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Developing the Common Marmoset (*Callithrix jacchus*) as a Model of Neurologic and Endocrine Aging

Amaya Madison Seidl
Trinity University, amayaseidl@gmail.com

DEVELOPING THE COMMON MARMOSET (*CALLITHRIX JACCHUS*) AS A MODEL OF NEUROLOGIC AND ENDOCRINE AGING

AMAYA SEIDL

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Kimberley A. Phillips
Thesis Advisor & Department Chair

Jennifer Henderson, AVPAA

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Preface

Over the past three years working in the Phillips Lab, I have contributed to investigations that aim to uncover the aging process of the common marmoset (*Callithrix jacchus*), a promising model for human endocrine and neurologic aging, in both pathological and healthy contexts. My thesis encompasses two major projects that examine marmoset endocrine and neurologic aging profiles, respectively. The first project (Chapter 1) documents the trajectory of cortisol across the common marmoset lifespan via measure of hair cortisol concentration (HCC) in a cohort of 50 captive animals subdivided into five age groups that span the entire marmoset lifespan. In doing so, this project evaluated whether marmosets display senescence of the hypothalamic-pituitary-adrenal (HPA) axis, signaled by deleterious cortisol accumulation late in life. A manuscript for this study has been submitted to the *Neurobiology of Aging*. The second project is an ongoing 10-month pilot study seeking to characterize aging profiles in a cohort of seven aged common marmosets in terms of neuroinflammation, cognition, and neurodegenerative biomarkers. Chapter 2 reviews the current literature with respect to neuroinflammation in neurodegenerative disease examined by study of microglia, evaluation of neurodegenerative biomarkers, and the promise that the common marmoset presents in delineating these dimensions as metrics of decline associated with age and disease. Chapter 3 details the progress made thus far in the pilot study in aged marmosets and future directions for the project. My work across these projects has been done with the goal of furthering our understanding of the aging process in the marmoset, with the hope that these findings may be applied to future efforts using the common marmoset as an model of human aging, serving to identify risk factors for decline associated with aging, and elucidate the underlying mechanisms of processes inherent in normal or pathological decline with age.

Submission to *Neurobiology of Aging*

CHAPTER 1

Cortisol Levels Across the Lifespan in Common Marmosets (*Callithrix jacchus*)

Lopez, M.G.[1], Seidl, A.[1], & Phillips, K.A.[1,2]

[1]*Department of Neuroscience, Trinity University, San Antonio, TX, USA*

[2]*Southwest National Primate Research Center, Texas Biomedical Research Institute, San Antonio, TX, USA*

Abstract

Human aging is associated with senescence of the hypothalamic-pituitary-adrenal (HPA) axis, leading to progressive dysregulation characterized by increased cortisol exposure. This key hormone is implicated in the pathogenesis of many age-related diseases. Common marmosets *(Callithrix jacchus)* display a wide spectrum of naturally-occurring age-related pathologies that compare similarly to humans, and are increasingly used as translational models of aging and age-related disease. Whether the marmoset HPA axis also shows senescence with increasing age is unknown. We analyzed hair cortisol concentration (HCC) across the lifespan of 50 captive common marmosets via a cross-sectional design. Samples were processed and analyzed for cortisol using enzyme immunoassay. HCC ranged from 1,416 to 15,343 pg/mg and was negatively correlated with age. We found significant main effects of age group and sex on HCC, and no interaction effects. Infants had significantly higher levels of HCC compared to all other age groups. Females had higher HCC than males. These results suggest marmosets do not show dysregulation of the HPA axis with increasing age.

Introduction

Glucocorticoids are steroid hormones involved in essential physiological processes, including metabolism (Vegiopoulos and Herzig, 2007), water and electrolyte balance (Hawkins et al., 2012), immune response (Cruz-Topete and Cidlowski, 2015), mood and cognitive functions (Farrell and O'Keane, 2016; Tatomir et al., 2014), and cardiovascular function (Cruz-Topete et al., 2016). Adrenal glucocorticoids are regulated by the hypothalamic-pituitary-adrenal (HPA) axis. Under basal conditions, glucocorticoids are released into the bloodstream in a circadian and ultradian rhythm, with peak levels during the morning in humans. When the HPA axis is stimulated due to physiological or psychological stress, glucocorticoid synthesis and secretion increases.

While a swift stress response via the HPA axis is a crucial survival mechanism, timely downregulation is important to avoid the negative effects of glucocorticoid excess. Chronic exposure to stress leads to overactivity of the HPA axis, amplifying long-term glucocorticoid levels and triggering widespread consequences, increasing the risk of psychological, cardiovascular, and immune pathologies rooted in chronic HPA axis dysregulation. High glucocorticoid levels are associated with obesity, osteoporosis, hypertension, and diabetes mellitus (Bjorntorp and Rosmond, 2000; Manelli and Giustina, 2000). Furthermore, glucocorticoids, particularly cortisol, have been implicated in a variety of pathophysiologies associated with aging in humans (Gardner et al., 2013), including late-life depression and anxiety (Piazza et al., 2010), dementia of degenerative and vascular origins (Balldin et al., 1983), cardiovascular disease (Iob and Steptoe, 2019), diabetes, hypertension, and mortality risk (Schoorlemmer et al., 2009). In humans, senescence of the HPA axis leads to progressive dysregulation, which is marked by sustained elevated glucocorticoid production and exposure

(Moffat et al., 2020). A cross-sectional study in humans reported HCC increased with age (Feller et al., 2014).

Nonhuman primates are critical models for geroscience and the translation of the basic biology of aging to clinical applications. Although studies on aging of the HPA axis in non-human primates are limited, many species display age-related declines in cortisol concentration [rhesus macaques (*Macaca mulatta*), vervet monkeys (*Chlorocebus aethiops*), and baboons (*Papio hamadryas*)], as measured via plasma and hair (Dettmer et al., 2014; Fourie and Bernstein, 2011; Goncharova and Lapin, 2004; Lutz et al., 2021). While these studies generally have not included aged or geriatric subjects, a recent longitudinal study examined age-related changes in cortisol levels in wild chimpanzees ranging from approximately 10 – 63 years (Emery Thompson et al., 2020). Urinary cortisol displayed significant increases with age in these chimpanzees.

Common marmosets (*Callithrix jacchus*) have recently emerged as a valuable model for the study of aging and age-related diseases (Ross, 2019; Tardif et al., 2011). In addition to their small size and compatibility with laboratory housing in species-typical social groups, the marmoset is the fastest developing anthropoid primate, reaching sexual maturity by 2 years of age. Marmosets are considered aged around 7 years, and geriatric at around 10 years (Geula et al., 2002; Ross, 2019). Marmosets have been found to display many aging phenotypes that mimic human aging, including increased risk of cardiovascular changes, inflammatory disease, metabolic impairment, suppressed immune function, and impaired cognition (Ross, 2019; Ross et al., 2019; Ross et al., 2012).

Cortisol can be measured from blood, saliva, urine, feces, and hair; each of these provides insight into a different time frame of the sample. Cortisol obtained from blood and saliva are

considered to provide “point” measures, as the measurement indicates a time frame of minutes since the cortisol was excreted (Meyer, J. and Novak, M., 2012). Urine and feces are considered “state” measures and reflect activity of the HPA axis over several hours up to a few days (Meyer, J.S. and Novak, M.A., 2012). Long-term HPA axis activity over weeks or months, depending upon the length of hair analyzed, can be quantified via analysis of hair cortisol content.

Our previous work with marmosets demonstrated sex and age differences in HCC, with females presenting higher HCC than males, and juveniles presenting higher HCC than adults (Garber et al., 2020). However, there has not been an examination of HCC across the lifespan in common marmosets. Here, we conducted a cross-sectional study to investigate age-related changes in HCC in common marmosets. We sampled individuals ranging in age from 2 weeks (infancy) to 14.5 years (geriatric). We were particularly interested in determining whether marmosets exhibit senescence of the HPA axis, as indicated by an increase in HCC during the geriatric stages.

Method

Animals

Our sample consisted of 50 common marmosets (*Callithrix jacchus*; male $n = 25$, female $n = 25$) from five age groups: infant (0-3 months), juvenile (12–18 months), adult (3-5 years), aged (7-9 years), and geriatric (>10 years). Five males and five females were sampled from each age group. All animals were socially housed except one adult male and one adult female during the three months prior to hair collection. Infants, juveniles, and two adult male breeders were kept in their family groups at the time of collection. Subjects were housed at the Southwest National Primate Research Center, Texas Biomedical Research Institute, San Antonio, TX, USA and maintained in accordance with the *Guide for the Care and Use of Laboratory Animals*

(ILAR, 2011). Room temperatures were maintained at 81-91°F (27-33°C) and a 12h:12h light: dark cycle. Marmosets had fresh food *ad libitum* which consisted of a purified diet (Harlan Teklad TD130059 PWD) and Mazuri diet (AVP Callitrichid 5LK6), supplemented with fresh fruits, vegetables, seeds, and dairy.

Sample collection

Research staff collected approximately 100g of hair from the upper back of each animal using an electric shaver. Samples were opportunistically collected during routine health screenings. The electric shaver was cleaned with disinfectant after each use. Samples were stored, out of direct sunlight, in paper envelopes until processed and analyzed.

Cortisol assay

We used a previously validated procedure to extract and assay cortisol from marmoset hair (Meyer et al., 2014; Phillips et al., 2018). A step-by-step protocol for the extraction and assay procedure has been deposited at the protocols.io repository (**dx.doi.org/10.17504/protocols.io.bw8wphxe**). Marmoset samples were diluted 1:40 with phosphate buffer solution before being analyzed, in duplicate, using a commercially available enzyme immunoassay kit (Salimetrics, State College, PA). Resulting values, expressed as µg/dL, were converted into pg/mg for analysis.

Data analysis

All analyses were performed using R Statistical Software (v4.0.2; R Core Team, 2020). Intraassay coefficient of variation (CV) was 3% and the interassay CV was 5.3%. One subject, a male infant, was determined to be unhealthy from test results obtained during the health screening; thus, that animal's hair sample was excluded from analysis. Therefore, there were 9 animals (male n=4, female n=5) in the infant age group. We performed the Shapiro-Wilks test of

normality for HCC concentration within each age group before statistical analysis. As this indicated HCC concentrations within age groups were normally distributed, we conducted a two-way analysis of variance, testing for main effects of sex and age group, and an interaction effect. We also performed correlational analysis on the HCC values and age (in months). Alpha was set at 0.05.

Results

We analyzed hair cortisol concentration (HCC) via enzyme immunoassay in 50 captive common marmosets divided into five age groups spanning the entire lifespan. Overall, HCC in our sample of common marmosets ranged from 1,416 to 15,343 pg/mg (see Table 1 and Figure 1).

HCC displayed a moderate negative correlation with animal age (in months), $r(47) = 0.51$, $p < 0.05$ (see Figure 2). Examination of these data revealed large variation in HCC within the infant group in comparison to those of other age groups, leading us to question whether survival was related to HCC in infancy. We did not find survival or health outcomes associated with HCC among these infants.

An analysis of variance showed a main effect of age group, $F(4, 39) = 17.32$, $p < 0.001$. Post hoc analyses using the Bonferroni post hoc criterion for significance indicated that infants had significantly higher HCC compared to all other age groups. No other pairwise comparisons were significant. Additionally, a main effect of sex was found, $F(1, 39) = 4.66$, $p = 0.037$, with females having higher HCC than males. The interaction between sex and age group was not significant.

Table 2. Mean hair cortisol concentration (pg/mg +/- SE) in captive common marmosets across the lifespan.

	Cortisol Concentration (pg/mg)	
	Male	**Female**
Infant (0-3 months)	9,513 ± 1563	9,408 ± 1620
Juvenile (12-18 months)	3,336 ± 518	4,686 ± 867
Adult (3-5 years)	3,267 ± 277	6,585 ± 597
Aged (7-9 years)	4,027 ± 327	5,213 ± 339
Very Aged (> 10 years)	2,923 ± 381	2,837 ± 799

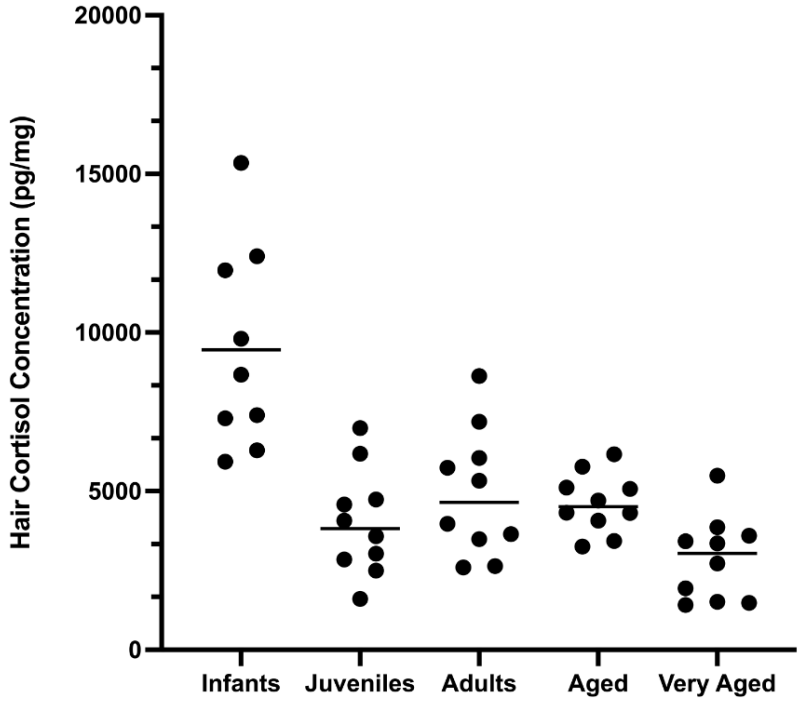

Figure 1. The distribution of hair cortisol concentration in each age group, showing the median, first quartile, third quartile, and minimum and maximum values. Mean values (+/- SE) are provided in Table 1.

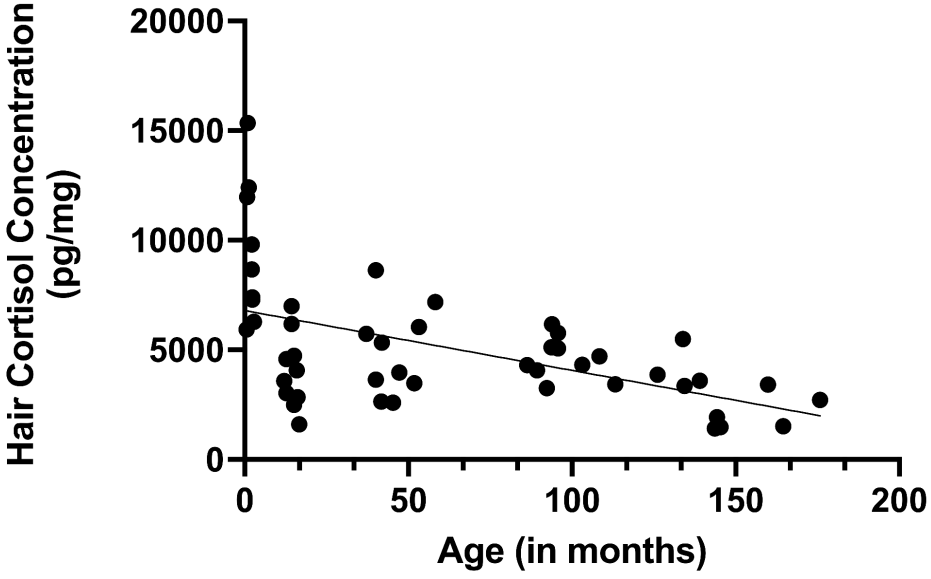

Figure 2. The association between hair cortisol concentration (HCC) and age in common

Marmosets, $r(47) = -0.51$, $p < 0.05$.

Discussion

Our results indicate marmosets display age-related changes in HCC, with HCC decreasing significantly from the infant to juvenile stage and remaining low throughout adult, aged, and geriatric age groups. We also found a significant effect of sex, with females having higher HCC than males across age groups.

Our finding of female common marmosets having higher HCC than males is consistent with Garber et al. (2020)'s investigation of HCC in wild common marmosets. Other nonhuman primate species also show sex differences in HCC, though the influence of sex on cortisol is inconsistent. In baboons, macaques, and vervet monkeys, females showed significantly higher cortisol than males (Dettmer et al., 2014; Laudenslager et al., 2012; Lutz et al., 2021). However, studies of chimpanzees (Yamanashi et al., 2013) and orangutans (Carlitz et al., 2014) did not find significant differences in HCC between sexes.

Our data support hypercortisolism in infants brought on by postnatal HPA hyperactivity and a suppression of the negative glucocorticoid feedback system. High circulating glucocorticoid levels in infancy followed by sharp decline is particularly well established in marmosets (Pryce et al., 2002) as well as baboons (Gesquiere et al., 2005), and demonstrated among many platyrrhine taxa (*i.e.* Central and South American monkeys) (Fourie and Bernstein, 2011). Certain platyrrhines exhibit glucocorticoid resistance arising from varying glucocorticoid receptor binding affinities that influence circulating hormone levels, which may contribute to differing long standing patterns between species (Pryce et al., 2002). These distinctions in endocrine physiology position the common marmoset as a model for evaluating long-term

consequences of high postnatal glucocorticoid exposure and identifying developmental distinctions across species in the hypothalamic system.

HCC was negatively correlated with age, and we did not detect an increase in HCC in aged or geriatric marmosets. This pattern of declining cortisol from young to adult ages has been reported in numerous species, including vervet monkeys (*Chlorocebus aethiops*), rhesus monkeys (*Macaca mulatta*), baboons (*Papio* spp.) (Dettmer et al., 2014; Fourie and Bernstein, 2011; Laudenslager et al., 2012).

Normal aging in humans is characterized by a general increase in mean daily serum cortisol concentrations in elderly. Sustained increase in circulating cortisol is associated with several detrimental outcomes, including accelerated aging, impairment in memory and cognitive function, and disrupted sleep cycles.

Studies examining cortisol trends across the human lifespan have shown mixed results. While some demonstrate increases in basal cortisol production with age (Moffat et al., 2020; Nicolson et al., 1997; Pavlov et al., 1986), others only demonstrate increased HPA axis responsiveness or diminished negative feedback control (Born et al., 1995; Otte et al., 2005; Rohleder et al., 2003).

To date, among primates only chimpanzees have been found to show senescence of the HPA axis (Emery Thompson et al., 2020). This study found a significant increase in urinary cortisol in adult wild chimpanzees over a period of 20 years, suggesting that increasing cortisol concentrations may be an aspect of the normal aging process in chimpanzees.

Two characteristics of the present study should be noted: 1) we utilized a cross-sectional design to investigate cortisol concentration across age groups; and 2) we assayed cortisol via a

state measure (hair) rather than a point measure (e.g., serum or urine). While previous studies on humans and nonhuman primates utilizing cross-sectional designs have reported similar findings of cortisol concentration decreasing with age (Feldman et al., 2002; Lutz et al., 2021)(Lutz et al., 2021), Feller et al. (2014) and Nicolson et al. (1997) found HCC to be positively correlated with age in humans. Some longitudinal approaches have reported a different association of cortisol with age. Moffat et al. (2020), using a longitudinal approach to study cortisol changes across the lifespan in humans, reported sustained elevated increased glucocorticoid concentrations. Additionally, Emery Thompson et al. (2020) reported similar findings in a longitudinal study of adult wild chimpanzees. Thus, it is feasible that longitudinal study would reveal different age-related patterns of HCC in marmosets. A second consideration concerns our use of hair to evaluate cortisol concentration. HCC is regularly used as a noninvasive measure to assess retrospective HPA axis activity (Heimburge et al. 2019).

Considering the differing findings in human and nonhuman primate investigations into cortisol concentrations with respect to age, it seems plausible that senescence of the HPA axis may not be characteristic or an inevitable consequence of aging, but rather may arise in certain populations due to variation in life course. Numerous studies have demonstrated individual variations in cortisol concentration trajectories, and these differences are associated with cognitive decline trends. For example, Franz et al. (2011) reported a significant association between cortisol levels and cognition in men aged 51 to 60, such that higher cortisol output was linked to poorer cognitive performance. In a 35-year longitudinal component of their study, cognitive ability at age 20 predicted midlife salivary cortisol levels, suggesting that factors early in life significantly impact aging outcomes, which vary among individuals. Specifically, Franz et al. (2011) cite the vulnerability hypothesis, which posits that cortisol levels and cognitive effects

observed late in life originate from pre-existing likely genetically-mediated risk factors. As such, primates such as the common marmoset may be an important model for identifying key mechanisms of HPA axis maintenance during aging aside from dysregulation.

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Author Contributions

Matthew Lopez: Conceptualization, Investigation, Formal Analysis, Writing - Original Draft.

Amaya Seidl: Investigation, Writing - Original Draft.

Kimberley Phillips: Conceptualization, Investigation, Writing - Review and Editing, Funding Acquisition.

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CHAPTER 2

Literature Review

The following review encompasses the current body of literature in neuroscience and geroscience research toward the goal of characterizing phenotypic changes over the course of normal and pathological aging. Delineating healthy and pathological aging profiles and, further, identifying the mechanisms underlying these differential trajectories is foundational for the development of interventions that combat age-associated deterioration and neurodegenerative diseases early in their course. To begin, it will describe the complementary nature of neuroscientific and geroscientific approaches in efforts to understand and address neurodegenerative disease, given that age is the single greatest risk factor for the development of neurodegenerative disease. The review will then transition to cover two blooming areas of investigation into neurodegeneration: neuroinflammation and blood biomarkers, summarizing current insights and room for further applications. Repeated failures of interventions based in rodent models to produce effective clinical tools necessitate the use of a more promising translational model. The final section of this review explains the great potential for neuroscience and particularly aging research that the common marmoset (*Callithrix jacchus*) presents as an anthropoid primate that demonstrates human-like aging phenotypes spontaneously over a short lifespan. Next, this section will detail existing investigations of neuroinflammation and neurodegenerative biomarkers in this non-human primate model, and room for future developments in these areas and beyond.

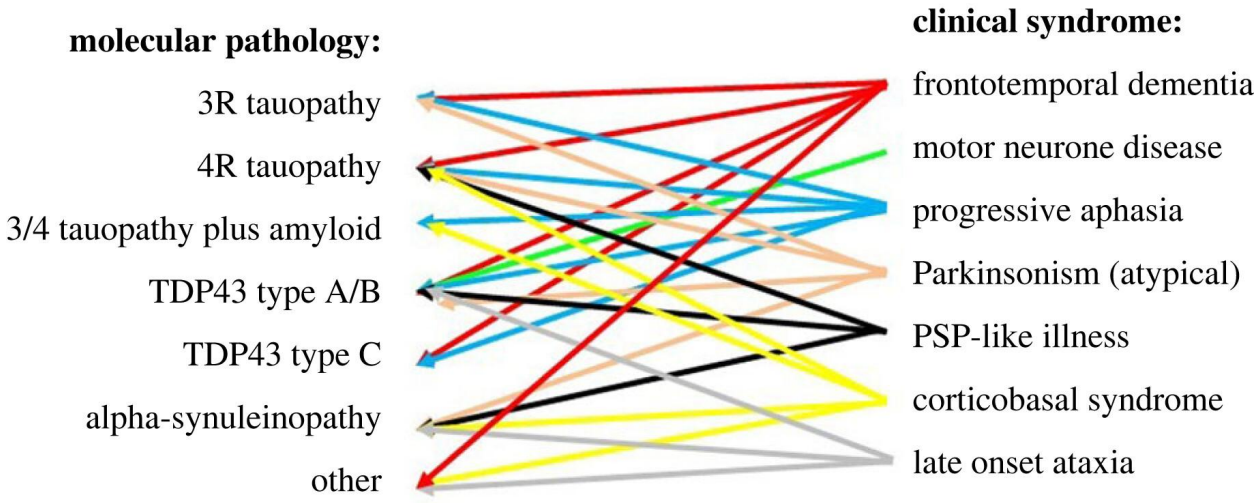
1.0 Neuroscience and Geroscience: A Necessary Blend of Research

As human longevity increases and population pyramids across the globe are inverting, the compounding societal burden of age-related disease has necessitated a shift in biomedical research toward understanding the aging process and its consequences: pathological and fatal (Jin et al., 2015).

Over the past few decades, tremendous efforts have been expended to investigate specific neurodegenerative diseases in in-vitro, animal, and clinical settings. Nevertheless we still lack any cures, and there are little to no effective treatments to halt or slow the progression of these conditions beyond symptom management. These diseases exhibit substantial overlap of both phenotypic manifestation and molecular underlyings. A well-studied example is the accumulation of tau isoforms, which is a prevalent primary and secondary hallmark of various diseases, including Alzheimer's disease (AD), Progressive supranuclear palsy (PSP), corticobasal degeneration (CBD) and chronic traumatic encephalopathy (CTE) (Orr et al., 2017; Zhang et al., 2022). Also among the usual molecular suspects in prevalence is transactive response DNA binding protein of 43 kDa (TDP-43), an intranuclear protein with a major regulatory role and involvement in RNA processing (Meneses et al., 2021). Disruption of this protein is evident in amyotrophic lateral sclerosis (ALS), AD, and a subset of frontotemporal lobar degeneration (FTLD) (Meneses et al., 2021). Neurodegenerative diseases also share genetic factors that predispose individuals to developing them (Pickrell et al., 2016; Arneson et al., 2018) and similar gene expression signatures (Li et al., 2014; Grünblatt et al., 2007; López González et al., 2016; Arneson et al., 2018). Despite numerous common pathological components, clinical diagnoses are diverse, as is summarized in Figure 1.

Figure 1.

Molecular Pathologies vs Clinical Syndromes of Neurodegeneration



Note. Neurodegenerative diseases share numerous underlying molecular elements, despite being diverse in clinical diagnosis. PSP = Progressive supranuclear palsy. From "Neurodegenerative disease of the brain: a survey of interdisciplinary approaches. Journal of the Royal Society Interface" by Davenport, F., Gallacher, J., Kourtzi, Z., Koychev, I., Matthews, P. M., Oxtoby, N. P., Parkes, L. M., Priesemann, V., Rowe, J. B., Smye, S. W., Zetterberg, H. (2023). *20*(198), 20220406. Published by the Royal Society under the terms of the Creative Commons Attribution License.

The greatest risk factor for developing neurodegenerative diseases is increasing age. The failure of translational models focused on specific diseases, in light of crossover among pathological elements in various forms of these conditions, and the common primary risk factor of age, suggest a need for research focused on demystifying the physiological mechanisms of the aging process that leave the nervous system susceptible to neurodegenerative disease ("Overcoming Gaps in the Treatment of Neurodegenerative Disease," 2020). Thus, an integration of research in both neuroscience and geroscience, the biology of aging, is a crucial

strategy in the combat against neurodegeneration (Hernandez et al., 2022). There is much room to explicate the complex and interacting effects of environmental and genetic factors leading to the development of neurodegenerative diseases. This is pertinent given the need for methods of early disease detection and, in complement, interventions that are effective in stopping or slowing disease progression. Toward this goal, two major research areas are characterizing neuroinflammatory and prognostic blood biomarker profiles in neurodegenerative diseases.

2.0 Neuroinflammation

2.1 Neuroinflammation in Neurodegenerative Disease

Inflammation is a nonspecific defensive response coordinated by the innate immune system in the event of injury or the presence of pathogens. Neuroinflammation describes a variety of immune responses occurring in the central nervous system (CNS). Accumulating evidence has established that these processes are implicated in neurodegenerative disease, both in triggering the onset of these conditions and accelerating their progression (Chen et al., 2016; Lyman et al., 2014). Specifically, neuroinflammation is linked to synaptic dysfunction (Mishra et al., 2012; Stark and Bazan, 2011), the modulation of neurogenesis (Rolls et al., 2007), tau hyperphosphorylation (Lee et al., 2010), and neuronal death (Harry et al., 2008; Y.P. Liu et al., 2005). There remains much room to demystify the mechanisms underlying these associations and, more broadly, the multidimensional role of neuroinflammation in neurodegenerative disease.

2.2 Microglia: Orchestrators of the Neuroinflammatory Response

Microglia are the resident macrophages that survey the CNS for damage and debris (Ransohoff and Perry, 2009). They make up 10 to 15% of all cells in the brain (Nayak et al., 2014). The characteristic processes of microglia make them highly extensive and mobile to

accomplish their responsibility of surveillance (Nimmerjahn et al., 2005). Microglia serve to maintain homeostasis in the microenvironment of the brain as primary immune responders and their activity is heavily implicated in the neuroinflammatory process. Neuroinflammatory changes are pervasive across a wide variety of neurodegenerative diseases, and this is suspected in many cases to be associated with prolonged microglial overactivity (Rauf et al., 2022).

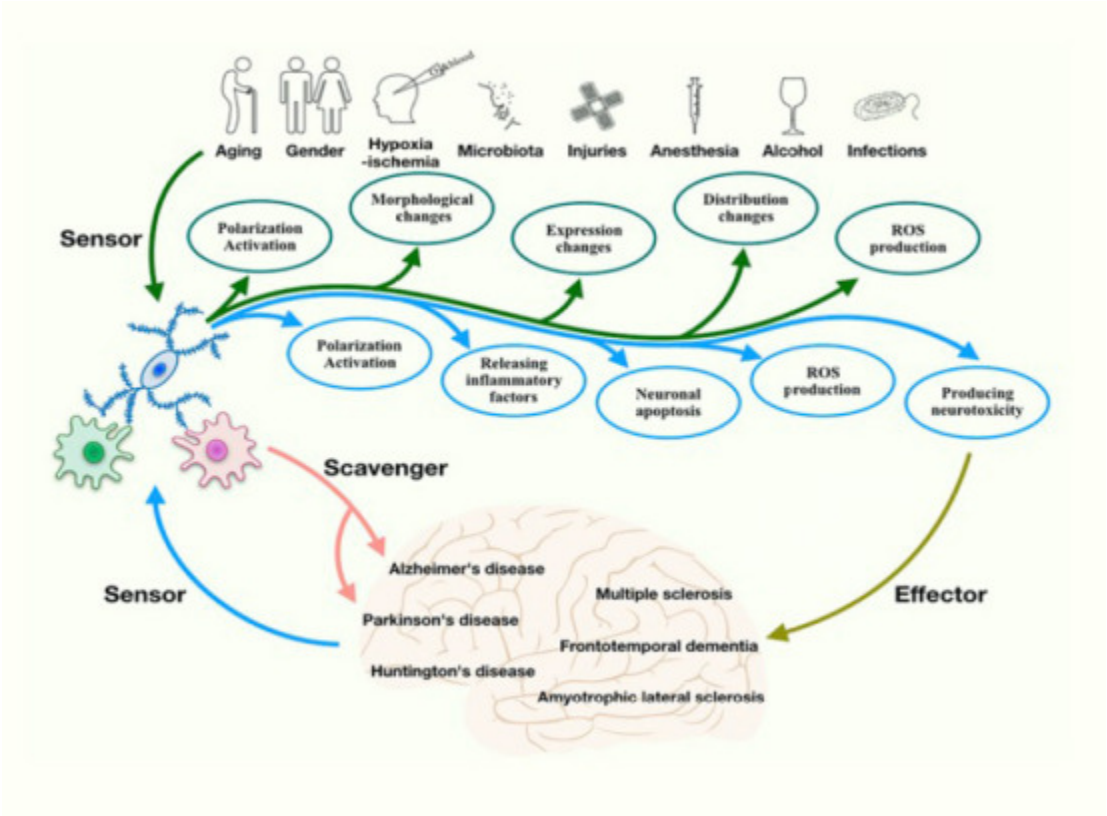
The microglial population is diverse in terms of morphology, phenotype, function, associated biomarkers, and signaling pathways (Orihuela et al., 2016; Kierdorf and Prinz, 2017). Variation across these respects occurs differentially across brain regions and arises from genetic, epigenetic, intrinsic, and extrinsic influences (Grubman et al., 2016; Thion et al., 2018).

Microglia may function as both sensors and effectors in a manner that is reflective of and responsive to microenvironmental changes in the CNS (Xu et al., 2021). In doing so, their activity is interlaced in the progression of various neurodegenerative pathologies (Figure 2). Microglia display three major states: resting, activated, and ameboid or phagocytic (Figure 3, left panel). In a healthy CNS environment, microglia are considered to be in a resting state, and present as structurally ramified cells with somas (Das Sarma, 2014; Glenn et al., 1992; Edler et al., 2021). In this state, cell somas remain in place while cell processes extend and retract as a means of surveillance of the surrounding environment, interacting with neighboring neurons and other glial cells. In the event that microglia detect any deviations from homeostasis in their surroundings, such as mechanical injury, invasion, or disease, they become activated, which induces morphological transformation manifesting as enlargement, retraction of cell processes, and migration (Mrak, 2012; Bolmont et al., 2008; Edler et al., 2021). The ameboid state is characterized by an enlarged soma and loss of processes, and is adopted to fulfill a scavenging role and undergo phagocytic clearance activity (Cai et al., 2013; Edler et al., 2021).

Aged microglia begin to display a unique dystrophic phenotype (Figure 3, middle panel), distinguished morphologically by enlarged soma, cytoplasmic abnormalities, altered processes, and nonuniform tissue distribution (Streit et al., 2004). Microglial functioning also deteriorates with age, as microglial processes slow and become less efficient, impairing the microglial response to damage and disease (Damani et al., 2011; Hefendehl et al., 2014).

Figure 2.

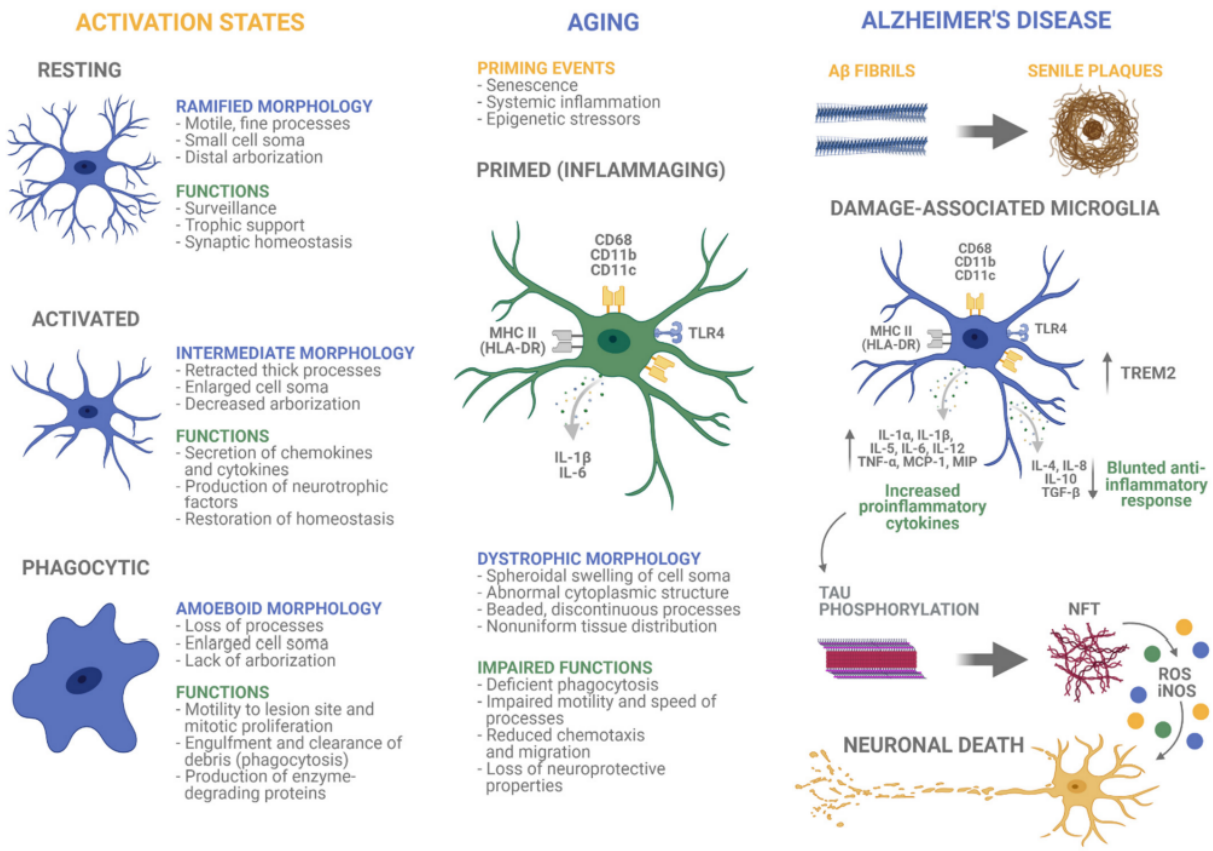
Microglia as sensors and effectors in neurodegenerative disease



Note. Microglia are activated under various conditions, and their activation contributes to inflammation that drives neurodegenerative processes. ROS = Reactive oxygen species. From Figure 2 in "Microglia in neurodegenerative diseases" by Xu, Y., Jin, M. Z., Yang, Z. Y., & Jin, W. L. (2021). *Neural regeneration research*, *16*(2), 270–280.

Figure 3.

Microglial Activation States in Aging and Alzheimer's Disease



Note. Three microglial activation states are distinguished morphologically, phenotypically, and functionally. Aging is associated with chronic microglial activation, leading to the inception of distinctive dystrophic morphology and diminished function. In Alzheimer's disease (AD), damage-associated microglia accumulate near the sites of amyloid and tau hallmarks and are associated with an aggravated and sustained pro-inflammatory state. From Figure 1 in

Activated microglia are key orchestrators of neuroinflammation via cytokine release (See Section 3.3). They also may produce reactive oxygen species (ROS) which in turn induce neurotoxicity and apoptosis in impacted neurons (Naik & Dixit, 2011; Yang et al., 2011; Kierdorf & Prinz, 2017). These effects may aggravate neurodegenerative processes. Neurodegenerative diseases are widely associated with microglial hyperactivity, leading to excessive, prolonged neuroinflammation.

The role of microglia in Alzheimer's disease (AD) progression has been heavily studied and may serve as an exemplary model of altered microglial activity (Figure 3, right panel) in response to and potentially exacerbating the diseased state. The amyloid deposition characteristic of AD has been found to provoke the activation of microglia, which results in the proliferation of proinflammatory molecules, promoting a magnified inflammatory response (Cameron & Landreth, 2010). The Alzheimer's brain displays a presence of cytokines derived from microglia as well as additional immune mediators, suggesting a chronic inflammatory state thought to have neurotoxic consequences (Wyss-Coray and Mucke, 2002). In addition to the clearance of amyloid plaques via phagocytic activity, microglia serve to clear the remains of damaged or dying cells, suggesting a major role for these macrophages to play in neurodegenerative environments. There is also genetic evidence of the association of microglia with AD pathology in that the majority of risk genes for the disease are highly expressed, and many selectively expressed, by microglia in the brain (Hansen et al., 2018; Sarlus & Heneka, 2017). Conversely,

microglia are also thought to function protectively to prevent the onset of AD by preventing the accumulation of amyloid plaques (Hansen et al., 2018)

2.3 TSPO: A Metric of Microglial Activation

18 kDa Translocator protein (TSPO) is an outer mitochondrial membrane protein heavily expressed in activated microglia that has been employed in research as a metric of neuroinflammation. Presence of this protein has been positively correlated with amyloid deposits (Kreisl et al., 2013a, 2017) and negatively correlated with cognition (Yokokura et al., 2017).

TSPO positron emission tomography (PET) ligands have been employed in research examining neuroinflammation over the past two decades, and have been developed in three generations. First-generation tracer [^{11}C]-PK11195 has some drawbacks, most notably low signal-to-noise ratio. Second- and third generation TSPO tracers are not subject to the same limitations but are susceptible to the rs6971 polymorphism in humans (Kreisl et al., 2010; Kreisl et al., 2013b; Crawshaw & Robertson, 2017). This polymorphism arises from a nonconservative Ala147Thr mutation and gives rise to three phenotypes with differential affinity to second generation TSPO radiotraders (Owen et al., 2012). These are high-affinity (Ala/Ala), mixed-affinity (Ala/Thr), and low-affinity binding (Thr/Thr) (Owen et al., 2012).

Coughlin and colleagues, who characterized the rs6971 polymorphism and the resultant phenotypes, have conducted two studies of neuroinflammation in young and retired NFL players. In these projects, participants were genetically screened for TSPO binding affinity phenotype and low-affinity binders were ineligible for participation (2015, 2017). In their 2015 pilot study utilizing [^{11}C]-DPA-713 PET imaging, they found higher DPA-signal among 9 former NFL players in comparison to 9 age-matched healthy controls in regions such as the right amygdala and the supramarginal gyrus. They applied the same radiotracer in their 2017 study, which

evaluated data from 12 active or former NFL players and 11 matched controls. They found that the NFL players displayed higher total distribution volume in 8 of the 12 investigated brain regions, including the hippocampus, left entorhinal cortex, and supramarginal gyrus.

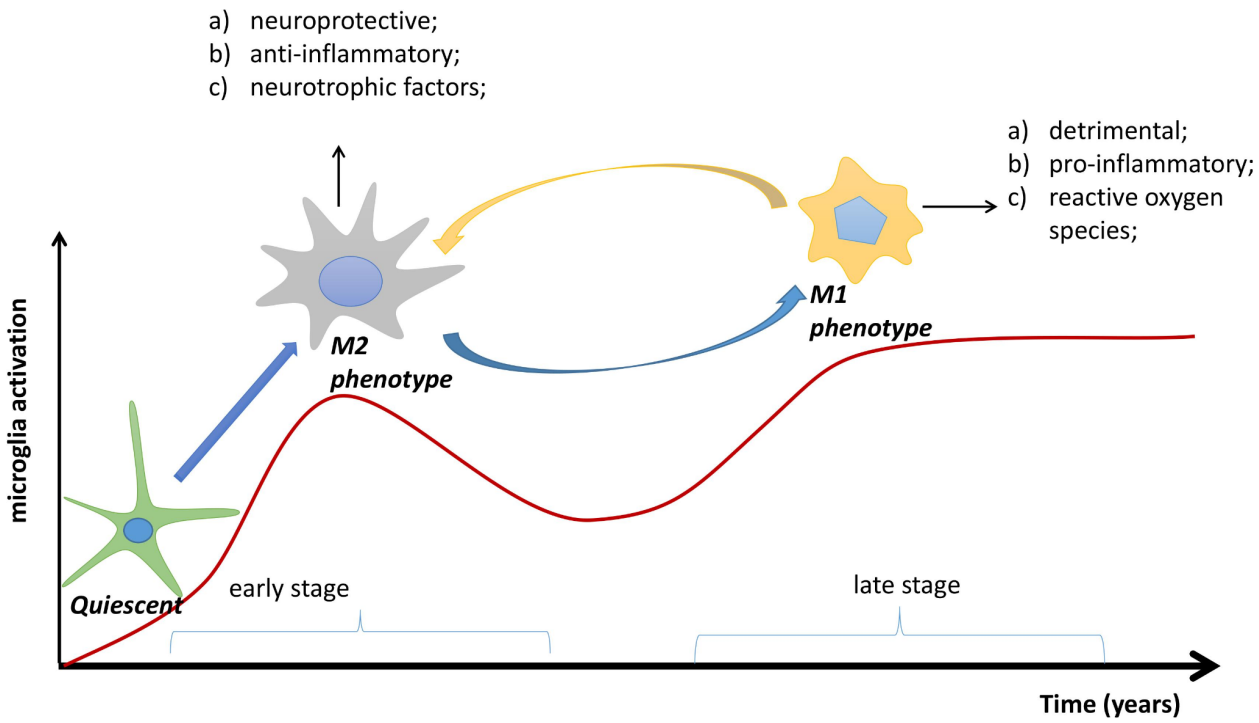
Over half of the population is estimated to exhibit either high- or mixed-affinity binding to rs6971-sensitive TSPO radiotracers (Owen et al., 2015). Importantly, no association between TSPO binding affinity phenotype and amyloid load has been found in AD and mild cognitive impairment (MCI) patients (Fan et al., 2015). Thus, findings about microglial activity from studies that exclude low-affinity binding individuals may be reasonably generalized.

2.4 Microglial M1 and M2 Phenotypes

Active microglia may be categorized into two major phenotypes with opposing effects and separate roles in the progression of diseases such as AD (Subramaniam & Federoff, 2017; Wes et al., 2016). The M1 phenotype is associated with pro-inflammatory activity and is most abundant in clinical state AD, whereas the M2 phenotype is associated with neuroprotection and is most prominent in preclinical stage AD, as Aβ begins to accumulate but patients are not yet symptomatic (Tang & Le, 2016). Microglia are thought to transform into these two phenotypes differentially depending on disease stage, and the distribution of M1 versus M2 microglia over the course of AD is illustrated in Figure 4. In preclinical AD, Aβ plaque accumulation detected by surveying microglia leads to activation into the protective M2 phenotype; during clinical AD, the M2 phenotype may be converted into the detrimental M1 phenotype (Shen et al., 2018).

Figure 4.

M1 and M2 Microglial Phenotypes Differ in Presence and Impact in Alzheimer's Disease



Note. From Figure 1 of "Clinical PET Imaging of Microglial Activation: Implications for Microglial Therapeutics in Alzheimer's Disease" by Shen Z, Bao X and Wang R (2018). doi: 10.3389/fnagi.2018.00314 Published in Frontiers in Aging Neuroscience, an open-access journal and distributed under the terms of the Creative Commons Attribution License (CC BY).

Activated microglia of both M1 and M2 types exhibit increased expression of the mitochondrial 18kDa translocator protein (TSPO), making TSPO a target for PET imaging of microglial activation (Venneti et al., 2009). Quantifying TSPO to measure microglial activation does not allow for the distinction between M1 and M2 subtypes. However, integration of other data indicative of neurologic state such as cognitive performance and additional biomarker data with PET imaging of microglial activation may allow for the determination of AD disease progression in individual subjects.

2.5 Study of Microglial Activity in Neurodegenerative Disease via TSPO-PET

Studies evaluating TSPO as a measure of neuroinflammation via PET imaging are accumulating across various disease models. In the case of AD, most TSPO PET studies demonstrate elevated binding in AD participants compared to controls, specifically in fronto-temporal regions, with more modest increases shown in neocortical regions in individuals with mild cognitive impairment (Bradburn et al., 2019). Transgenic AD mouse models evaluated via PET imaging have also shown increased TSPO binding (Mirzaei et al., 2016; Brendel et al., 2016; B. Liu et al., 2015). The precise relationship between AD pathology and these observed increases in TSPO expression remains unclear. High TSPO binding has been found in asymptomatic individuals that demonstrated incidental amyloid positivity (Zou et al., 2020; Hamelin et al., 2016) as well as in individuals that meet clinical criteria for mild AD or amnestic MCI, but lack amyloid binding on PET (Zou et al., 2020; Okello et al., 2009). This suggests that TSPO may increase in response to both amyloid deposition and amyloid-independent neurodegeneration.

In multiple sclerosis (MS), elevated TSPO expression has been found in white matter lesions in individuals with relapsing-remitting MS or secondary progressive MS (Bodini et al., 2020; Datta et al., 2017b; Debruyne et al., 2003; Oh et al., 2011). Non-lesional white matter and cortical gray matter in MS also exhibit greater TSPO signal than in age-matched controls (Bodini et al., 2020; Herranz et al., 2016; Rissanen et al., 2014; Colasanti et al., 2014), and these higher levels are correlated with greater brain atrophy and more severe disability. This increase is associated with greater brain atrophy and worse disability (Datta et al., 2017a; Datta et al., 2017b; Colasanti et al., 2014). Higher TSPO signal in MS is also predictive of new lesions,

worsening brain atrophy, and a more severe disability trajectory within a year after measurement (Datta et al., 2017b; Bodini et al., 2020).

Microglial activation is also suspected to contribute to cognitive impairment in HIV. A [^{11}C]-DPA-713 PET study comparing brains of 23 human subjects with HIV who were effectively treated with combination antiretroviral therapy to those of 12 healthy controls, significantly higher volume-of-distribution ratios were uncovered in white matter, the cingulate cortex, and supramarginal gyrus, which suggested localized glial cell activation in susceptible regions. This study also revealed an increase in the volume-of-distribution ratio within the frontal cortex found in individuals with HIV-associated dementia (Coughlin et al., 2014).

3.0 Blood Biomarkers of Neurodegeneration

3.1 The Current Prospect of Neurodegenerative Biomarkers

Biomarkers are key tools for the identification and decoding of diseases. In the context of neurodegenerative disease, biomarkers present the opportunity to quantify the pathological processes that lead to characteristic dysfunctions (Ehrenberg et al., 2020). These biomarkers may be byproducts of disease progression itself, or reflective of general damage brought on by disease. Biomarkers that originate from the CNS are present at high concentrations in the cerebrospinal fluid (CSF) and at much lower concentration in the blood. Progress in the development and refinement of immunoassays and mass spectrometry-based methods over recent decades opens a window of opportunity for fine-tuned detection of blood-based biomarkers that could revolutionize neurodegenerative disease patient monitoring (Barro & Zetterberg, 2021).

3.2 Glial Fibrillary Acidic Protein (GFAP)

Glial fibrillary acidic protein (GFAP) has emerged among the first neurodegenerative biomarkers to be assessed reliably in blood. GFAP is an astrocyte cytoskeletal protein that is associated with CNS damage and astrogliosis in the context of traumatic brain injury (TBI) and neurodegenerative disease (Abdelhak et al., 2022).

Early evaluations of this biomarker are concentrated in the area of TBI research. GFAP has been found to be predictive of CT abnormalities, with reported area under the ROC curve (AUC) values reliably above 0.77 (Papa et al., 2012, 2014, 2016; Bogoslovsky et al., 2017; Bazarian et al., 2018; Frankel et al., 2019; Yue et al., 2019; Huebschmann et al., 2020). In a 2020 study by Czeiter and colleagues comparing predictive values of biomarkers alongside CT scans after injury, GFAP from CSF was found to have a higher AUC (0.89) than five other biomarkers associated with neurodegeneration: S100B, NSE, UCH-L1, NfL and t-tau.

Elevated GFAP levels have also been revealed in some of the most prevalent neurodegenerative diseases. High CSF-GFAP has been documented in AD (Jesse et al., 2009; Abu-Rumeileh et al., 2019; Ishiki et al., 2016). Ishiki and colleagues found CSF GFAP levels were also increased in the cases of dementia with Lewy bodies (DLB) and frontotemporal lobar degeneration (2016).

GFAP levels in the blood across various neurodegenerative diseases were investigated by Oeckl et al. in 2019. They found blood GFAP levels to be higher in participants with AD, DLB, or Parkinson's disease dementia (PDD) than controls, participants with behavioral variant frontotemporal dementia (bvFTD) or participants with PDD, whereas there was no difference in blood biomarker levels among the control, PDD, and bvFTD groups (Oeckl et al., 2019a). A key finding of this study was that all neurodegenerative disease groups exhibited similar CSF levels

of GFAP. For AD participants, higher CSF to serum ratios were attributed to the varying distribution of neuroinflammation and distinct appearances of astrogliosis being displayed in neurodegenerative diseases (Oeckl et al., 2019a,b; Benussi et al., 2020).

Another study of blood GFAP found levels to be higher in participants with AD in comparison to cognitively healthy participants (Elahi et al., 2020). In a recent study by Chatterjee and colleagues, GFAP level assessed from blood was found to be associated with AD and elevated in cognitively healthy older adults at risk of developing AD based on brain Aβ load (2021). Importantly, Asken et al. found plasma GFAP levels to be positively correlated with cortical Aβ deposition in symptomatic AD participants during early disease stages, with these associations diverging across individuals during later stages (2020). These findings regarding the relationship between blood GFAP levels and amyloid deposition over the course of AD progression suggest that astrocytic activation or damage begins during the presymptomatic disease phase.

3.3 Anti- and Pro-Inflammatory Cytokines

Cytokines are acute phase proteins that are secreted from glial cells, including microglia and astrocytes. These small proteins are diverse in form and in the activities they induce via cell-signaling. Cytokines are involved in autocrine, paracrine and endocrine signaling, and have demonstrated immunomodulating effects. They may serve to both boost and suppress the inflammatory response.

Pro-inflammatory cytokines such as tumor necrosis factor (TNF), interleukin-1β (IL-1β), interleukin 6 (IL-6) and interferon γ (IFN-γ) are released by microglia (Wang et al., 2015). While these factors are released with the purpose of preventing extensive CNS damage, they may also cause damage to neurons and glial cells. Accumulated studies demonstrate that chronic

microglial activation, as is suspected in various neurodegenerative pathologies, is a key contributor to the appearance and progression of such diseases (Smith et al., 2012).

In AD, it has been found that elevated levels of pro-inflammatory cytokines, most notably such as TNF, IL-1β, IL-6 and IFN-γ, may prevent phagocytosis of amyloid plaques, promoting pathogenesis of the disease (Stamouli & Politis, 2016). Several post-mortem studies have detected high levels of pro-inflammatory cytokines near AD lesion sites (Dickson, 1997; Griffin et al., 1995; Hüll et al., 1996). Genetic analyses performed by Di Bona and colleagues demonstrated associations between AD progression and certain genetic polymorphisms in the genes that encode TNF-α and IL-1β (2008, 2009).

4.0 The Common Marmoset as a Model of Human Aging

4.1 Advantages of the Common Marmoset as a Non-human Primate Model

The common marmoset has emerged as a key nonhuman primate model in neuroscience and more precisely in investigations of age-related pathologies (Abbott et al., 2003). Transgenic rodent models have been crucial in furthering our understanding of AD, however clinical trials with these foundations have repeatedly failed over almost two decades (King, 2018). To bridge this gap between animal modeling and clinical translation, there is great need for animal models that undergo cerebral and behavioral changes that more closely resemble those of humans.

Marmosets are practical for study in that they are small in physical size and may be group housed in lab settings that simulate their natural habitat, in a manner that is far less difficult to maintain than for comparable Old World monkeys (Ross et. al, 2017). This South American monkey reaches sexual maturity at around 1.5 years of age, is considered aged around 7, geriatric around 10, and reaches maximum length of lifespan around 20 (Guela et al., 2002). Although there is a vast body of research about age‑related cognitive decline in humans, the majority of

existing studies are cross‐sectional in design and compare younger versus older adults. The common marmoset's short lifespan, the shortest among anthropoid primates, is the key advantage of the species as an aging model for the examination of lifetime aging in longitudinal designs. Further, as these primates age, they demonstrate naturally-occuring phenotypes that resemble aging phenotypes in humans.

4.2 Human-like Aging Phenotypes in Marmosets

The common marmoset spontaneously exhibits various aging phenotypes mirroring those of humans, such as increased risk of cardiovascular conditions, inflammatory disease, metabolic impairment, suppressed immune function, impaired cognition, and impaired motor behavior (Ross, 2019; Ross et al., 2019; Ross et al., 2012). Marmosets display β-amyloid deposition as they age (Geula et al.,2002; Ridley et al., 2006) as well as diminished adult neurogenesis (Leuner et al., 2007). Marmosets also exhibit presbycusis, a relevant demonstration of sensory decline with age (Harada et al., 1999; Sun et al., 2021). Importantly, marmosets have presented with highly variable patterns of cognitive aging trajectories and degrees of neuropathology across individuals (Rothwell et al., 2021), signaling their utility in examining both pathological and normal aging processes.

Thus, this model may be instrumental in identifying factors that predict susceptibility to age‐related cognitive decline and the development of neurodegenerative pathologies in certain individuals. Considering the benefits of the common marmoset as a model in neuroscience research, especially in the context of lifelong aging, much work has accumulated that utilizes this primate as a model of human neural qualities and processes that coincide with aging.

4.3 Age-related Cognitive Decline in Marmosets

A substantial portion of research in marmosets is focused on assessing elements of cognition, such as motivation and attention, simple discrimination learning (LaClair et al., 2019; Munger et al., 2017; Sadoun et al., 2019; Sadoun et al., 2015), and delayed match-to-position (Spinelli et al., 2004; Takemoto et al., 2011; Takemoto et al., 2015; Workman et al., 2019). However, investigation of cognitive changes with age in marmosets is less abundant.

Among existing work, two studies have demonstrated a decline in reversal learning abilities, reflective of executive function, in old compared to young marmosets in a small cohort (Munger et al., 2017; Sadoun et al., 2019). Rothwell and colleagues (2021) also evaluated reversal learning in marmosets as they aged from about 5 to 9 years old, and observed a decline in performance at around 8 years old, on average. In addition, degeneration of myelin in the corpus callosum was found to be predictive of quality of executive functioning in a small cohort of geriatric marmosets (Phillips et al., 2019). Sadoun et al. (2019) also found decline in spatial working memory in marmosets with age. In a longitudinal cognitive profiling study of 16 marmosets, Glavis-Bloom et al. (2022) found delayed onset of learning, slowed learning rate, and decreased asymptotic working memory performance in aged marmosets. In the first and only study to date examining episodic-like memory in marmosets, Decastro and Girard document declines in location and temporal memory of objects in a cohort of 20 marmosets including 7 aged subjects (2021). Impairments in inhibitory control in aged (7-8 years) marmosets were also reported in a study of 35 marmosets aged 3 to 14 years (Sadoun et al., 2019).

4.4 Assessing Neuroinflammation in Marmosets

Examination of neuroinflammation in marmosets is limited and highly concentrated in induced diseased states, most prominently in the case of experimental autoimmune

encephalomyelitis (EAE), an induced model of MS (Merkler et al., 2006; Jagessar et al., 2015; Leibovitch et al., 2018). In this model, altered microglial activation states have been observed in neocortical EAE lesions (Merkler et al., 2006). A 2016 study investigated the role of inflammation on AD progression in six adult and aged marmosets that were injected intracranially with Aβ fibrils in three right hemisphere cortical sites (Philippens et al.). Half of the animals received co-injections of lipopolysaccharide (LPS) to induce a neuroinflammatory condition, and these marmosets demonstrated accelerated amyloidosis, whereas the other group did not exhibit plaques (Philippens et al., 2016).

Morphological changes in microglia have been observed as marmosets age in a study by Rodriguez-Callejas et al. (2016). This group compared distribution of microglia across inactive, active, and dystrophic morphologies in different age groups. They found that resting microglia decreased in presence over the course of aging, and a greater presence of dystrophic microglia in aged animals versus adults and adolescents. Also noteworthy from this study is the finding that dystrophic microglia were present in the brains of adolescent and adult marmosets in the absence of amyloid deposition (Rodriguez-Callejas et al., 2016). More recently, this study group analyzed brains from male marmosets and documented increased dystrophic microglia in old (mean age 11.25 years) animals (Rodriguez-Callejas et al., 2019).

There is a gap in the current body of research for noninvasive, longitudinal assessment of neuroinflammatory activity in marmosets as they age and exhibit spontaneous, non-induced pathological profiles.

4.5 Assessing Neurodegenerative Biomarkers in Marmosets

In marmosets, blood-based biomarkers have been substantially studied only in the context of demyelinating diseases (Lalive et al., 2006). Notably, Kalinichenko et al. (2020) recently

found higher levels of neutral ceramidase, a key regulator of cellular homeostasis, growth, and death, in blood serum that was correlated to superior long-term memory performance in adult black tufted-ear marmosets (*Callithrix penicillata*). It has been established that inflammatory cytokines can be quantified from marmoset plasma samples, and geriatric marmosets have been found to exhibit altered profiles (Ross et al., 2019). Pro- and anti-inflammatory cytokine profiles have yet to be established in healthy versus impaired marmosets.

4.6 Further Applications of the Marmoset Aging Model

Once we are able to characterize marmoset aging profiles, a multimodal approach should be employed to investigate risk factors for certain aging phenotypes and elucidate underlying mechanisms of such progressions.

There is an exciting potential for the application of transgenic methods to the common marmoset in research of aging and neurodegeneration. Marmosets are an ideal nonhuman primate target for transgenic and knock-out production, as they are the anthropoid primate with the highest fertility rate and most rapid maturation. Transgenic marmoset lines were first created and remain primarily produced in Japan (Sasaki et al., 2009). The Japanese Brain/MINDS national brain project, which began in 2014, has led much of the progress toward the generation of transgenic lines for brain disease modeling in marmosets (Okano et al., 2016). Developments are currently in progress in the United States and Europe. The popular target for these projects aimed to produce an AD model is alteration of genes associated with Aβ processing (Kishi et al., 2014). Other methods have also been used in marmosets to successfully modify Aβ load in the brain, including direct injection of Aβ fibrils (Baker et al., 1993; Ridley et al., 2006) or inducing an inflammatory state in the brain causing an increase in Aβ and tau that results in plaque formation (Philippens et al., 2016).

Based on aging profiles that may be established by a synthesis of the findings across accumulating longitudinal studies of marmosets, in-vitro investigations may offer insight into the precise mechanisms underlying different aging profiles exhibited in the marmoset that may lead to the identification of therapeutic targets. However, cell cultures derived from the common marmoset are not very well developed. Dorigatti and colleagues have recently published protocols for neuron and glial cell cultures derived from the postnatal common marmoset (Dorigatti et al., 2021). Application of this in-vitro complement to longitudinal in-vivo study of marmosets is a key step in translational modeling with the species. This modality presents the opportunity for highly specialized investigation of the underlying cellular mechanisms of aging, and the factors that distinguish variable aging phenotypes, which could assist in identifying precise targets for diagnosis and treatment of age-associated conditions.

The common marmoset is a promising model of human aging and, in an interwoven fashion, neurodegenerative processes. This is due to key inherent qualities of the species, most importantly the presentation of human-like aging phenotypes over the course of a short lifespan. As the body of research in this non-human primate model builds, there will be growing opportunity for the development of interventions that help to capture and address the inevitable and pathological consequences that come with age.

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CHAPTER 3

Pilot Study: Evaluating Neuroinflammatory, Cognitive, and Neurodegenerative Biomarker Profiles in Aged Marmosets

Introduction

1.0 The Intersection of Neurodegenerative Disease and Aging

Aging is the greatest known risk factor for neurodegenerative disease. We have yet to understand how mechanisms of aging leave the brain susceptible to the inception and progression of these pathologies. We also currently lack effective treatments that stunt the typically irreversible progression of neurodegenerative pathologies associated with aging. As our population ages and the societal burden imposed by neurodegenerative disease intensifies, the need for insight into this relationship to inform the development of therapeutic and preventive treatments is ever present. There is a great room for multimodal investigation in the fields of neuroscience and geroscience in order to understand and combat neurodegenerative disease and other deleterious consequences of aging.

2.0 Microglial Activation as a Metric of Neuroinflammation

Microglia are the surveying macrophages of the central nervous system (CNS), and the primary instigators of neuroinflammation. Sustained microglial overactivity is associated with a neuroinflammatory element in numerous neurodegenerative diseases (Lyman et al., 2014). Active microglia may be categorized into two major phenotypes with opposing effects and roles in the progression of diseases such as Alzheimer's disease (AD) (Subramaniam & Federoff, 2017; Wes et al., 2016). The M1 phenotype is associated with damaging, pro-inflammatory activity and is most abundant in clinical state AD. In contrast, the M2 phenotype is associated with neuroprotection and is most prominent in preclinical stage AD (Tang and Le, 2016).

Activated microglia of both M1 and M2 types exhibit increased expression of the mitochondrial 18kDa translocator protein (TSPO), making TSPO a target for PET imaging of microglial activation (Venneti et al., 2009). Quantifying TSPO as a metric of microglial

activation does not allow for the distinction between M1 and M2 subtypes. However, integration of cognitive performance data with PET imaging of this microglial activation as well as data for neuroinflammatory biomarkers in the present study may allow for the determination of AD disease progression in individual subjects.

In humans, the affinity of second generation radiotracers such as [^{18}F]-DPA-714 for TSPO depends on a polymorphism in the TSPO gene, which categorizes individuals into three phenotypes: high-affinity, mixed-affinity and low-affinity (Kreisl, 2020; Owen et al., 2012, 2015). These expression levels may be evaluated in humans from blood samples given consistent expression across individuals (Piras et al. 2021).

3.0 Evaluation of Neurodegenerative Biomarkers

Neurodegenerative biomarkers present the opportunity to quantify the pathological processes that lead to characteristic dysfunctions across various neurodegenerative diseases and monitor disease progression beginning in asymptomatic preclinical stages (Ehrenberg et al., 2020). Among these is glial fibrillary acidic protein (GFAP), an astrocyte cytoskeletal protein that is reliably analyzed from blood, has demonstrated association with CNS damage and astrogliosis in the cases of traumatic brain injury (TBI) and neurodegenerative disease (Abdelhak et al., 2022).

In the present project, established and novel biomarkers associated with neuroinflammation, aging, and AD, including GFAP and cytokines will be quantified from the serum and plasma of eight aged marmosets. We will investigate whether these biomarkers are correlated with neurological biomarkers such as microglial activation, assessed via DPA-PET signal, and with cognitive impairments in aged marmosets, assessed via two cognitive tasks.

4.0 Assessing Aging Profiles in Marmosets

Marmosets are demonstrating promise and prominence as a translational neuroscience and geroscience model, particularly with the aim of understanding neurodegenerative disease. This is given that marmosets spontaneously exhibit age-associated phenotypes mirroring those of humans such as cognitive decline (Sadoun et al., 2019), amyloid deposition (Geula et al.,2002; Ridley et al., 2006), and diminished adult neurogenesis (Leuner et al., 2007). The key advantage of this model is a short lifespan– the shortest among anthropoid primates.

This project will evaluate neuroinflammation and cognitive performance in a parallel fashion in aged marmosets to inform discernment between M1 and M2 microglial activity measured broadly by DPA-PET signal. To evaluate for potential changes in microglial activation patterns in the aged marmosets, two rounds of PET imaging will be conducted: first halfway through the study during the fifth month, then just before project conclusion during the tenth month.

This pilot study will compile comparative analyses of microglial activation, cognitive performance, peripheral inflammation, and pro-/anti-inflammatory cytokine profiles in a small cohort of aged marmosets. In doing so these data will provide foundational evidence contributing to the goal of fully characterizing normal and pathological endocrine, brain, and cognitive aging in marmosets, which will inform the use of this model in furthering our understanding of human neuroendocrine aging and ultimately in combating neurodegenerative disease. We anticipate three possible distinct aging profiles that we may detect based on cross-analysis of DPA-PET signal, cognitive performance, and biomarker levels. These are 1) clinical pathological aging, 2) preclinical pathological aging, and 3) normal aging.

Method

1.0 Study Overview

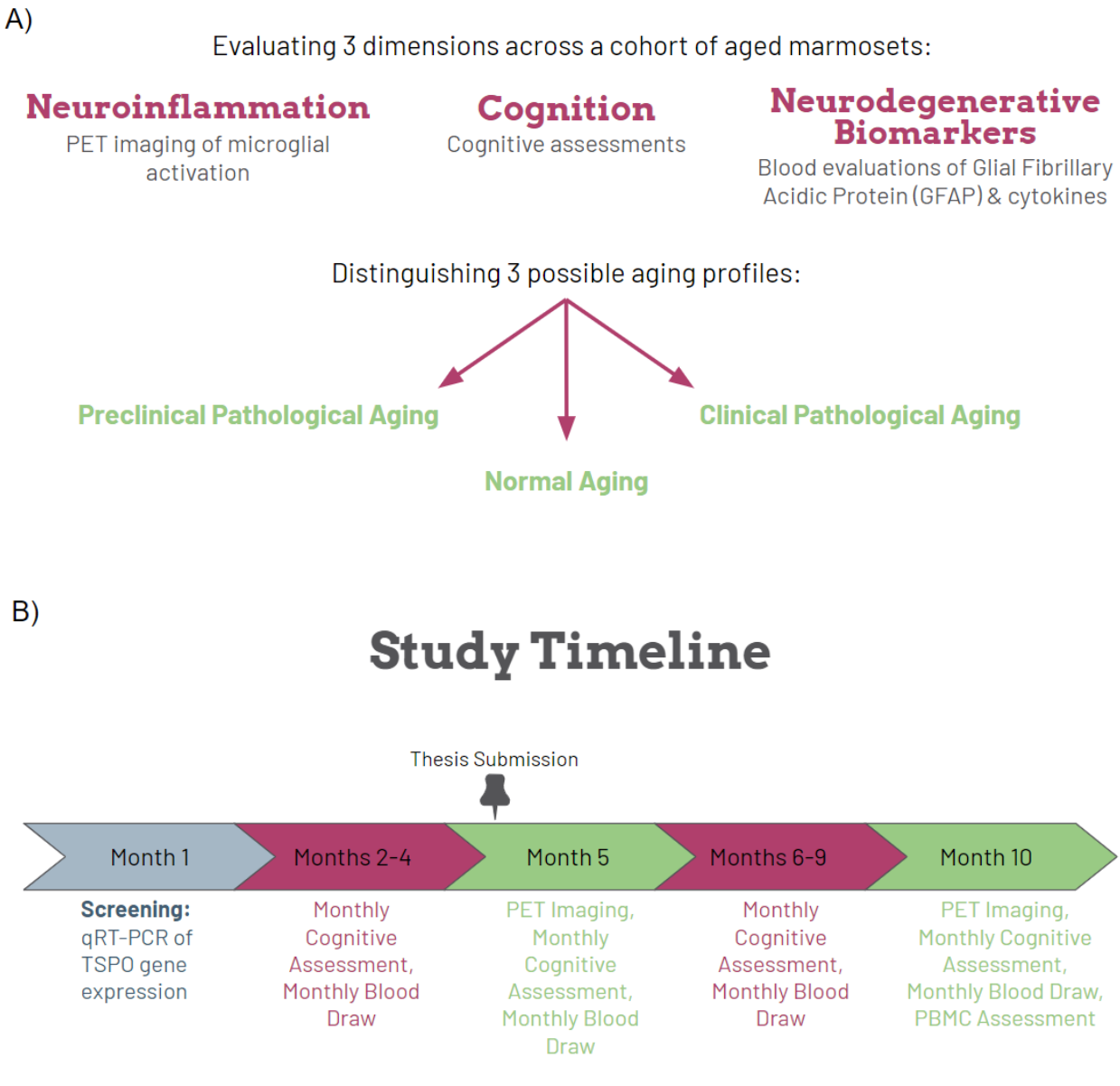
A schematic of this 10-month pilot study design and timeline is provided in Figure 1. Over the course of the study period, a parallel analysis will be conducted across three dimensions: 1) neuroinflammation, 2) cognition, and 3) neurodegenerative biomarkers, in order to characterize aging profiles in the common marmoset.

In vivo examinations of aged common marmosets are being conducted at the Southwest National Primate Research Center (SNPRC), Texas Biomedical Research Institute, San Antonio, TX. The SNPRC is an Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC) accredited program. This work is in adherence to the provisions of the Animal Welfare Act of 1966 (Public Law 89-544), as well as its subsequent amendments, and the standards established in the eighth edition of "The Guide for the Care and Use of Laboratory Animals". This project is being conducted with the approval of the Texas Biomedical Research Institute Institutional Animal Care and Use Committee (IACUC), in adherence to the Health Research Extension Act of 1985 (Public Law 99-158).

This written thesis is being submitted during Month 5., the week of the initial round of DPA-PET imaging collection (Figure 1). To follow, all work conducted and data obtained thus far will be detailed.

Figure 1.

Pilot Study Overview



Note. A) A schematic overview of this pilot study. B) The 10-month study timeline. This written thesis has been submitted entering Month 5, prior to the first round of PET imaging analysis.

2.0 Selection of Subjects

Eighteen aged marmosets underwent a screening process to evaluate their compatibility for this project.

Although expression of the TSPO gene in marmoset blood samples was previously unknown, we verified in the development of this study that marmosets do in fact express the TSPO genes. We predicted that they exhibit the same genetic polymorphism found in humans that gives rise to varying binding affinities to second generation TSPO radiotracers, such as [^{18}F]-DPA-714 used in this study. To distinguish these phenotypes, qRT-PCR annotating the TSPO gene was performed on blood samples from the eighteen candidates at Texas Biomedical Research by the Molecular Core Laboratory, which revealed their TSPO expression affinities relative to multiple reference genes.

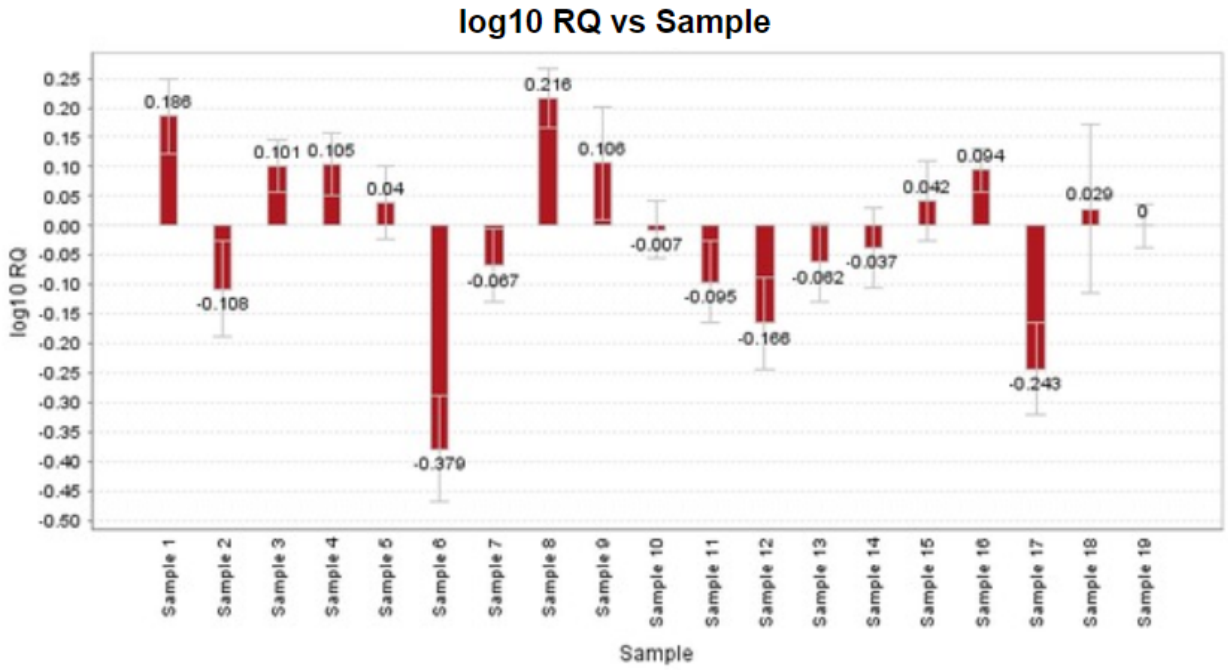
qRT-PCR results were analyzed using double delta Ct analysis to calculate relative gene expression (RQ). In this procedure, software measures amplification of the target (in this case, TSPO) in comparison to multiple endogenous control targets (Schmittgen & Livak, 2008). Whether marmosets exhibit distinct TSPO binding phenotypes as humans do is unknown. To account for this possibility, we assessed degrees of binding affinity by comparing each individual RQ to the pooled mean of RQs from all 18 candidates. The results of this analysis are summarized in Figure 2.

Aged marmosets with moderate–high relative TSPO expression affinity among our candidates, based on comparison to the pooled mean expression fold change (Figure 2, Sample 19), were eligible for this project. To meet this criterion, we aimed to admit animals with expressions above the pooled average (positive values in Figure 2). Candidates also underwent health evaluations to evaluate their compatibility for PET study. Also taken into consideration were any existing cognitive performance data for these animals, in order to enroll a balanced sample of cognitively normal and impaired subjects. At the conclusion of the screening phase,

seven of the eighteen marmosets were enrolled in the study: 3 males, and 4 females, with ages ranging 7.49–9.92 years at the time of screening.

Figure 2.

qRT-PCR Analysis of TSPO Expression



Note. Summary of qRT-PCR evaluating TSPO gene expression in study candidates, analyzed using double delta Ct analysis. Expression fold change (RQ) transformed logarithmically to illustrate relative expressions among the evaluated samples. Sample 19 represents a pooled mean of TSPO RQs derived from all 18 samples. Samples 1-18 are from the 18 marmosets considered for this study. Error bars represent minimum and maximum RQ determined for each sample.

3.0 DPA-PET Imaging

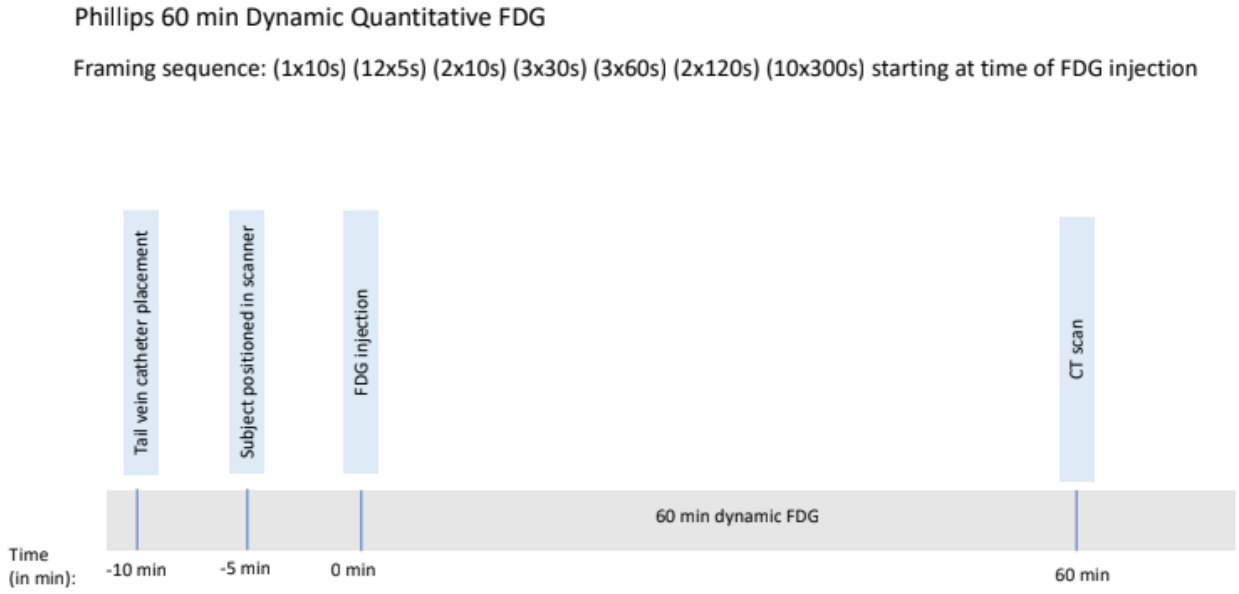
Subjects will undergo two rounds of dynamic PET imaging at the SNPRC PET/CT suite in this study, six months apart, using the [^{18}F]-DPA-714 radiotracer. [^{18}F]-DPA-714 (N,

N-Diethyl-2-(2-(4-(2-[^{18}F] fluoroethoxy) phenyl)-5, 7-dimethylpyrazolo [1, 5-a] pyrimidin-3-yl) acetamide) is being synthesized by Dr. Sid Kumarapperuma at the Research Imaging Institute, UT Health San Antonio, using a GE TRACERlab™ FX2N radiosynthesis module with minor modifications from the procedure published by Keller and colleagues (2017).

The Phillips Lab dynamic PET imaging protocol has been established for use in FDG-PET studies examining brain glucose metabolism, and will be applied to the DPA-PET element of this project. The timeline of this process is summarized in Figure 3. Animals will be anesthetized prior to imaging sessions with isoflurane. They will then undergo 60-minute dynamic imaging concluded with capture of a CT scan. Image binning during dynamic capture is detailed in the framing sequence in Figure 3.

Figure 3.

Dynamic PET Imaging Timeline



Note. Phillips lab protocol for 60-minute dynamic PET imaging. This FDG-PET protocol will be adapted for the use of radiotracer [^{18}F]-DPA-714 to quantify microglial activation.

4.0 PET Image Analysis

DPA-PET images will be analyzed using the PMOD software. PET images will be registered with CT scans, which will provide anatomical orientation for DPA-PET signal assessment. A marmoset brain atlas will be overlayed onto these fused images in order to discern specific regions for analysis. Regions of interest for this study include the corticostriatal system and the prefrontal cortex, as these will be assessed via the two cognitive tasks incorporated into this study. We are also interested in examining DPA-PET signal in the hippocampus in this cohort of aged marmosets.

5.0 Cognitive Evaluation

Cognitive assessment is being conducted in a unit that is attached onto the balcony (dimensions: 11" x 11" x 10.5") of our subjects' home cages. Our subjects' participation in cognitive tasks is always voluntary. After undergoing a habituation period for each of the cognitive tasks, subjects are being assessed monthly to track longitudinal cognitive trajectories. Subjects are being evaluated via two cognitive tasks: staircase and detoured reach. Photos of the setups for each of these tasks are included in Figure 4.

5.1 Visuospatial Integration Assessment via Staircase Task

The staircase task is conducted in two forms with inverted designs: "Hill" (Figure 4A) and "Valley" (Figure 4B). In this task, marmosets reach through two outer plexiglass slots (Hill) or a central plexiglass slot (Valley) in order to retrieve treats that are positioned on four ascending steps. For successful retrievals of treats from the lowest to highest step, animals receive one, two, three, and four points toward their score out of a possible ten for a given session. All trials are recorded on video and scored by an individual who is blind to subject age

or status. The use of Hill and Valley apparatuses and whether the treats are placed on the right or left sides of the staircase is balanced across trials.

A successful retrieval consists of a marmoset reaching through a plexiglass slot toward a treat, successfully grabbing the treat, and pulling it back through the slot without any drops. In the case that an animal knocks a treat off of a step before grabbing it, the attempt is marked as unsuccessful, and the animal does not receive points for that treat. Neither the order in which treats are retrieved (e.g. from the lowest to highest step), nor the speed of treat retrieval are incorporated in the scoring of this task. Rather, each retrieval attempt is recorded as successful or unsuccessful; successful attempts add points to the animal's score according to the height of the step, and unsuccessful attempts result in no points added to the animal's score.

Performance on the staircase task is indicative of visuospatial integration and has been associated with corticostriatal functioning in marmosets (Henderson et al., 1998; Marshall & Ridley, 2003; Phillips et al., 2017).

5.2 Latent Inhibition Assessment via Detoured Reach Task

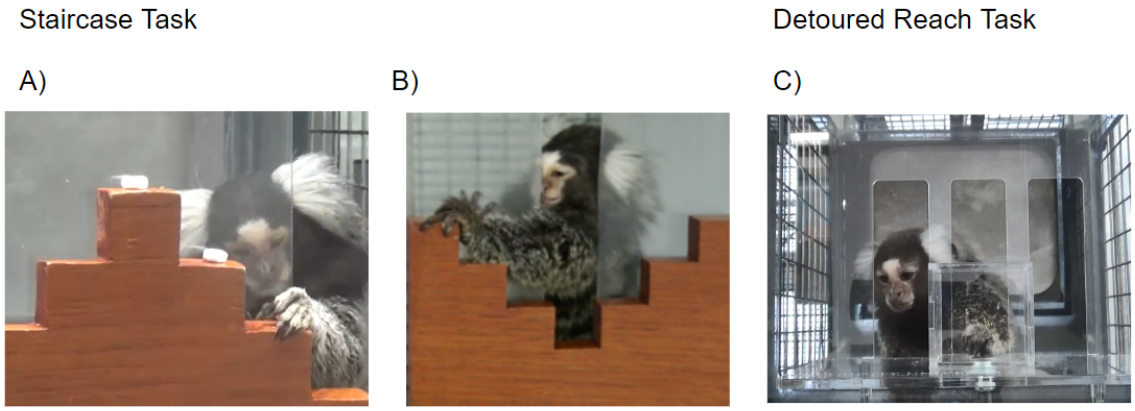
The detoured reach task (Figure 4C) will be used in complement to the staircase task during this pilot study to assess cognition. The detoured reach task evaluates an animal's capacity for latent inhibition and executive function as they retrieve a reward using line of sight reaching. In this task, marmosets are presented with a clear box with one open side which they may reach through to retrieve a treat. The box will contain a treat either in the center or outside edge of the box.

Animals undergo 20 trials for each session of this task, and are allotted a maximum of 30 seconds to retrieve the treat from the box. During the week-long habituation period for this task, marmosets underwent 10 trials per session, all with the open side of the box in the front-facing

position toward them. During test sessions of this task, the orientation of the open side of the box is randomized in order across all 20 trials in front-, left-, and right-facing positions relative to the marmoset. Trials are scored as successful or unsuccessful, and the overall percent of correct initial attempts to retrieve the treat is recorded for each session. All trials are recorded on video and scored by an individual who is blind to subject age or status. This task assesses prefrontal dysfunction in monkeys, including marmosets (Dias et al., 1996), and has also distinguished cognitive deficits in some geriatric marmosets (Ross et al., 2019).

Figure 4.

Cognitive Assessment Setups



Note. Cognitive assessment apparatus for the staircase task in the A) Hill and B) Valley formats, and C) for the detoured reach task.

6.0 Evaluation of Neurodegenerative Biomarkers

Monthly blood samples will be collected for analysis of various biomarkers throughout the study, beginning at baseline. Marmoset blood samples are being collected from the femoral vein by trained personnel in accordance with SNPRC protocol. In this process animals are not

sedated and are restrained for about 3-5 minutes using a marmoset restraint device (9 cm length x 7 cm diameter PVC tube) with Velcro straps across the abdomen and thighs allowing free movement of the head, arms and tail. The amount of blood obtained from each draw is based on the following blood volume calculations: Animal body weight [kg] x 60 mL x .10 = maximal volume of blood to be drawn at one time [in mL].

Blood serum will be assessed for GFAP via an enzyme-linked immunosorbent assay (ELISA) on two occasions throughout the study duration, spaced four months apart (Figure 1). Blood plasma will also be assessed for the presence of pro-inflammatory cytokines including IL-1B, IL-8, and TNF; and anti-inflammatory cytokines including IL-4, IL-10, and TGFβ via Luminex assay on two occasions throughout the study duration, spaced four months apart. Samples are stored at -80°C prior to analysis. In addition, peripheral blood mononuclear cells (PBMCs) will be purified from blood samples, and a portion of these will be used to determine lymphocyte subsets and cell activation on two separate occasions throughout the study. The remaining cells will be stored in liquid nitrogen for future use.

Anticipated Results and Future Directions

1.0 Data Analysis

We will conduct comparative analyses of longitudinal assessments of cognitive performance, peripheral inflammation, pro- and anti-inflammatory cytokine profiles, and microglial activation. We will analyze the DPA-PET neuroinflammatory data via the PMOD software to quantify microglial activation. We are particularly interested in examining DPA-PET signal to illustrate microglial activity in the corticostriatal system and prefrontal cortex, as functioning of these regions is associated with performance on the staircase and detoured reach tasks, respectively. Multiple regression via R/RStudio will be used to determine the degree to

which changes in cognitive performance are correlated with blood analytes implicated in neuroinflammation and microglial activation. M1 and M2 microglial activity indicated by DPA-PET signal will be distinguished phenotypically based on consideration of cognitive performance trajectories and neurodegenerative biomarker levels.

2.0 Anticipated Aging Profiles

We anticipate three possible aging profiles displayed among our cohort of aged marmosets over the course of this pilot study: clinical pathological aging, preclinical pathological aging, and normal aging. A clinical pathological aging profile would be characterized by high DPA-PET signal in our regions of interest, concurrent with decline in cognitive performance across our tasks and high levels of GFAP and pro-inflammatory cytokines from our blood assessments. The DPA-PET signal in this profile would be indicative of M1 pro-inflammatory, detrimental microglial activity in clinical disease states. A preclinical aging profile would similarly display high DPA-PET signal and inflammatory biomarker levels, but this will not be alongside a decline in cognitive performance across our assessments. The DPA-PET signal in this profile would be indicative of neuroprotective, anti-inflammatory activity of the M2 microglial phenotype. Finally, there is potential that we observe a normal aging profile, which will not exhibit a substantial DPA-PET signal, nor significant levels of neurodegenerative biomarkers, nor a decline in cognition demonstrated by performance on our two tasks.

3.0 Considerations and Extensions Moving Forward

3.1 DPA-PET Imaging of Aging Marmosets

This pilot project is evaluating [^{18}F]-DPA-714 as a method of detecting microglial activity to characterize neuroinflammation in aged marmosets. To our knowledge, it is the first study to

document marmoset expression of the TSPO gene and utilize [^{18}F]-DPA-714 in dynamic PET imaging of this species. If we are able to validate this methodology, and demonstrate its utility in distinguishing neuroinflammatory profiles in marmosets, future applications may expand our understanding of neuroinflammation in this key non-human primate model. Neuroinflammation is a dynamic process, and potential further studies using this technique should aim to capture a wider temporal window of microglial activity. It is possible that the 6-month period in between the two dynamic PET sessions in this study will be too brief to capture marked differences in microglial activity in some marmosets. A major goal of future applications of this imaging should be pinpointing the initiation of neuroinflammatory activity, both protective and detrimental, in relation to other pathological elements and manifestations of decline. These efforts should also aim to characterize neuroinflammatory trajectories over time.

A key element for consideration in the use of the [^{18}F]-DPA-714 TSPO radiotracer is the need to account for the genetic polymorphism that gives rise to the three binding phenotypes. We found variation in TSPO binding affinity among our pool of 18 aged marmosets during the screening process. However, further work to identify thresholds delineating high- mixed- and low-binding affinity phenotypes in the common marmoset is an important area in the future expansion of this methodology.

3.2 Characterizing Cognitive Profiles in Aging Marmosets

Decline in executive function, visuospatial integration, and inhibitory control, which we are assessing in this study via staircase and detoured reach tasks, are established aspects of cognitive decline associated with aging and neurodegeneration. However, a further critical area of focus with respect to assessing cognitive trajectories and discerning cognitive decline is hippocampal functioning. The Phillips Lab has previously employed a computerized task that is

established to assess hippocampal activity in marmosets. This task was not feasible during this 10-month pilot study, as it requires about 6 months for training. However, future developments from this work would benefit from incorporating a method of hippocampal functional assessment to inform comparative analysis of hippocampal functioning and neuroinflammation in marmosets as they age.

3.3 Characterizing Neurodegenerative Biomarker Profiles in Aging Marmosets

There is a substantial gap in the literature of assessment of neurodegenerative biomarkers in marmosets, and to our knowledge, this study is the first to do so in a longitudinal fashion. In furthering this model of human aging, a major area for development is uncovering the extent to which marmosets display elevated levels of anti- and pro-inflammatory biomarkers in a manner that reflects diseased state. A greater goal in this realm should be to establish standard biomarker ranges in marmosets and the trajectories of these levels over time across diseased and normal aging profiles.

3.4 Outlook

This pilot project incorporates several novel means of assessing aging profiles in the common marmoset with the objective of contributing to the development of this promising non-human primate model of human neurologic aging. Findings from this study will inform future extensions and ultimately translational applications, for which there is a wide window of opportunity, and mounting societal need.

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