THESIS

CHARACTERIZATION OF THE DUNKIN HARTLEY GUINEA PIG AS A NON-TRANSGENIC AND MULTIMORBID MODEL OF BRAIN AGING

Submitted by

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ABSTRACT

CHARACTERIZATION OF THE DUNKIN HARTLEY GUINEA PIG AS A NON-TRANSGENIC AND MULTIMORBID MODEL OF BRAIN AGING

Alzheimer's Disease and Alzheimer's Disease Related Dementia (AD/ADRD) affect an estimated 55 million people worldwide; a staggering figure that is expected to grow in the coming years. With this projection looming, we have yet to identify any effective cures, treatments, or preventative strategies. Historically, AD/ADRD research is conducted using genetically engineered pre-clinical models, that express a specific brain aging pathology. Recent discoveries, however, have identified a dynamic whole-body "inflammaging" phenotype that exists with, and likely contributes to, AD/ADRD onset and progression. Currently, we do not have an accessible and tractable preclinical model that naturally mimics the age-related, systemic and progressive neurodegenerative phenotype present in humans. Recent findings, however, suggest the Dunkin Hartley guinea pig (HGP) may address this need. HGPs are known to develop systemic inflammation and progressive age-related comorbidities characteristic of human aging. The presence of this whole-body aging phenotype prompted investigation into the brain. Genetic and transcriptomic analyses found aged HGPs exhibit strong sequence homology, and similar protein expression patterns to human brain aging and AD. Further, immunohistochemical assessment found aged HGPs express markers of neuroinflammation and misfolded proteins in the hippocampus. To further interrogate these novel findings, we examined the histopathology of 4 brain regions often implicated in neurodegenerative decline

for evidence of progressive neuropathology. Our results identify the presence of an age related neuroinflammatory and phosphorylated tau phenotype. Findings from this study contribute to the overarching hypothesis that AD/ADRD is a whole-body disease, and ultimately support the goal of closing the existing translational gap between preclinical and clinical neurodegenerative research.

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CHAPTER I – INTRODUCTION

Global improvements to medical practice, research technique, and health education, successfully extended the human lifespan – but how are we spending those extra years? Currently, 33% of people aged 45-64 are living with two or more chronic health conditions. By 65, that percentage nearly doubles, with 63% of older adults living with two or more chronic conditions [1, 2]. This age-associated accumulation of chronic disease, known as the multimorbidities of aging, is an important consideration as the global population of older adults continues to grow. For the first time in history, people aged 65+ make up the fastest growing age-bracket, outnumbering children aged 5 and under [3]. Without intervention, these co-evolving health trends will result in a large population of chronically ill individuals. Global health 'wins' led us to the successful extension of lifespan; now it's time to shift our focus toward the extension of healthspan.

The mismatch between our lifespan - the number of years from birth until death, and our healthspan - the number of years for which we are disease free, is the impetus for modern approaches to age related disease. Common medical practice treats age related chronic diseases one-by-one, following a "disease-first" framework. Though this approach has had success, for many older adults this point-blank treatment has resulted in an ever-expanding list of daily medications, frequent trips to health centers, and an overall reduced quality of life. A "patient-first" framework, which emphasizes proactive disease prevention, was developed to address these shortcomings [4].

Aligned with this shift in clinical practice, is a shift in research perspectives on aging and age-related disease. New evidence suggests that by targeting the systemic cellular processes that contribute to the aging process, we can compress the amount of time spent with multimorbidities of aging at the end of life [5]. This systemic approach may be a particularly effective strategy for the treatment and prevention of neurodegenerative diseases, such as Alzheimer's Disease and Alzheimer's Disease Related Dementia (AD/ADRD). Previously, AD/ADRDs were thought to be the result of one specific pathological 'driver' [6, 7]. Though this theory is not incorrect, new evidence suggests it is overly simplistic. It appears multiple systemically dysregulated drivers contribute to age related neurodegenerative decline [8, 9]. Epidemiological evidence indicates AD/ADRD is not only comorbid with other multimorbidities of aging [10], but individuals diagnosed with multimorbidities of aging earlier in life (<55 years) have a significantly increased risk of developing AD/ADRD [11]. This chronological accumulation of disease implies that long-term systemic dysregulation may ultimately lead to accelerated neurodegeneration and AD/ADRD [12].

One challenge currently limiting the wide-spread adoption of this whole-systems approach is the lack of accessible and tractable preclinical AD/ADRD models. Often, specific pathological features of AD/ADRD are induced in genetically identical rodent models via exogenous chemicals, or genetic modification. These results are often biased towards the specific pathological mechanism induced, and therefore do not capture the complex nature of the multimorbid neurodegenerative phenotype [13]. Other preclinical models such as non-human primates [14] and canines [15] do naturally display the multimorbid phenotype,

however, their relatively long lifespans, slow disease progression, and the cost of large animal husbandry deter many research institutions from using these models.

The Dunkin Hartley guinea pig (HGP) has emerged as a pre-clinical model that may address these limitations. HGPs are a non-transgenic animal model, that naturally accumulate multimorbidities of aging. Throughout their relatively short lifespan, HGPs develop progressive musculoskeletal dysfunction, metabolic dysfunction, and mobility impairments similar to the human aging phenotype [16-21]. In addition to this peripheral aging phenotype, HGPs also express strong sequence homology and similar transcriptomic mechanisms to human AD-related genes [22, 23]. Further, pilot data identified evidence for spontaneous neuroinflammation-associated microgliosis, astrogliosis, accumulation of misfolded proteins, and neuron loss in the hippocampus [22]. These findings are promising; however, more analysis is needed to characterize the HGP neurodegenerative phenotype.

The goal of this research is to further characterize the neurodegenerative phenotype of young and aged HGPs. We will assess tissue samples across four different brain regions for biomarkers of pathological neuroinflammation, misfolded protein aggregation, and gross tissue morphology. We hypothesize aged HGPs will display advanced brain-aging pathology when compared to young HGPs. This research will contribute to the growing body of literature surrounding the use of the HGP as a translational model of aging and neurodegeneration.

CHAPTER II – REVIEW OF THE LITERATURE

POPULATION AGING

Rapid industrialization and widespread technological advancements have revolutionized the global health network [24]. We share data, update best-practices, and communicate research faster than ever before. These advancements have led to improved health literacy, increased access to care, and widespread adoption of public health practices; all of which have contributed to an increased global lifespan [25]. In fact, since 1950 the average global life expectancy has nearly doubled, causing a shift in global age demographics [26]. For the first time in history people aged 65+ make up the fastest-growing age group [27], currently outnumbering children under five years old and predicted to outnumber the youth age bracket (15-24) by 2050 [3]. This shift is not inherently bad; however, our current societal infrastructure was not designed to withstand this growth spurt. Without appropriate reform to our current healthcare, economic, and social systems, this 'ticking time bomb' of aging could become a crisis of equal magnitude to climate change [28].

Global trends in aging are reflected in the United States (U.S.). As our largest generation, the Baby Boomer's, age into the 65+ category and birth rates remain steady or decline [29], the total proportion of older adults has increased [30]. In the U.S. 65 marks the retirement age, and the point at which both Social Security benefits, and Medicare health insurance become ubiquitously available [31, 32]. Social Security and Medicare are designed to support older adults in their retirement and are primarily funded through federal payroll tax. These programs have worked well, given the historically robust workforce supporting a relatively small, retired

population. However, this careful balance is askew. In 2005, 3.3 working adults contributed enough income tax to support 1 retired adult. By 2040, it is predicted that tax from 2.1 workers will need to support 1 retiree [33]. Managing this shift will be a generation defining challenge. Working-class taxpayers may end up contributing a greater percentage of their income to support this system, thereby reducing their net-savings and ultimately hurting their ability to retire comfortably by 65. Meanwhile, older adults now face the risk of running out of savings given they need more money to fund both a longer retirement and their growing health needs [34].

Healthcare costs nearly double between the ages of 70 and 90 [35]. Though Medicare covers 65% of these costs, the 65+ age bracket still saw a 41% increase of inflation-adjusted out-of-pocket healthcare expenses between 2009 and 2019, while costs for the population under 65 were maintained [36]. The escalating health expenses among those 65+ is largely related to the increasing rate of chronic disease among this population. Nearly two-thirds of adults aged 65 and older have two or more chronic health conditions [2]. This age-associated increase in chronic disease not only places a large burden on the individual and families of those struggling with disease, but it also places higher demand and fiscal stress on the healthcare system [37].

Though necessary, healthcare reform is a complex and multifaceted endeavor, that perhaps falls a *bit* outside this thesis's scope. Governmental reform, however, is only one piece of the puzzle. Understanding how the accumulation of chronic disease is effecting overall age-related decline [38] is crucial to maintain societal health. Research efforts are beginning to focus on addressing the underlying cellular mechanisms that drive age associated health

decline, in an attempt to improve the overall aging process. If we can increase the number of years we spend in good health, not only could we improve our quality of life, but we could also alleviate some of the strain on our healthcare system. In the last one hundred years we successfully extended our life *quantity*; now it is time to focus on improving the *quality* of our extra time.

HEALTHSPAN

Increased longevity has historically been an esteemed marker of societal health; if we are living for longer, we must be healthier, right? Unfortunately, it's not that simple. While lifespan refers to the total number of years we are alive, healthspan describes the subset of those years for which we are free from disease [39]. Ideally the gap between the end of our lifespan and healthspan is minimal – meaning most of our years are spent in good health, free from the burden of age-related chronic conditions. Those with the smallest gap seem to glide into their 9th and 10th decades with minimal overall decline [40], while those with a larger gap spend a greater proportion of their life with poor health [41]. Unfortunately, most people currently fall into the second category; spending on average 18% of their total lifespan with at least one, but often two or more age-related morbidities [42]. To address this gap, aging research has shifted its focus to the extension of healthspan through the compression of morbidity.

To understand what drives healthful aging, we must first define what it means to be healthy. Throughout the first half of the 20th century, health was described simply as the absence of disease [43]. Given it was uncommon to survive disease, let alone live *with* or *beyond* them, this straightforward definition was sufficient. Recent research and medical

advancements, however, have progressed our understanding of diseases and their treatment. Now, it is common to live with and beyond many previously lethal diseases [44]. To reflect these advancements, the World Health Organization redefined health as "a state of complete physical, mental, and social well-being" [45]. Instead of viewing health as a simple binary, we now understand it to be a complex and variable spectrum. This evolved perspective accurately reflects the dynamic nature of health by considering both macro- and microscopic dimensions. Through this framework we can create and apply integrative 'best health practices' throughout our lives, potentially attenuating overall age-related decline [46].

GEROSCIENCE

Research in the field of Geroscience focuses on the progressive physiological decline associated with aging [39]. Like lifespan and healthspan, geroscientists look at chronological and biological age to assess a person's health. Chronological age describes time elapsed since birth, while biological age refers to age-related epigenetic, cellular, physiological, and functional changes [47]. A significantly older biological age than chronological age is associated with earlier functional decline and a shortened healthspan [48, 49]. Biological ag*ing* is therefore defined as "decline that (1) simultaneously involves multiple organ systems and (2) is gradual and progressive" [50]. Importantly, there is evidence that the rate at which we biologically age is modifiable and potentially reversable [51].

Efforts to capture and characterize biological drivers of aging have been successful. In 2013, López-Otín and team described nine "hallmarks of aging" which are age-associated cellular and mechanistic processes that contribute to biological aging in a causative manner [52]. These original hallmarks were updated and expanded in 2023 to reflect the

interconnected and systemic nature of biological aging [53]. Though individual hallmarks are often the target of experimental therapeutic interventions, the updated hallmarks highlight the importance of viewing the aging process as a whole-body and multi-system event, instead of one isolated process.

The 2023 hallmarks are organized into a three-tiered functional hierarchy, including primary, antagonistic, and integrative drivers. The primary hallmarks are composed of broad, systemic drivers that directly influence the aging process through progressive and accumulative damage. These 'feedforward' drivers include genomic instability, telomere attrition, epigenetic alterations, loss of proteostasis, and disabled macroautophagy. If dysregulated, each of these primary hallmarks can initiate a cascade of feedback responses. The feedback responses are captured in the second-tier antagonistic drivers, which include mitochondrial dysfunction, deregulated nutrient sensing, and cellular senescence. The antagonistic hallmarks appear to amplify the accumulated damage caused by dysregulated primary drivers. Third-tier integrative drivers describe the late-stage effects of the primary and antagonistic drivers. These include microbial dysbiosis, chronic inflammation, altered intracellular communication, and stem cell exhaustion. Integrative drivers act to compound the damaging effects of primary and secondary drivers, ultimately accelerating the rate of biological aging. Importantly, the relationship between each tier should be considered bidirectional. Dysregulation can occur and accumulate at any of the three tiers and ultimately contribute to an overall accelerated rate of biological age and age-related decline.

Though the list of hallmarks is surely not conclusive, it has provided a framework upon which new mechanistic research and clinical application can stem. In fact, characterization of

these drivers and their associated biomarkers have already made an impact. Epidemiological and comparative clinical studies have found biomarkers associated with the hallmarks of aging accurately predict older adult functional outcomes [54], hospital mortality among critically ill patients [55], and all-cause mortality among community dwelling individuals [56, 57]. Novel healthspan extending interventions have also been developed utilizing this framework. A few promising candidates include therapeutic strategies that target mechanisms of macro-autophagy and nutrient sensing, such as metformin, rapamycin, and caloric restriction [58-60], as well as therapeutics targeting cellular senescence and the senescence-associated secretory phenotype [61]. Interestingly, these therapeutic strategies all appear to indirectly attenuate inflammation, an integrative hallmark of aging.

BRAIN AGING

As with all organs, the brain begins to show initial signs of aging shortly after maturation that progressively accumulate with age [62, 63]. Pathologically, these changes are characterized by altered neural circuitry, reduced plasticity, slowed signal transmission, and diminished structural integrity of white matter [64-67]. *Relax,* though these changes sound daunting, they often occur slowly over the course of decades. Cognitively, normal brain aging may present as slowed word recall, reduced ability to multitask, and shortened attention span – all of which have little to no impact on the livelihood and independence of older adults [68, 69]. Unfortunately, there is not yet a specific protocol that guarantees normal brain aging. Some people experience these progressive changes earlier and/or faster than others, leading to more severe neurological degeneration and potentially resulting in disorders such as Alzheimer's disease and Alzheimer's disease-related dementias (AD/ADRD) [70].

The mechanisms that lead to accelerated neurodegeneration and ultimately AD/ADRD are not fully understood. Over the last 30 years, AD/ADRD research and pharmaceutical development focused largely on single-cause-single-effect hypotheses [71, 72], such as the cholinergic hypothesis [73], the metal ion hypothesis [74], and perhaps most notably the Amyloid-Beta (Aβ) hypothesis [6]. Though these hypotheses are not inherently incorrect, new evidence suggests they do not effectively capture the multiplex nature of neurodegeneration and AD/ADRD [8, 9, 75]. Instead, they appear to over-emphasize the role of singular pathologies that contribute to but are not solely causative of AD/ADRD.

Epidemiological research has shown AD/ADRD rarely occurs in isolation. Older adults with AD/ADRD are more likely to have multiple chronic conditions than their cognitively healthy counterparts [76], and diagnosis is often subsequent to the accumulation of other multimorbidities of aging [10]. In fact, people who have more than one chronic condition before the age of 55 have a significantly increased risk of developing AD/ADRD [11, 77] when compared to those without the multimorbid phenotype [78]. Not only does the accumulation of chronic diseases correlate with the onset of AD/ADRD, but a 2-year longitudinal study found presence of the multimorbid phenotype appears to accelerate the rate of cognitive decline among individuals already diagnosed with AD [79]. This apparent chronological and compounding accumulation of systemic diseases implies there is a relationship between long-term peripheral dysregulation and AD/ADRD onset.

Chronic inflammation (CI) is one common link between multimorbidity, AD/ADRD, and aging. CI increases with age and occurs in the absence of overt infection or active disease [80]. Systemic CI is an integrative hallmark of aging; known to contribute to various age-related

chronic diseases characterized by the 'inflammaging' phenotype [81]. Inflammaging is implicated in diseases ranging from energetic disorders such as metabolic syndrome, obesity, and type 2 diabetes mellitus [82]; to various cancers [83], cardiovascular disease, atherosclerosis [84], sarcopenia [85], osteoporosis [86], and behavioral and mental health disorders [87].

CI in the brain was first recognized by Alois Alzheimer, who identified colocalized neuroimmune cells with the amyloid-beta plaques [88, 89]. Though this observation was made in the original AD case studies, only recently has it been considered at length. In the central nervous system, CI is evidenced by increased transcriptomic expression of inflammation-related proteins [90] and morphological changes to microglia and astrocytes, the resident immune cells of the brain [91, 92]. Microglia are the multi-functional macrophages of the brain, working to degrade and clear dysfunctional proteins and cells [93]. In response to insults, microglia shift from a resting state to an activated one. When microglia activate, they undergo morphological and phenotypic changes that mediate an inflammatory immune response. In healthy conditions microglia activation acute and transient; addressing the specific insult before returning to a resting state. With age and under chronic stress, however, microglia shift from a resting morphological state to a primed one, meaning their threshold for activation is lowered [94]. In these stressed states, microglia become chronically activated and inflammatory [95], and begin to display upregulated expression of multiple AD related genes [96]. The identification of functionally unique microglia opposes the historical belief that activated microglia are phenotypically homogenous and could provide insight to the not-well understood role of microglia in neurodegeneration.

Astrocytes also become activated with AD/ADRD progression [97]. Astrocytes support neuronal communication and homeostasis by synthesizing and degrading neurotransmitters, supporting synapse formation, and maintaining the blood brain barrier [98]. There appears to be a close relationship between activated astrocytes and Aβ accumulation [99], though the interaction is not fully understood. Chronically activated astrocytes also contribute to inflammation via dysregulation of the blood brain barrier [100], which is an established feature of AD/ADRD [101]. It is hypothesized that both microglia and astrocytes are initially protective [99], however, under chronic activation they begin to release detrimental factors that act to compound AD pathology [102].

Though it is well established that CI is present both centrally and peripherally [103] in AD/ADRD, and there is correlative evidence for cross-talk between the two systems [104-106], the mechanism of communication and potentially temporal nature of CI onset is not fully lucid [107]. Some hypothesize CI begins in the periphery *first,* and then progresses centrally [108] while others hypothesize the opposite [109, 110]. Despite these amounting 'chicken-or-egg' opinions, there appears to be consensus that once onset has occurred, CI participates in a positive feedback loop in which inflammation acts to compound the progression of other mechanistic drivers of AD/ADRD. Therefore, CI should be considered core to AD/ADRD pathophysiology.

Loss of protein homeostasis, or protein dyshomeostasis, is another hallmark that links multimorbidity, AD/ADRD, and age. Protein homeostasis (proteostasis) is a highly conserved and fundamental system, responsible for the dynamic regulation of protein synthesis, folding, chaperoning, and degradation [111]. A diverse system of quality control checkpoints,

collectively referred to as the proteostasis network, maintain tissue-specific protein integrity by promoting protein translation, preventing misfolded protein accumulation, maintaining protein chaperone molecules, and improving protein binding capacity [112].

The proteostasis network is essential for normal cellular function, however, under chronically high levels of cellular, metabolic, and environmental stress, it is susceptible to dysregulation [113, 114]. These chronic stressors appear to accumulate with advanced age, and result in systemic protein dyshomeostasis [115, 116]. Loss of proteostasis in the brain leads to the accumulation of misfolded proteins and protein aggregation [117], colloquially referred to as junk proteins. These junk proteins, most often Aβ and phosphorylated tau (p-Tau), are a well described feature of AD/ADRD pathology [118]. Accumulation of Aβ and p-Tau contributes to a proteo-toxic and neuro-toxic micro-environment, which appears to further contribute and compound the accumulation of misfolded proteins in the brain [7, 12].

The role of misfolded proteins in AD/ADRD pathogenesis and progression has been a focal point of research. Specific emphasis was placed on the 'Aβ hypothesis' after Hardy and Allsop (1991) put forth compelling findings identifying a pathogenic mutation in the β-amyloid precursor protein gene [119]. They proposed this mutation, which leads to the accumulation of Aβ, initiated the pathological cascade of AD by triggering the misfolding and aggregation of p-Tau and eventually causing neuronal death. This initial cascade hypothesis initiated research that has expanded our understanding of protein dyshomeostasis in AD/ADRD [7], aided the development of novel diagnostic biomarkers of AD such as the ATX(N) system [120], and provided the basis for the anti-amyloid pharmaceutical Lecanemab (Leqembi®) which modestly improves cognitive function among individuals with AD/ADRD [121]. The Aβ hypothesis

substantially progressed AD/ADRD research, however, as we learn more about the etiology, it seems early isolation of a singular mechanism may have contributed to an overly narrow scope of AD/ADRD research that has neared the point of dogmatism.

Exploring alternative hypotheses is crucial, especially considering the substantial evidence that Aβ accumulation is not solely contributive to AD/ADRD progression. For example, post-mortem studies have found extensive Aβ accumulation in cognitively healthy individuals [122, 123], and numerous clinical trials for pharmaceuticals that successfully clear Aβ plaques from the brain, but do not improve the functional outcomes of those with AD/ADRD [71]. Beyond this, promising results from therapeutics targeting p-Tau – a previously overlooked aspect of AD/ADRD, appear to effectively slow the progression of AD [124]. Taken in-step, these findings highlight the need for continued interrogation of both the seemingly well characterized aspects of AD/ADRD, like p-Tau, and the less-well understood concurrent symptoms and drivers of dysregulation that may be contributing to disease.

RESEARCH GAPS

Characterization and application of the hallmarks of aging have undoubtedly reshaped our approach and understanding of AD/ADRD. As research moves away from single-cause-single-effect hypotheses, and toward hypotheses that emphasize the multi-faceted and potentially systemic nature of AD/ADRD; a clear gap in research methodology has formed. Most often, single AD/ADRD phenotypes are induced in rodent models via genetic engineering or exogenous chemical treatment [13]. Given the AD/ADRD phenotype must be artificially introduced in these models, it is easy to selectively bias results towards the specific pathway we *think* we know the most about; thereby limiting our overall understanding of the complex and

progressive biological processes at play. A systematic review by Kim et al. found that of the 2,700 AD clinical trials put forth between 2004 and 2021, less than 2% were *potentially* successful at reducing AD/ADRD symptoms in humans [72]. This failure to translate successful preclinical pharmaceuticals into clinically meaningful results, exemplifies the perils of one-dimensional and homogenous experimental design [13]. To begin to close this translational gap, we need to utilize preclinical models that mimic the genetic variability and age-related progressive decline present in humans.

Over the last decade, compelling research has identified canine [15] and non-human primate species [14] as pre-clinical models that could help to shrink this translational gap. Both species have genetic variability similar to humans and appear to develop a similar age-related neurodegenerative phenotype [125-128]. Research in these large animals, however, can be challenging. These animals are relatively long-lived and have a late onset of disease [129]. These features make them particularly useful for longitudinal and observational study. However, large animal research is typically high cost given the unique animal care and husbandry needs. This demand and diligence can be a major - if not impossible - barrier to overcome, especially for smaller institutions with limited access to funding and/or space. Limited accessibility to research models diminishes the opportunity to reproduce research, and often results in publication bias. The Dunkin Hartley guinea pig has emerged as a potential solution to address these shortcomings and help bridge the existing translational gaps.

THE DUNKIN HARTLEY GUINEA PIG

The Dunkin Hartley guinea pig (HGP) is an accessible and tractable model of naturally occurring, age-related, multimorbidity. HGPs are a non-transgenic outbred strain of guinea pig that spontaneously develop a peripheral age-related multimorbid phenotype similar to humans. Starting at 3mo of age, the HGP begins to develop inflammation-driven musculoskeletal decline and joint degeneration [130]. Musculoskeletal remodeling and associated metabolic dysfunction occur from 9-15mo [18], and by 15-24mo, mobility is greatly impaired [17]. Along with structural changes to their musculoskeletal system and overall mobility, HGPs also develop whole-body metabolic dysfunction with age and naturally occurring obesity [20]. Lastly, they are a known model for inducible glucose intolerance [19], and atherosclerosis [21]. The accumulation of comorbidities with age closely mimics the inflammaging phenotype seen in humans and has prompted further inquiry into the HGP neurodegenerative phenotype.

Recent research found HGPs have strong sequence homology to human AD-related genes [23]. Further, a transcriptomic analysis found they mirror degenerative mechanisms characteristic of human brain aging [22]. Immunohistochemical analysis of the hippocampal region of 5 and 15mo HGP brain tissue showed signs of progressive neuroinflammation, as evidenced by microgliosis, astrogliosis, and accumulation of the misfolded proteins Aβ and p-Tau [22]. These exciting findings prompted further investigation into the HGP as a preclinical model for neurodegeneration and AD/ADRD pathology.

STUDY PURPOSE

The purpose of this study is to further characterize the neuropathological phenotype of HGPs by comparing markers of brain aging and neurodegeneration in the brain tissue of young and aged guinea pigs across four brain regions.

HYPOTHESIS

We hypothesize that 15mo male and female HGP brains will exhibit greater markers of neuroinflammation and protein dyshomeostasis compared to 5mo male and female HGPs.

CHAPTER III – METHODS

STUDY DESIGN AND ANIMAL CARE

Animal procedures were approved by the Colorado State University Animal Care and Use Committee (19-9129A) in accordance with the National Research Council's Guide for the Care and Use of Laboratory Animals in compliance with the ARRIVE guidelines. Male and female HGPs were purchased from Charles River Laboratories (Wilmington, MA, USA) at 1 and 4mo of age (n=16 total; n=4/sex/age group). Animals were maintained at the Colorado State University Laboratory Animal Resources housing facilities and were monitored daily by a veterinarian. Guinea pigs were singly housed in solid bottom cages and provided ad libitum access to water and regular chow diet (Teklad Global Guinea Pig Diet 2040; Envigo, Madison, WI). Once the animals reached 5 or 15mo of age, they were anesthetized according to the American Veterinary Medical Association, with a mixture of isoflurane and oxygen. Blood was collected via direct cardiac puncture. The guinea pigs were then transferred to a carbon dioxide chamber for euthanasia. Following euthanasia, brains were collected from each animal.

TISSUE PROCESSING FOR HISTOPATHOLOGY AND IMMUNOHISTOCHEMISTRY

Whole brains were fixed in 10% buffered formalin or 4% paraformaldehyde at room temperature for at least 48h. Tissue was processed using the Leica TP1020 Automatic Benchtop Tissue Processor and embedded in paraffin wax (Cancer Diagnostics, Cat #: EEPAR56). The tissue was sectioned using the Thermo Scientific HM 325-2 Manual Microtome at 5µm thickness and mounted on positively charged glass slides (Superfrost Plus, Cancer Diagnostics, Cat #: 4951).

HISTOPATHOLOGY

Whole brain tissue sections were deparaffinized in xylene and rehydrated through graded ethanol. After rinsing with tap water, samples were submerged in diluted hematoxylin (1:1 hematoxylin to 1% acetic acid [diluted with 70% ETOH]) for 6 minutes. Samples were rinsed under tap water, then differentiated in 0.5% acetic acid solution before they were submerged in Eosin. Samples were rinsed again in tap water, dehydrated in graded ethanol, and finally submerged in xylene. Slides were preserved with mounting media and a coverslip and left at room temperature until imaging.

Hematoxylin and Eosin (H&E) staining on whole brain sections was utilized for the assessment of spongiotic tissue. Spongiosis is a gross morphological pathology evidenced by holes in the brain tissue. It is not a known feature of AD/ADRD pathology, however, during pilot data collection it was observed in the cerebellum of HGPs. This novel finding prompted further exploration in other brain regions. H&E is an acid-base stain, in which basic hematoxylin dyes basophilic (acidic) structures blue, and acidic eosin counterstains the basic elements red.

IMMUNOHISTOCHEMISTRY

Whole brain tissue sections were stained via immunohistochemistry (IHC) for protein biomarkers characteristic of neurodegeneration including reactive microglia, activated astrocytes, and abnormal protein accumulation.

Whole brain tissue sections were deparaffinized in xylene and rehydrated through graded ethanol. EDTA buffer (1mM EDTA disodium salt dihydrate, 0.05% Tween; pH 8.0) was heated for 20 minutes at 100^{o}C to retrieve sample antigens for enhanced antibody binding. Sections were incubated at room temperature in 0.3% hydrogen peroxide (H2O2) buffer for

30mins to remove endoperoxides, and then washed with TrisA/2% Bovine Serum Albumin (BSA) 3 times for 2 minutes each. The tissue was blocked and incubated for 1 hour with TrisA/2%BSA+10% goat or rabbit serum diluted in 1 M TBS. Samples were coated in primary antibody, which was prepared to their optimum concentration in TrisA/2%BSA and left to incubate at 4^{o}C overnight. A goat anti-ionized calcium binding adaptor molecule 1 (Iba-1) (1:400; Abcam, Cat #: ab5076) was used to identify microglia. A rabbit anti-S100 calcium-binding protein β (S100β) (1:750; Abcam, Cat #: ab41548) was used for astrocyte identification. For the assessment of misfolded tau proteins, two antibodies were utilized to identify phosphorylated tau isoforms: anti-phospho-Tau-T217 (1:200; ABclonal, Cat #: AP1233) antibody to identify cytosolic phosphorylated tau, and anti-phospho-Tau (Thr181) (1:800; Invitrogen, Cat #: MN1050) to identify p-Tau fibrils. Post incubation, wash steps were performed with TrisA/2%BSA, and then blocked for 1 hour at room temperature with an ABC HRP peroxidase detection kit (Vector Laboratories, Cat #: pk-4,000) and ImmPACT DAB Substrate, Peroxidase (HRP) Kit (Vector Laboratories, Cat #: sk-4,105) was used as a chromogen. Slides were counterstained with hematoxylin (Thermo Fisher Scientific, Cat #: 7231) and bluing solution (Cancer Diagnostics, Cat #: FX2107). The immunoreaction period for slides of the same antigen was kept consistent across batches. Slides were preserved with a coverslip in mounting medium and left at room temperature until imaging.

As a negative control, four slides (n=2 young, 2 aged) were stained without primary antibody to ensure binding specificity. One whole brain tissue section was stained following the IHC protocol described above. During the overnight primary antibody + TrisA/2%BSA incubation, control slides were coated in only TrisA/2%BSA and no primary antibody and left

overnight. Secondary antibody was diluted to the standard concentration in either goat (Figure 1A) or rabbit (Figure 1B) serum.

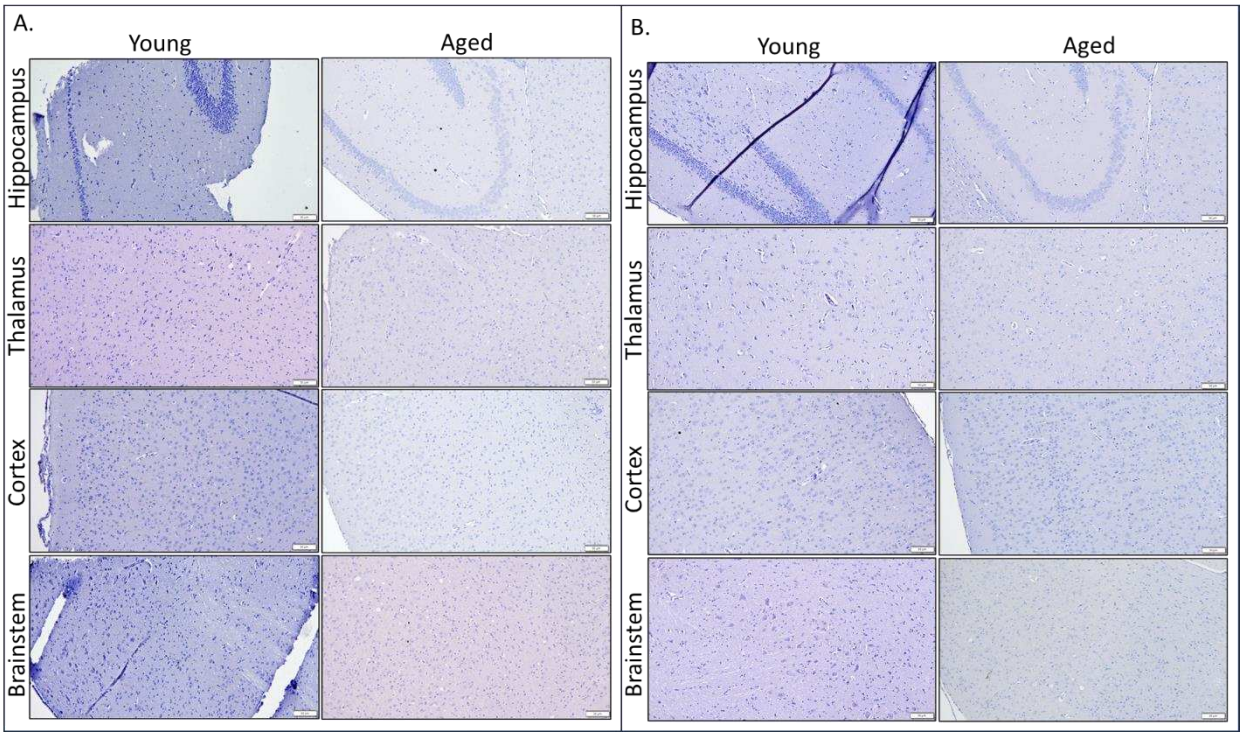

Figure 1. Secondary only control slides confirm primary antibody binding specificity. Representative images of young and aged secondary only slides in goat (A) and rabbit (B) serum. No evidence of brown ImmPACT DAB substrate indicates primary antibody binding specificity.
Images acquired at 10x magnification, scale bar= 50µm

IMMUNOHISTOCHEMICAL ANALYSIS

Imaging

Whole brain images were taken using an Olympus BX53 microscope with an Olympus DP70 camera using an Olympus UPlanApo20x objective (N.A.= 0.75). Olympus CellSens software (v.1.18) was then used to quantify positive staining in the images. Brain regions of interest (ROIs), were manually drawn on each tissue sample for the four brain regions assessed,

including: the hippocampus, thalamus, cortex, and brainstem. The ROIs were then analyzed for the pathology via cellular quantification or pathological scoring.

Regional Analysis

This study assessed the hippocampus, thalamus, cortex, and brainstem. AD is strongly associated with degeneration of the hippocampus, which is associated with learning and memory consolidation. The cortex and brainstem are most often implicated in ADRDs, such as Lewy body dementia (LBD) which typically begins in the brainstem, or frontal/temporal dementia (FTD) which is localized to the frontal and temporal cortices. The thalamus is not known to be directly involved in AD/ADRD pathology, however, its crucial role in the relay and integration of sensory and motor information warranted interrogation. Regions were defined by gross anatomical location, and assessment cellular/tissue morphology. A representative image containing the four determined regions is shown in Figure 2. The total ROI area for each region was kept as consistent as possible to control overall cell count variability.

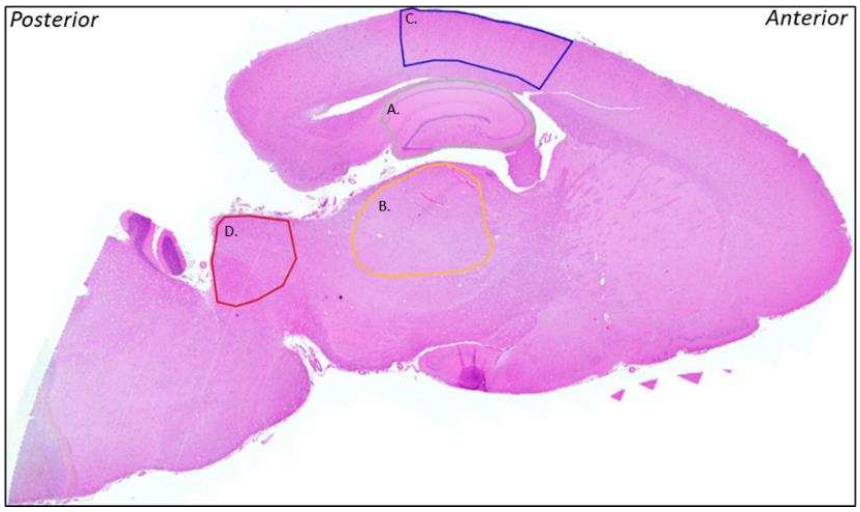

Figure 2. Whole brain sections were cut in the sagittal plane. The four brain regions analyzed included the hippocampus (A; green), thalamus (B; yellow), cortex (C; blue), and brainstem (D; red). If the region could not be clearly identified it was excluded from analysis.
Image acquired at 20x magnification.

Cellular Quantification

ROIs for Iba-1 and S100β were quantified using the 'manual threshold' function in CellSens analysis software and then further refined utilizing the sphericity, shape, and area (µm) tools. With the appropriate filters in place, images were manually assessed to verify the accuracy of the software count; manual adjustments were made accordingly. Raw positive cell counts were normalized to the total ROI area (mm^2).

Pathological Scoring

Pathological scoring was employed for the morphological assessment of spongiosis via H&E stains, and the abnormal phosphorylated tau protein markers p-Tau (Thr181), and p-Tau 217. Animal IDs were blinded and independently scored by three researchers. H&E and p-Tau 217 stains were scored between 1 and 5, with 5 indicating extreme pathology and 1 indicating

no pathology. P-Tau (Thr181) was scored on a 1-3 scale, with 3 representing extreme pathology. The mean of the score was calculated for each region and stain.

H&E scoring was utilized to assess spongiosis, or holes, in the brain tissue. Size and quantity of the holes was used to determine pathological severity. A score of 5 indicated large holes evenly distributed throughout the tissue, while a score of 1 indicated no noticeable holes of any size. The representative images used to determine the scoring scale are shown in Figure 3A.

P-Tau 217 scoring was based on the quantity of cells with cytosolic staining, and the intensity of the stain compared to non-specific background staining. An image with a score of 1 indicated little to no accumulation of cytosolic p-Tau and a very light stain, while a score of 5 indicated intense and abundant staining in the cytosol. The representative image scale used to score p-Tau 217 is shown in Figure 3B.

P-Tau (Thr181) scoring was based on the presence of p-Tau fibrils or tangles, and the visible intensity of the stain compared to the background staining. A scale of 1 - 3 was utilized for this stain to best capture the diverse appearance of the protein marker across the four regions. Figure 3C shows the representative images used to gauge p-Tau (Thr181) scoring, where 1 exhibited little to no p-Tau fibril staining, 2 described moderate accumulation, and a score of 3 indicated severe pathology.

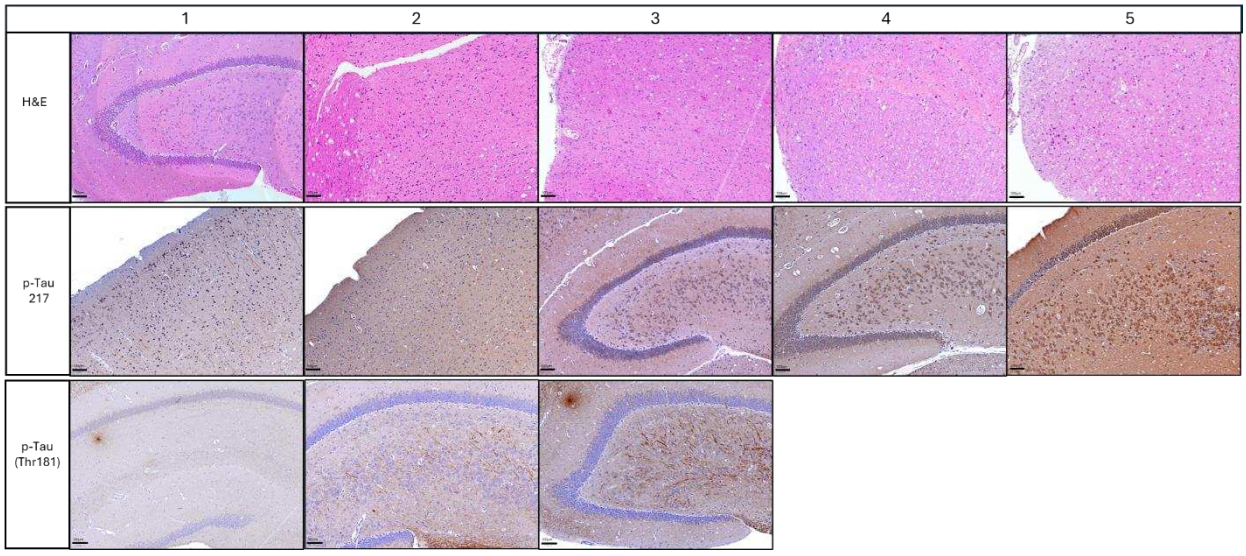

Figure 3. Scales used to score each immunohistochemical protein expression. Pathological score scales used to assess pathological scores for H&E (A), p-Tau 217 (B), and p-Tau (Thr181) (C). Each pathologist received a copy of the scale and written instructions before scoring to maintain consistency throughout the scoring session.
Images acquired at 20x magnification, scale bar =100µm.

STATISTICAL ANALYSIS

Statistical analysis was performed using PRISM GraphPad 10 Software (GraphPad Software, La Jolla, CA). For both the young and aged cohorts, cellular quantification and pathological score data was acquired simultaneously and blinded. Descriptive statistics are reported in Table 1. No outliers were identified using the ROUT method with Q=1%. Unpaired t-tests with Welch's *post hoc* correction were used to compare the young vs aged guinea pigs. Exploratory two-way ANOVAs were performed to visually assess the effect of age, sex, and/or an interaction for each stain. Critical value for significance was set *a priori* to $\alpha=0.05$ for both the t-tests and two-way ANOVAs. Variability was assessed by comparing the standard deviations in the young and aged cohorts for each region and stain.

CHAPTER IV – RESULTS

INCREASED VARIABILITY AMONG AGED HGPS

Standard deviation was used as a rough assessment of genetic variability among the HGPs. In 14 of the 20 total stain/region measurements, aged HGPs had a larger standard deviation than the young.

AGE ASSOCIATED INCREASE IN GLIOSIS AMONG FOUR BRAIN REGIONS.

Compared to young HGPs, aged HGPs had greater overall cell counts of astrogliosis (Figure 4) and microgliosis (Figure 5) in the hippocampus, thalamus, cortex, and brainstem. Astrogliosis in the hippocampus (Figure 4 A-C) and thalamus (Figure 4 D-F) was significantly higher in the aged HGPs compared to the young HGPs ($p=0.03$; $p=0.04$). Microgliosis in the aged brainstem (Figure 5 J-L) was significantly higher ($p=0.02$) than the young HGPs. Astrogliosis in the cortex (Figure 4. G-I) and brainstem (Figure 4 J-L), and microgliosis in the hippocampus (Figure 5 A-C), thalamus (Figure 5 D-F), and cortex (Figure 5 G-I), was visibly increased with age, however, these results were not statistically significant.

AGED ASSOCIATED INCREASE OF P-TAU FIBRILS (THR181).

The pathological score of p-Tau fibrils (Thr181) was greater in the aged compared to the young HGPs in all four regions (Figure 6). This age associated increase reached significance in the hippocampus ($p=0.003$) (Figure 6 A). Interestingly, there was limited evidence of age associated accumulation of cytosolic p-Tau 217 (Figure 7). In the hippocampus and cortex (Figure 7 A,C) aged HGPs had somewhat greater p-Tau 217 protein expression, however, neither reached significance. In the thalamus and brainstem (Figure 7 B, D) there was either no

change, or a decrease in cytosolic p-Tau 217 scores. p-Tau fibrils (Thr181) displayed more score variability in the aged HGPs than the young. Both the young and aged HGPs displayed high variability of cytosolic p-Tau 217 across each brain region.

SPONGIOSIS IS NOT PRESENT IN ALL BRAIN REGIONS OF THE HGP BRAIN AGING PHENOTYPE.

H&E staining showed an age-associated increase of spongiosis in the brainstem; however, there was no pathological change in the hippocampus, thalamus, or cortex (Figure 8. D). Increased presence of spongiosis in the HGP brainstem could indicate the presence of specific ADRD pathology, however, analysis of other related protein markers is necessary to address this question.

EARLY RESULTS SUGGEST SEX DIFFERENCES MAY INFLUENCE THE HGP NEURODEGENERATIVE PHENOTYPE.

A two-way ANOVA was conducted on all data sets to assess the influence of sex and age on outcomes. Small sample sizes for each sex/age limited statistical power, however, gross visual assessment and interpretation of these data is meaningful for the overall characterization of the HGP model. Females appeared to influence the age-associated increase in astrogliosis in the hippocampus, thalamus, and brainstem to a greater degree than males (Figure 9). In the cortex, however, males appeared to have the greatest effect on age-associated differences.

In the brainstem, there was a significant effect of sex on microgliosis in the aged males. Iba-1 protein was significantly greater in the aged males ($p=0.004$) than the young males, which contributed to the main effect of age ($p=0.014$) (Figure 10 D). In the thalamus and cortex (Figure 10. B,C), males also appeared to contribute to age associated change to a greater

degree than female HGPs. The hippocampus did not show any evidence of age or sex associated changes.

No relationships emerged relating sex and age to the presence of cytosolic p-Tau 217 (Figure 11). Females appeared to have a greater degree of whole-brain variability than males, however, this variability is evident in both young and aged female HGPs. Increased p-Tau (Thr181) in the hippocampus is significantly driven by aged female HGPs ($p=0.02$) (Figure 12 A). Females also saw a greater degree of change from young to old than the male HGPs in the thalamus, cortex, and brainstem (Figure 12. B-D).

Finally, no significant whole-brain effects of sex emerged in the assessment of spongiosis. In the brainstem, aged male HGPs appeared to influence the effect of age in the spongiotic brainstem (Figure 13. D), however, no other relationships between age and sex are apparent (Figure 13).

TABLES AND FIGURES

Table 1. Descriptive statistics of young and aged HGP. Sample size (n), mean, and standard deviation (sd) for each young/aged cohort for each region and stain. Yellow cells indicate instances in which the standard deviation in the aged cohort was greater than the young. Bold text indicates a significant difference between the young and aged means (*=p≤0.05, **=p≤0.01)

Stain	Age Group	Hippocampus			Thalamus			Cortex			Brainstem		
		n	mean	sd	n	mean	sd	n	mean	sd	n	mean	sd
S100β	Young	8	147.6	29.32	7	102.4	13.23	8	115.1	33.06	7	120.4	26.02
	Aged	8	**186.4***	37.36	7	**142.7***	41.04	6	148.6	41.95	6	159.4	44.87
Iba-1	Young	8	61.15	14.64	8	41.42	9.45	7	50.02	12.54	8	47.88	8.86
	Aged	7	**66.4**	16.28	7	**46.12**	10	5	57	8.5	6	73.27*	19.51
p-Tau 217	Young	8	3.29	1.07	7	3.24	0.78	8	2.58	0.92	5	3.067	0.59
	Aged	6	**3.61**	0.74	6	**3**	0.84	6	3.16	1.03	6	3.056	1.48
Thr(181)	Young	8	2.16	0.47	8	2.08	0.55	8	1.52	0.58	7	1.9	0.93
	Aged	6	**2.89****	0.27	6	**2.5**	0.83	6	2.33	0.81	6	2.27	0.8
H&E	Young	8	1.67	0.17	7	1.52	0.46	8	1.2	0.35	8	1.75	0.66
	Aged	8	**1.13**	0.17	8	**1.58**	0.66	7	1.19	0.26	6	2.77	1.51

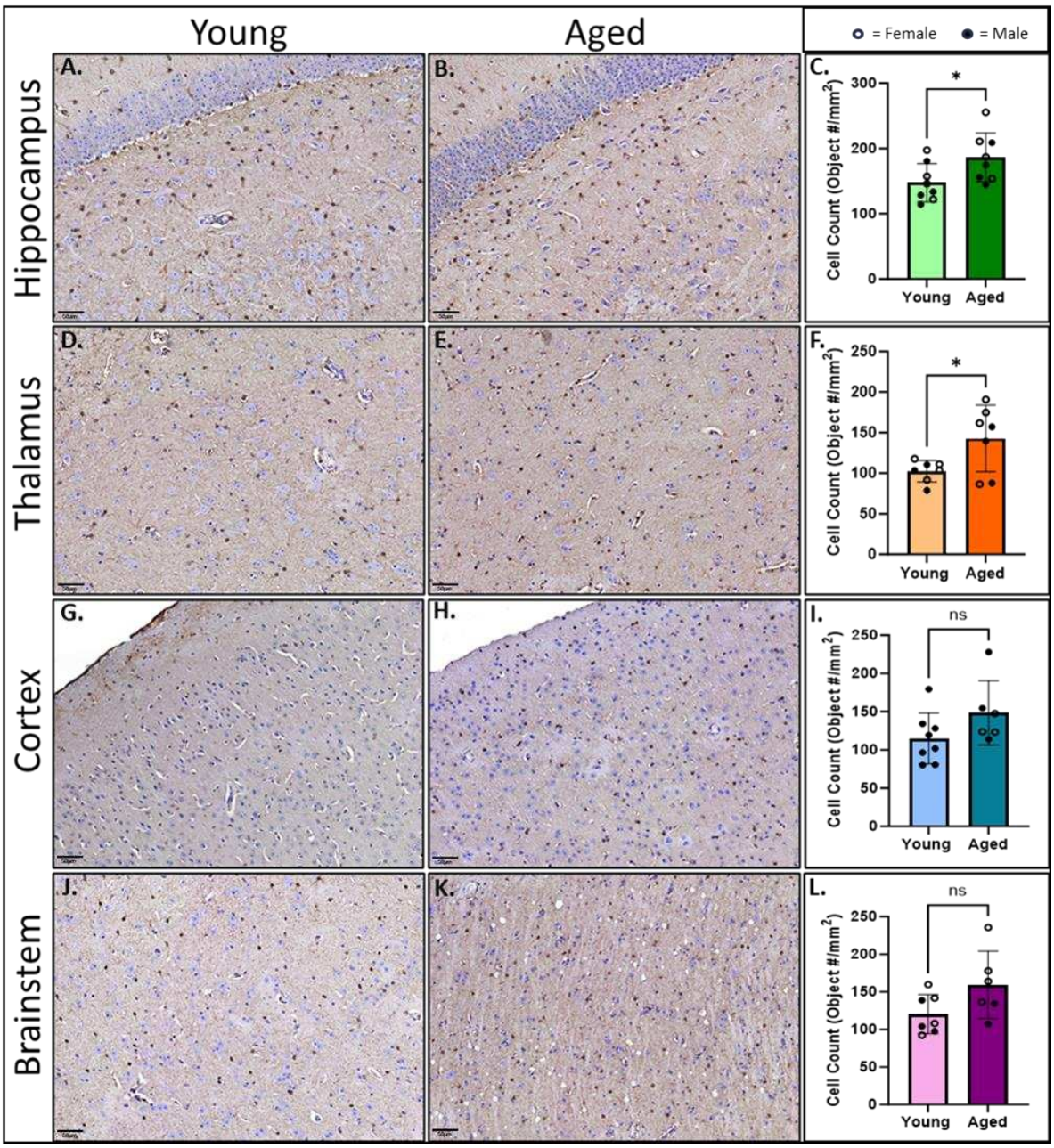

Figure 4. Age associated change of S100β protein marker in all four brain regions. The aged hippocampus (panels A-C; p=0.03*) and thalamus (panels D-F; p=0.04*) displayed significantly higher S100β expression compared to the young HGPs. Increased cell counts in the cortex (panels G-I; p=0.14) and brainstem (panels J-L; p=0.09) are evident but do not reach statistical significance.

Images acquired at 20x magnification, scale bar =50µm.

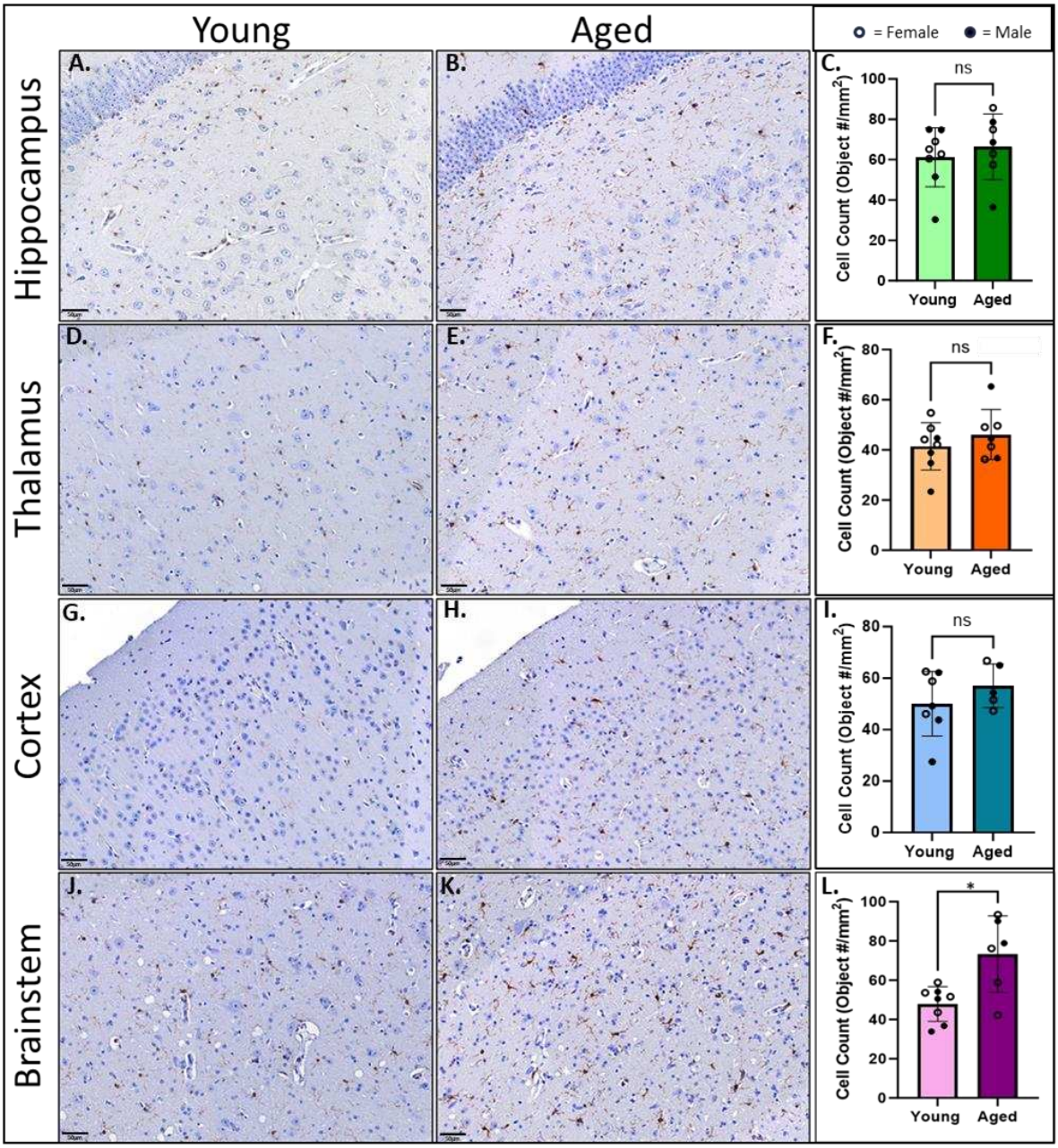

Figure 5. Significantly greater Iba-1 protein marker in the aged HGP brainstem. The hippocampus (panels A-C; p=0.52), thalamus (panels D-F; p=0.37), and cortex (panels G-I; p=0.22) show greater cell counts in the aged HGPs, however, do not reach statistical significance. Aged HGPs had significantly higher positive cell counts in the brainstem than young HGPs (panels J-L; p=0.02*).
Images acquired at 20x magnification, scale bar =50µm.

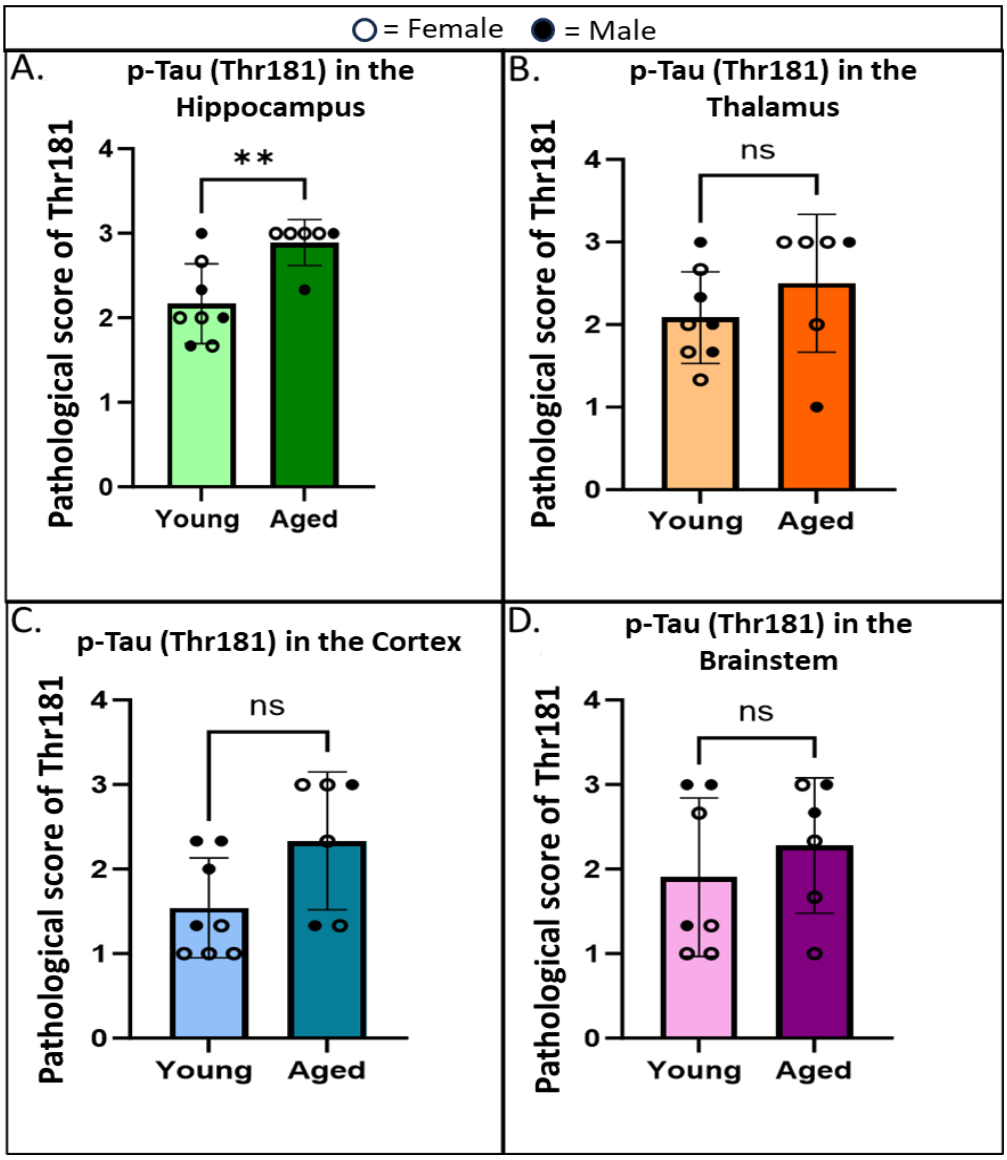

Figure 6. Tau fibril protein marker significantly greater in the HGP hippocampus. Pathological scoring for Thr-181(AT270) showed more severe pathology in aged HGP (A) hippocampus (p=0.003**). Apparent age associated change in the (B) thalamus (p=0.32), (C) cortex (p=0.07), and (D) brainstem (p=0.45) did not reach significance.

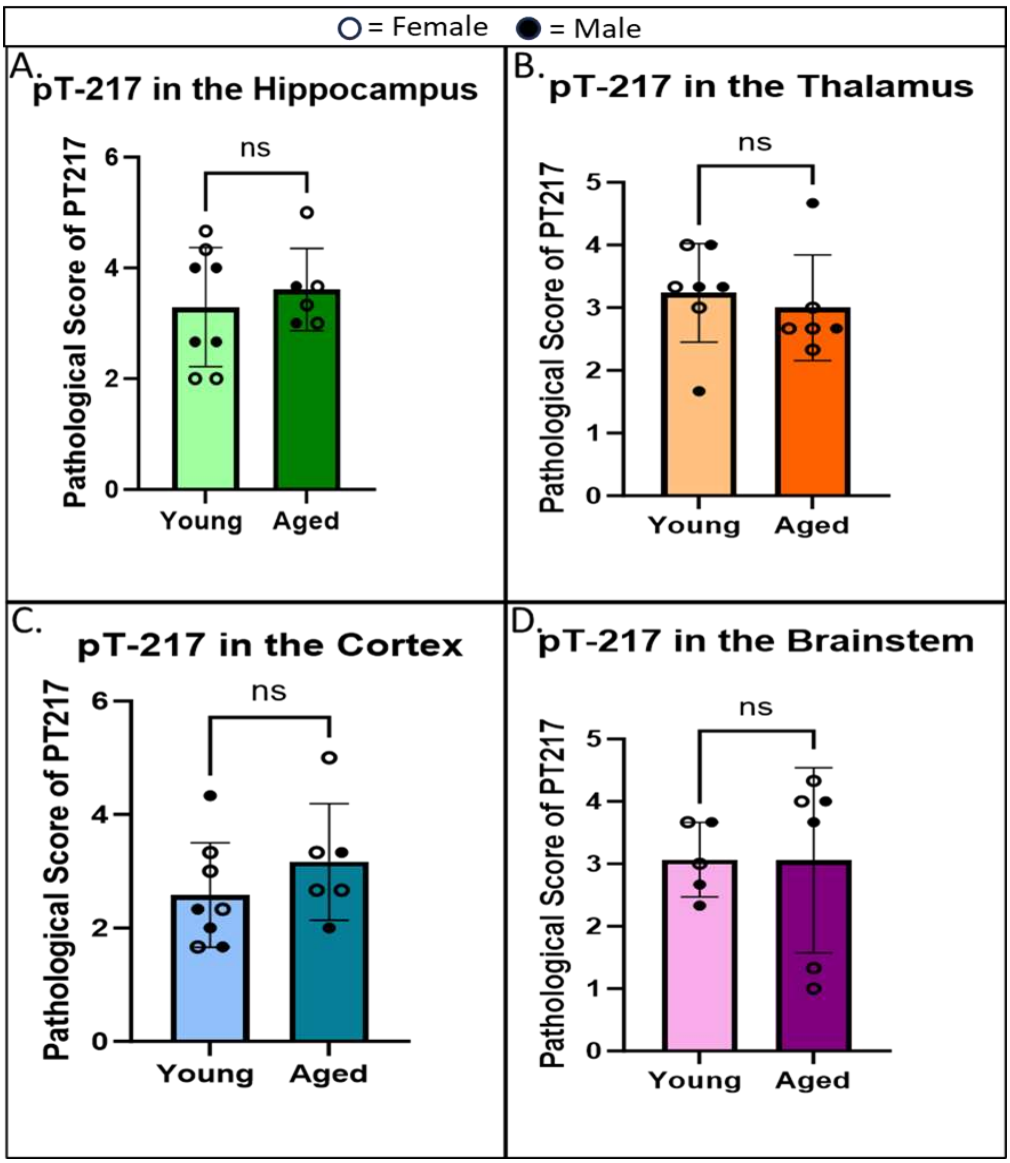

Figure 7. No evidence of age-related change in p-Tau 217 protein expression. Pathological scoring results for cytosolic p-Tau 217 protein did not show evidence of age-related changes in the (A) hippocampus (p=0.52), (B) thalamus (p=0.61), (C) cortex (p=0.29) or (D) brainstem (p=0.98). Both the young and aged HGP groups displayed wide standard deviations, which indicates a wide range of pathological variability.

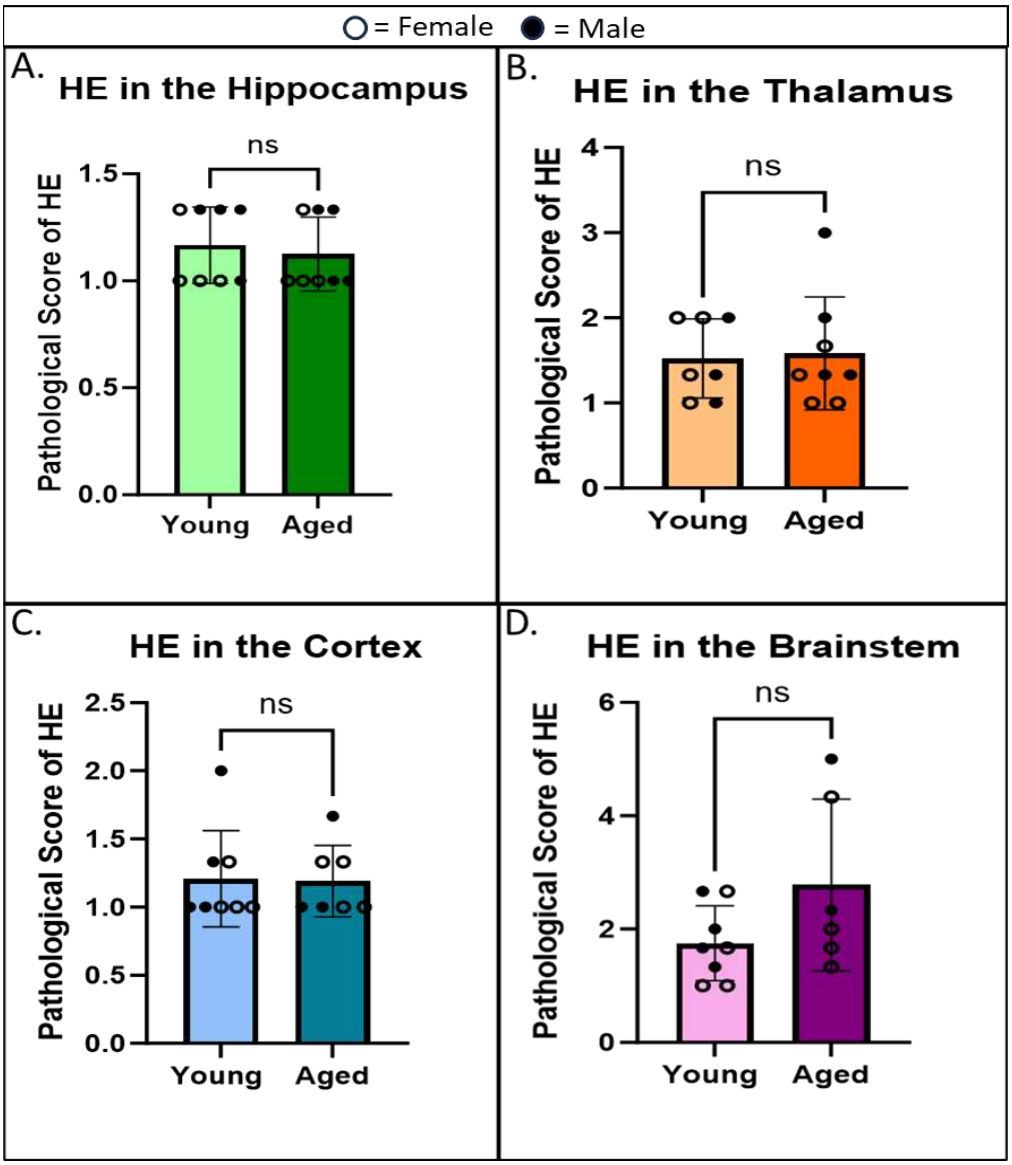

Figure 8. H&E staining reveals age-related change in spongiosis in the brainstem. No apparent relationship between age and spongiosis in the (A) hippocampus (p=0.16), (B) thalamus (p=0.84), or (C) cortex (p=0.91). Aged HGP brainstems (D) show evidence of age-related spongiotic pathology, however it did not reach statistical significance (p=0.16).

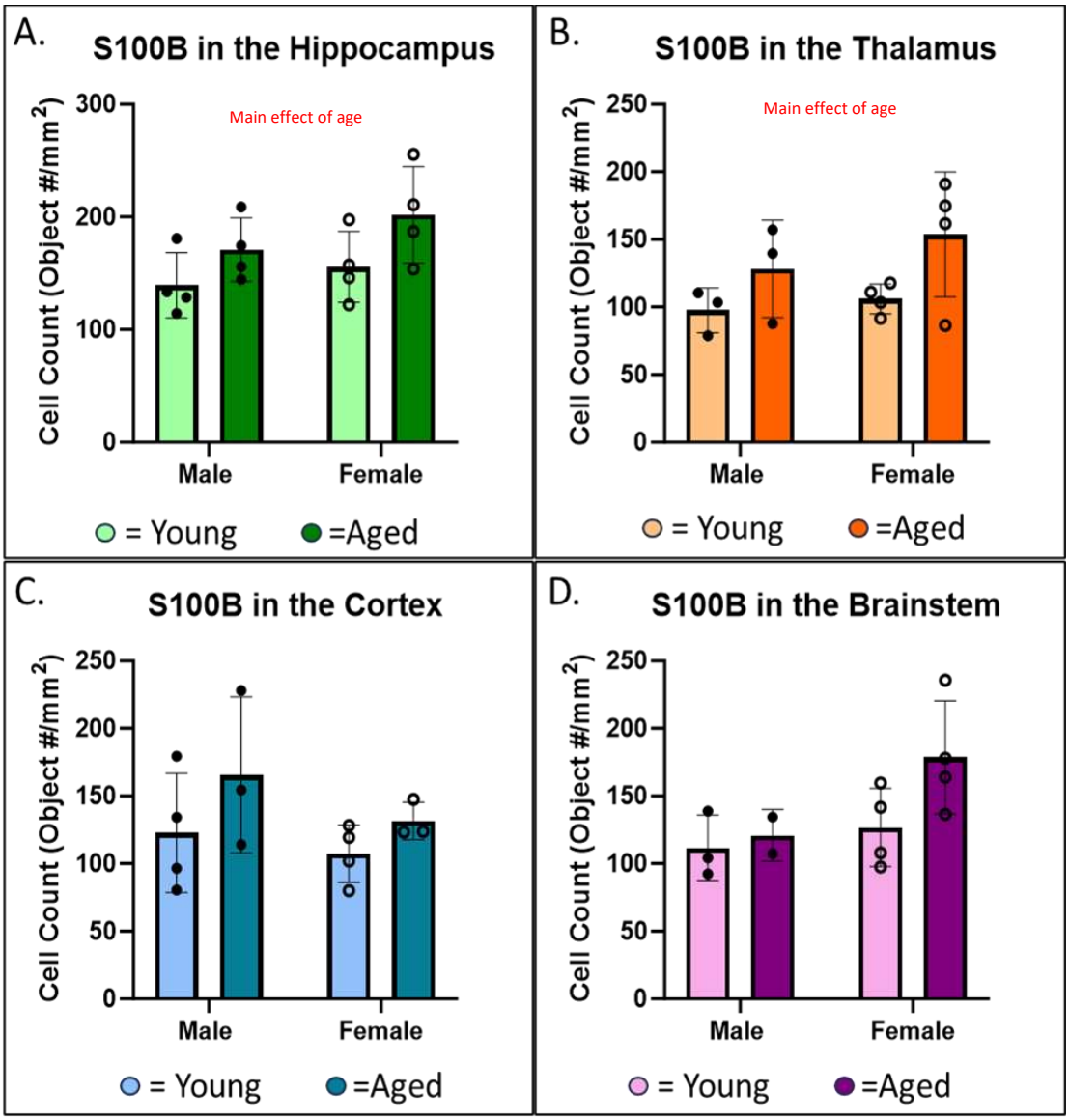

Figure 9. **No evidence of sex associated change in S100β protein expression.** The hippocampus and thalamus had a main effect of age (p=0.03*, p=0.04*). Females appeared to influence age related change to a greater degree in the (A) hippocampus, (B) thalamus, and (D) brainstem; while male HGPs appeared to have a greater influence on age associated changes in the (C) cortex. Analyses did not identify a significant effect of sex or an interaction between sex and age.

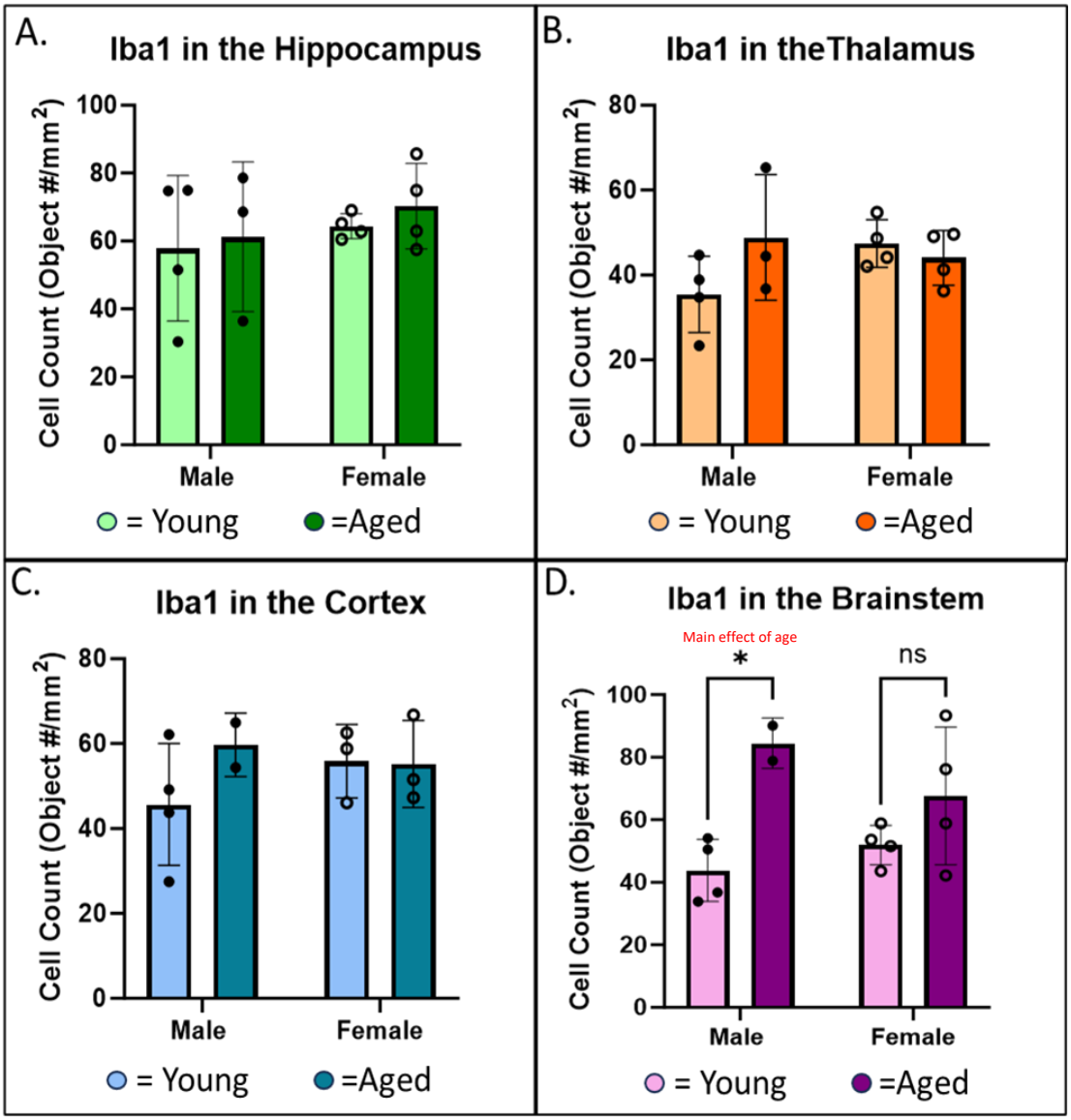

Figure 10. Assessment of sex and age relationship for Iba-1 protein is statistically increased in the brainstem of male guinea pigs. Aged males appeared to contribute to greater microglia counts in the (B) thalamus and (C) cortex. A significant overall effect of age in the (D) brainstem (p=0.004**) which is driven by aged male HGPs (p=0.014).

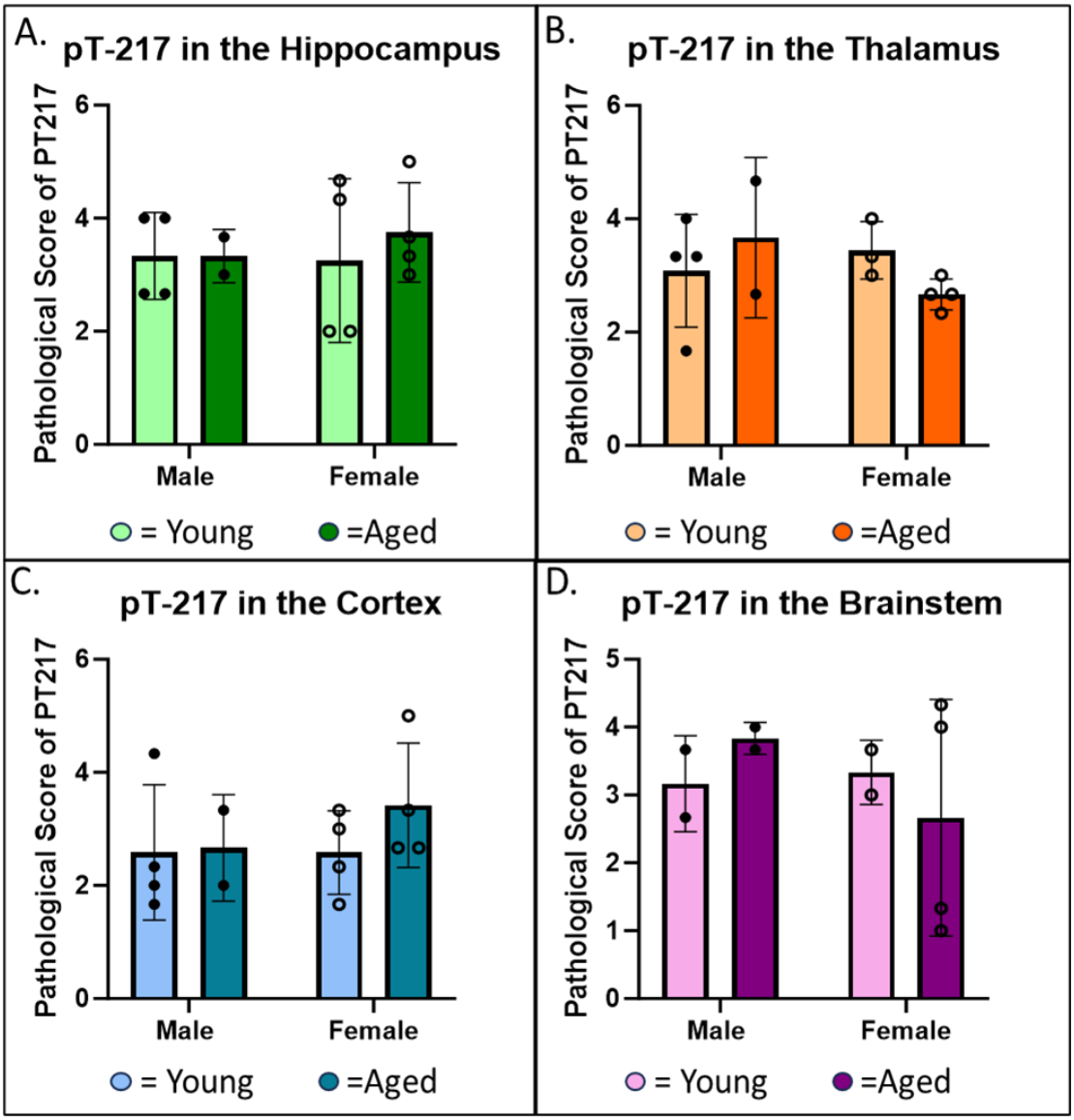

Figure 11. No apparent effect or interaction between age and sex on p-Tau 217 expression. Large variability and moderate to high pathological score data could imply p-Tau 217 accumulated before 5mo in the HGPs.

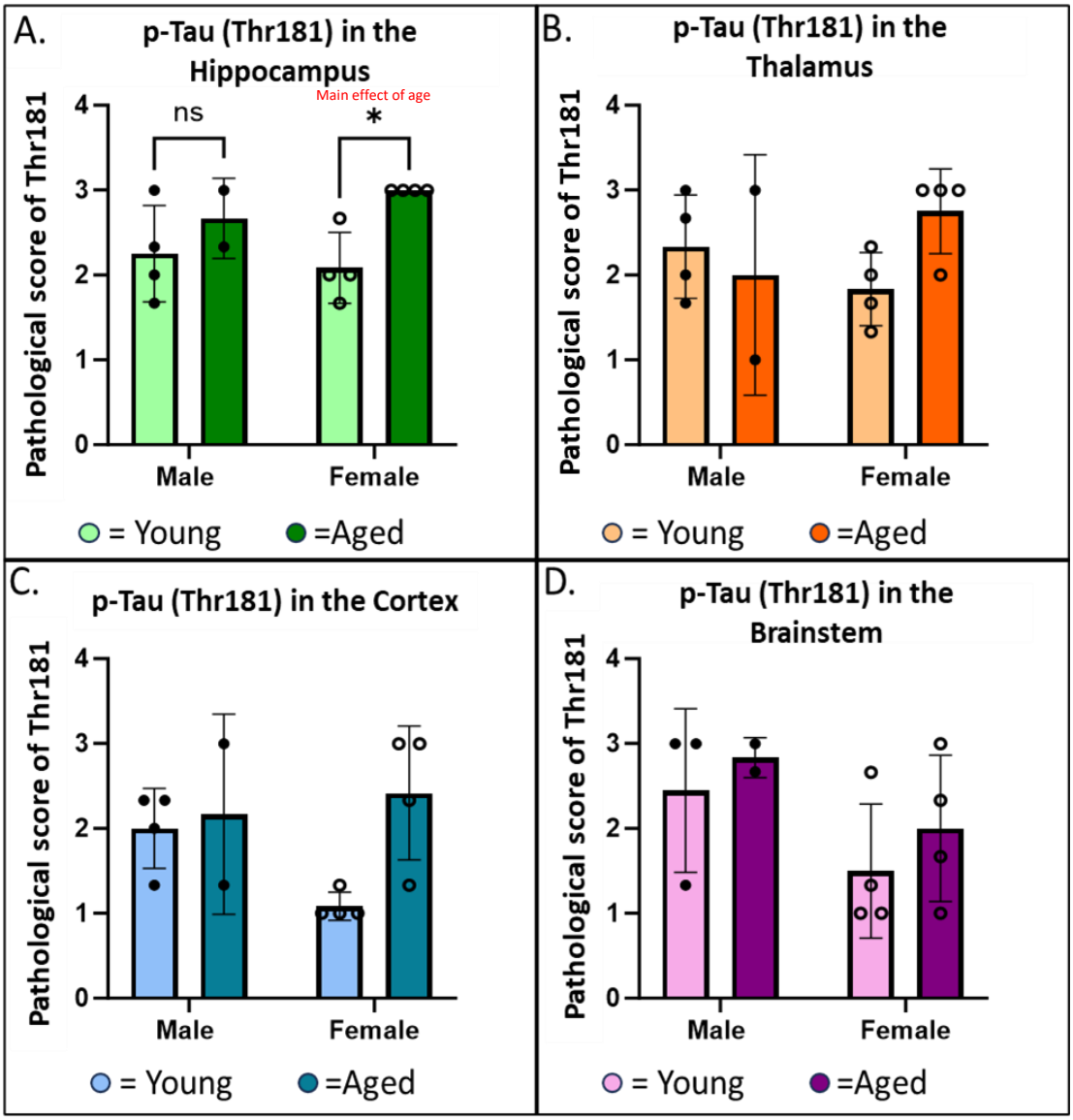

Figure 12. Aged female HGPs significantly contribute to age-associated p-Tau fibril (Thr181) expression in the hippocampus. p-Tau (Thr181) in the (A) hippocampus had a main effect of age (p=0.01**), with female HGPs significantly driving this relationship (p=0.02*). Females also appeared to contribute to an age associated increase of Thr181 in the (B) thalamus, and (C) cortex. In the (D) brainstem, males exhibited a higher pathological score, however, females had a greater degree of change between the young and aged HGPs.

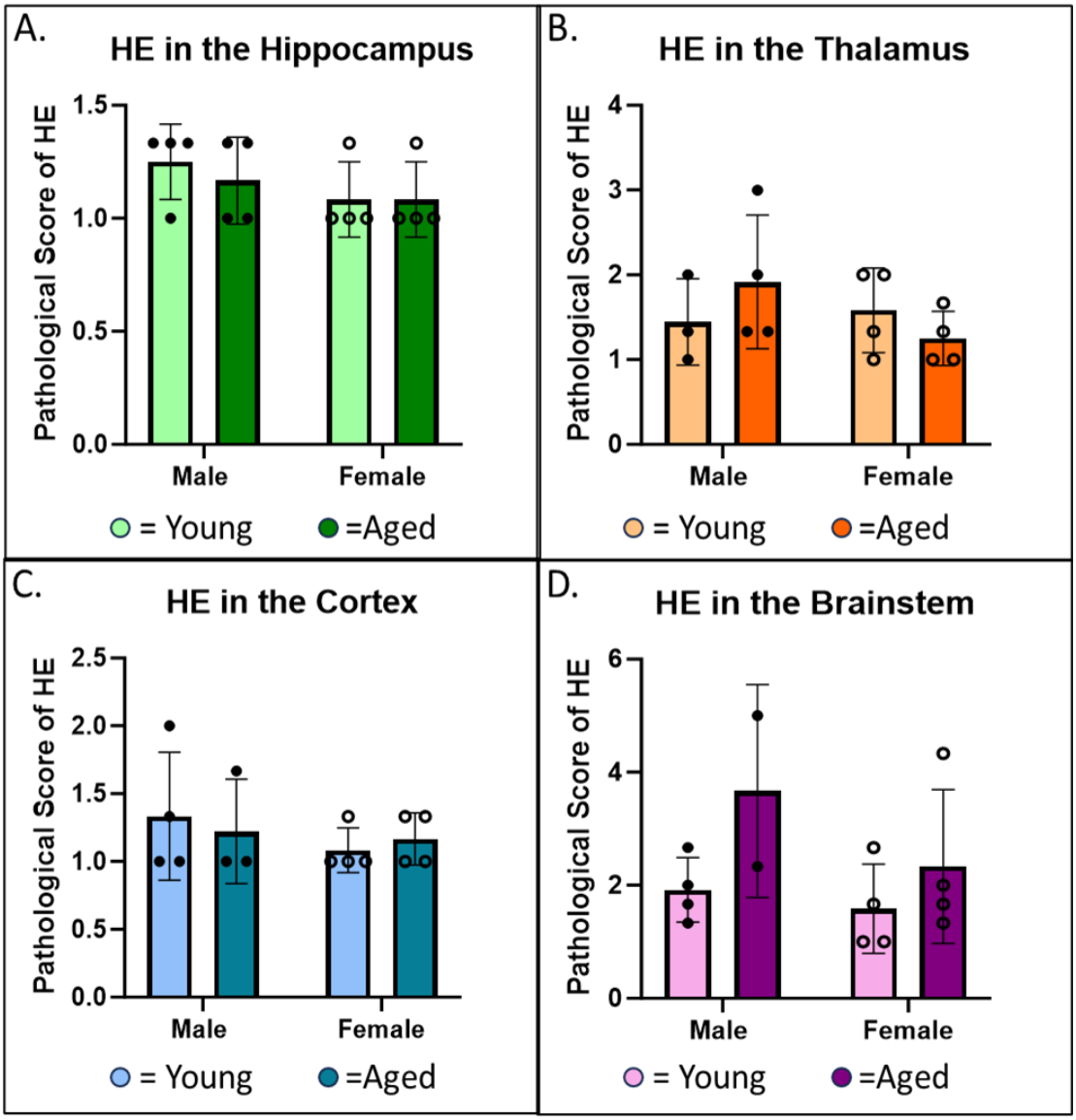

Figure 13. Age-associated increase of spongiosis in the brainstem is driven by male HGPs. No other age or sex related trends were apparent.

CHAPTER V – DISCUSSION

SUMMARY OF FINDINGS

The objective of this study was to characterize the neurodegenerative phenotype of the Hartley Guinea Pigs (HGPs) across four brain regions implicated in age-related neurodegenerative disease. The primary findings show evidence of whole-brain, age-related neuroinflammation and accumulation of p-Tau fibrils. Further, aged HGPs display a greater degree of variability when compared to young HGPs, and early evidence suggests sex differences may also be a feature of the HGP neurodegenerative phenotype. These findings mimic characteristics of human cognitive diseases like Alzheimer's disease (AD) and AD-related diseases (ADRDs) and highlight the potential for the HGP as a spontaneous and progressive model for neurodegenerative decline.

These data support our hypothesis that HGPs naturally develop an age-related increase in markers of neuroinflammation and protein dyshomeostasis. Though further assessment is needed to fully elucidate if the HGPs mimic a specific AD/ADRD phenotype, these findings demonstrate a meaningful and spontaneous age-associated phenotype that is not present in common rodent models. Cumulatively, these data contribute to the overall characterization of the HGPs brain aging phenotype and support the HGP as a potentially useful preclinical model of whole-body *and* brain aging.

NEUROINFLAMMATION

Chronic inflammation (CI) is an established hallmark of aging [53]. Though it is classified as an integrative third-tier hallmark, it is not clear if CI is a cause or effect of aging. It seems that

CI participates in the aging process at multiple levels in a circular mechanism, both initiating and responding to age-related changes [81]. Regardless of the directionality of the relationship, CI is a core feature of the aging phenotype, and directly influences the overall progression of age-related diseases [131]. Understanding the role of CI in the aging process may help identify novel therapeutic targets.

Concurrent peripheral and central CI are known features of AD/ADRD disease progression [103, 132]. Evidence suggests a relationship between the two systems exists, however, a specific cellular signaling mechanism of 'cross-talk' is not known [107]. Utilization of a preclinical model that expresses a similar inflammaging phenotype to humans, could help identify the role of CI in neurodegeneration. The HGP experiences a human-like pro-inflammatory phenotype in peripheral systems, including joint degeneration, metabolic dysfunction of skeletal muscle, and mobility impairments [16-18]. Given this phenotype, and the hypothesized relationship between CI in the peripheral and central systems, we aimed to assess if an age-related neuroinflammatory phenotype exists in the HGP.

To do this we quantified common neuroinflammatory protein markers. We compared the activated astrocyte marker S100β [133] , and the activated microglia marker Iba-1 [134] in young and aged HGPs. Our results found a greater number of positive cells in both neuroinflammatory proteins in all four brain regions in the aged HGPs. These results are the first to demonstrate a whole-brain, age-related neuroinflammatory phenotype in the HGP.

Follow-up studies should aim to identify the progression of neuroinflammation throughout the HGP lifespan. A longitudinal study design utilizing plasma biomarkers for peripheral [135] and central [136] inflammation would effectively map the progression of CI in

each system. Establishing the biomarker kinetics of peripheral and central CI would identify how CI progresses throughout the whole body, supporting the hypothesis that neurodegeneration and AD/ADRD are multi-systems diseases [9]. These findings may also support the use of the HGP as an effective preclinical model to study mechanisms of crosstalk between the central and peripheral systems.

In summary, aged HGPs express greater evidence of neuroinflammation than young HGPs. These findings support the use of the HGP as a model of neuroinflammation and prompt further study into the phenotypic progression throughout the lifespan. Utilization of a preclinical model that naturally develops the inflammaging phenotype could expand our understanding of CI in aging, and potentially identify novel therapeutic AD/ADRD targets.

PROTEIN MISFOLDING AND NEURON LOSS

Increased expression of p-Tau protein is a common pathological change in patients with AD/ADRD. Tau is a broadly incorporated microtubule-associated protein that promotes the formation, stabilization, and growth of axons [137, 138]. In a healthy brain, tau protein is spliced into 6 unique isoforms. Each isoform contains a unique microtubule-binding domain, which is composed of phosphorylation sites that determine where and how tau is incorporated into neuronal microtubules [139]. Under pathological conditions, the microtubule binding domain on each isoform is hyperphosphorylated. Hyperphosphorylation reduces the binding capacity of tau, ultimately leading to neuronal and axonal instability and accumulation of non-functional tau aggregates [140].

Our present study looked at two hyperphosphorylated tau isoforms, p-Tau 217 and Thr-181, both of which are strongly associated with AD pathology. In IHC experiments, p-Tau 217 is

expressed in the cytosol of the neuron, while Thr-181 is associated with tau fibril accumulation located primarily in the axon. We hypothesized that both tau proteins would have a greater pathological score in the aged brains when compared to the young. Our data, however, did not fully support this. We found a greater pathological score of p-Tau fibrils (Thr-181) in all four aged brain regions, while cytosolic p-Tau 217 did not show any age-related change. Importantly, cytosolic p-Tau 217 scores did not show an absence of pathology in the young HGPs as we hypothesized. Instead, the scores indicated that young HGPs already express an advanced tau pathology at 5mo, indicating they may already be experiencing age related neuropathology.

This explanation is supported by the HGPs developmental timeline. At 5mo HGPs are still maturing and growing, however, they begin experiencing age-related musculoskeletal decline as early as 3mo [17, 18]. Given the hypothesized relationship between musculoskeletal and neurodegenerative decline in humans, 5mo may have been too late to fully capture a significant age-related change. Further, studies comparing p-Tau 217 and Thr-181 in cerebrospinal fluid and plasma, indicate p-Tau 217 may be more sensitive to early tau accumulation and detection [120, 141].

To further assess this hypothesis, an IHC assessment of 2mo or 3mo HGPs for both tau proteins could establish a more accurate timepoint to assess age-related change. Alternatively, a longitudinal study assessing the blood plasma concentration of p-Tau 217 and Thr-181 would demonstrate the pathological progression of each protein throughout HGP development and lifespan.

SEX DIFFERENCES

Though females have historically been considered at higher risk for developing AD/ADRD than males, limited research has been conducted to assess this claim [142]. This oversight is surprising given the known differences in male and female brain anatomy [143], and some epidemiological evidence that supports the claim [144]. Recently, however, there has been a push to include sex and gender variables in research studies – stimulated in part by the demand for precision medicine, and in part by an NIH policy change that formally imposed this expectation.

Women have a greater life-time risk of developing AD than men [145]. This statistic is largely informed by a greater life expectancy among females, combined with advanced age being the primary risk factor of AD. When survivorship bias is controlled, in the U.S. diagnosis of AD/ADRD past the age of 85 is equal between males and females [146]. Interestingly, in other countries such as Europe [147] and Asia [148], similar studies have shown females are at an increased risk throughout their lifespan. These contradictory findings that appear to be contingent of geographical location, imply that both sex and gender may influence AD/ADRD diagnosis.

Sex is related to biological and physiological differences between males and females, as informed by genetic and chromosomal differences [149]. Sex-specific differences that could influence AD/ADRD diagnosis are factors that uniquely effect one sex. For example, pregnancy and menopause exclusively effect females, while prostate cancer exclusively effects males. Alternatively, gender describes personal, social, and cultural beliefs surrounding sex [149]. Gender differences include AD risk factors like lower education level, or caregiver status;

neither of which are unique to a specific sex but have historically been associated with women [150, 151]. Gender specific factors are an important consideration for public health scientists, as they are typically informed by social and cultural beliefs and are thereby more likely to experience variability throughout a person's life. These differences are unique to the human experience, and therefore cannot be accurately captured by a pre-clinical model. Sex differences, however, are related to underlying physiological interactions between sex-specific features and 'universal' disease pathology. Given their mechanistic and ubiquitous nature, sex differences can be studied using pre-clinical animal models.

Early evidence from this study, suggests the HGP may exhibit sex differences. Though our sample size had limited statistical power, our results show apparent differences in protein expression between the male and female groups. Future studies should continue to include equal populations of male and female HGPs, to fully establish if sex differences are present. If so, the HGP could help further our understanding of how sex-specific differences may interact with and contribute to the pathophysiological progress of AD/ADRD.

BIOLOGICAL VARIABILITY

Variability within each HGP age group is proposed to be reflective of the biological variability between each guinea pig. Unlike other models, the HGPs are non-transgenic – meaning they are genetically dissimilar. As seen in our data, variability was greater in the aged HGPs when compared to the young HGPs. This aspect of the HGP is a strength of the model. Humans are genetically variable, and even seemingly well-defined AD/ADRD pathologies progress at different rates and to different degrees in different people [152]. With age, variations in our genome and proteome interact with our environment to influence our

capacity to adapt to stress. In humans, therefore, we'd expect to see greater variability among protein expression with age – which is reflected throughout our data set. Utilization of a preclinical model that mimics heterogeneity is essential to the production of effective diagnostics, preventative treatments and pharmaceutical interventions.

Though biological variability is an important aspect of human health, it is often overlooked in common pre-clinical animal models. Most often, AD/ADRDs are modeled in transgenic mouse models. These genetically identical models are induced to express a specific AD/ADRD pathology. Though these models are beneficial to determine a specific biological mechanism, these studies do not translate well into human therapeutics. AD/ADRD is dynamic and likely involves many systems. By inducing a singular, one-dimensional pathology not only is selection bias inherently present, but the exploration and investigation of potentially novel, multi-dimensional mechanisms is limited.

NOVEL FINDINGS IN THE BRAINSTEM

This research identified increased evidence of spongiotic tissue in the aged HGP brainstem. These findings are novel and may indicate the presence of a specific ADRD pathology. One possible ADRD our results could point to is Lewey Body Dementia (LBD). LBD is the third most prevalent form of dementia, currently encompassing dementia with Lewy bodies and Parkinson's Disease [153]. LBD results in similar cognitive impairments to AD, as well as mobility and neuromuscular deficits. Pathologically, LBD is characterized by the accumulation of Lewy bodies, which are neuronal cytoplasmic inclusion bodies composed of α-synuclein protein and ubiquitin in the brainstem [154]. Along with Lewy body accumulation, microvacuolation, and misfolded proteins p-Tau and Aβ are also known pathological features of LBD [155].

Microvacuolation is associated with cell death and is characterized by the presence of small vacuoles in brain tissue [156] which may present as spongiosis on H&E staining. Our data showed significantly greater spongiosis and phosphorylated-Tau fibrils (Thr181) in the brainstem of aged males, similar to the known pathology of LBD. Furthermore, LBD is known to disproportionately effect males [157], which is also reflected in our data.

Taken together, we can preliminarily speculate that the HGP may model pathologies characteristic of LBD. These data are not conclusive, however. Future analysis should include Aβ and α-synuclein protein staining to fully identify if this specific pathology exists in the HGP.

A MODEL OF AGING?

Comparative animal models have been utilized for research since 6th c. BCE. These early studies aimed to understand the gross anatomy, and function of the human body. As our understanding of the human body advanced, so did our use of animal models. By the 20th century, various animal models of disease had been widely incorporated into research practice to demonstrate biological significance [158]. During this time, research practices shifted away from observational and toward experimental studies. Transgenic animal models, specifically rodents, were developed to control genetic and biological variability, thereby improving experimental reproducibility. Creation of these modes improved our capacity to study specific mechanistic pathways *in vivo*, however, they do not fully recapitulate the progression of age-related disease in humans.

Human aging is complex. Multiple factors interact with one another to various degrees at various times, to ultimately drive human aging. Specific age-related diseases, therefore, result from dysregulation of these interacting systems. Despite the complexity of this process,

transgenic rodent models are still overwhelmingly favored by researchers [159]. These models do not naturally age similarly to humans and, when modified to express certain age-related phenotypes, tend to over emphasize one specific mechanistic pathway. This clear limitation of rodent models informs the substantial translational gap between pre-clinical and clinical pharmaceutical efficacy. In response to these shortcomings, some researchers have begun to shift back towards observational studies in non-transgenic [160]. This approach aims to re-introduce the lost genetic variability, and thereby more accurately mimic human disease progression.

The HGP may fit into this framework as a non-transgenic model of natural and spontaneous aging. Though further assessments are needed, shifting the focus away from transgenic models and toward models that naturally capture the diverse aging processes may be essential to the discovery of novel therapeutics and truly effective preventative strategies.

LIMITATIONS

The limitations present in this study are primarily methodological. One of the major barriers present was related to inconsistent brain region presence. Many of the mounted tissue samples did not include the brainstem or were sliced too laterally to include the thalamus. This limitation reduced our initial sample size (n=8 young/aged, 4=female/male), to an n=2 for some of the aged male staining data. Though this limitation reduced our statistical power, visual trends still allowed for meaningful data interpretation to compel further research into the HGP model.

Another related limitation is the lack of precision when identifying brain regions. For example, though the brainstem is one region; it is not homogeneous. Instead, it is composed of

functionally unique regions, which may be relevant for identifying specific pathology. This regional heterogeneity extends to each of the four regions assessed. Given the inconsistency of the level at which the tissue section was cut and mounted, this lack of regional and structural precision may have introduced excess variability.

Lastly, the present study did not contain a control organism. The PigmEnTed (PET) guinea pig strain has previously been used as a comparison strain, given it does not accumulate progressive aging pathology at the same rate as the HGP. Our pilot data utilized both the PET and HGP strain to compare young and aged cell counts for S-100β, Iba-1, Aβ, and Thr-181 protein markers. These results identified a more severe pathology for all markers in the HGP than the PET strain at both 5 and 15mo [22]. With these results in mind, use of the PET strain could have potentially helped contextualize our p-Tau 217 data. We would expect to see that at 5mo the PET strain would have significantly lower cytosolic tau protein expression than the HGP. This would support our hypothesis that the HGP is experiencing an advanced cytosolic tau pathology at a young age.

FUTURE DIRECTIONS AND CONCLUSIONS

Results from this study continue to establish the HGP as a preclinical model of brain aging. We've shown that various age-related markers of aging are naturally and progressively increased among aged HGPs. Stemming from these findings, future research could interrogate functional outcomes of the guinea pigs used in this study. Determining if a relationship exists between pathological severity and function would strengthen the use of the HGP as a model of aging, as opposed to isolated pathology. Future studies should also include a larger array of protein markers, such as Aβ or α-synuclein to elucidate if a specific aging pathology exists.

The HGP should be considered a useful model of preclinical brain and whole-body aging. Utilization of a progressive and non-transgenic model will help bridge the existing translational gap and promote the advancement of healthspan extending interventions.

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