EFFECTS OF VARYING LEVELS AND DIFFERING DURATIONS OF CALORIE RESTRICTION ON CELLULAR AND SYNAPTIC PROTEINS, AS WELL AS THE INFLAMMATORY STATE OF THE FEMALE MOUSE BRAIN

A THESIS SUBMITTED TO

THE GRADUATE SCHOOL OF ENGINEERING AND SCIENCE

OF BILKENT UNIVERSITY

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR

THE DEGREE OF

MASTER OF SCIENCE

IN

NEUROSCIENCE

By

Duygu Macaroğlu

September 2020

EFFECTS OF VARYING LEVELS AND DIFFERING DURATIONS OF CALORIE RESTRICTION ON CELLULAR AND SYNAPTIC PROTEINS, AS WELL AS THE INFLAMMATORY STATE OF THE FEMALE MOUSE BRAIN

By Duygu Macaroğlu

September 2020

We certify that we have read this thesis and that in our opinion it is fully adequate, in scope and in quality, as a thesis for the degree of Master of Science.

Michelle Marie Adams(Advisor)

Soner Doğan

Hacı Hulusi Kafalıgönül

Approved for the Graduate School of Engineering and Science:

Ezhan Karaşan
Director of the Graduate School

ABSTRACT

EFFECTS OF VARYING LEVELS AND DIFFERING DURATIONS OF CALORIE RESTRICTION ON CELLULAR AND SYNAPTIC PROTEINS, AS WELL AS THE INFLAMMATORY STATE OF THE FEMALE MOUSE BRAIN

Duygu Macaroğlu
M.S. in Neuroscience
Advisor: Michelle Marie Adams
September 2020

Aging is an inevitable and complicated process leading to functional decline. Regarding brain aging, cognitive decline takes place in multiple domains, including learning, memory, executive functions, motor coordination, and language. At the cellular and molecular level, age-related cognitive decline is elucidated with certain hallmarks, including aberrant neuronal network, stem cell exhaustion, glial cell activation, and inflammation. Calorie restriction (CR) is a widely-utilized approach for coping with aging's detrimental effects even though there is no one agreed way for the application of CR. In this study, varying levels of CRs were applied for differing durations to MMTV-TGF-alpha female mice. The study initiated when mice were 10-weeks of age (Baseline) and carried out until 49/50 weeks of age and 81/82 weeks of age. There were four dietary groups named Ad-libitum (AL; control), Chronic Calorie Restriction (CCR), Intermittent Calorie Restriction - Restriction (ICR-R), and Intermittent Calorie Restriction -Refeed (ICR-RF).

The study's first aim was to show age-related changes in the cellular and synaptic proteins and the inflammatory state of the female mice's brains. The second aim of the study is to demonstrate the effects of varying levels of CR implemented for the short-term and the long-term manner on the same hallmarks. Our findings showed both chronic- or intermittent- CR altered the synaptic integrity proteins against brain aging at the long-term period (81/82 weeks) compared to the short-term (49/50 weeks) period except for PSD-95. Similarly, both chronic- or intermittent- CR showed an attenuative impact on the pro-inflammatory markers, but IL-6 was affected only by CCR at the same periods. Furthermore, an age-related imbalance between neurogenesis and astrogliogenesis was shown based on DCX and GFAP. Both chronic- or intermittent- CR showed a compensatory effect on it acting through astrogliogenesis, even though it was not statistically significant.

Keywords: brain aging, calorie restriction, proliferation, synaptic integrity, neuroinflammation, western blot, female mouse .

ÖZET

DEĞİŞEN DÜZEY VE FARKLI SÜRELERDEKİ KALORİ KISITLAMASININ HÜCRESEL VE SİNAPTİK PROTEİNLER VE DİŞİ FARE BEYNİNİN İNFLAMATUVAR DURUMU ÜZERİNE ETKİLERİ

Duygu Macaroğlu
Nörobilim, Yüksek Lisans
Tez Danışmanı: Michelle Marie Adams
Eylül 2020

Yaşlanma, fonksiyonel gerilemeye sebep olan kaçınılmaz ve karmaşık bir süreçtir. Beyin yaşlanması ile ilgili olarak, bilişsel gerileme; öğrenme, hafıza, yürütücü işlevler, motor koordinasyon ve dil dahil olmak üzere birçok farklı alanda gerçekleşir. Hücresel ve moleküler düzeyde ise, yaşa bağlı bilişsel gerileme; anormal nöronal ağ, kök hücre tükenmesi, glial hücre aktivasyonu ve iltihaplanma gibi belirli özelliklerle açıklanmaktadır. Kalori kısıtlaması (KK), yaşlanmanın zararlı etkileriyle başa çıkmada yaygın olarak kullanılan bir yaklaşım olmasına rağmen uygulanma yöntemi üzerine uzlaşılmış tek bir yöntem yoktur. Bu çalışmada, MMTV-TGF-alfa dişi farelere değişen düzey ve farklı sürelerde kalori kısıtlaması uygulanmıştır. Çalışmalar, fareler 10 haftalıkken (Başlangıç) başlatılmıştır ve 49/50 haftalık ve de 81/82 haftalık olana kadar sürdürülmüştür. Çalışma, Ad-libitum (AL; kontrol), Kronik Kalori Kısıtlaması (KKK), Aralıklı Kalori Kısıtlaması - Kısıtlama (AKK-K) ve Aralıklı Kalori Kısıtlaması -Yeniden Besleme (AKK-YB) olarak adlandırılan dört farklı diyet grubunu içermektedir.

Çalışmanın ilk amacı, hücresel ve sinaptik proteinlerdeki yaşa bağlı değişiklikleri ve dişi farelerin beyinlerinin iltihaplanma durumunu göstermektir. Çalışmanın ikinci amacı ise, kısa- ve uzun süreli olarak uygulanan değişen düzeydeki kalori kısıtlamalarının belirtilen özellikler üzerindeki etkilerini göstermektir. Bulgularımız, hem kronik hem de aralıklı KK'nın, sinaptik integrite proteinlerini , PSD-95 hariç, beyin yaşlanmasının etkilerine karşı uzun-süreli (81/82 hafta) uygulamada kısa-süreli (49/50 hafta) uygulamaya kıyasla değiştirdiğini göstermiştir. Benzer şekilde, hem kronik hem de aralıklı KK, proinflamatuvar belirteçler üzerinde zayıflatıcı bir etki göstermiştir, fakat IL-6 aynı dönemlerde yalnızca KKK'den etkilenmiştir. Ayrıca, nörojenez ve astrogliojenez arasındaki yaşa bağlı imbalans, DCX ve GFAP temel alınarak gösterilmiştir. Hem kronik hem de aralıklı KK, istatistiksel olarak anlamlı olmamasına rağmen astrogliogenez yoluyla etki eden telafi edici bir etki göstermiştir.

Anahtar sözcükler: beyin yaşlanması, kalori kısıtlaması, proliferasyon, sinaptik integrite, nöroinflamasyon, western blot, dişi fare.

Acknowledgement

First of all, I would like to thank my supervisor Prof. Dr. Michelle M. Adams, for allowing me to be part of her laboratory and research team. And also, I am so grateful to her for giving me this opportunity to work on such a great project.

Secondly, I would like to thank Assoc. Prof. Soner Doğan and Asst. Prof. Bilge Güvenç Tuna and their team for sharing their samples generously and helping me all the time.

I would like to thank Assoc. Prof. Soner Doğan and Asst. Prof. Hacı Hulusi Kafalıgönül for taking part in my thesis committee and their help.

I would like to thank all past and current members of Adams Lab, namely Begün Erbaba, Beyza Özen, Bilge Aşkın, Duygu Mutlu, Ergül Dilan Çelebi-Birand, Elif Tuğçe Karoğlu-Eravşar, Hande Özge Aydoğan, Melek Umay Tüz-Şaşik, Narin Ilgım Ardıç, Naz Mengi, and Zeynep Gültekin for their guidance, assistance, and supports. Most especially, I would like to express my deepest gratitude to Melek Umay Tüz-Şaşik for her guidance throughout my thesis. Her comments, feedback, and help mean a lot to me. Also, I would like to thank Elif Tuğçe Karoğlu-Eravşar for her help in statistical analysis. In this regard, I would like to thank Hande Özge Aydoğan as well. She also helped me a lot with the statistical analysis, but more importantly, I am so grateful for her friendship and support all the time.

I would like to express my deepest gratitude to Bilgen Işık-Karaşahin, Murat Karaşahin, and their lovely children Doğa and Defne! They became my family in Ankara. Also, I would like to express my deepest gratitude to Clive Topley. I cannot thank enough for his friendship, support, and encouragement all the time.

Last but not least, I would like to express my deepest gratitude to my lovely big family, in particular my mom Şerife Macaroğlu, my dad Fahri Macaroğlu and my lovely sister Yaren Macaroğlu. I could not have done it without them. I am so lucky to be part of this family and so grateful for their unconditional love and support all the time. My most exceptional thank is to Uzay Gökay. Words cannot describe how grateful I am for being in my life. You are the pillar of strength in every part of my life.

This thesis was financially supported by The Scientific and Technological Research Council of Turkey (TUBITAK) to establish and maintain the experimental animals with the grant no 119S238 and The European Molecular Biology Organization (EMBO) Installation Grant through TUBITAK to Michelle M. Adams for the Western blot experiments.

Contents

List of Figures

List of Tables

Chapter 1

Introduction

1.1 Brain Aging

Aging is an inevitable and complicated process. Most organisms experience age-related imbalance in the homeostatic state of the body, therefore a decline in body systems' functioning. Although people may have experienced normal aging, which is not accompanied by pathological conditions, age-related impairments increase the risk factors for multiple diseases, including cardiovascular diseases, neurodegenerative diseases, cancer [1]. The lifespan of the human has been rising with the advancement in the field of health. According to the United Nations (UN), the percentage of older people (over 65 years) in the global population will reach over 15 percent by 2050 [2]. An increase in humans' life expectancy has caused people to suffer from age-related dysfunctions for a longer time. Therefore, it created the need for improvement in the healthspan of people as that of lifespan.

Regarding normal brain aging, age-related deterioration in the homeostasis is a multifactorial process associated with cognitive impairments in multiple domains. As people get older, they experience mild cognitive decline, including learning, memory, executive functions, motor coordination, language [1, 3]. There are well-accepted brain aging hallmarks at the cellular and molecular levels, including aberrant neuronal network, stem cell exhaustion, glial cell activation, and inflammation [1]. On the other hand, aging hallmarks are categorized as primary hallmarks (that are the primary causes of aging), antagonistic hallmarks (that are the responses to primary hallmarks), and the integrative hallmarks (that are the response to primary and antagonistic hallmarks and the reason behind the phenotypically observable alterations during aging) [4]. Due to the aging process's complexity, it is not possible to attribute age-related alterations to one single hallmark. Instead, most of the brain aging process pathways show a complex interplay between each other, which makes it difficult to understand. Thus, it is essential to understand possible interaction among the hallmarks and how the aging cascades proceed and contribute to cognitive decline.

All in all, brain aging is a complex process, during which more and more people suffer from age-related deteriorations with the expansion of lifespan. It is a process that negatively affects not only the suffered individuals but also economics. Comprehension of complex interplays between hallmarks and the interventions to cope with age-related decline in functions of the brain provides older people with an independent life, as well as may protect them from age-related pathologies.

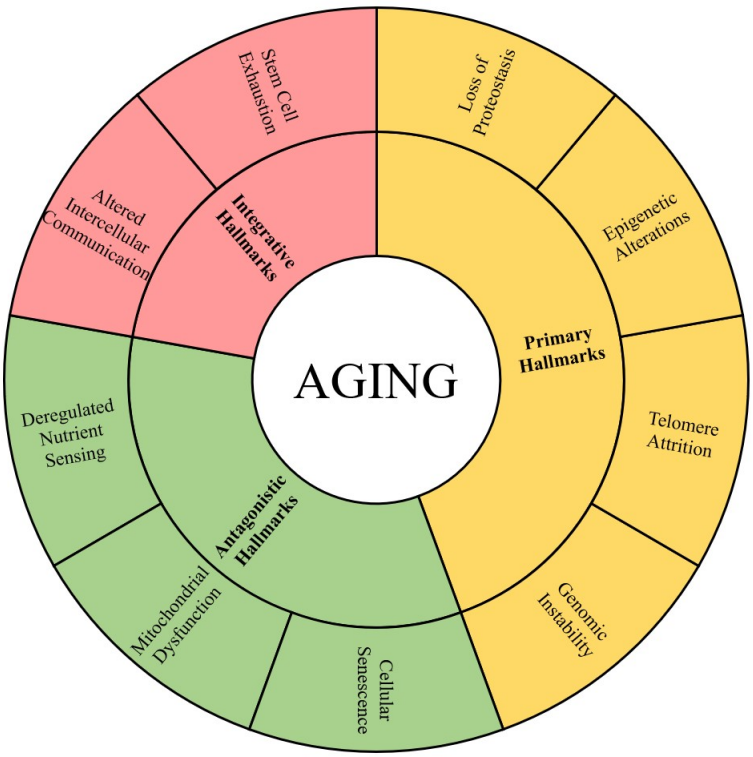

Figure 1.1: Interconnections among hallmarks of aging [4]

1.2 Proliferation and Brain Aging

Neurogenesis is a process of forming new neurons from neural stem cells (NSCs), which was thought to be restricted only to the embryonic development of the central nervous system (CNS). Starting with Altman's studies on the postnatal rat hippocampus in 1965, multiple studies were carried on the generation of new neurons after the perinatal period[5, 6, 7]. After the second half of the 1990s, it became evident that neurogenesis is not limited to the central nervous system's developmental stages in mammals. Instead, the mammalian brain is capable of

producing new neurons throughout adulthood in two well-defined regions: the subgranular zone (SGZ) of the dentate gyrus in the hippocampus and the subventricular zone (SVZ) of the lateral ventricles. The newly born neurons in SGZ of the dentate gyrus remain in the hippocampus and form the dentate granule cells after differentiation. The newly born neurons in SVZ of the lateral ventricles migrate through the rostral migratory stream (RMS) to the olfactory bulb, where they form olfactory interneurons [8, 9].

There are various methods to measure the rate of neurogenesis and cell proliferation in the postnatal period. 5'-Bromo-2'-Deoxyuridine (BrdU) is an analog of thymidine, which is able to incorporate into the deoxyribonucleic acid (DNA) during the replication of DNA. Since it is an exogenous marker, it has to be injected generally through intraperitoneally. Immunohistochemical detection of BrdU through fluorescently tagged 2^{o} antibodies is widely used to trace and count the number of dividing cells. It is also possible to get an idea of the proliferation in different cell types using the cell-specific markers with BrdU. In addition to exogenous markers, there are endogenous markers, including Ki-67 and PCNA. Ki-67 is a nuclear protein that is expressed at different stages of the cell cycle. Since it does not need to be injected exogenously, it is used as an alternative to BrdU in neurogenesis studies. Similar to BrdU, it can be detected by immunohistochemistry [10]. PCNA (proliferating cell nuclear antigen) is an essential factor for the function of DNA polymerases during DNA replication [11]. It is expressed during all cell cycle stages and able to mark dividing cells in the neurogenic regions. In addition to addressing neurogenesis in the dividing cells, other markers can also assess the source and stages of dividing cells. As mentioned above, co-labeling of actively dividing cells with cell- or stage-specific markers

provides broader comprehension for adult neurogenesis and cellular proliferation. To illustrate, glial fibrillary acidic protein (GFAP) distinguish astrocytes from other glial cells in the central nervous system [12], and ionized calcium-binding adapter molecule 1 (Iba-1) is used for marking the microglial cells [13]. Besides, neuronal cells can be traced at different stages until they become a mature functional neuron. Doublecortin is a guider protein during the migration that is used as an immature neuron marker [14]. On the other hand, the neurons' maturation can be detected by the expression of neuronal nuclei (NeuN) [15].

Even though neurogenesis goes on during adulthood, there are age-related alterations in the process, including a decrease in the rate of neurogenesis and proliferation, as well as the changes in the microenvironment of the neural stem cells [16] Age-related alterations in the neurogenesis and proliferation has been attributed to cognitive decline [1]. However, certain studies reported an inverse relationship between cognitive abilities and neurogenesis in aged-rats [17]. That is to say, with increasing age, subjects could perform well in behavioral tasks meaning intact cognitive ability, but still, they could not have preserved neurogenesis. Therefore, decreased neurogenesis and proliferation can have a different meaning at different ages and needs to be further investigated for the comprehension of this complex topic [18]

1.3 Synaptic Alterations and Brain Aging

Communication between neurons is not provided by physical contact; instead, it occurs by releasing neurochemicals into a specialized gap named synaptic cleft. The synaptic regions have a highly specialized structure to facilitate synaptic transmission. Essential components for a synaptic transmission include the presynaptic-, postsynaptic cells, and a synaptic cleft [19]. Presynaptic cells are responsible for the production, storing, and secretion of neurotransmitters in a controlled manner. Neurotransmitters are not found dispersed in the presynaptic terminal; instead, they are stored in synaptic vesicles initially formed from the Golgi apparatus. There are specialized regions named active zones on the presynaptic terminal, in which Ca $^{+}2$ channels and SNARE proteins are concentrated. The fusion of synaptic vesicles to the membrane of presynaptic cells is managed through the SNARE, and chaperone proteins that are also found on the vesicular membrane, so active zones are the predetermined regions synaptic vesicles are docked, primed, and fused to release neurotransmitters [19, 20, 21]. Each protein involved in the process is essential for proper synaptic transmission to take place at the right time and place.

Similar to presynaptic cells, postsynaptic cells have specialized regions where the receptors are concentrated through structural proteins. Namely, postsynaptic density (PSD) regions are suited right across the active zones to facilitate the neurotransmission in the excitatory synapse. In contrast to excitatory synapses, inhibitory synapses do not have PSD but still have specialized composition [22].

Depending on either excitatory or inhibitory synapse, synaptic region composition differs in terms of receptors, structural- and cell adhesion proteins. Excitatory postsynaptic density regions are rich in ionotropic- (AMPA and NMDA receptors) and metabotropic glutamatergic receptors (mGlu receptors) and their corresponding structural proteins for stabilization [23, 24]. On the other hand, inhibitory postsynaptic regions are rich in ligand-gated chloride ion channels that are GABA- and glycine receptors. In addition to structural proteins for the localization and stabilization of the postsynaptic receptors, intracellular signaling pathways play a role in the dynamics of receptors on the membrane [25, 26, 27]. The synaptic cleft is the physical gap between presynaptic- and postsynaptic neurons. The extracellular matrix fills the synaptic cleft, which includes the cell adhesion molecules and fibrils considered essential for stabilizing synapses at the right positions [28]. Besides, glial cells are located around the synaptic cleft, where they reuptake of neurotransmitters and endocytose into the presynaptic cells.

Age-related changes in cognitive impairments have been attributed to the neuronal and synaptic loss during healthy aging. Contrary to this, recent studies with the improvement in the stereological methods showed that neuron and synapse numbers preserved with aging [29, 30, 31, 32]. Therefore, cognitive impairments can be associated with the subtle molecular and cellular changes in the key synaptic proteins, as well as the alterations in the excitatory/inhibitory (E/I) balance instead of due to the alterations in neuron or synapse numbers [33, 34].

1.4 Neuroinflammation and Brain Aging

The blood-brain barrier shields the central nervous system (CNS) from the circulatory lymphocytes, cytokines, and chemokines under homeostatic conditions. The specialized endothelial cell (EC) forming the blood vessel wall around the brain provides a tight regulation for movements of molecules in and out and protect the CNS from infectious or toxic agents [35]. The innate immunity in the central nervous system is provided by CNS-specific cell types: microglia and astrocytes. Microglia are the pivotal immune cells of the CNS, whose progenitor cells originate from the yolk sac during the embryonic developmental stage[36]. They constitute around 10-15% of the total cells in the CNS [37]. After completion of embryonic development and BBB construction, they become resident innate immune cells of the CNS. Microglial cells have a highly plastic feature, which is tightly regulated with the interaction in their microenvironment as well as other CNS cells, including astrocytes, neurons, and oligodendrocytes. High plasticity provides microglial cells with morphological changes in response to a variety of threats, including aging, infectious agents, and trauma [38, 39]. Under normal physiological conditions, they are found in a ramified form. In response to various threats, they undergo morphological and molecular alterations named primed state. They become de-ramified morphologically and more responsive to the insults with elevated expression of neuroinflammatory markers and lowered threshold for their activation. Also, they demonstrate age-related alterations in the transcriptional profile [39].

On the other hand, astrocytes are the most abundant cell types in the CNS, whose progenitor cells originate from neural stem cells (NSCs) differently from the microglia [40]. They provide various functions with neurons, including physical support, protection against excitotoxicity by removing excess neurotransmitters from the synaptic cleft, and maintaining the blood-brain barrier [40, 41]. As in the microglial cells, they display high plasticity and go through morphological and molecular alterations as a response to changes in their microenvironments due to various insults. The reactive form of astrocytes in the aged brain has short and stubby processes compared to that of young ages [42]. Upregulation of glial fibrillary acidic protein (GFAP) is considered as a marker for activated astrocytes [12], and the interplay among the astrocytes and microglial cells through cytokines play a significant role in the fate of neuroinflammation [43].

With advancing age, dysregulation of homeostasis in the immunity of CNS leads to polarization in the phenotype of microglia and astrocytes to activated reactive form. Phenotypic alterations in the glial cells are accompanied by the elevation in pro-inflammatory cytokines, including IL-6, TNF-alpha, IL-1beta, which ultimately lead to chronic low-level inflammation [38] Persistent inflammation in CNS is thought to be one of the reasons behind age-related cognitive deficits, as well as predispositions to neurodegenerative diseases [44].

1.5 Calorie Restriction and Brain Aging

Caloric restriction (CR) is a well-studied approach, which increases both lifespan and healthspan of the organisms without genetic interventions to cope with the detrimental effects of aging [80]. Even though there are controversial results in CR's effectiveness on the extension of lifespan and healthspan of the living beings, its effectiveness was shown on various living beings, including yeast, nematodes, fruit flies, and rats [45, 46, 47]. It is based upon varying levels of reduction (20-40%) in the daily caloric intake of organisms without causing an imbalance or malnutrition in macronutrients, including carbohydrate, protein, fat, as well as vitamin and mineral sources [48]. However, there is no one agreed way for applying CR regarding the period, amount, and the way of restriction.

There are two widely used approaches in applying CR: Chronic Calorie Restriction (CCR) and Intermittent Fasting (IF). In the context of brain aging, both ways have been proven to alleviate age-related deterioration [49]. The basis of CCR is the sustained restriction in the daily calorie intake throughout the study, whereas IF is based on every-other-day feeding. IF has started to gain attention since it can be easily sustained throughout the lifetime compared to the constantly applied CR approaches. In addition to the every-other-day feeding way of IF, there is a unique approach, which is a cyclic manner regimen based on a restriction period (severe than CCR) followed by a non-restricted (ad-libitum) period and named as Intermittent Calorie Restriction (ICR) [48, 50]. Generally, CR has been studied in the cross-sectional studies in a life-long manner [51, 52, 53, 54, 55]. But still, short-term CR applications have gained considerable attention for the understanding mechanism of dietary restrictions as well as

determine the shortest period to have significant impacts against the detrimental effects of aging. Such studies are especially important for the translational potential of CR applications.

Although CR's impacts on the lifespan and healthspan have been introduced in many organisms, CR's molecular mechanism has not been elucidated completely. It has been proposed that it alters the molecular pathways that are involved in energy metabolism, including Mammalian Target of Rapamycin (mTOR), Insulin-like Kinase-1 (IGF-1) signaling, and Adenosine Monophosphate-Activated Protein Kinase (AMPK) signaling [56, 57, 58]. In addition to them, CR attenuates aging's detrimental effects by reducing ROS, oxidative stress, and inflammation. It also leads to the epigenetic modifications on both histone proteins and DNA that ultimately affect the autophagy pathways against aging [56, 57].

On the other hand, there have been findings suggesting a stabilizing effect of CR on the key synaptic proteins, including synaptophysin, NR2A, NR2B, GluR1, and GluR2 [47]. Its neuroprotective effect has been suggested through multiple studies, in which CR increased brain-derived neurotrophic factor (BDNF) level. Due to the role of BDNF in neuronal survival, beneficial effects of CR have been associated with the adult neurogenesis as well [59]. Hence, CR has been implementing widely to ameliorate age-related deteriorations by altering multiple molecules and pathways.

1.6 Mouse as a Model Organism

There are a variety of different model organisms in gerontology studies. In a broad sense, it can be divided into two: mammalian model organisms and non-mammalian model organisms. Each model organism, being either mammal or non-mammal, has its advantages and disadvantages depending on the research questions. Regarding translational research, mammalian model organisms have the priority due to commonalities with humans, including a high percentage of genome similarity, conserved genome sequences, and protein domains [60].

The prevalence of mice as a model organism in aging studies has started around the 1970s after caloric restriction experiments, which were initially done for cancer tumorigenesis studies. Those studies facilitated the availability of the colonies of the aged-rodents, thereby they become prevalent in aging studies as well [61]. However, because of the concerns about the fluctuation in the female mice's hormonal level, scientific research in the aging has been dominated exclusively by male mice. Although it secures against hormonal changes, it stunts the understanding of brain-aging comprehensively.

In this study, we utilized healthy MMTV-TGF-α (C57BL/6) female mice. Those mice overexpress human transforming growth factor-α (TGF-α) under the control of mouse mammary tumor virus promoter (MMTV). TGF-α acts through epidermal growth factor-receptors (EGF-R/ErbB), which initiates a signaling cascades controlling cell proliferation, survival, migration, and differentiation [62] . The overexpression of TGF-α in mammary epithelium is associated with tumor development in the mammary glands around their middle-ages [63, 64]. In that sense, it is a widely used mouse model in breast cancer studies.

Regarding brain aging studies, it is also an important model organism since aging does not come about alone. In fact, aging is a process accompanied by a variety of predispositions to diseases. Hence, it is essential to understand how we age when we have a genetic susceptibility to illness.

All in all, mice as a mammalian model organism have various advantages, including high similarity in terms of the genome sequence, protein structures, and conserved pathways [60]. The inclusion of females and transgenic strains into aging studies is essential to comprehend the brain's aging process widely. Hence, the knowledge acquired from animal studies can be more comprehensive when the most suitable model organism is utilized.

1.7 Aim of the Study

Nowadays, there is a gradual increase in the lifespan of humans following the advancement in the field of medicine [65]. However, extension in the lifespan is not followed by that of healthspan. People with a higher life expectancy suffer from age-related deterioration for a more extended period. Hence, comprehension of how healthier brain aging can be achieved becomes significant for people to sustain independent and disease-free life.

Calorie restriction is a well-studied approach, which trustily increases both lifespan and healthspan of the organisms without genetic interventions [80]. Although we have non-negligible knowledge regarding CR and its impacts on the brain, it is still not understood fully. Also, there is no one agreed way for the application of CR in terms of restriction amount and period of application. On

the other hand, both brain aging and CR studies have been male-dominated due to the concerns about the hormonal fluctuation in females, which stunts the understanding of brain-aging comprehensively.

Here in this thesis, we firstly aimed to show age-related changes in the cellular and synaptic proteins as well as the inflammatory state of the female mice's brains. The second aim of the study is to demonstrate the effects of varying types of dietary restrictions implemented for the short-term and the long-term manner on the same topics and the potential of dietary regimens to slow down the age-related alterations.

Chapter 2

Methods

Section titles marked with an asteriks (*) indicates that those prodecures were performed by the team of Doğan-Lab at Yeditepe University.

2.1 *Animals

MMTV-TGF-α (C57BL/6) male mice were donated by Dr. Margot Cleary, Hormel Institute Medical Research Center, University of Minnesota. The breeding colony was established at Yeditepe University, YUDETAM. MMTV-TGF-α positive female mice were obtained by crossing the following mice: MMTV-TGF-α (C57BL/6) positive male with MMTV-TGF-α (C57BL/6) negative female. During the experiments, animals were subjected to a controlled environment ($21 - 24^oC$ temperature and 12h/12h light cycle). Also, they had free access

to water. Their medical conditions were checked on a daily basis. All the experimental procedure applied to animals was approved by Yeditepe University Animal Care and Use Committee.

2.2 *Experimental Setup

In this study, thirty-six female MMTV-TGF-α (C57BL/6) mice were used. The distribution of the animal numbers by experimental groups was indicated in Table 2.1. Feeding experiments were started when mice were 10 weeks old (Young Adult). A set of mice was sacrificed at the beginning of the feeding experiments to form a baseline. They were also named as Baseline (10-weeks). Other animals were randomly assigned to three different diet groups: Ad libitum(AL), Chronic Caloric Restriction (CCR), and Intermittent Caloric Restriction(ICR). Mice were fed with a standard pathogen-free food – Altromin TPF 1414, provided from Kobay A.Ş (Ankara, Turkey).

Mice in the AL group had free access to food until they were sacrificed. Mice in the CCR group were subjected to 15% restriction in their daily caloric intake compared to AL-fed mice. Mice in the ICR group were subjected to 60% restriction for a week, then 60% restriction was followed by three-week ad-libitum feeding. This four-week cyclic regimen was applied until the designated time-points. Mice in the ICR group was further divided into two groups based on which stage of the cyclic regimen they were sacrificed. A group of mice being sacrificed right after the 60% restriction period was named ICR-Restriction (ICR-R); on the other hand, a group of mice being sacrificed after the ad-libitum period was called ICR-Refeed (ICR-RF).

Animals were subjected to the feeding regimens as mentioned above until two designated time points: 49/50-weeks (Middle-Aged) and 81/82-weeks (Old). All mice were sacrificed after overnight fasting. Mice in the ICR-RF group were sacrificed in either 49-weeks or 81-weeks, which correspond to the end of the refeeding period. Mice in the ICR-R group were sacrificed in either 50-weeks or 82-weeks, which correspond to the end of the restriction period. Mice in the AL and CCR groups were sacrificed in all four designated weeks (49-week,50-week,81-week, and 82-week). Since the animals sacrificed at consecutive weeks did not show a significant difference in terms of weight, size, or medical conditions, they were considered as one time period as 49/50-weeks and 81/82-weeks (Figure 2.1).

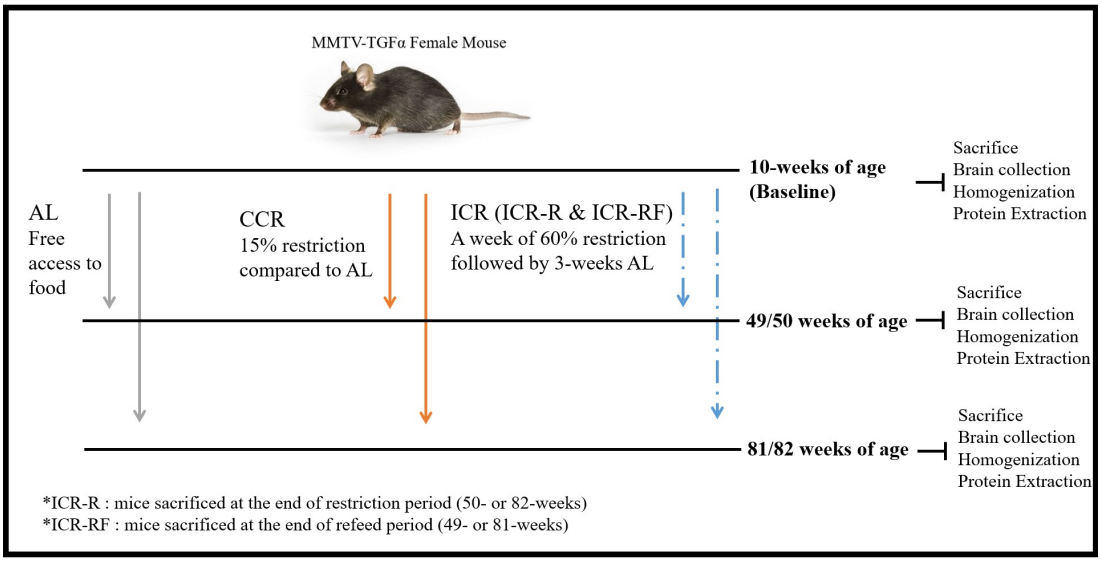

Figure 2.1: Figurative Representation of Experimental Setup

Table 2.1: Number of animals by experimental groups

	Ad Libitum	CCR	ICR-R	ICR-RF
Baseline (10-week)	4	-	-	-
49/50 week	4	4	4	4
81/82 week	4	4	4	4

2.3 *Sample Homogenization

Mice were sacrificed at the designated time-points. First of all, whole brains were collected then they were cut into half through a sagittal plane. One-half of the brains were immediately frozen via dry-ice, and the other halves were utilized for the pathological examinations.

Frozen brain tissues were used for homogenization. Firstly, brain tissues were weighed then were cut into small pieces to ease the homogenization. And then, they were washed with 600 μL of 1X PBS to remove surface contaminants. The washing step was performed three times and through pipetting up and down. After the washing, a spoon of 0.5 mm zirconium oxide beads (ZROB05) was placed on the samples. 1X PBS Buffer was used as a homogenization buffer. The volume of PBS Buffer was determined according to the weight of the brain samples (that is, 400 μL of PBS was added for 200 mg of brain sample). Then the cap of eppendorf was closed tightly and covered by using parafilm. Homogenization

was performed by means of Bullet Blender at Speed 8 for 3 min. When homogenization was accomplished, zirconium beads were removed by centrifugation at max rpm for 10 min at +4°C. Homogenized brain tissues were collected into a new 1.5 mL eppendorf and stored at -86°C for further experiments.

2.4 Protein Extraction from Mouse Brain Homogenates

The brain homogenates which were homogenized in ice-cold, sterile 1X PBS by the members of Doğan Lab in Yeditepe University were taken from -80°C refrigerator. For each group of samples, 50 μL of brain homogenates were used for protein extraction purposes. They were thawed on ice then mixed well by pipetting before centrifugation. First of all, PBS was removed from each of the samples by centrifugation at 14.000 rpm for 10 min at +4°C. Resulting supernatants were discarded then pellets were dissolved in 300 μL of radioimmunoprecipitation assay (RIPA) buffer [0.25%(w/v) Sodium Deoxycholate, 150-mM Sodium Chloride, 50-mM Tris-HCl pH 8.0, %1(v/v) NP-40, 1.0-mM EDTA, 1X Protease Inhibitor (05 892 970 001, Roche) and 1%(v/v) Phosphatase Inhibitor (P0044, Sigma)]. The amount of RIPA buffer was decided based on the previous optimization experiments. Afterward, each sample was waited on ice for 30 min and tapped gently every 10 min interval. They were then centrifuged at 13.000 rpm for 20 min at +4°C, and supernatants were carefully collected into new eppendorfs. Supernatants were aliquoted to decrease the negative effect of the thaw/freeze cycle on proteins. The recipe of RIPA Buffer is shown in Table 2.2

Table 2.2: Radioimmunoprecipitation Assay (RIPA) Buffer Recipe

RIPA Buffer	
Materials needed	Volume(16 mL)
2M NaCl	1.2 mL
1M Tris-HCl, pH 8	800 µL
100% NP-40	160 µL
0.5M EDTA	32 µL
Na-Deoxycholate(powder)	0.04 g
2X Protease Inhibitor	8 mL
100% Phosphatase Inhibitor	160 µL
Double-Distilled Water	5.648 mL

2.5 Bradford Assay

To determine the concentration of soluble protein extracts, Bradford Assay was used. The assay was performed on a 96-well plate. As a standard, Bovine Serum Albumin (BSA, 1mg/mL) was used in different concentrations with a range of 2 μg/mL – 20 μg/mL.

First of all, ddH_20 was added into the 96-well plate, as indicated in Table 2.3. After then, BSA (A7906, Sigma-Aldrich, St. Louis, MO, USA) and the unknown protein samples were loaded accordingly. Blank, standards, and samples were loaded in duplicates. Lastly, 250 μL of Bradford Assay Reagent (B6916, Sigma, St. Louis, MO, USA) was added onto each well. The plate was shaken on an orbital shaker at 250 rpm for 45 seconds at room temperature then placed

into a dark place for 10 min incubation without shaking. In the meantime, the Microplate Reader (SpectraMax M5, Molecular Devices, Sunnyvale, CA, USA) was turned on and was allowed for being calibrated. Before taking measurements, the presence of air-bubbles on each well was checked, and they were blown up, if any. The absorbances were obtained at 595 nm wavelength. The absorbance of the blank was substrated automatically by the SoftMax-Pro Software. A standard curve was plotted based on the net absorbances of standards (Figure 2.2). Then the concentrations of the unknown proteins were calculated according to the linear equation of the standard curve.

Table 2.3: Standard and unknown protein preparations for Bradford Assay

	BSA(1mg/mL) (µL)	ddH_20 (µL)
Blank	0	5
Standard 1	0.5	4.5
Standard 2	1	4
Standard 3	2	3
Standard 4	3	2
Standard 5	4	1
Standard 6	5	0
Unknown Protein	0.5	4.5

Table 2.4: Representative plate design for Bradford Assay

	1	2	3	4	5	6	7	8
A	Blank	Blank	Sample 1	Sample 1	Sample 8	Sample 8	Sample 15	Sample 15
B	Standard 1	Standard 1	Sample 2	Sample 2	Sample 9	Sample 9	Sample 16	Sample 16
C	Standard 2	Standard 2	Sample 3	Sample 3	Sample 10	Sample 10	Sample 17	Sample 17
D	Standard 3	Standard 3	Sample 4	Sample 4	Sample 11	Sample 11	Sample 18	Sample 18
E	Standard 4	Standard 4	Sample 5	Sample 5	Sample 12	Sample 12		
F	Standard 5	Standard 5	Sample 6	Sample 6	Sample 13	Sample 13		
G	Standard 6	Standard 6	Sample 7	Sample 7	Sample 14	Sample 14		
H								

Table 2.5: Absorbance values of standards and samples obtained at 595 nm

	1	2	3	4	5	6	7	8
A	-0.003	0.003	0.167	0.206	0.155	0.154	0.180	0.189
B	0.021	0.029	0.154	0.163	0.157	0.162	0.189	0.167
C	0.066	0.057	0.136	0.146	0.168	0.153	0.166	0.192
D	0.128	0.124	0.133	0.131	0.113	0.132	0.180	0.165
E	0.197	0.207	0.153	0.154	0.139	0.161		
F	0.275	0.270	0.122	0.152	0.175	0.141		
G	0.341	0.343	0.142	0.163	0.185	0.141		
H								

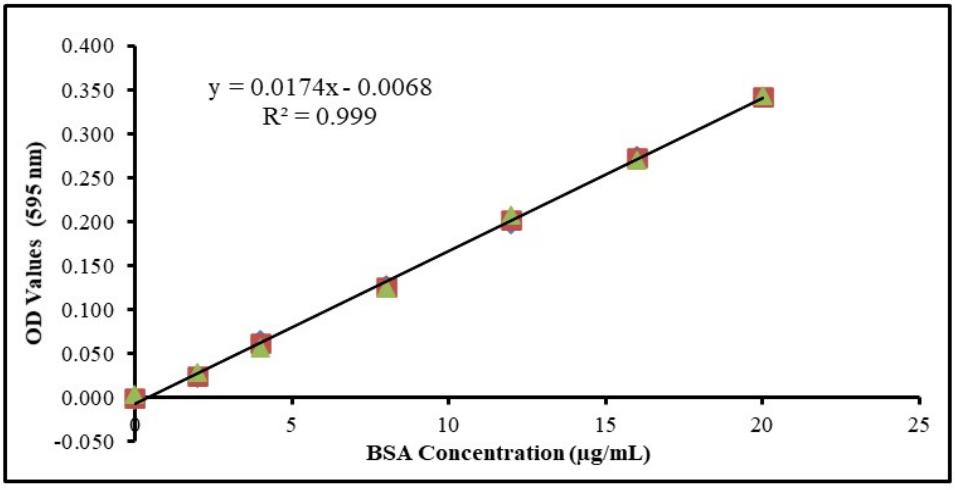

Figure 2.2: Standard curve of BSA Standards

2.6 Western Blot

Western blotting is a widely used laboratory method to differentiate a protein of interest from a mixture and/or to see the changes in the amount of protein across treatments [66].

2.6.1 Sample Preparation for Western Blot

The amount of protein loaded into the wells was determined by optimization experiments. To avoid any saturation on the band intensity, proteins were prepared in two different quantities as 20 μg for GEP [67] and SYN [68], and as 40 μg for PSD-95 [69], DCX [70], IL-6 [71], TNF-alpha [72], and GFAP [73]. Loading control - Actin [74] worked well in both quantities without saturation. According to the required amount of proteins, proteins were diluted with ddH_20, then mixed with 2X Loading Dye (Table 2.6). 10% DTT was added freshly into 2X Loading Buffer in the ratio of 1:9. Samples were boiled at 95°C for 5 min before loading into wells. After heating, they were quick spun and waited on ice.

Table 2.6: 2X Loading Buffer Recipe

2X Loading Buffer	
Materials needed	Volume(mL)
10% SDS	4
20% Glycerol	2
4% BPB	0.1
0.125-M Tris, pH 6.8	2.5
10% DTT to be added each time freshly	

2.6.2 SDS-PAGE Gel Preparations

According to the size of the protein of interests, two different separating gel concentrations were used. 10% separating gel was used to separate the size of proteins in the range of 110 kDa - 35 kDa, and 12% separating gel was used for the proteins whose sizes are in the range of 50 kDa - 15 kDa. The stacking gel concentration was 5% for both. For both concentrations of separation gel, glass plates with 1.5 mm integrated spacers were used. They were covered with a short plate then slid into the casting frame as the short plate facing forward. Before pouring the gel mixture, gel cassettes were checked against any leakage. Depending on the protein of interests, either 10% or 12% of separating gel was prepared, as shown in Table 2.7. The gel was poured into the gel cassette via pipette. Isopropanol alcohol was added on top of the separating gel in order to flatten the gel and aid polymerization. When the gel was polymerized, isopropanol alcohol was discarded. Then the stacking gel solution, which was prepared, as indicated in Table 2.8, was poured over the separating gel. Air-bubble was blown up, if any. A 15-well comb was placed carefully. The gels were left to be polymerized for about 20 min.

Table 2.7: Separating Gel Recipe for 10% and 12% concentrations

Separating Gel (10%)	
Materials needed	Volume (18 mL)
ddH2O	8.6 mL
1.5 M Tris pH 8.8	4.5 mL
40 % Acrylamide	4.5 mL
10% SDS	180 µL
10% APS	180 µL
TEMED	18 µL

Separating Gel (12%)	
Materials needed	Volume (18 mL)
ddH2O	7.7 ml
1.5 M Tris pH 8.8	4.5 mL
40 % Acrylamide	5.4 ml
10% SDS	180 µL
10% APS	180 µL
TEMED	18 µL

Table 2.8: Stacking Gel Recipe for 5% concentration

Stacking Gel (5%)	
Materials needed	Volume (10 mL)
ddH2O	6.0 mL
0.5 M Tris pH 6.8	2.5 mL
40 % Acrylamide	1.25 mL
10% SDS	100 µL
10% APS	100 µL
TEMED	10 µL

2.6.3 Sample Loading and Electrophoresis

Gel cassette sandwiches were removed from the casting frame carefully. Residual polymerized gel outside of the gel cassette sandwiches was cleaned with double-distilled water. Then they were placed into the electrode assembly as the short plate facing inward. Any leakage was checked, then the tank was filled with 1X Running Buffer (Table 2.9) until a two gel level. The combs were taken out carefully without disrupting the wells. Before loading the samples, they were mixed well then were quick spun. Each sample was loaded carefully in order not to introduce any bubble into wells. A protein ladder (26616, Thermo Scientific Paisley, UK) was loaded as 2μL before the first sample and after last the sample. The remaining empty wells were loaded with 2X loading dye so that samples could run in a balance. As an electrophoresis system, Mini-PROTEAN Tetra Cell (BioRad, CA, USA) was utilized. Samples were run for 30 min at 90V to align perfectly before being separated according to size. After then, the voltage was increased to 120V at which the samples were run until loading dyes reach the end of the gel. The entire running process had lasted around 2-2.5 hours.

Table 2.9: 10X Running Buffer Recipe

10X Running Buffer, pH 8.3	
Materials needed	Volume (1L)
250 mM Tris	30.285 g
1.9 M Glycine	144.134 g
10% SDS	100 mL
ddH2O	Up to 1 L

2.6.4 Electrotransfer of Samples

Ten to fifteen minutes before the end of the electrophoresis, Polyvinylidene difluoride (PDVF) membranes were soaked into 100% methanol for the activation. After 3 min incubation in the 100% methanol, the membranes were transferred into ice-cold 1X Transfer Buffer (Table 2.10). In the meantime, transfer equipment: Whatman filter papers, gel holder cassette, and foam pads were also equilibrated in ice-cold 1X Transfer Buffer. Upon completing the electrophoresis, gel cassette sandwiches were taken from the chamber, and then the short plates were removed via gel releasers. After discarding the stacking gel, the transfer sandwich was assembled, as indicated in Figure 2.3.

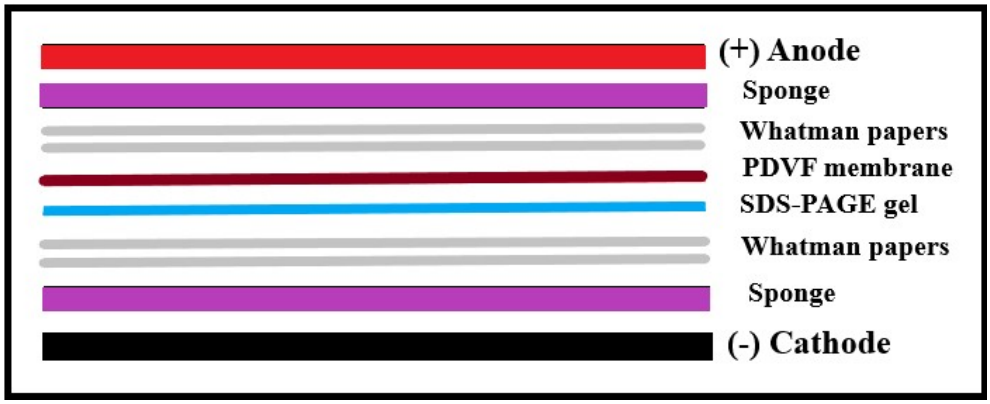

Figure 2.3: Figurative Representation of Electrotransfer Assembly in Western Blot

The transfer sandwich was slid into the electrode assembly. The cooling unit was placed into the tank. The tank was filled with ice-cold 1X Transfer Buffer then covered by the lid. The transfer was carried out in the Mini Trans-Blot Cell Module (BioRad, CA, USA) in a cold room. Transfers of PSD-95 [69], GEP [67], DCX [70], and SYN [68] were performed at 100 V for 90 min, whereas transfers of IL-6 [71], TNF-alpha [72], and GFAP [73] were performed at 90 V for 75 min.

Table 2.10: 1X Transfer Buffer Recipe

1X Transfer Buffer	
Materials needed	Volume (2L)
25-mM Tris	6 g
195-mM Glycine	28 g
Methanol	400 mL
ddH2O	Up to 1.6 L

2.6.5 Blocking and Antibody Incubations

Around thirty minutes before the end of the electrotransfer, 5% blocking solution was prepared in a 50 mL falcon. The type of blocking solution was determined according to the optimization experiments of antibodies. In this study, all antibodies performed better with 5% non-fat dry milk in 1X-TBS-T (Tris Buffered Saline - 3% Tween 20) (Table 2.11). Depending on the required volume of the blocking solution, non-fat dry milk powder was weighed and dissolved in 1X TBS-T. Then it was left on the shaker in the cold-room.

Table 2.11: 10X TBS Recipe

10X TBS, pH 7.6	
Materials needed	Volume (2L)
100 mM Tris	12 g
1.5 M NaCl	88 g
ddH2O	Up to 1L

After the electrotransfer of the proteins, PDVF membranes were taken from the transfer system and then was put into 1X-TBS-T. Membranes were cut into smaller pieces according to the position of protein interests. In this way, more than one protein of interest could be checked per each running. Then they were blocked by 5% dry-milk in 1X-TBS-T for an hour at room temperature on a shaker. After the blocking step, they were washed with 1X TBS-T for 5 min on a shaker then incubated with the primary antibody (Table 2.12) at $+4^{o}C$ for overnight.

Table 2.12: Primary antibody list used in western blot experiments

Antibody Name	**Product Code**	**Working Concentration(v/v)**	**Host Species**
Abcam anti-PSD-95 Antibody	ab18258	1:10000	Rabbit polyclonal
Abcam anti-Gephyrin Antibody	ab185930	1:2000	Rabbit polyclonal
Abcam anti-Double Cortin Antibody	ab18723	1:1000	Rabbit polyclonal
Abcam anti-Synaptophysin Antibody	ab32594	1:20000	Rabbit polyclonal
Abcam anti-GFAP antibody	ab53554	1:2000	Goat polyclonal
Novus Biologicals IL-6 Antibody	NB600 - 1131	1:4000	Rabbit polyclonal
Novus Biologicals TNF-alpha Antibody - Azide Free	NB600 - 587	1:2000	Rabbit polyclonal
Abcam anti-Actin antibody -Loading Control	ab1801	1:2000	Rabbit polyclonal

After overnight incubation with the primary antibody, membranes were washed three times with 1X TBS-T for 10 min. Then they were incubated with secondary antibody(Table 2.13) at room temperature for an hour. After secondary antibody incubation, membranes were washed three times with 1X TBS-T for 10 min and become ready for chemiluminescent detection.

Table 2.13: Secondary antibody list used in the western blot experiments

Antibody Name	Product Code	Working Concentration(v/v)	Host Species
Cell Signalling Technology Anti-rabbit IgG, HRP-linked Antibody	CST-#7074S	1:5000	Goat
Abcam Rabbit Anti-Goat IgG H&L (HRP)	ab97100	1:5000	Rabbit

2.6.6 Chemiluminescent Detection

Around ten minutes before taking images, the Biorad-ChemiDocTM XRS+ imaging system (Biorad, CA, USA) was turned on. Then, the tray was cleaned by 70% Ethanol. For the detection purpose, Supersignal West Femto Maximum Sensitivity Substrate (Thermo-scientific, Rockford, IL, USA: 34095) was used.

When the imaging system becomes ready, the chemiluminescent solution was prepared by mixing the enhancer solution and the peroxide solution in a ratio of 1-1. The membrane was placed onto a clean and flat surface; then, it was covered by the chemiluminescent solution. After 5 min incubation of the membrane with the chemiluminescent solution at dark, the membrane was interlaid in a clean

piece of a sheet protector. After the excess solution was dried by a towel paper, the protein of interest was visualized. The exposure time was varied depending on the antibody and protein of interests. It was optimized for intense band by Image Lab 6.1 Software to avoid signal saturation.

2.7 Image Quantification

All image quantifications were performed in a double-blind manner. Gel images were exported from Image Lab 6.1 Software for quantification. Band intensity measurements were done through the ImageJ program (NIH, Bethesda, MD, USA). Exported images were opened by the ImageJ, and then their types were changed to 16-bit from the RGB scale. Each band was enclosed with a rectangle. The size of the rectangle was the same for all the bands in the same image. After the selection of band areas, each lane was plotted. A straight line was drawn to the bottom side of the curve to define the measurement area. It is also essential to eliminate any background effect on the band intensity measurement. The area under the curve was measured, then normalization steps were followed.

There were two different normalization approaches applied to data. The first one was within gel normalization. The aim of within-gel normalization was to minimize the differences among technical replicates, which might have happened due to variation in exposure time or experimental process. Firstly, all band intensities of an antibody in the same gel were averaged. Then each band intensity of that antibody, which was in the same blot, was divided by the averaged value. This procedure was applied to all antibodies, including the loading control. The second normalization approach was related to the internal loading control, where

each band intensity of an antibody was divided by band intensity of its corresponding loading control (i.e., Actin). Loading-control normalization was applied after the within-gel normalization. The second normalization is essential for eliminating errors that might have occurred during the sample loading part.

2.8 Statistical Analysis

Statistical analysis was performed by using IBM SPSS Statistics 22 Software (IBM, USA). The results were shown as mean ± SEM . The sample size for each experimental group was four (n=4). For each biological replicate, there were two technical replicates.

The homogeneity of variance and the normality were checked by Levene's Test and Kolmogorov-Smirnov Test, respectively. Analysis of Variances(ANOVA) was implemented when both assumptions were not violated. One-Way ANOVA was performed with a factor of duration with three levels (10 weeks, 49/50 weeks, and 81/82 weeks) to see whether the duration has an impact on the protein level of ad-libitum fed mice. Two-Way ANOVA was carried out with a factor of duration with two levels (49/50 weeks and 81/82 weeks) and a diet factor with four levels (AL, CCR, ICR-R, and ICR-RF). Pairwise comparisons were performed via Tukey's Post Hoc Test whenever required. A simple effect test was followed whenever there was a statistically significant main effect (either duration or diet) or interaction.

Kruskal Wallis Test was applied to a group of protein data when both assumptions were violated. Three Kruskal Wallis Tests were used to those data to analyze the effects of independent variables. The first one was performed with a factor of duration with three levels (10 weeks, 49/50 weeks, and 81/82 weeks) to examine if the duration affects the protein level of AL-fed mice. Since the Kruskal Wallis Test is not able to deal with two independent variables together, the effects of duration and diet were analyzed one by one. Whenever there was a statistically significant duration or diet effect, Kruskal Wallis Test was followed by Mann-Whitney U Test for pairwise comparison. P-value adjustment was done and stated where necessary so that the accumulation of Type I error could be eliminated.

Chapter 3

Results

3.1 Cellular markers for proliferation

3.1.1 Doublecortin (DCX)

Doublecortin (DCX) is an essential protein guiding the immature neurons throughout their migration during developmental stages and adult neurogenesis [14]. The protein level of DCX was investigated to comprehend the age- and caloric restriction (CR) -related alterations in the proliferative state of the brain.

Following the validation of the normality and equal variances, Analysis of Variances (ANOVAs) was performed on normalized protein data. One-way ANOVA test showed no significant effect of duration on the protein level of DCX on ad-libitum fed mice at the $p < .05$ level ($F_{(2,9)} = 0.185$, $p = .835$). This result indicates that the protein level of DCX tends to be stable in the whole brain of ad-libitum fed mice with advancing duration/age.

On the other hand, Two-way ANOVA revealed a significant main effect of duration on the protein level of doublecortin on mice subjected to different dietary regimens at the $p < .05$ level ($F_{(1,24)} = 6.010$, $p = .022$). A simple effect analysis was carried out to understand better the effect of duration on different dietary regimen groups. It was revealed that the effect of duration was statistically significant on the Chronic Caloric Restriction (CCR) group at the $p < .05$ level ($F_{(1,24)} = 9.490$, $^{*}p = .005$). In fact, the protein level of doublecortin tends to decrease with increasing duration of the aforementioned dietary regimen, whereas other dietary regimens tend to be stable with advancing duration/age.

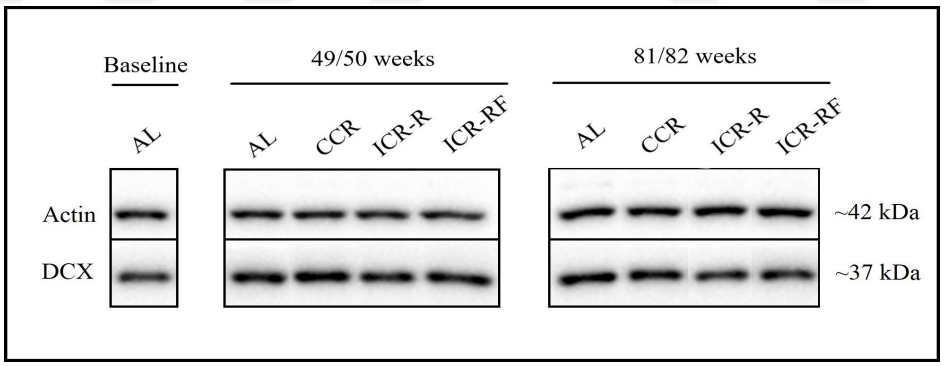

Figure 3.1: Representative immunoblot image of Doublecortin and Actin (loading control). Each band was labeled according to the duration and dietary regimen.

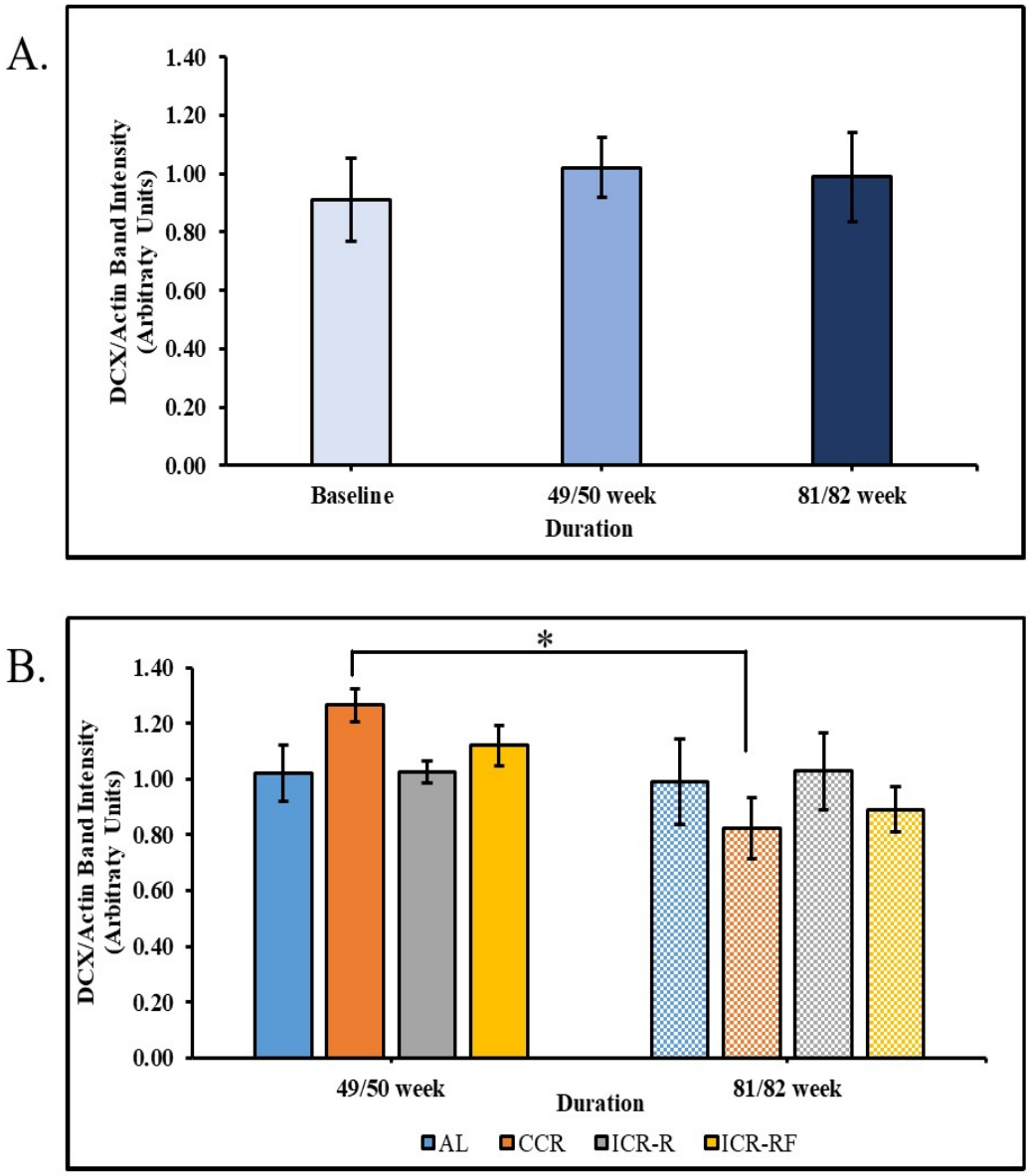

Figure 3.2: **Effects of varying levels of calorie restriction at differing durations on the protein level of Doublecortin in the whole brain of MMTV-TGF-α female mice.**(A) Bar graph for normalized band intensities of DCX for ad-libitum fed animals (n=4 per group; 2 technical replicates for each animal). Each bar represents the mean± SEM.(B) Bar graph for normalized band intensities of DCX for ad-libitum, CCR, ICR-R, and ICR-RF fed animals at 49/50 weeks and 81/82 weeks (n=4 per group; 2 technical replicates for each animal). Each bar represents the mean± SEM.

3.1.2 Glial fibrillary acidic protein (GFAP)

Glial fibrillary acidic protein (GFAP) is a structural protein that belongs to class III intermediate filaments. It is used to distinguish astrocytes from other glial cells in the central nervous system. In addition to being a marker for astrocytes, its increasing level is associated with neuroinflammation [12]. The protein level of GFAP was investigated to comprehend the age- and caloric restriction (CR) -related alterations in both the proliferative- and inflammatory state of the brain.

Following the violation of the normality and equal variances, Kruskal Wallis H Test was performed on normalized protein data. Kruskal Wallis Test applied to ad-libitum fed mice data showed a statistically significant effect of duration on the protein level of glial fibrillary acidic protein (GFAP) at the $p < .05$ level ($\chi^2(2) = 8.115$, $p = 0.017$). Following the significant p-value, Mann Whitney U Test was applied to see which groups have the statistical significance. It was revealed that the protein level of GFAP tends to be stable until the age of 49/50-weeks (U=1.000, $^{\#}p = 0.043$); however, it tends to increase after 49/50-weeks of age where there was a statistically significant difference among 49/50-weeks- and 81/82-weeks of age at the $p < .025$ level (U=0.000, $^{*}p = 0.021$).

To understand the effects of different dietary regimens (AL, CCR, ICR-R, and ICR-RF) and their durations (49/50-weeks and 81/82-weeks), two more Kruskal Wallis H Test were performed. The test results revealed a significant main effect of duration on the protein level of glial fibrillary acidic protein (GFAP) at the $p < .05$ level ($\chi^2(1) = 23.273$, $p = 0.000$). Following the significant p-value, Mann Whitney U Test was applied to see which dietary groups have the statistical significance by increasing duration. Regardless of the type of dietary regimens, the protein level of GFAP was in a tendency to rise by increasing duration/age in all dietary regimens at the $p < .025$ level. (U=0.000, $^*p$ =0.021)

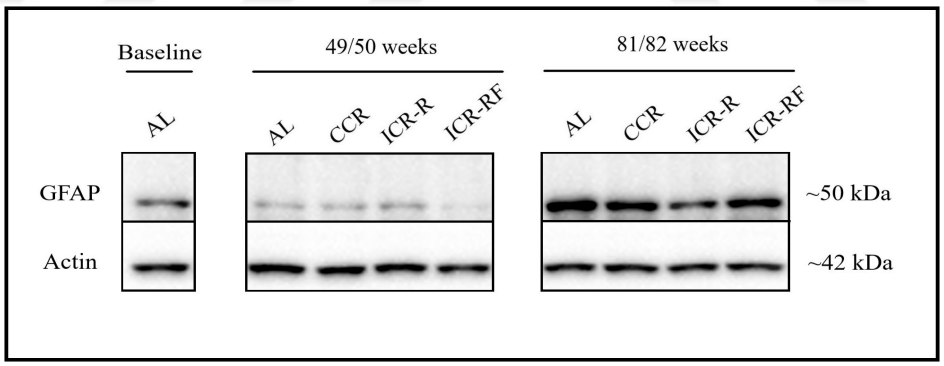

Figure 3.3: Representative immunoblot image of GFAP and Actin (loading control). Each band was labeled according to the duration and dietary regimen.

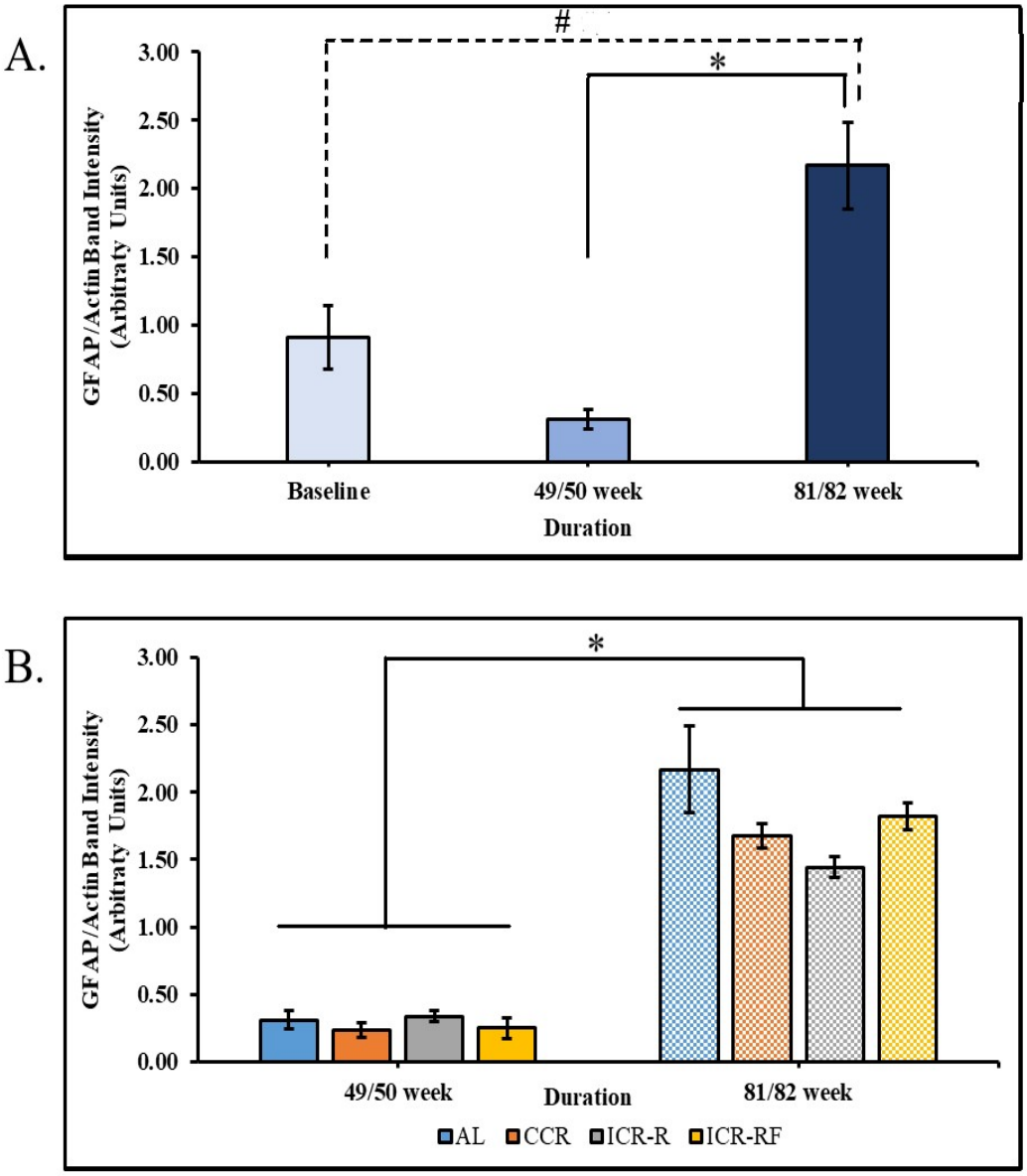

Figure 3.4: **Effects of varying levels of calorie restriction at differing durations on the protein level of GFAP in the whole brain of MMTV-TGF-α female mice.**(A) Bar graph for normalized band intensities of GFAP for ad-libitum fed animals (n=4 per group; 2 technical replicates for each animal). Each bar represents the mean± SEM.(B) Bar graph for normalized band intensities of GFAP for ad-libitum, CCR, ICR-R, and ICR-RF fed animals at 49/50 weeks and 81/82 weeks (n=4 per group; 2 technical replicates for each animal). Each bar represents the mean± SEM.

3.2 Synaptic integrity markers

3.2.1 Postsynaptic Density Protein-95 (PSD-95)

Postsynaptic Density Protein-95 (PSD-95) is one of the main scaffolding proteins localized at the postsynaptic density regions of the excitatory synapses. It regulates the clustering of the key components at the postsynaptic sites, including NMDA receptors, K-ion channels, and cell adhesion molecules [23, 24, 75, 76]. The protein level of PSD-95 was investigated to comprehend the age- and caloric restriction (CR) -related alterations in the integrity of postsynaptic excitatory regions.

Following the violation of the normality and equal variances, Kruskal Wallis H Test was performed on normalized protein data. Kruskal Wallis Test applied to ad-libitum fed mice data showed that there was not a statistically significant effect of duration on the protein level of PSD-95 at the $p < .05$ level ($\chi^2(2) = .346$, $p = .841$). This result indicates that the protein level of PSD-95 tends to be stable in the whole brain of ad-libitum fed mice with increasing duration/age.

To understand the effects of different dietary regimens (AL, CCR, ICR-R, and ICR-RF) and their durations (49/50-weeks and 81/82-weeks), two more Kruskal Wallis H Test were performed. The test results revealed no significant main effect of duration or diet on the protein level of PSD-95 at the $p < .05$ level ($\chi^2(1) = 2.750$, $p = 0.097$, $\chi^2(3) = 5.287$, $p = .152$,respectively). Hence, it indicates that PSD-95 at the protein level tends to be stable regardless of the dietary regimens and their application periods in the whole brain of MMTV-TGF-alpha female mice.

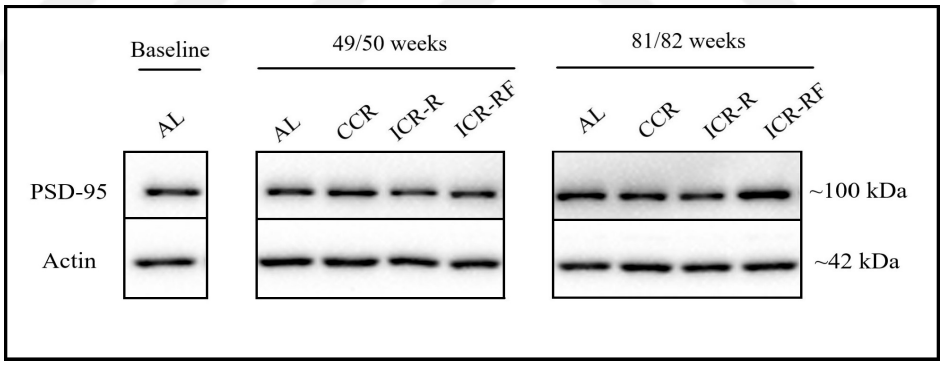

Figure 3.5: Representative immunoblot image of PSD-95 and Actin (loading control). Each band was labeled according to the duration and dietary regimen.

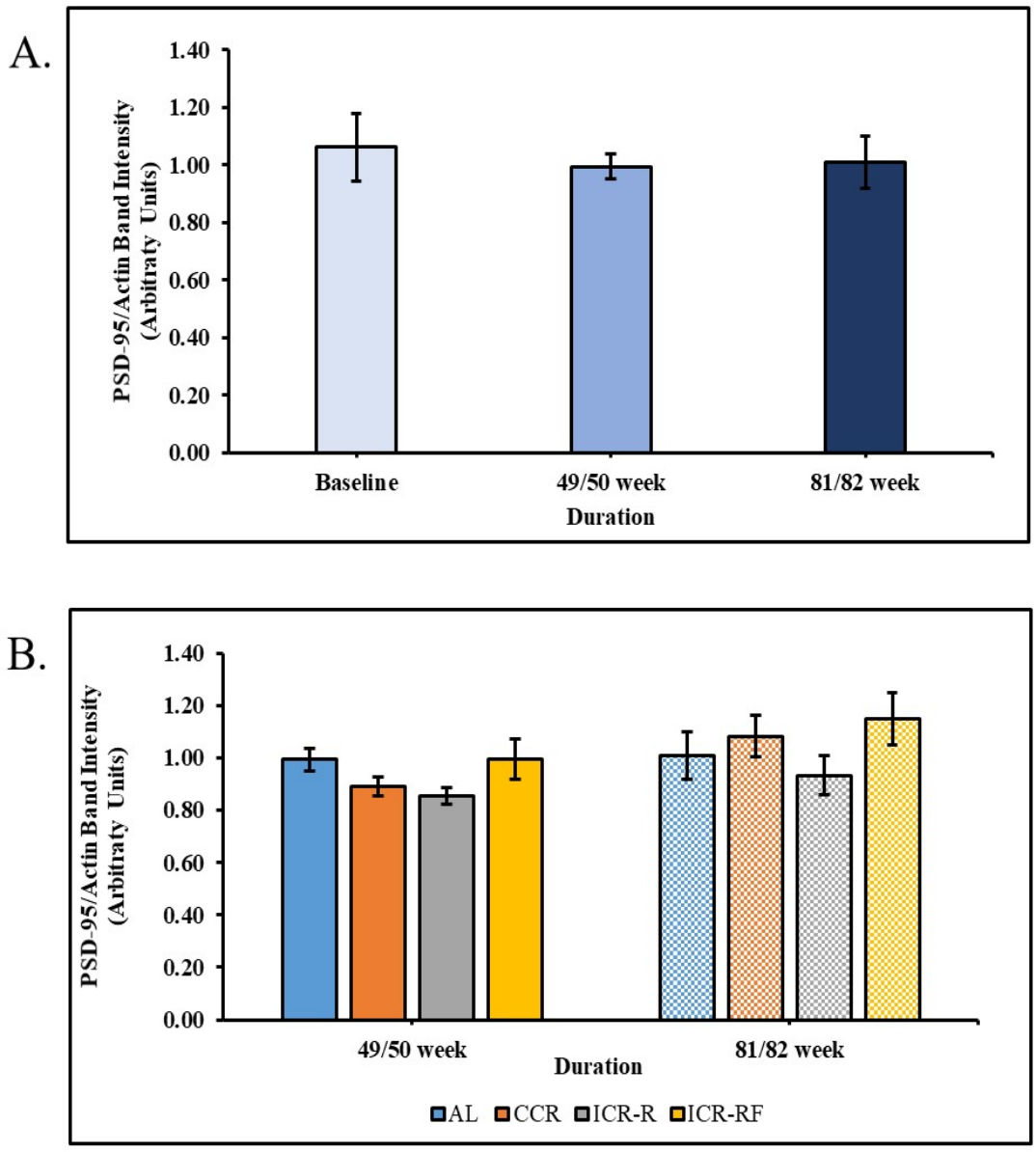

Figure 3.6: **Effects of varying levels of calorie restriction at differing durations on the protein level of PSD-95 in the whole brain of MMTV-TGF-α female mice.**(A) Bar graph for normalized band intensities of PSD-95 for ad-libitum fed animals (n=4 per group; 2 technical replicates for each animal). Each bar represents the mean± SEM.(B) Bar graph for normalized band intensities of PSD-95 for ad-libitum, CCR, ICR-R, and ICR-RF fed animals at 49/50 weeks and 81/82 weeks (n=4 per group; 2 technical replicates for each animal). Each bar represents the mean± SEM.

3.2.2 Gephyrin (GEP)

Gephyrin (GEP) is one of the essential inhibitory postsynaptic scaffolding protein that takes a role in the regulation of the anchoring and stabilization of $GABA_AR$ and Glycine Receptor at the postsynaptic density sites [26, 25]. The protein level of GEP was investigated to comprehend the age- and caloric restriction (CR) -related alterations in the integrity of postsynaptic inhibitory regions.

Following the validation of the normality and equal variances, Analysis of Variances (ANOVAs) was performed on normalized protein data. One-way ANOVA test showed a statistically significant effect of duration on the protein level of GEP on ad-libitum fed mice at the $p < .05$ level ($F_{(2,9)} = 4.312$, $p = .049$). Following the significant ANOVA test result, Tukey's HSD Post Hoc Test revealed a statistically significant difference among Baseline (10-weeks of age) and 49/50 weeks of age at the $p < .05$ level ($^*p = 0.043$) . This result indicates that the protein level of GEP tends to decrease in the whole brain of ad-libitum fed mice with increasing age even though the reduction at 81/82 weeks of age was not statistically significant compared to Baseline ($p = .187$).

On the other hand, Two-way ANOVA revealed that there was a significant main effect of duration on the protein level of GEP on mice, which were subjected to different dietary regimens, at the $p < .05$ level ($F_{(1,24)} = 35.772$, $p = .000$). Simple effect analysis was carried out to better understand the effect of duration on different dietary regimen groups. It was revealed that the simple effect of duration was statistically significant on three of the diet groups as follows: Chronic Caloric Restriction (CCR) group at the $p < .05$ level ($F_{(1,24)} = 15.915$, $^{**}p = .001$), Intermittent Caloric Restriction-Restriction(ICR-R) at the $p < .05$ level

($F_{(1,24)}$= 8.157, $^{**}p$ = .009) and Intermittent Caloric Restriction-Refeed (ICR-RF) at the p <.05 level ($F_{(1,24)}$ = 17.232, $^{***}p$ = .000). In fact, the protein level of GEP tends to increase with increasing duration of caloric restriction treatments regardless of type. In contrast, ad-libitum feeding tends to be stable with increasing duration.

Furthermore, the simple effect of diet was statistically significant at the duration of 81/82 weeks at the p <.05 level ($F_{(3,24)}$= 4.272, p = .015). The pairwise comparisons on dietary regimens in 81/82 weeks revealed a statistically significant increase in the protein level of GEP in the ICR-RF group compared to AL feeding at the p <.05 level (^{b}p = 0.020, Bonferroni adjusted). Besides, there was also a marginally significant difference in the CCR group compared to AL feeding in the duration of 81/82 weeks (^{a}p =0.064, Bonferroni adjusted)

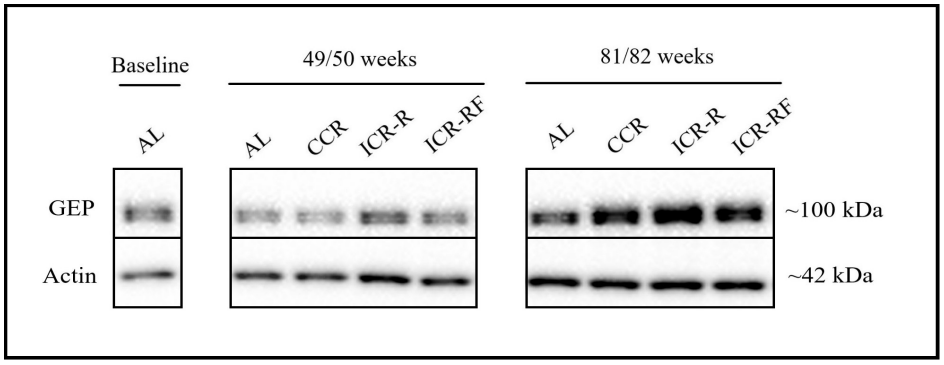

Figure 3.7: Representative immunoblot image of GEP and Actin (loading control). Each band was labeled according to the duration and dietary regimen.

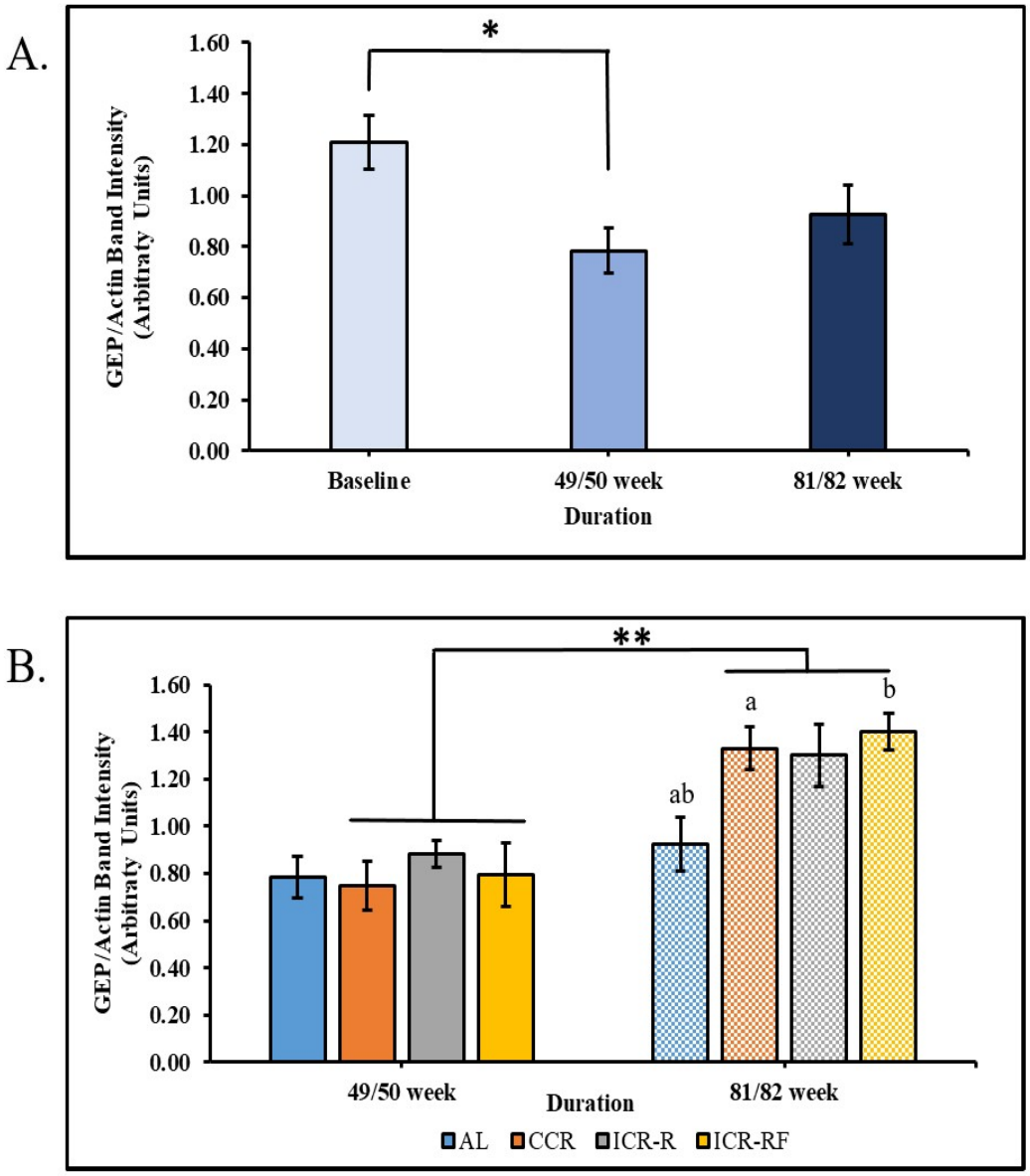

Figure 3.8: **Effects of varying levels of calorie restriction at differing durations on the protein level of GEP in the whole brain of MMTV-TGF-α female mice.**(A) Bar graph for normalized band intensities of GEP for ad-libitum fed animals (n=4 per group; 2 technical replicates for each animal). Each bar represents the mean± SEM.(B) Bar graph for normalized band intensities of GEP for ad-libitum, CCR, ICR-R, and ICR-RF fed animals at 49/50 weeks and 81/82 weeks (n=4 per group; 2 technical replicates for each animal). Each bar represents the mean± SEM.

3.2.3 Synaptophysin (SYN)

Synaptophysin (SYN) is a vesicle-associated protein located in the presynaptic synapse. It plays a role in the fusion of the vesicles to the membrane of the presynaptic neurons and the reuptake of neurotransmitters by endocytosis [77]. The protein level of SYN was investigated to comprehend the age- and caloric restriction (CR) -related alterations in the integrity of presynaptic regions.

Following the violation of the normality and equal variances, Kruskal Wallis H Test was performed on normalized protein data. Kruskal Wallis Test applied to ad-libitum fed mice data showed that there was not a statistically significant effect of duration on the protein level of synaptophysin at the $p < .05$ level ($\chi^2(2) = 2.192$, $p = 0.334$). This result indicates that the protein level of synaptophysin tends to be stable in the whole brain of ad-libitum fed mice with increasing duration/age.

To understand the effects of different dietary regimens (AL, CCR, ICR-R, and ICR-RF) and their durations (49/50-weeks and 81/82-weeks), two more Kruskal Wallis H Test were performed. The test results revealed a significant main effect of duration on the protein level of synaptophysin at the $p < .05$ level ($\chi^2(1) = 16.568$, $p = 0.000$). Following the significant p-value, Mann Whitney U Test was applied to see which dietary groups have the statistical significance by increasing duration. It was revealed that the effect of duration was statistically significant

on three of the diet groups as follows: Chronic Caloric Restriction (CCR) group at the p <.025 level (U=0.000, $^{*}p$ =0.021), Intermittent Caloric Restriction-Restriction(ICR-R) at the p <.025 level (U=0.000, $^{*}p$ =0.021) and Intermittent Caloric Restriction-Refeed (ICR-RF) at the p <.025 level (U=0.000, $^{*}p$ =0.021). In fact, the protein level of synaptophysin tends to decrease with increasing duration of caloric restriction treatments regardless of type. In contrast, ad-libitum feeding tends to be stable with increasing duration.

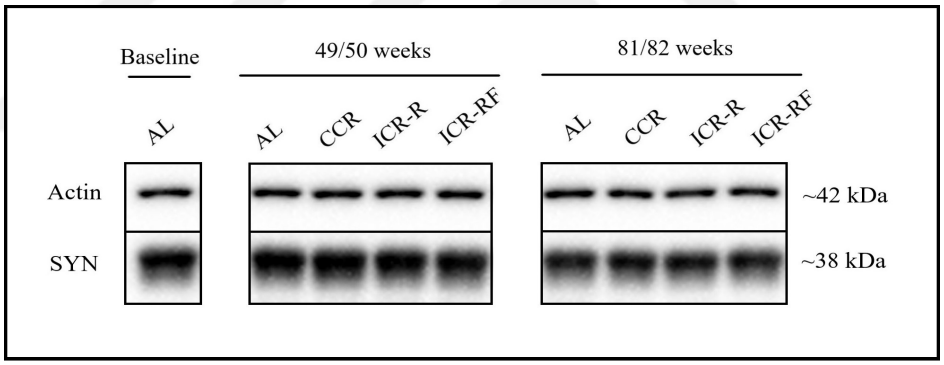

Figure 3.9: Representative immunoblot image of SYN and Actin (loading control). Each band was labeled according to the duration and dietary regimen.

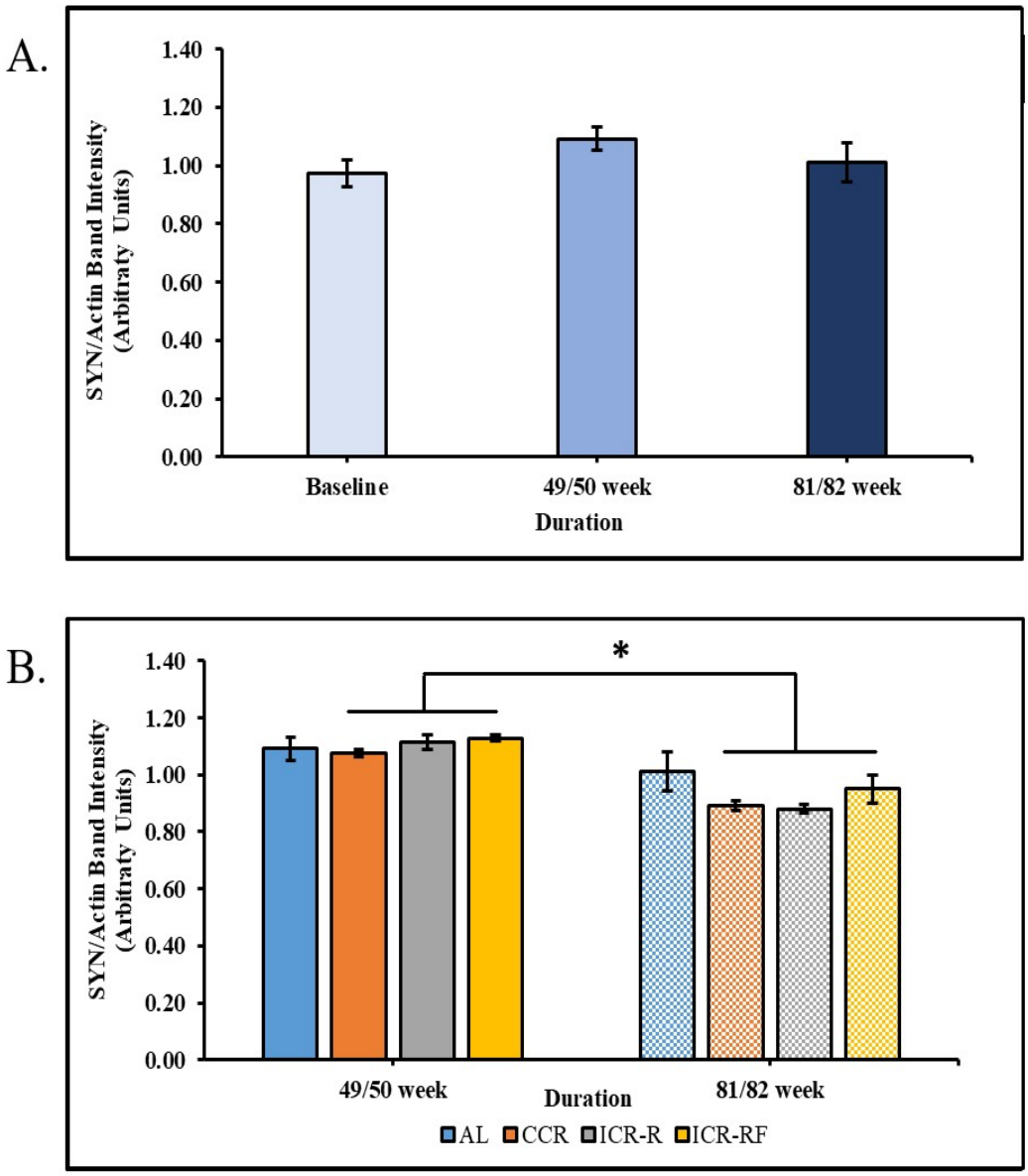

Figure 3.10: **Effects of varying levels of calorie restriction at differing durations on the protein level of SYN in the whole brain of MMTV-TGF-α female mice.**(A) Bar graph for normalized band intensities of SYN for ad-libitum fed animals (n=4 per group; 2 technical replicates for each animal). Each bar represents the mean± SEM.(B) Bar graph for normalized band intensities of SYN for ad-libitum, CCR, ICR-R, and ICR-RF fed animals at 49/50 weeks and 81/82 weeks (n=4 per group; 2 technical replicates for each animal). Each bar represents the mean± SEM.

3.3 Inflammatory markers

3.3.1 Interleukin-6 (IL-6)

Interleukin 6 (IL-6) is another main pro-inflammatory cytokine secreted by various central nervous system cells, including neurons, astrocytes, microglial cells, and endothelial cells [78]. The protein level of IL-6 was investigated to comprehend the age- and caloric restriction (CR) -related alterations in the neuroinflammatory response of the brain.

Following the validation of the normality and equal variances, Analysis of Variances (ANOVAs) was performed on normalized protein data. One-way ANOVA test showed a significant effect of duration on the protein level of IL-6 on ad-libitum fed mice at the p <.05 level ($F_{(2,9)}$= 7.693, p = .011). Following the significant ANOVA test result, Tukey's HSD Post Hoc Test revealed a statistically significant difference among Baseline (10-weeks of age) and 49/50 weeks of age at the p <.05 level ($^{*}p$ = 0.015) and between Baseline and 81/82 weeks of age p <.05 level ($^{*}p$ = 0.027) This result indicates that the protein level of IL-6 tends to increase compared to the Baseline (10-weeks of age) in the whole brain of ad-libitum fed mice with increasing age. However, there was not a statistically significant difference between 49/50-weeks- and 81/82-weeks of age animals. Therefore the increase in the protein level of IL-6 was not perpetual by the duration; instead, it stays stable between 49/50-weeks- and 81/82-weeks of age.

On the other hand, Two-way ANOVA revealed a significant main effect of duration on the protein level of IL-6 on mice, which were subjected to different dietary regimens, at the $p < .05$ level ($F_{(1,24)} = 8.378$, $p = .008$). A simple effect analysis was carried out to better understand the effect of duration on different dietary regimen groups. It was revealed that the effect of duration was statistically significant on the Chronic Caloric Restriction (CCR) group at the $p < .05$ level ($F_{(1,24)} = 4.963$, $^{*}p = .036$). In fact, the protein level of IL-6 tends to decrease with increasing duration of the aforementioned dietary regimen. Differently from this, other dietary regimens tend to be stable with increasing duration.

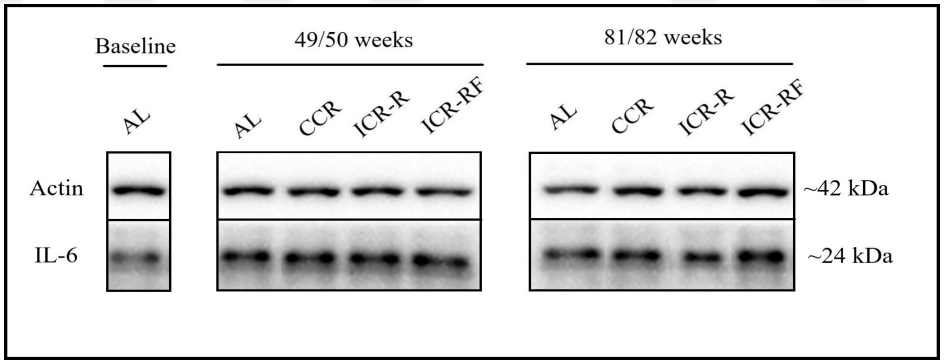

Figure 3.11: Representative immunoblot image of IL-6 and Actin (loading control). Each band was labeled according to the duration and dietary regimen.

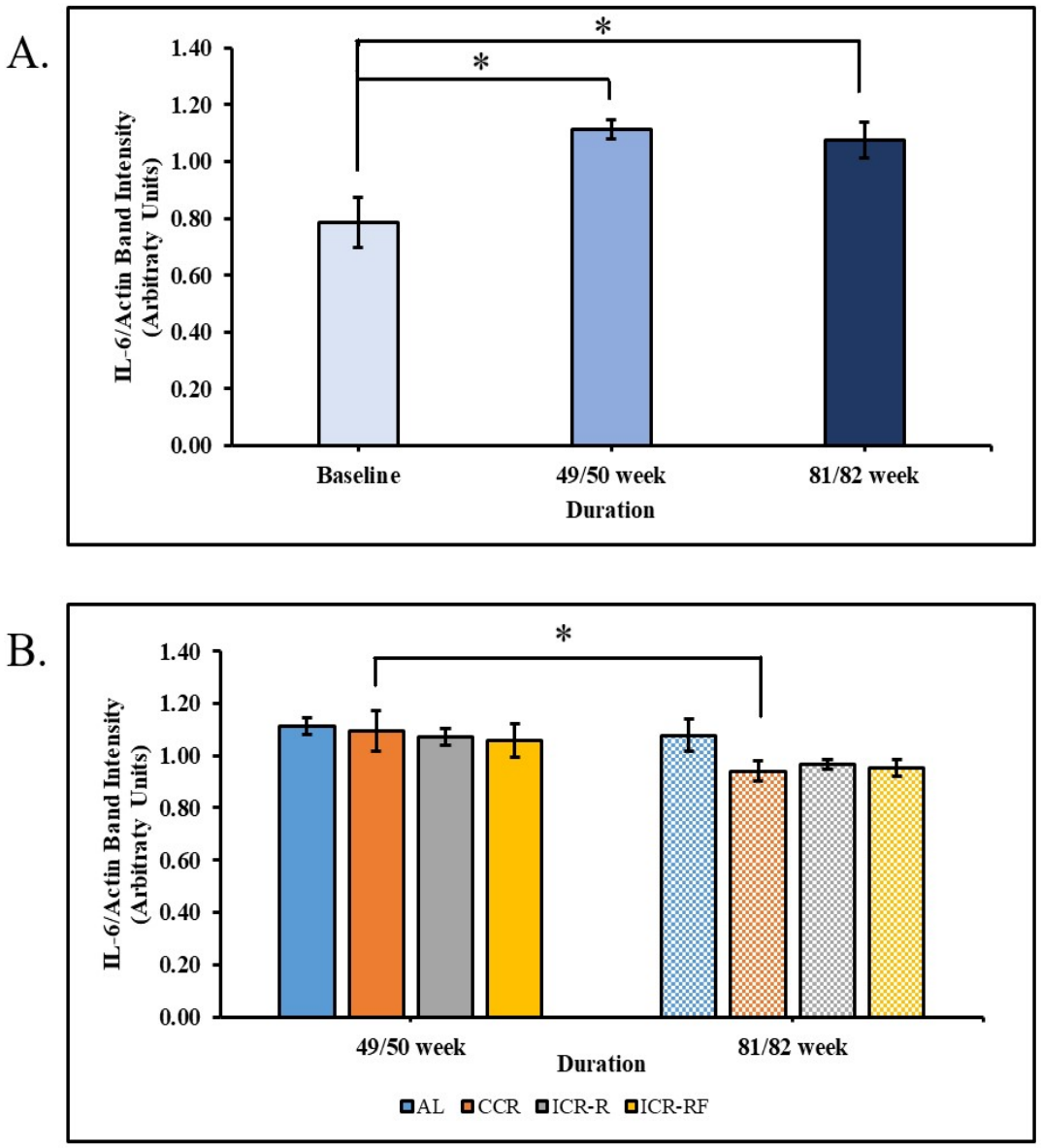

Figure 3.12: **Effects of varying levels of calorie restriction at differing durations on the protein level of IL-6 protein in the whole brain of MMTV-TGF-α female mice.**(A) Bar graph for normalized band intensities of IL-6 for ad-libitum fed animals (n=4 per group; 2 technical replicates for each animal). Each bar represents the mean± SEM.(B) Bar graph for normalized band intensities of IL-6 for ad-libitum, CCR, ICR-R, and ICR-RF fed animals at 49/50 weeks and 81/82 weeks (n=4 per group; 2 technical replicates for each animal). Each bar represents the mean± SEM.

3.3.2 Tumor necrosis factor-alpha (TNF-α)

Tumor necrosis factor-alpha (TNF-α) is a pro-inflammatory cytokine secreted mainly by glial cells, including astrocytes and microglial cells, as well as neurons in the central nervous system [79]. The protein level of TNF-α was investigated to comprehend the age- and caloric restriction (CR) -related alterations in the neuroinflammatory response of the brain.

Following the validation of the normality and equal variances, Analysis of Variances (ANOVAs) was performed on normalized protein data. One-way ANOVA test showed that there was no significant effect of duration on the protein level of TNF-α on ad-libitum fed mice at the $p < .05$ level ($F_{(2,9)} = 0.450$, $p = .651$). This result indicates that the protein level of TNF-α tends to be stable in the whole brain of ad-libitum fed mice with increasing duration/age.

On the other hand, Two-way ANOVA revealed that there was a significant main effect of duration on the protein level of TNF-α on mice, which were subjected to different dietary regimens, at the $p < .05$ level ($F_{(1,24)} = 26.988$, $p = .000$). Simple effect analysis was carried out to better understand the effect of duration on different dietary regimen groups. It was revealed that the effect of duration was statistically significant on three of the diet groups as follows:

Chronic Caloric Restriction (CCR) group at the $p < .05$ level ($F_{(1,24)} = 10.651$, $^{**}p = .003$), Intermittent Caloric Restriction-Restriction(ICR-R) at the $p < .05$ level ($F_{(1,24)} = 12.603$, $^{**}p = .002$) and Intermittent Caloric Restriction-Refeed (ICR-RF) at the $p < .05$ level ($F_{(1,24)} = 8.718$, $^{**}p = .007$). In fact, the protein level of TNF-alpha tends to decrease with increasing duration of caloric restriction treatments regardless of type. In contrast, ad-libitum feeding tends to be stable with increasing duration.

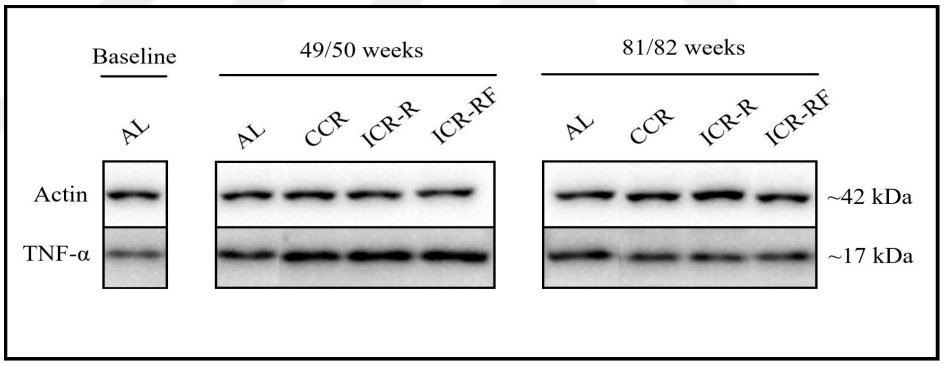

Figure 3.13: Representative immunoblot image of TNF-α and Actin (loading control). Each band was labeled according to the duration and dietary regimen.

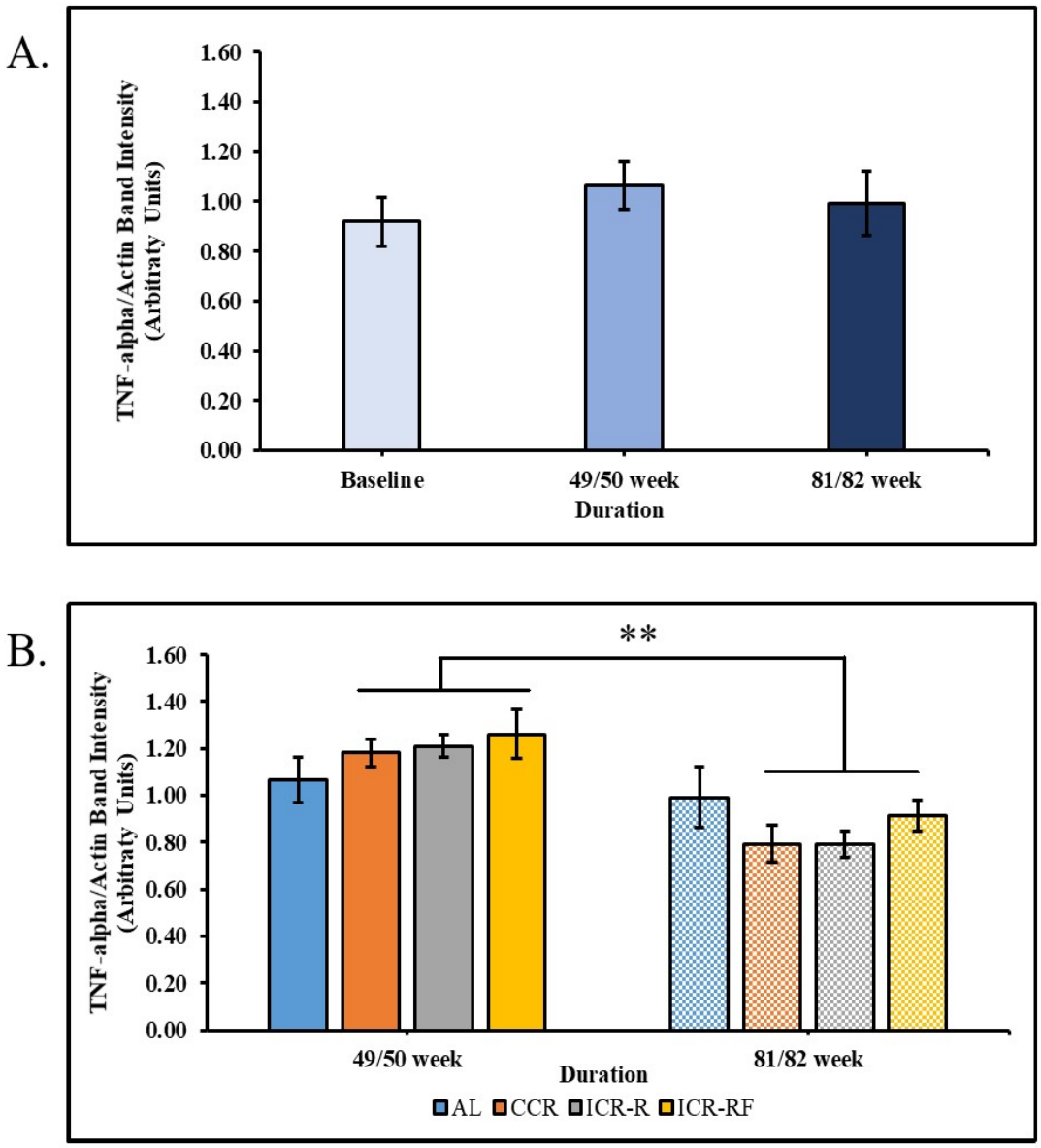

Figure 3.14: **Effects of varying levels of calorie restriction at differing durations on the protein level of TNF-α protein in the whole brain of MMTV-TGF-α female mice.**(A) Bar graph for normalized band intensities of TNF-α for ad-libitum fed animals (n=4 per group; 2 technical replicates for each animal). Each bar represents the mean± SEM.(B) Bar graph for normalized band intensities of TNF-α for ad-libitum, CCR, ICR-R, and ICR-RF fed animals at 49/50 weeks and 81/82 weeks (n=4 per group; 2 technical replicates for each animal). Each bar represents the mean± SEM.

3.3.3 Glial fibrillary acidic protein (GFAP)

In this study, Glial fibrillary acidic protein (GFAP) was used for the assessment of both proliferation and neuroinflammation. In order not to present same results twice, the graphs are not added under the section 3.3.3 . Please see the section **3.1.2 Glial fibrillary acidic protein (GFAP)** for the results.

Chapter 4

Discussion

The extension in the human lifespan with advancing technology in health creates a need for studies in aging. In fact, the main reason for this need is that extension of lifespan is not followed by the expansion of healthspan in human life. People with higher life-expectancy mean that they spend more time at the older ages; therefore, they suffer from age-related dysfunctions for a longer time. Taking into account all of these, interventions for delaying the age-related dysfunctions come to the fore

Caloric restriction (CR) is a well-trusted approach that increases the lifespan and healthspan of the organisms without genetic interventions to cope with aging's detrimental effects. It is based upon the reduction in the daily caloric intake of organisms without causing an imbalance in carbohydrate, protein, fat, and mineral sources [49, 80, 48]. The effectiveness of the CR approach was shown on various living beings, including yeast, nematodes, fruit flies, and rats [45, 46, 47].

Aging alters the neural stem cells' proliferative potential, hence decreasing the neurogenesis, which is associated with age-related cognitive decline [81]. Upon certain manipulations, including exercise and dietary interventions, proliferation ultimately neurogenesis can be stimulated at the older ages [82]. In our study, we checked the protein level changes of immature neuron marker, Doublecortin (DCX) and astrocytes marker, Glial fibrillary acidic protein (GFAP) to investigate age-related changes in the proliferative state of the central nervous system and effects of CR as an intervention to cope with age-related alterations. According to our results, there was no age-dependent alteration in the DCX level with advancing age (10 weeks-, 49/50 weeks- and 81/82 weeks of age) in ad-libitum fed mice. Previous studies on rhesus monkeys reported a sharp decline in the number of DCX^+ cells in the dentate gyrus [83]. Studies on postmortem human hippocampi' also reported a sharp decline in DCX expression at mRNA level [84]. Since the expression of DCX is not restricted to neurogenic areas [85, 86], whole-brain measurements could mask the alterations in the hippocampus and olfactory bulb. Also, we measured the protein expression, but it is unknown whether the number of cells expressing DCX or the amount of protein expressed by each cell was altered.

In terms of the effects of varying levels of CR that were applied for differing durations on the DCX level, our data showed dietary regimens, including all CRs and AL, did not differ from each other statistically. But yet, duration has a statistically significant effect on the protein level alterations of DCX, which lowered the DCX level only in CCR at 81/82 weeks compared to 49/50 weeks. In many studies, DCX expression is also suggested as an indicator for the level of neurogenesis in adulthood [87, 88], and it has been stated CR has an impact so

as to increase the neurogenesis [59, 82, 89]. Despite the previous studies, other studies suggested that neurogenesis could have a different meaning at different ages and need to be further investigated to comprehend this complex topic [17, 18]. Although our result seems to conflict with the previous studies, as it is explained above, the protein level may not be a representative for neurogenesis since the alterations in the number of immature neurons were unknown. Also, it was not known whether immature neurons go through maturation or not. Hence, the decreased protein level of DCX driven by CCR may not suggest a decrease in neurogenesis. Further studies, including immunohistochemistry, BrdU injection, or additional stage-specific markers, can help the interpretation of the result more accurately.

For the assessment of age-related alterations in the proliferation of astrocytes, the protein level of GFAP was investigated. According to our study results, the GFAP level stayed stable between 10-weeks of age and 49/50-weeks of age. In contrast to no change until 49/50-weeks of age, the protein level of GFAP increased at the age of 81/82 weeks compared to 49/50 weeks- and 10-weeks of age even though the latter was marginally significant. Our results show parallelism in terms of age-related increases in the expression level of GFAP with the previous studies [90, 91, 92, 93, 94]. A study on rhesus macaques reported an increase in the immunoreactivity of GFAP in the aged animals compared to middle-aged and mature ones in the hippocampus and prefrontal cortex. The study also reported there was no significant age-related change in the number of cells expressing GFAP [93]. In the context of proliferation, we did not count the number of cells expressing GFAP. So the alteration in the GFAP expression may be associated with an increased number of astrocyte processes and cell size on

the resident astrocytes, as suggested in the study on rhesus macaques [93] or may be related to an increased number of astrocytes indicating the increased proliferation. Other studies regarding age-related alterations in astrocytes revealed a significant increase in the number of cells [95]. Hence, it is essential to include immunohistochemistry experiments to see the correlation between protein level and the number of cells, if any.

In terms of the effects of varying levels of CR that were applied for differing durations on GFAP level, our study results revealed that dietary regimens did not statistically differ from each other, but yet duration had a significant impact on GFAP levels, which increased in all CR and AL feeding groups at the age of 81/82 weeks compared to the age of 49/50 weeks. Also, there was a trend at the age of 81/82 weeks, in which all CRs tended to decrease compared to their AL counterparts even though the differences were not statistically significant. High variance in the ad-libitum fed animal data at 81/82 weeks of age potentially impedes statistical significance. Age of 81/82 weeks at which most of the mice are expected to enter an anestrus stage where mice experience persistent estrus. Still, the trajectory for the transition of reproductive senescence is alternating, which might be the source of variance in the data [96]. Generally, elevation in GFAP expression is associated with activated astrocytes, so neuroinflammation. Therefore, it is essential to determine if the elevated level arises from changes in the number. If so, whether those cells are in the reactive form or not should be answered. There have been studies reporting the age-related changes in the fate of neural stem cells in which NSCs are redirected to the glial lineage at the older ages. Therefore, neurons and glial cells, particularly astrocytes, may be affected differently by CR, and the meaning of proliferation is different [17, 18, 97]

Age-related decline in cognitive abilities has also been attributed to the neuronal and synaptic loss during healthy aging. However, recent studies showed no significant loss in the number of neurons or synapse in healthy aging [29, 30, 31, 32]. So, the reason behind the age-related decline in cognitive abilities may be subtle molecular and cellular changes in the key synaptic proteins, as well as the alterations in the ratio of excitatory/inhibitory (E/I) synapses [33, 34]. To investigate age-related the molecular changes and effects of CR as an intervention to cope with age-related alterations in the synaptic regions, the following synaptic integrity proteins were measured: Postsynaptic Density Protein 95 (PSD-95) – postsynaptic excitatory marker, Gephyrin (GEP) – postsynaptic inhibitory marker, and Synaptophysin (SYN) – presynaptic integrity marker.

For the assessment of alterations on postsynaptic excitatory synapses, the protein level of PSD-95, which one of the main scaffolding protein localized at the postsynaptic density regions of the excitatory synapses, was investigated [23]. It regulates the clustering of the key components at the postsynaptic sites, including NMDA receptors, K-ion channels, and cell adhesion molecules [24, 75, 76]. In our study, the protein level of PSD-95 stayed stable across three age groups (10 weeks-, 49/50 weeks- and 81/82 weeks of age) in ad-libitum fed animals. In contrast to our results, a previous study on the hippocampal synaptosome of male rats demonstrated a significant decrease in the protein level of PSD-95 with increasing age [98]. Another study which was done on male mice showed the similar results with rats in which the hippocampus and cortex showed an age-dependent decrease in the protein level of PSD-95 in 30 months old mice compared to 3 months old mice while 18 months-and 24 months-old mice were stable compared to 3 months old mice [99]. Having no significant alterations on the PSD-95 level

could be linked to sex differences since previous studies reported the alterations in regards to males. It is well documented that estrogen is a key steroid having neuroprotective and neurotrophic impacts on the brain and taking a role in the translation of PSD-95 through non-genomic response mechanism [100, 101]. It is also known that reproductive aging is accompanied by decreased systemic estrogen levels in mice, similar to humans [61]. Despite the decline in the systemic level of estrogen, the endogenous estrogen level in the brain may be sustained as a compensatory mechanism against brain aging, which ultimately stabilized the PSD-95 level across age groups. Furthermore, previous studies showed age-related alterations in a region-specific manner. It was already known that brain aging is not a uniform process that each region is affected in different ways and varying levels [102]. Therefore, alterations at the whole-brain protein level could not be in parallel with the different regions of the brain, or whole-brain measurements might not be able to detect subtle changes. On the other hand, synaptic regions are highly dynamic sites where essential components such as receptors and synaptic proteins can be internalized through endocytosis or concentrated at the active sites through exocytosis or lateral diffusion in the membrane depending on the activity of pre- and postsynaptic neurons [103, 104]. So, there might be functional changes at the excitatory synapses through receptor/synaptic protein trafficking by phosphorylation/dephosphorylation [104] without altering the total protein amount.

In terms of the effects of varying levels of CR that were applied for differing durations on PSD-95 level, our study demonstrated that there were no statistically significant alterations related to diet types and durations. To some extent,

our results contradict with the previous studies, in which they reported CR attenuated the age-related decline in the protein level of key synaptic components of the excitatory synapse, including NR1, NR2B, GluR1, and GluR2 compared to AL animals [32]. As indicated above, one potential reason could be the obscuring effect of whole-brain measurements where subtle alterations could be masked. Another reason could be sexual dimorphism, meaning that CR's action mechanism may be differential in males and females regarding excitatory synapse or specifically on PSD-95.

For the assessment of alterations in the inhibitory synapses, the protein level of GEP, which is one of the essential inhibitory postsynaptic scaffolding protein, was investigated. It takes a role in the regulation of the anchoring and stabilization of $GABA_AR$ and Glycine Receptor at the postsynaptic density sites [26, 25]. According to our study results, there was an age-related decreasing trend in the protein level of GEP in AL-fed mice even though the decrease at the age of 81/82 weeks was not statistically significant compared to Baseline (10-weeks of age). This tendency to decrease in the GEP level with stable PSD-95 level indicates that there may be an age-related alteration in the E/I ratio in favor of excitation. Although age-related alterations in the expression of GEP is not well-studied, one of the previous studies on the visual cortex of humans and rats reported a diminishing pattern in the protein level of GEP with advancing aging starting from early childhood [105]. In contrast to this study, another study on the dorsal cochlear nucleus (DCN) of rats showed an age-related increase in GEP at both mRNA and protein levels [106]. It seems that alterations in the GEP depend on

the brain areas being investigated, and global changes may be in the direction of decrease, as shown in our study, which could potentially affect the overall E/I balance.

In terms of the effects of varying levels of CR that were applied for differing durations on the GEP level, the overall effect of dietary regimens as the main factor did not show statistical differences. Despite this, there was an increase in the protein level of GEP in ICR-RF and CCR animals compared to AL-fed animals at the 81/82 weeks; the latter was marginally significant. In fact, the increasing trend was observed in all CR animals at the 81/82 weeks of age. However, relatively high variances in CCR and ICR-R animals compared to ICR-RF animals reduces the power for detection of statistical significance. Furthermore, duration has a statistically significant impact on the protein level alterations of GEP, which increased the GEP level in all CRs (CCR, ICR-R, and ICR-RF) at 81/82 weeks compared to 49/50 weeks counterparts. This result indicates that the effects of CR on the whole brain could be more prominent on inhibitory synapses compared to that of excitatory. Furthermore, CR altered the E/I ratio towards inhibition in contrast to healthy brain aging, during which alteration in the E/I ratio favored the excitation. Therefore, the upregulation of GEP with the effect of CR could be a compensatory mechanism against age-related increased excitation.

To assess alterations in the presynaptic regions, the protein level of synaptophysin, which a vesicle-associated protein located in the presynaptic synapse, was investigated. It plays a role in the fusion of the vesicles to the membrane of the presynaptic neurons and the reuptake of neurotransmitters by endocytosis [77]. In our study, data revealed no significant change in the protein level of

synaptophysin with advancing age (10 weeks-, 49/50 weeks- and 81/82 weeks of age) in ad-libitum fed mice. In contrast to our results, a previous study on the hippocampal synaptoproteome of male mice reported that synaptophysin tended to decrease with aging [98]. However, another study on male mice showed no age-dependent alterations in the protein level of synaptophysin in the hippocampus and cortex [99]. Similar to PSD-95, the translation of synaptophysin is also regulated by the non-genomic response mechanism of estrogen. Thereby stabilization of synaptophysin levels across age groups could be due to gender, as explained above in the PSD-95 alterations. But still, to answer sexual dimorphism in terms of synaptophysin level, further studies are needed.

In terms of the effects of varying levels of CR that were applied for differing durations on the SYN level, our data showed dietary regimens, including all CRs and AL, did not differ from each other statistically. But yet, duration has a statistically significant effect on SYN's protein level alterations, which lowered the SYN level in all CRs (CCR, ICR-R, and ICR-RF) at 81/82 weeks compared to 49/50 weeks. Even though some studies correlated the decreased level of SYN with the cognitive decline in pathological conditions including Alzheimer's disease and Schizophrenia [107, 108, 109], in our case, it could be associated with altered inhibition in the postsynaptic region. That is to say, due to GEP's role in the localization of $GABA_AR$ to the right across of presynaptic region, alterations in the GEP level is associated with changes in the synaptic transmission [110, 111]. For this reason, increased inhibition might affect the presynaptic compartments in the form of decreased SYN level. Hence, alterations in the synaptophysin level could have different meanings depending on the conditions and should be investigated more comprehensively.

Glial cell activation and inflammation is another hallmark of brain aging [1]. Due to the impairments in CNS homeostasis, the balance among pro-inflammatory and anti-inflammatory responses shifted towards pro-inflammation in healthy aged brains. The shift in favor of pro-inflammation leads to persistent low-level chronic inflammation in the brain, which can potentially be detrimental for the proper function of brains [38]. Age-related low-level chronic inflammation is revealed by the increase in the inflammatory cytokines, as well as the activated phenotype of astrocytes and microglial cells.

Our study checked the protein levels of two pro-inflammatory markers, TNF-alpha and IL-6, and an astrocyte marker - GFAP to assess how neuroinflammation changes with age and varying levels of CRs that were applied for the differing duration in female mice. In parallel with the previous studies, our study revealed that IL-6 tended to increase with age (49/50 weeks- and 81/82 weeks of age) compared to Baseline (10-weeks of age) in ad-libitum fed groups, but there was no additional increase between 49/50 weeks- and 81/82 weeks old mice. In the previous studies, age-related changes in the level of IL-6 was investigated on male mice and rats [112, 113]. On male BALB/c mice, one study showed an age-dependent rise in the IL-6 level in old mice compared to young and adult mice [112].

In terms of varying levels of CR and differing durations effects on the IL-6 protein level, diets as the main factor did not differ from each other statistically. But still, a statistically significant decrease in IL-6 protein level was observed in the CCR group at 81/82 weeks of age compared to 49/50 weeks of age counterpart. The attenuation effect of calorie restriction (CR) on the age-related increase in the pro-inflammatory cytokines at the systemic- and local levels, including mouse

hippocampus and rat hypothalamus, has been shown [114, 115]. Although a significant effect was seen only in CCR animals, ICR-R and ICR-RF were also showed a slight decrease at the age of 81/82 weeks compared to 49/50 weeks. CR's hormetic action mechanism might be one of the reasons why we only have seen CR-driven attenuation in the CCR group. A week of 60% restriction in ICR-R and ICR-RF could induce so much stress on the mice, ultimately halting the beneficial effect of CR [116]. However, it seems the hormetic and toxic action mechanisms of CR differ depending on the protein of interests, where we have seen the hormetic effects of ICR-R and ICR-RF in the synaptic regions. Also, IL-6 is not only related to the neuroinflammatory response but also manages the redirection of neural progenitor towards glial lineage in aged animals [117]. Hence almost preserved level might be due to its role in the gliogenesis, in which our data showed that GFAP expression was increased at the 81/82 weeks of age compared to 49/50 weeks of age. Lastly, as indicated before, the global alterations might be different from the regional alterations.

When it comes to TNF-alpha, our data revealed that the TNF-alpha protein level stayed stable across age groups (10 weeks-, 49/50 weeks- and 81/82 weeks of age). This result conflicts with the previous studies showing an age-related increase in the pro-inflammatory cytokine levels [38, 118]. As it is indicated before, global protein expression could be different than the region-specific profile of TNF-alpha. Further experiments could tell more about the age-related alterations in the TNF-alpha protein level. Another explanation for why TNF-alpha stabilized across age groups could be sex-dependent differences. Inhibitory effect of estrogen on TNF-alpha secretion has been shown in many studies [119, 120, 121]. Hence, the endogenous estrogen level of the brain might prevent the age-related

increase in TNF-alpha even though we did not know how the local estrogen level altered in our animals. So, it is essential to measure the local estrogen level of mice in the following studies.

In terms of varying levels of CR and differing durations effects on the TNF-alpha protein level, diets as the main factor did not differ from each other statistically. But yet duration had a significant impact on TNF-alpha levels such that it decreased in all CR animals (CCR, ICR-R, and ICR-RF) at the age of 81/82 weeks compared to the age of 49/50 weeks. As indicated above, CR has growing evidence for the amelioration of low-chronic inflammation [114, 115] that are also supported by our findings. A previous study on the rat hippocampal neuron culture showed that TNF-alpha exposure modulates the inhibitory synapses by internalization of $GABA_AR$, which is followed by a decrease in gephyrin level [111]. In parallel with the previous findings, our study demonstrated that CR alters the expression level of TNF-alpha, which also affects the inhibitory synapses such a way that gephyrin expression increased. Hence neuroinflammatory- and synaptic alterations seem to be closely interplayed, and molecular mechanisms yet to be determined. The trend in the CR-driven alterations of GFAP level seems to be involved in this interplay. It is needed to check the microglial response to CR in order to understand the potential mechanism.

Regarding the neuroinflammation, age-related alterations in the protein level GFAP, which is widely used astrocyte markers in brain studies, were also investigated. The results were as indicated in the proliferation part. Age-related increase in the GFAP expression level in AL animals was in parallel with the previous studies [90, 91, 92, 93, 94]. As mentioned before, elevation in the GFAP expression is an indicator of astrogliosis [12]. Specific to our study, since we did

not count the number of cells expressing GFAP, we could not make a conclusion on how astrocytes enhance the neuroinflammation; that is, they might undergo morphological alterations accompanied by elevated GFAP level with no change in cell number or might be driven directly by the increased number of astrocytes. In addition to the suggestion in the proliferation part, the neuroinflammatory response of female brains might be different from that of males, so understanding the gender difference can help arrive at a more accurate conclusion. Concerning CR impacts, although the high variance in AL animals at the age of 81/82 weeks impedes the statistical significance, there was a trend at the age of 81/82 weeks, in which all CRs tended to decrease compared to their AL counterparts. In parallel to previous studies, all CRs seem to have the potential to alleviate the neuroinflammation via decreasing the GFAP expression when applied in a long-term manner [122, 123]. However, region-specific investigation of alterations in the protein level of GFAP can potentially be more efficient in revealing subtle changes since brain aging is not a homogenous process, so each region may be affected by aging at a varying level, as well as responded to CR interventions differently.

Chapter 5

Conclusion and Future Prospectives

In this thesis, the effects of varying levels of CRs applied for different durations were investigated to understand the potential ameliorative impacts of CRs on brain aging. For this purpose, MMTV-TGF-α female mice were utilized. In addition to the contribution of this thesis to uncovering the potential differences of varying levels of CRs and their application periods on brain aging, it is also an important study due to the utilization of both female and transgenic strain mice. It is a pioneer study in order to understand how we age when we have a disease susceptibility. Nonetheless, further studies can help us find more precise answers to our research questions as well as can potentially open new discussion topics.

In the context of alterations in neurogenesis and proliferation, we have measured the global protein level of an immature neuronal- and astrocyte marker. Our study revealed that there were age-related alterations in astrocytes in terms

of protein level. Although our findings were not statistically significant, CR could potentially slow down age-related alteration. Further studies should focus on the number of astrocytes through immunohistochemistry experiments in addition to the protein level. Since we did not count the number of astrocytes, we did not know whether the increase in the GFAP level is due to the increase in the number of cells or an increase in the amount of protein expressed by each cell. When it comes to the immature neuronal marker, it did not show age-related alterations, whereas only CCR lowered the expression of DCX with aging. Further studies may include a mature neuron marker, NeuN, to see whether immature neurons go through the maturation. Also, as in GFAP, assessment of proliferation or neurogenesis could be supported with alterations in the number of cells, so immunohistochemistry experiments, BrdU injection, or other proliferation markers could be included.

We have measured the global protein level of presynaptic and excitatory- and inhibitory-postsynaptic integrity markers in the context of alterations in the synaptic integrity proteins. Our study revealed the age- and CR-related alterations in the presynaptic and inhibitory postsynaptic regions. Even though the global alterations could give an idea about the integrity of synapses, we still did not know whether the alterations are due to changes in the number of synapses, active zones, or the amount of proteins expressed by each active zone. To expand understanding, brain-region-specific alterations and their correlation with the global changes could be checked. Synaptoproteome isolation in a region-specific manner can provide more extensive information for synaptic regions [124]. Immunohistochemistry can be applied to see if alterations in the protein levels

are correlated with the number of synapses. In addition to all of these, changes in the synaptic receptor markers can be included in further studies, including $GABA_AR$, Glycine, AMPA, NMDA receptors and their subunits. There may be other alterations in the synaptic regions in addition to protein level changes. Synaptic regions are highly dynamic sites where essential components such as receptors and structural proteins can be internalized through endocytosis or concentrated at the active sites through exocytosis or lateral diffusion in the membrane depending on the activity of pre- and postsynaptic neurons [103]. Hence, although there were no alterations in the total protein amount of, e.g., PSD-95 and SYN, there might be changes in synapses' activity. So, the experiments (Ca $^{+}2$ imaging, EPSP & IPSP records) to assess the changes in synaptic plasticity during aging, and CR treatment can be included. In this regard, the epigenetic regulation of mRNA encoding the protein of interests through miRNA can help interpret conflict results.

In the context of the neuroinflammation, we have measured the global protein level of two pro-inflammatory markers: IL-6 and TNF-alpha, and an astrocyte marker – GFAP. Our data showed that there were age-related alterations in the inflammatory state of the female mice's brains, and CRs could be useful in ameliorating the chronic-low level inflammation globally. To have more comprehensive interpretations, the alterations in pro-inflammatory cytokine protein levels should be supported by the anti-inflammatory response of the brain to both aging and calorie restriction. Comprehension of how the shift occurs between pro- and anti-inflammation may help determine the potential mechanisms and crosstalks essential for modulating inflammatory responses in the CNS. Even though the blood-brain barrier (BBB) shields the CNS from systemic cytokines, it is known

that it is exposed to age-related alterations [125]. Age-related leakage through BBB could contribute to the local levels of inflammatory cytokines, which may impede the understanding. Evaluation of the potential contributions of systemic pro-inflammatory cytokines by perfusing the blood from the cerebral vasculature could be the right approach for this purpose [126]. Our study investigated alterations in the global protein level that are valuable but still limited since the source of the pro-inflammatory and anti-inflammatory cytokines is unknown. Also, we did not count the number of astrocytes, so we did not know whether the increase in the GFAP level is due to the increase in the number of cells or an increase in the amount of protein expressed by each cell. Although the GFAP level increase is associated with the activated phenotype of astrocytes [127], immunohistochemistry could tell more about the correlation between protein amount and morphological alterations related to astrogliosis. Furthermore, we have seen the more apparent changes in GFAP and TNF-alpha in the context of neuroinflammation and the potential ameliorative effect of CR on them. For this reason, the drugs targetting these proteins can be potential to mimic the long-term CR effects hence may reverse the age-related detrimental alterations during normal brain aging.

In addition to all the discussed points above, there might be an impact of mice strain. It has been shown that overexpression of TGF-alpha is limited to mammary glands and its action mechanism autocrine or paracrine manner [128, 129]. Also, TGF-alpha is not able to cross BBB during the postnatal period [130]. However, it might have some sort of influence on the brain during the brain's developmental stage. It is necessary to include wild-type animals to comprehend whether the natal predisposition to breast cancer may alter the brain's response to aging and CR. Systemic and brain's local estrogen levels measurement show

the potential to explain the conflicting results in PSD-95, SYN, and TNF-alpha. So, brain homogenates can be used to assess local estrogen levels in the brain. Furthermore, the inclusion of male animals (both wild-type and transgenic) can help to understand the gender-specific differences in the context of brain aging and CR's impacts.

To conclude, our findings showed that either chronic- or intermittent- CR altered the synaptic integrity proteins at the long-term period (81/82 weeks) compared to the short-term (49/50 weeks) period except for PSD-95. Similarly, CRs showed an attenuative impact on the pro-inflammatory markers where IL-6 was affected only by CCR at the same periods. CR also affected the brain's proliferation, but based on limited markers; it seems glial cells are more affected than neurons. These are the critical initial findings obtained in transgenic female mice. Follow-up studies based on these results are of great importance in understanding brain aging and CR's potential as an intervention to it in female mice.

Bibliography

[1] M. P. Mattson and T. V. Arumugam, "Hallmarks of brain aging: Adaptive and pathological modification by metabolic states," *Cell Metabolism*, vol. 27, no. 6, p. 1176–1199, 2018.

[2] "World population prospects 2019: Highlights," *Statistical Papers - United Nations (Ser. A), Population and Vital Statistics Report*, 2019.

[3] J. H. Morrison and M. G. Baxter, "The ageing cortical synapse: hallmarks and implications for cognitive decline," *Nature Reviews Neuroscience*, vol. 13, no. 4, p. 240–250, 2012.

[4] C. López-Otín, M. A. Blasco, L. Partridge, M. Serrano, and G. Kroemer, "The hallmarks of aging," *Cell*, vol. 153, no. 6, p. 1194–1217, 2013.

[5] J. Altman and G. D. Das, "Autoradiographic and histological evidence of postnatal hippocampal neurogenesis in rats," *The Journal of Comparative Neurology*, vol. 124, no. 3, pp. 319–335, 1965.

[6] J. Paton and F. Nottebohm, "Neurons generated in the adult brain are recruited into functional circuits," *Science*, vol. 225, no. 4666, pp. 1046–1048, 1984.

[7] L. J. Richards, T. J. Kilpatrick, and P. F. Bartlett, "De novo generation of neuronal cells from the adult mouse brain.," *Proceedings of the National Academy of Sciences*, vol. 89, no. 18, pp. 8591–8595, 1992.

[8] L. E. Mello and B. M. Longo, *Neurogenesis: A Change of Paradigms*, p. 11–33. Springer Netherlands, 2010.

[9] G.-L. Ming and H. Song, "Adult neurogenesis in the mammalian brain: Significant answers and significant questions," *Neuron*, vol. 70, no. 4, p. 687–702, 2011.

[10] N. Kee, S. Sivalingam, R. Boonstra, and J. Wojtowicz, "The utility of ki-67 and brdu as proliferative markers of adult neurogenesis," *Journal of Neuroscience Methods*, vol. 115, no. 1, p. 97–105, 2002.

[11] W. Strzalka and A. Ziemienowicz, "Proliferating cell nuclear antigen (pcna): a key factor in dna replication and cell cycle regulation," *Annals of Botany*, vol. 107, no. 7, p. 1127–1140, 2010.

[12] E. M. Hol and M. Pekny, "Glial fibrillary acidic protein (gfap) and the astrocyte intermediate filament system in diseases of the central nervous system," *Current Opinion in Cell Biology*, vol. 32, p. 121–130, 2015.

[13] Y. Imai, I. Ibata, D. Ito, K. Ohsawa, and S. Kohsaka, "A novel gene iba1 in the major histocompatibility complex class iii region encoding an ef hand protein expressed in a monocytic lineage," *Biochemical and Biophysical Research Communications*, vol. 224, no. 3, p. 855–862, 1996.

[14] S. Couillard-Despres, B. Winner, S. Schaubeck, R. Aigner, M. Vroemen, N. Weidner, U. Bogdahn, J. Winkler, H.-G. Kuhn, L. Aigner, and et al.,

"Doublecortin expression levels in adult brain reflect neurogenesis," *European Journal of Neuroscience*, vol. 21, no. 1, p. 1–14, 2005.

[15] V. V. Gusel'Nikova and D. E. Korzhevskiy, "Neun as a neuronal nuclear antigen and neuron differentiation marker," *Acta Naturae*, vol. 7, no. 2, p. 42–47, 2015.

[16] H. Kuhn, H. Dickinson-Anson, and F. Gage, "Neurogenesis in the dentate gyrus of the adult rat: age-related decrease of neuronal progenitor proliferation," *The Journal of Neuroscience*, vol. 16, no. 6, p. 2027–2033, 1996.

[17] J. L. Bizon, "More is less: Neurogenesis and age-related cognitive decline in long-evans rats," *Science of Aging Knowledge Environment*, vol. 2005, no. 7, 2005.

[18] A. Arslan-Ergul, A. T. Ozdemir, and M. M. Adams, "Aging, neurogenesis, and caloric restriction in different model organisms," *Aging and Disease*, vol. 4, no. 4, p. 221–232, 2013.

[19] M. E. Burns and G. J. Augustine, "Synaptic structure and function: Dynamic organization yields architectural precision," *Cell*, vol. 83, no. 2, p. 187–194, 1995.

[20] R. G. Zhai and H. J. Bellen, "The architecture of the active zone in the presynaptic nerve terminal," *Physiology*, vol. 19, no. 5, p. 262–270, 2004.

[21] T. C. Südhof, "Neurotransmitter release: The last millisecond in the life of a synaptic vesicle," *Neuron*, vol. 80, no. 3, p. 675–690, 2013.

[22] D. Keith, "Excitation control: balancing psd-95 function at the synapse," *Frontiers in Molecular Neuroscience*, vol. 1, 2008.

[23] M. B. Kennedy, "The postsynaptic density at glutamatergic synapses," *Trends in Neurosciences*, vol. 20, no. 6, p. 264–268, 1997.

[24] H. Kornau, L. Schenker, M. Kennedy, and P. Seeburg, "Domain interaction between nmda receptor subunits and the postsynaptic density protein psd-95," *Science*, vol. 269, no. 5231, p. 1737–1740, 1995.

[25] W. Yu, M. Jiang, C. P. Miralles, R.-W. Li, G. Chen, and A. L. D. Blas, "Gephyrin clustering is required for the stability of gabaergic synapses," *Molecular and Cellular Neuroscience*, vol. 36, no. 4, p. 484–500, 2007.

[26] M. Sassoè-Pognetto and J.-M. Fritschy, "Gephyrin, a major postsynaptic protein of gabaergic synapses," *European Journal of Neuroscience*, vol. 12, no. 7, p. 2205–2210, 2000.

[27] J. W. Hell, "Camkii: Claiming center stage in postsynaptic function and organization," *Neuron*, vol. 81, no. 2, p. 249–265, 2014.

[28] B. Zuber, I. Nikonenko, P. Klauser, D. Muller, and J. Dubochet, "The mammalian central nervous synaptic cleft contains a high density of periodically organized complexes," *Proceedings of the National Academy of Sciences*, vol. 102, no. 52, p. 19192–19197, 2005.

[29] P. R. Rapp and M. Gallagher, "Preserved neuron number in the hippocampus of aged rats with spatial learning deficits.," *Proceedings of the National Academy of Sciences*, vol. 93, no. 18, p. 9926–9930, 1996.

[30] M. E. Calhoun, D. Kurth, A. L. Phinney, J. M. Long, J. Hengemihle, P. R. Mouton, D. K. Ingram, and M. Jucker, "Hippocampal neuron and synaptophysin-positive bouton number in aging c57bl/6 mice," *Neurobiology of Aging*, vol. 19, no. 6, p. 599–606, 1998.

[31] P. R. Rapp, "Neuron number in the parahippocampal region is preserved in aged rats with spatial learning deficits," *Cerebral Cortex*, vol. 12, no. 11, p. 1171–1179, 2002.

[32] I. G. Newton, M. E. Forbes, M. C. Linville, H. Pang, E. W. Tucker, D. R. Riddle, and J. K. Brunso-Bechtold, "Effects of aging and caloric restriction on dentate gyrus synapses and glutamate receptor subunits," *Neurobiology of Aging*, vol. 29, no. 9, p. 1308–1318, 2008.

[33] J. Luebke, Y.-M. Chang, T. Moore, and D. Rosene, "Normal aging results in decreased synaptic excitation and increased synaptic inhibition of layer 2/3 pyramidal cells in the monkey prefrontal cortex," *Neuroscience*, vol. 125, no. 1, p. 277–288, 2004.

[34] M. Majdi, A. Ribeiro-Da-Silva, and A. C. Cuello, "Cognitive impairment and transmitter-specific pre- and postsynaptic changes in the rat cerebral cortex during ageing," *European Journal of Neuroscience*, vol. 26, no. 12, p. 3583–3596, 2007.

[35] R. Daneman and A. Prat, "The blood–brain barrier," *Cold Spring Harbor Perspectives in Biology*, vol. 7, no. 1, 2015.

[36] M. Prinz, D. Erny, and N. Hagemeyer, "Ontogeny and homeostasis of cns myeloid cells," *Nature Immunology*, vol. 18, no. 4, p. 385–392, 2017.

[37] M. Mittelbronn, K. Dietz, H. J. Schluesener, and R. Meyermann, "Local distribution of microglia in the normal adult human central nervous system differs by up to one order of magnitude," *Acta Neuropathologica*, vol. 101, no. 3, p. 249–255, 2001.

[38] E. Bok, M. Jo, S. Lee, B.-R. Lee, J. Kim, and H.-J. Kim, "Dietary restriction and neuroinflammation: A potential mechanistic link," *International Journal of Molecular Sciences*, vol. 20, no. 3, p. 464, 2019.

[39] D. J. Disabato, N. Quan, and J. P. Godbout, "Neuroinflammation: the devil is in the details," *Journal of Neurochemistry*, vol. 139, p. 136–153, 2016.

[40] M. Nedergaard, B. Ransom, and S. A. Goldman, "New roles for astrocytes: Redefining the functional architecture of the brain," *Trends in Neurosciences*, vol. 26, no. 10, p. 523–530, 2003.

[41] R. Siracusa, R. Fusco, and S. Cuzzocrea, "Astrocytes: Role and functions in brain pathologies," *Frontiers in Pharmacology*, vol. 10, 2019.

[42] A. L. Palmer and S. S. Ousman, "Astrocytes and aging," *Frontiers in Aging Neuroscience*, vol. 10, 2018.

[43] M. K. Jha, M. Jo, J.-H. Kim, and K. Suk, "Microglia-astrocyte crosstalk: An intimate molecular conversation," *The Neuroscientist*, vol. 25, no. 3, p. 227–240, 2018.

[44] D. M. Norden and J. P. Godbout, "Review: Microglia of the aged brain: primed to be activated and resistant to regulation," *Neuropathology and Applied Neurobiology*, vol. 39, no. 1, p. 19–34, 2013.

[45] P. Kapahi, M. Kaeberlein, and M. Hansen, "Dietary restriction and lifespan: Lessons from invertebrate models," *Ageing Research Reviews*, vol. 39, p. 3–14, 2017.

[46] G. D. Lee, M. A. Wilson, M. Zhu, C. A. Wolkow, R. D. Cabo, D. K. Ingram, and S. Zou, "Dietary deprivation extends lifespan in caenorhabditis elegans," *Aging Cell*, vol. 5, no. 6, p. 515–524, 2006.

[47] M. M. Adams, L. Shi, M. C. Linville, M. E. Forbes, A. B. Long, C. Bennett, I. G. Newton, C. S. Carter, W. E. Sonntag, D. R. Riddle, and et al., "Caloric restriction and age affect synaptic proteins in hippocampal ca3 and spatial learning ability," *Experimental Neurology*, vol. 211, no. 1, p. 141–149, 2008.

[48] M. P. Mattson and V. D. Longo, "Fasting: Molecular mechanisms and clinical applications," *Cell Metabolism*, vol. 19, p. 181–192, Feb 2014.

[49] B. Martin, M. P. Mattson, and S. Maudsley, "Caloric restriction and intermittent fasting: Two potential diets for successful brain aging," *Ageing Research Reviews*, vol. 5, no. 3, p. 332–353, 2006.

[50] Y.-L. Chen, L. Ling, G. Su, M. Han, X. Fan, P. Xun, and G. Xu, "Effect of intermittent versus chronic calorie restriction on tumor incidence: A systematic review and meta-analysis of animal studies," *Scientific reports*, vol. 6, p. 33739, 09 2016.

[51] R. J. Mccarter and J. Palmer, "Energy metabolism and aging: a lifelong study of fischer 344 rats," *American Journal of Physiology-Endocrinology and Metabolism*, vol. 263, no. 3, 1992.

[52] T. M. Mcshane, "Life-long moderate caloric restriction prolongs reproductive life span in rats without interrupting estrous cyclicity: effects on the gonadotropin-releasing hormone/luteinizing hormone axis," *Biology of Reproduction*, vol. 54, no. 1, p. 70–75, 1996.

[53] L. K. Heilbronn and E. Ravussin, "Calorie restriction and aging: review of the literature and implications for studies in humans," *The American Journal of Clinical Nutrition*, vol. 78, no. 3, p. 361–369, 2003.

[54] L. Fontana, T. E. Meyer, S. Klein, and J. O. Holloszy, "Long-term calorie restriction is highly effective in reducing the risk for atherosclerosis in humans," *Proceedings of the National Academy of Sciences*, vol. 101, no. 17, p. 6659–6663, 2004.

[55] M. J. Bonorden, O. P. Rogozina, C. M. Kluczny, M. E. Grossmann, J. P. Grande, A. Lokshin, and M. P. Cleary, "Cross-sectional analysis of intermittent versus chronic caloric restriction in the tramp mouse," *The Prostate*, vol. 69, no. 3, p. 317–326, 2008.

[56] C. V. Cauwenberghe, C. Vandendriessche, C. Libert, and R. E. Vandenbroucke, "Caloric restriction: beneficial effects on brain aging and alzheimer's disease," *Mammalian Genome*, vol. 27, no. 7-8, p. 300–319, 2016.

[57] M. Kaeberlein, "Longevity and aging," *F1000Prime Reports*, vol. 5, 2013.

[58] M. R. Klass, "A method for the isolation of longevity mutants in the nematode caenorhabditis elegans and initial results," *Mechanisms of Ageing and Development*, vol. 22, no. 3-4, p. 279–286, 1983.

[59] J. Lee, W. Duan, J. M. Long, D. K. Ingram, and M. P. Mattson, "Dietary restriction increases the number of newly generated neural cells, and induces bdnf expression, in the dentate gyrus of rats," *Journal of Molecular Neuroscience*, vol. 15, no. 2, p. 99–108, 2000.

[60] J. R. Harris and V. I. Korolchuk, *Biochemistry and Cell Biology of Ageing, Clinical science*. Springer, 2019.

[61] P. M. Conn, *Handbook of models for human aging*. Elsevier Academic Press, 2006.

[62] M. J. Wieduwilt and M. M. Moasser, "The epidermal growth factor receptor family: Biology driving targeted therapeutics," *Cellular and Molecular Life Sciences*, vol. 65, no. 10, p. 1566–1584, 2008.

[63] G. Smith, R. Sharp, E. Kordon, C. Jhappan, and G. Merlino, "Transforming growth factor-alpha promotes mammary tumorigenesis through selective survival and growth of secretory epithelial cells," *The American journal of pathology*, vol. 147, pp. 1081–96, 11 1995.

[64] B. G. Tuna, P. B. Atalay, M. Altunbek, B. M. Kalkan, and S. Dogan, "Effects of chronic and intermittent calorie restriction on adropin levels in breast cancer," *Nutrition and Cancer*, vol. 69, no. 7, p. 1003–1010, 2017.

[65] M. Kyriazis, "Ageing throughout history: The evolution of human lifespan," *Journal of Molecular Evolution*, vol. 88, no. 1, p. 57–65, 2019.

[66] P.-C. Yang and T. Mahmood, "Western blot: Technique, theory, and trouble shooting," *North American Journal of Medical Sciences*, vol. 4, no. 9, p. 429, 2012.

[67] A. Swaminathan, R. Hassan-Abdi, S. Renault, A. Siekierska, R. Riché, M. Liao, P. A. D. Witte, C. Yanicostas, N. Soussi-Yanicostas, P. Drapeau, and et al., "Non-canonical mtor-independent role of depdc5 in regulating gabaergic network development," *Current Biology*, vol. 28, no. 12, 2018.

[68] A. Toledo, F. Lang, M. Doengi, H. Morrison, V. Stein, and S. L. Baader, "Merlin modulates process outgrowth and synaptogenesis in the cerebellum," *Brain Structure and Function*, vol. 224, no. 6, p. 2121–2142, 2019.

[69] M.-J. You, M. Bang, H.-S. Park, B. Yang, K. B. Jang, J. Yoo, D.-Y. Hwang, M. Kim, B. Kim, S.-H. Lee, and et al., "Human umbilical cord-derived mesenchymal stem cells alleviate schizophrenia-relevant behaviors in amphetamine-sensitized mice by inhibiting neuroinflammation," *Translational Psychiatry*, vol. 10, no. 1, 2020.

[70] S.-S. Jiao, L.-L. Shen, C. Zhu, X.-L. Bu, Y.-H. Liu, C.-H. Liu, X.-Q. Yao, L.-L. Zhang, H.-D. Zhou, D. G. Walker, and et al., "Brain-derived neurotrophic factor protects against tau-related neurodegeneration of alzheimer's disease," *Translational Psychiatry*, vol. 6, no. 10, 2016.

[71] S. Yue, X. Zheng, and Y. Zheng, "Cell-type-specific role of lamin-b1 in thymus development and its inflammation-driven reduction in thymus aging," *Aging Cell*, vol. 18, no. 4, 2019.

[72] K. A. Rodriguez, Y. H. Edrey, P. Osmulski, M. Gaczynska, and R. Buffenstein, "Altered composition of liver proteasome assemblies contributes to enhanced proteasome activity in the exceptionally long-lived naked mole-rat," *PLoS ONE*, vol. 7, no. 5, 2012.

[73] A. J. Hughes, D. P. Spelke, Z. Xu, C.-C. Kang, D. V. Schaffer, and A. E. Herr, "Single-cell western blotting," *Nature Methods*, vol. 11, no. 7, p. 749–755, 2014.

[74] E. Bocksteins, G. V. D. Vijver, P.-P. V. Bogaert, and D. J. Snyders, "Kv3 channels contribute to the delayed rectifier current in small cultured mouse

dorsal root ganglion neurons," *American Journal of Physiology-Cell Physiology*, vol. 303, no. 4, 2012.

[75] E. Kim, M. Niethammer, A. Rothschild, Y. N. Jan, and M. Sheng, "Clustering of shaker-type k channels by interaction with a family of membrane-associated guanylate kinases," *Nature*, vol. 378, no. 6552, p. 85–88, 1995.

[76] M. Irie, "Binding of neuroligins to psd-95," *Science*, vol. 277, no. 5331, p. 1511–1515, 1997.

[77] S. E. Kwon and E. R. Chapman, "Synaptophysin regulates the kinetics of synaptic vesicle endocytosis in central neurons," *Neuron*, vol. 70, no. 5, p. 847–854, 2011.

[78] M. Erta, A. Quintana, and J. Hidalgo, "Interleukin-6, a major cytokine in the central nervous system," *International Journal of Biological Sciences*, vol. 8, no. 9, p. 1254–1266, 2012.

[79] G. Olmos and J. Lladó, "Tumor necrosis factor alpha: A link between lammation and excitotoxicity," *Mediators of Inflammation*, vol. 2014, p. 1–12, 2014.

[80] E. J. Masoro, *Caloric restriction: a key to understanding and modulating aging*. Elsevier, 2008.

[81] Y. Takei, "Age-dependent decline in neurogenesis of the hippocampus and extracellular nucleotides," *Human Cell*, vol. 32, no. 2, p. 88–94, 2019.

[82] J. Lee, K. B. Seroogy, and M. P. Mattson, "Dietary restriction enhances neurogenesis and up-regulates neurotrophin expression in the hippocampus of adult mice," *Journal of Neurochemistry*, vol. 81, p. 57–59, 2008.

[83] L. B. Ngwenya, N. C. Heyworth, Y. Shwe, T. L. Moore, and D. L. Rosene, "Age-related changes in dentate gyrus cell numbers, neurogenesis, and associations with cognitive impairments in the rhesus monkey," *Frontiers in Systems Neuroscience*, vol. 9, 2015.

[84] K. J. Mathews, K. M. Allen, D. Boerrigter, H. Ball, C. S. Weickert, and K. L. Double, "Evidence for reduced neurogenesis in the aging human hippocampus despite stable stem cell markers," *Aging Cell*, vol. 16, no. 5, p. 1195–1199, 2017.

[85] J. Nacher, C. Crespo, and B. S. Mcewen, "Doublecortin expression in the adult rat telencephalon," *European Journal of Neuroscience*, vol. 14, no. 4, p. 629–644, 2001.

[86] H. Koizumi, T. Tanaka, and J. G. Gleeson, "Doublecortin-like kinase functions with doublecortin to mediate fiber tract decussation and neuronal migration," *Neuron*, vol. 49, no. 1, p. 55–66, 2006.

[87] J. P. Brown, S. Couillard-Després, C. M. Cooper-Kuhn, J. Winkler, L. Aigner, and H. G. Kuhn, "Transient expression of doublecortin during adult neurogenesis," *Journal of Comparative Neurology*, vol. 467, no. 1, p. 1–10, 2003.

[88] S. Couillard-Despres, B. Winner, S. Schaubeck, R. Aigner, M. Vroemen, N. Weidner, U. Bogdahn, J. Winkler, H.-G. Kuhn, L. Aigner, and et al., "Doublecortin expression levels in adult brain reflect neurogenesis," *European Journal of Neuroscience*, vol. 21, no. 1, p. 1–14, 2005.

[89] L. Bondolfi, F. Ermini, J. M. Long, D. K. Ingram, and M. Jucker, "Impact of age and caloric restriction on neurogenesis in the dentate gyrus of c57bl/6 mice," *Neurobiology of Aging*, vol. 25, no. 3, p. 333–340, 2004.

[90] C. E. Finch, "Neurons, glia, and plasticity in normal brain aging," *Neurobiology of Aging*, vol. 24, 2003.

[91] D. M. Norden, M. M. Muccigrosso, and J. P. Godbout, "Microglial priming and enhanced reactivity to secondary insult in aging, and traumatic cns injury, and neurodegenerative disease," *Neuropharmacology*, vol. 96, p. 29–41, 2015.

[92] S. G. Kohama, J. R. Goss, C. E. Finch, and T. H. Mcneill, "Increases of glial fibrillary acidic protein in the aging female mouse brain," *Neurobiology of Aging*, vol. 16, no. 1, p. 59–67, 1995.

[93] G. E. Haley, S. G. Kohama, H. F. Urbanski, and J. Raber, "Age-related decreases in syn levels associated with increases in map-2, apoe, and gfap levels in the rhesus macaque prefrontal cortex and hippocampus," *Age*, vol. 32, no. 3, p. 283–296, 2010.

[94] I. Jalenques, A. Burette, E. Albuisson, and R. Romand, "Age-related changes in gfap-immunoreactive astrocytes in the rat ventral cochlear nucleus," *Hearing Research*, vol. 107, no. 1-2, p. 113–124, 1997.

[95] M. L. Cotrina and M. Nedergaard, "Astrocytes in the aging brain," *Journal of Neuroscience Research*, vol. 67, no. 1, p. 1–10, 2001.

[96] C. E. Finch, L. S. Felicio, C. V. Mobbs, and J. F. Nelson, "Ovarian and steroidal influences on neuroendocrine aging processes in female rodents," *Endocrine Reviews*, vol. 5, no. 4, p. 467–497, 1984.

[97] J. M. Encinas, T. V. Michurina, N. Peunova, J.-H. Park, J. Tordo, D. A. Peterson, G. Fishell, A. Koulakov, and G. Enikolopov, "Division-coupled astrocytic differentiation and age-related depletion of neural stem cells in the adult hippocampus," *Cell Stem Cell*, vol. 8, no. 5, p. 566–579, 2011.

[98] H. D. Vanguilder, H. Yan, J. A. Farley, W. E. Sonntag, and W. M. Freeman, "Aging alters the expression of neurotransmission-regulating proteins in the hippocampal synaptoproteome," *Journal of Neurochemistry*, 2010.

[99] J. T. Rogers, C.-C. Liu, N. Zhao, J. Wang, T. Putzke, L. Yang, M. Shinohara, J. D. Fryer, T. Kanekiyo, G. Bu, and et al., "Subacute ibuprofen treatment rescues the synaptic and cognitive deficits in advanced-aged mice," *Neurobiology of Aging*, vol. 53, p. 112–121, 2017.

[100] D. Dumitriu, P. R. Rapp, B. S. Mcewen, and J. H. Morrison, "Estrogen and the aging brain: an elixir for the weary cortical network," *Annals of the New York Academy of Sciences*, vol. 1204, no. 1, p. 104–112, 2010.

[101] K. T. Akama and B. S. Mcewen, "Estrogen stimulates postsynaptic density-95 rapid protein synthesis via the akt/protein kinase b pathway," *The Journal of Neuroscience*, vol. 23, no. 6, p. 2333–2339, 2003.

[102] D. R. Riddle, *Brain aging: models, methods, and mechanisms*. CRC, 2007.

[103] J. M. Henley and K. A. Wilkinson, "Ampa receptor trafficking and the mechanisms underlying synaptic plasticity and cognitive aging," *Dialogues Clin Neurosci.*, vol. 15, p. 11–27, Mar 2013.

[104] I. Song and R. L. Huganir, "Regulation of ampa receptors during synaptic plasticity," *Trends in Neurosciences*, vol. 25, no. 11, p. 578–588, 2002.

[105] J. G. A. Pinto, D. G. Jones, C. K. Williams, and K. M. Murphy, "Characterizing synaptic protein development in human visual cortex enables alignment of synaptic age with rat visual cortex," *Frontiers in Neural Circuits*, vol. 9, 2015.

[106] H. Wang, J. Turner, L. Ling, J. Parrish, L. Hughes, and D. Caspary, "Age-related changes in glycine receptor subunit composition and binding in dorsal cochlear nucleus," *Neuroscience*, vol. 160, no. 1, p. 227–239, 2009.

[107] C.-I. Sze, J. C. Troncoso, C. Kawas, P. Moution, D. L. Price, and L. J. Martin, "Loss of the presynaptic vesicle protein synaptophysin in hippocampus correlates with cognitive decline in alzheimer disease," *Journal of Neuropathology and Experimental Neurology*, vol. 56, no. 8, p. 933–944, 1997.

[108] S. Eastwood, P. Burnet, and P. Harrison, "Altered synaptophysin expression as a marker of synaptic pathology in schizophrenia," *Neuroscience*, vol. 66, no. 2, p. 309–319, 1995.

[109] F. W. Hopf, J. Waters, S. Mehta, and S. J. Smith, "Stability and plasticity of developing synapses in hippocampal neuronal cultures," *The Journal of Neuroscience*, vol. 22, no. 3, p. 775–781, 2002.

[110] R. Pizzarelli, M. Griguoli, P. Zacchi, E. M. Petrini, A. Barberis, A. Cattaneo, and E. Cherubini, "Tuning gabaergic inhibition: Gephyrin molecular organization and functions," *Neuroscience*, vol. 439, p. 125–136, 2020.

[111] H. Pribiag and D. Stellwagen, "Tnf- α downregulates inhibitory neurotransmission through protein phosphatase 1-dependent trafficking of gabaa receptors," *Journal of Neuroscience*, vol. 33, no. 40, p. 15879–15893, 2013.

[112] S.-M. Ye and R. W. Johnson, "Increased interleukin-6 expression by microglia from brain of aged mice," *Journal of Neuroimmunology*, vol. 93, no. 1-2, p. 139–148, 1999.

[113] S.-M. Ye and R. W. Johnson, "Regulation of interleukin-6 gene expression in brain of aged mice by nuclear factor kb," *Journal of Neuroimmunology*, vol. 117, no. 1-2, p. 87–96, 2001.

[114] M. Prechel, "Increased interleukin-6 production by cerebral cortical tissue of adult versus young mice," *Mechanisms of Ageing and Development*, vol. 92, no. 2-3, p. 185–194, 1996.

[115] D. Horrillo, J. Sierra, C. Arribas, M. G.-S. Frutos, J. M. Carrascosa, N. Lauzurica, T. Fernández-Agulló, and M. Ros, "Age-associated development of inflammation in wistar rats: Effects of caloric restriction," *Archives of Physiology and Biochemistry*, vol. 117, no. 3, p. 140–150, 2011.

[116] M. B. Cicekdal, B. G. Tuna, M. Charehsaz, M. P. Cleary, A. Aydin, and S. Dogan, "Effects of long-term intermittent versus chronic calorie restriction on oxidative stress in a mouse cancer model," *IUBMB Life*, vol. 71, no. 12, p. 1973–1985, 2019.

[117] J. Godbout and R. Johnson, "Interleukin-6 in the aging brain," *Journal of Neuroimmunology*, vol. 147, no. 1-2, p. 141–144, 2004.

[118] G. Paolisso, M. R. Rizzo, G. Mazziotti, M. R. Tagliamonte, A. Gambardella, M. Rotondi, C. Carella, D. Giugliano, M. Varricchio, F. D'Onofrio, and et al., "Advancing age and insulin resistance: role of plasma tumor necrosis factor-α," *American Journal of Physiology-Endocrinology and Metabolism*, vol. 275, no. 2, 1998.

[119] S. H. Ralston, R. G. G. Russell, and M. Gowen, "Estrogen inhibits release of tumor necrosis factor from peripheral blood mononuclear cells in postmenopausal women," *Journal of Bone and Mineral Research*, vol. 5, no. 9, p. 983–988, 1990.

[120] J. An, R. C. J. Ribeiro, P. Webb, J.-A. Gustafsson, P. J. Kushner, J. D. Baxter, and D. C. Leitman, "Estradiol repression of tumor necrosis factor-alpha transcription requires estrogen receptor activation function-2 and is enhanced by coactivators," *Proceedings of the National Academy of Sciences*, vol. 96, no. 26, p. 15161–15166, 1999.

[121] M. Kipp, S. Karakaya, S. Johann, E. Kampmann, J. Mey, and C. Beyer, "Oestrogen and progesterone reduce lipopolysaccharide-induced expression of tumour necrosis factor-alpha and interleukin-18 in midbrain astrocytes," *Journal of Neuroendocrinology*, vol. 19, no. 10, p. 819–822, 2007.

[122] G. Pani, "Neuroprotective effects of dietary restriction: Evidence and mechanisms," *Seminars in Cell and Developmental Biology*, vol. 40, p. 106–114, 2015.

[123] M. Kaur, S. Sharma, and G. Kaur, "Age-related impairments in neuronal plasticity markers and astrocytic gfap and their reversal by late-onset short term dietary restriction," *Biogerontology*, vol. 9, no. 6, p. 441–454, 2008.

[124] M. Laßek, J. Weingarten, and W. Volknandt, "The proteome of the murine presynaptic active zone," *Proteomes*, vol. 2, no. 2, p. 243–257, 2014.

[125] M. Erickson and W. Banks, "Age-associated changes in the immune system and blood–brain barrier functions," *International Journal of Molecular Sciences*, vol. 20, no. 7, p. 1632, 2019.

[126] M. M. Adams, M. E. Forbes, M. C. Linville, D. R. Riddle, W. E. Sonntag, and J. K. Brunso-Bechtold, "Stability of local brain levels of insulin-like growth factor-i in two well-characterized models of decreased plasma igf-i," *Growth Factors*, vol. 27, no. 3, p. 181–188, 2009.

[127] L. F. Eng and R. S. Ghirnikar, "Gfap and astrogliosis," *Brain Pathology*, vol. 4, no. 3, p. 229–237, 1994.

[128] Y. Matsui, S. A. Halter, J. T. Holt, B. L. Hogan, and R. J. Coffey, "Development of mammary hyperplasia and neoplasia in mmtv-tgfα transgenic mice," *Cell*, vol. 61, no. 6, p. 1147–1155, 1990.

[129] E. Di Marco, J. H. Pierce, T. P. Fleming, M. H. Kraus, C. J. Molloy, S. A. Aaronson, and P. P. Di Fiore, "Autocrine interaction between tgf alpha and the egf-receptor: quantitative requirements for induction of the malignant phenotype," *Oncogene*, vol. 4, no. 7, p. 831–838, 1989.

[130] W. Pan, K. Vallance, and A. J. Kastin, "Tgfα and the blood–brain barrier: Accumulation in cerebral vasculature," *Experimental Neurology*, vol. 160, no. 2, p. 454–459, 1999.