GENETIC AND ENVIRONMENTAL INTERVENTIONS ALTERING THE COURSE OF BRAIN AGING: EVIDENCE FROM THE ZEBRAFISH (*DANIO RERIO*) MODEL

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By

Elif Tuğce Karoğlu Eravşar

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By Elif Tuğce Karoğlu Eravşar

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

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ABSTRACT

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Elif Tuğce Karoğlu Eravşar

PhD in Neuroscience

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Age-related cognitive decline occurs during normal aging, although there is no prominent neural loss in the brain. Subtle molecular alterations in synaptic and cellular dynamics are likely underlying these cognitive alterations. One challenge is the widely heterogeneous profile regarding age-related behavioral changes and neurobiological underpinnings. Therefore, it is crucial to characterize how individual factors can contribute to successful or unsuccessful aging and whether these factors can induce shared patterns of alterations in the cellular and synaptic dynamics. Three different intervention approaches were utilized in the current study. The first intervention was a genetic manipulation in the cholinergic system component acetylcholinesterase (AChE), which results in reduced levels of this enzyme in the *ache*$^{sb55/+}$ mutants. Previous studies have characterized this model as a delayed aging model because of its preserved cognitive abilities at an older age. The current study

was the first study analyzing the neurobiological changes in this mutant model within the context of aging. It was shown that reduced brain AChE activity levels persist in different age groups, including old age in the mutant animals. This reduction was accompanied by subtle decreases in the other elements of the cholinergic system, including acetylcholine and nicotinic acetylcholine receptor subunit alpha-7. Genotype significantly altered key glutamatergic receptor subunits such as *N*-methyl *D*-aspartate-type receptor subunit 2B (NR2B) and glutamate receptor subunits 2 and 3 (GluR2/3), with these markers significantly reduced in the *ache*$^{sb55/+}$ mutants and likely maintaining homeostatic synaptic scaling. At old age, a significant age-related elevation was observed in the synaptophysin levels (SYP) of the old *ache*$^{sb55/+}$ mutants, and this mutation prevented an age-related decline in the gephyrin (GEP) levels which was evident in the wild-type controls. This mutation also altered the cellular dynamics; an immature neuronal marker, embryonic lethal abnormal-vision (ELAV Drosophila) like-3 (HuC) was significantly upregulated in the *ache*$^{sb55/+}$ mutants at all ages. In contrast, the levels of inflammation-related markers, glial fibrillary acidic protein (GFAP) and reactive oxygen species (ROS), were downregulated subtly in the mutants. It can be concluded that reduced levels of brain AChE can be associated with altered excitatory homeostasis and preserved levels of GEP and SYP through aging. At the same time, the neuronal marker was upregulated, and inflammation-related markers were downregulated. The second intervention was applying short-term environmental enrichment using the sensory cues to young and old zebrafish to induce successful aging. It was shown that environmental enrichment increases the brain weight in old zebrafish, prevents age-related decrements in the levels of synaptic proteins, including SYP and NR2B, and doublecortin-like kinase

(DCAMKL1). Additionally, environmentally enriched old zebrafish had elevated levels of GEP while applying this environmental intervention did not modulate the age-related increases in oxidative stress indicators. The third intervention was also a non-genetic approach. Two short-term opposing dietary treatments, such as caloric restriction (CR) and over-feeding (OF), were applied to young and old zebrafish and an *ad-libitum* diet. It was demonstrated that a short-term CR regimen upregulated the glutamatergic components of neurotransmission such as GluR2/3 and post-synaptic density 95 (PSD95). Significant age-related decline in GEP levels was observed in old zebrafish in the OF dietary condition. Expression levels of synaptic and regulatory genes were relatively stable, while inflammation-related gene *tnfa* was altered in an age-dependent manner. Additionally, in the young zebrafish, a significant elevation of trunk cortisol was demonstrated in the OF group compared to CR-fed young zebrafish. Taken together, evaluating different components such as the cholinergic system, diet, and environment can provide us insights into the neurobiological underpinning of successful aging and possible determinants of unhealthy aging.

Keywords: cholinergic system, aging brain, environmental enrichment, synaptic proteins, calorie intake, zebrafish

ÖZET

BEYİN YAŞLANMASINI DEĞİŞTİREN GENETİK VE ÇEVRESEL MÜDAHALELER: ZEBRABALIĞI *(DANIO RERIO)* MODELİNDEN BULGULAR

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Yaşlanan beyinde belirgin bir nöral kayıp olmamasına rağmen, yaşa bağlı bilişsel düşüş normal yaşlanmada ortaya çıkmaktadır. Sinaptik ve hücresel dinamiklerdeki moleküler değişiklikler muhtemelen bu bilişsel değişikliklerin altında yatan nedenlerdir. Yaşa bağlı davranış değişimleri ve nörobiyolojik parametrelerde oldukça heterojen bir profil gözlemlenmektedir, bu durum da karakterizasyon ve tedavi geliştirme amaçlı çalışmaları zorlaştırmaktadır. Bu nedenle, bireysel faktörlerin başarılı veya başarısız yaşlanmaya nasıl katkıda bulunabileceğini ve bu faktörlerin hücresel ve sinaptik dinamiklerde ne gibi değişimlere sebep olabileceğini karakterize etmek oldukça önemlidir. Bu çalışmada yaşlanma sürecini değiştiren üç farklı müdahale yaklaşımı uygulanmıştır. İlk müdahale bir genetik modeli içermektedir, düşük asetilkolinesteraz (AChE) seviyelerine sahip genç ve yaşlı *ache*$^{sb55/+}$ mutantları kullanılmıştır. Önceki çalışmalar, bu modeli ileri yaşlarda gözlemlenen korunmuş bilişsel yeteneklerinden dolayı gecikmiş yaşlanma modeli olarak nitelendirmiştir ve bu çalışma, yaşlanma bağlamında bu mutant modelindeki nörobiyolojik değişiklikleri

analiz eden ilk çalışmadır. Mutant hayvanlarda farklı yaş gruplarında azalmış beyin AChE aktivitesi seviyelerinin devam ettiği ve bu azalmaya asetilkolin ve nikotinik asetilkolin reseptörü alt birimi alfa-7 dahil olmak üzere kolinerjik sistemin diğer elementlerinde hafif düşüşlerin eşlik ettiği gösterilmiştir. Genotipin etkisi, *N*-metil *D*-aspartat tipi reseptör alt birimi 2B (NR2B) ve glutamat reseptör alt birimleri 2 ve 3 (GluR2/3) gibi anahtar glutamaterjik reseptör alt birimlerini önemli ölçüde değiştirmiştir, bu belirteçler *ache*$^{sb55/+}$ mutantlarında anlamlı olarak azalmıştır. Yaşlanmayla birlikte, *ache*$^{sb55/+}$ mutantlarının sinaptofizin (SYP) seviyelerinde önemli bir artış gözlemlenmiştir. Yabanıl-tip kontrol grubunda gefirin (GEP) seviyelerinde yaşa bağlı azalmalar meydana gelirken; *ache*$^{sb55/+}$ mutantlarında GEP seviyelerinde yaşlanmaya bağlı değişikler önlenmiştir. Mutasyonun ayrıca hücresel dinamikler üzerinde de etkileri vardır, nöronal belirteç HuC, *ache*$^{sb55/+}$ mutantlarında tüm yaş gruplarında önemli ölçüde artmıştır, enflamasyon ile ilgili belirteçlerden olan glial fibril asidik protein (GFAP) ve reaktif oksijen türleri (ROS) seviyeleri mutantlarda azalan bir trende sahiptir. İkinci müdahale, başarılı yaşlanmayı teşvik etmek için genç ve yaşlı zebra balıklarına duyusal elemanlar kullanarak kısa süreli çevresel zenginleştirme uygulamasıdır. Çevresel zenginleştirmenin yaşlı zebra balıklarında beyin ağırlığını artırdığı, SYP, NR2B ve DCAMKL1 gibi proteinlerin düzeylerindeki yaşa bağlı düşüşleri önlendiği gösterilmiştir. Ek olarak, çevresel olarak zenginleştirilmiş yaşlı zebra balıkları yüksek GEP seviyelerine sahipken, bu çevresel müdahalenin uygulanması oksidatif stres göstergelerindeki yaşa bağlı artışları modüle etmemiştir. Üçüncü müdahale de genetik olmayan bir müdahaledir. Kalori kısıtlaması (CR) ve aşırı beslenme (OF) gibi kısa süreli karşıt diyet tedavileri, genç ve yaşlı zebra balıklarına uygulanmıştır. Kısa süreli CR rejiminin, GluR2/3 ve

post sinaptik yoğunluk 95 (PSD95) gibi nörotransmisyonun glutamaterjik bileşenlerini artırabileceği gözlemlenirken; OF diyet grubunda GEP seviyelerinde yaşa bağlı anlamlı bir düşüş gösterilmiştir. Sinaptik ve düzenleyici genlerin ekspresyon seviyeleri nispeten sabitken, enflamasyon ile ilgili gen *tnfa* yaşa bağlı bir şekilde değişim göstermiştir. Ek olarak, CR ile beslenen genç zebra balıklarına kıyasla OF grubunda önemli bir kortizol yükselmesi bulunmuştur. Birlikte ele alındığında, kolinerjik sistem, diyet ve çevre gibi farklı bileşenlerin değerlendirilmesi, bize başarılı yaşlanmanın nörobiyolojik temelleri ve sağlıksız yaşlanmanın olası belirleyicileri hakkında önemli bilgiler sağlayabilmektedir.

Anahtar Sözcükler: kolinerjik sistem, beyin yaşlanması, çevresel zenginleştirme, sinaptik proteinler, kalori alımı, zebra balığı

To my beloved family…

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CHAPTER 1

INTRODUCTION

1.1 Aging and cognitive alterations

Normal aging without the presence of neuropathological conditions such as Alzheimer's disease can lead to alterations in the cognitive abilities of older adults. These alterations are associated with improvements in specific cognitive ability categories such as language skills. In contrast, in a group of cognitive domains, aging can lead to deteriorations which can be referred to as aging-related cognitive decline. One domain that stays relatively stable and even improved with aging is language skills. The specific categories of this cognitive ability, such as vocabulary knowledge and reserve, show improvements with aging. On the other hand, even within the same cognitive ability, age-related changes can occur differentially, and certain categories can present more vulnerability against age-related changes. To illustrate, language-related processes, which were mainly modulated by the processing speed, such as word generation and verbal fluency, can demonstrate significant declines and deteriorations with aging [1], [2].

Another cognitive ability is a speed of processing, and dysregulation in this domain can impair multiple related cognitive skills. Deteriorations in the speed of processing can alter multiple cognitive categories such as speed of motor activities, response latency, and perception. Processing speed is vulnerable against aging, and at older ages, people tend to have disrupted overall processing speed compared to younger people [3]. Furthermore, it was proposed that deteriorations in processing

speed can underlie cognitive decline in different cognitive skills and have an explanatory role in these changes [3]. Age-related changes can also be observed in attention and its categories like selective attention and divided attention. Old adults tend to perform drastically slower in conditions demanding selective attention, focusing on the particular features of the task while ignoring the others [4]. Additionally, another category of attention, divided attention which refers to directing the attentional sources to multiple cues or tasks, is significantly impaired with aging [5].

Memory-related deficits are pronounced within the context of aging. It has been shown that encoding and retrieval steps were generally jeopardized with aging while no evident changes were reported in retention processes [6],[7]. Additionally, the different categories of memory are selectively impaired while the other categories remain relatively stable. Studies have reported that semantic and implicit memory components were stable through aging, while episodic memory raising from an individual's collection of experiences was impaired significantly at older ages [2],[8].

Overall, cognitive decline can occur in normal aging, but this decline is not a unitary process indicating impairments in all measures. Certain cognitive skills are selectively affected with normal aging, while others tend to remain stable and spared. However, these declining components can impair the self-sufficiency, well-being, and mind-span of older adults. Therefore, characterization of the neurobiological underpinning of these cognitive changes is very crucial to understand the mechanism of age-related changes and develop intervention strategies.

1.2 Aging and neurobiological alterations

Normal aging does not promote global loss of neurons and synapses in the cortex and subcortical regions, unlike age-related neuropathological conditions such as Alzheimer's disease characterized by progressive neuronal death and synaptic loss as well as the existence of neuropathological profile [9]. Despite the fairly maintained number of neurons and synapses, age-related cognitive decline still occurs in normal aging. It affects the cognitive domains, including episodic memory, executive functions, and processing speed [2]. Therefore, it is crucial to dissect the elements contributing to normal aging-related cognitive decline and target these elements for further interventions to ameliorate the aging phenotype.

In normal aging, there are no apparent histopathological alterations; yet changes occur in more subtle levels. These changes can include disturbances in synaptic integrity and content, decreased neurogenesis, increased inflammatory markers, altered metabolic activity, and transcriptional changes [10]. It is crucial to note that these elements are not totally disconnected from each other. For example, it was shown that with normal aging, the transcriptional profile could be skewed on genes regulating synaptic plasticity, inflammation, and metabolic activity [11], which in turn can alter all the elements. Therefore, there is no single contributing factor; instead, there is extensive cross-talk between multiple systems. Complexity is not limited to the cross-talk between different components; each system, such as different neurotransmitter systems, is not equally vulnerable against age-related alterations. Therefore, it can be promising to focus on the more vulnerable systems to have effective interventions.

1.2.1 Altered synaptic proteome with aging

Age-related disturbances in synaptic integrity and plasticity can be considered as a more direct cause of age-related cognitive decline. Analysis of age-related changes in the synaptic proteome has crucial contributions to age-related cognitive decline. The levels of some synaptic proteins can give information about the age-related impairments on neurotransmission. Post-synaptic 95 (PSD95) is a clustering and scaffolding protein, and it is a member of the membrane-associated guanylate kinase family; with its PDZ domain, it can cluster glutamate receptors; α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptors (AMPARs), and N-methyl-D-aspartate receptors (NMDARs) on the post-synaptic site. It also clusters cytoskeletal elements, ion channels, and molecules responsible for signal transduction depending on the synaptic activity [12]. It has roles in the maturation of the synaptic elements, including terminals and spines. Additionally, PSD95 has a crucial role in the maintenance of excitatory-inhibitory balance. Overexpression studies showed that while glutamatergic excitatory clusters increased in terms of their size, numbers of gamma-Aminobutyric acid (GABA)ergic synapses become reduced. Parallel to that, knock-down studies with PSD95 revealed a decreased number of glutamatergic synaptic innervations, whereas the number of GABAergic innervations was increased [13]. In the aging literature, it was shown that hippocampal levels of PSD95 were reduced with aging, and this decrease was more prominent in the old groups with impaired cognitive performance [14].

PSD95 can cluster different glutamate receptors such as AMPA and NMDA; it indirectly clusters the AMPA type receptors with additional proteins such as TARP

and Stargazin while directly binds to the NMDA receptors. Glutamate receptor subunit 2 (GluR2) is a subunit in AMPA receptors expressed abundantly in most AMPA receptors [13]. The presence or absence of this subunit can alter the calcium regulation, to illustrate if GluR2 was in lower levels or lacking cell becomes more permeable to calcium [15]. Similarly, if GluR2 was expressed in high levels, cells become less calcium-permeable. Additionally, GluR2 was exposed to RNA editing unedited glutamine (Q) transformed to arginine (R) in an edited form; and this RNA editing can also regulate the calcium homeostasis while unedited form was permeable to calcium and edited form renders low calcium permeability [15]. However, this editing occurs in almost all the cases when GluR2 was expressed. Declines in GluR2 levels were reported with aging; reduced levels of GluR2 make the cell more vulnerable to neurotoxic damage because of the elevated permeability to calcium [16]. Glutamate receptor subunit 3 (GluR3) is another AMPA type receptor whose functions are relatively enigmatic compared to the GluR2 subunit. GluR3 is involved in the insertion and trafficking of AMPA receptors and the induction of fast currents of glutamatergic transmission and responsible for regulating short-term plasticity dynamics [17].

NMDA-type receptor subunit 2B (NR2B) is one of the receptor subunits that contribute to channel formations, and also, this subunit can prolong and sustain the response of the receptor. NR2B subunit expression can be altered developmentally, while its expression levels are very high in the post-natal brain, decrements in NR2B subunit levels are evident at the adult ages [18]. Additionally, over expression studies targeting NR2B showed that elevations in NR2B levels are associated with promoted

synaptic plasticity as well as memory and learning performances, and age-related declines were previously reported with regards to this subunit [19][20]. There are different views concerning this age-related decline in NR2B levels. The first view suggests that this reduction in NR2B manifests protection at older ages against the excitotoxicity since NR2B is associated with high calcium influx. The other opinion indicates that higher expression of NR2B is beneficial in all age groups, including old and young ages, and NR2B enhancement at an older age is inducing promoted plasticity and improved behavioral performance [21].

Gephyrin (GEP) can be considered as an inhibitory counterpart of the PSD95. GEP is found in the GABAergic and glycinergic synapses; it anchors and clusters $GABA_{A,}$ the ionotropic GABA receptors, and glycine receptors. Knock-down studies indicated that reduced GEP expression is associated with larger glutamatergic terminals and PSD95-NMDA clusters while GABAergic innervations decrease. Overexpression of GEP induces smaller glutamatergic terminals, PSD95-NMDA clusters, while $GABA_A$ and GEP clusters become larger [22]. It was shown that GEP levels could be altered depending on the age and cognitive status of the animal; it was reported that in the old and cognitively impaired groups, GEP levels were significantly increased in the parietal cortex, whereas no change was observed in the prefrontal cortex [23]. Additionally, evidence suggested that GEP levels were downregulated in neuropathological conditions, while maintained and elevated levels of GEP were protective against harmful effects of these insults [24]. $GABA_A$ receptors are the clustering partners of GEP, and these receptors have several subunits. One of these subunits is alpha 1 (GABA-A-a1). GABA-A-a1 is one of the

predominant subunits widely expressed in the central nervous system and obtaining binding sites for psychiatric drugs like benzodiazepines [25]. In terms of aging literature, it was shown that GABA-A-a1 subunit levels are relatively stable at different age groups [26].

Synaptophysin (SYP) is a transmembrane glycoprotein, and SYP can be found in the presynaptic region and on synaptic vesicles. It was suggested that SYP could have potential roles in the trafficking of the synaptic vesicles and endocytosis [27]. In aging literature, it was demonstrated that increased SYP levels were significantly correlated with intact cognitive performance at older ages [28], and age-related decreases at SYP levels in the hippocampus of old animals were reported [20].

To conclude, aging is associated with subtle alterations in synaptic integrity and plasticity associated with age-related cognitive decline. When synaptic integrity and plasticity elements were evaluated together, one of the most intriguing shared features of PSD95 and GEP is their heterotypic effects. Their effects are not limited to their clustering targets, but they can alter the strength of synaptic innervations of their inhibitory or excitatory counterparts. PSD95 and GEP can have combinatory contributions to excitatory/inhibitory balance, which can be disturbed with aging. Taken together, the protein levels of PSD95, GEP, and their clustering partners GluR2/3, NR2B, and GABA-A-a1, as well as SYP, can give general information regarding excitatory/inhibitory balance and presynaptic integrity, which can be disturbed with aging.

1.2.2 Vulnerability of cholinergic system with aging

One of the most studied neurotransmitter systems in the scope of aging research is the cholinergic system. Bartus and his colleagues suggested that dysregulation in cholinergic neurotransmission contributed to age-related cognitive decline and Alzheimer's disease [28]. Acetylcholine is the neurotransmitter of cholinergic neurotransmission; it is composed of two elements; choline and acetate. Acetate is carried by coenzyme A, and this complex consisting of acetate and coenzyme A is called acetyl coenzyme A. The choline acetyltransferase enzyme (ChAT) transfers the acetate from the complex of acetyl coenzyme A to the choline, and at the end of this synthesis, acetylcholine is obtained. After acetylcholine is released from the cholinergic terminals, termination of this neurotransmitter is conducted with the enzyme acetylcholinesterase. Acetylcholinesterase breaks acetylcholine in the synaptic cleft into choline and acetate, and after this enzymatic degradation, choline molecules are taken up by choline transporters back to the cell. This reuptake process has high efficiency, and it is a rate-limiting factor for the acetylcholine synthesis; because the amount of choline in the cell body which is transported by axonal transport to the terminals is not adequate when it is compared with the amount of released choline; that is why uptake of choline is a crucial step [29].

After the release, acetylcholine can stimulate its receptors; there are two types of acetylcholine receptors. The first class is the nicotinic acetylcholine receptors (nAChRs); they are ionotropic receptors and coupled with ligand-gated ion channels. There are two subunits of nAChRs, α and β, coded by different genes in the central nervous system. In mammalian brain 12 subunits of nAChRs were identified; α-2, 3,

4, 5, 6, 7, 8, 9, 10; and β-2, 3, 4. These ionotropic receptors in the central nervous system can be permeable to calcium, potassium, and sodium. Additionally, it was suggested that nAChRs were anchored and clustered at the pre-synaptic region with rapsyn, a cytoskeletal protein [30]. The second class is the muscarinic acetylcholine receptors (mAChRs); they are metabotropic receptors coupled with class A G proteins. Different subtypes of mAChRs were reported; M1, M2, M3, M4, and M5; and their activation associated with the second messenger system and protein kinase activity. Knock-out studies revealed their various functions: regulation of dopaminergic release, learning, locomotion, activating MAPK signaling, and regulating stress response [31]. With all this information, it could be said that cholinergic neurotransmission has multiple components, and age-related hypothesized dysfunctions can alter these various elements.

Deficiencies in acetylcholine biosynthesis were reported in old mice with a 75% reduction in the biosynthesis of acetylcholine compared to the younger ages; additionally, a parallel decrease was reported in the release of acetylcholine with increasing age following the stimulation [32]. Besides the synthesis and release of the neurotransmitter of acetylcholine, dysfunctions can occur in the other components, including; the high-affinity choline uptake, which was considered a rate-limiting process for the acetylcholine synthesis and alterations in the expressions of the critical subunits in the nAChRs and mAChRs. Interestingly, it was reported that different subunits of the nAChRs could have selective vulnerabilities depending on aging and age-related diseases. For example, in Alzheimer's disease, the α4 subunit of

nAChRs was severely reduced, whereas the affected subunit is α7 in Lewy body dementia [33].

1.2.3 Oxidative stress and aging

Besides alterations in the main neurotransmitter systems and synaptic dynamics, aging is also associated with low-grade inflammation, which persists through aging and becomes chronic. Aging-related changes in oxidative stress mediating mechanisms can also contribute to this low-grade inflammation [34]. Accumulation of these chronic detrimental processes can lead to dysregulated cellular and synaptic dynamics, as well as neuropathological conditions. It was reported that aging disrupts the glial dynamics and can lead to aberrant changes and reactivity in the regulation of microglial cells and astrocytes [10]. Astrocytes are also closely related to the glutamatergic neurotransmission system. When glutamate was released to the synapse, astrocytes were involved in the uptake of glutamate. Deficiencies in this system might lead to the accumulation of glutamate and elevated excitation, which can be detrimental in the long term [35]. Aberrant glial reactivity can be accompanied by elevation on the levels of inflammatory cytokine tumor necrosis factor-alpha (TNF-alpha) in the aging brain.

Aging triggers the accumulation of oxidative materials such as reactive oxygen species (ROS) and induces oxidative alterations and peroxidation in certain cellular elements such as lipids. The brain can have selective vulnerabilities against these age-related changes in oxidative stress mechanisms because of its higher metabolic demands and oxygen consumption compared to other organs [36]. Elevated ROS levels can increase the incidences of lipid peroxidation. The brain is vulnerable to

lipid peroxidation due to the larger surface area of the membranes contributed by dendrites and extended axons [37]. Increases in these measures can lead to membrane instability and elevated ROS damage, altering the cellular and synaptic dynamics in the aging brain [38].

1.3 Age-related sex differences

Another crucial and generally overlooked factor is gender in the aging literature. Gender can alter the course of aging at many levels, molecular to behavioral, and the course of age-related neurodegenerative diseases as well. For example, male subjects tend to experience steeper declines in cognitive performance than females [40]. The alterations associated with aging in glucose metabolism and blood flow rate are sex-specific [39]. The levels of circulating sex hormones such as estrogen are associated with lower peroxide production, regulating cellular aging [38]. It was reported that age-related gene expression changes could occur in a sexually dimorphic pattern. In humans, it was shown that critical time windows in which more prominent changes were observed in the gene expression were different between males and females; more drastic changes occur in females at 80-90 years of age, whereas this window was 60-70 years of age for males. Interestingly altered genes were skewed in different categories between sexes; in females, gene groups associated with immune activation were selectively increased, whereas, in males, gene groups related with protein transport-synthesis and energy production were decreased with aging [11].

Sexual dimorphism can also be observed in the dynamics of brain mitochondria. Mammalian studies indicated that in adulthood, females tend to have significantly

lower peroxide production than males, but after the operation of ovariectomy, peroxide levels of brain mitochondria have become similar to males, and interestingly with the estradiol treatment, this effect was reversed in the females. Additionally, when males were treated with estradiol, a similar improvement, reduction in the peroxide production, was also observed [38].

The effects of the circulating sex hormones are not limited only to mitochondrial integrity; they can exert their effects on synaptic integrity. It was shown that estradiol treatment on ovariectomized rats increased the NMDR1 subunit of NMDA receptors at protein and mRNA levels in the hippocampus and increased dendrites in the CA1 region [40]. It could be said that circulating sex hormones can profoundly affect mitochondrial exhaustion, synaptic integrity, and gene expression patterns associated with age-related symptoms.

1.4 Zebrafish as a model organism to study aging

Zebrafish have high physiological and genetic homology to humans. The developmental and mutagenesis studies are benefited robustly from this model; one pair can produce approximately 200 embryos; since their development occurs ex Utero, transparent embryos can be observed and manipulated easily [41]. Various genetic strains are available in the zebrafish, and some strains can be very effective compared to the other models. In terms of aging research, zebrafish is a valuable model. A gradual cognitive decline was observed in the aging zebrafish, which is very similar to humans [42]. Beyond that, aging markers, including reactive oxygen species, senescence-associated beta-galactosidase (SABG) activity, lipofuscin

deposits, and telomere attrition, were demonstrated parallel changes in response to aging as in humans [43]. Moreover, sexual dimorphism in terms of age-related synaptic protein alterations, gene expression changes, and neurogenesis was observed in the zebrafish brain [44]–[46].

1.5 Genetic interventions to alter the course of aging

Genetic interventions can give information about the importance of the interfered system and present more causative explanations. Due to the fact that the cholinergic system has a selective vulnerability against age-related changes, it might be promising to focus on the cholinergic system as an intervention strategy. Generally, acetylcholinesterase inhibitors were used widely to reduce Alzheimer's disease-related cognitive deficits and pathology, although this treatment's efficiency is still questionable [47]. Reducing acetylcholinesterase levels could be promising. Conceptually, it will increase acetylcholine levels in the synapse and lead to its stimulation. These changes might have the potential to prevent age-related cholinergic hypofunction. Knock-out studies with mice targeting acetylcholinesterase did not reveal prominent impacts. Homozygous mutants had severe developmental muscle abnormalities, while heterozygous mutants were viable. However, other compensatory cholinesterase activities were observed in the heterozygous mutants, despite the reduced acetylcholine levels [48]. In mammals, acetylcholinesterase is not the only cholinesterase responsible for the termination of acetylcholine; butyrylcholinesterase can also cleave acetylcholine at lower rates. Therefore, when mutation reduces acetylcholinesterase levels, compensatory increases occur at the butyrylcholinesterase levels [49].

In that case, to see more noticeable changes, vertebrate models could be a better option. To illustrate, in zebrafish, there is no functional butyrylcholinesterase, and acetylcholinesterase is the only cholinesterase responsible for cleaving the acetylcholine. Homozygous mutation in the acetylcholinesterase coding gene (*ache*$^{sb55/sb55}$ line) results in the complete abolishment of acetylcholinesterase functions, yet it is associated with abnormalities in the development of musculature; sensory neurons, and more importantly, lethal [50]. On the other hand, heterozygous mutants (*ache*$^{sb55/+}$ line) show no developmental abnormality, characterized by decreased brain levels of acetylcholinesterase and increased acetylcholine levels [51]. More importantly, these heterozygous mutants were behaviorally characterized as a delayed aging model. After 24 months of age, wild-type zebrafish demonstrated a significant cognitive decline in the domains of entrainment to temporal-spatial cues, learning performance in conditioned place preference tests, and flexibility of the learning strategies. On the other hand, 24 months old *ache*$^{sb55/+}$ mutants showed comparable performance with the young (6 months of age) groups, with intact behavioral performance [42]. Analysis of the neurobiological basis of this improvement can have explanatory roles in the contribution of altered cholinergic neurotransmission to age-related cognitive decline.

1.6 Environmental interventions to alter the course of aging

Genetic models of interventions are powerful in terms of the establishment of a more focused relationship between the gene of interest and its targets, but since they were induced at very early stages of the development; they can alter the developmental dynamics; change the microenvironment of the brain and lead to

different base-line compared to the wild-type controls. At this point, along with the genetic interventions, environmental interventions can create a more holistic view.

1.6.1 Environmental enrichment

Environmental enrichment is one of the environmental interventions, and it is referring to exposure to perceptually, socially, physically, and cognitively stimulating environments. It could be thought that the level of stimulation may not be so comparable between humans and laboratory animals; an enriched environment would be associated with high-level tasks such as increased intellectual activities, participating in group activities in humans. On the other hand, in laboratory animals, the setup is generally achieved by introducing large cages, new toys, mazes, wheels, and group housing [52]. To understand the underlying mechanisms and distinguish neurobiological contributors of the ameliorative effects of environmental enrichment, animal models are still required. Environmental enrichment was carried out with success in the previous works using different model organisms, including zebrafish.

In the aging literature, it was shown that environmental enrichment could reduce cognitive decline in the old groups in terms of their performances in spatial memory acquisition [53], radial arm maze, and Morris water maze tasks [54]. Neurobiological changes have accompanied these improvements. To illustrate, enriched and old animals had higher neurogenesis, higher cortical thickness, increased spine density, increased branching of dendrites than old control groups raised in a barren environment [52]. Additionally, an increased expression of neurotrophins, including BDNF and NGF, was reported in the rats exposed to an enriched environment [55].

Also, it was indicated that environmental enrichment could increase the levels of the synaptophysin in the hippocampus and frontoparietal cortex in old rats [53]. Moreover, in the literature, pioneering studies indicated that environmental enrichment could modulate the activity of particular neurotransmitter systems. It was noted that brain acetylcholine and acetylcholinesterase levels were increased in the enriched animals [56].

Evidence suggested that the application of environmental enrichment can be associated with better cognitive performance and altered neurobiological dynamics. However, the literature is still inadequate to point out certain factors that might alter or potentiate the impacts of environmental enrichment. The first factor is the ideal time to initiate this treatment. Environmental enrichment can be started at very young, adult, or old ages, and the obtained effects may not be so comparable. For example, it was shown that old subjects could be benefitted from the environmental enrichment, while behavioral and neurobiological measures were more drastically affected at very young ages [57][58]. Another critical factor is the gender, effectiveness and neurobiological outcomes of the environmental enrichment can be differentially affecting the males and females, to illustrate in the rat models, it was shown that old female animals with enrichment could have higher levels of SYP, which is an overall synaptic protein; but another study demonstrated no profound effects of enrichment in male animals [53][54]. Finally, the components through which the EE is working need to be determined. Traditionally these interventions were applied in a mixed fashion, and the individual components of the EE such as sensory, exercise, and socialization were blended. A recent study indicated that the

magnitude of the effects of EE differed depending on these individual factors. For example, environmental (sensory) enrichment and physical exercise have prevented amyloid-beta infusion-related memory deficits and decreased lipid peroxidation in the brain, while social enrichment merely altered social recognition performance antioxidant capacity [59]. Therefore, dissecting out the components of EE in terms of their specific molecular mechanisms within the context of aging is essential for translational applications.

1.6.2 Dietary interventions

Another environmental intervention that can alter the course of aging is dietary interventions. Dietary interventions can have opposing actions on achieving successful aging. For example, one strategy applied as a dietary intervention is restricting the total calorie intake, and the application of the caloric restriction (CR) can delay age-related impairments. Effects of caloric restriction can alter many different pathways, including; nutrient-sensing signaling, autophagy, survival, growth, and so on [60]. Evidence suggested that the CR diet could improve old rats' behavioral performance and prevented declining levels of glutamatergic proteins such as NR2B and GluR2 and general synaptic protein SYP [61]. Besides its effects on the synaptic proteins, the application of CR can reduce the ROS activity and the lipid peroxidation in the aging brain, which can manifest the further protective effects of the CR on brain metabolism [62].

While CR has possible ameliorating impacts on the aging brain, other dietary interventions can accelerate brain aging. These dietary interventions generally consist

of increased fat content in the regular diet by inducing higher calorie intake. Evidence suggested that elevated intake in the total calorie amount could cause cognitive deteriorations at adult age and the older ages [63]–[66]. Additionally, it was indicated in the human studies that people with high BMI scores in their middle age had an increased risk of the incidence of dementia and neuropathological diseases at older ages than people having normal BMI scores [67].

Higher calorie intake leads to detrimental changes in neurobiological components. Evidence suggested that elevated calorie consumption inducing obesity led to significant reductions in the brain levels of vesicular glutamate and GABA transporters, altering the syntheses of these key neurotransmitters [68]. It was demonstrated that the levels of PSD95 and Activity Regulated Cytoskeleton-associated protein (Arc) declined with the overfeeding regimen in the mammalian models [64]. Previous studies showed that a high-calorie diet with elevated fat intake could increase the protein levels of inflammatory markers, microglial markers and decrease Brain-derived neurotrophic factor (BDNF) on the brain [63], [69].

1.7 Aims and Hypothesis

The main aim of this thesis is to characterize the synaptic proteome and cellular markers with aging. In this respect, genetic and environmental interventions were utilized to decelerate or accelerate the aging profile in the zebrafish brain. Possible sexually dimorphic patterns were also taken into account by incorporating females and males in equal ratios to all designated experimental designs. A genetic intervention mentioned in Chapter 3 was targeting the cholinergic system, and the

levels of the main enzyme AChE responsible for the degradation of ACh were reduced with mutation. Previous studies with mammalian models indicated that disruptions in the cholinergic neurotransmission system could occur even in normal aging without an evident neuropathological profile [70]. Therefore, interventions targeting the cholinergic system could be promising for further investigations.

On the other hand, little work has focused on the interactions between cholinergic system activity and aging in the zebrafish literature. Still, they were only assessing age-related behavioral characteristics with less emphasis on the neurobiological basis of these changes [42]. Therefore, the first part of this thesis aims to focus on the alterations in the cholinergic system components induced by genetic mutation with response aging and their possible interactions with the synaptic and cellular proteins and oxidative stress markers. Another focus was on environmental interventions aim to change the course of aging. The first environmental intervention mentioned in Chapter 4 was an application of environmental enrichment, which was short-term and using sensory elements and synaptic/cellular proteins in addition to oxidative stress mediatory markers were checked in this part. The second environmental intervention was induced by dietary regimens, and the effects of overfeeding were compared with caloric restriction within the aging domain. Effects of different diets were assessed on synaptic protein and gene expression profile as well as cortisol levels which was the main focus in Chapter 5.

It is hypothesized that genetic interventions focusing on the cholinergic system can lead to changes in the cholinergic system components, synaptic protein levels,

cellular and inflammatory markers. For environmental enrichment, it is expected that the application of environmental enrichment prevents aging-related deteriorations and can help to induce a healthy aging profile. Likewise, similar ameliorative effects are expected with caloric restriction, while the altered profile in the synaptic markers is anticipated in the overfeeding intervention.

Taken together, aim one was focusing on genetic interventions targeted to the cholinergic system and its effects on aging. This aim consisted of profiling the cholinergic system elements and the related pathways by analyzing key proteins regulating cholinergic neurotransmission. These elements were including acetylcholine (ACh), acetylcholinesterase (AChE), and nicotinic receptor subunit levels, as well as synaptic, neuronal, glial, inflammatory, and proliferative protein markers expressed in young-old, male-female, and wild-type- *ache*$^{sb55/+}$ mutants. Aim two was investigating the effects of short-term environmental enrichment in age and gender-dependent manner on the levels of critical proteins regulating synaptic, neuronal, glial, and proliferative dynamics as well as oxidative stress. Lastly, aim three was to analyze the effects of feeding interventions, caloric restriction, and over-feeding in an age-dependent manner on the levels of synaptic proteins and genes along with the trunk cortisol levels.

CHAPTER 2

METHODS

2.1 Animals

All zebrafish were maintained and raised in a controlled and recirculating housing system, ZebTec (Techniplast, Italy), in a zebrafish facility at Bilkent University Molecular Biology and Genetics Department, Ankara, Turkey. This system enables stable and adjusted water quality parameters such as the constant temperature of 27.5, pH of 7.5, and conductivity which ensures the wellbeing of the zebrafish. Fish were kept with a light: dark cycle of 14 hours at a light and 10 hours at dark with an automated lighting system in the fish facility. In the standard system conditions, zebrafish were fed twice with dry flakes and once with an *Artemia* for a day.

In Chapter 3, the brain proteome of the cholinergic mutants *ache*$^{sb55/+}$which lacks acetylcholinesterase (AChE) was characterized and compared with wild-type animals. These mutants were obtained from the European Zebrafish Resource Center-Karlsruhe Institute of Technology. A total of 60 *ache*$^{sb55/+}$ and wild-type zebrafish were utilized for this study, young animals were 10-11 months old, and old animals were 30-31 months old. These ages were selected by considering the progression of age-related cognitive decline in zebrafish [71]. For the confirmatory analyses of acetylcholine (ACh) and AChE levels, an additional very young age group whose age was 3.5-5 months old was incorporated into the study. The male and female ratio in each age and genotype group was 1:1. All fish for this aim was maintained in the

system until euthanization. The experimental protocols in Chapter 3 were approved by Bilkent University Laboratory Animals Local Ethics Committee with dates: June 15, 2016, and July 31, 2019, and decision numbers of 2016/21 and 2019/23.

In Chapter 4, the effects of environmental enrichment (EE) were investigated in young-old and male-female animals as an environmental intervention that can result in decelerated aging phenotype. In this study, 93 wild-type zebrafish were used, the young group was 6-months old, and the old group was 27-months old. Both males and females were utilized in this study. These fish were maintained in standard system conditions until they were exposed to enriched or barren environmental conditions outside of the system for five weeks. For this chapter, experimental procedures were approved with date: September 12, 2018, and no: 2018/28 by the Bilkent University HADYEK.

In Chapter 5, two opposing dietary regimens were compared within the context of aging. A total of 72 wild-type male and female zebrafish were utilized in this study. Before the 12 weeks of dietary interventions were started, all zebrafish were maintained at the standard system conditions. Before the feeding intervention protocol, the young group was 9-months old, and the old group was 20-months old. When the intervention was ended young group was 12-months old, and the old group was 23-months old. Bilkent University HADYEK approved all experimental procedures for this chapter with the date: September 6, 2017, no: 2017/12.

2.2 Environmental Interventions

2.2.1 Environmental Enrichment

Environmental enrichment (EE) setups were conducted in Chapter 4. Bottom gravel, swimming tubes, and artificial plants were used in the EE setup [57]; this intervention was done outside of the ZebTec system due to these additional materials. A quarantine room with the same lighting cycle system as the ZebTec system has (14L: 10D) was used for this study. The biggest challenge in the EE of the aquatic species, including zebrafish, is keeping water quality parameters the same in the enriched and barren-control environments. Previous studies utilizing the zebrafish model for EE-related interventions generally used separate tanks for enriched and control environments [57][72]. Still, artificial plants, bottom gravel, and additional plastic objects can release some materials into the water, which might change the water quality and be a confounding variable in this kind of experimental setup. One study had a resolution for that problem; they used a big tank equipped with the plastic plants and stones involved in the enrichment setup. This tank was used as a supply tank, and water was taken from this tank to the enriched and barren-control tanks [73]; this resolution can eliminate some problems. Another problem is that; bottom stones increase the surface area accumulating the biological waste, which can change water quality, such as nitrite/nitrate levels differentially in the enriched and control environments. Considering all these variables, we have designed a novel tank for EE in the zebrafish model for this aim.

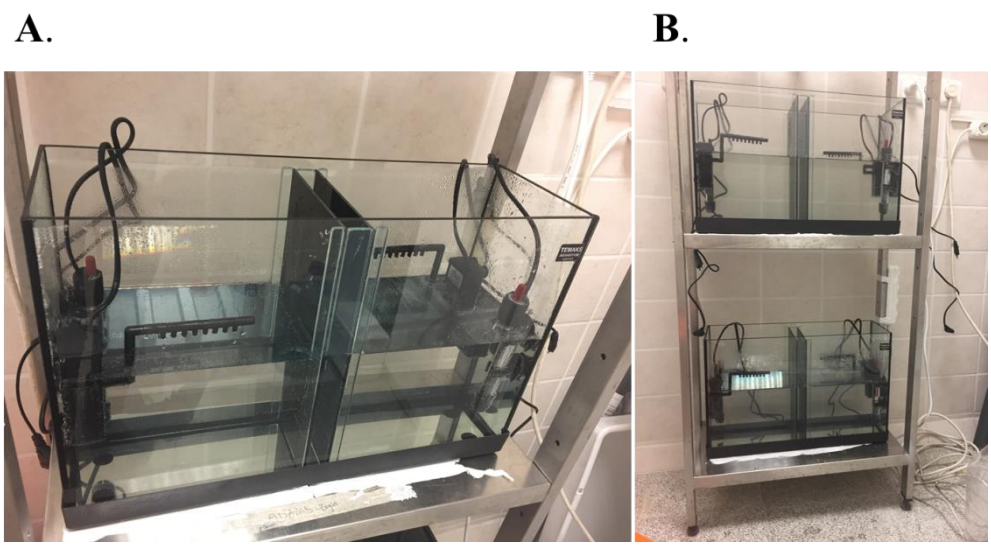


Figure 2.1 Representative pictures of the tank design equipped with air pumps and heaters (A) a view of experimental tanks (B). Adapted from Karoglu-Eravsar et al., 2021 [74].

A standard glass aquarium with the dimensions of 50x15x30 cm was used. The length aquarium was divided into two equal compartments; the dimensions of these divided compartments were 23x15x30 cm. Two Plexiglas black and opaque separators were used for the tank's division (3mm thickness, 15x30 cm). Separators were stabilized with the attachment of the glass notches to the aquaria. Holes (1.5 mm radius) were drilled onto the Plexiglas separators to enable the water passage between the experimental compartments. To eliminate the visual contact between the compartments through the holes drilled in separators, two different separators with holes at not overlapping locations were utilized. There was a small gap (3 cm) between these separators; this gap was used to fill and empty water for cleaning purposes without inducing any stress in the zebrafish. Heaters and air pumps were placed in both of the divided compartments (Figure 2.1).

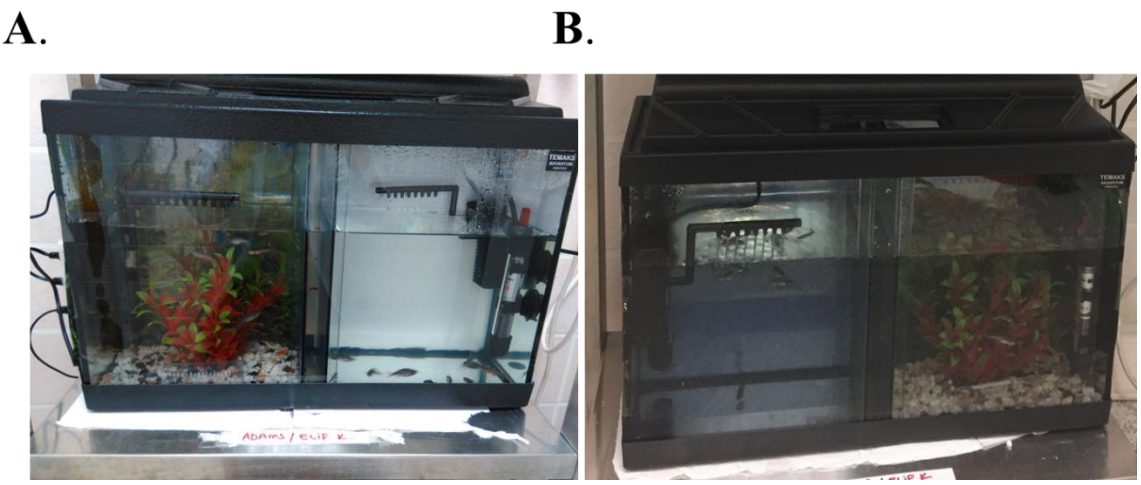


Figure 2.2 A pilot study with white background (A). Used experimental setup for enrichment study with dark blue background (B) Adapted from Karoglu-Eravsar et al., 2021 [74].

As enrichment materials, artificial plastic plant, bottom gravel, 50 ml falcon tubes (with two open ends) were put into the EE compartment (Figure 2.2). Three walls of tanks were coated to prevent irrelevant visual interference from the laboratory environment. EE compartments were covered with sea and plants' sight; barren-control environments were coated with white paper in the pilot studies, but on the animals, less locomotion was observed with white background (Figure 2.2.A). It was previously shown that light background colors could increase stress levels in zebrafish [75]. Therefore in the actual experimental setup, the barren-control environment was covered with dark blue paper, which resembles the color of the standard ZebTec system (Figure 2.2.B). Tanks were filled with 15L of system water. Since this setup was not a recirculating system, one-third of the water was emptied, and fresh system water was added with intervals of 2 days; and air pumps were washed thoroughly during the cleaning. Each time just before the cleaning, water

samples from the tanks were collected, and these samples were exposed to pH and nitrate tests to check the water quality parameters. Temperatures of tanks were recorded daily, and experimental fish were fed with dry flakes two times a day and an *Artemia* once a day. Dry flakes for each meal were weighed to eliminate the potential variance between the feeding regimens of EE and control fish. This experimental protocol lasted for four weeks with an additional one more week of habituation at the beginning that makes five weeks in total.

2.2.2 Feeding Interventions

In Chapter 5, feeding interventions were conducted, and these interventions were applied in the ZebTec system since this intervention was not introducing any foreign substance. Young-old and male-female zebrafish were divided into three feeding regimens; *ad-libitum* (AL), overfeeding (OF), and caloric restriction (CR). The *ad-libitum* group was fed twice with dry flakes on a day with a total amount of 180 mg (this amount was for 16 fish in the young group and 13 fish in the old group). The caloric restriction group was fed 90 mg of dry flakes once in two days (this amount was for 16 fish in the young group and 13 fish in the old group). The over-feeding group was fed with dry flakes on a day with a total amount of 360 mg (this amount was for 15 fish in the young group and 13 fish in the old group) Table 2.1. *Artemia* was given to the *ad-libitum* group 3 times a week, the caloric-restriction group once a week, and the over-feeding group twice a day Table 2.1. This feeding regimen was applied for 12 weeks.

Table 2. 1 Dietary regimens for feeding interventions

Diet Group	Dry Flakes (for two days)	*Artemia* (for one week)
AL	360 mg	3 times
CR	90 mg	Once
OF	720 mg	14 times

2.3 Euthanization and Dissection of Experimental Subjects

All zebrafish that were subjected to the experimental protocols were euthanized through submersion in cold system water with ice for 10 minutes [76]. After cessation of the operculum movements, animals were decapitated with a scalpel blade. The weight and length of the animals were recorded. For the dissection of the brain tissue, heads were opened from the ventral surface. Following the removal of gills, eyes were detached from the optic nerves, and fatty tissues surrounding the skull were cleaned. Skull was opened from the ventral side very carefully, and the brain was extracted and weighed. Trunks were opened to confirm the gender; gender was determined by the presence of testes or ovaries. The brain, eyes, gills, and trunk of each zebrafish were put into Eppendorf tubes and immediately exposed to snap freezing with liquid nitrogen. All tissues were stored in -80°C.

In Chapter 3, genotyping experiments were performed to distinguish between *ache*$^{sb55/+}$ and their wild-type siblings. Tail tissues were used for the genomic DNA extraction. Tails of the animals that were utilized for Chapter 3 were separated from

the trunk, put into an Eppendorf tube, and stored separately for DNA extraction procedures.

2.4 Protein Extraction for Western Blot Experiments

For the samples that were used in Chapter 4 and Chapter 5, proteins were isolated with RIPA buffer containing 150 mM NaCl, 50 mM Tris-HCl pH: 8, 0.1% SDS, 1% NP-40, and 2x protease inhibitor. Brain tissues were homogenized in 300 µl of RIPA buffer with an insulin syringe by passing through 15-20 times on the ice. Then lysates were incubated on ice for 30 minutes; they were mixed few times during this incubation. Following the incubation, samples were centrifuged at 13,000 rpm for 20 minutes at 4°C. Supernatants were collected carefully and aliquoted, and stored at -80°C for the western blot experiments.

For the samples that were used in Chapter 3, we wanted to analyze ACh and AChE measurements and protein expression changes from the same biological samples since the mutation has causal impacts on AChE. Brain tissues were homogenized in ice-cold 200 µl of 50 mM Tris-HCl (pH:8) with an insulin syringe. Homogenized samples with 50 mM Tris-HCl (pH:8) were divided into two. The first part was homogenized further with a sonicator (4 cycles of 2 seconds of the pulse; amplitude 0.8) and then incubated on the ice for 30 minutes. The second part was incorporated with 25 µl of 5XRIPA buffer (750 mM NaCl, 50 mM Tris-HCl pH: 8, 0.5% SDS, 5% NP-40, and 10x protease inhibitor) and incubated on ice for 30 minutes. Following the incubation, both homogenates were centrifuged at 13,000 rpm at 4°C for 20 minutes; supernatants were collected, aliquoted, and stored at -

80°C. Homogenates with 50 mM Tris-HCl were used for ACh/AChE assays, while homogenates incorporated with RIPA buffer were used for Western Blot experiments. An initial aliquot is made for the ACh/AChE assays because the detergent in the RIPA buffer interferes with the reagents in the kits. Therefore, after an aliquot of the sample protein homogenate is prepared using the 50-mM Tris, pH: 8, the RIPA buffer with protease inhibitors was added to the sample to eliminate any potential degradation of proteins of interest.

2.5 Protein Extraction for Biochemical Assays

For the biochemical assays measuring ACh, AChE, and reactive oxygen species (ROS), RIPA extracted samples could not be used since the RIPA's detergent content was interfering with these assays. Therefore, different samples or different combined extraction methods were used for these assays. For Chapter 3, since mutations were directly altering AChE activity, we wanted to compare ACh, AChE, and brain protein levels from the same biological samples. Brain samples used for Chapter 3 were subjected to combined extraction as mentioned in the previous part. Brains were homogenized in ice-cold 200 µl of 50 mM Tris-HCl (pH:8) with an insulin syringe. Homogenized samples with 50 mM Tris-HCl (pH:8) were divided into two; the first part was used to measure ACh and AChE levels, this part was homogenized further with a sonicator and incubated on ice for 30 minutes; following the incubation, samples were centrifuged at 13,000 rpm at 4°C for 20 minutes; supernatants were collected, aliquoted and used for ACh and AChE levels.

For the measurement of ROS activity levels in Chapter 3 and Chapter 4, brain samples were homogenized in 150 µl cold phosphate-buffered saline (PBS) by passing through the syringe. These homogenates were then centrifuged for 10 minutes at 10,000 g in a refrigerated centrifuge at 4 ° C. Additionally, these samples were also utilized in AChE activity measurements in Chapter 4.

2.6 Determination of Total Protein Amount

The Bradford assay was utilized to determine the total protein amounts of the protein samples extracted with RIPA, Tris buffer, MDA lysis buffer, or PBS. The Bradford assay was done on 96 well-plates. The assay was performed by following the manufacturer's instructions. Briefly, ddH_2O was distributed to the wells, as shown in Table 2.2. After that, bovine serum albumin (BSA), (A7906, Sigma-Aldrich, St. Louis, MO, USA), which was used as standard in this assay, was added between the concentration range of 2-20 µg/ml to the wells allocated for standards, and the unknown protein samples were added. After the standards and unknown protein samples were loaded into 96 well-plates, Bradford Reagent (B6916, Sigma, St. Louis, MO, USA) in the volume of 250 µl were added to the wells. Plates were shaken in the orbital shaker for one minute at 250 rpm and incubated for 10 minutes at room temperature. Absorbance values were assessed at 595 nm with spectrophotometry SpectraMax (M5, Molecular Devices). For the concentration calculations, optical densities of blanks were subtracted from the standards and unknown protein samples. Linear curve fitting was applied between BSA concentrations of the standards and optical density values of the standards, equation obtained from this curve fitting was

conducted on the optical densities of the unknown protein samples to calculate their concentrations.

Table 2. 2 Reactions for Bradford assay

Samples	**ddH_2O (µl)**	**BSA(µl) (1mg/ml stock)**	**Sample**
Blank	5	-	-
Standard 1	0.5	0.5	-
Standard 2	1	1	-
Standard 3	2	2	-
Standard 4	3	3	-
Standard 5	4	4	-
Standard 6	5	5	-
Unknown Samples	4.5	-	0.5

2.7 DNA Extraction from Tail Samples

For the genotyping experiments in Chapter 3, genomic DNA was extracted from the tail samples. Lysis buffer in a volume of 200 µl of containing 100 mM Tris pH: 8.2, 10 mM EDTA, 200 mM NaCl, 0.5% SDS, and proteinase K was added onto the tail samples. They were incubated at 55°C with shaking overnight. On the following day, samples were incubated at 95°C for 20 minutes to deactivate the proteinase K. Then, homogenates were centrifuged at 13,000 rpm at 4°C for 20 minutes. Supernatants were collected and transferred into the new tubes; 175 µl of Isopropanol was added onto them and mixed by passing through the pipette. Samples were

centrifuged at 13,000 rpm for 20 minutes at 4°C, supernatants were discarded, and pellets were washed with 500 µl of 70% Ethanol. Following the addition of 70% Ethanol, samples were centrifuged one more time at 13,000 rpm for 20 minutes at 4°C. Supernatants were discarded, and pellets were air-dried for 20 minutes. After this evaporation step, pellets were resuspended with 20 µl of DNase/RNase free water (AM9937, ThermoFisher Scientific, MA, USA), and genomic DNA concentrations were measured with NanoDrop 2000 (ThermoScientific, Rockford, IL, USA).

2.8 RNA isolation, DNase treatment, and cDNA Synthesis

RNA isolation procedure was performed for the gene expression experiments in Chapter 5. Total RNA was isolated with Trizol Reagent (15596026, ThermoScientific, Rockford, IL, USA) from the snap-frozen brain tissues. For the extraction protocol, the manufacturer's instructions were followed with slight modifications. Snap-frozen brain tissues were put into Eppendorf tubes with 250 µl of Trizol reagent, and homogenization was performed by sonication on ice. Samples were centrifuged at 12,000 g for 5 minutes at 4°C to reduce the homogenates' fat content. Following the centrifuge, supernatants were collected into a new tube and incubated for 5 minutes at room temperature to dissociate. Fifty-µl of chloroform was added onto the homogenates mixed with gentle finger tapping for few times and then incubated at room temperature for 5 minutes. Homogenates mixed with chloroform were further centrifuged at 12,000 g for 15 minutes at 4°C. After this step, phase separation was observed, and RNA containing an aqueous phase was collected carefully into a new tube. This aqueous phase was mixed with 125 µl of isopropanol and incubated at room temperature for 10 minutes for the precipitation process.

Centrifugation was done following this step at 12,000 g for 10 minutes at 4°C. Supernatants were discarded carefully, and 250 µl 75% ethanol was added onto pellets for washing. Samples containing ethanol were vortexed a few times and then centrifuged at 12,000 g for 5 minutes at 4°C. Following this step, supernatants were discarded carefully, and pellets were air-dried for 10 minutes. RNA pellets were resuspended in 20 µl of DNase/RNase free water (AM9937, ThermoFisher Scientific, MA, USA) then incubated at 55°C for 10 minutes to solubilize. RNA concentrations were determined by NanoDrop 2000 (ThermoScientific, Rockford, IL, USA), and RNA samples were diluted into a concentration of 200 ng/µl.

DNase treatment was applied following the RNA extraction procedure to the RNA samples. For this step, 20 µl of RNA was utilized from samples whose concentrations were 200 ng/ µl. DNase treatment kit was used for this step, and the manufacturer's instructions were followed (AM1907, ThermoFisher Scientific, MA, USA). A total of 20 µl of RNA samples were mixed with 2.33 µl of 10Xbuffer, a component of the kit, and 1 µl of DNase. Mixed samples were incubated at 37°C for 30 minutes. After this incubation, 2.6 µl of inactivation buffer was mixed with the samples to inactivate the DNase; they were incubated at room temperature for 5 minutes and then centrifuged at 12,000 g for 1 minute. Following this step, supernatants were collected, and their concentrations were determined by using NanoDrop 2000 (ThermoScientific, Rockford, IL, USA).

After the DNase treatment procedure, cDNA synthesis was performed (1708891 iScript cDNA synthesis kit, Biorad, Hercules, CA, USA). For this step, 500 ng of DNase-treated RNA samples were used by following the manufacturer's protocol.

Samples were mixed with 15 µl of DNase/RNase free water, 4 µl of 5X buffer, and 1 µl of reverse transcriptase. Mixed samples were incubated at 25°C for 5 minutes, 42°C for 30 minutes, 85°C for 5 minutes. After these cycles, cDNA samples were aliquoted and stored at -80°C for gene expression experiments.

2.9 Cortisol Extraction

Cortisol measurements were conducted for the experiments in Chapter 5. Snap frozen trunks were weighed and put into 50 ml falcon tubes with 5 ml of PBS. Trunks were homogenized with a homogenizer (VDI25, VWR, Radnor, PA, USA). Between the different samples, the homogenizer was cleaned with 70% Ethanol and double-distilled water. Diethyl ether was added to samples at a volume of up to 30 ml. The falcon tubes were vortexed thoroughly and centrifuged at 5,000 rpm for 15 minutes at room temperature. The upper phase containing diethyl-ether and lipids was carefully collected with serological pipettes and then transferred to new falcon tubes. The tubes were incubated in the hood without a cap at room temperature overnight to allow for the evaporation of diethyl ether. Following the evaporation, 500 µl of the assay diluent was added to the pellets and mixed thoroughly using a pipette.

2.10 Cortisol Determination

Cortisol measurements were conducted for the experiments in Chapter 5. A commercially available cortisol kit was used to determine the cortisol levels of the samples (KGE008B, R & R&D systems, Minneapolis, MN, USA). The manufacturer's instructions were followed for the protocol. Each sample was tested in duplicates with dilutions of 1:2, 1:4, and 1:8. Optical densities (ODs) were

measured at 450 nm with background correction at 570 nm with Spectramax M5 microplate reader (Molecular Devices, Sunnyvale, CA, USA). Mean ODs of blanks were subtracted from each well, and a 4-parameter logistic (4-PL) curve fitting was carried out to the ODs and known concentration of the standards, then the equation was used for calculation of the concentrations of the unknown samples. These concentration values were divided by the animal's trunk weight and indicated as ng/g trunk weight. This method has been used in zebrafish studies [77], including our group [78].

2.11 Western Blot

Protein samples that were extracted by using RIPA buffer were used for the Western Blot analysis. Different concentrations of total protein amounts were used for the detection of proteins of interest that was shown in Table 2.3. Protein samples were first diluted to the required concentration for detection and then mixed with 2X Laemmli buffer containing 4% SDS, 20% glycerol, 0.125-M Tris-HCl pH: 6.8, 0.004% Bromophenol Blue and 10% dithiothreitol, which was freshly added each time. Protein samples mixed with 2X Laemmli buffer were incubated at 95°C for 10 minutes, and then samples were loaded into the acrylamide gels.

The resolving gel for separation was prepared 10% which was containing 2.5 ml of 1.5-M Tris-HCl, pH: 8.8, 100 µl of 10% SDS, 2.5 ml of 40% Bisacrylamide, 100 µl of 10% Ammonium Persulfate, 10 µl of Tetramethylethylenediamine and 4.8 ml double distilled water for the total volume of 10 ml. Stacking gel was prepared 5% including 2 ml of 0.5-M Tris-HCl, pH: 6.8, 80 µl of 10% SDS, 1 ml of 40%

Bisacrylamide, 80 µl of 10% Ammonium Persulfate, 8 µl of Tetramethylethylenediamine and 4.8 ml double distilled water for the total volume of 8 ml. After gels were cast and prepared, samples were loaded into gels and run under denaturing and reducing conditions at 90 V for 30 minutes, followed by 120 V for 120 minutes at room temperature by using a 1X running buffer containing 25-mM Tris-HCl, 190-mM Glycine, and 1% SDS as a final concentration with pH: 8.3. A prestained protein ladder (26616, ThermoScientific, Rockford, IL, USA) was used as a marker to assess the corresponding molecular weights of the protein, and for electrophoresis, Mini Protean Tetra system was utilized (BioRad, CA, USA).

Following the electrophoresis, SDS-PAGE gels were transferred to PVDF membranes (88518, ThermoScientific, Rockford, IL, USA) by using an ice-cold transfer buffer containing 25-mM Tris, 195-mM Glycine, and 20% Methanol as final concentrations. First PVDF membrane was activated with 100% Methanol by incubation for 2 minutes at room temperature, and then SDS-PAGE gels and PVDF membranes were balanced with incubation in transfer buffer, and the wet transfer was applied with Mini Protean Tetra system instrument (BioRad, CA, USA). The transfer chamber was placed on ice, and ice packs of this system were placed into the chamber to prevent overheating; transfer was carried out at 90 V for 90 minutes.

After the transfer, membranes were incubated with a blocking solution. Blocking solution was prepared using TBS-T containing 20-mM Tris, 150-mM NaCl, and 0.3% Tween-20 with pH adjusted to 7.6. For the blocking solution, either bovine serum albumin or non-fat milk powder was utilized and mixed with TBS-T at a concentration of 5%.

Membranes were incubated with blocking solution at room temperature with gentle agitation for 50 minutes. Following the blocking, membranes were washed with TBS-T quickly and placed into primary antibody solutions. Primary antibodies that were used in Chapters 3, 4, and 5 were listed in Table 2.3 in detail. Primary antibody incubation was done at 4°C with gentle agitation overnight. Membranes were washed with TBS-T 5 times after the primary antibody incubation. At the first two and last two steps, membranes were washed for 5 minutes, and at the third washing step, membranes were washed for 10 minutes. All washing steps were completed at room temperature and with gentle agitation. Secondary antibodies listed in Table 2.3 were mixed with blocking buffer at the indicated concentrations. Secondary antibody solutions were incubated with membranes for 55 minutes at room temperature with gentle agitation. Incubation was followed by 5 times washing steps with TBS-T. After the last washing step, membranes were incubated with ultra-sensitive enhanced chemiluminescent substrate (34095, ThermoScientific, Rockford, IL, USA) for 5 minutes at dark developed bands were visualized by using ChemiDoc MP System (BioRad, Hercules, CA, USA).

Table 2.3 List of the antibodies utilized in the current study

Antigen	Supplier	Catalog #	Use	Dilution	Total Prot. Amt.	Chapter
Doublecortin-like kinase 1	Abcam	ab109029	Primary	1:1000	30 µg	3, 4
Embryonic lethal abnormal vision (ELAV; Drosophila)-like 3	Abcam	ab78467	Primary	1:2000	30 µg	3, 4

Gamma-Aminobutyric acid type-A alpha-1 subunit	Abcam	ab211131	Primary	1:1000	30 µg	3, 4, 5
Gephyrin	Abcam	ab185930	Primary	1:2500	30 µg	4
Gephyrin	Santa Cruz	sc-6411	Primary	1:1000	30 µg	3, 5
Glial fibrillary acidic protein	Abcam	ab53554	Primary	1:2000	30 µg	3, 4
Glutamate Receptor 2 and 3	LSBio	LS-C15368	Primary	1:1000	15 µg	3, 4, 5
Nicotinic Acetylcholine Receptor alpha-7	Abcam	ab216485	Primary	1:1000	20 µg	3
N-methyl D-aspartate receptor-type subunit 2B	LSBio	LS-C25797	Primary	1:1000	30 µg	3, 4, 5
Post-synaptic Density 95	Abcam	ab18258	Primary	1:5000	15 µg	3, 4, 5
Proliferating cell nuclear antigen	Abcam	ab18197	Primary	1:1000	30 µg	3, 4
Synaptophysin	Abcam	ab32594	Primary	1:10000	15 µg	3, 4, 5
Actin	Abcam	ab1801	Primary	1:1000	20 µg	3
β-Tubulin	CST	#2146	Primary	1:5000	15-30 µg	3, 4, 5
Goat-HRP	Abcam	ab97100	Secondary	1:10000	-	3, 4, 5
Rabbit-HRP	CST	#7074	Secondary	1:5000	-	3, 4, 5

Band intensities were quantified blindly by using ImageJ software (NIH, Bethesda, MD, USA). Two sequential normalization methods have been utilized. Gels in all Chapters were prepared so that one gel contains at least one individual from the comparison groups, and all gels were run as cohorts. Each band intensity was averaged to the mean of their within gel cohorts. This method minimizes the systemic variation among the blots due to exposure or other factors. Then these values were divided by corresponding housekeeping protein intensities. Additionally, each biological replicate has two technical replicates, and the places of these technical replicates were switched to eliminate any systematical variance.

2.12 Biochemical Assays

Commercially available kits were utilized for the measurement of acetylcholine and acetylcholinesterase levels. Additionally, to have the relative measurement of free-radical content in the brain lysates, a method using 2′,7′-Dichlorofluorescein was carried out.

2.12.1 Acetylcholine Assay

ACh levels were analyzed for the experiments in Chapter 3. A commercially available Red Amplex Acetylcholine Detection Kit was used by following the manufacturer's instructions (A12217, ThermoFisher Scientific, MA, USA) to investigate ACh levels in the samples. Previous studies indicated that this kit could reliably measure ACh levels in the zebrafish brain extracts [51]. Briefly, for the detection, 30 μg of protein was used from each sample, and Tris-extracted samples mentioned in the Methods section were used. Standard concentrations used were 1

μM, 2 μM, 4 μM, 6 μM, 8 μM, 10 μM and 20 μM. A 30 μg of samples were mixed with 1X reaction buffer at the final volume of 100 μl. Then, 100 μl working solution containing 400 μM Amplex Red reagent, 1U/mL acetylcholinesterase, 2U/mL horse radish peroxide, and 0.2U/mL choline oxidase were added to the samples and known standards. Each sample and standards were tested in duplicates. The plate was incubated in the dark at room temperature for 30 minutes. Fluorescence of plates was read with microplate reader SpectraMax (M5, Molecular Devices) with 544 nm excitation and 590 nm emission. The mean of plate blanks was subtracted from each value, and linear curve fitting was applied between the standards' fluorescence readings and their known concentrations. The equation was extracted from this curve fitting and used to determine the concentrations of the samples.

2.12.2 Acetylcholinesterase Assay

For the AChE levels assessments, a colorimetric enzymatic assay kit (ab138871, Abcam, Cambridge, UK), using DTNB based quantification, was utilized. This product was not shown previously, working accurately and optimally with zebrafish samples in the literature. Therefore protocol for this assay was optimized. Extraction with either 25-mM Tris-HCl or PBS was utilized rather than the usage of conventional lysis buffers like RIPA, which contains SDS and interferes with the reagents of these kinds of assays. In the optimization experiments, embryos with *ache*$^{sb55/sb55}$ homozygous genotype, brain tissues of *ache*$^{sb55/+,}$ and control group, wild-type age-matched zebrafish utilized. The optimization experiments were conducted by using these samples; since complete abolishment of acetylcholinesterase was expected at 4 days post fertilization (dpf) [50], and lower levels of brain

acetylcholinesterase were reported previously in *ache*$^{sb55/+}$ adult young mutants [51]. Our optimization experiments indicated that homozygous embryos had completely abolished AChE activity at 4-dpf. In contrast, in the 4-dpf wild-type controls, enzymatic activity was drastically higher, and heterozygous *ache*$^{sb55/+}$ mutants had prominently reduced AChE activity as compared to their wild-type siblings Figure 2.3.

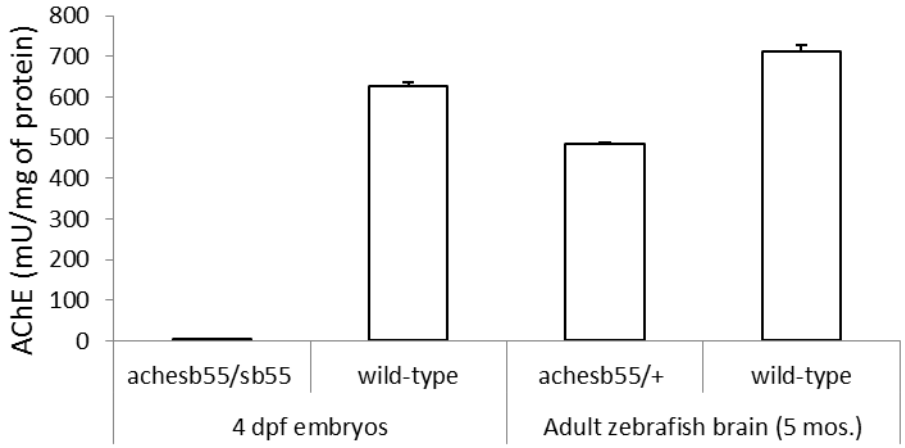


Figure 2.3 Optimization of AChE assay by using *ache*$^{sb55/sb55}$ embryos *and* *ache*$^{sb55/+}$ brains.

This assay was used for the experiments in Chapters 3 and 4. The manufacturer's instructions were followed, and 20 µg of total protein from samples were loaded into a 96 well-plate. AChE standards were prepared by serial dilution; the range for the standards' concentrations was between 0-1000 mU/ml. Assay buffer was added into the samples up to the volume of 50 µl. Then 50 µl of working solution containing DTNB and acetylthiocholine were added to the wells; the plate was incubated in the dark at room temperature for 15 minutes. After the incubation, absorbance values were assed with microplate reader SpectraMax (M5, Molecular Devices) at 410 nm.

The mean value of the blanks was subtracted from each optical density readings; linear curve fitting was carried out between the absorbance values of the standards and their known concentrations. An equation was extracted from this curve fitting and applied for the determination of the concentration of the unknown samples.

2.12.3 Detection of Reactive Oxygen Species

Protocol to measure reactive oxygen species (ROS) by utilizing 2′,7′-Dichlorofluorescin diacetate (D6883, Sigma-Aldrich, St. Louis, MO, USA) in the zebrafish brain samples was kindly provided by Dr. Angelo Piato. This method was being used for the experiments in Chapters 3 and 4. Briefly, brain samples were homogenized in 150 µl cold phosphate-buffered saline (PBS) by passing through the syringe. The homogenates were then centrifuged for 10 minutes at 10,000 g in a refrigerated centrifuge (4° C). Supernatants were collected into new tubes for use in experiments. PBS amounts specified in Table 2.4 were put into the 96-well microplate, and then the samples were added. Since DCF is a light-sensitive material at dark, the specified amount of DCF was added to the reaction. The microplate was transferred into the microplate reader in the dark environment. It was incubated in the reader at 37 ° C for 30 minutes for de-esterification. Then, fluorescence was measured from the wells at 488 nm excitation and 525 nm emission.

2.12.4 Detection of Lipid Peroxidation

These experiments were conducted for Chapter 4. The lipid peroxidation content of the samples was investigated by measuring the malondialdehyde content (MDA) of the samples; MDA is a very reactive molecule and produced by lipid peroxidation

[79]. A commercially available colorimetric kit was used to investigate the samples' MDA content (ab118970, Abcam, Cambridge, UK), and the manufacturer's instructions and protocol were followed with slight modifications at the extraction and lysis processes. Snap-frozen brain tissues were put into 300 µl of lysis buffer and 3 µl of 100X BHT solution, and tissues were homogenized by passing through a syringe 15-20 times on the ice. Homogenates were centrifuged at 10,000 g in a refrigerated centrifuge (4° C). Supernatants were collected, and the total protein content of these supernatants was calculated by using the Bradford assay. From each supernatant, 200 µl volume was mixed with 600 µl of TBA solution. These mixed samples and standards were incubated at 95° C for 60 minutes, and following this incubation, samples were put into ice for 10 minutes. After this on-ice incubation period, from the mixtures, 200 µl were loaded into a 96 well-plate in duplicates, and absorbance values were assed with microplate reader SpectraMax (M5, Molecular Devices) at 532 nm. Mean optical densities (ODs) of blank wells were subtracted from each OD value, and linear curve fitting was applied between the ODs of the standards and their known concentrations. An equation was obtained from this curve fitting and used to calculate the unknown samples' concentration.

Table 2.4 Reaction volumes for controls and unknown samples

	PBS (µl)	**Sample (µl)**	**DCF (1 mM)(µl)**
Control 1	195	-	5
Control 2	175	25	-
Control 3	190	5 (0.4 mM H_2O_2)	5
Samples	170	25	5

2.13 Genotyping Experiments with Quantitative PCR (qRT-PCR)

The mutant line was continued by crossing two heterozygous parents. Out of this cross, 1/4 of embryos become paralyzed at 72 hours post-fertilization (hpf); 2/4 of embryos are heterozygous mutants, and 1/4 of embryos are wild-type siblings. Another crossing strategy was outcrossing the known heterozygous fish with a wild-type animal. The outcome of this cross 1/2 of the animals will be wild-type, while the other half is heterozygous mutants. Therefore, to discriminate heterozygous mutants and their wild-type siblings, genotyping is required. The genotyping of *ache*$^{sb55/+}$ mutants was carried out with the qPCR method, which was optimized and developed with members of the Özlen Konu group [80]. These experiments were conducted for Chapter 3.

Two forward primers were used for the genotyping. The first primer, primer W, recognizes the wild-type sequence; the second primer M, identifies the point mutation in heterozygous and homozygous mutants. Primer sequences were shown in Table 2.5. Primer M was not efficiently amplified in the wild-type animals. Since heterozygous mutants have both copies, both primers amplified the genomic DNA. The amplification difference between the two primers is close to 0 in heterozygous mutants, and in the wild-type siblings, approximately 10 ct differences were observed between primer M and primer W.

Table 2.5 Primer sequences utilized in genotyping experiments

Primer	Forward Primer 5′-3′	Reverse Primer 5′-3′
Wild-type Primer (W)	ACACGTGCCATATTGCAGAG	CTGCTCCAGGGAAGAACTTG
Mutant Primer (M)	ACACGTGCCATATTGCAGAA	CTGCTCCAGGGAAGAACTTG

For the protocol, genomic DNAs extracted from the tail samples were diluted into a concentration of 100 ng/µl; the final concentration was10 ng/µl in the reaction. The total reaction was performed in 10 µl of the final volume. SYBR Green I Master mix solution (04887352001, Roche, Mannheim, Germany) was used by following the manufacturer's instructions. Experiments were conducted by using the LightCycler 480 System (Roche, Mannheim, Germany). Mutant and wild-type primer sequences were indicated in Table 2.5.

2.14 Quantitative PCR (qRT-PCR)

Gene expression analyses were investigated by using qPCR. These experiments were utilized for Chapter 5. Obtained cDNA samples were diluted into 1:2 concentrations for the *tnfa* and *il10* expression analyses, 1:4 for *igf1* expression level investigation, and 1:8 for the remaining genes indicated in Table 2.6. Primer sequences and their final concentrations in the reaction were demonstrated in Table 2.6. The total reaction was performed in 20 µl of the final volume. SYBR Green I Master mix solution (04887352001, Roche, Mannheim, Germany) was used by following the manufacturer's instructions. Experiments were conducted by using the LightCycler 480 System (Roche, Mannheim, Germany).

Table 2.6 Sequences and concentrations of primers used in the gene expression analyses

Gene Symbol	Forward Primer 5′-3′	Reverse Primer 5′-3′	Final Concentration (µM)
bdnf	AGAGCGGACGAATATCGCAG	GTTGGAACTTTACTGTCCAGTCG	0.5
sypa	CAGGGTGCTGAAAGTCCCAT	TTCTTGCACTCCACGCTCAT	0.5
gepb	GGTTCTCTACCTTGGACCAGC	GGTGATGTCCACCGCACTAT	0.5
dlg4	GGAAGGGAAGAGCCAGTACG	CTTCACCGGGCCCAGAATAA	0.5
dlg4b	TGAACGATGACCTCCTCTCC	TCCGTCCATCTCGTACTCG	0.5
tnfa	AGGAACAAGTGCTTATGAGCCATGC	AAATGGAAGGCAGCGCCGAG	0.5
il10	TCACGTCATGAACGAGATCC	CCTCTTGCATTTCACCATATCC	0.5
igf1	GGGCATTGGTGTGATGTCTT	CCAGTGAGAGGGTGTGGGTA	0.5
rpl13a	TCTGGAGGACTGTAAGAGGTATGC	AGACGCACAATCTTGAGAGCAG	0.5

For the analyses, two sequential normalization methods have been utilized. First, delta-ct values were calculated by subtracting the individual ct's from the average ct of the gene of interest from the entire samples. Then delta-delta-ct values were calculated by subtracting these normalized ct values from their corresponding normalized *rpl13a* values, which were used as a housekeeping gene based on the previous works [81]. Fold change was calculated by the formula: 2^(-(delta-delta-ct)), then for the analyses and graphical visualization, log2 transformation was applied, and relative gene expression levels were indicated as logFC, which is log2 transformed fold change values.

2.15 Statistical Analyses

Assumptions of normality, including normal distribution and homogeneity of variance, were checked in all of the analyses in Chapters 3, 4, and 5. These assumptions were checked with Kolmogorov-Smirnov/ Shapiro-Wilk tests and Levene's test, respectively. When these assumptions were fulfilled, parametric tests were conducted. In the cases which violated these assumptions, non-parametric equivalents of these parametric tests were carried out. All significance levels were set as $p<0.05$ except for the cases in which the *p*-value was adjusted based on the number of the comparisons. All analyses were conducted using SPSS (IBM, Istanbul, Turkey) and graphical demonstrations were prepared with GraphPad Prism 9 (GraphPad Software, San Diego, CA, USA).

In Chapter 3, both 2-way with the factor of genotype with two levels (wild-type and $ache^{sb55/+}$) and age with two levels (young and old); and also 3-way ANOVA with the factor of genotype with two levels (wild-type and $ache^{sb55/+}$) age with two levels (young and old), and gender with two levels (female and male) were carried out. In the cases of no gender-dependent main effects or interactions were demonstrated a 2-way ANOVA results were reported, while in the cases of significant gender main effects or gender-dependent interactions, both 2-way and 3-way ANOVA results were reported. These analyses were conducted on the levels of synaptic proteins, cellular proteins, and inflammatory proteins. Further multivariate principal component analysis (PCA) was performed for this chapter by incorporating all synaptic, cellular, and inflammatory proteins. Correlation matrices were used for the PCA, and as a rotation method, Varimax was applied.

For the case of Chapter 4, a 3-way ANOVA with the factors of an environment with two levels (enriched and barren), gender with two levels (female and male), and age with two levels (young and old) was applied for the analyses of protein expression difference, AChE activity and ROS content along with the body parameter measurements such as body mass index (BMI) and wet brain weight. Significant main effects were followed by Bonferroni posthoc test, and for the cases of the significant interactions, simple effects analyses were conducted. For the instances in which assumptions of normality were violated, separate Kruskal-Wallis tests for the effects of the environment, age, and gender were applied. Mann-Whitney U tests with adjusted p values were used if the significant effect of environment, age, or gender were observed as a pairwise comparison. Further multivariate principal component analysis (PCA) was performed for this chapter by incorporating the brain protein expression data. Covariance matrices were used for the PCA, and as a rotation method, Varimax was utilized.

In Chapter 5, the effect of gender was not statistically significant on the dependent measures, so males and females clustered together for this chapter. A 2-way ANOVA with the factors of the diet with three levels *(ad-libitum*, caloric-restriction, and over-feeding) and age with two levels (young and old) was applied for the analyses of protein expression differences and gene expression patterns along with the body parameter measurements such as body weight, body mass index (BMI) and trunk cortisol levels. Significant main effects were followed by Bonferroni posthoc test, and for the cases of the significant interactions, simple effects analyses were conducted. For the instances in which assumptions of normality were violated,

separate Kruskal-Wallis tests for the effects of diet and age were applied. Mann-Whitney U tests with adjusted p values were used if the significant effect of diet or age were revealed. Further multivariate principal component analysis (PCA) was performed for this chapter as well by incorporating the brain protein expression data. Covariance matrices were used for the PCA, and as a rotation method, Varimax was utilized.

CHAPTER 3

THE EFFECTS OF GENETIC CHOLINERGIC MANIPULATION ON SYNAPTIC, CELLULAR AND INFLAMMATORY PROTEIN LEVELS WITHIN THE CONTEXT OF AGING

3.1 General body parameters were comparable between *ache*$^{sb55/+}$ mutants and wild-type zebrafish, while *ache*$^{sb55/+}$ mutants had lower brain weight at the younger ages

This mutant model was created by using genetic interventions [50]. Because the affected gene was expressed widely in the neuromuscular junctions, general body parameters were assessed in the mutants to demonstrate any potential alterations in overall body parameters. A significant main effect of age was shown on the body weight measure, $\chi^2(1)=17.177$, $p<0.0005$. At older ages, zebrafish had significantly higher body weights. This increase depending on age, was statistically significant in *ache*$^{sb55/+}$ mutants ($p= 0.001$), while this difference was marginally significant in the wild-type group ($p= 0.070$), Figure 3.1. Besides, the marginally significant main effect of genotype was demonstrated; *ache*$^{sb55/+}$ mutants tend to have lower total body weight than the control group, $\chi^2(1)=2.364$, $p=0.054$.

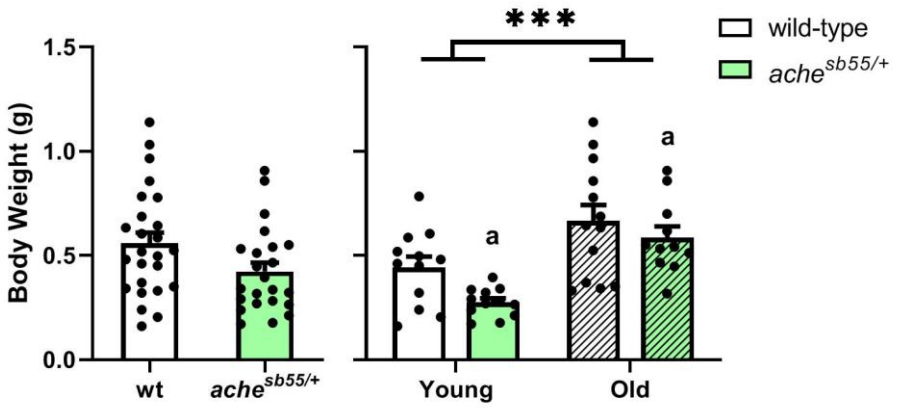


Figure 3.1 Body weight assessments across genotype and age groups. A significant main effect of age was shown altering body weight. a: Significant main effect of age was observed in a genotype group. ***: $p < 0.005$. Error bars = +Standard Error Mean.

Body length measure was also compared between the age and genotype groups to show mutation is not altering the developmental dynamics significantly. The effect of age was statistically significant, and at the older ages, zebrafish had significantly increased body length, $\chi^2(1)=22.003$, $p<0.0005$. Pairwise comparisons indicated that this age effect was significantly changing the body length in wild-type animals ($p= 0.020$) and *ache*$^{sb55/+}$ mutants ($p< 0.0005$), Figure 3.2. In terms of genotype effects, no significant difference was revealed, $\chi^2(1)=1.276$, $p= 0.259$. These observations indicate that mutation is not severely altering the developmental dynamics, and mutants had comparable body parameters to the control group.

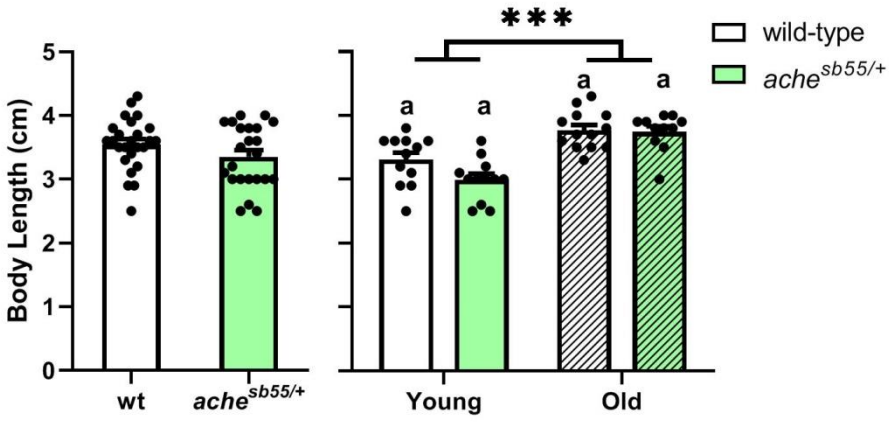


Figure 3.2 Body length assessments across genotype and age groups. A significant main effect of age was shown altering body length. a: Significant main effect of age was observed in a genotype group. ***: $p < 0.005$. Error bars = +Standard Error Mean.

Besides body weight and length measures, body mass index (BMI) was calculated and compared between the groups. It was revealed that age significantly altered the BMI scores, $\chi^2(1)=8.215$, $p=0.004$. Multiple comparisons indicated that this significant increase was mostly contributed by *ache*$^{sb55/+}$ mutants in which old animals had significantly higher BMIs ($p=0.008$). In contrast, in the wild-type group, no significant difference was observed in BMI scores ($p= 0.268$), Figure 3.3. The genotype effect was not significantly changing the BMI values across the groups, $\chi^2(1)=2.364$, $p=0.124$.

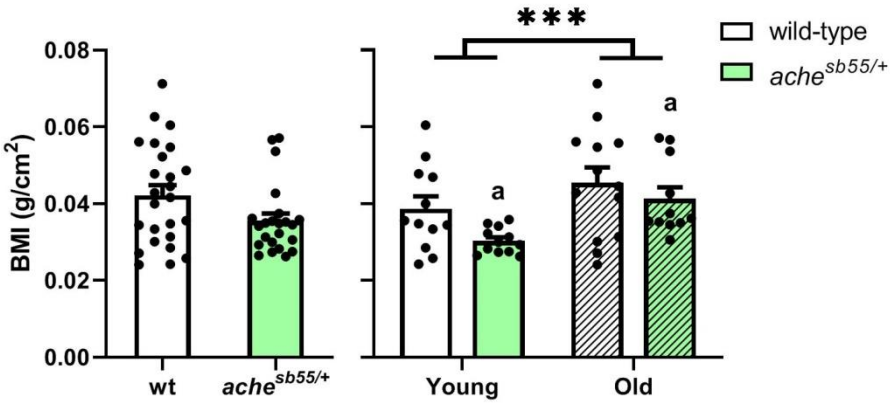


Figure 3.3 Body mass indices (BMI) across genotype and age groups. A significant main effect of age was shown altering BMIs. a: Significant main effect of age was observed in a genotype group. ***: $p < 0.005$. Error bars = +Standard Error Mean.

As the last body parameter, wet brain weights were compared between the groups. An age effect was statistically significant, with older animals having increased wet brain weight measures than the young group, $\chi^2(1)=9.074$, $p=0.003$. It was indicated that old mutants had significantly higher brain weight than the young mutant group ($p= 0.020$), and this age difference was not significantly affecting the wild-type group ($p= 0.248$), Figure 3.4. Additionally, a significant main effect of genotype was demonstrated on the brain weight, $\chi^2(1)=9.531$, $p=0.002$. The mutant group had lower brain weight measures compared to the wild-type group. Pairwise comparison indicated that genotype affected the brain weight at a young age significantly ($p= 0.016$), while no significant genotype differences were observed at old age ($p= 0.250$).

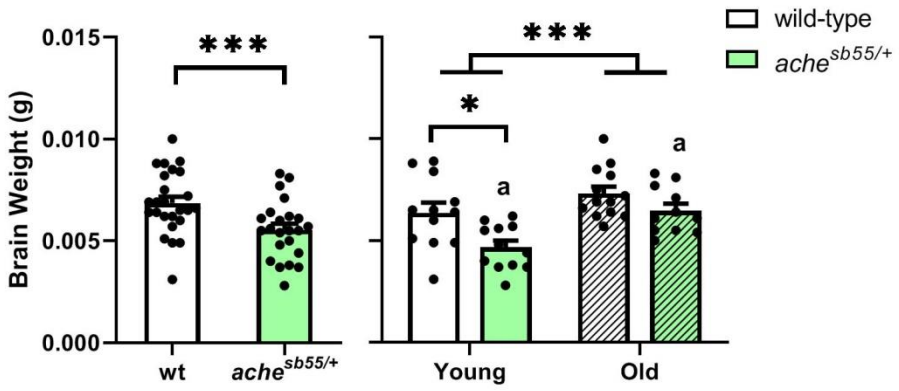


Figure 3.4 Brain weight measures across genotype and age groups. Significant main effects of age and genotype were shown, which altered brain weight. a: Significant main effect of age was observed in a genotype group. *: $p < 0.05$, ***: $p < 0.005$. Error bars = +Standard Error Mean.

Since gender-driven patterns were not so robust, in this chapter, males and females were clustered together in age and genotype groups, and their distribution was equal in each group. Body parameters were also checked for the factor of gender and previously reported results for the male and female differences were confirmed [82]. Gender significantly altered body weight, $\chi^2(1)=3.478$, $p=0.049$, and BMI, $\chi^2(1)=6.966$, $p=0.008$, with females with significantly higher body weight and BMI measures. Additionally, gender was significantly changing the brain weight, $\chi^2(1)=5.058$, $p=0.025$. Females had lower brain weight values compared to males. No significant effect of gender was seen in the body length measure, $\chi^2(1)=0.003$, $p=0.959$.

3.2 Protein markers of cholinergic neurotransmission were significantly altered in the brains of *ache*$^{sb55/+}$ mutants as compared to age-matched wild-type controls

Since the point mutation directly affected the acetylcholinesterase (AChE) coding gene *ache*, markers of cholinergic neurotransmission were investigated comparatively in the brains of *ache*$^{sb55/+}$ and the wild-type animals at both young and old ages. Three cholinergic markers have been assessed, including the main enzyme, AChE, responsible for the enzymatic deactivation of the acetylcholine in zebrafish brain [50]; ACh, which was the primary neurotransmitter acting in the cholinergic neurotransmission. Lastly, an alpha-7 subunit of the nicotinic ACh receptors (nAChR-a7), one of the predominant subunits of nicotinic ACh receptors in the zebrafish brain was investigated. This subunit had implications in cognitive processes [83].

On the AChE levels, a significant main effect of genotype was revealed, $F(1,20)=87.649$, $p<0.0005$. In each age and gender group, *ache*$^{sb55/+}$ animals had significantly lower levels of AChE. These groups in which significant effect of genotype was revealed with Bonferroni tests were including; young *ache*$^{sb55/+}$ as compared to wild-type ($p<0.0005$), and old *ache*$^{sb55/+}$ as compared to old wild-type groups ($p<0.0005$), Figure 3.5. These observations were parallel to the literature. Although there is no similar age by gender experimental design was published in the literature, Ninkovic et al. indicated that 3-6 months old female *ache*$^{sb55/+}$ animals had

significantly decreased AChE levels compared to their wild-type siblings[51]. Behra et al. showed that in heterozygous mutant embryos, AChE levels were significantly lower than the wild-type embryos[50]. Overall, the mutation is directly affecting the AChE coding *ache* gene. In all groups, a significant decrease in the mutants' AChE levels was observed independently from the age or gender of the animal. Additionally, there was a significant main effect of age altering the levels of AChE, $F(1,20)= 4.388$, $p=0.049$, Figure 3.5. Pairwise comparisons revealed no significantly different pairs regarding age difference, so it was an overall effect. At older ages, animals had lower levels of brain AChE, which can have possible implications on age-dependent cholinergic hypofunction as proposed in the literature[28]. Furthermore, no significant interaction was found between the factors of age and genotype, $F(1,20)=0.378$, $p=0.546$.

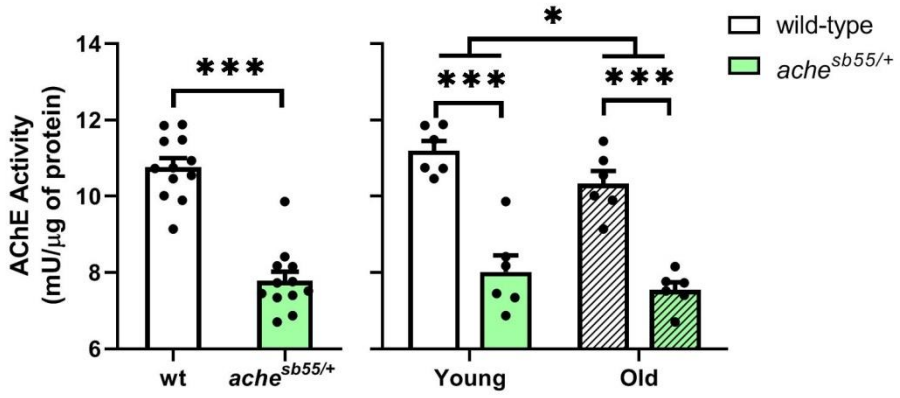


Figure 3.5 Brain AChE levels across genotype and age groups. Significant main effects of genotype and age were shown altering the levels of AChE. *: $p < 0.05$, ***: $p < 0.005$. Error bars = +Standard Error Mean.

On the acetylcholine (ACh) levels, a significant main effect of genotype was observed, $F(1,20)=5.033$, $p=0.036$. Mutant animals had lower ACh levels than wild-type controls; posthoc analyses revealed no significantly different pairs at young or old ages when both genders were merged, Figure 3.6. Moreover, there was no significant main effect of age, $F(1,20)= 3.363$, $p=0.082$, and age by genotype interaction, $F(1,20)=0.056$, $p=0.815$, on ACh levels. It was expected that since *ache*$^{sb55/+}$ mutants have significantly lower levels of AChE due to the mutation, these animals tend to have increased levels of ACh. Because AChE is only functional cholinesterase responsible for the enzymatic degradation of the ACh, and when there are lower levels of AChE, neurotransmitter ACh will not be degraded and may result in increased levels of ACh. In the literature, only one study has measured the brain levels of ACh in *ache*$^{sb55/+}$ mutants. Ninkovic et al. indicated that in the 3-6 months old *ache*$^{sb55/+}$ females, there was a significant, approximately 1.5 fold, increase in the brain levels of ACh compared to their wild-type controls [51]. The difference between our results and Ninkovic et al. might be related to the age of the animals. In the current study, the young group was 10-11 months old, and the old group was 30-31 months old, while the age that Ninkovic et al. used was 3-6 months old fish [51]. There might be alteration with the course of development and aging, so 12 more *ache*$^{sb55/+}$ mutants and wild-type controls which were 3.5-5 months old were incorporated into the current work to understand the observed differential pattern.

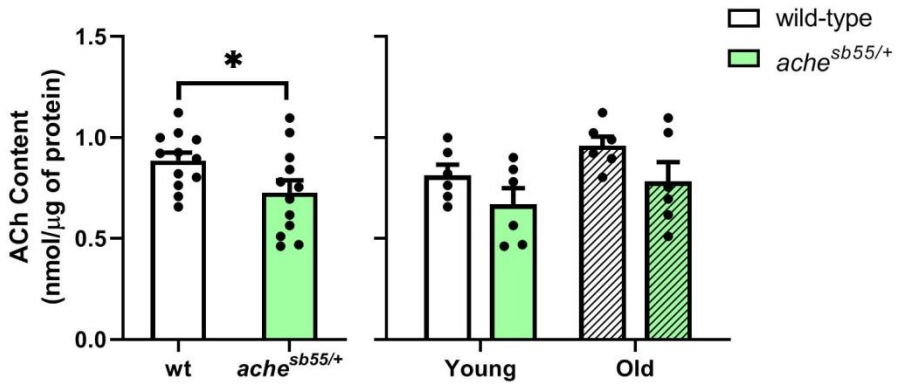


Figure 3.6 Brain ACh levels across genotype and age groups. A significant main effect of genotype was shown altering the levels of ACh. *: $p < 0.05$. Error bars = +Standard Error Mean.

ACh and AChE assays were performed with samples from 3.5-5 months old *ache*$^{sb55/+}$ and the wild-type fish. Data merged with gender groups segregated since Ninkovic et al. was utilized the female animals only in their study [51]. On the AChE levels, a significant main effect of the genotype was confirmed again, $F(1,24)=127.096$, $p<0.0005$. Compared to their wild-type control *ache*$^{sb55/+}$ mutants had significantly lower levels of AChE activity; genotype effect was significant in all age and gender groups including; 3.5-5 months old females ($p<0.0005$); 3.5-5 months old males ($p<0.0005$); 10-11 months old females ($p=0.001$); 10-11 months old males ($p<0.0005$); 30-31 months old females ($p=0.002$) and 30-31 months old males ($p=0.001$), Figure 3.7. Moreover, a significant main effect of age was also revealed after the inclusion of 3.5-5 months old groups, $F(2,24)=64.745$, $p<0.0005$. Overall, the age difference was driven by the 3.5-5 months old group; in the other age groups, there was no significant effect of the age. In female wild-type groups, 3.5-5 months

old animals had significantly higher AChE activity compared to 10-11 months old (p<0.0005) and 30-31 months old (p<0.0005) groups. Likewise, in female *ache*$^{sb55/+}$ groups, 3.5-5 months old animals had significantly higher AChE activity compared to 10-11 months old (p=0.01) and 30-31 months old (p<0.0005) groups. Similar patterns were observed for both *ache*$^{sb55/+}$ and wild-type males; 3.5-5 months old group had significantly higher AChE activity compared to 10-11 months old group in wild-type (p<0.0005) and *ache*$^{sb55/+}$ (p=0.002) fish; and 30-31 months old group in wild-type (p<0.0005) and *ache*$^{sb55/+}$ (p=0.003), Figure 3.7. No significant gender effect was observed in the data, $F(1,24)=0.087$, $p=0.770$. Furthermore, no significant interaction was demonstrated among the factors including genotype by age, $F(2,24)=2.645$, $p=0.092$; genotype by gender, $F(1,24)=0.989$, $p=0.330$; age by gender, $F(2,24)=0.857$, $p=0.437$; and genotype by age by gender, $F(2,24)=0.047$, $p=0.954$).

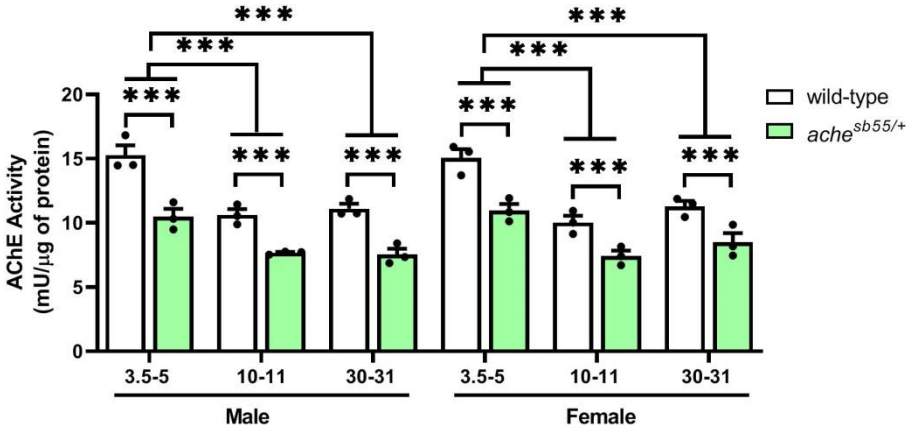


Figure 3.7 Brain AChE levels across genotype and age groups with very young age was incorporated. Significant main effects of genotype and age were shown altering the levels of AChE. ***: $p < 0.005$. Error bars = +Standard Error Mean.

On the ACh levels, a significant main effect of genotype was revealed, $F(1,24)=5.626$, $p=0.026$, Figure 3.8. Genotype effect was only significant in the female 30-31 months old group ($p=0.012$). Moreover, a significant main effect of age was revealed on the ACh data, $F(2, 24)=19.843$, $p<0.0005$, Figure 3.8. The age effect was observed between wild-type females between 3.5-5 months old and 30-31 months old groups ($p<0.0005$); wild-type males between 3.5-5 months old and 10-11 months old ($p=0.046$) and 3.5-5 months old and 30-31 months old ($p=0.036$); and lastly in *ache*$^{sb55/+}$ males between 3.5-5 months old and 10-11 months old ($p=0.011$) and 3.5-5 months old and 30-31 months old ($p=0.002$) groups, Figure 3.4. Also, there was no significant main effect of gender on the ACh levels, $F(1,24)=.163$, $p=0.690$; in addition to no significant interaction among the factors including genotype by age, $F(2,24)=0.764$, $p=0.477$; genotype by gender, $F(1,24)=0.218$, $p=0.645$; age by gender, $F(2,24)=1.761$, $p=0.193$; and genotype by age by gender, $F(2,24)=2.855$, $p=0.077$.

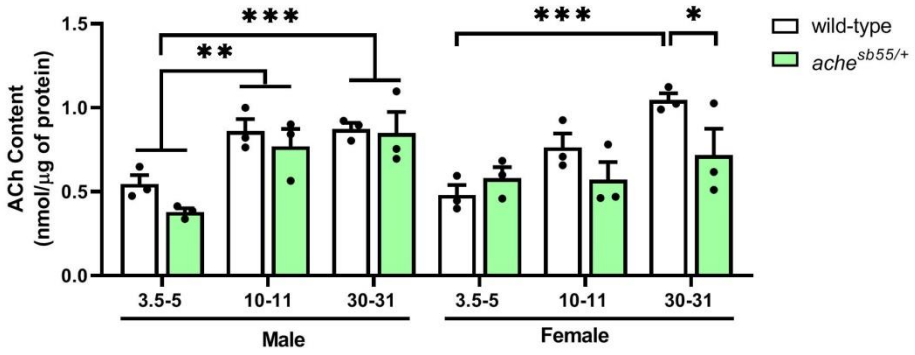


Figure 3.8 Brain ACh levels across genotype and age groups with very young age was incorporated. Significant main effects of genotype and age were shown

altering the levels of ACh. *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.005$. Error bars = +Standard Error Mean.

In addition to the primary neurotransmitter and its deactivator enzyme involved in the cholinergic system, nAChR-a7 was assessed among the age and genotype groups. On the brain levels of nAChR-a7, a marginally significant main effect of genotype was observed, $F(1,20)=3.647$, $p=0.070$, as *ache*$^{sb55/+}$ animals having lower levels of nicotinic nAChR-a7 compared to wild-type animals, and this difference was more pronounced at the younger ages, Figure 3.9. The effect of age was not significantly altering the levels of nAChR-a7, $F(1,20)= 1.430$, $p=0.246$. Lastly, no significant interaction was demonstrated between age and genotype, $F(1,20)=1.271$, $p=0.273$.

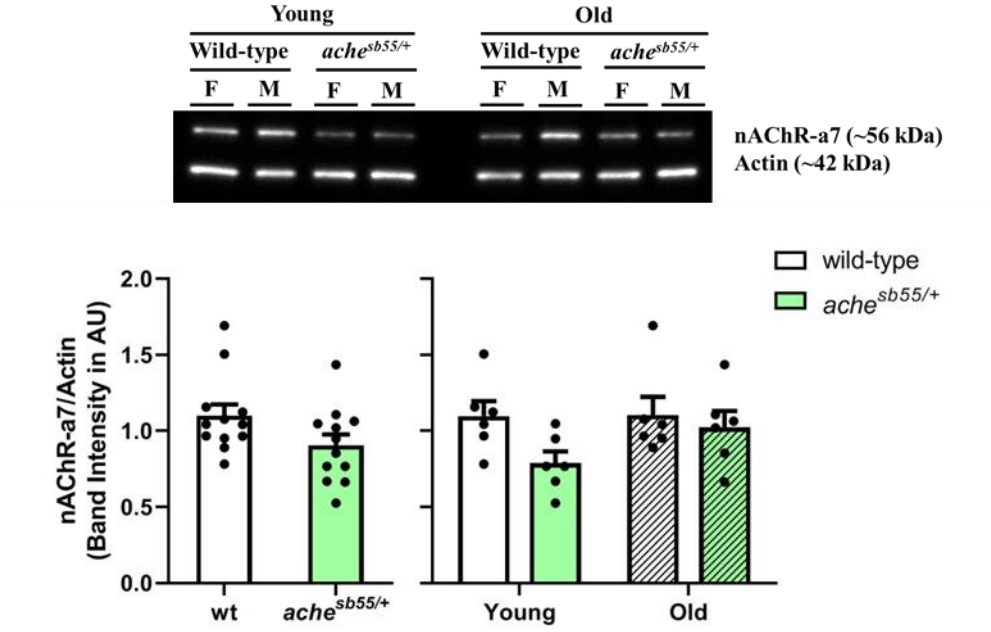


Figure 3.9 Representative western blot image was indicated for nAChR-a7 antibody. nAChR-a7 levels across genotype and age groups. Error bars = +Standard Error Mean. F: Female; M: Male.

3.3 Excitatory, inhibitory, and presynaptic protein levels were affected in *ache*$^{sb55/+}$ mutants differentially in age and gender-dependent way

Samples used for the analyses of cholinergic markers were also tested for the examinations of synaptic proteins. The levels of Post-synaptic Density-95 (PSD-95), Glutamate receptor subunits 2 and 3 (GluR2/3), N-methyl D-aspartate receptor subtype 2B (NR2B), Gephyrin (GEP), Gamma-aminobutyric acid receptor subunit alpha-1 (GABA-A-a1), and Synaptophysin (SYP) were assessed. Representative blots were demonstrated in Figure 3.10 for synaptic markers.

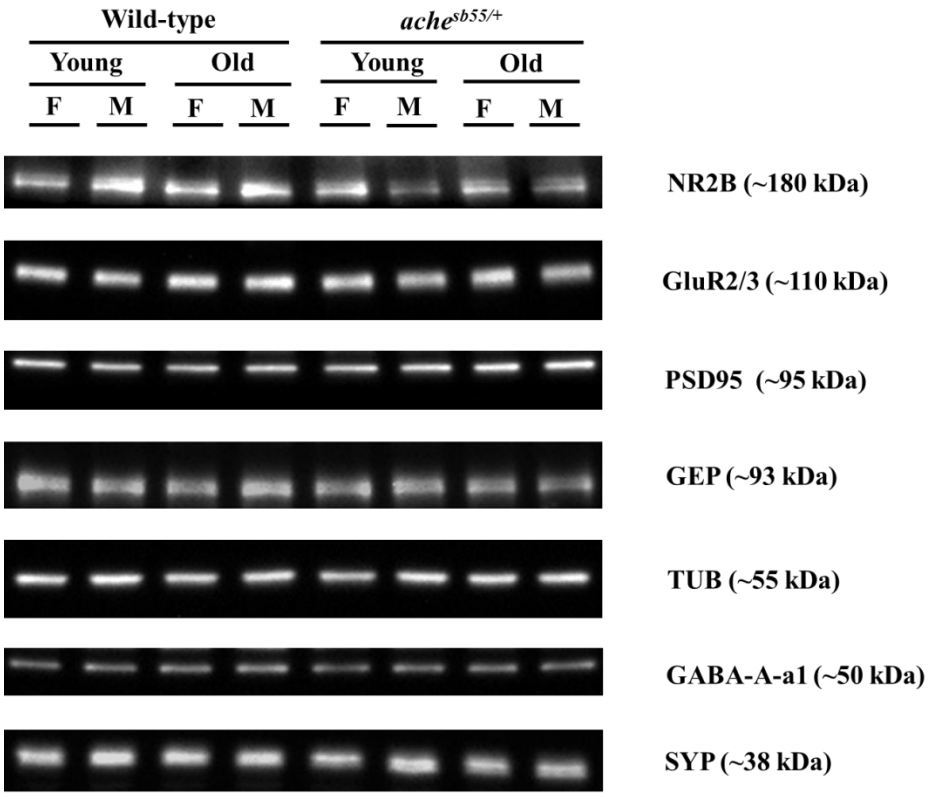


Figure 3.10 Representative pictures from one cohort for Western blot experiments of synaptic protein levels examined in the current Chapter for effects of age and genotype. Bands were obtained at the expected molecular weights for all antibodies. F: Female; M: Male.

There was no significant main effect of genotype on PSD95 levels, which was the primary scaffolding and clustering protein found at excitatory glutamatergic post-synaptic regions, $F(1,20)=2.174$, $p=0.156$, Figure 3.11. Additionally, there was no significant effect of age altering the levels of PSD95, $F(1,20)= 1.584$, $p=0.223$. Moreover, no significant interaction was revealed between the factors of genotype and age, $F(1,20)=1.474$, $p=0.239$, Figure 3.11.

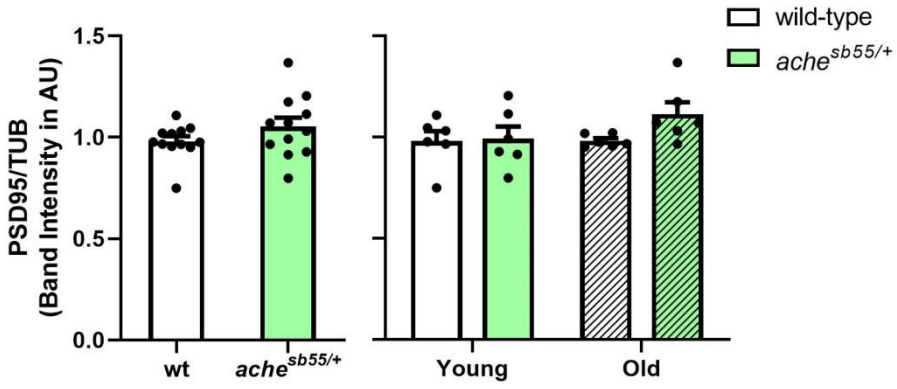


Figure 3.11 PSD95 levels across genotype and age groups. Error bars = +Standard Error Mean.

On the GluR2/3 levels, which are the subunits of AMPA type glutamate receptors and control the calcium regulation significant main effect of genotype was revealed, $F(1,20)=5.265$, $p=0.033$. Multiple comparisons demonstrated no significant

change in the young group with genotype as the levels of GluR2/3 in young wild-type animals were not significantly different from young *ache*$^{sb55/+}$ animals (p=0.521). On the other hand effect of genotype was statistically significant at the old age; the old *ache*$^{sb55/+}$ group had significantly lower levels of GluR2/3 compared to wild-type old animals (p=0.017), Figure 3.12. There was no significant main effect of age altering the GluR2/3 levels, $F(1,20)= 0.446$, p=0.512, and no significant genotype by age interaction was found as well, $F(1,20)=1.880$, p=0.186, Figure 3.12.

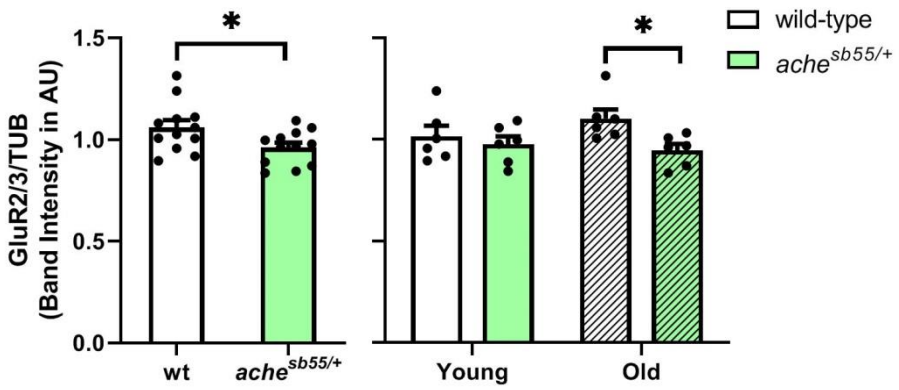


Figure 3.12 GluR2/3 levels across genotype and age groups. GluR2/3 levels were altered significantly by genotype. *: $p < 0.05$. Error bars = +Standard Error Mean.

NR2B was one of the main subtypes of NMDA receptors and on its levels. On NR2B levels, a significant main effect of genotype was observed, $F(1,20)=13.816$, p=0.001, Figure 3.13. Moreover, this genotype difference was significant between old wild-type and *ache*$^{sb55/+}$ groups (p=0.007), and at a young age, the difference between young wild-type and young *ache*$^{sb55/+}$ groups was also statistically significant (p=0.036). Overall, NR2B data indicated that *ache*$^{sb55/+}$ animals tend to have lower

levels of NR2B compared to the wild-type controls. However, expression of NR2B seems to be more stable through aging, $F(1,20)= 0.132$, $p=0.720$, and no significant genotype by age interaction was revealed, $F(1,20)=0.288$, $p=0.597$, Figure 3.13.

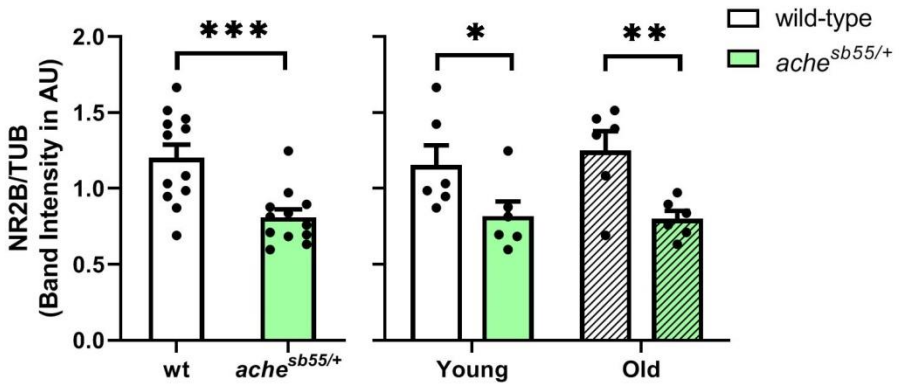


Figure 3.13 NR2B levels across genotype and age groups. NR2B levels were altered significantly by genotype. *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.005$. Error bars = +Standard Error Mean.

SYP was analyzed as a marker of pre-synaptic integrity. The genotype effect was not statistically significant on the SYP levels, $F(1,20)=0.001$, $p=0.978$, Figure 3.14. On the other hand, there was a significant main effect of age, altering SYP levels, $F(1,20)= 6.435$, $p=0.020$. Pairwise comparisons did not demonstrate a significant age difference in the wild-type group ($p=0.223$); on the other hand, the age effect was significant in the *ache*$^{sb55/+}$ mutants; as older mutants had significantly higher SYP levels compared to young mutants, Figure 3.14. Therefore, it could be said that the mutants rather than the wild-type group majorly drive the age effect. Lastly, the interaction between the age and genotype was not significant, $F(1,20)=0.639$, $p=0.434$.

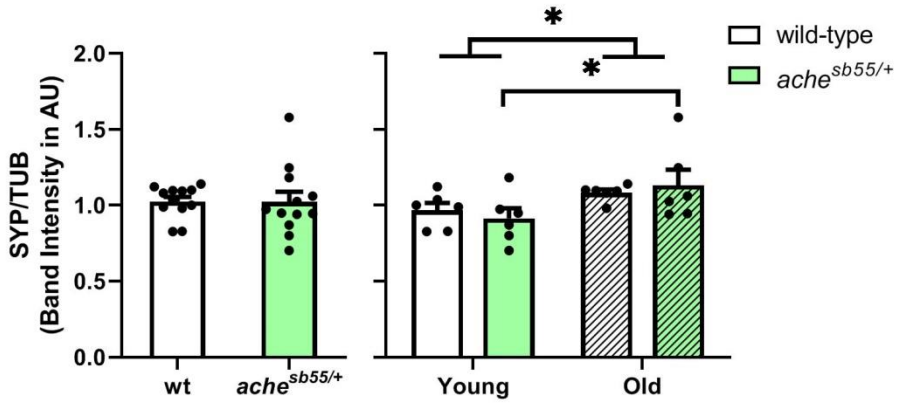


Figure 3.14 SYP levels across genotype and age groups. SYP levels were altered significantly by age. *: $p < 0.05$. Error bars = +Standard Error Mean.

On the levels of GEP, which is the main clustering protein found at inhibitory GABAergic synapses, no significant main effect of genotype was demonstrated, $F(1,20)=0.000$, $p=0.982$. The main effect of age was marginally significant on the GEP levels, $F(1,20)= 3.936$, $p=0.061$, while there was no significant genotype by age interaction, $F(1,20)=2.058$, $p=0.167$, Figure 3.15.A. Moreover, gender-specific modulations were observed on the levels of GEP, and additionally, data were analyzed with three-way ANOVA as well. Three-way ANOVA demonstrated a significant main effect of age, $F(1,16)=5.891$, $p=0.027$, Figure 3.15.B. With aging, GEP levels were reduced specifically in the wild-type genotype; Bonferroni tests indicated a significant reduction in GEP levels ($p=0.043$). Moreover, a significant gender by genotype interaction was revealed on the GEP levels, $F(1,16)= 9.897$, $p=0.006$. In *ache*$^{sb55/+}$ females, GEP levels were relatively stable with increasing age. In contrast, there was a significant reduction in the wild-type females, and at younger ages, *ache*$^{sb55/+}$ females had significantly lower levels of GEP compared to wild-type

females (p=0.007), and *ache*$^{sb55/+}$ females' GEP levels tend to be maintained. On the other hand, *ache*$^{sb55/+}$ males at the younger ages tend to have higher levels of GEP compared to the young female *ache*$^{sb55/+}$ group (p=0.004). It could be said that in *ache*$^{sb55/+}$ mutants, gender-specific maintenance or alterations on GEP levels are implicated.

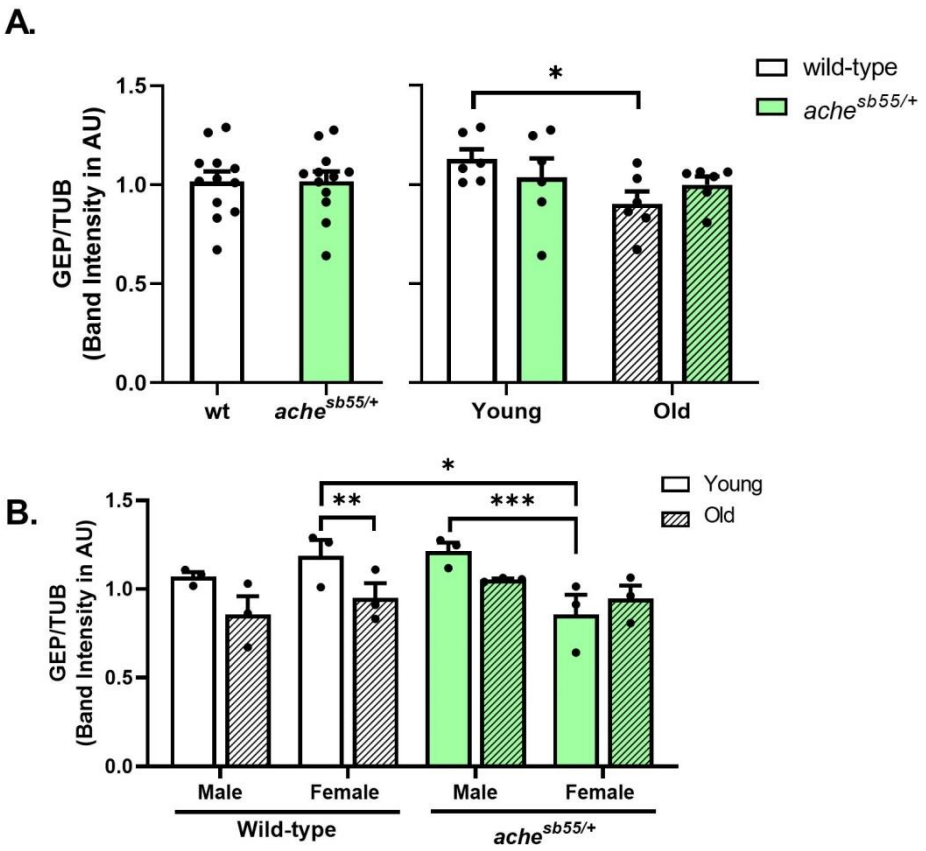


Figure 3.15 GEP levels across genotype and age groups (A). Effect of age and gender by genotype interaction was demonstrated (B). *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.005$. Error bars = +Standard Error Mean.

GABA-A-a1, which was a subunit of the $GABA_A$ receptor, was investigated as an indicator of inhibitory neurotransmission. On the levels of GABA-A-a1, no

significant main effects of age, $F(1,20)= 0.889$, $p=0.357$, or genotype, $F(1,20)=0.289$, $p=0.597$, and no significant genotype by age interaction was shown, $F(1,20)=0.278$, $p=0.604$, Figure 3.16.A. However, as in the case of the GEP, gender-specific modulations were also observed on the levels of GABA-A-a1. Additionally, data were analyzed with three-way ANOVA as well. Three-way ANOVA demonstrated a significant main effect of gender, $F(1,16)= 6.417$, $p=0.022$, Figure 3.16. Overall, males had higher levels of GABA-A-a1, but *ache*$^{sb55/+}$males mainly drive the effect. Post-hoc comparisons indicated that the gender effect was significant between young *ache*$^{sb55/+}$ females and young *ache*$^{sb55/+}$ males ($p=0.015$); young *ache*$^{sb55/+}$males have significantly higher levels of GABA-A-a1, Figure 3.16.B.

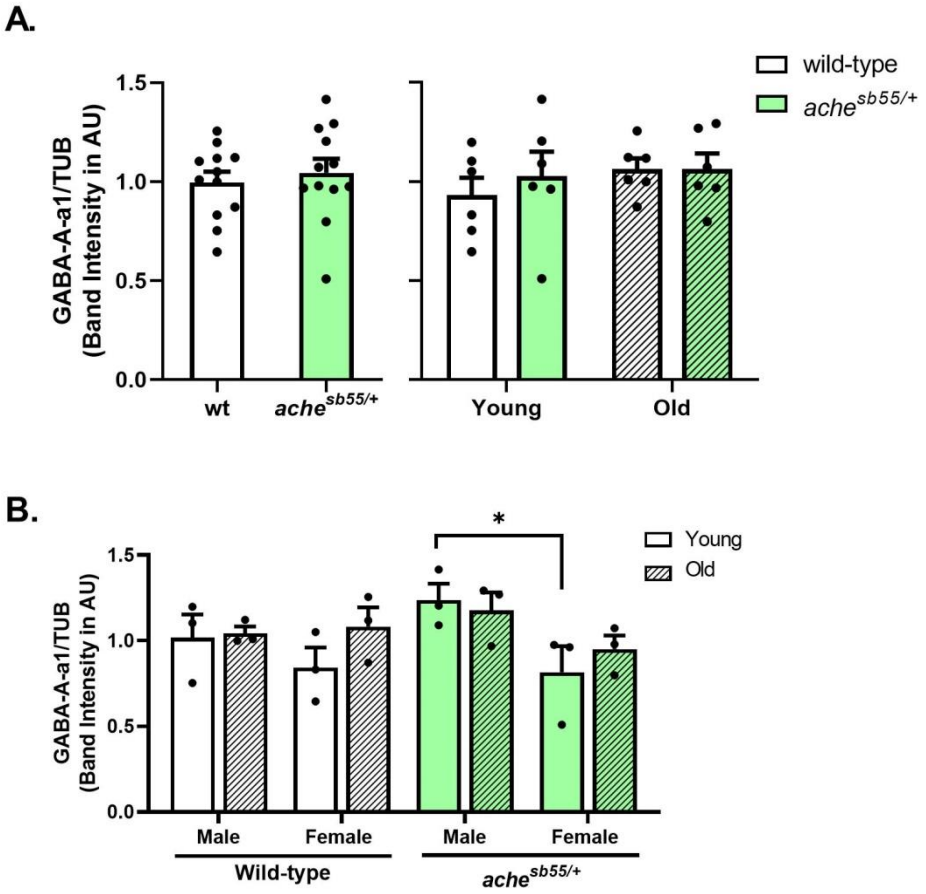


Figure 3.16 GABA-A-a1 levels across genotype and age groups (A). The effect of gender was demonstrated (B). *: $p < 0.05$. Error bars = +Standard Error Mean.

3.4 The brain levels of the immature neuronal marker were promoted while glial marker was downregulated slightly in the *ache*$^{sb55/+}$ mutants

Alterations on the synaptic markers dependent on the genotype of the animal were demonstrated as a following step cellular integrity markers were investigated within the context of aging and genotype. Neuronal markers, embryonic lethal, abnormal vision (ELAV; Drosophila) like 3 (HuC) and doublecortin like kinase 1 (DCAMKL1), a glial marker, glial fibrillary acidic protein (GFAP), a proliferation marker, proliferating cell nuclear antigen (PCNA) were investigated within this context. Representative blots for cellular markers were indicated in Figure 3.17.

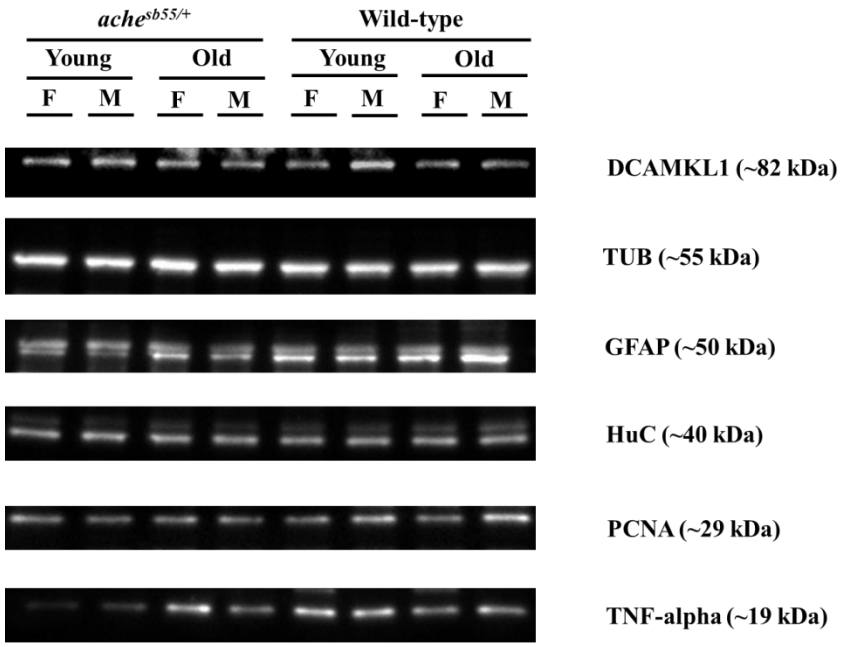


Figure 3.17 Representative pictures from one cohort for Western blot experiments of cellular and inflammatory protein levels examined in the current

Chapter for effects of age and genotype. Bands were obtained at the expected molecular weights for all antibodies. F: Female; M: Male.

HuC levels were investigated as an indicator of the immature neuronal population. Age was not significantly altering the global protein levels of HuC in the brain, $F(1,20)= 0.802$, $p=0.381$, Figure 3.18. However, a significant main effect of genotype was revealed in HuC levels, $F(1,20)=10.377$, $p=0.004$. *ache*$^{sb55/+}$ mutants have significantly elevated levels of HuC compared to the wild-type group, and this genotype difference was significant in the young group ($p=0.022$) and significant in the old group ($p=0.050$). Moreover, no significant interactions were found on the HuC data, $F(1,20)=0.077$, $p=0.785$.

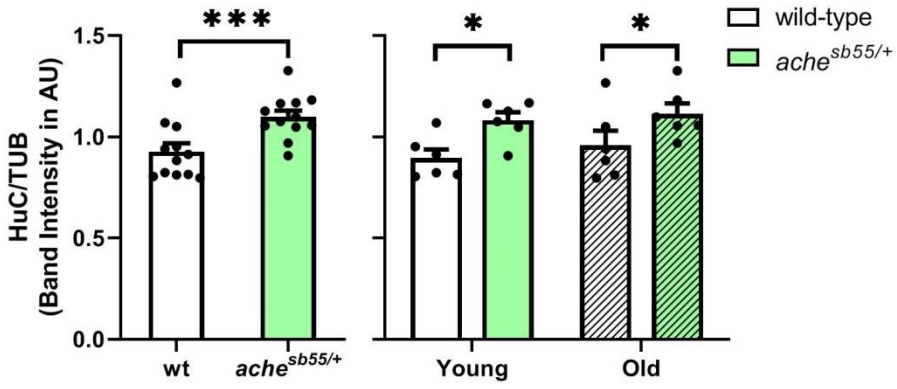


Figure 3.18 HuC levels across genotype and age groups. Effect of genotype was demonstrated. *: $p < 0.05$, ***: $p < 0.005$. Error bars = +Standard Error Mean.

DCAMKL1 was used as a post-mitotic neuronal marker which is expressed in migrating neurons. No significant main effect of age, $F(1,20)= 0.407$, $p=0.531$, or

genotype, $F(1,20)=2.687$, $p=0.117$, was observed in the DCAMKL1 data, while there was no significant genotype by age interaction, $F(1,20)=2.479$, $p=0.131$, Figure 3. 19.

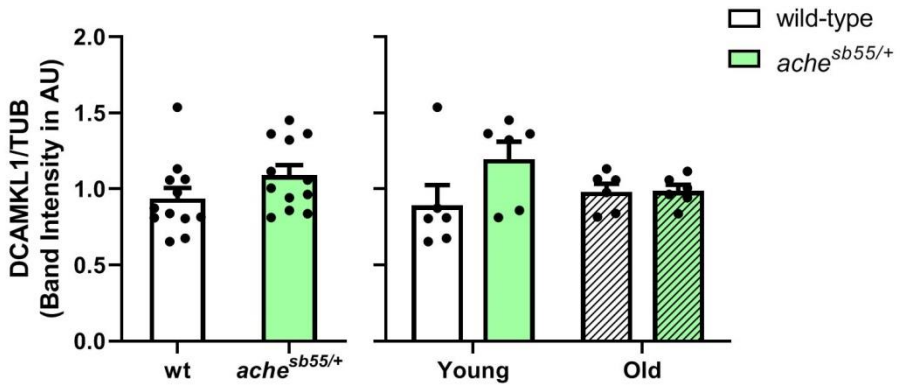


Figure 3.19 DCAMKL1 levels across genotype and age groups. Error bars = +Standard Error Mean

GFAP was used as a glial marker to gain insights regarding the glial population concerning age and genotype. Main effect of age, $F(1,20)= 0.787$, $p=0.385$, was not statistically significant. However, the genotype effect was marginally significant, $F(1,20)=4.111$, $p=0.056$, and *ache*$^{sb55/+}$ animals had lower levels of GFAP compared to wild-type animals, Figure 3.20. No statistically significant interaction was observed between age and genotype, $F(1,20)=0.063$, $p=0.805$.

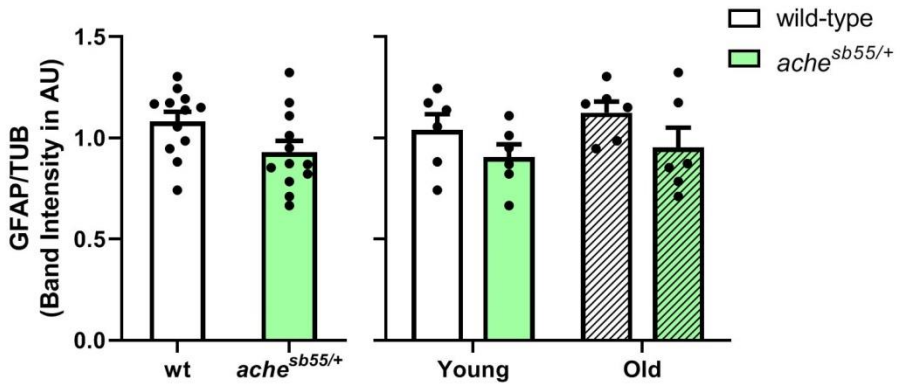


Figure 3.20 GFAP levels across genotype and age groups. Error bars = +Standard Error Mean

As a last cellular marker, PCNA was used as a global proliferation marker. In the PCNA levels, no significant main effect of age was demonstrated, $F(1,20)= 0.065$, $p=0.801$. The genotype effect was not statistically significant, $F(1,20)=0.435$, $p=0.517$. Moreover, there was no significant interaction between genotype and age altering the levels of PCNA, $F(1,20)=2.010$, $p=0.172$, Figure 3.21.

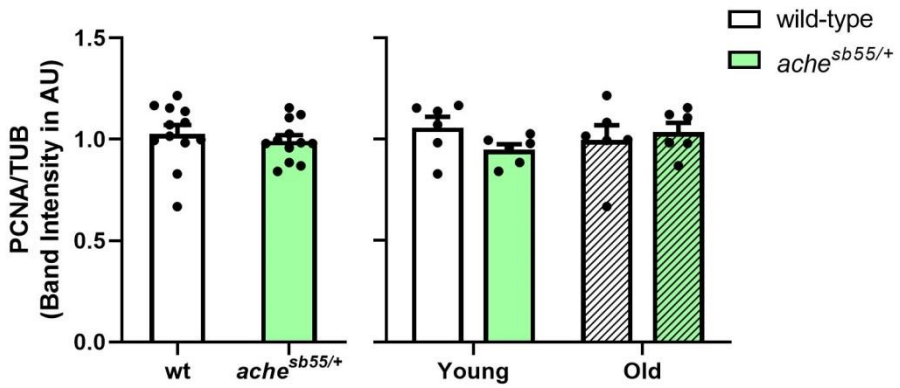


Figure 3.21 PCNA levels across genotype and age groups. Error bars = +Standard Error Mean

3.5 Subtle decreases were observed on the free radical content in the brain of *ache*$^{sb55/+}$ mutants while no evident alterations in the brain levels of inflammatory protein

To understand whether mutation and reduced levels of AChE can have further implications of the inflammatory profile in the brain, and indicators of inflammation were assessed. The first marker was tumor necrosis factor-alpha (TNF-alpha), a pro-inflammatory protein associated with increased inflammation. The second indicator was reactive oxygen species (ROS) activity which can demonstrate the free-radical content and elevated levels can have further implications on increased inflammation.

TNF-alpha was used as an indicator of increased inflammation. On its levels no significant main effect age was demonstrated, $F(1,20)= 2.180$, $p=0.155$. Additionally, there was no significant main effect of genotype altering the levels of TNF-alpha, $F(1,20)=1.120$, $p=0.302$, and interaction between the factors of genotype and age, $F(1,20)=0.007$, $p=0.934$, Figure 3.22.

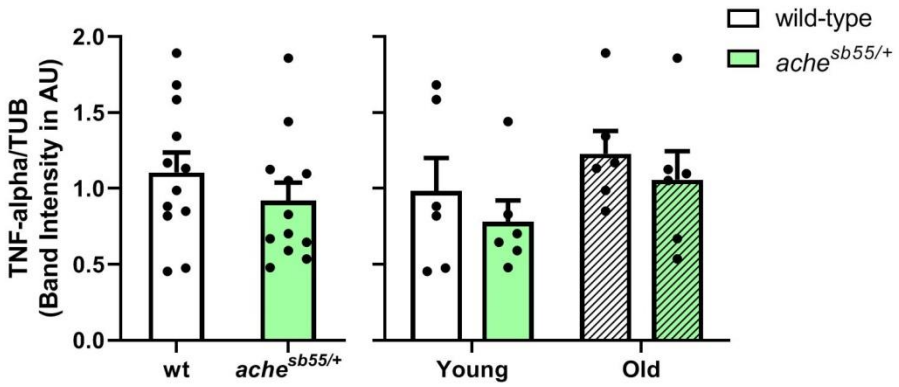


Figure 3.22 TNF-alpha levels across genotype and age groups. Error bars = +Standard Error Mean

In the ROS levels main effect of genotype, $F(1,20)=0.326$, $p=0.575$, and age, $F(1,20)= 2.896$, $p=0.104$, were not statistically significant and no significant interaction was observed between age and genotype, $F(1,20)=1.086$, $p=0.310$. However, in the case of ROS activity levels, gender-dependent modulations were observed, and in addition to two-way ANOVA, a three-way ANOVA was also utilized. Three-way ANOVA revealed no significant main effect of genotype, $F(1,16)=0.440$ $p=0.517$, and gender, $F(1,16)= 3.200$, $p=0.093$. On the other hand, the main effect of age was marginally significant, $F(1,16)=3.910$, $p=0.066$; at older ages, animals tended to have increased ROS activity levels, Figure 3.23. Interestingly, a significant gender by genotype interaction was revealed on ROS activity levels, $F(1,16)=6.281$, $p=0.023$. Simple effects analysis was carried out to break this significant interaction into its parts. It was found that the effect of gender was significant in wild-type animals ($p=0.008$) in which males have higher ROS activity compared to females, Figure 3.23. Moreover, the effect of genotype in the male

animals was significant (p=0.040); male *ache*$^{sb55/+}$ mutants tend to have lower ROS activity levels compared to wild-type males, Figure 3.23. Interactions among other factors were not statistically significant (Genotype by Age: $F(1,16)=1.466$, p=0.244; Age by Gender: $F(1,16)=0.554$, p=0.468; Age by Gender by Genotype: $F(1,16)=0.961$, p=0.341).

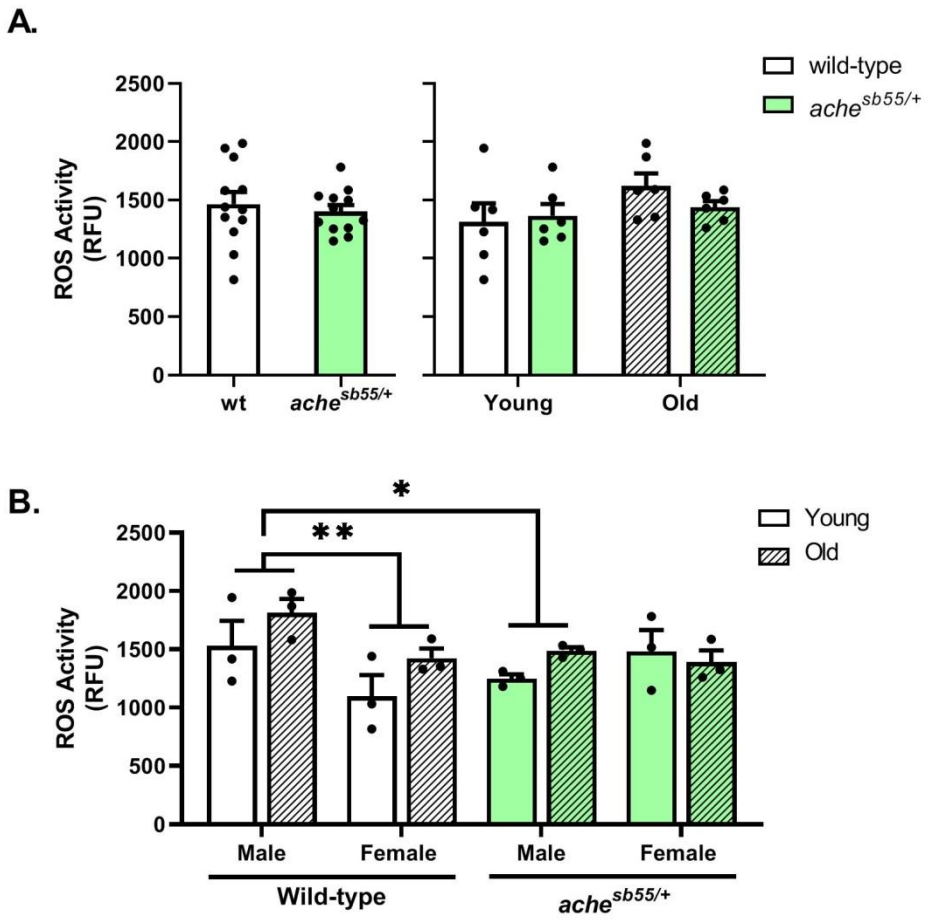


Figure 3.23 ROS activity levels across genotype and gender groups. Gender groups collapsed together (A). Age, gender, and genotype groups were separated gender by genotype interaction was revealed (B). *: $p < 0.05$, **: $p < 0.01$. Error bars = +Standard Error Mean

3.6 Multivariate analyses revealed genotype and age-dependent clustering patterns on the synaptic, cellular, and inflammatory proteins

Correlation analyses were conducted among the synaptic, cellular, and inflammatory proteins. Significant correlations were revealed among these proteins. First correlations driven by the cholinergic elements can be analyzed; the ACh levels were positively correlated with cholinergic receptor subunit nAChR-a7 ($r(22)=0.556$, $p=0.005$) and also positive correlations were found between ACh and glial marker GFAP ($r(22)=0.637$, $p=0.001$) and inflammatory marker TNF-alpha ($r(22)=0.532$, $p=0.007$). Another cholinergic marker, AChE, was positively correlated with excitatory marker NR2B ($r(22)=0.466$, $p=0.022$); and it was negatively and significantly correlated with immature neuronal marker HuC ($r(22)=-0.597$, $p=0.002$). The last cholinergic marker which was the receptor subunit nAChR-a7 was positively correlated with presynaptic marker SYP ($r(22)=0.420$, $p=0.041$), glial marker GFAP ($r(22)=0.727$, $p<0.0005$) and inflammatory marker TNF-A ($r(22)=0.589$, $p=0.002$). Among the excitatory markers, a significant and positive correlation was found between GluR2/3 and NR2B ($r(22)=0.469$, $p=0.021$), while GluR2/3 was negatively correlated with PSD95 ($r(22)=-0.460$, $p=0.024$). Additionally, excitatory marker PSD95 was positively correlated with presynaptic marker SYP ($r(22)=0.596$, $p=0.002$). A significant positive correlation was observed between the two inhibitory markers GEP and GABA-A-a1 ($r(22)=0.416$, $p=0.043$), and also it was demonstrated that GABA-A-a1 was positively correlated with pre-synaptic element SYP ($r(22)=0.508$, $p=0.011$). When the cellular markers were assessed, two neuronal markers HuC and DCAMKL1, were positively and

significantly correlated ($r(22)=0.478$, $p=0.018$). Moreover, inflammatory marker TNF-alpha was positively and significantly correlated with glial marker GFAP ($r(22)=0.689$, $p<0.0005$) and excitatory marker NR2B ($r(22)=0.430$, $p=0.036$). Correlation maps can be seen in Figure 3.24.

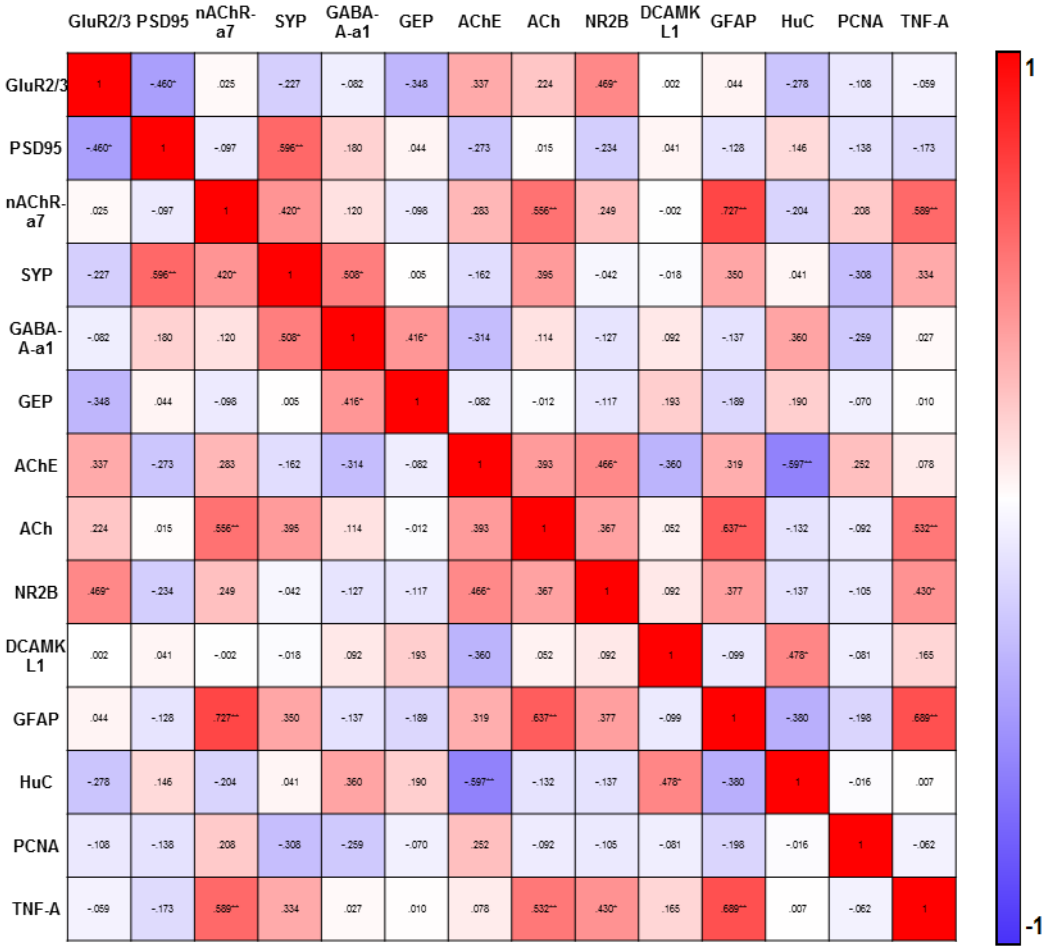


Figure 3.24 Analyses revealed significant correlations among the proteins. *: $p<0.05$, **: $p<0.001$.

As a further step, principal component analysis was conducted to understand the data in a reduced dimension and clustering pattern of the proteins of interest. Two components were extracted; in total, these two components were explaining 46.6% of

the total variance. The extracted first component (PC1) was associated with 26.5% of the variance, and this component was controlled by nAChR-a7, ACh, GFAP, and TNF-alpha. The other component, PC2, was associated with a 20.1% variance in the data and controlled by GluR2/3 and NR2B positively and negatively contributed by PSD95 and SYP, Figure 3.25. Scores for individual data points were visualized for PC1 and PC2, and age groups were shown by the different panels in Figure 3.26. It can be observed that genotype segregation was very prominent in PC1. PC1 was associated with the cholinergic markers ACh and receptor subunit nAChR-a7 and also the inflammation-related markers GFAP and TNF-alpha; in the *ache*$^{sb55/+}$ mutants at both young and old ages, lower PC1 scores were observed genetic manipulations on AChE levels can also be associated with lower levels of the other cholinergic markers while inflammatory markers tended to be reduced with the lower levels of AChE.

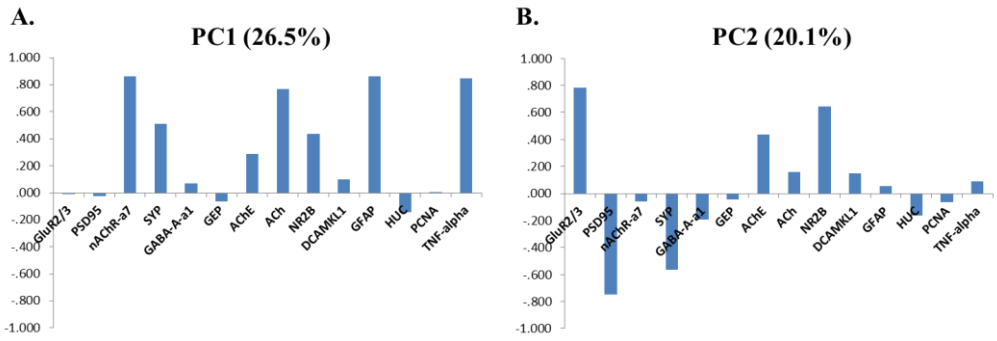


Figure 3.25 Factor loading scores for two principal components with respect to proteins. PC1 was contributed by nAChR-a7, ACh, GFAP and TNF-alpha positively (A). PC2 was contributed by GluR2/3 and NR2B positively while negatively controlled by PSD95 and SYP (B).

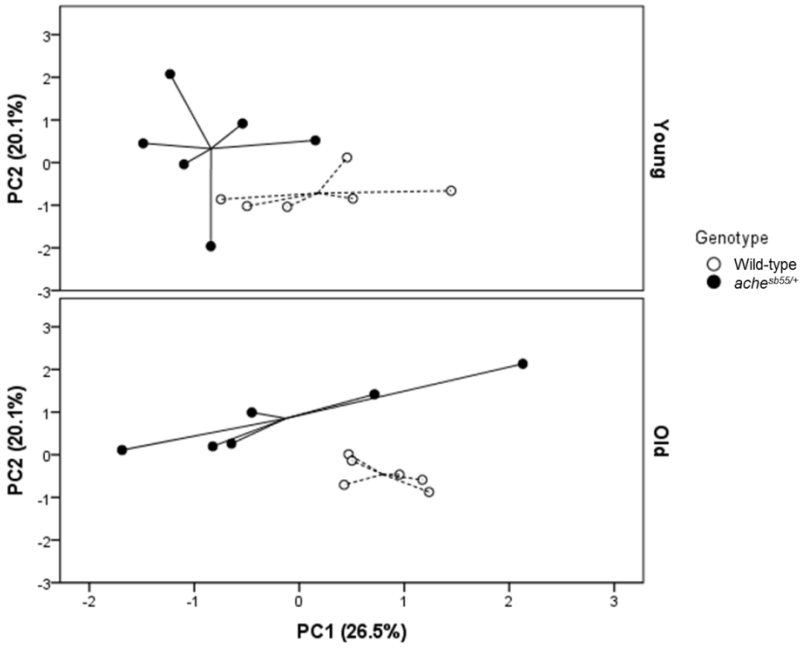


Figure 3.26 Clustering profiles of components in which both age groups and genotype groups were separated. Age groups were shown in separate panels while genotype groups were denoted by different colors Wild-type: White, *ache*$^{sb55/+}$: Black.

3.7 Discussion and Conclusions

In this chapter, *ache*$^{sb55/+}$ mutants were characterized as compared to their age-matched wild-type controls. This mutant model was previously characterized as a delayed aging model in the behavioral measures, but neurobiological changes in this model were only investigated in the young zebrafish groups. This chapter indicated that AChE levels were declined significantly in all of the analyzed age groups in the mutants. Additionally, an overall decrease in the neurotransmitter ACh was found in the mutants. Although the decreasing pattern of cholinergic receptor subunit nAChR-a7 was demonstrated in mutants as compared to wild-type, this difference was not

statistically significant. In terms of synaptic elements, significant declines in the glutamate receptors, including GluR2/3 and NR2B subunits, were demonstrated in the mutant animals. These decreases were accompanied by age-related elevation at the levels of presynaptic protein SYP and inhibitory marker GEP. Cellular markers were also assessed. It was shown that immature neuronal marker HuC was significantly elevated in the *ache*$^{sb55/+}$mutants, which was significant at both young and old ages. Lastly, marginally significant decreases were seen in the glial marker GFAP and oxidative stress marker ROS levels in mutants compared to wild-type animals. It can be concluded that mutation leading to lower levels of AChE is increasing the proliferation in mutant animals, which have higher levels of HuC at both young and old ages, and tunes the elements of synaptic transmission by reducing the excitatory elements and increasing the presynaptic and inhibitory elements which can shift the excitatory and inhibitory balance to more inhibitory side at the older ages. This modulation can be protective against age-related excitotoxic damages in these mutants.

A point mutation in the *ache*$^{sb55/+}$ affected the *ache* gene, which codes AChE and leads to the production of non-functional AChE protein. This mutation reduces the AChE activity in heterozygous *ache*$^{sb55/+}$ mutants while it is lethal and led to complete abolishment of the AChE enzyme in the homozygous *ache*$^{sb55/sb55}$ mutants [50]. In this mutant model cholinergic system was altered, and studies utilizing this specific mutant model have investigated the neurobiological alterations at young adult zebrafish between 3.5-6 months old [51], [84]. Therefore, we have analyzed additional cholinergic neurotransmission elements and assessed the levels of these

markers in different age groups. Previous literature lacks characterization of the neurobiological alterations at adult and old ages, and there was limited knowledge in this model whether the effects of mutation persist in older age groups. First, we have assessed the levels of AChE, which was the direct target of the mutation. Our results indicated that *ache*$^{sb55/+}$ mutants had significantly reduced levels of AChE in the brain in all age groups. Effects persisted at the old ages, which was parallel to the previous studies utilizing the embryos and young adult groups [50], [51], [84]. As the second element of cholinergic neurotransmission, we have checked the levels of neurotransmitter ACh in the brain tissues because it was previously reported that elevated ACh accompanied decreases in AChE in this model [51]. Our observations showed a different pattern, and even our results demonstrated an overall significant declining trend in ACh levels of the *ache*$^{sb55/+}$ mutants. One possible explanation for this difference can be the mutants' age range that was 3-6 months old female zebrafish utilized in a previous study [51]. The experimental design of the current research young age was selected between the 10-11 months old fish since it was previously reported that cognitive decline in zebrafish is evident when they were around 2-years-old compared to 1-year-old fish [42]. Therefore, we have included a younger age group, 3.5-5 months old, to the data to have a more comparable age range to the study of Ninkovic and his colleagues [51] for the measurements of AChE and ACh levels. They have shown that ACh levels increased significantly in the *ache*$^{sb55/+}$ females, which were 3-6 months old compared to their wild-type controls. Although we could not show a significant increase in *ache*$^{sb55/+}$ female 3.5-5 months old animals in our data, there is a numerical increase in the *ache*$^{sb55/+}$ females in this age group. The reason that we could not find any significant difference might be

relevant to our sample size. In our 3.5-5 months old ache female group, there were three animals, and in the wild-type controls, there were three animals; but the sample size in that study was 9 for the *ache*$^{sb55/+}$ group and 12 for the controls. Although we could not replicate Ninkovic and his colleagues' results exactly, there is a numerical increase in our data. The important thing is that with age and gender, this pattern has been changing, which was not previously shown in the literature. It was previously discussed and interpreted as *ache*$^{sb55/+}$ mutants might have elevated ACh levels with advancing age, explaining their preserved cognitive abilities at the older ages. However, none of these previous works measured the ACh levels in *ache*$^{sb55/+}$ animals older than 6 months. As a third component, a subunit of ionotropic nicotinic ACh receptor was measured. The alpha-7 subunit was selected since it is one of the predominant subunits and has a potentially ameliorating impact on cognitive performance, as shown in previous works [83]. Additionally, when the cholinergic system was manipulated with this mutation, alterations in the receptor stoichiometry dynamics can be observed. Alpha-7 subunit can form ion-channels in homomers, and changes at the nAChR-a7 levels can also reflect the possible changes in the stoichiometry due to the reduced levels of AChE. Our data showed no significant change on nAChR-a7 levels, but in the *ache*$^{sb55/+}$ mutants, there was a decreasing trend regarding the levels of this subunit. Overall, our data was indicating that elevated levels of ACh may not be the only determinant of the preserved cognitive abilities at older ages; decreased levels of AChE may alter the dynamics of pathways including neuro-inflammation or micro-environment of the brain which might explain behavioral delayed aging profile in the *ache*$^{sb55/+}$ mutants.

Synaptic protein levels were assessed as a next step. Modulatory impacts can be seen at other neurotransmission systems since the cholinergic system was manipulated. Firstly, elements of excitatory glutamatergic neurotransmission were analyzed, including NR2B, GluR2/3, and PSD95. Previously, it was reported that treatments with AChE inhibitors, reducing the levels of AChE, could enhance the elements of excitatory neurotransmission [85]. However, it is essential to note that these pharmacological treatments applied in a specific time and effects are relatively transient and did not alter the developmental dynamics very robustly compared to the genetic interventions against similar targets. Our data indicated that genotype significantly altered the levels of NMDA receptor subunit NR2B and AMPA receptor subunits GluR2/3, and *ache*$^{sb55/+}$ mutants had significantly lower levels of these synaptic proteins. NR2B subunit levels were lower in the *ache*$^{sb55/+}$ mutants at both young and old ages. Therefore, it could be said that this expression profile is not altered through aging and is probably associated with the effect of mutation on the micro-environment of the brain. NMDA receptors are calcium and sodium permeable receptors. When NR2B subunit levels were decreased, it might have shown reduced glutamatergic activity and calcium influx. Some studies indicated that significant elevations in this subunit might be associated with oxidative damage [86]. However, in our case, it was a persistent reduction in NR2B levels occurring through development.

Moreover, GluR2/3 levels also showed a significant overall decline in *ache*$^{sb55/+}$ mutants, and this decrease was prominent at the older ages. GluR2/3 subunits are inversely regulating the calcium permeability, Glur2 lacking AMPA receptors are

more permeable to calcium, and during the long term potentiation, AMPA receptors that lack GluR2 are inserted at the membrane [15]. Therefore, decreased levels of this subunit can be associated with elevated calcium permeability. It can be considered as a compensatory mechanism to decline NMDA subunits to maintain homeostatic synaptic scaling. Additionally, as another component of excitatory neurotransmission, PSD95, the clustering, and scaffolding protein binds to NMDA and AMPA receptors, was investigated [13]. We have observed no significant change in the levels of this excitatory scaffolding protein. Overall, these results indicated that mutation could be associated with persistent changes in decreases in the NMDA subunits, but this decline can be compensated by the lower levels of GluR2/3 subunits enabling more calcium permeability to maintain stable and comparable homeostatic synaptic scaling.

Additionally, elements of inhibitory GABAergic neurotransmission were also assessed between age and genotype groups. GEP is a scaffolding and clustering protein that binds to the $GABA_A$ receptors, which are ionotropic receptors [87]. Age-related declines in the GEP levels were previously reported, and higher levels of GEP can be associated with less susceptibility against aging-related excitotoxicity [24], [88]. Our data showed a significant age-related decline in the GEP in wild-type zebrafish, while *ache*$^{sb55/+}$ mutants had stable and non-fluctuating levels of GEP through aging. As another inhibitory element, the alpha-1 subunit of $GABA_A$ receptors was investigated, and no significant age or genotype effect was demonstrated on this marker as parallel to the previous work [26]. Overall, our data have shown that stable levels of inhibitory protein GEP were maintained at both young and old ages in the mutants. On the other hand, in the wild-type animals, a

significant reduction in GEP at old ages was seen, which can increase animals' susceptibility against detrimental effects of aging.

In addition to the excitatory and inhibitory markers and overall synaptic marker SYP was also investigated. Previously studies have shown that aging can lead to decreases in the levels of SYP [89]. Although a significant decline at old age was not seen in the wild-type animals, it was observed that the old mutant group had significantly elevated SYP levels with aging, which also can show increased resilience and synaptogenesis in the mutant group.

Apart from the synaptic markers, mutation can also alter cellular dynamics. For that reason, neuronal markers, a glial marker, and a global proliferation marker have been investigated in the current chapter. As neuronal markers, HuC and DCAMKL1 were investigated. HuC is considered one of the earliest neuronal markers expressed in the brain of the zebrafish; its expressions were observed in the neuronal precursor cells [90]. Previous studies reported a decline in HuC positive cell number in specific brain regions such as subthalamic nuclei [91]. Regarding the effects of aging, we did not see a significant decrease in old age. Still, previous reports were generally focusing on specific regions in the brain, and in this study, we have assessed the global levels of this neuronal protein. Interestingly, we have observed the effect of genotype on this marker at both young and old ages of the mutant groups. Mutants had significantly elevated levels of this marker, implying elevated neuronal cells and elevated neuronal proliferation in the mutants. As second neuronal marker levels of DCAMKL1 were investigated, this marker was expressed in the migrating neurons and zones of proliferation, and it shares homology to doublecortin [92]. While no

significant alterations were evident on DCAMKL1, there was a trend in the young group, and mutants tend to have elevated levels of this marker compared to wild-type animals. This trend is in parallel to increases seen in HuC levels in the mutant groups.

Additionally, glial marker GFAP levels were also investigated as a cellular marker. GFAP levels can manifest glial population, and increased levels of this marker can be seen in pathological brain conditions and when the glial cells' functioning was disrupted [93]. On the GFAP levels, a marginally significant main effect of genotype was observed, and in the mutants, GFAP levels tend to be reduced compared to wild-type animals. This observation can indicate that reduced levels of AChE can mediate glial modulation and prevents the elevation in these proteins. As a last cellular marker, PCNA was assessed as an overall proliferation marker expressed in the proliferating cells of different origins like neuronal and glial [94]. On the PCNA levels, no specific difference was found between the genotype and age groups; this observation might be associated with the analyses of the global levels of PCNA rather than focusing on region-specific alterations.

Subtle reductions were observed on the GFAP levels in the *ache*$^{sb55/+}$ mutants, which can protect against insults associated with inflammatory markers. These detrimental markers, including TNF-alpha and inflammatory marker and ROS, an oxidative stress marker, were assessed. Modest decreases were seen in the *ache*$^{sb55/+}$ mutants regarding the levels of TNF-alpha, but these differences were not statistically significant. ROS levels can indicate the contents of free radicals in the brain, showing an elevation in brain insults and aging [95]. In this chapter, the effect of aging was marginally significant, and ROS levels showed an increasing trend with aging.

Specifically, in the male animals, the genotype was altering the ROS levels. In the male *ache*$^{sb55/+}$ mutants, ROS levels were reduced significantly compared to wild-type zebrafish, demonstrating that *ache*$^{sb55/+}$ mutants tend to have subtly reduced levels of inflammation-related markers such as GFAP and oxidative stress such as ROS. These observations were parallel to the previous work utilizing the AChE inhibitors. It was shown that this pharmacological intervention is associated with lower levels of plasma markers of inflammation in the blood [96]. Moreover, multivariate analyses supported this observation that reduced levels of AChE were associated with declined levels of inflammatory markers such as GFAP and TNF-alpha.

Another crucial point is that this mutation was induced at a very early stage, and it can alter the overall developmental dynamics. In this respect, its effects can be considered long-term. However, it is still unclear whether short-term exposure to reduced levels of AChE can be effective in delaying age-related deteriorations. Pharmacological interventions utilizing AChE inhibitors can provide insights in terms of possible short-term modulations because of reduced levels of AChE. It was reported that in 12 months old zebrafish groups, treatment with AChE inhibitor, donepezil, led to decreased locomotor activities and increased anxiety-related behaviors as measured by the light-dark paradigm in an exposure dose-dependent manner [97]. Additionally, behavioral alterations resulted from donepezil treatment were accompanied by elevations in the whole body cortisol levels which can indicate that acute donepezil treatment at a young age can impair the behavioral measures as well as alter the physiological responses. Another study has compared the effects of

reduced AChE levels between different intervention procedures in young zebrafish groups, a genetically induced reduction that utilized the *ache*$^{sb55/+}$ mutants were compared with acute exposure to AChE inhibitors [84]. Results indicated that mutant groups showed reduced impulsivity as measured by the 5-choice serial reaction time paradigm, while in the drug groups, no significant improvement was observed with short-term exposure [84]. These results suggest that acute fluctuations in the levels of AChE may not present prominent benefits at behavioral and physiological measures; on the other hand, developmental or long-term manipulations can improve some of these parameters.

Inducible mutations at a specific time frame can enlighten these issues regarding the duration-specific effects of AChE reduction. Additionally, literature is sorely lacking in terms of the manipulations of AChE at an older age in the zebrafish model organism; at old age, behavioral and physiological alterations can show a different profile. For further research, inducible mutagenesis approaches can be complementary. They can be created via Tol2 transposon-mediated strategy to knockdown the *ache* gene; knockdown can be induced at any stage of development through exposure to the designated stimulus, such as heat shock [98]. AChE levels can be altered after the maturation period, which can eliminate the effects of AChE reduction on the developmental dynamics in the brain, as well as at the older ages through this methodology. This methodology can complement the observations seen in the *ache*$^{sb55/+}$ mutants in which alterations in the developmental dynamics can occur, and the effects were long-term.

To conclude, the current chapter showed that *ache*$^{sb55/+}$ mutants have significantly lower levels of AChE at different age groups, and lower levels of AChE were associated with the lower levels of the other cholinergic markers, including ACh and nAChR-a7. This observation can indicate that although lower levels of AChE can be related to the elevation of ACh with the application of pharmacological treatments [99], the mutation is having long-lasting effects on development, and the system should maintain its homeostasis. Therefore, it was expected to see an overall reduction in the other cholinergic elements in the *ache*$^{sb55/+}$ mutants. Moreover, in *ache*$^{sb55/+}$ mutants, excitatory neurotransmission markers decreased evidently, while inhibitory neurotransmission elements tended to be stable through aging. At the same time, a significant age-related decline was observed in inhibitory clustering protein levels of the wild-type zebrafish. Additionally, in the mutants, levels of presynaptic marker SYP were promoted with aging. Interestingly, neuronal marker HuC was upregulated significantly in *ache*$^{sb55/+}$ mutants in all investigated age groups indicating the possible effects of AChE reductions in overall neuronal populations. Lastly, inflammation-related markers GFAP and ROS levels were subtly declined in the mutant groups compared to wild-type zebrafish. It could be said that permanent and long-lasting AChE reductions can upregulate the neuronal markers, prevent the age-related decline in inhibitory neurotransmission components, and downregulate the excitatory markers with aging.

CHAPTER 4

THE EFFECTS OF SENSORY ENVIRONMENTAL ENRICHMENT ON BODY PARAMETERS, SYNAPTIC AND CELLULAR PROTEINS WITHIN THE CONTEXT OF AGING AND GENDER

4.1 Effects of environmental enrichment, aging, and gender on body parameters including body weight, length, body mass index (BMI), and wet brain weight

In the enrichment setup, since the animal was introduced into a novel environment, body parameters were recorded very carefully to ensure no eating or growth problem might be related to stress due to a novel environment. A significant main effect of age on the body weight of the animals was revealed, $F(1,85)=58.038$, $p<0.0005$, Figure 4.1. As expected, old groups had significantly increased body weight compared to young groups, and this increase was significant in all comparison groups such as female-barren-young vs. female-barren-old ($p<0.0005$); female-enriched-young vs. female-enriched-old ($p<0.0005$), male-barren-young vs. male-barren-old ($p=0.035$) and male-enriched-young vs. male-enriched-old ($p=0.001$) groups. A significant main effect of gender was also observed on the body weight of the animals, $F(1,85)=71.023$, $p<0.0005$. Females had significantly elevated body weights than males in young-barren ($p<0.0005$), young-enriched ($p=0.015$), old-barren ($p<0.0005$), and old-enriched ($p<0.0005$) conditions. No significant main effect of the environment was found on the body weights, $F(1,85)=0.551$, $p=0.460$, indicating that an enriched environment does not force animals to severe swimming or stress, leading to weight loss. In terms of interactions, no significant environment

by gender, $F(1,85)=1.104$, $p=0.296$; age by environment, $F(1,85)=2.525$, $p=0.116$; and age by gender by environment, $F(1,85)=0.191$, $p=0.663$, were demonstrated. Moreover, a significant age by gender interaction was revealed on body weight data, $F(1,85)=4.196$, $p=0.044$.

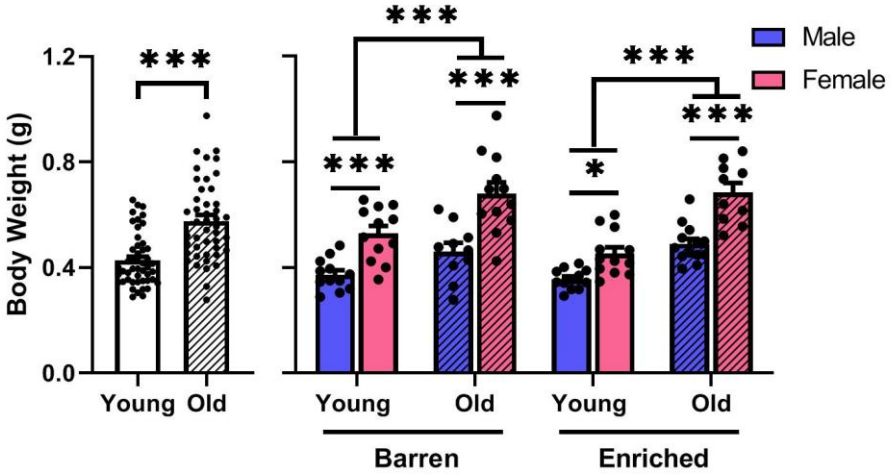


Figure 4.1 Body weight of the zebrafish was altered by the factors of gender and age while no specific effects of environment were revealed. *: $p < 0.05$, ***: $p < 0.005$. Error bars = +Standard Error Mean. Adapted from Karoglu-Eravsar et al., 2021 [74].

On the body length data only observed significant main effect was the main effect of age, $F(1,85)=141.137$, $p<0.0005$, Figure 4.2. Old animals had significantly higher body lengths compared to the young animals; and this difference is significant in barren-female ($p<0.0005$), barren-male ($p<0.0005$), enriched-female ($p<0.0005$) and enriched-male ($p<0.0005$) groups. The main effects of age and gender were not statistically significant and additionally no significant interaction was found affecting

the body length of the zebrafish (gender: $F(1,85)=0.021$, $p=0.844$; environment: $F(1,85)=0.006$, $p=0.940$; age by gender: $F(1,85)=0.744$, $p=0.391$; gender by environment: $F(1,85)=0.014$, $p=0.908$; age by environment: $F(1,40)=2.362$, $p=0.128$; age by gender by environment: $F(1,40)=0.014$, $p=0.908$).

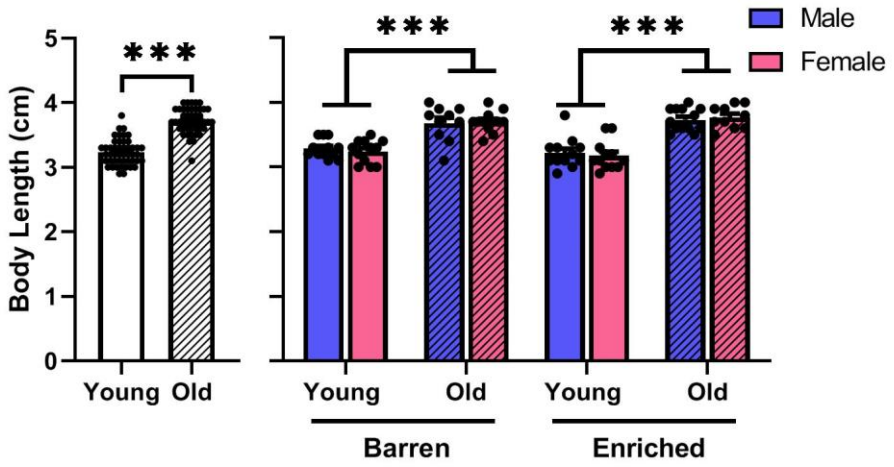


Figure 4.2 Body length of the zebrafish was altered by the factor of age only while no specific effects of environment or gender were revealed. ***: $p < 0.005$. Error bars = +Standard Error Mean. Adapted from Karoglu-Eravsar et al., 2021 [74].

The only observed significant main effect was the main effect of gender on the BMI levels, $\chi^2(1)=63.253$, $p<0.0005$, Figure 4.3. Females had significantly higher BMIs compared to males, and this difference was significant in barren-young ($p<0.0005$), barren-old ($p<0.0005$), enriched-young ($p=0.003$), and enriched-old ($p=0.001$) groups. The main effects of environment and age were not significantly

altering the BMI levels (environment: $\chi^2(1)=0.901$, $p=0.343$; age $\chi^2(1)=0.003$, $p=0.954$).

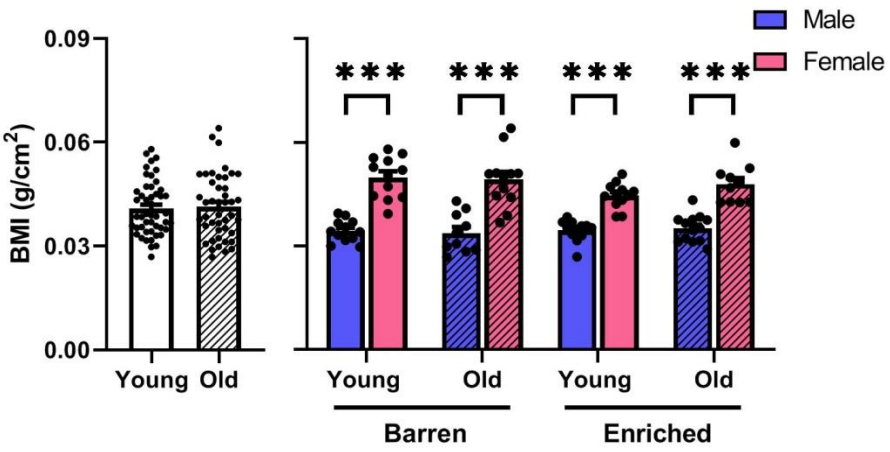


Figure 4.3 Body mass index values were significantly altered by a factor of age; no gender or environment-dependent alterations were observed. ***: $p < 0.005$. Error bars = +Standard Error Mean. Adapted from Karoglu-Eravsar et al., 2021 [74].

In the enrichment studies conducted with other model animals like mice, it was indicated that environmental enrichment could increase the animal's total brain weight [52]. Moreover, it would be interesting to see whether these kinds of observations will be similar in zebrafish and whether they depend on the factors of age and gender of the animal. A significant main effect of age was revealed $F(1,85)=129.579$, $p<0.0005$, Figure 4.4. It was shown that with aging brain weight of the animals increased significantly. This increase was significant between female-barren-young vs. female-barren-old ($p<0.0005$); male-barren-young vs. male-barren-old ($p<0.0005$); female-enriched-young vs female-enriched-old ($p<0.0005$) and male-

enriched-young vs male-enriched-old (p<0.0005). A significant main effect of gender was observed, F(1,85)=42.622, p<0.0005, Figure 4.4. Males had significantly higher brain weights compared to females; this gender difference was significant in all groups, including female-barren-young vs. male-barren-young (p=0.004), female-enriched-young vs. male-enriched-young (p=0.003), female-barren-old vs. male-barren-old (p=0.002), and female-enriched-old vs. male-enriched-old (p<0.0005), Figure 4.4.

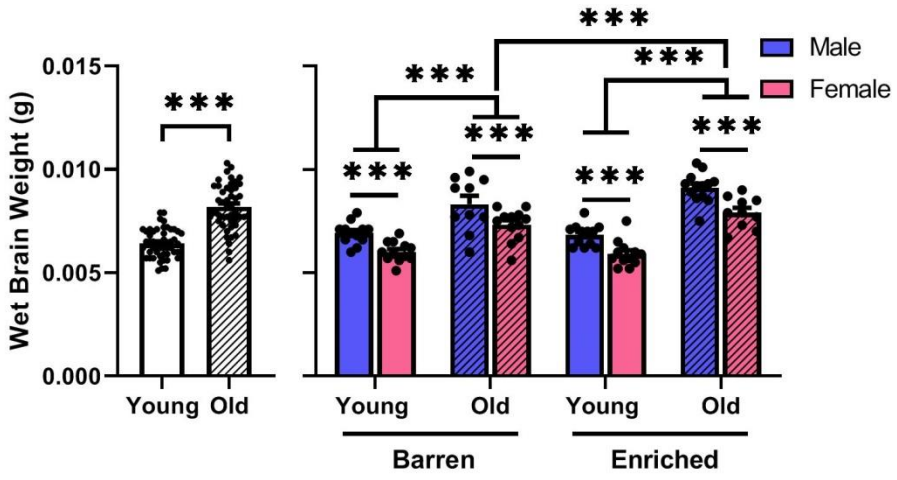


Figure 4.4 Wet brain weight measures were significantly altered by factors of age and gender, and a significant environment by age interaction was revealed. ***: p < 0.005. Error bars = +Standard Error Mean. Adapted from Karoglu-Eravsar et al., 2021 [74].

Additionally, in the brain weight data marginally significant main effect of the environment was observed, F(1,85)=3.782, p=0.055, as well as a significant environment by age interaction was revealed, F(1,85)=6.594, p=0.012. In the overall

data, zebrafish exposed to an enriched environment tended to have higher brain weight than the barren controls. Still, the effect of environment was significant in the old group (p=0.002) compared to the young. Moreover, when this environmental effect in the old group was further analyzed for gender factor, it was observed that the male-enriched-old group had significantly higher brain weight compared to the male-barren-old group (p=0.011). This increase in the enriched group was marginally significant in the old female animals (p=0.068), Figure 4.4. No significant interaction was observed between the factor of age and gender, $F(1, 85)=0.422$, p=0.518; factors of gender and environment, $F(1, 85)=0.135$, p=0.714; and lastly among all three factors, $F(1, 85)=0.116$, p=0.734, Figure 4.4.

4.2 Environmental enrichment alters the synaptic protein levels in an age-dependent manner

Western blot analyses were conducted with the selected markers to understand the effects of environmental enrichment on the synaptic elements and key neurotransmission elements. Post-Synaptic Density 95 (PSD95), Glutamate receptor subunit 2/3 (GluR2/3), and N-methyl D-aspartate receptor subtype 2B (NR2B) were used as excitatory neurotransmission markers. On the other hand, Gephyrin (GEP) and Gamma-aminobutyric acid receptor subunit alpha-1 (GABA-A-a1) were utilized as inhibitory neurotransmission markers. Lastly, presynaptic and synaptic vesicle marker Synaptophysin (SYP) was used in this study. Representative blots for the synaptic proteins of interest were presented in Figure 4.5.

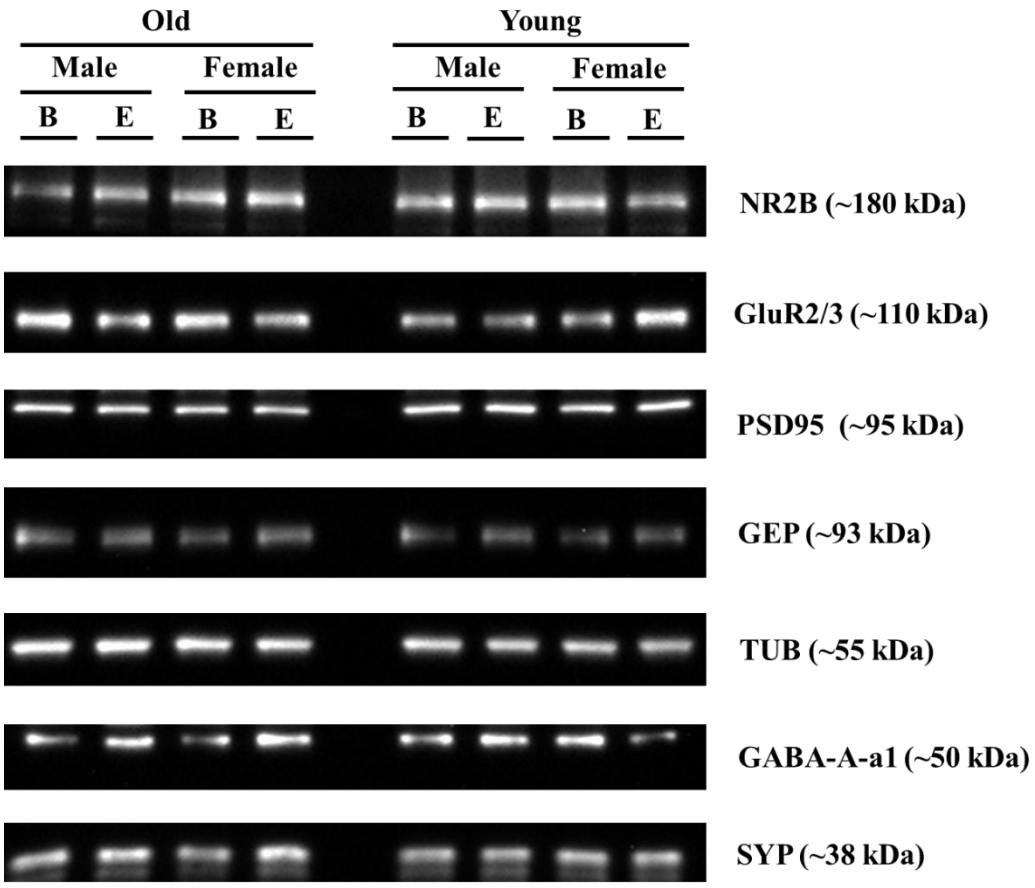


Figure 4.5 Representative pictures from one cohort for Western blot experiments of synaptic protein levels examined in the current Chapter for effects of age, gender and the environmental condition. Bands were obtained at the expected molecular weights for all antibodies. B: Barren environmental condition; E: enriched environmental condition. Adapted from Karoglu-Eravsar et al., 2021 [74].

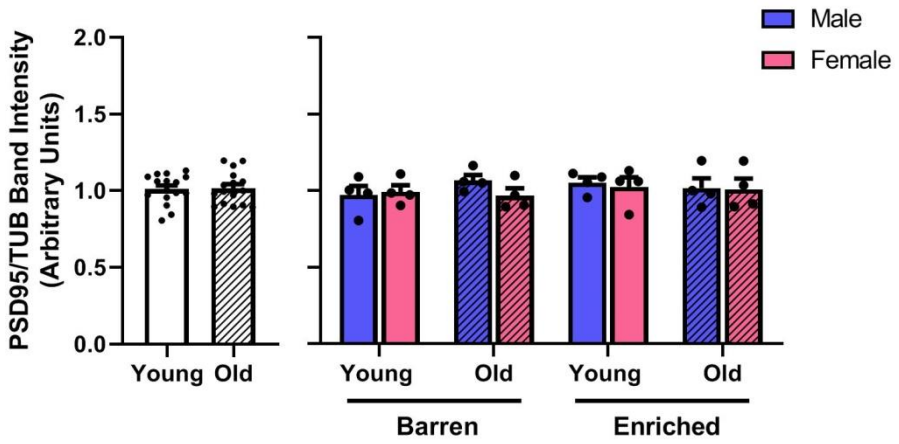


Figure 4.6 PSD95 levels were not altered significantly. Error bars = +Standard Error Mean. Adapted from Karoglu-Eravsar et al., 2021 [74].

On the PSD95 level there was no significant main effect of age, $F(1,24)=0.019$, $p=0.891$, gender, $F(1,24)=0.571$, $p=0.457$, and environment, $F(1,24)=0.484$, $p=0.493$, Figure 4.6. Moreover, no significant interaction was revealed on the data including age by gender, $F(1,24)=0.429$, $p=0.519$; age by environment, $F(1,24)=0.642$, $p=0.431$; gender by environment, $F(1,24)=0.077$, $p=0.784$; and lastly age by gender by environment, $F(1,24)=0.870$, $p=0.360$, Figure 4.6.

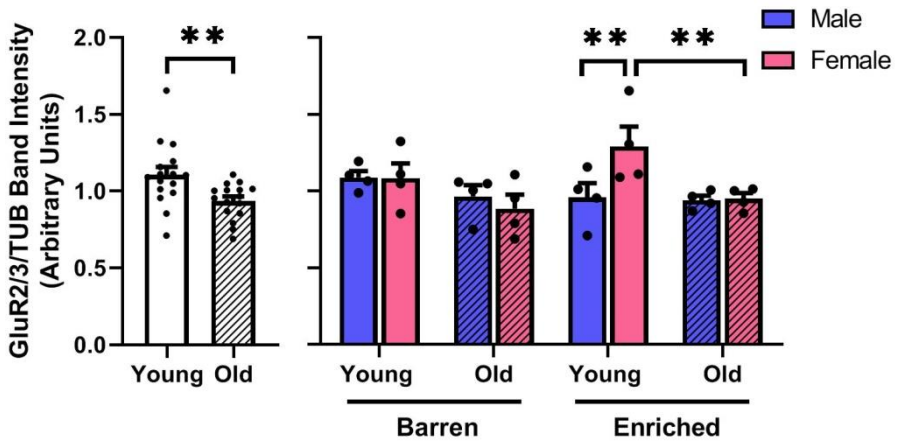


Figure 4.7 GluR2/3 levels were changed significantly by age and gender-dependent interactions. **: $p < 0.01$. Error bars = +Standard Error Mean. Adapted from Karoglu-Eravsar et al., 2021 [74].

On GluR2/3 levels, there was a significant main effect of age, F(1,24)=8.736, p=0.007; there was an overall significant pattern of decrease in the levels of GluR2/3 with increasing age. Pairwise comparisons showed that in the enriched females, this age-mediated decrease was significant (p=0.007). Other factors were not significantly changing the GluR2/3 levels, such as gender, F(1,24)=1.232, p=0.278; and environment, F(1,24)=0.259 , p=0.615. Additionally, age by gender interaction, F(1,24)=3.023 , p=0.095; age by environment interaction, F(1,24)=0.025, p=0.876; and age by gender by environment interaction, F(1,24)=1.140, p=0.296, were not statistically significant. However, a marginally significant gender by environment interaction was revealed, F(1,24)=3.428, p=0.076, Figure 4.7. Multiple comparisons demonstrated that the gender effect was significant on the young animals exposed to an enriched environment (p=0.008), indicating that young females in the enriched

environment tend to have higher GluR2/3 levels than males. Moreover, a significant difference between young enriched females and young enriched males was observed (p=0.008), Figure 4.7.

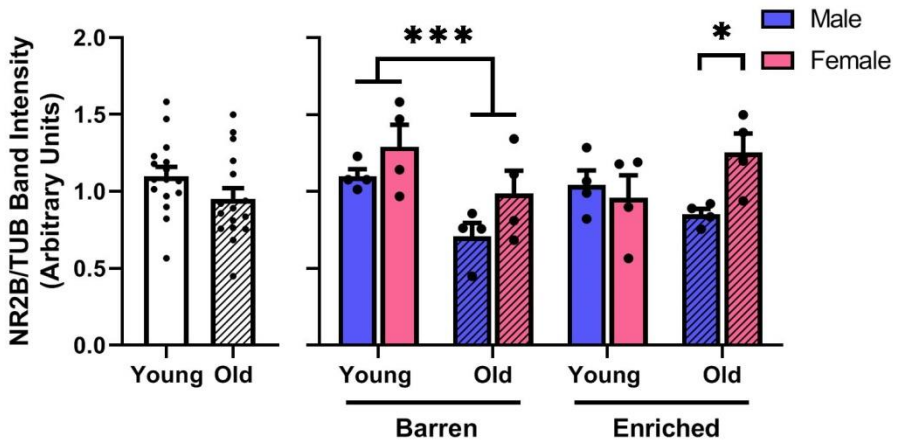


Figure 4.8 NR2B levels were altered by the age-environment interaction and gender. *: $p < 0.05$, ***: $p < 0.005$. Error bars = +Standard Error Mean. Adapted from Karoglu-Eravsar et al., 2021 [74].

NR2B is one of the predominant subunits of the excitatory NMDA receptors. The main effects of environment, $F(1,24)=0.005$, $p=0.946$, and age, $F(1,24)=3.505$, $p=0.073$, were not statistically significant. On the other hand, a significant main effect of gender was observed on NR2B levels, $F(1,24)=6.450$, $p=0.018$, as females having higher levels of NR2B compared to males, Figure 4.8. Posthoc analyses indicated a significant gender difference in the old-enriched group (p=0.017). A significant age by environment interaction was demonstrated altering the levels of NR2B, $F(1,24)=6.450$, $p=0.018$. While maintained levels of NR2B were observed in the enriched animals with aging, a significant reduction in NR2B levels was

demonstrated at old age in subjects assigned to the barren environment (p=0.005). Interactions between the other factors were not statistically significant including age and gender, $F(1,24)=3.324$, p=0.081; gender and environment, $F(1,24)=0.230$, p=0.636; and age and gender and environment, $F(1,24)=1.592$, p=0.219.

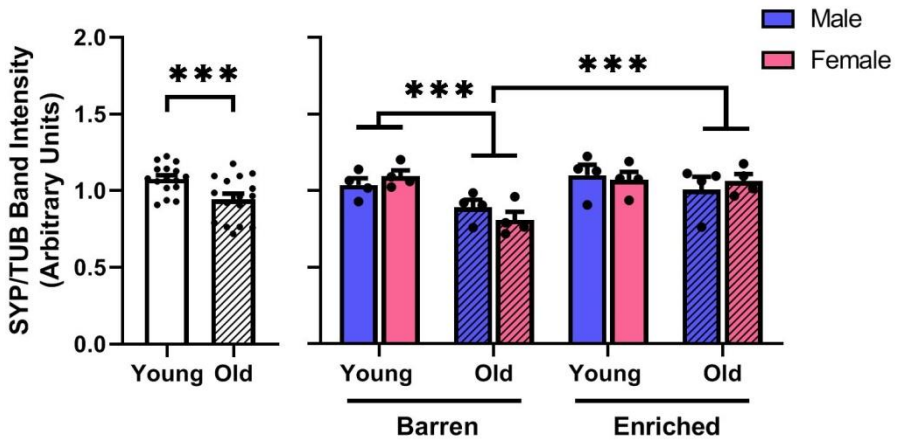


Figure 4.9 SYP levels were altered significantly by age and age by environment interaction. ***: $p < 0.005$. Error bars = +Standard Error Mean. Adapted from Karoglu-Eravsar et al., 2021 [74].

A significant main effect of age was revealed on SYP levels, $F(1,24)=11.351$, p=0.003, Figure 4.9; showed that old animals tended to have reduced SYP levels. Also, a significant main effect of environment was observed on SYP levels; animals in the enriched environment tended to have higher levels of SYP compared to animals in the barren environment, $F(1,24)=6.706$, p=0.016, Figure 4.9. Moreover, a significant age by environment interaction was found, $F(1,24)=4.362$, p=0.048; multiple comparisons revealed that the effect of age is significant in the barren environment (p=0.001), and the effect of environment is significant in the old group

(p=0.003). It could be observed that with aging, animals in the barren environment tended to have lower levels of SYP, and this decrease was robust and significant in females (p=0.001) and marginally significant in males (p=0.079). However, animals in the enriched environment tended to have significantly higher SYP levels at older ages compared to animals maintained in the barren environment; this increase was significant in both males (p=0.039) and females (p=0.004). Additionally, on the SYP levels there was no significant main effect of gender, $F(1,24)=0.0001$, p=0.003; and no significant interaction between age and gender, $F(1,24)=0.130$, p=0.722; gender and environment, $F(1,24)=0.110$, p=0.743; and age and gender and environment, $F(1,24)=2.048$, p=0.165.

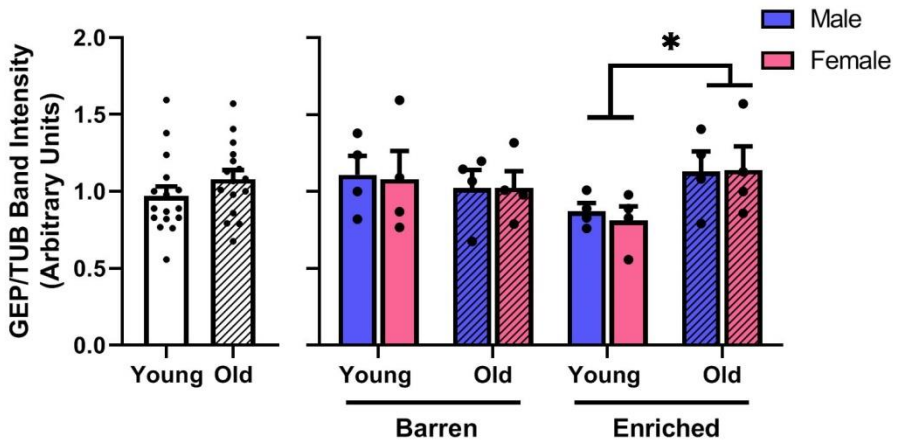


Figure 4.10 GEP levels were elevated in the enriched environment with aging. *: $p < 0.05$. Error bars = +Standard Error Mean. Adapted from Karoglu-Eravsar et al., 2021 [74].

GEP is a major scaffolding protein found at the inhibitory synapses. On the GEP levels no significant main effect was revealed including the factors of age,

$F(1,24)=1.536$, $p=0.227$; gender, $F(1,24)=0.047$, $p=0.831$; and environment, $F(1,24)=0.613$, $p=0.441$, Figure 4.10. Moreover, interactions among these factors were also not reaching the threshold for significance such as age and gender, $F(1,24)=0.078$, $p=0.782$; gender and environment, $F(1,24)=0.004$, $p=0.951$; and age and gender and environment, $F(1,24)=0.011$, $p=0.917$. However, a significant interaction between the factors of age and environment was observed, $F(1,24)=4.196$, $p=0.05$. In the enriched environment, GEP levels were elevated significantly with aging ($p=0.029$), Figure 4.10.

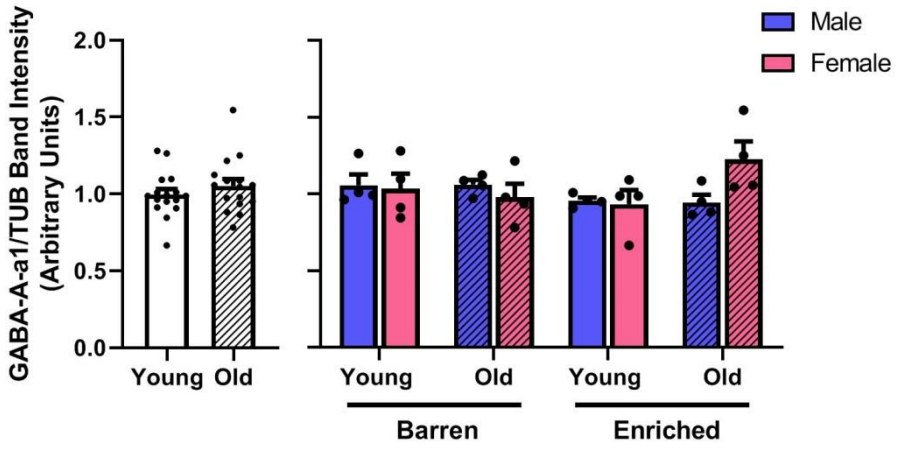


Figure 4.11 GABA-A-a1 levels tend to be stable across the environment, age, and gender groups. Error bars = +Standard Error Mean. Adapted from Karoglu-Eravsar et al., 2021 [74].

On the GABA-A-a1 levels no significant main effect was revealed for the factors of age, $F(1,24)=1.071$, $p=0.311$; gender, $F(1,24)=0.456$, $p=0.506$; and environment, $F(1,24)=0.006$, $p=0.759$, Figure 4.11. Moreover, there was no significant interaction between age and gender, $F(1,24)=1.233$, $p=0.278$; age and environment

$F(1,24)=2.277$, $p=0.144$; gender and environment, $F(1,24)=2.672$, $p=0.115$; and gender-age-environment, $F(1,24)=2.674$, $p=0.115$, Figure 4.11.

4.3 Environmental enrichment altering post-mitotic neuronal marker in an age-dependent manner

In addition to the synaptic integrity markers, cellular markers were assessed regarding the factor of age, gender, and environment. Two neuronal markers; embryonic lethal, abnormal vision (ELAV; Drosophila) like 3 (HuC) and doublecortin like kinase 1 (DCAMKL1); a glial marker of glial fibrillary acidic protein (GFAP), and a proliferation marker, proliferating cell nuclear antigen (PCNA) were investigated. Representative blots for the examined cellular markers were demonstrated in Figure 4.12.

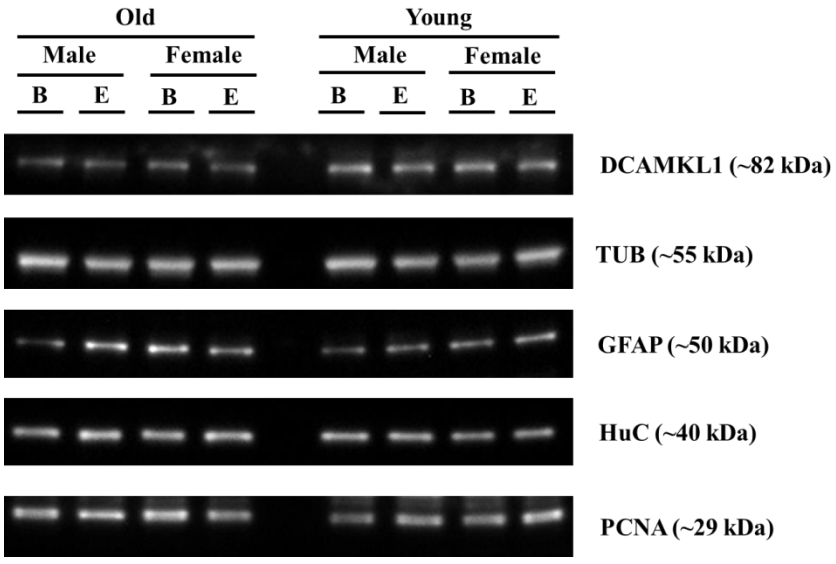


Figure 4.12 Representative pictures from one cohort for Western blot experiments of cellular protein levels examined in the current Chapter for effects of age, gender and the environmental condition. Bands were obtained at the

expected molecular weights for all antibodies. B: Barren environmental condition; E: enriched environmental condition. Adapted from Karoglu-Eravsar et al., 2021 [74].

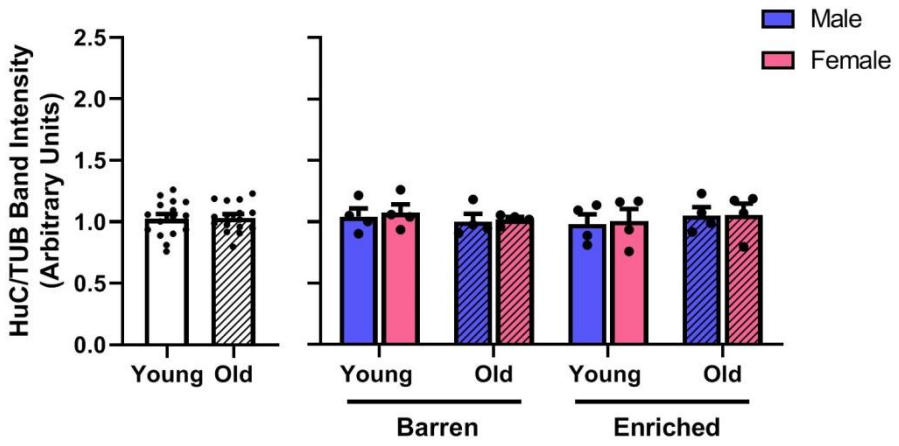


Figure 4.13 HuC levels tend to be stable across the environment, age, and gender groups. Error bars = +Standard Error Mean. Adapted from Karoglu-Eravsar et al., 2021 [74].

HuC was used as an immature neuronal marker in this study. No significant main effects of age, $F(1,24)=0.015$, $p=0.904$; or gender, $F(1,24)=0.135$, $p=0.716$; or environment, $F(1,24)=0.041$, $p=0.841$; was observed on the HuC expression levels, Figure 4.13. Additionally, no significant interaction was revealed among the factors including age and gender, $F(1,24)=0.029$, $p=0.866$; age and environment, $F(1,24)=1.119$, $p=0.301$; gender and environment, $F(1,24)=0.008$, $p=0.930$; and age and gender and environment, $F(1,24)=0.0001$, $p=0.988$.

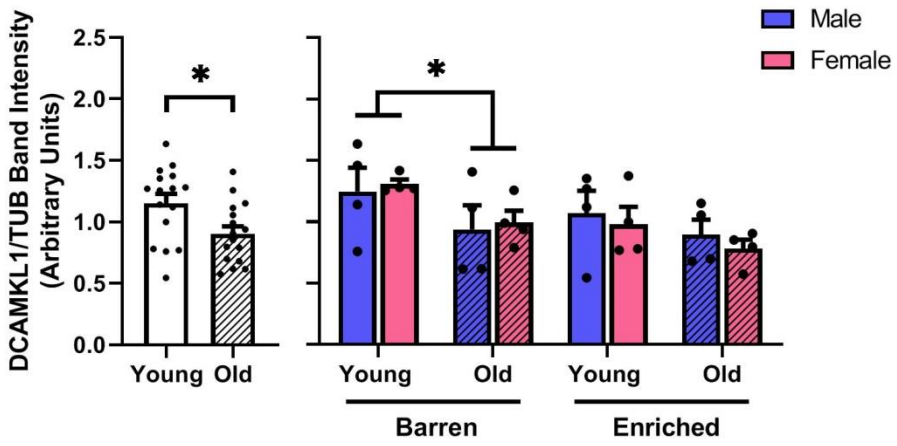


Figure 4.14 DCAMKL1 levels decreased with aging in the barren environmental condition. *: $p < 0.05$. Error bars = +Standard Error Mean. Adapted from Karoglu-Eravsar et al., 2021 [74].

DCAMKL1 is a marker expressed in migrating neurons [100] and was used as another neuronal marker. As in the case for the HuC, no significant changes depending on the environment, $F(1,24)=3.589$, $p=0.070$; or gender, $F(1,24)=0.052$, $p=0.822$, were observed in DCAMKL1 levels, Figure 4.14. On the other hand, a significant main effect of age was demonstrated, $F(1,24)=6.206$, $p=0.020$. Multiple comparisons indicated an age-related reduction in DCAMKL1 levels was significant in the barren environment ($p=0.038$), whereas no significant decline was observed in the enriched environment with aging. Additionally, no significant interaction was revealed among the factors including age and gender, $F(1,24)=0.004$, $p=0.950$; age and environment, $F(1,24)=0.388$, $p=0.539$; gender and environment,, $F(1,24)=0.638$, $p=0.432$; and age and gender and environment, $F(1,24)=0.002$, $p=0.962$.

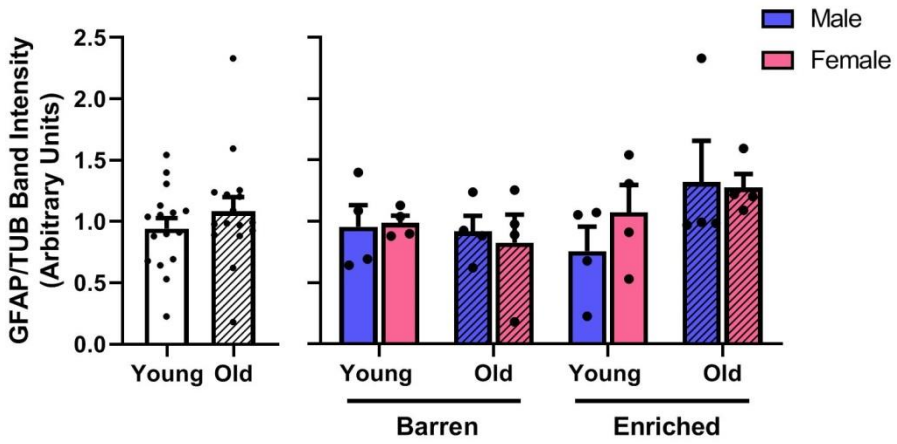


Figure 4.15 GFAP levels tend to be stable across the environment, age, and gender groups. Error bars = +Standard Error Mean. Adapted from Karoglu-Eravsar et al., 2021 [74].

On the GFAP levels, the effects of age, $\chi^2(1)=0.688$, $p=0.407$; and gender, $\chi^2(1)=0.818$, $p=0.366$; and environment, $\chi^2(1)=1.945$, $p=0.163$, was not statistically significant, and no evident trend was observed regarding the GFAP levels, Figure 4.15.

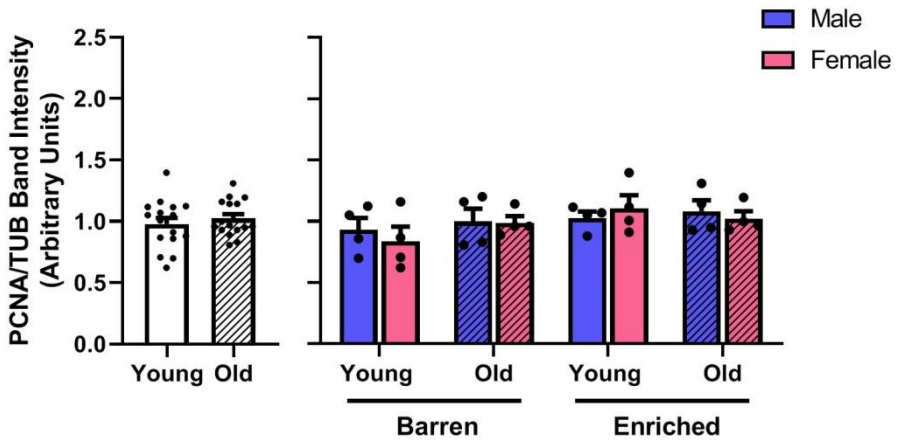


Figure 4.16 PCNA levels tend to be stable across the environment, age, and gender groups. Error bars = +Standard Error Mean. Adapted from Karoglu-Eravsar et al., 2021 [74].

On the PCNA levels there was no significant main effect of age, $F(1,24)=0.533$, $p=0.472$; and gender, $F(1,24)=0.120$, $p=0.732$; but there was a marginally significant main effect of environment, $F(1,24)=3.740$, $p=0.065$. Animals in the enriched environment tended to have higher levels of PCNA, especially in the young groups, Figure 4.16. Also, no significant interactions between the factors age and gender, $F(1,24)=0.053$, $p=0.820$; age and environment, $F(1,24)=0.974$, $p=0.334$, gender and environment, $F(1,24)=0.246$, $p=0.625$; and age and gender and environment, $F(1,24)=0.793$, $p=0.382$, were revealed , Figure 4.16.

4.4 Age is significantly increasing the activity of reactive oxygen species (ROS) and lipid peroxidation while no environment-dependent modulations are evident

AChE activity was investigated to decipher the effects of the environment on the cholinergic system and its integrity concerning aging and gender differences. The main effect of age, $\chi^2(1)=1.138$, p=0.286; gender, $\chi^2(1)=0.045$, p=0.832; and environment, $\chi^2(1)=0.427$, p=0.514, was not statistically significant on the AChE activity levels, Figure 4.17.

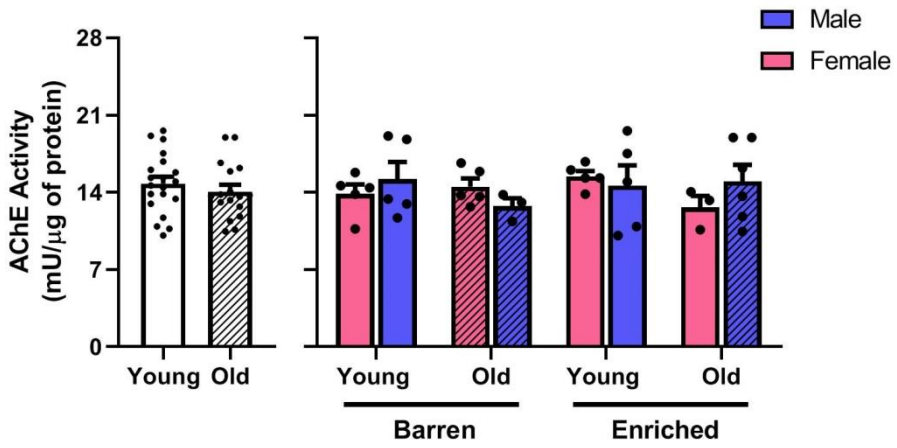


Figure 4.17 AChE activity levels tend to be stable across the environment, age, and gender groups. Error bars = +Standard Error Mean. Adapted from Karoglu-Eravsar et al., 2021 [74].

ROS levels were assessed to reveal possible environment-driven effects on the free-radical content within the context of aging and gender differences. A main effect of age on the ROS levels was statistically significant, $\chi^2(1)=6.554$, $p=0.010$. However, multiple comparisons did not reveal the significantly different pairs

regarding age, so it was an overall effect. Additionally, the effect of gender on the ROS levels was statistically significant, $\chi^2(1)=7.815$, $p=0.005$. Females had lower ROS activity levels than males, and pairwise comparisons indicated that this gender difference was significant in the enriched young group ($p=0.033$), Figure 4.18. On the other hand, the effect of environment was not significant, $\chi^2(1)=0.023$, $p=0.879$.

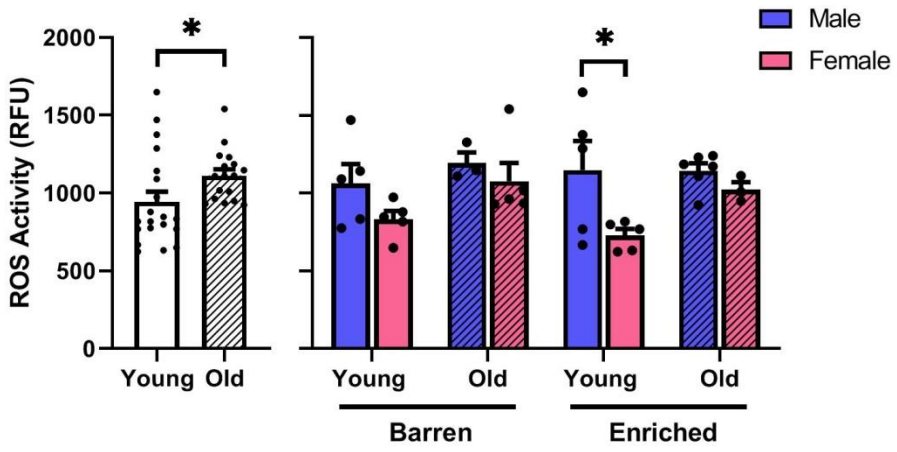


Figure 4.18 ROS activity levels were significantly altered by the factors of age and gender. *: $p<0.05$. Error bars = +Standard Error Mean. Adapted from Karoglu-Eravsar et al., 2021 [74].

MDA levels were investigated as a marker of the lipid peroxidation content. A significant main effect of age was demonstrated on the MDA content, $F(1,16)=5.772$, $p=0.029$, Fig. 4.19. MDA content was accumulating significantly with aging, but multiple comparisons did not reveal significantly different pairs; there was an overall aging effect. Other main effects and interactions did not demonstrate a significance including gender, $F(1,16)=0.014$, $p=0.909$; environment, $F(1,16)=0.073$, $p=0.791$; age and gender, $F(1,16)=0.001$, $p=0.977$; age and environment, $F(1,16)=0.149$,

p=0.705; gender and environment, F(1,16)=3.385, p=0.084; age and gender and environment, F(1,16)=0.275, p=0.607.

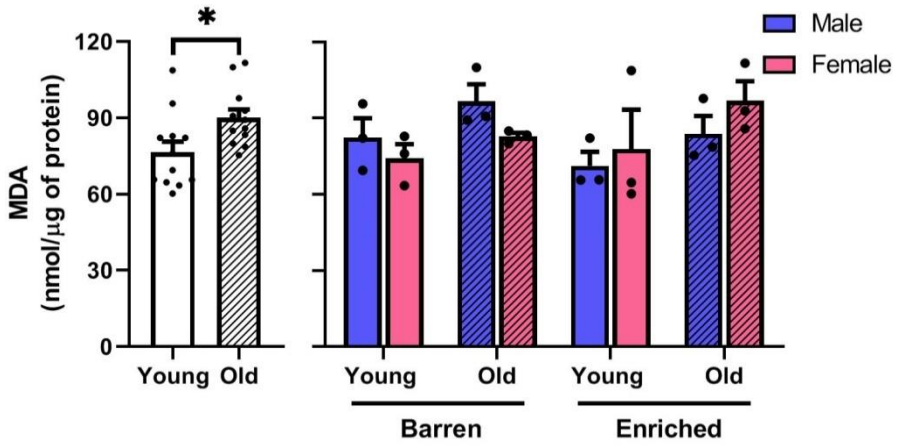
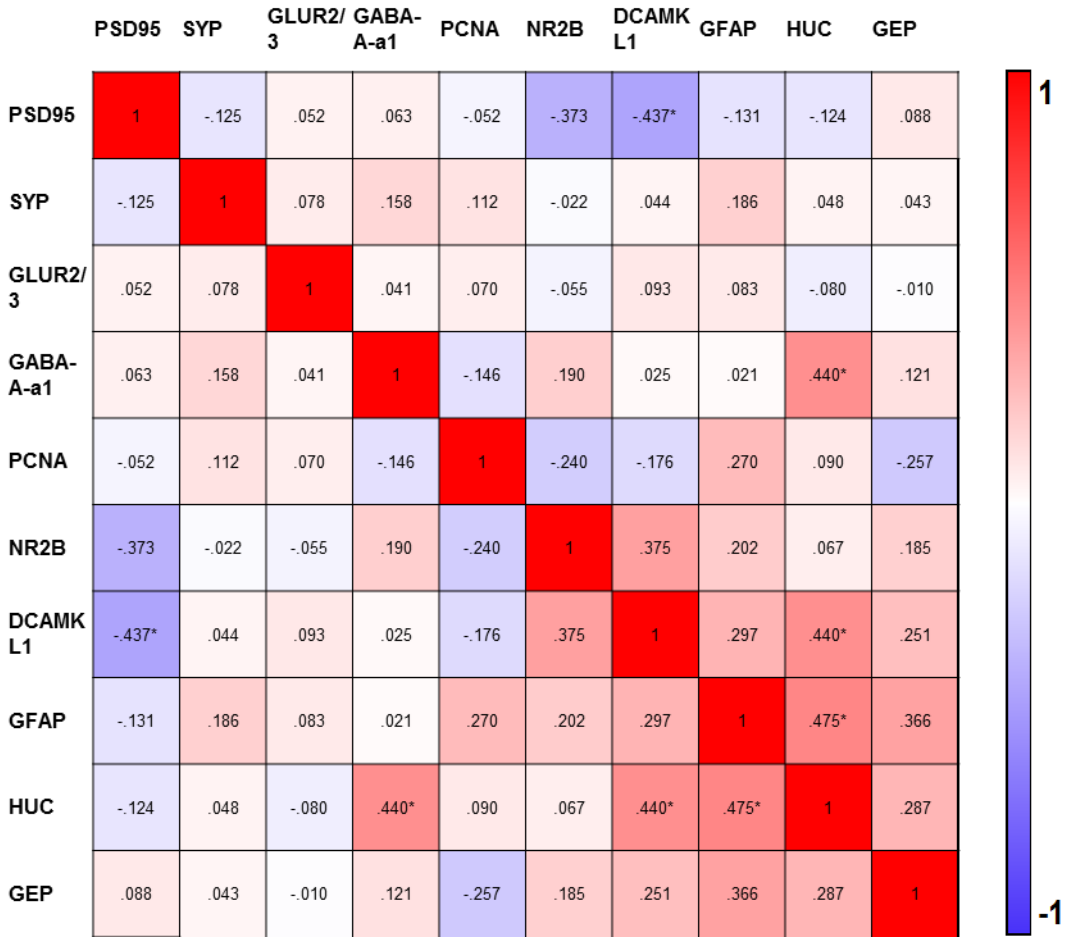


Figure 4.19 MDA levels were significantly altered by the factor of age. *: p<0.05. Error bars = +Standard Error Mean. Adapted from Karoglu-Eravsar et al., 2021 [74].

4.5 Multivariate analyses showed age and environment-dependent clustering on synaptic and cellular proteins

Correlation analyses were conducted among the synaptic and cellular proteins before the further multivariate analyses. Significant correlations were revealed among cellular and synaptic proteins. DCAMKL1, a post-mitotic neuronal marker, was significantly and negatively correlated with the excitatory clustering marker PSD95 (r(22)=-0.437, p=0.033). Also, DCAMKL1 was significantly and positively correlated with another neuronal marker which was HuC, giving information about immature neuronal population (r(22)=0.440, p=0.032). In addition to this correlation

HuC was significantly and positively correlated with inhibitory synaptic marker GABA-A-a1 ($r(22)=0.440$, $p=0.031$); and glial marker GFAP ($r(22)=0.475$, $p=0.019$), Figure 4.20.



	PSD95	SYP	GLUR2/3	GABA-A-a1	PCNA	NR2B	DCAMKL1	GFAP	HUC	GEP
PSD95	1	-.125	.052	.063	-.052	-.373	-.437*	-.131	-.124	.088
SYP	-.125	1	.078	.158	.112	-.022	.044	.186	.048	.043
GLUR2/3	.052	.078	1	.041	.070	-.055	.093	.083	-.080	-.010
GABA-A-a1	.063	.158	.041	1	-.146	.190	.025	.021	.440*	.121
PCNA	-.052	.112	.070	-.146	1	-.240	-.176	.270	.090	-.257
NR2B	-.373	-.022	-.055	.190	-.240	1	.375	.202	.067	.185
DCAMKL1	-.437*	.044	.093	.025	-.176	.375	1	.297	.440*	.251
GFAP	-.131	.186	.083	.021	.270	.202	.297	1	.475*	.366
HUC	-.124	.048	-.080	.440*	.090	.067	.440*	.475*	1	.287
GEP	.088	.043	-.010	.121	-.257	.185	.251	.366	.287	1

Figure 4.20 Analyses revealed significant correlations among the synaptic and cellular proteins. *: $p<0.05$.

A multivariate analysis incorporating all synaptic and cellular protein levels was performed using principal component analysis (PCA). The three components were obtained by using PCA. The explained total variance by summation of these three components was 73.017%. The first principal component explained (PC1) 41.679% of the variance. When its contributors were analyzed, it was revealed that DCAMKL1 and NR2B affect PC1 positively, while PSD95 has inverse contributions (component loading scores>0.5 or <-0.5). The second component (PC2) was associated with 18.277% of the variance, and it was significantly driven by glial marker GFAP positively (component loading score> 0.5). In addition to GFAP, immature neuronal marker HuC and proliferation marker PCNA also contributed to PC2. However, their contributions were slightly weaker (component loading score> 0.4). Lastly, the third (PC3) component was accounted for 13.061% of the total variance, and PC3 was mainly driven by GEP positively (component loading scores>0.5), while PCNA has a negative contribution to PC3 (component loading scores<-0.5). Factor loadings of each component with respect to synaptic and cellular protein were shown in Figure 4.21.

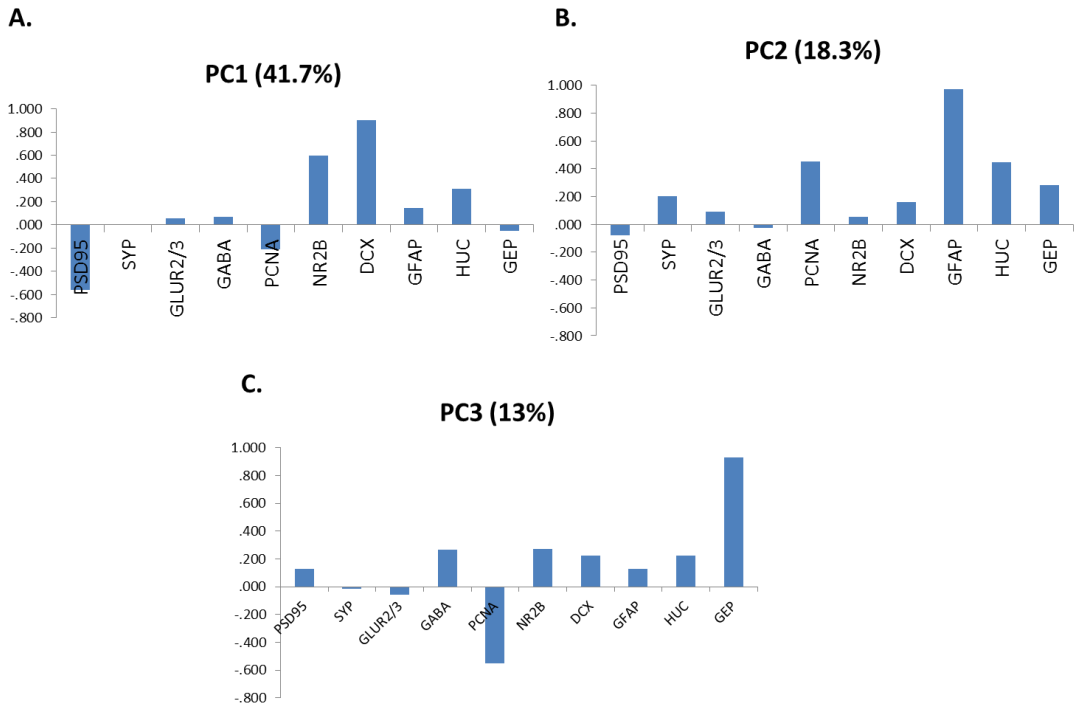


Figure 4.21 Factor loading scores for three principal components with respect to proteins. PC1 is contributed by DCAMKL1 and NR2B positively and PSD95 negatively (A). PC2 was contributed by GFAP positively (B). PC3 was altered by GEP positively and PCNA negatively (C).

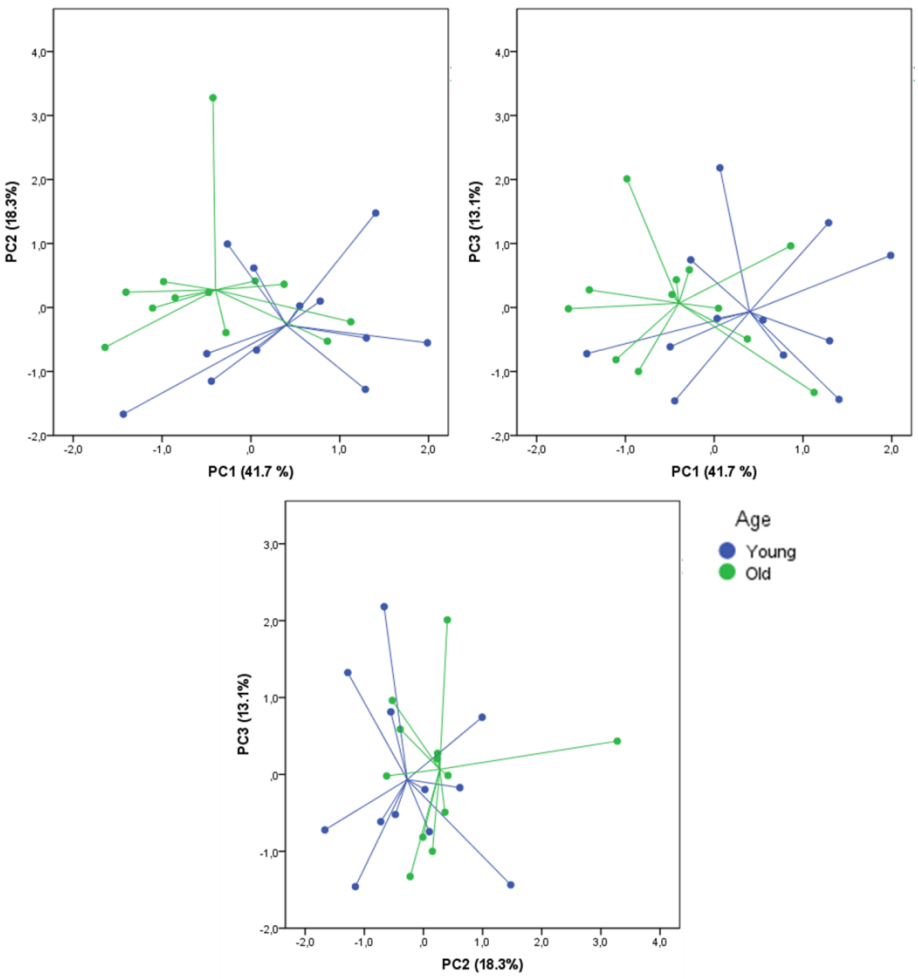


Figure 4.22 Three components were visualized by separating the age groups; PC1 showed an age-specific clustering profile. Age groups were denoted by different colors. Young: Blue, Old: Green.

The age-dependent clustering profile of these three components was shown in Figure 4.22. It can be observed that more prominent age-specific clustering was found in the first component, PC1, which was positively regulated by NR2B and DCAMKL1 and negatively contributed by PSD95. The age-specific trend indicated that young animals tended to have higher PC1 scores, while zebrafish have lower PC1 scores at older ages. Moreover, groups were separated solely depending on the

factor of environment, but no strong clustering trend was observed on these data Figure 4.23. However, when both age and environment groups were segregated, environmental effects that are dependent on the animal's age become more evident Figure 4.24. It can be observed that old animals in the enriched environment tended to have higher PC2 scores, which were driven by glial, immature neuronal, and proliferative markers positively. Young animals assigned to an enriched environment tend to have lower PC3 scores positively modulated by inhibitory marker GEP and negatively contributed by proliferation marker PCNA, Figure 4.24.

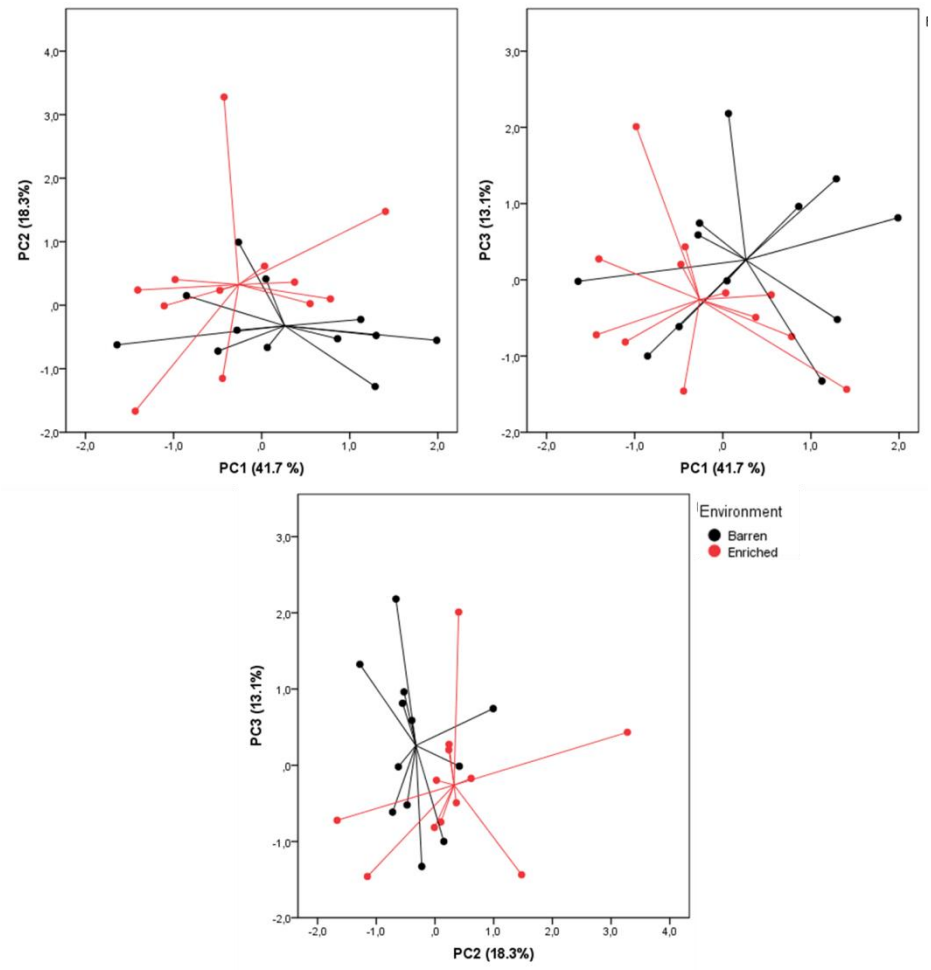


Figure 4.23 Three components were visualized by separating the environmental conditions, which were denoted by different colors. Barren: Black, Enriched: Red.

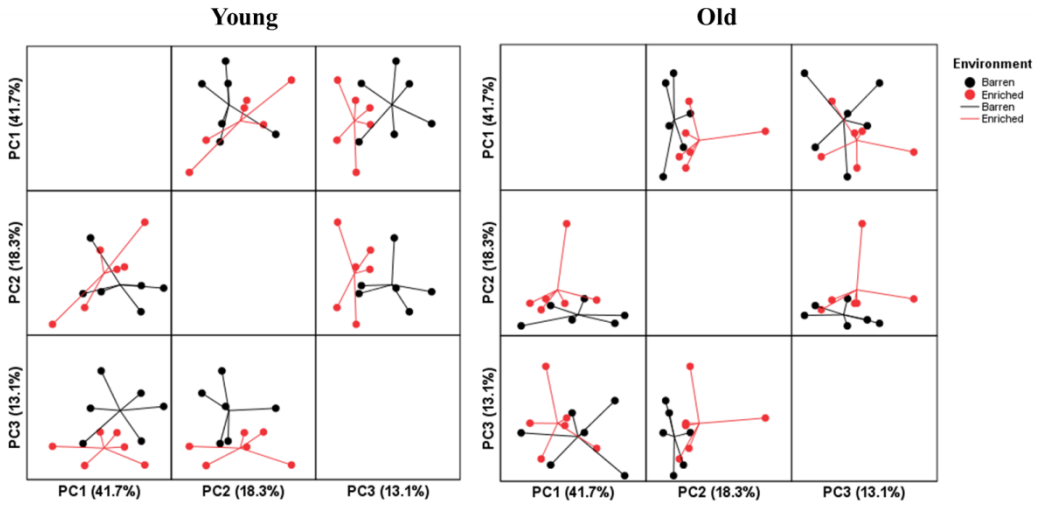


Figure 4.24 Clustering profiles of components in which both age groups and environmental conditions were separated. Age groups were shown in separate panels while environmental conditions were denoted by different colors Barren: Black, Enriched: Red.

4.6 Discussion and Conclusions

This chapter focused on showing the ameliorating effects of sensory EE against alteration occurring in the zebrafish brain. In the literature, these positive effects of EE were evaluated within the mammalian models mainly. It has been shown that EE can alter the behavior, increase the learning performance and resilience of the organism against external factors in the zebrafish model organism [57],[72],[101]. However, most of these studies were conducted using young adult groups, and there is a relative lack of evidence about the effects of EE in the aging domain in this model. Also, in the current design, apart from including two age groups and comparing them within the same experimental conditions, both female and male subjects were included and we were able to distinguish certain age and gender

mediated patterns in few of the markers which can provide insights about gender-specific vulnerabilities and developing gender-specific strategies to prevent neurobiological deteriorations occurring with aging. Our finding demonstrated that with aging synaptic markers including excitatory elements of neurotransmission such as NR2B and GluR2/3, synaptic integrity indicator SYP, and post-mitotic marker expressed majorly at the zones of neurogenesis DCAMKL1 were significantly decreased, and also it was shown that oxidative stress markers such as the levels of lipid peroxidation and ROS activity were significantly elevated in the aging brain of the zebrafish. Application of EE prevented the age-related decline in the levels of NR2B and DCAMKL1 and also elevated levels of SYP and GEP in the old zebrafish exposed to short-term EE with sensory elements. It can be concluded that old animals can benefit from application of EE and older age groups can also be the targets of this intervention since at this age group parameters regulating the activity-dependent synaptic plasticity are maintained and restored by EE.

In the current Chapter, enrichment intervention was designated as short-term by exposing the animals to a novel and enriched environment for 4-weeks apart from the initial one-week period in the habituation phase. This duration, 4-weeks, was chosen because it was previously demonstrated that it could lead to improvements in behavioral measures, altered expression profiles of the genes regulating neuroprotective effects in the brain, and increased resilience against external factors such as unpredictable chronic stress application by altering the oxidative stress-dependent components [57], [72]. The paradigm utilized in this study focused on EE's sensory components and forced physical exercise was not introduced as a part of this

paradigm. Exercise as an intervention can be applied to mammalian models and zebrafish, which was achieved with forced swimming under conditions where water flow velocities were increased experimentally [102]. Conventional EE paradigms were conducted by combining components like the sensory, exercise, and socialization factors [52]. However, recent studies indicated that different aspects and components of the EE could induce differential outcomes by affecting dissociated pathways. These studies have been evaluated within the context of aging, as it was shown that short-term EE without physical exercise could reverse age-related behavioral deficits, including anxiety-related behavior and spatial memory impairments in middle-aged and old mice. In contrast, the application of physical exercise without EE did not lead to behavioral improvements in these age groups [103].

Interestingly, physical exercise alone and physical exercise combined with EE are associated with gene expression alterations in middle-aged mice. Still, an EE without physical exercise does not result in gene expression changes, although the effects were evident on the behavior [103]. Another study using a longitudinal design in a rat model showed that life-long EE in the absence of physical exercise prevents age-related cognitive declines and reverses structural and molecular alterations in the brain occurring with aging [104]. Moreover, the components of EE differentially alter cellular mechanisms in the scope of normal aging and within the context of age-related neurodegenerative diseases. It has been demonstrated that both EE in the absence of physical exercise and physical exercise on its own could prevent the detrimental effects of amyloid-beta infusion-related memory impairment and

decrease lipid peroxidation in the brain. At the same time, social enrichment only alters social recognition performance and antioxidant capacity [59].

The recent converging lines of evidence suggest that delineation of specific components of EE in terms of their dissociable effects on specific molecular mechanisms within the context of aging is crucial to understand the differential ameliorative aspects of this intervention. In this study, we observed brain weight and synaptic protein differences in the EE groups in the absence of a forced physical exercise component, providing insights regarding the variable effects of sensory enrichment regarding age and gender. For further research, specific EE components can be investigated separately and with full-factorial design, such as sensory and exercise enrichment without social components or social and exercise enrichment without sensory components within the context of aging. Additionally, each component can be systematically investigated. For example, the intensity and frequency of the sensory stimuli can be altered gradually by using computerized systems; and social components of EE can be investigated by demonstrating the virtual fish compared to maintaining the fish in social groups to investigate its effects systematically.

General body parameters were investigated, such as body weight, length, and BMI. In the body weight parameter, age and gender were significantly changing the body weight. This observation was parallel to zebrafish' developmental milestones, and old animals have higher body weight values than younger ages [105]. Female zebrafish had higher body weights than males, which was again in parallel to observations in the literature because female zebrafish had ovaries and eggs

comprising nearly 30% of their body [82]. In the length parameters, no difference was seen between the females and males, and only significant changes were seen for the age factor. Old zebrafish had significantly higher body length measurements than young zebrafish; again, these observations were parallel to the literature [82]. On the BMI measure, no difference was found between old and young zebrafish. The reason for that was in both length and weight measures; significant increases were seen in old zebrafish, and BMI was calculated based on these two measures because increases in these two measures were proportional and significant, no difference was seen in BMI. The only difference in the BMI was seen between female and male zebrafish, and male zebrafish had significantly lower than the female zebrafish due to the eggs and ovaries found in female zebrafish [82]. The rationale to have these measurements was to show whether sensory EE alters these parameters and the well-being of fish by inducing stress or overall changes in general locomotor activity or eating problems. No environmental effect was seen on these body measures showing that EE intervention did not lead to significant changes in the well-being of the fish.

EE can also regulate the stress response; previous studies have shown that EE can put mild stress on the animals with exposure to a novel environment, and a mild level of stress can contribute to possible protective effects of the EE [60]. In the current work, stress responses were not evaluated since there was no significant change observed on the body parameters such as body weight, body length, and BMI, which can reflect the overall well-being of the animals. Another reason was that previous work has shown that without applying the additional stressor, there was no significant change in the body cortisol levels with 4-weeks of EE compared to the

barren environmental condition [72]. However, when the additional stressor like unpredictable chronic stress was introduced in the EE setup, it was shown that stress response was blunted in the animals with EE. At the same time, in the control environment, there was a significant upregulation in the body cortisol levels reflecting elevated levels of stress [72]. Since there was no additional external stressor in the current paradigm, we did not expect a significant change in the cortisol response, and body cortisol levels were not analyzed. Also, although our intervention was considered short-term, after 4-weeks, body cortisol levels can be decreased to baseline. For further research, duration-dependent effects of EE can be analyzed concerning cortisol levels, and shorter durations of EE such as 1-week and 2-weeks can be compared with the longer durations such as 4-weeks. In this way, possible changes in the cortisol response at the initial period of EE can be determined, and the time required for the adaptation of the stress response can be determined.

The protein expression levels of PSD95, GluR2/3, and NR2B were analyzed to investigate the changes in the excitatory synaptic markers. PSD95 is a membrane-associated guanylate kinase and located in the post-synaptic site of the excitatory glutamatergic neurons. It scaffolds the primary receptors of the glutamate, including N-methyl D-aspartate (NMDA) and alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA); it binds to NMDA receptors directly while indirectly regulate the AMPA type receptors [13]. The current study's results demonstrated no significant effects or interaction among the factors, including age, gender, or environmental intervention. Previous studies utilizing the mammalian model demonstrated that the application of EE with physical exercise (PE) components

could increase the protein expression levels of PSD95 [106]. However, it was also demonstrated that PE application on its own could also elevate PSD95 [107], [108]. Since we did not observe any elevation of the PSD95 levels with the application of sensory, short-term EE, it could be said that most modulations on the PSD95 levels can be mediated by the PE components of the EE rather than its sensory components.

Another component of excitatory neurotransmission analyzed in the current study was GluR2/3, which are the two major subunits of ionotropic AMPA receptors. These proteins can regulate the RNA editing process, calcium permeability, and trafficking of AMPA receptors [109]. It was demonstrated that GluR2/3 levels reduced significantly with aging; this observation was parallel to the literature [61], [110]. Additionally, differential effects of age and gender type were demonstrated under EE conditions. Young female fish with EE had elevated GluR2/3 levels to the old female fish with EE and young male fish with EE. EE can present more advantage for young female zebrafish concerning GluR2/3, and higher levels of GluR2/3 can show buffer mechanism against aging-related deteriorations where GluR2/3 levels decrease in cases of increased calcium influx and increased oxidative stress [111], [112]. In this regard, in the present study, ROS activity alterations were parallel to GluR2/3 changes, as demonstrated increases seen in GluR2/3 subunits parallel decreases in ROS in young zebrafish with EE. Therefore, benefits of this subunit were observed in young female zebrafish with EE. Our results demonstrated that susceptibility and benefits obtained from EE could depend on age and gender regarding this specific subunit.

The third excitatory synaptic component analyzed was the subunit of NMDA receptors NR2B. NR2B has regulatory roles, mainly in synaptic plasticity and also synapse maturation and facilitation of responses such as long-term potentiation (LTP) [19], [113]. Interestingly, it was demonstrated that in the control environment, NR2B protein expression levels decrease significantly at old zebrafish; this declining trend was also demonstrated in the other model organisms in aging research [20], [114]. Also, the exposure to EE kept NR2B levels from declining with age, and EE can be associated with stable levels of NR2B at different ages, which can increase the subjects' brain resistance [21]. Besides the effects of age, an effect of gender was found and demonstrated that NR2B levels were higher in female zebrafish than that of males, and this difference was significant. Previous studies reported that sex hormones, including estradiol, have increased abundancy of NR2B receptor levels, and estradiol facilitated expression levels of NR2B and long-term potentiation [151]. In the light of these findings, it was also reported that estradiol levels are higher in female zebrafish [152], and the elevated protein levels of NR2B can be modulated in a sex hormone-dependent manner.

In addition to the excitatory synaptic markers, SYP, a general synaptic marker and an indicator of pre-synaptic integrity, was investigated. In the literature, it was shown that the protein levels of SYP reduced at older ages as compared to young ages in multiple model organisms of human aging [14], [20], [44], [88]. Results indicated a parallel decline in SYP, and animals exposed to the control environment drove this overall significant decline. Previous studies showed that EE could elevate the levels of SYP in rodent models [53], [106], [115]–[117]; similarly, our results

indicated a significant increase in SYP levels in zebrafish with EE as compared to zebrafish maintained in the control environment. More importantly, the data showed an interaction between environment and age; SYP levels were relatively stable with aging in the zebrafish exposed to EE; old zebrafish with EE had significantly elevated SYP than older fish in the control conditions. It can be concluded that EE with sensory components can promote SYP levels, but also, this elevation occurs depending on the age of the zebrafish.

GEP and GABA-A-a1 were investigated as the elements of inhibitory synaptic transmission. GEP is a clustering and scaffolding protein like PSD95. On the contrary, it clusters the proteins at inhibitory synapses to illustrate one of its clustering partners as the subunits of ionotropic $GABA_A$ receptors [87]. Different subunits constitute $GABA_A$ receptors, and GABA-A-a1 is one of these subunits required for the functional channel. Additionally, expression levels of this subunit can be associated with resistance against stress [118]. An age-related decline in the GEP protein expression levels was reported in the literature, while no specific fluctuation was reported in GABA-A-a1 levels with aging [88], [26]. Our data indicated an interaction between environment and age regarding the levels of GEP; the old zebrafish with EE had elevated levels of GEP than young zebrafish in the EE. Increased levels of GEP can protect the brain from increased excitotoxicity arising at older ages. A previous study indicated that reduced protein levels of GEP could be associated with elevated levels of excitotoxic insults [24]. No age difference was demonstrated on GABA-A-a1 protein expression; previous evidence suggested that

this inhibitory subunit maintained without significant fluctuation at different ages [26].

Apart from the synaptic markers, cellular markers were also investigated concerning gender, age, and EE. For this purpose, two markers expressed in the neural cells were assessed, such as HuC and DCAMKL1. Both markers were expressed in post-mitotic neuronal cells; HuC was expressed in immature cells while DCAMKL1 was expressed in migrating neurons [90], [92]. We did not observe a significant effect of EE on HuC and DCAMKL1 levels. In this respect, one important factor needs to be considered: the physical exercise component of the EE paradigms utilized in the previous studies. It is well-known that short-term physical exercise on its own could induce neurogenesis and significantly increase DCX positive cells [119]. Therefore, EE's observed effects on neuronal markers and neurogenesis indicators might be driven solely by its physical exercise component. Since our EE paradigm did not include any forced exercise, this is likely why no alterations in the global levels of these neuronal markers were observed.

On the other hand, current results also demonstrated a significant decrease in DCAMKL1 levels with aging in the control environment driving the overall significant declining pattern in DCAMKL1 levels. However, in the EE condition, no age-specific reduction was seen. To conclude, with the EE exposure, DCAMKL1 levels were stabilized with aging, while noticeable fluctuations were observed in the control environment.

In addition to neuronal markers, glial and proliferation indicators were also investigated. GFAP, which was used as a glial marker, could be altered and elevated in the cases of brain insults such as dysregulated functioning of astrocytes [93]. However, it should be noted that glial cell populations are not only altered and activated during these detrimental conditions but also changed after exposure to improved and stimulating environmental conditions such as EE [120], [121]. Upregulation in the glial populations indicated by increased GFAP may be associated with increased neuronal support by glia and synaptic tuning, crucial for cognitive processing [121]. No significant difference was found regarding this glial marker. The proliferating cell nuclear antigen (PCNA) levels, which is an endogenous marker of cell division and labels cycling progenitor cells, were investigated as an indicator of global proliferation [94]. Although there was no difference in PCNA, zebrafish with EE had a tendency to had elevated levels of PCNA, which may show subtle increases in the proliferation in zebrafish with EE.

The further aim was to reveal whether the environment and age-mediated changes occurred in the oxidative stress markers and mediatory counterparts. For this aim, two oxidative-stress markers were investigated, such as ROS and lipid peroxidation levels. In the literature, increases in these markers were reported with aging in the brain in different model organisms [122]. No other effect such as EE was seen on these markers. Evidence suggested that EE could reduce oxidative stress markers when pathological brain conditions and applications of stressors were conducted [72], [123]. Nonetheless, when the environmental or biological insult was not present, no specific EE modulation was reported [59], [72]. Therefore, our results

showed comparable changes to previous findings [59], [72]. Additionally, in the ROS levels, gender effect was seen, and female zebrafish had lower ROS levels than male zebrafish. This difference can be modulated by sex hormones [124].

In addition to oxidative markers, a cholinergic marker, AChE, was investigated since its levels were changing due to changes in the oxidative status levels [37], [125]. No significant differences were revealed on the AChE levels. For future studies, more specific mechanisms related to other critical components of the cholinergic system, such as alpha-7 nicotinic acetylcholine receptors, which directly affect cognitive abilities [83], will be further investigated within the context of EE.

Protein expression levels of the markers checked with Western blot analysis were further combined in multivariate analysis by using principal component analysis (PCA). In the PCA, we have observed age- and environment-specific clustering profiles in these markers. Age-related changes were mostly seen in the first component (PC1), which was driven positively by DCAMKL1 and NR2B and negatively by PSD95, with old groups tending to have lower PC1 scores compared to young animals. This observation may imply aging-related plasticity changes since both DCAMKL1 and NR2B are associated with increased plasticity [19], [92]. The expression levels of the NMDA receptor subunits NR2A and NR2B shifts across development. This shift can differentially regulate synaptic plasticity, with maturation and pruning processes happening when PSD95 occludes NR2B, and this occurs with significant elevations in the levels of PSD95 and NR2A [126]. Additionally, it was shown that DCAMKL1 could alter post-synaptic content with its kinase domain by negatively regulating elements such as PSD95 [127]. Therefore, this negative

relationship among these markers likely reflects maturation- and pruning-mediated alterations. Environment-dependent clustering profiles were observed in the other two components, PC2, and PC3. Young and old enriched groups tend to have higher PC2 scores compared to animals in the barren environment. PC2 was controlled by GFAP, HuC, and PCNA positively. Higher PC2 scores might indicate overall increases in immature neuronal and glial populations, which can potentially exert more plasticity in enriched environmental conditions. In terms of PC3, differences were observed between young enriched and barren environmental groups. Enriched young subjects tend to have lower PC3 scores than the young group in the barren environment, which was positively driven by GEP and negatively regulated by PCNA. Low PC3 scores in young enriched animals may show increased overall proliferative capacity along with decreased elements of inhibitory neurotransmission, and these changes can modulate the plasticity dynamics in enriched young groups. These data suggest that changes in the levels of excitatory and neuronal proteins altering the plasticity mechanisms may occur with aging. Moreover, environment-specific modulations may likely be observed in the clustering profile of immature neuronal, glial, proliferative, and inhibitory markers.

To conclude, the current study indicated that at older ages, synaptic markers such as excitatory markers like NR2B and GluR2/3, synaptic integrity indicator SYP, and post-mitotic neuronal marker DCAMKL1 were significantly declined. Also, it was found that oxidative stress markers were significantly increased in the brains of the old zebrafish. Exposure to sensory EE attenuated the age-related decrease in the levels of NR2B and DCAMKL1. Also, it increased the levels of SYP and GEP in the

old zebrafish groups exposed to short-term EE with sensory components. To conclude, old zebrafish can benefit from short-term exposure to EE, and old ages can also be the targets of this intervention. At this age, parameters controlling the activity-associated synaptic plasticity are maintained and restored by EE.

CHAPTER 5

THE EFFECTS OF OPPOSING DIETARY TREATMENTS OF CALORIC RESTRICTION AND OVERFEEDING ON BODY PARAMETERS, SYNAPTIC PROTEIN LEVELS, AND GENE EXPRESSION PATTERNS ON THE BRAIN WITHIN THE CONTEXT OF AGING

5.1 Dietary interventions have altered the general body parameters as well as cortisol levels

Dietary interventions have lasted for 12 weeks, and when these interventions were terminated, and fish were euthanized, body weight and length of the animals were measured and recorded individually. These measures were analyzed within the context of diet and aging, and they were used to calculate the body mass index of the zebrafish. A significant main effect of age was revealed on the body weight measurements, $\chi^2(1)=9.301$, $p=0.002$, Figure 5.1. As animals got older, they had significantly higher body weight measures as compared to their younger counterparts. Pairwise comparisons indicated that this age effect was statistically significant in *ad-libitum* (AL) ($p=0.001$) and caloric restriction (CR) ($p<0.0005$) regimens; old animals in these dietary regimens had significantly higher body weight as compared to young animals of these feeding conditions, Figure 5.1. However, in the over-feeding (OF) regimen, no significant effect of age was revealed by posthoc analyses Figure 5.1. Additionally, the dietary condition's effect was significantly changing the body weight of the animals, $\chi^2(2)=30.745$, $p<0.0005$, Figure 5.1. Pairwise comparisons indicated that at young age OF group had significantly higher body weight compared

to young AL (p=0.044) and young CR (p<0.0005) animals. At a young age, the CR-fed group had significantly lower body weight than the young AL group (p<0.0005). When body weight measures were investigated at older ages among the diet groups, it was observed that CR old animals had significantly reduced body weight compared to AL old (p<0.0005) and OF old (p<0.0005) zebrafish. However, at old age, no significant difference in the body weight was observed between AL and OF groups, Figure 5.1.

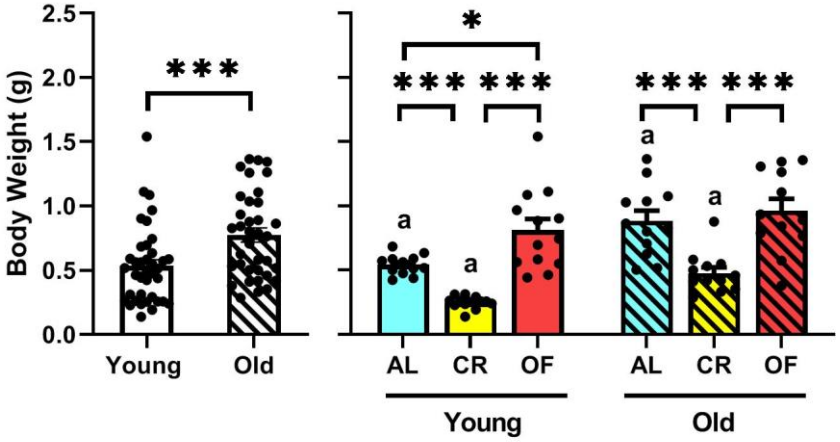


Figure 5.1 Total body weight measurements across age and diet groups. Significant effects of age and diet were demonstrated on the body weight measures. a: Significant main effect of age was observed in a diet group. *: p < 0.05, ***: p < 0.005. Error bars = +Standard Error Mean. Adapted from Karoglu-Eravsar et al., 2021 (under-review) [128].

In terms of a measure of length, a significant main effect of age was demonstrated, $\chi^2(1)=18.424$, p<0.0005, Figure 5.2. As a similar pattern to weight measures, animals had significantly increased body length when they became older.

Post-hoc analyses indicated that this age effect is significantly affecting the AL (p<0.0005) and CR (p<0.0005) groups. On the other hand, no significant length difference was observed between OF-young and OF-old animals, Figure 5.2. In addition to the age effect, a significant main effect of dietary condition was seen on the length measures, $\chi^2(2)=26.873$, p<0.0005. Multiple comparisons among the diet groups showed that CR animals had lower body length as compared to AL (p<0.0005) and OF (p<0.0005) groups at a young age. Similar differences were revealed at old age, AL (p<0.0005) and OF (p=0.001) animals had significantly increased body length as compared to the CR group. No significant difference was found between AL and OF animals in terms of length measures at both young and old ages, Figure 5.2.

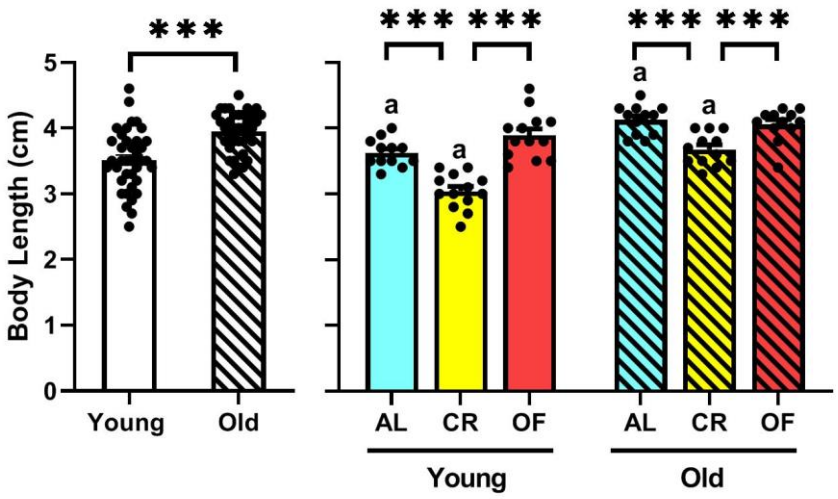


Figure 5.2 Total body length measurements across age and diet groups. Significant effects of age and diet were demonstrated on the body length measures. a: Significant main effect of age was observed in a diet group. ***: p <

0.005. Error bars = +Standard Error Mean. Adapted from Karoglu-Eravsar et al., 2021 (under-review) [128].

In conjunction with alterations seen in body weight and length measurements, similar trends were also observed on the body mass index (BMI) measure, calculated by dividing body weight in grams to squared body length in centimeters. BMI generally was utilized as another body measure to indicate the animals' well-being in response to external factors and used in different model organisms, including zebrafish [129]. A significant age effect was also observed on BMI, $\chi^2(1)=4.725$, $p=0.030$, Figure 5.3. However, the age effect was solely seen in the CR diet group; young CR animals had significantly lower BMI than old CR subjects ($p=0.004$). On the other hand age effect on BMI was not significant in AL-fed and OF-fed animals. Additionally, a significant effect of dietary condition was also observed on the BMI, $\chi^2(2)=39.337$, $p<0.0005$. Pairwise comparisons conducted among the dietary regimens have shown that at a young age, animals in the CR regimen had significantly lower BMI compared to AL ($p<0.0005$) and OF ($p<0.0005$) -fed zebrafish ($p<0.0005$). This pattern was the same in the old age group as well AL animals ($p=0.002$) and OF ($p<0.0005$) animals had significantly increase BMI as compared to aged CR zebrafish, Figure 5.3.

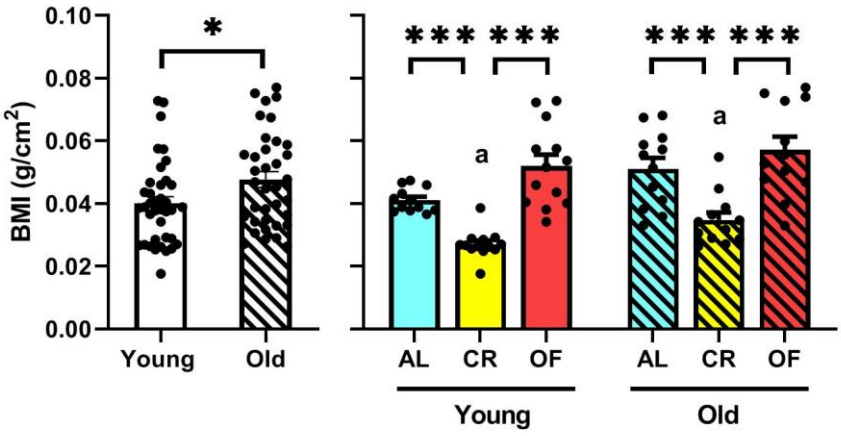


Figure 5.3 Total body mass index (BMI) measurements across age and diet groups. Significant effects of age and diet were demonstrated on the BMI. a: Significant main effect of age was observed in a diet group. *: $p < 0.05$, ***: $p < 0.005$. Error bars = +Standard Error Mean. Adapted from Karoglu-Eravsar et al., 2021 (under-review) [128].

As a last general body parameter, brain weight measurements were compared between age groups and dietary conditions. A significant main effect of age was demonstrated in the brain weight data, $F(1,66)=25.899$, $p<0.0005$. Increasing age was associated with elevated brain weight, and this age-specific increase was significant in AL ($p= 0.001$), CR ($p< 0.0005$), and OF ($p= 0.015$) dietary regimens, Figure 5.4. Additionally, the dietary condition's main effect was significantly changing the wet brain weight of the zebrafish, $F(2,66)=17.771$, $p<0.0005$. Pairwise comparisons indicated that at a young age, CR zebrafish had lower brain weight values compared to AL ($p= 0.001$) and OF ($p< 0.0005$)-fed zebrafish groups. Likewise, at old age, CR

zebrafish had lower brain weights than AL old zebrafish (p= 0.005) and OF old zebrafish (p= 0.001), Figure 5.4. No significant interaction was demonstrated between age and dietary condition concerning wet brain weight measurements, $F(2,66)=0.289$, $p=0.750$.

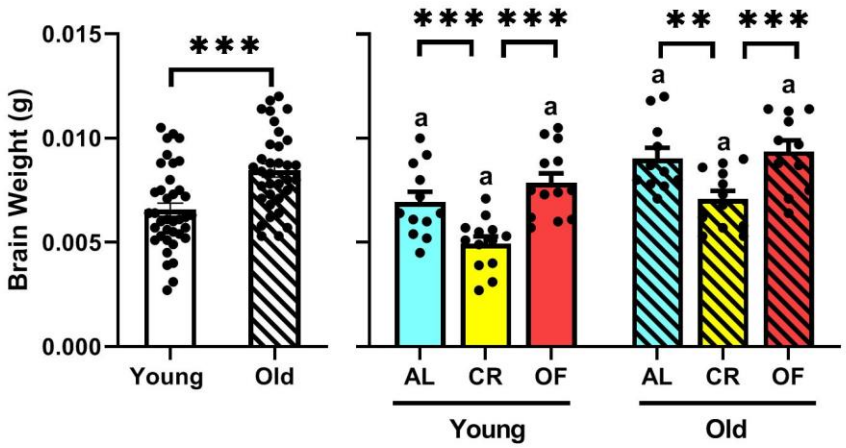


Figure 5.4 Wet brain weight measurements across age and diet groups. Significant effects of age and diet were demonstrated on brain weight. a: Significant main effect of age was observed in a diet group. **: $p < 0.01$, ***: $p < 0.005$. Error bars = +Standard Error Mean. Adapted from Karoglu-Eravsar et al., 2021 (under-review) [128].

Trunk cortisol was assessed to understand the diet or age-related fluctuations in a stress response marker in zebrafish. On the trunk cortisol levels, the effect of age was not significant, $F(1,47)=1.326$, $p=0.255$. However, a significant main effect of diet was demonstrated affecting the trunk cortisol levels, $F(2,47)=3.856$, $p=0.028$. Posthoc tests were carried out to distinguish significantly different groups; it was observed that there was a significant difference between OF-young animals as

compared to CR-young animals (p=0.040). However, this increasing pattern, seen in OF dietary regimen at a young age, was not demonstrated at the older ages, Figure 5.5. Moreover, an interaction between age and diet factors did not significantly affect the trunk cortisol levels, $F(2,47)=0.729$, p=0.488, Figure 5.5.

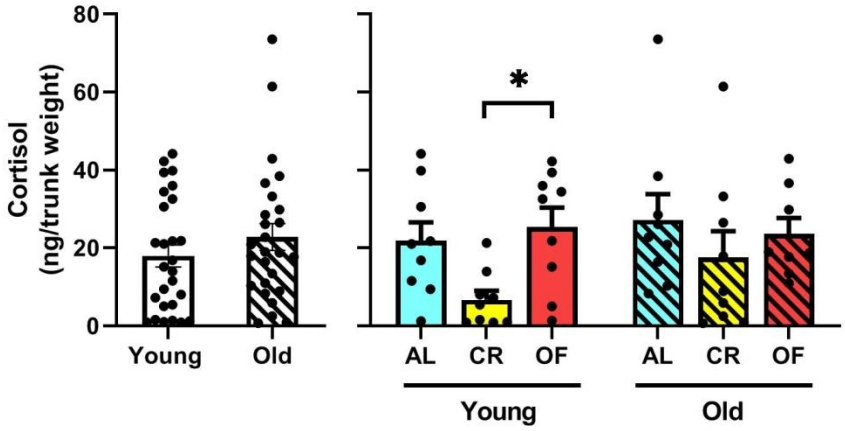


Figure 5.5 Trunk cortisol levels across age and diet groups. A significant effect of diet was demonstrated on trunk cortisol levels. *: $p < 0.05$. Error bars = +Standard Error Mean. Adapted from Karoglu-Eravsar et al., 2021 (under-review) [128].

5.2 Dietary interventions were altering excitatory synaptic markers while aging changed the inhibitory and presynaptic indicators

Synaptic proteins that can provide insights about the specific neurotransmission components were investigated concerning aging and different feeding regimens. As elements of excitatory synaptic integrity synaptic proteins; Post-synaptic Density 95 (PSD95), Glutamate receptor subunit 2/3 (GluR2/3), and N-methyl D-aspartate receptor subtype 2B (NR2B) were selected. Gephyrin (GEP) and Gamma-aminobutyric acid receptor subunit alpha-1 (GABA-A-a1) were investigated in terms of inhibitory markers of neurotransmission. Additionally, presynaptic and synaptic vesicle marker Synaptophysin (SYP) was used in this study. Representative blots of the cohorts were indicated in Figure 5.6.

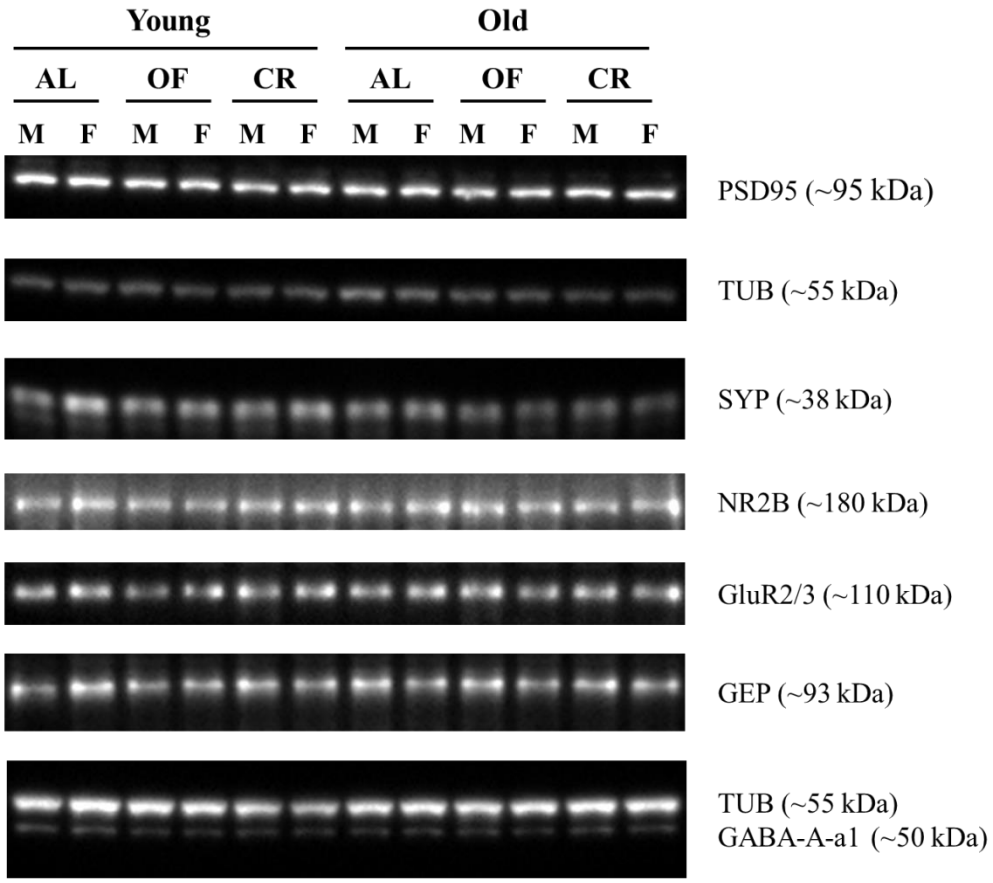


Figure 5.6 Representative pictures from one cohort for Western blot experiments of synaptic protein levels examined in the current Chapter for effects of age and dietary interventions. Bands were obtained at the expected molecular weights for all antibodies. AL: *Ad-libitum*; OF: overfeeding; CR: Caloric restriction; M: Male; F: Female. Adapted from Karoglu-Eravsar et al., 2021 (under-review) [128].

PSD95 was a scaffolding protein found at excitatory synapses and anchors and cluster glutamate receptors at the postsynaptic region [13]. Effect of age was marginally changing the PSD95 levels, $\chi^2(1)=2.919$, $p=0.088$, Figure 5.7. At the older age, brain levels of PSD95 were showing a decreasing tendency. In terms of the

dietary regimen's effects, a significant main effect of diet was revealed on PSD95 levels, $\chi^2(2)=10.613$, $p=0.005$. In the overall data, the CR group tended to have higher levels of PSD95 compared to the other feeding groups. Moreover, this difference is significant between old AL and old CR groups ($p=0.020$) and marginally significant between old OF and old CR groups ($p=0.074$), Figure 5.7.

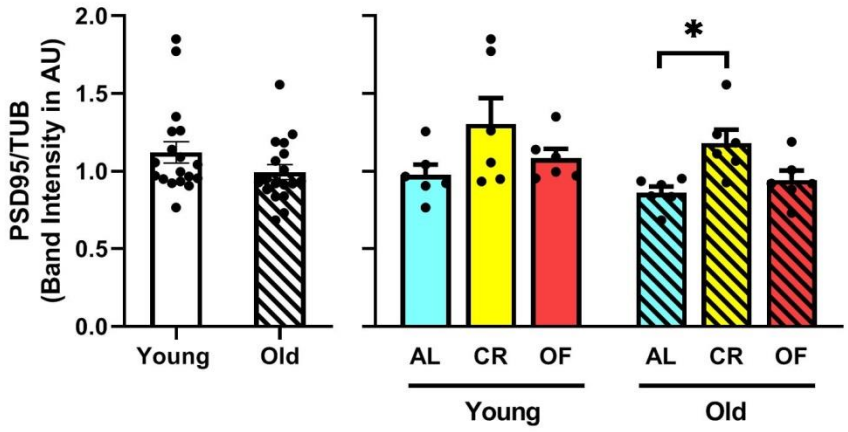


Figure 5.7 PSD95 levels across age and diet groups. A significant effect of diet was demonstrated on PSD95 levels. *: $p < 0.05$. Error bars = +Standard Error Mean. Adapted from Karoglu-Eravsar et al., 2021 (under-review) [128].

GluR2/3 were the AMPA receptor subunits and controlled the local calcium influx [15]. A significant main effect of age was found on the levels of GluR2/3, $F(1,30)=4.716$, $p=0.038$, levels of GluR2/3 tended to reduce in the old groups compared to young. Pairwise comparisons revealed no significantly different pairs but rather was an overall effect of a factor of age, Figure 5.8. Similar to the PSD95, a similar significant main effect of diet was also observed for the GluR2/3 levels, $F(2,30)=9.913$, $p<0.0005$. Like in the case of the PSD95, CR groups tended to have

significantly higher levels of GluR2/3. This difference was significant between young-AL and young-CR groups (p=0.006) and young-OF and young-CR groups (p=0.034). In the old group, parallel to this trend, the CR animals tended to have higher GluR2/3 levels than OF group (p=0.062), and the AL group (p=0.080) differences observed in the old group were not statistically significant, Figure 5.8. Lastly, there was no significant interaction between these factors altering the GluR2/3 levels, $F(2,30)=0.301$, p=0.742.

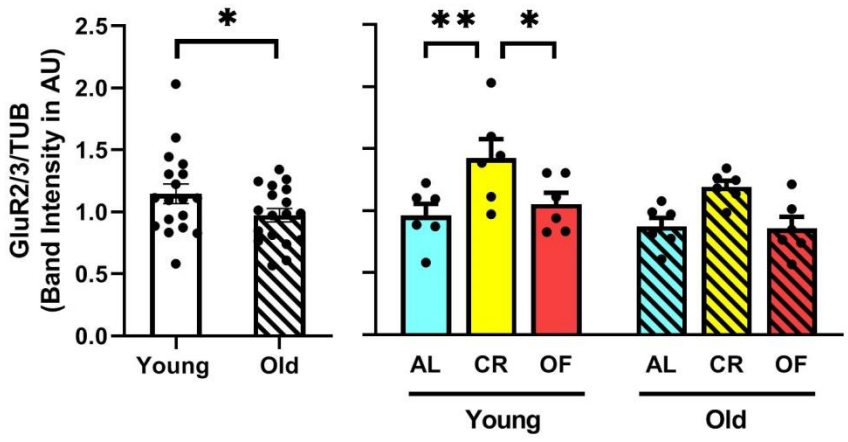


Figure 5.8 GluR2/3 levels across age and diet groups. Significant main effects of diet and age were demonstrated on GluR2/3 levels. *: $p < 0.05$, **: $p < 0.01$. Error bars = +Standard Error Mean. Adapted from Karoglu-Eravsar et al., 2021 (under-review) [128].

The last excitatory marker investigated in the current study was NR2B, a subtype of NMDA receptors, and its increased expression is associated with promoted synaptic plasticity [130]. The effect of age was not significantly altering the levels of

NR2B, $\chi^2(1)=1.445$, $p=0.229$, Figure 5.9. Additionally, the effect of diet was not statistically significant for the NR2B, $\chi^2(2)=3.047$, $p=0.218$, Figure 5.9.

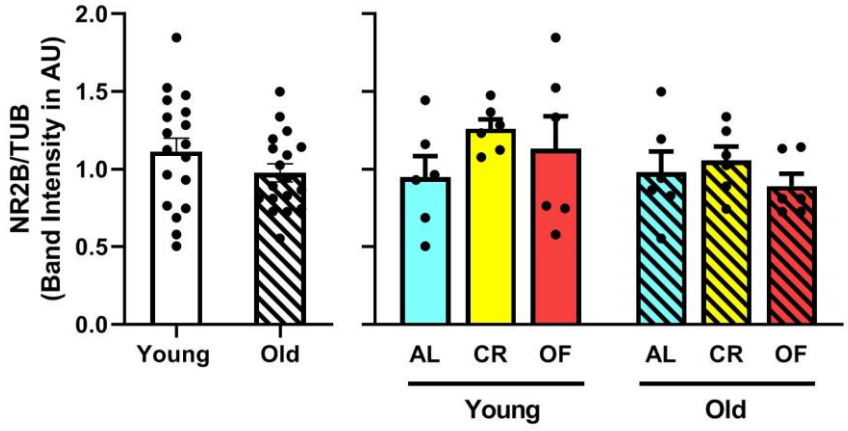


Figure 5.9 NR2B levels across age and diet groups. Error bars = +Standard Error Mean. Adapted from Karoglu-Eravsar et al., 2021 (under-review) [128].

In terms of inhibitory neurotransmission, scaffolding and clustering protein found at GABAergic synapses GEP and one of its clustering partners and predominant form of GABA-A receptor in the central nervous system GABA-A-a1 were further investigated. On the GEP levels, a significant effect of age was revealed, $\chi^2(1)=5.481$, $p=0.019$. Young groups had significantly higher levels of GEP compared to the old groups, Figure 5.10. Pairwise comparisons were conducted to differentiate the significant difference between the pairs. The age factor was robustly affecting the OF group, as they showed a significant age-related decline in GEP levels ($p<0.050$). Additionally, no significant diet effect was observed on GEP levels, $\chi^2(2)=.416$, p=.812, Figure 5.10.

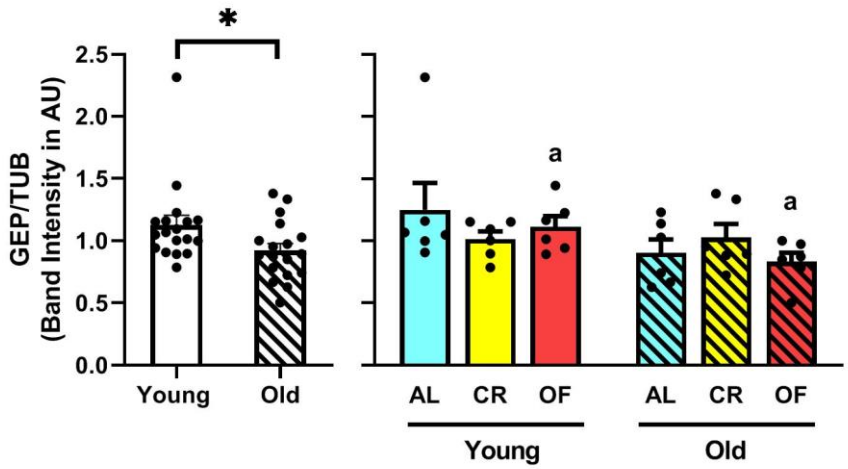


Figure 5.10 GEP levels across age and diet groups. A significant effect of age was demonstrated on the GEP. a: Significant main effect of age was observed in a diet group. *: $p < 0.05$. Error bars = +Standard Error Mean. Adapted from Karoglu-Eravsar et al., 2021 (under-review) [128].

On the expression levels of the GABA-A-a1, which was the clustering partner of the GEP, no significant age effect was shown, $\chi^2(1)=0.842$, $p=0.359$, Figure 5.11. Moreover, dietary interventions were not significantly altering the levels of the GABA-A-a1 on the zebrafish brain, $\chi^2(2)=1.272$, $p=0.529$, Figure 5.11.

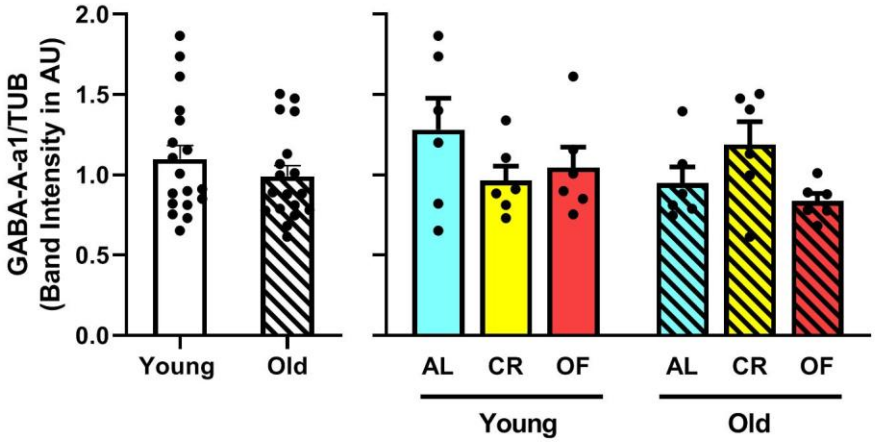


Figure 5.11 GABA-A-a1 levels across age and diet groups. Error bars = +Standard Error Mean. Adapted from Karoglu-Eravsar et al., 2021 (under-review) [128].

As the last synaptic protein marker, SYP was analyzed as an indicator of pre-synaptic integrity. A significant effect of age was revealed, $\chi^2(1)=7.929$, $p=0.005$, on the SYP levels. An overall trend showed that with increasing age, SYP levels tended to be reduced, Figure 5.12. Moreover, no pairs significantly different from each other were differentiated across the diet groups by multiple comparisons, so the factor of age was altering the SYP levels in an overall pattern that was not specific to a particular dietary regimen. In terms of effects-driven by dietary regimen, no significant effect was demonstrated on SYP levels, $\chi^2(2)= 0.038$, $p=0.981$, Figure 5.12.

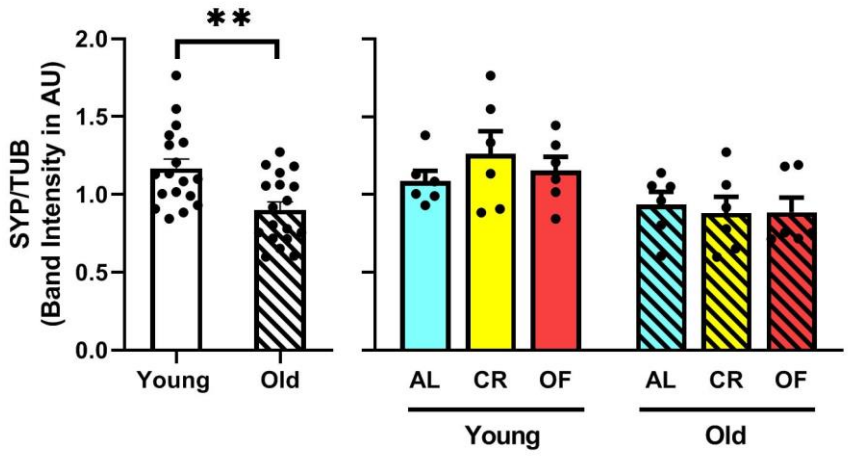


Figure 5.12 SYP levels across age and diet groups. A significant effect of age was demonstrated on the SYP levels. **: $p < 0.01$. Error bars = +Standard Error Mean. Adapted from Karoglu-Eravsar et al., 2021 (under-review) [128].

5.3 Genes related to synaptic neurotransmission, neurotrophic factors, and inflammation were not altered significantly by dietary interventions

Alterations concerning dietary condition and age of an animal were observed on the synaptic protein expression levels. Further gene expression analyses were conducted to show whether changes in the brain protein levels are in concordance with gene expression alterations. For this purpose, genes of interest that were coding synaptic proteins in the zebrafish brain were selected. These genes were *dlg4* and *dlg4b* which were the paralogs, and functionally code the PSD95 protein in zebrafish, *sypa,* which codes SYP protein, and lastly, *gepb* coding the protein GEP. Additionally, genes that are potentially regulating neurotransmission and synaptic plasticity were also assessed, including *igf1* and *bdnf* having roles in growth and

neurotrophic signaling, *tnfa*, a pro-inflammatory marker, and *il10*, an anti-inflammatory marker.

The genes *dlg4* and *dlg4b* coding the excitatory protein PSD95 and regulating the excitatory neurotransmission were analyzed. In the *dlg4* levels, no significant effect of age was observed, $F(1,30)=0.419$, $p=0.522$, and the same pattern for the effect of age was observed in *dlg4b* as well, $F(1,30)=1.510$, $p=0.229$, Figure 5.13.A and B, respectively. In terms of the effects of the dietary regimen, in the *dlg4* expression levels, a marginally significant main effect of the dietary condition was observed, $F(2,30)=2.654$, $p=0.087$, as a trend animals with OF regimen tend to have lower *dlg4* expression levels as compared to AL and CR subjects, Figure 5.13.A. Diet effect for *dlg4b* was not statistically significant, $F(2,30)=1.140$, $p=0.333$, Figure 5.13.B. Additionally, no significant age by diet interaction was revealed altering the expression *of dlg4*, $F(2,30)=0.152$, $p=0.860$; and *dlg4b*, $F(2,30)=0.458$, $p=0.637$, 5.13.A and B, respectively.

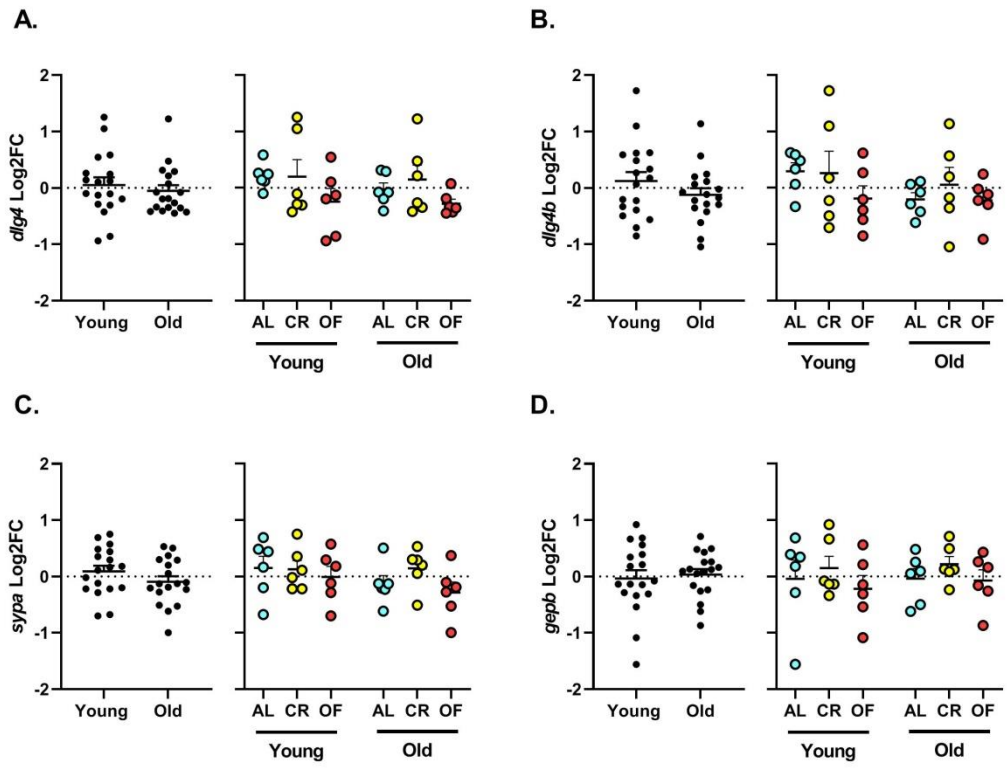


Figure 5.13 Relative expression levels of the synaptic genes across age and diet groups. (A) *dlg4,* (B) *dlg4b*, (C) *sypa* and (D) *gepb*. Error bars = +Standard Error Mean. FC: Fold Change

On the relative expression levels of *sypa* which is encoding presynaptic protein SYP no main effect of age, $F(1,30)=1.656$, $p=0.208$; diet, $F(2, 30)=1.352$ $p=0.274$; or significant interaction between age and diet, $F(2,30)=0.502$, $p=0.610$, were revealed, Figure 5.13.C. In terms of *gepb* expression patterns, which have potentially regulating roles o inhibitory neurotransmission, no significant main effect of age, $F(1, 30)=0.157$, $p=0.695$; or diet, $F(2,30)=1.154$, $p=0.324$, were observed, Figure 5.13.D. Likewise, no significant age by diet interaction was revealed on the relative expression levels of *gepb, *$F(2,30)=0.053$, $p=0.948$, Figure 5.13.D.

In terms of regulatory genes of neurotrophic signaling, *igf1* and *bdnf* were investigated. On the levels of *igf1,* a main effect of age was not statistically significant, $F(1,30)=1.693$, $p=0.203$. As a trend, with aging *igf1* expression levels showed a decreasing pattern. No main effect of diet was observed on the *igf1* data set, $F(2,30)=0.324$, $p=0.726$, Figure 5.14.A. Moreover, the interaction between age and diet was not statistically significant, $F(2,30)=0.546$, $p=0.585$. On the relative expression levels of *bdnf,* no main effect of age, $F(1,30)=1.893$, $p=0.179$; no main effect of diet, $F(2,30)=1.334$, $p=0.279$; and no significant diet by age interaction, $F(2,30)=0.211$, $p=0.811$, were revealed, Figure 5.14.B. However, as an overall trend in the data, it could be said that aging leads to decreasing trend considering the expression levels of *bdnf.*

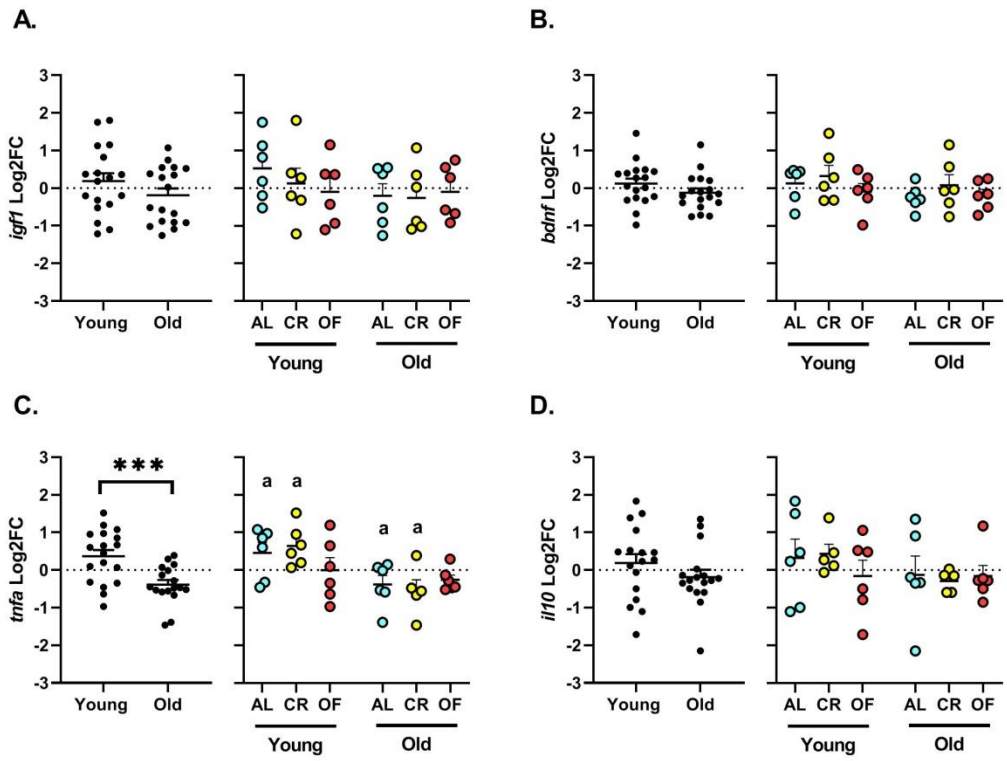


Figure 5.14 Relative expression levels of the regulatory genes across age and diet groups. (A) *igf1,* (B) *bdnf,* (C) *tnfa* and (D) *il10*. ***: $p < 0.005$. a: Significant main effect of age was observed in a diet group. Error bars = +Standard Error Mean. FC: Fold Change

The other two regulatory genes were involved in inflammatory signaling. The first marker was *tnfa,* and it was regulating the pro-inflammatory signaling. It was revealed that age affected the expression level of *tnfa* significantly, $F(1,30)=13.496$, $p=0.001$, Figure 5.14.C. At older ages, zebrafish had significantly lower expression levels of *tnfa* in the brain than animals at younger ages. Moreover, multiple comparisons revealed that this age effect was statistically significant in AL ($p=0.025$) and CR ($p=0.003$) dietary conditions while the age effect was not observed in the OF-

fed group specifically (p=0.474). The main effect of diet was not significant, F(2,30)=0.303, p=0.741, and no significant interaction was found between the age and diet, F(2,30)=1.725, p=0.196, on the *tnfa* gene expression levels. In contrast to *tnfa*, the second gene *il10* was regulating anti-inflammatory processes. On the expression levels of *il10* main effects of age, F(1,30)=1.503, p=0.230, and diet, F(2,30)=0.269, p=0.766, were not significantly altering its levels, and no significant interaction was revealed on *il10* as well, F(2,30)=0.429, p=0.655, Figure 5.14.D.

5.4 Further correlational and multivariate analyses revealed a diet-dependent clustering in synaptic proteins

Synaptic proteins were analyzed together to understand the correlations and clustering profiles for age and dietary status. An overall correlation was conducted using Pearson correlation coefficient r, and significant correlations among the synaptic proteins were revealed, Figure 5.15. In the overall data, all age and diet groups were collapsed together. Excitatory neurotransmission elements were positively and significantly correlated with each other. Significant correlations were demonstrated between PSD95 and GluR2/3, (r(34)=0.744, p<0.0005); PS95 and NR2B, (r(34)=0.379, p=0.023) and lastly GluR2/3 and NR2B (r(34)=0.683, p<0.0005), Figure 5.15. Moreover, pre-synaptic protein SYP was significantly and positively correlated with excitatory protein markers PSD95, (r(34)=0.461, p=0.005), and GluR2/3, (r(34)=0.431, p=0.009). Inhibitory markers of neurotransmission GEP and GABA-A-a1 were positively and significantly correlated with each other (r(34)=0.486, p=0.003), but there was no correlation between the inhibitory markers and other synaptic proteins, Figure 5.15.

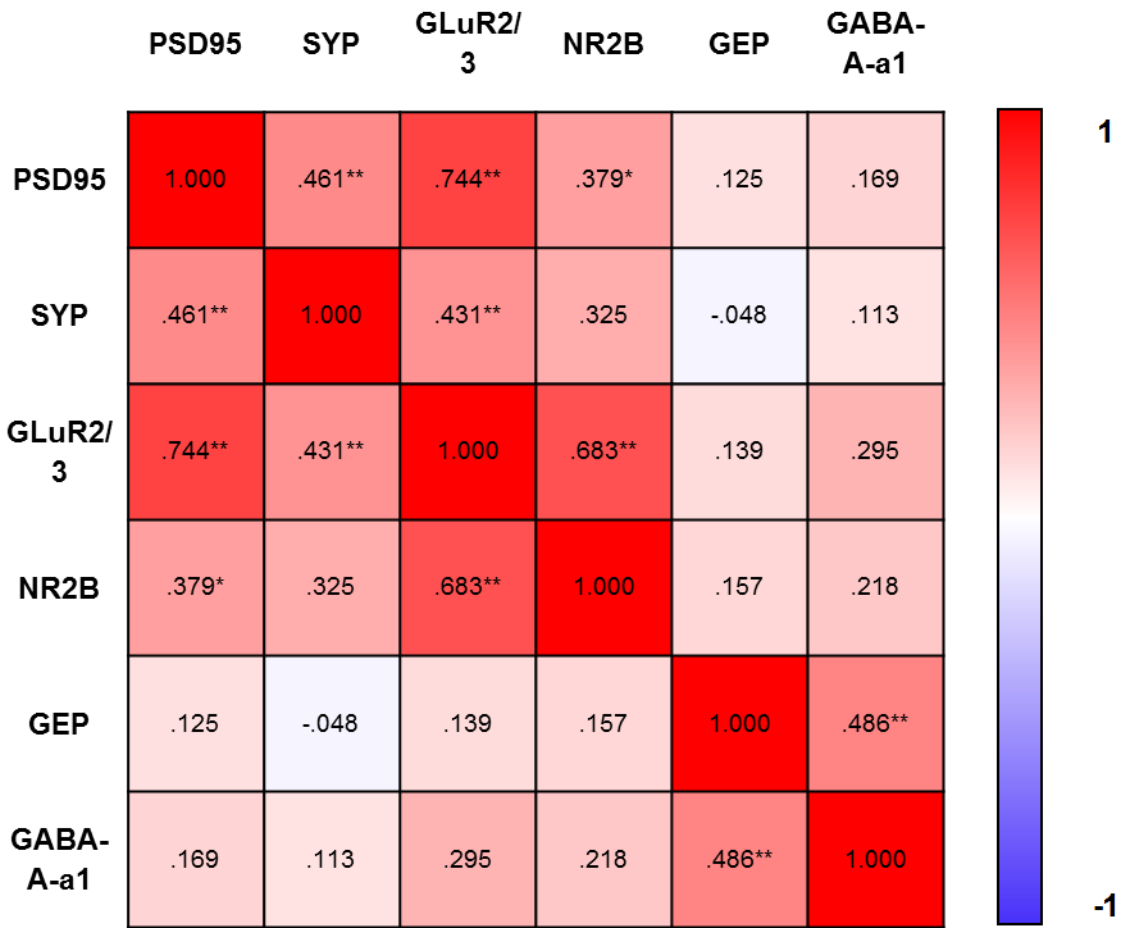


Figure 5.15 Analyses revealed significant correlations among the synaptic proteins. *: $p<0.05$, **: $p<0.001$.

A further multivariate analysis was conducted to reveal the clustering profile using principal component analyses; as an outcome of this PCA, two principal components were extracted. The first principal component was explaining 45.046% of the total variance. It was driven by excitatory markers PSD95, NR2B, GluR2/3, and pre-synaptic marker SYP positively (component loading scores>0.6), Figure 5.16. The second principal component was accounted for 22.866% of the total variance and contributed by the inhibitory markers GEP and GABA-A-a1 positively

(component loading scores>0.6). Together, these two components were explaining 67.912% of the variance in the protein data.

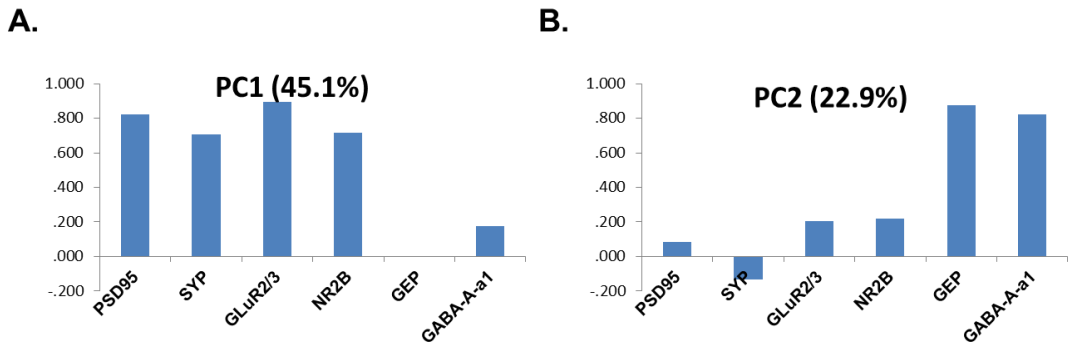


Figure 5.16 Factor loading scores for two principal components for synaptic proteins. PC1 is contributed by PSD95, GluR2/3, NR2B, and SYP positively (A). PC2 was contributed by GEP and GABA-A-a1 positively (B).

These two components were visualized for each individual separately by dividing the age and dietary groups. Age and diet-specific clustering patterns can be observed in the PC1. At younger ages, animals in the CR group tend to have higher PC1 scores than AL and OF animals at a young age. Excitatory and presynaptic markers drove this component. Higher PC1 scores in the CR group at a young age can imply that CR can facilitate synaptic plasticity at younger ages by altering these excitatory and presynaptic elements, Figure 5.17. Additionally, age by diet-driven profile can also be observed in PC2 controlled by inhibitory components. At older ages, both AL and OF dietary groups are showing to have lower PC2 scores than younger ages; this can imply possible comprises in the inhibitory neurotransmission occurring with aging, and animals can be vulnerable to excitotoxicity during the aging due to repressed inhibition, Figure 5.17. However, in the old age group of the CR animals is

comparable to young. There was no robust change occurring with aging in the inhibitory markers of neurotransmission, but elevations of these markers with CR can promote more resilience in the CR group against aging-related molecular changes.

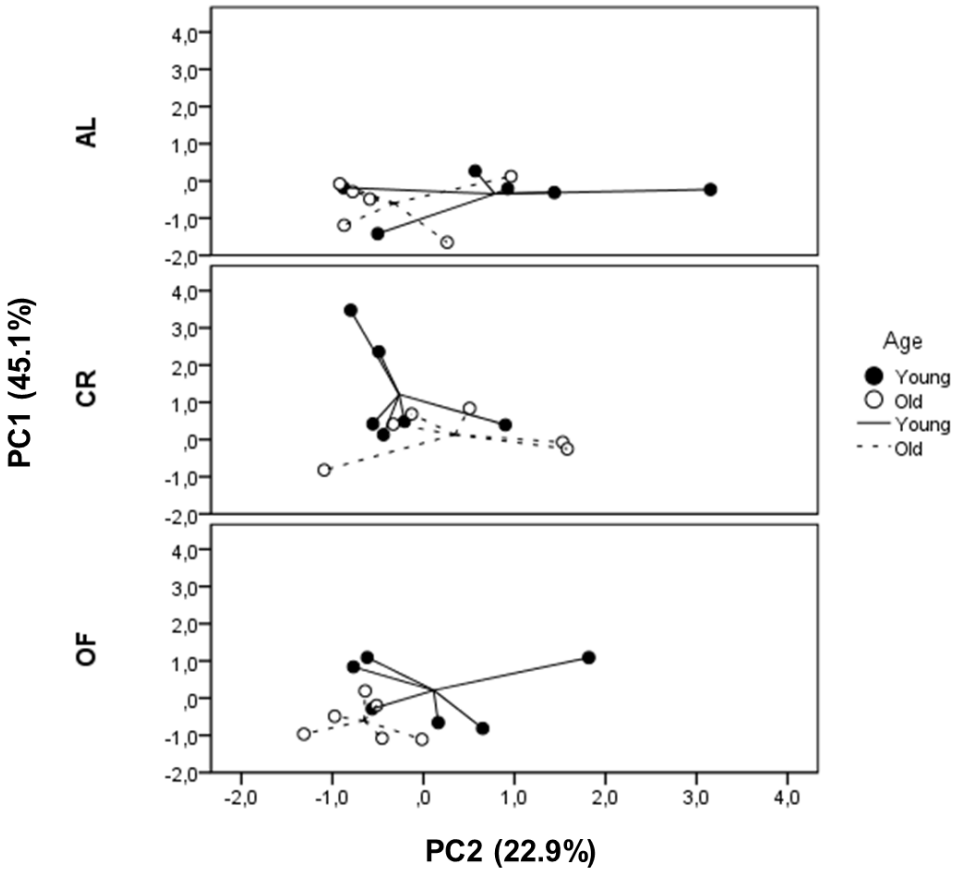


Figure 5.17 Clustering profiles of components extracted from protein expression data in which both age groups and dietary conditions were separated. Diet groups were shown in separate panels, while age groups were denoted by black and white. Young: Black, Old: White.

5.5 Correlations and multivariate analyses revealed positive correlations among synaptic and regulatory genes and diet-driven clustering pattern

All genes analyzed together to reveal possible interactions and shared clustering patterns among them. Correlational analyses indicated that *bdnf* which is a regulatory gene controlling neurotrophic signaling is significantly and positively correlated with synaptic elements including *sypa*, ($r(34)=0.735$, $p<0.0005$); *gepb* ($r(34)=0.698$, $p<0.0005$); *dlg4*, ($r(34)=0.759$, $p<0.0005$); *dlg4b*, ($r(34)=0.845$, $p<0.0005$); *igf1*, ($r(34)=0.412$, $p=0.013$); and *tnfa*, ($r(33)=0.343$, $p=0.044$). Moreover all synaptic genes were significantly and positively correlated with each other, significant correlations were revealed between *sypa* and *gepb*, ($r(34)=0.610$, $p<0.0005$); *sypa* and *dlg4*, ($r(34)=0.585$, $p<0.0005$); *sypa* and *dlg4b*, ($r(34)=0.626$, $p<0.0005$); *gepb* and *dlg4*, ($r(34)=0.537$, $p=0.001$); *gepb* and *dlg4b*, ($r(34)=0.557$, $p<0.0005$); and *dlg4* and *dlg4b*, ($r(34)=0.918$, $p<0.0005$), Figure 5.18. Additionally, regulatory gene *igf1* was positively and significantly correlated with excitatory synaptic genes *dlg4*, ($r(34)=0.396$, $p=0.017$); and *dlg4b*, ($r(34)=0.420$, $p=0.011$); and also presynaptic gene *sypa*, ($r(34)=0.444$, $p=0.007$), Figure 5.18. Lastly, *tnfa* was significantly and positively correlated with excitatory synaptic genes *dlg4*, ($r(33)=0.502$, $p=0.002$); and *dlg4b*, ($r(34)=0.448$, $p=0.007$), Figure 5.18. No significant correlation was observed for the case of regulatory gene *il10*.

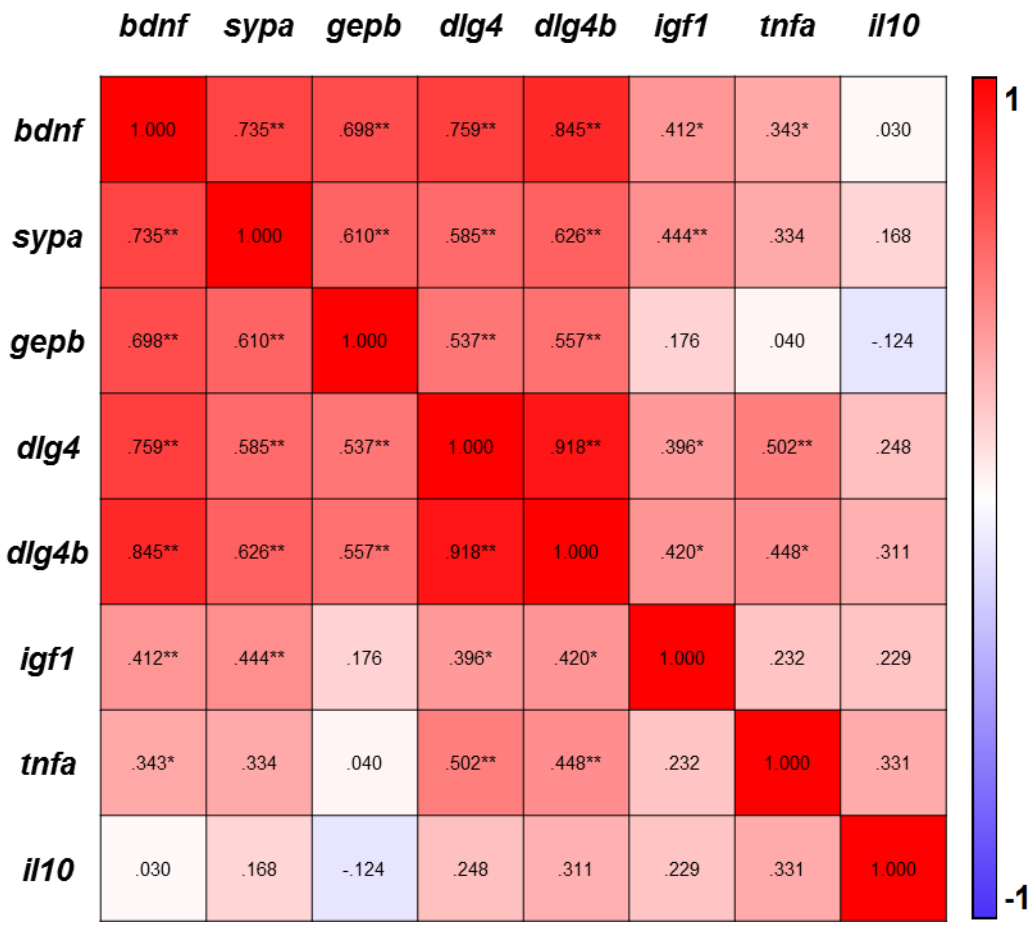


Figure 5.18 Analyses revealed significant correlations among synaptic and regulatory genes. *: $p<0.05$, **: $p<0.001$.

Multivariate analyses were conducted by using principal component analysis (PCA). As a result of PCA, two principal components were extracted from the gene expression dataset. These two components were accounted for 67.034% of the total variance in the data. The first component (PC1) was explaining 48.743% of the variance in the gene expression data. It was driven by synaptic genes *sypa, gepb, dlg4,* and *dlg4b* in addition to neurotrophic signaling regulating gene *bdnf*. The

second component (PC2) accounted for 18.291% of the variance in total and was driven by inflammation regulatory genes *il10* and *tnfa* positively, Figure 5.19.

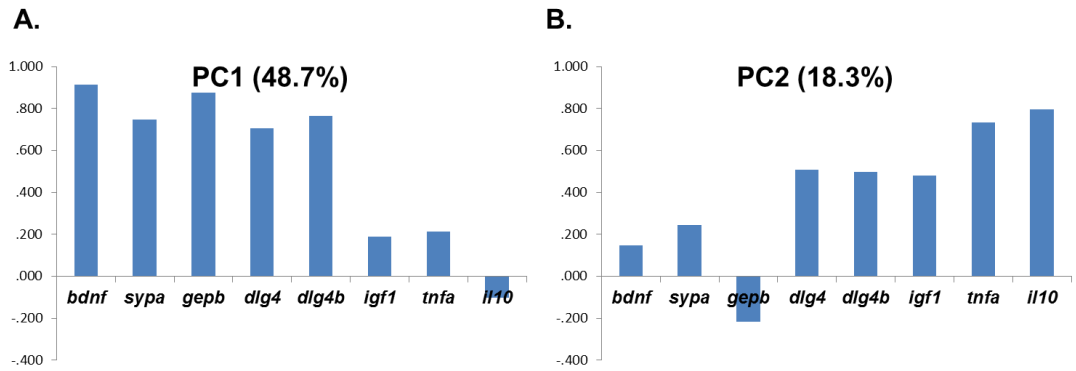


Figure 5.19 Factor loading scores for two principal components for synaptic proteins. PC1 is contributed by *bdnf*, *sypa*, *gepb*, *dlg4* and *dlg4b* positively (A). PC2 was contributed by *tnfa* and *il10* positively (B).

Following the analysis scores, individual data points were visualized in the dimensions of PC1 and PC2 to detect any possible age or diet-driven influences, Figure 5.20. In the PC1 score driven by all synaptic genes and *bdnf*, no age-specific patterns were seen, but in the OF group, PC1 scores tend to be lower than both AL and CR animals Figure 5.20. Additionally, in terms of PC2 regulated by genes involved in the inflammatory signaling pathway, prominent segregation between the age groups was shown in the CR group, Figure 5.20. In the CR group, younger animals had lower PC2 scores as compared to old CR subjects. However, this does not mean that CR old animals had elevated inflammatory signaling since the old CR PC2 levels were quite comparable to AL and OF old groups; but the CR young group

had a lower PC2 score. This pattern can indicate that CR can alter the inflammatory signaling in an age-dependent way which was more prominent at younger ages than old.

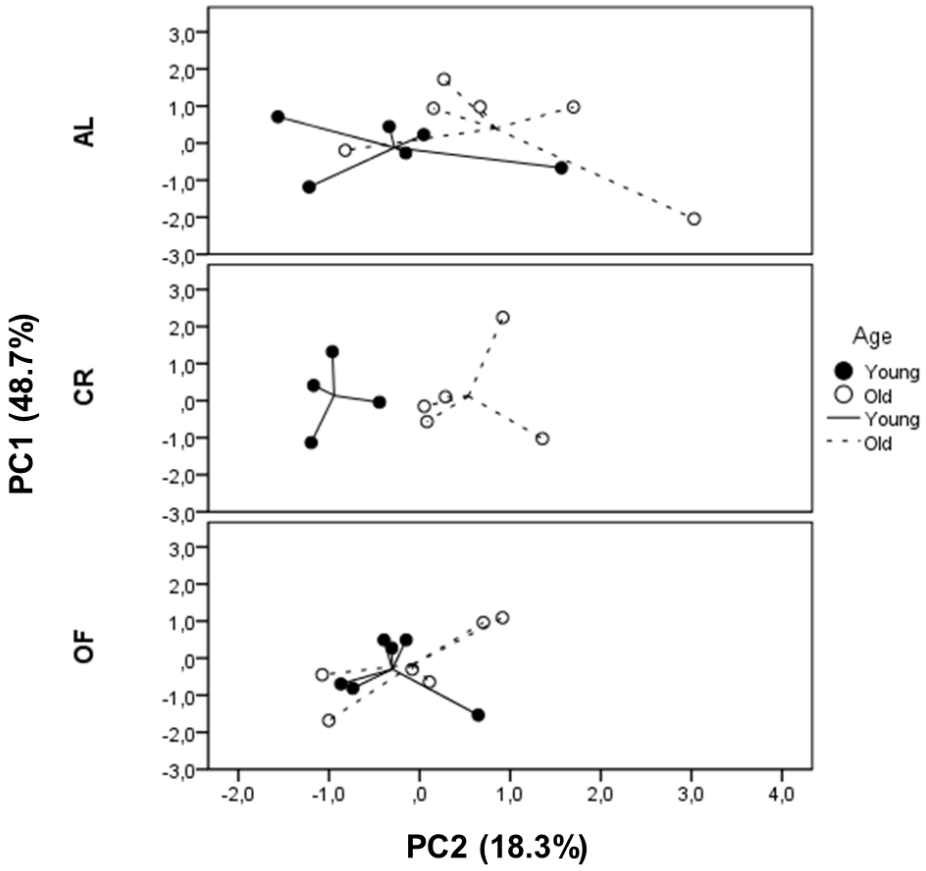


Figure 5.20 Clustering profiles of components extracted from gene expression data in which both age groups and dietary conditions were separated. Diet groups were shown in separate panels, while age groups were denoted by black and white. Young: Black, Old: White.

5.6 Discussion and Conclusions

This Chapter indicated that feeding interventions had changed body parameters in young and old zebrafish, including body weight and length and BMI. Significant BMI, weight, and length reductions in young and old CR groups were observed compared to the other dietary regimens; in OF group, we have observed significant increases in body weight as compared to the AL group at a young age. In the OF group, a significant increase in trunk cortisol measures was observed at a young age compared to the CR young zebrafish, reflecting metabolic alterations depending on the calorie intake. This study was aimed to show global changes and fluctuations in the zebrafish brain depending on diet in an age-dependent perspective. Age-related reductions were demonstrated in the synaptic protein levels of excitatory post-synaptic protein GluR2/3, inhibitory clustering protein GEP, and presynaptic integrity protein SYP. At the transcriptional level, age-related reduction in the levels of inflammatory gene *tnfa* was revealed. Besides the effects of aging, dietary intervention-dependent alterations were found on excitatory neurotransmission elements, including PSD95 and GluR2/3. Their levels were increased in the CR group compared to the other dietary regimens, and in the CR group, excitatory/inhibitory balance tends to be shifted towards excitation. These results demonstrated that changes in calorie intake by applying different dietary regimens with respect to aging altered the levels of excitatory synaptic proteins that change the excitatory/inhibitory balance and local regulation of calcium, which can change the resilience of zebrafish against aging.

In the one-week-long habituation period, age-matched zebrafish were randomly assigned to diet groups. After this habituation period, three dietary regimens, AL, OF, and CR, were applied for 12 weeks. At the end of these interventions, zebrafish were euthanized, and their dry weight and length measurements were recorded. Old zebrafish had significantly higher body weight and body length measurements compared to their younger zebrafish. These observations were parallel to the literature in which it was demonstrated that zebrafish was continuously growing at the advancing ages as well [131], [132]. In addition to the body length and weight measurements, BMIs were also calculated and investigated because the effect of dietary interventions cannot be only understood with weight and length parameters. When the effects of the diet were evaluated, it was revealed that CR animals had lower body weight in the young group than AL and OF young zebrafish. A significant increase in body weight was seen in young OF zebrafish compared to the young AL group. At old age, a significant decrease was seen in CR zebrafish compared to other dietary regimens. On the length measure, only diet-related changes were seen in the CR zebrafish, which has a shorter body length than other dietary conditions at young and old ages. Although differences were significant, the baseline values differed subtly among the diet groups, which would not indicate a severe problem in developmental dynamics. In terms of BMI measures, OF and AL zebrafish had significantly higher BMI scores than CR zebrafish in both young and old ages. However, in the young OF group marginally significant difference was seen compared to AL zebrafish. Weight and BMI score differences seen in the CR regimen were parallel to the previous work [133]. In the OF group, we have observed a significant increase at young age considering the body weight and a marginally

significant increase in BMI compared to young AL fish. However, this increasing pattern was not very evident at older ages; this can be associated with altered responsiveness against nutrient cues with aging [134].

Another purpose of the current study was to assess trunk cortisol levels among age and dietary interventions to gain insights regarding the regulation of stress and its interactions with metabolism. Cortisol is a predominant glucocorticoid in zebrafish as in humans and the main product of the stress response. In literature, it was demonstrated that trunk cortisol levels could be increased by exposure to stressors such as social, chemical, physical, and environmental in the zebrafish model organism [135]. Also, increased cortisol levels were associated with anxiety, erratic behavior, and cognitive deteriorations in zebrafish [136]. Moreover, cortisol levels can be investigated within the context of feeding regimens and their metabolic interactions. Evidence suggested that in humans, obesity is associated with elevated levels of serum cortisol, and also the administration of cortisol can elevate glucose production through the stimulation of gluconeogenesis [137]. In the zebrafish literature, studies indicated that high-calorie intake could increase anxiety behavior and cognitive impairments, which might be with the elevated levels of cortisol [66], [138]. Still, few studies have measured trunk cortisol levels with regards to manipulations of calorie intake. In one study, our group showed that 10-weeks of CR regimen did not lead to significant fluctuations in trunk cortisol levels compared to AL control in both young and old zebrafish groups [78]. In another study, it was indicated that exposure to a high-calorie diet enriched with cholesterol and glucose content for 19 days had elevated the levels of trunk cortisol significantly in adult

zebrafish; this increase was accompanied by higher anxiety behavior and cognitive impairments [136]. Our results showed that there was no significant difference between AL and CR groups; this observation was similar to our previous study [78], but the CR group tend to have lower levels of trunk cortisol.

Moreover, in the young age groups, we have found a significant elevation of trunk cortisol in OF group compared to the CR group. This finding might reflect that high-calorie intake can induce increased trunk cortisol levels at young ages, as indicated in the previous study [136]. Interestingly, at older ages, the effect of diet was not so prominent in our data, although the decreasing pattern of cortisol levels was maintained in the CR group. Evidence suggested that normal aging could be associated with subtle alterations in the physiological stress response and disturbed older individuals' normal stress response [139]. Therefore, becoming less responsive to nutrient cues in cortisol regulation might occur with advancing age in zebrafish the model. Unfortunately, in the zebrafish literature, cortisol regulation in aging is sorely lacking. Studies mainly utilized zebrafish at larval and adult ages [133], and zebrafish at older ages were being less emphasized. Cortisol levels can reflect the possible changes in the metabolism and stress regulation dynamics. Within the scope of the feeding interventions, more direct markers of metabolism can be investigated in the zebrafish model. Other nutrient-responsive signaling pathways such as leptin, glucose, and insulin can be studied for further research with the blood samples collected from the groups of zebrafish.

. One of this chapter's main aims was to assess the changes in the protein expression levels of the synaptic plasticity modulating proteins and demonstrate

whether they are showing age or diet-specific expression patterns. Synaptic proteins of PSD95, GluR2/3, and NR2B were investigated as markers of the excitatory synaptic transmission system's integrity. PSD95 was the main scaffolding protein found at glutamatergic synapses; it has implications in clustering glutamatergic receptors such as AMPA and NMDA indirectly and directly, respectively [13]. In our data, we have observed a marginally significant age-related decreasing trend on PSD95 levels. A previous study with a rat model indicated an age-related decrease in PSD95 levels in the hippocampus [140], which was parallel to our observations.

Moreover, we have demonstrated a significant main effect of dietary condition altering the PSD95 levels; CR zebrafish tended to have elevated levels of PSD95 compared to other diet groups at both young and old ages. One study showed that adult mice fed with a high-fat diet for 14 weeks have significantly lower whole-brain levels of PSD95 [64]. These observations might support our results. Young and old zebrafish in the feeding groups of AL and OF containing higher calorie amounts have lower levels of PSD95 levels compared to CR groups, and elevated levels of PSD95 were observed in the CR group containing the lowest calorie amount.

GluR2/3 was used to assessed alterations in the AMPA type receptors; these subunits regulate the trafficking of the AMPA receptors and control calcium dynamics [15]. In the literature, it was shown that with normal aging, these subunit levels decreased significantly. Still, the application of lower-calorie diets like CR stabilized their levels, and the decline was prevented at old age groups in the rat models [20], [110]. In this chapter, the mammalian models' results were replicated, GluR2/3 levels reduced significantly with aging. Also, dietary regulation on GluR2/3

levels was revealed; CR groups tend to have maintained and elevated levels of GluR2/3 at both young and old ages compared to AL and OF dietary regimens.

Lastly, the third excitatory marker, NR2B, is one of the predominant forms of NR2 subtype of NMDA receptor and has implications on induction of LTP [19]. Experiments conducted with rat hippocampus suggested that NR2B levels tend to be decreased at older ages [20], [110]. Additionally, it has been shown that diet can differentially regulate NR2B levels depending on age and region. One study showed that both AL and CR rats have age-related decreases in NR2B levels in the CA1 region of the hippocampus [110]. In contrast, another study indicated that age-related decrease occurs in the AL group; but in the CR groups, NR2B levels tend to be maintained at older ages in the CA3 region of the hippocampus [20]. We have observed no main effect of diet and age on NR2B levels; one reason might be that any significant effects on NR2B levels could be the region-specific expression patterns of this synaptic marker. As indicated above, even between the different hippocampus subregions, diet-driven effects were differential on NR2B expression levels [20], [110]. Overall data indicates that excitatory synaptic integrity markers of PSD95 and GluR2/3 demonstrated a decreasing pattern with aging, while in CR groups, these excitatory markers tend to be elevated compared to the other dietary regimens.

In addition to the excitatory neurotransmission integrity markers, inhibitory components were also investigated for age and dietary condition factors. GEP and GABA-A-a1 were assessed for this purpose. GEP was a scaffolding protein at the GABAergic synapses. It clusters GABA receptors, more specifically $GABA_A$

ionotropic receptors on the post-synaptic site. GABA-A-a1 is a subunit of $GABA_A$ receptors and has regulatory roles in channel formation [87]. This chapter demonstrated that GEP levels significantly declined at older ages, and this decrease was very robust in OF group compared to the other groups. Moreover, there was no effect of diet on the GEP levels. Previous work indicated that GEP levels could be changed in a region-specific manner, and age-related declines were previously characterized in specific human brain regions such as the visual cortex [88]. Results of this chapter regarding the GEP levels were demonstrating a comparable age-related trend to the previous findings.

In terms of GABA-A-a1, it was demonstrated that this subunit is not changing robustly in the hippocampal regions regulating the behavior with aging [26].In this chapter, we also showed no age or diet-specific regulation of this inhibitory marker. Furthermore, these excitatory and inhibitory markers of neurotransmission can be conceptualized as a whole; they can regulate excitatory-inhibitory balance together. In terms of diet groups, excitatory components were elevated in the CR group, while inhibitory GEP tended to be stable. In the CR group, this balance tends to be shifted toward increased excitation compared to the other dietary regimens. This shift might underlie CR's possible beneficial effects on cognitive abilities as indicated in different model organisms [20], [141]. Future studies could focus on assessing cognitive and behavioral effects of CR and OF on the zebrafish model and matching these synaptic changes altered by age and diet to the behavioral profile.

We used the SYP as a general indicator of synaptic and pre-synaptic integrity. SYP is a glycoprotein with transmembrane domains; it can be located in synaptic

vesicles and pre-synaptic regions [27]. In the literature studies were demonstrating declining levels of SYP with increasing age [14], [20], [44], [88]. This chapter demonstrated a comparable reduction in SYP levels with aging; we have observed overall significant reductions. However, the dietary condition's effect was not found in this chapter regarding the levels of SYP. In the literature, it was demonstrated that decline in the SYP levels could be prevented by applying CR in specific brain regions of the rat models [20]. However, another evidence using whole-brain rather than a specific region indicated no diet-dependent regulation on the SYP levels in the mice model [64]. This observation can explain why we did not see any significant diet modulation on SYP levels using whole-brain lysates from zebrafish. For future studies, these possible region-specific alterations on SYP levels can be investigated considering the dietary condition within the aging research scope.

Multivariate analysis was performed to gain more insights into the relationships among these synaptic and cellular proteins. Age-dependent clustering was primarily observed in the first component (PC1), which was driven by excitatory markers PSD95, GluR2/3, and NR2B and presynaptic marker SYP, with old groups having lower PC1 scores as compared to young. This pattern might reflect the alterations in the elements of excitatory neurotransmission and presynaptic integrity during aging. Interestingly, CR animals tend to have higher PC1 scores than the AL and the OF groups, which likely supports the idea that CR-fed animals have preserved synaptic elements and plasticity. Moreover, the second (PC2) and third (PC3) components demonstrated both age- and diet-mediated clustering profiles. PC2 was regulated by DCAMKL1 and PCNA in both the AL and CR diets. In this case, the aged animals

had decreased PC2 scores compared to the young fish, while OF-fed groups had an opposite pattern of increased scores with aging, which may imply that high-calorie intake affects the brain's cellular dynamics. PC3 was regulated by inhibitory synaptic markers GEP and GABA-A-a1. In the AL and OF dietary regimens, age-dependent decreasing patterns were demonstrated in PC3. Still, in CR-fed animals, the PC3 scores seem to be maintained at old ages, showing that inhibitory neurotransmission elements are likely maintained with a CR dietary regimen. These results suggest that synaptic changes underlying plasticity likely occur with aging. CR-dependent alterations can be observed in the excitatory and inhibitory synaptic markers and a presynaptic marker, which can potentially contribute to the possibly ameliorative effects of a CR diet.

Complementary gene expression analyses were performed for two reasons; alterations in the synaptic proteins' levels as a response to dietary manipulations started at different ages can be accompanied by similar alterations in the related genes. We did not find any significant change regarding the expression levels of synaptic genes, which corresponds to changes occurring in the synaptic proteins. However, several patterns have been shared. For example, PSD95 protein levels were elevated by the CR diet, specifically, and its coding paralog genes *dlg4* and *dlg4b* were showing similar increasing trends in the CR diet groups at both young and old ages. Moreover, neurotrophic signaling modulatory genes *igf1* and *bdnf* were also analyzed. No significant differences were revealed on their levels, although age-related declining trends were shown in their levels, which was parallel to the previous observations [78]. Also, inflammation regulating genes were analyzed, such as *tnfa*

and *il10*. Interestingly on the *tnfa* levels, an age-related significant declining trend was seen; importantly, significant declines were found at AL and CR feeding regimens. Aging is associated with elevated inflammation and increased protein levels of TNF-alpha [34]. Therefore, an overall declining pattern with aging in the gene expression levels of *tnfa* was not similar to the changes reported at the protein levels. However, alterations in the mRNA expression patterns and protein expression levels can be differential. The post-translational mechanism can alter the protein levels. Adaptations to a state such as aging can lead to the lower gene expression levels *tnfa* to balance the increased abundance of its protein levels with aging. In the data, it seems like this type of adaptation is not occurring in the OF group, increasing the susceptibility of this diet group.

Multivariate analyses were also applied to the gene expression data. It was revealed that all synaptic genes and *bdnf* drove the PC1 component. With the diet group comparisons, it was demonstrated that zebrafish in the OF regimen had lower PC1 values when compared with the other dietary regimens. The second component, PC2, was regulated by inflammation-related genes, and the difference between the age groups was demonstrated in the CR group. In CR dietary regimen, young zebrafish had lower PC2 values than old zebrafish in the same dietary regimen. Nonetheless, this age difference does not show that old zebrafish with CR diet had an increased inflammatory signaling/activity because the old zebrafish with CR had similar PC2 values to AL and OF old zebrafish, while young zebrafish with CR diet tend to have lower PC2 scores. This observation can demonstrate that CR might

change the inflammatory components, which were more evident in young age groups when they were compared with old zebrafish.

In conclusion, zebrafish literature was demonstrated that zebrafish is an appropriate model organism to investigate the effects of dietary manipulations since it is sharing a relatively high homology to humans regarding the effects of dietary interventions on the body [142], [143]. This chapter investigated the molecular changes occurring in the aging brain and response to dietary interventions such as the CR and the OF. Results demonstrated that aging is associated with a significant reduction in synaptic proteins, including GluR2/3, GEP, and SYP. The application of the CR diet can elevate the levels of excitatory synaptic markers of GluR2/3 and PSD95. Apart from effects on brain aging, dietary interventions can alter body parameters such as body weight, body length, BMI, and cortisol levels, which indicate stress regulation. These results obtained in this chapter can increase our knowledge about the possible impacts of dietary interventions on the molecular changes in the brain, establish a zebrafish model to study these diet and age-dependent effects.

CHAPTER 6

OVERALL CONCLUSIONS AND FUTURE PROSPECTS

Age-related behavioral and cognitive decline occurs in normal aging, and detrimental changes were seen in neuropathological conditions. Subtle molecular alterations in synaptic and cellular dynamics can explain the neurobiological underpinnings of these changes. However, this research area is still limited since there is a heterogeneous profile in cognitive and behavioral decline progression and neurobiological alterations with aging. Treatment and prevention strategies were not equally effective in each case. Therefore, it is essential to understand how individual factors can contribute to successful or unsuccessful aging and whether these factors can induce shared patterns of alterations in the cellular and synaptic dynamics. Three interventions were evaluated within the current work to shed light on these questions.

In the first approach, a genetic intervention was used, and neurobiological elements were characterized in a mutant model with lower levels of AChE. This model can provide insights into the possible treatment strategies to prevent aging-related deteriorations because AChE inhibitors were used in different model organisms and humans to reduce the effects of neurodegenerative conditions such as Alzheimer's disease [85]. Additionally, utilized zebrafish mutants were characterized as a delayed aging model in terms of the behavioral phenotype in the previous work [71], [144]. Therefore, we can understand the neurobiological correlates of successful aging by analyzing neurobiological changes in this model. The second and third approaches were involving environmental interventions. Thanks to these

interventions, we can understand the factors associated with heterogeneous profiles with aging regarding the health-span and mind-span. In the first intervention, the sensory environment was manipulated, and more stimulating environments were arranged experimentally to show whether the differences arise from the environment that an individual is experiencing can alter the course of aging and the neurobiological correlates of these changes. The other environmental intervention was systemically analyzing the effects of total calorie intake, explaining the heterogeneous profile in aging. This intervention can also help unveil the neurobiological correlates of successful aging associated with lower calorie intake and unsuccessful aging that may be associated with higher calorie consumption. These three intervention approaches utilizing different elements were investigated and compared in the current work using zebrafish model organisms.

In these three intervention approaches, general body parameters such as body weight, length, BMI, and brain weight were investigated. Alterations in these body parameters were in conjunction with all of these interventions. Body weight was significantly increasing with aging, and this significant pattern was shown in all three approaches. Additionally, females had higher body weight than males, and this difference was significant in environmental enrichment and feeding interventions, while it was marginally significant in the results of the genetic intervention. Regarding the effects of treatment, it was shown that environmental enrichment is not changing the total body weight while applying the CR diet has significantly reduced the total body weight at both young and old ages. Also, it was shown that the application of OF was increasing the total body weight in young animals compared to

AL. Lastly, in the mutants marginally significant decrease in body weight measure was observed. Alterations in the body weight, in terms of the shared effects of age and gender as well as diet, were similar to previously reported works [45], [82], [143], [145].

Body length was investigated as another body parameter. In all three chapters, it was shown that with aging, the zebrafish' length was significantly increased, and these observations were also parallel to the previous studies [45], [143]. Also, on the length, the effect of the dietary regimen was found. Zebrafish with the CR regimen had lower body length measures than other diet conditions at both young and old ages. It might show that short-term application of CR might alter the growth dynamics in the zebrafish; no further effects of enrichment or genotype were revealed on the body length measurements in the other chapters. BMI was also calculated by using the weight and length measures to indicate the overall status of the animal because in some cases, usage of body length and weight might be insufficient. In all these three interventions, female animals had significantly higher BMI than males; this observation was parallel to the previous work since female zebrafish had ovaries and eggs, which comprise approximately 30% of the total body volume [82]. In environmental enrichment intervention, no effect of age was seen in the BMI. Both length and weight increases were very proportional through aging; in genetic and feeding interventions, a significant elevation in BMI scores with aging was demonstrated. When these age effects on BMIs were investigated, it was revealed that this age-related increase was contributed by *ache*$^{sb55/+}$ mutants in the genetic intervention part and CR animals in the feeding intervention part. Wild-type and AL-

fed animals that were the control groups in these chapters show no age-dependent increase in BMI scores similar to the animals investigated in environmental enrichment intervention. BMI changes dependent on age in *ache*$^{sb55/+}$ mutants and CR animals can indicate that these animals tend to have lower BMI scores at younger ages, which can make the age effect more prominent.

As a last general body parameter, wet brain weight measures were analyzed in all three interventions. Age and gender effects were significant and in the same direction in all three chapters. In old animals, higher brain weights were observed, and also, males had higher brain weights than females. Observations regarding age were following the same pattern as shown that brain volume was increasing with aging in zebrafish [78]. Additionally, differences in brain weight measures were also seen in the treatment groups. For example, enriched old animals had elevated brain weight compared to old control groups. On the contrary, it was shown that *ache*$^{sb55/+}$ mutants had lower brain weight than wild-type controls, and this difference was prominent at young ages. Similarly, the CR regimen was reducing the brain weight of the animals as compared to other diet groups at young and old ages. These reductions in the brain weight with CR and mutation would less likely show any possible detrimental effects. It could be said that these reductions in the brain weight measures were proportional to the overall body growth changes since in these mutant and CR groups, significant or marginally significant differences were seen in the body weight measures.

Overall, alterations in body weight, body length, BMI, and brain weight were consistent among three intervention approaches regarding the effects of age and gender. On the body measures, decreases were seen in the CR group in terms of

weight, length, BMI, and brain weight measurements; and also in *ache*$^{sb55/+}$ mutants, reductions in the body weight and the brain weight were seen. These can show that these interventions lead to subtle alterations in the growth dynamics. Further studies can benefit from more sophisticated measurement methods such as body fat volume, muscle volume assessments, and metabolic parameters such as oxygen consumption to gain more insights [146].

Synaptic markers that have a crucial role in synaptic plasticity and excitatory/inhibitory balance were investigated within these three intervention approaches. Synaptic markers PSD95, NR2B, and GluR2/3, regulating the excitatory glutamatergic system, were investigated in all three chapters. In environmental enrichment and genetic intervention parts, no specific alterations were seen in the levels of PSD95. PSD95 was the clustering protein in the glutamatergic synapses and had heterotypic effects of inhibitory neurotransmission [13]. On the other hand, it was shown that the CR diet is upregulating the PSD95 levels compared to the other regimens and this effect is very prominent in the old zebrafish. It could be said that PSD95 is more responsive to nutrient and metabolism-related alterations rather than sensory changes or cholinergic modulations.

Another excitatory marker was NMDA receptor subunit NR2B. In the environmental enrichment intervention environment and age-specific changes were observed in the levels of this subunit. In the control environment, NR2B was decreased significantly in old zebrafish, while no significant decrease at old age was evident in the zebrafish with enriched environment treatment. Genotype-specific effects on NR2B levels were revealed in the genetic intervention part, and mutants

had significantly lower levels of NR2B at both young and old ages. Observed patterns can show that environmental enrichment stabilizes the NR2B and eliminates its age-specific decline. At the same time, in the mutants, synaptic components were altered developmentally to maintain homeostasis, and mutation is associated with the decrements in NR2B independent from the factor of age.

In addition to the NMDA receptor subunits, AMPA subunits GluR2/3 were assessed. In the environmental enrichment and feeding intervention parts, an overall significant reduction in GluR2/3 levels was observed at old zebrafish. Additionally, CR was associated with higher levels of this protein. In the genetic intervention part only observed effect was the effect of genotype, and in mutants, GluR2/3 levels were significantly lowered, and these decrements were prominent at older ages. GluR2/3 levels modulate calcium permeability while elevated levels of this subunit can decrease the calcium permeability, declines can increase the calcium influx. For the environmental interventions parts, age-related decline in GluR2/3 can indicate an increased vulnerability against neurotoxicity due to elevated calcium influx. However, in the mutants, another glutamate subunit was already downregulated, controlling and increasing the calcium influx. Therefore, the decline in GluR2/3 can balance the decrease in NR2B by increasing the calcium influx to retain the synaptic homeostasis. For further studies, other predominant forms of receptor subunits such as NR2A for NMDA receptors and GluR1 for AMPA receptors can be investigated for these interventions. Subunit expression dynamics can alter the overall function [147], and a more comprehensive perspective can be obtained with the assessments of these additional subunits.

Inhibitory synaptic components were investigated for environmental and genetic interventions. GEP is an inhibitory clustering partner found in the GABAergic synapses having heterotypical effects on the excitatory synapses. GABA-A-a1 is a subunit of the $GABA_A$ receptor expressed abundantly in the central nervous system [22]. In the feeding intervention part, an overall reduction in the GEP levels was observed. When the diet groups were examined separately, it was revealed that this age-related decline was prominent in the OF dietary regimen. Similar to this observation in the genetic intervention part, a significant age-related decrease was observed in GEP levels in wild-type animals. In contrast, in the mutant group, GEP levels were maintained through aging. No age-dependent decline was seen in the environmental enrichment intervention part; however, it was observed that zebrafish in the enriched environment had significantly elevated levels of GEP at old age.

There is an overall shared pattern in the GEP levels among these interventions; significant decreases in GEP are prominent in normal aging and detrimental aging induced by OF diet. However, maintained and elevated levels of GEP are induced in the mutants and environmentally enriched zebrafish, which can manifest protective effects. Previous works indicated that GEP levels could modulate the alterations in the excitatory elements in the aging brain. Lower levels of GEP could be observed in the neuropathological conditions characterized by elevated excitotoxicity [24]. In terms of the second inhibitory synaptic marker, no treatment or age-dependent modulations were seen in the GABA-A-a1 levels after these mentioned interventions. This observation was in conjunction with the literature GABA-A-a1 levels tend to demonstrate a stable expression profile with aging [26]. For further investigation,

other predominant subunits of GABA receptors such as β2/3 and ϒ1/3, which were severely altered in age-related neuropathological conditions [148], can be investigated.

SYP was assessed as a general presynaptic marker. In the feeding intervention part, SYP levels were decreased significantly at old ages. Similarly, in the environmental enrichment part, an overall age-dependent decline was seen in SYP levels. More specifically, in the control environment, this decrease was significant in old zebrafish. In contrast, old zebrafish in the enriched environment had significantly elevated levels of SYP compared to old animals in the control environment. In the genetic intervention part, a reverse pattern was observed in which an overall age-related increase was observed in SYP levels. However, when this increase was analyzed in the genotype groups, it was shown that mutants, not the wild-type animals, significantly drove this age-related increase. Therefore, elevated levels of SYP with aging in the mutants can be associated with healthy aging. It can be said that elevated levels can be associated with successful aging, as seen in the old animals exposed to the enriched environment and *ache*$^{sb55/+}$ mutants. For further research, potential contributors of the age-related presynaptic decline can be characterized by assessing the levels of specific presynaptic markers including, vesicular transporters of GABA and glutamate.

Alterations in the cellular dynamics were investigated for interventions; for this purpose, the global levels of two neuronal markers were analyzed. These markers were HuC, an immature marker of neuronal cells, and DCAMKL1, a post-mitotic marker expressing in migrating neurons. An age-related decrease was demonstrated

in the neuronal marker DCAMKL1 in the environmental enrichment part; more specifically, the decline was seen in the control environment. In contrast, DCAMKL1 levels were stable with aging in zebrafish maintained in an enriched environment. In the genetic intervention part, genotype-specific changes were seen in the levels of HuC, and *ache*$^{sb55/+}$ mutants had significantly elevated levels of HuC in both young and old age groups. Taken together, environmental enrichment and *ache*$^{sb55/+}$ mutants were proposed as delayed aging models, and it was shown that global levels of neuronal markers were maintained or elevated in these delayed aging models.

An astrocyte marker GFAP was also investigated in our interventions, while no specific alteration was observed in environmental enrichment intervention, a marginally significant decrease in the *ache*$^{sb55/+}$ mutants was shown. As the last cellular marker, an overall proliferation marker PCNA was assessed, and no significant alterations were revealed for factors utilized in the interventions of the current study. In this work, the global levels of the cellular markers were analyzed by using Western Blot experiments. However, we did not see very robust changes; one reason could be evaluating these markers at a global level in which region-specific or very subtle fluctuations can be overlooked. Therefore, further work was designated to investigate these possible region-specific and subtle differences using similar markers with the immunohistochemistry technique.

ROS activity was investigated to infer the accumulation of the cellular damage. In the environmental enrichment chapter, a significant main effect of age was shown. Old zebrafish had elevated brain ROS levels, and the factor of environmental condition did not modulate this effect. Additionally, ROS levels were affected by

gender, and male zebrafish had higher brain ROS levels compared to females. A similar trend was also observed in the genetic intervention part. Still, in this intervention, the effects of aging were marginally significant, with old zebrafish having elevated ROS levels compared to young. Interestingly, in this chapter, genotype and gender-dependent modulations were observed. In the wild-type animals, males had significantly increased ROS levels compared to wild-type females; and more importantly, in the male *ache*$^{sb55/+}$ mutants, significantly decreased ROS levels were evident compared to wild-type males. Overall, aging can be considered as the primary determinant of ROS accumulation, which can induce gender-specific vulnerabilities, and environmental interventions are not robustly changing the ROS activity. On the other hand, a genetic intervention targeting the cholinergic system has reduced this ROS accumulation in a gender-dependent manner. For further studies, anti-oxidant enzyme levels can be investigated within a similar scope, and sex hormone levels with their receptor expression patterns can be checked to unveil gender-specific alterations in oxidative stress mediating mechanisms.

Overall changes observed in the synaptic, cellular, and oxidative stress markers were subtle within the context of aging and the interventions. In this study, global levels of these markers were assessed from the whole brain tissues. There can be robust region-specific alterations occurring with respect to aging and genetic or environmental interventions, which cannot be evaluated through Western Blot analyses. Similar markers can be utilized with the immunohistochemistry experiments in which region-specific changes can be investigated reliably for further

research. Additionally, in the current work, protein lysates extracted from the total homogenate were examined; and these global changes may not present information regarding the compartment-specific alterations, which can be more prominent. Analysis of specific compartments such as synaptosomes can provide insights regarding the distinct changes occurring in the synaptic membranes. Lastly, proteins of interest can be exposed to post-translational modification due to exposure to a new state induced by mutation or environmental intervention. One approach to distinguish these mechanisms can be investigating the phosphorylated forms of the synaptic proteins for future work.

Taken together, results obtained from different interventions targeting the age-related changes point out shared underlying mechanisms. Elevated levels of SYP and GEP can have further protective implications at older ages, and significant age-dependent reductions on their levels can be observed in accelerated aging models such as the OF diet. Additionally, maintained or elevated global levels of neuronal markers with aging can induce protective effects. Both genetic and environmental interventions were associated with comparable alterations in the synaptic dynamics in a more comprehensive view. At first glance, more robust changes can be expected with genetic interventions than environmental interventions. Genetic interventions are induced at a very early stage of development. They can alter the baseline, and capacity of the system, regarding neuro-developmental dynamics and the microenvironment of the brain. This altered baseline may serve as a buffer mechanism to reduce vulnerabilities against aging-related deteriorations. At the same time, even short-term environmental interventions can drastically change the

dynamics concerning synaptic activity and integrity and have a significant potential to alleviate age-specific susceptibilities. To conclude, the genetic background can determine the baseline conditions of the system, which can act as a buffer mechanism, but also environmental contributors can attenuate the vulnerabilities of the system; can change the course of the aging.

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