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Effect of Reduced Neurogenesis on Microglial Activation

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Effect of Reduced Neurogenesis on Microglial Activation

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I. Introduction

Socio-Economic Impact of Cognitive Decline:

As the baby boomer generation reaches retirement, geriatric Americans are becoming a much larger portion of the United States' population. The 2018 U.S. Census estimated that 15.6% of the American population is over the age of sixty-five. This number translates to over 51 million people in the United States over sixty-five years old (U.S. Census Bureau, 2018). As this group ages, they will experience degradations of various health aspects; one of which is a decline in cognitive function. Cognitive function is a term encompassing a person's capacity for learning, memory, and other nervous system-driven tasks essential for normal, functional living (Albert, 1997). When cognitive function begins to decline, activities of daily living (ADLs) become increasingly difficult if not impossible. It is this incapability to perform ADLs that often results in a dementia diagnosis.

Cognitive decline is exceedingly prevalent in the aging population. Cognitive decline will present itself in much of the elderly population as a reduced ability to learn or to remember (Albert, 1997). In the United States, 1 in 9 people over the age of sixty-five report confusion or memory loss (Taylor, 2018). Often, cognitive decline is the result of a neurodegenerative disease (Deuschi, 2009). As a neurodegenerative disease progresses, patients lose the ability to care for themselves. They cannot feed or clothe themselves, and they can even become a hazard to themselves. Their families are often left with the difficult task of finding/providing around-the-clock care for their loved one. For many, the solution could be a nursing facility, in-home care, or a move into a relative's home. Those who do not have family to take care of them are left to rely upon Medicare for their end-of-life needs. Those needs can become quite costly with the

various conditions and injuries that tend to accompany dementia. With Medicare being constantly used as a political weapon, its existence is indefinite. In 2010, it was estimated that each adult with dementia accumulated $56,290 in care-related costs annually. Dementia is one of the most expensive pathologies with the total cost for care in America reaching $109 billion in 2010; $7 billion more than the national costs related to heart disease in that same year (Hurd, 2013). The economic, sociological, and individual burden of dementia would be alleviated by treatments for the above-mentioned cognitive decline.

None of the treatment options available for cognitive decline have been entirely successful. This failure could be due to incomplete information regarding the aging brain. It is impossible to completely understand cognitive decline if the normal aging process itself is not yet entirely understood. To execute applied research aimed at alleviating dementia symptoms, basic research on aging and the brain must first be done to help comprehend the cause of those symptoms.

Effects of Aging on Hippocampus:

The hippocampus is the brain region most involved in memory formation, retention, and recollection (Scoville, 1957). Memory loss is one of the key symptoms of cognitive decline, and since memory is the main function of the hippocampus, the hippocampus is an essential brain region to study in order to understand cognitive decline (Wimmer, 2012). Even in the absence of cognitive decline, the hippocampus undergoes structural and functional changes as aging progresses.

The aging hippocampus has been shown to experience structural changes which negatively impact function. For example, shrinkage of the hippocampus has been linked

with memory loss (Erikson, 2011; Schuff, 1998) with the shrinkage being even more drastic under dementia-like conditions (Perrson, 2006). Structural synaptic abnormalities in the hippocampus have been associated with increased severity of cognitive decline. Specifically, a reduced amount of the protein synaptophysin in hippocampal neurons, is linked with worsened abilities to learn or remember (Sze, 1997). In normal aging, dendritic changes in the hippocampus are conducive to plasticity; however, in those afflicted with cognitive decline, dendritic proliferation decreases, indicating a reduction in neuroplasticity (Flood, 1990). The structural and biochemical changes of the hippocampus experienced during aging engender alterations in hippocampal functionality, and therefore cognitive function.

Synaptic transmissions are reduced as aging progresses, resulting in less neuronal connectivity, possibly contributing to lower abilities of information processing (Vanguilder, 2010). The rate of cognitive decline is directly proportional to the amount of hippocampal activation in adults with mild cognitive impairment (Miller, 2008). Hippocampal activity implies action potentials and the release of neurotransmitters. Neurotransmitters are the molecules of communication released into the synaptic cleft following an action potential. Acetylcholine is a neurotransmitter involved in memory. Age-related deficiencies of the cholinergic system within the hippocampus is a known contributor to cognitive decline (Gallagher, 1995; Pyapali, 1996). A reduction of protein synthesis in the aging hippocampus has deleterious effects on memory and long-term potentiation (Mullany, 1997; Pang, 2004). Particularly, subunits of the NMDA receptor, an ionotropic glutamate receptor involved in memory function, are diminished in aging (Eckles-Smith, 2000).

Aside from synaptic abnormalities, aging can cause changes to the general structure of the hippocampus. As aging progresses, the production of novel neurons decreases (Apple, 2017). While the exact function of new neurons in the adult brain is unclear, they are implicated to be involved in learning, memory, and plasticity. Since learning and memory are reduced in unsuccessful aging, new neurons are a promising avenue to encourage successful aging, and to combat neurodegenerative diseases such as Alzheimer's Disease (Jessberger, 2008; Barnes, 2011).

Alzheimer's Disease:

Although Alzheimer's disease (AD) is not the focus of this study, the extensive research on AD provides potential mechanisms to study in relation to aging in general. Being the most common form of dementia, Alzheimer's Disease affects over five million people in the United States (Mayeux, 2010). Lapses in memory, reported by the patient or the caregiver, are often the primary reason a person with early Alzheimer's first seeks the advice of a physician (Carr, 2000). Not surprisingly, the hippocampus is the brain area first affected by Alzheimer's. Hippocampal atrophy occurs before symptoms of Alzheimer's are even expressed (Fox, 1996). Specifically, the CA1 area of the hippocampus atrophies prior to the presentation of symptoms (Apostolova, 2010). The CA1 subregion is involved in contextual memory retrieval; therefore, the atrophy experienced is likely a large contributor to the memory deficits seen in Alzheimer's patients (Ji, 2008).

Another early event in the progression of AD is neuroinflammation. In AD, neuroinflammation is prompted by the degradation of neurons in the hippocampus, and will subsequently promote further hippocampal atrophy (Heneka, 2007). The cyclic

nature of inflammatory effects makes inflammation one of the most detrimental mechanisms of AD. It is arguably the largest contributor to AD pathogenesis (Akiyama, 2000). Anti-inflammatories such as NSAIDs have been tentatively shown to prevent the onset of Alzheimer's disease (McGreer, 1996). Attempts to directly target the cells involved in inflammation are underway, but none have proved entirely successful (Seo, 2018). Inflammatory changes happen even in normal aging of the hippocampus. As aging progresses, pro-inflammatory chemicals are increased while anti-inflammatory chemicals are decreased, resulting in an overall increase of inflammation which makes the brain more susceptible to neurodegenerative diseases, cognitive decline, and memory dysfunction (Silva, 2013).

As a result of the increased inflammation within the hippocampus during the progression of AD, neurogenesis decreases (Lazarov, 2010). Neurogenesis is a mechanism of neuroplasticity, so a decline in neurogenesis could explain some of the cognitive deficits seen in Alzheimer's Disease. Reversing this decline in neurogenesis has been examined as an avenue of treatment for AD. Increasing neurogenesis could potentially replace the neurons lost over the course of AD, or at least mitigate the effects of neuronal atrophy (Kunlin, 2004). Clinical trials for a neurosteroid, a drug used to promote neurogenesis, are proving hopeful in combating AD (Chen, 2018). Even in the absence of a neurodegenerative disease, promoting neurogenesis could help facilitate successful aging.

Neurogenesis:

It is widely understood that after the embryonic and early postnatal period, lost neurons will not be innately replaced. Yes, the brain will compensate for the death of a

neuron to retain function, but in the case of neurodegenerative diseases, injuries, and widespread atrophy, this compensation is often not adequate to maintain a normal level of function (Grade, 2017). Some areas of the brain have more compensatory mechanisms than others. The dentate gyrus, in particular has been extensively studied for its unique ability to produce new neurons throughout adulthood (Altman, 1965).

The dentate gyrus (DG) is the region within the hippocampus believed to be the first location on the path to episodic memory within the hippocampus (Amaral, 2008). The DG receives information from the entorhinal cortex, and sends the received information to the CA3 area of the hippocampus for further processing. The structure of the dentate gyrus is important to recognize when considering its functions. The DG is made of three layers. The molecular layer holds the extensions from the entorhinal cortex as well as the dendrites of granule cells. The polymorphic layer houses mossy fibers, unmyelinated axons of granule cells. The largest layer is the granule cell layer. As its name suggests, the granule cell layer is made of the cell bodies of granule cells and is the area of interest when considering neurogenesis (Amaral, 2008).

It is the granule cells of the dentate gyrus that can be created throughout the lifetime. The process of neurogenesis occurs in the subgranular zone of the dentate gyrus (Altman, 1965). It is in the subgranular cells that precursor cells are found. The precursor cells develop to form granular neurons to occupy the granular zone. GFAP+ astrocytes are involved in precursor cell proliferation and differentiation (Ming, 2005). Astrocytes may even be the precursor cells found in the subgranular zone (Doestch, 1999; Seri, 2001). Once the precursor cell is formed from the astrocyte, the cell's fate must be decided. Glial cells are cells that support the neuronal function of the brain, and

gliogenesis occurs throughout the nervous system for the duration of a mammal's life. A precursor cell can either become a glial cell or a neuron. When exposed to bone morphogenic protein, the precursor cell will become a glial cell (Campanellia, 2008). Since the astrocytes in the subgranular zone secrete neurogenesin-1, a bone morphogenic protein antagonist, the precursor cells will become neurons, not glial cells (Ueki, 2003). Once the precursor cell, becomes a neuroblast, it migrates to the granular zone where it continues to mature.

As neurogenesis is a naturally-occurring process, it must have functional relevance. Determining that functional relevance has been the aim of neuroscientists around the world for the past fifty years. Several hypotheses have permeated the literature. The most popular of these is the idea that the new neurons contribute to learning and memory (Ming, 2005). New neurons certainly contribute to neuroplasticity, and plasticity is a mechanism of learning and memory, so this theory is well-founded.

Some clues as to the function of neurogenesis lie in the mechanisms that influence it. Exercise and learning can increase neuronal production (Fabel, 2009). Aging reduces the number of novel granule cells in the hippocampus (Apple, 2017). In older adults, neurogenesis has been found to be reduced by up to 80% of that experienced in younger adults (Riddle, 2007). This reduction of neurogenesis could contribute to the cognitive impairment that older individuals experience more often than their younger counterparts. This, and the fact that neurogenesis could be used to reverse neurodegeneration, makes it an essential mechanism to study.

Studying neurogenesis is not a simple task. Studying neurogenesis in humans is difficult, because often immunocytochemistry and Western Blot are the techniques used

to determine the amount and location of neurogenesis. Both techniques require tissue to be removed from the organism in a manner that preserves the protein composition of cells. Inevitably, the organism must be sacrificed for the purpose of science, something completely unethical to do to a human. Additionally, animal models have been created that can manipulate neurogenesis, or the factors that influence it, to better determine causation without extensive confounding variables. Directly manipulating the brain of a human in this manner is unethical.

TK Model:

Amazing advancements in medicine and neurological knowledge have been made over the past century. Much of this progress was made using rats. Rats were the very first mammal used for scientific research beginning in the 1850s (Jacob, 1999). Using rats to model humans in research provides several advantages. Rats have shorter lifespans than humans, so research projects with rats are much more efficient. Additionally, rats are genetically similar to humans, allowing for human conditions to be modelled in rats. For example, a strain of rats has been bred to exhibit an Alzheimer's-like pathology (Vandamme, 2014). Using rats enables scientists to make structural or chemical manipulations that would be unethical to make in the brain of a human. Since the brains of rats have many of the same processes and connections as human brains, the results of a rat experiment can be postulated to be true in the human brain. Thus, through studying the rat brain, the mechanisms of the human brain become more clear.

Rats are especially useful in studying neurogenesis, given that up to 9,000 new neurons can be created in the adult rat hippocampus each (Cameron, 2001). Thus, a transgenic rat model designed to model an inhibition of neurogenesis has been created.

Inhibiting neurogenesis allows researchers to gain a better understanding about the function and impact of the new neurons on the extracellular environment. The model used to do this involves a transgenic rat with herpes simplex virus thymidine kinase (TK) in cells with glial fibrillary acid protein (GFAP+) (Snyder, 2016). GFAP is found in abundance in the progenitor cells that become the new neurons. Stopping the GFAP-expressing cells from dividing has been shown to stop the production of new neurons in the subgranular zone (Garcia, 2004). So, at eight weeks old, this transgenic model is treated with valganciclovir (VGCV). VGCV is an antiviral treatment that kills cells in the S-phase of mitosis if the cell expresses thymidine kinase. Since only the GFAP cells in this model have TK properties, only the GFAP progenitor cells are ablated. Thus, neurogenesis is halted following the VGCV treatment (Schloesser, 2009). While it is GFAP-expressing cells that are targeted, astrocytes remarkably remain unaffected by this treatment, making it a true model for stopping the differentiation of stem cells; or ablating neurogenesis.

In an analysis of the TK hippocampal tissue where neurogenesis was stopped at eight weeks, the hippocampus shrunk and dendritic losses were found - not unlike the changes that occur in aging (Schoenfeld, 2017). This indicates that halting neurogenesis could potentially hasten the aging process of the hippocampus. Therefore, there is validity in understanding the cellular mechanisms occurring in the TK rats as they age without neurogenesis.

Effects of Inflammation:

Inflammation is an immune response to a stressor such as injury, toxins, or infection. Inflammation occurs when immune cells swarm the afflicted area. The rapid

increase of immune-cell presence can “suffocate” neurons and lead to neuronal death. Inflammation is increased in the brains of aging individuals. The term “inflammaging” was coined to describe the chronic, low-grade inflammation found in the brains of aging individuals (Franceschi, 2007). One proposed cause for inflammaging is the fact that senescent cells release pro-inflammatory cytokines (Chinta, 2015). A cytokine is a signaling protein released by immune system-related cells to regulate inflammation or immunity.

The constant release of pro-inflammatory cytokines can lead to chronic inflammation. Chronic inflammation has some functional benefits, but it can also be detrimental to neurological health. Low-grade inflammation can induce the activity of immune cells in the brain to help fight infection (Deleidi, 2015). Alternatively, increased inflammation has been linked to depression (Loftis, 2008). This could correlate to the increased depression experienced by the aging population in contrast to younger groups of people (Fiske, 2003). Inflammation increases with aging, and this causes behavioral and cognitive changes. The regulatory bodies of inflammation in the brain are microglia, a type of glial cell. Glial cells are the supporting cells for the neurons. They facilitate brain health and neuronal function. Changes to microglial function could impact brain health and cognitive function in even normal aging, nevertheless abnormal aging.

Microglia:

Microglia are implicated in both cell generation and cell degeneration in the hippocampus through inflammation and phagocytosis. Microglia are a part of the brain’s immune system; they clean cellular debris, and regulate neuroinflammation (Rothwell, 1995). Microglia working as phagocytes have been linked with a reduction of synaptic

connection (Yoshiyama, 2007). To regulate inflammation, microglia can be activated to produce either pro-inflammatory cytokines or anti-inflammatory cytokines. For example, when activated by liposaccharides, they release pro-inflammatory cytokines such as interleukin-18 (IL-18) (Kyoungho, 2000; Rothwell, 1995). Expression of the genes responsible for creating inflammatory factors such as interleukin increase with age (Terao, 2002). If inflammation is higher in the aging brain, as it most often is, then it follows that microglia might be more activated as well.

Microglia and the regulatory tasks they typically accomplish are affected by aging. As the hippocampus gets older, the number of microglial cells is increased (Mouton, 2002); as is the density of microglia in the hippocampus (Gebara, 2013). The amount of debris in the hippocampus due to apoptosis, cell death, is greatly increased, and microglia are responsible for the clearing of this debris resulting in an increase of the microglial activation ratio (Cerbrai, 2012). Additionally, microglia were found to be more activated in the entorhinal cortex, the main pathway between the hippocampus and the neocortex, in humans with Alzheimer's disease (Cagnin, 2001; Hopperton, 2018). This increased microglial activation could be correlated with the increased inflammation also experienced by Alzheimer's disease patients and the aging population in general.

Microglia's Effect on Neurogenesis:

Microglia's inflammatory and phagocytic properties also affect neurogenesis. Microglia monitor the inflammation of the extracellular environment, and this regulation decides if new neurons will live. Microglia can reduce the number of new neurons through phagocytosis; the process of cellular eating. It has been found that although neuron production decreases with age, the proportion of new neurons microglia consume

remains the same (Sierra, 2010). So, despite reduced production, microglia do not allow for increased novel neuron survival. Additionally, microglia in the subgranular zone of the dentate gyrus that express presenillin variants (involved in AD) were found to negatively impact neurogenesis more than normal (Choi, 2008).

Alternatively, microglia have been found to promote neurogenesis under certain conditions. Living in an enriched environment is known to induce increased neurogenesis, and this increased neurogenesis is also accompanied by an increase in the total number of inactive microglia (Ziv, 2006). Extracellular environment has also been found to influence the effect microglia have on surrounding processes such as neurogenesis. Pro-inflammatory cytokine activated microglia have been found to reduce neurogenesis through inflammatory response (Ekdahl, 2003). Alternatively, microglia activated by anti-inflammatory cytokines were shown to promote neurogenesis (Butovsky, 2005). Microglia can either restrict or encourage neurogenesis dependent upon the activating agent. But does ongoing neurogenesis regulate microglial activity, thus assisting in slowing aging? This is just one of the countless uncertainties surrounding the relationship between microglia and neurogenesis.

Neurogenesis's Effect on Microglia:

The existing literature is very one-sided in that it focuses on the effect glial cells or proteins have on neurogenesis. The directionality of the relationship between glial cells and neurogenesis may seem inconsequential, but a huge gap in research exists when attempting to discern how neurogenesis affects the extracellular environment. This presents a problem when considering neural stem cells as a potential route of treatment for neurodegenerative diseases like Alzheimer's. Inflammation is a key part of

Alzheimer's disease, and since microglia are the regulators of neuroinflammation, it is crucial to understand how changes to neurogenesis affect microglial activation. Therefore, using the TK rat model, I inhibited neurogenesis for various durations to determine how losing the ability to produce new neurons may accelerate the aging process through changes of hippocampal volume and microglial activation.

Hypothesis:

I expect both aging and neurogenesis ablation to result in a reduction of hippocampal volume and an increase in microglial activation.

II. Materials and Methods

A) Materials

i) Genotypes and Age Groups

A transgenic Long-Evans rat model was used to determine how microglial activation and hippocampal volume are affected by neurogenesis and age. Rat brains were donated by Heather Cameron of the National Institute of Mental Health (NIMH). Some of these rats had been involved in experiments previously conducted at NIMH, but being in control groups, they underwent no experimental manipulation other than the genotype manipulation from which this experiment benefits. Dr. Cameron donated thirty-six brains in three different stages of maturity at the time of sacrifice. The nine rats in the young group were twelve weeks old, the eleven in the middle group were twenty-one weeks old, and the sixteen in the older group were thirty-two weeks old. Each age group contains two conditions. The experimental group is the transgenic rat with herpes simplex virus thymidine kinase (TK) in cells with glial fibrillary acid protein (GFAP+). The TK rats in each age group were treated with valganciclovir (VGCV) at 8 weeks old

to stop neurogenesis in the subgranular zone of the hippocampus (Snyder, 2016). Thus, the three age groups reflect pharmacological inhibition of adult neurogenesis for 4, 13, and 22 weeks, respectively. The control condition is the wild type (WT) rats. These rats underwent the same VGCV treatment as the TK rats; however, due to an inactive transgene, adult neurogenesis is unaffected in WT rats. Genotypes (WT vs. TK) were originally determined through RT-PCR of ear samples, performed and provided by Heather Cameron's lab at the NIMH.

Figure 1: Experimental groups

	WT (n)	TK (n)
12 weeks	5	4
21 weeks	5	5
30 weeks	8	8

ii) Tissue Retrieval and Staining Devices

All brain tissue was preserved though normative fixation procedures using 4% paraformaldehyde. After a long period of fixation, tissue was shipped to Belmont with a cryoprotectant, 20% sucrose, from which the tissue was processed for immunohistochemical staining and microscopy analysis. Once thoroughly cryoprotected, the brains were separated into two hemispheres; one of which remained in the cryoprotectant for use in later studies. The remaining hemisphere was cut into three coronal sections. The anterior and posterior sections were deposited back into the cryoprotectant. The middle piece containing the hippocampal area of interest was sliced using a sliding Microtome (American Optical). The 40 μm thick slices were systematically placed into a 12-well plate filled with phosphate buffer solution (PBS) to

obtain twelve representative samples of brain tissue. These samples were stored in the PBS-filled well plates until staining.

B) Methods

i) Verifying Genotype

Representative slices of the entire hippocampus from each brain were processed with a Doublecortin (DCX) antibody (Cell Signaling Technologies) to verify the genotype. DCX is an endogenous protein marker of immature neurons, so WT rats should have ambient DCX expression within the hippocampus, while TK rats should have complete inhibition of DCX expression. To begin, the tissue underwent three rinses in PBS. Once rinsed, the tissue was soaked in a blocking agent comprised of a dilution of Tween20 (ThermoFisher Scientific) in PBS (1:5) and normal donkey serum (Millipore Sigma). The tissue soaked in the blocking agent for twenty minutes at room temperature to improve the signal-to-noise ratio. It was immediately moved to the primary stain. This primary stain consisted of PBS and Rabbit anti-DCX primary antibody. The tissue soaked in the primary stain for five days at 4°C following which it was rinsed in PBS three more times. The tissue then soaked in the fluorescent secondary antibody solution (ThermoFisher Scientific) for one hour in the dark at room temperature. From this point on the tissue was kept in the dark as much as possible using aluminum foil wrapped around the well plates to maintain the integrity of the fluorescence. After an hour passed, the tissue was again rinsed by PBS before being immersed in a Hoescht stain (ThermoFisher Scientific) at a 1:1000 ratio with PBS for ten minutes. The process concluded with a final three rinses of PBS. The hippocampal tissue was then mounted

onto frosted microscope slides, cover slipped using glycerol (70%), and sealed using clear nail polish.

Doublecortin is a protein expressed in immature neurons, and the DCX stain illuminates immature neurons visible under a fluorescent microscope. The presence of new neurons in tissue either confirms that the rat was a WT rat, or refutes the validity of the TK rat. The DCX stain verified the genotype of each brain, and the Hoescht stain provided a background of cell-bodies. An epifluorescent BX-51 microscope (Olympus) was used for histological analysis of tissue stained with fluorescent antibodies. The DCX stain showed new neurons in the dentate gyrus formation as bright green, and any tissue with neurogenesis were recorded as being a WT rat. The TK rats treated with valganciclovir should not show any new neurons with the DCX stain. Presence of DCX staining in TK-genotyped rats can mean either an error in genotyping, drug treatment, or immunohistochemical reaction. To rule out the last option, staining was reperformed on any potential false positive tissue. Otherwise, TK tissue with positive DCX staining were to be thrown out from analysis.

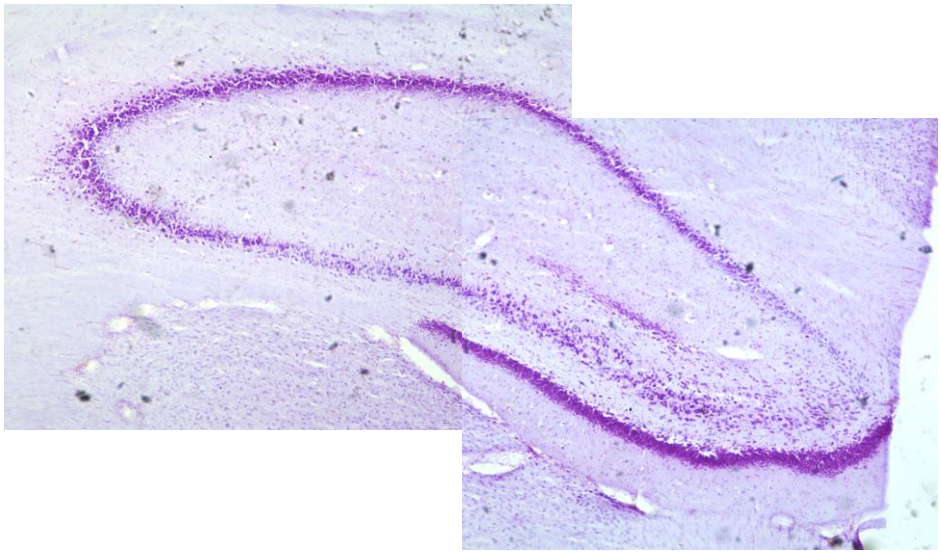
ii) Measuring Hippocampal Volume

A cresyl violet (Sigma) stain was used for hippocampal volume analysis. Ten hippocampal slices from one well containing a full representation of each brain were mounted on SupraFrost slides (ThermoFisher Scientific). These slides were left to dry overnight. Once dry, the slides were moved under a fume hood, placed in a vertical slide holder, and dipped in 250 mL of double-deionized water. The slide holder was soaked in 250mL of cresyl violet stain for approximately six minutes. Following incubation, the tissue underwent a series of increasing concentrations of ethanol; starting with 50% and

ending with 100% (with the 70% interval containing one drop of acetic acid). The slides were soaked in the clearing solution, Xylene. Once five minutes had passed, slides were cover-slipped using Permount and allowed to dry overnight before removal from the hood for analysis.

The cresyl violet stain can be viewed under a BX-45 light microscope (Olympus). Seven hippocampal images of each brain were captured at a 4x objective(figure 2). These images were then analyzed using the software ImageJ (NIH). This software allowed for the entirety of the hippocampal slices to be traced and calculated an area for each traced section. The hippocampal areas of the seven images from each brain were recalculated to be a volume based on the thickness of each slice (40 μm), and these values were averaged to obtain an estimated value for hippocampal volume of each brain which were then compared across genotypes and age groups.

Figure 2: Cresyl Violet-stained hippocampus



iii) Determining Microglial Activation

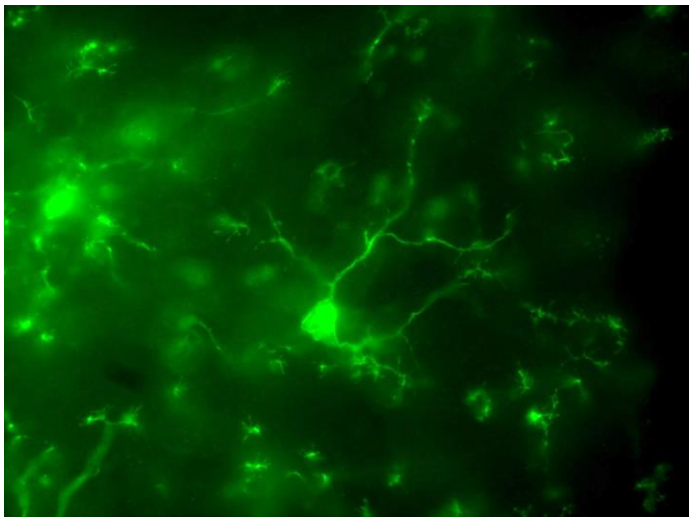
When microglia are activated, they become smaller and more amoeboid. Ionized calcium-binding adaptor molecule-1 (iba1) is a marker expressed by all microglia regardless of activation level (Figure 3). Staining iba1 molecules would allow for the morphology of the microglia to be analyzed. Iba1 staining is conducted through an immunofluorescent reaction, meaning that similar to the DCX reaction, a primary and secondary antibody was be used. In addition to the iba1 stain, a Hoescht stain was conducted on all tissue to provide a background and ease navigation during analysis.

To begin the staining process, representative sample from each brain was rinsed three times with PBS. The tissue was then placed in a blocking solution consisting of 25 μL 20% Tween-20 and 30 μL normal donkey serum for every 1mL of PBS. The tissue remained in this solution at room temperature for twenty minutes after which it was transferred directly into the primary stain. The primary stain is 1:500 rabbit anti-iba1 in blocking solution. The tissue remained in the primary stain overnight at 4°C. The tissue was rinsed in PBS three times before being placed in the secondary antibody solution which consisted of 5μL Donkey anti-rabbit Alexa 488 (Invitrogen) for every 1mL of PBS. Once in the secondary solution, the well plate was wrapped in aluminum foil to maintain the integrity of the fluorescence. After one hour of incubation in the secondary solution at room temperature, the tissue was rinsed in PBS three times, and Hoescht stained. The tissue was then mounted onto frosted slides, cover-slipped with glycerol, and sealed with clear nail polish.

The morphology of the microglia could then be measured by tracing the area of each microglia using ImageJ (NIH). Microglia shrink their processes and become

amoeboid in shape when activated, so reduction in microglia morphology is one indication of microglia activation. Additionally, when viewed from a lower objective, the density of microglia in the area surrounding the dentate gyrus can be measured. The amount of microglia in an area could be indicative of the amount of work they are conducting in that area.

Figure 3: Iba1 stain



iv) Statistical Analysis

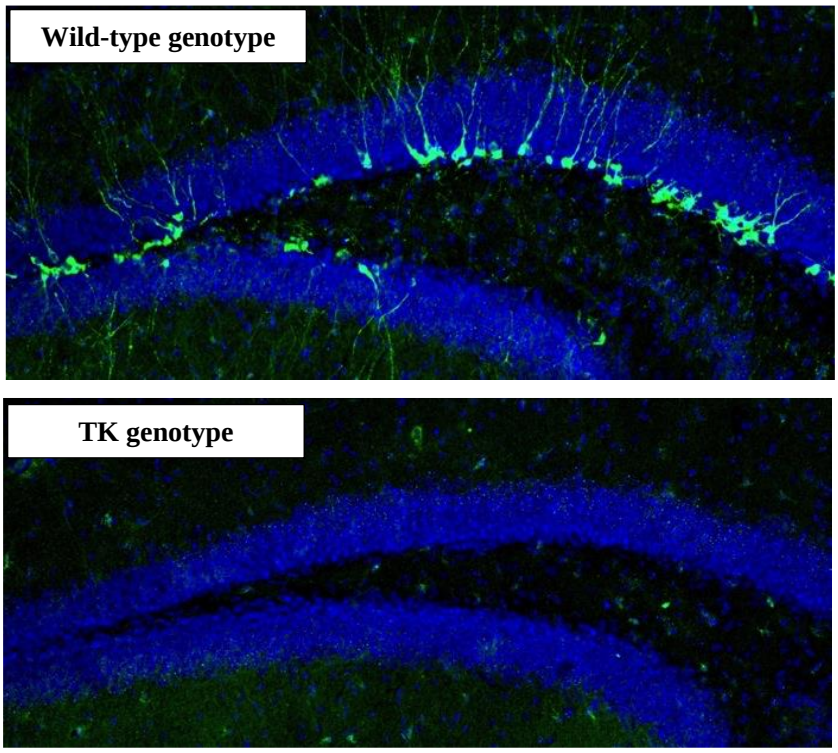
A 2x3 (TK/WT x Age) between-subjects ANOVA test was the statistic run for each dependent variable (microglia morphology/density and hippocampal volume) using SPSS. Each ANOVA test revealed the interaction between aging and reduced neurogenesis on the dependent variables. We expected to see the oldest TK group exhibit the most microglial activation within the dentate gyrus while also demonstrating the smallest hippocampal volume.

III. Results

Verifying Genotype:

Genotypes were verified using a blind analysis of the DCX stained tissue. The genotype of each brain was verified, and no exclusions were necessary. Figure 4 shows the visual contrast between a DCX-positive cell and a DCX-negative cell. The new neurons born in the WT group show up as a bright green. This makes the WT dentate gyrus easily distinguishable from the dull, TK dentate gyrus that has no bright staining other than the Hoescht cell-body staining used to show the structure of the DG.

Figure 4: TK vs. WT DCX stain



Hippocampal Volume:

In order to examine the effects of neurogenesis ablation and age on hippocampal volume, hippocampal volume from all six treatment groups was obtained using ImageJ and analyzed with a 2x3 (genotype x age) between-subjects ANOVA. There was no main effect of genotype ($F_{1,29} = .77$, $p = .39$), but a significant main effect of age ($F_{2,29} =$

4.50, p = .02). Tukey's post-hoc tests showed that hippocampal volume was decreased in the oldest group, compared to the youngest group ($p < .05$) as shown in Figure 5. Lastly, there was no significant genotype x age interaction on hippocampal volume ($F_{2,29} = 1.51$, $p = .24$).

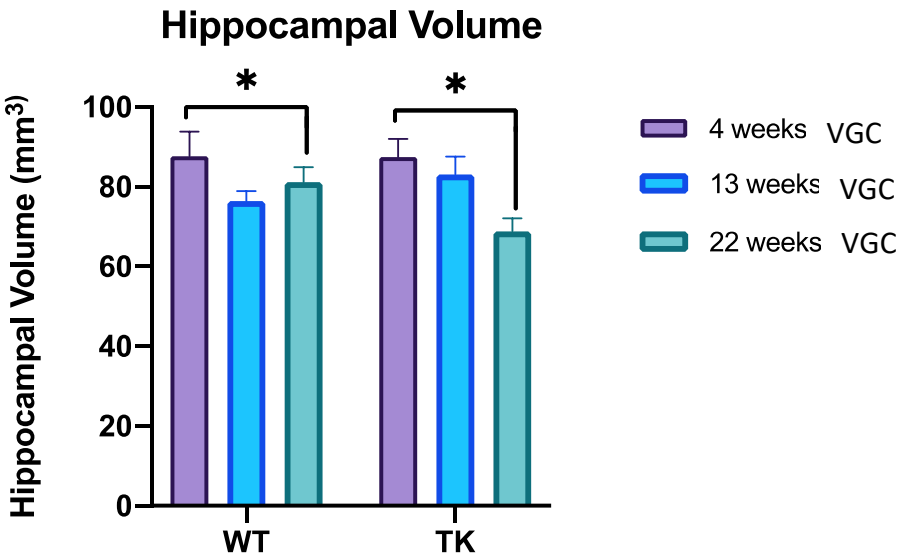
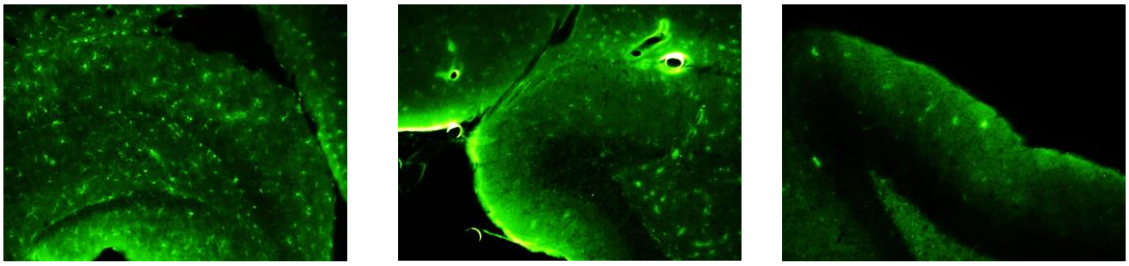


Figure 5: Effect of Age on Hippocampal Volume. Hippocampal volume was not affected by inhibiting neurogenesis, but age did have a significant effect between the oldest and youngest groups. Error bars represent S.E.M. * $p < .05$.

Determining Microglial Activation:

It was visually obvious that the microglial density was much higher in the younger rats than the older group even before statistical analyses took place. The images below demonstrate the disparity between the amount of microglia in each age group.

Figure 6: Microglial Density



4 Weeks VGC **13 Weeks VGC** **22 Weeks VGC**

To determine the effects of neurogenesis ablation and age on microglia density within the hippocampus, iba1-stained microglia from all six treatment groups were counted and compared across groups using a 2x3 (genotype x age) between-subjects ANOVA. There was no main effect of genotype ($F_{1,29} = .12$, $p = .73$), but a significant main effect of age ($F_{2,29} = 4.06$, $p = .03$). Tukey's post-hoc tests showed that microglial density was decreased in the oldest group, compared to the youngest group ($p < .05$) as shown in Figure 7. Lastly, there was no significant genotype x age interaction on microglial density ($F_{2,29} = .31$, $p = .74$).

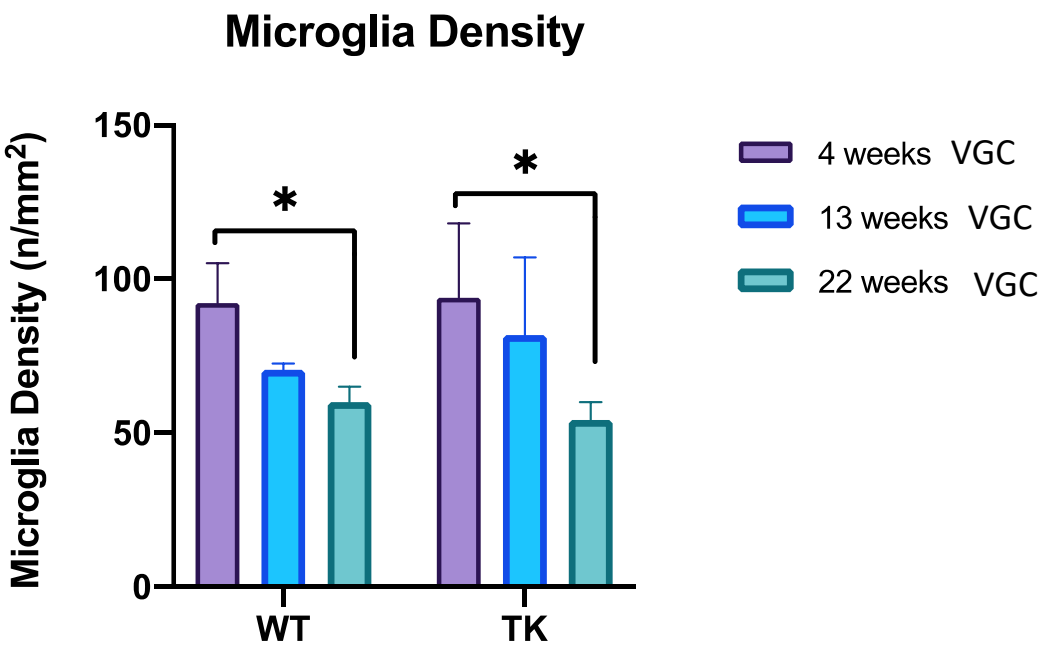


Figure 7: Effect of Age on Microglial Density: Microglial density was not affected by inhibiting neurogenesis, but age did have a significant effect between the oldest and youngest groups. Error bars represent S.E.M. * $p < .05$

In addition to influencing microglial density, aging also affected the area of the microglia found in the dentate gyrus. Changes in microglia morphology implicate

changes in microglial activation. Since activated microglia are smaller than quiescent microglia, smaller microglial area implies more microglial activation. To determine the effects of neurogenesis ablation and age on microglia activation, microglia areas from all six treatment groups were obtained using ImageJ and analyzed with a 2x3 (genotype x age) between-subjects ANOVA. There was no main effect of genotype ($F_{1,29} = 2.14$, $p = .15$), but a significant main effect of age ($F_{2,29} = 15.78$, $p < .001$). Tukey's post-hoc tests showed that microglia area was decreased in the oldest group, compared to the youngest group ($p < .01$), and the middle group compared the youngest group ($p < .001$) as shown in Figure 8. Lastly, there was no significant genotype x age interaction on microglia area/activation ($F_{2,29} = 2.40$, $p = .11$).

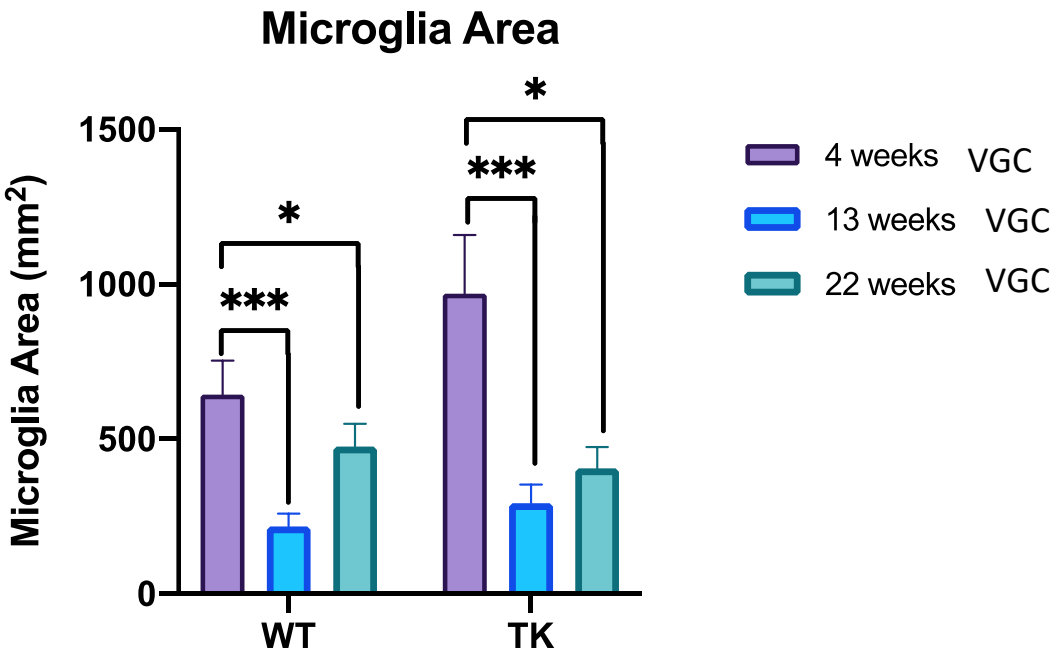


Figure 8: Effect of Age on Microglia Area: Microglia area was not affected by inhibiting neurogenesis, but age did have a significant effect between the oldest and the youngest groups as well as the middle and the youngest groups. Error bars represent S.E.M., * $p < .05$, *** $p < .001$.

IV. Discussion

The results of each measure indicate that stopping new neurons from growing has no significant effect on the brain's immune system in the dentate gyrus. Completely inhibiting neurogenesis had no effect on hippocampal volume or microglia activity/density. While neurogenesis did not correlate with any of the measures, aging negatively affected all three (hippocampal volume, microglial area, and microglial density). Despite the significant effects of aging, there was no interaction between neurogenesis loss and aging, meaning that the ablation of neurogenesis may not be an adequate model for aging.

As seen in figure 5, aging had a significant effect on hippocampal volume. The oldest brains of both genotypes had the smallest volumes while the youngest brains had the largest. Decline in hippocampal volume has been linked with the decline in spatial learning and memory often experienced during aging (Hamazeh, 2017). The exact cause of hippocampal shrinkage in late adulthood remains unclear.

One proposed mechanism for the reduction of hippocampal volume involves brain-derived neurotropic factor. Lower levels of brain-derived neurotropic factor (BDNF), a neuron growth and proliferation promoter, have been linked with the reduced hippocampal volume (Erickson, 2010). BDNF is essential for successful hippocampal neurogenesis (Rossi, 2006). Increasing BDNF levels increases the amount of new neurons (Scharfman, 2005). Reducing BDNF levels reduces differentiation of new neurons following neurogenesis (Taliaz, 2009). BDNF affects neurogenesis, and

neurogenesis maintains hippocampal volume; perhaps it is the reduction of BDNF that causes a reduction of neurogenesis that results in a smaller hippocampal volume.

Since neurogenesis did not affect hippocampal volume in this experiment, an alternative explanation for hippocampal shrinkage has been found. Microglia regulate inflammation within the brain. As hippocampal brain cells age, they release pro-inflammatory cytokines (Chinta, 2015). This causes an increase in microglia presence within the hippocampus as aging progresses (Gebara, 2013). More microglia in the hippocampus leads to more inflammation, causing cell death. Cell death causes more microglial activation due to the phagocytic nature of microglia (Cerbrai, 2012). The cycle of microglial upregulation causing neuronal death causing microglial upregulation is also a possible mechanism for the reduction of hippocampal volume in the aging brain. It is possible to end this cycle, at least in an animal model. The overreactive microglia population could be eliminated by inhibiting colony-stimulating factor 1 receptors, receptors necessary for microglial survival. The inhibition can then be removed, and the microglia allowed to repopulate. The new microglia do not show the aggressive behaviors of the old, and increases in neurogenesis, synaptic complexity, and hippocampal volume follow this treatment (Elmore, 2018).

It was expected that both microglia density and activation would increase as age increased (Gebara, 2013; Elmore, 2018; Kaneshwaran, 2019). Microglia density typically increases in areas experiencing inflammation or cell death such as the aging hippocampus (Pearson-Leary, 2017). Microglial activation, measured by the morphology of the microglia, typically increases with age as well (Mangold, 2017). Increases in microglial density and activation typically happen in conjunction (Appel, 2018; Gebara,

2013). The oldest rats did express the most microglial activation, as was expected, but they also had the least microglial density. While the cause of these results is unclear, two hypotheses as to why there might be fewer, but more activated microglia in the oldest age group, barring experimental error, emerged upon further examination of the literature.

Experimental error is the first thing to consider when attempting to explain the discrepancy in our results. The density of microglia in the area surrounding the dentate gyrus was significantly lower in the older age group indicating that despite the microglia being more activated in the older age group, there were less microglia present in the hippocampus. One explanation for this could have been that the 10x magnification was too wide to view the much smaller, activated microglia as opposed to the larger microglia of the younger rats.

Microglia in the hippocampus remain in a more immune-vigilant state than microglia in the rest of the nervous system (Grabert, 2016). Microglia are especially primed for an immune response in aging (Perry, 2013). Perhaps at the time of sacrifice, the microglia in the hippocampus of the aged rats had just begun an immune response, making them more activated, but not yet increased in number.

Microglia have a relatively long lifespan compared to other glial cells (~4 years), but a third of the brain's microglial population are still replenished each year (Reu, 2017). Interferon regulatory factor 8 (IRF-8) is protein essential for the development of microglia. In a mouse model with reduced IRF-8, microglial density was also reduced (Kierdorf, 2013; Yin, 2017). IRF-8 is found to be reduced with aging (Zhou, 2019). Since aging causes a reduction of IRF-8 and a reduction of IRF-8 causes a decrease in

microglial density, this could explain the small density found in the aged rats despite the greater activation.

One final hypothesis for the concurrent smaller microglia and smaller amount of microglia in the oldest age group could be microglial senescence. Microglia themselves experience functional and structural changes associated with senescence (Spittau, 2017). Most notably, microglia become dystrophic as they undergo senescence; meaning they lose the processes that greatly contribute to their area, making them appear more activated when in fact they are just senescing (Luo, 2010). Further analysis of the tissue is needed to confirm any of these hypotheses.

The effects of aging on microglial activation, density, and hippocampal volume were significant, but the ablation of neurogenesis did not have a significant effect on any measure regardless of duration. Looking at previous studies, the TK rats should have shown a smaller hippocampal volume than the WT rats (Schoenfeld, 2017). One potential confound of this study could have been where the volume was analyzed. Representative slices of the entire hippocampus were measured and considered in the overall volume. Neurogenesis only occurs in the dentate gyrus, so if neurogenesis is ablated it might have only caused a shrinkage of the dentate gyrus, and not the entire hippocampus. Thus, measuring the entire hippocampus instead of just the dentate gyrus may have diluted any dentate shrinkage caused by the prolonged ablation of neurogenesis.

The TK rats also did not show a change in microglial activation. It was hypothesized that reducing neurogenesis might increase microglial activation in the dentate gyrus because of the apoptosis of the proliferating neurons due to the VGC

treatment. However, no change to microglial activation was found, other than that linked to aging. In a previous study using the same transgenetic model, there was no TK effect on astrocytes (Snyder, 2016). This suggests that the ablation of neurogenesis may just have no effect on glial cells. Additionally, since microglia have both positive and negative regulatory effects on neurogenesis, these processes could be neutralizing, and completely removing neurogenesis would not change the overall "work" of the microglia (Morrens, 2011). Despite neurogenesis ablation not affecting microglial activation, reductions in neurogenesis, as seen during aging, have other implications.

Neurons born in the developed dentate gyrus are important for spatial problem solving, learning, pattern separation, and mood regulation (Seib, 2015; Snyder, 2016). Aging is linked with changes in hormones such as greater levels of glucocorticoids, stress hormones. Increasing glucocorticoids reduces neuronal birth in the dentate gyrus (Veena, 2011). The reduction of neurogenesis in aging leads to a reduction of the beneficial effects of neurogenesis: plasticity, learning, memory, and mood regulation. Neurogenesis was found to be reduced by up to 80% in aged rats (Jen, 2003). The largest implication of this reduced neurogenesis is cognitive decline. Excessive decline in neurogenesis is linked with increased risk for dementia (Mathews, 2017).

Microglial activation is also associated with an increased risk for dementia. Microglial activation is caused by neuronal death, blood-brain barrier disruption, or invading lymphocytes (Finch, 2002). Typically, microglial activation increases with age, as supported by the results of this experiment (Mouton, 2002). It is the changes that accompany senescence that causes microglia to go from neuroprotective in youthful individual to neurotoxic in older individuals (Luo, 2010). Specifically, the chronic

inflammation that occurs during aging results in an overactivation of microglia that negatively impacts learning and memory. The overactivation of microglial becomes a cycle wherein inflammation causes microglial activation which causes inflammation which causes more microglial activation, and further declines in learning and memory.

Learning and memory deficits are associated with both increased microglial activation and decreased neurogenesis during aging. These deficits can be reversed or prevented by targeting either mechanism. The microglial overactivation cycle could potentially be broken by consuming an antioxidant-rich diet (Wu, 2016). Microglial activation can also be reduced using herbal compounds such as resveratrol (grapes, peanuts, berries), trans-cinnamaldehyde (cinnamon), and curcumin. These natural remedies work mostly through anti-inflammatory methods to reduce microglia activation and decrease cognitive decline (Fu, 2018). Increasing neurogenesis, or preventing its reduction is an alternative method of staving off learning and memory deficits. Exercise has been shown to increase neurogenesis to near-normal levels in aged subjects (Fabel, 2009; Erikson, 2011). Exercise is useful to neurogenesis by increasing BDNF levels and blood flow to the neurogenic niche (Sieb, 2015). Exercise is currently the least invasive treatment with the most potential to reverse/prevent cognitive decline in humans. A more invasive option for reversing neurogenesis-related cognitive decline involves chemically targeting stress-induced changes to the hippocampal protein composition. This method has been shown to counteract neurogenesis reduction and the cognitive decline that this reduction induces (Seib, 2013). Cognitive decline is a huge problem associated with aging, but mechanisms designed to promote neurogenesis and reduce microglial activation have proved useful in mitigating the effects of aging.

V. Literature Review: Response to Senescence and Mortality

In the absence of events or illnesses leading to a premature abbreviation of life, senescence is inevitable for almost all organisms. Humans have the privilege of being the only organisms to be cognitively aware of their inevitable decline in function, fertility, and ultimate mortality. The response to such knowledge is different for each person or group with cultural values influencing a person's attitude towards impending death (Howard, 1965). Societal responses to mortality and senescence include stereotyping aging individuals, increasing research efforts, and adjusting life to accommodate those experiencing senescence.

Stereotyping

Age stereotyping is not only a poor system for understanding people of different demographics but can also have detrimental effects for those experiencing the stereotyping (Levy, 2009). In one study surveying people over sixty-five years old, 84% of the participants had experienced some form of ageism (McGuire, 2008). Holding age-related stereotypes contributes to a phenomenon referred to as the "Generation Gap," a divide between members of different generations resulting in decreased ability to exist cohesively within the same environment (Wood, 2005). Age stereotypes not only affect relationships between members of different generations, but also influence those targeted by the stereotype. When primed with a negative age-related stereotype, aging people perform worse on both memory and physical tasks (Hess, 2003; Emile, 2014). Self-stereotyping can be influential in the aging process. People who hold positive beliefs and attitudes about aging were found to live an average of 7.5 years longer than people with negative concepts regarding aging (Levy, 2000). Holding negative age stereotypes about

oneself can also impact a person's self-concept, an important aspect of mental wellness (Zhang,2017). Stereotypes are abundant throughout society, and they can greatly impact peoples' attitudes towards their own, and other's journey through senescence and mortality.

Ageism can be combatted through several different methods, but it will never fully be eradicated as humanity's underlying fear of death breeds prejudice against those closest to it. Intergenerational connections, group setting in which older generations interact with younger generations, were shown to decrease ageist attitudes (Grefe, 2011). Education is one of society's most powerful tools. Implementing curriculum designed to dispel myths about aging improved students' attitudes toward aging and older people (Gleberzon, 2002). Ageism is especially pronounced in the workplace, but implementing mentoring and awareness programs help to reduce corporate age discrimination (Gibson, 2010). Stereotypes surrounding aging will continue to prevail as will research aimed to combat these harmful stereotypes.

Researching

The endless cycle of aging and dying has undoubtedly led to endless research studies aimed at understanding, improving, and preventing senescence. Various health problems accompany aging with some being more serious than others. Even in "successful aging," aging undergone without concurrent disease, death is inevitable (Rowe, 1997). Thus, even "normal" aging is studied extensively by researchers around the world.

While aging undoubtedly impacts the entire body, its effect on the brain is perhaps the most devastating. In order to understand how aging directly influences the

brain's structure and function, researchers have turned to animal models. With animal models, researchers overcome ethical and technical restrictions posed by studying humans. With animals, researchers can directly manipulate the brain's structure, or genetically modify the organism to experience the disease or condition of interest. Manny different animals have been used in the endeavor to understand aging, with each species having its own benefits. Aging canines have neurological and psychological changes like that of humans including β-amyloid deposition and age-related cognitive dysfunction; however, they do not experience neurofibrillary tangles, making them useful only as models for early aging (Cummings, 1996). Mice models have proved endlessly useful in researching both normal and diseased aging. Often, it can be costly and time-consuming to wait for an animal model to reach an age appropriate for an aging study. To alleviate the cost and time burden, a mouse model that ages rapidly has been created. The Senescence-Accelerated Mouse strains (SAM) experience pathological and structural changes like that seen in humans, such as decline in learning in memory, but at a faster rate than typical mice (Hosokawa, 1999). Using rodents, researchers can do things such as induce DNA breakage or ablate neurogenesis to learn how these occurrences might contribute to aging effects in humans (Katyal, 2008; Schoenfeld, 2017).

In addition to using animal models to understand the aging brain, research has been done on both humans and animals aiming to prevent the effects of senescence from occurring. Cognition is a person's capability for learning, memory, and other nervous-system driven tasks essential for normative, functional living (Albert, 1997). Losing cognitive function is a prevalent fear amongst the aging population, thus preventing this loss from occurring is the goal for which many neuroscientists are striving. The exact

cause of the cognitive decline has proved difficult to pinpoint. Abnormalities in the neurotransmitter, serotonin (5-HT), have been studied as a potential cause for cognitive decline. Specifically, in the hippocampus, serotonin was found to have reduced postsynaptic effects in an aged model (Baskys, 1987). 5-HT uptake inhibitors were found to improve memory in aging subjects likely due to the prolonged presence of serotonin in the synaptic cleft, allowing for normal postsynaptic action to occur (McEntee, 1991). Additionally, cognitive decline has been associated with increased hippocampal activation. A mouse model with increased hippocampal activation was treated with levetiracetam, an antiepileptic drug to reduce overactivation. Following the treatment, the mice experienced a reversal in cognitive decline when treated early enough (Devi, 2013). Serotonin and levetiracetam are only two of the countless mechanisms/treatments being studied in hopes of preventing/reducing cognitive decline.

While pharmacological substances have proved useful in preventing senescence, a person's life-style can also play a role in their well-being as they age. Diets, regimens, and specific foods have been found to have anti-aging qualities. Dark chocolate, fish, vegetables, nuts, and garlic are among the foods being proclaimed as anti-aging largely due to their anti-oxidant and anti-inflammatory properties (Sabate, 2010; Corti, 2009; McAllister Pryade, 2011). Specifically, a Mediterranean diet, a diet containing foods high in monounsaturated fatty acids, has been linked with lower mortality, and less instances of neurodegenerative or cardiovascular disease (Chrysohoou, 2013). Restricting calories has shown to correlate with a longer life, delayed senescence, and reduced risk for chronic diseases. While restricting calorie intake can prove to be a

difficult regimen to implement, introducing aerobic activities can have the same beneficial effects (McCartney, 2012).

Research has undoubtedly proved successful since the human lifespan continues to rise, and longevity has become mankind's greatest achievement (Kirkwood, 2008). With the population living longer, the elderly population is growing larger and larger. A growing number of people out of the workforce with increased health problems has caused a need for social accommodations for these people.

Accommodating

Geriatric Americans are becoming a much larger portion of the United States' population. The 2018 U.S. Census estimated that 15.6% of the population is over the age of sixty-five. This number translates to over 51 million people in the United States over sixty-five years old (U.S. Census Bureau, 2018). Sixty-five years is the average age of retirement for people in America, and accommodations have had to be made to facilitate the housing and care of the growing elderly population. The most notable accommodation came from the United States government: Medicare.

In the 1950s and 60s, the newly formed health insurance companies were reluctant to insure people over sixty-five. In fact, in 1961 56% of people over sixty-five did not have health insurance. It was not until 1966 that Medicare health coverage was provided to beneficiaries over sixty-five years old. By 2019, 60.6 Medicare had 60.6 beneficiaries costing $705.9 billion. Medicare has undoubtedly helped millions of aging Americans in its fifty years of existence, but it is anticipated that the Medicare Part A trust fund will be depleted by 2026 (Medicare Resources, 2019). Following the

depletion, claims filed will have to be covered by payroll taxes, an amount insufficient to support the health needs of the growing elderly population.

The lack of funding for healthcare for the elderly is especially concerning since the average life expectancy is projected to exceed ninety years for women by 2030 (Kontis, 2017). With people expected to continue living older, their quality of life in their old age must be taken into consideration. Factors essential to a maintaining good quality of life in aging include: nutrition, relative involvement, minimal physical problems, love and attachment, continued learning, and attitude towards aging (Imanzadeh, 2018). Life is not quantifiable, but the quality of it seems to be so. Unfortunately, quality of life for the aging seems to be on a decline (Gurwitz, 2019). Efforts have been made to improve quality of life in both chronically ill and healthy individuals. Combining dance and relaxation therapy has shown to reduce depression and anxiety thereby improving the quality of life of elderly patients (Adam, 2016). Alternatively, pet therapy has proved hopeful in bettering the lives of the aging (Meretti,2010). Accommodating the growing aging population is a challenge, but with economic and therapeutic adaptions, aging can be made a more comfortable experience.

Each person's experience and attitude towards their own mortality is unique, but societal responses to senescence are widespread. Stigmas surrounding the elderly are prevalent, as are research endeavors to understand the aging process, and efforts aimed at improving it. Endless are the questions and uncertainties surrounding aging, and endless will be the endeavor to prevent and improve the process.

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