AGE AND GENDER ALTER SYNAPTIC PROTEINS IN ZEBRAFISH (*DANIO RERIO*) MODELS OF NORMAL AND DELAYED AGING

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Elif Tuğce Karoğlu

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July 2017

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

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ABSTRACT

AGE AND GENDER ALTER SYNAPTIC PROTEINS IN ZEBRAFISH (*DANIO RERIO*) MODELS OF NORMAL AND DELAYED AGING

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MSc in Neuroscience

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Cognitive decline occurs during normal aging in some specific domains of cognitive abilities including but not limited to episodic memory, divided attention and executive functions, however, it is not a unitary decline since some cognitive domains, including vocabulary and implicit memory tend to be preserved and even improved at older ages. Normal aging is not associated with global and significant neuronal and synapse loss, yet subtle molecular alterations occurring in gene expression patterns, protein homeostasis, mitochondrial dynamics and hypofunction in the cholinergic system may account for the age related decline in some cognitive abilities. Additionally, males and females showed differential vulnerabilities against age-related alterations in the cognitive abilities, physiological integrity and subtle molecular dynamics. More direct relationships can be established between the age-related cognitive decline and subtle molecular changes by analyzing the elements of synaptic integrity, which could alter synaptic plasticity and result in the changes in learning and memory abilities. Post-synaptic 95 (PSD-95), gephyrin (GEP) and synaptophysin (SYP) are integral synaptic proteins and they could be attributed as

indicators of excitatory post-synaptic, inhibitory post-synaptic and pre-synaptic integrities, respectively. The first aim of this study was to show effects of age and gender on the expression levels of PSD-95, GEP and SYP in young, middle-aged and old, female and male zebrafish cohorts. Significant age by gender interactions were revealed in the levels of PSD-95 and SYP. It was shown that PSD-95 and SYP levels tend to be preserved and increased in the female groups throughout the aging process, whereas, in male groups, expression levels of these proteins tend to be reduced at older ages. The second aim was to investigate whether ameliorating the cholinergic hypofunction might have beneficial effects on the aging-related protein expression alterations and check for sexually dimorphic patterns. For this aim old male and female zebrafish from a mutant line (ache), which has decreased levels of acetylcholinesterase and increased levels of acetylcholine, were compared with old male and female wildtype animals. In the ache old groups, significant increases in the expression levels of SYP and GEP were revealed compared to the wildtype, and also in the old ache females SYP expression was higher than the other groups. These studies emphasized the importance of gender and sexually dimorphic patterns in the context of aging andcholinergic manipulations could be a promising target of intervention to attenuate the effects of age-related synaptic alterations, which could have possible contributions to age-related cognitive decline.

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Key words: zebrafish, aging, sexual dimorphism, synaptic proteins, acetylcholine, acetylcholinesterase

ÖZET

YAŞ VE CİNSİYETİN NORMAL VE GECİKTİRİLMİŞ YAŞLANMA ZEBRABALIĞI (*DANIO RERIO*) MODELLERİNDE SİNAPTİK PROTEİNLERİ DEĞİŞTİRMESİ

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Normal yaşlanmayla birlikte; epizodik hafıza, bölünmüş dikkat ve yürütme işlevleri de dahil olmak üzere bilişsel işlevlerin bazı belirli alanlarında bilişsel gerileme gözlenmektedir. Normal yaşlanma, büyük çaplı ve anlamlı nöronal kayıpla ve sinaps kaybıyla ilişkili değildir, ancak, gen ekspresyon motiflerinde, protein homeostazında, mitokondriyal dinamiklerde ortaya çıkan küçük moleküler değişiklikler ve kolinerjik sistem hipofonksiyonu yaşlanmaya bağlı bilişsel gerilemeyi fizyolojik düzlemde açıklayıcı role sahiptirler. Buna ek olarak, dişi ve erkeklerde yaşlanmanın fizyolojik ve bilişsel integriteye olan etkilerinin farklılık gösterdiği belirtilmiştir. Sinaptik integrite elementlerini analiz ederek, yaşlanmaya bağlı bilişsel gerileme ve küçük moleküler değişiklikler arasında daha doğrudan ilişkiler kurulabilir. Post-sinaptik 95 (PSD-95), gefirin (GEP) ve sinaptofizin (SYP) anahtar sinaptik proteinlerdir ve sırasıyla eksitatör post-sinaptik, inhibitör post-sinaptik ve pre-sinaptik integritelerin göstergeleri olarak atfedilebilirler. Bu proteinler nöral hücrelerin indüksiyon kapasitelerini değiştirerek öğrenme ve hafıza

gibi bilişsel işlevlere etki edebilirler. Bu çalışmanın ilk amacı yaş ve cinsiyetin PSD-95, GEP ve SYP'nin protein ekspresyonu düzeyleri üzerindeki etkilerini genç, orta-yaşlı ve yaşlı, dişi ve erkek zebrabalığı kohortlarında göstermektir. Bulgularda, PSD-95 ve SYP düzeylerinde istatistiksel olarak anlamlı cinsiyet yaş etkileşimi gözlemlenmiştir. PSD-95 ve SYP düzeylerinin dişi gruplarda artan yaşla birlite seviyelerinin korunduğu ve arttığı gözlemlenirken, erkek gruplarda PSD-95 ve SYP ekspresyon seviyeleri yaşlanmayla birlikte azalma eğilimi göstermektedir. Çalışmanın ikinci amacı, kolinerjik hipofonksiyonun iyileştirilmesinin, yaşlanmaya bağlı protein ekspresyonu değişiklikleri üzerinde faydalı etkileri olup olmadığını araştırmak ve olası cinsel dimorfik farklılıkları kontrol etmektir. Bu amaçla, düşük asetilkolinesteraz seviyesine sahip olan dişi-erkek yaşlı mutant hattı (ache), dişi-erkek yaşlı yabanıl-tip zebrabalığı grupları ile karşılaştırıldı. Bulgularda, yaşlı ache mutantlarında yaşlı yabanıl–tip kontrol grubuna nazaran ,SYP ve GEP'in ekspresyon düzeylerinde istatistiksel olarak anlamlı bir artış gözlemlenmiştir. SYP ekspresyon seviyelerinin, dişi-yaşlı ache mutant grubunda diğer gruplara kıyasla daha yüksek olduğu gözlemlenmiştir. Bu çalışma yaşlanma bağlamında cinsiyetin etkilerin ve cinsel dimorfik farklılıkların önemini vurgulanmaktadır. Kolinerjik manipülasyonlar, yaşla ilişkili bilişsel gerilemeye olası katkılar sağlayabilecek yaşa bağlı sinaptik değişikliklerin etkilerini hafifletmek için umut verici bir hedef olabilir.

.***Anahtar kelimeler:*** zebrabalığı, yaşlanma, seksüel dimorfizm, sinaptik proteinler, asetilkolin, asetilkolinesteraz.

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CHAPTER 1

Introduction

Thanks to advances in technology, medicine and life styles, the older population, who are over 65 years old, is increasing. Reports indicated that, in 2010 the older population was estimated to be 40.2 million; and in 2050 a distinct increase was projected, with older individuals comprising 88.5 million in US [1]. Yet it is evident that increased lifespan does not always correspond to better health and mind-span, it is crucial to maintain them all to have a good quality of life and to achieve successful aging. Advancing age is associated with increased prevalence of cognitive impairments and approximately 50% of the older population suffers from cognitive decline associated with normal aging and age-related pathologies [2]. Therefore, studies to understand molecular mechanisms and high-level alterations in behavior and brain activity associated with aging; and interventions focusing on ameliorating those components become promising and required areas of research. To understand the mechanisms of normal aging, distinctions should be made between the normal aging-associated alterations and changes, which are the result of pathological age-related conditions including Alzheimer's disease (AD), Parkinson's disease (PD), frontotemporal dementia (FD) and so forth. After the distinction between normal and pathological brain aging; molecular and structural changes that support normal aging-related cognitive alterations that lead to modifications in the higher order brain systems will give a unified picture to understand the mechanisms of aging.

1.1 Normal Aging vs. Pathological Aging

Although pioneering studies indicated that there is significant neuronal loss in the brain with advancing age even in the absence of pathological conditions, subsequent studies reported that in normal aging there is no significant neuronal loss [3], [4]. Furthermore, it was indicated that there is no significant global synapse loss during normal aging [5], [6]. The most evident distinction between normal and pathological aging could be made based on selective neuronal and synaptic losses and vulnerabilities, specific for each pathological age-related condition neuropathological profile.

Alzheimer's disease (AD) is one of the most common progressive neurodegenerative diseases affecting the older individuals. In AD impairments in memory and higher cognitive abilities are more prominent than the normal aging-related cognitive decline. Episodic memory impairments, delayed recall deficits, semantic coding and semantic memory deficits were evident in AD [7]. In terms of neurodegeneration, progressive neural loss occurs in the neocortex and hippocampus, which are crucial regions for memory encoding and higher level cognitive abilities like executive functioning. Also, global degeneration was observed in the interconnections and the projections between the association areas of the brain in AD patients [8], [9]. Additionally, cholinergic system has selective vulnerability in the context of AD, as degeneration was evident in the cholinergic projection from the nucleus basalis of Meynert [10]. Amyloid β (Aβ) protein accumulation in the extracellular space and the intracellular inclusions of the neurofibrillary tangles, which contain the abnormally hyper-phosphorylated tau proteins, are the neuropathological hallmarks of AD [8]. Although some non-demented older adults

also Aβ protein accumulation and the presence of neurofibrillary tangles, the distribution profile of those hallmarks differs between the AD patients and non-demented older adults. Those neuropathological hallmarks are present in restricted brain regions in the non-demented older adults, whereas they are wide-spread in the AD patients, which has synaptotoxic effects and eventually results in wide-spread synaptic loss and disturbances in axonal dynamics [11].

Parkinson's disease (PD) is another age-related progressive neurodegenerative disease, which has devastating effects on the motor system. Clinical features of this disease include tremors, slowness of movement, abnormal posture like disturbed axial position of the body and rigidity of movement [12]. Besides the evident motor symptoms, cognitive impairments including dementia and affective disorders including anxiety, depression and anhedonia were observed in PD patients [12]. In PD dopaminergic neural loss occurs, especially in the substantia nigra of the basal ganglia, and this critical loss is associated with the motor symptoms during the onset of PD [13]. In terms of neuropathological profile, neural loss is accompanied with the aggregation and accumulations of Lewy bodies and neurites, which are the aggregated, as well as accumulated forms of alpha-synuclein [13].

Frontotemporal dementia (FD) is characterized by significant atrophy in the frontal and the temporal lobes of the brain. Compared to AD, in FD personality problems, which are due to the disturbed dynamics of the frontal lobe, are prominent. There are more impairments in executive functions, whereas less impairment in memory performance in FD [7]. In terms of the neuropathological profile, severe neural loss in the frontal and temporal lobes accompanied with proteinopathies are

present and not related with tau or alpha-synuclein proteins but rather ubiquitin. This distinguishes the pathology of the FD from the other age-related neuro-degenerative pathologies [14].

To summarize age-related pathological conditions like AD, PD and FD, have differential neurodegenerative profiles and specific brain regions are more vulnerable. This is not the case during normal aging, which is not accompanied by significant global neuronal loss. Furthermore, the pathological pattern and distribution of those pathologies across the brain regions differentiate neurodegenerative disorders among themselves and normal aging.

1.2 Cognitive and Neurocognitive Alterations in Normal Aging

Older adults experience cognitive decline without an existence of evident neuropathology or dementia. However, this decline is not a unitary process, rather multidimensional. While specific cognitive domains are declining with increased age; some cognitive domains are stable and even improved in older adults compared to the young group.

Cognitive abilities can be evaluated under the more general domains, which are crystallized and fluid intelligence. Crystallized intelligence is based on the well-learned and practiced information and access to that information; vocabulary and the general knowledge about the concepts could be examples in this category. On the contrary fluid intelligence requires more active monitoring, evaluation and the reasoning about the concepts which were novel and not based on the previous experiences; cognitive abilities, which require more flexibility, such as, working memory, processing speed and executive functions can be an examples for this category [15]. Pioneering studies indicated that fluid intelligence is more vulnerable

to normal aging, as normal functioning older adults tend to show lower performance with the tasks under this category compared to younger adults; whereas in the tasks that require crystallized intelligence, older adults showed similar and even improved performance compared to the young controls [16]. Therefore, cognitive abilities can be analyzed with respect to these two domains, to see the pattern of age-related cognitive alterations.

Processing speed refers to the speed of the cognitive skills in addition to the speed of the motor responses. This ability includes many components like speed of the decision making processes, perceptual processes, motor processes and reaction time. It was shown that processing speed is impaired in older adults compared to the younger subjects, and theories suggested that a slower speed of processing can be an underlying factor for decline in the varieties of the cognitive skills in older adults [17]. Processing speed requires evaluation and the reaction in unfamiliar tasks and the weight of the previous experiences is not that strong for this ability; so processing speed can be attributed to part of the fluid intelligence and it is plausible to expect age-related decline in this ability.

Attention basically refers to concentrating on a relevant stimulus while ignoring the irrelevant distractor stimuli in the context. Yet, this cognitive domain includes multiple components. Selective attention is the process in which a specific feature of a stimulus was attended to like the visual shape of the letter while ignoring the irrelevant sets of the other stimuli like the color of the letter. Older adults performed slower in the tasks requiring selective attention compared to the young groups, which can be attributed to impaired processing speed [18]. Another and the most affected component is divided attention; this ability requires processing two or

more information simultaneously. Divided attention is distinctly impaired with advancing age, which might be due to inappropriately and inefficiently allocating and dividing the attentional resources in the demanding tasks in older adults [19]. Sustained attention is another component and refers to focus and attention to certain stimuli over a period of time and studies indicated that the sustained attention performances of the older adults are similar as the young groups [20].

Memory is one of the most well-studied cognitive skills of the aging domain because of the distinguishing deteriorations that results from age-related pathological conditions or normal aging. Memory has many subcategories but firstly the processes of forming a memory can be analyzed with respect to aging. The first step is the acquisition, which requires encoding of the novel knowledge and information. Studies indicated that encoding declines with increasing age [21]. Retention, which is recalling the previously encoded information, is another process of memory, and in the older adults this process is stable with subtle problems in the source information [22]. Retrieval is recalling and accessing the newly encoded information and this process is also impaired in the older adults compared to young [21]. Memory can be subdivided into two categories, explicit and implicit memory. Explicit memory includes the collection of the information that is consciously acquired; and explicit memory is divided into two categories; episodic and semantic. Episodic memory is the collection of the memories of personal experience and this memory category declines with aging [23], whereas semantic memory refers to the information collection about facts, concepts, ideas and so forth, compared to episodic memory in which there is a life-long decline, in semantic memory there is a late-life decline in this ability [23]. Implicit memory refers to the unconscious collection of the

information; one of the examples could be priming; which occurs unconsciously and affects the successive encoding of the new information and manipulates the reaction, implicit memory skills are preserved at the older ages too compared to explicit memory [15].

Language abilities tend be stable and preserved in the older ages. Vocabulary knowledge is one of the constituents of the language and research indicates that it is stable and there are even improved patterns with an increasing age. Language processes, which depend on speed of processing, show subtle declines with increasing age in this category, verbal fluency can be an exemplar that requires active search for and relevant generation of a word [24], [15].

Additionally to the age-related cognitive alterations, thanks to the advancing technology in brain imaging and manipulation tools, neural correlates of cognitive decline have been investigated. It is evident that in the specific cognitive domains, aging is associated with deficits and impairments, yet it is crucial to understand what kind of activity alterations occurred in the brain in response to aging. Studies indicated that during the verbal tasks associated with long-term and working memory, younger adults have increased activity in the left prefrontal cortex. While older adults were engaging with the same task; different from the younger adults, bilateral activation in the prefrontal cortex was observed [25], [26]. The underlying reason for the recruitment of the additional regions and activity was speculated many times; the reason was attributed to the inefficient processing of the inhibitory mechanisms; while it was argued that this additional recruitment is supportive and functional (for review see [27]). It was revealed that older adults with better behavioral and cognitive performance showed significant bilateral activity, whereas

the neural activity in the low-performing older adults was unilateral and the activity pattern was similar as the young control groups [28]. Additionally repetitive transcranial magnetic stimulation (rTMS), which temporarily disrupts the neuronal function in the area of interests according to the applied signal frequency, showed that when the rTMS was applied to the left dorsolateral prefrontal cortex in the young adults, the performance and the accuracy in the memory tasks was significantly impaired compared to the condition when rTMS was applied to the right dorsolateral prefrontal cortex. However, in the older adults there was no difference in whether rTMS was applied to the right or left dorsolateral prefrontal cortex, behavioral performance is significantly impaired in both of the conditions [29]. Those studies indicated that increased bilateral activation and the recruitment of the additional brain regions during the task performance are functional and supportive against age-related cognitive decline.

To conclude, cognitive decline is experienced by older adults, yet there is no unitary decline. Cognitive domains based on the usage of well-learned knowledge and skills were preserved at even older ages, whereas decline is evident in the cognitive domains that are more flexible and require active reasoning and manipulation of the novel information. Additionally this decline is associated with the alterations in the brain activity, and the compensatory recruitment of the additional brain areas during the cognitive performance serve as scaffolding against aging.

1.3 Structural and Cellular-Molecular Alterations during Normal Aging

1.3.1 Structural Alterations

Age-related structural alterations should be investigated to have insights about underlying factors contributing to cognitive decline. Although, initial studies indicated that there is an age-related significant neural loss in the brain, later work demonstrated that there is no significant neural loss during the course of normal aging [3], [4]. Additionally, it was shown that there is no global synapse loss in the aging brain [5], [6], yet there are region specific synaptic vulnerabilities (for review see, [30]).

Although there is no neuronal and synaptic loss during normal aging, cortical volumetric measurements indicated that after age of 60, shrinkage was observed in the regions including frontal and parietal cortices, and white matter deficiencies were observed in the frontal regions [27]. A comprehensive study, revealed that volume loss without a neural loss can be explained by the reduction in the neuronal volume and disturbance in the dendritic architecture [31]. Therefore, it could be said that significant neuronal or synaptic loss are not underlying factors for cognitive decline, yet there is alterations in the cellular and synaptic dynamics which could give insights about it.

1.3.2 Cellular and Molecular Alterations

Due to the situation that there are no major structural changes or significant neuronal or synapse loss during aging, it is likely that subtle cellular and molecular alterations underlie the changes in cognitive abilities. So examining how physiological integrity is changed during the course of the normal aging would give

insight into possible contributors of cognitive alterations. Aging-related subtle cellular and molecular alterations may lead to impairments in the function of the cellular dynamics. Moreover, these targets could be used for future drug manipulations to improve the course of cognitive declines.

The first factor could be epigenetic alterations occurring throughout the aging process. Transcriptional changes are one of the contributors to epigenetic differences in aged brains and it was revealed that age-related alterations exist in the encoding of the genes that are the crucial elements of lysosomal clearance, inflammatory pathways and mitochondrial regulation. Moreover, overexpression in the genes, which are regulating the inflammatory and immune responses, was found, while there is a lower expression of the genes regulating energy metabolism and apoptosis in the older subjects including humans, rats and mice compared to the young groups [32]. Therefore, these kinds of common motifs in gene expression profile might give information about age-related alterations in the transcriptional integrity.

Another crucial component is protein homeostasis and aging can disturb the dynamics of it. Heat shock proteins, chaperones and ubiquitin proteasomes are the key elements of the control mechanism for protein folding and renewal processes; so disturbances in this control mechanism result in aggregated and abnormal protein accumulation. During aging there is a functional decline in the crucial components of this control machinery [33].

Mitochondrial function and reactive oxygen species (ROS) were broadly investigated in the aging domain. According to first observations, advancing age is associated with mitochondrial dysfunction and increased production of ROS, which eventually increases the mitochondrial damage and leads to disturbances in the

cellular dynamics [34]. However, studies indicated that increased production of ROS is for the compensation of the homeostatic disturbance, but if it exceeds a certain threshold during aging this compensatory response has a detrimental effects on mitochondrial and cellular integrity (for review see [35]).

1.4 Aging and Dysregulation of Synaptoproteome

Aging alters the cellular and molecular dynamics, which eventually could lead to declines in cognitive abilities. As indicated previously, there are no gross structural alterations in the aging brain but some molecular alterations like epigenetic changes, mitochondrial dysfunction and loss of protein homeostasis might account for age-related cognitive impairments. However, more direct relationships can be established to find the basis of the age-related cognitive decline by investigating the synaptic integrity in the aging brain. Studies have indicated that aging is also associated with dysregulated synaptic protein expression and these alterations have deteriorating impacts on neurotransmission, vesicle trafficking, induction of long-term potentiation synaptic plasticity, which are crucial for learning and memory formation. Therefore, in this thesis the synaptic proteins; postsynaptic density 95 (PSD-95), gephyrin (GEP) and synaptophysin (SYP) were investigated as indicators of excitatory post-synaptic integrity, inhibitory post-synaptic integrity and pre-synaptic integrity, respectively through the course of normal aging and in delayed aging model.

1.4.1 Postsynaptic density-95

Postsynaptic density-95 (PSD-95) is one of the major scaffolding proteins in post-synaptic densities. It has roles in anchoring and localizing the receptors into

post-synaptic regions, regulating the trafficking of the synaptic and adhesion proteins, and controlling activity-dependent synaptic responses [36], [37]. PSD-95 is a member of the membrane-associated guanylate kinase family (MAGUK), it consists of 3 PDZ domains, which serves as protein interaction sites by enabling the anchoring of key proteins and receptor subunits, 1 Src homology 3 domain (SH3), and 1 guanylate kinase domain (GK) [38]. PSD-95 directly binds to the GluN2 subunits of the N-methyl-aspartate receptor (NMDAR)-type of glutamate receptors and regulates their localization. Through stargazin and TARP, it clusters and binds to the α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA)-type of glutamate receptors [38]. Both NMDA and AMPA are the ionotropic receptors for glutamate, which is the primary excitatory neurotransmitter in the central nervous system. Those receptors are key to induce and modulate activity dependent synaptic responses including long-term potentiation (LTP) which is the progressive strengthening of the synapses due to experience based frequent stimulation, and long-term depression (LTD), which is the weakening of the synapses due to the activity and experience based stimulation [39]. Both of these processes, LTP and LTD, are strongly associated with learning and memory. Studies conducted with a PSD-95 knock-out mice model indicated that those mice show an enhanced LTP pattern, whereas there was impairment in LTD, and behaviorally those mice showed spatial learning and memory deficits [40].Additionally it was shown that in the hippocampal cell cultures, PSD-95 mimics the effects of the LTP and changes the silent synapses through AMPA receptor mediated insertion to make functional synapses [41].

Studies focusing on the alterations in the levels of the PSD-95 throughout aging revealed no consistent results. Whereas, some works have indicated that the levels of PSD-95 are significantly reduced in the old and behaviorally-impaired groups compared to old-non impaired and young controls [37], some studies have reported an increase in the levels of the PSD-95 in the old-behaviorally impaired groups compared to the controls [42]. Inconsistencies can result from potential region-specific expression profile changes in PSD-95 and region-specific vulnerabilities with aging.

1.4.2 Gephyrin

Gephyrin (GEP) is also a major scaffolding protein at post-synaptic sites, but it clusters glycinergic and ionotropic γ-aminobutyric acid $_A$ ($GABA_A$) receptors, which are the crucial inhibitory components of the neurotransmission. Structurally, as a scaffolding protein, GEP lacks PDZ domains which serve as a connection region for cytoskeletal elements or synaptic proteins, but it has auto-oligomerization and by this process it serves as clustering protein. It has three domains, one G domain, one E domain that has high affinity binding sites for other synaptic proteins and cytoskeletal elements, and one C domain that links E and G domains and some cases binds to the synaptic proteins [43].

Brain sections and cultured hippocampal cells taken from GEP knock-out mice indicated that in the synaptic region crucial subunits of the $GABA_A$ receptor were missing and increased amount of $GABA_A$ receptor subunits were detected in the intracellular compartments, which were supposed to anchor at synaptic sites [44]. On the other hand overexpression of GEP resulted in little increases in the $GABA_A$ and GEP clusters, and decreases in the PSD-95 clusters and the sizes of the glutamatergic

pre-synaptic regions were observed [45]. Those studies indicated that GEP is crucial for the localization and integrity of the $GABA_A$ receptors, but also there is a cross-talk between the GEP and glutamatergic transmission through PSD-95 and other mechanisms.

Aging studies have reported that in the old-impaired groups of rats GEP levels were increased in the parietal lobe compared to the old-unimpaired and young groups, but this pattern was not observed in the prefrontal cortex [46]. Also it studies using a rat model of aging have indicated that the excitatory-inhibitory ratio, which was derived from PSD95 divided by GEP values, showed decreased patterns in the old impaired group in both prefrontal and parietal areas, and an increased expression of the GEP was found in the old impaired groups [47]. On the other hand, studies conducted with the human V1 reported an opposite pattern with GEP levels significantly decreased at older ages [48]. These kinds of inconsistencies in the aging literature may show region-specific vulnerabilities and expression differences of GEP.

1.4.3 Synaptophysin

Synaptophysin (SYP) is a transmembrane glycoprotein that has four trans-membrane domains which span the vesicles. It localizes in the synaptic vesicles and was used as a marker of the density and integrity of the pre-synaptic regions [49]. SYP has potential roles in the processes of endocytosis and synaptic vesicle trafficking [50] but the exact roles of SYP in the synaptic function are still unclear. Studies conducted with a SYP knock-out mouse indicated that hippocampal neurons have increased synaptic depression and the recovery of the synaptic vesicle pool took an extended period of time [50].

In terms of aging literature, it was shown that the levels of the SYP are significantly and positively correlated with the behavioral performance, while the levels of the SYP are reduced in the impaired old groups, the levels were stable between the old unimpaired and young groups [51]. Additionally it was shown that, in hippocampus there was a decrease in the expression levels of SYP with increasing age [52]. Therefore, besides its roles in synaptic trafficking, preserved levels of the SYP can be interpreted as the indicator of the preserved cognitive abilities.

The aim of the present study in this thesis was to show whole brain and region-specific expression profiles of the three synaptic proteins; PSD-95, GEP and SYP, which are regulating the differential aspects of the synaptic function and integrity, to obtain possible age-related alterations in the expression profiles, and lastly to decipher the possible role of the gender, which has been underemphasized in many aging studies. Moreover, these data are among the first to examine these proteins in the context of aging in the zebrafish brain. It was hypothesized that there would be age-related changes in the levels of the PSD-95, GEP and SYP; and those alterations in the protein expression profile would depend on the gender of the animal.

1.5 Sexual Dimorphism and Aging

Gender is one of the most important variables that affect the course of aging in behavioral, molecular and cellular aspects, yet gender tends to be underestimated in the aging studies. In terms of structural sexual dimorphism, there are sexual volumetric differences in the adult human brain. Imaging studies conducted with functional magnetic resonance imaging (f-MRI) indicated that cerebrum-normalized volumes of the cortical regions including frontal and medial-paralimbic areas were

larger in women, whereas, in men cerebrum-normalized volumes of the hypothalamus, amygdala and fronto-medial area were larger [53]. Additionally, imaging studies indicated that non-demented old male groups and those old males with mild cognitive impairment had significantly more microstructural deficits, which were revealed by diffusion tensor imaging, than the non-demented old female groups and those old females with mild cognitive impairment [54]. Also, circulating sex hormones affects the architecture of the key regions like the hippocampus, which controls learning and memory functions. It was shown that estrogen activates stronger excitatory dendrites in the CA1 region of the hippocampus and this induction takes place in a N-methyl-D-aspartate (NMDA) receptor-dependent way [55]. Additionally, estrogen replacement therapy in the gonadectomised rodents increases the NR1 subunit of the NMDA receptors in CA1 neurons and also manipulates and increases the responses of those neurons [56], [57].

Epigenetic alterations occurring during normal aging show distinct sexually dimorphic patterns. Gene expression studies conducted in intact individuals with an age range of 20-99, indicated that the forebrain showed distinct alteration profiles during the ages of 60-70 compared to the other age ranges; more interestingly gene categories altered in both an age and a gender dependent manner. To illustrate in the males down regulation of the genes, which are crucial for the energy metabolism, protein synthesis and transportation, were more prominent than in the females, whereas up regulation of the genes crucial for the immune response are more prominent in the females. Altered expression profiles peak at different time points in both genders; in males more prominent changes were observed between 60-70 years of age and the expression profile was stable after 80 years of age; whereas there are

dynamic alterations in the gene expression profiles during aging in females and this alterations continued until 80-90 years of age [58]. Those observations which showed that age-related gene expression changes were sexually dimorphic were also consistent with the studies conducted with animal models including zebrafish [59], [60]. Taken together this evidence suggests that age-related alterations exert their effects in a gender-dependent manner and changes in each gender may not be similar. Therefore, both gender groups should be included in aging studies to get insights about the whole picture.

1.6 Zebrafish as a Gerontological Model

The zebrafish has been a popular model organism for developmental and biological studies, due to its large fecundity, easy and inexpensive maintenance, transparent embryos and observable development, and available mutant and transgenic lines, which can provide more causative observations. As a model organism zebrafish has conserved physiological and genetic homology to humans [61]. Also, zebrafish have an integrated nervous system. The architecture of areas like spinal cord cerebellum; olfactory bulb, spinal cord and retinal cells are similar to their mammalian counterparts. However, the morphology of telencephalon and optic tectum show some differences. In the embryonic stage, the telencephalon undergoes an eversion and extends dorsally, which is different than the mammalian brain development [62]. Due to this eversion process, finding homologous structures which correspond to the telencephalic regions in the zebrafish become a challenging work. Yet recent ablation studies with zebrafish and other teleost models indicated that specific telencephalic structures likely correspond to mammalian hippocampus and amygdala [63], [64]. To illustrate, when lesions were induced in the ventrolateral

portion of the dorsal telencephalon, deficits in spatial learning and memory were observed, which is similar to hippocampal lesions in mammalian brain; and lesions in the medial portion of the dorsal telencephalon resulted in impairments in fear conditioning and learning so the behavioral phenotype of the medial portion of dorsal telencephalon shows resemblance to amygdala lesions in mammalian models [64]. Although, ablation studies give general information about behavioral deficit and phenotype, expanded work on neuro-architecture and circuit structure of those telencephalic segments are still required. Another distinct and different region in zebrafish brain is the optic tectum. The optic tectum is a predominant structure in the diencephalon of the zebrafish like in the other teleost species, which is different from mammals [62]. Tectal neurons in this region are responsible for the basic visual processing includes motion, direction and color detection. Moreover, besides first-order motion detection, which are dependent to the luminescence differences, they are also detecting the apparent and second-order motion like in mammals, which are based on the second-order visual features including contract, flickering, texture etc. differences [65]. This kind of high level visual processing might indicate that the optic tectum is likely very similar to mammalian visual cortical areas such as V1 and V2.

The zebrafish is also a promising gerontological model since the lifespan of zebrafish is around 3-5 years [66], which is longer than conventional animal models like mice. The longer lifespan of zebrafish might be seen as drawback for aging studies yet it is crucial to observe gradual age-related differences. One of the most important findings about this model is that zebrafish shows gradual cognitive decline with an increasing age, which is also observed in humans [67]. Moreover, age-related

cellular and molecular alterations can also be observed in the aged zebrafish. To illustrate accumulation of the senescence-associated beta-galactosidase and oxidized proteins were observed in old zebrafish tissues including brain and muscle [68], [66]; those markers are considered as the hallmarks of cellular senescence. Because of the accumulated DNA damage with an increasing age some chromosomal regions become susceptible to those changes, one of those regions are the telomeres and telomere shortening was observed in the aged humans and mammalian models [35]. This phenomenon was also observed in the zebrafish, age-related shortening of telomeres and decreases in the activity of telomerase were observed in previous studies [69], [68]. Finally, compared to the mouse, zebrafish show higher resemblance to humans in terms of telomere length [69]. Therefore, it can be concluded that hallmarks of the aging in the humans and mammals are also observed in the zebrafish and it represents a promising model to study human aging.

1.7 Conclusion

Normal aging differs from the age-related pathological conditions, with the most evident features of the age-related pathological conditions as progressive neurodegeneration, and abnormal accumulation of proteins and global distribution of those proteinopathies across brain regions, which result in severe deficits in cognitive abilities [7], [8], [14], [70]. However, during normal aging cognitive decline is also prominent in some cognitive domains including processing speed, episodic memory, divided attention and executive functions, which are less severe than the cognitive decline occurred in the pathological conditions and they affect differential aspects of the cognitive domains [15]. Yet, normal aging-related cognitive decline is not a

unitary process; whereas some cognitive abilities decline with an advancing age; some cognitive domains including sustained attention, implicit memory, and vocabulary show stable and even improved profiles in the older adults [20]. There are no gross structural alterations including global neuronal and synapse loss occurring throughout the normal aging process [3], [6] that accounts for age-related cognitive decline. However, subtle molecular alterations including age-related gene expression changes [58], disturbances in mitochondrial dynamics [32], protein homeostasis [33], and hypofunction in the cholinergic system [10] were reported in previous studies. Disturbances in neurotransmission regulating proteins might have a direct explanatory role in those age-related cognitive decline and alterations; it was also reported that age-related changes exert their effects differentially in males and females [58]. In the present thesis, this possible dysregulation in the levels of the key synaptic proteins PSD95, GEP and SYP which might have contributions to the age-related cognitive decline, was investigated in different age and gender groups of wild-type zebrafish and in an old mutant line, which has been proposed as a delayed aging model in a previous study [67].

CHAPTER 2

Methods

To investigate the possible age-related dysregulation in the key synaptic proteins that have possible contributions to the age-related alterations in synaptic composition that might underlie cognitive decline, Western blotting was used to measure the relative expression levels of the selected synaptic proteins among different groups. This was done in both wild-type and delayed aging zebrafish models. In this Chapter, information is provided about the subjects, dissections, protein isolation procedure, Western blot protocol and image quantification procedure. These common methods are applied in Chapters 3 and 4, and any specific methods are indicated in those Chapters.

2.1. Subjects

All fish were raised and maintained in standard conditions in the Bilkent University Molecular Biology and Genetics Department Zebrafish Facility. They were kept in a recirculating and controlled ZebTec housing system (Tecniplast, Italy) with the temperature of the system water at 28°C. In this system, pH was between 7.0-7.5 and the water quality parameters were frequently checked by the readings of the mechanical and the carbon filters. Based on the stocking density of 3 fish per liter, which has been shown to not create increased levels of stress [71], animals were maintained in 3.5 L or 8 L tanks with a light-dark cycle of 14L:10D. Fish were fed with dry flakes twice a day and live food; artemia once a day. All the birth dates were recorded and labeled on the tanks, and the fish which had the same date of birth

were kept together. Age groups were young (6-8 months old), middle-aged (11-14.5 months old), old (27-30) and very old (34-43 months old) and in each Chapter chosen age groups are indicated. Two different euthanasia methods were employed in Chapters 3 and 4 and those methods are explained in each Chapter.

2.2 Dissections

Following euthanasia, heads were decapitated using a scalpel blade. With the use of a stereomicroscope, the head was placed dorsal side down. Gills and the connective tissues were removed from the ventral side to reach the skull, after visualization of the optic chiasm; the eyes were removed from the optic nerves by surgical scissors. The skull was exposed after the removal of eyes, gills and connective tissues, and it was broken carefully and the brain was extracted from the ventral surface. After the visualization of anterior structures like telencephalon, the brains and the tissue of interests were put into 1.5 ml tubes and tissues were snap frozen by immersing the tubes into the liquid nitrogen to prevent further degradation of samples. To determine the gender, the peritoneal cavity was opened and when eggs were visualized the gender was determined as female and in the presence of testis the gender was designated as male, and bodies were subjected to the same snap freezing protocol as the other tissues. All of the samples were stored at -80°C for the subsequent protein analysis experiments.

2.3. Protein Isolation from Whole Brain of the Zebrafish

Stored whole brain tissues were taken from -80°C for the protein isolation protocol; they were put into 1.5 ml Eppendorf tubes. Approximately 60 µl of radioimmunoprecipitation assay (RIPA) buffer [50-mM TrisHCl, pH 8.0, containing

150-mM NaCl, 1% NP40, and 0.1% SDS and protease inhibitor (2× stock, 05 892 970 001, Roche)] was added for each 1 mg of tissue. This lysis buffer is suitable for target proteins that are membrane bound, nuclear or found in the whole cell. The recipe for the preparation of 1 ml RIPA buffer is found in Table 2.1.

Table 2.1. Preparation of 1 ml RIPA solution

Stock Solution	Amount (µl)
2-M NaCl	75
1-M TrisHCl pH:8	50
NP40 (100%)	10
SDS (10%)	10
ddH_2O	355
Protease Inhibitor (2x)	500

After tissues were suspended in the required amount of the RIPA buffer, they were homogenized with a 25-gauge, 2-mL syringe by passing through it 10-12 times on ice. Homogenates were incubated on ice for 30 minutes, during that period they were gently mixed three times. After the incubation, samples were centrifuged at 13,000 rpm for 20 minutes at 4°C, and the supernatant was collected and aliquoted. Following the determination of the soluble protein concentrations, protein samples were stored in -80°C.

2.4. Bradford Assay

To determine the concentration of the soluble proteins a Bradford assay was performed in a 96-well plate. Firstly, ddH_2O for blanks, standards and unknown samples was loaded into the 96-well plate, amounts were indicated in the Tables 2.2

and 2.3. After that standards (bovine serum albumin (BSA, A7906, Sigma-Aldrich, St. Louis, MO, USA) ranging from 2-20 μg/ml) and unknown protein samples were loaded into the plate in duplicates, template designs is illustrated in Table 2.4. After ddH_2O, BSA standards and protein samples were loaded, 250 μl of Bradford Reagent (B6916, Sigma, St. Louis, MO, USA) was added into the wells, and a 96-well plate was mixed using the orbital plate shaker at 300 rpm at room temperature for 2 minutes. Then plate was incubated without shaking at room temperature for an additional 10 minutes. The plate was checked whether there were any air-bubbles in each well that could affect the absorbance values of the samples. In cases where there were air bubbles they were popped with a sterile syringe tip. Absorbance values were measured at 595 nm with a multi-plate reader (SpectraMax M5, Molecular Devices, Sunnyvale, CA, USA), and examples of the net absorbance values are found in Table 2.5. Blanks were assigned in the template design and the absorbance values of the blanks were subtracted from the absorbance values of the each well. Net absorbance of the standards and the concentrations were plotted, and a linear curve fit was applied to those data points and standard curve was created which was used for the concentration calculations of the unknown protein samples, which are shown in Figure 2.1.

Table 2.2. Preparations of the standards

	BSA (µl) (1mg/1ml stock)	**ddH_2O (µl)**	**Final Conc. (µg/ml)**
Blank	0	5	0
Standard 1	0.5	4.5	2
Standard 2	1	4	4
Standard 3	2	3	8
Standard 4	3	2	12
Standard 5	4	1	16
Standard 6	5	0	20

Table 2.3. Preparation of the unknown protein samples

	Protein (µl)	**ddH2O (µl)**
Unknown Protein Samples	0.5	4.5

Table 2.4 Illustration of the template design for 96-well plate (loaded partially)

	1	2	3	4
A	STD0	STD0	f-tel-old	f-tel-old
B	STD1	STD1	f-op-old	f-op-old
C	STD2	STD2	f-cer-old	f-cer-old
D	STD3	STD3	m-tel-old	m-tel-old
E	STD4	STD4	m-op-old	m-op-old
F	STD5	STD5	m-cer-old	m-cer-old
G	STD6	STD6		
H				

Table 2.5. Illustration of the net absorbance values for the standards and samples

	1	2	3	4
A	0	0	0.058	0.055
B	0.054	0.036	0.123	0.111
C	0.055	0.062	0.133	0.12
D	0.112	0.097	0.082	0.078
E	0.17	0.171	0.152	0.145
F	0.237	0.253	0.135	0.125
G	0.289	0.327		
H				

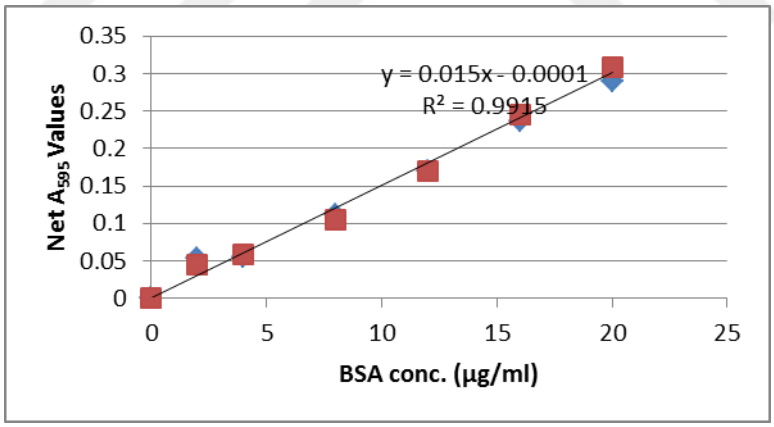

Figure 2.1. Illustration of the standard curve. Red and blue symbols are showing net absorbance values of the duplicates of the standards along with the corresponding concentration

2.5. Western Blot Protocol

Sodium dodecyl sulfate polyacrylamide gels (SDS-PAGE) were prepared for the gel electrophoresis, which was used to segregate the proteins according to their molecular weights. For the cohorts in which Postsynaptic density-95 (PSD-95) and the synaptophysin (SYP) levels were quantified, 10% resolving gels were prepared at a 1.0 mm thickness; 10% resolving gels were prepared at a 1.5 mm thickness for the cohorts which were used for the gepyhrin (GEP) quantification, the recipe of the 10% resolving gel is shown in Table 2.6. To eliminate the formation of the air bubbles in the resolving gel, 2-propanol was added onto the gels. After, resolving gels were polymerized, 2-propanol was discarded and the surface was rinsed with ddH2O and blotted with filter paper. Then 5% stacking gels were poured above the resolving gels, and the proper combs were placed carefully without inducing any air bubbles, the recipe of the 5% stacking gel is indicated in Table 2.7. Running buffer with a concentration of 10X (recipe is shown in Table 2.8) was diluted to a 1X concentration, gels were placed into the electrophoresis chamber, which was filled with ice-cold 1X running buffer, and then combs were removed from the wet surface to eliminate breakage in the stacking gel.

Table 2.6. 10% Resolving Gel Recipe

Materials	Amount (ml) (for 10 ml)
ddH_2O	4.8
1.5-M Tris, pH 8.8	2.5
10% SDS	0.1
40% Bis-acrylamide	2.5
10% APS	0.1
TEMED	0.01

Table 2.7. 5% Stacking Gel Recipe

Materials	Amount (ml) (for 8 ml)
ddH_2O	4.824
0.5-M Tris, pH 6.8	2
10% SDS	0.08
40% Bis-acrylamide	1
10% APS	0.08
TEMED	0.016

For the detection of the levels of PSD-95 and SYP 10 µg of proteins were used; and for the detection of GEP levels 40 µg of proteins was used. Each gel was run in cohorts that are indicated in the each Chapter in order to have reliable comparisons among the groups. After required concentrations were calculated, samples were mixed with 2X loading buffer (recipe is indicated in Table 2.9), they were incubated on dry bath at 95°C for 10 minutes, quick spanned afterwards; and waited on ice until the loading of the samples to the SDS-PAGE gels.

Table 2.8. 10X Running Buffer, pH 8.3

Materials	Amount (For 1L)
250-mM Tris	30.285 g
1.9-M Glycine	144.134 g
10% SDS	100 ml
ddH_2O	Up to 1 L

Table 2.9. 2X Loading Buffer

Stock Solution	Amount (ml)
10% SDS	4
20% Glycerol	2
4% BPB	0.1
0.125-M Tris, pH 6.8	2.5
Each time 10% DTT was added freshly	

Samples were loaded with fine pipette tips without inducing air-bubbles into the wells, and as a marker pre-stained protein ladder (26616, Thermo Scientific Paisley, UK) with the size range of 10-180 kDa was used to determine the molecular weights of the selected proteins and loaded into each gel. A Mini-PROTEAN Tetra Cell electrophoresis system (BioRad, CA, USA) was used for the electrophoresis. Gels were run at 90 volts (V) for 30 minutes, after samples reached to the resolving gel they were run at 120V for approximately 100 more minutes for adequate separation.

Gels were removed from electrophoresis chamber and glass holders, and put into the 4°C transfer buffer to equilibrate. Polyvinylidene difluoride (PVDF) membranes and the whatman filter papers were prepared in the required dimensions, which was 7.9x9.8 cm. PVDF membrane was activated with 100% Methanol (MeOH) for one minute, and then put into 4°C transfer buffer (recipe is shown in Table 2.10). Materials of the transfer system like sponges and whatman paper were soaked in the 4°C transfer buffer. They were placed into the Mini Trans-blot Electrophoretic Transfer Cell module (BioRad, CA, USA) as indicated in Figure 2.2. Proteins segregated in the SDS-PAGE gel were transferred into the PVDF membrane at 100V for 90 minutes in the cold room, which was at a temperature of 4°C; milliampere (mA) readings were checked during the transfer to eliminate increased resistance, which can occur due to content of the buffers or heating of the system during the transfer.

Table 2.10. Transfer Buffer

Materials	Amount (For 2L)
25-mM Tris	6 g
195-mM Glycine	28 g
Methanol	400 ml
ddH_2O	Up to 1.6 L

Figure 2.2. Illustration of the transfer assembly

Following the transfer, PVDF membranes were removed from the transfer chamber and the holders, and incubated with the blocking buffer for one hour at room temperature with rocking motion. The content of the blocking buffer was chosen according to the information sheet of the each antibody and the optimization experiments. For the cohorts in which the levels of PSD-95 and SYP were analyzed, non-fat milk powder was used as a blocking solution in the amount of 5% dissolved in Tris Buffered Saline with Tween 20 (TBS-T). TBS-T was prepared by diluting 10X TBS stock to 1X and adding 0.3% of Tween 20 to the diluted solution, the recipe for 10X TBS is indicated in Table 2.11. For the cohorts that were analyzed for the GEP levels, blocking solution was prepared with bovine serum albumin (BSA) (Sigma-Aldrich, St. Louis, MO, USA), at the amount of 5% dissolved in TBS-T.

Table 2.11. 10X TBS, pH 7.6

Materials	Amount (For 1L)
100-mM Tris	12 g
1.35-M NaCl	88 g
ddH_2O	Up to 1L

Following one hour of blocking at room temperature, membranes were quickly washed with TBS-T. Primary antibodies which were used in the Western blot experiments were anti-PSD-95 (abcam, Cambridge, UK: ab18258, 1:5000 dilution), anti-SYP (abcam, Cambridge, UK: ab32594, 1:20000 dilution) and anti-GEP (Santa Cruz Biotechnology, Santa Cruz, CA, USA: sc-6411, 1:1000 dilution). Anti-PSD-95 and anti-SYP were incubated simultaneously, and in this mix anti-β tubulin (Cell Signaling Technology, Danvers, AM, USA: #2146, 1:5000 dilution) was used as a housekeeping control. Anti-PSD-95, anti-SYP and anti-TUB primary antibodies mix were prepared with 5% non-fat milk powder in TBS-T. For anti-GEP incubations, different methods were used which are indicated in each Chapter; anti-GEP primary antibodies were prepared in 5% BSA in TBS-T. Membranes were incubated with primary antibodies for 18 hours at $4^{o}C$ with rocking motion.

Membranes were taken from $4^{o}C$ and the primary antibodies were collected. Membranes were washed with TBS-T for five times at room temperature with rocking motion for a duration of the following washing steps: 5, 5, 10, 5 and 5 minutes, respectively. After the washing steps were completed, membranes were incubated with appropriate secondary antibodies for 55 minutes at room temperature with rocking motion. Primary antibodies including anti-PSD-95, anti-SYP, anti-TUB and anti-ACTIN (abcam, Cambridge, UK: ab1608, 1:1000 dilution) were raised in

rabbit and for those antibodies anti-rabbit-HRP (Cell Signaling Technology, Danvers, AM, USA: #7074, 1:5000 dilution) was used as a secondary antibody. In contrast anti-GEP was raised in goat and for the detection of the anti-GEP; anti-goat-HRP (abcam, Cambridge, UK: ab97100, 1:10000 dilution) was used as a secondary antibody. When the incubation with the secondary antibodies was completed, secondary antibodies were collected and membranes were washed with TBS-T for five times at room temperature with rocking motion and a duration of the following washing steps: 5, 5, 10, 5 and 5 minutes, respectively.

To get chemiluminescent signals from the horseradish peroxidase (HRP) conjugated secondary antibodies, Supersignal West Femto Maximum Sensitivity Substrate (Thermoscientific, Rockford, IL, USA: 34095) was used. This kit constitutes of two solutions, which are Luminol/Enhancer Solution and Stable Peroxide Solution. Those solutions were mixed in the proportion of 1:1 and immediately put onto the membranes. Membranes were incubated in a dark chamber for 5 minutes and then visualized. For the experiments in the Chapter 3, to detect the chemiluminescent signal, an automatic film processor was used (Hyperprocessor, Amersham Life Sciences, UK), and after the visualization the x-ray films were photographed. For the experiments in Chapter 4, a ChemiDoc™ XRS+ imaging system was used (Biorad, CA, USA) with the ImageLab software (Biorad, CA, USA).

2.6. Image Quantification

All images acquired with the photographed x-ray film for the blot sets in Chapter 3 or with the ImageLab software for the blot sets in Chapter 4 were quantified with ImageJ software (NIH, Bethesda, MD, USA). The first step was

converting those images into 8-bit from the RGB scale. A rectangular selection tool was used for the selection of each of the bands of interest. For each band in the blot, the same sized rectangular selection area was used. After all the lanes in the cohort were selected, the intensity difference of each lane in the rectangular selection area was plotted, in the plot the area between two peaks was calculated as the intensity measurement. Intensity measurements were performed in an unbiased manner as the person quantifying the images was blind to the age, gender and/or genotype of the groups. Blot sets in Chapter 3 were quantified by Dilara Özge Halim and in Chapter 4 were quantified by Melek Umay Tüz-Şaşik.

Two different normalization methods were employed for all the data sets. The first one was the within-blot normalization, which is referred to as "normalized values." The aim of this normalization method was to reduce exposure and inter-assay differences that might occur between the blots because for each subject within the groups minimally three replicates were performed. In this normalization method the individual band intensity of the selected band was divided by the averaged intensity of that band in the same blot cohort [52], [6], [72]. This normalization procedure allows the expression pattern of each protein of interest, including the housekeeping proteins like TUB and ACTIN, to be distinguished individually based on gender, age and/or genotype. The second normalization procedure used was a standard normalization with the synaptic protein of interest normalized by the corresponding house-keeping protein within the blot. For this protocol after the within blot normalization, each band intensity of the protein of interest was divided by the corresponding housekeeping protein like TUB or ACTIN intensity. This second procedure was referred to as "TUB-normalized values" or "ACTIN-

normalized values". Yet, the drawback of the second normalization procedure was that housekeeping proteins were considered to be stable across different age, gender and/or genotype groups. Therefore, results derived from both normalization procedures were included and represented in this thesis.

CHAPTER 3

Expression of Postsynaptic Density-95, Gephyrin and Synaptophysin in Young, Middle-Aged and Old Male and Female Zebrafish

3.1. Introduction

Aging alters the cellular and molecular dynamics, which eventually could lead to declines in cognitive abilities. As indicated in the Chapter 1, there are no gross structural alterations in the aging brain but some molecular alterations like epigenetic changes, mitochondrial dysfunction and loss of protein homeostasis might account for age-related cognitive decline. However, more direct relationships can be established to find the basis of the age-related cognitive decline by investigating the elements of synaptic integrity in the aging brain. Studies have indicated that aging is also associated with dysregulated synaptic protein expression and these alterations have possible deteriorating impacts on neurotransmission, vesicle trafficking, induction of long-term potentiation and synaptic plasticity, which are crucial for learning and memory formation. Therefore, in Chapter 3, the synaptic proteins postsynaptic density 95 (PSD-95), gephyrin (GEP) and synaptophysin (SYP) were investigated as indicators of excitatory post-synaptic integrity, inhibitory post-synaptic integrity and pre-synaptic integrity, respectively.

The aim of the present study in this thesis was to show whole brain and region-specific expression profiles of the three synaptic proteins; PSD-95, GEP and SYP, which are regulating the differential aspects of the synaptic function and integrity, to obtain possible age-related alterations in the expression profiles, and

lastly to decipher the possible role of the gender, which has been underemphasized in many aging studies. Moreover, these data are among the first to examine these proteins in the context of aging in the zebrafish brain. It was hypothesized that there would be age-related changes in the levels of the PSD-95, GEP and SYP; and those alterations in the protein expression profile would depend on the gender of the animal.

3.2. Materials and Methods

3.2.1. Subjects

A total of 52 zebrafish was used for this study. For the whole brain protein expression analysis, young (6–8 months old), middle-aged (MA) (11–14.5 months old), and old (24–30 months old) female and male zebrafish were used, distribution of the groups is indicated in Table 3.1. To investigate further changes during the course of aging very-old (VO) females (34–43 month old) were added to the study, age-matched VO males could not be added to this study due to availability of zebrafish stocks. For the protein expression analysis of the micro-dissected brain regions, young and old male and female zebrafish were used with the distribution of the groups shown in Table 3.2. For this analysis, two age groups, young and old were selected because it was hypothesized to observe a more significant difference between them. All fish were maintained at standard conditions as mentioned in Chapter 2.1.

Table 3.1. Distribution of age and gender groups for whole brain protein expression analysis

	Female	Male
Young	6	6
MA	6	6
Old	6	6
VO	3	-

Table 3.2. Distribution of age and gender groups for the protein expression analysis from micro-dissected regions

	Female	Male
Young	3	3
Old	3	3

For the euthanasia, Tricaine (Sigma-Aldrich, St. Louis, MO, USA) was used at a concentration of 0.3 g/L of Tricane was dissolved in the ddH_2O. This solution was put into a plexiglass holding tank, fish were put into the holding tank and remained there until gill movements stopped, which took approximately 10-15 minutes. The animal protocol of this study was approved by the Bilkent University Local Animal Ethics Committee (HADYEK) with approval date: Feb 9, 2010 with the no: 2010/1 and updated on Jul 15, 2016 with the no:2016/22.

3.2.2. Dissections

Dissections of the whole brain and the other tissues, and the gender determination were performed as indicated in Chapter 2.2. For the micro-dissection

sets, after the extraction of the whole brain, the brain was placed ventral side down. Three regions were separated with the guidance of zebrafish brain atlas [62]. Micro-dissected regions were 1) telencephalon, which includes, the ventral and dorsal telencephali and olfactory bulbs, functional studies indicated that those regions in the teleost brain correspond to hippocampus, amygdala and cortex [63], 2) optic tectum, which corresponds to the mammalian visual cortex and sensitive to first- and second-order motions [65], and 3) cerebellum and medulla spinalis. A representation of the micro-dissection and region separation is indicated in Figure 3.1. Because the sizes of the micro-dissected regions were smaller than the whole brain, to recover a sufficient amount of protein each gender and age groups were pooled together. Micro-dissected and pooled regions were not exposed to snap freezing, they were directly subjected to protein isolation protocol.

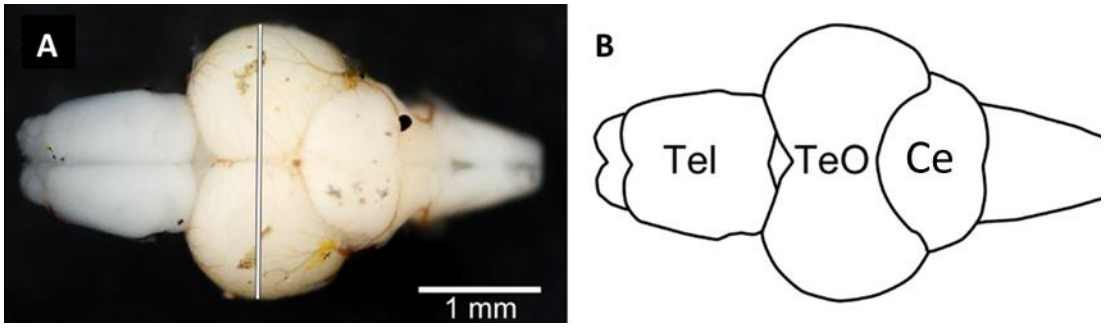

Figure 3.1. Illustration of the microdissection procedure on zebrafish brain, zebrafish brain (A), schematic representation of microdissection (B). Tel: telencephalon; TeO: optic tectum; Ce: cerebellum. (adapted from Naslund et al., 2014, [73])

3.2.3. Protein Isolation

The protein isolation procedure for the whole brain sets followed the same protocol as indicated in the Chapter 2.3. Micro-dissected regions were put into the 100 µl of RIPA buffer directly, and during the micro-dissections each region was pooled with 3 animals in each group. Then the additional RIPA buffer was added into the tubes when there is more tissue, the proportion was similar with approximately 60 µl of RIPA buffer used for each 1 mg of tissue. The subsequent steps were performed in the same manner as indicated in Chapter 2.3.

3.2.4. Western Blot

Experiments followed the procedure indicated in Chapter 2.5. In each gel representative samples of the each group were included to have reliable comparisons between the age and gender groups. Anti-PSD-95, anti-SYP and anti-TUB primary antibodies were blotted simultaneously; anti-GEP and anti-TUB primary antibodies were blotted sequentially because those antibodies were raised against different secondary antibodies. For each sample, experiments were performed minimally in triplicates and for each sample approximately 3 measurements were taken. Therefore, biological and the technical replicates ensure that the alterations of the protein expression pattern are likely due to the true biological alterations based on advancing age or gender. Blots were visualized and developed Image quantification was performed by Dilara Özge Halim and she does not have information about the age or gender groups of samples. Procedure was mentioned in the Chapter 2.6.

3.2.5. Statistical Analysis

Assumptions of normality were checked with Kolmogorov-Smirnov and Levene's test in terms of normal distribution and homogeneity of variance respectively, and the assumptions of normality were fulfilled. A two-way analysis of variance (ANOVA) was carried out with the factors of age with three levels (young, middle-aged (MA) and old) and gender with two levels (female and male) on the expression levels of the key synaptic proteins; PSD-95, SYP, and GEP. Significance level was set to $p<.05$. Bonferroni correction, which has more stringent cutoff value,s was used as a post-hoc comparison. In the cases of the significant interactions, simple effects analysis was carried out to break down the levels of the one independent variable compared to its effects on the levels of the other independent variable. For the validation of ANOVA results and to perform principle component analysis a multivariate analysis of variance (MANOVA) with the factors, age and gender was performed on the expression levels of the three synaptic proteins. Principal component analysis was applied to the whole brain expression for PSD-95, SYP and GEP. Components were extracted, if the Eigen values were greater than 0.5. In the cases in which component loadings were greater than 0.5 ($r >0.5$), correlations were considered as significant. To investigate region-specific alterations of the expression of those key synaptic proteins, a three-way ANOVA with the factors of age with two levels (young and old); gender with two levels (female and male) and region with three levels (telencephalon (Te), optic tectum (TeO) and cerebellum/medulla/spinal cord (Ce)) was carried out and micro-dissected and pooled brain lysate set was used for this part. Significance level was set to $p<.05$. For the analysis of the very old female group; a one way ANOVA with the factor of age

with four levels (young, MA, old and very-old) was applied to the data. Significance level was set to $p<.05$. All of the analyses mentioned were carried out with SPSS 19. software (IBM, Istanbul, Turkey).

3.3. Results

3.3.1 Antibodies Yield Expected Bands in the Zebrafish Brain Protein Lysates

A large problem for use of zebrafish samples in protein studies is to find the appropriate antibodies as most of the antibodies are not working or require extensive optimizations. The antibodies used in the present study were selected based on epitope homology. In the Western blot experiments with zebrafish brain lysates, anti-PSD-95 antibody gave an expected band at approximately 100 kDa; anti-TUB antibody gave an expected band at approximately 55 kDa; anti-SYP antibody gave an expected band at approximately 38 kDa and lastly anti-GEP antibody gave an expected band at 93 kDa; expected molecular weight of the bands were determined according to the information sheet of the each antibody. To ensure the specificity of the observed band, zebrafish brain lysates were subjected to the Western blot experiments with the positive control protein lysates which were extracted from the cortex of the mouse. In Figure 3.2, the bands of interests for the zebrafish and mouse brain lysates are shown, and it could be observed that zebrafish brain lysates yield specific bands for those antibodies, which were consistent with the positive control. Additionally blots of the experimental cohorts are shown in Figure 3.3.

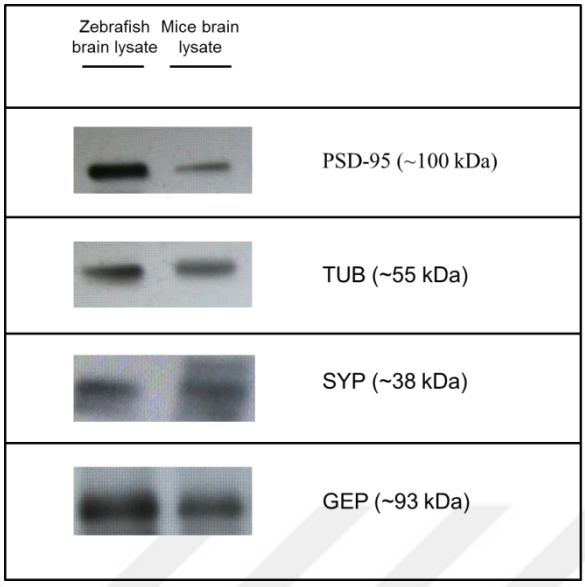

Figure 3.2. Observed bands for the protein samples extracted from zebrafish brain lysate and mouse cortical lysate as positive control.[74] (adapted from Karoglu et al., 2017, Reprinted with permission from Elsevier)

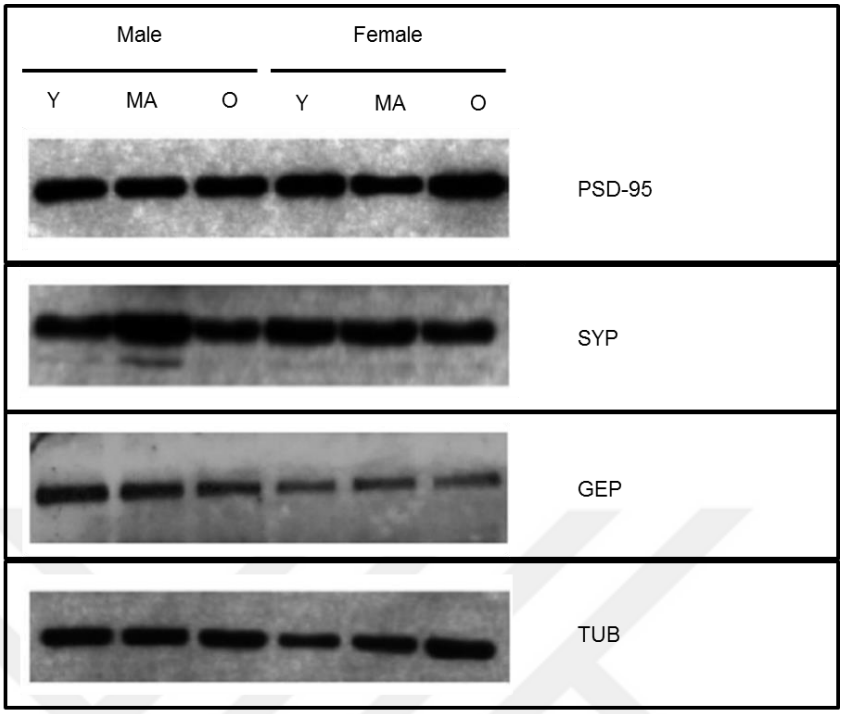

Figure 3.3. Illustration of the experimental cohorts in each western blot for PSD-95, SYP, GEP and TUB. Y: young; MA: middle-aged; O: old. [74] (adapted from Karoglu et al., 2017, Reprinted with permission from Elsevier)

3.3.2. The Expression of the Neurotransmission Regulating Proteins were Affected Differentially by Age and Gender

3.3.2.1. Postsynaptic Density-95 (PSD-95)

Postsynaptic density-95 (PSD-95) is one of the components of the excitatory and activity-dependent neurotransmission [37] and gives information about the integrity of the post-synaptic regions. Trends in the both normalized (Figure 3.4.A) and TUB-normalized (Figure 3.4.B) data indicated that PSD-95 levels tend to be increased with an advancing age in the female group, whereas there is a decreasing pattern in the male group in terms of the expression levels of PSD-95. Two-way

ANOVA revealed a significant gender by age interaction in the normalized data ($F_{(2,30)} = 5.43$, $p = 0.010$). To investigate this interaction further, simple effects analysis carried out to break the levels each gender and age groups and understand the interaction between them. It was shown that, in the normalized data effect of gender within the young group was significant ($p = 0.011$), and the effect of age within the female group was significant ($p = 0.027$). In the TUB-normalized data a significant gender by age interaction was also maintained ($F_{(2,30)} = 4.38$, $p = 0.022$). Simple effects analysis revealed that gender has a significant effect on the old group in terms of the expression levels of PSD-95 ($p = 0.049$), and the effect of age was close to be counted as significant in the females ($p = 0.059$). Therefore, it could be said that both normalized and TUB-normalized data indicated that the effects of aging on the expression levels of PSD-95 depends on the gender, and in females this excitatory neurotransmission component increases through advancing age whereas the expression pattern was tend to be more stable and even decreasing in the males.

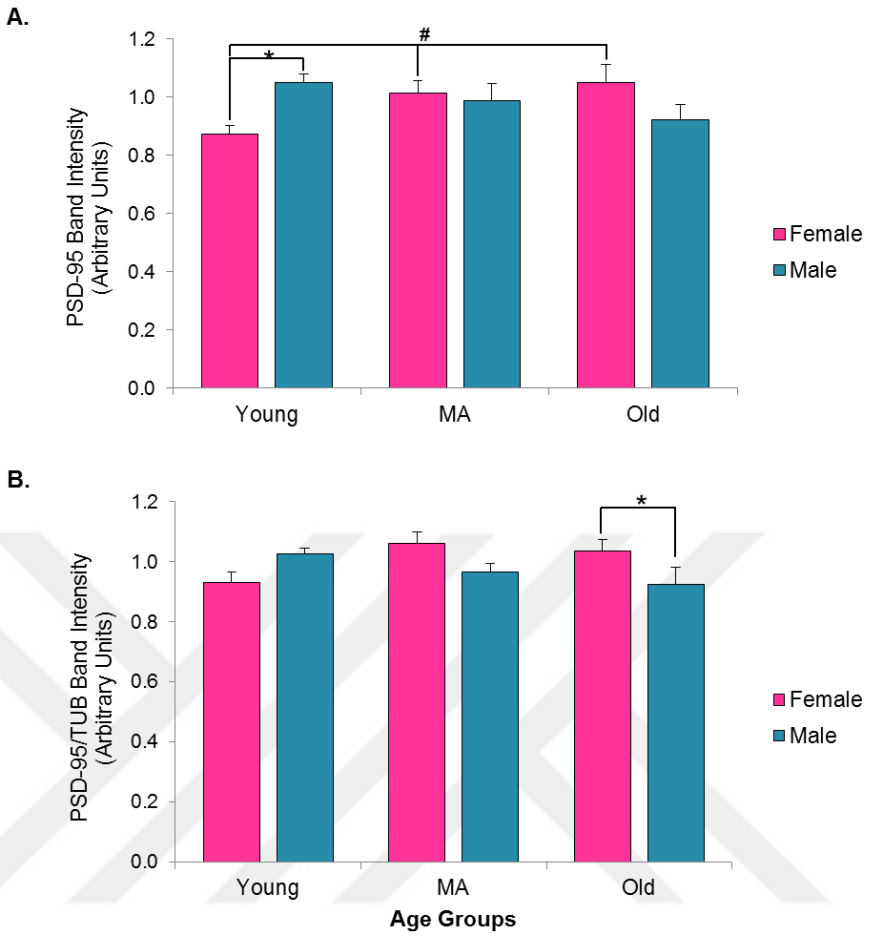

Figure 3.4. PSD-95 expression across gender and age groups; normalized data (A), TUB-normalized data (B). MA: middle-aged. Error bars show +SE, *gender within age, #age within gender, symbols p<0.05.[74] (adapted from Karoglu et al., 2017, Reprinted with permission from Elsevier)

3.3.2.2. Synaptophysin

Synaptophysin (SYP) is the pre-synaptic vesicle glycoprotein and an indicator of the pre-synaptic integrity [51]. Both normalized (Figure 3.5.A) and TUB-normalized (Figure 3.5.B) data indicated that in the male group the expression levels of SYP increase during the middle age and decrease at the old ages, whereas

the expression levels of the SYP tend to be more stable in the female group. Statistical analyses were confirmed the observations since in the normalized data a significant gender by age interaction was revealed ($F_{(2, 29)} = 8.006$, $p = 0.002$, Figure 3.5.A) and consistent with that a significant gender by age interaction is also revealed in the TUB-normalized data ($F_{(2, 29)} = 4.469$, $p = 0.020$, Figure 3.5.B). Simple effects analyses were carried out on these both data sets to break those significant interactions into their parts. In the normalized data (Figure 3.5.A) it was shown that gender has significant effects on middle-aged ($p = 0.004$) and old ($p = 0.017$) age groups; and the effect of the age was significant in the male group ($p = 0.01$) in terms of the expression levels of the SYP. In the TUB-normalized (Figure 3.5.B) data significant effect of gender on the middle-aged group ($p = 0.019$), and a significant effect of age was observed in the male group ($p = 0.001$), which was consistent with the normalized data. Additionally in the TUB-normalized data (Figure 3.5.B), a significant main effect of the age was revealed ($F(2, 29) = 4.666$, $p = 0.018$), and to find the differences a Bonferroni test was applied as a multiple comparison, and the expression level of SYP was significantly reduced in the old-male group compared to the middle-aged-male group ($p = 0.004$).

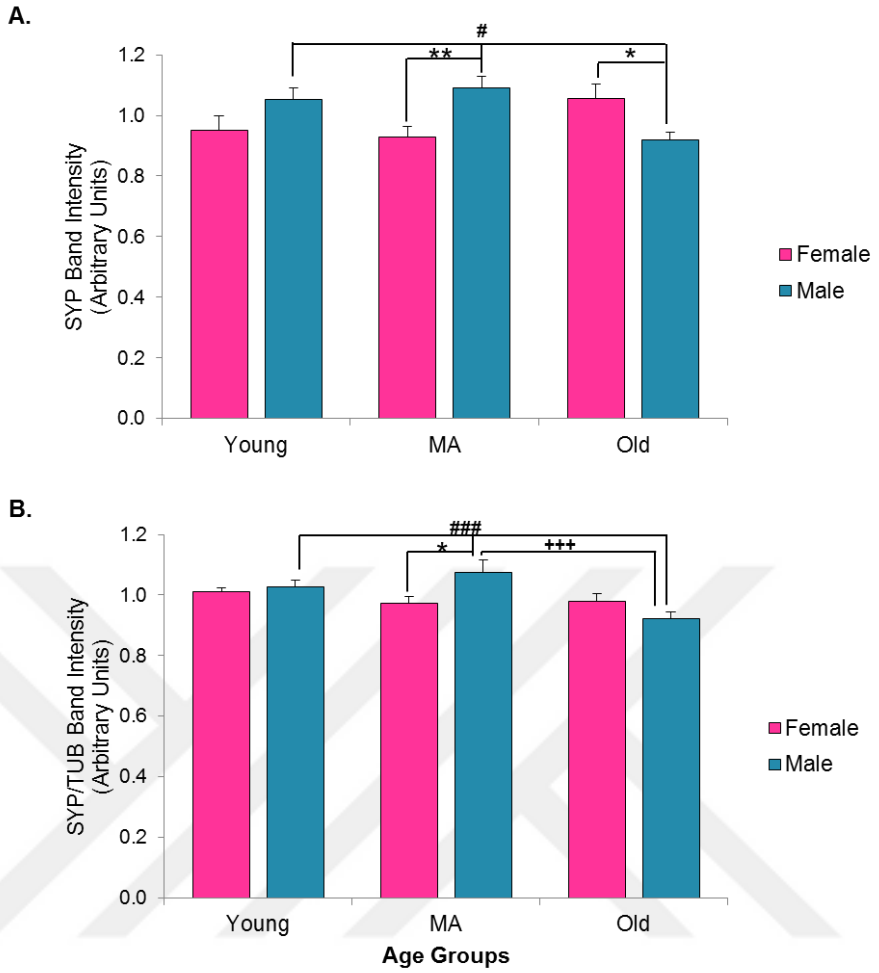

Figure 3.5. SYP expression across gender and age groups; normalized data (A), TUB-normalized data (B). MA: middle-aged. Error bars show +SE, *gender within age, #age within gender, + significant difference in Bonferroni. One symbol: p<0.05; two symbols p<0.01; three symbols p<0.0005 [74] (adapted from Karoglu et al., 2017, Reprinted with permission from Elsevier)

3.3.2.3. Gephyrin

Gephyrin is a major scaffolding protein at inhibitory synapses it clusters $GABA_A$ and glycine receptors at post-synaptic regions [75], and it could be considered as a component of the inhibitory neurotransmission and inhibitory post-

synaptic integrity. Pattern of the expression alterations of the GEP levels seem to be similar between normalized (Figure 3.6.A) and TUB-normalized data (Figure 3.6.b). In the males it increases in the middle-aged group, yet there is a reduction of the expression of GEP at the old males; this pattern seems to be more stable in the female group. No significant main effect was found in both normalized ($F_{(5,30)} = 2.012$, $p = 0.106$; Figure 3.6.A) and TUB-normalized ($F_{(5,30)} = 1.766$, $p = 0.150$, Figure 3.6.B) data sets. However, gender by age interaction in the normalized data might be showing a distinct trend towards significance ($F_{(2,30)} = 2.840$, $p = 0.074$; Figure 3.6.A).

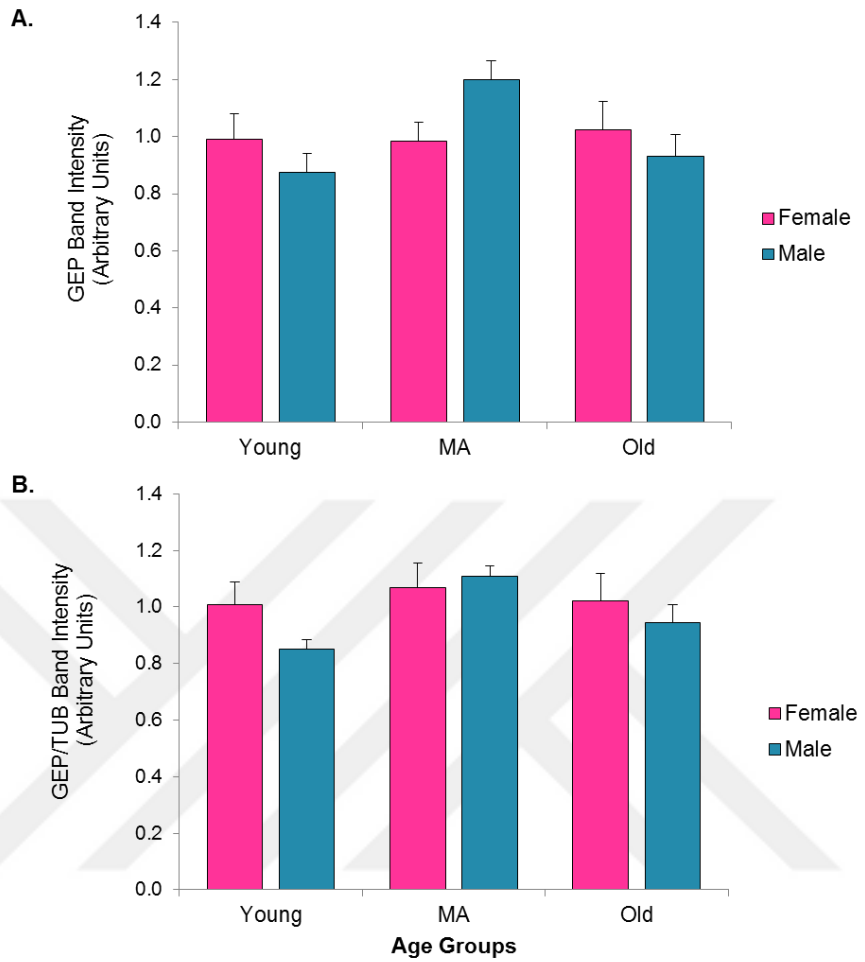

Figure 3.6. GEP expression across gender and age groups; normalized data (A), TUB-normalized data (B). MA: middle-aged. Error bars show +SE, [74] (adapted from Karoglu et al., 2017, Reprinted with permission from Elsevier)

3.3.3. Principle Component Analysis and Investigating Clustering Profiles

The purpose of this analysis was to reduce the dimensions of the data, retain the variation through the clustering profile of the samples, and detect the dominant patterns in the expression profile. Firstly, significant gender by age interactions which were found with the separate two-way ANOVAs were confirmed with the

MANOVA which demonstrated a significant gender by age interaction on PSD95, GEP and SYP levels as a whole, $F_{(6, 54)} = 4.850$, $p = 0.001$; Wilk's $\Lambda = 0.422$. After this validation with MANOVA, principle component analysis was carried out. Significant correlations were revealed after this analysis; it was found that expressions of PSD-95 and SYP ($r = 0.57$, $p < 0.0005$) and expressions of GEP and SYP ($r = 0.42$, $p = 0.007$) were significantly correlated (Figure 3.7.A). Two components were extracted after this analysis; PC1 and PC2 which explains the 87.5% variation in the whole data set (Figure 3.7.B).

A.

Correlation Matrix[a]

		psd95	syp	Gephyrin
Correlation	psd95	1,000	,568	,182
	syp	,568	1,000	,416
	Gephyrin	,182	,416	1,000
Sig. (1-tailed)	psd95		,000	,147
	syp	,000		,007
	Gephyrin	,147	,007	

a. Determinant = .557

B.

Total Variance Explained

Component	Initial Eigenvalues			Extraction Sums of Squared Loadings
	Total	% of Variance	Cumulative %	Total
1	1,798	59,931	59,931	1,798
2	,828	27,585	87,516	,828
3	,375	12,484	100,000	

C.

Component Matrix[a]

	Component	
	1	2
syp	,886	-,081
psd95	,777	-,519
Gephyrin	,639	,743

Extraction Method: Principal Component Analysis.

a. 2 components extracted.

D.

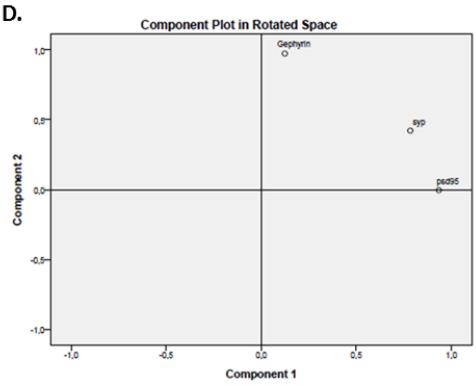

Figure 3.7. Principal component analysis for overall data. Correlation matrix (A), extracted components with their Eigen values (B), component matrix (C), distribution of the synaptic proteins according to their component loadings (D).

Component matrix indicated that PC1 is strongly and positively correlated with all three variables PSD-95, SYP and GEP. PC1 increases with increasing levels of PSD95, SYP and GEP, in this respect they may vary together. PC2 is strongly correlated with the increasing GEP levels, whereas it is negatively correlated with the PSD95 levels. So this correlation could be considered interesting, because the GEP is major scaffolding-clustering protein at the inhibitory synapses and PSD95 has the same roles in the excitatory synapses. Expression levels of the PSD-95, SYP and GEP were plotted against the orthogonal components; PC1 and PC2 to observe the general pattern (Figure 3.7.D). Regression factor scores were calculated for each sample and with those values; each sample were plotted against the PC1 and PC2 (Figure 3.8.A). Age groups were plotted separately to analyze the complex age related patterns (Figure 3.8.B, C, D). It can be interpreted that PC1 gives information about the general integrity of the synapses, and PC2 gives information about the inhibitory/excitatory balance more on to the inhibitory contributions.

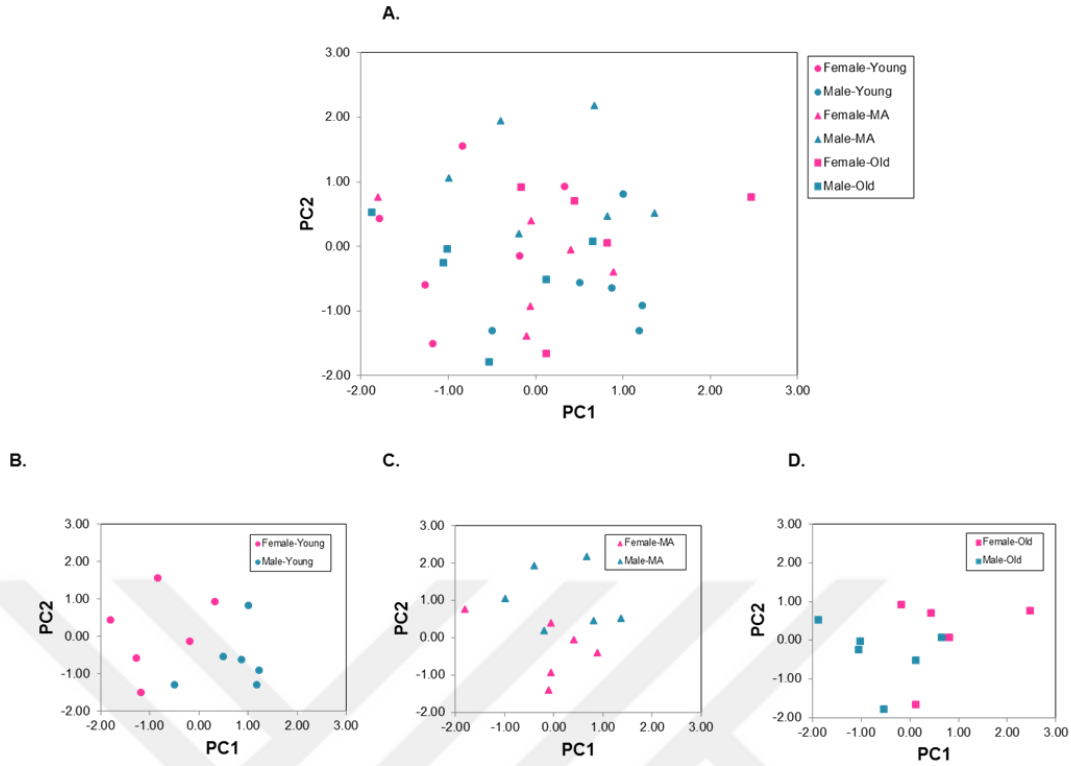

Figure 3.8. Plotting each sample with respect to orthogonal components PC1 and PC2. All groups were included (A), young groups were included (B), middle-aged groups were included (C), old groups were included (D). MA: middle aged. Each group was denoted with different color or symbol as indicated in legends. [74] (adapted from Karoglu et al., 2017, Reprinted with permission from Elsevier)

From the Figure 3.8.B, C, and D, clustering profiles of the young and old groups showed distinct differences between female and male groups, and a shift could be observed between the young and the old ages; yet the pattern of middle-aged group indicated a more complex pattern. Therefore separate PCAs were applied to the individual age groups.

A.

Correlation Matrix[a,b]

		psd95	syp	Gephyrin
Correlation	psd95	1,000	,790	-,088
	syp	,790	1,000	,410
	Gephyrin	-,088	,410	1,000
Sig. (1-tailed)	psd95		,001	,392
	syp	,001		,093
	Gephyrin	,392	,093	

a. Only cases for which AgeM = young are used in the analysis phase.
b. Determinant = .142

B.

Total Variance Explained[a]

Component	Initial Eigenvalues			Extraction Sums of Squared Loadings
	Total	% of Variance	Cumulative %	Total
1	1,856	61,880	61,880	1,856
2	1,072	35,733	97,613	1,072
3	,072	2,387	100,000	

C.

Component Matrix[a,b]

	Component	
	1	2
syp	,981	,060
psd95	,866	-,472
Gephyrin	,380	,920

Extraction Method: Principal Component Analysis.

a. 2 components extracted.
b. Only cases for which AgeM = young are used in the analysis phase.

D.

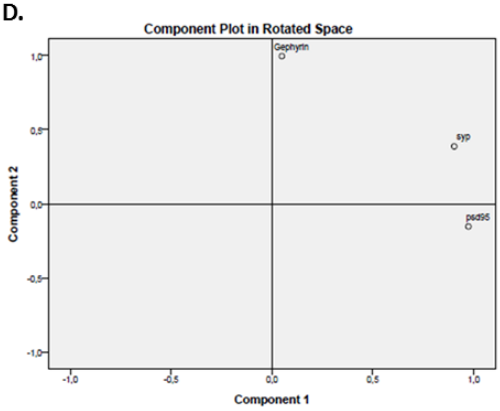

Figure 3.9. Principal component analysis for young group. Correlation matrix (A), extracted components with their Eigen values (B), component matrix (C), distribution of the synaptic proteins according to their component loadings (D).

In the young group, a general pattern was similar as the overall data. The expressions of PSD-95 and SYP were significantly correlated (r = 0.79, $p < 0.001$, Figure 3.9.A), but the significant correlation between SYP and GEP was not observed compared to the overall data. PC1 was driven by all synaptic proteins of interest; PSD-95, SYP and GEP, and PC2 was mainly driven by GEP and negatively correlated with the PSD-95 (Figure 3.9.C). Clustering profile of the young age group shows similar trends as the overall data (Figure 3.9.D).

A. Correlation Matrix[a,b]

		psd95	syp	Gephyrin
Correlation	psd95	1,000	,176	-,075
	syp	,176	1,000	,474
	Gephyrin	-,075	,474	1,000
Sig. (1-tailed)	psd95		,293	,408
	syp	,293		,060
	Gephyrin	,408	,060	

a. Only cases for which AgeM = ma are used in the analysis phase.
b. Determinant = .726

B. Total Variance Explained[a]

Component	Initial Eigenvalues			Extraction Sums of Squared Loadings
	Total	% of Variance	Cumulative %	Total
1	1,485	49,507	49,507	1,485
2	1,048	34,947	84,454	1,048
3	,466	15,546	100,000	

C. Component Matrix[a,b]

	Component	
	1	2
syp	,876	,117
Gephyrin	,826	-,343
psd95	,189	,958

Extraction Method: Principal Component Analysis.

a. 2 components extracted.
b. Only cases for which AgeM = ma are used in the analysis phase.

D.

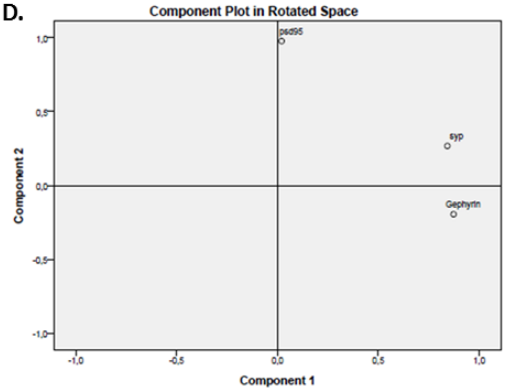

Figure 3.10. Principal component analysis for middle aged group. Correlation matrix (A), extracted components with their Eigen values (B), component matrix (C), distribution of the synaptic proteins according to their component loadings (D).

In the middle-aged group no significant correlation between the synaptic proteins was found; but the correlation between the GEP and SYP was marginally significant ($r = 0.47$, $p < 0.06$, Figure 3.10.A). Component matrix indicated a really distinct pattern for the middle-aged group with most of the variance driven by GEP and SYP through PC1, and PC2 was mainly driven by PSD-95, which was negatively correlated with GEP, Figure 3.10.D. This profile showed an opposite pattern compared to the overall data.

A. Correlation Matrix[a,b]

		psd95	syp	Gephyrin
Correlation	psd95	1,000	,789	,505
	syp	,789	1,000	,398
	Gephyrin	,505	,398	1,000
Sig. (1-tailed)	psd95		,002	,057
	syp	,002		,113
	Gephyrin	,057	,113	

a. Only cases for which AgeM = old are used in the analysis phase.
b. Determinant = .281

B. Total Variance Explained[a]

Component	Initial Eigenvalues			Extraction Sums of Squared Loadings
	Total	% of Variance	Cumulative %	Total
1	2,146	71,545	71,545	2,146
2	,654	21,787	93,332	,654
3	,200	6,668	100,000	

C. Component Matrix[a,b]

	Component	
	1	2
psd95	,924	-,193
syp	,885	-,361
Gephyrin	,714	,697

Extraction Method: Principal Component Analysis.

a. 2 components extracted.
b. Only cases for which AgeM = old are used in the analysis phase.

D.

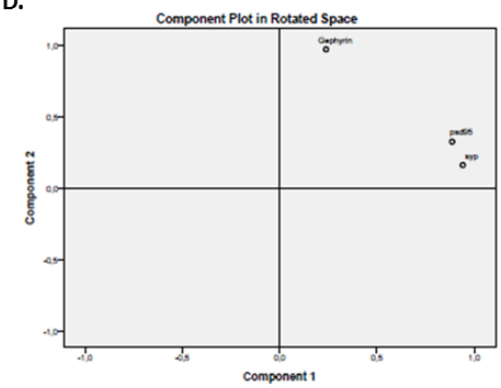

Figure 3.11. Principal component analysis for old group. Correlation matrix (A), extracted components with their Eigen values (B), component matrix (C), distribution of the synaptic proteins according to their component loadings (D).

In the old group, a significant correlation between the expression levels of PSD-95 and SYP (r = 0.79, $p < 0.002$, Figure 3.11.A) was found. PC1 was driven by the all PSD-95, SYP and GEP; on contrary PC2 was mainly driven by GEP and which negatively correlated with the other elements. The clustering profile of the old group showed a similar pattern with the overall data, Figure 3.11.D.

After separate PCA analyses, it could be concluded that young and old ages showed resemblance to the overall data and the variance between them driven by PSD-95 and SYP and GEP was orthogonal. In the middle-aged group, the pattern

changed, and the variance was mainly driven by GEP and SYP and PSD-95 became orthogonal to them. These results indicated that middle-age could serve as a break-point for the age-related alterations and important to be included in the aging studies to differentiate age-related complex patterns.

3.3.4. Expression Alterations at Very Old Ages in Female Group

To evaluate the expression levels of the key synaptic proteins further, a very old group was also included into the present study. Due to the availability of the very old fish in our fish facility, only a female very old group was added and the expression levels were analyzed among the female groups throughout aging. Four very old female zebrafish whose age range was 34-43 months old were included.

One-way ANOVA revealed that there are no significant age-related alterations in the both normalized and TUB-normalized expression levels of PSD-95 ($F_{(3,18)} = 2.831$, $p = 0.068$; Figure 3.12.A), ($F_{(3,18)} = 2.400$, $p = 0.102$; Figure 3.12.B); normalized and TUB-normalized expression levels of GEP ($F_{(3,17)} = 0.045$, $p = 0.987$; Figure 3.12.C), ($F_{(3,17)} = 0.284$, $p = 0.836$; Figure 3.12.D); and normalized and TUB-normalized expression levels of SYP ($F_{(3,17)} = 1.389$, $p = 0.280$; Figure 3.12.E), ($F_{(3,17)} = 1.364$, $p = 0.287$; Figure 3.12.F). Those observations suggested that the expression levels of PSD-95, GEP and SYP were not changing and stable even at the very old ages in the female group.

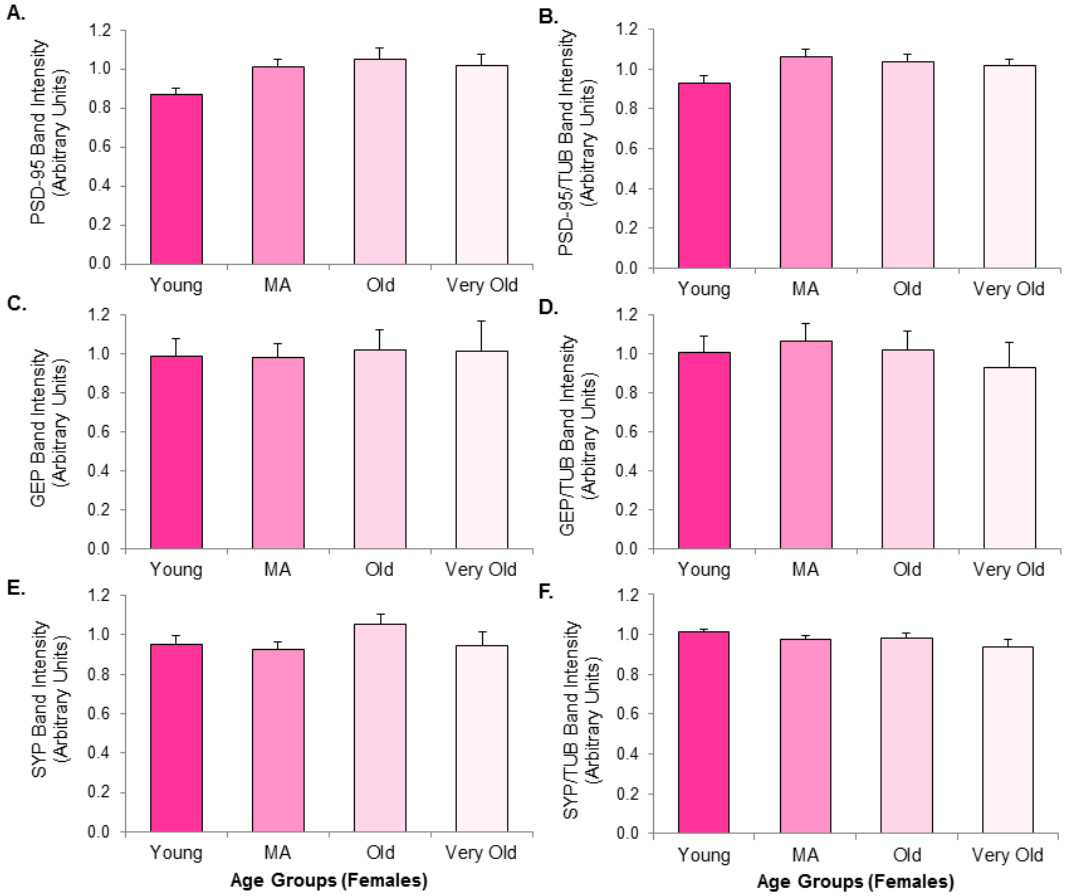

Figure 3.12. Expression of the synaptic proteins through aging in female group. Normalized (A) and TUB-normalized (B) PSD-95 data; normalized (C) and TUB-normalized (D) GEP data; normalized (E) and TUB-normalized (F) SYP data. MA: middle-aged. Error bars show +SE, [74] (adapted from Karoglu et al., 2017, Reprinted with permission from Elsevier)

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3.3.5. Region-specific Alterations of the Key Synaptic Proteins According to Age and Gender

All the data shown was derived from the whole brain analysis of the expression levels of the PSD-95, GEP and SYP. Studies showed that specific regions

like hippocampus and medial prefrontal cortex (mPFC) have differential vulnerabilities to aging and dynamics of the synaptic integrity are differentially affected by advancing age [30]. Therefore, it is important to focus on region-specific alterations at the levels of PSD-95, GEP and SYP to see the whole pattern. For that reason, zebrafish brains were micro-dissected following the procedure indicated in the Figure 3.1.B. Since, zebrafish has a small brain, to recover the sufficient protein concentrations required for the Western blot experiments, 3 animals in each group (female-young, female-old, male-young and male-old) were pooled group-wise and region-wise [telencephalon (Te), optic tectum (TeO), cerebellum/medulla/spinal cord (Ce)] after the micro-dissection procedure. Results showed that, the expression levels of the PSD-95 in both normalized (Figure 3.13.A) and TUB-normalized (Figure 3.13.B) did not show any significant differences between the age and gender groups ($F_{(11,24)} = 2.173$, $p = 0.054$; Figure 3.13.A); ($F_{(11,24)} = 1.086$, $p = 0.412$; Figure 3.13.B). Yet, TUB-normalized PSD-95 values of the Te region show a resemblance to the overall data in which an increasing expression pattern of PSD-95 was observed in the old females (Figure 3.13.B). No significant main effect or interaction was found in both normalized and TUB-normalized expression levels of the GEP between the age and gender groups among the different regions ($F_{(11,24)} = 0.224$, $p = 0.993$; Figure 3.13.C), ($F_{(11,23)} = 0.656$, $p = 0.763$; Figure 3.13.D). In terms of SYP expression levels both normalized and TUB-normalized data were not significantly different between the age-gender groups among the regions ($F_{(11,24)} = 0.513$, $p = 0.876$; Figure 3.13.E), ($F_{(11,24)} = 1.015$, $p = 0.463$; Figure 3.13.F). However, the pattern of normalized SYP expression in the Te region (Figure 3.13.E) show similar patterns as the overall data in which there is an increasing pattern in the females but a

more stable pattern in the male. Although, there is no significant difference, expression patterns of Te region show promising results and it is indicated the expression profile of the Te region shows resemblance to the whole brain expression data; and the whole brain expression alterations might be driven by this region.

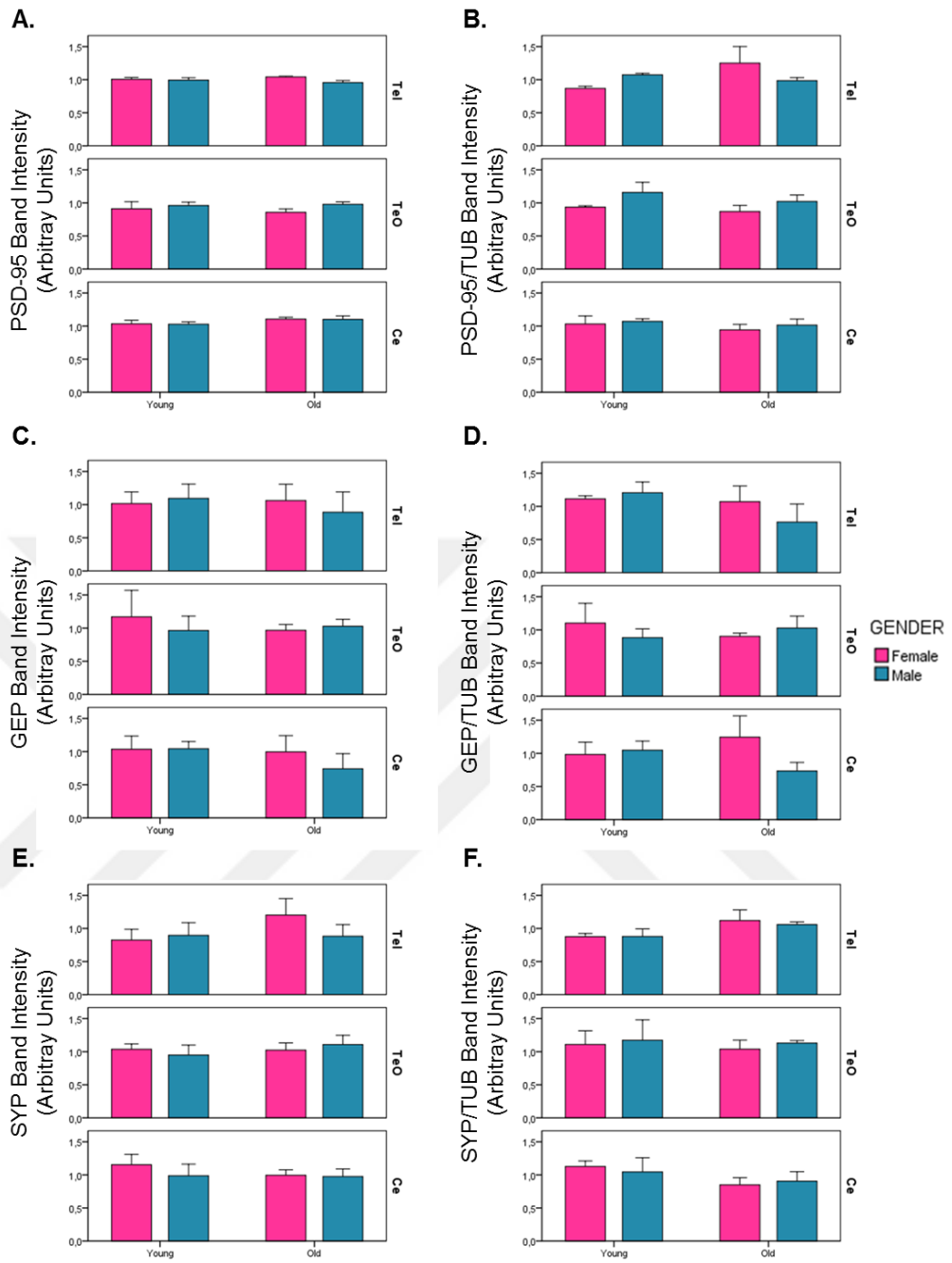

Figure 3.13. Region-specific expression of the key synaptic proteins in gender and age groups. Normalized (A) and TUB-normalized (B) PSD-95 data; normalized (C) and TUB-normalized (D) GEP data; normalized (E) and TUB-normalized (F) SYP data. Tel: telencephalon; TeO: optic tectum; Ce: cerebellum/medulla spinalis; MA: middle-aged. Error bars show +SE [74] (adapted from Karoglu et al., 2017, Reprinted with permission from Elsevier)

3.3.6. Alterations in the House-keeping Protein: β- Tubulin

Tubulin (TUB) was used as a housekeeping protein whose expression supposed not to change between different tissues, age and gender groups. TUB expression levels were analyzed in all the experiments to ensure that no loading, pipetting or transfer problems in the Western blot experiment. Overall, TUB showed a stable pattern and no significant difference was found ($F_{(5,30)} = 1.616$, $p = 0.186$). Additionally, TUB values were used for the data series of "TUB-normalized" data. Procedure was followed the protocol described in Chapter 2.6. TUB values for each data set are shown with the sample size and the standard errors at the Table 3.3.

Table 3.3. Mean values of TUB for each group[74]

Group	N	Mean	Std. error
Female-young	6	0.96	0.029
Male-young	6	1.025	0.018
Female-middle-aged	6	0.946	0.036
Male-middle-aged	6	1.051	0.039
Female-old	6	1.02	0.036
Male-old	6	0.988	0.029
Female-very old	4	1.048	0.025
Tel-female-young	3	1.163	0.073
Tel-male-young	3	0.924	0.024
Tel-female-old	3	0.888	0.14
Tel-male-old	3	0.972	0.04
TeO-female-young	3	0.97	0.099
TeO-male-young	3	0.849	0.08
TeO-female-old	3	0.996	0.046
TeO-male-old	3	0.975	0.091
Ce-female-young	3	1.016	0.065
Ce-male-young	3	0.962	0.043
Ce-female-old	3	1.19	0.087
Ce-male-old	3	1.094	0.058

(adapted from Karoglu et al., 2017,
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3.4. Discussion

The present study was designated to reveal the effects of age and gender on the PSD-95, SYP and GEP expression levels. In terms of the expression levels of the PSD-95, which is considered as an indicator of excitatory post-synaptic integrity, a significant gender by age interaction was revealed in the both normalized and TUB-normalized data sets, and the general pattern indicated that the older ages females tend to have higher levels of the PSD-95 compared to the male groups whose PSD-95 expression tend to be more stable through aging. In the previous studies using the rat male subjects indicated that, PSD-95 levels were significantly reduced in the old cognitively impaired groups in regions including hippocampus, specific laminar layers of frontal and parietal cortices compared to the old behaviorally intact and young groups [37], [47]. In that sense, in the present study significant main effect of age was not observed as those studies; yet old group in the current study was more heterogeneous which includes both cognitively intact and impaired fish possibly. Therefore, if the old fish can be characterized according to their behavioral and cognitive phenotype, similar results in the rat models might be replicated. Another difference was that in the mentioned rat studies, specific regions and even specific laminar layers were investigated in terms of age related expression profile of the PSD-95. To focus region specific alterations, micro-dissection was performed into the subset of animals indicated in Table 3.2, and no significant pattern of age or gender was revealed on this data; because the micro-dissected regions were smaller, to recover the sufficient concentration of proteins pooling procedure was applied. However, the downfall of the pooling might be the blurring the effect of the individual variances, that could be the reason why differences were not significant.

Although, no significant difference was observed in PSD-95 expression on the groups of the micro-dissected regions, a general trend in the whole brain data was preserved in the TUB-normalized PSD-95 values in the Tel region. It was interesting because Tel constitutes 10% of the total brain weight, yet studies indicated that Tel showed some homologies and functional similarities to mammalian hippocampus, amygdala and cortex [63], [64].Therefore, it could be considered that alterations in the whole brain expression levels of PSD-95 might be driven by the Tel.

GEP was considered as an indicator of an inhibitory post-synaptic integrity, in the current study no significant main effect or interactions were revealed with respect to age and gender groups in both whole brain expression and region-specific expression of the GEP. However, expression alterations in GEP might have contributions to the excitatory and inhibitory balance in the context of changes in PSD-95. Therefore, this balance might be altered through the course of aging as indicated by the previous studies [47], [48].

In terms of SYP, which was considered as an indicator of the pre-synaptic integrity and general synaptic integrity, a significant age by gender interaction was revealed in the whole brain expression levels. Results demonstrated that, whereas, females tend to have stable levels of the SYP throughout the aging, the expression of the SYP in the males tends to be reduced. Previous studies conducted with a mouse model reported significant reduction of the SYP levels in the hippocampus of the male mice [52], [51]. In the TUB-normalized data, SYP levels were significantly reduced in the old males compared to the middle-aged males, this pattern might be considered as consistent with the previous studies in the literature. In terms of region

specific SYP expression levels normalized SYP expression in the Tel showed resemblance to the whole brain expression data.

Overall data suggested that age and gender specific alterations occurred in the elements of pre-synaptic integrity and the excitatory components of post-synaptic integrity whereas element of inhibitory post-synaptic integrity showed stable pattern. Current study is valuable due to analyzing the effects of gender on the aging domain because in the most of the mammalian studies gender differences were underestimated and key protein expression levels were determined only in the male groups. Additional studies like immunohistochemistry are required to investigate region-specific alterations in more specific manner such as analyzing the expression profile in the specific portions of Tel and different laminar portions.

CHAPTER 4

Expression of Postsynaptic Density-95, Gephyrin and Synaptophysin in Old Wild-type and Delayed Aging Mutant Zebrafish Models

4.1. Introduction

It has been indicated that age-related cognitive decline occurs in some cognitive abilities without presence of neurodegeneration or age-related pathology. In the previous Chapters evidence suggests that subtle molecular alterations in the synapses and cellular dynamics might have an explanatory role in cognitive decline. Although, there are no gross age-related structural alterations, age-related hypofunction in some neurotransmitter systems were reported. The cholinergic system is one of the susceptible systems to age-related changes and hypofunction was prominent in this system at older ages [10]. Maintenance and the effects of cholinergic system on the aging process are so crucial because especially basal-forebrain cholinergic system has extensive innervations with hippocampal formation, which has significant contributions to the learning and memory processes. In this Chapter the effects of cholinergic system on the aging and possible interventions which manipulate the cholinergic transmission were focused.

4.1.1 Cholinergic System

4.1.1.1 Structure of the Acetylcholine and its Receptors

Acetylcholine (ACh) is the neurotransmitter of the cholinergic system and it is circulated in both peripheral and central nervous systems. It acts as a neuromodulator, and according to the context it can lead to excitation or inhibition.

ACh consists of lipid-derived choline and acetate. For the synthesis of the ACh, choline and acetate cannot be directly linked to each other, for the attachment of these two elements enzymes coenzyme A (CoA) synthesized in the mitochondria and choline acetyltransferase (ChAT) are required. Acetate is transferred via CoA, and the complex of acetate bound to the CoA is named as acetyl-CoA. ChAT breaks down this complex; takes acetate and transfers it to the choline. At the end of these processes choline and acetate attached together which constitute the ACh [76].

There are two types of ACh receptors. The first of these are nicotinic acetylcholine receptors (nAChRs) which are the ionotropic type of receptors. They form ion channels permeable to Ca^{2+}, Na^{+} and K^{+}. The two major subunits of the nAChRs are an alpha and a beta subunits. Differential participation of these two subunits for the formation of nAChRs results in diverse functions of the nAChRs. The second is muscarinic acetylcholine receptors (mAChRs), which are the metabotropic type of receptors. mAChRs consist of five subunits which are m1-m5; they control the ion channels and modify the neural activity through intracellular signaling and alterations in the Ca^{2+} homeostasis. Because of their metabotropic nature, mAChRs have slower impacts on the neural activity and response compared to the ionotropic nAChRs, yet they exert more prolonged effects [76]. After ACh was released to the synaptic cleft it is removed and recycled by enzymatic

deactivation. There are few cholinesterases playing role in the degradation of the ACh like butyrylcholinesterase (BuChe) yet the main enzyme is the acetylcholinesterase (AChE). AChE breaks the ACh down into the two main components; choline and acetate. After this enzymatic degradation choline is transported back in the cell through choline transporters [77].

4.1.1.2 Cholinergic System and its Pathway

Although, cholinergic neurons and projections are widely distributed throughout many brain regions, several cholinergic neuron clusters have more prominent projections. The first cluster is the cholinergic system of the basal forebrain; in terms of morphology they are large neurons. Their projections start from septum of the brain and extend to the nucleus basalis of the Meynert along the Broca's diagonal band. Besides these extensions, projections from the cholinergic neurons in the medial septum reach to the subcortical regions including amygdala and hippocampus; which are crucial areas for the cognitive processes including learning, memory formation-modulation and emotions. This interaction emphasize the importance of the cholinergic input and cross-talks between the cholinergic input and cognitive processes which are vulnerable to the ag-related alterations [10]. The second cholinergic cluster is the cholinergic system of pedunculopontine nuclei; cholinergic projections start from the laterodorsal tegmental portion of these nuclei and extend through the thalamic regions [78].

4.1.2 Cholinergic System and Normal Aging

Cholinergic system is vulnerable against aging-related alterations, age-related impairments in the cholinergic neurons of the basal forebrain have been reported, but

this deterioration was not limited with only this cholinergic population, impairments in the cholinergic neurons of the striatum and lateral dorsal tegmental nuclei have also been observed [10]. Thus, impairments in the cholinergic neurons of other regions as well as the cholinergic system of the basal forebrain may contribute to age-related alterations in the brain and cognitive decline.

Studies in which young, old impaired and old intact rat groups were utilized; poor cognitive performance was associated with the loss and the reduction of the choline acetyltransferase (ChAT), playing role in the synthesis in the acetylcholine (ACh), positive neurons. In the old impaired groups, fewer numbers and greater atrophy in ChAT positive neurons were observed, whereas, in old intact groups state and the numbers of the ChAT positive neurons were similar to the young control groups [78].

However, these studies were correlational and more causal evidences are required to create more robust relationships between the age-related alterations in the cognitive and molecular dynamics and the integrity of the cholinergic system. In this context, it has been shown that blocking the muscarinic acetylcholine receptors (mAChRs) temporarily in young groups came with impairments in learning and memory which showed resemblance to the cognitive decline occurring in aging [79]. While functional impairments of the cholinergic activity may play an important role in age-related cognitive impairments, alleviating the cholinergic dysfunction may attenuate the severity of the age-related cognitive decline. It has been demonstrated that administration of the oxotremorine which is a mAChR agonist, attenuated the age related cognitive impairments in spatial learning and memory as well as increased the slope of hippocampal excitatory post-synaptic potentials in the old rats

whose potentiation was jeopardized during aging [80]. Taken together this evidence suggests that the integrity and the function of the cholinergic system change during the aging, but interventions aimed at the activity of the cholinergic system might cause the effects on the age-related cognitive decline to be ameliorated.

4.1.3 Cholinergic System and Pathological Aging

In addition to normal aging, cholinergic system is one of the neurotransmission systems selectively impaired from pathological conditions that induce neurodegeneration. A significant reduction in choline acetyltransferase (ChAT) activity in the hippocampus and cortex was reported in Alzheimer's disease (AD). In particular, selective cholinergic loss in the basal forebrain especially in the nucleus basalis of Meynert took place throughout the progression of AD. Because of the prominent cholinergic susceptibility in AD; treatments targeting the dysfunction in the cholinergic system attenuated the progression of the AD. Usage of the inhibitors of cholinesterases which break ACh down and remove the ACh from the synaptic cleft were one of the commonly-used interventions for the AD to increase cholinergic activity [70].

Parkinson's disease (PD) is another progressive neurodegenerative disease affecting the integrity of the cholinergic system. Imbalance hypothesis indicated that the balance between dopamine and ACh is impaired in PD. In this context, studies have indicated that low dopamine levels in PD occur with increased ACh levels; whereas, in the healthy adults activation of the dopaminergic neurotransmission inhibits the ACh release. Therefore, abnormally increased activation of the ACh during the onset of the PD, disrupted the inputs from dopaminergic circuitry to the motor areas [81].

Finally in terms of frontotemporal dementia (FD), it has been shown that treatments with the cholinesterase inhibitors aggravated the symptoms of the FTD in the patients, in this respect it was proposed that FTD occurs with the dysregulations in the serotonergic and dopaminergic circuitries whereas the cholinergic system remained intact [82]. Overall, these data suggest that cholinergic system and neurotransmission have selective vulnerabilities to the normal aging and the nature of the neurodegeneration.

4.1.4 Cholinergic System and Synaptic Alterations

Acetylcholine is a neuromodulatory neurotransmitter; it exerts its effects through modulating the both excitatory and inhibitory elements of neurotransmission. Therefore, age related alterations in the functionality and integrity of the cholinergic system would result in the disturbance in the both inhibitory and excitatory neurotransmission.

For the excitatory neurotransmission, there are robust interactions between cholinergic system and the glutamatergic receptors. Both behavioral and electrophysiological studies have shown that NMDA receptors are required for the cholinergic receptor agonists to show their beneficial effects; when NMDA receptors were blocked ameliorative effects of the cholinergic receptor agonists diminished [83], [84]. Additionally, this close relationship is not only limited with the NMDA receptor, but also it has been indicated that kainite and AMPA receptors are needed for the ACh release in the hippocampus [85]. Apart from excitatory neurotransmission elements; ACh interacts with the inhibitory GABAergic neurotransmission. Studies utilizing the optogenetics method revealed that neurotranmitters of ACh and GABA co-released from the neurons located in the

basal forebrain cholinergic system and those neurotransmitters regulated each other's responses [86]. Taken together these evidences cholinergic system interacts with the glutamatergic and GABAergic systems which are the main excitatory and inhibitory sources of the neurotransmission respectively. Therefore, age-related alterations in the cholinergic system might result in the disturbances of the dynamics of glutamatergic and GABAergic neurotransmission.

4.1.5 Cholinergic System and Sexual Dimorphism

It has been shown that age related alterations affect males and females differentially, age-related sexually dimorphic patterns were documented in gene expression profile, brain activity and connectivity patterns and expression of the key synaptic proteins as indicated in the Chapter 1.4. Such dimorphism can also be encountered in the context of the age-related alterations in the cholinergic system. It was reported that estradiol treatment resulted in the increase in the levels of the degradative enzymes acetylcholinesterase (AChE) and choline acetylase (CAT) in diagonal band of Broca of the gonadectomized females; whereas in gonadectomized males CAT activity showed a decreasing pattern in response to estradiol treatment [87].. It has also been supported that estradiol protected the cognitive processes vulnerable to aging such as memory, attention and learning along with the cholinergic function.[88]. Overall, it is suggested that activity of the cholinergic system might be affected by the hormonal state and the circulating hormones in the context of the age related changes.

4.1.6. Zebrafish Mutant Line with Manipulated Cholinergic System Activity

To investigate causative relationships between the cholinergic system and age-related alterations, a mutant line in which there is a single nucleotide in *ACHE* protein coding gene was used. This single nucleotide mismatch results in loss of function of acetylcholinesterase (AChE); and it was reported that AChE activity is completely abolished in the homozygous mutants and their results indicated that this enzyme is crucial for the development and maintenance of primary sensory neurons and muscles [89]. Although, a homozygous mutation is lethal in these mutants, heterozygous mutants have intact development of the axial muscles and sensory neurons, and it was shown that in the heterozygous mutants, AChE activity is significantly reduced; and because the role of this enzyme was degrading the acetylcholine (ACh) in the synaptic cleft to the choline and acetate, a significant 1.5 fold increase in the brain levels of ACh was reported in the heterozygous mutants [90].

The cholinergic hypothesis of aging emphasized the hypofunction in the cholinergic system; and theoretically because the heterozygous mutants have significantly increased levels of ACh in the brain, this hypofunction can be ameliorated by the increased activity of the cholinergic system. Behavioral studies conducted with young and old heterozygous mutant (ache) fish and wild-type controls indicated that the learning curve, which is based on anticipatory increase in the motor activities, was better in young wildtype fish compared to the old wildtype after 7 days of adaptation and the performances of old ache mutants were similar to the young groups, which are significantly different from the performance of the old wildtype group. Additionally, both ache young and old fish showed superior

performance in the extinction phase of the conditioned place preference paradigm, which could show that ache fish can learn and re-do this process faster [67]. Results of this study indicates that ache mutants can serve as a delayed aging model because of their altered and more active cholinergic system and superior behavioral performance.

The aim of this study in this thesis was to reveal the difference among the expression levels of the key synaptic protein; PSD-95, GEP and SYP, which were mentioned in the previous Chapter, between the wild-type old group and ache old group, which was considered as a delayed aging model. Many studies have indicated that the activity and the function of the cholinergic system can be influenced by circulating sex hormones, and age-related alterations could occur in a sexually dimorphic way so gender differences interacting with the genotype may exert their effects on the protein expression profile.

4.2. Materials and Methods

4.2.1. Subjects

All animals were maintained at standard conditions as mentioned in the Chapter 2.1. For this part of the study an *ache* ^{sb55}/+ (ache) line was used apart from the wild-type zebrafish. In this line there is a loss of function mutation in the gene which encodes acetylcholinesterase. There is a single nucleotide mismatch (Ser>Asn at position 226) [89]. This mutation is lethal in the homozygous mutants *ache*^{sb55}/ *ache* ^{sb55}, so heterozygous mutants were used. The transgenic line was obtained from Karlsruhe Institute of Technology (KIT). To maintain the heterozygous mutants, systematic sets of breeding setups were prepared. After the collection of the embryos, parents were held in the smaller tanks for 3 days, which were cleaned daily.

At 72 hours post-fertilization (hpf), homozygous embryos are completely paralyzed, so a tail test was applied. The tail-test is based on a touch-evoked response, the tail of the embryo was touched gently by a fine surgical tweezers, and normally they are mobile and active at 72 hpf as they swim away after this stimulation. Because homozygous mutants are completely paralyzed at this time point they did not swim away or move after the touch-evoked stimulation, to be sure that they are not dead but paralyzed with the microscope their heart beats were detected. In the cases where paralyzed embryos were observed in a ratio 1/4, parents were identified as heterozygous mutants, and from this cross 1/2 of the embryos were heterozygous ache and 1/4 of the embryos were the wild-type siblings. For the continuation of the heterozygous line, from the breeding crosses in which 1/4 ratio of immobile paralyzed embryos were observed remaining heterozygous ache (2/3) and wild-type siblings (1/3) were raised as a new generation, to distinguish ache and their wildtype siblings, systemic breeding setups were prepared as indicated above and then after the euthanasia tail samples were collected and stored for further genomic DNA analysis.

A total 12 zebrafish was used for this part of the study. Since ache has been proposed as a delayed aging model [67], the most distinguishing differences were expected at the old ages and so those ages were compared. Therefore all the groups were selected from the old age group (30 months old), the distribution of the gender and the genotype groups were indicated in Table 4.1.

Table 4.1. Distribution of the gender and the genotype groups

	Female	Male
Wild-type	3	3
Ache	3	3

For the euthanasia ice-water was used. Ice was put into the small plexiglass holding tank, and system water was added onto the ice, and the temperature of the ice-system water was between 4-7°C. Fish were put into the holding tank and held in there until gill movements stopped, which took approximately 10-15 minutes. The animal protocol of this study was approved by the Bilkent University Local Animal Ethics Committee (HADYEK) with approval date: Feb 9, 2010 and no: 2010/1 and updated on Jul 15, 2016 and no:2016/22.

4.2.2. Dissections

Dissections of the whole brain and the other tissues, and the gender determination were performed as indicated in Chapter 2.2. Additionally, after the decapitation of the head and the extraction of the whole brain, tails were separated from the body with surgical scissors for the further genomic DNA extraction to confirm the genotype of the heterozygous mutants.

4.2.3. Protein Isolation

Protein isolation procedure for the whole brain sets followed the same protocol as indicated in the Chapter 2.3.

4.2.4. Western Blot

Experiments followed the procedure indicated in Chapter 2.5. In each gel representative samples of the each group were included to have reliable comparisons between the age and the genotype groups, blot cohorts were indicated in Figure 4.1. Anti-PSD-95, anti-SYP and anti-TUB primary antibodies were blotted simultaneously; for the anti-GEP detection after the transfer membrane was cut from the 70 kDa band, upper membrane was incubated with primary antibody anti-GEP, which gives band at approximately 93 kDa and the lower portion of the band was blotted with anti-ACTIN, which gives band at 42 kDa. For each sample, experiments were performed minimally in triplicates and for each sample approximately 3 measurements were taken. Therefore, biological and the technical replicates ensure that the alterations of the protein expression pattern are likely due to the true biological alterations based on genotype and the gender differences. Blots were visualized and developed with ChemiDoc™ XRS+ imaging system (Biorad, CA, USA) and the images were acquired with the software system of the machine, ImageLab (Biorad, CA, USA). Image quantification was performed by Melek Umay Tüz-Şaşik and she was blind to the gender and the genotype groups. The detailed procedure is mentioned in the Chapter 2.6.

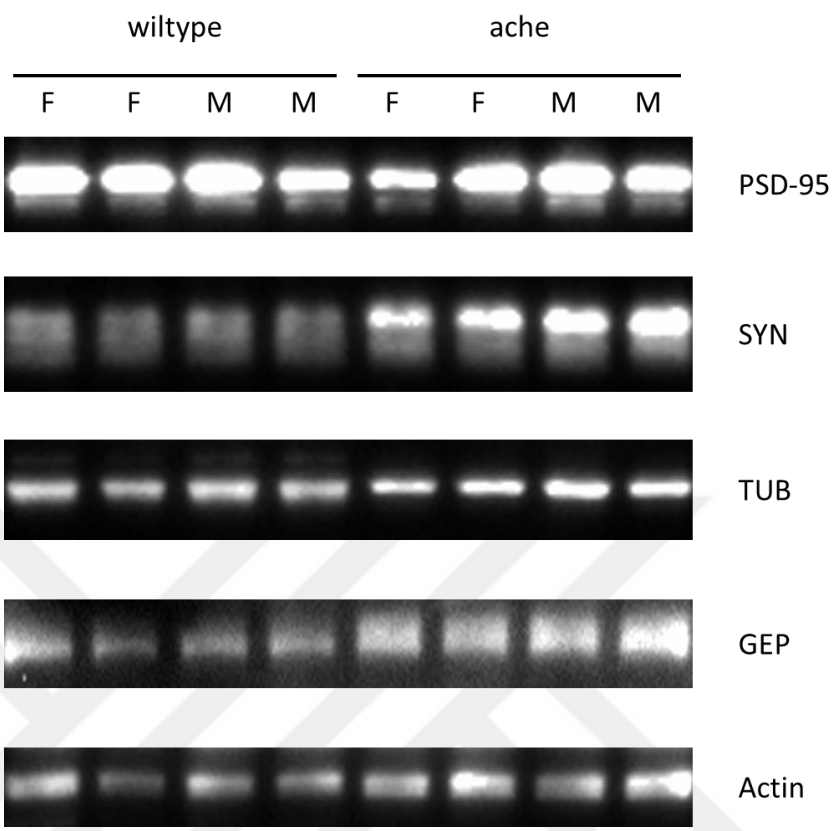

Figure 4.1. Representation of the experimental cohorts in the blots

4.2.5. DNA Extraction from Tail

To genotype heterozygous ache mutants and their wildtype siblings, after euthanasia of zebrafish by immersion in ice-water, tails were separated from the body with scissors and then snap-freezed with the liquid nitrogen. Tail samples were stored at -80°C in 1.5 ml tubes until DNA extraction. Tails were put into the 200 µl of DNA extraction buffer; 100-mM Tris, pH 8.2 containing 10-mM EDTA, 200-mM NaCl and 0.5% SDS, just before putting the tail into the extraction buffer 2mg/ml proteinase K was added to the solution(2 µl from 100X stock solution). Tail samples

were incubated at 55°C with rocking overnight. On the next day samples were incubated at 95°C for 20 minutes to inactivate proteinase K. After samples were cooled down, they were centrifuged at 13,000 rpm for 20 minutes at 4°C. Supernatants were transferred into the new Eppendorf tube, 175 µl of 2-propanol was added to the supernatants and mixed by pipetting through. 2-propanol and supernatant mixture was centrifuged at 13,000 rpm for 20 minutes at 4°C. After centrifugation, supernatants were discarded and to wash the pellet 500 µl of 70% EtOH was added. The pellet and 70% EtOH were centrifuged at 13,000 rpm for 20 minutes at 4°C. Supernatants were discarded, and pellets were air-dried. Pellets were resuspended in 20 µl of nuclease free water and DNA concentrations were measured with NanoDrop 2000. DNA samples were stored at -20°C.

4.2.6. Touch-down Polymerase Chain Reaction

In the ache mutants with single nucleotide mismatch, Ser226 is replaced with Asn226 (Figure 4.2.). To detect this small mismatch replacement, 3 primers were designed (Table 4.2); one forward primer detecting the wildtype Ser226 (S), one forward primer detecting the mutant Asn226(N), and one reverse primer (R). Sequences of the primers are shown in the Table 4.2. For each sample 2 reactions were prepared as indicated in the Table 4.3; in the wild-type samples a band was expected from the reaction prepared with S forward primer and no band was expected from the reaction prepared with the N forward primer. In the ache heterozygous samples, bands were expected from the both reactions prepared with N and S primers.

```
781 AGACAGCCGCCCCCTCTTCACACGTGCCATATTGCAGAGTGGTGTTCCAAATACACCCTG
                        >>>>>>>>>>>>>>>>>>>>(S)
                        ACACGTGCCATATTGCAGAA
                        >>>>>>>>>>>>>>>>>>>>(N)

841 GGCCACTGTTACCTTTGATGAGGCCCGTCGGAGAACCACCAAGTTGGGCAAACTGGTTGG

901 TTGCACCTGGGGCAATGATACTGAACTGATTGATTGTCTGAGGAATAAACATCCACAGGA

961 ACTAATTGATCAAGAGTGGCAAGTTCTTCCCTGGAGCAGCCTTTTCCGCTTTTCCTTTGT
                        <<<<<<<<<<<<<<<<<<<<(R)
```

Figure 4.2. Representation of the mutation and the primers on the partial sequence

Table 4.2. Sequences of the primers

N	ACA CGT GCC ATA TTG CAG AA	Forward
S	ACA CGT GCC ATA TTG CAG AG	Forward
R	CTG CTC CAG GGA AGA ACT TG	Reverse

Table 4.3. Prepared PCR reactions for each sample

	Forward	Reverse	Wild-type	sb55/+	PCR product (bp)
Reaction-1	N	R	-	+	200
Reaction-2	S	R	+	+	200

After DNA was extracted from the tail and concentrations were measured, 100 ng/µl concentrations were calculated for each sample. Reactions were prepared for each sample as indicated in Table 4.4. For each sample two reactions were prepared with N and S forward primers, and because the mutation is so small based on one nucleotide mismatch, Touch-down PCR protocol was adapted from Korbie& Mattick (2008) [91] as this protocol increases the specificity.

Table 4.4. PCR mix for each sample

Materials	Amount (ul)
10-uM Forward primer	0.25
10-uM Reverse primer	0.25
10-mM dNTP	0.5
10x Taq buffer	2.5
Taq polymerase	0.25
25-mM MgCl	1.5
DNA and nuclease free water	19.75

Table 4.5. Touch-down PCR phases

		Temperature	Duration	
Phase 1	Denature	95°C	3 mins	
	Denature	95°C	30 seconds	10 cycles
	Anneal	65°C *	45 seconds	
	Elongate	72°C	60 seconds	
Phase 2	Denature	95°C	30 seconds	
	Anneal	55°C	45 seconds	
	Elongate	72°C	60 seconds	
Termination	Elongate	72°C	5 minutes	
	Halt Reaction	4°C	15 minutes	
	Hold	23°C	Forever	

* In each cycle the temperature was reduced 1°C so that the final temperature of this cycle was 55°C

After the Touch-down PCR, 4 µl of 6X loading dye (B7021S, New England Biolabs, Kocaeli, Turkey) was added into the 25 µl of PCR products and mixed by pipetting. Throughout this mix 12 µl was loaded in to the agarose gel. The recipe is indicated in Table 4.7. The recipe for the 1X TAE, used as the running buffer, is shown in Table 4.6. In all cases the gel was run for 20 mins at 100V and then visualized.

Table 4.6. Recipe of 50X TAE buffer

Materials	Amount
2-M Tris	242 g
50-mM EDTA	18.61 g
Acetic acid	57.1 ml
ddH_2O	Up to 1 L

Table 4.7. Recipe of 1% Agarose gel

Materials	Amount
Agarose	1 g
1XTAE	100 ml
Ethidium Bromide Solution (10 µg/ml)	2 µl

4.2.7. Statistical Analysis

The first step was the checking the assumptions of normality with Kolmogorov-Smirnov and Levene tests in terms of normal distribution and homogeneity of variance. After those assumptions were fulfilled, a two-way

ANOVA with the factors of gender with two levels (female and male) and genotype with two levels (wild-type and ache) was carried out on the expression levels of key synaptic proteins. Significance level was set to $p<.05$. A Bonferroni correction, which has more stringent cutoff values, was used as a post-hoc test. In the cases of the significant interactions, simple effects analysis, which break down the levels of the one independent variable and compare its effects on the levels of the other independent variable, followed the two-way ANOVA. For the validation of ANOVA results and to perform principle component analysis a multivariate analysis of variance (MANOVA) with the factors, genotype and gender was performed on the expression levels of the three synaptic proteins

Although, the sample size was limited as compared to the previous Chapter, in order to see the dominant patterns in the variation of the PSD-95, SYP and GEP expressions, a principal component analysis (PCA) was applied. Components were extracted, if the Eigen values were greater than 0.5. In the cases in which component loadings were greater than 0.5 ($r >0.5$), correlations were considered as significant.

4.3. Results

4.3.1. Postsynaptic Density-95

Postsynaptic density-95 (PSD-95) was considered as an indicator of excitatory neurotransmission and post-synaptic integrity. Old groups were analyzed based on their gender and genotype designations. No significant main effect or interaction was observed after a two-way ANOVA was applied to the data set on both normalized ($F(3,8)=0.211$, $p=0.886$, Figure 4.3.A) and TUB-normalized ($F(3,8)=1.068$, $p=0.415$, Figure 4.3.B) PSD-95 values.

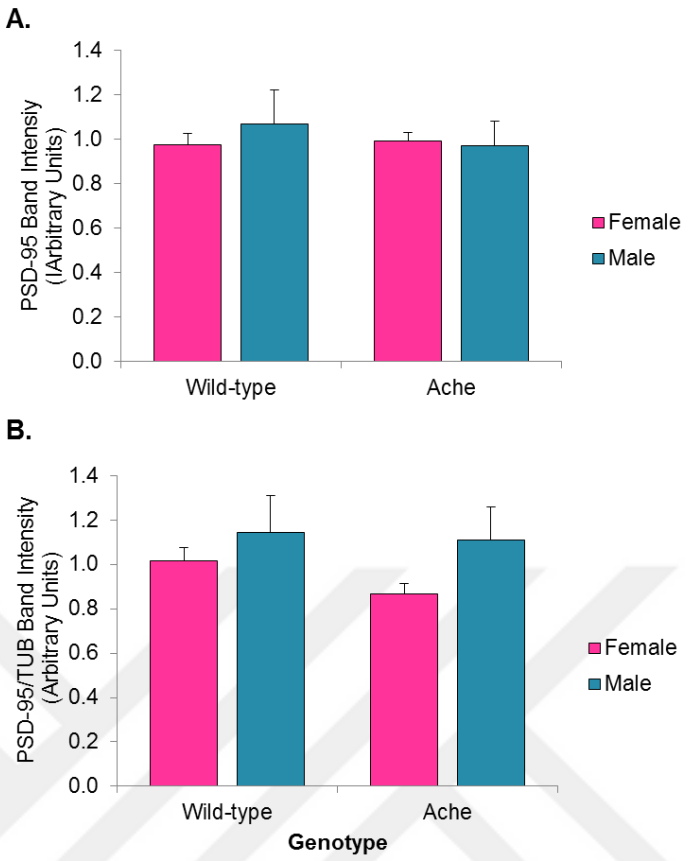

Figure 4.3. PSD-95 expression across gender and genotype groups; normalized data (A), TUB-normalized data (B). Error bars show +SE,

4.3.3. Synaptophysin

Synaptophysin (SYP) was used as a marker of pre-synaptic integrity, and studies have indicated that increased SYP levels were correlated with a better cognitive performance [51]. Significant main effects of genotype was revealed in the normalized data ($F(1,8)=38.419$, $p<0.0005$, Figure 4.4.A),with the ache group having significantly higher levels of the SYP compared to the wild-type group. Bonferroni

correction indicated significant differences between wild-type-female and ache-female ($p<0.0005$), wild-type-male and ache-female ($p=0.004$), ache-male and ache-female ($p=0.007$) groups. Moreover, a significant gender by genotype interaction was found in the normalized data ($F(1,8)=29.401$, $p=0.001$, Figure 4.4.A) and to break this interaction into the components simple effects analyses were carried out. Simple effects analyses showed that genotype has significant effects in the female group ($p<0.0005$)

In the TUB-normalized data in terms of the main effect of the genotype, a distinct trend towards significance was found ($F(1,8)=4.250$, $p=0.073$, Figure 4.4.B). However, gender by genotype interaction was not significant in the TUB-normalized data in the expression levels of SYP ($F(1,8)=2.811$, $p=0.132$, Figure 4.4.B). However, eventhough there was a trend it is not likely to represent a true statistically-significant difference,

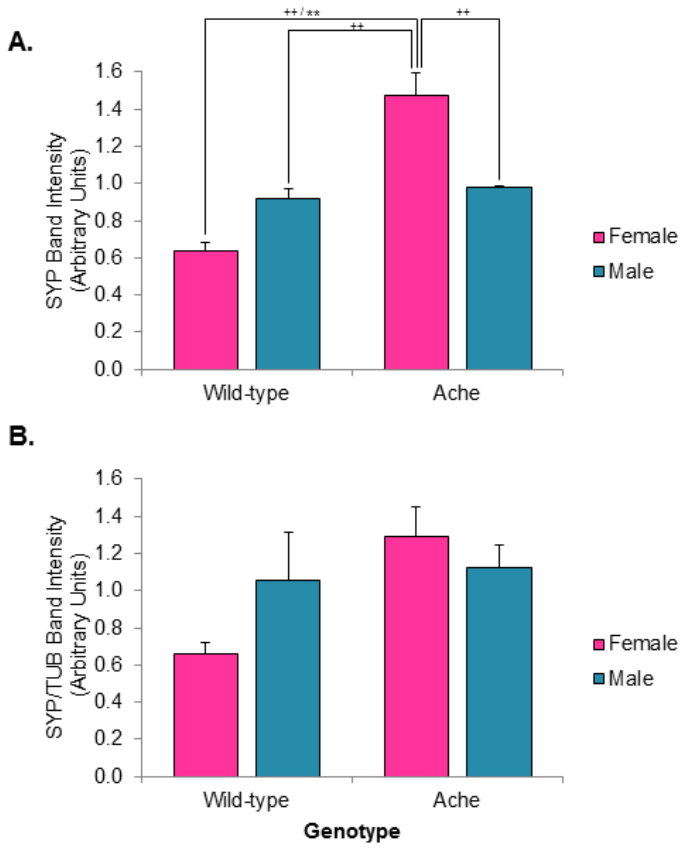

Figure 4.4. SYP expression across gender and genotype groups; normalized data (A), TUB-normalized data (B). Error bars show +SE, *gender within genotype, $^{+}$significant difference in Bonferroni. Two symbols p<0.01.

4.3.3. Gephyrin

Gephyrin (GEP) which is the major scaffolding protein, has roles in the clustering of inhibitory $GABA_A$ receptors and is considered as an component of inhibitory neurotransmission and inhibitory post-synaptic integrity. In the normalized data significant main effect of genotype was revealed ($F(1,8)=13.890$, $p=0.006$, Figure 4.5.A). It was shown that, in old ages, expression of GEP was significantly increased in the ache (both gender groups) compared to the wild-type controls.

However, ACTIN-normalized data did not show the similar pattern in terms of GEP expression and no significant main effect or interaction was found ($F(3,8)= 0.948$, $p=0.462$, Figure 4.5.B). For that reason to eliminate the probability of an experimental error, housekeeping protein expressions were analyzed.

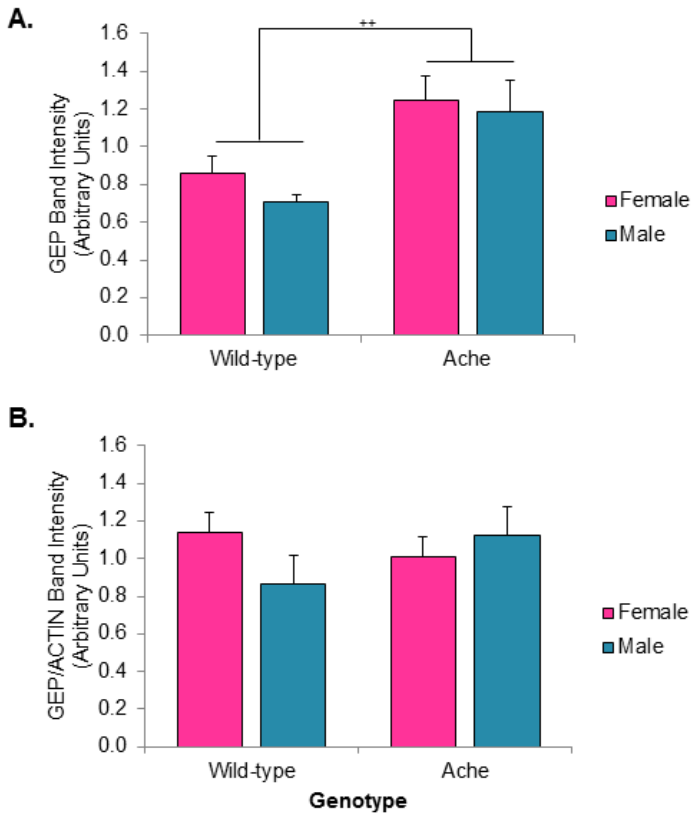

Figure 4.5. GEP expression across gender and genotype groups; normalized data (A), TUB-normalized data (B). Error bars show +SE; $^{+}$significant main effect. Two symbols: $p<0.01$.

4.3.4. Analysis of the House Keeping Proteins

For the sets of PSD-95 and SYP blots TUB was used as a housekeeping protein, and the general expression pattern was similar in the TUB-normalized data compared to the normalized data. TUB levels of the groups were shown in the table with the standard errors and no significant main effect or interaction was found ($F(3,8) = 0.573$, $p=0.649$). In the GEP sets, bands of interests were overlapping with TUB whose molecular weight was 55 kDa, therefore ACTIN whose molecular weight was 42 kDa was used instead of TUB. Although, the concentration measurements were based on the same Bradford assay and the samples were the same, in terms of expression levels of ACTIN significant main effect of genotype was found ($F(1,8)=7.969$, $p=0.024$) and in the ache group ACTIN levels were significantly higher than the wildtype group. Whereas, there is a stable TUB expression, increased expression of the ACTIN in the mutant ache group might indicate that mutation could alter the cytoskeletal elements and increased the levels of the ACTIN which was considered a more plastic compared to the TUB [92]. To support this idea, with the remaining samples one last Western blot experiment was carried out and the membrane was incubated for GEP, TUB and ACTIN. Normalized GEP data (Figure 4.6.A), TUB-normalized GEP data (Figure 4.6.B) and ACTIN-normalized data were shown (Figure 4.6.C). Whereas, TUB-normalized GEP data showed resemblance to the normalized GEP data in which GEP has increased levels of expression in the ache group, ACTIN-normalized data indicated an opposite trend in which GEP has decreased levels of expression in the ache group. Therefore, it could be considered that because of the more plastic nature, ACTIN levels may show

instability due to the mutation, though TUB has more stable expression pattern which would be a better housekeeping control compared to the ACTIN.

Table 4.8. Mean values of TUB and ACTIN for each group

Group	N	TUB	Std. Error	ACTIN	Std. Error
female-wildtype	3	0.972	0.103	0.781	0.158
male-wildtype	3	0.990	0.250	0.856	0.096
female-ache	3	1.149	0.052	1.236	0.026
male-ache	3	0.889	0.084	1.072	0.154

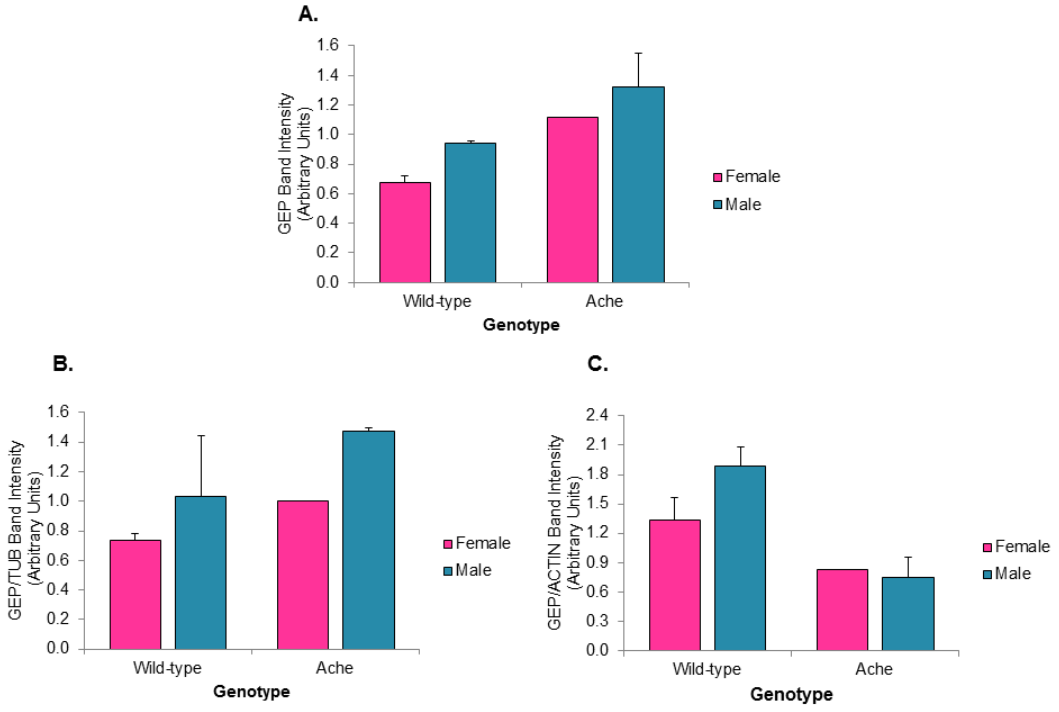

Figure 4.6. Analysis of the different housekeeping controls, on the one set of a blot. Normalized GEP values (A), TUB-normalized GEP values (B) and ACTIN-normalized GEP values (C). Error bars show +SE.

4.3.5. Principle Component Analysis and Investigating Clustering Profiles

The purpose of this analysis was same as previous Chapter, which was to reduce the dimensions of the data, retain the variation through the clustering profile of the samples, and detect the dominant patterns in the expression profile. Although the sample size is lower than in the previous Chapter, dominant patterns in the expression profile could be promising to analyze. Firstly, a significant gender by genotype interaction and a significant main effect of the genotype which were found

with the separate two-way ANOVAs were confirmed with the MANOVA. MANOVA demonstrated a significant gender by genotype interaction on PSD95, GEP and SYP levels as a whole, $F_{(3,6)} = 9.117$, $p = 0.012$; Wilk's $\Lambda = 0.180$. Additionally this analysis revealed a significant main effect of the genotype on the expression levels of PSD95, GEP and SYP as a whole, $F_{(3,6)} = 12.025$, $p = 0.006$; Wilk's $\Lambda = 0.143$. After this validation with MANOVA, principle component analysis was carried out. A significant correlation between SYP and GEP was revealed (r=0.56, p=0.029, Figure 4.7.A). Two components were extracted after this analysis, PC1 explained the 52.5% of variation and PC2 explained the 33.8% of variation in the data set, together these two components explained the 86.3% of variation in the current data set (Figure 4.7.B). A component matrix indicated that PC1 was strongly and positively correlated with SYP and GEP, and the PC2 which was orthogonal to the PC1 was strongly and positively correlated with PSD-95 (Figure 4.7.C). Expression levels of the PSD-95, SYP and GEP were plotted against the orthogonal components, PC1 and PC2, to observe the general pattern (Figure 4.7.D). In this plot SYP and GEP were clustered together, which indicated that they vary together, and PSD-95 was in the orthogonal PC2 direction. Regression factor scores were calculated for each sample and with those values each sample were plotted against the PC1 and PC2 (Figure 4.8) to analyze the pattern between genotype groups. Segregation in the genotype groups were distinct in terms of clustering against PC1, whose variation was positively driven by SYP, and GEP in the wild type group samples were clustered in the negative portion of the PC1. This was in contrast to the ache group clustered in the positive portion of the PC1.

Clustering against PC2, whose variation was driven by PSD-95, indicated a similar profile between the wild-type and ache groups.

A.

Correlation Matrix

		PSD95	SYN	GEP
Correlation	PSD95	1,000	-,030	,154
	SYN	-,030	1,000	,561
	GEP	,154	,561	1,000
Sig. (1-tailed)	PSD95		,463	,316
	SYN	,463		,029
	GEP	,316	,029	

B.

Total Variance Explained

Component	Extraction Sums of Squared Loadings			Rotation Sums of Squared Loadings		
	Total	% of Variance	Cumulative %	Total	% of Variance	Cumulative %
1	1,575	52,505	52,505	1,560	51,997	51,997
2	1,015	33,843	86,348	1,031	34,351	86,348

Extraction Method: Principal Component Analysis.

C.

Component Matrix[a]

	Component	
	1	2
PSD95	,194	,971
SYN	,861	-,265
GEP	,892	,045

Extraction Method: Principal Component Analysis.

a. 2 components extracted.

D.

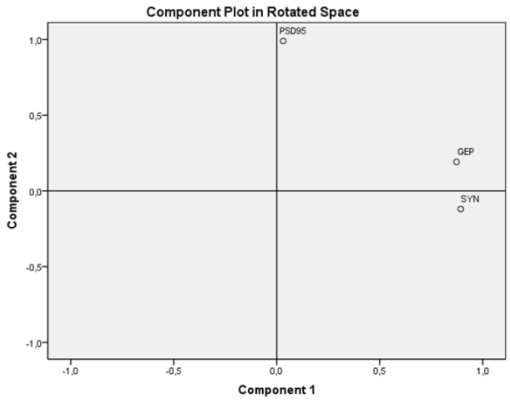

Figure 4.7. Principal component analysis for all groups together. Correlation matrix (A), extracted components with their Eigen values (B), component matrix (C), distribution of the synaptic proteins according to their component loadings (D).

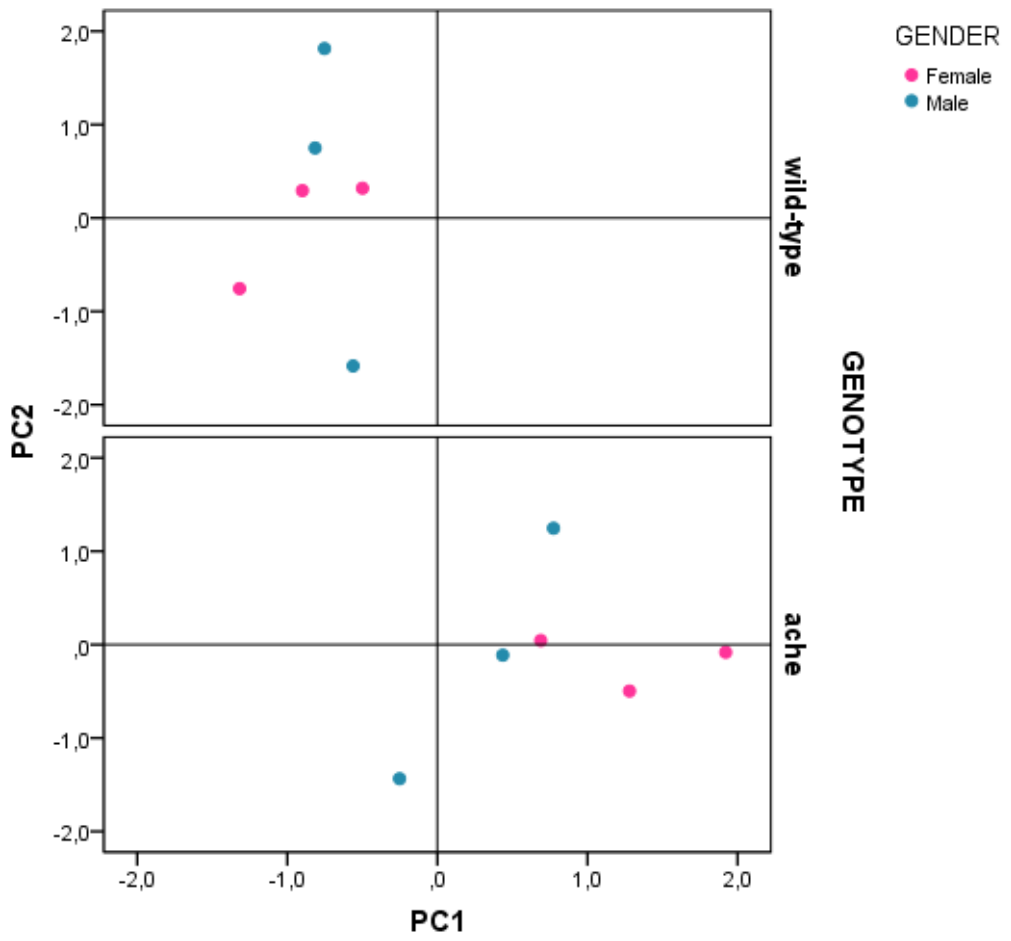

Figure 4.8. Plotting each sample with respect to orthogonal components PC1 and PC2. All groups were included. Gender groups denoted with different colors indicated in the legend. Genotype groups were shown as separate panels

4.3.6 Clustering Profiles of Combined Datasets from Chapter 3 and 4

The purpose of this analysis was to reduce the dimensions of the data, retain the variation through the clustering profile of the samples, and detect the dominant patterns in the expression profile. To have an overall perspective, the dataset from Chapter 3, which consists of alterations in the expression profiles of PSD95, GEP

and SYP of young, middle aged and old; female and male zebrafish, was combined with the dataset of this chapter, which includes expression profiles of PSD95, GEP and SYP in wildtype old and ache old; female and male zebrafish. The purpose of this integration was to analyze the overall clustering profile, and investigate the resemblance between the mentioned groups. Principle component analysis was applied to the combined datasets. A significant correlation was revealed after this analysis; it was found that expressions of GEP and SYP ($r = 0.47$, $p < 0.0005$) and were significantly correlated (Figure 4.9.A). The three components were extracted after this analysis; PC1, PC2 and PC3 which explains the 100% variation in the combined dataset (Figure 4.9.B).

A.

Correlation Matrix[a]

		psd95	syp	Gephyrin
Correlation	psd95	1,000	,226	,171
	syp	,226	1,000	,467
	Gephyrin	,171	,467	1,000
Sig. (1-tailed)	psd95		,063	,125
	syp	,063		,000
	Gephyrin	,125	,000	

a. Determinant = .737

B.

Total Variance Explained

Component	Initial Eigenvalues			Extraction Sums of Squared Loadings		
	Total	% of Variance	Cumulative %	Total	% of Variance	Cumulative %
1	1,599	53,310	53,310	1,599	53,310	53,310
2	,872	29,069	82,378	,872	29,069	82,378
3	,529	17,622	100,000	,529	17,622	100,000

Extraction Method: Principal Component Analysis.

C.

Component Matrix[a]

	Component		
	1	2	3
syp	,823	-,212	-,527
Gephyrin	,795	-,348	,496
psd95	,537	,840	

Extraction Method: Principal Component Analysis.

a. 3 components extracted.

D.

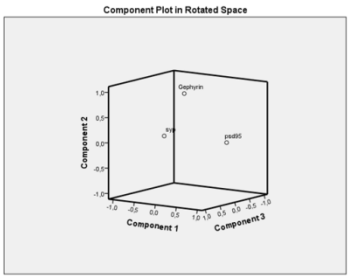

Figure 4.9. Principal component analysis for combined datasets from Chapter 3 and 4. Correlation matrix (A), extracted components with their Eigen values (B), component matrix (C), distribution of the synaptic proteins according to their component loadings (D).

The component matrix indicated that PC1 is strongly and positively correlated with all three variables PSD-95, SYP and GEP. PC1 increases with increasing levels of PSD95, SYP and GEP, in this respect they may vary together. PC2 is strongly correlated with the increasing PSD95 levels, whereas it is negatively correlated with the GEP and SYP levels (Figure 4.9.C.). Expression levels of the PSD-95, SYP and GEP were plotted against the orthogonal components; PC1, PC2 and PC3 to observe the general pattern (Figure 4.9.D). Regression factor scores were calculated for each sample and with those values; each sample were plotted against the PC1, PC2 and PC3. Panels were separated with respect to age groups and ache, to check the similarities between the clustering profiles of young middle-aged and old groups compared to the ache (Figure 4.10.A, B, C and D). The clustering profile of the female ache group showed resemblance to the young female group (Figure 4.10.A and D), there was no distinct similarities between the clustering profile of the male ache group compared to age groups (Figure 4.10.D). A larger sample size is required to further investigate the clustering profile of the ache group. However, a similar clustering profile of the ache females to the young female group is promising because it shows that there is a possible delayed aging phenotype of the ache group and sexually dimorphic patterns in this delayed aging model.

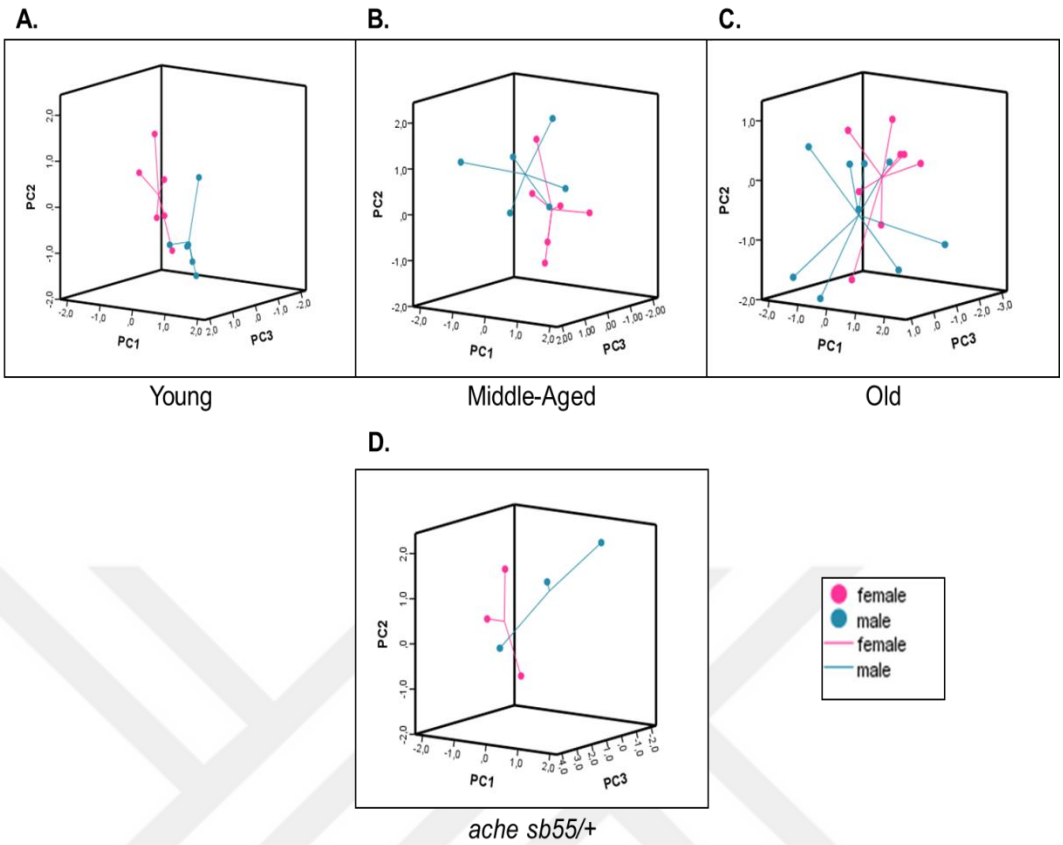

Figure 4.10. Plotting each sample with respect to orthogonal components PC1, PC2 and PC3 in 3D space. Data was combined from both Chapter 3 and 4. Clustering profiles of young (A), middle-aged (B), old (C) and *ache sb55/+* (D). Gender groups were denoted with different colors indicated in the legend.

4.3.7 Screening the Mutation to Differentiate Wild-type and Heterozygous Mutants

Two reactions were prepared for each sample and the first reaction was with the primer N, which recognizes the mutated Asn226, and the second reaction was with the primer S, which recognizes the wildtype Ser226. In the wildtypes, bands were expected only in the reactions prepared with the S primer at 200 bp, and in the

ache group, since they were heterozygous mutants, bands were expected from the both reactions prepared with N and S primer. After many optimizations and the trial of different methods, touch-down PCR conditions indicated in the Chapter 4.2.6 and their products gave the most optimal results. In the wildtype expected bands were observed from the reactions with S primer, and no bands were observed from the reactions with N primer; and in the ache group bands were observed from the both reactions prepared with N and S primers (Figure 4.9). The results were were consistent with our expectations. Therefore, this method and protocol could be used for the further genotyping of the ache adult fish.

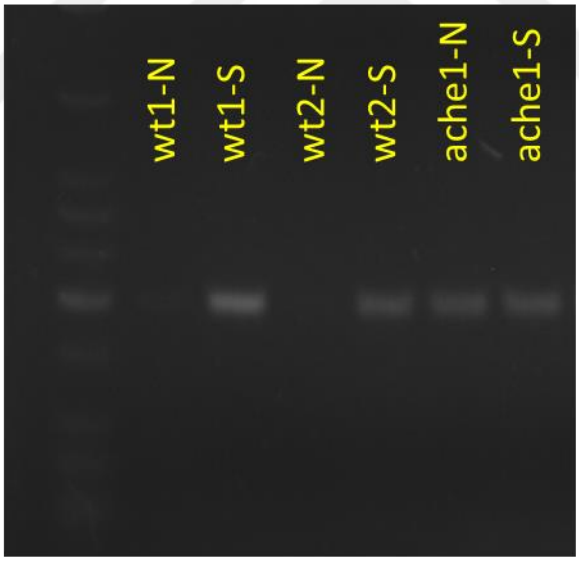

Figure 4.11. Gel image of the PCR products from wildtype and ache genomic DNA samples. wt: wild-type; ache: *ache* $^{sb55/+}$ (This experiment was performed with Ayşe Gökçe Keşküş)

4.4. Discussion

The present study was designated to investigate the effects of the altered and increased activity of the cholinergic system on the expression of the key synaptic proteins PSD-95, GEP and SYP in the old animals. This age group was chosen because the most distinguishing changes should be observed between these groups. Moreover, both males and females were included in order to determine if there is asexually dimorphic pattern among these groups.

Current data revealed no significant alterations in the levels of PSD95 between gender and genotype groups. Although, previous studies indicated that increased activity of cholinergic system exerted its ameliorative effects NMDARs dependent manner [84], significant alterations in PSD-95 which clusters and scaffolds the NMDARs directly were not observed in the ache mutants. To establish a direct relationships rather than the key scaffolding protein, Western blotting of the subunits of the NMDARs could be analyzed for the further investigation. Additionally, the current data was based on the whole brain expression profile of PSD-95, and there could be region-specific alterations in the PSD-95 levels. In this respect, micro-dissections and immunohistochemistry experiments could be performed.

In terms of GEP expression, significant increases in the ache group were observed in the normalized data. GEP serves as the indicator of the inhibitory neurotransmission and previous studies suggested different results in the age-related expression profile of the GEP. To illustrate, whereas a significant age-related reduction was reported in human V1 [48], a significant age-related increase was reported in the cognitively impaired old rats in parietal and frontal cortices [47].

However, the exact role of the GEP is still enigmatic, and crosstalk between GABAergic and cholinergic system was expected because in the basal forebrain cholinergic neurons, which constitute the large portion of the cholinergic system, co-release of GABA and ACh, as was detected following optogenetic stimulation [86]. Additionally, increased levels of GEP in the ache group might serve as buffer against age-related excitotoxicity.

In the SYP expression profile, a significant increase was revealed in the ache group. Previous studies indicated that SYP reflects synaptic integrity and was significantly correlated with better cognitive performance in mice [51]. In this respect according to their behavioral phenotype ache fish was proposed as a delayed aging model by Yu et al (2006) [67]. Therefore, this increase in the SYP levels might be confirming that the ache mutants are an appropriate delayed aging model. Moreover, different from the previous studies female ache fish were included into the present study and the significant interaction was observed between the genotype and gender of the fish. The SYP increase is much more prominent in the female ache group compared to the males. This sexually dimorphic pattern indicated that activation of the cholinergic system might exert its effects differentially in males and females.

Overall the data suggested that pre-synaptic and inhibitory post-synaptic integrity elements were affected by the activation of the cholinergic system in the old ache group compared to the wild-type control, whereas the excitatory post-synaptic integrity element showed a more stable pattern. Most interestingly, the impacts of the cholinergic system on the pre-synaptic integrity elements might depend on the gender, and males and females might have differential susceptibilities.

CHAPTER 5

Conclusions and Future Prospects

Normal aging cognitive decline is prominent among some cognitive domains including processing speed, episodic memory, divided attention and executive functions [15]. However, age-related cognitive decline is not a unitary process; whereas some cognitive abilities decline with an advancing age, some cognitive domains including sustained attention, implicit memory, and vocabulary show stable and even improved profile in the older adults [20]. There are no gross structural alterations, including neuronal and synapse loss, occurring through normal aging [3], [6] that can account for the age related cognitive decline. Moreover, subtle molecular alterations including age-related gene expression changes [58], disturbances in mitochondrial dynamics [32], protein homeostasis [33], and hypofunction in the cholinergic system [10] were reported by previous studies. Disturbances in neurotransmission regulating proteins might have a direct explanatory role in age-related cognitive decline and alterations; it was also reported that age-related changes exert their effects differentially in males and females [58]. In this thesis, three key synaptic proteins were focused as the indicators of the differential parts of synaptic integrity, whose disturbance might be associated with the age-related alterations and cognitive decline. The first one was postsynaptic integrity-95 (PSD-95), since it clusters the glutamatergic receptors, NMDA, directly, and AMPA, indirectly, in post-synaptic sites, which are crucial for the excitatory neurotransmission and induction of LTP [36]. The second synaptic protein was gephyrin (GEP), since it is the major scaffolding protein in inhibitory synapses and it clusters $GABA_A$ and glycine

receptors [75]. The third synaptic protein focused on was synaptophysin (SYP), which is localized in the synaptic vesicles and pre-synaptic sites, and preserved levels of SYP at older ages are correlated with better cognitive performance [51]. These proteins were investigated in the experimental setups of Chapters 3 and 4.

In Chapter 3, the main aim of the current study was to analyze the levels of neurotransmission regulating proteins among age groups and to decipher possible existence of sexual dimorphism on those expression levels with respect to aging. In the both normalized and TUB-normalized data it was revealed that expression levels of PSD-95, which gives information about the excitatory post-synaptic integrity, and SYP, which indicates the pre-synaptic integrity, were significantly affected by age and gender; this significant gender by age interaction was marginally significant in terms of the expression levels of the GEP. It was shown that PSD-95 and SYP levels tend to be preserved and increased in the female groups throughout aging, whereas, in male groups, expression levels of these proteins tend to be reduced. Thus, the data indicate that in zebrafish age-related changes in synaptic protein levels are modified in a sexually-dimorphic manner.

Although, a significant gender by age interaction on the expression levels of the key synaptic proteins was observed; the main effect of age was not observed in a similar manner compared to the studies conducted with the rat models [37], [93], [47]. This might be due to the heterogeneous old group used in the present study, which was explained in Chapter 3.4, the Discussion section. For further studies old groups can be divided into two categories; cognitively impaired and cognitively intact according to their behavioral performance based on standard tests, including T-maze, which is also applicable in the zebrafish model [94]. After this segregation in

the old group according to the cognitive performance, levels of the key synaptic proteins can be analyzed again, different and meaningful expression trends may be revealed.

Additionally, previous studies indicated that age-related alterations observed in the protein levels were not observed in the gene expression patterns [93], [37]. Therefore, alterations in the protein expression may not be parallel and seen in the transcriptome. For the further experiments, gene expression levels could be measure for the selected key synaptic proteins, and the results would be promising and establishing unitary and accurate prospects for normal aging-related alterations.

In Chapter 4, it was investigated whether ameliorating the cholinergic hypofunction through the loss of function mutation in the coding gene acetylcholinesterase, which breaks down the acetylcholine in the synaptic cleft, might have beneficial effects on the aging-related protein expression alterations and to check whether a possible ameliorative impact of the increased cholinergic transmission exerts its effects differentially in old males and females. In the normalized data significant increases in the expression levels of SYP and GEP was found, whereas no alteration was observed in the expression levels of PSD-95 in the ache groups compared to the wildtype. Additionally was a significant gender by genotype interaction in the SYP expression levels. Therefore, manipulation in the cholinergic system and the results might be dependent on the gender. Additionally, housekeeping normalized data did not show significant differences although the patterns were similar in terms of SYP expression. However, this situation brings into the question of whether the mutation disrupts the cytoskeletal dynamics.

To eliminate this question in future experiments, membranes can be stained with the reversible total protein kits, which has higher sensitivity compared to the Ponceau staining, and in it was showed that normalization done with the total protein stain gave more reliable results compared to the housekeeping proteins [95]. In the subset of the experiments reversible protein stain (24585, Thermo Scientific, Paisley, UK) kit was also tried along with the incubation with the housekeeping proteins TUB and ACTIN, most fluctuations were detected in the ACTIN and least fluctuations were detected in the total protein stain kit between wildtype and ache mutants. Therefore, in the future experiments including ache mutants, this staining procedure can be used as a regular protocol along with the other housekeeping proteins, because the effects of the mutation and effects on the cytoskeletal proteins are unclear.

Addiction studies conducted with the ache mutants indicated that better behavioral performance was observed in the ache mutants but other kinds of chemical interventions targeting the cholinergic system including acetylcholinesterase inhibitors and muscarinic receptor agonist did not result in the better behavioral phenotype [90]. Therefore, this mutation might exert its effects through the developmental dynamics, and zebrafish can be treated with the chemical targeting the cholinergic system and expression levels of the synaptic proteins can be compared with the ache mutants. These studies will be important for discriminating between developmental and adult effects of changes in the cholinergic system.

Ache mutants were proposed as the delayed aging model because of their preserved cognitive abilities at older ages compared to their wild-type siblings [67]. To confirm those observations in addition to the key synaptic protein expression

analysis as indicated in this thesis, other markers of the senescence like measuring the telomere length, and the levels of senescence associated beta galactosidase and neurogenic capacities can be investigated to give more concerete information about the proposed delayed aging phenotype of ache mutants, and perviously those markers were analyzed by our group between the young and old wild-type zebrafish [68].

To conclude, in this thesis it was revealed that age and gender have combinatory effects on the expression profile of key synaptic proteins and in the old ache mutants with increased cholinergic activity, the expression profile of these synaptic proteins was changed compared to the wild-type controls but this change is dependent on the gender in terms of a pre-synaptic integrity marker. This study emphasized the importance of gender and sexually-dimorphic patterns in the context of aging and cholinergic manipulations could be a promising target of interventions to attenuate the effects of age-related synaptic alterations, which could have possible contributions to age-related cognitive decline.

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