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REJUVENATION OF THE EPIGENETIC LANDSCAPE OF THE AGED BRAIN

THROUGH MANIPULATION OF CIRCULATING FACTORS

by

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REJUVENATION OF THE EPIGENETIC LANDSCAPE OF THE AGED BRAIN THROUGH MANIPULATION OF CIRCULATING FACTORS

A

DISSERTATION

Presented to the Faculty of

The University of Texas

MD Anderson Cancer Center UTHealth

Graduate School of Biomedical Sciences

in Partial Fulfillment

of the Requirements

for the Degree of

DOCTOR OF PHILOSOPHY

by

Edward Charles Koellhoffer, B.S.

Houston, Texas

May 2019

Dedication

This thesis is dedicated to my parents, who have always supported me without question in all of my endeavors.

Acknowledgements

First and foremost, I would like to acknowledge my parents who have always been supportive of me in all of my endeavors. They have never deterred me from pursuing my dreams or attempted to limit my aspirations. Instead they have always done their absolute best to help me. When I was a child, my brother and I would collect various items from the woods on hikes to keep on our “science table” in the basement. I was easily fascinated by the world around me, and my parents have allowed me to develop my scientific curiosity from a very young age. I owe all of my accomplishments to their continued support over the years. I know this has been a long journey for me since graduating high school, but they have always given me all the support I have ever needed.

I am forever grateful to my partner, Justin Harpie, who has been by my side throughout my graduate school training. He has been by my side and has helped me through the frustrations that can come with scientific research, but I have also had the pleasure of celebrating all of my successes with him. He has been by my side throughout my journey, and I would not have been able to accomplish all that I have without his continued, unwavering support.

I would like to thank the many members of our lab who have offered advice and assistance with my work. In particular, I would like to thank John d’Aigle, who joined our lab as an inexperienced research technician looking to gain laboratory experience. I have had the honor of mentoring him in numerous laboratory skills and have had the pleasure of watching him become a skilled, independent researcher. John has been essential in helping me in numerous aspects of my thesis research, and I am forever

indebted to him. I know that John will continue to grow and develop into a very strong, independent scientist and wish him the best of luck on his endeavors.

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I am thankful for the support and advice from my Advisory Committee, Drs. Robert Bryan, Andrew Bean, Jarek Aronowski, and Terry Walters. Transferring from one institution to another halfway through a thesis research project was no easy feat, but their support has allowed me to continue to complete my thesis research.

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I am grateful for the support of the National Institutes of Health, for having the confidence in me and support me in my MD/PhD training.

Finally, I would like to thank my mentor, Dr. Louise D. McCullough, who has provided me with continued support throughout my dissertation research. She gave me the academic freedom to pursue an area of research that interested me, and provided me with all the resources and connections I would need to successfully

pursue my interests. Louise takes her role as a mentor very seriously, and I would not have been able to achieve my academic accomplishments without her advice. I am forever grateful to Louise and am incredibly proud to call her my mentor.

REJUVENATION OF THE EPIGENETIC LANDSCAPE OF THE AGED BRAIN THROUGH MANIPULATION OF CIRCULATING FACTORS

Edward Charles Koellhoffer, B.S.

Advisory Professor: Louise Deborah McCullough, M.D., Ph.D.

The aging population of the United States is expanding at an alarming rate. The Center for Disease Control and Prevention estimates that the population of those age 65 years and older will reach over 50 million by 2020 and will double to 100 million by 2060. This will not only put a massive strain on national healthcare resources, but will also increase the number of those who are not able to live and function independently. It is becoming increasingly vital to understand how the brain changes with age and mechanisms to possibly protect and rejuvenate the aged brain to a younger, healthier phenotype to promote healthy aging.

In this work, we found that there is an increase in the number and amount of pro-inflammatory cytokines in the brain with age, demonstrating that the brain becomes progressively pro-inflammatory with age. Notably, we observed an increase in IFNγ and GM-CSF which are two cytokines implicated in the detrimental priming phenotype of the aged brain. Additionally, we found that the aged brain becomes epigenetically dysregulated, with an increase in Ezh2 function and simultaneous loss of the opposing function of Jmjd3, thereby leading to a respective increase in H3K27me3 and decrease in H3K27me1 in the brain with age. Furthermore, when we examined the role of Ezh2 in primary microglia cultures *in vitro*, we found that inhibition of Ezh2 could

simultaneously abrogate pro-inflammatory polarization and enhance anti-inflammatory polarization. Together, this data suggests that increasing function of Ezh2 may directly contribute to the pro-inflammatory phenotype of the brain with age.

We also found that culturing primary microglia with plasma from healthy aged mice resulted in up-regulation of pro-inflammatory cytokines *Il1b* and *Il6 in vitro*, suggesting that circulating peripheral factors may directly influence the transition of the brain to a pro-inflammatory phenotype with age. We tested if the age-associated epigenetic dysregulation and pro-inflammatory phenotype could be reversed by utilizing the surgical model of heterochronic parabiosis. In this model, a young and aged animal are surgically attached so that the two come to share a common blood supply. Using young-young and aged-aged isochronic surgical controls, we found that young blood rejuvenates the levels of H3K27me3 to those of a younger animal. Additionally, when we induced a neuroinflammatory response in the heterochronic and aged isochronic parabionts, we found that the neuroinflammatory response of aged heterochronic animals was rejuvenated and reduced compared to aged isochronic controls.

This work is the first to investigate the role of epigenetic dysregulation of Ezh2 and Jmjd3 in the brain with age. Additionally, this is the first work to examine the ability of the circulating peripheral immune system to rejuvenate the epigenetic landscape of the aged brain and functional response to a pro-inflammatory stimulus. Future identification of the specific circulating peripheral factor(s) responsible for brain aging and rejuvenation may allow for therapeutic intervention to promote healthy brain aging in older individuals.

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Chapter 1:
Introduction

This chapter is based upon:
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Introduction

By 2050 the global life expectancy is expected to increase by nearly eight years. However, the quality of life of the estimated ~17% of world's population soon to be over 65 years will not necessarily be any better due to the increasing burden of age-related diseases such as stroke and other neurodegenerative diseases. The rate of dementia will increase from 9.4% to 23.5% by the mid-century, and the number of people living with Alzheimer's disease in the U.S. is expected to grow by nearly 10 million (1). By 2030, nearly 4% of the U.S. population is projected to have had a stroke, straining our already limited resources and health infrastructure (2). Our scientific understanding of normal aging processes is incomplete and the mechanisms leading to age-related disability with the advent of age-mitigating/rejuvenation therapies needs further exploration.

The effects of aging on the central nervous system (CNS) are widespread, as are systemic changes in peripheral tissues. The importance of communication between the CNS and the periphery is increasingly recognized, and may be mediated by systemic factors, the autonomic nervous system, commensal bacteria (i.e., the microbiome) and/or the neuro-immune axis. Age-related changes in CNS homeostasis are not solely intrinsic in nature, but are mediated through bidirectional communication between the CNS and the systemic environment (**Figure 1.1**). Differences in neuronal function have been observed in the CNS with age, but it is becoming increasingly apparent that it is possible to slow, or even reverse, aging by restoring "youthful" peripheral tissue compartments (3, 4). This includes the bone marrow niche that gives rise to the body's immune system, which can have a beneficial positive feedback effect on distant areas including the CNS.

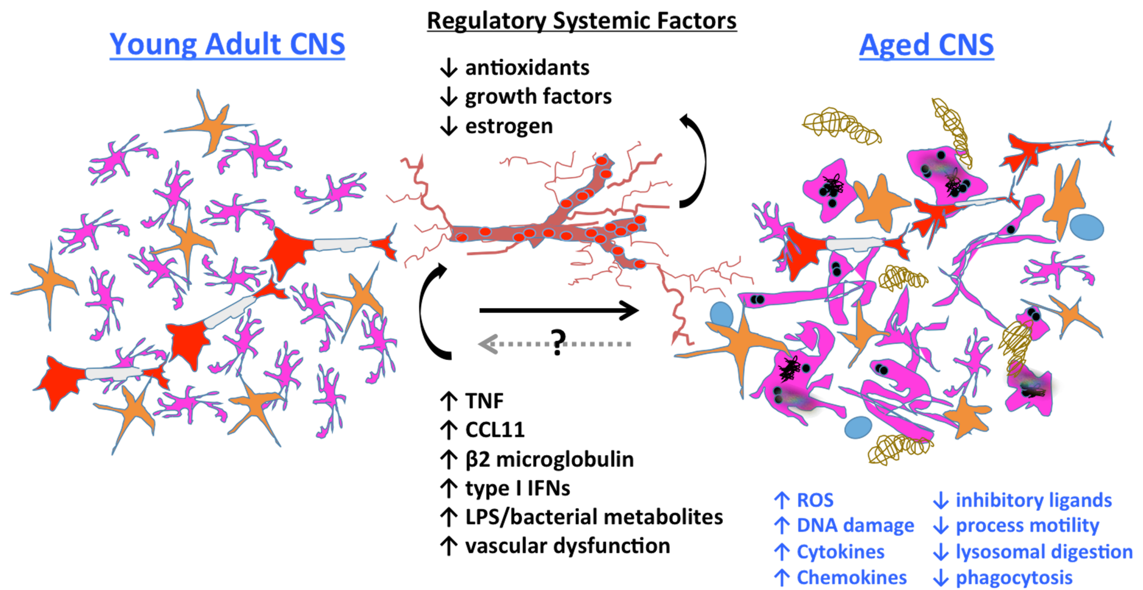

Figure 1.1. The impact of aging on microglia function and its systemic regulation. Young microglia (in pink) gradually transition from a ramified morphological state to a deramified, spheroid formation with abnormal processes with chronological age. Several cytoplasmic features are hallmarks of microglial senescence including increased granule formation, autofluorescent pigments such as lipofuscin, and process fragmentation. Age-related neuronal loss (in red) reduces the overall level of immunoinhibitory molecules (e.g., CD200, CX3CL1) required to maintain microglia in a quiescent state. Basal increases in inflammatory signaling are associated with enhanced ROS production which results in the generation of free radicals, lipid peroxidation, and DNA damage. This positive feedback loop is further compounded by defects in lysosomal digestion and autophagy, resulting in the potentially toxic buildup of indigestible material. Concurrent reductions in process motility and phagocytic activity lead to decreased immune surveillance and debris clearance, resulting in plaque formation (beige). In turn, microglia activation triggers astrocyte activation (in orange) and promotes the recruitment of T cells (in blue) into the aging brain. These pathological features of microglial aging are highly influenced by the systemic

environment. Diminished levels of circulating anti-aging factors in conjunction with increased concentrations of pro-aging factors are critical drivers of microglial senescence. For example, diminished estrogen levels in older (menopausal) females are associated with elevated expression of macrophage-associated genes in the brain. Therapeutic interventions intended to increase anti-aging factors and decrease pro-aging factors appear to be able to halt or delay microglia aging, enhance neurogenesis, and improve cognitive function.

Inflammation is viewed as a central driver of aging and/or age-related dysfunction. The term ‘inflamm-aging’ was coined to describe the ever subtle but gradual increase in inflammatory signaling with age (5, 6). Although inflamm-aging is primarily macrophage-driven, the accrued effects of this are widespread, affecting nearly all cells at either the intrinsic or extrinsic level—to an extent that fundamentally alters normal physiological behavior—as evidenced by the overall age-related decline in normal function. Characteristics of the aged brain such as gray matter loss and cortical thinning, shrinkage in hippocampal volume, deficits in learning and memory, and decreased remyelination (see **Figure 1.2**) are all processes that have been empirically proven to involve inflammation, the severity of which likely depends on the level of degeneration and how it is modulated.

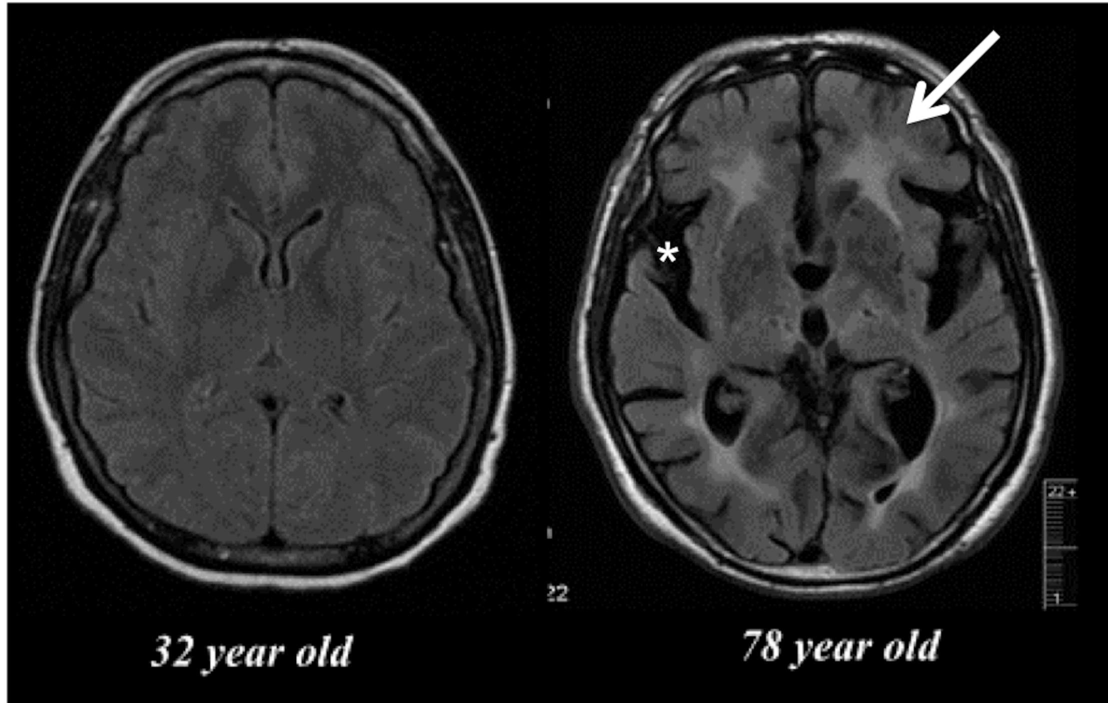

Figure 1.2. A: MRI of a normal 32-year-old woman. There is no evidence of atrophy or white matter disease. B: MRI of a 78-year-old woman with mild cognitive impairment. There is considerable frontal temporal atrophy as seen by an enlarged Sylvian fissure (asterisks) and white matter disease (white arrow).

No cell is protected from the detrimental effects of aging, and this includes the primary immune cell of the CNS, the resident tissue macrophages known as microglia. These cells represent 5-15% of all brain cells, and are considered to be the housemaids of the CNS, providing nourishment and support to neighboring neurons, clearing debris, and being the first responders to foreign stimuli (7). Like their neuronal counterparts, microglia are believed to be post-mitotic and long-lived, with minimal, if any, turnover. Although recent depletion studies imply the existence of latent microglia progenitors, it is not clear what role this proposed population of cells may have in replenishing microglia populations under normal homeostatic conditions across the lifespan (8). Thus, these cells may still be viewed as especially vulnerable to the cumulative effects of aging, and thus, poised to negatively impact the neurovascular niche as a result of a compromised ability to perform essential 'house-keeping' functions. While the role of aging on circulating macrophages and other lymphoid-associated myeloid cells has received significant attention in recent years, our understanding of the age-related changes in the function of CNS-resident microglia is less clear. This review highlights current findings and concepts on the effects of aging on microglia and stresses their potential contribution to inflamm-aging and age-related stress.

Age-related changes in microglia phenotype

Aged microglia exhibit increased soma volume, a retraction in processes, and a loss in uniform tissue distribution (9). Moreover, microglial process speed is significantly slower with age in healthy and injured animals, resulting in less active tissue sampling and impaired synaptic contact (10). These age-related abnormalities in

cytoplasmic structure, deramification, and process fragmentation were collectively termed ‘microglial dystrophy’, and are more indicative of a senescent rather than classical activation phenotype (11, 12). The newly coined ‘dark’ microglia phenotype defined by condensed electron-dense cytoplasm and nucleoplasm, nuclear chromatin remodeling, and high levels of synaptic stripping activity and oxidative stress applies not only to microglia populations associated with pathological states such as chronic stress and Alzheimer’s disease, but to the microglia that are observed in normal aging (13). While these alterations at the ultrastructural level are only now beginning to be described, it has been well known that aged microglia are highly granular and atypically dark in appearance in immunohistological preparations. Defects in lysosomal digestion can result in the progressive accumulation of indigestible material largely composed of lipofuscin and other autofluorescent pigments (14, 15). Researchers studying protein expression in aged microglia using immunofluorescence or flow cytometry techniques are likely familiar with the high degree of autofluorescence in these cells. Age-related microglia-associated autofluorescence is often viewed as a technical nuisance as it is difficult to differentiate changes in the normal CNS or those due to severe injury. The accumulation of lipofuscin and other non-degradable autofluorescent byproducts is believed to be due to impairment in disposal mechanisms and has also been implicated in several neurodegenerative diseases including Alzheimer’s disease (16, 17).

Strategies to investigate functional characteristics of microglia

Not all aged microglia exhibit an “aged” phenotype, and it is unclear whether autofluorescence is confined to dystrophic (i.e., dysfunctional) microglia populations in select regions or if it is a more widespread phenomena, as it is currently difficult to

isolate these cells and evaluate their function relative to their non-autofluorescent counterparts. Using flow cytometry, our lab has recently identified a significant population of side scatter-^{high} microglia in the aged brain that exhibit a surprising level of both granular content and autofluorescence background (18). These cells display functional abnormalities when compared to young microglia and more importantly, to side scatter-^{low} microglia that co-exist alongside them in the aged CNS (see **Figure 1.3**). These functional abnormalities include higher production of reactive oxygen species (ROS) and pro-inflammatory cytokines, increased mitochondrial content, and poor phagocytic ability—all features of a senescent or dystrophic macrophage phenotype. However, if the proper precautions are not taken, aged microglia can appear to stain positive for nearly any antibody or fluorescent label. Because of this artifact, the high level of background exhibited by aged microglia requires the use of fluorescence-minus one (FMO) or isotype controls specifically using aged brains and subsequently gating on aged microglia to determine the true background level—which in studies from our laboratory appear to be greater than that of any other immune cell in the body. Other methods of masking or quenching autofluorescence such as Sudan Black B have been developed and are often employed during histological preparations (19). Given the clinical ramifications of lipofuscin accumulation in both neurons and glia, future efforts to develop brain-imaging techniques which can exploit the naturally occurring level of autofluorescence (i.e., lipofuscin-like material) in the CNS may prove highly useful in predicting or diagnosing neurological disease states in humans.

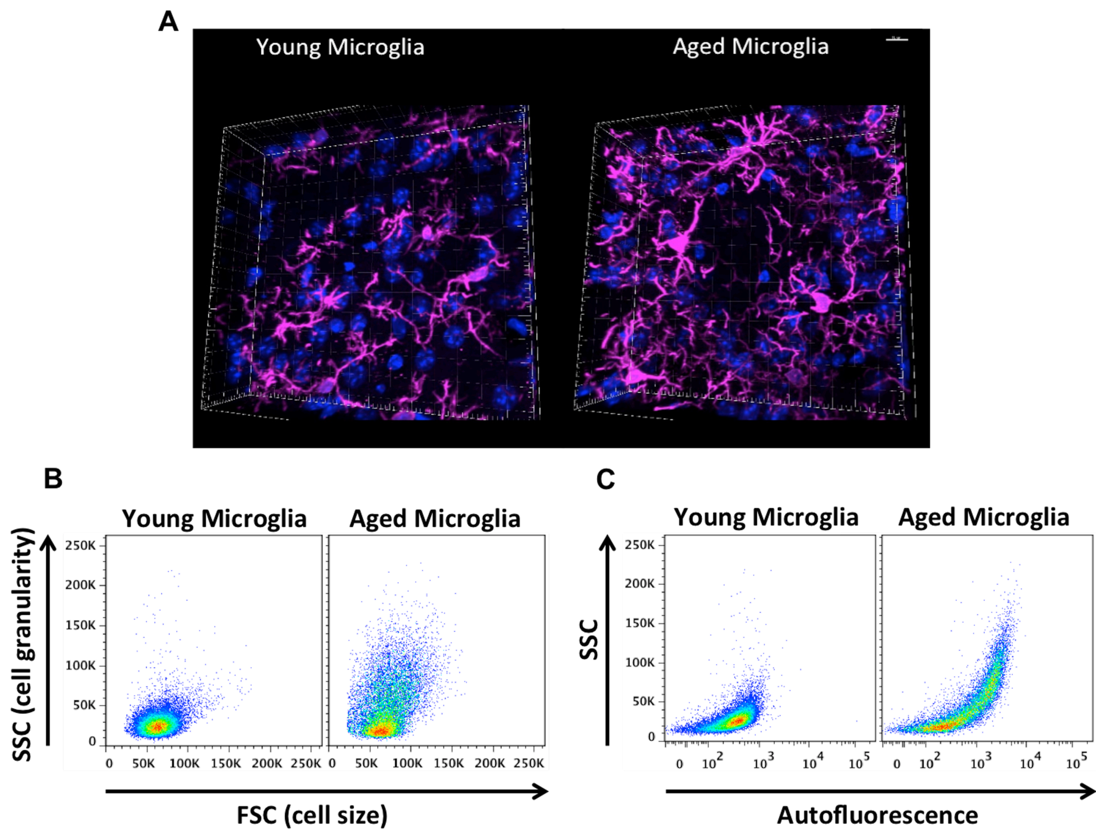

Figure 1.3. Age-related microglial dystrophy. Confocal microscopy images of DAPI-counterstained (blue) Iba1-positive cortical microglia (pink) highlight the enlarged soma and abnormal, twisted cytoplasmic processes of aged microglia (A). Flow cytometry preparation of $CD45^{int}CD11b^{+}Ly6C^{-}$ microglia demonstrate a significant increase in cellular granularity and size with age (B). A population of side scatter-high aged microglia exhibits high levels of autofluorescence (FITC channel) compared to their younger counterparts and is indicative of lysosomal dysfunction (C).

Despite several reports demonstrating prolonged maintenance of aged microglia *in vitro* (20, 21), for many, the long-term culture of microglia isolated from aged brains can be technically challenging if not impossible. These methods are invaluable to the study of intrinsic age-related changes in microglia function. One alternative approach is the immediate *ex vivo* functional assessment of freshly harvested cells. Although some activation is induced during the mechanical/enzymatic digestion procedure via crude extraction from their native microenvironment (e.g., loss of contact-inhibitory signaling), this confounder is seen by many to be unavoidable and perhaps necessary in order to understand their functional activity. *Ex vivo* functional testing is advantageous for aging studies because it allows the investigator to probe for intrinsic microglia activity in a very acute time window (within hours), obtaining as close to their presumed *in vivo* functional identity as possible with minimal artifact introduced by standard cell culture systems. Indeed, a recent report suggests that neonatal microglia undergo dramatic 'age-like' changes in as short of time from 2 days *in vitro* to 16 days *in vitro* (22). Nonetheless, *in vitro* approaches are currently indispensable and the advent of more efficient long-term culture methods will hopefully allow researchers to address many important questions that *ex vivo* testing is not well suited for.

Another issue regarding the activation status of microglia is the suitability of the existing nomenclature and its application to age-related microglial phenotypes. The long-held M1/M2 convention for describing macrophage polarization may be more applicable to *in vitro* systems than for far more complex *in vivo* environments, as mixed phenotypes are commonly seen (23). The M1/M2 *in vitro* paradigm, originally premised on infection studies, attempts to explain the predisposition for peripheral macrophages to respond as either 'inflammatory' (M1) or 'reparative' (M2) subsets depending on their exposure to the cytokine byproducts of polarized T cell subsets (Th1: IFNγ or Th2: IL-

4). Subsequent transcriptomic profiling has demonstrated significant differences between bone marrow-derived monocyte populations and CNS-resident microglia, which may be due in part to the age (high vs. low turnover) and environment (circulation vs. brain) of the cells being profiled, and any interactions between the two. An expert analysis of this controversy was recently discussed by Ransohoff (24). Among the many salient points expressed was the lack of predicted transcriptional organization found between polarization states induced in several disease models as demonstrated by *ex vivo* expression profiling of microglia, indicating that microglial reactivity is multifactorial and injury-specific, and thus, unlikely even to fall along a linear continuum. The application of M1/M2 markers for the *in vivo* description of microglia activation states is then seemingly inadequate in defining the injury-resolving capacity of these cells and their associated functions. Thus, it would seem that attempting to classify the pro-inflammatory phenotype of aged microglia as M1 may be too simplistic in that it ignores the adaptive requirement of these cells to respond to the demands of a changing microenvironment over the lifespan (25). In recent years, the senescence-associated secretory phenotype, or SASP, has been utilized to more accurately describe aged senescent cells, however these criteria may vary depending on cell type, especially as not all aged cells are senescent *per se* (26, 27). Aged microglia are likely to exhibit many of the same phenotypic features as other aged post-mitotic tissue-resident macrophages. Although SASP criteria have yet to be established specifically for microglia, emerging studies suggest a framework for one will emerge in the next few years.

Reactive oxygen species-mediated damage in the aging brain:

One of the most profound changes that occurs with aging is the gradual increase in reactive oxygen species (ROS) generation. Indeed, glial cell activation and elevated oxidative stress burden are hallmarks of CNS aging and manifest during the course of many if not all neurodegenerative diseases (**Figure 1.4**). As the predominant myeloid cell in the nervous system, microglia are the main source of oxidation products and inflammatory mediators during aging. Elevated microglia ROS production (e.g., superoxide anion, hydroxyl radical, and lipid hydroperoxides) can impose a hazard to nearby neurons either through direct release (i.e., neurotoxicity) or via second messenger signaling pathways such as PKC, MAPK, and NF-κB activation which serve to intensify the pro-inflammatory response (28). The importance of ROS to age-related neuropathology is evidenced in its key capacity to mediate the detrimental effects of amyloid beta (Aβ) and lipopolysaccharide (LPS)-induced CNS injury (29, 30). LPS induces the generation of ROS from the actively respiring mitochondria as well as NADPH oxidase (NOX). Superoxide production via NOX has been shown to be the main contributor of ROS in several age-related neurodegenerative diseases and is linked to the classical activation of microglia (31). Ansari and Scheff reported an inverse correlation between NOX activity and cognitive impairment, in which higher NOX activity was associated with worse cognitive performance in individuals of all stages of Alzheimer's disease (32). Indeed, NOX2-deficiency has been shown to reduce oxidative stress, leading to improved cerebrovascular function and behavior in a mouse model of Alzheimer's disease. For example, chronic treatment of apocynin, a NOX inhibitor, reduced plaque size and microglia number in hAPP(751)(SL) mice (33).

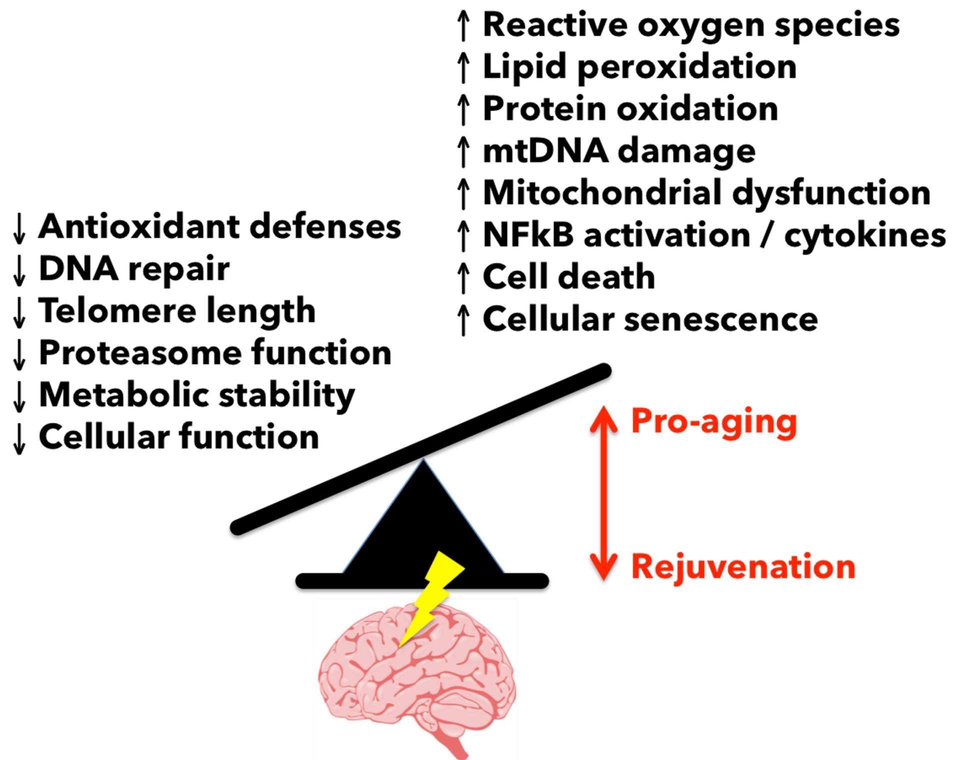

Figure 1.4. Imbalance between oxidative stress and antioxidant defenses in the aging brain. Oxidative stress arises when there is an excess of free radicals over antioxidant defenses. This imbalance leads to an inability to detoxify the reactive intermediates and results in oxidative damage of genes and proteins. Oxidative stress is a consequence of the aging process and is involved in many diseases such as Alzheimer's disease, stroke, and atherosclerosis. Stress-activated pathways impact gene expression and alter the normal function of cells, often resulting in respiratory chain dysfunction, altered proteostasis, telomere shortening, apoptosis, and cellular senescence.

One of the hallmarks of Alzheimer's disease is cerebral amyloid angiopathy (CAA), which is characterized by the deposition of Aβ within the walls of cerebral arterioles. Treatment with apocynin and tempol, a non-specific ROS scavenger, attenuated ROS production and improved cerebrovascular function in aged Tg2576 mice (34). Treated mice exhibited a reduction in CAA formation and CAA-related microhemorrhages, indicating that NADPH oxidase-derived ROS are a key contributor to CAA formation and associated vascular dysfunction. Together these findings suggest that the age-related increase in microglial ROS production has widespread effects on the neurovascular niche and may be a key accelerant of neurodegenerative disease and cognitive impairment.

Microglia are thought to be the main mediator of ROS-induced neuronal injury and several studies have demonstrated that NADPH oxidase-deficient primary microglia exhibit blunted levels of intracellular ROS, extracellular superoxide, TNF expression, and neurotoxicity following LPS stimulation *in vitro* (30, 35). NOX may even play a critical role in the development of chronic inflammation as previous work has shown that NOX2-deficient mice exhibit less dopaminergic neurodegeneration in the substantia nigra 10 months after a single systemic LPS injection compared to $NOX2^{+/+}$ controls (35). It is possible that loss of NOX function attenuates ROS production by the infiltrating neutrophils and monocyte-derivatives in the hours and days following injury. Interestingly though, microglia from NOX2-deficient mice fail to show any increase in activation morphology as early as 1 hour following injection, implying that it is the early wave of ROS production that determines the severity of disease course.

As noted above, both NOX and ROS levels increase in the CNS with normal aging and following injury. ROS production and lipid peroxidation is significantly

elevated in older mice after contusion spinal cord injury compared to young controls (36). NOX2 expression was greater in ROS-producing microglia/macrophages in the lesion site of older mice. Interestingly, aging is also associated with a loss of free radical scavenging mechanisms. Antioxidant defenses have been shown to be attenuated in aged microglia, as evidenced by reduced cellular levels of glutathione and dysregulation of heat shock proteins such as heme-oxygenase 1 (37, 38). The potential for antioxidant therapy to improve microglia aging and, in turn, brain aging has recently been reviewed (39). As of yet it is still unclear whether worsening injury with age is primarily a result of exacerbated or chronic microglial production of ROS or if aging neurons are just more susceptible to ROS-mediated damage.

Neuronal-glial interactions and immunoinhibitory signaling in the aging brain:

In addition to intrinsic age-related stress, microglia are highly responsive to environmental stimuli throughout the lifespan, including extrinsic immunological stressors. As guardians and primary caretakers of the more vulnerable neuronal populations, the manner in which microglia respond to these stressors is critical for normal neuronal function. Activated microglia exhibit augmented production of inflammatory cytokines, ROS, and metabolic byproducts known to be neurotoxic. Thus, given their complex array of activation-sensing receptors and complementary inhibitory receptors, microglia are tightly regulated to deliver calibrated responses to any given stimulus across space (gradient effects) and time (from pathogenesis to resolution phase). To avoid over-activation and any resultant bystander damage, the requirement for microglial inhibitory receptors is essential to not only prevent the generation of unwanted inflammation, but to also ramp down injury-driven inflammatory

responses once they are largely resolved. However, with age, the ability of these inhibitory receptors to maintain microglial quiescence is impaired in part due to reductions in the expression of their cognate ligands.

CD200/CD200R1:

The CD200-CD200R1 immunoinhibitory signaling axis in the CNS is comprised of gray matter neurons that ubiquitously express CD200 both stably on their surface and in secreted form, and microglia/macrophages which express the receptor for this ligand, CD200R1. Microglia/macrophage immune responsiveness is believed to be constitutively down-regulated under normal conditions via direct interactions with neighboring neurons, leading to microglial quiescence. Interestingly, young adult mice that are deficient in CD200, exhibit many features seen in normal aging mice such as basal increases in microglia activation, T cell infiltration, blood-brain barrier permeability, impaired long-term potentiation (LTP), and exacerbated responses to injury and disease (18, 40–42) implying that CD200 levels may naturally diminish with normal aging (43). Concomitant increases in transcriptional expression of pro-inflammatory genes and decreases in anti-inflammatory genes such as *Cd200* have since been demonstrated in the substantia nigra and hippocampus of older animals (44, 45) leading one to speculate that the lack of microglia inhibition leads to greater inflammation which is detrimental to learning and memory.

CD200 expression is decreased in the brain of Alzheimer's patients and Aβ-challenged mice (46). Subsequent *in vivo* studies demonstrated that intrahippocampal administration of CD200 fusion protein decreased microglial activation and decreased LTP deficits in both aged and LPS-treated rats (47). Activation of CD200R1 by CD200

fusion protein inhibited Aβ-induced increases in IL-1β, TNF, CD40, and CD68 (48). Moreover, Aβ-induced deficits in LTP were attenuated by CD200 fusion protein in hippocampal slice culture. Consistent with these findings, the delivery of an adeno-associated virus expressing CD200 into the hippocampus of APP mice for a period of 6 months restored neurogenesis and reduced diffuse plaques in 12-month-old mice (49). Additionally, the authors found that *in vitro* stimulation of microglial CD200R1 promoted neuronal growth and despite being anti-inflammatory, resulted in greater Aβ internalization. This is in contrast to a new report that has shown microglia from CD200-deficient mice exhibit increased lysosomal and phagocytic activity in response to Aβ challenge (50). This effect was mediated in part by mTOR inhibition and implies that CD200 normally functions to suppress immune functions such as phagocytosis. Alternatively, it is possible that the chronic loss of functional CD200 in these knockout models over the lifespan could prime microglia to respond in a manner that is not consistent or comparable to microglia from otherwise normal, healthy wildtype mice. As is the delicate balance of any regulatory system, the potential to increase inhibitory signaling may be offset the adaptive requirement for activation (e.g., injury-sensing/stimulus recognition, migration to injury sites, debris clearance and phenotype switching). Thus, it is not yet clear whether pro-inflammatory-induced activation of microglia is required to drive phagocytic activity and a return to homeostasis or if chronic inflammation impedes normal function as appears to be the case in the aging brain.

CX3CL1/CX3CR1:

The chemokine fractalkine (CX3CL1) which is expressed on neurons in membrane-bound form or secreted by neurons functions similarly to CD200, suppressing activation by binding to its receptor, CX3CR1, expressed on microglia. This interaction is important for downregulating microglial activation and maintaining CNS homeostasis (51, 52). Fractalkine signaling is similarly impaired with normal aging, following LPS challenge, and in APP(swe) transgenic mice, as expression levels for both the ligand and the receptors have been shown to be significantly decreased and inversely associated with inflammatory activity (52–55). The age-related loss of fractalkine ligand in the rodent hippocampus is associated with decreased neurogenesis; however the survival and proliferation of neuronal progenitor cells was restored by exogenous fractalkine, an effect that was not seen in young animals suggesting that this was an age-dependent mechanism (56). Protracted downregulation of the fractalkine signaling pathway is associated with delayed recovery from sickness behavior, elevated IL-1β levels, and decreased TGFβ production in the aged brain (57). However predictable the outcome of fractalkine receptor activation on microglia might seem, several reports highlight the complex nature of this immune inhibitory signaling. For example, intrahippocampal injection of Aβ fibrils was found to upregulate CX3CR1 expression on activated microglia and increase synaptic dysfunction and cognitive impairment (58). It is not surprising that CX3CR1 expression was enhanced by Aβ stimulation, as many immune inhibitory receptors are known to upregulated in classically activated microglia/macrophages in an effort to counteract the pro-inflammatory state. Both CX3CL1- and CX3CR1-deficiency have been shown to reduce Aβ deposition in APPPS1 mice, and an increase in microglial p38MAPK activation and cytokine production (59, 60). In other studies, CX3CR1-deficiency resulted in worsened neuronal and memory deficits in hAPP mice independent of

plaque load (61). Despite the reduction in Aβ plaque deposition, CX3CR1-deficient mice exhibited exacerbated Tau pathology, an effect that was subsequently shown to be suppressed by fractalkine overexpression (62). Together, these findings suggest that a delicate balance of activating and immunoinhibitory signaling is likely required to perform the full spectrum of function required to maintain homeostasis in the aged CNS environment.

Phagocytosis in the aging brain:

Debris clearance is an essential role of microglia. Normal aging has significant effects on endocytic pathways, including the phagocytic uptake of debris. Transcriptional analysis of acutely isolated microglia from APPswe/PS1dE9 Alzheimer's disease (AD) mice reveal diminished expression of genes associated with phagocytosis (63). At the functional level, young microglia (1 month old mice) internalize ~50% more Aβ42 than aged microglia (15 month old mice), demonstrating an age-related decrease in phagocytic behavior beginning at birth (< 8 days old mice) (37). Data from our laboratory has shown age-related deficits in the phagocytosis of physiological (Aβ) and non-physiological (latex beads) cargo not only at baseline, but also following *ex vivo* stimulation with PMA/ionomycin (18). While aging negatively affects the ability of microglia to phagocytose Aβ, it does not appear to limit their ability to adhere to amyloid plaques or *in vitro* fibrillized Aβ (64). Moreover, aging did not affect the functional uptake of bacterial bioparticles, and others have reported that aged microglia exhibit greater uptake of quantum dots (65), implying that aging may differentially affect phagocytic pathways at various stages (adherence, internalization, digestion, etc.) or with different substrates. In an interesting study by Hendrick in 2014,

the authors showed that while aging enhanced microglial capacity for myelin phagocytosis, it simultaneously reduced myelin's susceptibility for uptake, suggesting that age-related phagocytic impairment may be mediated both by intrinsic and extrinsic factors, depending on the nature of the substrate (66).

Inhibitory 'don't eat me' signals that prevent host attack such as CD47 have also been shown to prevent microglia phagocytosis of healthy cells via activation of the immune inhibitory receptor signal regulatory protein-α (67), although it is unclear what role if any molecules like these have in aging. The ability of a given phagocytic substrate to induce microglia activation either through toxicity or via receptor-mediated signaling may in turn alter the phagocytic potential of that cell (68). For example, aging decreases microglia and monocyte uptake of α-synuclein oligomers and is associated with increased TNF secretion (69). The age-related increase in microglia cytokine production, specifically TNF family members, has been demonstrated numerous times and is a hallmark of CNS aging. However, the nature of the relationship between increased TNF production and microglia phagocytosis is one that warrants greater examination, as several studies support an inverse relationship with age. For example, TGFβ-induced phagocytosis is abolished in aged microglia compared to their younger counterparts, indicating that receptor-signaling pathways are significantly altered with age and may underlie endocytic impairment (70). The interactions between cytokine signaling and phagocytosis have proven to be highly complex and provocative, as newly emerging data suggests that microglial phagocytosis and plaque clearance may be suppressed as result of an overproduction of anti-inflammatory molecules such as IL-10 and arginase-1, rather than mediated by pro-inflammatory dysfunction (71–73). While our understanding of age-related changes in microglial phagocytosis and changes in the expression of scavenger receptors (i.e., CD36) is infancy, the

development of novel drugs that are capable of directly modulating phagocytic activity and reducing plaque load is becoming a realistic goal.

Microglial depletion and implications to aging:

Just as conditional genetic targeting approaches have advanced our understanding of the molecular mechanisms underlying microglial activation, recent pharmacological and genetic microglia depletion studies have aided our understanding of the net effect of these cells on aging and age-related cognitive decline. These innovative strategies take advantage of several known aspects of microglial identity and requirements for survival. For instance, colony-stimulating factor 1 receptor (CSF1R) is essential for the growth and survival of microglia and other monocyte-derived cells. CSF1R blockade with PLX5622 eliminates microglia for sustained periods of time, allowing for the long-term investigation of microglia in neurodegenerative disease models (74). PLX5622-induced depletion of microglia prevented their association with plaques, but did not alter amyloid-β levels or plaque load. Importantly, this strategy was able to reduce overall neuroinflammation and attenuated contextual memory deficits in 10-month-old 3xTg-AD mice, without having any overt effect on behavior or cognition in normal adult wild type mice. It is unclear whether microglial depletion in wild-type aged animals has the potential to have a positive cognitive benefit. Administration of the CSF1R inhibitor PLX3397 improved functional recovery and spared neuronal loss in part by reducing chronic inflammation in 5-8 month old mice following a hippocampal lesion induced by diphtheria toxin exposure (75). This strategy, similar to what was seen with other CSF1R treatments, was accompanied by an increase in dendritic spine density, suggesting these cells

have critical roles in sculpting synapses even after development. PLX5622 also prevented whole-brain irradiation-induced memory deficits in young mice in part by limiting microglia proliferation and monocyte infiltration (76, 77). These studies have enhanced our knowledge of both the homeostatic role of microglia and their functional contribution to disease pathology. Insights into the global effects of dystrophic microglia in normal aging using similar depletion approaches could prove extremely useful to our understanding of the contribution of microglial senescence to age-related cognitive decline.

Systemic regulation of microglia aging:

Although it is true that the CNS is largely protected from the systemic environment, it is not impermeable to it. Indeed, systemic administration of LPS has been found to induce microglia activation, neurodegeneration, and sickness behavior (78). Thus, it is evident that the brain is influenced by changes in peripheral homeostasis. Although the brain is protected by the blood-brain barrier, there are several conduits through which systemic messages reach the CNS, including the vascular and lymphatic networks, and via the choroid plexus and cerebrospinal fluid. These anatomical interfaces, which likely convey vital information under healthy conditions, may also predispose the brain to the detrimental effects of systemic aging. Work by Baruch *et al* demonstrated that aging induces a type I interferon (IFN)-dependent gene expression profile in the choroid plexus (79). Interestingly, blocking IFN-I signaling in the aged brain down-regulated IFN-I-dependent gene expression in the choroid plexus, restored hippocampal neurogenesis, improved cognitive function, and partially reversed age-related glial activation. These findings suggest that age-

related microglial dystrophy (i.e., senescence) can be reversed by external modulation. Other blood-brain borders may similarly affect microglia function with age in a location-dependent fashion. Regional heterogeneity in microglia function and differences in immune vigilance are likely to become exacerbated with age as neurovascular function and blood vessel integrity has been reported to be selectively compromised in the hippocampus and frontoparietal cortex (80–82). Pioneering studies using heterochronic parabionts and plasmapheresis support a strong link between the systemic environment and brain aging. Systemic immune factors such as CCL11 and β2-microglobulin are elevated in an age-dependent manner in the plasma and hippocampus, and impair neurogenesis and cognitive function (83, 84). Systemic exposure to either molecule induced similar deficits in learning and memory that were reversed by antibody blockade. These studies convincingly demonstrate the important link between the brain and the periphery. How this bi-directional communication occurs is a very active and novel area of research, as drug and antibody delivery is much easier in the periphery due to poor blood-brain penetrance. Manipulating systemic factors to influence brain inflammation in very appealing for therapeutic development.

One example of bidirectional communication is illustrated by studies examining the gut-brain axis. Recent work has demonstrated a modulatory effect of gut microbiota on microglia function (85). Germ-free mice exhibited altered microglia morphology and impaired responses to LPS stimulation and viral infection (86). Similar changes in microglial phenotype were seen following partial ablation of microbiota by oral antibiotics that were normalized following recolonization with microbiota from specific-pathogen free mice. Mice deficient in the short-chain fatty acid (SCFA) receptor FFAR2 had similar deficits in microglia function as did germ-free mice, suggesting that microbiota-derived SCFA exert systemic control over microglial

development and homeostasis. While compositional shifts in microbiome complexity have been shown to occur with age, the significance of these changes and their effect on microglial behavior remains to be seen. Nevertheless, these studies highlight a critical role for the systemic environment in regulating microglia activity and even suggest that brain aging may be mitigated by healthy lifestyle choices and dietary manipulations (87–90).

The role of aging on the microglial response to brain injury and disease

As reviewed above, microglia undergo drastic phenotypic changes with age. These molecular and cellular changes are many, and their summation leads to a dysregulated microglia phenotype. Additionally, these phenotypic changes may be influenced by the changes in the periphery, including microbiome changes (86), age-associated perfusion deficits within the brain itself (91), and even diet (92). Aging is, no doubt, a complex process and takes its toll on microglia phenotype and function in homeostasis. What is also crucial to consider are the effects of aged microglia in the context of injury and disease in both humans and the animal models we use to study them. Here we now review the effects and noteworthy issues raised by these age-associated changes in microglia function in a number of neurological injuries and diseases.

Aging exacerbates lipopolysaccharide-induced pro-inflammatory microglial response:

As animals and humans age, there are notable changes in cognition and reaction times even in healthy subjects, and may be an unavoidable consequence of

aging (93). However, studies in animals have shown that age-related cognitive decline is correlated with higher levels of pro-inflammatory cytokines produced by microglia and that the aging brain is sensitized to immune challenges (94). Aged individuals are more susceptible to delirium when ill with a peripheral infection, stress, or following surgery, recently reviewed elsewhere (95–98). This is also observed in animal models (see **Table 1.1**). These symptoms are associated with activation of the peripheral innate immune system, and in turn leads to an exacerbated inflammatory responses in the aged brain (99–101).

Stressor	Study	Animals	Age(s)	Sex	Model	Notable findings
Peripheral infection	(99)	BALB/c mice	Young 3-6m Aged 20-24m	Male	LPS i.p. injection	• Exaggerated ↑ IL-1β, IL-6, lipid peroxidation in aged brain • ↓ social behavior, food intake, weight loss in aged
	(102)	F344XBN rats	Young 3m Aged 24m	Male	Live *E. coli* i.p. injection	• At baseline: ↑ hippocampal HMGB1 protein, mRNA in aged; ↑ HMGB1 protein in CSF of aged • Following i.p. *E. coli* injection: Prolonged ↑ expression of IL-1β, IL-18, TNF in aged; prolonged sucrose anhedonia (depression) and ↓ juvenile social exploration in aged • Inhibition of HMGB1: abrogated primed phenotype of aged brain to peripheral *E. coli* injection, restoring behavior to that of young animals
Central innate immune activation	(103)	BALB/c mice	Young 3-4m Aged 20-22m	Male	LPS i.c.v. injection	• Prolonged ↓ locomotor activity, social behavior, and food intake in aged • ↑ cerebellar and hippocampal IL-1β, IL-6, and TNF expression in aged
Surgery	(104)	BALB/c mice	Young 4-6m Aged 23-25m	Male	1.5 cm abdominal incision and gentle manipulation of internal organs for 1 min	• Anesthetic and analgesics: no effect on hippocampal IL-1β, IL-6 and TNF mRNA expression • Surgery: ↑ IL-1β expression in aged hippocampus; locomotor activity unchanged in in young or aged mice
	(105)	C57Bl6/J mice	4m	Female	0.5cm abdominal incision	• Surgery ↑ anxiety, ↓ special memory
	(106)	C57Bl6/J mice	2m-8m	Female	Simple laparotomy	• Surgery ↑ total alpha-synuclein and S100beta in the cortex, ↓ attention
Stress	(107)	BALB/c mice	Young 3-5m Aged 22-24m	Male	30min restraint stress daily for 4d	• Stress ↑ weight loss, exaggerated ↑ hippocampal and hypothalamic IL-1β mRNA expression in aged; exaggerated ↑ corticosterone in aged • Higher hippocampal MHCII mRNA and IHC staining in aged mice at baseline, and increased in aged mice following stress

Table 1.1. Summary of animal studies comparing young and aged differences in cognitive function following stress. Various models of stress and immune system activation examined in young and aged animals show that aged animals have an exaggerated neuroinflammatory response and prolonged behavioral deficits compared to young animals.

Peripheral infection may be modeled in rodents using intraperitoneal injections of lipopolysaccharide (LPS), which is a component of the cell wall of gram-negative bacteria and a potent stimulator of Toll-like receptor (TLR) 4 signaling. This model rapidly induces a pro-inflammatory response. Additionally, some labs have begun modeling infection with peripheral injection of *Escherichia coli* to control for weight-dependent dosing, as aged mice are heavier and require larger quantities of LPS (102). LPS can be injected peripherally and rapidly induces a neuroinflammatory response with associated sickness behaviors in rodents, characterized by lethargy, reduced activity, fever, and social withdrawal (108). This behavior is an evolutionarily preserved response, as social withdrawal during infection is a protective mechanism to prevent the spread of illness to others. This behavioral response may be in part due to the elevated production of cytokines and systemic stress factors that enter the brain parenchyma via the circumventricular organs or indirectly through activation of endothelial cells and nearby perivascular macrophages. While this is a seemingly positive protective mechanism, the CNS of aged animals respond in an exaggerated manner to peripheral LPS injection, with higher immediate expression of pro-inflammatory genes and delayed behavioral recovery (99). This demonstrates that aged microglia are "primed" to respond more robustly to a pro-inflammatory stimulus.

While the phenomena of microglia priming with age has been well-established, the molecular mechanisms underlying this phenotype have only recently been investigated. Recently, it was found that high mobility group box 1 (HMGB1) protein levels are elevated in the hippocampi and cerebrospinal fluid (CSF) of aged rats. HMGB1 is secreted by various immune cells including microglia as a danger signal (102) and in turn stimulates cells via the TLR4 and receptor for advanced glycation end-products (RAGE) receptors to activate pro-inflammatory gene expression.

Blockade of HMGB1 with a pharmacological inhibitor, Box-A, was able to abrogate the priming phenotype of microglia in aged rats *in vivo* through reductions of MHCII and TLR4 expression in the hippocampus (102), two notable molecules up-regulated in microglial priming. Treatment with Box-A also improved functional recovery in social exploration tasks in aged animals (102) and enhanced freezing behavior in contextual fear conditioning tests, showing an improvement in cognitive function (102). Further work examining strategies to reverse the primed phenotype of aged microglia will provide novel targets to reduce dysfunctional neuroinflammatory responses in the elderly after neurological injury.

Aged microglia contribute to enhanced pathology following traumatic brain injury (TBI):

Elderly patients are more vulnerable to traumatic brain injury (TBI), with a doubling of TBI incidence every 10 years beginning at the age of 65, mostly related to increased falls (109, 110). Age is an independent predictor for mortality after TBI (111, 112) and older patients that survive their injury have reduced functional recovery compared to younger individuals (113, 114). Additionally, adults age 55 years or older suffering a moderate to severe TBI or those 65 or older suffering a mild TBI have an increased risk of developing dementia compared to younger patients (115). Together, these clinical findings suggest that the brain becomes more sensitive to TBI as we age, and that the pathological consequences of injury are much graver. Many factors contribute to this age-related vulnerability including increased amyloid deposition (116), however, it is not clear whether inflammation drives the exacerbation of injury or if it is secondary or correlative to the increase in neuronal death seen in older individuals.

TBI may be modeled in animals using a variety of methods (117–119). In rodents administered a controlled cortical impact (CCI), microglia are activated and express higher levels of pro-inflammatory genes and lower or undetectable levels of anti-inflammatory genes even months after the initial injury, suggesting an overall shift in microglia phenotype toward a pro-inflammatory status with time following TBI. Aged mice that underwent CCI displayed higher MHCII expression (120) and had higher expression of NADPH oxidase subunits $p22^{phox}$ and $gp91^{phox}$ Aged mice also had a corresponding reduction in the expression of antioxidant enzymes superoxide dismutase 1 (SOD1) and glutathione peroxidase 1 (GPX1) following TBI compared to young mice, indicative of higher ROS production in aged mice. These results corresponded with larger lesion volume and reduced cellular density in the hippocampus and thalamus of aged mice 7 days after CCI (120). Together, these results suggest that microglia in the aged brain are more detrimental in TBI and may predispose the aged to impairments in phagocytosis and increased accumulation of toxic waste products such as amyloid.

In addition to contributing to the excessive pro-inflammatory status in the aged brain, microglia have been shown to be directly involved in post-TBI recovery, including neurite outgrowth. Nogo receptor 1 (NgR1) signals through the RhoA-ROCK pathway and results in the collapse of the growth cone of neurites (121). Thus, higher levels of NgR1 are associated with reduced neurite outgrowth. NgR1 was found to be highly expressed in $Iba1^{+}$ microglia in the cortex and basal forebrain beginning at P1 and declined until stable baseline adult levels were achieved by P21 (122). However, expression levels were increased in mice at 17 months of age (122), suggesting that aged microglia may predispose the aged brain to decreased neurite outgrowth following an acute injury. In young animals with TBI from a stab wound model, the

number of Iba1⁺ NgR1⁺ microglia increased relative to sham controls, although total levels of NgR1 measured by Western blot did not increase 7 days post-injury (122). However, given the different phenotype of microglia in the aging brain, future studies in aged mice will be necessary to confirm the effects of elevated NgR1 in aged microglia following TBI.

It is not difficult for one to imagine the synergistic effects of aging and a prior TBI, as aging itself primes microglia in a similar manner as TBI. Thus, the response to TBI in aged animals is noticeably worse, and the response of repetitive TBI in aged animals is worse still. With the observation of increased incidence of TBI with each decade of life after the age of 65, repetitive TBIs are not only more common, but the consequences of each additional TBI are likely more severe than the previous TBI, leading to progressive deficits and cerebral atrophy.

Aged microglia contribute to worse recovery and functional outcomes following stroke:

Age is the leading non-modifiable risk factor for stroke. While preclinical studies in animal models have mostly been performed in young animals, there are many laboratories that are now transitioning their studies into aged animals. Popa-Wagner's group in particular has demonstrated profound changes in microglia activation after stroke with age (123, 124). While more expensive and surgically challenging, this is an increasingly important consideration as the response to stroke differs in young and aged animals (18, 125, 126). Perhaps most importantly, aged animals have comorbidities that resemble those seen in clinical populations, including obesity (127) and hypertension. Thus, modeling stroke in aged animals allows for a more accurate

modeling of stroke (124). However, variations in stroke models (e.g. transient vs. permanent occlusion of vessels, time duration of infarction, age of “aged” animals, strain of mice, etc.) make it difficult for laboratories from different groups to compare studies.

Interestingly, despite the high level of cell death that occurs following cerebral ischemia, one study reported that microglia have a more anti-inflammatory phenotype with higher expression of CD206, IL-10, YM1/2, TGFβ, ARG1, and CCL22 in the first 3 to 5 days following stroke, with a transition to higher expression of pro-inflammatory genes CD16, CD32, and iNOS at 7 and 14 days post-stroke (128). While this study examined RNA expression from an entire stroke hemisphere, the phenotype of microglia following stroke is likely influenced by their relative location to the infarcted region where cell death and pro-inflammatory stimuli are abundant. However, these authors found corresponding patterns of expression of CD16/32$^+$ Iba$^+$ and CD206$^+$ Iba1$^+$ microglia using immunofluorescence in the peri-infarct region of the stroke consistent with their gene expression findings (128). The timing of phenotype switching likely depends on the stroke model used, and the brain area assessed.

Similar findings were also found in aged animals, with higher Iba1$^+$ CD206$^+$ microglia peaking at 7 days post-stroke, and then transitioning to elevated Iba1$^+$ CD16/CD32$^+$ microglia at 14 days post-stroke (129). However, despite this pattern of early anti-inflammatory polarization followed by delayed pro-inflammatory polarization in both young and aged mice, aged mice had reduced anti-inflammatory polarization relative to young animals (129). The authors also found a positive correlation between the number of Iba1$^+$ CD206$^+$ microglia and improved cognitive and motor performance, suggesting that the reduced ability of aged microglia to efficiently polarize to an anti-inflammatory phenotype may be responsible for poorer functional recovery following

stroke (129). However, the exact mechanism linking the inflammatory status of microglia to functional outcomes remains to be elucidated.

As noted earlier, microglia of aged animals have reduced cellular motility and phagocytosis, and augmented production of pro-inflammatory cytokines. These baseline differences have implications for microglial activation after stroke. In a distal MCAO (dMCAO) model of stroke, aging increased microglial proliferation in the peri-infarct area (130). Additionally, while aging was not shown to reduce proliferation of neuroblasts within the subventricular zone, there was an age-related reduction of migration of these neuroblasts to the peri-infarct area (131). Perhaps the enhanced proliferation of microglia (and astrocytes) in the peri-infarct area of aged animals is detrimental to neuronal repair processes following stroke leading to worse behavioral outcomes.

Interestingly, aging is associated with an accumulation of resident memory CD8 T cells in the brain parenchyma (42). At baseline, higher numbers of CD8 T cells correlate with reduced pro-inflammatory functions of microglia in the aged brain; however, following tMCAO these CD8 T cells had increased TNF, IFNγ and CCL2 as determined by intracellular cytokine staining (42). Together, these results suggest that CD8 T cells within the aging brain modify microglia homeostasis under naïve conditions but may be another source of priming the aging brain and potentiating damage following tMCAO. The role of these resident memory CD8 T cells in the aging brain will need to be evaluated in other disease models to determine their importance and relevance to disease.

It has also become increasingly apparent that inflammatory changes following ischemic stroke are not limited to the CNS but are evident throughout the body, including peripheral immune organs like the spleen. Following tMCAO both young and

aged mice have elevated gut permeability and translocation of intestinal microbes, but aged mice had prolonged loss of body weight, severe hypothermia, and persistent elevation of plasma IL-6 production at 72 hours post-stroke indicating that aged animals were not able to resolve the infection (132). While not directly assessed in this study, bacterial byproducts themselves (e.g. LPS) may be elevated in the blood of aged mice following stroke and, given the increased level of blood-brain barrier breakdown after ischemic injury, may directly activate microglia and further potentiate the pro-inflammatory polarization of microglia in the aged brain. Further studies investigating these mechanisms are necessary to understand how peripheral factors could influence microglia polarization and function, both under healthy conditions and in response to injury and disease.

The role of aged microglia in Alzheimer's disease:

Alzheimer's disease (AD) is a neurodegenerative disease primarily affecting older individuals and is the most common cause of dementia (133, 134). The earliest clinical manifestation of AD is memory impairment, and this is usually present at the time of clinical presentation/diagnosis of the disease. Currently available treatments only target the symptoms of AD, as there are no disease-modifying therapies available for the treatment of AD. New therapeutic approaches include vaccines targeted against Aβ and infusions of antibodies targeting Aβ such as Bapineuzumab; however, these approaches have failed in clinical trials to improve clinical outcomes (135) despite being well-tolerated by patients (136–138). While these results were disappointing, there is renewed hope in the clinical effectiveness of aducanumab, an antibody that selectively targets aggregated Aβ which appears to show promise in

preclinical studies and clinical trials (139). However, even if these new biological therapeutics slow or even halt the progression of AD, aged patients have other etiologies for cognitive decline beyond amyloid-beta pathology, such as vascular dementia, for which these drugs are likely to be less effective.

The AD brain has many specific pathological findings (140). These include aggregations of Aβ, hyperphosphorylated tau, neurofibrillary tangles, and glial cell activation. While the exact cause of Alzheimer's disease pathogenesis remains debatable, it is becoming clear that microglia play a crucial role in disease pathology, which has been recently reviewed elsewhere (141–143). The hippocampus is the area of the brain most densely populated by microglia, and is also one of the brain regions that is affected early in AD and leads to many of the clinical symptoms (38, 134, 144). The exact trigger for microglia activation remains unclear, but Aβ itself is capable of directly activating microglia (145–147). Accumulation of extracellular Aβ may be due to impaired phagocytosis of abnormal proteins by aged microglia. Aged microglia have poorer phagocytosis compared to microglia from young mice (18), potentially leading to accumulation of Aβ in the extracellular environment, further formation of Aβ aggregates, and a subsequent further activation of microglia (64, 148).

The reduced ability of microglia to phagocytose Aβ may be due to a decreased ability of microglia to directly bind and degrade Aβ. One study specifically comparing phagocytosis of Aβ by microglia in young and aging PS1-APP mice found that microglia from 8-month-old PS1-APP mice demonstrated significantly reduced RNA expression of Aβ binding receptors SRA, CD36, and RAGE relative to age-matched wildtype littermates, and levels declined even further by 14-months of age (149). Furthermore, reduced expression of Aβ degrading enzymes insulysin, neprilysin, and MMP9 was seen by 14-months of age (149). These findings suggest that in the setting

of AD, microglia become increasingly inefficient at their ability to clear Aβ with age, highlighting yet another functional failure of aged microglia.

The inflammatory nature of aged microglia likely also plays a role in AD pathogenesis. Microglia in naïve wildtype aged mice express elevated levels of IL-1β and TNF (18) compared to young microglia, suggesting that the pro-inflammatory "activated" phenotype occurs in the absence of AD or any other pathology and are a hallmark of aging in the CNS. In the setting of AD pathology, microglia PS1-APP mice were found to have elevated RNA expression of IL-1β and TNF at 8-months of age with further elevation in expression by 14-months (149). N9 microglia cells treated with TNF were found to have decreased expression of phagocytic receptors SRA and CD36, and in turn also had decreased uptake of Aβ (149). This suggests that elevation of inflammation from microglia within the aging brain reduces their ability to effectively phagocytose Aβ, thereby contributing to the pathogenesis and/or progression of Alzheimer's disease.

Recently, experiments have been performed using plasma transfers and parabiosis models to examine the contribution of peripheral cells and circulating factors in AD pathology, as summarized in **Table 2**.

Study	Strain	Age(s)	Model	Duration	Notable findings
(150)	APP on C57Bl/6 background	Young 2-3m Aged 16-20m	Heterochronic parabiosis • Aged APP – Young WT • Aged APP – Aged APP • Aged WT – Aged WT	5 weeks	• In the hippocampus: rejuvenation of synaptophysin and calbindin immunoreactivity; no change in total Aβ or Aβ-42 levels; no effect of CD68 immunoreactivity
			Plasma transfer • PBS • Young plasma	Administration twice weekly for 4 weeks	• In the hippocampus: rejuvenation of synaptophysin and calbindin immunoreactivity; no effect of CD68 immunoreactivity • Improved memory, spatial learning memory with young plasma transfer
(151)	APPswe/PS1dE9 Tg	Young 3m Tg 3m	Heterochronic parabiosis • Young Tg – Young WT • Age-matched Tg • Age-matched WT	6 months	In heterochronic Tg parabionts: • ↓ Aβ-40, Aβ-42, total Aβ, and Congo Red plaques in brain • ↓ CAA vessel number and area • Alleviation of neuronal degeneration and apoptosis
(152)	B6.CD45.1 5XFAD (CD45.2)	4m or 8m	Parabiosis B6.CD45.1 - 5XFAD	4 weeks	• No recruitment of CD45.1 WT monocytes to brains of 5XFAD parabionts • Brain-resident microglia associate with amyloid plaques, not peripheral monocytes
	B6.CD45.1 APP-PS1 (CD45.2)	3.5m	Parabiosis B6.CD45.1 - APP-PS1	9 weeks	• No recruitment of CD45.1 WT monocytes to brains of APP-PS1 parabionts

Table 1.2: Summary of studies examining the role of the peripheral immune system on AD pathology. Recent experiments utilizing models of parabiosis and plasma transfers are beginning to address the role of and extent that the peripheral immune system and soluble plasma factors may be manipulated in modifying AD pathology and cognition.

The most recent study utilized a model of heterochronic parabiosis to explore the possibility that circulating factors in young blood may prevent AD pathology and progression. Heterochronic parabiosis is a model in which a young animal is surgically attached to an aged animal and through anastomoses of the wound healing process come to share a common blood supply. This model has been shown to have significant effects on neurogenesis and identified circulating molecular markers of aging, particularly CCL11 and β2-microglobulin (83, 153). Heterochronic parabiosis with young 2-3-month-old wildtype mice and old 16-20-month-old APP transgenic mice showed that after 5 weeks of shared circulation, no reduction in total Aβ or Aβ-42 was seen in the hippocampus of aged APP heterochronic mice compared to APP isochronic mice (150). Interestingly, there was no difference in CD68 immunoreactivity (a lysosomal protein enriched in myeloid cells including microglia (154)) in the hippocampi of these mice, suggesting that once pathology is established the potential for reducing progression or reversing plaque burden may be limited. However, despite the inability of heterochronic parabiosis to delay or reverse disease pathology, APP mice had increased synaptophysin and calbindin immunoreactivity in the hippocampus, suggesting that circulating factors in young blood may at least be able to restore synaptic protein levels despite a lack of reduction in total Aβ or Aβ-42 plaque levels (150). While behavioral outcomes cannot be assessed in parabionts due to the surgery, these may be more accurately assessed in similar studies using plasma transfer experiments (83, 153). Similar to heterochronic parabionts, APP mice receiving blood from young wildtype donors also demonstrated increased synaptophysin and calbindin immunoreactivity relative to APP mice receiving PBS. APP mice receiving plasma from young wildtype donors were shown to have improved performance on Y-maze and contextual fear conditioning tests (150). Together, these

results suggest that factors from young healthy donors may be able to improve functional performance in diseased animals despite the inability to delay or reverse disease progression. Further investigation of potential “rejuvenation factors” in young plasma will be necessary to determine which factors may be responsible for these changes.

Epigenetic regulation of microglia polarization

As microglia are cells poised to respond to changes in their environment, determination of the overall phenotype of microglia is multifactorial and complex. Numerous environmental factors play a role. More specifically, damage associated molecular patterns (DAMPs), pathogen associated molecular patterns (PAMPs), ionic imbalances, and signals through various Toll-like receptors (TLRs) are all independently capable of initiating signaling cascades.

Additionally, while microglia phenotypes were initially believed to be analogous to those of peripheral macrophages of the hematopoietic myeloid lineage, it has been more recently believed that microglia phenotypes should not be thought of as mutually exclusive pro-inflammatory or anti-inflammatory phenotypes. Instead, many authors believe that microglia should be defined by the inciting stimulus to allow the scientific community to interpret results appropriately (155). Such definitions of specific stimuli, concentrations, or design and duration of exposures in vivo will allow for others to interpret and replicate the results (155, 156).

Depending on the signaling molecules present and the amount likely determine the overall phenotype of microglia. Thus, if predominantly TLR4 signaling molecules are present in the surrounding microenvironment, those microglia will be predominantly pro-inflammatory. However, if a mixture of pro- and anti-inflammatory signaling

molecules are simultaneously present in the microenvironment (which is more indicative of what occurs in the in vivo environment) a single microglia cell may simultaneously express both pro- and anti-inflammatory genes. However, the overall phenotype of microglia may be generalized by examining the levels of common markers of both pro- and anti-inflammatory phenotypes.

However, while the more realistic polarization of microglia occurs in vivo, the need to manipulate the microenvironment to elicit precise conditions is ideal for studying molecular mechanisms in vitro. While a number of stimuli may be used to manipulate the microenvironment, lipopolysaccharide (LPS) and interferon (IFN)-γ are commonly used in combination to stimulate a pro-inflammatory phenotype polarization whereas interleukin (IL)-4 is used to stimulate an anti-inflammatory phenotype (155). Using these conditions, one may specifically isolate microglia and polarize their phenotypes to better understand the molecular mechanisms of microglia phenotype polarization.

Epigenetic regulation is becoming increasingly implemented in the regulation of phenotype polarization of T cells (157, 158), B cells (159–162), and more recently identified in macrophages and microglia (61, 163–168). The term “epigenetics” is derived from Greek, and in translation means “above the gene.” As such, epigenetics refers to the study of changes in gene expression that occur outside of the DNA sequence itself, and include methods of modifying the physical chromatin structure which may allow for transcriptional activation or suppression (169). Understanding the role of epigenetic regulation in microglia phenotype polarization is particularly intriguing because many times a single epigenetic regulator may simultaneously regulate the expression of sets of genes (163, 169, 170). Thus, the potential to target one molecule to control the expression of an entire phenotype is particularly attractive.

There are many elements to epigenetic regulation, including methylation of DNA strands themselves, modifications of histone tails, and nucleosome positioning. The modification of histone tails provides an elaborate "histone code," allowing cells to gain control of their gene expression over a wide range of conditions (169, 171, 172). However, as each nucleosome can be composed of various types of histones, and each histone tail may have numerous modifications simultaneously, understanding the effective summation of various combinations of modifications in various specific cell types is only beginning to be understood.

Recently it was discovered that epigenetic regulation plays an essential role in microglia polarization. Tang *et al* discovered that Jumonji Domain Containing 3 (Jmjd3) was essential to microglia anti-inflammatory polarization (163). Jmjd3 is a histone lysine demethylase that specifically removes methyl groups from transcriptionally repressive histone H3 lysine 27 trimethylation (H3K27me3) marks to monomethylation marks (H3K27me1) (173). The histone modification H3K27me1 established by Jmjd3 allows for transcriptional activation (173). Tang *et al* found that when Jmjd3 was inhibited there was a significant reduction in H3K27me1 levels at the promoter of *Irf4*, a transcription factor heavily involved in microglia anti-inflammatory polarization (163, 174, 175). Furthermore, inhibition of Jmjd3 they found a significant reduction in the expression of Irf4 (163). These authors concluded that Jmjd3 is essential for microglia to fully polarize to an IL-4-induced anti-inflammatory phenotype (163).

Interestingly, contrasting the enzymatic effects of Jmjd3 is Enhancer of Zeste Homologue 2 (Ezh2). Ezh2 is a histone lysine *N*-methyltransferase that establishes H3K27me3 marks (176). This particular histone modification represses gene transcription by condensing the chromatin structure and preventing RNA Polymerase II and transcription factors from interacting with promoters (176–178). As Ezh2 and

Jmjd3 perform antagonizing functions, we hypothesize that while Jmjd3 is essential for anti-inflammatory polarization that Ezh2 may be essential for pro-inflammatory polarization (**Fig. 1.5**).

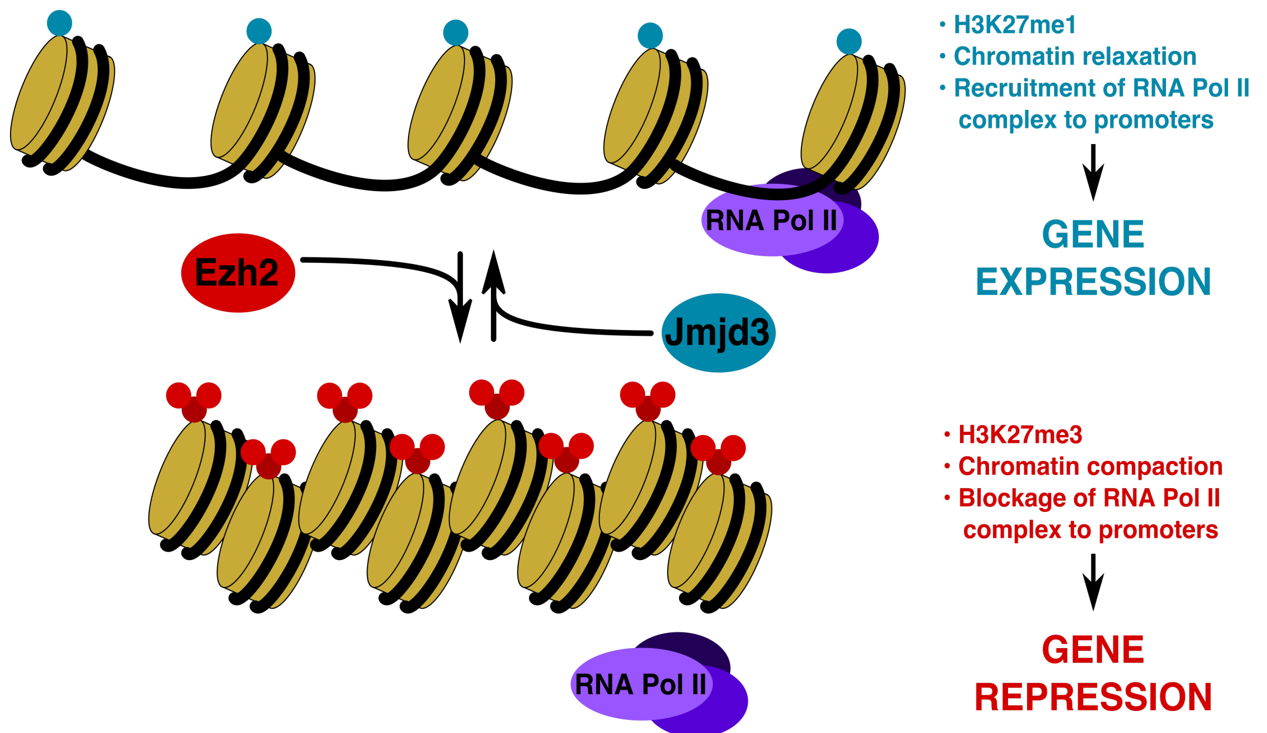

Figure 1.5. Functional epigenetic antagonism of Ezh2 and Jmjd3. Ezh2 deposits methyl groups to establish H3K27me3 marks, leading to compaction of chromatin and displacement of RNA Polymerase II transcription complex to repress gene transcription. In contrast, Jmjd3 demethylates H3K27me3 marks to H3K27me1 which allows for chromatin relaxation and the ability for RNA Polymerase II complex to activate gene transcription.

As discussed above, microglia phenotype changes drastically with age and acquires a pro-inflammatory phenotype at baseline. Additionally, we understand that epigenetic regulation plays a significant role in microglia polarization. We hypothesize that peripheral factors in aged mice are responsible for increasing Ezh2 function in the brain with age, causing the aged brain to acquire a dysfunctional pro-inflammatory aged phenotype. We further hypothesize that these epigenetic modifications, which by definition are reversible, may be rejuvenated through manipulation of the circulating peripheral immune system.

Conclusion

The U.S. population is aging at an alarming rate and the increase in age-related diseases such as stroke, TBI, and Alzheimer's disease will place an increasing strain on our healthcare system. Chronic age-related increases in inflammation exist in both the periphery and CNS. Microglia, like other long-lived cells may be especially vulnerable to the detrimental effects of aging, as reflected by changes in their molecular and cellular phenotype, decreased phagocytic potential, and increased production of ROS. These pro-inflammatory and primed microglia play a substantial role in the pathogenesis and progression of age-related neurological diseases and in the exaggerated response to injury and infection seen in the aged. Understanding the mechanisms by which microglia age will enhance the identification and development of novel intervention strategies to reduce the burden of age-related neurological diseases.

Chapter 2:
Materials and Methods

Mice

All animal experiments and terminal endpoints were conducted in compliance with approved protocols from the Institutional Animal Care and Use Committee of the University of Texas Health Science Center. Animal numbers of each group were calculated by power analysis and animals were randomly assigned to each group where applicable. All animals were either bred in-house or were allowed to rest for at least one month prior to experimentation. For naïve aging studies, C57Bl/6 mice at 3-, 6-, 12- (Charles River), 18-, or 24-months of age (NIA) were used. For parabiosis surgeries, young 2- to 3-month-old GFP mice (C57BL/6-Tg(UBC-GFP)30Scha/J, Stock 004353, Jackson Labs) or wildtype C57Bl/6 mice (Charles River) were used, as well as aged 18- to 20-month-old wildtype C57Bl/6 mice (NIA). For primary microglia cell culture, C57Bl/6 (Charles River) P0.5-P3 mouse pups were used. Sex was determined using the black dot method and confirmed with DNA from tail snips. Tails were genotyped for *SRY*, the male-specific testis determining gene, and *MYOG* was used as a positive PCR genotype control.

Parabiosis Surgery

For parabiosis surgeries, pairs consisted of young (2- to 3-month) GFP and young WT (young isochronic), young GFP and aged (18- to 20-month) WT (NIA) (heterochronic), and aged WT and aged WT (aged isochronic). Parabiosis was performed as previously described with some modifications (83, 179) . Briefly, animals were randomly assigned to surgical groups and pair-housed 1 pair per cage for at least 1 week prior to surgery. Pairs noted to have excessive fighting were excluded from the study. Mice were anesthetized with isoflurane, ophthalmic ointment was applied, and mice were administered slow-release buprenorphine pre-operatively (Buprenorphine

SR-LAB, 1mg/mL, 5mL, ZooPharmBZ8069317). Fur of adjacent surgical sides was clipped. Mice were transferred to a disinfected surgical station where they were aseptically prepared. Identical skin incisions were made on opposing flanks from the olecranon, along the length of the side, to the just anterior to the patella. 4-0 nylon sutures (Ethicon) were used to secure the humerus and femur of parabionts. Skin flaps of parabionts were joined using 6-0 Vicryl sutures (Ethicon) with simple continuous stitching. Prior to final skin closure, wounds were flushed with 5mL sterile saline. Stitches were secured with 9mm wound clips (Reflex) placed at regular intervals. In our model design, parabionts were not administered antibiotics in an attempt to maintain the integrity of the intestinal microbiome. Pairs were administered 1mL of sterile saline subcutaneously for 7 days following surgery, and were given softened food in their cages. Paired weights were monitored daily for 7 days, and then twice a week thereafter. Chimerism was confirmed by cheek bleed and analysis of percent GFP+ cells by flow cytometry after 2 weeks. This control was not possible for aged isochronic pairs as aged GFP mice are not commercially available, although others have reported efficient chimerism in aged isochronic pairs (180). Pairs were humanely euthanized after 8 weeks and samples were collected for further analysis.

Multiplex cytokine analysis

Deeply anesthetized mice were perfused with ice-cold 1X PBS and whole hemispheres were extracted and flash frozen. Tissue samples were homogenized in NP-40 lysis buffer (Thermo Fisher) and sonicated for 6 cycles of 5” on, 5” off on ice at 60% amplitude (QSonica). Lysates were centrifuged for 20 minutes at 4°C, 28,000rpm (Thermo Fisher). Supernatants were aliquoted and frozen at -80°C. Samples were thawed once for use in assays. Protein concentration was determined by BCA

(Thermo Fisher). The following multiplex immunoassays were used: Bio-Plex Pro Mouse Chemokine 33-plex Assay (Bio-Rad, 12002231), Bio-Plex Pro Mouse Cytokine 23-plex Assay (Bio-Rad, M60009RDPD), Bio-Plex Pro Mouse Cytokine Group II 9-plex Assay (Bio-Rad, MD000000EL), Bio-Plex Pro Mouse Cytokine Group III 8-plex Assay (Bio-Rad), Bio-Plex Pro Mouse TGFβ 3-plex Assay (Bio-Rad, 171W4001M), Milliplex MAP Mouse Kidney Injury Magnetic Bead Panel 2 (MKI2MAG-94K, Millipore), Milliplex MAP Mouse MMP Magnetic Bead Panel 3 (MMMP3MAG-79K, Millipore), Milliplex MAP Mouse Angiogenesis/Growth Factor Magnetic Bead Panel (MAGPMAG-24K, Millipore), Milliplex MAP Mouse Soluble Cytokine Receptor Magnetic Bead Panel (MSCRMAG-42K, Millipore). Samples were run in duplicate and assays were performed according to the manufacturer's protocols with some minor modifications. Assays were analyzed using a Bio-Plex 200 (Bio-Rad) with a minimum of 50 beads per bead region required for a valid read. Samples with a percent coefficient of variation >15% were excluded from analysis.

Primary microglia culture

P0.5-P3 mouse pups were humanely euthanized on ice and brains were removed for dissection. Cortices were isolated and enzymatically digested in 1.5mL sterile tubes using an enzymatic digestion kit (Neural Dissociation Kit (P), Miltenyi Biotec). Cortices were homogenized using sterile 1000uL and then 200uL pipet tips (USA Scientific). Individual cortices were plated in 25cm^2 flasks (Falcon) coated with poly-D-lysine (PDL)-coated (Millipore) in 4mL of media and cells were allowed to adhere overnight. To study sex differences, cells were cultured in Sex Differences Murine Microglia Media (SD mMG Media: phenol red-free DMEM (Gibco), 10% charcoal-stripped fetal bovine serum (Sigma), glutamine (Gibco), and 1% penicillin / streptomycin (Gibco). After 24hr

media was aspirated, adherent cells were gently rinsed in 1mL of media to remove debris, and 4mL of fresh media was added. On day in vitro (DIV) 5 and DIV 10, L929 conditioned media was added to 30%. On DIV 14, cultures were supplemented with HEPES and sodium bicarbonate to maintain pH while shaking at 150rpm for 2-3 hours on a 37°C incubated shaker. Microglia were counted and plated at 60-80,000 cells / well of PDL-coated 24-well plates (Corning) in L929 conditioned microglia media and allowed to rest for at least 2 days prior to experimentation.

BV-2 microglia cell culture

BV-2 murine microglia cell line cultures were maintained in Dulbecco's Modified Eagle's Medium, 10% heat-inactivated fetal bovine serum, and 1% penicillin / streptomycin (Gibco).

L929 conditioned media

L929 cell line (ATCC) were cultured to full confluency on 75cm^2 flasks (Corning) in DMEM, 10% heat-inactivated FBS, 1% penicillin / streptomycin. Media was removed, cells were rinsed in SD MMG Media, and then cultured in 35mL of SD mMG Media. After 2 weeks, L929 conditioned media was collected, filtered, and frozen at -80°C.

Stimulation of primary microglia with young or aged murine plasma

Primary microglia were obtained and cultured as above. Plasma was isolated and pooled from 3 healthy male C57Bl/6J mice of 3 months or 18 months of age. SD mMG media was prepared substituting 10% FBS with 10% murine plasma, and cultures were stimulated for 96 hours before isolating cells for RNA for RT-PCR analysis as described below.

RNA Isolation and Reverse Transcription PCR (RT-PCR)

For RT-PCR analysis, RNA was isolated using the RNEasy Micro Plus kit (Qiagen) RNEasy Lipid Mini Kit (Qiagen) and RNA purity was assessed by Nanodrop (Thermo). RNA was DNase-treated (NEB) and cDNA and no reverse transcriptase controls were prepared using iScript cDNA Synthesis Kit (Bio-Rad). Samples were diluted and PrimePCR Arrays (Bio-Rad) were performed according to the manufacturer's instructions using (Supermix name) Bio-Rad on a CFX384 Touch (Bio-Rad). Genes of interested were validated by individual real-time PCR (RT-PCR) using (Supermix name) (Bio-Rad) on a CFX96 Touch or CFX384 Touch (Bio-Rad). Primer sequences were designed using the PrimerQuest Tool available from IDT DNA (https://www.idtdna.com/Primerquest/Home/Index) with attempts to design primer pairs around exon boundaries. Sequences were validated and are listed in **Table 2.1**. Data was analyzed using the ΔΔCt method.

Target	Forward primer	Reverse primer	Accession
Il1b	GGCAGGCAGTATCACTCATT	GAGGATGGGCTCTTCTTCAAA	NM_008361
Il6	AGGAGACTTCACAGAGGATACC	GAATTGCCATTGCACAACTCTT	NM_031168
Tnfa	CCTCTTCTCATTCCTGCTTGT	TGGGAACTTCTCATCCCTTTG	NM_019467
Nos2	CCACAGTCCTCTTTGCTACTG	TGCAGACAACCTTGGTGTT	NM_010927
Arg1	CACCCTGACCTATGTGTCATTT	TCTGGGAACTTTCCTTTCAGTTC	NM_007482
Aif1	GAAGCCTTCAAGGTGAAGTACA	TTCAGCTCTAGGTGGGTCTT	NM_019467
Itgam	CCGGAAAGTAGTGAGAGAACTG	ATCCAAGGGATCACCGAATTT	NM_001082960

Table 2.1. RT-PCR primers used for gene expression analysis.

Western Blotting

Protein samples were prepared using Lamelli buffer (Bio-Rad) supplemented with beta-mercaptoethanol, heated, and loaded onto 4-15% TGX polyacrylamide gels (Bio-Rad) and transferred onto PVDF (Bio-Rad). Blots were blocked using 5% milk in Tris-Buffered Saline with Tween-20 (TBS-T). Blots were probed with anti-histone H3 (Abcam), anti-beta-actin-HRP (Sigma), anti-H3K27me1 (Millipore), anti-H3K27me3 (Millipore), anti-Jmjd3 (Abcam), anti-Ezh2 (Cell Signaling). Horseradish peroxidase-conjugated secondary antibodies were used to detect primary antibodies (Vector Labs). Images were taken using a (Bio-Rad G-box) (Bio-Rad). Selected images were quantified using Image Lab (Bio-Rad, Version 5.2.1).

Statistics

Statistics are presented as mean ± standard error of the mean (SEM) for all experiments using Prism 7 (GraphPad). Interval power analysis was performed to determine group size. For comparisons between 2 groups, Student's t-test was performed. For comparisons of multiple groups in naïve aging experiments, one-way ANOVA was performed using the youngest 3-month-old animal group as a control group. If an interaction was statistically significant, then Dunnett post-hoc test was performed. For comparisons involving multiple groups in heterochronic parabiosis experiments, two-way ANOVA was performed. If an experiment was found to be statistically significant, then Tukey post-hoc test was performed. For evaluation of mortality and weight loss of our model of parabiosis, log-rank Mantel Cox analysis was performed.

Chapter 3:
The Aging Brain is Epigenetically Dysregulated

Rationale

Other labs have shown that the histone demethylase Jmjd3 is essential for microglia anti-inflammatory polarization by demethylating H3K27 modification to a transcriptionally active H3K27me1 modification (163, 173). However, Ezh2 is a histone lysine N-methyltransferase which performs opposing functions to Jmjd3 by establishing transcriptionally repressive H3K27me3 histone modifications (176). Dr. Rodney Ritzel from our laboratory has shown that microglia become more pro-inflammatory in the brain with age (181). We hypothesized that Ezh2 function may be enhanced in the brains of mice with age and may be associated with the pro-inflammatory phenotype of the naïve aged brain. We first investigated the brains of aged mice to determine if the brain becomes epigenetically dysregulated with age.

Ezh2 and Jmjd3 become imbalanced in the brain with age

To investigate if levels of Ezh2 and Jmjd3 change in the brain with age, we analyzed protein levels of Ezh2 and Jmjd3 relative to β-actin by Western blot in whole murine brains at 6, 12, 18, and 24 months of age (**Fig. 3.1**). Relative to 6-month-old mice, Jmjd3 was significantly decreased by 12 months and remained decreased at 18 and 24 months (**Fig. 3.1B**). Ezh2 decreased by 12 months but levels of Ezh2 at 18 and 24 months of age were not significantly different from the levels of 6-month-old mice (**Fig. 3.1C**). Additionally, as Ezh2 and Jmjd3 perform antagonizing functions, we analyzed the ratio of Ezh2 levels relative to Jmjd3 levels. Our analysis revealed that Ezh2 was significantly elevated relative to Jmjd3 by 18 months and remained increased at 24 months (**Fig. 3.1D**). Additionally, the ratio of Ezh2:Jmjd3 demonstrated a progressive stepwise increase with age (**Fig. 3.1D**). Furthermore, the level of Ezh1 does not

change with age (**Fig. 3.2**). These findings suggest that the brain becomes epigenetically dysregulated in regards to Ezh2 and Jmjd3 with age.

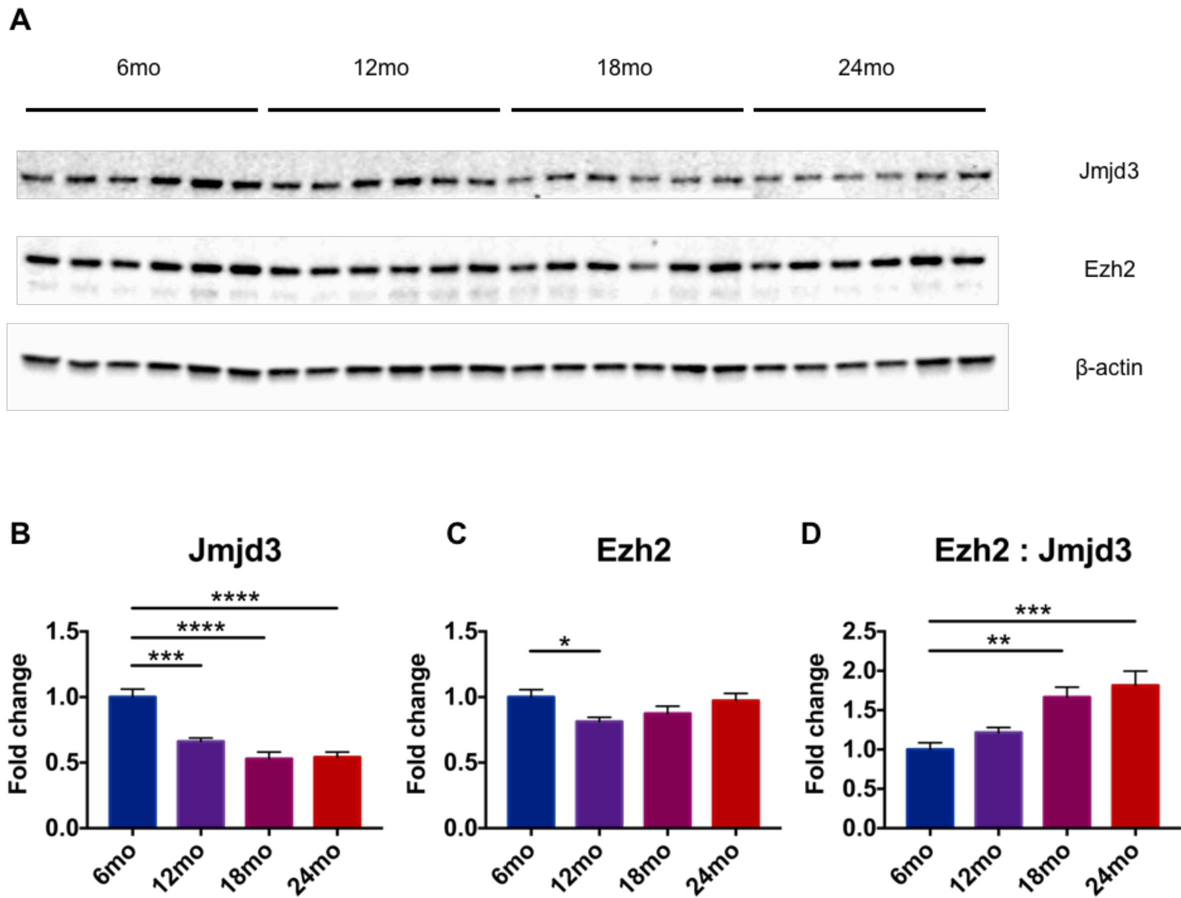

Figure 3.1. Jmjd3 decreases with aging and results in an increased Ezh2:Jmjd3 ratio with age. (A) Western blot of Whole brain protein extracts of naïve 6-, 12-, 18-, and 24-month-old male mice of Jmjd3, Ezh2, and β-actin loading control immunoreactivity. Quantification of Jmjd3 **(B)**, Ezh2 **(C)**, and calculated ratio of Ezh2:Jmjd3 quantification **(D)**. Data are presented as the mean +/- SEM, one-way ANOVA with Dunnet post-hoc test, n = 6/group, * $p<0.05$, ** $p<0.01$, *** $p<0.005$, **** $p<0.001$.

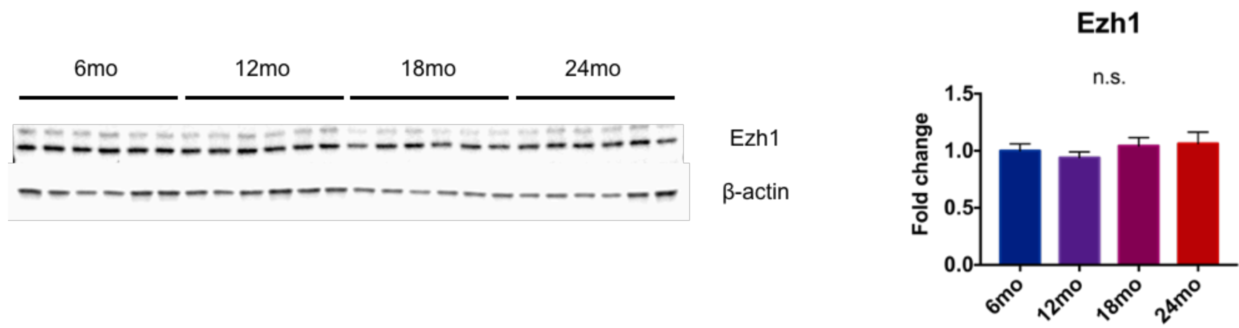

Figure 3.2. Ezh1 levels do not change in the brain with age. Western blot of Whole brain protein extracts of naïve 6-, 12-, 18-, and 24-month-old male mice of Ezh1 and β-actin loading control immunoreactivity. Quantification of Ezh1 relative to β-actin. Data are presented as the mean +/- SEM, one-way ANOVA with Dunnett post-hoc test, n = 6/group, n.s. not significant.

H3K27me3 and H3K27me1 become imbalanced in the brain with age

We then examined the levels of the histone modifications established by Ezh2 and Jmjd3, H3K27me3 and H3K27me1, respectively. Western blot analysis of whole brain murine protein samples revealed that with age mice undergo a decrease in H3K27me3, H3K27me2, and H3K27me1 relative to total histone H3 (**Fig. 3.3**). The levels of H3K27me3 and H3K27me1 correspond with the changes in the levels of Ezh2 and Jmjd3, respectively. Analysis of the ratio of H3K27me3:H3K27me1 demonstrates that the amount of H3K27me3 relative to H3K27me1 significantly increases by 18 months and remains elevated at 24 months (**Fig. 3.3**). Together, these results suggest that the brain becomes epigenetically dysregulated with age.

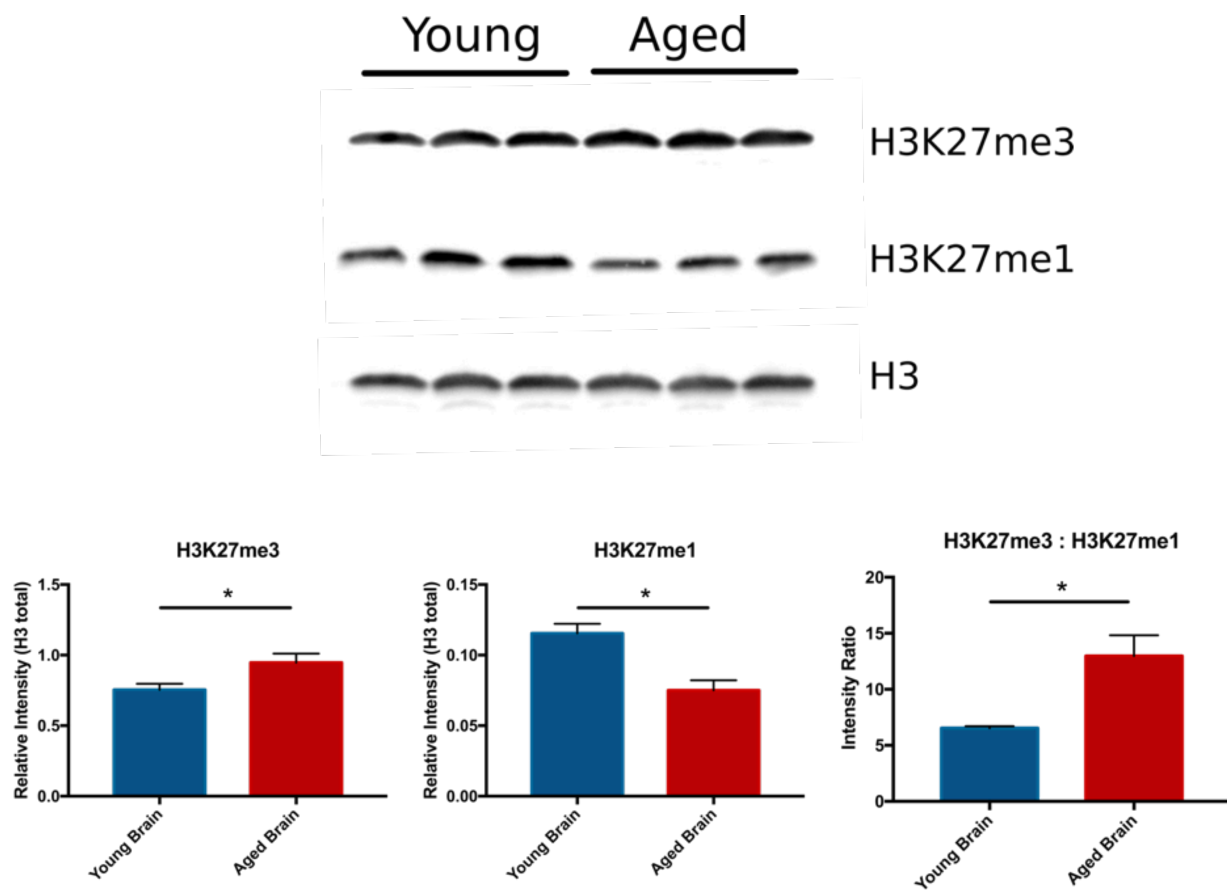

Figure 3.3. The brain becomes epigenetically dysregulated with age. Western blot of whole brain protein extracts of naïve 3- and 18-month-old male mice for H3K27me3, H3K27me1, and H3 total loading control. Quantification of H3K27me3, H3K27me1, and calculated ratio of H3K27me3:H3K27me1. Data are presented as the mean +/- SEM, Student's t-test, n = 3/group, * $p<0.05$.

The brain becomes progressively inflammatory with age

To examine if the baseline inflammatory status changes in the brain with age, we examined whole brain protein extracts of 3-, 6-, 12-, 18-, and 24-month-old mice by multiplex cytokine analysis. Analysis revealed that the levels of 24 cytokines, chemokines, and other analytes begin to be elevated at 6 months of age and progressively accumulate at 12, 18, and 24 months of age (**Fig. 3.4A**). Additionally, within our data analysis, we found it particularly notable that IFNγ and GM-CSF, which are implicated in the primed phenotype of microglia, were found to be significantly elevated in the brain with age (**Fig. 3.4B, 3.4C**). Overall, this analysis demonstrates that the brain acquires an inflammatory status that progressively increases with age and may promote a primed phenotype of microglia with age.

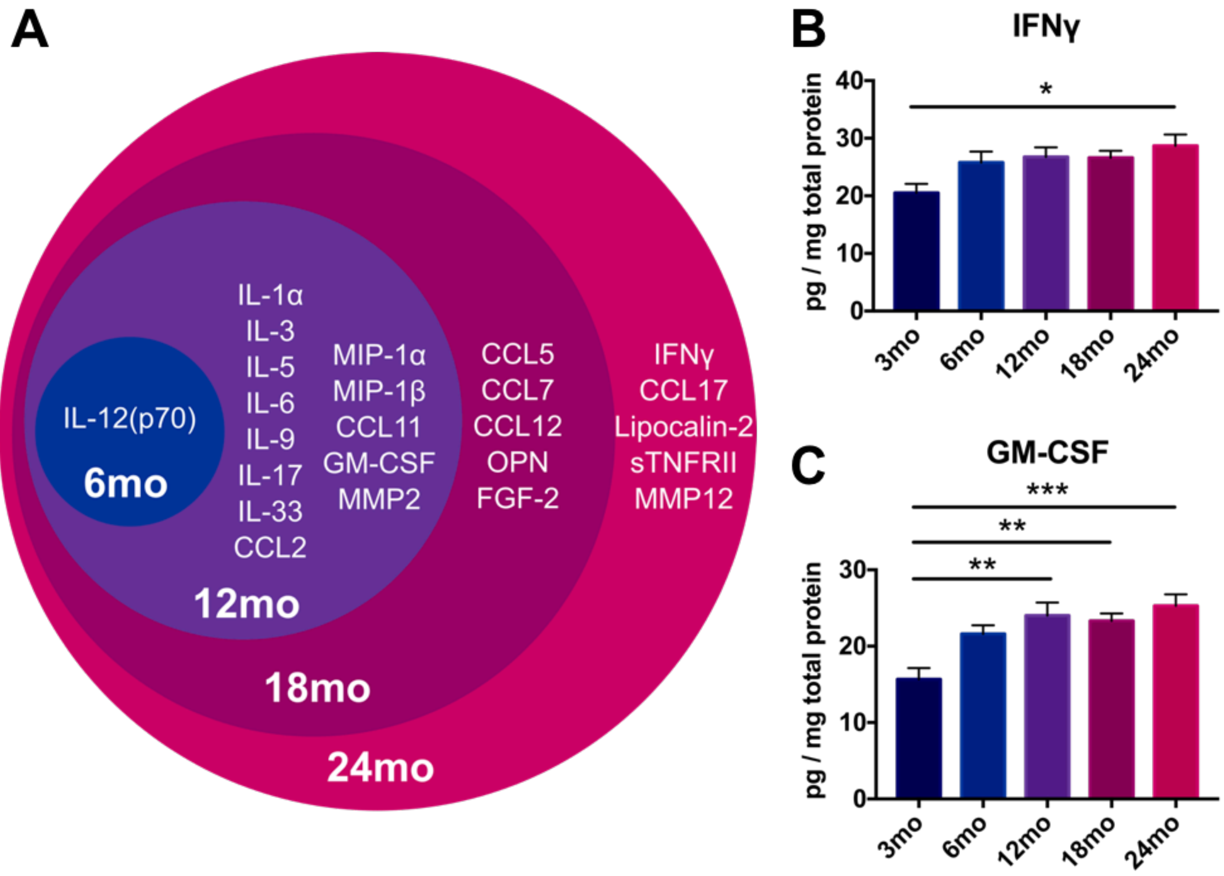

Figure 3.4. Age-associated increase in inflammatory cytokines in the naïve murine brain. (A) Whole brain extracts were examined in the brains of naïve mice at 3, 6, 12, 18, and 24 months of age by multiplex cytokine analysis. Each circle designates the age of the mice examined, and the analytes significantly elevated at that age are present in the circle and remain significantly elevated at later ages. The individual plots for IFNγ **(B)** and GM-CSF **(C)** are shown. Data are presented as the mean +/- SEM, one-way ANOVA with Dunnett post-hoc test, n = 5-9/group, * $p<0.05$, ** $p<0.01$, *** $p<0.005$.

The circulating peripheral factors

We wanted to examine if the circulating peripheral factors also increased in naïve mice across the healthy aging lifespan. We isolated plasma from naïve 3-, 6-, 12-, 18-, and 24-month-old mice and examined levels of circulating peripheral factors by multiplex cytokine ELISA analysis. We found a that mice demonstrate a significant increase in CXCL13, CCL11, CX3CL1, monocyte chemoattractant proteins MCP-3 and MCP-5, and macrophage inflammatory proteins MIP-1α, MIP-2, and MIP-3α. Notably, our results independently replicate those of the Wyss-Coray laboratory which found an increase in circulating CCL11 with age (180). Moreover, our results also demonstrate that circulating IFNγ does not increase with age, and levels of circulating GM-CSF were undetectable. This suggests that the age-associated increase of these two cytokines observed in the brain are not directly due to increased circulating levels of these factors.

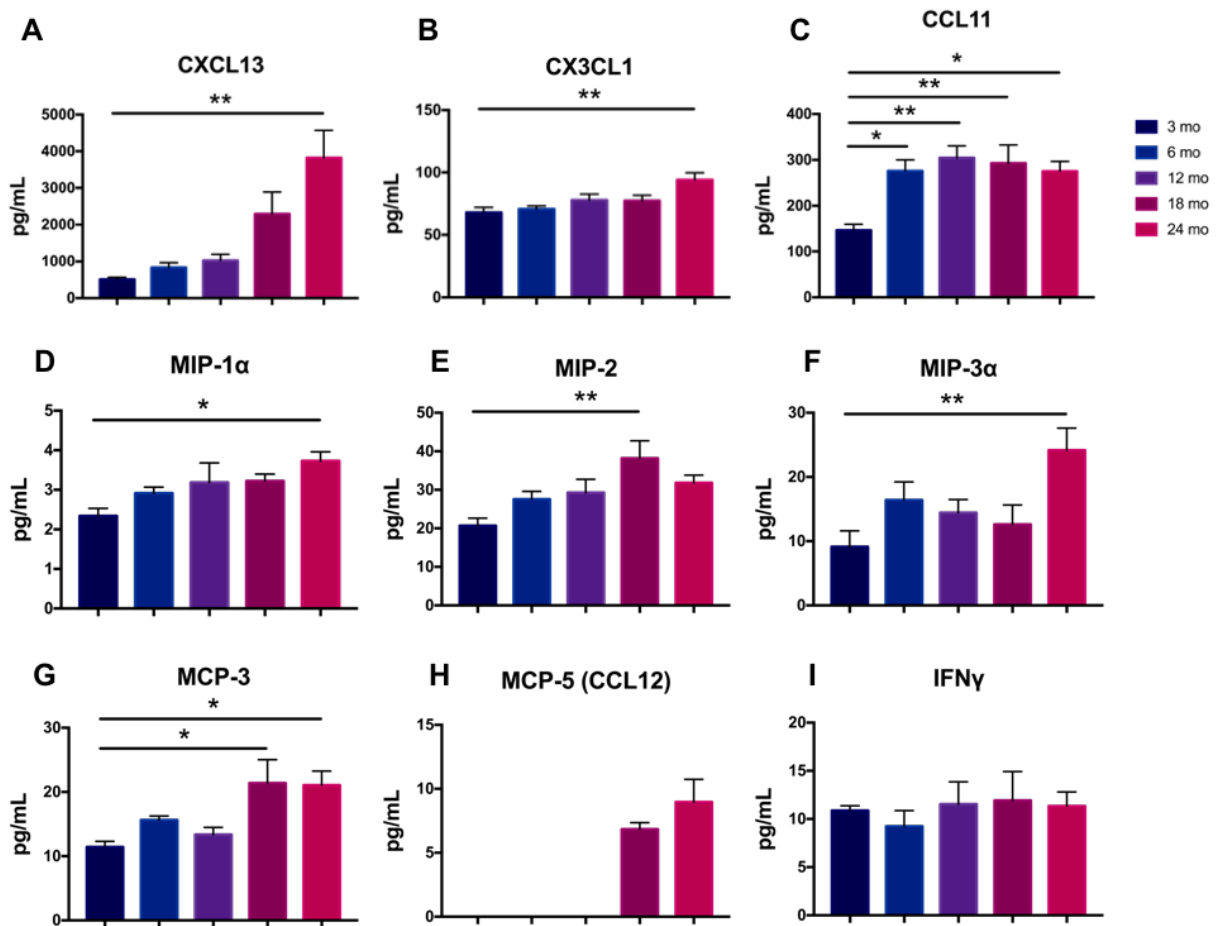

Figure 3.5. Increase in circulating plasma factors with age. Multiplex cytokine ELISA analysis of plasma isolated from naïve 3-, 6-, 12, 18-, and 24-month old mice. Significant results were found for elevations in circulating **(A)** CXCL13, **(B)** CXCL1, **(C)** CCL11, **(D)** MIP-1α, **(E)** MIP-2, **(F)** MIP-3α, (**G)** MCP-3, and **(H)** MCP-5. Levels of IFNγ **(I)** were not significantly different from levels at 3 months of age. Data are presented as the mean +/- SEM, one-way ANOVA with Dunnett post-hoc test, n = 5-9/group, * p<0.05, ** p<0.01.

The aged hippocampus has elevated expression of *Aif1* and *Itgam*

Iba1 and CD11b and encoded by genes *Aif1* and *Itgam*, respectively, and in the absence of injury and peripheral cellular infiltration are two microglia-specific genes. Age-associated microglia priming is associated with up-regulation of both *Aif1* and *Itgam*. We found that the expression of *Aif1* and *Itgam* increase in the hippocampus of mice with age (**Fig. 3.6**). This further supports our observation that the aged brain becomes progressively inflammatory with age.

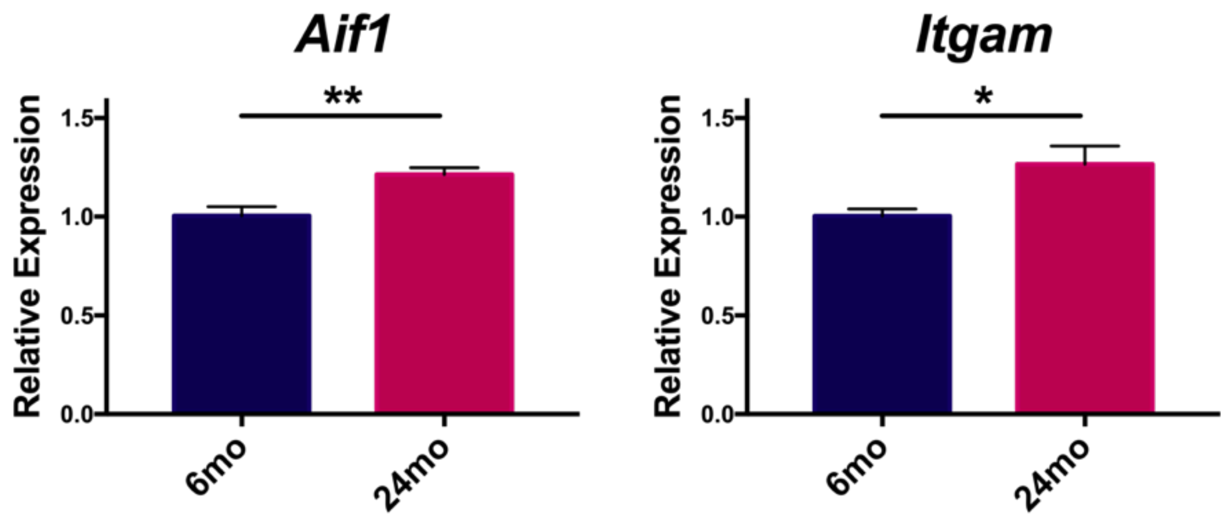

Figure 3.6. *Aif1* and *Itgam* expression increases in the hippocampus of naïve mice with age. RT-PCR analysis using the ΔΔCt method for *Aif1* and *Itgam* expression in the hippocampus of naïve mice at 6 and 24 months of age. Data are presented as the mean +/- SEM, Student's t-test, n = 6-8/group, * $p<0.05$, ** $p<0.01$.

Aged plasma factors directly influence microglia phenotype *in vitro*

To determine if peripheral factors can influence microglia phenotype, we cultured primary microglia cultures using plasma isolated from young 3-month-old or aged 18-month-old mice. We found that the microglia stimulated with aged plasma demonstrated up-regulation of pro-inflammatory genes *Il1b* and *Il6*, but not *Tnfa* (**Fig. 3.7**). This demonstrates that an aged plasma factor(s) may be directly responsible for influencing a pro-inflammatory phenotype microglia in the brain with age.

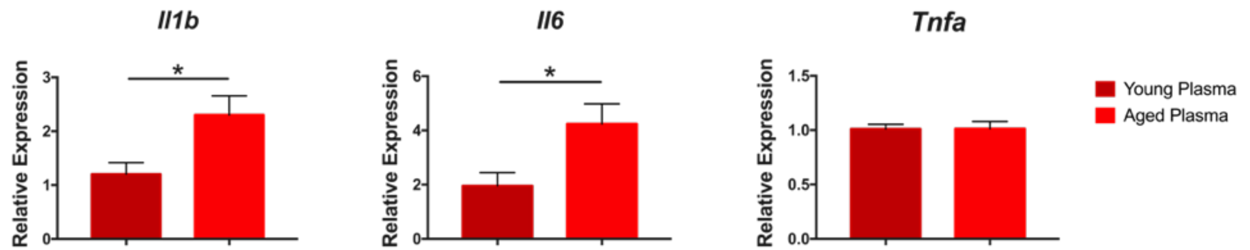

Figure 3.7. Aged plasma increases expression of *Il1b* and *Il6* in primary microglia *in vitro*. Primary microglia were cultured in serum-free media containing plasma isolated from young 3-month-old or aged 18-month old male mice. RT-PCR analysis using the ΔΔCt method was performed. Data are presented as the mean +/- SEM, Student's t-test, n = 4/group, * $p<0.05$.

Conclusions

This work provides valuable insight into how the brain and periphery change with healthy aging in the absence of any inciting stimulus. First, this data demonstrates that the brain becomes epigenetically dysregulated and pro-inflammatory with age. The aged brain exhibits higher levels of ratio of Ezh2:Jmjd3 and corresponding increase in H3K27me3 and decrease in H3K27me1 with age. Additionally, the brain becomes more pro-inflammatory with age and increases progressively throughout the lifespan of the healthy naïve mouse. This suggests that the aging process is cumulative and consistently progresses with time. Additionally, our corresponding studies of aging plasma demonstrates that some factors increase in the circulating periphery with age, but these do not directly reflect those that increase in the brain. Of those elevated in the circulating periphery, notably macrophage inflammatory proteins and monocyte chemoattractant proteins, suggests a progressive inflammatory environment. However, levels of major pro-inflammatory cytokines were not significantly elevated. For example, levels of IL-1β, IL-6, and TNF-α were not significantly elevated in the circulating plasma with age. When examining the plasma and brain multiplex cytokine ELISA data together, this suggests that the pro-inflammatory environment of the aging brain is potentially an independent inflammatory process and not reflective of ongoing systemic inflammation.

Furthermore, we questioned whether circulating peripheral factors may influence the inflammatory phenotype of the aging brain. We cultured primary microglia and stimulated cells with plasma isolated from naïve young or aged mice. Of those cultured with plasma from aged animals, there was increased expression of pro-inflammatory *Il1b* and *Il6*. This suggests that circulating peripheral factors may directly influence the

phenotype of the aged brain. Together, this work demonstrates that there is an imbalance in epigenetic regulation between Ezh2 and Jmjd3 and this is accompanied by a significant transition of the aged brain to a pro-inflammatory phenotype.

Chapter 4:
Ezh2 is Essential for Pro-Inflammatory Microglia Polarization

Rationale

Being the resident innate immune cells of the central nervous system, microglia have the potential to initiate immune responses through polarization of their phenotype. While multiple phenotypes have been characterized in response to particular stimuli, the two predominant phenotypes are the pro-inflammatory and anti-inflammatory phenotypes. As we age, Dr. Rodney Ritzel from our laboratory has shown that microglia in the brains of aged mice become more pro-inflammatory at baseline and produce more IL-1β and TNFα (181). However, how these aged microglia become more pro-inflammatory remains unknown.

Many laboratories have investigated if microglia turnover in the brain throughout the healthy lifespan of an individual, but it is largely believed that the microglia that populate our CNS during embryonic development may reside there for the lifetime of the mouse and even for several decades in humans (182, 183). Essentially, as we age so do the microglia in our CNS. These microglia, however, alter their gene expression with age to assume a more pro-inflammatory phenotype at baseline in a naïve CNS (181). We hypothesized that microglia alter their epigenetic regulation to assume a pro-inflammatory phenotype with age.

It has been shown that epigenetic regulation is essential to appropriate microglia phenotype polarization. The histone demethylase, Jumonji Domain Containing 3 (Jmjd3, *Kdm6b*), is essential for anti-inflammatory phenotype polarization (163). Jmjd3 functions by removing histone H3 lysine 27 trimethylation (H3K27me3) marks to a monomethylation mark (H3K27me1). This modification allows chromatin to go a from a condensed structure with H3K27me3 marks to a more relaxed chromatin structure with H3K27me1 (173). This change in chromatin structure allows for RNA Polymerase II and transcription factors to bind to promoters and transcribe anti-inflammatory genes.

The function of Jmjd3 is antagonized by Enhancer of Zeste Homologue 2 (Ezh2), which is a histone lysine N-methyltransferase that functions to transfer methyl groups from SAM molecules to H3K27me1 modifications to establish H3K27me3 marks (176). In theory, this would reverse the effects of Jmjd3 to repress gene targets. Thus, we hypothesize that Ezh2 and Jmjd3 lie in a functional antagonism and that Ezh2 is essential for pro-inflammatory phenotype polarization.

Pro-inflammatory polarization of microglia results in increased deposition of H3K27me3

We hypothesized that Ezh2 is essential to microglia pro-inflammatory polarization. We first wanted to examine the possible role for Ezh2 in polarization of microglia. We cultured BV-2 microglia cell line and then stimulated cells with LPS+IFNγ, IL-4, or PBS vehicle control. We then collected cells 4 hours and 24 hours after stimulation for analysis by Western blot. We found that following 24 hours of stimulation that there was significant increase in H3K27me3 (**Fig. 4.1**). Additionally, we did find a significant decrease in Ezh2 but not homologue Ezh1 at 24 hours of stimulation with LPS+IFNγ (**Fig. 4.1**). We also further examined if LPS and/or IFNγ were independently responsible for the increased H3K27me3 deposition that we observed following 24hr of stimulation, however, we found H3K27me3 to be similarly increased at 24 hours of stimulation with either LPS alone, IFNγ alone, or LPS+IFNγ combined (**Fig. 4.2**). Thus, our results demonstrate that Ezh2 function may play a role in pro-inflammatory polarization of microglia.

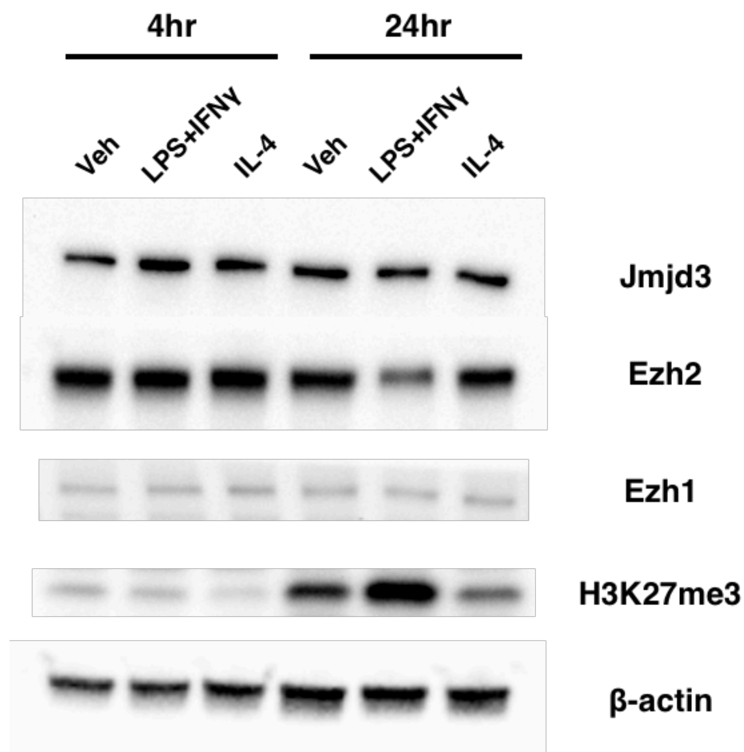

Figure 4.1. Stimulation of BV-2 microglia with LPS+IFNγ results in increased deposition of H3K27me3. BV-2 microglia cultures were stimulated with vehicle control, 100ng/mL LPS + 10ng/mL IFNγ, or 20ng/mL IL-4 for 4 or 24 hours. Representative Western blot of Jmjd3, Ezh2, Ezh1, H3K27me3, and β-actin loading control immunoreactivity of 3 independent experiments.

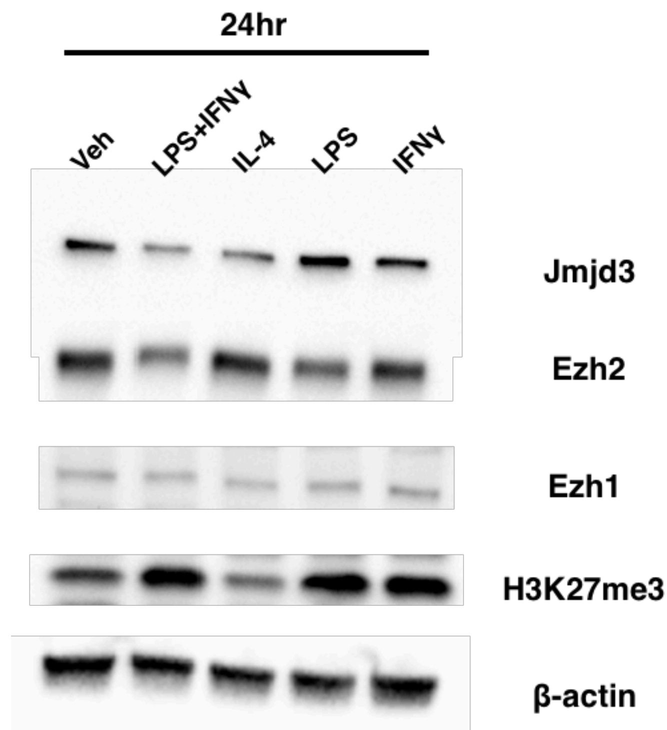

Figure 4.2. Stimulation of BV-2 microglia with LPS, IFNγ, or combined LPS+IFNγ results in increased deposition of H3K27me3. BV-2 microglia cultures were stimulated with vehicle control, 100ng/mL LPS + 10ng/mL IFNγ, or 20ng/mL IL-4 for 24 hours. Representative Western blot of Jmjd3, Ezh2, Ezh1, H3K27me3, and β-actin loading control immunoreactivity of 3 independent experiments.

Inhibition of Ezh2 results in abrogation of pro-inflammatory polarization

We wanted to investigate if inhibition of Ezh2 could block pro-inflammatory polarization of microglia. Primary microglia were cultured and pre-treated with GSK343 or DMSO vehicular control, and subsequently stimulated with LPS+IFNγ, IL-4, or 0.1% PBS vehicular control. RT-PCR analysis demonstrated that inhibition of Ezh2 abrogated LPS+IFNγ-induced up-regulation of pro-inflammatory genes *Il1b*, *Tnfa*, *Il6*, and *Nos2* (**Fig. 4.3**). Additionally, inhibition of Ezh2 resulted in enhanced up-regulation of IL-4-induced anti-inflammatory gene *Arg1* (**Fig. 4.4**). These results suggest that Ezh2 is essential for microglia pro-inflammatory phenotype polarization and may be involved in repressing genetic transcription of anti-inflammatory genes.

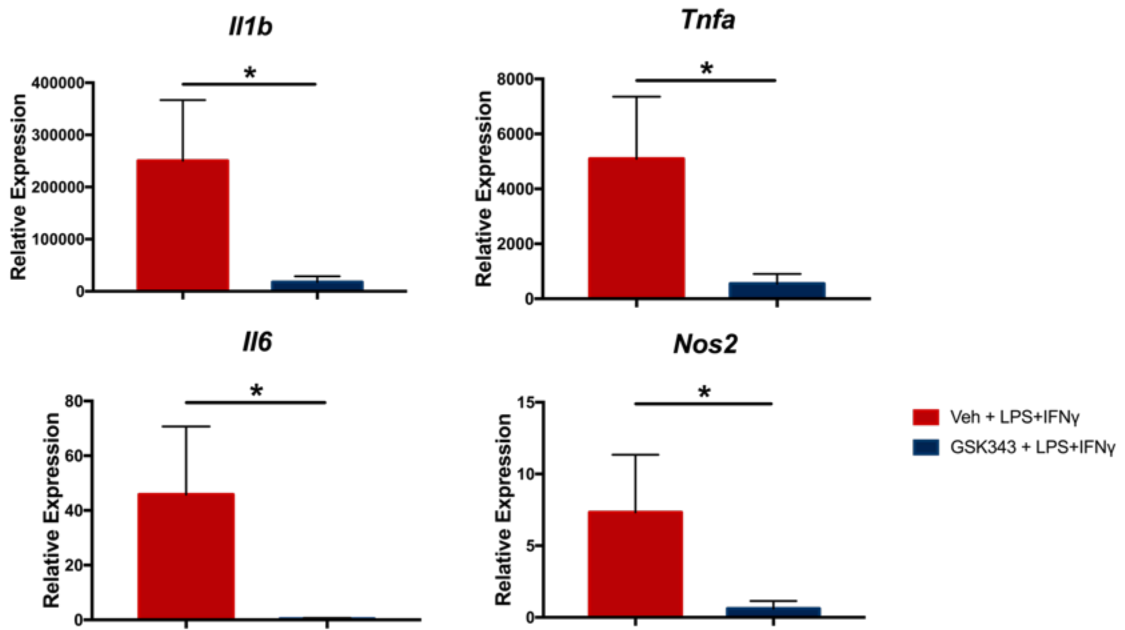

Figure 4.3. Pharmacological inhibition of Ezh2 results in decreased expression of pro-inflammatory molecules. Primary microglia were cultured with 6uM GSK343 or DMSO vehicle control for 24 hours prior to stimulation with 100ng/mL LPS + 10ng/mL IFNγ, or 20ng/mL IL-4 for 24 hours in the presence of 6uM GSK343 or DMSO vehicle control. Data are presented as the mean +/- SEM, n = 6/group, * $p<0.05$.

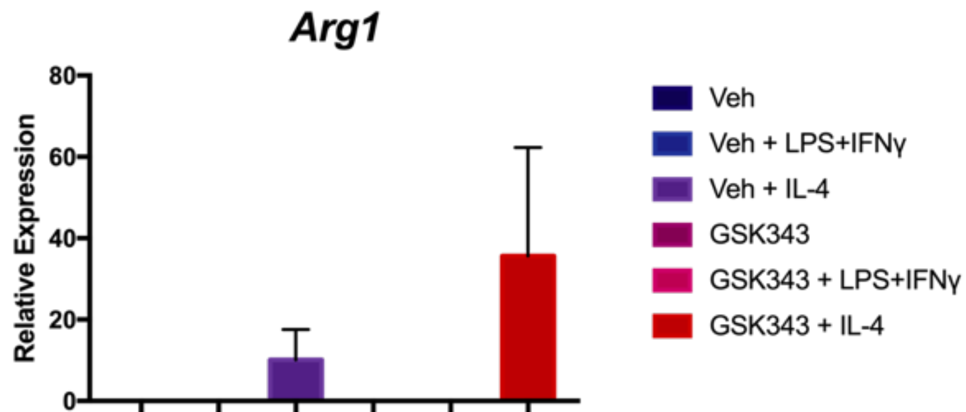

Figure 4.4. Pharmacological inhibition of Ezh2 results in increased expression of anti-inflammatory marker and *Arg1*. Primary microglia were cultured with 6uM GSK343 or DMSO vehicle control for 24 hours prior to stimulation with 100ng/mL LPS + 10ng/mL IFNγ, or 20ng/mL IL-4 for 24 hours in the presence of 6uM GSK343 or DMSO vehicle control. RT-PCR analysis using the ΔΔCt method was performed. Data are presented as the mean +/- SEM, n = 6/group, * $p<0.05$, ** $p<0.01$.

Inhibition of Ezh2 results in decreased production of pro-inflammatory cytokines

To determine if Ezh2 is not only responsible for increased gene expression of pro-inflammatory genes, we cultured primary microglia as before and pre-treated with GSK343 or DMSO vehicular control, and subsequently stimulated with LPS+IFNγ for 24 hours. We then collected media for multiplex cytokine analysis. We found that pharmacological inhibition of Ezh2 results in decreased production of several pro-inflammatory cytokines, including IL-1β, IL-6, and TNFα (**Fig. 4.5**). These results demonstrate that Ezh2 function is essential for pro-inflammatory phenotype polarization of microglia.

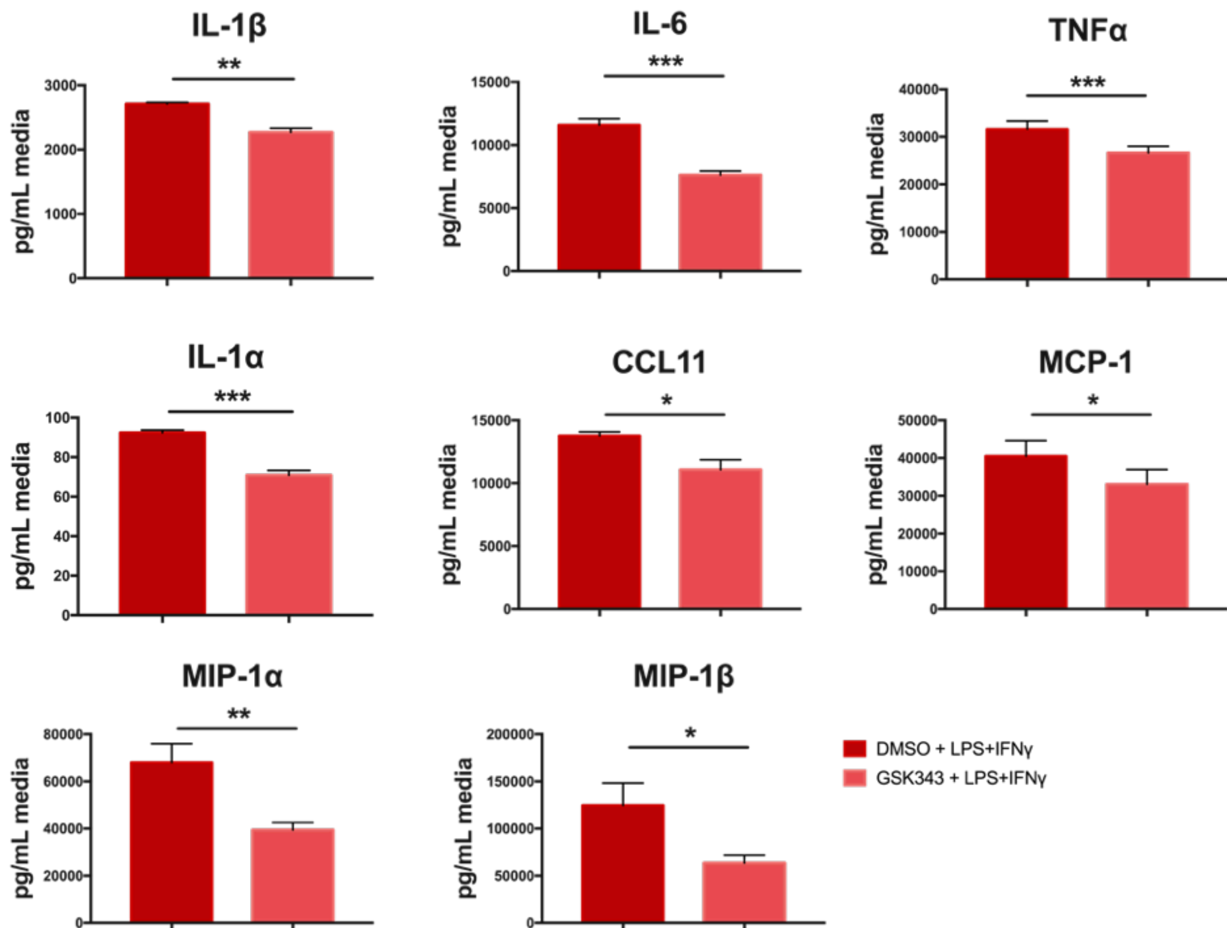

Figure 4.5. Pharmacological inhibition of Ezh2 results in decreased production of pro-inflammatory cytokines. Primary microglia were cultured with 6uM GSK343 or DMSO vehicle control for 24 hours prior to stimulation with 100ng/mL LPS + 10ng/mL IFNγ for 24 hours in the presence of 6uM GSK343 or DMSO vehicle control. Multiplex cytokine analysis was performed. Data are presented as the mean +/- SEM, Student's paired t-test, n = 6/group, * $p<0.05$, ** $p<0.01$, *** $p<0.005$.

Conclusion

We found that polarization of microglia to a pro-inflammatory phenotype results in increased deposition of H3K27me3 following 24 hours of stimulation with LPS+IFNγ, a histone methylation mark established by Ezh2. Additionally, we found this mark to increase following 24 hours of stimulation with LPS, IFNγ, or a combination of LPS+IFNγ. As LPS and IFNγ are capable of independently polarizing microglia to a pro-inflammatory phenotype (155, 156), this suggests that H3K27me3 is important to the acquisition of a pro-inflammatory phenotype.

When we inhibited Ezh2 using the functional inhibitor GSK343, we found that in the setting of a pro-inflammatory stimulus using LPS+IFNγ that the expression several major pro-inflammatory molecules were decreased, notably *Il1b*, *Il6*, *Tnfa*, and *Nos2*. This suggests that Ezh2 function is essential to the expression of pro-inflammatory genes. Additionally, when we inhibited Ezh2 and stimulated cultures with IL-4 to induce an anti-inflammatory phenotype, we found a significant increase in the expression of anti-inflammatory marker *Arg1*. This suggests that Ezh2 has a role in the expression of both pro- and anti-inflammatory gene sets, and functions to promote a pro-inflammatory phenotype by simultaneously down-regulating anti-inflammatory genes.

When we examined the media of cultures following pro-inflammatory stimulation with LPS+IFNγ with or without inhibition of Ezh2 with GSK343, we found that those cultures with Ezh2 inhibition has significantly less pro-inflammatory cytokine production. Collectively, our results demonstrate that Ezh2 is essential for pro-inflammatory phenotype polarization of microglia.

Chapter 5:
Heterochronic Parabiosis Rejuvenates the Epigenetic Status of the Aging Brain

Rationale

Our results have shown that Ezh2 promotes pro-inflammatory polarization in microglia (Chapter 4), and that the brain becomes epigenetically imbalanced with age to promote a predominant pro-inflammatory phenotype (Chapter 3). Additionally, our work *in vitro* demonstrates that plasma factors may promote a pro-inflammatory polarization of microglia. Epigenetic modifications are, by definition, reversible and may be influenced by response to the surrounding microenvironment. We questioned if the epigenetic imbalance observed in the aging brain is 1) influenced by circulating peripheral factors and 2) is potentially reversible. We chose to utilize a model of heterochronic parabiosis to investigate the role of the circulating peripheral factors on the epigenetic landscape of the aging brain.

Characterization of murine heterochronic parabiosis model

To determine if the epigenetic imbalance that occurs in the brains of mice with age can be rejuvenated through manipulations in peripheral factors, we utilized a model of heterochronic parabiosis in which young and aged mice are surgically joined and come to share a common blood supply. This model has been well-established by other labs and has been shown to have significant rejuvenation effects in the aged brain (83, 153).

Numerous studies and work from our own laboratory have demonstrated that the intestinal microbiome can influence behavior tests (132, 184). In our model, we perform all surgeries under strict sterile technique and intentionally do not use antibiotics as part of our post-operative care in an effort to preserve the integrity of the intestinal microbiome. This modification allows us to effectively study the mutual

sharing of circulating cells, factors, metabolites from the intestinal microbiome, and the interplay of all of these factors together with each other rather than individually assessing the role of each potential factor (**Fig. 5.1**).

	Bone marrow chimera	Plasma transfer	Microbiome transfer	Parabiosis
Cells	X			X
Peripheral factors		X		X
Microbiome			X	X
Interplay of above factors				X

Figure 5.1. Model of heterochronic parabiosis. Young and aged animals are surgically attached and allowed to share a common blood supply through anastomoses. The model allows for the simultaneous transfer of cells, circulating peripheral factors, and intestinal microbiome metabolites which provides substantial power in conjunction with other animal models such as bone marrow chimerism, plasma transfer, and microbiome transfer studies.

We did not observe any mortality post-operatively due to infection, but rather all mortality observed was due to parabiotic illness as determined by autopsy (**Fig. 5.2**). Parabiotic illness is a condition which is believed to be caused by the development of unequal anastomoses create an imbalance in the shared blood supply and one parabiont accumulates blood while the other is depleted, and is essentially analogous to twin-twin transfusion syndrome of monochorionic gestational twins(185). Interestingly, the mortality from parabiotic illness was rare in young isochronic pairs but equally observed between heterochronic and aged isochronics pairs (**Fig. 5.2B, C**). Additionally, we found that nearly all mortality occurred by 4 weeks after surgery, suggesting by this time post-operatively that anastomoses were well-established (**Fig. 5.2B**). We then allowed parabionts to live conjoined until 8 weeks post-operatively before use in our studies. Weight loss of combined parabionts was highest in the aged isochronic mice, but they maintained their post-operative weight loss by the time of sacrifice (**Fig. 5.2D**). This is similar to our work with aged animals, in which aged animals lose more weight post-operatively compared to young animals (125).

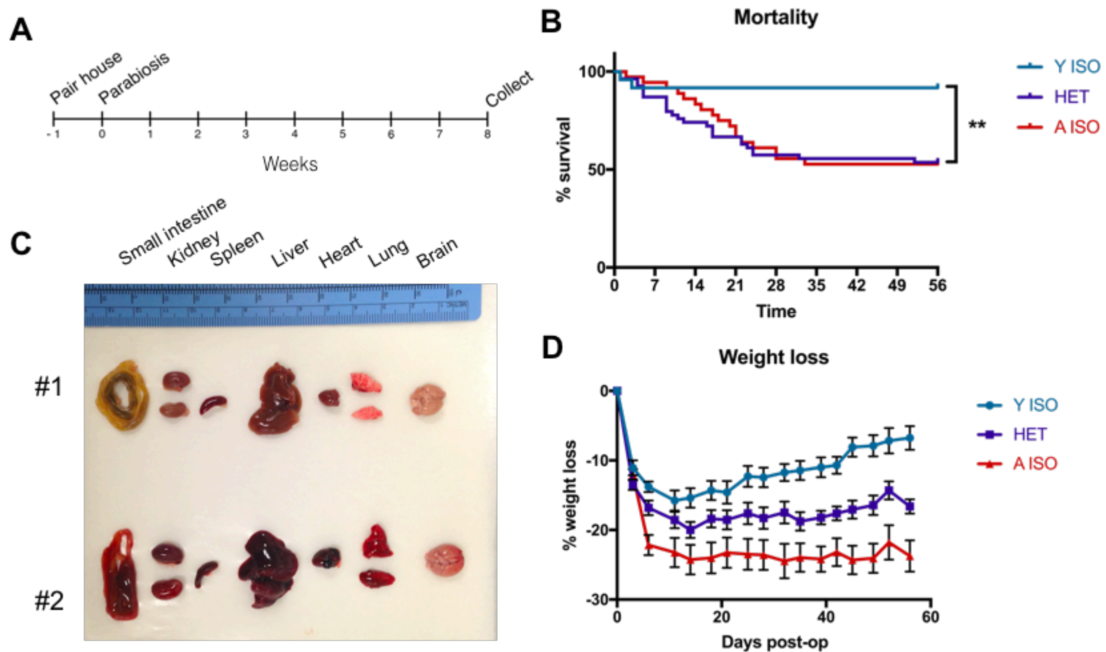

Figure 5.2. Characterization of the heterochronic parabiosis model. (A) Experimental timeline of the heterochronic parabiosis model. Mice were randomly assigned to experimental groups and were pair-housed together for at least 1 week prior to surgery. Parabionts were joined for 8 weeks prior to collection of samples for further investigation. **(B)** Mortality of parabionts. We found heterochronic (HET) and aged isochronic (A ISO) pairs to experience significant mortality compared to young isochronic (Y ISO) pairs. **(C)** Parabiotic illness. Autopsy of deceased parabionts demonstrates parabiotic illness, in which parabionts experience unequal blood circulation. In the autopsy of the pair shown, the organs of one mouse were pale and appear depleted of circulating blood (#1, top) while the other appears saturated with blood (#2, bottom row), thus demonstrating an unequal share of the circulating blood supply. **(D)** Weight loss of parabionts following surgery. Data are presented as a **(B)** Kaplan-Meier curve, log-rank Mantel Cox analysis, ** $p<0.01$; **(D)** mean +/- SEM, log-rank Mantel Cox analysis, ** $p<0.01$.

Heterochronic parabiosis rejuvenates H3K27me3 in the aged brain

We investigated if heterochronic parabiosis can reverse the epigenetic dysregulation that occurs in the brain with age (**Fig. 5.3**). Western blot analysis of whole brain protein extracts revealed that the age-associated increase in H3K27me3 and decrease in H3K27me1 was maintained (Y ISO vs. A ISO, **Fig. 5.3A, B**). This suggests that the parabiosis surgery itself does not disturb the epigenetic modifications that occur with age in naïve animals (**Fig. 3.3**). Interestingly, we found that heterochronic parabionts have levels of H3K27me3 that are reduced in the brains of A HET mice relative to those of A ISO parabionts (**Fig. 5.3A, B**). However, the levels of H3K27me3 were similar between Y ISO and Y HET animals, suggests that the rejuvenation effect observed in A HET mice is not from a dilution effect of a detrimental factor into Y HET animals. Together, this suggests there is a peripheral factor from the young parabiont that is beneficial in reducing the levels of H3K27me3 in the aged brain.

Examination of H3K27me1 in parabionts demonstrated maintenance of the age-associated decrease in our parabiosis surgical model (Y ISO vs. A ISO, **Fig. 5A, C**). However, heterochronic parabiosis failed to increase levels of H3K27me1 in the A HET brain (i.e. failed rejuvenation effect) and also failed to decrease levels in the Y HET brain (i.e. failed accelerated aging effect) (A HET and Y HET, respectively, **Fig. 5.3A, C**). This suggests that peripheral factors do not directly influence levels of H3K27me1.

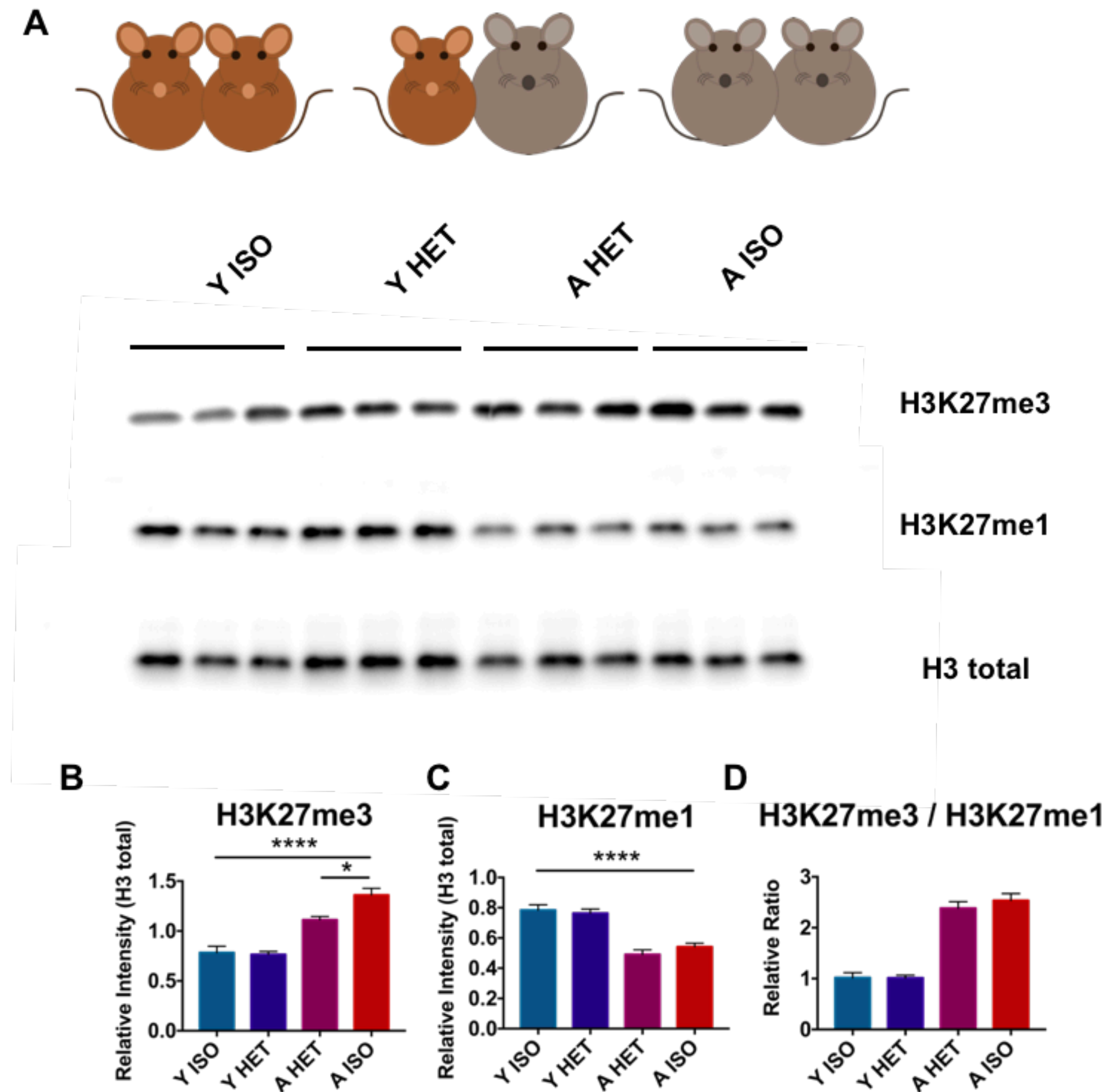

Figure 5.3. Heterochronic parabiosis rejuvenates age-associated increase of brain H3K27me3. (A) Representative Western blot of whole brain samples of naïve parabionts stained for H3K27me3, H3K27me1, and H3 total. **(B)** Quantification of intensity of H3K27me3 relative to H3 total. **(C)** Quantification of intensity of H3K27me1 relative to H3 total. **(D)** Analysis of the ratio of H3K27me3 relative to H3K27me1. Data are presented as the mean +/- SEM, two-way ANOVA with Tukey post-hoc test, n=9/group, * $p<0.05$, **** $p<0.001$.

Heterochronic parabiosis rejuvenates brain levels of IFNγ and GM-CSF

The primed phenotype of microglia is characterized by an increase in receptors for signaling (e.g. MHC II, TLR4) and a decrease in molecules tampering the immune response (e.g. CX3CR1, CD200R). However, while aged microglia are primed to respond to pro-inflammatory signaling, others have found that there is no substantial increase in basal production of inflammatory cytokines in naïve aged brains. Our data presented here demonstrates that there is a significant increase in the basal levels of inflammatory cytokine production in naïve brains with age when comparing brains of mice of increasing age relative to 3-month-old mice (**Fig. 3.4**). Others in our lab have used flow cytometry to demonstrate that aged microglia produce more TNFα and IL-1β at baseline. It is important to note that these increases, while significant, are subtle and thus detection of these differences likely depends on both the age of the young and aged mice as well as the technique used.

Thus, given our results demonstrating an increase in basal production of inflammatory cytokines with age, we first sought to characterize the levels of cytokines in naïve parabionts brain and plasma using multiplex cytokine analysis. It is important to note that at the time of sacrifice the Y ISO mice are similar to a 5- to 6-month-old mouse and the A ISO are similar to a 20- to 22-month-old mouse. Thus, we anticipated that any differences in baseline levels of cytokines in naïve parabionts would be subtle. However, we found that there was a significant age-associated increase in IFNγ and GM-CSF between Y ISO and A ISO groups (**Fig. 5.4**), which is consistent with our findings in naïve mice. Importantly, this age-associated significant increase in IFNγ and GM-CSF was not observed in A HET mice (**Fig. 5.4**). Thus, loss of this age-associated increase in IFNγ and GM-CSF may suggest that the primed phenotype of aged brain may be reversed in the brains of aged heterochronic parabionts.

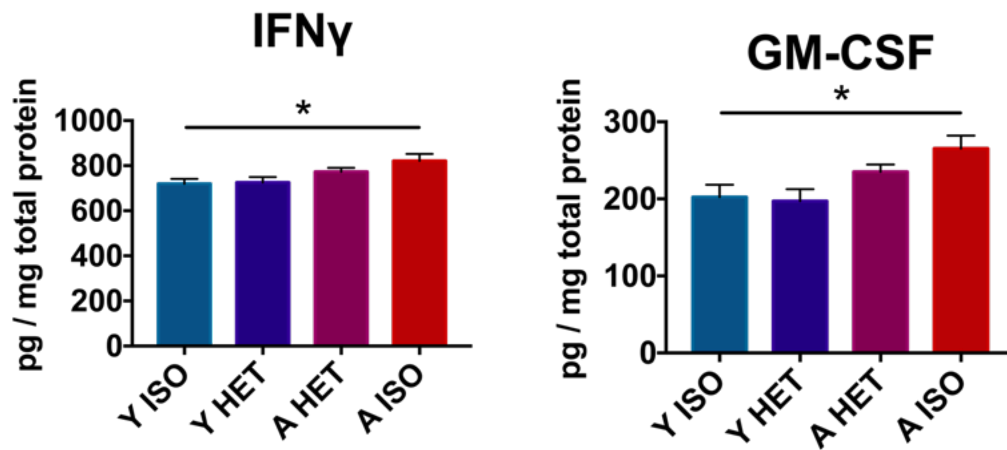

Figure 5.4. Heterochronic parabiosis reverses age-associated increase in brain IFNγ and GM-CSF. Multiplex cytokine ELISA analysis results for IFNγ **(A)** and GM-CSF **(B)** levels observed in the brains of naïve parabionts. Data are presented as the mean +/- SEM, two-way ANOVA with Tukey post-hoc test, n=9-10/group, * $p<0.05$.

Heterochronic parabiosis restores *Aif1* and *Itgam* expression to younger levels

As we have shown, the expression of *Aif1* and *Itgam* (which encode Iba1 and CD11b, respectively) increase in the hippocampus of naïve mice with age (**Fig. 3.5**). Increased expression of these molecules is associated with the primed phenotype of microglia. We wanted to investigate if heterochronic parabiosis could reverse the age-associated increased expression of these genes. In our model of heterochronic parabiosis, we found that the aged hippocampus demonstrates an age-associated increase in both *Aif1* and *Itgam* (Y ISO vs. A ISO, **Fig. 5.5**). Interestingly, this up-regulation was not observed in A HET mice (Y ISO vs. A HET, **Fig. 5.5**). This suggests that heterochronic parabiosis restores the expression of *Aif1* and *Itgam* to those of younger animals. This also suggests that heterochronic parabiosis may reverse age-associated microglia priming phenotype.

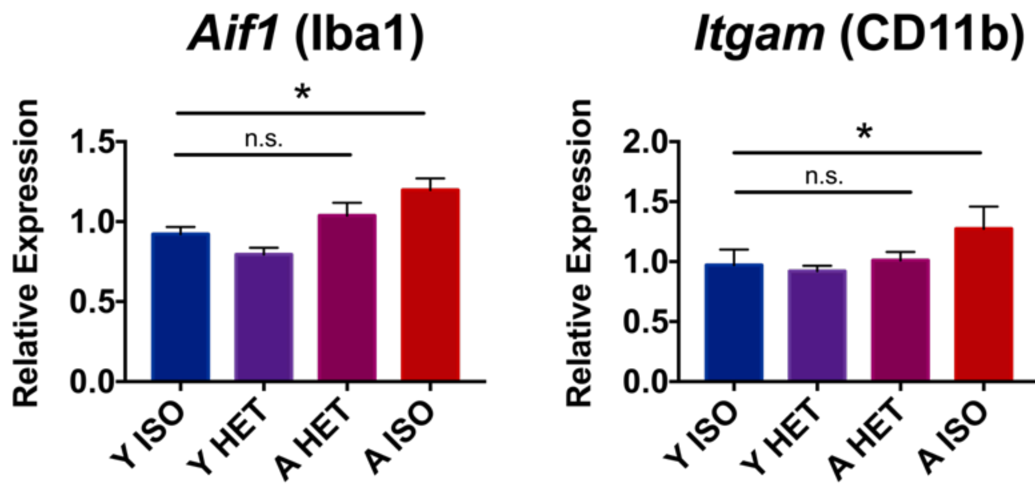

Figure 5.5. Heterochronic parabiosis reverses age-associated increase in brain *Aif1* and *Itgam*. RT-PCR of hippocampus samples of naïve parabionts for *Aif1* and *Itgam*. Data are presented as the mean +/- SEM, two-way ANOVA with Tukey post-hoc test, n=8/group, * $p<0.05$.

Heterochronic parabiosis reverses the primed phenotype of aged brain

We observed rejuvenation of H3K27me3 in the A HET brain, which our *in vitro* work suggests is involved in pro-inflammatory phenotype polarization. We also observed rejuvenation of age-associated increase in brain levels of IFNγ and GM-CSF in the A HET brain, both of which have been associated with the primed phenotype of aged microglia. Together, these results suggest that the primed phenotype of aged brain may be rejuvenated in the brains of A HET mice. To test this hypothesis, we developed a novel model of neuroinflammation in heterochronic parabiosis. This model utilizes the well-characterized neuroinflammatory model of peripheral stimulation by intraperitoneal LPS injection. However, LPS cannot be accurately dosed by weight in parabionts as the two animals are surgically attached to one another. Instead, we used a consistent total amount of total LPS injected into all parabionts. If anything, aged mice are heavier than young parabionts and in turn would receive a smaller dose of LPS by weight. However, we hypothesize that aged animals will have a more exaggerated neuroinflammatory response to LPS even in the setting of a smaller dose by weight. Therefore, if anything, our model is conservative.

We injected parabionts with 100ug LPS intraperitoneally and collected samples 24 hours later. We found that the production of 14 of the 45 cytokines investigated were elevated in brains of A ISO parabionts relative to A HET parabionts (**Fig. 5.6**). Of particular interest, heterochronic parabiosis reduced the levels of IL-1β and IL-6, two major cytokines involved in the pro-inflammatory response (**Fig. 5.6**). GM-CSF was found to be significantly reduced in the brains of A HET mice following peripheral stimulation with LPS (**Fig. 5.6**). CCL11, which has been found to be detrimental to neurogeneration in the hippocampus with age, was also found to be reduced in brains of A HET parabionts compared to A ISO surgical controls following LPS intraperitoneal

stimulation (**Fig. 5.6**). This data suggests that heterochronic parabiosis rejuvenates the age-associated primed phenotype of microglia.

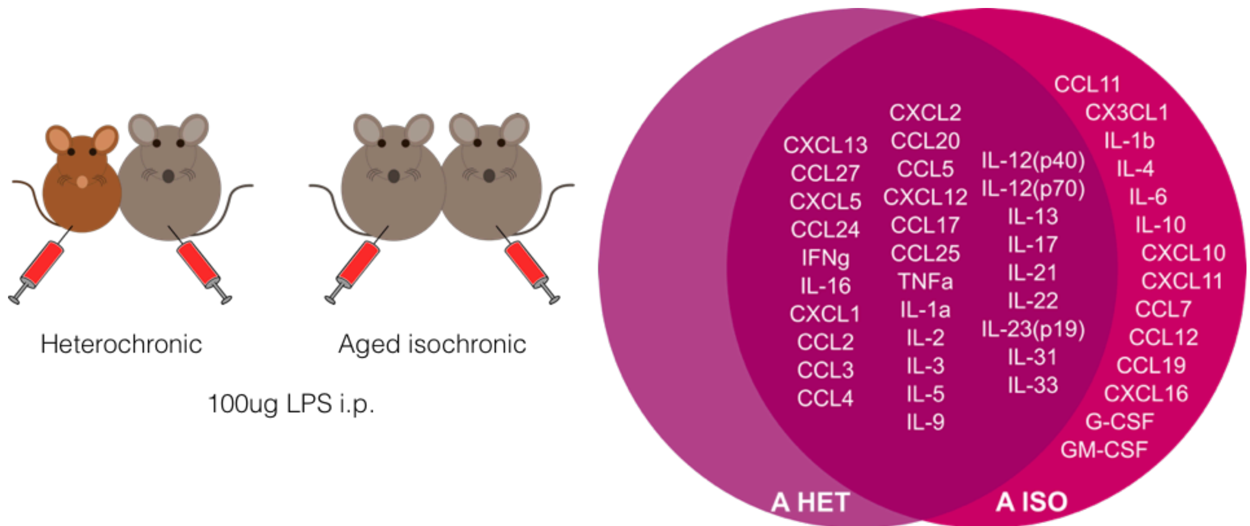

Figure 5.6. Heterochronic parabiosis reverses the age-associated primed phenotype of the aging brain. Heterochronic and aged isochronic parabionts were injected with 100ug LPS intraperitoneally and samples were collected 24 hours later. Brain protein lysates were analyzed by multiplex cytokine ELISA analysis. The circles represent those that were elevated in the A HET or A ISO parabionts, and the overlapping region represents those analytes that were statistically similar between parabionts. The 14 analytes that were found to be significantly elevated in the A ISO parabionts relative to the A HET parabionts are shown. No analytes were found to be significantly elevated in the A HET parabionts. Data are presented as the mean +/- SEM, Student's unpaired t-test, n=4-5/group, significance defined as $p<0.05$.

Conclusions

Being the resident innate immune cell of the central nervous, microglia have the potential to regulate homeostasis in the CNS as well as be some of the first cellular responders to neurological injury. Microglia are plastic cells that reside in a resting, surveillant phenotype and monitor the microenvironment for signals that may trigger an immunological response. In the setting of an appropriate stimulus, microglia may polarize their phenotype to a predominantly pro-inflammatory or anti-inflammatory state. However, what is notable about microglia, is that they have a tendency to transition from a resting, surveillant phenotype toward a pro-inflammatory phenotype with age. However, the molecular mechanisms underlying this transition in baseline phenotype with age have not been fully investigated.

This work first examines how the environment of the brain changes with age. We first began by investigating how the inflammatory environment of the brain changes with age. We developed a method to isolate protein samples in their native structure for analysis by multiplex cytokine analysis, which requires their native structure to be preserved for accurate and precise quantification. When we compare the levels of analytes in the brains of mice across their healthy, naïve lifespan from 3-, 6-, 12-, 18-, and 24-months of age, we find that many inflammatory molecules increase in the brain over the life of mice (**Fig. 3.4**). These analytes increase compared to 3-month-old naïve mice beginning at 6 months of age and progressively accumulate at 12-, 18-, and 24-months of age. These results suggest that the brain becomes progressively inflammatory with age. This finding also corresponds with our finding that the brain becomes epigenetically imbalanced with age. Our results demonstrate that there are higher levels of H3K27me3 and lower levels of the antagonizing H3K27me1 histone modification. This also corresponds with our finding that there is a decrease in Jmjd3

levels in the brain with age, and a predominant increase in the ratio of Ezh2:Jmjd3 in the brain with age as well. Together, these results suggest that the epigenetic imbalance between Ezh2 and Jmjd3 in the brain with age is associated with the accumulation of pro-inflammatory molecules in the brain.

However, epigenetic modifications are, by definition, reversible. Also, we found that plasma from aged mice could induce up-regulation of pro-inflammatory cytokines in primary microglia cultures (**Fig. 3.7**). We hypothesized that through heterochronic parabiosis that the aged brain could be rejuvenated to a less pro-inflammatory status at baseline. Our results demonstrate that exposure of the aged brain to young circulating systemic factors rejuvenates the levels of H3K27me3 in the brain. Additionally, brain levels of IFNγ and GM-CSF in A HET parabionts are also statistically similar to those of Y ISO animals, signifying that exposure of the aged brain to the young periphery reduces the levels of these two particular cytokines which are involved in age-associated microglia phenotype priming. Finally, when we test this experimentally through intraperitoneal injection with LPS into heterochronic and aged isochronic parabionts, we find that there is a significant reduction of pro-inflammatory cytokines in the brains of A HET mice relative to A ISO surgical controls. Collectively, this data suggests that exposure of the aged brain to young circulating systemic factors rejuvenates the primed phenotype of the aged brain. Ultimately, these findings suggest that possible exposures in our environment may subtly

Chapter 6:
Discussion and Future Directions

The United States population is aging at an alarming rate. The Center for Disease Control (CDC) estimates that the population of those age 65 and older in will reach 30 million by 2020 and will double to 60 million by 2050 (186). This will place a massive strain on the healthcare system, and interventions to assist in healthy aging will become of greater importance in the very near future.

The brain withholds some of our body's greatest functions and allows us to function independently as individuals. It is the center of our thought processes and cognition, and allows us to function as independent adults. However, aging exposes us to the risk of age-associated neurological diseases which threaten our ability to think for ourselves and may result in our dependence on others. Being able to maintain healthy brain function will become essential to promote healthy aging in the United States and around the world.

Our work investigates how the brain changes with age and possible mechanisms that may partially responsible for brain aging. We are particularly focused on the role of microglia in brain aging, which have been implicated in numerous neuroinflammatory and neurodegenerative diseases, including Alzheimer's and Parkinson's diseases. Being able to understand and potentially control microglia phenotype may allow us to possibly prevent, delay, and/or treat these devastating illnesses and allow people to age healthy and gracefully.

This work demonstrates how the brain changes epigenetically with age. The epigenetic landscape is partially responsible for regulating gene expression, and dysfunctional epigenetic regulation can lead to a dysfunctional and detrimental phenotype. Our studies have identified that the ratio of global levels of Ezh2 and Jmjd3 and their respective histone modifications H3K27me3 and H3K27me1 become imbalanced with age. Additionally, there is age-associated inflammation in the brain

with age that progressively accumulates throughout the lifespan of healthy mice used in our studies. Suspecting that microglia may be responsible for this dysfunctional phenotype, we manipulated primary microglia cells in vitro using a pharmacological inhibitor of Ezh2 in the setting of a pro-inflammatory stimulus. We found that inhibition of Ezh2 diminishes the polarization of microglia to a pro-inflammatory phenotype and enhances expression of anti-inflammatory genes. These findings suggest that Ezh2 lies at the balance of pro- and anti-inflammatory polarization, and promotes a pro-inflammatory phenotype by simultaneously up-regulating pro-inflammatory genes and down-regulating anti-inflammatory genes.

Next, we aimed to identify if the circulating peripheral factors could influence the epigenetic phenotype of the aging brain. To accomplish this, we used a surgical model of heterochronic parabiosis in which a young and old mouse are surgically attached and allow the two animals to share a common blood supply. The benefits of this model are profound, and allow not only circulating peripheral factors to be examined, but also the circulating peripheral cells, exposure to the intestinal microbiome of the other parabiont, as well as the interplay of all of these factors. Using this model, we were able to examine if the peripheral factors were responsible for brain aging or if modifications associated with brain aging could be rejuvenated.

We found that exposure of the aged brain to a young periphery could reverse the levels of H3K27me3 which are associated with a pro-inflammatory phenotype in microglia. Additionally, aged animals exposed to a young parabiont (A HET) had similar levels of IFNγ and GM-CSF in the naïve brain which are two molecules implicated in microglia phenotype priming. We tested if the rejuvenation of H3K27me3, IFNγ, and GM-CSF were functionally significant by exposing heterochronic and aged isochronic parabionts to a neuroinflammatory stimulus via intraperitoneal LPS

injections. We found that aged animals exposed to a young parabiont had significantly reduced levels of numerous cytokines in the brain following a pro-inflammatory stimulus. Together, these results demonstrate that circulating peripheral factors can effectively rejuvenate the epigenetic landscape of the aged brain which results in physiological improvement in the setting of a neuroinflammatory stimulus (**Fig. 6.1**).

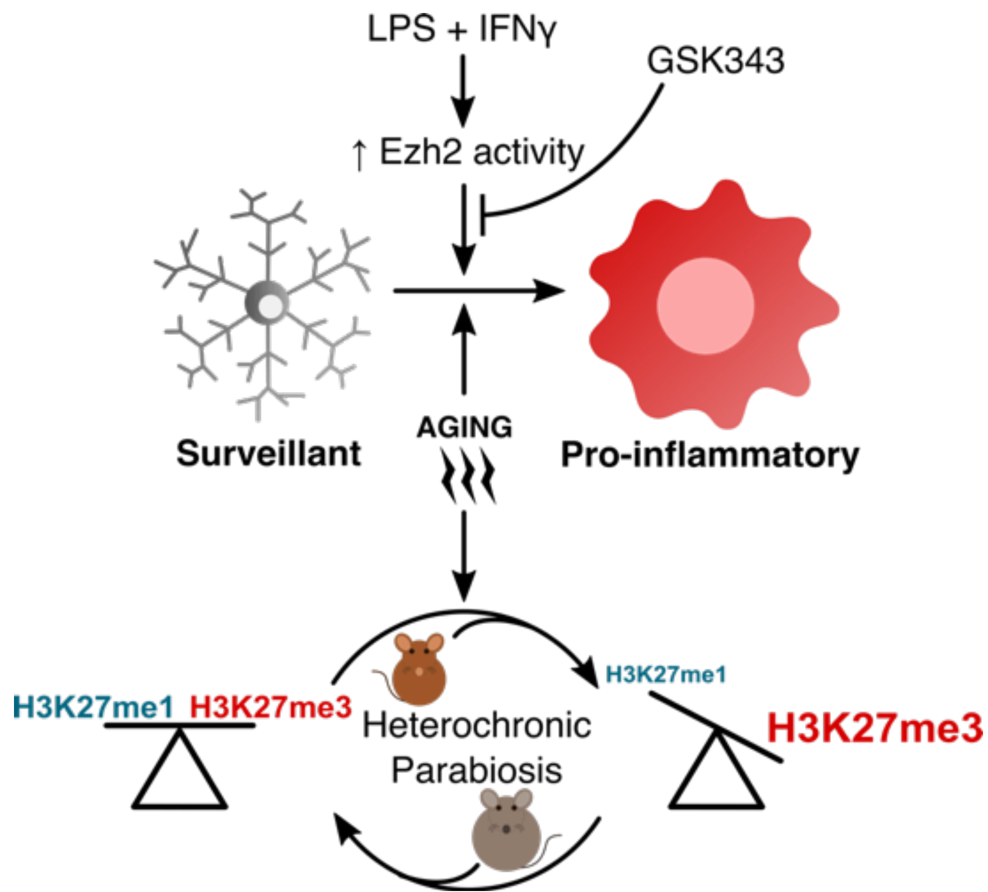

Figure 6.1. Summary of conclusions. This work demonstrates that through pro-inflammatory stimulation with LPS+IFNγ there is increased Ezh2 activity and transition of microglia from a surveillant to a pro-inflammatory phenotype. Additionally, naïve aging transitions the brain to a pro-inflammatory phenotype at baseline with associated epigenetic imbalance between H3K27me3 and H3K27me1, with a predominance for imbalance favoring H3K27me3. Heterochronic parabiosis can at least partially reverse this effect of aging, thereby partially restoring the aged brain to a younger phenotype.

Our studies are not without their limitations. Our studies have primarily focused on the epigenetic changes and alterations in the environment of whole brain samples, and have not specifically identified the age-associated epigenetic dysregulation of microglia specifically. However, our *in vitro* studies begin to provide insight that pro-inflammatory polarization increases the deposition of H3K27me3 globally. Additionally, we also found that inhibition of Ezh2 reduces the level of LPS+IFNγ-associated deposition of H3K27me3 and up-regulation of pro-inflammatory genes (**Figure 4.3**).

To identify the role of H3K27me3 and its antagonizing activating histone modification H3K27me1 in microglia specifically, flow assisted cytometric sorting (FACS) analysis of these specific histone modifications in $CD45^{int}$ $CD11b^{+}$ microglia is limited. Histones interact to form an octameric nucleosome, which has been found to be approximately 110 Å in diameter (187). Antibodies themselves are calculated to be of similar size at approximately 100 Å (188). Thus, it is reasonably possible that the physical size of antibodies (which likely have different binding affinities for their specific targets) may perhaps interfere with each other from binding to nearby histones. In the setting of flow cytometry multiple antibodies are simultaneously competing for their targets. Thus, flow cytometry would likely give inaccurate results in an attempt to quantify total levels of this histone proteins in microglia at the single cell level.

Our studies are also limited by currently available technology to identify if specific epigenetic modifications at promoters of particular pro- and anti-inflammatory genes are affected. Ideally, we would prefer to quantify the levels of H3K27me3, H3K27me1, total histone H3, Ezh2, and Jmjd3 at the sites of promoters of pro-inflammatory and anti-inflammatory associated genes using chromatin immunoprecipitation (ChIP). Given that pro- and anti-inflammatory phenotypes are

defined by sets of genes as opposed to a single particular target, we would ideally utilize ChIP-sequencing (ChIP-seq) technology. Unfortunately, these experiments are limited by current available methods as the scientific community strives to reduce the quantity of input required for ChIP-seq.

ChIP-seq technology is currently limited by the quantity of input in two ways. First, one limitation is the quantity of the total input. If there is insufficient total input DNA, detection of even abundant DNA-associated proteins will be difficult to detect. In our lab, naïve young and aged animals have approximately 30-40,000 live $CD45^{int}$ $CD11b^{+}$ microglia cells per whole brain (125, 181, 189). Samples yielding these quantities of microglia are fixed with paraformaldehyde which stabilizes antibody binding while analyzing samples on the cytometer. However, to sort microglia for RNA use (e.g. mRNA sequencing) or DNA uses (e.g. ChIP-seq), fresh cells must be used. Microglia, in our experience, are fragile cells and do not tolerate *in vitro* or artificial environments after extraction from the brain (18). Given these additional challenges, we obtain approximately 10-15,000 live $CD45^{int}$ $CD11b^{+}$ microglia cells per whole brain (data not shown), which reduces the total available input for ChIP-seq. ChIP-sequencing with low cellular input of approximately 10,000 cells per highly abundant DNA-associated target proteins (e.g. total histone H3) is just beginning to become possible and reliably reproducible with new techniques (190–192); however, performing ChIP-sequencing for multiple targets from a single 10,000 cell sample is still not within reach using the most up-to-date techniques at the writing of this thesis. As such these experiments for our purposes are still not possible.

A second limitation of current ChIP-seq technology is the available DNA-associated quantity of the target molecule, such that if there is an insufficient level of the DNA-associated protein within the sample, then there will be insufficient associated

DNA to detect following immunoprecipitation. Ezh2 and Jmjd3 are likely transient DNA-associated protein molecules, making their detection in small sample sizes especially challenging typically require the use of millions of cells for ChIP (163, 170). Due to these cumulative limitations, we cannot directly address our hypothesis that aging increases Ezh2 localization to specific DNA promoters of anti-inflammatory genes to increase H3K27me3 locally resulting in their transcriptional down-regulation *in vivo* in naïve animals or parabionts. Improvements in future techniques that allow for reduction of sample input down to approximately 1,000 cells may allow for these ChIP-sequencing experiments to be completed and provide insight into the underlying molecular mechanisms of epigenetic regulation of aging.

Additionally, the model of heterochronic parabiosis is not without complications. As discussed above in Chapter 5, heterochronic parabiosis is powerful in that it examines the roles of circulating cells, soluble factors, and the simultaneous interplay and interaction with each other. However, at the same time, this is a disadvantage because it does not provide insight into a specific mechanism providing a significant effect.

Also, as this is an *in vivo* surgical model, there are possible environmental effects that may be influencing our results. One such possible effect may be the relative physical activity of the animals within parabiont pairs. Young animals are physically more active than their aged counterparts (193, 194); however, the relative activity of young isochronic, heterochronic, and aged isochronic parabionts has not been evaluated in this present study. This may be entirely plausible, as others have shown in mice and in humans that those who exercise are generally healthier, with improvements in mitochondrial function (195–197), neurogenesis (4, 193, 194, 198, 199), clearance of amyloid (80, 200), prevention of age-associated whole brain volume

loss (201), and even reduction in microglia activation (88). While this is interesting to speculate the role of exercise on brain aging and the possible role within the heterochronic parabiosis model, evaluation of the role of exercise on the epigenetic regulation of the aged brain is beyond the scope of this study but would be an exciting extension of the work presented here.

Our studies presented did not specifically identify that the decrease in whole brain H3K27me3 levels in aged heterochronic parabionts was due to a soluble plasma factor. To address this question, this would require plasma transfer studies in which the isolated plasma pooled from naïve young and naïve aged animals is transferred into naïve aged and naïve young animals, respectively, as well as isochronic transfer controls. These experiments require an extensive supply of animal resources and require pooling plasma from approximately 500-2000 naïve animals of the same age (83, 202). These experiments have been performed by others to demonstrate the specific role of circulating soluble factors in reversing some aspects of brain aging (83, 202). However, perhaps more interestingly, transfer of umbilical plasma from human samples has demonstrated a rejuvenating effect in aged mice in the setting of ischemic stroke (203), olfactory function (204), and hippocampal function (205). We propose that it may be a more cost-efficient and translational model to isolate plasma from umbilical sources or healthy young donors as well as healthy aged donors to transfer into young and aged mice. For the quantities of plasma required for these experiments, human plasma is more readily available from healthy donors and would also reduce the numbers of animals required for use in these studies. The use of human plasma would also demonstrate a more translational approach to identify the role of soluble factors on brain aging.

Our studies identified that the circulating peripheral environment is at least partially responsible for brain aging and may be a possible therapeutic route to promote healthy brain aging. However, which particular factor(s) has not been identified in this work. Ongoing studies are further examining plasma via mass spectroscopy, fecal samples via 16S rRNA sequencing and metabolomic profiling, further examination of the structural integrity of the intestine, as well as many others. We hope to be able to identify select molecules that are responsible for brain aging that we may pharmacologically target, as well as those beneficial molecules that are lost with brain aging that we could potentially supplement.

However, these possible weaknesses of the heterochronic parabiosis model could perceived as strengths. Aging is not a homogenous process. Most *in vivo* studies use young animals, in part because they are less costly but also because there is less variation in experimental values due to the homogeneity of the animals used to represent the population. However, mice (as used in this study) and humans both die at varying ages from biological processes that are heterogenous. Aging is complex, and it is doubtful that one factor is responsible for the entirety of the aging process. Similarly, it is equally doubtful that one factor is responsible for the maintenance of youth. Thus, the model of heterochronic parabiosis may have weaknesses that may be interpreted as strengths, or strengths that may be interpreted as weaknesses. Ultimately, this is one experimental method that provides experimental evidence that must be interpreted and discussed by the collective scientific community. We believe the power of the model lies within the interplay of all factors, and the results could be interpreted on a broader scale, such that being exposed to multiple youthful factors—blood, social partner, microbiome, physical activity, etc.—may collectively be rejuvenating to the aged brain.

We do not intend to discover the fountain of youth. Rather we aim to identify mechanisms that may promote healthy aging and possibly prevent onset of neurological disease in high-risk individuals or those with an extensive family history. By enabling the aging population to maintain their cognition and recovery from possible neurological injuries, we may be able to enhance the quality of life and allow many to continue to live independently. Life should be worth living, and maintaining healthy neurological functions will allow us to continue to lead productive and enjoyable lives until we must finally depart from this Earth.

List of abbreviations

A HET	aged heterochronic
A ISO	aged isochronic
AD	Alzheimer’s disease
AIF-1	allograft inflammatory factor 1
APP	amyloid precursor protein
ARG1	Arginase-1
ATP	Adenosine triphosphate
Aβ	amyloid β
CAA	Cerebral amyloid angiopathy
CCI	controlled cortical impact
CCL	chemokine (C-C motif) ligand
CD	cluster of differentiation
ChIP	chromatin immunoprecipitation
CNS	central nervous system
CSF	cerebrospinal fluid
CSF1R	colony stimulating factor 1 receptor
CXCL	chemokine (C-X-C motif) ligand
DIV	day *in vitro*
DNA	deoxyribonucleic acid
EED	embryonic ectoderm development
Ezh1	enhancer of zeste homologue 1
Ezh2	enhancer of zeste homologue 2
FFAR2	free fatty acid receptor 2
FMO	fluorescence minus one
GFP	green fluorescent protein
GM-CSF	granulocyte macrophage colony stimulating factor
GPX1	glutathione peroxidase 1
H3K27me1	histone H3 lysine 27 monomethylation
H3K27me2	histone H3 lysine 27 dimethylation
H3K27me3	histone H3 lysine 27 trimethylation
HDAC	histone deacetylase
HMGB1	high mobility group box 1 protein
Iba1	ionized calcium binding adaptor molecule 1
IFN	interferon
IL	interleukin
iNOS	inducible nitric oxide synthase
Int	intermediate
IRF4	interferon regulatory factor 4
IRF5	interferon regulatory factor 5
Jmjd3	jumonji domain containing 3
KDM	lysine demethylase
LPS	lipopolysaccharide
LTP	long-term potentiation
MAPK	mitogen-activated protein kinase
MCP1	monocyte chemotactic protein 1
MHC	major histocompatibility complex
MIP	macrophage inflammatory protein

MMP9	matrix metalloproteinase 9
MRI	magnetic resonance imaging
mTOR	mechanistic target of rapamycin
NADPH	nicotinamide adenine dinucleotide phosphate
NFκβ	nuclear factor κ β
NgR1	nogo receptor 1
NO	nitric oxide
NOX	NADPH oxidase
PBS	phosphate-buffered saline
PKC	protein kinase C
PRC1	polycomb repressive complex 1
PRC2	polycomb repressive complex 2
RAGE	receptor for advanced glycation end products
RNA	ribonucleic acid
ROS	reactive oxygen species
ROS	reactive oxygen species
SCFA	short chain fatty acids
Seq	sequencing
SOD1	superoxide dismutase 1
SRA	scavenger receptor A
Suz12	suppressor of zeste 12
TBI	traumatic brain injury
TGFβ	transforming growth factor β
TLR	toll-like receptor
tMCAO	transient middle cerebral artery occlusion
TNF	tumor necrosis factor
Y HET	young heterochronic
Y ISO	young isochronic

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