The Role of Nrf2 in Brain Cellular Senescence

by
Grace Ross

A THESIS

submitted to

Oregon State University

Honors College

in partial fulfillment of
the requirements for the
degree of

Honors Baccalaureate of Science in Biochemistry and Molecular Biology
(Honors Scholar)

Presented February 26, 2020

Commencement June 2020

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Grace Ross for the degree of Honors Baccalaureate of Science in Biochemistry and Molecular Biology presented on February 26, 2020. Title: The Role of Nrf2 in Brain Cellular Senescence.

Abstract approved:_____

Viviana Perez

Aging is a major risk factor for the deterioration of biochemical pathways that lead to many chronic neurodegenerative disorders (i.e. Parkinson's and Alzheimer's Disease). Cellular senescence is a hallmark of aging, a condition characterized by stable growth arrest resulting in a cell that no longer divides, but is metabolically active. A senescent cell leaks a mixture of proinflammatory cytokines and other factors, collectively called the senescence-associated secretory phenotype (SASP), that become toxic to neighboring cells. Removal of senescent cells has previously been shown to promote healthy tissue function and delay age-associated pathologies in mice. Nrf2 is a transcriptional factor involved in regulating the cell's response to stress. Mice without the gene for Nrf2 (Nrf2KO) show increased age-related diseases, including cognitive decline. Previous studies in our lab have shown that Nrf2KO mice have higher levels of cellular senescence in many tissues. This study addressed the hypothesis that an absence of Nrf2 will lead to an increase in cellular senescence in the hippocampus, and be a driver for age-associated cognitive decline and neurodegeneration in mice. Via SA-β-galactosidase staining, qPCR, Western Blotting and Immunohistochemistry, the levels of senescent cell markers (p16, p21, SA-β-gal, and SASP members) were experimentally determined to be higher in the hippocampus of Nrf2KO mice compared to WT. Treatment with Rapamycin (a mTOR inhibitor that inhibits cellular senescence) and Senolytics (drugs that selectively kill senescent cells) had a mild effect on levels of senescence in the Nrf2KO and WT mice. Behavioral tests demonstrated Nrf2KO mice had less cognitive flexibility, and that Rapamycin significantly increased the performance of both Nrf2 KO and WT groups. Our data suggests Nrf2 is a powerful influencer of cellular senescence in the hippocampus of mice, and Rapamycin and Senolytics may be promising therapies for cognitive decline by targeting cellular senescence.

Key Words: cellular senescence, Nrf2, aging

Corresponding e-mail address: rossgr@oregonstate.edu

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APPROVED:

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Viviana Perez), Mentor, representing Biochemistry and Biophysics

_____

Kathy Magnusson, Committee Member, representing Biomedical Sciences, College of Veterinary Medicine

_____

Kari Van Zee Committee Member, representing Biochemistry and Biophysics

_____

Toni Doolen, Dean, Oregon State University Honors College

I understand that my project will become part of the permanent collection of Oregon State University, Honors College. My signature below authorizes release of my project to any reader upon request.

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Grace Ross, Author

Introduction

Aging as a Risk Factor for Chronic Diseases

The U.S Census Bureau reported a startling conclusion that by 2035, individuals 65 years or older will outnumber the population under the age of 18, for the first time in history (Meinert, 2018). The impact of this demographic shift is undeniable, and the pressure is on for the scientific community to make quick headway in defining and slowing down the biological aging process. Researchers in the past have definitely been up for the challenge, and have succeeded in increasing longevity in invertebrate animal models (i.e. yeast, fruit fly and worms) and rodents using caloric restriction, genetic manipulation and small molecule drugs (McCay et al., 1935; Harrison et al., 2009, 2014; Kanfi et al., 2012). Unfortunately, the period of morbidity continues to match the increase of lifespan in humans, and aging remains the largest risk factor for the majority of chronic diseases. Among these are cardiovascular disease, diabetes, glaucoma and neurodegenerative disorders (i.e. dementia, Parkinson's Disease and Alzheimer's Disease).

It can be easy to take for granted that aging is a process which can be studied and manipulated much in the same way as any other biological process. However, it was a defining moment for the healthy aging field when scientists first accepted that aging was amenable to scientific study (Zainabadi, 2018). This realization came from recognizing divergent organisms show similar survival curves, with survival decreasing with time **(Fig 1)**. Additionally, variation in lifespan within a species was observed in honeybees and rodents. For example, when comparing similar body size rodents, the naked mole rat has an unprecedented lifespan of 30 years, while a house mouse only makes it to four (Lewis et al., 2012). Additionally, while genetically identical, the queen honey bee lives up to ten times longer than the workers (Winston 1987; Keller and Genoud 1997; Page and Peng 2001). This led researchers to believe that the aging process could be scientifically studied, and that model organisms would be fundamental in

investigating the human aging process (Kaeberlein et al., 2001). By decreasing food intake by a specified amount (30-40% below ad libitum for mice) researchers reported the first case of lifespan extension in rodents, a key discovery in the field, in 1935 (McCay et al., 1935). There have since been numerous studies in which caloric restriction has been successful in increasing longevity in a diverse group of organisms (Weindruch et al., 1986; Fernandes et al., 1976; Kubo et al., 1984).

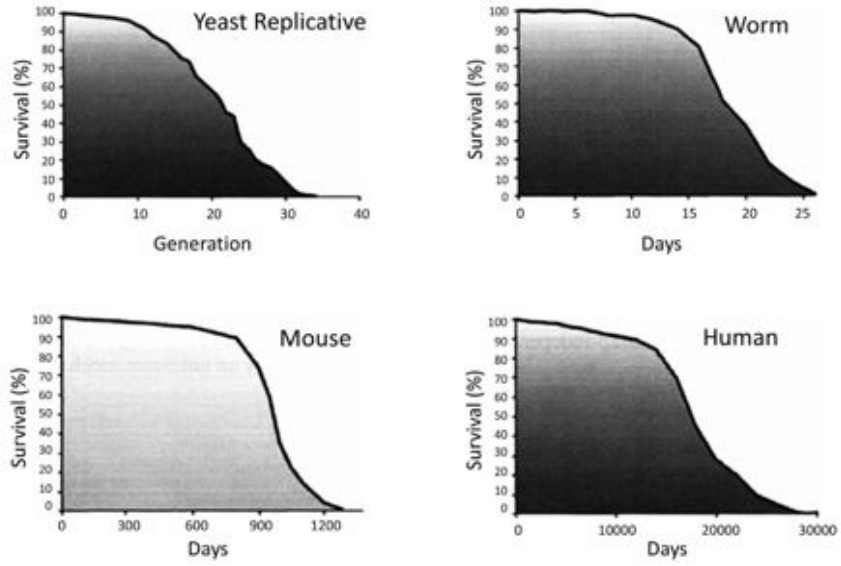

Figure. 1. Similar mortality curves are observed in divergent organisms. Kaplan-meier survival curves demonstrate a consistent trend in organism survival with time, hinting at a commonality in the aging process (Kaeberlein et al., 2001; Zainabadi, 2018).

Hallmark of Aging

There have been many hypotheses developed to describe the aging process, including the oxidative stress theory (Harman, 1956; Kirkood, 2000). However, the significance of other processes have been revealed in subsequent studies and a set of cellular and molecular hallmarks have been identified to achieve the goal of extension in health life expectancy, or healthspan (Sierra, 2016 Lopez-Otin et al., 2013). A hallmark or a pillar of aging, is defined as a common denominator of normal aging in a variety of organisms. Ideally, experimental worsening of the hallmark should speed up the aging process, whereas experimental enrichment should extend lifespan (Lopez-Otin et al., 2013). The extent to which any of the

hallmarks follow this pattern is in part contingent on the interconnectedness of all the aging hallmarks. Identifying hallmarks is an organizational strategy to provide some structure for an incredibly complex field, as well as direct further studies.

There are nine identified hallmarks of aging as can be seen in **Figure 2**. The following processes were identified as individual hallmarks in 2013: genomic instability, telomere attrition, epigenetic alterations, loss of proteostasis, deregulated nutrient-sensing, mitochondrial dysfunction, cellular senescence, stem cell exhaustion and altered intercellular communication (Lopz-Otin et al., 2013).

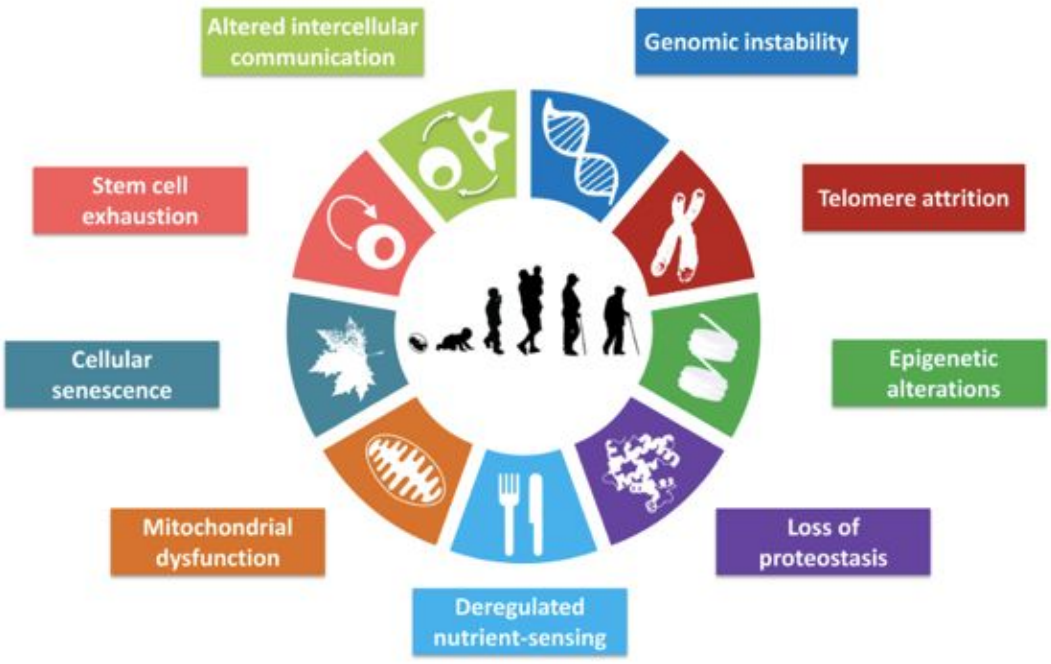

Figure 2. The nine identified hallmarks of aging. The 2013 review identified the biological processes shown in the figure (genomic instability, telomere attrition, epigenetic alterations, loss of proteostasis, deregulated nutrient-sensing, mitochondrial dysfunction, cellular senescence, stem cell exhaustion and altered intercellular communication) to be common aspects of the aging process in varied organisms (Lopez-Otin et al., 2013).

More recently, the field of geroscience has emerged with the purpose of defining the mechanisms of aging by identifying potential drivers of the aging process (Sierra, 2016). Seven pillars of aging were identified and include inflammation, response to stress, epigenetics, metabolism, macromolecular damage, proteostasis and stem cells. In this case a pillar of aging may involve one or more hallmarks of aging (**Fig. 3)**. The 'other biology' category was included to acknowledge unidentified biological processes that contribute to aging, but have not yet been detailed.

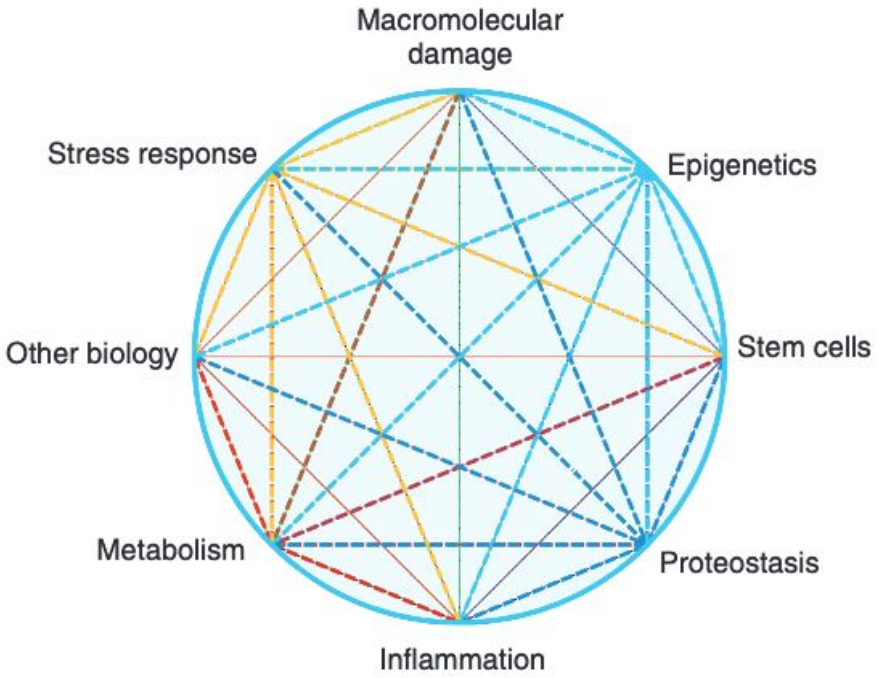

Figure 3. The main pillars of geroscience research. The above schematic represents the potential drivers of the aging process, with connecting lines highlighting that these processes are intricately connected (Sierra, 2016).

Cellular Senescence

Cellular senescence is a condition characterized by stable growth arrest resulting in a cell that no longer divides, but remains metabolically active. Cellular senescence was first identified in human fibroblasts by Hayflick and Moorhead in 1961, and while they did not know it then, the senescence they observed was

caused by telomere shortening (Bodnar et al., 1998). Mitochondrial dysfunction and DNA damage have also been identified as causes of cell senescence (Campisi, 2014).

Cellular senescence is a normal and even healthy process that occurs throughout life. The negative effects of senescence result from net accumulation over time, which compromises tissue functionality and may even limit the regenerative potential of adult stem cells, as can be seen in **Figure 4** (Collado et al., 2007). A senescent cell is dangerous to its environment largely because it leaks a mixture of proinflammatory cytokines and other factors that become toxic to neighboring cells. The factors leaked by senescent cells are collectively called the senescence-associated secretory phenotype (SASP; Rodier & Campisi, 2011), which can induce inflammation, a concurrent phenotype that accompanies aging in mammals (**Figure 4**; Salminen et al, 2012). However, the exact composition of the SASP can vary significantly, depending on the cell type, origin of the stress that causes senescence, and potentially other factors not well defined (Maciel-Baron et al., 2016; Wiley et al., 2016). Interestingly, inflammation is a characteristic of many neurodegenerative diseases and it is thought that senescent cells contribute via the SASP, which includes a mixture of proinflammatory cytokines.

In our lab, we focus on characterizing the impact of cellular senescence on age-related chronic diseases, such as neurodegeneration. Previous studies have investigated these similar questions, and when taken together show that by removing senescent cells, researchers have been successful in both delaying and preventing the onset of age-related disease phenotypes in various mouse models (Baker et al., 2011, 2016). Using the transgene INK-ATTAC to eliminate $p16^{Ink4a}$ positive cells (common biomarker for senescence), it was shown that removal of these cells delayed the onset of the disease phenotype in BubR1 progeroid mice. Additionally, late-life clearance of the $p16^{Ink4a}$ positive cells attenuated already established age-related disorders (Baker et al., 2011). In 2016, the Kirkland group came to a similar

conclusion in WT C57BL/6 mice, demonstrating that even partial removal of senescent cells using Senolytics (described later) can decrease age-related phenotypes in mice that have not been modified to be a model for a specific disease (Zhu et al., 2016).

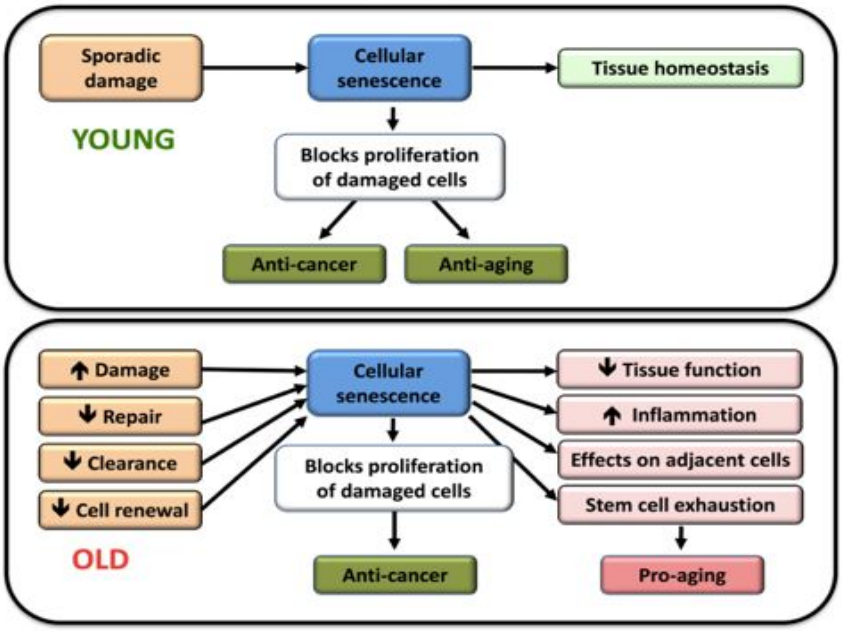

Figure 4. Causes and effects of cellular senescence in young and old age. Cellular senescence is an advantageous cellular pathway for young organisms, as senescence can block the proliferation of sporadically damaged cells and support tissue homeostasis. The surplus of damaged cells in older organisms causes an accumulation of senescent cells that lead to deleterious effects and accelerate aging.

Taken together, these studies demonstrate the role of senescent cells in age-related chronic diseases, and our current study aims to add to these findings with a focus on neurodegeneration.

Nuclear factor erythroid 2-related factor (Nrf2)

Nrf2 is a transcriptional factor involved in regulating the cell's response to stress, and it has been defined as a pro longevity signaling pathway (Harman, 2006; Steinbaugh M, et al; 2012). Levels of Nrf2 decreases with age in several species, and *in vitro* during replicative senescence (Suh et al., 2004; Sykiotis et al., 2008; Shih & Yen, 2007; Duan et al., 2009; Kapeta et al., 2010). Additionally, Nrf2 was found to be a mediator in the lifespan extension achieved following caloric restriction in mice and treatment with small molecule drugs in *C. elegans* (resveratrol, metformin, Rapamycin, etc) (Chen et al., 2005 Kode et

al., 2008 Onken and Driscol., 2010). It has been observed that mice without the gene for Nrf2 (Nrf2KO) develop several age-related diseases, including cognitive decline, a common symptom for neurodegeneration (Calkins et al., 2009; Ramsey., 2007; Motohashi et al., 2004). Taken together, the level of Nrf2 in an organism is a promising target for developing therapies and furthering our understanding of age-related chronic diseases.

Rapamycin

Since the discovery that Rapamycin extends lifespan in mice (Harrison et al ., 2009), the pharmacological agent has been a popular drug trial candidate in longevity studies. Rapamycin inhibits the mTOR pathway, which has significant effects on mammalian metabolism and physiology (Saxton et al., 2017). Inhibiting the mTOR pathway has also been heavily researched as a cancer therapeutic, as upregulation of the mTOR pathway promotes tumor growth through diverse mechanisms including lipid metabolism, glycolytic metabolism and suppression of autophagy (Yin et al., 2016; Saxton et al., 2017). Additionally, our data shows treatment with Rapamycin increases levels of Nrf2 in mice (Wang et al., 2017), and although the exact mechanism by which Nrf2 is elevated with Rapamycin treatment is unknown, it is thought to be regulated at the protein level, because a decrease in Keap1, a protein that targets Nrf2 degradation, is observed (Kobayashi et al., 2004, Wang et al 2017). Another effect of Rapamycin is its ability to prevent the induction of cellular senescence (Demidenko et al., 2009; Cao et al., 2011; Lerner et al., 2013). The fact that Rapamycin promotes healthy tissue function by eliminating senescent cells, possibly through Nrf2 activated pathways, led us to investigate treatment with Rapamycin as a possible therapy in this study.

Senolytics

After the seminal paper by the Kirkland lab showing that Senolytics improved health in mice, Senolytic drugs have become a popular pharmacological agent in aging research. Senolytic drugs interfere with the anti-apoptotic, pro-survival mechanisms in senescent cells to eliminate them from the cellular environment. The research studies using Senolytics, use a mixture of Dasatinib and Quercetin. Dasatinib eliminates senescent human fat cell progenitors, while Quercetin is more effective against senescent human endothelial cells and mouse bone marrow mesenchymal stem cells (BM-MSCs). The combination was successful in reducing senescent cell burden in chronologically aged, radiation-exposed and progeroid mice (Kirkland et al., 2015, Baker et al., 2011, 2016). Due to the variety of senescent phenotypes, it is difficult to establish if all senescent cells are cleared in a study using Senolytics. However, Dasatinib and Quercetin have been shown to have the ability to alleviate multiple aging phenotypes, as would be predicted if they truly did act by eliminating senescent cells (Kirkland, 2013a; Kirkland & Tchkonia, 2014).

Hypothesis and overall goal

Previous data in our lab showed that mice without the gene for Nrf2 (Nrf2KO mice) develop increased cellular senescence in several tissues, including the brain (Wang et al., 2017). Furthermore, decreased levels of Nrf2 were identified in mice modeling AD (Kanninen et al., 2008), as well as in samples from human AD patients (Ramsey et al., 2007). Intriguingly, senescent cells have ~65% less Nrf2 protein than normal cells, and their removal has already been noted to promote healthy tissue function and delay age-related pathology (Kapeta et al., 2010). The **objective** of this study is: i) to address the hypothesis that an absence of Nrf2 will lead to an increase in brain cellular senescence, specifically in the hippocampus, and be a driver for age-associated cognitive decline and neurodegeneration in mice. And ii) to determine

whether treatment with Rapamycin or Senolytics attenuates observed cellular senescence and improves cognitive decline and neurodegeneration in mice.

Materials and Methods

Animals

This study received prior approval from Oregon State University Institutional Animal Care and Use Committee. Three to four-month-old WT and Nrf2KO mice were divided into six groups including wild-type (WT) and Nrf2KO mice with or without Rapamycin (7-8 mice per group (Rapamycin and Vehicle) and per sex (female and male); or Senolytics (8-9 animals per group (Senolytcs or Vehicle) and per sex (female and male). The Rapamycin mice received IP injection at a dose of 4 mg kg^{-1} body weight (dissolved in ethanol then diluted with vehicle containing 5% Tween-80 and 5% PEG400) or same volume ethanol diluted with vehicle as controls. Mice were treated every other day for 14 weeks, beginning when mice were 3-4 months old. Senolytic mice received gavage of Dasatinib (LC Laboratories) and Quercetin (Dasatinib (D) 12 mg kg^{-1} and Quercetin (Q) 120 mg kg^{-1} 3 times/week, for 3 weeks). The mice were euthanized at 48 or 72 hr after the last dose of Rapamycin or Senolytics, respectively, and a thorough necropsy was performed. Tissues were snap frozen in liquid nitrogen, then stored at -80 °C for subsequent molecular work.

Cell Culture

Astrocytes were isolated with the Anti-ACSA-2 MicroBead Kit from Miltenyi Biotec, following the protocol for Isolation and Cultivation of Astrocytes from adult mouse brain. Two hemi-brains of mice from the same genotype (9-11 month old) were homogenized and transferred to a prepared enzymatic mix to digest the extracellular matrix (prepared from provided solutions). Hemi brains were harvested from mice treated with Senoylitcs for six months (D 8 mg kg^{-1} , Q 80 mg kg^{-1} , 3 times/week for 4 weeks followed by D 12 mg kg^{-1} , Q 120 mg/kg 3 times/week for 5 months). After dissociation, myelin and

cell debris were removed using the Debris Removal System, and erythrocytes were next removed using Red Blood Cell Removal Solution (10X). The Anti-ACSA-2 MicroBead Kit was then used to isolate the astrocytes from the single cell suspension via magnetic separation. Dead cells are removed using AstroMACS Separation Buffer.

After astrocytes were isolated from mouse brain, cells were seeded in 6-well plate, and maintained in DMEM: F12 media supplemented with 10% FBS, 100 units mL^{-1} penicillin, and 100 μg mL^{-1} streptomycin at 37 °C in 5% CO_2 for 10 days.

Quantitative Reverse Transcription Polymerase Chain Reaction (RT-PCR)

RT-PCR was used to analyze mRNA concentrations of SASP proteins i.e., TNFα, IL6, CCL4, VCAM, IL1α, IL1β, ICAM, and cell cycle arrest markers p16 and p21, in hippocampal samples from mice (6-8 month). The mRNA was extracted using the RNeasy kit (Qiagen, Valencia, CA, USA). cDNA was reverse-transcribed from RNA using SuperScript® III First- Strand Synthesis SuperMix following manufacturer's instructions (Invitrogen). Target mRNA levels were measured by qPCR and normalized to actin.

Senescence Associated (SA)-β-Galactosidase Staining

Brain Cryostat Sections: Prepared cryostat slide samples (5-10 microns) of mouse brain were removed from -80 °C freezer and rinsed in 1X PBS. Slides were then transferred to a 4% PF fixing solution for 15 min, then rinsed in 1X PBS three times. Reagent mixture of pH 5.5 was prepared by adding pH buffers, 5ul/mL 10% Tween and 2ul/mL Spider SA-β-gal reagent from Dojindo Laboratories and applied to the slides. Samples were then placed in a covered box with ½ inch water, and set in a 37 °C incubator for two hours. Dapi stain was applied after incubation, and slides were stored overnight at room temperature. Images were taken the next day on Keyence fluorescence microscope.

Fat Tissue: Subcutaneous fat tissue was isolated from the stomach of mice, snap frozen in liquid nitrogen and stored in -80 ℃ freezer. The experiment was carried out using the SA-β-Galactosidase Staining kit

from Cell Signaling Technology, and corresponding protocol. Tissue was cut into similar sized pieces and fixed in 4% PF solution for 15 min. The SA-β-gal stain was prepared with 10X stain (included in kit) and X-gal, prepared in DMF. Tissues were added to the staining solution and placed in a 37 °C incubator overnight to be imaged the next day.

Astrocytes: Same procedure detailed for cryostat sections was applied to cultured astrocytes. Astrocytes were isolated from 9-12 month mouse brain, seeded in 6-well plate, and maintained in Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM: F12 ATCC Cat# 30-2006) supplemented with 10% FBS, 100 units mL^{-1} penicillin, and 100 μg mL^{-1} streptomycin at 37 °C in 5% CO_2 for 10 days. Then SA-β-gal activity was analyzed by using a senescence detection kit from Thermofisher.

Immunohistochemistry

Immunohistochemistry was carried out for the identification of p16 (p16-INK4A from Protein Tech) using a fluorescent secondary antibody (Alexa Fluor (R) Cell Signaling). Prepared cryostat slide samples of mouse brain(5-10 microns) were retrieved from -80 °C freezer and rinsed in 1X PBS, then fixed in 3% PF fixing solution for 15 min. Slides were removed from fixing solution, rinsed in 1X PBS and set in Triton solution for 15 min. Samples were subsequently blocked with goat serum mixture for 45 min. Primary antibody was applied to the samples and left overnight at 4 °C. Secondary antibody was added the next day for one hour. Dapi was applied and samples were left overnight at room temperature. Imaging with Keyence fluorescence microscope took place the next day.

Morris Water Maze

5-6 male mice, 6m old, for each group were subjected to Morris Water Maze to measure: long term memory and cognitive flexibility. The tank was 4ft in diameter and filled with opaque 18 °C water. Acclimatization occurred in the first two days; mice were given one minute to swim without a platform or spatial cues present. Platform was then placed in the center of the tank and mice remained on the platform for 30 seconds, either on their own or with help from the researcher. To test long term memory, the mice

underwent a number of probe and place trials with spatial cues on the walls and a consistently placed platform. Mice had an initial probe trial to rule out any bias for specific locations in the tank, followed by four place trials, a one-hour break, four more place trials, and then a final rest before a probe trial at the end of the day. This was repeated for two days. Reversal trials were carried out to assess cognitive flexibility. In the reversal trial, spatial cues were present, but the platform was placed in the opposite quadrant.

Fear Conditioning

Fear Conditioning experiments were carried out at the University of Texas Health Science Center at San Antonio. 4-5 mice (5 month old) were subjected to a fear conditioning test to measure their ability to learn an aversive stimulus. Mice were subjected to training and contextual testing, recording freezing times, defined as the absence of any movement except for respiratory-related movements. On training day, animals were loaded into Coulbourn boxes and received a shock lasting two seconds after a 30 second tone played in the box. Freezing to context was recorded on the second day, where mice were subjected to contextual testing, meaning the 30 second tone was played without a corresponding shock. Freezing times were recorded and the average % freezing time for each animal was recorded and reported.

Statistics

Data are means +/- SEM of 5-6 animals and were analyzed using one-way ANOVA followed by Holm-Sidak. *=$p<0.05$. For Behavior and cognition, a Repeated Measures ANOVA, data are means +/- SEM of 4-5 animals *=$p<0.05$

Results

Increase in cellular senescence markers in the hippocampus of Nrf2KO mice

Previous data in the lab demonstrated that Nrf2KO mice showed increased levels of cellular senescence in all tissues examined, such as adipose tissue, lungs, and liver and treatment with Rapamycin was

successful in reducing the number of senescent cells (**Fig. 5**). Therefore, we wanted to determine if deficiency in Nrf2 increased senescent cells in the brain, specifically in the hippocampus due primarily to its association with memory loss and rapid deterioration in neurodegenerative diseases, including Alzhiermer's Disease.

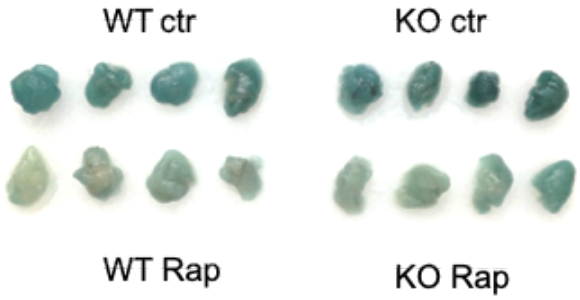

Figure 5. β-gal staining in fat tissues. Subcutaneous fat tissue from B6 Nrf2 WT & KO mice treated with Rapamycin (4mg/kg every other day for 14 weeks) were isolated following sacrifice 48 hours after last injection, and stained with SA-β-galactosidase.

We isolated hippocampal tissue from mice (6-7 months old) treated with Rapamycin for 14 weeks, and prepared cryostat sections to experimentally determine levels of cell senescence in this region of the brain. To determine levels of cellular senescence in the hippocampus of the Nrf2KO compared to the WT, we measured SASP factors with qPCR, p16 levels with fluorescent immunohistochemistry (IHC) and intensity of SA-β-gal staining. Our results demonstrate a significant increase in mRNA levels of several proinflammatory cytokines (members of the SASP) such as, TNFα, CCL4 and VCAM in the Nrf2KO compared to the WT (**Fig. 6*A***). SA-β-gal staining showed a clear increase in red fluorescence in the CA3 region of the hippocampus (indicated by purple circles) of the Nrf2KO (**Fig. 6*B***) indicating an increase in senescent cells. Finally, fluorescent IHC showed elevated expression of p16 in the CA3 region, as can be seen in the images and corresponding quantification (**Fig. 6*C***). Thus, all three widely used markers of cellular senescence were found to be elevated in the hippocampus of Nrf2KO compared to the WT.

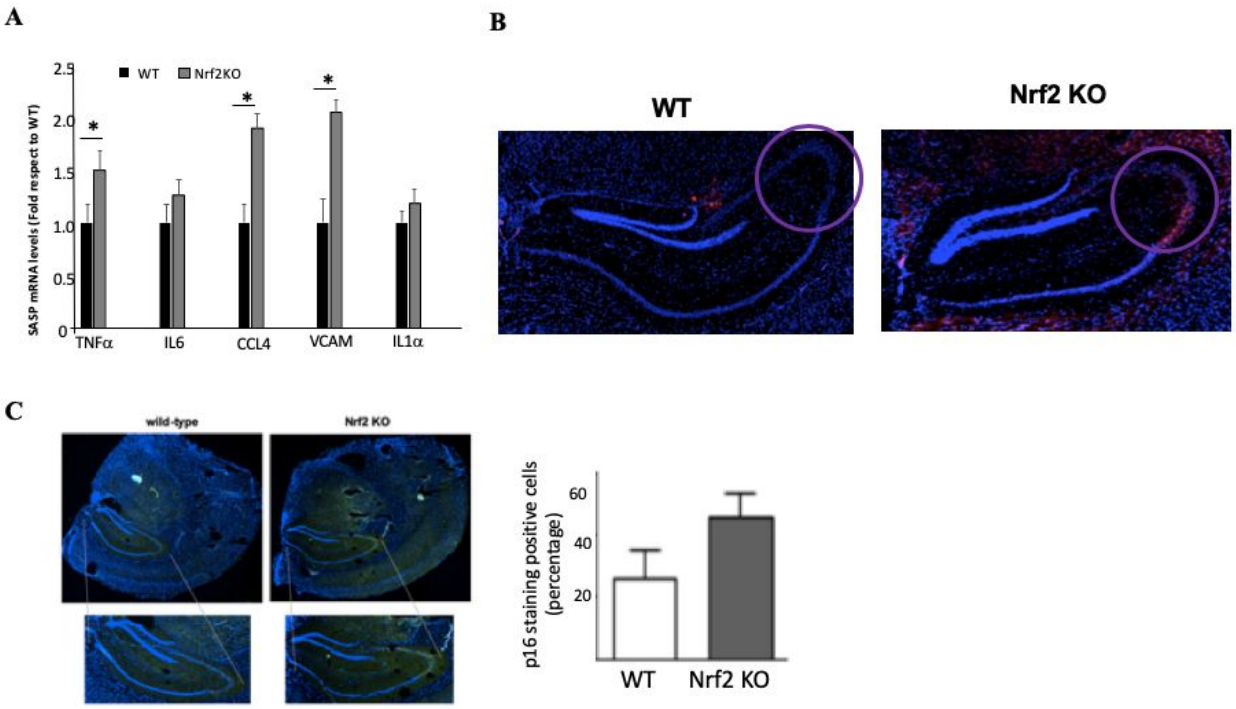

Figure 6. Increases in cellular senescence in the hippocampus of Nrf2KO mice. **(A)** RT-PCR shows Nrf2KO mice exhibit higher levels of SASP cytokines with significance for TNFα, CCL4 and VCAM. **(B)** An increase in SA-β-galactosidase staining is observed in the hippocampus of Nrf2KO mice, **(C)** fluorescent immunohistochemistry images and quantification for p16 demonstrates an increase in p16 positive cells in Nrf2KO mice.

Rapamycin Treatment have a mild effect on senescent markers in the hippocampus

Previous data, including data from our lab, have shown that Rapamycin decreased cellular senescence *in vitro* and *in vivo*. In this study, we wanted to test the effect of Rapamycin in clearing senescent cells in the hippocampus of Nrf2KO and WT mice. Mice treated with Rapamycin (4mg kg^{-1} every other day for 14 weeks), were sacrificed and hippocampal tissue were subjected to similar assessments of cellular senescence as noted before. Measurement of SASP factors using qPCR shows that levels of the SASP markers were lowered post Rapamycin treatment (**Fig. 7**), although only the mRNA levels of IL1α, IL6 and CCL4 were significantly reduced post treatment with Rapamycin (**Fig. 7*A***). SA-β-gal staining demonstrated once again an increase in fluorescence in the Nrf2KO compared to the WT, and treatment with Rapamycin showed a decrease in b-gal staining (**Fig. 7*B***). p16 mRNA levels were also measured and

we observed a significant decrease in p16 level was observed after Rapamycin treatment (**Fig. 7*C***).

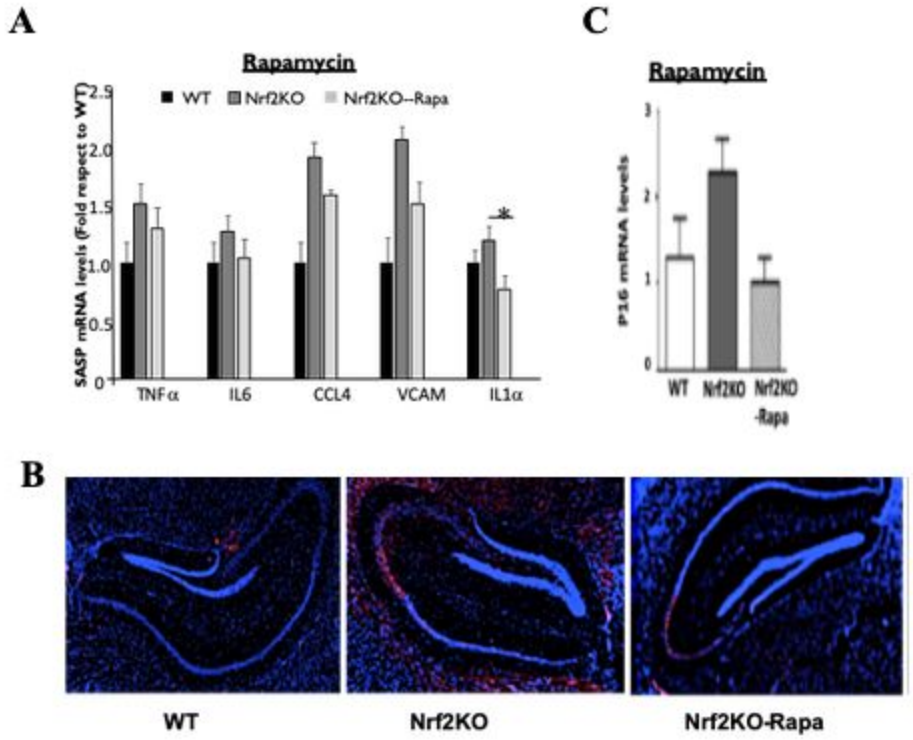

Figure 7. Treatment with Rapamycin has a mild effect on senescent markers in the hippocampus of Nrf2 KO mice. **(A)** RT-PCR show levels of SASP factors increase in Nrf2KO mice, and a trend in reduction following treatment with Rapamycin (4 mg kg^{-1}) every other day for 14 weeks can be observed, **(B)** visible reduction in SA-β-galactosidase staining fluorescence and **(C)** results of p16 immunohistochemistry are not significant, but fit the trend that Rapa have a mild effect on senescent markers the hippocampus.

Treatment with Senolytics have a mild effect on senescent markers in the hippocampus

To test the effect of clearing senescent cells in the hippocampus using Senolytics, we treated groups of mice with Senolytic drugs (12mg kg^{-1} D, 120mg kg^{-1} Q) three times/week for three weeks and hippocampal tissue were subjected to similar assessments of cellular senescence as described previously. We observed that Senolytic treatment lowered the levels of some components of the SASP, but not all of them (**Fig. 8**). Only the mRNA levels for TNFα, IL6 and CCL4 were significantly decreased (**Fig. 8*A***). Senolytic treatment has a mild effect in decreasing the SA-β-gal staining in the hippocampus (**Fig. 8*B***).

Similarly, Senolytic treatment reduces the p16 mRNA levels in a similar fashion to what we observed with Rapamycin (**Fig. 8*C***).

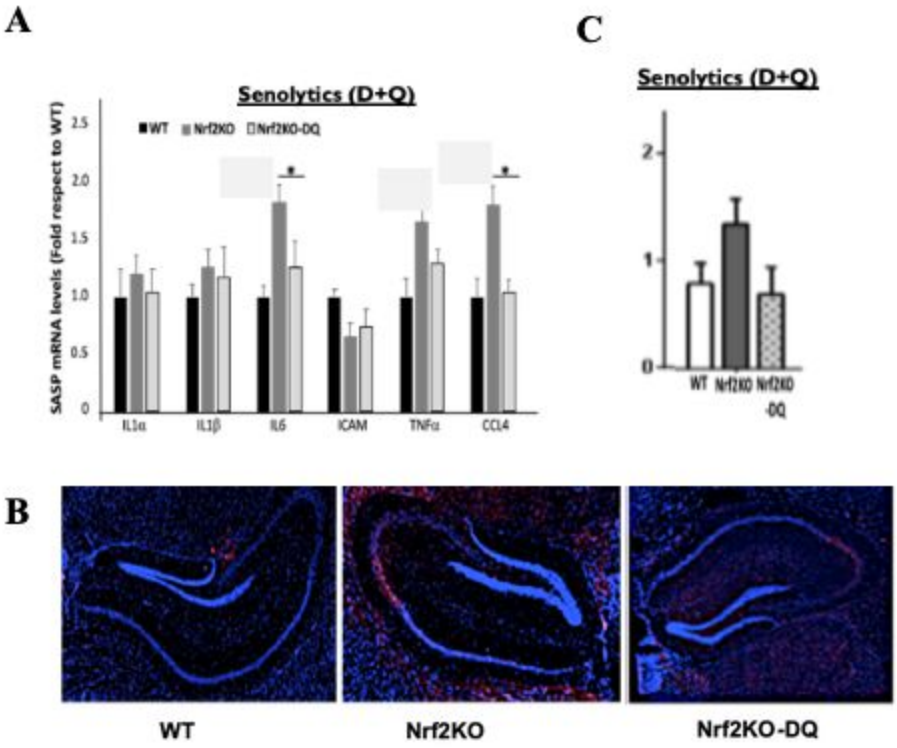

Figure 8. Senolytic treatment mildly reduces senescence markers in the hippocampus of Nrf2 KO mice compared to WT. (A) RT-PCR show levels of SASP factors increase in Nrf2KO mice, and a trend in reduction following treatment with Senolytics Dasatinib (12 mg kg^{-1}) and Quercetin (120 mg kg^{-1}) (D+Q) 3 times/week, for 3 weeks, **(B)** visible reduction in SA-β-galactosidase staining fluorescence and **(C)** results of p16 immunohistochemistry are not significant, but fit the trend that Senolytics have a mild effect on senescent markers the hippocampus.

Treatment with Senolytics in isolated astrocytes has a dramatic reduction in senescent markers

To investigate whether the effect of the Senolytic drugs was cell specific, we isolated astrocytes from the whole brain and performed qPCR and SA-β-gal staining analysis. Levels of SASP members show dramatic increase in expression in the Nrf2KO compared to WT mice, and Senolytic treatment had a significant reduction in the SASP levels, being close to WT levels (**Fig. 9*A***). We found similar results with SA-β-gal analysis, where our results demonstrate a stronger fluorescent signal in astrocytes from

Nrf2 KO compared to wild-type, and a significant decrease in fluorescence signal following treatment with Senolytics (**Fig. 9*B***). Results from the images were quantified (number of cells expressing SA-β-gal/total number of cells) to further show that Nrf2 deficiency increased cellular senescence in 66% of isolated astrocytes compared to WT mice, and Senolytic drugs decreased SA-β-gal expression in 74% of senescent cells compared to astrocytes isolated from Nrf2KO mice (**Fig. 9*C*)**.

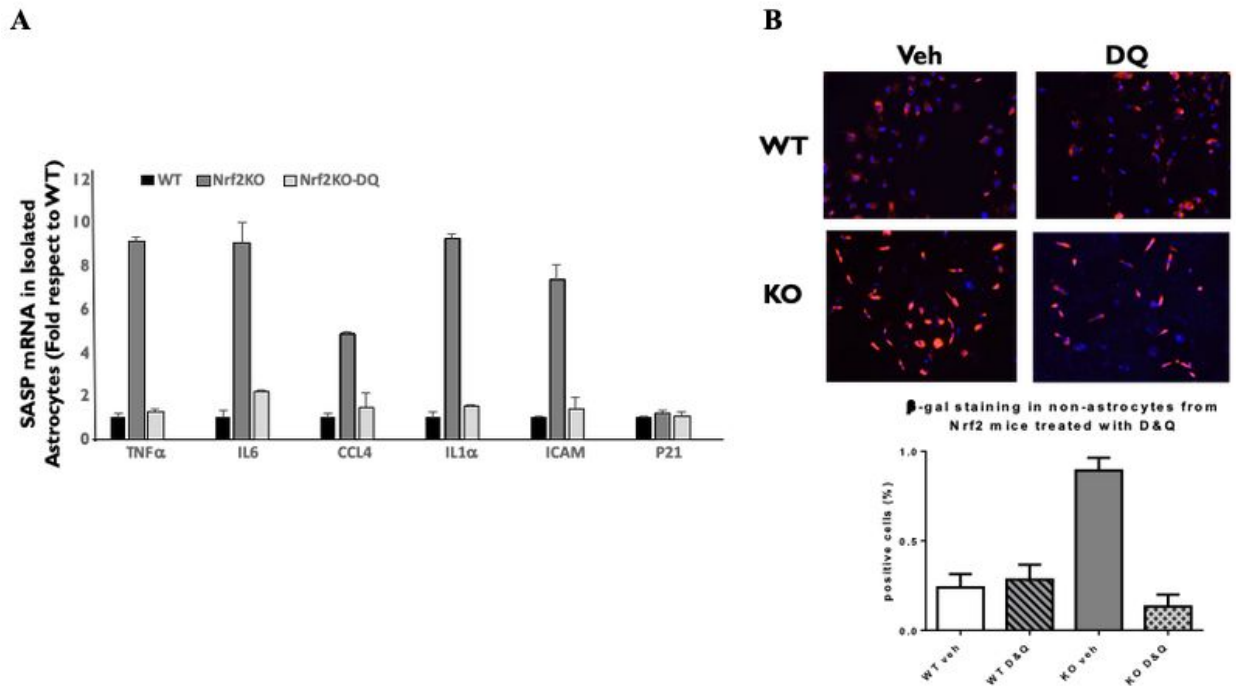

Figure 9. Senolytics has a dramatic effect on senescent markers in isolated astrocytes. **(A)** Levels of six members of the SASP show dramatic increase in expression in Nrf2KO compared to the wild-type and a large reduction post treatment with Senolytics, close to wild-type level. **(B)** SA-β-gal results demonstrate a stronger fluorescent signal in astrocytes from Nrf2KO compared to WT, and a decrease in fluorescence signal following treatment with Senolytics. Results from the images were quantified to further show the dramatic effect of Nrf2 and Senolytic drugs on senescent cell markers on isolated astrocytes (N = 4-5).

Nrf2KO mice have less cognitive flexibility than the wild type mice

Behavioral testing was carried out to assess cognitive differences in the Nrf2KO and WT mice and investigate the effect of treatment with Rapamycin. Using Fear Conditioning Testing to assess the ability of the mice to learn and remember an association between environmental cues and adverse stimuli, the Nrf2KO mice do not learn how to respond (freezing) to the adverse stimulus (**Fig. 10*A*)**. Additionally,

during the second probe trial, where the mice are allowed to free swim for 30 seconds in the absence of the platform, Nrf2KO mice had a significantly lower average proximity to the platform than the WT (**Fig. 10*B*)**, suggesting the WT mice have better developed long term memory than the Nrf2KO. Lastly, we treated the mice with Rapamycin to see whether this treatment can improve the observed deficits in behavior to be similar to the WT. Mice, both WT and Nrf2KO, treated with Rapamycin had higher average proximity to the old platform position, suggesting that Rapamycin improves cognitive flexibility of both genotypes, the Nrf2KO and WT (**Fig. 10*C***).

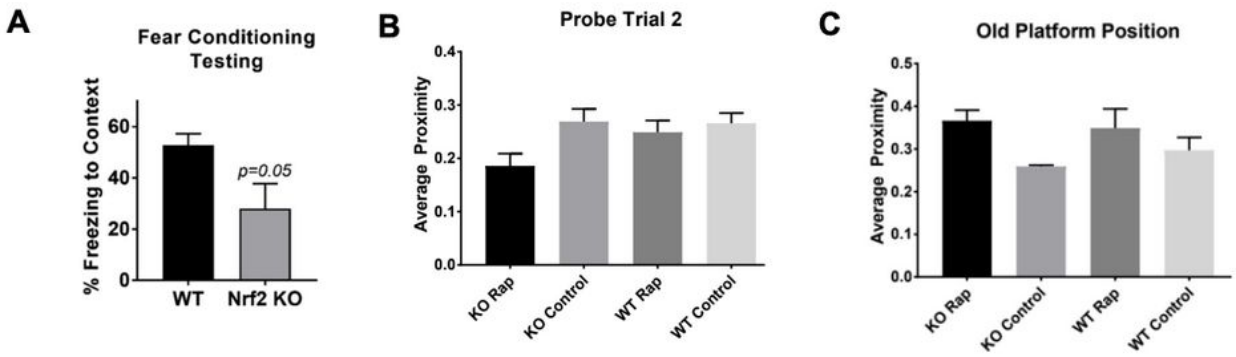

Figure 10. Behavior and cognitive assessments in Nrf2KO mice. (A) Nrf2KO mice showed a decrease in performance in Fear Conditioning Testing, indicating that Nrf2 KO mice do not learn how to respond (freezing) to adverse stimulus as well as WT. **(B)** Nrf2 KO mice had a significantly lower average proximity to the platform than the WT mice during probe trials (p= 0.0477, n =5) indicating that Nrf2 KO mice have better long-term memory than the WT. **(C)** Rapamycin mice show a significant effect (p=0.0134, n=5) in improving the average proximity to the old platform position indicating that Rapamycin improves cognitive flexibility in both mice.

Discussion

Multiple studies have demonstrated the positive effects of removing senescent cells on delaying and attenuating age-related pathologies, corroborating the classification of cellular senescence as a hallmark of aging (Lopez-Otin et al., 2013; Kapeta et al., 2010; Baker et al., 2011, 2016; Zhu et al., 2016). There are several stressors that induce cellular senescence, for example telomere shortening, a natural effect of cellular aging, can cause cells to enter the stable growth arrest that is observed in senescence (Bodnar et al., 1998). Moreover, mitochondrial dysfunction and DNA damage are also causes of senescence, and

cells with a weakened response to these stresses may be further vulnerable to senescence (Campisi, 2014). Interestingly, this is what is observed in mice without the gene for Nrf2, which is intricately involved in a cell's response to stress. Knocking out Nrf2 leads to an increase in senescence *in vivo*, as well as *in vitro* (Wang et al., 2017; Suh et al., 2004; Sykiotis et al., 2008; Shih & Yen, 2007; Duan et al., 2009; Kapeta et al., 2010). It is known that cellular senescence is a hallmark of aging in humans, however the role of Nrf2 in brain cellular senescence has not yet been detailed.

Characterization of the effect of age and cellular senescence on the hippocampus is important in understanding hippocampal involvement in neurodegenerative diseases, like Alzheimer's Disease (AD), one of the most common neurodegenerative disorders and early diagnosis is vital for treatment. Furthermore, the hippocampus is implicated in all neurodegenerative disorders, and it is well known that changes in the hippocampus contribute to the memory impairment observed in AD and other conditions of dementia (Moodley & Chan, 2014). This is at least in part because the dentate gyrus region of the hippocampus is one of only two regions of the brain that can generate new neurons, however its neurogenic capacity declines with age (Vivar, 2015).

Our lab previously reported that in the absence of Nrf2, there is an increase in cellular senescence in multiple tissues including whole brain, however the effect of Nrf2KO in the hippocampus of mice has not been reported (Wang et al., 2017). Using a Nrf2KO to probe the relationship between Nrf2 and senescence in the hippocampus, we demonstrated an increase in senescent markers in the absence of Nrf2 in this region of the brain (**Fig. 2**). Knowing that accumulation of senescent cells leads to tissue damage, our results could broaden the knowledge surrounding how the hippocampus loses function in neurodegenerative diseases. Further studies must be completed to strengthen the connection between this increase in senescence in the hippocampus, and other characteristics of neurodegenerative disorders (i.e.

activation of microglia, increased toxicity to tau or Aβ plaques, etc).

We know that Rapamycin increases levels of Nrf2 and prevents induction of cellular senescence (Wang et al., 2017). It has also been shown that Senolytic drugs effectively remove senescent cells and alleviate multiple aging phenotypes (Kirkland, 2013a; Kirkland & Tchkonia, 2014). However, the extent to which these therapies affect senescence in the hippocampus of mice in the absence of Nrf2 is unknown. Our results from RT-PCR on SASP members and p16, as well as SA-β-gal staining, provide evidence that treatment with Rapamycin and Senolytics have mild effects on senescence in the hippocampus of mice (**Fig. 3)**.

The mild effect observed in our data called our attention because it is known that Rapamycin has decreased cellular senescence in other tissues, but we think that it is possible that the Rapamycin does not cross the blood brain barrier (BBB) efficiently enough to reach the hippocampus in sufficient concentrations. While much concern has been raised about the efficiency of Rapamycin crossing the BBB, multiple studies have shown Rapamycin is able to cross over the BBB (Kwon CH et al., 2003; Litt L et al., 2001). Furthermore, in one study, Rapamycin was identified in brain tumors extracted from patients with neuroblastoma, who had been treated with the drug (Cloughesy et al., 2008). Clearly Rapamycin can cross the BBB, we may need to adjust to a higher dose, similar to other groups to see an effect in the hippocampus.

Also, it has been shown that Senolytic treatment has a significant effect in the clearance of senescent cells in the hippocampus of WT and AD mouse models (Kirkland et al., 2016; Baker et al., 2011, 2016). Moreover, a study in APP/PS1 AD mice did show that Senolytic drugs were readily detectable with mass spectrometry two hours after administration (Zang et al 2019). However, given the variety of dosage and

duration of treatment between experiments with Senolytics, there may be corresponding differences in the ability of these compounds to cross the BBB as well as the rate of their subsequent metabolism that we do not yet understand. Thus, we believe that by using a higher dose over a longer period of time, more similar to previous publications, may result in significant results.

Another possibility for the observed mild-effect of Rapamycin and Senolytics on senescent markers in the hippocampus, is that the Nrf2KO mice have too much cellular damage due to the absence of Nrf2, that it is more difficult for either drug to rescue. Also, another consideration is that the age we started the treatment (3-4 months of age), may already be too old and the mice may have developed senescence, so we may need to start the treatment at younger age to have a better response. Overall, these considerations could explain why other groups have shown a significant effect of Rapamycin and Senolytics on senescent markers, but we observed a mild-effect (Baker et al., 2011, 2016; Kirkland et al., 2015) .

To strengthen the connection between Nrf2, cellular senescence and neurodegeneration, it is important to ask if there is a connection between senescence in the hippocampus and cognitive performance in mice. Our results from behavioral testing suggest that Nrf2KO mice have less cognitive flexibility than the WT mice. Additionally, Rapamycin treatment has a significant effect in increasing cognitive flexibility performance in both the Nrf2KO and WT mice. We carried out behavioral testing (Morris Water Maze, Fear Conditioning) on Nrf2KO and WT mice treated with Rapamycin or Senolytics. Rapamycin treatment has a significant effect in increasing cognitive flexibility performance of both Nrf2KO and WT mice, however a bigger difference is observed in the Nrf2KO. Because significant effects on cognition were only observed in mice treated with Rapamycin, in both the Nrf2KO and the WT, it is possible that alternative effects of Rapamycin positively impacted the performance of the mice. We are also aware that we had a significantly low number of animals per group (N = 5-6) for the behavioral and cognitive

analysis (i.e. Morris Water Maze and Fear Conditioning experiments). We are in the process of generating a new cohort of animals (N = 12) to repeat these experiments with a larger number of mice per group.

It is known that mTOR (molecular target of Rapamycin) signaling is involved in longevity regulation, shown by the inhibition of mTOR signaling resulting in extended lifespan in multiple invertebrates (i.e. yeast, nematodes and fruit flies; Lamming et al., 2013). However, pharmacological inhibition of mTORC1 with Rapamycin also provides neuroprotection, as has been shown in several *in vivo* models of neurodegenerative diseases (i.e. Alzhiermer's Disease, Parkinson's Disease and Huntington's Disease; Bove et al 2011). It was noted in the 2011 review from Bove *et al* that the accumulation of misfolded proteins and increased protein aggregation was a common characteristic of the aforementioned neurodegenerative diseases. One possible explanation is that inhibition of mTORC1 pathway with Rapamycin decreases protein aggregation because mTORC1 pathway both drives protein synthesis and causes defects in cellular autophagic pathways (Bove et al., 2011). Therefore, it is also reasonable that Rapamycin has this effect by both preventing aggregation and cellular senescence. Future studies examining the levels of protein aggregates in addition to senescence should be conducted to draw further conclusions.

When it was evident that Rapamycin and Senolytic therapies had mild non-significant effects in whole brain tissue, we were curious if we could find a more pronounced effect if we look in specific cells, like hippocampal astrocytes. Our data show a dramatic increase in both SA-β-gal expression and mRNA levels of SASP factors in astrocytes (**Fig. 6**), supporting our hypothesis that the activity of Senolytic drugs is somewhat stronger in specific cells. Future studies examining different brain cell types (i.e. microglia and neurons) from Nrf2KO and WT mice treated with Senolytics must be completed to strengthen this finding. Additionally, we need to determine whether Rapamycin treatment has a similar effect in astrocytes to those observed after treatment with Senolytics.

In summary, our data provides important advancements to the understanding of the interconnectedness of Nrf2 and brain cellular senescence. We demonstrated cellular senescence increases in the hippocampus of the Nrf2KO mice compared to WT, which may have broader implications as an early indication of neurodegenerative diseases, including Alzhiermer's Disease. Rapamycin and Senolytics have previously been implicated in attenuating and delaying age-related pathologies, and our data suggest these therapies may also decrease cellular senescence in the hippocampus of Nrf2KO compared to WT mice. Finally, we showed that Rapamycin improves cognitive performance of both Nrf2 KO and WT mice, possibly by reducing senescence in the hippocampus.

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