EXPRESSION OF KEY SYNAPTIC PROTEINS IN ZEBRAFISH (*DANIO RERIO*) BRAIN FOLLOWING CALORIC RESTRICTION AND ITS MIMETIC AND THEIR RELATIONSHIP WITH GENDER

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

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January 2017

EXPRESSION OF KEY SYNAPTIC PROTEINS IN ZEBRAFISH (DANIO RERIO) BRAIN FOLLOWING CALORIC RESTRICTION AND ITS MIMETIC AND THEIR RELATIONSHIP WITH GENDER

By Ayşegül Dede
January 2017

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.

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ABSTRACT

EXPRESSION OF KEY SYNAPTIC PROTEINS IN ZEBRAFISH (*DANIO RERIO*) BRAIN FOLLOWING CALORIC RESTRICTION AND ITS MIMETIC AND THEIR RELATIONSHIP WITH GENDER

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MSc in Neuroscience

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January 2017

Aging is a progressive decline of physiological functioning and metabolic processes. Among all the organs, the brain seems to be the most vulnerable part of the body to the age-related changes because of the relatively high consumption of oxygen and glucose as compared to other organs. Both structural and cognitive changes occur during the aging process. A great effort has been spent to ameliorate the outcomes occurring within the brain as a result of aging. Caloric restriction (CR) is considered to be the only non-genetic intervention which decreases age-related cognitive decline. Rapamycin (RAP) has become a candidate drug which was shown to mimic the effects of CR by blocking the nutrient-sensing pathway, the mammalian target of Rapamycin, (mTOR) pathway. The first aim of this study was to investigate the expressions of key synaptic proteins; gephyrin, PSD-95 and synaptophysin, which are involved in the synaptic plasticity, after short-term (4 weeks) CR and RAP interventions in young and old, male and female zebrafish. The second aim was to investigate whether the expression of glutamate receptor subunits, NR2B and

GluR2/3, display a sexually dimorphic pattern in middle age zebrafish. It was found that there was no significant difference in the expression of key synaptic proteins between the CR and RAP animal groups as compared to the *ad libitium* (AL) fed group and also no significance was found in the expression of NR2B and GluR2/3 in middle-aged male and female zebrafish. Highlighted studies in this thesis demonstrate that short-term (4 weeks) of CR and RAP treatments were too short to observe an effect in the expression level of gephyrin, synaptophysin, and PSD-95, and in the middle age, expression of NR2B and GluR2/3 did not display sexually dimorphic pattern. Our initial results of key synaptic protein levels indicate that they are stable throughout aging with respect to gender and CR interventions.

Keywords: aging, zebrafish, brain aging, caloric restriction, rapamycin, sexual dimorphism, synaptic proteins, glutamate receptor subunits

ÖZET

ZEBRA BALIĞI (*DANIO RERIO*) BEYNİNDEKİ ANAHTAR SİNAPTİK PROTEİN İFADELERİNİN KISA SURELİ KALORİ KISITLAMASI VE KALORİ KISITLAMASI TAKLİTÇİSİ VE CİNSİYETLE İLİŞKİSİ

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Nörobilim Lisansüstü Programı, Yüksek Lisans

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Ocak 2017

Yaşlanma, fizyolojik işlevler ve metabolik süreçlerdeki azalmadır. Vücuttaki organlar arasında beyin, diğer organlara kıyasla nispeten yüksek oksijen ve glikoz tüketimi nedeniyle yaşla ilişkili değişikliğe karşı vücudun en savunmasız organıdır. Yaşlanma sürecinde yapısal ve kognitif değişiklikler meydana gelir. Yaşlanma ile beyninde ortaya çıkan sonuçların iyileştirilmesi için büyük bir çaba harcanmaktadır. Kalori kısıtlaması (KK), yaşla ilişkili olarak azalan kognitif fonksiyonları geliştirmede genetik olmayan tek müdahale olarak düşünülmektedir. Rapamisin (RAP), rapamisin memeli hedefi (mTOR) besin sinyal yolağını bloke ederek, KK'nın etkilerini taklit ettiği gösterilen aday bir ilaçtır. Bu çalışmanın ilk amacı, kısa süreli (4 hafta) KK ve RAP uygulamasının yaşlı ve genç, dişi ve erkek zebra balıklarındaki önemli sinaptik proteinler olan sinaptofizin, post-sinaptik yoğun protein 95 kD (PSD-95) ve gephyrinin protein ifadesine etkisine bakmaktır. Bu çalışmanın ikinci amacı ise glutamat reseptör alt birimlerinin (NR2B ve GluR2 / 3) ifadelerinin orta yaş zebra balığında cinsel dimorfik bir model gösterip göstermediğini araştırmaktır. Sonuçlar,

önemli sinaptik proteinlerin ifadelerinin, KK ve RAP hayvan grupları, kontrol grubu ile karşılaştırıldığında anlamlı bir fark bulunmadığı ve glutamat reseptör alt birimlerinin ifadelerinin orta yaşlı dişi ve erkek zebra balıklarında birbirinden anlamlı olarak farklı olmadığını göstermiştir. Bu tez çalışmasında vurgulanan çalışmalar kısa süreli KK ve RAP tedavisinin, gephyrin, sinaptofizin ve PSD-95'in ifade düzeyinde bir etki gözlemlemek için çok kısa olduğunu ve orta yaşlı zebra balıklarındaki NR2B ve GluR2/3'ün ifadesinin cinsel dimorfik özellik göstermediğini göstermektedir. Başlangıç sonuçlarımız, anahtar sinaptik protein seviyelerinin yaşlanma süresince cinsiyetlerde ve KK müdahaleleri açısından sabit kaldığını göstermektedir.

Anahtar kelimeler: Yaşlanma, zebrabalığı, beyin yaşlanması, kalori kısıtlaması, rapamisin, cinsel dimorfik özellik, sinaptik proteinler, glutamat reseptör alt birimleri

ACKNOWLEDGEMENT

First and foremost, I would like to express my gratitude to my advisor Dr. Michelle Adams for accepting me as her student and for her patience, guidance, knowledge and support, without her I would not have performed any of these experiments.

I'd like to thank Dr. Ayşe Begüm Tekinay, Dr. Çağdaş Devrim Son, Dr. Özlen Konu and Dr. Tülin Yanık for their kindness on accepting to take part in my thesis committee.

I'd like to acknowledge my dear collages in Adams lab; Dr. Ayça Arslan Ergül, Dr. Fusun Doldur-Ballı, Melek Umay Tüz, Begün Erbaba, Naz Şerifoğlu for their guidance and friendship. I acknowledge Narin Ilgım Ardıç for the western blot experiments in the Chapter 2. I'd like to express my sincere gratitude to Elif Tugce Karoglu and Zeynep Erbas for their help in statistics and experiments.

I'd like to acknowledge Tulay Arayici for the zebrafish brain dissections.

I'd like to thank Dr. Esra Yuca for her advices, kindness and inspiration.

I would like to thank my dearest friends Pelin Özge Burhan, Ergül Dilan Çelebi, and Göksemin Şengül for their full-time friendship and support.

I would also like to thank my amazing friends Selin Su Yirmibesoglu, Seren Hamsici, Melike Sever and Gokhan Gunay for their support, belief, inspiration, love and always reminding me what it means to be a friend, in good times and bad times.

I'd like to convey my sincere thanks for my friends Selen Erpalak, Ozge Ozguc, Tayanc Gozel, Hazal Sena Metin, Ceren Ozen, Esin Gulce Seza, Hulya Copoglu,

Ebru Okur, Gokhan Mamak for their friendship since the very first day of my undergraduate years.

I’d like to thank Dr. Sertac Onde for all the support, food and best wishes throughout my undergraduate and graduate years.

I’d like to express my sincere appreciation to my family and my family to-be Egemen Deniz Eren. Without their support, love and encouragement, none of them could have happened. I’d like to dedicate my thesis to my Father whom I want to be the most proud of me.

I would like to acknowledge The Scientific and Technological Research Council of Turkey (TUBITAK) grant no 214S236 for funding my research and financially supporting me.

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Abbreviations

AL	*Ad-libitum*
ANOVA	Analysis of Variance
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
APS	Ammonium per sulfate
BDNF	Brain-derived neurotrophic factor
CR	Caloric Restriction
DG	Dentate Gyrus
DMSO	Dimethyl sulfoxide
GABA	γ-Aminobutyric acid
kDa	kilo Dalton
LTP	Long Term Potentiation
MANOVA	Multiple Analysis of Variance
MRI	Magnetic resonance imaging
mTOR	mammalian target of rapamycin
NMDA	N-Methyl-D-aspartete
PCA	Principal Component Analysis
PKB	Protein kinase B
PSD-95	postsynaptic density protein 95
RAP	Rapamycin
SDS-PAGE	Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis
TBS-T	Tris-Buffered Saline and Tween 20
TEMED	Tetramethylethylenediamine
TOR	target of rapamycin

CHAPTER 1

Introduction

1.1.Aging

Aging is described as a continuous deterioration of physiological and metabolic functions [1,2]. It is a multisystemic process and all systems within the organism are affected; muscles become weaker, bones become more fragile, skin shrinks, immunity and memory become weaker [2]. In order to explain the reasons of aging, many hypotheses were developed such as deteriorations in the biological membranes, attack of free radicals, impairment in calcium regulation, and, particularly in mammals a negative effect of glucocorticoids and stress [3–8].

Decrease in walking speed and increase in the duration of step timing are among the physical dysfunctions that elderly people experience [9,10]. Falling incidence is one of the physical irregularities that come upon with age [10]. Tromp *et al.* (2001) showed that 30% of elderly people who are 65 years of age fall at least once per year and this rate increases with age [11]. Weakening of muscle strength and disruption in muscle joints are also related to decreases in the physical functioning [12]. In addition to the muscular disturbances; deteriorations in vision, prolonged reaction times, reductions in learning ability are experienced by the elderly [10]. Cross-section studies has reported that in human populations, the remembering of verbal information is decreased with normal aging [13]. Moreover, changes in the age-related memory deprivation is conserved among mammalian species; in humans, monkeys, dogs, and mice, the decline in spatial memory has been

observed [13–16]. About 2% of the body weight is represented by the average adult brain and despite its comparatively small size, the brain consumes 20% of the total oxygen [17]. Therefore, the brain is more prone to oxidative damage that occurs with aging.

1.1.1. Brain aging

The influence of aging on the brain is widespread [18]. Effects of aging on the brain can be characterized by a change in the molecules, cells, gross morphology, cognitive functions and metabolism [18]. During the course of aging, a number of changes which influence the structure of the brain occur. Along with the neuroanatomical changes, neurochemical and cognitive changes are also observed during the course of aging [19].

1.1.1.1.Structural Changes

Not all changes associated with aging occur in the same way in all brain regions [20]. Some of the structural changes associated with aging are the reductions in the volume of gray matter (in the frontal lobes, temporal lobes, the hippocampus, and cerebellum), the susceptibility of prefrontal white matter volume, and the decline in the volume of cerebellum and hippocampus [21, 22]. Svennanholm *et al.* (1997) showed that volume and/or weight of brain decreases by 5% every 10 years after the age of 40 [23]. The loss in the volume of gray matter and striatal volume are also critical components that are associated with aging [24–26]. Reductions in the white matter display correlations with alterations in processing speed, short-term recall and executive function in aged individuals [13]. Magnetic Resonance Imaging (MRI) analysis on the measurement of brain volume has shown that decreases in the volume

of frontal cortex occur more than other cortical and subcortical areas [27,18]. It was concluded that all cortical and subcortical areas become smaller with aging and frontal and striatal regions lose more volume compared to other regions. In addition to the change in the volume of brain regions, the neuronal volume is another indicator of aging. Earlier studies reported that age-related cognitive decline was a consequence of a significantly reduced number of cells and synapses [28–34]. However, more recent studies showed that this decline does not occur as a result of aging and stated that synaptic structures or molecules actually do change with age [35–38]. Pioneering work by Rapp *et al.* (2002) shows that the neuron death does not occur by aging [39]. Similarly, Murphy *et al.* (1996) demonstrated that with aging, neuronal volume, rather than number, decreases [40]. Additionally, Barnes *et al.* (2003), described the decrease in the number of dendritic synapses or synaptic plasticity during aging [41].

1.1.1.2.Neurochemical and Metabolic Changes

In rodents, aging-associated reductions in the brain-derived neurotropic factor (BDNF) might contribute to age-dependent changes in the cognitive impairment [42–44]. Decreases in the secretion of growth hormones was a consequence of aging [45]. Also, in the aging brain glucose metabolism becomes disrupted; glucose and oxygen input decreases [46]. Impairment in calcium metabolism, dysfunction in mitochondrial functioning and increases in the production of reactive oxygen species are other consequences of brain aging [18].

1.1.1.3.Cognitive Changes

Cognitive aging is characterized by decreases in learning abilities, poor attention, and deteriorations in memory [47]. As a result of aging, reductions in cognitive function can be observed in most individuals who are between 65-84 years old, and the prevalence rate in the reductions in the cognitive functions was shown to be 28% [48]. When a task involving executive function is given, prefrontal cortex and hippocampus activation were higher in young adults compared to older adults [49], [50]. Particularly, the hippocampus is a crucial brain region for learning and memory and it is vulnerable to aging [51]. Rodents with damaged hippocampus display impairments in learning and memory [52]. Also in humans with damaged temporal lobe, similar to rodents, spatial information processing is impaired [53]. Moreover, Burke and Barnes *et al.* (2006) showed that hippocampal volume and neuronal plasticity decreased with healthy aging and which explains hippocampus-dependent functional reduction [54]. Ojo *et al.* (2015) suggested that impaired hippocampal synaptic integrity and signaling and increased neuroinflammation can explain this functional reduction [55]. Loss of synaptic connections is another potential reason for cognitive decline [13]. In aged monkeys and humans, a decrease in synaptic density was observed, and this is correlated with reduced activation in prefrontal cortex while performing an executive function [56, 57]. Also, decreases in the number of synaptic connections in the dentate gyrus region of hippocampus in old rats explain spatial learning impairments [58]. In addition to synaptic connections, decreases in the glutamate uptake and release with age were reported. Glutamate is the major excitatory neurotransmitter in the central nervous system and during aging, density of its receptors N-methyl-D-aspartate (NMDA) receptor and α-

amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor display reduction in the number of their subunits [59]. Also, down-regulation of regulatory proteins involved in neurotransmission in the hippocampus was reported [60].

1.2. Caloric Restriction

Caloric restriction (CR) is defined as the reduction in the total calorie intake without any decline in the micronutrients [61]. The favorable results of a CR regimen can be listed as reduced age-related lipid alterations, triggering of protective stress proteins, repression of the age-related decreases in vascularity, as well as a repression of the age-related reduction in neural progenitor cell division, and glutamate receptor subunit levels [61–66]. CR can prolong the life expectancy in a wide range of species including rodents by 20% to 40% and predicates a daily reduction in all dietary components in a certain proportion [67, 68].

Studies showed that CR affects the regulation of adult neuronal stem cells; it leads to an increment of neurogenesis in young adult rats, and in old adults, and it enhances the neurogenesis which normally declines with aging [69–71]. Lee *et al.* (2002) tested the hypothesis that 3 months of CR improves neurogenesis and reported that there was an increase in the number of newly generated neurons in dentate gyrus (DG) region of hippocampus in adult animals under a CR-feeding regimen and this increase was due to the enhancement of survival [71].

In rodents, nutritional supplementation positively affects memory and learning. Kuhla *et al.* (2013) reported improvements in learning and memory in animals with lifelong CR diet [72] . Also, levels of key synaptic proteins which change with age and thought to be related to synaptic plasticity in hippocampus have been shown to

be stabilized with CR treatment. Adams *et al.* (2008) showed that in the CA3 region of hippocampus ionotropic type of glutamate receptor subunits displayed age-related reductions in their protein expression level and this reduction was rescued with CR [61]. In the same study, in addition to glutamate receptor subunits, a presynaptic vesicle protein, synaptophysin, was reported to be stabilized in CR animals compared to control animals [61].

Also, CR protects neurons from exotoxins, oxidative and metabolic damages and also enhances neurotransmitter systems particularly in the hippocampus [62], [73]. Metabolic stability has been shown to be a better indicator of longevity than metabolic rate. McCarter and McGee (1989) reported that loss of functions associated with aging is related to impaired homeostatic state and capacity of preserving a steady metabolic state is a determinant of longevity [74]. In addition to its positive effects on health, late onset CR was proposed to extend lifespan; CR regimen conducted on 12 months old rats showed reduction in age-related oxidative stress [75].

Although CR is an important approach, development of potential CR mimetics could be more applicable to humans. Recent studies showed that the positive effects of CR result from the blocking of mammalian target of rapamycin (mTOR) nutrient-sensing pathway both *in vivo* and *in vitro*. For example, CR has been shown to alter mTOR signaling in adipose tissue and mammary tumors, and it has been reported that CR regimen changed the expression of genes associated with mTOR signaling in mice hypothalamus [76, 77].

1.3. Rapamycin

A National Institute of Aging Intervention Testing Programme has been launched with the purpose of finding compounds which are safe, non-invasive and effective in order to increase the lifespan of model organisms and to date, rapamycin was the most effective FDA approved agent [78]. Because of its involvement in metabolism and regulation of growth in response to aminoacid and nutrient uptake, the target of rapamycin (TOR) became a well-studied pathway in relation to CR and CR mimetics [79]. TOR is an important signaling node which controls cellular metabolism and life span in both invertebrates and vertebrates [80]. It is also involved in the control of cell division, proliferation and survival and regulation of long-lasting synaptic plasticity [81,82]. mTOR is the mammalian homolog of the TOR pathway and is also involved in nutrient sensing pathway, therefore it is thought that rapamycin and CR exert their effects through similar mechanisms [83]. Genetic and pharmacologic blockage of mTOR was shown to increase lifespan in yeasts, worms, fly and mice and this results in the question of the relationship between mTOR and aging [84–88]. For example, 16 weeks or longer rapamycin treatment results in 30% reduction in mTOR activity in mice [89].

In recent years, rapamycin has been shown to significantly increase both median and maximal lifespan in mice [87,88]. As rapamycin can be applied for the long term, it can also be applied for short-term; life span of old mice was found to increase after short-term (6 weeks) rapamycin treatment [90]. Recently, chronic rapamycin treatment has been shown to delay aging by inhibiting the mTOR activity [87].

Chronic inhibition of mTOR by rapamycin modulates cognitive and non-cognitive components of behavior throughout lifespan in mice. Compared to control groups, mice under rapamycin treatment displayed some distinct behaviors; they spent significantly more time on voluntary wheel running [91]. Sixteen weeks or longer rapamycin treatment resulted in 30% reduction in mTOR activity in mice [79]. Also, compared to control fed littermates, mice with at least 16 weeks of rapamycin treatment displayed enhancement in age-related cognitive decline [89]. Parallel to those results, in many but not in all rodents; CR activity decreased mTOR activity, similar to rapamycin, and improved cognition [61,76,92–94]. Therefore, reduction in mTOR activity with rapamycin and CR treatment; improves cognition in young mice and maintains cognitive performance in old mice [79].

1.4. Zebrafish as a Model Organism

Many fish species were used as model organisms for aging studies but recently zebrafish (*Danio rerio*) were designated as a potential organism in order to understand the mechanism of diseases as well as vertebrates development and genetic studies [95]. It is a relatively small fish (1-2 inch), and a fresh water animal native to India [96]. Also, the average lifespan of zebrafish is between 36-42 months and maximum lifespan can reach up to 66 months and its generation time is 3-4 months [95].

For developmental biology studies, it has a couple of advantages; developmental genes of zebrafish are conserved across vertebrates; its small size enables many animals to be hosted even in small places; it is easy to feed them with a water soluble chemical or drug and it is easy to make mutation studies [95]. Also, it is an ideal model for mutation analysis [96]. Since its embryos are transparent;

morphology of zebrafish embryos can be monitored with gross or visual examinations [96]. A female zebrafish can produce hundreds of eggs in a clutch and this provides many numbers of offsprings [96]. Fast ex-utero development enables behavioral and morphological observations within 4-7 days [96]. Also, their sizes are large enough to dissect enough tissues from specific organs [96]. Zebrafish possess an integrated nervous system and similar to mammals, they have high behavioral abilities such as learning and memory, and social behaviors [97,98]. Aging zebrafish exhibit phenotypes similar to aging mammals such as the appearance of senescence-associated beta-galactosidase staining and oxidized proteins [99]. Furthermore, aging zebrafish develops spinal curvature morphology [100]. Also, as in most vertebrates, zebrafish shows gradual senescence features during aging [99].

1.5. Gender Difference

Many of sexually dimorphic characteristics were reported at anatomical, molecular and cellular levels in vertebrates including humans and rodents and invertebrates such as drosophila [101,102]. Differences in brain organization and function between genders can result from many regulatory systems; sex-specific genomes of males and females, hormonal regulation, gene expression in various regions of the brain and epigenetic changes [103]. In *Drosophila melanogaster*, it was reported that 50% of the genes are sexually dimorphic [101]. Similarly, Santos *et al.* (2008) showed that in zebrafish, there are 42 genes, which are expressed differently in males and females and, protein synthesis is more active in males than females [104]. This diversity also differs depending on the brain region and the age of the subjects. Ampantsiz *et al.* (2012) showed that in zebrafish, the number of cycling cells differs in males and females depending on the brain region; in medial

zone of dorsal telencephalon, periventricular nucleus of the posterior tuberculum and ventral part of periventricular pretectal nucleus the number of cycling cells is more in females [105]. On the contrary, in dorsal zone of periventricular zone number of cycling cells in more in males [105]. The *in-situ* hybridization method employed by Bayless and Shah (2016) showed that the upregulation and downregulation of genes show variation based on the region and gender in mice which were explained by the sex hormones, and contribution of sexually dimorphic gene expression to sexually dimorphic social behaviors [106]. Similarly, neuroimaging techniques showed that in mice, sexual dimorphism exists in brain areas such as amygdala and hypothalamus [102]. In humans, gene expression in male brain changes until the age of 60 and 70 and is stabilized in the following years; however, in female brains, change in gene expression proceeds until the age of 80 and 90 [107].

Sexual dimorphism manifests itself at the structural level as well. For example, a study conducted on 380 adult subjects showed that the male brains were structurally more asymmetric in the majority of the brain regions [108]. It was also reported that in male mice, the total area of corpus callosum was larger than the female mice of the same age [102]. Moreover, it was reported that men display higher brain atrophy and increased cerebrospinal fluid in frontal and temporal lobes compared to women [107]. Likewise, it was shown that brain volume of a male mouse is 2.5% larger as compared to a female mouse. Also, in terms of age, it was indicated that men display sharper changes compared to women as they get older [109]. In addition to the structural differences, brain physiology was shown to differ in cognitive tasks between genders [102]. Brain activity, task performance, and memory function were reported to change depending on the gender as well [107].

Therefore, the investigation of sexual dimorphism is vital in order to have a comprehensive understanding of the differences in genders, as it would make future studies more comprehensive.

CHAPTER 2

Expression of PSD-95, Synaptophysin and Gephyrin in Calorically Restricted and Rapamycin Treated Young and Old, Male and Female Zebrafish

2.1.Introduction

Among the non-genetic interventions, CR has been shown to be the only way to postpone aging and age-related cognitive decline in mammals [110]. It has been investigated since the late 1900s-; McCay *et al.* (1935) showed that decline in the amount of food intake in rats leads to an increment in maximum lifespan and also it was reported that mice under caloric restriction showed enhanced motor and learning performances [111,112]. Furthermore, mice and rats which were given a CR diet displayed decelerated aging and developed protection to the impairments in the tissues occurring with age [110]. The purpose of CR is to reduce the overall calorie intake up to 30-40% without causing undernourishment; therefore, CR does not mean starvation [110]. Duration, regime, age or gender of the organism and organism itself are the key variables for CR studies. CR can be applied for short-term (4- 10 weeks) or longer terms (3 months, 6 months and more) and dietary regimen can be applied with the alternate day feeding method or by reducing the daily portion of the meal [110]. For example, Lee *et al.* (2002) used alternate day feeding method and 3 months of CR and showed alterations in neurotrophin expression and neurogenesis [71]. Another study investigated the effects of 4, 20 and 74 weeks of CR on the working memory in mice and showed that lifelong CR enhanced the working

memory [72]. When 30% CR was applied to 3 months old rats for 6 months and 14 months, expression of PKB and S6 which are the downstream elements in the mTOR pathway, were reduced [113]. However, when 35-40% CR was applied for 6 months to 3-4 months old animals, expression of several proteins involved in this pathway did not change [113]. This indicates that age or the organism, duration of CR and also percentage of the calorie intake is important. Furthermore in CR studies, AL group is important to examine and understand the effects of CR, since it acts as a control group.

Although CR has been shown to increase lifespan and enhance cognitive and physical activities, it is difficult to apply a true CR regimen to humans. Therefore, raising CR mimetics has become an intriguing issue for pharmaceutical companies [110]. Among the candidate drugs, rapamycin, which blocks the mammalian target of rapamycin (mTOR) nutrient sensing pathway, has become the most efficient and well-studied CR mimetic [110].

Rapamycin was shown to increase medial and maximal lifespan in male and female mice [88]. It can be applied short term or long term; for example, Chen *et al.* (2009) showed that 6 weeks (short term) of rapamycin treatment increase the lifespan of old mice [90]. Not only life span but also cognitive functions can be enhanced with rapamycin treatment. At least 16 weeks of rapamycin treatment has been shown to ameliorate the age-related cognitive decline [89]. Similarly, 16 months and 3 months of rapamycin treatment were reported to enhance cognitive functions by increasing the NMDA signaling [114].

Expression of key synaptic proteins can be investigated as indicators of pre- and postsynaptic integrity. The three synaptic proteins; postsynaptic density protein 95

(PSD-95), synaptophysin, and gephyrin are the markers of synaptic integrity. PSD-95 is largely present in the excitatory central nervous system synapses and was first identified as a Type-I or asymmetric PSD. It appears as 90–95 kDa on SDS-polyacrylamide gels [115]. Due to its PDZ domains, it acts as a scaffold protein and is crucial for the anchoring of NMDA- and AMPA-type glutamate receptors and therefore involved in the synaptic plasticity [116]. PSD-95 mutant mice displayed abnormal long-term potentiation (LTP) with impaired learning and memory functions [117]. Because of its role in synaptic functioning on excitatory postsynaptic membranes, PSD-95 is a critical component. Gephyrin is a key, highly conserved scaffold protein that is present in the postsynaptic membrane of the inhibitory synapses and extensively expressed in vertebrates, and it is analogous of PSD-95 in inhibitory synapses [118–120]. It coordinates the organization of postsynaptic density which includes glycine, γ-aminobutyric acid type A ($GABA_A$) receptors as well as synaptic transmission and long-term potentiation [118]. It has been found to be expressed in diverse tissues involving skeletal muscle, heart, liver and brain with different expression patterns which indicates its decisive role in both central nervous system and metabolism [118]. Therefore, investigation of the expression of gephyrin can provide information about the inhibitory synapses and clustering of inhibitory synaptic proteins. Synaptophysin is a leading synaptic vesicle glycoprotein and expressed in neurons and endocrine cells [121]. Mutant mice which lack the synaptophysin displayed deteriorations in synaptic vesicle formation, maturation and endocytosis; therefore it is considered the molecular marker of synaptic vesicles [121]. Also, the role of synaptophysin in the neurotransmitter release has been investigated widely. It was reported that antisense oligonucleotides

or antibodies directed against synaptophysin resulted in the excessive decline in the evoked release reconstituted in *Xenopus* oocytes [122]. Also, the levels of synaptophysin were reduced in aged rats and resulted in the defects in learning [123]. Similarly, Calhoun *et al.* (1998) showed the correlation between synaptophysin levels and learning and memory abilities [124]. Therefore, the synaptophysin level is a valid signal of presynaptic integrity.

It has been shown that males and females differ in their brain biochemistry, structure, and function [125]. Alterations in the gene profile across lifespan were also reported to be gender as well as brain region specific [107]. Similar to the gene expression, structural and functional changes, neurogenic capacities of neurons in the cerebellum also differ between genders [126]. In addition to the cerebellum, hippocampus displays sexual dimorphism in terms of its structure, response to stimuli as well as neurochemical make-up [127]. Separating animal groups based on gender will provide answers to the given questions for both genders. For example, females and males express different levels of dendritic spine density under stressed conditions and they respond in opposite ways to the same stimuli which would be a more accurate answer to the question of how do the spine density change under stress conditions [128].

This chapter is designated to demonstrate the expression levels of synaptophysin, PSD-95 and gephyrin after short-term (4 weeks) of CR and rapamycin (RAP) interventions in young and old, male and female zebrafish.

2.2. Materials and Methods

2.2.1. Subjects

In the experiments, 12 zebrafish (*Danio rerio*) were used, these animals were one cohort of a larger study of 48 animals. The Bilkent University Local Animal Ethics Committee (HADYEK) approved the zebrafish protocols of this study with the numbers of 2012/6 and 2012/30. Animals were distributed as illustrated in the Table 2.1.

Table 2.1 Distribution of subjects

Western blot Cohort	**Number**	**Total Study**	**Number**
Young-Female	3	Young-Female	15
Old- Female	3	Old- Female	15
Young- Male	3	Young- Male	9
Old- Female	3	Old- Female	9
TOTAL	**12**		**48**

2.2.2. Housing

Subjects were housed at 28 °C with a 14 hours of light and 10 hours of dark cycle and kept within a recirculating tank system in the zebrafish facility of Molecular Biology and Genetics Department, Bilkent University, Ankara, Turkey. Animals were kept in 4 L glass aquaria (Pasabahce, Turkey) and aquaria were cleaned and filled with fresh water twice a week. Before starting the treatment period, the animals were habituated to the glass aquaria.

2.2.3. Treatment

Animals were treated either with rapamycin (RAP) or dimethyl sulfoxide (DMSO) (CR and AL groups). The stock solution was prepared by dissolving rapamycin (Fluka, Sigma, Germany) in DMSO (Applichem, Germany) to 500 μM concentration and stored at -20°C. Fifty μL from stock solution was added to the water of RAP group, and 50 μL of DMSO was added to the water of CR and AL groups in order to obtain a final concentration of 100 nM. The drugs and treatments were administered during light cycles.

2.2.4. Dietary Regimen

Ad-libitum (AL) feeding and caloric restriction (CR) were the two dietary conditions. AL animals were fed with 20 mg of food (TetraMin, Germany) and CR animals were fed with 1 mg of food per fish per day on weekdays and animals were fed individually in 600 mL glass beakers (Isolab, Germany) with 250 mL system water. On weekends, fish were not separated from the housing tanks and AL and RAP group were fed with 40 mg of food and CR group were fed with 2 mg of food.

2.2.5. Weighing and Transferring

In order to transfer the fish from aquaria to the beakers, separate nets were used for the each animal group. During weekdays, fish were transferred to the beakers for treatment, housed there for 5 hours and taken back to its aquaria. On Mondays and Fridays fish were weighed in a 250 mL beaker.

2.2.6. Euthanasia

Subjects were euthanized in a tank filled with distilled water and ice. After euthanasia, removal of the head from the body was performed by using a scalpel.

The eyes and optic nerves were taken out to allow separation of brain from the skull. The brain was dissected from the skull and put in a 0.5 mL microtube and thrown in liquid nitrogen for snap freeze and stored at -80°C. In order to be able to identify the gender of animals, the abdomen areas of each animal were dissected. Presences of eggs were an indication of females and presence of testes were an indication of male zebrafish. In the cases of which no visible eggs or testes could be observed, animals were excluded from the study.

2.2.7. Protein Isolation

Before homogenization of the brain tissues, lysis buffer solution which is composed of 75 µL of 2M NaCl, 50 µL of 1M TrisHCl pH:8, 10 µL of 100% NP-40, 10 µL 10% SDS, 355 µL sddH_2O and 500 µL of protease inhibitor (2x stock, 05 892 970 001, Roche) for 1 mL was prepared. Brain samples were placed another 1.5 µL Eppendorf tube. Sixty µL of lysis buffer was added for each 1 mg tissue. Samples were homogenized by using a 2 mL syringe by pawing through 5-10 times. Homogenates were incubated on ice for 30 minutes by gently mixing twice and centrifuged at 13.000 rpm for 20 minutes at 4^{o}C. Supernatants of samples were taken and aliquoted.

2.2.8. Protein Quantification

Bradford assay (Bradford Reagent, B6916, Sigma, St. Louis, MO, USA) was employed in order to determine the soluble protein concentration. Bovine serum albumin (BSA; Sigma, St. Louis, MO, USA) was used as a standard in order to construct a standard curve. The samples were added according to Table 2.2.

Table 2.2 Amounts of solutions required to be added into each tube from 1 mg/mL BSA.

(µL)	**0**	**1**	**2**	**4**	**6**	**8**	**10**
Bradford	400	400	400	400	400	400	400
BSA	0	1	2	4	6	8	10
ddH_2O	100	99	98	96	94	92	90

Bradford, sample proteins, and ddH_2O were added into each tube according to Table 2.3 in order to quantify of the sample proteins.

Table 2.3 Bradford assay for protein samples.

For Sample Protein	**Volume**
Bradford	400 µL
Total Protein	1 µL
ddH_2O	99 µL

Into the 1.5 µL Eppendorf tube, first 99 µL of ddH_2O and then 1 µL of protein were added for each sample and tubes were kept in ice. Finally, Bradford reagent was added and all samples were incubated for 5 min at room temperature**.** Beckman DU-640 Spectrophotometer (6511 Bunker Lake Blvd. Ramsey, Minnesota, 55303 USA) was employed to quantify proteins in the lysates. The wavelength of the Spectrophotometer was set to 595 nm= λ. First, blank samples were measured. Based on the data, the graph of the A595 vs. BSA variables was drawn and the linear graph and the equation validating the graph were supplied by using Excel file.

2.2.9. Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE enables protein separation according to their size. Two types of agarose gels were prepared for the SDS-PAGE, the one in the bottom is the basic (pH 8.8) resolving gel, which has high acrylamide content and the one in the top is the slightly acidic (pH 6.8) stacking gel and composed of low acrylamide content.

2.2.9.1. Preparation of Resolving and Stacking Gel

In order to prepare 10 mL 10% Resolving Gel, 15 mL empty falcon was used. Two different sized glass plates (Biorad Life Science 1000 Alfred Nobel Drive Hercules, California 94547 USA) and 1 spacer 1.0 mm thick (Biorad Life Science 1000 Alfred Nobel Drive Hercules, California 94547 USA) were taken and each glass plate was attached to spacers. After mixing all the reagents according to the Table 2.4 by slowly swirling, the mixture was poured immediately between the glass holders till the line of the holder by Pasteur pipette. In order to remove bubbles, isopropanol was gently added. After polymerization occurred, isopropanol was cleared out and the gel was washed with ddH_2O.

Table 2.4 10 mL 10% Resolving Gel

Order	**Substance**	**Amount (mL) For 1 Gel**
1	ddH_2O	4.65 mL
2	1.5 M Tris (pH=8.8)	2.5 mL
3	10% SDS	100 µL
4	40 % Acrylamide Mix	2.65 mL
5	10% APS	100 µL
6	TEMED	10 µL

In order to prepare 8 mL 10% Stacking Gel, 15 mL empty falcon was used. Gel was prepared by adding the given order in Table 2.5 and mixed by using pasteur pipette and the comb was placed on it. After polymerization occurred, the slips, comb, and bottom spacer were removed.

Table 2.5 8 mL 4% Stacking Gel

Order	**Substance**	**Amount (mL) For 1 Gel**
1	ddH_2O	4.764 mL
2	0.5 M Tris (pH=6.8)	2.0 mL
3	10% SDS	80 µL
4	40 % Acrylamide Mix	1.060 mL
5	10% APS	80 µL
6	TEMED	16 µL

Reagents:

1. **Separating Buffer (1.5 M Tris-HCl, pH 8.8):**

For 100 mL, 18.171 g of Tris Base (FW:121.14) was dissolved in 50 mL of water. pH was adjusted to 8.8 with HCl then add full volume of water.

2. **Stacking Buffer (0.5 M Tris-HCl, pH 6.8):**

For 100 mL, 6 g Tris Base (FW:121.14) was dissolved in 60 mL water. pH was adjusted to 6.8 with HCl then volume was adjusted with water.

3. **Sodium Dodecyl Sulfate SDS (10%):**

10 g lauryl sulfate was dissolved in 90 mL ddH_2O dissolve, brought to 100 mL.

4. **Ammonium per sμL fate APS: (10%, Fresh):**

30 mg ammonium persulfate was dissolved in 300 μL sddH_2O.

2.2.9.2. Preparation of Protein Samples and Loading & Running

Protein concentration was calculated according to the Bradford Quantification assay and appropriate volumes of the samples were diluted with loading buffer; 25 μL of protein plus ddH_2O, and 25 μL of loading buffer were mixed and the Eppendorf tubes were incubated at 95^oC for 10 minutes to boil the proteins within the samples and denature them but keeping sulfide bridges. Also, this provides negatively charged amino acids not being neutralized and allow running of the proteins in the electric fields. Then they were quickly span down by centrifuge machine. Samples were kept on ice till the loading protocol. In total 50 μL of was loaded into the wells.

Mini-PROTEAN 4- gel electrophoresis system was used for casting and running 1.0 mm thickness gels. The system comes with a tank, lid with power cables, 1 electrode assembly, 1 companion running module 1 minicell buffer dam, 2 casting stands, 4 casting frames, 5 10-well combs, and 5 sets of glass plates. Glasses were placed on cassette and cassettes were placed into the tank. The tank was loaded with running buffer till '2 gels' level. The comb was removed and wells were washed with 1X Running buffer by using 2mL syringe. Samples were loaded into the wells and empty wells were loaded with sample buffer. Thermo Scientific PageRuler Prestained Protein Ladder (2x250 μL 10 to 170 kDa- with product number "26616")

was used as a marker. Its formulation is: dye- stained proteins in 62.5 mM Tris-phosphate(pH= 7.5), 1 mM EDTA, 2% SDS, 10 mM DTT, 1 mM NaN3 and 33% glycerol. The tank was connected to the Bio-Rad power supply and set 90V at room temperature for 30 minutes for stacking and 120 V and kept working till the blue dye reached the bottom for separation of the proteins.

Reagents:

1. **Loading Buffer:**

4 % SDS (4 mL from % 10 SDS), 10 % B-ME, 20 % glycerol (2 mL), 0.004 % BPB (100 ul from 4% stock) and 0.125 M Tris-HCl pH= 6.8 were mixed.

2. **10x Running Buffer:**

25 mM TrisBase (stock powder FW121.14), 195 mM Glycine (stock powder MW75.07) and SDS (from stock 10%). For 1 L of 10X running buffer, 30.285 gr of 25 mM TrisBase and 144.134 gr of 195 mM Glycine were dissolved in 500 mL ddH_2O. After, 100 mL of 10% SDS was added and mixed gently in order to prevent bubble formation. pH was adjusted to 8.3 and bottle was filled till 1L. Before using it in SDS-PAGE, 10X running buffer was diluted to 1X and kept in 4^0C.

2.2.10. Western Blot

2.2.10.1. Transfer

Transfer is the primary step for Western blotting. It uses electric field perpendicular to the surface of the gel and results in the transfer of proteins on the gel to the membrane. When running was completed, cassettes were removed from the tank. The glass holders were separated from each other. The gel was cut by razor

blade from the line, which separates the resolving gel from the stacking gel and equilibrated in transfer buffer for 10 minutes. One piece of nitrocellulose PVDF membrane (Immubilon-P Transfer Membrane MD Millipore, Billerico, MA, USA) which is activated with 100% methanol, was cut in appropriate dimensions (i.e., same size of the gel), incubated in 100% methanol for 15 seconds and incubated in cold transfer buffer for 5 minutes. For each membrane, four Whatman papers were cut in appropriate dimensions (i.e., same size of the gel) incubated in cold transfer buffer for 10 minutes. The PVDF membrane, four Whatman papers, gel, and two fiber pads were placed in transfer sandwich as described in Figure 2.1. For each step of the sandwich preparation, a 10mL tube was used to remove any air bubbles between the layers by gently rolling over the sandwich and transfer buffer were poured gently to keep PVDF membrane, Whatman papers and fiber pads wet.

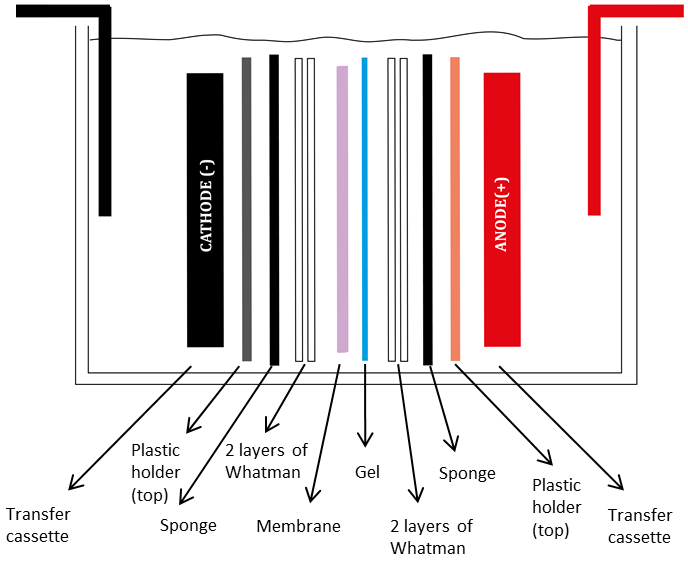

Figure 2.1 Preparation of transfer sandwich. Black plastic cassette was placed at the bottom, on top of it, sponge pad and two Whatman papers which were incubated in the transfer buffer for two minutes were placed. Gel was put on the Whatman papers. PVDF membrane was carefully put on the gel by holding with tweezers. Two Whatman papers were placed on the PVDF membrane and sponge pad was placed at the top and through clamps cassette was closed carefully. After each step, transfer buffer was slowly added so that PVDF membrane, Whatman papers, and fiber pads were kept wet. Cassette was situated into the slots by facing the black plastic support to the positive (+) electrode side and-and red plastic support to the negative (-) electrode side.

The cassettes were placed into the tank slots by considering black sides were looking the same negative electrode side. The transfer buffer was poured into the tank until it reached the 4 gels level of the tank. The tank was closed by lid and power supply connection was provided. The transfer was done at 90 V for 90 minutes at 4 ^{o}C.

Reagents:

1. **Transfer Buffer:**

25 mM TrisBase (stock powder FW121.14), 195 mM Glycine (stock powder FW75.07), Methanol (from 100% stock). For 2 L of Transfer Buffer, 6 g of 25 mM TrisBase, 28 g of 195 mM Glycine were dissolved in 1 L of ddH_2O. After 400 mL of 100% methanol was added and the volume was completed to 2 L with ddH_2O. It was kept in 4 ^oC before using.

2. **10 X Tris-Buffered Saline:**

25 mM TrisBase and 80 g NaCl were dissolved in 600 mL of ddH_2O. pH was adjusted to 7.6 with 1 N HCl/ NaOH and volume was completed to 1L with ddH_2O.

3. **Tris-Buffered Saline and Tween 20 (TBS-T):**

10X TBS was diluted to 1X TBS and 3 mL of Tween-20 was added by using a Pasteur pipette

4. **Blocking Solution: 5% Non-Fat Dry Milk (Stock)**

50 mL of TBS and 250 μL of Tween-20 were mixed and 2.5 g milk powder was dissolved in the solution via using vortex.

2.2.11. Primary and Secondary Antibody Incubation

After the transfer finished, the PVDF membrane was taken carefully by using plastic forceps and incubated in the 5% blocking buffer for an hour in room temperature by shaking. Blocking is important because it prohibits non-specific binding of antibodies. After blocking, the membranes were incubated with primary

antibodies for 16 hours at 4^{o}C on the shaker in slow motion. Primary antibodies were α-synaptophysin (Abcam, Cambridge, UK: ab32594, 1:20000 dilution), α -PSD-95 (Abcam, Cambridge, UK: ab18258, 1:5000 dilution), α -gephyrin (Santa Cruz Biotechnology, Santa Cruz, CA, USA: sc-6411, 1:1000 dilution) and α-β tubulin (Cell Signaling Technology, Danvers, AM, USA: #2146, 1:5000 dilution), which was the loading control. All antibodies were prepared in 5% milk powder (Blocking Buffer) + TBS-T. After overnight incubation, membranes were washed with TBS-T for five times in order to remove excess, unbound primary antibody. Duration of the washings was determined according to Table 2.6. Antibodies PSD 95- Tubulin – Synaptophysin were prepared as a mixture by mixing antibodies in BSA with respectively 1:5000 / 1:5000 / 1:20000 dilutions. The optimizations of the antibodies showed that the best signal from zebrafish taken from10 μg concentration. In total, 20 μL of the volume of the samples were loaded into the wells in equal volumes. Goat anti-Rabbit IgG H&L (HRP) was used as the secondary anti- body for anti-synaptophysin, anti-PSD-95 and anti-β tubulin.

Secondary antibodies were anti-rabbit (Cell Signaling Technology, Danvers, AM, USA: #7074, 1:2500 dilution) and, for gephyrin, an anti-goat (Abcam, Cambridge, UK: ab97100, 1:10000 dilution). The membranes were incubated with secondary antibodies for 1 hour at room temperature. All antibodies were prepared in 5% milk powder + TBS-T. Duration of the washings was determined according to Table 2.6.

Table 2.6 Duration of primary and secondary antibody washings.

Washing Time	**1st**	**2nd**	**3rd**	**4th**	**5th**
Duration (min)	5	5	10	5	5

2.2.12. Chemiluminescent Detection

Membranes were put on a clean surface and bands of interests were covered with 500 μL of chemiluminescent kit which consists of 2 different buffers, one of which is the Luminol/ Enhancer and other is the Stable Peroxide Buffer. Each one was used in 1:1 ratio system. So for 500 μL of the mixture, the consisted of 250 μL of one buffer and 250 μL of the other buffer, and they were mixed in an Eppendorf. Membranes were incubated for 5 minutes in a dark, light-proof chamber. After the incubation of membranes in the chemiluminescent kit, the chemical was drained off from the membrane by holding one corner of the membrane with a forceps to a paper towel. Membranes then were put on a clean surface and covered with the light-proof chamber and taken to imaging.

2.2.13. Imaging

A Biorad-Chemidoc™ MP imaging system which employs image lab software was used in order to image the membranes. In the software, the multichannel program was chosen and exposure duration was set to either ‘faint bands’ for the strong signals or ‘intense bands’ for the weak signals.

2.2.14. Quantification

Band densities were quantified by employing ImageJ program (NIH, Bethesda, MD, USA). Image of the membrane was opened in the ImageJ program and band

densities were quantified. Given data were copy-pasted to the Microsoft excel program. Data was normalized by dividing the quantified data of the band of interest to the corresponding tubulin in order to have average intensities and calculate the variations.

2.2.15. Statistical Analysis

Quantified data and body weight data was first grouped and tested for normality and homogeneity with Shapiro-Wilk Test and Levene's Test, respectively, by employing the SPSS (IBM, Istanbul, Turkey) statistics program was performed. In the cases where assumptions for parametric tests were fulfilled, two-way ANOVAs with the factors of age with two levels (young and old) and treatment groups (CR, RAP, AL) and factors of gender with two levels (male and female) and treatment groups (CR, RAP, AL) and were performed. When results of ANOVA were significant, Bonferroni Test and Tukey HSD Test were performed for further post hoc analysis. The significance level was accepted as $p<0.05$. Graphs of the data were piloted by using GraphPad Prism (version 4.00 for Windows, GraphPad Software, San Diego CA, USA).

2.3.Results

2.3.1. Body Weight

Changes in the initial body weights of animals in four groups are displayed in Figure 2.2. The percentages of the lost weight were calculated by subtracting initial body weight from final body weight and dividing the result to a hundred. Over a period of 4 weeks, *ad libitum*-fed group (AL) lost 6%, rapamycin-treated group

(RAP) lost 10% and caloric restriction group (CR) lost 18% of their initial body weight.

Descriptive statistics of young and old animals based on treatment groups are given in Table 2.7A. For young animals, the average of initial body weight changed from 0.72±0.0 g to 0.64±0.03 g in the AL group, and from 0.88±0.28 g to 0.79±0.16 g in RAP group, and from 0.71±0.13 g to 0.47±0.04 g in CR group. For old animals, the average of initial body weight changed from 0.83±0.28 g to 0.75±0.05 g in AL group, and from 0.68±0.16 g to 0.58±0.05 g in RAP group and from 0.84±0.04 g to 0.68±0.06 g in CR group. Descriptive statistics of male and female animals based on treatment groups are illustrated in Table 2.7B. For males, the initial body weight changed from 0.68±0.06 g to 0.65±0.01 g in AL group, and from 0.63±0.08 g to 0.60±0.09 g in RAP group, and from 0.83±0.04 g to 0.58±0.19 g in CR group. For females, the initial body weight changed from 0.88±0.22 g to 0.77±0.18 g in AL group, and from 0.94±0.20 g to 0.77±0.20 g in RAP group, and from 0.72±0.13 g to 0.57±0.11 g in CR group (Data was stated as mean±std.Deviation).

Table 2.7 Descriptive statistics of young and old **(A)**, and male and female **(B)** animals' initial and final body weights based on treatment groups.

Treatment	Body Weight	Age	Mean	Std. Deviation	N
CR	Initial	Young	,7100	,12728	2
		Old	,8350	,03536	2
		Total	,7725	,10500	4
	Final	Young	,4650	,03536	2
		Old	,6800	,05657	2
		Total	,5725	,12997	4
	Total	Young	,5875	,16070	4
		Old	,7575	,09743	4
		Total	,6725	,15295	8
RAP	Initial	Young	,8800	,28284	2
		Old	,6850	,16263	2
		Total	,7825	,21945	4
	Final	Young	,7900	,16971	2
		Old	,5800	,05657	2
		Total	,6850	,15927	4
	Total	Young	,8350	,19740	4
		Old	,6325	,11644	4
		Total	,7338	,18500	8
AL	Initial	Young	,7200	,00000	2
		Old	,8300	,28284	2
		Total	,7750	,17521	4
	Final	Young	,6400	,02828	2
		Old	,6800	,05657	2
		Total	,6600	,04320	4
	Total	Young	,6800	,04899	4
		Old	,7550	,18771	4
		Total	,7175	,13318	8

A

Treatment	Body Weight	Gender	Mean	Std. Deviation	N
CR	Initial	Female	,7150	,13435	2
		Male	,8300	,04243	2
		Total	,7725	,10500	4
	Final	Female	,5650	,10607	2
		Male	,5800	,19799	2
		Total	,5725	,12997	4
	Total	Female	,6400	,13140	4
		Male	,7050	,18574	4
		Total	,6725	,15295	8
RAP	Initial	Female	,9400	,19799	2
		Male	,6250	,07778	2
		Total	,7825	,21945	4
	Final	Female	,7650	,20506	2
		Male	,6050	,09192	2
		Total	,6850	,15927	4
	Total	Female	,8525	,19311	4
		Male	,6150	,07047	4
		Total	,7338	,18500	8
AL	Initial	Female	,8750	,21920	2
		Male	,6750	,06364	2
		Total	,7750	,17521	4
	Final	Female	,6700	,07071	2
		Male	,6500	,01414	2
		Total	,6600	,04320	4
	Total	Female	,7725	,17802	4
		Male	,6625	,04031	4
		Total	,7175	,13318	8

B

Also, in Table 2.8, percentages of the lost body weight of each animal are illustrated.

Table 2.8 Loss of the body weight of each animal as percentages. The percentages of the lost weight were calculated by subtracting initial body weight from final body weight and dividing the result to a hundred.

Age	Gender	CR	RAP	AL
Young	Female	13%	10%	1%
Young	Male	28%	10%	6%
Old	Female	17%	18%	8%
Old	Male	14%	3%	0%

The differences between initial and final body weights as a function of age and gender were tested and illustrated in Figure 2.2. Two-way ANOVAs were conducted firstly to investigate whether different treatment groups or age had a significant effect on the difference between the initial and final body weights then, to investigate

whether different treatment groups or gender had a significant effect on the difference between the initial and final body weights. Results indicated that there was no significant main effect of treatments and age on the initial and final body weight ($F(2, 12) = 0.396$, $p=0.682$ and ($F(1, 12) = 0.059$, $p=0.812$, respectively). Also, there was no interaction of treatment by age on the initial and final body weight of the animals ($F(2, 12) = 3.68$, $p=0.067$) (Figure 2.2A and Figure 2.2B). Furthermore, results showed that there was no significant main effect of treatment and gender on the initial and final body weights of the animals ($F(2, 12) = 0.433$, $p=0.658$ and ($F(1, 12) = 2.859$, $p=0.117$, respectively). Also, no significant interaction of treatment by gender was observed ($F(2, 12) = 2.478$, $p=0.126$). The results indicated that the change in the body weights among age and gender groups was not dependent on different treatments.

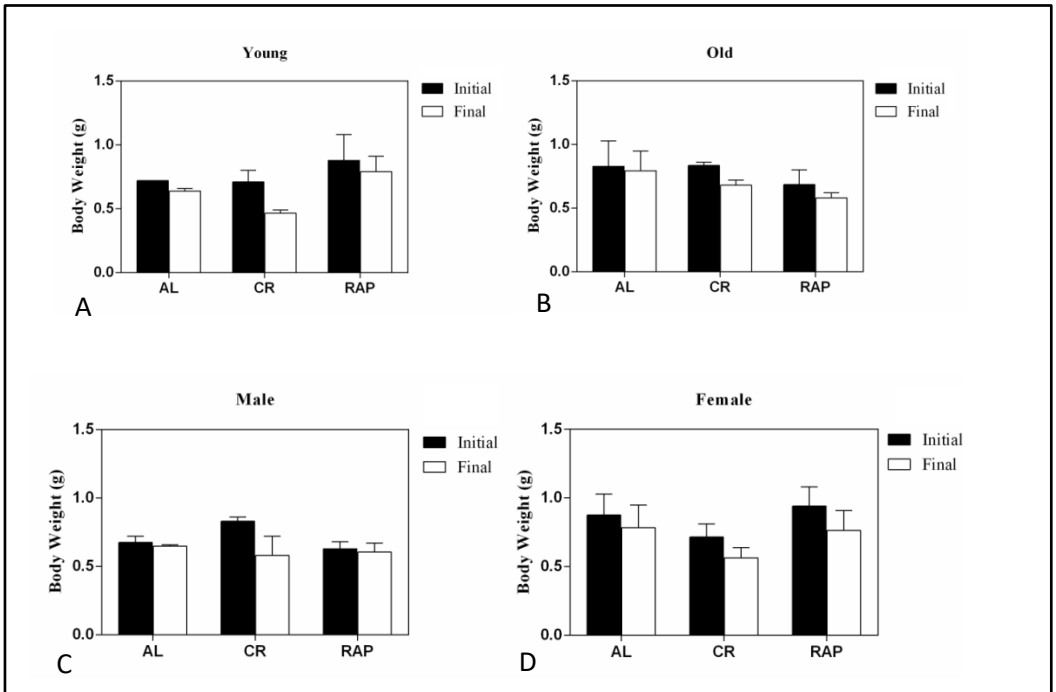

Figure 2.2 Change in the initial body weight of **(A)** old, **(B)** young, **(C)** male and **(D)** female animals. There is no significant interaction of treatment, on age and gender groups. Data were analyzed by two-way ANOVA, and presented as means ± SEM

2.3.2. Synaptophysin-PSD-95-Tubulin and Gephyrin Expression

Lysates from CR, RAP and AL treated animal brains were loaded into the gels as shown in Figure 2.3. Figure 2.3 shows the representative image of the western blot results of the relative expression of the key synaptic proteins postsynaptic density protein (PDS-95) and synaptophysin. Tubulin was used as the loading control. A two-way ANOVA was conducted in order to investigate whether different treatment groups had a significant effect on the expression of PSD-95, synaptophysin, and gephyrin.

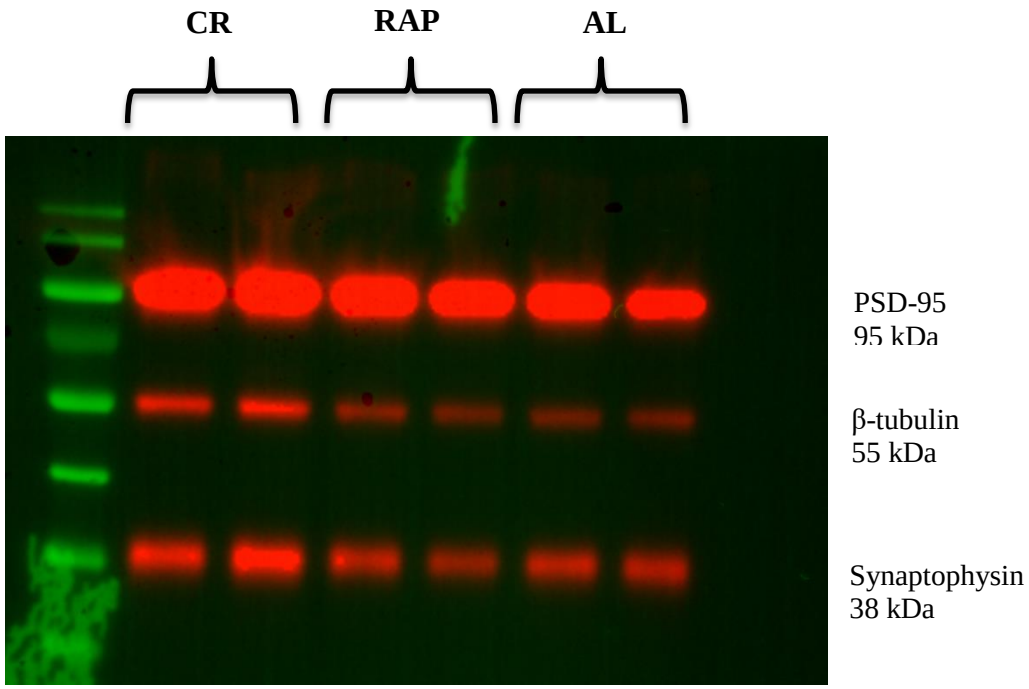

Figure 2.3 Representative image of the blots incubated with PSD-95 / TUB / SYN antibody. (This experiment was performed by Narin Ilgim Ardic.)

PSD-95 is a postsynaptic scaffold protein its expression was shown to be highest in glutamatergic synapses; it binds scaffold adaptor proteins and facilitates an indirect interaction between scaffold proteins and glutamate receptors, as well as

other signaling proteins [60]. PSD-95 is expressed in the 95 kDa band on the PVDF membrane. In this study, it was used as postsynaptic marker of excitatory synapses. Relative expression of PSD-95 over three groups in gender and age level is shown in the Figure 2.4.

Relative expression of PSD-95 was calculated by performing a two-way ANOVA. It was found that there was no statistically significant effect of CR and RAP treatments on the expression of PSD-95 in young ($F(2, 3) = 0.032$, $p=0.918$) and old ($F(2,3) = 0.071$, $p=0.933$) animals (Figure 2.4A). Also, no significant interaction between AL, CR and RAP treatments and young and old in the expression of PSD-95 ($F(2,9)=0.101$, $p=0.906$) was found (Figure 2.4A). Likewise, no statistically significant effect of CR and RAP treatments on the expression of PSD-95 in female ($F(2,3)=0.110$, $p=0.883$) and male ($F(2,3)=0.016$, $p=0.984$) animals was calculated (Figure 2.4B). In addition, no significant interaction between gender groups and treatment ($F(2,9)= 0.002$, $p=0.99$) was found (Figure 2.4B).

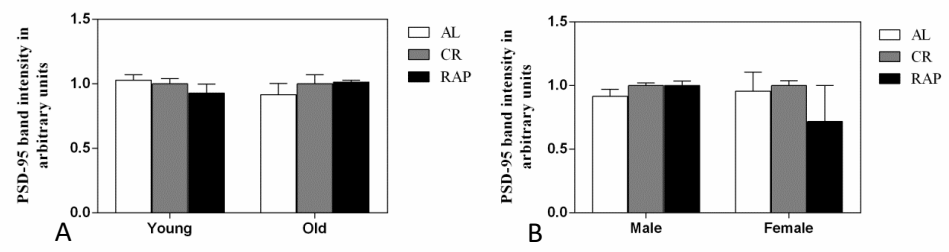

Figure 2.4 The relative expression of PSD-95 protein levels across gender, age and treatment groups. **(A)** The relative expression of PSD-95 in young and old groups across AL, CR and RAP treatments. **(B)** The relative expression of PSD-95 in male and female animal groups across AL, CR and RAP treatments. There was no significant change in the expression of PSD-95 in male and female, and old and young animals after CR and RAP treatments. Data were analyzed by two-way ANOVA, and presented as means ± SEM

Synaptophysin is an integral membrane protein and present in the presynaptic vesicles. It is essential for endocytosis and vesicle trafficking in presynaptic membrane. It is expressed in the 38 kDa band on the PVDF membrane. Synaptophysin was used as an indicator of presynaptic integrity. Relative expression graphic of Synaptophysin over CR, RAP and AL groups is shown in Figure 2.5. It was shown that the expression of synaptophysin was not significantly different in young ($F(2,3)=0.158$, $p=0.860$) and old ($F(2,3)=0.010$, $p=0.990$) animals (Figure 2.5A). Additionally, no statistically significant interaction between age and treatment groups ($F(2,9)=0.084$, $p=0.921$) was calculated (Figure 2.5A). Likewise, no statistically significant effect of treatments on the expression of synaptophysin was observed in female ($F(2,3)=0.010$, $p=0.990$) and male ($F(2,3)=0.025$, $p=0.976$) animals (Figure 2.5B). Similarly, no statistically significant interaction between gender and treatment groups ($F(2,9)=0.010$, $p=0.990$) was calculated (Figure 2.5B).

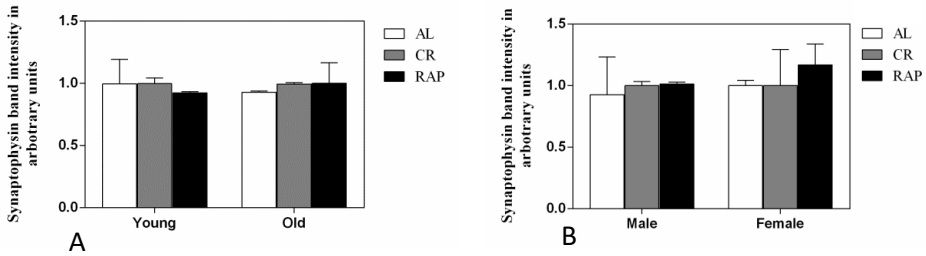

Figure 2.5 The relative expression of synaptophysin protein levels across gender, age and treatment groups. **(A)** Relative expression of Synaptophysin in young and old groups across AL, CR and RAP treatments. **(B)** Relative expression of synaptophysin in male and female animal groups across AL, CR and RAP treatments. There was no significant change in the expression of synaptophysin in male and female, and old and young animals after CR and RAP treatments. Data were analyzed by two-way ANOVA, and presented as means ± SEM

β-tubulin is a cytoskeletal protein and used a housekeeping protein control in

many studies. In this study, it was as well used as the loading control in order to identify any loading mistakes and in order to normalize the quantified data of the samples of interest. It is expressed in the 55 kDa band on the PVDF membrane. Analysis of the statistics of the tubulin was performed by employing a two-way ANOVA. It was demonstrated that expression of tubulin was not significantly different in young ($F(2,3)=2.60$, $p=0.22$) and old ($F(2,3)=1.06$, $p=0.47$), and male ($F(2,3)=0.32$, $p=0.75$) and female ($F(2,3)=0.66$, $p=0.59$) animals across treatment groups. Also, in Table 2.9 the descriptive statistics of the expression levels of tubulin across three groups is illustrated. This data indicated that no significant pipetting or loading mistake occurred during the experiment.

Table 2.9 Descriptive statistics of β-tubulin expression levels across AL, CR and RAP groups. Expression of tubulin was not significantly different across all groups. Data were analyzed by two-way ANOVA.

Group	Treatment	Mean	Std. Deviation	N
Young	CR	1.60	0.68	2
	RAP	0.75	0.17	2
	AL	1.18	0.61	2
Old	CR	0.77	0.47	2
	RAP	1.04	0.01	2
	AL	0.64	0.08	2
Male	CR	1.05	0.87	2
	RAP	0.96	0.12	2
	AL	0.66	0.11	2
Female	CR	0.32	0.29	2
	RAP	0.83	0.28	2
	AL	1.16	0.64	2

Gephyrin is a key postsynaptic protein present in the inhibitory synapses and it modifies the inhibitory synaptic neurotransmission. In this study, gephyrin was used as a postsynaptic integrity marker of inhibitory synapses. Anti-gephyrin antibody gives numbers of non-specific bindings on the membrane, therefore; membrane was cut from 70 kDa band. Representative image of relative expression of gephyrin and β-tubulin was illustrated in Figure 2.6A.

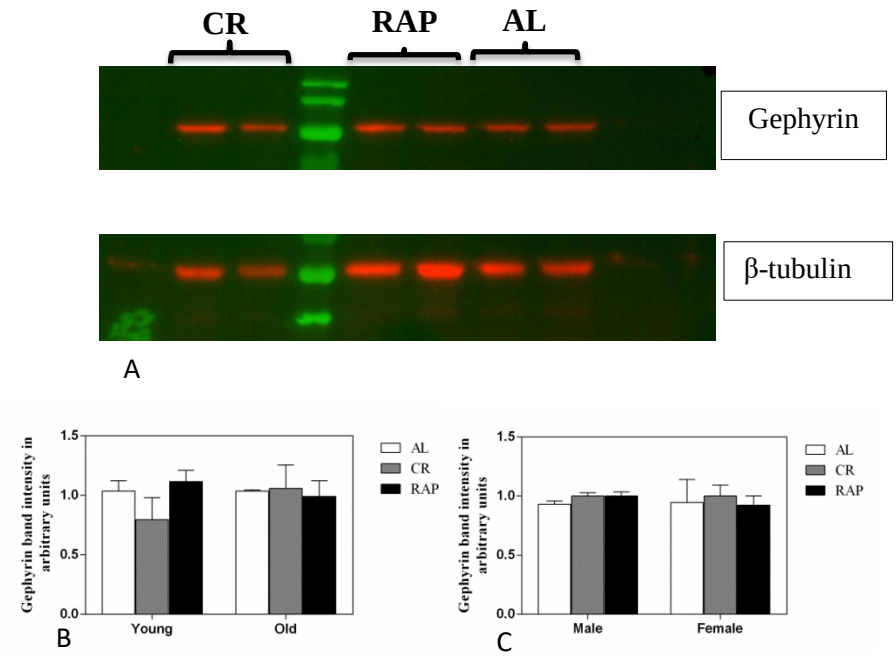

Figure 2.6 The relative expression of gephyrin across groups. **(A)** Representative image of gephyrin and Tubulin bands. **(B)** Relative expression of gephyrin in young and old animals across AL, CR and RAP groups. **(C)** Relative expression of gephyrin in male and female animals across AL, CR and RAP groups. There was no significant effect of CR and RAP treatments on the expression of gephyrin in young and old and male and female animals. Data were analyzed by two-way ANOVA, and presented as means ± SEM

No significant difference in the expression of gephyrin in young $F(2, 3) = 0.158$, $p= 0.860$) and old animals ($F(2,3) = 0.010$, $p= 0.990$) was calculated (Figure 2.7B). Also, it was calculated that age and treatment was not significantly interacting ($F(2, 9) = 2.918$, $p= 0.130$ (Figure 2.7B). Expression of gephyrin in female animals ($F(2,3) = 0.010$, $p= 0,990$) and male animals ($F(2,3) = 0.025$, $p= 0.976$) animals under CR and RAP treatment was shown to be not significantly different (Figure 2.7C). Also, gender and treatment was not significantly interacting ($F(2, 9) = 1,072$, $p= 0.400$ (Figure 2.7C).

β-tubulin was used as a loading control and descriptive statistics of each animal group under AL, CR and RAP treatments is illustrated in the Table 2.10. It was found that expression of tubulin was not significantly different in young ($F(2,3)=1.89$, $p=0.29$) and old ($F(2,3)=6.41$, $p=0.082$), and male ($F(2,3)=0.52$, $p=0.63$) and female ($F(2,3)=0.18$, $p=0.88$) animals across treatment groups. Results indicated that no significant loading or pipetting error took place.

Table 2.10 Descriptive statistics of β-tubulin expression levels across AL, CR and RAP groups. Expression of tubulin was not significantly different across all groups. Data were analyzed by two-way ANOVA.

Group	Treatment	Mean	Std. Deviation	N
Young	CR	0.79	0.25	2
	RAP	1.11	0.13	2
	AL	1.03	0.01	2
Old	CR	1.05	0.04	2
	RAP	0.95	0.02	2
	AL	1.17	0.09	2
Male	CR	1.17	0.27	2
	RAP	1.09	0.16	2
	AL	1.06	0.05	2
Female	CR	0.89	0.39	2
	RAP	1.00	0.01	2
	AL	1.14	0.13	2

Further analysis was performed with employing MANOVA with the two factors; age with two levels (young and old) and treatment with three levels (CR, RAP and AL) and also for protein expressions, gender with two levels (male and female) and treatment. A MANOVA was employed since there were three independent variables of which the main effects as well as interaction effects needed to be investigated. According to the results, no significant interaction between treatments and protein expression was found in young and old animals ($F(6, 2) = 0.397$, $p = 0.839$; Wilk's $\Lambda = 0.238$ and $F(6, 2) = 0.357$, $p = 0.862$; Wilk's $\Lambda = 0.233$, respectively). Also, no significant interaction between treatments and protein

expression was found in male and female animals ($F(6, 2) = 1.000$, $p = 0.575$; Wilk's $\Lambda = 0.062$ and $F(6, 2) = 1.486$, $p = 0.455$; Wilk's $\Lambda = 0.054$, respectively).

2.4 Discussion

In this study, the effects of a short-term (4 weeks) caloric restriction (CR) and rapamycin (RAP) treatments on the expression of key synaptic proteins (synaptophysin, PSD-95 and gephyrin) in young (9-11 months old) and old (27-33 months old), and male and female zebrafish was investigated. Here, it was hypothesized that zebrafish shows age and gender related decrease in the expression of key synaptic proteins and this can be prevented by CR and it's mimetic, RAP. We grouped animals into four groups; young and old, male and female animals. Since there were just one animal for a specific treatment, age and gender, (for example; there were just one young female CR animal, one young male CR animal, one old female in CR group, one old female in CR group) groups were heterogeneous in terms of age and gender which also explains the relatively higher error bars in the graphs (Figure 2.2).

In this study, we aimed to perform 20% CR. There are some other studies involving 30-40% or 15% of CR [113]. It was shown that in all CR treatments, animals have lost their body weight depending on the age and diet duration [113]. According to the body weight results, RAP group lost 10% of their body weight; AL group lost 6% of their initial body weight. In CR group total reduction in the initial body weight was 18% (Table 2.8). Also, results showed that CR animals lost more of their initial body weight that old CR animals did (Figure 2.2). Parallel to our results, an unpublished cohort study (N=48) from our group which performed

MANOVA with three factors; age with two levels (young and old), gender with two levels (male and female) and treatments with three levels (CR, RAP and AL) showed that there's a significant main effect of age and gender on the initial body weight of the animals, and also showed an interaction of treatment by age and gender and further Tukey HSD test and Bonferroni tests showed that reduction in the initial reduction of the initial body weights of young CR groups was statistically significant ($p<0.05$) [129]. Similarly, Arslan-Ergul *et al.* (2016) also reported that after 10 weeks of caloric restriction, young animals lost more of their initial body weight than old animals [130]. They also indicated that rodents display similar trend in terms of body weight loss. Due to the given food to CR animals, we expected the reduction in the body weight, however; all groups lost their initial body weight regardless of the amount of food they fed with. Therefore, we speculated that animals were stressed out during the housing conditions. For example, it was previously shown that repeated stress caused decline food intake and reduction on body weight in the adult rats, which might explain the reduction of the body weight in the all groups [131]. Furthermore, it was reported that long term starvation resulted in reduced physical activity in zebrafish [132]. In our feeding set-up, as animals ate less food, the physical activity was reduced, and in time, animals could not put much effort to reach the food. However, our speculations upon stress are required further investigation with cortisol tests.

Zebrafish is a social animal and produce a hierarchy in their society [133]. We separated animals for feeding during weekdays for five hours and place them back into their aquaria. The reason behind the separating animals was to feed each animal equal amounts of food. That is, due to the social hierarchy, animals with higher

social hierarchy get food more than the rest, therefore; we separated the animals in order to avoid unequal feeding and have all the animals get amount of interest. Except for the feeding times, animals were housed as two in one aquarium. Based on housing conditions, we speculate that this method resulted in the social isolation of the animals and eventually created stress upon animals. In their study, Arslan-Ergul *et al.* (2016) kept the animals in the same tank during the whole experiment in order not to disturb the social network of the animals and measured the stress levels with the cortisol tests and cortisol tests revealed no sign of stress [130]. Net stress was one another factor that our animals faced with during transportation from the aquaria to the beakers. Ideally, for the husbandry of the zebrafish, 5 animals per liter should be housed in order not to create stress upon animals [134]. During the periods of which any treatment was applied, animals were housed as 2 fish per liter. During feeding times, the housing situation was 1 fish per 0.25L. The ratio of the per fish per liter that we had was described as stress inducing crowding density [134]. Ideal husbandry should be 5-6 fish per liter. Therefore, even though further investigation of cortisol tests is needed, ideal husbandry conditions supports our speculation.

CR and RAP are acting upon the same pathway, which is mammalian target of rapamycin (mTOR) pathway and mTORC1, which is one of the two complexes of mTOR pathway that CR and RAP affect, was shown to involve various important mechanisms including synaptic plasticity, adult neurogenesis, learning and memory and also function as nutrient and growth sensor [135]. Due to the activity of mTORC1 in the nutrient sensing mechanisms, it has become the center of CR studies, including ours. Although we expect the similar results of the CR and RAP treatments, there are some speculations about the similarity of effects of CR and

RAP treatments. It was described that both treatments downregulated the mTORC1 pathway and increase medial and maximal life span but they displayed different impacts on the insulin sensitivity, expression of cell cycle [136]. Similarly, Karoglu *et al.* (2014) investigated the expression levels of 9 selected genes in the general cohort that also include our set of animals and showed that two genes *p27* and *Rab1a* expressions were not similarly affected by RAP and CR treatment although their expressions showed age and gender related alterations after RAP and CR treatments [137].Therefore, in order to ensure if our experimental setup with CR and RAP treatments indeed blocked the mTORC1 pathway, the expression of downstream elements of mTORC1 pathway such as S6K1 should be investigated [138].

The regime and duration of CR vary depending on the studied model organism. Reducing the daily proportion of meal or feeding animals every other day are the different regimes used in the CR studies [110]. Depending on the regime, results may differ. In our study, we followed a daily reduction in the calorie intake and feed the animal every day. In their study, Arslan-Ergul *et al.* (2016) followed the every-other-day feeding dietary regimen in which animals were fed once in two days [130]. Compared to the reduction in the daily calorie intake, which was the CR regimen we followed, every-other-day feeding dietary regimen eliminates the competition to the food, also animals were fed with more food than they were fed with our method.

In our preliminary study, we reported that in the expression level of key synaptic proteins, no significant difference between CR and RAP group was observed in any age and gender group compared to the AL group. It was indicated that mild CR (15% reduction) did not significantly alter the expression of genes which are involved in the mTORC1 pathway in hippocampus and neocortex [113]

However, the same study showed that 30% reduction in the calorie intake did significantly change the expression of downstream elements of mTOR pathway in young and middle-aged mice and 35-40% CR did not change the expression level of same proteins in cerebellum and cortex in 6 months old mice [113]. This data indicates that duration of CR, age of the animal and the brain region differentially affects the mTOR pathway. Similarly, we found that expressions in the AL group were also not significantly different in age and gender groups. Here, we speculate that these non-significant results can be explained by the stress, duration of CR, dose of RAP as well as using whole brain lysates. In our study, we used whole brain lysates instead of studying in a region-specific manner. Zebrafish possess a small size of brain compared to mammals and has basic vertebrate brain structure such as retina, olfactory bulb and spinal cord, yet some structures are present as homolog, for example telencephalon in zebrafish is mammalian homolog of cortical and subcortical regions or pallium in zebrafish is mammalian homolog of hippocampus and amygdala [139]. Therefore, in the future studies; housing, feeding regimen and also the duration of the treatments that we performed as well as the number of animals and brain regions must be taken into account.

In our study, we obtained 100 nM of RAP concentrations for the RAP treatments. In their study, Goldsmith *et al.* (2006) investigated the fin growth in adult and juvenile zebrafish reported that in adult fish, 4 weeks of fasting and 6 days of 5 nM, 50 nM and 100 nM concentrations of RAP treatment showed similar effects by reducing the expression of growth marker and number of actively dividing cells, however; juvenile animals affected mildly from fasting and 5 nM, 50 nM and even 100 nM of RAP concentrations [140]. Taking the treatment protocol of this study as

a reference, we selected 100nM concentration of RAP and performed the RAP and CR treatments for 4 weeks with the desire of creating optimal treatment conditions. However, according to the results of the experiments, we speculate that 5 hours of RAP treatment was not efficient enough for the blocking of the mTOR pathway as we hypothesized. Also, 100nM concentration of RAP may not be efficient enough to down regulate mTOR pathway.

In this study, we used postsynaptic density protein 95 (PSD-95) as postsynaptic integrity marker in excitatory synapses, synaptophysin as presynaptic integrity marker, and gephyrin as postsynaptic integrity marker in inhibitory synapses. We investigated the expression of these key synaptic proteins in CR and RAP treated young and old, male and female zebrafish. Although the zebrafish have been using as a model organism for many years for aging and aging-associated disorder studies, there still are a lot of unknown information about zebrafish and its aging process as well as gender difference in the protein and gene expression level. Also, CR studies are common in many organisms but information about its effects on zebrafish should be investigated, which is the main object of this study. Understanding the molecular mechanisms in zebrafish will help to build better experimental setups for mammals, particularly for humans. Zebrafish has become a popular organism due to its homology between mammals, and also it is easy to conduct experiments which cannot be readily conducted on humans.

PSD-95 is a postsynaptic scaffold protein which binds adaptor proteins and facilitates an indirect or indirect interaction with glutamate receptors; AMPA and NMDA [141–143]. In this preliminary study, we reported that expression of PSD-95 did not significantly change in age groups under CR and RAP treatments (Figure

2.4A). This is the first study that investigates effects of CR and RAP interventions on the expression of PSD-95 in different age and gender groups. According to our results, in young animals, the expression of PSD-95 was numerically smaller in RAP animals compared to the AL and CR animals (Figure 2.4A). Although we expected similar results after CR and RAP treatments, due to the potential failure of the treatments resulted from the stress upon young animals as well as the inefficiency of CR and RAP treatments, we did not observe likewise expressions between CR and RAP treated animals. Also, we observed that numerical reduction in the expression of PSD-95 with age in AL group was stabilized with CR and RAP treatments (Figure 2.4A). Adams *et al.* (2008) showed that in young rats the expression of NR1, NR2A, and NR2B, which are the glutamate receptor subunits that PSD-95 interacts with, was statistically higher in AL young groups compared to young CR groups and [143,61]. They further showed that decreased expressions were stabilized with the CR treatments, which indicates the significant rescuing effect of CR treatment on the expression of PSD-95. One of the reasons that significant results they showed and we didn't show could be the number and gender of the animals per group. In their study, they used only male animals and groups were composed of 8-10 animals. However, our age groups composed of both male and female animals and each group was composed of 2 animals. Also, duration of CR treatments was longer in their study. According to our results, expression of PSD-95 was numerically decreasing with age in AL group and increasing with age in RAP group which may lead to the conclusion of a slight enhancement of the expression of PSD-95 with mild blockage of the mTORC1 pathway through RAP treatment. In order to confirm this speculation, expression of the downstream elements of mTORC1 pathway needs further

investigation.

In terms of gender, we found no statistically significant difference in the expression of PSD-95 in male and female animals under CR and RAP treatments (Figure 2.4B). Numerically, in male CR and RAP groups, expression of PSD-95 was higher compared to the AL group, which might be the indication of a mild effect of CR and RAP treatments. In the future studies, this should be confirmed with the expression studies conducted on the downstream elements of the mTORC1 pathway. However, in a female RAP group, expression was numerically smaller compared to the female CR and AL groups as well as compared to the male AL, CR and RAP groups. This could result from the stress that we speculate particularly on old female animals were suffered from. It was reported that stress upon the animals interfered with the function of the mTOR pathway, therefore; we may have failed to block mTOR pathway as we hypothesized, particularly in female groups [113], [144]. According to our results, for the AL and CR groups, expression of PSD-95 was numerically similar in male and female groups (Figure 2.4B). However, unpublished data from our laboratory showed that in young females, expression levels of PSD-95 was significantly higher than in young males, and expression was increased in females as they age but it was stable in males as they age [145]. The controversial results can be explained with the number of sample size as well as the effect of stress that we speculated.

In this study, gephyrin was used as a postsynaptic protein marker in inhibitory synapses. Gephyrin is selectively located at GABAergic and glycinergic postsynaptic sites [134]. In this study, we demonstrated that expression levels of gephyrin were not significantly different in age groups under CR and RAP treatments (Figure 2.7B).

We found that in young animals, expression of gephyrin was numerically and statistically similar in AL and RAP groups and higher than in CR group. In old animals, although expression of gephyrin was not significantly different between AL, CR and RAP groups, in RAP group, expression level is numerically less than AL and CR groups which also raises questions about RAP treatment. However, unpublished data from our lab suggested that expression of gephyrin did not statistically change throughout aging [145]. Therefore, we can speculate that since expression of gephyrin does not decrease overtime, CR and RAP treatments do not influence the expression of gephyrin by blocking the mTOR pathway. However, the association between mTORC1 pathway and gephyrin was demonstrated [146]. Therefore, in order to confirm this speculation, the mTORC1 pathway can be investigated further in the future studies. Also, instead of gephyrin another inhibitory scaffold protein can be used. Similarly, it was previously shown that gene expression of α_1 subunit of $GABA_A$ did not change after CR feeding in old rats compared to the AL old rats but gene expression of α_2 subunit of $GABA_A$ was less in old AL rat compared to the old CR rat [147]. Yet, gene expression studies may have different results from the protein expression studies because genes may undergo posttranslational modifications which can affect the protein expression. Moreover, protein expressions give more clear information about the function of the protein of interest. Furthermore, expressions of subunits of $GABA_A$ display different expression profiles which leads to the conclusion of the fact that expression of the receptors subunits may be distributed in a region-specific manner and this may provide a functional diversity in different brain regions [147].

In terms of the gender, statistical analysis showed that expression of gephyrin

was not significantly different gender groups regardless of the treatments (Figure 2.7C). Particularly, expression of gephyrin was numerically less in male AL animals compared to CR and RAP animals and it was statistically and numerically similar to female AL group (Figure 2.7C). In male group, expression of gephyrin was similar in CR and RAP groups. In terms of females, expression in CR group was numerically higher than in RAP and AL group. According to the unpublished data from our laboratory, except for the middle age group, expression of gephyrin was numerically higher in females than in males throughout aging [145]. The reason we have not observed the same pattern could be the number of sample size and also the stress that we speculate. By investigating the mTORC1 pathway more in detail in the future studies, we can confirm the effects of CR and RAP treatments on the gephyrin expression.

Previous studies showed that gephyrin and PSD-95 are both located in the postsynaptic cleft and their expression follows an excitatory/inhibitory balance and this balance changes depending on the age of the organism as well as brain region [148]. In this study, we reported that expression of both PSD-95 and gephyrin did not significantly change depending on the treatment and also age and gender, and we found no trend between the expression of these two proteins; in other words, expression of PSD-95 and gephyrin was statistically similar in each gender, age and treatment group. Considering the sample size of groups, stress upon the animals and duration and treatment approaches of CR and RAP, our observations might not be informative about this balance.

In this study, in addition to postsynaptic proteins, synaptophysin was used as presynaptic protein marker which is involved in vesicle trafficking and recycling in

the presynaptic terminals [121]. In our study, we report that expression of synaptophysin was not significantly affected by CR and RAP treatments in different age and gender groups (Figure 2.5). Our results showed that in young animals, expression of synaptophysin was numerically similar in AL and CR groups and higher than in RAP group. In old animals, expression of synaptophysin was similar after CR and RAP treatments and numerically higher compared to AL group. We found that decreased expression of synaptophysin with aging was stabilized with CR and RAP interventions. Similarly, Adams *et al.* (2008) showed that expression of synaptophysin was significantly higher in young animals than old animals but it was decreased with age, and CR treatment stabilized the decreased expression [61]. Likewise, Djordjevic *et. al* (2010) investigated the both gene and protein expression of presynaptic proteins including synaptophysin in the cortex and hippocampus of young and old CR and AL rats and demonstrated that decreased synaptophysin expression was stabilized with CR [149]. Our results indicated the mild effect of CR and RAP treatments on the synaptophysin expression but this should be confirmed with the effect of the interventions on the mTORC1 pathway in these animal groups. Here, we speculate that animals used in this study were stressed out due to the housing and treatment conditions and stress have affected the expression of synaptophysin. However, Müller *et al.* (2011) investigated the expression of genes which are associated with synaptic vesicle including synaptophysin in the prefrontal cortex and hippocampus of the rat after chronic stress and they reported that synaptophysin expression did not change after chronic stress in the prefrontal cortex [150]. Additionally, Thome *et al.* (2001) showed that stress significantly reduces the expression of synaptophysin in cerebral cortex but not in prefrontal cortex or

hippocampus [151]. This indicates that expression of synaptophysin changes depending on the brain region after stressful conditions.

In terms of gender, even though the not statistically significant results between treatment and gender groups, expression of synaptophysin was numerically less in male AL group, compared to the male CR and RAP group. This data indicated that, even though the speculations about stress, CR and RAP interventions may have worked in male groups. Indeed, expression of the synaptic proteins we investigated similar expression profile after CR and RAP treatments. In female group, expression of synaptophysin was numerically higher in RAP group compared to the AL and CR groups and also we found no trend of the effect of the treatments in the expression of synaptic proteins. We claim that this was a result of particular effect of stress upon females.

In order to ensure the accuracy of the results, housekeeping proteins are used in the expression studies. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), β-actin, β-tubulin are commonly used housekeeping genes in these studies [152]. Using housekeeping genes helps to eliminate variations taking place during experiments such as loading mistakes or transfer efficiency and normalize the target protein. Among them, we preferred β-tubulin. In our study, we have reported no significant differences between the loaded samples which indicate no loading or pipetting errors took place during the experiment (Figure 2.6, Table 2.10).

In this study, one of the aims was observing the expression of key synaptic proteins under different treatment conditions in male and female zebrafish. For many years, most of the studies used male subjects, or do not specify the gender in the studies. Questioning the hypothesis by investigating the each sex would provide

making fewer assumptions about the results and interpretation would be more correct. Although we reported no significant change or difference in the expression of synaptic proteins in male and females, there's an increasing evidence that male and female brains do exhibit sexually dimorphic characters in terms of biochemistry, structure as well as function [125]. Here we claim that the way of housing animals caused stress on the animals. Also, duration of CR and treatment way of RAP may not be efficient enough to observe a statistically significant result. Supportive findings indicate that housing animals individually along with chronic stress significantly decreased the BrdU labeling, which is a proliferation marker, but not in socially housed male rats [153]. Also, another study conducted on birdsongs indicated that nutritional stress similarly impaired telencephalon in males and females but in the three song control regions, significant differences in males and females were reported [154]. Another study showed that in females, levels of corticosteroid increases faster in males than females after chronic stress which indicates that females handle stress better than males do [155]. These data indicate that stress also works in sexually dimorphic pattern. Therefore, not only the gender but also the brain regions should be investigated individually.

In this study, data were first tested for normality and homogeneity of variance Shapiro-Wilk Test and Levene's Test. After having the conformation of the parametric tests, we performed two tests, a two-way ANOVA with the factors of treatment with three levels (CR, RAP and AL), age with two levels (young and old) for the age groups and gender with two levels (male and female) for the gender groups on the analysis of body weight data and also analysis of the each protein expression and MANOVA with three factors of treatment with three levels, age with

two levels and age with two levels for the data for the general cohort. Another approach for the investigation of such datasets is the clustering of the data. Principal Component Analysis (PCA) can be employed in order to investigate the expression levels of the proteins relative to each other. However, clustering data is applicable when the sample size is large. In our dataset, the main problem we faced with was the small sample size. Small sample size increases the variation within the data and therefore statistical analysis results in nonsignificant values, similar to our case. Therefore, in the future studies sample size should be increased in order to have statistically healthier results.

To conclude, this study aimed to demonstrate the expression of key synaptic proteins which are gephyrin, PSD-95 and synaptophysin in young and old, male and female zebrafish which are under short-term (4 weeks) CR and RAP treatments. Here, we report that our initial results of key synaptic protein levels indicated that they are stable throughout aging with respect to gender and CR interventions. Based on the unexpected loss in the body weight and information in the current literature, we speculated that animals used in this study were stressed during housing conditions. Therefore, our results do not indicate a comprehensive expression profile. In addition to stress, a number of animals used in the experiments was another limitation of sampling animals and distributing onto groups. Since our results indicated a trend towards significance, by increasing the sample size, results will be more accurate. Also, 4 weeks of CR and RAP treatments would not be enough to block mTORC1 pathway. As well as the duration, exposure to the treatments was not enough to block mTOR pathway as we hypothesized. Furthermore, animal groups used in the study were not homogeneous enough to obtain comprehensive results.

This grouping problem also resulted from the limited number of animal numbers. Another important parameter was the duration of CR. Therefore; regardless of stress factors, we have concluded that 4 weeks of CR and RAP treatments was too short. In addition to the duration, due to the numerical differences between the expressions in different treatment groups, we speculated that 5 hours of RAP treatment was not long enough to affect mTORC1 pathway as we speculated. In order confirm the speculation of ours, expression studies such as microarray, qPCR or western blot experiments should be performed in the future studies. Also, expression of key synaptic proteins changes depending on the brain region, in other words, expression follows a region-specific pattern. Here, in our study we used whole brain lysates due to the small size of the zebrafish brain and also zebrafish do not possess brain structures such as the hippocampus, it has brain regions homolog to the mammalian brain region. Additionally, the housing of the animals is a key variable in the CR and RAP treatments. Animals should not be replaced between the treatments in order to avoid creating a stressful environment for the animals. Here, by investigating the male and female animal groups, we aimed to observe differences in genders because many studies have indicated the sexual dimorphism on gene and protein and also cognitive level, from zebrafish to human. However, gender groups were composed of old and young animals, therefore results were not informative. We used zebrafish as a model organism in order to reveal more information about its aging and sexual dimorphism as well as effects of treatments which are inefficient or rather difficult when conducted on higher primates such as humans. Due to the similarities between zebrafish and mammals in terms of their homolog genes, organs, and aging patterns, it has become a very favorable model organism.

CHAPTER 3

Expression of Glutamate Receptor Subunits NR2B and GluR2/3 in Middle Aged Male and Female Zebrafish

3.1.Introduction

Glutamate, which is the leading excitatory neurotransmitter in the mammalian brain, regulates the excitatory neurotransmission via two types of glutamate receptors; metabotropic glutamate receptors (mGluRs) and ionotropic glutamate receptors (iGluRs) [156,157]. Metabotropic glutamate receptors work through G protein coupled receptors and change the metabolism and gene expressions of neurons and [156-158]. Ionotropic glutamate receptors, however, constitute of ion channels and change the membrane potential of postsynaptic neurons [156]. Ionotropic glutamate receptors are composed of three classes depending on their pharmacological and electrophysiological characterization; α-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA), kainate and N-methyl-D-aspartate (NMDA) receptors [156].

AMPA receptors (AMPARs) provide the fast excitatory neurotransmission in central nervous system and are involved in the synapse formation, synaptic plasticity and stabilization [159]. AMPARs present as heterodimers with the different combination of four subunits; GluR1, GluR2, GluR3, and GluR4 but majorly containing GluR2 [159,160]. Expression and turnover of subunits changes depending on the neuron and glia as well as developmental stage of the organism [161,162]. For example, GluR1 and GluR2 are expressed overwhelmingly in the for brain which

covers hippocampus and cerebral neocortex and GluR3 and GluR4 have lower expressions [160]. Also, during development, the GluR1 expression is higher compared to GluR2 but its expression increases as the development proceeds [163], [164]. Therefore, investigation of the changes in the expression of subunits with age and also gender might help to reveal the synaptic changes taking place during aging because of the involvement of AMPARs in synaptic plasticity.

Similar to AMPARs, NMDA receptors (NMDARs) are composed of different subunits based on the biophysical and pharmacological properties, interacting partners and the subcellular localization of the each subunit and their expression changes in the central nervous system during development [165]. In addition to developmental stage, even in mature neurons, the composition of NMDARs varies based on the neuronal activity [165]. There are seven subfamilies of NMDARs and present as heterodimers and each subfamily is composed of subunits. GluN1 (NR1), GluN2 (NR2) which is composed of four subunits GluN2A, GluN2B, GluN2C and GluN2D and GluN3 which are composed of two subunits GluN3A and GluN3B [165]. NMDARs can generally be in the form of heterotetrameric assemblies in which subunits of GluN1 and subunits of GluN2 are associated or they present as GluN2 and GluN3 subunit mixture [165]. For example, GluN1 subunits express in all ages including embryonic stage till adulthood [165]. Also, expression of the subunits changes across the brain regions; although GluN1-2 expression is widespread, GluN1-1 and GluN1-4 expression follows a complementary distribution pattern, that is, GluN1-1 is expressed in more rostral regions. In addition, expression of GluN1-a and GluN1-b overlaps but their relative expression changes depending on the region [165].

Among the NMDARs, GluN2 subunits become prominent due to their involvement in the receptor's functional heterogeneity and they display different spatiotemporal expression profiles [166,167]. For example, expression of GluN2A begins shortly after birth and in adulthood, it is expressed abundantly across the CNS [165]. Similarly, expression of GluN2B is kept at high levels after birth, makes a peak around the first postnatal week [165]. Particularly the expressions of GluN2B, GluN2D, and GluN3A subunits in the early developmental stages and the expression of GluN2A and GluN2B in higher brain structures lead to the conclusion of their importance in synaptogenesis and synaptic maturation as well as in the synaptic function and synaptic plasticity [168,169].

Investigation of the differences between male and female brains has been becoming an important topic. Particularly, zebrafish have been shown to display sexually dimorphic pattern in the structure, morphology, behavior and also protein and gene expression [170-171,104]. For instance, Arslan-Ergul and Adams (2014) reported that there are more than 500 genes, which were related to the neurogenesis and cell proliferation, expressed differentially and there was a male dominance in the gene expression profile [172]. Similarly, Small *et al.* (2009) showed male-biased patterns of gene expression [173]. In opposite to these results, Chatterjee *et al.* (2016) indicated that at transcriptome level, differential gene expressions were female biased [174]. In addition to gene expressions, Vizziano-Cantonnet *et al.* (2010) showed that the expressions of certain enzymes also differ in male and female zebrafish brains [175]. Furthermore, Albasanz *et al.* (2016) reported that male zebrafish possess more metabotropic glutamate receptor 5 ($mGlu_5$), which is a G protein-coupled receptor, compared to female zebrafish [176]. These results underlie

the importance of the examination of different genders in order to have a comprehensive understanding of the expression of genes related to cell proliferation and neurogenesis, as well as certain enzymes. This preliminary study will provide information regarding the sexual dimorphism in terms of the expressions of proteins involved in the synaptic neurotransmission

In this chapter, we hypothesize that expression of glutamate subunits will show a sexually dimorphic pattern in middle aged zebrafish.

3.2.Materials and Methods

3.2.1. Subjects

Animals (AB strain) which were used in the preliminary study were composed of middle-aged (18 months old) three females and five males and study was approved by The Bilkent University Local Animal Ethics Committee (HADYEK) in the date of date: June 15, 2016, and no: 2016/21.

3.2.2. Housing

Animals were fed twice a day and reserved 14 hours of light and 10 hours of a dark cycle within 24 hours at 28 °C and kept within a recirculating tank system in the zebrafish facility of Department of Molecular Biology and Genetics, Bilkent University, Ankara, Turkey.

3.2.3. Euthanasia

Subjects were euthanized by following the instructions in Section 2.2.6.

3.2.4. Protein Isolation

Brain samples dissected from the zebrafish were homogenized by following the protocol in the Section 2.2.7.

3.2.5. Protein Quantification

Protein quantification was held by following the instructions which were mentioned in Section 2.2.8. and in Table 2.2 and Table 2.3.

3.2.6. Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis (SDS-PAGE)

3.2.6.1. Preparation of Resolving and Stacking Gel

SDS-PAGE was conducted by following the instructions mentioned in the Section 2.2.9. Two acrylamide gels having different percentages were prepared for the optimization purposes. First one was prepared by following the instructions mentioned in section 2.2.9.1. and in Table 2.4 and Table 2.5. The second acrylamide gel percentage was adjusted to 4% stacking and 8% resolving gel. Preparation of the gels is described in Table 3.1 and Table 3.2. Same reagents mentioned in the section 2.2.9.1. were used.

Table 3.1 10 mL 8% Resolving Gel

Order	**Substance**	**Amount (mL) For 1 Gel**
1	ddH_2O	5300 mL
2	1.5 M Tris (pH=8.8)	2.5 mL
3	10% SDS	100 µL
4	40 % Acrylamide Mix	2.0 mL
5	10% APS	100 µL
6	TEMED	10 µL

Table 3.2 8 mL 4% Stacking Gel

Order	**Substance**	**Amount (mL) For 1 Gel**
1	ddH_2O	4.764 mL
2	0.5 M Tris (pH=6.8)	2.0 mL
3	10% SDS	80 µL
4	40 % Acrylamide Mix	1.060 mL
5	10% APS	80 µL
6	TEMED	16 µL

3.2.6.2. Preparation of Protein Samples and Loading & Running

Fifty µL of 20 ug, 40 ug, and 80 ug of the samples were loaded into the wells in order to see the expression curve and find out in which concentration protein expression is optimum. In order to make samples ready to load in the wells, the procedure mentioned in the Section 2.2.9.2. was followed.

Electrophoresis tools which were mentioned in 2.2.10.1 were employed. Two running protocols were used. The first one is mentioned in 2.2.10.1. In the second protocol, the tank was connected to the Bio-Rad power supply and set 80 V at room temperature till the blue dye reached the bottom for separation of the proteins. Miniamper reading was recorded in order the system to heat up. Same reagents mentioned in the section 2.2.9.2. were used.

3.2.7. Western Blot

3.2.7.1.Transfer

Transfer of the proteins was completed by following the protocol mentioned in the section 2.10.1. Same reagents mentioned in the section 2.2.10.1. were used.

3.2.8. Primary and Secondary Antibody Incubation

The transfer step was followed by blocking the membrane in blocking solution for 70 minutes in order to get rid of non-specific bindings. After, the membranes were incubated with primary antibodies for 16 hours at 4^{o}C on the shaker in slow motion. Primary antibodies were anti-NR2B (LifeSpan Biosciences, Seattle, WA, USA: LS-C25797), anti-GluR2+GluR3 (LifeSpan Biosciences, Seattle, WA, USA: LS-C15368), and anti-β tubulin (Cell Signaling Technology, Danvers, AM, USA: #2146,) which was the loading control. None of the mentioned antibodies were studied in zebrafish so far. Figure 3.1 was followed for the antibody optimization experiments.

Name	Antibody name	Stock concentration g/mL	Volume from stock µL	Final concentration µg/mL	Final solution volume mL	Dilution
NR2B	Anti-GRIN2B antibody	1 g/mL	5 µL	0.5 µg/mL	10 mL	1:2000 dilution
		1 g/mL	10 µL	1 µg/mL	10 mL	1:1000 dilution
		1 g/mL	10 µL	2 µg/mL	5 mL	1:500 dilution
		1 g/mL				
GLUR2+ GLUR3	Anti-GLUR2+GLUR3 antibody	1 g/mL	5 µL	0.5 µg/mL	10 mL	1:2000 dilution
		1 g/mL	10 µL	1 µg/mL	10 mL	1:1000 dilution
		1 g/mL	10 µL	2 µg/mL	5 mL	1:500 dilution

Figure 3.1 Optimization plan for the antibodies.

All antibodies were prepared in 5% milk powder (Blocking Buffer) + TBS-T. After overnight incubation, membranes were washed with TBS-T for six times as shown in Table 3.3.

Table 3.3 Washing durations of the primary antibodies.

Washing Time	**1st**	**2nd**	**3rd**	**4th**	**5th**	**6th**
Duration (min)	5	5	10	5	5	10

Secondary antibodies were anti-rabbit (Cell Signaling Technology, Danvers, AM, USA: #7074). All antibodies were prepared in 5% milk powder + TBS-T. Duration of the washing was determined according to Table 3.4. After one-hour incubation at room temperature, membranes were washed with TBS-T for six times as shown in Table 3.4 and incubated for 40 additional minutes in TBS-T.

Table 3.4 Washing durations of the secondary antibodies.

Washing Time	**1st**	**2nd**	**3rd**	**4th**	**5th**	**6th**
Duration (min)	5	5	10	5	5	10

3.2.9. Antibodies

3.2.9.1. Anti-NR2B

Its brand name is Anti-GRIN2B/NMDAR2B/NR2B antibody. It is a polyclonal antibody, and produced in Rabbit, and rose against Mouse. It recognizes NR2B at 180 kDa. According to the datasheet, it shows reactivity with Human, Monkey,

Mouse, Bat, Dog Hamster, Horse, Pig, Rabbit, Chicken, Xenopus, and Zebrafish. It was stored in -20^{o}C for long term usage and came with 1mg/mL concentration.

3.2.9.2. Anti-GluR2+GluR3

Its brand name is Anti-GLUR2+GLUR3 Antibody. It is a polyclonal antibody, and produced in Rabbit, and rose against Rat. It recognizes NR2B at 110 kDa. According to the datasheet, it shows reactivity with Human, Monkey, Mouse, Rat, Bovine, Dog, Hamster, Horse, Pig, Rabbit, Chicken, Xenopus and Zebrafish. It was stored in -20^{o}C for long term usage and came with 1mg/mL concentration.

3.2.10. Chemiluminescent Detection

Membranes were taken from the TBST were put on parafilm and bands of interests were covered with 500 μL of chemiluminescent kit which consists of 2 different buffers, one of which is Luminol/ Enhancer and other is Stable Peroxide Buffer. Each one was used in 1:1 ratio system. So for 500 μL mixture in total was consisting of 250 μL of one buffer and 250 μL of the other buffer, and they were mixed in an Eppendorf. Membranes were incubated for 5 minutes in a dark, light-proofed chamber.

3.2.11. Imaging

Imaging was performed by following the instructions in the Section 2.2.13.

3.2.12. Quantification

Western blot results were quantified by following the instructions mentioned in the Section 2.2.14.

3.2.13. Statistical Analysis

Quantified data were first tested for normality and homogeneity of variance Shapiro-Wilk Test and Levene's Test, respectively, by employing SPSS (IBM, Istanbul, Turkey) statistics program and the cases where assumptions were fulfilled for the parametric tests, t-test was performed. The significance level was accepted as $p<0.05$. Graphs of the data were piloted by using GraphPad Prism (version 4.00 for Windows, GraphPad Software, San Diego CA, USA).

3.3.Results

3.3.1. Optimization of Antibodies

3.3.1.1. Anti-NR2B Optimization

The optimization experiment was started with the SDS-PAGE and blotting with the protocol in Chapter 2 section 2.2.9 and 2.2.10. Two dilutions of anti-NR2B primary antibody were tried which were 1:500 and 1:1000 dilutions. Male and female zebrafish brain homogenates were used in order to characterize the anti-NR2B antibody in zebrafish and mouse brain homogenate were used as positive control. As a secondary antibody, α- Rabbit in 1:5000 dilution were used. Results are shown in Figure 3.2 and Figure 3.3. Membranes were washed 30 minutes as shown in Table 2.6 and incubated in blocking solution at room temperature for 50 minutes. We selected the mouse brain protein lysates as a positive control.

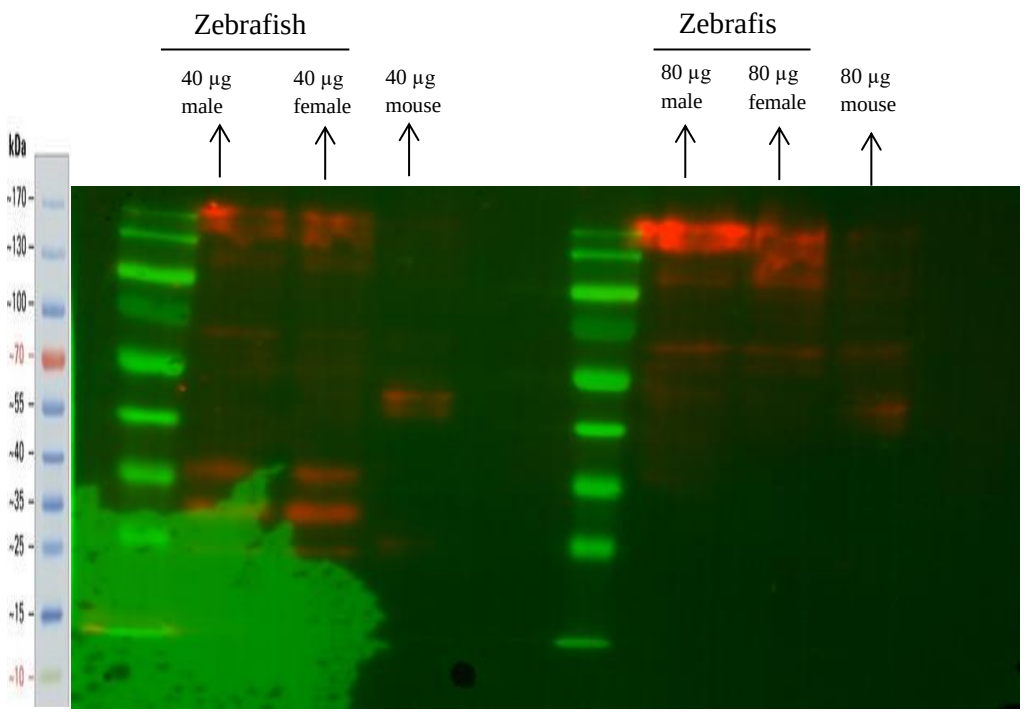

Figure 3.2 Representative image of 1:500 dilution of α-NR2B antibody.

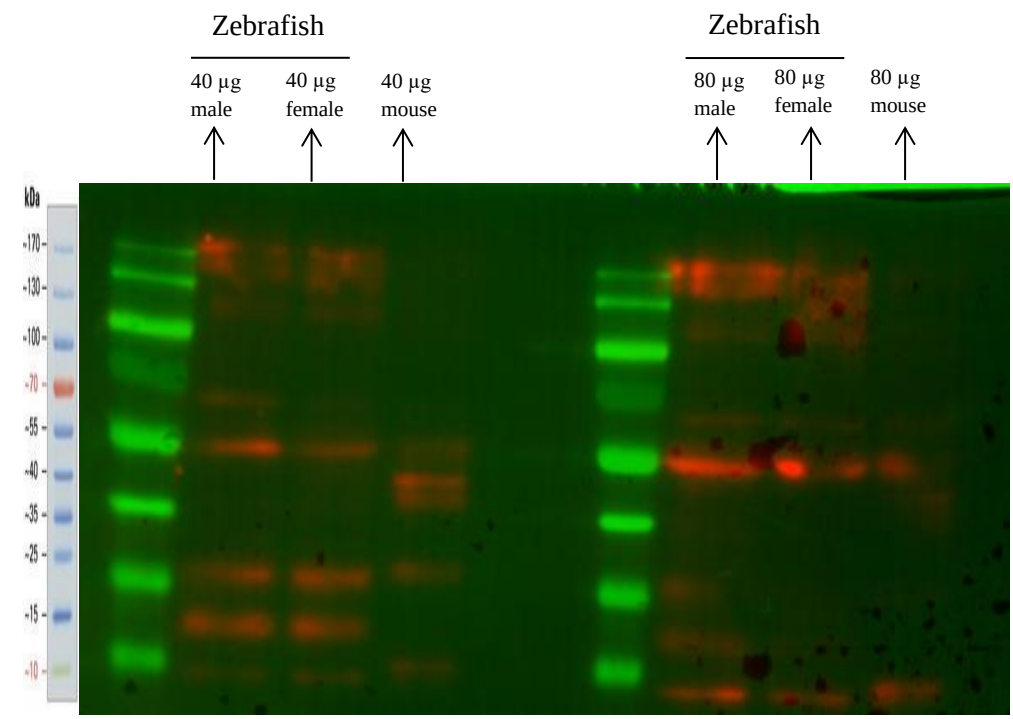

Figure 3.3 Representative image of 1:1000 dilution of α -NR2B antibody.

NR2B gives band at 180 kDa and 1:500 dilution gave more expression than 1:1000 dilution expression did, which was expected. Also, 80 µg protein concentration gave more expression than 40 µg protein concentration. Although anti-NR2B worked, there were non-specific bindings on the membrane. Also in both dilutions of the antibodies, bands of the positive control were faded.

In the second experiment, two membranes were prepared and same concentrations of α–NR2B antibody were tested; 1:500 and 1:1000. However, instead of using male and female zebrafish brain homogenates separately, only one gender of the brain of the zebrafish was used. In addition to 40 µg and 80 µg protein concentrations, 20 µg protein concentrations were tested. The reason behind the using only one gender' brain protein lysates was that, for the optimization experiments, lysates of one gender was enough to find the optimal antibody dilution. The first gel was incubated with 1:500 dilution of anti-NR2B and the second gel was incubated with 1:1000 dilution of anti-NR2B. In order to get rid of the non-specific bindings, gels were incubated in blocking buffer for 70 minutes at room temperature and the washing of the secondary antibody was performed for 40 minutes as shown in Table 3.4. Results are shown in Figure 3.4 and Figure 3.5.

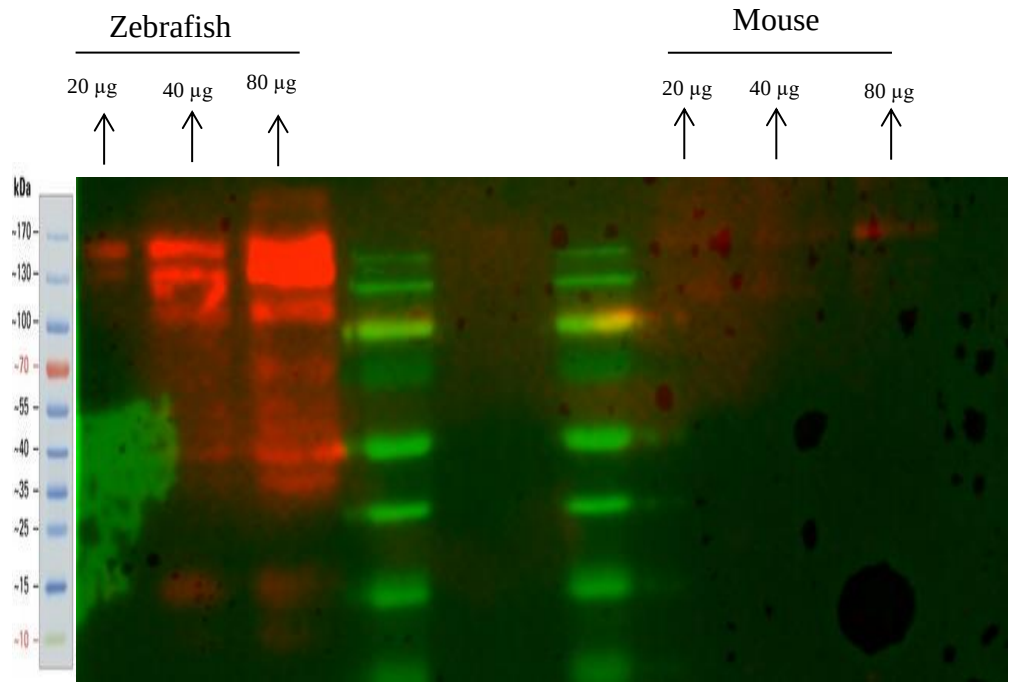

Figure 3.4 Representative image of the first membrane with 1:500 dilution of α -NR2B antibody.

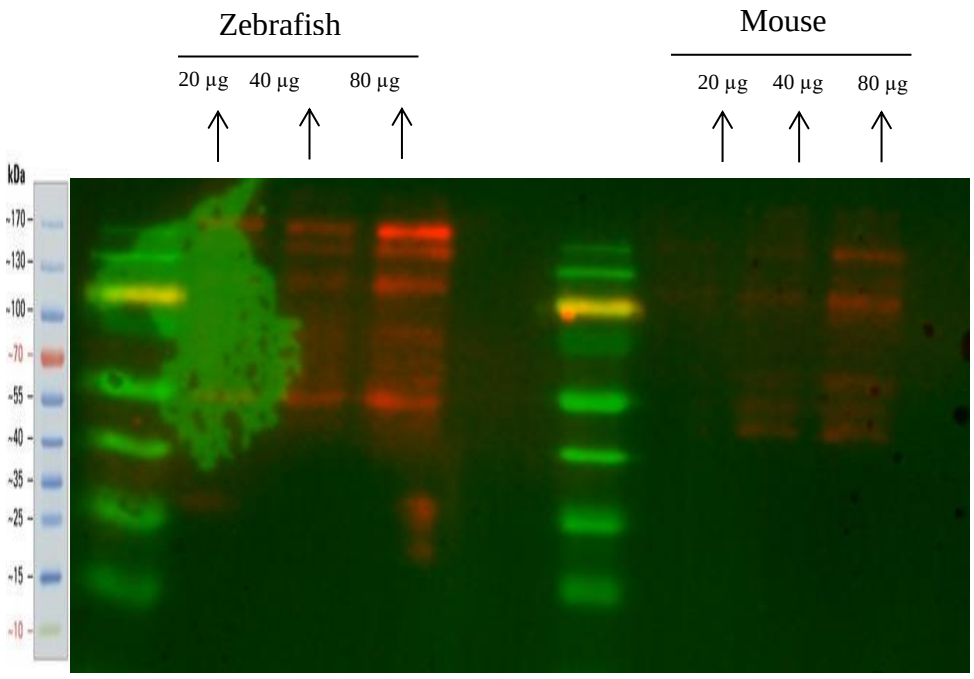

Figure 3.5 Representative image of the second membrane with 1:1000 dilution of α-NR2B antibody.

Compared to 1:500 dilution of –NR2B antibody, 1:1000 dilution gave more clear results. In 1:500, wells which were loaded with 40 µg and 80 µg protein concentrations gave very strong signals and cannot be quantified. Also, positive control gave expression with 80 µg protein concentration in the both membranes. In the membrane incubated with 1:1000 dilution of anti-NR2B, none of the bands were exploded and all bands were clear. But in both membranes, non-specific bindings were present. In order to get rid of these, both membranes were incubated in TBS-T for 45 minutes at room temperature. Results were shown in Figure 3.6 and Figure 3.7.

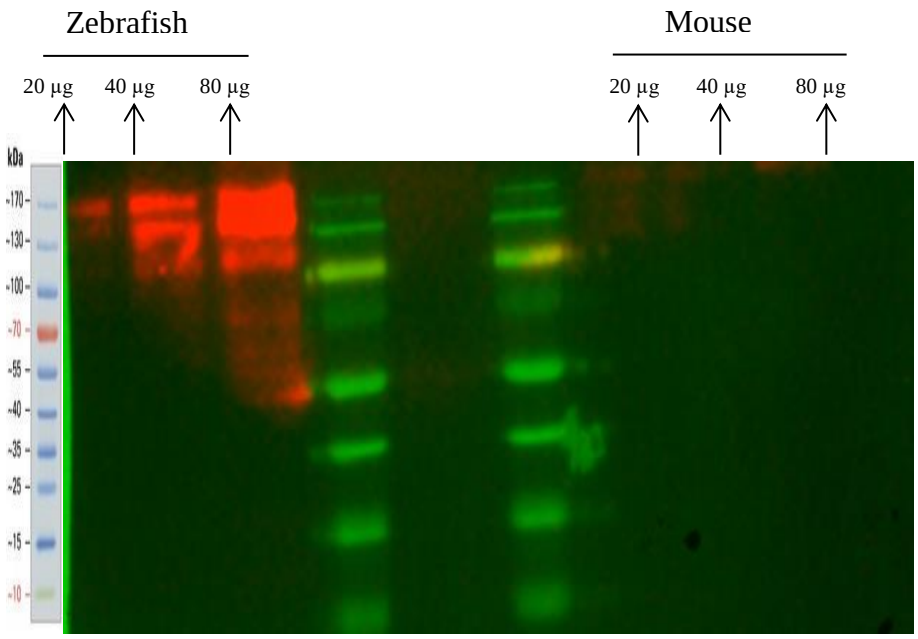

Figure 3.6 Representative image of the membrane with 1:500 dilution of NR2B after 45 minutes of TBS-T incubation.

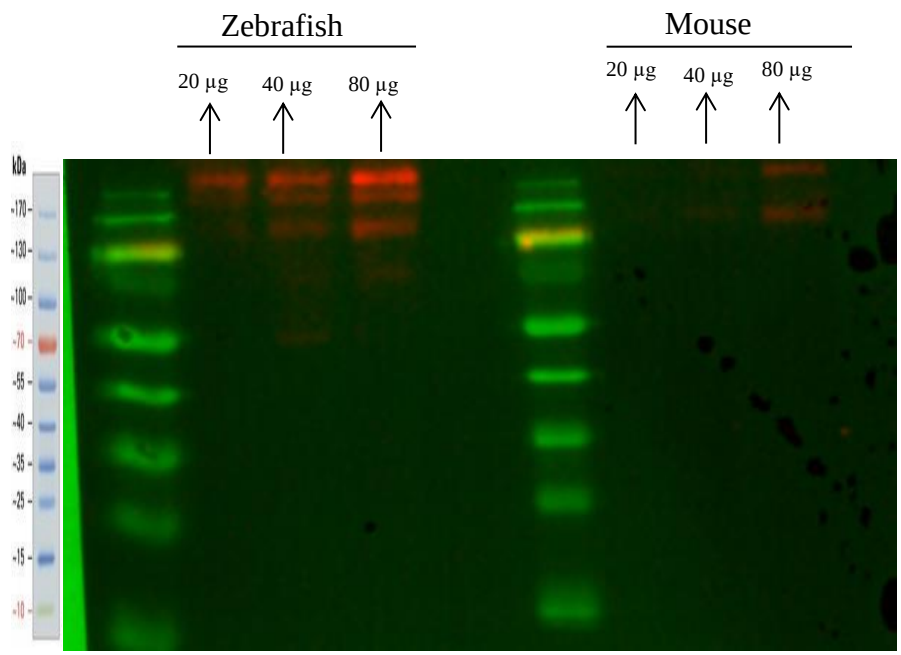

Figure 3.7 Representative image of the membrane with 1:1000 dilution after 45 minutes of TBS-T incubation.

As shown in Figure 3.6 and Figure 3.7, most of the non-specific bindings were cleared after 45 minutes TBS-T incubation. Also, in the membrane which was incubated with 1:1000 dilution, bands were more visible. Here, we concluded that that 1:500 anti-NR2B primary antibody dilution and 80 µg protein concentration was too high for the observation of NR2B expression. Also, TBS-T incubation resulted in the decrease in the band intensity of positive control.

In the following experiment, 20 µg and 40 µg concentrations of proteins were loaded on one gel and after transfer, the membrane was cut into two, right-hand side was incubated with 1:2000 dilution of –NR2B and left-hand side were incubated with 1:1000 dilution of –NR2B. Membranes were incubated in blocking

buffer for 70 minutes and washed 40 minutes after the secondary antibody incubation and kept in TBS-T for additional 45 minutes. Results are shown in Figure 3.8.

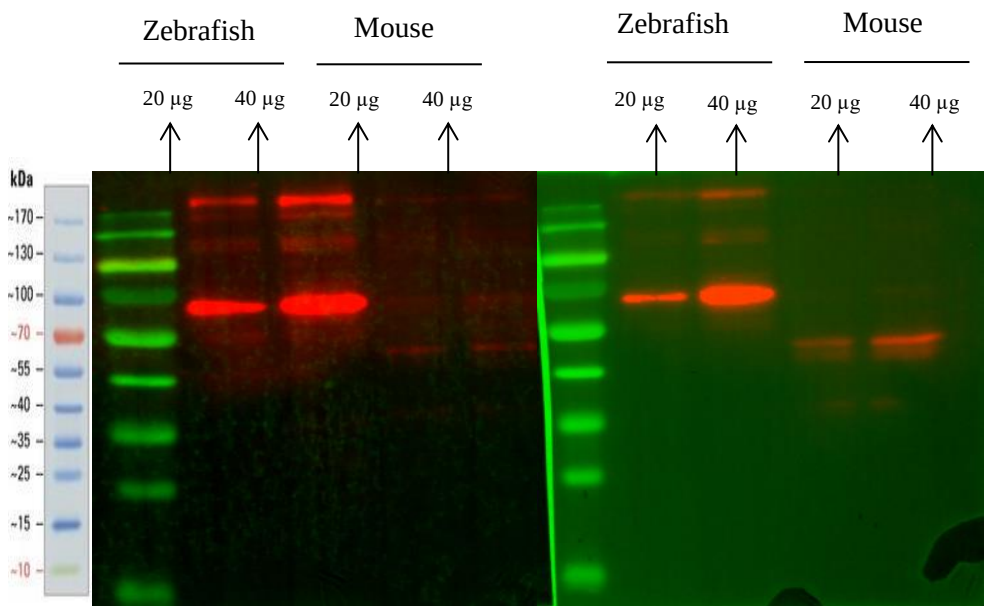

Figure 3.8 Representative images of the two membranes. Left-hand side membrane was incubated with 1:1000 dilution of the anti-NR2B and right-hand side membrane was incubated with 1:2000 dilution of the anti-NR2B.

Compared to the 1:2000 dilution, 1:1000 dilution of the α-NR2B antibody gave more clear expression in the NR2B protein band. Also, 40 µg concentrations of protein gave better expression than 20 µg concentrations of protein. However, between 70 kDa and 55 kDa, there was a non-specific band which was not observed in the former experiments. It was concluded that 40 µg concentrations of protein and 1:1000 dilution is the best concentration and dilution for the observation of anti-NR2B. Also, we cut the membrane from 130 kDa band in order to ensure that no non-specific bindings occurred on our bands of interest.

3.3.1.2. Anti-GluR2+ GluR3 Optimization

Optimization of anti-GluR2+ GluR3 antibody was started with by following the protocol mentioned in the Section 3.2.6. This antibody was expected to express the bands with 110 kDa. Two antibody concentrations 1:1000 and 1:2000 were tested. After transfer protocol, the membrane was cut into two and the right-hand side was incubated with 1:2000 dilution of anti-GluR2+ GluR3 antibody and left-hand side were incubated with 1:1000 dilution of anti-GluR2+ GluR3 antibody. Membranes were incubated in the blocking solution for 70 minutes and β-tubulin with 1:5000 dilution was used as the secondary antibody. Membranes were washed for 40 minutes after the secondary antibody incubation. Mouse brain homogenates were used as a positive control. Results are shown in Figure 3.9.

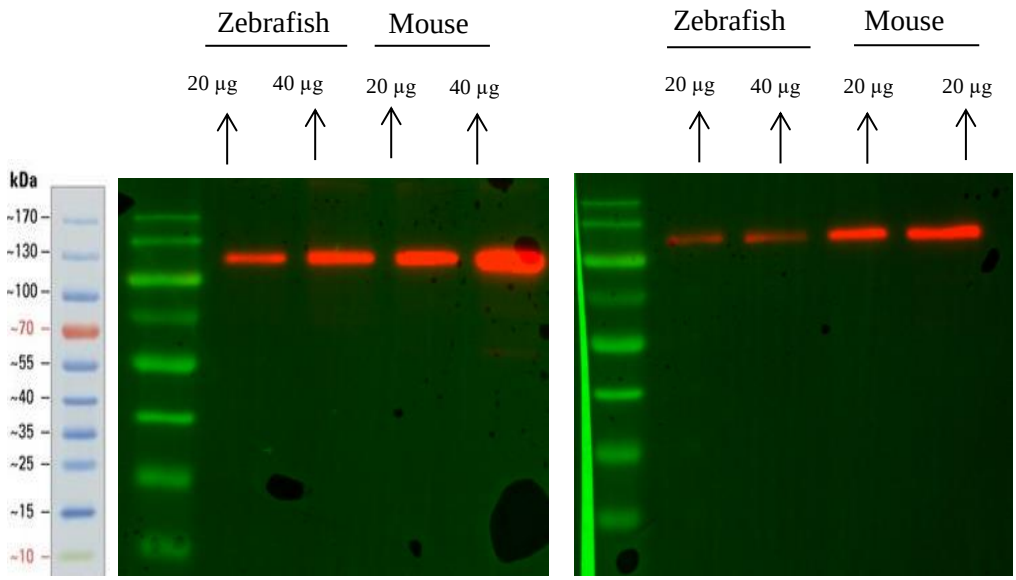

Figure 3.9 Representative image of the expression of anti-GluR2+ GluR3. The left-hand side shows membrane incubated with 1:1000 dilution of anti-GluR2+ GluR3 and right-hand side shows the membrane incubated with 1:2000 dilution of anti-GluR2+ GluR3.

Anti-GluR2+ GluR3 antibody did not give any non-specific bindings in both dilutions. As shown in Figure 3.9, 1:1000 dilution gave clear expression and 40 µg of protein concentration gave better expression compared to 20 µg of protein concentration. 1:2000 dilution of anti-GluR2+ GluR3 also worked compared to the 1:1000 dilutions, expression of both 20 µg and 40 µg of protein concentrations expression was weaker. In addition, positive control expressed anti-GluR2+ GluR3 in both antibody dilutions and protein concentrations. We concluded that the optimal dilution of anti-GluR2+ GluR3 was 1:1000 and loading of 40 µg of protein concentration.

3.3.2. Expression of anti-NR2B and anti-GluR2+GluR3 in Middle-Aged Male and Female Zebrafish: Preliminary Study

Five male and three female middle-aged (18 months old) zebrafish were used in the preliminary study. In order to avoid the non-specific binding occurred during the optimization experiments, membranes were cut from the 70 kDa and just above the 130 kDa lines. All experiments were conducted as technical triplicates. Representative image of the results is shown in the Figure 3.10.

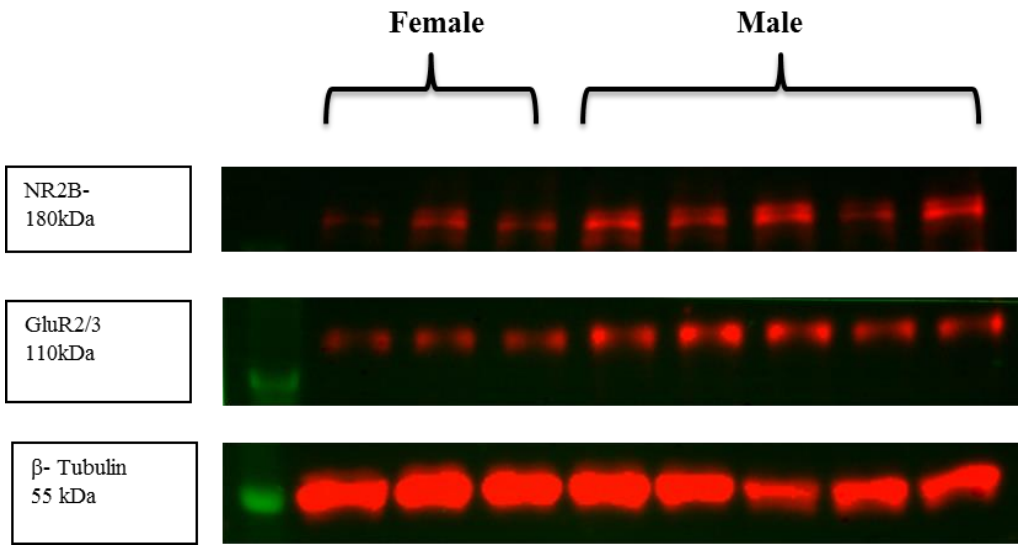

Figure 3.10 Representative picture of the expression of NR2B, GluR2+GluR3, and β-Tubulin.

Relative expression of NR2B in male (1.062 ± 0.1805 N=5) and female (0.8968 ± 0.2794 N=3) animals are shown in the Figure 3.11A. For the statistical analysis of the expression of NR2B in male and female animals, independent samples *t-test* was carried out. It was calculated that expression of NR2B was not significantly different between male and female animals ($t(6)=0.5235$, $p=0.6119$). Relative expression of GluR2/3 in male (1.054 ± 0.1617 N=5) and female (0.9102 ± 0.1622 N=3) are displayed in the Figure 3.11B. In order to perform statistical analysis for the expression of GluR2/3 in male and female animals, independent samples *t-test* was employed. It was found that expression of GluR2/3 was not significantly different between male and female animals ($t(6)= 0.5842$, $p=0.5804$).

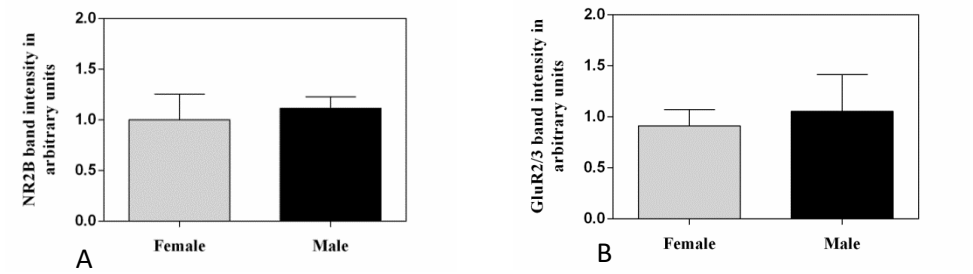

Figure 3.11 Relative expressions of NR2B **(A)** and GluR2/3 **(B)** in male and female zebrafish. Data were analyzed by independent samples *t-test*., and presented as means ± SEM

Tubulin, as a loading control, was shown to not significantly different in any experimental group (1.00± 0.2078 N=8, 0.99± 0.3061 N=8, 0.99± 0.3061 N=8, 1.00± 0.1267 N=8), indicating that no loading, pipetting or transfer mistake took place during the experimental procedure.

3.4.Discussion

This study was designated to investigate the expression of NMDA and AMPA receptor subunits NR2B and GluR2/3, respectively, in middle age (18 months old) male and female zebrafish. We selected these subunits for their function in the glutamatergic neurotransmission and therefore, synaptic plasticity because aging brain experiences structural and functional alterations and these ultimately results in a reduction in the cognitive functioning even in healthy individuals. Learning and memory are particularly dependent on the NMDA neurotransmission [114]. Particularly, NR2B is involved in the receptor endocytosis and receptor biophysics [114]. Similarly, AMPA receptors participate in the receptor trafficking and dysfunctions of the receptor subunits lead to diseases, as well as deteriorated synaptic neurotransmission [177]. Most importantly, we selected NR2B and GluR2/3 for their

direct interaction with PSD-95 with is the scaffold protein in excitatory neurons [141–143].

For this purpose, we selected anti-NR2B and anti-GluR2+GluR3 antibodies. Optimization experiments are the key steps for all the experimental setups. It is important for determining optimum dilutions, durations, and experimental conditions. To our knowledge, these two antibodies have never been tested in zebrafish brain lysates. Although zebrafish has been using widely as a model organism, still many antibodies have not been tested or specifically targeted zebrafish tissues. Mainly, based on the protein homology between targeted organisms for the antibody, antibodies are purchased and optimized. Reactivity of the antibody in the organism is another parameter in the choosing the antibody of interest. However, antibody may not work even though the similarities or reactivity, and also may work even though absence of similarity or reactivity. Also, monoclonal and polyclonal feature of the antibody of interest may result in non-specific result which is something undesired. NR2B and GluR2+3 are involved in neurotransmission in glutamatergic neurons and hence; it is important to point out their expression level.

NR2B is an 180 kDa protein, and NR2B band is represented in the highest band in the protein marker. Therefore, we considered it as ‘high molecular weight protein’. During SDS-PAGE step, adjustment of the percentage of the acrylamide gel is valid since, the smaller the percentage of acrylamide gel, the more likely the high molecular weight proteins run through the gel. We started with 10% of acrylamide gel but due to the molecular weight of the protein, we prepared 8% of acrylamide gel. Also, duration of running is another important parameter. High molecular weight

proteins tend to run slower, therefore; we run the samples in 90V till the dye reaches the bottom which took almost 4 hours. However, in such long SDS-PAGE cases, due to the elevations in the temperature of the buffer in the tank, the gel may melt. In order to prevent this, we kept the tank in the cold room. Also, longer duration in the SDS-PAGE step provides the extension of the distance between bands which is useful if the membrane is going to be cut.

Selection of the antibodies depends on of several parameters. Reactivity, specificity, clonality are among these parameters. Even though the fact that zebrafish have been studied widely, many antibodies are not produced particularly for zebrafish. Therefore; by controlling reactivity and specificity, we chose our antibodies. During optimization experiments, the problem we mainly faced with was the non-specific bindings. Both of the antibodies we optimized were polyclonal which tend to bind more than one epitope of the protein of interest as well as other proteins. In addition, the concentration of the primary and particularly secondary antibody may lead such non-specific bindings. To prevent it, we tried different concentrations of the antibody of interest. Blocking is an important step for western blotting since it prevents the antibody from binding to the membrane nonspecifically. For this purpose, we selected 5% non-fat dry milk for blocking. Another alternative blocking solution used for blocking is Bovine Serum Albumin (BSA) but due to its lower protein concentration compared to non-fat dry milk, we selected non-fat dry milk. In addition to selection of blocking buffer, durations of washings of the primary and secondary antibodies is important to reduce the number of nonspecific bindings. In our experiment, we started with 30 minutes washing of primary and secondary antibodies but in order to reduce the density of non-specific bindings; we

increased the duration 40 minutes which resulted in more clear images. Also, incubating the membranes after secondary antibody washing in the TBS-T, which is a detergent by which we prepare also blocking solutions, for 50 minutes and we observed decreased density of non-specific bindings.GluR2+GluR3 is an 110 kDa protein and during optimization experiments. We performed western blot with the optimized conditions for –anti NR2B and anti GluR2+GluR3 antibody displayed no non-specific bindings. When both antibodies blotted together, we still observed nonspecific bindings, therefore, we decided to cut the membrane from the 70 kDa and just above the 130 kDa lines in order to get rid of non-specific bindings occurred after anti-NR2B incubations.

In preliminary study, we aimed to demonstrate the expression of NR2B and GluR2/3 in middle-aged male and female zebrafish. Here, reported that expression of NR2B was not significantly different between males and females (Figure 3.11). However, expression of NR2B in males was numerically higher in males than in males. Unpublished data from our laboratory indicated that expression of PSD-95 in females was significantly higher than in males [145]. When we consider the direct interaction of PSD-95, this data contradicts with our results. In their study, they had an equal number of males and females yet in our experiment, we had 3 females and 5 males which might explain the different results. Also, NR2B phosphorylation reduces with age, hence; phosphorylated levels of NR2B should be investigated in the future studies [114]. Also, NR2B has been reported to be expressed predominantly in prefrontal cortex where working memory functions take place [163]. Monaco and Gulchina (2015) showed that the ratio of NR2A/NR2B changes depending on the brain region and in mature hippocampus and primary sensory

cortex, this ratio becomes higher, however, in prefrontal cortex; this balance is kept stable during development and in the adulthood state, and NR2B expression becomes higher than NR2A expression [178]. Therefore, we can conclude that expression level of NR2B subunit might change based on the neuronal structure or synaptic maturation, brain region. Additionally, in our study we used individual brains instead of pooling the brain lysates in contrary to many other studies which provide a distinct advantage. Also, in many studies, animal groups are generally composed of only young and old animals. Yet middle age, which can be considered as a transition state and we can characterize and estimate when expressions do start to change.

Demonstration of the expression levels of glutamatergic receptor subunits which are involved in the synaptic transmission and synaptic responses was the aim of this preliminary study. Accordingly, among the AMPA receptor subunits, we particularly showed the expression level of GluR2/3 due to their distinctive functions in synaptic responses [179]. It was previously shown that expression of synaptic proteins and receptor subunits display sexually dimorphic pattern [170,180–182]. However, here we claim that GluR2/3 expression is not significantly different between males and females (Figure 3.12). According to the data of this preliminary study, we proposed that expression level of GluR2/3 is similar between middle-aged male and female animals although in males expression is numerically more than in females. However, as mentioned above, expression of PSD-95 is higher in females than in males according to the unpublished data from our laboratory [145]. Some studies indicate the direct interaction of PSD-95 with AMPA receptors and some others show that direct interaction is between NMDA receptors [141,143]. Given the literature information and our results, this requires further investigation. Since PSD-

95 expression increases with age, by adding young and old animals to this study, future studies can address the association between PSD-95 and NR2B and GluR2/3.

In many studies, animal groups are generally composed of only one gender, however sexual dimorphism in aging or gene expressions between genders are poorly understood. In this study sexual dimorphism in the context of expression of glutamate receptor subunits has been studied in order to contribute the information to the literature about the understanding of the differences between the genders. It was previously shown that aging and synaptic plasticity is sexually dimorphic in humans as well as zebrafish [164–166]. However, considering the local expressions and the mechanism they participate in; investigation of the expression levels in a region-specific manner can provide more accurate information. Glutamatergic neurotransmission is involved in the neuronal migration, proliferation, survival and learning and memory, and therefore; NMDARs and AMPARs become the center of such studies [183]. Also, a malfunction in glutamate receptor subunits was linked with many neurodegenerative disorders; therefore, investigation of these subunits will help to understand the gender and age-specific approaches for the future studies.

CHAPTER 4

Conclusion & Future Prospects

Aging is defined by the continuing decline in the physiological and metabolic functioning [2]. Brain, compared to the other organs in the body, is affected to the greatest extent due to the high consumption of glucose and oxygen. Structural and cognitive changes take place as a consequence of aging. Great efforts have been put on understanding and delaying the effects of aging in the brain. Caloric restriction (CR), which refers to the decrease in the daily calorie intake, is said to be the only non-genetic intervention which can reduce the age-related cognitive decline as well as structural deteriorations [184]. Although it has been shown to enhance cognitive and structural functions, most people would feel reluctant or incapable of adapting such treatment, therefore; the idea of CR mimetic was put forward. Since CR acts upon the mTOR nutrient-sensing pathway, rapamycin (RAP) has become a candidate mimetic for CR [185]. Both approaches were shown to enhance medial and maximal life span, and also improve cognitive functions. Therefore, they have attracted attentions for the aging and age-related disorder studies.

Zebrafish has become a promising model organism for genetic, developmental and aging researchers due to the homology of genes and brain structure with mammals. Due to its relatively small size, zebrafish is easy to house and feed. Also, relatively short lifespan and generation time enable aging studies in a shorter time. Male and female zebrafish were shown to display sexual dimorphism in molecular,

cellular and behavioral level. Therefore, it is an ideal organism for the investigation of the gender differences.

In this present work, two preliminary studies were conducted. In the first study, effects of short-term (4 weeks) CR and RAP treatments on the expression of key synaptic proteins in young (9-11 months old) and old (27-33 months old), male and female zebrafish was investigated. Body weight data indicated reduction in the body weight in AL and RAP groups was a sign of stress. Also, handling and housing of the animals for treatments resulted in social stress and net stress upon the animals. These speculations need further investigation with cortisol tests. In the expression studies, we found no statistically significant effect of the CR and RAP treatments on the expression of PSD-95, gephyrin and synaptophysin in age and gender groups compared to the AL animals. Taken numerical differences into account, we claimed that; 4 weeks is a short time in order to observe a significant effect of CR and RAP treatments on the synaptic proteins compared to the AL groups. In addition to the duration of the treatments, we speculate that exposure to RAP for 5 hours was not efficient enough for the drug to interfere with the mTORC1 pathway, as we speculated. In the future studies, these speculations can be verified by investigating the downstream elements of mTORC1 pathway by performing expression studies such as microarray and in-situ hybridization for gene expressions or western blot and immunohistochemistry for the protein expressions. Also, in the future studies experimental conditions can be modified so that any kind of factors that cause social stress is eliminated and animals exposed to the treatments in the optimal levels.

In the present work, we used whole brain lysates in the protein expression studies, however; region-specific distribution of the many of the proteins was

reported. Therefore, working on particular brain region such as prefrontal cortex or hippocampus, where cognitive and motor functions are administered, will provide more detailed and clearer information in the future studies. As in young and old animals; in male and female animals gene expressions and protein expressions show differences. Therefore, investigation of gender differences is very important in order to have accurate and essential ideas. In order to have statistically more significant outcomes, and also for the sake of the outcome of the experiments, sample size should be increased in the future studies. Given the studies showing that CR and RAP treatments improve cognitive functions, cognitive tests can be performed in the future studies in order to confirm both molecular and behavioral effects of these treatments. CR and RAP treatments are particularly important because they increase medial and maximal lifespan by postponing the age-related diseases such as diabetes, cardiac diseases. Therefore, investigations of such treatments are not only crucial for brain and cognitive aging, but also for other life-threatening diseases.

In the second study, we investigated the expression of two glutamate receptor subunits NR2B and GluR2/3 in middle-aged (18 months old) male and female zebrafish. We focused on glutamate receptors because they are involved in the glutamatergic neurotransmission which is important for the synaptic plasticity. We selected anti-NR2B and anti-GluR2+GluR3 antibody as antibody. These two antibodies have never been tested for zebrafish before; therefore we started with the optimization experiments. Optimizing the gel percentage, SDS-PAGE duration, blocking duration, duration of the washing times and dilutions of primary and secondary antibodies were the key parameters that we have changed for the optimization experiments. The antibodies we used in these experiments were

polyclonal. Polyclonal antibodies tend to generate large numbers of non-specific bindings and recognize multiple epitopes on the antigen they bind. Therefore we observed a number of non-specific bindings on the membranes. In the preliminary study which aimed to investigate the expression of NMDA and AMPA glutamate receptor subunits NR2B and GluR2/3, respectively, in middle-aged (18 months old) male and female zebrafish, we reported that the expression of NR2B was not significantly different between male and female groups. Similarly, expression of the GluR2/3 was reported to be non-significant between male and female groups. We particularly chose middle age in this study it is a transition state between young and old ages and it can give information about the trend of the change.

Here, the reason for selecting these particular subunits was their role in the glutamatergic neurotransmission. For example, NR2B gives the receptor various biophysical properties and regulates the receptor affinity to the glutamate [186]. This feature of the NR2B makes it important for pharmacologic studies as well as synaptic plasticity studies. Also, AMPA receptor trafficking was shown to be involved in the regulation of synaptic plasticity, learning and memory [187]. In addition to the synaptic plasticity, dysregulation of NMDA and AMPA receptors are involved in the neurodegenerative diseases such as Alzheimer's Disease, and multiple sclerosis [177], [188].

In both studies, we focused on the proteins and receptor subunits that are involved in the synaptic plasticity, which eventually affects cognitive functions. Also, subjects which were used in both studies were divided based on their gender because understanding the response of different genders to particular treatments will help to create more reliable approaches and experimental setups for the following

experiments. In terms of age, in the first study we divided our subjects into two age groups; young and old. Although using young and old animals in order to explain the effects of aging on the subjects, including an age group such as middle age would provide more detailed information such as the trend of the change as mentioned above. In the first study, we treated animals with short-term (4 weeks) CR and RAP and concluded that 4 weeks of CR was a short time to observe the effects of the treatments. In the second study, without any treatment, we demonstrated the expression of glutamate receptor subunits in male and female middle-aged animals. We did not observe statistical significance in the expression, however; it may be due to the age of the animals. In other words, in middle ages, expression of glutamate receptor subunits maybe stabilized in both genders. Future studies can add young and old age groups to the same study, and make conclusions about the general profile.

PSD-95 is the scaffold protein and its direct and indirect interaction with NMDA and AMPA receptors was previously shown [141]–[143]. Here, we showed that expression of PSD-95 numerically decreases with age, and numerically similar in males and females in AL group. As a next step, we investigated the expression in the subunit level in middle-aged animals. It is important because the demonstration of the tendency of the PSD-95 on a particular subunit in specific age and gender will provide creating better approaches for the understanding of subunit and protein specific diseases. Also, having particular age and gender groups will decrease the number of assumptions and provide healthier results. The expression of NR2B and GluR2/3 in the middle-aged male and female brains which are treated with CR and RAP can be investigated in the future studies in order to have a more comprehensive understanding about glutamatergic neurotransmission following CR interventions.

Further, in addition to the gephyrin; γ-aminobutyric acid (GABA) receptor subunits can be investigated in order to reveal the mechanism of inhibitory neurotransmission and have a better understanding of excitatory/inhibitory balance in the brain.

In this present work, our initial results of key synaptic protein levels indicate that they are stable throughout aging with respect to gender and CR and RAP interventions. Proteins and receptor subunits used in the both of the studies are related to the cognitive functions. The trend of the change in their expressions between genders during aging will provide a better approach to improving decreased cognitive functions with certain treatments such as CR and RAP. Also, impairments of these proteins and subunits result in the neurodegenerative diseases such as Alzheimer's Disease or dementia. Due to the neurological, developmental and genetic similarities between zebrafish and mammals, we believe that these results contribute to the creation of better approaches to the studies on mammals. Therefore, information provided by this study will contribute to the understanding of the molecular mechanisms as well as will help to find better treatment approaches to the diseases.

Bibliography

[1] M. Pollack, S. Phaneuf, and A. Dirks, “The Role of Apoptosis in the Normal Aging Brain , Skeletal Muscle , and Heart,” *Ann. N.Y. Acad. Sci.*, vol. 959, pp. 93–107, 2002.

[2] J. A. Bergado and W. Almaguer, “Aging and synaptic plasticity: A review,” *Neural Plast.*, vol. 9, no. 4, pp. 217–232, 2002.

[3] K. Abe and H. Saito, “Tyrosine kinase inhibitors, herbimycin A and lavendustin A, block formation of long-term potentiation in the dentate gyrus in vivo,” *Brain Res.*, vol. 621, no. 1, pp. 167–170, 1993.

[4] M. A. Lynch, “Analysis of the mechanisms underlying the age-related impairment in long-term potentiation in the rat,” *Rev.Neurosci.*, vol. 9, no. 3, pp. 169–201, 1998.

[5] J. F. Disterhoft, J. R. Moyer, Jr, and L. T. Thompson, “The Calcium Rationale in Aging and Alzheimer’s Disease. Evidence from an Animal Model of Normal Aging,” *Ann. N. Y. Acad. Sci.*, vol. 747, pp. 382–406, 1994.

[6] P. W. Landfield and J. C. Eldridge, “Evolving aspects of the glucocorticoid hypothesis of brain aging: Hormonal modulation of neuronal calcium homeostasis,” *Neurobiol. Aging*, vol. 15, no. 4, pp. 579–588, 1994.

[7] P. W. Landfield, “Aging-related increase in hippocampal calcium channels,” *Life Sci.*, vol. 59, no. 5–6, pp. 399–404, 1996.

[8] M. L. Michaelis, D. J. Bigelow, C. Schoneich, T. D. Williams, L. Ramonda,

D. Yin, A. F. R. H??hmer, Y. Yao, J. Gao, and T. C. Squier, “Decreased plasma membrane calcium transport activity in aging brain,” *Life Sci.*, vol. 59, no. 5–6, pp. 405–412, 1996.

[9] L. Cai, J. S. Y. Chan, J. H. Yan, and K. Peng, “Brain plasticity and motor practice in cognitive aging,” *Front. Aging Neurosci.*, vol. 6, no. MAR, pp. 1–12, 2014.

[10] M. Iosa, A. Fusco, G. Morone, and S. Paolucci, “Development and decline of upright gait stability,” *Front. Aging Neurosci.*, vol. 6, no. FEB, pp. 1–12, 2014.

[11] A. M. Tromp, S. M. F. Pluijm, J. H. Smit, D. J. H. Deeg, L. M. Bouter, and P. Lips, “Fall-risk screening teest: a prospective study on predictors for falls in community dwelling elderly,” *J. Clin. Epidemiol.*, vol. 54, no. 8, pp. 837–844, 2001.

[12] G. Pichierri, P. Wolf, K. Murer, and E. D. de Bruin, “Cognitive and cognitive-motor interventions affecting physical functioning: a systematic review.,” *BMC Geriatr.*, vol. 11, no. 1, p. 29, 2011.

[13] and P. L. Bruce A. Yankner, Tao Lu, “The Aging Brain,” *Annu. Rev. Pathol. Dis.*, vol. 3, pp. 41–66, 2008.

[14] E. B. Montgomery Jr, W. C. Koller, T. J. K. LaMantia, M. C. Newman, E. Swanson-Hyland, A. W. Kaszniak, and K. Lyons, “Early detection of probable idiopathic Parkinson’s disease: I. Development of a diagnostic test battery,” *Mov. Disord.*, vol. 15, no. 3, pp. 467–473, 2000.

[15] Z. C. Lai, M. B. Moss, R. J. Killiany, D. L. Rosene, and J. G. Herndon, "Executive system dysfunction in the aged monkey: Spatial and object reversal learning," *Neurobiol. Aging*, vol. 16, no. 6, pp. 947–954, 1995.

[16] M. E. Bach, M. Barad, H. Son, M. Zhuo, Y. F. Lu, R. Shih, I. Mansuy, R. D. Hawkins, and E. R. Kandel, "Age-related defects in spatial memory are correlated with defects in the late phase of hippocampal long-term potentiation in vitro and are attenuated by drugs that enhance the cAMP signaling pathway.," *Proc. Natl. Acad. Sci. U. S. A.*, vol. 96, no. 9, pp. 5280–5, 1999.

[17] M. E. Raichle and D. a Gusnard, "Appraising the brain's energy budget.," *Proc. Natl. Acad. Sci. U. S. A.*, vol. 99, no. 16, pp. 10237–10239, 2002.

[18] R. Peters, "Ageing and the brain," *Postgr. Med*, vol. 82, no. 2, pp. 84–88, 2006.

[19] F. Mora, "Successful brain aging: plasticity, environmental enrichment, and lifestyle.," *Dialogues Clin. Neurosci.*, vol. 15, no. 1, pp. 45–52, 2013.

[20] J. N. Trollor and M. J. Valenzuela, "Brain ageing in the new millennium," *Aust. N. Z. J. Psychiatry*, vol. 35, no. 6, pp. 788–805, 2001.

[21] F. M. Gunning-Dixon, A. M. Brickman, J. C. Cheng, and G. S. Alexopoulos, "Aging of cerebral white matter: a review of MRI findings," *Int. J. Geriatr. Psychiatry*, vol. 24, no. 2, pp. 109–117, Feb. 2009.

[22] N. Raz, U. Lindenberger, K. M. Rodrigue, K. M. Kennedy, D. Head, A. Williamson, C. Dahle, D. Gerstorf, and J. D. Acker, "Regional brain changes in aging healthy adults: General trends, individual differences and modifiers,"

Cereb. Cortex, vol. 15, no. 11, pp. 1676–1689, 2005.

[23] L. Svennerholm, K. Boström, and B. Jungbjer, "Changes in weight and compositions of major membrane components of human brain during the span of adult human life of Swedes," *Acta Neuropathol.*, vol. 94, no. 4, pp. 345–352, 1997.

[24] D. H. Salat, J. A. Kaye, and J. S. Janowsky, "Prefrontal gray and white matter volumes in healthy aging and Alzheimer disease," *Arch Neurol*, vol. 56, no. 3, pp. 338–344, 1999.

[25] N. Raz, F. M. Gunning, D. Head, J. H. Dupuis, J. McQuain, S. D. Briggs, W. J. Loken, A. E. Thornton, and J. D. Acker, "Selective aging of the human cerebral cortex observed in vivo: differential vulnerability of the prefrontal gray matter," *Cereb Cortex*, vol. 7, no. 3, pp. 268–282, 1997.

[26] N. Raz, K. M. Rodrigue, K. M. Kennedy, D. Head, F. Gunning-Dixon, and J. D. Acker, "Differential aging of the human striatum: Longitudinal evidence.," *Am. J. Neuroradiol.*, vol. 24, no. 9, pp. 1849–1856, 2003.

[27] N. Raz, "Aging of the brain and its impact on cognitive performance: integration of structural and functional findings BT - Handbook of Aging and Cognition," vol. 2, no. June, 2000, pp. 1–90.

[28] Y. Geinisman, R. W. Berry, J. F. Disterhoft, J. M. Power, and E. a Van der Zee, "Associative learning elicits the formation of multiple-synapse boutons.," *J. Neurosci.*, vol. 21, no. 15, pp. 5568–5573, 2001.

[29] H. Brody, "Organization of the cerebral cortex. III. A study of aging in the

human cerebral cortex," *J. Comp. Neurol.*, vol. 102, no. 2, pp. 511–556, 1955.

[30] K. O. Devaney and H. A. Johnson, "Neuron Loss in the Aging Visual-Cortex of Man," *Journals Gerontol.*, vol. 35, no. 6, pp. 836–841, 1980.

[31] G. Henderson, B. E. Tomlinson, and P. H. Gibson, "Cell counts in human cerebral cortex in normal adults throughout life using an image analysing computer," *J. Neurol. Sci.*, vol. 46, no. 1, pp. 113–136, 1980.

[32] W. Bondareff, "Synaptic atrophy in the senescent hippocampus," *Mech. Ageing Dev.*, vol. 9, no. 1–2, pp. 163–171, 1979.

[33] C. A. Curcio and J. W. Hinds, "Stability of synaptic density and spine volume in dentate gyrus of aged rats," *Neurobiol. Aging*, vol. 4, no. 1, pp. 77–87, 1983.

[34] Y. Geinisman, W. Bondareff, and J. T. Dodge, "Partial deafferentation of neurons in the dentate gyrus of the senescent rat," *Brain Res.*, vol. 134, no. 3, pp. 541–545, 1977.

[35] H. Haug and R. Eggers, "Morphometry of the human cortex cerebri and corpus striatum during aging," *Neurobiol. Aging*, vol. 12, no. 4, pp. 336–338, 1991.

[36] A. Peters, J. H. Morrison, D. L. Rosene, and B. T. Hyman, "Feature article are neurons lost from the primate cerebral cortex during normal aging?," *Cereb. Cortex*, vol. 8, no. 4, pp. 295–300, 1998.

[37] P. R. Rapp and M. Gallagher, "Preserved neuron number in the hippocampus

of aged rats with spatial learning deficits.," *Proc. Natl. Acad. Sci. U. S. A.*, vol. 93, no. 18, pp. 9926–30, 1996.

[38] L. Shi, M. M. Adams, M. C. Linville, I. G. Newton, M. E. Forbes, A. B. Long, D. R. Riddle, and J. K. Brunso-Bechtold, "Caloric restriction eliminates the aging-related decline in NMDA and AMPA receptor subunits in the rat hippocampus and induces homeostasis," *Exp. Neurol.*, vol. 206, no. 1, pp. 70–79, 2007.

[39] P. R. Rapp, P. S. Deroche, Y. Mao, and R. D. Burwell, "Neuron number in the parahippocampal region is preserved in aged rats with spatial learning deficits.," *Cereb. Cortex*, vol. 12, no. 11, pp. 1171–1179, 2002.

[40] D. G. M. Murphy, C. DeCarli, A. R. McIntosh, E. Daly, M. M. J., P. Pietrini, J. Szczepanik, M. B. Schapiro, C. L. Grady, B. Horwitz, and S. I. Rapoport, "Sex Differences in Human Brain Morphometry and Metabolism: An In Vivo Quantitative Magnetic Tomography Study on the Effect of Aging Resonance Imaging and Positron Emission," *Arch Gen Psychiatry*, vol. 53, pp. 585–594, 1996.

[41] C. A. Barnes, "Long-term potentiation and the ageing brain.," *Philos. Trans. R. Soc. Lond. B. Biol. Sci.*, vol. 358, no. 1432, pp. 765–72, 2003.

[42] F. Mora, G. Segovia, and A. del Arco, "Aging, plasticity and environmental enrichment: Structural changes and neurotransmitter dynamics in several areas of the brain," *Brain Res. Rev.*, vol. 55, no. 1, pp. 78–88, 2007.

[43] M. Gooney, E. Messaoudi, F. O. Maher, C. R. Bramham, and M. A. Lynch,

"BDNF-induced LTP in dentate gyrus is impaired with age: Analysis of changes in cell signaling events," *Neurobiol. Aging*, vol. 25, no. 10, pp. 1323–1331, 2004.

[44] P. A. Adlard, V. M. Perreau, and C. W. Cotman, "The exercise-induced expression of BDNF within the hippocampus varies across life-span," *Neurobiol. Aging*, vol. 26, no. 4, pp. 511–520, 2005.

[45] P. S. Van Dam and A. Aleman, "Insulin-like growth factor-I, cognition and brain aging," *Eur. J. Pharmacol.*, vol. 490, no. 1–3, pp. 87–95, 2004.

[46] V. Ibáñez, P. Pietrini, M. L. Furey, G. E. Alexander, P. Millet, A. L. W. Bokde, D. Teichberg, M. B. Schapiro, B. Horwitz, and S. I. Rapoport, "Resting state brain glucose metabolism is not reduced in normotensive healthy men during aging, after correction for brain atrophy," *Brain Res. Bull.*, vol. 63, no. 2, pp. 147–154, 2004.

[47] A. Schättin, K. Baur, J. Stutz, P. Wolf, and E. D. de Bruin, "Effects of Physical Exercise Combined with Nutritional Supplements on Aging Brain Related Structures and Functions: A Systematic Review," *Front. Aging Neurosci.*, vol. 8, no. July, 2016.

[48] E. Scafato, C. Gandin, L. Galluzzo, S. Ghirini, F. Cacciatore, A. Capurso, V. Solfrizzi, F. Panza, A. Cocchi, D. Consoli, G. Enzi, G. B. Frisoni, C. Gandolfo, S. Giampaoli, D. Inzitari, S. Maggi, G. Crepaldi, S. Mariotti, P. Mecocci, M. Motta, R. Negrini, D. Postacchini, F. Rengo, and G. Farchi, "Prevalence of aging-associated cognitive decline in an italian elderly population: results from cross-sectional phase of Italian PRoject on

Epidemiology of Alzheimer's disease (IPREA).," *Aging Clin. Exp. Res.*, vol. 22, no. 5–6, pp. 440–449, 2010.

[49] J. M. Logan, A. L. Sanders, A. Z. Snyder, J. C. Morris, and R. L. Buckner, "Under-recruitment and nonselective recruitment: Dissociable neural mechanisms associated with aging," *Neuron*, vol. 33, no. 5, pp. 827–840, 2002.

[50] J. Persson, C. Y. C. Sylvester, J. K. Nelson, K. M. Welsh, J. Jonides, and P. A. Reuter-Lorenz, "Selection requirements during verb generation: Differential recruitment in older and younger adults," *Neuroimage*, vol. 23, no. 4, pp. 1382–1390, 2004.

[51] L. Verret, S. Trouche, M. Zerwas, and C. Rampon, "Hippocampal neurogenesis during normal and pathological aging," *Psychoneuroendocrinology*, vol. 32, no. SUPPL 1, pp. 26–30, 2007.

[52] S. Corkin, "Lasting Consequences of Bilateral Medial Temporal Lobectomy: Clinical Course and Experimental Findings in H.M.," *Seminars in Neurology*, vol. 4, no. 2. pp. 249–259, 1984.

[53] R. G. Morris, P. Garrud, J. N. Rawlins, and J. O'Keefe, "Place navigation impaired in rats with hippocampal lesions.," *Nature*, vol. 297, no. 5868. pp. 681–683, 1982.

[54] S. N. Burke and C. A. Barnes, "Neural plasticity in the ageing brain," *Nat Rev Neurosci*, vol. 7, no. 1, pp. 30–40, 2006.

[55] J. O. Ojo, P. Rezaie, P. L. Gabbott, and M. G. Stewart, "Impact of age-related

neuroglial cell responses on hippocampal deterioration," *Front. Aging Neurosci.*, vol. 7, no. APR, pp. 1–16, 2015.

[56] J. P. Bourgeois and P. Rakic, "Synaptogenesis in the occipital cortex of macaque monkey devoid of retinal input from early embryonic stages.," *Eur. J. Neurosci.*, vol. 8, no. 5, pp. 942–50, 1996.

[57] X. Liu, C. Erikson, and A. Brun, "Cortical Synaptic Changes and Gliosis in Normal Aging, Alzheimer's Disease and Frontal Lobe Degeneration," *Dementia*, vol. 1, no. 7, pp. 128–134, 1996.

[58] P. W. Landfield and G. Lynch, "Impaired monosynaptic potentiation in in vitro hippocampal slices from aged, memory-deficient rats.," *J. Gerontol.*, vol. 32, no. 5, pp. 523–533, 1977.

[59] G. Segovia, A. Porras, A. Del Arco, and F. Mora, "Glutamatergic neurotransmission in aging: A critical perspective," *Mech. Ageing Dev.*, vol. 122, no. 1, pp. 1–29, 2001.

[60] 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," *J. Neurochem.*, vol. 113, pp. 1577–1588, 2010.

[61] 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 J. K. Brunso-Bechtold, "Caloric restriction and age affect synaptic proteins in hippocampal CA3 and spatial learning ability," *Exp. Neurol.*, vol. 211, no. 1, pp. 141–149,

2008.

[62] K. Eckles-Smith, D. Clayton, P. Bickford, and M. D. Browning, “Caloric restriction prevents age-related deficits in LTP and in NMDA receptor expression,” *Mol. Brain Res.*, vol. 78, no. 1–2, pp. 154–162, 2000.

[63] C. D. Lynch, P. T. Cooney, S. A. Bennett, P. L. Thornton, A. S. Khan, R. L. Ingram, and W. E. Sonntag, “Effects of moderate caloric restriction on cortical microvascular density and local cerebral blood flow in aged rats,” *Neurobiol. Aging*, vol. 20, no. 2, pp. 191–200, 1999.

[64] B. Martin, M. Pearson, R. Brenneman, E. Golden, A. Keselman, T. Iyun, O. D. Carlson, J. M. Egan, K. G. Becker, W. Wood, V. Prabhu, R. de Cabo, S. Maudsley, and M. P. Mattson, “Conserved and differential effects of dietary energy intake on the hippocampal transcriptomes of females and males,” *PLoS One*, vol. 3, no. 6, 2008.

[65] K. B. Aly, J. L. Pipkin, W. G. Hinson, R. J. Feuers, P. H. Duffy, L. Lyn-Cook, and R. W. Hart, “Chronic caloric restriction induces stress proteins in the hypothalamus of rats,” *Mech. Ageing Dev.*, vol. 76, no. 1, pp. 11–23, 1994.

[66] M. T. Tacconi, L. Lligona, M. Salmona, N. Pitsikas, and S. Algeri, “Aging and food restriction: effect on lipids of cerebral cortex,” *Neurobiol Aging*, vol. 12, no. 1, pp. 55–59, 1991.

[67] B. P. Yu, E. J. Masoro, and A. McMahan, “Nutritional influences on aging of Fischer 344 rats: II. Pathology.,” *J. Gerontol.*, vol. 40, no. 6, pp. 671–688, 1985.

[68] G. S. Roth, D. K. Ingram, and M. A. Lane, “Slowing ageing by caloric restriction,” *Nature medicine*, vol. 1, no. 6, pp. 546–551, 1995.

[69] C. W. Levenson and N. J. Rich, “Eat less, live longer? New insights into the role of caloric restriction in the brain.,” *Nutr. Rev.*, vol. 65, no. 9, pp. 412–415, 2007.

[70] 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,” *Neurobiol. Aging*, vol. 25, no. 3, pp. 333–340, 2004.

[71] J. Lee, K. B. Seroogy, and M. P. Mattson, “Dietary restriction enhances neurotrophin expression and neurogenesis in the hippocampus of adult mice,” *J. Neurochem.*, vol. 80, no. 3, pp. 539–547, 2002.

[72] A. Kuhla, S. Lange, C. Holzmann, F. Maass, J. Petersen, B. Vollmar, and A. Wree, “Lifelong Caloric Restriction Increases Working Memory in Mice,” *PLoS One*, vol. 8, no. 7, 2013.

[73] M. Okada, H. Nakanishi, T. Amamoto, R. Urae, S. Ando, K. Yazawa, and M. Fujiwara, “How does prolonged caloric restriction ameliorate age-related impairment of long-term potentiation in the hippocampus?,” *Mol. Brain Res.*, vol. 111, no. 1–2, pp. 175–181, 2003.

[74] R. J. McCarter and J. R. McGee, “Transient reduction of metabolic rate by food restriction,” *Am. J. Physiol. - Endocrinol. Metab.*, vol. 257, no. 2, p. E175 LP-E179, Aug. 1989.

[75] H. F. Poon, H. M. Shepherd, T. T. Reed, V. Calabrese, A. M. G. Stella, G.

Pennisi, J. Cai, W. M. Pierce, J. B. Klein, and D. A. Butterfield, “Proteomics analysis provides insight into caloric restriction mediated oxidation and expression of brain proteins associated with age-related impaired cellular processes: Mitochondrial dysfunction, glutamate dysregulation and impaired protein synthesis,” *Neurobiol. Aging*, vol. 27, no. 7, pp. 1020–1034, 2006.

[76] S. Dogan, A. C. Johannsen, J. P. Grande, and M. P. Cleary, “Effects of intermittent and chronic calorie restriction on mammalian target of rapamycin (mTOR) and IGF-I signaling pathways in mammary fat pad tissues and mammary tumors.,” *Nutr. Cancer*, vol. 63, no. 3, pp. 389–401, 2011.

[77] P. Wu, C. Jiang, Q. Shen, and Y. Hu, “Systematic gene expression profile of hypothalamus in calorie-restricted mice implicates the involvement of mTOR signaling in neuroprotective activity,” *Mech. Ageing Dev.*, vol. 130, no. 9, pp. 602–610, 2009.

[78] N. L. Nadon, R. Strong, R. A. Miller, J. Nelson, M. Javors, Z. D. Sharp, J. M. Peralba, and D. E. Harrison, “Design of aging intervention studies: The NIA interventions testing program,” *Age (Omaha).*, vol. 30, no. 4, pp. 187–199, 2008.

[79] S. Calvert, R. Tacutu, S. Sharifi, R. Teixeira, P. Ghosh, and J. P. de Magalhães, “A network pharmacology approach reveals new candidate caloric restriction mimetics in C. elegans,” *Aging Cell*, vol. 15, no. 2, pp. 256–266, 2016.

[80] K. Pankaj and Z. Brian, “TOR Ppathway: Linking Nutrient senseing to life span,” *Sci. Aging Knowl. Enviorment*, vol. 2004, no. 36, pp. 1–7, 2004.

[81] D. E. Martin and M. N. Hall, “The expanding TOR signaling network,” *Curr. Opin. Cell Biol.*, vol. 17, no. 2, pp. 158–166, 2005.

[82] J. Halloran, S. A. Hussong, R. Burbank, N. Podlutskaya, K. E. Fischer, L. B. Sloane, S. N. Austad, R. Strong, A. Richardson, M. J. Hart, and V. Galvan, “Chronic inhibition of mammalian target of rapamycin by rapamycin modulates cognitive and non-cognitive components of behavior throughout lifespan in mice,” *Neuroscience*, vol. 223, pp. 102–113, 2012.

[83] M. Kaeberlein and B. K. Kennedy, “A midlife longevity drug?,” *Nature*, vol. 460, no. August, pp. 331–332, 2009.

[84] K. Pankaj, Z. Brian, T. Harper, D. Koslover, V. Sapin, and S. Benzer, “Regulation of Lifespan in Drosophila by Modulation of Genes in the TOR Signaling Pathway,” *Div. Biol.*, vol. 14, no. 2, pp. 189-885–890, 2004.

[85] R. W. Powers, M. Kaeberlein, S. D. Caldwell, B. K. Kennedy, and S. Fields, “Extension of chronological life span in yeast by decreased TOR pathway signaling,” *Genes Dev.*, vol. 20, no. 2, pp. 174–184, 2006.

[86] M. Kaeberlein, “Response to Nutrients Regulation of Yeast Replicative Life Span by TOR and Sch9 in Response to Nutrients,” vol. 1193, no. 310, 2005.

[87] D. E. Harrison, R. Strong, Z. D. Sharp, J. F. Nelson, C. M. Astle, K. Flurkey, N. L. Nadon, J. E. Wilkinson, K. Frenkel, C. S. Carter, M. Pahor, M. a Javors, E. Fernandez, and R. a Miller, “Rapamycin fed late in life extends lifespan in genetically heterogeneous mice.,” *Nature*, vol. 460, no. 7253, pp. 392–395, 2009.

[88] R. A. Miller, D. E. Harrison, C. M. Astle, J. A. Baur, A. R. Boyd, R. De Cabo, E. Fernandez, K. Flurkey, M. A. Javors, J. F. Nelson, C. J. Orihuela, S. Pletcher, Z. D. Sharp, D. Sinclair, J. W. Starnes, J. E. Wilkinson, N. L. Nadon, and R. Strong, "Rapamycin, but not resveratrol or simvastatin, extends life span of genetically heterogeneous mice," *Journals Gerontol. - Ser. A Biol. Sci. Med. Sci.*, vol. 66 A, no. 2, pp. 191–201, 2011.

[89] J. Halloran, S. Hussong, R. Burbank, N. Podlutskaya, L. Sloane, S. N. Austad, R. Strong, A. Richardson, M. Hart, and V. Galvan, "Chronic inhibition of mTOR by rapamycin modulates cognitive and non-cognitive components of behavior throughout lifespan in mice," *Neuroscience*, vol. 223, pp. 102–113, 2012.

[90] C. Chen, Y. Liu, Y. Liu, and P. Zheng, "mTOR regulation and therapeutic rejuvenation of aging hematopoietic stem cells.," *Sci. Signal.*, vol. 2, no. 98, p. ra75, 2009.

[91] J. M. Flynn, M. N. O'Leary, C. A. Zambataro, E. C. Academia, M. P. Presley, B. J. Garrett, A. Zykovich, S. D. Mooney, R. Strong, C. J. Rosen, P. Kapahi, M. D. Nelson, B. K. Kennedy, and S. Melov, "Late-life rapamycin treatment reverses age-related heart dysfunction," *Aging Cell*, vol. 12, no. 5, pp. 851–862, 2013.

[92] T. Komatsu, T. Chiba, H. Yamaza, K. Yamashita, A. Shimada, Y. Hoshiyama, T. Henmi, H. Ohtani, Y. Higami, R. de Cabo, D. K. Ingram, and I. Shimokawa, "Manipulation of caloric content but not diet composition, attenuates the deficit in learning and memory of senescence-accelerated mouse

strain P8," *Exp. Gerontol.*, vol. 43, no. 4, pp. 339–346, 2008.

[93] B. Martin, M. Pearson, L. Kebejian, E. Golden, A. Keselman, M. Bender, O. Carlson, J. Egan, B. Ladenheim, J. L. Cadet, K. G. Becker, W. Wood, K. Duffy, P. Vinayakumar, S. Maudsley, and M. P. Mattson, "Sex-dependent metabolic, neuroendocrine, and cognitive responses to dietary energy restriction and excess," *Endocrinology*, vol. 148, no. 9, pp. 4318–4333, 2007.

[94] M. P. Mattson, W. Duan, S. L. Chan, A. Cheng, N. Haughey, D. S. Gary, Z. Guo, J. Lee, and K. Furukawa, "Neuroprotective and neurorestorative signal transduction mechanisms in brain aging: Modification by genes, diet and behavior," *Neurobiol. Aging*, vol. 23, no. 5, pp. 695–705, 2002.

[95] E. T. Keller and J. M. Murtha, "The use of mature zebrafish (Danio rerio) as a model for human aging and disease.," *Comp. Biochem. Physiol. C. Toxicol. Pharmacol.*, vol. 138, no. 3, pp. 335–41, 2004.

[96] G. S. Gerhard, "Comparative aspects of zebrafish (Danio rerio) as a model for aging research," *Exp. Gerontol.*, vol. 38, no. 11–12, pp. 1333–1341, 2003.

[97] P. Blader and U. Strähle, "Zebrafish developmental genetics and central nervous system development.," *Hum. Mol. Genet.*, vol. 9, no. 6, pp. 945–951, 2000.

[98] R. Gerlai, "Zebra fish: An uncharted behavior genetic model," *Behav. Genet.*, vol. 33, no. 5, pp. 461–468, 2003.

[99] S. Kishi, J. Uchiyama, A. M. Baughman, T. Goto, M. C. Lin, and S. B. Tsai, "The zebrafish as a vertebrate model of functional aging and very gradual

senescence," *Exp. Gerontol.*, vol. 38, no. 7, pp. 777–786, 2003.

[100] G. S. Gerhard and K. C. Cheng, "A call to fins! Zebrafish as a gerontological model.," *Aging Cell*, vol. 1, no. 2, pp. 104–111, 2002.

[101] E. Jazin and L. Cahill, "Sex differences in molecular neuroscience: from fruit flies to humans.," *Nat. Rev. Neurosci.*, vol. 11, no. 1, pp. 9–17, 2010.

[102] S. Spring, J. P. Lerch, and R. M. Henkelman, "Sexual dimorphism revealed in the structure of the mouse brain using three-dimensional magnetic resonance imaging," *Neuroimage*, vol. 35, no. 4, pp. 1424–1433, 2007.

[103] N. Jahanshad and P. M. Thompson, "Multimodal neuroimaging of male and female brain structure in health and disease across the life span," *J. Neurosci. Res.*, vol. 95, no. 1–2, pp. 371–379, 2017.

[104] E. M. Santos, P. Kille, V. L. Workman, G. C. Paull, and C. R. Tyler, "Sexually dimorphic gene expression in the brains of mature zebrafish," *Comp. Biochem. Physiol. - A Mol. Integr. Physiol.*, vol. 149, no. 3, pp. 314–324, 2008.

[105] K. Ampatzis, P. Makantasi, and C. R. Dermon, "Cell proliferation pattern in adult zebrafish forebrain is sexually dimorphic," *Neuroscience*, vol. 226, pp. 367–381, 2012.

[106] D. W. Bayless and N. M. Shah, "Genetic dissection of neural circuits underlying sexually dimorphic social behaviours.," *Philos. Trans. R. Soc. Lond. B. Biol. Sci.*, vol. 371, no. 1688, p. 20150109-, 2016.

[107] N. C. Berchtold, D. H. Cribbs, P. D. Coleman, J. Rogers, E. Head, R. Kim, T. Beach, C. Miller, J. Troncoso, J. Q. Trojanowski, H. R. Zielke, and C. W. Cotman, “Gene expression changes in the course of normal brain aging are sexually dimorphic,” 2008.

[108] V. A. Kovalev, F. Kruggel, and D. Y. Von Cramon, “Gender and age effects in structural brain asymmetry as measured by MRI texture analysis,” *Neuroimage*, vol. 19, no. 3, pp. 895–905, 2003.

[109] N. Raz, F. Gunning-Dixon, D. Head, K. M. Rodrigue, A. Williamson, and J. D. Acker, “Aging, sexual dimorphism, and hemispheric asymmetry of the cerebral cortex: Replicability of regional differences in volume,” *Neurobiol. Aging*, vol. 25, no. 3, pp. 377–396, 2004.

[110] A. Arslan-Ergul, A. T. Ozdemir, and M. M. Adams, “Aging, neurogenesis, and caloric restriction in different model organisms.,” *Aging Dis.*, vol. 4, no. 4, pp. 221–32, 2013.

[111] C. M. McCay, M. F. Crowel, and L. . Maynard, “The Effect of Retarded Growth upon the Length of Lifespan and upon the Ultimate Body Size,” *J. Nutr.*, 1935.

[112] D. K. Ingram, R. Weindruch, E. L. Spangler, J. R. Freeman, and R. L. Walford, “Dietary restriction benefits learning and motor performance of aged mice,” *J Gerontol*, vol. 42, no. 1, pp. 78–81, 1987.

[113] B. V Phillips-Farfan, C. Rubio Osornio Mdel, V. Custodio Ramirez, C. Paz Tres, and K. G. Carvajal Aguilera, “Caloric restriction protects against

electrical kindling of the amygdala by inhibiting the mTOR signaling pathway," *Front Cell Neurosci*, vol. 9, no. March, p. 90, 2015.

[114] S. Majumder, A. Caccamo, D. X. Medina, A. D. Benavides, M. A. Javors, E. Kraig, R. Strong, A. Richardson, and S. Oddo, "Lifelong rapamycin administration ameliorates age-dependent cognitive deficits by reducing IL-1B and enhancing NMDA signaling," *Aging Cell*, vol. 11, no. 2, pp. 326–335, 2012.

[115] M. B. Kennedy, "The postsynaptic density at glutamatergic synapses.," *Trends Neurosci.*, vol. 20, no. 6, pp. 264–268, 1997.

[116] M. Sheng, "Molecular organization of the postsynaptic specialization.," *Proc. Natl. Acad. Sci. U. S. A.*, vol. 98, no. 13, pp. 7058–7061, 2001.

[117] M. Migaud, P. Charlesworth, M. Dempster, L. C. Webster, a M. Watabe, M. Makhinson, Y. He, M. F. Ramsay, R. G. Morris, J. H. Morrison, T. J. O'Dell, and S. G. Grant, "Enhanced long-term potentiation and impaired learning in mice with mutant postsynaptic density-95 protein.," *Nature*, vol. 396, no. 6710, pp. 433–439, 1998.

[118] G. Choii and J. Ko, "Gephyrin: a central GABAergic synapse organizer.," *Exp. Mol. Med.*, vol. 47, no. 4, p. e158, 2015.

[119] W. Yu and A. L. De Blas, "Gephyrin expression and clustering affects the size of glutamatergic synaptic contacts," *J. Neurochem.*, vol. 104, no. 3, pp. 830–845, 2008.

[120] S. K. Tyagarajan and J.-M. Fritschy, "Gephyrin: a master regulator of

neuronal function?," *Nat. Rev. Neurosci.*, vol. 15, no. 3, pp. 141–56, 2014.

[121] O. H. Shin, "Exocytosis and synaptic vesicle function," *Compr. Physiol.*, vol. 4, no. 1, pp. 149–175, 2014.

[122] J. Alder, B. Lu, F. Valtorta, P. Greengard, and M. Poo, "Calcium-Dependent Transmitter Secretion Resonctituted in Xenopus Oocytes: Requirement for Synaptophysin," vol. 257, 1992.

[123] T. D. Smith, M. M. Adams, M. Gallagher, J. H. Morrison, and P. R. Rapp, "Circuit-specific alterations in hippocampal synaptophysin immunoreactivity predict spatial learning impairment in aged rats.," *J. Neurosci.*, vol. 20, no. 17, pp. 6587–93, 2000.

[124] 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," *Neurobiol. Aging*, vol. 19, no. 6, pp. 599–606, 1998.

[125] R. M. Shansky and C. S. Woolley, "Considering Sex as a Biological Variable Will Be Valuable for Neuroscience Research," *J. Neurosci.*, vol. 36, no. 47, pp. 11817–11822, 2016.

[126] K. Ampatzis and C. R. Dermon, "Sex differences in adult cell proliferation within the zebrafish (Danio rerio) cerebellum," *Eur. J. Neurosci.*, vol. 25, no. 4, pp. 1030–1040, 2007.

[127] M. D. Madeira and A. R. Lieberman, "Sexual dimorphism in the mammalian limbic system," *Prog. Neurobiol.*, vol. 45, no. 4, 1995.

[128] T. J. Shors, C. Chua, and J. Falduto, “Sex differences and opposite effects of stress on dendritic spine density in the male versus female hippocampus.,” *J. Neurosci.*, vol. 21, no. 16, pp. 6292–6297, 2001.

[129] A. Arslan-Ergul, A. T. Ozdemir, and M. M. Adams, “Experimental Set-up for Caloric Restriction and Its Possible Mimetics in Young and Old Female and Male Zebrafish (Danio rerio),” *Unpubl. Manuscr.*

[130] A. Arslan-Ergul, B. Erbaba, E. T. Karoglu, D. O. Halim, and M. M. Adams, “Short-term dietary restriction in old zebrafish changes cell senescence mechanisms,” *Neuroscience*, vol. 334, pp. 64–75, 2016.

[131] R. B. Harris, J. Zhou, B. D. Youngblood, I. I. Rybkin, G. N. Smagin, and D. H. Ryan, “Effect of repeated stress on body weight and body composition of rats fed low- and high-fat diets.,” *Am. J. Physiol.*, vol. 275, no. 6 Pt 2, pp. R1928-38, 1998.

[132] C. M. Novak, X. Jiang, C. Wang, J. A. Teske, C. M. Kotz, and J. A. Levine, “Caloric restriction and physical activity in zebrafish (Danio rerio),” *Neurosci. Lett.*, vol. 383, no. 1–2, pp. 99–104, 2005.

[133] S. Kishi, B. E. Slack, J. Uchiyama, and I. V. Zhdanova, “Zebrafish as a genetic model in biological and behavioral gerontology: Where development meets aging in vertebrates - A mini-review,” *Gerontology*, vol. 55, no. 4, pp. 430–441, 2009.

[134] M. Pavlidis, N. Digka, A. Theodoridi, A. Campo, K. Barsakis, G. Skouradakis, A. Samaras, and A. Tsalafouta, “Husbandry of Zebrafish, Danio

Rerio , and the Cortisol Stress Response," vol. 0, no. 0, 2013.

[135] C. Garza-lombó and M. E. Gonsebatt, "Mammalian Target of Rapamycin : Its Role in Early Neural Development and in Adult and Aged Brain Function," vol. 10, no. June, pp. 1–14, 2016.

[136] W. C. Fok, Y. Zhang, A. B. Salmon, A. Bhattacharya, R. Gunda, D. Jones, W. Ward, K. Fisher, A. Richardson, and V. I. Pérez, "Short-term treatment with rapamycin and dietary restriction have overlapping and distinctive effects in young mice," *Journals Gerontol. - Ser. A Biol. Sci. Med. Sci.*, vol. 68, no. 2, pp. 108–116, 2013.

[137] E. T. Karoglu, "Caloric Restriction and Its Possible Mimetics in Young-Old and Male-Female Zebrafish," *Sr. Thesis*, 2004.

[138] C. Selman, J. M. A. Tullet, D. Wieser, E. Irvine, S. J. Lingard, A. I. Choudhury, M. Claret, and H. Al-qassab, "Regulates Mammalian Life Span," *Science (80-.).*, vol. 461, no. October, pp. 140–144, 2009.

[139] R.-K. Cheng, S. J. Jesuthasan, and T. B. Penney, "Zebrafish forebrain and temporal conditioning.," *Philos. Trans. R. Soc. Lond. B. Biol. Sci.*, vol. 369, no. 1637, p. 20120462, 2014.

[140] M. I. Goldsmith, M. K. Iovine, T. O'Reilly-Pol, and S. L. Johnson, "A developmental transition in growth control during zebrafish caudal fin development," *Dev. Biol.*, vol. 296, no. 2, pp. 450–457, 2006.

[141] G. A. Yudowski, O. Olsen, H. Adesnik, K. W. Marek, and D. S. Bredt, "Acute Inactivation of PSD-95 Destabilizes AMPA Receptors at Hippocampal

Synapses," *PLoS One*, vol. 8, no. 1, pp. 1–9, 2013.

[142] X. Chen, J. M. Levy, A. Hou, C. Winters, R. Azzam, A. A. Sousa, R. D. Leapman, R. A. Nicoll, and T. S. Reese, "PSD-95 family MAGUKs are essential for anchoring AMPA and NMDA receptor complexes at the postsynaptic density.," *Proc. Natl. Acad. Sci. U. S. A.*, vol. 112, no. 50, pp. E6983-92, 2015.

[143] S. Won, S. Incontro, R. A. Nicoll, and K. W. Roche, "PSD-95 stabilizes NMDA receptors by inducing the degradation of STEP 61," *Proc. Natl. Acad. Sci.*, p. 201609702, 2016.

[144] J. Luo, L. Zhang, N. Ning, H. Jiang, and S. Y. Yu, "Neotrofin reverses the effects of chronic unpredictable mild stress on behavior via regulating BDNF, PSD-95 and synaptophysin expression in rat," *Behav. Brain Res.*, vol. 253, pp. 48–53, 2013.

[145] E. Karoglu, D. Halim, B. Erkaya, F. Altaytas, A. Arslan-Ergul, O. Konu, and M. M. Adams, "Aging Alters The Molecular Dynamics Of Synapses In A Sexually Dimorphic Pattern In Zebrafish (Danio rerio)," *Unpubl. Manuscr.*

[146] C. O. F. Machado, K. Griesi-Oliveira, C. Rosenberg, F. Kok, S. Martins, M. Rita Passos-Bueno, and A. L. Sertie, "Collybistin binds and inhibits mTORC1 signaling: a potential novel mechanism contributing to intellectual disability and autism.," *Eur. J. Hum. Genet.*, vol. 24, no. 1, pp. 59–65, 2015.

[147] M. C. Mhatre and M. K. Ticku, "Caloric restriction retards the aging associated changes in g -aminobutyric acid A receptor gene expression in rat

cerebellum," pp. 270–275, 1998.

[148] 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," vol. 26, no. May, pp. 3583–3596, 2007.

[149] A. Djordjevic, M. Perovic, V. Tesic, N. Tanic, L. Rakic, S. Ruzdijic, and S. Kanazir, "Long-term dietary restriction modulated the level of presynaptic proteins in the cortex and hippocampus of the aging rat," *Neurochem. Int.*, vol. 56, no. 2, pp. 250–255, 2010.

[150] H. K. Müller, G. Wegener, M. Popoli, and B. Elfving, "Differential expression of synaptic proteins after chronic restraint stress in rat prefrontal cortex and hippocampus," *Brain Res.*, vol. 1385, pp. 26–37, 2011.

[151] J. Thome, B. Pesold, M. Baader, M. Hu, J. C. Gewirtz, R. S. Duman, and F. A. Henn, "Stress differentially regulates synaptophysin and synaptotagmin expression in hippocampus," *Biol. Psychiatry*, vol. 50, no. 10, pp. 809–812, 2001.

[152] R. E. Ferguson, H. P. Carroll, A. Harris, E. R. Maher, P. J. Selby, and R. E. Banks, "Housekeeping proteins : A preliminary study illustrating some limitations as useful references in protein expression studies," pp. 566–571, 2005.

[153] C. Westenbroek, J. A. Den Boer, M. Veenhuis, and G. J. Ter Horst, "Chronic stress and social housing differentially affect neurogenesis in male and female rats," *Brain Res. Bull.*, vol. 64, no. 4, pp. 303–308, 2004.

[154] I. F. MacDonald, B. Kempster, L. Zanette, and S. A. MacDougall-Shackleton, “Early nutritional stress impairs development of a song-control brain region in both male and female juvenile song sparrows (Melospiza melodia) at the onset of song learning.,” *Proc. Biol. Sci.*, vol. 273, no. 1600, pp. 2559–2564, 2006.

[155] B. B. Turner and D. a Weaver, “Sexual dimorphism of glucocorticoid binding in rat brain.,” *Brain Res.*, vol. 343, no. 1, pp. 16–23, 1985.

[156] R. H. Scannevin and R. L. Huganir, “Postsynaptic organization and regulation of excitatory synapses.,” *Nat. Rev. Neurosci.*, vol. 1, no. 2, pp. 133–141, 2000.

[157] E. Karakas, M. C. Regan, and H. Furukawa, “Emerging structural insights into the function of ionotropic glutamate receptors,” *Trends Biochem. Sci.*, vol. 40, no. 6, pp. 328–337, 2015.

[158] S. Nakanishi, “Molecular diversity of glutamate receptors and implications for brain function.,” *Science*, vol. 258, no. 5082, pp. 597–603, 1992.

[159] J. M. Henley and K. A. Wilkinson, “Synaptic AMPA receptor composition in development, plasticity and disease.,” *Nat. Rev. Neurosci.*, vol. advance on, 2016.

[160] J. T. R. Isaac, M. Ashby, and C. J. McBain, “The Role of the GluR2 Subunit in AMPA Receptor Function and Synaptic Plasticity,” *Neuron*, vol. 54, no. 6, pp. 859–871, 2007.

[161] K. Archibald, M. J. Perry, E. Molnár, J. M. Henley, E. Molnar, and J. M. Henley, “Surface expression and metabolic half-life of AMPA receptors in cultured rat cerebellar granule cells.,” *Neuropharmacology*, vol. 37, no. 10–

11, pp. 1345–53, 1998.

[162] a L. Mammen, R. L. Huganir, and R. J. O'Brien, "Redistribution and stabilization of cell surface glutamate receptors during synapse formation.," *J. Neurosci.*, vol. 17, no. 19, pp. 7351–8, 1997.

[163] W. Wisden and P. H. Seeburg, "Mammalian ionotropic glutamate receptors," *Curr Opin Neurobiol*, vol. 3, no. 3, pp. 291–298, 1993.

[164] H. Monyer, P. H. Seeburg, and W. Wisden, "Glutamate-operated channels: Developmentally early and mature forms arise by alternative splicing," *Neuron*, vol. 6, no. 5, pp. 799–810, 1991.

[165] P. Paoletti, C. Bellone, and Q. Zhou, "NMDA receptor subunit diversity: impact on receptor properties, synaptic plasticity and disease.," *Nat. Rev. Neurosci.*, vol. 14, no. 6, pp. 383–400, 2013.

[166] C. Akazawa, R. Shigemoto, Y. Bessho, S. Nakanishi, and N. Mizuno, "Differential expression of five N-methyl-D-aspartate receptor subunit mRNAs in the cerebellum of developing and adult rats.," *J. Comp. Neurol.*, vol. 347, no. 1, pp. 150–60, 1994.

[167] H. Monyer, N. Burnashev, D. J. Laurie, B. Sakmann, and P. H. Seeburg, "Developmental and regional expression in the rat brain and functional properties of four NMDA receptors," *Neuron*, vol. 12, no. 3, pp. 529–540, 1994.

[168] M. A. Henson, A. C. Roberts, I. P??rez-Ota??o, and B. D. Philpot, "Influence of the NR3A subunit on NMDA receptor functions," *Prog. Neurobiol.*, vol.

91, no. 1, pp. 23–37, 2010.

[169] S. Pachernegg, N. Strutz-Seebohm, and M. Hollmann, “GluN3 subunit-containing NMDA receptors: Not just one-trick ponies,” *Trends Neurosci.*, vol. 35, no. 4, pp. 240–249, 2012.

[170] A. Arslan-ergul and M. M. Adams, “Gene expression changes in aging Zebrafish (Danio rerio) brains are sexually dimorphic,” 2014.

[171] K. Ampatzis and C. R. Dermon, “Sexual dimorphisms in swimming behavior, cerebral metabolic activity and adrenoceptors in adult zebrafish (Danio rerio),” *Behav. Brain Res.*, vol. 312, pp. 385–393, 2016.

[172] A. Arslan-Ergul and M. M. Adams, “Gene expression changes in aging Zebrafish (Danio rerio) brains are sexually dimorphic,” *BMC Neurosci.*, vol. 15, no. 1, p. 29, 2014.

[173] C. M. Small, G. E. Carney, Q. Mo, M. Vannucci, and A. G. Jones, “A microarray analysis of sex- and gonad-biased gene expression in the zebrafish: evidence for masculinization of the transcriptome,” *BMC Genomics*, vol. 10, 2009.

[174] A. Chatterjee, M. Lagisz, E. J. Rodger, L. Zhen, P. A. Stockwell, E. J. Duncan, J. A. Horsfield, J. Jeyakani, S. Mathavan, Y. Ozaki, and S. Nakagawa, “Sex differences in DNA methylation and expression in zebrafish brain: A test of an extended ‘male sex drive’ hypothesis,” *Gene*, vol. 590, pp. 307–316, 2016.

[175] D. Vizziano-Cantonnet, I. Anglade, E. Pellegrini, M. M. Gueguen, A. Fostier,

Y. Guiguen, and O. Kah, “Sexual dimorphism in the brain aromatase expression and activity, and in the central expression of other steroidogenic enzymes during the period of sex differentiation in monosex rainbow trout populations,” *Gen. Comp. Endocrinol.*, vol. 170, no. 2, pp. 346–355, 2011.

[176] J. L. Albasanz, S. Santana, F. Guzman-Sanchez, D. León, J. S. Burgos, and M. Martín, “2-Methyl-6-(phenylethynyl)pyridine Hydrochloride Modulates Metabotropic Glutamate 5 Receptors Endogenously Expressed in Zebrafish Brain,” *ACS Chem. Neurosci.*, p. acschemneuro.6b00213, 2016.

[177] G. Whitehead, P. Regan, D. J. Whitcomb, and K. Cho, “Ca2+-permeable AMPA receptor: A new perspective on amyloid-beta mediated pathophysiology of Alzheimer’s disease,” *Neuropharmacology*, vol. 112, pp. 221–227, 2017.

[178] S. A. Monaco, Y. Gulchina, and W.-J. Gao, “Neuroscience and Biobehavioral Reviews NR2B subunit in the prefrontal cortex : A double-edged sword for working memory function and psychiatric disorders,” *Neurosci. Biobehav. Rev.*, vol. 56, pp. 127–138, 2015.

[179] Y. Meng, Y. Zhang, and Z. Jia, “Synaptic Transmission and Plasticity in the Absence of AMPA Glutamate Receptor GluR2 and GluR3,” vol. 39, pp. 163–176, 2003.

[180] J. Sajjad, V. D. Felice, A. V Golubeva, J. F. Cryan, and S. M. O. Mahony, “SEX-DEPENDENT ACTIVITY OF THE SPINAL EXCITATORY AMINO ACID TRANSPORTER : ROLE OF ESTROUS CYCLE,” *Neuroscience*, vol. 333, pp. 311–319, 2016.

[181] K. Ampatzis, P. Makantasi, and C. R. Dermon, “CELL PROLIFERATION PATTERN IN ADULT ZEBRAFISH FOREBRAIN IS SEXUALLY DIMORPHIC,” *Neuroscience*, vol. 226, pp. 367–381, 2012.

[182] K. Ampatzis and C. R. Dermon, “Sex differences in adult cell proliferation within the zebrafish (Danio rerio) cerebellum,” vol. 25, no. December 2006, pp. 1030–1040, 2007.

[183] F. P. Menezes and L. W. Kist, “Evaluation of Age-Dependent Response to NMDA Receptor Antagonism in Zebrafish,” vol. 12, no. 2, pp. 137–143, 2015.

[184] A. Arslan-Ergul, A. T. Ozdemir, and M. M. Adams, “Aging, neurogenesis, and caloric restriction in different model organisms,” *Aging Dis*, vol. 4, 2013.

[185] J. Bové, M. Martínez-Vicente, and M. Vila, “Fighting neurodegeneration with rapamycin: mechanistic insights.,” *Nat. Rev. Neurosci.*, vol. 12, no. 8, pp. 437–52, 2011.

[186] G. France, D. Fernandez-Fernandez, E. S. Burnell, M. W. Irvine, D. T. Monaghan, D. E. Jane, Z. A. Bortolotto, G. L. Collingridge, and A. Volianskis, “Multiple roles of GluN2B-containing NMDA receptors in synaptic plasticity in juvenile hippocampus,” *Neuropharmacology*, vol. 112, pp. 76–83, 2016.

[187] V. Anggono and R. L. Huganir, “Regulation of AMPA receptor trafficking and synaptic plasticity,” *Curr. Opin. Neurobiol.*, vol. 22, no. 3, pp. 461–469, 2012.

[188] R. Macrez, P. K. Stys, D. Vivien, S. A. Lipton, and F. Docagne, “Mechanisms of glutamate toxicity in multiple sclerosis: biomarker and therapeutic opportunities,” *Lancet Neurol.*, vol. 15, no. 10, pp. 1089–1102, 2016.