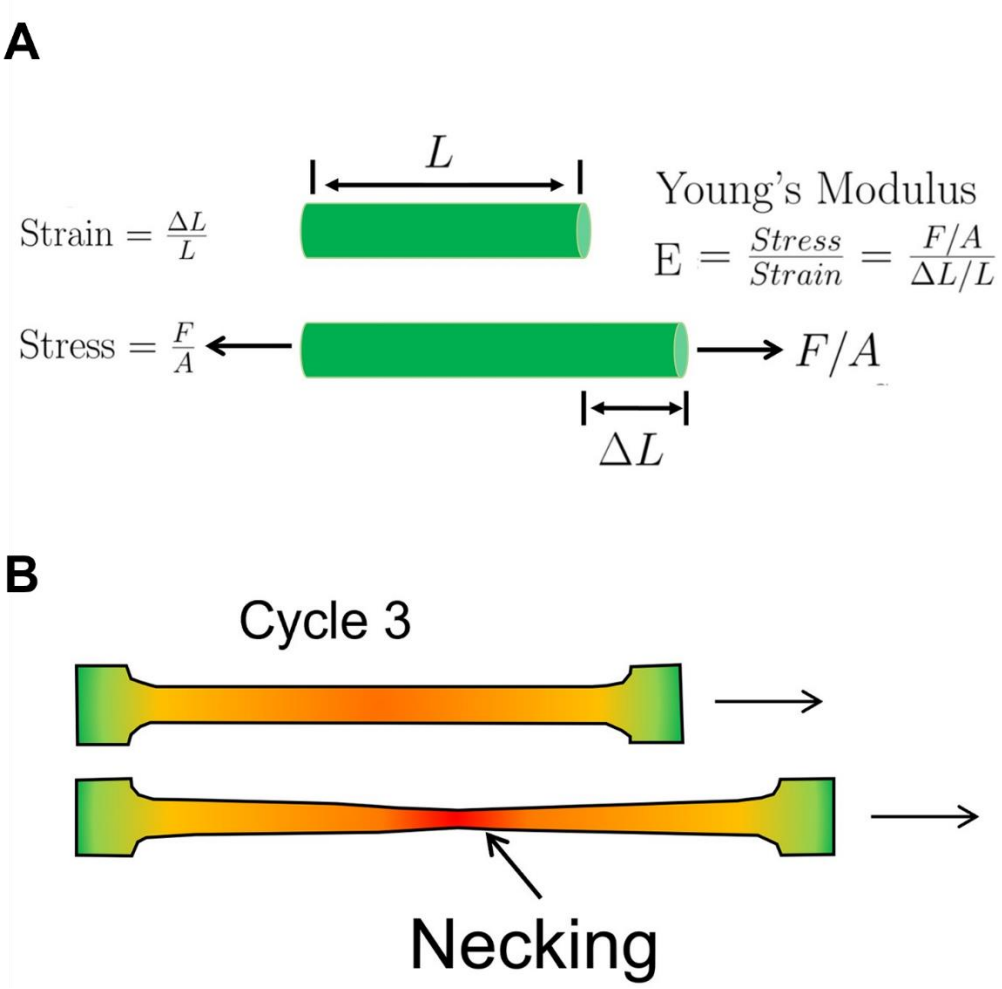


Figure 8: Model of Acute Stress Response.

A. Application of Young's Modulus is used to predict microglial responses as stress intensifies across successive ramified microglial cycles. The varying stress levels are represented, providing insights into the dynamic mechanical properties of microglia during acute stress. **B.** Diagram of the Necking Phenomenon, an irreversible point of deformation, is depicted in the final ramified microglial cycle. The inclusion of necking highlights a crucial aspect of the acute stress response model, emphasizing the irreversible structural changes microglia undergo under increasing stress conditions. The combination of Young's Modulus prediction and the identification of necking contributes to a comprehensive understanding of the mechanical behavior of microglia during acute stress.

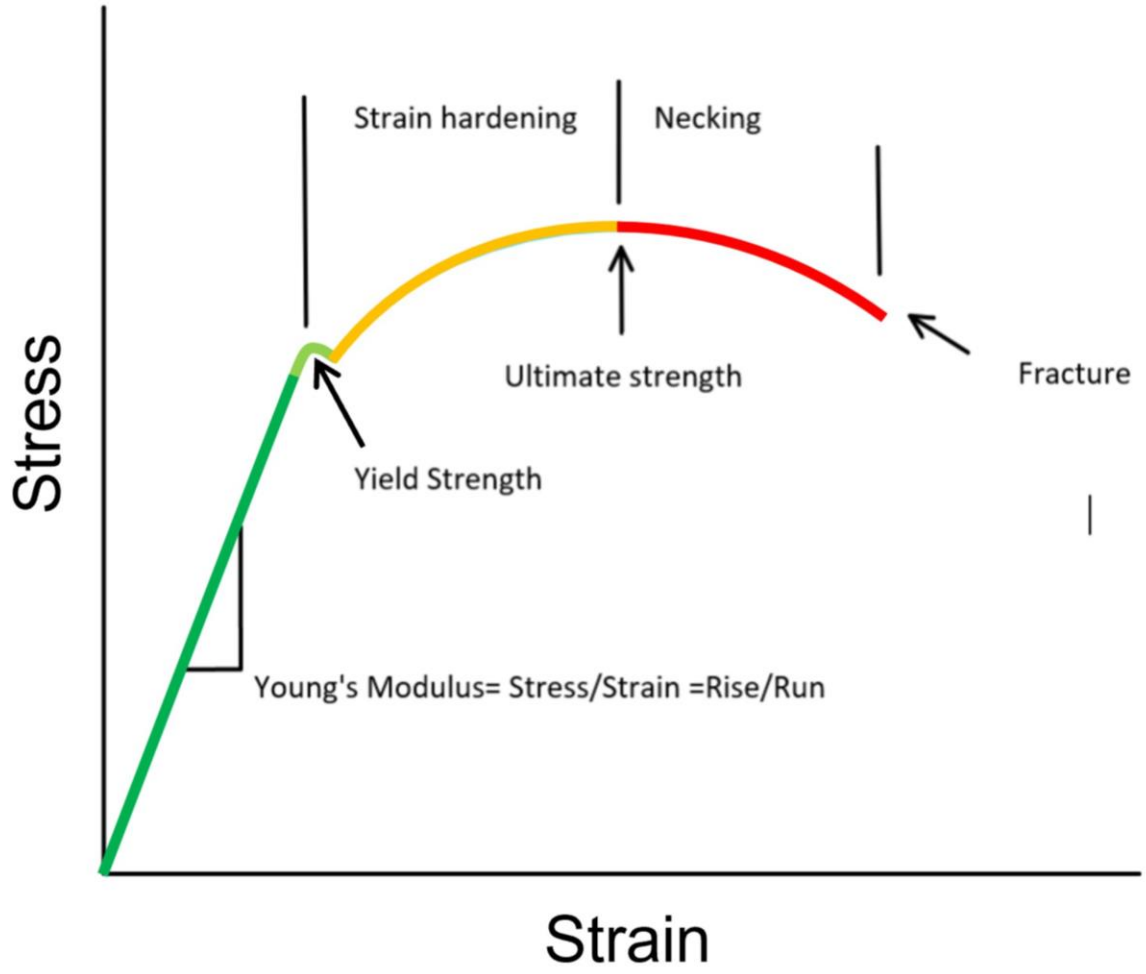


Figure 9: The Relationship of Stress and Strain in Young's Modulus Highlights Important Points of the Microglial Stress Response.

Young's Modulus slope regarding increasing stress levels during successive microglial cycles. It highlights distinct regions: the yield strength (green), strain hardening (yellow), and critical stages leading to necking and fracturing (red), with the point before necking indicating ultimate strength. The green region signifies initial deformation akin to microglial response to acute stress, while yellow reflects their capacity for adaptation and resistance. The red portion denotes critical stages, including irreversible deformation resembling the point of no return during acute stress responses. Understanding these mechanical properties aids in predicting microglial reactions to varying stress levels, vital for modeling acute stress responses.

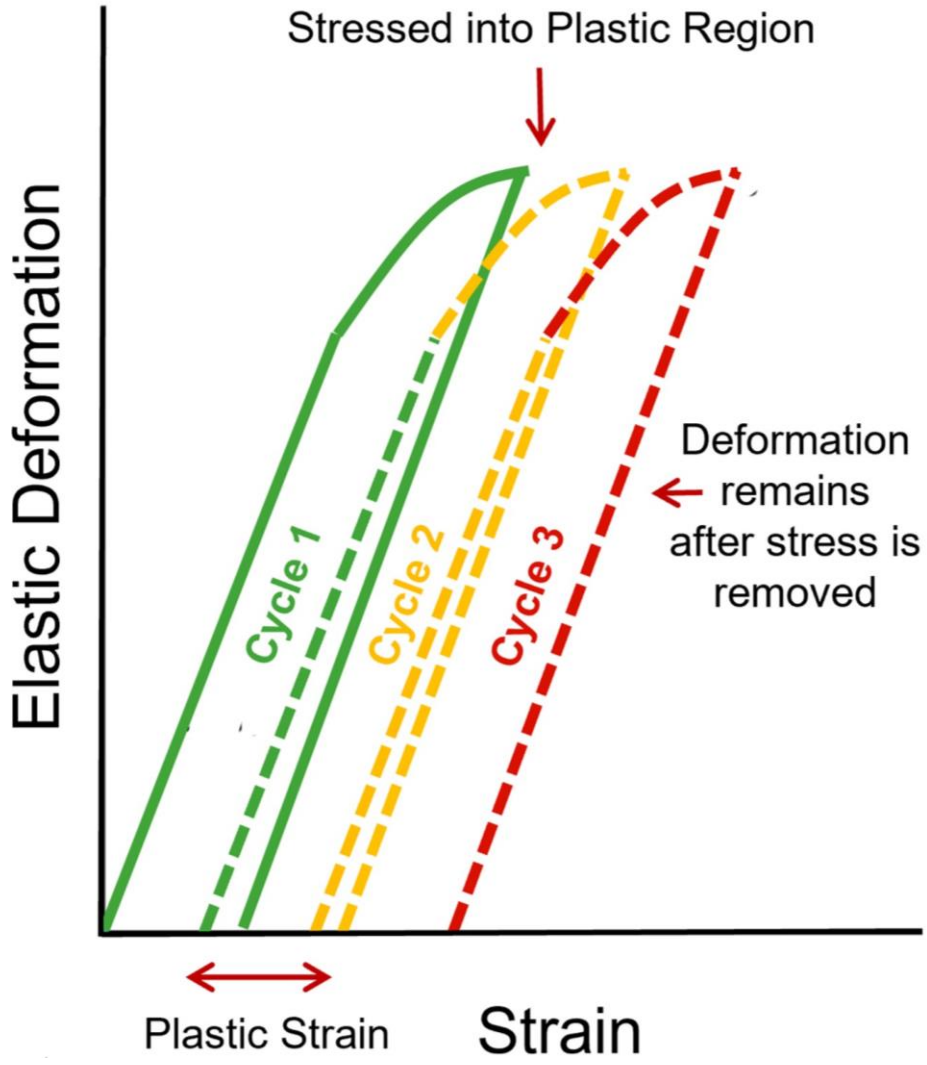


Figure 10: Microglial Plasticity and Deformation Across Successive Cycles.

Diagram of the alterations in plastic deformation as microglia progress through successive cycles. Examining elastic deformation changes with increasing strain, microglia demonstrate distinctive plastic regions of deformation during cycle 1 (green), cycle 2 (yellow), and cycle 3 (red). Once cycle 3 is reached, the deformation persists after stress has been removed, emphasizing the lasting impact of microglial plasticity.

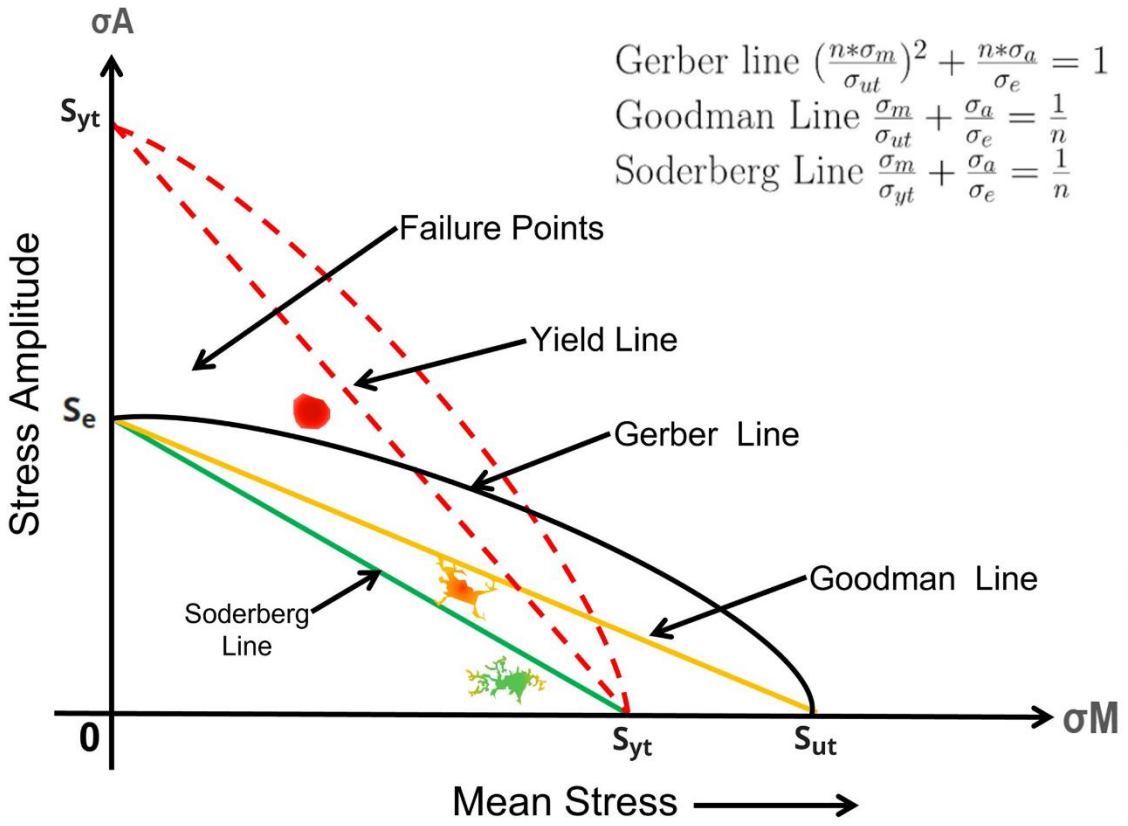


Figure 11: Predictive Endurance Limits in Microglial Stress Response.

Predictions of stress amplitude over mean stress in microglial cycles using three critical lines: the Gerber line (Black), the Goodman Line (Yellow), and the Soderberg Line (Green). The Gerber line predicts fatigue failure based on alternating stress levels, while the Goodman line predicts failure due to fluctuating stresses. The Soderberg line estimates failure from a combination of mean and alternating stresses. Each line aids in predicting the endurance limit of microglia to ensure structural integrity across successive cycles.

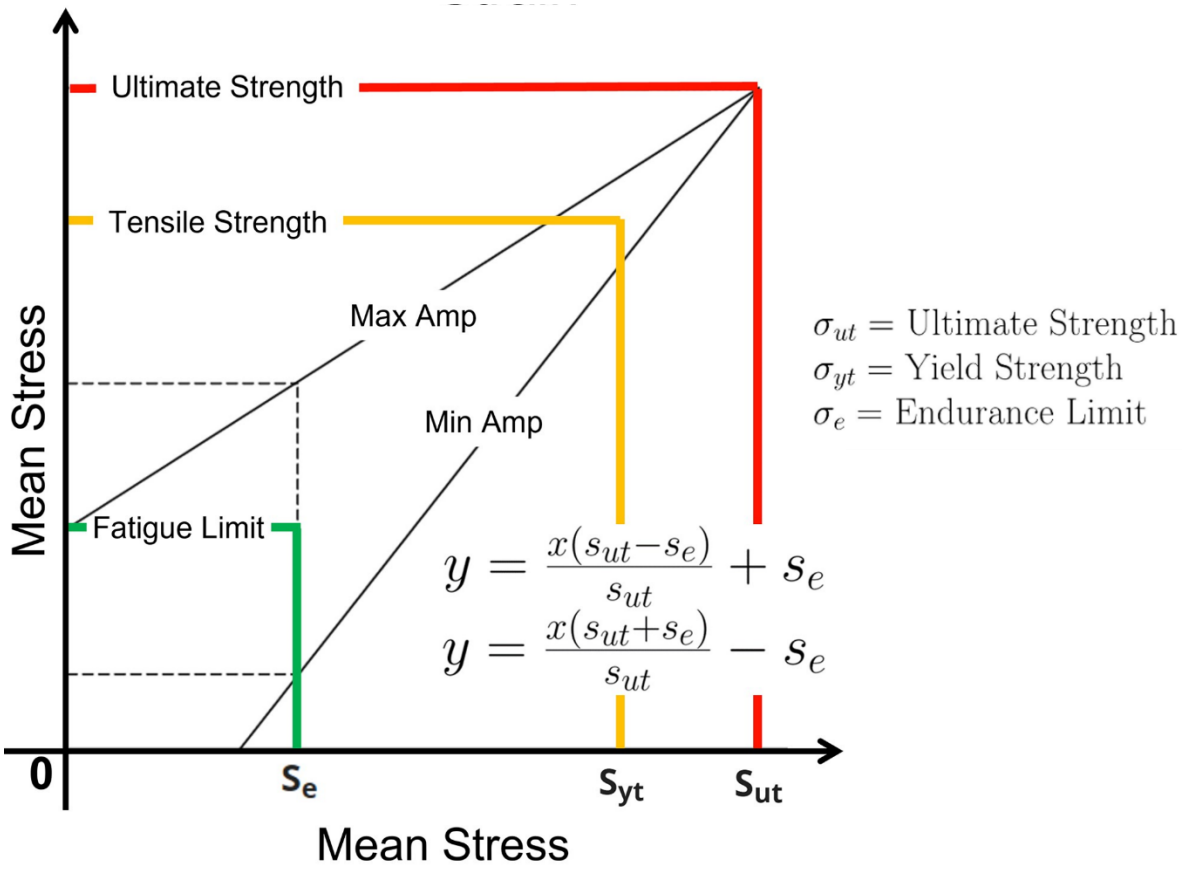


Figure 12: Predictions of Microglial Fatigue Limits.

The Modified Goodman diagram provides estimations for the maximum and minimum stress amplitude that microglia can endure when subjected to a given mean stress during a fatigue test. Three critical points are highlighted: the fatigue limit (green), representing the stress level below which microglia can endure an infinite number of cycles without failure; the tensile strength (yellow), indicating the maximum stress amplitude for a given mean stress; and the ultimate strength (red), signifying the absolute maximum stress that microglia can withstand. These parameters are utilized to predict the fatigue limits of microglia and provide insight into their stress response and structural resilience over multiple cycles.

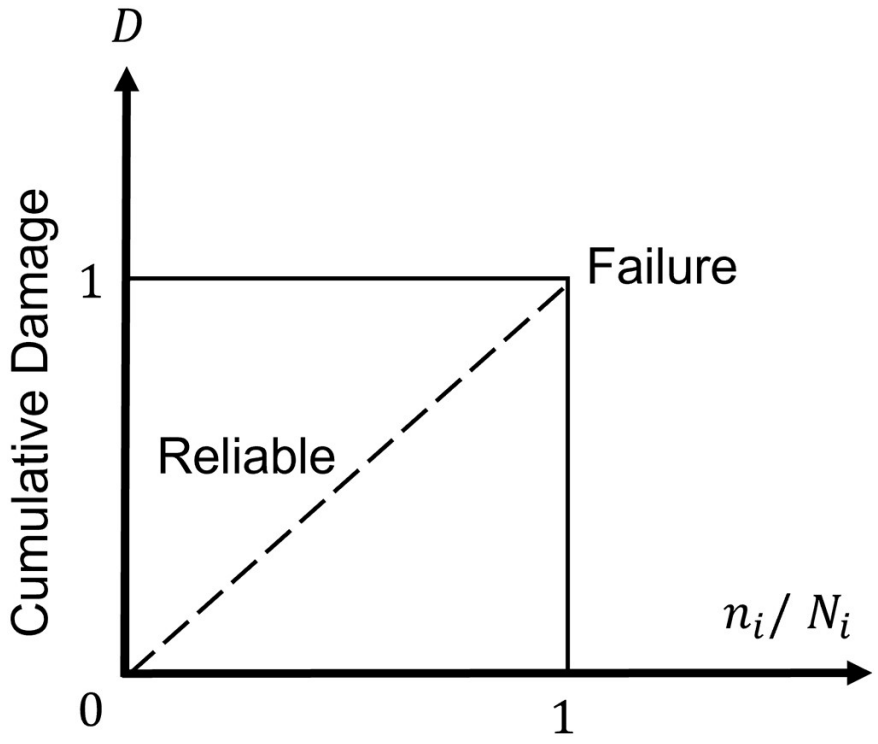


Figure 13: Cumulative Damage Prediction Model.

The graph illustrates the predictive capacity of the model in distinguishing between reliable and failed outcomes as cumulative damage accumulates. It showcases the relationship between increasing cumulative damage and the eventual transition to failure, marked by irreversible deformation.

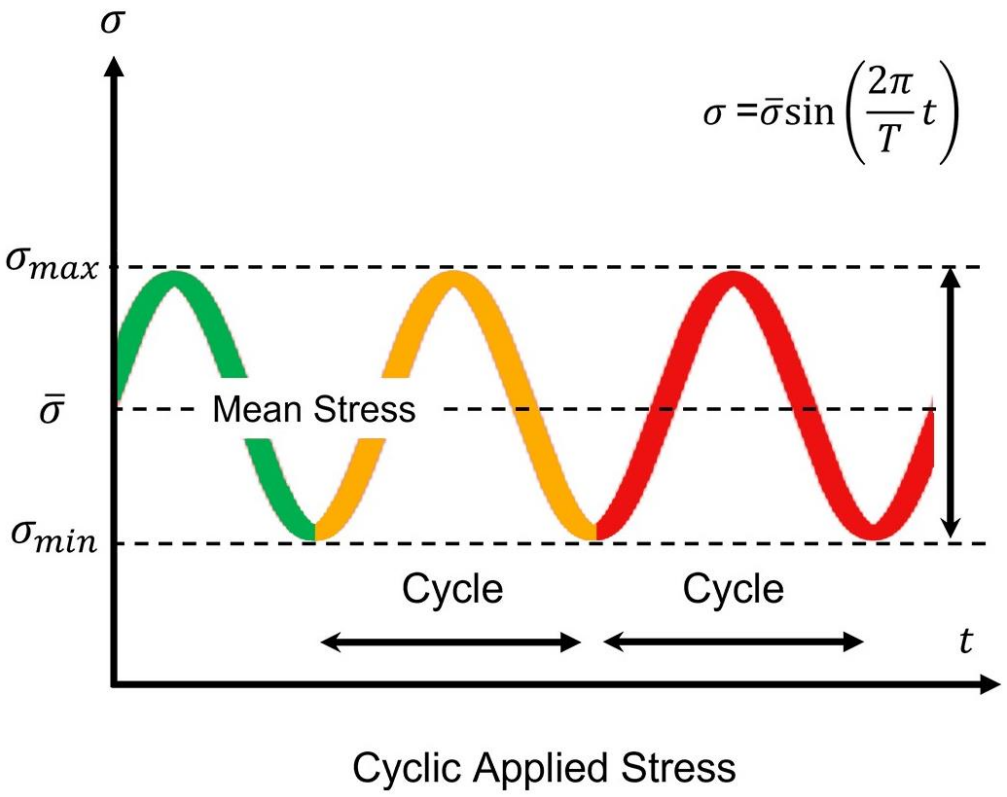


Figure 14: Microglial Response Across Cyclic Applied Stress Dynamics.

Representative diagram of cyclic applied stress based on mean stress amplitude. The diagram depicts the minimum, maximum, and mean amplitudes of stress changes over successive cycles, highlighted by distinctive colors representing Cycle 1 (green), Cycle 2 (yellow), and Cycle 3 (red). This representation captures the fluctuations and patterns in stress amplitudes throughout the cyclic stress application, providing insight into the microglial response over successive cycles.

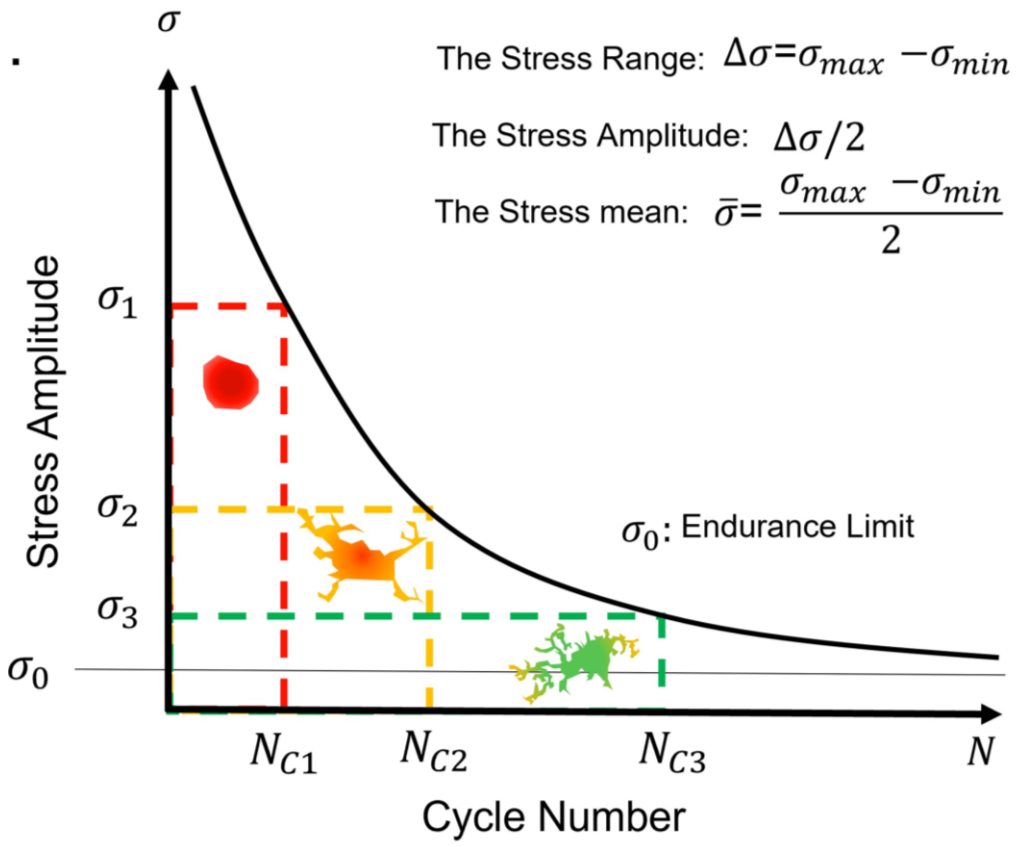


Figure 15: Predictions of Acute to Chronic Inflammation.

Representative image of the dynamic relationship between stress amplitude, cycle number, and the estimated endurance limit. By incorporating stress range, stress amplitude, and stress mean, the graph provides a comprehensive view of how microglial response evolves over successive cycles, shedding light on the critical point at which the system transitions from sustainable stress levels to potential failure.

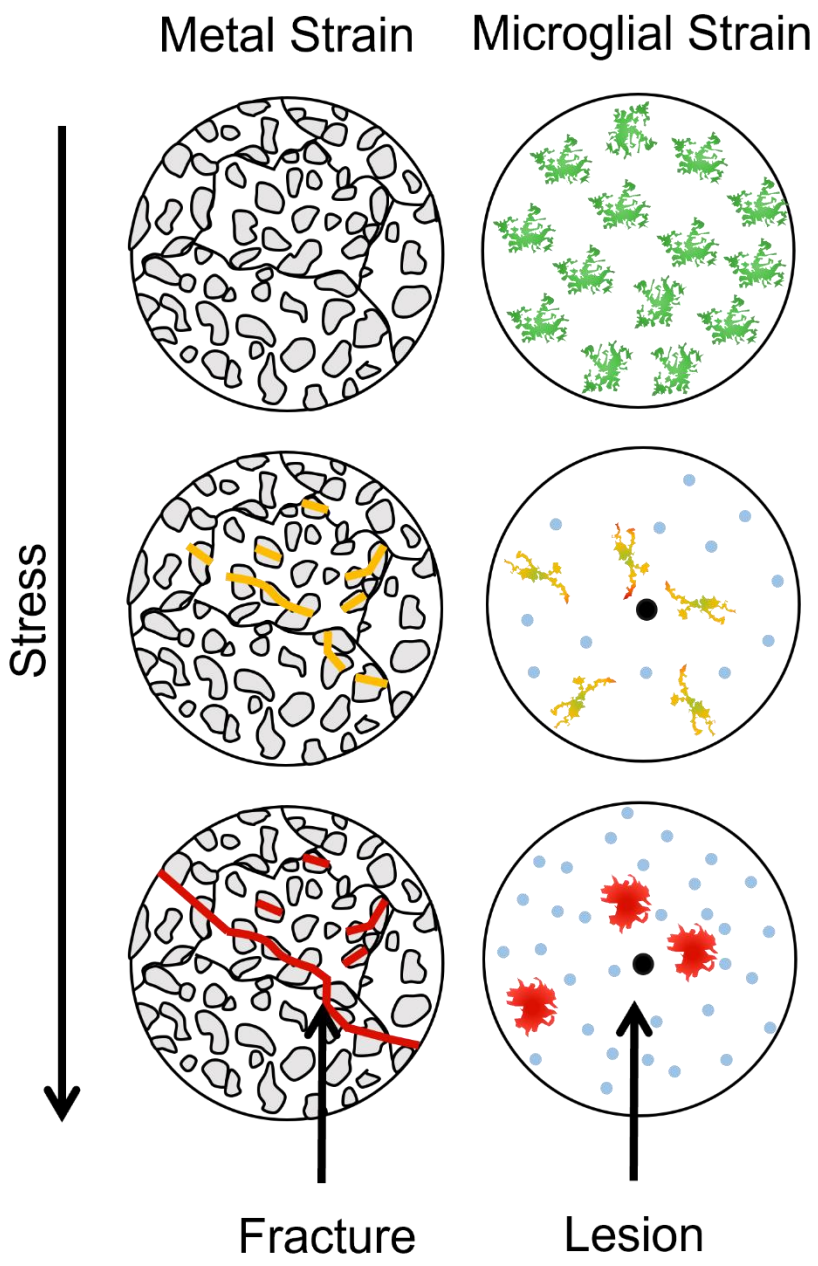


Figure 16: Comparative Responses in Metal and Microglial Stress-Strain Dynamics.

Representative comparative diagram of increasing stress over metal vs tissue. Illustrates the progressive stress increase in both metal and microglial tissue, highlighting distinct responses — metal undergoes macroscopic failure, while microglial tissue exhibits microlesions, triggering prolonged microglial responses.

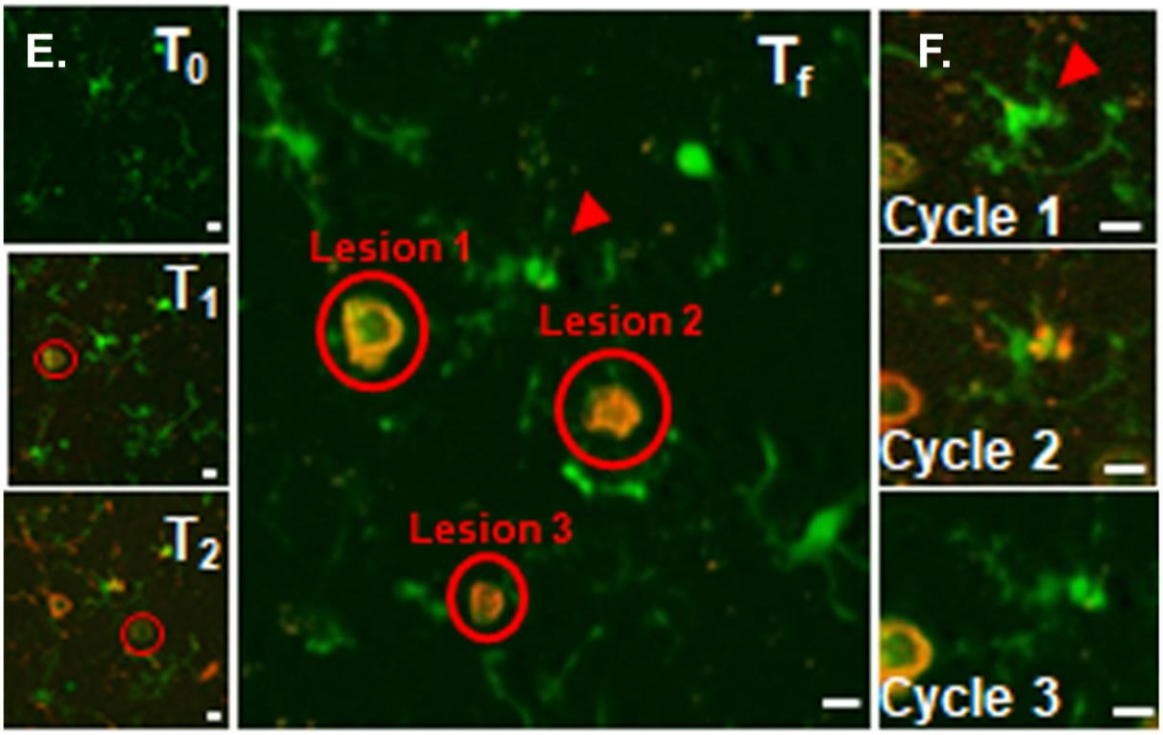


Figure 17: Microglial Stress Response to Cycles of Laser-Induced Microlesions.

A. Representative time-course imaging from Time 0 to Time final depicts two-photon laser micro-lesions initiating cycles of microglial stress. **B.** In vivo representative images capture dynamic morphological changes in microglia during each cycle of stress, providing insights into the temporal progression of microlesion-induced stress responses.

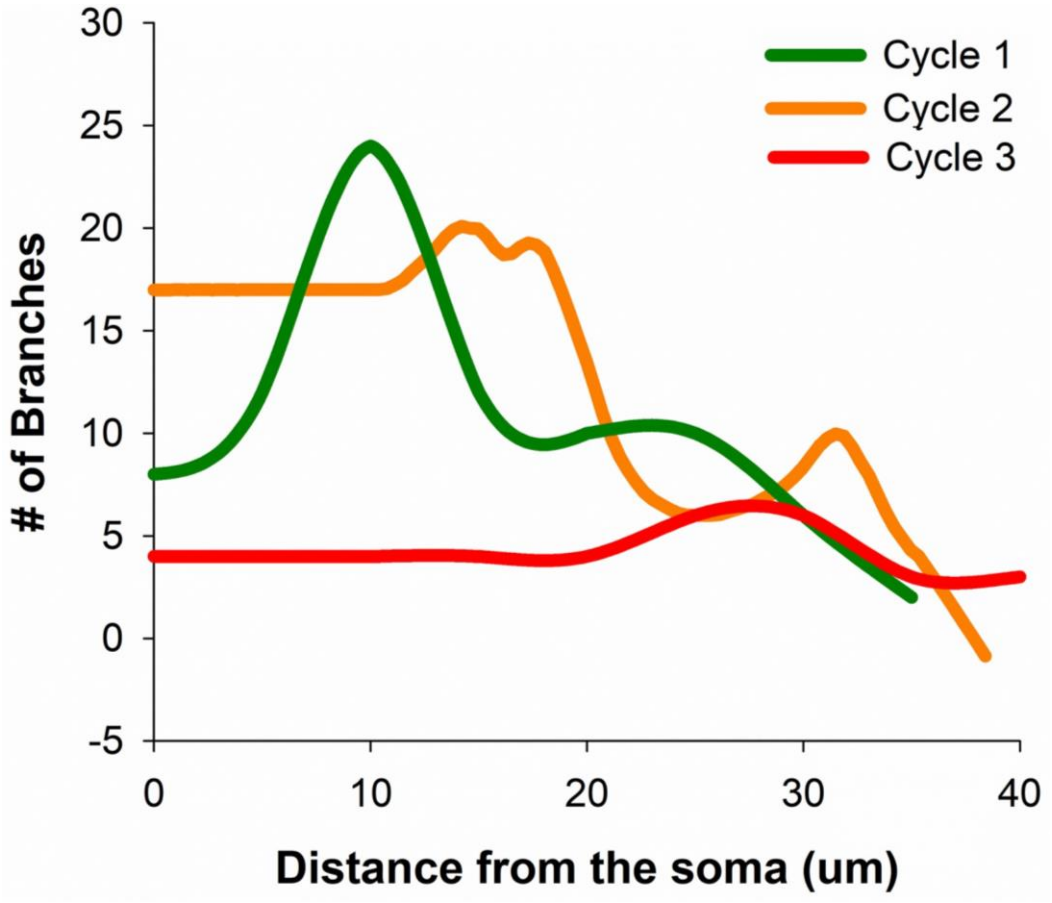


Figure 18: Microglial Morphological Changes Over Progressive Cycles of Stress.

Representative Sholl analysis shows a diminishing trend in the number of microglial branches with successive cycles of stress. The graph illustrates this phenomenon over the course of three cycles, indicating the impact of stress on microglial morphology and branching complexity.

Results for Aim #2: Outward Depolarization of the Microglial Mitochondrial Membrane Potential Following LPS Administration

To begin investigating the utility of using the ΔΨm to indicate the responsiveness of microglia to immune stimuli, we bath applied LPS to acute cortical slices prepared from CX3CR1-GFP mice loaded with tetramethylrhodamine ethyl ester (TMRE) to indicate changes in the ΔΨm. Timelapse recordings were taken over a duration of 5 minutes baseline followed by LPS (10ug/mL) exposure for an additional 30 minutes (Figure 19A, 20A). We traced microglial cell profiles based on the GFP signal and examined changes in TMRE over the LPS time course compared to baseline. We found that LPS exposure resulted in a progressive increase in ΔΨm in microglia, with successive stages of escalating intensity (Figure 21A). On average, LPS exposure resulted in a 253.12% increase in intensity in microglia (Figure 21A inset). As a point of comparison, we also traced the profiles of surrounding neuronal somas and examined changes in TMRE over the LPS time course. In contrast to microglia, neurons showed a more gradual rise in ΔΨm (Figure 21A). LPS resulted in an average 50% increase in TMRE intensity in neuronal somas (Figure 21A inset). Microglia underwent a significant increase in ΔΨm following LPS exposure compared to ΔΨm in adjacent neurons ($p = 0.038$, $n = 10$; Figure 21A).

The microglial ΔΨM progressively increased in distinctive states, characterized by a steep rise in ΔΨm and separated by a brief plateau. The first state (S1) begins within 6.06 minutes of LPS application, with an average duration of 2.02 minutes. The second state (S2) starts within 14.06 minutes of LPS application, with an average duration of 2.07 minutes. The third state (S3) begins within 23.50 minutes of LPS application, with

an average duration of 3 minutes. We found that the initial state was characterized by the steepest rise in ΔΨm, with a significantly greater ΔΨm slope angle compared to state 3 ($p= 0.007$, $n=10$; Figure 22B). The increase in the intensity of TMRE in microglia following LPS suggests more metabolic demand within microglia compared to the surrounding neuronal population. The progressive states in microglial ΔΨm indicate that these cells are responding to the immune stimulus in a regulated fashion. Dynamic depolarizations were recorded in the ΔΨm of microglia following LPS application (Figure 22A,B), characterized by temporally separated progressive states (Mean 365.5, 844, and 1,410s).

To examine the subcellular regulation of microglia in response to LPS, we examined ΔΨM in the soma, branches, and endfeet of microglia (Figure 23A) in acutely prepared slices from CX3CR1-GFP mice loaded with TMRE. When reviewing the LPS-induced changes in ΔΨM in microglia somas, we found that the sharpest rise occurred early following application, within 6.06 minutes, which was followed by a more gradual rise throughout the remainder of the 30-minute time course (Figure 23B). Analysis of ΔΨM in microglia branches following LPS application showed a delayed increase, commonly characterized by a sharp rise between 13.03- 15.10 minutes (Figure 23C). Microglia endfeet showed the most delayed change in ΔΨM following LPS, with the sharpest rise occurring between 22- 25 minutes (Figure 23D).

Next, we examined the average percent change within each subcellular domain related to the progressive states we identified. We found that the microglia soma undergoes a significant shift in ΔΨM during state 1 following LPS, while the microglia endfeet undergo a substantial shift in the ΔΨM during state 3 (Figure 24A). Analysis of the

maximum ΔΨM slope angle of each subcellular domain in each state revealed that the branches undergo the steepest slope changes during state 2 and state 3. In contrast, microglia endfeet undergo the steepest slope change during state 3 (Figure 25C). We also examined the rate of change ΔΨM in each microglia subcellular domain with respect to the progressive states and found that the endfeet show the most significant rate of change during state 3 (Figure 26C). These data reflect a progressive depolarization of the ΔΨM in microglia following exposure to an immune stimulant, which can be separated temporally into distinct states and spatially into subcellular domains. Changes in microglia ΔΨM occur first in the soma, then radiate outward through the branches and finally to the endfeet over 30 minutes following LPS application.

We next wanted to determine if the depolarization of microglial ΔΨm in response to LPS could be modulated. The outer mitochondrial membrane protein TSPO is known to signal inflammatory transcriptional pathways and interact with ROS as a vital part of the microglial inflammatory response (Cosenza-Nashat et al. 2009) (Cosenza-Nashat et al. 2009). We examined the effects of the well-characterized TSPO inverse agonist, emapunil, which has previously been demonstrated to exert neuroprotective effects. For these studies, acutely prepared slices from CX3CR1-GFP mice were loaded with TMRE and pretreated with emapunil before LPS exposure. Timelapse imaging of microglia pretreated with emapunil showed a visible decrease in the intensity of TMRE following LPS exposure compared with LPS alone (Figure 27A). The change in intensity of the ΔΨM in the microglia soma is substantially attenuated following LPS when slices were pretreated with emapunil (Figure 27B). The state 1 rise in ΔΨm of the microglia soma is

notably attenuated by 7 minutes following LPS exposure (red arrow Figure 28A). On average, the percent change in ΔΨm in the microglia soma following LPS was significantly attenuated by emapunil pretreatment (Figure 28B).

The effects of emapunil on the microglial ΔΨm following LPS exposure reveal nuanced alterations in different subcellular compartments. While the ΔΨm of microglia branches did not exhibit a statistically significant decrease with emapunil treatment, there was a notable trend towards decreased intensity (Figure 29A, B). In contrast, the ΔΨm of microglia endfeet showed a distinct response, with emapunil exerting an inverse effect during different states of depolarization induced by LPS. Specifically, during the earlier states (S1 and S2), there was a higher ΔΨm observed in the emapunil-treated group compared to the LPS-only group, while during the late state (S3), there was a lower ΔΨm (Figure 30A, B). This differential response suggests a complex modulation of microglial mitochondrial function by emapunil, with varying effects depending on the subcellular region and the stage of immune stimulation.

Furthermore, the analysis of the rate of change in ΔΨm in different subcellular domains during distinct states provides valuable insights. Significant alterations in the rate of change were observed in the soma and branches during early states (S1 and S2) (Figure 31A, B), while the endfeet exhibited significant changes during later states (S2 and S3) (Figure 31C). Moreover, a direct comparison between the ΔΨm slope angle of the microglial soma and endfeet revealed an inverse relationship. Whereas the ΔΨm in the soma decreased over each state, the ΔΨm in the endfeet increased, and emapunil attenuated this effect (Figure 32). These findings underscore the intricate regulation of microglial mitochondrial dynamics in response to immune stimuli and the potential of

emapunil to modulate these responses. Understanding these subcellular dynamics and the effects of pharmacological interventions like emapunil could offer new avenues for therapeutic strategies targeting neuroinflammatory processes implicated in various neurological disorders. These results highlight the importance of considering subcellular compartmentalization and temporal dynamics in studying mitochondrial responses in microglia and suggest avenues for further investigation into the mechanisms underlying emapunil's effects on microglial function.

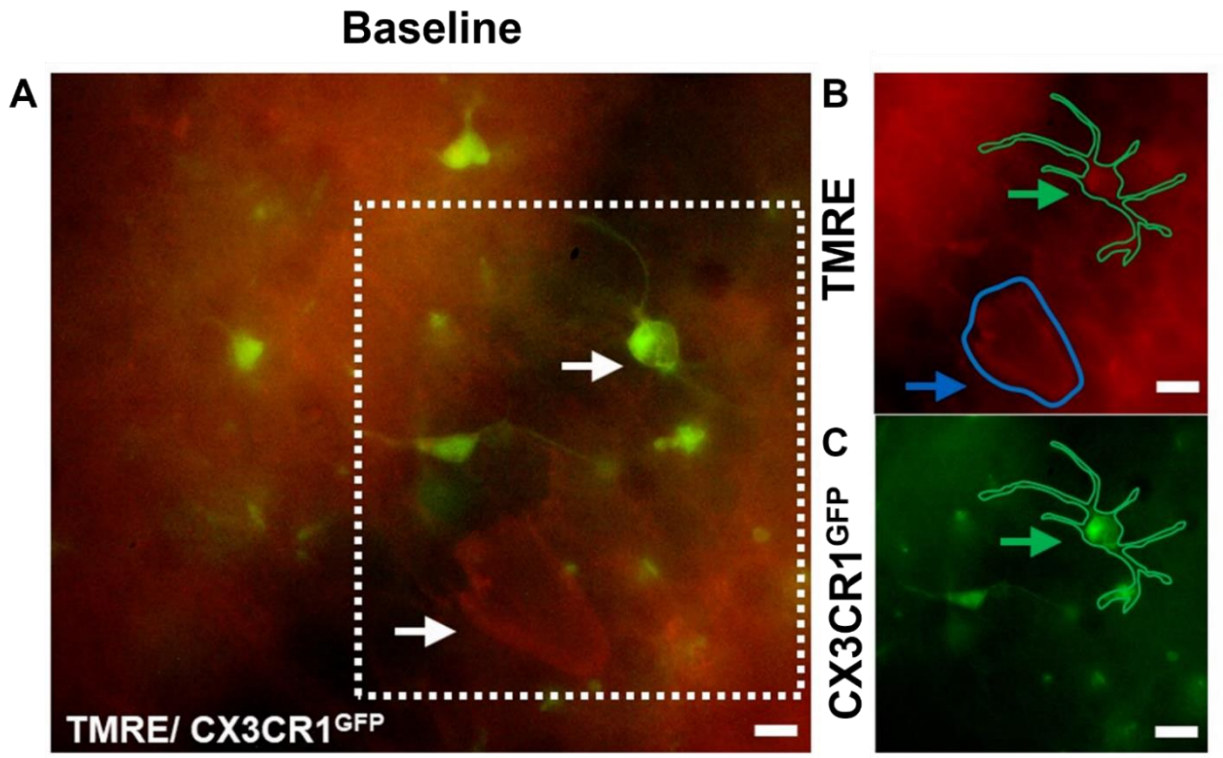


Figure 19: Visualization of Microglia-Specific Mitochondrial Membrane Potential (MMP) in Acute Hippocampal Slices.

A. Representative images of CX3CR1GFP acute hippocampal slices dye loaded with TMRE. **B.** The red channel highlights increased ΔΨM in both microglia (outlined in green) and adjacent neurons (outlined in blue). **C.** The green channel depicts the expression of EGFP, specifically in microglia. This figure aims to provide specificity in the overlap of TMRE and EGFP for accurate measurement of microglia-specific mitochondrial activity.

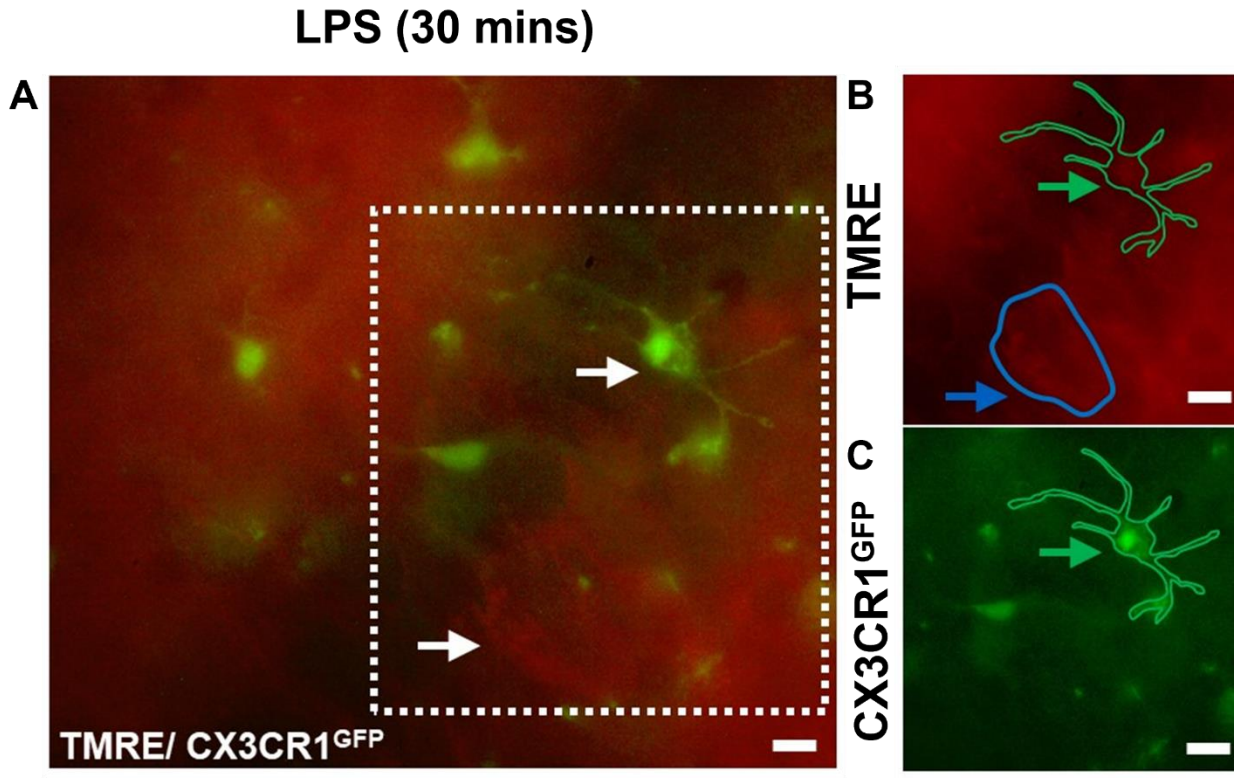


Figure 20: Visualization of Microglial Mitochondrial Membrane Potential (ΔΨM) Changes in Acute Hippocampal Slices Following LPS Administration

A. Representative images of CX3CR1GFP acute hippocampal slices dye loaded with TMRE 30 mins following LPS administration. **B.** The red channel highlights increased ΔΨM in microglia (outlined in green) and a plateau in the adjacent neurons (outlined in blue) **C.** The green channel depicts the expression of EGFP, specifically in microglia following LPS administration.

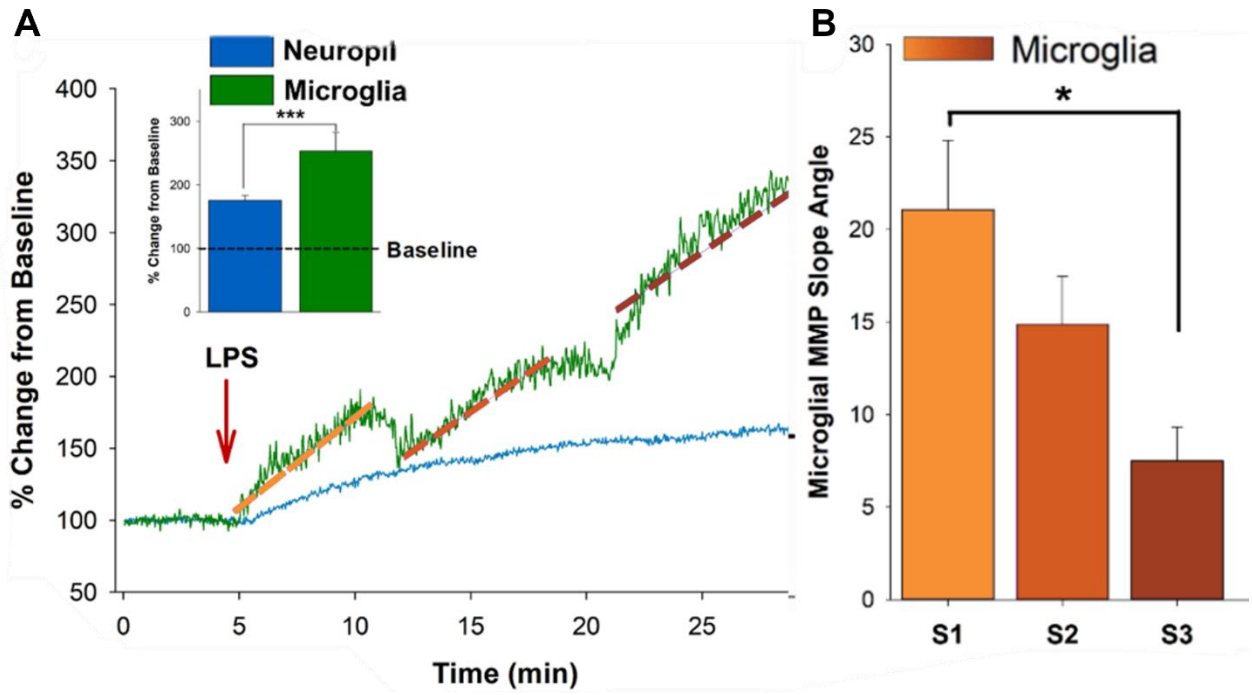


Figure 21: Microglia-Specific Increases in ΔΨM Occur in Progressive Stages Following LPS Administration.

A. Representative traces show that following the application of the ΔΨM in microglia dynamically increased in three progressive states (S1-3) over 30 minutes of exposure to LPS. By contrast, the adjacent neurons showed only a slow and gradual rise. On average, microglia ΔΨM increases by close to 300%, while the adjacent neuronal ΔΨM increases by ~50% ($p = 0.038$). **B.** The initial rise in microglial ΔΨM was the sharpest, and the slope angle decreased over the two following states (states begin at ~5, 13, and 23 mins following LPS administration). On average, the slope angle of the first state was significantly greater than the slope angle of the third state ($p = 0.007$) ($n=10$).

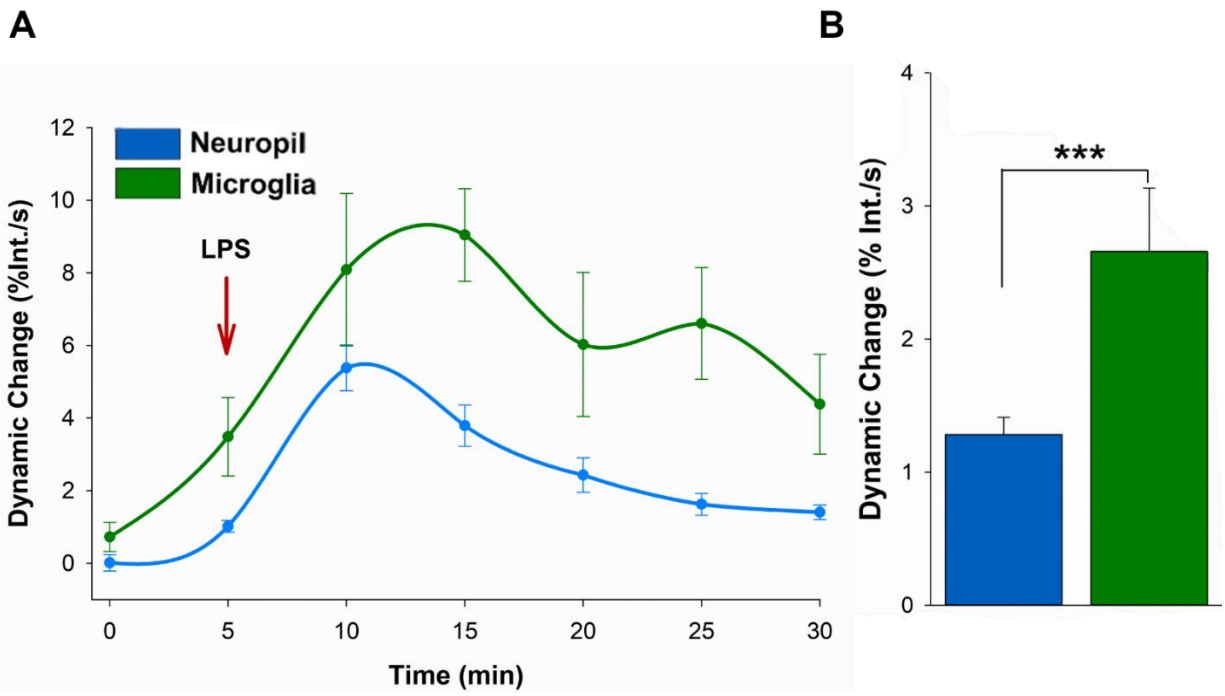


Figure 22: Microglia-Specific Temporal Dynamics of ΔΨM Following LPS Administration.

A. Illustration of the dynamic and faster rate of microglial ΔΨM increase compared to adjacent neurons. The adjacent neuronal ΔΨM, in contrast, exhibits a single earlier increase, emphasizing the differential temporal patterns between microglia and neurons in response to LPS **B.** The total dynamic change confirmed the significantly higher microglial ΔΨM compared to adjacent neurons (p=0.031) (n=10).

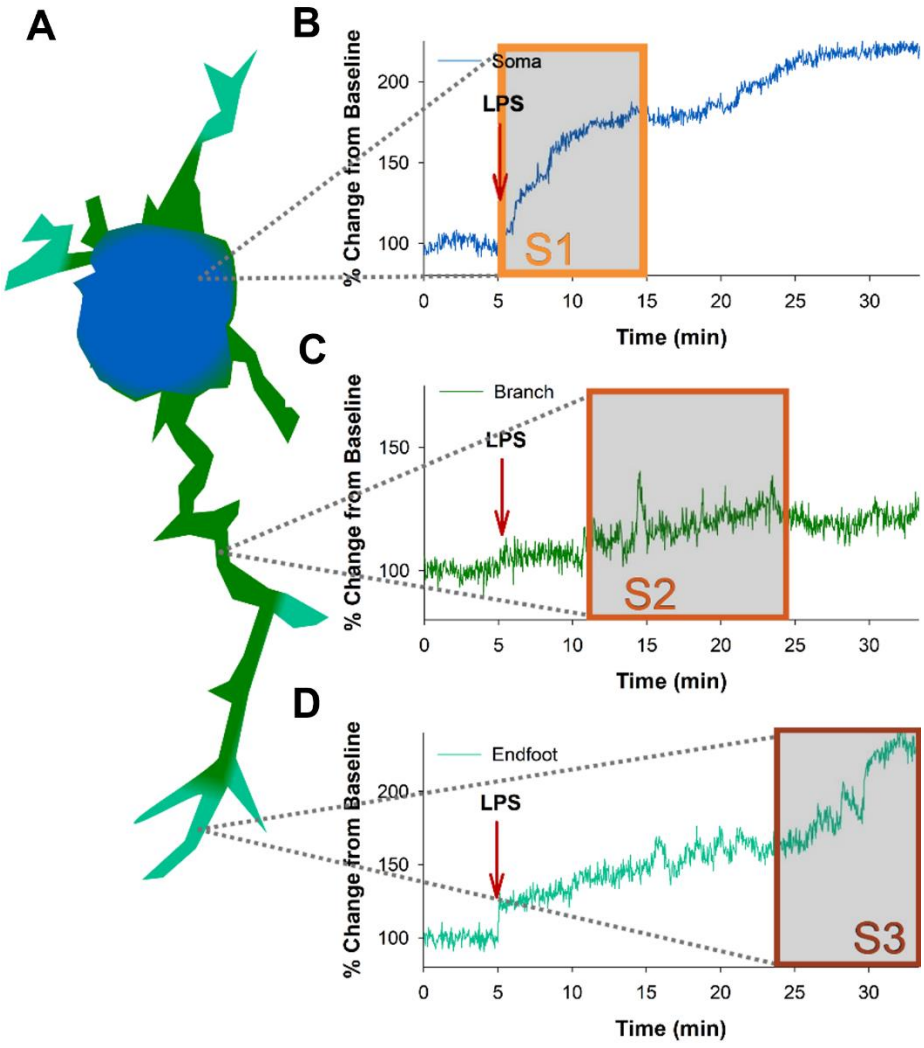


Figure 23: The Progressive States Observed Following LPS Administration Represent Radiating Depolarization of the Microglial ΔΨM, Beginning in the Soma and Progressing to the Endfeet.

A. Schematic of the subcellular progression of microglial ΔΨM changes in regions of interest following LPS administration. **B.** Representative trace of the intensity of the ΔΨM in the microglial soma climbed rapidly between 5-15 mins post LPS administration, which temporally matches S1. **C.** The sharpest rise in microglial branch ΔΨM occurs after 11 minutes of LPS administration. **D.** The intensity of the ΔΨM in the microglial endfeet climbed rapidly between 24-30 mins post LPS administration, which temporally matches S3.

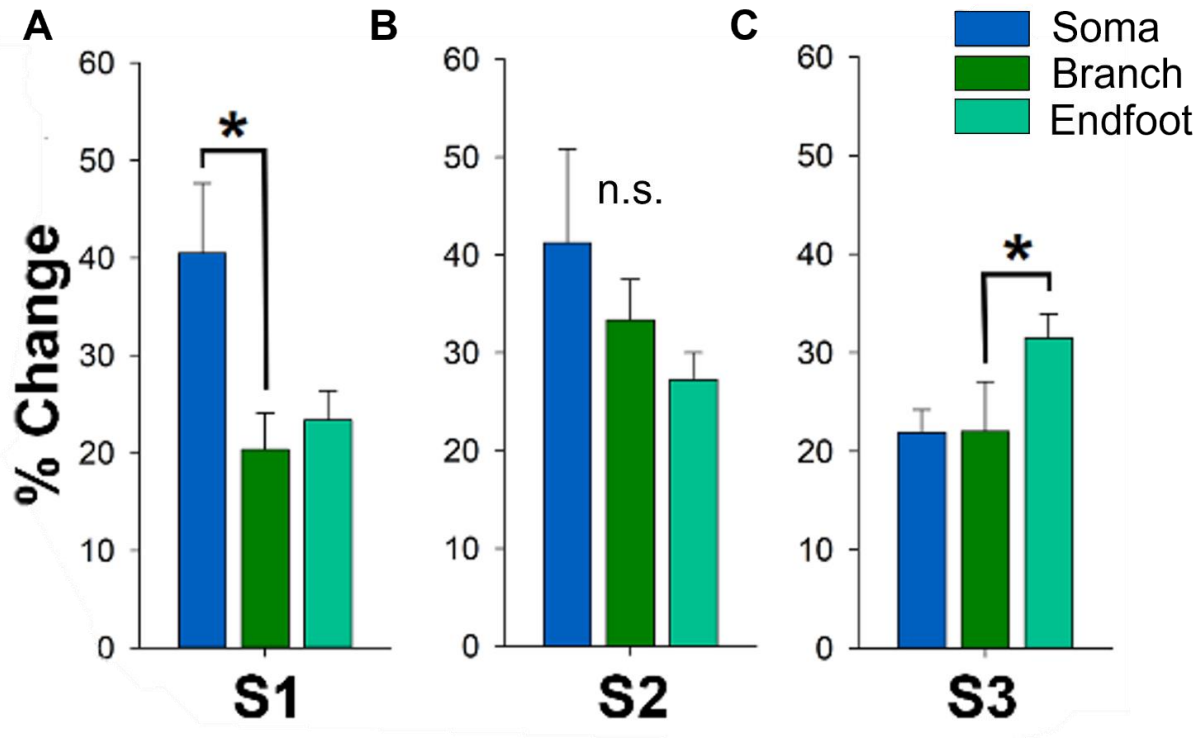


Figure 24: Subcellular Dynamics of Microglial ΔΨM Across Progressive Stages.

A. Examines the average percent change from baseline in each subcellular domain during the three progressive stages (n=10), highlighting the largest magnitude change in the soma during S1 (p= 0.025). **B.** Displays a downward trend in S2 of the percent change from baseline, with no significant difference between regions. **C.** Illustrates that during S3, the largest magnitude change occurs in the endfeet (p=0.013).

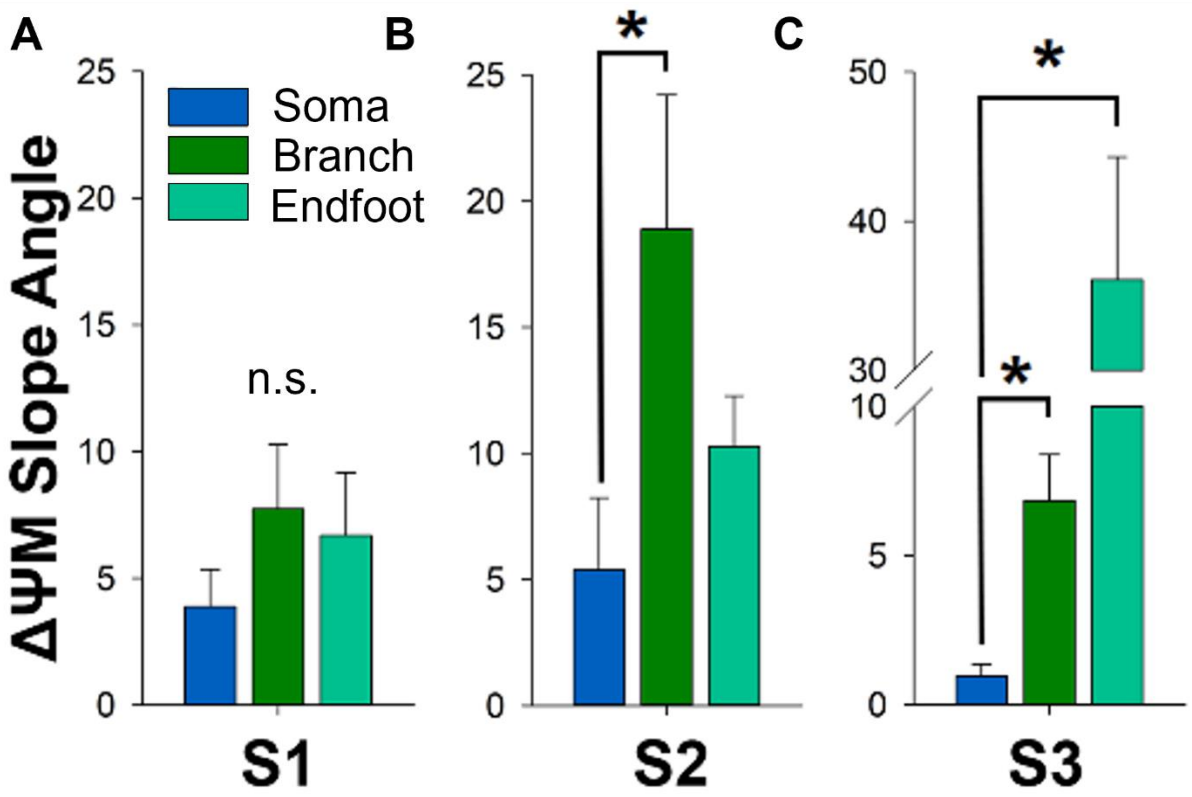


Figure 25: Microglial ΔΨM Slope Angle Dynamics Across Progressive States.

A. No significant difference in the slope angle of ΔΨM is noted in S1 between regions (n=10). **B.** Quantification of the maximum ΔΨM slope angle indicates that microglial branches exhibit the sharpest rise during S2 (p=0.008). **C.** Both branches and endfeet demonstrate significantly greater slope angles during S3 compared to the soma (p=0.001).

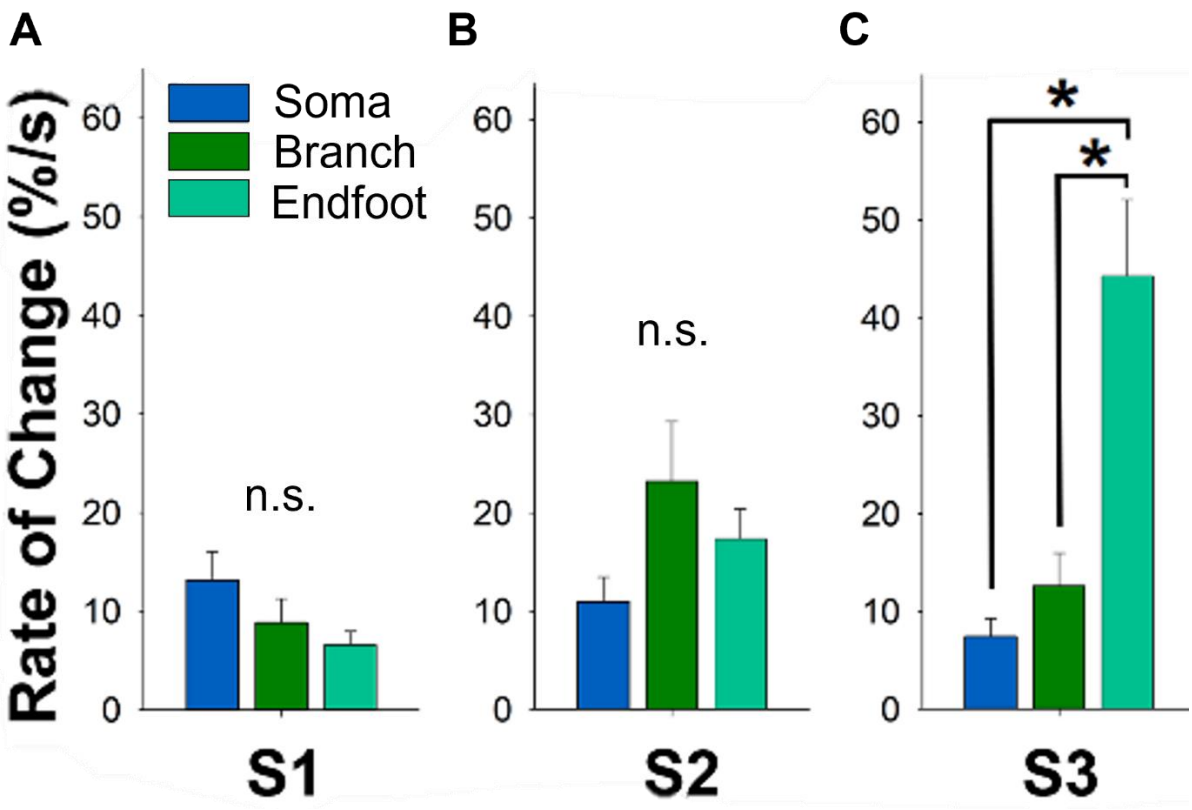


Figure 26: Temporal Dynamics of Microglial ΔΨM Rate of Change Across Progressive States.

A.-B. No significant difference in the rate of change during S1 and S2. **C.** Significant divergence observed in the rate of change in microglial endfeet during S3 ($p < 0.001$) (n=10). Emapunil intervention redirects the depolarization of MMP from the soma to the endfoot.

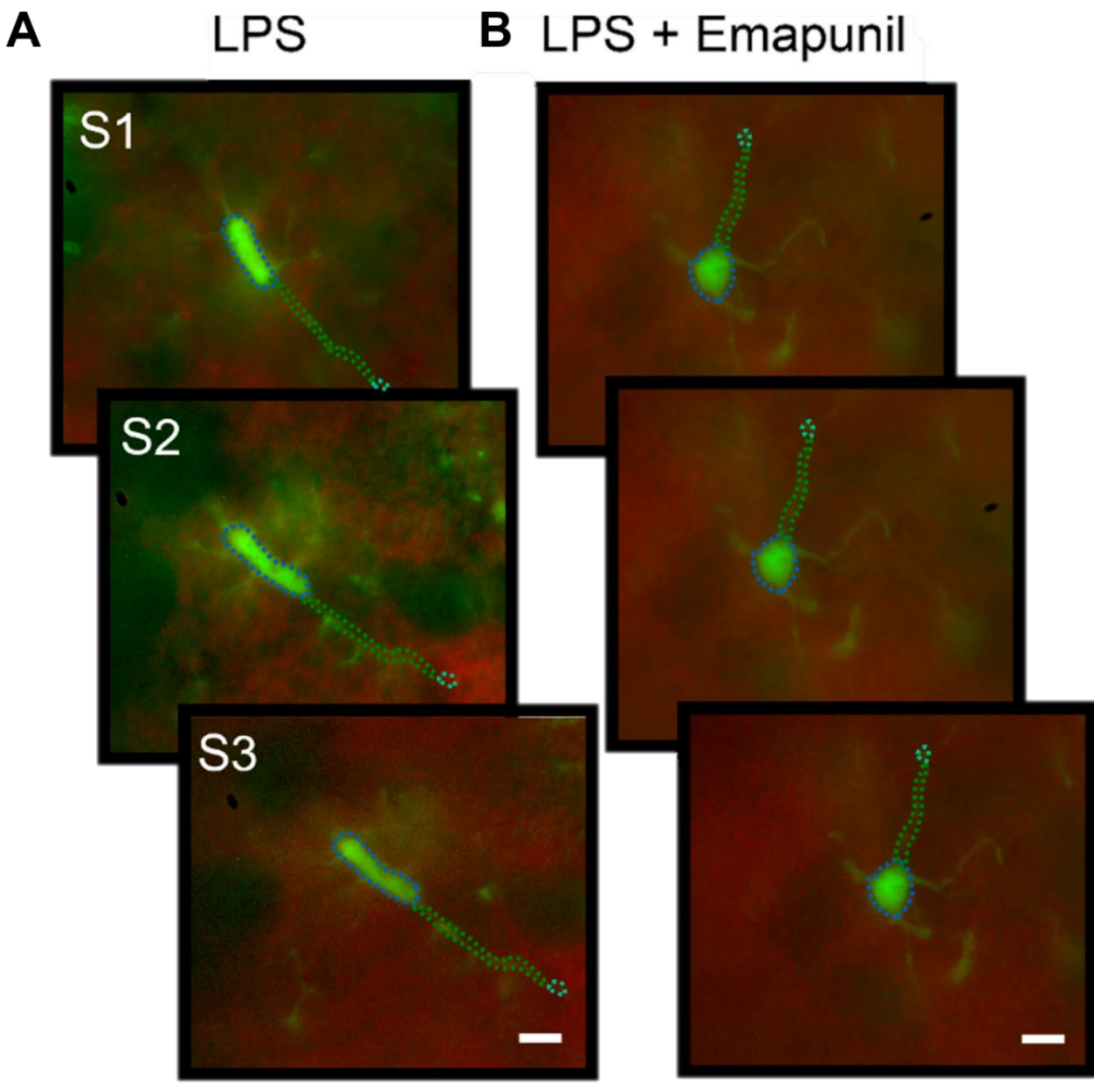


Figure 27: Morphological Changes in Microglia During Progressive States of LPS Treatment.

A. Representative timelapse images capturing the morphological transitions of slices treated with LPS during states 1-3. **B.** Impact of LPS and emapunil administration on microglial morphology during states 1-3, showcasing a shift from a polarized morphology in the LPS group to a more ramified morphology following the combined LPS and emapunil treatment.

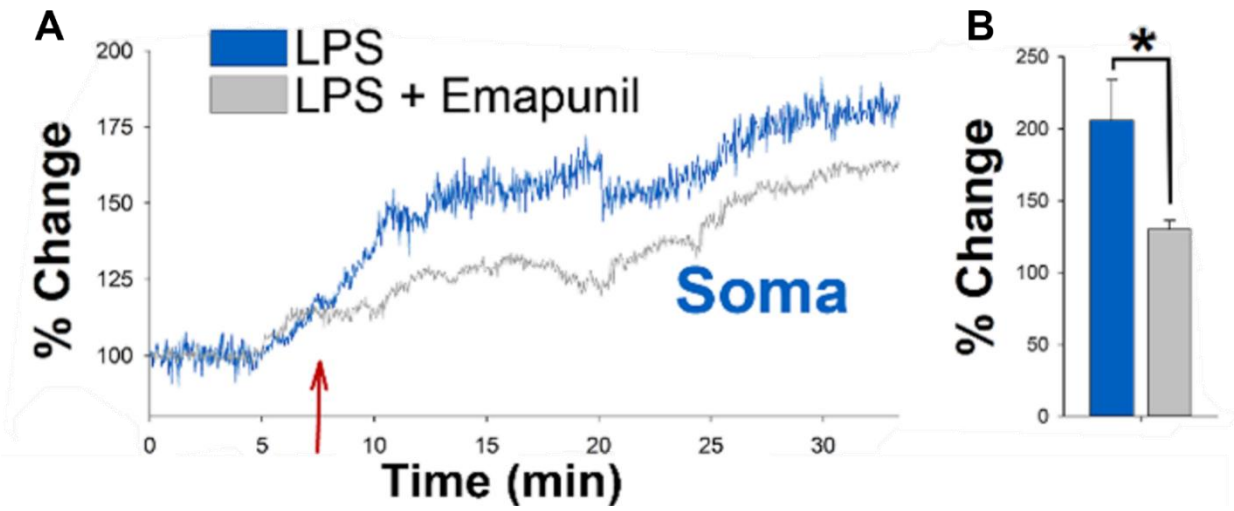


Figure 28: Emapunil Modulation of Microglial Soma ΔΨM in Response to LPS.

A. Representative traces of the microglial soma showed that emapunil attenuated the percent change from baseline of the ΔΨM caused by LPS within 7 minutes of administration. B. On average, treatment with emapunil resulted in a significantly smaller change in the soma ΔΨM caused by LPS compared with LPS alone (p= 0.026) (n=10).

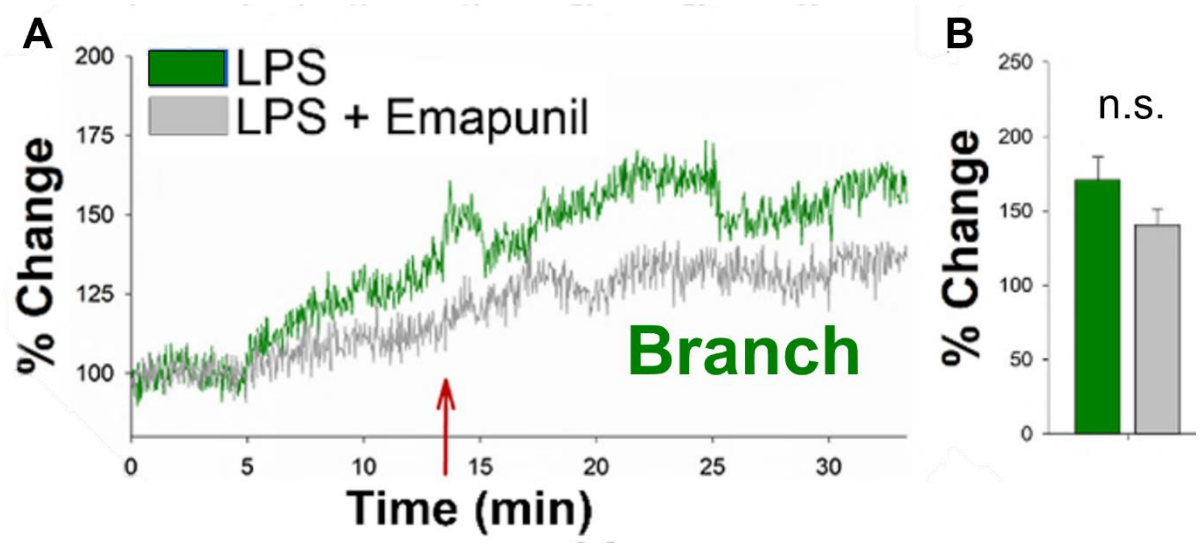


Figure 29: Modulation of Microglial Branch ΔΨM by Emapunil in Response to LPS Administration.

A. Representative traces reveal that emapunil did not significantly attenuate the change in microglial branch ΔΨM induced by LPS. **B.** A slight decrease in branch ΔΨM from the LPS to the LPS and Emapunil group is reflected in the total percent change from the baseline (n=10).

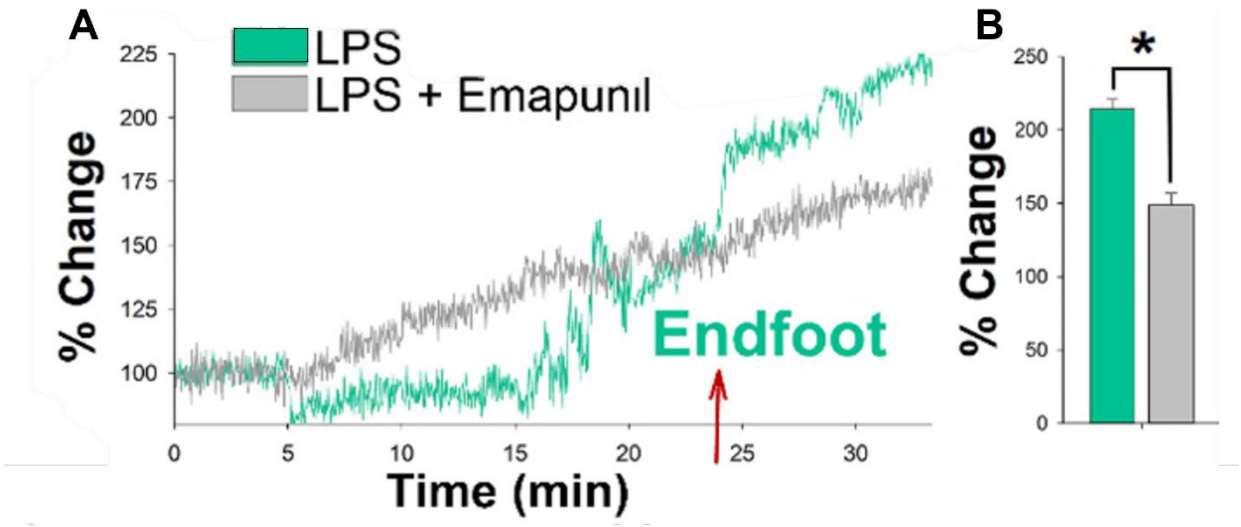


Figure 30: Emapunil Modulation of Microglial Mitochondrial Membrane Potential in Endfeet Following LPS Administration.

A. Representative raw traces of microglial ΔΨM in LPS-treated groups with and without TSPO Ligand Emapunil. The trace illustrates the attenuation of the effect of LPS on ΔΨM in the presence of Emapunil. **B.** Quantification of the total percent change in microglial ΔΨM from baseline, showing a significant decrease in the average percent change in Emapunil-treated groups compared to LPS-only groups, indicating the suppressive influence of Emapunil on the LPS-induced changes in microglial ΔΨM in endfeet ($p=0.001$) ($n=10$).

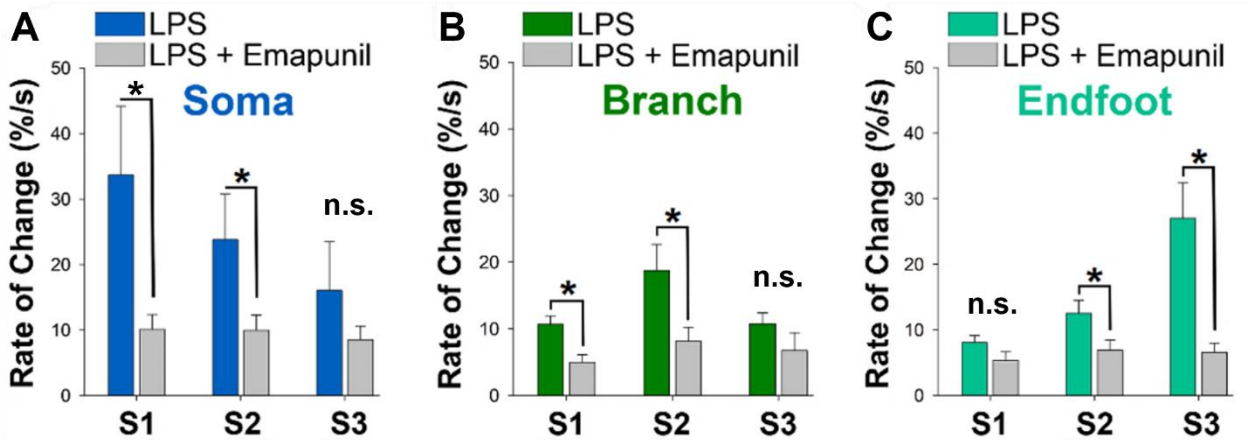


Figure 31: Microglial ΔΨM Dynamics in Soma, Branches, and Endfeet Across Progressive States Following LPS and Emapunil Treatment.

A. Quantification of the rate of change comparing LPS and LPS plus emapunil treated slices in the microglia soma. This graph depicts the difference between the LPS groups and the LPS plus Emapunil groups over states (S1, S2, and S3) (p=<0.001). **B.** Quantification of the rate of change comparing LPS and LPS plus emapunil treated slices in the microglia branches. This graph illustrates the distinct progression in microglia branches over the S1 and S2 (p=<0.001). **C.** Quantification of the rate of change comparing LPS and LPS plus emapunil treated slices in the microglia endfeet. The TSPO ligand emapunil shunts the somatic to endfoot progression of ΔΨM depolarization in microglial endfeet, highlighting differences in response to LPS between the two conditions in S2 and S3 (p=<0.001) (n=10).

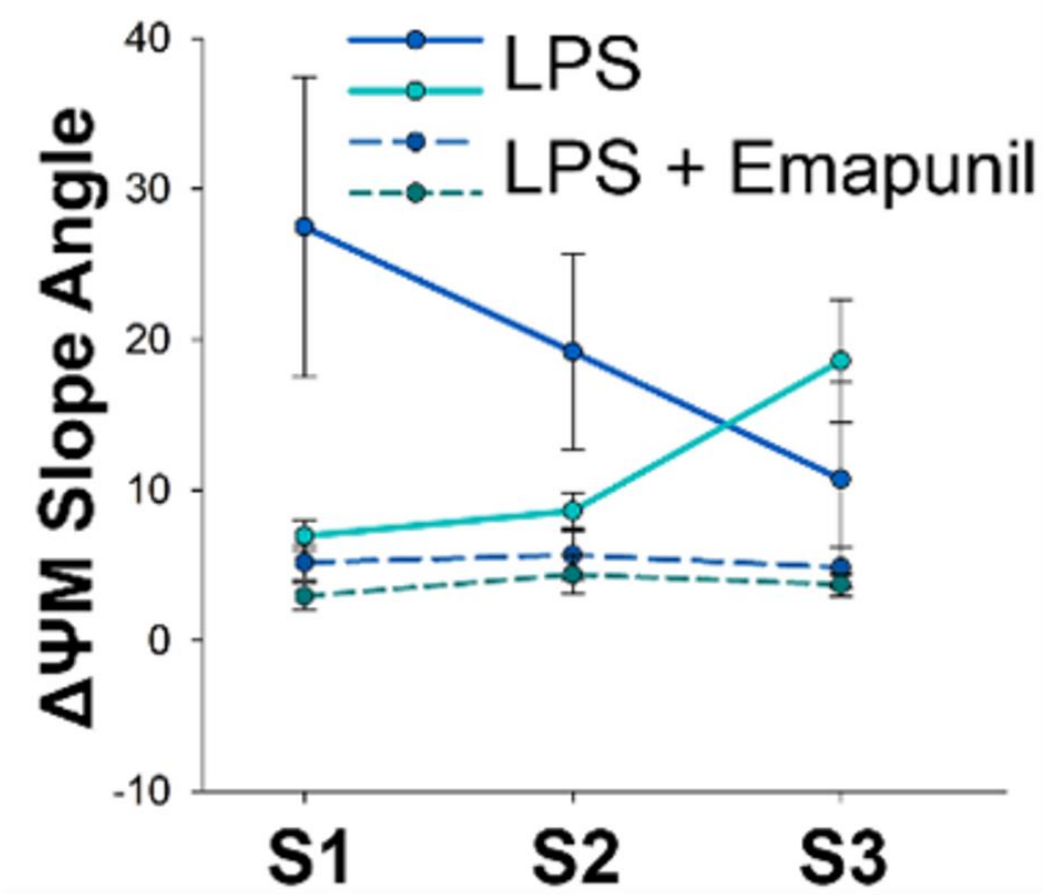


Figure 32: Emapunil Modulates Microglial ΔΨM Slope Angles Across Progressive States.

In the microglial soma, emapunil significantly attenuated the slope angle of ΔΨM during states 1 ($p=0.007$) and 2 ($p=0.001$). Conversely, in the microglial endfeet, emapunil exhibited a pronounced impact during state 3 ($p<0.001$). These findings highlight the dynamic regulatory effects of emapunil on microglial ΔΨM slope angles across different states, shedding light on its nuanced modulation of mitochondrial membrane potential under varying conditions.

Chapter Five: Discussion

Discussion for Aim #1

Historical Binary Categorization of Microglia

Microglia have historically been oversimplified into a binary classification, failing to capture their intricate and dynamic behavior. This classification dichotomized microglia into "resting" and "activated" states, portraying them as either dormant observers or reactive responders to injury. However, this simplistic framework, prevalent in late 20th-century research, limited the understanding of microglial functions. It portrayed microglia as static entities transitioning between two discrete states in response to specific stimuli, obscuring their true diversity and plasticity.

Microglial research has undergone a significant transformation with recent advancements in technology and methodology. The traditional binary classification of microglia into "resting" and "activated" states has been challenged by studies employing cutting-edge techniques such as single-cell transcriptomics and advanced imaging. These approaches have revealed a continuum of microglial states characterized by diverse transcriptional profiles and dynamic morphological changes, challenging the oversimplified view of microglial behavior.

The emergence of single-cell transcriptomics has provided unprecedented insights into the molecular heterogeneity of microglia. Contrary to the static dichotomy of resting and activated states, single-cell RNA sequencing has uncovered a spectrum of gene expression patterns across individual microglial cells. This continuum suggests a dynamic and context-dependent response to the microenvironment, with microglia exhibiting varying degrees of activation and functional specialization.

Moreover, advanced imaging techniques have complemented transcriptomic studies by visualizing the dynamic behavior of microglia in real-time. These imaging approaches have revealed a spectrum of morphological changes and migratory behaviors, challenged the notion of a static resting state, and highlighted the complexity of microglial responses. These findings underscore the need for a more nuanced understanding of microglial biology, acknowledging their dynamic nature and diverse functional states in health and disease.

The historical binary classification of microglia has shaped academic perceptions and influenced experimental methodologies, potentially biasing results by overlooking the intricacies of microglial responses. Assuming that "activated" microglia exclusively demonstrate pro-inflammatory behavior, potential oversights in acknowledging their diverse functions, such as roles in tissue repair and maintaining homeostasis, could have arisen. Consequently, this oversimplified framework may have obscured the true complexity of microglial behavior and its implications in health and disease.

Recent research has shed light on the multifaceted roles of microglia, revealing their involvement in various physiological processes and pathological conditions. Beyond their traditionally recognized functions, such as synaptic pruning and neuronal support, microglia play crucial roles in cellular debris clearance and immune regulation within the central nervous system. However, the binary paradigm failed to capture the intricate interactions between different microglial subtypes and their dynamic adaptation to the neural microenvironment. As a result, the true extent of microglial diversity and functionality may have been underestimated, hindering our comprehensive understanding of their contributions to neural homeostasis and disease progression.

Acknowledging the limitations of the simplistic binary categorization is crucial for advancing microglial research and refining our understanding of their multifaceted roles within the central nervous system. This acknowledgment enhances our comprehension of normal microglial function and reshapes our perspective on their involvement in neuroinflammation and neurodegenerative disorders. Consequently, therapeutic strategies targeting microglia must now embrace this heterogeneity to develop interventions that selectively modulate specific microglial functions without globally suppressing their diverse roles. This paradigm shift signifies a pivotal moment in our understanding of microglial biology, urging the adoption of a more dynamic framework that appreciates the complexity of microglial states and paves the way for innovative therapeutic approaches.

The historical constraints of the binary classification have steered academic perceptions and potentially skewed experimental methodologies, thereby obscuring the nuanced nature of microglial responses. By transcending the simplistic view that "activated" microglia solely manifest pro-inflammatory behavior, we can begin to appreciate their diverse functions, including roles in tissue repair and the maintenance of homeostasis. Recent research has illuminated the intricate involvement of microglia in various physiological processes and pathological conditions, revealing their dynamic adaptation to the neural microenvironment. Microglia's remarkable ability to transition between ramified, amoeboid, and hyper-ramified morphological states highlights their dynamic responsiveness to environmental stimuli, allowing them to adapt swiftly to changing conditions and be primed for subsequent encounters with stressors (Hines et al. 2013). Embracing the concept of context-dependent microglial states, wherein microglia exhibit

distinct functional profiles based on environmental cues, offers a more comprehensive understanding of their contributions to neural homeostasis and disease progression.

Context-Dependent Microglial States

Recent research has revealed a dynamic spectrum of functional states among microglia, challenging oversimplified concepts of their activation and highlighting the need for a more nuanced understanding of their diverse functions. This paradigm shift emphasizes the multifaceted nature of microglial behavior, which is intricately shaped by the local environment and specific stimuli. Studies across various disciplines, including proteomics, transcriptomics, morphology, epigenetics, and metabolomics, have provided compelling evidence supporting this view, revealing the coexistence of multiple microglial states.

Studies in proteomics have been instrumental in revealing the multidimensional integration of microglial states, showcasing a diverse array of protein expression patterns within these immune cells. Proteomic analyses have uncovered a diverse array of protein expression patterns within microglia, revealing distinct molecular signatures associated with different functional states (Mrdjen et al. 2023). These findings suggest that microglia can exist in a spectrum of activation states, each characterized by unique protein profiles that reflect their specific roles and responses to environmental cues. Additionally, proteomic studies have identified key signaling pathways and regulatory mechanisms governing microglial function, providing valuable insights into the underlying molecular mechanisms driving their dynamic behavior (Butovsky et al. 2014). These findings underscore the complex nature of microglial behavior and highlight the importance of considering various factors in characterizing their functional diversity.

Transcriptomic studies have significantly contributed to our understanding of the diverse functional states of microglia, shedding light on the complex landscape of gene expression patterns within these immune cells. By analyzing gene expression profiles across individual microglial cells, transcriptomic analyses have revealed a continuum of states rather than discrete categories, challenging the traditional binary classification (Ochocka and Kaminska 2021). These studies have identified distinct transcriptional signatures associated with different microglial states, indicating their capacity to coexist in multiple functional states simultaneously. For example, research has shown that microglia exhibit unique gene expression patterns in response to various stimuli, such as injury, infection, or neurodegenerative processes (Hammond et al. 2019). Moreover, transcriptomic analyses have elucidated the regulatory networks and signaling pathways that govern microglial function, providing insights into their dynamic behavior's molecular mechanisms.

Studies in epigenetics have provided compelling evidence for the coexistence of multiple functional states among microglia, unveiling the intricate regulatory mechanisms that govern their dynamic behavior. Epigenetic modifications, such as DNA methylation and histone acetylation, play pivotal roles in modulating gene expression and shaping cellular identity. Research has demonstrated that gene expression profiles do not solely determine microglial states but are also influenced by epigenetic changes that confer long-lasting alterations in cellular function (Xiaoyu Li et al. 2023). For instance, studies have shown that exposure to environmental stimuli can induce specific epigenetic modifications in microglia, leading to persistent changes in their functional

states (Catale et al. 2020). Additionally, epigenetic studies have revealed the existence of epigenetic signatures associated with different microglial states, highlighting their capacity to adopt diverse functional phenotypes in response to varying environmental cues (Petralla et al. 2021). Furthermore, investigations into the crosstalk between epigenetic regulators and signaling pathways have elucidated the complex regulatory networks that orchestrate microglial responses in health and disease.

Research in morphology has provided valuable insights into the diverse functional states of microglia, revealing the dynamic nature of their cellular structure in response to various stimuli. Microglia exhibit a wide range of morphological phenotypes, reflecting their ability to adapt to different microenvironments and functional requirements (Salter and Stevens 2017). Studies employing advanced imaging techniques, such as two-photon microscopy and electron microscopy, have unveiled the complexity of microglial morphology and its correlation with their functional states (Davalos et al. 2005). In the healthy brain, microglia display a ramified morphology characterized by long, branching processes that continually survey their microenvironment (Nimmerjahn, Kirchhoff, and Helmchen 2005). Upon activation, microglia undergo dynamic morphological changes, including retraction of processes, enlargement of cell bodies, and altered motility, reflecting their transition to a reactive state (Kettenmann et al. 2011). Furthermore, studies have demonstrated that microglial morphology is influenced by various signaling pathways and environmental cues, highlighting the intricate interplay between cellular morphology and functional states (Vidal-Itriago et al. 2022). Understanding the relationship between microglial morphology and functional states is essential for elucidating their roles in health and disease. However, it is important to note that

morphology alone cannot determine the state of activation, as microglia exhibit diverse morphological phenotypes even within the same functional state (Torres-Platas et al. 2014). Exploration of complementary approaches, such as metabolomics, has suggested a promising and comprehensive understanding of the microglial function and its modulation in different physiological and pathological conditions.

Metabolomic studies are beginning to emerge as a powerful tool for unraveling the intricate metabolic landscape of microglial cells and their functional diversity. By analyzing the comprehensive profile of small molecules and metabolites within microglia, metabolomic studies have revealed distinct metabolic signatures associated with different microglial states (Hou et al. 2024). These findings indicate that microglia can exist in a spectrum of metabolic states, each characterized by unique metabolic profiles reflecting their specific functions and responses to environmental cues. For example, research has demonstrated that microglial activation is associated with alterations in various metabolic pathways, including glycolysis, oxidative phosphorylation, and lipid metabolism (Lauro and Limatola 2020). Additionally, metabolomic analyses have highlighted the role of metabolites such as lactate, glutamate, and ATP in modulating microglial function and immune responses (Monsorno, Buckinx, and Paolicelli 2022). Moreover, metabolomic studies have provided insights into the metabolic interactions between microglia and other cell types within the central nervous system, further elucidating the complex metabolic networks underlying neuroinflammatory processes (Drougard et al. 2023). Overall, metabolomic approaches offer a comprehensive understanding of microglial metabolism and its

contribution to health and disease, paving the way for the development of novel therapeutic strategies targeting metabolic pathways in neuroinflammatory disorders.

The predictive model of microglial stress introduced in this study fills a critical gap by providing insights into the context-dependent nature of microglial states, offering a nuanced understanding of their functional diversity. This model facilitates the development of tailored therapeutic interventions by elucidating how microglial responses vary in different pathological contexts. Instead of adopting a broad approach to targeting microglial activation, future strategies can leverage this model to modulate specific microglial functions based on the specific disease or injury scenario. This precision targeting has the potential to mitigate harmful effects while preserving beneficial microglial functions. Ultimately, understanding the context-dependent nature of microglial states and their contributions to chronic neuroinflammation is crucial for devising effective therapeutic strategies aimed at resolving prolonged neuroinflammatory cascades.

Chronic Inflammatory Cascades

Chronic neuroinflammation, characterized by persistent and sustained inflammation in the brain, poses a significant threat to neurological health. Research has shown that chronic inflammation in the brain involves a complex interplay of various immune cells, signaling molecules, and glial cells (Sun, Koyama, and Shimada 2022). Microglia can transition into a persistent pro-inflammatory state in response to repeated or chronic stress. Studies, such as those utilizing advanced imaging techniques like positron emission tomography (PET) scans, have provided visual evidence of increased

microglial activation in individuals experiencing chronic stress, showcasing the tangible impact on brain inflammation (Kreisl et al. 2020).

Furthermore, the molecular events driving chronic inflammatory cascades often involve the dysregulation of cytokines and chemokines—key signaling molecules in the immune response. Experimental models utilizing rodent subjects subjected to chronic stress paradigms have demonstrated elevated levels of pro-inflammatory cytokines in the brain, contributing to a sustained inflammatory milieu (Munshi et al. 2020). These findings emphasize the molecular complexity of chronic inflammatory cascades and highlight the need for targeted interventions to modulate these signaling pathways.

Moreover, studies examining the impact of chronic neuroinflammation on neuronal health have revealed a reciprocal relationship. Chronic inflammation can lead to synaptic dysfunction and neurodegeneration, further perpetuating the inflammatory cascade (W. Zhang et al. 2023). Research employing neuroimaging techniques, such as magnetic resonance imaging (MRI), has demonstrated structural changes in the brains of individuals with chronic inflammatory conditions, underscoring the destructive consequences of persistent inflammation (Quarantelli 2015).

Investigations into the molecular signaling pathways implicated in chronic neuroinflammation have unveiled several key mediators. Among these, the nuclear factor-kappa B (NF-κB) pathway emerges as pivotal in regulating immune and inflammatory responses within the brain. Repeated or inadequately terminated stressors have been shown to activate NF-κB signaling (Koo et al. 2010), perpetuating the sustained expression of pro-inflammatory genes. This sustained activation of NF-κB contributes significantly to the maintenance of chronic inflammation in the brain.

The role of stress as a trigger for these cascading events is a focal point in the predictive model in an effort to understand the etiology of chronic neuroinflammation. Whether psychological or physiological, chronic stress has been consistently linked to the dysregulation of the immune response in the brain. Research has demonstrated that stress hormones, such as cortisol, can modulate microglial activity and influence the release of inflammatory mediators (Sugama and Kakinuma 2020), providing a mechanistic link between stress exposure and chronic neuroinflammation. The exploration of chronic inflammatory cascades in the context of prolonged neuroinflammation unveils a complex web of molecular and cellular events. Advanced imaging technologies and molecular studies have contributed to our understanding of microglial activation, signaling pathways, and the impact of stress on these processes. This research enhances our comprehension of the mechanisms sustaining chronic neuroinflammation and lays the groundwork for potential therapeutic interventions aimed at disrupting these cascades and mitigating the long-term consequences of neuroinflammatory states.

Chronic inflammation induced by prolonged stress represents a significant risk factor for neurodegenerative processes, significantly impacting various aspects of brain health, such as neuronal integrity, synaptic function, and overall neurological well-being. Numerous studies have provided compelling evidence linking chronic inflammation to neurodegenerative disorders such as Alzheimer's disease, Parkinson's disease, and Huntington's disease (Amor et al. 2010). These investigations underscore the importance of understanding the nuanced interactions between chronic inflammation and neurodegeneration. In Alzheimer's disease, for instance, chronic neuroinflammation

is implicated in the accumulation of beta-amyloid plaques and tau protein tangles, hallmark pathological features of the disease (Kinney et al. 2018). Research utilizing advanced imaging techniques, including positron emission tomography (PET) scans, has demonstrated a correlation between elevated inflammatory markers and increased neurodegenerative burden in affected individuals (Kreisl et al. 2020).

The impact of chronic inflammation on neuronal health is multifaceted. Inflammatory mediators, including cytokines and chemokines released during chronic inflammation, can exert toxic effects on neurons. These molecules contribute to excitotoxicity, oxidative stress, and mitochondrial dysfunction, collectively promoting neuronal damage and apoptosis. Furthermore, chronic inflammation disrupts the delicate balance of neurotrophic factors, crucial for neuronal survival and plasticity, further exacerbating the neurodegenerative cascade.

Synaptic integrity, critical for efficient neuronal communication, is another casualty of chronic inflammation. Research utilizing electron microscopy and electrophysiological techniques has revealed that sustained inflammatory responses lead to the loss of synapses, impairing the intricate network of connections between neurons. This synaptic dysfunction is implicated in cognitive decline observed in various neurodegenerative conditions associated with chronic inflammation.

Overall brain function bears the brunt of chronic inflammation, with studies indicating cognitive decline and behavioral changes as sequelae. Animal models subjected to prolonged inflammatory challenges exhibit deficits in learning and memory tasks, providing valuable insights into the cognitive consequences of chronic neuroinflammation. Human studies, including longitudinal assessments of cognitive

function in individuals with chronic inflammatory conditions, further support the link between sustained inflammation and cognitive impairment.

Research into the neurodegenerative consequences of chronic inflammation underscores the gravity of its impact on neuronal health, synaptic integrity, and overall brain function. Recognizing the irreparable damage incurred as a result of sustained inflammatory responses emphasizes the urgent need for targeted interventions to disrupt these detrimental processes and mitigate the long-term consequences on brain health. The predictive model of microglial stress cycles presented in this study offers a promising avenue for understanding and potentially intervening in these cascades, providing a valuable tool to guide future research and therapeutic strategies to preserve brain health in the face of chronic inflammation.

Microglial Morphology and Organized Microdomains

The investigation of microglial morphology, often regarded as a hallmark of microglial activation and chronic neuroinflammation, served as an initial metric for constructing the predictive model of microglial stress presented in this study, as elucidated in previous research. With their slender, ramified processes, microglia actively survey the neural microenvironment, exhibiting a dynamic and motile nature that adapts to their surroundings. Studies exploring microglia morphology have revealed an interconnected network of slender processes forming an elaborate mesh covering the entire neural parenchyma. This structural complexity is more than aesthetic; it plays a crucial role in microglial interactions with neurons, synaptic elements, and blood vessels, allowing them to sense and promptly respond to changes in the neural environment.

Organized microdomains contribute significantly to the harmonious microglial landscape observed in healthy tissues. The tiled arrangement of microglial territories ensures comprehensive coverage of the neural parenchyma, preventing redundancy in surveillance and minimizing the risk of excessive responses to stimuli. This organized structure optimizes the spatial coverage of microglia and modulates their responses, allowing for swift communication and coordination in responding to changes in the neural microenvironment.

An intricate cellular mosaic emerges within these tiled microdomains, showcasing the interconnectedness and dynamic interactions among microglial cells. The continuous communication and interaction observed at the borders of microglial territories create a network-like structure within the tissue. The collaborative efforts of microglia within these organized microdomains are crucial for efficiently surveying the neural parenchyma, exchanging signals, and coordinating responses to maintain tissue homeostasis. This cellular mosaic extends beyond communication, potentially involving collaborative phagocytosis, enhancing the efficiency of waste removal and contributing to overall tissue health.

The predictive model of microglial cycles of stress leverages the understanding of microglial morphology, organized microdomains, and intricate cellular interactions. Drawing parallels with models commonly used to predict metal fatigue, the study hypothesizes that as microglia are exposed to increasing cycles of stress, irreversible deformations occur, making the cells less capable of enduring future stress. The application of a predictive model based on these structural and organizational aspects provides a novel approach to understanding the transition between acute and chronic

inflammation in the brain, offering insights that may contribute to the development of innovative therapeutic interventions for neuroinflammatory diseases.

Dynamic Interplay of Microglia and Astrocytes in Chronic Neuroinflammation

In the intricate landscape of the central nervous system, the role of microglia extends beyond mere implications in inflammatory processes, urging us to broaden our perspective to include the dynamic interplay with other crucial glial cells, notably astrocytes. While microglia were specifically targeted in the model for their pivotal role in sensing and responding to inflammatory signals, acknowledging the intricate involvement of astrocytes is paramount for a comprehensive understanding of neuroinflammatory states. Under normal conditions, microglia maintain homeostasis through vigilant surveillance in a quiescent state. However, chronic inflammation disrupts this delicate balance, pushing microglia into a hyperactivated state marked by morphological changes. This hyperactivation leads to the sustained release of inflammatory mediators, underscoring microglia's significant role in perpetuating the inflammatory response. This hyperactivation of microglia also triggers a cascade effect on astrocytes, prompting their transition into a reactive state characterized by hypertrophy and increased secretion of pro-inflammatory cytokines, thus intensifying the neuroinflammatory environment in the brain.

Astrocytes, traditionally known as the " support cells " of the brain, surpass their conventional immune functions, actively contributing to maintaining the metabolic environment. Their roles in nutrient supply, energy metabolism, and neurotransmitter regulation position them as essential players in overall brain homeostasis. Recognizing this dynamic collaboration becomes crucial for comprehending the comprehensive

metabolic responses during neuroinflammation. Astrocytes undergo substantial changes in response to chronic inflammation, transitioning into a reactive state characterized by hypertrophy and increased expression of pro-inflammatory cytokines. This astrocytic activation contributes to an amplification of the inflammatory milieu in the brain, establishing a positive feedback loop with microglia. The crosstalk between these glial cell types is a crucial aspect of glial activation overdrive, with each influencing the activation state of the other. The potent trigger for this overdrive lies in repeated or inadequately terminated stress, which releases glucocorticoids and other stress-related molecules, priming glial cells for exaggerated responses to subsequent inflammatory stimuli. Chronic stressors have been linked to sustained activation of both microglia and astrocytes, perpetuating a neuroinflammatory environment. Insights from animal models subjected to chronic stress paradigms provide valuable information on the molecular signaling pathways involved in stress-induced glial activation overdrive.

Recognizing the dynamics of glial cells, particularly the interplay between microglia and astrocytes, would likely enrich the predictive model of microglial cycles of stress by incorporating the reciprocal influence between these cell types. Understanding how astrocytes respond to microglial activation and vice versa could refine the model's predictions, providing a more comprehensive view of the neuroinflammatory processes and their implications for brain health over time. Integrating data on astrocyte reactivity and its relationship with microglial stress cycles could enhance the model's predictive accuracy and offer insights into potential therapeutic interventions targeting glial cell interactions in neuroinflammatory conditions. The complexity of glial responses

underscores the need for continued exploration, paving the way for targeted interventions to restore glial homeostasis and preserve brain health.

Within the framework of interconnected glial dynamics, a focus can be made on the communication networks and signaling mechanisms orchestrating the interplay between microglia and astrocytes. Signaling molecules such as cytokines, chemokines, and neurotransmitters act as messengers in this intricate conversation, resembling a complex code that holds the key to understanding how glial cells collaborate in response to neuroinflammatory cues. Going beyond the individual responses of microglia and astrocytes, this exploration ventures into the collective behavior of the glial network during neuroinflammation. Treating neuroinflammation as a collaborative effort rather than a series of isolated responses is emphasized as essential for gaining a holistic understanding. This intricate web of interactions likely shapes the trajectory of neuroinflammation, influencing both immediate responses and long-term consequences for neuronal function and overall brain health.

Beyond exploring glial interactions in neuroinflammation, potential limitations still lie in adapting predictive models to capture the nuanced transition between acute and chronic inflammation. Similar to predicting metal fatigue, the dynamic nature of glial processes necessitates addressing challenges in distinguishing healing from harmful states. Nevertheless, the study's call for a comprehensive understanding of glial dynamics provides opportunities to refine predictive models, offering transformative insights for therapeutic interventions in neuroinflammatory disorders. The present model focused on microglia due to the intricate relationship between microglial morphology and stress. Studies have revealed that alterations in cellular structure serve as robust indicators of

microglial responses to stressors, offering a visual representation of the dynamic interplay between the nervous and immune systems. Research has demonstrated that exposure to acute or chronic stressors induces distinct changes in microglial morphology (Schramm and Waisman 2022). In response to stress, microglia can transition from their characteristic ramified morphology to an activated state characterized by retracted processes and an amoeboid shape. This morphological transformation is often associated with functional changes, including the release of pro-inflammatory cytokines and other immune mediators. The predictive model's integration of parameters reflecting both morphological and functional alterations becomes even more powerful with the incorporation of advances in imaging technologies, such as in vivo two-photon microscopy and high-resolution confocal imaging. These techniques allow for real-time visualization of microglial dynamics in live brain tissue, providing crucial insights into the temporal aspects of microglial responses to stress and highlighting the rapid morphological changes they undergo in response to environmental challenges. This comprehensive approach enhances our understanding of microglial behavior and empowers the predictive model to accurately capture the dynamic nature of microglial responses and their functional implications in neuroinflammatory processes.

Microglial Morphology as a Dynamic Indicator of Stress Responses

Understanding microglial morphology as a stress indicator involves acknowledging the heterogeneity of microglial responses. Not all stressors elicit the same morphological changes, and the temporal dynamics of these responses can vary. For instance, acute stress may trigger reversible alterations in microglial morphology, while chronic

stressors may induce sustained changes that contribute to long-term neuroinflammation. This recognition of what shapes microglial responses underscores the intricate interplay between environmental factors ranging from toxin exposure to neuroplasticity, highlighting the importance of understanding their collective impact on microglial morphology and subsequent neuroinflammatory outcomes.

Environmental Impact on Microglial Morphology

The relationship between microglial morphology and the external environment is a dynamic interplay that has garnered attention in recent research. Prolonged exposure to stressors, such as social isolation or chronic unpredictable stress, induces alterations in microglial structure. These changes include modifications in cell shape, processes, and the overall complexity of the microglial network. The ability of microglia to morphologically adapt to chronic stress highlights their responsiveness to environmental challenges. Exposure to environmental toxins, such as heavy metals or pollutants, has been implicated in altering microglial morphology. Research has demonstrated that microglia respond to environmental toxins by undergoing morphological changes indicative of activation (Wendimu and Hooks 2022). The adaptability of microglial morphology in the face of environmental challenges underscores their role as sentinels that dynamically respond to external threats.

Moreover, the concept of neuroplasticity extends beyond neurons to include glial cells, with microglia playing a pivotal role. Previous studies have explored how experiences and environmental stimuli associated with neuroplasticity impact microglial morphology. Neuronal activity and synaptic changes can trigger microglial responses, leading to alterations in morphology that are intertwined with the brain's adaptive processes.

In conclusion, the adaptability of microglial morphology in response to environmental modulators is a fascinating aspect of their functional repertoire. Chronic stress, environmental toxins, and neuroplasticity collectively can contribute to the dynamic nature of microglial states. The induced microlesions methodology provides a pertinent means to validate the predictive model, as it simulates an aspect of the dynamic and diverse environmental challenges microglia encounter. By inducing controlled stressors akin to those observed in chronic neuroinflammatory conditions, this approach offers a relevant context for assessing the model's ability to predict microglial responses to varying stress stimuli, thus enhancing its translational relevance and applicability in understanding neuroinflammation.

Discussion for Aim #2

In the pursuit of understanding the transition from acute to chronic inflammation, the initial aim of this study focused on modeling microglial cycles of stress as a means to predict this shift. While microglial morphology serves as a valuable indicator of changes in the microenvironment indicative of inflammation, it became evident that relying solely on morphology could not definitively determine the state of activation. Consequently, the exploration expanded to include the search for more refined metrics of microglial function, particularly during escalating neuroinflammatory states.

This aim investigates the mitochondrial dynamics of activated microglia to examine if the microglial ΔΨM can be used as an assay to indicate microglia signaling and function. We found that microglial-specific increases in the ΔΨM occurred in progressive states following LPS administration. The initial (S1), intermediate (S2), and late (S3) microglial ΔΨM states reflect a radiating depolarization that begins in the soma

and progresses to the endfeet. The application of a TSPO inverse agonist, Emapunil, shunts the somatic to endfoot progression of the ΔΨM. A significant difference in the microglial ΔΨM is eminent in the transition between states and indicates a divergence in metabolic activity during the initial (S1) and late states (S3) of microglial activation.

Recent advancements in our understanding of neuroimmunology have departed from the traditional dualistic classifications of microglial activation (Paolicelli et al. 2022). In the past, microglial activation was categorized as either resting or activated, failing to account for the intricate and multifaceted nature of microglial responses. Microglia have context-dependent differential states (Tay et al. 2017); however, morphology alone cannot determine the state of activation. This study demonstrates that microglial metabolic states based on mitochondria can be quantified. Further, the divergence in the ΔΨM highlights the temporal dynamics of microglial activation, revealing the nuanced metabolic changes that occur during different phases.

The ΔΨM serves as a marker of the intricate electrochemical gradient activity across the inner mitochondrial membrane and provides valuable insights into the energy status of mitochondria. It is a dynamic indicator, reflecting the balance between proton pumping and the permeability of the inner mitochondrial membrane. ΔΨM arises from the separation of charges across the inner mitochondrial membrane, driven by proton pumping through the electron transport chain (ETC). High ΔΨM is generally associated with increased mitochondrial activity, efficient ATP synthesis, and a well-coupled ETC. Conversely, a decrease in ΔΨM may indicate mitochondrial dysfunction or altered metabolic conditions. This snapshot captures the dynamic interplay of charged particles and molecular events that govern cellular bioenergetics. However, there is a need to

acknowledge the limitations inherent in relying solely on ΔΨM for a comprehensive understanding of cellular metabolism. While offering a snapshot into mitochondrial function, ΔΨM represents only a momentary glimpse and cannot encapsulate the complexity of ongoing cellular metabolic activities.

The fluctuations of ΔΨM in response to cellular demands and environmental conditions highlights the dynamic nature of ΔΨM. This includes factors such as nutrient availability, cellular stress, and metabolic state influence ΔΨM. This transient nature should be considered in future research, as with a single measurement it will provide only a snapshot and may not capture the full spectrum of mitochondrial and cellular dynamics over time. It will be important to approach the interpretation of the ΔΨM with awareness of its limitations and need for a holistic understanding of cellular metabolism. Future studies should approach the ΔΨM as one piece of the puzzle, to provide an investigation that integrates diverse measures to grasp the nuanced and multifaceted nature of mitochondrial function within the broader cellular context.

While acknowledging the significance of ΔΨM, it is crucial to recognize its limitations in assessing the overall cellular metabolic landscape. A singular parameter and does not encapsulate the complexity of cellular metabolism, which involves various pathways, compartments, and dynamic responses to environmental cues. Relying solely on ΔΨM may overlook essential aspects of cellular bioenergetics beyond the mitochondrial electrochemical gradient. To overcome the limitations of ΔΨM future research may include monitoring ATP levels, assessing oxygen consumption rates, or examining specific metabolites. By combining ΔΨM data with information from complementary

measurements, a more comprehensive understanding can be gained of cellular bioenergetics.

The Nature of Dye-Induced Cellular Toxicity

This study uses the common practice of dye loading TMRE to visualize the ΔΨM and places a focus on the mitochondrial dynamics of microglia. Similarly, to any methodological approach the use of dyes can introduce challenges if not monitored closely such as dye-induced cellular toxicity. Fluorescent indicators and contrast agents play a pivotal role in elucidating brain anatomy and function, however the cellular toxicity associated with the overuse of dyes in acute brain slices has the capacity to impact brain parenchyma. Dyes such as calcium indicators, neuronal tracers and rhodamine dyes have been shown to exert cytotoxic effects on neuronal and glial cells (Cameron et al. 2016). This toxicity can manifest in a variety of forms, ranging from alterations in cell morphology to compromised cell viability. The chemical properties of specific dyes, particularly those with pronounced affinity for cellular structures, can lead to disruptions in cellular membranes, ion homeostasis, and metabolic processes. Neurons, being the primary functional units of the brain, are susceptible to alterations in membrane integrity and signaling cascades induced by cytotoxic dyes. The homeostatic roles of glial cells, including astrocytes and microglia, can also be disrupted and further influence neuronal health and synaptic communication.

The use of dyes often poses significant challenges in experimental designs that require prolonged exposure to the dye for real-time imaging or long-term observations. However, the benefits of using dyes to gather dynamic data can often out-weight the potential harm that they can inflict on the cellular health over extended periods. In an

effort to mitigate dye-induced cellular toxicity, future research should consider the use of alternative dyes with lower cytotoxic profiles, optimizing the dye concentration, and develop strategies to minimize the exposure duration of the dye while imaging.

A critical consideration that must be made when utilizing dyes is the effect on cellular physiology. Alterations in cell physiology can pose a formidable challenge that can impact the accuracy and interpretation of the data. The application of various dye aid in the visualization of dynamic cellular processes such as changes in the ΔΨM, neuronal activity, synaptic transmission, or morphological changes. Studies have suggested that dyes with affinities for specific cellular structures or organelles may interfere with normal physiological process, which has the capacity to influence membrane potentials, ion fluxes, and cellular signaling cascades (Loew 1992).

The impact of dyes on cellular physiology has been reported on the effect of calcium indicators neuronal excitability and synaptic transmission. Calcium indicators, commonly used to monitor intracellular calcium levels, can influence the activity of voltage-gated ion channels disrupting the delicate balance of neuronal excitability. This interference can confound the interpretation of recorded synaptic transmission, neuronal firing patterns, or network activity.

The confounding factors of dyes have also found in morphological studies that involve neuronal tracers that are designed to highlight intricate details of cellular architecture . The use of dyes has been suggested to affect processes like axonal transport, dendritic arborization, and neurite outgrowth. In addition, acute brain slice studies often involve the exploration of signal transduction pathways critical for cellular communication. The application of dyes may interfere with these pathways, potentially modulating

downstream signaling events. Although the use of dye on acute slices presented in this study provides a novel tool to examine microglial metabolomic activity, it is important to maintain the balance between disentangling the genuine cellular dynamics from the complexities introduced by the application of dyes. To mitigate the potential of TMRE altering microglial physiology, the dye concentration was optimized, and the experimental protocol was designed to minimize the exposure duration to ensure a balance between effective visualization and preserving the integrity of microglial functions. By refining the outlined methodology, we have adopted an innovative imaging technique that balances the duration of observations and the preservation of cellular health. Furthermore, by doing so, have also ensured a reliable and physiological relevant tool that can be extended over varying experimental timelines.

Mitochondrial Function Beyond Membrane Potential

The ΔΨM, while indicative of membrane potential, does not fully encapsulate the diverse metabolic activities occurring within mitochondria. The exploration of mitochondrial function beyond the ΔΨM delves into the intricate and multifaceted roles that mitochondria play within the cellular landscape. While ΔΨM provides a valuable glimpse into the electrochemical gradient, in future research, it will be beneficial to examine processes such as the TCA cycle, ETC activity, and ATP production.

The Tricarboxylic Acid (TCA) Cycle, also known as the citric acid cycle or Krebs cycle, stands as a cornerstone of mitochondrial function and parallels the scope of ΔΨM. Within this metabolic hub, mitochondria, revered as cellular powerhouses, actively engage in a series of intricately orchestrated reactions. The TCA cycle commences with the oxidative decarboxylation of acetyl-CoA, a process that serves both as a central

point for the breakdown of various nutrients and generates critical reduced coenzymes—NADH and FADH2. These coenzymes emerge as pivotal players in the subsequent electron transport chain, navigating a complex journey through protein complexes embedded in the inner mitochondrial membrane. In its cyclic and dynamic nature, the TCA cycle not only furnishes intermediates for biosynthetic pathways but also sets the stage for the efficient production of ATP through the interconnected dance of biochemical reactions, revealing the integral role of mitochondria in cellular bioenergetics.

Electron Transport Chain (ETC) Dynamics represents another facet of mitochondrial function that highlights the intricate orchestration within the inner mitochondrial membrane. The interdependence of the ΔΨM and ETC activity transcends the electrochemical gradient's mere representation. A symphony of biochemical events ensues as electrons journey through the series of protein complexes—ranging from complexes I to IV—embedded in the inner mitochondrial membrane. This orchestrated flow not only propels the pumping of protons across the membrane but also intricately contributes to the establishment and modulation of ΔΨM. The dynamic interplay between ΔΨM and ETC, a dance of charged particles and molecular machinery, highlights the complex machinery at the heart of cellular bioenergetics. This connection underscores the significance of understanding the individual components and the synergistic relationship between membrane potential and electron transport, which could offer a nuanced glimpse into the multifaceted nature of mitochondrial function.

ATP Production and Cellular Energetics have long been shown to unveil the pivotal role of mitochondria as the powerhouses of the cell. Mitochondria aid in the coupling of

protons to flow back into the mitochondrial matrix, which is propelled by ΔΨM with the phosphorylation of adenosine diphosphate (ADP) to yield ATP. This enzymatic process occurs within the inner mitochondrial membrane, where ATP synthase, a molecular motor, harnesses the potential energy stored in ΔΨM to drive the synthesis of ATP. The integration of ΔΨM with ATP production in future research could emphasize the orchestration of cellular energetics by mitochondria and underscore their function beyond establishing electrochemical gradients. This symbiotic relationship between membrane potential and ATP synthesis could reveal a nuanced interplay within mitochondria and offer a novel understanding of how these organelles are central architects in sustaining the cellular powerhouse during progressive neuroinflammatory states.

Beyond serving as energy hubs, mitochondria actively oxidize fatty acids, breaking down fatty acids to generate acetyl-CoA for ATP production. Additionally, they contribute to amino acid metabolism, participating in the catabolism and synthesis of amino acids vital for cellular functions. Furthermore, mitochondria play a pivotal role in the regulation of reactive oxygen species (ROS), managing the delicate balance between oxidative stress and cellular health. Understanding this metabolic diversity becomes paramount, as it unveils mitochondria as dynamic organelles that generate energy and intricately respond to the ever-changing metabolic landscape of the cell. This comprehensive role positions mitochondria as central orchestrators in cellular homeostasis, navigating a complex interplay of pathways to meet the diverse metabolic demands of the cell.

Focusing exclusively on ΔΨM may oversimplify the intricate landscape of mitochondrial function. Integrating measurements of membrane potential with assessments of TCA cycle intermediates, ETC activities, and ATP levels could provide a holistic strategy that ensures a nuanced understanding of mitochondrial function and the multifaceted roles these organelles play in microglial metabolomics.

Microglial ΔΨM Dynamics Under Acute Inflammatory Conditions

While the focus on investigating microglial mitochondrial membrane potential (ΔΨM) under acute inflammatory conditions is undoubtedly crucial for understanding the immediate responses of microglia, there are limitations that can be inherited with this singular perspective. The study's primary concentration on acute inflammation may offer valuable insights into the initial phases of microglial activation, but it may fall short of providing a holistic understanding of the broader spectrum of microglial behavior across varying inflammatory states. One significant limitation lies in the potential oversimplification of microglial responses. Acute inflammation represents only a partial view of the neuroinflammatory spectrum that makes up neuroinflammatory processes' dynamic and evolving nature. Relying solely on observations during acute phases may lead to a skewed understanding, overlooking the subtleties and complexities that characterize chronic inflammatory conditions. Microglial responses to acute stimuli may differ significantly from their behavior in prolonged or recurrent inflammatory settings, where adaptive changes and regulatory mechanisms could come into play. To overcome these limitations, future research endeavors should consider incorporating longitudinal studies that span acute and chronic phases of inflammation. Adopting a more comprehensive approach that includes diverse inflammatory stimuli and extends

the observation period presented in this research can capture the dynamic evolution of microglial responses over time.

Harnessing Mito::mKate2 for Dynamic Insight into Microglial Mitochondria

Specifically, the integration of mito::mKate2 or mKate, a mitochondrial red fluorescent protein, within the context of microglial studies emerges as a powerful strategy to address the limitations associated with examining mitochondrial membrane potential (ΔΨM) solely under acute inflammatory conditions. This innovative approach holds the potential to revolutionize the exploration of microglial behavior by allowing for more nuanced and comprehensive investigations, bridging the gap between acute and chronic inflammatory states. One primary advantage of mKate lies in its ability to serve as a reliable marker for mitochondria within microglial cells. By exploiting the genetic modification of microglial cells to express mKate, the unprecedented visualization of mitochondrial dynamics can be seen in real-time. These transgenic models, expressing mKate specifically within microglial cells, provide unparalleled precision insight into the spatial and temporal distribution of mitochondria. The red fluorescence emitted by mKate facilitates the tracking of individual mitochondria within microglia, allowing for the observation of their movement, fusion, and fission dynamics. This dynamic insight into mitochondrial behavior during acute inflammatory conditions provides a holistic understanding of how microglia navigate their environment over time.

Incorporating mKate into longitudinal studies enhances the spatial resolution of mitochondrial dynamics and enables a more comprehensive assessment of microglial responses. The red fluorescence emitted by mKate serves as a versatile marker that can be coupled with other fluorescence-based probes to examine additional aspects of

microglial behavior, such as changes in mitochondrial membrane potential, oxidative stress, or calcium signaling. This approach opens the door to longitudinal studies that extend beyond the immediate response to acute inflammation, providing a continuous and detailed assessment of microglial mitochondria behavior throughout the entire inflammatory continuum. Such longitudinal studies offer the opportunity to capture not only the initiation but also the evolution and potential resolution of microglial responses, shedding light on the adaptive mechanisms that may arise in chronic inflammatory settings.

Mitochondrial Dynamics Modulation by TSPO Ligand

In this study, the ligand of a mitochondrial protein known as the 18 kDa Translocator Protein (TSPO) was utilized to modulate mitochondrial dynamics and neuroinflammatory states. Studies suggest that TSPO, by interacting with specific ligands, may serve as a modulator, fine-tuning the balance between fusion and fission events. The subcellular localization of mitochondria is intricately linked to cellular function, and TSPO's involvement suggests a level of coordination that extends beyond individual organelles, potentially impacting broader cellular processes. The concept of TSPO-mediated mitochondrial dynamics modulation expands our understanding of the diverse functions of this protein. TSPO's influence on the movement and distribution of mitochondria hints at its role as a cellular orchestrator, responding to internal and external cues to optimize mitochondrial function. Further investigations into the molecular mechanisms through which TSPO influences mitochondrial dynamics, potentially uncovering novel therapeutic avenues for conditions associated with mitochondrial dysfunction.

TSPO, through its interaction with ligands, is also proposed to act as a modulator of ROS production within mitochondria. ROS are byproducts of cellular respiration, and while they play essential roles in cellular signaling, their excessive production can lead to oxidative stress, causing damage to cellular components. This modulation is not a simple on-off switch but a nuanced adjustment that responds to the cellular context. TSPO's regulatory role in ROS production hints at its adaptive function, allowing cells to fine-tune their response to changing environmental conditions or internal cues. Future exploration into the underlying mechanisms of TSPO-mediated ROS regulation in various cellular processes could broaden the current understanding of redox signaling, apoptosis, and inflammation. The regulatory role of TSPO in mitochondrial ROS production adds a layer of sophistication to its functions, presenting opportunities for targeted interventions in conditions where ROS dysregulation plays a pivotal role.

The ΔΨM serves as a vital barometer of the energetic status of mitochondria and is intricately linked to the electron transport chain and ATP production, making it a central player in cellular bioenergetics. By interacting with specific ligands, TSPO is suggested to contribute to the modulation of ΔΨM. This regulation is not merely a passive adjustment but an active participation in the orchestration of mitochondrial vitality. TSPO's influence on ΔΨM reflects its role in maintaining the delicate balance between energy production and consumption within mitochondria, impacting cellular functions ranging from metabolism to apoptosis. The exploration of the modulatory role of TSPO ligands on ΔΨM regulation also sheds light on the potential consequences for cellular health. A finely regulated ΔΨM is essential for the efficient functioning of mitochondria,

influencing processes such as ATP synthesis, calcium homeostasis, and the production of reactive oxygen species. Contributing to the control of ΔΨM, is a key player in the broader cellular landscape, influencing cellular responses to both physiological and pathological stimuli. Diseases such as neurodegenerative disorders and metabolic diseases often involve disruptions in mitochondrial function, including altered ΔΨM. Investigating TSPO's role in ΔΨM regulation opens avenues for understanding its potential contribution to these conditions and exploring therapeutic strategies that target TSPO to restore mitochondrial health.

Specificity Challenges in TSPO- Ligand Binding Therapies

Challenges have been associated with achieving the desired specificity in ligand binding, emphasizing the potential consequences of unintended off-target effects that could compromise the precision of therapeutic interventions. Understanding these specificity challenges is paramount for assessing the overall effectiveness and safety of utilizing TSPO ligands in the modulation of neuroinflammation. While TSPO is a promising target for regulating neuroinflammation due to its association with activated microglia, there is a potential for ligands to bind to other receptors or molecular targets, introducing the risk of unintended consequences.

The therapeutic effects of various TSPO ligands were considered in this study to mitigate the challenges posed by the structural diversity of TSPO ligands, which may contribute to variations in binding affinities and selectivity across different compounds (Table 1). TSPO-ligand Emapunil showed a high affinity for TSPO in a variety of experimental conditions that included electroencephalogram activity, exploratory behavior, anxiety, Depression, and PTSD.

Table 1: The Therapeutic Effect of TSPO Ligands in Models of Neurological Conditions

Experiment	Species	Compound	Dosage	Result	Citation
Electroencephalogram Activity and Exploratory Behavior	Mice	Ro5-4864 XBD-173 PK11195	5 mg/kg and 1 mg/kg	Higher power in the δ and θ and dose-dependent variations in the α and γ. The least noticeable behavioral effects of XBD-173 were a dose-dependent decrease in locomotor activity in the OFT, all the while maintaining a robust EEG modulation.	Hines et al. 2022
Alzheimer's disease	Mice	Ro5-4864 PK11195	3 mg/kg once per week for 4 weeks.	3x TgAD animals showed reduced neuropathology and enhanced cognitive function; wild-type mice showed decreased soluble β-amyloid.	Barron et al. (2013)
Anxiety	Rats	Alprazolam XBD-173	1 mg/kg intraperitoneally every 5 days and 0.1, 1, or 10 mg/kg p.o.	In rats that were prone to panic, an infusion of sodium lactate induced panic behavior, which was inhibited by both XBD-173 and alprazolam. XBD-173 administration did not result in any drowsiness, but alprazolam significantly decreased locomotor activity.	Rupprecht et al. (2009)
Brain ischemia	Rats	Diazepam	30 and 90 minutes after the insult, 10 mg/kg i.p.	Compared to just 12.8 ± 0.3% of living neurons in the untreated ischemic brains, 72.0 ± 14.5% of CA1 hippocampal neurons survived the lesion.	Sarnowska et al. (2009)
Depression	Mice	ZBD-2	For two weeks, take 2 or 4 mg/kg, p.o. once daily.	ZBD-2 administration prevented BDNF and CREB, two signaling proteins linked to synaptic plasticity, from declining in expression. Furthermore, the persistent SCI-induced gliocyte activation at the lesion site was abolished by ZBD-2 treatment.	Li et al. (2017b)

Anxiety & Depression	Mice Rats	XBD-173 PK11195 Ro5-4864	1 ml kg^{-1} 10 ml kg^{-1}	Through MBR (TSPO), XBD-173 has anti-anxiety and antidepressant-like actions, but it doesn't have the negative side effects of traditional benzodiazepines.	Kita et al. (2004)
Multiple sclerosis	Mice	Etifoxine	50 mg/kg for 22 days postpartum.	Reduced penetration of peripheral immune cells into the spinal cord and enhanced regeneration of oligodendroglia following inflammatory demyelination.	Daugherty et al. (2013)
Neuroinflammation	Mice	PK 11195	I.P. at 3 mg/kg for 14 days.	Reduced content of β-amyloid1–42, elevated progesterone and allopregnanolone levels in the brain, and defense against cognitive impairments brought on by repeated treatment of LPS (500 μg/kg, intraperitoneally for 11 days).	Ma et al. (2016)
Post-traumatic stress disorder	Mice	YL-IPA08	Over 18 days, at doses of 0.1, 0.3, and 1 mg/kg.	Enhanced anxiety and contextual terror in the inescapable electric foot-shock-induced mice model of PTSD were suppressed by YL-IPA08.	Li et al. (2014)
Post-traumatic stress disorder	Mice	XBD-173	1 mg/kg, p.o. for 14 days, and 0.03, 0.1, 0.3, and mg.	Repeated treatments with XBD-173 improved the mice's long-term behavioral deficits, such as freezing and anxiety-like behavior, caused by foot shocks. However, these treatments had no effect on spontaneous locomotor activity or body weight.	Rocca et al. (1998)
Post-traumatic stress disorder	Rats	Midazolam	i.p. injections of 0.125, 0.25, 0.5, and 1 mg/kg were given 30 minutes prior to testing.	Through combined TSPO and CBR and neurosteroidogenesis, midazolam ameliorates the behavioral impairments in rats' PTSD behavior as measured by a single chronic stress scenario.	Miao et al., (2017

The dynamic nature of neuroinflammation poses significant challenges for interventions involving TSPO ligands, emphasizing that not all such ligands may offer optimal interventions. Research has delved into the intricacies of neuroinflammatory processes, emphasizing their multifaceted and evolving nature over time. This complexity arises from the intricate interplay of immune responses and molecular cascades within the central nervous system, influenced by factors such as the type of insult, disease progression, and involvement of different cell types. The evolving landscape of neuroinflammation introduces complexities for static interventions involving TSPO ligands, highlighting the need for a nuanced understanding of these dynamic processes.

One key challenge highlighted in the exploration is the temporal dynamics of neuroinflammation and its impact on the efficacy of TSPO ligands. Unlike static conditions, where a consistent target is present, the ever-changing nature of neuroinflammation may influence the effectiveness of TSPO ligands at different stages. This emphasizes the necessity for interventions that can adapt to the evolving landscape, considering the varying phases of neuroinflammation. While some TSPO ligands may still hold therapeutic potential, recognizing these temporal nuances becomes crucial for optimizing their anti-inflammatory actions.

Furthermore, the spatial dynamics of neuroinflammation present another layer of complexity for TSPO ligand interventions. Different brain regions may exhibit distinct inflammatory profiles, posing challenges for uniform interventions. The effectiveness of certain TSPO ligands may vary across regions with differing degrees of inflammation, highlighting the importance of tailoring interventions to specific anatomical contexts. While this spatial heterogeneity introduces challenges, it does not necessarily discredit

the use of TSPO ligands; instead, it emphasizes the need for a more targeted and personalized approach.

The exploration of the dynamic nature of neuroinflammation underscores the challenges faced by TSPO ligand interventions, emphasizing that not all such ligands may offer optimal interventions. While acknowledging these limitations, it is essential to recognize that this does not entirely discount the potential therapeutic benefits of certain TSPO ligands. Understanding the temporal and spatial dynamics of neuroinflammation provides valuable insights for refining interventions involving TSPO ligands, ensuring their optimal use in modulating inflammatory responses within the central nervous system. Future research may focus on addressing these challenges to enhance the effectiveness of specific TSPO ligands and their potential interventions in navigating the complexities of neuroinflammation.

Immunomodulators and Microglial ΔΨM: Mechanistic Insights for Neuroinflammatory Therapies

In the context of neuroinflammatory disorders, such as MDD and PTSD, the intricate interplay between immune activation, cellular metabolism, and mitochondrial function plays a critical role. One of the key pathways involved in neuroinflammation is the NF-κB pathway, which is activated in response to immune stimuli such as LPS, leading to increased cytokine production and inflammatory responses. This activation cascade underscores the importance of understanding the molecular mechanisms that regulate microglial activation and subsequent neuroinflammatory processes (Figure 33). TSPO expression has been linked to increased neuroinflammation and cytokine production. During inflammatory responses, TSPO expression is upregulated, suggesting its

involvement in modulating immune activation and inflammatory signaling pathways. This upregulation highlights TSPO as a potential pharmacological target for regulating neuroinflammatory processes.

In the present study, emapunil treatment, acting as an inverse agonist of TSPO, showed promise in attenuating the increase in the microglia ΔΨM induced by LPS. This observation suggests that emapunil may exert its therapeutic effects through modulating microglial function and mitochondrial dynamics. However, the underlying mechanisms by which emapunil influences microglial MMP remain to be fully elucidated. One potential mechanism by which emapunil may exert its effects is through the activation of AMP-activated protein kinase (AMPK) in microglial cells. AMPK, a key cellular energy sensor, regulates energy metabolism and maintains cellular homeostasis. Activation of AMPK has been shown to influence mitochondrial function, including mitochondrial biogenesis, oxidative phosphorylation, and quality control mechanisms.

The effects of emapunil-induced AMPK activation on microglial ΔΨM are likely multifaceted. AMPK activation may enhance mitochondrial function and biogenesis, leading to an increase in ΔΨM, reflecting improved mitochondrial health and functionality in microglial cells. Additionally, AMPK activation may influence mitochondrial dynamics and morphology, further impacting microglial responses to stimuli and overall cellular function. The modulation of AMPK activity by emapunil could have significant functional consequences in neuroinflammatory disorders. It may regulate microglial activation states, inflammatory responses, and cellular metabolism. Moreover, alterations in mitochondrial function and ΔΨM could impact microglial

functions such as phagocytosis, cytokine production, and neuroinflammatory processes, ultimately influencing the progression of neuroinflammatory diseases.

The observed effects of emapunil on microglial ΔΨM provide valuable insights into the potential of utilizing ΔΨM as a screening tool for assessing the therapeutic efficacy of various immunomodulators in neuroinflammatory disorders. Immunomodulators encompass a diverse range of compounds, including anti-inflammatories, antioxidants, thiazolidinediones (TZDs), metformin, and adiponectin, each with distinct mechanisms of action impacting mitochondrial biogenesis and function.

Anti-inflammatories, such as nonsteroidal anti-inflammatory drugs (NSAIDs) and cyclooxygenase-2 (COX-2) inhibitors, exert their effects by dampening the inflammatory response. By reducing inflammation, these agents indirectly influence mitochondrial function, as chronic inflammation is known to impair mitochondrial biogenesis and function. By mitigating inflammation, anti-inflammatories may contribute to the preservation of mitochondrial integrity and function, thereby potentially impacting microglial ΔΨM.

Antioxidants play a crucial role in combating oxidative stress, a common feature of neuroinflammatory disorders. Oxidative stress can lead to mitochondrial dysfunction and damage, contributing to the pathogenesis of neuroinflammation. Antioxidants, such as vitamin C, vitamin E, and glutathione, protect mitochondria from oxidative damage and support their function. By preserving mitochondrial integrity, antioxidants may contribute to maintaining microglial ΔΨM and overall cellular homeostasis in neuroinflammatory conditions.

TZDs, including rosiglitazone and pioglitazone, are peroxisome proliferator-activated receptor gamma (PPARγ) agonists with anti-inflammatory properties. TZDs have been shown to enhance mitochondrial biogenesis and function by activating PPARγ, which in turn stimulates the expression of genes involved in mitochondrial biogenesis and oxidative metabolism. By promoting mitochondrial health, TZDs may positively influence microglial ΔΨM and contribute to the resolution of neuroinflammation. Similarly, metformin, an antidiabetic drug, has been found to activate AMPK, leading to enhanced mitochondrial biogenesis and function. By modulating AMPK activity, metformin may indirectly impact microglial MMP and cellular energy metabolism, thereby exerting therapeutic effects in neuroinflammatory disorders. Finally, adiponectin, an adipokine with anti-inflammatory and insulin-sensitizing properties, has been shown to enhance mitochondrial biogenesis and function in various cell types. By promoting mitochondrial health, adiponectin may contribute to maintaining microglial ΔΨM and modulating neuroinflammatory responses.

Overall, the findings suggest that emapunil-mediated modulation of microglial ΔΨM reflects changes in cellular energy metabolism and response to immunostimulation. This underscores the potential of microglial ΔΨM as a screening tool for indicating the therapeutic potential of other immunomodulators in neuroinflammatory disorders. Understanding the molecular mechanisms underlying these interactions could pave the way for the development of novel therapeutic strategies targeting microglial function and mitochondrial dynamics in neuroinflammatory diseases.

Contribution to a Comprehensive Understanding of Microglial Metabolism

Recognizing the intricate interactions between immunomodulators and neuroinflammatory processes provides a foundation for exploring their broader impact on cellular functions, particularly within the realm of microglial metabolism. Microglial metabolism stands at the intersection of immune response, energy production, and cellular homeostasis. The conventional understanding of microglial activation often hinges on immediate and observable changes in cellular morphology or cytokine release. However, this study breaks new ground by venturing into the subtler realm of microglial metabolism, recognizing that the interplay between mitochondrial dynamics and cellular energetics is a critical determinant of overall cellular function.

The investigation into ΔΨM dynamics serves as a key entry point into this metabolic exploration. ΔΨM is not merely a static parameter but a dynamic reflection of mitochondrial health, bioenergetic status, and cellular demand. By scrutinizing the fluctuations in ΔΨM, researchers gain a window into the energetic transactions occurring within microglial mitochondria. This dynamic insight enables the study to decipher how microglial cells respond metabolically to the cues of inflammation, revealing potential adaptations and regulatory mechanisms.

The emphasis on mitochondrial behavior broadens the scope of the investigation. Mitochondria, beyond being the cellular powerhouses, actively engage in shaping microglial responses. The study implements a novel tool that aids in understanding how alterations in mitochondrial behavior, distribution, or turnover, correlate with shifts in microglial metabolic states. This holistic approach recognizes that microglial metabolism

is not confined to energy production alone but is intricately linked to other vital cellular functions that contribute to the overall immune response.

The potential contributions of this study are far-reaching. By providing a more holistic view of microglial metabolism, future research can advance the understanding of the underlying mechanisms driving neuroinflammation. Unraveling the metabolic nuances of microglial responses may uncover novel therapeutic targets for neurological disorders where dysregulated inflammation plays a pivotal role.

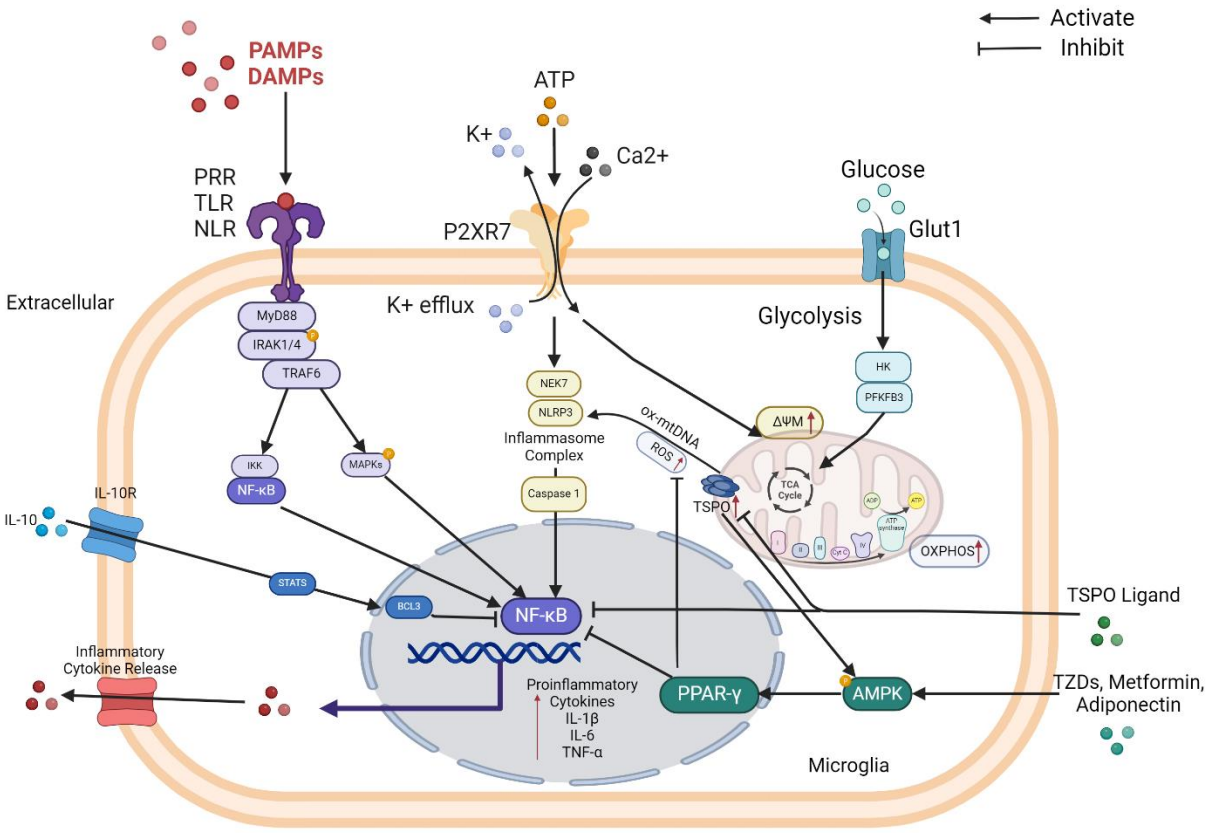


Figure 33:Exploring the Underlying Mechanisms of the Microglia ΔΨM as a Screening Tool for Immunostimulation.

This schematic illustrates the complex relationship between microglial activation pathways and mitochondrial membrane potential (ΔΨm) dynamics, serving as a screening tool for immunostimulation. Pathogen- or damage-associated molecular patterns (PAMPs/DAMPs) trigger inflammatory signaling pathways via pattern recognition receptors (PRRs), leading to inflammasome assembly and cytokine release. Simultaneously, metabolic reprogramming shifts from oxidative phosphorylation (OXPHOS) to glycolysis, influencing ΔΨm and reactive oxygen species (ROS) generation. The regulatory role of the IL-10 pathway on NF-κB signaling is highlighted, balancing neuroinflammation. Notably, TSPO ligands, such as emapunil, may modulate AMP-activated protein kinase (AMPK) activation, influencing mitochondrial biogenesis and function, which could impact microglial responses and the progression of neuroinflammatory diseases.

Chapter Six: Conclusion

In conclusion, this dissertation has delved into the pressing healthcare challenges posed by neuroinflammatory disorders, emphasizing conditions like Major Depressive Disorder (MDD) and Post Traumatic Stress Disorder (PTSD) and their profound impact on individuals and society at large. The recognition of the intricate interplay between neuroinflammation, glial cell metabolism, and underlying energy dynamics has emerged as a central theme, challenging the historical prioritization of neurons in neurological research. Microglia, as key players in orchestrating immune responses within the central nervous system, have taken center stage in this study, revealing their dynamic roles in immunological balance, synaptic maintenance, and tissue repair.

Despite the growing acknowledgment of microglia's significance, the development of targeted therapeutic interventions has been hindered by limited investigative tools and a critical knowledge gap. This dissertation aims to address these challenges by focusing on the intricate relationship between neuroinflammation, microglial metabolic activities, and their impact on the progression of neuroinflammatory disorders. The experimental hypotheses put forth suggest that microglial mitochondrial membrane dynamics are crucial for their rapid response to immune stimuli, with potential implications for novel therapeutic interventions. The study not only seeks to provide fresh insights into the complex network of interactions within the central nervous system but also aims to contribute innovative approaches to combat the global health challenge posed by neuroinflammatory disorders.

These findings could reshape our understanding of microglial responses to immune stimuli, offering potential avenues for more effective treatments for inflammatory brain

conditions. The proposed exploration of Emapunil as an inverse agonist holds promise for domain-specific regulation of microglial metabolic activity, presenting a valuable tool for targeted interventions. This dissertation marks a step forward in bridging knowledge gaps, emphasizing the need for a holistic approach that considers the intricate cellular community within the brain to pave the way for transformative advancements in neuroinflammatory disorder research and therapeutics.

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Curriculum Vitae

Kendra McGlothen
Neuroscience/Psychology Instructor
Kimcglothen@gmail.com

EDUCATION

BACHELOR OF ARTS IN PSYCHOLOGY

University of Nevada Las Vegas, January 2017

DOCTOR OF PHILOSOPHY IN NEUROSCIENCE,

University of Nevada Las Vegas, (PhD Candidate)
Expected Spring 2024

PROFESSIONAL SUMMARY

Neuroscience and Psychology Instructor with a proven track record in developing course content and delivering high-quality undergraduate courses. Adept at instructing traditional and online students. Experienced in teaching a diverse range of courses, from introductory to upper-division levels, including general psychology and neuroscience, which encompass research methods, cognitive, development, perception, psychopathology, and social psychology. Committed to fostering an inclusive academic environment that promotes the success of faculty, staff, and students from diverse backgrounds. Eager to contribute to the growth and success of the department through continued professional development and service contributions. Ready to make a positive impact in a dynamic academic setting with a forward-looking vision.

SKILLS

- Lesson Planning
- Online Teaching
- Lecture Presentation
- Assignment Grading
- Facilitating Group Discussion

RELEVANT EXPERIENCE

GRADUATE TEACHING ASSISTANT

University of Nevada Las Vegas, August 2020-Present

- Developed and implemented lesson plans for a variety of learning styles.
- Provided individualized instruction to meet the needs of struggling students.
- Facilitated small group discussions on topics related to the subject matter.
- Encouraged critical thinking by posing questions during lectures and discussions.
- Promoted an inclusive atmosphere where all students felt respected and valued.
- Graded exams and other coursework to reflect students' mastery of material accurately.

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GRADUATE RESEARCH ASSISTANT

University of Nevada Las Vegas, August 2018-Present

- Conducted behavioral assessments on both wildtype and genetically modified mice (GCamp6f, hM3Dq, Cx3cr1) using tasks including open field, string pull, and the rung walk.
- Performed in vivo surgical techniques, including craniotomies, intracranial injections of Adeno-associated viral vectors, and EEG/EMG implantation.
- Executed brain extraction and preservation techniques, utilizing methods such as drop fix and acute slice preparation.
- Utilized various microscopy techniques, including light, confocal, and two-photon microscopy, to examine glia morphology and activation.
- Conducted immunohistochemistry and Rhodamine staining as part of the microscopy analysis process.
- Created detailed project plans that included tasks, timelines, and resources needed.
- Analyzed and interpreted data analysis results to draw inferences and conclusions.
- Summarized information and provided briefings to leadership on accomplishments and project updates.
- Carried out lengthy research projects to develop new products, processes, and applications.

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SERVICE AND OUTREACH

Promoting Diversity in STEM (AccesSTEM)
Assisted the Hines Group Laboratory as a coordinator for a STEM outreach program that provides primary school students the opportunity to gain interactive experience in a neuroscience lab.

The Chemical Collective Radio Show
Co-hosted a UNLV KUNV radio show that delved into the fascinating and complex world of drugs and drug culture. This show covered a range of topics, including the latest research on drug use, the neural mechanism of drug action and abuse, and the cultural significance and stigmatization of drugs in society.

Marc Kahre Elementary School Science Fair Organizer and Judge
Annually partners with Marc Kahre Elementary School to volunteer as a science fair organizer and judge. I assist with evaluating and scoring the students' projects based on criteria like scientific method, creativity, and presentation, providing constructive feedback and recognizing outstanding achievements.

AWARDS

UNLV Graduate College Rebel Grad Slam Scholarship (2023)
UNLV College of Liberal Arts Summer Stipend (2023)
Graduate & Professional Student Research Forum Presentation Award (2023)
UNLV Graduate College Patricia Sastaunik Scholarship (2022)
UNLV Graduate College Summer Session Scholarship (2021)
Western Association of Graduate Schools Honorable Mention (2021)
Western Association of Graduate Schools 3MT Competition (2021)
UNLV Graduate College Rebel Grad Slam Scholarship (2020)
Graduate & Professional Student Research Forum Presentation Award (2020)

PRESENTATIONS

McGlothen, K. I., Cutler, M., & Hines, D.J. (2023, April). Predictions of the Future: Modeling microglial cycles of stress based on engineering predictions of metal fatigue. *Talk presented at UNLV's Graduate and Professional Student Research Forum.*

McGlothen, K. I., Hines, R.M. & Hines, D.J. (2022, November). Modeling cycles of microglial stress based on engineering predictions of metal fatigue. *Poster presented at the Society for Neuroscience Conference.*

McGlothen, K. I., & Hines, D.J. (2022, April). Boldly Going Where No One Has Gone Before: A Novel Approach to Examining Metabolic Processes in the Brain. *Talk presented at UNLV's Inspiration, Innovation, and Impact: Graduate Student Research Showcase.*

McGlothen, K. I., & Hines, D.J. (2021, April). Outward Depolarization of Microglial Mitochondria Membrane Potential Following LPS Administration. *Talk presented at UNLV's Graduate and Professional Student Research Forum.*

McGlothen, K. I., & Hines, D.J. (2021, April). Outward Depolarization of Microglial Mitochondria Membrane Potential Following LPS Administration. *Talk presented at UNLV's Inspiration, Innovation, and Impact Event*

McGlothen, K. I., Hines, R.M. & Hines, D.J. (2021, November). Emapunil attenuates microglial mitochondria membrane potential following LPS administration. *Talk presented at the Society for Neuroscience Conference.*

McGlothen, K. I. & Hines, D.J. (2021, March). Astrocytes Affect on Cortical Circuitry and Motor Behavior. *Talk presented at the WAGS 3 Minute Thesis.*

McGlothen, K. I. & Hines, D.J. (2020, November). Astrocytes Affect on Cortical Circuitry and Motor Behavior. *Talk presented at the Rebel Graduate Slam 3 Minute Thesis.*

McGlothen, K. I. & Hines, D.J. (2020, November). The Impact of Astrocytes on Cortical Circuitry and Motor Behavior. *Talk presented at the Graduate and Professional Student Research Forum.*

McGlothen, K. I. & Hines, D.J. (2020, February). DREADD Activation of Cortical Astrocytes Effect Motor Behavior. *Talk presented at the Rebel Graduate Slam 3 Minute Thesis.*

PUBLICATIONS

McGlothen, K. I., Cutler, E., Hines, R.M. & Hines, D.J. (2024). Modeling Cycles of Microglial Stress Based on Engineering Predictions of Metal Fatigue. Frontiers (In preparation)

McGlothen, K. I., Hines, R.M. & Hines, D.J. (2024). Outward Depolarization of the Microglial Mitochondria Membrane Potential Following LPS Administration. Frontiers (In preparation)

Lopez, A, Strong, H.N., **McGlothen, K. I.**, Hines, D.J., & Baker, J.R. (2022). A Compact Avalanche-transistor Based Pulse Generator for Transcranial Infrared Light Stimulation (TILS) Experiments. MDPI

MEETINGS AND CONFERENCES

Black in Neruo Conference 2023
Society for Neuroscience Conference 2022
Black in Neruo Conference 2022
Society for Neuroscience Conference 2021
Black in Neruo Conference 2021
Western Association of Graduate Schools Annual Conference 2021
SFN Global Connectome 2020
Neuro TC Symposium: Optogenetics Conference 2020