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AGING, A PATHOLOGICAL FACTOR IN NEUROLOGICAL INJURY

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Aging, a pathological factor in neurological injury

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ABSTRACT

Aging, a pathological factor in neurological injury

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One of the main reasons for CNS drugs to fail in clinical development is not considering age as a risk factor while studying chronic age-related neurological/neurodegenerative diseases in preclinical studies. We first set out to gain a comprehensive understanding of the impact of age on various aspects (anatomical, immunological, and biochemical) in rodents that play a key role in determining the onset, progression, and evolution of disease severity. With advancing age, the vascular structure and function are compromised which is hypothesized to accelerate cognitive decline. The initial step toward developing novel therapeutics is to characterize the age-related vascular modifications. Utilizing a vessel painting technique, we labelled the surface cortical vessels of young and aged Sprague-Dawley rats and analyzed for classical angiographic features (junctions, lengths, end points, density, etc). We found significant decrease in vascular components while vascular complexity and lacunarity were significantly increased in the aged brain compared to young brain. These age-dependent changes were prominent at the level of right and left middle cerebral artery (MCA) as well as on a global scale. Next, we investigated the changes on the peripheral immune response following lipopolysaccharide (LPS) induced acute systemic inflammation in young and aged Sprague Dawley rats. We observed age-related immunosuppression in the splenic leukocytes indicative of reduced ability of the spleen to retain the immune cells. We also found dysregulated cytokine/chemokine expression in the plasma following LPS stimulation in aged and young animals. Interestingly, we noticed significant increase in circulatory neutrophil population in the aged animals compared to young animals in response to LPS at 24h. Taken together, these studies confirm the presence of age-related modifications in the vasculature as well as immune system suggesting altered response to injury/infection and thus emphasizing the need to utilize age-appropriate models when studying diseases of the elderly. Lastly, we wanted to test the therapeutic effect of a novel agent in case of brain injury model in aged rodents. Previous studies by our lab and others have showed that targeting mitoNEET using NL-1 was neuroprotective following brain injury models. We wanted to investigate if administration of NL-1 could improve functional outcomes following stroke in an aged rodent model of cerebral ischemia reperfusion injury. We found significant decrease in infarct volume and edema index at 24h post stroke. We also saw enhanced survival and reduced behavior deficits. Moreover, we showed improved BBB integrity, reduced oxidative stress and apoptosis at 72h post stroke. Interestingly, PLGA encapsulated NL-1 at 0.25mg/kg (which is 40-fold lesser dose than NL-1 at 10mg/kg) produced better therapeutic effects. Future studies should focus on understanding the mechanism underlying the biology of aging thus enabling the development of novel therapeutic targets for neurological disorders/diseases.

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ABBREVIATIONS

4-HNE: 4-hydroxynonenal
ACA: Anterior Cerebral Artery
AD: Alzheimer's Disease
ADME: Absorption, Distribution, Metabolism and Excretion
AIDS: Acquired Immunodeficiency Syndrome
AIS: Acute Ischemic Stroke
ALS: Amyotrophic Lateral Sclerosis
ANOVA: Analysis of Variance
APCs: Antigen Presenting Cells
ApoE: Apolipoprotein E
APP: Amyloid Precursor Protein
ARV: Antiretroviral Drugs
ATP: Adenosine Triphosphate
Aβ: Amyloid Beta
BBB: Blood-Brain Barrier
BDNF: Brain Derived Neurotrophic Factor
bEnd3: Murine Brain Vascular Endothelial Cells
BETi: Bromodomain Inhibitors
cART: Combination Antiretroviral Therapy
CBF: Cerebral Blood Flow
CH: Contralateral Hemisphere
CIV: Corrected Infarct Volume
CNS: Central Nervous System
CVD: Cerebrovascular Diseases
DAG: Diacylglycerol
DAPI: 4',6-diamidino-2-phenylindole
DCs: Dendritic Cells
DiI: 1,1'-dioctadecyl-3,3,3',3' tetramethylindocarbocyanine perchlorate
EAE: Experimental Autoimmune Encephalomyelitis
ELISA: Enzyme-linked Immunosorbent Assay
FDA: Food and Drug Administration
FITC: Fluorescein Isothiocyanate
GPCR: G-protein coupled receptors
HAND: HIV-Associated Neurocognitive Disorders
HIV: Human Immuno deficiency virus
HRP: Horseradish Peroxidase
HT: Hemorrhagic Transformation
ICU: Intensive Care Unit

IFN-γ: Interferon-gamma
IH: Ipsilateral Hemisphere
IHC: Immunohistochemistry
IL: Interleukin
IL1β: Interleukin 1 beta
IRI: Ischemia-reperfusion injury
LC-MS/MS: Liquid Chromatography-Mass Spectrometry
LPS: Lipopolysaccharide
LRA: Latency Reversal Agent
MCA: Middle Cerebral Artery
MMP-9: Matrix Metalloproteinases-9
MMSE: Mini-Mental State Examination
mN: MitoNEET
MnSOD: Manganese Superoxide Dismutase
mNSS: Modified Neurological Severity Scores
MS: Multiple sclerosis
NK: Natural Killer
NL-1: NEET Ligand 1
NP: Nanoparticles
NVU: Neurovascular Unit
PBS: Phosphate Buffered Saline
PCA: Posterior Cerebral Artery
PD: Parkinson's disease
PFA: Paraformaldehyde
PKC: Protein kinase C
PLGA: poly D,L-lactic-co-glycolic acid
PPARγ: Peroxisome Proliferator-Activated Receptor Gamma
ROS: Reactive Oxygen Species
rTEM: Reverse Transendothelial Migration
SD: Standard Deviation
SEM: Standard Error of the Mean
TBI: Traumatic Brain Injury
TLR-4: Toll-like Receptor 4
tMCAO: Transient Middle Cerebral Artery Occlusion
TNF-α: Tumor Necrosis Factor α
tPA: Tissue Plasminogen Activator
TTC: Triphenyltetrazolium Chloride
TUNEL: Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling
ZO-1: Zonula Occludens-1

CHAPTER ONE

Introduction

LITERATURE REVIEW

Aging epidemiology

The world's population is aging at an alarming rate. In 2019, it was estimated that there were 703 million people aged 65 or over worldwide and this number is expected to surpass 1.5 billion by 2050, that is about 25% of the world's population (Nations *et al.*, 2019). In the US alone, the older population (aged≥65 years) is projected to expand from 53 million in 2018 to 88 million in 2050 (Hou *et al.*, 2019). In addition to well developed nations, less developed nations will also see a surge in their older population (Preston and Stokes, 2012; Shetty, 2012; Fernández-Ruiz, 2019). Reports predict one in five people in underdeveloped nations will be over 60 years old by 2050 (Shetty, 2012). The sources of such growth in aging population are found to be due to changing patterns of fertility, mortality, and migration (Preston and Stokes, 2012). Additionally, the average human life expectancy due to improvements in health care has drastically increased over the years (Cuny, 2012; Fernández-Ruiz, 2019; Aburto *et al.*, 2020). The life-expectancy of Americans is estimated to increase by about 7.4% in the next ~40 years, i.e., from 79.7 in 2017 to 85.6 in 2060 with women expected to live longer than men on an average, in 2060 (Medina *et al.*, 2020). However, this rise in life span is not directly proportional to the quality of life, showing only a modest increase in the years spent without disability (Aging and Metabolism: Two Sides of the Same Coin, 2017). This is due to the fact that there is a surge in global burden of late-life diseases and is associated with increased incidence and prevalence of geriatric neurodegenerative diseases in older individuals (Cuny, 2012; Callixte *et al.*, 2015; Baker and Petersen, 2018; Partridge *et al.*, 2018;

Fernández-Ruiz, 2019). As we progress towards increasingly aged population, mortality due to all causes increases exponentially (Parsons, 2007). In the recent years, the number of critically ill elderly patients admitted to the intensive care unit (ICU) have not only increased (Esme *et al.*, 2019) but also have low hospital survival rate when compared to young patients (Topeli and Cakir, 2013). The financial cost of providing long-term care for geriatric patients imposes a huge economic burden on the health care sector (Florence *et al.*, 2018). Therefore, understanding the biology of aging and age-related diseases is of utmost importance and emphasize the need for developments and advances to improve the quality of life in elderly by slowing the biological aging process.

Hallmark signs of biological aging

Biological aging is clearly distinct from chronological aging and is considered as a complex, dynamic physiological process. It is linked to physical, metabolic, cognitive, and emotional aspects of individual function (Bland, 2018). With progression of time, genetic, epigenetic and environmental factors lead to accumulation of aberrant molecular mechanism at cellular, molecular and organization level thereby altering the physical appearance, functionality and regenerative capacity of an organism (Khan *et al.*, 2017; Hou *et al.*, 2019). Hence, it is necessary to identify the molecular and cellular processes underlying the complex physiological process of biological aging. Over the years, studies have identified nine hallmark signs of aging which are classified into three categories namely, the primary, antagonistic and integrative (López-Otín *et al.*, 2013). The primary hallmark constitutes the main driving force of aging, consisting of genomic instability, telomere attrition, epigenetic alterations, and loss of proteostasis. The second

antagonistic hallmarks, associated with mitochondrial dysfunction, cellular senescence and deregulated nutrient sensing, provide benefits at low levels and become detrimental at high levels. Finally, the integrative hallmarks which comprise stem cell exhaustion and altered intercellular communication induce damage beyond repair due to pro-longed accumulation (López-Otín *et al.*, 2013, 2016; Hou *et al.*, 2019). This system of identification of the hallmark signs of aging has aided in the development of novel therapeutic targets aiming to restore the functional decline seen in aged individuals.

Aging and neurological/neurodegenerative disorders

Neurological/Neurodegenerative disorders are diseases affecting the central nervous system (CNS) classified by death of neurons leading to functional and cognitive decline (Mayne *et al.*, 2020). Neuronal loss may be progressive as in the case of Alzheimer's Disease (AD), Parkinson's disease (PD), and amyotrophic lateral sclerosis (ALS) (Dugger and Dickson, 2017) or can be caused due to primary insult such as demyelination, ischemia or trauma leading to secondary neurodegeneration as in the case of multiple sclerosis (MS), acute ischemic stroke (AIS) and traumatic brain injury (TBI) (Mayne *et al.*, 2020). Neurodegenerative diseases are one of the most life-threatening disorders around the world, predominantly affecting the elderly (Niccoli and Partridge, 2012a; Harris *et al.*, 2014; Fülöp *et al.*, 2016; Kowalska *et al.*, 2017; Hou *et al.*, 2019; Azam *et al.*, 2021). Among the several risk factors for neurodegenerative/neurological disorders, the aging process itself imposes a significant impact (Niccoli and Partridge, 2012a; López-Otín *et al.*, 2013; Callixte *et al.*, 2015; Hou *et al.*, 2019; Azam *et al.*, 2021; Wrigglesworth *et al.*, 2021).

There is a close association between aging and age-related diseases at molecular, cellular and organization level. With advancing age, the brain undergoes several changes (Figure 1.1). Normal brain aging is characterized based on progressive changes in local reductions in glucose metabolism, impaired cellular calcium signaling, oxidative stress, accumulation of damaged and misfolded proteins, mitochondrial dysfunction leading to aberrant energy metabolism, chronic low grade inflammation, and neuronal atrophy driven by defective activity at synapses (e.g. receptors, transporters, ion channels and synthetic enzymes) (Rango and Bresolin, 2018; Erickson and Banks, 2019; Fernández-Ruiz, 2019; Hou *et al.*, 2019; Mayne *et al.*, 2020; Blinkouskaya *et al.*, 2021). Additionally, there is increased glial reactivity (increased astrogliosis and microglia activation) exacerbating neuroinflammation as well as decreased neurogenesis concurrent with significant loss of neurons (Fernández-Ruiz, 2019; Hou *et al.*, 2019; Mayne *et al.*, 2020; Blinkouskaya *et al.*, 2021). The microvasculature of the CNS is composed of tightly regulated endothelial cells forming the blood brain barrier (BBB) whose function is to protect the brain against circulating toxins or pathogens (Daneman and Prat, 2015; Profaci *et al.*, 2020). Recent studies have demonstrated increased blood brain barrier permeability in certain brain regions of older cohorts suggesting BBB disruption as a part of the normal aging process (Goodall *et al.*, 2018; Verheggen *et al.*, 2020). Moreover, noninvasive brain imaging studies have revealed age-related changes on the brain morphology (specific structures as well as different anatomical regions), along with vascular morphology (Abdelkarim *et al.*, 2019; Nyberg and Wåhlin, 2020; Beishon *et al.*, 2021). Several studies showed the rate of increase in cerebral atrophy, the most important morphological change, with aging (Blinkouskaya and Weickenmeier, 2021;

Blinkouskaya *et al.*, 2021). This is associated with decline in global brain volume, white matter volume, brain folding, emergence of white matter lesions, gray matter shrinking, sulcal widening, thinning of the cortex, and ventricular enlargement (Blinkouskaya and Weickenmeier, 2021; Blinkouskaya *et al.*, 2021). Apart from global structural alterations, the brain vascular components undergo remodeling with age resulting in changes in cerebral blood flow (CBF) and concurrent neurovascular coupling dysfunction (Abdelkarim *et al.*, 2019; Beishon *et al.*, 2021; Mokhber *et al.*, 2021). In addition, recent studies demonstrated higher activity in certain brain regions during rest and cognitive tasks which might be a compensatory mechanism for reduced functional connectivity in older subjects (Sala-Llonch *et al.*, 2015; Hughes *et al.*, 2020). Consequently, disrupted functional architecture of brain networks along with vascular dysfunction leads to the cognitive impairment in the elderly (Sala-Llonch *et al.*, 2015; Damoiseaux, 2017; Abdelkarim *et al.*, 2019; Hughes *et al.*, 2020; Beishon *et al.*, 2021; Mokhber *et al.*, 2021). Taken together, these normal age-related physiological changes are exacerbated during pathological conditions leading to onset and rapid progression of the disease state. Understanding the biology of normal aging is not only useful in predicting the disease severity but also important for successful development of therapeutic interventions for the treatment of neurodegenerative/neurological diseases in the elderly.

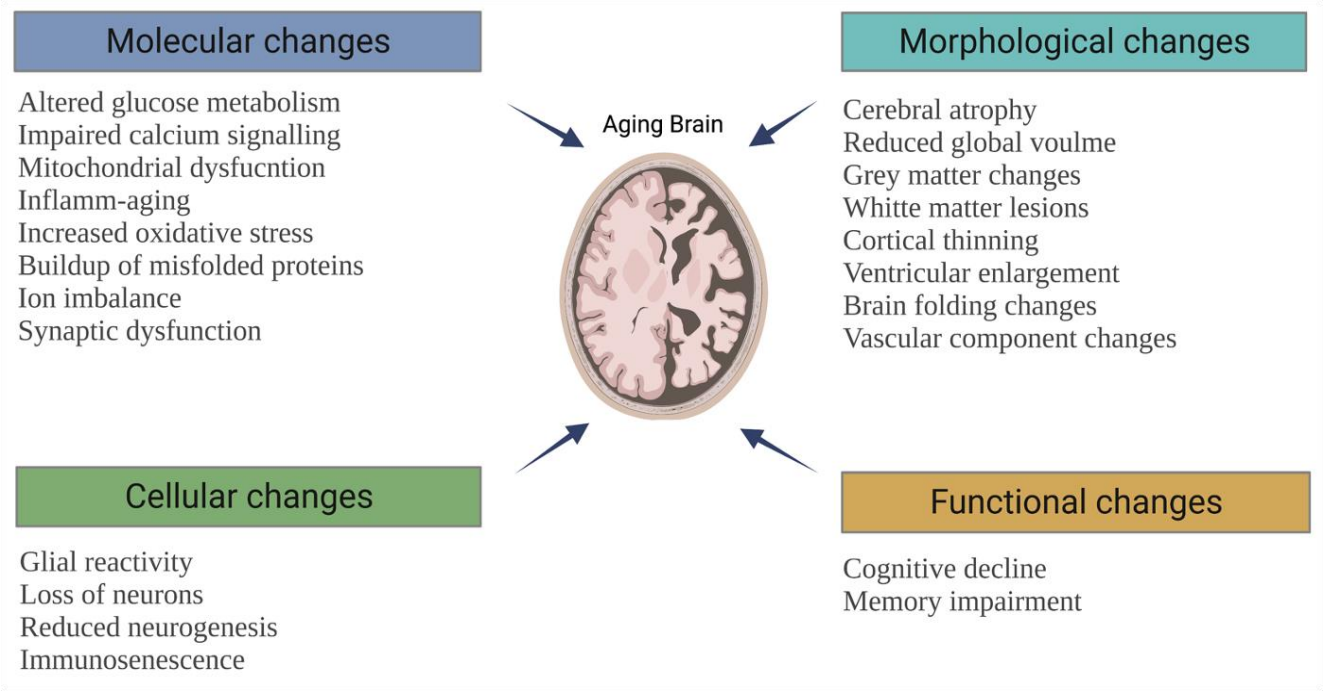

Figure 1.1: Age-related physiological changes occurring in the healthy brain. *Created with BioRender.com*

Therapeutic potential of pharmacological agents in age-related CNS diseases

The pipeline of drug discovery and development process is an expensive one apart from being time consuming. Currently, it is estimated about $113 million to over $6 billion dollars required on an average for each novel drug to be approved for clinical use (Rennane *et al.*, 2021). However, studies reported that 90% of the drug candidates fail to advance during clinical trials and drug approval even after rigorous optimization and validation at preclinical stage (Hingorani *et al.*, 2019; Sun *et al.*, 2022). In general, the failure rate is higher (close to 100%) for CNS targeting drugs especially in case of major neurodegenerative diseases, in both preclinical and clinical studies (Gribkoff and Kaczmarek, 2017; Cummings *et al.*, 2019; Howes and Mehta, 2021). For example, till date the only food and drug administration (FDA) approved pharmacological agent to treat ischemia stroke is reperfusion with tissue plasminogen activator (tPA) despite 1000+

successful preclinical studies (O'Collins *et al.*, 2006; Dhir *et al.*, 2020; Lourbopoulos *et al.*, 2021). Owing to the high failure rate, longer time for drug development process and post-development regulatory review, major pharmaceutical companies are moving away from CNS drug development process (Gribkoff and Kaczmarek, 2017; Howes and Mehta, 2021). In case of AD, there are currently only 126 molecular entities in clinical trials as opposed to 3558 agents in cancer clinical trials (Moser and Verdin, 2018; Cummings *et al.*, 2021). As a consequence of expensive drug development failure, a huge burden is imposed on not only the healthcare providers but also the citizens through healthcare taxation (Hingorani *et al.*, 2019). With the growing older population and increased prevalence of chronic neurodegenerative diseases (Baker and Petersen, 2018; Partridge *et al.*, 2018), there is an urgent need to develop CNS drugs in a timely manner by reducing the cost and time associated with the clinical trials (Cummings *et al.*, 2021). Additionally, the success rate of clinical trials can be improved by identifying the right drug, druggable target, biomarkers, recruiting the right participants, predicting those at the high risk category for disease development as well as starting treatment at the right age (Baker and Petersen, 2018; Cummings *et al.*, 2019).

Aging is associated with increased co-morbidities. The prevalence of multimorbidity (the coexistence of multiple chronic diseases) in older individuals ranges from 55 to 98% from a global perspective (Marengoni *et al.*, 2011). In the US, it is said that six in ten adults have at least one chronic disease and four in ten adults have two or more (Maresova *et al.*, 2019). As a consequence of higher prevalence of chronic disease, the number of drugs taken by aged individuals tend to be higher than younger individuals. A recent survey showed that about 69% of aged adults (40-79 years) consumed at least

one prescription drug and 22.4% consumed at least 5 prescription drugs in the US (Hales *et al.*, 2019). Studies have shown that aging alters the pharmacokinetic and pharmacodynamic properties of a drug (Mangoni and Jackson, 2004; Drenth-van Maanen *et al.*, 2020). A study conducted in rats showed decreased clearance, volume of distribution, potency and increased elimination half-life for a serotonin receptor antagonist, lerisetron with aging (Jauregizar *et al.*, 2003). In addition, age-related differences exist in disease-relevant systems and thus significantly influence the outcomes of studies examining the mechanism of drug action, drug efficacy, drug toxicity as well as biology of disease (Jackson *et al.*, 2017). Apart from the underlying mechanism of biological aging, several age-related physiological changes accompanying dementia and frailty can alter the pharmacokinetics of medications (Drenth-van Maanen *et al.*, 2020). Loss of body weight and muscle mass can inadvertently decrease the volume of distribution of hydrophilic drugs while increasing the volume of distribution of hydrophobic drugs (Drenth-van Maanen *et al.*, 2020). Additionally, age related dementia is associated with increased BBB permeability and decreased efflux pump activity which results in neurotoxicity for certain drugs (Reeve *et al.*, 2015, 2017). Despite these differences, aged individuals are frequently excluded from clinical trials. For example, only a small fraction of people >50 years have been included in human trials while testing antiretroviral drugs (Budak, 2020).

RATIONALE

Despite the existence of several animals to study human diseases, finding the right model that fully recapitulates the disease pathophysiology or phenotype continues to be

a problem (Nielsch *et al.*, 2016). Rodent models are the most commonly used mammals in biomedical research and non-clinical drug trial (Jackson *et al.*, 2017). Multitude of factors affect the outcome of a study of which age of the animals have a significant impact on new drug candidates and is associated with altered drug handling, physiological reserve, and pharmacodynamic responses (McLean and Le Couteur, 2004; Ettlin *et al.*, 2010). Inconsistent choice of age while studying specific age-related diseases in rodent models negatively impacts the quality of the research and leads to failure of translation of drugs from bench to bedside (Jackson *et al.*, 2017). Therefore, there is an urgent need to understand the influence of age on various aspects (anatomical, immunological, and biochemical) in rodents that play a key role in determining the etiology, progression, prognosis of age-related chronic disease.

OBJECTIVES

The overarching goal of this dissertation was to characterize the influence of age on anatomical, immunological, and biochemical aspects and its impact on therapeutic outcomes in the context of age-related neurological disorders.

Chapter 2: Pharmacological profile of bryostatin-1 in various CNS diseases/disorders

Our lab is primarily interested in testing the therapeutic potential of pharmacological agents in aged rodent models of brain injury/neurodegenerative diseases. One such pharmacological agent that our lab focused on was bryostatin-1, an ultra-potent protein

kinase C (PKC) modulator. The pharmacological profile of bryostatin-1 in various CNS diseases/disorders has been well studied by our lab and others and thus reviewed here.

Chapter 3: Characterize the effect of aging on cerebrovascular architecture

Age-related changes on the vascular morphology have been implicated in several cerebrovascular and neurodegenerative diseases. Reduction in vessel density with aging is correlated with reduced cerebral blood flow (CBF), neuro vascular coupling, impaired cellular metabolism. However, it is not known at what level these changes are taking place. To investigate that, we employed a vessel painting technique to label the surface cortical endothelial cells. Using aged and young Sprague Dawley rats, we measured age-related changes on the classical vessel parameters, complexity, vessel diameter and order of branching of the middle cerebral arteries as well as global surface cortical vessels. Given the association between cerebral vessel structure and cognition, the results of the study would provide a detailed characterization of vessel architecture with age in vivo and may help elucidate the cognitive changes during senescence.

Chapter 4: Characterize the effect of age on peripheral immune response following lipopolysaccharide (LPS) induced systemic inflammation

With advancing age, our immune system undergoes cellular and functional alterations characterized by immunosenescence and chronic low-grade inflammation termed as inflamm-aging. Several studies have reported age-related changes in innate and adaptive arms of immunity. However, changes in the composition of leukocytes in response of injury/infection in an aged subject is unknown. We used LPS induced systemic

inflammation model to determine the influence of age on acute immune response at 3 and 24h in young and aged Sprague Dawley rats. We performed flow cytometry to investigate the age-related changes in major leukocyte population in blood and spleen. We also analyzed the cytokine expression following systemic inflammation using enzyme-linked immunosorbent assay (ELISA). Given the importance of peripheral immune system in the pathological progression of neurodegenerative diseases, the results of this study would provide evidence for age-related alterations in blood and splenic leukocyte composition as well circulating cytokine/chemokine profiles. Given the role of peripheral immune system in driving neuroinflammation, the knowledge gained from this study would aid in comprehending the pathological progression of neurodegenerative diseases in elderly.

Chapter 5: Investigate the therapeutic potential of a novel mitoNEET ligand, NL-1 in an aged rodent model of brain injury (cerebral ischemia/reperfusion injury model)

Cerebral ischemia is the leading cause of mortality and morbidity predominantly affecting the elderly. While recanalization of the ischemic brain with tissue plasminogen activator (tPA) and endovascular thrombectomy can restore the blood flow and salvage the brain tissue, they can do more harm than good due to ischemia-reperfusion injury (IRI). Among the various pathological mechanisms of ischemia-reperfusion injury, mitochondrial dysfunction due to free radical production plays a pivotal role in orchestrating neuronal damage and serves as a potential therapeutic target. Prior studies demonstrated that targeting MitoNEET (mN), an iron- sulfur cluster protein located in the outer mitochondrial

membrane that acts a redox senor, by using the ligand NL-1 produces significant tissue sparing and neuroprotective effects following ischemic stroke, traumatic brain injury & Parkinson's disease. Here we hypothesized that targeting mN activity following cerebral ischemia reperfusion injury would reduce ischemic brain injury & improve neuronal survival in the penumbra region surrounding the infarct by reducing lipid peroxidation induced cellular damage. We evaluated the efficacy of NL-1 and a nanoparticle formulation of NL-1 in treating cerebral ischemia/reperfusion injury using aged female rats following a 2 h middle cerebral artery occlusion (MCAO) with reperfusion. The results of this study would demonstrate that mN is a novel druggable target in the case of cerebral ischemia/reperfusion injury that requires further investigation.

In summary, this dissertation evaluates the function of age as a non-modifiable risk factor for neurological disorders. This dissertation emphasizes the need to understand and characterize the effect of age on brain vessel structure as well as the peripheral immune system following systemic inflammation in a quantitative manner. Additionally, this body of work also documents the therapeutic efficacy of a novel ligand NL-1, targeting mitochondrial protein mN in an aged rodent model of cerebral ischemia reperfusion injury. Overall, this work underscores the importance of using aged rodents while studying age-related diseases to enhance the success rate of clinical drug development.

CHAPTER TWO

Evaluating use of bryostatin-1 for the treatment of neurological diseases and injury

ABSTRACT

Protein kinase C (PKC) isozymes are a family of kinases that regulate many downstream signaling processes and play vital roles in human health and disease. Owing to their involvement in key biological events such as signal transduction, cell proliferation and apoptosis, PKC isozymes have become attractive drug targets. Bryostatin-1, a macrocyclic compound obtained from the marine organism *Bugula neritina*, is an ultra-potent PKC modulator with high affinity for PKC isozymes alpha, delta and epsilon. In recent years, preclinical and clinical studies have shifted away from probing bryostatin-1 as an anti-cancer drug and instead have shifted focus to the promising pharmacological benefits of bryostatin-1 as a neuroprotective agent for central nervous system diseases. The purpose of this mini-review is to discuss the latest findings advancing the pharmacological potential of bryostatin-1 for the treatment of neurological diseases, including Alzheimer's disease, traumatic brain injury, ischemic stroke, multiple sclerosis and neuroAIDS.

INTRODUCTION

Bryostatins, isolated from marine bryozoans *Bugula neritina*, are highly complex oxygenated macrocyclic lactones that were initially studied as anti-cancer compounds due to their anti-tumorigenic and cytotoxic profiles in murine lymphocytic leukemia cells (Pettit *et al.*, 1982). Bryostatins demonstrate a wide range of anti-cancer effects, including immune stimulation, growth inhibition and antineoplastic effects on multiple malignancies (Wu *et al.*, 2020). Of the isolated 21 analogs, bryostatin-1 is the most well-studied and pharmacologically characterized (Wu *et al.*, 2020). Bryostatin-1 is a potent PKC regulator with strong binding affinity (K_i=0.6-2.1nM) for PKC isozymes alpha (α), beta (β), delta (δ), gamma (ν), epsilon (ε), eta (η), theta (θ) which are determined using a cell free assay (Ly *et al.*, 2020). In particular, bryostatin-1 has strong efficacy towards PKCε with displacement of phorbol ester, the prototypical exogenous ligand for PKC, occurring at sub-nanomolar concentrations in rodents and humans (Hess *et al.*, 1988). Both conventional and novel PKC isozymes have shown varied function and dose-dependent regulation when exposed to bryostatin-1 while atypical PKC isozymes do not bind to bryostatins (Szallasi *et al.*, 1994; Mutter and Wills, 2000).

The pharmacological activity of bryostatin-1 involves allosteric modulation of PKC by binding to their regulatory C1 domains ((C1a and C1b), mimicking the action of endogenous ligand diacylglycerol (DAG). This binding drives the conformational change of the PKCs thus potentiating the transient binding of PKC to the plasma membrane and increasing the activation of downstream signaling cascades (Wender and Staveness, 2014). While low concentrations of bryostatin-1 stimulate PKC activity, studies on cancer cells show that upon prolonged exposure to bryostatin-1 at high concentrations, PKC

expression undergoes downregulation via ubiquitin-mediated proteasomal degradation (Mutter and Wills, 2000; Sun and Alkon, 2006). This unique biological activity of bryostatin-1, as an activator and inhibitor of PKC activity, at different concentrations and exposure intervals, provides unique opportunities for studying PKC activity and should be considered when developing dosing schemes for bryostatin-1.

After bryostatin-1 is administered, it is widely distributed in the body and well tolerated with the most common adverse effect being myalgia (Zhang *et al.*, 1996; Mutter and Wills, 2000; Sun and Alkon, 2006). For more than 20 years, multiple Phase I/II clinical trials for solid and hematological malignancies have been conducted with bryostatin-1 alone and in combination with other chemotherapeutic agents (Kollár *et al.*, 2014). While bryostatin-1 showed absence of tumor-promoting activity in contrast to other PKC activators, it did not progress to Phase III clinical trial as an anti-cancer drug (Kollár *et al.*, 2014; Raghuvanshi and Bharate, 2020).

In the early 2000s, studies began showing promising results with bryostatin-1 as a CNS drug (Figure 2.1). These pharmacological studies of bryostatin-1 focused on its action in stimulating synapse formation, release of neurotrophic factors, enhanced learning capacity and consolidation of memories (Sun and Alkon, 2006, 2014; Kollár *et al.*, 2014). Bryostatin-1 has also been reported to have anti-inflammatory and anti-oxidative properties that mitigate cellular stress and neuroinflammation (Kornberg *et al.*, 2018; Safaeinejad *et al.*, 2018). Recently, studies using bryostatin-1 treatment following ischemic stroke and traumatic brain injury demonstrated pronounced reductions in edema formation and marked decreases in blood-brain barrier (BBB) disruptions (Tan *et al.*, 2013, 2015; Lucke-Wold, Logsdon, *et al.*, 2015), which led our lab to postulate that

bryostatin-1 confers neuroprotection following brain injury, in part, by restoring functional integrity of the BBB (Tan *et al.*, 2013, 2015; Lucke-Wold, Logsdon, *et al.*, 2015). In this review, we discuss recent advances in the therapeutic use of bryostatin-1 to treat neurological diseases/disorders with a specific focus on Alzheimer's disease (AD), traumatic brain injury (TBI), acute ischemic stroke (AIS), multiple sclerosis (MS) and reversing latency of human immunodeficiency virus (HIV).

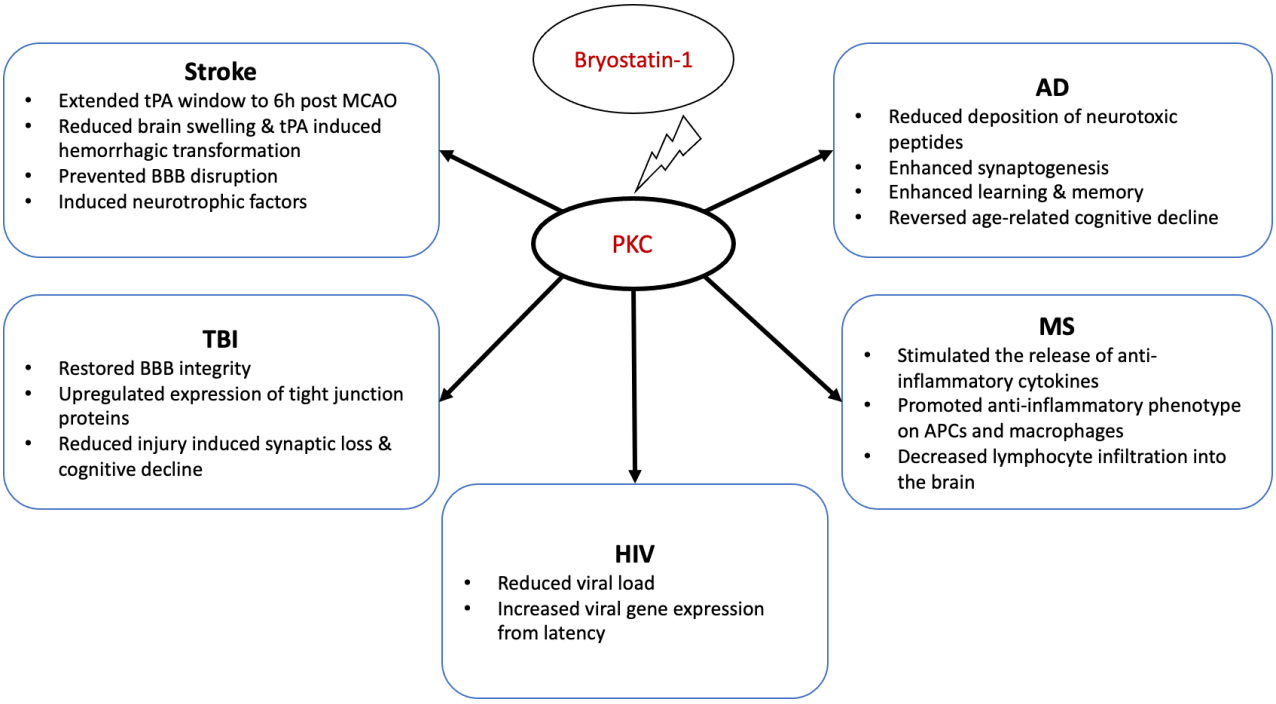

Figure 2.1: Possible clinical applications of bryostatin-1 as a CNS drug. A number of preclinical and clinical studies have recently focused on the therapeutic applications of bryostatin-1 in Alzheimer's disease (AD), traumatic brain injury (TBI), acute ischemic stroke, multiple sclerosis (MS) and neuroAIDS. *See text for detail.*

BRYOSTATIN-1 IN AD

AD is a chronic neurodegenerative disease characterized by progressive dementia that affects over 17 million people globally (Schrott *et al.*, 2015). With advancing age, the incidence and prevalence of AD is increasing and thus causing a surge in mortality and morbidity worldwide (Sawda *et al.*, 2017). The progression of age-related incidence of AD

is 15% in the age group of 60–64 to 85–89 years (Fernández-Ruiz, 2019). In the US, it is estimated that 6.2 million individuals are currently living with AD dementia, and is predicted to increase to more than 13.8 million by 2060 (2021 Alzheimer's disease facts and figures, 2021). Pathological hallmarks associated with AD include deposition of amyloid beta (Aβ) plaques, formation of neurofibrillary tangles, progressive loss of synapses and marked neurodegeneration (Sadigh-Eteghad *et al.*, 2015; Nelson *et al.*, 2017).

Secretases (α-, β- and γ-) are proteases that process amyloid precursor protein (APP) and play a pivotal role in generation and modulation of Aβ peptide (Ahmad *et al.*, 2019). A leading hypothesis for the pathogenesis of AD posits that AD-associated neuronal dysfunction and cognitive decline is due to extracellular accumulation of Aβ protein resulting from aberrant proteolytic processing of APP via β-secretase (Sadigh-Eteghad *et al.*, 2015). A prior study demonstrated that bryostatin-1, through activation of PKCε, attenuated Aβ plaque deposition by potentiating the secretion of a soluble, non-toxic form of APP α-secretase through a non-amyloidogenic pathway (Etcheberrigaray *et al.*, 2004; Hongpaisan, M-K Sun, *et al.*, 2011) (Figure 2.2). Additionally, repeated administration of bryostatin-1 (40 μg/kg; i.p.) in double transgenic AD mice reduced the deposition of neurotoxic peptides- $A\beta_{40}$ and $A\beta_{42}$ with concomitant improvement in survival and behavioral outcomes (Etcheberrigaray *et al.*, 2004). Furthermore, bryostatin-1 treatment stabilized and prolonged activity of neprilysin, a potent Aβ-degrading enzyme, in the brain through upregulation HuD, an mRNA binding protein responsible for neural development and neuronal plasticity (Lim and Alkon, 2014).

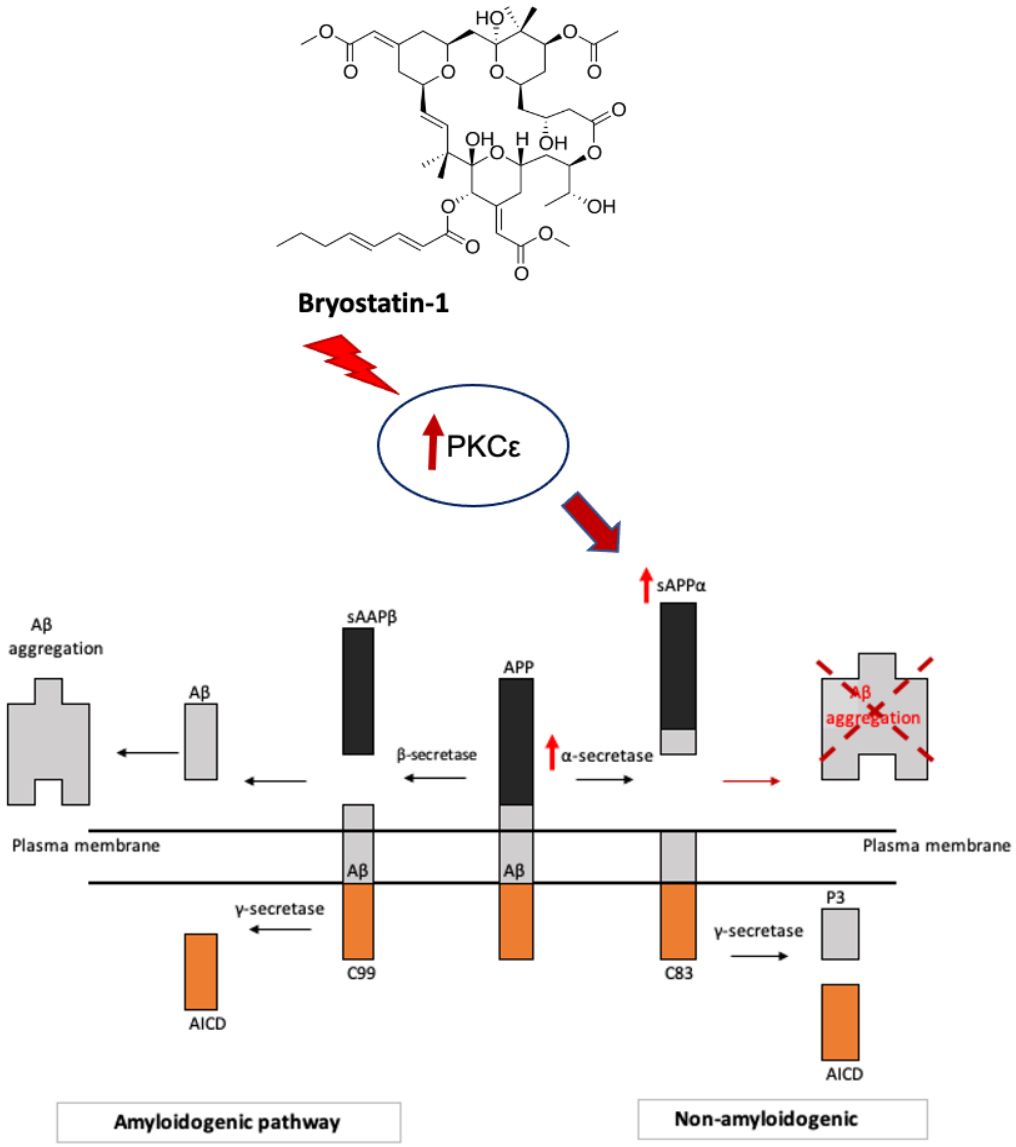

Figure 2.2: Bryostatin-1 attenuated Aβ plaque deposition in Alzheimer's disease in a PKCε dependent pathway. AD pathophysiology is widely characterized by the deposition β-amyloid peptide (Aβ) in the brain which ultimately results in neurodegeneration and cognitive defects. The fate of amyloid precursor protein (APP) cleavage by secretases (proteolytic enzymes) determines the severity of disease progression. In, the amyloidogenic pathway, APP is sequentially cleaved by β- and γ-secretase resulting in the generation of the neurotoxic Aβ plaques. Whereas in the non-amyloidogenic pathway, α-secretase cleaves the APP, generating soluble α-APP fragments (sAPPα) and C-terminal fragment (C83), which is further cleaved by γ-secretase, producing non-toxic P3 and APP intracellular domain (AICD). Evidence suggest that PKC is instrumental in the processing APP. Activating protein kinase C epsilon (PKCε) by bryostatin-1 is shown to enhance the α-processing of APP as indicated by increased production of sAPPα while decreasing the formation of Aβ plaques in the brain.

Analysis of autopsy-confirmed AD brains revealed elevated levels of Aβ along with reduced PKCε, manganese superoxide dismutase (MnSOD), and brain derived neurotrophic factor (BDNF) in hippocampal CA1 pyramidal neurons (Sen *et al.*, 2018). The same association was noticed in in vitro using cultures of human primary hippocampal neurons. These effects were reversed by bryostatin-1 administration. A preclinical study using 8-month-old transgenic AD mice showed that bryostatin-1 (30 µg/kg; i.p) administration for 12 weeks restored the levels of PKCε with concomitant decline in soluble amyloid beta protein levels (Hongpaisan, M-K Sun, *et al.*, 2011). In addition, the authors found that bryostatin-1 prevented the inhibition of BDNF levels associated with synaptic loss even before the deposition of Aβ plaques in the hippocampal neurons (Hongpaisan, M-K Sun, *et al.*, 2011). Moreover, in vitro studies showed that treatment with tert-butyl hydroperoxide (TBHP) induced increased reactive oxygen species (ROS) while decreased the levels of PKCε, MnSOD, BDNF as well as neuronal density (Sen *et al.*, 2018). These effects were recused by bryostatin-1 administration (Sen *et al.*, 2018). Studies showed that aging results in decreased proteosome activity (Khan and Nelson, 2018), which plays a central role in degrading and clearing Aβ plaques in the brain (Saez and Vilchez, 2014; Cao *et al.*, 2019). However, bryostatin-1 at sub-nanomolar to nanomolar concentrations (0.3-30 nM) enhanced proteasomal activity in the ubiquitin-proteasome pathway in PKCε dependent manner (Khan and Nelson, 2018). Taken together, bryostatin-1 improved neuronal survival and reduced oxidative stress in the hippocampal neurons indicating attenuation of neuronal dysfunction as seen in AD.

PKC-mediated signaling pathways play significant roles in AD pathophysiology. PKC activation mediates both excitatory and inhibitory synaptogenesis, which is vital for the neuronal plasticity involved in memory and learning (Sun and Alkon, 2005; Hongpaisan *et al.*, 2013; Nelson *et al.*, 2017). Bryostatin-1 administration (10 μg/kg; i.p.) to young adult rats increased the density of mushroom spines, a PKC dependent long-term associative memory storage unit, on dendrites (Hongpaisan and Alkon, 2007). In addition, bryostatin-1 treatment increased double-synapse presynaptic boutons that synapse with mushroom spines, and pre-synaptic vesicles indicating enhanced memory-induced synaptogenesis (Hongpaisan and Alkon, 2007). Bryostatin-1 induced upregulation of mushroom spine synapses and BDNF release in apical dendrites of CA1 pyramidal neurons, which reversed age-dependent cognitive decline (Hongpaisan *et al.*, 2013). Furthermore, bryostatin-1 improved learning and memory by significantly increasing the frequency and amplitude of GABAergic inhibitory postsynaptic currents as well as increasing the firing rate of GABAergic interneurons, both of which indicate enhanced neurotransmission in hippocampal neurons (Xu *et al.*, 2014).

Acute oral administration of bryostatin-1 (5 μg/mouse in vehicle oil control) to transgenic AD mice for less than two weeks significantly improved learning and memory performance while reducing plaque deposition in the cortex/hippocampus when compared to i.p administration (Schrott *et al.*, 2015). Recently, a nanoparticle-encapsulated bryostatin-1 formulation showed an increased efficacy when compared to unmodified bryostatin-1 (Schrott *et al.*, 2021). The study showed that AD transgenic mice (6.5 to 8 months of age) treated with nanoparticle encapsulated bryostatin-1 formulation (1, 2.5, or 5 μg/mouse) showed improvements in spatial memory on Morris water maze

indicating the effectiveness of the novel nanoparticle-encapsulated formulations of the drug in treating cognitive deficits associated with AD (Schrott *et al.*, 2021).

The first clinical study of bryostatin-1 in AD patients was conducted by Nelson et al., 2017. Bryostatin-1, administrated at a dose of 25µg/m^2; i.v in a double blind Phase IIa trial, showed improvement in Mini-Mental State Examination (MMSE) score between treatment and placebo groups (Nelson *et al.*, 2017). The data also showed that bryostatin-1 was well tolerated in AD patients (Nelson *et al.*, 2017). Following the favorable pharmacokinetic profile of bryostatin-1 from Phase IIa trials and promising results from compassionate use trials, a double-blind, randomized, placebo-controlled Phase II trial to investigate the safety, tolerability, and efficacy of bryostatin-1 in advanced AD patients was conducted (Farlow *et al.*, 2019). Although there was no significant difference in the prime outcome between the placebo and treated groups, the study provided a dose limit for future clinical trials as <40µg (which corresponds approximately to < 25µg/m2 doses). In addition, the authors suggested that memantine, a conventional medication used to treat moderate-to-severe Alzheimer's disease, blocked bryostatin-1 induced potential therapeutic effects (i.e., decrease in Severe Impairment Battery (SIB) scores in AD patients). Future trials should focus on testing the efficacy of bryostatin-1 at 20µg in the treatment of moderate to severe AD in the absence of memantine thus preventing any compounding factors (Farlow *et al.*, 2019).

BRYOSTATIN-1 IN TBI

The incidence of TBI in the US is estimated to be 1.7 million incidents per year and occurs mainly among adolescents (15-19 years) and older populations (>65 years) (Alan Georges; James G. Booker, 2019). However, it imposes significant problem in older

patients with increased emergency department visits, hospitalizations, and mortality (Thompson *et al.*, 2006; Gardner *et al.*, 2018; Peters and Gardner, 2018). Most TBIs are classified as mild and are mainly caused by closed brain injuries such as a concussion resulting from a car or sports accident or a fall (Alan Georges; James G. Booker, 2019). In case of older individuals, falls are considered to the primary mechanism of TBI affecting more women than men (Peters and Gardner, 2018). Age-related increased prevalence of co-morbidities, pre-existing health conditions, consumption of certain drugs such as warfarin along with intracranial changes such as dura adherence to skull, cerebrovascular atherosclerosis and bridging vein fragility are some factors that associated with increased disease severity and higher rate of mortality as seen in older adults (Thompson *et al.*, 2006). The pathophysiology of TBI is complex resulting from primary impact and secondary injuries, both of which can exacerbate cerebral damage. The underlying mechanism(s) involve activation of various biochemical and molecular pathways resulting in neural depolarization, ionic disturbances, glutamate excitotoxicity, mitochondrial dysfunction, increased oxidative stress, inflammatory response and neuronal death (Kinoshita, 2016; Galgano *et al.*, 2017). Disruption of the BBB may worsen the injury by increasing cerebral edema and intracranial pressure and reducing cerebral perfusion pressure (Kinoshita, 2016; Galgano *et al.*, 2017). Additionally, aging can negatively impact the outcomes following TBI (Thompson *et al.*, 2006). Without proper interventions, these consequences can lead to permanent brain injury and a higher predisposition to worsened outcomes following a repeated head injury (Dewan *et al.*, 2018).

Experimental studies demonstrate that shockwave-induced TBI disrupts the BBB and increases deposition of Aβ as a consequence of secondary damage (Itoh *et al.*, 2009;

Abdul-Muneer *et al.*, 2013; Hue *et al.*, 2014). Administration of bryostatin-1 (2.5 mg/kg; i.p) to young adult male Sprague-Dawley rats 5 mins after shockwave-induced TBI significantly reduced BBB permeability by upregulating expression of the tight junction proteins, zonula occludens-1 (ZO-1), occludin, and VE-cadherin (Lucke-Wold, Logsdon, *et al.*, 2015). In addition, bryostatin-1 was able to decrease toxic PKCα protein levels while increasing PKCε expression levels in isolated cerebral microvessels, thereby maintaining the integrity of BBB after injury (Lucke-Wold, Logsdon, *et al.*, 2015). Furthermore, extended treatment of bryostatin-1 starting at 8h following mild TBI (mTBI) over 2 weeks protected the brain from synaptic loss and cognitive decline in male C57b6/J mice (Zohar *et al.*, 2011). This rescue was possibly mediated through bryostatin-1 binding to the regulatory domain of PKC (Kortmansky and Schwartz, 2003) to increase ADAM10 (putative α-secretase) levels while decreasing BACE-1 (β-secretase) levels. Altering the expression of both ADAM10 and BACE-1 would ultimately lower the rate of Aβ deposition leading to reduced plaque formation and possibly enhanced memory and cognition (Zohar *et al.*, 2011). This study also indicated that repeated application of bryostatin-1 rescued the TBI-induced reduction in pre and post synaptic neural connections in the CA1 region of the hippocampus (Zohar *et al.*, 2011). Taken together, bryostatin-1 is a potential treatment for both short and long term TBI sequelae. However, these protective effects are not observed when bryostatin-1 is administrated at sub-acute time points such as 14 days post injury (Zohar *et al.*, 2011). Identifying the appropriate therapeutic window of bryostatin-1 for maximum neuroprotective effect following brain injury is warranted. Future studies should investigate the effect of co-treatment of bryostatin-1 and current treatment strategies for TBI.

BRYOSTATIN-1 IN AIS

AIS is a disease of the elderly. In the US, it is the fifth leading cause of death and major cause of adult disability (Yang *et al.*, 2017). About 75% of the strokes occur in patients above 65 years (Yousufuddin and Young, 2019). As the number of aged population (>65 years) is expected to rise, there is increased prevalence of hypertension, diabetes, obesity, and cardiovascular diseases coupled with lifestyle changes that significantly contributes to the growing incidence of stroke in aging population (Kowalska et al., 2017). It is estimated that the incidence of stroke will double over the next 40 years (2010–2050) affecting the elderly (aged ≥75 years) and minority groups (Benjamin *et al.*, 2018). Elderly patients (>85years) are associated with longer hospitalization and thereby increase the burden on healthcare system (Benjamin *et al.*, 2018). Especially in this age group (>85 years), women are prone to increased stroke mortality and disability than men due to increased life span for women (Rexrode *et al.*, 2022). So far, reperfusion with tissue plasminogen activator (tPA) has been the gold standard to treat patients suffering from ischemic stroke but the treatment has a narrow therapeutic widow requiring administration of tPA within 3-4.5 h after onset of stroke (Peña *et al.*, 2017). Moreover, not all AIS patients are eligible for tPA treatment due to tPA associated intracerebral bleeding and risk of hemorrhagic transformation (HT) (Peña *et al.*, 2017). These shortcomings emphasize the need for developing novel pharmacological agents that act as adjuvants with the existing therapy to prolong the therapeutic window and efficacy of tPA by reducing its adverse effects.

Studies indicate that co-administration of bryostatin-1 with tPA exhibited neuroprotective effects following ischemic stroke (Tan *et al.*, 2013, 2015). Administration of tPA (5 mg/kg; i.v.) adjuvated with bryostatin-1 (2.5 ug/kg; i.v.) every third day over a three-week course demonstrated enhanced survival rates and reduced brain swelling in aged female Sprague-Dawley rats with experimentally induced ischemic stroke (Tan *et al.*, 2013). Furthermore, bryostatin-1 treatment not only extended the time window for tPA administration to 6 h post stroke, but also reduced mortality, hemispheric swelling and tPA induced-HT (Tan *et al.*, 2015). Bryostatin-1 at nanomolar concentrations prevented degradation of the active, membrane-bound PKC enzymes (Sun *et al.*, 2008). Tan and coworkers demonstrated that bryostatin-1 through PKC activation prevented BBB disruption via downregulation of matrix metalloproteinases-9 (MMP-9) (Tan *et al.*, 2015). In addition, the authors showed that bryostatin-1 differentially regulated the expression of PKC isozymes predominantly present in the brain following ischemic stroke (Tan *et al.*, 2015). Bryostatin-1 initially caused upregulation of PKCα and PKCε; however, at 24 h post-stroke the expression of PKCα was downregulated while PKCε expression levels remained elevated (Tan *et al.*, 2013).

Post-ischemic chronic administration of bryostatin-1 in rats rescued ischemia induced spatial learning and memory impairment but not sensorimotor ability in a PKC dependent fashion (Sun *et al.*, 2008). In addition, impaired rats that were administered bryostatin-1 following AIS displayed retrieved learned spatial experiences i.e. pretrained water maze (Sun *et al.*, 2009). Moreover, bryostatin-1 treatment was able to induce neurotrophic factors (such as BDNF) and synaptogenesis while preventing neuronal loss, loss of dendritic spines and presynaptic vesicles in pyramidal neurons of rat hippocampus

(Sun *et al.*, 2008, 2009). In combination with voluntary exercise, bryostatin-1 was shown to improve functional recovery after cerebral infarct in male Sprague-Dawley rats (Mizutani *et al.*, 2015, 2016). Bryostatin-1 in adjuvant with exercise increased phosphorylation of α-amino3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR) subunit GluR1 at the serine-831 residue in the perilesional cortex, which is essential for neuronal plasticity through long-term potentiation (Mizutani *et al.*, 2015). In addition, the treatment of bryostatin-1 and exercise showed increased levels of serotonin (5-HT), an important neurotransmitter involved in synaptic plasticity (Lesch and Waider, 2012), and decreased 5-HT turnover in the perilesional cortex indicating a positive correlation between serotonin dynamics and motor performance (Mizutani *et al.*, 2016).

BRYOSTATIN-1 IN MS

MS is a progressive, autoimmune disorder that leads to progressive degeneration of myelinated neurons in the CNS (Oh *et al.*, 2018). Initially, MS was considered a disease of the young adults with a typical disease onset between 20-40 years (Shirani *et al.*, 2015). However, data from recent reports showed a significant increase in the mean age of onset, with a notably higher incidence observed in patients over 60 (Solaro *et al.*, 2015; Koch-Henriksen *et al.*, 2018). This is associated with severe disability (Confavreux and Vukusic, 2006) as well as higher risk of losing all income from earnings thereby exacerbating the socioeconomic status of MS patients (Wandall-Holm *et al.*, 2022). MS is characterized by neuroinflammation followed by deposition of CNS plaques that are comprised of debris from immune cells, demyelinated axons, axonal injury and glial scar tissue that ultimately results in neuronal dysfunction (Ghasemi *et al.*, 2017). The initial

relapse-remitting phase of MS is mediated by the migration of autoreactive T-cells, specifically Th1 cells, across the BBB into the CNS leading to demyelination followed by progressive secondary axonal death (Losy, 2013). Age-related mechanisms such as immunosenescence may accelerate this transition from acute multifocal recurrent inflammation to diffuse chronic neurodegeneration (Confavreux and Vukusic, 2006; Musella *et al.*, 2018; Vaughn *et al.*, 2019; Ostolaza Ibáñez *et al.*, 2022). The existing treatment options for MS do not focus on preventing disease progression related disability as seen in the case of elderly (Vaughn *et al.*, 2019) resulting in increased proportion of older patients not receiving treatment for MS (Wandall-Holm *et al.*, 2022). This imposes a significant challenge on the elderly.

Bryostatin-1 has been shown to be a promising lead drug for MS based on its immune-potentiating properties (Ariza *et al.*, 2011; Safaeinejad *et al.*, 2018). *In vitro* studies indicated that bryostatin-1 was capable of facilitating the switch from Th1 to Th2 mediated immune responses by acting like a toll-like receptor 4 (TLR-4) ligand and stimulating the release of anti-inflammatory cytokines (Ariza *et al.*, 2011). Emerging evidence showed that bryostatin-1 (30 µg/kg) administered 3 d per week had beneficial therapeutic effects on MS complications in a mouse model of experimental autoimmune encephalomyelitis (EAE) (Kornberg *et al.*, 2018). This study showed that upon bryostatin-1 treatment with concurrent EAE induction in mice, there was a diminished peripheral immune response and decreased lymphocyte infiltration into the brain with significant reductions in total CD4+ and Th1 lymphocyte populations (Kornberg *et al.*, 2018). Although dynamic changes in BBB permeability were not measured in these studies, it stands to reason based of the effects of bryostatin-1 on BBB integrity in other neurological

injury models (Tan *et al.*, 2013, 2015; Lucke-Wold, Logsdon, *et al.*, 2015) that the beneficial effects of bryostatin-1 noted in these studies were due, at least in part, by reducing the degree of BBB compromise following induction of EAE in mice.

In addition, bryostatin-1 administration was shown to modulate antigen presentation of dendritic cells (DCs) through upregulation of CD86 but not CD40, another costimulatory protein expressed on antigen presenting cells (APCs) that is needed for T cell activation (Kornberg *et al.*, 2018). Another possible mode for the pharmacological action could be demonstrated by the ability of bryostatin-1, at low doses, being able to upregulate IL-10, an anti-inflammatory cytokine and arginase-1, a classic M2a macrophage marker, both of which are indicative of wound healing and tissue repair in the CNS (Kornberg *et al.*, 2018). In addition to macrophages and DCs, future studies should focus on the immunologic actions of bryostatin-1 on microglia, the prominent immune cells of the central nervous system (CNS). Understanding the detailed molecular mechanism underlying the immunologic actions of bryostatin-1 in multiple sclerosis model is warranted.

BRYOSTATIN-1 IN NEUROAIDS

HIV infects various cells of the body causing acquired immunodeficiency syndrome (AIDS). It is well known that HIV-1 crosses the BBB leading to HIV-associated neurocognitive disorders (HAND) such as HIV-associated dementia, neuroAIDS and opportunistic CNS infections (Eisfeld *et al.*, 2013). Owing to the effectiveness of antiretroviral therapies, the number of aged individuals (45 years and above) living with HIV infection has increased to 50% in the high income countries like US (Collaboration,

2008; Cohen *et al.*, 2015). However, response to antiretroviral treatment is different between young and aged cohorts with aged patients showing reduced immune recovery possibly due to age-related immunosenescence (Mpondo, 2016). Older patients are at higher risk of mortality due to HIV (Jiang *et al.*, 2013). Additionally, the prevalence of HIV-associated neurocognitive disorders may be accelerated by the person's age (especially above 50 years) (Mackiewicz *et al.*, 2019). Recent reports have demonstrated the association of apolipoprotein E (ApoE) in the pathogenesis of HAND along with increased secretion of the beta-amyloid peptide and presence of amyloid plaques in the brains of HIV positive patients (Geffin and McCarthy, 2018; Fulop *et al.*, 2019; Mackiewicz *et al.*, 2019). These studies further strengthen the hypothesis that aged individuals bearing HIV infection are increasingly susceptible to developing AD and other AD-related dementias (Fulop *et al.*, 2019; Mackiewicz *et al.*, 2019). Given the importance of influence of age on HIV disease progression (Jiang *et al.*, 2013), it would be important to identify therapeutic agents targeting novel mechanisms underlying the pathogenesis of age-related HIV neurodegeneration (Mackiewicz *et al.*, 2019).

Although, combination antiretroviral therapy (cART) can efficiently control the virus to almost undetectable systemic levels, cART has been unable to completely eradicate HIV from the body due to viral sequestration and rebound stores, such as occurs in CNS reservoirs (Eisfeld *et al.*, 2013; Dahabieh *et al.*, 2015). CNS-derived HIV viral strains have unique features when compared to those found in peripheral compartments that differentially modulate viral replication, latency and resistance to drugs, such as latency reversal agents (LRA) (L. R. Gray *et al.*, 2016; Lachlan R. Gray *et al.*, 2016). This difference in response poses a significant limitation for the body's ability to eradicate brain

reservoirs of HIV. Thus, an urgent need exists to develop strategies to purge CNS viral reservoirs.

In the CNS, microglia and astrocytes are major sites for HIV replication and sequestration (Marban *et al.*, 2016). Studies indicate that administration of bryostatin-1 reduces viral load by improving the induction of latent HIV to enhance effectiveness of cART (Marsden *et al.*, 2017, 2018). Bryostatin-1 functioned in synergy with several LRAs, including histone deacetylase inhibitors, bromodomain inhibitors (BETi) and polyanionic carbosilane dendrimers, to fAIS (Perez *et al.*, 2010; Higashida *et al.*, 2011; Darcis *et al.*, 2015; Albert *et al.*, 2017; Heffern *et al.*, 2019; Relaño-Rodríguez *et al.*, 2019; Peng *et al.*, 2020). When co-administered, bryostatin-1 and JQ1 (BETi) exhibited a highly efficient, synergistic activity for HIV reactivation in latently infected microglial cells *in vitro* (Darcis *et al.*, 2015). The mechanism for this synergistic activity of bryostatin-1 was found to be mediated through increased PKCα and PKCδ activities, which is indicative of the need for higher doses (Díaz *et al.*, 2015). From a therapeutic perspective, this finding is troubling as higher doses of bryostatin-1 have been shown to lead to astrogliosis, disturbances in astrocytic glutamate uptake/release balance, chronic neuroinflammation and increased neuronal excitotoxicity (Proust *et al.*, 2017). Furthermore, higher doses of bryostatin-1 have been shown to increase BBB permeability by upregulating the expression of pro-inflammatory cytokines (Dental *et al.*, 2017). A prior study found this pro-inflammatory activity of bryostatin-1 could be attenuated by co-administration of JQ1 but no other LRAs (SAHA and BIX-01294) (Proust *et al.*, 2017). Therefore, it is important to carefully evaluate the choice of LRA in combination with bryostatin-1 to mitigate the potential risk of neurotoxicity associated with bryostatin-1. Future studies should also

focus on developing multifunctional nano therapies comprising antiretroviral drugs (ARV) and LRAs to overcome problems associated with HIV latency in the CNS and aid in targeted drug adherence (Jayant *et al.*, 2018).

DISCUSSION

To ensure maximum pharmacological effect, it is important to understand the pharmacokinetics of bryostatin-1 as well as its exposure time and concentration-dependent effects on various PKC isozymes. Up until early 2000s, there was not an established method to reliably quantify the levels of bryostatin-1 in human plasma which ultimately hindered the progress of bryostatin-1 research in cancer treatment (Blackhall *et al.*, 2001). Over the decades, several quantitative methods have been developed to measure bryostatin-1 levels in with high sensitivity. In 2005, Zhao et al., developed a novel assay using triple quadrupole mass spectrometer to measure bryostatin-1 in human plasma sample with a detection limit of 50pg/ml. In this study, bryostatin-1 was administered over 2 weeks as continuous intravenous infusion at a single dose level of 20 ug/m^2 and the half-life of the drug was found to be 9 h (Zhao *et al.*, 2005). Recently, Nelson and colleagues demonstrated that bryostatin-1 detection limit can be improved by adduct formation by adding sodium acetate (Nelson *et al.*, 2014). They developed an ultrasensitive mass spectrometric method detecting bryostatin-1 sodium adducts at low pmol/l concentrations and enabled measurement in brain and other tissues without the use of radioactive labels (Nelson *et al.*, 2014). It is known that bryostatin-1 is relatively stable in vivo, widely distributed but predominately present in lung, liver, gastrointestinal tract, and fatty tissue (Sun and Alkon, 2006). It has also been shown to cross the blood

brain barrier in mice following iv administration despite being a large molecule (MW = 905.03 g/mol) (Nelson *et al.*, 2014). The study showed that brain uptake is saturated at doses of 10μg/m^2 and the maximum brain concentration (C_{max}) of bryostatin-1 was 0.20nM (Nelson *et al.*, 2014). The plasma disappearance curve of bryostatin-1 is fitted into one compartment model for i.p. administration and into a two-compartment pharmacokinetic model for i.v. administration (Sun and Alkon, 2006; Nelson *et al.*, 2014, 2017). A single dose (25 μg/m^2) of bryostatin-1 showed maximum levels of bryostatin-1 and its target PKCε within 1h after the onset of infusion (Nelson *et al.*, 2017) with rapid elimination rate (22.97h and 28.76h for i.v. and i.p. administration respectively) (Sun and Alkon, 2006). In addition, pharmacokinetic profiles demonstrated increased plasma drug concentration upon repeated and continuous infusions of bryostatin-1 (Zhao *et al.*, 2005), indicating accumulation of the drug possibly due to decreased elimination rate constant (0.027 min^{-1}) while increased elimination half-life (32-200h) (Nelson *et al.*, 2017). This prolonged exposure of bryostatin-1 is due to enterohepatic circulation as the drug is present in higher concentrations in the liver and gastrointestinal tract and cleared initially (within 12h) through urine and later (within 72h) through urine and feces (Sun and Alkon, 2006).

One main reason for the failure of bryostatin-1 as a chemotherapeutic agent is because PKC isoenzymes are involved in both oncogene and tumor suppressor gene activation (Garg *et al.*, 2013) and the variable expression of PKC isozymes in different types of tumor (Isakov, 2018) affects the efficacy of bryostatin-1. Administration of bryostatin-1 may only be effective when treating tumors harboring a specific PKC isoenzyme profile (Blackhall *et al.*, 2001). Despite the differences in pathology, various

age-related CNS disorders share some degree of similarity in PKC dependent mechanisms (Sun and Alkon, 2006; Lucke-Wold, Turner, *et al.*, 2015). Preclinical studies showed that bryostatin-1 crosses the BBB and specifically activates brain PKCε (Nelson *et al.*, 2014). PKCε is a promising biomarker of AD (Khan *et al.*, 2015). Research showed that PKCε levels in skin fibroblasts could serve as potential evaluation tools for the detection of early AD by reflecting changes in brain PKCε levels (Khan *et al.*, 2015). Investigating the use of other PKC isozymes as peripheral biomarkers for the diagnosis and prognostication of neurological diseases is warranted.

The exact mechanism of action of bryostatin-1 is unclear. Bryostatin-1 has been shown to induce biphasic effects on PKC activity (Nelson *et al.*, 2017; Ly *et al.*, 2020). At low concentration (<30µg/m2), bryostatin-1 leads to PKC translocation from the cytosol to the membrane, which is a measure of its activation. This is followed by a brief period of downregulation characterized by ubiquitination of PKC and degradation by proteasomal machinery (Sun and Alkon, 2006). A previous study showed that low doses of Bryostatin-1 (0.1-0.25 ng/ml) with short exposure times, resulted in PKCε induced prolonged protein synthesis necessary for long-term memory in a mollusc Hermissenda model (Alkon *et al.*, 2005). The authors showed that this prolonged protein synthesis lasting for at least one week is due to bryostatin-1 induced PKCε activation, followed initially by down-regulation and then de novo synthesis of PKC (Alkon *et al.*, 2005). Thus, PKC modulators can produce long-term changes even after complete elimination of the drug from the system (Nelson *et al.*, 2017). More recently, mammalian unc13 isoform 1 (Munc13-1), a C1 domain-containing protein which shares activators with novel and conventional PKC isoforms, has high binding affinity (Ki of 0.45 ± 0.04 nM) for bryostatin-

1and is identified as a molecular target in vitro (Blanco *et al.*, 2019). Thus future studies should investigate other neuronal signaling targets of bryostatin-1 such as munc 13, chimaerins and RasGRPs along with that of PKCs (Blanco *et al.*, 2019).

Majority of the oncology clinical trials administered IV infusions of bryostatin-1 at high doses (>30ug/m2) or over long periods of time (Nelson *et al.*, 2017). Although, bryostatin-1 showed promising anti-neoplastic activity in rodent models of cancer, it failed to progress beyond Phase I/II clinical trials (Kollár *et al.*, 2014). Increased drug concentration and/or prolonged exposure resulted in the halt of the clinical trials due to adverse effects, most notably myalgia and cancer progression (Kollár *et al.*, 2014; Farlow *et al.*, 2019). Some of the toxicities associated with bryostatin-1 administration include but not limited to phlebitis, lymphopenia, infection without neutropenia, hypophosphatemia, seizure, hypotension, diarrhea, and dehydration (Blackhall *et al.*, 2001; Kollár *et al.*, 2014). The exposure of bryostatin-1 is critical in designing its mechanism of action in neurological disorders (Table 2.1). At higher doses of bryostatin-1, the brain drug concentration is saturated (Nelson *et al.*, 2014). Sub-optimal concentrations are well tolerated by the body (Nelson *et al.*, 2017; Farlow *et al.*, 2019). Phase II clinical trial in AD patients demonstrated the dose limit of bryostatin-1 to be 40µg (25 µg/m^2) (Farlow *et al.*, 2019). Recently Cogram et al showed that chronic treatment (13 weeks) of bryostatin-1 (20 µg/m^2, i.v., 2 doses/week) resulted in significant therapeutic effects along with no evidence of adverse effects in a mouse model of fragile X syndrome (Cogram *et al.*, 2020).

Owing to its short half-life and rapid elimination rate from plasma after single bolus dose (Lucke-Wold, Turner, *et al.*, 2015), low dose continuous infusions over extended

period is necessary for improved efficacy (Nelson *et al.*, 2017; Farlow *et al.*, 2019). However, continuous IV infusions can be painful and lead to phlebitis and adverse reactions at the site of infusions (Blackhall *et al.*, 2001; Kollár *et al.*, 2014; Farlow *et al.*, 2019). This could be overcome by acute oral administration of bryostatin-1 (Schrott *et al.*, 2015). The authors showed that gavaged bryostatin-1 is not metabolized by liver enzymes as evidenced by in vitro absorption, distribution, metabolism and excretion (ADME) studies using human hepatocyte cells, and produced superior efficacy and significant functional outcomes compared to the intraperitoneal administration in a rodent model of AD (Schrott *et al.*, 2015). Pharmacokinetic analysis revealed that <5% of bryostatin-1 is taken up in the circulation when administered orally suggesting that lower concentrations of bryostatin-1 are sufficient to induce a therapeutic effect in vivo (Schrott *et al.*, 2015). Understanding the minimum blood/brain drug concentration required to produce neuroprotection is warranted. Furthermore, the authors developed a hydrophilic nanoparticle formulation of bryostatin-1 that increased its bioavailability and demonstrated enhanced PKC-ε and PKC- δ activity along with improved cognitive scores in a rodent AD model (Schrott *et al.*, 2020). Future studies should focus on developing newer formulations as well as optimizing the route of administration and the dosing strategy to maximize the therapeutic benefits of bryostatin-1 to treat neurological disorders.

CONCLUSION

Accumulating evidence from cellular and animal models demonstrates that the neuroprotective effects of bryostatin-1 is exerted by pharmacological modulation of various PKC isozymes (Sun and Alkon, 2006). Since PKC signaling plays a crucial role in several CNS related diseases (Battaini, 2001), bryostatin-1 is indeed an attractive candidate in the treatment of several neurological injuries/diseases including but not limited to AD, TBI, ischemic stroke, MS and neuroAIDS. Bryostatin-1 is currently being investigated in several Phase I and II clinical trials, assessing the safety, tolerability, and long-term efficacy profiles in the treatment of AD and as a LRA in HIV infected patients (Gutiérrez *et al.*, 2016; Nelson *et al.*, 2017; Farlow *et al.*, 2019) (Table 2.2). Recently, bryostatin-1 received organ drug status by the FDA as a possible treatment for Fragile X syndrome (Cogram *et al.*, 2020; Raghuvanshi and Bharate, 2020). So far it has demonstrated favorable safety profile at low doses in humans (Sun and Alkon, 2006) and has the potential to advance to Phase III trials as CNS therapeutic agent, alone or in combination with other neuroprotective agents.

FUTURE STUDIES

Despite being an important lead candidate, the advancement of bryostatin-1 has been hindered by an inability to scale up extraction to meet clinical demand. While a scalable synthesis of bryostatin-1 has been reported, a more promising approach has been the development of novel analogs, known as bryologs (Wender and Baryza, 2005; Wender *et al.*, 2005, 2006; Wender and Reuber, 2011). The bryologs have allowed for customization based on type of therapeutic intervention sought and to limit off target effects while retaining the same pharmacophoric functionalities of naturally occurring

bryostatins (Staveness *et al.*, 2016; Marsden *et al.*, 2018). In a nutshell, bryostatin-1 and its analogs hold significant promise for treating CNS diseases and also provide hope for enhancing targeted therapy.

Table 2.1: **Bryostatin-1 doses tested in rodent model of neurological diseases/disorders**

Disease model	Dose regimen	Route of administration	Mechanism of action	Treatment outcome	Reference
AD mouse model	30 µg/kg 2 doses/week for 12 weeks	i.p.	Activation of PKC isoforms (α and ε)	Prevents loss of hippocampal synapses and the memory impairment; reduces Aβ plaques	(Hongpaisan, MK Sun, *et al.*, 2011; Sen *et al.*, 2018)
	40 µg/kg 3/week from 3 weeks-6 months	i.p.	Activation of α-secretase and PKC isoforms	Reduces Aβ plaques and mortality rates	(Etcheberrigaray *et al.*, 2004)
	25 µg/m^2 - weekly; 15 and 20 µg/m^2-2/weekly	i.v.	Activation of PKCε	Increases BDNF	(Nelson *et al.*, 2017)
	0.5, 1, 5 µg/mouse	Oral; i.p.	Activation of α-secretase and PKC isoforms	Improves learning deficits; reduces plaque burden dose dependently	(Schrott *et al.*, 2015)
	1, 2.5, 5 µg/mouse nanoparticle encapsulated formulation; 3/week before testing and then daily for 5 days	oral	Activation of α-secretase and PKC isoforms	Improves cognitive deficits	(Schrott *et al.*, 2021)
TBI rodent model	30 µg/kg injection followed by 5× injections over a period of 14 days	i.p	ADAM10 activation and BACE1 deactivation	Protects against the cognitive and synaptic pathologies	(Zohar *et al.*, 2011)
	2.5 µg/kg	i.p.	Increased PKCε and decreased PKCα	Decreases BBB breakdown	(Lucke-Wold, Logsdon, *et al.*, 2015)
	20 µg/kg repeated dosing	i.p.	Activation of PKCε	Improves cellular as well as motor and cognitive behavior outcomes	(Giarratana *et al.*, 2020)
Cerebral ischemia rat model	2.5 µg/kg at 6h +rtPA at 2h post stroke; doses every 3 days for a total of 7 doses over 21 days	i.v. (tail vein)	Increased PKCε	Increases survival, reduces infarct volume, decreases hemispheric swelling/atrophyand improves neurological function	(Tan *et al.*, 2013)
	2.5 µg/kg at 2h +rtPa at 6h post stroke	i.p.	Increased PKCε and decreased MMP-9	Ameliorates BBB disruption and reduces the risk of HT	(Tan *et al.*, 2015)
	15 µg/m^2 2 doses/week for 10 doses	i.v. (tail vein)	PKC dependent	Antiapoptosis, synaptogenesis, and spinogenesis	(Sun *et al.*, 2008)
	15 µg/m^2 2 doses/week for 10 doses	i.v. (tail vein)	PKC-mediated	Enhances neurotrophic activity, induces synaptogenesis, and preserves spatial learning and memory	(Sun *et al.*, 2009)

	20 µg/m^2 Acute study: two doses Chronic study: 2 doses/week for 10 doses	i.v. (tail vein)	Possibly through increase in BDNF	Reverses the increased sensitivity and depressive immobility induced by cerebral ischemia	(Sun and Alkon, 2013)
	15, 10, 7.5, and 5 µg/m^2 + exercise	i.v. (tail vein)	Possibly through increase in p-GluR1	Improves exercise induced functional recovery at 15 and 10 µg/m^2	(Mizutani *et al.*, 2015)
	10 µg/m^2 At 5days after infarct +exercise	i.v. (tail vein)	Increased 5-HT and decreased 5-HT turnover	Improves ischemia induced motor dysfunction	(Mizutani *et al.*, 2016)
Mouse model of EAE	30 µg/kg dosed at 3 d/week	i.p.	Possibly through the modulation of TLR-4	Promotes anti-inflammatory phenotype on innate myeloid cells and reverses neurologic deficits	(Kornberg *et al.*, 2018)

Table 2.2: Bryostatin-1 doses tested in CNS clinical trials

Disease model	Doses tested	PKC activation	Peak time	Peak conc. in plasma	Outcomes	Reference
HIV_ phase I	10 and 20 µg/m^2 single doses, infusion over one hour	failed to activate PKC	0.5-h after infusion and gradually decayed in the first 8 h	under 1 nmol/l	Safe and well tolerated	(Gutiérrez *et al.*, 2016) (NCT02269605)
AD_ double-blind Phase IIa	25 µg/m^2 single doses, infusion over one hour	increased PBMC PKCε levels at 1 h followed by long-term downregulation 12-72h	Within 1-2h after infusion	1ng/ml	Safe and well tolerated; increases MMSE score at 3 h after the end of infusion	(Nelson *et al.*, 2017) (NCT02221947)
AD_ Randomized, Double-Blind, Placebo-Controlled, Phase II	20 µg and 40 µg, infusion over a period of 12 weeks	-	-	-	40ug- adverse effects with no efficacy and increased dropouts. 20ug- same adverse effects as that of placebo; SIB scores showed benefits from baseline at week 15	(Farlow *et al.*, 2019) (NCT02431468)

CHAPTER THREE

Characterization of age-related changes on cerebrovascular topology in Sprague Dawley rats

ABSTRACT

Aging-related anatomical and physiological changes have been implicated in the pathogenesis of many neurological diseases, including Alzheimer's and Parkinson's diseases and ischemic stroke. Altered function of reliable homeostatic pathways for glucose control, energy metabolism, cellular replication and repair have been found to play important roles in the pathogenesis of many of these age-related diseases. Another age-related finding has been the marked decrease in vessel density, which pose to be the underlying factor for reduced cerebral blood flow, impaired bioenergetics and altered cellular response found in aged rodents when compared to younger rodents. What remains unknown is, at what level of vessels are these changes occurring and how does this change in density relates to adequate perfusion of the brain in aged rodents. In the present study, we seek to gain a better understanding of the age-related anatomical changes in the surface vessels of the brain. Using young (3-4 months old) and aged (18-20 months old) female Sprague-Dawley rats, we employed a vessel painting technique to specifically label cerebral endothelium so that we could visualize these vessels using fluorescent microscopy. From the images obtained we used a vessel counting software to perform a series of measurements to detail the absolute number and complexity of the vessel architecture. Using this method, we found that aged animals showed significant loss in vascular components along with 50% increased lacunarity. In contrast, young animals exhibited increased cortical branching, capillary number, and complexity. Therefore, a detailed characterization of vascular architecture reiterates the importance of age in selecting animal models of ischemic stroke, which may subsequently improve therapeutic success rate.

INTRODUCTION

The human brain is particularly susceptible to the effects of aging. Independent of other risk factors, such as hypertension, atherosclerosis, and diabetes, a person's advancing age is the predominant risk factor for many debilitating diseases, including Alzheimer's and Parkinson's diseases, other dementias, ischemic stroke and cancers (Kowalska *et al.*, 2017; Bland, 2018; Franceschi *et al.*, 2018; Hou *et al.*, 2019). Billions of dollars have been spent in pursuit of treatments to improve the overall quality of life for persons suffering from these diseases; yet, age-related diseases are among the most difficult to treat with few effective treatments available (Kirkland, 2016).

Case in point is ischemic stroke, which over the past three decades has seen numerous promising preclinical neuroprotectant drugs fail to translate into a single clinically viable therapeutic approach (Fluri *et al.*, 2015; Shi *et al.*, 2018; Xiong *et al.*, 2018; Lee *et al.*, 2021). Many reasons have been posited for this disconnect, including differences in complexity of human brain versus lower species, appropriate pharmacokinetic characterization of drug penetrance and delivery into the brain, lack of clinically relevant time courses for drug administration, lack of polypharmacy approaches, flawed construction of clinical trial exclusion/inclusion criteria and inherent inconsistencies within animal models of cerebral ischemia (i.e. anesthetic used, occlusion material, unnatural reperfusion profile, acute / chronic assessments of functional recovery, disconnect between infarct size and functional recovery (Lapchak *et al.*, 2013; Shi *et al.*, 2018; Xiong *et al.*, 2018; R Ma *et al.*, 2020; Lee *et al.*, 2021). While these issues are valid considerations and provide excellent means to improve the rigor of research being conducted, none of these approaches broaches the physiological consequences of

aging as being the single most predictive risk factor for prevalence and severity of ischemic stroke. A glance through the preclinical stroke literature demonstrably shows the majority of preclinical stroke studies used rodents under the age of 6 months, with most being 3 to 4 months old and some being as young as only a few weeks old (Kahle and Bix, 2012; Fluri *et al.*, 2015; Jackson *et al.*, 2017; Narayan *et al.*, 2021).

In this study, we investigated anatomical and physiological differences between young and aged female Sprague-Dawley rats in surface topology and complexity of cerebral vessels with a focus on the right and left middle cerebral arteries. We hypothesized that cerebral blood vessel length, diameter, branching, and network complexity differ substantially between old and young rats. Preliminary results of the computations performed on these models indicate that vasculature in older brains is less complex, includes less branching, and contains shorter vessels with greater diameter compared to vascular networks in the brains of young rats. The higher average vessel diameter in older brains is likely a compensatory mechanism for the inadequate perfusion of these smaller, less branched vessels. Given the importance of vascular structure to ischemic stroke outcomes, these structural differences suggest that age is an important factor when selecting animal models of ischemic stroke (Jackson *et al.*, 2017).

MATERIALS and METHODS

Animal Care and Use Statement. All procedures involving animals were approved by the West Virginia University Animal Care and Use Committee and abided by ARRIVE guidelines. 20 female Sprague-Dawley rats (3-4 and 20-22 months old; Hilltop Animal Laboratories; Scottdale, PA) were acquired and housed in the vivarium at the West

Virginia University (WVU) Health Sciences Center animal facility with food and water accessible ad libitum. Rats were acclimated upon arrival for at least 7 d prior to testing. All procedures were performed by investigators blinded to the age of the rats. All procedures involving animals were approved by the WVU Animal Care and Use Committee.

Serial Vessel Perfusion. Rats were euthanized by cardiac perfusion of 150 ml of warmed 1X phosphate buffered saline (37°C; pH 7.4) with heparin (0.02 mg/g body weight; Sigma-Aldrich; St. Louis, MO) and sodium nitroprusside (0.075 mg/g body weight; Sigma-Aldrich) for 15 min under deep anesthesia using 4% inhaled isoflurane (Patterson). Rats were then perfused with 50 ml of 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI; Life Technologies; Carlsbad, CA) followed by 200 ml of 4% paraformaldehyde (PFA; Sigma-Aldrich). Care was taken to ensure no air bubbles were trapped within the perfusion system, during each perfusion and switching between perfusates. A peristaltic pump was used to deliver perfusates at a rate of 5.2 ml/min into the left ventricle of the heart using a 25G needle. After perfusion with 4% PFA, rats were decapitated and brains carefully excised and post-fixed in 4% PFA overnight.

Image Acquisition & Vessel Measurements. Vessel painted brains were imaged using a widefield fluorescence microscope Olympus MVX 10. Whole brains were imaged under 1x objective and dorsal, ventral, and lateral surface images were captured to document labeling efficiency. The captured images were then analyzed using ImageJ software using

quantitative tools to obtain measurement of the physical parameters of the cerebral vessels.

Vessel Measurement. Basic morphometrics were performed using ImageJ (NIH, Bethesda, MD) software. In brief, microscopic images were preprocessed in ImageJ tool and then analyzed using the open-source software AngioTool® (National Institute of Health, National Cancer Institute, Bethesda, MD, USA). AngioTool® is a user-friendly software AngioTool® used to measure several morphological and spatial parameters. Using this tool, we calculated the total brain area, total area covered by the vessels, vessel density, number of vessels, vessel length, number of junctions, junction density, and number of endpoints. We also calculated the fractal dimensions and lacunarity, which are used to analyze structural complexity and heterogeneity of patterns, for each image using FracLac plugin for ImageJ. Branch order information was calculated using the same software. Middle cerebral artery was considered the primary branch and branches stemming of the primary were noted as secondary and so on. Additionally, we performed diameter analysis using Python with python script packages Tiffile and Scipy from the open-source Anaconda Distribution.

Statistical Analysis. All measurements and analyses were performed without knowledge of group. Data were reported as mean ± standard deviation (SD). For classical angiographic features measured from surface cortical vessels, unpaired t -test was used to determine statistical significance between aged versus young animals. All statistical analyses were performed using Graph Pad Prism software.

RESULTS

Global reduction of surface cortical vessels in aged brain. In order to visualize the cerebral vascular network, we employed vessel painting technique using 1,1' dioctadecyl-3,3,3',3' tetramethylindocarbocyanine perchlorate (DiI), dye to successfully label the endothelial cells of the surface cortical vessels in rats (Hughes *et al.*, 2015). We imaged the whole brains under Olympus MVX 10 widefield microscopy showing dorsal, ventral, and lateral aspects of the brains (Figure 3.1). Using this technique, 66% of young and 78% of aged brains were successfully perfused as determined by uniform pink staining with absence of any white/blanched patches. Upon visual inspection, we noticed densely packed microvascular organization in young cortex while the microvasculature was sparse and disorganized in the aged cortex (Figure 3.1). In addition, there was substantial reduction in surface cortical vessel as well as anastomosis in the aged brain (Figure 3.1B) as opposed to young brain (Figure 3.1A).

Age-related changes on the vascular architecture of the surface cortical vessels. It is well known that aging has a profound effect on the cerebral vasculature. We first wanted to characterize age-related changes on the basic morphological and spatial parameters of the surface cortical vessel on the axial plane. Classical vessel measurement parameters using AngioTool identified a marked difference in cortical vessel topology between young and aged female rats. Figure 3.2 demonstrates that aged rats displayed significant reductions in (i) total brain surface area ($p=0.045$; versus in young rats), (ii) vessel surface area ($p=0.001$; versus in young rats) and (iii) vessel density ($p=0.0001$;

versus in young rats). Comparison of vessels (Figure 3.2) between young and aged showed that aged rats displayed vessels with significant reductions in (iv) junctions (p=0.0003), (v) junction density (p=0.0004) and (vi) number of endpoints (p=0.003). Furthermore, evaluation of vessel measurements (Figure 3.2) found a significant difference between aged and young cerebral blood vessels in (vii) total vessel length (p=0.008) and (viii) lacunarity (p=0.0007) but no difference in (ix) average vessel length (p=0.1563).

Age-related changes on the vascular architecture of the Middle Cerebral Artery (MCA). The middle cerebral artery is the major artery supplying oxygenated blood and nutrients to frontal, parietal, temporal areas of the brain (Gao *et al.*, 2022). MCA is clinically significant as occlusion of the artery results in cerebral ischemia leading to infarct and necrosis of brain parenchyma (Gao *et al.*, 2022). Age is a non-modifiable risk factor for cerebral ischemia (Yousufuddin and Young, 2019) and preclinical studies have demonstrated severe stroke outcomes and reduced functional recovery following experimental stroke in aged rodents as opposed to young rodents (Rosen *et al.*, 2005; DiNapoli *et al.*, 2008; Manwani *et al.*, 2011). Therefore, we wanted to perform morphometric analysis on the left and right MCA in young and aged rats using the AngioTool. Figure 3.3 demonstrates significant decrease in (i) total brain surface area (p=0.025; p=0.0003) (ii) vessel surface area (p<0.0001; p<0.0001) and (iii) vessel density (p<0.0001; p<0.0001) in both right and left MCA of aged rats when compared to young rats. Analysis of both (right and left) MCA vessel metrics between young and aged displayed significant reductions in (iv) junctions (p=0.0005; p=0.0025), (v) junction density (p=0.0076; p=0.013) but not (vi) number of endpoints (p=0.3044; p=0.2596).

Furthermore, evaluation of vascular parameters (Figure 3.3) of the right and left MCA found a significant difference between aged and young cerebral blood vessels in (vii) total vessel length (p=0.0006; p=0.0001) and (viii) lacunarity (p=0.0494; p=0.0048) but no difference in (e) average vessel length (p=0.6194; p=0.4071).

Age-related decrease in vascular complexity. Next, we wanted to determine if the age-related reduction in vessel length could be compensated by changes in vessel diameter. The output skeleton image generated from AngioTool (Figure 3.3B) was fed into Python to create a histogram of surface cortical vessel diameters of aged and young animals (Figure 3.4A). We observed a significant reduction in the number of small diameter vessel in the aged animals versus young animals. Aged animals displayed about 50% reduction in vessels with diameters less than 40 μm and 80% reduction in vessels with diameters less than 10 μm. However, no differences were observed for large diameter surface cortical vessels (>90 μm) between young and aged animals (Figure 3.4A). Additionally, we wanted to determine vascular complexity in aged and young animals by analysis the branches arising from the MCA. Figure 3.4B demonstrated age-dependent decline in the number of higher order branches stemming from the MCA indicating decreased collaterals and anastomoses due to aging.

DISCUSSION

With greater understanding of the implications of cellular senescence on physiological function and the identification of critical time periods of changes in cellular signaling occurring at all levels, from gene expression to post-translational modification,

there is mounting evidence that (1) aged brains handle stress and trauma vastly different than younger brains, (2) this difference in response is not linear, and (3) the degree and expectation of recovery from brain injury is different in the aged versus the young. Taking these considerations into mind along with the lengthy list of caveats already associated with preclinical stroke models, it is difficult to ascertain the long-term value of using younger animals to explore the mechanisms of ischemic stroke progression and recovery.

In the present study, we performed morphometrics analysis of the cerebral vasculature of healthy young and aged female Sprague Dawley rats. With the aid of vessel painting technique, we found that aged rats have reduced surface cortical vessels. We demonstrated age related reduction in total brain area, classical vessel parameters and increased lacunarity in axial surface cortical vessels as well as the MCA. In addition, we demonstrated an overall decrease in small diameter vessels in aged brains compared to young brains. Finally, we observed an age dependent decline in higher branch orders sprouting from the main MCA indicating reduced vascular complexity in case of aged animals.

Several techniques have been developed to visualize the structure of brain vasculature. These include injection of various compounds such as resins, gelatin, latex, dyes, and fluorescently labelled substances into the circulation to stain the vessels and quantify morphological parameters like vessel density, length, and branch points (Lugo-Hernandez *et al.*, 2017). However, some of the drawbacks associated are use of expensive reagents, difficulty in perfusion, incomplete vascular filing, and vascular leakage of the dye into brain tissue (Konno *et al.*, 2017; Lugo-Hernandez *et al.*, 2017). Herein, we employed vessel painting technique with a lipophilic carbocyanine dye 1,1′-

dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI), that binds selectively to the lipid membranes via insertion of its alkyl chains and commonly used for tract tracing in the brain (Hughes *et al.*, 2015). Using this simple, and economical method, we were able to successfully label 66% of young and 78% of aged cerebral surface arteries. This method is a reliable way of visualizing the smallest vascular elements with high resolution using fluorescent microscopy (Hughes *et al.*, 2015).

The cerebral vasculature controls blood supply to the brain and is responsible for the maintenance of microenvironment for proper neuronal function (Bogorad *et al.*, 2019). With advancing age, the brain cerebrovascular architecture undergoes several changes. In the present study, we demonstrated global reduction in surface cortical vessels in the aged animals when compared to young animals which is accordance to previous reports (Jin, 2019). Indeed, age-related cerebral vessel loss has been reported across multiple species (Schager and Brown, 2020). Characterization of surface cortical vessel parameters showed significant age-dependent reduction in vessel area, vessel density, and total vessel length. This finding is consistent with previous reports demonstrating age associated decline in cerebral microvascular architecture (Sonntag *et al.*, 1997; Xu *et al.*, 2017; Li *et al.*, 2018; Bálint *et al.*, 2019; Lowerison *et al.*, 2022). This age-related vessel loss could be due to vessel pruning that is not counterbalanced by formation of vessels (Cudmore *et al.*, 2017), and/or due to reduced levels of proangiogenic factors leading to decreased angiogenesis (Sadoun and Reed, 2003). Additionally, aging is associated with global decreases in blood flow velocity, cerebral perfusion and global increases in vessel tortuosity (Xu *et al.*, 2017; Li *et al.*, 2018; Lowerison *et al.*, 2022). Taken together, it can be inferred that age-related decline in cerebral microvascular network can lead to

impairment in vascular supply due to reduced cerebral blood flow (CBF). Furthermore, brain micro vascular changes is associated with impaired neurovascular coupling (Lipecz *et al.*, 2019; Shaw *et al.*, 2021), a process which largely depends on the underlying vascular structure to match the energy demands of the activated neurons (Lecrux and Hamel, 2011; Iadecola, 2017; Tarantini *et al.*, 2017). Recent studies have shown link between neurovascular dysfunction and cerebral blood flow with worse cognitive performance (Tarantini *et al.*, 2017; Toth *et al.*, 2017; Rensma *et al.*, 2020; Bracko *et al.*, 2021). These findings strengthen the association between microvascular changes leading to compromised cerebral blood flow and age-related memory and cognitive impairment.

With increasing age, our brain undergoes structural and functional changes. At 65 years, it is estimated that majority of the brain regions decrease in area by 1% to 5% over a span of 7 years and this decline becomes steeper with advancing age (Sele *et al.*, 2021). Using a surface-based reconstruction approach, the average total surface area of a human was found to be 1,692 ± 117 cm^2 and the global reduction in total surface area was measured to be 3.68 cm^2/year (Lemaitre *et al.*, 2012). Accordingly, we showed significant reduction in total brain area of aged animals compared to young animals. Sele et al., 2021 demonstrated a strong co-relation between changes in anatomical structures with changes in cognitive scores and estimated sharp declines in cognitive performance with increasing age (Sele *et al.*, 2021). In addition, we showed age-dependent increase in lacunarity, which is a measure of gaps in patterns (Karperien *et al.*, 2011). This finding is consistent with previous reports and highlights the importance of such a parameter in predicting the diseases outcomes (disability accumulation) in chronic neurodegenerative

diseases such as Multiple Sclerosis (MS) and in the diagnosis of cognitive impairment (Karperien *et al.*, 2011; Arthur *et al.*, 2019; Roura *et al.*, 2021). Taken together, changes in surface cortical area and lacunarity can be used as a cortical biomarker of cognitive healthy aging.

The middle cerebral artery is the primary artery supplying blood to the vast majority of the brain and has significant clinical relevance (Gao *et al.*, 2022). The MCA and its cortical branches carry oxygenated blood and nutrients to areas such as primary motor and somatosensory cortex, insular, auditory cortex, basal ganglia, internal capsule, speech and language centers of the brains (Navarro-Orozco and Sánchez-Manso, 2021). It is the most common blood vessel that is occluded by an embolus or thrombus in case of ischemic stroke resulting in cerebral edema and neuronal apoptosis (Navarro-Orozco and Sánchez-Manso, 2021). Understanding the age-related remodeling of cerebral arteries and arterioles is important as it may aid in predicting the risk of cerebrovascular diseases (Diaz-Otero *et al.*, 2016). In the present study, we observed a significant age-related reduction in morphometric measures such as vessel area, vessel density, vessel length, number of junctions and junction density of both right and left MCA of the rat brain. This is because of reduced cerebral arterial anastomoses occurring between the branches (Sonntag *et al.*, 1997). Aged animals showed decreased ratio of arteriolar anastomoses to arterioles (1:6.6) compared to young animals (1:5) (Sonntag *et al.*, 1997). Additionally, we showed that aged brains have less complex vascular metrics as evidence by reduced number of distal branches while no changes in the proximal vessels sprouting from the main MCA. This is consistent with reports demonstrating more pronounced age-related vessel loss owing to reduced perfusion in distal brain regions like somatosensory,

motor and retrosplenial cortices in contrast to proximal brain regions like striatum and thalamus (Schager and Brown, 2020). As the complexity of the MCA vascular network decreases with aging, the role of individual vessels will be important in determining the degree of perfusion after occlusion as in the case of cerebral ischemia.

The brain microvascular network consists of communicating arteries that loop to form the Circle of Willis at the base of brain, giving rise to the anterior, middle, and posterior cerebral arteries (Schaffer *et al.*, 2006; Bogorad *et al.*, 2019; Navarro-Orozco and Sánchez-Manso, 2021). Pial or leptomeningeal collaterals are small diameter vessels on the cortical surface that connect distal branches of the anterior cerebral artery (ACA) and posterior cerebral artery (PCA) with distal branches of the middle cerebral artery (MCA) (Liebeskind, 2003). These collaterals are clinically significant as they determine the redistribution of blood flow to distal regions following an occlusion and define the degree of ischemic insult (Schaffer *et al.*, 2006). In the present study, we found about 50 % reduction in small diameter vessels (<40 μm) and about 80% decrease in vessels with diameter <10 μm on the axial surface of aged brain compared to young brain. With reduced tissue perfusion due to reduced small diameter vessels, regions supplied by the MCA in aged animals may have less resilience to interruptions or changes in blood flow leading to exacerbated ischemic damage than young animals (Faber *et al.*, 2011; J Ma *et al.*, 2020a). In addition, aged rats showed significant impairment in collateral dynamics leading to increased severity of collateral failure as opposed to young rats (J Ma *et al.*, 2020a). Therefore improving collateral circulation may aid as adjuvant therapy to recanalization therapy (Cuccione *et al.*, 2016; J Ma *et al.*, 2020b).

Some drawbacks associated with this study is that the dye DiI preferentially labels arteries, leaving out veins and venules (Hughes *et al.*, 2015; Konno *et al.*, 2017). In addition, the hydrophobic dye can form aggregates during perfusion causing occlusion of small diameter blood vessels resulting in some degree of heterogeneity in labelling and not suitable for microvessels staining (Konno *et al.*, 2017). Furthermore, DiI dissolves in alcohol making this technique not suitable to be combined with other immunohistochemical methods (Hughes *et al.*, 2015). However, this method was chosen as it provides a simple, cost-effective and efficient alternative to complex techniques such as corrosion casting for analysis of vascular parameters (Hughes *et al.*, 2015; Konno *et al.*, 2017). Another limitation of this study is the imaging depth was limited to surface cortical vessels allowing us to perform two-dimensional analysis of the vascular structures. To further investigate three-dimensional capillary network characteristics within the deeper brain regions at high resolution, histological analyses combined with light sheet microscopy is warranted. This study was conducted in female rats. The rationale for using female animals is women have increased life span compared to men and hence have increased risk of chronic cerebrovascular diseases (CVD) such as stroke and Alzheimer's (Alharbi *et al.*, 2020; Kumar and McCullough, 2021). In addition, the aging blood brain barrier (BBB) of females shows ultrastructural modifications that might contribute to altered capillary blood flow thereby contributing to vascular diseases (Frías-Anaya *et al.*, 2021). Sex hormones such as estrogens, progestins, and androgens have shown to regulate vascular functions by acting on the cerebral vasculature (Robison *et al.*, 2019). A recent study demonstrated sex differences on the intrinsic structure of rat MCA (Wang *et al.*, 2020) possibly altering neurovascular coupling responses and

cognition with aging. Therefore, understanding the structural characteristics of vessels in young and aged male animals and its causative role in CVD will be useful in developing therapeutic interventions for pre-clinical studies in the future.

CONCLUSION

In summary, we have systematically investigated age-related changes in surface cortical vessels of young and aged rat brain using a simple yet relatively robust vessel painting technique. We found global reduction in vascular parameters and significant decline in the number of branches from the main middle cerebral artery indicative of decreased cerebral blood flow to the aged brain consistent with previously published reports. Given the importance of vascular structure in ischemic stroke, the compromise in surface cortical vessel is suggested to contribute to worse functional outcomes following cerebral ischemia observed in aged animals. This study sheds light on understanding the biology of vascular aging and highlights the importance of using age-appropriate animals while studying specific diseases of aging. The selection of more appropriate models will likely increase the success rate of stroke intervention research that aims to translate preclinical findings.

FUTURE DIRECTIONS

Some unanswered questions in the field are 1) why these changes occur in the aged brain and what propels them? 2) why are there region-specific microvascular loss and what are the regional cues that could help predict heterogeneous vessel loss across brain regions? 3) what are the cellular and molecular mechanisms underlying the biology

of vascular aging? 4) how are the components of the neurovascular unit (NVU) altered due to aging?

A

B

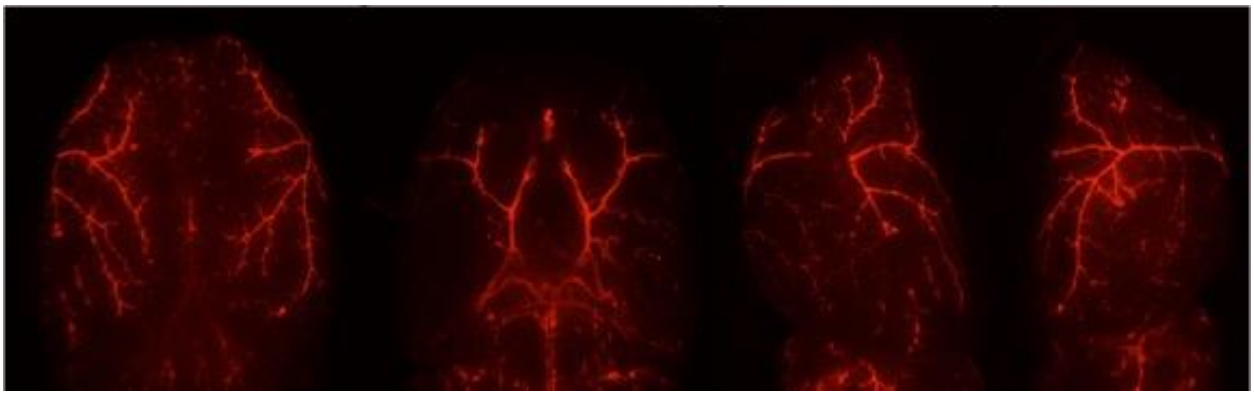

Figure 3.1: Cerebral vasculature of rat brain visualized by vessel painting. Representative images of cerebral vascular architecture showing dorsal, ventral, left and right side view of A) Young and B) Aged rat brain. (n=6-7 animals/group)

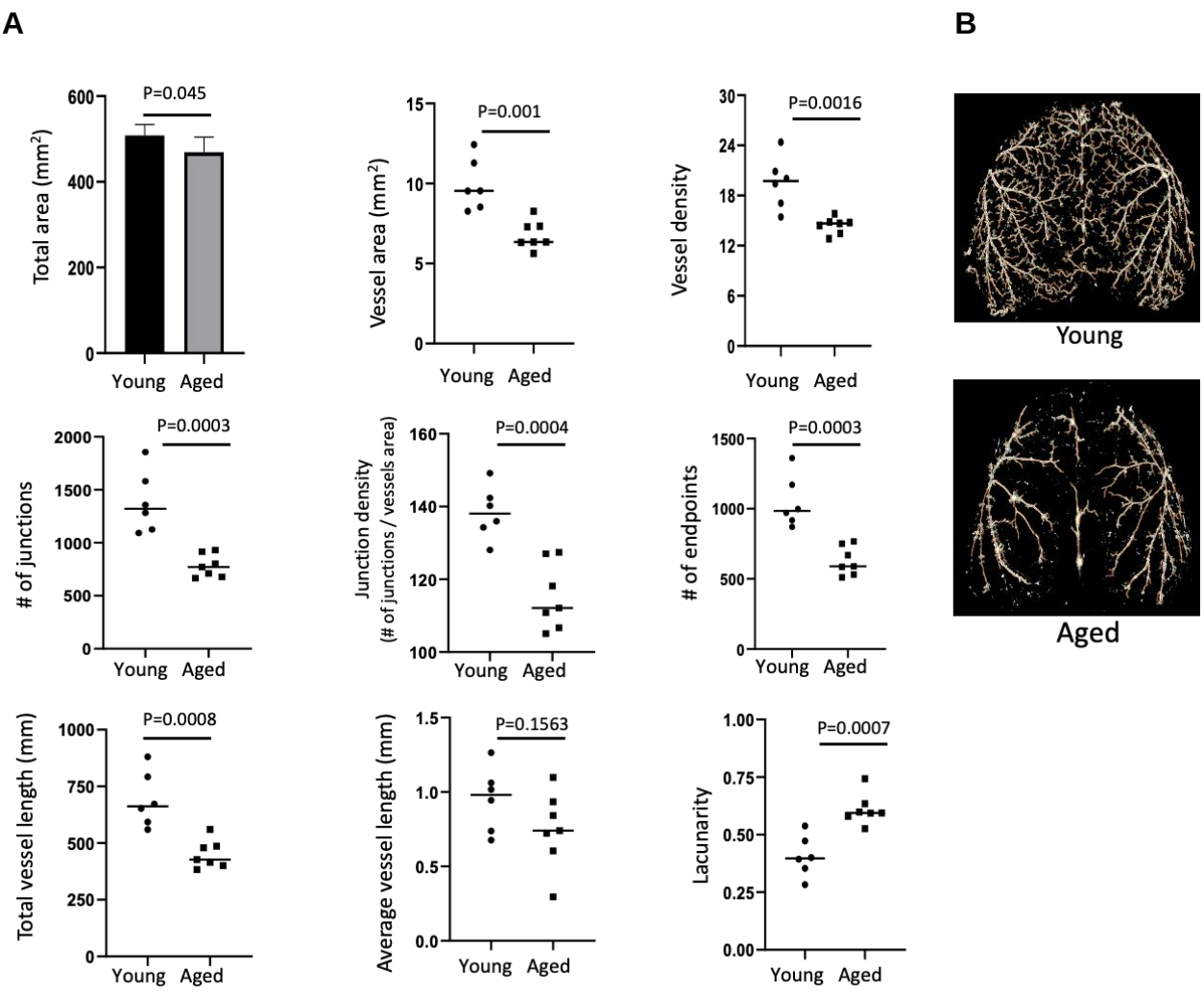

Figure 3.2: Surface Cortical Analysis of Total Brain. A) Basic morphometric and spatial parameters- total area, vessel area, vessel density, number of junctions, junction density, number of endpoints, total vessel length, average vessel length and lacunarity calculated for surface cortical vessel of aged and young brain in the axial plane. B) Representative skeleton image of young and aged brain after processing using the AngioTool. (n=6-7 animals/group). Data reported as mean ± S.D.

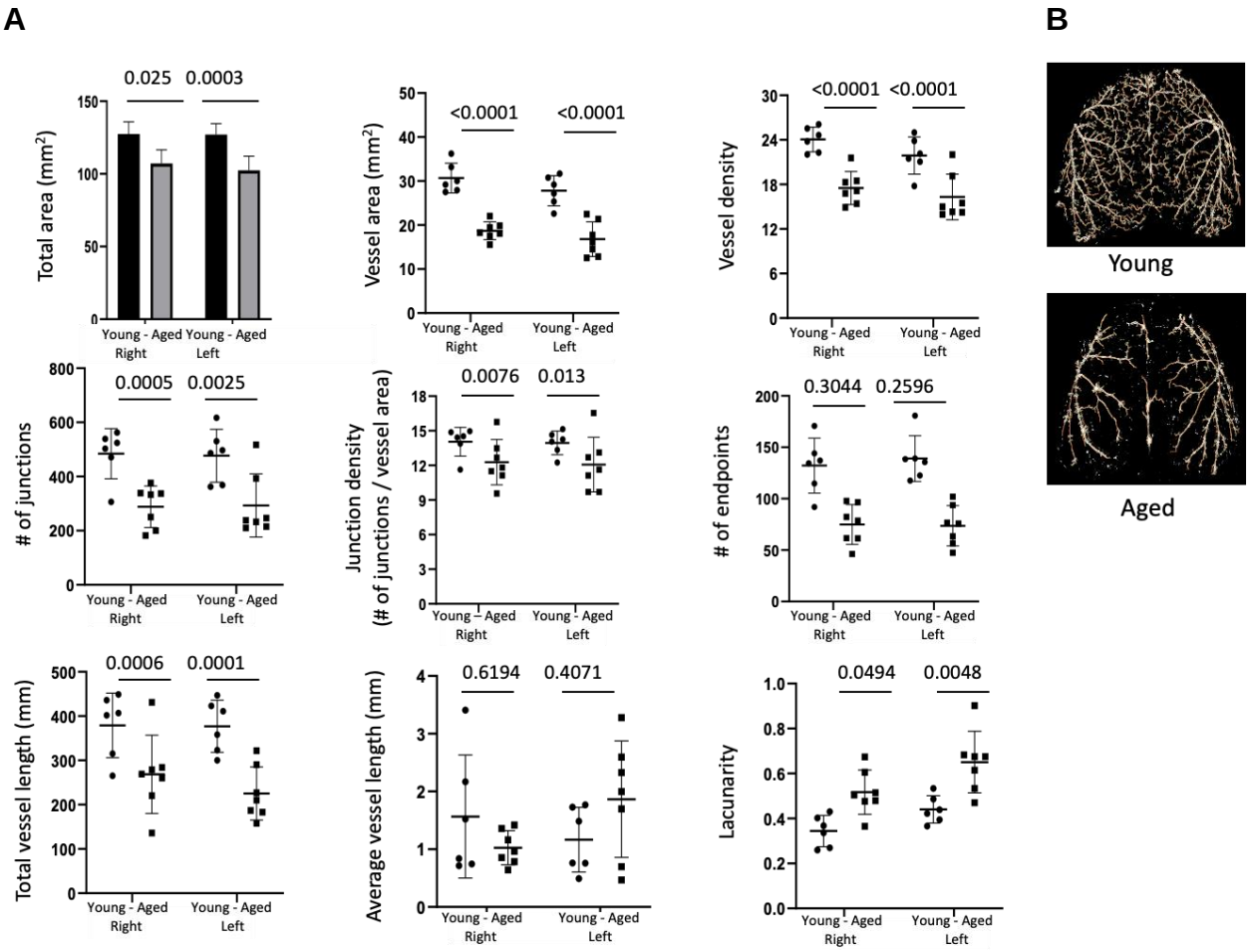

Figure 3.3: Surface Cortical Analysis of Middle Cerebral Artery (MCA). A) Basic morphometric and spatial parameters- total area, vessel area, vessel density, number of junctions, junction density, number of endpoints, total vessel length, average vessel length and lacunarity calculated for right and left MCA of aged and young animals. B) Representative skeleton image of young and aged brain after processing using the AngioTool. (n=6-7 animals/group). Data reported as mean ± S.D.

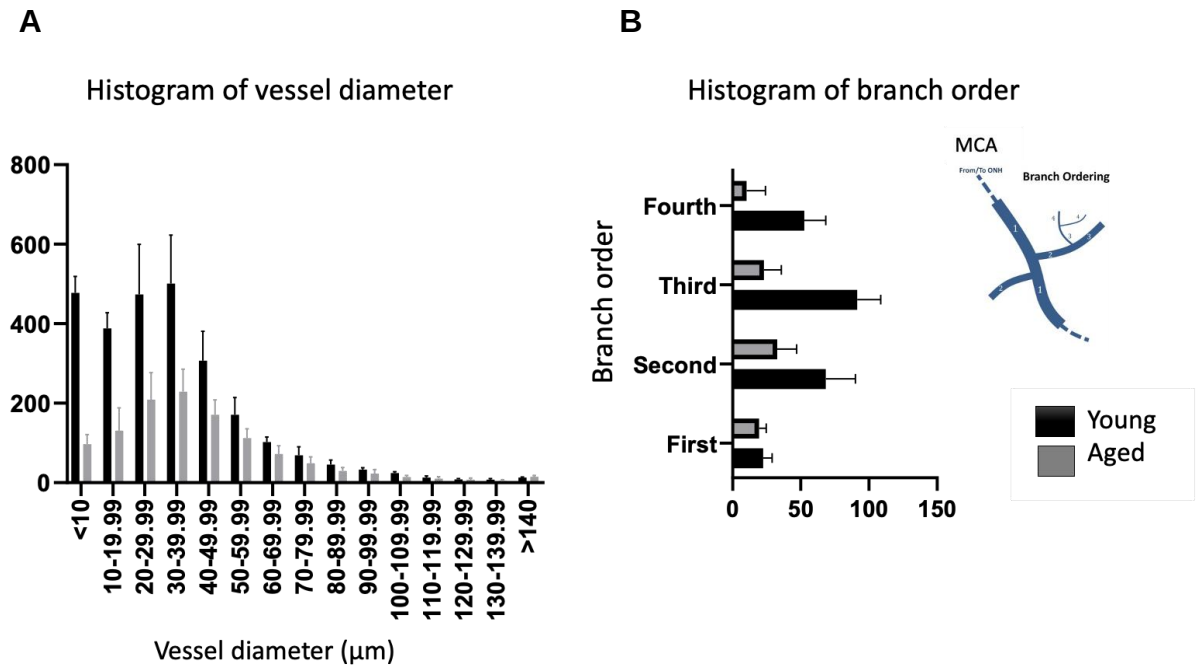

Figure 3.4: Vessel Diameter and Branch Order Analysis. A) Histogram of vessel diameter in aged and young brains B) Histogram of branch order information of aged and young MCA. (n=6-7 animals/group).

CHAPTER FOUR

Characterization of age-related changes on leukocyte populations following acute systemic inflammation in Sprague Dawley rats

ABSTRACT

As we age our immune system gradually degrades in function leading to immunosenescence, which is associated with increased incidence and severity of several chronic diseases, including cardiovascular, neurodegenerative diseases, cancer & diabetes. Given the clinical implications of age-related changes in the immune system, a critical gap in knowledge exists regarding how age changes immune response when stimulated. Previous studies have identified many hallmark signs of immune aging. However, what remains unknown are how aging changes the composition and quantitative response of leukocytes in response to injury/disease. In the present study, we investigated the impact age has on changes in major leukocyte populations in the blood and spleen of young (3-4 months old) and aged (18-20 months old) female Sprague-Dawley rats. We employed a robust flow-cytometric protocol to characterize leukocyte populations in rats after subjecting them to lipopolysaccharide (LPS) challenge. Rats were exposed to 0.9% saline or LPS (1 mg/mL) for 3 or 24 h after which, blood and spleen were collected and analyzed. Results demonstrated an overall decline in leukocyte numbers and increased numbers of B cells and natural killer (NK) cells in aged animals as compared to young animals. Interestingly, while neutrophils saw modest increases in expression in young LPS-treated rats at 24 h, a multi-fold increase in neutrophil counts were measured in aged LPS-treated rats at 24 h. This study affirms that age provokes several changes in the systemic immune response that can have dramatic effects on response to injury/disease and underscores the importance of using age-appropriate animals when exploring injury/disease.

INTRODUCTION

A prominent feature of biological aging in mammals is a gradual but progressive deterioration of cellular function over time that occurs across multiple organ systems (Pawelec, 2018). This process, known as cellular senescence, is an influencing factor in many age-related pathologies (Pawelec, 2018). Aging is a primary risk factor for several chronic diseases, including cancers, cardiovascular disease, diabetes, stroke, and neurodegenerative disorders (Niccoli and Partridge, 2012b; Haynes, 2020). Yet, when researching the pathophysiology and treatment effectiveness in these diseases, consideration of processes underlying normal aging are often ignored. This failure to account for age-related changes, often referred to as aging hallmarks, may be an underlying factor in the failure of so many treatments, especially in the neurological area, to translate from promising preclinical studies into clinical practice (Kirkland, 2016). Therefore, studies should evaluate age as a variable when studying animal models of specific diseases (Jackson *et al.*, 2017).

The relationship between biological aging and cellular senescence is complex as the interaction between these two processes can, at times, be difficult to differentiate (Fülöp *et al.*, 2016; Pawelec, 2018). For example, as an individual ages, time-dependent hallmark signs of aging, such as telomere erosion, metabolic slowing and increased genomic instability begin to emerge as driving forces in perpetuating cellular senescence, which, in turn, contributes to the development of other aging hallmarks, such as low-grade inflammation, stem cell exhaustion, mitochondrial dysfunction and altered intercellular communication (Fülöp *et al.*, 2016; Pawelec, 2018). The interplay between different aging hallmarks creates a vicious cycle resulting in aberrant responsiveness of cells to external

stimuli and ultimately tissue dysregulation (Fülöp *et al.*, 2016; Pawelec, 2018). A prime example of this can be seen in the relationship between low-grade inflammation, often referred to as inflamm-aging, and immunosenescence leading to progressive deterioration in the ability to respond to infection in the older population (Fülöp *et al.*, 2016; Pawelec, 2018; Bischof *et al.*, 2019). This age associated chronic systemic inflammation not only increases mortality but also contributes to the development of several neurological injuries and neurodegenerative diseases in the elderly (Ritzel *et al.*, 2018; Bischof *et al.*, 2019; Barbé-Tuana *et al.*, 2020; Chee and Solito, 2021; Finger *et al.*, 2021; Sykes *et al.*, 2021).

For the past two decades, our lab has studied the effects of age on ischemic stroke severity and recovery. Results of our studies, using comparative indicators of ischemic stroke injury (infarct volume, brain swelling, functional recovery, mortality), definitively show that aged rodents have worsened outcomes after ischemic brain injury than their younger counterparts (Rosen *et al.*, 2005; Lucke-Wold *et al.*, 2012). In addition, several studies have demonstrated how the age-related changes alter peripheral immune response after stroke and hence influence stroke risk and outcome (Shaafi *et al.*, 2014; Kim *et al.*, 2018; Ritzel *et al.*, 2018; Aref *et al.*, 2020; Sykes *et al.*, 2021).

The objective of this study was to determine the effect(s) of aging on the acute systemic immune response following lipopolysaccharide (LPS) challenge. We hypothesized that immunosenescence in aged rats would lead to greater difficulty in mounting an effective acute response following LPS administration. To assess response, we stimulated an immune challenge by administering a single dose of LPS (1 mg/kg; i.p.) in female Sprague-Dawley rats (2-3 and 18-20 months). Using flow cytometry and ELISA,

we measured changes in leukocyte populations in blood and spleen & inflammatory cytokine expression in plasma at 3 and 24 h following LPS administration.

MATERIALS & METHODS

Animals. Studies using rats were approved by the West Virginia University (WVU) Animal Care and Use Committee and abided by ARRIVE guidelines. Forty-two female Sprague-Dawley (SD) rats were used in this study (20 young; 22 aged). Aged female SD rats (20-22 months) were acquired from our aging colony (Hilltop Laboratories; Scottdale, PA) and young female SD rats (3-4 months) were purchased from Hilltop Laboratories. Upon arrival, rats were housed in the WVU animal facility with food and water supplied ad libitum.

Experimental design. Upon arrival, rats were randomly assigned to one of four treatment groups within age category: Group 1- administered 0.9% sterile saline intraperitoneally (i.p.) with tissue collection at 3 h after injection; Group 2- administered 0.9% sterile saline (i.p.) with tissue collection at 24 h after injection; Group 3- administered lipopolysaccharide (LPS; 1 mg/kg; i.p.; Sigma Chemicals; St. Louis, MO) with tissue collection at 3 h after injection; Group 4- administered LPS (1 mg/kg; i.p.) with tissue collection at 24 h after injection. Saline and LPS were filtered through a 22 mm syringe filter before use. At specified time point, rats were anesthetized with 4% isoflurane (Patterson) and blood collected (~5 ml) in citrated tubes from the left ventricle. After blood draw, rats were cardiac perfused with ice-cold 0.9% saline for 10 min, at which time the spleen was excised and weighed.

Tissue processing. After collection, 2 ml of citrated blood was separated for flow cytometry and the remaining blood centrifuged at 1500 x g for 10 min at 4°C. Plasma was collected and frozen at -20°C until assayed. Leukocytes from the blood were isolated using Lympholyte cell separation media for mammals (Cedarlane) according to manufacturer's instructions. Spleen was cut into small cubes, homogenized using a dounce homogenizer, and centrifuged at 1300g for 10 mins. Cells were strained using a 40μM nylon mesh into a 50 ml conical tube. Red blood cells were lysed using 1x RBC Lysis Buffer (Multi-species) from eBioscience™. Cell pellets were resuspended, and Trypan blue dye was used to quantify the live cells using an automated cell counter (Countess from Invitrogen) (as shown in Figure 4.1).

Flow cytometry. Cell lysates from blood and spleen were assessed using a 9-color staining panel (Barnett-Vanes *et al.*, 2016) with antibodies as detailed in Table 4.1. First, 5 x 10^5 cells were washed and stained with Live/Dead dye (eBioscience™; 1:1000) in 1X phosphate buffered saline (PBS; pH=7.4). Next, cells were blocked with anti-CD32 to mitigate Fc-mediated non-specific binding for 30min at 4C. Cells were then incubated in antibody cocktail in 1X PBS containing 1% bovine serum albumin and 0.1% sodium azide for 1h at 4C. At end of incubation, cells were washed and fixed in 0.4% paraformaldehyde. Flow cytometric normalization was performed using fluorescent compensation beads (AccuCount Blank Particles, Spherotech) and cells were analyzed on a multicolor flow cytometer (LSRFortessa™, Becton-Dickinson). The fluorescence minus one principle

was used to account for background antibody fluorescence in our positive and negative gating of cell populations.

Multiplex Cytokine ELISA. Circulating cytokines (interleukin (IL)-1 beta (1b), IL-4, IL-5, IL-6, IL-10, IL-13, interferon-gamma (IFN-ɣ), keratinocyte chemoattractant/growth regulated oncogene (KC/GRO) & tumor necrosis factor alpha (TNF-α) were measured in plasma samples collected at termination according to manufacturer's instructions (Pro-inflammatory Panel 2 V-Plex kit (K15059D-1); MSD; Rockville, MD). Cytokine assays were read using a MESO QuickPlex SQ 120 and data were analyzed using the MSD Discovery Workbench software version 4.0. Lower limits of detection for cytokines (pg/mL) were IL-4 (0.16), IL-5 (6.89), IL-6 (7.18), IL-10 (6.18), IL-13 (0.45), TNF-α (1.04), IFN-ɣ (1.48). Due to several samples in the LPS-treated groups reaching upper limits of detection for IL-6 & KC/GRO, these samples were repeated using a 1:10 dilution.

Statistical analysis. For leukocytes, raw counts were normalized using compensation beads (10,000 counts for blood; 7,500 counts for spleen) and expressed as mean ± SD. For comparative evaluation of T lymphocytes, a percent of total cell-type population was calculated by taking a ratio of CD4+, CD8+, CD4-/CD8- & CD4+/CD8+ counts to compensated total T lymphocytes. For cytokine levels in plasma using multi-plex ELISA, cytokine concentration in experimental groups was normalized to cytokine concentration in plasma of naïve young and aged rats & expressed as mean ± SD. Since variance in all treatment groups were normally distributed, a three-way analysis of variance (ANOVA) was used to determine statistical significance on main effects of treatment, time point &

age followed by Tukey's HSD post-hoc analysis to identify inter-group differences. Using an *a priori* power analysis to calculate sample sizes, an n of 5 rats per treatment group was deemed sufficient to calculate statistical significance with a $P<0.05$. All statistical analyses were performed using Graph Pad Prism software.

RESULTS

LPS sickness behavior and mortality. Regardless of age, rats exposed to LPS (1 mg/kg; i.p.) exhibited signs of sickness behavior, including anhedonia, irritability and lethargy that continued out to 24 h. At 24 h, other signs of sickness behavior were observed, including loss of appetite, piloerection, and increased aggression. No young rats experienced mortality during the 24 h of LPS exposure; however, aged rats experienced 2 deaths out of the 12 aged rats exposed to LPS used in the study. Both rats were in the LPS 24 h group. This 29% mortality rate in the LPS 24 h group was significantly higher than observed in the aged PBS 24 h group and the combined 17% mortality rate in the aged LPS group (3 & 24 h) was significantly higher than shown in the young LPS group.

Gating strategy and characterization of leukocyte profiles using FACS. To obtain a population of live single leukocytes (Figure 4.2), we employed a gating strategy in blood and spleen that removed debris & not "live" cells (live/dead dye), gated to singlets using FSC-H & FSC-W parameters to filter out doublets & clumps of cells and finally gated for $CD45^+$ (pan-leukocyte) cells, resulting in a population of live, single leukocytes for sequential separation. T lymphocytes were gated as $CD3^+$, natural killer (NK) cells were

gated as $CD161^{+}$, B cells as $CD45R^{+}$; neutrophils as $CD43^{+}$ and higher granularity and finally monocytes as differential expression of CD43 ($CD43^{Hi}$ and $CD43^{Lo}$).

Age-related changes in spleen and body weight. Next, we wanted to investigate the age-related changes in body & spleen weights and splenocyte count after LPS challenge in rats (as shown in Table 4.2). We observed increase in spleen weight upon LPS administration irrespective of the age. Spleen weight was increased in aged LPS 3h compared to young LPS 3h group. We also noticed that the body weight increased upon aging. We then normalized spleen weights to body weights and noticed that spleen/body weight ratio was increased upon LPS treatment. Overall, there was a decrease in the number of splenocytes due to aging. In addition, aged animals showed an increase in spleen count upon LPS treatment at 3h. The number of splenocytes plummeted at 24h post LPS treatment irrespective of age. Further, the number of splenocytes was normalized to spleen weight and we noticed LPS induced decrease in the total spleen count by spleen ratio in the young animals while an increase in the total spleen count by spleen ratio in aged LPS 3h.

Quantification of leukocyte populations in blood. Using flow cytometry, we isolated & quantified leukocyte populations from whole blood in young and aged female rats at 3 and 24 h after LPS challenge. Regardless of the age, LPS challenge significantly reduced total leukocytes ($p<0.01$), T cells ($p<0.0001$ for young; $p<0.05$ for aged), B cells ($p<0.05$ for young; $p<0.0001$ for aged) in the blood at 3 and 24 h when compared to age-matched PBS treated rats (as shown in Figure 4.3). As Figure 4.3A indicates there was no difference in total white blood cell ($CD45^{+}$) counts measured between PBS-treated rats

at 3 and 24 h in young (p=0.9998) or aged (p=0.2361). Likewise, comparison of young PBS-treated rats to aged PBS-treated rats showed no difference (p=0.9995) in circulating total leukocytes. In aged rats, a significant (p=0.01) increase in total leukocytes was observed at 24 h after LPS challenge in the aged rats when compared to 24 h in young rats. Regardless of age, no difference (p>0.05) in total leukocytes was measured within LPS-treated rats between the 3 and 24 h time points. As Figure 4.3B illustrates there was a no difference (p>0.05) in circulating T lymphocytes ($CD3^+$) between PBS-treated rats at 3 and 24 h in young or aged. Similarly, no difference (p>0.05) in T lymphocyte counts were demonstrated between LPS-treated rats at 3 and 24 h after LPS challenge in young or aged rats. A significant (p<0.05) age effect was shown in PBS-treated rats with a marked reduction in circulating T lymphocytes observed in aged rats at 3 and 24 h when compared to young rats. Conversely, Figure 4.3C indicates increased B lymphocytes in aged PBS treated rats when compared to young PBS treated rats (p=0.0056). As Figure 4.3D shows NK cells were significantly increased (p<0.05) at 24h compared to 3h LPS treatment in the aged animals. Additionally, there was a significant difference (p=0.0021 for PBS; p=0.0040 for LPS) in the main effect of age with aged rats having increased circulating NK cells regardless of treatment (Figure 4.3D). A similar trend was observed for monocytes which showed a significant increase (p<0.0001 for PBS; p=0.0078 for LPS) in number due to aging, irrespective of the treatment (Figure 4.3F). In addition, Figure 4.3F shows that monocytes population was significantly decreased (p<0.01) at 3h compared to 24h LPS treatment in the aged animals. It is also interesting to note that the neutrophil count was significantly increased (p<0.0001) in aged LPS treated rats at 24 h (Figure 4.3E).

Quantification of leukocyte populations in spleen. The spleen leukocyte populations were isolated & quantified in young and aged female rats at 3 and 24 h after LPS challenge using flow cytometry. Overall, aged rats exhibited reduced leukocyte counts at both 3 and 24 h in the spleen when compared to young rats irrespective of the treatment (Figure 4.4). Aged animals showed a significant decrease in white blood cells ($p<0.0001$), T lymphocytes ($p<0.0001$), B lymphocytes ($p<0.0001$), NK cells ($p<0.05$ for LPS and PBS) and monocytes ($p<0.0001$ for PBS; $p=0.013$ for LPS) when compared to young animals (Figures 4.4A, B, C, D & F). Additionally, monocyte population was significantly decreased ($p<0.001$) at 24h compared to 3h LPS treatment in aged animals (Figure 4.4F). Interestingly, neutrophil counts in the spleen were significantly increased ($p<0.01$) in young LPS treated rats at 24 h while the numbers were significantly decreased ($p<0.0001$) in aged LPS treated rats at 24 h (Figure 4.4E).

Measurement of inflammatory cytokines in plasma. Using MSD multiplex rat ELISA kit, we measured pro-inflammatory cytokine expression in the plasma of aged and young rats at 3 h and 24 h after LPS challenge, according to manufacturer's instructions. Regardless of age, LPS challenge significantly increased IL-4 levels ($p<0.0001$ in case of young; $p<0.01$ in case of aged) in the plasma at 3 and 24 h compared to PBS treatment (Figure 4.5A). In case of young animals, this effect was prominent at 24h ($p<0.0001$ versus 3h) upon LPS stimulation (Figure 4.5A). However, aged animals had significantly reduced ($p<0.0001$) IL-4 expression than young animals at 24h after LPS administration as seen in Figure 4.5A. Conversely, aged animals showed significant increase ($p<0.05$)

in IL-5 expression at 24h post LPS stimulation compared to young rats (Figure 4.5B). Additionally, this increase was significantly higher at 24h ($p<0.01$) compared to 3h post LPS treatment in the aged animals. Regardless of age, IL-5 expression in plasma is significantly increased ($p<0.01$ for young; $p<0.0001$ for aged) upon LPS challenge versus PBS treatment at 24h (Figure 4.5B). Similarly, IL-10 & IL-1β expression was significantly increased upon LPS stimulation irrespective of age at 3h and 24h (Figure 4.5C & F). This increase was significantly higher ($p<0.05$) at 24h compared to 3h in LPS treated aged animals in case of IL-10 expression as seen in Figure 4.5C. Figure 4.5 C & F shows no age-related change in expression of IL-10 ($p=0.8651$) & IL-1β ($p=0.3675$) while Figure 4.5 D & E shows no significant changes ($p>0.05$) in the expression of IFNγ & IL-13 across the groups. There was significant difference in the main effect of time point with increased ($P<0.0001$) IL-6, CXCL-1 & TNFα expression at 3h while decreased ($p<0.0001$) expression at 24h upon LPS challenge irrespective of age (Figure 4.5G, H &I). However, at 24h post LPS treatment IL-6 and CXCL-1 levels were significantly increased in aged ($p<0.05$) compared to young animals (Figure 4.5 G & H). Apart from treatment & time point related effect, TNFα also showed age related effect with significantly decreased ($p<0.01$) levels in aged LPS treated rats as opposed to young LPS treated rats at 3 h (Figure 4.5 I).

DISCUSSION

The immune system is a dynamic network that is tightly regulated to ensure effective protection against foreign antigens. With increasing age, it undergoes a remodeling process characterized by a decline in immune cell function due to cell intrinsic

and extrinsic factors (termed as immunosenescence) accompanied by chronic low-grade inflammation (termed as inflamm-aging). This dysregulation of the immune system seen in elderly increases their susceptibility of developing chronic, infectious diseases and decreases the effectiveness of vaccines (Ciabattini *et al.*, 2018). With rising life expectancy of the global population, understanding the impact of age on our immune system is utmost priority to improve the quality of life and decrease the burden on the healthcare. While it has been established that the aging process undergoes several changes and thus does not produce adequate response against infection, data regarding the cellular analysis in response to acute systemic inflammation is limited. Peripheral blood leukocyte count is routinely examined to detect systemic infection and is useful for determining the underlying pathological conditions (W Chen *et al.*, 2021). In this study, we investigated the age-related differences in leukocyte response following LPS induced acute systemic inflammation in female Sprague Dawley rats. We administered the endotoxin Lipopolysaccharide (LPS), an important component of Gram-negative bacterium cytoderm (Ronco *et al.*, 2014) to mimic systemic inflammation in humans. We employed a robust 9-colour flow cytometric method (Barnett-Vanes *et al.*, 2016) to effectively characterize the major rat leukocyte population in the blood and spleen. Herein, we report an age-related increase in NK cells, monocytes and neutrophils in the circulation accompanied by a decline in major leukocyte population in the spleen. In addition, we observed a shift in cytokine expression towards a pro-inflammatory phenotype in the aged animals compared to young animals treated with LPS.

White blood cells of the immune system are derived from the progenitor or precursor cells called the hematopoietic stem cells in the bone marrow. In the context of

healthy aging, there is a shift towards myeloid cell populations relative to lymphoid cell populations in circulation (Mogilenko *et al.*, 2021). This gradual expansion of myeloid cell populations is partly due to age-related thymic involution resulting in alteration of T-cell composition and T-cell related immuno-incompetence (Gui *et al.*, 2012). In addition, there is skewing of the hematopoietic compartment towards myelopoiesis concomitant with the loss of lymphoid-committed progenitors in the aging bone marrow thereby decreasing the peripheral adaptive immune populations (Almanzar *et al.*, 2020; Krishnarajah *et al.*, 2021). Accordingly, we noticed a decline in the overall number of lymphocytes (T cells + B cells + NK cells) while the number of monocytes were increased in the circulation of aged animals compared to young animals. Taking a closer look at the individual lymphocytes, we found significant age-related increase in the population of NK cells in circulation. NK cells are the primary population of innate lymphoid cells that protects against viral infection. We distinguished rat NK cells based on CD 161 expression, which marks innate proinflammatory cytokine-responsive subtype of NK cells (Kurioka *et al.*, 2018). Recent evidence showed that aged individuals had a diminished proportion of the immature (CD56bright) NK population while increased population of mature (CD56dim), late, low cytotoxic NK cells (Zheng *et al.*, 2020). Thus, increased number of proinflammatory NK cell subtype is required by the aged animals to induce cell cytotoxicity. However, immunosenescence of NK cells due to aging causes the NK cells to lose their capacity for antiviral activity thereby increasing the incidence of infections in elderly (Zheng *et al.*, 2020). With respect to adaptive immune system, we observed a significant decrease in CD3+ T cells which may be due to shortening of telomere length leading to delayed clearance of pathogens and prolonged duration of infection (Yu and

Zheng, 2019). On the other hand, the circulatory B cells were significantly increased in the aged rats when compared to young rats. It is known that aging promotes the polarization of naive T cells and B cells to age-associated, exhausted and regulatory phenotype (Frasca *et al.*, 2011; Lin *et al.*, 2016; Zheng *et al.*, 2020). Additionally, aging causes slow turnover and inability of new B cells to effectively replenish the peripheral mature B cell populations (Johnson *et al.*, 2002). This leads to rapid decline in humoral response and correlate with poor response to vaccination in addition to shortening the time interval between booster doses in the elderly (Ciabattini *et al.*, 2018). Although the number of circulating monocytes is increased with age, they are not capable of clearing bacterial infection due to premature release from the bone marrow (Puchta *et al.*, 2016). We also observed significant increase in monocytes levels in circulation of aged animals compared to young animals after LPS stimulation at 24h. In older subjects, monocytes are increased upon chronic inflammatory conditions and produce increased proinflammatory cytokines such as IL6, TNF, IL1β (Puchta *et al.*, 2016) consistent with our observation. Furthermore, aging alters the gene expression in monocytes leading to increased risk of cardiovascular mortality, coronary artery plaque formation and atherosclerosis (Choi *et al.*, 2017; Saare *et al.*, 2020). Such age-related physiological changes in the immune system predisposes the elderly to infectious diseases (Esme *et al.*, 2019). A recent study showed a strong correlation between the ratios of circulating myeloid cells and lymphocytes with blood coagulation parameters and disease severity in case of COVID-19 (Ma *et al.*, 2021), which predominantly affected individuals 65 years and above (Yanez *et al.*, 2020; Guerrero and Wallace, 2021). The authors found increased neutrophil-to-lymphocyte ratio (NLR), monocyte-to-lymphocyte ratio (MLR),

and basophil-to-lymphocyte ratio (BLR) in critically ill/deceased patients (Ma *et al.*, 2021). Thus, careful attention should be paid to changes in WBC count in elderly subjects as leukocyte count has a potential clinical implication in predicting mortality as well as long-term survival (Nilsson *et al.*, 2014).

Neutrophils are the most abundant cellular component of the innate immune system that provide protection against bacterial or fungal infection (Butcher *et al.*, 2000). They are primary cellular responders that are recruited at the site of injury/infection through extravasation across the vascular barrier in response to specific chemotactic and pro-inflammatory signals (Fine *et al.*, 2020). Neutrophils levels are increased in response to sterile and nonsterile inflammation. Nishio and colleagues reported increased numbers of neutrophils required for wound repair in elderly subjects (Nishio *et al.*, 2008). During systemic inflammatory conditions such as sepsis, the mean volume of neutrophils was increased and can be used a promising hematologic parameter for differentiating elderly patients with and without sepsis (Lee and Kim, 2013). Overall, elevated neutrophil levels negatively impact the health-related quality of life (Wouters *et al.*, 2021). In the context of healthy aging, neutrophil counts were found to be increased in circulation of aged subjects irrespective of sex (Valiathan *et al.*, 2016; Menees *et al.*, 2021; Serre-Miranda *et al.*, 2022). This may be offset by decreased phagocytic ability leading to impairment in the ability to clear pathogens and thus increased susceptibility to infection in the aged population (Butcher *et al.*, 2000). Additionally, hyper segmented, functionally compromised neutrophil populations were accumulated in the splenic white pulp due to decreased apoptosis with advancing age (Tomay *et al.*, 2018). In accordance, although not significant, we noticed an increasing trend for neutrophils in aged rats compared to

young rats upon vehicle treatment suggesting a compensatory mechanism for the reduced functionality of aged neutrophils. Furthermore, neutrophil diapedesis and chemotaxis is impaired with aging (Brubaker *et al.*, 2013; Uhl *et al.*, 2016; Barkaway *et al.*, 2021). Studies have demonstrated that neutrophils exhibit reverse transendothelial migration (rTEM) by traversing the endothelium in the reverse direction and thus re-entering the circulation (Burn and Alvarez, 2017). While some studies showed rTEM of neutrophils have anti-inflammatory effects and resolve chronic tissue inflammation others demonstrated its role in disease progression (Burn and Alvarez, 2017). A recent report from Barkaway et al., 2021 demonstrated increased levels of neutrophil reverse transendothelial migration (rTEM) in aged mice due to dysregulated local inflammatory milieu allowing pathogenic neutrophils to disseminate to distal organs and cause damage (Barkaway *et al.*, 2021). The authors showed that rTEM is propelled by increased expression of CXCL-1 produced by mast cells located at the endothelial cell junction (Barkaway *et al.*, 2021). Consistent with this report, we saw elevated expression of CXCL-1, a major chemokine for neutrophil attraction, in the plasma at 24h post stimulation with LPS. In the present study, we showed age-related significant increase in neutrophil counts in peripheral blood at 24h after LPS induced systemic inflammation, which is the major finding of the paper. This age-related neutrophilia observed in circulation is merely not a response to systemic inflammation but also due to release of immature neutrophils from marginated pools such as spleen demonstrated by decreased splenic neutrophils at 24h after LPS induced acute systemic infection. Recent reports showed that neutrophils are heterogeneous, with specific neutrophil sub-populations in the marginated pools (lungs, liver and spleen, in addition to bone marrow) and executing distinct functions

(Christoffersson and Phillipson, 2018; Herrero-Cervera *et al.*, 2022). For example, recently Deniset and colleagues identified a novel neutrophil subsets Ly6G^{hi} and Ly6G^{intermediate} residing in the murine splenic red pulp region and demonstrated their role in the clearance of systemic S. pneumoniae by eliciting rapid innate immune response in vivo (Deniset *et al.*, 2017). Taken together, it will be interesting to know if a specific population of neutrophil is associated with rTEM and thus help identify novel therapeutic targets aimed at preventing distant organ damage especially in the older adults.

The spleen is the second largest lymphoid organ and plays an important role in the maintaining the homeostasis by acting as a blood filter (Lewis *et al.*, 2019). The spleen is considered the storage unit primary for T cells, B cells, NK cells, macrophages, and DCs whose function is to eliminate encapsulated bacteria as well as elicit central and peripheral immune tolerance (Aw *et al.*, 2016; Turner and Mabbott, 2017). More recently, the existence of monocytes and neutrophils reserves in the spleen were discovered (Swirski *et al.*, 2009; Puga *et al.*, 2011). Aging causes splenic structural modifications noticeably in the marginal zone and white pulp region (Turner and Mabbott, 2017). This is indicative of changes in the number and distribution of immune cell population housed within the microarchitecture of the spleen thereby affecting its function (Turner and Mabbott, 2017). Accordingly, we showed overall immunosuppression in the total number of leukocytes, T cells, B cells, monocytes, and NK cells in the spleen of aged animals compared to young animals leading to deterioration of cellular and humoral immune system with age. This is accompanied by significant increase in B cells, monocytes, and NK cells in the circulation. We also observed decreased spleen to body weight ratio along with reduced number of splenocytes per gram of spleen due to aging consistent with

previous findings (El-naseery *et al.*, 2020; Menees *et al.*, 2021). Taken together, our data suggests age-related impairment in the ability to retain immune cell reservoirs in the spleen. Several studies have investigated the role of spleen in initiating peripheral immune response to brain injury such as ischemic stroke (Liu *et al.*, 2015; Pennypacker and Offner, 2015). In vivo studies showed that splenectomy prior to cerebral ischemia significantly decreased neurodegeneration by blunting systemic inflammation in young and aged animals (Ajmo *et al.*, 2008; Chauhan *et al.*, 2018; Seifert and Offner, 2018). Additionally, splenectomy reduced peripheral inflammatory cytokine levels as well as decreased macrophages, neutrophils, B cells, and T cells infiltration into the brain at the site of injury thereby attenuating ischemic brain damage (Ajmo *et al.*, 2008; Chauhan *et al.*, 2018; Seifert and Offner, 2018). Given the fact that the incidence and mortality rate of stroke is higher in the aged population (Avan *et al.*, 2019; Yousufuddin and Young, 2019), the effect of age on splenic response following splenectomy in contributing cerebral ischemia induced brain damage is warranted.

Aging is characterized by disruption of the cytokine homeostasis and skewing towards a proinflammatory state termed as inflamm-aging (Rea *et al.*, 2018). Overall, we saw increase in cytokine levels upon LPS stimulation irrespective of age and time (Seemann *et al.*, 2017). Our data showed elevated levels of pro inflammatory cytokines (TNF-α, IL-1β, IL-6, CXCL-1) in the plasma upon LPS challenge at 3h and the values seemed to return to baseline at 24h after LPS treatment. On the other hand, we noticed significant changes in anti-inflammatory cytokines with increased IL-10 and IL-5 expression and concomitant decrease in IL-4 levels upon LPS stimulation at 24h in the aged rats. IL-6, TNF-α and IL-1β are key pro-inflammatory cytokines that have been

implicated in autoimmune diseases, bacterial infections, cardiovascular disease, (Kany *et al.*, 2019) and more recently COVID-19 (Ragab *et al.*, 2020). Understanding the cytokine profiles of aged individual is particularly important in diseases like COVID-19, that predominantly affects the older populations resulting in increased hospitalization and mortality, to help clinicians classify patients based on the severity of infections (Angioni *et al.*, 2020). Elevated levels of pro-inflammatory cytokines are known to increase the risk of cardiovascular diseases, frailty, as well as cognitive impairment (Ferrucci and Fabbri, 2018). Our data showed elevated levels of IL-6 and CXCL-1 at 24h after LPS treatment in the aged group compared to the young LPS 24h group indicating prolonged systemic inflammation after LPS stimulation upon aging. Increased expression of plasma/serum IL-6 levels positively correlated with poor stroke outcomes and may serve as a prognostic marker (Shaafi *et al.*, 2014; Aref *et al.*, 2020; Mosarrezaii *et al.*, 2020). Additionally, prolonged LPS induced systemic inflammation is dangerous as it results in disruption of the blood brain barrier thereby allowing infiltration of inflammatory immune cells (Fu *et al.*, 2014). This in turn can drive neuroinflammation leading to secondary neurodegeneration following stroke (Stuckey *et al.*, 2021). Accumulating evidence has shown that chronic inflammation is associated with age-related memory and cognitive decline (Sartori *et al.*, 2012; Fielder *et al.*, 2020). All these events negatively impact the CNS and contribute to disease pathologies such as Alzheimer disease, Parkinson disease, and other neurodegenerative diseases which predominantly affect the elderly (Hou *et al.*, 2019). Given the importance of chronic inflammation in driving multiple CNS pathologies, anti-inflammatory and immunomodulating agents appear to be attractive treatment options in providing neuroprotection.

One particular caveat is that this study was conducted in female rats. Growing body of literature demonstrated that immune cell profile and responses to both self and foreign antigens is altered based on sex differences (Gubbels Bupp, 2015; Klein and Flanagan, 2016; Díaz *et al.*, 2020; Márquez *et al.*, 2020; Breznik *et al.*, 2021; Serre-Miranda *et al.*, 2022). Future study will focus on investigating the cellular composition and the cytokines levels following infection using male animals. In vivo endotoxemia model using LPS, an important component of Gram-negative bacterium cytoderm (Ronco *et al.*, 2014), was employed to mimic acute systemic inflammatory response. However, varying dosages of LPS differentially regulated cytokine expression indicating a dose-dependent accumulation of leukocytes in vivo (Larsson *et al.*, 2000; Morris *et al.*, 2014). Along those lines, a recent study conducted in SD rats tested three different concentrations of LPS and found 10mg/kg to be effective in inducing sepsis as evidenced by significant modulation of blood, biochemical and molecular markers (Bhardwaj *et al.*, 2020). Additionally, the study confirmed that male rats are more susceptible to infections than female rats (Bhardwaj *et al.*, 2020) indicating the need for increased dose of LPS to elicit the same response in females. In the present study we used a single dose of 1mg/kg LPS, which probably was not significant to induce a robust immune response in the female SD rats. A more extensive evaluation of peripheral immune system components following chronic LPS exposure is warranted. Studies showed that aged animals showed phenotypic changes in both lymphoid and myeloid lineages in a diverse range of tissue and organs (Almanzar *et al.*, 2020; Krishnarajah *et al.*, 2021), and thus leading to potential differences in cell cytotoxicity in response to injury/disease. For example, dentate gyrus resident NK cells are increased in the brains of healthy aged adults and result in NK

dependent depletion of neuroblasts leading to cognitive decline and reduced neurogenesis (Jin *et al.*, 2020). However, in the current study we focused on changes in major leukocyte population specifically in the blood and spleen in the presence and absence of LPS induced systemic infection. It would be interesting to understand age related alteration in other tissues following LPS challenge as well. Future studies should focus on delineating the mechanism of immunosenescence in each tissue following acute infection with advancing age.

CONCLUSION

In summary, our study provides a reference data set for major leukocyte populations and pro- and anti-inflammatory cytokines in blood and spleen of young and aged rats with and without LPS treatment. The response to brain injury following neurological disorders such as stroke is greatly determined by peripheral immune system. Age-associated changes in the systemic immune response can alter brain vasculature and drive chronic neuroinflammation which worsens stroke outcome in the elderly. This study highlights the importance of age-related changes on systemic immune response and underscore the importance of choosing the correct age based on development or cellular aging of the system being studied. Future studies should focus on development of novel therapies to target the peripheral immune system such as bone marrow rejuvenation, which holds great promise for the treatment of neurological diseases.

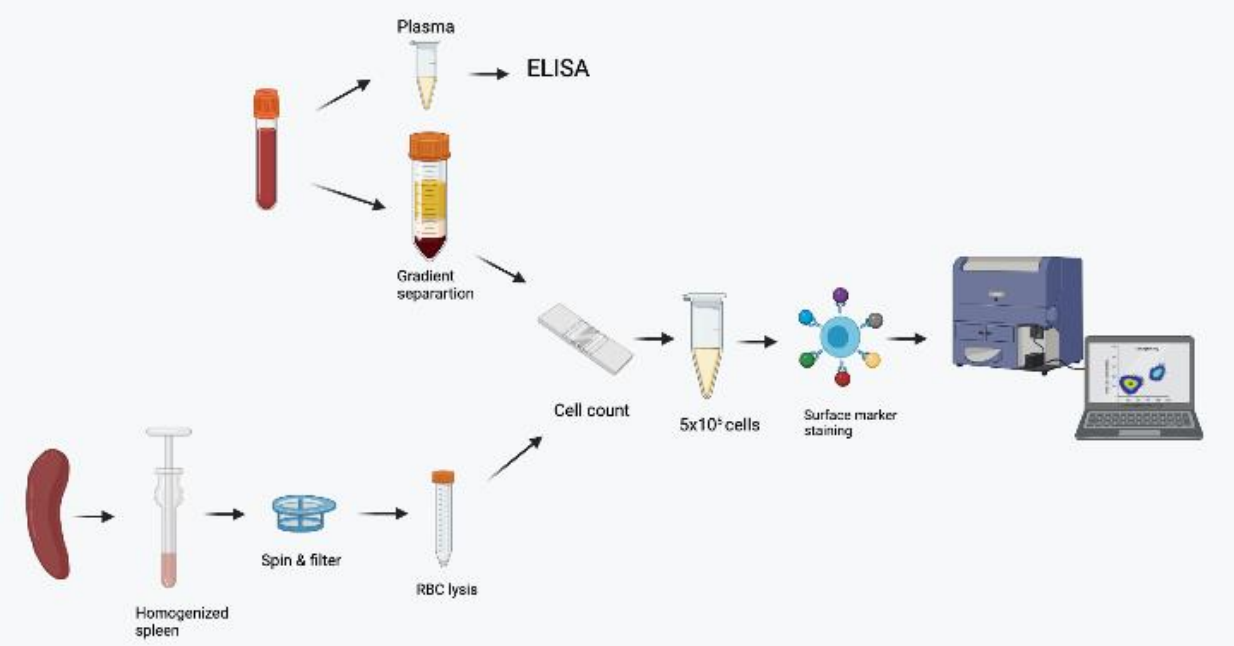

Figure 4.1: Schematic showing the processes involved in sample processing and analysis. Major leukocyte population in the blood and the spleen were analyzed by flow cytometry and the cytokines/chemokines in the plasma were analyzed by ELISA (Created in BioRender.com).

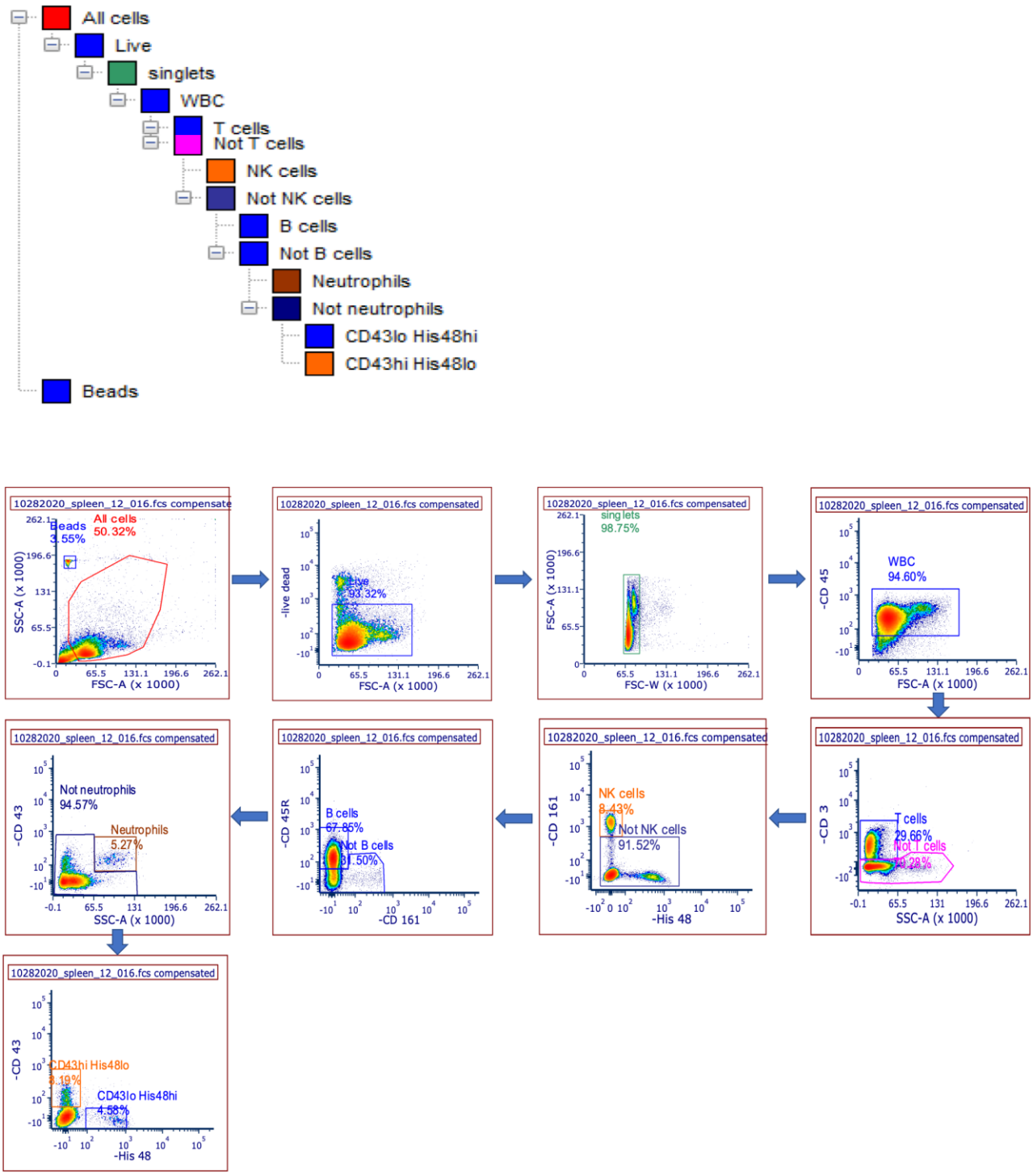

Figure 4.2: Gating strategy to identify major leukocyte populations in aged and young rats. Blood (not shown) and spleen were processed, analyzed and the relative proportion of each leukocyte population presented was presented. (n=5 animals/group).

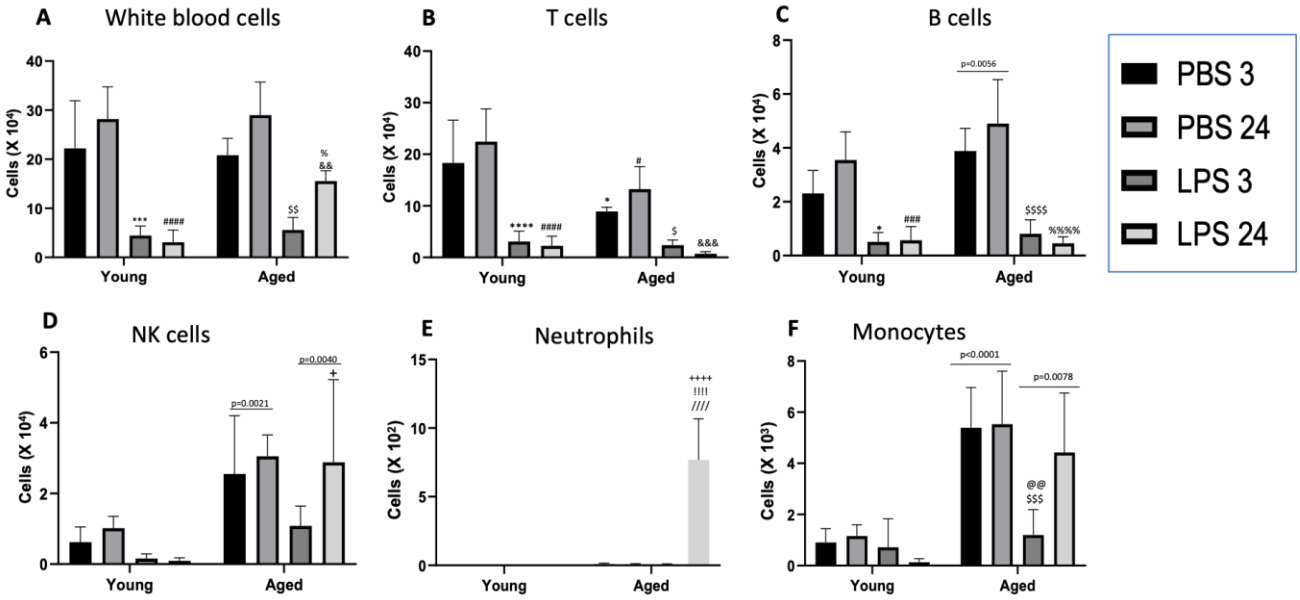

Figure 4.3: Changes in circulating leukocytes after LPS challenge. Rats were administered LPS or saline, followed by which blood was collected either 3h or 24h, processed and analyzed. (* denotes difference compared to young PBS 3h, $^{\#}$ denotes difference compared to young PBS 24h, $^{\$}$ denotes difference compared to aged PBS 3h, $^{\%}$ denotes difference compared to aged PBS 24h, $^{\&}$ denotes difference compared to aged PBS 24h, $^{/}$ denotes difference compared to young LPS 24, $^{+}$ denotes difference compared to aged LPS 3h, $^{@,!}$ denotes difference compared to aged PBS 24h; $^{\%,*,\ \#,\ \$,\ +}p < 0.05$, $^{\#\#,\$\$,\ \&\&}p < 0.01$, $^{***,\ \&\&\&,\ \#\#\#}p < 0.001$, $^{****,\ \$\$\$\$,\ \%\%\%\%,\ ////,\ !!!!,\ ++++,\ \#\#\#\#}p < 0.0001$). Data are presented as mean ± SD; n=5 animals/ group.

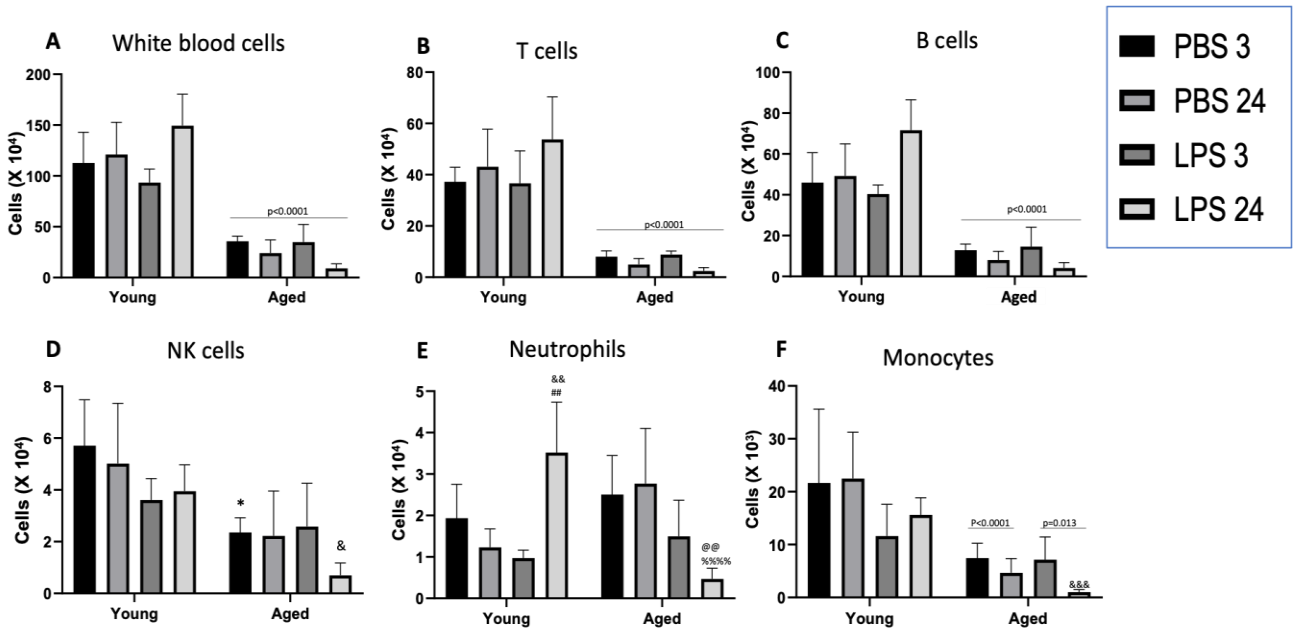

Figure 4.4: Changes in splenic leukocytes after LPS challenge. Rats were administered LPS or saline, followed by which spleen was collected either 3h or 24h, processed and analyzed. Data are presented as mean ± SD; n=5 animals/ group. (* denotes difference compared to young PBS 3h, $^{\#}$ denotes difference compared to young PBS 24h, $^{\$}$ denotes difference compared to aged PBS 3h, $^{\%}$ denotes difference compared to aged PBS 24h, $^{\&}$ denotes difference compared to aged PBS 24h, $^{/}$ denotes difference compared to young LPS 24, $^{+}$ denotes difference compared to aged LPS 3h, $^{@,!}$ denotes difference compared to aged PBS 24h; $^{\%,*,\ \#,\ \$,\ +}$ $p < 0.05$, $^{\#\#,\$\$,\ \&\&}p < 0.01$, $^{***,\ \&\&\&,\ \#\#\#}p < 0.001$, $^{****,\ \$\$\$\$,\ \%\%\%\%,\ ////,\ !!!!,\ ++++,\ \#\#\#\#}p < 0.0001$). Data are presented as mean ± SD; n=5 animals/ group.

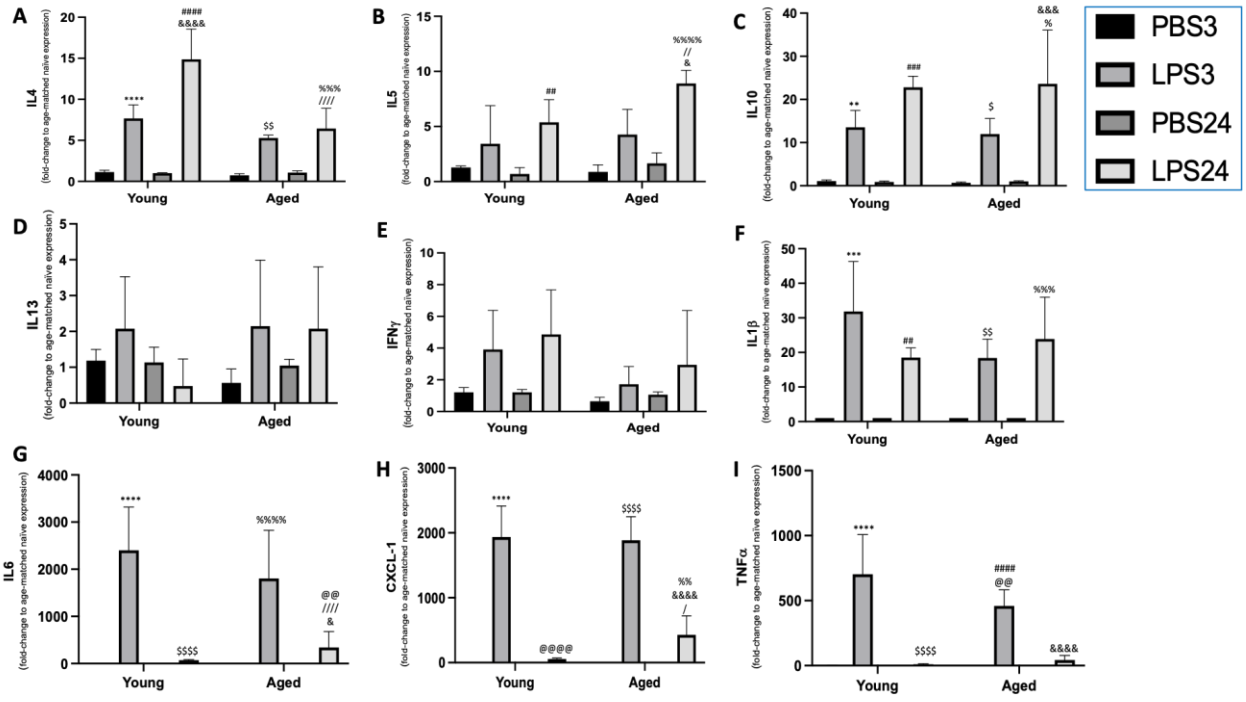

Figure 4.5: Plasma cytokine expression after LPS stimulation. Rats were administered LPS or saline, followed by which changes in plasma pro-and anti-inflammatory cytokine levels were measured at 3 h and 24 h. Data are presented as mean ± SD; n=5 animals/ group. (* denotes difference compared to young PBS 3h, # denotes difference compared to young PBS 24h, $ denotes difference compared to aged PBS 3h, % denotes difference compared to aged PBS 24h, & denotes difference compared to aged PBS 24h, / denotes difference compared to young LPS 24, + denotes difference compared to aged LPS 3h, @,! denotes difference compared to aged PBS 24h; %,*, #, $, + $p < 0.05$, ##,$$, &&p < 0.01, ***, &&&, ###$p < 0.001$, ****, $$$$, %%%%, ////, !!!!, ++++, ####$p < 0.0001$). Data are presented as mean ± SD; n=5 animals/ group.

Table 4.1: Monoclonal antibodies for leukocyte characterization

Antibody	Flurochrome	Dilution	Supplier
Live-Dead	eFluor® 780	1:1000	eBioscience
CD 32	N/A	1:200	BDPharmingen
CD 45	Alexa-Fluor®700	1:100	Biolegend
CD 3	BV 421	1:100	BDBioscience
CD 43	PE	1:200	Biolegend
His 48	FITC	1:200	eBioscience
CD 161	APC	1:200	Biolegend
CD 45R (B220)	PE-Cy7	1:200	eBioscience

Table 4.2: **LPS induced changes in body and spleen weight in the context of aging**

	Vehicle _3h		LPS _3h		Vehicle _24h		LPS_24h	
	Aged	Young	Aged	Young	Aged	Young	Aged	Young
Spleen weight (g)	0.61614	0.61658	0.82656	0.75804	0.71804	0.62462	0.7639	0.74136
Body weight (BW) (g)	363.6	278	352.8	278.4	384.2	281	394.4	278.2
Spleen weight /BW (g/g)	0.0016904	0.002216	0.0023354	0.00272	0.0018607	0.002221	0.0019459	0.002672
Total splenocytes	1.06E+08	1.98E+08	1.94E+08	1.88E+08	1.84E+08	2.29E+08	1.00E+08	1.46E+08
Total splenocytes/spleen weight (cells/g)	1.72E+08	3.25E+08	2.41E+08	2.49E+08	2.61E+08	3.62E+08	1.33E+08	1.98E+08

CHAPTER FIVE

Novel mitoNEET ligand NL-1 improves therapeutic outcomes in an aged rat model of cerebral ischemia/reperfusion injury

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ABSTRACT

Cerebral ischemic stroke is a leading cause of mortality and disability worldwide. Currently, there are a lack of drugs capable of reducing neuronal cell loss after ischemia/reperfusion-injury after stroke. Previously, we identified mitoNEET, a [2Fe-2S] redox mitochondrial protein, as a putative drug target for stroke. In this study, we tested the novel mitoNEET ligand, NL-1, in a preclinical model of ischemic stroke with reperfusion using aged female rats. Using a transient middle cerebral artery occlusion (tMCAO), we induced a 2 h ischemic injury and then evaluated the effects of NL-1 treatment on ischemic/reperfusion brain injury at 24 and 72 h. Test drugs were administered at time of reperfusion via IV dosing. Results demonstrated that NL-1 (10 mg/kg) treatment markedly reduced infarct volume and hemispheric swelling in the brain as compared rats treated with vehicle or a lower concentration of NL-1 (0.25 mg/kg). Surprisingly, the protective effect of NL-1 was significantly improved when encapsulated in PLGA nanoparticles, where a 40-fold lesser dose (0.25 mg/kg) of NL-1 produced an equivalent effect as the 10 mg/kg dose. Evaluation of changes in blood-brain barrier (BBB) permeability and oxidative stress using immunohistochemical staining corroborated the protective actions of NL-1, showing reduced extravasation of IgG and decreased levels of 4-hydroxynonenal (4-HNE) in the brains of aged female rats at 72 h after tMCAO with reperfusion. Our studies indicate that targeting mitoNEET following ischemia/reperfusion-injury is a novel drug target pathway that warrants further investigation.

INTRODUCTION

Ischemic stroke is a leading cause of death and disability in the United States; yet, with the exception of reperfusion of infarcted tissue by thrombectomy, no other options are approved for stroke treatment. Early reperfusion of infarcted tissue using tissue plasminogen activator or endovascular mechanical devices is associated with lower rates of death and improved health outcomes compared to stroke patients not receiving treatment (Yang *et al.*, 2021). However, reperfusion is not without risks and can result in reperfusion injury, which is characterized by increases of reactive oxygen species (ROS) formation, oxidative stress, blood-brain barrier (BBB) disruption & neuroinflammation which leads to greater loss of vulnerable neurons adjacent to the infarct core (Jurcau and Ardelean, 2021; Yang *et al.*, 2021). Reperfusion injury following ischemia is clinically noted by hemorrhagic transformation, increased vasogenic edema, infarct expansion and neurologic worsening (Mandalaneni *et al.*, 2020; Imran *et al.*, 2021; Jurcau and Ardelean, 2021).

Recent studies demonstrate that cerebral ischemia and post-ischemic reperfusion causes a wide array of mitochondrial dysfunction resulting in a sequelae-of-events leading to ATP depletion, increased oxidative stress, changes in mitochondrial dynamics and ultimately neurodegeneration (Mandalaneni *et al.*, 2020). Conceptual views of mitochondrial function have changed due to advances in our understanding of how mitochondria work together within an integrated network to dynamically remodel and reorganize to meet energetic demands of the cell. When these changes cannot be accomplished, mitochondrial dysfunction results in a diminished ability of cells to meet

energetic demand (Wu et al., 2021). Within the past decade, ischemic stroke has gained greater attention for effects of mitochondrial dysfunction on neuronal death following ischemic/reperfusion insult. Targeting mitochondria for treatment of post-stroke brain injury has mainly focused on reducing neuron destruction by attenuating ROS formation (Galkin, 2019).

MitoNEET is an iron-sulfur [2Fe-2S] cluster protein embedded in the outer mitochondrial membrane that functions to regulate oxidative capacity within mitochondria by acting as a pH and redox sensor (Tamir *et al.*, 2015). Prior studies indicate that mitoNEET plays a protective role in the brain following neurological injury and disease and demonstrate that targeting mitoNEET may be a valuable therapeutic approach to mitigate the severity of reperfusion injury following ischemic stroke (Geldenhuys *et al.*, 2014; Tamir *et al.*, 2015).

NL-1 (Figure 5.1) is a first-in-class compound designed based off of the thiazolidinedione structure of pioglitazone, a drug marketed for its function as a nuclear peroxisome proliferator-activated receptor gamma (PPARγ) agonist (Geldenhuys *et al.*, 2010). Pioglitazone, an anti-diabetic drug, has demonstrated neuroprotective activity in several neurological disorders, including Parkinson's disease, Alzheimer's disease and ischemic stroke (Yonutas and Sullivan, 2013). In preclinical neurodegenerative models, pioglitazone administration reduced oxidative stress, decreased ROS formation and diminished neuroinflammation. Following ischemic stroke, pioglitazone and rosiglitazone protected neurons in the brain from mitochondrial dysfunction and pioglitazone prevented mitochondrial-associated apoptosis (Culman *et al.*, 2012; J Chen *et al.*, 2021). These promising findings were found to be an off-target effect of thiazolidinedione compounds

at mitoNEET; however, due to unfavorable side effects (edema formation, bone loss and heart failure) of the glitazones, it has necessitated the development of lead compounds, such as NL-1, that selectively target mitoNEET without PPARγ activity (Saralkar *et al.*, 2020, 2021).

In the current study, we evaluated the efficacy of NL-1 treatment on ischemia/reperfusion injury in aged female rats following a transient middle cerebral artery occlusion (tMCAO). Using a 2 h ischemia followed by reperfusion, we assessed neurologic outcome, infarct volume and hemispheric swelling at 24 h and indices of BBB disruption, oxidative stress and apoptosis at 72 h after tMCAO. Results of this study validated that NL-1 has a dose-dependent therapeutic potential to improve tissue sparing following ischemia/reperfusion injury in the brain by decreasing vasogenic edema, reducing BBB disruption and attenuating neuroinflammation and provides compelling supportive evidence that mitoNEET functions as a therapeutic target for treatment of ischemic stroke.

MATERIALS & METHODS

Drugs and chemicals. All chemicals were obtained from commercial sources. The mitoNEET agonist, NL-1, was synthesized using Knoevangel condensation as previously described (Geldenhuys *et al.*, 2010). Prior to administration, NL-1 was freshly prepared by dissolving a known amount of NL-1 in 100% methanol then evaporating the solvent. Dried NL-1 was then reconstituted in ethanol (2% v/v) in phosphate buffered saline (PBS; pH 7.4) with Tween-80 (2% w/v) to final doses of 0.25 and 10 mg/kg. PBS with 2% ethanol and 2% Tween-80 served as vehicle (negative control). NL-1 (0.25 mg/kg) loaded poly

D,L-lactic-co-glycolic acid (PLGA) nanoparticles (NL-1 NP) were prepared using emulsification and solvent evaporation as previously described (Geldenhuys *et al.*, 2011). Unentrapped (free) NL-1 was separated from NL-1 loaded nanoparticles by gel filtration. The dose of NL-1 NP was restricted to 0.25 mg/kg due to physical limitations of the encapsulation process used and to ensure volume of drug administered i.v. through the tail vein did not exceed 0.5 ml. Based on these constraints, we tested free NL-1 at 0.25 mg/kg and 10 mg/kg. Experimental compounds and vehicle were filtered through a 0.22 µm syringe filter prior to administration.

Animals and experimental design. All animal procedures were approved by the West Virginia University Animal Care and Use Committee prior to experimentation and abided by ARRIVE 2.0 guidelines (Percie du Sert *et al.*, 2020). Female Sprague-Dawley rats (22–24 months) were acquired from our aging colony located at Hilltop Laboratories (Scottdale, PA) and housed in the West Virginia University animal facility under 12 h light-dark conditions with food and water available ad libitum. Upon arrival, rats were randomly assigned to one of four treatment groups (vehicle, NL-1 (0.25 mg/kg; i.v.), NL-1 (10 mg/kg; i.v.) or NL-1 NP (0.25 mg/kg; i.v.) at one of two time points (24 h (n = 5 rats/group) or 72 h (n = 3–4 rats/group)). A sham group was included for 4-HNE studies (n = 3) and a naïve group was included for the iron accumulation study (n = 3). Rats at 24 h after tMCAO were assessed for changes in neurological function, infarct volume and hemispheric swelling, while rats at 72 h after tMCAO were qualitatively assessed for changes in IgG extravasation (a marker of BBB disruption), 4-HNE, a marker of lipid peroxidation or TUNEL positive cells, a marker of cell death. To reduce bias and improve scientific rigor,

dose administered was randomly assigned, surgeon was blinded to treatment groups, immunohistochemistry was visualized and assessed separately by 2 investigators blinded to treatment groups and data analysis was performed by investigator blinded to treatment groups.

MCAO procedure. Using aged (22–24 months) female Sprague-Dawley rats, ischemic brain injuries were induced on anesthetized (2% isoflurane) rats using a tMCAO model of ischemic stroke as previously described (DiNapoli *et al.*, 2008, 2010; Kelly *et al.*, 2009; Tan *et al.*, 2013, 2015). Briefly, a 4–0 suture (#403956; Doccol Corp; Sharon, MA) was inserted through the internal carotid artery into the right MCA to produce an ischemic brain injury. Suture was removed after 2 h of ischemia to allow for reperfusion of the infarcted area. After surgery, rats were administered 0.5 ml saline (i.p.) for fluid replenishment and bupivacaine (1%; Sigma Chemicals; St. Louis, MO) was injected (s.c.) at incision sites for pain relief. Rats were housed singly on fresh bedding with moistened food and water placed on the floor of the cage overnight for ease of access. Laser Doppler was used to establish relative cerebral blood flow (CBF) baseline and monitor for changes in CBF over the 2 h ischemia. Inclusion/exclusion criteria were: (1) rat experienced less than a 75% reduction in CBF from baseline after suture insertion; (2) rat experienced restoration of CBF less than 80% of baseline upon reperfusion; (3) rat died before assigned time point (age-related or MCAO-related); (4) perforation of cerebrovasculature during suture insertion; (5) tumor present in location that prohibited successful MCAO surgery; (6) rat placed on clinical call by West Virginia University vet staff after arrival but before MCAO.

Neurologic outcomes. Neurological functional assessments were carried out by investigators blinded to treatment groups using standard operating procedures in the WVU rodent behavior core. A modified Neurological Severity Scores (mNSS) and a health/sickness screen were performed on rats (n = 12–13 rats per treatment) at 24 h post-MCAO to evaluate impairments in motor, sensory, balance and reflex measures during the acute period recovery following tMCAO, as previously described (Zhang *et al.*, 2002). The scoring range was from 0 to 20 points for the health/sickness behavior screen and 0 to 18 points for the mNSS, with higher scores indicative of more severe impairment (See Table 5.1 and 5.2).

Infarct volume and hemispheric swelling measurement. At 24 h after tMCAO, aged female rats (5 rats per treatment) were anesthetized with 2% isoflurane, euthanized by cardiac perfusion with 1× PBS, decapitated, brains removed and sliced coronally at 2 mm intervals. Sections were incubated in 2% 2,3,5-triphenyltetrazolium chloride (TTC; Sigma Chemicals) for 20 min at 37 °C. Following TTC staining, infarct volumes were quantified according to method previously described (Isayama *et al.*, 1991). For anterior and posterior side of each brain section, ischemic area, ipsilateral area and contralateral area were outlined and infarct volume was calculated. Corrected infarct volumes were calculated to avoid overestimation of infarct size using the equation: IV = (LA-[RA-RI]) x d, where LA = area of left hemisphere (mm2), RA = area of right hemisphere (mm2), RI = infarcted area (mm2) and d = slice thickness (2 mm). Hemispheric swelling (%) was calculated, as an indicator of edema formation, according to previously described method

(Isayama *et al.*, 1991). Briefly, hemispheric swelling = (RV-LV) / LV. X 100%, where RV = volume of right hemisphere (mm3) and LV = volume of left hemisphere (mm3).

Brain extraction, fixing and sectioning for microscopy studies. Rats (3–4 rats per treatment) were deeply anesthetized with inhaled 4% isoflurane and transcardially perfused with 1× PBS for 10 mins followed by perfusion with 4% paraformaldehyde (PFA) for 5 mins before decapitation. The brains were extracted and sectioned using a brain matrix. Each 2 mm section was post-fixed in 4% PFA overnight and cryoprotected in 30% sucrose for 2 d. Sections were paraffin embedded, sectioned (5 µm) using a microtome and plated onto microscope slides. For qualitative assessment, three to four slides containing coronal sections from each subject in each treatment group were deparaffinized and rehydrated using xylene and gradient ethanol (100%, 95%, 85%, 70%, 50%) baths. Section preparation and microscopic visualization was carried out as described below. Sections were stored at −80 °C until used. For all assessments, perilesional areas were determined using H&E-stained adjacent sections.

Iron content in the brain after tMCAO. At 72 h following tMCAO, female aged rats were evaluated for iron content in the brain using brightfield microscopy of Perl's stained sections with 3,3′ diaminobenzidine (DAB) enhancement. Re-hydrated sections were rinsed three times in 0.1× PBS for 5 min and then stained in 1% potassium ferrocyanide/ 0.1 N HCl for 40 min. Sections were rinsed three times in distilled water for 5 min and then incubated in MeOH containing 0.01 M NaN3 and 0.3% H2O2 for 75 min. Sections were rinsed twice in 0.1× PBS for 5 min then incubated in 0.025% 3,3’-DAB and 0.005%

H2O2 in 0.1 N HCl for 40 min. Reaction was stopped by washing sections in 0.1× PBS. Sections were mounted on slides, dehydrated in serial EtOH (25% to 100%) and cleared with xylenes. Slides were cover slipped and visualized using an Olympus VS120 slide scanning microscope. Once stitched images of brain sections were acquired, 4 images of perilesional areas around the infarct from each section were viewed at 20× magnification and non-heme iron visualized as dark brown/black granular staining.

Qualitative assessment of BBB impairment after tMCAO. At 72 h following tMCAO, female aged rats were evaluated for changes in BBB extravasation of IgG using immunohistochemistry. For IgG assessment of BBB leakage, re-hydrated sections were fixed in 100% methanol for 3 min and then washed in 1× PBS. Endogenous peroxidases were quenched by incubating sections in 3% v/v hydrogen peroxide in 1× PBS with 4% v/v horse serum for 30 min. Sections were then washed 3 times in 1× PBS. Subsequently, brain sections were permeabilized in 1% w/v TritonX-100 in PBS with 4% horse serum for 1 h followed by washing 3 times in 1× PBS. Brain sections were stained for IgG by incubating sections in horseradish peroxidase linked rat anti-IgG (1:500; HAF005; R&D Systems; Minneapolis, MN) in 1× PBS with 4% horse serum for 2 h. Sections were washed 3 times with 1× PBS. After staining, slides were cover slipped using Flouromount-G (Southern Biotech; Birmingham, AL) and allowed to dry prior to imaging. Slides were visualized by bright field microscopy using an Olympus MVX10 microscope (Pittsburgh, PA).

Qualitative assessment of lipid peroxidation and cell death after tMCAO. At 72 h following tMCAO, aged female rats were evaluated for lipid peroxidation and apoptosis using immunohistochemistry.

For assessment of lipid peroxidation, re-hydrated brain sections were fixed in 100% methanol for 3 min and washed in 1× PBS. Brain sections were permeabilized using 1% Triton X-100 in PBS with 4% horse serum for 1 h followed by washing 3 times in 1× PBS. Brain sections were immunostained using a primary antibody for 4-HNE, a marker of lipid peroxidation, (1:200; mouse, MAB3249; R&D Systems) and then incubated in the dark overnight at 4 °C. The following day, the primary antibody was removed and slides were washed 3 times in 1× PBS, prior to secondary antibody incubation. Sections were incubated in secondary antibody (anti-mouse AlexaFluor 568 (1:1000; Invitrogen) for 2 h in the dark at room temperature, washed 3 times in 1× PBS, cover slipped using Flouromount-G with DAPI and imaged using an MIF Olympus Slide Scanner with a 20× objective.

Apoptosis was assessed via terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) using a TUNEL system kit (DeadEnd™ Fluorometric; Promega) according to manufacturer's instructions. The re-hydrated sections were washed in 0.85% NaCl for 5 min followed by 1× PBS for 5 min. For apoptosis detection, the slides were fixed with 4% PFA for 15 min followed by washing with 1× PBS two times for 5 min each. The sections were permeabilized using 100 µl of Proteinase K solution for 10 min, washed with 1× PBS for 5 min, fixed again in 4% PFA for 15 min and then washed with 1× PBS for 5 min. Brain sections were allowed to equilibrate using 100 µl of equilibration buffer for 10 min followed by labeling using 50 µl of TdT reaction mix containing nucleotide mix

and rTdT enzyme for 1 h at 37 °C in a dark, humidified chamber. After incubating, the reaction was stopped using 2xSSC for 15 min, followed by washing 3 times with 1× PBS for 5 min. Finally, slides were mounted using the ProLong™ Glass Antifade Mounting media with NucBlue™ stain containing DAPI and visualized using a Zeiss LSM 710 confocal microscope using 40× objective with constant exposure time for each marker in all analyzed sections.

Microvessel isolation and brain distribution of NL-1 and NL-1 NP. To determine the distribution of NL-1 and NL-1 NP into the brain, naïve rats were injected with NL-1 (10 mg/kg) or NL-1 NP (0.25 mg/kg) intravenously through the tail vein. After 1 h, rats (n = 3 rats per treatment) were anesthetized using 4% isoflurane, euthanized and brain collected. The cerebellum olfactory bulbs, meninges and choroid plexus were discarded and the left and right cortices underwent microvessel isolation as previously described (Huber *et al.*, 2002). Briefly, right and left cortex of each brain was dissected, weighed, placed into a Dounce homogenizer with 4 ml of microvessel isolation buffer and homogenized using 10 strokes with the pestle. The brain homogenate was centrifuged at 500 xg at 4 °C for 5 min to remove debris. Four ml of 26% dextran was added to the brain homogenate and vortexed. The brain homogenate was centrifuged at 10,000 xg at 4 °C for 30 min. One ml of the brain supernatant was placed into a 1.5 ml microfuge tube to serve as microvessel-depleted brain homogenate. The remaining supernatant was discarded and 4 ml of microvessel isolation buffer with 0.5% bovine serum albumin (BSA) was added to resuspend the vascular pellet and filter the pellet through a 100 µm mesh strainer. The filtered pellet solution was then filtered over a 40 µm mesh strainer and the

vessels on the strainer were collected with 8 ml of microvessel isolation buffer with 0.5% BSA. The resuspended pellet was centrifuged at 5000 xg at 4 °C for 10 mins. The supernatant was discarded and microvessel pellet resuspended in 1 ml of microvessel isolation buffer with 0.5% BSA and placed into a pre-weighed 1.5 ml microfuge tube. The tube was then centrifuged at 5000 xg at 4 °C for 5 min. Tube weight was recorded and isolated microvessel pellet was resuspended in 1 ml of microvessel isolation buffer. All samples were stored at −80 °C until used.

Once all samples were obtained, samples were thawed and sonicated 3 times for 20 s. For NL-1 isolation from samples, an equivalent volume of ethyl acetate was added, vortexed and centrifuged at 5000 xg at 4 °C for 10 min. The ethyl acetate fraction was then dried under flowing air and resuspended in 200 mL of acetonitrile for LC-MS/MS quantification. Samples were analyzed using an ABSciex 5500 LC-MS/MS and compared to a standard curve as described earlier (Culman et al., 2012). The standard curve regression (r2) for this study was 0.99.

Statistical analysis. All data were acquired and analyzed by investigators blinded to treatment groups. Data were reported as mean ± standard deviation (SD). Comparison of mortality between groups was analyzed using the log-rank Mantel-Cox test with live denoted as 0 and dead as 1. Infarct volume and hemispheric swelling were compared between groups using one-way analysis of variance (ANOVA) with significance determined using Tukey's HSD post hoc analysis. Health/sickness behavior screen and mNSS were compared between groups using one-way ANOVA with repeated measures (before and after MCAO) with significance determined using Tukey's multiple

comparisons post hoc analysis. TUNEL positive cells that co-localized with DAPI positive nuclei in the ipsilateral hemisphere were counted in selected area of the 40× micrograph for each rat in each treatment. The total number of TUNEL positive cells from each micrograph for each rat was combined and a mean of TUNEL positive cells for all rats within a treatment were analyzed and plotted. Level of significance was set at $p < 0.05$.

RESULTS

Mortality and exclusion of rats from study. A total mortality rate of 30% from experimental ischemic stroke with reperfusion was observed in this study with a total of 76 rats (23 vehicle, 22 NL-1 (0.25 mg/kg), 15 NL-1 (10 mg/kg) and 16 NL-1 NP) were needed to complete the study. We used 49 rats (n = 12 for vehicle, NL-1 (0.25 mg/kg) and NL-1 (10 mg/kg) and n = 13 for NL-1 NP) in the study and excluded 27 rats from the study (21 for deaths before assigned time point after tMCAO (mortality) and 6 for other reasons). Mortality rates at 24 h resulting from tMCAO with reperfusion for the four treatment groups were: vehicle (45%; 10 of 22)), NL-1 (0.25 mg/kg) (40% (8 of 20)), NL-1 (10 mg/kg) (14%: 2 of 14)) and NL-1 NP (7%; 1 of 14). A significant difference ($p = 0.007$) in mortality was noted between treatment groups. Pairwise comparison showed significant differences ($p < 0.01$) between both the NL-1 (10 mg/kg) and NL-1 NP groups as compared to the vehicle group. Additionally, the NL-1 (10 mg/kg) and NL-1 NP groups showed a significant ($p < 0.01$) decrease in mortality compared to the NL-1 (0.25 mg/kg) group. No differences ($p > 0.05$) were observed between the NL-1 (0.25 mg/kg) and vehicle groups or between the NL-1 (10 mg/kg) and NL-1 NP groups. For the 6 rats excluded from the study for other reasons, 1 from the NL-1 (10 mg/kg) group was

excluded for having a tumor in a location that infringed on MCAO surgery, 3 (2 NL-1 (0.25 mg/kg) and 1 NL-1 NP) were assigned into treatment groups but excluded due to vet staff issuing a clinical call before an MCAO surgery was performed and 2 (1 vehicle and 1 NL-1 NP) were excluded for not meeting inclusion/exclusion criteria of achieving a ≥ 75% decrease in CBF from baseline.

Changes in neurological functional assessment post-MCAO. Using the mNSS and health/sickness behavior screen, we assessed the effects of NL-1 administration on neurological recovery at 24 h after tMCAO. Results indicated no difference ($p > 0.05$) in mNSS scores between either NL-1 (0.25 mg/kg) or NL-1 (10 mg/kg) and vehicle treatment group (Figure 5.2A). A significant difference ($p < 0.05$) was found for treatment with NL-1 NP and vehicle treated rats. No difference ($p > 0.05$) in mNSS between NL-1 (10 mg/kg) and NL-1 NP was shown (Figure 5.2A). A significant ($p < 0.05$) difference in the health/sickness behavior screen was noted between both NL-1 (10 mg/kg) and NL-1 NP when compared to vehicle treated rats (Figure 5.2B) but no difference ($p > 0.05$) between NL-1 (0.25 mg/kg) and vehicle treated rats was found (Figure 5.2B).

Changes in infarct volume and hemispheric swelling post-MCAO. We assessed effects of NL-1 administration on infarct volume and hemispheric swelling in aged female rats at 24 h after 2 h tMCAO with reperfusion. Using 2 mm brain sections stained in TTC (Figure 5.3A), we determined that NL1 (10 mg/kg) and NL-1 NP treated rats had significantly reduced infarct volumes by 45% and 69%, respectively, when compared to vehicle treated rats (Figure 5.3B). No difference ($p > 0.05$) in infarct volume was observed

between NL1 (0.25 mg/kg) and vehicle treated rats (Figure 5.3B). A significant decrease in hemispheric swelling of 35% in NL1 (10 mg/kg) and 59% in NL-1 NP treated rats was demonstrated when compared to vehicle treated rats (Figure 5.3C).

Iron accumulation in the brain post-MCAO. Using Perl's stained brain sections with DAB enhancement, we evaluated the effect of NL-1 and NL-1 NP treatment on accumulation of non-heme iron in the brain at 72 h post-tMCAO. Figure 5.4A is a representative micrograph of iron staining in the naïve brain of an aged rat. As the figure depicts, there are a few areas of iron staining localized within neurons and around blood vessels, which is a typical observation seen in aged rodents (Hagemeier *et al.*, 2014). Figure 5.4B illustrates a profound increase in iron accumulation in the brain at 72 h post-MCAO in rats treated with vehicle. Rats treated with low dose of NL-1 (0.25 mg/kg) showed increased iron accumulation in perilesional areas surrounding the infarct at levels comparable to vehicle treated rats (Figure 5.4C). Figure 5.4D and E reveal a marked decrease in iron staining in brain sections surrounding the infarct at 72 h post-stroke in rats treated with NL-1 (10 mg/kg) and NL-1 NP, respectively.

Changes in BBB permeability post-MCAO. Changes in BBB permeability were assessed at 72 h after post-MCAO using IgG immunostaining in brain sections. After the brain was perfused, the only detectable IgG would be due to IgG that left the vascular space. Figure 5.5 demonstrates a marked reduction in IgG levels after treatment with NL-1 (10 mg/kg) and NL-1 NP as compared to vehicle. In contrast, NL-1 (0.25 mg/kg) showed no reduction in IgG staining. To quantitatively assess improved BBB permeability

observed in the NL-1 (10 mg/kg) and NL-1 NP treated rats at 72 h post-stroke, we measured extravasation of sodium fluorescein (230 Da) and albumin (~65 kDa) using fluorimetry analysis of brain homogenate. Results indicated no difference ($p > 0.05$) in transient BBB permeability to sodium fluorescein or albumin at 72 h post-stroke in any of the treated groups as compared to sham rats.

Changes in lipid peroxidation post-MCAO. At 72 h after tMCAO, levels of lipid peroxidation were visualized by fluorescent immunostaining using 4-HNE as a protein adduct marker. Figure 5.6 shows that 4-HNE staining was noticeably decreased in aged rats treated with NL-1 (10 mg/kg) or NL-1 NP. NL-1 (0.25 mg/kg) did not provide noticeable protection against lipid peroxidation as indicted by 4-HNE staining when compared to vehicle treated rats.

Changes in TUNEL positive cells post-MCAO. At 72 h after tMCAO, the number of TUNEL positive cells was markedly reduced in the NL-1 (10 mg/kg) and NL-1 NP treated rats as compared to vehicle treated rats (Figure 5.7A). No change in the number of TUNEL positive cells was visualized between NL-1 (0.25 mg/kg) treated rats and vehicle treated rats (Figure 5.7A). Aggregate counting of TUNEL positive cells demonstrated that treating rats with NL-1 (10 mg/kg) or NL-1 NP significantly ($p < 0.0001$) reduced the number of TUNEL positive cells in rats as compared to vehicle and NL-1 (0.25 mg/kg) treated rats (Figure 5.7B). Vehicle and NL-1 (0.25 mg/kg) treated rats showed a significantly ($p < 0.0001$) higher number of TUNEL positive cells as compared to sham rats (Figure 5.7B). No difference ($p > 0.05$) in TUNEL positive cells was measured

between vehicle and NL-1 (0.25 mg/kg) treated rats. No difference ($p > 0.05$) in TUNEL positive cells was shown for NL-1 (10 mg/kg) or NL-1 NP treated rats when compared to sham rats (Figure 5.7B).

NL-1 concentration in rat brain microvessels. A starting bolus dose of NL-1 or NL-1 NP was administered through the tail vein to provide a starting NL-1 blood concentration of 3.7 μM (NL−1) and 0.09 μM (NL-1 NP) for distribution. At 1 h after administration, the brain was excised and microvessels were isolated from the brain parenchyma. Results showed the brain concentration of NL-1 to be 287 ± 125 ng/ml in the NL-1 (10 mg/kg) treated rats and 143 ± 29 ng/ml in the NL-1 NP treated rats. The concentration of NL-1 in the isolated microvessels was 55 ± 10 ng/ml in NL-1 treated rats and 1693 ± 671 ng/ml in NL-1 NP treated rats.

DISCUSSION

This was the first study to investigate the use of a mitoNEET ligand as an ischemic stroke therapeutic in aged animals. Results from the study demonstrate that the mitoNEET ligand, NL-1, significantly improved survival, reduced ischemia/reperfusion injury at 24 h and decreased accumulation of parenchymal protein extravasation, lipid peroxidation and cell death around the infarct area at 72 h after a 2 h ischemia with reperfusion. Moreover, this study revealed that by nano-encapsulating NL-1 using PGLA, an equivalent degree of protection as seen with NL-1 at 10 mg/kg could be produced using a 40-fold lower dose of (0.25 mg/kg).

MitoNEET is an outer mitochondrial membrane protein belonging to the zinc finger protein family. The presence of a [2Fe-2S] cluster in the dimer allows for mitoNEET to act as a redox active sensor that modulates mitochondrial bioenergetic function (Saralkar *et al.*, 2021). Previous studies showed that if mitoNEET were reduced in vivo, mitochondrial deficits were observed, including disrupted oxidative phosphorylation, electron transport chain deficits, increased ROS (hydrogen peroxide and superoxide) production and ultrastructural changes in the mitochondrial cristae (Maier *et al.*, 1998; Wiley *et al.*, 2007). These changes in mitochondrial function were accompanied by reduced expression of the antioxidant defense proteins, superoxide dismutase 2 and glutathione peroxidase (Geldenhuys *et al.*, 2021). On the other hand, transgenic overexpression of mitoNEET provided for improved mitochondrial bioenergetics, reduced inflammation and decreased ROS production, as has been shown in other aged models of disease (Geldenhuys *et al.*, 2017; Chang *et al.*, 2018; Joffin *et al.*, 2021).

The mitoNEET ligand NL-1 was designed as a tool compound to study mitoNEET physiology without PPAR-γ agonist activity (Colca *et al.*, 2004, 2013; Kusminski *et al.*, 2012; Divakaruni *et al.*, 2013; Nadareishvili *et al.*, 2019). NL-1 was found to provide tissue sparing effects in a mouse model of traumatic brain injury, an effect which was lost in mitoNEET knockout (−/−) mice, as demonstrated by both immunohistochemistry and behavioral measures; thus, supporting a mitoNEET-based mechanism of action (Yonutas *et al.*, 2020). Furthermore, mitoNEET ligand, TT01001 (Takahashi *et al.*, 2015), was recently shown to provide neuroprotection against subarachnoid hemorrhage in a rodent model by preventing mitochondrial dysfunction (Shi *et al.*, 2020). These findings provide a robust scientific foundation for evaluating mitoNEET as a therapeutic target using a

clinically relevant preclinical ischemic stroke model. In a previous study, we reported that NL-1 administration provided protection against ischemia/reperfusion injury post-stroke in young male mice (Saralkar *et al.*, 2021). Furthermore, secondary off-target pharmacology revealed that NL-1 did not appreciably interact with known kinases or GPCR targets (Saralkar *et al.*, 2021). These findings prompted us to evaluate NL-1 in a model of cerebral ischemia/reperfusion injury using aged female rats. Since stroke is an age-associated disease, it is critical to evaluate the effectiveness of any novel therapeutic agent in an aged model of disease. As we have previously reported along with others, brain injury and recovery after ischemic stroke is markedly different between aged and young animals (DiNapoli *et al.*, 2008, 2010; Kelly *et al.*, 2009; Tan *et al.*, 2013, 2015; Tang *et al.*, 2016; Spychala *et al.*, 2018; Balseanu *et al.*, 2020; Davis *et al.*, 2020; Panta *et al.*, 2020; Banerjee *et al.*, 2021). In the current study, we found that NL-1 was able to provide significant protection in aged female rats against cerebral tissue damage induced by tMCAO with reperfusion. A caveat to our findings is that we only evaluated aged female rats due to reduced sexual dimorphism in weight between aged females as compared to aged males. Whereas, young males are often preferred over young females due to confounding issues with estrus cycle, in studies using aged females, which are reproductively senescent, they are more comparable in weight to younger females; thus, reducing confounding factors of needing to adjust surgical parameters (i.e. filament diameter and length, percentage of anesthesia, volume of fluid replacement) as would need to be done in comparative studies between aged female and aged male rats. In follow-up experiments, aged male rats will be included to evaluate for sex as a biological variable, as several recent studies suggest important differences in ischemic stroke

progression and recovery are sex dependent (Manwani *et al.*, 2015; Cohan *et al.*, 2019; Seifert *et al.*, 2019; El-Hakim *et al.*, 2021). As an aside, we have now reported positive findings of NL-1 as a central acting mitoceutical in young and aged, male and female, mice and rats and following ischemic stroke and traumatic brain injury; thus, suggesting that modulation of mitoNEET effectively reduces mitochondrial dysfunction post-neurological injury, regardless of these variables (Yonutas *et al.*, 2020; Saralkar *et al.*, 2021).

A common measure of neuroprotection following preclinical stroke is functional improvement as measured using a composite neurological scale. These measures are typically performed starting at 24 h after MCAO and can be as simple as the Bederson scale (Bederson *et al.*, 1986), which evaluates forearm flexion, resistance to lateral push and circling to more sophisticated neurological scales, such as the mNSS, which evaluates multiple neurological parameters, including motor, sensory, reflex and balance (Zhang *et al.*, 2002). Unfortunately, these neurological scales, which rely heavily on changes in sensorimotor performance, have proven to be ineffective at discerning post-stroke improvements in stroke outcomes in aged rodents (Turner *et al.*, 2012). The sickness behavior scale reduces the impact that diminished motor function and coordination has on post-stroke functional assessments in aged rodents. The sickness behavior scale tracks changes in physical status (body weight/temperature and food/water consumption), self-care (appearance, grooming, posture) and social parameters (home cage activity and social interactions). By tracking changes in these measures, more subtle changes in neural injury progression and recovery can be discerned even by aged rodents. In this study, scores on the mNSS indicated that only

the NL-1 NP group displayed any improved functional outcome at 24 h post-tMCAO with reperfusion; however, the overall improvement in health of the rat at 24 h post-tMCAO was potentially masked in the NL-1 (10 mg/kg) and NL-1 NP treatment groups as motor and balance assessments were obscured by the lack of movement and hesitancy observed in the aged rats even before MCAO. The sickness scale scores showed a more robust improvement post-stroke for both the NL-1 (10 mg/kg) and NL-1 NP groups as compared to vehicle treated rats.

NL-1 has been previously reported to provide protection against oxygen-glucose deprivation, mimicking reperfusion injury, in murine vascular endothelial cells (bEnd3 cells) (Saralkar *et al.*, 2020). The bEnd3 cells are routinely used as a BBB model, as they form similar tight junctions and selective permeability of small organic molecules (Geldenhuys *et al.*, 2015; Dubey *et al.*, 2019). In our stroke model using aged rats, we found similar evidence of BBB augmentation with use of NL-1. The vascular marker, IgG, was elevated at 72 h after experimental stroke with reperfusion in the brains of rats treated with vehicle, which is indicative of increased IgG accumulation due to BBB disruptions over the 72 h. NL-1 and NL-1 NP treatments attenuated extravasation of IgG at the BBB, suggesting that the protection seen after ischemic stroke may, in part, be due to preservation of BBB function. Increased hemispheric swelling, as indicated by IgG accumulation in the infarct, post-stroke has been associated with deleterious effects on neuronal survival (DiNapoli *et al.*, 2008), as well contributing to the vascular dementia seen in patients who survive a stroke (Nwafor *et al.*, 2019; Sarvari *et al.*, 2020). Due to NL-1 being shown to have moderate BBB permeability in the mouse, we formulated NL-1 into PLGA-based nanoparticles to improve tissue distribution due to a short half-life (<3

h) (Saralkar *et al.*, 2020, 2021). The promising results in vitro led us to evaluate the impact formulation of NL-1 into a NP would have on preventing ischemia/reperfusion post-stroke. NPs have been shown to improve the neuroprotective activity of select agents by several other groups, providing support for pursuing this avenue of drug delivery to the CNS (Mdzinarishvili *et al.*, 2013). We found that NL-1 NP allowed for a 40-fold reduction in drug dosing, from 10 mg/kg to 0.25 mg/kg with equivalent protection post-stroke. The improved outcomes observed with the lower dose NPs suggest that NL-1 is either not reaching the CNS in sufficient concentration as in vitro studies indicated or delivery to endothelial cells that comprise the BBB is more substantive in the mechanism of action than initially postulated. Since mitochondrial dysfunction in cerebral microvessels has been shown to play a role in BBB breakdown and increased permeability, targeting mitochondrial dysfunction as a therapeutic target to improve bioenergetics could be a novel approach for ischemic stroke treatment. While evidence suggests that NL-1 improves endothelial cell function, it is also possible that the therapeutic effect of NL-1 occurs at other brain cell types, such as neurons or astrocytes, and that improvements in BBB functional integrity and diminished edema formation are an indirect beneficiary of these effects; Prior data indicates that cerebral endothelial cells are almost 4 times more susceptible than neuronal cells and 16 times more susceptible than astrocytes to iron-induced mitochondrial dysfunction (Gaasch *et al.*, 2007); thus, the findings that NL-1 and NL-1 NP were found in high concentration in the isolated cerebral microvessels, 16% of total brain NL-1 concentration in NL-1 (10 mg/kg) treated rats and 92% of total brain NL-1 concentration in NL-1 NP treated rats, may point to improved endothelial cell function playing a role in the beneficial effects of NL-1 treatment post-stroke. Future studies will

probe, at a mechanistic level, how the modulation of mN within cerebral endothelium impacts mitochondrial bioenergetics and antioxidant capacity, especially as it applies to barrier functions and gain a clearer understanding of the pharmacokinetic profile of nanoencapsulated NL-1.

Mitochondrial dysfunction occurring following ischemic stroke with reperfusion results in a significant increase in lipid peroxidation. A primary driver of cellular lipid peroxidation and an indicator of mitochondrial dysfunction is excessive iron accumulation in the brain. Results of our study, show a profound increase in iron accumulation in the perilesional area in vehicle and NL-1 (0.25 mg/kg) treated rats as compared to naïve control rats. This marked increase was effectively mitigated in rats treated with NL-1 (10 mg/kg) or NL-1 NP; thus, suggesting that mitochondrial performance in cells near the infarct were improved with NL-1 treatment. One of the byproducts of oxidative stress is 4-HNE, which contains aldehyde functionality to form protein adducts (Osakada *et al.*, 2021). Proteomic analysis has indicated that mitoNEET, which is rich in lysines, binds covalently with 4-HNE resulting in changes in the redox character of the [2Fe-2S] clusters (Arnett *et al.*, 2019). When rats were treated with NL-1 (10 mg/kg) or NL-1 NP, there was a noticeable reduction in 4-HNE staining. Since elevated levels of 4-HNE contribute to neuronal cell loss, this reduction in 4-HNE observed may be a prognostic biomarker of the overall tissue sparing seen with NL-1 and NL-1 NP treatment.

To limit confounding factors, such as increased hemorrhage risk and spontaneous recanalization, these studies targeted a 2 h ischemic brain injury with reperfusion. Thus, these studies modeled an ischemic brain injury eligible for recanalization with tissue plasminogen activator, which accounts for 3–7% of stroke patients. Future studies will be

performed to establish applicable therapeutic time window out to at least 6 h after onset of ischemia. In addition, we will probe the effects of permanent occlusion (majority of ischemic strokes) on NL-1 efficacy; however, we postulate the effects will be diminished based on prior results of permanent MCAO in young male mice (Saralkar *et al.*, 2021). Finally, future studies will extend the post-stroke evaluation period out to several weeks to months to evaluate long-term recovery. Based on the results of these studies, especially the marked reductions in mortality and edema observed, we posit that it is highly likely these changes in the degree of ischemic brain injury will not be transient but will represent marked improvements in recovery that extend into the chronic phase of post-stroke recovery.

CONCLUSION

In this study, we report for the first time that the therapeutic use of the mitoNEET ligand NL-1 at a dose of 10 mg/kg resulted in a marked improvement in survival, attenuated infarct volume and reduced hemispheric swelling at 24 h after tMCAO with reperfusion in aged female rats. Moreover, when NL-1 was encapsulated in PGLA nanoparticles, this therapeutic benefit was again produced at a dose that was 40-fold less than unencapsulated NL-1. The improved measures of functional outcome using a sickness scale along with improved BBB function and reduced indicators of oxidative stress reinforce the therapeutic potential of probing mitoNEET for treatment of ischemic stroke with reperfusion. Showing the robust efficacy of NL-1 in aged rats is particularly important as ischemic stroke is a disease of the elderly and the prior failures of many stroke neuroprotectants was due, in part, to use of animal models that did not reflect the

population most vulnerable to ischemic stroke. Taken together, mitoNEET represents a novel drug target in reperfusion-injury post stroke that warrants further investigation.

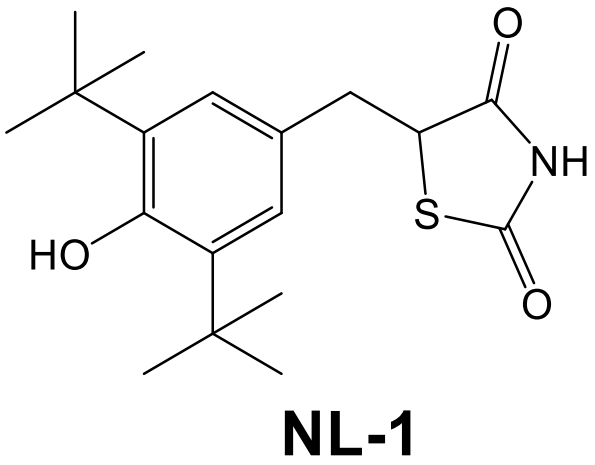

Figure 5.1. Structure of the mitoNEET ligand, NL-1.

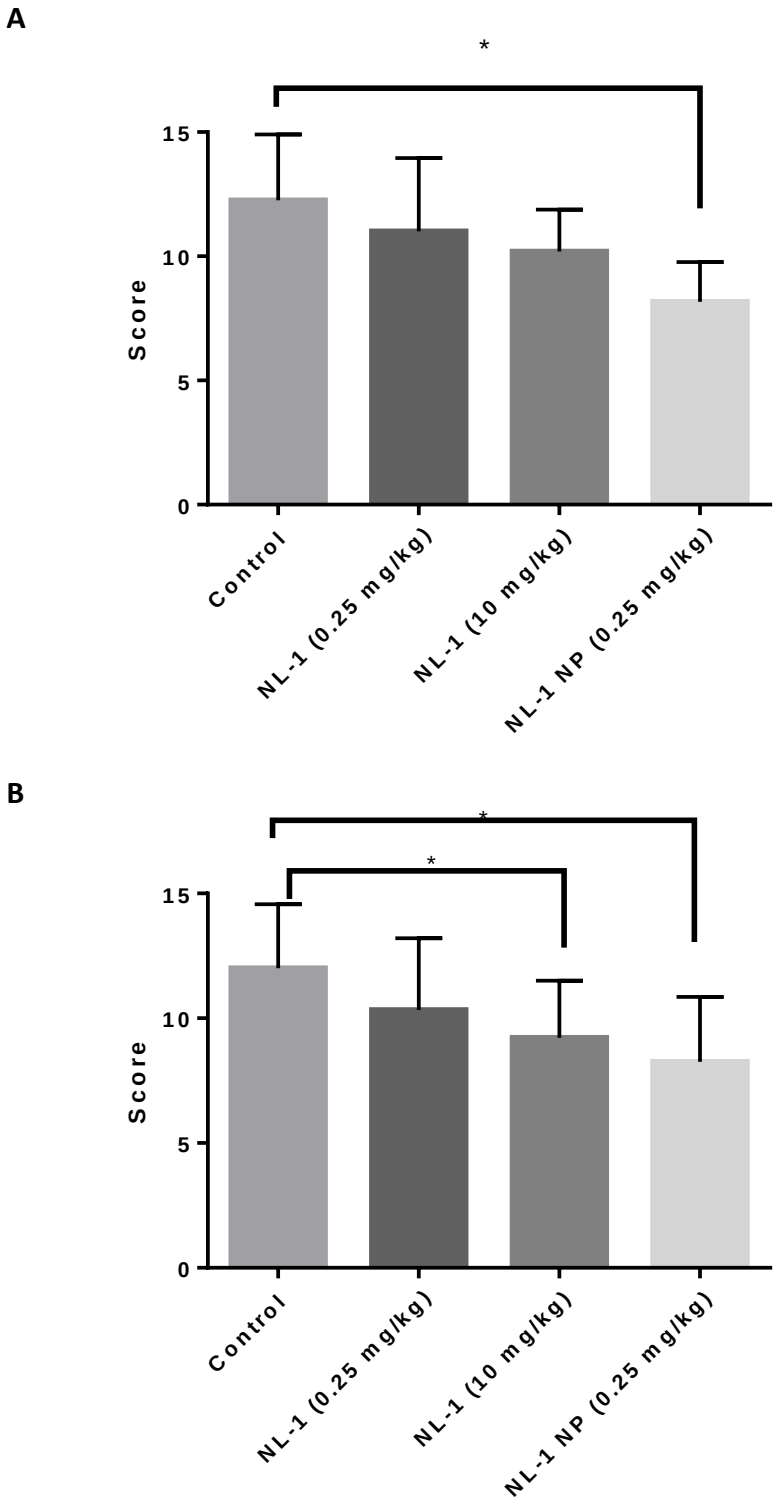

Figure 5.2. Neurological assessment of NL-1 treatment at 24 h post-tMCAO with vehicle, NL-1 (0.25 mg/kg), NL-1 (10 mg/kg) and NL-1 nanoparticles (0.25 mg/kg). (A) mNSS behavior and (B) health/sickness scale ($*p < 0.05$). Data reported as mean ± S.D.

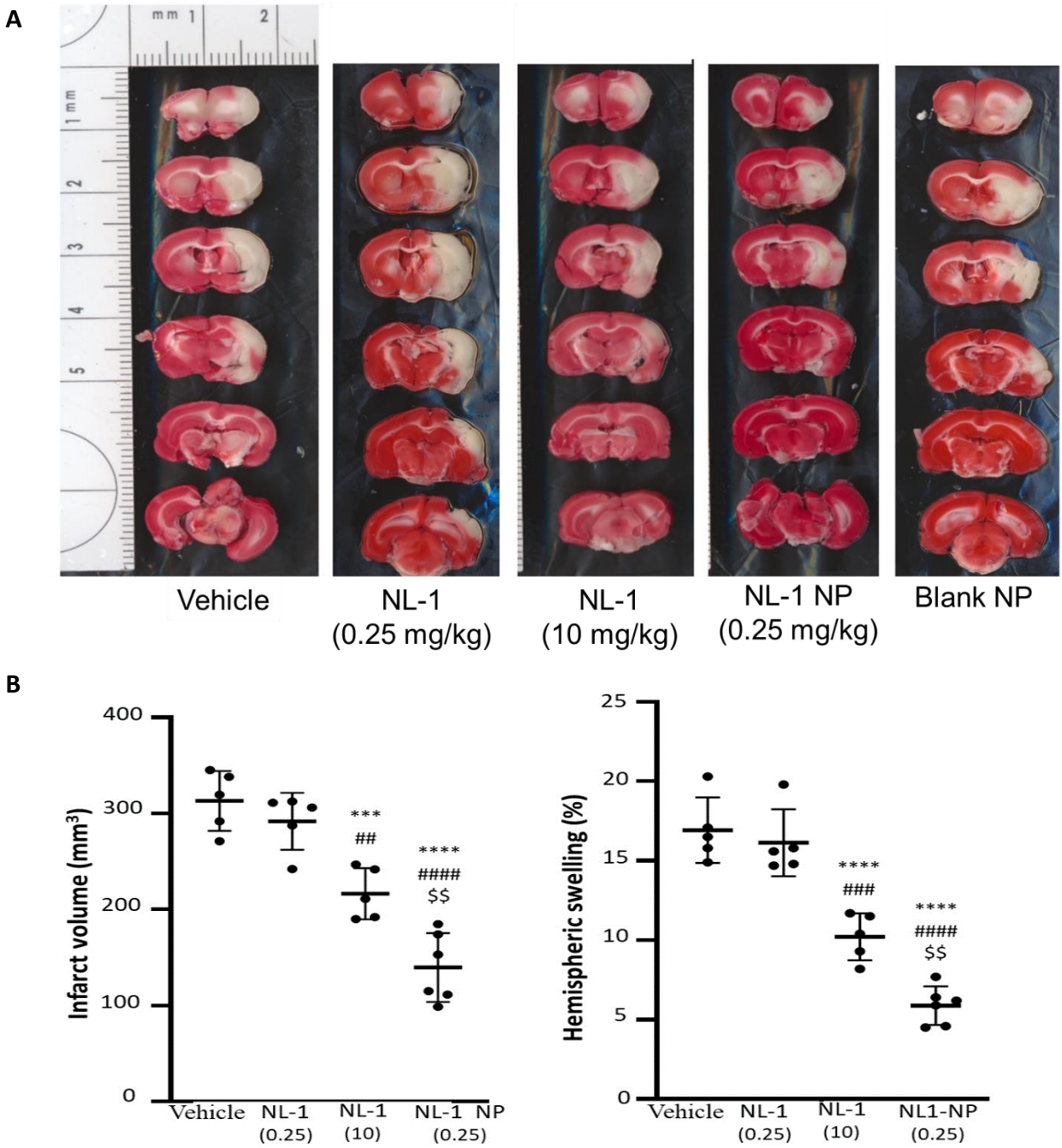

Figure 5.3 Effect of NL-1 treatment on infarct volume and hemispheric swelling. (A) Representative TTC stained brain sections for vehicle, NL-1 (0.25 mg/kg), NL-1 (10 mg/kg) and NL-1 nanoparticles. Effect of NL-1 and NL-1 nanoparticle treatment on (B) infarct volume and (C) hemispheric swelling at 24 h after tMCAO (* denotes difference compared to vehicle, $^{\#}$ denotes difference compared to NL-1 (0.25 mg/kg) and $^{\$}$ denotes difference compared to NL-1 (10 mg/kg); $^{\#\#,\$\$}p < 0.01$, ***, $^{\#\#\#}p < 0.001$, ****, $^{\#\#\#\#}p < 0.0001$). (n = 5 rats/treatment). Data reported as mean ± S.D.

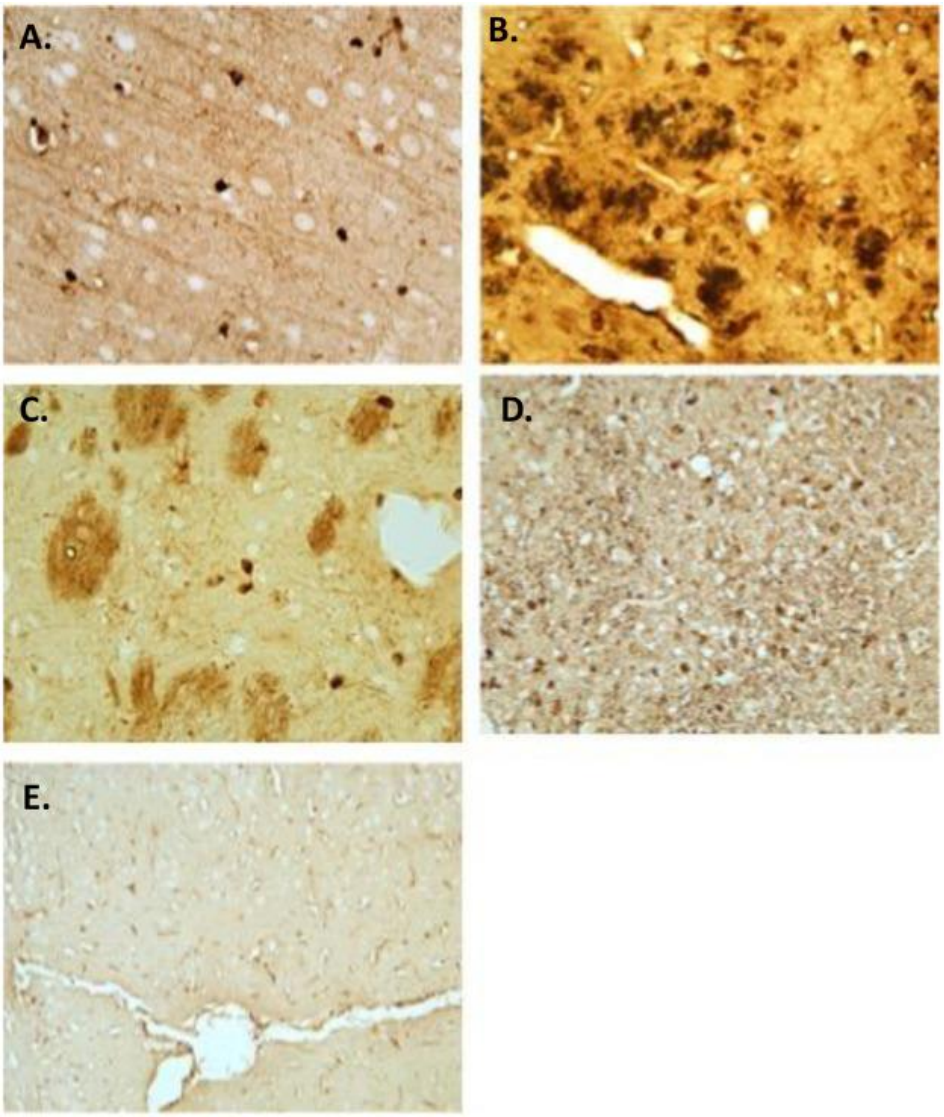

Figure 5.4. Effect of NL-1 on iron accumulation in the brain. At 72 h following tMCAO, iron content in perilesional areas of the brain were visualized in sections stained using Perl's method with DAB enhancement. All brain sections were assessed in comparison to (A) sham and (B) vehicle treated rats. Treatment groups consisted of (C) NL-1 (0.25 mg/kg), (D) NL-1 (10 mg/kg) and (E) NL-1 NP treated rats. Sections were visualized at 20× using a brightfield microscope. n = 3–4 rats/treatment.

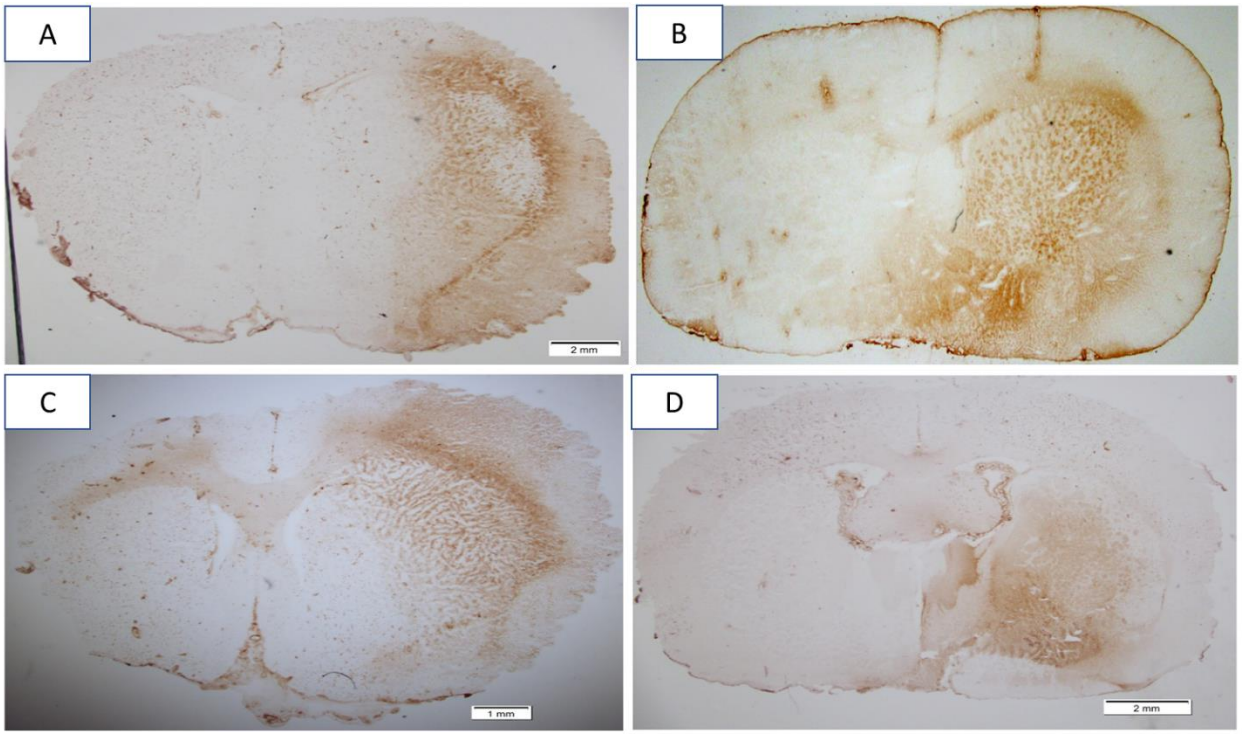

Figure 5.5. Qualitative assessment of NL-1 treatment on IgG extravasation. At 72 h following tMCAO, extravasation of IgG in the brain was visualized in sections using immunohistochemistry. Brain sections were assessed in comparision to (A) vehicle treated rats. Treatment groups consisted of (B) NL-1 (0.25 mg/kg), (C) NL-1 (10 mg/kg) and (D) NL-1 NP treated rats. Sections were visualized at 20× using a brightfield microscope. N=3-4 rats/treatment.

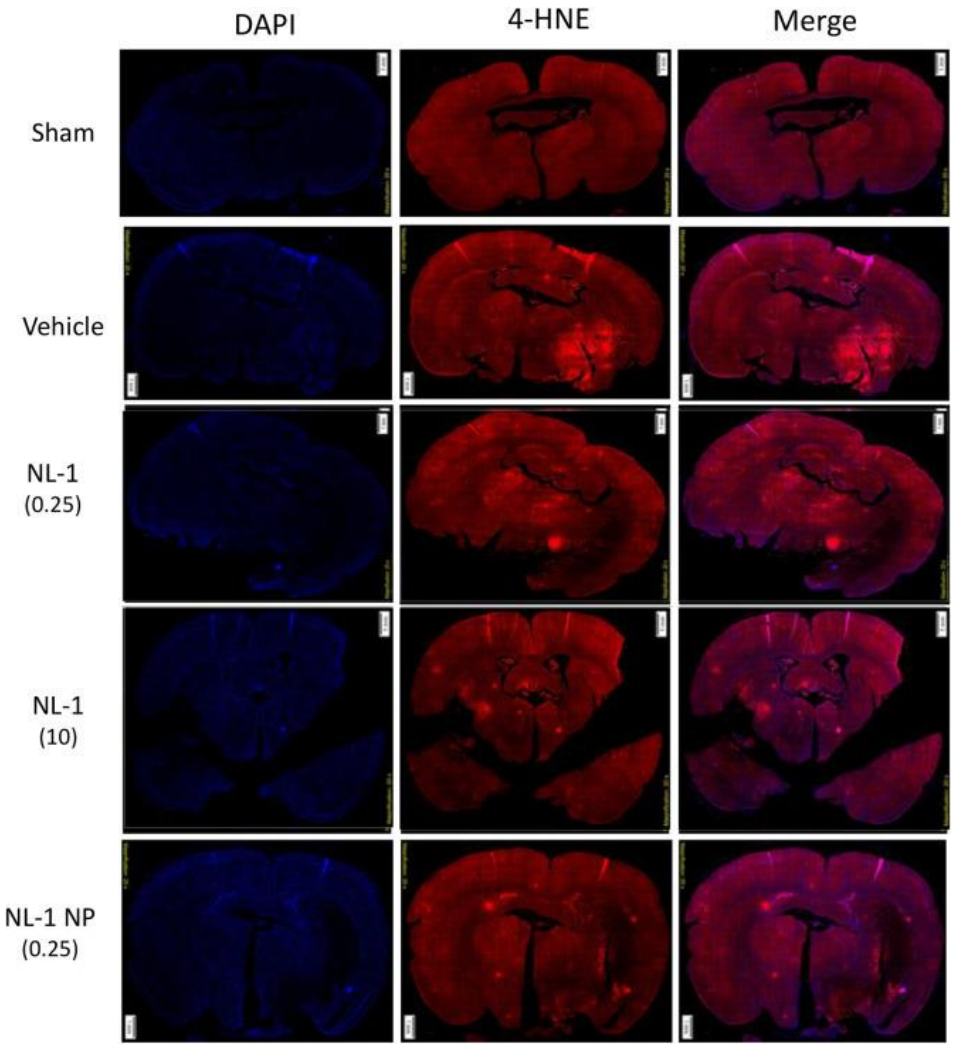

Figure 5.6. Qualitative assessment of NL-1 treatment on 4-HNE protein adduct staining. At 72 h following tMCAO, lipid peroxidation in the brain was visualized using immunofluorescence for 4-HNE. Brain sections were assessed in comparison to (A) sham and (B) vehicle treated rats. Treatment groups consisted of (C) NL-1 (0.25 mg/kg), (D) NL-1 (10 mg/kg), and (E) NL-1 NP. Sections were visualized at 20× using an epifluorescent microscope. n = 3–4 rats/treatment.

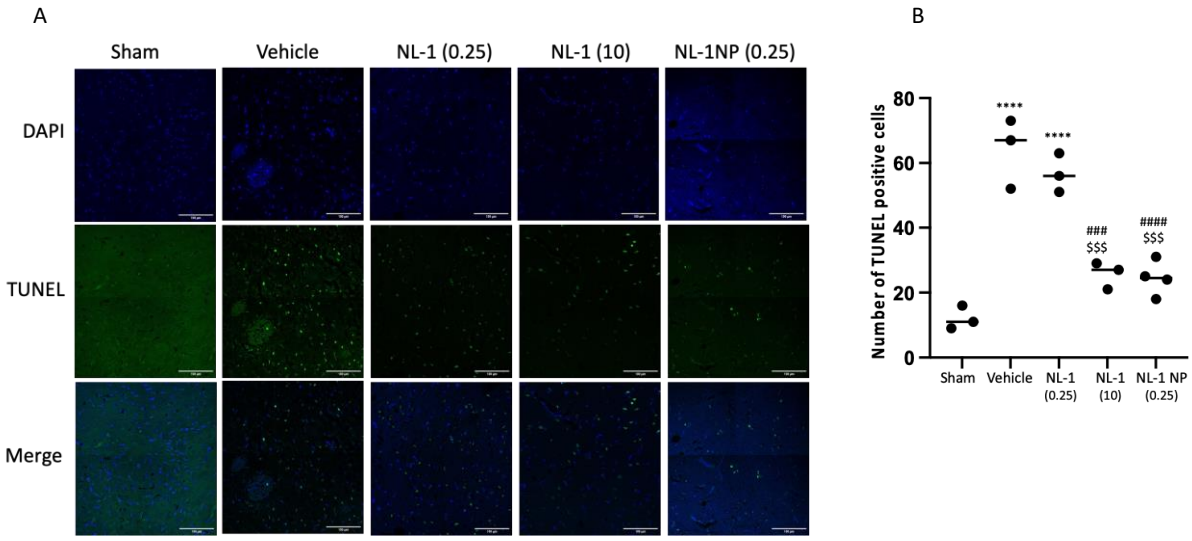

Figure 5.7. Effect of NL-1 treatment on apoptosis. At 72 h following tMCAO, cell death (apoptosis) in the perilesional areas surrounding the core of the infarct were (A) visualized using an immunofluorescent TUNEL staining kit. Brain sections were assessed in comparison to vehicle treated rats. Treatment groups consisted of NL-1 (0.25 mg/kg), NL-1 (10 mg/kg), and NL-1 NP. Sections were visualized at 40× using a confocal microscope. n = 3–4 rats/treatment. (B) Quantitation of number of TUNEL positive cells was performed. **** denotes $p < 0.0001$ difference compared to sham. ### and #### denotes $p < 0.001$ and $p < 0.0001$ difference compared to vehicle treated rats. $$$ denotes $p < 0.001$ difference compared to NL-1 (0.25 mg/kg). n = 3–4 rats/treatment. Data reported as mean ± S.D.

Table 5.1 Health/sickness screen scale

Parameter	Observation	Score
General Appearance	Normal Groomed, healthy appearing fur, pink mucous membranes and ear lobes	0
	Mild Abnormal Mildly rough/scruffy/dull fur, slightly less well-groomed, light pink mucous membranes/ear lobes, minimal porforin staining, slightly squinted eyes	1
	Moderate Abnormal Rough/scruffy fur, piloerection, poor grooming, pale mucous membranes, and ear lobes, squinted eyes	2
	Severe Abnormal Very rough fur, no evidence of grooming, white mucous membranes, and ear lobes, substantial porforin staining, severely squinted or closed eyes	3
Posture	Normal	0
	Slight Hunch Spine slightly curved	1
	Moderate Hunch Spine curved, paws slightly under body	2
	Severe Hunch Spine dramatically curved, paws tucked under body, head angled downward	3
Body Condition	Normal	0
	Thin Slight segmentation of vertebrae, dorsal pelvic bones are more prominent, slight dehydration (skin pinch test response is slightly delayed)	1
	Emaciated Prominent vertebrae and skeletal bones that are readily palpable, dehydrated (skin pinch test results in skin remaining tented)	2
Respiration	Normal	0
	Altered Increased rate and/or effort	1
	Abnormal/Distressed Very increased rate or gasping/labored breathing, irregular	2
Body Temperature	Normal/No change	0
	1-4 degree C	1
	5-8 degree C	2
	9-12 degree C	3
Body Weight	0-5% change	0
	5.1-10% change	1
	10.1-15% change	2
	15.1-20% change	3
	> 20.1% change	4
Spontaneous Locomotion/Social Interaction	Normal Active and interacting with cage-mate(s)	0
	Mild Abnormal Still spontaneous activity and some peer interaction but reduced	1
	Moderate Abnormal Lethargic (may need probing via tapping on cage or cage tilt) and minimal peer interaction	2
	Severe Abnormal Immobile and no peer interaction	3

Table 5.2: Modified Neurologic Severity Scale (mNSS)

Subtest		Score
1. Reflex test		**3**
	Corneal Reflex – lack of eye blink when cornea is touched	1 (0.5/eye)
	Pinna Reflex – lack of head shake when pinna is touched	1 (0.5/ear)
	Startle – lack of jumping, freezing, moving when loud noise is heard	1
2. Walking test		**3**
	Normal	0
	Inability to walk straight	1
	Circling/falling toward paretic side	2
	Immobile (despite probing)	3
3. Placing test		**3**
	Visual- lack of placement	1 (0.5/paw)
	Tactile – lack of placement	1 (0.5/paw)
	Proprioceptive – lack of placement	1 (0.5/paw)
4. Beam balance test		**6**
	Balances steadily or traverses beam to clamp	0
	Grasps sides of beam but is generally steady	1
	Balances unsteadily, hugs beam and/or 1 limb slips/falls down	2
	Balances unsteadily, hugs beam, 2 limbs slip/fall down	3
	Attempts to balance but very unsteady, falls under beam but recovers	4
	Attempts to balance but spins, clings to underside of beam	5
	No attempts to balance	6
5. Inverted test		**3**
	Forelimb flexion or limb not moving to aid with balance	1
	Hindlimb flexion or limb not moving to aid with balance	1
	Head moved more than 10 degrees from vertical center or persistent spin	1
	Total	**18**

Chapter Six

GENERAL DISCUSSION

SUMMARY

In the last decades tremendous advances in the field of biomedical sciences have increased the life span of individuals. As a consequence, there is growing number of older people (>65 years) living with chronic diseases and disability. In spite of age being the most important risk factor for diseases such as cancer, heart disease, neurodegenerative conditions, osteoporosis, arthritis, diabetes, sarcopenia and macular degeneration (Kirkwood and Tipton, 2017), older patients are generally underrepresented in clinical trials. Drugs showing efficacy in younger population may have completely different outcomes (adverse reactions) when tested in elderly. This might be due innate irreversible physiological alterations occurring due to aging which may ultimately manifest in chronic diseases. Therefore, it is pivotal to understand the biology of aging as well as characterize the effects of aging in healthy and pathological conditions, which is the focus of this dissertation. This knowledge will aid in the drug development process and thus may significantly improve the translational potential of novel therapeutic agents for the treatment of age-related chronic diseases.

The process of CNS drug development is challenging and time consuming. The success of CNS clinical trials can be improved by gaining a clear understanding of pathophysiology of CNS diseases as well as adverse side effects of the drugs on the CNS. Additionally, evaluating the efficacy of drugs to cross the BBB is utmost important in determining CNS therapeutic outcomes. One of the focus areas of our lab is studying the in vivo efficacy of therapeutic candidates using clinically relevant neuronal injury models. In the past, we have extensively studied the neuroprotective effects of bryostatin-

1, a PKC modulator for the treatment of cerebral ischemia and TBI. In vitro and in vivo studies conducted by others reported enhanced synaptogenesis, improved cognition, ant-inflammatory effects. Additionally, bryostatin-1 has shown to be effective in treatment of HIV-1 brain infection by eradicating CNS viral reservoirs. Bryostatin-1 has not only shown to penetrate the BBB and exert its actions but also has minimal adverse effects at therapeutic (low-moderate) doses. Following favorable outcomes in pre-clinical studies, bryostatin-1 has recently progressed to human trials for the treatment of MS, AD and Fragile X syndrome.

While age is considered a primary risk factor for neurological diseases/disorders, it is also a major determinant in the development and testing of pharmacological compounds targeting the CNS by altering the drug exposure and therapeutic outcomes. The process of biological aging affects every cell, tissue, and organ leading to anatomical, immunological, and physiological changes in our body. Given the importance of structural and functional decline in the CNS as well as immunosenescence in impacting neurodegeneration, the main goal of this dissertation was to

1) Characterize the age-related differences on cerebrovascular topology
2) Characterize the age-related differences on peripheral immune response following systemic infection
3) Test the therapeutic efficacy of a novel ligand NL-1, in the aged rodent model of cerebral ischemia reperfusion injury

This chapter concludes with a brief summary and suggestions for future experiments.

Brain vasculature dictates the supply of oxygen and nutrients and is essential for normal cellular metabolism. However, with advancing age, the cerebral vascular network undergoes structural and functional remodeling affecting the neuronal microenvironment. Several studies have proposed that dysfunctional vasculature precedes cognitive decline as seen in case of AD. So, we first sought to characterize differences in the morphology of cortical vasculature of young versus aged female rats using classical angiographic methods. Additionally, we evaluated the complexity of cortical vessel network using lacunarity measures. Once we analyzed differences in global cortical vessel structure, we also wanted to quantify the degree to which these classic vessel parameters are altered in the middle cerebral artery (MCA), the major artery supplying blood to the brain. We found reduced vessel density, increased lacunarity and decreased complexity at global level as well as at MCA level. The results from this study confirmed that the cerebral vasculature structure is modified due to aging which could potentially alter brain perfusion contributing to the pathophysiology of age-related neurological disorders.

After understanding the changes occurring at the cerebrovascular architecture with increasing age, we next moved on to quantifying age-related alterations in the peripheral immune system. The immune system is vital in maintaining homeostasis and providing protection against foreign pathogens. With advancing age, the immune system undergoes a remodeling process termed as immunosenescence which contributes to increased vulnerability to cancer, autoimmune diseases, and infections. Additionally, aged immune system is prone to systemic chronic low-grade inflammation – termed as inflamm-aging. Still, what remains unknown is, how the immune system reacts to systemic inflammation in aged subjects. Therefore, we wanted to determine the

composition of key leukocyte population in circulation as well as spleen due to aging in rodents. We also investigated the expression levels of cytokines and chemokines in plasma following LPS induced acute systemic inflammation at two different time points - 3 and 24h. Given the importance of spleen in hosting a range of immunological functions, we found significant immunosuppression in the spleen of aged female rats irrespective of treatment suggesting reduced capacity to retain the immune cells due to aging. We also revealed dysregulated cytokine/chemokine levels in the aged animals confirming the presence of age-related immunosenescence and inflamm-aging phenotype. Taken together, these findings are in line with previous literature and underscore the importance of using age-appropriate animals while studying specific diseases.

Lastly, we wanted to test the therapeutic efficacy of a novel pharmacological agent in an age-related neurological disease in vivo. Case in point is cerebral ischemia, which disproportionally affects the elderly leading to increased death and disability. Till date, reperfusion with tPA remains the golden standard for the treatment of stroke. However, it has narrow therapeutic window (3-4.5h after onset) and not everyone is eligible to receive the drug. Over the years, several pharmacological agents have been developed and yet not a single candidate has shown efficacy in the clinics. Therefore, there exists an urgent need to develop therapeutics alone or in combination with tPA for the treatment of cerebral ischemia. Mitochondrial dysfunction plays a central role in the neurodegeneration following ischemic-reperfusion injury. MitoNEET (mN), an outer mitochondrial membrane protein has been shown to regulate bioenergetic capacity by acting as a redox and pH sensor. Previous studies showed that targeting mN using a novel ligand called NL-1 has demonstrated neuroprotective effects following cerebral

ischemia, traumatic brain injury and Parkinson's disease. In the current study, we hypothesized that by potentiating mitochondrial activity, neuronal survival in the penumbra will be enhanced leading to smaller infarcts and improved functional outcomes. We tested the therapeutic outcomes of two doses of NL-1 as well as nanoparticle formulation of the drug in an aged female rat model of cerebral ischemia/reperfusion injury. We found significant reduction in infarct volume and hemispheric swelling in a dose-dependent manner. Interestingly, the nanoparticle encapsulated NL-1 at 0.25mg/kg showed improved therapeutic outcomes. In addition, NL-1 administration decreased oxidative stress as well as neuronal apoptosis in the penumbra region at 72h following stroke indicating its neuroprotective effects. This correlated with possible rescue of BBB function as evidenced by reduced IgG accumulation in the brain parenchyma upon NL-1 treatment. These results demonstrated that targeting mitoNEET via NL-1 holds promise as a novel strategy for the treatment of cerebral ischemia reperfusion injury.

FUTURE DIRECTIONS

Owing to various ethical reasons, studying aging on humans is difficult and so rodents are the preferred models to study age-related diseases. Future studies should focus on better modelling of neurological diseases in order to improve the translational potential of preclinical research. With advancing age, a great body of work research is a being performed to understand the evolution and mechanism of biological aging. The initial step toward developing novel therapeutics is to gain a detailed understanding of the cellular and molecular processes that promote or delay aging. Additionally, follow up studies are required to identify the potential links between neurological

disorders/diseases and hallmarks of aging. Elucidation of these pathways will open up new treatment avenues. However, therapeutic interventions targeting a single pathway may not be successful. A combination of improved multimodal approach will be necessary for effective treatment of age-related chronic diseases.

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